

STEM CELLS AND CARDIOVASCULAR DISEASES

EDITED BY: Shijun Hu, Lei Ye, Mingtao Zhao and Feng Lan
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STEM CELLS AND CARDIOVASCULAR DISEASES

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Editorial: Stem Cells and Cardiovascular Diseases

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Editorial on the Research Topic

Stem Cells and Cardiovascular Diseases

Cardiovascular disease remains the leading cause of death worldwide despite significant advances in our understanding of the disease and its treatment. Consequently, the therapeutic potential of stem cells has stimulated a recent surge of translational research and clinical trials aimed at addressing this challenge. In this Research Topic, an overview regarding stem cells and cardiovascular diseases has been provided through 28 articles contributed by 219 authors. Among which, 2 mini reviews, 6 review articles, and 20 original research papers were included.

Huang et al. summarized the applications of human pluripotent stem cells (hPSCs) in heart disease modeling, cell therapy, and next-generation drug discovery. Elde et al. reviewed cell therapy and paracrine signaling approaches to myocardial regeneration. Lin et al. discussed the fascinating perspectives on using patient-specific induced pluripotent stem cells (iPSCs) and clustered regularly interspaced short palindromic repeats (CRISPR) genome editing to functionally study the genetic and epigenetic determinants of congenital heart disease. Gao et al. summarized the protocols of differentiating hPSCs into cardiovascular cells, highlight their therapeutic applications for the treatment of cardiac diseases in large animal models, and discussed the challenges and limitations in using hPSC-derived cardiac cells for a better clinical application of cardiac cell therapy. Meng et al. summarized the current methods of constructing tissue blood vessels *in vitro* and *in vivo*. Liu et al. reviewed the current dilemmas in stem cell-based therapy for ischemic heart disease, including cell source and cell dosage, delivery method and timing of transplantation, cell tracing and the involved mechanisms. Jiang et al. summarized the genetic background of the most common inherited cardiomyopathy and the works have been done so far using hiPSCs for mechanistic research and symptom relief. Huang et al. reviewed the uses of nanoparticles as contrast agents for tracking mesenchymal stem cells (MSCs) and the challenges of clinical applications of MSCs in the treatment of cardiovascular diseases.

This Research Topic contains a series of original research papers related to stem cells and cardiovascular diseases covering methods establishment, mechanism exploration, and so on.

Two papers focus on gene editing. Qi et al. designed an all-in-one episomal vector that expressed a single guide RNA with an adenine base editor or a cytosine base editor. This system can efficiently introduce disease-specific mutations into hPSCs to display phenotype at the cellular level. Gao et al. characterized BlatCas9 as an alternative tool for mammalian genome editing which preferred a N₄CNAA protospacer adjacent motif and can be packaged into adeno-associated vector (AAV).

Several groups tried to reveal the underlying mechanisms of cardiovascular diseases using patient-specific iPSC derived cardiomyocytes/endothelial cells, such as Brugada syndrome (Li et al.),

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Duchenne muscular dystrophy related cardiomyopathy (Li et al.), uremic vasculopathy (Jang et al.), and type 2 diabetes mellitus (Su et al.). By using CRISPR/Cas9 technology, Li et al. created a *RRAD*^{-/-} H9 cell line to disrupt RAD to reveal mechanisms leading to cardiac hypertrophy.

As MSCs have great research value and broad application prospects in the cardiovascular disease, they have been a hot Research Topic. Yu et al. demonstrated that augmenting tripeptidyl peptidase 1 (TPP1) can improve the therapeutic efficacy of aged MSCs in myocardial infarction with enhanced DNA double-strand break repair through AKT/MRE11 pathway. Meng et al. showed that adiponectin modified bone marrow derived MSCs alleviate cardiac fibrosis via inhibiting TGF- β 1/Smad in diabetic rats. Li et al. demonstrated that CD73⁺ adipocytes derived MSCs showed a higher pro-angiogenic paracrine activity and displayed therapeutic efficacy for myocardial infarction therapy. Two independent groups developed similar model to visualize, trace, and quantify MSC based on cell-selective metabolic labeling (Zhang et al. and Du et al.). Briefly, mutant methionyl-tRNA synthetase (MetRS*) could utilize non-canonical amino acid azidonorleucine (ANL) instead of methionine during protein synthesis in MSCs. ANL can be covalently linked to alkyne-conjugated reagents via click chemistry. When incubated with ANL-supplemented media, MetRS* expressing MSCs can be detected. This may be leveraged to understand and improve stem cell therapy.

Besides MSCs, pericardial fluid cells (PFCs), perivascular adipose-derived stem cells (PVASCs), cardiac mesenchymal cells (CMCs), and REX-001 were also mentioned. PFCs exhibited progenitor cell features and could serve as an alternative cell source for myocardial tissue repair, engineering, and reconstruction (Sun et al.). PVASCs were capable of differentiating into multiple cell lineages and can contribute to vascular remodeling. As TBX20 inhibited smooth muscle cell differentiation and promoted endothelial cell differentiation from PVASCs, it could be used to inhibit neointima formation after vascular injury (Jie et al.). CMCs were proved to be effective in improving left ventricle function after myocardial infarction. Compared to atmospheric oxygen tension (21%), culturing CMCs at physiologic oxygen tension (5%) provided superior therapeutic efficacy in promoting cardiac repair *in vivo* (Bolli et al.). REX-001 is a kind of human bone marrow derived cell suspension enriched for mononuclear cells, granulocytes and CD34⁺ cells. REX-001 could enhance blood flow recovery after ischemic injury by inducing functional neovascularization (Rojas-Torres et al.).

As for engineered cardiac tissue, a layer-by-layer fabrication method was employed to produce large and thick cardiac tissue created from hiPSC-derived cardiomyocytes, endothelial cells, and fibroblasts. Layer-by-layer fabrication could be utilized to create prevascularized and functional cardiac tissue constructs for potential therapeutic applications (Pretorius et al.). Human ventricular cardiac anisotropic sheets derived from hiPSCs were used to investigate arrhythmogenicity, electrophysiological, and calcium transient changes induced by hypokalaemia (Gurung et al.).

What's more, Lin et al. revealed the protective effect of XIN against TNNT2 mutation-induced dilated cardiomyopathy. Yang et al. provided an energy-efficient strategy to promote the structural, metabolic and electrophysiological maturation of human embryonic stem cell-derived cardiomyocytes by intermittent starvation.

In summary, this topic provides a platform to exhibit a number of comprehensive reviews and original articles focusing on stem cell-based therapy for treating and modelling of cardiovascular diseases. We thank all authors for their support to make this topic a success.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Base Editing Mediated Generation of Point Mutations Into Human Pluripotent Stem Cells for Modeling Disease

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Human pluripotent stem cells (hPSCs) are a powerful platform for disease modeling and drug discovery. However, the introduction of known pathogenic mutations into hPSCs is a time-consuming and labor-intensive process. Base editing is a newly developed technology that enables facile introduction of point mutations into specific loci within the genome of living cells. Here, we design an all-in-one episomal vector that expresses a single guide RNA (sgRNA) with an adenine base editor (ABE) or a cytosine base editor (CBE). Both ABE and CBE can efficiently introduce mutations into cells, A-to-G and C-to-T, respectively. We introduce disease-specific mutations of long QT syndrome into hPSCs to model LQT1, LQT2, and LQT3. Electrophysiological analysis of hPSC-derived cardiomyocytes (hPSC-CMs) using multi-electrode arrays (MEAs) reveals that edited hPSC-CMs display significant increases in duration of the action potential. Finally, we introduce the novel Brugada syndrome-associated mutation into hPSCs, demonstrating that this mutation can cause abnormal electrophysiology. Our study demonstrates that episomal encoded base editors (epi-BEs) can efficiently generate mutation-specific disease hPSC models.

Keywords: human pluripotent stem cell, base editing, episomal vector, disease modeling, IPS, long QT syndrome, Brugada syndrome

INTRODUCTION

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) (Thomson et al., 1998) and the closely related human induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007), can self-renew and differentiate into various cell types. Due to these properties, these stem cells hold great promise for modeling mammalian organ development, studying disease mechanisms and pathways, and developing future therapies (Wang et al., 2012, 2014; Lan et al., 2013; Li et al., 2019). However, to realize these potential applications, genes of interest often need to

be engineered in the cell. For example, iPSCs derived from patients often contain genetic mutations that need to be corrected prior to conducting specific therapies (Maeder and Gersbach, 2016). For disease modeling with normal hPSCs, pathogenic mutations need to be introduced into cells to generate disease models (Wang et al., 2014; Xie et al., 2019). Therefore, it is crucial to develop methods and technologies for efficient genetic manipulation of stem cells.

The RNA-guided CRISPR-Cas9 system is a powerful tool for genome editing in hPSCs (Mali et al., 2013; Xie et al., 2017). In this system, a Cas9 nuclease and a single guide RNA (sgRNA) form a Cas9-sgRNA complex, which recognizes a specific DNA sequence that is complementary to the sgRNA, and subsequently generate a site-specific double-strand break (DSB) (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013). The DSBs are typically repaired by the cell's endogenous DNA repair machinery through non-homologous end-joining (NHEJ) or microhomology mediated end-joining (MMEJ), resulting in non-specific small insertions and deletions (indels) around the cut site, which can be useful for generating loss-of-function mutations (Kim and Kim, 2014). These DSBs can also be repaired by homology-directed repair (HDR) using an introduced DNA repair template, such as a double-stranded DNA donor plasmid or single-stranded donor DNA oligonucleotides (ssODN), resulting in the introduction of precise modifications (Wang et al., 2012; Kim and Kim, 2014). However, the HDR pathway is strongly disfavored in most cell types and requires special cellular machinery that is only present during cellular replication (Wang et al., 2012, 2014). Furthermore, the use of nuclease Cas9 to mediate DSBs results in potential undesired translocations or DNA rearrangements.

Base editing is a new genome-editing technology that directly generates precise point mutations without generating DSBs (Komor et al., 2016; Gaudelli et al., 2017). Base editors comprise fusions between a catalytically impaired Cas9 nuclease and a single-stranded DNA (ssDNA) specific deaminase enzyme. Upon binding to its target DNA, base pairing between the sgRNA and the target DNA strand results in the displacement of a small segment of single-strand DNA as an "R-loop" (Nishimasu et al., 2014; Rees and Liu, 2018). DNA bases within this ssDNA are therefore substrates for deamination and are subsequently modified by the deaminase enzyme. Two classes of DNA base editor have so far been developed—cytosine base editors (CBEs) convert a C-G base pair into a T-A base pair (Komor et al., 2016), and adenine base editors (ABEs) convert an A-T base pair into a G-C base pair (Gaudelli et al., 2017). Base editors hold great promise for the genetic modification of hPSCs (Chadwick et al., 2017).

The long QT syndrome (LQTS) is a heart disorder that is characterized by the prolongation of the QT interval of surface electrocardiograms and is associated with malignant arrhythmias, syncopal episodes, torsade de pointes form ventricular tachycardias, and an increased risk of sudden cardiac death (Moss, 2003). Mutations in the *KCNQ1* (LQT1), *KCNH2* (LQT2), and *SCN5A* genes (LQT3) account for the three most common pathogenic variants that are clinically annotated for LQTS (Moss, 2003). In addition to LQTS, mutations in *SCN5A*

have been shown to induce Brugada syndrome (BrS) (Wilde and Amin, 2018). We and others have demonstrated that hPSCs can be used for modeling LQTS (Liang et al., 2013; Wang et al., 2014).

We previously used an episomal vector to express Cas9 and sgRNAs (epiCRISPR), and achieved high efficiency of genome editing through the NHEJ/MMEJ pathway (Xie et al., 2017; Wang et al., 2019). This episomal vector can replicate during cellular division in eukaryotes (Van Craenenbroeck et al., 2000), permitting the continuous expression of Cas9 and sgRNAs in cells. The vector also contains a drug resistance gene, allowing for the enrichment of transfected cells by drug selection. In this study, we generate episomal vectors that express base editors (epi-BEs) and achieve high efficiency of precise genome editing in hPSCs. As a proof of concept, we first demonstrate that these epi-BEs can efficiently introduce mutations into hPSCs to generate LQTS models. We then evaluate a novel mutation on *SCN5A* for developing BrS. Our study demonstrates that these epi-BEs are a simple and efficient platform to generate human disease models in stem cells.

MATERIALS AND METHODS

Cell Culture

Human ESCs (H9, WA09, Wicell, Madison, WI, United States) and iPSCs were cultured on Matrigel-coated plates (ESC qualified, BD Biosciences, San Diego, CA, United States) using hESC mTeSR-1 cell culture medium (StemCell Technologies, Vancouver, Canada), whereas HEK293T and HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco). All media contained 100 U/ml of penicillin and 100 mg/ml of streptomycin. Cells were cultured at 37°C with 5% CO₂ in a humidified and mycoplasma negative incubator.

Plasmid Construction

Epi-ABEmax/epi-AncBE4max: KO plasmid (Addgene #135970) was PCR-amplified using primers EBV-F/EBV-R to generate Fragment 1, which contained oriP/EBNA1, gRNA expression cassette (hU6 promoter and gRNA scaffold) and EF1A core promoter. Fragment 2 was synthesized by GENEWIZ (Suzhou, China) and was used to express blasticidin S deaminase (BSD), and then Fragment 2 was PCR-amplified using primers BSD-F/BSD-R. Fragment 3 contained full ABE or CBE and was obtained from pCMV_ABEmax (Addgene #112095) or pCMV_AncBE4max (Addgene #112094); this was amplified by PCR using primers ABEmax-F/ABEmax-R or AncBE4-F/AncBE4-R. All three fragments were cloned together by NEBuilder Assembly Tool (NEB) following the manufacturer's protocol, resulting in epi-ABEmax-hU6 or epiAncBE4-hU6. Furthermore, hU6 was replaced by mU6 to eventually result in epi-ABEmax or epi-AncBE4max. mU6 was synthesized by GENEWIZ (Suzhou, China) and PCR-amplified using primers mU6-F/mU6-R, then cloned into the epi-ABEmax-hU6 or epi-AncBE4max-hU6 backbone, which was PCR-amplified using primers EBV-2-F/EBV-2-R. Sequences were verified by Sanger sequencing (GENEWIZ, Suzhou, China).

Epi-ABEmax-NG/epi-AncBE4max-NG: A portion of SpCas9-NG that contained the seven mutations (R1335V/L1111R/D1135V/G1218R/E1219F/A1322R/T1337R) was PCR-amplified from pX330-SpCas9-NG (Addgene #17919) using primers 1111-UP-F/1337-DOWN-R. This portion of SpCas9-NG was cloned into epi-ABEmax or epi-AncBE4max backbone, which was PCR-amplified using primers epi-backbone-F/epi-backbone-R, resulting in epi-ABEmax-NG or epi-AncBE4max-NG.

To express an sgRNA, the oligonucleotide duplexes were cloned into BspQI restriction sites of epi-ABEmax/epi-AncBE4max/epi-ABEmax-NG/epi-AncBE4max-NG.

All primers are listed in **Supplementary Table 1**.

Base Editing and Blasticidin Selection

Cells were plated into 48-well plates and transfected the next day at approximately 70% confluency. Briefly, 500 ng of epi-ABEmax/epi-AncBE4max/epi-ABEmax-NG/epi-AncBE4max-NG plasmid was transfected using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions at day 1. For H9 and iPSCs, cells were passaged on days 2, 6, and 11, respectively, and selected by blasticidin (Thermo Fisher Scientific) from day 2 to day 16. A 2.5 $\mu\text{g/ml}$ of blasticidin was added into the growth media, except on days 2, 6, and 11, where 10, 5, and 0 $\mu\text{g/ml}$ of blasticidin were used, respectively. For HeLa and HEK293T cells, 2.5 $\mu\text{g/ml}$ of blasticidin was used from day 2 to day 16. Transfected cells were harvested for analysis on days 6, 11, and 16. The antibiotic screening time can be shortened when editing efficiency is enough, which has been discussed in detail in a recent work (Geng et al., 2020).

The genomic DNA was isolated using QuickExtractTM DNA Extraction Solution (Lucigen) according to the manufacturer's instructions. Targets of base editing were amplified by PCR using KOD DNA Polymerase (Toyobo). The PCR products were sequenced using Sanger sequencing, and the editing efficiency was analyzed by EditR (Kluesner et al., 2018).

Off-Target Analysis

For each target site, five potential off-targets were selected based on Cas-Offinder¹ (Bae et al., 2014) and PCR-amplified for Sanger sequencing.

Single Cell-Derived Clone Screen

The iPSCs were passaged with ethylenediaminetetraacetic acid (EDTA). Then, 1×10^5 cells were seeded on a Matrigel (Corning, United States) pre-coated 10 cm dish using E8 media (Cellap, Beijing, China) with 2 μM of thiazovivin (TZV, Selleck, United States). Twenty-four hours later, the E8 media was replaced by new media without TZV. This media was changed every 2 days. Ten days after seeding, the single cell-derived clones were picked with a 1 ml sterile syringe and placed on a Matrigel pre-coated 24-well plate for cell expansion. Genomic DNA was extracted using the TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China) for DNA sequencing.

¹<http://www.rgenome.net/cas-offinder/>

Cardiomyocyte Differentiation

A chemically defined molecular-based method was applied for cardiomyocyte (CM) differentiation (Burridge et al., 2015). Briefly, cells (~90% confluency) were seeded on a Matrigel pre-coated 6-well plate at a ratio of 1:6 in E8 media. The media was changed to CDM3 (consisted of RPMI 1640, 500 $\mu\text{g/ml}$ of *Oryza sativa*-derived recombinant human albumin, and 213 $\mu\text{g/ml}$ of L-ascorbic acid 2-phosphate) supplemented with 6 μM of CHIR99021 when the cells reached ~75% confluency. After 48 h, the media was changed to CDM3 supplemented with 2 μM of Wnt-C59. After 2 days, the media was changed to CDM3 and refreshed every 2 days. After differentiation for 7–8 days, spontaneous contracting cells could be observed. On day 12, CMs were purified using a metabolic-selection method. Briefly, the medium consisted of RPMI 1640 without glucose, 213 $\mu\text{g/ml}$ of L-ascorbic acid 2-phosphate, 500 $\mu\text{g/ml}$ of *Oryza sativa*-derived recombinant human albumin, and 5 mM of sodium DL-lactate (Sigma, United States). After purification, CMs were cultured with RPMI 1640 and B27 (Life Technology, United States). For cellular maintenance, the medium was changed every 3 days.

Electrophysiological Test Using MEA

CMs were digested with Accutase (Thermo Fisher, United States). CytoView MEA24 plates (Axion Biosystems, Inc., Atlanta, United States) were pre-coated overnight by 0.5% Matrigel phosphate-buffered saline (PBS) solution. Then, 15,000 CMs were plated on each multi-electrode array (MEA) well with RPMI/B27 medium and cultured for 3 days. When the cellular electrophysiological activity became stable, the experimental data were recorded using Maestro EDGE (Axion Biosystems, Inc., Atlanta, United States) according to the MEA manual. The data were analyzed using the AxIS Navigator, Cardiac Analysis Tool, and IGOR software.

Clinical Data

The patient's data were provided by Xin Hua Hospital Affiliated to Shanghai Jiao Tong University.

Quantification and Statistical Analysis

The editing efficiency was analyzed by EditR, which is an algorithm for predicting potential editing in a guide RNA region from a single Sanger sequencing run (Kluesner et al., 2018). All data are shown as mean $\pm$ SD. Statistical analyses were conducted using Microsoft Excel. Two-tailed, unpaired Student's *t*-tests were used to determine statistical significance when comparing two groups. A value of $P < 0.05$ was considered to be statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

RESULTS

Episomal Vector-Based Base Editors for Efficient Base Editing

We designed an all-in-one episomal vector-based system to express an sgRNA with an ABE (ABEmax, Koblan et al., 2018),

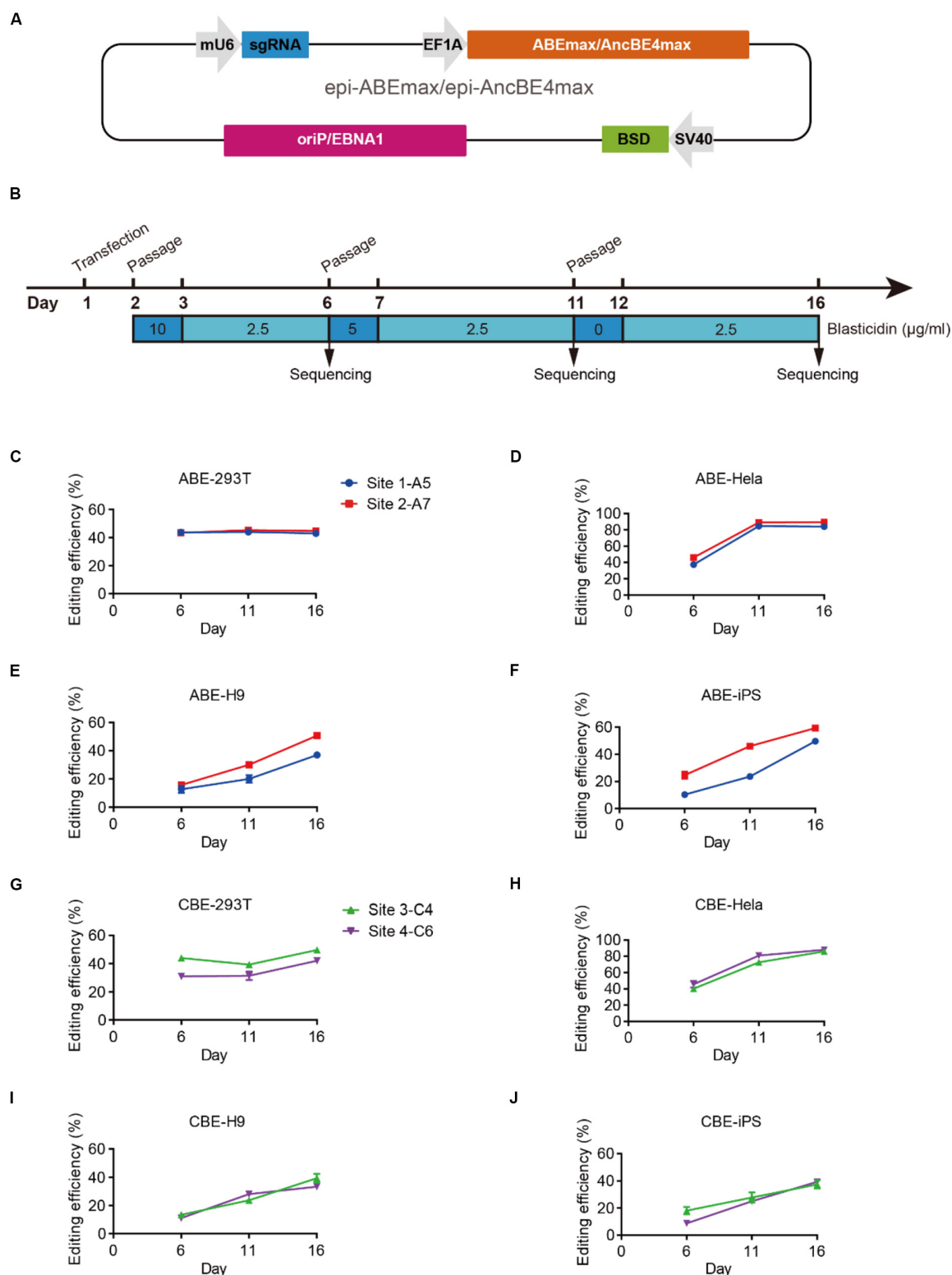
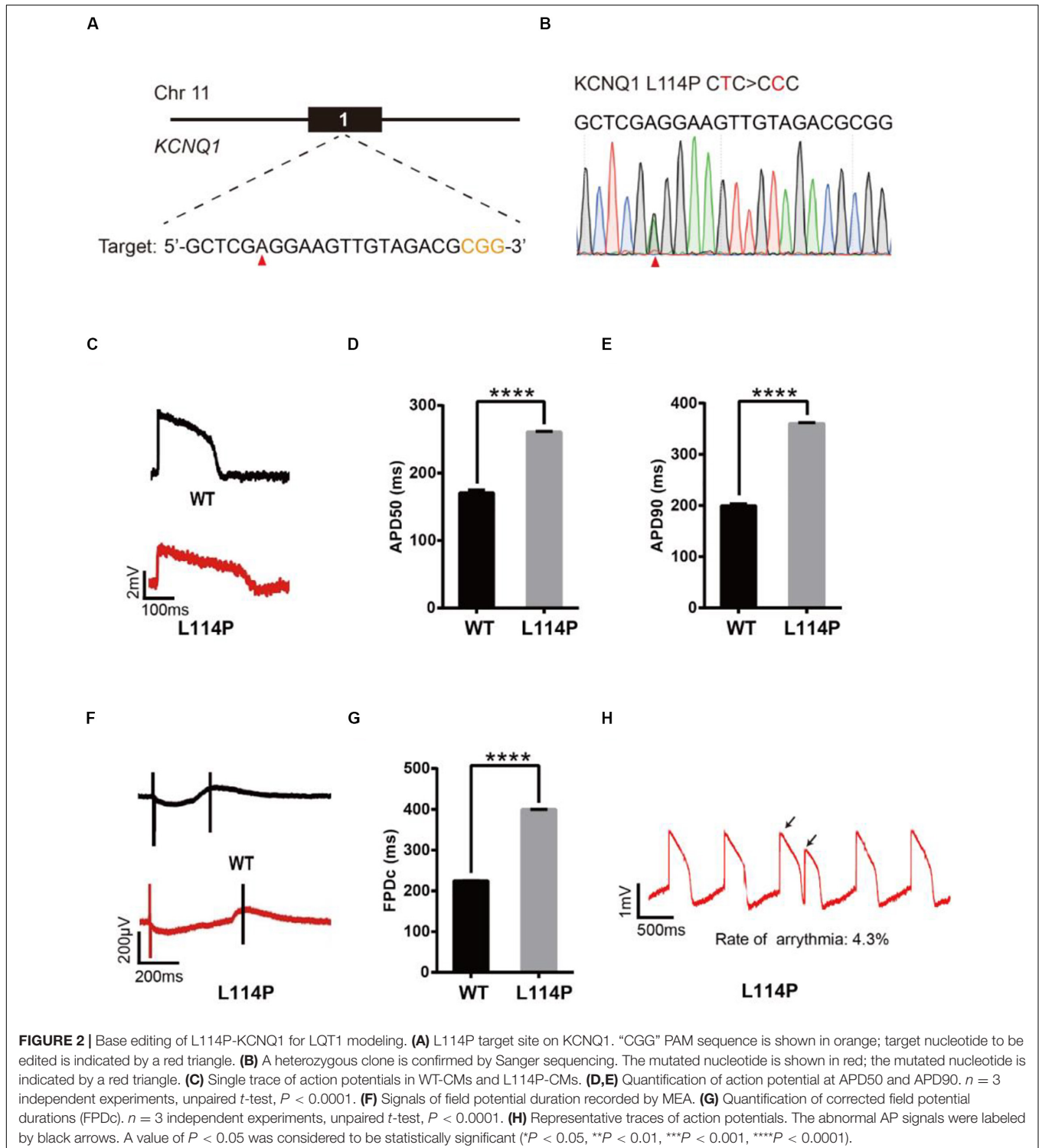


FIGURE 1 | Efficient base editing with epi-ABEmax and epi-AncBE4max. **(A)** Schematic of the epi-ABEmax and epi-AncBE4max plasmids. The vector contains a mouse U6 promoter-driven gRNA scaffold, an EF1a promoter-driven base editor, an SV40 promoter-driven a blasticidin S deaminase (BSD), and oriP/EBNA1 elements for the plasmid replication in eukaryotes. **(B)** Procedure of base editing with epi-ABEmax and epi-AncBE4max. **(C–F)** Editing efficiency of epi-ABEmax in HEK293T, HeLa, H9, and iPSCs was measured at three-time points, $n = 3$ independent experiments. **(G–J)** Editing efficiency of epi-AncBE4max in HEK293T, HeLa, H9, and iPSCs was measured at three-time points, $n = 3$ independent experiments.

which we named epi-ABEmax, or a CBE (AncBE4max, Koblan et al., 2018), which we named epi-AncBE4max. These vectors also express a BSD conferring resistance to blasticidin, which allows for enrichment of transfected cells through antibiotic selection (**Figure 1A**). First, we analyzed the capacity of the epi-ABEmax for base editing in two somatic cell lines

(HEK293T and HeLa) and two hPSC lines (H9 and iPSCs). We selected two previously reported sgRNAs, targeting Site 1 and Site 2 (Koblan et al., 2018), to determine adenine base editing efficiency. The plasmid was delivered into cells using a lipid-based transfection, followed by blasticidin selection (**Figure 1B**). Editing efficiency was measured on days 6, 11, and 16. The



maximum editing efficiencies were observed on days 6 and 11 for HEK293T and HeLa cells (**Figures 1C,D**). For H9 and iPSCs, the editing efficiency increased over time and seems to continue growing past day 16 (**Figures 1E,F**).

Next, we analyzed the capacity of the epi-AncBE4max for base editing in these four cell lines. We selected two gRNAs, targeting HEK3 (Site 3) (Komor et al., 2016) and FANCF (Site 4), to measure cytosine base editing efficiency. Similar to epi-ABEmax data, the maximum editing efficiencies were observed at days 6 and 11 for HEK293T and HeLa cells (**Figures 1G,H**). For H9 and iPSCs, the editing efficiency increased over time and seems to continue to increase past day 16 (**Figures 1I,J**). In summary, these epi-BEs enable efficient base editing in both somatic cells and hPSCs.

Base Editing of KCNQ1 for LQT1 Modeling

We next decided to show the utility of epi-BEs for modeling disease. For this, we introduced the pathogenic mutation L114P in KCNQ1, as annotated in previous literature (Jongbloed et al., 2002), into H9 cells. We designed a corresponding sgRNA (**Figure 2A**) that would install this mutation located at position 6 of the protospacer, counting the SpCas9 PAM as positions 21–23. Following a similar editing protocol as before, the editing efficiency reached up to 34% on day 16 (**Supplementary Table 2** and **Supplementary Figure 1A**). We screened 52 single cell-derived clones, from which there were 26 heterozygous clones and 3 homozygous clones (**Table 1**). To test whether base editing influenced the pluripotency of H9 cells, immunofluorescence staining was performed for the pluripotency markers OCT-4 and SSEA-4. Besides, a higher standard, teratoma formation experiment was performed. We note that base editing did not influence marker expression, and that the cells can differentiate into three germ layer lineages after base editing (**Supplementary Figure 2**).

Subsequently, heterozygous L114P-H9 cells were differentiated into CMs using a chemically defined, small molecule-based monolayer CM differentiation method (**Figure 2B** and **Supplementary Figure 2**) (Burridge et al., 2014). To test whether these CMs exhibited the LQT1 phenotype, electrophysiology was recorded using the local extracellular action potential (LEAP) assay, which is a high-throughput, non-invasive, label-free, and stable method to measure extracellular field potential (FP) from an intact CM syncytium (Hayes et al., 2019), which can be translated into an action potential (AP) (Hayes et al., 2019). Prolongation of the action potential duration (APD) and field potential duration (FPD) are the most important LQTS electrophysiological phenotypes at the cellular level (Itzhaki et al., 2011; Wang et al., 2014). APD at 50% (APD50) and 90% (APD90) in L114P-CMs were significantly longer than those in wild-type (WT)-CMs (**Figures 2C–E**). Corrected field potential duration (FPDc) in L114P-CMs was significantly longer than that in WT-CMs (**Figures 2F,G**). L114P-CMs also exhibited an abnormal AP profile, which resembles a closely coupled single triggered beat (**Figure 2H**).

TABLE 1 | Summary of base editing efficiency.

Gene	Mutation	Total number of clones	Heterozygous clones	Homozygous clones
KCNQ1	L114P	52	26 (50%)	3 (5.8%)
KCNQ1	R190Q	77	22 (28.6%)	6 (7.8%)
KCNH2	Y616C	4	4 (100%)	0 (0)
KCNH2	Y475C	41	18 (40.9%)	8 (19.5%)
SCN5A	E1784K	107	32 (29.9%)	5 (4.7%)
SCN5A	R1879W	47	2 (4.3%)	8 (17%)

To further test the capacity of these epi-BEs, we introduced another known pathogenic mutation (R190Q) (Barsheshet et al., 2012) into KCNQ1 to model LQT1 (**Supplementary Figures 3A,B**). The editing efficiency reached 31% on day 16 (**Supplementary Figure 1B** and **Supplementary Table 2**). We screened 77 single cell-derived clones, of which there were 22 heterozygous clones and 6 homozygous clones (**Table 1**). We differentiated heterozygous R190Q-H9 cells into CMs and recorded electrophysiology by using the LEAP assay (**Supplementary Figures 3C–G**). Compared with WT-CMs, R190Q-CMs displayed significantly longer APD and FPDc. R190Q-CMs also exhibited an abnormal AP profile, resembling that of a closely coupled single triggered beat (**Supplementary Figure 3H**). Taken together, these data demonstrated that both L114P-CMs and R190Q-CMs recapitulated LQT1 phenotype.

Base Editing of KCNH2 for LQT2 Modeling

To model LQT2, we introduced a known pathogenic mutation Y616C (Anderson et al., 2014) into the KCNH2 gene of H9 cells (**Figure 3A**). The editing efficiency reached 37% on day 16 (**Supplementary Figure 1C** and **Supplementary Table 2**). We screened four single cell-derived clones, all of which were heterozygous clones (**Table 1**). To test whether CMs exhibited the LQT2 phenotype, we differentiated the heterozygous Y616C-H9 cells into CMs, and electrophysiology was recorded by the LEAP assay (**Figure 3B**). APD50 and APD90 in Y616C-CMs were significantly longer than those in WT-CMs (**Figures 3C–E**). FPDc in Y616C-CMs was significantly longer than that in WT-CMs (**Figures 3F,G**). Y616C-CMs exhibited an abnormal AP profile, resembling that of a single triggered beat (**Figure 3H**).

We next introduced another known pathogenic mutation Y475C (Liu et al., 2006) into the KCNH2 gene to model LQT2 (**Supplementary Figures 4A,B**). We performed editing as described previously and note that the editing efficiency reached 53% on day 16 (**Supplementary Figure 1D** and **Supplementary Table 2**). We screened 41 single cell-derived clones, of which there were 18 heterozygous clones and 8 homozygous clones (**Table 1**). We recorded electrophysiology for the heterozygous Y475C-CMs by LEAP assay (**Supplementary Figures 4C–G**). Y475C-CMs showed longer APD and FPDc than WT-CMs. Y475C-CMs displayed triggered beats (**Supplementary Figure 4H**). Taken together, these data demonstrate that both Y616C-CMs and Y475C-CMs recapitulated LQT2 phenotype.

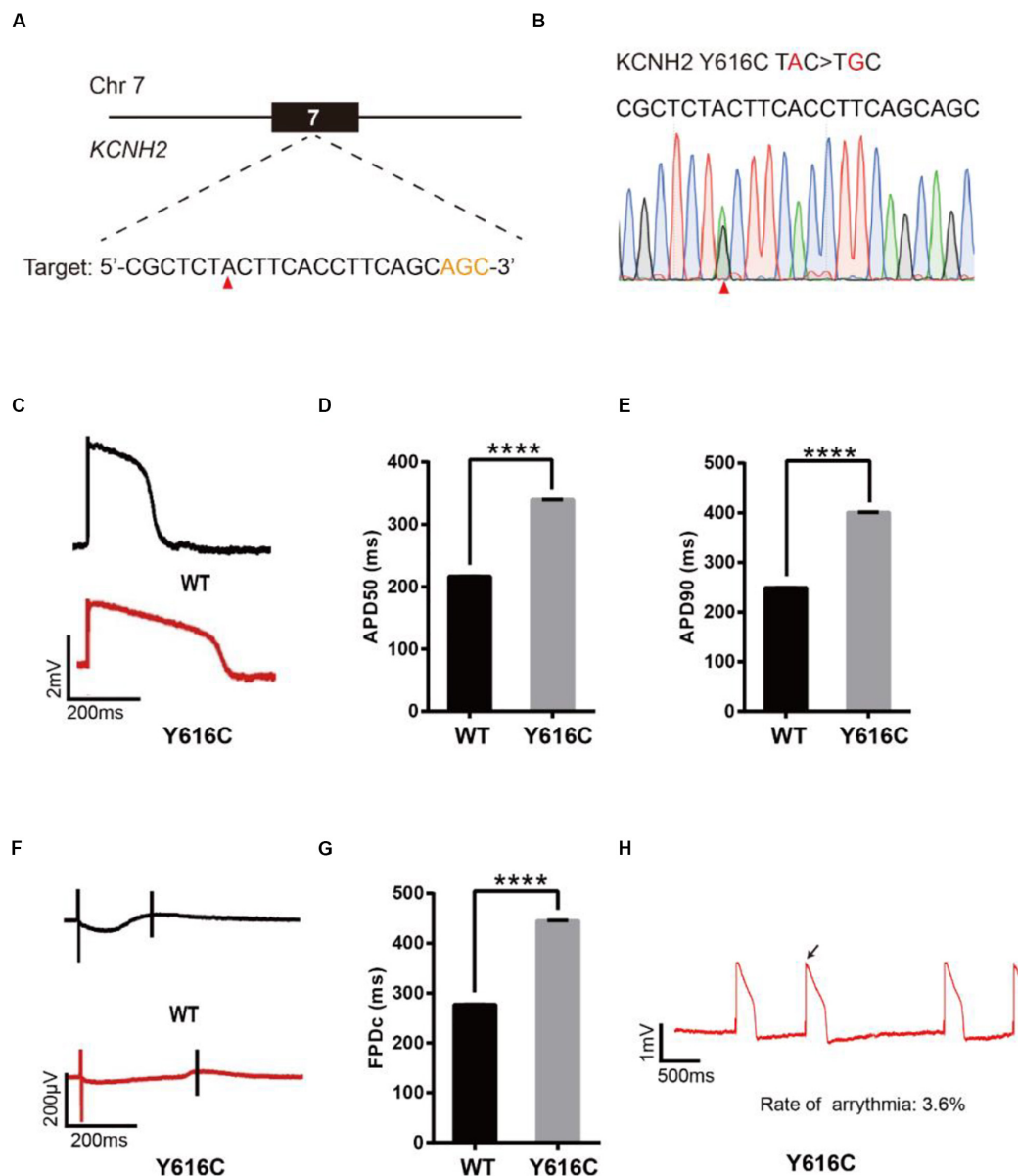


FIGURE 3 | Base editing of Y616C-KCNH2 for LQT2 modeling. **(A)** Y616C target site on KCNH2. “AGC” PAM sequence is shown in orange; target nucleotide is indicated by a red triangle. **(B)** A heterozygous clone is confirmed by Sanger sequencing. The mutated nucleotide is shown in red; the mutated nucleotide is indicated by a red triangle. **(C)** Single trace of action potentials in WT-CMs and Y616C-CMs. **(D,E)** Quantification of action potential at APD50 and APD90. $n = 3$ independent experiments, unpaired t -test, $P < 0.0001$. **(F)** Signals of field potential duration recorded by MEA. **(G)** Quantification of corrected field potential durations (FPDc). $n = 3$ independent experiments, unpaired t -test, $P < 0.0001$. **(H)** Representative traces of action potentials. The abnormal AP signals were labeled by a black arrow. A value of $P < 0.05$ was considered to be statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Base Editing of SCN5A for LQT3 Modeling

To model LQT3, we introduced a known pathogenic mutation E1784K (Makita et al., 2008) into the SCN5A gene of H9 cells (Figure 4A). The editing efficiency reached 21% on day 16 (Supplementary Figure 1E and Supplementary Table 2). We screened 107 single cell-derived clones, of which there were 32 heterozygous clones and 5 homozygous clones (Table 1). To test

whether CMs exhibited the LQT3 phenotype, we differentiated heterozygous E1784K-H9 cells into CMs, and electrophysiology was recorded by the LEAP assay (Figure 4B). APD50 and APD90 in E1784K-CMs were significantly longer than those in WT-CMs (Figures 4C–E). FPDc in E1784K-CMs was significantly longer than that in WT-CMs (Figures 4F,G). These data demonstrated that base-edited E1784K-CMs successfully recapitulated LQT3 phenotype.

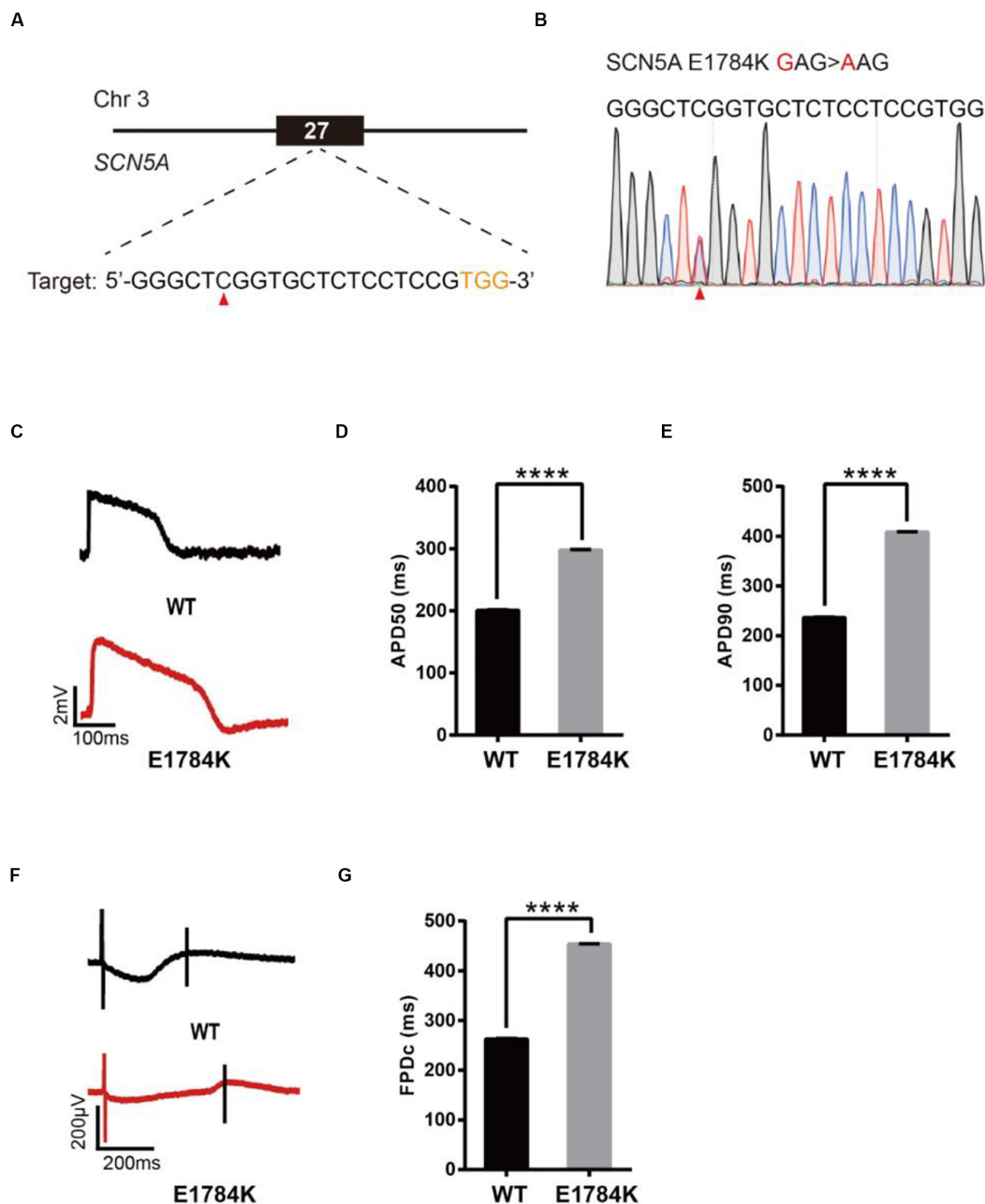


FIGURE 4 | Base editing of E1784K-SCN5A for LQT3 modeling. **(A)** E1784K target site on SCN5A. PAM sequence is shown in orange; target nucleotide is indicated by a red triangle. **(B)** A heterozygous clone is confirmed by Sanger sequencing. The mutated nucleotide is shown in red; the mutated nucleotide is indicated by a red triangle. **(C)** Single trace of action potentials in WT-CMs and E1784K-CMs. **(D,E)** Quantification of action potential at APD50 and APD90. $n = 3$ independent experiments, unpaired t -test, $P < 0.0001$. **(F)** Signals of field potential duration recorded by MEA. **(G)** Quantification of corrected field potential durations (FPDc). $n = 3$ independent experiments, unpaired t -test, $P < 0.0001$. A value of $P < 0.05$ was considered to be statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Evaluation of a Novel Mutation for Developing Brugada Syndrome

To demonstrate an additional application of epi-BEs, we use it to recapitulate and evaluate a novel mutation identified from a 62-year-old male patient who presented with sudden syncope. The patient's resting electrocardiogram (ECG) showed an ST elevation in leads V1–V3 with a saddle-back appearance

(Figure 5A), which is a characteristic pattern of BrS type 2 (Brugada et al., 2018). Genetic testing revealed a single missense mutation C5635T (R1879W) at the C-terminus of the SCN5A gene (Figure 5B).

To test whether R1879W is a pathogenic mutation, we used the epi-BEs to perform base editing and introduce it into H9 cells (Figure 5C). The editing efficiency reached 18% on day 16

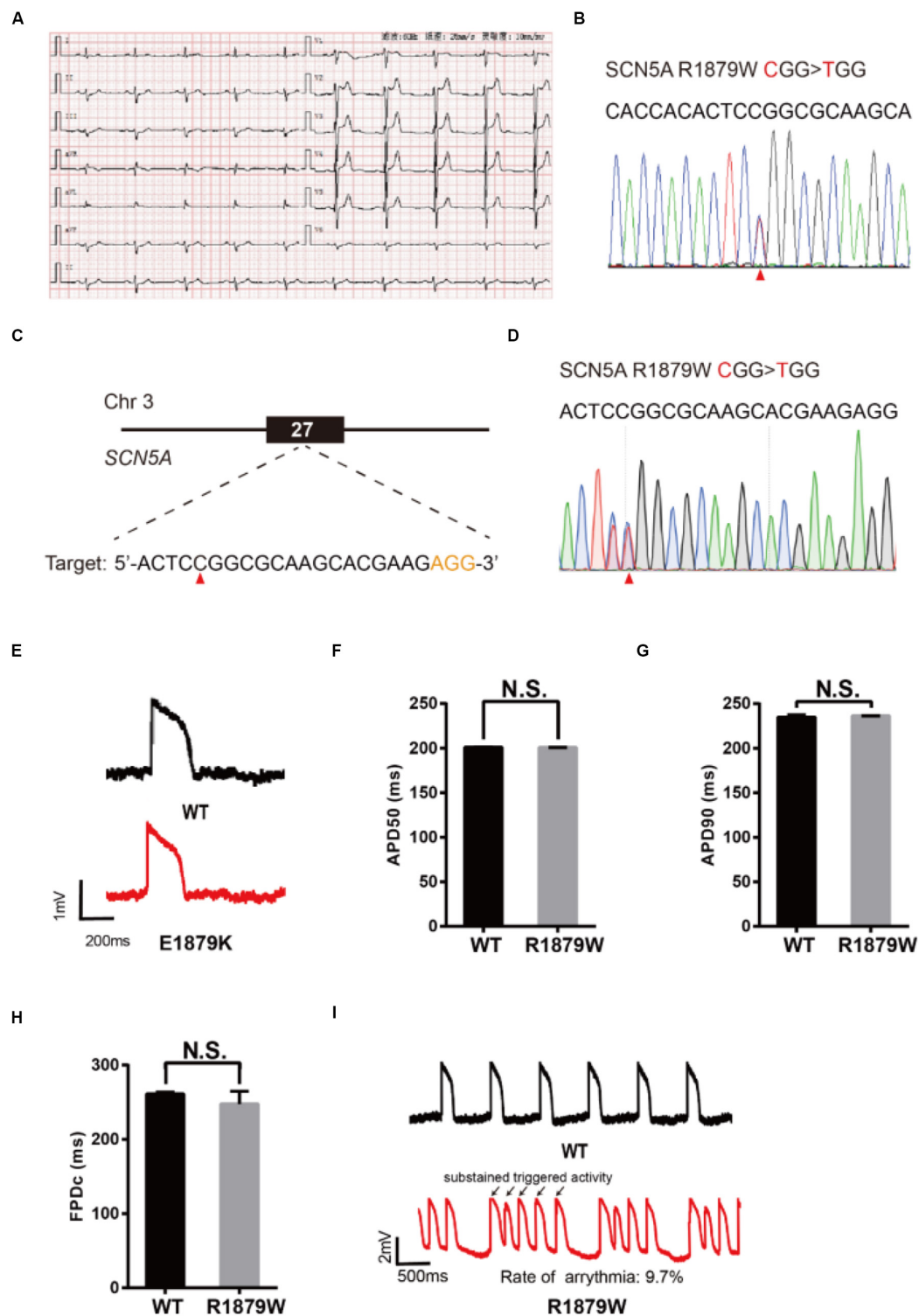


FIGURE 5 | Evaluation of R1879W-SCN5A mutation. **(A)** Patient's ECG results. The resting ECG showed an ST elevation in leads V1–V3 with a saddle-back pattern. **(B)** Sanger sequencing reveals that a C to T mutation occurs on SCN5A. **(C)** R1879W target site on SCN5A. PAM sequence is shown in orange; target nucleotide is indicated by a red triangle. **(D)** A heterozygous clone is confirmed by Sanger sequencing. The mutated nucleotide is shown in red; the mutated nucleotide is indicated by a red triangle. A neighbor nucleotide C is changed to T, but it does not change the amino acid. **(E)** Single trace of action potentials in WT-CMs and R1879W-CMs. **(F,G)** Quantification of action potential at APD50 and APD90. $n = 3$ independent experiments, unpaired t -test. **(H)** Quantification of corrected field potential duration (FPDc). $n = 3$ independent experiments, unpaired t -test. **(I)** Representative action potential traces with sustained triggered activity. Triggered beats were labeled by black arrows.

(Supplementary Figure 1F and Supplementary Table 2). We screened 47 single cell-derived clones, of which there were 2 heterozygous clones and 8 homozygous clones (Table 1). To test whether CMs exhibited BrS phenotype, we differentiated heterozygous R1879W-H9 cells into CMs, and electrophysiology was recorded by the LEAP assay (Figure 5D). APD50, APD90, and FPDc of R1879W-CMs showed no significant difference compared with WT-CMs (Figures 5E–H), consistent with previous studies (Miller et al., 2017; El-Battrawy et al., 2019). However, R1879W-CMs exhibited sustained triggered activity (Figure 5I), which is a typical characteristic of BrS patient-specific iPSC-derived CMs (Liang et al., 2016). Taken together, these results demonstrated that these epi-BEs could evaluate and recapitulate the functions of a novel mutation identified from a patient.

Off-Target Analysis

The epi-BEs enable long-term editing due to continued expression, which may increase undesired off-target effects. We analyzed a panel of potential off-target sites for each single cell-derived clone that was used for electrophysiological recording (Supplementary Table 3). These potential off-target sites were selected using an online tool that identifies potential off-target sites (see footnote 2) (Bae et al., 2014). These sites have at least three mismatches compared with the on-target sequences. The genomic DNA was extracted, and the potential off-target sites were PCR-amplified and subsequently sequenced by Sanger sequencing. Although cells were edited for 16 days, we did not detect any off-targets from these clones (Supplementary Figures 5–7). It is noteworthy to investigate the frequency of off-target cleavage by using whole genome sequencing in future studies.

DISCUSSION

hPSCs have proven to be a powerful tool for developing therapeutics and modeling disease (Lan et al., 2013; Wang et al., 2014). However, the generation of iPSCs from patients is a time-consuming and laborious process. Alternatively, mutations can be introduced into hPSCs by genome editing. Geng et al. (2020) developed a quick and efficient CRISPR/Cas9 genome editing procedure in iPSCs. We have previously demonstrated that the epiCRISPR system improves greatly the genome editing efficiency (Xie et al., 2017), but this technology can only introduce indels into cells for purposes of gene knockout. To overcome this limitation, we use the episomal vector to express base editors in this study, allowing the introduction of the pathogenic point mutations into hPSCs, which are more clinically relevant. This expanded technology further establishes the link between genome editing and hPSCs to enable future studies of diseases. Because the episomal vector can replicate in cells, there is an extended time possible for genome editing. As a result, higher editing efficiency has been achieved over time. Both heterozygous and homozygous mutations can be obtained and identified when analyzing single cells. The targeting range of ABEmax and AncBE4max is limited by the NGG PAM. To expand upon the

targeting range of these tools, we can generate other versions of the episomal vector, which expresses other versions of base editors, such as SpCas9-NG- and SauriCas9-derived base editors (Nishimasu et al., 2018; Hu et al., 2020).

Our engineered hPSC-derived CMs successfully recapitulate disease phenotypes at the cellular level. We generate a total of five LQTS models, including two LQT1 models, two LQT2 models and one LQT3 model. All of these disease models display significantly prolonged APDs and FPDc, consistent with those of previous studies (Liang et al., 2013; Wang et al., 2014; Yoshinaga et al., 2019). We finally evaluated a novel mutation identified from a BrS patient. Interestingly, this mutation does not influence APD and FPDc but induces sustained triggered beats, consistent with BrS phenotypes at cellular levels when studied previously (Liang et al., 2016; Miller et al., 2017; El-Battrawy et al., 2019). In summary, these data demonstrate the utility of epi-BEs and provide the biomedical research community with a useful tool for disease modeling and deciphering novel mutations.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Renji Hospital of Shanghai Jiao Tong University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.590581/full#supplementary-material>

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Characterization of *Brevibacillus laterosporus* Cas9 (BlatCas9) for Mammalian Genome Editing

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Compact CRISPR/Cas9 systems that can be delivered by AAV for *in vivo* genome editing hold great promise for clinical applications. *Brevibacillus laterosporus* Cas9 (BlatCas9) is a compact Cas9 nuclease that has been identified for plant genome editing. Here, we characterize BlatCas9 as an alternative tool for mammalian genome editing. We demonstrate that BlatCas9 prefers a N4CNAA protospacer adjacent motif (PAM), but N4C PAM is also editable in mammalian cells. We next demonstrate that BlatCas9 enables genome editing in a variety of cell types. Furthermore, BlatCas9 can be packaged into AAV for genome editing. Finally, we characterize the specificity of BlatCas9. In summary, BlatCas9 offers an alternative tool for both basic research and clinical applications.

Keywords: CRISPR/Cas9, genome editing, compact, aav, gene therapy

INTRODUCTION

The CRISPR/Cas9 system is a versatile tool for genome editing, and it has been rapidly and widely adopted by the scientific community (Cong et al., 2013; Hwang et al., 2013; Mali et al., 2013; Xie et al., 2017; Wang et al., 2019). This is a two-component system that contains a Cas9 nuclease and a guide RNA (gRNA) (Jinek et al., 2012). They form a Cas9–gRNA complex, recognizing a gRNA complementary DNA sequence and generating a site-specific double-strand break (DSB) (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013). The DSB is repaired by the cell's endogenous DNA repair machinery through either non-homologous end-joining (NHEJ) or homology-directed repair (HDR), resulting in site-specific mutations (Ran et al., 2013; Komor et al., 2017; Adli, 2018). By altering the 20-bp sequence at the 5' end of gRNA, one can easily modify a new target in the genome. However, target site recognition also requires a specific protospacer adjacent motif (PAM) (Jinek et al., 2012; Karvelis et al., 2015; Leenay et al., 2016), which limits the targeting scope of Cas9 for precise positioning. In the last few years, a number of CRISPR/Cas proteins have been repurposed for genome editing (Adli, 2018; Teng et al., 2018, 2019; Liu et al., 2019; Tian et al., 2020). These CRISPR/Cas systems recognize different PAMs, expanding the targeting scope.

The majority of Cas9 variants are large proteins, which adds particular limitation when they are required to be packaged into adeno-associated viruses (AAVs) for *in vivo* gene therapy. For example, the most extensively applied SpCas9 protein is 1,366 aa, which, together with its gRNA sequence, are too large to be packaged into AAV (Wu et al., 2010). To overcome the size limitation, five smaller Cas9 variants (<1,100 aa) have been discovered for mammalian genome editing,

including *Neisseria meningitidis* Cas9 (NmCas9, 1,082 aa) (Esvelt et al., 2013; Hou et al., 2013), *Staphylococcus aureus* Cas9 (SaCas9, 1,053 aa) (Ran et al., 2015), *Campylobacter jejuni* Cas9 (CjCas9, 984 aa) (Kim et al., 2017), *Neisseria meningitidis* Cas9 (Nme2Cas9, 1,082 aa) (Edraki et al., 2019), and *Staphylococcus auricularis* (SauriCas9, 1,061 aa) (Hu et al., 2020). However, only a portion of genome sites can be targeted by these smaller Cas9 variants due to the PAM limitation.

Exploration of Cas9 orthologs could offer a diversity of PAM sequences and novel biochemical properties that may be beneficial for genome editing applications. *Brevibacillus laterosporus* Cas9 (BlatCas9, 1,092 aa) is a compact Cas9 nuclease that has been identified for plant genome editing (Karvelis et al., 2015). Interestingly, BlatCas9 recognizes N4CNDD PAM, which is different from the existing Cas9. In this study, we characterized BlatCas9 for mammalian genome editing. We characterized PAM preference and gRNA length in mammalian cells. We demonstrated that BlatCas9 enabled genome editing in a variety of mammalian cell types.

RESULTS

BlatCas9 Enables Genome Editing in Mammalian Cells

To test whether BlatCas9 enables genome editing in mammalian cells, we employed a GFP-activation approach that allowed testing Cas9 activity in mammalian cells (Figure 1A) (Hu et al., 2020). We synthesized the gRNA scaffold and human-codon-optimized BlatCas9, flanked by nuclear localization signal sequences (NLS), and transfected them into the reporter cells (Supplementary Figures S1, S2). When we transfected BlatCas9 alone, no GFP-positive cells were observed; when we transfected BlatCas9 together with a 20-bp gRNA, GFP-positive cells were observed (Figure 1B). GFP-positive cells were sorted out, and sequences containing 7-bp randomized DNA were PCR-amplified for deep sequencing. Sequencing results revealed that insertions/deletions (indels) occurred (Figure 1C), demonstrating that BlatCas9 enables genome editing in mammalian cells.

Identification of BlatCas9 PAM Preference in Mammalian Cells

Protospacer adjacent motif sequences play a crucial role in target recognition. To characterize BlatCas9 PAM sequences in mammalian cells, we generated interactive visualization schemes (a WebLogo and a PAM wheel) based on deep-sequencing data (Leenay et al., 2016). Both of them revealed that BlatCas9 strongly preferred C at position 5, A at position 7, and accepted any nucleotide at positions 1–3 of PAM (Figures 2A,B). To test whether BlatCas9 requires longer PAM, we shifted the target sequence by three nucleotides in the 5' direction to allow PAM identification to be extended from 7 to 10 bp. The results revealed that BlatCas9 preferred N4CNAA PAM, where C was strongly preferred at position 5, and A was mildly preferred at positions 7 and 8 (Figures 2C,D). These results are very similar to an

in vitro PAM library screening results, which reveal that BlatCas9 prefers NNNNCNDD (N = G, C, A, or T; D = A, G, or T) PAM (Karvelis et al., 2015). We observed that A was preferred stronger in the first round of screening than that in the second round. In the first round of screening, a G was fixed at position 8, which may influence the nucleotide preference at position 7. BlatCas9 had no significant nucleotide preference at PAM longer than eight nucleotides.

To further test the PAM preference of BlatCas9, we constructed another PAM library with 8 bp of randomized DNA sequences (Supplementary Figure S3A). We fixed the first 3 bp of the PAM sequence as CTG and tested the PAM preference for other positions. The deep sequencing result revealed that BlatCas9 preferred N4CNAA PAM (Supplementary Figures S3B,C).

To test whether BlatCas9 can accept any nucleotide at positions 4 and 8 of PAM, we inserted a protospacer sequence with varied nucleotides at positions 4 and 8 of PAMs into GFP reporter plasmids and established stable cell lines (Figure 2E). Transfection of BlatCas9 with the corresponding gRNA induced GFP expression for all of them (Figure 2E), indicating that BlatCas9 can accept any nucleotide at these two positions. To test whether BlatCas9 can accept C at position 7 of PAM, we generated three PAMs with C at position 7. Transfection of BlatCas9 with the corresponding gRNA induced GFP expression for all of them (Supplementary Figure S4), indicating that BlatCas9 can accept C at position 7. In conclusion, N4C PAM is also editable by BlatCas9.

BlatCas9 Enables Editing Endogenous Genomic Sites

We next tested the genome-editing capability of BlatCas9 with a panel of seven endogenous gene targets in three cell lines, HEK293T, HCT116, and A375. The results showed that BlatCas9 could generate indels at all seven endogenous loci in HEK293T cells (Figure 3A) and varied indel efficiencies in HCT116 and A375 cells (Figures 3B,C). Importantly, BlatCas9 could be packaged into AAV for efficient genome editing in HEK293T cells (Figure 3D). In addition, we compared the efficiency of BlatCas9 with SpCas9 at three loci. BlatCas9 showed lower efficiency at ANAPC15_TS2, higher efficiency at ANAPC15_TS3, and similar efficiency at ANAPC15_TS4 compared to SpCas9 (Supplementary Figure S5). Taken together, BlatCas9 offers a novel platform for genome editing.

BlatCas9 Promotes Homologous Recombination

To test whether BlatCas9 can promote homologous recombination, we designed four gRNAs targeting the AAVS1 locus. We employed a donor plasmid containing a GFP reporter and a promoterless puromycin cassette, which expresses the puromycin resistance element only if inserted downstream of the PPP1R12C promoter (AAVS1 locus) (Figure 4A) (Wang et al., 2012; Chen et al., 2016). The donor plasmid with individual gRNA was transfected into cells followed by drug selection. Thirty days after transfection, stable GFP-expressing cell lines

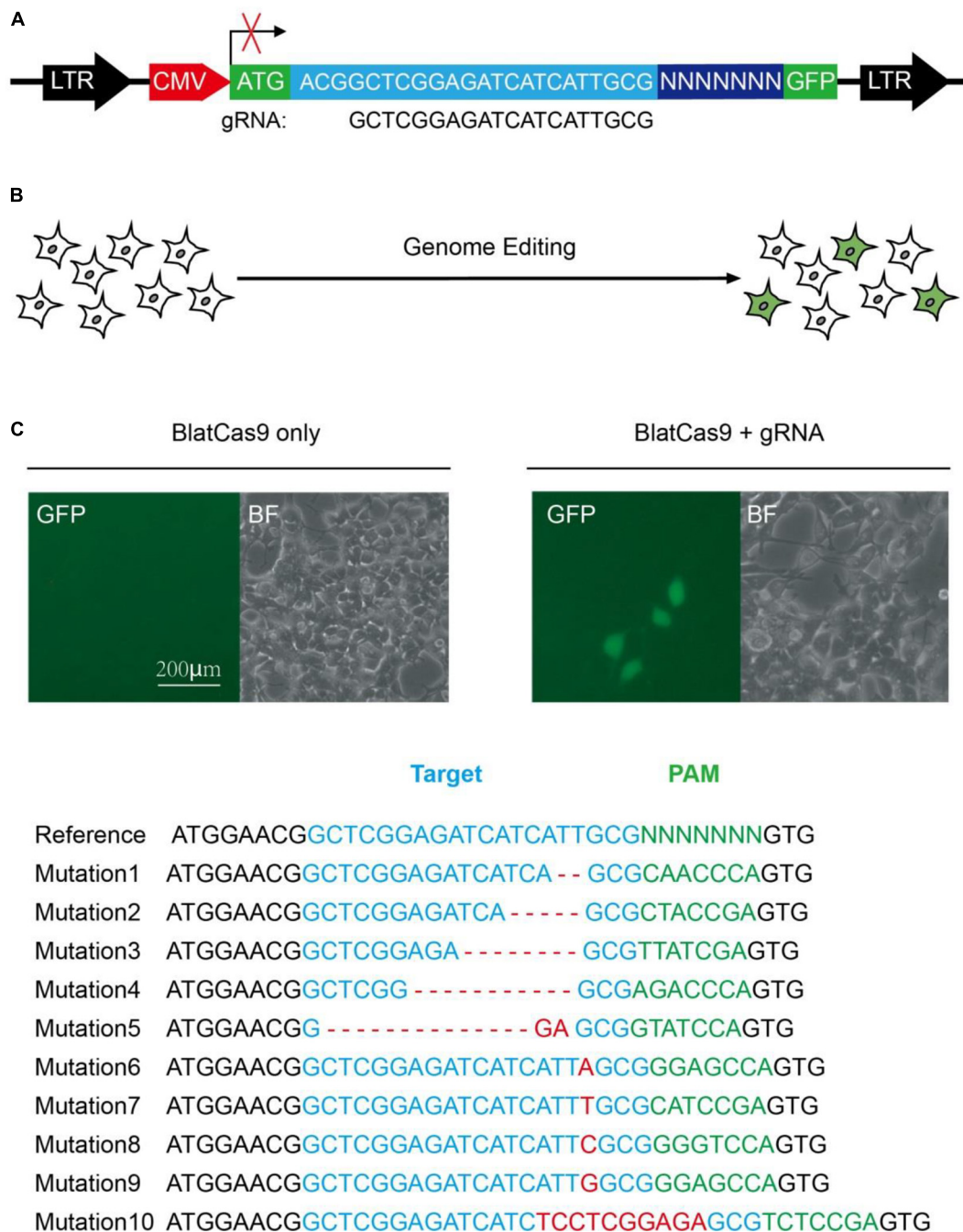


FIGURE 1 | A GFP reporter assay for protospacer adjacent motif (PAM) screening. **(A)** Schematic diagram of the GFP reporter assay. A lentiviral vector containing a CMV-driven GFP is disrupted by the insertion of a target sequence followed by a 7-bp random sequence between ATG and GFP coding sequence. The library DNA is stably integrated into the HEK293T cells. Genome editing generates in-frame mutations for a portion of cells, leading to GFP expression. **(B)** Transfection of *Brevibacillus laterosporus* Cas9 (BlatCas9) with guide RNA (gRNA) results in GFP expression, while transfection of BlatCas9 alone does not induce GFP expression. **(C)** Deep sequencing shows that the target sequences with various PAM sequences can be edited. The target sequence is shown in blue; indel mutations are shown in red; 7-bp random sequences are shown in green.

were established (**Figure 4B**). Very few GFP-positive cells could be observed for cells transfected with donor plasmid alone due to the random integration. To confirm that targeted integration occurred, genomic DNA was extracted for polymerase chain

reaction (PCR) detection. One primer targeted the GFP gene, and the other primer targeted the genomic DNA. If targeted integrations occur, a 959-bp band will be present. The results revealed that gRNA #4 (g4) induced efficient targeted integration

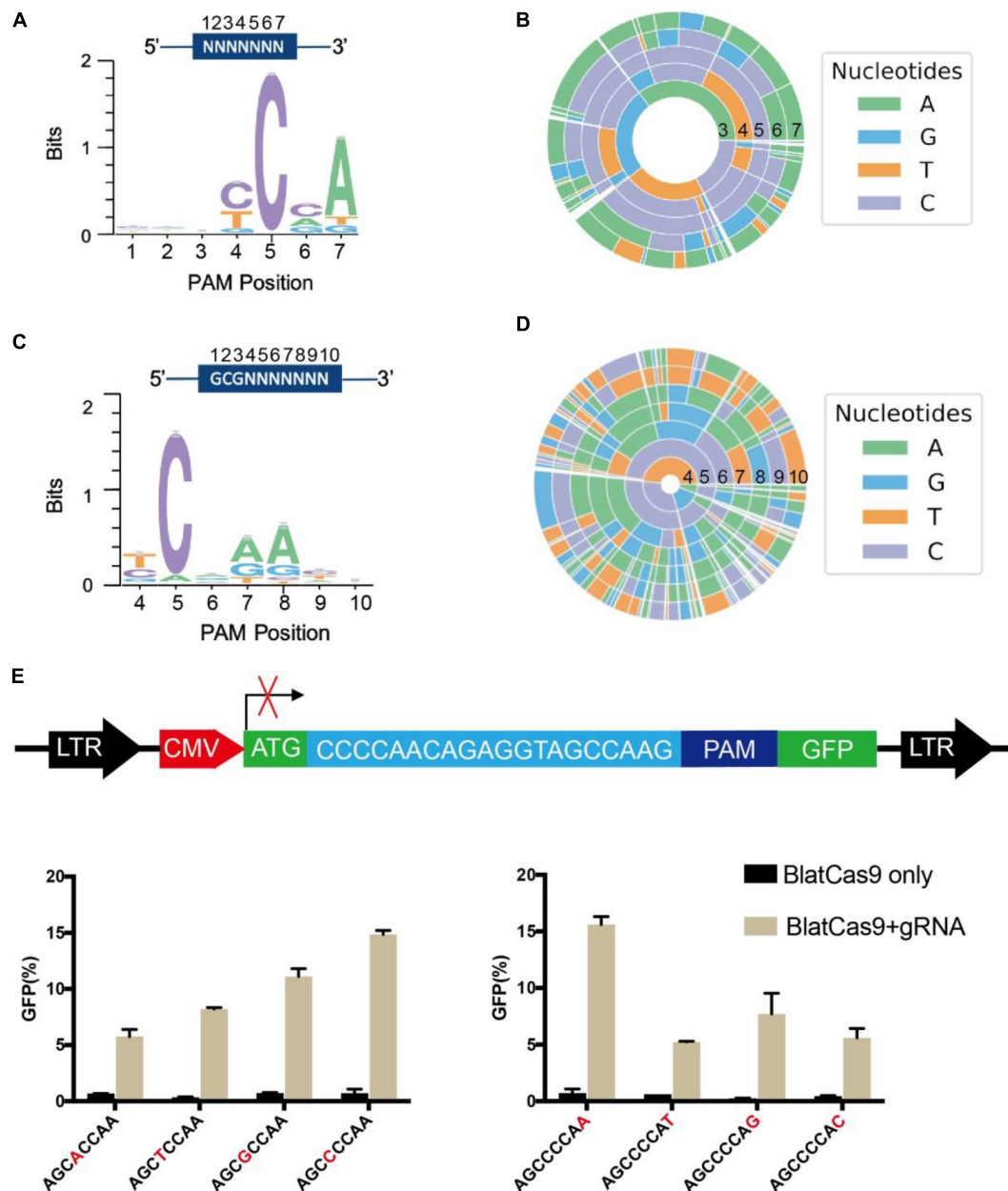


FIGURE 2 | PAM sequence analysis for BlatCas9. **(A,B)** WebLogo and PAM wheel are generated from the first round of PAM screening. **(C,D)** WebLogo and PAM wheel are generated from the second round of PAM screening. **(E)** Schematic diagram of the GFP reporter assay for PAM tests. BlatCas9 can accept any nucleotide at positions 4 and 8 of PAM. PAM sequences are shown below the figures; nucleotides at positions 4 and 8 are shown in red; data are shown as mean $\pm$ SD. $n = 3$.

(Figure 4C). For cells edited by g4, we further tested targeted integration for single cell-derived clones. Of 20 clones, 19 clones contained targeted integration (Figure 4D).

Optimization of BlatCas9 Guide Length

We next optimized the guide length of BlatCas9 for genome editing. We used a GFP-activation system to measure editing efficiency. A target sequence (site 1) was inserted into the GFP reporter to induce frameshift mutation and established a stable cell line (Figure 5A). When editing occurred, in-frame

mutation can occur, leading to GFP expression. We cotransfected BlatCas9 together with a series of gRNAs with variable guide lengths (17–24 bp) into cells and measured GFP-positive cells by fluorescence-activated cell sorting (FACS). The first nucleotide in the gRNAs was fixed to extra guanine (G) so that gRNAs can be transcribed by the U6 promoter. The results revealed that BlatCas9 was active for all gRNAs, but the 23-bp guide was the most active (28.3% GFP-positive cells) (Figure 5B). We tested three additional targets. However, the optimal guide length depends on the target sequences. For site 2, all gRNAs displayed

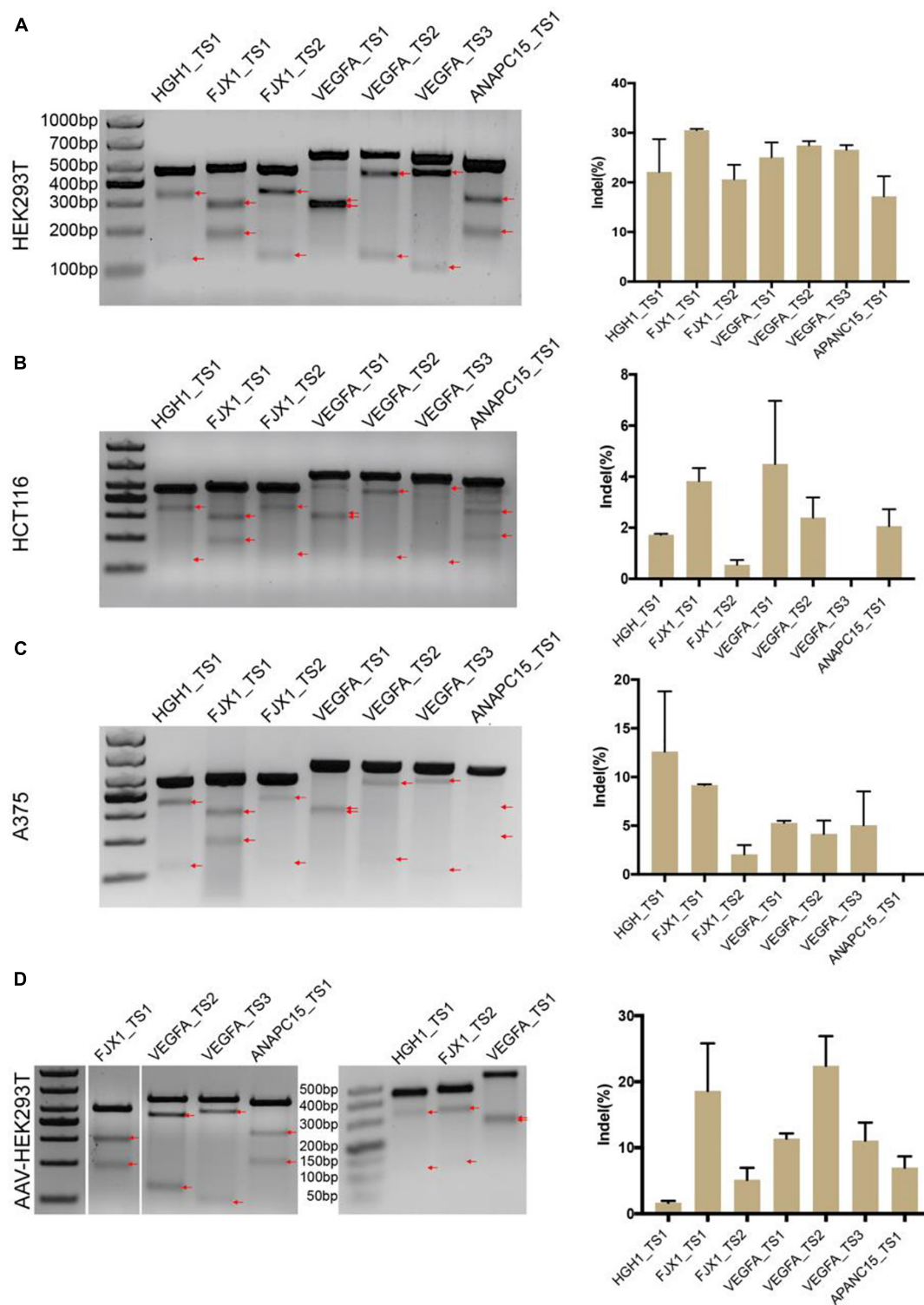


FIGURE 3 | Genome-editing capability of BlatCas9. BlatCas9 enables genome editing for a list of seven endogenous target sites in **(A)** HEK293T, **(B)** HCT116, and **(C)** A375 cells, respectively. **(D)** BlatCas9 enables genome editing for a list of seven endogenous target sites by adeno-associated virus (AAV) delivery in HEK293T cells. Gel pictures of T7E1 digestion are shown on the left; the bands generated by T7E1 digestion are indicated by red arrows. Indel frequencies measured by targeted deep sequencing are shown on the right. Data are shown as mean $\pm$ SD. $n = 3$.

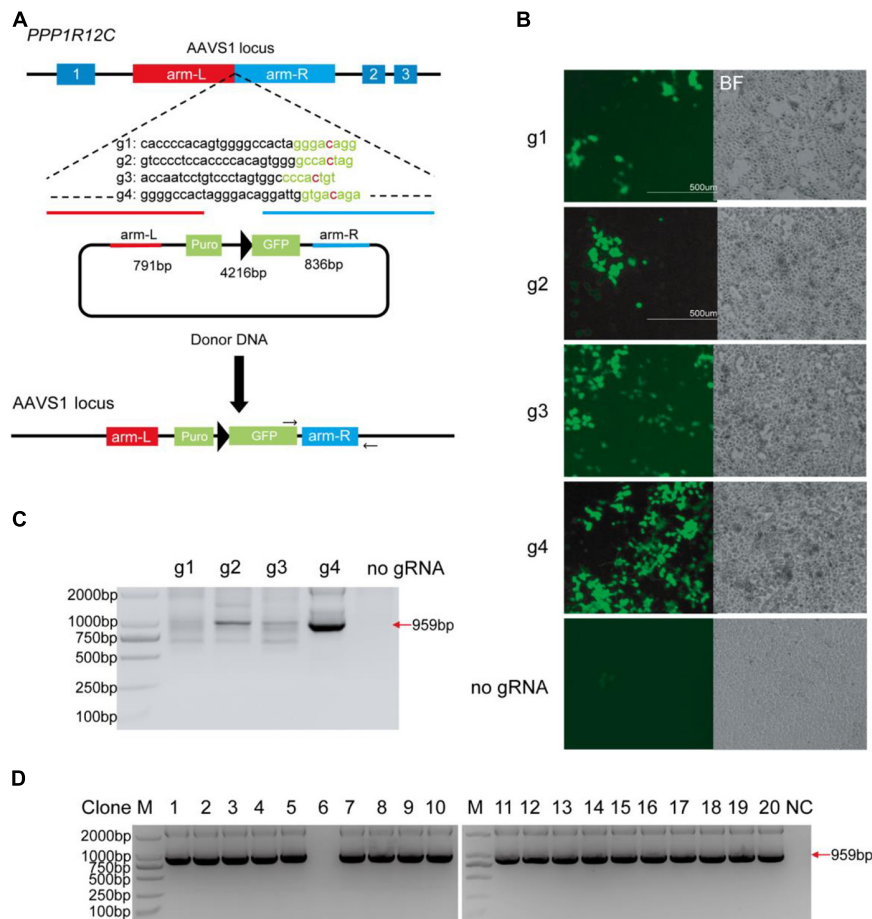


FIGURE 4 | BlatCas9 promotes homologous recombination. **(A)** A schematic diagram of homologous recombination using BlatCas9. **(B)** Thirty days after transfection, GFP-positive cells can be observed. Cells transfected with donor plasmid alone are used as control. Notably, control cells are not selected with drugs, and only a few GFP-positive cells can be observed. **(C)** Targeted integration in mixed cells is confirmed by polymerase chain reaction (PCR). **(D)** Targeted integration in the single cell-derived clones is confirmed by PCR. M, DNA marker; NC, negative control where genomic DNA was unedited.

similar efficiency. For site 3, the optimal guide length was 21 bp. For site 4, the optimal guide length was 18–21 bp (**Figures 5C–E**).

Specificity Analysis of BlatCas9

We next evaluated the off-target activity of BlatCas9 by using the GFP-activation cell line (**Figure 6A**). We initially generated a panel of 20-bp guides with single nucleotide mutation. BlatCas9 could tolerate single nucleotide mismatch at positions 1–18 but not position 20, counting the PAM as positions 21–28 (**Supplementary Figure S6**). We next generated a panel of 23-bp guides with dinucleotide mutations (**Figure 6A**). BlatCas9 showed reduced activity with mismatches at positions 1–11 and showed minimal or no activity with mismatches at positions 13–22, counting the PAM as positions 24–31.

To compare the genome-wide off-target effects of BlatCas9 to that of SpCas9, the genome-wide, unbiased identification of DSBs enabled by sequencing (GUIDE-seq) was performed (Tsai et al., 2015). We selected a target site containing a PAM that can be recognized by both SpCas9 and BlatCas9 on *ANAPC15*. Following transfections of Cas9 + gRNA plasmids and

GUIDE-seq oligos, we prepared libraries for deep sequencing. Sequencing and analysis revealed that on-target cleavage occurred for both Cas9 orthologs, reflected by GUIDE-seq read counts (**Figure 6B**). We identified 12 off-target sites for SpCas9. In contrast, we only identified one off-target site for BlatCas9. BlatCas9 requires longer PAM, which may contribute to less off-target effects in mammalian cells.

DISCUSSION

Small Cas9 nucleases (<1,100 aa) can be delivered by AAV for *in vivo* genome editing and hold great promise for gene therapy. Although five small CRISPR/Cas9 systems have been employed for mammalian genome editing, the targeting scope remains limited due to the requirement for a PAM sequence (Jinek et al., 2012; Karvelis et al., 2015; Leenay et al., 2016). These five small CRISPR/Cas9 systems include SaCas9 (Ran et al., 2015), NmCas9 (Hou et al., 2013), CjCas9 (Kim et al., 2017), Nme2Cas9 (Edraki et al., 2018), and SauriCas9,

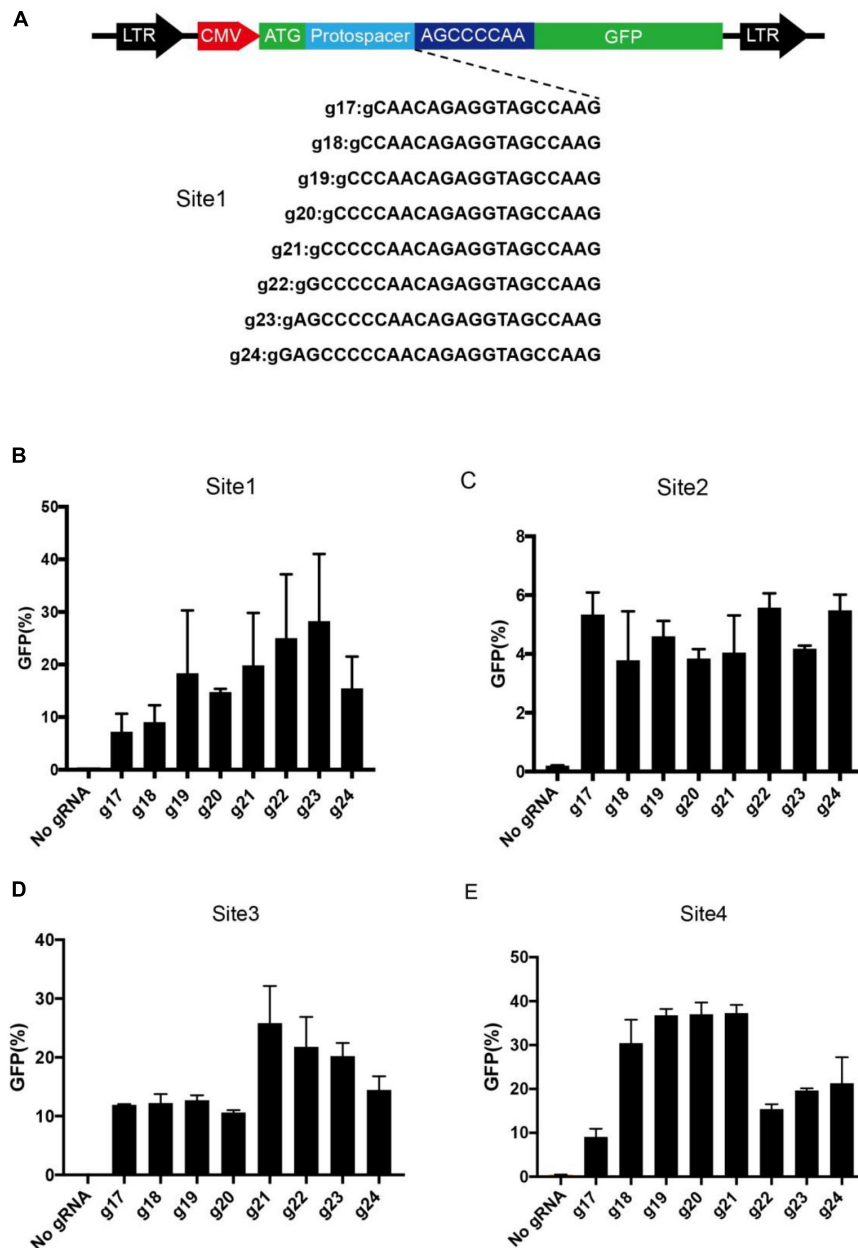


FIGURE 5 | Optimization of gRNA length. **(A)** A target sequence is inserted into the GFP reporter construct. A list of eight gRNAs varying from 17 to 24 bp is tested for genome editing. Additional G is added for transcription by a U6 promoter. **(B–E)** Quantification of GFP-positive cells for four different target sites. Data are shown as mean $\pm$ SD. $n = 3$.

which recognize target sequences associated with NNGRRT, N4GAYW/N4GYTT/N4GTCT, N4RYAC, N4CC, and NNGG PAMs, respectively. Exploration of Cas9 orthologs from different bacteria is another way to increase the targeting scope.

BlatCas9 is a compact Cas9 nuclease that has displayed activity *in vitro* and in plants (Karvelis et al., 2015). In this study, we demonstrate that BlatCas9 also enables genome editing in mammalian cells, extending the list of small CRISPR/Cas9 tools. Interestingly, our GFP reporter assay reveals that N4C PAM is

editable by BlatCas9, expanding the targeting scope. We observed that BlatCas9 tolerates dinucleotide mismatches at positions 1–11, indicating that the specificity remains to be improved. Several strategies, including rational design and directed evolution, have been used for Cas9 specificity improvement (Kleinstiver et al., 2016; Slaymaker et al., 2016; Chen et al., 2017; Casini et al., 2018; Vakulskas et al., 2018). These strategies can also be used to improve BlatCas9 specificity in future work. With further development, we anticipate that BlatCas9 can be an

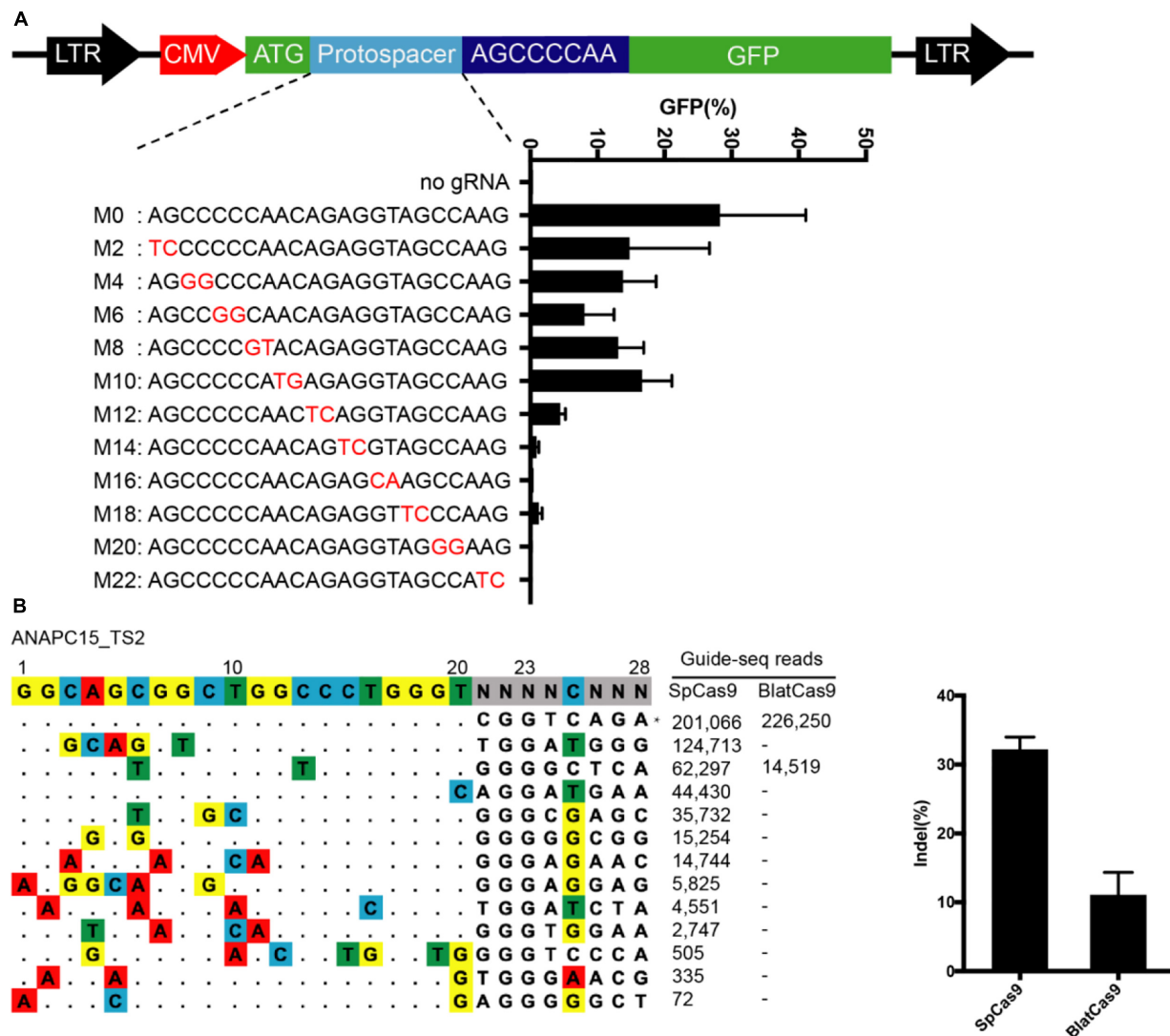


FIGURE 6 | Analysis of BlatCas9 specificity. **(A)** A target sequence is inserted between the ATG and GFP coding sequence, disrupting GFP expression. GFP expression can be induced by genome editing. A panel of gRNAs with dinucleotide mismatches (red), and each gRNA activity is shown below. $n = 2$. **(B)** GUIDE-seq is performed to compare the off-target effects of SpCas9 and BlatCas9. A target site (targeting *ANAPC15*) with a PAM compatible for both nucleases is selected. Read numbers for on and off targets are shown on the right. Mismatches compared to the on-target site are shown and highlighted in color. On-target site is indicated by “**”.

important genome editing tool for both basic research and clinical applications.

MATERIALS AND METHODS

Cell Culture and Transfection

A375 and HEK293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS (Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C and 5% CO₂. HCT116 cells were maintained in McCoy's 5A supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO₂. For BlatCas9

PAM sequence screening, HEK293T cells were plated in 10-cm dishes and transfected at ~60% confluency with BlatCas9-gRNA-expressing plasmid (15 µg) using Lipofectamine 2000 (Life Technologies). For genome editing of BlatCas9, cells were seeded on 12-well plates and transfected with BlatCas9-gRNA plasmid (1 µg) using Lipofectamine 3000 in Opti-MEM according to the manufacturer's instructions. The transfected A375, HEK293T and HCT116 cells were selected using media supplemented with 10, 10, 10, and 7 µg/ml of blasticidin, respectively.

Plasmid Construction

BlatCas9-gRNA expression plasmid construction: the vector backbone of pX601 (Addgene #107055) was used to express

Cas9. First, the miniCMV promoter on pX601 was replaced by normal CMV promoter as follows: pX601 was digested with *Xba*I/*Age*I to remove miniCMV promoter; normal CMV promoter was PCR-amplified from pCMV-ABE_{max} plasmid (Addgene #125648) using primers CMV-F/CMV-R, and cloned into linearized pX601 by T4 DNA ligation (NEB), resulting in the pAAV-CMV-SaCas9 plasmid. Second, the AAV-CMV-SaCas9 plasmid was PCR amplified by using primers pX601-F/pX601-R to remove SaCas9; human codon-optimized BlatCas9 gene was synthesized by HuaGene (Shanghai, China); BlatCas9 was cloned into the AAV-CMV-SaCas9 backbone by NEBuilder Assembly Tool (NEB) following the manufacturer's instructions, resulting in AAV-CMV-BlatCas9. For the genome editing of BlatCas9, the fragment of the human codon-optimized BlatCas9 gene and the blasticidin gene was synthesized by HuaGene (Shanghai, China); the fragment was cloned into the AAV-CMV-SaCas9 backbone by the NEBuilder Assembly Tool (NEB) following the manufacturer's instructions, resulting in AAV-CMV-BlatCas9-BSD. Sequences were verified by Sanger sequencing (GENEWIZ, Suzhou, China). The human-codon-optimized BlatCas9 sequence is available in **Supplementary Figure S2**. All target sequences can be found in **Supplementary Table S1**; all primers can be found in **Supplementary Table S2**.

PAM Sequence Analysis

Twenty base-pair sequences flanking the target sequence were used to fix the target sequence. Three nucleotides in front of a random sequence and GTGAGCAAGGGCG AGGAGCT were used to fix the 7-bp random sequence. Target sequences with in-frame mutations were used for PAM analysis. The 7-bp random sequence was extracted and visualized by WebLog3 (Crooks et al., 2004) and PAM wheel chart to demonstrate PAMs (Leenay et al., 2016).

Verification of PAM Sequence With GFP Reporter Constructs

Three GFP reporter plasmids containing different targets CTGGTCAGGAATGATCTGGAGACCCAGA, CCCCAACAG AGGTAGCCAAGAGCCCCAA and GGTCGAAGTTGGCCG TCAGGTGGTCGAA were constructed. Each plasmid was packed into a lentivirus to generate stable cell lines. To remove background mutations that induce GFP expression, the GFP-negative cells were sorted by the MoFlo XDP machine. The sorted cells were seeded into 24 wells and transfected with AAV-CMV-BlatCas9-BSD plasmid (800 ng) by Lipofectamine 2000 (Life Technologies). Three days after editing and selecting (10 µg/ml of blasticidin), the GFP-positive cells were analyzed on the Calibur instrument (BD). Data were analyzed using FlowJo.

Genome Editing of BlatCas9 at Endogenous Sites in Different Cell Lines

A375, HEK293T, and HCT116 cells were seeded into 12 wells and transfected with AAV-CMV-BlatCas9-BSD (1 µg)

by Lipofectamine 3000. The transfected A375, HEK293T, and HCT116 cells were selected using media supplemented with 10, 10, and 7 µg/ml of blasticidin, respectively. Cells were collected 3 days after transfection and selection. The genomic DNA was isolated, and the target sites were PCR amplified and extracted by Gel Extraction kit (QIAGEN). The PCR products were subjected to deep sequencing to check the editing efficiency.

Test of BlatCas9 Specificity

To test the specificity of BlatCas9, we generated a GFP reporter cell line with AGCCCCAA PAM. The HEK293T cells were seeded into 12 wells and transfected with AAV-CMV-BlatCas9-BSD (1 µg) by Lipofectamine 2000 (Life Technologies). Three days after editing and selecting (10 µg/ml of blasticidin), the GFP-positive cells were analyzed on a Calibur instrument (BD). Data were analyzed using FlowJo.

Adeno-Associated Virus Production

For the seven individual endogenous target site BlatCas9-gRNA packaging, HEK293T cells were seeded at ~40% confluency in a 6-cm dish the day before transfection. For each well, 2 µg of expressing plasmid, 2 µg of pAAV-RC (GenBank: AF369963), and 4 µg of pAAV-helper were transfected using 80 µl of PEI (0.1% m/v, Polysciences, Cat# 23966, pH 4.5). The media was changed 8 h after transfection. After 72 h, cells were scraped and poured into a 15-ml conical centrifuge tube. They were spun at 3,000 rpm, at 4°C for 10 min, and the supernatant was transferred into a new 15-ml tube. The cell pellets were resuspended in 1 ml of RB TMS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0), then transferred to a new 15-ml conical tube. They were frozen in a dry ice-ethanol bath for 10 min and thawed at 37°C for 10 min and repeated three times. The cells were spun at 3,000 rpm, at 4°C for 10 min. The two supernatants were mixed and filtered with a 0.45-µm polyvinylidene fluoride filter. One half of the volume of the mixed solution (1M NaCl + 10% PEG8000) was added and incubated at 4°C overnight. After centrifugation at 4°C for 2 h at 12,000 rpm, the flow-through was discarded, and 200 µl of chilled RB TMS was added. The quantitative PCR reveals that AAV titration is 6.3×10^8 copies/µl. Sixty microliters of the virus was added into a 12-well plate with ~80% confluency of HEK293T.

Guide-Seq

GUIDE-seq experiments were performed as described previously (Tsai et al., 2015), with minor modifications. Briefly, 2×10^5 of HEK293T cells were transfected with 1 µg of AAV-CMV-BlatCas9-BSD plasmid and 100 pmol of annealed GUIDE-seq oligonucleotides by electroporation, and then the cells were seeded into a 12-well plate. Electroporation parameters (voltage, width, number of pulses) were 1,150 V, 30 ms, and 1 pulse. Genomic DNA was extracted with a DNeasy Blood and Tissue kit (QIAGEN) 5 days after transfection according to the manufacturer's protocol. Library preparation

and sequencing were performed exactly as described previously (Tsai et al., 2015).

Quantification and Statistical Analysis

All the data are shown as mean $\pm$ SD. Statistical analyses were conducted using Microsoft Excel.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

NG, ZH, ML, and JW performed the experiments. CZ analyzed the data. YW designed the experiments and wrote the manuscript. YW revised the manuscript. YW and HL supervised

the project. HL applied for grants. All authors read and approved the final manuscript.

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The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.583164/full#supplementary-material>

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Disease Phenotypes and Mechanisms of iPSC-Derived Cardiomyocytes From Brugada Syndrome Patients With a Loss-of-Function SCN5A Mutation

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Brugada syndrome (BrS) is one of the major causes of sudden cardiac death in young people, while the underlying mechanisms are not completely understood. Here, we investigated the pathophysiological phenotypes and mechanisms using induced pluripotent stem cell (iPSC)-derived cardiomyocytes (CMs) from two BrS patients (BrS-CMs) carrying a heterozygous SCN5A mutation p.S1812X. Compared to CMs derived from healthy controls (Ctrl-CMs), BrS-CMs displayed a 50% reduction of I_{Na} density, a 69.5% reduction of $Na_v1.5$ expression, and the impaired localization of $Na_v1.5$ and connexin 43 (Cx43) at the cell surface. BrS-CMs exhibited reduced action potential (AP) upstroke velocity and conduction slowing. The I_{to} in BrS-CMs was significantly augmented, and the I_{CaL} window current probability was increased. Our data indicate that the electrophysiological mechanisms underlying arrhythmia in BrS-CMs may involve both depolarization and repolarization disorders. Cilostazol and milrinone showed dramatic inhibitions of I_{to} in BrS-CMs and alleviated the arrhythmic activity, suggesting their therapeutic potential for BrS patients.

Keywords: Brugada syndrome, disease modeling, induced pluripotent stem cells, SCN5A mutation, depolarization, repolarization

INTRODUCTION

Brugada syndrome (BrS), a genetic heart disease, is one of the major causes of sudden cardiac death in young people. The diagnosis of BrS is based on the changes in the electrocardiogram (ECG) with a transient or persistent ST-segment elevation in the right precordial leads and the right bundle branch block (Antzelevitch and Fish, 2006). In the past two decades, extensive research on BrS

has revealed parts of its genetic basis. To date, 293 different mutations in *SCN5A* encoding the α -subunit of the cardiac sodium channel ($Na_v1.5$) have been implicated as possible causes of BrS (Kapplinger et al., 2010). The implantation of an automatic implantable cardiac defibrillator (ICD) or the ablation of the arrhythmogenic substrate in the right ventricular outflow tract area have been proven effective in treating ventricular tachycardia and fibrillation and preventing sudden cardiac death in BrS patients. In addition, drug therapy with low-dose quinidine has been shown to prevent arrhythmic events in BrS patients after arrhythmic storm and should be considered according to current guidelines (class IIa indication). However, prophylactic use in asymptomatic patients is controversially discussed and worldwide commercial availability is limited. Thus, a detailed understanding of arrhythmic mechanisms in BrS is required to develop novel drug therapies that are urgently needed.

For more than a decade, the underlying pathophysiological mechanism of BrS has been a matter of debate between the depolarization versus repolarization hypotheses (Wilde et al., 2010). The depolarization theory relies on right ventricular conduction slowing and involvement of structural abnormalities including increased epicardial and interstitial fibrosis, and the reduced gap junction expression (Nademanee et al., 2015). The repolarization theory is mainly supported by the late fractionated low-voltage potentials found in right ventricle epicardium but not in endocardium by using canine heart wedge model, which are associated with repolarization defects (Szel and Antzelevitch, 2014; Patocskai et al., 2017). Nonetheless, there is a general consensus that *SCN5A* mutations resulting in $Na_v1.5$ loss-of-function either by the decreased expression of functional $Na_v1.5$ at the sarcolemma and/or by the altered channel gating properties lead to a diminution of I_{Na} and contribute to the development of arrhythmias in BrS (Patocskai et al., 2017). Recently, single cardiomyocytes (CMs) derived from patient-specific and genome-edited induced pluripotent stem cells (iPSCs) were used to recapitulate the phenotypes of patients with BrS and to study the underlying mechanism (Liang et al., 2016; Veerman et al., 2016; Ma et al., 2018). iPSC-CMs from BrS patients who tested negative for mutations in the known BrS-associated genes do not exhibit clear cellular electrophysiological abnormalities (Veerman et al., 2016). Patient-specific iPSC-CMs with a double missense mutation (p.R620H and p.R811H) or with a deletion (Δ 1397) in *SCN5A* could recapitulate single-cell phenotype features of BrS, including the blunted I_{Na} , an increased triggered activity, and an abnormal Ca^{2+} handling (Liang et al., 2016).

Interestingly, a more recent study reported a remarked lower I_{Na} density and an increased phase-1 repolarization at a slow pacing frequency (0.1 Hz) in BrS patient-specific iPSC-CMs with a compound *SCN5A* mutation (p.A226V and p.R1629X), leading to pro-arrhythmic action potential (AP) morphology (Ma et al., 2018). The dominant repolarizing currents in human CMs are the transient-outward potassium current (I_{to}) conducted by $K_v4.2$ and $K_v4.3$ channels which contribute to $I_{to,fast}$, and by $K_v1.4$ which accounts for $I_{to,slow}$ and the rapidly and slowly activating delayed rectifier potassium currents (I_{Kr} and I_{Ks}) conducted by HERG and $K_vLQT1/mink$ channels, respectively (Nerbonne and

Kass, 2005). Previous study identified gain-of-function mutations in *KCNB3* encoding $K_v4.3$ in patients with BrS as a result of the increased I_{to} (Giudicessi et al., 2011). These studies suggest that the underlying mechanism for BrS might be different in BrS patients with different mutations and both depolarization and repolarization mechanisms might coexist.

In the present study, we utilized iPSC-CMs from two BrS patients (BrS1-CMs and BrS2-CMs) with a heterozygous *SCN5A* point mutation p.S1812X causing a premature termination codon. We show that BrS-CMs from both patients reveal a reduced I_{Na} and a delayed sodium channel activation, which result in a slowed AP upstroke velocity and a reduced field potential (FP) conduction velocity (CV), similar to the phenotype observed in BrS patients with *SCN5A* loss-of-function mutations. Importantly, BrS-CMs also exhibited the enhanced I_{to} and an augmented I_{CaL} (L-type calcium current) window current, which may contribute to the arrhythmic phenotype. Exposure of BrS-CMs to cilostazol and milrinone, two clinically used phosphodiesterase (PDE) blockers, lowered I_{to} and alleviated arrhythmic activity, without affecting on the CV. These data indicate that the electrophysiological mechanisms underlying arrhythmia in BrS-CMs may involve an impaired coordination of I_{Na} , I_{to} , and I_{CaL} and cilostazol and milrinone can reduce the arrhythmia but have no effect on the CV.

MATERIALS AND METHODS

Generation of iPSCs

The study was approved by the Institutional Ethics Committee of University Medical Center Göttingen (approval number 21/1/11) and of Technical University of Dresden (approval number EK 422092019) and carried out in accordance with the approved guidelines. Bone marrow aspirate (10 mL) and peripheral blood (15 mL) were taken from the BrS1 and BrS2 patients, respectively, after obtaining written informed consent. For the Ctrl1, bone marrow aspirate of a 45-year-old female (without known cardiac disease) left over from diagnostic purposes was used. For the Ctrl2, skin biopsy was taken. Bone marrow-derived mesenchymal stem cells, peripheral blood mononuclear cells, and skin fibroblasts were cultured and reprogrammed into iPSCs using the STEMCCA lentivirus (BrS1-iPSCs, Ctrl1-iPSCs, Ctrl2-iPSCs) or Sendai virus system (BrS2-iPSCs), as described previously (Streckfuss-Bomeke et al., 2013; Cyganek et al., 2018). The generated iPSCs from the BrS patients and the healthy control were adapted to feeder-free culture conditions and cultivated on tissue culture plates pre-coated with Geltrex® (Thermo Fisher Scientific) in Essential 8™ medium (Thermo Fisher Scientific).

Karyotype Analysis

Karyotyping of BrS- and Ctrl-iPSCs was performed using standard methodology. Prior to karyotyping, the iPSCs were cultured on feeder-free conditions. The cells were treated with 100 ng/mL colcemid (Thermo Fisher Scientific) for 16 h. The metaphases were prepared according to standard methodology and subjected to Giemsa staining before analysis with a light

microscope (Zeiss Axio Imager.M2). The karyotype was analyzed and documented by using the software Case Data Manager 6.0 (Applied Spectral Imaging).

Genomic Sequencing

The genomic DNA of Ctrl- and BrS-iPSCs was isolated and purified using the automated Maxwell® 16 cell DNA purification kit (Promega) according to the manufacturer's instructions. DNA sequence comprising the *SCN5A* point mutation site in the generated Ctrl- and BrS-iPSCs was amplified by PCR. The primer set used is given in **Supplementary Table 3**. The PCR products were purified using the QIAquick® gel extraction kit (Qiagen) according to the manufacturer's instructions and sequenced by a commercial sequencing facility (Seqlab, Göttingen).

Spontaneous *in vitro* Differentiation

For spontaneous *in vitro* differentiation experiments, the cells were digested with 200 U/mL collagenase type 4 (Worthington Biochemicals) into big clusters and cultivated in suspension to form multicellular aggregates, known as embryoid bodies (EBs) for 8 days. The differentiation medium contained IMDM (Thermo Fisher Scientific), 20% fetal bovine serum, 1x non-essential amino acid (Thermo Fisher Scientific), and monothioglycerol (450 μ M, Sigma–Aldrich). EBs were thereafter plated onto 0.1% gelatin-coated tissue culture dishes, cultivated up to 1 month and used for further analyses.

Reverse Transcriptase-PCR (RT-PCR) and Quantitative Real-Time PCR

BrS1- and BrS2-iPSCs and Ctrl1-iPSCs were characterized for the expression of pluripotency genes *NANOG*, *LIN28*, *GDF3*, and *FOXD3* using reverse transcriptase-PCR (RT-PCR). Expression of tissue-specific genes *ALB* (albumin), α -*MHC* (myosin heavy chain), *TNNT2* [cardiac troponin T (cTnT)], and *TH* (thyroxine hydroxylase) was analyzed during EB differentiation. To assess the expression of *KCND2/3* and *KCNA4* genes, 3-month-old CMs derived from Ctrl- and BrS-iPSCs were collected as cell pellets for further use.

Total mRNA was isolated as described in the SV Total RNA Isolation System with on-column DNase digestion (Promega). DNase-treated RNA (200 ng) was used for the first-strand cDNA synthesis by using Murine Leukemia Virus Reverse Transcriptase and Oligo d(T)₁₆ (Thermo Fisher Scientific). One-tenth of the cDNA reaction was taken as PCR template and amplified for 25–35 cycles, denaturation at 95°C for 15 s, annealing at 52–64°C for 15–30 s depending on the primer melting temperatures, and elongation at 72°C for 30 s. The primer sequences (forward and reverse), annealing temperatures, and the cycles used for RT-PCR analyses are listed in **Supplementary Table 3**.

Quantitative real-time PCR were carried out using SsoAdvanced Universal SYBR Green PCR super mix, Hard-Shell Optical 96-well plates, and the BIO-RAD CFX96 Real-time PCR system (Bio-Rad) according to the manufacturer's instructions. Initial denaturation was performed at 95°C for 30 s, followed by 45 amplification cycles of 15 s at 95°C and 1 min elongation at 60°C. A melting curve was obtained for 65–95°C. PCR products

were confirmed by the melting temperature and agarose gel electrophoresis. Expression was quantified using $\Delta\Delta C_t$ method with *RPL32* as a reference. Sequences for *RPL32*, *TNNT2*, *KCND2/3*, and *KCNA4* real-time PCR analyses are listed in **Supplementary Table 3**.

Teratoma Formation Analysis

To investigate their differentiation potential, the generated iPSCs were differentiated into derivatives of the three germ layers *in vivo* as previously described (Streckfuss-Bomeke et al., 2013). Briefly, the undifferentiated iPSCs were subcutaneously injected into 8-week-old recombination activating gene 2 and gamma C deficient (RAGC) mice. The mice were sacrificed 8–12 weeks after injection and the resulting teratomas were examined with hematoxylin and eosin staining.

Directed Differentiation of iPSCs Into CMs

All iPSCs were cultured on Geltrex® in Essential 8™ medium as a monolayer with 80–100% confluence before the initiation of differentiation. At day 0, cells were cultured in RPMI1640 medium (Thermo Fisher Scientific) with Glutamax and HEPES (Thermo Fisher Scientific), 0.5 mg/mL human recombinant albumin, and 0.2 mg/mL L-ascorbic acid 2-phosphate and treated with 4 μ M CHIR99021 (Merck Millipore), a highly selective inhibitor of GSK3 β . After 48 h, CHIR99021 was removed and the cells were treated with 5 μ M Wnt antagonist II (IWP2, Merck Millipore). Another 48 h later, the medium was replaced without IWP2. From day 8 on, the medium was replaced by RPMI1640 with Glutamax and HEPES containing 1x B27 supplement with insulin (Thermo Fisher Scientific). First beating CMs appeared around day 8. At day 25, the CMs were first treated with collagenase 2 (200 U/mL, Worthington) for 30 min so that beating clusters could be detached from the culture dish. These clusters were collected and digested with 0.25% trypsin/EDTA solution (Thermo Fisher Scientific) into single cells, which were replated onto Geltrex®-coated culture dishes as a monolayer. CMs were kept in culture for 3 months for maturation and further electrophysiological and gene and protein expression analyses.

Allele-Specific Expression Analysis of the *SCN5A* Gene

For the allele-specific expression analysis of *SCN5A* in BrS-CMs, the mRNA was sequenced with primers containing specific barcodes using the Ion Torrent semiconductor sequencing system (Thermo Fisher Scientific). The genomic DNA was used as control.

Total mRNA was isolated and reverse transcribed into cDNA. The primer set (forward: 5'-GAG AGC ACC GAG CCC CTG AGT GAG G-3' and reverse: 5'-CAC CAT GGG CAG GTC CAT GTT GAT G-3') was used to amplify the region containing the mutation site. The PCR product was diluted 1/200 and used for a second PCR run with 12 cycles using the same forward primer and different custom-designed reverse primers (Sigma–Aldrich), which contained a unique barcode to distinguish individual samples. The DNA concentration of

each sample was measured with Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and 250 ng of each sample was pooled and electrophoretically separated on a 2% agar gel. The specific product was extracted from the gel in a QIAcube system using the QIAquick® gel extraction kit (Qiagen) and subsequently purified with Agencourt AMPure XP PCR purification kit (Beckman Coulter) according to the manufacturer's instructions and eluted in low TE buffer (Thermo Fisher Scientific). The DNA quantity was determined by real-time PCR analysis in a 384-well plate using the GeneRead Library Quant kit (Qiagen). 400 µL of 10 pmol PCR product was used for clonal amplification onto Ion Sphere particles accomplished by an emulsion PCR in the Ion OneTouch (Thermo Fisher Scientific) system according to the manufacturer's instructions. The Ion Sphere particles coated with the amplified template DNA were applied to an Ion Torrent sequencing chip and placed on the Ion Personal Genome Machine (Thermo Fisher Scientific) for sequencing.

Immunofluorescence Staining

Undifferentiated iPSCs or EB cultures at different differentiation stages as well as 3-month-old CMs were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS at room temperature for 20 min and then blocked with 1% bovine serum albumin (Thermo Fisher Scientific) at 4°C overnight. The primary and respective secondary antibodies used are listed in **Supplementary Table 4**. Nuclei were counter-stained with 4-6-diamino-2-phenylindole (DAPI, 0.4 µg/mL, Sigma-Aldrich). Samples were mounted with Fluoromount-G mounting medium (eBioscience). Antibodies staining against non-transmembrane proteins were additionally treated with 0.1% Triton X-100 (Sigma-Aldrich) in PBS to permeabilize the cell membrane. Fluorescent images were captured with a fluorescence microscope (Zeiss Observer.Z1 or Axio Imager.M2). For some images, the ApoTome modus (Zeiss) was used.

Western Blot

For analysis of protein levels, Ctrl- and BrS-CMs were solubilized in 0.5% digitonin, 1.5% NP-40, and 1.0% Triton X 100 solubilization buffer for 35 min at 4°C. Protein concentrations were determined with a BCA assay kit. Equal amounts of protein were loaded on biphasic 6 and 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and run at low voltage. After blotting to PVDF membranes, membranes were incubated with Nav1.5, Cx43, Cav1.2, Kv4.3, and cTnT antibodies overnight. The primary and respective secondary antibodies used for Western blots are listed in **Supplementary Table 4**. Western blots were developed with a peqlab Fusion FX Vilber Lourmat camera and analyzed using the FusionCapt Advance 16.12 software.

Patch-Clamp

Whole-cell clamp was used to measure membrane potential (current-clamp configuration), I_{Na} , late I_{Na} , I_{to} , and I_{CaL} (voltage-clamp configuration), as previously described (Wagner et al., 2006; Streckfuss-Bomeke et al., 2013). All experiments were conducted with 3-month-old Ctrl- and BrS-CMs at room temperature. The pipette and extracellular solutions for various

kinds of voltage and current patch-clamp recordings are listed in **Supplementary Table 5**.

Spontaneous APs were recorded immediately after rupture with the EPC10 amplifier (HEKA Elektronik) using the Patchmaster software (HEKA Elektronik). No current was injected into the cells. Signals were filtered with 2.9 and 10 kHz Bessel filters. At least 20 spontaneous APs in a row were analyzed and averaged using LabChart Pro software (ADInstruments) to determine V_{max} , APA, and RMP.

For current measurements, all recordings started at least 1 min after rupture with the EPC10 amplifier using the Patchmaster software. Signals were filtered with 2.9 and 10 kHz Bessel filters and recorded with an EPC10 amplifier. Membrane capacitance (C_m) and series resistance (R_s) were compensated automatically after rupture, and currents were normalized to C_m .

The pulse stimulation of sodium current recording was pictured in inset of **Figures 3B,D–F**. The current–voltage (I – V) relationship was determined by increasing the voltage stepwise from -95 mV to $+5$ mV in 5 mV steps at a holding potential of -100 mV. Each pulse lasted 50 ms. Steady-state inactivation was measured by a two-pulse protocol at a holding potential of -100 mV. The voltage of the first conditioning pulse (500 ms) was increased stepwise from -120 to -20 mV in 5 mV steps. The second pulse to -20 mV was used to measure I_{Na} and normalized to the maximum I_{Na} (which was usually after the first pulse). For the measurement of the recovery from inactivation of sodium channels, a two-pulse protocol was used, with an increasing delay between the two pulses ranging from 1 to 165 ms. The first conditioning pulse (1000 ms) is used to induce sodium channel inactivation and the second pulse is used to measure I_{Na} generated by sodium channels that recover from inactivation. The I_{Na} from the second pulse was normalized to the maximal I_{Na} initiated by the conditioning pulse.

To study the effect of milrinone on I_{Na} in Ctrl- and BrS-CMs, an automated patch-clamp system (Patchliner Quattro, Nanion Technologies GmbH) was used with low resistance NPC-16 chips and I_{Na} was recorded at room temperature. iPSC-CMs were dissociated into single cells for automated patch-clamp analysis, as previously described (Li et al., 2019). The liquid junction potentials and series resistance were not compensated. The holding potential was -100 mV. I_{Na} was recorded using voltage steps from -90 to $+70$ mV for 20 ms in 5 mV steps at an interval of 2000 ms. Currents were sampled at 25 kHz and low-pass-filtered at 2.9 kHz.

The pulse protocols for I_{to} current were shown in inset of **Figures 4B,C,F**. The current–voltage (I – V) relationship was determined by increasing the voltage stepwise from -40 to $+60$ mV in 10 mV steps at a holding potential of -90 mV with a 20 ms prepulse at -35 mV to inactivate sodium channel. Each pulse lasted 600 ms. Steady-state inactivation was measured by using a two-pulse protocol at a holding potential of -90 mV. The voltage of the first conditioning pulse (600 ms) was increased stepwise from -90 to $+40$ mV in 10 mV steps after a 20 ms prepulse at -35 mV to inactivate sodium channels. The second pulse of 600 ms at $+60$ mV was used to measure I_{to} and normalized to the maximum I_{to} . For the measurement of the recovery from inactivation of I_{to} , a two-pulse protocol was used,

with an increasing delay between the two pulses ranging from 0 to 10,240 ms. The pulse was depolarized from -90 to $+40$ mV and last for 600 ms. The I_{to} from the second pulse was normalized to the maximal I_{to} initiated by the conditioning pulse. The pulse intervals were 15 s for all the stimulations.

The impulse protocol for I_{CaL} was shown in inset of **Figures 4H,I,K**, as previously described (Luo et al., 2020). The current–voltage (I – V) impulse depolarized from -90 to -80 mV then every 10 mV as step to $+70$ mV with a duration of 600 ms. Steady-state inactivation was measured by using a two-pulse protocol at a holding potential of -90 mV. The voltage of the first conditioning pulse (600 ms) was increased stepwise from -90 to $+10$ mV in 10 mV steps. The second pulse of 600 ms at 0 mV was used to measure I_{CaL} and normalized to the maximum I_{CaL} . For the measurement of the recovery from inactivation of I_{CaL} , a two-pulse protocol was used, with an increasing delay between the two pulses ranging from 0 to 1460 ms. The pulse was depolarized from -90 to 0 mV and last for 600 ms. The I_{CaL} from the second pulse was normalized to the maximal I_{CaL} initiated by the conditioning pulse. The pulse intervals were 3 s for all the stimulations.

For the conductance (G/G_{max}) calculation, a custom built-in formula and add-in modular in excel were used. The same fit functions were used for analyzing the kinetics of I_{Na} and I_{CaL} . Steady-state activation and inactivation curves were fitted with a standard Boltzmann function: $Y = 1/[1 + \exp((V_{1/2} - V)/k_{\infty})]$, where $V_{1/2}$ is the half-maximal voltage of steady-state (in)activation and k_{∞} is the slope factor of the voltage dependence of (in)activation. The development of the recovery from inactivation was fitted with single exponential function: $Y(t) = Y_0 + A(1 - \exp(-t/\tau_{rec}))$, where A is the amplitude and τ_{rec} the time constant of recovery from inactivation. Persistent I_{Na} was measured by integrating the current between 50 and 450 ms of a 1000 ms pulse from a holding potential of -100 to -20 mV. The probability of I_{CaL} window current was calculated from steady-state (in)activation parameters through the following equation as previously described: Probability = $1/[1 + \exp((V_{1/2,act} - V)/k_{act})] * (1/[1 + \exp((V - V_{1/2,inact})/k_{inact})])$ (Huang et al., 2011).

The data were analyzed using Clampfit (Axon Instruments), Fitmaster (HEKA Elektronik), and GraphPad Prism (GraphPad Software, Inc.) software.

Multielectrode Array

The digested iPSC-CMs were seeded on Geltrex® -coated MEA chips. The final concentration was approximately 200,000 iPSC-CMs in 50 μ L RPMI1640 medium supplemented with 1x B27. The medium was replenished to 1 mL after seeding for around 30 min. In this study, 60MEA200/30iR-Ti-gr chips were used (Multichannel Systems). The medium was changed one day after digestion and thereafter every two days until day 6, which performed the recording. All the recordings were carried out at 35–37°C by using Cardio 2D software (Multichannel Systems). The sample rate was 10,000 Hz. The spontaneous beating frequency and inter-beat interval were automatically generated by Cardio 2D. At least five stable continuous CV values were averaged from spontaneous beatings. For FP metrics [peak-to-peak amplitude (PPA), peak-to-peak duration (PPD), and

peak-to-peak slope (PPS)] analysis, the averaged measurements (3 min) of the electrodes (Pin numbers: 22, 33, 44, 55, 66, and 77) were analyzed by Cardio 2D+ software. The rate of arrhythmia was quantified as standard deviation (SD) of the inter-beat interval, which was normalized to averaged inter-beat intervals.

Statistics

Data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism 7 using the two-tailed unpaired Student's t -test to compare differences between two independent groups, one-way ANOVA followed by Tukey's *post hoc* test to compare more than two groups, and the paired Student's t -test to compare differences between two dependent groups or the two-way ANOVA test for comparison of more groups and conditions. Results were considered statistically significant when the P value was <0.05 (* $P < 0.05$; # $P < 0.01$; \$ $P < 0.001$).

RESULTS

Generation and Characterization of iPSCs

We recruited two BrS patients, a 50-year-old male (BrS1) and his biological sister (BrS2) in the study. The ECG recordings of both patients at rest displayed the BrS-typical pattern, a coved-type ST-segment elevation in the right precordial leads V_1 and V_2 and a more saddleback pattern in V_3 followed by a negative T wave (**Supplementary Figure 1A**). In addition, both patients revealed first-degree AV block. In long-term ECG recordings of patient BrS1, multiple episodes of polymorphic ventricular tachycardia appeared during stair climbing (**Supplementary Figure 1B**). Both patients received an ICD for the treatment. Genetic screening for possible mutations in several cardiac-specific genes (*KCNQ1*, *KCNQ2*, *KCNQ3*, *KCNQ4*, *KCNQ5*, *KCNQ6*, *KCNQ7*, *KCNQ8*, *KCNQ9*, *KCNQ10*, *KCNQ11*, *KCNQ12*, *KCNQ13*, *KCNQ14*, *KCNQ15*, *KCNQ16*, *KCNQ17*, *KCNQ18*, *KCNQ19*, *KCNQ20*, *KCNQ21*, *KCNQ22*, *KCNQ23*, *KCNQ24*, *KCNQ25*, *KCNQ26*, *KCNQ27*, *KCNQ28*, *KCNQ29*, *KCNQ30*, *KCNQ31*, *KCNQ32*, *KCNQ33*, *KCNQ34*, *KCNQ35*, *KCNQ36*, *KCNQ37*, *KCNQ38*, *KCNQ39*, *KCNQ40*, *KCNQ41*, *KCNQ42*, *KCNQ43*, *KCNQ44*, *KCNQ45*, *KCNQ46*, *KCNQ47*, *KCNQ48*, *KCNQ49*, *KCNQ50*, *KCNQ51*, *KCNQ52*, *KCNQ53*, *KCNQ54*, *KCNQ55*, *KCNQ56*, *KCNQ57*, *KCNQ58*, *KCNQ59*, *KCNQ60*, *KCNQ61*, *KCNQ62*, *KCNQ63*, *KCNQ64*, *KCNQ65*, *KCNQ66*, *KCNQ67*, *KCNQ68*, *KCNQ69*, *KCNQ70*, *KCNQ71*, *KCNQ72*, *KCNQ73*, *KCNQ74*, *KCNQ75*, *KCNQ76*, *KCNQ77*, *KCNQ78*, *KCNQ79*, *KCNQ80*, *KCNQ81*, *KCNQ82*, *KCNQ83*, *KCNQ84*, *KCNQ85*, *KCNQ86*, *KCNQ87*, *KCNQ88*, *KCNQ89*, *KCNQ90*, *KCNQ91*, *KCNQ92*, *KCNQ93*, *KCNQ94*, *KCNQ95*, *KCNQ96*, *KCNQ97*, *KCNQ98*, *KCNQ99*, *KCNQ100*) revealed the heterozygous *SCN5A* point mutation C > A at position c.5435 in both patients (Schulze-Bahr et al., 2003). This non-sense mutation results in a premature termination codon causing the protein truncation at amino acid 1812 (p.S1812X). Genetic tests of their family members identified the mother as an asymptomatic carrier of the mutation, showing first-degree AV block, but no BrS-specific ECG pattern at rest. A brother died from sudden cardiac death at the age of 15. The father and another brother do not carry the mutation and show no BrS phenotype (**Supplementary Figure 1C**).

We generated iPSCs from these two patients (BrS1 and BrS2) by overexpression of the four Yamanaka factors *SOX2*, *KLF4*, *OCT4*, and *c-MYC* in somatic cells (**Supplementary Figure 2**). Three clones per individual were selected for further characterization (BrS1.1-3 and BrS2.1-3). In addition, we included control iPSC lines (Ctrl1.1-3 also known as MSC3-iPS1-3; and Ctrl2.1 and 2 also named as FB2-iPS1 and 2) from two independent healthy donors Ctrl1 and Ctrl2, which were described in our previous studies (Streckfuss-Bomeke et al., 2013; Cyganek et al., 2018). All generated BrS-iPSCs showed the typical morphology similar to Ctrl-iPSCs, were positive for pluripotency markers (**Supplementary Figure 2A**), showed

expression of endogenous pluripotency genes (**Supplementary Figure 3A**), and revealed normal chromosome numbers in more than 90% of cells (**Supplementary Figure 2C**). Genomic sequencing confirmed the presence of the *SCN5A* mutation c.C5435A (p.S1812X) in BrS1- and BrS2-iPSCs and its absence in both Ctrl1- and Ctrl2-iPSCs (**Supplementary Figure 2B**). Furthermore, Ctrl- and BrS-iPSCs were differentiated into derivatives of all three embryonic germ layers *in vitro* (**Supplementary Figures 3B,C**) and developed teratomas in immune-deficient mice (**Supplementary Figure 2D**). To reduce the phenotypic variability among different cell lines, all three lines of every individual were used for the following studies.

Reduced $\text{Na}_V1.5$ and Cx43 Expression in BrS-CMs

We differentiated Ctrl- and BrS-iPSCs into ventricular CMs using the directed differentiation method, as described previously (Cyganek et al., 2018). This method gave rise to the differentiation efficiency with more than 90% of cells positive for cTnT at day 25 after the initiation of differentiation. No significant difference in cardiac differentiation efficiency was observed between Ctrl- and BrS-iPSCs (data not shown). Immunofluorescence analyses using antibodies against the cardiac-specific proteins cTnT and α -actinin revealed well-organized cross-striations in both 3-month-old BrS- and Ctrl-CMs (**Figures 1A–C**). To study whether the p.S1812X mutation affected the cellular localization of $\text{Na}_V1.5$ in BrS-CMs, we applied an antibody that detects both full-length and truncated proteins of $\text{Na}_V1.5$. We found a different distribution of $\text{Na}_V1.5$ in Ctrl- and BrS-CMs (**Figure 1B**). Whereas equal distribution of $\text{Na}_V1.5$ along cell membrane was clearly observed in Ctrl-CMs, $\text{Na}_V1.5$ appeared irregular and intermittent on the cell surface. Similar to the findings of others (Malan et al., 2011; Davis et al., 2012), $\text{Na}_V1.5$ was also expressed in a diffused or a fine granular-like pattern in the cytosol of both Ctrl- and BrS-CMs. In some Ctrl- and BrS-CMs, we observed that $\text{Na}_V1.5$ was localized in a striated pattern, mostly overlapped with α -actinin (**Figure 1B**), as described in adult CMs (Mohler et al., 2004; Xi et al., 2009; Shy et al., 2014). The expression of connexin 43 (Cx43) was detected at the intercellular gap junctions in both Ctrl- and BrS-CMs (**Figures 1C,D**), indicating a cell-to-cell coupling. However, the co-staining of Cx43 with $\text{Na}_V1.5$ revealed the strong overlap and interaction of Cx43 with $\text{Na}_V1.5$ in Ctrl-CMs, but less in BrS-CMs (**Figure 1D**).

Western blot analyses using an antibody that only detects the full-length protein of $\text{Na}_V1.5$ showed a significantly reduced expression of $\text{Na}_V1.5$ in BrS-CMs by 69.5% compared to Ctrl-CMs ($P = 0.0093$; **Figures 1E,F**). Interestingly, Cx43 expression in BrS-CMs was also reduced by 34%, but not significantly, compared to Ctrl-CMs ($P = 0.1443$; **Figures 1E,G**). Furthermore, we assessed the expression of $\text{Ca}_V1.2$ and $\text{K}_V4.3$ in Ctrl- and BrS-CMs and did not observe significant differences between Ctrl- and BrS-CMs (**Figures 1E,H,I**). Analysis of the allele-specific *SCN5A* expression in BrS1- and BrS2-CMs revealed the balanced expression of *SCN5A* between the wild-type and mutated alleles (**Supplementary Figure 3D**).

Conduction Slowing in BrS-CMs

Clinically, both BrS patients experience severe arrhythmias and reveal first-degree AV block in the ECGs. To investigate whether the arrhythmic predisposition is exhibited in the monolayer culture of iPSC-CMs, we applied multi-electrode array (MEA) to compare the conduction properties of spontaneous electrical excitations in 3-month-old Ctrl- and BrS-CMs (**Figure 2**). The differences between Ctrl- and BrS-CMs in the FP metrics PPD (ms), PPA (mV), and PPS (V/s) were distinct after enlargement (**Figure 2A**). The beating frequency of BrS1-CMs (0.46 ± 0.07 Hz, $n = 23$) and BrS2-CMs (0.44 ± 0.02 Hz, $n = 19$) was comparable with Ctrl-CMs (0.45 ± 0.03 Hz, $n = 37$, **Figure 2C**). However, BrS-CMs revealed a smaller PPA and PPS but a prolonged PPD when compared to Ctrl-CMs (**Figures 2D–F**). In **Figure 2B**, typical excitation patterns and local activation times in reference to the trigger point were shown for both Ctrl2- and BrS1-CMs. The excitation of Ctrl2-CMs spread from one corner (the lower left corner as the trigger point) of the MEA to the other in around 10 ms (**Figure 2B**) whereas BrS1-CMs needed around 45 ms for the same distance (**Figure 2B**), indicating pronounced conduction slowing in BrS-CMs when compared to Ctrl-CMs. Quantitative analysis revealed that the CV of Ctrl-CMs was 15.47 ± 1.05 cm/s ($n = 37$), whereas 4.7 ± 0.44 and 4.4 ± 0.49 cm/s were observed in BrS1-CMs ($n = 23$, $P < 0.001$) and BrS2-CMs ($n = 19$, $P < 0.001$), respectively (**Figure 2G**). This dramatic difference can be vividly observed in our recorded movies (**Supplementary Videos 1, 2**). Moreover, BrS-CMs exhibited increased inter-beat interval variability compared to Ctrl-CMs, as demonstrated by the SD of the inter-beat interval normalized to the mean of the inter-beat intervals (**Figure 2H**).

Reduced Peak I_{Na} and Delayed Steady-State Activation of Sodium Channels in BrS-CMs

To assess the effect of the *SCN5A* mutation p.S1812X on the electrophysiological properties of BrS-CMs, I_{Na} density and gating properties of sodium channels were compared in 3-month-old BrS-CMs versus Ctrl-CMs using the whole-cell voltage-clamp technique (**Figure 3**). We observed no differences in the current density, voltage dependence, and gating properties of sodium channels between Ctrl1- and Ctrl2-CMs (**Supplementary Figure 4**). For a better overview and comparison of the data, we pooled the data of Ctrl1- and Ctrl2-CMs, as shown in **Figure 3**. Representative examples of I_{Na} recordings were shown in **Figure 3A**. Compared to the Ctrl-CMs, the I_{Na} densities were significantly reduced in BrS1- and BrS2-CMs (**Figure 3B**). The maximal peak I_{Na} in Ctrl-CMs ($n = 60$) at -35 mV showing -38.3 ± 1.8 A/F was reduced by more than 50% in BrS-CMs showing -18.1 ± 1.5 A/F in BrS1-CMs ($n = 37$, $P < 0.001$) and -16.7 ± 1.4 A/F in BrS2-CMs ($n = 19$, $P < 0.001$). There was no significant difference in the current-voltage relationship and the I_{Na} densities between BrS1- and BrS2-CMs (**Figure 3B**). However, current-voltage relationships normalized to the maximum peak I_{Na} showed a shift of peak

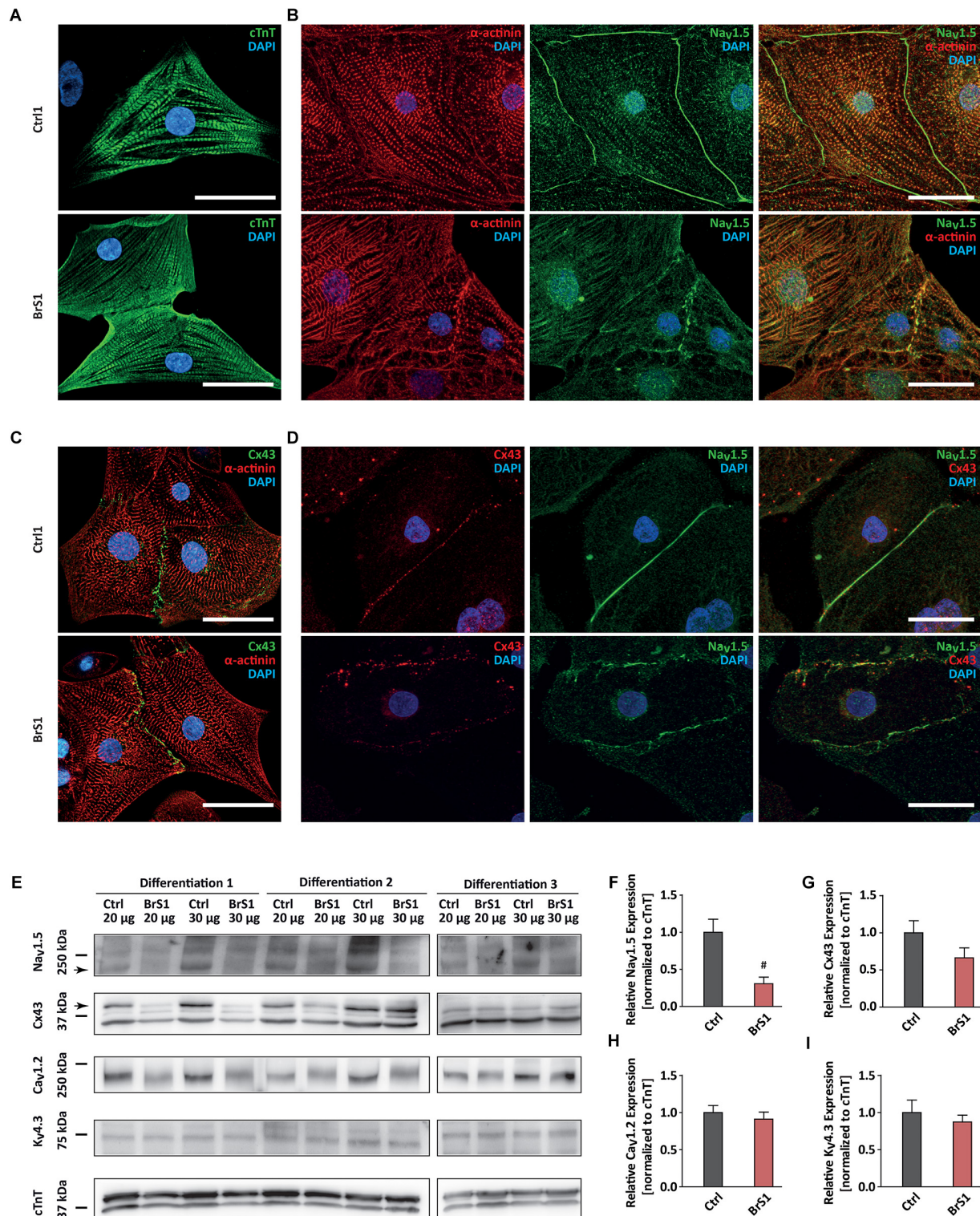


FIGURE 1 | The expression of cardiac-specific proteins in Ctrl- and BrS-CMs. **(A)** Immunostaining of Ctrl- and BrS-CMs for cardiac markers cTnT. Scale bar, 20 μ m. **(B)** Double-immunostaining of α -actinin and Nav1.5. Scale bar, 20 μ m. **(C)** Double-immunostaining of Cx43 and α -actinin. Scale bar, 20 μ m. **(D)** Double-immunostaining of Ctrl- and BrS-CMs with antibodies against Cx43 and Nav1.5. Scale bar, 20 μ m. **(E)** Shown are original western blots for the decoration of Nav1.5 α subunit, Cx43, Cav1.2, Kv4.3, and cTnT. The arrows represent the target bands used for quantification. Quantitative analysis of Nav1.5 α subunit **(F)**, Cx43 **(G)**, Cav1.2 **(H)**, and Kv4.3 **(I)** expression (normalized to cTnT). Data are presented as mean $\pm$ SEM. Two-tailed unpaired Student's *t*-test was used for statistical analysis: $\#P < 0.01$.

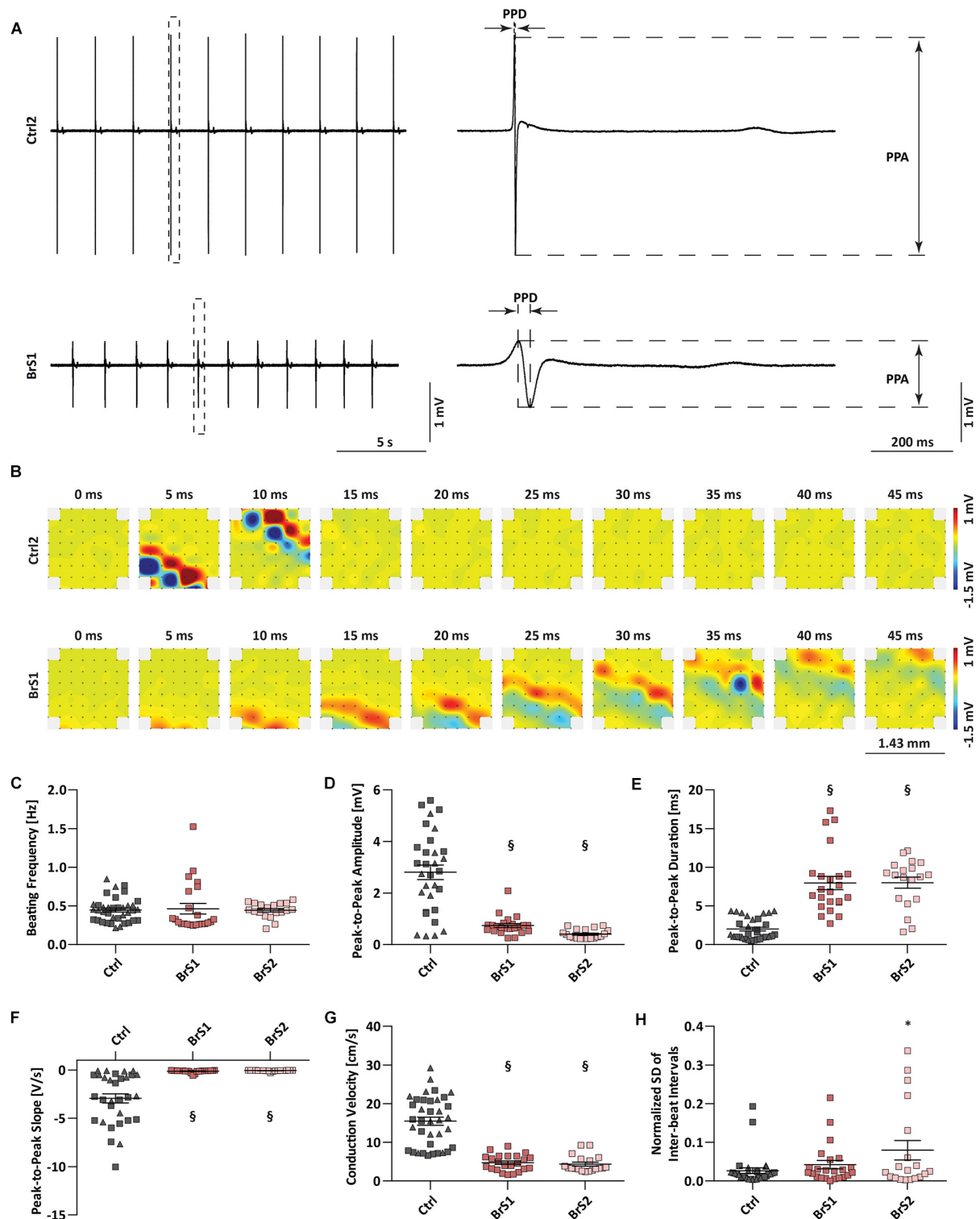
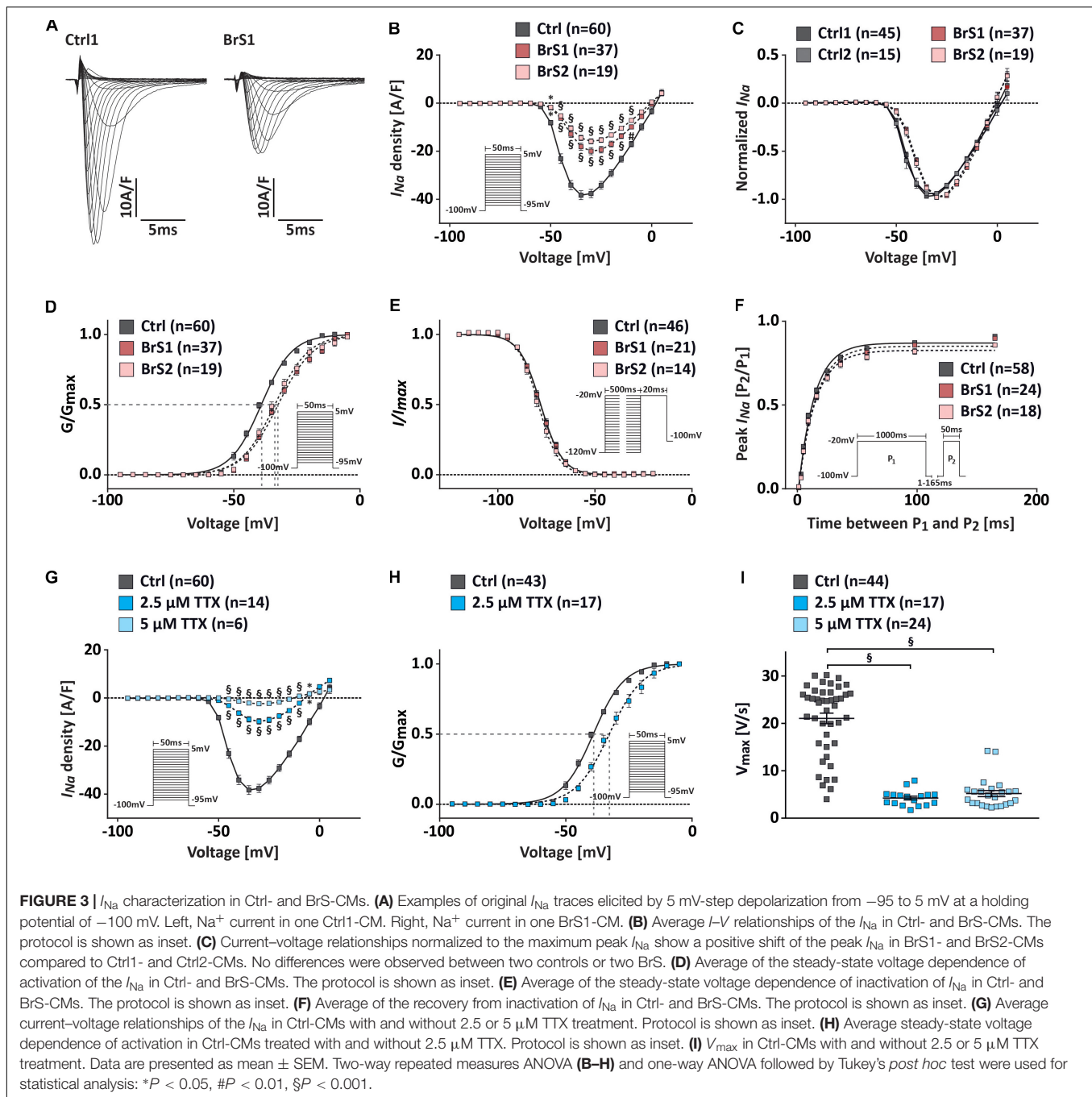


FIGURE 2 | Field potential and conduction velocity of Ctrl- and BrS-CMs. **(A)** Represented original field potential traces of Ctrl2- and BrS1-CMs were enlarged to show PPA, PPD, and PPS. **(B)** Represented beats of Ctrl2- and BrS1-CMs were dissected to 10 frames (0, 5, 10, 15, 20, 25, 30, 35, 40, 45 ms), respectively. The width and length of the electrodes distributed area were 1.43 mm. The metrics of MEA measurements including beating frequency **(C)**, PPA **(D)**, PPD **(E)**, PPS **(F)**, CV **(G)**, and normalized SD of inter-beat interval **(H)** in Ctrl-CMs, BrS1-CMs, and BrS2-CMs. Gray triangle represents Ctrl1 and gray rectangle represents Ctrl2. Data are mean $\pm$ SEM. One-way ANOVA followed by Tukey's *post hoc* test was used for statistical analysis: * $P < 0.05$, § $P < 0.001$. PPA, peak-to-peak amplitude; PPD, peak-to-peak duration; PPS, peak-to-peak slope; CV, conduction velocity; SD, standard deviation.



current toward the positive direction in BrS-CMs compared to Ctrl-CMs (Figure 3C).

To study the changes in channel kinetics, we compared the steady-state (in)activation and the recovery from inactivation of the sodium channel in BrS-CMs versus Ctrl-CMs. The steady-state activation curve was significantly shifted in a depolarizing direction in BrS-CMs compared to Ctrl-CMs (Figure 3D). $V_{1/2}$ was -39.1 ± 0.2 mV for Ctrl-CMs ($n = 60$), -32.4 ± 0.2 mV for BrS1-CMs ($n = 37$, $P < 0.001$), and -33.7 ± 0.3 mV for BrS2-CMs ($n = 25$, $P < 0.001$). Slope factor k_{∞} was 6.1 ± 0.2 mV

for Ctrl-CMs, significantly smaller than 6.9 ± 0.2 mV for BrS1-CMs ($P < 0.001$) and 6.7 ± 0.2 mV for BrS2-CMs ($P < 0.05$) (Supplementary Table 1). The steady-state inactivation of sodium channels in BrS-CMs did not differ significantly from Ctrl-CMs (Figure 3E and Supplementary Table 1). Next, we determined the fraction (A) of sodium channels recovered from an inactivation pulse (1000 ms at -20 mV) and the time constant (τ_{rec}) for this recovery. We found a slight but not significant slowing of the recovery from the inactivation in BrS-CMs versus Ctrl-CMs (Figure 3F and Supplementary Table 1).

However, no difference in persistent I_{Na} was detected in our study (**Supplementary Figures 4F,G**).

To assess whether the reduced I_{Na} density in BrS-CMs is related to the positive shift of the steady-state activation, we treated Ctrl-CMs with the Na^+ channel blocker tetrodotoxin (TTX; 2.5 and 5 μ M, **Figures 3G–I**). The treatment with 5 μ M TTX resulted in a robustly reduced I_{Na} , decreasing maximal I_{Na} to 5% of the basal level (**Figure 3G**). The peak I_{Na} density at -35 mV in Ctrl-CMs treated with 2.5 μ M TTX (-9.7 ± 1.2 A/F, $n = 14$) showed a significant reduction compared to non-treated Ctrl-CMs (-38.3 A/F, $n = 60$) (**Figure 3G**). We also observed a significant positive shift in the steady-state activation, as demonstrated by the change in $V_{1/2}$ in Ctrl-CMs treated with 2.5 μ M TTX (-32.7 ± 0.3 mV, $n = 14$) compared to non-treated Ctrl-CMs (-39.1 ± 0.2 mV, $n = 60$, $P < 0.001$) (**Figure 3H**). These data suggest that the reduced I_{Na} density in BrS-CMs is associated with the positive shift of the steady-state activation.

To study whether the blockage of Na^+ channel by TTX can mimic the FP properties observed in BrS-CMs, we treated Ctrl-CMs with 2.5 μ M TTX for 10 min. We found that the application of TTX in Ctrl-CMs led to a significantly reduced PPA, PPS, and CV, but a prolonged PPD (**Supplementary Figure 5**). In addition, TTX reduced beating frequency (**Supplementary Figure 5**). Taken together, these data indicate that BrS-CMs recapitulate the conduction abnormalities seen in patients with loss-of-function sodium channel mutations, and the conduction slowing can be evoked in Ctrl-CMs by TTX and is associated with the diminution of I_{Na} .

Augmented I_{to} Current and Altered Window Current of I_{CaL} in BrS-CMs

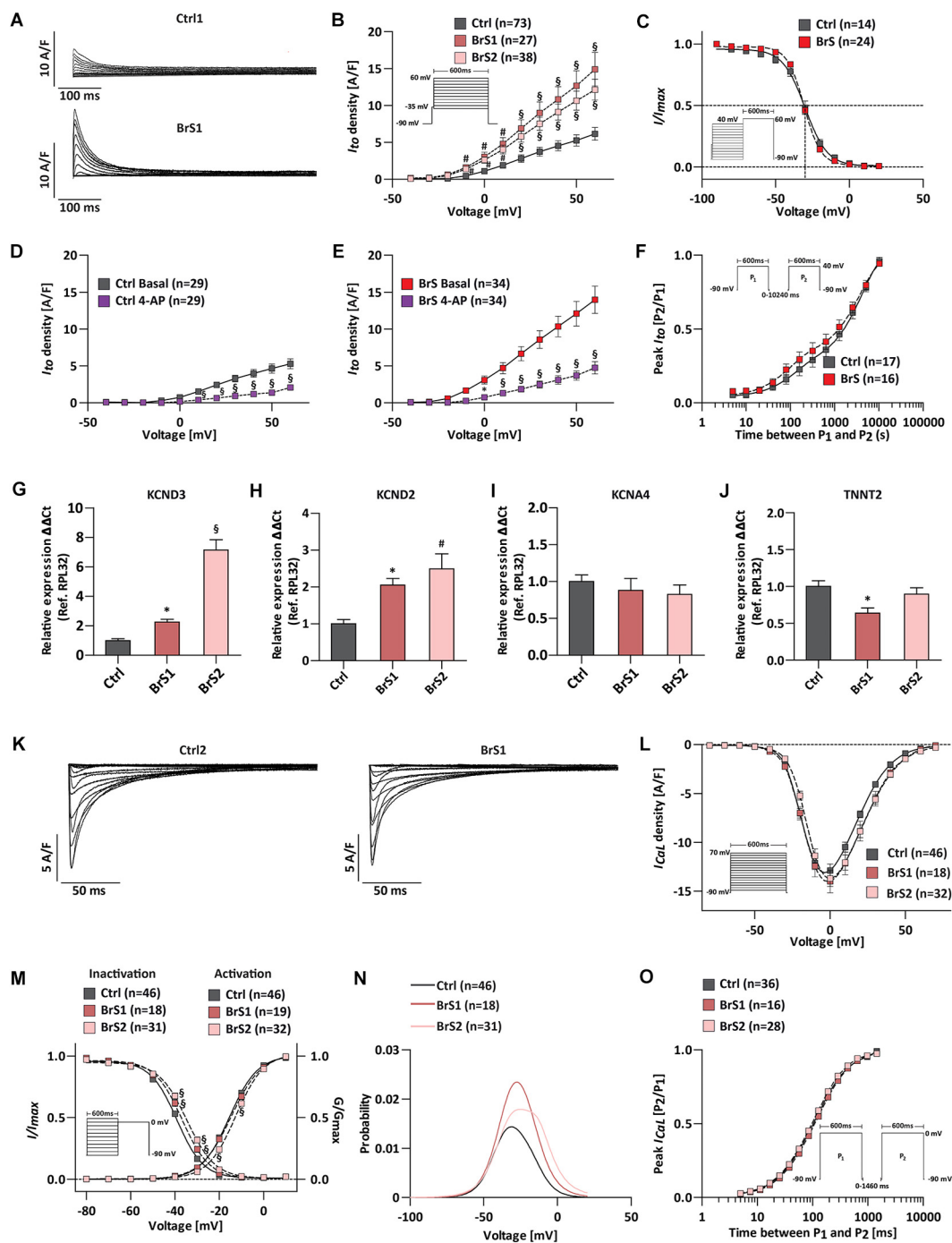
Previous studies reported that in the presence of weak I_{Na} , the unopposed outward K^+ current I_{to} cause accentuation of the AP notch in the right ventricular epicardium, resulting in accentuated J wave and ST-segment elevation associated with the Brugada pattern (Szel and Antzelevitch, 2014). To test whether I_{to} is pivotal in generating the disease phenotype in BrS, we recorded I_{to} from single iPSC-CMs (**Figure 4**). The representative I_{to} traces of Ctrl1-CMs and BrS1-CMs were shown in **Figure 4A**. Surprisingly, the BrS1-CMs and BrS2-CMs exhibited significantly larger I_{to} in comparison to Ctrl-CMs (**Figure 4B**). The maximum peak currents at $+60$ mV in BrS1-CMs (14.91 ± 2.27 A/F, $n = 27$, $P < 0.001$) and BrS2-CMs (12.14 ± 1.42 A/F, $n = 38$, $P < 0.001$) were 2.4 and 1.9 times bigger than those in Ctrl-CMs (6.14 ± 0.85 A/F, $n = 73$) (**Figure 4B**). When the cells were treated with 4-aminopyridine (4-AP), an I_{to} inhibitor, 60.9% reduction of the currents was observed in Ctrl-CMs (from 5.29 ± 0.67 to 2.07 ± 0.24 A/F, paired, $n = 29$; **Figure 4D**), which is comparable to 65.9% reduction in BrS-CMs (from 13.97 ± 1.8 to 4.8 ± 0.84 A/F, paired, $n = 34$; **Figure 4E**), indicating the measured currents are the 4-AP-sensitive I_{to} . Nevertheless, we did not detect any significant differences in $V_{1/2}$ for the steady-state inactivation between Ctrl-CMs (-30.41 ± 1.6 mV, $n = 14$) and BrS-CMs (-30.42 ± 0.72 mV, $n = 24$) (**Figure 4C** and **Supplementary Table 1**). Recovery from the inactivation of I_{to} in Ctrl-CMs showed fast (fast τ_{rec} , 279.7 ± 121.1 ms) and slow

phases (slow τ_{rec} , 2854 ± 581.7 ms), which were comparable to the fast (fast τ_{rec} , 211.9 ± 135.8 ms) and slow phases (slow τ_{rec} , 2773 ± 462.6 ms) in BrS-CMs, respectively (**Figure 4F** and **Supplementary Table 1**). Using real-time PCR technique, we assessed the expression of I_{to} -related genes *KCND2/3* and *KCNA4*. It turned out the expressions of *KCND2* and *KCND3* were significantly higher in BrS1- and BrS2-CMs when compared to Ctrl-CMs (**Figures 4G,H**). However, no difference regarding the expression of *KCNA4* was found between Ctrl- and BrS-CMs (**Figure 4I**). The *TNNT2* expression was normalized to reference gene *RPL32* like the other three genes (**Figure 4J**).

We further checked the I_{CaL} and did not detect significant differences in the peak I_{CaL} at 0 mV in BrS1-CMs (-13.99 ± 1.18 A/F, $n = 18$) or BrS2-CMs (-13.71 ± 0.8 A/F, $n = 32$) compared to Ctrl-CMs (-12.88 ± 0.66 A/F, $n = 46$) (**Figures 4K,L**). The curve of the steady-state activation was shifted to more depolarizing voltage in BrS2-CMs ($V_{1/2} = -12.51 \pm 0.50$ mV, $n = 32$, $P < 0.001$) but not in BrS1-CMs ($V_{1/2} = -14.45 \pm 0.84$ mV, $n = 19$, $P > 0.05$) when compared to Ctrl-CMs ($V_{1/2} = -15.54 \pm 0.38$ mV, $n = 46$) (**Figure 4M** and **Supplementary Table 1**). The steady-state inactivation curves were also significantly shifted toward more depolarizing voltage for BrS1-CMs ($V_{1/2} = -36.83 \pm 0.38$ mV, $n = 18$, $P < 0.001$) and BrS2-CMs ($V_{1/2} = -34.61 \pm 0.40$ mV, $n = 31$, $P < 0.001$) when compared to Ctrl-CMs ($V_{1/2} = -39.18 \pm 0.22$ mV, $n = 46$) (**Figure 4M** and **Supplementary Table 1**). Notably, the shifts in the voltages for half-(in)activation $V_{1/2}$ and a slight increase in slopes of (in)activation k_{∞} resulted in the significant increases in the window current (area under the curves in **Figure 4N**). After calculating with a window current probability formula, we found the probabilities of the I_{CaL} window current increased 1.59 times for BrS1-CMs and 1.56 times for BrS2-CMs when compared to Ctrl-CMs (**Figure 4N**). As window currents are steady-state currents, bigger window currents indicate the increased Ca^{2+} influx that may contribute to the formation of afterdepolarization and arrhythmia in BrS-CMs. The maximum ratio of peak I_{CaL} [P_2/P_1] of the recovery from the inactivation for Ctrl-CMs is comparable to those for BrS1-CMs and BrS2-CMs (**Figure 4O** and **Supplementary Table 1**). Taken together, our results demonstrate that BrS-CMs exhibit distinct anomalies not only in Na^+ channel function but also in K^+ and Ca^{2+} channel functions compared to Ctrl-CMs, which may result in the abnormal AP phenotype.

Reduced V_{max} and Increased Irregular Repolarization in APs of BrS-CMs

According to the following stringent criteria, the majority of 3-month-old iPSC-CMs (81%) reveal ventricular-like APs by using the C-clamp mode of patch-clamp technique (**Figure 5** and **Supplementary Table 2**). Ventricular-like APs exhibit a relatively negative resting membrane potential (RMP; < -60 mV), a rapid maximal upstroke velocity (V_{max}), a prominent plateau phase, and an AP amplitude (APA) over 95 mV. Atrial-like APs reveal similar properties as ventricular-like APs but lack the plateau phase. Nodal-like APs exhibit a more positive RMP (≥ -55 mV),



a slow $V_{\max}$ (≤ 5 V/s), and an APA < 85 mV. Overall, no significant differences in RMP and APA were observed in BrS-CMs compared to Ctrl-CMs (**Supplementary Table 2**). Since sodium channels are responsible for the fast upstroke of the atrial and ventricular APs, as expected, the $V_{\max}$ was significantly slower in spontaneously beating ventricular- and atrial-like BrS1- and BrS2-CMs compared to Ctrl-CMs (**Figure 5A**). Similarly, Ctrl-CMs after the treatment with TTX showed a slower $V_{\max}$ (**Figure 3I**). We also observed a significantly slower $V_{\max}$ in BrS2-CMs than in BrS1-CMs (**Figure 5B**), which is consistent with the more pronounced I_{Na} reduction in BrS2-CMs than in BrS1-CMs. Additionally, we found a pronounced AP notch in BrS-CMs compared to Ctrl-CMs (**Figure 5C**), which is in line with the augmented I_{to} density in BrS-CMs. Notably, we detected prominent irregularities in spontaneous AP recordings in BrS-CMs, including early afterdepolarization (EAD), EAD-triggered activities, delayed afterdepolarization (DAD), and DAD-triggered activities (**Figure 5C**). We observed the irregularities in 40% of BrS1-CMs ($n = 43$; $P < 0.001$, Fisher's exact test) and 42% of BrS2-CMs ($n = 31$; $P < 0.001$) compared to 11% of Ctrl-CMs ($n = 74$, **Figure 5D**). Taken together, these results demonstrate that BrS-CMs exhibit distinctly abnormal AP phenotype compared with Ctrl-CMs.

Assessment of Potential Pharmacotherapies Using BrS-CMs

Given that two clinically used PDE inhibitors cilostazol and milrinone have been shown to suppress the hypothermia-induced ventricular tachycardia/ventricular fibrillation by reversing the repolarization abnormalities (Gurabi et al., 2014), we tested their effects on our iPSC-CMs at therapeutically relevant concentrations (Szel et al., 2013). After 10 μ M cilostazol treatment of BrS-CMs for half an hour, we observed a 50.9% reduction of I_{to} in BrS1-CMs (from 14.91 to 7.3 A/F) and a 35.4% reduction in BrS2-CMs (from 12.14 to 7.8 A/F) at +60 mV pulse stimulation, whereas only a slight but not significant I_{to} reduction of 14.4% was observed for the Ctrl-CMs (from 6.14 to 5.25 A/F) (**Figures 6A–C**). The similar pattern was found when we tested the treatment with 2.5 μ M milrinone at the same condition: I_{to} was significantly reduced from 14.91 to 9.5 A/F in BrS1-CMs (36.3% reduction) and from 12.14 to 7.3 A/F in BrS2-CMs (40% reduction), but only a 10.9% reduction in Ctrl-CMs (from 6.14 to 5.7 A/F) (**Figures 6A–C**).

Moreover, we assessed whether these two drugs can alleviate the arrhythmic beating degree of BrS-CMs. We found that the treatment of BrS-CMs with cilostazol led to the decrease of cells with arrhythmic beating from 40.5% ($n = 74$) to 21% ($n = 14$) (**Figures 6D,E**). We detected the similar effect of milrinone on BrS-CMs and the arrhythmic beating degree of BrS-CMs dropped to 17% ($n = 12$) (**Figures 6F,G**). Both cilostazol and milrinone treatment had no obvious effect on Ctrl-CMs (**Figures 6D–G**). The most interesting thing is that no EAD- or EAD-triggered activities were observed in these AP recordings.

We further studied the effect of milrinone on I_{Na} using the automated patch-clamp system and found no significant changes in I_{Na} in both Ctrl- and BrS-CMs after 2.5 μ M milrinone

treatment for half an hour (**Figures 6H–J**). In line with these data, there were no significant effects of cilostazol and milrinone on the CV in Ctrl-, BrS1-, and BrS2-CMs (**Figures 6K,L**), but inter-beat interval variability was slightly reduced (**Supplementary Figures 4H–K**).

DISCUSSION

In this study, we applied patient-specific iPSC-CMs carrying the non-sense mutation p.S1812X in *SCN5A* and investigated the pathophysiological phenotypes and possible pharmacological therapy. Our results demonstrate the ability of patient-specific BrS-CMs to recapitulate the loss-of-function of $Nav1.5$ resulting from the truncation of *SCN5A* as evidenced by the significant reduction of peak I_{Na} , the significantly slower of the steady-state activation and the 69% reduction of $Nav1.5$ α -subunit protein expression. In line with these findings, we also observed the decreased AP upstroke velocity and the conduction slowing in BrS-CMs. Notably, we also found the increased I_{to} and the augmented window current of I_{CaL} in BrS-CMs when compared to Ctrl-CMs. We believe that the impaired interplay between I_{Na} , I_{to} , and I_{CaL} may be involved in the generation of arrhythmia in BrS-CMs. Furthermore, we showed the positive effect of PDE inhibitors cilostazol and milrinone on the reduction of the arrhythmia in BrS-CMs.

In this study, we, for the first time, applied the MEA technology and demonstrated the conduction slowing in BrS-CMs. It is well known that the I_{Na} and the electrically coupling degree of gap junctions are the key determinants of cardiac conduction (Kucera et al., 2002). We demonstrated that the I_{Na} density was reduced in BrS-CMs, which is consistent with other observations in iPSC-CMs with BrS-related mutations in *SCN5A* (Liang et al., 2016; Ma et al., 2018). Sodium channels play an important role in phase 0 of the cardiac AP and determine the upstroke velocity (Satin et al., 2004). In BrS-CMs, $V_{\max}$ was significantly lower compared to Ctrl-CMs, consistent with observed reduction in peak I_{Na} density. These findings were also demonstrated in patient-specific iPSC-CMs harboring the *SCN5A*^{L795insD} mutation (Davis et al., 2012). Our findings that the I_{Na} activation curve shifted toward more positive potential with an increased slope factor are in accordance with the later activation of sodium channels resulted from loss-of-function mutations in *SCN5A* as previously reported for BrS patients (Wilde and Amin, 2018). Additionally, the diminution of I_{Na} and the altered gating properties are clearly associated with the expression reduction of full-length $Nav1.5$ protein and the disrupted localization at the cell surface due to the non-sense mutation p.S1812X in *SCN5A* in BrS-CMs. Our data are in line with the previous study demonstrating that in HEK293 cells overexpressing the wild-type and the BrS-associated mutant p.V2016M *SCN5A* together, the surface expression of $Nav1.5$ is reduced due to its disturbed interaction with SAP97 via a PDZ domain (the last three residues SIV of $Nav1.5$), with a subsequent decrease of I_{Na} (Shy et al., 2014).

In line with previous studies (Kucera et al., 2002; Edokobi and Isom, 2018), we found the co-localization of $Nav1.5$ and

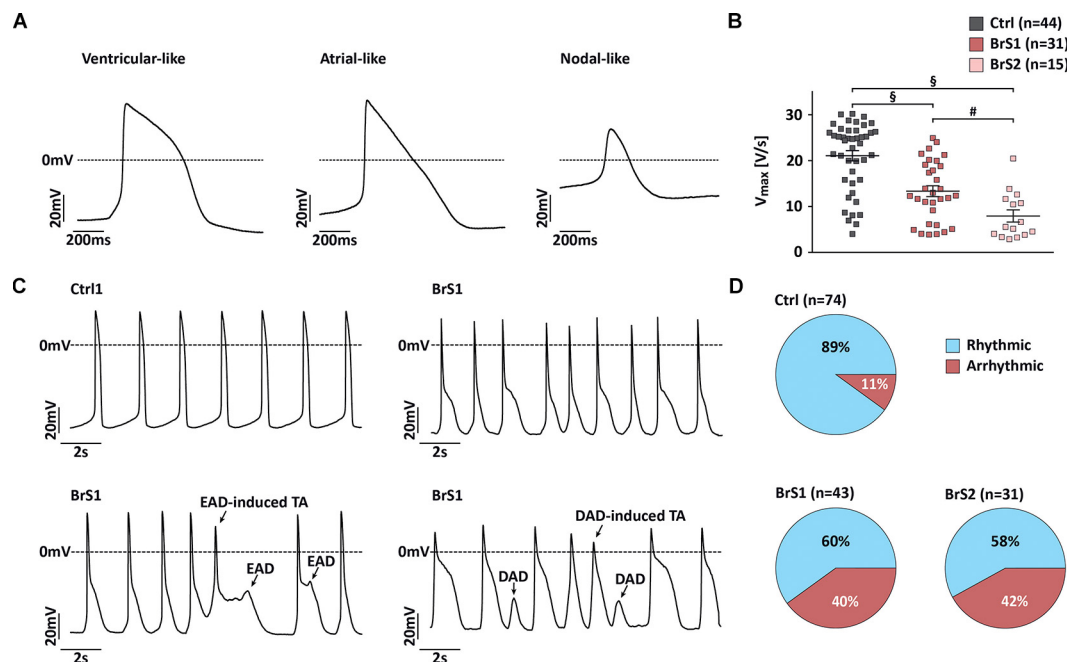


FIGURE 5 | Action potential characterization of Ctrl- and BrS-CMs. **(A)** Representative traces of spontaneous action potentials (APs) measured in ventricular-, atrial-, and nodal-like CMs. **(B)** V_{max} in ventricular- and atrial-like BrS- and Ctrl-CMs. Data are presented as mean $\pm$ SEM. One-way ANOVA followed by Tukey's *post hoc* test was used for statistical analysis: # $P < 0.01$, § $P < 0.001$. **(C)** Representative traces of spontaneous AP recordings from Ctrl- and BrS-CMs. **(D)** Percentage of Ctrl- and BrS-CMs with spontaneously (ar)hythmic beating. Two-tailed Fisher's exact test was used for statistical analysis.

Cx43 at the sites of cell-to-cell appositions in both Ctrl- and BrS-CMs, however, in BrS-CMs the co-localization of $Na_V1.5$ and Cx43 seems altered. Physical and functional interactions of sodium channel complex with gap junction and desmosomal components at intercalated discs in CMs are essential for cardiac excitability and conduction (Delmar, 2012; Cerrone and Delmar, 2014). Previous study demonstrated that loss of the gap junction protein Cx43 resulted in diminished $Na_V1.5$ expression at intercalated discs (Jansen et al., 2012). In our study, we observed a reduced expression of Cx43 in BrS-CMs, this brings up an open question: how the reduced expression of $Na_V1.5$ regulates the Cx43 expression, which should be clarified in future studies. Nonetheless, our data indicate that BrS-CMs with loss-of-function mutation p.S1812X in *SCN5A* reveal some pathophysiological phenotypes including the conduction slowing, supporting the “depolarization disorder” theory as the underlying mechanism of BrS.

Notably, we show that I_{to} is present in 3-month-old iPSC-CMs and, to our surprise, the I_{to} was significantly larger in BrS-CMs than in Ctrl-CMs. However, genetic analysis in both patients did not find mutations in *KCND3*, *KCNQ1*, *HERG*, and *KCNE1* (Schulze-Bahr et al., 2003). The 4-AP sensitivity experiments confirmed that the currents we recorded were I_{to} . Moreover, we observed a prominent AP notch in BrS-CMs. Previous studies showed that I_{to} is pivotal in generating the disease phenotype in BrS (Di Diego et al., 2002; Giudicessi et al., 2011). The direct relationship between I_{to} and BrS was first reported by applying the I_{to} activator NS5806 in isolated canine

ventricular wedge preparations. NS5806 could increase phase 1 and notch amplitude of the AP in the epicardium, but not in the endocardium, and accentuate J-wave in the ECG (Calloe et al., 2009). It is well accepted that the orderly sequence of repolarization in the heart is linked to heterogeneous distribution of the fast component of I_{to} in right ventricle epicardium and endocardium. The highest density of I_{to} is seen in epicardial CMs, whereas the lowest density is observed in endocardial CMs (Litovsky and Antzelevitch, 1988; Wettwer et al., 1994; Costantini et al., 2005). The changes in repolarization might lead to the so-called phase 2 re-entry and polymorphic ventricular tachycardia. One limitation of our study is that the *in vitro* BrS-CM model cannot recapitulate the *in vivo* heterogeneous distribution of I_{to} in right ventricular epicardium and endocardium. Nonetheless, our data demonstrate that the increased I_{to} in BrS-CMs is associated to the disease phenotype, and are in line with the previous studies (Delpon et al., 2008; Giudicessi et al., 2011; Perrin et al., 2014). Gain-of-function mutations in *KCND3* encoding $K_V4.3$ were found in patients with BrS, leading to the loss of the AP dome in the modified Luo-Rudy II AP model as a result of the increased I_{to} (Giudicessi et al., 2011). A mutation in *KCNE3* encoding a β -subunit of the $K_V4.3$ channel was found in a patient with BrS, which resulted in the reduction of its inhibitory effect on the $K_V4.3$ channel, leading to an increase in I_{to} (Delpon et al., 2008). Additionally, the mutation p.D612N in *KCND2* coding for $K_V4.2$ was identified responsible for the increased J-wave manifestation (Perrin et al., 2014). On the contrary, two recent studies reported some different findings. Different

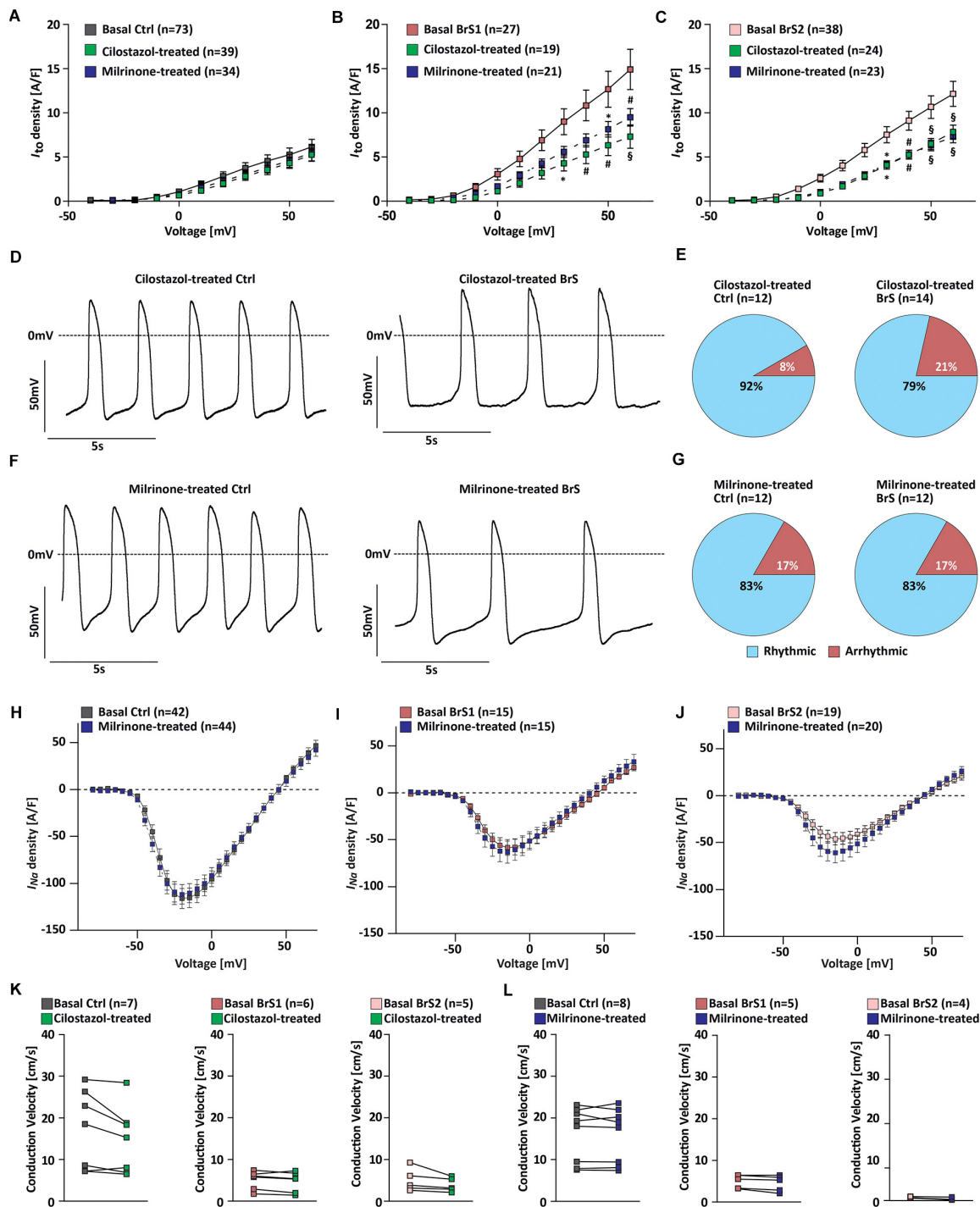


FIGURE 6 | Effects of PDE inhibitor cilostazol or milrinone on electrophysiological properties of Ctrl- and BrS-CMs. Average current–voltage relationships of I_{to} in Ctrl-CMs (A), BrS1-CMs (B), and BrS2-CMs (C) with and without cilostazol (10 μ M) or milrinone (2.5 μ M) treatment for half an hour. Data are presented mean $\pm$ SEM. (D) Representative original action potential (AP) traces of Ctrl- and BrS-CMs after 10 μ M cilostazol treatment. (E) Percentages of Ctrl- and BrS-CMs with rhythmic and arrhythmic APs after 10 μ M cilostazol treatment. (F) Representative original AP traces of Ctrl- and BrS-CMs after 2.5 μ M milrinone treatment. (G) Percentages of Ctrl- and BrS-CMs with rhythmic and arrhythmic APs after 2.5 μ M milrinone treatment. (H) Average I – V curves of I_{Na} (automated patch-clamp) in Ctrl-CMs (H), BrS1-CMs (I), and BrS2-CMs (J) with and without 2.5 μ M milrinone treatment for half an hour. No statistically significant differences were observed. (K) Quantitative analysis of the conduction velocity in Ctrl-, BrS1-, and BrS2-CMs with and without cilostazol (10 μ M) treatment. No statistically significant differences were observed. (L) Quantitative analysis of the conduction velocity in Ctrl-, BrS1-, and BrS2-CMs with and without 2.5 μ M milrinone treatment. No statistically significant differences were observed. Two-way repeated measures ANOVA (A–C,H–J) and two-tailed paired Student's t -test (K,L) were used for statistical analysis: * $P < 0.05$, # $P < 0.01$, § $P < 0.001$.

with our increased mRNA expression of *KCND2/3*, a significant decrease in the gene expression of *KCND3* was revealed in CMs derived from iPSCs generated from two BrS patients carrying the *SCN5A* R620H-R811H mutation and *SCN5A* Δ 1397 mutation, respectively (Liang et al., 2016). Moreover, the I_{to} current density remained unaltered in BrS iPSC-CMs with *SCN5A* A226V-R1629X variant (Ma et al., 2018). Although our data showing the increased I_{to} in BrS-CMs support the “repolarization disorder” theory of BrS, further studies need to investigate how the *SCN5A* mutation p.S1812X affects the transient-outward potassium channels, especially when we did not observe changes in Kv4.3 protein expression in BrS-CMs. We postulate that the augmented I_{to} may result from the post-translational modification of Kv4.3 channel and impaired protein-protein interaction. It is worth mentioning that there is an inverse relationship between the maximum velocities of depolarization and repolarization, as demonstrated in murine isolated ventricular CMs as well as in HEK293 cells overexpressing both Kv4.3 and Nav1.5 (Portero et al., 2018). Therefore, it is of vital importance to further study the entire molecular complexity, in which Nav1.5 is naturally embedded in human CMs and to assess the functional interactions of different ion channels.

Moreover, in our study higher arrhythmic tendencies in BrS-CMs were observed. Spontaneous AP recordings revealed a higher percentage of BrS-CMs with arrhythmogenic events, and FP recordings displayed the increased SD of the inter-beat intervals. Unlike type 3 long QT syndrome, in which most of the *SCN5A* mutations cause a significantly enhanced persistent I_{Na} contributing to the life-threatening ventricular arrhythmias (Malan et al., 2011; Ma et al., 2013), the mutation p.S1812X in our study did not alter the persistent I_{Na} in BrS-CMs. To our surprise, the window current probability of I_{CaL} was significantly increased in BrS-CMs compared to Ctrl-CMs, which is associated with the alterations in the steady-state activation and steady-state inactivation of the L-type calcium channels. Previous studies demonstrated that the increased I_{CaL} window current contributed to the EAD formation and EAD-mediated arrhythmias (Madhvani et al., 2015). In iPSC-CMs derived from BrS patients without identified mutations, no significant changes in the steady-state activation and steady-state inactivation of the L-type calcium channels were observed, but the probability of I_{CaL} window current was not calculated (Veerman et al., 2016). Future studies should investigate how the different channels in CMs, including the channels responsible for I_{CaL} , I_{to} , and I_{Na} , co-operate and regulate the arrhythmia in BrS-CMs.

Based on the I_{to} augment in our BrS-CMs, we speculate that the alleviation of AP arrhythmia could be possibly achieved through the reduction of I_{to} currents. Cilostazol and milrinone are two oral PDE3 inhibitors, which might be potentially antiarrhythmic drugs for BrS patients (Tsuchiya et al., 2002). Previous study reported that the ventricular fibrillation of a 67-year-old patient with BrS was prevented by oral cilostazol administration (Tsuchiya et al., 2002). In the setting of an arterially perfused right ventricular wedge, cilostazol and milrinone could suppress arrhythmogenesis associated with BrS (Szel et al., 2013; Szel and Antzelevitch, 2014). The effects of cilostazol and milrinone may be related to an elevation of

intracellular cyclic AMP concentration via inhibition of PDE activity, and consequently to suppression of I_{to} and to an increase in I_{Ca} , as previously discussed (Tsuchiya et al., 2002; Szel et al., 2013; Szel and Antzelevitch, 2014). Patocskaï et al. (2016) demonstrated that both cilostazol and milrinone reduced I_{to} in canine single left ventricular CMs, and addition of cilostazol or milrinone to the coronary perfusate restored the AP dome at all epicardial sites, reduced epicardial and transmural dispersion of repolarization, decreased J point and ST segment elevation, and terminated all arrhythmic activity in an experimental model of early repolarization syndrome. In our study, inhibition effects of cilostazol and milrinone on I_{to} and arrhythmogenic events in BrS-CMs suggest a new therapeutic potential to alleviate BrS probably via I_{to} reduction. Nevertheless, cilostazol and milrinone do not have effects on I_{Na} or CV.

CONCLUSION

By using iPSC-CMs from two BrS patients harboring the *SCN5A* mutation p.S1812X, we demonstrate here that the mutation results in the reduced I_{Na} but augmented I_{to} and increased I_{CaL} window current probability as well as conduction slowing, indicating that both repolarization and depolarization disorders coexist in BrS-CMs. Our findings indicate that the electrophysiological mechanisms underlying conduction slowing and arrhythmia in BrS-CMs involve not only the Nav1.5 loss-of-function but also an impaired coordination of I_{Na} , I_{to} , and I_{CaL} . Moreover, pharmacological treatments with the PDE3 inhibitors cilostazol and milrinone reduce the I_{to} and proarrhythmic events in BrS, suggesting their therapeutic potential for BrS patients.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/Supplementary Material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee of University Medical Center Göttingen (approval number 21/1/11) and of Technical University of Dresden (approval number EK 422092019) and carried out in accordance with the approved guidelines. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WL, MiS, SW, and KG conceived the study and designed experiments. WL, MiS, XJL, MV, CSM, MaS, SC, GW, and S-MH performed experiments and acquired data. WL, MiS, XJL, SW, MV, MaS, and KG analyzed the data. WL, MiS, SW, LC, GH, LSM, AE-A, and KG contributed to the interpretation of the data. WL, MiS, and

KG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.592893/full#supplementary-material>

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RAD-Deficient Human Cardiomyocytes Develop Hypertrophic Cardiomyopathy Phenotypes Due to Calcium Dysregulation

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Ras associated with diabetes (RAD) is a membrane protein that acts as a calcium channel regulator by interacting with cardiac L-type Ca^{2+} channels (LTCC). RAD defects can disrupt intracellular calcium dynamics and lead to cardiac hypertrophy. However, due to the lack of reliable human disease models, the pathological mechanism of RAD deficiency leading to cardiac hypertrophy is not well understood. In this study, we created a $RRAD^{-/-}$ H9 cell line using CRISPR/Cas9 technology. RAD disruption did not affect the ability and efficiency of cardiomyocytes differentiation. However, RAD deficient hESC-CMs recapitulate hypertrophic phenotype *in vitro*. Further studies have shown that elevated intracellular calcium level and abnormal calcium regulation are the core mechanisms by which RAD deficiency leads to cardiac hypertrophy. More importantly, management of calcium dysregulation has been found to be an effective way to prevent the development of cardiac hypertrophy *in vitro*.

Keywords: $RRAD$ knockout, RAD deficiency, HCM, L-type Ca^{2+} channels, calcium handling, calcium channel blocker

INTRODUCTION

Cardiac hypertrophy is a significant adaptive change in response to various stimuli from inside and outside the body. Physiological hypertrophy can preserve cardiac function, while pathological hypertrophy is often accompanied by some adverse events, such as arrhythmia, heart failure, sudden cardiac death (SCD), thus becomes an independent risk factor for cardiac mortality

(Shimizu and Minamino, 2016; Nakamura and Sadoshima, 2018). It is said that some regulatory genes related to calcium handling have been found to be involved in the pathogenesis of cardiac hypertrophy (Chang et al., 2007).

Ras associated with diabetes (RAD), a membrane protein consists of 308 amino acids, is encoded by human *RRAD* gene and is highly expressed in cardiomyocytes (Reynet and Kahn, 1993; Maguire et al., 1994). It acts as a calcium channel regulator by interacting with cardiac L-type Ca^{2+} channels (LTCC), which play a fundamental role in normal heart (Finlin et al., 2003). Previous evidence has shown that elevated intracellular Ca^{2+} and abnormal calcium regulation are the central mechanism for inducing cardiac hypertrophy (Lan et al., 2013; Monteiro da Rocha et al., 2016; Dewenter et al., 2017; Li et al., 2019). Studies on mice indicated that deficiency of RAD function in cardiomyocytes, which can lead to an increased L-type Ca^{2+} current ($I_{\text{Ca-L}}$) via upregulation of LTCC expression in the plasma membrane (Yada et al., 2007), significantly increased stress-induced cardiac hypertrophy and remodeling *in vitro* (Chang et al., 2007) and cardiac fibrosis *in vivo* (Zhang et al., 2011). In addition, RAD is significantly decreased in human failing hearts (Chang et al., 2007). Recently, a new study identified a rare missense *RRAD* mutation (p.R211H) in Brugada syndrome patient, which can disturb Na^+ current (I_{Na}) and $I_{\text{Ca-L}}$ thus leads to structural and electrical defects in cardiomyocytes (Belbachir et al., 2019). However, due to the lack of a human heart disease model, the role of RAD functional defects in the human heart is unclear.

Although efforts over past few decades have revealed the molecular function and pathogenic mechanism of RAD to a certain extent, these results are basically derived from the mouse model. However, due to species differences, cardiac electrophysiological characteristics are significant different between humans and mice. For example, mouse have a higher resting heart rate (500–700 bpm), a more negative action potential plateau, and a shorter action potential duration (APD) compared to human (Watanabe et al., 2011). Thus, mouse models could not effectively mimic the pathological process of human heart diseases. It is of great significance to establish a reliable human heart disease model to study RAD function. Fortunately, the rapid development of human pluripotent stem cells (PSCs) technology in recent years has provided a powerful tool for studying human cardiovascular diseases (Monteiro da Rocha et al., 2016; Chelko et al., 2019; Park et al., 2019).

Thus, we created a human embryonic stem cell line (hESCs) with RAD deficiency (*RRAD*^{-/-}) using CRISPR/Cas9 technology to explore the function of *RRAD* gene. After differentiated both wild type (WT) and *RRAD*^{-/-} hESCs (KO) into cardiomyocytes *in vitro*, we were surprised to find that cardiomyocytes derived from *RRAD*^{-/-} hESCs exhibited a distinct hypertrophic phenotype compared to wild type. Further studies confirmed that abnormal regulation of intracellular calcium level may be a major mechanism of cardiac hypertrophy. Based on the results, early interventions for abnormal calcium handling can prevent this pathological process.

MATERIALS AND METHODS

Human Embryonic Stem Cell Culture and Cardiac Differentiation

This study was approved by the Ethics Committee of Anzhen Hospital, Capital Medical University (#134/18). Human embryonic stem cell H9 (hESC-H9) were cultured on Matrigel-coated (Corning, United States) feeder-free plates with E8 medium (Cellapy, China). Cells were changed with fresh medium every day and passaged routinely using 0.5 mM EDTA without MgCl_2 or CaCl_2 (HyClone, United States) when confluence reaches 80%. Cells were maintained at 37°C, 5% CO_2 incubator.

Human embryonic stem cell H9 were differentiated into cardiomyocytes using a chemically defined small molecule-based protocol as previously reported (Burridge et al., 2014). Purification of cardiomyocytes using a metabolic-selection method as previous described (Tohyama et al., 2013).

Genome Editing

Single guide RNA (sgRNA) (TGCAGGTCGCGCTCGTCCAC) targeting *RRAD* gene were designed for next gene knock-out. The sgRNA was then ligated into the epiCRISPR plasmid as previously described (Xie et al., 2017). After disassociated into single cells, about 1×10^6 H9 cells were electroporated with 2–5 μg epiCRISPR plasmid containing the sgRNA using 4D nucleofector system (Lonza, Germany). Cells were then cultured with E8 medium supplemented with 10 μM Y-27632 (Rho kinase inhibitor) at the first 24 h and then selected with puromycin for 3–5 days. The positive clones were picked into 24-well plate for sequencing identification.

The protocol of both H9 and H9-*RRAD*^{-/-} GCaMP reporter cell lines were generated as previously reported (Li et al., 2019).

Immunostaining and Imaging Analyses

Cells were fixed with 4% PFA for 30 min, permeabilized with 0.5% Triton X-100 (Sigma, United States) for 15 min, and blocked with 3% BSA (Sigma) for 30 min. Cells were then incubated with primary antibodies overnight. After washed with PBS 3 times, cells were then incubated with secondary antibodies for 45 min at 37°C and counterstained with DAPI (Invitrogen, United States) for 10 min. Fluorescence images were performed under Leica DMI 4000B. Both primary and secondary antibodies were listed at an appropriate dilution in **Supplementary Table S2**.

Flow Cytometry

Both WT and KO cardiomyocytes were dissociated into single cells using CardioEasy CM dissociation buffer (Cellapy), and fixed with chilled fixation buffer (BD Biosciences) for 10–15 min at 25°C. Fixed cardiomyocytes were incubated with anti-cardiac troponin T antibody (cTnT) and then Alexa Fluor 488 secondary antibodies for 30 min, respectively. Cardiomyocytes were then washed with PBS three times and analyzed using FACS analysis equipment (EPICS XL, Beckman). Data were analyzed with FlowJo X software.

For multinuclear detection experiments, the cells were fixed with chilled 70% ethanol at -20°C for 24 h, washed with PBS one time, stained with 50 $\mu\text{g}/\text{mL}$ propidium iodide (Becton, Dickinson and Company, Franklin lake, NJ, United States) at room temperature for 15–20 min and analyzed using EPICS XL (Beckman Coulter). Data were analyzed with Modfit LT Software.

Cellular Ca^{2+} Imaging

Cardiomyocytes derived from H9-GCaMP and $\text{RRAD}^{-/-}$ -GCaMP lines were seeded onto Matrigel-coated confocal dishes or eight-well chambers. Intracellular Ca^{2+} flux was imaged at $40\times$ using a confocal microscope (Leica, TCS5 SP5, Germany). Spontaneous intracellular Ca^{2+} transients were recorded at 37°C , 5% CO_2 using standard line-scan mode. A total of 8192 line-scans were acquired for 8.192 s recording durations. For caffeine-induced Ca^{2+} release, 20 μM caffeine was used to release SR Ca^{2+} . Results were analyzed using Image J and Igor software.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from $(0.5-1) \times 10^6$ cells with TRIzol Reagent (Life Technologies, United States) and then treated with DNase I (Life Technologies) for 30 min at 37°C to remove DNA contamination. cDNA was prepared using the PrimeScriptTM reverse transcription system (Takara, Japan) as manufacturer's instructions. The gene expression levels were examined by quantitative real-time polymerase chain reaction (qRT-PCR) using the iCycler iQ5 (Bio-Rad) with $2 \times$ SYBR Master Mix (Takara) and the relative quantification was analyzed according to the ΔCT method. All primer sequences used were listed in **Supplementary Table S1**.

RNASeq Processing and Biological Information Analysis

Construction and sequencing of transcriptome libraries were executed by Annoroad Gene Technology Corporation (Beijing, China). Quality-qualified libraries were selected for sequencing using the Illumina platform. The sequencing strategy was PE150. The Raw Reads sequence from the Illumina platform was sequenced to obtain high-quality sequences (Clean Reads) by de-sequencing low-quality sequences and de-junction contamination, etc. All subsequent analyses were based on Clean Reads. Gene differential expression analysis were performed using DESeq2. The differential gene screening was mainly based on the difference fold (Fold change value) and the q value (padj value, P value after correction). Differential genes with $|\log_2 \text{Fold change}| \geq 1$ and $q < 0.05$ were usually selected as differentially expressed genes (DEGs). Heatmaps of hierarchical clustering analysis of DEGs was performed using R-package. Gene ontology (GO) enrichment analysis was performed on these DEGs, and a false discovery rate (FDR) of less than 0.05 was considered to be significantly enriched. For KEGG functional analysis, enrichment analysis was performed on each pathway using hypergeometric tests to identify pathways for significant enrichment in DEGs.

Electrophysiological Recording

L-type calcium current ($I_{\text{Ca-L}}$) in hESCs-CM was recorded by using EPC-10 amplifier system (HEKA Elektronik, Lambrecht, Germany) under whole-cell patch-clamp configuration. Firstly, hESCs-CMs were plated onto 13-mm glass coverslips and current recording was performed 48–72 h later. For recording of the $I_{\text{Ca-L}}$ in hESCs-CMs, the bath solution contained (in mM): 140 TEA, 5 BaCl_2 and 10 HEPES adjusted to pH 7.3 with CsOH. The pipette solution contained (in mM): 145 CsCl, 145 Cs-MeSO₃, 1 MgCl_2 , 4 Mg-ATP, 0.5 EGTA and 10 HEPES, adjusted to pH 7.3 with CsOH. Series resistance (R_s) was compensated by about 70% and less than $10 \text{ M}\Omega$ to minimize voltage errors. $I_{\text{Ca-L}}$ was measured at the holding potential of -80 mV followed by 300 ms depolarization test pulse steps from -50 to $+60 \text{ mV}$ in 10 mV increments with 1 s test interval. 200 nM nifedipine, a specific blocker of L-type voltage-gated Ca^{2+} channels, was added to the external solution to identify the current. All experiments were performed at room temperature, $22 \pm 1^{\circ}\text{C}$.

Western Blot Analysis

Cells to be tested were washed with pre-cooled PBS 3 times and then lysed with Mammalian Protein Extraction Reagent (Thermo, #78501, United States) containing 5 mM EDTA (Thermo, #1861275), protease inhibitor cocktail (Thermo, #1861278) and phosphatase inhibitor cocktail (Thermo, #1862495). Cell lysates collected were then vibrated three times every 10 min and centrifuged at 12,000–15,000 rpm for 15 min. For the extraction of membrane proteins, we used the Membrane Protein Extraction Kit (Thermo, #89842, United States) following the manufacturer's instructions. The concentration of supernatant protein was determined by PierceTM BCA Protein Assay Kit (Thermo, #23227). Next, 20–40 μg denatured protein samples were detected by electrophoresis on 6–12% [depending on the molecular weight of the protein to be tested. For proteins with a molecular weight greater than 250 KD, we used 6% sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE), added 0.5% SDS to the electrophoretic buffer, and reduced the methanol concentration to 10%]. SDS-PAGE and transferred to PVDF membrane or nitrocellulose membrane at 300 mA for 90–180 min (depending on the molecular weight of the protein to be tested) using gel transfer device (Bio-Rad). After blocked with 5% skimmed milk powder for an hour at room temperature, membranes were incubated with primary antibodies at 4°C overnight and then detected with corresponding secondary antibody. Both primary and secondary antibodies were listed in the **Supplementary Table S2** at an appropriate concentration.

Statistical Analysis

All experiments were repeated at least three times and data were shown as mean $\pm$ standard errors of the means (S.E.M.). Statistical comparisons were determined using two-sided Student's t -test between two groups or one-way ANOVA tests followed by Tukey's Multiple Comparison Test among multiple groups. Statistically significant was determined as a value of $P < 0.05$.

RESULTS

Establishment of *RRAD*^{-/-} hESC Line

To establish a genetical platform for exploring the function of *RRAD* gene, a sgRNA targeting exon 2 of *RRAD* were designed (Figure 1A). After electroporated with plasmid (containing sgRNA and epiCRISPR/Cas9) and selected with puromycin, we screened twelve clones for genotyping. Among these, one was no editing, six were heterozygous (with an unedited wild type allele) and five were homozygous (biallelic mutations). However, of the five homozygous, the sequencing results of two clones showed no frameshift mutations (one was edited with -21 bp and the other with +3 bp), so it was not used in the next experiment (data not shown). The sequencing results of the other three homozygous were: #7: -55 bp; #9: -14 bp; #12: +1 bp, -8 bp (Figure 1B and Supplementary Figure S1A). We selected #7, with 55 base pairs deletion resulting in frame-shifted coding sequence, for subsequent research (Figure 1B). Besides, in order to determine whether the *RRAD* gene knockout played the same role in different stem cell lines, we also established a *RRAD*^{-/-} hESCs-NKX2-5-GFP cell line using the same method. We selected five clones for genotyping. Among these, two were no editing, two were heterozygous (with an unedited wild type allele) and one were homozygous (biallelic mutations). This homozygous was edited by inserting 7 base pairs near the PAM region, causing a frameshift mutation (Supplementary Figure S1B). In addition, we analyzed the top ten off-target sites of sgRNA with online analysis software¹, and no off-target mutations were found identified by DNA sequencing (data not shown) (Pankowicz et al., 2016). *RRAD*^{-/-} line exhibited normal morphology (Supplementary Figure S1C), expressed pluripotency markers OCT4 and SSEA4 (Figure 1C), and had no chromosomal abnormalities (Supplementary Figure S1D). Teratoma formation assay generated cellular derivatives of three germ layers *in vivo* indicated that *RRAD* knockout did not affect the pluripotent nature of hESCs (Supplementary Figure S1E).

Differentiation of *RRAD*^{-/-} hESCs Into Cardiomyocytes

Both established *RRAD*^{-/-} line and WT hESCs were then differentiated into cardiomyocyte lineages (hESC-CMs) using standard small molecule-based protocols (Figure 1D). From 7 days after initiation of differentiation, spontaneous beating could be detected in WT (60 ± 5 beats per min) and KO (57 ± 7 beats per min) cardiomyocytes and no significantly difference in beating rates between the two groups (Supplementary Figures S2A,B). At day 15, we identified by western blot that cardiomyocytes derived from *RRAD*^{-/-} line exhibited complete loss of RAD protein (Figure 1E). Flow cytometry for cardiac Troponin T indicated that cardiomyocytes both derived from WT and KO were reached more than 85% (Figures 1F,G). Immunostaining for MLC2v and MLC2a demonstrated that about 80% cardiomyocytes of both WT and KO were positive for ventricular-specific marker MLC2v (Figures 1H,I). Taken

together, these findings suggested that *RRAD* knockout did not affect the ability and efficiency of myocardial differentiation.

RRAD^{-/-} Cardiomyocytes Recapitulate Hypertrophic Phenotype *in vitro*

Clinically, dysfunction in cardiac hypertrophy is mainly owing to thickening ventricular wall and cardiomyocytes hypertrophy (Harvey and Leinwand, 2011). As present study shows, both hESC-H9 and hESCs-NKX2-5-GFP-derived *RRAD*^{-/-} cardiomyocytes were noticeably larger than WT at day 40 post-induction (Figure 2A and Supplementary Figure S2C). Further verification by flow cytometry, measured with forward scatter of calibration spheres, also validated this conclusion (Figures 2B,C). In addition to increased size of cardiomyocytes, multi-nucleation (Arad et al., 2002) and disorganized arrangement of myofibrils (Kraft et al., 2013; Tanaka et al., 2014) are also common features of cardiac hypertrophy. Further immunofluorescence staining for cardiac Troponin T and α -actinin exhibited significantly higher frequencies of myofibrillar disarray (Figures 2D,E and Supplementary Figure S2C) and multinucleation (Figures 2F,G) in *RRAD*^{-/-} cardiomyocytes compared to WT. Also, flow cytometry shows an increased percentage of tetraploid and hexaploid in *RRAD*^{-/-} cardiomyocytes (Supplementary Figure S2D). Taken together, these findings suggested that cardiomyocytes derived from *RRAD*^{-/-} line could recapitulate hypertrophic phenotype *in vitro*.

Differential Transcriptome of *RRAD*^{-/-} Cardiomyocytes

To explore the potential molecular mechanisms of hypertrophic phenotype caused by RAD deficiency, we examined the transcriptome characteristics of *RRAD*^{-/-} cardiomyocytes in comparison to WT cardiomyocytes. We found a total of 2664 transcripts differentially expressed between *RRAD*^{-/-} and WT cardiomyocytes, from which, 1054 transcripts were upregulated, and 1610 transcripts were downregulated (Figure 3A). After GO enrichment analysis of differentially expressed transcripts, we identified 17, 25, and 12 categories in cellular component, biological process, molecular function, respectively (Figure 3B and Supplementary Figures S3A–C). Among them, ion binding and biological regulation were the most differentiated categories within molecular function and biological process, respectively, indicated that RAD plays a vital role in biological regulation and closely related to the function of ions, which is consistent with previous researches (Figure 3B and Supplementary Figures S3A–C; Yada et al., 2007; Belbachir et al., 2019). To explore the biological impacts of these differentially expressed transcripts, we next performed KEGG (Kyoto Encyclopedia of Genes and Genomes) functional analysis. Results as shown in Figure 3C, although “circadian entrainment” and “axon guidance” rank first and second in GO analysis of 15 significantly different pathways, they were involved in circadian rhythm control of organism (Golombek and Rosenstein, 2010) and neurological disorders (Van Battum et al., 2015), respectively. We focused on the third “calcium signal pathway” for it is closely related to the physiological function of *RRAD* gene and it has

¹<http://crispr.mit.edu>

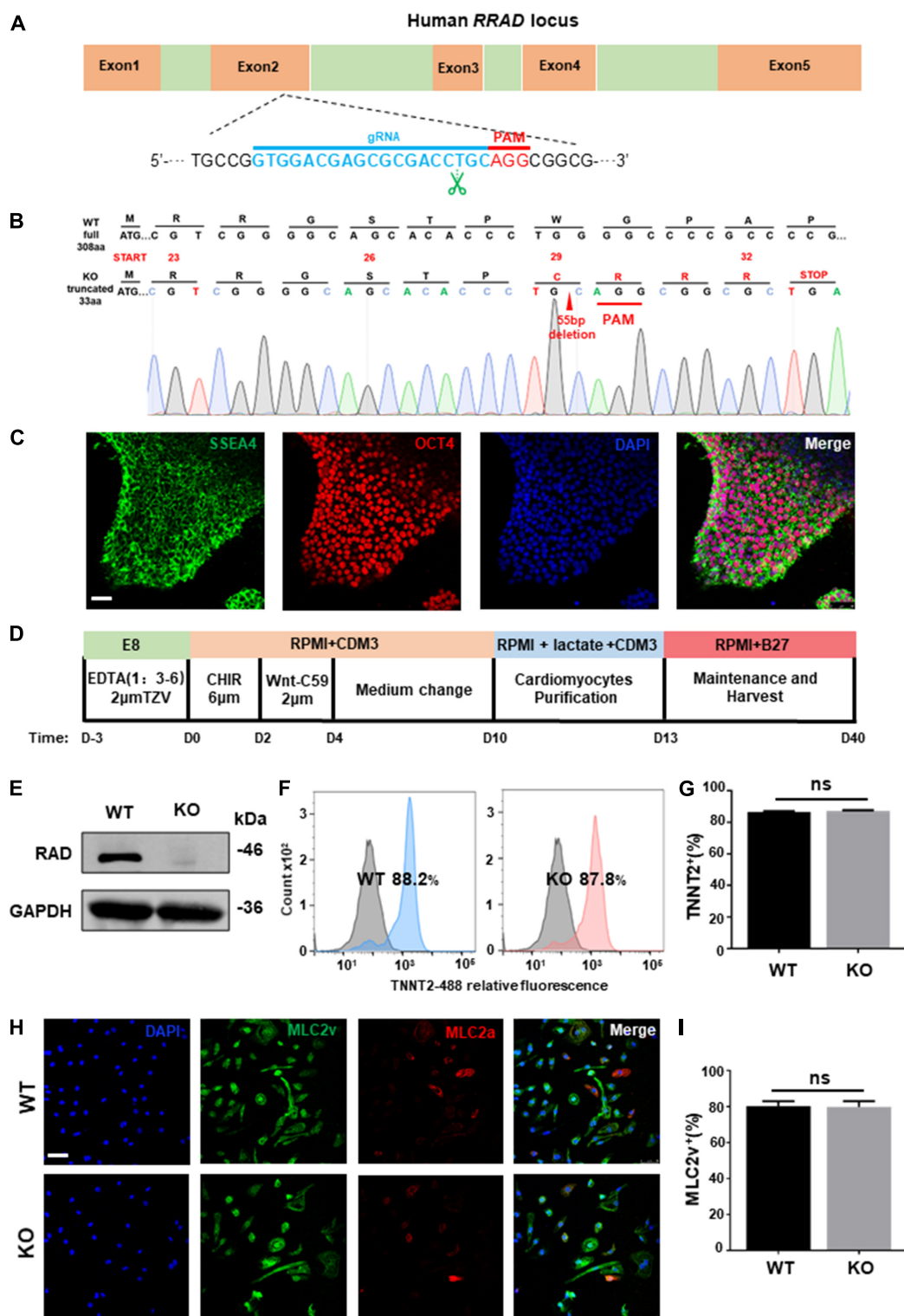


FIGURE 1 | *RRAD* knockout did not affect the pluripotency nature of hESC and the ability of cardiac differentiation. **(A)** Schematic of human *RRAD* locus and editing site of designed gRNA-epiCRISPR/Cas9. **(B)** Sequencing data shows deletion of 55 bases in *RRAD* knockout hESC lines. **(C)** Immunostaining for the pluripotency markers SSEA4 and OCT4 of *RRAD* knockout hESC lines. Scalebar, 50 μ m. **(D)** Schematic of cardiac differentiation using standard small molecule-based protocols. **(E)** Western blot shows RAD expression in WT and KO CMs at days 15. **(F,G)** Flow Cytometry for TNNT2 staining in representative WT and KO CMs at days 10 without purification. **(H,I)** Immunostaining for protein expression of MLC2v and MLC2a in WT and KO CMs at days 30. Scalebar, 50 μ m. Data are expressed as means $\pm$ S.E.M. of three independent experiments. ns, not significant.

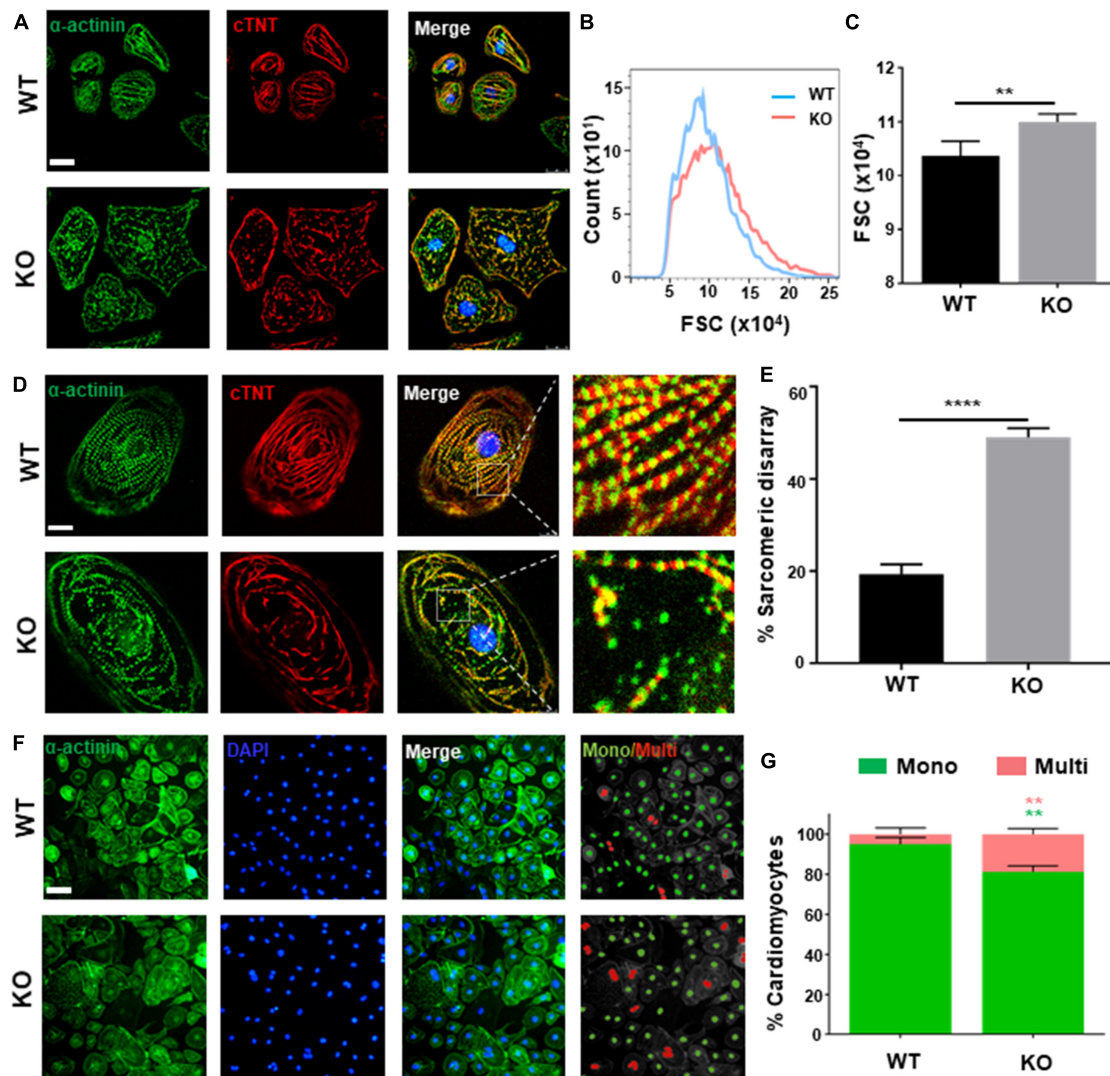


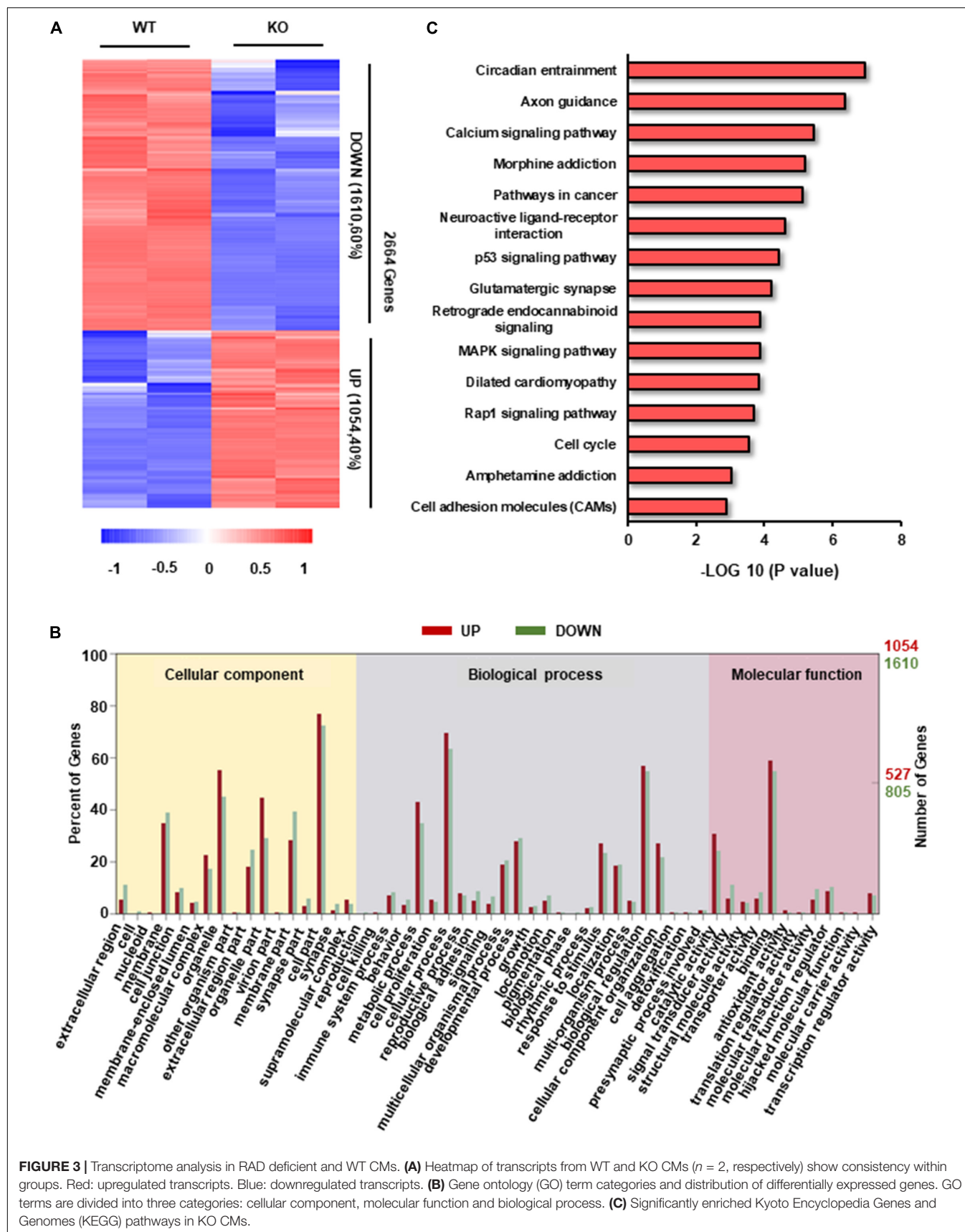
FIGURE 2 | Phenotyping cardiac hypertrophy in RAD deficient CMs. **(A)** Images of α -actinin (green) and cTnT (red) immunostaining show an increased size in KO CMs at days 40. Scale bar, 50 μ m. **(B,C)** Calibration of forward scatter (FSC; 10000 cells/sample, $n = 3$) shows an increased size in KO CMs at days 40. **(D)** Images of α -actinin (green) and cTnT (red) immunostaining show disorganized arrangement of myofibrils in KO CMs. Scale bar, 50 μ m. **(E)** Quantification of disorganized myofibrils in WT ($n = 206$) and KO CMs ($n = 198$). **(F)** Images of α -actinin/DAPI immunostaining. Scale bar, 50 μ m. **(G)** Quantification of mono- and multi-nucleation in WT ($n = 236$) and KO ($n = 262$) CMs. Data are expressed as means $\pm$ S.E.M. of three independent experiments. ** $P < 0.01$; **** $P < 0.0001$.

been confirmed as the central mechanism of cardiac hypertrophy in previous studies (Lan et al., 2013; Dewenter et al., 2017; Li et al., 2019). Taken together, these findings suggested that RAD is involved in the regulation of biological processes and is closely related to the regulation of calcium ion function.

RRAD^{-/-} Cardiomyocytes Display Abnormal Regulation of Intracellular Calcium

Homeostasis of calcium activity is crucial for cardiac excitation-contraction (EC) coupling and electrophysiological properties, early disruption of which, however, can lead to various dysfunctions such as cardiac hypertrophy and arrhythmia (Bers,

2008; Monteiro da Rocha et al., 2016). To explore the possible effects of RAD deficiency on calcium regulation, we generated GCaMP reporter cells as previously reported (Li et al., 2019) and assessed calcium transient of cardiomyocytes in both WT and KO lines at days 20, 30, and 40 post-induction. Results as shown in **Figures 4A,B**, cardiomyocytes derived from KO line showed smaller amplitude and significantly altered calcium transient which may be associated with arrhythmia-like voltage waveforms compared to control cells. Furthermore, abnormal calcium transients were observed at day 30 in KO CMs prior to the onset of hypertrophic phenotype, indicating that abnormal regulation of intracellular calcium level may be a critical factor in the pathogenesis of cardiac hypertrophy at cellular level (**Figure 4C**). Measurement of sarcoplasmic reticulum (SR) calcium stores



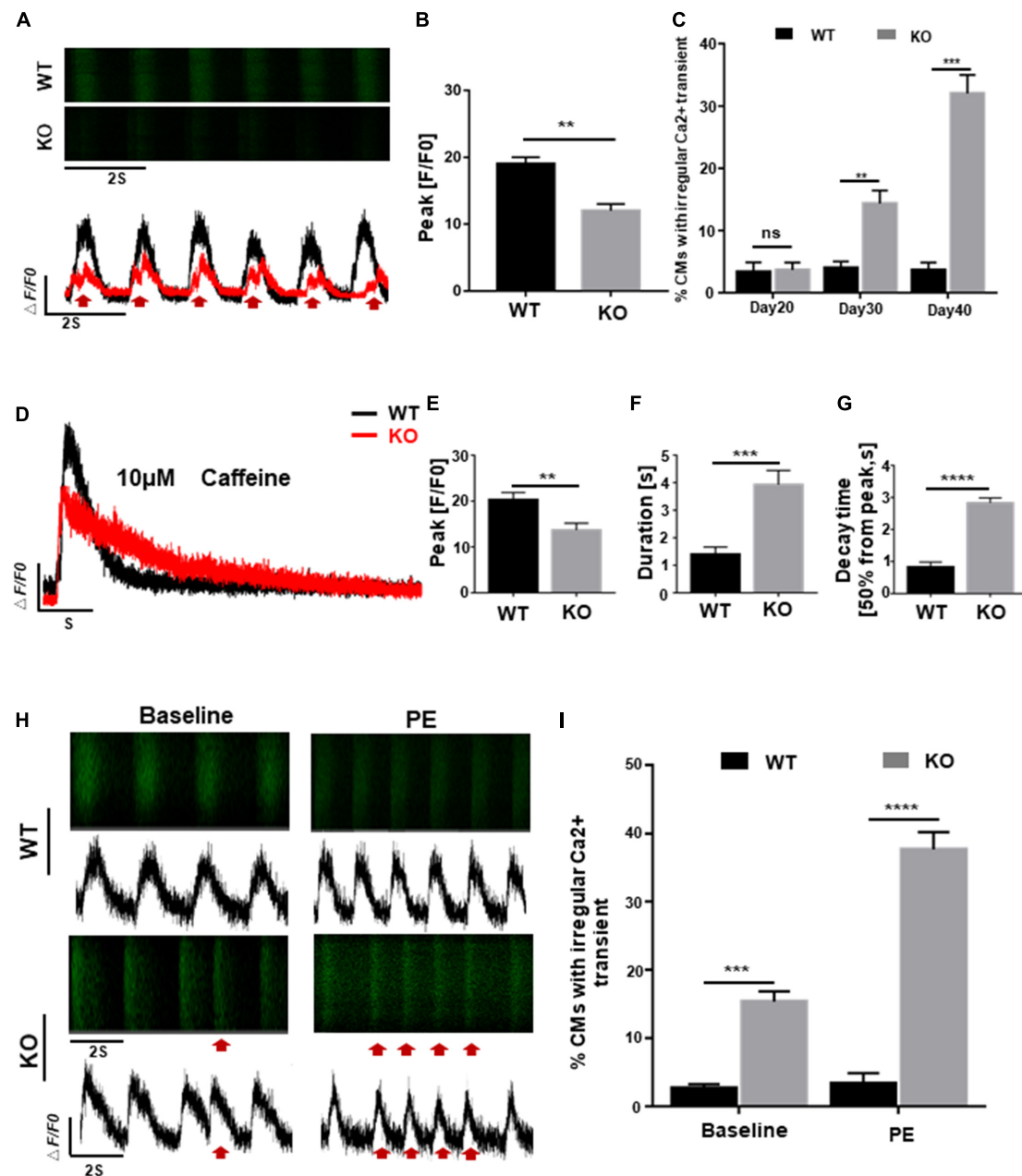


FIGURE 4 | RAD deficient CMs display abnormal regulation of intracellular calcium. **(A)** Representative line-scan images in WT and KO CMs. Red arrows indicate arrhythmia-like voltage waveforms observed in KO CMs but not in WT. **(B)** Peak amplitudes in WT and KO CMs ($n = 30$ cells per group). **(C)** Quantification of percentages for WT and KO CMs exhibit abnormal calcium regulation at days 20, 30, and 40 ($n = 30$ cells per group). **(D)** Representative Ca²⁺ transient from WT and KO CMs induced by caffeine exposure ($n = 5$ cells per group). **(E–G)** Peak amplitudes, duration and decay time after caffeine-induced Ca²⁺ transient in WT and KO CMs. **(H)** Representative line-scan images in WT and KO CMs after adrenergic stimuli by PE. Red arrows indicate arrhythmia-like voltage waveforms. **(I)** Quantification of WT and KO CMs exhibit arrhythmia-like voltage waveforms in response to PE. Data are expressed as means $\pm$ S.E.M. of three independent experiments. ns, not significant; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

induced by caffeine showed lower level of calcium release and prolonged decay time in KO CMs further prove that calcium dysregulation is occurring in the cell, which is consistent with the results above (Figures 4D–G and Supplementary Figure S3D).

Besides, fatal cardiac events in some patients with cardiac hypertrophy are often triggered by adrenergic stimuli, we

administrated 10 μ mol/L phenylephrine (PE) to both WT and KO CMs to mimic the stimulation. After 5 days of treatment, severely exacerbate presentation of multiple events were more frequently observed in KO CMs compared to WT which only showed an increased beating rate without rhythm disturbance (Figures 4H,I). These findings suggested

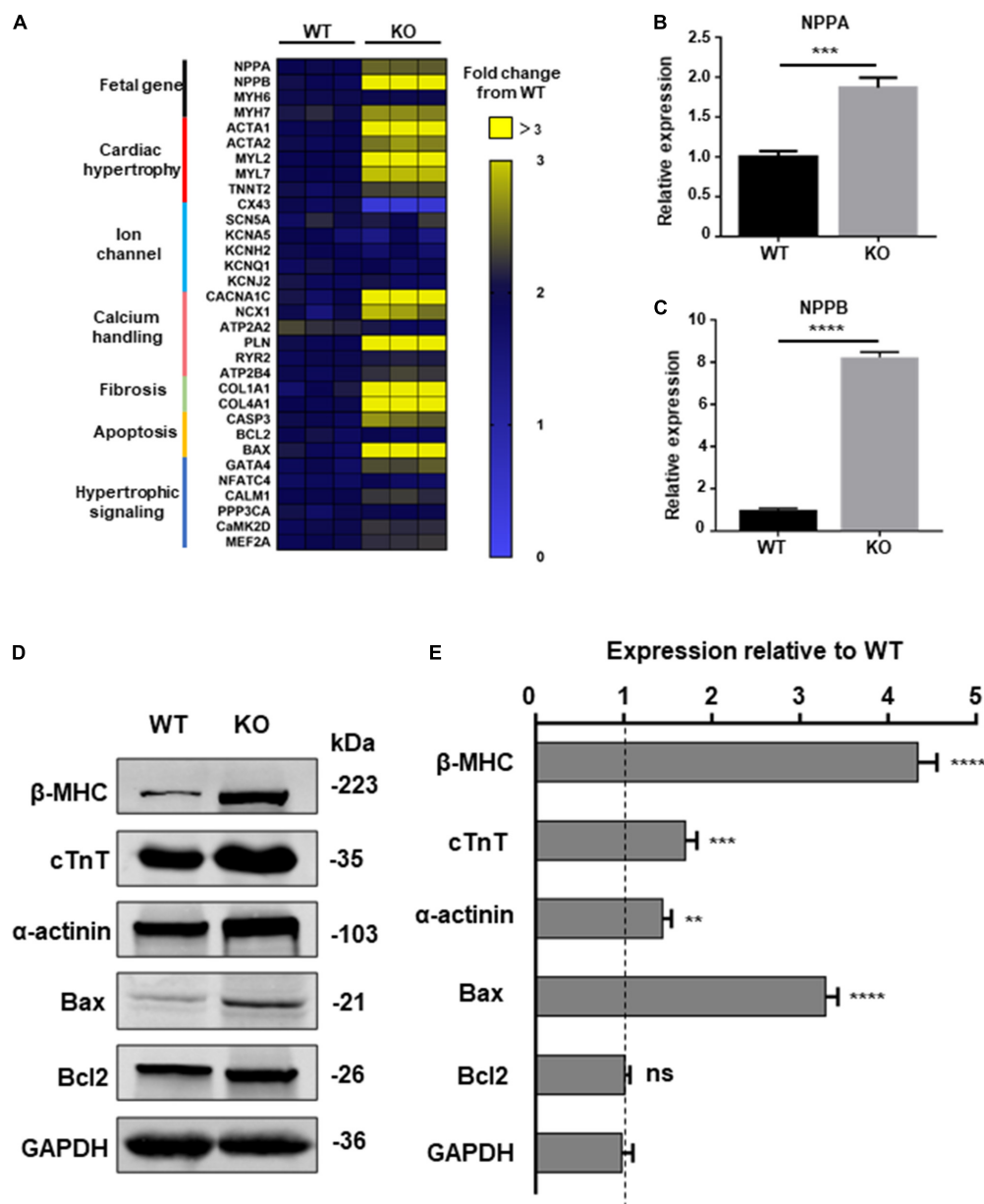


FIGURE 5 | Increased hypertrophic signaling in RAD deficient CMs. **(A)** Heatmap shows expression changes of genes related to cardiac hypertrophic signaling pathways in WT and KO CMs at days 40. **(B,C)** Specific markers for cardiac hypertrophy *NPPA*, *NPPB* are significant upregulated in KO CMs. **(D)** Western blot shows expression of hypertrophic related proteins in WT and KO CMs, respectively. **(E)** Quantification of protein expression normalized by GAPDH in WT and KO CMs. Data are expressed as means $\pm$ S.E.M. of three independent experiments. ns, not significant; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

that cardiomyocytes derived from *RRAD*^{-/-} line were more susceptible to phenylephrine-induced arrhythmias, one of the clinical manifestations of patients with cardiac hypertrophy.

RRAD^{-/-} Cardiomyocytes Exhibit Activation of Hypertrophy-Associated Genes

To further explore the relationship between dysfunction of RAD and pathological process of cardiac hypertrophy, a panel of genes

involved in hypertrophy include cardiac structure and function were measured by qPCR at day 20, 30, and 40 post-induction (Narsinh et al., 2011). Beginning at day 40, cardiomyocytes derived from KO line presented remarkably increased mRNA expression of fetal program, hypertrophy, hypertrophic signaling, fibrosis, calcium handling and apoptosis (*CASP3*, *BAX*) which has been identified to be closely related to cardiac hypertrophy (Figure 5A and Supplementary Figure S4A; Mosqueira et al., 2018). ANF, BNP, markers of cardiac hypertrophy, respectively encoded by *NPPA*, *NPPB*, were significant upregulated in

KO CMs (Figures 5B,C; Lowes et al., 1997; Gardner, 2003). Meanwhile, results of western blot exhibited increased protein expression of cardiac structure and apoptosis-associated proteins which matches the trend of the qPCR result (Figures 5D,E). Also, the results of trypan blue staining suggested an increased proportion of dead cardiomyocytes in the KO group, which may be a result of an increase in pro-apoptotic factors (Supplementary Figures S5A,B).

RRAD Knock-Out Increased LTCC Expression and I_{Ca-L} in the Cardiomyocytes

The homeostasis of intracellular Ca^{2+} concentration is maintained by a common regulation of the membrane and cytoplasmic transport systems. Among these, LTCC is the most important type of calcium channel located in the membrane of cardiomyocytes. Previous studies on mice have demonstrated that deficiency of RAD can lead to an increase of I_{Ca-L} via upregulation of LTCC expression in the plasma membrane (Yada et al., 2007). To determine the effect of RRAD gene knockout on I_{Ca-L} in cardiomyocytes derived from hESCs, we subsequently performed whole-cell patch-clamp experiments. Results as shown in Figures 6A–D, cardiomyocytes derived from KO line showed significantly increased membrane capacitance (pF) (Figures 6A,B). I-V curves I_{Ca-L} from the cardiomyocytes in each group show that I_{Ca-L} density (pA/pF) was significantly increased in KO CMs (Figures 6C,D).

Besides, western blot was performed to quantify the expression of $Ca_v1.2$ ($\alpha1c$ subunit of LTCC, encoded by *CACNA1C* gene) and a series of proteins closely related to intracellular calcium regulation, including RyR2, phosphorylated RyR2 (RyR2-Ser2808, RyR2-Ser2814), SERCA2a, PLN, IP3R and CACNB2 ($\beta2$ subunits of LTCC). Results as shown in Figures 6E,F, there was no difference in the expression of CACNB2 between two groups, but the expression of $Ca_v1.2$ was significantly increased in the KO CMs, which can be regarded as a potential evidence of increased intracellular calcium levels and could be correlated to various pathological process. In order to further detect the expression of $Ca_v1.2$ protein on the cardiomyocyte membrane, we extracted the total membrane protein of the WT and KO CMs. Result as shown in Supplementary Figures S4B,C, the expression of $Ca_v1.2$ was also significantly increased in the KO CMs. In addition, there was no difference in the expression of RyR2 between the two groups, while the expression of p-RyR2 (RyR2-Ser2808, RyR2-Ser2814) increased in the KO CMs, the expression of SERCA2a decreased, and the expression of PLN, which negatively regulates SERCA2a, also increased (Figures 6E,F). These results indicate that although the calcium released by SR increases, it cannot be effectively recovered due to impaired SERCA2a function, which explains the decrease of SR calcium to a certain extent and also becomes another important factor for the increase of intracellular calcium load. Furthermore, results of western blot at protein level were also consistent with the transcripts level of *CACNA1C*, *CACNB2*, *RYR2*, *ATP2A2*, *PLN*, and *ITPR1* (Supplementary Figure S6A).

RRAD Knock-Out Increased CaMKII Activity in the Cardiomyocytes

Calcium is a universal intracellular second messenger and can interact with a variety of intracellular molecules (Bers, 2008). Among these, CaMKII (Ca^{2+} /calmodulin-dependent kinase II) and calcineurin (Ca^{2+} /calmodulin-dependent serine/threonine phosphatase calcineurin) have been widely studied on their role in cardiac hypertrophy (Dewenter et al., 2017). Moreover, our data have shown that RRAD knockout can lead to an increased expression of LTCC in cardiomyocytes, which can in turn result in an increased cytoplasmic calcium level. To reveal the consequences of elevated intracellular calcium toward downstream molecules, we performed a western blot to determine which downstream pathway is affected by the change. Results as shown in Figures 6G,H, expression of activated (phosphorylated) CaMKII (p-CaMKII, CaMKII-Thr286), a strong inducer of hypertrophy pathways (Zhang et al., 2003), was significantly increased. However, expression of calcineurin, another common activator for inducing hypertrophy, did not show any alteration. Altogether, these findings implicated that Ca^{2+} -calmodulin-CaMKII may be the key pathway in mediating cardiomyocytes hypertrophy of RAD deficiency, whereas calcineurin does not play a major role.

Management of Calcium Dysregulation Prevents the Development of Cardiac Hypertrophy

Previous data have shown that elevated I_{Ca-L} of cardiomyocytes and dysregulation of intracellular calcium are the mechanisms responsible for cardiac hypertrophy caused by RAD deficiency. In order to block this pathological process, both WT and KO CMs at day 20 post-induction were treated with verapamil (LTCC blocker) at therapeutic dosages (100 nM). Starting after 10 days of pretreatment, homeostasis of calcium handling (Figures 7A,B), recovered storage of SR calcium (Figures 7C–E and Supplementary Figure S7A), and significantly decreased cell size (Figures 7E,G) could be observed in the treated KO CMs. Consistently, expression of several representative cardiac hypertrophy genes has shown no significant difference to WT after pharmaceutical inhibition of excessive calcium entry at early stage (Figure 7H and Supplementary Figure S7B). In addition, results of western blot shown the expression of SERCA2a, $Ca_v1.2$, and p-RyR2 (RyR2-Ser2808, RyR2-Ser2814) were significantly restored. More importantly, expression of p-CaMKII (CaMKII-Thr286), a strong inducer in mediating cardiac hypertrophy, also showed a significant decrease (Figures 7I,J). Altogether, these findings suggested that early treatment of calcium dysregulation prevents the development of cardiac hypertrophy caused by RAD deficiency.

DISCUSSION

In the present study, we reported an *in vitro* RAD deficient cardiomyocyte model derived from *RRAD*^{-/-} hESCs using CRISPR/Cas9 for the first time. Based on this model, functional

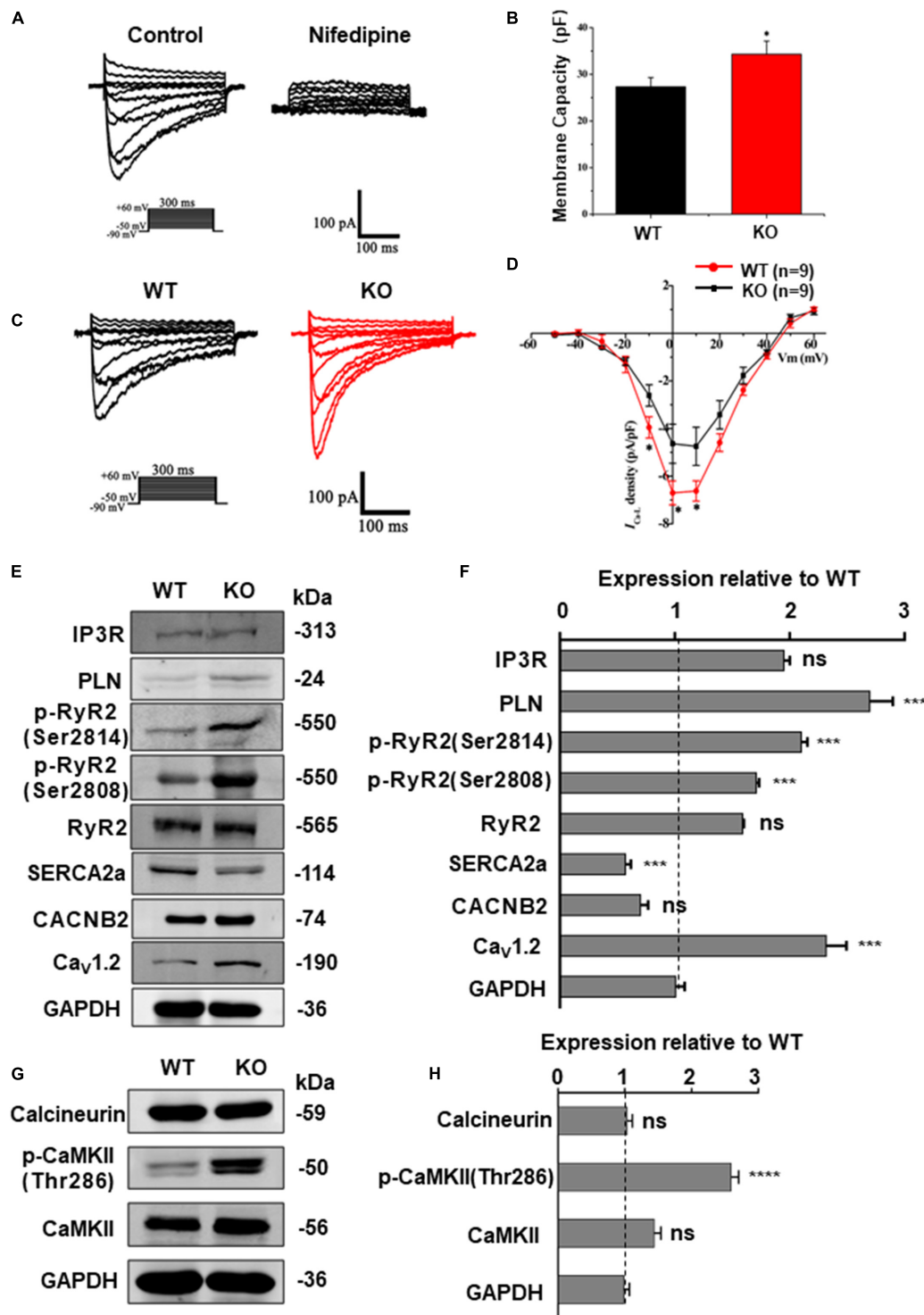


FIGURE 6 | RAD deficient CMs show increased I_{Ca-L} and CaMKII activity. **(A)** Recording and identification of representative I_{Ca-L} from a series of step pulses between -50 mV to $+60$ mV from a holding potential of -80 mV. 200 nM nifedipine, a specific blocker of L-type voltage-gated Ca^{2+} channels, blocked the current. **(B)** Histogram analysis showing that the cell membrane capacity (pF) was larger in KO CMs than that in WT CMs. **(C)** Typical I_{Ca-L} recording from WT CMs (black traces) and KO CMs (red traces). **(D)** I-V curves I_{Ca-L} showing that I_{Ca-L} density was significantly increased in KO CMs ($n = 9$, respectively). **(E)** Western blot shows expression of protein related to calcium regulation in WT and KO CMs, respectively. **(F)** Quantification of protein expression normalized by GAPDH in WT and KO CMs. **(G)** Western blot shows expression of calcineurin A, CaMKII and phosphorylated CaMKII (Thr286) in WT and KO CMs, respectively. **(H)** Quantification of protein expression normalized by GAPDH in WT and KO CMs. Data are expressed as means $\pm$ S.E.M. of three independent experiments. ns, not significant; * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$.

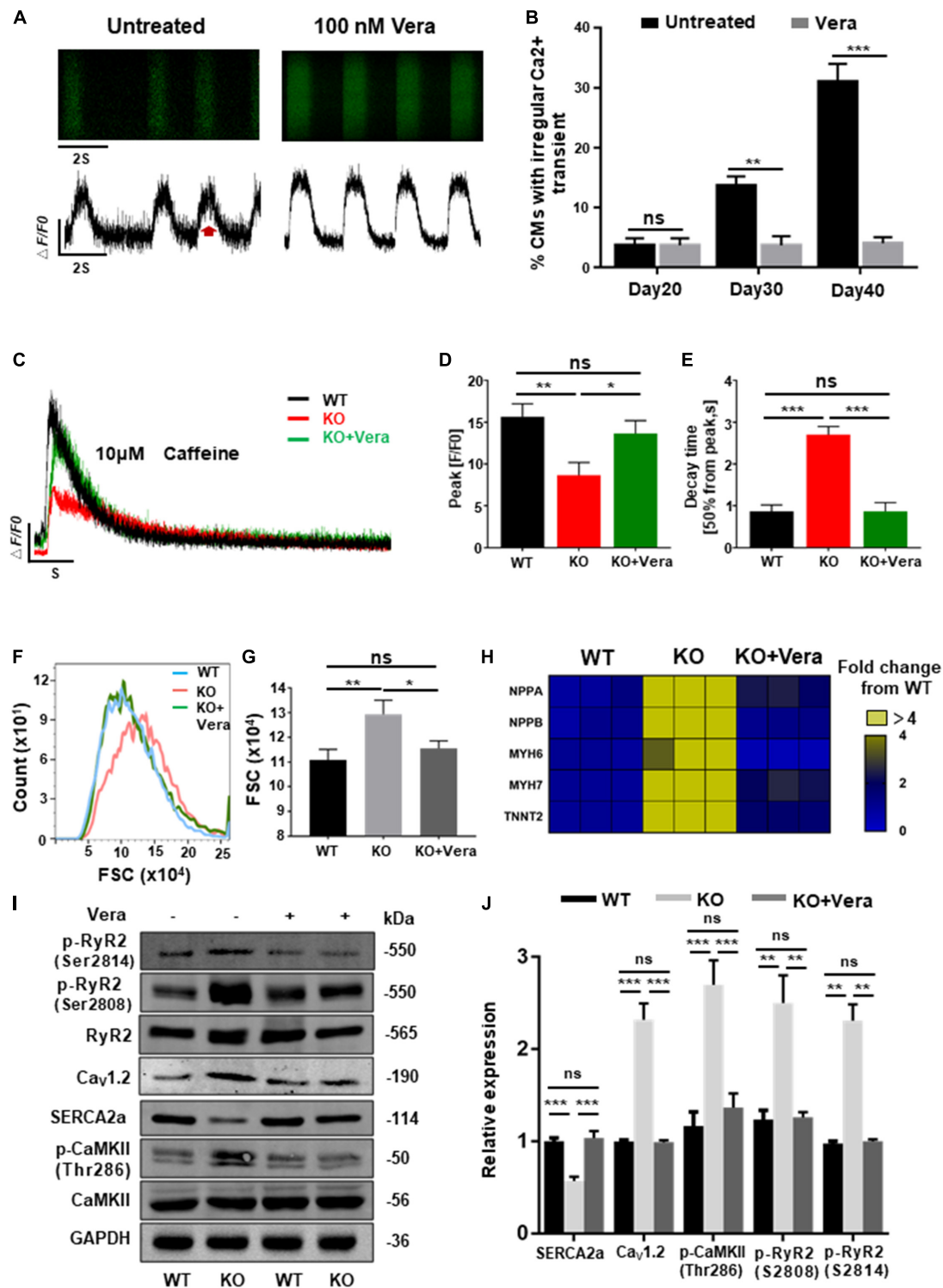


FIGURE 7 | Calcium channel blocker prevents the development of cardiac hypertrophy in RAD deficient CMs. **(A)** Representative line-scan images of KO CMs treated with 100 nM verapamil for 10 days. **(B)** Quantification of percentages of KO CMs found to exhibit abnormal calcium regulation after treatment with verapamil ($n = 30$ cells per group). **(C)** Representative Ca^{2+} transient induced by caffeine of KO CMs after treated with verapamil ($n = 5$ cells per group). **(D,E)** Peak amplitudes and decay time of KO CMs after treated with verapamil. **(F,G)** Flow cytometry shows changes in cell size of WT, KO and verapamil treated KO CMs. **(H)** Heatmap shows expression changes of genes related to hypertrophy in WT, KO, and verapamil treated KO CMs. **(I,J)** Western blot shows expression of Ca v 1.2, SERCA2a, p-RyR2 (Ser2808, Ser2814), and p-CaMKII (Thr286) in WT, KO, and verapamil KO CMs. Quantification of protein expression normalized by GAPDH. Vera, verapamil. Data are expressed as means $\pm$ S.E.M. of three independent experiments. ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

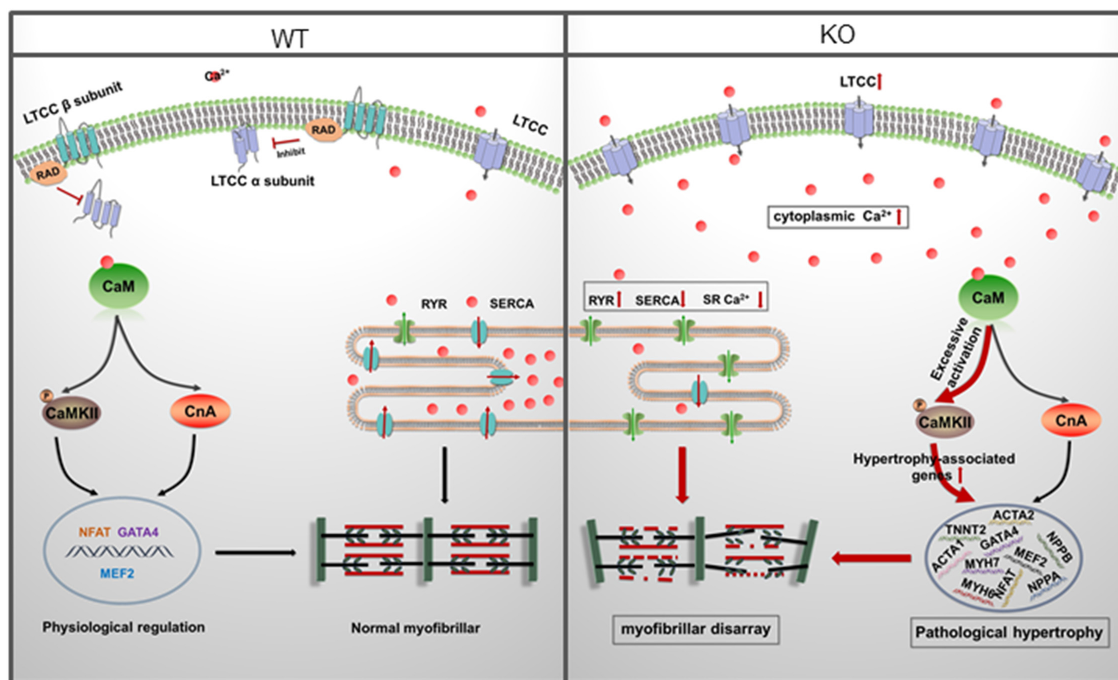


FIGURE 8 | A model for mechanisms of cardiac hypertrophy in RAD deficient CMs. RAD deficiency triggers hypertrophy-associated pathway via modulation of the cytoplasmic Ca^{2+} . NCX, sodium-calcium exchanger; LTCC, L-type calcium channel; CaM, Calmodulin; CaMKII, calmodulin-dependent kinase II; CnA, Calcineurin; SR, sarcoplasmic reticulum.

changes, underlying mechanisms and potential therapies of RAD-deficiency could be well studied in a human cardiac background. After verification by multiple methods, we found that RAD deficient cardiomyocytes exhibited significant hypertrophic phenotypes and activated hypertrophy-associated genes. Further investigation revealed that increased LTCC expression in the membrane of cardiomyocytes and abnormal regulation of intracellular calcium level were the central mechanism of the phenotypes. Management of calcium dysregulation with LTCC blocker, verapamil, prevents the development of cardiac hypertrophy caused by RAD-deficient CMs (Figure 8).

Ras associated with diabetes, a member of the RGK subfamily in the Ras-related GTPase (Wang et al., 2010), is encoded by *RRAD* gene and abundantly expressed in cardiomyocytes (Reynet and Kahn, 1993; Maguire et al., 1994). It comprises multiple functional domains, which can interact with a variety of signal transduction molecules including Rho kinase, calmodulin, and 14-3-3 protein. In addition, RAD could impact various aspects of cardiac physiological functions and pathological processes such as cytoskeletal regulation, neointimal formation after balloon injury in fibroblastic cells, and diabetic cardiomyopathy (Ward et al., 2002; Beguin et al., 2005; Fu et al., 2005; Ilany et al., 2006). Previous studies on mice have demonstrated that deficiency of RAD significantly increases stress-induced (thoracic transverse aortic constriction or phenylephrine) cardiac hypertrophy and remodeling both *in vivo* and *in vitro* (Chang et al., 2007). However, our data demonstrated that cardiomyocytes derived

from *RRAD*^{-/-} hESCs can spontaneously develop hypertrophic phenotypes such as increased size of cardiomyocytes, multi-nucleation, and disorganized arrangement of myofibrils 40 days post-induction without external factors. We speculate for two reasons. First, our cardiomyocytes are cultured at the cellular level in 2D mode, and lack the overall level of organ and system interaction. The course of cardiac hypertrophy is a slowly progressing process and is affected by various factors throughout the body. However, our model cannot simulate the pathological process at the tissue and organ level, which is also one of the limitations of this paper. Second, due to species differences, the electrophysiological characteristics of human and mice will also differ significantly, and the biological functions performed by them will also be different, which may be another reason for this result.

Calcium (Ca^{2+}) is a universal intracellular signaling molecule and is essential for the maintenance of normal functioning of the heart (Wang et al., 2010). Upon depolarization of the cardiomyocyte membrane, LTCC located on T-tubules (invaginations of the plasma membrane of the cell) is activated to allow Ca^{2+} to enter the cytoplasm, which triggers a more substantial SR calcium release (known as Ca^{2+} -induced Ca^{2+} release, CICR) and thus initiate a series of physiological activities such as electrophysiology, EC coupling, energy consumption (Servili et al., 2018). Consistently, on repolarization of the cardiomyocyte membrane, elevated cytoplasmic Ca^{2+} are restored to resting state through various routes such as sarcoplasmic reticulum SERCA (SR Ca^{2+} ATPase, transported

cytoplasmic Ca^{2+} to the SR), cell membrane sodium-calcium exchangers (NCX) and calcium pumps (NCX and Ca^{2+} ATPases, transported cytoplasmic Ca^{2+} to the extracellular). However, when this balance is disrupted by various factors resulting irregular Ca^{2+} regulations or abnormally elevated intracellular Ca^{2+} levels, it may become the initiator for many cardiac diseases such as cardiac hypertrophy or arrhythmia (Monteiro da Rocha et al., 2016; Dewenter et al., 2017). Previous studies have revealed that Rad can act as a negative regulator of LTCC activity by directly binding to their β -subunit and overexpression of S105N Rad (dominant negative mutant Rad) in guinea pig ventricular cardiomyocytes can lead to an increased intracellular Ca^{2+} via upregulation of LTCC expression in the plasma membrane (Finlin et al., 2003; Yada et al., 2007). In our study, cardiomyocytes derived from $RRAD^{-/-}$ hESCs exhibited significantly increased expression of *CACNA1C* and $\text{Ca}_v1.2$ (gene and protein of LTCC $\alpha 1c$ subunit, respectively) on transcription and protein levels, which lead to an increased $I_{\text{Ca-L}}$ (pA/pF). Measurement of intracellular Ca^{2+} regulation has shown that significant abnormal Ca^{2+} transient such as events associated with arrhythmia-like voltage waveforms were more frequently appeared in cardiomyocytes derived from $RRAD^{-/-}$ hESCs. Accordingly, we speculate that the electrophysiology of KO CMs should also be changed, but for some reason, we failed to record the action potentials of hESC-CMs. In addition, SR Ca^{2+} stores induced by caffeine showed a smaller calcium release and prolonged decay time in KO CMs supported another finding of impaired function of calcium regulation. Moreover, severely exacerbate presentation of multiple events induced by positive inotropic PE frequently observed in KO CMs were also compatible with the clinical manifestations of patients with cardiac hypertrophy. Interestingly, all these abnormalities above occur prior to the onset of hypertrophic phenotype indicated that overexpressed LTCC and abnormal Ca^{2+} handling may be the initiator of cardiac hypertrophy caused by RAD deficiency.

As an important intracellular second messenger, activity-induced Ca^{2+} influx through LTCC can initiate multiple Ca^{2+} -dependent signaling cascades and then lead to the activation of specific transcription programs, a process known as excitation-transcription (ET) coupling which play a critical role not only in cardiac homeostasis but also in cardiac disease development, since diseased cardiomyocytes show vast alterations in Ca^{2+} -handling and Ca^{2+} -dependent transcriptional patterns (Kim and Kim, 2018). Among these, calmodulin, CaMKII, and calcineurin are well-studied signaling molecules in cardiomyocytes (Dewenter et al., 2017). Generally, Ca^{2+} entering the cardiomyocytes initially binds to calmodulin and then activates Ca^{2+} -dependent cascades in the form of Ca^{2+} -calmodulin complex, such as Ca^{2+} -calmodulin-CaMKII and Ca^{2+} -calmodulin-calcineurin pathways, which are major signal mediators of cardiac hypertrophy and remodeling (Lehman et al., 2018). In addition, Ca^{2+} combined with calmodulin can induce the expression of ANF via interacting with CAMTA2, known as calmodulin-binding transcriptional activator 2 (Long et al., 2014). Meanwhile, CAMTA2 can also bind to NKX2-5 and participate in the pathogenesis of cardiac hypertrophy (Dewenter

et al., 2017). In our study, further investigation for time-based gene expression profiling revealed that cardiomyocytes derived from $RRAD^{-/-}$ hESCs exhibited significant upregulation of hypertrophy-associated genes which occurred right after the disorder of intracellular Ca^{2+} homeostasis. Also, increased cell death in the $RRAD^{-/-}$ cardiomyocytes may be a result of increased intracellular Ca^{2+} (Harvey and Leinwand, 2011; Dewenter et al., 2017). Furthermore, the increased expression of p-CaMKII, a potent inducer of hypertrophy pathways, revealed a critical role for the Ca^{2+} -calmodulin-CaMKII pathway in the hypertrophic phenotype caused by RAD deficiency.

A majority of patients with cardiac hypertrophy have varying degrees of cardiac dysfunction, usually caused by the thickening ventricular wall, which could lead to notably elevated ventricular pressures (Maron et al., 2003, 2014). ANF (atrial natriuretic factor) and BNP (brain natriuretic peptide), are diagnostic indicators of cardiac function secreted by atrial and ventricular myocytes, respectively. Both ANF and BNP are elevated more than 100 times in patients with cardiac hypertrophy to compensate for increased blood volume and pressure caused by thickening of the ventricular wall (Rodeheffer et al., 1986; Gardner, 2003; Taylor et al., 2018). Consistently, our data has shown that the expression levels of *NPPA* and *NPPB* (coding genes for ANF and BNP, respectively) are significantly increased, which reflects the impaired function of cardiomyocytes caused by RAD deficiency to some extent. In addition, upregulated β -myosin expression (β -MHC, encoded by *MYH7*), accompany with downregulated α -myosin expression (α -MHC, encoded by *MYH6*) is a sensitive indicator of cardiac hypertrophy (Lowes et al., 1997; Miyata et al., 2000). Furthermore, the shift toward β -MHC may also be the main cause of decreased contractile function and prolonged relaxation of cardiomyocytes during the transition from compensatory cardiac hypertrophy to end stage of heart failure (Nagata et al., 1998). Our data has shown that expression of *MYH7* is significantly increased in cardiomyocytes derived from $RRAD^{-/-}$ hESCs, both at the transcriptional and protein level. The result is also consistent with the pathological process in patients with cardiac hypertrophy.

The relationship between disorders of intracellular Ca^{2+} and cardiac hypertrophy has been confirmed for over a decade (Fatkin et al., 2000). Also, our data demonstrated that abnormal Ca^{2+} handling caused by overexpressed LTCC in cardiomyocytes may be the initiator of cardiac hypertrophy caused by RAD deficiency. Subsequently, inhibiting calcium dysregulation at early stage could prevent the development of cardiac hypertrophy further reinforce the hypothesis above.

However, it is important to notice that there are some limitations in our study. Firstly, maturity is critical for studying the function of hESC-CMs. Genetically modified hPSCs have been commonly used to study the gene function in cardiomyocytes, indicating that these cells are excellent models for cardiovascular research (Lan et al., 2013; Fogarty et al., 2017; Mosqueira et al., 2018; Li et al., 2019). In this study, we found that $RRAD^{-/-}$ cardiomyocytes were more prone to hypertrophic phenotype, whereas, WT cardiomyocytes were also exhibited a certain phenotype such as low frequencies of myofibrillar disarray, multi-nucleation under the same culture conditions.

This may be closely related to the immaturity of hESC-CMs. Therefore, more technologies aimed at promoting the maturation of hESC-CMs, such as increasing culture time (Kamakura et al., 2013), mechanical stimulation (Mihic et al., 2014), and tissue engineered tissues (Feric and Radisic, 2016) were necessary for future research. Secondly, hESCs differentiated cardiomyocytes cannot mimic disease phenotypes at the tissue and organ levels is another limitation of this article. Even though we hypothesized that the knockout of *RRAD* would cause an abnormal increase in Ca^{2+} in the cytoplasm of cardiomyocytes, activate the hypertrophy-associated Ca-CaM-CaMKII pathway, and result in increased expression of a series of hypertrophy genes including *NPPA*, *NPPB*, future researches aimed at the overall level will be necessary (Carvajal-Vergara et al., 2010; BurrIDGE et al., 2012). Despite this, our study reported potential functional changes, underlying mechanisms associated with RAD-deficiency for the first time in a human cardiac background which may provide novel insights for the study of the pathogenesis and treatment of cardiac hypertrophy.

DATA AVAILABILITY STATEMENT

The raw data generated during this study can be found in the SRA database, accession number PRJNA661724.

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AUTHOR CONTRIBUTIONS

FL, YW, XT, YL, and XIL designed the experiments. YL, XwL, FW, RB, YZ, SM, TD, YC, SZ, and W-JL performed the experiments. YL, XIL, XwL, FW, XT, YZ, YC, and W-JL analyzed the data. YL, FL, JG, XIL, YZ, and YC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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TPP1 Enhances the Therapeutic Effects of Transplanted Aged Mesenchymal Stem Cells in Infarcted Hearts via the MRE11/AKT Pathway

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Background: Poor cell survival after transplantation restricts the therapeutic potential of mesenchymal stem cell (MSC) transplantation into infarcted hearts, particularly in older individuals. TPP1, a component of the shelterin complex that is involved in telomere protection, is highly expressed in young MSCs but declines in aged ones. Here, we explore whether TPP1 overexpression in aged mouse MSCs improves cell viability *in vivo* and *in vitro*.

Methods: Aged mouse MSCs overexpressing TPP1 were injected into the peri-infarct area of the mouse heart after left anterior descending coronary artery ligation. In parallel, to evaluate cellular-level effects, H₂O₂ was applied to MSCs *in vitro* to mimic the microenvironment of myocardial injury.

Results: *In vivo*, the transplantation of aged MSCs overexpressing TPP1 resulted in improved cell survival, enhanced cardiac function, and reduced fibrosis compared to unmodified aged MSCs. *In vitro*, TPP1 overexpression protected aged MSCs from H₂O₂-induced apoptosis and enhanced DNA double-strand break (DSB) repair. In addition, the phosphorylation of AKT and the key DSB repair protein MRE11 were both significantly upregulated in aged MSCs that overexpressed TPP1.

Conclusions: Our results reveal that TPP1 can enhance DNA repair through the AKT/MRE11 pathway, thereby improving the therapeutic effects of aged MSC transplantation and offering significant potential for the clinical application of autologous transplantation in aged patients.

Keywords: stem cells therapy, myocardial infarction, aging, DNA repair, telomere

Abbreviations: MSC, mesenchymal stem cell; OMSC, aged MSC; OMSC^{null}, OMSC infected with Len-null; OMSC^{TPP1}, OMSC infected with Len-TPP1; OGD, oxygen glucose deprivation; YMSC, young MSCs; YMSC^{null}, YMSC transfected with null-siRNA; YMSC^{TPP1si}, YMSC transfected with TPP1-siRNA.

INTRODUCTION

Mesenchymal stem cells (MSCs) are a promising cell type for treating ischemic heart diseases due to their multipotency, capacity for self-renewal, and immune-privileged status (Williams et al., 2011; Broughton and Sussman, 2016; Golpanian et al., 2016). MSCs from older patients (the source of autologous stem cell therapy) have shorter telomere length than those of their young, healthy counterparts, as well as impaired stem cell properties, including proliferation, differentiation, and paracrine secretion. These observations likely explain the fact that clinical trials of autologous cell transplantation show limited therapeutic effects in the setting of myocardial infarction in older patients (Nurkovic et al., 2016). Because older patients are the target population for this therapy, novel strategies are needed to enhance the therapeutic potential of aged MSCs for autologous cell transplantation in myocardial ischemia.

The shortening of telomeres plays a crucial role in the cellular aging process (Shay, 2018). Aging is accompanied by increased DNA damage and diminished DNA repair capacity, which is counteracted in part by telomeres. Human telomeres are DNA sequences at the end of chromosomes, capped by six kinds of associated proteins called shelterin, which protect the telomeric DNA from being recognized as double-strand DNA breaks and leading to end-to-end fusions (Cosme-Blanco and Chang, 2008; Palm and de Lange, 2008; de Lange, 2009). The shelterin complex is composed of six core proteins: RAP1, TRF1, TRF2, TPP1, POT1, and TIN2 (Palm and de Lange, 2008). Among these, TPP1 actively recruits telomerase to telomeres, and together with POT1, it protects chromosome ends and regulates telomere length (Kibe et al., 2010; Rajavel et al., 2014). Defects in its protection lead to the initiation of DNA damage and cell apoptosis (Guo et al., 2007). Telomere length decreases with aging, and some premature aging syndromes have been linked to shelterin protein mutations, including in TIN2, TRF1, and TRF2 (Carroll and Ly, 2009). However, it is unknown whether TPP1 plays a part in DNA damage and repair in aged MSCs or whether targeting damage and repair impacts the therapeutic efficacy of these cells.

Herein, we demonstrate that augmenting TPP1 can enhance the therapeutic efficacy of aged MSCs in myocardial infarction. The underlying mechanisms in this process involve enhanced DNA repair. These findings provide new insights into strategies to deploy autologous stem cell transplantation therapy in aged patients.

MATERIALS AND METHODS

Cell Culture

hMSCs were isolated from patients undergoing hip replacement surgery (with young donors aged below 40 years and aged donors aged above 65 years). Informed consent was collected from each donor. The study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Life Technologies, United States) with 10% fetal bovine

serum (FBS, Gibco, United States) added. The culture was maintained in a humidified atmosphere containing 95% air/5% CO₂ at 37°C. Using 0.25% trypsin with 0.2% EDTA, cells were passaged at a ratio of 1:2 when they reached 70–80% confluence. Passage was performed with 4–6 cells in this study.

Cell Identification

The antigen profile of the MSCs was determined by flow cytometry. Passages 4–6 of hMSCs were stained at room temperature for 1 h with surface molecular-specific antibodies (FITC-CD45 for hematopoietic surface marker; PE-CD29, APC-CD90, PE-CD44 for mesenchymal surface markers; and FITC-CD34 for endothelial cell surface marker and isotype-matched control). The expression of the cell surface markers was quantified with flow cytometry (BD Biosciences, CA, United States).

Adipogenic, chondrogenic, and osteogenic differentiation of hMSCs was conducted as previously reported (Hu et al., 2016). hMSCs were seeded at density of $1.5\text{--}2 \times 10^5$ per well in a six-well plate overnight to allow attachment. For osteogenesis, hMSCs were incubated with osteogenic medium for 3 weeks. Differentiation was evaluated by Alizarin-Red Staining. For chondrogenesis, hMSCs were treated with chondrogenic medium for 3 weeks and assessed with toluidine blue staining. Adipogenic differentiation was induced with adipogenesis induction medium for 23 days. Oil Red O solution was used to visualize the fat vacuoles. Images were obtained from a light microscope.

Most of the MSCs were positive for CD90 (97.3%), CD29 (99.6%), and CD44 (99.7%) expression, and negative for the hematopoietic markers CD34 (0.2%) and CD45 (0.5%) (Supplementary Figures 1A–E). The multiple differentiation potential of adipogenesis, chondrogenesis, and osteogenesis in the MSCs were also identified (Supplementary Figures 1F–H).

Lentivirus Infection

Recombinant lentivirus containing TPP1 tagged with a flag was constructed by Hanbio (Shanghai, China), and an empty vector served as control. Each group of lentivirus expressed green fluorescent protein (GFP). hMSCs were seeded at a density of 6×10^4 in a six-well plate. After cell attachment, cells were infected with lentivirus (MOI = 20) in the presence of 8 µg/mL polybrene (Sigma) overnight. Then, the medium was replaced with fresh medium, and cells were cultured for another 24 h. The transduction efficiency was evaluated by Western blotting and fluorescence microscopy.

Small Interfering RNA Transfection

Small interfering RNA (siRNA) targeting human TPP1 (siTPP1) or MRE11 (siMRE11) and control siRNA (si-con) were obtained from Ribobio (Shanghai, China). When the MSCs reached 75% confluence, cells were transfected with siRNA using Lipofectamine RNAi MAX (Invitrogen, CA, United States) according to the manufacturer's protocol. After 8 h incubation (37°C), the transfection mixture was replaced by fresh medium; 48 h after transfection, the cells were harvested for further experiments.

Cell Apoptosis Model

To mimic the hypoxic aspects of the *in vivo* ischemic condition, an H₂O₂-induced apoptosis model was used for hMSCs. After transfection by lentivirus for 48 h, OMSC^{null} (Aged-MSCs infected with Len-null) or OMSC^{TPP1} (Aged-MSCs infected with Len-TPP1) were treated with 1,000 μ M H₂O₂ in DMEM without FBS (5% CO₂, at 37°C) for 2 h *in vitro*. An OGD apoptosis model was also used for prolonged cell stress. After transfection, the hMSC medium was replaced by DMEM without glucose and FBS, and the cells were cultured under hypoxic conditions (0.5% O₂/5% CO₂) at 37°C for 24 h.

Tube Formation

In the normoxia group, hMSCs (2×10^5 cells) transfected with lentivirus (Len-null or Len-TPP1) were incubated with DMEM and without FBS under normoxic culture. In the OGD group, cells were cultured with DMEM without glucose and FBS under hypoxic conditions. After 24 h incubation, the conditioned medium was collected from both groups. Additionally, DMEM without FBS and hMSCs incubation was also prepared to serve as the control. Human umbilical vein endothelial cells (HUVECs) transfected with GFP at 1.5×10^4 /well were plated in the above different conditioned medium in 96-well plates that were precoated with 50 μ L Matrigel (BD, NJ, United States). After 4–8 h plantation, the tube formation of the HUVECs was captured with a fluorescence microscope and was quantified by counting the number of tubes in each well.

Cardiomyocytes Apoptosis

The conditioned medium of OMSC^{null} or OMSC^{TPP1} after OGD and DMEM without glucose and FBS was collected, as previously described. H9C2 cardiomyocytes in the 6-well plate overnight for cell adherent at a density of 2×10^5 . Then, the H9C2 medium was replaced with the above-conditioned medium and cultured under hypoxic conditions (0.5% O₂/5% CO₂) at 37°C for 24 h. Following that, the apoptosis rate of H9C2 was assessed using Annexin V-APC/PI staining and flow cytometry.

Annexin V-APC/PI Staining

Cells were suspended in PBS and stained with a mixture of Annexin V-APC and propidium iodide (PI) dye for 30 min according to the manufacturer's protocol of Annexin V-APC/PI Apoptosis Detection Kit (SunGENE, Tianjin, China) and detected by flow cytometry (BD Biosciences, CA, United States).

Echocardiography

Heart function was assessed by transthoracic echocardiography before MI and at 3, 7, 14, and 28 days after it. Left ventricular (LV) M-mode and two-dimensional images were obtained, and cardiac function was analyzed with a Vevo 2100 system (Visual Sonics, Toronto, ON, Canada). The LV parameters measured included ejection fraction (EF), fractional shortening (FS), end-diastolic dimension, and end-systolic dimension. All parameters were measured in at least three separate cardiac cycles.

Quantitative PCR

RNA was extracted from cells with the Trizol reagent (Invitrogen, Thermo Fisher Scientific). Primers were purchased from Tsingke (Shanghai, China). Quantitative PCR was performed using SYBR Green PCR Master Mix (Takara, Japan) on a 7500 Fast Real Time PCR System (Applied Biosystems, Carlsbad, CA, United States). β -actin was used as a housekeeping gene, and data were determined with the comparative $\Delta\Delta$ Ct method. The primers are shown in **Supplementary Table 1**.

Immunofluorescence Staining

Tissue slices and cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-100 and blocked with 3% BSA in PBS for 1 h. Immunohistochemistry was performed with the following primary antibodies: Phospho-Histone H2A.X (Ser139) rabbit antibody (1:1000, CST, #9718), GFP primary antibody (1:400, Abcam), α -SMA (1:400, Abcam), CD31 (1:200, BD Bioscience), Cardiac Troponin I (1:200, Abcam), and anti-rabbit, anti-goat, and anti-mouse secondary antibody (1:400, CST). It was incubated with conjugated secondary antibody for 1 h at room temperature. Finally, diamidino-2-phenylindole (DAPI; blue) was used to stain the nucleus after washing twice by PBS. Fluorescent imaging was performed via a fluorescent microscope (Leica, Wetzlar, Germany).

γ -H2AX Foci Assay

Cells were seeded into a 24-well plate and exposed to 1,000 μ M H₂O₂ in DMEM without FBS for 1 h at 37°C. Then, they were recovered in fresh medium for 1 h. The imaging was captured by fluorescent microscope. Cells with 10 or more foci were counted as positive cells, which we interpreted as cells with unrepaired DNA double-strand breaks.

Alkaline Comet Assay

The alkaline comet assay was performed using the Trevigen Comet Assay kit (Trevigen, Gaithersburg, MD, United States). Cells at 1×10^5 /mL were suspended in PBS and mixed with molten LMAgarose at a ratio of 1:10 (v/v), and 50 μ L mixture was immediately pipetted onto the slide. The slide was incubated at 4°C for 10 min to accelerate gelling and was then immersed into cold lysis solution overnight at 4°C. Next, excess buffer was drained from the slides, and they were immersed into alkaline unwinding solution for 1 h at 4°C, in the dark. The slides were subjected to electrophoresis with 4°C Alkaline Electrophoresis Solution at 21 V for 30 min and immersed twice in H₂O for 5 min each and then in 70% ethanol for 5 min, following which, they were air dried. DNA was stained with 100 μ L SYBR GREEN (Invitrogen, 1:10,000) for 10 min and was immediately rinsed with H₂O and dried. The slides were viewed with a fluorescence microscope and analyzed with Comet Score Freeware v. 1.5 (TriTek, Niigata, Japan).

Sirius Red Staining

Then, 28 days after MI, all mice were sacrificed for histological study. The hearts were embedded into a Tissue Tek OCT

compound (Sakura Finetek, Torrance, CA, United States) and cut into 7 μm thick sections. Fibrotic area was assessed using Sirius red staining kit (Solarbio Life Sciences, Beijing, China) according to the manufacturer's instructions. The percentage of the infarct area was quantified with ImagePro-Plus software (Media Cybernetics, Rockville, MD, United States) and calculated according to the following the formula: infarct heart area (%) = (the sum of endocardial length and epicardial length of the infarcted area/the sum of endocardial and epicardial length of whole left ventricle) $\times$ 100%.

MI Model and MSC Transplantation

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 86-23, revised 1996) and were approved by the Animal Care and Use Committee of Zhejiang University. Myocardial infarction was established in male C57BL/6J mice (20–25 g, 10–12-weeks old) according to previously described methods (Hu et al., 2014). MI was induced by ligation of the left anterior descending coronary artery with a 6-0 silk suture. 10 min after the ligation, OMSC^{null} (1.5×10^5 cells in 20 μL DMEM per mouse), OMSC^{TPP1} (1.5×10^5 cells in 20 μL DMEM per mouse), or DMEM alone was intra-myocardially injected into the ischemic border zone of hearts at five sites. The control animals underwent MI surgery and were injected with DMEM only.

TUNEL Staining

The apoptosis of cells was detected with an *In Situ* Cell Death Detection Kit (Roche United States). The samples were fixed in 4% paraformaldehyde, permeabilized with 0.2% TritonX-100, and incubated with deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) reaction at 37°C for 1 h in the dark. Nuclei were stained with DAPI for 10 min. The images were observed under a fluorescence microscope, and the apoptotic ratio was calculated as the number of TUNEL-positive cells/total number of cells.

Western Blot

Proteins were separated by SDS-PAGE gel with a current of 30 mA and transferred onto PVDF membrane with a current of 300 mA. After blocking with 5% milk for 1 h, they were incubated overnight with the following primary antibodies: anti-MRE11 (1:1000, Abcam), anti-AKT (1:1000, CST); anti-pAKT (phosphor s473) (1:1000, CST), anti-PTOP (1:1000, Abcam), anti-cleaved caspase-3 (Asp175) (1:1000, CST), and anti-caspase-3 (1:1000, CST) at 4°C. Then, the membranes were washed three times with PBST and incubated with HRP-conjugated secondary antibodies (1:5000). After being washed by PBST, the protein bands were treated with ECL and detected with the Gel Doc EZ Imaging System (Bio-Rad, Berkeley, CA, United States).

β -actin was obtained from BD Biosciences, (San Jose, CA, United States); GAPDH, β -Tubulin, and HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Cell Signaling Technology.

PI3K Inhibitor Treatment

PI3K inhibitor LY294002 was obtained from Selleckchem (Houston, TX, United States). For the pretreatment, cells were cultured with DMEM containing 10% FBS and 10 μM LY294002 under 5% CO₂ at 37°C for 24 h. When cells were treated with H₂O₂, 10 μM LY294002 was also persistently contained in the DMEM with it for the PI3K inhibition group.

Telomerase Activity Assay

Telomerase activity assay was performed with the Telo TAGGG telomerase PCR ELISA kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Cells were harvested, centrifuged, and resuspended by 200 μL lysis reagent. The lysate was then centrifuged at 16,000 $\times g$ at 4°C for 20 min, and the supernatant was transferred to a fresh tube to perform the TRAP reaction. After amplification, the amplification product was added to the mixture of denaturation reagent and hybridization buffer. Next, 100 μL mixture was transferred into each well of the precoated MP modules supplied with the kit, and the MP modules were incubated at 37°C on a shaker (300 rpm) for 2 h. The hybridization solution and anti-DIG-POD working solution were added and removed, one after the other. Finally, TMB substrate solution with stop reagent was added, and a microplate (ELISA) reader was used to measure the absorbance of the samples at 450 nm. Hela cells were served as positive control.

Statistical Analyses

Data are presented as mean $\pm$ S.D. Student's *t*-test was performed to analyze the differences between two groups. For more than three groups, one-way ANOVA was used, followed by Tukey's post-test. All data were analyzed and presented by GraphPad Prism 6. The threshold for statistical significance was set at $P < 0.05$.

RESULTS

TPP1 Protects OMSCs Against Apoptosis *in vitro*

In our previous study, TPP1 expression was found to be upregulated in SIRT1-overexpressing OMSCs, which significantly enhanced the ability of these cells to survive after injection following myocardial infarction (Chen et al., 2014). Thus, we considered that TPP1 likely plays a vital anti-apoptotic role. We found that the level of TPP1 expression was significantly decreased in OMSCs compared to young MSCs (Supplementary Figure 2A); therefore, we chose to overexpress TPP1 in OMSCs (Supplementary Figure 2B). To mimic the microenvironment of myocardial infarction, apoptosis was induced in the OMSCs with 1,000 μM H₂O₂ for 2 h. Overexpression of TPP1 significantly attenuated apoptosis of OMSCs, as seen in the reduced apoptotic rate indicated by TUNEL (Figures 1A,B) and Annexin APC/PI assay (Figures 1C,D). Overexpression of TPP1 also significantly decreased cleaved caspase-3 expression through Western blotting

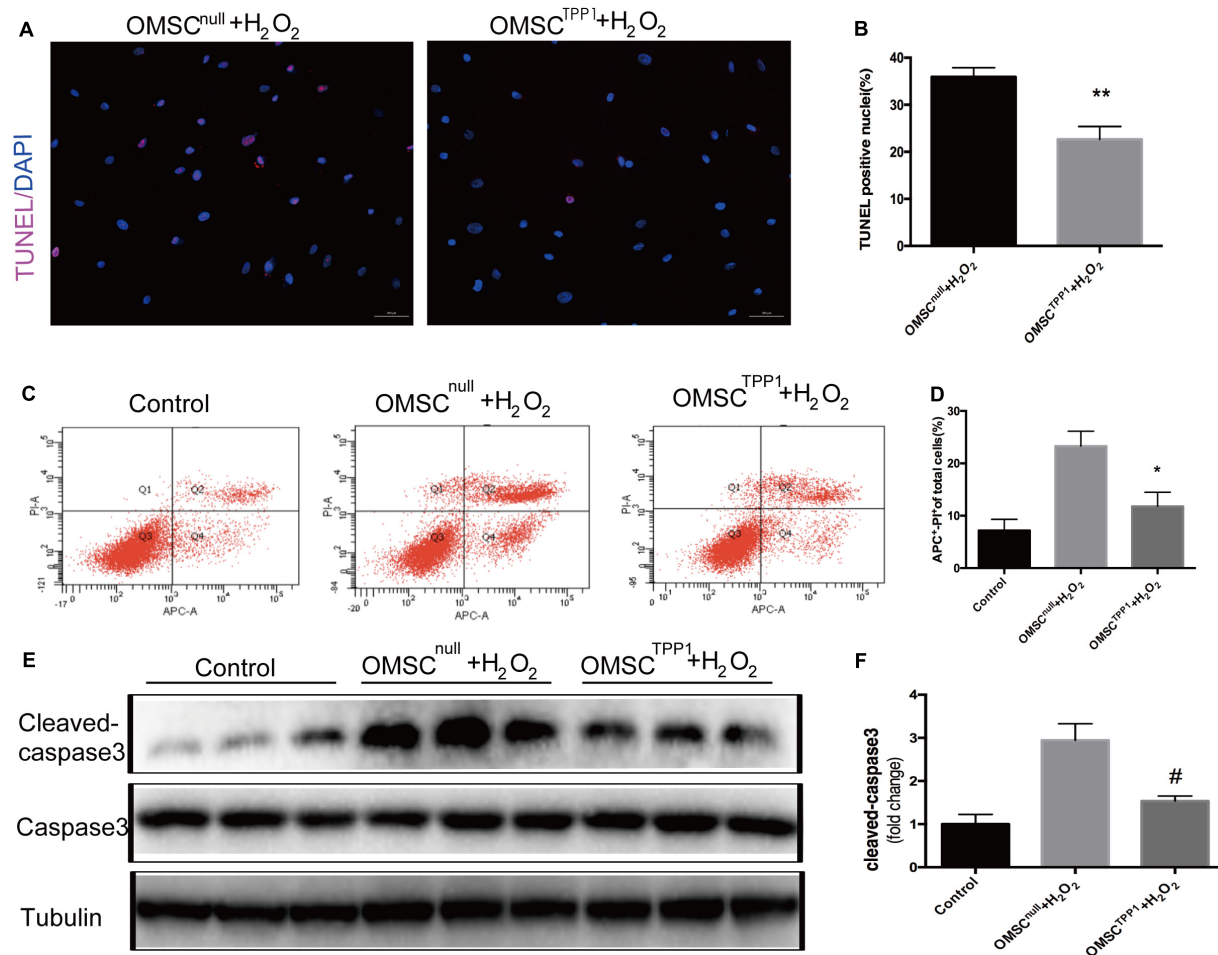


FIGURE 1 | TPP1 protects OMSC from apoptosis *in vitro*. **(A,B)** Representative images of TUNEL staining; the percentages of apoptotic cells were calculated as follows: (TUNEL-positive cells/total nuclei counted)*100%. Five or six fields were randomly selected from the well (at least five wells) in each group to count the TUNEL-positive cells. **(C,D)** Annexin APC/PI staining was analyzed for cell apoptosis and the apoptotic rate of quantification was measured. **(E,F)** The protein level of apoptotic marker cleaved caspase-3 was measured using western blotting. An independent *in vitro* experiment was repeated three times. Data are shown as mean $\pm$ S.D. * $P < 0.05$, # $P < 0.05$, ** $P < 0.01$.

(Figures 1E,F). These results demonstrate that TPP1 plays a vital role in protecting OMSCs against apoptosis.

TPP1 Overexpressing Aged MSC (OMSC^{TPP1}) Transplantation Results in Better Recovery of Cardiac Function

Next, we employed an MI model in mice (Figure 2A) to determine whether TPP1 overexpression in OMSCs results in better cardiac function. Myocardial contractile parameters were measured at 3, 7, 14, and 28 days after MI (Figure 2B and Supplementary Figures 4E,F). An echocardiographic image at day 28 (Figure 2C) revealed that the transplantation of OMSC^{TPP1} significantly improved systolic function with higher EF and FS than the OMSC^{null} and DMEM groups (Figure 2D). The results from Sirius red staining showed a reduction in infarct scarring (Figures 2E,F). Together, these results confirmed that transplantation of OMSC^{TPP1} could produce a significantly

smaller infarct area and better cardiac function than the OMSC^{null} group.

Enhancing Aged MSC Survival Through TPP1 Overexpression Results in Cardiac Cell Protection and Angiogenesis in Ischemic Hearts

The efficacy of aged MSC therapy is thought to be limited by the exceptionally small number of transplanted cells remaining in the target tissue. To address this question, we investigated whether TPP1 overexpression could improve OMSC survival *in vivo*. OMSCs were labeled with GFP for transplantation. The results indicated that the OMSC^{TPP1} transplantation group had a higher survival rate than the OMSC^{null} group (Figure 3A).

To assess whether enhanced aged MSC survival mediated by TPP1 could result in greater angiogenesis and less cardiomyocyte death, we detected the density of microvessels and cardiomyocyte

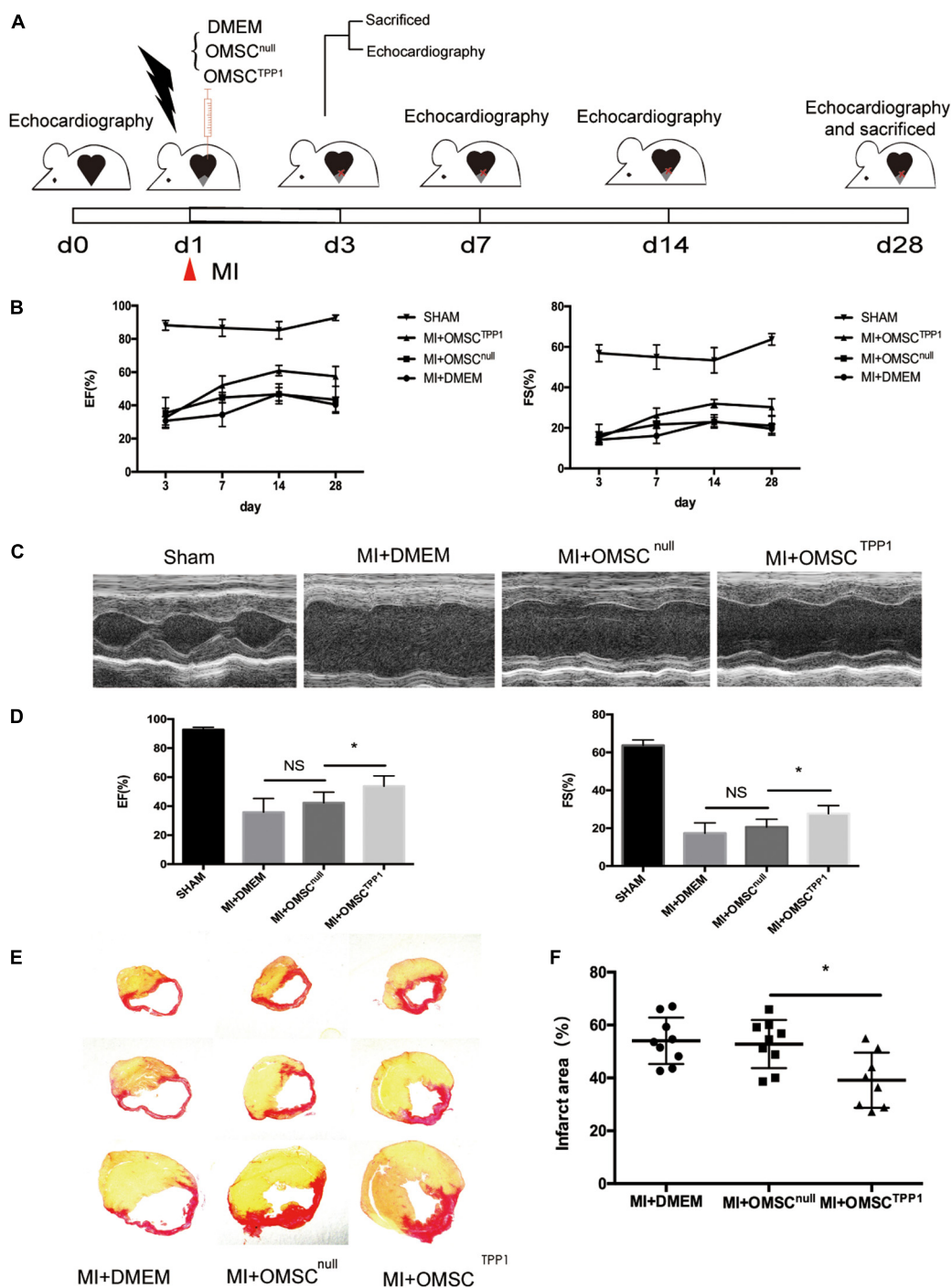


FIGURE 2 | OMSC^{TPP1} improves cardiac functional recovery and reduced cardiac fibrosis after MI. **(A)** Schematic representation of 28-day study of a myocardial infarction mouse model. Echocardiography was performed at days 3, 7, 14, and 28. Animals ($n = 4-8$ per group) were euthanized for tissue harvesting at days 3 and 28. Sham-operated mice served as controls. **(B)** Cardiac function, including ejection fraction (EF) and fraction shortening (FS), at days 3, 7, 14, and 28 after MI. **(C)** Representative echocardiographic images at day 28 after MI. **(D)** EF and FS at day 28 after MI. **(E,F)** The infarct area was determined by Sirius red staining ($n = 4-8$). All data are expressed as mean $\pm$ S.D. * $P < 0.05$.

apoptosis in the peri-infarct zones by CD31, smooth muscle actin (SMA), and TUNEL staining, respectively. CD31- and SMA-positive vessels were significantly more common in the

OMSC^{TPP1} group than in the OMSC^{null} group (Figures 3B,C). TUNEL staining was conducted to detect the cardioprotective effects of OMSC^{TPP1} therapy *in vivo*. The numbers of apoptotic

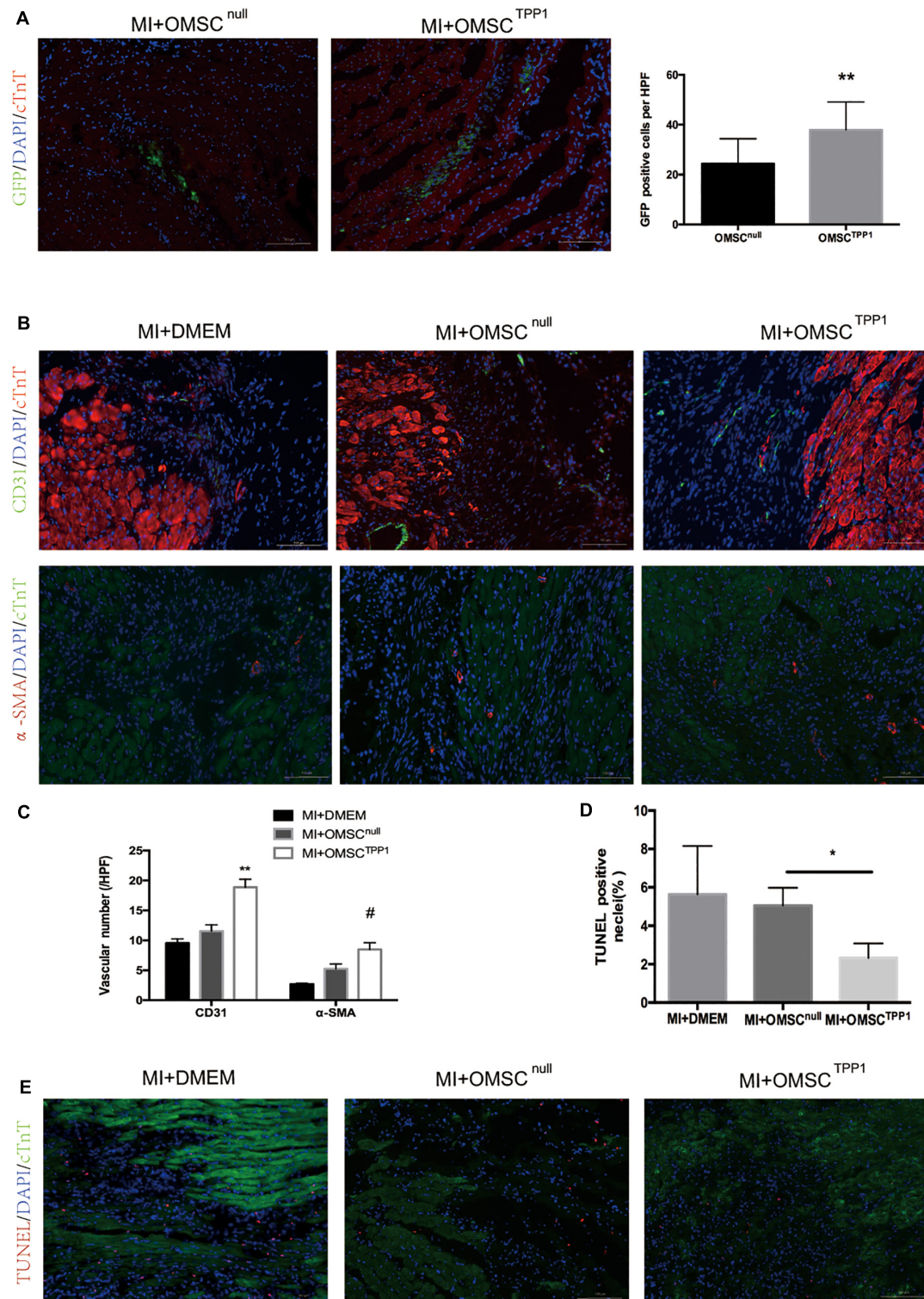


FIGURE 3 | Enhanced OMSC survival after TPP1 overexpression leads to reduced cardiomyocyte loss and enhanced angiogenesis after MI *in vivo*.

(A) Representative images showing cell survival of GFP-OMSC^{null} and GFP-OMSC^{TPP1} at day 3 post cell transplantation. GFP-positive cells were counted as surviving cells in the infarcted area, $n = 15\text{--}20$ from five mice in each group. **(B,C)** Representative immunofluorescence of α -SMA, and CD31 in the infarct border zone of ischemic hearts at day 28 post MI. Angiogenesis was identified by 5–6 high-power fields (HPFs) for each mouse ($n = 5\text{--}6$ for each group). **(D,E)** Representative images of a TUNEL staining assay in infarct border zone 3 days after MI; TUNEL-positive cardiomyocytes were quantitatively measured. At least five fields for each mouse were selected to count in each group, $n = 5\text{--}6$ mice. Data are shown as mean $\pm$ S.D. * $P < 0.05$, # $P < 0.05$, ** $P < 0.01$.

cells in the peri-infarct zone were significantly decreased in OMSC^{TPP1} group compared to the OMSC^{null} and DMEM group (Figures 3D,E).

Furthermore, to investigate the angiogenesis effects of TPP1-modulated OMSCs *in vitro*, we conducted a tube formation assay of HUVECs, which revealed significantly better tube information in the OMSC^{TPP1} and OGD medium group than in the OMSC^{null} and OGD medium group, and there were no statistical differences between the OMSC^{TPP1} and normoxia medium and OMSC^{null} and normoxia medium groups (Supplementary Figures 4A,B). We also evaluated the effects of hMSCs on cardiomyocyte apoptosis *in vitro*. The results demonstrate that less apoptotic cardiomyocytes were found in the OMSC^{TPP1} and OGD medium group than in the OMSC^{null} and OGD medium group (Supplementary Figures 4C,D).

These data indicate that prolonged OMSC survival in oxidative stress results in stronger angiogenesis and cardiomyocyte protection, together with improved myocardial function.

TPP1 Promotes DNA Repair via MRE11 Without Affecting Telomerase and Other Shelterin Components

Telomerase activity and shelterin components play a pivotal role in telomere length, telomere stability, and cell aging, particularly in stem cells (Blasco, 2007; Rossi et al., 2007). Numerous studies have found that telomerase and shelterin components have a positive impact on cell apoptosis (Arish et al., 2015; Wang et al., 2019). We checked whether TPP1 overexpression affects other shelterin components and found that the transduction of TPP1 did not affect the expression level of other shelterin genes (Supplementary Figures 3A,B). TPP1 overexpression also did not influence telomerase activity (Supplementary Figures 3C,D).

A recent study demonstrated the key role of TPP1 in telomere function and the DNA damage response (Qiang et al., 2014). To examine whether TPP1 is involved in DNA repair, γ -H2AX, which usually appears soon after DNA damage and demarcates the sites of unrepaired double-stranded breaks, was used to evaluate the effects of TPP1 on OMSCs after H₂O₂ stimulation. After 1 h H₂O₂ treatment, cells were able to repair oxidative damage for 2 h, and the remaining foci were detected by γ -H2AX immunofluorescence. Compared to OMSC^{null}, γ -H2AX foci were remarkably decreased in cells transfected with TPP1 after 2 h recovery (Figures 4A,B). MRE11 plays a vital role in DNA repair. Thus, we detected its expression in OMSCs that overexpress TPP1. A significant increase in the protein level of MRE11 was found in OMSC^{TPP1} relative to OMSC^{null} (Figures 4C,D). In addition, MRE11 was downregulated when TPP1 was knocked down by TPP1 siRNA (Supplementary Figure 2C) in young MSCs (YMSCs) (Figures 4E,F).

TPP1 Promotes DNA Repair Through Activation of AKT Phosphorylation in OMSCs

AKT might augment telomere protection by promoting homodimerization of TPP1 (Han et al., 2013). Therefore, we

speculated that TPP1 may induce AKT phosphorylation. As expected, TPP1 enhanced AKT phosphorylation in the presence of H₂O₂ in OMSCs (Figures 5A,B), and a decrease in AKT phosphorylation was observed after H₂O₂ exposure with TPP1 depletion by TPP1 siRNA (Figures 5C,D). Moreover, a DNA repair assay showed that DNA repair capacity of OMSC^{TPP1} was arrested by LY294002 (PI3K inhibitor), proving that TPP1 facilitates DSB repair through AKT activation (Figures 5E,F).

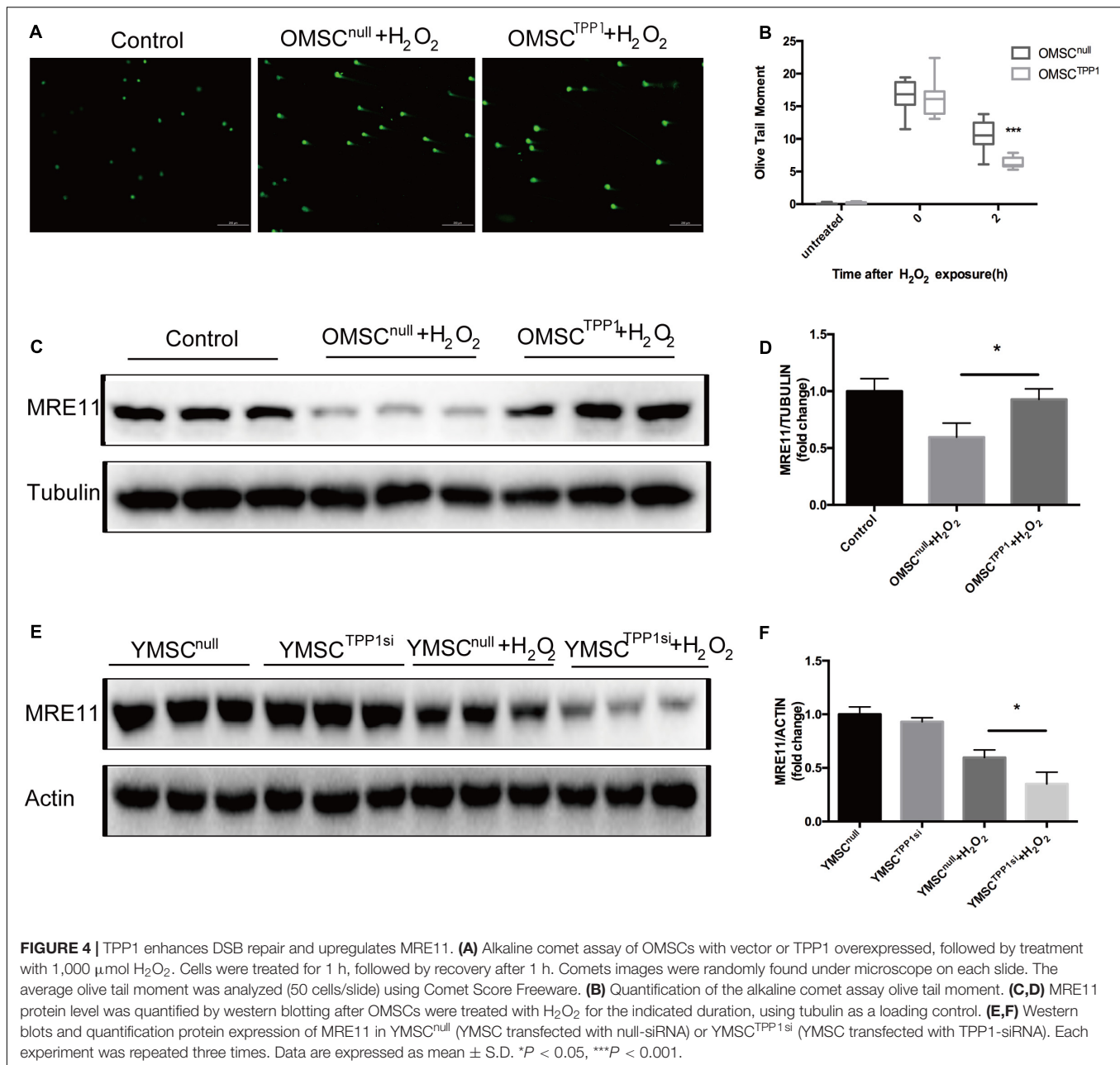
MRE11 Is Downstream of AKT and Responsible for TPP1-Induced DNA Repair

To further evaluate the role of MRE11 as the mediator of the effects of TPP1 on DNA repair, we investigated the interaction between AKT and MRE11. Previous studies have found that AKT promotes DNA repair in cancer cells by upregulating MRE11 expression following exposure to ionizing radiation (Deng et al., 2011). We wondered whether the upregulation of MRE11 by TPP1 was mediated by the AKT pathway. Interestingly, MRE11 was downregulated by the PI3K inhibitor LY294002 even after TPP1 overexpression (Figures 6A,B), which means that AKT phosphorylation is necessary for TPP1-induced MRE11 upregulation. To confirm whether MRE11 affects AKT phosphorylation, we transfected siRNA against MRE11 into TPP1-overexpressed OMSCs. The results indicated that MRE11 knockdown did not influence AKT phosphorylation (Figures 6C,D). These findings suggest that TPP1 activates AKT phosphorylation upstream of MRE11 to mediate DNA repair, thereby inducing greater therapeutic effects of OMSCs after their transplantation in MI (Figure 6E).

DISCUSSION

Mesenchymal stem cell-based cell therapy for ischemic heart disease has made great advances in recent decades. However, improving the therapeutic effects of OMSCs remains a major challenge. In our study, gene expression analyses of YMSCs versus OMSCs revealed that TPP1 was likely to be involved in the process of aging (Supplementary Figure 2A). Numerous studies have found that the levels of shelterin proteins that directly interact with TPP1, such as TRF1 and POT1, are decreased in aging (Derevyanko et al., 2017; Hosokawa et al., 2017). In the present study, the key role of TPP1 in enhancing therapeutic properties of OMSCs was demonstrated. We treated MSCs with H₂O₂ to induce oxidative stress to mimic the microenvironment of myocardial infarction, and we confirmed the importance of TPP1 in protecting OMSCs from H₂O₂-induced apoptosis. This led to enhanced MSC survival following stresses associated with ischemia, with potential implications for cell therapy in myocardial infarction. Furthermore, our results support a pathway wherein TPP1 activates AKT phosphorylation and then upregulates MRE11 to mediate DNA repair and enhanced the therapeutic properties of OMSCs.

The repair capacity of DSBs usually declines with aging (Pan et al., 2016) and is associated with a decreased expression of



DNA-repair proteins binding with the telomere (Ju et al., 2006). A previous study revealed that TPP1 blocks DSB resection, mediated by ATR (Kibe et al., 2016). MRE11, an important component of DNA repair, is critical for DNA end resection and regulating DSB repair. It is also a key part of the MRN complex, which is involved in non-homologous end-joining (NHEJ) and homology-directed repair (Stracker and Petrini, 2011). In addition, MRE11 had frequently been found to decrease in aged humans and other animals, with reduced NHEJ activity as well (Ju et al., 2006; Vyjayanti and Rao, 2006). It has also been found that MRE11 downregulation impairs the repair capacity of radiation-induced DSB in cancer cells (Nicholson et al., 2017). In addition, MRE11 interacts with TRF2 to play a protective role

in telomere fusion after replication (de Lange and Petrini, 2000; Deng et al., 2009). Here, we found that TPP1 could maintain MRE11 stabilization under oxidative stress and is the critical factor in the process of TPP1 enhancement of DSB repair and cell anti-oxidative stress capacity in human MSCs.

Many cellular processes can be attributed to AKT signaling, including apoptosis, proliferation, migration, and cell cycle regulation (Manning and Toker, 2017). In addition, several studies have demonstrated that AKT activity is involved in telomere protection and DNA repair (Han et al., 2013; Liu et al., 2014). AKT inhibition has also been shown to disrupt TPP1 and POT1 recruitment to the telomere (Han et al., 2013). Here, we revealed non-telomeric mechanisms of AKT, which

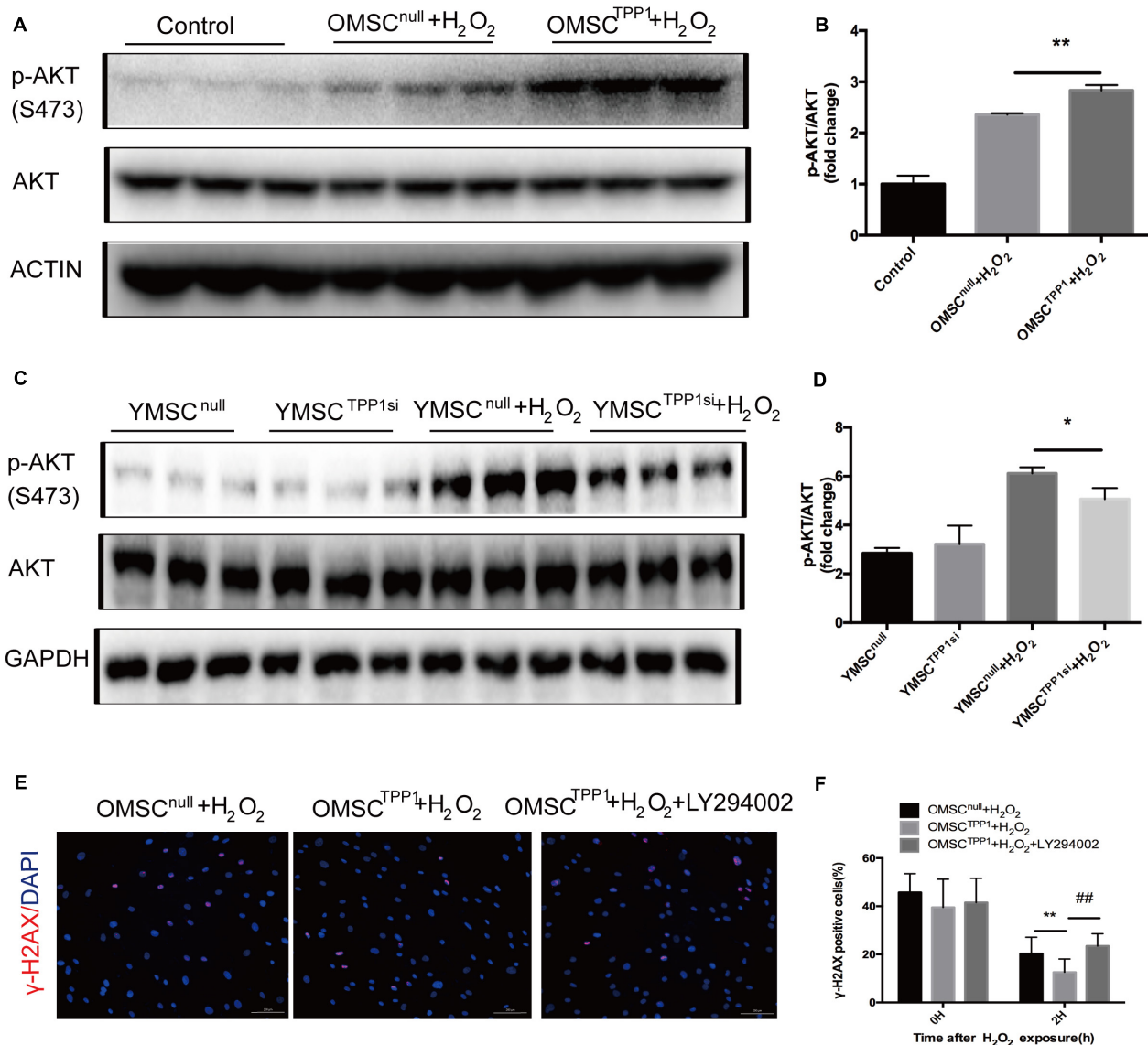


FIGURE 5 | TPP1 enhances DSB repair by AKT activation. **(A,B)** Western blots and quantification of p-AKT (s473) protein expression in OMSCs. **(C)** Western blots and quantification of p-AKT(s473) protein expression in YMSCs. **(D)** Western blots and quantification of p-AKT (s473) and TPP1 protein expression in OMSCs. **(E)** After transfection with lentivirus 24 h, OMSCs were pretreated with DMSO or LY294002 for 24 h and then treated with H₂O₂ for 1 h with another 1 h recovery. After this, the cells were fixed and stained for γ-H2AX and nuclei (DAPI) through immunofluorescence. Representative fluorescence graphs show the accumulation of γ-H2AX in cells, magnification 100×. γ-H2AX (red)-positive cells were counted as unrepaired cells through 5–6 high-power fields (HPFs) per well, $n = 3$ well in each group. **(F)** Quantification of γ-H2AX-positive cells in OMSCs after H₂O₂ treatment at the indicated times. Data are expressed as mean ± S.D. * $P < 0.05$, ** $P < 0.01$, ## $P < 0.01$.

are partly in accordance with a previous study that found that AKT interacts with TPP1 and regulates TPP1 homodimerization (Han et al., 2013). Our data showed that TPP1 activates AKT, which consequently upregulates MRE11 to enhance DSB repair. It is remarkable that the AKT-MRE11 pathway in OMSCs with overexpressed TPP1 was not activated in the absence of oxidative stress (**Supplementary Figure 2D**). Based on this molecular mechanism, it is reasonable to explain the *in vitro* results of tube formation, such that no difference was found between the OMSC^{TPP1}

and normoxia medium group and the OMSC^{null} and normoxia medium group.

Taken together, our results highlight the non-telomeric mechanisms by which TPP1 regulates aged MSC function and DSB repair capacity. We provide evidence that the mechanism involves the mutual regulation between TPP1 and AKT, and activation of MRE11 to enhance DNA repair. This work provides new mechanistic insight into autologous MSC transplantation therapy in aged individuals and indicates TPP1's role as a key element promoting DNA repair in the AKT/MRE11 pathway.

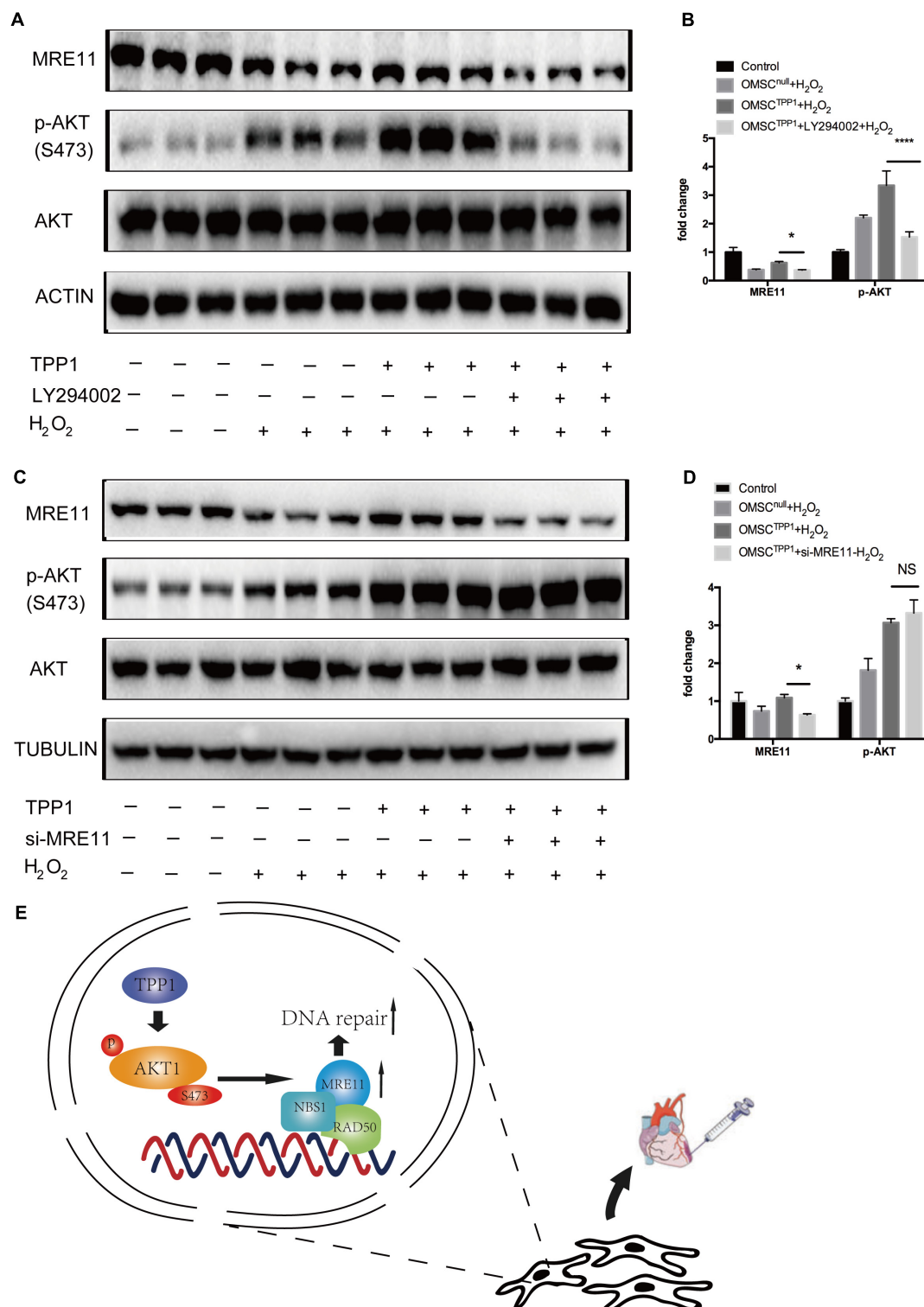


FIGURE 6 | The AKT/MRE11 pathway is responsible for the protective effects of TPP1 in response to oxidative stress. **(A,B)** OMSC^{null} and OMSC^{TPP1} were treated with DMSO or LY294002 for 24 h and treated with H₂O₂. Then, the cells were pretreated and lysed, and the levels of the p-AKT, AKT, and MRE11 proteins were detected by western blotting. Actin served as the loading control. **(C,D)** OMSC^{null} and OMSC^{TPP1} were transiently transfected with siRNA (control) or MRE11-siRNA for 48 h and then exposed to H₂O₂. The Western blots and quantification for MRE11, p-AKT, and AKT were analyzed. Each experiment was repeated three times. The data are represented as mean ± S.D. **P* < 0.05, *****P* < 0.0001. **(E)** Schematic diagram showing that TPP1 elevated DSB repair through the AKT-MRE11 pathway to enhance the therapeutic effects of OMSCs transplantation in MI.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the animal care and use Committee of Zhejiang University.

AUTHOR CONTRIBUTIONS

KY and ZZ designed the study. SC, WH, CG, and FL performed the analyses and produced the initial draft of the manuscript. JC, YQ, DX, and JZ contributed to the drafting of the manuscript. XL and JW provided conception and design, administrative support, financial support, and final approval of

the manuscript. All authors have read and approved the final submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.588023/full#supplementary-material>

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REX-001, a BM-MNC Enriched Solution, Induces Revascularization of Ischemic Tissues in a Murine Model of Chronic Limb-Threatening Ischemia

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Background: Bone Marrow Mononuclear Cells (BM-MNC) constitute a promising alternative for the treatment of Chronic Limb-Threatening ischemia (CLTI), a disease characterized by extensive blockade of peripheral arteries, clinically presenting as excruciating pain at rest and ischemic ulcers which may lead to gangrene and amputation. BM-MNC implantation has shown to be efficient in promoting angiogenesis and ameliorating ischemic symptoms in CLTI patients. However, the variability seen between clinical trials makes necessary a further understanding of the mechanisms of action of BM-MNC, and moreover, to improve trial characteristics such as endpoints, inclusion/exclusion criteria or drug product compositions, in order to implement their use as stem-cell therapy.

Materials: Herein, the effect of REX-001, a human-BM derived cell suspension enriched for mononuclear cells, granulocytes and CD34+ cells, has been assessed in a murine model of CLTI. In addition, a REX-001 placebo solution containing BM-derived red blood cells (BM-RBCs) was also tested. Thus, 24 h after double ligation of the femoral artery, REX-001 and placebo were administrated intramuscularly to Balb-c nude mice (n:51) and follow-up of ischemic symptoms (blood flow perfusion, motility, ulceration and necrosis) was carried out for 21 days. The number of vessels and vascular diameter sizes were measured within the ischemic tissues to evaluate neovascularization and arteriogenesis. Finally, several cell-tracking assays were performed to evaluate potential biodistribution of these cells.

Results: REX-001 induced a significant recovery of blood flow by increasing vascular density within the ischemic limbs, with no cell translocation to other organs. Moreover, cell tracking assays confirmed a decrease in the number of infused cells after 2 weeks post-injection despite on-going revascularization, suggesting a paracrine mechanism of action.

Conclusion: Overall, our data supported the role of REX-001 product to improve revascularization and ischemic reperfusion in CLTI.

Keywords: Chronic limb-threatening ischemia, critical limb ischemia, stem cell therapy, revascularization, angiogenesis, BM-MNC, bio-distribution assay

INTRODUCTION

Chronic limb-threatening ischemia (CLTI) results from the narrowing and obstruction of major arteries of the limb, usually correlated with the formation of atherosclerotic plaques (Conte and Vale, 2018; Uccioli et al., 2018). The incidence of CLTI is ~500–1,000 per million per year (Nehler et al., 2014), 10–15% of which are older adults (Fowkes et al., 2013). CLTI patients suffer from chronic rest pain, ischemic ulcers which may lead to gangrene, and an eventual amputation of toes or extremities. Also, due to associated comorbidities, CLTI patients are at a greater risk of experiencing myocardial and cerebral vascular infarctions (Norgren et al., 2007; Walter et al., 2011; Simpson et al., 2014; Conte and Pomposelli, 2015). Therefore, CLTI is a debilitating disease which significantly impacts patient's quality of life by leading to dependency on caregivers, permanent local wound treatment, and the chronic use of pain-relieving medications (Lawall et al., 2012).

To date, the ultimate treatment of CLTI is a surgical revascularization through bypass grafting or angioplasty, and amputations in case of non-salvageable limbs (Lichtenberg et al., 2018), although success rate of treating CLTI is highly variable and, in many situations, suboptimal (Patel, 2016). Moreover, only 30% of patients are suitable for a surgical revascularization due to high comorbidities (Adam et al., 2005; Goodney et al., 2012). As a result, amputation rates are unacceptably high in CLTI patients, typically exceeding 15–20% at 1 year of intervention and can vary according to additional comorbidities (Duff et al., 2019) such as diabetes mellitus (DM), which elevates the amputation rate to 50% (Spren et al., 2016). In general, DM patients are at a higher risk of developing CLTI or progressing to the severest stages of the disease, due to impaired vasculogenesis and vessel remodeling mechanisms (Howangyin and Silvestre, 2014; Thiruvoipati et al., 2015).

Thus, without effective treatments for CLTI, the prevalence of this debilitating disease may remain constant or increase with time (Duff et al., 2019). It is therefore imperative to identify alternative therapies to treat CLTI, to improve the quality of life of patients by reducing the need for multiple surgeries and/or amputations.

Novel cell therapies based on the administration of bone marrow-derived mononuclear cells (BM-MNC) have become a promising alternative to conventional surgery or angioplasty for the treatment of CLTI (Huang et al., 2005; Bartsch et al., 2007; Cobellis et al., 2008; Lu et al., 2011; Davies, 2012; Fowkes et al., 2017; Kondo et al., 2018). BM-MNC consist of a heterogeneous mix of mesenchymal stem cells (MSC), hematopoietic progenitor cells (HPC), endothelial progenitor cells (EPC), immature monocytes and lymphocytes, and pluripotent stem cells (Ratajczak et al., 2008; Franz et al.,

2009). Since the first implantation of autologous BM-MNC in 2002 (Tateishi-Yuyama et al., 2002), different pre-clinical and clinical studies have reported the beneficial effects of different combinations of BM-MNC in CLTI (Kalka et al., 2000; Hamano et al., 2001; Franz et al., 2009; Fujita and Kawamoto, 2017; Rigato et al., 2017). Overall, sufficient evidence has demonstrated autologous BM-MNC (aBM-MNC) therapies to be safe and effective in promoting new vessel formation, and thus, reversal of CLTI (Fadini et al., 2010; Idei et al., 2011; Murphy et al., 2011; Liang et al., 2016; Guo et al., 2018; Wahid et al., 2018), through improved perfusion, ankle brachial index (ABI), wound healing, pain at rest, pain free walking distance, and amputation free survival (Amann et al., 2009; Cobellis et al., 2010; Fadini et al., 2010; Ruiz-Salmeron et al., 2011; Yusoff et al., 2019). However, the results found in clinical trials are variable and moreover, cell survival is usually poor under ischemic environments (Brenes et al., 2012; MacAskill et al., 2018; Qadura et al., 2018; Beltran-Camacho et al., 2020), being necessary a deeper understanding of the mechanism of action of BM-MNC in order to improve their use as cell therapy to reverse CLTI.

In this study we investigated the regenerative effect of REX-001, an adult human bone marrow (BM)-derived cell suspension enriched for MNC, when injected in a murine model of CLTI, in order to understand the mechanisms potentially involved in BM-MNC induced revascularization within the ischemic tissues, as well as to evaluate REX-001 potential bio-distribution after intramuscular administration.

MATERIALS AND METHODS

Cell Isolation and Culture

REX-001 consists in a cell suspension of adult human BM derived cells enriched for MNC, containing a population of lymphocytes (20–51%), monocytes (4–22.3%) as well as granulocytes (20–67.7%) and hematopoietic stem cells expressing CD34 (1.4–10%). The final formulation of REX-001 can be found in the patent (US 2018/0055884 A1). For this study, BM-MNC were isolated from heparinized BM of healthy human donors (purchased from Hemacare, Charles-River). Briefly, manufacturing is performed with an initial BM volume reduction, including plasma, and red blood cell (RBC) removal. The intermediate sample bag containing volume-reduced BM goes through an automated density gradient centrifugation, followed by two washes of the MNC suspension. Approximately about 45 ml of BM-MNC product is collected in the output bag and the other components are removed to the disposable bag. The drug substance is centrifuged and the pellet is resuspended in the final formulation mix (hereafter adjuvant), a lactated ringer's solution with 1% w/v HSA and 2.5% w/v glucose in a volume of 5–30 ml.

On the other hand, the REX-001 Placebo Product (hereafter Placebo), a cell suspension of BM-derived red blood cells (BM-RBCs), is also collected from the RBC fraction during volume reduction step, formulated in 20 ml of adjuvant solution. Placebo is visually indistinguishable from fresh active product.

Animals

Female Balb-C Nude (CAnN.Cg-*Foxn1*^{nu}/Crl) mice (n:70) (see **Figure 1** for mice distribution), age 9 weeks, were obtained from Charles River Laboratories. Mice were allocated in individual ventilation cages inside special monitored rooms. Animals were fed sterile standard chow diet ad libitum and had free access to sterile water. Additionally, animals were constantly monitored for signs of ill-health for euthanasia in case of excessive suffering or presence of symptoms which would likely affect the experiment results. No animal was sacrificed prematurely during the experiment. Animal experiments were approved by the Ethical committee of the University of Cadiz, as well as the Andalusian Committee of animal experimentation (registration number ES110120000210 and project number 07-04-2016-043). This study followed the standard guidelines for animal research included in the Spanish laws included into the RD 53/2013 as well as the European Regulations (2012/707/UE).

Experimental Design

In order to evaluate the effect of REX-001 on revascularization in a murine model of CLTI, and also determine the presence of these cells within the ischemic tissues and/or their bio-distribution, different assays were carried out, as described in **Figure 1**.

Evaluation of Revascularization Effect

Briefly, three separated assays using REX-001 cell suspensions from healthy human donors were performed, with the same strategy and follow-up in all of them (**Figure 1A**). For the CLTI model, Balb-c nude mice (n:51) were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally before surgery, and double ligation of the left femoral artery was performed, occluding the distal and proximal ends with double knots (non-absorbable 6/0) of suture, as described (Niiyama et al., 2009; Beltran-Camacho et al., 2020). Mice received Ketoprofen (2 mg/kg) intraperitoneally as analgesic for three consecutive days.

Mice were then equally distributed between groups, based on blood flow levels and ischemic status registered 24 h after surgery, prior cell administration. Thus, mice received either REX-001 cells (an equivalent amount of REX-001 to obtain $1 \cdot 10^6$ MNC) in 50 μ l of adjuvant solution (R n:21), or 50 μ l of the Placebo solution (P, n:15). In addition, another set of mice received 50 μ l of the adjuvant, vehicle solution (A, n:15). Administration was done through 3-4 intramuscular injections in different sites of the left limb muscles: low back, low frontal, and middle muscles. Two mice died a few days later after infusion (from the A and R groups), and no measurements were registered from them. The total number and distribution of mice within the groups/assay can be found in **Figure 1A**.

Follow up of Physiological Changes in Response to Hind Limb Ischemia and Cell Administration

Blood flow was measured for both paws, on day 0 (before and after surgery), day 1, day 7, day 14, and day 21, using a Laser Doppler system (Periflux System 5000; Perimed). The right limb was taken as control, not-injured limb, and perfusion was expressed as the ratio of left (ischemic) vs. right (non-ischemic) limb. In addition, ischemic symptoms such as motility impairment, inflammation, ulceration, and necrosis were also registered for all mice during the entire assay according to Tarlov and ischemia scores (Tarlov, 1954; Yu et al., 2005; Brenes et al., 2012), registered in **Supplementary Tables S1–S3**.

Tissue Extraction and Processing

Mice (n:49) were sacrificed in a CO₂ chamber on day 21 after surgery. Low frontal muscles (tibialis) and middle muscles (bicep femoris, adductor, and semi-membranous) of the left limb were extracted and fixed for 15 days embedded in 4% formaldehyde prior dehydration in 30% sucrose during 24 h. Tissues were then frozen in OCT before immunohistochemistry (IHC).

Cell Pre-Labeling Assay

In addition to the groups described in section Evaluation of revascularization effect, another set of Balb-C Nude mice (n:6) were employed to confirm the presence of human cells within the injured area, by using a pre-labeling approach (**Figure 1B**). Thus, REX-001 cells were pre-labeled with biocompatible organic fluorescent nanoparticles (LuminiCell Tracker™ 540, SCT010 Sigma-Aldrich), 1 h at 37°C, 5% CO₂, and washed several times with PBS 1X before being administered ($1 \cdot 10^6$ cells/mouse) to mice that underwent femoral ligation 24 h earlier, as described above. Mice were sacrificed in a CO₂ chamber at different times after surgery: day 4 (n:2), day 14 (n:2), and day 21 (n:2), and muscles from low frontal limb and medium limb were extracted and processed as described before.

Immunohistochemistry (IHC)

Different tissue sections from all mice were employed to calculate the number of vessels and diameter size changes in response to cell administration and also to detect pre-labeled human cells within the ischemic limbs. Thus, tissues embedded in OCT were cut in consecutive sections of 8 μ m and placed in poly-lysine slides. In total, 5 tissue sections (low frontal), separated by 32 μ m each, were employed for cellular and vessel detection, while 3 tissue sections (middle muscles) were used to measure vascular diameters. All sections were pretreated for antigen retrieval and permeabilization with 1% SDS during 5 min and with 1% triton, 20 min, followed by a blocking step with 5% goat serum (S-1000, Vector Laboratories) and 0.1% triton for 1 h. Tissues were then incubated with anti- α -actin smooth muscle (α -SMA, 1:400, A5228 Sigma-Aldrich) at 4°C, over-night. Tissue auto-fluorescence was prevented by incubation with 0.3% Sudan Black B-70% ethanol for 20 min and incubation with specific secondary antibodies for 1 h in the darkness at room temperature (RT) were performed. Finally, nuclear staining with DAPI (0.2 μ g/ml) was carried out.

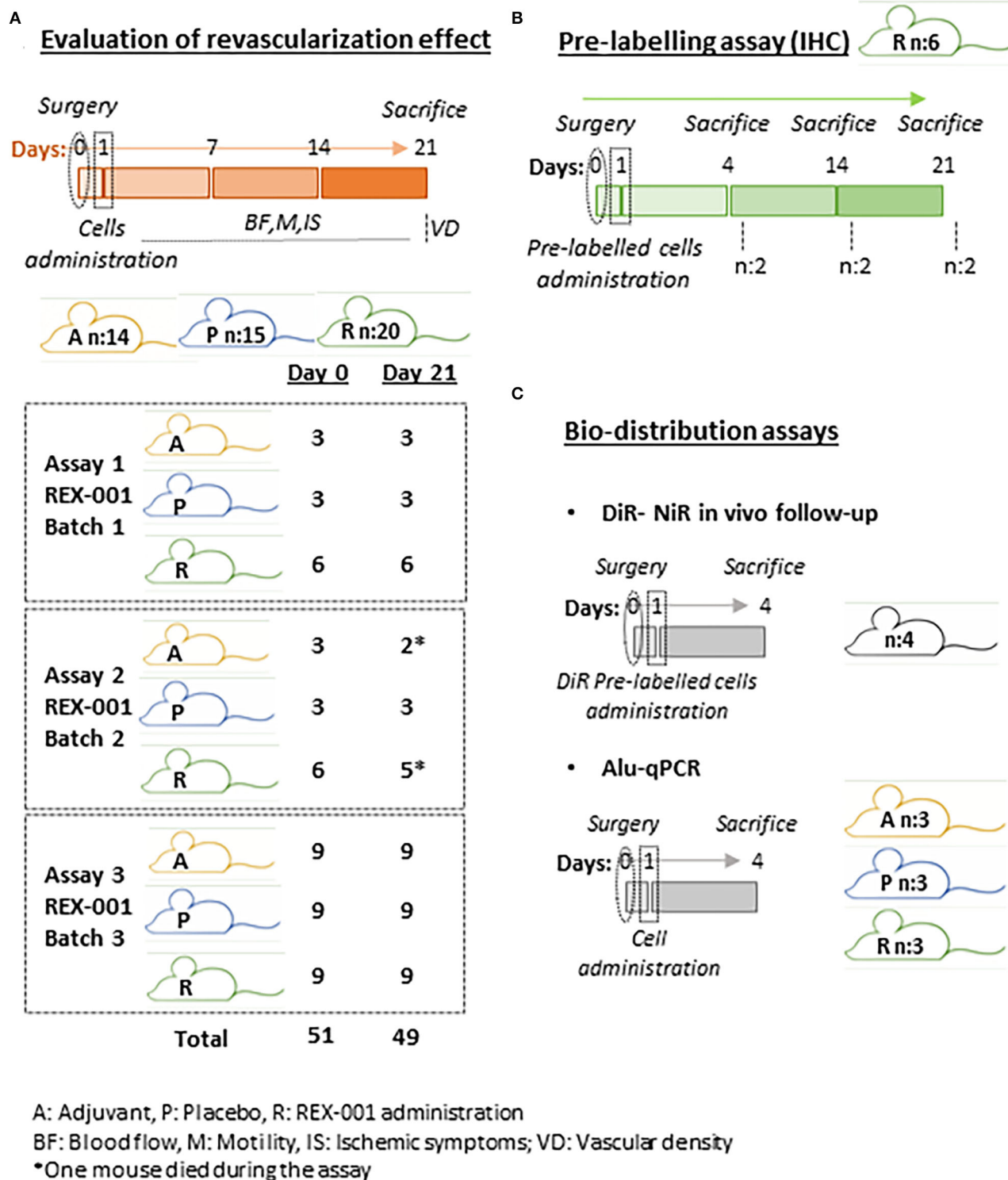


FIGURE 1 | Schematic representation of experimental distribution. **(A)** Schematic representation of evaluation of revascularization effect assay distribution. Three independent assays were performed equally with REX-001 cells obtained from healthy human batches. Balb-c nude received either adjuvant solution **(A)**, placebo **(P)** or REX-001 cells **(R)**. **(B)** A pre-labelling assay was performed to confirm the presence of REX-001 cells within the ischemic tissues, sacrificing Balb-c mice at different times, as shown. **(C)** Finally, REX-001 biodistribution was evaluated after intramuscular administration with DiR cell pre-labelling and NiR *in vivo* and *ex vivo* detection, and also by detection of specific human Alu sequences by qPCR in several organs (limbs, spleen, kidneys, lungs, liver). The number of mice per assay and total numbers per group is shown.

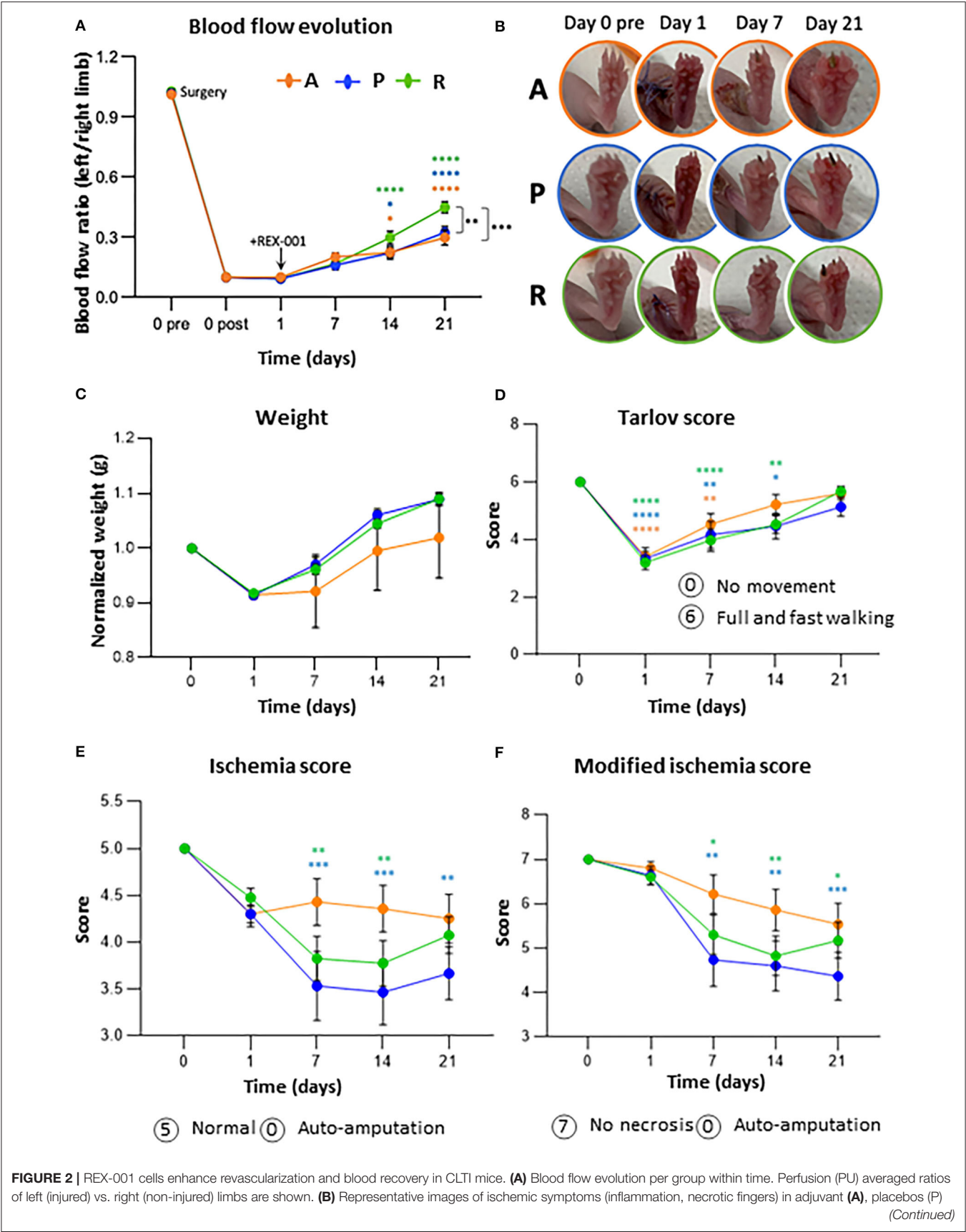


FIGURE 2 | and REX-001 (R) treated mice. **(C)** Weight changes, normalized vs. averaged values on day 0, pre-surgery. **(D)** Motility changes within time, according to Tarlov score values. Ischemic changes (ulceration, necrosis) detected along the assay, measured with both, the Ischemia **(E)** and modified ischemia scores **(F)**. Groups tested: Balb-c nude mice injected with Adjuvant (vehicle-only) (n:14), Placebo (P, n:15) and REX-001 (R, n:20, from different donors, R1, n:6, R2, n:5, R3, n:9). The averaged values from the three assays is shown as mean $\pm$ SE. Complete scores meaning are described in **Supplementary Tables 1–3**. Significant differences were calculated by two-way ANOVA and Tukey *post-hoc*, represented as: * with colors compared to post-surgical ratios at day 0 and * in black between groups in the same day (**p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001, and *****p*-value < 0.0001).

The entire tissue area of each section was analyzed by fluorescence microscopy, acquiring images at 20x and 40x using MMI CellCut Plus (Olympus) and visualizing them with the Zen 2 (Zeiss) software. Results were expressed as the number of blood vessels per cm² or blood vessels diameter (μ m). Additionally, the number of vessels containing pre-labeled cells vs. the total number of vessels were also quantified, as described (van Weel et al., 2008; Beltran-Camacho et al., 2020). Results were expressed as the mean $\pm$ SEM.

Bio-Distribution Assay

In order to confirm the presence of human (h) cells within the ischemic tissues and moreover, evaluate potential bio-distribution after intramuscular administration, two complementary approaches were carried out (**Figure 1C**): the application of an *in vivo* assay labeling cells with the lipophilic dye 1,1-dioctadecyl 3,3,3,3 tetra-methyl-indo-tricarbocyanine iodide (DiR) and Near Infrared (NiR) detection (Kalchenko et al., 2006; Bulte and Daldrup-Link, 2018), and further quantification of human DNA (hu-DNA) by q-PCR with specific Alu sequences (Funakoshi et al., 2017).

DiR Labeling

REX-001 cells ($3 \cdot 10^6$) were labeled with 6.67 μ M of DiR (Biotium #60017) according to manufacturer's instructions, 25 min at 37°C, 5% CO₂, centrifuged and washed three times with PBS 1X to discard excess or unbound dye. The final cell pellet was then resuspended in 50 μ l adjuvant solution before being administered intramuscularly to Balb-c nude (n:3) mice 24 h after femoral ligation, as described before ($1 \cdot 10^6$ /mouse). In addition, a negative control consisting in a mouse injected with unlabeled cells was used to discard any background signal. Anesthetized mice were scanned using NiR LI-COR Odyssey system (LI-COR) at days 1 and 4 after cell infusion. Parameters used for the scan: intensity 3, detectors in 700 nm and 800 nm activated, $\sim$ 30 min. Mice were sacrificed on day 4 and organs (lungs, kidneys, liver, spleen, right, and left limb muscles) were extracted and scanned again *ex vivo* to evaluate the presence of cells in individual organs. Intensity values were normalized vs. the negative control, taken their background signal as zero.

Alu-Based qPCR Quantitative Assay

Finally, the presence of human cells in different organs was also quantified by amplification by qPCR of Alu specific sequences (**Figure 1C**), as described (Beltran-Camacho et al., 2020). Thus, 24 h after femoral ligation, another set of CLTI mice (n:9) received intramuscularly 50 μ l of adjuvant solution (A, n:3), placebo solution (P, n:3) or $1 \cdot 10^6$ REX-001 cells (R, n:3). Animals were sacrificed 4 days after cell transplantation and organs (lungs,

kidneys, liver, spleen, right, and left limb muscles) were extracted, directly frozen in liquid N₂ and stored at -80°C . Biopsies ($\approx$ 40–50 mg) were crushed using liquid N₂ into a fine powder. Genomic DNA was isolated from tissue samples using the E.Z.N.A.® Tissue DNA Kit (Omega-biotek D3396-01). Human DNA (hu-DNA) was quantified using the Alu detection approach, as described (Funakoshi et al., 2017; Beltran-Camacho et al., 2020).

Each qPCR reaction employed 100 ng of genomic DNA in which Alu sequences were amplified using TaqMan Universal Master Mix II (ThermoFisher 4440043), 0.2 μ M primers and 0.25 μ M hydrolysis probes designed by (Funakoshi et al., 2017): forward primer 5'-GGTGAAACCCGCTCTACT-3', reverse primer 5'-GGTTCAAGCGATTCTCCTGC-3' and label probe 5'-(6-FAM)-CGCCCGGCTAATTTTGTAT-(BHQ-1)-3' (synthesized by Metabion). qPCR was carried out using a CFX Connect Real-Time System (Biorad) with the following protocol: 1 cycle of 95°C/10 min and 50 cycles of 95°C/15 s, 56°C/30 s, and 72°C/30 s.

Linearity and resolution limits were determined by diluting known amounts of hu-DNA (from 5 ng to 1 pg) in murine DNA, as described (Funakoshi et al., 2017; Beltran-Camacho et al., 2020). qPCR was performed in triplicates and C_t mean values were plotted to obtain the lineal equation as well as the R² values (**Supplementary Figure S2**). The sensitivity of the assay is such that one human cell among 10,000 mouse cells could be detected.

The amount of hu-DNA detected in 100 ng of total genomic DNA extracted was measured by qPCR, and further extrapolation of the total hu-DNA extracted was then calculated per mg of tissue. Finally, the amount of DNA (ng) per cells was calculated considering the relation of 5 pg of DNA per human cell (Dolezel et al., 2003). C_t values were analyzed with Bio-Rad CFX manager software (Biorad). Results were expressed as the mean $\pm$ SE of human cells detected per mg of tissue.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism v.8 software. Data were verified for normal distribution using Shapiro-Wilk normality test. Experiments with two different categorical independent variables (blood flow measurements within time) were analyzed with a two-way ANOVA test and Tukey's multiple comparison test for *post-hoc* analyses. Differences between three groups was tested with either One-way ANOVA test and Tukey's multiple comparisons test for *post-hoc* analysis or Kruskal-Wallis test and Dunn's test as *post-hoc*. Finally, the Pearson Coefficient value was calculated to check for potential correlations between the variables tested. Differences were statistically significant with *p* < 0.05.

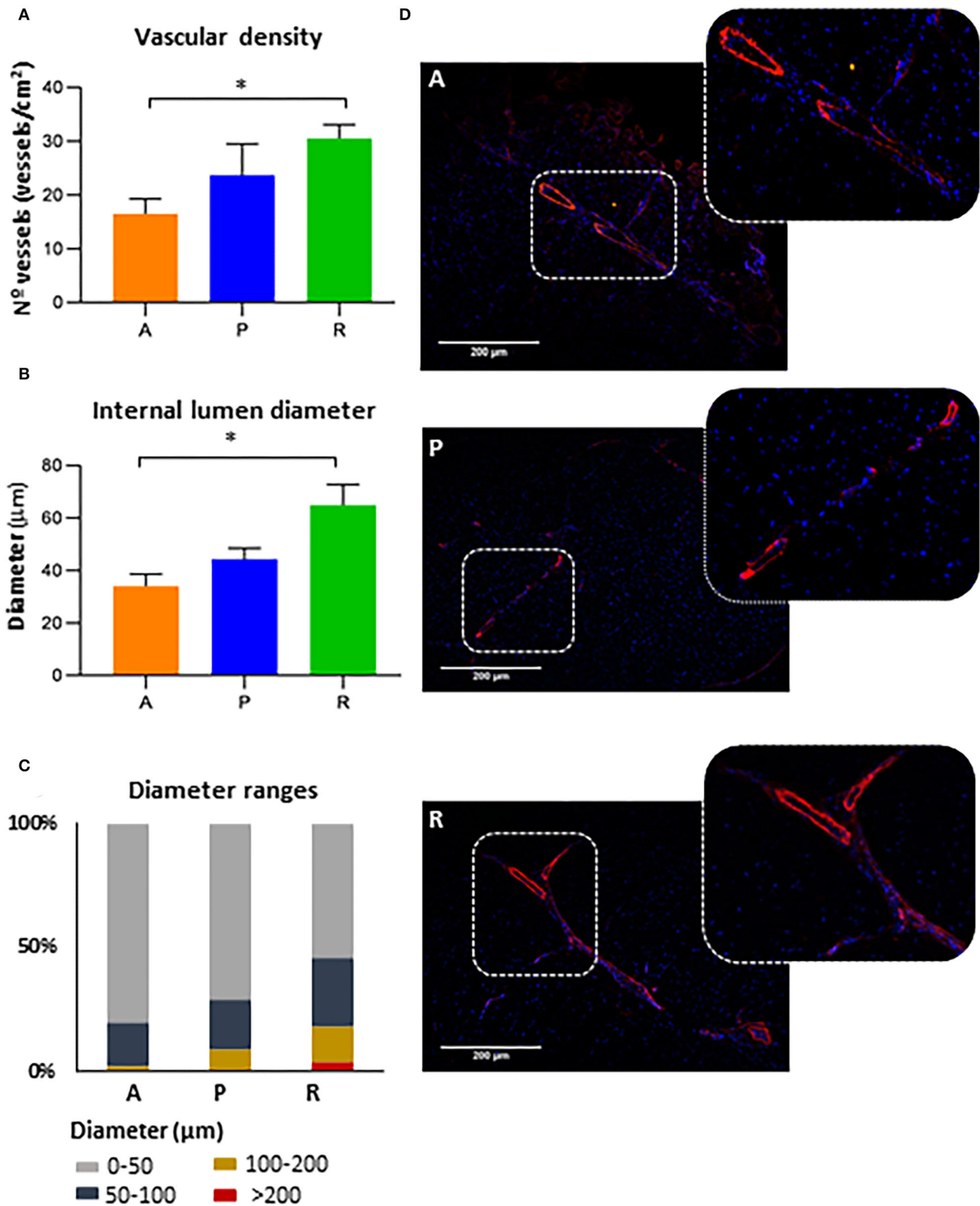


FIGURE 3 | Vascogenesis and arteriogenesis. **(A)** The number of blood vessels (vessels/cm²) and **(B)** diameter sizes (μm) were measured for mice administered with adjuvants (A, n:14), placebos (P, n:15) or REX-001 cells (R, n:20). **(C)** Vessel classification based on abundance percentage of different ranges of internal lumen (Continued)

FIGURE 3 | diameter (μm). **(D)** Representative IHC images to measure vascular density and diameter size are shown, using anti-mouse smooth muscle α -actin (red) and DAPI (blue). Data were presented as mean $\pm$ SEM. Significant differences were seen between the REX-001 (R) and adjuvant (A) treated mice (* p -value < 0.05), calculated with a one-way ANOVA, and Tukey *post-hoc* tests.

RESULTS

REX-001 Promotes a Significant Blood Flow Recovery in CLTI Mice

Immediately after femoral ligation, a significant decrease of blood flow was observed in all groups compared to pre-surgical values ($>85\%$ reduction), detecting a slight recovery in the three groups by day 7 (**Figure 2A**). Major changes were detected after day 14, with the group of mice treated with REX-001 (R) already showing a significantly higher recovery of blood flow ($p < 0.0001$) after surgery, compared to placebo- (P) and adjuvant-treated animals (A), whose blood flow rates also increased vs. baseline ($p < 0.05$) although remained below that of REX-001-treated mice. By day 21, differences were more pronounced, with the REX-001 group presenting blood flow ratios significantly higher than that of P group (p -value < 0.01) and the group of adjuvant-treated mice (p -value < 0.001). Remarkably, mice with placebo (P) and adjuvant solutions (A), showed similar blood flow ratios after 21 days of follow up.

In order to evaluate individual variability, coefficient of variations (CVs %) were calculated per day in each group of mice and human donor material, as indicated in **Supplementary Figure S1**. The highest variations were seen on day 7 (39.07–50.66% on average) and day 14 (38.10–55.06% on average). After 21 days post-surgery, mice treated with REX-001 cells showed a more defined pattern, with higher increase of blood flow ratios and lower variability (27.10%CV) than placebos and adjuvant groups (36.08 and 47.39%CV, respectively).

Evaluation of Ischemic Symptoms

All mice were evaluated periodically, and images were taken of all mice on days 0, 1, 7, 14, and 21 (**Figure 2B**). In terms of body weight, no differences were observed between all groups (**Figure 2C**) or between different REX-001 batches, losing weight right after surgery (on day 1 they lost on average 1.5 ± 0.06 g) but with tendency to recover, and to gain weight by day 21 in the case of the REX-001 and P groups. Although the adjuvant group (A) recovered less body weight than the others, weight changes were likely due to the surgical intervention and not due to the treatment applied.

Similarly, a significant mobility impairment was observed for all mice 24 h post-surgery ($p < 0.0001$), the majority of them not bearing weight properly on the injured toe or limping in some cases, probably as a result of the inflammation related to the surgical procedure (**Figure 2D**). Mobility-related symptoms continued unchanged after day 7, although most mice, mainly the ones treated with REX-001 (R), appeared to recover certain grade of mobility by day 21 independent of their ischemic outcomes. Finally, in response to femoral ligation, mice began showing symptoms of inflammation and ischemia (reddish area and black nails) by day 2, progressing to black necrotic fingers in some

cases (**Figures 2E,F**). Overall, the placebo group (P) showed the worst symptoms along the assay, while the adjuvant group (A) showed a slower progression but worsening status from day 7 onwards, with some mice losing several digits by the end of the study. REX-001 treated mice (R), however, began showing some improvement after day 7, with less inflammation and, despite showing nails or even necrotic toes, there was not worsening along the time of the assay, suggesting that cell administration might have stopped the ischemic progression.

REX-001 Promotes Collateral Vessel Formation and Arteriogenesis

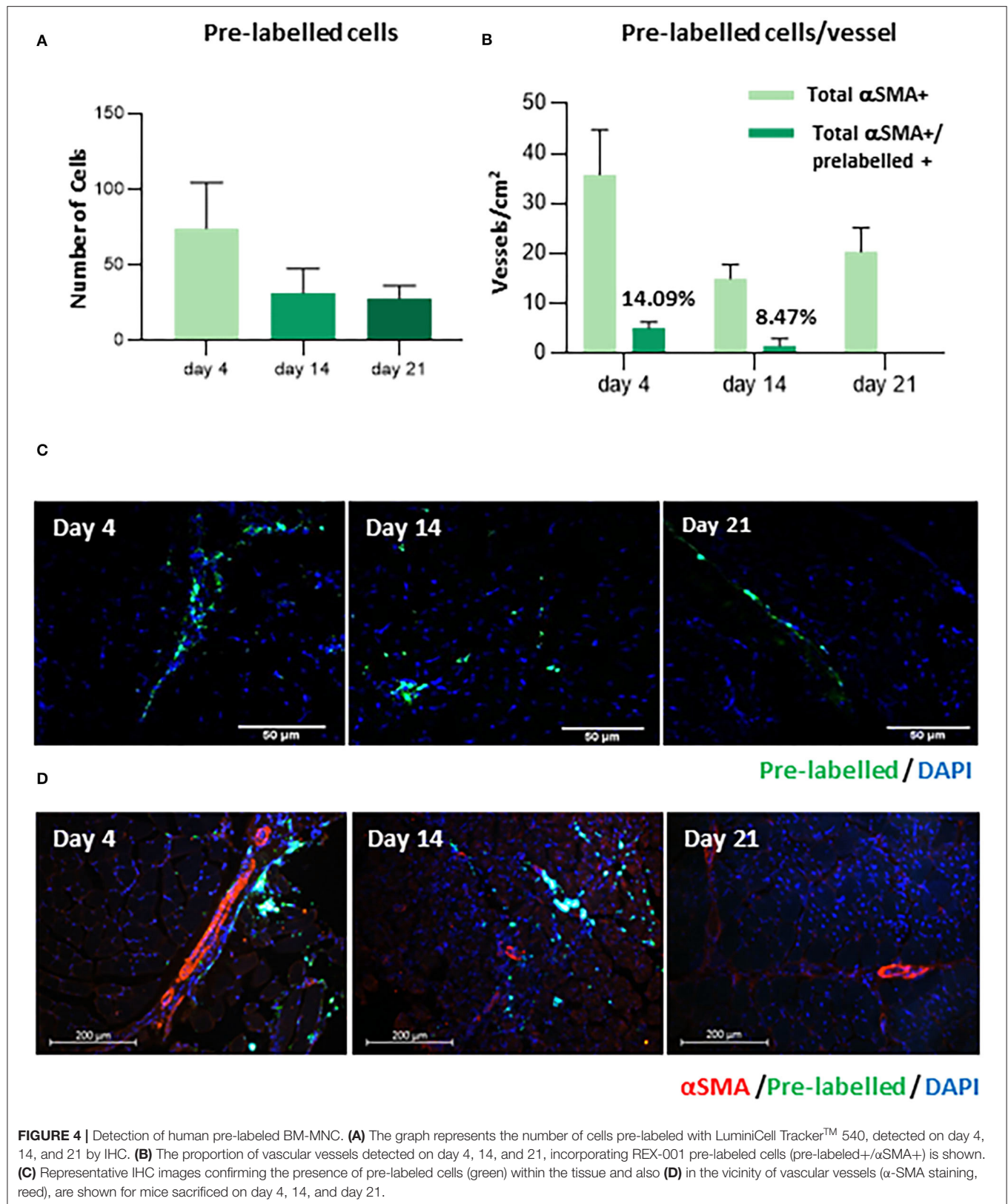
We subsequently tested whether blood flow recovery was related to an increased vascularization (**Figure 3**). Overall, our results indicated that administration of REX-001 promoted a significant increase in the number of μ -SMA positive vessels (**Figure 3A**) and vessel diameter (**Figure 3B**) compared to the adjuvant group (p -value < 0.05). Conversely, the placebo group (P) showed a slight increase in the number and diameter of vessels than the adjuvant group (A), although these changes were not significant compared to adjuvants or REX-001 treated mice.

Further classification of vascular vessels per diameter size (**Figure 3C**), indicated that mice treated with either adjuvant or placebo solution had higher number of vessels (80.3% and 70.7% from the total diameters measured) with lower diameters (0–50 μm) compared to the REX-001 group (54.1%). Conversely, REX-001 treated mice (R) showed more vessels with larger diameters (14% ranged between 100 and 200 μm and 3.8% diameters >200 μm) than the other groups (Adjuvants, with only 1.6% of capillaries between 100 and 200 μm ; Placebos, with 8.6% between 100 and 200 μm and only 0.3% of diameters >200 μm). Several examples are shown in **Figure 3D**.

In summary, the overall data related to vessel formation were in agreement with the results seen for blood flow after surgical ligation and ischemia, in which, by day 21, only the REX-001 group presented a significant perfusion recovery compared to the adjuvant and placebo groups (p -value < 0.01). Considering these data, we evaluated whether there was any relationship between vessel formation, diameter, and blood flow ratios, by applying a Pearson correlation test to the measurements obtained at day 21. Remarkably, we found significant correlations between blood-flow ratios and vessel number (p -value: 0.03, R: 0.599), but not between blood flow and internal diameter sizes (p -value: 0.21, R: 0.28) or between vascular density and diameters (p -value: 0.087, R: 0.372).

REX-001 Migrates to Vasculature of Ischemic Tissues After Intramuscular Administration

IHC assays confirmed the presence of pre-labeled REX-001 within the ischemic tissues surrounding the femoral artery,



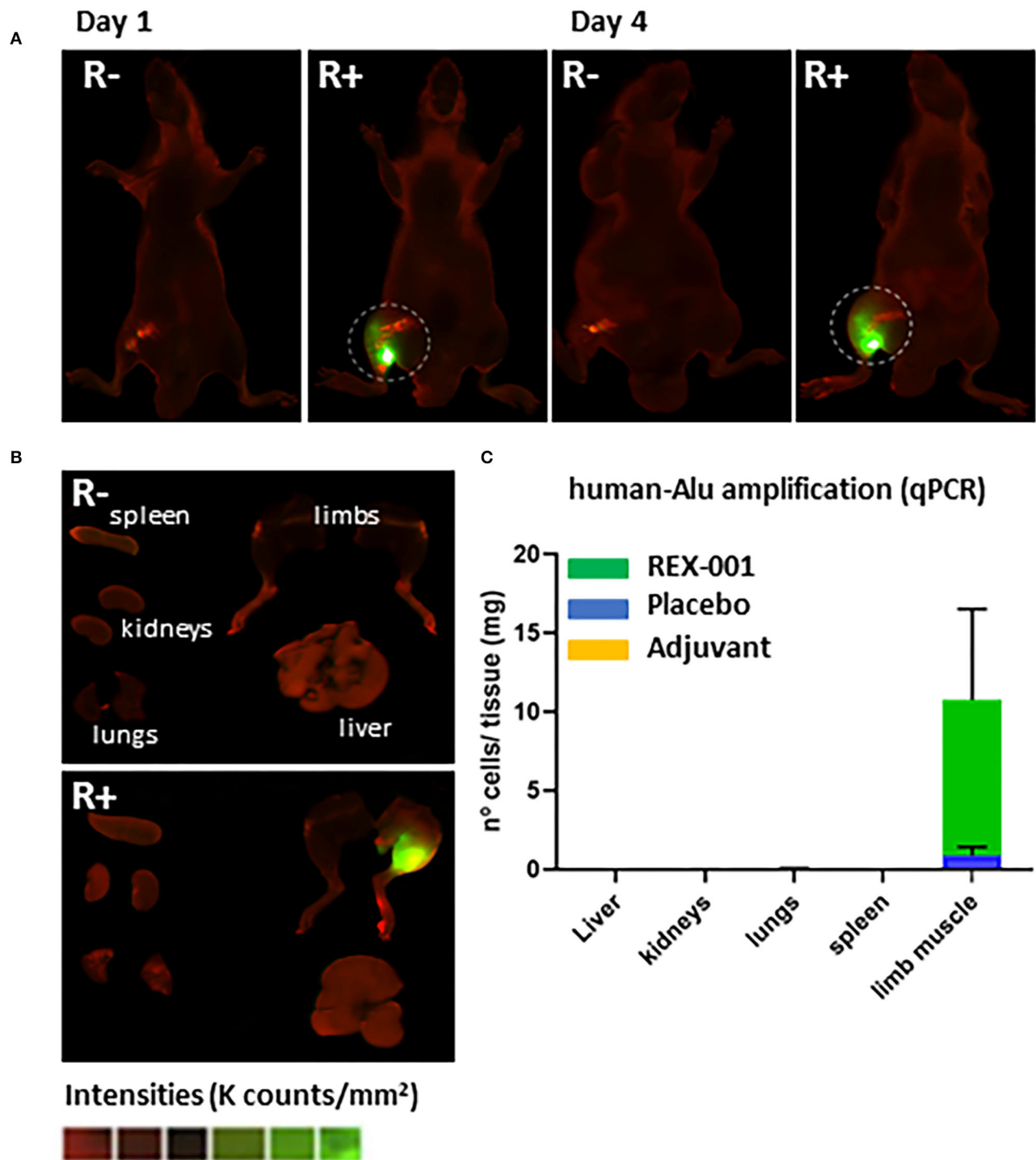


FIGURE 5 | Biodistribution assay. **(A)** Representative images of NiR scanning performed *in vivo* on day 1 (after cell administration) and day 4 in Balb-c nude mice with DiR pre-labeled REX-001 (R+, n:3) or with un-labeled cells (R-), both administered intramuscularly. **(B)** Representative images of *ex vivo* NiR scanning for the organs extracted on day 4 for the R+ and R- mice (lungs, spleen, kidneys, liver, and limbs). Intensity values were calculated as K counts/mm², with final R+ intensities calculated after subtracting background, negative signal from R- mice. **(C)** The number of cells detected per mg of organ tissue analyzed on day 4 (after cell administration) in Balb-c nude treated with either REX-001 cells (R, n:3), placebos (P, n:3) or adjuvants (A, n:3) was calculated after measuring the presence of human DNA with specific Alu sequences and qPCR analysis, in several organs (spleen, kidneys, lungs, liver, left limb).

mainly on day 4, but also on day 14 and day 21 after femoral ligation, although in lower levels than at early dates (**Figure 4A**). Moreover, pre-labeled REX-001 were found in the vicinity of vascular vessels (pre-labeled+/ α SMA+) mainly on day 4 (14.09%), with fewer numbers at day 14 (8.47%) and scarcely detected at day 21 (**Figure 4B**). Thus, REX-001 cells migrated to the damaged vasculature after intramuscular injection, although their numbers significantly decreased after 2–3 weeks post-injection (**Figures 4C,D**).

Cell Bio-Distribution

Next, we subsequently tracked the route of migration of cells post-intramuscular administration, in order to explain the decrease in the number of cells in ischemic areas after elapsed time. Two independent cell-tracking assays were carried out. First, according to *in vivo* (**Figure 5A**) and *ex vivo* (**Figure 5B**) NiR scans, our data corroborated that after intramuscular administration, DiR-prelabelled cells were mainly allocated in the ischemic areas of the limb, with almost no presence of REX-001 4 days after cell administration in lungs, kidneys, liver nor the spleen (**Figure 5B**). Similarly, amplification of specific human Alu sequences by qPCR (Funakoshi et al., 2017; Beltran-Camacho et al., 2020) confirmed the presence of human DNA, mainly in the hind limb muscle (**Figure 5C**) (9.90 ± 5.76 cells/mg of tissue). Also, a small percentage of human DNA was detected in the hind limb when the placebo solution was administered (<1 human cells/mg tissue).

DISCUSSION

To date, different studies have demonstrated the potential of using autologous BM-MNC to treat CLTI patients, as a safe and efficient strategy to achieve therapeutic angiogenesis and prevent amputation (Idei et al., 2011; Murphy et al., 2011; Liang et al., 2016; Guo et al., 2018). However, the urgent need to translate BM-MNC therapy into clinic may be reflected by the few pre-clinical assays testing BM-MNC in animal models of CLTI, and moreover, by the high variability seen between clinical trials (e.g., different study designs, dosing, routes of administration). This, in turns, could explain the scarce knowledge regarding the underlying mechanisms of action of these cells as well as the cell migration routes (Fadini et al., 2010; Idei et al., 2011; Pignon et al., 2017; Qadura et al., 2018). In addition, the selection of an optimal investigational product composition (bone marrow vs. peripheral/blood origin); isolated (MSC, CD34+, and EPC) or combined (mixed *ex vivo* or selected from an original cell source, such as BM-MNC); un-stimulated or pre-conditioned with VEGF, FGF-2, G-CSF, is still under intensive research (Zhang et al., 2008; Layman et al., 2011; Brenes et al., 2012; Gremmels et al., 2014; Beegle et al., 2016; Dong et al., 2018; MacAskill et al., 2018). Therefore, major effort is required to reach a consensus regarding these factors in order to standardize cell therapy in ischemic diseases such as CLTI (Brenes et al., 2012).

REX-001, a solution enriched with human BM-derived MNC, is intended for treatment of CLTI, and is currently under investigation in a two pivotal Phase III clinical trials

(NCT03174522 and NCT03111238). Herein, we have shown, for the first time, the pre-clinical results evaluating the effect of REX-001 product on revascularization in a murine model of CLTI, using the Balb-c nude strain, as well as the cellular biodistribution of this product as result of intramuscular administration. Furthermore, the comparative effect of REX-001 vs. the placebo solution, specifically designed and formulated for the double-blinded aforementioned Phase III clinical trials, has been also analyzed for the first time in an animal model.

In our study, a significant decrease of blood flow ($>85\%$ reduction) was seen after double ligation of the femoral artery, leading to inflammation and reduced mobility (most probably associated with the surgical procedure) as well as other ischemic symptoms (ulceration, necrotic digits), which were detected in most mice, with no clear differences between groups treated with either REX-001, placebos or adjuvants (vehicle only). The ischemic symptoms worsened with time, leading to progression of necrosis and digits falling off due to severe necrosis in several cases, although in general, REX-001 treated mice showed a less worsening status in the last 2 weeks, while mice in the adjuvant group showed slower disease progression, yet, presented the worst ischemic conditions on day 21. Regarding blood flow recovery, all three groups showed a slight increase of blood flow after 7 days of femoral ligation, although the REX-001 group showed a significant recovery by day 14 and 21 compared to both placebos (p -value < 0.01) and adjuvant treated mice (p -value < 0.001).

Balb-c nude mice are known to show poor perfusion recovery and slower revascularization response compared to other strains, such as C57BL/6 (Fukino et al., 2003; Nossent et al., 2017; Aref et al., 2019). As a result, the recovery seen in these mice (after 14–21 days) was slower than in other models (7–14 days), corroborating a dependency in the results with the strain used, as suggested (Fukino et al., 2003; Saqib et al., 2011; Aref et al., 2019). Nevertheless, the model applied seemed adequate to evaluate CLTI (Saqib et al., 2011; Nossent et al., 2017), and our data supports the positive effect of REX-001 product by enhancing blood flow perfusion after the ischemic injury induced.

Furthermore, the increase of blood flow in REX-001 treated mice was accompanied by a significant increase in vascular density and a higher percentage of vessels with wider diameters than placebos and moreover, than the adjuvant treated mice. The placebo solution promoted an increase of the number of vessels compared to the vehicle solution, although this was insufficient in achieving a significant recovery of blood flow. Therefore, similar to human trials, and in line with pre-clinical studies with intramuscular or intra-arterial administration of BM-MNC (Shintani et al., 2001; Yoshida et al., 2003), our data supports that administration of REX-001 promotes an increase of blood reperfusion due to increased vascular density.

We also confirmed the presence of human cells near the vascular vessels in ischemic tissues (14.9% of vessels contained REX-001 on day 4), supporting the hypothesis that cells administered intramuscularly do indeed reach the vasculature in ischemic areas adequately (Beltran-Camacho et al., 2020). Moreover, *in vivo* and *ex-vivo* DiR-pre-labeling assays and qPCR amplification of human Alu sequences, indicated that

REX-001 remained in the ischemic limbs, with no apparent cell translocation to other organs. Remarkably, the fact that we detected human DNA traces of the placebo solution (a residual RBC fraction derived from the human BM initial source) in the ischemic limbs, even at very low levels, supported the sensitivity of the strategy followed.

Conversely, the percentage of REX-001 in the ischemic limbs decreased within time, with no detection 21 days post-surgery. Different studies have reported such decrease, using among others, autologous cells expressing green fluorescent protein (GFP) and firefly luciferase (Fluc) reporter genes (van der Bogt et al., 2012), with a significant cell loss from the ischemic tissues after 4 weeks (van der Bogt et al., 2012). In this sense, future efforts should be made to implement transplanted BM-MNC survival and/or the ratio of local delivery in order to enhance the revascularization orchestrated by these cells. We have demonstrated here, in agreement with several clinical trials using this approach (Higashi et al., 2004; Cobellis et al., 2008; Matoba et al., 2008; Motukuru et al., 2008; Iafrati et al., 2011), the efficiency of intramuscular administration to maximize the local concentration of stem cells in the ischemic area. Perhaps, as suggested (Davies, 2012; Qadura et al., 2018), the combination of intra-muscular and intra-arterial administration may be an even better strategy to allow stem cells to reach additional areas, at higher concentrations, including ischemic muscle regions that still are perfused (Van Tongeren et al., 2008; Franz et al., 2009) and thus, achieve a higher and faster blood perfusion recovery.

In our study, the decrease in cell numbers with time did not correlate with cell translocation to other organs, suggesting that infused REX-001 might not proliferate after having promoted angiogenesis, so these cells may not be directly involved in vessel formation (given the small percentage of REX-001+ vessels), but they most probably contribute to revascularization in a paracrine fashion (van Weel et al., 2007; Burdon et al., 2010; van der Bogt et al., 2012). In this regard, some of the cells included in REX-001 product (enriched for MNC, as well as granulocytes and hematopoietic stem cells expressing CD34) might participate in the paracrine effect. For instance, circulating BM-derived EPC (CD34+CD45+) have been described as powerful angiogenic agents (Yanishi et al., 2020). These cells release angiogenic and chemo-attractant factors once migrated into the ischemic tissues, recruiting immune cells (neutrophils, monocytes) and activating other cells that will participate in the inflammatory response and also contribute to vascular remodeling (Beltran-Camacho et al., 2020). In addition, BM-immune precursors cells are thought to have an active role in angiogenesis and/or arteriogenesis itself (Nossent et al., 2017). For instance, neutrophils not only participate in inflammation but they can also promote vascularization by inducing angiogenesis via a pro-angiogenic phenotype (Lin et al., 2017; Seigne and Phillipson, 2017; Beltran-Camacho et al., 2020).

Preliminary results obtained in our laboratory indicated that REX-001 cultured *ex vivo* releases angiogenic cytokines such as CXCL4/PF4, metalloproteinase-8 (MMP-8) or Interleukin-8 (IL-8) to the conditioned medium. Moreover, the levels of MMP-8 seem to increase exponentially after several hours of culturing

these cells in basal media at 37°C, 5% CO₂. The involvement of MMP-8 as well as other metalloproteinases in angiogenesis has been already described (Lin et al., 2008; Deryugina and Quigley, 2010; Fang et al., 2013; Quintero-Fabian et al., 2019). Studies with MMP-8 and MMP-2 knock-out mice have shown an *in vitro* diminishment of cell proliferation and neo-capillary network growth, as well as a reduction in HUVEC migration and impaired angiogenesis *in vivo* (Cheng et al., 2007; Fang et al., 2013). MMPs not only contribute to the remodeling/degradation of the extracellular matrix (ECM), but also participate in many biological processes involved in stroke, cardiovascular diseases or arthritis (Chuang et al., 2019). Moreover, macrophage activation results in MMP secretion. Remarkably, some MMPs are related to the transition from M1 to M2 macrophage phenotypes, associated with immunomodulatory processes (Berg et al., 2019), suggesting that this transition might be promoted in presence of REX-001 cells. Future assays should be performed to confirm such phenomenon. Apart from MMP-8, IL-8 was also detected as released by REX-001 *in vitro*. IL-8, also named neutrophil chemotactic factor, is a soluble chemoattractant produced mainly by macrophages, but also by other cell types such as epithelial and endothelial cells (Oude Nijhuis et al., 2003). Notably, not only does IL-8 boost phagocytosis in macrophages, it also stimulates angiogenesis (Koch et al., 1992; Wu et al., 2018; Fousek et al., 2020). Thus, although preliminary, these results indicate that REX-001 release certain factors such as MMP-8 and IL-8 that might contribute to the vascular restoration induced by this cell product. Further studies are required to confirm these data and moreover, to complete the information regarding the molecular mechanisms of action of these cells. Despite this, our results confirmed that the administration of REX-001, and therefore the combined effect of such populations, has proven to be effective in promoting revascularization after CLTI.

CONCLUSION

Overall, our findings demonstrate the efficacy of REX-001 implantation to enhance blood flow recovery after ischemic injury, by inducing functional neovascularization in a murine model of CLTI. Moreover, detailed cell-tracking corroborated the efficiency of intramuscular administration, with REX-001 exerting a focalized action within the ischemic tissues and no apparent translocation to other organs. The decrease seen in cell numbers within time, despite promoting an increased revascularization, suggests a paracrine mechanism of action for these cells. Future research should now be focused on analyzing the molecular mechanisms of action of REX-001 cells as well as to evaluate their effect at the clinical level.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal experiments were approved by the Ethical committee of the University of Cadiz, as well as the Andalusian Committee of animal experimentation (registration number ES110120000210 and project number 07-04-2016-043).

AUTHOR CONTRIBUTIONS

MR-T and MJ-P: experimental assays, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript. LB-C: support in experimental assays and data collection, manuscript writing, and data analysis and interpretation. IS-G, AR-V, and SE-A: support in animal experimental assays and data collection. JM-R and SG: provision of study materials, conception, design, data analysis and interpretation, and final approval of manuscript. MD-R: conception and design, financial support, administrative support, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.602837/full#supplementary-material>

Supplementary Materials includes **Supplementary Tables 1–3**, with Tarlov scores (motility changes), ischemia, and modified ischemia scores, respectively; **Supplementary Figure 1** (with individual variations in blood flow) and **Supplementary Figure 2** (with linear equation applied to calculate human DNA).

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Modeling Uremic Vasculopathy With Induced Pluripotent Stem Cell-Derived Endothelial Cells as a Drug Screening System

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Background: Cardiovascular complications are the leading cause of mortality in patients with chronic kidney disease (CKD). Uremic vasculopathy plays a crucial role in facilitating the progression of cardiovascular complications in advanced CKD. However, the improvement of conventional research methods could provide further insights into CKD.

Objectives: In this study, we aimed to develop a novel model of uremic vasculopathy as a potential drug screening system.

Methods and Results: The effects of uremic serum and different combinations of uremic toxins on induced pluripotent stem cell (iPSC)-derived endothelial cells (ECs) of a normal control and a CKD patient were investigated using several functional assays. We found that a mixture of uremic toxins composed of high urea, creatinine, uric acid, and indoxyl sulfate exerted deleterious effects on normal control iPSC-ECs that were comparable to uremic serum by increasing reactive oxygen species and apoptosis, as well as suppression of tube formation. Additional characterization revealed a potential involvement of dysregulated TGF- β signaling as treatment with either losartan or TGF- β inhibitors led to the attenuation of adverse effects induced by uremic toxins. Importantly, impaired wound healing potential seen in CKD patient-specific iPSC-ECs was rescued by treatment with losartan and TGF- β inhibitors.

Conclusion: Our study demonstrated that simplified uremic toxin mixtures can simulate the uremic microenvironment reproducibly and CKD patient-specific iPSC-ECs can potentially recapitulate susceptibility to uremic vasculopathy. This novel model of uremic vasculopathy may provide a new research tool as a drug screening system.

Keywords: endothelial cells, induced pluripotent stem cells, uremic vasculopathy, uremic toxin, chronic kidney disease

INTRODUCTION

Cardiovascular complications are the leading cause of mortality and major morbidities in patients with chronic kidney disease (CKD) (Go et al., 2004; Tonelli et al., 2006; Stenvinkel, 2010). Uremic vasculopathy is the cornerstone of cardiovascular complications acting as the primary factor that accelerates atherosclerosis and heart failure (Levin et al., 1990; Deppisch et al., 2001; Jourde-Chiche et al., 2011; Sallée et al., 2014). It also remains an important hurdle for kidney transplantation in end-stage renal disease (ESRD) patients undergoing longstanding dialysis by causing severe atherosclerosis and heavy vascular calcification (Jourde-Chiche et al., 2011; Torres and de Broe, 2012). Although epidemiologic research can provide insightful results from CKD patients in the real world, large sample sizes with very long follow-up periods are required and the results can often be confounded by factors such as comorbidities other than CKD (Ballew and Matsushita, 2018). Therefore, studies revealing the pathophysiology of uremic vasculopathy and investigation of potential new drugs or the adequacy of current drugs are required to improve overall outcomes for CKD patients.

Previous studies using genetically engineered or surgically simulated murine CKD models have provided significant insights into the pathophysiology of uremia-induced cardiovascular complications (Baraka and El Ghotny, 2012; Xie et al., 2015). However, these murine models have limitations for investigating the effects of uremia on vessels because of interspecies differences and methodological limitations in simulating CKD or evaluating changes in vessels. To investigate the pathophysiology of uremic vasculopathy thoroughly in the hope of identifying new drugs, a novel model of uremic vasculopathy with human endothelial cells (ECs) that reflects genetic susceptibility to uremic toxins is essential.

In this study, we developed a novel model of uremic vasculopathy using ECs differentiated from human induced pluripotent stem cells (iPSCs). The effects of several uremic toxins on iPSC-ECs were compared with those of uremic serum from ESRD patients. The feasibility of this new model as a novel drug screening tool was also investigated.

MATERIALS AND METHODS

Study Participants and Design

Serum and peripheral blood mononuclear cells (PBMCs) were collected from 5 healthy volunteers and 5 ESRD patients receiving hemodialysis for more than a year. Informed consent was obtained from all subjects. The Institutional Review Board of the Samsung Medical Center approved the study protocol in compliance with the Declaration of Helsinki (October 2008), and informed consent was waived because of the retrospective and non-interventional design of the study (IRB number: 2016-11-025).

Several functional assays for comparing the effects of serum from normal controls and ESRD patients as well as simplified uremic toxin mixtures on iPSC-ECs were performed for establishing a new model of uremic vasculopathy.

The wound healing potential of normal control iPSC-ECs and ESRD patient-specific iPSC-ECs was also compared by a scratch migration assay.

Generation of iPSC-ECs

PBMCs from a normal control and an ESRD patient were reprogrammed into iPSCs using Sendai virus (Takahashi et al., 2007; Churko et al., 2013). ECs were differentiated from iPSCs according to the protocol validated in previous studies (Sa et al., 2017; Bezenah et al., 2018; Lee et al., 2019). Briefly, the iPSCs were treated with a differentiation induction medium (RPMI and B-27 minus insulin, Thermo Fisher Sci, Waltham, MA) supplemented with 6 μ M and 2 μ M of glycogen synthase kinase 3- β inhibitor, CHIR-99021 (Selleck Chemicals, Houston, TX), on day 0 and day 2, respectively. From day 4 to 12 of differentiation, cells were cultured in different combinations of differentiation induction media and EGM-2 media from Lonza (100% differentiation medium on day 4, 50% differentiation and 50% EGM-2 media on day 6, 25% differentiation and 75% EGM2 media on day 8, and 100% EGM2 medium on day 10) with growth factors including 50 ng/ml VEGF, 20 ng/ml FGF2, and 20 ng/ml BMP4 (PeproTech, Rocky Hill, NJ). On day 12 post-differentiation, cells were sorted by a magnetic cell sorting (MACS) system (Miltenyi Biotech, San Diego, CA) using magnetic beads conjugated to human CD144 antibodies, as directed by the manufacturer, and expanded on 0.2% gelatin-coated plates. iPSC-ECs were then cultured in EGM2 medium at 37°C and 5% CO₂ in a humidified incubator with medium changes every other day. The experiments described in this manuscript were performed between passages 2–4. The phenotypes of iPSC-ECs used in this study were characterized in previous studies (Lee et al., 2019; Ong et al., 2019).

Serum Sample Preparation and Analytic Procedure

For serum preparation, blood was separated by centrifuging clotted blood at 3,500 rpm for 15 min at 4°C and was aliquoted before storing at –80°C until needed for experiments. Serum uric acid, creatinine, and blood urea nitrogen were measured using Fuji Dri-Chem 7000i (Fujifilm Corporation, Tokyo, Japan).

Preparation of Uremic Toxin Mixtures

Urea, creatinine, indoxyl sulfate (Sigma-Aldrich, Louis, MO), and advanced glycation end-products (AGEs, BioVision, San Francisco, CA) were dissolved in Dulbecco's phosphate-buffered saline and uric acid (Sigma-Aldrich) was dissolved in 1 M NaOH, then the solutions were filtered (0.22 μ m pore size) before experiments.

Luminex Multiplex Assay

Serum samples were mixed with antibody-linked polystyrene beads on 96-well filter plates and incubated at room temperature for 2 h, followed by overnight incubation at 4°C on an orbital shaker at 500–600 rpm. After washing plates twice with wash buffer, a biotinylated detection antibody was added for 2 h at room temperature with shaking. Samples were then filtered, washed twice, and resuspended in streptavidin-PE for 40 min

at room temperature. Two additional vacuum washes were performed before adding the reading buffer. All standards and samples were measured in duplicate. Plates were read using a Luminex 200 instrument with a lower bound of 100 beads per sample per cytokine. For quality control, custom assay control beads (Radix Biosolutions, Georgetown, TX) were added to all wells.

Assays for Cellular Viability, Reactive Oxygen Species (ROS), and Caspase 3/7 Activity

iPSC-ECs plated on 96-well plates were subjected to plate-based assays after serum (15%) or uremic toxin mixture treatments. Assays using CellTiter-Glo 2.0, ROS-Glo H_2O_2 , and Caspase-Glo 3/7 (Promega, Madison, WI) were performed following the manufacturer's instruction and the luminescence signal was recorded on the GloMax-Multi detection system (Promega). Cells in the original sample plate were kept for measuring the total cell number by calcein-AM (Thermo Fisher Scientific) to allow normalization.

Measurement of Endothelial Function

An *in vitro* endothelial tube formation assay was carried out following the manufacturer's instructions. Briefly, after coating the 15-well μ -Slice Angiogenesis (Ibidi GmbH, Gräfelfing, Germany) with Corning Matrigel Basement Membrane Matrix, iPSC-ECs pre-treated with serum or uremic toxin mixture (UT)-H with or without drugs for 48 h were seeded at 1×10^4 cells/well. After 16 h incubation, capillary network images were taken using a Revolve microscope and quantitation was made using ImageJ.

For the *in vitro* migration assay, normal control iPSC-ECs and ESRD patient-specific iPSC-ECs were treated with drugs for 48 h before seeding in 24-well plate (5×10^4 cells/well). When cells reached 80% confluence, the culture medium was replaced overnight for serum starvation. A straight line was scraped in the cell monolayer with a sterile 200 μ l pipette tips and the debris was removed by washing twice with media. Cells were then incubated with EGM2 media and imaged at 0, 4, and 10 h after the scratch. The area and width of the scratch were analyzed with ImageJ. Losartan (a well-known angiotensin-receptor blocker; Sigma), SD-208 (a selective TGF- β receptor I kinase (ALK5) inhibitor; Selleckchem, Houston, TX), and LY2109761 (TGF- β receptor I and type II dual inhibitor; Selleckchem) were used for drug treatment.

Human TGF- β Pathway Protein Phosphorylation

After treating iPSC-ECs with either the control or UT-H for 24 h, the relative levels of phosphorylation of 8 TGF- β pathway proteins in cell lysates were quantified using human TGF- β pathway phosphorylation array (C1 series, RayBiotech, Peachtree Corners, GA) following the manufacturer's instructions. Briefly, after blocking each well, samples were incubated for 2 h at room temperature with membranes arrayed with antibodies against 8 TGF- β phosphorylated proteins. After washing twice in wash buffer, a detection antibody cocktail was added into each well

for 2 h at room temperature. After washing, membranes were then incubated with horseradish peroxidase (HRP)-rabbit IgG for 2 h, washed, and placed in the chemiluminescence detection buffer. FluorChem E (Protein Simple, San Jose, CA) was used to detect signal chemiluminescence intensities from protein array membranes. Quantitative analysis for chemiluminescent intensity was conducted using Image J.

RNA-seq Library Preparation and Analysis

Total RNA was first extracted from iPSC-ECs using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and 100 ng RNA was used to construct sequencing libraries. Libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA) and the raw 150 bp paired-end RNA-seq reads sequenced by Illumina HiSeq 2000 were trimmed by TrimGalore version 0.4.2 to exclude adapter sequences and bases with Phred scores of <20 , providing a low probability of base-calling error ($p < 1\%$) equivalent to $>99\%$ accuracy. The reads were aligned to the human genome hg38 by Hisat2 (Pertea et al., 2016), and annotated by FeatureCounts of the Subread package (Liao et al., 2014). Differentially expressed genes were detected by DESeq2 (Love et al., 2014). The gene ontology enrichment analyses were implemented by Metascape (Zhou et al., 2019).

Statistical Analyses

Data are expressed as the mean $\pm$ standard error of the mean (SEM) or mean $\pm$ standard deviation (SD). Group means were compared with the Mann-Whitney test using SPSS 12.0 K or analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* analysis using GraphPad Prism version 8 (GraphPad, San Diego, CA). Statistical significance was determined when the *P*-value was <0.05 .

RESULTS

Uremic Toxin Mixtures Mimic the Effects of Uremic Serum on iPSC-ECs

To establish a baseline for studying uremic vasculopathy, we first examined the effects of uremic serum collected from ESRD patients or normal serum from healthy volunteers on iPSC-ECs. Several parameters including cell viability, generation of reactive oxygen species (ROS), and apoptosis were measured following exposure of iPSC-ECs to uremic or normal serum. The blood urea nitrogen and serum creatinine concentrations of uremic serum collected immediately before hemodialysis are summarized with the baseline characteristics of ESRD patients in **Table 1**. The mean duration of dialysis was 9.8 years and hypertension was present in all ESRD patients.

iPSC-ECs treated with uremic serum exhibited a significant decrease in cell viability (**Figure 1A**) accompanied by a significant increase in ROS generation measured by H_2O_2 production (**Figure 1B**) compared to iPSC-ECs exposed to normal serum. In addition, the degree of apoptosis measured by caspase 3/7 activity in iPSC-ECs treated with uremic serum was not discernible at 4 h compared to those cultured in normal serum but was significantly enhanced by 24 h post-treatment

TABLE 1 | Baseline characteristics of normal controls and ESRD patients.

Characteristics	Normal controls (n = 5)	ESRD patients (n = 5)	P-value
Age, years	32 ± 7.6	49 ± 11.1	0.0317
Male: female	3: 2	3: 2	>0.9999
BMI (kg/m ²)	23.9 ± 4.43	24.5 ± 6.52	>0.9999
Blood urea nitrogen (mg/dL)	12.48 ± 2.119	51.04 ± 11.32	0.0079
Serum creatinine (mg/dL)	0.62 ± 0.110	10.44 ± 3.480	0.0079
Serum uric acid (mg/dL)	5.24 ± 1.322	7.72 ± 2.100	0.0635
Causes of ESRD, n (%)	NA	NA	NA
Diabetes mellitus		1 (20%)	
Hypertension		1 (20%)	
Glomerulonephritis		2 (40%)	
ADPKD		1 (20%)	
Cardiovascular diseases, n (%)	NA	NA	NA
Hypertension		5 (100%)	
Pericardial effusion		2 (40%)	
Diastolic dysfunction		1 (20%)	
Aortic dissection		1 (20%)	
Duration of hemodialysis, years	NA	9.8 (2–15)	NA

Laboratory values are mean ± standard deviation.

ADPKD, autosomal dominant polycystic kidney disease; BMI, body mass index; ESRD, end-stage renal disease; NA, not applicable.

(Figure 1C). Collectively, these results demonstrated that iPSC-ECs exposed to uremic serum collected from ESRD patients exhibited hallmarks of endothelial dysfunction.

Having demonstrated that uremic serum induces endothelial dysfunction in iPSC-ECs, we then sought to identify a minimal *in vitro* mixture of uremic toxins that would elicit similar effects on iPSC-ECs. To achieve this, we tested 25 combinations of substances known to be present in uremic serum (Table 2) based on three parameters: cell viability, ROS production, and apoptotic activity. We first compared control conditions and found that there were no significant differences in cell viability, ROS levels, and caspase 3/7 activity between control (CTL) 1 consisting of media only and CTL4 (media containing physiological levels of urea, creatinine, and uric acid). Subsequent testing revealed that compared with CTL1 and CTL4, exposure of iPSC-ECs in two different combinations of uremic toxin mixture, namely UT6 (containing a high concentration of urea, creatinine, uric acid, indoxyl sulfate, and AGE) and UT-H (containing a high concentration of urea, creatinine, uric acid, and indoxyl sulfate) led to significantly lower cell viability (Figure 1D). Likewise, both ROS production (Figure 1E) and apoptosis (Figure 1F) were significantly elevated in cells exposed to UT6 and UT-H.

Effects of Uremic Serum and Uremic Toxin Mixtures on the Angiogenic Ability of iPSC-ECs

We next sought to explore how uremic serum or uremic toxin mixture affect the angiogenic capability of iPSC-ECs. As shown

TABLE 2 | Composition of *in vitro* uremic toxin mixtures.

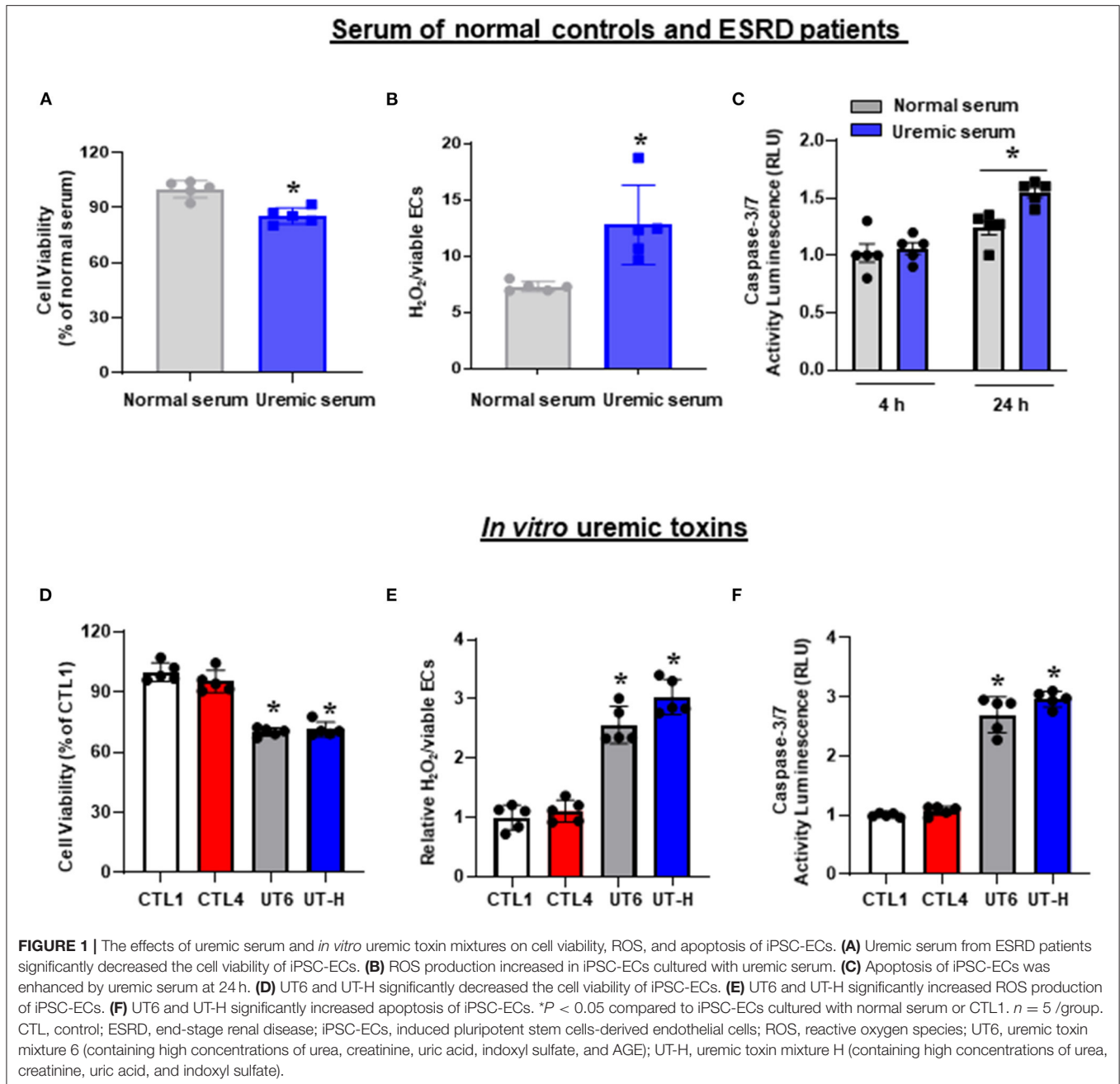
Groups	Urea, mM (BUN, mg/dL)	Creatinine, mM (mg/dL)	Uric acid, mM (mg/dL)	Indoxyl sulfate (mM)	AGE (mg/L)	NaOH (μL/mL)
Control 1 (media only)						
Control 2	5 (14.01)	0.1 (1.13)				
Control 3	5 (14.01)	0.1 (1.13)	0.25 (4.20)			11
Control 4	5 (14.01)	0.1 (1.13)	0.25 (4.20)			
Control 5						16
UA 1			0.5 (8.41)			
UA 2			0.8 (13.45)			
UA 3			1 (16.81)			
Urea	50 (142.86)					
Creatinine		1 (11.30)				
IS			1 (16.81)			
UT 1	50 (142.86)	1 (11.30)				
UT 2	50 (142.86)	1 (11.30)	1 (16.81)			
UT 3	50 (142.86)	1 (11.30)	1 (16.81)	0.5		
UT 4	50 (142.86)	1 (11.30)	1 (16.81)	0.5	1	
UT 5	25 (71.43)	1 (11.30)	1 (16.81)	0.5	10	
UT 6	50 (142.86)	1 (11.30)	1 (16.81)	0.5	10	
UT-A	25 (71.43)	1 (11.30)	0.5 (8.41)			
UT-B	25 (71.43)	1 (11.30)	1 (16.81)			
UT-C	50 (142.86)	1 (11.30)	0.5 (8.41)			
UT-D	50 (142.86)	1 (11.30)	1 (16.81)			
UT-E	25 (71.43)	1 (11.30)	0.8 (13.45)			
UT-F	25 (71.43)	1 (11.30)	0.8 (13.45)	1		
UT-G	50 (142.86)	1 (11.30)	0.8 (13.45)			
UT-H	50 (142.86)	1 (11.30)	0.8 (13.45)	1		

AGE, advanced glycation end-products.

in Figures 2A,B we observed that uremic serum significantly suppressed tube formation of iPSC-ECs determined by the number of nodes and meshes and the total area of meshes compared to normal serum. Similar findings were also revealed in iPSC-ECs supplemented with UT-H mimicking the effect of uremic serum. High concentrations of urea, uric acid, or indoxyl sulfate consistently suppressed tube formation of iPSC-ECs (Figure 3).

Activation of Intracellular TGF-β Pathway in iPSC-ECs by *in vitro* Uremic Toxin Mixtures and the Different Expression of Inflammatory Cytokines in Uremic Serum

TGF-β has been implicated as a major regulatory cytokine playing important and diverse roles in CKD (Patel and Dressler, 2005). To elucidate the mechanisms underlying the reduction of viability and angiogenesis and the elevation of ROS production and apoptosis in iPSC-ECs treated with uremic toxin mixtures or uremic serum, we first determined whether uremic toxin mixtures induce activation of TGF-β signaling in ECs. Phosphorylation levels of 8 TGF-β pathway proteins were measured in iPSC-ECs after



treating cells with either CTL4 or UT-H (Figure 4A). We found that UT-H treatment significantly increased the phosphorylation of all 8 TGF- β pathway proteins, ATF2, C-FOS, C-JUN, SMAD1, SMAD2, SMAD4, SMAD5, and TAK1, compared to CTL4 treatment (Figure 4B), suggesting that the inhibition of TGF- β signaling may protect against uremic toxins.

In addition, to analyze the differences in humoral factors between normal serum and uremic serum, we measured the levels of 62 human inflammatory cytokines in serum from normal

controls and ESRD patients (Supplementary Table 1). Of the 62 inflammatory cytokines measured, brain-derived neurotrophic factors (BDNF) and platelet-derived growth factor –BB (PDGF BB) were significantly decreased in uremic serum compared with normal serum, whereas interleukin (IL)-12 p40, leptin, resistin, and vascular cell adhesion molecule 1 (VCAM1) were significantly increased in uremic serum (Figure 4C). In addition, most other cytokines including TGF- β (*P* = 0.057) demonstrated an increasing trend in uremic serum compared to normal serum (Supplementary Table 1).

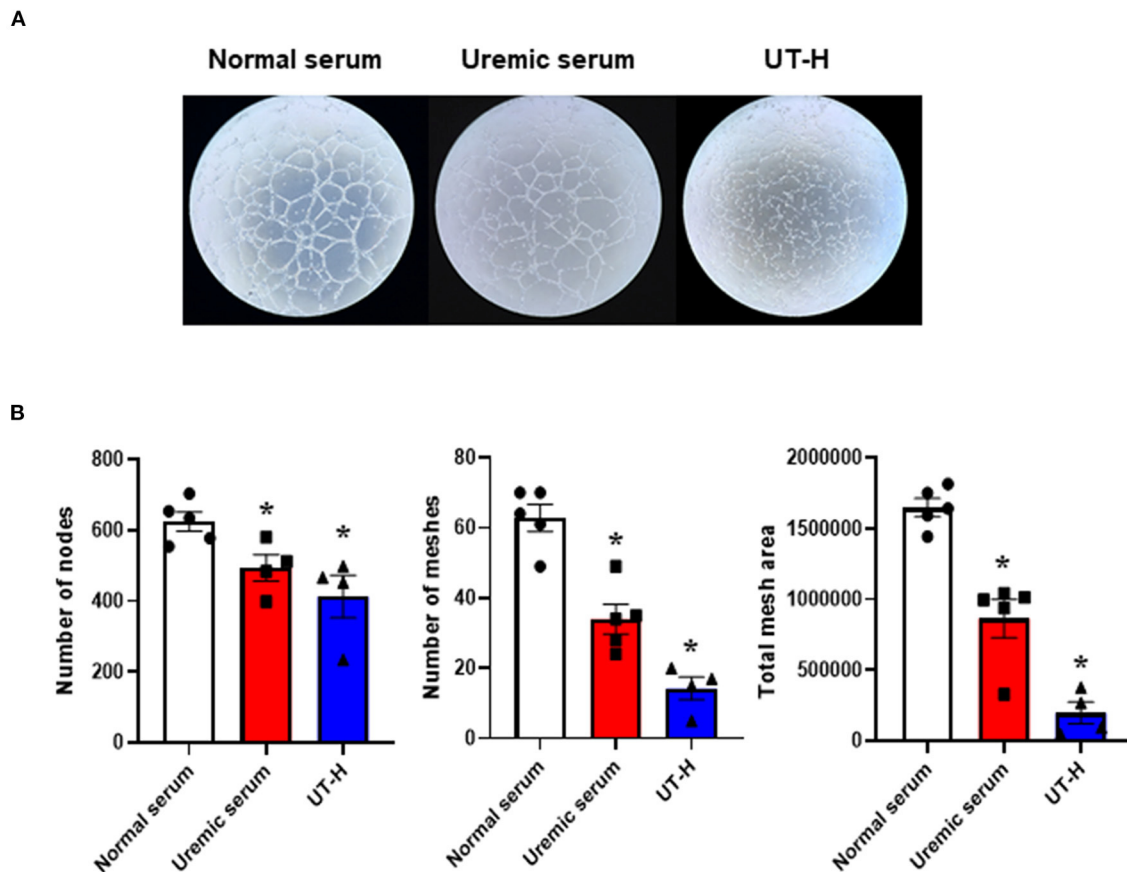


FIGURE 2 | The effects of uremic serum and *in vitro* uremic toxin mixture on vessel formation ability of iPSC-ECs. **(A)** Tube formation of iPSC-ECs was suppressed by uremic serum or UT-H. **(B)** Quantitative data from the tube formation assay. Uremic toxin mixtures exerted comparable effects with uremic serum on tube formation of iPSC-ECs. * $P < 0.05$ compared to normal serum. $n = 4$ –5/group. UT-H, uremic toxin mixture H (containing high concentrations of urea, creatinine, uric acid, and indoxyl sulfate).

Drug Screening Using a Simplified Uremic Vasculopathy Model With iPSC-ECs and Uremic Toxin Mixture

We next sought to test whether the inhibition of TGF- β signaling reduces uremic toxin-induced impairment of endothelial function. iPSC-ECs were incubated with three drugs targeting the TGF- β pathway, namely SD-208 (TGF- β type 1 receptor inhibitor), losartan (TGF- β signaling blocker), and LY2109761 (dual inhibitor of TGF- β type 1 and 2 receptor inhibitor) before TGF- β stimulation *via* treatment with uremic serum or UT-H. Treatment with SD-208 and LY2109761 significantly restored the formation of tubes in iPSC-ECs incubated with uremic serum and UT-H (Figure 5), which suggests that the inhibition of TGF- β signaling is a protective mechanism against uremic vasculopathy.

To further validate our *in vitro* model as a drug screening model, we generated iPSCs from an ESRD patient and compared the newly generated ESRD patient-specific iPSC line against normal control iPSCs to determine whether patient-specific

iPSC-ECs can potentially recapitulate susceptibility against uremic vasculopathy. ESRD iPSCs exhibited typical expression of pluripotency markers (Supplementary Figure 1A). The differentiation efficiency and expression of endothelial markers of ESRD patient-specific iPSC-ECs were also comparable to those of normal control iPSC-ECs (Supplementary Figure 1B). The functional integrity of normal control and ESRD patient-specific iPSC-ECs was then compared by tube formation assay and migration assay. ESRD patient-specific iPSC-ECs showed impaired tube formation compared to normal control iPSC-ECs (Supplementary Figure 2). In the migration assay, the degree of wound healing was significantly lower in ESRD patient-specific iPSC-ECs (Figure 6A) compared to control iPSC-ECs. Interestingly, losartan and SD-208 significantly mitigated the impairment of wound healing in ESRD patient-specific iPSC-ECs (Figure 6B) demonstrating that patient-specific iPSC-ECs represent a model that may be used for personalized drug screening in the future.

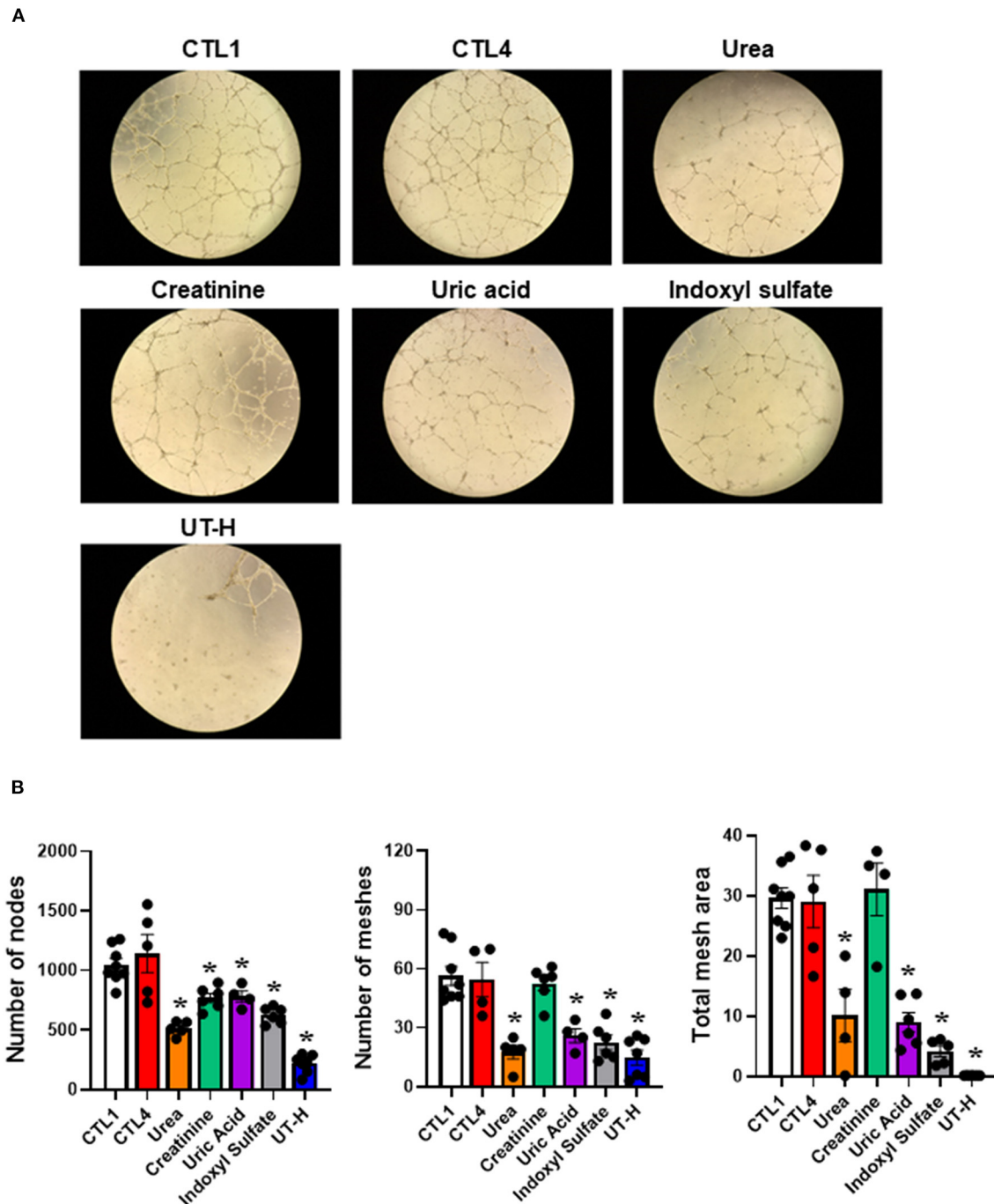


FIGURE 3 | The effects of each uremic toxin on vessel formation ability of iPSC-ECs. **(A)** Tube formation of iPSC-ECs was suppressed by each uremic toxin or UT-H. **(B)** Urea, uric acid, or indoxyl sulfate consistently suppressed tube formation of iPSC-ECs. * $P < 0.05$ compared to CTL1. $n = 4-5$ /group. CTL1, control 1; CTL4, control 4; UT-H, uremic toxin mixture H (containing high concentrations of urea, creatinine, uric acid, and indoxyl sulfate).

Gene and Functional Enrichment Analysis With the Addition of Uremic Toxin Mixture

Finally, to better understand the effects of UT-H on the transcriptome profiles of iPSC-ECs (GEO accession number: RNA-seq num GSE155969), RNA-sequencing was conducted.

Principal component analysis (PCA) was first conducted to examine the main source of variation in the data. PC1 reflected a closer correlation in iPSC-ECs from the same patient whereas PC3 identified distinct clusters corresponding to UT-H treated control samples (**Figure 7A**). Hierarchical clustering

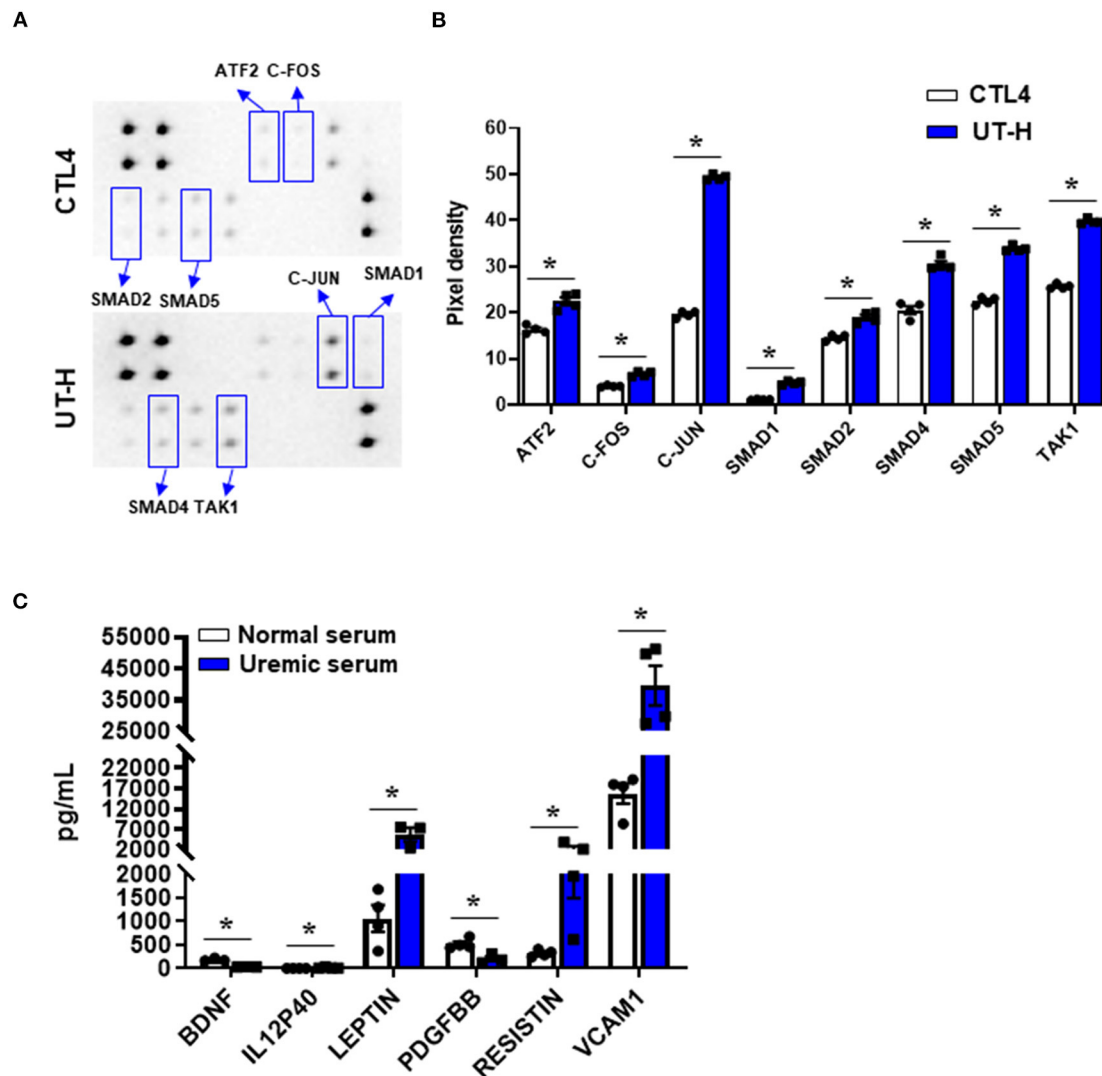


FIGURE 4 | The effects of uremic toxin mixtures on the intracellular TGF- β pathway of iPSC-ECs and inflammatory cytokine levels in uremic serum. **(A)** Duplicated signals of 8 TGF- β pathway phosphorylated proteins. **(B)** Quantitative and statistical analyses of each detected target. * $P < 0.05$ compared to CTL4. $n = 4$ /group. CTL4, control 4 (media containing normal physiologic levels of urea, creatinine, and uric acid); UT-H, uremic toxin mixture H (containing high concentrations of urea, creatinine, uric acid, and indoxyl sulfate). **(C)** Luminex multiplex assay of normal and uremic serum. IL-12P40, leptin, resistin, and VCAM1 were higher in uremic serum than in normal serum. * $P < 0.05$ compared to normal serum. $n = 4$ /group. BDNF, Brain-derived neurotrophic factor; IL-12P40, interleukin-12 P40; PDGF BB, platelet-derived growth factor –BB; VCAM1, vascular cell adhesion molecule 1.

analysis (**Figure 7B**) of differentially expressed genes (DEGs) revealed different gene expression patterns after exposure to UT-H compared with control groups. In addition, a functional enrichment analysis was conducted to reveal the biological process and molecular function involved in a simplified *in vitro* uremic vasculopathy model. The most preserved expression profiles between control and UT-H treated samples were related to the amino acid transport across the plasma membrane, neutral amino acid transport, L-alpha-amino acid transmembrane

transport, L-amino acid transport, as well as amino acid transmembrane transport (**Figure 7C**).

DISCUSSION

Complex and diverse systemic dysfunction involving nearly all major organs is common in ESRD patients, with cardiovascular complications being the leading cause of death (Tonelli et al.,

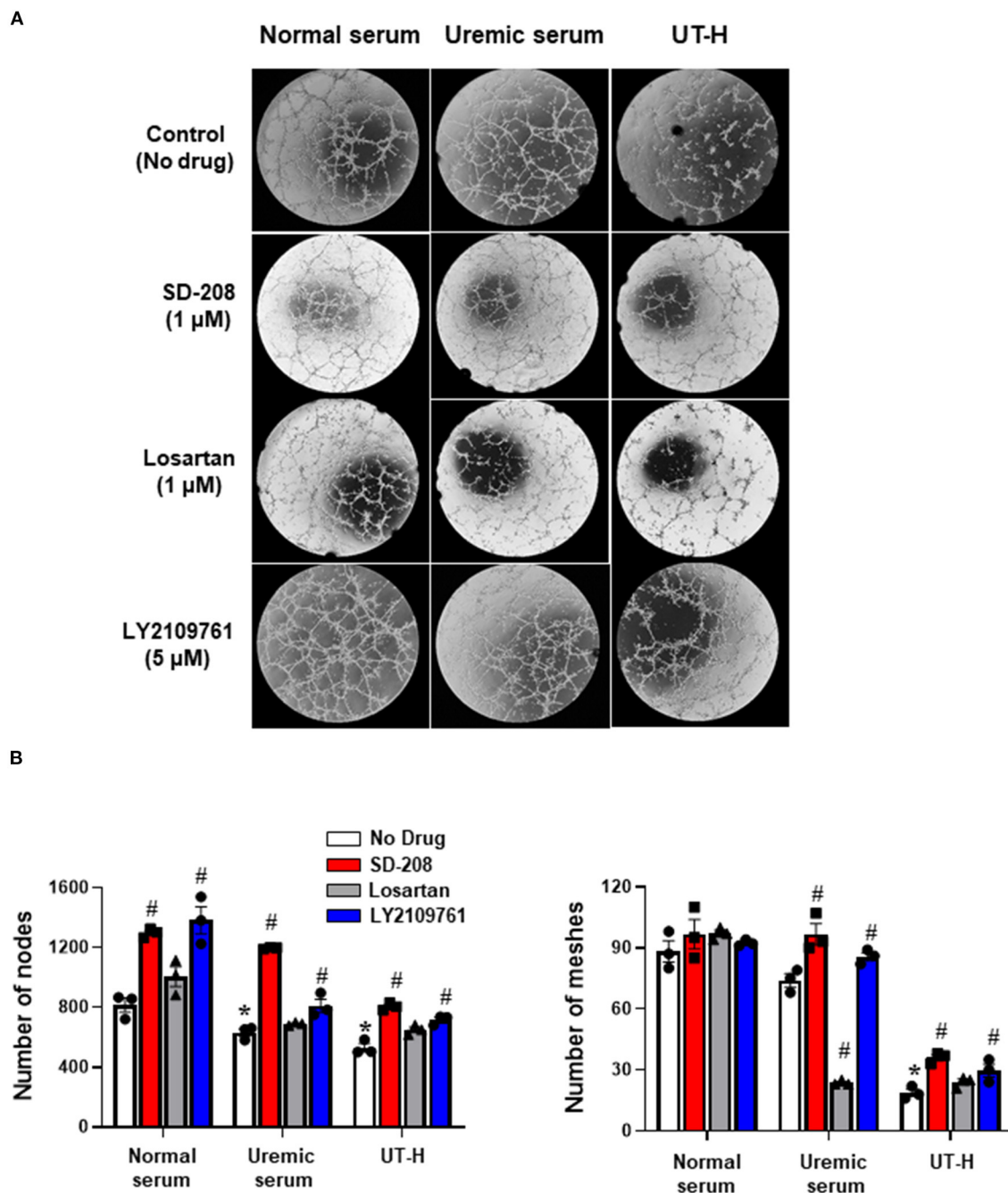


FIGURE 5 | Drug screening using a simplified uremic vasculopathy model. **(A)** The tube formation of iPSC-ECs was significantly suppressed in the uremic serum and UT-H groups. SD-208 and LY2109761 attenuated the adverse effects of uremic serum and UT-H on iPSC-ECs. The formation of capillary-like structures was imaged by phase-contrast microscopy ($\times 20$). **(B)** Quantitative data from the tube formation assay. Both SD-208 and LY2109761 showed favorable effects on the tube formation of iPSC-ECs treated with uremic serum or UT-H. Losartan also exerted favorable effects on iPSC-ECs treated with the UT-H but not significant. $*P < 0.05$ compared to normal serum group with no drug. $\#P < 0.05$ compared to no drug within the same group. $n = 3/\text{group}$. UT-H, uremic toxin mixture H (containing high concentrations of urea, creatinine, uric acid, and indoxyl sulfate).

2006; Stenvinkel, 2010). Endothelial dysfunction is not only an initiating pathogenic factor but also the cornerstone in the progression of cardiovascular complications in CKD (Aznar-Salatti et al., 1995; Merino et al., 2008; Kramann et al., 2012).

Uremic vasculopathy is an accelerated atherosclerotic disease frequently accompanied by severe vascular calcification under the uremic micromilieu of CKD patients (Martin-Rodriguez et al., 2015; Guo et al., 2017). Although a few uremic toxins are

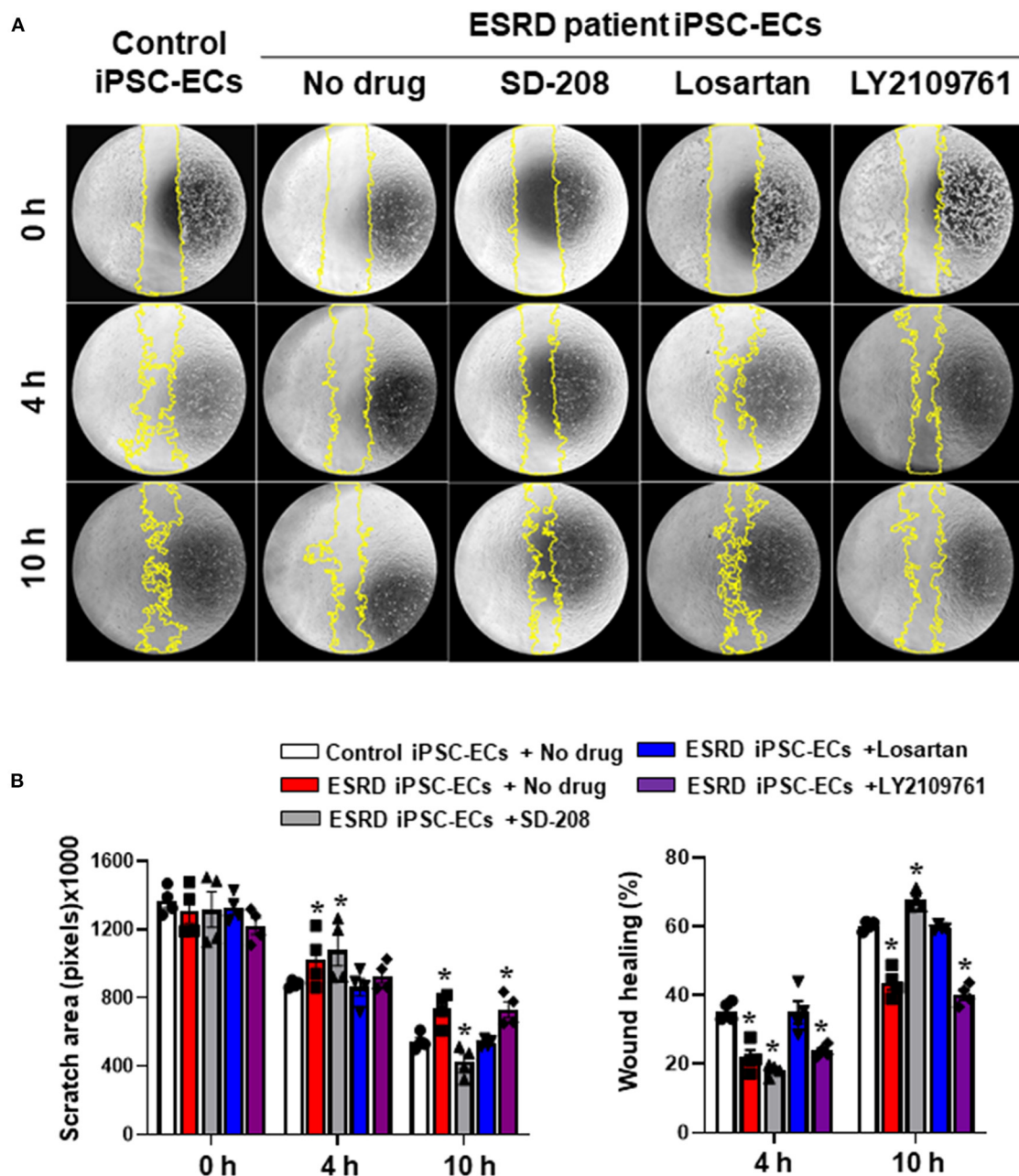
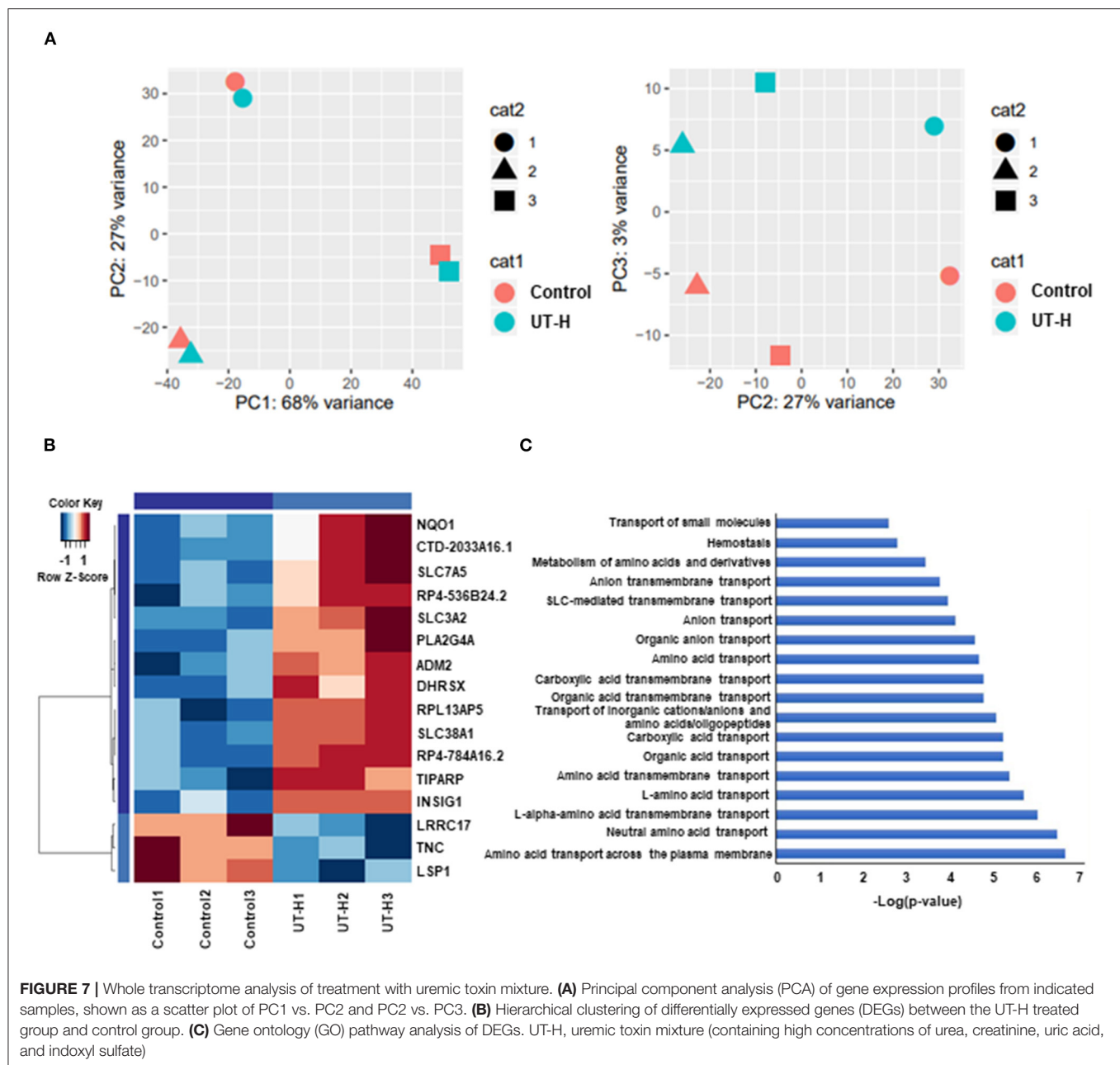


FIGURE 6 | Preliminary drug screening using ESRD patient-specific iPSC-ECs. **(A)** The degree of wound healing evaluated by a migration assay was significantly impaired in the ESRD patient-specific iPSC-ECs compared to the control iPSC-ECs. **(B)** Quantitative data from the migration assay. Losartan and SD-208 facilitated wound healing of the ESRD patient-specific iPSC-ECs. * $P < 0.05$ compared to the control iPSC-ECs with no drug. Control iPSC-ECs, iPSC-ECs from a normal control; ESRD, end-stage renal disease; ESRD iPSC-ECs, iPSC-ECs from an ESRD patient receiving hemodialysis.

known to cause endothelial damage (Glorieux and Vanholder, 2011), the types or concentration of uremic toxins triggering uremic vasculopathy is not fully understood. In this study, we have demonstrated that an *in vitro* uremic milieu comprising of urea, creatinine, uric acid, and indoxyl sulfate leads to impairment of iPSC-EC functional integrity. Importantly, these effects were comparable to the effects of uremic serum collected from ESRD patients but are more reproducible and consistent.

We have also shown that this simplified uremic vasculopathy model is potentially amenable to drug screening by revealing that TGF- β signaling inhibitors are capable of partially reversing the defects seen in iPSC-ECs exposed to uremic toxin mixtures. Lastly, we have shown that patient-specific iPSC-ECs derived from an ESRD patient exhibited a basal dysfunctional state compared to normal control iPSC-ECs, which can also be rescued by TGF- β inhibitors.



Cell culture models are often used for simulating uremic vasculopathy in humans. For instance, human umbilical vein endothelial cells (HUVECs) are commonly exposed to 10–20% diluted serum of CKD patients (Chitalia et al., 2011; Lanza et al., 2015). HUVECs have been most frequently used in cell culture models of uremic vasculopathy. However, these cells often do not reflect disease susceptibility because of their unique characteristics mimicking stem cells such as hematopoietic support (Yamaguchi et al., 1998; Bal et al., 2012, 2013). iPSC-ECs in our study may be superior to HUVECs in reflecting genetic susceptibility to uremic vasculopathy. It remains a challenge to discern the effects of each uremic toxin *per se* on ECs using the

serum of patients since serum usually contains a complex mixture of confounders such as cytokines and parathyroid hormones (Castillo-Rodríguez et al., 2017). In addition, differences in the concentration of each uremic toxin between individuals as well as temporal difference within the same patient also limit the usefulness of serum as a research tool. Moreover, there is also the ethical issue of acquiring large volumes of serum since most CKD patients are anemic. Based on these limitations, in this study, we investigated the effects of a simplified mixture of several well-known uremic toxins on iPSC-ECs, which were chosen by clinical criteria used to evaluate the degree of uremia as well as the pathogenic roles reported in previous studies.

Urea and creatinine are typical uremic toxins, most commonly used to evaluate CKD patients in clinics (Lau and Vaziri, 2017). Urea substantially contribute to the progression atherosclerosis by inducing endothelial dysfunction, endothelial progenitor cells senescence, and apoptosis of vascular smooth muscle cells (Giardino et al., 2017). Uric acid is known as an important uremic toxin contributing to CKD progression (Gustafsson and Unwin, 2013; Tsai et al., 2017) and increasing risk of cardiovascular mortality in CKD patients (Luo et al., 2019). Urea, creatinine, and uric acid are widely used as essential parameters to evaluate CKD patients in nephrology clinics since these toxins are easily measurable under internationally standardized protocols. Indoxyl sulfate and AGE were reported to increase cardiovascular risk in CKD (Wang et al., 2005; Ito et al., 2013; Barisione et al., 2015). Indoxyl sulfate, the most representative gut-derived uremic toxin, shows endothelial toxicity and subsequently plays a substantial role in the progression of atherosclerosis by inhibiting endothelial proliferation and repair (Hung et al., 2017; Lano et al., 2020). Indoxyl sulfate also impaired the functional ability of human mesenchymal stem cells (Wang et al., 2016). Our data showed that a mixture of high concentrations of urea, creatinine, uric acid, and indoxyl sulfate can consistently mimic the detrimental effects of uremic serum on iPSC-ECs. Treatment with high concentrations of urea, uric acid, or indoxyl sulfate also significantly impaired tube formation of iPSC-ECs, supporting the vascular toxicity of these uremic toxins reported in previous studies. The physiologic concentrations of urea, creatinine, and uric acid on the other hand did not cause adverse effects on iPSC-ECs, supporting our hypothesis that a simplified mixture of highly concentrated urea, creatinine, uric acid, and indoxyl sulfate can exert adverse effects on iPSC-ECs mimicking uremic serum.

Using this *in vitro* uremic vasculopathy model, we successfully elucidated that impaired TGF- β signaling is a potential pathogenic mechanism leading to impaired endothelial function in iPSC-ECs. This is in accordance with previous reports demonstrating that perturbed TGF- β signaling induces endothelial-mesenchymal transition enhancing fibrosis and non-hereditary disorders such as atherosclerosis and cardiac fibrosis (Pardali et al., 2017; Goumans and Ten Dijke, 2018). Mutations in the TGF- β superfamily have also been shown to disrupt endothelial patterning and induce arteriovenous malformations (Sugden and Siekmann, 2018). We also tested the feasibility of this simplified uremic vasculopathy model of iPSC-ECs for the screening of potential therapeutics. Losartan and TGF- β inhibitors were chosen for drug screening based on the previous studies reporting cardiovascular protective effects of angiotensin receptor blockers in CKD (Kuriyama et al., 2006; Verbeke et al., 2014; Kim-Mitsuyama et al., 2018) and our data showing the activation of intracellular TGF- β pathway in iPSC-ECs by *in vitro* uremic toxin mixtures. Both drugs attenuated the adverse effects of uremic toxins on normal control iPSC-ECs which is in line with a previous study reporting that the inhibition of TGF- β signaling leads to the improved vascular network formation by iPSC-ECs (Kurokawa et al., 2017). As a further extension of our work, we also demonstrated that ESRD patient-specific iPSC-ECs revealed impaired wound healing ability compared

to normal control iPSC-ECs demonstrating patient-specific iPSC-ECs recapitulate clinical susceptibility to a certain extent. Accordingly, the degree of wound healing was improved by losartan and SD-208 in ESRD iPSC-ECs. These results support the promising role of a simplified uremic vasculopathy model of iPSC-ECs as a novel drug screening tool reflecting disease susceptibility to uremic toxins.

Our study has a few limitations that should be addressed by future studies. First, more quantitative methodological approaches including functional genomics are required for improving the clinical usefulness of this model as a drug screening system. Although several quantitative or semiquantitative methods were used for analyzing phenotypic changes of iPSC-ECs in this study, more sensitive, as well as quantitative methods, are required for high throughput drug screening. Second, our study did not implement a standard method for evaluating the functional integrity of iPSC-ECs and there were some variations in evaluating the adverse effects of uremic toxins depending on the methods. A standardization process will be required for applying this approach in clinical practice. Third, we did not investigate aging effects on iPSC-ECs. Since the incidence of CKD and the risk of CKD progression to ESRD usually increase with age, further studies investigating differences in iPSC-ECs from young or old CKD patients would be required to improve clinical feasibility of this model.

CONCLUSION

Collectively, this study reports a novel platform of using iPSC-ECs to study uremic vasculopathy induced by a simplified uremic toxin mixture that can be applied as a potential drug screening system. Our data demonstrate that a simplified uremic toxin mixture can simulate the uremic microenvironment more reproducibly and consistently than uremic serum from ESRD patients. The deleterious effects of uremic toxins on iPSC-ECs were partly attenuated by an angiotensin-receptor blocker and TGF- β inhibitors supporting the potential usefulness of this model as a drug screening system. This new model of uremic vasculopathy represents a novel way to investigate the pathophysiology behind CKD and can be used to screen both conventional and new drugs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI GEO [accession: GSE155969].

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Samsung Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HRJ, HJC, WHL, and Y-GK wrote the first draft of the manuscript. HRJ, HJC, WH, S-GO, WHL, and Y-GK supervised the experiments. HRJ, YZ, N-YS, KL, JJ, JEL, and HL analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.618796/full#supplementary-material>

Supplemental Figure 1 | iPSCs and iPSC-ECs of a normal control and an ESRD patient. **(A)** ESRD patient-specific iPSCs showed comparable expression of iPSC pluripotency markers, such as Nanog, COT4, TRA-1-60, and SSEA-4, compared to the normal control iPSCs. **(B)** ESRD patient-specific iPSC-ECs had a similar expression of endothelial markers including VWF, CD144 (VE-Cadherin), and CD31 compared to control iPSC-ECs. DAPI, 4',6-diamidino-2-phenylindole; ESRD, end-stage renal disease; OCT4, octamer-binding transcription factor 4; SSEA4, stage-specific embryonic antigen 4; TRA-1-60, podocalyxin; VE-Cadherin, vascular endothelial cadherin; VWF, von-Willebrand factor.

Supplementary Figure 2 | Tube formation of iPSC-ECs from a normal control and an ESRD patient. **(A)** Vessel structure formation ability of iPSC-ECs from a normal control and an ESRD patient was compared with tube formation. **(B)** Tube formation was significantly impaired in ESRD patient-specific iPSC-ECs compared to normal control iPSC-ECs. * $P < 0.05$ compared to the control iPSC-ECs. ESRD, end-stage renal disease.

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Decoding Genetics of Congenital Heart Disease Using Patient-Derived Induced Pluripotent Stem Cells (iPSCs)

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Congenital heart disease (CHD) is the most common cause of infant death associated with birth defects. Recent next-generation genome sequencing has uncovered novel genetic etiologies of CHD, from inherited and *de novo* variants to non-coding genetic variants. The next phase of understanding the genetic contributors of CHD will be the functional illustration and validation of this genome sequencing data in cellular and animal model systems. Human induced pluripotent stem cells (iPSCs) have opened up new horizons to investigate genetic mechanisms of CHD using clinically relevant and patient-specific cardiac cells such as cardiomyocytes, endothelial/endocardial cells, cardiac fibroblasts and vascular smooth muscle cells. Using cutting-edge CRISPR/Cas9 genome editing tools, a given genetic variant can be corrected in diseased iPSCs and introduced to healthy iPSCs to define the pathogenicity of the variant and molecular basis of CHD. In this review, we discuss the recent progress in genetics of CHD deciphered by large-scale genome sequencing and explore how genome-edited patient iPSCs are poised to decode the genetic etiologies of CHD by coupling with single-cell genomics and organoid technologies.

Keywords: congenital heart disease, human induced pluripotent stem cells, NOTCH signaling, hypoplastic left heart syndrome, genetic models of CHD

INTRODUCTION

Congenital heart disease (CHD) is a leading cause of birth defect-related death and affects ~1% of live births in the United States (Hoffman and Kaplan, 2002; Nees and Chung, 2019). CHD is characterized by morphological abnormalities in the cardiac chambers, septa and valves as well as the great vessels arising from the heart. Congenital malformations of all aspects of the heart have been described but the most common types of CHD can be classified into the following categories: (1) cardiac septation defects, (2) conotruncal and aortic arch artery anomalies, (3) right- and left-sided outflow tract obstructive defects, and (4) left-right abnormalities (heterotaxy)

(Garg, 2006; Bruneau, 2008). Septation defects consist of atrial septal defects (ASD), ventricular septal defects (VSD) and atrioventricular septal defects (AVSD) while common conotruncal and aortic arch artery anomalies include tetralogy of Fallot (TOF), persistent truncus arteriosus and interrupted aortic arch. Right-sided outflow tract obstructive lesions include pulmonary stenosis and pulmonary valve atresia with intact ventricular septum (PA-IVS), whereas hypoplastic left heart syndrome (HLHS), aortic valve stenosis (AVS) and bicuspid aortic valve (BAV) are common left-sided outflow tract obstructive defects. Abnormalities in left-right signaling in the developing embryos affect cardiac looping, which is critical for proper alignment of the atria chambers to their appropriate-sided ventricles and great vessels. This disruption in proper signaling is associated with complex forms of CHD, such as double outlet right ventricle and double inlet left ventricle, clinically termed as heterotaxy syndrome (Kathiriyaa and Srivastava, 2000). Other major CHD that does not fit into the abovementioned categories includes isolated valve anomalies (e.g., Ebstein's anomaly of the tricuspid valve and mitral valve prolapse), total anomalous pulmonary venous connection, anomalous coronary artery and patent ductus arteriosus.

Epidemiologic studies reveal that genetic factors are the predominant cause of CHD whereas environmental factors (exposures, maternal conditions, intrauterine environment, etc.) are also important contributors (Liu et al., 2013; Pierpont et al., 2018). In total, specific genetic and environmental factors can be identified in 20–30% of all CHD cases. Genetic mechanisms underlying the development of CHD are complex and remain elusive using current genetic approaches (Liu et al., 2017; Pierpont et al., 2018). There are limited animal models to study the developmental genetics of CHD, and transgenic mice carrying human variants do not always recapitulate the clinical phenotypes of CHD (Majumdar et al., 2019). Human iPSCs are derived from somatic cells (such as skin fibroblasts or peripheral blood mononuclear cells) and have the potential to generate all cell types in the body originated from the three germ layers (Takahashi et al., 2007; Yu et al., 2007). Compared to animal models, patient iPSCs are clinically relevant and also include the genetic background of the affected individuals in a disease-specific manner, thus providing a powerful tool for studying the contribution of a given genetic variant to CHD. Patient-specific iPSCs can be differentiated into cardiomyocytes, endothelial/endocardial cells, cardiac fibroblasts and smooth muscle cells, which makes it feasible to study complex genetic regulation and gene-environment interactions simultaneously in multiple cell types in the heart (Hu et al., 2016; Zhao et al., 2017a; Gifford et al., 2019). Recent studies demonstrate that genome-edited iPSCs are ideal platforms to elucidate the regulatory roles of non-coding genetic variants in the risk of coronary artery disease and to investigate the contribution of combinatorial interactions of multiple genetic variants to complex cardiovascular disease (Lo Sardo et al., 2018; Deacon et al., 2019).

In this review, we discuss the latest progress on genetic etiologies of CHD uncovered by the state-of-the-art technologies such as whole genome sequencing (WGS) and whole exome

sequencing. We explore the fascinating perspectives on using patient-specific iPSCs and CRISPR genome editing to functionally study the genetic and epigenetic (environmental) determinants of CHD.

GENETICS OF CHD

With the advance of massively parallel sequencing, genetics of CHD have been aggressively explored in the past decade. Large scientific efforts such as NIH-funded Pediatric Cardiac Genomics Consortium (PCGC) have been established to coordinate the investigation of genetic variants present in CHD patient population relevant to clinical outcomes (Pediatric Cardiac Genomics Consortium et al., 2013; Jin et al., 2017). The genetic basis of CHD can be grouped into two categories: syndromic CHD and non-syndromic (isolated) CHD (Pierpont et al., 2018). Syndromic CHD is defined as CHD with other congenital anomalies, neurodevelopmental defects and/or dysmorphic features. Syndromic CHD may be caused by aneuploidy, copy number variants (insertions or deletions > 1,000 nucleotides), or single gene defects. Down syndrome (trisomy 21) is a common chromosome anomaly, and 40–50% of these patients have various types of CHD, with cardiac septation defects being the most common. Turner syndrome is caused by complete or partial loss of an X-chromosome, and left-sided defects (coarctation of the aorta, COA), BAV and HLHS are present in 30% of these patients. 22q11.2 deletion syndrome is one of the most common copy number variants with deletion of more than 40 genes on chromosome 22. Outflow tract defects are present in 75–80% of 22q11.2 patients. Syndromic CHD caused by single-gene defects includes Alagille syndrome (variants in *JAG1* and *NOTCH2*) and Holt-Oram syndrome (variants in *TBX5*) (Basson et al., 1997; Li et al., 1997b; **Table 1**). Genetic contributors of isolated CHD have been emerging in the past two decades and most variants are located in genes that are involved in the molecular regulation of cardiac development. Syndromic and isolated CHD display distinct genetic architectures: *de novo* protein-truncating variants (PTVs) are significantly enriched in syndromic CHD whereas inherited PTVs are mostly derived from unaffected parents in isolated CHD (Sifrim et al., 2016; Jin et al., 2017).

Pathogenic variants linked to isolated CHD primarily encode transcription factors, signaling molecules, structural proteins and epigenetic modifiers that are essential for normal cardiac development (Zaidi et al., 2013; Pierpont et al., 2018; Nees and Chung, 2019; **Table 1**). For instance, genetic variants in highly conserved transcription factors critical for cardiac development are found in both familial and sporadic cases of CHD. *NKX2-5* variants are present in patients with TOF and ASD with conduction delay (Schott et al., 1998; Benson et al., 1999; Goldmuntz et al., 2001; Stallmeyer et al., 2010). Pathogenic *GATA4* variants are associated with ASD, VSD, AVSD, pulmonary stenosis (PS), and TOF (Garg et al., 2003; Okubo et al., 2004; Hirayama-Yamada et al., 2005; Sarkozy et al., 2005; Tomita-Mitchell et al., 2007). A small subset of *GATA4* variant-induced cardiac malformations in humans are recapitulated in transgenic

TABLE 1 | A summary of single-gene variants underlying CHD.

Gene	CHD	Discovery methods	References
GATA4	Atrial septal defect	Linkage analysis	Garg et al., 2003
	Atrioventricular septal defect	PCR	Hirayama-Yamada et al., 2005
	Pulmonary stenosis	Targeted sequencing	Okubo et al., 2004
	Tetralogy of Fallot		Sarkozy et al., 2005
	Ventricular septal defect		Tomita-Mitchell et al., 2007
JAG1	Pulmonary artery stenosis	BAC	Eldadah et al., 2001
	Tetralogy of Fallot	FISH	Li et al., 1997a
		Linkage analysis	Mcdaniell et al., 2006
		PCR	Oda et al., 1997
		SSCP	
MIB1	Left ventricular non-compaction	Targeted sequencing	
		PCR	Luxan et al., 2013
		Targeted sequencing	
		Transgenic mice	
NKX2-5	Atrial septal defects	Zebrafish reporter assays	
		FISH	Benson et al., 1999
		Linkage analysis	Goldmuntz et al., 2001
		PCR	Schott et al., 1998
NOTCH1	Tetralogy of Fallot	Targeted sequencing	Stallmeyer et al., 2010
	Aortic valve stenosis	<i>In vitro</i> expression assay	Durbin et al., 2017
	Bicuspid aortic valve	Luciferase reporter assay	Garg et al., 2005
	Coarctation of the aorta	Microarray	Mcbride et al., 2008
	Hypoplastic left heart syndrome	Whole exome sequencing	Kerstjens-Frederikse et al., 2016
PCDHA13 SAP130	Hypoplastic left heart syndrome	Whole genome sequencing	Stittrich et al., 2014
			Zahavich et al., 2017
TBX5	Hypoplastic left heart syndrome	Mouse forward genetics	Liu et al., 2017
		Whole exome sequencing	
		Enhancer reporter assay PCR	Basson et al., 1997
		Targeted sequencing	Li et al., 1997b; Mcdermott et al., 2005
	Ventricular septal defect	Transgenic mice	Smemo et al., 2012
		Zebrafish reporter assay	

BAC, bacterial artificial chromosome; FISH, fluorescence in situ hybridization; SSCP, single-strand conformation polymorphism.

mouse models harboring the mutant human *GATA4* variants (Misra et al., 2012; Han et al., 2015).

Components of the NOTCH signaling pathway are linked to both syndromic and isolated CHD. *JAG1* variants are observed in ~90% of Alagille syndrome patients whereas *NOTCH2* variants account for additional 1–2% of individuals with Alagille syndrome (Li et al., 1997a; Oda et al., 1997; Mcdaniell et al., 2006; Kamath et al., 2012). Loss-of-function variants in *JAG1* cause pulmonary artery stenosis and TOF with or without pulmonary atresia (Eldadah et al., 2001). Heterozygous mutations in *DLL4* (ligand) and *NOTCH1* (receptor) lead to Adams Oliver syndrome with CHD present in about 25% of these patients (Stittrich et al., 2014; Meester et al., 2015). Variants in *RBPJ* which interacts with the cleaved NOTCH1 protein to form a transcriptional complex, are also linked to Adams Oliver syndrome (Hassed et al., 2012). Of note, pathogenic *NOTCH1* mutations are linked to BAV, HLHS, AVS, COA, and TOF (Garg et al., 2005; Mcbride et al., 2008; Kerstjens-Frederikse et al., 2016; Durbin et al., 2017; Zahavich et al., 2017). Mechanistically, *NOTCH1* mutations reduce the ligand binding ability, interrupt the S1 cleavage of NOTCH receptor in the Golgi, and impair the epithelial-to-mesenchymal transition (Riley et al., 2011). In addition, germline mutations in *MIB1* which encodes an E3 ubiquitin ligase that promotes endocytosis of NOTCH ligands, lead to left ventricular non-compaction

(LVNC) in autosomal-dominant pedigrees (Luxan et al., 2013). Myocardial *Mib1* mutations in mice cause the expansion of compact myocardium to proliferative immature trabeculae and interruption of chamber myocardium development.

The encyclopedia of DNA elements (ENCODE) project suggests that more than 80% of human genomic DNA has a biochemical function (Consortium, 2012). The majority of disease-causing variants identified by genome-wide association studies (GWAS) are located in non-coding DNA elements, many of which are embedded in the DNase I hypersensitive (open chromatin) regions (Maurano et al., 2012). GWAS in CHD have similar findings (Cordell et al., 2013; Hu et al., 2013; Mitchell et al., 2015; Hanchard et al., 2016). *De novo* variants in enhancer elements have been found in several human developmental defects including CHD and neurodevelopmental disorders (Short et al., 2018). For example, sequence variants in a limb-specific enhancer ZRS which is located nearly 1 Mb from its target gene *sonic hedgehog* (*Shh*) result in limb malformations such as preaxial polydactyly (Lettice et al., 2003). Copy number variants affecting topological associated domains have also been implicated in disrupting enhancers and causing developmental defects (Lupianez et al., 2015). Distal *cis*-regulatory elements have been identified in *TBX5*, of which variants are responsible for Holt-Oram syndrome (Mcdermott et al., 2005; Smemo et al., 2012). Among patients with Holt-Oram syndrome, three quarters

have CHD, with ASD and VSD as the most common cardiac defects. A homozygous variant found in an enhancer about 90 kb downstream of *TBX5* is associated with isolated ASD and VSD in a cohort of non-syndromic CHD patients. This single-nucleotide variant compromises the enhancer activity driving expression of *TBX5* in the heart in both mouse and zebrafish transgenic models (Smemo et al., 2012). Recent WGS and chromatin immunoprecipitation sequencing have enabled researchers to expand the genetic variants in non-coding DNA elements that may have a regulatory role in controlling gene transcription during heart development (Zhao et al., 2017b; Richter et al., 2020). Non-coding *de novo* variants (DNVs) are significantly enriched in individuals with CHD and potentially exhibit transcriptional and post-transcriptional regulatory effects on genes critical for normal cardiac morphogenesis. Genetic architecture of CHD in cardiac regulatory non-coding DNVs will be further elucidated with the advance of WGS and precise genome editing technologies.

PATIENT-SPECIFIC IPSCS FOR MODELING GENETICS OF CHD

Although a genetic etiology is identified in about 1/3 of CHD patients, experimental models to functionally validate genetic variants associated with CHD are far from perfect. Genetically engineered mice have been used for studying fundamental genetics of cardiac development for more than 25 years. Murine models are able to recapitulate some aspects of human cardiac development due to their similar stages of cardiac morphogenesis and adult cardiac structure (Majumdar et al., 2019). However, there are substantial differences in genomic content and physiology between humans and mice. Orthologous heterozygous variants sometimes do not reproduce similar CHD phenotypes when introduced into the mouse genome. Patient-derived iPSCs appear to provide a unique platform to study the genetic mechanisms of CHDs as they retain all the genetic information of the original affected individuals. Combined with CRISPR/Cas9 genome-editing, single-cell genomics, and cardiac organoid engineering technologies, patient-specific iPSCs would greatly complement the murine genetic models of CHD and illustrate novel perspectives on genetic etiologies of CHD for future precision diagnosis and treatment.

Human iPSCs are promising models for studying genetic mechanisms of isolated CHD caused by single-gene defects. In addition to cell-autonomous inherited cardiac disease such as long QT syndrome (Moretti et al., 2010; Itzhaki et al., 2011), ventricular tachycardia (Zhang et al., 2014; Sleiman et al., 2020) and dilated cardiomyopathy (Sun et al., 2012; Hinson et al., 2015), patient iPSCs have been employed to model several types of CHD, including BAV and calcific aortic valve disease (CAVD) (Theodoris et al., 2015), supraventricular aortic stenosis (SVAS) (Ge et al., 2012), cardiac septal defects (Ang et al., 2016), Barth syndrome (Wang et al., 2014), and HLHS (Hrstka et al., 2017; Yang et al., 2017; Miao et al., 2020; **Table 2**). Human iPSCs can be differentiated to the desired cardiovascular cell types relevant for studying different CHD (Protze et al., 2019), though

the immaturity of iPSC-derived cardiomyocytes (iPSC-CMs) continues to be a challenge for recapitulating the physiological scenarios in the heart (Karbassi et al., 2020; Zhao et al., 2020b). Robust cardiac differentiation protocols have been optimized to generate subtype-specific (atrial, ventricular and nodal) cardiomyocytes for precision disease modeling (Zhang et al., 2011; Lee et al., 2017; Protze et al., 2017; Ren et al., 2019; Liang et al., 2020; Zhao et al., 2020a).

Human iPSC models of CHD have employed major cardiac cell types such as cardiomyocytes (CMs), vascular smooth muscle cells (SMCs), and endothelial/endocardial cells (ECs) that can be derived from patient-specific iPSCs for laboratory research. These patient-derived cardiac cells carrying genetic variants enable researchers to study the disease mechanisms in a petri dish (**Table 2**). For example, pathogenic *GATA4* variants cause cardiac septal defects and cardiomyopathy. A heterozygous variant in *GATA4* (G296S missense) is linked to 100% penetrant ASD, VSD, AVSD or PS (Garg et al., 2003). Human iPSC-CMs from heterozygous *GATA4*-G296S patients display impaired contractility, defects in calcium handling ability and abnormal mitochondrial functions (Ang et al., 2016). Molecular analysis reveals that mutant *GATA4* disrupts the recruitment of *TBX5* which binds to cardiac super-enhancers and leads to dysregulation of genes related to cardiac septation. In another study, Theodoris et al. (2015) have derived iPSCs from patients with BAV and CAVD which are linked to *NOTCH1* haploinsufficiency. In iPSC-derived endothelial cells (iPSC-ECs), *NOTCH1* heterozygous nonsense variants disrupt the epigenetic architecture of *NOTCH1*-bound enhancers and cause the depression of anti-osteogenic and anti-inflammatory gene regulation networks in response to hemodynamic shear stress (Theodoris et al., 2015). Furthermore, the same group have recently utilized a combination of human iPSC technology, machine learning and network analysis to identify an efficacious therapeutic candidate XCT790 for preventing and treating aortic valve disease in a mouse model, demonstrating the prospective pharmacogenetic applications of CHD patient-specific iPSCs (Theodoris et al., 2020). Ge et al. (2012) have employed iPSC-derived smooth muscle cells (iPSC-SMCs) to investigate how elastin (*ELN*) gene variants lead to narrowing or blockage of the ascending aorta in SVAS. SVAS iPSC-SMCs harboring *ELN* variants are less mature and contractile, and show fewer networks of smooth muscle actin filament bundles compared to healthy controls. These SVAS iPSC-SMCs have a higher proliferation ability and migration rate in response to platelet-derived growth factor (PDGF), indicating that SVAS iPSC-SMCs recapitulate the pathological features of SVAS patients and may provide novel insights for future therapies.

Human iPSCs have been utilized to study complex genetics in CHD together with transgenic mouse models and clinical genetics. A recent study reveals that *NKX2-5* variants serve as a genetic modifier of a familial LVNC cardiomyopathy with variable age of presentation from childhood to incidental asymptomatic finding in adulthood (Gifford et al., 2019). Human iPSCs were created carrying the inherited compound heterozygous variants in *MKL2*, *MYH7* and *NKX2-5* while genetically engineered mice carrying the orthologous variants

TABLE 2 | Current iPSC models for studying disease mechanisms of CHD.

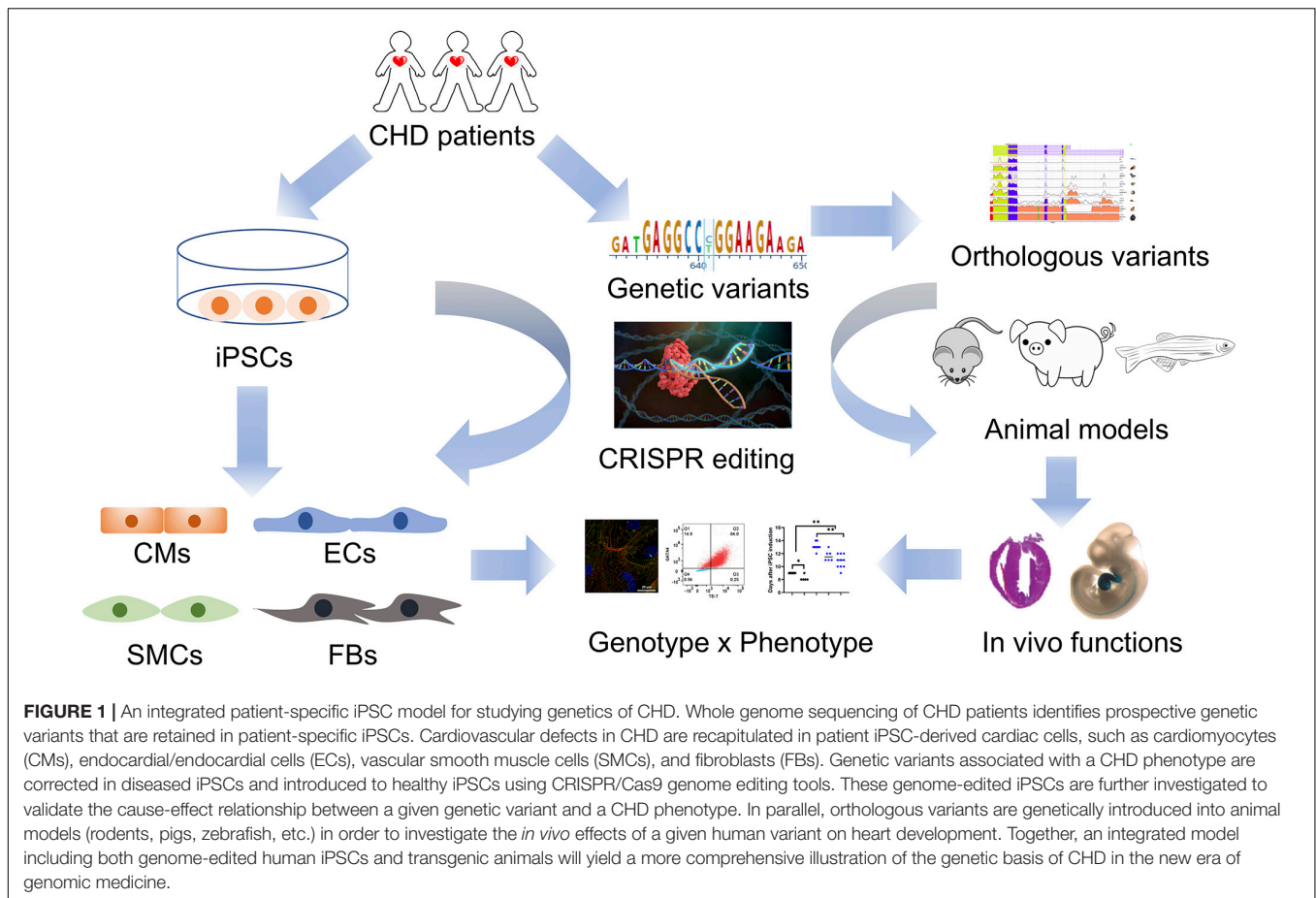
CHD	Variants	Cell types	Disease phenotypes	References
ASD VSD AVSD	<i>GATA4</i>	Cardiomyocytes	Impaired contractility Defects in calcium handling Abnormal mitochondrial functions	Ang et al., 2016
BTHS	<i>TAZ</i>	Cardiomyocytes	Irregular sarcomeres Abnormal myocardial contraction Excessive ROS generation	Wang et al., 2014
CAVD	<i>NOTCH1</i>	Endothelial cells	Defective epigenetic architecture Disrupted transcriptional response	Theodoris et al., 2015
HLHS	<i>NOTCH1</i> Unknown	Cardiomyocytes	Abnormal gene expression NO signaling deficiency Disorganized sarcomeres Reduced contraction force Decreased metabolic activity	Hrstka et al., 2017 Paige et al., 2020 Yang et al., 2017
HLHS	Unknown	Endothelial cells	Endocardial differentiation defects	Miao et al., 2020
LVNC	<i>MKL2</i> <i>MYH7</i> <i>NKX2-5</i>	Cardiomyocytes	<i>NKX2-5</i> is a genetic modifier Abnormal gene expression	Gifford et al., 2019
LVNC	<i>TBX20</i>	Cardiomyocytes	Defects in cardiac proliferation Abnormal TGF- β signaling	Kodo et al., 2016
PA-IVS	Unknown	Cardiomyocytes	Abnormal developmental trajectory Reduced contractility	Lam et al., 2020
SVAS	<i>ELN</i>	Smooth muscle cells	Less mature and contractile Higher proliferation ability in response to PDGF	Ge et al., 2012
VSD	<i>TBX5</i>	Cardiomyocytes	<i>TBX5</i> haploinsufficiency Disrupted gene regulatory network	Kathiriya et al., 2020

The CHD subtypes, genetic variants, relevant cell types, and disease phenotypes are listed. ASD, atrial septal defect; AVSD, atrioventricular septal defect; BTHS, Barth syndrome; CAVD, calcific aortic valve disease; HLHS, hypoplastic left heart syndrome; LVNC, left ventricular non-compaction; PA-IVS, pulmonary atresia with intact ventricular septum; SVAS, supraaortic stenosis; VSD, ventricular septal defect.

were also generated. By analyzing the phenotypes from transgenic murine hearts and patient iPSC-CMs, *NKX2-5* variants are identified as a genetic modifier for this cardiomyopathy with oligogenic inheritance. In another study, LVNC iPSC lines were generated from patients with *TBX20* variants (Kodo et al., 2016). LVNC iPSC-CMs show defects in proliferation which is caused by the abnormal activation of TGF- β signaling. In mice, overexpression of TGF- β 1 leads to arrest in cardiac development, disturbed expansion of embryonic cardiomyocytes and trabecular/compact layer ratio in the left ventricle. Mostly recently, Kathiriya and colleagues have generated *TBX5* knockout human iPSC lines with different dosages (heterozygous and homozygous) and performed single-cell RNA sequencing and gene regulatory network analysis. *TBX5* haploinsufficiency alters the expression of CHD-related genes and reduced *TBX5* gene dosage disrupts gene regulatory networks in human iPSC-CMs. Abnormal genetic interaction between *Tbx5* and *Mef2c* leads to ventricular septation defects in transgenic mice with reduced *Tbx5* gene dosage (Kathiriya et al., 2020). These studies further highlight the combinatorial advantages of using human iPSCs and transgenic mouse models to reveal genetic mechanisms of CHD pathogenesis (Figure 1).

Hypoplastic left heart syndrome is a severe form of CHD characterized by aortic and mitral valve atresia or stenosis, leading to a hypoplastic left ventricle and aorta (Saraf et al., 2019). Though HLHS has a strong genetic component, the genetic etiology of HLHS is complex (Mcbride et al., 2005). Further, mouse models are not able to fully recapitulate

the clinical phenotype (Liu et al., 2017; Grossfeld et al., 2019). Using HLHS patient-derived iPSC-CMs, multiple studies demonstrate the pathogenic link of *NOTCH1* variants to HLHS (Theis et al., 2015; Durbin et al., 2017; Hrsta et al., 2017; Yang et al., 2017). HLHS iPSCs harboring *NOTCH1* variants exhibit compromised ability to generate cardiac progenitors and HLHS iPSC-CMs show disorganized sarcomere structures and sarcoplasmic reticulum as well as a blunted drug response (Yang et al., 2017). Another independent study confirms that HLHS iPSCs have a deficiency in cardiomyocyte differentiation and NOTCH signaling pathway (Hrsta et al., 2017). Additionally, abnormalities in the nitric oxide (NO) pathway are found in the cardiac lineage specification of HLHS iPSCs with *NOTCH1* mutations. Small molecule supplementation could restore the cardiogenesis, implying a potential therapeutic target for HLHS patients. This study is consistent with the congenital cardiac abnormalities observed in *Notch1*^{+/-}; *Nos3*^{-/-} transgenic mice and demonstrates that interaction between NO pathway and NOTCH signaling is required for proper development of the left-sided cardiac structures including the aortic valve (Bosse et al., 2013; Koenig et al., 2016). Recently, Miao et al. (2020) have highlighted the contribution of endocardial defects to the pathogenesis of HLHS using patient iPSCs and single-cell RNA sequencing of human fetuses with under developed left ventricles. Although the genetic causes of these HLHS iPSCs are unclear, endocardial defects lead to abnormal endothelial-to-mesenchymal transition, reduced cardiomyocyte proliferation and maturation, and disrupted fibronectin-integrin



signaling. Another study by Mikryukov et al. (2021) has identified a critical role of BMP10 in the specification and maintenance of endocardial cells from human iPSCs. These iPSC-derived endocardial cells can induce trabeculation in iPSC-CMs and generate valvular interstitial-like cells, which are promising *in vitro* models for studying cardiac valve defects and LVNC. As the intercellular communication between endocardium and myocardium is essential for normal ventricular development (Macgrogan et al., 2018), further investigation would be warranted to illustrate how the abnormal crosstalk signaling leads to hypoplasia of the left ventricle using HLHS iPSC-CMs and iPSC-ECs.

The major challenge for studying genetics of CHD is lack of reliable models to functionally validate genetic variants that are discovered by massive genome sequencing. Although iPSC models are increasingly being used to study the contribution of genetic variation in the development of CHD, limitations should be carefully considered before any translational applications move forward. Human iPSC-CMs are fetal-like cardiomyocytes and show immature structural and physiological characteristics. For example, iPSC-CMs do not have mature structures of myofibrils and T-tubule, and they are misaligned compared to rod-shape adult cardiomyocytes (Karbassi et al., 2020; Zhao et al., 2020b). Enormous efforts have been made to promote the structural and functional maturation of iPSC-CMs, including

the addition of thyroid and glucocorticoid hormones (Parikh et al., 2017), physical and electrical conditioning (Ronaldson-Bouchard et al., 2018), and co-culture with stromal cells in 3D cardiac microtissues (Giacomelli et al., 2020). In addition, iPSC-CMs are mostly cultured as a 2D structure which differs from the 3D structure of the human heart. Patient iPSC-derived cardiac organoids may be better models as a 3D substitute for the human heart (Rossi et al., 2018; Richards et al., 2020). However, it is still undetermined whether cardiac organoids can recapitulate the developmental scenarios of CHD pathogenesis. After all, any iPSC-based models are *in vitro* systems, which are fundamentally distinct from the *in vivo* environment. Although animal models best represent the *in vivo* environment, animals are different from humans in terms of physiology and genomics, and may not be clinically relevant. Therefore, we propose an integrated model which incorporates patient-specific iPSCs with transgenic animals (Figure 1). We envision that genetic variants associated with a CHD phenotype are tested in genome-edited iPSCs which are patient-derived and clinically relevant, while orthologous variants are also genetically introduced to animal models (rodents, pigs, zebrafish, etc.) to investigate the *in vivo* functions. The combination of human iPSCs and transgenic animals will provide us a more comprehensive illustration of pathogenic mechanisms of CHD.

OUTLOOK

Recent advances in CRISPR/Cas9 genome editing (Adli, 2018), single-cell genomics (Tanay and Regev, 2017) and organoid (Rossi et al., 2018) technologies further propel the discovery of novel mechanisms of CHD development using patient- and disease-specific iPSCs. Precise genome editing technologies can be used to correct a given variant in patient iPSCs and then study whether the disease phenotypes can be rescued in genetically corrected isogenic cardiac cells (Hockemeyer and Jaenisch, 2016; Deacon et al., 2019). Concomitantly, this variant can be introduced to a healthy iPSC line with new genetic background to test whether it is sufficient to cause the disease phenotypes. Moreover, oligogenic inheritance in CHD may be studied in patient iPSCs by simultaneous correction or introduction of a combination of multiple genetic variants (Gifford et al., 2019). Single-cell RNA-seq analysis of human and mouse hearts has provided unprecedented resources on the trajectory of cardiac development *in vivo* at single-cell resolution and revealed a blueprint on how normal cell fate determination is altered under genetic perturbation and pathological conditions such as CHD (Cui et al., 2019; De Soysa et al., 2019; Litvinukova et al., 2020; Paik et al., 2020). Single-cell transcriptional profiling of healthy and diseased iPSCs during cardiac differentiation would decipher how a given genetic variant affects cardiac differentiation and developmental trajectories, and uncover new molecular insights in the pathogenesis of CHD (Churko et al., 2018; Kathiriya et al., 2020; Lam et al., 2020; Miao et al., 2020; Paige et al., 2020). As heart development is dependent on interaction among multiple cell types in the embryo, cardiac organoids and 3D bio-printing may serve as another tier of disease modeling using patient iPSCs (Lee et al., 2019; Nugraha et al., 2019). Cardiac organoids contain the spatial information of multiple cardiac

cell types and lay out a 3-D platform to study the complex interactions between genotypes and phenotypes under normal and diseased conditions using patient-specific iPSCs. Although cardiac organoids have been used for modeling drug-induced toxicity and myocardial infarction (Richards et al., 2020), it is still challenging to generate cardiac structures such as heart valves and septa that can represent the developmental defects in CHD using the current cardiac organoid technologies. Future therapeutic breakthroughs in precision medicine of CHD would require the convergence of precision genome editing, single-cell genomics and cardiac bioengineering, which is built upon clinically relevant and patient-specific iPSC platforms.

AUTHOR CONTRIBUTIONS

HL and M-TZ: conception and design, figure preparation, manuscript writing, and final approval of manuscript. KM and VG: manuscript writing and final approval of manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Isolation and Multiple Differentiation of Rat Pericardial Fluid Cells

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Objective: The aim of the present study is to isolate and analyze the characterization of pericardial fluid cells (PFCs) from rat and provides a morphological basis for the basic research and clinical application of PFCs.

Methods: After aseptic thoracotomy was performed, normal saline was injected into the pericardial cavity of 50 adult Sprague–Dawley rats. The mixture of diluted pericardial fluid was extracted, centrifuged, and cultured. The cell morphology of different generations in the pericardial fluid was observed on an inverted microscope. The expression levels of CD44, CD29, CD90, and pan-hematopoietic marker CD45 were analyzed via flow cytometry. The third-generation cells were used for osteogenic, adipogenic, and cardiac differentiation.

Results: PFCs were successfully isolated and subcultured. PFCs were predominantly circular in shape after 24 h of culture. Following subculture for 3 days, the cells demonstrated a spindle shape. The rat pericardial fluid contains cell populations with uniform morphology, good growth state, and strong proliferation ability. Flow cytometry results showed that CD29 (100%) and CD90 (99.3%) were positively expressed, whereas CD45 (0.30%) and CD44 (0.48%) were negatively expressed. The PFCs could differentiate into osteoblasts and adipocytes after being induced. Cardiac differentiation was also confirmed by cardiac troponin T (cTnT) and α -sarcomeric actin (α -SA) staining.

Conclusion: This study revealed that a subpopulation of cells was isolated from pericardial fluid, which exhibited progenitor cell features and multiple differentiation potency. PFCs could serve as an alternative cell source for myocardial tissue repair, engineering, and reconstruction.

Keywords: CD90, CD44, CD29, CD45, cell differentiation, pericardial fluid

INTRODUCTION

The pericardium consists of two layers, the outer fibrous layer and the inner serous layer (Buoro et al., 2020). The pericardial fluid is a serous fluid that exists between the serous pericardial viscera and the parietal layer. This fluid also serves as a cushion and reduces friction between the central viscera and the wall during the cardiac cycle. The normal pericardial fluid volume of adult human is approximately 50 mL (Phelan et al., 2015). The pericardium protects the heart by preventing aberrant motion, excessive dilation due to acute volume overload, and the spread of infections from other organs (Beltrami et al., 2017; Sahoo et al., 2017). Therefore, pericardial mesothelial

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cells could also synthesize and secrete certain vascular active substances into the pericardial cavity. Recent study revealed that mesothelial cells are abundant in pericardial fluid, which included other kinds of cells, such as neutrophils, lymphocytes, monocytes, and macrophages (Buoro et al., 2020). In the case of disease, the composition of the cellular fluid could be changed. High concentrations of vascular-derived growth factor accumulate in the pericardial fluid of patients with ischemic heart disease. This phenomenon may have a self-protective mechanism against myocardial ischemia (Fujita et al., 2001). Studies have shown that stem cells exist in various body fluids. The mesenchymal progenitor cells in the synovial fluid of patients with arthritis possess multiple mesenchymal differentiation potentials (Jones et al., 2004). Cells isolated from urine could differentiate into several bladder cell lineages expressing markers of urothelial, endothelial, and interstitial cells (Zhang et al., 2008). These cells are currently being investigated for tissue engineering applications. In the present study, flow cytometry, cell culture, and induction were used to detect the cellular components and differentiation potential of rat pericardial fluid.

MATERIALS AND METHODS

Experimental Animals

Fifty adult male Sprague–Dawley rats between 8 and 12 weeks old with an average body weight of 120 ± 20 g were housed with a 12-h light/dark cycle (lights on from 7:00 to 19:00) at constant temperature (25°C). All animal protocols were conducted in accordance with the guidelines of the Ministry of Science and Technology of the People's Republic of China [(2006)398] and approved by the Animal Care Committee of Xinxiang Medical University (No. 030032).

Extraction, Cell Isolation, and Culture of Rat Pericardial Fluid Cells

The thorax of healthy adult rats was opened in the middle of the sternum under sterile conditions after anesthesia and disinfection. The hooks were fixed to expose the pericardium. A 2 mm incision was cut near the pericardial fat, and 0.5 mL of normal saline was drawn with a trocar and injected into the pericardial cavity. The pericardial fluid was diluted, and a tube of cell suspension was taken from the pericardial fluid of approximately five rats. After the suspension was filtered, pericardial fluid cells (PFCs) were collected. The PF samples were centrifuged at 4°C (1200 r/min, 5 min), the cell pellets were washed with phosphate-buffered saline, and the supernatant was discarded. The cells were resuspended in complete medium (low-glucose DMEM, 30% fetal bovine serum, 100 U/mL of penicillin, and 100 U/mL of streptomycin) and inoculated for 24 h. On day 3, fluid exchange was observed, and the non-adherent cells were discarded. The fluid was changed every 3 days thereafter. When the cells were approximately 70–80% of the bottom of the bottle, they were digested with 0.25% protease (containing 0.02% EDTA, 0.04 mL/cm²) and passaged. The passage ratio was 1:2.

Flow Cytometric Identification

P3 cells were seeded in a T75 cell culture flask. After 3 days, the old culture solution was discarded, and the cells were thoroughly washed to remove the suspended cells. The cells were digested with 0.25% trypsin (containing 0.02% EDTA, 0.04 mL/cm²), resuspended, and counted in PBS. Fluorescence-labeled CD45-FITC (BioLegend, 1:100, United States), CD90-PE (BioLegend, 1:100, United States), CD44-PE (eBioscience, 1:100, United States), and CD29-FITC (BioLegend, 1:100, United States) monoclonal mouse antibodies were mixed, stored at 4°C in the dark for 30 min, and centrifuged at 1200 r/min for 5 min. The supernatant was discarded, and the excess antibodies were eluted three times. Isotype-identical antibodies were served as controls (BD Pharmingen). Detection was performed using the BD FACSCanto flow cytometry system (BD Corporation, United States).

Differentiation of Induced and Cultured PFCs Into Osteoblasts

The isolated P2 cells were seeded in a 24-well plate at a density of 1.2×10^6 /cm² and divided into six experimental groups ($n = 3$) and one control group ($n = 3$). When approximately 60–70% of the cells adhered to the other cells, the culture medium was replaced with bone culture medium (high-sugar DMEM containing 10% fetal bovine serum, 0.1 μmol of dexamethasone, 20 mmol/L of β-phosphate glycerol, and 50 μmol/L of ascorbic acid). The control group was cultured in complete medium. Cell morphology was regularly observed on an inverted microscope. After approximately 7 days, the protruding cells were retracted and connected tightly to form cell clusters. The experiment was repeated three times. The culture medium in each group was changed every 3 days. After 21 days of induction, the cells were fixed with paraformaldehyde for Alizarin red staining as previously reported (Zhang et al., 2008).

Differentiation of Induced and Cultured PFCs Into Adipocytes

The isolated P2 cells were seeded in a 24-well plate at a density of 1.2×10^6 /cm² and divided into six experimental groups ($n = 3$) and one control group ($n = 3$). When approximately 60–70% of the cells adhered to the other cells, the culture medium was replaced with an adipocyte-induction solution (low-sugar complete medium containing 1 μmol/L of dexamethasone, 0.01 g/L of insulin, 200 μmol/L of indomethacin, and 0.5 of mmol/L isobutyl methyl xanthine). The control group was cultured in complete medium, and the cells were cultured at 37°C in 5% CO₂ for 21 days, during which the solution was completely replaced every 3 days. Cell morphology was regularly observed on an inverted microscope. Some cells showed increased dendritic bifurcations after approximately 7 days. The experiment was repeated three times. Subsequently, the adipocytes were washed with PBS, fixed with paraformaldehyde for 20 min, stained with Oil Red O solution for 20 min at room temperature, rinsed twice with 60% isopropanol, and washed again with PBS, followed by observation under the microscope and image acquisition (Wei et al., 2019; Hou et al., 2020).

Multiple Differentiation of PFCs

The isolated P2 cells were seeded in a 24-well plate at a density of $1.2 \times 10^6/\text{cm}^2$ and divided into six experimental groups ($n = 3$) and one control group ($n = 3$). When approximately 60–70% of the cells adhered to the cell, the culture medium was replaced with myocardial culture medium (DMEM low-sugar medium containing 10% fetal bovine serum with 5 μmol of E61541, 5 μmol of phenylcyclopropylamine, 10 μmol of CHIR99021, 10 μmol of forskolin, 1 μmol of dorsomorphin, and 2 μmol of IWR-1). The control group was cultured in complete medium and co-cultured for 7 days. Cell morphology was observed on an inverted microscope every 3 days. After 3 days, some cells exhibited increased dendritic protrusions, and all cells showed concentric circles or clumps. Afterward, the culture medium was changed every 3 days. After 7 days of induction, cells were fixed with 4% paraformaldehyde for 20 min and then immersed in PBS three times for 5 min each time. Subsequently, 0.3% Triton X-100 was added to the permeable membrane for 20 min. The cells were immersed with PBS three times for 5 min each time and added with goat serum blocker (Bosterbio) for 60 min. The blocking solution was aspirated without washing, and primary anti-mouse polyclonal antibody cTnT (Abcam, 1:200) mix with/without Rabbit polyclonal to sarcomeric alpha actinin antibody (α -SA; Abcam, 1:500, United States) was added at 4°C overnight and used the next day. The primary antibody was washed with PBS three times. Afterward, secondary antibody Cy3/Alexa Fluor 488 goat anti-mouse/rabbit (Biyuntian, 1:500, United States) was added, and the mixture was incubated at room temperature for 2–4 h. Finally, nuclei were counter stained with DAPI.

Quantification of positive induced cells was performed by counting adipocytes (at least five lipid droplets observed in a cell), osteoblasts (at least larger than 5 μm calcified nodules), and cardiac-like cells (red and green) from nine randomly selected fields (three fields/well). Mean positive cell numbers were calculated for each group. ImageJ software was used to calculate cell numbers.

Statistical Analysis

All results presented are from at least three independent experiments for each condition. Data are expressed as scatter plots with mean $\pm$ standard deviation (SD). Statistical analysis was performed by one- or two-way ANOVA using GraphPad (PRISM software). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Morphological Characteristics of Rat Primary PFCs

The PFCs were attached to the surface of the plate and predominantly circular in shape with uniform size. After the PFCs were cultured for 24 h, they turned into spindle or star shaped. The nucleus was located at the center of the cell. During the growth process, the nucleus was gradually vortexed. After the cells were passed, they gradually shrank, and many

of them exhibited a polygonal angle (Figures 1A,B). They grew rapidly at the first, second, and third passages, reaching 100% confluence in 2–3 days. The cell morphology was uniform, mainly polygonal cells (fibroblast-like cells, Figure 1C), which is a typical morphology of PFCs (Figure 1D).

Flow Cytometry of Identified Surface Markers

Flow cytometry was used to assess the marker expression. FACS revealed that the PFCs of third passage were positive for some surface markers, which is a characteristic of other various stems/progenitor populations. As shown in Figure 2, these cells were CD90 and CD29 positive, markers for mesenchymal stem cells (MSCs) (Zhao et al., 2019), but negative of MSCs marker CD44. Moreover, this population cells were negative for pan-hematopoietic cell markers CD45, indicating that these cells were not hematopoietic derived cells and also different from MSCs. Immunofluorescence staining showed the same result with flow cytometry (Supplementary Figure 1).

Multipotent Differentiation of PFCs *in vitro*

To investigate the multilineage differentiation of PFCs, osteogenic, adipogenic, and cardiac differentiation were done. The accumulation of lipid vacuoles during adipogenic induction was observed in PFC cultures. Oil Red O staining showed intracellular lipid red staining (Figure 3A). In contrast to the negative control, all PFCs were grown in osteogenic medium, the number of cells was decreased, and the cell volume was gradually increased. Small nodules and granules were appeared in the cells, and floating matter was precipitated. Alizarin red staining showed red clumps or strip-shaped precipitates with obvious calcified nodules (Figure 3B). To determine the cardiac differentiation of PFCs, PFCs were cultured in cardiomyocyte differentiation medium and immunofluorescent staining confirmed cardiomyocyte marker cTnT expression after 7 days (Figure 3C). In the media without differentiation supplements, osteogenic, adipogenic, and cardiac differentiation was not detected (Supplementary Figure 2). Quantitative analysis of the multipotent differentiation of PFCs showed the percentage of differentiation toward adipocytes, osteoblasts, and cardiac-like cells (Figure 3D).

Alpha-sarcomeric actin (α -SA) proteins represent the structural building blocks of heart muscle (van der Velden and Stienen, 2019), which are essential for contraction and relaxation. Double staining revealed the α -SA expression in some cTnT positive cells (Figure 4). However, no beating cells were detected in the present study.

DISCUSSION

Hematologic and biochemical analyses of the pericardial fluids showed that the concentration of small molecules corresponded to a plasma ultrafiltrate (Buoro et al., 2020). LDH and protein levels are higher than plasma ultrafiltrate (Ben-Horin et al., 2005). The pericardial fluid could reflect the immediate changes in the

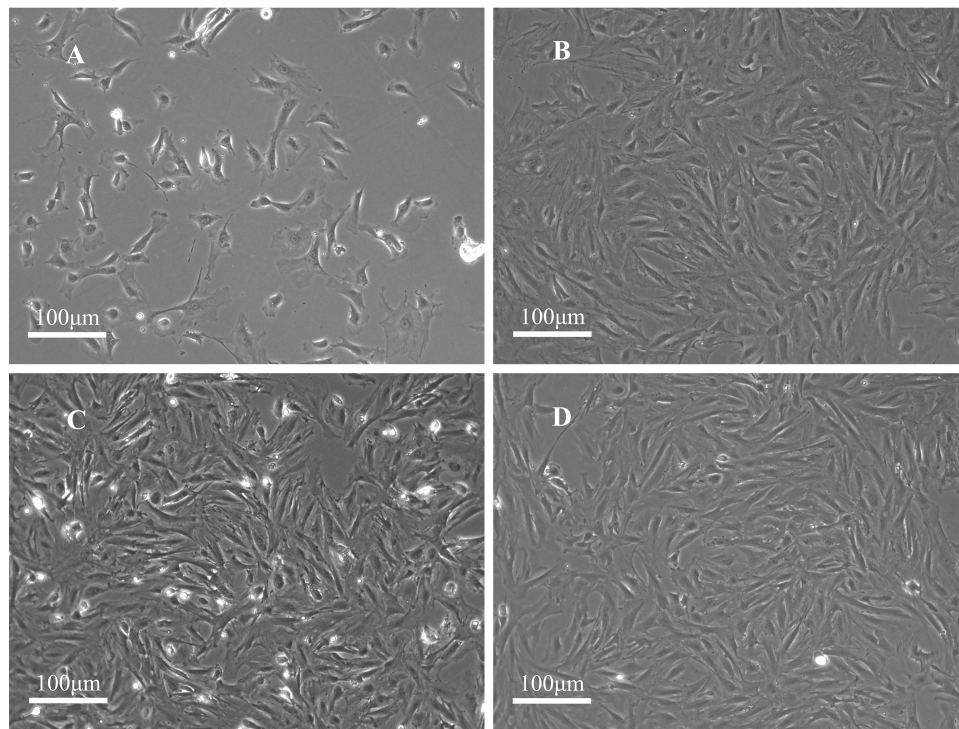
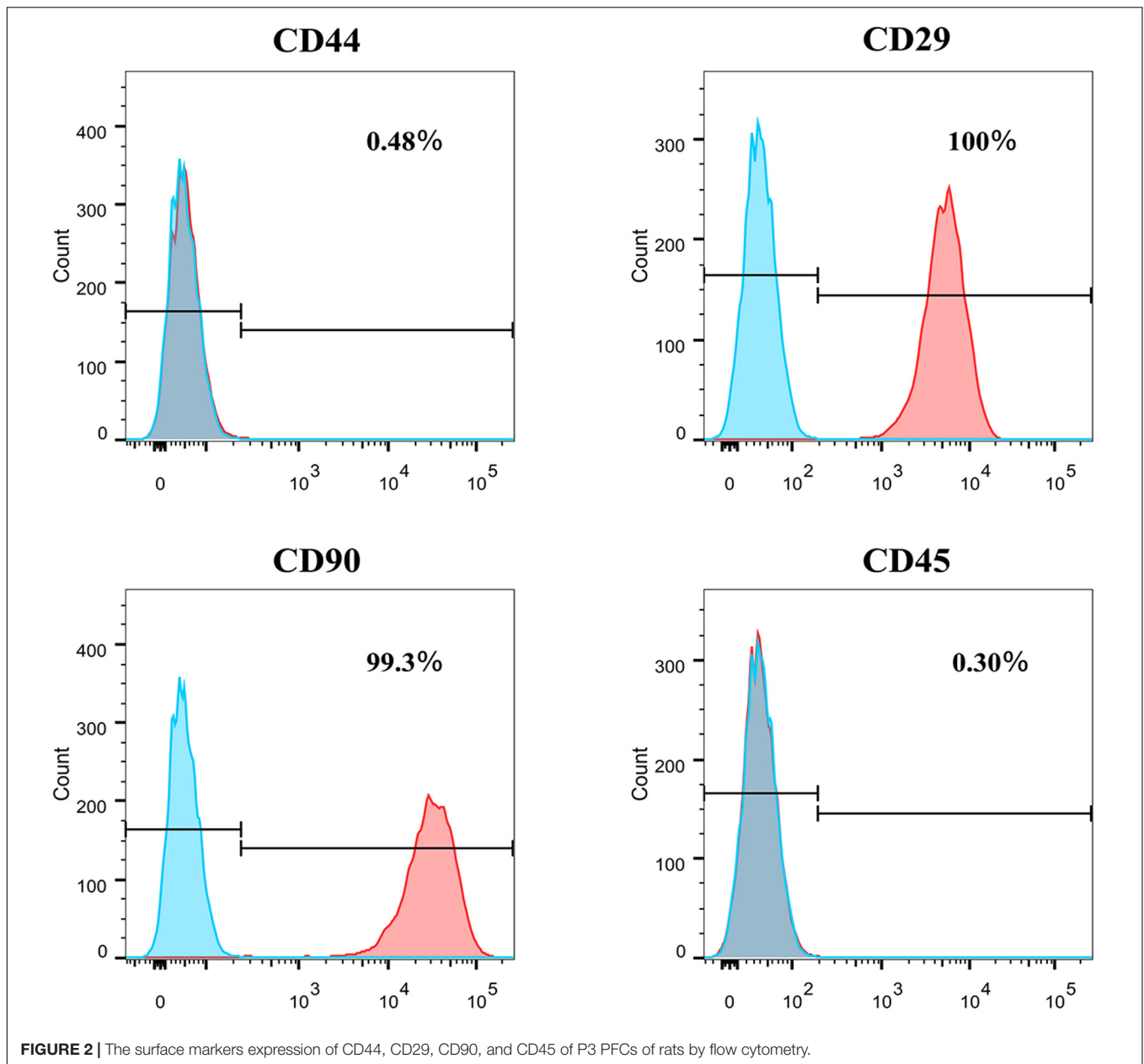


FIGURE 1 | Morphology of rat PFCs. **(A)** Primary PFCs were cultured for 1 day and circular-shaped cells of uniform size. **(B)** PFCs at the first passage were cultured for 3 days. **(C)** PFCs at the second passage were cultured for 3 days. **(D)** PFCs at the third passage were cultured for 3 days. PFCs were spindle-shaped (fibroblast-like cells) in first, second, and third passages after cultured for 3 days. Scale bar: 100 μ m.

molecular environment around the myocardium after ischemia; it could be used as an important clinical research object (Fujita et al., 2001). In a recent study, the plasma exosomes of pericardial fluid were digested with proteinase K and RNase to prove that they contain at least 16 miRNAs. Pericardial fluid exosomes can improve the survival, proliferation, and angiogenesis of endothelial cells cultured *in vitro* (Beltrami et al., 2017). Soluble mediators and regulatory factors can be released in the pericardial fluid, thereby inducing the growth of coronary artery blood vessel after myocardial infarction (Limana et al., 2007). After myocardial infarction, the exosomes in the pericardial fluid can induce endothelial–mesenchymal transition of the epicardium, enhance arteriogenesis, and reduce cardiomyocyte apoptosis. Pericardial fluid could also be used as a signal to evaluate cardiac pathological changes (Xiang et al., 2013; Foglio et al., 2015). A large amount of T cell infiltration and increased cytokines could be observed in the pericardial fluid of patients with chronic heart failure, leading to changes in the myocardial structural matrix and cause myocardial cell apoptosis that results in inflammation, which reflects T cell activation (Iskandar et al., 2017). The tissue origins of PFCs remain unclear. However, these findings suggested that pericardial fluid serves as a lubricating agent during the cardiac cycle and has many known and unknown physiological effects.

The majority of body fluids contain stem cells, and these cells are important for tissue regeneration and repair (Jones et al., 2004; Zhang et al., 2008; Ji et al., 2017). This observation serves

as a reminder whether there are cellular components similar to stem cells in pericardial fluid. In the present study, the flow cytometry results showed that almost all cells expressed CD29 (100%) and CD90 (99.3%), proving that rat PFCs are MSCs like stem cells. Some cells in the rat pericardial fluid could differentiate into adipogenesis, osteogenesis, and cardiomyocytes via induction, thereby reflecting the characteristics of these cells as stem cells. This study was the first to prove that the PFCs from pericardial fluid contain stem cells that are capable of proliferation *in vitro*, and their multipotentiality was investigated. These stem-cell characteristics of PFCs may play a role in the regeneration of myocardial or pericardial tissue; they may also participate in the formation of pericardial fluid components through their autocrine and paracrine effects, which in turn affect the metabolism of surrounding tissues. The CD90 molecule is a cell surface glycoprotein anchored by glycosphosphatidylinositol. CD90 has a single V-like immunoglobulin domain and is considered to be a surrogate marker for stem cells, such as hematopoietic stem cells (HSCs). CD90 is often used in combination with CD29, CD44, and CD45 to label stem cells. It is expressed in the human body in neurons and HSCs. It is also considered to be the main marker of HSC pluripotency consistent with CD34 (Craig et al., 1993; Mestas and Hughes, 2004; Majeti et al., 2007; Boitano et al., 2010). In addition, CD90 is a marker of other types of stem cells, such as MSCs, liver stem cells (Masson et al., 2006), epidermal stem cells (Nakamura et al., 2006), and endometrial progenitor/stem cells



(Gargett, 2006). The present study demonstrated that rat PFCs highly expressed CD90, indicating that these cells were likely to be stem cells. CD29 is a cell surface receptor involved in cell adhesion and recognition in various processes, including embryogenesis, hemostasis, tissue repair, immune response, and metastatic spread of tumor cells (Burrige and Chrzanowska-Wodnicka, 1996). The PFCs isolated and extracted in the present study highly expressed CD29, suggesting that integrin may act as a bidirectional transmission signal between the extracellular matrix and the cytoplasmic domain and participate in the metabolism of macromolecules in the pericardial fluid. CD44 is a receptor for hyaluronic acid, a cellular protein mainly expressed in immune cells and expressed in blood, epithelial, and endothelial cells and cartilages (Patouraux et al., 2017). The CD45

molecule is expressed in all leukocytes and is called leukocyte common antigen. It is a type of transmembrane protein with a similar structure and large molecular weight. It is important for maturation, function regulation, and signal transmission. It is generally used as a marker for bone marrow cells. In the present study, the expression of CD45 in PFCs was negative, suggesting that these cells were not derived from bone marrow.

The source of PFCs is unclear. PFCs may be derived from mesothelial cells of the pericardium or from tissues other than the pericardium passing through the mesothelial cells into the pericardial fluid. We proposed an alternative cell source and the biological meaning of these cells. Our results demonstrated that PFCs express the multipotency markers CD29 CD90. After induced, PFCs has higher osteogenic but lower adipogenic

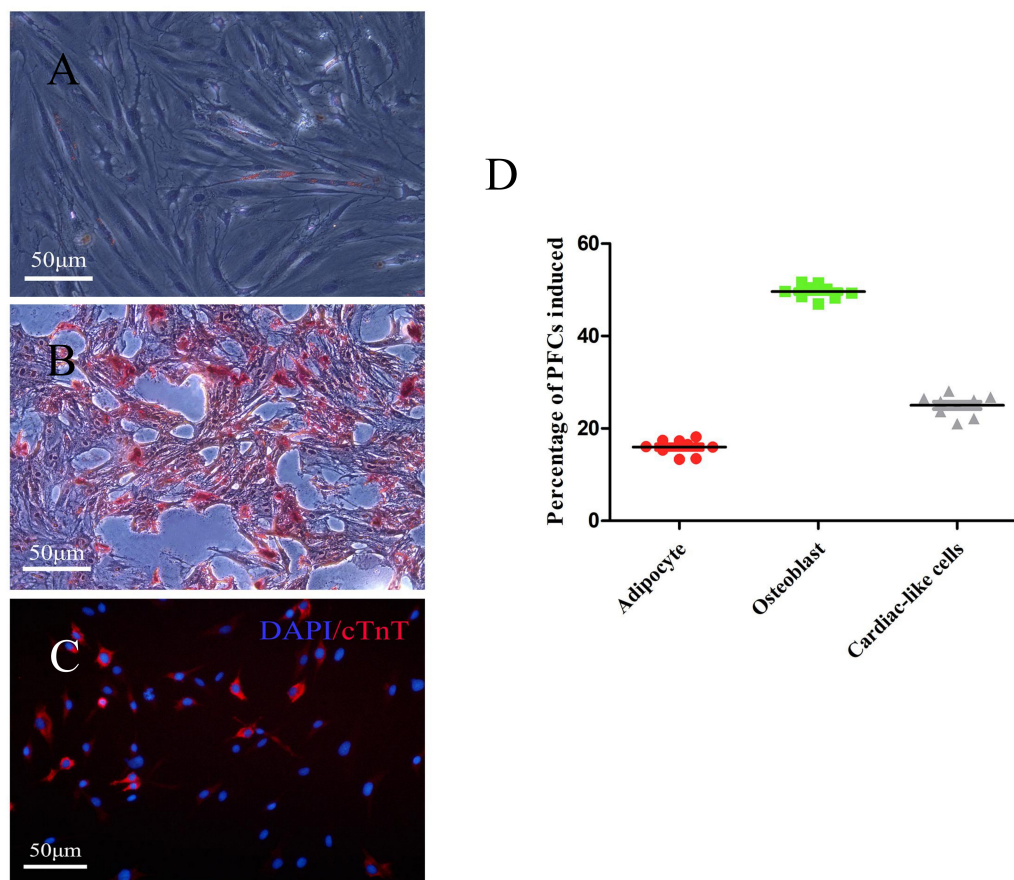


FIGURE 3 | Representative images showing differentiation of PFCs. **(A)** Adipogenic differentiation with oil-red O staining. **(B)** Osteogenic differentiation with Alizarin red staining. **(C)** Cardiac differentiation with cTnT staining. Scale bar: 50 μm. **(D)** The relative percentage of PFCs induced is shown as a scatter plot. Lines in scatter plot represent individual data points with indicated mean ± SD.

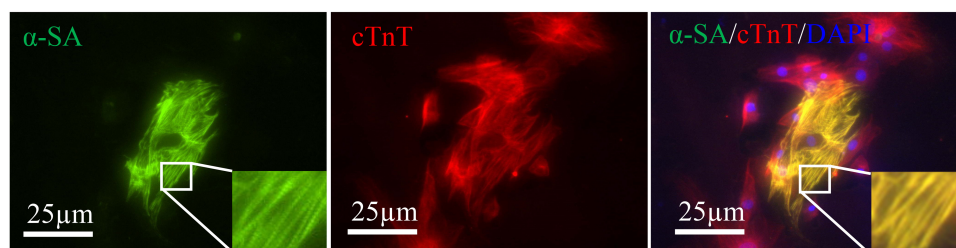


FIGURE 4 | Induced cardiac-like cells expressed cTnT (red) together with α-SA (green). Sarcomeres are magnified and highlighted in rectangle. Scale bar: 25 μm.

potential. Meanwhile, in the process of inducing cardiac-like cells *in vitro*, pericardial fluid-derived cells apparently lacked contractile function *in vitro*. Cellular fluid is a stable biological fluid with low clearance rate and a reservoir of biologically active substances that regulate heart function. Therefore, it should have a certain importance on the diagnosis, prognosis, and treatment of heart or pericardial diseases. However, due to the relatively difficult collection of cell fluids and the ethical limitations of samples from healthy individuals, routine research and studies like those of other biological fluids (e.g., serum, plasma, saliva,

urine, and joint fluid) have not been performed. Examination may be one of the reasons behind the low number of researches on the cellular composition of pericardial fluid.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Xinxiang Medical University.

AUTHOR CONTRIBUTIONS

YS, ZG, and ZL conceived and designed the study. YS and YW performed the experiments. YS wrote the manuscript. ZG, ZL, and YW reviewed and edited the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.614826/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Adiponectin Modified BMSCs Alleviate Heart Fibrosis via Inhibition TGF-beta1/Smad in Diabetic Rats

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Background: Accumulating evidence suggested that bone marrow mesenchymal stem cells (BMSCs) have therapeutic potential for diabetes and heart diseases. However, the effects of BMSC on reducing myocardial fibrosis need to be optimized. This study aimed to investigate the mechanism of adiponectin (APN) modified BMSCs on myocardial fibrosis in diabetic model *in vivo* and *in vitro*.

Methods: The high-fat diet combined with streptozotocin (STZ) injection were used to induced diabetic rat model. H9c2 cells were cultured under a high glucose medium as *in vitro* model. The BMSCs were modified by APN plasmid or APN small interfering RNA (siRNA), then transplanted to the diabetic rats by a single tail-vein injection, or co-cultured with H9c2 cells.

Results: We demonstrated that diabetic rats showed typical diabetic symptoms, such as decreased cardiac function, accumulation of pathological lesions and collagen expression. However, these impairments were significantly prevented by the APN modified BMSCs treatment while no effects on APN siRNA modified BMSCs treated diabetic rats. Moreover, we confirmed that APN modified BMSCs could attenuate the expression of TGF-beta1/smad to suppress the myocardial fibrosis in the diabetic rats and high glucose induced H9c2 cells.

Conclusion: The present results for the first time showed that APN modified BMSCs exerted protection on cardiac fibrosis via inhibiting TGF-beta1/smad signal pathway in diabetic rats. Our findings suggested that APN modified BMSCs might be a novel and optimal therapy for the diabetic cardiomyopathy in future.

Keywords: diabetic cardiomyopathy, adiponectin, bone marrow mesenchymal stem cells, TGF-beta1, myocardial fibrosis

Abbreviations: APN, adiponectin; BMSCs, bone marrow mesenchymal stem cells; DCM, diabetic cardiomyopathy; DMEM, Dulbecco's modified Eagle's medium; FS, fractional shortening; GFP, green fluorescent protein; HE, hematoxylin and eosin; LV, lentivirus vector; LVIDD, left ventricular internal diameter end diastole; LVIDs, left ventricular internal diameter end systole; LVEF, left ventricular ejection fraction; MOI, multiplicity of infection; SD, Sprague-Dawley; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; siRNA, small interfering RNA; STZ, streptozotocin; TGF- β , transforming growth factor-beta.

INTRODUCTION

Diabetes mellitus and its complications become a dominant public health problem (American Diabetes Association, 2012). Clinical cases suggested that heart failure incidence was significantly higher in patients with diabetes (Cavender et al., 2015). The studies revealed that hyperglycemia could directly injure cardiomyocytes leading to diabetic cardiomyopathy (DCM) (Seferovic and Paulus, 2015; Jia et al., 2018b). DCM is one of diabetic complications that causes heart failure in patients with diabetes (Wang et al., 2018). The pathogenesis of DCM is complex, and cardiomyocyte fibrosis is the main distinct pathological features of DCM (Chang et al., 2016; Faramoushi et al., 2016). Biopsies from diabetic patients with heart failure indicated that cardiac fibrosis might play a vital role in the DCM (Falcao-Pires et al., 2011). Cardiac fibrosis contributes to cardiac remodeling and finally leads to the progression of heart failure (Jia et al., 2018a). The transforming growth factor-beta (TGF- β) superfamily serves multiple roles in cell differentiation, proliferation and fibrosis. The TGF- β s have three isoforms (β 1, β 2, and β 3) in mammals (Travis and Sheppard, 2014), and TGF- β 1 is implicated in fibrosis (Oruqaj et al., 2015). TGF- β 1 over-expressed in myocardium induced stable hypertrophy to heart failure. Upregulation of TGF- β 1 was related to fibrosis in diabetic heart (Koitabashi et al., 2011). High glucose could increase TGF- β 1 activity and downstream canonical Smad signaling that increased interstitial fibrosis and cellular hypertrophy in the heart of diabetic rats (Bugyei-Twum et al., 2014). Moreover, TGF- β 1/Smad may play an important role in fibrogenesis of DCM (Shen et al., 2014).

Bone marrow mesenchymal stem cells (BMSCs) are multipotent stem cells and emerged protection through paracrine related factors and immunomodulatory to host cells (Jing et al., 2014). The scientists found that BMSCs could improve cardiac functions against DCM (Li et al., 2008). However, the effects of BMSCs on reducing cardiac fibrosis still need to be optimized. Interestingly, the adiponectin (APN) that secreted by adipocytes had anti-insulin resistance, anti-atherosclerotic, and anti-inflammatory effects (Wang et al., 2013). Furthermore, APN-knockout mice could enhance carbon tetrachloride-induced liver fibrosis (Nishihara et al., 2006) and renal fibrosis (Tian et al., 2018), while increasing APN expression attenuated liver and renal fibrosis. Therefore, we hypothesized a novel therapeutic effect of the APN modified BMSCs on DCM. The present study aimed to investigate the effects and possible signal pathways involved in APN modified BMSCs on cardiac structural and functional improvement of diabetic models *in vivo* and *in vitro*.

MATERIALS AND METHODS

Ethics Statement

The animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use and approved by the Committee of Animal Experiment Center of Zhejiang

University (Hangzhou, China). The best efforts have been made to minimize the number and the suffering of the rats.

Primary BMSCs Culture and Characterization

As our previous study reported (Wang et al., 2016), the primary BMSCs were harvested from the femurs of 2-week-old Sprague-Dawley (SD) female rats that purchased from Shanghai laboratory animal center (Shanghai, China). The rats were anesthetized with sodium pentobarbital (40 mg/kg). The bone marrow cavity was flushed and then cultured in Dulbecco's modified Eagle's medium (DMEM, Catalog: 11885-084, Gibco, Invitrogen, United States) with 10% (V/V) fetal bovine serum (Catalog: 10099-141, Gibco, Invitrogen, United States) and a mixture of 1% penicillin and streptomycin (Catalog: 10378016, Invitrogen, United States) and maintained in a tri-gas incubator (Thermo Fisher Scientific, Marietta, OH, United States) composed of 5% CO₂ and 95% air at 37°C and changed the fresh medium every 2 days.

Lentivirus Vector Construction and Infection of BMSCs

Recombinant lentivirus vector (LV) containing green fluorescent protein (GFP) as LV5-APN-GFP and LV3-APN-small interfering RNA (siRNA)-GFP, they were designed and constructed by Shanghai GenePharma Technology Co., Ltd. (Shanghai, China), and LV3-vehicle-GFP virus that expresses GFP only was also constructed as a control (Sanber et al., 2015). The target gene APN (Gene Bank accession NM_031012.1) fragment was inserted into the *NotI/BamHI* site on the LV5 vector. The target siRNA against rat APN gene was designed as following: 5'-GGATCTCGTTGTTGGGCTTTA-3'. The lentivirus was determined to be 1×10^8 PFU/ml by using a titration kit, and the lentivirus vector was stored at -80°C until use. BMSCs (5×10^5 cells) were seeded into the 6-well plate, incubated overnight, and then transfected with LV3-GFP, LV5-APN-GFP, and LV3-APN-siRNA-GFP for 72 h as BMSCs/Vehicle, BMSCs/APN+, and BMSCs/APN- separately (Sanber et al., 2015). The multiplicity of infection (MOI) was 100. Transduction efficiency of APN was examined after 48 h by fluorescence microscope (BX-53, Olympus Corp., Tokyo, Japan). The expression of APN was detected by western blot analysis.

Animal Models Induction

Male SD rats were obtained from Shanghai laboratory animal center (Shanghai, China). The rats were divided into six groups at random: the control group, the DM (diabetic rat) group, the BMSCs/Vehicle group, the BMSCs/APN+ group, and the BMSCs/APN- group ($n = 10$). After 18 h of fast, the rats were given a single intraperitoneal injection of freshly prepared streptozotocin (STZ; 65 mg/kg body weight) (Catalog: S8050, Solarbio, Beijing, China) dissolved in 0.01 M citrate buffer (pH 4.5) for diabetes modeling, while the control rats were intraperitoneally injected with equal amounts of citrate buffer (pH 4.5). A total of 72 h after injection, the fasting blood glucose was determined using an analyzer

(Accu-Chek®, Roche, Mannheim, Germany). Rats with blood glucose levels above 16.7 mmol/L were considered successful diabetic models for further study (Hasegawa et al., 2010). One week after STZ injection, the 200 µl PBS including 4×10^6 BMSCs, BMSCs/Vehicle, BMSCs/APN+, and BMSCs/APN– were injected into the diabetic rat by a single tail-vein injection as the BMSCs group, the BMSCs/Vehicle group, the BMSCs/APN+ group, and the BMSCs/APN– group, respectively.

Echocardiography Assay for the Cardiac Function and Metabolic Indexes Analysis of All Groups Rats

Six weeks after transplantation, the cardiac function was estimated through a M9 echocardiography machine (Mindray, Shenzhen, China). We anesthetized all rats with an intraperitoneal sodium pentobarbital (40 mg/kg) and removed the chest hair by a pet razor. A 30-MHz probe was then used to carry out the exam (Scherrer-Crosbie et al., 1998). The left ventricular internal diameter end diastole (LVIDD), left ventricular internal diameter end systole (LVIDS), left ventricular ejection fraction (LVEF), and fractional shortening (FS) were measured by an echocardiography doctor who blinded to all groups. After echocardiography assay, the body weight and fasting blood glucose were recorded on the basis of fasting for 8 h. All rats were anesthetized with an intraperitoneal sodium pentobarbital (40 mg/kg). We removed all rat's hearts, and recorded heart weight, then for subsequent analysis.

Masson, Hematoxylin and Eosin and Immunohistochemistry Staining

For pathological analysis, the heart sections were respectively subjected to Masson and hematoxylin and eosin (HE) staining (Wang et al., 2014). The hearts of five animals from each group were removed and fixed in 4% paraformaldehyde, then embedded in paraffin and sliced into sections (8 µm thick). We used HE (Catalog: G1005, Solarbio, Beijing, China), Masson's trichrome staining (Catalog: G1006, Servicebio, Wuhan, China), and immunohistochemistry staining to determine the left ventricular wall thickness, the collagen area, and the cross-sectional area of the cardiomyocytes. All images of the sections were collected by a light microscope (BX-53, Olympus Corp., Tokyo, Japan), and analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, MD, United States) for positive area measurement of whole vision field in each figure under 400× microscope to analyze the positive area of Masson's trichrome staining that relative to whole myocardium area (%).

Expression and localization of the target protein were observed by immunohistochemistry methods. The serial heart sections were incubated with primary antibodies overnight at 4°C, including TGF-β1 (Catalog: GB11179, 1:100, Boster Bio, Wuhan, China); Smad2 (Catalog: BA4557, 1:100, Boster Bio, Wuhan, China); Smad3 (Catalog: BA4559, 1:100, Boster Bio, Wuhan, China); Collagen I (Catalog: BA0325, 1:100, Boster Bio, Wuhan, China), and Collagen III (Catalog: BA0326, 1:100, Boster Bio, Wuhan, China). Sections were washing with PBS

and subsequently incubated with biotinylated and affinity-purified IgG secondary antibodies at room temperature for 4 h. Images were visualized by a light microscope (BX-53, Olympus Corp., Tokyo, Japan) and analyzed by Image-Pro Plus 6.0 (Media Cybernetics, Maryland, United States) for positive area measurement of whole vision field in each figure under 400× microscope to analyze the positive area of collagen I and collagen III Immunostaining that relative to whole myocardium area (%).

H9c2 Co-cultured With Modified BMSCs Under High Glucose Medium

Rat cardiomyocyte cell line H9c2 cells were cultured in DMEM medium (Catalog: 11885-084, Gibco, Invitrogen, United States) with 10% (V/V) fetal bovine serum (Catalog: 10099-141, Gibco, Invitrogen, United States), and supplement with 25 mM glucose (control group) or with 50 mM high glucose (DM group), at a humidified atmosphere containing 5% CO₂ and 95% air. The above BMSCs (5×10^5 /well) were seeded in the insert chamber of 6-well 0.4 µm transwell system, then co-cultured with 5×10^5 /well H9c2 in DMEM medium with 50 mM high glucose as DM+ BMSCs group, DM+ BMSCs/Vehicle group, DM+ BMSCs/APN+ group, and DM+BMSCs/APN– group, respectively. After 7 d, H9c2 cells were washed twice with PBS, lysed in Western and IP buffer (Catalog: P0013, Beyotime Institute of Biotechnology, Shanghai, China), then stored at –80°C for western blot analysis.

Western Blot Analysis

Proteins expression of rat's hearts and co-cultured cells were analyzed by western blot. The BCA protein concentration assay kit was used for protein quantification. Equal amounts of protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred from the gel to nitrocellulose membrane, blocked for 1 h with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature. The membranes were incubated overnight at 4°C with the primary antibodies TGF-β1 antibody (Catalog: 3711, 1:1000); Smad2 (Catalog: 5339, 1:1000); phospho-Smad2 (Catalog: 3108, 1:1000); Smad3 (Catalog: 9523, 1:1000), and phospho-Smad3 (Catalog: 9520, 1:1000) purchased from Cell Signaling Technology (Danvers, MA, United States). Actin (Catalog: A2228, 1:5000) was purchased from Sigma-Aldrich (Merck, KGaA, Darmstadt, Germany). After incubation for 16 h, the membranes were washed with TBST and incubated with infrared labeled secondary antibody for 1 h at room temperature and washed three times with TBST. Immunoblotted bands were analyzed by CLx Odyssey infrared imaging system (Li-COR biosciences, United States).

Statistical Analysis

Values were expressed as mean ± standard deviation (SD). All parameters were statistically performed by one-way analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant.

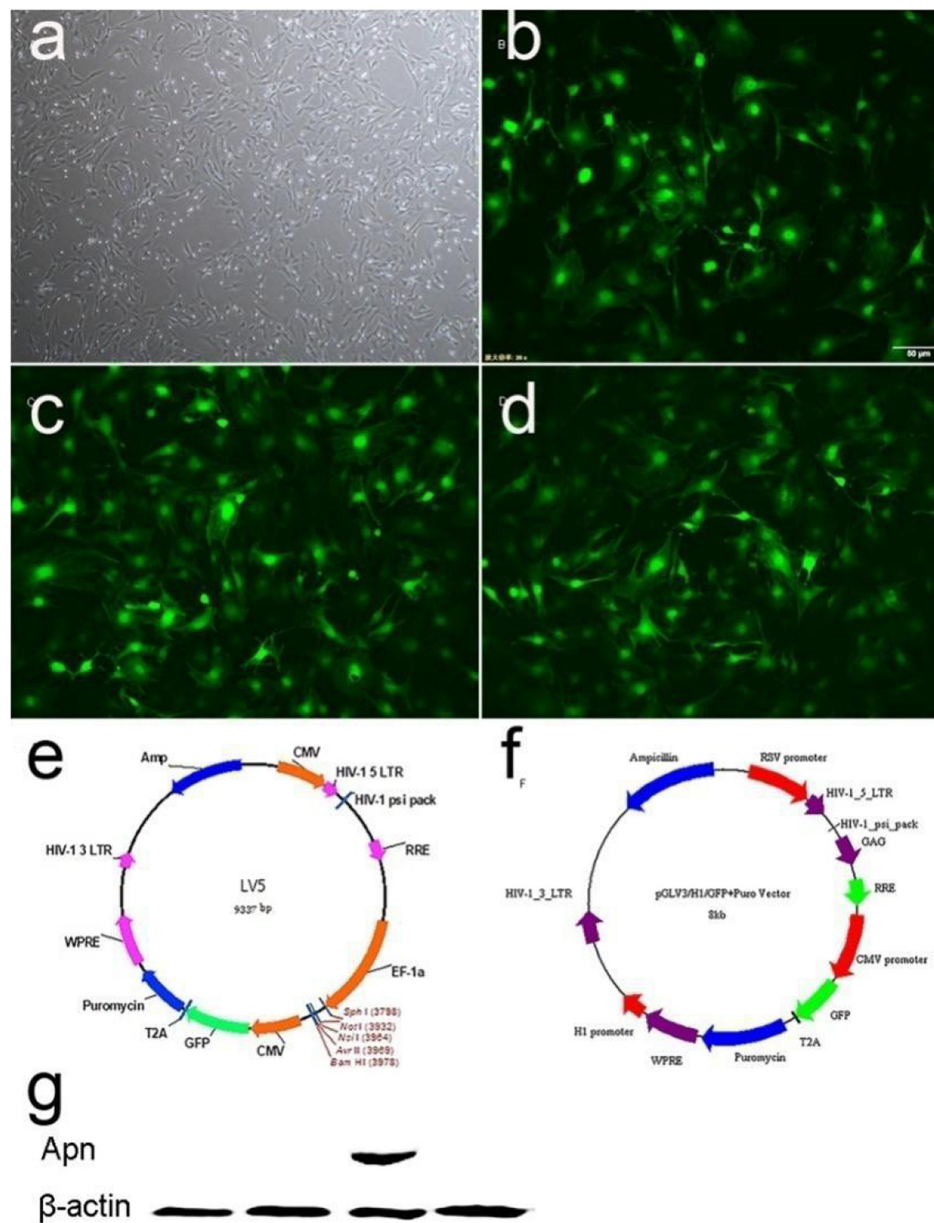


FIGURE 1 | The APN plasmid construction and expression. **(a)** Morphology characterization of cultured BMSCs (light microscopy). **(b)** GFP expression in BMSCs 48 h after transduction of empty vector into BMSCs. **(c)** GFP expression in BMSCs 48 h after transduction of APN into BMSCs. **(d)** GFP expression in BMSCs 48 h after transduction of APN-siRNA into BMSCs. **(e)** Maps of LV5. **(f)** Maps of LV3. **(g)** Western blotting detection of APN protein expression.

RESULTS

The BMSCs Isolation and APN Modification

The primary BMSCs were isolated and identified as our previous study (Wang et al., 2016). The GFP was used to detect the transfection efficiency of the virus (Figures 1e,f). A total of 48 h after infection, BMSCs were infected by lentivirus with GFP, and normal morphology was showed in three groups by fluorescence microscope (BX-53, Olympus

Corp., Tokyo, Japan) (Figures 1a–d). The results indicated over 80% transduction efficiency in BMSCs. A total of 72 h after transfection, the expression of APN was significantly increased in the BMSCs/APN+ group, while almost no detectable levels in the BMSCs, the BMSCs/Vehicle, and the BMSCs/APN– group (Figure 1g).

The Metabolic Indexes in All Groups

Six weeks after treatment, the body weight of DM rats was lower than that of the control rats (Figure 2A, $p < 0.01$), which

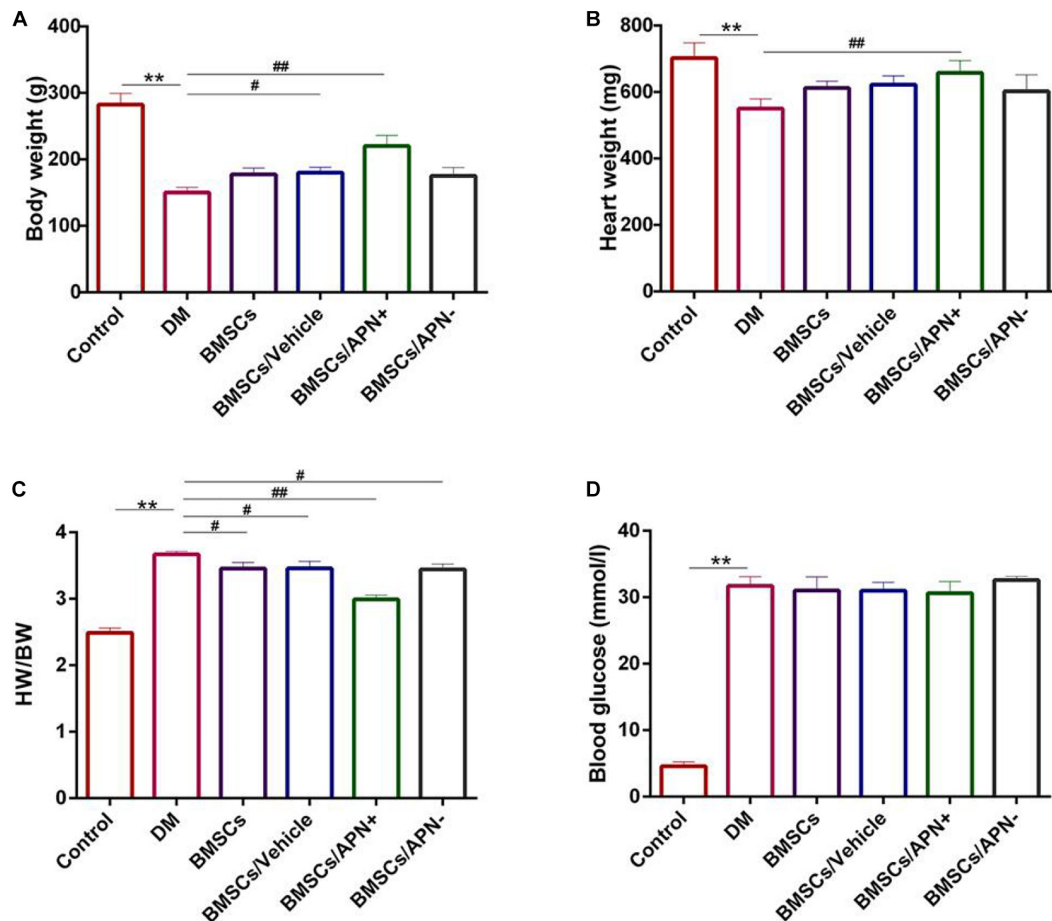


FIGURE 2 | Basic characteristics of experiment groups changes at 6 weeks. **(A)** Body weight (g). **(B)** Heart weight (mg). **(C)** Heart weight to body weight ratio (HW/BW). **(D)** Blood glucose (mmol/l). Data are mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, # $p < 0.05$, vs DM, ## $p < 0.01$, vs DM.

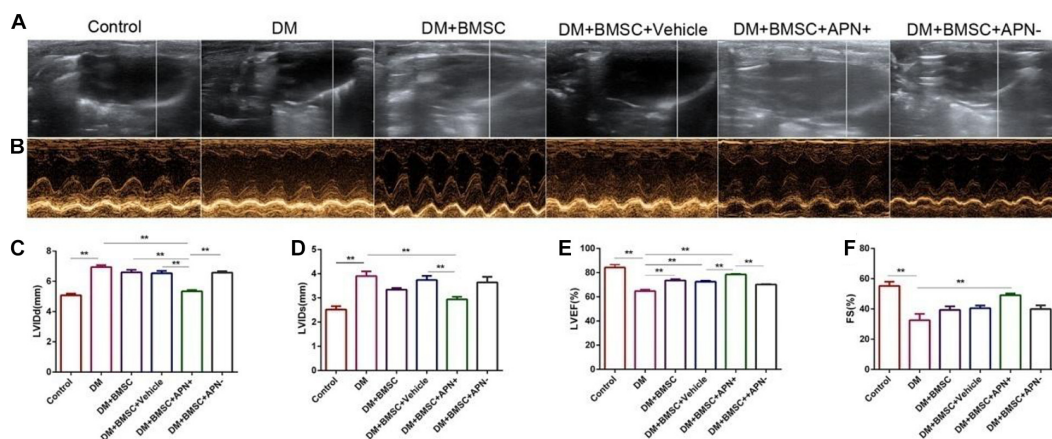


FIGURE 3 | Effects of APN on diabetes-induced LV dysfunction. **(A)** Two-dimensional echocardiograms. **(B)** M-mode echocardiograms. **(C)** Left ventricular internal diameter at end-diastole (LVIDd). **(D)** Left ventricular internal dimension systole (LVIDs). **(E)** LV ejection fraction (LVEF). **(F)** Fractional shortening (FS). Data are mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$.

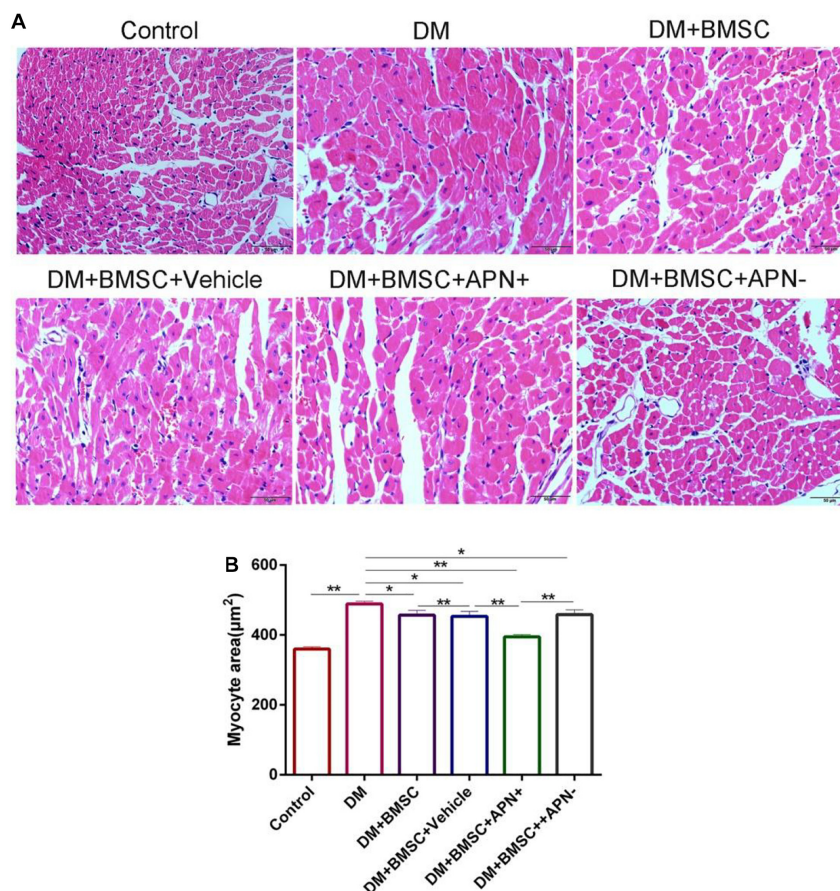


FIGURE 4 | APN attenuated pathological changes in the hearts of diabetic rats. **(A)** Representative micrographs of myocardial tissue sections stained with hematoxylin and eosin (scale bar: 50 μ m). **(B)** Quantitative analysis of myocyte size area. Data are mean $\pm$ SD; * p < 0.05, ** p < 0.01.

accompanied by the decrease of the heart weight (**Figure 2B**, p < 0.01). There is no remarkable differences in the BMSCs group, the BMSCs/Vehicle group and the BMSCs/APN– group. However, both of body weight and heart weight showed a significant increase in the BMSCs/APN+ group while comparing to the diabetic rats (**Figures 2A,B**, p < 0.01).

The ratio of heart weight to body weight (mg/g) was increased in the DM group and decreased in the BMSCs, the BMSCs/Vehicle, the BMSCs/APN– group, and BMSCs/APN+ group (**Figure 2C**, p < 0.01). After STZ injection, the blood glucose increased while compared to the control rats. There are no significant differences in the rest groups (**Figure 2D**, p > 0.05).

APN Modified BMSCs Attenuated Diabetes-Induced Cardiac Dysfunction

The cardiac structure and systolic/diastolic function in all rats were assessed by echocardiogram. Six weeks after treatment, the LVEF ratio was above 70, and the FS ratio was more than 40 in the normal rats. The DM rats exhibited a larger LVIDD/s diameter (p < 0.01, **Figures 3A–D**) and LVEF. However the FS ratio decreased (p < 0.01, **Figures 3A,E,F**) when compared to the control rats. BMSC/APN+ treatment dramatically improved

diameter in LVIDD, LVIDs, LVEF, and FS ratio (p < 0.01, **Figure 3**). There is no improvement of LVIDD and LVIDs in rats of BMSCs, BMSCs/Vehicle, and BMSCs/APN– group (**Figure 3**, p > 0.05). Moreover, the LVEF ratio of the BMSCs group and BMSCs/Vehicle group also improved significantly (**Figure 3**, p < 0.01) while the FS ratio of the BMSCs group, the BMSCs/Vehicle group, and the BMSCs/APN– group did not show significant alterations (**Figures 3A,F**, p > 0.05).

APN Modified BMSCs Improved Pathological Injury of Diabetic Heart

We analyzed the pathological changes of myocardial tissue to investigate the benefit of BMSCs/APN+ on diabetes-induced myocardial structural injury. The results demonstrated that diabetic heart exhibited cardiomyocyte hypertrophy, irregular myocardial arrangements, increased interstitial spaces, and myofibrillar discontinuation (**Figure 4A**). The normal control heart had regular myocardial structures and clear visible nuclei. Furthermore, BMSCs/APN+ group rats could improve these pathological changes. Cross-sections of left ventricular myocardial were examined at a magnification of 400 $\times$ (**Figure 4B**). The myocyte areas of LV were significantly

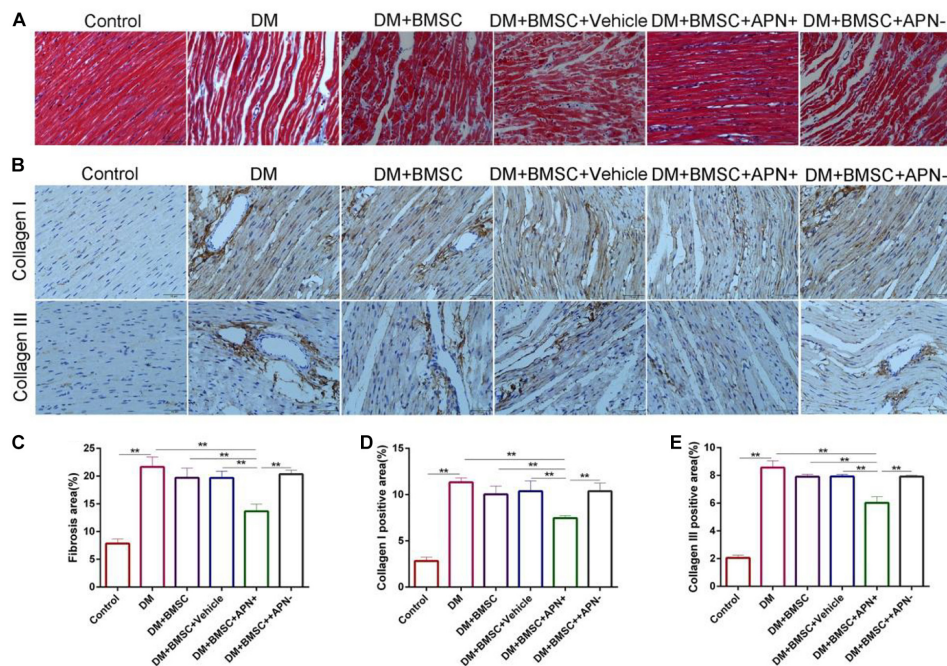


FIGURE 5 | Effect of APN on collagen deposition. **(A)** Representative Masson's trichrome staining. **(B)** Immunostaining of collagen I and collagen III. **(C)** Quantitative analysis of Masson's trichrome staining. **(D)** Immunohistochemistry analysis of collagen I. **(E)** Immunohistochemistry analysis of collagen III. Data are mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$.

increased in the DM rats, while compared with the control rats (Figure 4B, $p < 0.01$). The results indicated that transplanted BMSCs could reduce the increasing myocyte size, especially in the BMSCs/APN+ treatment groups (Figure 4C, $p < 0.01$).

APN Modified BMSCs Improved Diabetes-Induced Myocardial Fibrosis

Myocardial fibrosis is the major pathological lesion of DCM. Masson's trichrome staining of the heart showed certain irregularity of the myocardial fibrosis and increased collagen accumulation in the diabetes rats when compared with the normal heart. However, BMSCs/APN+ treated rats showed a remarked improvement for reduction collagen accumulation when compared to the diabetes rats (Figures 5A,C, $p < 0.01$). The collagen I and III, as cardiac fibrotic markers, were augmented under diabetic conditions and BMSCs/APN+ could reduce collagens I and III expression (Figure 5, $p < 0.01$). There is no significant differences of collagens I and III expression in the rest groups (Figure 5, $p > 0.05$). These results demonstrated that BMSCs/APN+ could attenuate myocardial fibrosis and improve the collagen deposition in diabetic hearts.

APN Modified BMSCs Alleviates Diabetes-Induced TGF- β 1/Smad 2/3 Signaling Pathway Activation

To verify the possible signaling pathway involved in myocardial fibrosis of diabetic heart, we analyzed the expression of TGF- β 1/Smad2/3 by immunohistochemistry. The results showed

their expression in the nucleus (Figure 6A) and the TGF- β 1/Smad2/3 protein increased in diabetic hearts (Figure 6, $p < 0.01$), while the BMSCs/APN+ treatment can reverse the these upregulation (Figure 6, $p < 0.01$). However, there is no detectable difference in the rest three groups (Figure 6, $p > 0.05$). The western blot analysis demonstrated that the TGF- β 1/Smad2/3 protein had significant reduction in the BMSCs/APN+ group (Figure 6, $p < 0.01$), and no marked alteration in the BMSCs group, the BMSCs/Vehicle group, and the BMSCs/APN- group as an assumption (Figure 6, $p > 0.05$). We also detected the expression of p-smad 2/3 protein, and found it phosphorylation was positively stressed in the BMSCs/APN+ diabetic rats (Figure 6, $p < 0.01$), but there is no significant improvement in the rest three groups (Figure 6, $p > 0.05$). These results indicated that BMSCs/APN+ could inhibit cellular TGF- β 1/Smad2/3 related signal pathways, which may alleviate diabetes-induced myocardial fibrosis in diabetic rats.

BMSCs/APN+ Co-cultured With H9c2 Inhibited High Glucose Induced TGF- β 1/Smad2/3 Activation

To clarify the role of BMSCs/APN+ treatment in collagen deposition *in vitro*, we cultured H9c2 in normal glucose (25 mM) as the control group and high glucose (50 mM) as the DM group. H9c2 in high glucose co-cultured with the BMSCs, the BMSCs/Vehicle, the BMSCs/APN+, and the BMSCs/APN- for 7 days, respectively. The western blot analysis showed

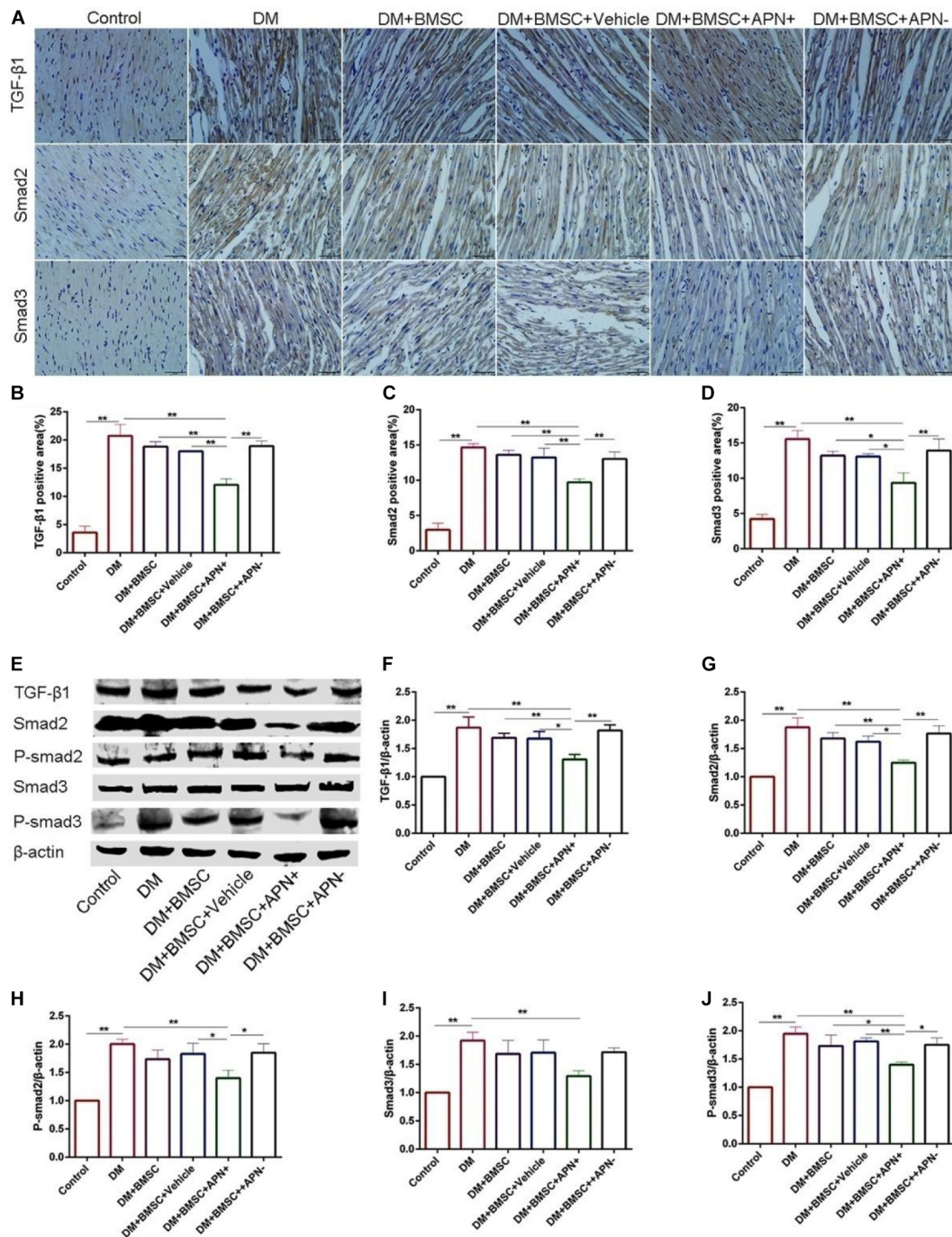


FIGURE 6 | The expression of TGF-β1/Smad 2,3 in all groups rats. **(A)** Immunostaining of TGF-β1, Smad2 and Smad3. **(B)** Quantitative analysis of TGF-β1. **(C)** Quantitative analysis of Smad2. **(D)** Quantitative analysis of Smad3. **(E)** Representative western blot: TGF-β1, Smad2, P-smad2, Smad3, and P-smad3. **(F)** The western blot assay of TGF-β1. **(G)** The western blot assay of Smad2. **(H)** The western blot assay of P-smad2. **(I)** The western blot assay of Smad3. **(J)** The western blot assay of P-smad3. Data are mean ± SD; * $p < 0.05$, ** $p < 0.01$.

that H9c2 cultured in high glucose significantly increased the TGF-β1 Smad2, P-smad2, Smad3, and P-smad3 when compared with the normal control group (Figure 7, $p < 0.01$). BMSCs/APN+ could inhibit the expression of TGF-β1/Smad2/3 (Figure 7, $p < 0.01$). There were no significantly altered in

the BMSCs, the BMSCs/Vehicle, and BMSCs/APN- treated H9c2 cells while compared with the high glucose cultured cells (Figure 7, $p > 0.05$). The present results demonstrated that BMSCs/APN+ could alleviate high glucose-induced TGF-β1/Smad2/3 activation.

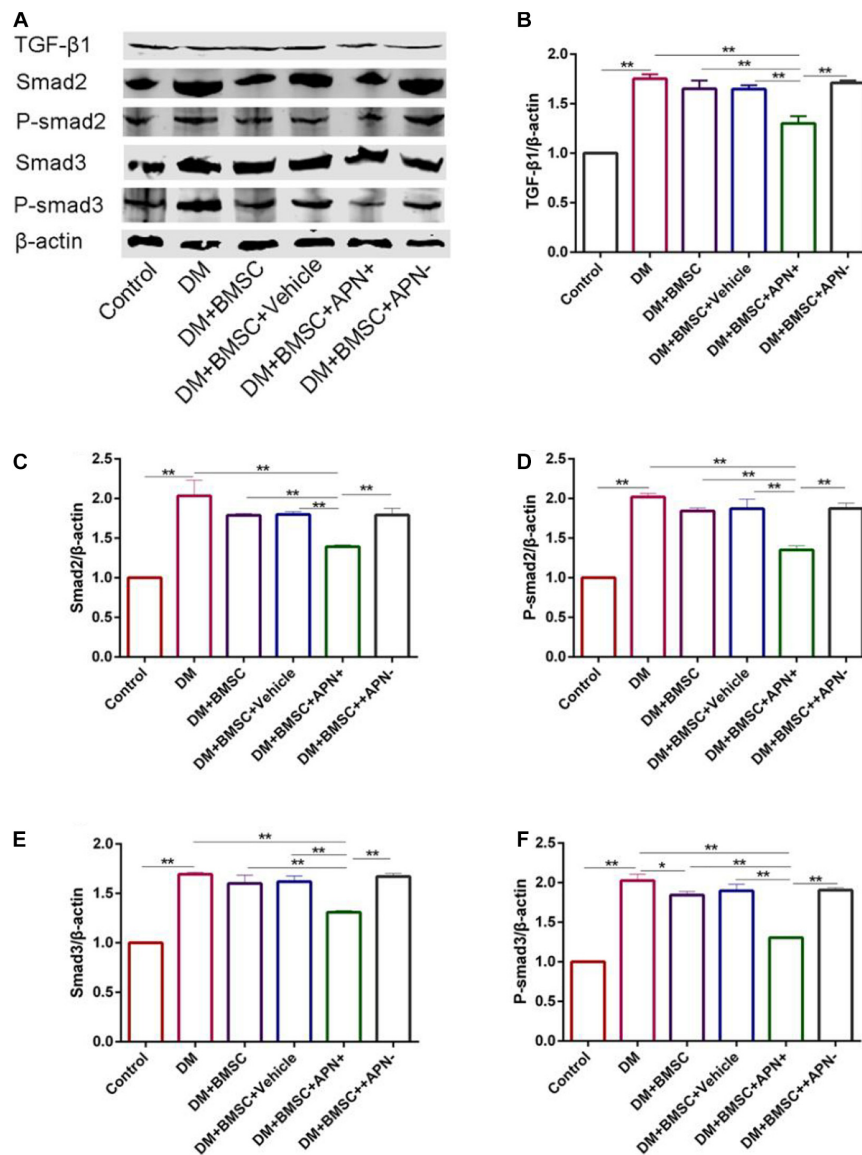


FIGURE 7 | The expression of TGF-β1/Smad 2,3 in high glucose cultured H9c2 cells. **(A)** Representative western blot: TGF-β1, Smad2, p-smad2, Smad3, and p-smad3. **(B)** The western blot assay of TGF-β1. **(C)** The western blot assay of Smad2. **(D)** The western blot assay of p-smad2. **(E)** The western blot assay of Smad3. **(F)** The western blot assay of p-smad3. Data are mean ± SD; **p* < 0.05, ***p* < 0.01.

DISCUSSION

Cardiac fibrosis is one of the most common pathological lesions of DCM, ultimately leading to heart failure (Huyhn et al., 2014). Therefore, reducing the excessive proliferation of collagen in the myocardium is necessary for preventing DCM and heart failure (Talior-Volodarsky et al., 2012). The present study for the first time suggested that APN modified BMSCs could improve fibrosis in diabetic heart *in vitro* and *in vivo*. During a 6-week follow-up period, we found a significant increase of collagen fibers and pathological changes in diabetic heart. The expression of collagen I and III also increased significantly in diabetic myocardium. The echocardiographic results showed that sustained increasing

collagen fibers in the myocardium might damage the cardiac function. Interestingly, a tail-vein injection of BMSC/APN+ caused significant improvement of body weight, cardiac function, collagen accumulation, and attenuated the pathological lesions in diabetic hearts.

Myocardial fibrosis is a chronic and progressive process characterized by an excessive accumulation of collagen fibers in diabetic hearts (Luo et al., 2013). Cardiac fibrosis contributes to cardiac remodeling, and finally leads to the progression of heart failure. Under diabetes conditions, myocardial fibrosis is regulated by various factors. The previous study revealed that TGF-β1 is the most important one (Sui et al., 2015). The studies of diabetic animals revealed that increased TGF-β1 was

associated with cardiac fibrosis. Moreover, TGF- β inhibition can attenuate cardiac fibrosis in animal models (Avila et al., 2014). The scientists found that high glucose could increase TGF- β 1 activity and downstream canonical Smad signaling that increased interstitial fibrosis and cellular hypertrophy in diabetic hearts (Shen et al., 2014). Activated TGF- β 1 binds to the TGF- β 1 receptor, it will allow the phosphorylation of the Smad 2/3 proteins. These Smads can be directly activated through phosphorylation by the TGF- β type I receptor kinase. Smad3 has been recognized as a key mediator of TGF- β -induced pro-fibrotic outcomes. Both Smad2 and Smad3 have been implicated in ECM production and tissue fibrosis (Yuan et al., 2013). Subsequently, these Smads complexes can be translocated into the nucleus and then regulate the transcription of the target genes, such as pro-fibrotic genes, that play crucial roles in the development of myocardial fibrosis (Zhang et al., 2014).

In the present study, the immunohistochemistry and western blot assay results demonstrated that the expression of TGF- β 1, Smad2/3, and p-Smad2/3 protein in the diabetic hearts tended to increase. Furthermore, BMSCs/APN+ treatment could decrease Smad2/3 phosphorylation, be consistent with the improvement of collagen formation and cardiac function while BMSCs/APN- have no effects on cardiac fibrosis, functional recovery in the diabetic hearts. Under the condition of long-term high glucose stimulation, the expression of TGF- β 1 in cardiomyocytes increases, which may induce fibroblasts to synthesize a large amount of collagen, promote fibroblast proliferation, and transform into myofibroblasts (Zhang et al., 2014). The APN modified BMSCs may reduce myofibrillar disorder and inhibit the proliferation and transformation of cardiac fibroblasts, as well as the interaction between fibroblasts and cardiomyocytes via TGF- β 1/Smad signal pathway.

H9c2 cell line showed an undifferentiated phenotype, which was similar to the physiological characteristics of normal cardiomyocytes in morphology, biochemistry and other characteristics, and was easy to obtain, passable and stable, which makes up for the poor repeatability of primary cardiomyocytes. H9c2 cardiomyocytes also have some limitations when used in isolated heart models. The present data demonstrated that high glucose increased expression of TGF- β 1 and p-Smad2/3 in H9c2 cells while co-cultured with the BMSCs/APN+ could reduce the expression of TGF- β 1 and p-Smad2/3. These results were consistent with the animal studies.

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CONCLUSION

In summary, the present data demonstrate a protective role of BMSCs/APN+ on diabetic hearts. Interestingly, high glucose increased TGF- β 1, p-Smad2/3, and collagen fibers deposition while BMSCs/APN+ could suppress these trend and improve functional recovery in the hearts of diabetic rats. Therefore, the present study provided a novel strategy for the treatment of DCM in patients with diabetes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee of Animal Experiment Center of Zhejiang University (Hangzhou, China).

AUTHOR CONTRIBUTIONS

KM performed the cells culture and animal model. KM and HC contributed to writing the manuscript and the data analysis. SC assisted with the experiments. YH and XZ conceived the study and contributed to the manuscript preparation. All authors read and approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Targeting of CAT and VCAM1 as Novel Therapeutic Targets for DMD Cardiomyopathy

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Duchenne muscular dystrophy (DMD) related cardiomyopathy is the leading cause of early mortality in DMD patients. There is an urgent need to gain a better understanding of the disease molecular pathogenesis and develop effective therapies to prevent the onset of heart failure. In the present study, we used DMD human induced pluripotent stem cells (DMD-hiPSCs) derived cardiomyocytes (CMs) as a platform to explore the active compounds in commonly used Chinese herbal medicine (CHM) herbs. Single CHM herb (DaH, ZK, and CQZ) reduced cell beating rate, decreased cellular ROS accumulation, and improved structure of DMD hiPSC-CMs. Cross-comparison of transcriptomic profiling data and active compound library identified nine active chemicals targeting ROS neutralizing Catalase (CAT) and structural protein vascular cell adhesion molecule 1 (VCAM1). Treatment with Quecetin, Kaempferol, and Vitamin C, targeting CAT, conferred ROS protection and improved contraction; treatment with Hesperidin and Allicin, targeting VCAM1, induced structure enhancement via induction of focal adhesion. Lastly, overexpression of CAT or VCAM1 in DMD hiPSC-CMs reconstituted efficacious effects and conferred increase in cardiomyocyte function. Together, our results provide a new insight in treating DMD cardiomyopathy via targeting of CAT and VCAM1, and serves as an example of translating Bed to Bench back to Bed using a multi-omics approach.

Keywords: DMD cardiomyopathy, CAT, VCAM1, CHM herbs, natural compounds, hiPSC-CMs

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked genetic disease affecting ~1:3500 to 1:5000 males worldwide, caused by loss of dystrophin expression (Lapidos et al., 2004; Verhaart and Aartsma-Rus, 2019). Dystrophin, a protein that provides anchorage of intracellular cytoskeleton to extracellular matrix via formation of the dystrophin glycoprotein complex (DGC), confers mechanical stability, cellular signaling and cellular integrity in skeletal and cardiac muscles (Koenig et al., 1988; Petrof et al., 1993; Talsness et al., 2015). Patients born with this disease experience

progressive muscle loss leading to muscle weakness, and by their late 20 s, succumb to dilated cardiomyopathy and respiratory failure. DMD cardiomyopathy is progressive and the present cardiac phenotypes include: arrhythmia, ECG abnormalities, diastolic dysfunction, fibrosis, gradual ventricular dilation, systolic dysfunction, and end-stage heart failure (Finsterer and Cripe, 2014; Yucel et al., 2018). As a leading cause of death in DMD patients, there is an urgent need to develop effective therapies for treating DMD cardiomyopathy and prevent the onset of heart failure.

Currently, Duchenne patients are prescribed with steroids (to slow down muscle deterioration) and general β -blockers (to prolong cardiac function) (Birnkrant et al., 2018). Chronic steroid usage is associated with significant side effects including increase in weight, growth arrest, and the increase risk of bone fracture (Angelini, 2007; Angelini and Peterle, 2012). Besides standard treatment, Chinese herbal medicine (CHM) has always been used as an adjunct therapy for diseases in Taiwan's healthcare system since 1995. CHM has also been used for physical frailty and showed anti-muscular atrophy effects in mouse models (Moorwood et al., 2011; Zhang et al., 2014; Zeng et al., 2019).

Previous studies have showed human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes (CMs) from DMD patients (DMD hiPSC-CMs) as a valid model to study mechanisms and treatment approaches for DMD cardiomyopathy (Lin et al., 2015; Chang et al., 2018). In this study, using DMD hiPSC-CMs as a validation platform to find the effective natural compounds in CHMs, we validate and identify active components of these CHM herbs. Cross-comparison of transcriptomic profiling data and active compound library identifies nine active chemicals targeting Catalase (CAT) and vascular cell adhesion molecule 1 (VCAM1). Through treating DMD hiPSC-CMs with these active chemicals, we show that Quercetin, Kaempferol, and Vitamin C confer increased CAT activity and were able to decrease H_2O_2 and increase DMD hiPSC-CMs contraction function; Hesperidin and Allicin induced VCAM1 expression in cytosol and enhanced sarcomere structure. Overexpression of either CAT or VCAM1 resulted in restoration of DMD hiPSC-CMs function. Together, our data provide an efficient Bed to Bench back to Bed translational avenue for DMD drug development.

MATERIALS AND METHODS

Ethics

This study was approved by the Human Studies Committee of China Medical University Hospital, Taichung, Taiwan [approval number: CMUH107-REC3-074(CR1)]. All protocols using human iPSC were reviewed and approved by the Ethics Review committee at Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (2018-207-K32).

Cell Culture and Cardiac Differentiation

Human iPSCs were cultured on Matrigel (Corning) coated plates with Nutristem hPSC XF medium (Biological Industries)

and passaged every 2–3 days by performing a 1:6 dilution. At 70–90% confluency, hiPSCs differentiation was induced to generate beating CMs as described previously (Chang et al., 2018). Briefly, hiPSC were treated with 4–6 μ M CHIR-99021 (SelleckChemicals) for 2 days, followed by a Wnt inhibitor IWR-1 treatment (5 μ M; Sigma) for another 2 days, in RPMI 1640 medium supplemented with B27 minus insulin (Thermo Fisher Scientific). On day 5, medium were changed to fresh RPMI 1640 medium supplemented with B27 minus insulin for 2 days and switched to RPMI 1640 medium supplemented with B27 until day 10. HiPSC-CMs were then purified using a metabolic-selection medium which consisted of RPMI 1640 without glucose, B27 supplement (Life Technology) and 4 mM of sodium DL-lactate (Sigma). Medium was changed every 2 days for the maintenance of cardiomyocytes. DMD and Healthy hiPSC lines were characterized in **Supplementary Table 1**.

Chinese Herbs Powder Extraction Preparation

Chinese herbal medicine stock solution was generated by dissolving 1 g Chinese herb extract (Jiangyin Tianjiang Pharmaceutical Co. Ltd) in 40 ml sterilized water and shook overnight at room temperature. The solution was centrifuged at 3000 rpm for 30 min. The supernatant was then filtered using 0.22 μ m filter, aliquoted, and stored at -20°C . Cell were treated using 0%/2%/5%/10% v/v concentration prior to downstream assays.

Real Time-Quantitative PCR (RT-qPCR)

Total RNA was extracted using TransZol Up Plus RNA Kit (TransGen Biotech) according to the manufacturer's instructions. Hundred nanogram RNA was used to generate cDNA by using the AMV Reverse Transcription System (TOYOBO). Real-time quantitative PCR was carried out with LightCycler[®] 480 II using a ChamQ Universal SYBR qPCR Master Mix (Vazyme) with GAPDH as a reference. Expression was quantified using $\Delta\Delta C_t$ method and expressed as fold enrichment. Primer sequences are listed in **Supplementary Table 2**.

Bioinformatic Analysis

For the cross comparison, we first generated a list of differentially regulated genes in DMD hiPSC-CMs compared to WT hiPSC-CMs. Next, we accessed three publicly available CHM active compound databases for this study (SuperTCM¹; SymMap²; TCMSP³). Active compounds and its gene targets for DaH, ZK, and CQZ were manually searched, and a list of active compounds to target genes was compiled. A Venn comparison was performed, and the results are shown below and now supplemented as a **Supplementary Table 3**.

Active Compounds Treatment

All concentrations of the active compounds were derived from previous published studies and DMD hiPSC-CMs were treated

¹<http://bioinf-applied.charite.de/supertcm/>

²<https://www.symmap.org/>

³<https://tcmspw.com/tcmsp.php>

for 5 days prior to functional assay. Specifically: Quercetin, Naringenin, Kaempferol, and Hesperidin were used at 10 μ M (Gutiérrez-Venegas et al., 2014; Denaro et al., 2021); Diosgenin at 200 ng/mL (Zhao et al., 2018); D-lactic acid at 10 μ M (Pohanka, 2020); Vitamin C at 10 μ M (Parra-Flores et al., 2019); Oxalic acid (10 μ M) (Liu et al., 2018); and Allicin at 10 μ M (Xiang et al., 2020).

Cardiomyocyte Contractility Assay

Single hiPSC-CMs were seeded on micropatterns 3 days prior to assay as previously described (Chang et al., 2020). Analysis of spontaneous beating of hiPSC-CMs was performed using video microscopy. Cardiomyocytes were maintained at 37°C and 5% CO₂ to keep physiologic conditions and bright-field videos (captured at 60 fps) were acquired using an Olympus IX83 microscopy. Contraction speed and beating frequency were extrapolated using an established Conklin method algorithm. Quantification of hiPSC-CMs contraction and beating rate was calculated using Matlab-based motion-tracking software as previously described (Huebsch et al., 2015).

Measurement of Intracellular ROS and Mitochondrial ROS

Total ROS levels in cardiomyocytes were measured using the CellroX Oxidative Stress dye per manufacture' instructions (Invitrogen). Mitochondrial ROS levels in cardiomyocytes were measured using a MitoSox Red Mitochondrial Superoxide Indicator (Invitrogen). Briefly, hiPSC-CMs were incubated with CellroX/MitoSox and Hoechst 33342 for 30 min at 37°C, washed once with PBS, and the fluorescent signal intensity was measured with Operetta CLS High Content Imaging System (PE) using a 20 $\times$ numerical aperture.

Immunofluorescence Staining

Day 30 cardiomyocytes grown on Matrigel covered coverslips were washed with PBS and fixed in 4% formaldehyde in PBS for 10 min at room temperature. After three PBS washes, cells were permeabilized and blocked with staining solution (0.1% Triton X-100 and 20% FBS in PBS) for 1 h at room temperature. Rabbit monoclonal anti-phospho-histone H2AX (Ser 139) (1:1000 dilution, Santa Cruz), mouse monoclonal anti-cTnT (1:400 dilution, Abcam), rabbit polyclonal anti-ACTN2 (1:200 dilution, proteintech), mouse monoclonal anti-Integrin beta 1 (1:300 dilution, Abcam), or rabbit polyclonal anti-VCAM1 (1:200 dilution, Proteintech) antibodies were diluted in staining solution and samples were incubated overnight at 4°C. Cells were washed in staining solution three times for 10 min each at room temperature and incubated with Alexa Fluor 594 or 488 conjugated secondary antibodies (1:1000 dilution, Invitrogen) at room temperature for 1–2 h. Samples were then washed three times with staining solution and then incubated with PBS containing 4',6-diamidino-2-phenylindole (DAPI) for 10 min and mounted with VECTASHIELD (VECTOR). Image acquisition was performed using a Zeiss LSM880 microscope and analyzed using ZEN software (Zeiss).

RNA-Sequencing

RNA-sequencing was performed by GENEWIZ company using the Illumina HiSeq instrument. Total RNA from day 30 WT and DMD hiPSC-CMs were isolated using the TRIzol Reagent (Life Technologies). One microgram total RNA (RIN value above 7) was used for library construction using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina®.

Libraries were multiplexed and loaded onto Illumina HiSeq instrument (Illumina). Sequencing was carried out using a 2 $\times$ 150 bp paired-end (PE) configuration; image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPIipeline-1.6 (Illumina) on the HiSeq instrument. Differential expression analysis was carried out using the DESeq Bioconductor package (Anders and Huber, 2010). After adjusting with Benjamini and Hochberg's approach for accounting the false discovery rate, *P*-value of *p* < 0.05 was deemed statistically significant and was used to identify differential expressed genes.

Transmission Electron Microscopy

HiPSC-derived cardiomyocytes were fixed using 2.5% glutaraldehyde solution for 2 h at room temperature inside a fume hood, washed three times with PBS, and post-fixed with 1% osmium tetroxide for 4 h. Next, samples were washed with PBS for three times, then dehydrated in pure ethanol two times (each for 30 min) and infiltrated with ethanol: epoxy 812(2:1,1:1,1:2, each for 30 min). Next, samples were embedded with Epon resin, sectioned at 70 nm thickness. The thin sections were mounted onto formvar-coated copper grids, counterstained with 3% uranyl acetate in 70% methanol and 30% water for 7 min, followed by lead citrate for 3 min. Micrographs were captured using a FEI (Tecnai G2 Spirit 120 kV) electron microscope.

IMP and EFP Measurements

Impedance (IMP) and extracellular field potential (EFP) measurements were conducted according to Nanion's standard procedures for the CardioExcyte 96. Briefly, CardioExcyte 96 Sensor Plates (Nanion Technologies) were pre-coated overnight with 1% Fibronectin phosphate-buffered saline (PBS) solution. Cardiomyocytes were seeded at 50,000 viable cells per well. Extracts/small compounds and hiPSC-CMs were incubated for 5 days prior to data acquisition. Medium was exchanged every other day. On the day of measurement, fresh medium was changed 2 h prior to assay. DMSO (0.01%) on the same plate was used as vehicle control. Data was analyzed using DataControl 96 software (Nanion).

Lentivirus Production and Gene Overexpression in DMD hiPSC-CMs

CAT or VCAM1 cDNA was PCR amplified and cloned into the target gene lentiviral plasmid (pLVX-ZsGreen1 backbone). HEK293T cells were plated in a 10 cm dish and transfected with packaging plasmids using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies). The supernatant of transfected cells was collected after 24 h. After centrifuged at 6000 g for 15 min, the supernatant containing viruses was

concentrated using Lenti-X Concentrator (Clontech). DMD hiPSC-CMs were seeded into 6-well plates and infected with lentiviruses for 24 h and then cultured 5 days prior to functional assay.

Statistical Analysis

All experimental data are presented as mean $\pm$ SD. Statistical significance between two groups was determined using two-tailed Student's *t*-test. For multiple group comparison, One-way ANOVA with Tukey's multiple comparison was used. *p*-value less than 0.05 was considered statistically significant. Data were analyzed and represented with GraphPad Prism. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

RESULTS

Identification of 4 CHM Herbs in the Treatment of Muscular Dystrophic Patients in Taiwan

Chinese herbal medicine has been used as an adjunct treatment for muscular dystrophy patients, including DMD, and is offered in Taiwan's medical healthcare system. To track the outcome of long-term CHM usage, we analyzed and compared non-CHM users to CHM users on overall mortality in patients with muscular dystrophies (Figure 1). A total of 581 patients with muscular dystrophies was identified in the Registry for Catastrophic Illness Patients of Taiwan's National Health

Insurance Research Database (NHIRD⁴) from the National Health Insurance (NHI) program between 2003 and 2013. Among these patients, patients exhibiting myocardial infarction, congestive heart failure, or any other malignancies were excluded (Figure 1A). Total of 80 patients that received more than 14 cumulative CHM days within 1 year after diagnosis were defined as CHM users. Total of 201 patients that received no CHM during the study period were defined as non-CHM users (Figure 1A). Kaplan–Meier survival analysis showed that the cumulative incidence of overall survival was significantly higher in CHM users compared to non-users (*p* = 0.0081, log-rank test). CHM users showed a lower risk of overall mortality than non-CHM users after adjusting for age, gender, prednisolone use, and comorbidities (OR: 0.393, 95% CI: 0.21–0.75, *p* = 0.0044) (Figure 1B). Using association rule mining and network analysis, we identified top 4 single herbs prescribed for the CHM user group: Yu-Xing-Cao (YXC; *Houttuynia cordata* Thunb.), Da-Huang (DaH; *Rheum palmatum* L.), Zhi-Ke (ZK; *Citrus aurantium* L.) and Che-Qian-Zi (CQZ; *Plantago asiatica* L.) (Figure 1C).

Verification of CHM's Therapeutic Potential Using DMD hiPSC-Derived Cardiomyocytes

To verify the effect of these CHMs on DMD cardiomyocyte function, we used DMD hiPSC-CMs. To detect the effect of

⁴<http://nhird.nhi.org.tw/>

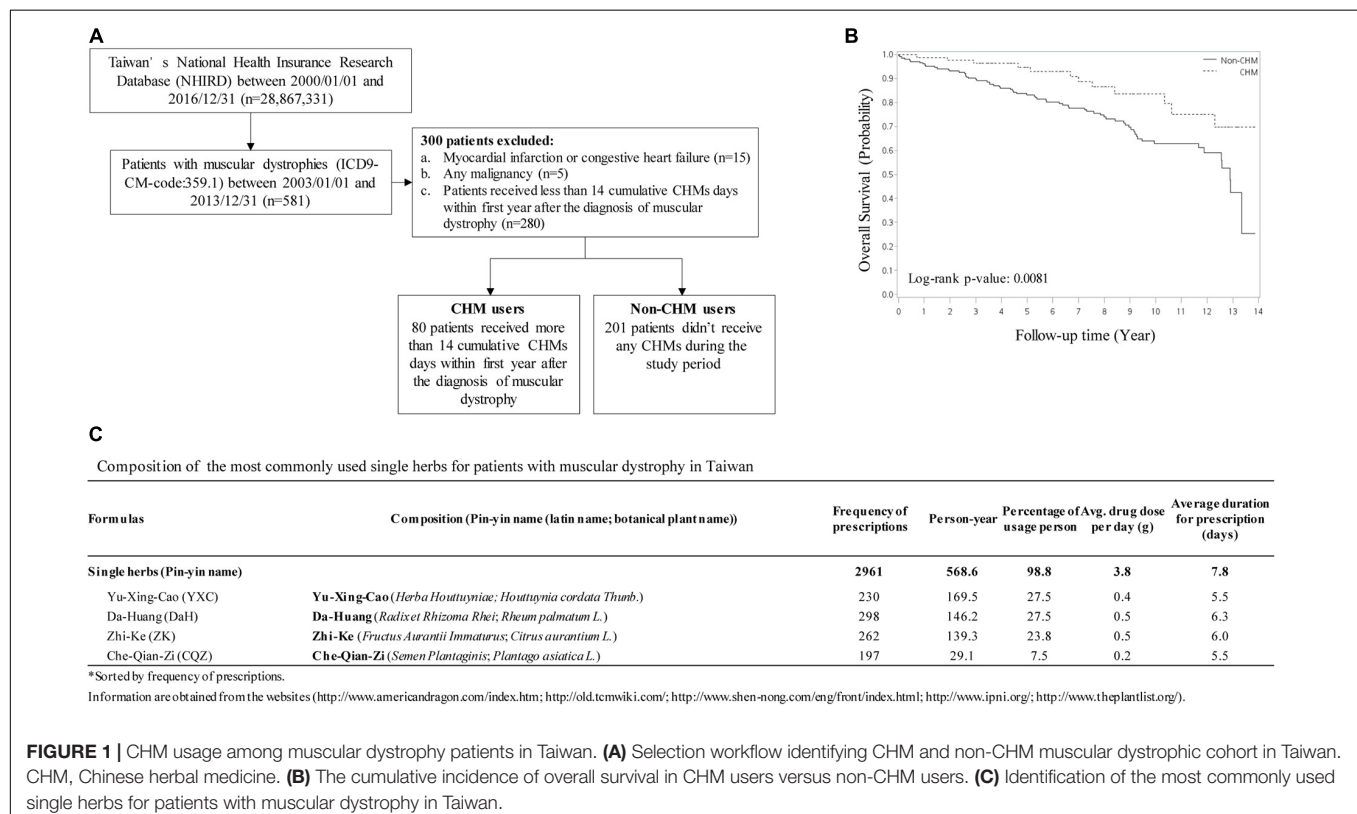


FIGURE 1 | CHM usage among muscular dystrophy patients in Taiwan. **(A)** Selection workflow identifying CHM and non-CHM muscular dystrophic cohort in Taiwan. CHM, Chinese herbal medicine. **(B)** The cumulative incidence of overall survival in CHM users versus non-CHM users. **(C)** Identification of the most commonly used single herbs for patients with muscular dystrophy in Taiwan.

CHM herbs on single cardiomyocyte contraction, hiPSC-CMs were grown into rod-shape single cells using microprinting (Chang et al., 2020). Micropatterned hiPSC-CMs exhibited well-aligned sarcomere structures after 3 days (**Figure 2A**). Compared to WT hiPSC-CMs, DMD hiPSC-CMs exhibited

aberrant electrophysiology with increased beating rate (**Figure 2B**). DaH, ZK, and CQZ treatments reduced beating rate in DMD hiPSC-CMs compared to vehicle (**Figures 2B,C**). Moreover, DaH and ZK treatments increased contraction velocity of DMD hiPSC-CMs (**Figure 2D**). However, YXC-treated

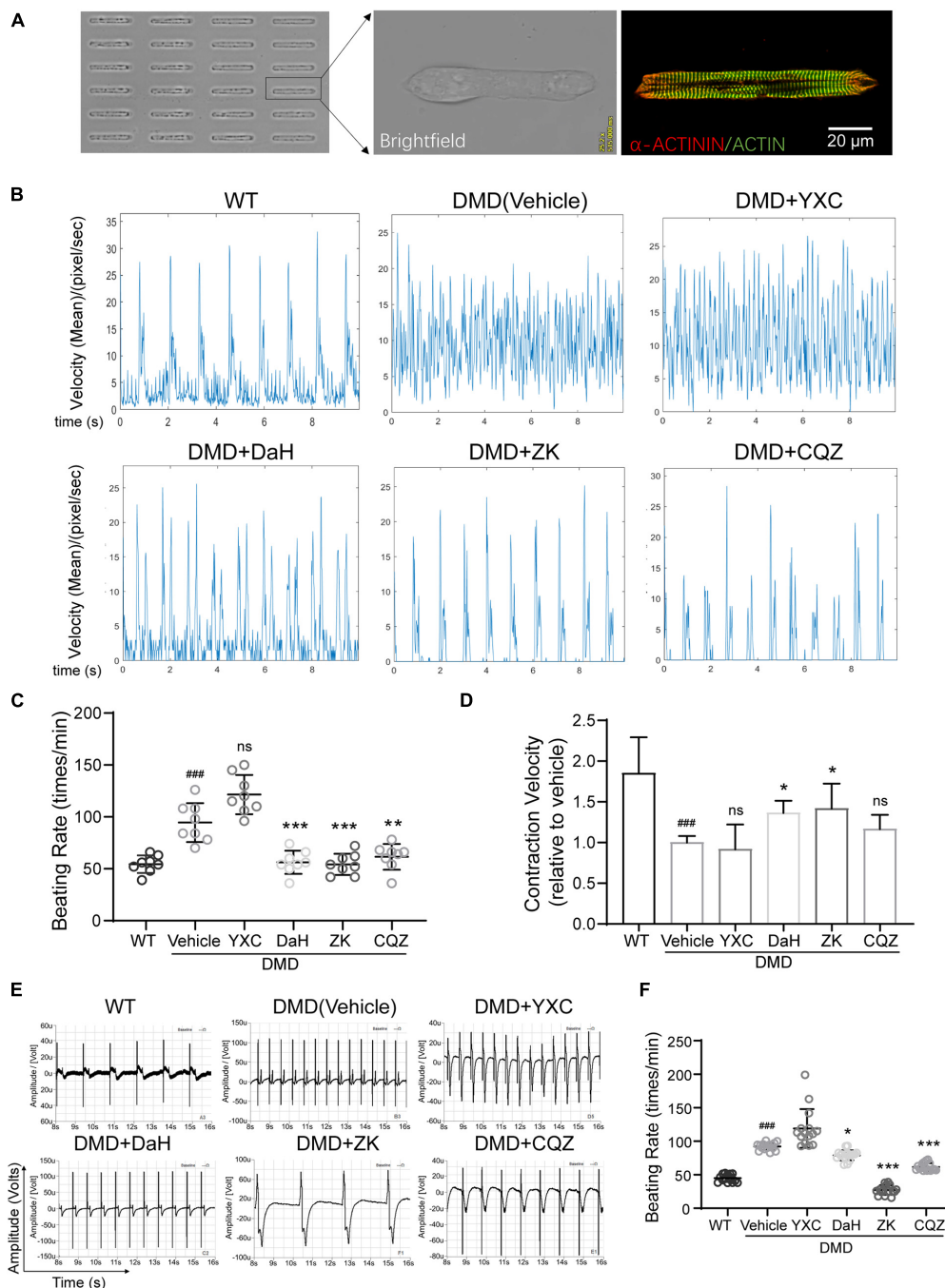


FIGURE 2 | Effect of DaH, ZK, and CQZ treatment on DMD cardiomyocyte function. **(A)** Representative image of micropatterned hiPSC-CMs, showing single cell with aligned sarcomere. **(B)** Representative motion tracing of single wild type (WT) hiPSC-CMs and DMD hiPSC-CMs. **(C)** Beating rate and **(D)** contraction velocity of single DMD hiPSC-CMs treated with YXC, DaH, ZK, CQZ, or Vehicle ($n = 8$). $###P < 0.001$ versus WT; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus vehicle. **(E)** Representative extracellular field potential (EPF) traces and **(F)** quantification of monolayer DMD hiPSC-CMs treated with YXC, DaH, ZK, CQZ, or Vehicle. WT hiPSC-CMs served as healthy control ($n = 12$). $###P < 0.001$ versus WT; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus vehicle; ns = no statistical significance.

DMD hiPSC-CMs exhibited intensified beating patterns, suggesting cardiotoxicity (**Figures 2B–D**). Next, we evaluated the effects of the four CHM herbs on cardiac electrophysiology using hiPSC-CM monolayers on the Nanion CardioExcyte96 platform (**Figure 2E**). Similar to single cell observations, we observed reduced beating rate in DMD hiPSC-CMs treated with DaH, ZK, and CQZ compared to vehicle (**Figures 2E,F** and **Supplementary Figure 1**). YXC treatment disrupted electrophysiology evident by aberrant EFP readings, which is in accordance with our single cell results (**Figures 2E,F**). DaH, ZK, and CQZ treatments significantly strengthened cardiac function (reduced beating rate and increased contraction velocity) in DMD cardiomyocytes. Subsequent experiments were carried out using DaH, ZK, and CQZ.

Identification of CAT and VCAM1 as Therapeutic Targets for CHM Herbs

To identify the active compounds in the three CHM herbs, we performed transcriptomic profiling of control and DMD hiPSC-CMs and cross-referenced differentially expressed genes with CHM active compound databases (SuperTCM, SymMap, TCMSP). Whole-transcriptome RNA sequencing analysis identified a total of 1099 upregulated and 604 downregulated differentially expressed genes in DMD hiPSC-CMs compared to WT hiPSC-CMs (**Figure 3A**). Gene ontology analyses revealed that genes involved in TCA cycle and mitochondrial electron transport, cardiac muscle contraction, and structure development were significantly affected in DMD hiPSC-CMs (**Figure 3B**). Next, we downloaded active compounds and corresponding gene targets of our CHM herbs from databases mentioned above. Using cross-comparison of transcriptomic profiling data and CHM database, we identified CAT and VCAM1 as major targets for our three CHM herbs (**Figure 3C**). Using RT-qPCR, we confirmed that CAT and VCAM1 are downregulated in DMD hiPSC-CMs compared to control hiPSC-CMs (**Figures 3D,E**). Next, we sought to determine which active compounds targeted CAT or VCAM1 in DMD hiPSC-CMs. Active compounds targeting CAT in Da-Huang included: Oxalic acid (Oxl), Vitamin C (VitC), D-lactic acid (Dla); in Zhi-Ke included: Naringenin (Nar), Kaempferol (Kae); and in Che-Qian-Zi included: Diosgenin (Dios), Quercetin (Que) (**Figure 3F**). Active compounds targeting VCAM1 included: Vitamin C (VitC) in Da-Huang; Hesperidin (Hes) in Zhi-Ke; Quercetin (Que) and Allicin (Alli) in Che-Qian-Zi (**Figure 3F**).

Single Herbs Decreased ROS Accumulation Through Decomposing H_2O_2 and Improved Structure of DMD hiPSC-CMs

Catalase plays a major role in protecting the adverse effects of accumulating peroxides via hydrogen peroxide (H_2O_2) hydrolysis (Dai et al., 2009). We used intracellular ROS and H_2O_2 levels as a readout for CAT activity. Compared with WT hiPSC-CMs, DMD hiPSC-CMs showed increased ROS levels at baseline (**Figures 4A,B**). However, we observed a drastic decrease in intracellular ROS in DMD hiPSC-CMs treated with DaH, ZK,

and CQZ (**Figures 4A,B**). Similarly, DaH, ZK and CQZ treatment also reduced mitochondrial superoxide levels (**Figures 4C,D**) in DMD hiPSC-CMs compared to vehicle. Together, these results show that DaH, ZK, or CQZ alone was able to reduce ROS burden in DMD hiPSC-CMs.

Focal adhesion is dynamic and integrins aggregates are formed to promote cellular adhesion by strengthening the connection between cytoskeleton and extracellular matrices. Vascular cell adhesion molecule 1 (VCAM1), located at cell membrane, plays an important role in the binding of integrin beta 1 (ITGB). To evaluate the impact of CHM herbs on myofilament structure of DMD hiPSC-CMs, we performed immunofluorescence staining on CHM-treated DMD hiPSC-CMs. At baseline, DMD hiPSC-CMs exhibited decreased VCAM1 protein distribution compared with WT hiPSC-CMs (**Figures 4E,F**). Treatment with DaH, ZK, and CQZ increased VCAM1 expression and protein levels in DMD hiPSC-CMs significantly (**Figures 4E,F** and **Supplementary Figure 2B**). Interestingly, reduced VCAM1 levels were accompanied with reduced cardiomyocyte surface area in DMD hiPSC-CMs and treatment with DaH, ZK, and CQZ reversed this phenotype (**Figure 4G**). Using transmission electron microscopy, DaH, ZK, and CQZ treated DMD hiPSC-CMs exhibited wider sarcomere structures compared to untreated DMD hiPSC-CMs (**Figure 4H**). These data suggest that DaH, ZK, and CQZ treatment improves DMD cardiomyocyte ultrastructure through induction of VCAM1 protein.

CAT Targeting Compounds Protect DMD Cardiomyocytes From Oxidative Damage and Rescue Cardiomyocyte Function

To test the antioxidant efficacy of the seven single natural compound targeting CAT, we measured intracellular ROS levels using Cellroxx and MitoSox (**Figure 5A**). Compared to vehicle treatment, Kae, Que, VitC, and Dla treatment significantly decreased total ROS accumulation in DMD hiPSC-CMs while Dios, Nar, Oxl only showed a trend (**Figures 5A,B**). Production of mitochondrial superoxide was reduced when DMD hiPSC-CMs were treated with Kae, Que, VitC, and Dla but not Dios, Nar, and Oxl (**Figures 5A,C**). Kae, Que, VitC, Oxl, and Dla treatment also showed decrease in H_2O_2 level (**Figure 5D**), which is in agreement with our cellular ROS results (**Figures 5A,B**). Compared to vehicle treated DMD hiPSC-CMs, Que, Kae, VitC, and Dla treatments decreased $\gamma H2AX$ foci accumulation in nuclei (**Figures 5E,F**), a readout for DNA damage.

Next, we examined contraction and electrical conduction of DMD hiPSC-CMs using Nanion (**Figure 6A**). Using cardiomyocyte impedance as a readout of contraction, DMD hiPSC-CMs treated with Que, Kae, VitC, and Nar exhibited a significant increase in contractility compared to vehicle (**Figure 6B**). Treatment with Que, Kae, and VitC also decreased cell beating rate in DMD hiPSC-CMs compared to vehicle (**Figure 6C**). It has been demonstrated that high oxidative stress can lead to mitochondrial dysfunction in dystrophic cardiomyocytes (Chang et al., 2016). Among these compounds, Dios showed cytotoxicity (data not shown). Mitochondrial function of DMD hiPSC-CMs treated with the

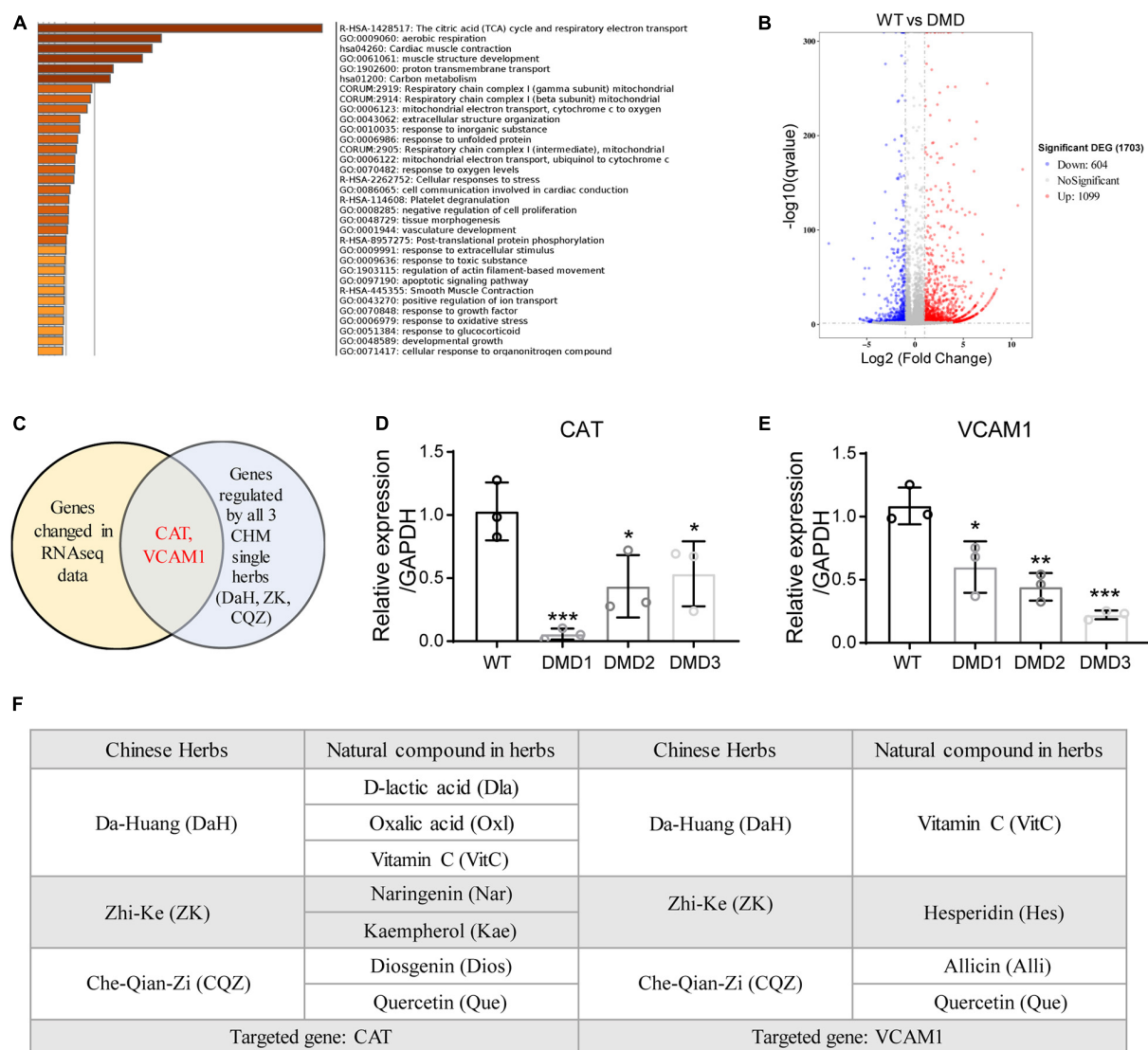


FIGURE 3 | Identification of CAT and VCAM1 as therapeutic targets for DaH, ZK, and CQZ. **(A)** GO term analysis of differentially expressed genes between WT and DMD hiPSC-CMs. Top enrichment clusters were shown, discrete color scale represented statistical significance. **(B)** Volcano plot of differentially expressed genes between WT and DMD hiPSC-CMs. **(C)** Venn diagram showing overlapping genes between differentially expressed gene list and CHM target genes. Relative expression of **(D)** CAT and **(E)** VCAM1 were measured by RT-qPCR ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. **(F)** Summary of active compounds in DaH, ZK, and CQZ targeting CAT and VCAM1, respectively.

other six compounds were evaluated using Seahorse bioanalyzer (**Figure 6D**). DMD hiPSC-CMs treated with Que, Kae, VitC, and Dla exhibited higher basal mitochondrial respiration compared to vehicle (**Figure 6E**); moreover, Que, Kae, and VitC treatments displayed significantly higher maximal respiratory capacity (**Figure 6F**).

Structural Fortification via VCAM1 Modulation Improves DMD hiPSC-CM Contractility

Besides ROS modulation through CAT, our analysis also identified VCAM1 targeting compounds. To evaluate the

impact of four natural compounds targeting VCAM1, we performed immunofluorescence stainings for ITGB and VCAM1 protein in hiPSC-CMs (**Figure 7A**). Compared to vehicle treatment, we observed an increase in VCAM1 protein levels when DMD hiPSC-CMs were treated with Alli, Hes, and Que (**Figures 7A,B**). Moreover, cell size and sarcomere lengths were increased after Alli/Hes and Alli/Hes/Que treatment, respectively (**Figures 7C,D**). Using transmission electron microscopy, Alli, Hes, and Que treated DMD hiPSC-CMs exhibited wider sarcomere structures compared to vehicle (**Figure 7E**). In conclusion, the results exhibited that Allicin and Hesperidin play an obvious role in the morphology maintain of DMD hiPSC-CMs.

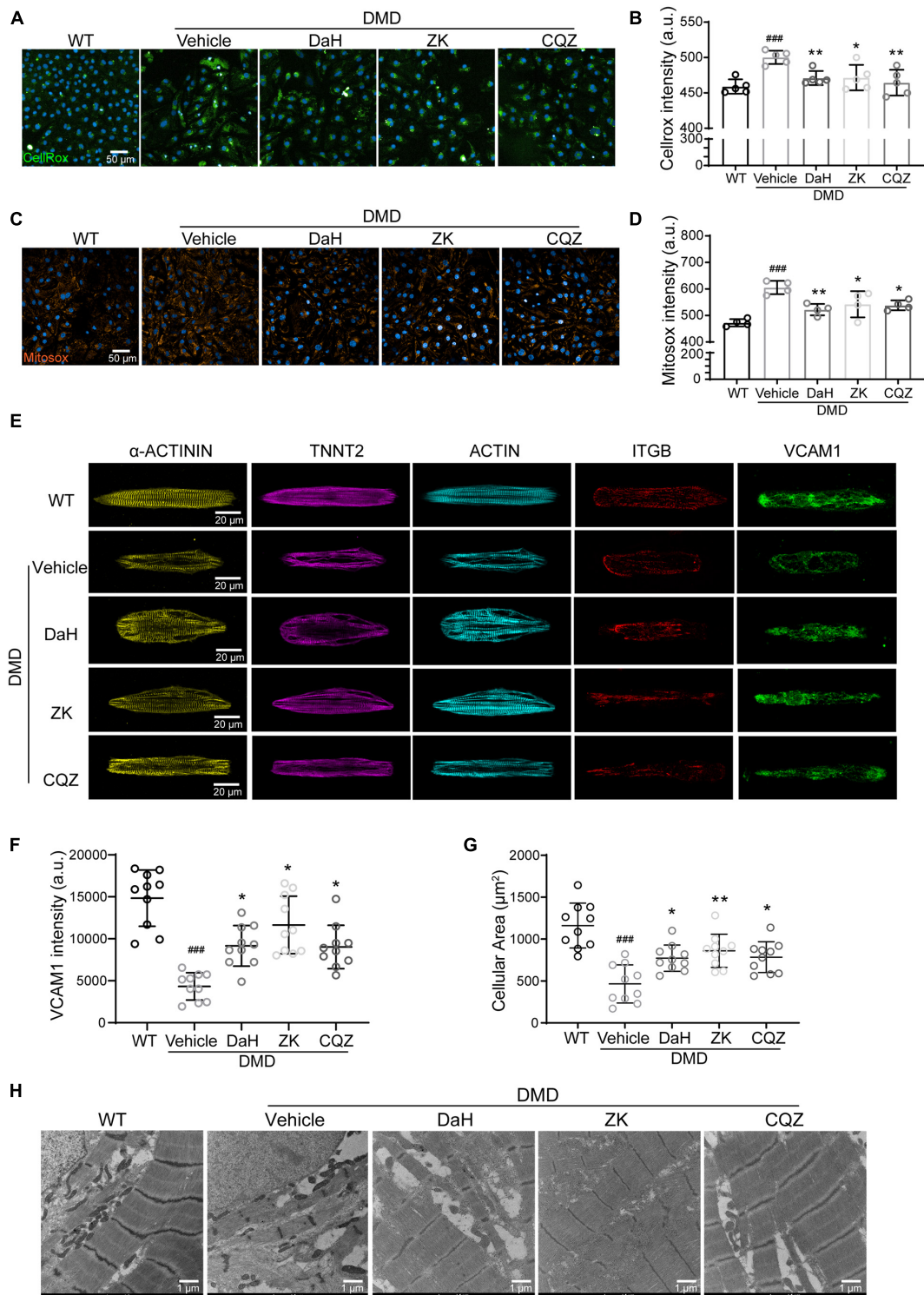


FIGURE 4 | DaH, ZK, and CQZ treatments decreased ROS accumulation and improved cytoskeletal structure of DMD hiPSC-CMs. **(A)** Representative micrographs of WT and DMD hiPSC-CMs treated with DaH, ZK, CQZ or vehicle stained for cellular ROS (CellRox). **(B)** Intracellular ROS levels were determined by measuring the level of fluorescent CellRox ($n = 5$). *** $P < 0.001$ versus WT; * $P < 0.05$, ** $P < 0.01$ versus vehicle. **(C)** Representative micrographs of WT and DMD hiPSC-CMs treated with DaH, ZK, CQZ or vehicle stained for mitochondrial ROS (Mitoxox). **(D)** Mitochondrial ROS levels were determined by measuring the level of fluorescent Mitoxox ($n = 4$). *** $P < 0.001$ versus WT; * $P < 0.05$, ** $P < 0.01$ versus vehicle. **(E)** Immunofluorescence micrographs of focal adhesion related proteins. **(F)** Quantification of total VCAM1 levels and **(G)** cell size of WT and DMD hiPSC-CMs ($n = 10$). *** $P < 0.001$ versus WT; * $P < 0.05$, ** $P < 0.01$ versus vehicle. **(H)** Representative transmission electron microscopy images of WT and DMD hiPSC-CMs.

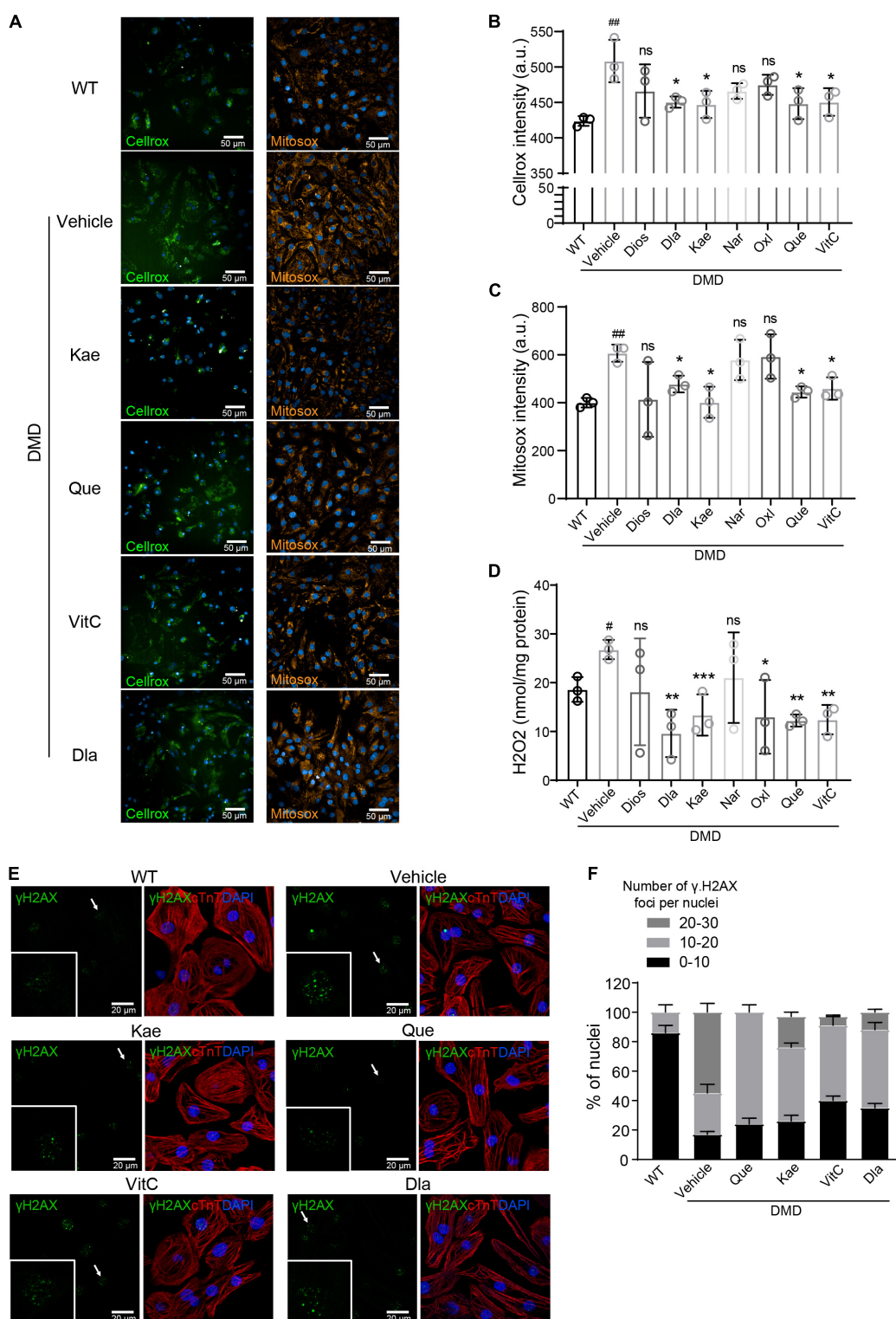
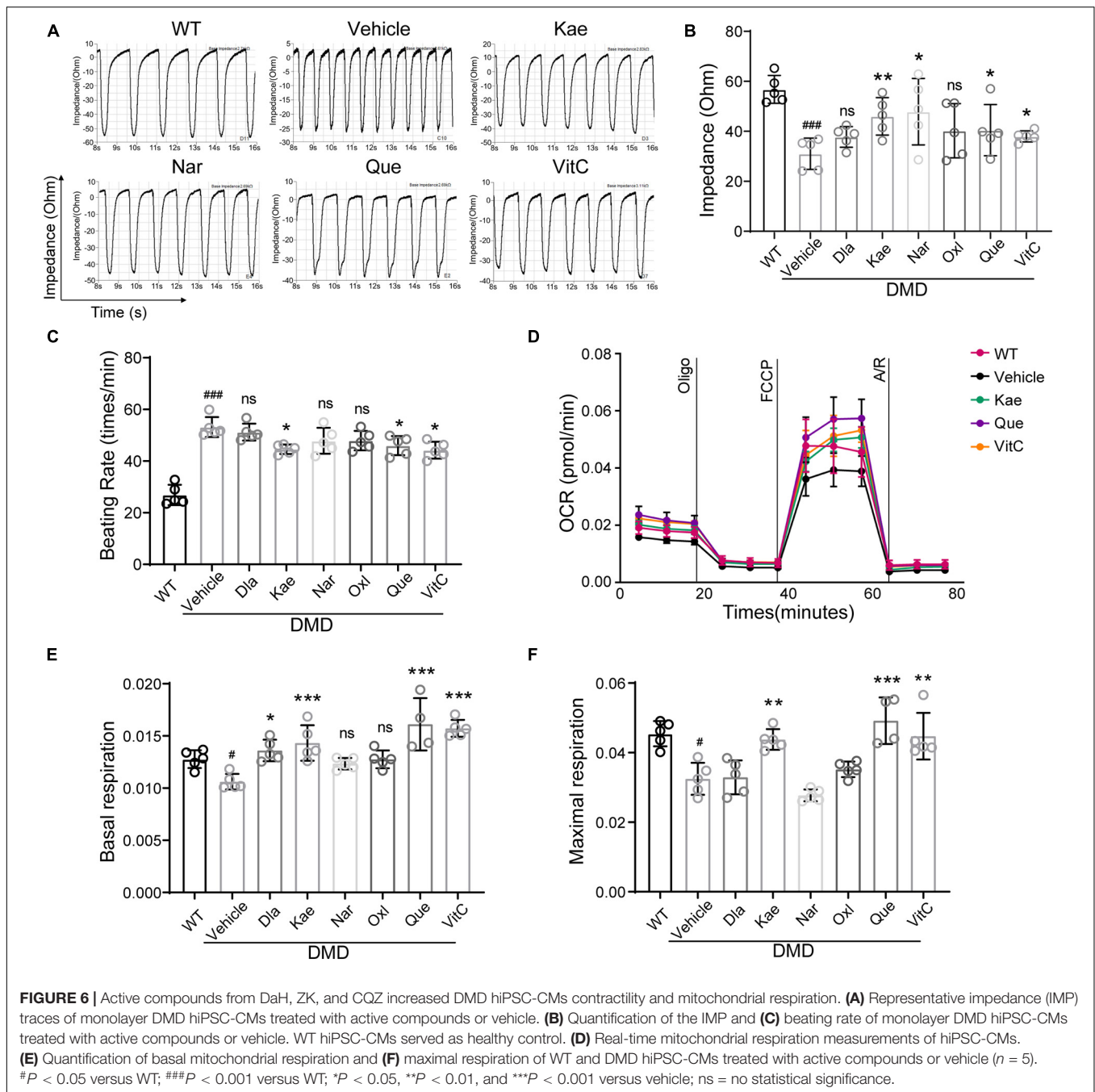


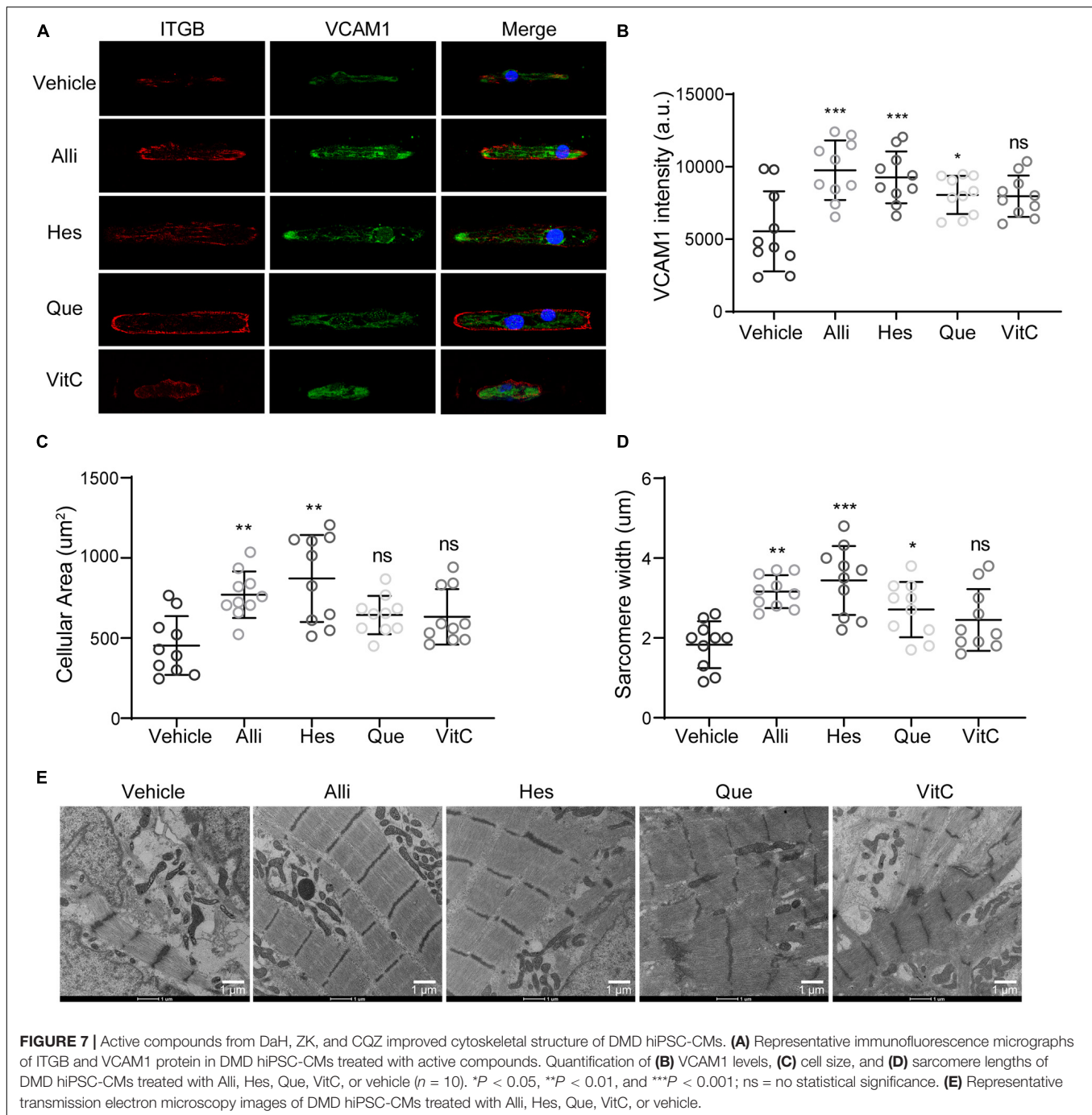
FIGURE 5 | Active compounds from DaH, ZK, and CQZ decreased intracellular ROS levels and protect cells from oxidative DNA damage. **(A)** Representative micrographs of WT and DMD hiPSC-CMs treated with active compounds or vehicle stained for cellular ROS (Cellroxi) and mitochondrial ROS (Mitosox). **(B)** Intracellular ROS levels (Cellroxi), **(C)** mitochondrial ROS levels (Mitosox), and **(D)** H₂O₂ levels in WT and DMD hiPSC-CMs were measured ($n = 3$). $^{\#}P < 0.05$, $^{##}P < 0.01$ versus WT; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus vehicle; ns = no statistical significance. **(E)** Representative images of γ H2AX staining in WT and DMD hiPSC-CMs treated with active compounds or vehicle, only active compounds showing statistical significance were shown. **(F)** Quantification of percentage of γ H2AX foci per nucleus in hiPSC-CMs.



Overexpression of CAT or VCAM1 Restores Mitochondrial Function, Contractility, and Structural Integrity in DMD hiPSC-CMs

To show cause and effect in improving myocardial function, we overexpressed CAT or VCAM1 in DMD hiPSC-CMs (Supplementary Figure 3). CAT overexpression significantly reduced intracellular H_2O_2 level (Figure 8A) and mitochondrial superoxide (Figures 8B,C) in DMD hiPSC-CMs compared to untreated. Compared to vehicle control, CAT overexpressing

DMD hiPSC-CMs exhibited increased basal mitochondrial respiration and maximal respiration compared to untreated, respectively (Figures 8D–F). By measuring impedance as a readout of contractility, we found that CAT induction improved DMD hiPSC-CMs contractility and decreased beating rate (Figures 8G–I). Similar to active compound results, VCAM1 overexpression (Figures 8J,K) resulted in increased cell size and sarcomere lengths of DMD hiPSC-CMs (Figures 8L,M). Together, our results show that restoration of either CAT or VCAM1 significantly lowers ROS levels and improves cardiac contractility, mitochondrial respiration, and sarcomere structure.



DISCUSSION

Clinically, DMD patients succumb to dilated cardiomyopathy and respiratory complications at second or third decade of life. However, our treatment options for DMD patients are still very limited. Although corticosteroids can slow down muscle loss in DMD patients, weight gain and bone fracture risks often drive patients into non-compliance. Despite muscle and respiratory interventions have significantly increased the life expectancy of DMD patients, however, dilated cardiomyopathy is now

the leading cause of death for DMD patients (Spurney, 2011). Results of AAV and micro-dystrophin clinical trials showed significant increase in micro-dystrophin expression but very limited improvement in mobility as per the NAAA evaluation (Mendell et al., 2020). Balancing viral dosage and DMD protein expression remains a challenge for DMD gene therapy. Treatment using autologous or allogeneic stem cells injection remains to be translated (Meregalli et al., 2013). Therefore, there is an urgent need for better biomarker monitoring and alternative treatment options for DMD patients.

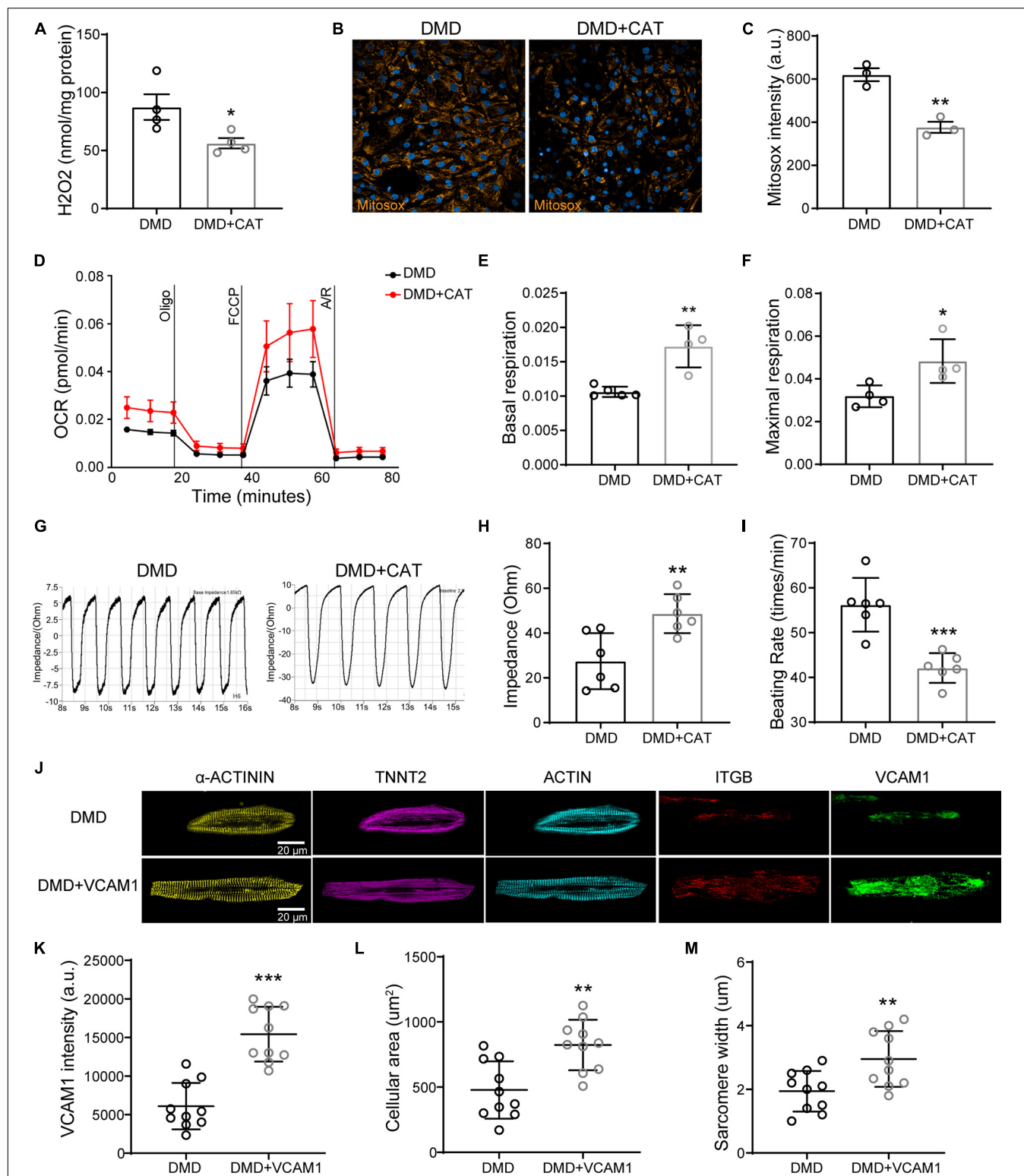


FIGURE 8 | Overexpression of CAT or VCAM1 in DMD hiPSC-CMs. **(A)** H₂O₂ levels, **(B)** Mitosox, and **(C)** mitochondrial ROS levels were quantified in DMD hiPSC-CMs overexpressing CAT. **(D)** Mitochondrial respiration was measured in CAT overexpressing DMD hiPSC-CMs. **(E)** Quantification of basal respiration and **(F)** maximal respiration. **(G)** Representative impedance traces of monolayer DMD hiPSC-CMs treated with CAT lentivirus or control and quantification of the **(H)** impedance and **(I)** beating rate ($n = 6$). ** $P < 0.01$ and *** $P < 0.001$. **(J)** Immunofluorescence micrographs of focal adhesion related proteins and VCAM1. Quantification of **(K)** VCAM1 levels, **(L)** cell size, and **(M)** sarcomere length in DMD hiPSC-CMs treated with CAT lentivirus or control ($n = 10$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Patients induced pluripotent stem cells derived cardiomyocytes (hiPSC-CMs) offer a scalable platform that can be used for drug screening and validation (Sallam et al., 2014). Availability of DMD cardiomyocytes enables us to study DMD cardiac functions such as electrophysiology and contractility. By cross-comparing clinical, RNAseq and CHM databases, we used DMD hiPSCs-derived cardiomyocytes as a surrogate to screen for active compounds in CHM prescription. Based on our multi-omics analysis, we identified and validated CAT and VCAM1 as novel targets for treating DMD cardiomyopathy.

Catalase is a major antioxidant enzyme which hydrolyze hydrogen peroxide (H_2O_2) into water and O_2 . H_2O_2 is an ubiquitous ROS in biological systems, which is produced as a byproduct of oxidative metabolism in peroxisomes and mitochondria (Dubreuil et al., 2020). Previous studies showed catalase plays a key role in cellular oxidative balance and cellular redox signaling regulation (Young and Woodside, 2001). Cardiac catalase overexpression has been shown to protect against ROS-induced cell death and oxidant-mediated activation of inflammatory signaling pathway (Dai et al., 2009; Otera and Fujiki, 2012; Cong et al., 2015). Catalase has also been shown to restore cardiac contractile dysfunction and intracellular Ca^{2+} mishandling induced by ethanol (Zhang, 2003) or LPS (Turdi et al., 2012). In our study, we found that DaH, ZK, and CQZ could protect DMD hiPSC-CMs from oxidative damage, improve mitochondrial functions, and cell contraction. Based on our multi-omics analysis, we further validated these finding by validating active compounds as well as CAT and VCAM1 overexpression.

Sarcomere integrity is critical to cardiomyocytes structural adaptation during cell development and disease process. Dystrophin deficiency results in calcium overload, increased reactive oxygen species, and mitochondrial dysfunction (Gonzalez et al., 2014; Chang et al., 2016). Cardiomyocytes continuously produce moderate levels of ROS and multiple antioxidant systems are needed to maintain a redox homeostasis (Santos et al., 2011; Bertero and Maack, 2018). In absence of dystrophin, stretch induced NOX2 increases ROS burden in cardiomyocytes (Prosser et al., 2011) and skeletal muscles (Petrillo et al., 2017). Mechanical distortion during physiologic stretch in muscles would induce NOX2-dependent ROS production via an intact microtubule network (Prosser et al., 2011; Khairallah et al., 2012). Given full-length dystrophin restoration is yet to be realized, drugs targeting ROS and/or promoting structure support could potentially slow down DMD progression.

Here, we provide evidence where CAT and VCAM1 are two key genes downregulated in DMD cardiomyocytes. By leveraging on clinical prescription and outcome data, we identified three CHM herbs that lowered ROS levels, increased mitochondrial respiration, reduced beating frequency, increased contraction

velocity, and boosted structural integrity in dystrophin-deficient cardiomyocytes. Further, we identified and showed active compounds that targeted CAT and VCAM1 provided equal efficacy. Lastly, we demonstrate that overexpression of CAT or VCAM1 conferred similar benefits. Together, our data identifies CAT and VCAM1 as novel targets and demonstrate that increase in activity or protein levels can restore cardiac function in DMD cardiomyocytes. We also demonstrate the feasibility in using clinical data with transcriptomic data to identify and validate new drug candidates using hiPSC-CM technology.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE166560>.

AUTHOR CONTRIBUTIONS

BL, Y-JL, and AC conceived the research and contributed to the writing of the manuscript. BL and WX performed the research and analyzed the data. W-ML and J-SC contributed to the analysis of clinical data in Taiwan. All the authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.659177/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Human Pluripotent Stem Cell-Derived Cardiac Cells: Application in Disease Modeling, Cell Therapy, and Drug Discovery

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Cardiac diseases are the leading cause of deaths worldwide; however, to date, there has been limited progress in the development of therapeutic options for these conditions. Animal models have been the most extensively studied methods to recapitulate a wide variety of cardiac diseases, but these models exhibit species-specific differences in physiology, metabolism and genetics, which lead to inaccurate and unpredictable drug safety and efficacy results, resulting in drug attrition. The development of human pluripotent stem cell (hPSC) technology in theory guarantees an unlimited source of human cardiac cells. These hPSC-derived cells are not only well suited for traditional two-dimensional (2-D) monoculture, but also applicable to more complex systems, such as three-dimensional (3-D) organoids, tissue engineering and heart on-a-chip. In this review, we discuss the application of hPSCs in heart disease modeling, cell therapy, and next-generation drug discovery. While the hPSC-related technologies still require optimization, their advances hold promise for revolutionizing cell-based therapies and drug discovery.

Keywords: pluripotent stem cell, cell therapy, drug discovery, cardiac cell, disease modeling

INTRODUCTION

Although cardiac diseases, such as myocardial infarction and heart failure, have been the leading cause of deaths worldwide, few drugs are being approved yearly compared with many other disease area, resulting in a huge gap between clinical need and drug development (Fordyce et al., 2015). The regenerative capacity of the heart is quickly lost during mammalian postnatal development. Pathological insults, such as ischemia, almost invariably lead to irreversible cardiac cell loss, which poses the greatest challenge to the treatment of cardiac diseases. In the past decades, rapid progress in human pluripotent stem cell (hPSC) technology enabled the generation of major cardiac cell types, thus unlocking new possibilities to treat patients with the most debilitating forms of heart disease using cell-based therapies (Mummery et al., 2012; Protze et al., 2019; Williams and Wu, 2019). To date, several studies have shown that transplantation of hPSC-derived cardiomyocytes holds great promise for attenuating cardiac dysfunction and reducing consequent fibrotic scarring (Gao et al., 2018; Liu et al., 2018).

Animal models are arguably the most widely used method to model disease onset and progression, providing valuable mechanistic insights in an *in vivo* setting. However, the extrapolation of translatable data from animals data to guide the treatment of human heart disease has been difficult due to considerable species differences (Matsa et al., 2014). hPSCs can potentially bridge this transitional gap by providing an unlimited source of human cardiac cells for biomedical research and drug discovery (Matsa et al., 2014). Protocols that enable the generation of cardiomyocyte subtypes such as ventricular, atrial, and sinoatrial pacemaker cells, as well as non-myocyte cell types including endothelial cells and fibroblasts have been developed (Mummery et al., 2012; Weng et al., 2014; Lee et al., 2017; Protze et al., 2017; Williams and Wu, 2019; Zhang et al., 2019). These highly enriched populations of specific cardiomyocyte subtypes facilitate disease modeling and drug testing by recognizing cellular, molecular and functional heterogeneity within the heart. Further, considering the spatial complexity of the heart, hPSC-based 3-D multicellular systems, including human heart organoids (Mills et al., 2017; Giacomelli et al., 2020), engineered heart tissues (EHTs) (Mannhardt et al., 2016), and heart-on-chip models (Zhang et al., 2016) have been shown to more accurately predict human cardiac biology and pathophysiology (Sharma et al., 2020). Incorporation of electrophysiology, epigenomics, transcriptomics, proteomics, metabolomics and imaging techniques will equip these hPSC-based platforms with additional tools to accomplish patient-specific disease modeling and personalized drug response screening.

In this review, we summarize the current progress of the application of hPSCs in cardiac disease and cell therapies (Figure 1). We also highlight the application of available hPSC platforms for the pharmacologic evaluation and cardiac safety assessment of drugs (Figure 1).

CARDIAC DISEASE MODELING USING hPSCs

Modeling heart disease is central to the understanding of the pathological processes. Conventional models of cardiovascular disease rely mostly on animals. However, the inherent differences between species render elucidation of human heart pathology unexpectedly difficult (Savoji et al., 2019). Therapeutics with promising results in animals often failed to show any improvement in clinical trials. Therefore, human-derived disease models bear the unique advantage to more faithfully represent human disease, thus potentially providing a more refined comprehension of disease mechanisms, paving the way to new therapeutic options (Zhao et al., 2020).

One of the major causes of cardiac disorders is genetic mutations (Dell'Era et al., 2015). The advent of iPSC technology provides a powerful means to model genetic cardiac diseases by using somatic cells directly from patients. In the past decade, various genetic cardiac disorders have been successfully modeled using iPSCs. For example, Timothy syndrome is caused by a missense mutation in L-type calcium channel $Ca_v1.2$ that leads to excess Ca^{2+} influx, prolonged action potential and irregular

contraction. Yazawa et al. (2011) reported the generation of iPSC-CMs derived from patients with Timothy syndrome, and these cells demonstrated irregular electrical activity and abnormal Ca^{2+} signaling similar to the cardiac phenotype found in the patients. iPSCs have also been used to model LEOPARD syndromes with *PTPN11* gene mutation (Carvajal-Vergara et al., 2010), long QT syndromes with a mutation in *KCNQ1* (Friedrichs et al., 2013), arrhythmogenic right ventricular dysplasia with *PKP2* mutations (Kim et al., 2013), and dilated cardiomyopathy with *TTN* mutations (Hinson et al., 2015).

One potential drawback of such application is that differences in the genetic background among cell lines may conceal the true phenotype induced by a single mutation. To address this challenge, the introduction of genome-editing techniques, such as clustered regularly interspaced short palindromic repeats-(CRISPR-) associated protein 9 (Cas9) system, help to generate isogenic iPSC lines, and allows researchers to study the precise effect of a mutation on the onset or progression of cardiac diseases while avoiding confounding genetic factors (Seeger et al., 2017; Lam and Wu, 2018). By correcting mutations or insertions, CRISPR/Cas9 further facilitates the elucidation of the causal role of the mutation. For example, CRISPR/Cas9 was successfully used to identify *SCN5A* as a causative mutation of arrhythmogenic right ventricular cardiomyopathy (ARVC), and corrected cells showed normal channel activity (Te Riele et al., 2017).

The heart is composed of multiple cell types, including cardiomyocytes and non-cardiomyocytes, and the intricate crosstalk and multifaceted regulation among cells are central to heart homeostasis and disease. In this perspective, co-culture of hiPSC-derivatives, 3-D organoids, EHTs and microfluidic organ-chips exhibit distinct advantages over mono-lineage cultures (Figure 2). Using relatively immature iPSC-CMs to represent adult-onset disorders remains challenging. Recent studies demonstrated that co-culture of fibroblasts, epicardial cells, or endothelial cells with hiPSC-CMs enhanced the maturity of hiPSC-CMs, which improved modeling of cardiac diseases (Bargehr et al., 2019; Giacomelli et al., 2020). Still, there are some limitations regarding regular 2-D culture, such as lacking a 3D extracellular matrix and a defined auxotonic load. 3-D cardiac constructs including organoids and EHTs overcome these limitations and improve representation of the *in vivo* cardiovascular environment. Cardiac organoids are generated from hPSCs that self-assemble and -organize into complex native-like organ structures (Richards et al., 2020). Voges et al. (2017) reported successful generation of an AMI model in human ESC-derived cardiac organoids. Cryoinjury induced local tissue damage, while the adjacent cells remained viable. Functional evaluation showed that tissue regeneration accompanied by functional recovery was seen 14-days post-injury (Voges et al., 2017). Another hiPSC-derived cardiac organoids that cooperate an oxygen-diffusion gradient and that are stimulated with neurotransmitter noradrenaline was shown to recapitulate the infarcted, border and remote zones of myocardial infarction in the human heart, with concurrent modeling of the hallmarks of AMI, including metabolic shifts, fibrosis and aberrant calcium handling (Richards et al., 2020).

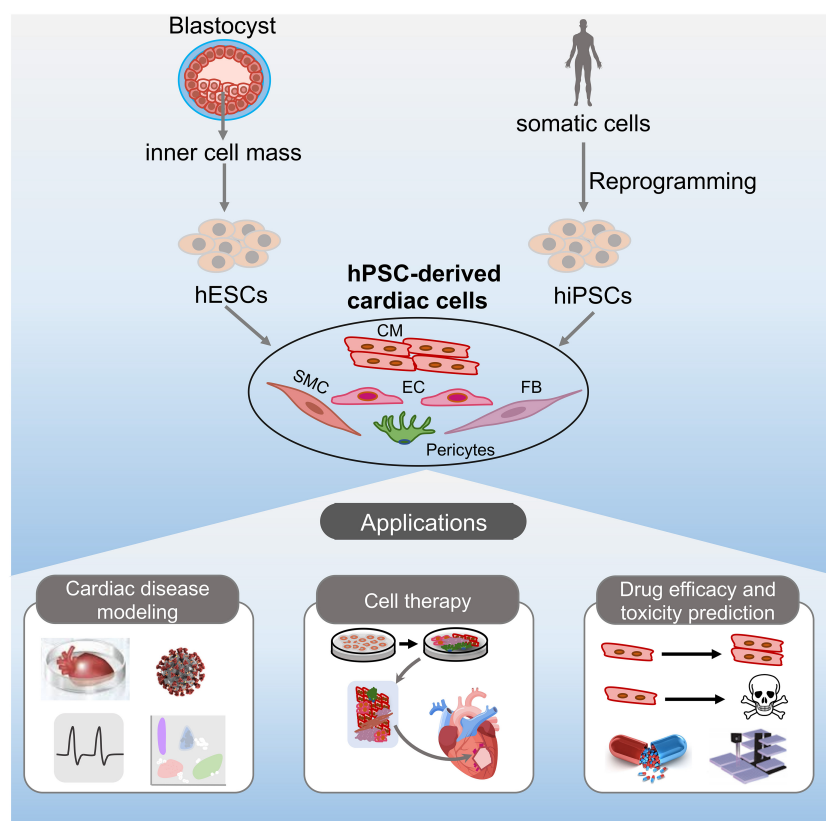


FIGURE 1 | Potential applications of hPSC-derived cardiac cells. hESCs or hiPSCs can differentiate into various cardiac cell types *in vitro*, including cardiomyocytes, endothelial cells, fibroblasts, smooth muscle cells, and pericytes. The resulting cells can be potentially used for disease modeling, cell therapy, and drug discovery.

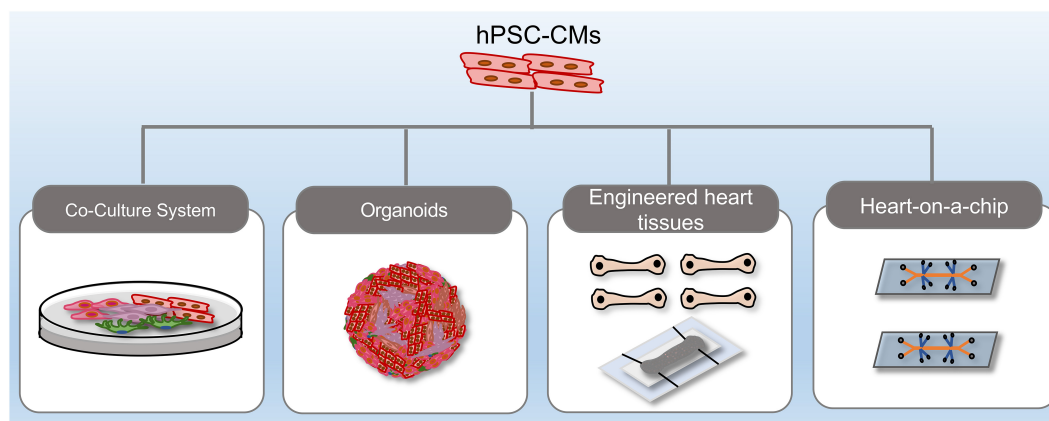


FIGURE 2 | Recent methodological advances in hPSC-derived complex platforms. hPSC-derived platforms have grown in complexity from simple, two-dimensional cultures into multi-lineage co-cultures, heart organoids, engineered heart tissues, and heart-on-a-chip systems.

Engineered heart tissues can be generated by mixing hPSC-derived cardiomyocytes with extracellular matrix components such as fibrinogen, collagen or Matrigel (Breckwoldt et al., 2017). The generation of EHTs also requires a casting mold that determines the 3-D shape of the heart tissue, and a support structure that provides mechanical restraint of the developing

heart tissue (Eder et al., 2016). EHTs are well suited to evaluate the effects of mechanical stimulation, and therefore can mimic afterload enhancement-induced disease conditions such as aortic valve stenosis, chronic hypertension, and cardiac hypertrophy (Hirt et al., 2012; Tzatzalos et al., 2016). Since EHTs are little heart muscles, they allow measurements of all essential parameters of

heart muscle function, including, but not restricted to, contractile force, conduction velocity, beating rate, rhythm, diastolic tension, passive tension, and intracellular Ca^{2+} transients (Hirt et al., 2014). Besides, EHTs are also compatible with histological analyses, protein detection, and sequencing techniques, such as single-cell RNA-sequencing.

The development of 3D stamping and bioprinting techniques provides scaffolds to generate heart-on-a-chip. EHTs and heart-on-a-chip methods allowed modeling of specific cardiac diseases, including Barth-syndrome-associated cardiomyopathy (Wang et al., 2014), Duchenne muscular dystrophy (Long et al., 2018) and primary hypertension-induced left ventricular hypertrophy (Zhao et al., 2019). Zhao et al. (2019) took advantage of organ-on-a-chip engineering and organoid self-assembly to generate mature ventricular tissue, and to perform electrical conditioning for up to 8 months, allowing modeling of chronic, polygenic conditions. With the generation of a wide variety of human organ-on-a-chip models, great efforts have been made to integrate multisensory systems. Zhang et al. (2017) reported two multiorgan models, liver- and heart-on-a-chip and heart-liver-cancer-on-a-chip, for automated and continual *in situ* monitoring of organoid behaviors.

Recently, hPSCs have also been proven quite useful in the study of COVID-19-related heart disorders. To date, this pandemic has led to more than 121 million infections and 2.6 million deaths worldwide. Apart from respiratory complications, COVID-19 is also known to induce cardiac complications, including myocardial injury, arrhythmias, acute coronary syndrome, which are major indicators of poor prognosis (Nishiga et al., 2020; Zheng et al., 2020). Since one of the major receptors for COVID-19 is not recognized by the virus in mice, hPSCs were rapidly recognized as an invaluable tool to address this problem, and has already yielded important insight into virus-host interactions, immune responses and the cytokine storm (Yang et al., 2020; Yiangou et al., 2020). Combined with various biochemical, cellular, molecular and genetic studies, these newly developed hPSCs-cardiac models will likely contribute much more to uncovering key mechanisms and therapeutic strategies of COVID-19-induced cardiac damage (Yiangou et al., 2020).

hPSC-BASED CELL THERAPY

Aside from mechanistic studies of cardiac disease, replenishing damaged myocardium with healthy cells (i.e., cell therapy) was another hotly pursued area of application. Transplantation of hESC-derived cardiomyocytes (hESC-CMs) was found to improve cardiac function in postinfarct rats and pigs (Kehat et al., 2004; Caspi et al., 2007; Laflamme et al., 2007). More importantly, Chong et al. (2014) showed that exogenously transplanted hESC-CMs remuscularized the infarcted area in a non-human primate model. However, hESCs may not be the best option for clinical treatment due to ethical concerns and immune rejection. Ethical concerns arise from their origins, since these cells are isolated from the inner cell mass of the human embryo, leading to the destruction of the latter. Immune rejection occurs due to allogenic transplantation of cells. By

contrast, iPSC technology, in which pluripotent stem cells are directly reprogrammed from the same patient's somatic cells, effectively circumvents the ethical issues associated with the use of ESCs. Additionally, iPSCs are considered autologous, and are believed not to require immunosuppression. Gene editing in hiPSC further helps to reverse disease phenotype by correction of the pathogenic mutation or variant, raising the possibility of personalized therapies for autologous stem cell transplantation.

Injecting hPSC-derived cardiac cells into the myocardium to replace dead cells appears to be a very straightforward concept. However, in practical terms, controlling the exact cell number, limited efficiency in cell delivery to target sites, and batch differences, all pose great challenges to the application of hPSC-based cell therapy (Matsuura et al., 2013; Guo et al., 2020). With the development of tissue engineering, stem cell-derived cell sheets gradually showed their superiority over direct injection in heart tissue repair (Matsuura et al., 2013). Kawamura et al. (2012) showed that highly pure (almost 90%) hiPSC-CMs sheets were able to attenuate left ventricular remodeling, increase neovascularization as well as inhibit fibrosis 8 weeks after cell transplantation into 12-week porcine models of myocardial infarction. The same group demonstrated that the combined use of cell sheets and the omental flap technique was beneficial even in treating severe heart failure (Kawamura et al., 2017). Most recently, it was reported that the transplantation of clinically relevant dimensions ($4\text{ cm} \times 2\text{ cm} \times 1.25\text{ mm}$) of human cardiac muscle patches significantly improved left ventricular function and reduced infarct size in infarcted swine (Gao et al., 2018).

Mechanistically, transplantation of hiPSCs-derived cardiac cells may provide a favorable microenvironment for pre-existing cells in the infarcted zone to proliferate, thus preventing serious post-MI events (Ye et al., 2014). The mechanisms by which injected cells exert these effects are still not fully clear. It has been shown that only a limited number of transplanted cells can differentiate and retain in the host myocardium after delivery, suggesting that the beneficial effects of cell therapy are mediated by the activation of paracrine pathways which leads to endogenous regeneration (Tachibana et al., 2017). The autocrine and paracrine factors facilitate angiogenesis, promote vascularization, attenuate fibrosis and relieve inflammation. Still, the clinical translation of hPSC-based cell therapy is facing a major problem, i.e., the immaturity of hPSC-derived cells, which can cause life-threatening arrhythmia, and teratoma formation (Cambria et al., 2017). Even vascularized cell sheets co-cultured with different types of cells exhibit an immature phenotype. As a source of *de novo* cardiomyocytes, hPSC-CMs have so far yielded only a short-term improvement in cardiac function ranging from weeks to months. Therefore, more advanced strategies to induce maturation, vascularization, and to improve durable cardiac function recovery are worth investigation.

hPSCs-BASED DRUG DISCOVERY

Human pluripotent stem cell technology is also widely used in cardiovascular drug discovery, providing pharmacologic and toxicologic predictions. Yazawa et al. (2011) found

that roscovitine, a compound that increases the voltage-dependent inactivation of CaV1.2, rescued the cardiomyocyte phenotypes in Timothy syndrome. Using hPSC-derived cardiomyocytes and organoids, compounds that promoted human heart muscle cell proliferation, but minimally affected heart rhythm and contractility, were identified (Mills et al., 2019). Although the identified compounds displayed poor pharmacokinetic properties, and were only maintained at pro-proliferative doses *in vivo* for a short period, they led to the elucidation of mevalonate pathway important for cardiomyocyte proliferation. Most recently, Theodoris et al. (2020) reported that a network-based screen in iPSC-derived cells revealed therapeutic candidates for a common form of heart disease involving the aortic valves, suggesting that the combined use of network-based screening, iPSC technique, and machine learning may represent an effective approach for drug discovery. Together, these inspiring hPSC-based drug studies offer meaningful pipelines to identify drug candidates that may lead to new therapeutic options for heart disease.

Unexpected cardiotoxicity is a major cause of drug attrition and drug withdrawal from the market (Laverty et al., 2011). Traditional methods of preclinical cardiac safety evaluation mainly rely on animal models, which tend to be expensive, low-throughput, and exhibit species differences in cardiac physiology (Mercola et al., 2013). Alternative methods to identify cardiotoxic drugs involve the heterologous expression of cardiac ion channels in non-cardiac cells. However, these non-cardiac cells lack CM-specific structural components such as sarcomeres, and inhibition of specific ionic currents alone cannot accurately measure the effects of drug candidates, which could miss potential arrhythmogenic effects or generate false-positive readings. Other cells, such as healthy and primary human CMs, may be used for preclinical evaluation of drug cardiotoxicity. However, sample access and cellular abundance may be prohibitive for its use in an industrial setting. Regulatory agencies around the globe, including US FDA, European EMA, Health Canada, and Japan NIHS, have recognized the lack of standardized assessment of cardiac safety, and thus encouraged the development of the Comprehensive *In Vitro* Proarrhythmia Assay (CiPA), which aims to explore the utility of hPSC-derived cardiomyocyte assays in evaluating cardiac safety and arrhythmogenesis (Sager et al., 2014). Data from recent studies support the utility of hPSC-derived cardiomyocytes for predicting drug-induced arrhythmia (Sager et al., 2014; Gintant et al., 2016; Blinova et al., 2018). Besides electrophysiology, hiPSC-CMs are also used to measure drug-induced alterations in cellular contractility and viability, thus broadening the scope of cardiac safety evaluation. Sharma et al. (2018) developed a detailed methodology to generate hiPSC-CMs and subsequently use these cells to evaluate drug-induced cardiotoxicity by using contractility and cytotoxicity assays. Using this platform, they performed cardiotoxicity screening of tyrosine kinase inhibitors, with results correlating with clinical phenotypes (Sharma et al., 2017). They also demonstrated that the observed toxicities could be ameliorated with cardioprotective insulin/IGF signaling.

Although hPSC-CMs raised hopes that this human test bed could broaden drug discovery approaches and improve preclinical drug testing, they are not identical with mature adult CMs. Important distinctions in ion channel function, gene expression, structural organization and functional responses to drugs limit their application for drug testing. Compared to standard 2D culture formats, engineered 3D heart tissues improve CM maturity, and exhibit a more physiological 3D muscle environment, longitudinal alignment, and easy access of measurements of force, which is one of the most important parameters of heart function (Eder et al., 2016). However, one major drawback of EHTs is their inability to scale up for the high-throughput screening of multiple samples in parallel. To some extent, EHTs in 24-well format or cardiac tissue miniaturization might provide possible solutions for drug testing purposes (Thavandiran et al., 2013; Eder et al., 2016).

Zhao et al. (2019) developed a scalable cardiac tissue cultivation platform that reconstructs a specific anatomic portion of the heart to facilitate more directed and accurate measurements of drug responses. “Human-heart-in-a-jar” is also a related technology, which involves embedding ventricular-like hiPSC-CMs in hydrogel to create an electromechanically coupled cardiac organoids chamber that is capable of pumping fluid. This organoid chamber is amenable to clinically relevant functional measurements, such as pressure-volume loop analysis, cardiac output, and ejection fraction (Li et al., 2018). As the field of iPSC technology continues to grow, to increase the efficiency of drug discovery and to reduce the cost, an established set of standards for the comparison of the quality of iPSC-CMs, quantification of cardiac contraction and electrophysiology, and validation of data and reproducibility, is desperately needed.

CHALLENGES AND FUTURE OUTLOOK

Despite major advancements in the application of PSC technology in cardiac diseases, several challenges remain, and require further in-depth research. As the structure, signaling, metabolism, and function in immature CMs are distinct from their adult counterparts, iPSC-CMs derived disease models might not be able to accurately reflect the true disease phenotype, making it difficult to confidently assess the efficacy or toxicity of drug candidates. Recently, various studies have tried to enhance the maturity of hiPSC-CMs via different means, including using T3 hormone, metabolic maturation, 3-D construction, mechanic stress, electrical stimulation, long-term culturing, and co-culture with other cell types such as cardiac fibroblasts and endothelial cells (Ahmed et al., 2020). However, it is worthwhile to point out that these “matured” cells still differ from adult human cardiomyocytes in many aspects, and therefore require careful data interpretation. Other limitations, such as scalability and clinical compatibility, also need to be addressed before one can faithfully extrapolate findings from such models. Developing optimal methods to efficiently generate large-scale mature hPSC-CMs is therefore of high importance and priority.

Furthermore, limited blood perfusion of cells within complex structures, especially for cell patches, is another issue yet to be

overcome. Otherwise, oxygen, nutrient and drug delivery, which all depend on the microvasculature, would be compromised. Simple co-culture of cardiomyocytes with endothelial cells only generates premature micro-vasculatures that are not sufficient to support perfusion, leading to the small size of cardiac organoids (i.e., 100 μm). To introduce microvascularization into cell patches, Schaefer et al. (2018) engineered a bilayer patch composed of a layer of human iPSC-CMs and a layer of human blood outgrowth endothelial cells. Implantation of this bilayer patch into infarcted rat hearts resulted in better CM survival compared to CM-only patch control. Importantly, after 4 weeks *in vivo*, the engrafted microvessels sprouted into the accompanying CM layer, and even became inosculated with the host vasculature. Qian et al. (2019) co-cultured human mesenchymal stem cells and endothelial cells in decellularized human dermal fibroblast sheets. However, without actual implantation *in vivo*, one can hardly infer the reparative effects of such patches purely based on biochemical data. Clearly, engineering heart-like tissues with intact microvasculature is still in its infancy. Once solved, *in vitro* cardiac models would be able to accommodate even more diversified functional assessments, offering greater versatility and yielding higher predictability.

CONCLUSION

In this review, we outlined the current applications of hPSC-CMs in disease modeling, cell therapy and drug discovery.

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- While hPSC technology has gained momentum in cardiac repair over the years, efforts to enhance the maturity of derived cells and to increase the complexity of tissue structure are still underway. Integrating genetic-, computer-, and bioengineering-based approaches will further empower this technique with greater precision, breadth and depth. It can be foreseen that through these interdisciplinary endeavors, hPSC technology will redefine *in vitro* cardiac modeling, promote personalized treatment, and increase the efficiency but reduce the cost of drug discovery.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Visualization and Identification of Bioorthogonally Labeled Exosome Proteins Following Systemic Administration in Mice

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Exosomes transport biologically active cargo (e.g., proteins and microRNA) between cells, including many of the paracrine factors that mediate the beneficial effects associated with stem-cell therapy. Stem cell derived exosomes, in particular mesenchymal stem cells (MSCs), have been shown previously to largely replicate the therapeutic activity associated with the cells themselves, which suggests that exosomes may be a useful cell-free alternative for the treatment of cardiovascular disorders. However, the mechanisms that govern how exosomes home to damaged cells and tissues or the uptake and distribution of exosomal cargo are poorly characterized, because techniques for distinguishing between exosomal proteins and proteins in the targeted tissues are lacking. Here, we report the development of an *in vivo* model that enabled the visualization, tracking, and quantification of proteins from systemically administered MSC exosomes. The model uses bioorthogonal chemistry and cell-selective metabolic labeling to incorporate the non-canonical amino acid azidonorleucine (ANL) into the MSC proteome. ANL incorporation is facilitated via expression of a mutant (L274G) methionyl-tRNA-synthetase (MetRS*) and subsequent incubation with ANL-supplemented media; after which ANL can be covalently linked to alkyne-conjugated reagents (e.g., dyes and resins) via click chemistry. Our results demonstrate that when the exosomes produced by ANL-treated, MetRS*-expressing MSCs were systemically administered to mice, the ANL-labeled exosomal proteins could be accurately and reliably identified, isolated, and quantified from a variety of mouse organs, and that myocardial infarction (MI) both increased the abundance of exosomal proteins and redistributed a number of them from the membrane fraction of intact hearts to the cytosol of cells in infarcted hearts. Additionally, we found that Desmoglein-1c

is enriched in MSC exosomes and taken up by ischemic myocardium. Collectively, our results indicate that this newly developed bioorthogonal system can provide crucial insights into exosome homing, as well as the uptake and biodistribution of exosomal proteins.

Keywords: exosomes, extracellular vesicles, mesenchymal stem cells, bioorthogonal labeling, click reaction, cardiac ischemia

INTRODUCTION

The therapeutic benefits of stem cells are mediated primarily via the secretion of paracrine factors (Bagno et al., 2018), and recent evidence suggests that the exosomes produced by mesenchymal stem cells (MSCs) largely replicate the improvements associated with administration of the cells themselves in large-mammal models of myocardial injury (Charles et al., 2020). Thus, while exosomes could be a useful cell-free alternative to MSC therapy, little is known of how exosomes are directed toward injured tissue, the distribution of the exosomal cargo in target organs and cells, or the physiological mechanisms that are induced by exosome uptake. This information is crucial for identifying the appropriate dose, timing, and route of administration for exosome therapy but remains difficult to decipher, as techniques for tracking exosomes and the exosome cargo are limited. Radiolabeled exosomes have been used to determine the half-life of exosomes in the circulation and to identify some of the major sites of exosome uptake (Morishita et al., 2015), but they cannot track the presence of exosome proteins in target organs and are dependent on half-life of the radiolabel. Thus, methods for accurately and reliably identifying both the source and targets of the exosomal cargo in living organisms will dramatically advance exosome research (Mathieu et al., 2019).

Cell-selective metabolic labeling generates proteins containing non-canonical amino acids with functional groups that can be linked to affinity reagents or fluorescent dyes for subsequent identification, isolation, and imaging. It was previously shown that the mutant *E. coli* methionyl-tRNA synthetase (MetRS^{L274G} or MetRS*) selectively appends the azide-bearing non-canonical amino acid azidonorleucine (ANL) to tRNA. Thus, when cells that express MetRS* are incubated with ANL, they produce proteins that contain ANL in lieu of endogenous methionine (Ngo et al., 2009) and can be covalently linked to alkyne-tagged reagents (e.g., dyes and resins) via click chemical reactions, such as copper-catalyzed azide-alkyne cycloaddition. These non-canonical amino acids are not produced *in vivo* and as such are excluded during normal protein synthesis.

For this study, we used non-canonical amino-acid labeling to develop an *in vivo* model that enables us to visualize, track, and measure the biodistribution of proteins from systemically administered MSC exosomes. MSCs were transfected with a lentiviral vector coding for MetRS* and incubated with ANL-supplemented media; then, exosomes were isolated from the MSC-conditioned media and intravenously administered to mice. Our results demonstrate that the ANL-labeled exosomal proteins can be accurately and reliably identified, isolated, and quantified from a variety of mouse organs, and that

surgically induced myocardial infarction (MI) both increased the abundance of labeled exosomal proteins and redistributed the localization from the membrane to the cytosol within organs following systemic administration.

MATERIALS AND METHODS

MSC Culture and Characterization

Mouse (C57BL/6) bone marrow MSCs were purchased from Cyagen Biosciences (Sunnyvale, CA, United States, Catalog Number: MUBMX-01001), cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and passaged 5–8 times before use. MSC identity was confirmed via flow-cytometry analyses of MSC surface marker expression and assessments of osteogenic and adipose differentiation potential. Differentiation assays were performed by following the manufacturer's protocols (Cyagen Biosciences, United States). Briefly, the cells were cultured on 0.1% gelatin-coated six-well plates (2×10^4 cells/cm²) in complete medium until confluent and then treated with osteogenic differentiation medium for 3 weeks or with adipocyte induction medium and maintenance medium for 2–3 weeks; osteogenic and adipose differentiation were analyzed by Alizarin staining and by Oil Red O staining (for lipid droplets), respectively.

Flow Cytometry

Flow cytometry was performed as previously described (Cheng et al., 2019). Briefly, 1×10^6 cells were incubated in 200 μ L cold phosphate-buffered saline (PBS) with Fc block (1:100 dilution), washed three times, stained with phycoerythrin (PE)-conjugated antibodies for mouse CD34 (1:50) or CD44 (1:50), or with fluorescein isothiocyanate (FITC)-conjugated antibodies for mouse CD11b (1:50), CD45 (1:50), or Sca-1 (1:50) (ebioscience, Germany). Data were acquired with a BDLSR II (BD Biosciences, United States) and analyzed with Cell Quest Software (Becton Dickinson, United Kingdom).

qRT-PCR

Quantitative reverse transcription PCR (qRT-PCR) was performed as previously described (Tang et al., 2009). Briefly, total MSC RNA was reverse transcribed with random hexamer primer, and 25 ng of the reverse-transcribed product was used for RT-PCR; results with the random primer were consistent with those obtained previously with a primer common to the 3' untranslated region (UTR) of Dsg1-a,b, and -c. Values were normalized to equivalent assessments of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA abundance.

Western Blotting

For protein extraction, 1×10^7 cells or 100 mg of frozen tissue were homogenized in 1 mL RIPA lysis buffer containing protease inhibitors (Sigma, 4693132001) and phosphatase inhibitors (Sigma, 4906837001). Samples were incubated with agitation for 30 min at 4°C and centrifuged at 13,000 rpm for 10 min at 4°C; then, the protein concentration in the supernatant was determined via bicinchoninic acid (BCA) assay (Pierce). For immunoblotting, proteins in the supernatant were denatured by heating at 95°C for 10 min, separated by SDS-PAGE, and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was incubated in 5% nonfat milk blocking buffer (tris-buffered saline [TBS]) for 1 h, incubated with primary antibody in TBS-containing 3% bovine serum albumin (BSA) overnight at 4°C, washed three times with TBS (0.5% Tween 20), incubated with secondary antibody, washed with TBS Tween 20, and then developed with Enhanced Chemiluminescence Detection Reagents (ECL, Thermo Fisher). Protein signals were imaged with a Bio-Rad ChemiDoc System.

Generation of Lenti-CAG-MetRS^{L274G}-mCherry Vector

The Lenti-CAG-MetRS^{L274G}-mCherry vector was newly engineered in our lab by using a pLenti.CAG.H2B-Dendra2.W (Addgene #51005) backbone and the MetRS^{L274G}-mCherry coding sequence from pMaRSC (Addgene #89189). Briefly, pLenti.CAG.H2B-Dendra2.W was amplified via high-fidelity PCR with 5' CAG-WPRE-5 primers (containing a sequence complementary to the WPRE region of the vector sequence with an AscI-AgeI-MluI-EcoRV restriction-site overhang) and 3' CAG-LINK-3 primers (containing a sequence complementary to the SalI and upstream region of the vector sequence with an EcoRV-MluI-AgeI-AscI restriction-site overhang). The 7.9-kb PCR product, which included the majority of the pLenti.CAG.H2B-Dendra2.W sequence but lacked the 1.1-kb H2B-Dendra2.W-coding region, was transformed into Top 10 competent cells (Thermo Scientific), where it was circularized via self-assembly cloning (Matsumoto and Itoh, 2011) into a pLenti.CAG.linker (containing multi-cloning sites SalI-AscI-AgeI-MluI-EcoRV) and verified by MluI enzyme digestion. To clone the MetRS^{L274G}-mCherry coding sequence, PCR was performed with 5' SalI-L274G-mCherry 5 primers, 3' AgeI-L274G-mCherry 3 primers, and the pMaRSC template (Addgene #89189) for 20 cycles; each cycle consisted of 30 s at 95°C, 30 s at 60°C, and 4 min at 72°C. The 4-kb PCR product, which contained the MetRS^{L274G}-mCherry coding sequence, was subsequently cloned into TA cloning vector PCRTM 2.1 (Thermo Scientific), and positive clones were verified by digestion with SalI and AgeI; then, the 4-kb MetRS^{L274G}-mCherry fragment was recovered with agarose gel and ligated into the SalI/AgeI-digested pLenti.CAG.linker to produce pLenti.CAG-MetRS^{L274G}-mCherry, which was confirmed by DNA sequencing. The Lenti.CAG-MetRS^{L274G}-mCherry vector was produced by co-transfecting pLenti.CAG-MetRS^{L274G}-mCherry with pMD2.G and psPAX2 into 293FT cells, and

then purified and concentrated as previously described (Cheng et al., 2010). All primer sequences are reported in **Supplementary Table 1**.

Lentivirus Transduction and ANL Labeling of Nascent Cellular Proteins

For lentivirus transduction, MSCs were seeded at ~80% confluence, and the lentivirus (MOI = 4) was applied with polybrene (8 µg/mL); transduction efficiency was evaluated 72 h later by monitoring mCherry fluorescence with a fluorescence microscope or via fluorescence-activated cell-sorting (FACS); control assessments were conducted with MSCs that had been transduced with Lenti.CAG.H2B-Dendra2.W. For ANL labeling, cells were washed twice in PBS and then suspended in methionine-, glutamine-, and cystine-depleted DMEM (Gibco, Cat. #21013024) containing 1 mM ANL (IRIS Biotech, Cat. #159610-92-1); the DMEM had been made complete with glutamine and cystine and supplemented with exosome-free FBS (Gibco, Cat. #A270801). Cells were cultured for 72 h before use in subsequent experiments.

Bio-Orthogonal Non-canonical Amino Acid Tagging (BONCAT)

MetRS*-transduced MSCs were incubated in 1 mM ANL for 24 h and lysed in RIPA buffer supplemented with protease inhibitors (Sigma, United States); then, total protein was collected, and the Click-iT reaction was performed with a Click-iT Protein Reaction Buffer Kit (Invitrogen, Waltham, MA, United States) as directed by the manufacturer's instructions. Briefly, up to 200 µg of ANL-labeled protein was reacted with 100 µL of Click-iT reaction buffer containing alkyne-Cy7 and copper-sulfate catalyst in a rotator for 20 min. Methanol and chloroform were added to remove residual reaction components and precipitate the proteins; then, the precipitated proteins were solubilized in Laemmli buffer and loaded for gel electrophoresis. Gels were immediately imaged with a ChemiDoc MP Imaging System (Bio-Rad, California, United States) under both stain-free mode and Cy7 mode.

Fluorescent Non-canonical Amino-Acid Tagging

For fluorescent non-canonical amino-acid tagging (FUNCAT) of MetRS*-transduced MSCs *in vitro*, MSCs were cultured in 1 mM ANL (Jena Bioscience, Jena, Germany) for 24 h, gently rinsed with PBS, fixed in 4% formaldehyde/PBS and permeabilized with 0.5% Triton X-100/PBS; then, the Click-iT reaction was performed with Click-iT reaction cocktails containing Alexa Fluor 488 alkyne (Click-iT Alexa Fluor 488 Protein Synthesis HCS Assay kit, Life Technologies, Waltham, MA, United States) as directed by the manufacturer's protocol. Cells were stained with Hoechst 33342 prior to imaging. For FUNCAT of MetRS* exosomal proteins *in vivo*, the general methodology was adapted from previous studies (Ngo et al., 2013). Briefly, mice were euthanized 2 h after exosome administration; then, the hearts were explanted, perfused, fixed

by incubation in 4% paraformaldehyde at room temperature for 4 h, cryoprotected in 30% sucrose/PBS solution overnight, frozen in OCT compound, cut into 5- μ M-thick sections, and stored at -20°C . Frozen sections were incubated at room temperature for 30 min, washed once with PBS, permeabilized in 0.25% Triton/PBS for 35 min, washed in PBS (3 washes, 5 min each), blocked in commercial blocking buffer (Thermo Scientific, TA-125-PBQ) for 7 min, and washed in PBS again (3 washes, 5 min each); then, the ANL-labeled proteins were conjugated to biotin-alkyne by incubating the sections at 4°C overnight in a 1:10,000 dilution of biotin-alkyne in 2 $\times$ Catalyst solution from the Click-iT Enrichment kit (Invitrogen, Cat. #C10416). Sections were thoroughly washed in PBS (5 washes, 5 min each) to remove residual biotin, sequentially incubated with primary biotin antibodies (1:1000 dilution) at 4°C overnight and with secondary antibodies at 4°C overnight, washed PBS (three times), incubated with Sudan Black for 30 min, and then washed again in PBS (three times).

Isolation and Characterization of MSC Exosomes

MetRS⁺ MSCs were plated at $\sim 50\%$ confluence in complete DMEM for 24 h, after which they were cultured in methionine-depleted DMEM supplemented with 1 mM ANL for 72 h. Conditioned media was collected from the MSCs, spun at 1,000 G to remove cell debris, passed through a 0.22-micron filter, and ultracentrifuged at 120,000 G for 2 h; then, the supernatant was removed and replaced with cold PBS, and the exosomes were ultracentrifuged at 120,000 G for 2 h. Exosome pellets were resuspended in 10 mL of cold PBS and stored at -80°C until use in subsequent experiments. The size and quantity of freshly isolated exosomes was estimated via nanoparticle-tracking analyses (NanoSight), and morphological assessments were conducted via electron microscopy as previously described (Cheng et al., 2019).

Mouse MI Model and Intravenous Exosome Delivery

All animal experiments in this report were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham and performed in compliance with the National Institutes of Health (NIH) publication *Guide for the Care and Use of Laboratory Animals*. Twelve- to 16-week-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, United States) were used for all experiments unless otherwise specified. For surgically induced MI, mice were intubated and connected to a ventilator, and anesthesia was initiated and maintained with inhaled 2% isoflurane USP (FlurisoTM, VetOne). The heart was exposed via left thoracotomy, and MI was induced by permanently ligating the left-anterior descending (LAD) coronary artery, as previously described (Qin et al., 2006); sham-operated animals underwent the identical surgical procedure, except LAD artery ligation was omitted. After chest closure, buprenorphine hydrochloride (0.1 mg/kg, Buprenex, Reckitt Benckiser Pharmaceuticals Inc.) and carprofen (5 mg/kg, Rimadyl, Zoetis) were administered via intraperitoneal

injection every 12 h for 3 days. Exosomes (10×10^9 in 200 μL PBS) were delivered via tail-vein injection 24 h after MI or Sham surgery, and mice were euthanized via CO_2 inhalation.

Isolation of ANL-Labeled Proteins From Whole Organs (Click-Catalyzed Alkyne-Agarose Capture)

Animals were euthanized via CO_2 inhalation and perfusion with 10 mL cold PBS; then, the heart, brain, lung, kidney, liver, and spleen were isolated, cut into ~ 20 -mg sections, rinsed twice in PBS, and flash frozen with liquid nitrogen. Membrane and cytosolic total protein fractions were isolated with a Mem-PER Plus Membrane Protein Extraction kit (Thermo Scientific, Cat. #89842), precipitated via methanol-chloroform extraction, and stored at -80°C . For ANL-labeling and MetRS⁺ protein capture, fractions were resolubilized in Click-iT buffer solution and processed as directed by the Click-iT Protein Enrichment Kit protocol (Invitrogen, Cat. #C10416). Captured peptides were stored at -80°C until identification via liquid chromatography mass spectrometry (LC-MS). To collect protein samples from non-administered exosomes, $\sim 1 \times 10^9$ exosomes were suspended in 100 μL PBS and prepared in parallel with organ samples for LC-MS identification.

Sample Preparation and Data Acquisition by Liquid Chromatography Mass Spectrometry

Dried peptides were reconstituted in 16 μL of 0.1% formic acid (FA); then, 8 μL of each sample was injected onto a 1,260 Infinity nano high-performance liquid chromatography (nHPLC) stack (Agilent Technologies, Santa Clara, CA, United States), and peptides were separated on a 75-micron (internal diameter) by 15-cm pulled tip C-18 column (Jupiter C-18 300 $\text{\AA}$, five micron, Phenomenex) that was attached to a Thermo Orbitrap Velos Pro hybrid mass spectrometer equipped with a nano-electrospray source (Thermo Fisher Scientific, Waltham, MA, United States). All data were collected in CID mode, and the nHPLC was configured with binary mobile phases consisting of solvent A (0.1%FA in ddH₂O) and solvent B (0.1% FA in 15% ddH₂O / 85% ACN) programmed as follows; 10 min at 2% solvent B; 90 min at 5–40% solvent B; 5 min at 70% solvent B; and 10 min at 0% solvent B. After each parent ion scan (300–1,200 m/z; 60 k resolution), fragmentation data (MS2) was collected for the 15 most-intense ions. For data dependent scans, charge state screening and dynamic exclusion were enabled with a repeat count of two, repeat duration of 30 s, and exclusion duration of 90 s.

MS Data Conversion and Searches

XCalibur RAW files were collected in profile mode, centroided, and converted to MzXML with ReAdWv.3.5.1; then, the mgf files were created by using MzXML2Search (included in TPP v. 3.5) for all scans. Data was searched via SEQUEST, which was set for two maximum missed cleavages, a precursor mass window of

20 ppm, trypsin digestion, and variable modifications C and M at 57.0293 and 15.9949, respectively. Searches were performed with a specific subset of the UniRef100 database.

Peptide Filtering, Grouping, and Quantification

The lists of peptide identifications (IDs) generated via SEQUEST (Thermo Fisher Scientific, Waltham, MA, United States) were filtered with Scaffold (Protein Sciences, Portland, Oregon, United States). Scaffold generates and retains only high-confidence IDs while producing normalized spectral counts across all samples, and the spectral counts can subsequently be used for relative quantification. Only peptides with charge states $\geq 2+$, minimum lengths of six amino acids, and nonzero quantities for all six mass tags were analyzed. For large datasets, Scaffold incorporates the two most common methods for statistical validation: false discovery rate (FDR), and protein probability; FDR was set at $<1\%$ cutoff, individual peptide probabilities were ≥ 0.8 , protein probabilities were ≥ 0.99 , and at least two peptides were assigned per protein. Relative quantification was performed via spectral counting and spectral count abundances were normalized between samples.

Bioinformatics and Statistics Analysis

For protein abundance data, the Pearson coefficient was calculated to evaluate correlations in protein expression between samples. Hierarchical clustering of samples and protein profiles was based on the complete linkage method, and differences in protein expression between the MI and Sham groups was evaluated with the Mann Whitney *U*-test. Differentially expressed proteins (DEPs) were defined as those with nominal *p*-value $< 5\%$ and fold-change > 1.5 , and were visualized via heatmaps. For

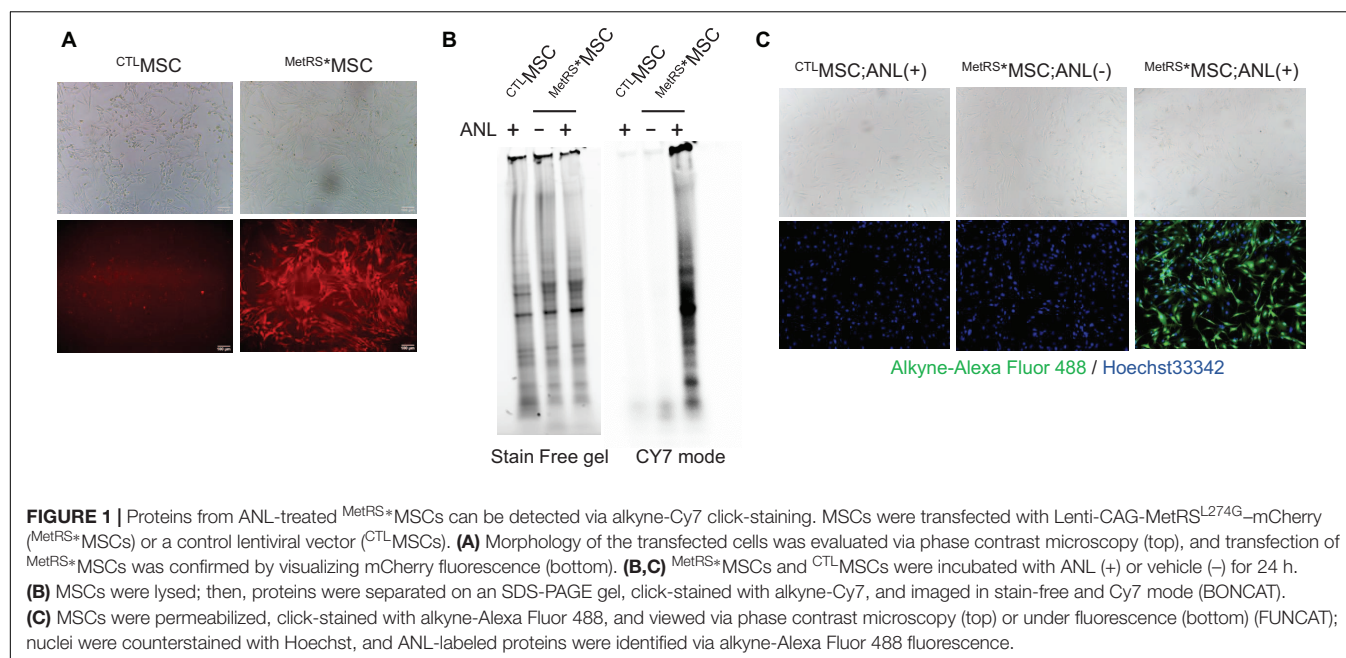
proteins that were detected in $\geq 60\%$ of one group and absent in the other group, we used the method developed by Kojima et al. (2012), with a more stringent cutoff ($> 60\%$ in only one group); proteins meeting this criteria were defined as “all-or-nothing” hits and included in the list of DEPs.

RESULTS

MetRS* Expression in MSCs Facilitates ANL-Labeling of Nascent MSC Proteins

Murine MSCs were obtained from a commercial vendor, and the characteristic pattern of MSC marker expression (i.e., positive for Sca-1 and CD44; negative for CD11b, CD45, and CD34) was verified via flow-cytometry (Supplementary Figure 1A). Phase-contrast microscopy confirmed that the cells were uniformly spindle-shaped, and robust multipotency was verified by differentiating the cells into adipocytes and osteocytes (Supplementary Figures 1B,C). To facilitate non-canonical amino acid tagging, MSCs were transfected with our newly generated MetRS* lentiviral vector (Lenti-CAG-MetRS^{L274G}-mCherry). The vector also coded for mCherry, which enabled stably transfected cells (MetRS* MSCs) to be selected via flow cytometry for mCherry fluorescence, and proteins produced by the MetRS* MSCs were labeled with ANL by incubating the selected cells in $1 \mu\text{M}$ ANL-supplemented media. Control assessments were conducted with MSCs that had been transfected with an equivalent vector that lacked the MetRS^{L274G}-mCherry cassette (CTL-MSCs).

Widespread mCherry expression was observed after just a single transfection (Figure 1A), and there were no apparent morphological differences between MetRS* MSCs and CTL-MSCs after 72 h of incubation in the ANL-supplemented media.



To confirm that ANL was incorporated into the ^{MetRS*}MSC proteome, BONCAT was conducted with lysates from ANL-treated ^{MetRS*}MSCs and ^{CTL}MSCs: proteins in the lysate were separated on a gel and covalently stained with alkyne-conjugated Cy7. Overall protein levels in the two MSC populations were equivalent; however, the alkyne-Cy7 stain was only observed in proteins from ^{MetRS*}MSC lysates, which spanned a wide spectrum of masses (**Figure 1B**). The results from our BONCAT assessments were also corroborated in intact cells via FUNCAT: the cells were permeabilized, and then click reactions were conducted with a cocktail containing Alexa Fluor 488 alkyne. The fluorescent signal (i.e., ANL-incorporated protein) was highly abundant in the cytosol of ^{MetRS*}MSCs but completely absent in ^{CTL}MSC cytosol (**Figure 1C**).

ANL-Alkyne Click Reactions Detect ANL-Labeled Proteins From ^{MetRS*}MSC Exosomes With High Sensitivity and Specificity

Assessments via nanoparticle-tracking analysis (**Figure 2A**) and transmission electron microscopy (**Figure 2B**) confirmed that treatment with ANL-supplemented media (1 μ M for 72 h) did not substantially alter the production, size, and ultrastructure of exosomes produced by ^{MetRS*}MSCs. Furthermore, when exosomes were collected from ^{MetRS*}MSCs (i.e., ^{MetRS*}MSC-Exos) and ^{CTL}MSCs (^{CTL}MSC-Exos) that had been grown

in media supplemented with or without ANL, BONCAT assessments of their protein cargo indicated that the total amount of protein in all four groups was similar, but alkyne-Cy7 fluorescence was only detected for proteins in exosomes from ANL-treated ^{MetRS*}MSC-Exos (**Figure 2C**). Thus, ANL labeling and subsequent detection was both sensitive and specific for proteins in ^{MetRS*}MSC-Exos.

ANL-Alkyne Click Reactions Detect ANL-Labeled Proteins From Systemically Administered ^{MetRS*}MSC-Exos in the Organs of Mice

To confirm that our method for detecting ANL-labeled ^{MetRS*}MSC-Exo proteins could effectively monitor the trafficking of proteins from systemically administered ^{MetRS*}MSC-Exos in vivo, exosomes were isolated from ANL- or vehicle-treated ^{MetRS*}MSCs and intravenously delivered into mice (20 μ g/mouse); 2 h later, cardiac tissues were harvested, sectioned, and analyzed via FUNCAT. The fluorescent signal was strongly observed in both the vasculature and cardiomyocytes of mice that received the ANL-treated ^{MetRS*}MSC-Exos but was completely absent in sections from mice that were administered vehicle-treated ^{MetRS*}MSC-Exos (**Figure 3**).

Having confirmed the fidelity of click-catalyzed ANL-staining in our *in vivo* model, we investigated whether ANL-labeled exosome proteins could be isolated from the organs of mice after

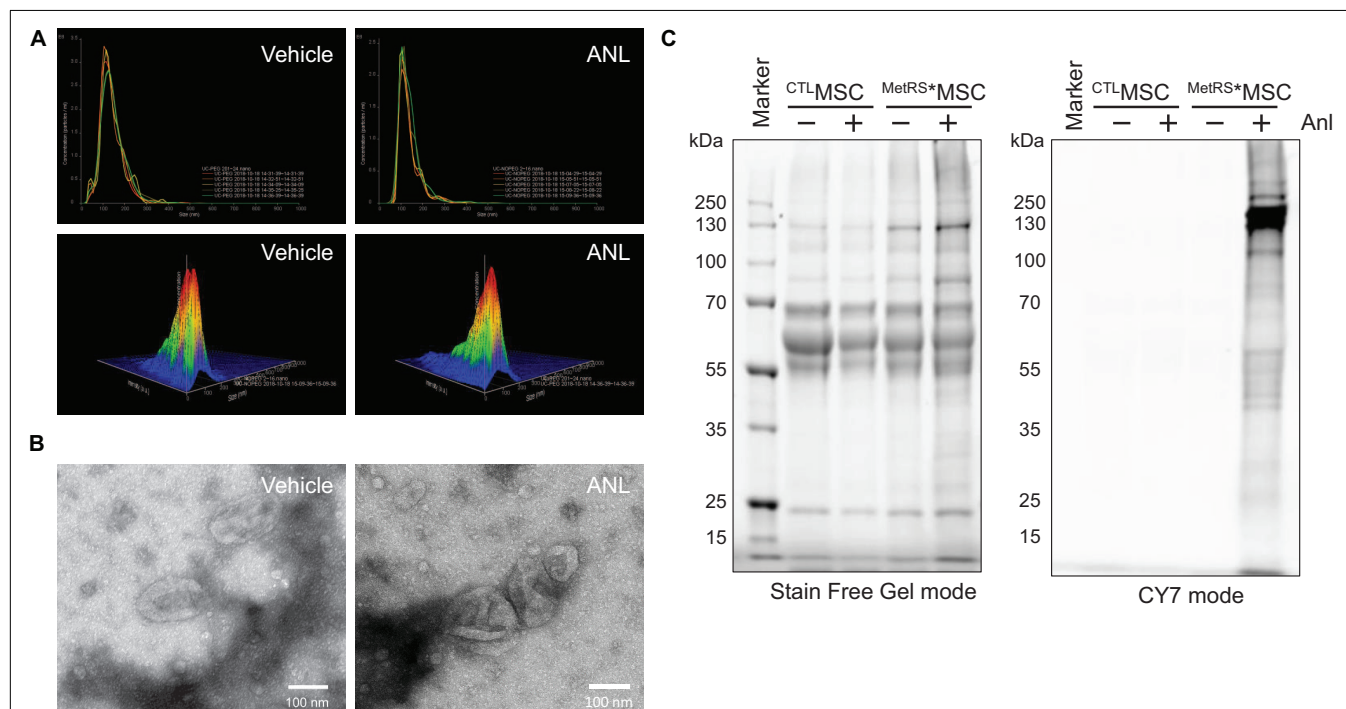


FIGURE 2 | Exosomal proteins from ANL-treated ^{MetRS*}MSCs can be detected via alkyne-Cy7 click-staining. **(A,B)** The ^{MetRS*}MSCs were incubated with ANL (+) or vehicle (–) for 24 h; then, exosomes were isolated from culture media and evaluated via **(A)** nanoparticle-tracking analysis and **(B)** transmission electron microscopy (TEM). **(C)** Exosomes were isolated from ^{CTL}MSCs and ^{MetRS*}MSCs that had been incubated with ANL (+) or vehicle (–) for 24 h; then, the exosomes were lysed, and proteins were separated on an SDS-PAGE gel, click-stained with alkyne-Cy7, and imaged in stain-free and Cy7 mode (BONCAT).

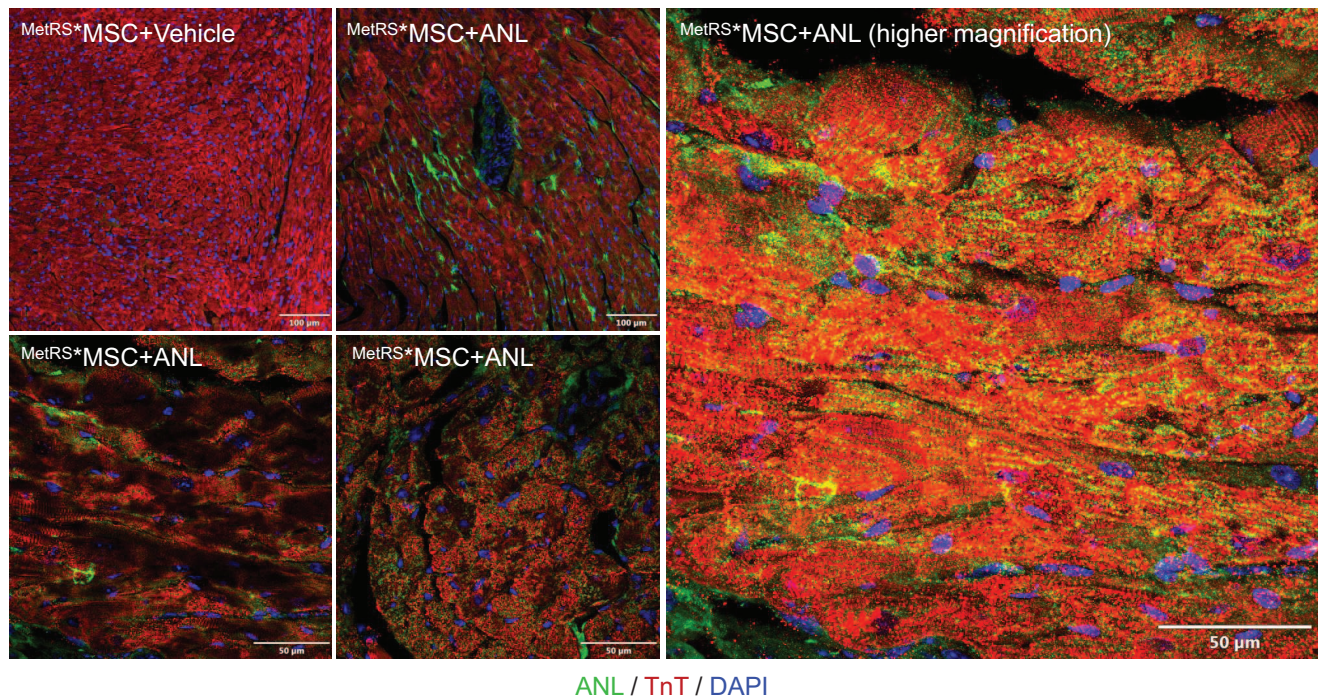


FIGURE 3 | Proteins from systemically administered ^{MetRS*}MSC-Exos can be visualized in mouse hearts via click-catalyzed alkyne-Alexa Fluor 488 staining. Exosomes were isolated from the culture media of ANL- or vehicle-treated ^{MetRS*}MSCs and injected into the tail vein of mice. Hearts were explanted 2 h later, sectioned, click-stained with reaction cocktails containing Alkyne-conjugated Alexa Fluor 488 (green), and counterstained with anti-Troponin T (red) and Hoechst (blue); regions of overlapping Alexa Fluor 488 and Troponin T fluorescence appear yellow.

^{MetRS*}MSC-Exo administration. Proteins were precipitated from whole mouse organs (heart, kidney, spleen, lung, liver, and brain), and then ^{MetRS*}MSC-Exo proteins (i.e., proteins containing the ANL-label) were captured from the precipitate with an agarose resin containing alkyne functional groups that, when catalyzed via click reaction, formed covalent links with the ANL label. Because the ^{MetRS*}MSC-Exo proteins comprised only a very small fraction of the total amount of protein in each organ, we confirmed the specificity and accuracy of click-catalyzed alkyne-agarose capture by comparing the data for each individual ^{MetRS*}MSC-Exo protein to data for the same protein in the corresponding organs from ^{MetRS*}MSC-Exo-treated animals when the click catalysis reaction was omitted and from animals that were not treated with ^{MetRS*}MSC-Exos.

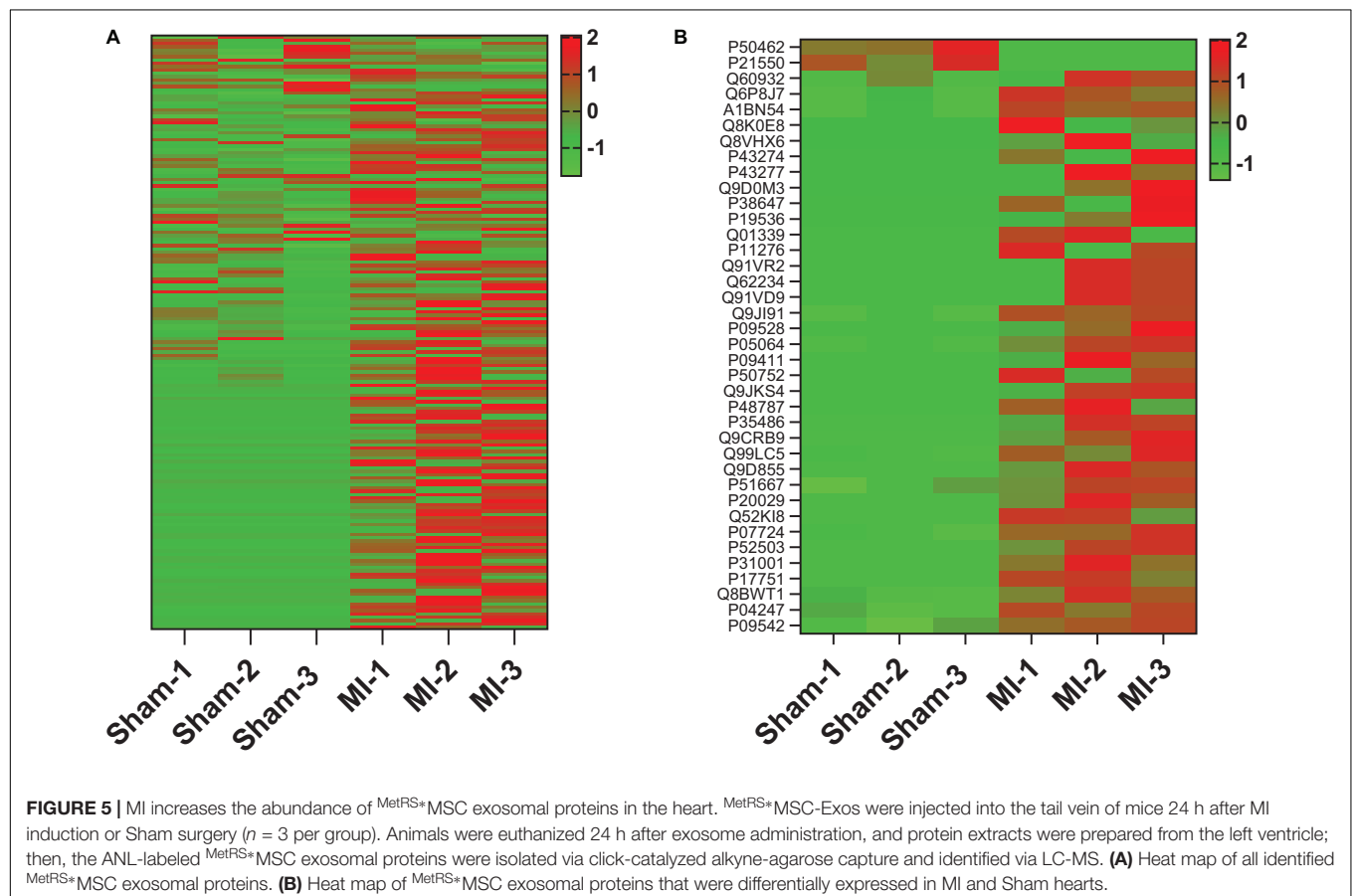
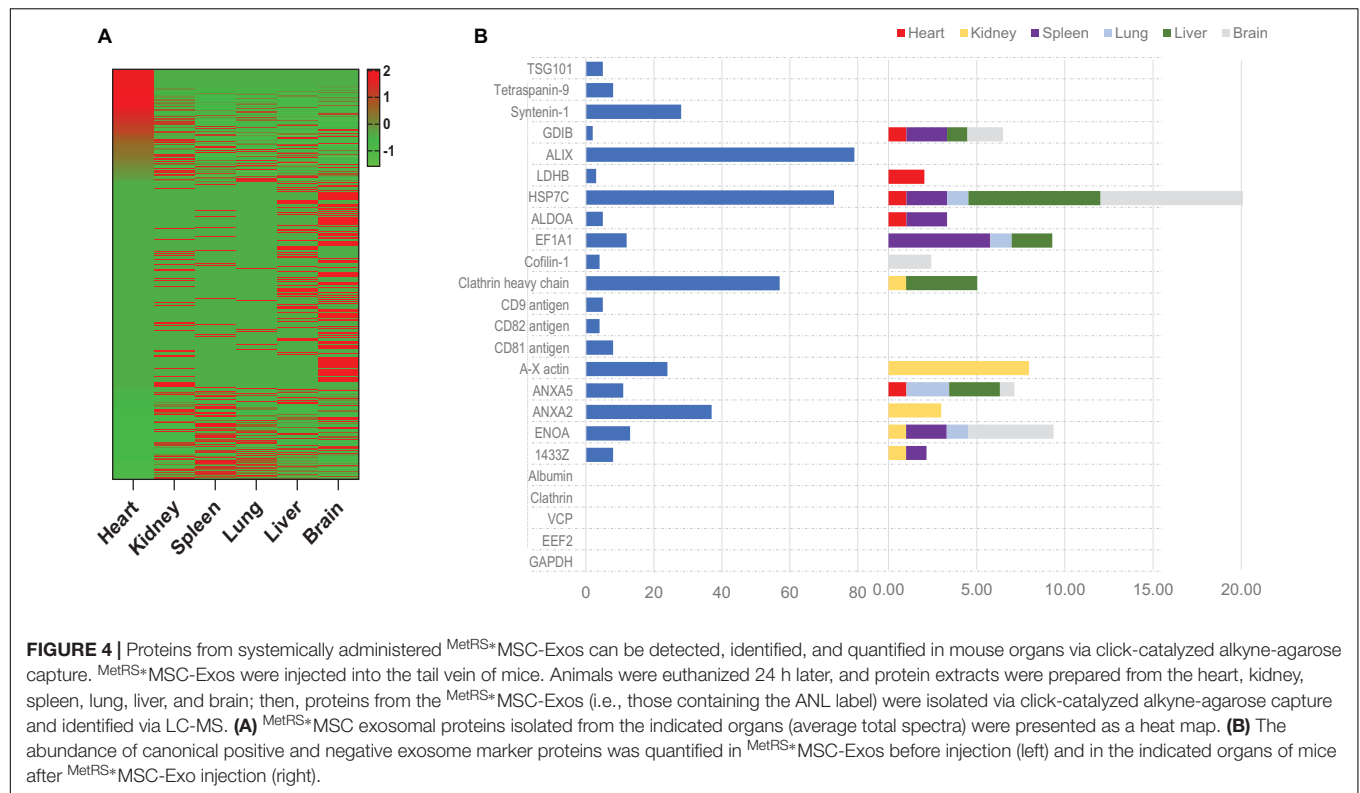
LC-MS analysis identified 205 ^{MetRS*}MSC-Exo proteins that were isolated from the hearts of ^{MetRS*}MSC-Exo-treated mice exclusively via click-catalyzed alkyne-agarose capture, compared to 29 proteins that were captured only when the click reaction was omitted, and 48 that were isolated both with and without click catalysis (Figure 4A); the abundance of each individual ^{MetRS*}MSC-Exo protein varied widely among organs (Figure 4B). Notably, most (but not all) of the exosome-marker proteins that were present in ^{MetRS*}MSC-Exos before administration (Supplementary Table 2) were also isolated from at least one of the organs of ^{MetRS*}MSC-Exo-treated mice, but they were not equally distributed across all organs (Figure 4C).

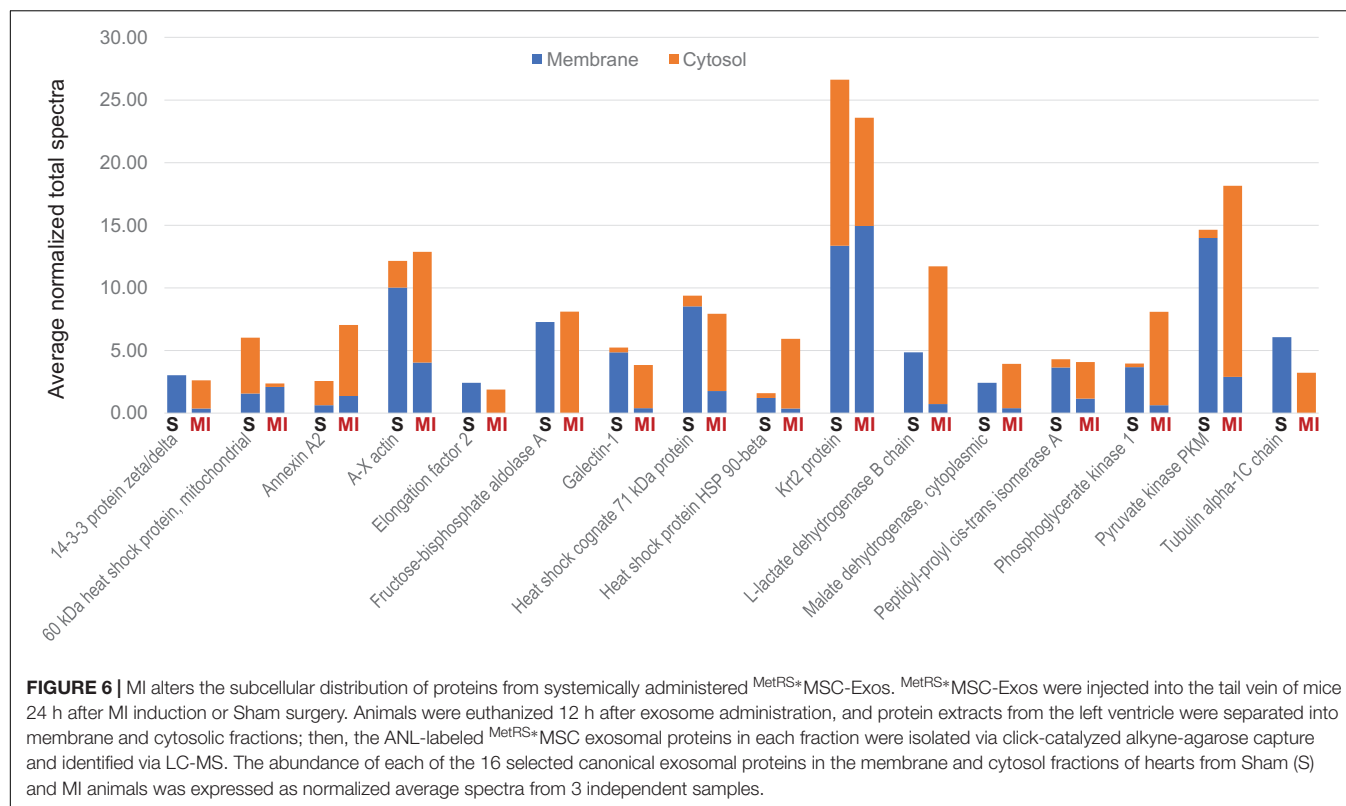
MI Increases Cardiac Uptake and Alters the Subcellular Distribution of ANL-Labeled ^{MetRS*}MSC-Exo Proteins

To determine whether tissue injury changes the biodistribution of MSC exosomes, ANL-labeled ^{MetRS*}MSC-Exos were administered to mice via tail-vein injection 24 h after surgically induced MI (the MI group) or sham surgery (the Sham group). Cardiac tissue was collected 12 h after ^{MetRS*}MSC-Exo administration, and ^{MetRS*}MSC-Exo proteins were isolated via click-catalyzed alkyne agarose capture. The number of ^{MetRS*}MSC-Exo proteins was dramatically greater in hearts from MI than from Sham animals (Figure 5A), and of those that were present in the hearts of both groups, nearly all were more abundant in MI hearts (Figure 5B). These observations are consistent with the MI-induced uptake of MSC exosomes in cardiac cells. Furthermore, when the membrane and cytosol fractions were evaluated separately, a number of ^{MetRS*}MSC-Exo proteins that were associated with the membranes of cells in the hearts of Sham animals were found primarily in the cytosol fraction of MI hearts (Figure 6).

Desmoglein-1c (DSG-1c) Is Expressed in MSCs, Enriched in MSC Exosomes, and Taken up by Ischemic Myocardium

The results from our click-catalyzed alkyne-agarose capture assessments indicated that desmoglein 1 (DSG-1) was more





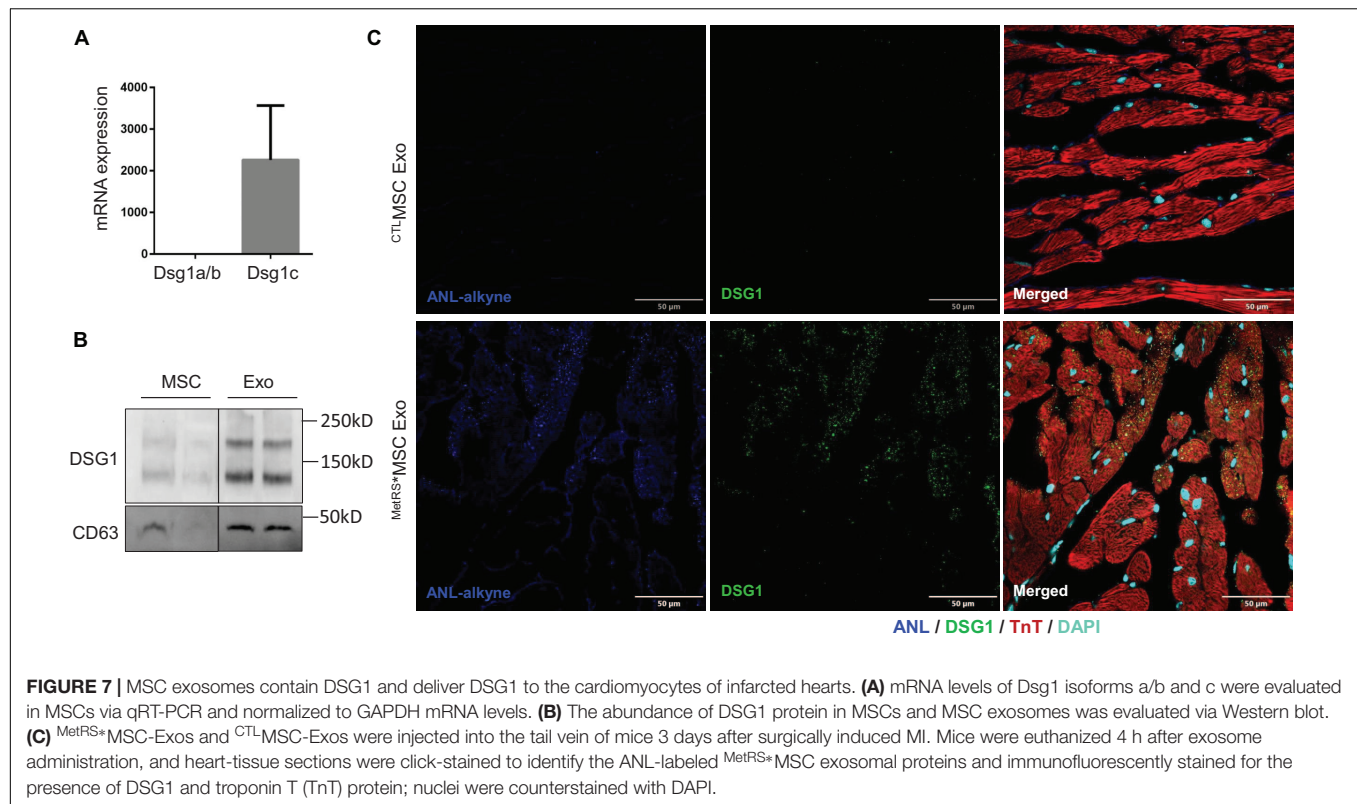
abundant in the heart than in other organs of both MI and Sham animals, and that cardiac DSG-1 levels increased in response to MI; however, to our knowledge, DSG-1 expression had not been previously reported in either MSCs or cardiomyocytes. Thus, we evaluated DSG-1 expression in MSCs via RT-PCR and in both MSCs and MSC exosomes via Western blot: our results confirmed that the DSG-1c isoform was expressed in MSCs (Figure 7A) and enriched in MSC exosomes (Figure 7B). To determine whether we could identify MSC-derived DSG-1 in the infarcted myocardium of ^{MetRS*}MSC-Exo-treated mice via FUNCAT, myocardial sections were click-stained with biotin-alkyne, sequentially stained with primary biotin antibodies and fluorescent secondary antibodies, and then counterstained for the presence of DSG-1 and troponin-T (TnT). When visualized via fluorescence, a number of TnT-expressing cells were positive for both the biotin-alkyne and DSG-1 labels (Figure 7C), which confirmed that the cardiomyocytes had taken up ^{MetRS*}MSC-Exo-derived DSG-1.

DISCUSSION

Because the benefits of stem-cell therapy for myocardial repair are primarily mediated via paracrine mechanisms, the factors produced by these cells, many of which are transported by exosomes, could serve as a potent, cell-free alternative for the treatment of patients with cardiovascular disease (Liu et al., 2018). Results from a number of preclinical studies indicate that MSCs and the exosomes produced by them are equally beneficial

(Xiao et al., 2018); however, the mechanisms that direct the homing of exosomes to injured tissues, as well as the uptake and biodistribution of their cargo, remain largely uncharacterized, because techniques for distinguishing between exosomal proteins and proteins from the targeted tissues are either unavailable or inefficient. Here, we demonstrate that when MSCs are transfected with a vector coding for MetRS* and cultured in ANL-supplemented medium, the proteins produced by the transfected MSCs are selectively labeled with ANL, which can be covalently linked (via click chemistry) to alkyne-tagged reagents for subsequent visualization, identification, and isolation. Thus, when exosomes produced by the ^{MetRS*}MSCs were systemically administered to mice, the protein components of their cargo could be accurately and reliably tracked, visualized, identified, and quantified in tissues from a variety of organs, including both intact and infarcted hearts. Notably, as bioorthogonal chemistry can occur in living systems without disrupting native biochemical processes (Prescher et al., 2004; Prescher and Bertozzi, 2005; Sletten and Bertozzi, 2011; Ngo et al., 2013; Han et al., 2020a), this approach can be applied to other cell populations and even extended to wholly *in vivo* investigations by generating MetRS*-transgenic mice (Alvarez-Castelao et al., 2017; Han et al., 2020a).

The overall pattern of ^{MetRS*}MSC-Exo protein distribution differed substantially between organs; thus, the mechanisms that govern exosome homing and the processing of exosomal proteins after uptake are likely cell- and tissue-type specific. Furthermore, our observation that a number of canonical exosome marker proteins (e.g., TSG101, Syntenin-1, ALIX, CD9, and CD81) were not present in any of the organs analyzed suggests that they



are not suitable for tracking exosome uptake; whether their absence is attributable to rapid degradation in the targeted tissues or another process will be investigated in future studies. We also observed that surgically induced MI led to an increase in the abundance of exosomal proteins in cardiac tissue, which is consistent with the therapeutic mechanism of systemically delivered exosomes (Xiong et al., 2021) but has not been explicitly demonstrated previously. MI was also associated with the redistribution of numerous exosomal proteins from the membrane fraction in intact hearts to the cytosol of cells in infarcted hearts. This observation was not anticipated but likely evolves from increases in membrane permeability, because the loss of cardiomyocyte membrane integrity is a hallmark of MI, and some proposed treatment strategies target membrane dysfunction (Han et al., 2020b). Our results also indicated that the desmosome protein DSG-1c was expressed in MSCs, enriched in MSC exosomes, and taken up by cardiomyocytes in response to MI and ^{MetRS*}MSC-Exo administration. DSG-1 expression has not been previously reported in cardiomyocytes; however, cardiomyocytes express DSG-2, which is critical for cardiac integrity and electric conduction. Notably, human DSG-2 mutations are associated with cardiac arrhythmia (Schinner et al., 2020), and genetic deletion of DSG-2 leads to arrhythmogenic cardiomyopathy and heart failure in mice (Pilichou et al., 2009); thus, whether the exosome-mediated transfer of DSG-1 to cardiomyocytes contributes to the benefit of MSC therapy warrants continued investigation, particularly since MSC exosomes appear to reduce the arrhythmogenicity of human cardiomyocytes (Sattayaprasert et al., 2020).

A limitation of this study is that N-terminal processing by methionyl aminopeptidases results in cleavage of the first residue during protein maturation, preventing identification of these proteins. Methionine is the first amino acid translated during protein synthesis in eukaryotes and the average methionine content of mammalian proteins is ~2.13% (Dieterich et al., 2006). Nevertheless, N-terminal posttranslational processing and acetylation occurs in ~30% of all mammalian proteins (Meinzel et al., 2005) and only ~1.02% of the open reading frames of the human database (predicted to be <25 residues and incomplete sequences) do not contain a single methionine residue (Dieterich et al., 2006).

In conclusion, when exosomes were collected from ^{MetRS*}MSCs that had been incubated with ANL and then systemically administered to mice, the ANL-labeled exosomal proteins could be accurately and reliably identified, isolated, and quantified from a variety of mouse organs, including both intact and infarcted hearts, via ANL-alkyne click chemistry. Thus, this bioorthogonal technique can provide crucial insights into the mechanisms that govern exosome homing and the uptake of exosomal proteins.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession numbers can be found below: European Bioinformatics

Institute (EBI) PRoteomics IDentifications Database (PRIDE) Archive, <https://www.ebi.ac.uk/pride/archive/>, PXD024482, PXD024483, PXD024484, and PXD024485.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham.

AUTHOR CONTRIBUTIONS

EZ and GQ conceptualized the study, interpreted the data, and wrote the manuscript. EZ, YL, CH, CF, LW, WC, YD, DH, SX, YW, and JM performed the experiments and analyzed the data. BA and JM made intellectual contributions and assisted in data interpretation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Layer-By-Layer Fabrication of Large and Thick Human Cardiac Muscle Patch Constructs With Superior Electrophysiological Properties

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Engineered cardiac tissues fabricated from human induced pluripotent stem cells (hiPSCs) show promise for ameliorating damage from myocardial infarction, while also restoring function to the damaged left ventricular (LV) myocardium. For these constructs to reach their clinical potential, they need to be of a clinically relevant volume and thickness, and capable of generating synchronous and forceful contraction to assist the pumping action of the recipient heart. Design prerequisites include a structure thickness sufficient to produce a beneficial contractile force, prevascularization to overcome diffusion limitations and sufficient structural development to allow for maximal cell communication. Previous attempts to meet these prerequisites have been hindered by lack of oxygen and nutrient transport due to diffusion limits (100–200 μ m) resulting in necrosis. This study employs a layer-by-layer (LbL) fabrication method to produce cardiac tissue constructs that meet these design prerequisites and mimic normal myocardium in form and function. Thick (>2 mm) cardiac tissues created from hiPSC-derived cardiomyocytes, -endothelial cells (ECs) and -fibroblasts (FBs) were assessed, *in vitro*, over a 4-week period for viability (<6% necrotic cells), cell morphology and functionality. Functional performance assessment showed enhanced *t*-tubule network development, gap junction communication as well as previously unseen, physiologically relevant conduction velocities (CVs) (>30 cm/s). These results demonstrate that LbL fabrication can be utilized successfully to create prevascularized, functional cardiac tissue constructs from hiPSCs for potential therapeutic applications.

Keywords: hearts, tissue engineering, layer-by-layer fabrication, stem cell, superior electrophysiology

INTRODUCTION

The use of engineered cardiac muscle patch constructs focusing on repair and functional restoration following an acute myocardial infarction (AMI), have shown promise in improving clinical outcomes for patients (Lake et al., 1985; Lucas and Szewda, 1999; Murphy and Steenbergen, 2008). Clinically, AMI is associated with postinfarction left ventricular (LV) remodeling and heart failure (Henkel et al., 2006; Gorenek et al., 2014). Cell therapy approaches utilizing products associated with human induced pluripotent stem cells (hiPSCs) have been examined pre-clinically as a viable therapeutic option (Xiaojun et al., 2012, 2014; Ye et al., 2014). Despite these promising results, the

search continues for an effective method of restoring both mechanical and electrical function to the AMI damaged region of the heart, which is necessary for a successful therapeutic application. Furthermore, electrical function is largely dependent upon the electrical excitability of the cells and subsequently the engineered tissue. Unfortunately, due to their immature phenotype, hiPSC-derived cardiomyocytes (iCMs) tend to have reduced electrical excitability compared to mature, adult CMs found in the native myocardium (O'Hara et al., 2011; Mummery et al., 2012).

One of the key indicators for CM functional maturity at the tissue level is the capacity to support fast action potential (AP) conduction. In cardiac tissue, AP represents the time-dependent changes in the transmembrane electrical potential in CMs, which occur during each heartbeat, and are functions of highly coordinated, time- and voltage-dependent changes in activity of various ion channels and transporters (Liu et al., 2016). Conduction velocities (CVs) of genetically purified iCMs in monolayer format have been reported as high as ~21 cm/s (Lee et al., 2012), with more complex 3D systems still lagging behind at reported CVs around 14–17 cm/s (Gao et al., 2018; Pretorius et al., 2020) with the potential of reaching up to 25 cm/s when highly organized (Gao et al., 2017). However, promising, the CVs reported for hiPSC-derived tissue still fall short of those observed in native adult LV tissue (~30–100 cm/s; Valderrábano, 2007; Yang et al., 2014). Some of the highest CVs reported for engineered cardiac tissue have been obtained with neonatal rat CMs, and was recorded at 32.3 ± 1.8 cm/s, yet at their thickest, these structures were recorded to only be 100 μ m (Jackman et al., 2018).

Utilization of promising fabrication strategies which yield highly organized structures, such as the layer-by-layer (LbL) approach, allow for superior control over a range of physicochemical properties. In a previous study, we demonstrated that our LbL fabrication method yields thick, vascularized engineered cardiac tissue, with CVs ranging between 17 and 19 cm/s, as well as viscoelastic properties that were similar to native murine myocardial tissue (Pretorius et al., 2020), yet this study only utilized CMs and endothelial cells (ECs). It is known, however, that fibroblasts (FBs) play key roles in the healthy native adult mammalian heart. FBs contribute to general heart function, homeostasis, and structure, most notably during the production and remodeling of extracellular matrix (ECM). Culturing CMs in the presence of FBs has also been shown to affect the electrophysiological properties of the CMs (Zhang et al., 2019).

In the present study, we aimed at developing a LbL approach to engineer large and thick tri-lineage (CM, EC, and FB) cardiac tissue constructs. The engineered cardiac tissue was characterized at various time points, over a 4 week period, in order to qualify and quantify the changes that occur in the structures *in vitro*, which ultimately lead to a better potential understanding of the remodeling that these structures undergo *in vivo*. We hypothesized that by mimicking the cellular composition of the native myocardium, i.e., utilizing CMs, ECs, and FBs, with a LbL fabrication process would not only allow for the production of thicker tissues but also

increase their ability to mimic the native myocardium more closely in terms of form and function. Characterization included (1) histology and immunostaining to analyze the cellular and structural morphology, (2) immunostaining to quantify viability, (3) immunostaining of 2D cryosections and 3D structures to analyze cell migration, potential vascularization, and final cellular fate, (4) immunostaining and quantification of ECM remodeling, (5) RNA analysis to quantify and corroborate the results obtained from immunostaining, and (6) optical mapping to determine the AP and CV to assess functionality.

MATERIALS AND METHODS

Cell Culture and Characterization

Human cardiac fibroblast induced pluripotent stem cells were reprogrammed from human cardiac FBs, and subsequently differentiated into hiPSC-cardiomyocytes (iCMs), as previously reported (Lian et al., 2012; Burrridge et al., 2014; Ye et al., 2014). Generally, spontaneous iCM contractions were observed between days 7 and 10 after commencement of the differentiation protocol, with beating numbers increasing up to day 12. Metabolic purification of iCMs was achieved via glucose deprivation (RPMI 1640 without glucose, supplemented with sodium DL-lactate and B27⁺, Gibco) for 3–6 days, initiated at day 9, allowed for a population of iCMs that yielded a minimum of 95% cTnT positive. hiPSCs were maintained at optimal conditions, as previously described (Zhu et al., 2017), on six-well plates coated with Matrigel (Corning), using mTeSR 1 maintenance media (STEMCell Technologies, Canada).

Human induced pluripotent stem cell-endothelial cells (iECs) were differentiated as described previously (Zhang et al., 2014; Gao et al., 2018; Su et al., 2018). Briefly, undifferentiated hiPSCs were seeded into 0.5 mL fibrin scaffolds and treated with CHIR99021 and U46619 in EBM2 medium (Lonza, United States) supplemented with B27[−] for 24 h. The medium was then replaced with EBM2 which was supplemented with B27[−], vascular endothelial growth factor (VEGF), erythropoietin (EPO), and transforming growth factor β 1 (TGF β 1), and then cultured for an additional 96 h with a media change midway through. Finally, the scaffolds were released and cultured in EGM2-MV medium (Lonza, United States) supplemented with B27⁺, VEGF, and SB-431542, with media changes every 2 days. hiPSC-ECs were purified and enriched via collection of cells positive for CD31 using fluorescence-activated cell sorting (FACSARIA II). Antibodies used for selection and cell characterization are listed **Supplementary Table 1**. See **Supplementary Figure 1** for cell characterization and **Supplementary Figure 2** for the proliferation assay showing the lack of tumorigenic properties of the iCMs utilized in this study.

Human induced pluripotent stem cell-FB (cFB) differentiation commenced using hiPSC-line DF19-9-11T with cells at 100% confluence (day 0) (Zhang et al., 2019). The hiPSC culture medium was changed to RPMI/B27[−] and supplemented with 12 μ M CHIR99021, with cells treated in this medium for 24 h (day 1). Medium was changed once again to RPMI/B27[−] for another 24 h (day 2). Within 24 h (before day 3), the medium

was changed to cardiac FB differentiation basal medium (CFBM, see **Supplementary Table 2**) supplemented with 75 ng/mL bFGF (WiCell Research Institute). Cells were then cultured with CFBM + 75 ng/mL bFGF every other day up to day 20 when they were utilized for flow cytometry analysis and passaged for subsequent use in tissue fabrication.

Fibrin Matrix Composition

The fibrin matrix used (per milliliter) used for the differentiation of the iECs as well as for the construction of each alternating layer of the cardiac tissue during the modular fabrication process consisted of the following components as was defined previously (Gao et al., 2018): 0.12 mL fibrinogen (25 mg/mL, Sigma-Aldrich, CAS# 9001-32-5), 0.02 mL Matrigel (Corning, # 356235), 0.56 mL of HEPES (20 mM, pH 7.4, Corning), 0.001 mL CaCl_2 (2 M), 0.3 mL DMEM (Gibco, High glucose, # 11965-118), and 0.006 mL thrombin (80 U/mL, MP Biomedicals).

Polydimethylsiloxane Platforms

Polydimethylsiloxane (PDMS) platforms were fabricated by mixing PDMS (Dow Corning Sylgard 184 Silicone, Product #2065622) in a 10:1 elastomer:curing agent ratio and poured into a 100 mm diameter Pyrex Petri dish (Corning, # 3160102). These were cured at 75°C for 2 h in an oven, after which custom platforms of 10 mm $\times$ 5 mm (2.5 mm thick) were cut. Prior to their use in the tissue fabrication and culture process, all PDMS structures were autoclaved.

Optimized LbL Engineered Tissue Fabrication

Following cell differentiation, cardiac tissue fabrication commenced (**Figure 1**). Petri dishes (BioLite Cell Culture Treated Dishes, Thermo Scientific) were coated with a 5% pluronic F-68 solution (Gibco, # 24040032) and incubated at 4°C overnight. The pluronic solution was removed, and a sterile polycarbonate frame (internal area: 1 $\times$ 2 cm²) was attached with a 2% agarose solution. Note that the frames were modified with channels to allow for maximal media contact following tissue fabrication. iCMs were dissociated (STEMdiff Cardiomyocyte Dissociation Medium, STEMCell) and mixed with the fibrin matrix at a concentration of 10×10^6 cells/mL. Note that the deposition of the iCM layer denotes “D0” for the remainder of the fabrication process. 400 μ L of this solution was quickly deposited into each mold to produce the first layer. Following complete polymerization, the culture medium was added (STEMdiff Cardiomyocyte Support Medium, 2 mg/mL ϵ -aminocaproic acid) and incubated at 37°C (5% CO₂) for 2 days. The next layer, comprised of iECs, was made in a similar fashion, with the following exceptions: iECs were dissociated using trypsin (0.25% trypsin, 0.1% EDTA, Corning, # 25053CI) and then mixed with the fibrin matrix at a concentration of 10×10^6 cells/mL. 200 μ L of this solution was quickly deposited into each mold, yielding a 2:1 ratio of iCMs:iECs (Gao et al., 2018). Fresh culture medium (10% fetal bovine serum, 2% B27⁺, and 2 mg/mL ϵ -aminocaproic acid, 10 μ M ROCK inhibitor in DMEM) was added, following layer polymerization. After

24 h, the frame containing the engineered cardiac tissue was lifted off of the dish surface and placed on top of custom-cut PDMS stilt, allowing for the tissue to be fully suspended in fresh culture media (2% fetal bovine serum, 2% B27⁺, and 2 mg/mL ϵ -aminocaproic acid in DMEM). After a further 48 h, the next layer, comprised of cFBs, was made in a similar fashion, with the following exceptions: cFBs were dissociated using TrypLE (Gibco, # 12604013) and then mixed with the fibrin matrix at a concentration of 10×10^6 cells/mL. 100 μ L of this solution was quickly deposited into each mold, yielding a 2:1:0.5 ratio of iCMs:iECs:cFBs (Pinto et al., 2016; Gao et al., 2018). Incubation continued for the desired period of time (1–4 weeks in total), with media replacement once per week.

Tissue Preservation

Fabricated tissue samples were fixed in 4% formaldehyde (Pierce, Thermo Scientific, # 28906) for 1 h prior to embedding in either optimal cutting temperature compound (OCT compound, Fisher Health Care, United States) or paraffin for histological analysis. Histological analyses were performed on 10 μ m sections. Whole-mount samples were stored in PBS until staining.

Histochemistry

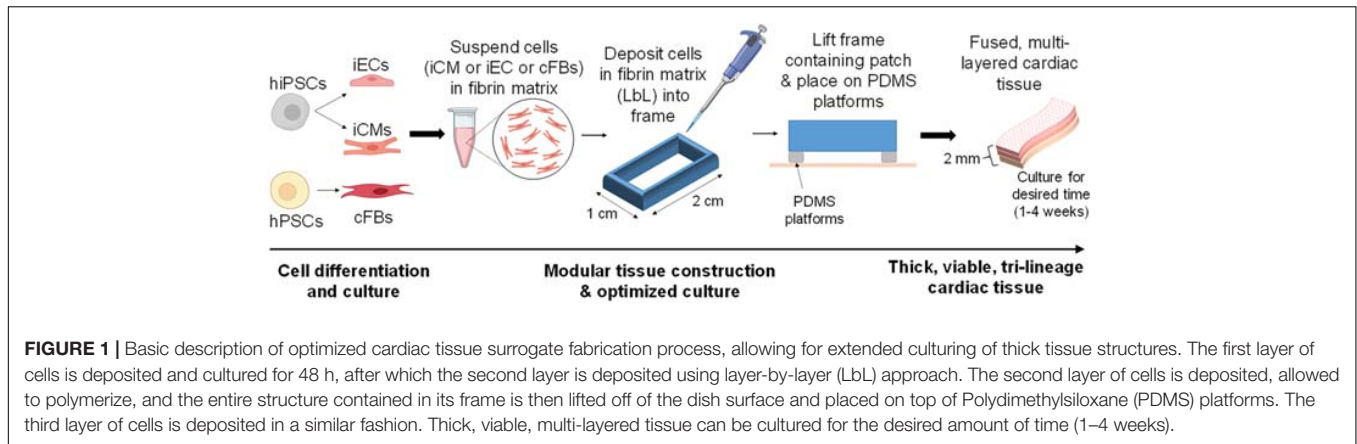
Deparaffinized and rehydrated sections were stained with hematoxylin solution (Mayer's, Merck, 3 min) followed by working eosin Y solution (2 min). Following subsequent dehydration, all samples were mounted with Permount and imaged using a bright field microscope (Olympus IX83 epifluorescent microscope).

Immunohistochemistry

Fixed OCT embedded sections were blocked and permeabilized for 30 min in 10% donkey serum, 3% BSA, and 0.05% Triton-X. Following addition of primary antibodies, samples were incubated for 1 h at room temperature (**Supplementary Table 1**). Samples were washed in PBS (3 $\times$ 5 min), and subsequently labeled with secondary antibodies with fluorescent tags and 4', 6-diamidino-2-phenylindole (DAPI, 100 ng/mL) and incubated for at room temperature for 1 h (**Supplementary Table 1**). Sections were mounted with VECTASHIELD Antifade Mounting Medium and visualized by confocal laser scanning (Olympus FV3000 confocal microscope).

Whole-Mount Staining

Fixed whole-mount samples were blocked and permeabilized in 10% donkey serum, 10% Tween-20, 3% BSA, 0.05% Triton-X, and 0.02% sodium azide in PBS overnight at 4°C. Then, primary antibodies were added and samples were incubated overnight at 4°C (**Supplementary Table 1**). Samples were washed in PBST (3 $\times$ 10 min) and fluorescently labeled secondary antibodies along with DAPI were added followed by another overnight incubation at 4°C. Following washing, tissue was cleared according to the previously described protocol (Li et al., 2019) with 3 h incubation in Ce3D clearing agent. Whole-mount constructs were transferred to an Ibidi μ -Slide (# 80286), covered with VECTASHIELD Antifade Mounting Medium,



and visualized via confocal laser scanning (Olympus FV3000 confocal microscope).

RNA Isolation and Analyses

Samples for RNA analysis were suspended in TRIZOL (Invitrogen) and homogenized. RNA extraction was completed using Direct-zolRNA MiniPrep Plus (Zymo Research Corporation) according to the manufacturer's protocol. Following concentration measurement on the Nanodrop device, 1 μ g of RNA was converted into complementary DNA (cDNA) through the reverse transcription reaction using the SuperScript IV VILO Master Mix (Thermo Fisher Scientific) then diluted to a final concentration of 5 ng/ μ L. qPCR analysis of each sample was performed on a QuantStudio 3 real-time PCR system using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Quantification of relative expression was done by normalizing to sample GAPDH expression. Primers used for RT-qPCR analysis are included in **Supplementary Table 3**.

Optical Mapping

To assess action potential duration (APD), CV, and minimum pacing cycle length, engineered LbL tissue samples were stained with a voltage-sensitive dye RH-237 (2.5 μ M) for 10 min and transferred to a perfusion chamber mounted on an inverted microscope. Control samples consisted of iCM:iEC bilayered tissue, while test samples consisted of iCM:iEC:cFBs. Samples were constantly perfused with Hank's balanced salt solution (HBSS) at approximately 37°C. Pacing/stimulation of all samples was done with a bipolar electrode consisting of a glass pipette filled with HBSS and a silver wire coiled around its tip. A micromanipulator was used to position the electrode tip at the sample's edge. Rectangular stimulation pulses, with a duration of 2 ms and current strength 1.5-times the excitation threshold were used. Fluorescence was excited with a 200-W Hg/Xe arc lamp and recorded with a 16 $\times$ 16 photodiode array (Hamamatsu) at a spatial resolution of 110 μ m per diode as previously described (Sowell and Fast, 2012). Excitation light was filtered at 532–587 nm, and emitted fluorescent light was filtered at >650 nm. The perfusion solution was supplemented with 5 μ M of blebbistatin to eliminate potential motion artifacts caused by the samples' spontaneous contractions. Isochronal

maps of the activation spread were constructed from activation times measured at 50% of the maximum AP amplitude. CV was calculated at each recording site from local activation times and averaged across the whole mapping area. AP duration was measured at 50 and 80% of signal recovery (APD₅₀) and (APD₈₀), respectively.

Statistical Analyses

All results are reported as mean $\pm$ standard error (mean $\pm$ SEM). Significant differences between two mean values were determined via the Student's two-tailed *t*-test; and ANOVA or repeated ANOVA with the Tukey post hoc test were used for multiple (more than two groups) comparisons or repeated measurements. *p*-values of less than 0.05 were considered statistically significant. These analyses were performed utilizing GraphPad Prism8 data analysis software package.

RESULTS

Layer-By-Layer Fabrication Produces Thick, Synchronously Beating, Fused Engineered Cardiac Tissue

Engineered cardiac tissue produced with the optimized method described in **Figure 1** yielded structures of 2.12 ± 0.083 and 1.38 ± 0.019 mm in thickness after 1 and 4 weeks in culture, respectively (**Figure 2A**, $n = 4$). The statistically significant ($p < 0.005$) reduction in tissue thickness between week 1 and week 4 are most likely due to compaction caused by the contractile forces exerted by both the iCMs as well as the cFBs. Following the deposition of the iCMs, synchronization of the iCM layer in all samples was noted after 48 h (on Day 2). The resulting beating-rate (per min) of each engineered tissue was determined (**Figure 2B**), yielding a rate of 109 ± 11.5 beats/min (bpm). Following the iEC layer addition on Day 3 and an additional 2 days of culture (Day 5), the beating-rate on the fifth day of culture, prior to cFB deposition had decreased significantly, to 47 ± 2.0 bpm. Following the deposition of the cFBs, and an additional 24 h of culture (Day 6), the beating-rate of the tissues was noted at 51 ± 3.9 bpm. After 4 weeks in culture, the beating-rate of the constructs were noted at 55 ± 1.8 bpm.

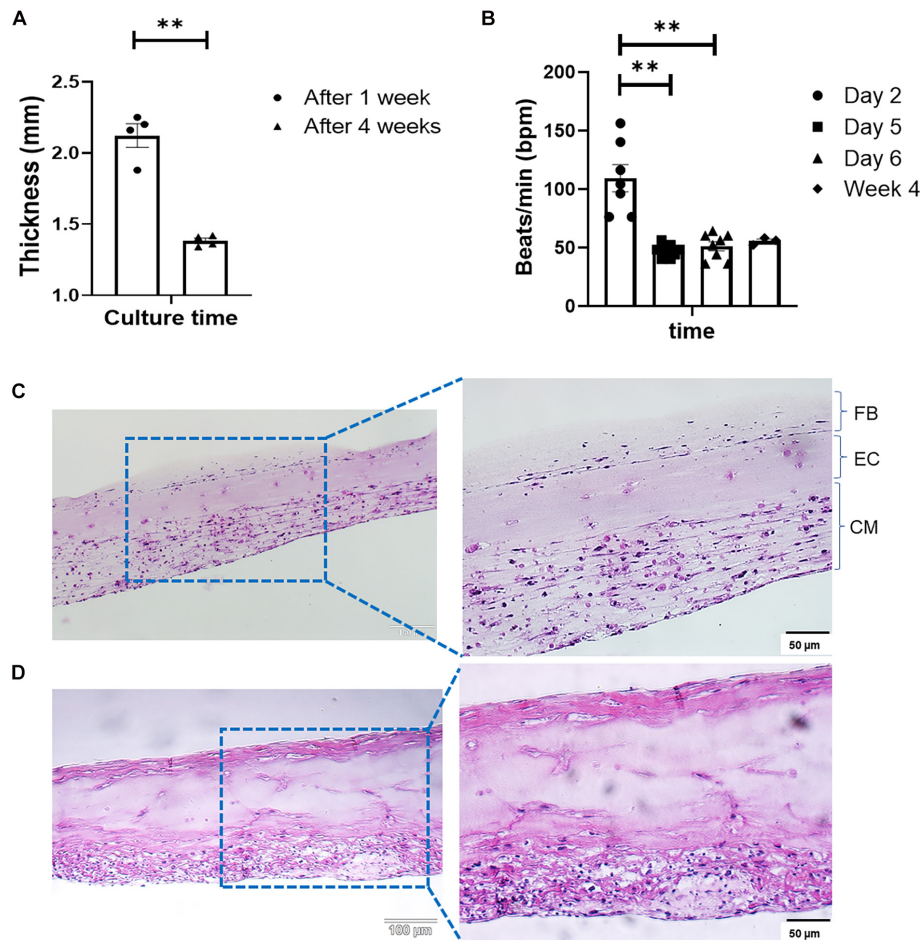


FIGURE 2 | (A) Change in tissue thickness between week 1 and week 4, measured with a caliper (Mitutoyo 500-196-30, Digimatic, $n = 4$, $**p < 0.005$). **(B)** LbL engineered tissue beat-rate over the first 6 days of culture ($n = 8$) as well as week 4 ($n = 3$), $**p < 0.005$. H&E staining of LbL engineered tissue at **(C)** Day 7, and **(D)** Day 28 of culturing.

Modification to the ECM composition between weeks 1 and 4 were observed via H&E staining (**Figures 2C,D**). Histology shows increases in structural ECM components such as collagen (increased intensity as well as the distribution of eosinophilic staining, **Figures 2C,D**). As sample time in culture increased, compaction of the structures the degree of compaction increased (**Figure 2D**), and structures, although initially somewhat disorganized (**Figure 2C**), began resembling *in vivo* muscle. The alterations in the ECM composition, specifically those associated with collagen deposition were attributed to both the culture of iCMs as well as the addition of cFBs, based on the strong eosinophilic staining noted in both the bottom as well as the top layers of the structure at week 4 (**Figure 2D**). Fusion and coupling of the structure layers was confirmed by not only the synchronous macroscopic beating of the engineered tissue (**Figure 2B**) but also via H&E staining. Another observation made via H&E was that of cell migration over the culture period. As with a previous study by this group (Pretorius et al., 2020), it was noted that iECs initiated migration and re-arrangement out of their originally deposited layer into the resident iCM

layer within less than 48 h post-deposition, as is clear from the somewhat acellular appearance of the middle layer of the engineered tissue (**Figure 2C**). The degree of cell migration and potential iEC re-arrangement was confirmed with cell-specific immunofluorescent markers (**Figure 4A**). It should be noted that structures visualized here via H&E staining appear thinner than those preserved in OCT or when measured with a caliper. This difference in overall tissue thickness is attributed to the multiple dehydration-related steps required during the H&E processing of the hydrogel structures leading to decreased preservation of the original architecture.

Layer-By-Layer Fabrication Results in Engineered Tissue With Limited Necrosis Markers

Tissue viability was determined via pMLKL staining (**Figure 3**), which specifically stains for cell necroptosis (Linkermann et al., 2014) and is associated with inflammatory markers (Negroni et al., 2017). The pMLKL-positive cells were quantified as

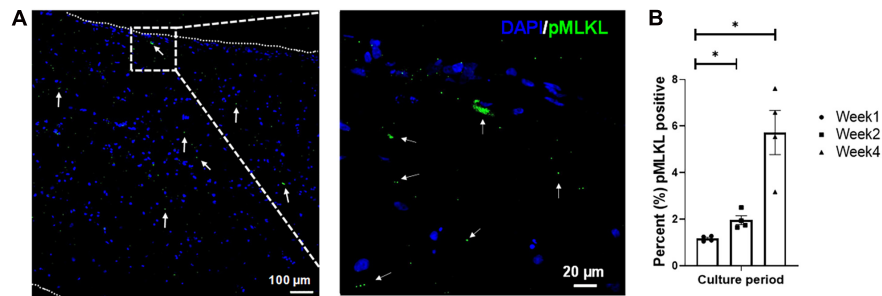


FIGURE 3 | Representative confocal micrograph of engineered LbL cardiac tissue displaying necrotic cells identified by the necrosis marker phosphorylated MLKL (Ser358, pMLKL) at (A) week 1 of culture, with (B) the percentage of necrotic cells in LbL engineered cardiac tissue ($n = 4$, $*p < 0.05$). Border indicated with short dashed line.

a percentage (%) of the total number of cells present (i.e., normalized to the DAPI staining, $n = 4$ with a minimum of five images taken per sample). The initial degree of necrosis noted in the engineered tissues was very low, and quantified at $1.17 \pm 0.06\%$. After an additional week of *in vitro* culture, the degree of necrosis increased to $1.96 \pm 0.19\%$, while the degree of necrosis after 4 weeks in culture was observed to be $5.73 \pm 0.95\%$. Even though the percentage of pMLKL positive cells increased significantly from week 1 to week 2 and week 4, respectively, it still compared well with previously published data from our group, showing $5.6 \pm 1.4\%$ after 4 weeks in culture of engineered structures that were initially ~ 1.73 mm thick at week 1 (Pretorius et al., 2020).

Cellular Migration and Fate

Imaging of the fluorescently stained sections (Figure 4Ai) showed migration of the iECs as well as the cFBs toward their iCM counterparts within a number of days after these layers had been deposited. The migration process continued over the 4 weeks of culture, yet leaving no area of the engineered structure completely acellular (Figure 4Aii).

Expression levels of various cell markers were monitored over the culture period, i.e., cTnT for iCMs, CD31 for iECs as well as α -SMA (alpha smooth muscle actin), periostin, vimentin and FAP (FB activation protein) for cFBs (Figure 4B). At each time point, at least three tissue samples were used to determine the respective expression levels. No statistically significant differences were noted in the expression levels of CD31, α -SMA, periostin, and vimentin or FAP, suggesting that the expression levels of these factors were stable throughout the culturing period. The slight decrease noted in vimentin expression can potentially be attributed to senescence of the cFB line, which is known to occur *in vitro* after a specific number of cell divisions and/or passages (Zhang et al., 2019). The slight increases in factors such as periostin, rather, could potentially be suggestive of an environment that is conducive to cellular motility (Gillan et al., 2002). The only statistically significant difference was noted in the expression level of cTnT when comparing week 1 to week 4. This increase could more likely than not be attributed to the maturation of the iCMs in the engineered tissue, since iCMs are terminally differentiated cells and minimal proliferation is

observed in these cell populations (see Supplementary Figure 2). To further investigate the potential maturation of the iCMs in the engineered tissues, the expression levels of known maturation markers as well as chamber-specific markers were examined (Figure 5).

Developmental and hormonal factors control the expression of alpha- and beta-myosin heavy chain (MHC), two functionally distinct, species-dependent cardiac MHC isoforms (Everett et al., 1984; Allen and Leinwand, 2001). Not only have these isoforms been shown to be correlated with contractile capabilities, but alterations in expression levels are also age-, and species-dependent (van der Velden et al., 1998). Switches in expression from one isoform to another have been highly referenced in literature in developing systems, with increases in β -MHC and more specifically β -MHC: α -MHC, being a key indicator of CM maturation (Reiser et al., 2001; Mihic et al., 2014; Yang et al., 2014; Ruan et al., 2015; LaBarge et al., 2019). During the culturing period, there was a statistically significant decrease in the expression levels of alpha-MHC (α -MHC), noted when comparing the expression levels of week 1 and week 2 as well as week 1 and week 4, respectively. Week 2 also saw an almost threefold increase ($p < 0.05$) in the expression level of beta-MHC (β -MHC) when compared to week 1. Analysis of this ratio showed an eightfold increase from week 1 to week 2 ($p < 0.05$), and a 19-fold increase from week 1 to week 4 ($p < 0.005$).

Expression levels for chamber-specific markers, i.e., atrial (MLC2a) and ventricular (MLC2v), were also compared (Ng et al., 2010). Analysis showed no statistically significant differences in MLC2a or MLC2v between any of the time points. Interestingly enough, when comparing the expression levels of ventricular:atrial markers, i.e., MLC2v:MLC2a, a 2.7-fold increase ($p < 0.05$) was noted between week 1 and week 2, while a 3.3-fold increase ($p < 0.005$) was observed between week 1 and week 4. With MI affecting the LV more prominently, having cells with a more ventricular-like phenotype present in the engineered tissues is highly advantageous.

Dynamic ECM Evolution

Throughout the 4-week culturing period, the engineered cardiac tissue structure ECM underwent remodeling to various degrees. As demonstrated in a previous study, due to fibrinolysis,

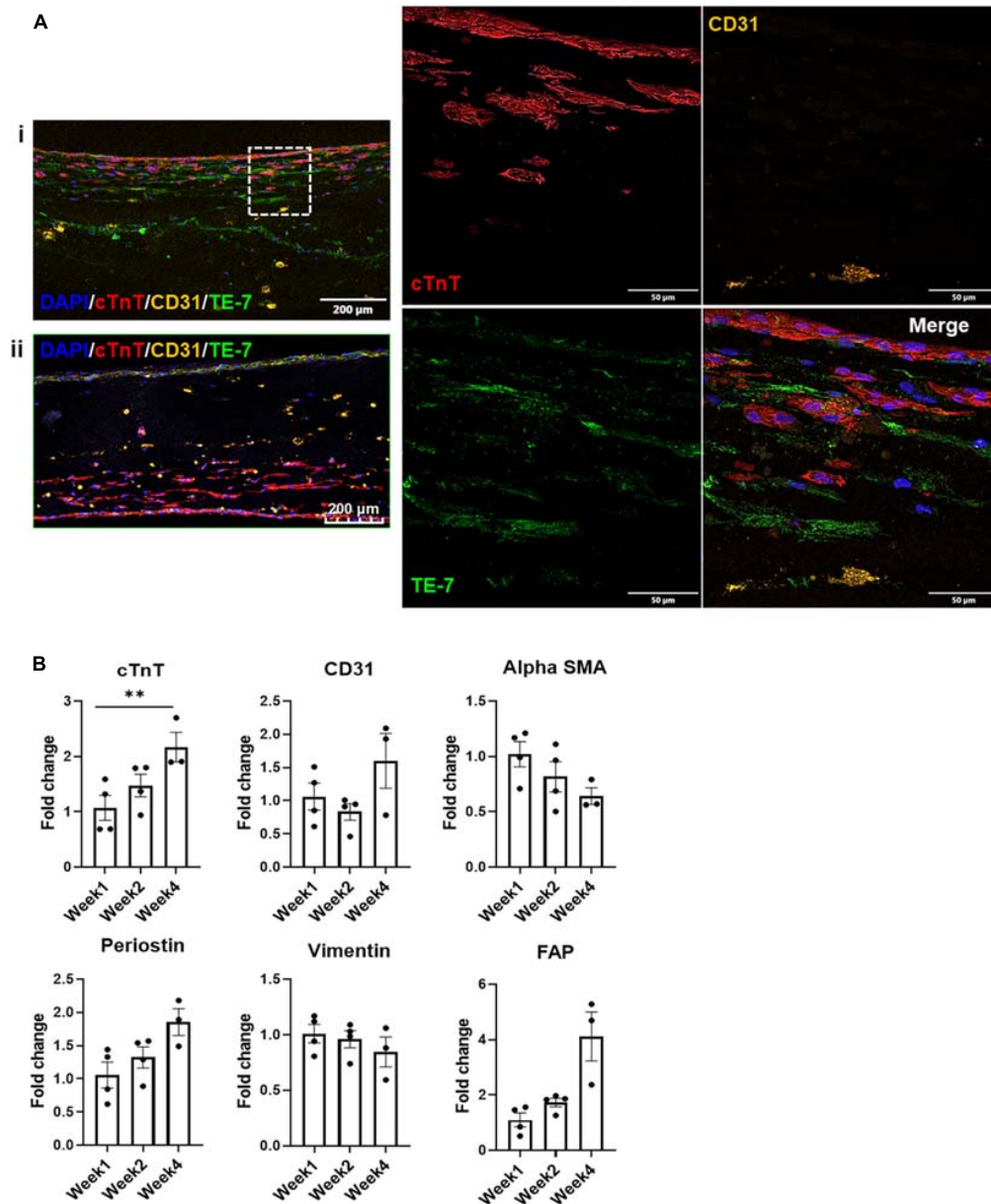


FIGURE 4 | (A) Fluorescent staining with cTnT, CD31, and TE-7 showing tri-lineage engineered tissue after (i) one and (ii) 4 weeks in culture, respectively, along with **(B)** expression levels of cell-specific markers at weeks one, two, and four, respectively ($n = 4$, $*p < 0.05$, $**p < 0.005$).

the majority of the fibrin matrix degrades within the first 2 weeks (Pretorius et al., 2020) (see **Supplementary Figure 3**). For this study, the degree to which the remodeling occurs, and how the addition of the cFBs affected potential matrix deposition compared to our previous study, was monitored and expression levels quantified via RNA analysis. For this analysis, at least three tissue samples were used per time point. With knowledge obtained from prior studies regarding the ECM remodeling during the culturing of iCMs (Bax et al., 2012; Wendel et al., 2015; Pretorius et al., 2020), samples were again analyzed for additional ECM components, including collagen 1 (Col1), collagen 3 (Col3), collagen 4 (Col4), laminin (Lam),

and fibronectin (FN) (**Figure 6**). Samples were also analyzed for elastin due to the incorporation of iECs and cFBs as well as the potential for vessel formation (Mecham et al., 1983; L'Heureux et al., 1998). Quantification showed a nearly fivefold increase in collagen 1 production from weeks 1 to 4. This trend compared well with our previous studies, where bi-layered structures consisting of only iCMs and iECs yielded a threefold increase in collagen 1 production (Pretorius et al., 2020). It is known that cFBs are vital in synthesizing ECM proteins, including, but not limited to, fibrillar collagen types 1 and 3, basement membrane type 4 collagen, FN, and laminin (Eghbali, 1992). Interestingly enough, the increasing trends in

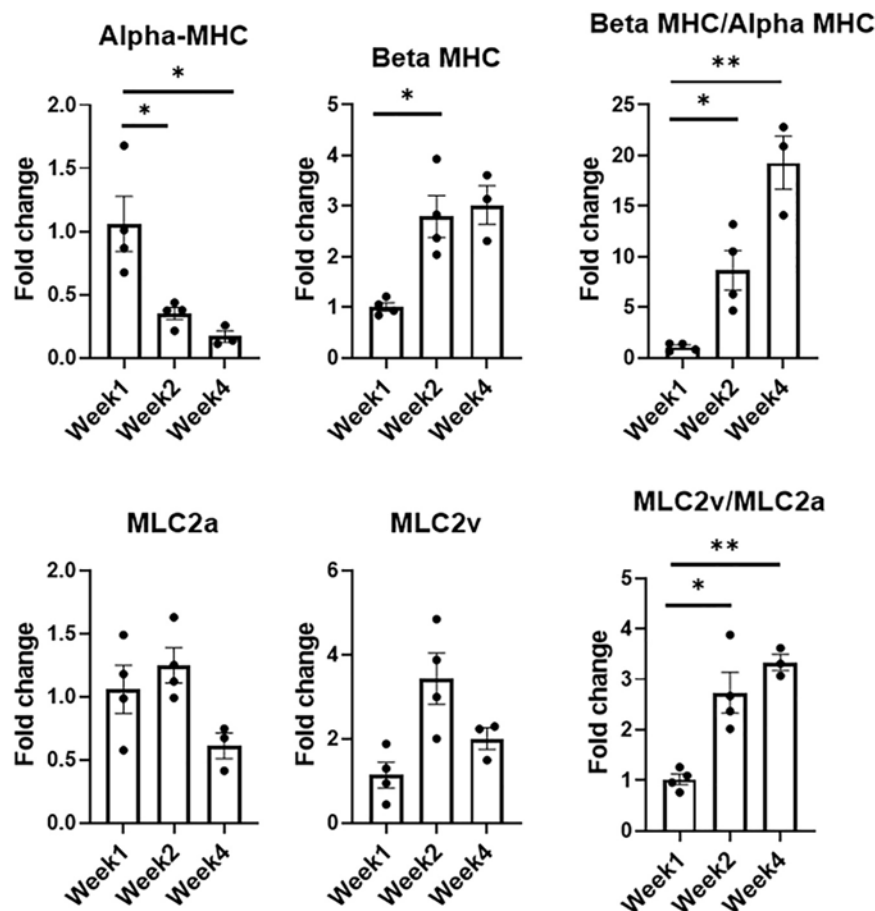


FIGURE 5 | Expression levels of CM-specific maturation and phenotypic markers at weeks one, two, and four, respectively ($n = 4$ for week 1 and week 2, $n = 3$ for week 4, * $p < 0.05$, ** $p < 0.005$).

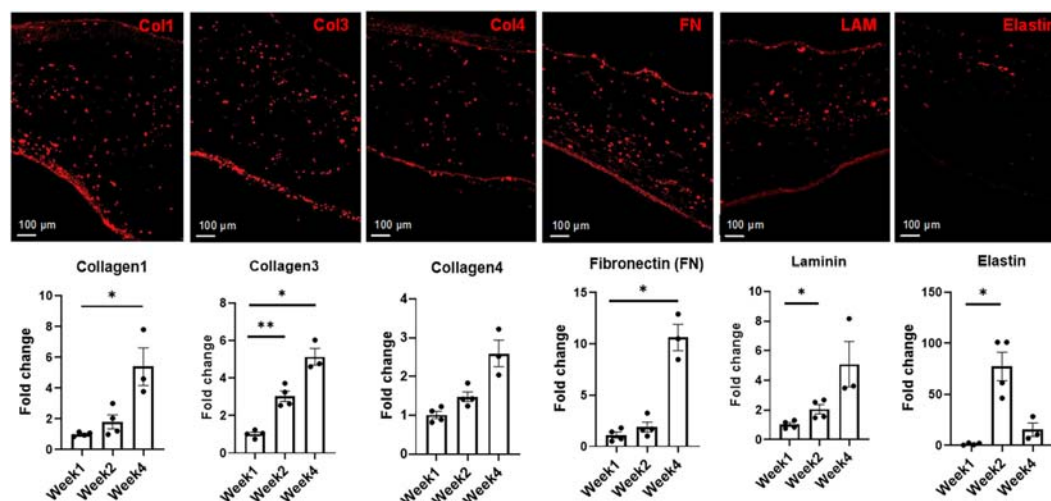


FIGURE 6 | Representative images and subsequent quantification of ECM evolution over 4 weeks in culture, showing the deposition of Collagen 1, Collagen 3, Collagen 4, Fibronectin (FN), Laminin (LAM), and Elastin at week 4. Expression levels of each extracellular matrix (ECM) marker were determined via RNA analysis. Scale bars = 100 μm , $n = 4$ for week 1 and week 2, $n = 3$ for week 4, * $p < 0.05$, ** $p < 0.005$.

collagen 1 production were also associated with the upregulation trends in periostin (**Figure 4B**), a known proponent of collagen fibrillogenesis and ECM remodeling (Takayama et al., 2006; Norris et al., 2007). Similarly, there was an upregulation trend in the expression levels of FAP. Previous studies have shown that collagen induces FAP expression via binding of $\alpha 3 \beta 1$ integrin (Kennedy et al., 2009).

Statistically significant increases were observed in the expression levels of collagen 3 when comparing week 1 to week 2 ($p < 0.005$) and week 1 to week 4 ($p < 0.05$), respectively. Expression of collagen 4 levels increased at both week 2 and week 4 compared to week 1, though this increase was not statistically significant. An almost 10-fold increase in the expression level of FN was noted when comparing week 1 to week 4. Statistically significant increases in the expression levels of laminin was noted between week 1 and week 2 ($p < 0.05$). An almost 70-fold increase in the expression level of elastin was observed from week 1 to week 2, followed by a decrease at week 4. One potential cause of this sharp decrease in the elastin expression level could be linked to bFGF production by the iECs in the engineered constructs (Savchenko et al., 2019). Previous studies have shown that free bFGF decreases the expression levels of elastin at the transcriptional level in culture (Rich et al., 1996).

Functional Performance of Tri-Lineage Engineered Tissue

Expression levels of factors associated with functional performance were assessed by RNA analysis (**Figure 7**) of developmental transcription factor Tbx20, calcium handling machinery (JP2, RyR2, Cx43, SERCA, and CACNA1C) and optical mapping (**Figure 8**) to determine CVs through the tissue. All mapping was performed over the 4 week culture period. From week 1 to week 2, there was a twofold increase in the expression level of RyR2, suggesting that some of the calcium handling machinery, specifically some of the machinery associated with sarcoplasmic reticulum (SR)-associated calcium release, improved (Ronaldson-Bouchard et al., 2018). A significant increase in Tbx20 expression was noted, with a 1.7-fold increase and a 2.5-fold increase observed from week 1 to week 2 and week 1 to week 4, respectively.

The conduction capabilities of the tri-lineage engineered cardiac tissues were evaluated over the 4 week period and compared to bilayered samples fabricated in a similar fashion, without any FBs (**Figure 8**). CVs were recorded between pacing cycle lengths of 200–800 ms, and average CVs are noted at the respective pacing cycle length in **Figure 8A** ($n = 4$ for each time point). The average CV trends show that increased time in culture, i.e., 2 weeks vs. 1 week, and 4 weeks vs. 2 weeks, allows for enhanced conduction capabilities. This trend holds true over the entire pacing frequency range, up to 200 ms (5 Hz). Maximum CVs noted at each time point, regardless of the pacing cycle length it was obtained at, are shown in **Figure 8B**. The maximum CV noted for each run was 25.6 ± 2.47 and 21.8 ± 0.97 cm/s for week 1, 37.3 ± 1.65 and 36.8 ± 2.59 cm/s for week 2, and 39.8 ± 4.35 and 30.0 ± 1.36 cm/s for week 4, for the tri-lineage and bilayered cardiac tissue structures, respectively. Of particular

note was that CVs as high as 49 cm/s were obtained after 4 weeks in culture (**Figure 8B**). The tri-lineage structures showed significant increases in CV from week 1 to week 2, as well as week 1 to week 4, with an overall increasing trend. Bilayered structures also showed a significant increase in the CV when comparing values obtained at week 1 to week 2, and week 1 to week 4, but overall maximum CVs were obtained after 2 weeks in culture. Tri-lineage engineered tissue structure APD values were obtained as functions of pacing cycle length (**Figure 8C**). Isochronal activation maps show uniform signal propagation across the engineered cardiac tissue after 1 week of culture at pacing cycle lengths of 200 and 800 ms (**Figure 8D**). Isochronal activation maps of engineered tissues paced at weeks two and four also show uniform conduction (**Supplementary Figure 4**). The lack of observed conduction heterogeneity or conduction blocks suggests low arrhythmic risk of the constructs.

DISCUSSION

In this study, a modular LbL fabrication method was developed to fabricate cardiac muscle constructs from three cardiac cell types derived from human pluripotent stem cell lines. Over a 4 week period in culture, it was shown that these large and thick cardiac tissue constructs were functionally superior to previously reported engineered structures from the perspective of their larger, thicker nature, as well as superior APD CV. This study is the first to our knowledge to demonstrate robust ($1 \times 2 \text{ cm}^2$) hiPSC-derived engineered cardiac tissues that surpass 2 mm in thickness ($\sim 2.12 \text{ mm}$), without significant apoptosis/necrosis. The physiologically relevant CVs ranging from around 25 cm/s after 1 week in culture to velocities as high as 49 cm/s after 4 weeks in culture, all lacking arrhythmogenic properties, demonstrate the significant clinical relevance of these cardiac muscle constructs. Previously, the best achievable thickness of engineered cardiac tissue constructs with this level of CV was only 100 μm (Jackman et al., 2018). Quantification of the temporal stability of the expression of cTnT, CD31, α -SMA, periostin, vimentin, and FAP suggested that all three cell populations (iCM, iEC, and cFBs) were stable. Additionally, this study also considered the temporal changes that occur during ECM remodeling in these engineered tissues, via immunofluorescent staining and RNA expression levels.

We have recently reported (Gao et al., 2018) that a novel dynamically cultured engineered human cardiac muscle patch (hCMP) demonstrated a significant advancement in the field of myocardial tissue engineering, due to its clinically relevant dimensions (2 cm $\times$ 4 cm $\times$ 1.2 mm) as well as contractile force generation capabilities were substantially greater than had been previously achieved (Ruan et al., 2016; Shadrin et al., 2017; Yang and Murry, 2017). However, these hCMPs were manufactured by *mixing* hiPSC-CMs, -ECs, and -SMCs into a single layer of cells. In the functional myocardium, CMs are typically found adjacent to one another as opposed next to the SMCs, FBs or ECs, and the mixing of cardiac cells in engineered cardiac tissue likely preventing the hiPSC-CMs from coalescing into a fully interconnected contractile apparatus in the past. This might

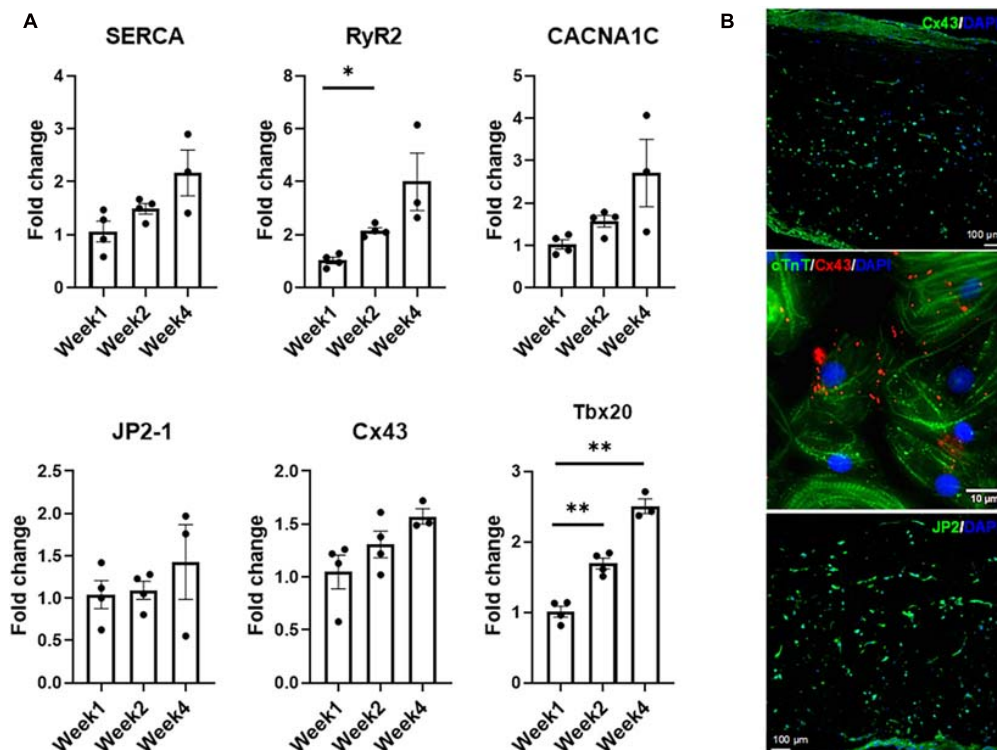


FIGURE 7 | (A) Expression levels of CM-specific functional markers at weeks one, two, and four, respectively ($n = 4$ for week 1 and week 2, $n = 3$ for week 4, $^*p < 0.05$, $^{**}p < 0.005$). **(B)** Representative confocal micrograph of tri-lineage engineered cardiac tissue at week 2 stained for Cx43 and JP2, respectively.

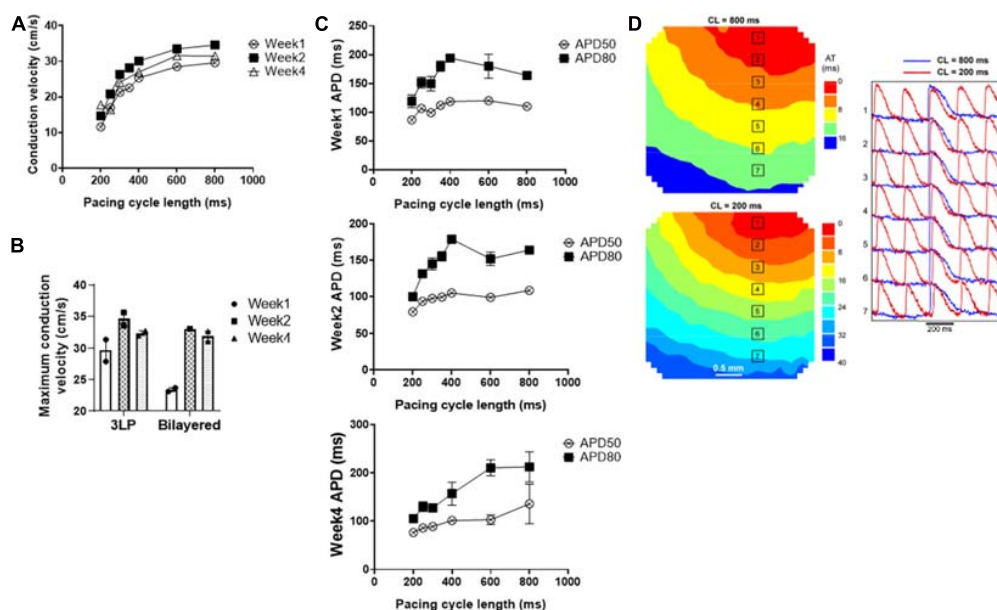


FIGURE 8 | (A) the average conduction velocity of the tri-lineage engineered cardiac tissue at over the 4-week culture period as a function of pacing cycle length ($n = 4$), **(B)** the maximum conduction velocity of the tri-lineage engineered cardiac tissue compared to bilayered control engineered cardiac tissue, at each time point, respectively ($n = 4$ with $^*p < 0.05$), **(C)** APD₅₀ and APD₈₀ of the tri-lineage engineered cardiac tissue as a function of pacing cycle length ($n = 4$). **(D)** Representative isochronal electrical activation maps of engineered cardiac tissue, with representative optical action potentials at locations indicated on maps pacing at 200 ms (red) and 800 ms (blue) cycle lengths after 1 week in culture.

partially explain why force generation measurements remained lower than in native heart tissue. In the current study with an hCMP composed of layers of hiPSC-CMs, -ECs, and -FBs sandwiched in a LbL fashion, compared to a single layer of hiPSC-CMs, the three-layered patch generated approximately twice the CV compared to the previous study, suggesting a significantly improved physiology using this LbL approach.

Cardiac FBs are known to contribute significantly to the normal ECM formation. During the 4 weeks in culture, ECM remodeling occurs. The increases in collagen 3 (**Figure 6**) are likely a precursor to collagen 1 in this case, as it is known to play a vital role in collagen 1 fibrillogenesis and is crucial for normal cardiovascular development (Liu et al., 1997). Fibronectin, is highly expressed in the heart during the early stages of embryogenesis and has furthermore been shown to be vital in the vasculogenesis process (George et al., 1993). It has also been suggested that increases in FN expression, as noted at week 4 could be related to the regulation of vessel formation in similar engineered cardiac tissues (Pretorius et al., 2020). Even though laminin is known to be a basal lamina protein and more closely associated with cellular differentiation, migration and adhesion, it has been suggested that manipulation of the ECM laminin content may be an effective means of inducing structural and functional changes at a cellular level by altering the cardiac titin isoform ratio (Hochman-Mendez et al., 2020a). Laminins and other proteins are connected to the cell surface by costameres, which are in turn assembled by a combination of integrins and dystroglycans, and serve as a structural and functional bridge. These costameres and their subsequent integrins contribute to signal transduction, transmitting force signals between the contractile apparatus and ECM via interactions with primarily titin and other Z-line associated structures (Peter et al., 2011). This process not only supports the mechanical integrity of the sarcolemma, contributes to mechanical signaling but also provides spatial prompts for muscle fiber organization (Hochman-Mendez et al., 2020b). The upregulation in the levels of laminin observed in the tri-lineage tissues could, thus, likely be associated with the structural and functional maturation of the iCMs, as well as the enhanced tissue compaction and alignment noted in **Figures 2C,D**. Increases in periostin are associated with significant increases in elastin production by cardiac-specific cell, and more specifically those cells negative for CD31 and CD45 (Kanisicak et al., 2016). Furthermore, the increased expression of elastin could likely be attributed to cellular migration and re-arrangement of the ECs into more organized structures as well (Mecham et al., 1983).

Even though the increases in junctophilin (JP2), sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), calcium voltage-gated channel subunit alpha1 C (aka $\text{Ca}_v1.2$, encoded by CACNA1C) and connexin 43 (Cx43) were not statistically significant, there were clear trends hinting at the development of the calcium handling machinery in the tri-lineage tissues (**Figure 7**). The multi-chambered mammalian heart has its origin from a simple tubular structure via polar elongation, myocardial differentiation and morphogenesis. The large family of T-box

(Tbx) transcription factors have been shown to be vital in their role as distinct subprograms during cardiac regionalization (Singh et al., 2005; Stennard et al., 2005). Tbx20 plays a central role in these pathways, and has important activities in both cardiac development and adult function, specifically affecting chamber differentiation and overexpression of Tbx20 has been shown to lead to increased expression of Cx43, and subsequent increases in CVs *in vivo* (Chakraborty and Yutzy, 2012). The expression of Tbx20 has also been associated with the activation of and maturation-associated genes in iCMs such as those recorded in **Figure 5** (Zhou et al., 2020). The tri-lineage LbL cardiac engineered tissues fabricated here are the first, to our knowledge, to be fabricated from hiPSC-CMs that have not only generated physiologically relevant CVs in excess of 30 cm/s (Yang et al., 2014), but generated velocities close to 50 cm/s with these cells (**Figure 8B**). Whether this increase in structure functionality is a function of cell-cell communication and paracrine signaling, a function of increased culturing time, or a combination of these factors remains to be seen. It has been shown, however, that cFBs do affect the maturation of hiPSC-CMs through secretion of growth-related cytokines when co-cultured (Ieda et al., 2009). These data suggest that superior electrophysiological function of the current LbL approach may be in part caused by the addition of the cFB layer.

It should be noted that, even though this 3D *in vitro* study enhanced our knowledge-base of thick, engineered cardiac tissue structures and some of their electrophysiological capabilities, further studies are required to better understand the mechanical aspects of the system. In order to better understand, model future system and avoid mechanical mismatch when implanting these structures, their viscoelastic properties need to be determined and related to the dynamic properties of the ECM (Little and Wead, 1971; Tsaturyan et al., 1984; Miller and Wong, 2000; Wang et al., 2010).

CONCLUSION

Here we have shown that a modular fabrication method, like LbL assembly, can be utilized to produce thick (~2.12 mm), viable engineered cardiac tissues from hiPSCs. The incorporation of cardiac FBs into the LbL assembly method allowed for superior, previously unobtainable CVs (>30 cm/s) along with a lack in arrhythmogenic potential, with tissues having the ability to be paced to 5 Hz (200 ms). *In vitro* characterization showed engineered tissue structures resembling those of native cardiac tissue along with minimal necrosis, even after 4 weeks in culture. Considering that the tissues engineered for this study have not undergone any form of maturation, nor have they been exposed to any external stimuli, suggests that their performance capabilities could be enhanced even further.

Future work will include the mechanical testing of the engineered cardiac tissues to determine viscoelastic properties to ultimately prevent mechanical mismatch between the engineered tissues and future host. *In vivo* studies in a large animal model will also commence in order to assess the potential clinical application

of these larger, thicker, engineered cardiac tissues in preventing LV dilatation of hearts with postinfarction LV remodeling.

DATA AVAILABILITY STATEMENT

The raw data generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

DP designed the LbL fabrication technique, designed the experiments, performed histology and imaging, sample preparation, and wrote the manuscript. AK-K performed the cell differentiation, sample staining, and imaging as well as RNA analysis. XL performed the cell differentiation, sample staining, and imaging. VF performed the optical mapping. JB reviewed the manuscript. TK supplied cFBs and reviewed the manuscript. JZ provided the project leadership, funding acquisition, method development, and manuscript revisions.

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Rebuilding the Vascular Network: *In vivo* and *in vitro* Approaches

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As the material transportation system of the human body, the vascular network carries the transportation of materials and nutrients. Currently, the construction of functional microvascular networks is an urgent requirement for the development of regenerative medicine and *in vitro* drug screening systems. How to construct organs with functional blood vessels is the focus and challenge of tissue engineering research. Here in this review article, we first introduced the basic characteristics of blood vessels in the body and the mechanism of angiogenesis *in vivo*, summarized the current methods of constructing tissue blood vessels *in vitro* and *in vivo*, and focused on comparing the functions, applications and advantages of constructing different types of vascular chips to generate blood vessels. Finally, the challenges and opportunities faced by the development of this field were discussed.

Keywords: vascular network formation, angiogenesis, cell therapy, decellularization, vessels on a chip

INTRODUCTION

The purpose of tissue engineering is to exploit engineering methods to overcome physiological or medical difficulties. Its main objective is to facilitate tissue or organ transplantation and scientific research constructing functional tissue models *in vitro*. After a long period of research and development, the technology of tissue engineering has achieved encouraging results and milestones. However, a significant challenge currently remains concerning how to generate a large volume ($>1\text{ cm}^3$) of viable, functional tissue. At present, the main difficulty in constructing larger tissues is the inability to form a vascularized network *in vitro* to supply oxygen and nutrition; thus, the central areas of fabricated tissues without vascularization tend to undergo rapid necrosis. Thus, tissue engineering research has focused on how to generate functional vascular networks. Herein, we will introduce the current progress of vascular network construction, including histological and cellular characteristics, using *in vivo* and *in vitro* strategies for angiogenesis, and the potential clinical applications. This review will first introduce the basic characteristics of blood vessels in the body and the mechanisms involved in angiogenesis of blood vessels *in vivo*. The current methods of constructing tissue vessels *in vitro* are also described within this review, including the use of vascular chips, and the functions, applications, and advantages of the vessels generated by these different methods are compared and analyzed.

STRUCTURAL CHARACTERISTICS OF THE VASCULAR NETWORK

Histological and Cellular Fundamentals of Peripheral Vessels

The peripheral vascular system (PVS) includes all the blood vessels that exist outside the heart. The classification of the PVS is described below. Arteries have larger calibers and a better capability to bear high perfusion pressure. When arteries reach a specific organ, they branch into smaller vessels. As a result, there are two main types of arteries in the body: (1) elastic arteries and (2) muscular arteries. Elastic arteries consist mainly of large arteries and directly connect to the heart (such as the aorta and pulmonary arteries) and present more elastic features. Muscle arteries mainly distribute as medium-sized vessels, and their walls primarily contain smooth muscle.

The arterioles are the main vessels present in different organs and present relatively limited amounts of elastic tissue. Capillaries are thin-walled blood vessels composed of a single layer of endothelial cells. Based on the histological and cellular characteristics of capillaries, the exchange of nutrients and metabolites is achieved mainly through diffusion. Unlike arteries, venous pressure is very low. The venous wall is thin and less flexible. The veins receive blood from capillaries to complete the circulation system.

The function and structure of each segment of the PVS depends on the organ which it supplies, varying across different tissues. Aside from capillaries, most blood vessels are made of three layers. The adventitia, or outer layer, is mainly composed of loose connective tissue, which provides structural support and maintains normal vessel shapes. The tunica media, or a middle layer, is composed of elastic and muscular tissue, which determines the internal diameter of the vessel. The inner layer is formed by a single layer of endothelial cells, which are surrounding by elastic fibers.

Vessel Formation: Morphological Changes and Signaling Cascades

There are several mechanisms involved in vessel formation and they are considered as different phases of the same biological process. Generally, vessel formation in an early development stage is defined as vasculogenesis, and describes the establishment of the first primitive vascular network. Next, angiogenesis follows the process of vasculogenesis, and consists of the formation of branches and vascular remodeling. Furthermore, angiogenesis is mainly used to describe vessel growth stemming from pre-existing ones, and represents the main vessel formation process in adulthood. Typically, vessel formation may be divided into three steps: (1) vasculogenic assembly, (2) vessel sprouting, and (3) vascular remodeling.

During embryogenesis, the formation of new blood vessels is a *de novo* process. Angioblasts (mesoderm-derived endothelial precursors) differentiate into a primitive vascular labyrinth (vasculogenesis) (Uhrin, 2019). Within this process, angioblasts differentiate into endothelial cells (ECs), and the ECs are recruited to form vascular cords (Baldwin, 1996). Next,

the vascular cords are further specified into hierarchically differentiated vessels of arteries, capillaries, and veins. To enable this process, several molecular signaling mechanisms have been implicated in the initial steps of vasculogenic assembly. Notch signaling is highly expressed in arteries, but poorly expressed in veins (Conway et al., 2001; Gridley, 2010). Blocking Notch signaling contributes to the genesis of more veins and disruption of arteries, which indicates that Notch signaling pathway is a key molecular mechanism in the induction of arteriogenesis. Furthermore, ephrin components are associated with Notch signaling; EphB2 is a target of Notch within arterial ECs and binds to EphB4 of venous ECs. Vascular endothelial growth factor (VEGF) is also a crucial factor that triggers arterial differentiation of ECs, and is a downstream component of the Hedgehog pathway (Kim et al., 2008). Moreover, transcription factors are also expressed by specific ECs. FOXC1 and FOXC2 drive the expression of arterial gene signatures under the guidance of VEGF and Notch signals (Hayashi and Kume, 2008). While COUP-TFII has been identified as a driving factor of venous cell fate. In addition, according to the different pressure loading profiles of the arterial and venous system, mechanical loading also contributes to the differentiation of specific vessels. Finally, the arterio-venous segregation is driven by VEGF-C.

A second process of vessel formation is defined as sprouting angiogenesis: there are three changes that define blood vessel morphology, sprouting, bridging, and intussusception, which is facilitated by two main types of cells: the tip cells and stalk cells (Conway et al., 2001). Essentially, the process could be described as (1) tip-stalk cell selection; (2) tip cell navigation and stalk cell proliferation; (3) branching coordination; (4) stalk elongation, tip cell fusion, and lumen formation; and (5) perfusion and vessel maturation. At the onset of sprouting, the tip cells secrete matrix metalloproteases (MMPs) such as MT-MMP1 to degrade basement membrane. Plasminogen activator inhibitor-1 (PAI1) controls the amount of MMPs secreted to prevent over-degradation. Furthermore, the antiangiogenic molecules, angiostatin and endostatin, are also involved in this process and ensure the optimal sprouting direction. Additionally, detachment of mural cells is stimulated by angiopoietin-2 (ANG2) for tip cells movement in response to stimulation by VEGF. Next, VEGF/Notch signaling selects tip and stalk cells. Stalk cells express enriched Notch signaling and tip cells express low levels. Conversely, tip cells express higher levels of the Notch ligand DLL4. JAGGED1 (JAG1) is expressed in stalk cells and acts to inhibit DLL4 signal activity and maintain Notch activity. Filopodia guide tip cells by sensing attractive and repulsive cues. Filopodia formation is stimulated by CDC42 and by the endocytosis of EphB2/VEGFR2 receptors. ROBO4/UNC5B signaling promotes stabilization of the endothelial layer through inhibition of SRC. Notch-regulated ankyrin repeat protein (NRARP) and SIRT1 guide stalk cells stabilization. Both WNT and Notch pathways are also involved in stalk cell stabilization through interaction with NRARP and LEF1/ β -CATENIN. Finally, the contraction of the cytoskeleton of ECs generates the vessel lumen. Besides ECs themselves, a few other cell types also make significant contributions to the angiogenesis process through their interaction with ECs. For example, macrophage

mediates the tip cells fusion and branch anastomosis, including VE-cadherin controlling cell adhesion (Potente et al., 2011; Hong and Tian, 2020). Furthermore, pericytes, vessel associated multi-functional support cells, promote endothelial sprouting during angiogenesis and provide guidance by spatially restricting VEGF signaling (Eilken et al., 2017). Pericytes can also penetrate tissues prior to EC ingrowth and create a path enabling subsequent effective angiogenic invasion by ECs (Ozerdem and Stallcup, 2003; Rajantie et al., 2004; Eilken et al., 2017).

The final phase is vascular remodeling, transitioning from a primitive towards a more stabilized and mature vascular plexus, involving steps such as adoption of a quiescent endothelial phalanx phenotype, basement membrane deposition, pericyte coverage, and branch regression. Stalk cells are regulated by blood flow shear stress-induced KLF2 signals. The up-regulation of KLF2 controls the remodeling of the vasculature. High KLF2 signaling ensures the endothelial quiescence cells form patent vessels, while low KLF activity induces ECs apoptosis and regression to terminate a vessel. The recruitment of pericytes is guided by platelet-derived growth factor receptor (PDGFR) β , S1PR1, EphB2, and Notch3 signaling. The process of pericyte coverage and acquisition of an endothelial phalanx phenotype is the typical sign of vessel maturation. Further, hypoxic conditions trigger the expression of HIF- α , leading to the over-expression of sVEGFR1 and VE-cadherin to force neoangiogenesis so as to improve oxygen perfusion.

Given the extensive interplay of ECs, pericytes and macrophages at different stages along blood vessel formation, recapitulating the spatial and temporal interaction of this multi-cell-lineage system is of critical importance for enabling effective vasculogenesis and angiogenesis in tissue-engineered models. All the molecular biological processes were summarized in **Figure 1**.

INDUCING VASCULAR REGENERATION *IN VIVO*

Growth Factors Stimulating Angiogenesis

From an angiogenesis perspective, growth factors play critical roles in directing cells to initiate and maintain angiogenesis, since they are responsible for transmitting signals between cells and between cells and their microenvironment (Elaimy and Mercurio, 2018; Omorphos et al., 2021). Several growth factors have been widely studied and proven to be effective in promoting vascular regeneration, including VEGF, basic fibroblast growth factor (bFGF), placental growth factor (PLGF) and platelet-derived growth factor-BB (PDGF-BB) (Elaimy and Mercurio, 2018; Hao et al., 2018; Kant and Coulombe, 2018).

Despite the therapeutic potential of growth factors in promoting tissue regeneration, their clinical application is hindered by their limited stability and systemic side effects *in vivo*. The stability of growth factors can be enhanced by their incorporation into polymers to form a delivery system, which extends the *in vivo* half-life while maintaining bioactivity (Wang et al., 2017; Caballero Aguilar et al., 2019). In addition,

delivery systems are able to provide spatial control of growth factor release, since they should remain at the target location long enough to induce a cellular response. After incorporation into hydrogel systems, growth factors, such as VEGF and bFGF showed improved ability to promote angiogenesis, long-term stability, and spatial localization *in vivo* compared to bolus injection or delivery in the aqueous solution of polymer (Adibfar et al., 2018; Shamloo et al., 2018).

Delivery Strategies for Growth Factors

Three main methods have been applied for delivering angiogenic growth factors: (1) physical entrapment; (2) immobilization by covalent conjugation; and (3) the use of biocompatible micro/nano-particles. Physical entrapment can be achieved either by mixing growth factors with polymers before gelation or by physical absorption after polymer scaffold manufacturing (Tayalia and Mooney, 2009). By incubating with growth factors, such as VEGF, PDGF-BB, and transforming growth factor beta (TGF- β 1), an alginate-sulfate hydrogel preparation triggered the formation of mature vascular structure (Freeman and Cohen, 2009; Sarker et al., 2019). Neovascularization responses may also be generated with hyaluronan hydrogel by mixing VEGF, bFGF, or KGF with polymers before gelation (Peattie et al., 2006; Pike et al., 2006; Graça et al., 2020). In order to achieve higher stability and prolonged release, growth factors can also be immobilized into polymers by covalent bonding. This usually requires chemical or enzymatic reactions between the growth factors and polymer scaffolds. For example, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was used to covalently immobilize VEGF and angiopoietin-1 onto collagen scaffolds with controlled angiogenic bioactivities (Chiu and Radisic, 2010; Enriquez-Ochoa et al., 2020). In addition, growth factors can also be encapsulated or conjugated with biocompatible micro/nanoparticles, which allows precise control of the growth factor release rate and cellular uptake efficiency by fine-tuning the particle size and the surface-to-volume ratio (Tayalia and Mooney, 2009; Oduk et al., 2018). Likewise, the release of growth factors can be prolonged in the local microenvironment, leading to improved cellular responses and stability (Chen et al., 2010). It has been reported that following treatment with nanoparticle-encapsulated VEGF, vascularization and angiogenic responses were achieved in the mouse hindlimb ischemia and rat myocardial infarction models (Golub et al., 2010; Oduk et al., 2018).

Since proteins generally lack stability *in vivo*, introducing growth factors by DNA transfer could provide sustained support for vascularization or angiogenesis. The delivery of VEGF gene using direct injection of naked plasmids or adeno-associated viruses (AAVs) has produced positive results in the activation of angiogenic genes and vascular regeneration, in critical limb ischemia and myocardial infarction models (Schwarz et al., 2000; Boden et al., 2016; Liu et al., 2017; Samatoshenkov et al., 2020). Endothelial progenitor cells (EPCs) with adenovirus transduction of the VEGF gene were tested in a mouse limb ischemia model and the implanted cells facilitated the neovascularization of the impaired region (Iwaguro et al., 2002).

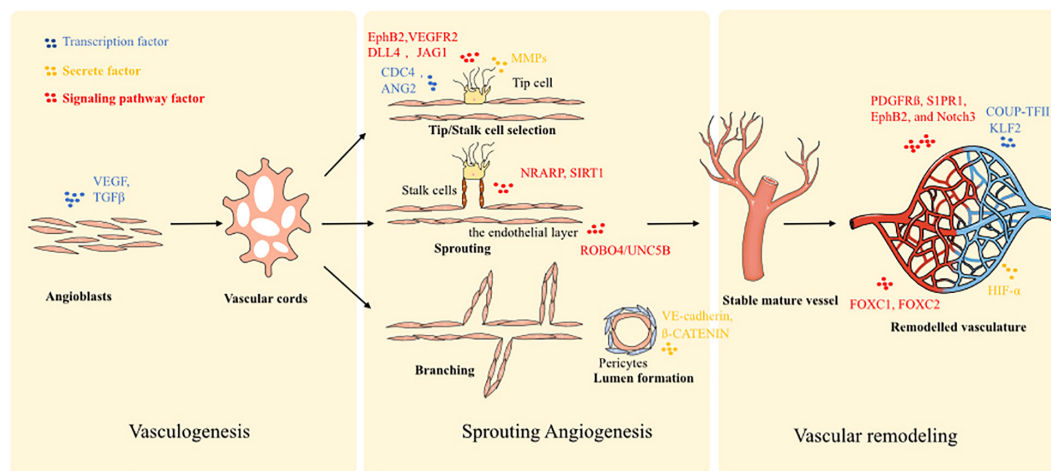


FIGURE 1 | The major molecular regulators in vasculogenesis, angiogenesis, and vascular remodeling.

Similarly, adipose stromal cells are able to serve as a vector for growth factor delivery because of the angiogenic growth factors they secrete, and have demonstrated remarkable angiogenic potential in a limb ischemia model (Rehman et al., 2004; Zhao et al., 2017). However, with DNA transfer it is difficult to control the exact dosage, and risk of mutagenesis from genome integration is also an important issue that needs to be taken into consideration. Compared to DNA delivery, RNA is an attractive alternative because of its ability to induce an exogenous transient expression of growth factors without mutagenesis risk (Lui et al., 2014). The mouse myocardial infarction model was used to test the transplantation of modified RNA encoding VEGF by intramyocardial injection. Injection of the modified RNA increased capillary density and reduced the area of infarction while inducing the differentiation of epicardial progenitor cells towards endovascular cells (Zangi et al., 2013).

Non-coding RNAs for Angiogenesis *in vivo*

Non-coding RNAs, which target mRNAs in a sequence-specific manner, contribute to the regulation of angiogenesis. miRNAs are a type of small endogenous non-coding RNA, which have been a focus of research for over a decade. A series of miRNAs have been shown to have pro- and anti-angiogenic characteristics. MiR-199a, miR-150, miR-145, and miR-153 interact with VEGF signaling, while miR-134, miR-153-3p, and miR-137 regulate Notch signaling activity, and are all involved in modulating angiogenesis (Zhao et al., 2020). MiR-320a and miR-27b have been administered in a preclinical study to re-establish neovascularization in retinopathy (Zampetaki et al., 2016). MiR-424 and miR-210 have been shown to have proangiogenic features in myocardial infarction (Hu et al., 2010), while miR-92 exerts antiangiogenic activity (Bonauer et al., 2009). Furthermore, several miRNAs (including miR-1, miR-133, and miR-126) have been used to inhibit angiogenesis as cancer therapy (Nohata et al., 2012).

In recent years, long non-coding (Lnc)-RNAs have also been discovered and their biological roles have been demonstrated more clearly. Lnc-SNHG1, H19, MIAT, ZFAS1, MEG8, MALAT1, NEAT1, and TUG1 have been identified for their ability to promote angiogenesis via targeting VEGF expression (Zhao et al., 2017; Hou et al., 2018; Long et al., 2018; Smith et al., 2018; Wang et al., 2018; Zhou et al., 2019; Barth et al., 2020; Li et al., 2020; Sui et al., 2020). Several *in vivo* delivery studies have confirmed their functional benefit in the prognosis of ischemic injury, especially of the heart and brain. Moreover, lnc-DIGIT, HIF1A, and XIST, targeting the Notch and DLL4 genes, modulate vascular network remodeling (Hayashi and Kume, 2008; Li et al., 2017; Miao et al., 2018; Tetzlaff and Fischer, 2018).

To deliver the non-coding RNAs *in vivo*, two common strategies are typically used: viral and non-viral introduction (Huang et al., 2020). For virus-based approaches, an AAV has been approved by the United States Federal Drug Administration for oligonucleotide delivery (Wang et al., 2019). Taking advantage of the AAV system, non-coding RNA inhibition and overexpression can be achieved by specific tissue infection based on different serotypes. Besides, adenovirus and lentivirus-based approaches have also been used to transfect animals in *in vivo* research studies. For the non-viral introduction, liposomes, nanoparticles, and hydrogels have been used to deliver non-coding RNAs for promoting vascular tissue regeneration (Li et al., 2018). Furthermore, chemical modifications of the oligonucleotide has been explored to further improve their *in vivo* stability and bioactivity, and include cholesterol modification, methoxyethyl modification, locked nucleic acid (LNA), and antagomirs strategies (Duygu et al., 2019).

CELL THERAPY *IN VIVO*

Besides biomolecular delivery, cell therapy is considered an alternative method for boosting angiogenesis. There are two

main strategies for implanting cells to target tissues, one is the direct cell delivery without material support (Hur et al., 2004), and the other involves embedding cells into a specific biomaterial matrix (Blache and Ehrbar, 2018), such as hydrogel, to generate a compound construct. EPCs are considered to possess the most appropriate features suitable for neo-vascularization in ischemia, as they reside in the bone marrow and retain the ability for self-renewal and transformation into mature ECs (Ingram et al., 2005; Bogoslovsky et al., 2010; Raval and Losordo, 2013). Thus, this type of cell source has been applied in several preclinical studies of functional vascular regeneration, including peripheral ischemia, myocardial infarction (NCT02174939 and NCT03216733) and ischemic cerebral stroke (NCT01289795, NCT01468064, NCT02157896, NCT02605707, and NCT02980354). The studies by Jeevanantham et al. (2012) and Erbs et al. (2007) demonstrate positive results for cell therapy after myocardial infarction in promoting heart function and rebuilding coronal microvascular circulation. Although no evidence has been presented indicating that EPCs can differentiate into other cell types beyond ECs in the heart, in the nervous system, EPCs can integrate into the vascular endothelium in ischemic areas and stimulate neurogenesis (Bogoslovsky et al., 2010).

The three-dimensional (3D) matrix of hydrogel has desired permeability and can effectively enable the diffusion of nutrients and metabolic wastes to the embedded cells, thereby supporting their survival and physiological functions, and has been extensively used for cell encapsulation and delivery (Schmidt et al., 2008). Hydrogel materials have become valuable 3D scaffolds for vascular engineering since they share many features with the natural extracellular matrix (ECM), including high water content and viscoelastic properties. Many hydrogel materials also offers tunable degradability, and can be gradually replaced by the host's ECM as the implanted tissues constructs mature *in vivo* (Langhans, 2018). Typically, the strategy includes four steps: (1) selection of cell sources: somatic cells, adult progenitor and stem cells, and pluripotent stem cells (PSCs) can be used to produce ECs; (2) establishment of hydrogel scaffolds: natural polymers, including collagen, poly(ethylene glycol) (PEG), gelatin methacrylate (GelMA), and synthetic polymers (including polylactic-co-glycolic acid) (PLGA), polycaprolactone (PCL), poly-L-lactic acid (PLLA), glycosaminoglycans (GAGs), hyaluronic acid (HA), and arginine-glycine-aspartic acid (RGD), have been used to manufacture hydrogel matrices for EC embedding and building micro-vessel systems (Khetan and Burdick, 2010; Chwalek et al., 2014; Blache and Ehrbar, 2018). (3) Compound functional graft building: the combination of scaffold, cells, growth factors, and mechanical stimuli recreates a functional microenvironment that stimulates tissue organization into an integrated engineered graft. (4) Graft implantation: the functional graft can be delivered in either in micro (10 μ m) and nano (10 nm) molecules of gels for skin wound repair and osteogenesis in bone loss (Shen et al., 2016; Alarçin et al., 2018). Furthermore, injectable hydrogels can be used for intra-vascular delivery, especially in myocardial infarction (Yang et al., 2018).

ENGINEERED RECAPITULATION OF THE VASCULAR CIRCULATION *IN VITRO*

Vascular Regeneration From Decellularized Matrix

The vasculature of native organs has complex spatial architecture that is essential for the organ-specific functions (Theocharis et al., 2016; Gilpin and Yang, 2017). The decellularized organ ECM also offers preserved essential ECM composition for supporting vessel formation, and serves as a reservoir for bioactive cues, such as growth factors and cell-adhesive mediators (Rhodes and Simons, 2007; Chan and Leong, 2008). Following decellularization, the porcine dermis showed retention of ECM proteins and expression of inherent growth factors, such as VEGF, TGF β , and FGF, which have bioactive potential to stimulate vascular regeneration (Hoganson et al., 2010). The closed circulatory circuit within an organ is composed of multiple types of blood vessels with diverse functions, which are supported by the surrounding ECM (Thottappillil and Nair, 2015). Via decellularization, it is possible to preserve the composition and the overall architecture of specific blood vessel types and to achieve organ-specific vascular regeneration (Rhodes and Simons, 2007; Thottappillil and Nair, 2015). For example, sinusoids are capillaries that exhibit an incomplete basal membrane and widely exist in the adrenal glands, liver, spleen, and bone marrow (Augustin and Koh, 2017). In the decellularized human liver, the sinusoid structures are preserved and allow the migration of LX2 cells during recellularization (Mazza et al., 2015).

The ECM scaffolds used for regenerating large-diameter vascular conduit can be obtained from both decellularized vascular and non-vascular structures. The decellularized ureter and umbilical artery are capable of supporting endothelialization, leading to durable blood vessel formation in *in vivo* animal models (Narita et al., 2008; Gui et al., 2009). Recellularization for vascular scaffolds can be accomplished either *in vivo* by recruiting host's cells following implantation or by *in vitro* culture with seeded cells (Yow et al., 2006). Following implantation, the decellularized allograft matrices demonstrated recellularization by recruiting host cells *in vivo* for ovine vessel and rat aortic conduit reconstructions (Ketchedjian et al., 2005; Assmann et al., 2013). The sources for *in vitro* recellularization can be mature vascular cells, such as ECs or smooth muscle cells, and EPCs (Zhang et al., 2007). For example, porcine iliac arteries can be decellularized and seeded with EPCs before transplantation, which allowed improved patency rates *in vivo* (Kaushal et al., 2001). However, efficient regeneration of complex microvascular structures, especially recapitulating their organotypic anatomy and functionality, remains challenging.

As an effort to address these challenges, advances in whole-organ perfusion decellularization in the past decade provide a convenient approach for producing organ-specific, acellular vascular bed allowing access to both macrovascular and microvascular compartments throughout the entire organ. For example, to rebuild the gas exchange function and attain vascularization, rat lungs were decellularized and seeded with

ECs and epithelial cells or adipose-derived stromal cells within a bioreactor, which simulated a biomimetic culture conditions. Following orthotopic transplantation, the mature vascularized lung constructs was capable of providing gas exchange function *in vivo* (Ott et al., 2010; Doi et al., 2017). For the recellularization of a liver graft, hepatocytes and ECs were used to reconstruct the vasculature in the decellularized liver matrix (Uygun et al., 2010). Using perfusion recellularization, the embryonic stem cells or iPSC-derived ECs can be efficiently retained in the decellularized kidney scaffold, resulting in a uniform distribution in the vascular network and in glomerular capillaries, together with site-specific EC specialization (Bonandrini et al., 2014; Ciampi et al., 2019).

Mimicking Angiogenesis *in vitro*

Compared to angiogenesis *in vivo*, models simulating angiogenesis *in vitro* can precisely control each component of vascular development, imitate the complex microenvironment and required factors in the body, with high repeatability. In current studies, *in vitro* vascular research usually uses differentiated ECs or adult EC lines and another type of cells (such as fibroblasts/mesenchymal cells/parenchymal cells, and even tumor cells) to be cultured under controlled 3D conditions. This creates a defined culture system for studying the effects of certain variables on angiogenesis. Two-dimensional (2D) *in vitro* models can be used to study the behavior of ECs, such as migration and proliferation; however, because 2D models lack the more physiological 3D environment, they cannot reflect the contribution of numerous typical characteristics of ECs, such as lumen formation and differentiation into tip and stalk cells. Therefore, constructing a 3D vascular model is particularly helpful for studying vascular tissue behavior with reproducible conditions of morphology and signals. Moreover, through reasonable engineering design, a high-throughput angiogenesis model has been developed, which facilitates screening of angiogenesis modulators.

Blood vessels are organs of luminal structure with essential transport and perfusion functions. Therefore, how to realize the perfusion and circulation function is a key task to accomplish for engineered vascular network *in vitro*. Tissue engineering methods for constructing 3D blood vessels *in vitro* mainly include self-organized cell sheets, 3D printed blood vessel networks and blood vessel chips (Sarker et al., 2018). In the self-organization approach, the growth and developmental process of the vascular system is stimulated, causing the EC to spontaneously form blood vessels in the hydrogel with native-like morphology and function. However, due to the randomness of self-organization, it remains difficult to perform fluid infusion. Although 3D printing blood vessels is convenient and automated, it is challenged by the difficulty to remove the hollow channels where the materials and blood vessel cells are fused, and the low spatial resolution.

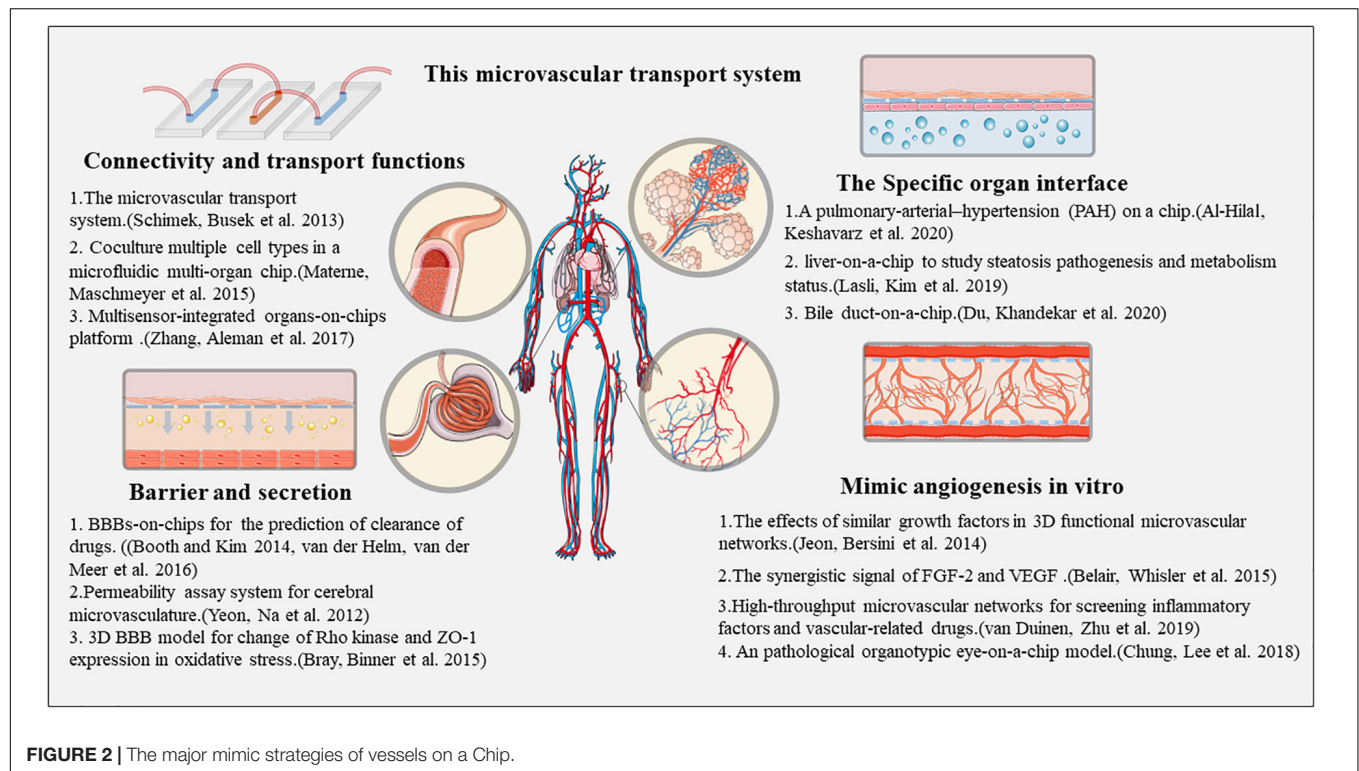
Jeon et al. (2014) combined ECs with the ECM, and observed the generation of microvascular networks, in which ECs began to aggregate into a solid tube-like shape, with buds extending outward while responding to the effects of native-like growth factor combination of VEGF, TGFβ1, and Ang1. Belair et al. (2015) implanted iPSC-ECs and lung fibroblasts into fibrin gels within microfluidic devices. At the beginning of the culture, iPSC-ECs showed budding, migration, and invasion behavior,

and were able to respond to fluid stimulation by extending in the direction of the fluid flow and forming perfusable lumens (Belair et al., 2015). van Duinen et al. (2017) developed a model of 96 individually addressable, 3D microvessels in a standardized microfluidic platform. It allows simultaneously analysis of the effects of different ECM/medium/cytokines (VEGF, TNFα, and IL-1β) on the barrier function of the engineered microvessels (van Duinen et al., 2017). The same group later refined this design and developed a more standardized high-throughput culture platform integrated into a 384-well plate, which formed 40 repeating units of three microfluidic channels. It can detect the effects of multiple factors and shows promise in application to screen vascular-related drugs (van Duinen et al., 2019). Kim et al. (2015) proposed a quantitative microfluidic angiogenesis screening platform (QMAS) that can monitor and quantify cell behavior from treatment with various drug concentrations, such as morphological changes, EC viability, and angiogenic bud formation. The platform has 14 connected chambers, with medium flowing through the gaps between each chamber to achieve a drug concentration gradient (Kim et al., 2015).

Establishing Different Types of Microvessels on a Chip

At present, there are several different constructions and design strategies that have generated blood vessel chips. Generally, there are five types of vessel chips available as *in vitro* models: (1) microfluidic membrane: a microfluidic device incorporating a mechanically stretchable membrane that can capture the organ-level function of vascular and epithelial interfaces; (2) microfluidic scaffolds: a standalone microfluidic network that can be fabricated using a synthetic polymeric elastomer serving as a scaffold for the construction of vascularized functional tissues; (3) microfluidic hydrogels: consisting of hydrogels with built-in microchannels, that offer an alternative method to model tissue interfaces; (4) self-assembled microvasculature: which establish proper connections to microchannels —such a self-assembled microvasculature can be perfused with a microfluidic circuit; (5) open-well design: uses preformed parenchymal tissues, such as tumor spheroids, that can be inserted on a preformed vascular bed in a multistep seeding approach —the versatility of this platform is that it allows potential integration of the microvasculature with various models of dense solid tissues (Zhang et al., 2018).

Initial efforts focusing on building microfluidic chip devices with only vascular structures, primarily for validating the vascular functions in response to fluid flow. To date, several cell sources have been explored to generate microvascular networks, including colony-forming cell-derived ECs isolated from the cord blood, human dermal microvascular ECs, kidney peritubular microvascular ECs and HUVECs. These artificial tissues model the formation of a vascular barrier with perivascular interaction, vascular sprouting, a basement membrane, and ECM formation, and cytokine responses. Elevated gene expression of VCAM1, E-selectin, ICAM1, and leukocyte recruitment in response to TNF-α stimulation allows modeling of vascular dysfunction due to sepsis and inflammation. Furthermore, several molecular tests could be performed to evaluate the inhibitory effects



on angiogenesis. Yasotharan et al. (2015) have built vessel chips using arteries from mice based on a microfluidic platform, and tested the calcium flux and dose-dependent vasoconstriction in response to phenylephrine, in the presence or absence of nifedipine.

Moving to engineering vascularized complex tissues, the blood-brain barrier (BBB) model has been generated by a hierarchical combination of ECs, neurons, and astrocytes based on a microfluidic platform (Andjelkovic et al., 2020). The sources of ECs for this purpose can be HUVECs, and primary brain-derived microvascular ECs. The BBB tissue models act as assays of drug activity, allowing evaluation of the flow, structure, and metabolic characteristics of brain vessels. Moreover, the incorporation of microvascular network is also important for studying tumor tissue biology as angiogenesis is essential for tumor growth. Thus, co-culture of various cancer cells with perfusable microvasculature allows generation of a virtual tumor tissue and provides a platform to observe tumor growth characteristics, screen drugs, and assess the efficacy of radiation therapy (Dijkstra et al., 2018). All the above information has been summarized in **Figure 2**.

PRACTICAL AND CLINICAL APPLICATIONS OF VESSELS ON A CHIP

Drug Testing

Preclinical drug screening is mainly performed using animal models. Although of great value, animal responses to drugs may show differences in terms of toxic doses and drug metabolism

compared to human patients. Disease models tissue-engineered with patient cells can be used to accurately predict drug responses on tissue or even organ scales. Many current *in vitro* disease models often contain only the organ parenchymal cells without vascular support; thus, lack the ability to investigate the transport and metabolism of drugs in the vascular system (Fernandez et al., 2016). Compared with animal models and 2D experimental platforms, microvascular chips can conduct more detailed experiments on factors affecting vascular formation and have greater potential for use in vascular disease models to study pathological events and develop drug screening platforms. Besides, the microvascular chip systems have already made into commercial application beyond laboratory, focusing on high-throughput drug screening, precise diagnosis, disease modeling, and targeted therapy verification, which provide essential models for clinical management of cardiovascular diseases with opportunities of new discoveries (Portillo-Lara et al., 2019; Savoji et al., 2019).

In general, drug testing focuses on three aspects: (1) effects of drugs on vascular function. Microvascular networks and artery tissue models provide a platform to test drug effects on vessel growth and contraction, such as inhibitors of Rho kinase (Y27633), tyrosine phosphorylation (genistein), SRC kinase (PP2), and nitric oxide (L-NAME) (Ribas et al., 2016). (2) Molecules for blood barrier transportation. BBB and blood-retina barriers are always impediments for drug transportation and cellular translocation to particular tissues of interest to reach a proper therapeutic dose. Booth and Kim (2012) published their μ BBB model, and demonstrated that co-culture with astrocytes resulted in an improved barrier functionality. Further,

they showed that permeability coefficients in their model under dynamic conditions correlated well with *in vivo* brain/plasma ratios, demonstrating the potential of this model to predict drug clearance by the BBB (Booth and Kim, 2014; van der Helm et al., 2016). Cho et al. (2015) published a 3D BBB model, with the ability to record transmigration of neutrophils across the endothelial barrier and through the side channels, which was used to study ischemia by exposing the endothelium to low oxygen and low glucose levels and subsequently allowing reoxygenation to normal conditions. Formation of reactive oxygen species and the activation of Rho kinases as a result of oxidative stress was confirmed, as well as a decrease in tight junction ZO-1 expression. These *in vitro* culture models (van der Helm et al., 2016) have been used in combination with animal models as a supplementary testing platform, as at this stage the *in vitro* platforms alone are not yet suitable to reproduce all functional activities. (3) Chemical medication screening. Co-culture of cancer cells and the permeable microvascular network could induce tumor tissue growth *in vitro* (Bray et al., 2015), and offers the possibility for screening of chemotherapeutic agents. Drug screening has been performed to evaluate the inhibition efficacy on tumor growth and on the associated vasculature, by agents such as bortezomib, vincristine, mitomycin C, gemcitabine, vorinostat, tamoxifen, linifanib, axitinib, and sorafenib, pazopanib, oxaliplatin, folinic acid (leucovorin), and 5-fluorouracil (Zhang et al., 2018).

Disease Modeling

Modeling a disease using patient-derived cells provides insight into the underlying pathology. Importantly, this approach provides huge advantages over the use of animal models, as animal models often cannot completely reproduce the physiological responses of human tissues (Cochrane et al., 2019).

An organotypic eye-on-a-chip model has been described, which mimics the retinal pigment epithelium (RPE)-choroid complex *in vitro*. This model consists of a RPE monolayer and adjacent permeable blood vessel network, which supports the barrier function of the outer blood-retina barrier (oBRB). The intact barrier function of the RPE-choroid complex is reconstituted while maintaining important structural features. Further, this model can successfully mimic the pathogenesis of the choroidal neovascularization (CNV). The alleviation of the pathological angiogenesis mechanisms can be modeled with bevacizumab, a clinical drug used in CNV treatment (Chung et al., 2018), utilizing blood outgrowth ECs (BOECs) as a disease-specific primary cell source to analyze vascular inflammation and thrombosis in vascular organ-chips or “vessel-chips” (Mathur et al., 2019). Similar concepts have been applied to other organ systems. Al-Hilal et al. (2020) reported a pulmonary-arterial-hypertension (PAH) model on a chip to elicit arterial remodeling under PAH. The authors fabricated a microfluidic device to emulate the luminal, intimal, medial, adventitial, and perivascular layers of a pulmonary artery. Three types of pulmonary arterial cells (PACs), endothelial, smooth muscle, and adventitial cells were seeded to form the model. Moreover, Lasli et al. used the human hepatocellular carcinoma (HepG2) cell line and HUVECs as spheroid cultures to establish a “liver-on-a-chip” model to study steatosis pathogenesis and metabolism status (Lasli et al.,

2019). In addition, Du et al. (2020) attempted to build a “bile duct-on-a-chip” to achieve organ-level function as a specific vessel device to evaluate bile duct pathophysiological processes.

Moreover, disease models have also been built to study the blood vessels themselves by examining endothelial dysfunction. TNF- α stimulation has been used to explore the biological procedure of thrombus formation as a mechanical cue. Further, ECs binding of T cells and neutrophil extravasation has been used to study the activation of endothelial inflammation. However, there is no suitable model to evaluate the features of Kawasaki disease. Thus, vessels chips may provide an opportunity to approach the pathogenesis of coronary lesions of Kawasaki disease. Furthermore, this platform may contribute to understanding mechanisms that promote thrombus formation, plaque geometry, and the high permeability observed in the atherosclerotic endothelium.

Tissue Regeneration and Repair

The limitations of organ transplantation due to insufficient organ supply have given rise to the development of regenerative medicine. Its main purpose is to replace missing or damaged tissues with materials and tissue-specific cells and their various combinations to promote structural and functional healing. Currently, only a few engineered tissues (skin, cartilage, and bladder) have reached clinical success, while biomaterials designed to replace more complex organs have not been commercialized because, unlike engineered skin, cartilage, or bladder tissue, cell viability and the optimal function of the construct cannot be maintained by diffusion alone, and oxygen and nutrients necessary for survival and organ integration cannot be effectively supplied following transplantation. Therefore, the generation of functional vasculature is critical to the clinical success of engineering and construction of organs (Bae et al., 2012).

The human body contains a rich blood vessel network, which is mainly used for supplying oxygen and nutrients. Therefore, for regenerative tissue culture models, a multi-organ chip system containing a blood vessel network chip is physiologically relevant. Schimek et al. (2013) established a human cardiovascular on a chip-based system, with the microvascular system co-cultured with skin and liver tissue, and demonstrated the ability of the microvascular chip to promote the survival of other tissues. Materne et al. (2015) proposed a protocol to co-culture multiple cell types in a microfluidic multi-organ chip, and successfully constructed a microenvironment of skin tissue and liver tissue and blood vessels, enabling the a circulation network. The development trend of organ chips is that of multi-organ combined chips; thus, researchers have attempted to integrate multiple types of chips into “body-chips.” Zhang et al. (2017) built an easy-to-use modular, sense-based, multi-organ platform, whereby multiple organoid models (human cardiac organoids and vascular organoid, and human liver organoids) are connected by micro-organ chip technology, which can systematically study and analyze the ability of multiple organ interactions, with the advantages of continuous, dynamic, and real-time monitoring (Zhang et al., 2017).

Engineering a functional alveolar-capillary interface (lung), large functional myocardial tissue with supplying vessels tissue

(heart), functional hepatic tissue combining hepatocytes and the bile duct (liver), functional renal tissue comprising permeability and filtering features (kidney) is the main goal for organ tissue construction from a regenerative perspective. Furthermore, mimicking the 3D arterial architecture, vascularized skin would also be more helpful for wound repair. Thus, the combination of functional artificial tissue with a vascularized network is a critical, desired objective for regenerative medicine. The establishment of blood vessels in different organs and different types of “organ on the chip” is summarized in **Table 1**.

DISCUSSION AND FURTHER CHALLENGES

Although rapid progress has been made in the field of vascular tissue engineering in the past decade, the lack of functional vasculature has been widely regarded as one of the main obstacles hindering the effective reproduction of *in vivo* physiology. So far, the method of vascular reconstruction using acellular matrix in the body is mainly aimed at the reconstruction of large blood vessels. The real perfusion function of the whole blood vessel network can only be achieved following transplantation into the body and anastomosis with the host blood vessels. Chips have many recognized advantages as a convenient *in vitro* model of organ function and disease. Therefore, *in vitro* engineered pre-vascularization followed by embedding other organ models into the pre-established capillary bed, can be regarded as a way to realize the co-cultivation of microvascular chips and other organs.

Bottleneck of Building Tissue-Engineered Blood Vessels

Building a functional vascular network has long been recognized as a major challenge in tissue engineering. Although we have now developed various types of vascular chips and printed vascular networks, there remains a lack of effective methods for regulating vascular network conformation and vascular scale, as well as a lack of commercially available models that are capable of supporting physiological perfusion and implantation. There is an unmet need to identify proper sources of patient-derived cells to be applied in organ-on-chip models to increase our mechanistic understanding of diseases and to assess the efficacy of drugs. The interactions between blood flow, vessel architecture, cells, and parenchymal tissues vary drastically from organ to organ. These differences largely arise from the structural, compositional and functional differences of the vasculature in different organs. The organ-specific origin of endothelial cells can have a profound effect on the biological activity. Many commonly used generic ECs, such as HUVECs, lack the characteristics presented by microvascular endothelial cells, which is an important feature for model systems. Thus, the choice of ECs will depend on the specific organ model and the corresponding parenchymal tissues. In the method of using acellular matrix and whole organ perfusion to build vascular network, the cell sources are always limited within a few cellular types, which is probably

insufficient to establish functional, organotypic circulation network. Advances in targeted differentiation of stem cells and primary tissue isolation techniques will help us develop more organ-specific ECs (Lin et al., 2019). Taking advantage of these innovations, we expect continuing progresses toward engineering organ-specific vascular systems for improved understanding of the physiological and molecular mechanisms of organ and tissue formation.

The application of acellular matrix for rebuilding vascular network is superior compared to other synthetic matrices, but it remains difficult to fully recapitulate the native growth factor environment. The material used for the blood vessel chip should also consider the characters of material to support cellular growth in tunnel formation. Besides natural scaffolds, polydimethylsiloxane (PDMS) is the one of most widely used synthetic materials, which demonstrates the features of insulation, non-conductivity, resistance to leakage, good biocompatibility, easy oxygen permeability, and good optical properties. However, PDMS is difficult to be used for regeneration and transplantation applications due to its non-degradability. With the development of new materials, acellular matrices, and artificial polymers should be designed in combination with each other to facilitate the building of functional vascular network (Zhang et al., 2018).

Materials for construction of chips, cellular fidelity, multiplexing, fluid handling, scalable production, and validation of organ-on-a-chip devices are all areas requiring further study (Zhang et al., 2018). Material modification techniques should be developed to enable the modulation of material biocompatibility as well as their function on proliferation and differentiation. In addition, materials to be in contact with blood, there has always been a challenge of potential thrombosis, thus material modifications that reduce thrombogenicity is an area of extensive research for future development of vascular interfaces (Greco Song et al., 2018). In addition, computational modeling that enables the design of physiologic network architecture, including channel size, hierarchical structure, flow rate control are desired (Chandra and Atala, 2019).

Cross-Cooperation Across Research Fields

To build vascular network mentioned in this article is a field that requires interdisciplinary methods, knowledge, and technologies in biotechnology, tissue engineering, vascular biology, biomaterials, cell engineering, and stem cell biology (Li et al., 2014). In order to achieve effective and functional vascularization of biological materials, it is necessary to make the combination and synchronization of several factors that effectively characterize tissue vascularization *in vitro*, incorporating the inherent characteristics of biological materials and adapting to scaffolds. The biological concerns include how to control vascular stimulation by pro-angiogenic molecules, appropriate culture conditions, and fluid shear stressor. When these factors are integrated on the chip, they may affect the

TABLE 1 | Build the blood vessels in different organ and different type of “organ on the chip.”

Chip type	Cell types	Hydrogel	Features	Reference
Angiogenic high-throughput platform	HUVEC-VeraVec™ human endothelial cells	4.0 mg/ml solution of collagen type I	High-throughput, perfusion rocker; optimize the combination of angiogenic factors	van Duinen et al., 2019
Angiogenic chip	Human umbilical vein endothelial cells or RFP-expressing HUVECs	2.5 mg/ml solution of type I collagen	Screen for anti-angiogenic therapeutic drugs	Kim et al., 2015
Blood–brain barrier chip (BBB chips)	The brain endothelial cell line bEnd.3 and the glial cell line C6	Collagen IV/fibronectin	Quantified trans-endothelial electrical resistance (TEER); the permeability of the blood-brain barrier was measured	van der Helm et al., 2016
Cerebral vasculatures chip	Primary cultured HUVECs and human astrocytes	Attachment	Testing for chip based permeability measurement of drugs	Yeon et al., 2012
Tumor angiogenesis chip	HUVECs and MSCs, cancer cell line	starPEG solution	Mimic tumor angiogenesis microenvironments <i>in vivo</i>	Bray et al., 2015
Wet-AMD on a Chip	HUVECs and normal human lung fibroblasts, ARPE-19 cell line	fibrinogen solution	Modeling the pathogenesis of CNV especially in terms of morphogenesis	Chung et al., 2018
Vessel-chips	Endothelial progenitor cells from blood	Type I rat-tail collagen	Modeling vascular inflammation and thrombosis	Mathur et al., 2019
Pulmonary-arterial-hypertension (PAH)-on-a-chip	ECs, SMCs, and ADCs isolated from healthy human and patients with idiopathic PAH (IPAH)	Type I collagen solution	Modeling the PAH pathophysiology on the device	Al-Hilal et al., 2020
Liver-on-a-chip	HepG2 (from ATCC HB-8065) and HUVECs (from ATCC PCS100-010)	Spheroid in suspension	Modeling the nonalcoholic fatty liver disease	Lasli et al., 2019
Microvascular transport system	Human dermal microvascular endothelial cells	Attachment	Modeling the transport function of the human cardiovascular system	Schimek et al., 2013

growth and function of blood vessels (Bramfeldt et al., 2010; Zhang et al., 2018).

Large or Small Vessels

At present, the application of large-caliber stent vessels in clinical applications will allow good blood flow perfusion and connectivity in the short-term, although in the long-term, further embolic events will often occur due to the deposition of blood components. The function, and often the transplantation of such large-caliber blood vessels, is limited to less than 1.5 cm in length, which is relatively costly (Fukunishi et al., 2016). The ideal vascular graft should be able to undergo rapid vascular remodeling by promoting host cell infiltration and encourage accompanying stent degradation. The perfusion function of small-caliber blood vessels and the maintenance of fluids is much more difficult to achieve. Therefore, to reconstruct large scale blood vessels, the acellular matrix-based angiogenesis is considered as optimal method with low rate of thrombosis occurrence and well anastomosis. Compared with the vascular chip, it can be used as a more convenient technology to verify or reproduce the function of part of the blood vessels in the body to simulate vascular-related diseases. In addition, due to the advantages of the repeatability and easy expansion of the

artificial vascular chip, it offers the promise to enable commercial applications in high-throughput drug screening.

AUTHOR CONTRIBUTIONS

DZ, YL, and XR conceived of the presented idea, supervised the project, and contributed equally to the final version of the manuscript. XM, YX, and JL summarized the reference and draft the manuscript. CD helped with collected the reference. XM draft the table. DZ organized the figure with online free material. All authors contributed to the article and approved the submitted version.

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The Current Dilemma and Breakthrough of Stem Cell Therapy in Ischemic Heart Disease

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Ischemic heart disease (IHD) is the leading cause of mortality worldwide. Stem cell transplantation has become a promising approach for the treatment of IHD in recent decades. It is generally recognized that preclinical cell-based therapy is effective and have yielded encouraging results, which involves preventing or reducing myocardial cell death, inhibiting scar formation, promoting angiogenesis, and improving cardiac function. However, clinical studies have not yet achieved a desired outcome, even multiple clinical studies showing paradoxical results. Besides, many fundamental puzzles remain to be resolved, for example, what is the optimal delivery timing and approach? Additionally, limited cell engraftment and survival, challenging cell fate monitoring, and not fully understood functional mechanisms are defined hurdles to clinical translation. Here we review some of the current dilemmas in stem cell-based therapy for IHD, along with our efforts and opinions on these key issues.

Keywords: stem cell, ischemic heart disease, cardiac repair, cellular therapy, mechanism

INTRODUCTION

Ischemic heart disease (IHD) is the leading cause of death worldwide (Moran et al., 2013), and it is estimated to have 11 million patients with IHD in China (The Writing Committee of the Report on Cardiovascular Health and Diseases in China, 2020). Myocardial ischemia causes irreversible myocardial cell death, which leads to mass loss of heart function, formation of fibrous scars, and adverse cardiac remodeling, eventually progress toward heart failure (Tao et al., 2015; Han et al., 2017). Although many advances have been made in the medical and surgical treatment for IHD, some IHD patients are not suitable for surgery and have no effective drug treatment, they are called “no-option” patients (Fuchs et al., 2003). Stem cell therapy has emerged as a new strategy for the treatment of IHD (Nguyen et al., 2016; Dou et al., 2018). However, clinical studies have not yet yielded the expected benefits, and multiple preclinical and clinical studies have shown paradoxical result (Pan et al., 2014; Bolli and Ghafghazi, 2017; Bagno et al., 2018). A meta-analysis by Gyöngyösi et al. (2016) assessed the efficacy of stem cells from different sources for the treatment of acute myocardial infarction (AMI). The results argued that stem cell therapy had

no significant effects on the improvement of myocardial contractility, ventricular remodeling and clinical prognosis. Several clinical trials found that stem cell transplantation had a moderate or subtle improvement in the heart function of patients with AMI and chronic heart failure after 1 year's follow-up (Cong et al., 2015; Henry et al., 2017). The ejection fraction of patients in the treatment group increased by 4–6% compared with the control group, and stem cell transplantation inhibited the early ventricular remodeling (Gyöngyösi et al., 2015). What do these discrepancies come from? The following unsolved issues of stem cell therapy might be able to answer this question.

CELL SOURCES AND DOSAGE

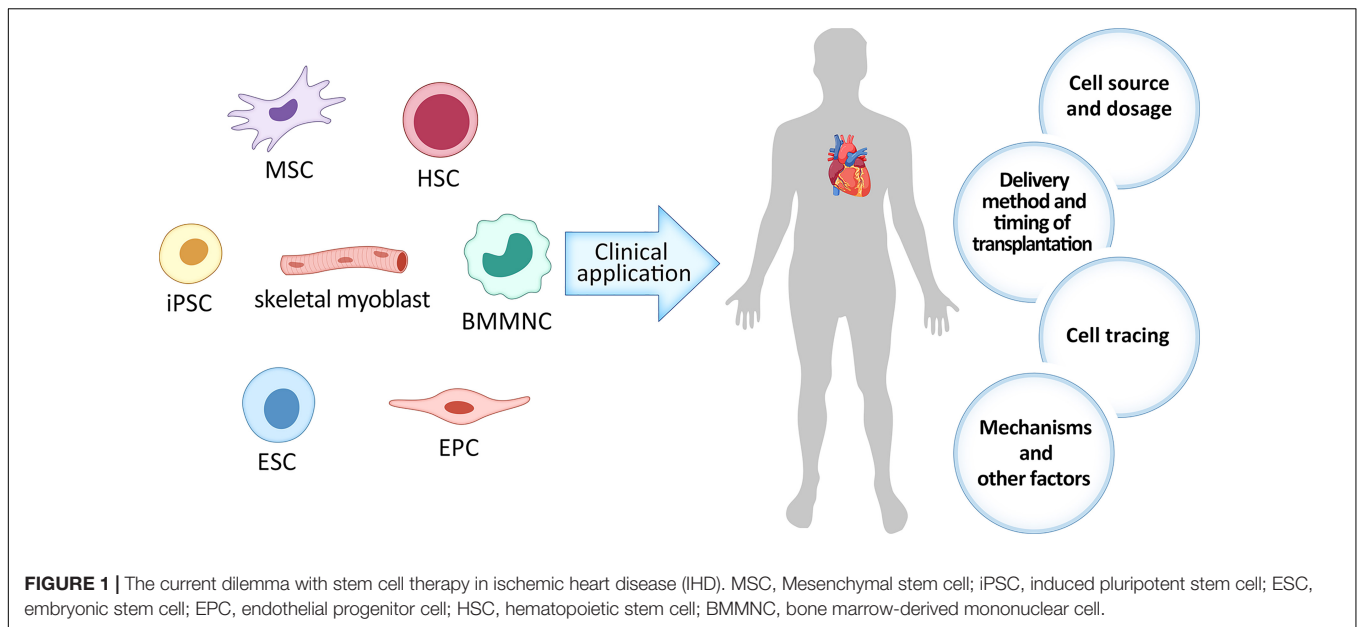
Plenty of studies have shown that various cell types exerted beneficial effects on cardiac repair (Figure 1), such as skeletal myoblast (Suzuki et al., 2000), bone marrow-derived mononuclear cell (BMMNC) (Cao et al., 2009), hematopoietic stem cell (HSC) (Garikapati et al., 2013), endothelial progenitor cell (EPC) (Babin-Ebell et al., 2010), mesenchymal stem cell (MSC) (Shabbir et al., 2009), embryonic stem cell (ESC) (Menasché et al., 2015), induced pluripotent stem cell (iPSC) (Mauritz et al., 2011), etc. Skeletal myoblast was the first cell type to be clinically tested in myocardial repair since researchers surmised that it can increase myocardial contractility. However, the efficacy was unsatisfactory mainly due to the high incidence of arrhythmias (Suzuki et al., 2000). BMMNC contains the undifferentiated HSC and MSC as well as other committed cells in various stages of maturation. Its abundance and easy accessibility allows for autologous implantation without expansion, which avoids the decline of stem cell differentiation and migration ability and negated the incidence of immune rejection. In our previous clinical trial, we found that intracoronary delivery of autologous BMMNC was safe and feasible for STEMI patients and can lead to long-term improvement in myocardial function as far as 4 years (Cao et al., 2009; Tao et al., 2015). HSC has multiple differentiation potentials and can be autologously transplanted, but they are limited in abundance, which leads to poor efficacy (Garikapati et al., 2013). EPC, isolated from peripheral blood and bone marrow, can give rise to vascular cells. Clinical application of EPC transplantation is expected to increase the capillary density and subsequently improve the microcirculation around the transplanted sites in infarcted heart. Studies have showed that EPC transplantation can also improve heart function, but its effect is restricted, which may result from its weak differentiation ability (Babin-Ebell et al., 2010). ESC have strong proliferation and differentiation capabilities, but it has ethical controversies and high risks of teratoma formation, which create hurdles to its clinical translation (Hentze et al., 2007; Wang et al., 2013).

MSC can be isolated from multiple tissues including, but not limited to, bone marrow, adipose tissue and umbilical cord, and can be easily expanded *in vitro*. MSC is the most widely studied cell type for its abundant source, simple operation, and immune exemption (Shabbir et al., 2009). Plenty of studies have confirmed its effectiveness and safety. Leistner et al. (2011) used autologous

BM-MSCs from 55 patients with AMI for vascular reconstruction after *in vitro* expansion and culture. Within 1 week, the cells were transplanted by means of intracoronary arterial injection. After 5 years of follow-up, no patient was hospitalized for treatment due to heart failure, and no cardiac muscle calcification or tumor formation was found. The ejection fraction of the patients increased from $(46 \pm 10)\%$ to $(57 \pm 10)\%$, and the infarct area decreased significantly. 116 patients with AMI received the treatment of umbilical cord mesenchymal stem cells *via* coronary artery injection for 4 months, the results showed that the myocardial survival area and myocardial perfusion in the treatment group were better than those in the control group. After 18 months, the cardiac function test showed that the ejection fraction in the treatment group increased by 7.8%, while that in the control group only increased by 2.8% (Gao et al., 2015). Another important cell type for cell therapy is iPSC. It can be derived from patients themselves and resembles the characteristics of ESC. A large number of preclinical studies have confirmed that iPSC-derived cardiomyocytes can improve cardiac function after AMI (Khatiwala and Cai, 2016). Besides, it is easy to obtain and can be expanded in a large amount in a short time *in vitro*, which stands out as the most promising cell population in the future.

As far as we are concerned, the selection of seed cells must follow the disease-driven principles firstly. For example, HSC is preferred for leukemia, MSC is preferred for graft-vs.-host disease (GVHD), etc. For IHD, MSC, and iPSC have a good prospect. Secondly, the ideal seed cells of IHD need to meet the following requirements: high availability (“off-the-shelf”), not functionally impaired by patient's comorbid conditions, consistent, convenient, and inexpensive large-scale expansion. According to the results of a number of stem cell clinical trials in the last decade, MSC and iPSC are the most promising cell types for IHD in clinical settings (Labusca et al., 2018).

Cell dose may be an important determinant of the efficacy of cell therapy. Excessive cells not only increase the costs, but also increase various risks, such as inducing vascular blockage, while too few cells may fail to achieve satisfactory results. However, there are few studies on the dose-effect relationship of cell transplantation and there is a lack of comparability between different studies due to differences in cell types, cell culture differences, transplantation methods, follow-up time, and evaluation indicators. A meta-analysis by Xu et al. (2016) involved the use of different dose of bone marrow stem cells (BMCs) for the treatment of AMI. The results showed that the LVEF change in the BMCs group was significantly higher than in the control group when the dose of BMCs was 1×10^8 and 1×10^9 , while no significant LVEF change was observed at a dose of 1×10^7 . In addition, when the dose of BMCs exceeds 1×10^9 , they did not manifest significant LVEF up-regulation ($P = 0.10$). Therefore, stem cells may be effective in patients with AMI with a dose between 1×10^8 and 1×10^9 . For the treatment of chronic IHD, Afzal et al. (2015) found that there was no significant improvement in cardiac parameters in trials that used fewer than 5×10^8 cells. Transplantation of 5×10^8 to 1×10^9 cells induced significant improvement in LVEF and left ventricular end systolic volume (LVESV). In addition, different



transplantation methods have posed different demands on cell dosage, and the optimal dosage remains to be further determined in each specific setting.

ROUTE OF CELL DELIVERY AND TIMING OF CELL TRANSPLANTATION

To date, delivery routes in cardiac cell therapy mainly includes thoracotomy injection, system infusion and imaging guide mini-invasive injection (Figure 2; Kanelidis et al., 2017).

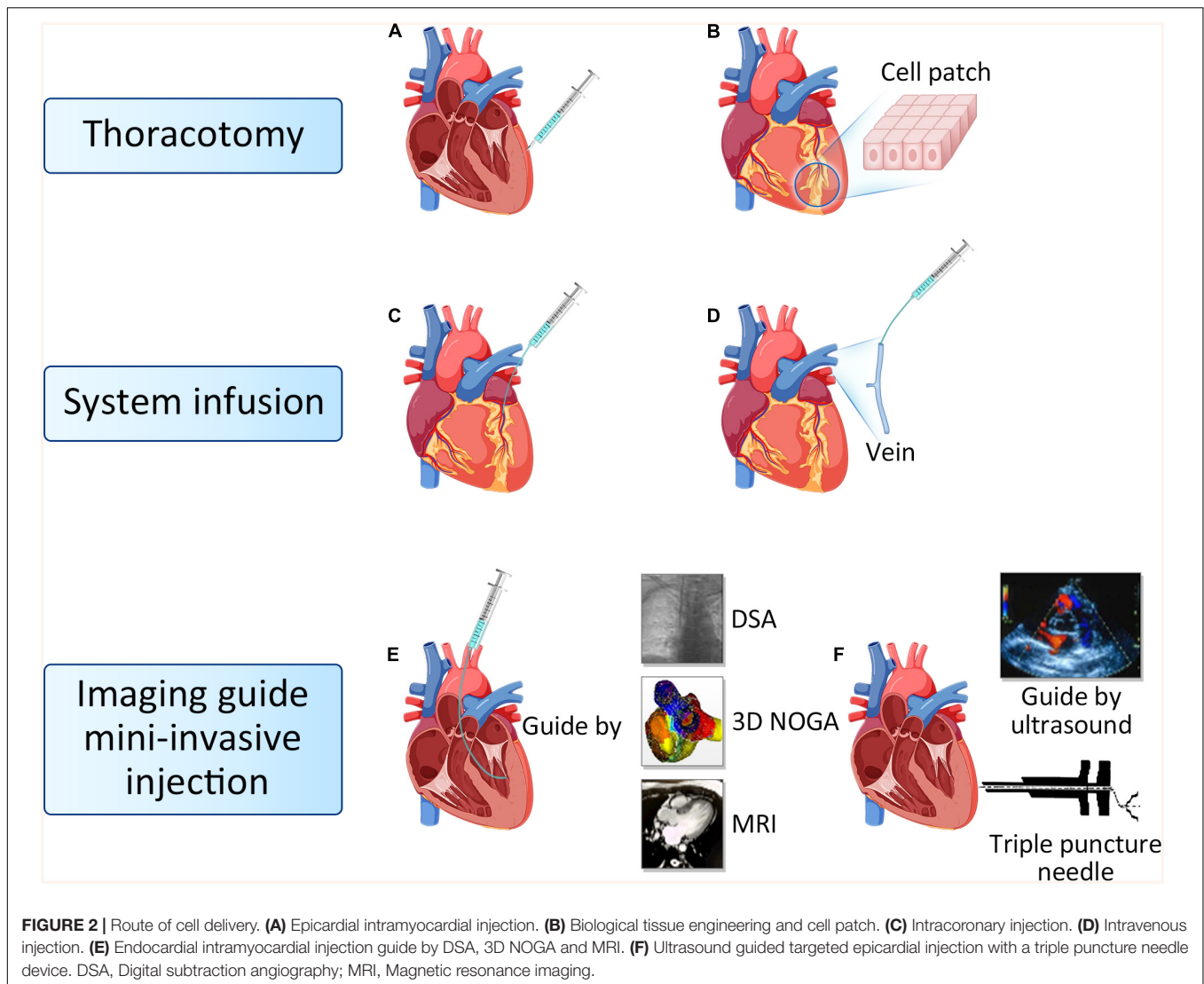
Thoracotomy Injection

Thoracotomy injection consists of epicardial intramyocardial injection and cell patch. The trans-epicardial intramyocardial injection is the most classical cell delivery method, which can inject cells into the targeted area directly to avoid cells loss (Hamano et al., 2001). However, this method usually requires general anesthesia and thoracotomy. Potential adverse effects are left ventricular perforation, bleeding from the myocardium and unbalanced ventricular motion caused by the uneven distribution of cells after injection. This transplantation method is suitable for patients undergoing coronary artery bypass surgery and simultaneous heart valve surgery. Tissue engineering technologies have been used to develop cell delivery methods for regenerative therapy (Avolio et al., 2015). Using this approach, stem cells are expanded and adhered to cardiac patch, and subsequently delivered onto the surface of the damaged heart *via* thoracotomy. In our previous research, we designed a cardiac patch fabricated with electrospinning cellulose nanofibers modified with chitosan/silk fibroin (CS/SF) multilayers, then engineered with adipose tissue-derived mesenchymal stem cells (AD-MSCs). We adhered the nano-patch to the epicardium of the infarcted region in rat hearts, found that CS/SF-modified nanofibrous patches promote the functional survival of engrafted

AD-MSCs (Chen et al., 2018). It has been reported that the cell patch improved cell survival and engraftment, resulting in a positive effect on cardiac function (Shimizu et al., 2009; Noguchi et al., 2016; Sugiura et al., 2016).

System Infusion

System infusion including intracoronary injection and intravenous injection. Intracoronary injection can increase the number of cells homing to the ischemic area of the myocardium, while avoiding the damage caused by direct injection in the myocardium (Diederichsen et al., 2008; de Jong et al., 2014). Patients do not need to open the chest when combined with PCI surgery, so it is the most commonly used approach in clinical practices. The disadvantage is that some stem cells can be lost through coronary circulation, and over dose of cell injection can cause coronary artery occlusion, resulting in regional myocardial infarction again (de Jong et al., 2014). Intravenous stem cell transplantation is a non-invasive, reproducible, economical and convenient clinical treatment strategy for patients with IHD. Although some studies have shown that systemic intravenous administration of cells could hardly home to the heart because of the pulmonary first-pass effect (Lin et al., 2015). However, other studies argued that intravenous delivery of MSC could limit infarct size and improve LV function, which could be ascribed to the secreted TSG6 from trapped cells in lungs. TSG6 was subsequently proved to promote the revascularization of damaged heart (Lee et al., 2009). In our previous study, we combined intracoronary delivery with multiple intravenous infusions in a porcine model of chronic myocardial ischemia, which was associated with improved cardiac function, increased perfusion, and alleviated ventricular remodeling, thus demonstrating the efficiency of combined delivery methods (Liu et al., 2016). Both intracoronary delivery and intravenous infusion are convenient in clinical practice, but



whether the combination of both is more superior than one alone and whether multiple transplantation is better than single transplantation still need further examination.

Imaging-Guided Mini-Invasive Injection

Imaging-guided mini-invasive injection includes trans-endocardial intramyocardial and trans-epicardial intramyocardial injection. Trans—endocardial intramyocardial injection guided by DSA, MRI, or three-dimensional (3D) NOGA electromechanical mapping system does not require thoracotomy, and stem cells can be transplanted directly into the target area, while it requires special positioning equipment and devices (Freyman et al., 2006; van der Spoel et al., 2012). During operation, there are potential risks such as myocardial cell damage, arrhythmia, and cell death of transplanted cells caused by high-pressure technology (Gyöngyösi et al., 2008). We have recently tested imaging-guided mini-invasive injection, and invented a triple puncture needle device that can perform intramyocardial injections through the thoracic epicardium

under ultrasound guidance. With this device, only 2 or 3 holes are punctured in the chest wall of the subject each time, and cells can be injected into the myocardium through the thoracic epicardium under the guidance of ultrasound, and the injection position can be accurately controlled. It has the advantages of less trauma, fewer complications, and multiple transplantation at different time points. The optimal transplantation method requires non or mini invasive, high cell retention. Imaging guide mini-invasive injection is hope to be widely used in clinical practices.

The optimal timing of cell therapy after AMI has been investigated in several trials using BM-MSC with catheter-based applications. Myocardial microenvironment at different time points after infarction has profound influences on stem cells survival, homing and differentiation (Traverse et al., 2012; Khodayari et al., 2019). In acute infarct stage, the microenvironment is not conducive to the survival and growth of stem cells because of the overwhelming inflammatory response in the myocardial injury area. It was found that inflammatory

reaction peaks at 1–4 days, some cytokines (such as VEGF) which were favorable to stem cells migration reached the peak of secretion at 7 days, and scars began to form at about 14 days after AMI (Traverse et al., 2009; Miettinen et al., 2012). In a recent systematic review, cardiac parameters [LVEF, LVESV, and left ventricular end-diastolic volume (LVEDV)] were significantly improved when stem cells were transplanted between 7 and 10 days after AMI (Traverse et al., 2011). For chronic IHD, there is no obvious time window problem, so we can select the time when the patients are in good condition (such as no angina attack and general physical activity without discomfort, which denotes that the heart blood supply and heart function are still good), suggesting that the patients' internal environment and myocardial microenvironment are relatively favorable for transplantation, so as to facilitate the survival, homing and differentiation of implanted cells.

Cell Tracing

The cell fate after transplantation is still unclear, which can be partially ascribed to the deficiency of optimal cell labeling and tracing methods. The ideal stem cell labeling method should have the characteristics of simplicity, strong specificity, high sensitivity, neglectable toxicity, good anti-interference performance and low false positive rate. Currently, cell labeling strategies can be mainly divided into exogenous labeling and endogenous labeling (Figure 3). Exogenous cell labeling transfer the molecular probe labels into cells for direct labeling, such as fluorescent dyes, ^{18}F -FDG radionuclide labels, nanoparticles, microbubble ultrasound contrast agents, etc. (Kircher et al., 2011; Qin et al., 2019). Fluorescein mainly bind to lipid molecules on the cell membrane, and the commonly used fluoresceins are DiI, CM-DiI, PKH26, PKH67 (Jiang et al., 2008; Weir et al., 2008). Fluorescent dye labeling is suitable for short-term tracing, because fluorescence will dilute and quench with the proliferation and differentiation of stem cells and the extension of time. The nucleic acid labeling method uses BrdU, DAPI, or Hoechst to label the DNA in the nucleus (Wang et al., 2016). With the increase of labeling time, nucleic acid labeling can also contaminate the surrounding cells. Besides, the labeled markers on transplanted cells may reduce or lost, which is very difficult to determine the stem cells that really need to be detected and observed. Radionuclide labeling has high resolution and accuracy, but its labeling time is limited by the short half-life, and radioactive elements may cause certain damage to stem cells and the human body (Hess et al., 2019). The method of labeling stem cells with nanoparticles is simple and can observe the migration of stem cells in the body non-invasively. However, there are some disadvantages. For example, the division of stem cells will dilute the concentration of nanoparticles in the cells, which will affect the long-term observation effect, and cell death will cause the dispersion of nanoparticles (Gallina et al., 2015; Zhu et al., 2016). In general, the exogenous labeling method is simple and direct, but it has disadvantages such as low labeling efficiency, unstable labeling process, short duration of the label in the cell, and difficulty in tracking the whole process.

Endogenous labeling use viral or non-viral vectors to transduce and integrate protein-encoding reporter genes into

the cell genome, and mark cells through the overexpression of specific reporter genes, such as GFP fluorescent protein (Hayashi and Hamachi, 2012; Tao et al., 2015). The signal of the reporter gene does not disappear with cell division and proliferation, and the tracking accuracy is high, which can achieve long-term cell tracking and overcome some deficiencies of exogenous labeling. However, reporter gene imaging requires genome manipulation, which has potential safety issues and deserves attention (Cao et al., 2006; Youn and Chung, 2013). Besides, the Y chromosome is a specific genetic structure of male individuals, which is stable and persistent. Labeling the specific gene sequence on the Y chromosome to prepare probes can trace the labeled stem cells for a long time without special treatment for the stem cells (Lu et al., 2010). However, the stability and sensitivity of the Y chromosome probe need further experimental research and verification.

In recent years, advances in imaging equipment have allowed monitoring of stem homing and survival. *In vivo* imaging technology is widely used for tracing after stem cell transplantation, which includes bioluminescence imaging (BLI), magnetic resonance tracing technology, nuclear medicine tracing technology, computer tomography tracing technology, ultrasonic tracing technology, and multimodal tracing technology (Figure 3).

BLI uses visible light produced by luciferase to produce visible light at a specific wavelength for visualization, which has high sensitivity, and can track and quantify cell survival. It also has a rapid response to environmental changes, fast imaging speed, and clear images. BLI also has advantages in *in vivo* imaging, which can obtain real-time information of complex pathophysiological processes of living tissue non-invasively (Zhang et al., 2017). Iwano et al. (2018) using a red-shifted and highly deliverable luciferin analog performed directed evolution on firefly luciferase and designed an all-engineered bioluminescence *in vivo* imaging system (named AkaBLI), which produces *in vivo* emission signals 100 to 1,000-fold brighter as compared with conventional technology. The AkaBLI allowing non-invasive visualization of single cells deep inside freely moving animals, and recorded video-rate bioluminescent signals from neurons in the striatum, a deep brain area, for more than a year. However, this technology is only used for animal study, not yet been applied to clinics.

Magnetic resonance tracer cell imaging technology uses MR contrast agent to label cells, Mn^{2+} , gadolinium chelate-based contrast agents, superparamagnetic iron oxide nanoparticles (SPIO), etc. MRI *in vivo* tracking of transplanted cells is a convenient, safe, non-invasive and sensitive method, which also has the advantages of high speed and high tissue resolution (Kim et al., 2007; Wallat et al., 2017). It can accurately locate and quantitatively analyze the lesions, and is non-radioactive. Skelton et al. (2015) found that the localization and dispersion of ferumoxytol-labeled cells could be effectively imaged and tracked at days 0 and 40 by MRI *in vivo*. However, MRI still has its own limitations: it cannot be dynamic in real time and not suitable for patients with any type of iron-based metal implants.

Nuclear medicine imaging technology refers to the use of radionuclide as tracers, which is suitable for functional and metabolic imaging. The most widely used radionuclide tracers include $^{99\text{m}}\text{Tc}$, ^{18}F -FDG, ^{125}I , etc., but these radionuclide

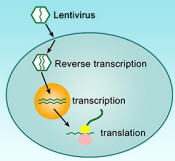
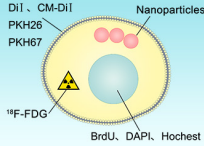
Cell labeling and tracing	Strength	Limitation
 Exogenous labeling	Simple Direct Convenient	Low labeling efficiency Unstable labeling process Short duration
 Endogenous labeling	High accuracy Long-term	Potential safety risks Complicated process
 Bioluminescence imaging	High sensitivity Real-time	Small animals only Low spatial resolution
 Magnetic resonance imaging technology	High tissue resolution Non-radioactive	Expensive Not suitable for patients with iron-based metal implants
 Nuclear medicine imaging technology	High sensitivity Quantification ability	Expensive Radioactive Half-life affect
 Ultrasound molecular imaging technology	Dynamic imaging Convenient Inexpensive	Low clarity Low resolution Operator dependency
 Multimodal imaging technology	High sensitivity High accuracy Quantification ability	Complicated process Expensive

FIGURE 3 | The strength and limitation of different cell labeling and imaging modality.

tracers have certain radioactive contamination and toxicity (Lezaic et al., 2013; Skelton et al., 2015; deKemp et al., 2016). The technology need two devices, single electron emission computed tomography (SPECT) and positron emission tomography (PET), both of which have high sensitivity (Katsikis and Koutelou, 2014).

Ultrasound molecular imaging technology connects specific ligands to the surface of ultrasound contrast agents smaller than red blood cells, accumulates in target tissues through blood circulation to observes its specific imaging (Lee et al., 2014). The key to this technology is ultrasound contrast agents, of which nano-scale ultrasound contrast agents are widely used.

Ultrasound imaging technology can trace monitor stem cells in real time, and is a non-invasive, non-toxic, non-radioactive, dynamic imaging method with high sensitivity. However, the image quality of ultrasonic tracing technology is inferior to other technologies in terms of clarity and resolution, and is greatly affected by gas, as well as the technical level of the operator (Li Y. et al., 2018; Brown and Lindner, 2019).

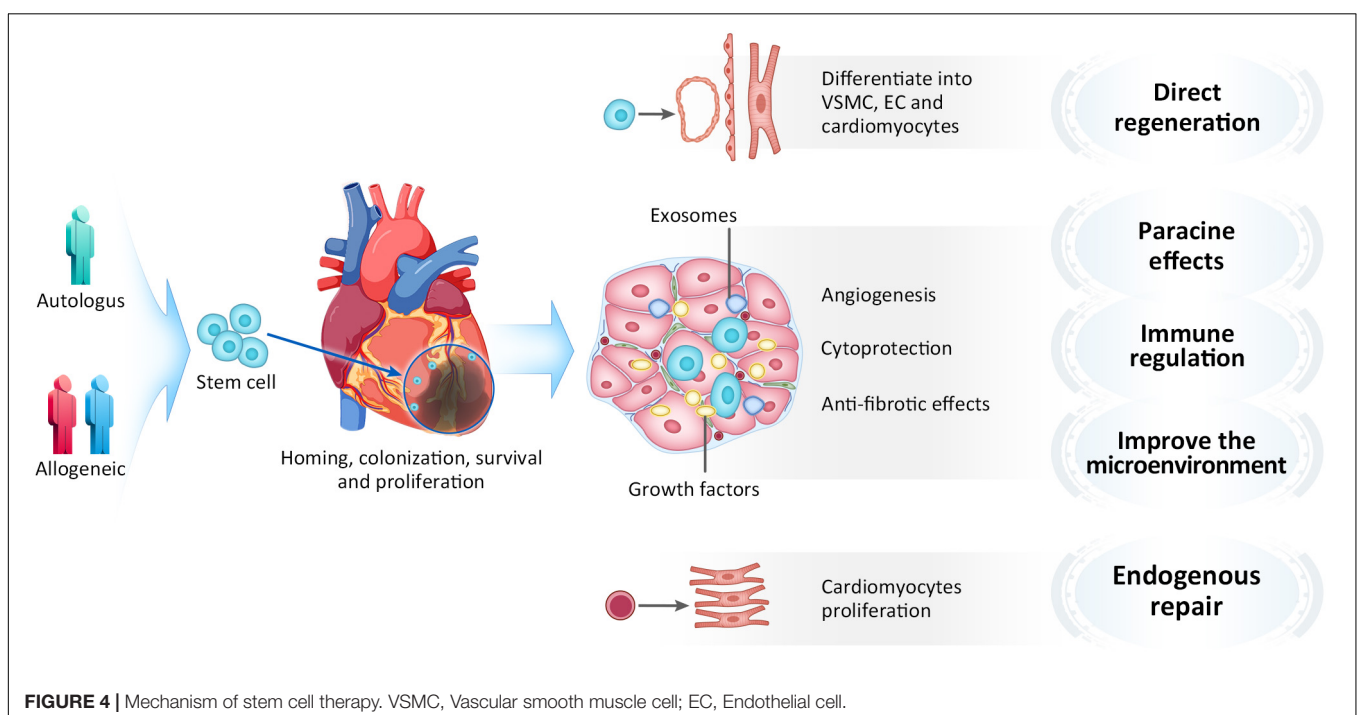
The above-mentioned molecular imaging technologies have their unique advantages for stem cell tracking. However, when one of these imaging technologies is used alone, its inherent shortcomings cannot be ignored for imaging. Therefore, some

scholars have proposed a multimodal imaging mode, which refers to the fusion of two or more imaging modes. The advantages of these combined modes are complementary, which can solve the shortcomings of a single imaging mode and improve the sensitivity and resolution of tissue imaging. Multimodal imaging technology has great advantages and potential for tracking stem cells *in vivo*. At present, PET/CT and SPECT/CT have been widely used in clinic. The key to multimodal imaging is multifunctional contrast agents. In recent years, due to the rapid development of multifunctional contrast agents, many scholars have studied ultrasound (US)/MRI (Mahmoudi et al., 2015), US/MRI/fluorescence (Sung et al., 2009), US/CT/MRI (Tang et al., 2014), and other multimodal stem cell tracing modes (Tao et al., 2016; Li et al., 2017; Qiao et al., 2017; Su et al., 2017). For pre-clinical research, the more detail the better, while for clinical applications, the more convenient the better. Therefore, in our opinion, multimodal molecular imaging will be the predominant tool for tracing and evaluating efficacy after cell transplantation.

MECHANISM OF STEM CELL THERAPY

The mechanism of cell-based myocardial repair is very complicated, and the underlying details remained to be uncovered. Attention to transplanted cells has shifted from the capacity of *in situ* differentiation toward cardiomyocytes and other vascular components, to their capacity of secreting factors that affects surrounding myocardium in a paracrine manner. The primary mechanisms by which cell therapy exerts beneficial effects is now believed to include direct regeneration, paracrine effects, immune regulation, microenvironment improvement and promoting endogenous cardiac repair (Ni et al., 2017;

Müller et al., 2018; **Figure 4**). Most of implanted cells were lost due to washing out from injected area and cell death from ischemia and inflammation. It is thought that very few numbers of stem cells might be able to directly contribute to vascular structures, and there are no clinical evidence that transplanted stem/progenitor cells differentiate into new mature myocardial so far. To date, paracrine effects is considered as important functional mechanism of cellular therapy (Gnecchi et al., 2008; Farzaneh et al., 2019). The transplanted stem cells released cytokines, chemokines and growth factors that inhibit apoptosis and fibrosis, enhance contractility (Han et al., 2017). Studies have revealed that stem cells can secrete a variety of cytokines, such as VEGF, epidermal growth factor (EGF), and hepatocyte growth factor (HGF) (Tang et al., 2015; Shafei et al., 2017), which play important roles in stem cell proliferation, chemotaxis, differentiation and anti-apoptosis. Besides, stem cells can also secrete anti-inflammatory factors, such as IL-10, which can regulate myocardial inflammation after myocardial infarction and improve the microenvironment (Bishopric, 2008; Li S. et al., 2018). Additionally, stem cells can secrete exosomes, which play important roles in cytoprotection, stimulation of angiogenesis, induction of antifibrotic cardiac fibroblasts, and modulation of M1/M2 polarization of macrophages infiltrating the infarcted region (Yu et al., 2014; Khan et al., 2015; Rosca et al., 2015; Zhao et al., 2015; Suzuki et al., 2016; Barile et al., 2017; Derkus et al., 2017; Jung et al., 2017; Menasché, 2018). Exosomes are membrane-bound extracellular vesicles with a diameter of 30–100 nm, contain biologically active proteins, RNAs and microRNAs, which involved in cell-to-cell communication. Studies have shown that exosomes secreted by mesenchymal stem cells can protecting myocardial cells from apoptosis and promoting cell proliferation and angiogenesis.



Recently, Gao et al. (2020) found that using the exosomes naturally produced from that mixture of heart muscle cells, endothelial cells and smooth muscle cells which were all derived from hiPSCs yields regenerative benefits equivalent to the injected hiPSC-CCs in a porcine model of myocardial infarction. Vagnozzi et al. (2019) recently found that transplanting live stem cells, dead stem cells, and zymosan (a chemical inducer of the innate immune response) can all provided a similar functional rejuvenation to the heart after ischemia-reperfusion injury in mice. Interestingly, injection of stem cells followed by immunosuppressant cyclosporine A eliminated this beneficial effect, which indicates that the immune regulation may be an important mechanism. Their study demonstrated that the functional benefit of cardiac cell therapy is due to an acute inflammatory-based wound healing response that rejuvenates the mechanical properties of the infarcted area of the heart. In addition, the heart itself has stem cells, which can slowly self-renew (Rafatian and Davis, 2018). A radioactive isotope study demonstrated that human cardiomyocytes gradually renew 1% of the cell population annually at the age of 25 years to 0.45% at the age of 75 years, which suggests that the heart has the limited ability of endogenous repair (Bergmann et al., 2009). However, some studies found that the stem cell transplantation could activate and promote endogenous myocardial repair (Wang et al., 2014; Wu et al., 2018; Sebastião et al., 2019). In our view, the limited self-renewal ability of the heart is obviously insufficient to support the improvement of short-term function, we believe that its repair mechanism may have a close association with paracrine effects and immune regulation.

FUTURE CLINICAL PERSPECTIVES

As the population ages, the number of IHD patients is increasing. Stem cell therapy, as a promising treatment method, will receive more and more attention. MSCs, especially umbilical cord mesenchymal stem cells (UC-MSCs), have the characteristics of multiple differentiation capabilities, immune exemption, easy access and large-scale expansion, and ethical requirements. In the future, they are expected to become a new drug for the treatment of IHD. iPSCs derived from patients themselves, are easy to obtain and do not have immune rejection. They can differentiate into a variety of cells and even organoids under the induction of small molecule compounds. They have been widely used in drug screening and efficacy evaluation. Generation of thick vascularized tissues that fully match the patient still remains an unmet challenge in cardiac tissue engineering. By means of 3D bioprinting, Noor et al. (2019) successfully print a cellularized human heart with major blood vessels using cardiac and endothelial cell-laden hydrogels, which demonstrate the potential of the approach for engineering personalized tissues and organs. Besides, mechanical and biochemical stimulation on stem cell improve the efficiency of tissue engineering, which provides new strategy for the current stem cell therapy dilemma (Rinoldi et al., 2019). In the future, stem cells can combined with tissue engineering and 3D printing technology, which will have broad application prospects in tissue regeneration and organ

transplantation. In addition, the exosomes provide an acellular therapeutic option for myocardial injury since they are acellular, consequently, easy to store and transport, which also avoiding the tumorigenic risks, arrhythmogenic complications, and immune rejection. With the development of imaging, minimally invasive and even non-invasive cell transplantation guided by imaging will be the trend. At the same time, multimodal imaging for cell tracing also provides convenient conditions for elucidating the mechanism of stem cell action.

THE RISKS OF STEM CELL THERAPY

As for the stem cell therapy, we should not only consider effectiveness and clinical accessible, but also pay more attention to the safety, including the risks of teratoma formation, arrhythmogenic complications, immune rejection, etc. The tumorigenesis post stem cells transplantation is one of the major concern to the clinical translation application, especially for ESC and iPSC. In order to reduce the risk of teratoma formation, there are three main strategies currently. Firstly, induce terminal differentiation or completely remove the residual pluripotent cells before transplantation. Secondly, interfere with the tumorigenic genes in the residual pluripotent stem cells (Hagan et al., 2018). Thirdly, do a good job of monitoring the formation of tumors after cell therapy, and clear them once they are found. For example, introducing suicide genes into stem cells, once tumors are formed, specific drugs can be used to safely remove tumors and prevent spreading. In addition, Tao et al. (2018) found that compared with ESC, parthenogenetic embryonic stem cell (pESC) have similar differentiating capacity but meanwhile with the risk reduction of teratoma formation and without ethical controversy, which provide a new choice for cell sources. Arrhythmogenic complications of cell therapy was firstly reported in skeletal myoblast (Suzuki et al., 2000). Majority studies have shown that MSC transplantation for IHD does not increase the risk of arrhythmia. Ramireddy et al. (2017) found that there was no evidence for ventricular proarrhythmia, manifested by sustained or non-sustained ventricular ectopy or worsened heart rate variability (HRV) in patients receiving MSCs. However, the long-term effects of cell therapy on ventricular arrhythmia need to be further studied. In addition, stem cell transplantation combined with amiodarone and ivabradine to prevent arrhythmia is also a feasible strategy.

CONCLUSION

Although stem cell therapy in IHD has achieved positive results and showed good prospects for clinical application, the optimum cell source and cell dosage, route of administration and timing of transplantation, cell tracing and the mechanism are parameters that still need to be addressed. Besides, we should also pay attention to the cell-preparation techniques, patients selection, follow up observation time, functional evaluation methods, and safety. Furthermore, disappointing results from clinical trials thus far suggest that we should perhaps reconsider fundamental

strategies to overcome the hurdles while continuing to expand the application of stem cell therapy for future clinical trials.

AUTHOR CONTRIBUTIONS

This work was collaboration among all of the authors. CL wrote the initial draft of the manuscript and prepared the figures. DH, PL, and YL reviewed the manuscript. FC proposed the original

idea and reviewed the manuscript. All authors read and approved the final submitted version of the manuscript.

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Navigating the Crossroads of Cell Therapy and Natural Heart Regeneration

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INTRODUCTION

Ischemic heart disease (IHD) affects more than 197 million people worldwide and accounts for the greatest number of years of life lost in the world (Virani et al., 2021). The cost of caring for these patients is expected to double over the next two decades (Heidenreich et al., 2011). Initial enthusiasm for cell therapy in IHD has been tempered by neutral clinical trial results despite signals of efficacy in animal models. Broadly, there are two cell therapy strategies being actively investigated for the potential treatment of IHD: (1) repopulating fibrotic myocardium with stem cells of various lineages, and (2) stimulating native cells of the myocardium to re-enter the cell cycle via paracrine signaling. Some of these approaches have shown potential for clinical utility, but the underwhelming translation of these therapies from animal models to patients suggest that large gaps remain in our mechanistic understanding of these processes. However, recent work has suggested an intricate relationship between inflammation, angiogenesis, and myocardial regeneration. This mechanism may explain the neutral results of cell therapy clinical trials and has sparked a new avenue for investigation that may have implications for future therapies aimed at activating natural regenerative pathways in humans.

STEM CELL THERAPY

There are two general strategies for cardiac regeneration after myocardial infarction (MI) currently under investigation. The first is direct transplantation of stem cells to the injured myocardium, which is an area of active study with many recent clinical trials (Ascheim et al., 2014; Menasché, 2018; Yau et al., 2019; He et al., 2020). The second strategy is to *redirect* resident cells of the

myocardium to adopt a cardiomyocyte (CM) fate themselves. The connection between the acute inflammatory response and myocardial regeneration is an emerging area of interest.

Stem Cell Transplantation in Animal Models

Efforts to capitalize on the regenerative potential of stem cells to repair or restore injured myocardium have been ongoing since the 1990s. After Loren Field's group demonstrated the feasibility of grafting syngeneic CMs into recipient myocardium without rejection, first in mice (Soonpaa et al., 1994) and then in a canine model (Koh et al., 1995), there was newfound optimism for the therapeutic implications. Shortly afterward, multiple studies demonstrated the ability of rat and rabbit skeletal myoblasts to engraft in injured myocardium, adapt to the cardiac workload, and showed some potential to augment myocardial performance (Chiu et al., 1995; Murry et al., 1996; Taylor et al., 1998). Eventually, human embryonic stem cell-derived CMs were studied in a non-human primate model of MI and showed robust remuscularization of the infarcted tissue with successful electromechanical coupling (Chong et al., 2014), but with an increased rate of ventricular arrhythmias. Subsequent non-human primate studies showed similar findings of modest myocardial regeneration with 11.6% of the infarct territory remuscularized (Shiba et al., 2016; Liu et al., 2018).

Clinical Trials

By the year 2000, trials in humans had begun. Phillippe Menasché's team injected skeletal myoblasts into the scarred myocardium of patients undergoing coronary artery bypass grafting (CABG) in a Phase 1 trial (Menasché et al., 2001). After achieving reassuring safety endpoints, a randomized, placebo-controlled, double-blind study named the Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial aimed to compare high versus low dose skeletal myoblast versus placebo injections during CABG (Menasché et al., 2008). The outcomes of this study were disappointing. The myoblast groups did not show any improvement in left ventricular (LV) and ejection fraction (EF) but did result in a decrease in LV volume and an increased rate of arrhythmias (although by 6 months the rate of arrhythmias was similar between groups). While Menasché's group reported an impressive durability of skeletal myoblasts in these trials (e.g., tissue samples from one patient showed evidence of engraftment 16 years after the initial treatment), other cell types were also being investigated.

Given the accessibility and low cost of bone marrow-derived stem cells, there was a rush to initiate clinical trials using these heterogeneous progenitors despite limited evidence that they could regenerate infarcted myocardium. Indeed, several of these trials showed no clinically significant improvement over CABG alone (Hendrikx et al., 2006; Ang et al., 2008; Pätälä et al., 2014). The reinfusion of enriched progenitor cells and infarct remodeling in acute myocardial infarction (REPAIR-AMI) trial enrolled 204 patients to investigate intracoronary infusion of bone marrow derived stem cells 3–7 days after revascularization for acute MI and showed a slight improvement in LV and EF.

However, a follow up study, the bone marrow transfer to enhance ST-elevation infarct regeneration (BOOST) trial, showed that the modest improvement in LV and EF was no longer evident by 18 months, tempering optimism. Despite disappointing results in these early trials, investigation continued as proponents wondered if the results were confounded by concurrent coronary revascularization and inconsistent proportions of subtypes of mononuclear stem cells within different autologous samples (Menasché, 2018). However, the FOCUS-CCTRN trial results published in 2012 addressed one of these concerns. The FOCUS-CCTRN trial compared catheter-deliverable transendocardial bone marrow-derived stem cells to patients with chronic heart failure, and showed no improvement in LV end systolic volume, maximal oxygen consumption, defect size, or reversible wall motion abnormalities (Perin et al., 2012). While the FOCUS-CCTRN trial eliminated the confounding variable of concurrent revascularization, the issue remained that many different subtypes of mononuclear cells (MNC), mostly of unknown significance, are being delivered simultaneously which may explain seemingly discordant results between comparably designed studies.

Nonetheless, some wondered if cell therapy may still have clinical utility that is simply not reflected in multifactorial metrics such as EF, and that perhaps including more acutely ill patients in future studies would reveal a signal (Rosenzweig, 2006). In 2014, the first randomized trial of intramyocardial injection of a low dose of allogeneic mesenchymal precursor cells (MPC) in patients undergoing left ventricular assist device (LVAD) implantation showed a signal of efficacy. Patients in the MPC group had a greater probability of temporary weaning of the LVAD at 90 days than the sham group. In 2019, the Cardiothoracic Surgical Trials Network published results from a follow up trial demonstrating that injection of high dose MPCs into the failing ventricle at the time of LVAD placement did not improve the proportion of patients able to successfully wean from the LVAD over 6 months, despite a signal in the earlier phase 2 trial mentioned above (Yau et al., 2019). However, when the subset of patients with IHD were analyzed in an exploratory *post hoc* analysis, this group did show a modest benefit in temporary weaning from LVAD compared to the control group, suggesting there is a differential response among the various etiologies of heart failure. The differential effect of MPCs on patients in this trial with ischemic versus non-ischemic heart failure implies that there may be potential therapeutic benefit, perhaps via different mechanisms than initially believed.

Inconsistent results from clinical trials of stem cell transplantation may be related to ambiguity about the putative mechanism. Evidence from several studies suggest that the beneficial effects of cell therapy may be attributable to paracrine signaling induced by the transplanted progenitor cells, rather than direct repopulation of the injured myocardium (Williams and Hare, 2011; Sanganalmath and Bolli, 2013). Specifically, one study using an ovine model of non-ischemic cardiomyopathy demonstrated sparse engraftment of green fluorescent protein-labeled MPCs throughout the myocardium, but an improvement in ventricular function (Psaltis et al., 2010). Another large animal study found that when MPCs were injected into the

infarct border zone at the time of acute MI, lower doses of MPCs resulted in the greatest improvement in LV remodeling and smaller infarct size relative to the control group and the high dose MPC group (Dixon et al., 2009). The results of these studies were discordant with the hypothesis that the therapeutic mechanism of cell therapy was engraftment and replacement of the non-viable CMs.

Suspecting alternative mechanisms for the findings of earlier studies on cell therapy for IHD, one of the most elegant studies to support the paracrine hypothesis came from Vagnozzi and Molkentin (Vagnozzi et al., 2020). Cellular debris from bone marrow mononuclear cells (bm-MNC) or cKIT+ cardiac progenitor cells (CPCs) were injected into the MI border zone in a mouse model. Not only did the cellular debris trigger a robust inflammatory immune response and improve fractional shortening and ventricular remodeling, but exogenous Zymosan – which activates sterile inflammation – had the same effect. In fact, the Zymosan group actually resulted in the greatest increase in CD31+ endothelial cell proliferation, compared to the injected bone marrow MNCs and CPCs. However, neither the Zymosan nor the CPCs or MNCs stimulated CM proliferation. Importantly, the improvement in ventricular function was abrogated by concurrent administration of cyclosporine, suggesting that activation of the inflammatory response after injury may underlie the potential of cell therapy rather than transdifferentiation of transplanted cells, as previously believed. Additional reports demonstrating the necessity of a sterile immune response in post-MI remodeling support this hypothesis and may partially explain the results from the human trial of MSC injections during LVAD implantation.

While Vagnozzi et al. (2020) showed that the acute inflammation triggered by cellular debris appears to modestly improve ventricular function, there was no evidence of CM proliferation. Similarly, a study examining the regenerative mechanisms in an axolotl cryoinjury model demonstrated that macrophage depletion abrogated myocardial regeneration despite cardiomyocyte proliferation (Godwin et al., 2017). Collectively, these findings suggest that the innate inflammatory response after myocardial injury is preserved across species and may result in recovery of ventricular function, but does not necessarily proceed to full scale myocardial regeneration. Additionally, applying principles from studies of regeneration in other organ systems, such as dental pulp (Marrelli et al., 2018), there is evidence to suggest that a coordinated microenvironmental remodeling response driven by the innate immune system may be essential to provide the appropriate biomechanical stimulus to drive regeneration. This informed many new lines of inquiry and will guide the design of future clinical trials.

It remains unclear whether the findings of the Vagnozzi study and those of previous clinical trials are on the mechanistic continuum of natural myocardial regeneration seen in neonatal small mammals, or if it is a non-specific antifibrotic damage-control strategy employed by the cell which is parallel to but does not result in natural regeneration, as it appears to be in the axolotl

(Godwin et al., 2017). Further studies investigating the transition from the acute inflammatory-based wound-healing response to angiogenesis and myocardial regeneration are needed.

NATURAL REGENERATION

The distinct challenge in reprogramming mature CMs in cell cycle arrest is our relative knowledge deficit surrounding the mechanisms for releasing CM cell cycle arrest. Native CM proliferation and migration into injured territories occurs physiologically in small and large mammalian neonates (Ingason et al., 2018; Ye et al., 2018; Das et al., 2019; Wang et al., 2020a,b). However, attempts to reactivate this pathway in adult mammals have proven challenging.

Hypoxia and Angiogenesis

Although the adult mammalian heart is unable to regenerate to any significant extent, neonatal mammals have the capacity to regenerate viable myocardium following MI in the early days of life (Haubner et al., 2012, 2016; Porrello et al., 2013; Wang et al., 2020a,b). It follows to reason that angiogenesis must precede myocardial regeneration. This is driven by the development of a network of collateral arteries formed by arterial endothelial cells (Ingason et al., 2018; Das et al., 2019), which are able to develop into arteries (Simons and Eichmann, 2015).

Efforts to harness the natural regenerative capacity have focused on releasing CMs from cell cycle arrest via a variety of approaches. Releasing cell cycle arrest has been attempted with overactivation of cell cycle activators such as cyclin D2, which led to enhanced angiogenesis and remuscularization in the MI border zone (Zhu et al., 2018). Similarly, the activation of Yap – a downstream effector of the Hippo pathway, involved in vascular remodeling and angiogenesis – has been shown to extend cell cycle activity and CM proliferation (Lin and Pu, 2014). Small molecules such as neuregulin also appear to promote CM proliferation in mice and in humans (Polizzotti et al., 2015), leading to improved ventricular function and reduced scar size after MI in small and large animal models (Cohen et al., 2014, 2020). It also appears possible to reprogram resident cardiac fibroblasts to adopt a CM fate by using small molecule inhibitors of transforming growth factor beta and WNT, which resulted in successful transdifferentiation and improved ventricular function in mice (Mohamed et al., 2017). Interestingly, zebrafish retain regenerative capacity throughout their life and have a relatively hypoxic circulatory system as a result of veno-arterial mixing in their two-chamber heart, similar to the shunt dependent phase of mammalian gestation (Cardoso et al., 2020). This hypoxic environment drives anaerobic glycolysis, the predominant energy source in the prenatal heart. However, upon rapidly transitioning to higher postnatal oxygen content levels, a metabolic transition to oxidative metabolism occurs. As the levels of glycolytic enzymes fall over the first postnatal week, those that are involved in oxidative metabolism increase reciprocally. The reactive oxygen species produced by the sudden spike in mitochondrial respiration induce damage to proteins, lipids, and DNA, which stimulates the DNA damage response

that ultimately leads to cell cycle arrest and polyploidization (Cardoso et al., 2020). The sudden oxidative stress and collateral damage that leads to cell cycle arrest appears to occur over the same timespan that neonatal mammalian hearts lose their regenerative capacity, and scavenging of reactive oxygen species appears to prolong the proliferative phase of the mammalian myocardium (Puente et al., 2014).

As evidence mounted that the findings in the cell therapy clinical trials were more likely related to paracrine signaling induced by transplanted cells rather than engraftment, some groups began attempting to recapitulate the responsible signaling pathways without the technical challenges of engrafting billions of cells. After MI, the acute inflammatory response attracts

macrophages, fibroblasts, and T cells to the infarct zone. These cells clear debris from apoptotic cells and remodel the extracellular matrix. In turn, potent chemoattractants such as stromal cell-derived factor 1 α (SDF) recruit endothelial progenitor cells to the infarct territory and promote angiogenesis (Frederick et al., 2010). By using advanced computer modeling, Hiesinger et al. (2011) developed an engineered SDF analog (ESA) which demonstrated improved efficiency in stimulating microvascularization in an ischemic cardiomyopathy mouse model, which was then subsequently validated in a rat hindlimb model and ovine MI model (Hiesinger et al., 2011; Macarthur et al., 2014; Edwards et al., 2016). Many groups began attempting cellular delivery of extracellular vesicles (EVs) loaded with

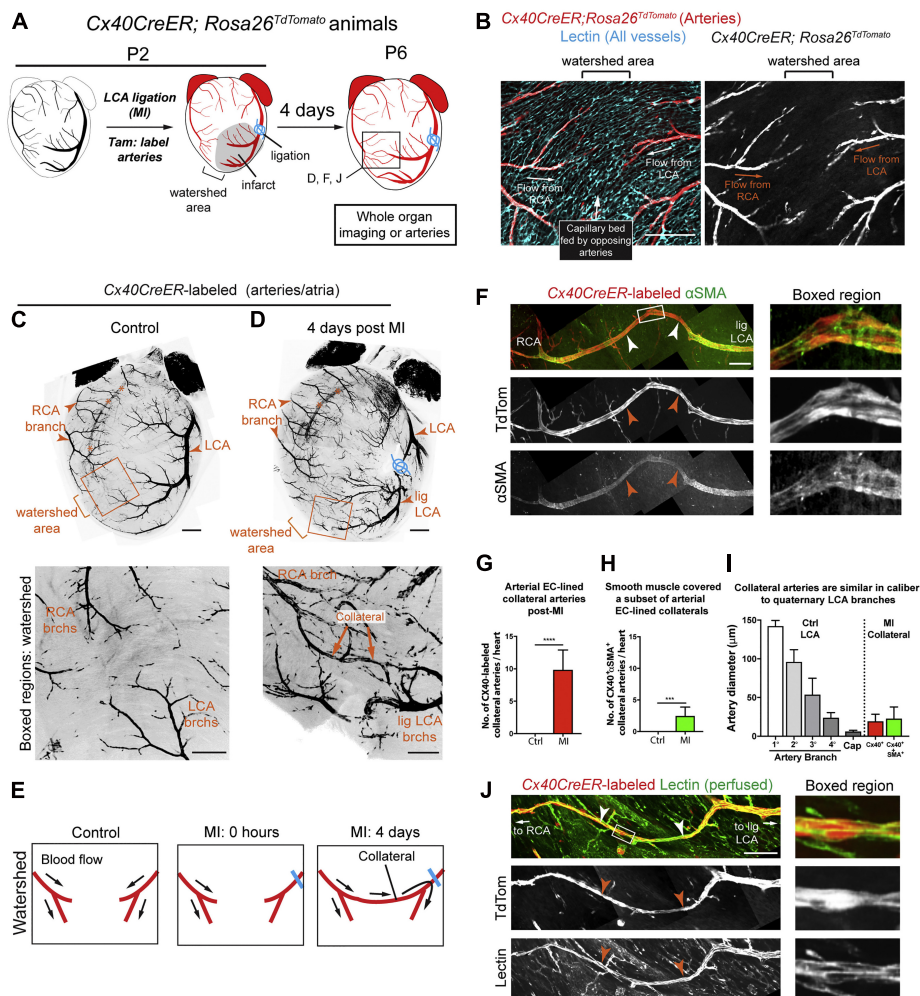


FIGURE 1 | Extensive Collateral Artery Formation in the Neonatal Mouse Heart 4 days after MI. **(A)** Experimental design where *Cx40CreER*-labeled arteries are shown in red. **(B)** Confocal image of a watershed area from a P2 control heart. The capillary bed (cyan) is fed by left coronary artery (LCA) and right coronary artery (RCA). **(C,D)** Confocal images (anterior views) of control **(C)** and myocardial infarction (MI) **(D)** hearts. Arterial ECs are shown in black. MI induced collateral arteries that connect ligated (lig) branches (brchs) of the LCA with RCA branches across the watershed area (boxed regions). Asterisks indicate the RCA showing through from the posterior heart wall. **(E)** Schematic of how collateral arteries restore blood flow (arrows) to injured myocardium. Arteries, red; ligation, blue. **(F)** Some *Cx40CreER*-labeled collateral arteries contain smooth muscle (white and red arrows). **(G,H)** Quantification of *Cx40CreER*-labeled **(G)** and smooth muscle-covered **(H)** collateral arteries. Hearts: $n = 8$ control, $n = 12$ MI. **(I)** Collateral arteries were similar in diameter to quaternary (4 $^{\circ}$) branches. Arteries: $n = 24$ *Cx40*⁺, $n = 9$ α SMA⁺. **(J)** Collateral arteries were perfused (white and red arrows). Cap, capillaries; P, postnatal; Tam, tamoxifen; EC, endothelial cells; TdTom, tdTomato. Scale bars: **(B)**, 200 μ m; **(C,D)** low magnification, 500 μ m; **(C,D)** boxed region, 200 μ m; **(F,J)**, 200 μ m. Error bars indicate SD. *** $p \leq 0.001$, **** $p \leq 0.0001$. Adopted from Das et al. (2019).

signaling molecules to the injured myocardium, activating the inflammatory and possibly regenerative responses. The cellular response to their cargo of proteins, lipids, and micro-RNAs (miRNAs) activates a variety of pathways depending on the specific composition of the EV cargo. EVs can also be enriched with pre-selected small molecules, proteins, or miRNA to modulate the intended response from the host cell, thus serving as an attractive vehicle to influence cell fate (de Abreu et al., 2020). In a porcine model of acute MI, human exosomes delivered intramyocardially decreased infarct size by about 20% and mildly improved LV remodeling (Gallet et al., 2017). While these approaches appear to hold potential to attenuate scar formation, the path toward a clinical trial with EVs most likely will involve rigorous purification, laboratory validation, and standardization testing, which is likely to be an expensive and lengthy process due to safety concerns regarding off-target effects.

Interpreting the Vagnozzi et al. (2020) study in this context suggests that a possible link between natural regeneration and cell therapy is the inciting inflammatory immune response. This is reinforced by recent work demonstrating that arterial endothelial cells in adult mice can be induced to undergo artery reassembly (i.e., neoangiogenesis) with the administration of exogenous SDF (Das et al., 2019; **Figure 1**). Exogenous administration of SDF or engineered analogs such as ESA have also been shown to augment ventricular function, reduce scar size, and restore myocardial biomechanics in both small and large animal models (Hiesinger et al., 2011; Macarthur et al., 2014; Wang et al., 2019). This is notable because SDF and its receptor CXCR4 have well-documented roles in immune activation and are upregulated by inflammatory cytokines such as VEGF, TGF β 1, and bFGF (García-Cuesta et al., 2019). Therefore, it appears plausible that we are getting closer to elucidating the transition between the acute inflammatory response and natural regeneration.

Unsurprisingly, excessive proliferation or inappropriate depletion of resident cardiac macrophages after acute MI can impair myocardial regeneration in mice (Aurora et al., 2014; Lavine et al., 2014; Dick et al., 2019) and result in an abnormal remodeling response. Specifically, macrophages in P1 mice seem to secrete soluble factors that stimulate angiogenesis (Aurora et al., 2014). In addition to macrophages, T-regulatory cells are also necessary for CM proliferation and myocardial regeneration (Li et al., 2019). Conversely, ablation of specific subsets of CD4 $^{+}$ T-cells actually improves cardiomyocyte proliferation and reduces fibrosis by directing macrophages away from the M2 phenotype (Bansal et al., 2019; Li et al., 2020). Neutrophils have traditionally been considered exclusively pro-inflammatory agents in the response to myocardial injury. However, neutrophil depletion studies have showed impaired ventricular functional recovery and an uncoordinated fibrotic response after MI (Puhl and Steffens, 2019). Neutrophils have been shown to play a role in the clearance of cellular debris and recruitment and activation of specific M2 macrophage subsets that promote clearance of apoptotic cells via neutrophil gelatinase-associated lipocalin (Horckmans et al., 2017). Given the importance of preserving the mechanical properties of native myocardium in recovery of ventricular function after MI (Wang et al., 2019), and to optimize the bioscaffold in which progenitor cells may adhere (Ballini

et al., 2017), it is plausible that an uncoordinated inflammatory or fibrotic response may result in an inadequate microenvironment for cardiomyocyte regeneration. Additionally, the complement pathway receptor 5a is evolutionarily conserved and promoted CM proliferation in a recent cross-species study (Natarajan et al., 2018; Vujic et al., 2020). The growing evidence for the role of inflammation and paracrine signaling recalibrated the focus of investigators toward endogenous pathways that regulate the stepwise progression from infarct maturation and innate wound healing to angiogenesis and subsequent myocardial regeneration.

CONCLUSION AND FUTURE DIRECTIONS

While this review focuses on cell therapy and paracrine signaling approaches to myocardial regeneration, innovative approaches in other fields such as hydrogels, biophysics, neurohormonal modulation, and tissue engineering have also yielded promising advancements toward the common goal of myocardial regeneration. The exponential pace of discovery of new genes, small molecules, and proteins that promote various steps of myocardial regeneration has identified many promising avenues of research. The neutral results of clinical trials investigating cell therapy for IHD may be partially explained by the emergence of an alternative inflammatory mechanism underlying the rescue of ventricular function after MI. It appears that the small therapeutic benefit of cell therapy is more likely related to endogenous inflammatory and wound-healing pathways, rather than direct replacement of the damaged myocardium with viable cardiomyocytes. This is a significant paradigm shift in the pursuit of myocardial regeneration after injury. Therapies targeting individual stimuli for regeneration have shown potential for success but have fallen short of the robust physiologic regenerative response seen in neonatal mammals. Nonetheless, repopulating the infarcted myocardium with viable, non-arrhythmogenic CMs remains the ultimate goal, and recent work in animal models on natural regeneration remains a promising field for further investigation. Future progress will likely depend upon interdisciplinary collaborations, including bioengineers, immunologists, cell biologists, and physicians to develop multifaceted therapeutic strategies that integrate bioengineering and developmental biology techniques.

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YJW and HW conceptualized the manuscript which was written by SE, and edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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CD73⁺ Mesenchymal Stem Cells Ameliorate Myocardial Infarction by Promoting Angiogenesis

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With multipotent differentiation potential and paracrine capacity, mesenchymal stem cells (MSCs) have been widely applied in clinical practice for the treatment of ischemic heart disease. MSCs are a heterogeneous population and the specific population of MSCs may exhibit a selective ability for tissue repair. The aim of our research was to adapt the CD73⁺ subgroup of adipose derived MSCs (AD-MSCs) for the therapy of myocardial infarction (MI). In this research, AD-MSCs were isolated from adipose tissue surrounding the groin of mice and CD73⁺ AD-MSCs were sorted using flow cytometry. To investigate the therapeutic effects of CD73⁺ AD-MSCs, 1.2×10^6 CD73⁺ AD-MSCs were transplanted into rat model of MI, and CD73⁻ AD-MSCs, normal AD-MSCs transplantation served as control. Our results revealed that CD73⁺ AD-MSCs played a more effective role in the acceleration function of cardiac recovery by promoting angiogenesis in a rat model of MI compared with mixed AD-MSCs and CD73⁻ AD-MSCs. Moreover, with the expression of CD73 in AD-MSCs, the secretion of VEGF, SDF-1 α , and HGF factors could be promoted. It also shows differences between CD73⁺ and CD73⁻ AD-MSCs when the transcription profiles of these two subgroups were compared, especially in VEGF pathway. These findings raise an attractive outlook on CD73⁺ AD-MSCs as a dominant subgroup for treating MI-induced myocardial injury. CD73, a surface marker, can be used as a MSCs cell quality control for the recovery of MI by accelerating angiogenesis.

Keywords: CD73, VEGF, adipose derived mesenchymal stem cells, angiogenesis, myocardial infarction

BACKGROUND

Various clinical trials have been conducted on patients with acute myocardial infarction (MI) using multiple cell lines, including mesenchymal stem cells (MSCs), bone marrow stem cells, and cardiac resident stem cells (Mayfield et al., 2014; Tao et al., 2016; Miao et al., 2017). These therapies utilizing stem cells to regenerate lost cardiac muscle are clinically attractive. The use of

Abbreviations: AD-MSCs, adipose derived mesenchymal stem cells; AMP, 5'-adenosine monophosphate; APCB, alpha, beta-methyleneadenosine-5'-diphosphate; bFGF, basic fibroblast growth factor; CXCR4, chemokine receptor-4; ECM, Extracellular matrix; FBS, Fetal bovine serum; HGF, hepatocyte growth factor; LAD, left anterior descending coronary artery; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESV, left ventricular end-systolic volume; MI, myocardial infarction; MSCs, mesenchymal stem cells; MOI, multiple of infection; SDF-1 α , stromal cell-derived factor-1 α ; VEGF, vascular endothelial growth factor.

MSCs, multipotent differentiation potential stem cells derived from mesoderm, for cell therapy might play a cardioprotective effect. This effect was not through the production of new cardiac tissue, but through the secretion of secondary paracrine factors that enhance the endogenous repair pathways (Bobi et al., 2017; Naji et al., 2019). Clinical and preclinical trials' results showed that the transplanting effect of MSCs was very limited due to its poor cell persistence and cardiomyocyte differentiation (Chen et al., 2018; Li et al., 2020).

Mesenchymal stem cells are heterogeneous and could be divided into many subgroups with different biological characteristics (Du et al., 2016; Mo et al., 2016; Chin et al., 2018; Liu et al., 2019). Morphologically, MSCs could be divided into three subgroups: a small fast self-renewing cell subgroup, a fibroblast-like subgroup, and a large slow-growing square or flat cell subgroup (Haasters et al., 2009). Functionally, CD56⁺ MSCs were proliferative, more chondrogenic, and less adipogenic than CD56⁻ MSCs (Elahi et al., 2016). Tseng reported that CD105⁺-AD-MSCs were inclined to differentiate into chondrocytes (Tseng et al., 2020). At present, bulk MSCs were used in most *in vivo* and *in vitro* studies, and the heterogeneity of unsorted MSCs compromised to comparability of cell therapy studies (Dromard et al., 2014; Fu et al., 2019).

Mesenchymal stem cells exhibit a variety of immunophenotypes. CD44, CD73, CD29, CD90, and CD105 are the most common markers of MSCs, which also lack the cell surface antigen CD45 (Petrenko et al., 2020). As reported by Florian et al., the expression patterns of CD73, CD105, CD90 varied in different morphological subsets. All three markers were stably expressed in rapid self-renewal cells whereas most flat cells lacked one or more MSC surface markers, predominantly CD73 (Haasters et al., 2009). In addition, there were significant differences in CD73 expression in MSCs from different sources (Darzi et al., 2016). We have found that CD73⁺ MSCs were the dominant subgroup regarding the potential for myocardial differentiation (Li et al., 2013).

CD73, an ecto-5'-nucleotidase, is a glycoprotein anchored to the cell membrane by glycosyl-phosphoryl inositol. It was widely expressed on the cell membrane and implicated in signal transduction (Allard et al., 2017; Kordass et al., 2018). Previous researches have proved that CD73 had the potency to promote cell proliferation and that CD45⁻CD14⁻CD73⁺ cell subsets had stronger clone forming ability (Suto et al., 2017, 2020). CD73 could promote tumor angiogenesis, release cytokines under ischemic conditions, and has the ability to make tumor cells escape immune recognition (Beavis et al., 2012; Ghalamfarsa et al., 2019). CD73 played an important role in myocardial protection during myocardial ischemic preconditioning (Wolff et al., 2015), and the expression of CD73 was upregulated in immune cells after MI and was linked to myocardial repair and improvement of cardiac function (Tan et al., 2019). In conclusion, CD73 may be a sensitive marker for screening subsets of MSCs and treating myocardial injury.

Adipose derived mesenchymal stem cells had many similarities with MSCs from alternative sources (Zuk et al., 2001). Among all transplantable seed cells, AD-MSCs had the peculiarities of simple sampling, multi-directional

differentiation, few ethical problems, rapid proliferation *in vitro*, and low immunogenicity (Bobi et al., 2017). Therefore, AD-MSCs have become a main choice for stem cell therapy of MI. Most studies have utilized unsorted AD-MSCs for research and injury treatment. Although CD73 has been used as an important criterion for the identification of AD-MSCs for many years, its specific role in the treatment of AD-MSCs transplantation is still unclear (Calloni et al., 2013; Ode et al., 2013). The role of CD73 in AD-MSCs cardiomyocyte differentiation and cytokine secretion also needs to be clarified.

In this study, CD73⁺ and CD73⁻ subsets of AD-MSCs were sorted by flow cytometry and their morphology and genomics were evaluated in detail. Lentivirus and APCP (inhibitor of CD73) were used to alter the expression or activity of CD73 and then the changes in cytokines secretion of these AD-MSCs were observed *in vitro*. We also evaluated the effect of CD73⁺-AD-MSCs transplantation on the repair of myocardial infarction in rats. The main objective of this study was to evaluate the utility of the dominant subset of AD-MSCs for improving MI treatment, and to provide theoretical and experimental basis for clinical application of CD73⁺ cells.

MATERIALS AND METHODS

Culture, Sorting, and Detection of AD-MSCs, CD73⁺, and CD73⁻ AD-MSCs

Adipose derived mesenchymal stem cells were isolated and cultured from the groin adipose tissue of adult C57BL/6 mice (body weight: 28.20 ± 0.66 g), which were obtained from the Experimental Animal Center of Xinxiang Medical University. The upon experimental methods and reagents used were referred to the previously published protocol (Zuk et al., 2001; Li et al., 2013). Cells were cultured with low sugar DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (Fetal bovine serum; Hyclone, United States). When cells reached 80% confluence, they were passaged using 1:2 splits. The morphology of the cells was observed by inverted microscope. Immunofluorescence and flow cytometry analysis were conducted on passage 3 AD-MSCs using monoclonal antibodies against CD29, CD44, CD73, and CD45 (BD Bioscience, 1:500).

CD73⁺-AD-MSCs and CD73⁻-AD-MSCs were isolated using flow cytometry and then cultured. With CD73 as the standard, flow cytometry (FACS Aria II, BD Bioscience) was used to sort out the two subgroups. The morphology of CD73⁺-AD-MSCs and CD73⁻-AD-MSCs in primary culture was determined by using HE staining. Subpopulations were further subcultured and the cells from the fourth to sixth passages were used for the follow-up experiments. Quantitative real-time PCR and Western blotting were used to detect the expression of CD73 in the two subgroups.

Real-Time PCR

Total RNA from CD73⁺-AD-MSCs and CD73⁻-AD-MSCs were extracted with Trizol (Invitrogen Corporation, China) according to the manufacturer's instructions. First-strand cDNA was synthesized with 50 ng of RNA (Takara Biomedical

Technology, China), which was used for each real-time PCR reaction. Relative quantitation of mRNA by real-time PCR was performed using Applied BeyoFast™ SYBR Green qPCR Mix (2X) (Beyotime Biotechnology, China). The primer sequences were as follows. CD73, 269bp, Forward primer: GGTGTGGGGATTGTTGGATA, Reverse primer: GCACTTCTTTGGAAGGTGGAT.

GAPDH, 231bp, Forward primer: TGGTGAAGGTC GGTGTGAAC, Reverse primer: GCTCCTGGAAGATGGT GATGG.

Western Blot Analysis

CD73⁺ AD-MSCs and CD73⁻ AD-MSCs were lysed in RIPA lysis buffer. Total protein was extracted and subjected to electrophoresis using 10% SDS-polyacrylamide gels and transferred to an apolyvinylidene difluoride (PVDF) membrane. The membrane was incubated with primary antibodies overnight and then incubated with secondary antibody for 2 h at room temperature. The primary antibodies included CD73 (ab175396, Abcam Biotechnology, United Kingdom, 1:500) and β -actin (AF0003, Beyotime Biotechnology, China, 1:1000). ECL Plus kit (P0018S; Beyotime Biotechnology, China) was used to detect signals according to the manufacturer's instructions.

Microarray Analysis of CD73⁺ and CD73⁻ AD-MSCs

The fifth passages of sorted subpopulations were used for microarray analysis. Gene chip was used to detect differentially expressed genes with consequent gene ontology (GO) analysis (Jikai Biotechnology Co., China). Genes in the GO term (including cellular components, biological processes and molecular functions) with $P < 0.05$ were considered significantly enriched. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource that clarifies the effects and advanced functions of biological systems. This resource was used to identify significant differences in differentially expressed genes in KEGG pathways. The datasets presented in this study can be found in online repositories: NCBI GEO; GSE167219

Overexpression and Inhibition the Expression of CD73

CD73 gene overexpression and CD73 gene siRNA lentivirus were constructed by Jikai Biotechnology Co., The cells in logarithmic growth phase (amount 5×10^4) were seeded in a 6-well cell culturing plate in complete culture medium in incubator supplied with 5% CO₂ at 37°C. Upon achieving 30–40% confluence, appropriate amount of medium virus enhanced infection solution (ENi.S.) and polybrene were added according to the MOI (Multiple of infection) value of cells. After 72–96 h infection time, lentivirus reporter gene GFP expression were detected. The Lentiviral transfected cells were used for subsequent VEGF detection by ELISA.

Paracrine Effects of CD73⁺ AD-MSCs

All experimental groups (AD-MSCs, CD73⁻ AD-MSCs, CD73⁺ AD-MSCs and CD73⁺ AD-MSCs + APCP) were

cultured in complete medium. Upon reaching 80–90% confluence, cells were cultured in serum-free DMEM. APCP (80 μ mol/L; alpha, beta—methyleneadenosine-5'-diphosphate) was used to inhibit the activity of CD73 in CD73⁺ AD-MSCs + APCP group. After 24 h, the concentration of cytokines in the conditioned medium was measured by ELISA. The selecting principles for cytokines were as follows: the cytokines which were secreted by MSCs and closely related to myocardial repair including vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 α (SDF-1 α), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) (Ho et al., 2016; Naji et al., 2019). Cytokine detection kit was purchased from Wuhan Huamei biotech Co., Ltd.

Scratch Wound Healing Assay

To test the ability of CD73⁺ AD-MSCs to induce endothelial migration, HUVEC cell line was used for scratch wound healing assay. 10⁶ cells HUVECs were seeded on a 6-well plate in Endothelial Cell Medium (ScienCell, United States) supplemented with 5% FBS. The cells were left 24 h to reach 80% confluence. The monolayer was disrupted by using a sterile 100 μ l tip and an acellular line was left in the middle of the well. Then HUVECs were treated with the conditioned medium of CD73⁺ AD-MSCs and CD73⁻ AD-MSCs. Images for five fields of views for each scratch were taken at 0 and 6 h. The percent of wound closure was calculated using Image J.

Myocardial Infarction Model and Cell Transplantation

Adult mix gender SD rats (body weight: 230 ± 18 g) were obtained from the Experimental Animal Center of Xinxiang Medical University and then used to replicate the MI model. The MI model was replicated as follows. After transnasal anesthesia with aether, the rats were immobilized in the supine position and anesthetized by intraperitoneal injection with 1% pentobarbital sodium (40 mg/kg). After skin preparation and disinfection, a median tracheotomy and intubation were performed. Make an incision between the third or fourth rib on the left to open the chest and expose the heart. At the lower 1–2 mm of the junction between the left atrial ear and the pulmonary artery cone, the left anterior descending coronary artery (LAD) was ligated by a 5/0 silk suture. Then the thorax was closed and the rats were injected 1.6 million units of penicillin into the abdominal cavity. After resumption of spontaneous breathing, the animal was monitored and tracheal intubation was removed. AD-MSCs were trypsinized, centrifuged, and resuspended before transplantation. 1.2×10^6 cells were injected into myocardial tissue of the infarcted areas in each group. The rats were randomly divided into sham operation group (SHAM), MI, mixed AD-MSCs transplantation group (MI + AD-MSCs), CD73⁺ AD-MSCs transplantation group (MI + CD73⁺ AD-MSCs) and CD73⁻ AD-MSCs transplantation group (MI + CD73⁻ AD-MSCs). Each group had 6 rats. All experiments complied with the Ethics Committee of Xinxiang Medical University (XYLL-2015023).

Color Doppler Ultrasound Detection of MI Postoperative Cardiac Function in Rats

PHILIPS iE33 ultrasound apparatus and S12-4 heart probe were used to evaluate the cardiac function every week from 1st to 4th week after MI. 1% pentobarbital sodium (40 mg/kg) was injected intraperitoneally before the analysis. The evaluated indicators of ultrasonic diagnosis were as follows: left ventricular ejection fraction (LVEF), left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV).

The Expression of VEGF and Factor VIII After MI *in vivo*

The myocardial tissue including infarcted area, infarcted marginal and peri-infarcted area were harvested, fixed in 4% buffered formalin, embedded in paraffin and then sliced continuously with the thickness of 8 μ m. VEGF and factor VIII were detected by immunohistochemistry staining and Western blotting. The primary antibodies were monoclonal antibody against VEGF (bs-1313R, Beijing BOOSEN Biotechnology Co., Ltd., China, 1:200) and polyclonal antibody against VIII factor (bs-2974R, Beijing BOOSEN Biotechnology Co. Ltd., China, 1:200). In brief, the sections were deparaffined, rehydrated, quenched with 3% H₂O₂, and blocked with 10% normal serum, then incubated with above primary antibodies overnight at 4°C, followed by incubation with secondary antibody and SABC-HRP Kit (Beyotime Biotechnology, China). The staining was developed by using 3,3'-diaminobenzidine Tetrahydrochloride and finalized with lightly counter staining with hematoxylin. 10 visual fields were randomly selected from each slide and analyzed by motic Images advanced software. Immunocytochemical staining positive products were quantitatively analyzed by HMIAS-2000 high definition medical image and text analysis system. The intensity of the positive reaction product was analyzed by the integral optical density (IOD) of the image processing system. Western blotting was used to detect VEGF and factor VIII in 120 mg myocardium from the edge of the left ventricular infarction at the end of the 4th week after MI.

Data Analysis

SPSS 23.0 statistical software were used for statistical analyses. The data were presented as means $\pm$ standard deviation (SD). The difference between groups was analyzed using one-way ANOVA and Tukey's *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Morphological Characteristics and Immunophenotyping of AD-MSCs

After the primary cells were cultured for 30 min, a large number of suspended circular cells were visible under the inverted microscope. After 2–3 days, the growth medium was replaced for the first time and AD-MSCs were assumed short rod or round shapes. After 7–10 days, AD-MSCs reached up to 80%

confluence. At passage 3 to 6 the cells were mostly spindle shaped and polygonal. The majority of passage 3 AD-MSCs expressed CD29 and CD44 (81.3 and 99.1%, respectively), while only 15.7% of cells expressed CD73 and CD45 was virtually undetectable (Figure 1 and Supplementary Figure 1).

Characterization of CD73⁺ and CD73[−] Subgroups

CD73⁺AD-MSCs exhibited mainly spindle and rod-like shapes, whereas CD73[−]AD-MSCs mainly polygonal large cells (Figure 2A). The expression of CD73 in CD73⁺AD-MSCs and CD73[−]AD-MSCs has statistically significant differences ($P < 0.05$) (Figures 2B,C). These subtypes also displayed significant differences in gene expression patterns. Based on GO analysis, the differences concerned 10 signaling pathways, including VEGF pathway which were associated with pro-angiogenesis of AD-MSCs (Figure 3).

Effect of CD73 on Cytokine Secretion of AD-MSCs *in vitro*

The secretion of VEGF, SDF-1 α , and HGF in CD73⁺AD-MSCs was significantly higher than that of CD73[−]AD-MSCs ($P < 0.01$) while secretion of bFGF remained similar between these two subgroups ($P > 0.05$). Inhibition of CD73 activity of CD73⁺AD-MSCs by APCP resulted in decreased secretion of VEGF ($P < 0.01$), SDF-1 α ($P < 0.05$), HGF ($P < 0.01$) and no effect on that of bFGF ($P > 0.05$). The expression of SDF-1 α and HGF in CD73[−]AD-MSCs was lower than that in unsorted AD-MSCs (Figure 4A). When CD73 expression was down-regulated in CD73 positive subset, VEGF secretion was decreased ($P < 0.01$) while it was vice versa in CD73 negative subset ($P < 0.05$) (Figure 4B). Scratch wound healing assay was used to evaluate the ability of cellular products to support angiogenesis *in vitro*. When HUVEC was treated with CD73⁺AD-MSCs and CD73[−]AD-MSCs conditioned medium for 6 h, the percent of wound closure was 51.8 and 30.2%, respectively. The difference between CD73⁺AD-MSC and CD73[−]AD-MSC induced wound closure was statistically significant (Figures 4C,D). These results indicate that CD73 promotes secretion of VEGF, SDF-1 α , and HGF factors.

Results of Cardiac Function Test

Compared with the sham group, the LVEF and LVEDV significantly decreased ($P < 0.01$) and the LVESV significantly increased at 1–4 weeks after MI ($P < 0.01$). Compared with MI group, however, the LVEF and LVEDV in MI rats from cell transplantation group were significantly increased ($P < 0.01$). Importantly, the LVEDV did not significantly increase at the 1st and 2nd weeks post-MI in CD73[−] cells transplantation group ($P > 0.05$). The LVESV significantly decreased at the 2nd and 4th weeks in all three cell transplantation groups ($P < 0.01$). Compared to AD-MSCs and CD73[−]AD-MSCs groups, CD73⁺AD-MSCs transplanted rats exhibited increase of LVEF (1st, 2nd, and 4th weeks) and LVEDV (4th week) ($P < 0.05$). When compared with AD-MSCs group, the LVESV of CD73⁺AD-MSCs group decreased on 2nd

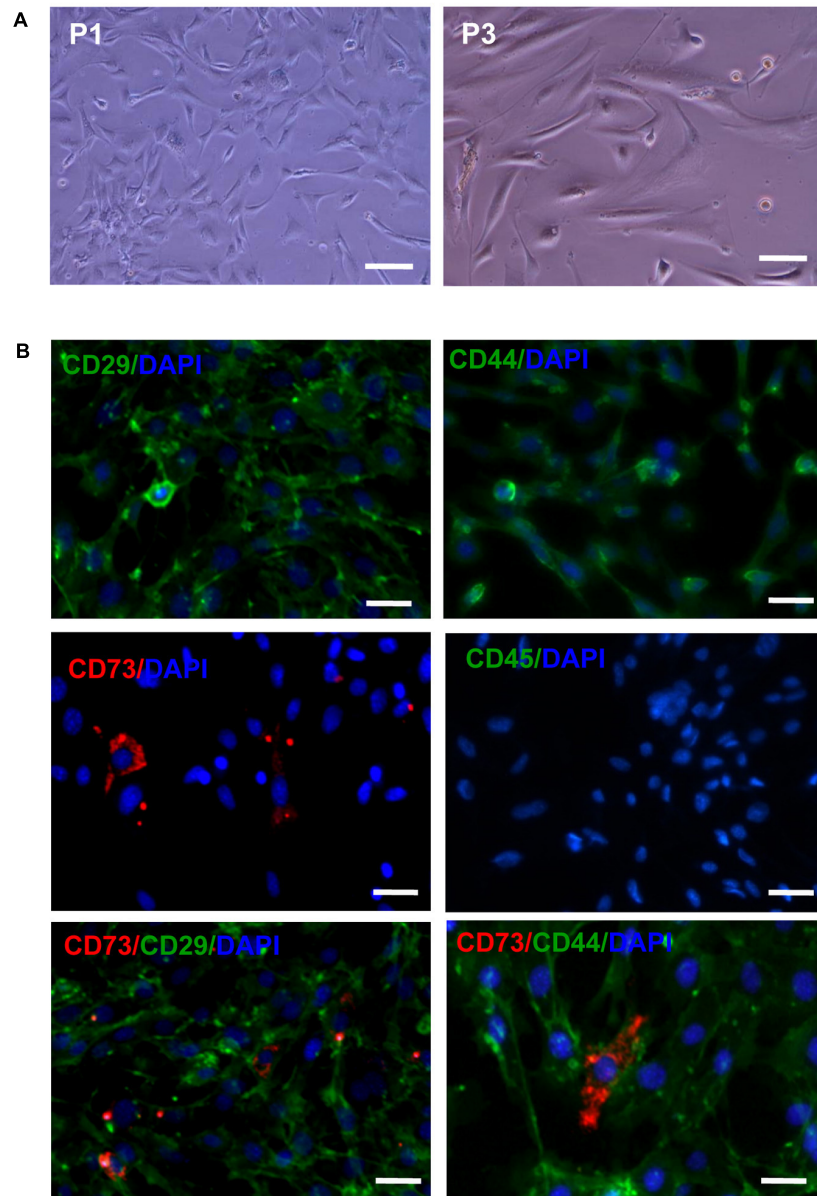


FIGURE 1 | The morphology and phenotypic characterization of AD-MSCs. **(A)** Morphology of AD-MSCs at different passages. P1: passage 1, P3: passage 3; Scale bar, 20 μ m. **(B)** Phenotypic characterizations of AD-MSCs using multicolor immunofluorescence. Immunofluorescence was performed for surface markers CD29, CD44, CD73, and CD45. Nuclei were counterstained with DAPI. Scale bars, 10 μ m.

weekend ($P < 0.05$). These results indicated that CD73⁺AD-MSCs, AD-MSCs and CD73⁻AD-MSCs could significantly improve cardiac function of rats with MI and CD73⁺AD-MSCs transplantation group appeared to be more effective (Figure 5 and Supplementary Figure 2).

Effect of CD73⁺ AD-MSCs Transplantation on Angiogenesis

Immunohistochemical staining and western blotting was used to detect the expression of factor VIII and VEGF, which marked vascular endothelial cells. The higher expression of

VEGF and factor VIII were detected in the myocardial infarction area in the cell transplantation group, and the highest expression was found in the CD73⁺AD-MSCs transplantation group ($P < 0.05$) (Figure 6). By the end of the 4th week post MI, the neovascularization density in all transplantation groups was significantly higher than in the untreated MI group ($P < 0.01$). However, the extent of increase in CD73⁺AD-MSCs group was higher than other transplanting groups ($P < 0.05$) (Supplementary Figure 3). These results indicate that transplantation of CD73⁺AD-MSCs were more effective in promoting myocardial angiogenesis.

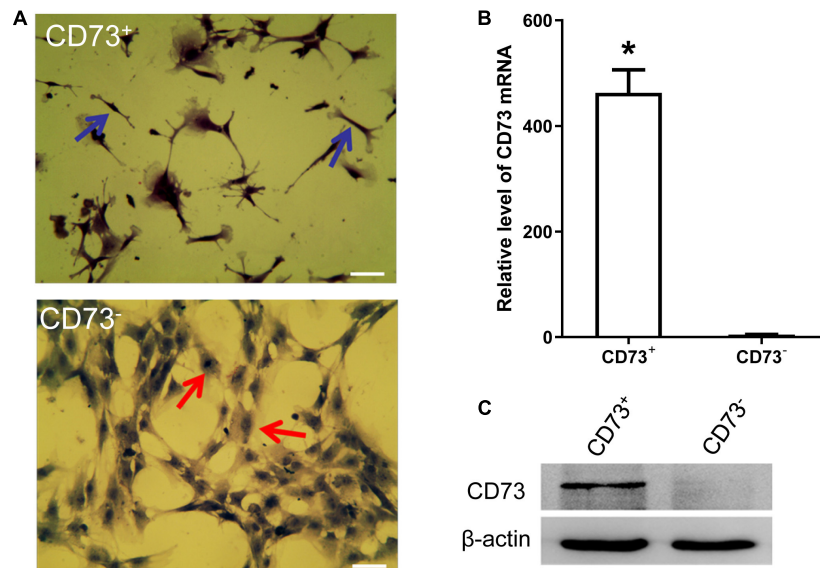


FIGURE 2 | AD-MSCs can be divided into CD73⁺ and CD73⁻ subpopulations. **(A)** HE staining was used to detect the morphological changes of CD73⁺ and CD73⁻ AD-MSCs. Blue arrows indicated spindle cells, red arrows showed big polygonal cells. Scale bars, 20 μ m. **(B)** Detection of CD73 mRNA in CD73⁺ and CD73⁻ AD-MSCs by Real-time quantitative PCR. Results are presented as mean $\pm$ SD. * $P < 0.05$, vs. CD73⁻ AD-MSCs. **(C)** The expression of CD73 in CD73⁺ and CD73⁻ AD-MSCs by Western blotting.

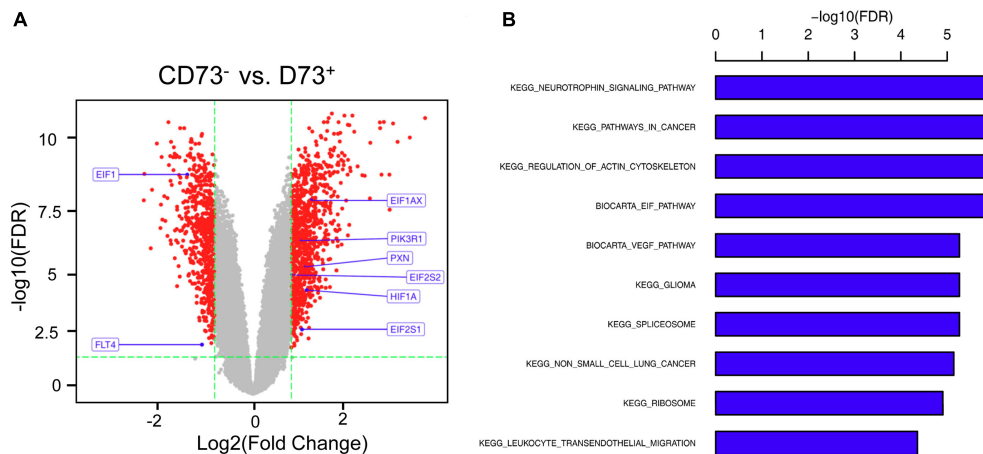


FIGURE 3 | Differential gene analysis of CD73⁺ vs. CD73⁻ AD-MSCs. **(A)** Volcano map of differential genes. **(B)** Go analysis for differentially expressed genes by KEGG.

DISCUSSION

Unlike most similar studies, the present study utilized CD73⁺ and CD73⁻ subsets of AD-MSCs for transplantation. We demonstrated that the presence of CD73 promotes the secretion of VEGF, SDF-1 α , and HGF factors by AD-MSCs *in vitro*. CD73⁺ AD-MSCs promoted vascular regeneration *in vivo*, which could be closely related to regulate the microenvironment of MI area. We also have showed that CD73⁺ AD-MSCs were more effective than bulk AD-MSCs in accelerating cardiac function recovery in the rat model of MI (Figure 7).

CD73 is a glycoprotein that acts as an exogenous 5'-nucleotide enzyme involved in signal transduction. CD73 hydrolyzes 5'-adenosine monophosphate (AMP) into adenosine and phosphoric acid and then interacts with adenosine receptors on the cell surface to modulate a variety of biological effects (Meng et al., 2019). CD73 also has non-hydrolase function, which is also a signal and adhesion molecule that regulates cell-extracellular matrix interaction (Yan A. et al., 2019). Moreover, anti-CD73 inhibitory antibody for cancer immunotherapy is already carried out in clinical trials (Minor et al., 2019). However, the role of CD73 in regeneration therapy of MSCs is rather limited.

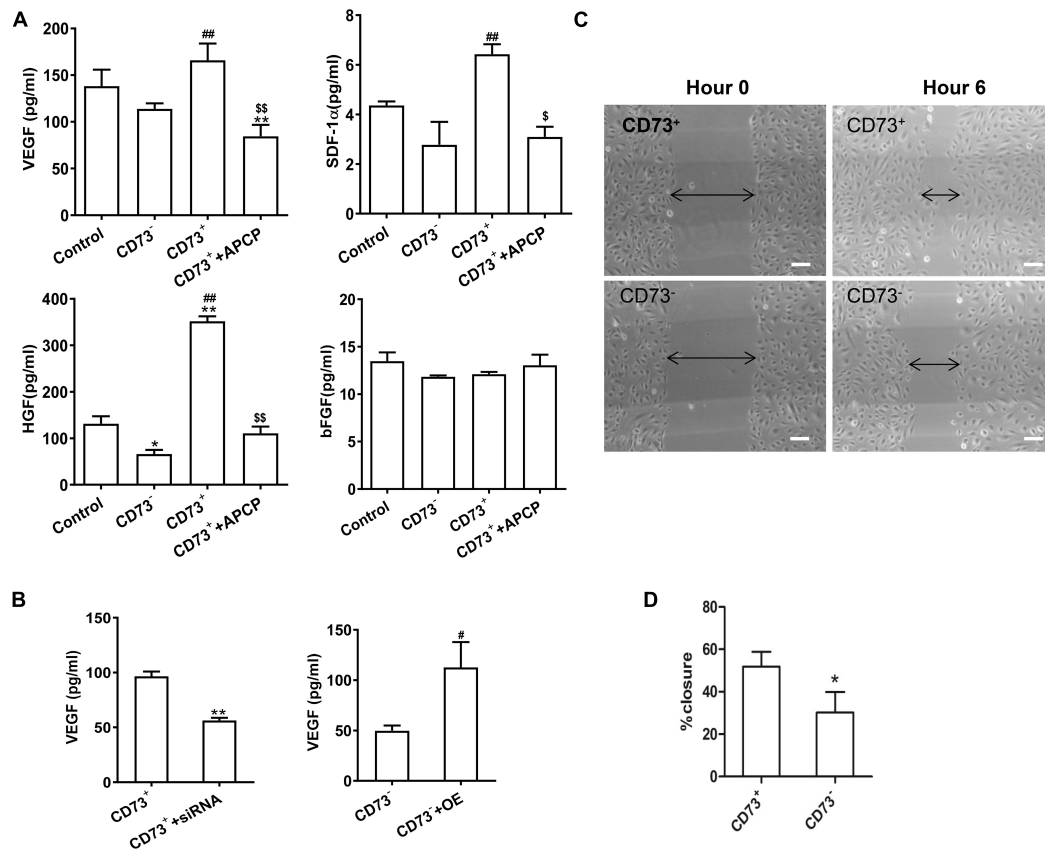


FIGURE 4 | Pro-angiogenic effects of CD73⁺ AD-MSCs. **(A)** Cytokine secretion analysis by ELISA in unsorted AD-MSCs (control), CD73⁺ AD-MSCs, CD73⁻ AD-MSCs, and CD73⁺ AD-MSCs + APCP. **P* < 0.05, ***P* < 0.01, vs. control; #*P* < 0.05, ##*P* < 0.01, vs. CD73⁻ AD-MSCs; \$*P* < 0.05, \$\$*P* < 0.01, vs. CD73⁺ AD-MSCs; **(B)** VEGF secretion of cytokines of AD-MSCs with changing CD73 expression. CD73⁺ + siRNA: CD73⁺ AD-MSCs were transfected with CD73 gene siRNA lentivirus; CD73⁻ + OE: CD73⁻ AD-MSCs were transfected with CD73 gene overexpression lentivirus. ***P* < 0.01, vs. CD73⁺ AD-MSCs; #*P* < 0.05, vs. CD73⁻ AD-MSCs. **(C)** Scratch wound healing assay of HUVEC treated with conditioned media of CD73⁺ AD-MSCs and CD73⁻ AD-MSCs. **(D)** The percent of wound closure was calculated. **P* < 0.05 vs. CD73⁻ AD-MSCs. Scale bars, 100 μm.

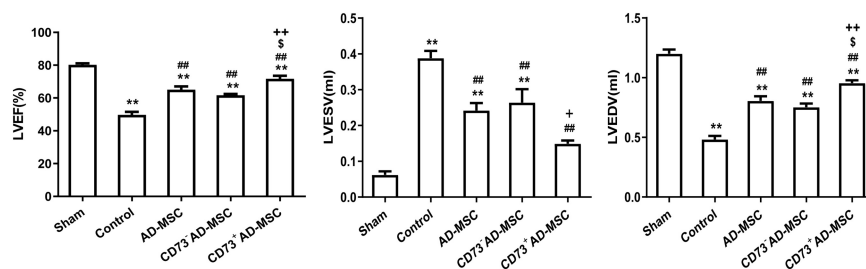
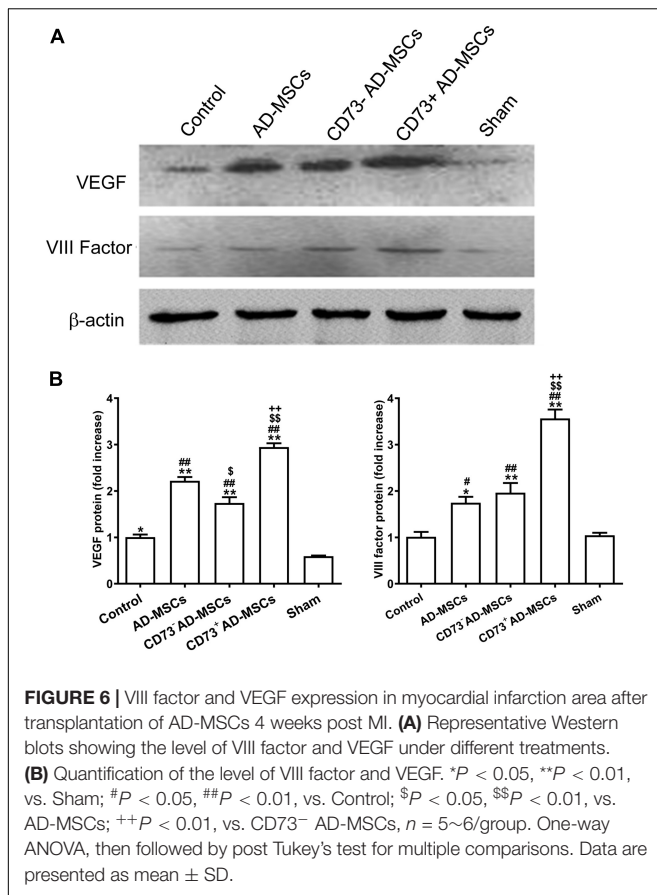


FIGURE 5 | Improvement of cardiac function after CD73⁺ AD-MSCs, CD73⁻ AD-MSCs, and AD-MSCs transplantation at week 4. Cardiac hemodynamic monitoring. LVEF: Left ventricular ejection fraction; LVESV (ml): left ventricular end-systolic volume; LVEDV (ml): left ventricular end-diastolic volume. **P* < 0.05, ***P* < 0.01, vs. Sham; ##*P* < 0.01, vs. Control; \$*P* < 0.05, vs. AD-MSCs; +*P* < 0.05, ++*P* < 0.01, vs. CD73⁻ AD-MSCs, *n* = 5–6. One-way ANOVA, then followed by post Tukey's test for multiple comparisons. Data are presented as mean ± SD.

Routinely, many laboratories tend to isolate and enrich MSCs through plastic adhesion when separating large numbers of MSCs (Li et al., 2016). In this study, CD73⁺ AD-MSCs and CD73⁻ AD-MSCs were separated by flow cytometry. The CD73⁺ cells had either spindle or rod shape, while CD73⁻ cells mostly large and polygonal, in line with previous observations (Haasters et al.,

2009). Although the transcriptome differences between MSCs from different sources are very small, they are sufficient to significantly affect cell behavior (Elahi et al., 2016). Gene chip analysis of this study revealed a number of differentially expressed genes in the subgroups. It showed differences between CD73⁺ AD-MSCs and CD73⁻ AD-MSCs when the transcription profiles



of these two subgroups were compared, especially in VEGF pathway. Therefore our data suggested that evaluation based on CD73, the surface marker, that can be used as a quality control identification standard for cell expansion *in vitro*.

Mesenchymal stem cells promote tissue repair by secreting soluble cytokines, promoting vascular regeneration, blocking apoptosis, suppressing inflammation, and stimulating the regeneration of host cells (Tsuji et al., 2010; Zhao et al., 2019). In this study, we transplanted cultured AD-MSCs into rats with MI and found that the purified CD73⁺ADMSCs significantly increased vascular density, promoted angiogenesis, improved ventricular remodeling and cardiac function when it was compared with mixed ADMSCs and CD73⁻ADMSCs. The therapeutic effect was good. Therefore the use of purified cell subsets could improve the pertinence of the study and the efficacy of the treatment.

Inhibition of CD73 with anti-CD73 antibody or APCP reduced tumor angiogenesis in mice (Allard et al., 2017). The mechanism was probably related to CD73's interaction with adenosine receptor A2B (Hesse et al., 2017). This interaction will stimulate vascular smooth muscle cells, endothelial cells, immune cells and tumor cells to secrete VEGF. Our results showed CD73⁺AD-MSCs were superior to unsorted AD-MSCs and CD73⁻AD-MSCs in promoting myocardial angiogenesis. ELISA results showed that CD73 expression could stimulate the secretion of VEGF, SDF-1 α , and HGF. The scratch

experiment results showed that CD73⁺AD-MSCs cellular products significantly promoted HUVEC migration and scratch wound healing to support angiogenesis. It is speculated that CD73 may contribute to angiogenesis in infarcted regions by upregulating the secretion of VEGF and SDF-1 α in AD-MSCs.

SDF-1 is a well-known chemokine. It played pivotal role in activation, mobilization, normalization, and retention of hematopoietic stem cells (Zhang et al., 2018). SDF-1 could induce VEGF secretion in cells. CXCR4 was the receptor of SDF-1. Then these actual roles effectively regulate the angiogenic activity and homing capacity of endothelial progenitor cells (Sordi et al., 2005; Lau and Wang, 2011). These cytokines not only promoted vascular regeneration, but also protected the myocardium from hypoxia (Lemcke et al., 2018). Our results indicated that CD73⁺ AD-MSCs, AD-MSCs and CD73⁻AD-MSCs could significantly improve the cardiac function of rats with MI, but that CD73⁺AD-MSCs transplantation appeared to be more effective. Exogenously expressed SDF-1 α /CXCR4 played a role in recruiting cardiac stem cells (CSC) and exogenous VEGF could accelerate cardiac repair partly through SDF-1 α /CXCR4 (Tang et al., 2011). MSCs promotes effective angiogenesis through overexpressing SDF-1. This effect can inhibit congestive heart failure caused by MI. *In vivo*, MSC-stimulated SDF-1 α expression in infarcted hearts resulted in massive mobilization and homing of bone marrow stem cells and CSCs (Tian et al., 2019). High expression of HGF also has an obvious cardioprotective effect, it can inhibit cardiocyte apoptosis, promote vascular regeneration, and act effectively in the process of anti-ventricular remodeling and repair (Sonnenberg et al., 2015; Chang et al., 2016). Gallo et al. reported that the HGF/Met axis also acts effectively in adapting myocardial regeneration and self-renewal. This axis could enhance the number of cardiac progenitor cells (Gallo et al., 2015).

The analysis of the effects of MSCs transplantation in the treatment of myocardial injury was mainly focused on cytokine secretion and the differentiation ability of transplanted cells (Yan W. et al., 2019). Clearly, transplantation of AD-MSCs triggers multiple mechanisms that collectively improve heart function. Cytokines secreted from MSCs exert protective effects by salvaging injured neighboring cells through regulation of apoptosis, inflammation, fibrosis, and angiogenesis (Jung et al., 2017). In recent years, studies have shown that MSCs may be involved in the regulation of immune responses (Hara et al., 2019). CD73 and CD39 can be regarded as "immune checkpoint mediators" and can regulate the function of various types of immune cells (Allard et al., 2014). Our study showed that the expression of CD73 stimulated the production of HGF *in vitro*. In addition, HGF has obvious regulatory effects on endothelial cells, epithelial cells, and hematopoietic progenitor cells. it also has been shown to regulate the chemotaxis of T cells in heart tissue (Komarowska et al., 2015). Promoting angiogenesis and antiinflammation can interact, and VEGF expression is higher at low doses of TNF- α (Gomzikova et al., 2019). It is essential for myocardial repairing cell transplantation that CD73⁺

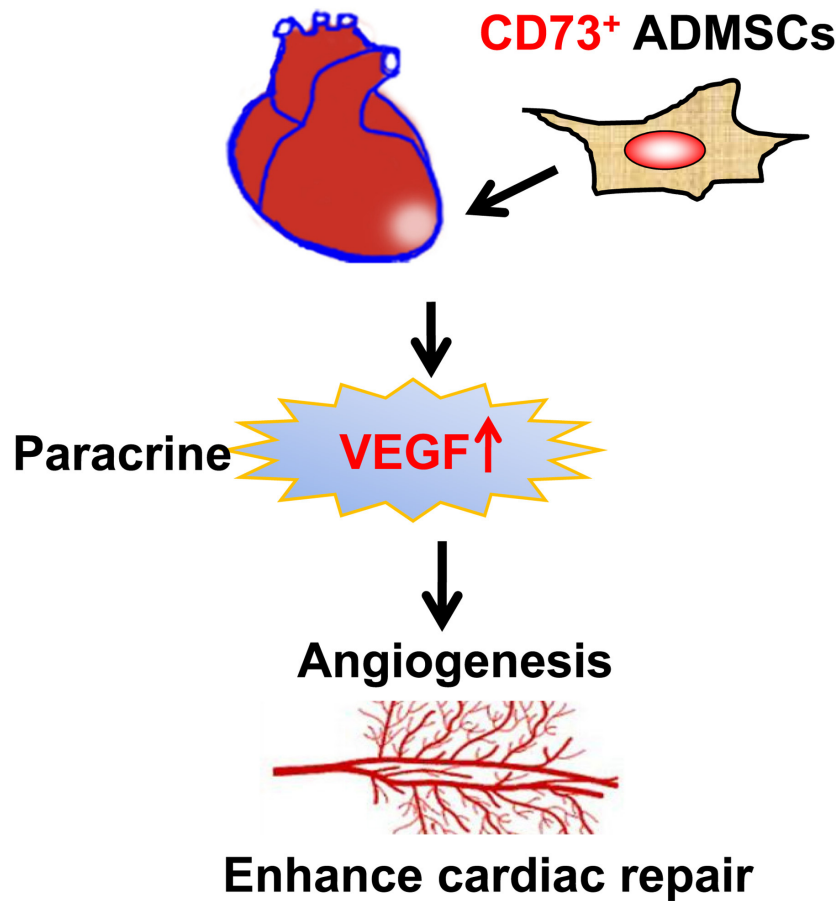


FIGURE 7 | The diagram of the mechanisms responsible for improved cardiac repair after myocardial transplantation of CD73 positive AD-MSCs.

AD-MSCs regulate myocardial microenvironment especially by promoting angiogenesis.

CONCLUSION

In the present study, our results demonstrated that CD73⁺ AD-MSCs revealed a higher pro-angiogenic paracrine activity and displayed therapeutic efficacy for MI therapy. These results suggested that CD73 could be used as a selection marker of MSCs for ischemic disease therapy. Further studies to investigate the intrinsic properties and therapeutic efficiency of CD73 and other subpopulations of MSCs will facilitate a new avenue for regenerative therapy. The strategy of using an MSC subpopulation offers a more effective and definitive methods for stem cell therapy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI GEO; GSE167219.

ETHICS STATEMENT

The animal study was reviewed and approved by the ethical review approval number: XYLL-2015023, Ethics Committee of Xinxiang Medical University.

AUTHOR CONTRIBUTIONS

QL and ZL conceived, designed the experiment, and wrote the manuscript. ZG administered the project and provided financial support. HH, JG, ML, XY, and HZ collected and analyzed data. MZ edited the manuscript. All authors checked and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.637239/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Differentiation and Application of Human Pluripotent Stem Cells Derived Cardiovascular Cells for Treatment of Heart Diseases: Promises and Challenges

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Human pluripotent stem cells (hPSCs) are derived from human embryos (human embryonic stem cells) or reprogrammed from human somatic cells (human induced pluripotent stem cells). They can differentiate into cardiovascular cells, which have great potential as exogenous cell resources for restoring cardiac structure and function in patients with heart disease or heart failure. A variety of protocols have been developed to generate and expand cardiovascular cells derived from hPSCs *in vitro*. Precisely and spatiotemporally activating or inhibiting various pathways in hPSCs is required to obtain cardiovascular lineages with high differentiation efficiency. In this concise review, we summarize the protocols of differentiating hPSCs into cardiovascular cells, highlight their therapeutic application for treatment of cardiac diseases in large animal models, and discuss the challenges and limitations in the use of cardiac cells generated from hPSCs for a better clinical application of hPSC-based cardiac cell therapy.

Keywords: human pluripotent stem cells (hPSCs), cardiovascular cells, differentiation, therapeutic application, large animal

INTRODUCTION

Cardiovascular diseases are the leading causes of death in the world. It is estimated that more than 5 million people die of myocardial infarction (MI) every year (Virani et al., 2020). Although thrombolysis, coronary intervention, and coronary artery bypass graft have significantly improved the prognosis, the high morbidity and mortality associated with MI indicate that current treatment strategy is far from satisfactory.

Cell transfer therapy is being explored as a potential approach to repopulate damaged cardiac tissue. In addition to skeletal myoblasts (Ye et al., 2007), bone marrow-derived cells (Assmus et al., 2010), and mesenchymal stem cells (Ye et al., 2013a), pluripotent stem cells (PSCs)-derived cardiovascular cells, including cardiovascular progenitor cells (CPCs), cardiomyocytes (CMs), endothelial cells (ECs), and smooth muscle cells (SMCs), have been extensively studied.

Human PSCs (hPSCs) include human embryonic stem cells (hESCs) and human induced PSCs (hiPSCs). Theoretically, they can differentiate into all somatic cells found in the human body and can be used as a disease model to explore the genetic mechanisms of diseases such

as congenital heart defects (Lin et al., 2021) or test drugs (Protze et al., 2019) or alternative cell sources for replacing diseased or damaged tissue or disease models *in vitro*. A variety of protocols have been developed to differentiate hPSC into cardiovascular lineages *in vitro*. In this review, we focus on ongoing progress in hPSC-based strategies for human cardiovascular cell derivation and application. This review summarizes the available protocols of differentiating hPSCs into cardiovascular cells, including CMs, ECs, SMCs, and CPCs, and highlights their therapeutic application for treatment of heart diseases in large animal models. Finally, the review discusses the challenges and limitations in the use of cardiac cells generated from hPSCs in the clinical perspective for the treatment of cardiac disease.

EMBRYONIC HEART DEVELOPMENT

The differentiation of hPSCs into CMs is similar to the process of the heart development and formation *in vivo* (Figure 1). Detailed signaling and transcriptional networks in heart development were described in a review by Bruneau (2013). During the embryonic period, Nodal is expressed in the epiblast and activates the distal visceral endoderm, which moves toward the oval to form the anterior–posterior axis (Perea-Gomez et al., 2002). Simultaneously, visceral endoderm secretes Nodal antagonists, including Cerberus, Lefty1, and Dickkopf-related protein 1, which make a gradient change of Nodal and WNT signals in the front and rear directions (Perea-Gomez et al., 2002). This promotes the development of primitive streak, which indicates the start of gastrulation, a process in which the inner cell mass is converted into the trilaminar embryonic disk (Perea-Gomez et al., 2002). This disk comprised the three germ layers: ectoderm, mesoderm, and endoderm. The induction of cardiac mesoderm and distinct populations of CPCs are primarily controlled by three families of extracellular signaling molecules: wntless integrated (WNT), fibroblast growth factor (FGF), and transforming growth factor β (TGF- β), including WNT3a, bone morphogenetic protein 4 (BMP4), Nodal, and activin-A (Spater et al., 2014). These signals induce the expressions of Brachyury and Eomes, which are markers of early mesoderm formation (Lim and Thiery, 2012). In the process of primitive streak migration, cells temporarily activate the transcription factor mesoderm posterior protein 1 (MESP1), which indicates entering the stage of cardiac mesoderm development (Lim and Thiery, 2012). Later, mesodermal progenitors commit to cardiac cells by WNT antagonist.

A subset of MESP1⁺ cells begin to transcribe the homeodomain transcription factors Nkx2.5, T-box 5 (Tbx5, a marker of the first heart field), and islet1 (Isl1) genes (a marker of the second heart field) (Evans et al., 2010). These factors represent cardiac lineage markers in the early developmental stages of the heart field. Nkx2.5 and Tbx5 are typical markers of primitive heart tube cells involved in the formation of the atria and left ventricular (LV) compartments, whereas the secondary heart field is mainly related to the development and formation of the right ventricle and outflow

tract (Evans et al., 2010). They are related to transcription factor GATA4/5/6 and serum response factor (SRF). Subsequently, genes related to the CMs are successively activated, such as α -actinin, myosin light chain, myosin heavy chain (MHC), and troponin, as well as myocyte enhancer factor-2 (MEF2) that regulates heart structural genes (Evans et al., 2010). These complexes process and lead to the proliferation and maturation of CMs.

In summary, heart development can be roughly divided into three stages: (1) gastrulation to cardiac specification during which mesodermal progenitors are developed; (2) heart development before beating during which cardiac progenitors are developed; and (3) heart development at beating during which myofibrillogenesis and trabeculation are developed.

DIFFERENTIATION OF hPSCs INTO CMs

Signaling Pathways Involved in Differentiation of hPSCs Into CMs

The basic principle of the current method of inducing hPSCs to differentiate into CMs *in vitro* is to simulate the heart development *in vivo*. The same differentiation regulation has been demonstrated in hESCs and hiPSCs. hPSCs are differentiated into CMs based on three stages through spatial-temporal modulation of signaling pathways, such as BMP, activin-A, WNT, etc. (Filipczyk et al., 2007; Laflamme et al., 2007; Kattman et al., 2011; Lian et al., 2012; Zhang et al., 2012; Fonoudi et al., 2015).

BMP-4 commits hPSCs into mesodermal lineage cells alone or in combination with activin-A. BMP signaling controls the expressions of GATA4, SRF, and MEF2C transcription factors (Klaus et al., 2012). Combination of activin-A and BMP4 induces KDR⁺PDGFR α ⁺ cardiogenic mesoderm in hPSCs, which expressed MESP1 between days 3 and 4 and Nkx2.5 by day 8 of differentiation (Kattman et al., 2011). Combining activin-A and BMP-4 with Matrigel-generated high purity (up to 98%) and yield (up to 11 CMs/input PSC) of CMs from hPSCs (Zhang et al., 2012).

WNT plays a bidirectional role in differentiation, depending on the time point of differentiation. At stage 1, both classical activation, which suppresses the catenin/GSK3 pathway, and non-classical signal transduction, which involves the C protein kinase C/C-Jun N-terminal kinase, have been shown to induce mesodermal lineage from hPSCs (Cohen et al., 2008). At stage 2, WNT antagonist, such as DKK1 and IWP, directs mesodermal progenitors to cardiac progenitors (Willems et al., 2011). Nkx2.5, Isl1, and Baf60c are controlled by WNT/ β -catenin signaling (Klaus et al., 2012).

Although either WNT activation/GSK3 inhibition (Ye et al., 2013b; Tan et al., 2019; Tao et al., 2020) or BMP4/activin-A (Laflamme et al., 2007; Hudson et al., 2012; Zhang et al., 2012; Ye et al., 2013b) has been shown to induce mesodermal lineage from hPSCs, WNT activator alone, such as CHIR99021, has gained popularity because of its cheap price and reproducible results (Lian et al., 2012; Su et al., 2018; Tan et al., 2019; Tao et al., 2020).

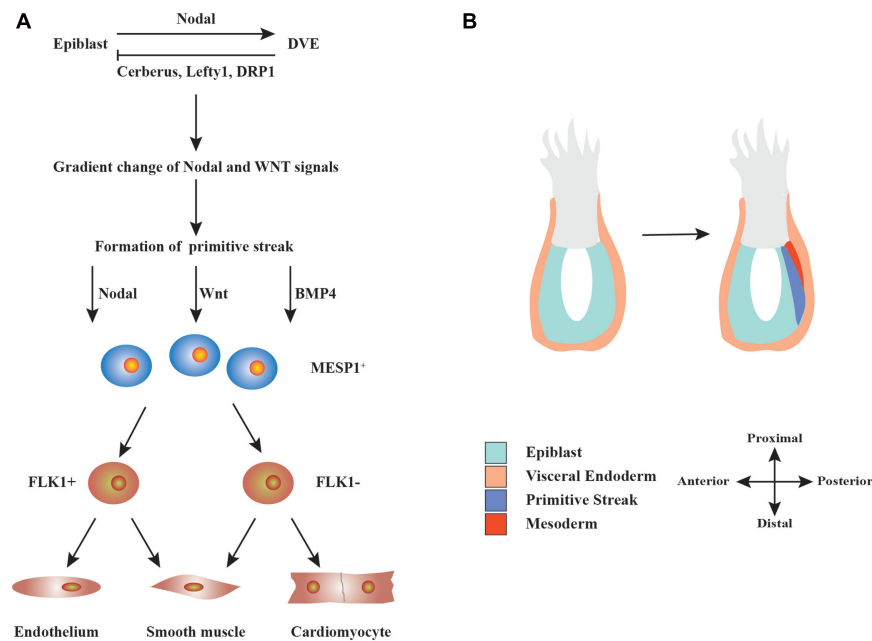


FIGURE 1 | Schematic diagram of the development of heart cells *in vivo*. **(A)** Mutual regulation between epiblast and distal visceral endoderm (DVE) through Nodal, Cerberus, Lefty1, and DRP1 signals leads to a gradient distribution of the concentrations of Nodal and Wnt, which results in the formation of primitive streak. During primitive streak migration, a small number of cells express mesoderm posterior protein 1 (MESP1), marking the beginning of heart development. MESP1⁺ cells finally differentiate into various cells that form the heart, such as endothelium, smooth muscle, and myocardium. **(B)** The migration of the primitive streak from posterior to anterior also marks the beginning of gastrulation, a crucial event in embryonic development. During this period, the embryo becomes a trilaminar embryonic disk, and the heart develops from the mesoderm.

Three-Dimensional Environment for CM Differentiation

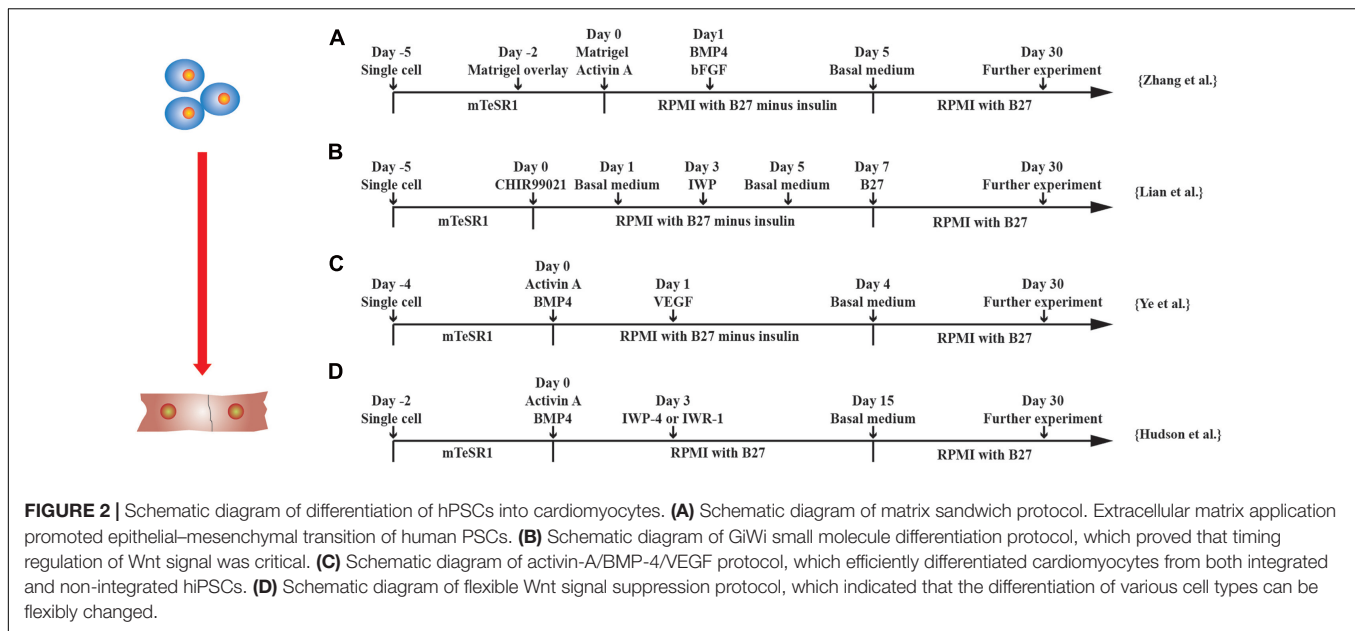
To mimic *in vivo* cardiac cell development, hPSCs were cultured in embryoid bodies (EBs) or spheroids (Itskovitz-Eldor et al., 2000; Kehat et al., 2001; Xu et al., 2002; He et al., 2003; Zhang et al., 2009; Kattman et al., 2011; Fonoudi et al., 2015; Kempf et al., 2015) or in suspended microcarriers (Ting et al., 2014) to differentiate into CMs. Differentiation of hPSCs in EBs results in three embryonic germ layer formation (Itskovitz-Eldor et al., 2000). Early studies showed that the cells in EBs generated spontaneous contraction and contained mixed cell populations of nodal-, atrial-, and ventricular-like cells, and the efficiency was quite low (Kehat et al., 2001; He et al., 2003; Zhang et al., 2009).

The size of EBs seems to be a critical factor that affects differentiation efficiency. Centrifugation (Ng et al., 2005; Burrige et al., 2007), engineered microwells (Mohr et al., 2006, 2010), and micropatterning (Bauwens et al., 2008) have been employed to produce more homogenously sized EBs, which is helpful for maximizing mesoderm formation and cardiac induction (Bauwens et al., 2008). More recently, spatial-temporal modulation of WNT signaling and activation of sonic hedgehog signaling in hPSCs, cultured in stirred suspension bioreactors, led to the generation of approximately 100% beating EBs containing highly pure (~90%) CMs in 10 days (Fonoudi et al., 2015). Using bioreactors, 4×10^7 to 5×10^7 CMs can be generated per differentiation batch at >80% purity in 24 days (Kempf et al., 2015).

Two-Dimensional Environment for CM Differentiation

Directed differentiation of hPSCs in monolayer is a more convenient method as compared with cardiac differentiation in three-dimensional (3D) environment. Two most efficient and popular CM differentiation methods are the GiWi small molecule differentiation protocol by Lian et al. (2012) and the matrix sandwich method by Zhang et al. (2012) (Figure 2). Lian et al. (2012) showed that temporal modulation of WNT signaling is essential and sufficient for efficient cardiac lineage induction in hPSCs under defined and growth factor-free conditions. WNT activation at the initial stage of hPSC differentiation enhanced CM generation, whereas shRNA knockdown of β -catenin during this stage fully blocked CM specification. Sequential treatment of hPSCs with GSK3, such as CHIR99021, followed by chemical inhibitors of WNT (IWP2) signaling in the later stage produced a high yield (up to 98%) and functional CMs from multiple hPSC lines. Zhang et al. (2012) demonstrated that extracellular matrix also plays an important role in hPSC differentiation. The Matrigel, an extracellular matrix, promotes an epithelial-to-mesenchymal transition, combined with activin-A, BMP4, and basic FGF (bFGF) generated high yield (up to 11 CMs/input PSC) and pure (up to 98%) CMs from hPSCs.

Other factors that affect the differentiation efficiency of hPSCs have been documented, including cell density, cell culture matrices, vascular endothelial growth factor 165 (VEGF₁₆₅), heparin, and insulin. A complete confluence of hiPSCs is required



during the differentiation process and increases the yield of CMs (Zhang et al., 2015). In addition to Matrigel, other extracellular matrices, such as recombinant human cadherin, vitronectin, laminin-521 and laminin-511, fibronectin, and a fibronectin mimetic, support efficient CM differentiation of hPSCs (Burrage et al., 2014). A low concentration of VEGF-A at differentiation stage 2 efficiently differentiated hiPSCs into CMs, especially the one reprogrammed from blood mononuclear cells (Ye et al., 2013b). Heparin can act as a WNT modulator to promote CM production condition (Lin et al., 2017). Insulin can redirect differentiation from cardiogenic mesoderm and endoderm to neuroectoderm in differentiating hESCs (Freund et al., 2008).

Xeno-Free and Chemically Defined Systems for CM Differentiation From hiPSCs

To be safe for clinical application, a xeno-free and chemically defined differentiation system is required. E8 medium and StemMACS iPS-Brew XF medium have been developed as xeno-free media for maintenance and expansion of hPSCs. Vitronectin XF and human recombinant laminin are coating matrix for maintaining hPSC growth and differentiation of hPSCs into cardiac cells (Hayashi and Furue, 2016; Yap et al., 2019). Transferrin has been used to replace B27 in a chemically defined medium for CM differentiation from hiPSCs (Zhang et al., 2020). hiPSC-CMs derived from transferrin-supplemented medium have similar transcriptome and the maturation level compared to those generated in B27 minus insulin medium.

High CM differentiation efficiency using xeno-free and chemically defined system have been reported (Burrage et al., 2014; Tan et al., 2018). Burrage et al. (2014) obtained contractile cell sheets of up to 95% cardiac troponin T (cTNT) CMs using RPMI 1640 medium supplemented with L-ascorbic acid 2-phosphate, recombinant human albumin, and small molecules.

Using a bovine serum albumin-free and chemically defined system, Tan et al. (2018) were able to differentiate hPSCs into clinical-grade CMs, which generated greater than 80% cTNT + CMs.

Purification of hPSC-Derived CMs

Although self-beating immature CMs can be obtained through the above methods, the differentiation efficiency is cell line dependent. There are still many unknown non-CMs, such as undifferentiated hPSCs or cell differentiation into other directions. In order to make the hPSC-CM have therapeutic value, non-CMs need to be removed. A detailed description of strategies for purification of hPSC-CM can be found in review by Ban et al. (2017).

Genetic Modification

Genetic method to enrich CM was first developed (Anderson et al., 2007; Xu et al., 2008; Kita-Matsuo et al., 2009; Ma et al., 2011). Cardiac-specific promoter, such as α MHC promoter, with puromycin or neomycin selection gene, was introduced into hESCs to generate stable transgenic cell lines (Xu et al., 2008; Kita-Matsuo et al., 2009). The drug selected CMs were 96% pure and could be cultured for over 4 months. Anderson et al. developed two genetic selection systems: (1) negative selection of proliferating cells with the herpes simplex virus thymidine kinase/ganciclovir gene system and (2) positive selection of CMs expressing a bicistronic reporter: α MHC promoter driven green fluorescent protein (GFP) with puromycin-resistance gene (Anderson et al., 2007). However, only the puromycin method enriched CMs up to 91.5% purity, which was about 2.7-fold that of the negative selection method.

Nkx2.5 is expressed in early cardiac mesoderm cells throughout the left ventricle and atrial chambers during embryogenesis (Evans et al., 2010). GFP was engineered to

the Nkx2.5 locus of hESC to facilitate the monitoring CM differentiation (Elliott et al., 2011). Den Hartogh et al. (2016) generated a dual fluorescent reporter MESP1 (mCherry)/Nkx2.5 (GFP) line in hPSC. This enabled the visualization of precardiac MESP1 + mesoderm and their further commitment toward the cardiac lineage through activation of Nkx2.5 (Den Hartogh et al., 2016). Jung et al. (2014) used nodal cell inducer TBX3, coupled with MHC6 promoter-based antibiotic selection, which can obtain 80% of functional sinus pacemaker cells. Although genetic modification seems to improve the purity of hPSC-CMs, it may be more useful for monitoring CM differentiation rather than for purifying hPSC-CMs.

Cell Surface Markers

Efforts have been made to identify cell surface markers on CMs. Dubois et al. (2011) screened 370 known CD antibodies and found that signal-regulatory protein α (SIRP α) is a marker specifically expressed on hPSC-CMs. Cell sorting targeting SIRP α can enrich cardiac precursors and hPSC-CMs up to 98% purity. In addition, vascular cell (VC) adhesion molecule 1 has been identified as a cell surface marker for cTnT expressing CMs from 242 antibodies by Uosaki et al. (2011).

Lin et al. (2012) developed a protocol to select CPCs based on cell surface markers during differentiation stages. hPSCs were cultured in EBs and dissociated. The low-KDR/c-Kit⁺ CPCs were isolated by fluorescence activated cell sorting. After culture with VEGF/DKK1, cells were further isolated based on CD166. The CD166⁺ cells were differentiated into CMs, and CD166⁻ cells were differentiated into SMCs.

Physical or Chemical Methods

Purification of CMs using a Percoll gradient or metabolic selection has been established. Early study using EBs for hPSC-CM differentiation results in cells from three embryonic germ layers (Itskovitz-Eldor et al., 2000). Percoll density centrifugation can enrich CMs reaching 70% (Xu et al., 2002). Because of the special metabolic mode of CMs, a medium containing lactate without glucose has been used to inhibit the growth of non-CMs, so that only CMs can survive, which increases CM purity up to 99% (Tohyama et al., 2013). Fluorescent molecular beacons targeting the mRNA of MHC6/7 in CMs have been developed to enrich cTnT⁺ CMs up to 97% (Ban et al., 2013).

More recently, it has been shown that synergy between CHIR99021 and concurrent removal of cell-cell contact can massively expand hiPSC-CMs *in vitro* (i.e., 100- to 250-fold) (Buikema et al., 2020). The lymphoid enhancer binding factor/T-cell-specific transcription factor activity and AKT phosphorylation are underlying mechanisms for a synergistic effect. The differentiated hPSC-CMs are often a mixture of several CM subtypes, such as atrial-, ventricular-, and pacemaker-like CMs, which cannot meet the requirements of precision medicine. A comprehensive description of how chamber-specific CMs are produced during development and how atrial-, ventricular-, and

pacemaker-like CMs are induced *in vitro*, can be found in review of Zhao et al. (2020).

DIFFERENTIATION OF hPSCs INTO ECs

Endothelial cells are a thin layer of specialized cells that directly contact with the blood flow, the circulatory system, and blood throughout the body. Therefore, the function of ECs involves multiple fields of vascular biology, such as nutrient exchange, immune cell adhesion and migration, and intercellular communication (Lerman and Zeiher, 2005). If ECs are damaged or dysfunctional, it is easy to cause atherosclerosis and other common cardiovascular diseases (Lerman and Zeiher, 2005).

Embryonic Origins of ECs

The development of blood vessels in the embryo is slightly different from CMs. The initial embryonic blood vessels come from the extraembryonic mesoderm of the yolk sac (Goldie et al., 2008). The progenitor cells differentiate to form a solid cell mass called "blood island," which will fuse to form a primitive network of tubules known as a vascular plexus. The outer layer of cells gradually becomes flattened to become the most primitive ECs, whereas the inner cells form the primitive hematopoietic stem cells (Goldie et al., 2008). These differentiated blood islands continue to fuse to form the vascular plexus, which is further remodeled to form arteries or veins. In addition, the endothelium of cardiac coronary arteries originates from the sinus venosus through VEGF-C-stimulated angiogenesis during the development of the heart (Chen et al., 2014). The coronary endothelium of interventricular septum is differentiated from the endocardium progenitor cells (Harris and Black, 2010).

Signaling Pathways in EC Differentiation

hPSCs need to be differentiated into mesodermal progenitor cells by regulating WNT signaling pathway followed by commitment to endothelial lineage principally by VEGF signaling (Su et al., 2018; Wang K. et al., 2020). VEGF is a key growth factor in EC differentiation from hPSCs (Olsson et al., 2006; Nourse et al., 2010). VEGF/VEGF receptor (VEGFR) signaling promotes vascular endothelial differentiation by up-regulating ETV2 expression (Liu et al., 2015). Synergistically using BMP4, FGF2, and VEGF up-regulate the mitogen-activated protein kinase (MAPK) and PI3K pathways to induce early vascular progenitors from hiPSC-derived mesodermal progenitors through regulation of the ETS family transcription factors, ETV2, ERG, and FLI1 (Harding et al., 2017).

ETV2 is a dispensable regulator for vascular EC development. It is expressed in hematopoietic and endothelial progenitors in the yolk sac (Koyano-Nakagawa et al., 2012). ETV2 acts downstream of BMP, Notch, and WNT signaling to regulate blood and vessel progenitor specification. Chromatin immunoprecipitation assay by Liu et al. (2015) showed that ETV2 can bind not only to promoters or enhancers of Flk1 and Cdh5, but also to other genes that perform

critical roles in vascular endothelial or hematopoietic cells, including GATA2, Meis1, Dll4, Notch1, Nr1p/2, Flt4, Flt1, RhoJ, and MAPK.

3D Environment for EC Differentiation

Similar to CM, 3D and two-dimensional (2D) culture systems have been applied to differentiate hPSCs into ECs (Levenberg et al., 2002; Li et al., 2011; Adams et al., 2013; Sahara et al., 2014; Zhang et al., 2014, 2017; Patsch et al., 2015; Sivarapatna et al., 2015; Gil et al., 2016; Liu et al., 2016; Harding et al., 2017; Su et al., 2018; Wang K. et al., 2020). 3D system includes EB formation or patch-mediated EC differentiation (Levenberg et al., 2002; Li et al., 2011; Adams et al., 2013; Zhang et al., 2014; Sivarapatna et al., 2015; Su et al., 2018).

Early studies cultured hESCs in EBs to facilitate formation of the three embryonic germ layers and purified differentiated ECs by cell sorting based on CD31 (Levenberg et al., 2002; Li et al., 2011; Adams et al., 2013; Gil et al., 2016). ECs can be differentiated from EBs of hESCs under hemangioblast differentiation conditions in two stages (Gil et al., 2016). EBs were cultured with BMP4 for 2 days and dissociated into single cells and cultured with BMP4, VEGF, stem cell factor, thrombopoietin, Flt-3 ligand, and bFGF for another 2 days to obtain ECs. Approximately 37% of hESCs differentiated into ECs as assessed by flow cytometry.

Adams et al. (2013) differentiated hiPSCs in EBs to get ECs. Although only 18% of cells differentiated into ECs, which have biological function to react to proinflammatory factors, such as interleukin 1 β (IL-1 β), tumor necrosis factor α , and lipopolysaccharide.

To monitor EC differentiation, an hESC cell line was engineered with VE-cadherin promoter-driven GFP EBs (Sahara et al., 2015). BMP4 and a GSK3 β inhibitor were applied in an early phase and followed by treatment with VEGF-A and inhibition of the Notch signaling pathway in a later phase for EC differentiation in EBs (Sahara et al., 2015). This resulted in differentiation efficiency up to 50% within 6 days.

Recently, ECs were more efficiently generated from EBs based on the modulation of signaling pathways involved in mesodermal progenitor cells in the early stage and endothelial specification at a later stage (Sivarapatna et al., 2015). Human EBs were first differentiated into mesoderm using BMP-4 followed by dissociation and cultured as monolayer and further treated with VEGF to specify EC fate (Sivarapatna et al., 2015). The differentiation protocol improved EC differentiation efficiency by greater than 50%.

It was found that 3D environment promoted hiPSC differentiation into ECs when hiPSCs were seeded into thrombin–fibrinogen patch (Zhang et al., 2014). 3D environment enhanced EC differentiation through up-regulation of p38MAPK and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways (Su et al., 2018). Synergistically using CHIR99021 with U-46619, a prostaglandin H2 analog that activates ERK1/2 and p38MAPK signaling, not only more efficiently induces mesodermal progenitors in early stage, but also enhances ETV2 transcription factor expression at later stage, which leads to >85% hiPSCs converted to EC fate (Su et al., 2018).

2D Environment for EC Differentiation

With the understanding of signaling pathways required in EC differentiation from hPSCs, 2D monolayer for EC differentiation gains popular. hPSCs are differentiated into intermediate mesodermal progenitor cells at early stage followed by commitment to endothelial specification at later stage by VEGF (Patsch et al., 2015; Sahara et al., 2015; Sivarapatna et al., 2015; Gil et al., 2016; Zhang et al., 2017; Su et al., 2018; Rosa et al., 2019; Wang K. et al., 2020) (**Figure 3**).

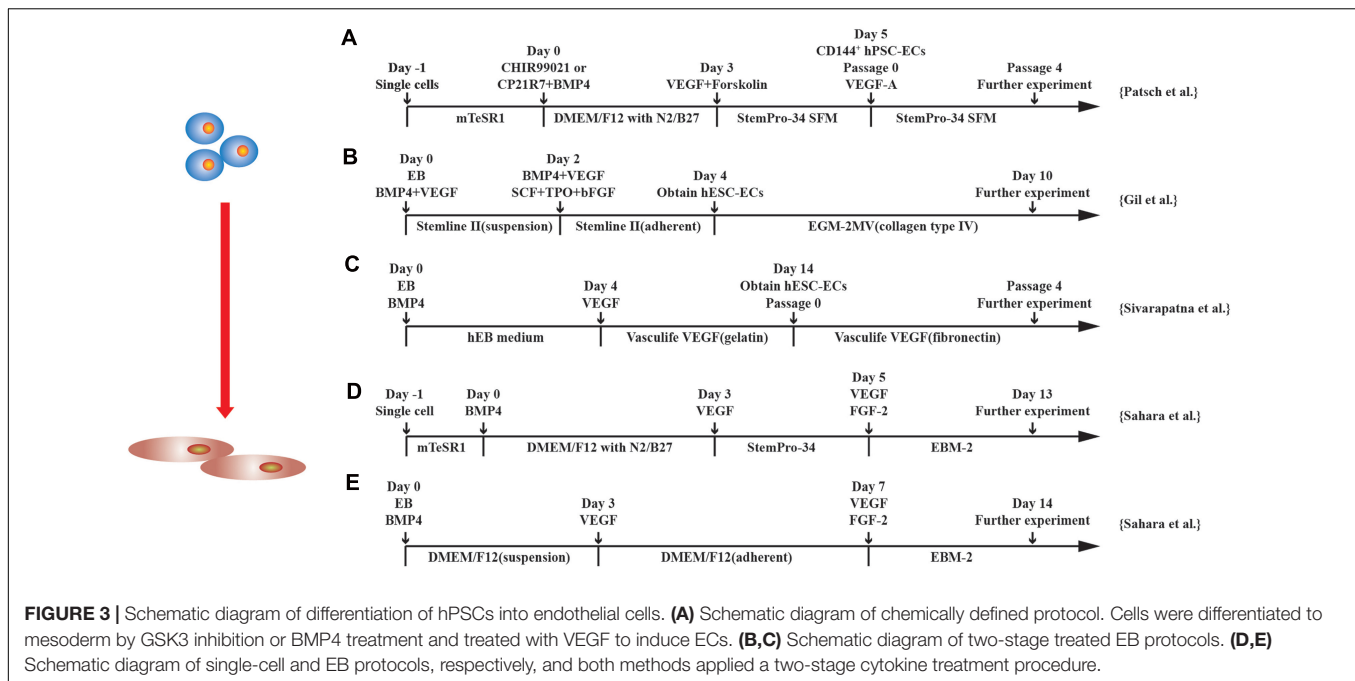
Although a combination of BMP4 and bFGF commits hPSCs into mesodermal lineage (Ikuno et al., 2017; Rosa et al., 2019), synergistically using GSK3 inhibitor with BMP4 and/or activin-A has been shown to more efficiently and rapidly commit hPSCs to a mesodermal fate, and subsequent exposure to VEGF-A resulted in efficient differentiation of hPSCs into ECs (Patsch et al., 2015; Sahara et al., 2015; Zhang et al., 2017). CHIR99021 alone has been used for induction of mesoderm at stage 1 of EC differentiation (Liu et al., 2016). At a later stage, VEGF alone or combined with other factors induces EC differentiation from mesodermal progenitors (Patsch et al., 2015). Combining cyclic adenosine monophosphate has been shown to efficiently induced EC differentiation as it increases the expression of VEGFR2 and another VEGFR, neuropilin1, through protein kinase A activation (Yamamizu et al., 2009; Ikuno et al., 2017). Synergistically using FGF2, VEGF, and BMP4 efficiently induced vascular progenitors from hiPSC-derived mesodermal progenitors through regulation of the ETS family transcription factors, ETV2, ERG, and FLI1 via MAPK and PI3k signaling (Harding et al., 2017). Combining VEGF with inhibitor of Notch signaling pathway in the second stage and converted > 50% hPSCs to ECs in 6 days (Sahara et al., 2015).

In addition to small molecules and growth factors, genetic modification has been applied to enhance EC differentiation. Wang K. et al. (2020) transfected mesodermal progenitors with modified mRNA encoding ETV2, a master transcription factor in EC development. This efficiently converted mesodermal progenitors into ECs rapidly and robustly. The implementation of exogenous ETV2 may overcome the issues of inefficient activation of ETV2 during EC differentiation and hiPSCs reprogrammed from various somatic cells.

Specification of Arterial, Venous, and Lymphatic Endothelial Cells

hPSC-derived ECs are heterogeneous (Rufaihah et al., 2013). They displayed arterial, venous, and, to a lesser degree, lymphatic lineage markers (Rufaihah et al., 2013). The traditional ECs isolated were based on CD31 and/or VE-cadherin, which cannot discern between EC subtypes. Therefore, it is necessary to develop methods to derive or purify iPSC-EC-specific subtypes. Several studies developed protocols to derive more homogenous hPSC-EC subtypes (Rufaihah et al., 2013; Sivarapatna et al., 2015; Zhang et al., 2017; Rosa et al., 2019).

VEGF concentration has been shown to affect differentiated EC subtypes. Rosa et al. (2019) demonstrated that modulation of VEGF concentration (10 vs. 50 ng/mL) can direct mesodermal



progenitor cell into venous-like versus arterial-like ECs in a chemically defined and serum-free condition. Rufaihah et al. (2013) confirmed this and further showed that hiPSC-derived ECs are mainly arterial subtype in the presence of high concentrations of VEGF-A (50 ng/mL) and 8-bromoadenosine-3':5'-cyclic monophosphate (0.5 mmol/L), as they expressed higher levels of ephrin B2, whereas lower concentrations of VEGF-A favored venous subtype and combination of VEGF-C, and angiopoietin-1 promoted the expression of lymphatic phenotype.

Biomimetic flow bioreactors have been employed to facilitate the induction of arterial ECs (Sivarapatna et al., 2015). hiPSC-ECs were purified by CD31⁺ magnetic beads and cultured on bioreactor membrane and enssembled into bioreactors. Flow generated shear stress on hiPSC-ECs, which induced the expressed arterial EC markers: ephrin B2, CXCR4, connexin40, and Notch-1. Zhang et al. (2017) demonstrated that combination of FGF2, VEGFA, SB431542, RESV, and L690 in the absence of insulin greatly improved arterial EC differentiation, whereas venous-like ECs were derived by treating cell with VEGF-A and BMP4 only. The arterial ECs expressed arterial genes, such as CXCR4, DLL4, Notch4, ephrin B2.

There are limited studies on lymphatic endothelial lineage differentiation from hPSCs. Rufaihah et al. (2013) showed that combination of VEGF-C and angiopoietin-1 promoted the expression of lymphatic phenotype. Lee et al. (2015) compared three different culture conditions: spontaneous differentiation through EB formation, coculture with OP9 cells, and a feeder-free culture with gelatin, and found that the coculture system most effectively induced lymphatic endothelial differentiation of hPSCs. Lymphatic ECs expressed key markers, including *PROX1*, *LYVE1*, *VEGFR3*, and *PODOPLANIN*. These cells promoted wound healing through lymphatic neovascularization. More

recently, it was shown that low-dose (<1 ng/mL) BMP9 promotes early lymphatic-specified ECs (Subileau et al., 2019).

DIFFERENTIATION OF hPSCs INTO SMCs

The sources of vascular smooth muscle in the embryonic development process are multiple lineages (Majesky, 2007; Sinha et al., 2014), such as neural crest (Jiang et al., 2000), secondary heart field (Waldo et al., 2005), proepicardial organ, lateral plate mesoderm (Mikawa and Gourdie, 1996), and the paraxial mesoderm (Wasteson et al., 2008). Detailed description of the embryonic origins of human vascular SMCs can be found in reviews by Majesky (2007) and Sinha et al. (2014).

Protocol-directed hiPSC-SMC differentiation is quite different in 3D (Xie et al., 2007; Ge et al., 2012; Kinnear et al., 2013; Wang et al., 2014; Kinnear et al., 2020) and 2D (Huang et al., 2006; Patsch et al., 2015; Yang et al., 2016) culture systems. Cells derived from the outgrowth of human EBs cultured in SMC differentiation condition, which was only composed of Dulbecco modified eagle medium (DMEM) + 5% fetal bovine serum (FBS) and a gelatin-coated surface, produced SMCs expressing smooth muscle MHC (SMMHC) and α -smooth muscle actin (α -SMA) (Xie et al., 2007). Surprisingly, when outgrowing cells were cultured in growth condition, which was composed of smooth muscle growth medium and Matrigel-coated surface, <10% of cells expressed SMMHC and α -SMA.

Ge et al. (2012) cultured hiPSC in EBs for 6 days in differentiating medium, which was composed of DMEM medium containing 10% FBS, 1% non-essential amino acids, 0.1 mM mercaptoacids, and 1% L-glutamine. Then, EBs were cultured on 0.1% gelatin-coated surface with fresh differentiation medium

for another 6 days. Furthermore, cells were dissociated and transferred to Matrigel-coated plates in SmGM-2 media for 1 week. At last, cells were passaged and cultured on 0.1% gelatin-coated culture dishes and cultured with 5% FBS differentiation medium for at least 5 days to complete differentiation. This produced highly homogenous SMC-like cells (around 96%) (Ge et al., 2012). This protocol has been used to differentiate elastin mutant hiPSC into SMCs to model elastin insufficiency phenotype in SMCs (Kinnear et al., 2020) and Williams–Beuren syndrome *in vitro* (Kinnear et al., 2013). Furthermore, the same protocol has efficiently induced SMCs used for manufacturing of macroporous and nanofibrous poly(L-lactic acid) scaffold (Wang et al., 2014). These studies suggest that human EB mediated SMC differentiation from hPSCs is highly efficient in the condition of DMEM supplemented with FBS and using gelatin as extracellular matrix.

In addition to the differentiation through induced EB, monolayer culture differentiation system has been explored (Figure 4). Huang et al. used 10 μ M all-*trans* retinoid acid to differentiate hESCs in monolayers. It was shown that > 93% of the cells expressed SMC-marker genes, such as SMMHC and α -SMA, and proteins and were able to contract (Huang et al., 2006). Combining GSK3 inhibition and BMP4 to commit hPSCs to mesodermal cells followed by platelet-derived growth factor two B subunits (PDGF-BB), SMCs can be generated > 80% efficiency within 6 days (Patsch et al., 2015).

Yang et al. (2016) used iPSCs and ESCs from different sources to obtain contractile and synthetic SMC by monolayer cell culture. CHIR99021 and BMP4 were used to induce mesodermal lineage from hPSCs followed by VEGF-A and TGF- β treatment to induce vascular progenitors. Differentiation medium was switched to PDGF- β and TGF- β either on day 7 or 10 to induce contractile or synthetic SMCs. Contractile SMCs expressed higher levels of MHC11 and calponin and had stronger contraction activity, whereas synthetic SMCs expressed more collagen and had stronger proliferation activity. Both protocols converted ~45% of hPSCs to SMC phenotypes, and the purity could be increased to ~95% in 4 mM lactate acid in RPMI1640 metabolic medium.

It seems that EB-mediated SMC differentiation can reach similar differentiation efficiency as monolayer differentiation by modulating WNT, PDGF- β , and TGF- β pathways. They may represent SMCs with different lineage background (Majesky, 2007; Sinha et al., 2014). Thus, it is necessary to identify signature markers in SMCs from different lineage and define the lineage specification of SMCs differentiated from protocols. This helps to apply hPSC-SMCs in understanding vascular development in embryo and use disease-specific hPSC-SMCs in disease modeling and drug screening.

DIFFERENTIATION OF hPSCs INTO CPCs

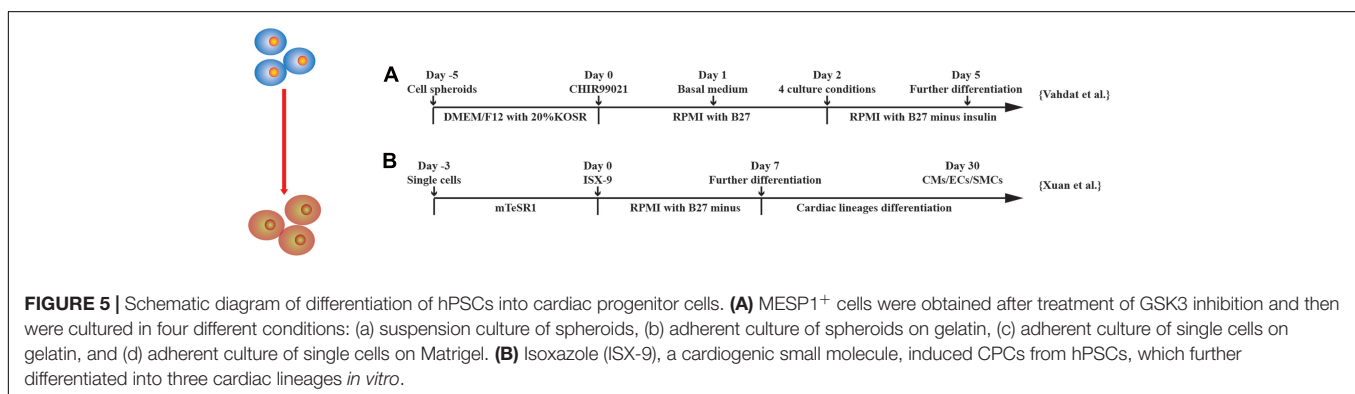
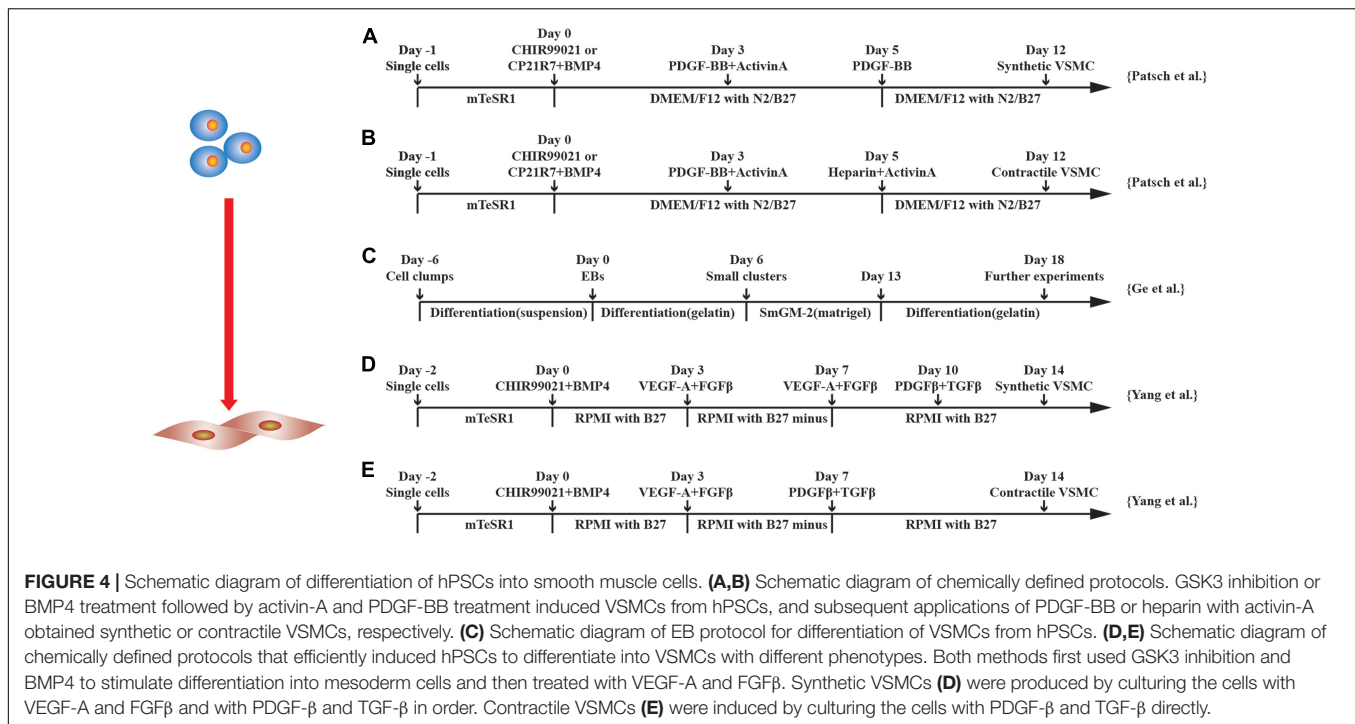
Recently, induction of CPCs is gaining attention as they are able to self-renew and predetermined to differentiate into cardiac lineage cells *in vitro* and *in vivo*. This saves time and is

cost-effective as compared to derive CMs, ECs, and SMCs *in vitro* and further transplantation *in vivo*. Various CPC markers, such as stage-specific embryonic antigen 1 (SSEA-1) (Bellamy et al., 2015), MESP1 (Bondue et al., 2008), and Nkx2.5 (Birket et al., 2015), have been investigated (Figure 5).

hPSCs treated with BMP2 give rise to an early population of cardiovascular progenitors, characterized by SSEA-1 (Brade et al., 2013; Bellamy et al., 2015). This progenitor population was multipotential and able to generate CMs, SMCs, and ECs *in vitro*. When purified SSEA-1⁺ progenitors implanted into non-human primates (NHPs), they differentiated into ventricular CMs. However, non-purified SSEA-1⁺ progenitor cell implantation resulted in teratomas in the scar tissue.

Cardiogenic mesodermal cells (CMCs) expressing MESP1 have been shown to differentiate into almost all cardiac cell types both *in vitro* and *in vivo* (Bondue et al., 2008; Brade et al., 2013; Den Hartogh et al., 2015; Lescroart et al., 2018). To monitor early cardiac mesoderm in hPSCs, a dual MESP1 (mCherry/w)–NKX2-5 (eGFP/w) reporter line was developed in hESCs (Den Hartogh et al., 2015). Induction of cardiac differentiation in this reporter line resulted in transient expression of MESP1-mCherry, followed by expression of NKX2.5-eGFP. MESP1-mCherry cells showed increased expression of mesodermal markers. Whole-genome microarray profiling and fluorescence-activated cell sorting analysis of MESP1-mCherry cells showed enrichment for mesodermal progenitor cell surface markers, such as PDGFR α , CD13, and ROR-2. MESP1-mCherry derivatives contained an enriched percentage of Nkx2.5-eGFP and CMs, SMCs, and ECs.

Vahdat et al. (2019a; 2019b) established a protocol for maintenance and large-scale expansion of early CPCs, so-called CMCs in a defined culture system. Through chemical screening, they developed a medium containing three factors, A83-01, bFGF, and CHIR99021, which generated CMCs expressing cardiac mesoderm markers and cardiac-specific transcription factors *MESP1*, *SSEA1*, *ISL1*, *PDGFR α* , *NKX2.5*, and *MEF2c*; 10¹⁴ CMCs were generated after 10 passages and were able to differentiate into CMs, ECs, and SMCs *in vitro*. To monitor CPC derivation, selection, and maintenance, Birket et al. (2015) engineered hPSCs to carry a cardiac lineage reporter to enable robust expansion of MYC expression primitive pre-NKX2.5⁺CPCs. Through regulation of FGF and BMP signaling, NKX2.5⁺CPCs can be differentiated into ventricular-like cells, pacemaker-like cells, ECs, and SMCs. Yap et al. (2019) developed a chemically defined, xeno-free, laminin-based differentiation protocol to generate CPCs from hESCs. Laminin-221, an abundant laminin isoform in heart extracellular matrix, induced a transcriptomic signature with up-regulated markers for cardiac development. CPCs appeared on day 9 or 11 of differentiation and highly expressed *ISL1*, *TBX5*, *MEF2C*, *C-KIT*, and *GATA3*. Single-cell RNA sequencing of CPCs identified three main progenitor subpopulations, including CMs, SMCs, and small population of epithelial cells. The CPCs generated human heart muscle bundles in mouse heart post-ischemia/reperfusion (I/R) injury. Uosaki et al. (2012) showed that coaggregation of endodermal cell line End2 with hESCs significantly promoted the induction of KDR⁺PDGFR α ⁺CPCs, suggesting a direct contact with endoderm-like cells can induce cardiac progenitors from hPSCs.



Bylund et al. (2017) found that BMP antagonist GREMLIN 2 is linked to cardiogenesis. Inhibition of canonical BMP signaling followed by JNK pathway activation by GREM2 induced cardiac differentiation of hiPSCs. Furthermore, GREM2 promoted proliferation of CPCs.

Other factors have been shown to be effective in deriving CPCs. Xuan et al. (2018) demonstrated that hiPSCs treated with isoxazole 9 (ISX-9), a potent inducer of adult neural stem cell differentiation, for 3 days stimulated hiPSCs to become CPCs expressing NKX2.5, GATA4, ISL1, and MEF2C and were able to generate CMs, SMCs, and ECs *in vitro* and *in vivo*. ISX-9 activated multiple pathways including TGF-β-induced epithelial-mesenchymal transition signaling and canonical and non-canonical WNT signaling at different stages of cardiac differentiation. Cyclosporin-A, an immunosuppression drug, has been shown to stimulate differentiation of FLK1⁺ mesodermal cells into FLK1⁺/CXCR4⁺/VE-cadherin⁻ CPCs

and CMs (Fujiwara et al., 2011). The beating colonies from hiPSCs were increased approximately 4.3 times by addition of cyclosporin-A at mesoderm stage.

One feature associated with hPSC-derived CPCs is their great extracellular vesicle (EV) secretory profile (El Harane et al., 2018). EVs are rich in miRNAs, and most of the 16 highly abundant, evolutionarily conserved miRNAs are associated with tissue-repair pathways. *In vitro*, EV increased cell survival, cell proliferation, and EC migration and stimulated tube formation. *In vivo*, EV significantly improved cardiac function through decreased LV volume and increased LV ejection fraction.

Although CPCs are emerging as a better option as compared to CM transplantation, several issues need to be solved before it can be fully translated into clinic: (1) Purified versus non-purified. Non-purified CPCs have the risk to form teratoma after implantation. It is possible that early CPC population may contain pacemaker cells to behave as foci of automaticity and

cause arrhythmias. Thus, purified cell population is preferred. (2) Purification method. Except for SSEA1, MESP1, and NKX2.5 are intracellular markers; if we use genetically modified CPC based on MESP1 or *Nkx2.5* expression, there is a concern of safety issue. (3) Electrical coupling: CPCs are not CMs. Although they will develop into CM in heart eventually after implantation, the early developed CMs may cause electrical uncoupling leading to ventricular arrhythmia.

CARDIOMYOPLASTY IN LARGE ANIMAL MODELS USING hPSC-DERIVED CARDIOVASCULAR CELLS

The continuous improvement of differentiation efficiency of hPSCs has made large quantities of human CPC, CM, EC, and SMC reality. They are being tested as cell transfer therapy for cardiac repair not only in small, but also in large animal heart models of heart diseases (Kawamura et al., 2012, 2013; Xiong et al., 2012; Chong et al., 2014; Ye et al., 2014; Shiba et al., 2016; Gao et al., 2018; Ishigami et al., 2018; Zhu W. et al., 2018; Ishida et al., 2019; Romagnuolo et al., 2019) (Table 1).

Two large animal models, pig and NHP, have been used as preclinical models to investigate feasibility, efficacy, and safety of hiPSC-derived cardiac cells. Among them, more studies used pigs as the pig's heart is very similar to human's in terms of morphology, size, electrophysiology, and metabolic physiology (Lelovas et al., 2014). Comparatively, relatively fewer studies with NHPs have been reported. Although NHPs are more closely resembled to human anatomy, physiology, function, and metabolism (Cox et al., 2017) and are more valuable from an experimental perspective, they are not cost-effective and are associated with ethical issues (Zhu K. et al., 2018; Cong et al., 2019).

Transplantation of hPSC-CMs Only

Cardiac cells differentiated from hiPSCs have been either directly intramyocardially injected or applied epicardially using cells sheets or patches. In many studies, transplantation of hPSC-CMs into cardiovascular disease model animals could improve heart function and reduce the ventricular remodeling. Kawamura et al. (2012) generated hiPSC-CM sheets using 6-cm thermoresponsive dishes. The cell sheets were approximately 30- to 50 μ m thick. Eight hiPSC-CM sheets were implanted through median sternotomy with chronic MI. The transplanted hiPSC-CM sheets attenuated LV remodeling and increased neovascularization without teratoma formation. To enhance survival of hiPSC-CMs posttransplantation, the same group implanted hiPSC-CM sheets with an omentum to enhance blood supply to cell sheets (Kawamura et al., 2013). Histology showed the transplanted tissues contained abundant cTnT⁺ cells surrounded by vascular-rich structures. In addition, it has been found that the transplantation of hESC-CMs can promote remuscularization to a certain extent in both pig and NHP models (Chong et al., 2014; Romagnuolo et al., 2019).

Transplantation of Multilineage Cardiac Cells

In addition of CMs, VCs, including ECs and SMCs, differentiated from hESCs (hESC-VCs) or hiPSCs (hiPSC-VCs), have been investigated. Implantation of VCs promoted survival of ischemic cells, angiogenesis, and antiapoptotic effect through paracrine factors released. Implantation of hESC-VCs or hiPSC-VCs seeded in fibrin/thrombin patch alleviated LV contractile dysfunction and wall stress and improved myocardial energetics (Xiong et al., 2012) and attenuated the reduction of ATP utilization at infarct border zone (Xiong et al., 2013) in porcine heart model of I/R.

On this basis, researchers began to combine these different cells and transplant them into animal models together. Ye et al. (2014) implanted trilineage cardiac cells, including CMs, ECs, and SMCs, derived from hiPSCs in combination with a fibrin patch loaded with insulin growth factor into porcine heart post-acute I/R. The transplantation of trilineage cardiac cells makes the efficacy of cell therapy more comprehensive and effective: hiPSC-CMs regenerated CM, whereas hiPSC-VC improved donor and host CM viability and stimulated neovascularization. Molecular factors having antiapoptotic (angiogenin, angiopoietin, IL-6, matrix metalloproteinase-1, PDGF-BB, TIMP Metalloproteinase Inhibitor 1, urokinase receptor, and VEGF), promoting cell homing (IL-8, monocyte chemoattractant protein-1, Monocyte chemoattractant protein-3, matrix metalloproteinase-9), and inducing cell division (angiogenin, angiopoietin, PDGF-BB, VEGF) properties were identified in paracrine factors released by hiPSC-CMs and hiPSC-VCs.

Gao et al. (2018) manufactured human cardiac muscle patch (hCMP) using 4 million hiPSC-CMs, 2 million each of hiPSC-ECs and hiPSC-SMCs. They implanted two hCMPs into pig heart model of acute MI. The hCMP transplantation was associated with significant improvements in LV function; reduced cardiac apoptosis, infarct size, and myocardial wall stress; and reversed some MI-associated changes in sarcomeric regulatory protein phosphorylation. Ishigami et al. (2018) generated cardiac tissue sheets using simultaneously induced hiPSC-CMs and hiPSC-VCs in temperature-responsive culture dishes. They transplanted four cardiac tissue sheets on the epicardium of infarcted myocardium in a porcine model of chronic MI. Transplantation resulted in significant increases of circumference strain and capillary density and reduction of fibrotic tissue in infarct and border regions after transplantation.

Limitations and Improvements in Transplantation Treatment of Animal Models

Histological analysis revealed that only a few implanted hiPSC-CMs survived at week 8 after implantation. Thus, the improved cardiac function was achieved mainly through the paracrine factors instead of regeneration of CMs (Kawamura et al., 2012). Therefore, the recovery of heart function involves many aspects, and it is necessary to use multilineage cell transplantation to improve blood vessel supply, inflammation regulation, and metabolism.

TABLE 1 | Cellular cardiomyoplasty in large animal models using hPSC-derived cardiovascular cells.

Animal model	Cell type	Cell number	Teratoma	Arrhythmia	
Pig model of acute ischemia/reperfusion	hiPSC-CMs	2.0×10^6	N.A.	Not detected	Ye et al., 2014
	hiPSC-ECs	2.0×10^6			
	hiPSC-SMCs	2.0×10^6			
Pig model of acute ischemia/reperfusion	hiPSC-CMs	4.0×10^6	N.A.	Not detected	Gao et al., 2018
	hiPSC-ECs	2.0×10^6			
	hiPSC-SMCs	2.0×10^6			
Pig model of acute ischemia/reperfusion	hESC-ECs	2.0×10^6	N.A.	During surgery	Xiong et al., 2012
	hESC-SMCs	2.0×10^6			
Pig model of chronic ischemia	hiPSC-CMs	2.5×10^7	Not detected	N.A.	Kawamura et al., 2012
Pig model of chronic myocardial infarction	hiPSC-CMs	1.0×10^7	Not detected	Not detected	Ishigami et al., 2018
	hiPSC-ECs				
	hiPSC-VMCs				
Pig model of chronic myocardial infarction	hiPSC-CMs	1.0×10^8	N.A.	N.A.	Ishida et al., 2019
Monkey model of chronic ischemia	hESC-CMs	1.0×10^9	Not detected	Within 24 h after delivery	Chong et al., 2014
Monkey model of chronic ischemia	hiPSC-CMs	4.0×10^8	N.A.	Within 4 weeks after delivery	Shiba et al., 2016

N.A. means that there was no relevant data or information in the article.

To improve cell engraftment and reduce immunogenicity of allogeneic iPSC-CMs, Kawamura et al. (2016) injected allogeneic monkey iPSC-CMs into MHC-matched or non-matched NHPs. The transplantation of allogeneic iPSC-CMs in MHC-matched NHP had increased cell engraftment with less immune-cell infiltration. Shiba et al. (2016) injected 4×10^8 major histocompatibility complex (MHC) matched allogeneic iPSC-CMs into NHPs post-chronic MI. Transplantation of the iPSC-CMs improved heart contractile function at 4 and 12 weeks posttransplantation. Although electrical coupling was established between donor and host CMs as assessed by use of the fluorescent calcium indicator G-CaMP7.09, the incidence of ventricular tachycardia was transiently, but significantly, increased when compared to control animal group. Furthermore, no macroscopic or microscopic tumor formation was detected. Wang et al. (2019) determined the efficacy of hESC-derived CPCs in NHPs. They found that implantation of hESC-CPCs into acutely infarcted myocardium significantly ameliorated the functional worsening and scar formation, concomitantly with reduced inflammatory reactions and CM apoptosis, as well as increased vascularization. Moreover, hESC-CPCs modulated cardiac macrophages toward a reparative phenotype in the infarcted hearts.

CHALLENGES OF hPSC TECHNOLOGY IN THE TREATMENT OF CARDIOVASCULAR DISEASES

Cell Quality

Although various protocols have been developed to induce hPSCs into CPCs, CMs, ECs, and SMCs, which have been extensively evaluated in small and large animal models of heart diseases,

there is a lack of commonly accepted standards to evaluate and control the quality of hPSC-derived cardiac cells. This especially applies to hiPSC-derived cardiovascular cells, as the reprogramming may change the genetic stability, and epigenetic memory may compromise therapeutic outcome.

Immunogenicity of hPSCs and Their Derivatives

The second issue is related to the immunogenicity of hPSCs. It has been shown that hESCs have low expression of MHCI and complete absence of MHCII antigens and costimulatory molecules, such as CD80 and CD86 (Li et al., 2004; Wu et al., 2008). The expression levels of the above molecules in hiPSCs are almost same as those in hESCs (Suarez-Alvarez et al., 2010). Thus, hPSCs may possess immune privilege property. However, increased MHC expression and immunogenicity have been documented after differentiation (Swijnenburg et al., 2005; Suarez-Alvarez et al., 2010). Although immunosuppressive drug regimens can be used to suppress recipients' immune response to transplanted allogeneic hPSC-derived cells, optimal dose and combination of different drugs to achieve minimal drug toxicity are still far from optimization. Universal hESC or hiPSC cell lines, which have human leukocyte antigen (HLA) class I (HLA-I) and II (HLA-II) knock-out (Han et al., 2019; Xu et al., 2019; Wang X. et al., 2020), may be a solution. HLA-I and HLA-II knockout hiPSCs can generate immunocompatible and ready-to-use cardiovascular cells.

Defects of hiPSCs Derivatives

The third issue is specifically related to hiPSCs. Although hiPSCs can differentiate into "personalized" patient-specific cells and

tissues to circumvent both immunogenicity barriers, they may have limited therapeutic potential if they are reprogrammed from patients with diseases caused by genetic mutations. Again, derivatives of universal hESCs or hiPSCs will be a good option for allogeneic transplantation.

Optimal Cell Types and Numbers for Cardiac Repair or Regeneration

The fourth issue is associated with cell type and cell dosing. Currently, most studies determined the efficacy of one cell type, either hPSC-CMs or CPCs, whereas only a few have compared different stage-specific cardiac cells. Thus, it is hard to provide unequivocal evidence for the superiority of one type over the other. In large animal heart models, transplanted hPSC-CM numbers ranged between 4×10^8 and 1×10^9 (Chong et al., 2014; Shiba et al., 2016; Liu et al., 2018; Romagnuolo et al., 2019). Although from a clinical perspective, a higher number of hPSC-CMs may be more

beneficial to cardiac function, a mixed cardiac cell population may be a cost-effective way as compared with pure CM transplantation. Genetic modification hPSC derivatives with genes to enhance their reparability may be a cost-effective option (Tao et al., 2020).

AUTHOR CONTRIBUTIONS

YG and JP conceived the design of the work. YG wrote the manuscript with support from JP. Both authors contributed to the article and approved the submitted version.

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Uncovering Inherited Cardiomyopathy With Human Induced Pluripotent Stem Cells

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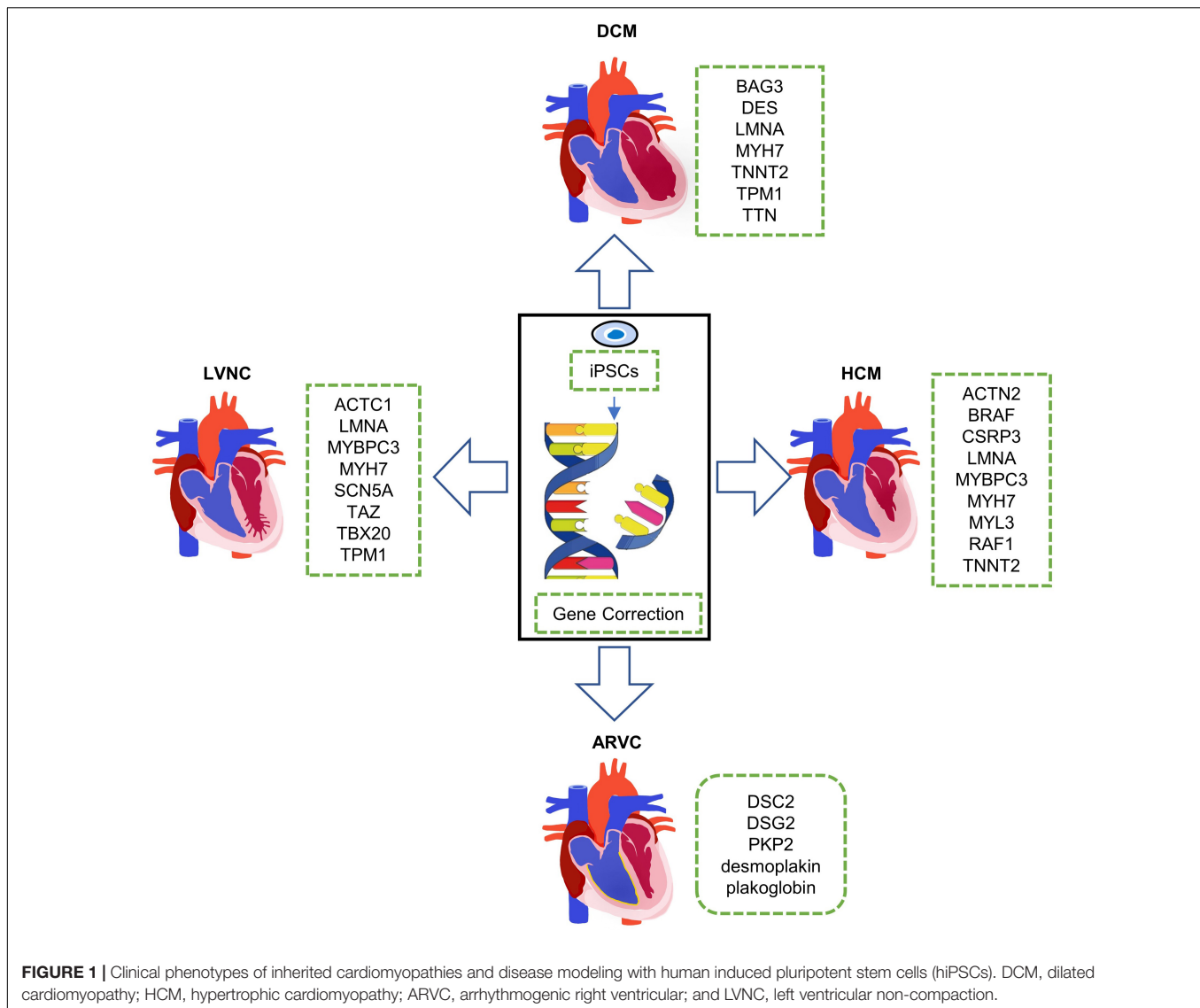
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In the past decades, researchers discovered the contribution of genetic defects to the pathogenesis of primary cardiomyopathy and tried to explain the pathogenesis of these diseases by establishing a variety of disease models. Although human heart tissues and primary cardiomyocytes have advantages in modeling human heart diseases, they are difficult to obtain and culture *in vitro*. Defects developed in genetically modified animal models are notably different from human diseases at the molecular level. The advent of human induced pluripotent stem cells (hiPSCs) provides an unprecedented opportunity to further investigate the pathogenic mechanisms of inherited cardiomyopathies *in vitro* using patient-specific hiPSC-derived cardiomyocytes. In this review, we will make a summary of recent advances in *in vitro* inherited cardiomyopathy modeling using hiPSCs.

Keywords: induced pluripotent stem cell, inherited cardiomyopathy, disease modeling, heart disease, pathogenic mechanism

INTRODUCTION

Inherited cardiomyopathy is a kind of myocardial disease with a range of different genetic disorders and epigenetic variations (Fatkin and Graham, 2002). Inherited cardiomyopathies mainly include dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), left ventricular non-compaction (LVNC), and arrhythmogenic right ventricular (ARVC) (Figure 1). Among these inherited cardiomyopathies, DCM is characterized by ventricular chamber dilatation and systolic dysfunction. It is usually related to mutations in genes such as *BAG3*, *LMNA*, *MYH7*, *TNNT2*, *TTN*, and so on. HCM is a disease with abnormal myocardial thickening. The cause of the disease is often closely related to the mutations of *ACTN2*, *BRAF*, *CSRP3*, *LMNA*, *MYL3*, and *TNNT2*, etc. Thickened myocardium makes it harder for the heart to pump blood. LVNC is characterized by the phenotype that the various tissue layers of the heart are not completely gathered together, resulting in a deep recess in the muscle wall. Patients with LVNC usually have symptoms of heart failure, arrhythmias, and thromboembolism and always accompanied by mutations in genes such as *ACTC1*, *MYBPC3*, *MYH7*, *SCN5A*, *TAZ*, *TBX20*, and so on. ARVC is a heart disease in which the right ventricular muscle is replaced by fat and/or scar tissue. With the aggravation of the degree, the right ventricle (RV) gradually loses its ability to pump blood, and it is mainly caused by mutations in genes such as *DSC2*, *PKP2*, and desmoplakin, etc. Patients with ARVC usually have arrhythmias and increased risk of cardiac arrest or death.



To reveal the pathogenesis of these genetic defects, a variety of disease models have been established. Human heart tissues and primary cardiomyocytes are the optimum choice undoubtedly, but they are difficult to obtain and culture *in vitro*, which hinders their application in cardiovascular research (Savla et al., 2014). Researchers then developed genetically modified animal models, among which mouse model is widely used. These animal models are crucial in progressing knowledge of the pathophysiology of heart disease because the basic principles of cardiac excitation and contraction are relatively conservative in the species. However, although animal models provide some useful results for the pathogenesis of cardiomyopathy, animals and humans are different at the molecular level, such as genetic background, epigenetic inheritance, transcriptional regulation, and protein modification. These, in turn, lead to significant differences between the functional and electrophysiological properties of the animal and human hearts, which limits the interpretation and applicability of these data (Ma et al., 2013). Therefore, the lack

of a good source of live human cardiomyocytes *in vitro* and the failure to establish variant patient-specific disease models have significantly stymied research on inherited cardiomyopathy. Fortunately, human induced pluripotent stem cells (hiPSCs) provided a promising platform for genetic disease modeling.

Human induced pluripotent stem cells can be reprogrammed from somatic cells, which are easily isolated from the skin, urine, blood, and more unexpected samples, by virus-dependent or independent overexpression of a set of reprogramming factors such as Oct3/4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). The hiPSCs can be indefinitely maintained and expanded in the pluripotent state *in vitro* and theoretically have the ability to generate any adult cell types (Kamp and Lyons, 2009). Through modulating BMP4 and WNT that are critical in heart development, hiPSCs are able to efficiently differentiate into cardiomyocytes (Qyang et al., 2007; Cao et al., 2013). Two major strategies have been developed for hiPSC-based inherited cardiomyopathy modeling: (1) reprogramming the

somatic cells from genetic disease patients into hiPSCs and then differentiating them into cardiomyocytes and (2) performing gene editing to introduce pathogenic mutations into hiPSCs of healthy donor and then differentiating them into cardiomyocytes. Three precise genome-editing techniques, including zinc finger nuclease (ZFN; Kim et al., 2010), transcription activator-like effector nuclease (TALEN; Schmid-Burgk et al., 2013), and clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISPR-Cas9; Wu et al., 2014), can be used to correct or introduce mutations in hiPSCs.

In this review, we will discuss the most common inherited cardiomyopathies abovementioned, including their genetic background and the work researchers have done so far using hiPSCs for mechanism research and symptom relief (Figure 1).

DILATED CARDIOMYOPATHY

Dilated cardiomyopathy is a common form of cardiomyopathy, which is characterized by ventricular chamber dilatation and systolic dysfunction (Maron et al., 2006). DCM contributes to hypertension, sudden cardiac death, progressive heart failure, and eventually leads to heart transplant (Wu et al., 2015). Recent studies have shown that cardiomyocytes from DCM patients often exhibit abnormal sarcomeric structure, which is likely due to pathogenic mutations in sarcomere protein-coding genes such as giant sarcomere protein titin (*TTN*), cardiac troponin T (*TNNT2*), tropomyosin 1 (*TPM1*), and desmin (*DES*; Fürst et al., 1988; Sehnert et al., 2002; Deacon et al., 2019). In recent years, the pathogenic genes of DCM and their pathological mechanism have been studied by establishing hiPSC-based cell models of DCM (Deacon et al., 2019).

Bearing significant roles in cardiac muscle, single *TTN* proteins extend from the Z-disk to the M-line, spanning the entire half-sarcomere length, and interact with the thick and thin filaments (Fürst et al., 1988). *TTN* truncating variants (TTNtv) are a major cause of genetic DCM, accounting for approximately 25% of familial cases (Herman et al., 2012). Hinson and his colleagues (Hinson et al., 2015) found that the TTNtv of DCM patients with *TTN* mutations in clinical practice are markedly enriched in the A-band. In order to understand the mechanism behind this phenotype, they generated patient-specific hiPSC-derived cardiomyocytes (hiPSC-CMs) with different *TTN* mutations, including two A-band TTNtvs (p.A22352fs or p.P22582fs) and one missense mutation (p.W976R) within the Z/I junction of *TTN* protein, as well as mutant isogenic hiPSC-CMs carrying CRISPR/Cas9-mediated TTNtvs in the I- or A-band exons. They observed considerable contractile dysfunction in both hiPSC-CM microtissues with I- or A-band TTNtvs, and attributed it to sarcomere insufficiency and aberrant responses to mechanical and adrenergic stress. However, previous studies revealed a tendency for *TTN* truncations in DCM to distribute to the A-band, while I-band TTNtvs are better tolerated and have been observed in healthy individuals (Herman et al., 2012; Roberts et al., 2015). Basing on RNA splicing analyses, Hinson and his colleagues further observed extraordinarily high rates of I-band exon incorporation into

TTN transcripts in hiPSC-CMs (88% of *TTN* transcripts), five times more than that in adult left ventricle tissue (18% of *TTN* transcripts; Hinson et al., 2015). Thus, alternative RNA splicing-mediated elimination of I-band exon in adult cardiomyocytes might be a major mechanism for low incidence of I-band TTNtvs in DCM patients.

Cardiac troponin T, encoded by *TNNT2*, is another cardiac-specific sarcomere protein that involves in sarcomere assembly and cardiomyocyte contraction. Mutations of *TNNT2* could cause abnormal calcium ion dynamics and sarcomeric α -actinin, decreased contractility, and affect β -adrenergic signaling of cardiomyocytes. *TNNT2* mutation is also the leading cause of heart failure and sudden death in young athletes (Sehnert et al., 2002). By using patient-specific hiPSC model, Sun et al. (2012) demonstrated that hiPSC-CMs carrying *TNNT2* p.R173W mutation could partially recapitulate the disease phenotype of DCM, as indicated in altered regulation of calcium ion (Ca^{2+}), decreased contractility, and abnormal distribution of sarcomeric α -actinin. Interestingly, metoprolol, a β_1 -selective β -blocker showing beneficial effect on the clinical symptoms of DCM patients, could improve the sarcomeric organization of DCM hiPSC-CMs (Sun et al., 2012). On this basis, Wu et al. found that p.R173W mutation could promote the nuclear localization of *TNNT2*, and alter the epigenetic regulation of key β -adrenergic signaling genes in DCM hiPSC-CMs (Wu et al., 2015). These studies provide novel targets for the treatment of DCM caused by *TNNT2* p.R173W mutation.

Bcl2-associated athanogene 3 (*BAG3*) encodes an anti-apoptotic co-chaperone protein, which is most highly expressed in skeletal and cardiac muscle and localizing to the sarcomeric Z-disk (Homma et al., 2006). Mutations in *BAG3* have been associated with DCM and muscular dystrophy (McDermott-Roe et al., 2019). However, heterozygous *BAG3* knockout mice failed to recapitulate the clinical phenotypes of heterozygous *BAG3* mutation-related cardiac disease (Homma et al., 2006). Two groups have explored the effects of DCM-related *BAG3* deficiency in cardiomyocytes, by using genome-editing approach to create both heterozygous and homozygous mutations in *BAG3* gene (Judge et al., 2017; McDermott-Roe et al., 2019). Despite the inconsistent observations at baseline, both groups demonstrated more serious fiber disarray in *BAG3*-deficient hiPSC-CMs under proteasome inhibition-induced proteotoxic stress. Consistently, nonsense mutations in *BAG3* locus have been shown to cause *BAG3* haploinsufficiency and disengagement of the *BAG3*-coordinated cardioprotective chaperone complex in hiPSC-CMs (Judge et al., 2017), which might be the pathologic mechanism of DCM-associated *BAG3* mutation. McDermott-Roe et al. demonstrated that stimulating the HSF1 (heat shock factor 1)-driven stress response pathway can reduce fiber disorder mediated by proteasome inhibition in hiPSC-CMs with *BAG3* p.R477H mutation (McDermott-Roe et al., 2019). This may help clinical treatment of DCM patients carrying *BAG3* mutations.

Some other sarcomeric genes have also been involved in DCM. Deacon et al. (2019), through the establishment of hiPSC-CMs containing both sarcomeric gene *TPM1* mutation (p.E33K) and the costameric gene *vinculin* mutation (p.N220fs), demonstrated that composite genetic variants can combine and interact to

induce DCM, especially when suffering from other disease-causing stressors. An unfamiliar heterozygous mutation of the desmin (*DES*), A285V mutation, was recently identified in DCM by Tse and colleagues through whole-exome sequencing (WES; Tse et al., 2013). They found that this mutation could cause three-dimensional structure changes of *DES* and then induce abnormal *DES* aggregations in hiPSC-CMs. Moreover, the mutation in β -myosin heavy chain 7 (*MYH7*) gene, which encodes myosin heavy chain (β -MHC), can also induce DCM. Yang et al. (2018) established an hiPSC-CMs line with *MYH7* mutation (p.E848G), and demonstrated that the reduced contractile function of single cells and engineered heart tissues with *MYH7* mutation (p.E848G) is due to the damaged interaction between *MYH7* and cardiac myosin-binding protein C.

In addition to mutations in sarcomere-related genes, mutations in nuclear membrane proteins can also cause DCM. The lamin A/C gene (*LMNA*) encodes two major nuclear lamina proteins lamins A and C through alternative splicing. Many *LMNA* mutations, including p.R225X, p.S143P, and p.K117fs, have been identified in familial DCM patients (Siu et al., 2012; Lee et al., 2019; Shah et al., 2019). The most common features of hiPSC-CMs derived from *LMNA* mutant DCM patients are arrhythmia, abnormal calcium handling, and increased sensitivity to electric and hypoxia stimulation (Shah et al., 2019). Many reports suggest that *LMNA* mutation-caused cardiac pathology is related to the activation of the PDGF pathway, and the symptoms caused by *LMNA* mutations can be moderated by the platelet-derived growth factor receptor β (PDGFRB) inhibitors and statins (Siu et al., 2012; Lee et al., 2019; Shah et al., 2019). Siu et al. (2012) demonstrated that under electrical stimulation, hiPSC-CMs carrying R225X or *LMNA* frameshift mutation present nuclear bleb formation and micronucleation. In the meanwhile, they found that the pro-apoptotic effects of field electric stimulation could be markedly attenuated by pharmacological blockade of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway with U0126 and selumetinib (AZD6244; Siu et al., 2012).

Consistent with clinical disease manifestations, hiPSC-CMs with DCM mutations often exhibit phenotypes such as altered regulation of calcium ion and abnormal myocardial sarcomere structure. The advantage of modeling diseases through hiPSCs is that they cannot only summarize the disease phenotype but also its pathogenesis, which will greatly promote and deepen our understanding of the underlying mechanisms of DCM.

HYPERTROPHIC CARDIOMYOPATHY

Hypertrophic cardiomyopathy is a disease of the cardiac sarcomere and tend to link to arrhythmia, electrophysiological abnormalities, and sudden cardiac death (Ramachandra et al., 2021). Practically, about 50% of the cases are caused by mutations either in *MYH7*, encoding β -myosin heavy chain, or in *MYBPC3*, encoding cardiac myosin-binding protein C (cMyBP-C; Birket et al., 2015), both of which are sarcomeric protein-encoding genes. Recently, researchers have also used hiPSCs to

conduct HCM research (Prajapati et al., 2018; Tang et al., 2019; Ramachandra et al., 2021).

The *MYH7* is an important part of myosin and is related to contractile capacity of cells (Fontaine et al., 2021; Li et al., 2021). Studies have found that hiPSC-CMs carrying a missense mutation (p.R663H) in *MYH7* displayed the phenotype of cellular enlargement, contractile arrhythmia at the single-cell level, and dysregulation of Ca^{2+} cycling and elevation in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$). The main cause of these defects is the continuous increase in $[\text{Ca}^{2+}]_i$, which leads to the activation of calcineurin that further dephosphorylates NFAT3 transcription factors, allowing them to translocate to the nucleus and interact directly with classical transcript factors such as GATA4 and MEF2 (Lan et al., 2013). In hiPSC-CMs carrying p.R442G mutation in *MYH7*, Han et al. (2014) observed sarcomere disorders, indicating the reduction of contractility in HCM hiPSC-CMs and an abnormal Ca^{2+} handling. Treatment of verapamil, a calcium channel blocker, successfully alleviated calcium handling abnormalities and arrhythmia in HCM hiPSC-CMs with *MYH7* p.R663H or p.R442G mutations (Lan et al., 2013; Han et al., 2014). In addition, some Na^+ channel blocker such as lidocaine, mexiletine, and ranolazine, but not drugs targeting K^+ channels, are also able to restore normal beat frequency in HCM hiPSC-CMs (Lan et al., 2013). These evidences provide some theoretical basis for the treatment of HCM caused by *MYH7* mutation.

The mutation of *MYBPC3*, encoding a thick filament contractile protein that regulates sarcomere organization and cardiac contractility, is another important cause of HCM, which in turn causes damage to the contractile ability of cardiomyocytes and ultimately leads to HCM (Jin et al., 2020). Birket et al. (2015) found that the amount of MyBPC3 protein was reduced by nearly half in *MYBPC3*-mutant (c.2373dupG) hiPSC-CMs when compared with the normal level. Due to the haploinsufficiency, the maximum Ca^{2+} -activated force markedly decreased. Premature termination codon (PTC) is another common mutation form of *MYBPC3* that causes HCM. Seeger et al. found that PTC mutation led to nonsense-mediated decay (NMD) and ubiquitin-proteasome system (UPS), leading to haploinsufficiency of healthy alleles and ultimately to HCM. Inhibition of the NMD pathway in HCM hiPSC-CMs carrying *MYBPC3* (p.R943X) mutation resulted in the restoration of molecular phenotypes and calcium-processing kinetics (Seeger et al., 2019). These studies have shown that when dealing with HCM caused by mutations in the *MYBPC3* gene, targeting the NMD pathway might be effective.

In addition to sarcomeric genes, mutations in some genes related to cell signaling pathways can also cause HCM. The BRAF protein is a RAF family serine/threonine protein kinase involved in RAS/MAPK signaling. Researchers have found that its mutations can cause pro-fibrotic phenotype, enhanced susceptibility to arrhythmias, aberrant MEK1/2 signaling and sarcomere disorders, and eventually lead to HCM (Cashman et al., 2016; Josowitz et al., 2016; Jaffré et al., 2019). Josowitz et al. used hiPSC-CMs to construct disease

models carrying *BRAF* p.T599R or p.Q257R mutations. They sorted out *SIRPα*⁺/*CD90*[−] and *SIRPα*[−]/*CD90*⁺ cardiomyocytes so as to examine hiPSC-derived cell type-specific phenotypes and cellular interactions underpinning HCM. They found that *BRAF*-mutant *SIRPα*⁺/*CD90*[−] cardiomyocytes displayed the phenotype of HCM, and *BRAF*-mutant *SIRPα*[−]/*CD90*⁺ cells exhibited a pro-fibrotic phenotype and partially modulated cardiomyocyte hypertrophy through transforming growth factor (TGF) paracrine signaling. These symptoms can be relieved by inhibition of TGFβ or RAS/MAPK signaling (Josowitz et al., 2016). Furthermore, hiPSCs from a patient with cardio-facio-cutaneous syndrome were also used to develop the 3D human-engineered cardiac tissue model of *BRAF* p.T599R mutation-induced HCM. This model reflects an enhanced susceptibility to arrhythmias (Cashman et al., 2016). The *RAF1* p.S257L mutation also demonstrated potential to cause cell-autonomous hypertrophy and myofibrillar disarray in hiPSC-CMs, through activation of ERK5 and MEK1/2 signaling, respectively, (Jaffré et al., 2019).

Moreover, researchers have also established disease models through hiPSCs to study HCM caused by mutations in genes such as SCO cytochrome oxidase-deficient homolog 2 (*SCO2*), *CSR3P3*, which encodes muscle LIM protein, and *ACTN2*, encoding α-actinin 2 (Hallas et al., 2018; Li et al., 2019; Prondzynski et al., 2019), which are also some common genetic mutations that cause HCM. These mutations can, respectively, result in reduced mitochondrial oxidative ATP production capacity, multinucleation, disorganized sarcomeric ultrastructure, and higher myofilament Ca²⁺ sensitivity (Hallas et al., 2018; Li et al., 2019; Prondzynski et al., 2019).

Diastolic dysfunction (DD) is commonly diagnosed in HCM patients, and is a major cause of morbidity and mortality among HCM patients. Wu et al. (2019) generated four hiPSC-CM models of DD that carry different mutations in sarcomeric proteins, including *MYH7* (p.R663H), *MYBPC3* (p.V321M), *MYBPC3* (p.V219L), and *TNNT2* (p.R92W). These human hiPSC-CM models recapitulated the disease phenotype of DD in HCM, as evidenced by shortened diastolic sarcomere length, decreased maximum relaxation rate, and prolonged relaxation duration. Both diastolic Ca²⁺ overload and enhanced myofilament Ca²⁺ sensitivity are involved in impaired diastolic function in HCM hiPSC-CMs, which can be alleviated by treatment with partial Ca²⁺ or late Na⁺ current inhibitors (Wu et al., 2019).

On the one hand, the above research shows that hiPSC-CMs with HCM pathogenic genes often have the characteristics of cellular enlargement, contractile arrhythmia, and disorganized sarcomeric, which are consistent with their arrhythmia and electrophysiological abnormalities in diseased organs. On the other, these studies point out the direction for further exploring the possible pathogenesis of HCM and provide a theoretical basis for the rational formulation of clinical protocols. The hiPSC-CM-based HCM studies have shown that abnormal calcium homeostasis is an important factor leading to inherited HCM. The development of drugs that inhibit Ca²⁺ and Na⁺ channels may provide a direction for HCM treatment.

LEFT VENTRICULAR NON-COMPACTION

Left ventricular non-compaction is caused by an arrest of normal endomyocardial morphogenesis. Patients with LVNC often have symptoms of heart failure, arrhythmias, and thromboembolism. LVNC is mainly related to mutations in genes encoding taffazin (*TAZ*), lamin A/C (*LMNA*), sodium channel type Vα (*SCN5A*), *MYH7*, *MYBPC3*, α-cardiac actin 1 (*ACTC1*), and α-tropomyosin (*TPM1*). Among them, researchers have established hiPSC-CMs models of *ACTC1*, *TPM1*, and *TBX20* genes with mutations to study the pathological mechanisms of LVNC.

Among them, *ACTC1* and *TPM1* are genes encoding sarcomere proteins. To elucidate the pathological role, hiPSCs from a patient carrying a E99K-*ACTC1* (α-cardiac actin 1) mutation has been developed and studied (Smith et al., 2018). Smith et al., through the study of two different training methods, 3D and 2D, found that E99K-*ACTC1* hiPSC-CMs have obviously arrhythmogenesis in both kinds of models, that is because of the abnormal Ca²⁺ handling, such as prolonged Ca²⁺ transients (Smith et al., 2018). Takasaki et al. found that hiPSC-CMs carrying a p.R178H mutation in *TPM1* exhibited obvious pathological changes, such as disturbed sarcomere structure and impaired calcium handling in cardiomyocytes. Microarray analysis showed that *TPM1* mutations lead to down-regulated expression of many genes involved in heart development and beneficial regulation cellular processes, particularly the calcium signaling pathway (Takasaki et al., 2018).

In addition, researchers have also studied many other mutations associated with LVNC by using patient-specific hiPSC-CMs. LVNC hiPSC-CMs carrying p.T262M mutation and stop-gain mutation p.Y317* in *TBX20* (T-box transcription factor 20) possess a defect of proliferative capacity due to abnormal activation of TGF-β signaling. Compared with mild DCM hiPSC-CMs, LVNC hiPSC-CMs have a significantly higher expression of TGF-β signaling pathway-related genes, while the expression of PRDM16, the repressor of TGF-β signaling, was significantly down-regulated in all LVNC hiPSC-CMs. These data indicate that the abnormal activation of TGF-β signaling by *TBX20* mutation is associated with the proliferation defect of LVNC hiPSC-CMs (Kodo et al., 2016). These studies provide more possibilities for the follow-up investigators' work and lay the foundation for the clinical treatment of LVNC.

ARRHYTHMOGENIC RIGHT VENTRICULAR

Arrhythmogenic right ventricular is an inherited primary heart muscle disorder characterized by fibrofatty infiltration of the myocardium and cardiomyocyte loss basically in the RV that may lead to sudden cardiac death (Chen et al., 2020; Jahng et al., 2021). More than 50% of the ARVC patients had mutations in the desmosome genes including plakophilin 2 (*PKP2*), desmoglein 2 (*DSG2*), desmoplakin, and plakoglobin (Lombardi and Marian, 2010).

Among them, the most common mutations occur in the *PKP2* gene (Calkins and Marcus, 2008), which is important for desmosomal protein localization, lipid formation, β -catenin activity, and the activity of Wnt signaling pathway. For this reason, in recent years, researchers have begun to use hiPSCs to construct disease models of ARVC caused by *PKP2* mutations. In the beginning, Ma and his colleagues investigated the role of 1841T > C mutation of *PKP2* gene in hiPSC-CMs. The *PKP2* mutant hiPSC-CMs exhibit reduced localization of desmosomal proteins on the cell surface, enlarged cardiomyocytes, and a more lipid formation. They also found that after exposure to adipogenic differentiation medium for 2 weeks, the lipid content of ARVC-hiPSC-derived cardiomyocytes was significantly higher than that in the control group (Ma et al., 2013). In hiPSC-CMs with the c.2484C > T mutation of *PKP2*, Kim and his colleagues manifested abnormal nuclear translocation of plakoglobin and decreased β -catenin activity in cardiogenic conditions (Kim et al., 2013). However, these hiPSC-CMs cannot reproduce the pathological phenotypes of ARVC in standard cardiogenic conditions only if adjusting the cultivation conditions to mature them (Kim et al., 2013). Khudiakov et al. (2015) using hiPSC-CMs with two mutations (c.23321del.T, p.K859R) in the *PKP2* gene, found that the *PKP2* mutations could decrease the activity of Wnt signaling pathway during adipogenic and cardiomyogenic differentiation. To improve this hiPSC-CMs model, Wen et al. developed a method to induce metabolic maturation of hiPSC-CMs. Moreover, they found that coactivation of normal peroxisome proliferator-activated receptor (PPAR)- α and abnormal PPAR- γ pathways could stimulate the pathological signatures such as excessive CM adipogenesis, CM apoptosis, Na^+ channel downregulation, and intracellular calcium deficiency of ARVC in hiPSC-CMs carrying two mutations (c.2484C > T, c.2013delC) in *PKP2* (Wen et al., 2015). The abovementioned studies indicate that induction of adult-like metabolic energetics is essential to model a cardiac disease with patient-specific hiPSCs.

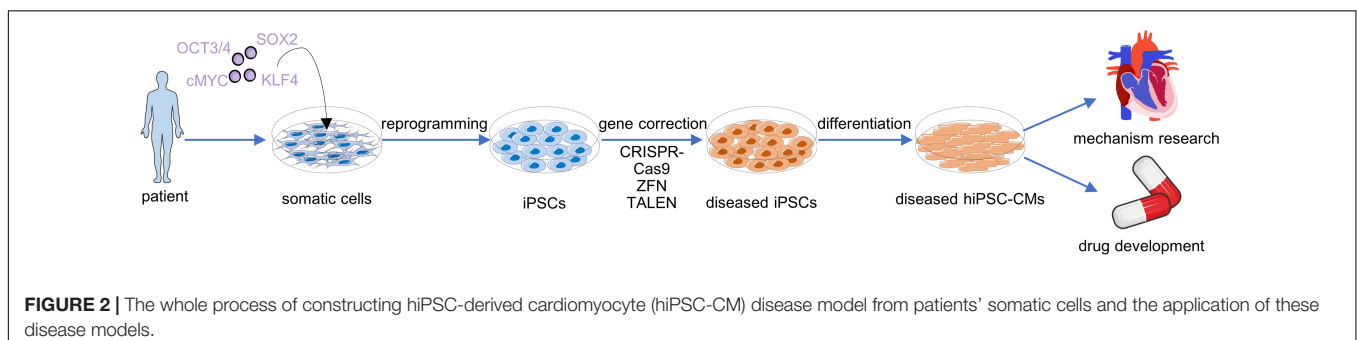
Besides, Padrón-Barthe et al. demonstrated the genetic mechanism of ARVC5, the most aggressive ARVC subtype, by using the hiPSC-CM model. ARVC5 is a devastating disease that is caused by a point mutation in *TMEM43*, a transmembrane protein that is located in the nuclear membrane. Their research demonstrated that the hiPSC-CMs with p.S358L mutation in *TMEM43* had an increased contraction duration, time to peak, and relaxation time in mutants, and these

contraction abnormalities could be relieved by inhibition of glucogen synthase kinase 3 β (Padrón-Barthe et al., 2019). In addition to mutations in desmosomal and nuclear membrane proteins that can cause ARVC, abnormalities in ion channels can also cause ARVC. Cardiomyocytes derived from *DSG2* mutant hiPSCs displayed multiple ion channel dysfunction, cellular electrophysiological abnormalities, and increased sensitivity to adrenergic stimulation, which may underlie ARVC patients' arrhythmias (El-Battrawy et al., 2018). In the above studies, it was found that hiPSC-CMs derived from patient's somatic cells showed various phenotypes such as enlarged cardiomyocytes, increased lipid formation, and abnormal cell contraction, which are related to the fibrofatty infiltration of the myocardium in ARVC patients. Therefore, patient-specific hiPSC-CMs have been shown to be beneficial in studying the pathogenesis of ARVC.

In addition to the abovementioned cardiomyopathies, researchers have also used iPSCs to study restrictive cardiomyopathy (RCM), a rare heart disease, which is usually caused by increased myocardial stiffness, which results in limited ventricular filling (Galea et al., 2020). Brodehl et al. constructed a disease model with p.Y122H mutation of *DSE* by using iPSCs and found that the mutant desmin with a severe filament assembly defect supports the pathogenicity of the RCM. By establishing a disease model of this mutation, they first classified the p.Y122H mutation of *DSE* as a novel pathogenic mutation (Brodehl et al., 2019).

OUTLOOK

As one of the increasingly serious problems of the 21st century, cardiomyopathy still has many difficulties waiting for us to overcome, for example, the pathogenesis of many genetic diseases has not yet been clarified, and the corresponding treatment options have not been studied. As we described above, hiPSC technology is a powerful means that can be used (Figure 2). Through this technology, researchers have established various disease models of cardiomyopathy, such as HCM, DCM, ARVC, and LVNC, and studied the mechanisms of pathogenesis caused by mutations of different genes. Therefore, hiPSC-based cardiovascular disease models provide more possibilities for the follow-up investigators' work and will greatly promote and deepen our understanding of the underlying mechanisms of cardiomyopathy. In addition to these 2D disease models,



researchers have also constructed 3D organoid models to simulate real human organs and relieve the limitations of spatial factors on disease models (Giacomelli et al., 2020). In addition, iPSC-CMs is also widely used in drug screening (Ito et al., 2020; Li et al., 2020), and iPSC-CM and its derivatives are also used to treat heart failure (Nakamura and Murry, 2019). These all lay the foundation for the clinical treatment of cardiac disease.

However, at present, hiPSC-CMs generally have the problem of immaturity. Mature and immature cells have huge differences in their cell morphology, number of mitochondria, sarcomere structure, calcium transient, and electrophysiology, etc. In addition, the maturation of cardiomyocytes will bring more detailed subtypes, such as ventricular myocardium, atrial myocardium, and sinoatrial node cells, etc. All of these have a significant impact on our research on specific diseases. If the subtypes of differentiated and mature cardiomyocytes can be obtained, we can make more detailed and precise studies on the physiological processes of diseases. Besides, the cellular and molecular crosstalk between cardiac cell lineages is difficult to model by using only hiPSC-derived cardiomyocytes in a two-dimensional model. Therefore, we should use hiPSCs to differentiate a variety of cardiac cells, such as endothelial cells, and construct a three-dimensional model of the cardiac cells to better simulate the overall changes in the heart and the interaction between cells when the disease occurs.

In summary, hiPSC technology will not only completely alter our understanding of diseases but also will eventually change the future of medical practice and make personalized medicine possible.

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XJ, YC, and XL mainly wrote the manuscript. LY, MY, and ZS revised the manuscript. WL and SH wrote and revised the manuscript, as well as provided the funding. All authors contributed to the article and approved the submitted version.

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Cardiac Mesenchymal Cells Cultured at Physiologic Oxygen Tension Have Superior Therapeutic Efficacy in Heart Failure Caused by Myocardial Infarction

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Stem/progenitor cells are usually cultured at atmospheric O₂ tension (21%); however, since physiologic O₂ tension in the heart is ~5%, using 21% O₂ may cause oxidative stress and toxicity. Cardiac mesenchymal cells (CMCs), a newly discovered and promising type of progenitor cells, are effective in improving left ventricle (LV) function after myocardial infarction (MI). We have previously shown that, compared with 21% O₂, culture at 5% O₂ increases CMC proliferation, telomerase activity, telomere length, and resistance to severe hypoxia *in vitro*. However, it is unknown whether these beneficial effects of 5% O₂ *in vitro* translate into greater therapeutic efficacy *in vivo* in the treatment of heart failure. Thus, murine CMCs were cultured at 21% or 5% O₂. Mice with heart failure caused by a 60-min coronary occlusion followed by 30 days of reperfusion received vehicle, 21% or 5% O₂ CMCs via echocardiography-guided intraventricular injection. After 35 days, the improvement in LV ejection fraction effected by 5% O₂ CMCs was > 3 times greater than that afforded by 21% O₂ CMCs (5.2 vs. 1.5 units, *P* < 0.01). Hemodynamic studies (Millar catheter) yielded similar results both for load-dependent (LV dP/dt) and load-independent (end-systolic elastance) indices. Thus, two independent approaches (echo and hemodynamics) demonstrated the therapeutic superiority of 5% O₂ CMCs. Further, 5% O₂ CMCs, but not 21% O₂ CMCs, significantly decreased scar size, increased viable myocardium, reduced LV hypertrophy and dilatation, and limited myocardial fibrosis both in the risk and non-infarcted regions. Taken together, these results show, for the first time, that culturing CMCs at physiologic (5%) O₂ tension provides superior therapeutic efficacy in promoting cardiac repair *in vivo*. This concept may enhance the therapeutic potential of CMCs. Further, culture at 5% O₂ enables greater numbers of cells to be produced in a shorter time, thereby reducing costs and effort and limiting cell senescence. Thus, the present study has potentially vast implications for the field of cell therapy.

Keywords: stem cells, heart failure, myocardial infarction, cardiac function, cardiac repair, cardiac mesenchymal cells, physiologic oxygen tension

INTRODUCTION

Cell therapy is emerging as a potentially useful approach to the treatment of heart failure. Cardiac mesenchymal cells (CMCs), a newly discovered and promising type of progenitor cells recently isolated from the heart of mice, rats, and humans, have shown significant efficacy in improving cardiac function in mice and rats that underwent myocardial infarction (MI) resulting in heart failure (Guo et al., 2017; Wysoczynski et al., 2017). In addition to their efficacy, CMCs offer several advantages over other cell types, including the fact that their isolation and expansion are considerably simpler and less expensive than other cardiac-derived cells (Wysoczynski et al., 2017).

Since the establishment of the first cell line culture for mouse fibroblasts in 1943 (Earle et al., 1943), cell culture at normoxic conditions (atmospheric oxygen tension, 21%) has been the standard method used by almost all investigators that use stem or progenitor cells in preclinical and in clinical studies. However, physiologic oxygen tension in living organs is only 3–12% (Mas-Bargues et al., 2019); in the heart, it is ~5% (Roy et al., 2003). This raises the concern that culturing cells at 21% O₂ may cause toxicity due to oxidative stress. Furthermore, cells grown at 21% O₂ may not survive well in the microenvironment of the heart where oxygen tension is 5%, and particularly in the scarred regions where O₂ is very low. Indeed, many studies in animal models have documented that the vast majority (>95%) of cells transplanted in the heart die or vanish shortly after administration (Wysoczynski et al., 2018). Previous studies of cells other than CMCs suggest that culturing cells at physiological oxygen tension is beneficial (Mohyeldin et al., 2010; Drela et al., 2014; Jagannathan et al., 2016). We have recently demonstrated that the use of physiologic (5%) oxygen tension to culture CMCs *in vitro* improves cell morphology, markedly decreases cell size, markedly increases cell proliferation, and greatly enhances cell resistance to severe hypoxic stress (Bolli et al., 2021). However, it is unknown whether these beneficial effects of 5% oxygen *in vitro* translate into greater therapeutic efficacy *in vivo* in the treatment of heart failure.

The present investigation was undertaken in follow-up to our previous work *in vitro* (Bolli et al., 2021). The goal of this study was to directly compare the therapeutic efficacy of CMCs cultured at physiologic oxygen tension (5%) with that of CMCs cultured with the commonly used atmospheric oxygen tension (21% O₂) in a mouse model of heart failure caused by an old MI. We found that CMCs cultured at 5% O₂ had significantly greater therapeutic efficacy; that is, they produced a greater improvement in left ventricular (LV) function, a greater increase in viable myocardium, and a greater decrease in myocardial fibrosis.

MATERIALS AND METHODS

Ethics Statement

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Publication No. [NIH] 86–23) and with the guidelines of the Animal Care and Use Committee

of the University of Louisville, School of Medicine (Louisville, KY, United States). The protocol was approved by the Institutional Animal Care and Use Committee of the University of Louisville. All the surgical procedures, measurements of LV function (echocardiography and hemodynamic studies) and pathological analyses were performed by investigators double-blinded to treatment allocation.

Isolation of Murine CMCs

Male C57BL/6J mice (The Jackson Laboratory), 12 ± 0.5 weeks old, were euthanized by sodium pentobarbital injection (100 mg/kg, i.p.). The hearts were excised, rinsed with PBS (pH 7.4) at room temperature, minced into small pieces, and then enzymatically dissociated with Collagenase II (5 µg/mL in PBS, ≥ 125 units per mg dry weight; Worthington) via gentle agitation at 37°C for 45 min. After Collagenase II inactivation with DMEM/F12 medium containing 10% FBS, cells were centrifuged at 600 g for 10 min at room temperature. The collected cell pellet was suspended in growth medium consisting of DMEM/F12 (Invitrogen), 10% FBS (Seradigm, VWR), bFGF (10 ng/mL, Invitrogen), EGF (10 ng/mL, Invitrogen), ITS (insulin/transferrin/selenium, Invitrogen), glutamine (Invitrogen), and penicillin streptomycin solution (Invitrogen). A single-cell suspension was plated into culture plates. The non-adherent cells were removed 2 h after initial plating, and the attached cells were gently rinsed with PBS and then continuously cultured in new growth medium. Collected floating cells were plated into new cell culture plates and cultured for the next 2 h. The procedure was repeated three more times at 24, 48, and 72 h, respectively. A total of five fractions were collected after myocardial digestion. The cell fractions that attached within the first 2 and 4 h were discarded, as most of them were cardiac fibroblasts, which can quickly attach on the culture plates, whereas the cells that attached on the culture plates at 24, 48, and 72 h were designated as cardiac mesenchymal cells (CMCs), whose properties and function have been identified previously (Wysoczynski et al., 2017).

Murine CMC Culture

Murine CMCs were cultured in growth medium (Wysoczynski et al., 2017; Schulman et al., 2018). Under a microscope, when cells reached ~80% confluence in the culture plate, cell images were acquired to monitor changes in cell morphology. Then, cells from one 100-mm culture plate were digested by trypsinization, counted with a hemocytometer under a microscope, split and seeded into new 100-mm culture plates at 5,000 cells/cm². Beginning at passage 2, cells were cultured either in a 21% O₂ or 5% O₂ incubator supplied with 5% CO₂ at 37°C. Cells at passages 5–6 were employed for cell transplantation *in vivo*. To control the quality of the cell batch, the same batch of cells at passage 2 characterized previously (Wysoczynski et al., 2017) was used to develop 21% and 5% O₂ CMCs for the *in vitro* studies of cell competence and functional properties (Bolli et al., 2021). The same batch of these 21% and 5% O₂ CMCs used in those *in vitro* studies was employed for the current study *in vivo*. In all experiments, CMCs were passaged <7 times (Bolli et al., 2021).

Murine Model of Heart Failure Caused by an Old MI

C57BL/6J female mice (weight, 22.4 ± 0.4 g; age 14–15 weeks) purchased from The Jackson Laboratory (Bar Harbor, ME, United States) were used. Mice were maintained in microisolator cages under specific pathogen-free conditions in a room with a temperature of 24°C, 55–65% relative humidity, and a 12-h light–dark cycle. Mice were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and ventilated using carefully selected parameters. The chest was opened through a midline sternotomy, and a non-traumatic balloon occluder was implanted around the mid-left anterior descending coronary artery using an 8-0 nylon suture. To prevent hypotension, blood from a donor mouse was given at serial times during surgery. Rectal temperature was carefully monitored and maintained between 36.7 and 37.3°C throughout the experiment. In all groups, MI was produced by a 60-min coronary occlusion followed by reperfusion (**Figure 1**). Successful performance of coronary occlusion and reperfusion was verified by visual inspection (i.e., by noting the development of a pale color in the distal myocardium after inflation of the balloon and the return of a bright red color due to hyperemia after deflation) and by observing ST-segment elevation and widening of the QRS on the ECG during ischemia and their resolution after reperfusion). Mice were then followed for 65 days (Guo et al., 2017; Wysoczynski et al., 2017; Guo et al., 2020).

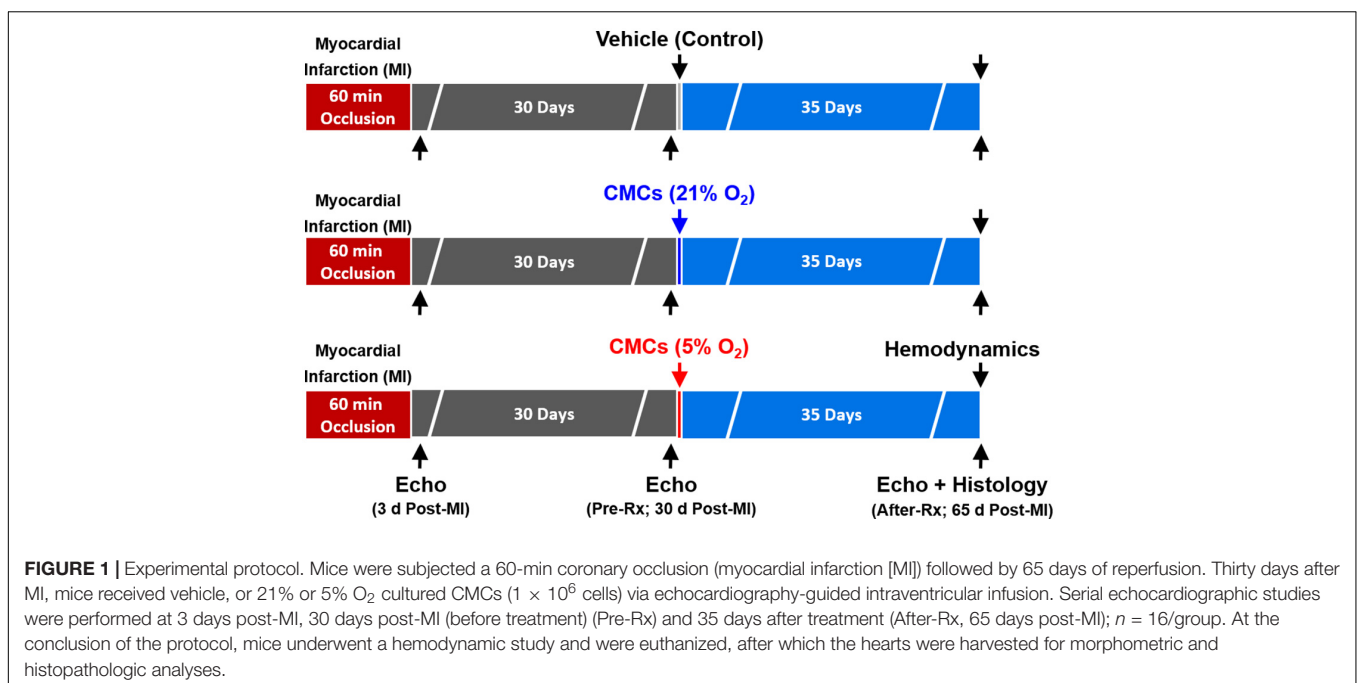
Echocardiography-Guided Intraventricular CMC Transplantation

Thirty days after MI, mice were randomly allocated to vehicle or different cell-treated groups. CMC or vehicle transplantation was performed using a Vevo 2100 Imaging System (VisualSonics, Inc.) equipped with a 30-MHz transducer, a Vevo Image Station

with Injection Mount, and micro-manipulation controls (Guo et al., 2017; Nong et al., 2020). Mice were reanesthetized with isoflurane (3% for induction, 1.5% for maintenance). The anterior chest was shaved and mice were placed on the imaging table in the right lateral decubitus position with the left lateral side facing the injection mount. After a good long-axis view of left ventricle was procured, the transducer was turned 90 degrees clockwise. The left ventricle was scanned in the short-axis view from apex to base to determine the optimal site for needle insertion. To prevent bleeding from the LV wall, it is crucial to find a site that avoids the infarct scar and coronary arteries. Under real-time B-mode view, a 0.5-inch 30 G needle connected to a 1.0 ml syringe was carefully inserted from the left lateral side of the chest and advanced into the center of the LV cavity. Successful penetration of the LV cavity was indicated by a small reflux of bright red blood from the needle into the syringe tip. CMCs (1×10^6 cells in 200 μ l of PBS) or vehicle (200 μ l of PBS) were infused at a steady rate of 2.2 μ l/s over 90 s. After the infusion, the needle was quickly withdrawn from the left ventricle. Using a rectal temperature probe, body temperature was controlled in the range of $37 \pm 0.2^\circ\text{C}$, and the electrocardiogram and respiration were monitored carefully during the whole procedure. Mice were allowed to recover in a temperature-controlled area (Guo et al., 2017).

Echocardiographic Studies

The echocardiographic studies were performed using the Vevo 2100 Imaging System (VisualSonics, Inc.) equipped with a 30-MHz transducer, as previously described (Li Q. et al., 2011). Serial echocardiograms were obtained at 3 days after the 60-min occlusion (Post-MI), 30 days after MI (before treatment, Pre-Rx), and 35 days after treatment (After-Rx, 65 days post-MI) (**Figure 1**) under isoflurane anesthesia (3% for induction, 1% for maintenance).



Using a rectal temperature probe, body temperature was carefully maintained at $37 \pm 0.2^\circ\text{C}$ throughout the study. The parasternal long axis and parasternal short-axis views were used to obtain 2D mode images for the measurement of LV mass, end-diastolic and end-systolic LV volume (LVEDV and LVESV), stroke volume (SV), and EF, as previously described (Dawn et al., 2006; Li Q. et al., 2011). Digital images were analyzed off-line by blinded observers using the Vevo 2100 software. Measurements were performed according to the American Society for Echocardiography (Gottdiener et al., 2004; Mehra et al., 2018). At least three measurements were acquired and averaged for each parameter (Gao et al., 2011).

Hemodynamic Studies

To avoid any potential effects of isoflurane anesthesia on cardiac function, hemodynamic studies were performed 4 days after the final echocardiography studies and just before euthanasia, as previously reported (Guo et al., 2017; Mehra et al., 2018). Mice were anesthetized with sodium pentobarbital (60 mg/kg i.p.), intubated, and ventilated with a positive pressure ventilator (Hugo-Sachs Elektronik D-79232 [Germany]; ventilation rate, 105/min; tidal volume 10.3 $\mu\text{l/g}$). Rectal temperature was kept at $37 \pm 0.2^\circ\text{C}$. A 1.0 French pressure-volume (PV) catheter (PVR-1035, Millar Instruments) was inserted into the left ventricle via the right carotid artery. The position of the catheter was carefully adjusted until typical and stable PV loop signals were acquired. After 30 min of stabilization, the PV signals were recorded continuously with an MPVS ULTRA Pressure-Volume Unit (Millar Instruments) coupled with a Powerlab 16/30 converter (AD Instruments), stored, and displayed on a computer with LabChart 7.0 software (AD Instruments). Inferior vena cava occlusions were performed with external compression to produce variably loaded beats for determination of the end-systolic PV relation and other derived constructs of LV performance. Parallel conductance from surrounding structures was calculated by a bolus injection of 5 μl of 30% NaCl through the jugular vein. Echocardiography-derived SV was used as outside reference for alpha calibration for LV volume. All hemodynamic data analyses were performed off-line using LabChart 7.0 software by an investigator blinded to the treatment allocation (Li Q. et al., 2011).

Histological Studies

At the conclusion of the protocol, the heart was arrested in diastole by an i.v. injection of 0.15 ml of CdCl_2 (100 mM), excised, and perfused retrogradely at 60–80 mmHg (LVEDP = 8 mmHg) with heparinized PBS for 2 min, followed by 10% neutral buffered formalin solution for 15 min. The heart was then sectioned into three slices from apex to base, fixed in formalin for 24 h, and subjected to tissue processing and paraffin embedding. Paraffin-embedded LV blocks were sectioned at a thickness of 4 μm for histological studies (Li Q. et al., 2011). Morphometric parameters, including LV cavity area, total LV area, risk region area, scar area, LV wall thickness, and infarct expansion index were measured in sections stained with Masson's trichrome as described previously (Tang et al., 2016). Myocardial collagen content was quantitated on picrosirius red-stained heart

images acquired with polarized light microscopy by determining collagen area per mm^2 of risk region or non-infarcted region (Tang et al., 2018). All acquired images were analyzed using NIH ImageJ software (version 1.52a) and measurements were averaged from three slides (trichrome) or two slides (collagen) per heart (Guo et al., 2017; Tang et al., 2018).

Statistical Analysis

The statistical methods were similar to those previously used by our group (Bennett et al., 1987; Triana et al., 1991; Li et al., 1993, 2001; Tang et al., 1996; Dawn et al., 2008). Data are presented as mean $\pm$ SEM. Data were analyzed with one-way repeated measures ANOVA as well as with one-way ANOVA for normally distributed data, or Kruskal–Wallis one-way analysis of variance on ranks for data that are not normally distributed, as appropriate, followed by unpaired Student's *t*-tests with the Bonferroni correction. A $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SigmaStat software system (3.5 V).

RESULTS

Exclusions

A total of 96 mice were used for this study. Forty-one mice died during or shortly after the surgical procedure of opening the chest and producing a 60-min coronary occlusion to induce MI. Seven mice were excluded because of technical problems, including body temperature out of normal range ($n = 1$), balloon occluder malfunction ($n = 1$), bleeding after echocardiography-guided LV cell injection ($n = 2$), and poor echocardiographic images for data analysis ($n = 3$). Thus, a total of 48 mice were included in the final analysis.

Fundamental Physiological Parameters

During the echocardiographic studies, heart rate and body temperature (fundamental physiological parameters that may impact myocardial function) were similar in the three groups. The heart rate was 516 ± 10 beats/min in the vehicle group, 520 ± 11 beats/min in the 21% O₂ cultured CMC group, and 495 ± 7 beats/min in the 5% O₂ cultured CMC group. By experimental design, rectal temperature remained within a narrow, physiologic range ($36.9\text{--}37.1^\circ\text{C}$) in all three groups (Table 1).

Effect of Physiologic Oxygen Tension on CMCs

After they were isolated from the mouse heart, CMCs were cultured in an incubator supplied either with 21% or 5% O₂ beginning at passage 2. As illustrated in Figure 2A, CMCs cultured at 21% O₂ for 4 days at passage 5 showed poor morphology and exhibited lower cell density; cells were large and elongated with varied shapes. In contrast, CMCs cultured at 5% O₂ for 4 days at passage 5 were small, showed higher density, and had round/oval and more uniform shape (Figure 2B). These observations are consistent with our previous studies

TABLE 1 | Rectal temperature and heart rate on the days of echocardiography assays.

	Post MI (3 days after MI)	Pre Rx (30 days after MI)	After Rx (35 days after Rx)
Temperature (°C)			
Vehicle Group	36.9 ± 0.1	37.1 ± 0.1	36.9 ± 0.0
21% O ₂ CMC Group	36.9 ± 0.1	36.9 ± 0.0	37.0 ± 0.0
5% O ₂ CMC Group	37.0 ± 0.1	37.0 ± 0.0	37.0 ± 0.0
Heart rate (beats/min)			
Vehicle Group	516 ± 10	487 ± 9	483 ± 9
21% O ₂ CMC Group	520 ± 11	499 ± 8	511 ± 7
5% O ₂ CMC Group	495 ± 7	482 ± 9	495 ± 10

Measurements of rectal temperature and heart rate were acquired during the echocardiographic studies ($n = 16/\text{group}$). Data are means $\pm$ SEM.

(Bolli et al., 2021) and suggest that physiological 5% O₂ is beneficial to CMC function.

Effect of CMCs Cultured at Physiologic Oxygen Tension on LV Function Measured by Echocardiography

Echocardiographic studies were performed 3 days after MI (Post-MI), 30 days after MI (before treatment) (Pre-Rx), and 35 days after treatment (After-Rx, 65 days after MI). According to our

inclusion criteria, only mice with severe LV dysfunction, i.e., EF < 35% at 30 days after MI, were included in the study (Figure 3). Representative echocardiographic images acquired from a mouse in the 5% O₂ CMC group before MI, 30 days after MI (Pre-Rx), and 35 days after cell treatment (After-Rx), are presented in Figure 2C.

As expected, the vehicle group showed a significant, progressive deterioration in LV function, with the average EF decreasing from 31.5 to 28.4% ($P < 0.05$) in the 30-day interval between 3 days post-MI and right before vehicle injection (Pre-Rx); in this group, LV EF continued to decline from 28.4 to 27.4% ($P < 0.05$) in the subsequent 35-day interval after vehicle injection (After-Rx) (Figure 4A).

At 30 days post-MI (before treatment) (Pre-Rx), there were no significant differences among the three groups with respect to LV end-diastolic volume (LVEDV), end-systolic volume (LVESV), stroke volume (SV), and EF (Figures 4A,B, 5A–C), indicating that the severity of post-MI LV remodeling and dysfunction was comparable in all groups. The pronounced LV dilatation in all groups, as shown by the increase in LVEDV and LVESV (Figures 5D,E), demonstrates that this murine model exhibits significant LV remodeling after MI. Thirty-five days after treatment (After-Rx), a significant improvement in LV EF was noted both in the 21% and 5% O₂ CMC groups compared with that in the vehicle group; however, the improvement was significantly more robust in the 5% O₂ CMC group. This is

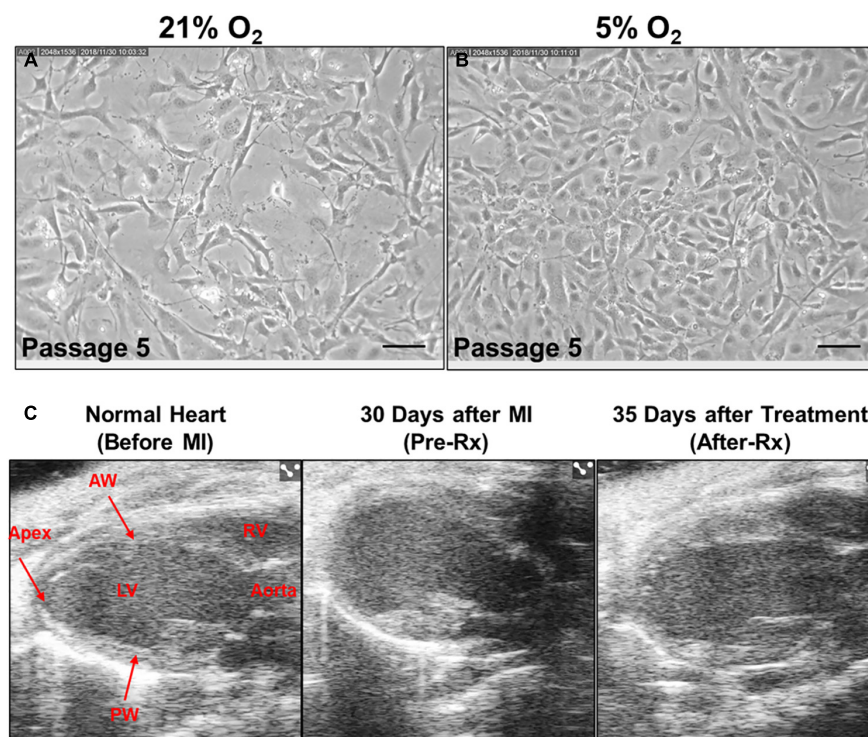
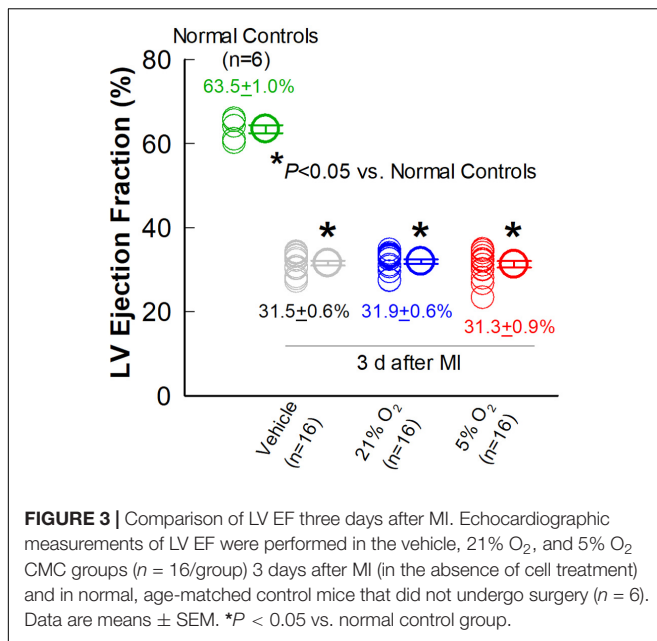


FIGURE 2 | Effect of CMCs cultured at 5% oxygen tension on cardiac function after MI. Representative microscopic images of CMCs acquired from 21% (A) and 5% (B) O₂ cultured CMCs at passage five under magnification $\times 200$. Scale bars, 100 μm . (C) Representative echocardiography B-mode long-axis images obtained before MI (left), 30 days after MI (Pre-Rx) (middle) and 35 days after 5% O₂ cultured CMC treatment (After-Rx) (right). LV, left ventricle; RV, right ventricle; AW, anterior wall; PW, posterior wall.



demonstrated by the fact that in this group, the increase in LV EF from pretreatment values (Pre-Rx) to 35 days after treatment (After-Rx) was $5.2 \pm 0.9\%$ (absolute units), whereas in the 21% O₂ CMC group it was only $1.5 \pm 0.7\%$ ($P < 0.05$ vs. the 5% O₂ CMC group) (in the vehicle group, LV EF actually decreased by $-1.0 \pm 0.5\%$) (Figure 4C). As a result, at 35 days after treatment LV EF was significantly ($P < 0.05$) greater in the 5% O₂ CMC group compared with the 21% O₂ CMC group (Figures 4A,B). A similar pattern was also observed in Δ LVEDV, Δ LVESV and Δ SV: both 21% O₂ CMCs and 5% O₂ CMCs improved LVEDV, LVESV and SV compared with vehicle (Figures 5D–F), but the improvement in the 5% O₂ CMC group was statistically greater than that in the 21% O₂ CMC group (Figures 5D,E). One-way repeated measures ANOVA showed that among the three groups, only 5% O₂ CMCs significantly improved LV EF (Figure 4A) and LV remodeling (EDV and ESV; Figures 5A,B) at 35 days after treatment as compared with pre-treatment values.

Taken together, these results indicate that 5% O₂ CMCs are superior to 21% O₂ CMCs in improving the function of the failing murine heart after a 30-day old MI and that the use of physiologic oxygen tension (5%) to culture CMCs increases their therapeutic efficacy.

Effect of CMCs Cultured at Physiologic Oxygen Tension on LV Function Measured by Hemodynamic Studies

To prevent any after-effects of the anesthesia used during the echocardiographic assessment 35 days after cell administration, a 4-day interval was allowed between echocardiographic and hemodynamic measurements; thus, the hemodynamic studies were performed just before euthanasia, 39 days after vehicle or cell administration. At this time, all LV functional parameters were markedly depressed in the vehicle, 21% and 5% O₂

CMC groups (Figure 6). Figure 6A illustrates representative LV pressure-volume (P-V) loops acquired in the vehicle, 21% and 5% O₂ CMC groups. The leftward shift of the volume signal and increased amplitude of the pressure signal in the P-V loops (resulting in a steeper end-systolic pressure-volume relationship) in the 5% O₂ CMC-treated mouse are indicative of reduced LV operating volumes and enhanced contractility. Compared with the vehicle group, hemodynamic parameters were improved in both cell-treated groups, but the improvement was more robust in the 5% O₂ CMC group than in the 21% O₂ CMC group. This was the case not only for load-dependent (LV EF and LV dP/dt) but also for load-independent (end-systolic elastance and Tau) indices of LV function (Figures 6E–H). Both LV dP/dt and end-systolic elastance were significantly greater in the 5% O₂ CMC group than in the 21% O₂ CMC group, and EF was improved in the former but not in the latter (Figures 6E–G). Thus, two independent methods of functional assessment (echocardiography and hemodynamic studies) consistently demonstrated that the functional benefits offered by 5% O₂ CMC treatment are superior to those of 21% O₂ CMC treatment.

Effect of CMCs Cultured at Physiologic Oxygen Tension on LV Structure

A hallmark of ischemic cardiomyopathy is the presence of compensatory hypertrophy in the surviving myocardium. As shown in Table 2, LV weight was significantly increased in vehicle-treated mice compared with normal controls, indicating the development of compensatory LV hypertrophy after MI in this murine model of chronic ischemic cardiomyopathy. Importantly, the ratios of LV weight to body weight and LV weight to tibial length were significantly reduced in the 5% O₂ CMC group ($n = 12$, $P < 0.05$) as compared with the vehicle group ($n = 12$, Table 2), demonstrating that administration of 5% O₂ grown CMCs ameliorated LV hypertrophy. In contrast, these parameters did not differ significantly between the 21% O₂ and vehicle groups. Although the differences among groups were not statistically significant, both the ratios of lung weight to body weight and lung weight to tibial length were nominally lower in the 5% O₂ CMC group (but not in the 21% O₂ CMC group) compared with the vehicle group, suggesting that 5% O₂ grown CMCs may alleviate pulmonary congestion.

The effects of CMC transplantation on LV remodeling were further assessed by Masson's trichrome staining. Figure 7 shows representative Masson's trichrome-stained myocardial sections from the vehicle, 21% and 5% O₂ CMC groups. Scar tissue and viable myocardium are identified in blue and red, respectively. There were no appreciable differences among the three groups with respect to the size of the region at risk (Figure 7B), suggesting that the magnitude of the ischemic insult was comparable in all groups. This is confirmed by the fact that LV EF was similar in all groups after MI, as shown in Figure 4B. Compared with the vehicle group, the 5% O₂ CMC-treated hearts exhibited a decrease in scar size and a concomitant increase in the amount of viable myocardium in the region at

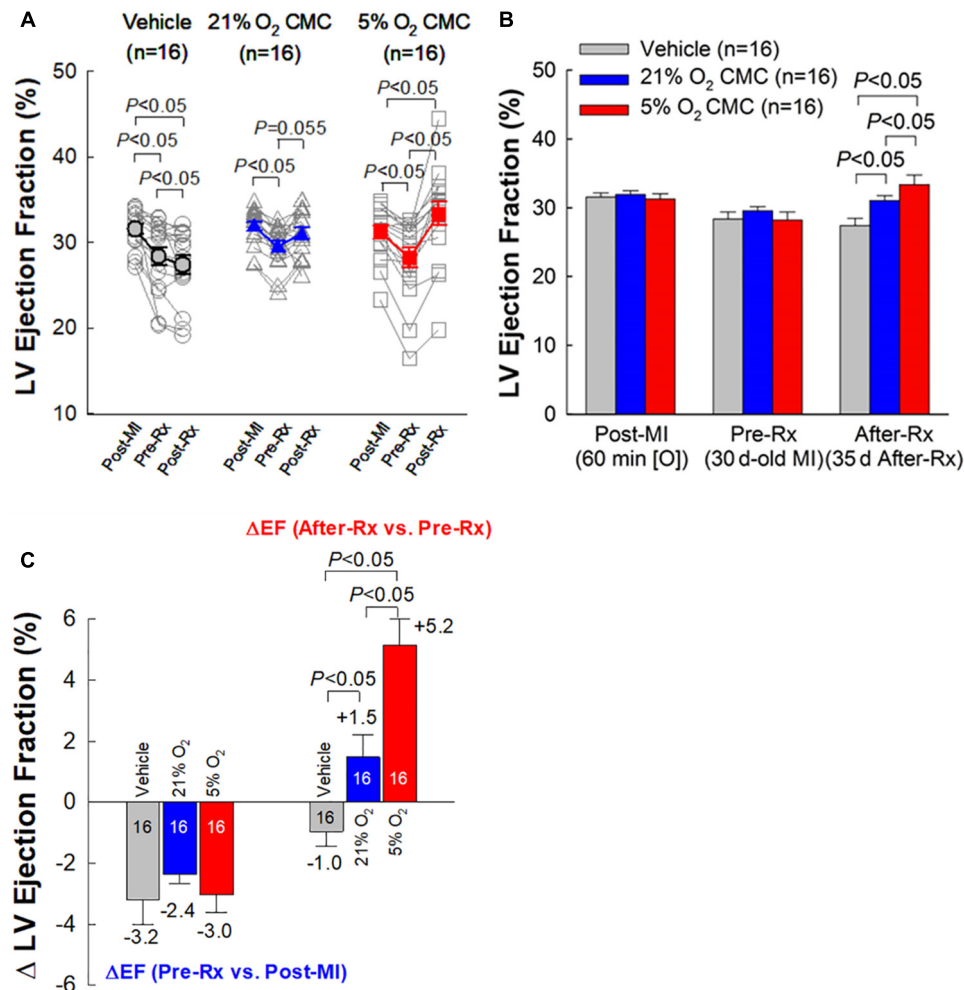


FIGURE 4 | Echocardiographic assessment of LV EF. **(A)** Values of LV EF acquired from each individual mouse at each time-point. **(B)** Mean values of LV EF at each time-point. **(C)** Changes in LV EF. On the *left* are depicted the changes in LV EF (absolute units) between 30 days after MI (before treatment) (Pre-Rx) and 3 days after MI (Post-MI). On the *right* are depicted the changes in LV EF (absolute units) between 35 days after treatment (After-Rx) and 30 days after MI (before treatment) (Pre-Rx). $n = 16$ /group. Data are mean $\pm$ SEM.

risk (**Figure 7B**), concomitant with an increase in LV anterior wall (infarct wall) thickness, a decrease in LV posterior wall (non-infarcted wall) thickness, and a decrease in LV expansion index (which is indicative of reduced LV dilatation) (**Figure 7C**). In contrast, the 21% O₂ CMC-treated hearts did not differ significantly from the vehicle-treated hearts with respect to scar size, viable myocardium, or non-infarcted wall thickness (**Figures 7B,C**). The increased amount of viable myocardium may have contributed to the increased cardiac function in mice that received 5% O₂ CMCs.

Effect of CMCs Cultured at Physiologic Oxygen Tension on Myocardial Collagen Content

One of the features of the failing heart is fibrosis, i.e., excess deposition of collagen in the extracellular matrix. We performed quantitative analysis of collagen content in the

heart using picrosirius red staining followed by imaging with polarized light microscopy (**Figures 8A–C**). Collagen content was evaluated based on stained pixel density using the NIH ImageJ software and expressed as a percentage of the risk or non-infarcted region. In the risk region (which is the sum of the infarcted and border regions), collagen content tended to be less in the 21% O₂ CMC group compared with the vehicle group but the difference was not statistically significant (**Figure 8D**). In contrast, collagen content in the risk region was significantly reduced in hearts treated with 5% O₂ grown CMCs as compared with the vehicle group ($40.1 \pm 3.8\%$ of risk region vs. $51.6 \pm 4.7\%$, respectively, $P < 0.05$; **Figure 8D**). Similarly, in the non-infarcted region, collagen content was significantly less in the 5% O₂ CMC-treated group ($9.4 \pm 2.1\%$ of non-infarcted region) as compared with the vehicle group ($15.7 \pm 1.9\%$, $P < 0.05$; **Figure 8E**)—a relative reduction of nearly 50%. In contrast, collagen

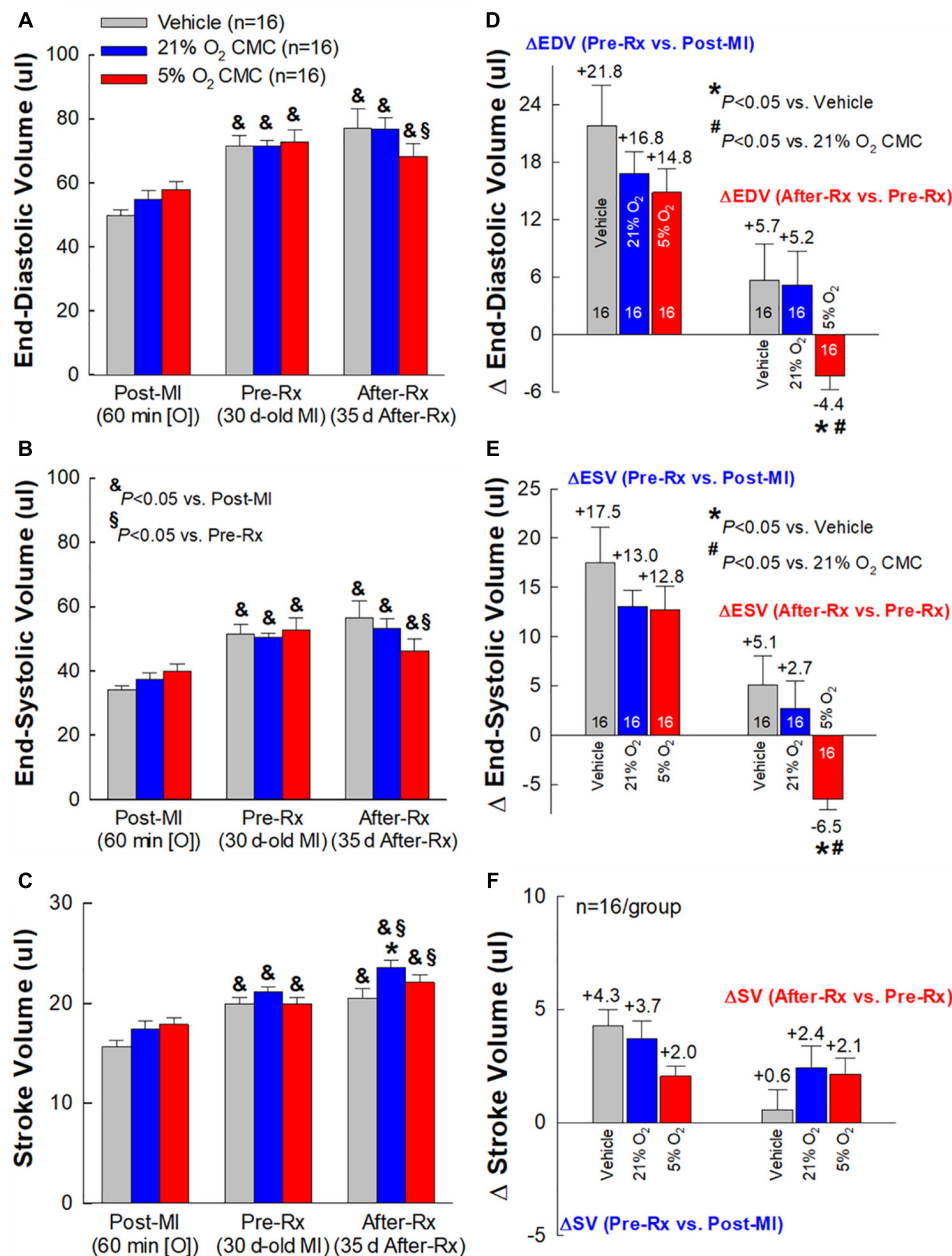


FIGURE 5 | Echocardiographic assessment of LV volumes. Serial echocardiographic measurements of LV end-diastolic volume (EDV) (A), LV end-systolic volume (ESV) (B) and LV stroke volume (SV) (C), were performed 3 days after a 60-min coronary occlusion/reperfusion (Post-MI), 30 days post-MI (before treatment) (Pre-Rx), and 35 days after treatment (After-Rx, 65 days post-MI). The right panels depict the changes in LV EDV (D), ESV (E), and SV (F) between 30 days post-MI (before treatment) (Pre-Rx) and 3 days after MI (Post-MI) (left), and between 35 days after treatment (After-Rx) and 30 days post-MI (before treatment) (Pre-Rx) (right). $n = 16$ /group. Data are means $\pm$ SEM. & $P < 0.05$ vs. the same group Post-MI; § $P < 0.05$ vs. the same group Pre-Rx; * $P < 0.05$ vs. vehicle group at the same time-point; # $P < 0.05$ vs. 21% O₂ CMC group at the same time-point.

content was not statistically significant different in hearts given 21% O₂ grown CMCs compared with vehicle, although it tended to be less in the former (Figure 8E). This reduced collagen deposition in the myocardial interstitial space of both risk and non-infarcted regions may have contributed, at least in part, to the functional benefits imparted by 5% O₂ CMC therapy.

DISCUSSION

The goal of this study was to compare the therapeutic efficacy of CMCs cultured at atmospheric oxygen tension (21%) (which is the standard method used by almost all investigators that study stem or progenitor cells) with that of CMCs cultured at physiologic oxygen tension (5%). We used a well-established

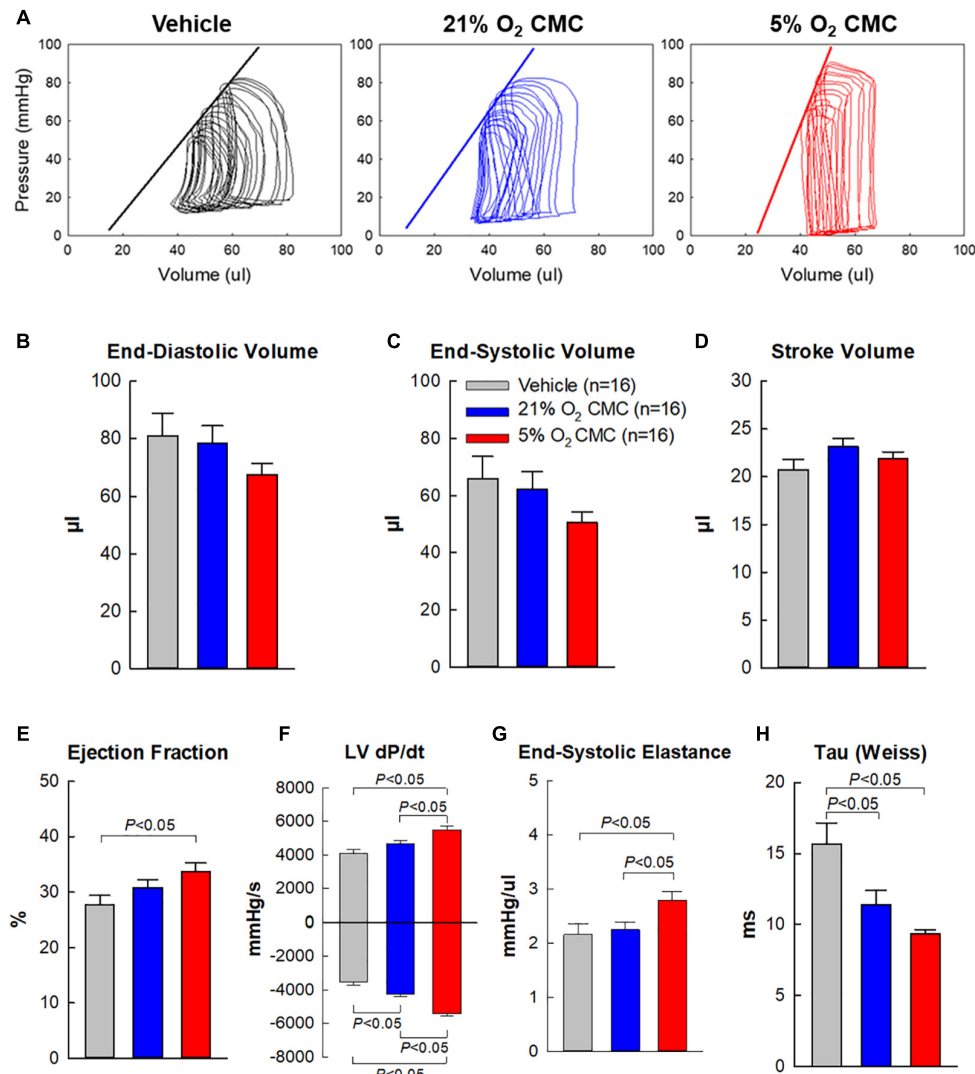


FIGURE 6 | Hemodynamic assessment of cardiac function. Hemodynamic studies were performed with a 1F Millar conductance catheter 39 days after treatment (4 days after the final echocardiographic study), just before euthanasia. **(A)** Representative LV pressure-volume loops recorded during preload manipulation by brief inferior vena cava occlusions. **(B–H)** Quantitative analysis of hemodynamic variables. Data are means $\pm$ SEM.

TABLE 2 | LV hypertrophy parameters.

	Normal control (n = 5)	Vehicle (n = 12)	21% O ₂ CMCs (n = 11)	5% O ₂ CMCs (n = 12)
LV weight (mg)	109.4 $\pm$ 7.5*	126.0 $\pm$ 3.0	115.8 $\pm$ 3.1*	112.2 $\pm$ 3.3*
Body weight (g)	24.8 $\pm$ 0.9	23.6 $\pm$ 0.2	23.1 $\pm$ 0.4	24.3 $\pm$ 0.4
LV weight/Body weight (mg/g)	4.5 $\pm$ 0.4*	5.4 $\pm$ 0.1	5.0 $\pm$ 0.2	4.6 $\pm$ 0.1*
LV weight/Tibia length (mg/mm)	5.9 $\pm$ 0.4*	7.0 $\pm$ 0.2	6.4 $\pm$ 0.2	6.2 $\pm$ 0.2*
Lung weight/Body weight (mg/g)	6.8 $\pm$ 0.3	7.4 $\pm$ 0.2	7.3 $\pm$ 0.2	7.1 $\pm$ 0.1
Lung weight/Tibia length (mg/mm)	9.1 $\pm$ 0.6	9.8 $\pm$ 0.2	9.6 $\pm$ 0.2	9.3 $\pm$ 0.2

*P < 0.05 vs. vehicle group. Data are means $\pm$ SEM.

murine model of chronic ischemic cardiomyopathy in which LV dysfunction is produced by a 30-day old MI (Guo et al., 2017; Nong et al., 2020). This model, characterized by severe and progressive LV dysfunction, LV hypertrophy, and myocardial fibrosis, mimics the common clinical setting of chronic HF

caused by old MIs, which accounts for approximately half of all cases of HF (Jaarsma and Stromberg, 2000; Amirrasouli and Shamsara, 2017; Benjamin et al., 2019).

The salient findings of this study can be summarized as follows. In mice with LV dysfunction caused by a 30-day

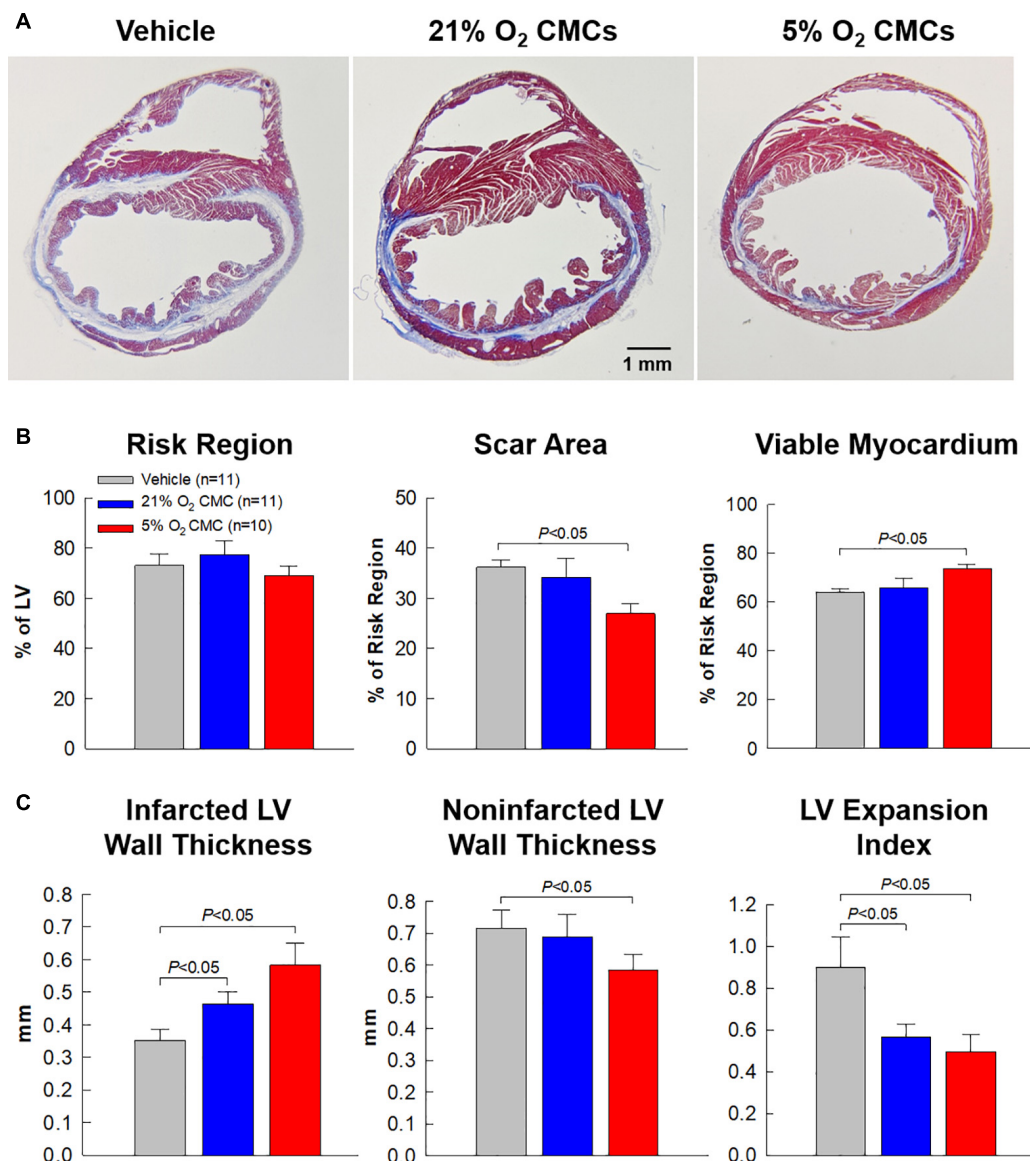
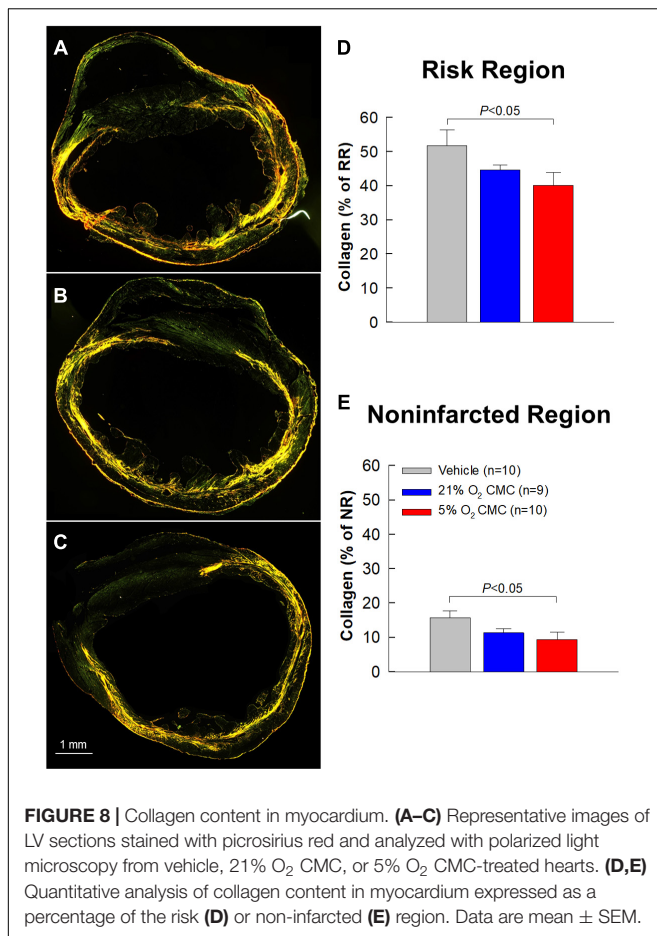


FIGURE 7 | Morphometric analysis of LV remodeling. **(A)** Representative Masson's trichrome-stained myocardial sections from vehicle, 21% O₂ CMC, or 5% O₂ CMC-treated hearts. Scar tissue and viable myocardium are identified in blue and red, respectively. **(B,C)** Quantitative analysis of LV morphometric parameters. Data are mean $\pm$ SEM.

old MI, both 21% and 5% O₂ CMCs improved LV function; however, the improvement was significantly greater with 5% O₂ CMCs than 21% O₂ CMCs. For example, relative to control mice, the increase in LV EF was approximately triple in mice treated with 5% O₂ CMCs compared with mice treated with 21% O₂ CMCs (5.2 ± 0.9 vs. 1.5 ± 0.7 absolute EF units, respectively; **Figure 4**). The evidence we provide for the superior therapeutic efficacy of 5% O₂ CMCs is robust, because it is based on two independent methods to measure LV function (echocardiography and hemodynamic studies) and on load-dependent as well as independent parameters, both of which point consistently to a greater improvement in hearts treated with 5% O₂ CMCs (**Figures 4–6**). This functional improvement

afforded by 5% O₂ CMCs was associated with a reversal or prevention of compensatory LV hypertrophy, such that the LV weight/body weight and LV weight/tibia length ratios were similar to those of normal mice (**Table 2**). The reduction in compensatory LV hypertrophy was likely secondary to improved LV function. The functional improvement in the 5% O₂ CMC group was also associated with improved LV structure, as shown by thicker infarct scars, thinner non-infarcted LV walls, and markedly reduced LV expansion index, which likely reflected more viable tissue, less compensatory hypertrophy, and less LV dilatation (**Figure 7C**). Unlike heart treated with 21% O₂ CMCs, hearts treated with 5% O₂ CMCs exhibited smaller scars and more viable myocardium (**Figure 7**). Since we have previously



demonstrated that CMCs do not engraft in the heart (Guo et al., 2017; Wysoczynski et al., 2017), these results suggest that administration of 5% O₂ CMCs may have reduced myocyte apoptosis and/or promoted endogenous myocyte proliferation. Finally, administration of 5% O₂ CMCs was associated with less fibrosis in the non-infarcted region (Figure 8E), suggesting that these cells were more effective at limiting extracellular matrix remodeling. Taken together, these results demonstrate that 5% O₂ CMCs are therapeutically superior to 21% O₂ CMCs in the setting of chronic ischemic cardiomyopathy. Previous studies have shown increased efficacy of other cell types cultured at physiologic oxygen tension (Li T. S. et al., 2011; Amirrasouli and Shamsara, 2017); however, to our knowledge, this is the first report that the therapeutic efficacy of CMCs *in vivo* is greatly enhanced by culturing them at physiologic oxygen tension (5%).

Some methodological aspects of this study deserve comment. Mice underwent a 60-min coronary occlusion followed by 30 days of reperfusion; this model of reperfused MI is more clinically relevant than models of permanent occlusion because in contemporary practice almost all patients with MI undergo spontaneous or iatrogenic reperfusion (Benjamin et al., 2019). Reperfusion dramatically alters the evolution and pathophysiology of MI (Basso and Thiene, 2006). The long (60-min) coronary occlusion produces large infarcts, sufficient to

cause heart failure. This is confirmed by the severe, progressive decline in LV function (Figures 4, 5) and the development of structural abnormalities characteristic of the failing heart, i.e., LV hypertrophy (Table 2) and myocardial fibrosis (Figure 8). We allowed mice to recover for 30 days in order to ensure that the infarct has healed completely and a stable scar has formed. This situation is analogous to that of patients with coronary artery disease who have scars caused by old MIs and develop heart failure as a consequence of the loss of cardiac muscle. At 30 days after MI, vehicle or CMCs were delivered directly into the LV cavity via percutaneous injection under echocardiographic guidance. This method is much less traumatic than repeated thoracotomies, results in much higher survival rates, and enables CMCs to be delivered close to the coronary arteries (Guo et al., 2017; Nong et al., 2020). Using this method, we have previously documented that a significant number of cells are retained in the heart at 1 day after injection, comparable to or greater than that seen after intramyocardial and intracoronary injection (Guo et al., 2017).

The robustness of our conclusions is supported by a number of considerations. In almost all rodent studies, LV function is assessed only with load-dependent measurements. However, there are distinct advantages in using load-independent parameters. Unlike EF or fractional shortening, load-independent parameters are not affected by uncontrollable variables such as venous return, ventricular dimensions, aortic pressure, LV compliance, peripheral resistance, etc., which vary continuously and unpredictably in intact animals (Schertel, 1998; Lindsey et al., 2018). Consequently, load-independent parameters are more reliable and meaningful. Among these, particular importance should be assigned to end-systolic elastance (Ees), which is often regarded as the gold-standard for assessing cardiac contractility (Schertel, 1998; Lindsey et al., 2018). Therefore, in this study the measurements of Ees could be viewed as the most important indicator of the therapeutic actions of transplanted cells. As shown in Figure 6G, in the 5% O₂ CMCs group Ees was significantly greater not only compared with the vehicle group but also compared with the 21% O₂ CMCs group. The superiority of 5% O₂ CMCs is further corroborated by the structural results. As shown in Figures 7, 8 and Table 2, structural improvement, i.e., a reduction in scar size, LV hypertrophy, and collagen content and an increase in viable myocardium, were observed only in hearts treated with 5% O₂ CMCs. Taken together, these diverse and independent results support the conclusion that 5% O₂ CMCs were more efficacious than 21% O₂ CMCs.

The mechanism(s) responsible for the superior therapeutic efficacy of 5% O₂ CMCs vs. 21% O₂ CMCs remain to be elucidated. We have previously demonstrated that CMCs do not engraft in the heart and do not differentiate into new cardiomyocytes (Guo et al., 2017; Wysoczynski et al., 2017) and thus (similar to other cell types) (Tokita et al., 2016) act via paracrine mechanisms. The exact nature of these paracrine actions has not yet been ascertained for any mesenchymal cell type used heretofore (Banerjee et al., 2018). The increased resistance of 5% O₂ CMCs to severe hypoxia and their much higher proliferation rate *in vitro* as well as their enhanced

telomerase activity and elongated telomere length (Bolli et al., 2021) imply that, after transplantation *in vivo*, 5% O₂ CMCs will be more likely to survive the harsh environment of the infarcted heart and will continue to multiply at a higher rate, which should result in greater cell numbers and thus greater therapeutic effects. Our finding that treatment with 5% O₂ CMCs was associated with an increase in viable myocardium (Figure 7) suggests possible antiapoptotic actions and/or increase proliferation of endogenous myocytes. Our finding of decreased collagen content in both the risk and non-infarcted regions of hearts treated with 5% O₂ CMCs (Figure 8) offers another possible explanation, since increased collagen deposition resulting in fibrosis impairs contractile function. Further studies will be necessary to test these hypotheses and determine the mechanisms that account for the superiority of 5% O₂ CMCs. This uncertainty regarding mechanism(s) of action is common to essentially all cell types that have been tested for the treatment of heart failure (Wysoczynski et al., 2018).

CONCLUSION

In conclusion, this study shows for the first time that, compared with the commonly used atmospheric oxygen tension (21%), the use of physiologic oxygen tension (5%) to culture CMCs markedly increases their therapeutic efficacy in a murine model of chronic ischemic cardiomyopathy. These results are important not only because they enhance the therapeutic potential of CMCs but also because culture at 5% O₂ enables greater numbers of cells to be produced in a shorter time, thereby reducing costs and effort and limiting cell senescence (Bolli et al., 2021). Due to the similarity between CMCs and other stem/progenitor cells, it is likely to these results may apply to other types of cells being studied in experimental or clinical trials but still cultured at atmospheric O₂ tension. Thus, the present study has potentially vast implications because it supports a paradigm shift in the field of cell therapy. The standard method to culture most stem/progenitor cells, including CMCs, is to use 21% O₂ tension rather than the physiologic oxygen tension in the tissue. Our results suggest this method needs to be changed and that for CMCs, and probably for other types of stem/progenitor cells as well, physiologic oxygen tension needs to be used to maximize therapeutic efficacy, both in preclinical and clinical studies.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, University of Louisville.

AUTHOR CONTRIBUTIONS

RB did cell culture, acquired cell images, and conducted echocardiographic studies and analyses as well as histological procedure. AA and SH performed histological sectioning, staining, and image analyses. CD did cell culture partially for cell transplantation. AT, YN, and YG performed the surgical procedures, echocardiography-guided intraventricular cell transplantation, echocardiographic and hemodynamic studies, and analyses. QL partially performed experiments, designed all the studies, analyzed and interpreted the data, prepared figures, and wrote and finalized the manuscript. All authors have reviewed and approved the final version of the manuscript and meet the International Committee for Medical Journal Editors (ICMJE) authorship criteria.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Diabetic Endothelial Cells Differentiated From Patient iPSCs Show Dysregulated Glycine Homeostasis and Senescence Associated Phenotypes

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Induced pluripotent stem cells derived cells (iPSCs) not only can be used for personalized cell transfer therapy, but also can be used for modeling diseases for drug screening and discovery *in vitro*. Although prior studies have characterized the function of rodent iPSCs derived endothelial cells (ECs) in diabetes or metabolic syndrome, feature phenotypes are largely unknown in hiPSC-ECs from patients with diabetes. Here, we used hiPSC lines from patients with type 2 diabetes mellitus (T2DM) and differentiated them into ECs (dia-hiPSC-ECs). We found that dia-hiPSC-ECs had disrupted glycine homeostasis, increased senescence, and impaired mitochondrial function and angiogenic potential as compared with healthy hiPSC-ECs. These signature phenotypes will be helpful to establish dia-hiPSC-ECs as models of endothelial dysfunction for understanding molecular mechanisms of disease and for identifying and testing new targets for the treatment of endothelial dysfunction in diabetes.

Keywords: endothelium, endothelial function, senescence, mitochondrial function, glycine

INTRODUCTION

Vascular endothelial cells (ECs) play an important role in maintaining vascular homeostasis (Rajendran et al., 2013). In addition to serving as a physical barrier between the vessel wall and blood, ECs regulate platelet aggregation, coagulation, fibrinolysis, and vascular tone through secreting nitric oxide (NO), cytokines, and growth factors (Kruger-Genge et al., 2019). Endothelial dysfunction is a condition in which the EC loses its physiological properties, promotes vasoconstriction, fibrinolysis, inflammation, and atherogenesis (Kruger-Genge et al., 2019).

Type 2 diabetes mellitus (T2DM) is characterized by hyperglycemia, insulin resistance, and relative lack of insulin secretion (DeFronzo et al., 2015). T2DM causes endothelial dysfunction,

induces abnormal angiogenesis, and changes serum amino acid metabolism, especially for glycine, which is significantly decreased, due to hyperglycemia (Thalacker-Mercer et al., 2014; Drabkova et al., 2015; Giraldo-Grueso and Echeverri, 2020). These factors constitute molecule and cellular basis for both microvascular and macrovascular complications in diabetes (DeFronzo et al., 2015; Palmer A.K. et al., 2015; Guo et al., 2017; Yan-Do and MacDonald, 2017; Alves et al., 2019; Rashvand et al., 2019; Giraldo-Grueso and Echeverri, 2020). Treatments to improve endothelial function, cell viability, and angiogenesis and correct glycine metabolism may be promising therapeutic targets for preventing microvascular and macrovascular complications in diabetes.

Although primary human ECs are the best choice of cells for studying endothelial dysfunction, senescence, and angiogenesis, they are limited by cell source and cell isolation technique. The discovery of human induced pluripotent stem cells (hiPSCs) has paved the way for EC generation (Patsch et al., 2015; Zhang et al., 2017; Christensen et al., 2019). Disease specific hiPSC lines can be reprogrammed from patient somatic cells and subsequently can be differentiated back into somatic cells for disease modeling and drug screening and discovering *in vitro* (Liu et al., 2018; Rowe and Daley, 2019; Williams and Wu, 2019).

In this study, we investigated hiPSC lines reprogrammed from dermal fibroblasts derived from patients with T2DM. ECs were generated from diabetic hiPSCs (dia-hiPSCs). We aim to identify signature and specific phenotypes in dia-hiPSC derived ECs (dia-hiPSC-ECs). This will be helpful to establish dia-hiPSC-ECs as models for understanding the molecular mechanism as well as screening and discovering drugs for treatment of endothelial dysfunction and senescence, abnormal angiogenesis, and disrupted glycine homeostasis in diabetes.

MATERIALS AND METHODS

hiPSC Culture and Differentiation

Four hiPSC lines used in this study were reprogrammed from dermal fibroblasts using Sendai virus and 4 reprogramming factors (Thermo Fisher, North Logan, UT, United States): OCT4, SOX2, KLF4, and C-MYC, as described previously (Ye et al., 2013). PCBC16iPS and P1C1iPS cells were reprogrammed from human neonatal and adult dermal fibroblasts of healthy subjects (Lonza, Switzerland), respectively (Zhang et al., 2014). DP2-C8iPS and DP3-C6iPS cells were reprogrammed from dermal fibroblasts of two adult patients with T2DM. PCBC16iPS and P1C1iPS are derived from male donors. DP2 donor was a female patient and 53 years old, while DP3 donor was a male patient and 51 years old. The procedures were approved by the ethics committee of Nanjing Hospital, Nanjing, China and the Centralised Institutional Review Board of Singapore Health Services Pte. Ltd, Singapore. Informed consent forms were signed by both patients. hiPSCs were cultured in a feeder-free system with a 1:1 mixture of E8/mTeSR (STEMCELL Technologies, Canada) and passaged every 4 days with Versene (Thermo Fisher Scientific, United States). PCBC16iPS was an established hiPSC

line and described previously (Ye et al., 2013, 2014; Zhang et al., 2014; Su et al., 2018; Tan et al., 2019; Tao et al., 2020).

Pluripotency of P1C1iPSC, DP2-C8iPSC, and DP3-C6iPSC were characterized by fluorescence immunostaining, teratoma formation assay, and karyotyping. For fluorescence immunostaining, hiPSCs were fixed with 4% paraformaldehyde for 20 min at room temperature followed by 0.1% triton X-100 permeabilization. After blocking, primary antibody, mouse anti-Oct3/4 (SC-365509, Santa Cruz Biotech, United States) or anti-TRA-1-60 (130-106-872, Miltenyi Biotec, Germany), at 1:100 dilution each was added into the DPBS containing 10% goat serum (Thermo Fisher Scientific, United States) (10% DPBS) and incubated overnight at 4°C. Then, cells were incubated with FITC-conjugated donkey anti-mouse IgG secondary antibody (Thermo Fisher Scientific, United States) in 10% DPBS for 1 h at room temperature, labeled with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, United States), washed, and viewed under a fluorescence microscope (Olympus, Japan).

For teratoma formation experiment, the animal experimental protocol and procedures were approved by the Institutional Animal Care and Use Committee of Singapore Health Services Pte. Ltd. 2×10^6 hiPSCs were injected into the flanks of NOD-SCID mice (Invivos, Singapore). Animals were followed-up to 3 months. Teratoma were explanted, processed, and embedded into paraffin. Tissue sections of teratoma were stained with hematoxylin and eosin and viewed under an Olympus microscope to identify endoderm, mesoderm, and ectoderm cells. For karyotyping, hiPSCs were assessed by Cytogenetics Lab. of Department of Molecular Pathology, Singapore General Hospital, Singapore or of KK Women's and Children's Hospital, Singapore.

The EC differentiation protocol was described previously by us with modification (Zhang et al., 2014; Su et al., 2018). Briefly, 2-3 days before initiating differentiation, hiPSCs were dissociated into single cells and seeded into 6-well plates. On day 0 of differentiation, cells were cultured in DMEM/F12 medium (Thermo Fisher Scientific, United States) supplemented with B27 without insulin (B27-, Thermo Fisher Scientific, United States) and 10 μ M CHIR99021 (CHIR, STEMCELL Technologies, Canada) and 1 μ M U46619 (Santa Cruz Biotech, United States) for 24 h. On day 1, medium was replaced with DMEM/F12 supplemented with B27-, 100 ng/ml vascular endothelial growth factor-165 (VEGF) (ABM, Canada), 10 ng/ml transforming growth factor β 1 (TGF β 1, ProSpec-Tany Tech., Israel), and 50 ng/ml erythropoietin (EPO, ProSpec-Tany Tech., Israel) for 48 h, the medium was refreshed on day 3, and the cells were cultured for another 48 h. On day 5, the differentiating hiPSCs were harvested and cultured in EGM2-MV (Lonza, Switzerland) supplemented with B27, 100 ng/ml VEGF, and 10 μ M SB-431542 (SB, MedChemExpress, United States) in flasks that were coated with 1 μ g/cm² fibronectin (Sigma-Aldrich, United States). The medium was changed every 2 days. ECs were purified on day 11 using fluorescence activated cell sorting (FACS). Cells positive for both CD31 and CD144 (both from BD Pharmingen, United States) expression were collected and expanded (Zhang et al., 2014; Su et al., 2018). ECs were cultured in endothelial growth medium (EGM) which was composed of EGM2-MV medium

supplemented with B27, 100 ng/ml VEGF, and 10 μ M SB (Zhang et al., 2014).

Characterization of hiPSC-ECs *in vitro*

Fluorescence Immunostaining

For characterization of hiPSC-ECs *in vitro*, cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After blocking, primary antibodies, mouse anti-CD31 or anti-CD144 (both from BD Pharmingen, United States), at 1:100 dilution was added into 10% DPBS and incubated overnight at 4°C. Then, cells were incubated with PE-conjugated goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific, United States) in 10% DPBS for 1 h at room temperature, labeled with DAPI, washed, and viewed under a fluorescence microscope (Olympus, Japan).

Cell Population Doubling Time

Endothelial cell population doubling time was calculated within 7 days post-sorting. Briefly, ECs were harvested on day 2 post-sorting and cultured in 6-well plates (2×10^5 cells/well) in EGM. The medium was changed every 2 days. ECs were harvested and counted on day 7.

Western Blot Analysis

Endothelial cells on day-7 post-sorting were used for Western Blot analysis as described previously (Ye et al., 2009; Su et al., 2018). For protein analysis using EC supernatant, 2×10^5 ECs/well were cultured in 1 ml EGM for 48 h. Then supernatant was collected to determine the endothelin-1 and Matrix metalloproteinase-1 (MMP-1) proteins secreted from ECs. Cell lysate was prepared using PhosphoSafe™ Extraction Reagent (Merck, Germany) and protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories, United States). Proteins from supernatant or cell lysate were separated, electrophoretically blotted onto nitrocellulose membranes, and washed with 10 mM Tris-HCl buffer (pH 7.6) containing 0.05% Tween-20; then, the membranes were incubated in blocking buffer (5% non-fat dry milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20) at room temperature for 1 h and with diluted primary antibodies for overnight at 4°C. Primary antibodies were detected with secondary antibodies conjugated with horseradish peroxidase (HRP) and visualized with a ChemiDoc™ MP Imaging System (Bio-Lab, United States) and Image Lab 5.1 software (Bio-Lab, United States). The detailed antibody information was provided in **Supplementary Table 1**.

Endothelial Function *in vitro*

Dil-Conjugated Acetylated Low-Density Lipoprotein (Dil-ace-LDL) Uptake and Tube Formation

Dil-ace-LDL uptake and tube formation were evaluated as described previously (Zhang et al., 2014; Su et al., 2018). For Dil-ace-LDL uptake assay, hiPSC-ECs were incubated with DAPI overnight (1:1,000 dilution) and then in EGM supplemented with 10 μ g/mL of Dil-ace-LDL (Life Technologies, United States) at 37°C for 12 h. For tube-formation assay, cells were seeded in 48-well plate that had been coated with Matrigel (Corning, United States) and incubated at 37°C for 24 h. Numbers of node, junction, and

branches and total branches length per magnification (10 $\times$) were quantified using angiogenesis analyzer of Image J.

Nitric Oxide (NO) Produced by ECs

To assess NO produced, hiPSC-ECs on day 7 post-sorting were seeded in 12-well plate and cultured in 1 ml EGM for 48 h. Supernatant was collected and stored at -80°C. Cells were washed with DPBS and lysed in 100 μ l PhosphoSafe™ Extraction Reagent. The concentration of NO was determined using Nitric Oxide Detection Kit as per instruction (SKT-212, StressMarq Biosciences Inc., United States) and normalized by protein quantity and expressed as μ M/mg.

Endothelin-1 Secreted by ECs

To determine secreted endothelin-1, a vasoconstrictor, hiPSC-ECs supernatant was collected for Western Blot analysis as described above. Endothelin-1 protein expression was presented as fold change after comparing with that of PCBC-EC which was considered as 100%. The detailed information on endothelin-1 antibody was provided in **Supplementary Table 1**.

Adhesion Molecule Expressed by ECs

Protein expression of adhesion molecule, including ICAM-1 and VCAM-1, was determined using Western Blot as described above. The protein expression level was normalized by GAPDH and expressed as percentage of GAPDH. The detailed information on ICAM-1, VCAM-1, and GAPDH antibodies was provided in **Supplementary Table 1**.

Senescence of ECs

To determine cell senescence, ECs on day 14 post-sorting were stained for β -galactosidase (β -gal) expression using Senescence β -galactosidase staining kit (98605, Cell Signaling, United States). EC lysates were used to assess the protein expression of p16, p21, and p53 using Western Blot. The protein expression level was normalized by GAPDH and expressed as percentage of GAPDH. The detailed information on p16, p21, and p53 antibodies was provided in **Supplementary Table 1**.

Mitochondrial Function of ECs

To determine EC mitochondrial membrane potential, after overnight cultured with EGM supplemented with DAPI, ECs on day-7 post-sorting were cultured with 1:1 ratio of fresh EGM and JC-1 dye solution (Mitochondria staining kit, Sigma-Aldrich, United States) for 30 min in an incubator at 37°C. Then, cells were washed with DPBS and cultured in fresh EGM. Images of red fluorescence were randomly taken at 50 ms at 20 $\times$ magnification using Olympus IX73 microscope and Cell Sens Standard software (Olympus, Japan). The fluorescence intensity of each cell was calculated as the overall fluorescence intensity divided by EC number in each image using Image J. Each cell line had three biological replicates and each replicate had 6–8 images analyzed.

To determine mitochondrial structural protein content, EC lysates were used to assess protein expression of mitofusin-1 (Mfn-1), mitofusin-2 (Mfn-2), fission-1 (Fis-1), and ATP5 using Western Blot. The protein expression level was normalized by

GAPDH and expressed as percentage of GAPDH. The detailed information on Mfn-1, Mfn-2, and Fis-1 antibodies was provided in **Supplementary Table 1**.

To determine ATP synthesis efficiency, ECs were cultured in white 96-well plate. On the day of assay, cell culture medium was removed and 100 μ L of ATPlite 1 step Luminescence Assay buffer (6016731, PerkinElmer, United States) was added into each well. The intensity of luminescence was determined using Infinite M200 (TECAN, Switzerland) and was normalized to cell number.

Glycine Homeostasis in ECs

Intracellular Glycine Content

Methods of extraction and metabolic profiling for amino acid were described (Newgard et al., 2009; Muoio et al., 2012). ECs on day-7 post-sorting were washed DPBS and were scraped off wells using 300 μ L of 0.6% formic acid (Sigma-Aldrich, United States). 100 μ L mixture was used for protein content measurement. The remaining mixture was mixed with 200 μ L acetonitrile (Sigma-Aldrich, United States). The amino acid extracts were derivatized with 3 M Hydrochloric acid in methanol or butanol (Sigma-Aldrich, United States), dried, and reconstituted in methanol for analysis in Liquid chromatography–mass spectrometry.

Methods of amino acid analysis were modified from Newgard et al. (2009). Briefly, amino acids were separated using a C8 column (Rapid Resolution HT, 4.5 $\times$ 50 mm, 1.8 μ m, Zorbax SB-C8) on an Agilent 1290 Infinity LC system (Agilent Technologies, CA, United States) coupled with quadrupole-ion trap mass spectrometer (QTRAP 5500, AB Sciex, DC, United States). Mobile phase A (10/90 Water/Acetonitrile) and Mobile phase B (90/10 Water/ Acetonitrile) both containing 10 mM of Ammonium formate were used for chromatography separation. The liquid chromatography run was performed at a flow rate of 0.6 mL/min with initial gradient of 20% B for 0.5 min, then ramped to 100% B in 2.5 min, maintained for 0.5 min, followed by re-equilibrated the column to the initial run condition (20% B) for 2 min. All compounds were ionized in positive mode using electrospray ionization. The chromatograms were integrated using MultiQuant 3.0 software (AB Sciex, DC, United States). The amino acid concentration was normalized by protein concentration and presented as μ M/mg/ml.

Protein Quantification of Glycine Transporters (GlyT) and Serine Hydroxymethyltransferase (SHMT)

Endothelial cell lysates were used to assess the protein expression of GlyT1, GlyT2, cytoplasmic SHMT (cSHMT), and mitochondrial SHMT (mSHMT) using Western Blot. The protein expression level was normalized by GAPDH and expressed as percentage of GAPDH. The detailed information on GlyT1, GlyT2, cSHMT, and mSHMT antibodies was provided in **Supplementary Table 1**.

Paracrine Factor Profile of ECs

To assess angiogenic factors released from hiPSC-ECs, 2×10^5 hiPSC-ECs/well were seeded in 12-well plate and cultured in 1 ml endothelial basal medium (EBM) (Lonza, Switzerland) supplemented with 100x B27 for 48 h. The supernatant was collected and used to determine the profiles of angiogenic factors

released from hiPSC-ECs using Human Angiogenesis Arrays Q3 kit (Raybio Tech., United States) as described (Ye et al., 2014).

Endothelial Cell Function *in vivo*

Murine Hind-Limb Ischemia (HLI) Model and Treatment

The animal experimental protocol and procedures were approved by the Institutional Animal Care and Use Committee of Singapore Health Services Pte. Ltd. The animal model was developed as previously described (Su et al., 2018). Briefly, 8-week-old NOD-SCID mice (InVivos, Singapore) were anesthetized with 1.5–2% isoflurane and the femoral artery of the right limb was ligated with 6-0 polypropylene sutures. Animals were randomly assigned to treatment with 1.2×10^6 hiPSC-ECs in 0.1 mL EBM (the hiPSC-EC groups, $n = 6$ for each group) or with 0.1 mL EBM (the basal medium group, $n = 6$). The hiPSC-ECs differentiated from the 4 hiPSC lines or basal medium were administered 3 days after HLI induction via 4 intramuscular injections into the center of the ligated area and the surrounding region along the femoral artery.

Laser Doppler Imaging

Mice were anesthetized with 1.5–2% isoflurane, their hind limbs were shaved, and laser Doppler imaging was performed using a PeriScan PIM 3 System (Perimed, Sweden) as described (Su et al., 2018). Measurements in the ischemic (right) limb were normalized to measurements in the non-ischemic (left) limb and expressed as a percentage.

Immunohistochemistry

To identify transplanted hiPSC-ECs, a primary antibody specifically against human CD31 (hCD31, mouse anti-human CD31-Biotin) was used and visualized by mouse anti-Biotin-VioBright 515 (both from Miltenyi Biotec, Germany). Fluorescence images were taken with an Olympus IX71 fluorescence microscope.

Neovascularization in ischemic limb was determined as described (Su et al., 2018). Cryosections were stained for CD31 expression (rabbit anti-CD31, Abcam, United States), which targets both human and mouse ECs), to evaluate total vessel density, and smooth muscle actin (SMA) expression (Cy3-conjugated mouse anti-SMA antibodies, Sigma-Aldrich), which targets SMCs, to evaluate arteriole density. Vascular structures that were positive for CD31 expression and for both CD31 and SMA expression were counted for all animals in each group.

Statistics

Data are presented as mean $\pm$ standard deviation (SD). Comparisons among groups were analyzed for significance via one-way analysis of variance (ANOVA) with the Tukey correction. Analyses were performed with SPSS software. A value of $p < 0.05$ was considered significant.

RESULTS

Characterization of hiPSCs

P1C1iPSCs, DP2-C8iPSCs, and DP3-C6iPSCs displayed typical morphological features of human embryonic stem cells (hESCs): colonies with a tightly packed appearance and a well-defined edge and cells have prominent nucleoli (**Supplementary Figure 1A**). They all expressed pluripotent cell markers, including Oct3/4 and TRA-1-60 (**Supplementary Figures 1B,C**). Karyotyping demonstrated that all three hiPSC lines had normal chromosome number and structure (**Supplementary Figure 2A**). Teratoma formation assay showed that all three hiPSC lines were able to form teratoma in NOD-SCID mice. Hematoxylin and eosin staining showed that mesoderm (cartilage), ectoderm (epidermis), and endoderm (gastrointestinal glands) lineage cells were formed in teratoma (**Supplementary Figure 2B**).

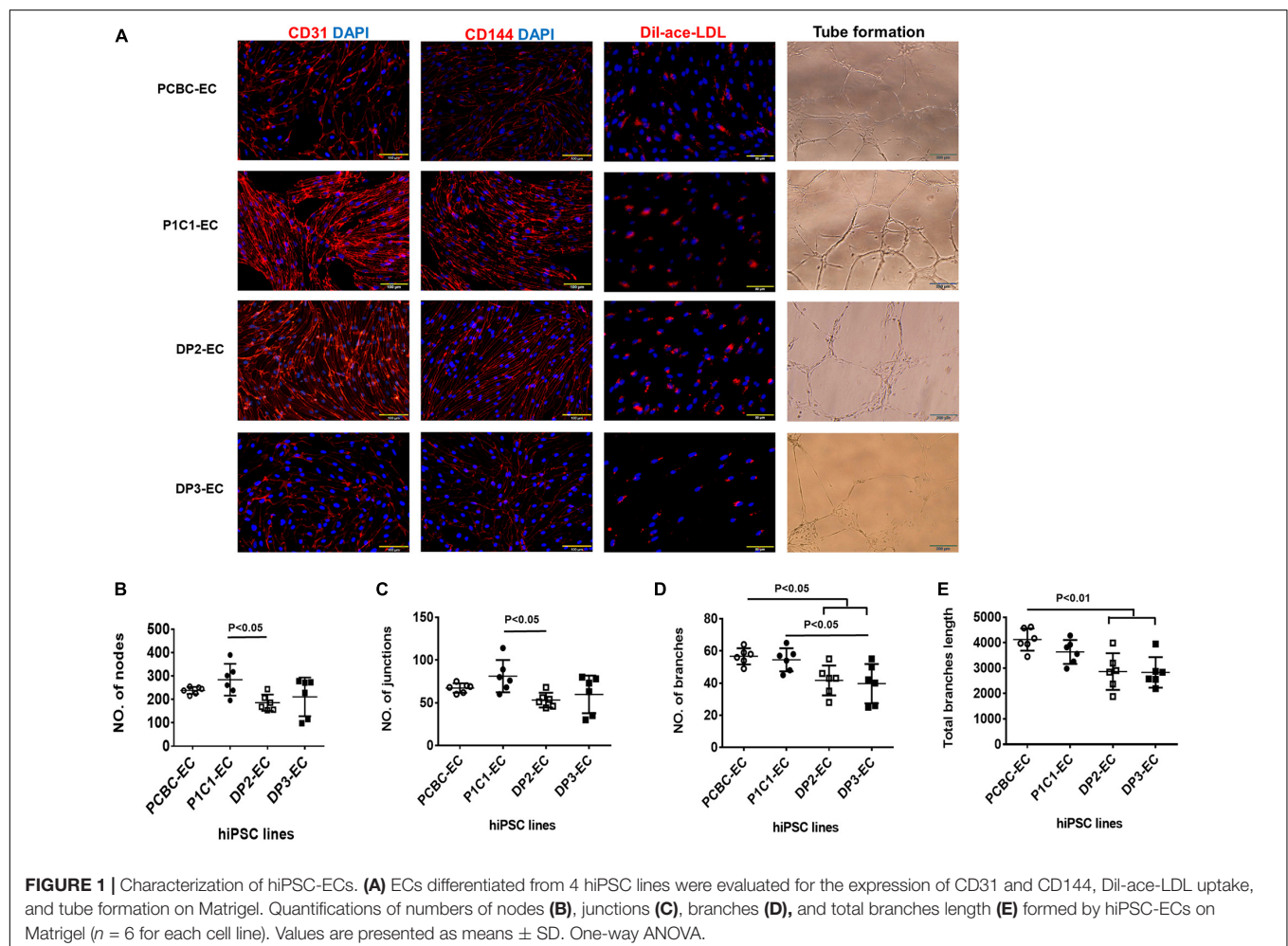
hiPSC-ECs Had Typical EC Characteristics, but dia-hiPSC-ECs Were Less Potent for *in vitro* Angiogenesis

Endothelial cells differentiated from 4 hiPSC lines co-expressed CD31 and CD144 (**Figure 1A**). Functional assessments

confirmed that hiPSC-ECs were able to take up DiI-ace-LDL and form tubular structures on Matrigel (**Figure 1A**). However, the formation of tubular structures was less extensive for DP2-ECs and DP3-ECs than PCBC-ECs and P1C1-ECs. P1C1-ECs formed the highest numbers of nodes (238.33 ± 15.4 , $p < 0.05$) and junctions (67.3 ± 5.5 , $p < 0.05$) which were significantly higher than DP2-ECs (185.8 ± 34.8 and 53.2 ± 8.6 , respectively, **Figures 1B,C**). PCBC-ECs formed the highest numbers of branches (56.7 ± 5 , $p < 0.05$) and had the longest total branch length ($4,122 \pm 437.2$, $p < 0.01$) which were significantly higher than DP2-ECs (41.7 ± 9.3 and 2864.3 ± 719.9 , respectively) and DP3-ECs (39.7 ± 12.2 and 2830.8 ± 600 , respectively), while P1C1-ECs (54.5 ± 7.2 , $p < 0.05$) formed significantly higher number of branches than DP3-ECs only (**Figures 1D,E**). These results suggest that ECs differentiated from dia-hiPSCs are less potent for *in vitro* angiogenesis.

Dia-hiPSC-ECs Had a Trend for Reduced NO Production and Increased Endothelin-1 Secretion

Reduced NO production and increased production of vasoconstrictor, such as endothelin-1, are typical phenotypes of



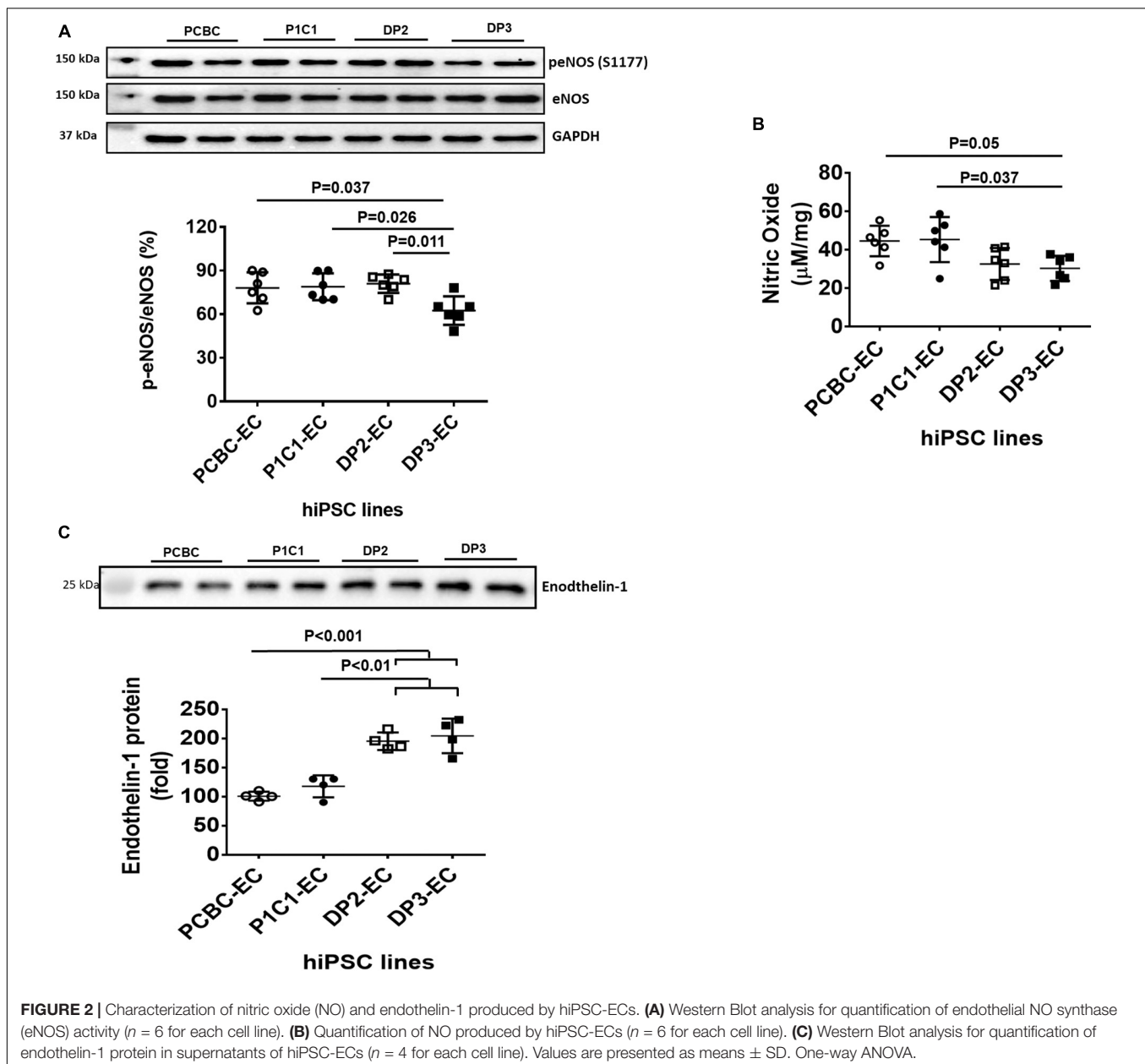
endothelial dysfunction. Thus, we measured and compared NO and endothelin-1 production among 4 hiPSC-EC lines. Western Blot showed that activities of eNOS in PCBC-EC ($78.1 \pm 11.7\%$) and P1C1-EC ($79.2 \pm 9.3\%$) were similar to that of DP2-EC ($77.1 \pm 11.1\%$) and were significantly higher than that of DP3-EC ($59.6 \pm 11.4\%$, $p < 0.05$ for both; **Figure 2A**). Quantification of NO measured by ELISA showed that although PCBC-EC ($44.6 \pm 7.9 \mu\text{M}/\text{mg}$) and P1C1-EC ($45.4 \pm 11.7 \mu\text{M}/\text{mg}$) had high and DP2-EC ($32.6 \pm 8.3 \mu\text{M}/\text{mg}$) and DP3-EC ($30.4 \pm 6.5 \mu\text{M}/\text{mg}$) had low NO productions, the significant difference was only seen between P1C1-ECs and DP3-ECs ($p < 0.05$; **Figure 2B**).

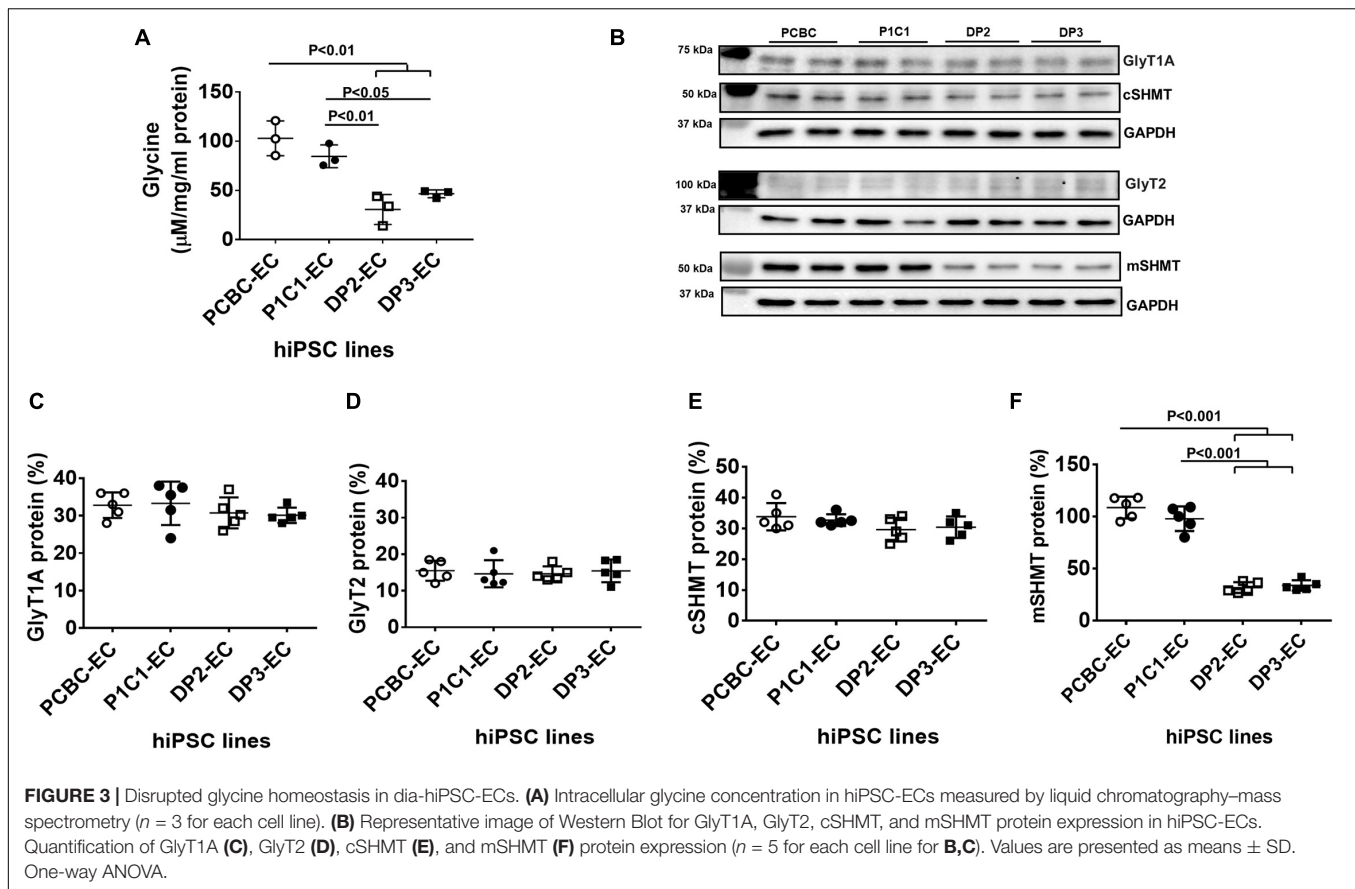
Endothelin-1 in supernatant was analyzed using Western Blot (**Figure 2C**). Endothelin-1 protein in supernatant is

significantly increased in DP2-EC (increased by 90 and 66%, respectively) and DP3-EC (increased by 104 and 74%, respectively) compared to PCBC-ECs and P1C1-ECs. This indicates that dia-hiPSC-ECs secrete higher levels of endothelin-1 compared to healthy hiPSC-ECs.

Disrupted Glycine Homeostasis in Dia-hiPSC-ECs

Metabolomic analysis of amino acid profiles showed that intracellular glycine was significantly lower in dia-hiPSC-ECs compared to healthy hiPSC-ECs (**Figure 3A**). The intracellular glycine concentration in DP2-EC was 29.6 or 36.2% and DP3-EC was 45.2 or 55% of PCBC-ECs or P1C1-ECs. As intracellular





glycine content is maintained by uptake from the media by the transporters GlyT1 and GlyT2 and also by conversion from serine by cSHM and mSHMT, we further measured their protein expression levels. Western Blot showed that the major isoform of GlyT1 is GlyT1A (70.5 kDa), not GlyT1C (78 kDa). Protein expression levels of GlyT1A, GlyT2, and cSHMT were similar among four cells lines (**Figures 3B–E**).

On the contrary, the protein expression levels of mSHMT were significantly lower in the dia-hiPSC-ECs (DP2-ECs = $32.7 \pm 4.4\%$ and $39.7 \pm 10.2\%$) than the healthy hiPSC-ECs (P1C1-EC = $107.1 \pm 9.1\%$ and P1C1-EC = $101.9 \pm 20.1\%$, $p < 0.001$ for both), indicating the low intracellular glycine level may be due to low protein expression of mSHMT (**Figures 3B,F**).

Dia-hiPSC-ECs Had Slow Growth Rates and Increased Senescence

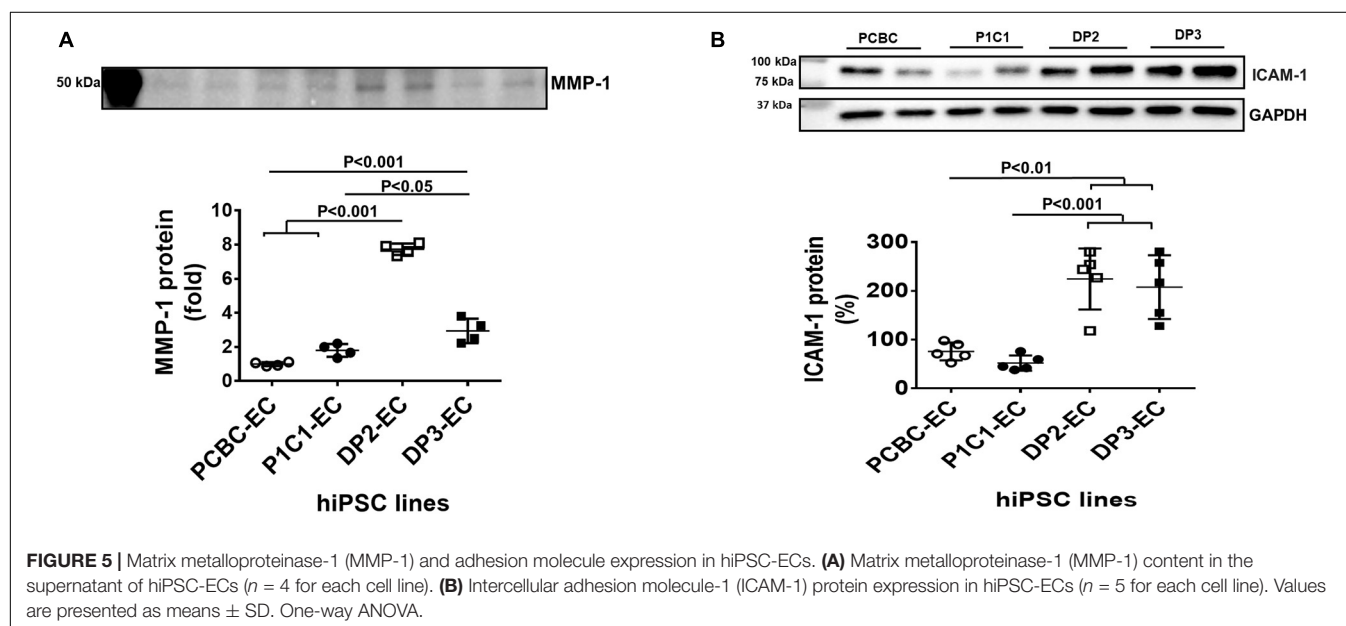
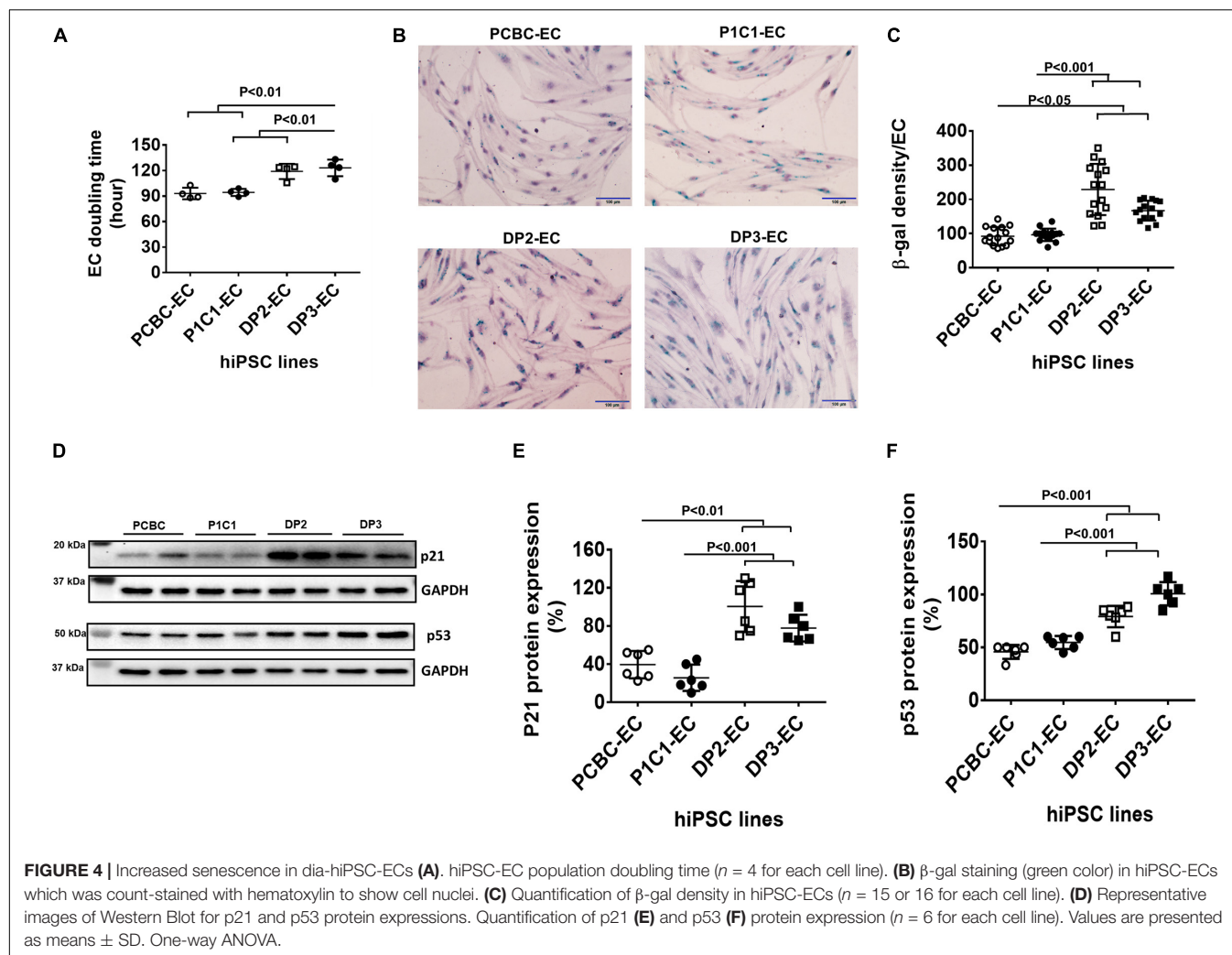
Cell doubling time increased by 26 and 30% in DP2-ECs and DP3-ECs, respectively, compared to PCBC-ECs and P1C1-ECs, indicating slower cell growth rate in dia-hiPSC-ECs than healthy hiPSC-ECs (**Figure 4A**). A cell senescence assay showed that β -gal protein expression significantly increased by 145 and 137%, respectively, in DP2-EC and by 88.9 and 82.7%, respectively, in DP3-EC compared to PCBC-EC and P1C1-EC (**Figures 4B,C**).

Western Blot showed that p21, not p16 (data not shown), protein expression significantly increased in DP2-ECs ($100.42 \pm 26.8\%$) and DP3-ECs ($77.8 \pm 14\%$) compared to PCBC-ECs ($39.5 \pm 14.4\%$, $p < 0.01$ for both) and P1C1-ECs

($25.7 \pm 13.8\%$, $p < 0.001$ for both; **Figures 4D,E**). Consistently, p53 protein expression also significantly increased in DP2-ECs (72.9 and 44.8%, respectively) and DP3-ECs (117 and 81.2%, respectively) compared to PCBC-ECs and P1C1-ECs (**Figures 4D,F**). These results suggest that dia-hiPSC-ECs have increased senescence which may be an underlying cause of slow cell growth rate of dia-hiPSC-ECs due to the accumulation of senescent ECs.

The Senescence-Associated Secretory Phenotype of Dia-hiPSC-ECs

We examined markers that make up the senescence associated secretory phenotype (SASP) (Zeng and Millis, 1996; Coppe et al., 2010; Freitas-Rodriguez et al., 2017). Using the Human Angiogenesis Arrays Q3 Kit, we found that angiopoietin-1 reduced and MMP-1, VEGF, and PECAM-1 increased in the supernatant of dia-hiPSC-ECs as compared to the supernatant of healthy hiPSC-ECs (**Supplementary Table 2**). Up-regulated MMP-1 and VEGF have been seen in senescent cells and belong to the senescence-associated secretory phenotype (Coppe et al., 2010). Furthermore, Western Blot showed that protein concentration of MMP-1 was significantly higher in the supernatant of the dia-hiPSC-ECs than the healthy hiPSC-ECs ($p < 0.001$: PCBC-EC vs. both DP2-EC and DP3-EC; $p < 0.001$: P1C1-EC vs. DP2-EC; $p < 0.05$: P1C1-EC vs. DP3-EC; **Figure 5A**). The MMP-1 concentration was about 0.8-folds in P1C1-EC supernatant,



6.74-folds in DP2-EC supernatant, or 1.94-folds in DP3-EC supernatant as compared to PCBC-EC supernatant which was considered as 100%.

Increased ICAM-1 Protein Expression

Adhesion molecules, including ICAM-1 and VCAM-1 were determined by Western Blot. Protein expression of ICAM-1 was significantly higher in DP2-EC ($224.6 \pm 62.6\%$) and DP3-EC ($207.8 \pm 65.3\%$) than in PCBC-EC ($75.8 \pm 18.2\%$, $p < 0.01$ for both) and P1C1-EC ($52.1 \pm 15.65\%$, $p < 0.001$ for both; **Figure 5B**). However, VCAM-1 was undetectable (data not shown).

Mitochondrial Dysfunction in Dia-hiPSC-ECs

JC-1 dye staining of hiPSC-ECs showed that mitochondria of dia-hiPSC-ECs had significantly low membrane potential in dia-hiPSC-ECs compared to PCBC-ECs and P1C1-ECs

(**Figure 6A**). The fluorescence intensity reduced significantly by 37.8 and 40.8%, respectively, in DP2-ECs and by 37 and 40.1%, respectively, in DP3-ECs compared to PCBC-ECs ($p < 0.05$ for both) and P1C1-ECs ($p < 0.05$ for both; **Figure 6B**).

Furthermore, Western Blot showed that although mitochondrial protein expression of Mfn-1 and Mfn-2 were similar among 4 hiPSC-EC lines, protein expression of Fis-1, a protein responsible for mitochondrial fission, was significantly higher in DP2-ECs ($82.5 \pm 8.8\%$) and DP3-ECs ($76.9 \pm 14.55\%$) compared to PCBC-ECs ($50.8 \pm 4.9\%$, $p < 0.01$ for both) and P1C1-ECs ($42.5 \pm 11\%$, $p < 0.01$ for both; **Figures 6C–F**).

ATP production significantly decreased in DP2-EC and DP3-EC as compared with PCBC-EC and P1C1-EC. ATP production indicated by luminescence intensity in DP2-EC was 34 or 35.1% and DP3-EC was 30.3 or 31.3% of PCBC-ECs ($p < 0.001$ for both) or P1C1-ECs ($p < 0.001$ for both;

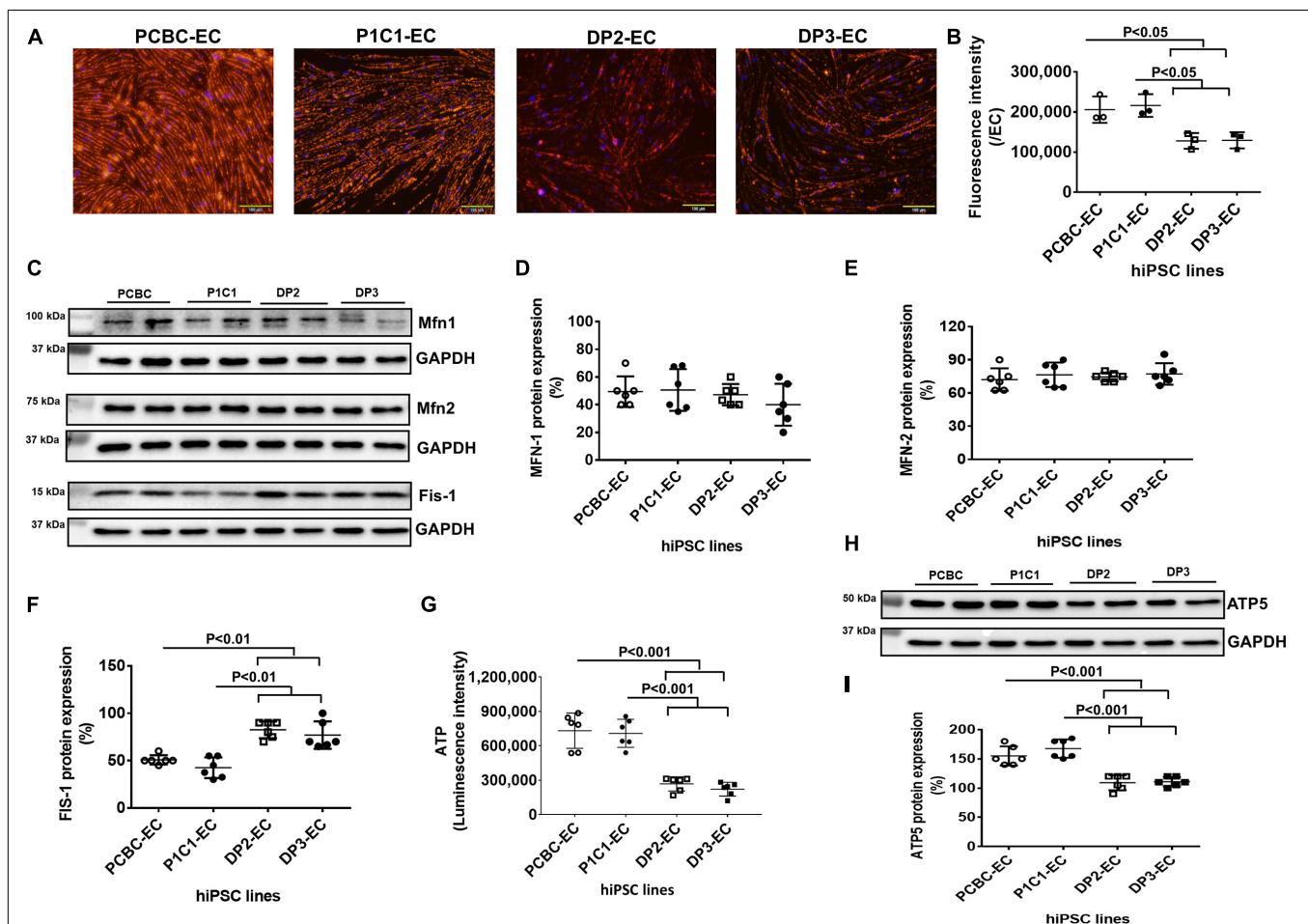


FIGURE 6 | Impaired mitochondrial function in dia-hiPSC-ECs. **(A)** JC-1 staining to visualize mitochondrial membrane potential. **(B)** Quantification of hiPSC-EC mitochondrial membrane potential. **(C)** Representative images of Western Blot for mitochondrial proteins in hiPSC-ECs, including mitofusin-1 (Mfn-1), mitofusin-2 (mfn-2), and Fission-1 (Fis-1). Quantification of MFN-1 **(D)**, MFN-2 **(E)**, and FIS-1 **(F)** protein expression. **(G)** Quantification of ATP production in hiPSC-ECs. **(H)** Western Blot analysis for ATP synthase 5 (ATP5) expression. **(I)** Quantification of ATP5 protein expression ($n = 6$ for each cell line). Values are presented as means $\pm$ SD. One-way ANOVA.

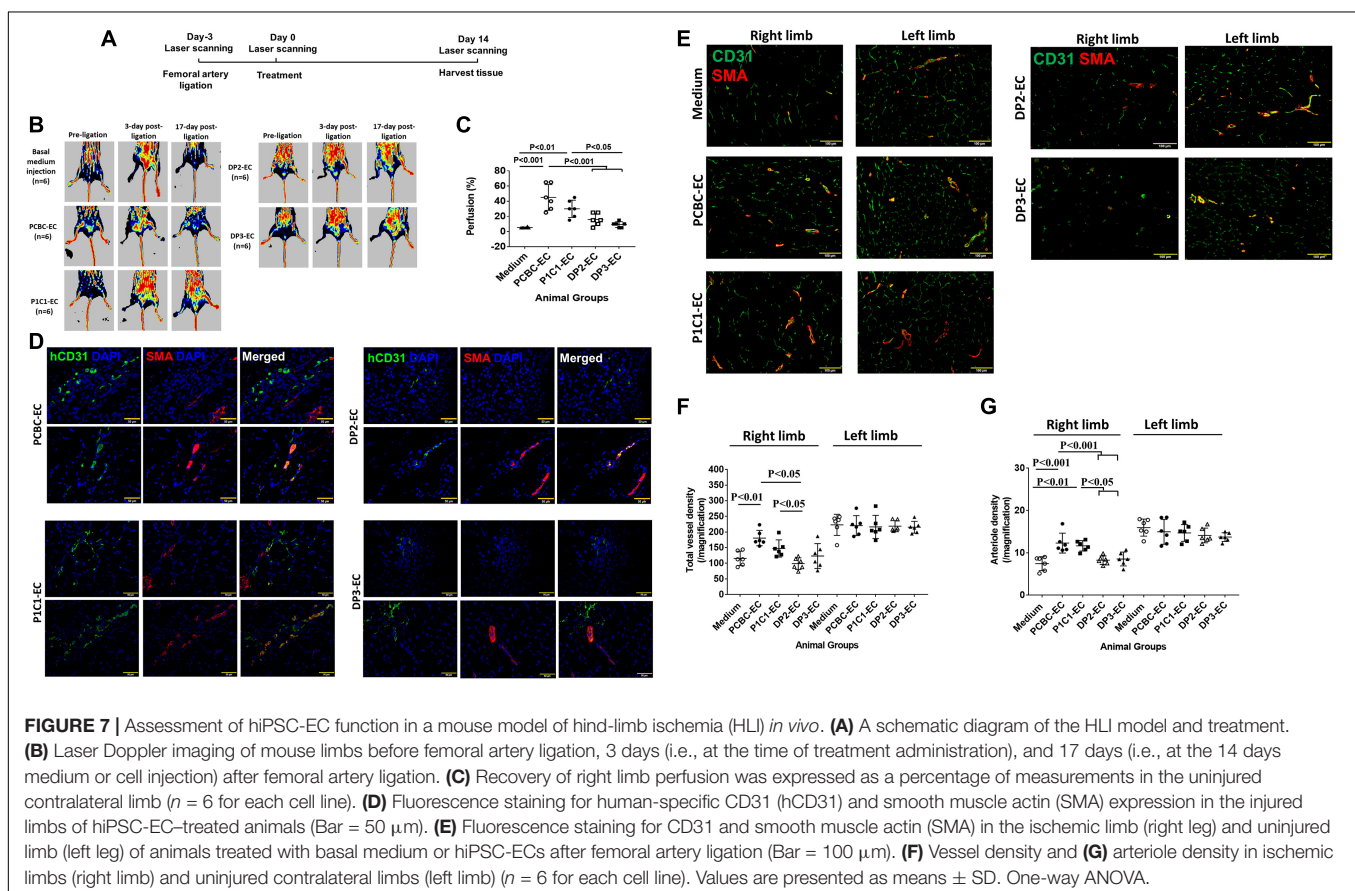
Figure 6G). Consistently, protein expression of ATP5, a subunit of ATP synthase, significantly decreased in dia-hiPSC-ECs (DP2-EC = $109.2 \pm 12.8\%$ and DP3-EC = $110.8 \pm 8\%$) compared to healthy hiPSC-ECs (PCBC-EC = $155 \pm 16.4\%$, $p < 0.001$ for both and P1C1 = $167.5 \pm 15.7\%$, $p < 0.001$ for both; **Figures 6H,I**). These results suggest that dia-hiPSC-ECs have impaired mitochondrial function as evidenced by low mitochondrial membrane potential and ATP production, decreased ATP5 protein expression, and increased Fis-1 protein expression.

dia-hiPSC-EC Have Poor Angiogenic Potential for Treatment of HLI

The potential of dia-hiPSC-ECs for treatment of ischemic disease was evaluated in a mouse model of HLI (**Figure 7A**; Su et al., 2018). Perfusion was barely detectable in mouse limbs of the basal medium group at 14 days post-treatment. However, perfusion was recovered to 45 or 35% in the ischemic limbs of the PCBC-EC or P1C1-EC group, which was not only significantly greater than the extent of recovery in the basal medium injected mice ($5.7 \pm 1\%$, $p < 0.001$ or $p < 0.01$), but also than that in the DP2-EC ($15.8 \pm 8\%$, $p < 0.001$ vs. PCBC-EC group) or DP3-EC group ($12.7 \pm 3.8\%$, $p < 0.001$ vs. PCBC-EC group or $p < 0.05$ vs. P1C1-EC group; **Figures 7B,C**).

Tissue sections stained for the specific hCD31 and SMA indicated that the transplanted hiPSC-ECs were present both in capillaries and smooth muscle cell coated arterioles in all animal groups that received hiPSC-EC transplantation (**Figure 7D**), suggesting that transplanted hiPSC-ECs can contribute to capillary and arteriole formation in ischemic limbs.

Assessments in sections stained for CD31 (detecting both hiPSC-EC and mouse EC) and SMA (**Figure 7E**) indicated that total vessel density was significantly higher in the ischemic limbs of animals in the PCBC-EC group (180.5 ± 24.7) than in the basal medium (115.5 ± 22.9 , $p < 0.01$) or DP2-EC (98.9 ± 18.5 , $p < 0.05$) group, while total vessel density of the P1C1 group (was only significantly higher than the DP2-EC group (**Figure 7F**). Arteriole densities in the ischemic limbs of animals were significantly higher in the PCBC-EC (12.3 ± 2.3) or P1C1 (11.6 ± 1.2) groups than the basal medium (7.4 ± 1.6 , $p < 0.001$ vs. PCBC-EC group or $p < 0.01$ vs. P1C1-EC group), DP2-EC (8.2 ± 1 , $p < 0.001$ or $p < 0.05$), and DP3-EC (8.5 ± 1.6 , $p < 0.001$ or $p < 0.05$) groups (**Figure 7G**). The increased arteriole density may be due to the angiopoietin-1 secreted by healthy hiPSC-ECs after transplantation (**Supplementary Table 2**). Collectively, these observations suggest that transplanted dia-hiPSC-ECs had poor angiogenic potential in restoring perfusion and stimulating neovascularization in ischemic limb.



DISCUSSION

We characterized hiPSC-ECs derived from patients with T2DM and identified feature phenotypes in two dia-hiPSC-EC lines: disrupted glycine homeostasis, increased senescence, and impaired mitochondrial function and angiogenic potential.

Diabetes causes endothelial dysfunction (Avogaro et al., 2011; Giraldo-Grueso and Echeverri, 2020). Some of the mediators of endothelial dysfunction include reduced bioavailable NO, increased production of vasoconstrictors, such as endothelin-1 and angiotensin II, and dysregulated inflammation or thrombosis (DeFronzo et al., 2015; Palmer A.K. et al., 2015; Yan-Do and MacDonald, 2017; Alves et al., 2019; Kruger-Genge et al., 2019; Rashvand et al., 2019; Giraldo-Grueso and Echeverri, 2020). Endothelial dysfunction is considered as an early step in the cascade of adverse events that lead to atherosclerosis and ischemic heart disease (Giraldo-Grueso and Echeverri, 2020). In addition, diabetes causes impaired angiogenesis (Kota et al., 2012; Fadini et al., 2019) and induces endothelial senescence (Shosha et al., 2018; Bitar, 2019) and mitochondrial dysfunction (Teodoro et al., 2018; Yarbeygi et al., 2019), all of which have been associated with diabetic complications and are being investigated as therapeutic targets.

hiPSCs generated human ECs not only can be used for treatment of ischemic diseases, but also can be used for modeling diseases and drug screening and discovering *in vitro* (Liu et al., 2018; Rowe and Daley, 2019; Williams and Wu, 2019). Although ECs differentiated from hiPSCs of pulmonary hypertension and Hutchinson–Gilford progeria syndrome have been studied (Zhang et al., 2011; Gu et al., 2017; Sa et al., 2017; Matrone et al., 2019), few studies have characterized the function of iPSC derived ECs in diabetes or metabolic syndrome in rodents (Takenaka-Ninagawa et al., 2014; Gu et al., 2015) and human (Vila Gonzalez et al., 2020). The endothelial dysfunction of mouse iPSC derived from obesity is due to decreased NO production (Gu et al., 2015). Only one study has investigated ECs derived from hiPSCs of patients with diabetic macular edema (Vila Gonzalez et al., 2020). The results of this work were able to recapitulate clinical evaluations of anti-VEGF responsiveness *in vitro*. Thus, feature phenotypes are largely unknown in hiPSC-ECs from patients with diabetes. In this study, we characterized dia-hiPSC-ECs and found that they not only recapitulated several phenotypes of endothelial dysfunction, such as increased vasoconstrictor (endothelin-1) and adhesion molecule (ICAM-1) production and impaired angiogenesis, but also showed disrupted glycine homeostasis, increased senescence, and impaired mitochondrial function.

Although decreased NO production is a feature phenotype of endothelial dysfunction, significantly decreased eNOS activity and NO production were only found in DP3-ECs in this study. However, endothelin-1 and ICAM-1 production significantly increased in both dia-hiPSC-EC lines. These results indicate that increased vasoconstrictor and adhesion molecule expression may be more common phenotypes than reduced NO production in dia-hiPSC-ECs.

One signature phenotype we found in this study is the disrupted glycine homeostasis, including low intracellular glycine

content. This is consistent with clinical findings of serum glycine level which is lower not only in T2DM patients, but also in patients with impaired glucose tolerance (Wang-Sattler et al., 2012; Drabkova et al., 2015; Palmer N.D. et al., 2015). Serum glycine concentration was negatively associated with steady-state plasma glucose (Seibert et al., 2015) and has been identified as a serum biomarker of both insulin sensitivity and regional fat mass in functionally limited older adults (Lustgarten et al., 2013). Low intracellular glycine level may compromise vascular endothelial growth factor signaling in ECs and lead to poor angiogenic potential and mitochondrial function (Guo et al., 2017). The observed low intracellular glycine content may be due to low protein expression levels of mSHTM. Intracellular glycine homeostasis is modulated by GlyT (GlyT1 and GlyT2) and SHMT (cSHMT and mSHMT). Except for mSHMT, the other three protein expression levels are similar between the healthy hiPSC-ECs and dia-hiPSC-ECs. Future studies are needed to explore the mechanism of how disrupted intracellular glycine homeostasis causes endothelial dysfunction in dia-hiPSC-ECs for a better understanding the role of glycine homeostasis in endothelial function in diabetes.

Increased senescence is found in dia-hiPSC-ECs. Feature characteristics of senescent dia-hiPSC-ECs include up-regulated β -gal, p21, and p53 protein expression and increased secretion of MMP-1 and VEGF which are components of a SASP (Coppe et al., 2010), and impaired mitochondrial function. Mitochondrial dysfunction is composed of up-regulated Fis-1 protein expression, decreased mitochondrial membrane potential, ATP synthase protein expression, and ATP production in dia-hiPSC-ECs. Although senescence may be a causal role in mitochondrial dysfunction (Korolchuk et al., 2017), it may be that mitochondrial dysfunction itself leads to senescence (Wiley et al., 2016). Increased senescence in dia-hiPEC-ECs may be due to the high protein expression levels of p53 and p21 as the p53/p21 pathway leads to the inhibition of Cdk4/6 activity and contributes to senescence (Sharpless and Sherr, 2015). Poor cell viability associated with senescent dia-hiPSC-ECs may result in poor angiogenic potential *in vitro* and *in vivo*.

One limitation of the current study is the number of hiPSC lines used. We only used 2 hiPSC lines each of healthy subjects and patients with T2DM. Although robust difference was observed in glycine homeostasis, endothelial senescence, mitochondrial function, angiogenic potential in dia-hiPSC-ECs as compared with healthy hiPSC-ECs, they may be limited in 2 dia-hiPSC-EC lines used in this study.

In conclusion, we identified feature phenotypes in dia-hiPSC-ECs: disrupted glycine homeostasis, increased senescence, impaired mitochondrial function and angiogenesis. Efforts are needed to find the key causal factors and the molecular mechanisms contributing to the onset and development of disrupted glycine homeostasis and senescence in dia-hiPEC-ECs. The features identified here may serve as targets for new therapeutic strategies for the treatment of endothelial dysfunction in diabetes.

DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and in its online **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Singapore Health Services Pte. Ltd.

AUTHOR CONTRIBUTIONS

LS, XK, SL, and YG: collection and/or assembly of data and data analysis and interpretation. J-PK: manuscript writing and editing. XS and JM: provision of study material and manuscript writing.

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TBX20 Contributes to Balancing the Differentiation of Perivascular Adipose-Derived Stem Cells to Vascular Lineages and Neointimal Hyperplasia

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Background: Perivascular adipose-derived stem cells (PVASCs) can contribute to vascular remodeling, which are also capable of differentiating into multiple cell lineages. The present study aims to investigate the mechanism of PVASC differentiation toward smooth muscle cells (SMCs) and endothelial cells (ECs) as well as its function in neointimal hyperplasia.

Methods: Single-cell sequencing and bulk mRNA sequencing were applied for searching key genes in PVASC regarding its role in vascular remodeling. PVASCs were induced to differentiate toward SMCs and ECs *in vitro*, which was quantitatively evaluated using immunofluorescence, quantitative real-time PCR (QPCR), and Western blot. Lentivirus transfections were performed in PVASCs to knock down or overexpress TBX20. *In vivo*, PVASCs transfected with lentivirus were transplanted around the guidewire injured femoral artery. Hematoxylin-eosin (H&E) staining was performed to examine their effects on neointimal hyperplasia.

Results: Bulk mRNA sequencing and single-cell sequencing revealed a unique expression of TBX20 in PVASCs. TBX20 expression markedly decreased during smooth muscle differentiation while it increased during endothelial differentiation of PVASCs. TBX20 knockdown resulted in the upregulation of SMC-specific marker expression and activated Smad2/3 signaling, while inhibiting endothelial differentiation. In contrast, TBX20 overexpression repressed the differentiation of PVASCs toward smooth muscle cells but promoted endothelial differentiation *in vitro*. Transplantation of PVASCs transfected with TBX20 overexpression lentivirus inhibited neointimal hyperplasia in a murine femoral artery guidewire injury model. On the contrary, neointimal hyperplasia significantly increased in the TBX20 knockdown group.

Conclusion: A subpopulation of PVASCs uniquely expressed TBX20. TBX20 could regulate SMC and EC differentiation of PVASCs *in vitro*. Transplantation of PVASCs after vascular injury suggested that PVASCs participated in neointimal hyperplasia via TBX20.

Keywords: Tbx20, perivascular adipose-derived stem cells, SMC differentiation, EC differentiation, neointimal hyperplasia

INTRODUCTION

Adipose tissue is important for cardiovascular homeostasis. Adipose tissue is initially thought as an organ for energy storage but was recently discovered to be rich in adipose-derived stem cells (ASCs). These cells express the surface markers of adult mesenchymal stem cells and can give rise to multiple lineages, such as cardiomyocytes, endothelial cells, smooth muscle cells, osteoblasts, chondrocytes, and adipocytes (Gimble et al., 2007; Bunnell et al., 2008). Previous studies mainly focused on subcutaneous and abdominal adipose tissue and their inner ASCs for their easy access and abundance in number (Pitrone et al., 2017). Little attention has been paid to characterizing the resident stem cells in perivascular adipose tissue (PVAT), named PVASCs. PVAT locates outside the adventitial layer of systemic blood vessels except cerebral vessels and participates in regulating vascular function (Rajsheker et al., 2010). In a murine femoral artery guidewire injury model, transplantation of PVAT accelerated neointimal hyperplasia whereas subcutaneous adipose tissue inhibited neointimal hyperplasia (Manka et al., 2014). Gu et al. (2019) demonstrated that PVASCs transferred to the perivascular side of grafted vein could migrate to neointima and subsequently differentiate into SMCs. Pan et al. (2019) confirmed that PVASCs from old mice could aggravate neointimal hyperplasia compared to those from the young in a murine guidewire femoral artery injury model. However, the mechanism of PVASCs differentiation toward SMCs is still unclear.

The differentiating capacity of ASCs from other depots toward ECs and SMCs *in vitro* has been verified (Ma et al., 2017; Arderiu et al., 2019). SMC differentiation is associated with a high expression of SMC-specific proteins, such as smooth muscle α -actin (α -SMA), Calponin, and SM22 α (Mack, 2011). Transforming growth factor- β 1 (TGF- β 1) was commonly applied for SMC differentiation in multiple pluripotent stem cells (Owens, 1995). In the Smad-dependent signaling pathway, TGF- β 1 activates the TGF- β receptor, leading to the phosphorylation of Smad2/3. The phosphorylated Smad2/3 forms a complex with Smad4 and translocates to the nucleus, subsequently modulating target gene expression (Derynck and Zhang, 2003). ECs are generally identified through a variety of specific markers, such as CD31, VE-cadherin, VEGFR1, and VEGFR2. Increasing evidence

indicated that ASCs from other depots could give rise to ECs *in vitro* and had proangiogenic effects in the hindlimb ischemia model (Planat-Benard et al., 2004; Hutchings et al., 2020). However, the differentiation of PVASCs toward ECs has not been investigated yet.

TBX20 is a crucial transcription factor for the heart, eyes, ventral neural tube, and limbs during embryonic development. Its deficiency could cause congenital heart disease (Meins et al., 2000). In humans, mutations in TBX20 were associated with a complex spectrum of developmental abnormalities, including defects in septation, chamber growth, and cardiomyopathy (Kirk et al., 2007). TBX20 knockout in mice results in embryonic lethality around E10.5 (Stennard et al., 2005). Overexpression of TBX20 in adult cardiomyocytes promoted proliferation and improved the myocardial infarction outcomes in mice (Xiang et al., 2016). TBX20 overexpression enhanced cardiomyogenic differentiation and inhibited osteogenic differentiation in human subcutaneous adipose-derived MSCs (Neshati et al., 2018; Mollazadeh et al., 2019). In endothelial cells, TBX20 upregulated peroxisome proliferator-activated receptor- γ , suppressed the generation of reactive oxygen species, and decreased the expression of adhesion molecules in response to oxidized low-density lipoproteins (Shen et al., 2013). In zebrafish endothelial development, transient knockdown of TBX20 impaired angiogenesis (Meng et al., 2018).

Our bulk RNA-seq confirmed that PVASCs uniquely expressed TBX20. Single-cell RNA-seq analysis revealed a unique subpopulation of PVASCs which highly expressed TBX20. These findings led us to investigate its role in PVASCs. In the present study, we verified that TBX20 expression significantly changed during PVASC differentiation toward SMCs and ECs. Furthermore, we confirmed that TBX20 regulated both SMC and EC differentiation simultaneously *in vitro*. In a mouse femoral artery guidewire injury model, overexpression of TBX20 in PVASCs decreased neointimal hyperplasia after vascular injury. These findings highlighted the importance of TBX20 for PVASC differentiation and vascular neointimal hyperplasia.

MATERIALS AND METHODS

Experimental Animals

All animal experiments were approved by the Animal Ethics Committee of Zhejiang University in accordance with the Guide for the Care and Use of Laboratory Animals. C57/BL6 mice were purchased from Shanghai Model Organisms Center. All animals were fed a chow diet in a 12-h light/dark environment at 25°C in the Animal Care Facility of the Second Affiliated Hospital, Zhejiang University School of Medicine.

Abbreviations: PVASCs, perivascular adipose-derived stem cells; SMCs, smooth muscle cells; ECs, endothelial cells; H&E, hematoxylin-eosin; PVAT, perivascular adipose tissue; α -SMA, smooth muscle α -actin; SM22 α , smooth muscle 22 alpha; TGF- β 1, transforming growth factor- β 1; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; IGF-1, insulin-like growth factor; BSA, bovine serum albumin; CCK8, Cell counting kit-8; PVDF, polyvinylidene difluoride.

Isolation and Culture of Adipose-Derived Stem Cells

ASCs were isolated from mouse subcutaneous, abdominal, and perivascular adipose tissue, as described previously (Gu et al., 2019). Briefly, adipose tissue was cut into 1-mm³ small pieces, which was then digested with 2 mg/mL collagenase type I (Gibco) and 1 mg/mL Dispase II (Sigma) in Hanks' balanced salt solution (HBSS) at 37°C for 30–45 min with occasional vortex. The digestion was stopped by DMEM/F12 with 10% FBS and subsequently passed through a 100-μm filter followed by a centrifugation at 300 g for 5 min. Cell precipitates were resuspended in a stem cell culture medium as the following: Minimal Essential Medium-α (Gibco) with 15% fetal bovine serum (Gibco), 10 ng/mL recombinant human leukemia inhibitory factor (Sigma), 5 ng/mL recombinant human FGF (R&D), 2 mmol/L L-glutamine (Sigma), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco); they were then incubated in a 5% CO₂ incubator. The cells were passaged at a ratio of 1:3 every 2 or 3 days. The medium was refreshed every 2 days.

Cell Identification

PVASCs at passages 3–6 were analyzed using a fluorescence-activated cell sorter (FACS). Briefly, cells were incubated for 30 min at 4°C with the following antibodies: Sca1-PerCP (abcam); CD105-FITC (abcam); CD90 (Invitrogen); CD45-FITC (abcam); and CD31-PE (BD Biosciences). The cells without any staining were served as blank. Flow cytometry was performed on a BD flow cytometer. FCS files were exported and analyzed using FlowJo V10 software.

The adipogenic differentiation medium, chondrogenic differentiation medium, and osteogenic differentiation medium were purchased from CHEM. PVASCs were seeded on gelatin-coated plates and then cultured with a specific differentiation medium and maintained for 2 weeks. The medium was changed every 3 days. After 2 weeks, cells were stained with Alcian Blue, Oil red O, or Alizarin Red S (CHEM) following the manufacturers' instructions, respectively, to assess adipogenic, chondrogenic, and osteogenic differentiation.

PVASCs are positive for Sca1, CD90, and CD105 and negative for CD45 and CD31 (**Supplementary Figures 1A,B**). PVASCs were capable of differentiating into adipocytes, chondrocytes, and osteoblasts, respectively (**Supplementary Figures 1C–E**).

Cell Differentiation

PVASCs from passages 3 to 6 were used for this study. For smooth muscle differentiation, 5 ng/mL Recombinant Mouse TGF-β1 (R&D) was added to induce the cell differentiation. For endothelial cell differentiation, cells were cultured in EGM-2 medium (EGM-2 Bullet kit; Lonza) enriched with growth factors such as VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor), EGF (epidermal growth factor), IGF-1 (insulin-like growth factor), ascorbic acid, hydrocortisone, and heparin, according to instruction provided by the medium manufacturers. Differentiated cells were analyzed by QPCR, Western Blot, and immunofluorescence within 7 days.

Lentiviral Vector Transduction

Lentiviruses containing full-length cDNA of TBX20 (LV-TBX20) or negative control viruses (LV-NC) were constructed by GeneChem (Shanghai, China). Lentiviral particles containing shRNA against TBX20 (sh-TBX20) or scramble shRNA (sh-scramble) were prepared by GeneChem (Shanghai, China). The lentiviruses were transfected into PVASCs with MOI 50, according to the manufacturer's protocol. The efficiency of lentiviruses was verified by QPCR and Western Blot analysis after 72 h.

Western Blot

Protein was extracted from ASCs using RIPA lysis buffer (Beyotime) and then was quantified using a BCA Protein Assay Kit (Thermo Fisher). Ten to fifty microgram of each protein sample was separated via SDS-PAGE and electro-transferred onto PVDF membranes. Following blockade with PBST containing 5% BSA, the membranes were incubated with the following primary antibodies overnight at 4°C: anti-β-Actin (Santa Cruz); anti-TBX20 (sigma); anti-α-SMA (abcam); anti-Calponin (abcam); anti-SM22α (abcam); anti-Smad2 (HuaBio); anti-Smad3 (HuaBio); anti-phospho-Smad2 (HuaBio); anti-phospho-Smad3 (HuaBio); anti-CD31 (HuaBio); anti-VE-cadherin (R&D); and anti-VEGFR1 (HuaBio). The membranes were then washed four times with Tris-buffered saline/0.5% Tween-20 and incubated at 37°C for 1 h with the secondary antibodies (1:3,000; Thermo Fisher). Antigen and antibody complexes were detected by using an ECL Kit (Millipore). Immunoblots were quantified using Image Lab (version 6.0) software.

Quantitative Real-Time PCR

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One thousand nanograms of total RNA was used for each reaction of reverse transcription by using PrimeScript RT reagent Kit (Takara) and then subjected to real-time PCR using TB Green Premix Ex Taq II (Tli RNase H Plus) kits (Takara) on an Applied Biosystems 7500 Fast Real-Time PCR System (ABI). Each sample was performed in triplicate, and all results were normalized to the expression of being normalized to β-actin (ACTIN). Data was analyzed using the $2^{-\Delta\Delta C_t}$ method against β-actin. Results were expressed as fold changes compared to the control. The sequences of PCR primers were listed in **Supplementary Table 1**.

Immunofluorescence Staining

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.05% Triton X-100 for 10 min. Then, cells were stained with the following primary antibodies overnight at 4°C: α-SMA, Calponin, SM22α, CD31, VEGFR1, and VEGFR2. After washing with PBS for three times (5 min each), the cells were stained with secondary antibodies (Life Tech; Alexa Fluor 488) diluted 1:500 in PBS. Nuclei were stained with DAPI (Sigma). Images were acquired using a fluorescence confocal microscope (Leica).

5-Ethynyl-20-Deoxyuridine (EdU) Cell Proliferation Assay

Cells were seeded on a 24-well plate and cultured overnight. The 5-ethynyl-20-deoxyuridine agent (EdU, Beyotime, Shanghai, China) was added to each well and allowed 2 h for incorporation. The cells were fixed in 4% PFA for 20 min and washed three times with PBS containing 3% bovine serum albumin (BSA). The cells were incubated in PBS containing 0.3% Triton X-100 for 15 min, followed by BSA-containing PBS washes three times. The cells were stained with 5 μ g/mL Hoechst 33342 at room temperature for 10 min and washed three times with PBS.

CCK-8 Assay

Cells were counted 24 h after adhering to the 96-well plate. The original culture medium was replaced with 100 μ L fresh culture medium containing 10 μ L CCK-8 reagent (Beyotime, Shanghai, China) and continued to culture for 2 more hours. The optical density (OD) at 450 nm was measured using an automatic plate reader (Bio-Rad, United States), which was used to determine cell proliferation.

Migration Assay

Migration of PVASCs was assessed by wound-healing scratch. Cells were seeded into six-well plates to confluence for 24 h. Then, an 800- μ m tip was used to produce a uniform linear scratch in the center of the wells. The images of cell migration across the wound were captured at 0 h and 24 h using a microscope with a digital camera.

Murine Femoral Artery Guidewire Injury Model and Cell Transplantation

The procedure used for this model is well-established based on our own previous work (Xie et al., 2017). Briefly, male mice were anesthetized with an intraperitoneal injection of pentobarbital sodium. An arteriotomy was performed in the epigastric branch of both sides of the femoral arteries. A 0.014" guide wire (Hi-Torque, Cross IT 200 XT) was penetrated into the femoral artery and subsequently pulled back and forth three times, resulting in endothelial denudation. The femoral artery was ligated after the retreat of a guidewire. Sham injury without guidewire injury was regarded as negative control. All successful models were transplanted with PVASCs (sh-scramble, sh-TBX20, LV-NC, LV-TBX20). 1×10^6 cells were suspended in 30 μ L Matrigel, which was applied at the adventitial side of the injured femoral artery. Twenty-eight days after cell transplantation, the animals were sacrificed and histologically examined.

Histology

The injured femoral arteries were carefully harvested 4 weeks post operation. Samples were subsequently embedded in paraffin and cut into 4- μ m-thick sections. The sections were then stained with hematoxylin and eosin (H&E) to analyze the neointimal areas and intima/media (I/M) ratios.

Single-Cell RNA-Sequencing Analysis

The single-cell RNA-seq dataset was obtained from Professor Pingjin Gao. Detailed information of this dataset can be found in the article named "Perivascular adipose tissue-derived stromal cells contribute to vascular remodeling during aging" (Pan et al., 2019). The Seurat V3 on RStudio was applied to perform single-cell analysis for cell clustering, differential gene expression analysis, and gene expression visualization. In terms of the analysis procedure, the data was filtered by standard Seurat functions of nFeature_RNA, nCount_RNA, and percent.mt (**Supplementary Figures 2A–G**). The data was then further refined by functions including NormalizeData(), FindVariableFeatures(), RunPCA(), RunTSNE(), and FindAllMarkers() in Seurat package. The data of single-cell analysis of the aorta was extracted from "Atheroprotective roles of smooth muscle cell phenotypic modulation and the TCF21 disease gene as revealed by single-cell analysis" from Nature Medicine 2 (GSE131778).

RNA Sequence Analysis

Bulk mRNA sequencing was performed by Novogene company. Subcutaneous, abdominal, and perivascular adipose-derived stem cells at passages 3–6 were harvested for the following sequencing. Subsequent analysis was achieved by applying RStudio. Pearson analysis, box plot, PCA plot, and violin plot of FPKM were performed for quality control (**Supplementary Figures 2H–K**).

Statistical Analysis

The number of replications applied in each respective experiment is displayed in each figure individually. All the results are presented as mean $\pm$ SEM. T-student test was used between two groups, and ANOVA was used among more than two experimental groups. Values of $P < 0.05$ were considered to be significant.

RESULTS

TBX20 Expressed Highly in Perivascular Adipose-Derived Stem Cells

Previous publications demonstrated the anatomical, physiological, cellular, clinical, and prognostic differences among adipose tissues present in subcutaneous areas, abdominal cavity, and outside the adventitial layer of artery (Rittig et al., 2012). Thus, we investigated the difference of ASCs from subcutaneous adipose tissue, abdominal adipose tissue, and perivascular adipose tissue at transcriptional expression. Here, we performed bulk RNA-Seq assay for the above three kinds of ASCs. A heatmap of differentially expressed genes (DEGs) indicated significant variances among PVASCs, Ab-ASCs, and Sub-ASCs (**Figure 1A**). A total of 1,942 genes were expressed particularly in PVASCs, 949 genes in AB-ASCs, and 1,844 genes in Sub-ASCs (**Figure 1B**). The top 10 most distinct genes were plotted in the heatmap (**Figure 1C**). Among all DEGs, TBX20 raised our interest, which was highly expressed in PVASCs and previously reported to be involved with endothelial

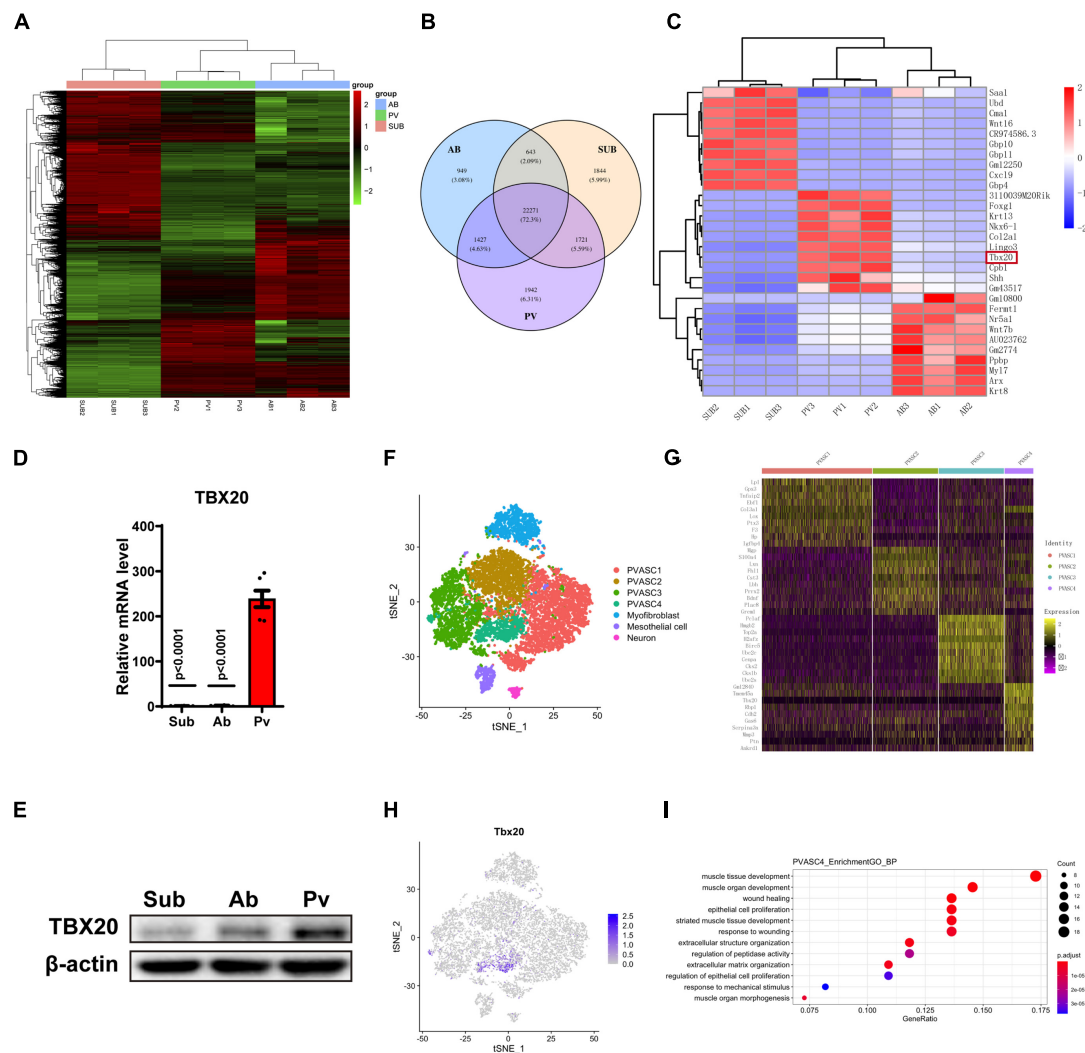


FIGURE 1 | TBX20 expressed highly in perivascular adipose-derived stem cells. **(A,B)** Bulk mRNA sequencing of subcutaneous (Sub), abdominal (Ab), and perivascular (Pv) ASCs. **(C)** Top 10 differentially expressed genes among subcutaneous, abdominal, and perivascular ASCs. **(D)** QPCR of TBX20 among subcutaneous, abdominal, and perivascular ASCs. **(E)** Western Blot of TBX20 among subcutaneous, abdominal, and perivascular ASCs. **(F–I)** Single-cell RNA-seq predicts the effect of TBX20 on capacities of PVASCs. **(F)** T-SNE map showing four subpopulations of PVASCs. **(G)** Heatmap displayed the expression of top 10 differential genes for four clusters. **(H)** Individual t-SNE visualization of TBX20 displayed the respective TBX20⁺ subpopulation. **(I)** Genotype ontology analysis represented the biological process for PVASC4.

development and angiogenesis (Meng et al., 2018). Quantitative real-time polymerase chain reaction (Figure 1D) and Western Blot (Figure 1E) validated such unique higher expression of TBX20 in PVASCs.

TBX20 May Be Involved in the Differentiation of PVASCs to SMCs and ECs

To further explore the characteristics of PVASCs, we analyzed single-cell RNA-seq data of PVASCs from a previous publication (Pan et al., 2019). The T-SNE map showed four subpopulations of PVASCs (Figure 1F and Supplementary Figure 3A). The heatmap of the top 10 DEG further displayed PVASCs'

heterogeneity, among which TBX20 was uniquely expressed in PVASCs' subpopulation 4 (Figures 1G,H). GO analysis further inferred that PVASC4 was the only subpopulation involved in both muscle development and epithelial cell proliferation, all of which contributed to vascular remodeling (Figure 1I and Supplementary Figures 3B–D). The Panther pathway confirmed that TGF-beta, FGF, Wnt, and integrin signaling pathways were among the top five correlated pathways (Supplementary Figure 3E), all of which were closely related to vascular remodeling.

Taking the analysis results and previous publications together, we hence doubted whether TBX20 regulated EC or SMC differentiation of PVASCs. To confirm this hypothesis, PVASCs were treated with TGF-β1 (5 ng/mL) for 1, 3, and 7 days to induce

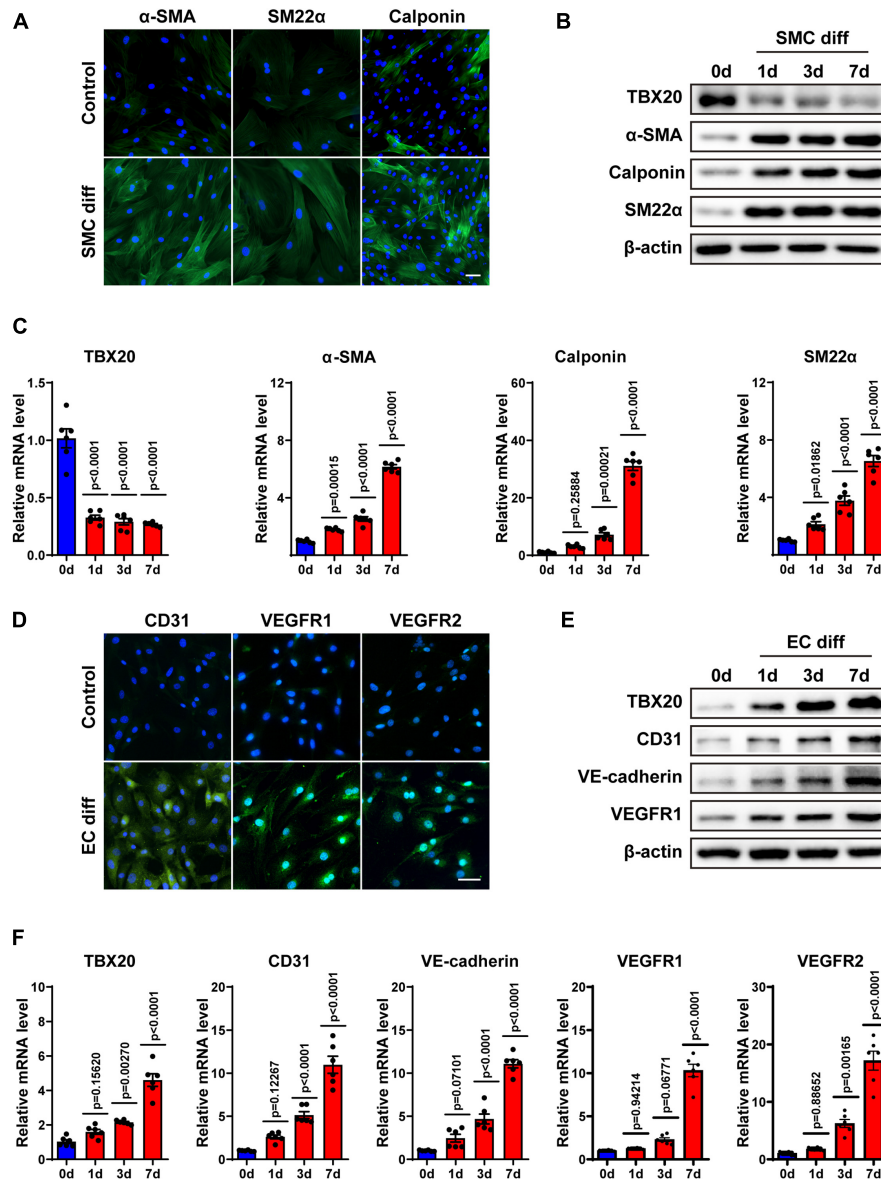


FIGURE 2 | TBX20 may play a role in the differentiation of PVASCs to SMCs and ECs. **(A)** PVASCs were treated with or without TGF- β 1 (5 ng/mL) for 7 days. Immunofluorescent staining showed the upregulation of SMC markers. **(B,C)** PVASCs were cultured in SMC differentiation medium for 0, 1, 3, and 7 days. Western Blot analysis and QPCR analysis of TBX20, α -SMA, SM22 α , and calponin. **(D)** PVASCs were cultured in the control medium or EGM-2 medium for 7 days. Immunofluorescent staining of EC markers showed upregulation in differentiated PVASCs toward endothelial cells. **(E,F)** PVASCs were cultured in the EGM-2 medium for 0, 1, 3, and 7 days. Western Blot analysis and QPCR analysis of TBX20, CD31, VE-cadherin, VEGFR1, and VEGFR2. Scale bar = 100 μ m. Cell nuclei were stained with DAPI.

SMC differentiation as previously described (Gu et al., 2019). SMC differentiation was confirmed by immunofluorescence staining (Figure 2A). As shown in Figures 2B,C, both the protein and mRNA levels of SMC differentiation-specific markers, α -SMA, SM22, and Calponin, were significantly increased in response to TGF- β 1 with the decreased TBX20 expression in a time-dependent manner.

For EC differentiation, PVASCs were induced in the EGM-2 medium for 1, 3, and 7 days. To confirm the endothelial phenotype, the induced cells were harvested and

analyzed for EC-specific markers (CD31, VEGFR1, VEGFR2) by immunofluorescence staining (Figure 2D). Real-time PCR and Western Blot showed that levels of EC markers such as CD31, VE-cadherin, VEGFR1, and VEGFR-2 were significantly increased in the differentiated cells (Figures 2E,F). Along with EC markers, in contrast to SMC differentiation, expression of TBX20 was upregulated during EC differentiation.

The above data proved that PVASCs could give rise to vascular lineages *in vitro*, establishing a theoretical foundation for its participation in vascular remodeling *in vivo*. Interestingly, the

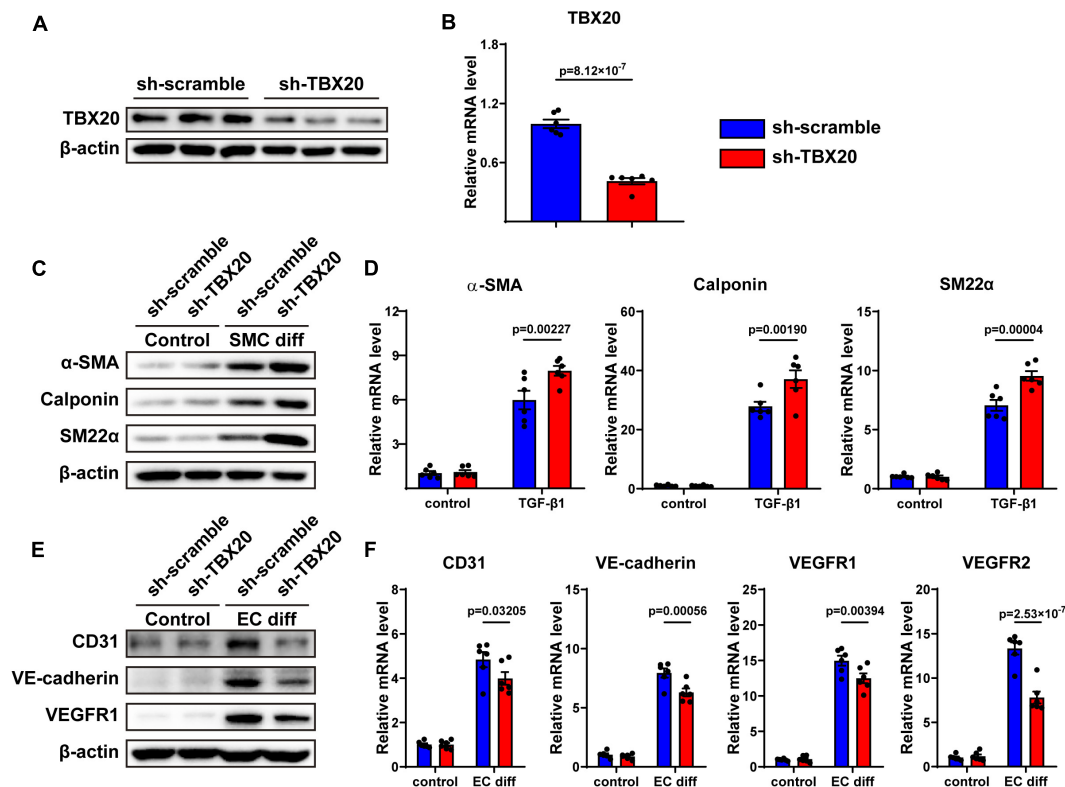


FIGURE 3 | Knockdown of TBX20 improved SMC differentiation and inhibited EC differentiation. (A,B) TBX20 level was significantly downregulated after shRNA TBX20 transfection, determined with Western Blot (A) and QPCR (B). (C,D) Transfection of PVASCs with sh-TBX20 lentivirus improved the level of SMC markers as shown by Western Blot (C) and QPCR (D). (E,F) Transfection of PVASCs with sh-TBX20 lentivirus inhibited the level of EC markers as shown by Western Blot (E) and QPCR (F).

expression of TBX20 was opposite in SMC and EC differentiation from PVASCs, which drove us to the following experiments.

Knockdown of TBX20 Improved SMC Differentiation and Inhibited EC Differentiation

To determine whether TBX20 regulated cell differentiation, PVASCs were infected with lentivirus-expressing shRNA sequences of TBX20 or sh-scramble (control) sequence. The efficiency of knockdown for TBX20 was confirmed by Western Blot and QPCR analysis (Figures 3A,B). We then treated control (sh-scramble) and TBX20 knockdown (sh-TBX20) PVASCs with TGF- β 1 to induce SMC differentiation. Both mRNA and protein levels of α -SMA, SM22 α , and Calponin were markedly increased in sh-TBX20 PVASCs (Figures 3C,D), indicating that TBX20 knockdown promoted SMC differentiation. On the contrary, after EC differentiation for 7 days, the mRNA levels and protein expressions of EC markers (CD31, VE-cadherin, VEGFR1 and VEGFR2) significantly declined upon the inhibition of TBX20 (Figures 3E,F).

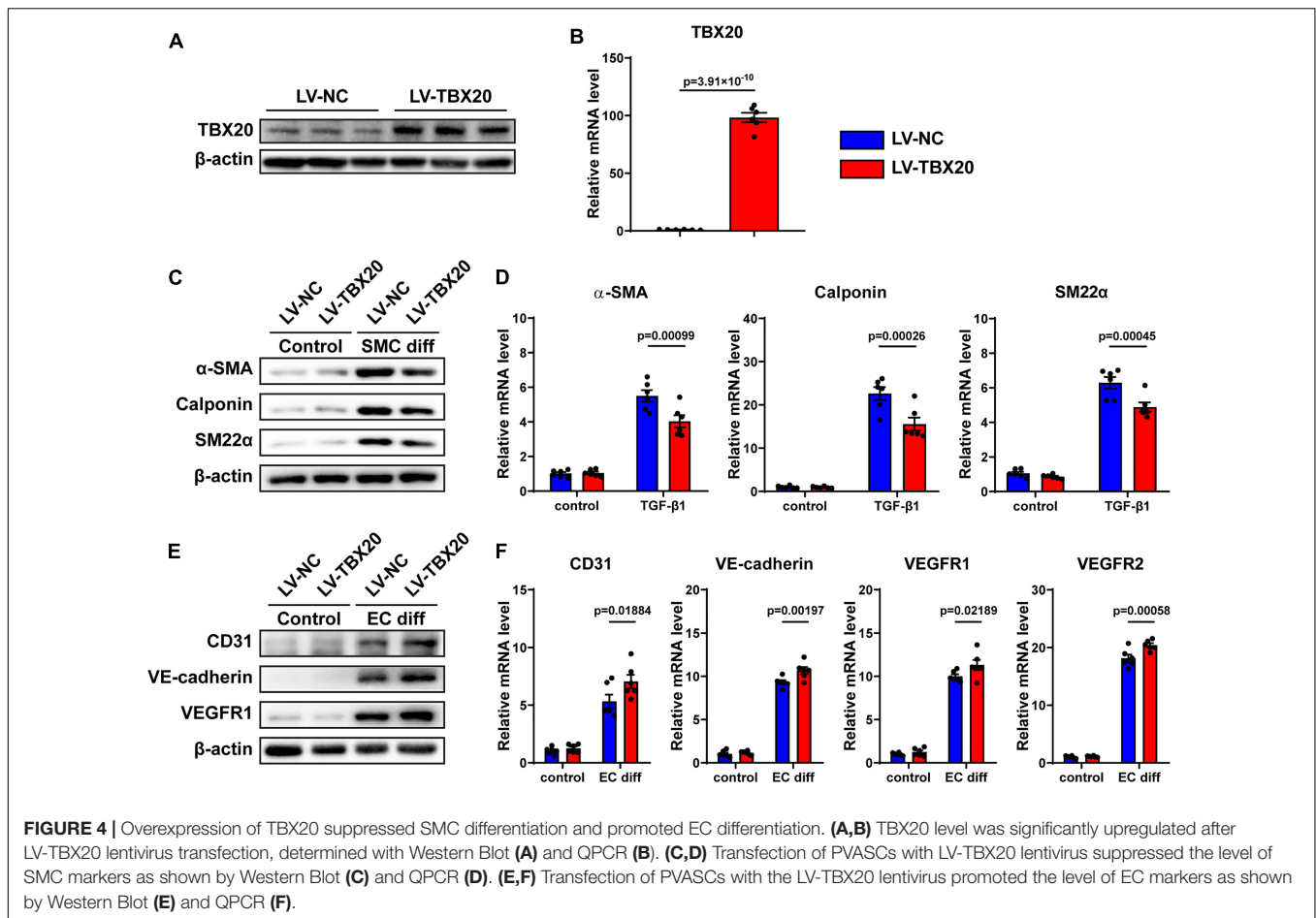
To further investigate whether TBX20 was involved in other cell biological processes, we also performed assays for cell migration and proliferation, both of which played crucial roles in vascular remodeling. However, we found that knockdown of

TBX20 did not affect migration and proliferation of PVASCs (Supplementary Figures 4A–E). In summary, TBX20 acted oppositely and regulated the cell differentiation of both SMC and EC from PVASCs.

Overexpression of TBX20 Suppressed SMC Differentiation and Promoted EC Differentiation

To further investigate whether TBX20 was involved in SMC and EC differentiation, PVASCs were transfected by the TBX20-overexpressing lentivirus vector and control vector. Compared with the LV-NC group, both mRNA and protein levels of TBX20 were significantly increased in the LV-TBX20 group (Figures 4A,B). After TGF- β 1 stimulation, QPCR and Western Blot analyses revealed that overexpressing TBX20 significantly suppressed SMC gene expressions (Figures 4C,D). After PVASCs were induced with the EGM-2 medium for endothelial differentiation for 7 days, a higher expression of EC markers was detected in the LV-TBX20 PVASC group compared with the LV-NC group (Figures 4E,F).

Taken together, these findings suggested that TBX20 suppressed SMC differentiation and promoted EC differentiation. In addition, similar to previous results, overexpression of TBX20 did not affect the migration and proliferation of PVASCs



(Supplementary Figures 5A–E). It is interesting that TBX20 could regulate both EC and SMC differentiation where the trends of TBX20 expression are totally inverse during cell differentiation, respectively.

TBX20 Was Important for the Activation of Smad2/3 Signaling During SMC Differentiation

Hinted by bioinformatic analysis, TBX20⁺ PVASC4 was the only subpopulation responsible for muscle development. We hence explored the underlying mechanism of TBX20 in PVASC differentiation toward SMCs. Control (sh-scramble) and TBX20-knockdown (sh-TBX20) PVASCs were starved for 24 h in the serum-free medium and subsequently treated with TGF-β1 for 15, 30, and 60 min prior to collection for Western Blot of Smad phosphorylation. TBX20 knockdown significantly increased the phosphorylation of Smad2/3 in response to TGF-β1 (Figure 5A). To further determine the role of TBX20 in Smad activation, we examined Smad2/3 nucleus translocation responses to TGF-β1 by immunofluorescence staining in control or TBX20-knockdown cells. We confirmed that Smad2/3 could translocate into the nuclei after TGF-β1 treatment in control cells, and Smad2/3 nuclear translocation was significantly

enhanced in TBX20-knockdown cells (Figure 5B). On the other hand, after TBX20 overexpression, phosphorylation and nuclear translocation of Smad2/3 were reduced, compared with the LV-NC group (Figures 5C,D). These suggested a potential mechanism for TBX20 as an effect mediator in regulating Smad2/3 signaling.

Role of TBX20 in Neointimal Hyperplasia After Vascular Injury

Firstly, to exclude the role of TBX20 in other vascular lineage cells, we analyzed single-cell sequencing data of the aorta. Our analysis revealed that all cell types within the aorta hardly expressed TBX20 (Supplementary Figures 6B–E). We also performed QPCR for PVASCs, vascular SMCs, and vascular tunica media, thus excluding the role of TBX20 in resident SMCs (Supplementary Figure 6A). To investigate the role of TBX20 in PVASCs in neointimal hyperplasia, we performed guidewire injury of the femoral artery in mice. Male mice underwent guidewire injury of the femoral artery, followed by transplantation of PVASCs transfected with sh-TBX20 lentivirus or sh-scramble lentivirus, LV-TBX20 lentivirus, or LV-NC lentivirus in Matrigel at the adventitial side of the injured vessel. Four weeks postsurgery, vascular histological staining indicated that TBX20 knockdown promoted intimal hyperplasia.

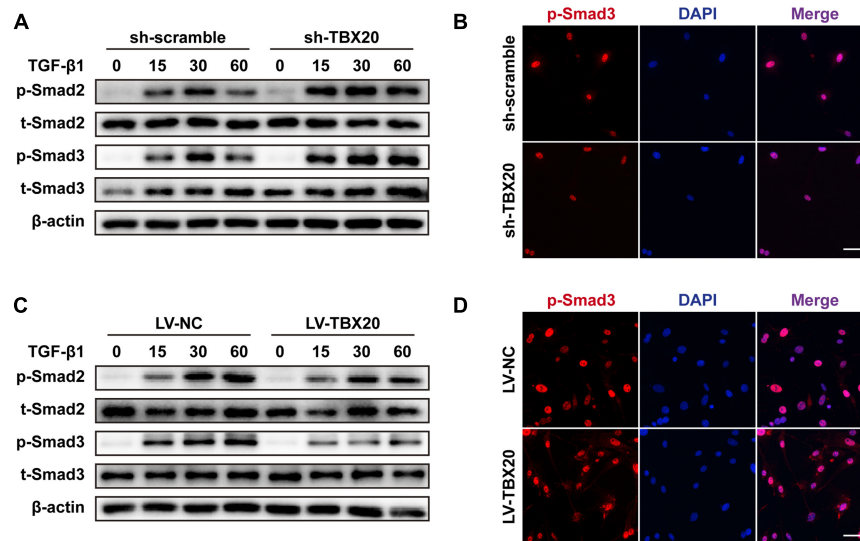


FIGURE 5 | TBX20 was important to the activation of Smad2/3 signaling during SMC differentiation. **(A,C)** PVASCs were incubated with 5 ng/mL TGF- β 1 after starving with 2% FBS medium for 24 h, and the samples were harvested at 15, 30, and 60 min. Western Blot analyses detected the phosphorylation of Smad2/3. **(B,D)** Immunofluorescence staining revealed that more Smad3 was translocated into the nuclei in the sh-TBX20 cells and less Smad3 was translocated into the nuclei in the LV-TBX20 cells. Scale bar = 50 μ m. Cell nuclei were stained with DAPI.

Compared with the sh-scramble group, the intima/media ratio in the sh-TBX20 group was increased (**Figures 6A,B**). In the transplantation of LV-TBX20 PVASCs after vascular injury, neointima formation was significantly inhibited. Compared with the LV-NC group, the intima/media ratio in the LV-TBX20 group was greatly reduced (**Figures 6C,D**). Altogether, these findings indicated that TBX20 played a crucial role in neointima hyperplasia in response to endovascular injury.

DISCUSSION

Adipose tissues are present at multiple locations. Adipose tissues at different locations are distinct in morphology and composition (Wu et al., 2012). Perivascular adipose tissue (PVAT) draws a lot of attention because of its anatomic location and environmental/metabolic context (Fitzgibbons et al., 2011; Chang et al., 2012, 2020; Padilla et al., 2013). PVAT harbors mesenchymal stem cells, which can be a promising source for stem cell therapies. In the present study, we performed RNA-Seq assay to measure the gene expression of ASCs from subcutaneous, abdominal, and perivascular adipose tissue. Among the top 10 genes specifically expressed in PVASCs, transcriptional factor TBX20 is the only one which had been proved closely related to vascular angiogenesis (Shen et al., 2013; Meng et al., 2018). With the permission of Professor Pingjin Gao, we reanalyzed her single-cell data of PVASCs and identified a TBX20⁺ subpopulation of PVASCs. Such finding and subsequent GO analysis further consolidated our confidence of TBX20's role in cell differentiation and vascular remodeling.

Many studies showed the roles of TBX20 regulating cardiac development (Meins et al., 2000; Stennard et al., 2005; Kirk et al.,

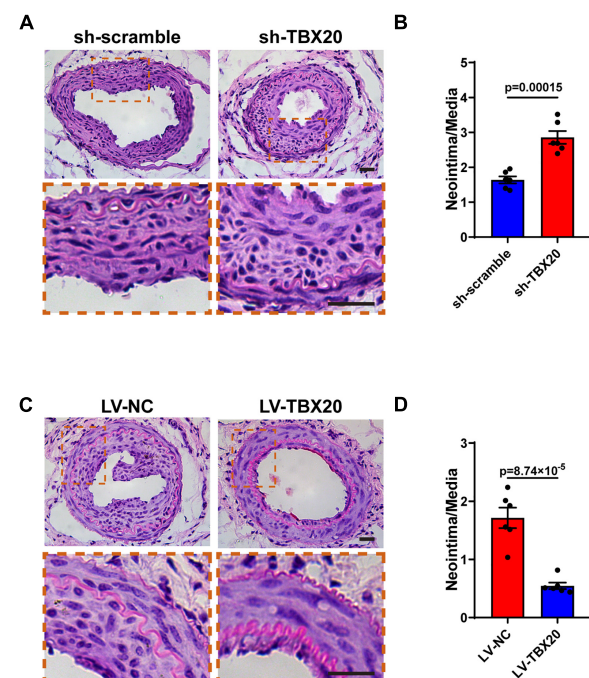


FIGURE 6 | Role of TBX20 in neointimal hyperplasia after vascular injury. **(A,C)** H&E staining of injured femoral arteries at 28 days post-PVASCs transplantation. **(B,D)** Quantitative analysis of the area ratio of neointima to media. Scale bar = 50 μ m.

2007). Few evidences addressing the vascular system indicated that TBX20 mediated angiogenic processes and suppressed endothelial cell injury (Shen et al., 2013; Meng et al., 2018).

Moreover, TBX20 overexpression enhanced cardiomyogenic differentiation and inhibited osteogenic differentiation in human adipose-derived MSCs (Neshati et al., 2018; Mollazadeh et al., 2019). In the present study, we demonstrate for the first time that TBX20 simultaneously mediates both SMC and EC differentiation of PVASCs *in vitro* and modulates the neointimal hyperplasia after vascular injury in mice. Our study revealed that TGF- β 1, which is of great potency for SMC differentiation (Chen and Lechleider, 2004), significantly downregulated TBX20 expression during PVASC differentiation into SMCs. Phosphorylation of Smad2/3 is one of the canonical pathways in TGF- β 1-induced SMC differentiation (Derynck and Zhang, 2003). In our study, we found that TBX20 inhibited the phosphorylation and nucleus translocation of Smad2/3 in response to TGF- β 1, suppressing SMC-specific gene expression in PVASCs. Moreover, TBX20 displayed opposite effects on differentiation of PVASCs to ECs and SMCs. TBX20 significantly increased during the differentiation of PVASCs into endothelial cells and TBX20 promoted EC differentiation. Such data suggested that TBX20 might act as a buffer and balance cell fates. Maintaining an appropriate level of TBX20 expression could be meaningful for maintaining homeostasis in vascular remodeling.

PVASCs can differentiate to SMCs, and cell transplantation assays confirmed their involvement in neointimal hyperplasia through migration from the adventitia side to the intima and SMC differentiation (Gu et al., 2019). In the present study, we tested the effects of TBX20 on intimal hyperplasia by means of cell transplantation in the murine femoral artery guidewire injury model. Our data showed that TBX20 overexpression in PVASCs through genetic modification significantly inhibited neointimal proliferation. Moreover, transplantation of TBX20-knockdown PVASCs increased the neointimal hyperplasia. There is still a debate on how mesenchymal stem cells participate in various pathological processes. Cell differentiation, cytokine secretion, and immunomodulation are three mainstream hypotheses. Underlying mechanism of PVASCs participation in neointimal formation requires additional work. Many strategies were implemented for the prevention of vascular stenosis. For instance, sirolimus-eluting stents have been widely used in coronary artery diseases which can prevent occlusion by inhibiting SMC proliferation (Suzuki et al., 2001). However, in-stent restenosis is still of common occurrence, implying that SMCs should not be the only one to blame. Here we provide a potential therapeutic target for vascular stenosis

diseases independent of SMCs, vascular adventitial stem cells, and macrophages.

In conclusion, our study demonstrated that TBX20 could inhibit SMC differentiation and promote EC differentiation *in vitro*. *In vivo*, TBX20 could effectively inhibit neointima formation after vascular injury, which provided a new intervention target for vascular diseases like restenosis.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the (GEO) repository, accession number (GSE171946).

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Zhejiang University.

AUTHOR CONTRIBUTIONS

YJ, YM, and JS performed the research and drafted the manuscript. HN and YL carried out the cell culture. YuZ and HM participated in the statistical analysis. CL, YiZ, and SD participated in the histological analysis. MX and YX conceived the study and participated in the study design. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.662704/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cardiac Overexpression of XIN Prevents Dilated Cardiomyopathy Caused by *TNNT2* ΔK210 Mutation

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TNNT2 mutation is associated with a range of cardiac diseases, including dilated cardiomyopathy (DCM). However, the mechanisms underlying the development of DCM and heart failure remain incompletely understood. In the present study, we found the expression of cardiac XIN protein was reduced in *TNNT2*-ΔK210 hESCs-derived cardiomyocytes and mouse heart tissues. We further investigated whether XIN protects against *TNNT2* mutation-induced DCM. Overexpression of the repeat-containing isoform XINB decreased the percentage of myofilaments disorganization and increased cell contractility of *TNNT2*-ΔK210 cardiomyocytes. Moreover, overexpression of XINB by heart-specific delivery via AAV9 ameliorates DCM remodeling caused by *TNNT2*-ΔK210 mutation in mice, revealed by partially reversed cardiac dilation, systolic dysfunction and heart fibrosis. These results suggest that deficiency of XIN may play a critical role in the development of DCM. Consequently, our findings may provide a new mechanistic insight and represent a therapeutic target for the treatment of idiopathic DCM.

Keywords: dilated cardiomyopathy, gene therapy, *TNNT2*, cardiac remodeling, XIN

INTRODUCTION

Heart failure due to cardiomyopathy is among the most common causes of cardiovascular mortality (Harmon et al., 2005). Numbers of mutations in genes encoding sarcomeric proteins have been identified in individuals with cardiomyopathy (Morita et al., 2005; Villard et al., 2011). Cardiac troponin T (cTnT), encoded by *TNNT2*, is a cardiac muscle specific sarcomere protein that is important for the cell morphology, sarcomere assembly and myofilament contraction in cardiomyocytes (Solaro and Rarick, 1998; Wei and Jin, 2011). Deletion of lysine 210 (ΔK210) in *TNNT2* gene has been found to cause familial dilated cardiomyopathy (DCM) (Kamisago et al., 2000), which is characterized by dilated ventricular chamber and reduced systolic function leading to progressive heart failure with high mortality (Du et al., 2007; Kimura, 2010; Sfichi-Duke et al., 2010). Attempts to elucidate the molecular mechanisms underlying DCM mutations showed multiple molecular mechanisms including Ca²⁺ sensitivity of myofilament (Morimoto et al., 2002; Sfichi-Duke et al., 2010), remodeled intracellular calcium handling (Inoue et al., 2013; Lan et al., 2013) or interactions among the thin filaments protein constituents (Morimoto et al., 2002;

Lombardi et al., 2008; Kimura, 2010) were involved in development of the disease. However, the detailed pathogenic mechanism underlying DCM disease remains poorly understood. Combined with human pluripotent stem cell (hPSC) technology and efficient cardiac differentiation protocols, human cardiomyocytes with corresponding gene mutations could be obtained *in vitro*, making it possible to expediently explore the effects of genetic mutations in the initial phases of disease development using isogenic human disease model systems (Sallam et al., 2014; Savla et al., 2014).

Xin actin-binding repeat-containing proteins (XIRPs) are a family of striated muscle-specific proteins defined by the presence of 15–28 copies of the conserved 16-aa XIN repeats. XIN is encoded by XIRP1 gene, also called *hXina* or *CMYA1*, is a striated muscle-specific gene and belongs to the XIRP family (Eulitz et al., 2013). Human XIRP1 gene gives rise to three XIN isoforms, known as XINA, XINB, and XINC (Molt et al., 2014). It is interesting that this gene has only been found in vertebrates with true chambered heart. XIN is expressed during early developmental stages of cardiac and skeletal muscles (Pacholsky et al., 2004). In cardiomyocytes, XIN is localized predominantly at intercalated discs (ICDs), which showed significance in the regulation of cardiac development and functions (Sinn et al., 2002). Multiple binding sites enabled XIN strengthen membrane apposition between cardiomyocytes by linking actin cytoskeleton to adherens junctions of ICDs (van der Ven et al., 2006; Wang et al., 2012, 2013). Additionally, its localization at adherens junctions of muscle cells and interaction with β -catenin, filaminC, and Mena/VASP imply a role in linking and transducing signals important for cardiac remodeling. Inactivation of *Xin* in chick embryos led to looping defects, abnormal beating behavior and edema (Wang et al., 1999), while *Xin*-deficient mice display myopathy, impaired contractility, and attenuated muscle repair (Al-Sajee et al., 2015), which indicating a crucial role in heart development and cardiac disease. Moreover, it has been observed that Xin may participate in a BMP-Nkx2.5-MEF2C pathway and promote the expression of ventricular myosin heavy chain (vMHC) gene to participate in cardiogenesis regulation (Wang et al., 1999, 2014). All these indicating an important role for XIN in early myofibrillogenesis and later in the maintenance of striated muscle integrity.

In the previous study, we characterized that the cellular phenotypes of *TNNT2*- Δ K210 hESC-CMs (human embryonic stem cells derived cardiomyocytes) recapitulated DCM disease features (Li et al., 2021). Through whole transcriptomic analysis, down-regulated expression of XIN was identified in day35 *TNNT2*- Δ K210 hESC-CMs. In this study, we further confirmed the decreased expression of XIN in DCM models and focused on the therapeutic effects of XIN. We found that overexpression of XINB improved sarcomere organization and contraction force of *TNNT2*- Δ K210 cardiomyocytes. *In vivo* cardiac-specific overexpression of XINB via AAV9 in neonatal *TNNT2*- Δ K210 DCM mice significantly rescued the DCM phenotypes. In conclusion, our findings uncovered the importance of XIN in *TNNT2* mutation induced dilated cardiomyopathy.

MATERIALS AND METHODS

Cell Culture and Cardiac Differentiation

The human ESC line H7 obtained from WiCell Research Institute under specific Material Transfer Agreement was used in this study. All human stem cells researches followed the ISSCR Guidelines for the Conduct of Human Embryonic Stem Cell Research. Human ESCs were cultured using mTesR human pluripotent stem cell culture medium (STEMCELL Technologies) on Matrigel-coated (BD Biosciences) 6-well or 12-well plates. Cardiac differentiation were performed according to previously described protocols (Lian et al., 2013). Each of three congenic lines were used in the following analysis. hESC lines between 70 and 80 passages were used in the following analysis.

RNA Extraction and Real Time Quantitative PCR

Total RNA was extracted using Trizol from Invitrogen according to the operating manual. The relative expression profiles of *XIRP1* were examined by TOYOBO real-time PCR chemistry. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as endogenous control gene. PCR reaction were run in a final volume of 20 μ l composed of 1 μ l of cDNA, 9 μ l of buffer Mix and 100 nM primer pairs. The PCR condition consisted of one initial denaturation at 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min, which were performed by Bio-Rad iQ5 Real Time PCR System. All reactions were performed in triplicate. The primer sequences used are provided in **Supplementary Table 1**.

Western Blot

Cells or heart tissue were lysed with RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) (Beyotime Biotechnology, P0013B) with protease inhibitor cocktail (APExBio, K1012). Protein concentrations were determined by BCA Protein Assay Kit (TIANGEN, PA115). Equal amounts of protein were loaded on 10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membrane (Millipore, IPVH00010). After blocked with 5% skimmed milk at room temperature, membranes were incubated with primary antibodies (Xirp1: sc-166658, Santa Cruz Biotechnology, diluted at 1:1,000; *GAPDH*: 60,004-1-Ig, Proteintech, diluted at 1:1,000; β -tubulin: 10068-1-AP, Proteintech, diluted at 1:2,000) at 4°C overnight and then incubated with corresponding secondary antibody. Western blots results were detected with Tanon 5200 Multi.

Immunofluorescence Staining

Morphological changes of hESC-cardiomyocytes were detected using immunostaining. After fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100, the cells were blocked with 4% goat serum, incubated with primary antibodies (cTnT: ab45932, Abcam, diluted at 1:400; Xirp1: sc-166658, Santa Cruz Biotechnology, diluted at 1:100) overnight at 4°C, and then visualized with secondary antibody. Frozen left ventricular tissue slides were incubated with Alexa FluorTM

488 Conjugated-Wheat Germ Agglutinin (Life Technologies, United States) and DAPI (Life Technologies) to visualize myocyte membranes. Fluorescence images were obtained with a confocal microscope (LEICA SP8).

Echocardiography

Echocardiography was performed at determined time. Mice were anesthetized by 1.5% isoflurane and M-mode images were captured and measured using the small animal echocardiography analysis system at the midventricular level. The echocardiographer was blinded to mouse genotypes or treatments.

Histological Analysis

Cardiac tissue from mice at 3 months of age was fixed in 4% formaldehyde and paraffin embedded. The sections were sliced at 5 μ m. Hematoxylin-eosin (H&E) and Masson staining were performed to examine general histology and analyze cardiac fibrosis, respectively. The sections were observed and photographed by the microscope. For the detection of transmission electronmicroscopy, heart tissues from ventricle were immediately fixed in 2.5% glutaraldehyde solution for about 1 h at room temperature and post-fixed for 3 h. Samples were embedded, sectioned at about 70 nm thickness, and stained with lead citrate for 8 min. Micrographs were captured using a PHILIPS CM-120 transmission electron microscope.

Lentivirus Production

XINB cDNA was cloned into target gene lentiviral vector (pCDH-EF1-MCS-T2A-copGFP) at the MCS locus. The HEK293T cells were transfected with the target gene lentiviral vector plasmid and packaging plasmids using Lipofectamine 3,000 (Life Technologies). The transfected cells were cultivated and collected supernatant every 24 h. Ultrafiltration was used to concentrate the medium. Lentiviruses expressing EGFP gene were used as the control. The efficiency of virus transduction was confirmed 24 h after infection.

Recombinant AAV9 Virus Production

We cloned XINB or Luciferase cDNA into ITR-containing AAV9 plasmid with cardiac specific TNNT2 promoter and obtained the pAAV:TNNT2:XINB and pAAV:TNNT2:Luciferase plasmids. AAV9 was packaged using HEK293 cells with AAV9:Rep-Cap and pHelper and purified by an iodixanol gradient centrifugation. AAV9 titer was determined by quantitative PCR. 50 μ l containing 1×10^{12} vg AAV9-Luci or AAV9-XINB virus were used for one mouse. Intraperitoneal injection of AAV9 was performed within 3 days of birth.

Contraction Force Measurements

HESC-cardiomyocytes were separated into single cell and cultured on a 0.4–0.8 mm thick mattress of undiluted Matrigel for 2–3 days. We used video-based edge detection to measure contractility, which were visualized using a Zeiss CFM-500 inversion fluorescence microscope. Spontaneous contraction traces were recorded and analyzed by FelixGX software (PTI).

In vivo Bioluminescence Imaging

In vivo bioluminescence imaging was performed to confirm AAV-9 mediated gene expression. 15 min after intraperitoneal injection of D-luciferin (Promega, 15 mg/ml, 0.2 ml) under anesthesia with inhalation of isoflurane, the mice were placed for imaging using an IVIS Lumina K system (PerkinElmer).

Animals

A knock-in mouse with deletion of Lys-210 mutation in cardiac troponin T gene (*TNNT2*- Δ K210) was used as the DCM model animal, which were constructed by VIEWSOLID biotechnology co., LTD. All procedures involving animal use, housing, and operations in this study were approved by the Institutional Animal Care and Use Committee of the Fudan University and met the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Statistical Analysis

All data are presented as mean $\pm$ SEM (standard error of the mean). Two normally distributed data sets analysis was performed using unpaired two-tailed Student's *t*-test. One-way ANOVA was used to test for significant differences in multiple group comparisons. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

XIN Deficiency Was Detected in the TNNT2- Δ K210 hESC-Derived Cardiomyocytes

HESCs (H7) lines with either a DCM-causing heterozygous Δ K210 (Δ K210/WT) or homozygous Δ K210 (Δ K210/ Δ K210) mutation in the *TNNT2* gene were generated as described before (Li et al., 2021). Through modification of the WNT signaling pathway, we differentiated the isogenic heterozygous and homozygous *TNNT2*- Δ K210 mutant hESCs into cardiomyocytes and beating cells were observed on day 8–9 after cardiac differentiation (Figure 1A). Immunostaining of WT, and *TNNT2*- Δ K210 hESCs-derived cardiomyocytes showed positive staining of cardiac markers cTnT, α -actinin and MLC2V (Supplementary Figure 1A). Fluorescent activated cell sorting (FACS) analyses for cTnT showed a $\sim$ 90% purity of cTnT + cardiomyocytes in our cardiac differentiation and similar efficiencies among H7 WT and *TNNT2*- Δ K210 hESC lines (Supplementary Figure 1B). In the previous study, we characterized that the cellular phenotypes, such as Ca^{2+} handling, and contractility of the *TNNT2*- Δ K210 cardiomyocytes recapitulated DCM disease features (Li et al., 2021). We further analyzed whole transcriptomic results of day35 WT (WT/WT) and *TNNT2*- Δ K210 cardiomyocytes (Supplementary Figure 2A). Since there are no reports of patients carrying homozygous *TNNT2*- Δ K210 mutation, heterozygous *TNNT2*- Δ K210 cardiomyocytes may have a stronger disease-related significance. Through comparing with WT cardiomyocytes and RT-qPCR analysis, we found

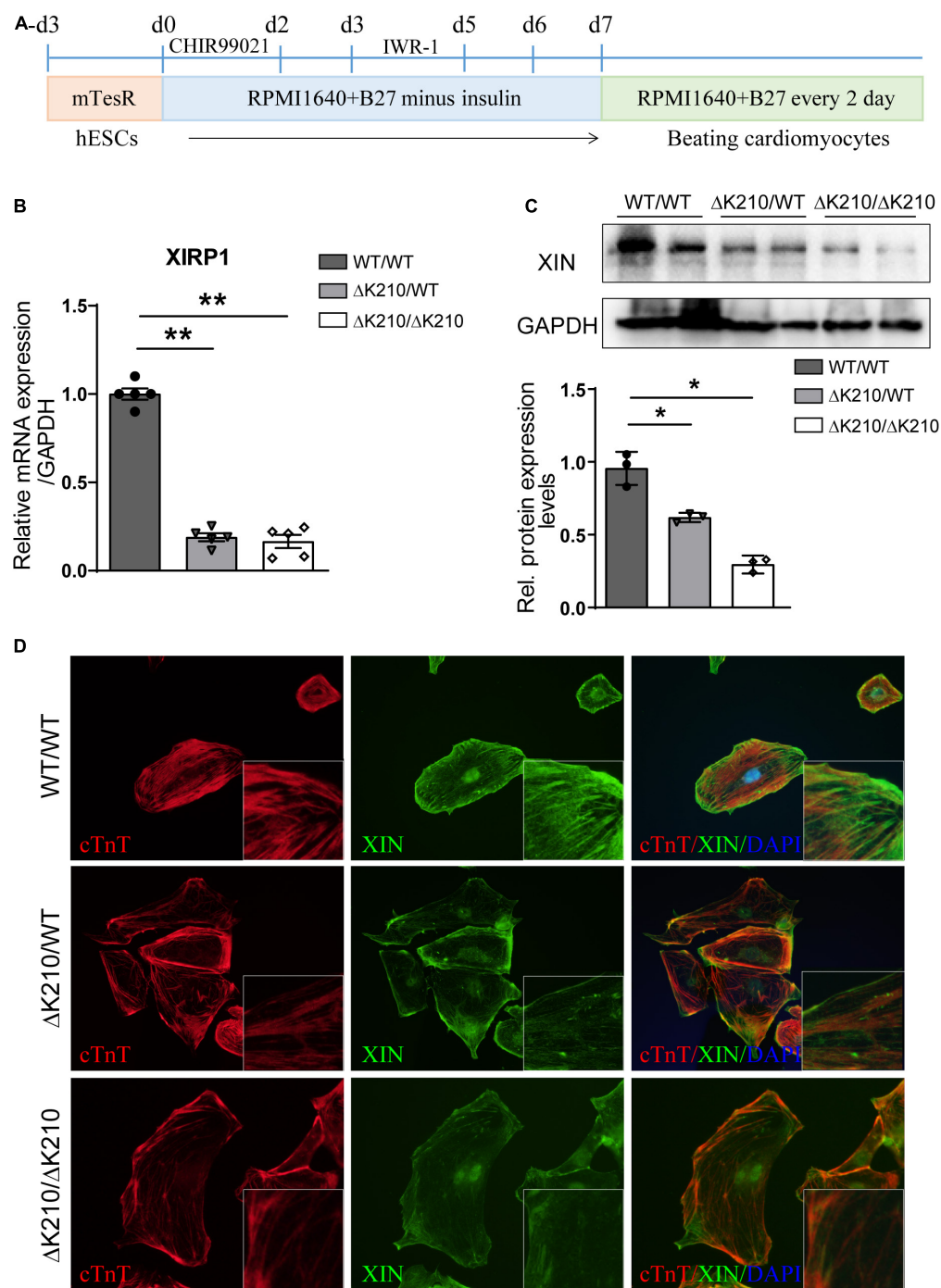


FIGURE 1 | Cardiac differentiation of *TNNT2*-ΔK210 mutant hESCs and the expression of XIN. **(A)** Schematics of optimized chemically defined cardiac differentiation protocol. **(B)** Quantitative PCR verification of *XIRP1* expression in wild type (WT/WT), *TNNT2*-ΔK210 (heter: ΔK210/WT, homo: ΔK210/ΔK210) hESC-cardiomyocytes at day35 post cardiac differentiation. **(C)** Detection of XIN protein expression at day35 post differentiation by Western blotting. GAPDH was used as the loading control. **(D)**, Immunostaining detection of XIN (green) and cTnT (red) in cultured WT/WT, WT/ΔK210 and ΔK210/ΔK210 cardiomyocytes. Data are represented as mean ± SEM. **P* < 0.05, ***P* < 0.01.

XIRP1 was sharply down-regulated and the encoded XIN also decreased in both heterozygous and homozygous *TNNT2*-ΔK210 cardiomyocytes at day35 post cardiac differentiation (Figures 1B,C and Supplementary Figures 2B,C). Previous

studies showed XIN is an F-actin-binding protein and expressed in the early stages of development of cardiac muscles, so we hypothesized that reduction of XIN participated in the development of dilated cardiomyopathy. Localization and

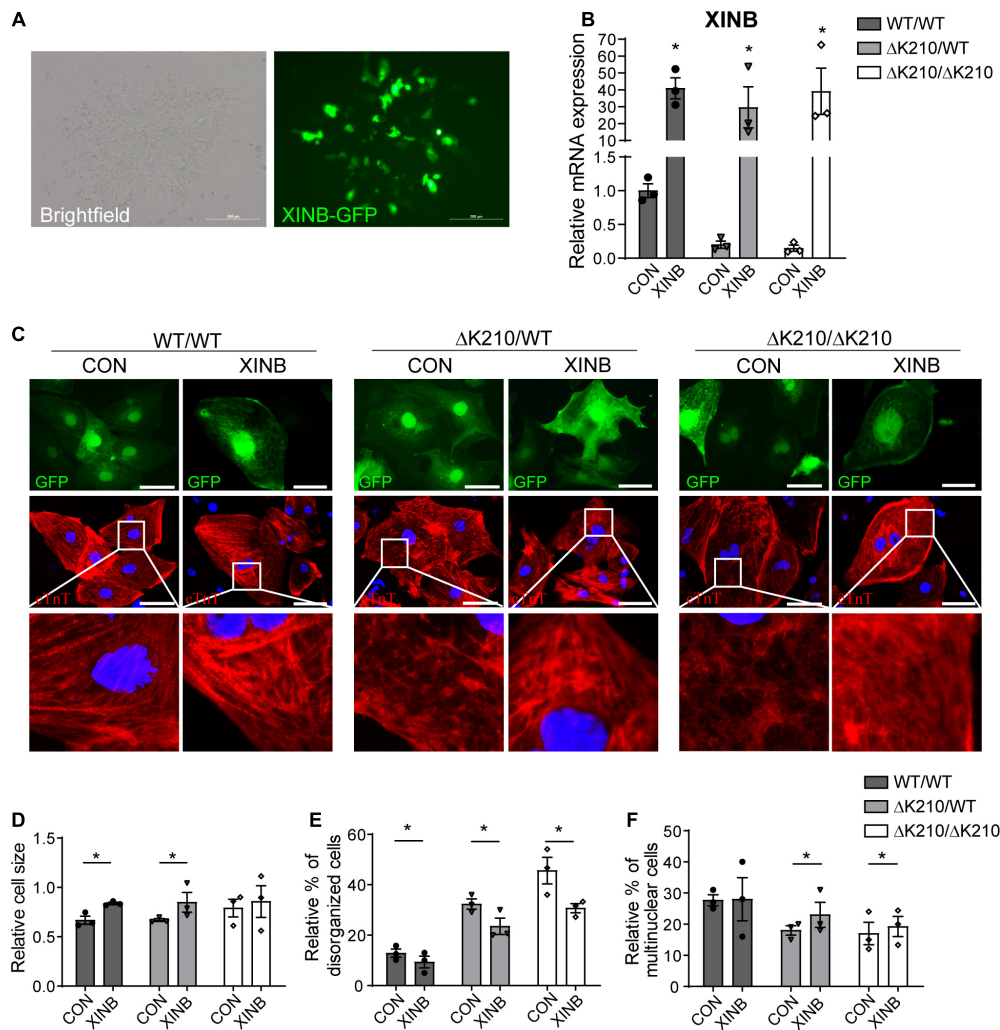


FIGURE 2 | Overexpression of XINB partially rescued the DCM phenotype of *TNNT2*-ΔK210 cardiomyocytes. **(A)** Brightfield and fluorescence images of hESC-cardiomyocytes illustrating green fluorescence protein (GFP) expression after XINB-lentivirus transfection. Scale bars, 200 μm. **(B)** Relative XINB expression in WT/WT, WT/ΔK210, and ΔK210/ΔK210 cardiomyocytes determined by quantitative PCR. **(C)** Representative immunostaining of cTnT (red) in cultured WT/WT, WT/ΔK210, and ΔK210/ΔK210 cardiomyocytes after XINB overexpression. Cells transfected with lentivirus expressing GFP were used as control. Scale bars, 50 μm. **(D)** Quantification of relative cell size and **(E)** statistics of the relative disorganized sarcomeric pattern for WT/WT, WT/ΔK210, and ΔK210/ΔK210 cardiomyocytes after overexpression of XINB. **(F)** Percent of multinuclear cells increased after XINB overexpression in *TNNT2*-ΔK210 cardiomyocytes. $n > 30$, 3 lines in each group; data are represented as mean $\pm$ SEM. * $P < 0.05$.

myofilament pattern of XIN in WT and *TNNT2*-ΔK210 cardiomyocytes were then further examined. Compared to WT cardiomyocytes, staining patterns of XIN combined with myofilaments cTnT were changed and weaker in *TNNT2*-ΔK210 cardiomyocytes (Figure 1D). These results suggested a possible role of XIN in the regulation of cardiomyocytes remodeling.

XINB Improved Myofilaments Organization in *TNNT2*-ΔK210 Cardiomyocytes

Because of the limitations of lentivirus and recombinant adeno-associated virus vector capacity, we used isoform of XINB (containing XIN-repeats, peptide motifs which bind actin

filaments by coiling around them) in the next *in vitro* and *in vivo* study. We overexpressed XINB in *TNNT2*-ΔK210 cardiomyocytes to check whether restore of XINB can rescue the DCM-like cellular phenotypes. Lentiviral vectors containing XINB-T2A-GFP were generated, with GFP cassette to indicate the transduced cells, while GFP only was used as the negative control. Cardiomyocytes were infected with XINB or control lentiviruses for 7 days. Obvious GFP (Figure 2A) and increased XINB (Figure 2B) were detected after transfection. As XIN binding to actin filaments and may play an important role in early myofibrillogenesis, we then used immunofluorescence to detect the myofilament structure after XINB overexpression. Compared to WT cardiomyocytes, myofibrils were poorly organized with diffusions and damages (myofilament staining

was weaker and less dense, punctate distribution of sarcomeric cTnT over one fourth of the total cellular area) (Sun et al., 2012) in $\Delta K210$ cardiomyocytes. While XINB overexpressed $\Delta K210$ cardiomyocytes showed better organized and dense myofilaments over the entire cellular area (Figure 2C). An increase in cell size of WT/WT and $\Delta K210$ /WT cardiomyocytes and a reduced percentage of *TNNT2*- $\Delta K210$ cardiomyocytes with disorganized cTnT distribution patterns were observed after XINB expression (Figures 2D,E). We also found higher frequency of multinucleation in XINB overexpressed *TNNT2*- $\Delta K210$ cardiomyocytes versus control (Figure 2F), which might suggest a further mature state. In conclusion, phenotypes of *TNNT2*- $\Delta K210$ cardiomyocytes such as dispersion and disorganization of myofilaments were improved after XINB overexpression. These results showed that XIN may play an important role in myofibrillar assembly and the maintenance of muscle integrity.

Overexpression of XINB Increased Contractile Force of hESC-Derived Cardiomyocytes

To further evaluate the effects of XINB overexpression, we next examined the contractile force of cardiomyocytes. Compared with control, XINB treated WT/WT, $\Delta K210$ /WT and $\Delta K210$ / $\Delta K210$ cardiomyocytes showed increased contractility (Figures 3A–D). It was previously reported that, in chicken, cXin participated in a BMP2–Nkx2.5–Mef2C–cXin–vMHC pathway to regulate cardiogenesis (Wang et al., 2014). Then we further examined expression of genes such as *TNNT2*, *ACTN2* and *MYH7*, which related to cardiac muscle contraction, and found they were also up-regulated after XINB overexpression (Figures 3E–G), suggesting an improvement in cardiomyocyte contractile function. And the increased expression of cardiac structural genes after XINB transfection may also contribute to the enlarged cell size, improved myofilaments organization and maturation of cardiomyocytes in Figures 2D–F.

XIN Was Down-Regulated in the *TNNT2*- $\Delta K210$ Mice Hearts

We next detected the expression of Xin in heart tissues of wild type and *TNNT2*- $\Delta K210$ mice at different days after birth. The results indicated that *Xirp1* expression was also down-regulated in the *TNNT2*- $\Delta K210$ mice since they were born (Figure 4A). Western blot analysis showed decreased level of XIN (encoded by *Xirp1*) protein in *TNNT2*- $\Delta K210$ mice hearts 7 days after birth (Figures 4B,C).

XINB Protected Against Dilated Cardiomyopathy in *TNNT2*- $\Delta K210$ Mice

To assess whether XINB could rescue the phenotype of DCM *in vivo*, we next used recombinant adeno-associated virus type 9 (AAV9) to specifically express XINB in the heart of neonatal *TNNT2*- $\Delta K210$ DCM mice (Figure 5A). Plasmid AAV9: *TNNT2*: XINB (AAV9-XINB) with XINB driven by *TNNT2* promoter was generated to express XINB specifically

in cardiomyocytes, while AAV9: *TNNT2*: Luciferase (AAV9-Luci), in which XINB was replaced by luciferase, was used as control. To validate the efficiency of viral delivery, uninjected mice were used as controls. The luciferase protein was detected in hearts but not in any other organs of the AAV9-Luci treated mice, and also not found in the uninjected mice (Figure 5B). Functions of the wild type and *TNNT2*- $\Delta K210$ transgenic mice were assessed using echocardiography at 1 and 3 months of age. The representative M-mode echocardiographic images at 3 months of age were shown in Figure 5C. LV ejection fraction (Figure 5D) and fractional shortening (Figure 5E) of the XINB-treated *TNNT2*- $\Delta K210$ mice were significantly increased by $64.3 \pm 3.7\%$ to $78.6 \pm 3.1\%$ and $28.8 \pm 3.1\%$ to $40.0 \pm 2.8\%$ compared with control. The left ventricular (LV) end systolic and diastolic diameter of the mice was decreased by 2.5 ± 0.2 mm to 2.0 ± 0.06 mm and 4.3 ± 0.2 mm to 3.8 ± 0.1 mm at 3 months of age compared with that of the AAV9-luciferase injected *TNNT2*- $\Delta K210$ transgenic mice (Figures 5F,G). These results suggested that the cardiac function of *TNNT2*- $\Delta K210$ mice was significantly improved after XINB overexpression.

XINB Improved Cardiac Pathological Phenotypes in the *TNNT2*- $\Delta K210$ Mice

The morphological changes of heart ventricles and cardiomyocytes were further determined by histological examination (Figures 6A–E). Overexpression of XINB in the *TNNT2*- $\Delta K210$ transgenic mice significantly reduced the ventricular dilatation and accumulation of collagen in the interstitial space compared with the typical histological changes observed in the heart tissues of the control *TNNT2*- $\Delta K210$ mice (Figures 6A,B). At 3 months of age, *TNNT2*- $\Delta K210$ mice also showed cardiomyocytes enlargement compared to WT mice. XINB overexpression prevented the development of heart dilation and hence recovered the size of myocytes (Figures 6C,D). Twisted Z-line, sparse myofilaments, and swollen mitochondria was also seen in the control *TNNT2*- $\Delta K210$ mice hearts. However, the disrupted sarcomeric structures of heart was improved by XINB overexpression, which showed relative dense and aligned myofilaments with mitochondria arranged in the gap (Figure 6E). These changes indicated that the morphological phenotypes of DCM in *TNNT2*- $\Delta K210$ mutant mice were partially rescued by cardiac-specific expression of XINB.

DISCUSSION

Detailed mechanisms for dilated cardiomyopathy caused by *TNNT2* gene mutation remain largely unknown. Current research using dilated myocardium from patients at a relative late disease stage might neglect the earliest molecular events which is key for DCM development. In this study, we used cardiomyocytes derived from hESCs which carried the DCM-causing *TNNT2*- $\Delta K210$ mutation and confirmed XIN decreased compared with control. Furthermore, XIN was also decreased in DCM mouse heart. Overexpression of the repeat-containing isoform XINB in $\Delta K210$ cardiomyocytes improved myofilaments

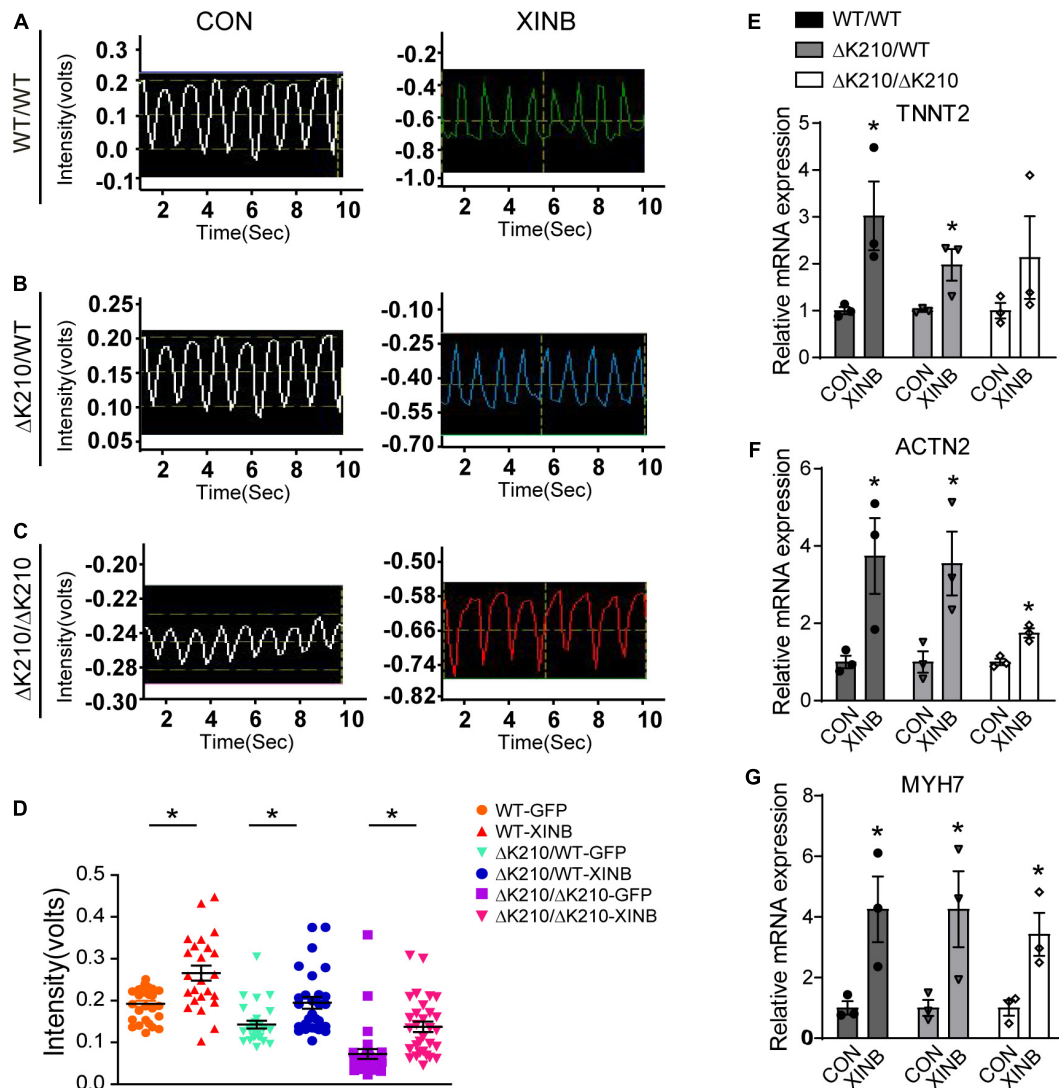


FIGURE 3 | XINB increased contractility of *TNNT2*- Δ K210 cardiomyocytes. **(A)** Representative video recording traces of spontaneous contraction of single WT/WT. **(B)** Δ K210/WT and **(C)** Δ K210/ Δ K210 cardiomyocytes in control or XINB overexpression group. **(D)** Statistics of video recording intensities showed increased contractile forces after XINB overexpression in WT/WT ($n = 29$), Δ K210/WT ($n = 30$), and Δ K210/ Δ K210 ($n = 30$) cardiomyocytes from 3 independent lines in each group. Relative expression of cardiac muscle contraction related genes **(E)** *TNNT2*, **(F)** *ACTN2*, and **(G)** *MYH7* increased after XINB overexpression. Data are represented as mean $\pm$ SEM. * $P < 0.05$.

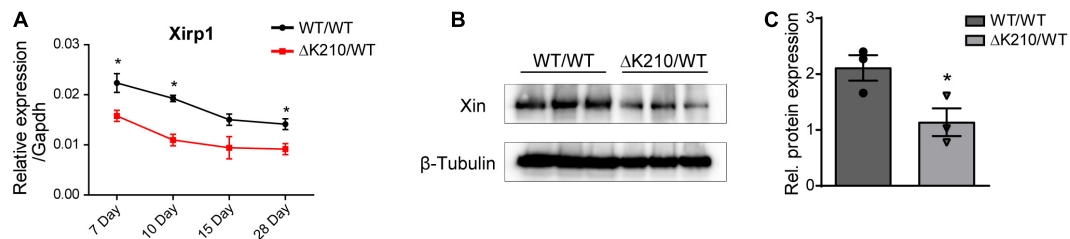


FIGURE 4 | Expression of *Xirp1* in the *TNNT2*- Δ K210 mice hearts. **(A)** Changes in temporal mouse *Xirp1* expression in wild type and *TNNT2*- Δ K210 mutant mice. **(B)** Western blot analysis showed decreased level of XIN protein in Day 7 *TNNT2*- Δ K210 mice hearts. **(C)**, Quantification of western blot signals in **(B)** and normalized to the loading control (β -Tubulin). Data are represented as mean $\pm$ SEM. * $P < 0.05$.

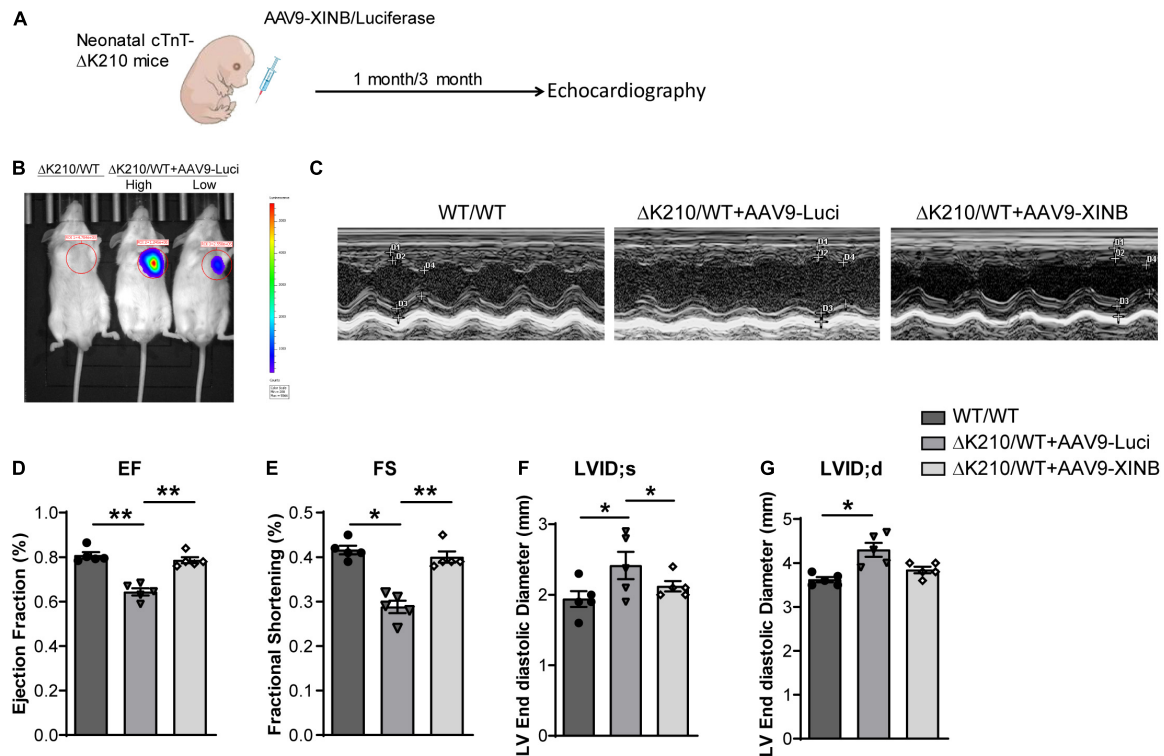


FIGURE 5 | Cardiac-specific expression of XINB improved cardiac function of *TNNT2*-ΔK210 mice. **(A)** Schematic illustration of the design of XINB delivery through AAV9. **(B)** *In vivo* bioluminescence imaging of mice tested the efficiencies of AAV9-Luci with the cTnT promoter for cardiomyocyte-specific gene expression. 3-day-old neonatal mice were intraperitoneally injected with AAV9-Luci viruses (1×10^{12} viral genomes per mouse). Mice without AAV injection were used as control. High: high dose. Low: low dose. **(C)** Representative M-mode echocardiography recordings of 3-months-old WT mice, AAV9-Luci injected *TNNT2*-ΔK210 mice, and AAV9-XINB injected *TNNT2*-ΔK210 mice. **(D)** Echocardiographic analysis of ejection fraction (EF), **(E)** fractional shortening (FS), **(F)** left ventricular end systolic internal diameter (LVID; s), and **(G)** left ventricular end diastolic internal diameter (LVID; d) of *TNNT2*-ΔK210 mice after XINB treatment ($n = 5$ per group). Data are represented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

organization and increased cell contractile force. Increase in the LVEF and LVFS after XINB treatment via AAV9 indicated that overexpression of XINB also improved heart functions *in vivo*. These results indicated XIN deficiency might be involved in the resulted loosely organized myofilament and systolic dysfunction caused by the *TNNT2*-ΔK210 mutation and overexpression of XINB could prevent DCM cardiac remodeling caused by the mutation.

Intra-exonic splicing of *XIRP1* gene resulted in three XIN isoforms: XINA (a full length protein), XINB (lacks filamin C-binding carboxy terminus) and truncated XINC that lacks XIN repeats, β -catenin-binding region and most of functional regions (van der Ven et al., 2006). Because of the limitations of lentivirus or recombinant adeno-associated virus vector capacity, we used XINB in this study. And we speculate XINA, as a full length protein, will work as well as XINB. Previous study revealed XINC protein only existed in hypertrophic tissues by comparing normal and hypertrophic human cardiac samples (Otten et al., 2010), suggesting that this isoform might also be involved in the pathogenesis of cardiomyopathy. Organization of individual components into thick or thin filaments and their integration into M-bands and Z-lines are important for myofibrillar assembly (Claeys et al., 2009; Sanger et al., 2010).

Consistent with our results, survey of microarray datasets in Gene Expression Omnibus (GEO) has revealed that *XIRP1* downregulated in failing hearts from human patients with diabetic or non-diabetic heart failure (GDS4314), and idiopathic DCM (GDS651) (Wang et al., 2014). XIN repeats as an actin-binding motif, directly bind actin filaments *in vitro* and cross link these filaments into networks in a similar manner as nebulin repeats. Within the XIN repeats, the overlapped β -catenin-binding domain, Ena/VASP binding domain DNA-binding domain and proline-rich region were identified from human XIN (van der Ven et al., 2006; Wang et al., 2012, 2013). XIN act as part of a complex that participate in the early morphogenesis of the heart and the maintenance of striated muscle integrity (Claeys et al., 2009). Deficiency of XIN might disrupt cytoskeletal protein arrangements and interactions, further weaken the stability of myofibril assembly and maintenance, thus leads to DCM.

In the present study, we were able to successfully rescue cardiac remodeling in a *TNNT2*-ΔK210 mouse model of dilated cardiomyopathy by overexpressing XIN. XIN deficiency might be a key mechanism of *TNNT2*-ΔK210 mutation-caused familial DCM and XIN could act as an interventional target for the relevant heart diseases.

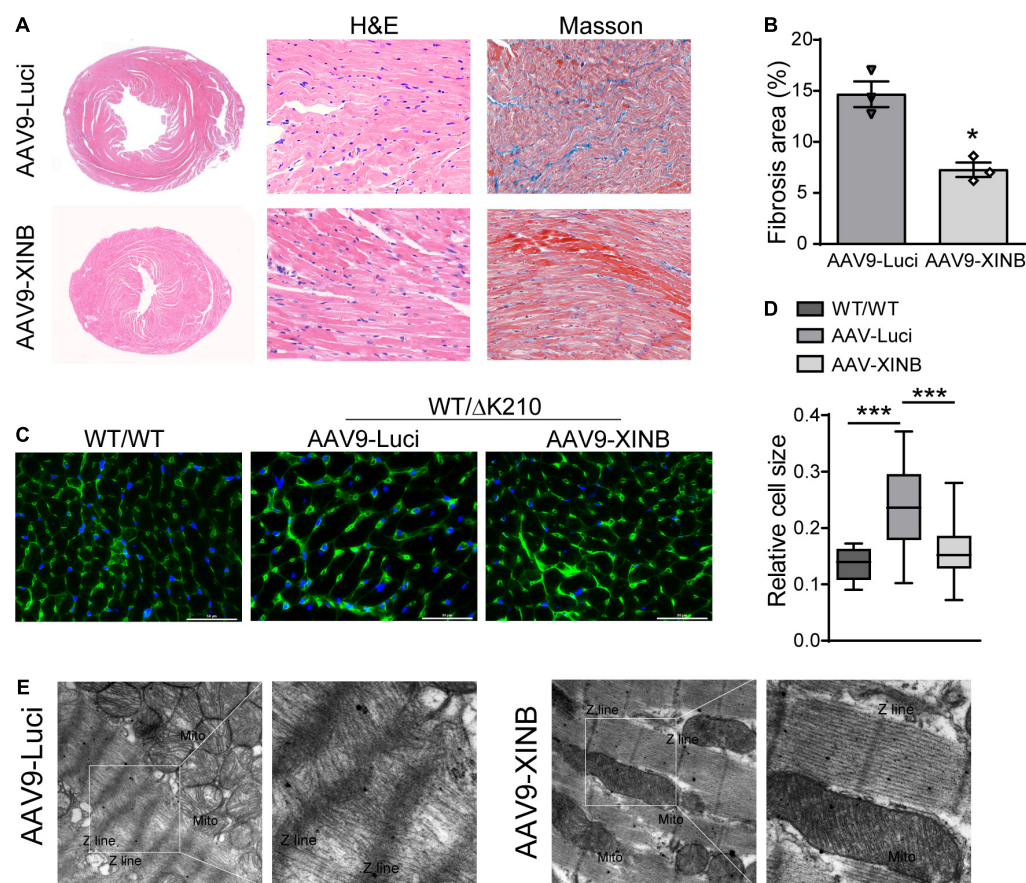


FIGURE 6 | Morphological studies showed cardiac-specific expression of XINB in *TNNT2-ΔK210* mice rescued DCM phenotypes. **(A)**, Representative H&E staining of the hearts from 3-months old control and AAV9-XINB injected *TNNT2-ΔK210* mice. Masson's trichrome staining showed less fibrosis in the mutant LV tissue when compared to control. **(B)** Quantification of interstitial fibrosis area in each group. **(C,D)** Wheat germ agglutinin (WGA; as an indicator of cardiomyocyte size) staining of left ventricle sections and quantification of myocyte sizes. Scale bars, 50 μm. **(E)** Ultrastructure of the left ventricular tissue sections from 3-months old mice revealed by transmission electron microscopy at $\times 6,000$ magnification. AAV9-XINB treatment significantly improved the sarcomeric structure of *TNNT2-ΔK210* mice. Data are represented as mean $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Fudan University.

AUTHOR CONTRIBUTIONS

BL, NS, QL, and CX conceived the research and wrote the manuscript. BL, YG, YZ, XZ, YL, and CZ performed the experiments and data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Deciphering the *in vivo* Dynamic Proteomics of Mesenchymal Stem Cells in Critical Limb Ischemia

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Objective: Regenerative therapy using mesenchymal stem cells (MSC) is a promising therapeutic method for critical limb ischemia (CLI). To understand how the cells are involved in the regenerative process of limb ischemia locally, we proposed a metabolic protein labeling method to label cell proteomes *in situ* and then decipher the proteome dynamics of MSCs in ischemic hind limb.

Methods and Results: In this study, we overexpressed mutant methionyl-tRNA synthetase (MetRS), which could utilize azidonorleucine (ANL) instead of methionine (Met) during protein synthesis in MSCs. Fluorescent non-canonical amino-acid tagging (FUNCAT) was performed to detect the utilization of ANL in mutant MSCs. Mice with hindlimb ischemia (HLI) or Sham surgery were treated with MetRS^{mut} MSCs or PBS, followed by i.p. administration of ANL at days 0, 2, 6, and 13 after surgery. FUNCAT was also performed in hindlimb tissue sections to demonstrate the incorporation of ANL in transplanted cells *in situ*. At days 1, 3, 7, and 14 after the surgery, laser doppler imaging were performed to detect the blood reperfusion of ischemic limbs. Ischemic tissues were also collected at these four time points for histological analysis including HE staining and vessel staining, and processed for click reaction based protein enrichment followed by mass spectrometry and bioinformatics analysis. The MetRS^{mut} MSCs showed strong green signal in cell culture and in HLI muscles as well, indicating efficient incorporation of ANL in nascent protein synthesis. By 14 days post-treatment, MSCs significantly increased blood reperfusion and vessel density, while reducing inflammation in HLI model compared to PBS. Proteins enriched by click reaction were distinctive in the HLI group vs. the Sham group. 34, 31, 49, and 26 proteins were significantly up-regulated whereas 28, 32, 62, and 27 proteins were significantly down-regulated in HLI vs. Sham

at days 1, 3, 7, and 14, respectively. The differentially expressed proteins were more pronounced in the pathways of apoptosis and energy metabolism.

Conclusion: In conclusion, mutant MetRS allows efficient and specific identification of dynamic cell proteomics *in situ*, which reflect the functions and adaptive changes of MSCs that may be leveraged to understand and improve stem cell therapy in critical limb ischemia.

Keywords: hind limb ischemia, mesenchymal stem cells, cell therapy, methionyl-tRNA synthetase, proteomics, mass spectrometry

INTRODUCTION

Peripheral artery disease (PAD) is caused by limited or restricted blood flow in peripheral arteries that can lead to limb pain, disability, or mortality. Critical limb ischemia (CLI) is the most serious form of PAD, which is characterized by a severe blockage in the arteries of the lower extremities and markedly reduced blood-flow (Dua and Lee, 2016; Campia et al., 2019). Cell-based therapies were regarded as an innovative way to improve blood flow recovery in order to prevent necrosis and amputation and limb-threatening complications (Tongers et al., 2014; Cho et al., 2015; Hou et al., 2016). Various stem and progenitor cells have been applied, including whole bone marrow cells, bone marrow mononuclear cells, hematopoietic stem cells, endothelial progenitor cells, and hemangiocytes (Padilla et al., 2007; Zhang et al., 2008; Kwon et al., 2011; Agudelo et al., 2012; Lee et al., 2013). Amid them, mesenchymal stem cells (MSCs) have been regarded as the most potential and promising cell types at both preclinical and clinical levels (Zhang et al., 2018; Jaillard et al., 2020; Yan et al., 2020; Yao et al., 2020).

MSCs are multipotent stem cells with the capacity of self-renewal and multilineage differentiation including mesodermal lineage, endodermal lineage and ectodermal lineage. Besides, they are easy to be obtained and expanded *in vitro*, marking its importance in cell therapy, regenerative medicine and tissue repair (Samsonraj et al., 2017; Trohatou and Roubelakis, 2017; Fu et al., 2019; Zhao et al., 2019). MSCs migrate toward and proliferate in response to chemokine gradients at sites of ischemia, where they aid in the promotion of neovascularization through paracrine effects or by terminally differentiating into vascular cells and myocytes (Lian et al., 2010; Hou et al., 2016; Gu et al., 2017; Shafei et al., 2017; Qadura et al., 2018; Park et al., 2019). However, the specific mechanisms how MSCs respond to and improve the ischemic microenvironment have yet to be fully characterized.

In this study, we utilized a metabolic labeling technique to tag and further enrich nascent proteins in the transplanted cells *in situ* (Erdmann et al., 2015; Sadlowski et al., 2018; Alvarez-Castelao et al., 2019; Han et al., 2020). Mutation of MetRS from L to G at 274 site allowed the utilization of ANL instead Met during protein synthesis. In this way, nascent proteins are tagged with ANL in cells transduced with mutant MetRS (Han et al., 2020). Further identification, enrichment and analysis of ANL tagged proteins will allow us to decipher the dynamic cell functions *in vivo*. We performed mass spectrometry and

extensive bioinformatics analysis to ANL tagged proteins in transplanted MSCs in HLI model. The metabolic labeling method doesn't modify the cells themselves, providing an efficient tool for in-depth and dynamic study of MSCs functions *in vivo*.

MATERIALS AND METHODS

MetRS^{L274G} MSC Transduction and Characterization

Bone marrow-derived mesenchymal stem cells (BMSCs), which were derived from C57BL/6J mice, was purchased from Cyagen Biosciences (Sunnyvale, CA, United States, Catalog Number: MUBMX-01001). (L274G) mutant-mCherry fusion protein was inserted into the cloning site of pMaRSC plasmid (Addgene, Plasmid #89189). BMSCs were seeded at 6 cm dish, and cultured with DMEM media supplemented with 10% FBS. When cells reached to ~80% confluence, lentivirus was applied with the addition of polybrene (10 µg/mL). Seventy-two hours after lentivirus infection, the transduction efficiency in BMSCs was evaluated by mCherry expression. The passage numbers of the MSCs used in all experiments were 6–10.

Flow Cytometry

Flow cytometry was performed as we previously described (Han et al., 2020). Briefly, BMSCs cells were digested by trypsin and collected by centrifugation. Cells pellet was resuspended in cold PBS and cells concentration was adjusted to 1×10^6 cells/200 µL. mCherry signals were acquired with BD TM LSR II (BD Biosciences, United States) and analyzed by FlowJo software.

Fluorescent Non-canonical Amino-Acid Tagging (FUNCAT)

MetRS^{L274G}-MSCs were cultured in DMEM media containing 10% FBS and 1mM ANL (Jena Bioscience, Jena, Germany) for 24 h. Then cells were gently rinsed with PBS and fixed in 4% formaldehyde in PBS. Click-iT reaction was performed with Click-iT reaction cocktails containing Alexa Fluor 488 alkyne (Click-iT Alexa Fluor 488 Protein Synthesis HCS Assay kit, Life Technologies, Waltham, MA, United States) according to the manufacturer's instructions. For FUNCAT of MetRS^{L274G}-MSCs in the mouse hind limbs, ANL was i.p. administrated at 15 mg/kg every 6 h for 4 times, then the animal was sacrificed. The gastrocnemius muscle was isolated and immediately placed

in pre-cooled 4% paraformaldehyde. After overnight fixation at 4°C, the tissue was dehydrated in 30% sucrose for another 12 h and embedded in OCT for preparation of frozen sections. The tissue sections were then evaluated by click-iT reaction as described above.

Limb Ischemia, Cell Transplantation, and Laser Doppler Imaging

Unilateral high femoral artery ligation and superficial femoral artery (SFA) excision was performed on 6- to 8-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, Me). Hindlimb ischemia surgery was performed as described previously (Niiyama et al., 2009). Briefly, mice were anesthetized with 2% isoflurane in 100% oxygen at a flow rate of 1 L/min. Ligations were placed at the femoral artery and its large branches in C57BL6 mice using 7-0 Prolene (Ethicon, Somerville, NJ) and the femoral artery was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries. Immediately after surgery, the mice were randomly assigned into four groups: the MSC + HLI group, the PBS + HLI group, the MSC + Sham group, the PBS + Sham group. In MSC group, a total of 1.0×10^6 cells in 100 μ L PBS was injected intramuscularly into 4 sites of the gastrocnemius muscle. Assessment of limb function and ischemic damage were performed as described previously. Tissue perfusion of the hind limbs was assessed with a laser Doppler perfusion imager (Moor Instruments, Devon, United Kingdom) on days 0, 1, 3, 7, and 14 days after treatment. The digital color-coded images were analyzed to quantify blood flow in the region from the knee joint to the toe.

HE Staining

The gastrocnemius muscle was isolated and prepared for frozen sections as in FUNCAT. Tissue sections of gastrocnemius muscle from the hind limbs were harvested at different time points. HE staining was performed as described before (Fischer et al., 2008). First, O.C.T. in tissue frozen sections was removed by soaking in ddH₂O for 5 min. Then the slice was applied into Harris Hematoxyllin solution (Fisher Chemical) for 30 s, then placed in 1% Li₂CO₃ (pH 8-8.2, diluted in ddH₂O) for 30 s after washing with ddH₂O, then placed in 80% ethanol for 1 min. Eosin was used to stain cytoplasm for 4 min, then dehydrated using 95% EtOH, 100% EtOH twice, and Xylene. At last, the slide was sealed with mounting medium and covered with a coverslip. The images were captured using Olympus IX83 microscope (Olympus, Japan).

Immunofluorescence

For capillary density analysis, sections were washed with ddH₂O and PBS. Blocking was performed by incubating sections with 5% BSA in PBST for 1.5 h. Tissue sections were incubated with rabbit polyclonal anti-mouse CD31 (1:100; PA5-38315, Thermo fisher) at 4°C for 12 h. After washing with PBST for three-times, sections were incubated with goat anti-rabbit conjugated with FITC (1:200; Invitrogen) and wash three times to remove free antibody. Then the slices were sealed with mounting medium

with DAPI. The images were captured using a laser-scanning confocal microscope, Fluoview FV300 (Olympus, Japan).

Isolation of ANL-Labeled Proteins From MetRS^{L274G} MSCs-Transplanted Hind Limb

Tissue sections from ischemic and healthy limbs were harvested at days 1, 3, 7, or 14 after HLI surgery, the mice were euthanized and gastrocnemius muscle were collected, ANL was i.p. administrated (15 mg/kg) at every 6 h for 4 times 1 day before collecting muscles. Gastrocnemius muscles were isolated and subjected to Click-iT reaction (Click-&-Go™ Dde Protein Enrichment Kit, click chemistry tools, United States) for enrichment of ANL-labeled proteins on an alkyne resin according to the manufacturer's instructions. Specifically, cell lysate from different gastrocnemius muscle samples was prepared with RIPA buffer (proteinase inhibitor should be protected from EDTA, which will inhibit click reaction). Then azide-modified proteins were enriched with Click-&-Go™ Dde Protein Enrichment Kit as recommend by manufacturer (Click Chemistry Tools, Scottsdale, AZ, United States). In brief, protein lysate was applied to click reaction with Dde Biotin Alkyne catalyzed with Copper (II) Sulfate. The biotinylated proteins were enriched with streptavidin agarose resin after removing the free Biotin Alkyne. Then peptides from biotinylated proteins were digested by Trypsin and desalt with C-18 desalting cartridges. The digested peptides were concentrated by Speedvac and used for the following MS analysis.

Sample Preparation and Data Acquisition by LC-MS

Cell Lysate from different samples was prepared with RIPA buffer (proteinase inhibitor should be protected from EDTA, which will inhibit click reaction). Then azide-modified proteins will be enriched with Click-&-Go™ Dde Protein Enrichment Kit as recommend by manufacturer (Click Chemistry Tools, Scottsdale, AZ, United States). In brief, protein lysate was applied to click reaction with Dde Biotin Alkyne catalyzed with Copper (II) Sulfate. The biotinylated proteins were enriched with streptavidin agarose resin after removing the free Biotin Alkyne. Then the peptide from biotinylated proteins will be digested by Trypsin and desalt with C-18 desalting cartridges. The digested peptide will be concentrated using a speedvac. Dried peptides were reconstituted in 20 μ L of 0.1% Trifluoroacetic acid. Eight microliter of each sample was injected into a 1260 Infinity nHPLC stack (Agilent Technologies, Santa Clara, CA, United States). This system runs with a Thermo Orbitrap Velos Pro hybrid mass spectrometer, equipped with a nano-electrospray source (Thermo Fisher Scientific, Waltham, MA, United States), and all data were collected in CID mode. The nHPLC was configured with binary mobile phases that included solvent A (0.1%TFA in ddH₂O), and solvent B (0.1% TFA in 15% ddH₂O/85% Acetonitrile), programmed as follows; 10 min at 2% solvent B; 90 min at 5–40% solvent B; 5 min at 70% solvent B; 10 min at 0% solvent B. Following each parent ion scan (300–1200 m/z @ 60k resolution), fragmentation data (MS2) was collected on the topmost intense

15 ions. For data dependent scans, charge state screening and dynamic exclusion were enabled with a repeat count of 2, repeat duration of 30 s, and exclusion duration of 90 s.

MS Data Conversion and Searches

The XCalibur RAW files were collected in profile mode, centroided and converted to MzXML using ReAdW v. 3.5.1. The mgf files were then created using MzXML2 Search (included in TPP v. 3.5) for all scans. The data was searched using SEQUEST engine, which was set max Missed Cleavages of 5, fragment tolerance of 0.00 Da, parent tolerance of 0.012 Da and variable modifications was + 16 on M (Oxidation), + 57 on C (Carbamidomethyl). Searches were performed by blasting the raw data with the Uniref100 database.

Peptide Filtering, Grouping, and Quantification

The lists of peptide IDs generated based on SEQUEST (Thermo Fisher Scientific, Waltham, MA, United States) search results were further analyzed using Scaffold viewer (Protein Sciences, Portland, Oregon, United States). Scaffold filters and groups all peptides to generate and retain only high confidence IDs while also generating normalized spectral counts across all samples for the purpose of relative quantification. Protein threshold was set to 99%, minimum Peptides to 2, and Peptide threshold to 80% to be consistent with the cutoff. Scaffold incorporates the two most common methods for statistical validation of large proteome data sets, false discovery rate (FDR), and protein probability. The FDR was set at <1% cutoff, with a total group probability of ≥ 0.8 , with at least two peptides assigned per protein. Relative quantification was performed via spectral counting and spectral count abundances were normalized between samples.

Bioinformatics and Statistics Analysis

Difference analyses in protein expression between the Sham and HLI groups were performed using a *t*-test, and $p < 0.05$ was taken to indicate statistical significance. 1.5-fold increases or reductions were defined as the standard for differentially expressed proteins (DEPs). Hierarchical clustering was performed to cluster proteins profiles based on complete linkage method. Gene Ontology (GO) analysis were conducted using the interproscan-5 program against the non-redundant protein database (including Pfam, PRINTS, ProDom, SMART, ProSiteProfiles, PANTHER). Functional pathway enrichment of DEPs were performed according to the Kyoto Encyclopedia of Genes and Genomes database (KEGG)¹. The enrichment pipeline was used to perform the enrichment analysis of GO and KEGG, PANTHER, REACTOME, and WIKI pathway, respectively. A protein-protein interaction network was extracted by matching all the differentially expressed proteins against the STRING-db server², a database of both known and predicted protein-protein interactions.

¹<http://www.kegg.jp/>

²<http://string.embl.de/>

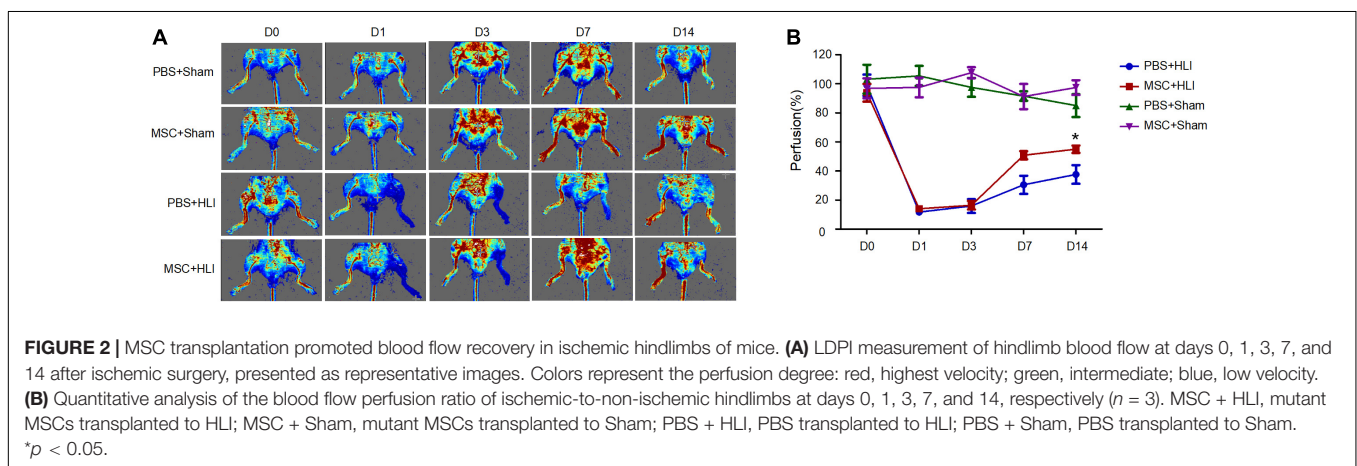
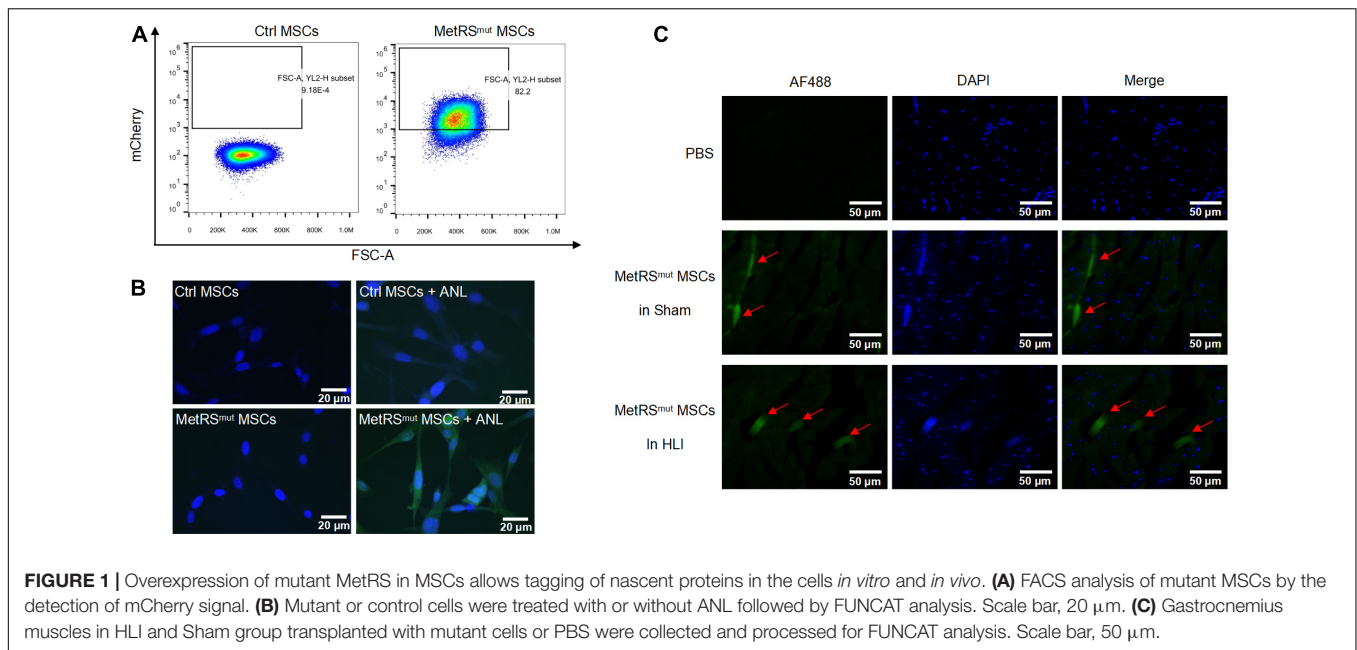
RESULTS

Mutant MetRS Labels Nascent Proteins in the MSCs by Incorporation of ANL

When BM-MSCs at passages 3–4 reached 80% confluence, cells were infected with lentivirus carrying MetRS^{L274G}-mCherry as previously described (Han et al., 2020). As shown in **Figure 1A**, 82.2% of the mutant cells expressed strong mCherry signal and mCherry positive cells were further sorted by flow cytometry. After treatment with 1 mM ANL for 24 h, Fluorescent non-canonical amino-acid tagging (FUNCAT) was performed according to the instructions (Han et al., 2020). Nascent proteins with ANL incorporation in mutant MSCs can be detected by click reaction with alkyne conjugated with Alexa Fluor 488, while no proteins were labeled with Alexa Fluor 488 in control groups, indicating a high labeling efficiency and a good sensitivity of this method (**Figure 1B**). MetRS^{L274G}-MSCs were administered into the gastrocnemius muscle immediately after the surgery, the samples were harvested at days 1, 3, 7, and 14. ANL (15 mg/kg) was i.p. injected every 6 h for 4 times at 24 h before sacrificing the mice. The tissue slices were subjected to click-iT reactions with alkyne-Alexa Fluor 488 (FUNCAT), and the MetRS^{L274G}-MSCs showed a strong FUNCAT signal, indicating nascent ANL-tagged proteins in these cells (**Figure 1C**). Thus, MetRS^{L274G} MSCs can be tagged *in situ* after injection into mouse hind limb by click-iT reaction.

MSC Treatment Improves Blood Reperfusion in the Mouse Ischemic Hindlimb

To determine the repair function of MSCs in ischemic tissues, 1×10^6 MSCs in 50 μ L of PBS or 50 μ L of PBS were transplanted into gastrocnemius muscles immediately after left hind limb ischemia of C57/BL6 mice. Then the blood perfusion was measured by laser doppler perfusion imaging. After ligation, the blood perfusion in left hindlimb severely diminished to $\sim 10\%$ compared to that of right hindlimb (**Figures 2A,B**). Mice treated with either MSCs or PBS showed equal proximal and distal blood flow on days 1 and 3 after femoral artery ligation. At day 7, the MSC group showed augmented proximal and distal perfusion, which was much more enhanced at day 14 (**Figures 2A,B**). Histologic staining were correlated with functional outcomes of blood perfusion in different groups. As shown in the **Figure 3A**, gastrocnemius muscle fibers in the MSC and PBS groups were seriously disrupted at 1 day after ligation; inflammatory cells were recruited to the ischemic muscle at days 3 and 7 after ischemia. At day 14, the MSC-treated muscles had less inflammatory cells and a relatively intact structure. Angiogenesis was also assessed by CD31 immunofluorescence staining. As shown in **Figure 3B**, capillary density was severely decreased at day 3 after ischemia. At day 7 after ischemia, vessels became dense where there are the most inflammatory cells. In addition, more larger vessels appeared in MSC group than PBS group at days 7 and 14 after ischemia. In total, the MSC-treated mice showed increased angiogenesis compared with the control mice, which is consistent with the data of blood reperfusion (**Figure 2**).



Analysis of the MSC Proteome in the Ischemic Muscle at Different Time Points

To delineate the beneficial effects of MSCs in ischemic hindlimb, adaptive changes of MSCs proteomics *in vivo* were detected by highly sensitive click reaction followed by LC/MS analysis. The gastrocnemius muscle lysates, comprised of three biologically independent samples for all groups at each time point, were subjected to isolation of ANL-labeled proteins by a cleavable alkyne-biotin-tag immobilized on streptavidin resin. We obtained $\sim 4 \mu$ g of ANL-labeled proteins from each mouse gastrocnemius muscle transplanted with 1×10^6 MSCs. Notably, a relatively large number of ANL-labeled proteins were identified in the MetRS^{L274G} MSC-injected Sham and HLI muscle at days 1, 3, 7, and 14 post-surgery/cell administration (182, 178, 189 and 175 for Sham and 175, 185, 170 and 167 for HLI, respectively) (**Figure 4A**). The proteins were analyzed by hierarchical analysis, MSC proteins in HLI group were distinctively different from

those in Sham group at four time points (**Figure 4B**). Moreover, the isolated proteins in HLI group were more pronounced in biological process, such as energy metabolic process and translation (**Supplementary Figure 1**).

To gather comprehensive information on differentially expressed proteins, we applied the volcano plots for all data in ischemic vs. normal muscle. Interestingly, 34, 31, 49, and 26 proteins were significantly up-regulated, whereas 28, 32, 62, and 27 proteins were significantly down-regulated in HLI vs. Sham group at days 1, 3, 7, and 14, respectively (**Figure 5**). All differentially expressed proteins at different time points were also displayed by heat map (**Figure 6**), which give us a better understanding of the functional dynamic MSC proteomes *in vivo*. For example, 60S ribosomal proteins had distinctive expression levels through days 1 to 14, suggesting their different roles at the progressing stages of hind limb ischemia. In addition, cytoskeletal proteins vimentin, A-X actin, and filamin were detected at a higher level in HLI group than in Sham group, suggesting

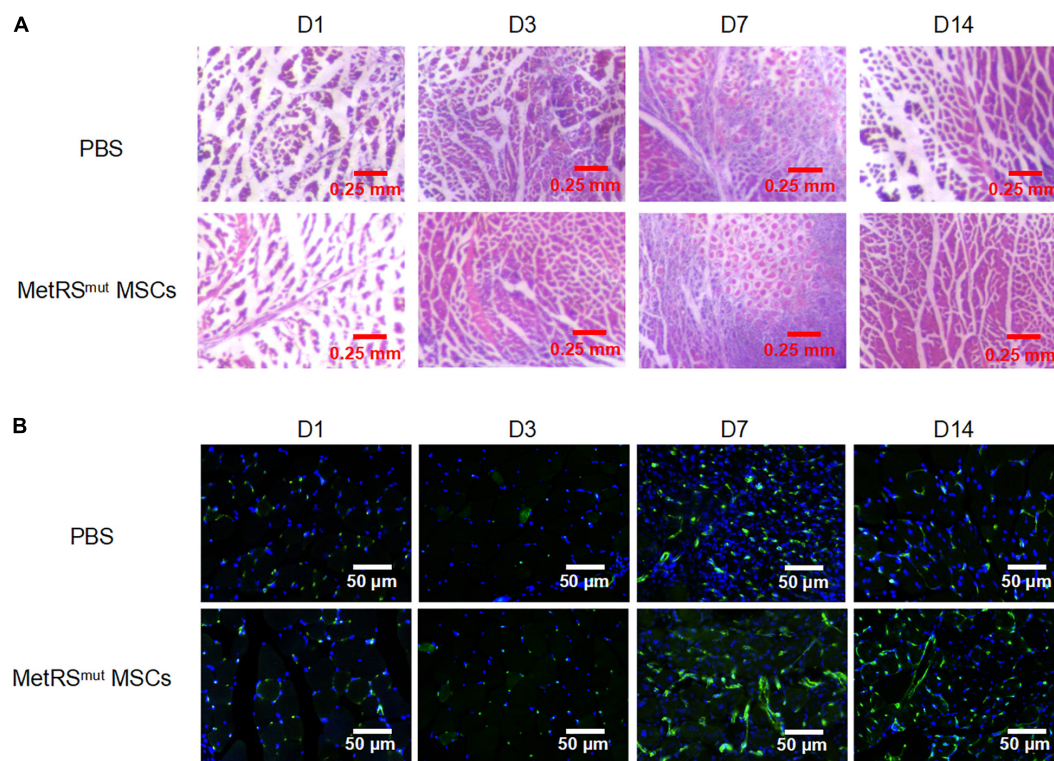


FIGURE 3 | Histological analysis of ischemic hindlimb by HE and vessel staining. Gastrocnemius muscles in HLI group transplanted with either mutant cells or PBS were collected and processed for HE staining (A) and CD31 staining (B). Scale bar, 250 μm for (A) and 50 μm for (B).

MSCs' contribution to the production of extracellular matrix in ischemic muscle.

MSCs Respond to the Ischemic Environment by Altering Proteins Involved in Apoptosis and Energy Metabolism

We performed KEGG pathway and enrichment analysis which allows us to identify the enriched biological pathways, and processes involved in the reparative process of MSCs in ischemic hind limb (Figure 7). At days 1 and 3, pathways and proteins related to apoptosis induced DNA fragmentation, activation of DNA fragmentation factor, formation of senescence-associated heterochromatin foci (Sahf), apoptotic execution phase were highly enriched in the HLI group as compared to the Sham group. The highly expressed apoptosis related proteins and pathway indicated a drastic change and adaptation of MSCs in ischemic environment, which can explain the loss of most injected MSCs after injection. At day 7, pathways and proteins related to energy metabolism induced ATP synthesis, glycolysis, TCA cycle, glycolysis/gluconeogenesis, biosynthesis of amino acids, amino acid metabolism were highly enriched in the HLI group as compared to the Sham group. Remarkably, the pathways of energy metabolism gradually possess a dominant position through days 7 and 14. Notably, proteins enriched for apoptosis and energy metabolism are reported to be associated

with inflammation and tissue regeneration (Kujoth et al., 2005; Abdel Malik et al., 2017), suggesting that MSC proteomics may be an indispensable resource for ischemic therapy.

Furthermore, we analyzed protein and protein interaction networks of the differentially expressed proteins from serial time-points (Figure 8) in order to gain an overall understanding of MSC-mediated beneficial effects during ischemic response. MSCs in HLI group expressed an abundance of proteins related to regulation of apoptosis (Atp5a1, Mdh2, Sdhb), which reflects the functional states of MSCs in adaptation to the ischemic environment by maintaining cell survival. In addition, the different proteomic changes of MSCs in HLI were pronounced in ribosome function, protein translation and energy metabolism related proteins (Gm6096, Rpl34, Rpl4, Eef2). Transplanted cells also secreted massive tissue remodeling proteins exerting cytoprotective effects through elevated expression of structure proteins (Mybpc2, Myl2, Myh4, Mylpf) following HLI.

DISCUSSION

The major findings of this study are: (a) Overexpression of mutant MetRS in MSCs provides a sensitive and specific method to analyze dynamic cell proteomes *in vivo*. (b) The expression of ribosomal proteins were changed at 1 day post-surgery and throughout to 14 days, suggesting disrupted protein translation *in situ* in the ischemic limb; (c) Substantial amount of apoptosis

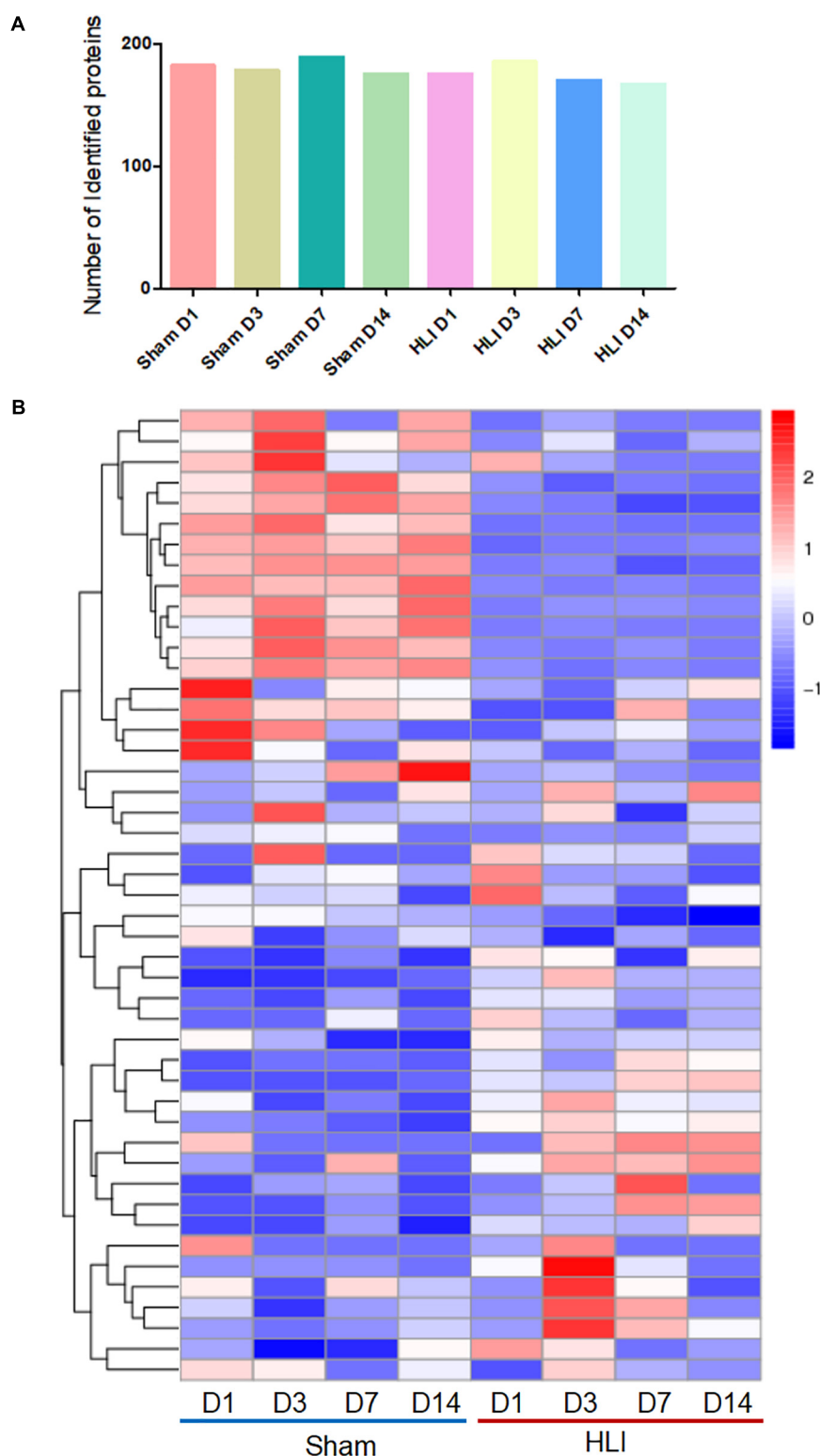


FIGURE 4 | Identification of MSC-specific proteome in the hindlimb. The mice were subjected to HLI or Sham surgery, and mutant cells were i.m. injected in the gastrocnemius muscle. Mice were then randomly grouped and received i.p. injections of ANL at days 0, 2, 6, and 13, gastrocnemius muscles were collect 24 h later for protein enrichment and biological analysis. $n = 3$ mice for each time point in each group. **(A)** Nearly 200 proteins were identified in the eight groups.

(B) Hierarchical clustering of proteins expressed in all groups. Each column corresponds to the mean expression levels of proteins in 3 independent biological samples, and each row corresponds to a protein.

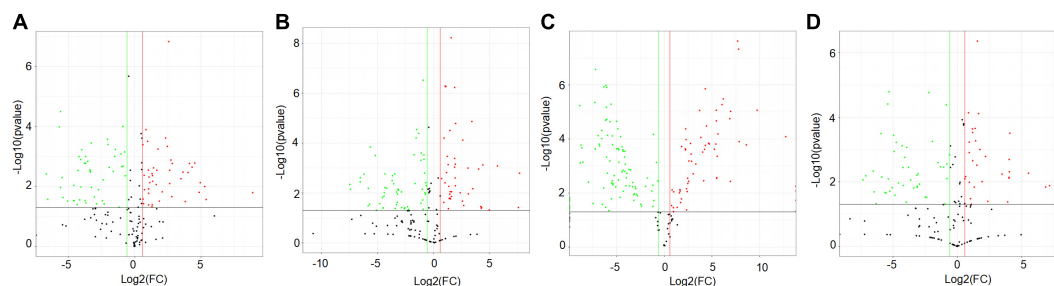


FIGURE 5 | Volcano plot showing the differentially expressed proteins at each time points. Proteins with statistically significant differential expression (≥ 1.5 -fold, $p < 0.05$) at days 1 (A), 3 (B), 7 (C), and 14 (D) located in the top right and left quadrants. Red dots indicate up-regulated proteins (ratio > 1.5 , $p < 0.05$), green dots indicate down-regulated proteins (ratio < 0.67 , $p < 0.05$).

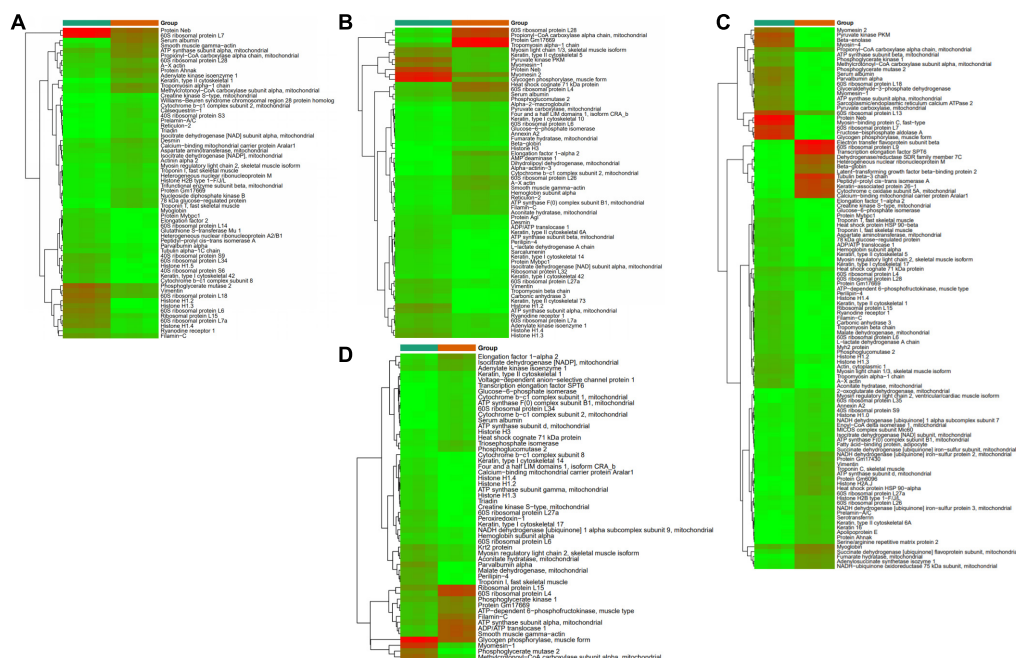


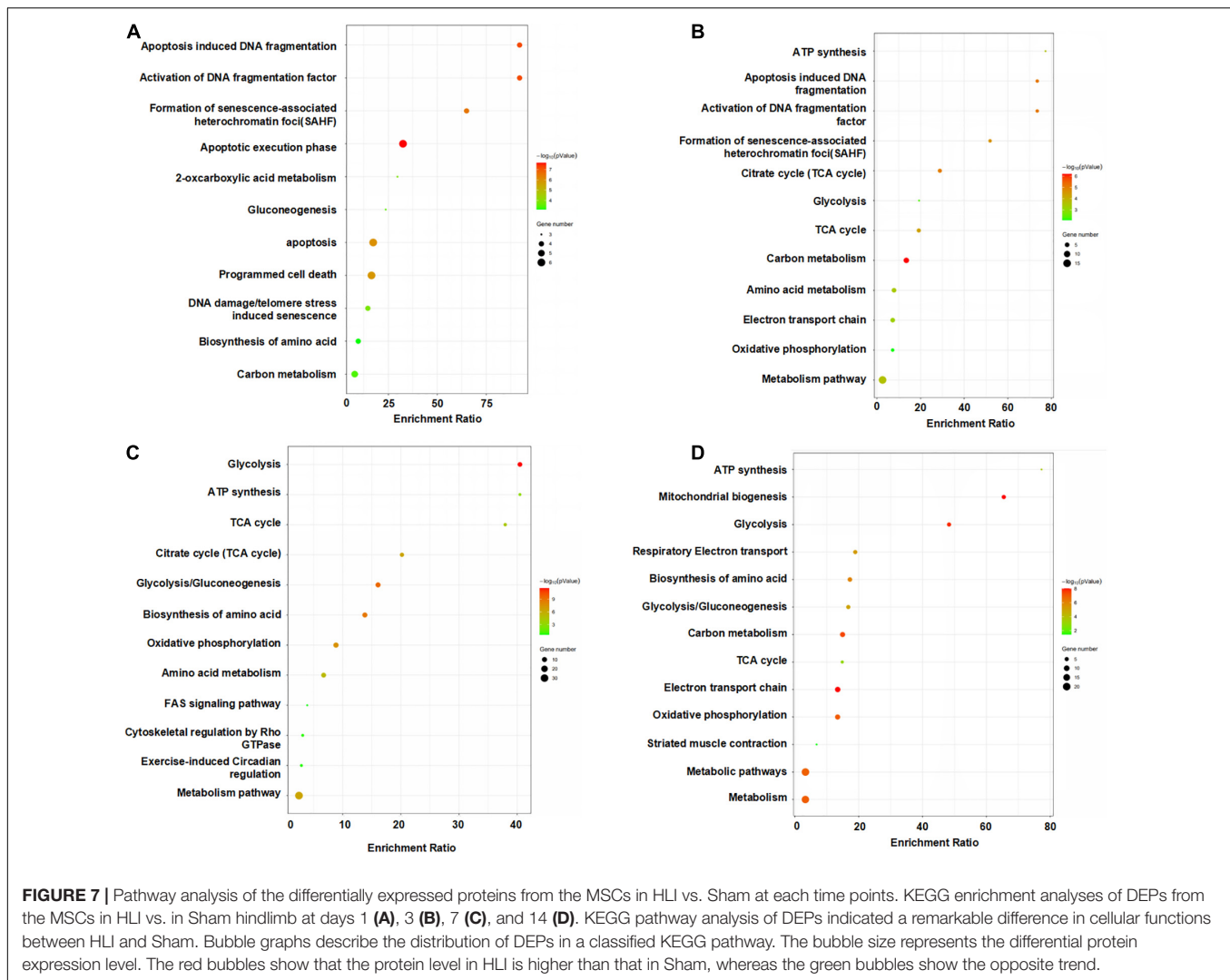
FIGURE 6 | Heat map showing the differentially expressed proteins at each time points. The heat map shows the differentially expressed proteins at days 1 (A), 3 (B), 7 (C), and 14 (D). Each row represents a differentially expressed protein and each column, sample replicate. The color scale illustrates the relative level of differentially expressed protein expression: red, higher than the reference channel; green, lower than the reference.

and energy metabolism related proteins were secreted from MSCs and involved in the repair process of HLI.

Metabolic Labeling of Nascent Proteins *in situ*

MSCs-mediated tissue repair is mainly through releasing growth factors, cytokines, and extracellular vesicles. Potential mechanisms include angiogenesis, beneficial tissue remodeling, anti-inflammatory responses, and finally promotion of tissue endogenous regeneration. However, most MSC proteome studies were performed in cell culture, and the identified factors may not represent their true function *in vivo*. So techniques that could identify cell-type specific proteomes *in situ* may help us better understand how the cells respond to the

ischemic microenvironment. Frequently used methods to isolate specific cell types are FACS sorting and laser microdissection of fluorescent labeled cells. Metabolic proteome labeling is a technique that could isolate specific cell proteomes while maintaining cellular integrity during isolation. The first study to perform *in situ* proteome labeling was termed BioOrthogonal Non-Canonical Amino acid Tagging (BONCAT) (Dieterich et al., 2006). In this method, expression of mutant MetRS is driven by a cell-specific promoter. Mutant MetRS could utilize non-canonical amino acid azidonorleucine during protein synthesis. After labeling, click reaction could be performed to biotinylate ANL residues, followed by enrichment via streptavidin beads. In our study, we used the previously established bio-orthogonal proteome labeling method to isolate and analyze cell-specific proteomes in ischemic hind limb.



MSC Therapy Improved Hindlimb Perfusion in a HLI Model

Intramuscular injection of MSCs increased vascular density and perfusion in the hind limb at 2 weeks post-treatment compared to PBS alone. It is likely that infused MSCs engraft and alter the parenchymal microenvironment and stimulate endogenous regenerative mechanisms. It is consistent with previous studies in mouse HLI model using MSC therapy regardless of stem cell types (allogeneic, autologous, or xenograph), the route of administration (IV, or IM), and the timing of cell transplantation following surgical ligation (Parolini et al., 2010). To investigate cell proteome profiles *in situ* could further reveal targets upon which MSCs alter and decipher mechanisms of action for MSCs in HLI.

Proteomic Changes of MSCs From HLI in Muscle Tissue

The change of ribosomal proteins to ischemic insults has been shown to persist for up to 14 days. We demonstrated that the

increased expression of RPL28 and RPL26 at days 1 and 3 post-surgery, consistent with other reports (Lee et al., 2013; Ong et al., 2013; Han et al., 2020), and elevations declined to normal values by day 14. RPL28 belongs to the ribosomal proteins family. These proteins constitute the large subunit and small subunit of the ribosome primarily responsible for mRNA translation and protein synthesis. In addition to their role in ribosomal biogenesis and protein production, ribosomal proteins possess ribosome-independent functions, especially in tumorigenesis, immune signaling and development. A recent study suggested that high RPL28 was associated with changes in extracellular matrix pathways, indicating a potential role for this protein in tissue remodeling (Labriet et al., 2019; Han et al., 2020). Indeed, we observed an up-expression of several ECM proteins such as filamin-C, and vimentin at different time points after surgery. ECM proteins do not simply serve as structural scaffold that determines the mechanical properties. Under conditions of stress, ECM proteins can drive cell biological responses with an important role in the tissue remodeling (Jourdan-Lesaux et al., 2010; Frangogiannis, 2017; Frangogiannis, 2019). Under hypoxic

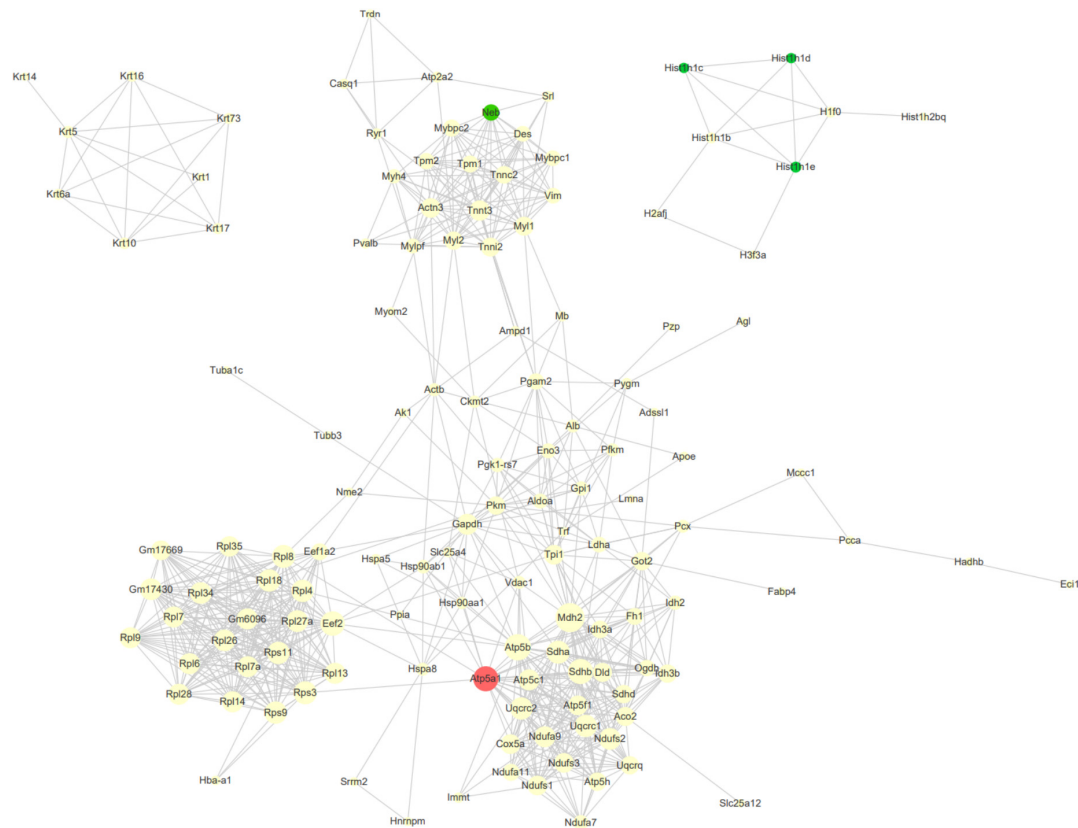


FIGURE 8 | Protein-protein interaction (PPI) network analysis of DEPs. Network analysis using CytoScape of the MSC proteins interactome reveals clustering around nodes involved in apoptosis, metabolism and structural maintenance. The degree of a node indicates the number of connections to other nodes, edge thickness indicates the degree of interactions. The red nodes indicated consistently upregulation DEGs at four time points, and the green nodes indicated consistently downregulation DEGs.

stress, filamin-C interacted with other molecules to restore cellular membrane integrity, thereby promoting cellular survival during these conditions (Leber et al., 2016; Neethling et al., 2016). Overall, it may be envisioned that ribosomal proteins, apoptosis related proteins and ECM proteins are secreted from MSCs and involved in the repair process of HLI.

There are several limitations of this study. Studies have shown that transplanted MSCs differentiate into endothelial cells, secrete angiogenic factors, and thereby induce neovascularization in ischemic tissue (Al-Khaldi et al., 2003a,b; Kinnaird et al., 2004). In the present study, MSC transplantation markedly increased blood perfusion and capillary density in the ischemic hindlimb compared with control group. However, we didn't identify many angiogenic factors secreted by MSCs to echo this phenomena. Methionine is the first amino acid of a protein polypeptide, and the signal peptide, which is in the first 16–30 amino acid, is snipped off during translation followed with transport through the endomembrane system. Therefore, secretory proteins lose the first methionine when signal peptide is cleaved off, leading to less ANL incorporation and tagging of secretory proteins. This might explain that secretory proteins are less tagged and enriched in our study. In addition, we did not explore the difference of intramuscular or intra-arterial

injections into the ischemic hindlimb that are common routes of cell transplantation. Direct implantation of MSCs has several advantages including increasing the local production of anti-inflammatory and angiogenic factors in the ischemic limb. However, cells remain localized near the site of injection with intramuscular injection, whereas intra-arterially implanted cells distribute broadly in the ischemic tissue, which might differ in their proteomes *in situ*. Further research should determine if different transplantation routes would alter function of MSCs and if some combination of administration routes would be optimal for their therapeutic effects.

CONCLUSION

This study provided evidences that overexpression of mutant MetRS in MSCs provides a sensitive and specific method to analyze dynamic cell proteomes *in vivo* in a mouse ischemic hindlimb model. MSCs respond to the ischemic environment by altering proteins involved in apoptosis and energy metabolism. Although we demonstrate a series of protein changes in the ischemic microenvironment, follow-up studies will be required to confirm the proteome profiling in our study.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD026954 and doi: 10.6019/PXD026954.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics committee of Southern Medical University.

AUTHOR CONTRIBUTIONS

YD, XL, and WY: study design, data acquisition and analysis, manuscript drafting. YD, DH, HO, and XP: data acquisition. ZZ, BL, BZ, QF, and DL: data analysis/interpretation. ZH and ZL: manuscript editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.682476/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Nanoparticles for Stem Cell Tracking and the Potential Treatment of Cardiovascular Diseases

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Stem cell-based therapies have been shown potential in regenerative medicine. In these cells, mesenchymal stem cells (MSCs) have the ability of self-renewal and being differentiated into different types of cells, such as cardiovascular cells. Moreover, MSCs have low immunogenicity and immunomodulatory properties, and can protect the myocardium, which are ideal qualities for cardiovascular repair. Transplanting mesenchymal stem cells has demonstrated improved outcomes for treating cardiovascular diseases in preclinical trials. However, there still are some challenges, such as their low rate of migration to the ischemic myocardium, low tissue retention, and low survival rate after the transplantation. To solve these problems, an ideal method should be developed to precisely and quantitatively monitor the viability of the transplanted cells *in vivo* for providing the guidance of clinical translation. Cell imaging is an ideal method, but requires a suitable contrast agent to label and track the cells. This article reviews the uses of nanoparticles as contrast agents for tracking MSCs and the challenges of clinical use of MSCs in the potential treatment of cardiovascular diseases.

Keywords: nanoparticles, stem cell therapy, mesenchymal stem cell, cardiovascular diseases, imaging technique

INTRODUCTION

In the medical world, stem cell-based therapies have emerged and expanded as a potentially promising therapeutic treatment (Heslop et al., 2015). In such a treatment process, stem cells, possessing properties of self-renewal and potency, play a significant role, and under appropriate conditions, these cells can be differentiated into a variety of cells (Srivastava and Ivey, 2006; Maumus et al., 2011; Hao et al., 2014; Aly, 2020). Compared with direct transplantation of tissue or organ for patients, stem cell-based therapies are more promising and potential because these cells can be feasibly and readily obtained avoiding donor shortage and immune incompatibility (Diekman and Guilak, 2013; Lilly et al., 2016). In general, stem cells can be divided into two major types, embryonic stem cells (ESCs) and adult stem cells (ASCs) (Lv et al., 2014). The transplantation of ESCs derived from the early embryo has been demonstrated to be effective in the treatment of many diseases, due to their being highly undifferentiated cells and having the ability of evolving into all tissues and organs (Krebsbach and Robey, 2002; Hao et al., 2009). However, the research for ESCs is controversial, being difficult to accepted by common people (Li et al., 2010). Compared with ESCs, ASCs, which originated from tissues, are easily accepted and have been widely applied

in preclinical and clinical trials, but leads to low capacity of differentiation (Young and Black, 2004; Ye et al., 2006; Alison and Islam, 2009). For example, the hematopoietic stem cells (bone marrow), originated from ASCs in the blood, can differentiate into various types of mature blood cells, and their transplantation has been used to treat all kinds of blood diseases (Carella et al., 2000; Schmitz et al., 2002; Mikkola and Orkin, 2006; DiPersio et al., 2009; Jaiswal et al., 2009). Neural stem cells, possessing the ability of differentiating into neurons and astroglia, are also derived from ASCs, and their transplantation may treat diseases of the nervous systems (Temple, 2001; Lee et al., 2005, 2009; Andres et al., 2008; Stenudd et al., 2015). In 1970, Friedenstein discovered a rare type of stromal cells in human bone marrow that are now known as mesenchymal stem cells (MSCs) (Friedenstein et al., 1970). Although MSCs have similar abilities to ASCs, there is still a lot of debate about their origin. Moreover, some researchers claimed that the name of MSCs should be changed to medicinal signaling cells in order to accurately reflect the fact that these cells play the role of constructing new tissues (Caplan, 2017). At present, MSCs are found in many tissues beyond the bone marrow, including adipose tissue, lung tissue, synovial membrane, endometrium, and peripheral blood. In different tissues, MSCs exhibit differences in immunophenotype, differentiation potential, and immunomodulatory. The researches show that the transplantation of MSCs is an effectively promising and potentially therapeutic treatment for various diseases including wound healing, ischemic encephalopathy, and ischemic heart disease (Grauss et al., 2007; Wu et al., 2007; Uccelli et al., 2008; Kim et al., 2015a; Xie et al., 2016).

To date, stem cell-based therapies have been intensively employed in the treatment of wounds, blood and cardiovascular diseases, cartilage defect, diabetes, etc. (Goradel et al., 2018; Vagnozzi et al., 2020). However, there are still much to be further explored for stem cell-based therapies, in order to identify therapeutic efficacy and further dosage (Yi et al., 2017). For these purposes, it is necessary to track stem cells *in vitro* and *in vivo* with high temporal and spatial resolution in a real-time manner during a prolonged period, which involves monitoring stem cells' behavior *in vivo*, identifying if they are capable of surviving, integrating with the host tissue, undertaking the desired cellular differentiation, and finally unveiling the responses and behaviors of transplanted cells upon exposure to various diseased environments.

THE MEANS OF TRACKING STEM CELLS AND THE IMPORTANCE OF NANOPARTICLES

For tracking stem cells, non-invasive cell imaging is currently a very hot field of research (Kircher et al., 2011). Compared with traditional histopathological techniques, non-invasive cell imaging can monitor the behaviors of the transplanted cells over time *in vivo* for further treatment offering detailed information, thus, showing more efficient therapeutic effects (Cahill et al., 2004; Walczak and Bulte, 2007; Bulte, 2017;

Meir and Popovtzer, 2018; Peng et al., 2018). Only by using non-invasive means can the viability of the transplanted cells be estimated in the clinical setting, showing potential prospects (Shang et al., 2017; Tkaczyk, 2017). Non-invasive cell tracking can be divided into direct and indirect labeling techniques. Compared with direct labeling, which can directly label cells, possessing easy operation but being unable to track daughter cells due to fast signal decay, indirect labeling obtains the ability of labeling cells by implanting an indicator into cells (Li et al., 2008; Kraitchman and Bulte, 2009; Kim et al., 2016a,b). Although indirect labeling is complex and expensive, it is more effective and stable for tracking the differentiation of the transplanted cells (Davis, 1972; Akins and Dubey, 2008; Hong et al., 2010). In general, indirect labeling can be divided into two major classes, gene modification and external label. Gene modification is suitable for long-term tracking because the labeled genes can be steadily expressed in the next generation (Chan et al., 2017; Su et al., 2019). However, the imaging technique of gene modification is single and only being detected by a tracking fluorophore leading to the limitation of tissue penetration (Herschman, 2004; Loewen et al., 2004). In addition, the labeled genes are derived from exosomes, leading to immune incompatibility (Zheng et al., 2017). Another indirect labeling method, external label, can overcome the obstacle of the foreign gene by introducing additional contrast agents, but it still has some limitations (Crabbe et al., 2010; Li et al., 2013; Hachani et al., 2017). Because the introduction of contrast agents brings some new problems, including the reduction effect of photo-signal over time, optical interference is induced by tissue autofluorescence, incompatibility, cytotoxicity, and low labeling efficiency (Bellin, 2006; Cosgrove, 2006; Lusic and Grinstaff, 2013). Thus, an appropriate contrast agent should be developed and utilized to break through these limitations.

Nanoparticles (NPs), the diameter of which ranges from 1 to 100 nm, cover a diverse range of chemical composition with an equally diverse number of applications (Khan et al., 2019). Due to their nanoscale dimensions, NPs can easily transport across cell membrane and reach the crucial organelles including the endoplasmic reticulum, mitochondria, and nucleus (McNamara and Tofail, 2017). Moreover, a high surface area-over-volume ratio augments their interaction with cellular components (Bhirde et al., 2011; Edmundson et al., 2013). Furthermore, the morphology and size of NPs determine their physicochemical properties leading to the differences in cellular uptake and interaction with biological tissues, which could not be achieved by bulk materials (Barua and Mitragotri, 2014). As a result, NPs have been regarded as a promising contrast agent. Nowadays, nanotechnology in medical science has been regarded as a great breakthrough in this century (Zhang et al., 2008; Lin, 2015). Importantly, some special NPs have unique magnetic and/or optical properties, which can offer real-time imaging *in vivo* for tracking the transplanted stem cells, showing a strong potential in breaking through the obstacle of external label (Rhyner et al., 2006; Hahn et al., 2011). These NPs for tracking stem cells include organic, inorganic, and composite NPs, and the representative examples have magnetic NPs and photoacoustic NPs (Van Den Bos et al., 2003; Ferreira, 2009; Wang et al., 2012b; Gao et al., 2013; Accomasso et al., 2016; Riera et al., 2019).

The transplanted stem cells labeled by these NPs can be detected by multiple imaging methods, such as magnetic resonance imaging (MRI), nuclear imaging [including single-photon emission computed tomography imaging (SPECT) and positron emission tomography-computed tomography (PET-CT)], and photoacoustic imaging (Sutton et al., 2008; Andreas et al., 2012; Cheng et al., 2016; Guo et al., 2019b; Patrick et al., 2020). However, there are still many problems to be addressed in the actual application process for NPs. First, NPs for tracking stem cells *in vivo* always require good cellular uptake and efficient internalization. Therefore, the surface chemistry of NPs should be importantly considered (Accomasso et al., 2016). In most cases, NPs with positive charge have the improvement of cellular uptake due to electrostatic interaction, leading to an accelerated internalization (Saha et al., 2013; Behzadi et al., 2017). Certainly, NPs with positive charge also may delay their internalization process, due to the influence on the adsorption of some specific proteins (Jambhrunkar et al., 2014). Second, importantly, before use, NPs should be comprehensively characterized for their composition and purity under different conditions, in order to evaluate their toxic effects on cells and ensure safety *in vivo* (Chandran et al., 2017). The reasons are that their toxic effects on cells may highly depend on the unique characteristics of NPs themselves. What is more, compared with bulk material, NPs have higher surface area-to-volume ratio and surface reactivity, which are more susceptible to the environment, so they should be modestly employed (Alkilany and Murphy, 2010; Lankoff et al., 2012). In addition, close attention should be paid to the agglomeration of NPs, which may lead to bad cellular uptake and further induced cytotoxicity (Gluga et al., 2014).

The internalization of NPs is influenced by their charge, size, shape, and the ability of cellular uptake, due to the force of their internalization driven by endocytosis. After internalization by endocytosis, NPs will pass environments of lysosomes and peroxisomes where the acidic material and oxidative substance could entirely degrade them. Possibly, a part of them may undergo exocytosis or escape to other intracellular locations (Chen et al., 2013; Calero et al., 2014).

NANOPARTICLES FOR TRACKING MSCS AND THE POTENTIAL TREATMENT OF CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVDs), with high incident, are one of the major causes of human deaths worldwide and responsible for more than 17.7 million deaths in 2015, according to the World Health Organization (Zampelas and Magriplis, 2020). Therefore, developing an effective means for treating CVDs is of paramount urgency (Behlke et al., 2020). Currently, the means for treating CVDs include, but are not limited to, surgical treatment, delivering the targeted drug, and the repair and regeneration of the cardiac tissues (Godin et al., 2010; Cui et al., 2016; Deng et al., 2020). However, surgical treatment and targeted drug delivery only can delay and relieve the progression of CVDs but are unable to solve the inherent problems due to the deficiency of the regeneration for dead heart cells. In regenerative medicine,

adult stem cell-based therapies have been shown as a promising potential for treating cardiovascular diseases in preclinical trials because the repair and regeneration of the cardiac tissues can be expected to solve the differentiation of stem cells into heart cells, thereby, offering improved efficacy (Kastrup, 2011). In these cells, MSCs play an important role in the therapy of CVDs, due to their ability of self-renewal and differentiating into various types of cells, including cardiovascular cells (Caplan, 2009; Ye et al., 2014). Moreover, after transplantation of MSCs, they can generate a structure resembling cardiac myocytes and exert anti-inflammatory effects, which can protect the myocardium, further improving the repair of the cardiovascular system. Notably, MSCs have been demonstrated by preclinical studies to have a strong ability to generate blood vessels with minimal adverse effects on the tissues near the injected region. Therefore, the transplantation of MSCs is potentially promising to improve the treatment of CVDs (Trivedi et al., 2010; Goradel et al., 2018; Guo et al., 2020). Despite its great potential for CVD treatment, MSC-based therapies still face some challenges, for example, the real-time tracking for MSC fate after their transplantation, which can provide information such as cell differentiation, the role that MSCs play in vascular repair, and mechanisms of cardiovascular regeneration to guide therapy process and improve therapeutic effect.

Cell imaging is an ideal method for real-time tracking of MSCs, but requires a suitable contrast agent to label MSCs. NPs are a group of contrast agent, which have been widely applied in MSC tracking (Zhang and Wu, 2007; Fu et al., 2011; Liang et al., 2013; Santoso and Yang, 2016; Yi et al., 2017; Guo et al., 2019a). Especially, MSCs labeled by magnetic and/or optical NPs can directly be imaged to offer a real-time tracking *in vivo* (Jing et al., 2008; Betzer et al., 2014; Wu et al., 2014; Perez et al., 2017). These NPs include superparamagnetic iron oxide (SPIO) NPs, gadolinium (Gd)-based NPs, gold (Au)-based NPs, quantum dots (QDs)-based NPs, upconversion (UC)-based NPs, silicon-based NPs, and several other classes of NPs. Here, we will summarize and discuss in detail NPs for labeling and tracking MSCs *in vivo*, shown in Table 1.

Superparamagnetic Iron Oxide Nanoparticles

Generally, SPIO NPs are a kind of NPs with a core-shell structure, in which the core is composed of an iron oxide and then is covered by a coating layer as the shell. The coatings layer alters the surface properties of SPIO NPs, and the iron oxide core provides its magnetic properties (Singh et al., 2010; Mahmoudi et al., 2011). Due to unique magnetic properties, SPIO NPs have the ability to generate a strong inhomogeneous field leading to the contraction of T2 relaxation time, thereby, generating obvious contrast in T2-weighted MRI, and are generally used as a T2-contrast agent (Andreas et al., 2012; Li et al., 2013; Bull et al., 2014; Santoso and Yang, 2016; Jiang et al., 2019). So far, SPIOs are the only commercial NPs, several formulations of which have already been approved by the Food and Drug Administration and have been regulated for clinical applications (Wang et al., 2013). MSCs labeled by SPIO NPs can be tracked by MRI, providing a

TABLE 1 | Comparison between nanoparticles for tracking mesenchymal stem cells (MSCs).

Example	Types of nanoparticles (NPs)	Preparation	Imaging modality	Types of MSCs	Advantages	Disadvantages	Main results
Lu et al., 2007; Andreas et al., 2012	Superparamagnetic iron oxide (SPIO) NPs	Easy	T2-weighted magnetic resonance imaging (MRI)	Human MSCs	Obvious contrast	Low cell-labeling efficiency for MSCs; necessary to functionalize SPIO NPs	No apparent influences on viability of MSCs
Babic et al., 2008; Kim et al., 2011; Szpak et al., 2013; Lewandowska-Łańcucka et al., 2014; Park et al., 2014; Liao et al., 2016; Rawat et al., 2019; Gu et al., 2018; Lee et al., 2020	SPIO NPs	Complex	T2-weighted MRI	Human MSCs; mouse MSCs	Obvious contrast; enhancement of cellular internalization	Induce precipitation of NPs; perturb the cell membrane	No apparent influences on viability of MSCs
Zhang et al., 2010	SPIO NPs	Easy	Proton (^1H) MRI images	Human MSCs	High sensitivity of cell detection	Difficult to accurately quantify cell population	No apparent influences on viability of MSCs
Sehl et al., 2019	SPIO NPs	Complex	Proton (^1H) MRI images	Mouse MSCs	High sensitivity of cell detection; quantify the persistence of transplanted MSCs		No apparent influences on viability of MSCs
Sherry et al., 1988; Ratzinger et al., 2010	Gd-based NPs	Easy	T1-weighted	Human MSCs	Distinguish some similar low signal	Low cellular uptake for MSCs	No apparent influences on viability of MSCs
Kim et al., 2015b; Santelli et al., 2018	Gd-based NPs	Easy	T1-weighted MRI	Human MSCs	Distinguish some similar low signal; long-term tracking		No apparent influences on viability of MSCs
Huang et al., 2020	Au-based NPs	Easy	Photoacoustic imaging and CT imaging	Mouse MSCs	Detected in deep tissue at a high resolution; directly labeled; excellent biocompatibility		No apparent influences on viability of MSCs
Chen G. et al., 2015; Li et al., 2016	QD-based NPs	Complex	Fluorescence imaging	Human MSCs	Longer lifetime than traditional fluorescence dye; photochemical stability	Unsatisfied cytotoxicity and stochastic blinking; the contradiction of sensitivity and definition	No apparent influences on viability of MSCs
Idris et al., 2009; Ang et al., 2011; Zhao et al., 2013; Chen X. et al., 2015; Ma et al., 2016	UC-based NPs	Complex	UC luminescence imaging	Human MSCs	Detected in deeper tissue; more stable and higher definition	Poor biocompatibility; low uptake for cellular	No apparent influences on viability of MSCs
Huang et al., 2005; Chen and Jokerst, 2020; Yao et al., 2020	Silicon-based NPs	Complex	Fluorescence imaging	Human MSCs	Excellent biocompatibility and chemical inertness; easily modified by bioconjugation; good cellular uptake		No apparent influences on viability of MSCs
Chen et al., 2019	Silicon-based NPs	Complex	Photoacoustic imaging	Human MSCs	Good biocompatible and cells tracking capacity; long time		No apparent influences on viability of MSCs
Gao et al., 2016	Other-based NPs	Complex	Aggregation-induced emission imaging	Mouse bone marrow-derived MSCs	Possessing long-term tracking and strong anti-photobleaching ability		No apparent influences on viability of MSCs
Yin et al., 2018	Other-based NPs	Complex	Photoacoustic imaging	Human MSCs	High signal-to-noise; deeper tissue imaging		No apparent influences on viability of MSCs

non-invasive method to monitor the fate of transplanted MSCs *in vivo* (Li et al., 2013; Bull et al., 2014; Guldreis et al., 2017; Xie et al., 2019; Elkhenany et al., 2020).

However, MSCs lack phagocytic capacity, and the surface charge of SPIO NPs are negative, which is a similar charge to that of the cellular membrane. Therefore, the internalization of SPIO NPs is limited, leading to a low efficiency of cell labeling. In order to solve these problems, it is necessary to functionalize SPIO NPs by introducing positive charges or other cell membrane-penetrating moieties on the surface (Lu et al., 2007; Andreas et al., 2012). Moreover, these unique functionalized surfaces also increase the stability of SPIO NPs in hydrophilic conditions, as well as are used as a coating layer for delaying the degradation of the iron oxide core (Barrow et al., 2015). For example, the uses of cationic polymers such as poly-L-lysine (PLL) and chitosan have commonly been utilized to improve the cellular internalization of SPIO NPs and protect them from being easily degraded *in vivo* (Babic et al., 2008; Szpak et al., 2013; Lewandowska-Łańcucka et al., 2014; Park et al., 2014). As an MRI probe for tracking MSCs, PLL-SPIOs were more efficient and safer than naked iron oxide NPs, which was in accordance with the fact that their R_2 value was higher than that of uncoated nanoparticles. However, the highly positive charge of PLL induced the precipitation of NPs and perturbed the cell membrane. Liu et al. (2011) used polyethylenimine with a low molecular weight (2 kDa) to wrap SPIO NPs and obtain PEI-SPIO NPs, which could be readily internalized by MSCs for long-term tracking (19 days). Compared with single SPIO NPs, the modified SPIO NPs hold a controlled clustering structure, leading to much higher T_2 relaxivity. Moreover, Lewin et al. (2000) used a short HIV-Tat peptide to functionalize SPIO NPs, which could be effectively engulfed by hematopoietic and neural progenitor cells. The results suggested that the cellular uptake of iron can reach an extremely high level (10–30 pg per cell) showing effective cell labeling and MRI tracking. However, NPs functionalized by cationic polymer or molecules may cause a certain level of toxicity, although it would drastically enhance cellular uptake of NPs. Therefore, some researchers (Kim et al., 2011; Liao et al., 2016) utilized 2-aminoethyl-trimethyl ammonium (TMA) to modified SPIO NPs. It was found that TMA-SPIO NPs could easily and efficiently label MSCs to monitor their fate *in vivo*, and the labeled MSCs showed low cytotoxicity. In another work, Gu et al. (2018) synthesized PA-SPIO NPs using a self-assembled lipopeptide amphiphile (PA) to modify the surfaces of SPIO NPs for labeling mouse MSCs. The modified NPs showed the enhancement of labeling efficiencies and the improvement of their contrast by shortening the relaxation time. In addition, these NPs exhibited excellent dispersibility and stability in water. More importantly, MSCs labeled by the PA-SPIO NPs were transplanted into mouse, showing no adverse effects on the osteogenic and adipogenic differentiation. Rawat et al. (2019) synthesized SPIO NPs and then used them to label MSCs. The labeled MSCs could be tracked by MRI to understand their fate *in vivo*. Moreover, these labeled MSCs still maintained differentiation potential. Lee et al. (2020) used poly lactic-co-glycolic acid (PLGA) to modify SPIO NPs, and then utilized fluorescent dye Cy5.5 to functionalize the prepared NPs

for labeling and tracking MSCs to investigate the interactions between PLGA-SPIO NPs and MSCs. The results showed that the prepared NPs had no apparent influences on the survival and differentiation of MSCs when the concentration was at 40 $\mu\text{g/ml}$ for more than 96 h, demonstrating that the internalization pathway of MSCs is via endocytosis.

In proton (^1H) MRI images, SPIO NP-labeled cells appear as signal void regions (Miguel et al., 2007). This effect gives rise to enhance the sensitivity of cell detection, but poses challenges for accurate quantification of the cell population as well. Another limitation for SPIO NP-based cell tracking is the lack of specificity in some tissues. It is ambiguous to identify these cells *in vivo*, as other regions in anatomic MRI appear as dark as well (Zhang et al., 2010). To overcome these limitations, Sehl et al. (2019) utilized the combination of iron-based MRI, ^{19}F MRI, and MPI cellular imaging technologies to monitor and quantify the persistence of the transplanted MSCs and infiltrate macrophages *in vivo*, shown in **Figure 1**. First, MSCs were labeled with iron oxide NPs (ferumoxylol) and then implanted into the hind limb muscle of 10 C57/6 mice. Finally, a perfluorocarbon agent was administered intravenously for uptake by phagocytic macrophages *in situ*. The ferumoxylol-labeled MSCs were detected by proton (^1H) MRI and magnetic particle imaging (MPI). Perfluorocarbon-labeled macrophages were detected by fluorine-19 (^{19}F) MRI. These three modalities are complementary and provide integrated information (specificity, sensitivity, and quantification of cell number). They proposed that these cellular imaging techniques could be used to monitor MSC engraftment over time and detect the infiltration of macrophages at transplant sites.

Gadolinium-Based Nanoparticles

T_1 -weighted sequences are often collected before and after infusion of T_1 -shortening MR contrast agents, so as to reduce the T_1 relaxation times of ^1H atoms in water molecules and produce hyperintensities in T_1 -weighted images appearing white (Na and Hyeon, 2009). Currently, Gd-based NPs are the most widely used T_1 -contrast agent for labeling and tracking stem cells (Ni and Chen, 2015). Compared with T_2 -contrast agent of SPIO NPs, T_1 -contrast agent of Gd-based NPs can distinguish some similar low signal resulting from intrinsic signal in tissue or hemorrhage, due to their capability of generating a bright positive signal. Therefore, a T_1 -contrast agent generally is predominately employed as a probe in detecting the transplanted stem cells in a low-signal region (Na and Hyeon, 2009; Zhu et al., 2013; Robert et al., 2015).

Gd-based NPs usually are complex NPs, being composed of Gd^{3+} and their chelating ligand of which diethylenetriamine pentaacetic acid (DTPA) is the most common (Sherry et al., 1988; Ratzinger et al., 2010). Modified by DTPA, Gd-based NPs reduced cytotoxicity due to the enhancement of hydrophilicity, but also weakened the interaction with the cell membrane leading to low cellular uptake for MSCs. Furthermore, their labeling efficiency was low resulting from the poor targeting ability of MSCs. Considering these problems, Tseng et al. (2010) prepared Gd-based NPs used as an MRI contrast agent to label and track human MSCs. Due to the modification of hexanedione, the

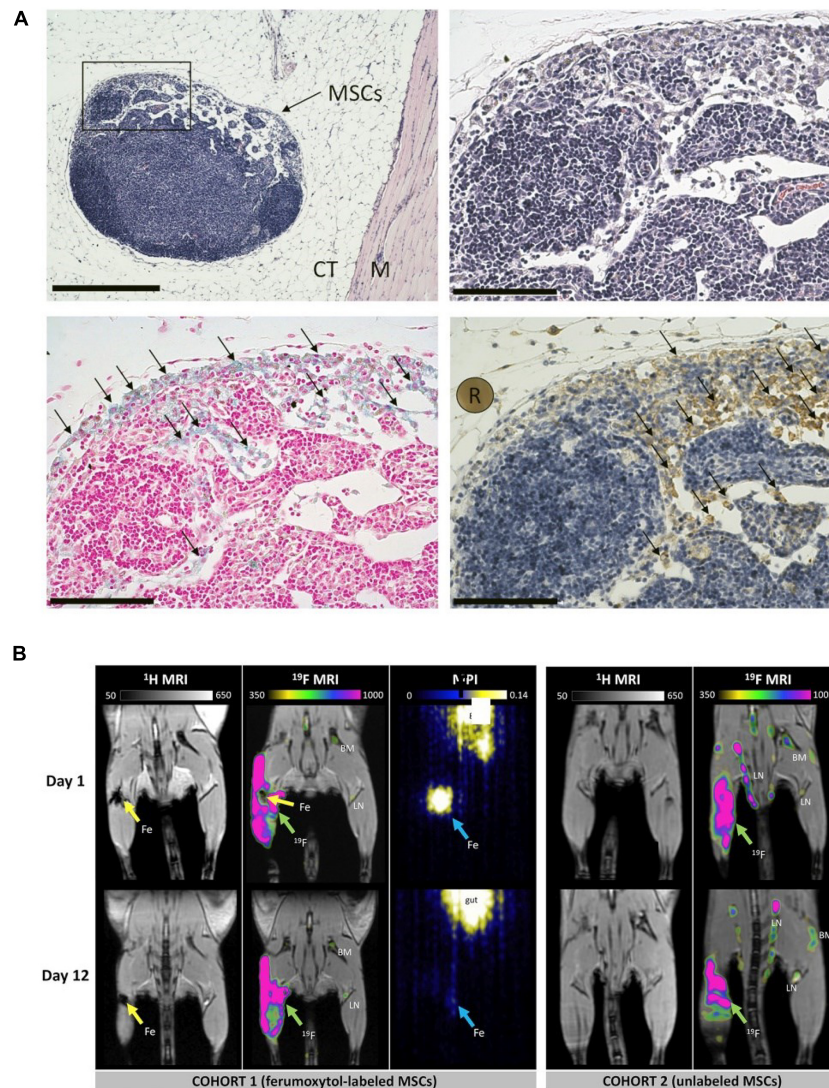


FIGURE 1 | (A) Histological validation showing the presence of MSCs surrounded in connective tissue (CT) and muscle (M) in hematoxylin and eosin at $\times 10$ magnification (scale bar 500 μm). **(B)** *In vivo* proton (^1H)/fluorine 19 (^{19}F) magnetic resonance imaging (MRI) and magnetic particle imaging (MPI) (adapted Sehl et al., 2019).

biocompatibility of the prepared Gd-based NPs was improved. Moreover, compared with Gd-based NPs modified by DTPA, the prepared Gd-based NPs showed more excellent cellular uptake for MSCs and could be easily detected *in vivo* due to the shortening of T1 relaxation times. In another work, Kim et al. (2015b) reported a Gd-based NP to label MSCs, in which Gd^{3+} was chelated with a pullulan derivative ligand. It was found that the prepared Gd-based NPs could dramatically enhance cellular uptake for MSCs, leading to the enhancement of internalization efficiency from 32 ± 2 to 98 ± 4 pg Gd/cell. Moreover, the labeled MSCs achieved a long-term tracking of 21 days *in vivo*, due to low signal attenuation. Cai et al. (2017) fabricated Gd-based NPs of 7 nm for tracking MSCs by utilizing melanin to modify Gd^{3+} . The results showed that Gd-based NPs modified by melanin increased the stability of MRI contrast agent and

shorten T1 relaxation time, compared with traditional Gd-based NPs modified by DTPA. The labeled MSCs achieved long-term tracking for more than 4 weeks, due to the enhancement of labeling efficiency. Importantly, the labeled MSCs had no apparent cytotoxicity at a proper concentration of 800 $\mu\text{g}/\text{ml}$. However, preclinical studies suggested that for tracking MSCs *in vitro* and *in vivo*, the single-imaging technique was difficult to complete, and the integration of multiple labeling means often was required. Consequently, Santelli et al. (2018) developed multimodality imaging to track MSCs labeled by Gd-based NPs, shown in Figure 2. The Gd-based NPs composed of spherical europium-doped gadolinium oxysulfide ($\text{Gd}_2\text{O}_2\text{S}:\text{Eu}^{3+}$) could be detected by MRI, X-ray imaging, and photoluminescence imaging. In the *in vitro* test, the number of MSCs labeled by $\text{Gd}_2\text{O}_2\text{S}:\text{Eu}^{3+}$ was up to 2,500, showing feasible cell tracking.

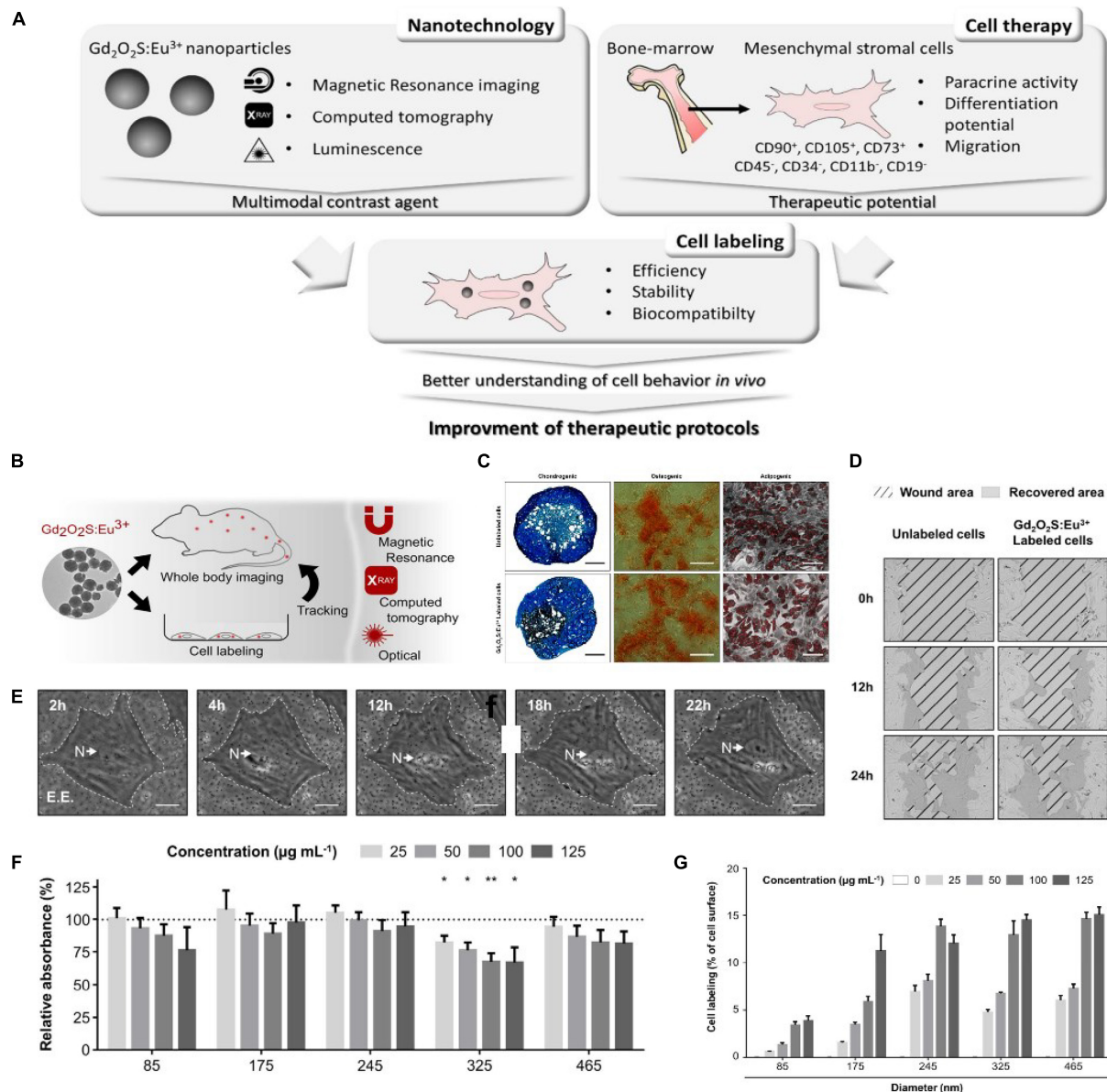


FIGURE 2 | (A) Schematic representation of nanoparticle potential in cell therapy and future impact. **(B)** Multimodality imaging for tracking mesenchymal stem cells (MSCs). **(C)** Differentiation of MSCs (scale bar 150 μm). **(D)** Representative phase-contrast images of wound closing. **(E)** Time-lapse of labeling extracted from video microscopy acquisition. **(F)** Viability of MSCs after 24-h $Gd_2O_2S:Eu^{3+}$ labeling evaluated by MTT. **(G)** Quantification of MSC labeling with $Gd_2O_2S:Eu^{3+}$ expressed as the percentage of cell surface area occupied by NPs (adapted by Santelli et al., 2018).

Moreover, the results suggested that the effects of the NPs on viability, proliferation, migration, and differentiation of the transplanted MSCs were innocuousness.

Gold-Based Nanoparticles

Due to their cytocompatibility and strong optical absorption in the near-infrared region, Au-based NPs are potential contrast agents of photoacoustic imaging. In addition, Au-based NPs can be detected in deep tissue at 2 cm at a high resolution of 100 μm , so they are emerging as an alternative method for tracking cells *in vivo* (Murphy et al., 2008; Giljohann et al., 2010).

More importantly, MSCs can be directly labeled by Au-based NPs, so their differentiation after transplantation *in vivo* can be detected using photoacoustic imaging *in vivo* (Turjeman et al., 2015). Donnelly et al. (2018) utilized Au-based NPs as a photoacoustic contrast agent for real-time tracking MSCs to guide their delivery. It was found that the labeled MSCs could be detected by ultrasound/photoacoustic imaging in the spinal cord. In another work, Dhada et al. (2019) prepared Au-based NPs to label and track MSCs by photoacoustic imaging, which was composed of gold nanorods and IR775. The results suggested that cell death also could be detected because IR775 was sensitive

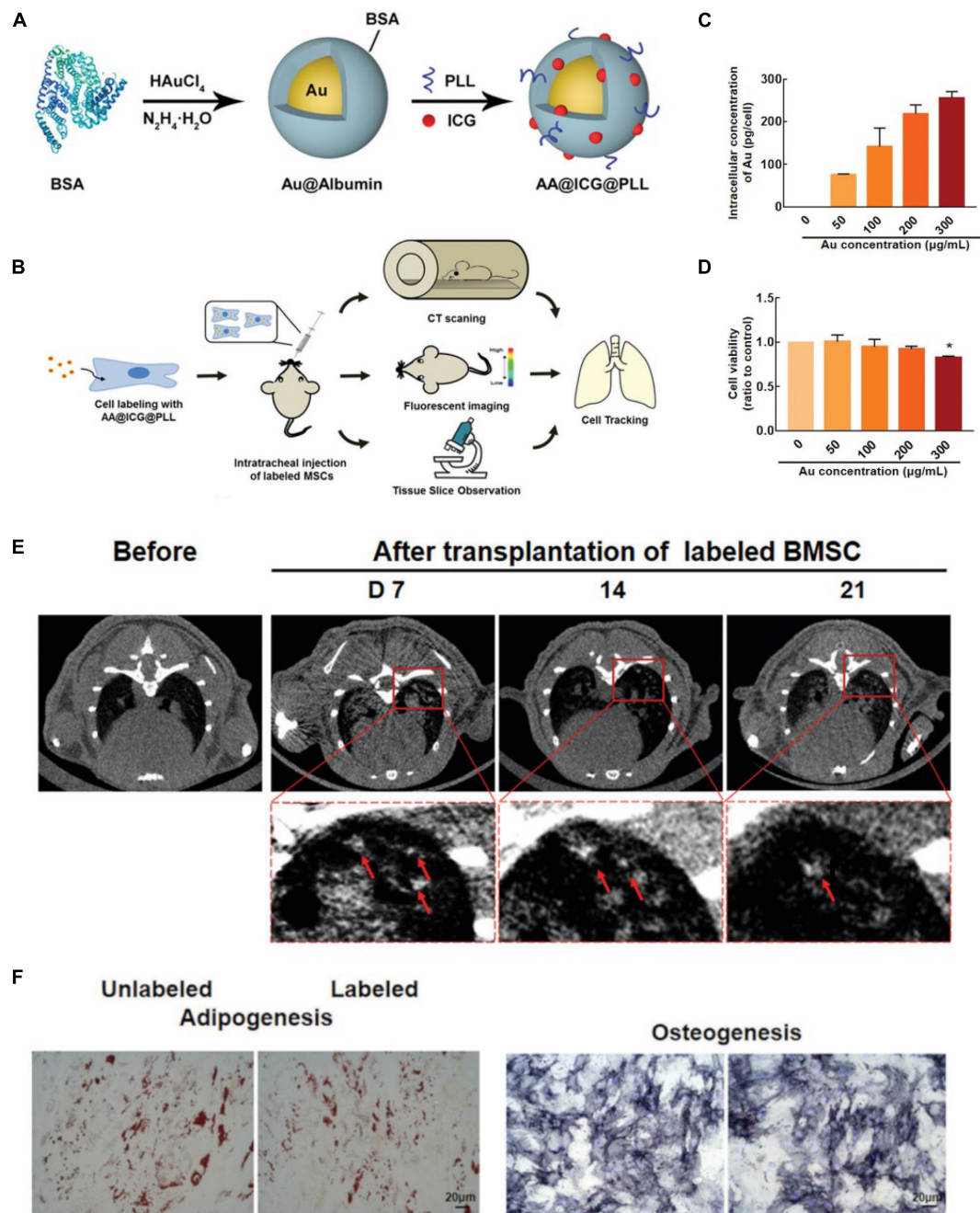


FIGURE 3 | (A) Schematic illustration of the synthesis of AA@ICG@PLL gold (Au)-based nanoparticles (NPs). **(B)** Experimental design for tracking AA@ICG@PLL-labeled MSCs in a silica-induced PF mouse model. **(C)** Intracellular Au content measured by ICP-MS. **(D)** Relative viability of BMSCs labeled with AA@ICG@PLL NPs at various Au concentrations. **(E)** *In vivo* computed tomography (CT) images of AA@ICG@PLL-labeled BMSCs at 7, 14, and 21 days after transplantation. **(F)** Bright-field images of Oil Red O staining and ALP staining of NP-loaded and unloaded BMSCs. Scale bar = 20 μm (adapted by Huang et al., 2020). * $p < 0.05$ compared with the unlabeled group.

to reactive oxygen species (ROS). Therefore, the viability of the transplanted MSCs *in vivo* was quantificationally measured. Moreover, Laffey et al. (2020) studied the viability of MSCs labeled by Au-based NPs in a frozen environment. The results showed that the labeled MSCs undergoing the process of freezing, storing, and thawing for 2 months still could be detected by

photoacoustic imaging, and their differential ability also had no apparent difference with the unlabeled MSCs.

Certainly, Au-based NPs also can be detected by computed tomography (CT), due to the high X-ray absorption. Thus, they are used as a CT maker to label and track MSCs. For example, Betzer et al. (2017) used Au-based NPs as labeling agents to

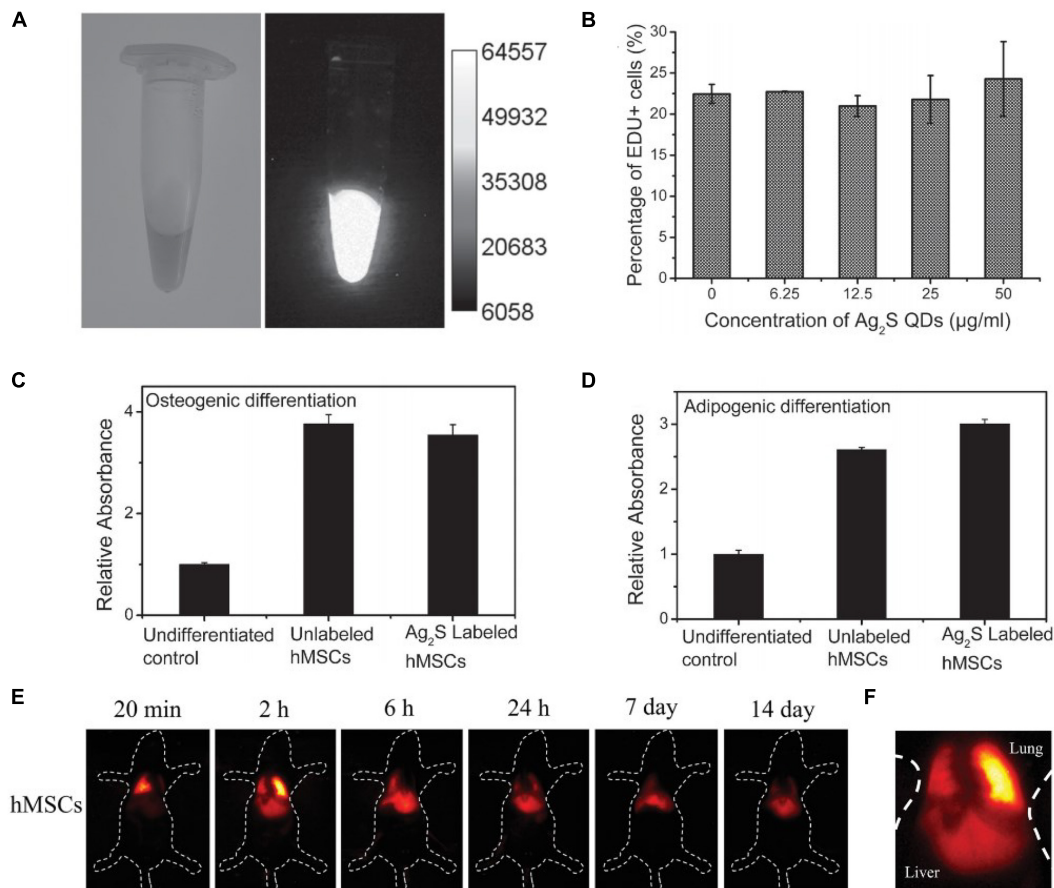


FIGURE 4 | (A) A photoluminescence image (PA) of the Ag₂S QD-labeled human MSC solution at a density of 4×10^6 cell ml⁻¹. **(B)** Cell proliferation. Quantification of **(C)** osteogenic and **(D)** adipogenic differentiation by measuring the absorbance of Oil-Red O and alizarin red extracted from cell lysates at 404 nm, respectively. **(E)** The time course of the *in vivo* near-infrared (NIR) PL images of a healthy mouse after transplantation of Ag₂S QD-labeled human MSCs. **(F)** Higher magnification NIR PL image of mice transplanted with human MSCs only after 2 h (adapted from Chen G. et al., 2015).

longitudinally and quantitatively track MSCs *in vivo*. It was found that the labeled MSCs were detected for up to 24 h by CT, showing a long-term tracking. Nafiujjaman and Kim (2020) reported a biocompatible Au-based NPs used as a CT maker, in which Au NPs were modified by poly-L-lysine (PLL) to change the charge properties of surface enhancing the cellular uptake. The MSCs labeled by Au-based NPs could be detected by CT and showed high labeling efficiency. Huang et al. (2020) synthesized Au-based NPs (AA@ICG@PLL) with dual-modal imaging (CT and near-infrared fluorescence) to label and track MSCs of mice *in vivo*, shown in **Figure 3**. Due to modification by indocyanine green (ICG) and poly-L-lysine (PLL), AA@ICG@PLL showed excellent cellular uptake for MSCs and biocompatibility. It was found that the labeled MSCs could be tracked via dual-modal imaging for more than 21 days, and importantly, the prepared NPs had anti-inflammatory properties.

Quantum Dot-Based Nanoparticles

QD-based NPs are semiconductor NPs with II–VI or III–V elements, which can emit particular frequency light after being stimulated (Jaiswal et al., 2004). The emitted light frequency

depends on the size and shape of QD-based NPs. Therefore, QD-based NPs show different colors (Seleverstov et al., 2006). In general, the fluorescence lifetime of QD-based NPs is longer than traditional fluorescence dye (Bailey et al., 2004). In addition, compared with fluorescent dye, QD-based NPs are easier to be detected due to the non-overlapping of absorption and emission (Mazumder et al., 2009). Specifically, QD-based NPs have broad absorption of continuous distribution, but show narrow and symmetrical properties (Yukawa and Baba, 2017). More importantly, they have the advantage of photochemical stability. As a result, QD-based NPs have been used as contrast agents for long-term tracking of MSCs *in vivo*. However, QD-based NPs also have some disadvantages, such as unsatisfied cytotoxicity and stochastic blinking. Moreover, the augmentation of QD-based NPs in dosage increases the sensitivity of cell imaging, but may decrease their definition (Shah et al., 2007; Liang et al., 2013).

Hsieh et al. (2006) reported a QD-based NP to label human MSCs, in which CdSe was used as the core, and the shell was covered by ZnS. In addition, the commercial QD-based NPs also were developed and utilized as contrast

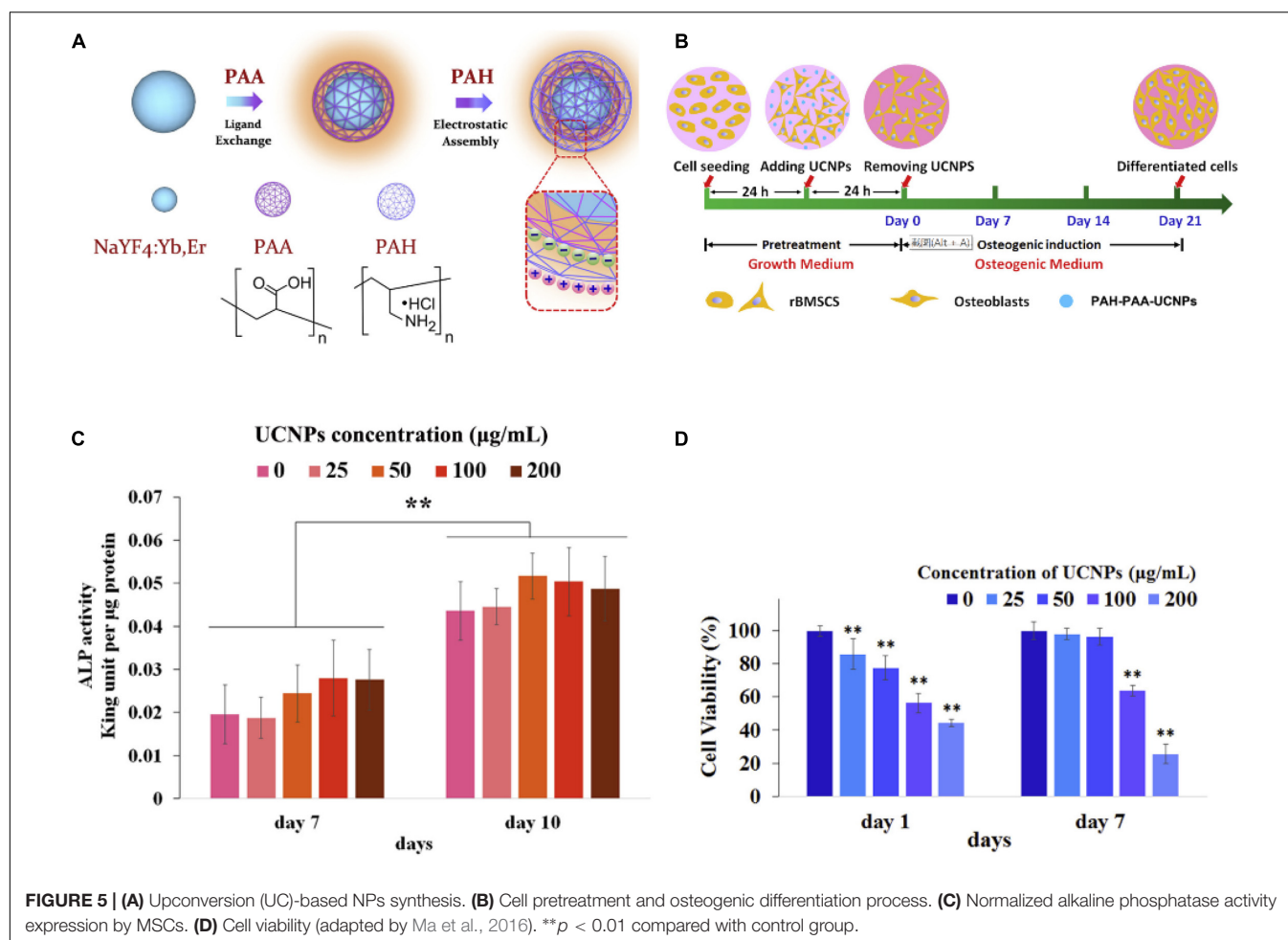


FIGURE 5 | (A) Upconversion (UC)-based NPs synthesis. **(B)** Cell pretreatment and osteogenic differentiation process. **(C)** Normalized alkaline phosphatase activity expression by MSCs. **(D)** Cell viability (adapted by Ma et al., 2016). ***p* < 0.01 compared with control group.

agents to label and track the cell distributions. However, these QD-based NPs showed unsatisfied biocompatibility and cytotoxicity, so they had to be modified before use. In recent years, Li et al. (2016) prepared QDs-based NPs (RGD-β-CD-QDs) to label and track human MSCs, which were composed of QDs, β-cyclodextrin (β-CD), and Cys-Lys-Lys-ArgGly-Asp (CKKRGD) peptide. The QDs modified by β-CD enhanced the cellular uptake and facilitated the differentiation of MSCs, due to the small molecule dexamethasone and siRNA carried by β-CD. More importantly, the labeled MSCs could be detected for up to 3 weeks. Chen G. et al. (2015) reported a AgS₂ QD-based NP for tracking human MSCs *in vivo* by utilizing fluorescence imaging, shown in Figure 4. It was found that the prepared NPs with good biocompatibility could be detected in the second near infrared, and the stability of fluorescence reached up to 30 days. Furthermore, the labeled MSCs was dynamically visualized at high resolution of 100 ms for more than 14 days. Chetty et al. (2019) prepared CuInS₂-ZnS QD-based NPs to label umbilical cord-derived MSCs, and the labeling efficiency reached 98% due to the prepared QD-based NPs displaying high photoluminescence quantum yield of 88%. In addition, the labeled MSCs showed no apparent influences on stemness and had the ability of long-term tracking.

Upconversion-Based Nanoparticles

UC-based NPs, usually being lanthanide-doped nanocrystals, can undergo a photon UC process in which the sequential absorption of two or more photons leads to anti-Stokes type emission of a single higher-energy photon (Wang et al., 2010). Due to the unique properties of being sensitive to the near-infrared light, UC-based NPs can be detected in deeper tissue compared with traditional contrast agents with fluorescence. More importantly, the imaging of UC-based NPs is more stable and has higher definition (Ang et al., 2011; Chen X. et al., 2015).

Idris et al. (2009) reported a contrast agent of UC-based NPs to label and track MSCs, which was composed of NaYF₄:Yb, Er NPs, and silicon. The labeled MSCs could directly be imaged *in vivo* to dynamically visualize their behaviors, but the region imaged by confocal microscopy was small. Wang et al. (2012a) fabricated UC-based NPs to label MSCs, which was modified by polyethylene glycol (PEG) and oligo-arginine. MSCs labeled by the UC-based NPs were detected by fluorescence imaging *in vivo*, and the introduction of oligo-arginine improved the cellular uptake of the NPs, showing excellent labeling efficiency and high sensitivity of 10 cells. In another work, Cheng et al. (2013) developed a multifunctional UC-based NP, integrating magnetic properties into UC luminescence NPs. Utilizing the

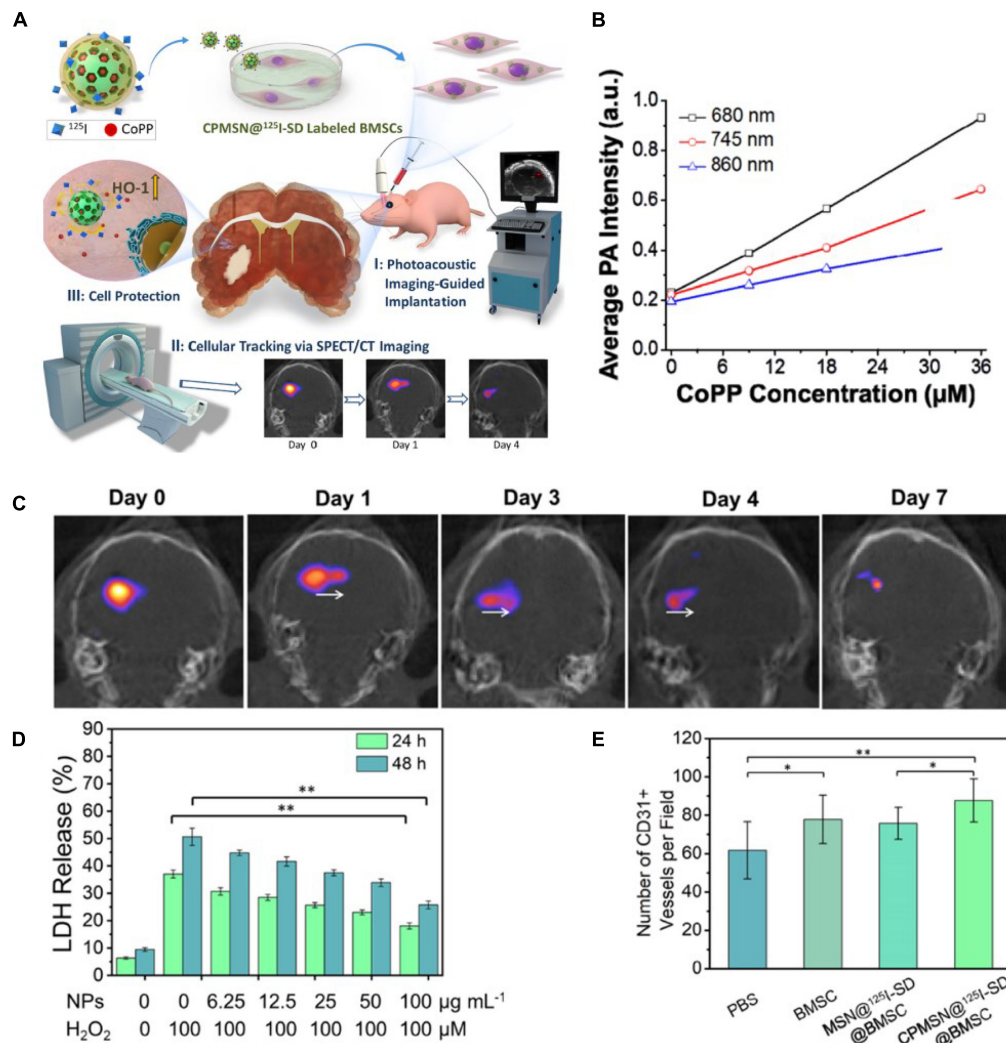


FIGURE 6 | (A) Schematic illustrating the triple functionality of CPMSN@125I-SD for stem cell therapy of brain ischemia. **(B)** Corresponding average intensities of CPMSN@125I-SD with various cobalt protoporphyrin IX (CoPP) concentrations in the NIR region. **(C)** Single-photon emission computed tomography imaging (SPECT)/CT images of ischemic mouse brain tissue on different days (0–7 days) after intracerebral injection of the labeled BMSCs (500,000 cells). **(D)** Cell death assessment for MSCs after treatment with different concentrations of CPMSN@125I-SD (0–100 μg mL⁻¹) and exposure to 100 μM H₂O₂ for 24 and 48 h. **(E)** Bar graph showing the quantification of the number of CD31⁺ cells (adapted from Yao et al., 2020). **p* < 0.05; ***p* < 0.01.

prepared NPs, the labeled MSCs can be tracked via fluorescence imaging and MRI, possessing highly effective sensitivity of 10 cells in mouse *in vivo*. Zhao et al. (2013) prepared a UC-based NP for labeling mouse MSCs, which was composed of polyethylenimine (PEI) and (α-NaYbF₄:Tm³⁺)/CaF₂, enhancing the detective depth *in vivo* due to absorption and emission for near-infrared light.

In recent years, Ma et al. (2016) reported a UC-based NP, which was composed of NaYF₄:Yb³⁺, Er³⁺ NPs, poly (acrylic acid) (PAA), and poly (allylamine hydrochloride) (PAH), as a fluorescence maker for tracking bone marrow MSCs *in vitro*, shown in Figure 5. The biocompatibility and cellular uptake of NaYF₄:Yb³⁺, Er³⁺ NPs were highly enhanced, due to electrostatic interaction. Moreover, it was found that the effects of cellular uptake of UC-based NPs (≤50 μg/ml) on the osteogenic

differentiation had no apparent difference by tracking the MSCs. Kang et al. (2018) prepared a UC-based NP with NIR-controllable properties to label MSCs. By a remote-controllable way, the stem cell differentiation was regulated. Furthermore, Ren et al. (2020) used ligand-free NaYF₄:Yb/Er UC-based NPs to label and track mouse bone MSCs, demonstrating that the UC-based NPs at a proper concentration could enhance osteogenic differentiation.

Silicon-Based Nanoparticles

Recently, silicon-based NPs have been demonstrated as a good contrast agent to label cells because they can be easily modified by bioconjugation (Shin et al., 2019). Furthermore, it has excellent biocompatibility and chemical inertness, thus, being able to serve as a probe for tracking cells *in vivo* (Huang et al., 2005; Hsiao et al., 2008). Silicon-based NPs, being simply divided into

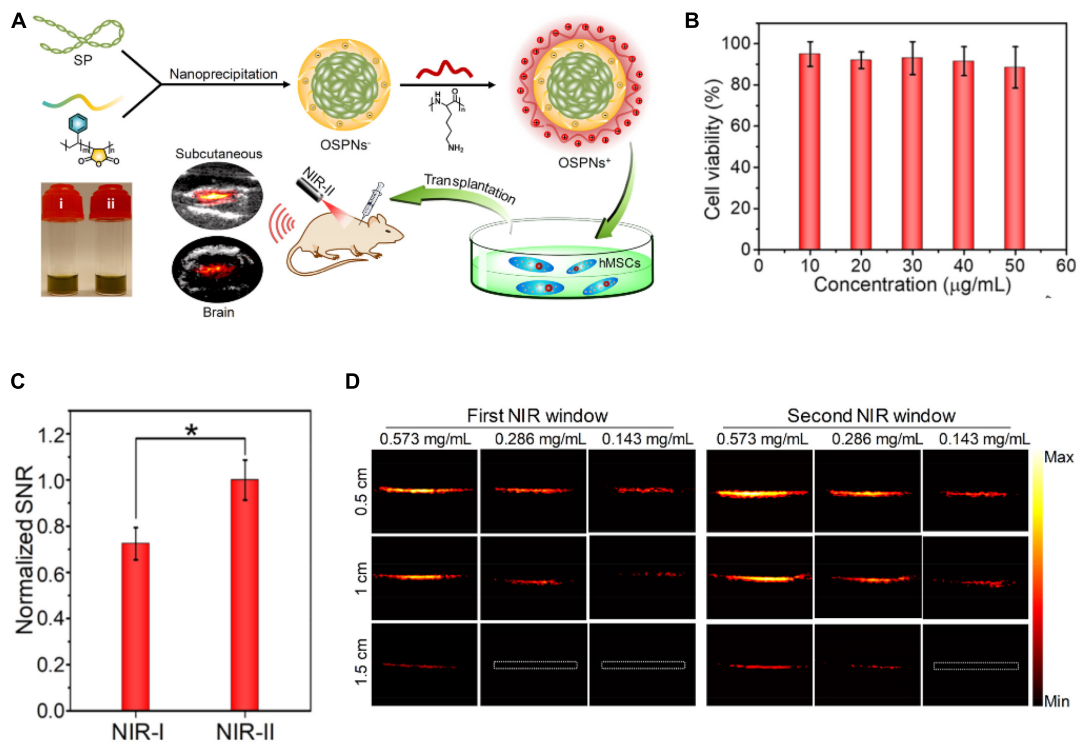


FIGURE 7 | (A) Illustration of the preparation procedure of OSPN⁺ and the photoacoustic labeling of human MSCs after transplantation. **(B)** The MTT assay of human MSCs treated by OSPN⁺ for 12 h under various concentrations. **(C)** Normalized PA SNR of OSPN⁺-labeled hMSCs implanted into mice brain under NIR-I (860 nm) or NIR-II (1,064 nm) light excitation (**p* < 0.05). **(D)** PA imaging of aqueous OSPN⁺ solutions (0.573, 0.286, and 0.143 mg/mL) (adapted from Yin et al., 2018).

silica NPs and silicon carbide NPs, not only contain fluorescent properties but also are used as ultrasound agents (Chen and Jokerst, 2020; Yao et al., 2020).

Huang et al. (2005) prepared a mesoporous silica NP modified by fluorescein isothiocyanate, and the labeled MSCs could be detected by imaging to track their viability *in vivo*. Due to clathrin-mediated endocytosis, the NPs could be internalized into MSCs and showed good cellular uptake. In addition, the highly efficient labeling had no apparent influences on the viability of MSCs. In another work, Chen and Jokerst (2020) used silica NPs to label MSCs and then track the MSCs *in vivo* by ultrasound imaging. The results showed that silica NPs could significantly increase the ultrasound signal of MSCs *in vivo*. Yao et al. (2020) reported a unique core-shell NP in which the core is composed of cobalt protoporphyrin IX (CoPP)-loaded mesoporous silica NPs, and the shell is a ¹²⁵I-conjugated/spermine-modified dextran polymer, to label and guide the transplantation of MSCs by PA imaging and SPCT nuclear imaging, shown in Figure 6. The obtained NPs not only could instantly image the transplantation of MSCs but also quantitatively tracked their migrations for a long time. Significantly, the NPs steadily released CoPP to increase the survival of MSCs in ischemic mice.

Another type of silicon-based NPs is silicon carbide NPs, which are commonly used as protecting layer due to their unique properties of being durable and chemically inert. Moreover, typically, silicon carbide NPs (≤10 nm) are utilized as contrast

agents to label and track cells *in vivo* because of their photoluminescence resulting from quantum confinement effects. Chen et al. (2019) used three sizes of silicon carbide NPs to label MSCs, showing dual modality imaging (photoluminescence and photoacoustic imaging). When the size of the NPs is 620 nm, the labeled MSCs could be detected *in vitro* for more than 20 days. Moreover, the NPs also exhibited good biocompatibility and the capacity of cell tracking because the differentiated cells also could be imaged.

Other Nanoparticles

Gao et al. (2016) prepared an aggregation-induced emission NP, the surface of which is modified by Tat peptide. The obtained NPs had highly efficient ability of labeling mouse bone marrow-derived MSCs, and the red emission could be detected for more than 12 passages, which were not achieved by traditional fluorescence, possessing long-term tracking and strong anti-photobleaching ability. More importantly, MSCs labeled by the NPs showed low cytotoxicity, and their viability and differentiation had no apparent influences. Lim et al. (2019) developed bicyclo nonyne (BCN)-conjugated glycol chitosan NPs (BCN-NPs) as a delivery system of dual-modal stem cell imaging probes. Yin et al. (2018) reported an organic semiconducting polymer NP (OSP⁺) as a PA contrast agent for tracking MSCs, utilizing a second near-infrared (NIR-II) adsorption to broaden

the limitation of conventional inorganic PA contrast agents and the narrow range of the wavelength in the first near-infrared (NIR-I) window, shown in **Figure 7**. The prepared cationic NPs showed the deeper tissue imaging due to the significantly higher signal-to-noise (SNR) and enhanced the cellular uptake for human MSCs because of their good biocompatibility, appropriate size, and optimized surface property.

PROBLEMS AND PROSPECTS

Mesenchymal stem cell transplantation has been shown to be a strong potential for the treatment of CVDs. Many NPs have been fabricated and used as contrast agents by non-invasive imaging to track and monitor the transplanted MSCs, for providing more information to guide further therapy. However, one big issue associated with NPs for labeled MSCs is that NPs released from dead cells will be uptaken by macrophages, which give false-positive information *in vivo*. Maybe, we can label dead cells with other methods to exclude the false-positive information. Moreover, the single-imaging technique can be inefficient to meet all needs for tracking, and each of those has its own disadvantages. Although there

are some multimodal imaging techniques, more multimodal labeling agent-based NPs should be developed in the future to optimize MSC dose and delivery route for the treatment of CVDs. Importantly, the poor targeted migration and low survival rate of MSCs transplanted into the cardiovascular should be solved.

AUTHOR CONTRIBUTIONS

HH completed the first draft of the manuscript. XD, ZH, and ZY offered the excellent advices and perfected the first draft. WH improved the first draft and finally finished the manuscript and provided the support of funds. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Intermittent Starvation Promotes Maturation of Human Embryonic Stem Cell-Derived Cardiomyocytes

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Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) represent an infinite cell source for cardiovascular disease modeling, drug screening and cell therapy. Despite extensive efforts, current approaches have failed to generate hPSC-CMs with fully adult-like phenotypes *in vitro*, and the immature properties of hPSC-CMs in structure, metabolism and electrophysiology have long been impeding their basic and clinical applications. The prenatal-to-postnatal transition, accompanied by severe nutrient starvation and autophagosome formation in the heart, is believed to be a critical window for cardiomyocyte maturation. In this study, we developed a new strategy, mimicking the *in vivo* starvation event by Earle's balanced salt solution (EBSS) treatment, to promote hPSC-CM maturation *in vitro*. We found that EBSS-induced starvation obviously activated autophagy and mitophagy in human embryonic stem cell-derived cardiomyocytes (hESC-CMs). Intermittent starvation, via 2-h EBSS treatment per day for 10 days, significantly promoted the structural, metabolic and electrophysiological maturation of hESC-CMs. Structurally, the EBSS-treated hESC-CMs showed a larger cell size, more organized contractile cytoskeleton, higher ratio of multinucleation, and significantly increased expression of structure makers of cardiomyocytes. Metabolically, EBSS-induced starvation increased the mitochondrial content in hESC-CMs and promoted their capability of oxidative phosphorylation. Functionally, EBSS-induced starvation strengthened electrophysiological maturation, as indicated by the increased action potential duration at 90% and 50% repolarization and the calcium handling capacity. In conclusion, our data indicate that EBSS intermittent starvation is a simple and efficient approach to promote hESC-CM maturation in structure, metabolism and electrophysiology at an affordable time and cost.

Keywords: embryonic stem cells, cardiomyocyte maturation, intermittent starvation, pluripotent stem cells, autophagy

INTRODUCTION

Cardiovascular disease has become the number one killer of human health (Tan and Ye, 2018). The most common form of cardiovascular disease is myocardial infarction, accompanied by the cumulative loss of functioning cardiomyocytes due to myocardial ischemia, which eventually leads to heart failure. Due to the limited regenerative capability of adult cardiomyocytes, current strategies, including pharmaceutical treatment and revascularization therapy, are ineffective in preventing disease progression. Meanwhile, the primary human cardiomyocytes are difficult to obtain and maintain *in vitro*, which has long been a major problem for mimicking and investigating human heart diseases. Cardiomyocytes (CMs) derived from human pluripotent stem cells (hPSCs), including embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), show enormous potential for the development of cardiac disease models, drug discovery and cell therapy (Ye et al., 2015; Gintant et al., 2017; Hu et al., 2018). However, these applications are hampered by the immature phenotypes of hPSC-derived cardiomyocytes (hPSC-CMs) that resemble fetal CMs.

The major hallmarks of cardiomyocyte maturation include maturational hypertrophy, well-formed and organized myofibrils, increased DNA content and mitochondrial density, as well as the ability to perform β -oxidation of fatty acids, functional electrophysiology and calcium handling (Matsui et al., 2007). Many approaches such as long-term culture, biophysical, biochemical, and bioelectrical stimulations, have been developed to enhance the maturity of hPSC-CMs (Hirt et al., 2014; Yang et al., 2014). For instance, we and others have reported that retinoic acid and fatty acid treatment could enhance the structural, metabolic and electrophysiological maturation of hPSC-CMs (Yang et al., 2019; Miao et al., 2020). In addition, tissue engineering, 3D culture and miRNAs were also used to promote hPSC-CMs maturation (Lee et al., 2015; Sun and Nunes, 2017). However, much simpler and more effective approaches are urgently needed to drive hPSC-CM maturation at an affordable time and cost.

The perinatal period has been regarded as a critical window for cardiomyocyte maturation *in vivo*. Instantly, after birth, cardiomyocytes experience a short starvation period, due to sudden interruption of nutrient supply and the switch from placental nutrition to nursing (Fang et al., 2014). This starvation period is accompanied by autophagy in the heart, and the morphologic and metabolic changes in cardiomyocytes. In the fetal stage, cardiomyocytes proliferate sharply and utilize glycolysis as a source of energy. After birth, cardiomyocytes undergo hypertrophic growth and show a metabolic shift from glycolysis to oxidative phosphorylation to produce ATP (Chung et al., 2010; Correia et al., 2017). Mitochondria are the key organelles of energy conversion, metabolism and signal amplification in cardiomyocytes. The metabolic switch of cardiomyocytes is closely related to the state of mitochondria. With the development of heart, mitochondria in cardiomyocytes become more elongated and branched, and establish densely distributed cristae (Martin et al., 2014). The selective autophagy of mitochondria, termed mitophagy, plays an important role

in the quality control of mitochondria by removal of damaged or immature mitochondria (Bravo-San Pedro et al., 2017). In this regard, starvation-induced autophagy may promote cardiomyocyte maturation both *in vivo* and *in vitro*.

To verify our hypothesis, we starved hESC-CMs with Earle's balanced salt solution (EBSS), and investigated the effects of starvation on the maturation of hESC-CMs. We observed a rapid activation of autophagy in hESC-CMs after EBSS treatment and found that the intermittent starvation promotes the structural, metabolic, and electrophysiological maturation of hESC-CMs. In summary, our results indicate that intermittent EBSS starvation provides a new direction for promoting the maturation of cardiomyocytes for better application in disease modeling, drug screening and cell therapy.

MATERIALS AND METHODS

Cell Culture and Cardiomyocyte Differentiation

Experiments with the human embryonic stem cells were approved by the Ethical Committee of Soochow University. The human embryonic stem cell lines H1 and hES3 were maintained in PSCeasy[®] medium (Cellap, China) on Matrigel-coated dishes. The hESCs at 80% confluence were passaged using 0.5 mM EDTA (Sigma, United States), and 2 μ M thiazovivin (Selleck Chemicals, United States) was supplemented for 24 h after cell passage. Cardiomyocyte differentiation was induced in the chemically defined medium CDM3 which is made of RPMI1640 (Thermo Fisher, United States), bovine serum albumin (Sigma-Aldrich, United States) and L-ascorbic acid 2-phosphate (Sigma-Aldrich, United States) as previously described (Miao et al., 2020). Briefly, hESCs at 95% confluence were first cultured in CDM3 supplemented with 5 μ M CHIR99021 (Sigma-Aldrich, United States) for 2 days to induce mesoderm differentiation, and the medium was then refreshed with CDM3. Two days later, cells were cultured in CDM3 supplemented with 2 μ M Wnt-C59 (Selleck chemicals, United States). The medium was subsequently changed to CDM3 and refreshed daily. Beating cardiomyocytes derived from hESCs were noted around days 9 to 10. The cardiomyocytes were passaged onto gelatin-coated plates and further purified for 4 days in glucose-free RPMI1640 (Thermo Fisher, United States) supplemented with bovine serum albumin, L-ascorbic acid 2-phosphate and sodium DL-lactate (Sigma-Aldrich, United States).

Cell Starvation

The purified cardiomyocytes were washed 3 times and incubated with EBSS (Sigma, United States) for the indicated times to induce starvation. Following starvation, cardiomyocytes were maintained in CDM3. To induce intermittent starvation, cardiomyocytes at day 15 were treated with EBSS 2 h per day for 5, 10 or 15 days, respectively.

Quantitative Real-Time PCR

Total RNAs were isolated from cells using TRIzol Reagent (Sigma-Aldrich, United States) and were reverse-transcribed into

cDNA using the Takara PrimeScript™ RT Reagent Kit (Takara, Japan). Quantitative real-time PCR (qPCR) was performed on the Applied Biosystems® StepOnePlus™ Real-Time PCR System (Thermo Fisher, United States). The mRNA expression data were normalized to the expression levels of 18S rRNA, and gene expression levels were calculated by the method of comparative threshold cycle ($2^{-\Delta \Delta C_t}$). All primers used for qPCR are listed in **Supplementary Table 1**.

Mitochondrial DNA Content Assay

Genomic and mitochondrial DNA (mtDNA) was extracted using the Genomic DNA Extraction Kit (Tiangen, China). Relative mtDNA content was measured by quantitative real-time PCR. The primers used for the mtDNA content assay are listed in **Supplementary Table 1**.

Immunofluorescence Staining

The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, United States) for 15 min at room temperature (RT), followed by permeabilization with 0.5% Triton X-100 (Sigma-Aldrich, United States) for 15 min. The permeabilized cells were blocked in 5% BSA (Sigma-Aldrich, United States) for 1 h at RT and then incubated at 4°C with the indicated primary antibodies overnight. The next day, the cells were washed with PBS-T (0.1% Tween 20 in PBS) and were incubated with the corresponding fluorescent secondary antibodies for 1 h at RT. Subsequently, the cells were stained with Hoechst 33342 for 15 min at RT. The images were captured under an LSM 880 confocal microscope (Carl Zeiss, Germany), and the ImageJ software was used for further analysis. All antibodies used in this study are listed in **Supplementary Table 2**.

Western Blotting

The cells were lysed with pre-cooled RIPA buffer containing the protease inhibitor (Roche, United States), sonicated on ice for 10 s, and then centrifuged (12,000 g) for 15 min at 4°C to collect the supernatant. The protein concentration was calculated with a BCA kit (Beyotime, China). The same amount of protein samples was separated by SDS-PAGE, and transferred to a PVDF membrane (EMD Millipore, Germany). Subsequently, the PVDF membrane was successively washed with TBS-T (0.1% Tween 20 in TBS) 3 times, blocked in the 5% solution of skim milk powder, and then incubated with the primary antibodies at 4°C overnight. After washing with TBS-T 3 times, the PVDF membrane was incubated with the corresponding HRP-conjugated secondary antibodies for 1 h at RT. The protein bands were visualized using a Western Blot Detection Kit (Cell Signaling Technology, United States) and further quantified using ImageJ software by normalizing the band intensity of interest to that of GAPDH. Antibodies used for western blotting are listed in **Supplementary Table 2**.

Flow Cytometric Analysis

The cells were trypsinized into single cells and successively fixed with 1% PFA for 15 min at RT and 90% cold methanol for 15 min at 4°C. The fixed cells were washed with 0.5% BSA in

PBS and subsequently incubated with the primary antibodies diluted in PBS containing 0.5% BSA and 0.1% Triton X-100 for 1 h at RT, followed by incubation with appropriate secondary fluorescent antibodies for 30 min. Finally, the cells were analyzed by a Guava easyCyte™ 8 flow cytometer (EMD Millipore, Germany). Antibodies used for flow cytometric analysis are listed in **Supplementary Table 2**.

Mitochondrial Staining

For mitochondrial staining, hESC-CMs cultured on coverslips were incubated with prewarmed medium containing 0.1 μM MitoTracker Red CMXRos (Thermo Fisher, United States) for 20 min. These cells were then stained with TNNT2 antibody, followed by incubation with Alexa Fluor 488-conjugated secondary antibody as shown in **Supplementary Table 2**. After nuclear staining with Hoechst 33342, cells were visualized under an LSM 880 confocal laser scanning microscope (Carl Zeiss, Germany). For the flow cytometric assay, the hESC-CMs incubated with 0.1 μM MitoTracker Red CMXRos were dissociated into single cells before subsequent TNNT2 and nuclear staining, and finally analyzed with Guava easyCyte 8 (EMD Millipore, Germany).

Mitochondrial Membrane Potential Assay

Mitochondrial activity was evaluated by using the mitochondrial membrane potential assay kit with JC-1 (Beyotime, China). Briefly, hESC-CMs were washed with D-PBS and incubated with JC-1 staining medium at 37°C for 20 min, followed by washing with JC-1 staining buffer and refreshing with cell culture medium. The cells were then observed under an Olympus DP73 fluorescence microscope (Olympus, Japan) to visualize the green JC-1 monomers (λ ex: 490 nm; λ em: 530 nm) representing predominantly depolarized mitochondria and red JC-1 aggregates (λ ex: 520 nm; λ em: 590 nm) forming in polarized mitochondria. In addition, in the shooting process, we carried out at the same time period and used the same exposure time and parameters to ensure the real changes of mitochondrial membrane potential in each group (Wang et al., 2020). Furthermore, the fluorescence intensity of each group was analyzed by ImageJ software. Carbo-nyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), a potent oxidative phosphorylation uncoupler, could efficiently eliminate the mitochondrial membrane potential. Cells treated with FCCP (10 μM) were used as a positive control showing the transition of red fluorescence to green fluorescence.

Patch Clamp for Action Potential Recordings

The cardiomyocytes were seeded on the gelatin-coated 35-mm dishes. After cell adherence for 48 h, spontaneous action potentials (APs) of cardiomyocytes were recorded using the patch clamp system. First, the medium was changed to an external bath solution containing 140 mM NaCl, 4 mM KCl, 1.2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and HEPES, adjust pH to 7.4 with NaOH. The cells were incubated for 15 min in a cell incubator.

The internal solution (115 mM potassium aspartate, 15 mM KCl, 4 mM NaCl, 1 mM MgCl₂, 5 mM Mg-ATP, 5 mM EGTA, and 5 mM HEPES adjusted pH to 7.2 with NaOH) was added into the electrode tube. Using an Axopatch 200B amplifier in the current clamp mode, the spontaneous APs were recorded with a current clamp. Finally, the data were collected and analyzed by pClamp 10 software.

Calcium Transient

The hESC-CMs growing on gelatin-coated coverslips were subjected to calcium imaging. Briefly, cells were incubated in Tyrode's solution supplemented with 5 μ M Fluo-4 AM (Thermo Fisher, United States) for 30 min at 37°C, followed by 3 washes with prewarmed Tyrode's solution. After immersion in Tyrode's solution for 5 min, the spontaneous calcium signaling in hESC-CMs was captured by an LSM 880 confocal laser scanning microscope (Carl Zeiss, Germany) with a 63 $\times$ oil immersion objective, using the line-scanning mode with a sampling rate of 1 ms/line. The calcium signals were analyzed to calculate the time to peak (s), Vmax ($\Delta F/F_0/s$) and decay time (s).

Seahorse XF24 Metabolic Flux Analysis

The cells were seeded onto the gelatin-coated XF24 cell culture microplates (Seahorse Bioscience, United States) at 7.5×10^4 cells/well. After 3 days of culture, the oxygen consumption rate (OCR) or extracellular consumption acid rate (ECAR) was detected using a seahorse XF24 analyzer. When the OCR was detected, 2 μ M oligomycin, 1 μ M FCCP and 0.5 μ M rotenone/antimycin A were sequentially added to the cell culture microplate, and the cardiomyocytes were cultured in base medium that contained the desired substrate. When ECAR was analyzed, 10 mM glucose, 2 μ M oligomycin and 50 mM 2-DG were sequentially injected into the special measuring solution containing L-glutamine. The OCR or ECAR was normalized to the protein concentration of cardiomyocytes.

Statistical Analysis

Comparisons between two groups were analyzed using Student's *t*-test. Comparisons among multiple groups were analyzed with one-way analysis of variance (ANOVA) with the Bonferroni *post hoc* test. Statistical significance was denoted by $p \leq 0.05$. All data are presented as the mean $\pm$ SEM. All experiments were performed at least three times.

RESULTS

EBSS Starvation Induces Autophagy and Benefits H1-Derived Cardiomyocytes

By using the chemically defined protocol presented in **Supplementary Figure 1A**, we successfully differentiated H1 into spontaneously beating cardiomyocytes (H1-CMs) with a high differentiation efficiency (**Supplementary Figure 1B**). The differentiated cardiomyocytes were further verified by abundant expression of cardiac markers including *TNNT2*, *MYH6*, *TNNI1*, *TNNI3* and α -actinin (**Supplementary Figures 1C–E**).

To induce cell starvation, we incubated the H1-CMs with EBSS for 1, 2, 3, 4, and 5 h. Because EBSS starvation is a classic and powerful inducer of autophagy, we first evaluated the autophagy levels in cardiomyocytes immediately after EBSS treatment. As shown in **Figures 1A,B**, EBSS treatment markedly increased the conversion of microtubule-associated protein light chain 3 (LC3) from LC3-I to LC3-II, an essential event for autophagy activation (Zhu et al., 2007). Our data indicated that EBSS treatments for 1 to 5 h efficiently induced autophagy in H1-CMs, and the 2-h treatment of EBSS was chosen for further studies. We then assessed mitophagy, the mitochondria-specific autophagy, in EBSS-treated cardiomyocytes. The protein expression of the mitophagy marker Parkin was also significantly upregulated in cardiomyocytes by EBSS treatment for 2 h (**Figures 1C,D**), indicating a successful induction of mitophagy.

To study the effect of starvation on cardiomyocyte maturation, we optimized the time window of EBSS treatment as indicated in **Figure 1E**. From day 15 of cardiomyocyte differentiation, the H1-CMs were intermittently starved with EBSS (2 h per day) for 5, 10 or 15 days and then maintained in CDM3 until subsequent analysis on day 35. Compared with other groups, H1-CMs intermittently treated with EBSS for 10 days showed the highest upregulation of the mitochondrial and metabolic marker *PGC1 α* (**Figure 1F**), as well as the late cardiac markers *MYH6* (**Figure 1G**) and *MYH7* (**Figure 1H**). These data indicated that intermittent starvation for 10 days could efficiently improve cardiomyocyte maturation, which was selected for further analysis.

Intermittent Starvation Promotes Structural Maturation of hESC-Derived Cardiomyocytes

To evaluate the effect of intermittent starvation on cellular morphology and structure, H1-CMs on day 35 were stained with the sarcomeric α -actinin antibody and Hoechst 33342 to visualize the sarcomere structures and nuclei, respectively (**Figure 2A**). Through statistical analysis, we found intermittent starvation for 10 days obviously increased the ratio of multinucleated cardiomyocytes (**Figure 2B**), a feature of cardiomyocyte maturation. Compared with the control group, H1-CMs subjected to EBSS-induced starvation exhibited more larger cell area (**Figure 2C**) and increased sarcomere length (**Figure 2D**). In addition, qPCR data showed that the mRNA expression of structural maturation-related genes *TNNI1*, *TNNI3*, *TTN N2B*, *TNNT2*, and *GJA1* were significantly upregulated in H1-CMs after EBSS treatment (**Figure 2E**). To investigate whether this effect is cell line specific, we further verified the effect of EBSS-induced starvation on the structural maturity in hES3-derived cardiomyocytes (hES3-CMs, **Supplementary Figure 2A**), as indicated by the increased multinucleation rate (**Supplementary Figure 2B**) and cell area (**Supplementary Figure 2C**), the elongated sarcomere (**Supplementary Figure 2D**), as well as the elevated expression of structural representative markers *MYH6* (**Supplementary Figure 2E**) and *MYH7* (**Supplementary Figure 2F**) and other structural maturation-relative genes (**Supplementary Figure 2G**). Taken together, these results

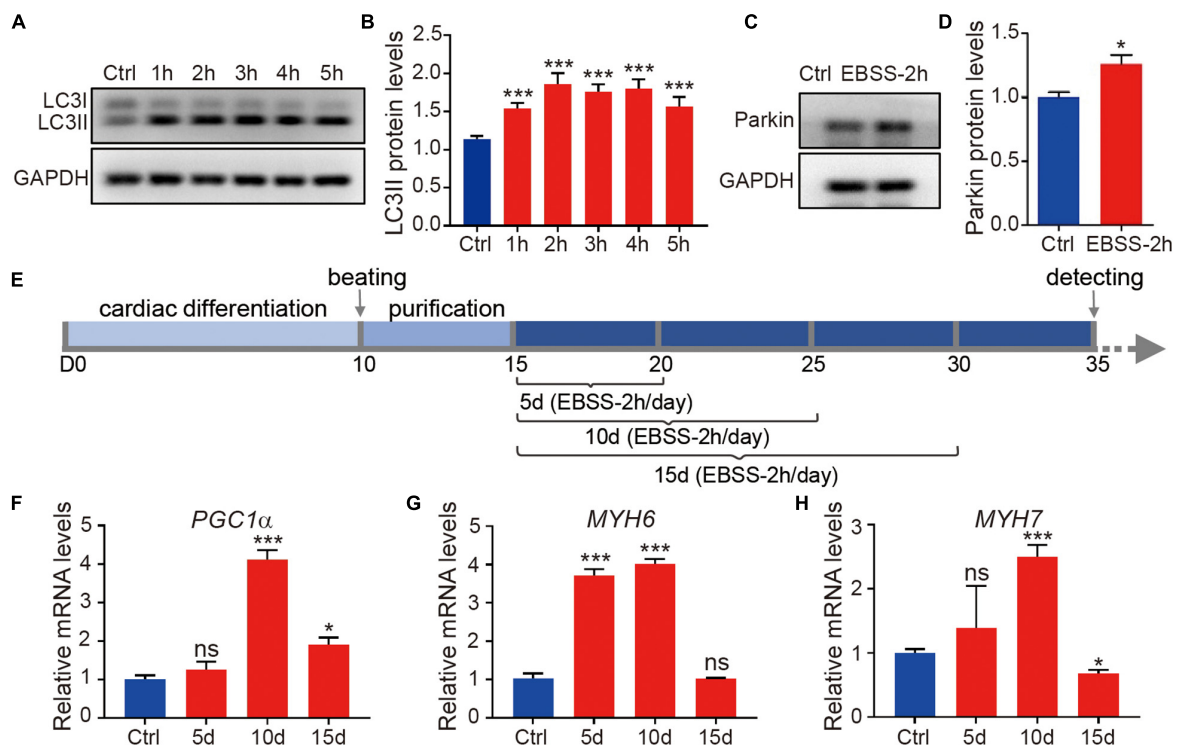


FIGURE 1 | Starvation induces autophagy of human embryonic stem cell-derived cardiomyocytes. **(A)** Western blotting analysis of LC3 expression in H1-CMs immediately after incubation with EBSS for 1 (1 h), 2 (2 h), 3 (3 h), 4 (4 h) and 5 h (5 h), respectively. **(B)** Statistical analysis of panel **(A)**. **(C)** Western blotting analysis of Parkin expression in H1-CMs immediately after EBSS incubation for 2 h. **(D)** Statistical analysis of panel **(C)**. **(E)** Schematic of EBSS-induced starvation (2 h per day) in H1-CMs for 5 (5 day), 10 (10 day) and 15 days (15 day), respectively. **(F–H)** The qPCR analysis of the metabolic marker *PGC1α* and cardiac markers (*MYH6* and *MYH7*) in EBSS-treated H1-CMs on day 35 of cardiomyocyte differentiation. Student's *t*-test or one-way ANOVA; **p* < 0.05, ****p* < 0.001, and ns, not significant.

demonstrated that intermittent starvation promoted the structural maturation of hESC-CMs.

Intermittent Starvation Enhances the Metabolic Maturation of hESC-Derived Cardiomyocytes

Mitochondrial biogenesis is closely linked with cell metabolism. While cardiomyocytes are more energy-consuming cells that need much more ATP to maintain survival, mitochondrial maturation is very important for the maturation of hESC-CMs. Therefore, we detected the indexes of mitochondrial biogenesis. MitoTracker staining showed a stronger red fluorescence intensity in EBSS-treated H1-CMs, while no obvious effect was detected in mitochondrial distribution by intermittent starvation (Figure 2F). By flow cytometric analysis, we further confirmed the increased fluorescence intensity of MitoTracker in EBSS-treated H1-CMs (Figure 2G). Additionally, as shown in Figures 2H,I, the relative mitochondrial DNA copy numbers, assessed by qPCR detection of the specific mitochondrial DNA regions encoding *mtCO1* and *mtND1*, were significantly increased in EBSS-treated H1-CMs. Meanwhile, we examined the expression of representative marker *PGC1α* (Supplementary Figure 3A) of cardiac metabolism and mitochondrial copy number gene *mtCO1*

and *mtND1* (Supplementary Figures 3B,C) in the control and EBSS-treated hESC-CMs, which is consistent with the data obtained in H1-CMs. Therefore, intermittent starvation treatment could increase the mitochondrial DNA content of hESC-CMs.

Mitochondrial membrane potential is an important parameter of mitochondrial activity. We thus analyzed the mitochondrial activity in the control and EBSS-treated H1-CMs by staining with the mitochondrial membrane potential indicator JC-1 and evaluating the resulting red/green fluorescence. After JC-1 incubation, both control and EBSS-treated H1-CMs displayed a strong red JC-1 fluorescence signal, indicating high mitochondrial membrane potential in these cells (Figure 2J). Compared to the control H1-CMs, the ratio of red/green fluorescent intensity was significantly increased in EBSS-treated H1-CMs (Figure 2K). Meanwhile, the qPCR data showed significantly increased expression of genes associated with mitochondrial structure and function, including *SDHA*, *SDHB*, *SDHC*, *SDHD*, *NDUFS1*, *NDUFS4*, *NDUFS6*, *NDUFA4*, and *NDUFV1* in EBSS-treated H1-CMs (Figure 2L).

The above data indicated the EBSS starvation could promote mitochondrial maturation and increase the mitochondrial activity of hESC-CMs, which is critical for ATP production through oxidative phosphorylation, the major energy source for contraction of the adult heart. We thus further assessed

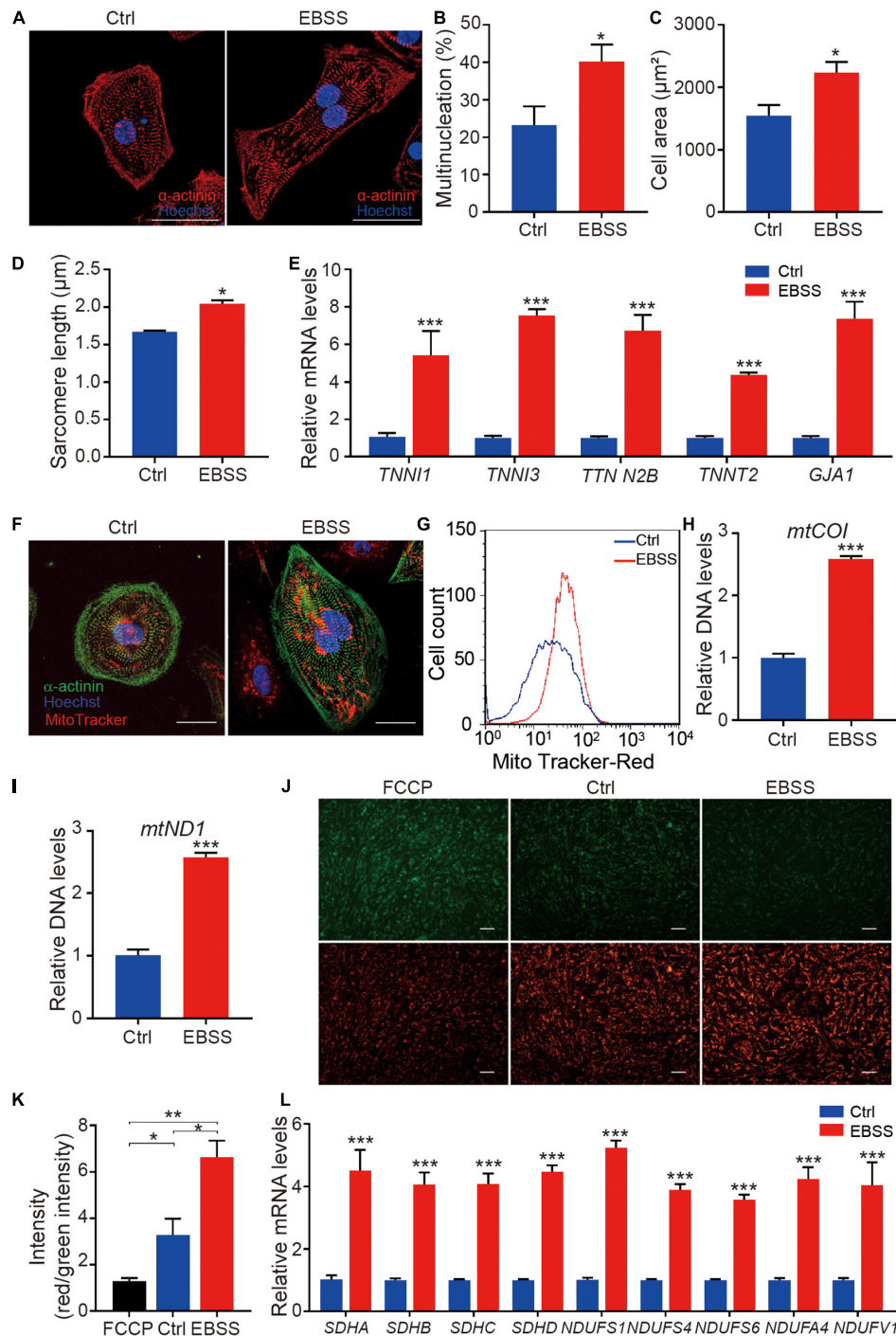


FIGURE 2 | Intermittent starvation promotes structural maturation and mitochondrial maturation of H1-derived cardiomyocytes. **(A)** Representative immunostaining images of α -actinin (red) in control and EBSS-treated H1-CMs. The nuclei were stained with Hoechst 33342 (blue). Scale bar, 50 μ m. **(B)** Statistical analysis of multinucleation in the control and EBSS-treated H1-CMs. **(C)** Statistical analysis of cell area in the control and EBSS-treated H1-CMs. **(D)** Statistical analysis of sarcomere length in the control and EBSS-treated H1-CMs. **(E)** The qPCR analysis of cardiac structural genes (*TNNI1*, *TNNI3*, *TTN N2B*, *TNNT2*, and *GJA1*) in control and EBSS-treated H1-CMs. **(F)** The mitochondria staining with MitoTracker Red CMXRos (red) in the control and EBSS-treated H1-CMs. The cytoskeleton and nuclei were stained with α -actinin antibody (green) and Hoechst 33342 (blue), respectively. Scale bar, 50 μ m. **(G)** Flow cytometric analysis showing more abundant mitochondrial content in EBSS-treated H1-CMs. **(H,I)** The qPCR data indicate significantly increased mitochondrial DNA copy number (*mtCO1* and *mtND1* DNA) in EBSS-treated H1-CMs. **(J)** Representative images of mitochondrial membrane potential assay using JC-1 dye in control and EBSS-treated H1-CMs. Green fluorescence signal indicates JC-1 monomers in cells with depolarized mitochondria and red signal indicates JC-1 aggregates forming in polarized mitochondria. Scale bar, 100 μ m. **(K)** Quantifications of mitochondrial membrane fluorescence intensity (red/green) in panel (J). **(L)** The qPCR analysis of markers of mitochondrial structure and function. Student's *t*-test; **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

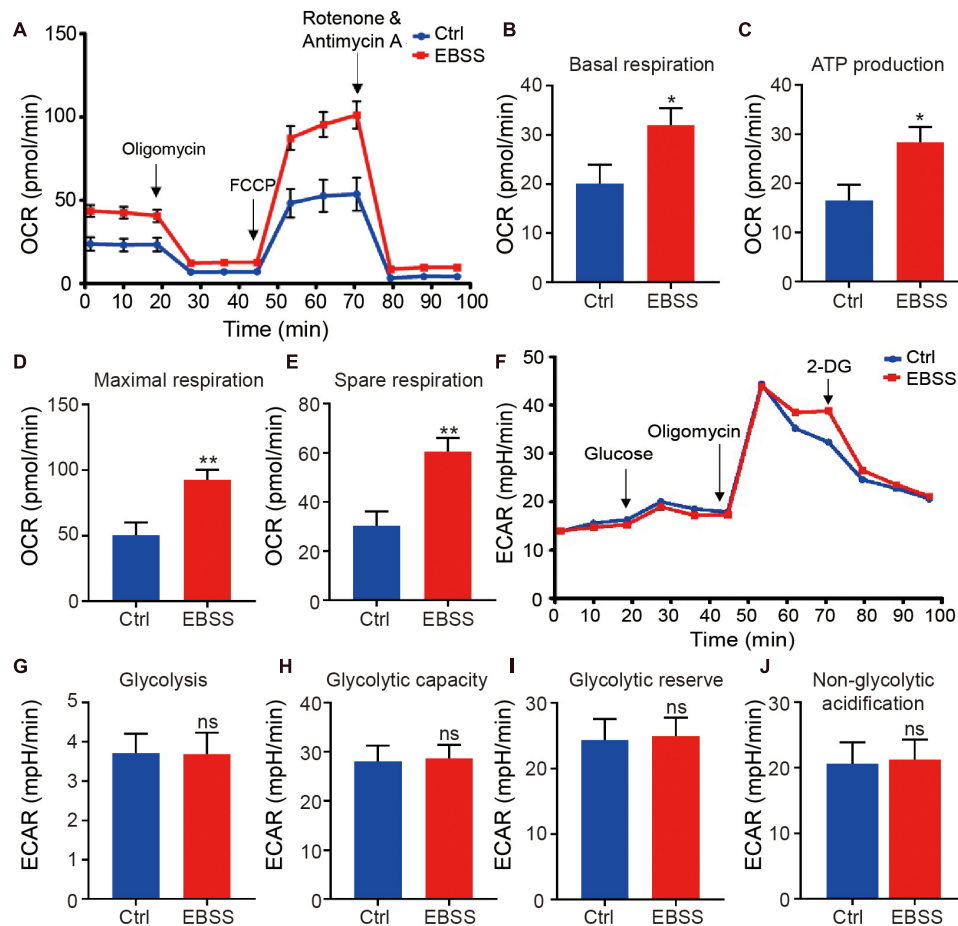


FIGURE 3 | Intermittent starvation enhances metabolic maturation of H1-derived cardiomyocytes. **(A)** Profile of average oxygen consumption rate (OCR) normalized to baseline as evaluated by a Seahorse XF24 Extracellular Flux Analyzer in control and EBSS-treated H1-CMs. **(B–E)** Assessment of basal respiration **(B)**, ATP production **(C)**, maximal respiration **(D)** and spare respiration **(E)** in control and EBSS-treated H1-CMs. **(F)** Representative ECAR traces of control and EBSS-treated H1-CMs obtained to investigate glycolysis using a Seahorse XF24 Extracellular Flux Analyzer. **(G–J)** Quantification of the glycolysis **(G)**, glycolytic capacity **(H)**, glycolytic reserve **(I)** and non-glycolytic acidification **(J)** in control and EBSS-treated H1-CMs. Student's *t*-test; **p* < 0.05, ***p* < 0.01, and ns, not significant.

the effect of intermittent starvation on mitochondrial oxidative metabolism in H1-CMs using the XF24 Extracellular Flux Analyzer. We observed a significant increase in the oxygen consumption rate (OCR) of EBSS-treated H1-CMs (**Figure 3A**). It was mainly reflected in the increased basal respiration, ATP production, maximal respiration and spare respiration (**Figures 3B–E**). However, no significant change in the extracellular acidification rate (ECAR), an indicator of glycolysis, was observed between the EBSS-treated group and the control group (**Figures 3F–J**). These results revealed that EBSS-induced starvation could promote the mitochondrial metabolic maturation of H1-CMs.

Intermittent Starvation Promotes Electrophysiological and Calcium Handling Maturation of hESC-CMs

We further assessed the functional characteristics of H1-CMs including the electrophysiological properties and calcium

handling. The patch-clamp technique was used to record the action potential, and the representative spontaneous action potentials from the control and EBSS-treated H1-CMs are clearly shown in **Figure 4A**. Statistical analysis showed that intermittent starvation significantly prolonged the action potential duration at 90% repolarization (APD90) and action potential duration at 50% repolarization (APD50) in H1-CMs, two parameters usually used to indicate electrophysiological maturation of cardiomyocytes (**Figures 4B,C**). Nevertheless, we did not observe significant changes in the V_{max} upstroke and resting membrane potential (RMP) in the control and EBSS-treated H1-CMs (**Figures 4D,E**).

Calcium handling is fundamental to cardiac function. In this study, we performed calcium imaging analysis on control and EBSS-treated H1-CMs in which the fluorescent calcium indicator Fluo-4 AM was loaded to detect intracellular free calcium. Compared to the control group, the EBSS-treated cardiomyocytes showed a prolonged calcium transient (**Figure 4F**), a shortened peak-reaching time (**Figure 4G**),

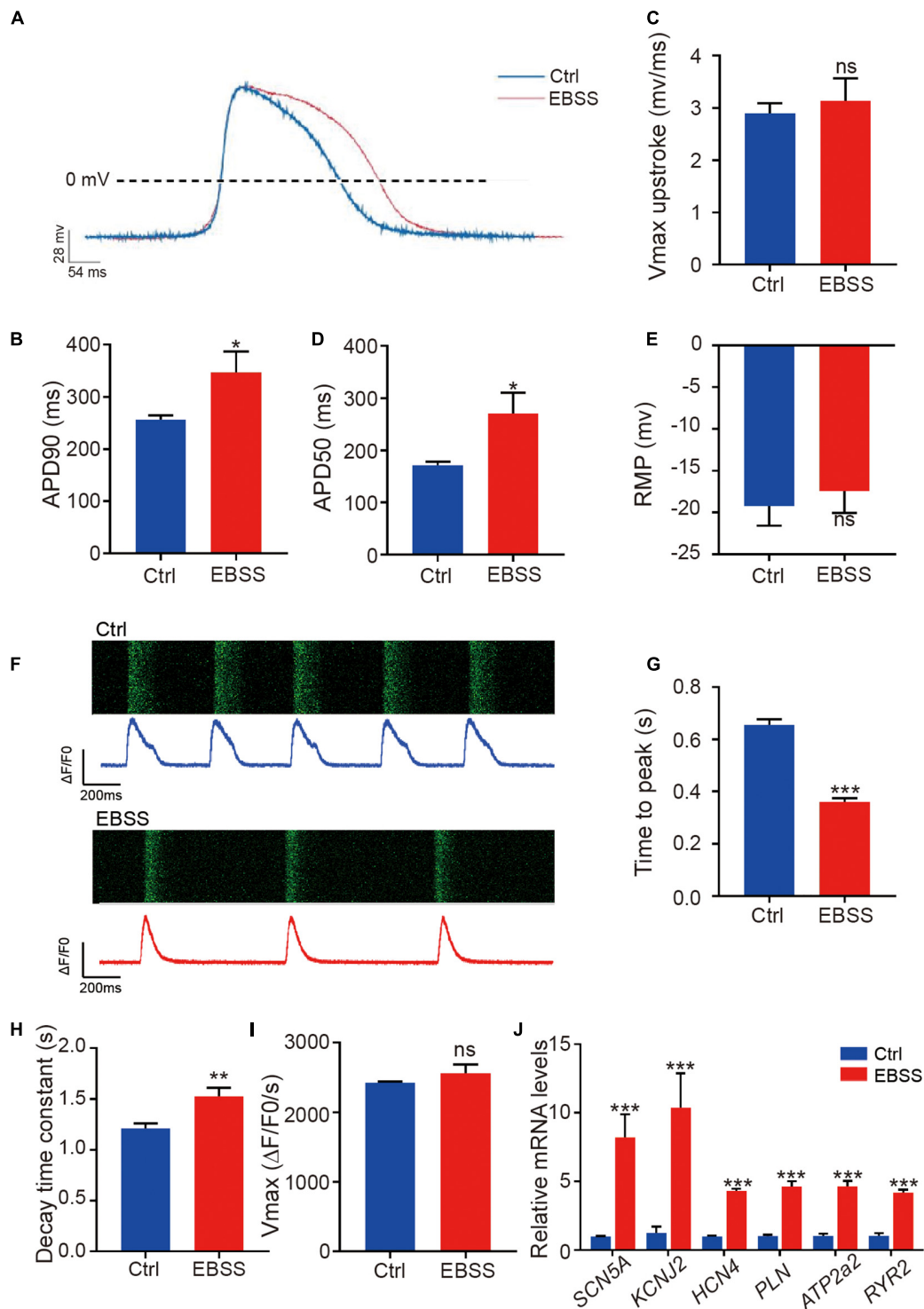


FIGURE 4 | Intermittent starvation promotes electrophysiological maturation and calcium handling of H1-derived cardiomyocytes. **(A)** Representative action potential traces from control and EBSS-treated H1-CMs. **(B–E)** Assessment of APD90 (action potential durations at 90% repolarization), APD50 (action potential durations at 50% repolarization), Vmax upstroke and RMP (resting membrane potentials). **(F)** Representative images showing the time-lapse recording of calcium activity (green) and the calcium handling traces from control (blue trace) and EBSS-treated (red trace) H1-CMs preloaded with the calcium indicator Fluo-4 AM. **(G,H)** Ca^{2+} transient properties of control and EBSS-treated H1-CMs, including the time to peak **(G)**, decay time constant **(H)**, and maximal velocity of upstroke **(I)**. **(J)** The qPCR analysis of electrical conduction (*SCN5A*, *KCNJ2*, and *HCN4*) and calcium handling (*PLN*, *ATP2a2*, and *RYR2*) markers in control and EBSS-treated CMs. Student's *t*-test; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and ns, not significant.

and a longer decay time (**Figure 4H**), while no change in $V_{\max}$ upstroke (**Figure 4I**) was observed in these cells. The data acquired from hES3-CMs also further validated the accelerative effect of EBSS-induced starvation on cardiac calcium handling (**Supplementary Figure 4**). Consistent with the prolonged interval of calcium transient interval, we observed a decreased spontaneous contraction rate, another feature of cardiac maturation, in EBSS-treated H1-CMs when compared with that in control cardiomyocytes (**Supplementary Movies 1, 2**). Meanwhile, the qPCR results demonstrated that the maturation marker genes related to electrical conduction (*SCN5A*, *KCNJ2*, and *HCN4*) and calcium handling (*PLN*, *ATP2a2*, and *RYR2*) were significantly upregulated in H1-CMs by EBSS-induced starvation (**Figure 4J**). Taken together, these data indicated that the intermittent starvation could facilitate the functional maturation of H1-CMs, including the electrophysiological properties and calcium handling.

DISCUSSION

Pluripotent stem cell-derived cardiomyocytes are a promising cell resource for cardiac disease modeling, drug screening and cell therapy (van Laake et al., 2008; Hartman et al., 2016). However, hPSC-CMs display immature structural, metabolic and electrophysiological features resembling embryonic or fetal cardiomyocytes, which severely obstruct accurate disease modeling and clinical applications. Prolonged culture (>90 days) has showed some positive effects on cardiomyocyte maturation; however, it is time-consuming and costly. Although a large number of studies have tried to improve the maturation of hPSC-CMs within a reasonable time frame by chemical, genetic, and biomechanical approaches, yet these approaches are not enough to efficiently mature these cells (Karbassi et al., 2020). In this study, we established a novel and accessible approach to promote the structural, metabolic and electrophysiological maturation of hPSC-CMs by EBSS-induced intermittent starvation. This approach is accompanied by autophagy and mitophagy in hPSC-CMs.

Despite a lack of knowledge regarding the regulatory mechanism, the prenatal-to-postnatal transition is believed to present a critical window for cardiomyocyte maturation (Neary et al., 2014; Gentillon et al., 2019). Immediately after birth, neonates sustain severe nutrient starvation and display massive autophagosome formation in their hearts, indicating a critical role of autophagy during heart development (Correia et al., 2017; Kretschmar et al., 2019). Our approach in this study is to mimic the *in vivo* starvation event through EBSS treatment to promote hESC-CM maturation. EBSS is a widely used inducer of autophagy (Bravo-San Pedro et al., 2017; Cai et al., 2019). We also observed dramatic induction of autophagy and mitophagy in EBSS-treated hESC-CMs, which displayed more mature features on day 35 of cardiomyocyte differentiation. Thus, we believe that starvation-induced autophagy is involved in cardiomyocyte maturation *in vitro*. As a highly conserved

cellular mechanism of protein recycling, autophagy is adaptively activated by nutrient deprivation, hypoxia, or other stress (Zhu et al., 2007). Numerous molecular mechanisms have been causally implicated in stress-induced autophagy. The nutrient sensors, sirtuin-1 (SIRT1) and adenosine monophosphate-activated protein kinase (AMPK) were recently shown to augment autophagy in cardiomyocytes, as master regulators of hundreds of genes and proteins (Packer, 2020). For instance, AMPK is activated in response to decreased level of ATP/ADP, and inhibits mTOR activity by phosphorylating the tuberous sclerosis complex 2 (TSC2), thereby stimulates autophagy (Matsui et al., 2007). While phosphorylation of AMPK promotes maturation of autophagosomes (Jang et al., 2018), SIRT1 primarily promotes selective clearance of damaged mitochondria (Fang et al., 2014). According to previous studies, we speculated that both AMPK2 and SIRT1 might mediate the effects of EBSS-induced starvation on cardiomyocyte maturation.

Previous studies indicated that the maturation process of hPSC-CMs is stagnated at days 20 – 30 of cardiomyocyte differentiation, when these hPSC-CMs reach the late embryonic stage rather than the neonatal stage (Lee et al., 2010; Uosaki et al., 2015). Thus, we preferred to induce cardiomyocyte starvation before maturation arrest. Through optimizing the time window, we found that intermittent starvation (a 2-h treatment of EBSS per day from day 15) for 10 days is more efficient in promoting the maturation of hESC-CMs. Our study also supports the existence of a critical window for promoting maturation in hPSC-CMs.

Cardiomyocytes undergo dramatic changes in structure, metabolism and function during the course of maturation *in vivo* (Mordwinkin et al., 2013). Notably, our results revealed that EBSS-induced starvation could promote the maturation of hESC-CMs, which was mainly reflected in the following three aspects. Structurally, the EBSS-treated hESC-CMs showed a larger cell size, more organized contractile cytoskeleton and a higher percentage of multinucleate cardiomyocytes. Metabolically, EBSS-induced starvation promoted oxidative phosphorylation. Functionally, EBSS-induced starvation strengthened electrophysiological maturation and the capacity of calcium handling. However, cardiomyocyte maturation is a complex process, involving a series of critical events (Fang et al., 2014; Chen et al., 2019). Despite of partial maturation, EBSS-treated hESC-CMs could not completely mimic adult cardiomyocytes. Thus, combinatorial approaches might be more efficient and necessary to accurately control the maturation of hPSC-CMs to meet different requirements in disease-modeling and clinical applications. Our recent study has indicated that retinoid acid (RA) treatment could also enhance hPSC-CM maturation (Miao et al., 2020), and in the future the combined treatment is expected to further optimize the protocol for hPSC-CM maturation.

In summary, EBSS-induced intermittent starvation is an efficient strategy to promote the structural, metabolic and functional maturation of hPSC-CMs at an affordable time and

cost. This new approach will help to improve the applications in cardiac disease modeling, drug screening, and cell therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

SH, WL, MY, ZY, and Z-AZ conceptualized and designed the study. SH, WL, MY, and JY wrote the manuscript. JY, ND, YY, CS, DZ, and XN acquired and analyzed the majority of the data. SH, WL, ZL, JC, and MY revised the manuscript critically for important intellectual content. SH supervised the study and provided main funding support. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.687769/full#supplementary-material>

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Arrhythmic Risk Assessment of Hypokalaemia Using Human Pluripotent Stem Cell-Derived Cardiac Anisotropic Sheets

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Introduction: Hypokalaemia, defined as an extracellular concentration of K^+ below 3.5 mM, can cause cardiac arrhythmias by triggered or re-entrant mechanisms. Whilst these effects have been reported in animal and human stem cell-based models, to date there has been no investigation in more complex structures such as the human ventricular cardiac anisotropic sheet (hvCAS). Here, we investigated arrhythmogenicity, electrophysiological, and calcium transient (CaT) changes induced by hypokalaemia using this bioengineered platform.

Methods: An optical mapping technique was applied on hvCAS derived from human pluripotent stem cells to visualize electrophysiological and CaT changes under normokalaemic (5 mM KCl) and hypokalaemic (3 mM KCl) conditions.

Results: Hypokalaemia significantly increased the proportion of preparations showing spontaneous arrhythmias from 0/14 to 7/14 (Fisher's exact test, $p = 0.003$). Hypokalaemia reduced longitudinal conduction velocity (CV) from 7.81 to 7.18 $\text{cm}\cdot\text{s}^{-1}$ ($n = 9, 7; p = 0.036$), transverse CV from 5.72 to 4.69 $\text{cm}\cdot\text{s}^{-1}$ ($n = 12, 11; p = 0.030$), prolonged action potential at 90% repolarization (APD_{90}) from 83.46 to 97.45 ms ($n = 13, 15; p < 0.001$), increased action potential amplitude from 0.888 to 1.195 ΔF ($n = 12, 14; p < 0.001$) and CaT amplitude from 0.76 to 1.37 ΔF ($n = 12, 13; p < 0.001$), and shortened effective refractory periods from 242 to 165 ms ($n = 12, 13; p < 0.001$).

Conclusion: Hypokalaemia exerts pro-arrhythmic effects on hvCAS, which are associated with alterations in CV, repolarization, refractoriness, and calcium handling. These preparations provide a useful platform for investigating electrophysiological substrates and for conducting arrhythmia screening.

Keywords: stem cells, ventricular arrhythmias, hypokalaemia, optical mapping, action potential

INTRODUCTION

Hypokalaemia is defined as an extracellular concentration of K^+ below the normal range of 3.5–5.3 mM (Macdonald and Struthers, 2004; Unwin et al., 2011). It is a common biochemical abnormality in cardiac patients that may represent a side effect of diuretic therapy or result from endogenous activation of the renin-angiotensin system and high adrenergic tone. Transient hypokalaemia is also induced by intense exercise during recovery (Atanasovska et al., 2018). It is a risk factor for ventricular arrhythmias that can predispose patients to sudden cardiac death (Osadchii, 2010; Chen et al., 2021). The mortality rate of hospitalized patients with hypokalaemia is tenfold higher than in the generalized hospital population, illustrating its potentially life-threatening consequences (Paltiel et al., 2001). Low levels of K^+ are replaced with supplements through oral or intravenous routes.

Hypokalaemia can induce several electrophysiological abnormalities, such as delayed ventricular repolarization, which in turn predisposes patients to triggered activity through the development of early afterdepolarizations (EADs) and re-entrant arrhythmias by increasing the dispersion of repolarization (Schulman and Narins, 1990; Macdonald and Struthers, 2004; Osadchii, 2010). Its pro-arrhythmic effects and mechanisms have been demonstrated in previous studies in animal and human stem cell-based models (Tse et al., 2016b; Kuusela et al., 2017). However, to date, there have been no investigations into more complex structures such as the human ventricular cardiac anisotropic sheet (hvCAS). hvCAS is a micro-patterned substrate that is specifically designed to reproduce the anisotropy of native human ventricles and allows for direct visualization of arrhythmic spiral re-entry (Chen et al., 2011; Luna et al., 2011; Wang et al., 2013; Shum et al., 2017; Wong et al., 2019). In this study, we investigated the electrophysiological and calcium transient (CaT) changes induced by hypokalaemia using this bioengineered platform (Wang et al., 2013; Shum et al., 2017). An optical mapping technique was applied on hvCAS derived from human pluripotent stem cells to investigate electrophysiological and CaT changes on normokalaemic (5 mM KCl) and hypokalaemic (3 mM KCl) preparations.

MATERIALS AND METHODS

This study has been approved by the University of Hong Kong/Hospital Authority Hong Kong West Cluster Institutional Review Board. Informed consent was given by the subject.

Reprogramming and Cell Culture

A peripheral blood sample was obtained from a normal healthy adult person. CD34⁺ cells were isolated from peripheral blood mononuclear cells (>95% purity) by using human CD34 Microbead Kit (130-046-702; Miltenyi Biotec) and MACS system (130-042-201; 130-098-308; 130-042-303; Miltenyi Biotec). They were expanded for 3 days in StemSpan™ H3000 (Stemcell Technologies) with the cytokine cocktail CC100 (Stemcell Technologies).

Nucleofection was performed on them with the non-integrated episomal vectors pCXLE-hOCT3/4-shp53, pCXLE-hSK, and pCXLE-hUL (Okita et al., 2011) using the Human CD34 Cell Nucleofector™ kit (Amaxa) according to the manufacturer's protocol for reprogramming.

Cells were then seeded to Geltrex (A1413302; Gibco)-coated plates in E8 medium (Life Technologies). After a few days, morphological changes were observed and human induced pluripotent stem (iPS) cell colonies typically started to appear on day 7. Tra-1-60 enrichment was performed on day 13 after nucleofection.

Human embryonic stem cells (hES2) (ESI, NIH code ES02) (passages 35–80) and human iPS cell (passages 50–90) lines were maintained in feeder- and serum-free conditions in mTeSR1 medium (STEMCELL Technologies) on Matrigel™ (BD Biosciences) and E8 medium on Geltrex respectively at 37°C in 5% CO₂ and 21% O₂ in a humidified normoxic environment and cell passaging was performed every 3, 4 days at 70–80% confluence using Accutase (A11105; Gibco). A tissue culture-treated six-well plate (3,506; Coster) was enrolled. The cells were negative in the mycoplasma test.

Human iPS Cells Validation

The human iPS cell colonies were treated with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min for fixation and then permeabilized by treating with 0.1% Triton X-100 for 15 min. They were then washed with PBS three times. Staining with anti-OCT4, SSEA-4, and Tra-1-81 for 2 h at room temperature were performed on the fixed samples. They were then treated with fluoroconjugated goat anti-rabbit or anti-mouse secondary antibodies for 1 h. For the pluripotency test, human iPS cells were differentiated to form Embroid Bodies (EB). The EB medium used was DMEM/F12 (Life Technologies) with 20% Knockout Serum Replacement (Life Technologies), 2 mM nonessential amino acids, 2 mM L-glutamine, and 0.1 mM β-mercaptoethanol. EBs were stained at day 15 for three germ layer markers (i.e., Tuji, SMA, and AFP). Karyotyping was performed as described by Bates (Bates, 2011).

Directed Cardiomyocyte Differentiation and Characterization

Human embryonic stem (ES) and iPS cells were digested into single-cells by Accutase and seeded onto Matrigel-coated and Geltrex-coated plates respectively at $\sim 3 \times 10^5$ cells/10 cm². An established directed cardiomyocyte differentiation protocol was used (Weng et al., 2014). ES cells were put in mTeSR1 medium and iPS cells were put in E8 medium for 4 days until ~ 80 –90% confluence. They were digested into single-cells by Accutase and transferred to ultralow-attachment six-well plates (Corning) for non-adherent culture in mTeSR1 medium (for ES cells) and E8 medium (for iPS cells), with Matrigel (40 μg/ml) with BMP4 (1 ng/ml; Invitrogen) and Rho kinase inhibitor (RI) (10 μM; R&D) under hypoxic conditions with 5% O₂. This was termed Day (D) 0 as it was the initiation of cardiomyocyte differentiation. On D1 (24 h later), the culture was changed to StemPro34 SFM (Invitrogen) with ascorbic acid (AA, 50 μg/ml; Sigma), 2 mM

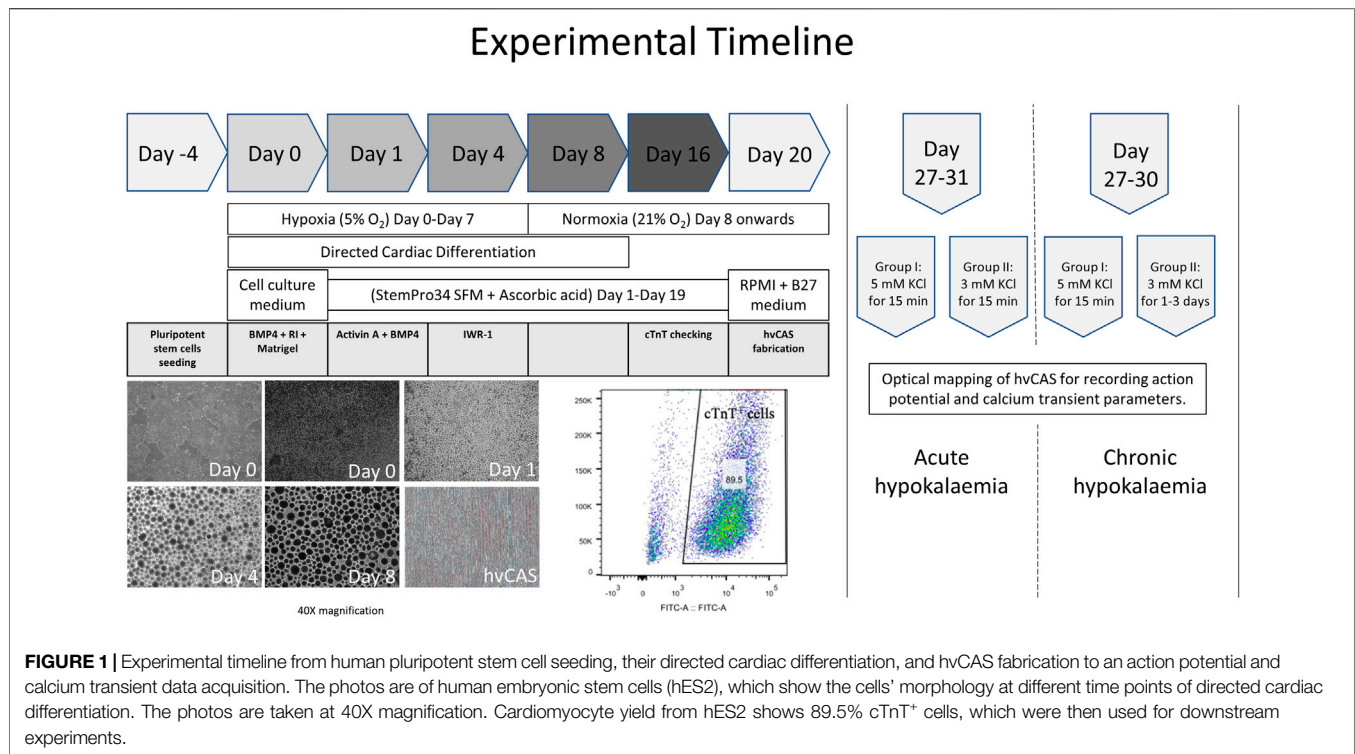


FIGURE 1 | Experimental timeline from human pluripotent stem cell seeding, their directed cardiac differentiation, and hvCAS fabrication to an action potential and calcium transient data acquisition. The photos are of human embryonic stem cells (hES2), which show the cells' morphology at different time points of directed cardiac differentiation. The photos are taken at 40X magnification. Cardiomyocyte yield from hES2 shows 89.5% cTnT⁺ cells, which were then used for downstream experiments.

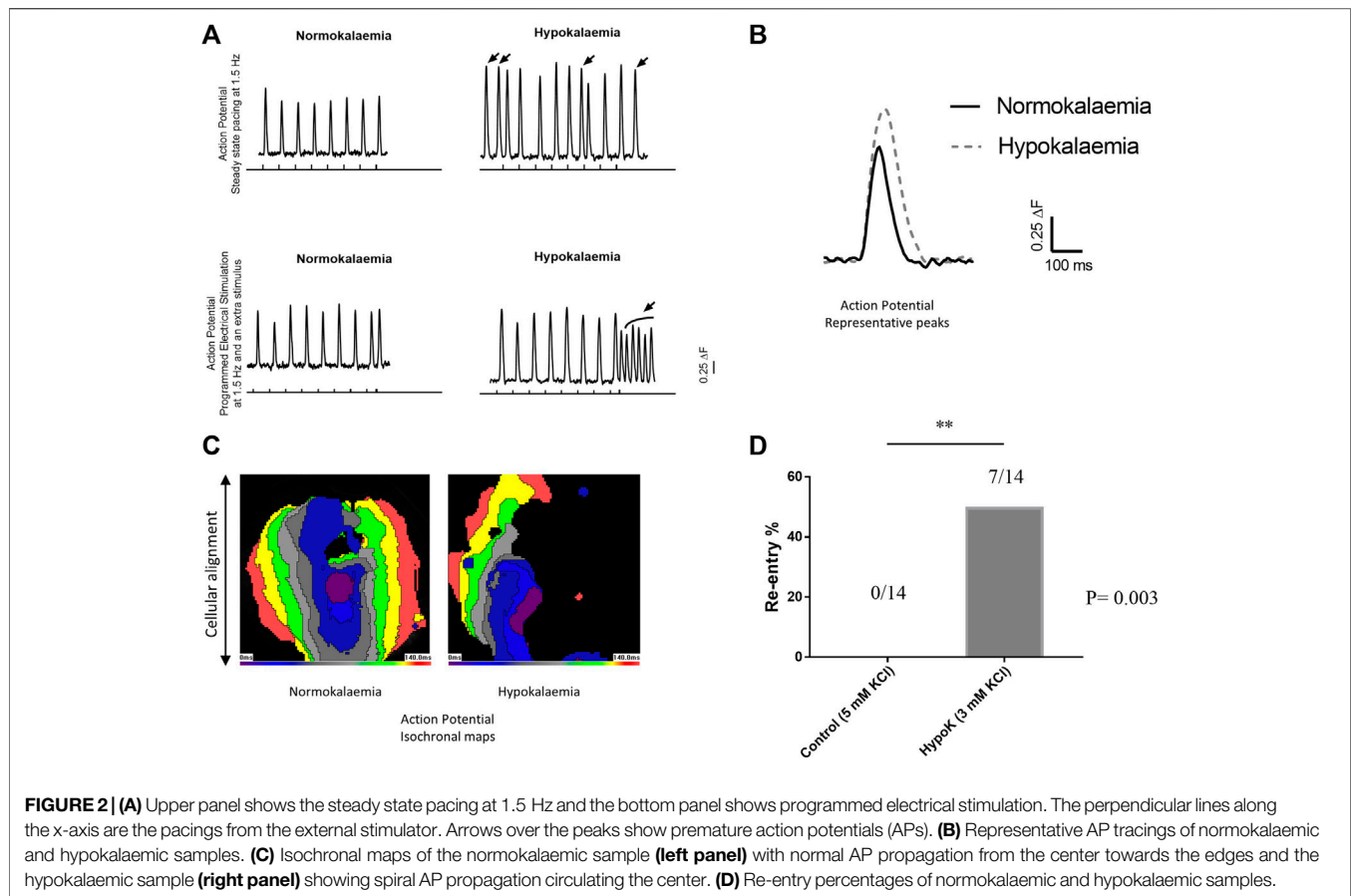
GlutaMAX-1 (Invitrogen), BMP4 (10 ng/ml), and human recombinant activin-A (10 ng/ml; Invitrogen). On D4, the culture was changed to StemPro34 SFM + AA, 2 mM GlutaMAX-1, IWR-1, a Wnt inhibitor (5 μ M; Enzo Life Sciences). Contracting clusters could be observed on D8 or later. On D8, the culture was changed to StemPro34 SFM + AA, 2 mM GlutaMAX-1, and transferred to a normoxic environment. From D8 onwards, cells were maintained in StemPro34 SFM + AA, 2 mM GlutaMAX-1, and replenished every 3–4 days for further characterization and downstream functional assays.

Flow cytometry analysis was performed on differentiated cells collected at D16–18 to quantify cTnT⁺ cells resembling cardiomyocyte yield.

Fabrication of hvCAS

hvCAS substrates were fabricated according to a published protocol (Shum et al., 2017). The mold was obtained from Ming Wai Lau Centre for Reparative Medicine, Karolinska Institutet of groove dimensions 15 μ m (R) $\times$ 5 μ m (D) $\times$ 5 μ m (W). Each substrate was 15 mm in diameter. Polystyrene shrink films (Clear Shrink Dinks) were hot-embossed at 180°C for 30 min. They were then treated with ultraviolet ozone (UVO) for 8 min (Jetlight UVO). Finally, they were submerged in 70% ethanol and kept under UV light for at least 15 min for sterilization. The substrates were coated with Matrigel and suctioning was performed for at least 15 min to remove air bubbles. The substrates were kept in a four-well plate (6900A07; Thermo Scientific Nunc) using vaseline.

Cardiomyocyte clusters (cTnT⁺ cells >60%) were digested into single-cells on D18–20 by treatment with 0.025% Trypsin-EDTA, phenol red (Gibco, Cat. No. 25300062, 500 ml, stored at -20°C) (4 ml per six-well plate) in PBS for 12 min at 37°C in a water bath with shaking at 90 rpm, which was then blocked by H1 medium with 20% Fetal Bovine Serum using twice the volume of 0.025% Trypsin-EDTA used. H1 medium with 20% Fetal Bovine Serum (FBS) medium was prepared by mixing 20% of FBS (Gibco, Cat. No. 1027016, 500 ml, stored at -20°C) in Dulbecco's Modified Eagle Medium (DMEM) (High Glucose) (Gibco, Cat. No. 11965118, 500 ml, stored at 4°C) with Penicillin-Streptomycin (P/S)(1X) (Gibco, Cat. No. 15140122, 100 ml, stored at -20°C), GlutaMAX (1X) (Gibco, Cat. No. 35050061, 100 ml, stored at room temperature) and MEM Non-Essential Amino Acids (NEAA) (1X) (Gibco, Cat. No. 11140-035, 100 ml, stored at 4°C). The solution was then filtered through a 0.22 μ m PES membrane. The digested cells were passed through 40 μ m cell strainer (BD Biosciences). Cell seeding on the Matrigel-coated polystyrene substrate was done at 250 K cm⁻² termed day 0 of hvCAS. The hvCAS were maintained on high glucose DMEM-based medium containing heat-inactivated 10% FBS, GlutaMAX (1X), MEM NEAA (1X) for 2 days at 37°C in 5% CO₂ in a humidified normoxic environment. The medium was changed to RPMI 1640 medium (72,400; Gibco) with B-27 (17,504; Gibco) with P/S (1X) on the third day of seeding and replenished every second day. Optical mapping was performed from D7–D11 post hvCAS fabrication for electrophysiological examination after the formation of intercellular electrical junctions. The experimental timeline from cell culture to data acquisition is shown in **Figure 1**.



Optical Mapping of hvCAS

Fresh normal Tyrode's solution consisting of, in $\times 10^{-3}$ M, 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 D-glucose, and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH 7.4 were prepared. For hypokalaemia, the freshly prepared Tyrode's solution contained 3×10^{-3} M KCl whereas the other composition remained the same. The solution was consumed for up to 7 days.

hvCAS preparations were loaded with RH237 (5×10^{-6} M; Lot: 1,890,562; Life Technologies, membrane voltage sensitive dye), Rhod-2 AM (10×10^{-6} M; R1245MP; Thermofisher, calcium dye), and Pluronic F-127 (0.04%; P-3000MP; Life Technologies) for 20 min at 37°C in normal Tyrode's solution (5 mM KCl for normokalaemic hvCAS) and hypokalaemic Tyrode solution (3 mM KCl for hypokalaemic hvCAS), followed by washing once with PBS and incubation for 10 min in Blebbistatin (50×10^{-6} M, B0560; Sigma-Aldrich) containing normal Tyrode's solution and hypokalaemic Tyrode's solution for the two subsets to minimize potential motion artifacts during optical mapping.

hvCASs were washed once with PBS and changed to normal Tyrode's solution (for normokalaemia hvCAS) and hypokalaemic Tyrode's solution (for hypokalaemia hvCAS) and maintained at 35–37°C using a culture dish incubator (Warners Instruments) for optical mapping. High-resolution optical mapping was

performed using a MiCAM05 (SciMedia, CA, United States) for Action Potential (AP) and CaT parameters. The field of view was 17.6 mm by 17.6 mm obtained by using 1× objective and a 1× condensing lens. The input light source was a high-powered LED illumination system (LEX2, SciMedia, CA, United States) filtered by a 515 ± 35 nm band-pass excitation filter, and the light was split into two using a splitter. One was passed through a >700 nm long-pass emission filter for RH237 (voltage sensitive dye) and the other through a 590 nm band-pass emission filter for Rhod2-AM (calcium sensitive dye). Data acquisition was done at a sampling rate of 5 ms/frame and BVAna software (SciMedia) was used for data analysis followed by clampfit (Axon Instruments). A programmable electrical stimulator (Master9; AMPI, Israel) was used for pacing (10 V, 10 ms pulse duration) through a unipolar point-stimulation electrode (Harvard Apparatus, MA, United States). The point-stimulation electrode was positioned perpendicular to the hvCAS surface at its center. Optical mapping was performed once per sample and unpaired.

Pacing Protocol for hvCAS

1) Steady state pacing was eight trains from 0.5 to 3 Hz (i.e., 30–180 bpm) at an increment of 0.5 Hz unless there was a loss of 1:1 capture. If they captured all the frequencies without arrhythmia, they were subjected to Programmed Electrical Stimulation (PES).

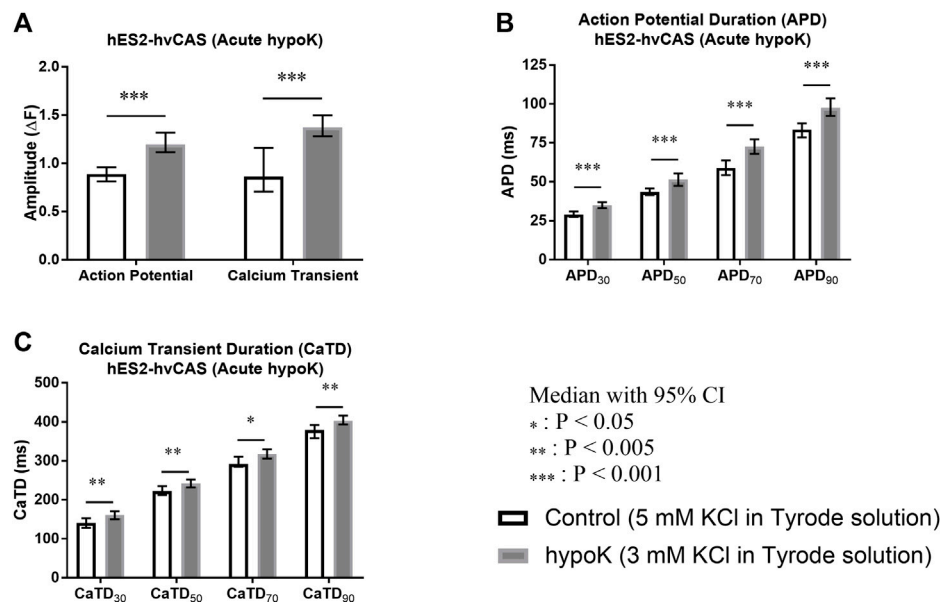


FIGURE 3 | Human embryonic stem cell (hES2)-derived human ventricular cardiac anisotropic sheet (hvCAS) paced at 1.5 Hz during acute hypokalaemic (hypoK) experiment where the treatment was for 15 min before data acquisition. Number of samples is represented by *n* where the first number refers to control (5 mM KCl in Tyrode solution) samples and the second number refers to hypoK (3 mM KCl in Tyrode solution) samples. **(A)** Action Potential (AP) Amplitude (*n* = 12, 14) and Calcium Transient (CaT) Amplitude (*n* = 12, 13). ΔF represents the change in fluorescence intensity. **(B)** AP Duration (APD) at 30, 50, 70 and 90% fluorescence decay intervals from the peak fluorescence intensity represented by APD₃₀, APD₅₀, APD₇₀ and APD₉₀. (*n* = 13, 15). **(C)** CaT Duration (CaTD) at 30, 50, 70 and 90% fluorescence decay intervals from the peak fluorescence intensity represented by CaTD₃₀, CaTD₅₀, CaTD₇₀ and CaTD₉₀. (*n* = 11, 10). Amplitude of AP and CaT increased in hypokalaemic condition (asterisks, *p* < 0.001) and also for the APD and CaTD at 30, 50, 70 and 90% decay (asterisks, *p* < 0.001 for APD and *p* < 0.005, *p* < 0.05 for CaTD).

2) PES used eight trains of 1.5 Hz (S1) followed by a premature trigger (S2), where S1-S2 intervals were gradually shortened to find the Effective Refractory Period (ERP). The starting S1-S2 interval was 550 ms, which was then consecutively shortened in steps by 50 ms until capture had failed. S1-S2 interval was then increased by 20 ms, and then by 2 ms decrements until the ERP was achieved, which is the maximum S1-S2 interval that failed to lead to AP propagation (Figure 5C).

hvCAS Subsets

Acute hypokalaemia: Optical mapping was performed on hvCAS for simultaneously measuring AP and CaT in normokalaemic and hypokalaemic conditions. All the hvCAS were maintained in the normokalaemic medium until optical mapping day (D7-D11 post hvCAS fabrication). On optical mapping day, normal Tyrode's solution was used for one of the subsets (normokalaemia) and hypokalaemic Tyrode's solution was used for another subset (hypokalaemia). The hvCAS were in respective Tyrode's solution from dye loading to data acquisition (30 min to <60 min). The unpaired test was chosen as repeated measurement, which led to a significant decrease in the signal to noise ratio.

Chronic hypokalaemia: The fabricated hvCAS were divided into two subsets (Subset I: control, Subset II: experimental). Both groups were kept at normokalaemic condition until D7 post hvCAS fabrication. From D7–D10, optical mapping was performed for AP and CaT in which subset I was in

normokalaemic condition throughout the experiment, whereas subset II was changed to hypokalaemic condition on D7–D10.

AP and CaT Parameters

The following parameters were obtained from the experimental records:

- 1) Activation Latency or time-to-peak: The time interval from the start of the stimulus to the peak of the AP or CaT;
- 2) Amplitude: The height of the peak;
- 3) AP Duration (APD_x) and CaT Duration (CaTD_x): The time interval from the peak of the AP or CaT at *x* = 30, 50, 70, and 90% repolarization or decay respectively;
- 4) ERP: The maximum S1-S2 interval that failed to elicit an AP;
- 5) Upstroke Velocity: The velocity at which the fluorescence reaches the peak from baseline;
- 6) Conduction Velocity (CV): The velocity at which AP propagates between two points;
- 7) Anisotropic Ratio (AR): The ratio of longitudinal CV to transverse CV.

Statistical Analysis

Prism (Version 7.0) and Microsoft Excel (Version 16.31) were used for statistical analysis. Values are in the median with lower and upper 95% Confidence Intervals. Different experimental groups were compared by Mann Whitney Test. Fisher's exact test was used to analyze spiral induction. *p* < 0.05 was considered

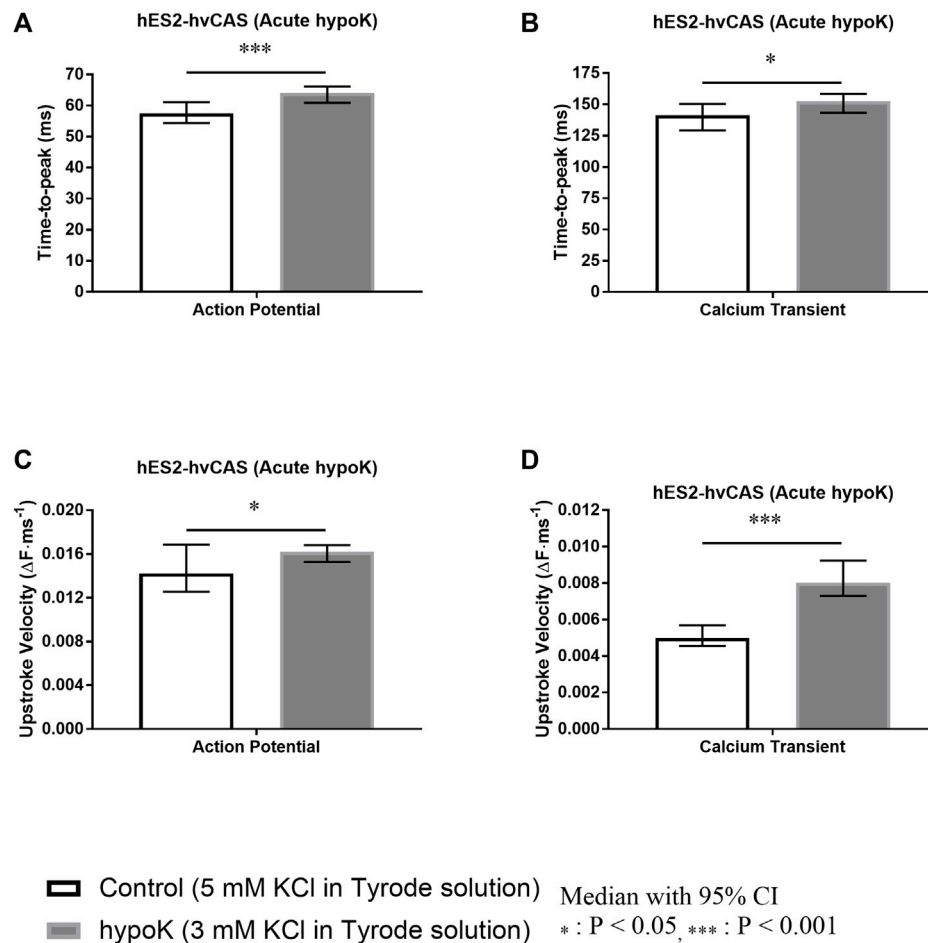


FIGURE 4 | Human embryonic stem cell (hES2)-derived human ventricular cardiac anisotropic sheet (hvCAS) paced at 1.5 Hz during acute hypokalaemic (hypoK) experiment where the treatment was for 15 min before data acquisition. Number of samples is represented by n where the first number refers to control (5 mM KCl in Tyrode solution) samples and the second number refers to hypoK (3 mM KCl in Tyrode solution) samples. **(A)** Action Potential (AP) Time-to-peak. ($n = 14, 17$). **(B)** Calcium Transient (CaT) Time-to-peak. ($n = 12, 11$). **(C)** AP Upstroke Velocity measured by the change in fluorescence intensity per millisecond. ΔF represents the change in fluorescence intensity. ($n = 9, 10$). **(D)** CaT Upstroke Velocity measured by the change in fluorescence intensity per millisecond. ΔF represents the change in fluorescence intensity. ($n = 11, 10$) Time-to-peak of AP and CaT increased in hypokalaemic condition (asterisks, $p < 0.001$, $p < 0.05$ respectively). Similarly Upstroke Velocity of AP and CaT increased in hypokalaemic condition (asterisks, $p < 0.05$, $p < 0.001$ respectively).

statistically significant. Data analysis was performed for 1.5 Hz electrical stimulation recordings.

RESULTS

Acute Hypokalaemia Leads to Higher AP and CaT Amplitudes, and Longer APD and CaTD

Arrhythmogenicity and its relationship to AP and CaT activation and recovery properties were examined under normokalaemic and hypokalaemic conditions in hvCAS using optical mapping. During regular steady-state pacing, reproducible AP waveforms could be observed under normokalaemic conditions. By contrast, acute hypokalaemia led to the generation of premature APs that likely reflected

triggered activity. Moreover, an S1S2 protocol, which delivered a successively premature S2 extra-stimulus after a regular train of S1 stimuli, was used to provoke arrhythmogenesis. With this protocol, an increase in the proportion of preparations exhibiting spiral wave re-entry was observed during hypokalaemia (hypokalaemia = 7/14, 50%; normokalaemia = 0/14, 0%; asterisks, $p = 0.003$ (Fisher's exact test); **Figure 2**).

Acute hypokalaemia significantly increased AP amplitude ($p < 0.001$), and CaT amplitude ($p < 0.001$; **Figure 3A**). It also produced AP prolongation, as reflected by increases in APD₃₀ ($p < 0.001$), APD₅₀ ($p < 0.001$), APD₇₀ ($p < 0.001$) and APD₉₀ ($p < 0.001$; **Figure 3B**). CaTs were similarly prolonged during hypokalaemia, as reflected by higher CaTD. Thus, hypokalaemia increased CaTD₃₀ ($p = 0.001$), CaTD₅₀ ($p = 0.003$), CaTD₇₀ ($p = 0.009$) and CaTD₉₀ ($p = 0.002$; **Figure 3C**).

TABLE 1 | hES2-hvCAS Action Potential (AP) Parameters in acute hypokalaemia. Data analysis was performed for 1.5 Hz electrical stimulation recordings.

Parameters		Sample	Median	Lower 95% CI	Upper 95% CI	N	p Value		
(a) Amplitude (ΔF represents the change in fluorescence intensity)		NormoK	0.888	0.81	0.958	12	***		
		hypoK	1.19	1.12	1.32	14	< 0.001		
(b) AP Duration (APD _x) (Time duration in millisecond from the peak fluorescence intensity to the corresponding fluorescence decay intervals)	x								
	30%	NormoK	29.1	27.6	31	13	***		
		hypoK	34.9	33	37	15	< 0.001		
	50%	NormoK	43.4	41.4	45.8	13	***		
		hypoK	51.5	47.4	55.3	15	< 0.001		
	70%	NormoK	59	54.4	63.7	13	***		
		hypoK	72.6	68	77.2	15	< 0.001		
	90%	NormoK	83.5	78.5	87.5	13	***		
	hypoK	97.4	92.2	104	15	< 0.001			
(c) Time-to-peak/Activation Latency (Time duration in millisecond from the start of the rise of the fluorescence intensity to the peak fluorescence intensity)		NormoK	57.4	54.4	61	14	***		
		hypoK	64.1	60.9	66.1	17	< 0.001		
(d) Upstroke Velocity (ΔF /millisecond; ΔF represents the change in fluorescence intensity)		NormoK	0.0142	0.0126	0.0169	9	*		
		hypoK	0.0162	0.0153	0.0168	10	= 0.023		
(e) Conduction Velocity (CV) (cm·s ^{−1} ; AP propagation between two points)	Longitudinal (L) CV	NormoK	7.81	7.01	10.6	9	*		
		hypoK	7.18	4.99	7.62	7	= 0.036		
	Transverse (T) CV	NormoK	5.72	4.83	7.02	12	*		
		hypoK	4.69	3.93	6.34	11	= 0.030		
(f) Anisotropic Ratio (Ratio of LCV to TCV)		NormoK	1.47	1.14	1.77	9	ns		
		hypoK	1.38	1.11	2.09	8	0.444		
(g) Effective Refractory Period (millisecond)		NormoK	242	200	275	12	***		
		hypoK	165	156	220	13	< 0.001		
(h) hvCAS statistics		Sample	Spontaneous	Maximum Capture Frequency (Hz)					Spiral Induction
				N/A (capture at higher frequency)	1	1.5	2	2.5	3
		NormoK	0% (0/14)	1/14 (NC: 0.5, 1), (C: 1.5–3)	0/14	0/14	0/14	1/14	12/14
		hypoK	21% (3/14)	5/14 (NC: 0.5–1.5 = 1), (NC: 0.5 = 3), (NC: 0.5–1 = 1)	0/14	0/14	0/14	3/14	6/14

NormoK, normokalaemia; hypoK, hypokalaemia; NC, non-capture; C, capture.

TABLE 2 | hES2-hvCAS Calcium Transient (CaT) Parameters in acute hypokalaemia. Data analysis was performed for 1.5 Hz electrical stimulation recordings.

Parameters		Sample	Median	Lower 95% CI	Upper 95% CI	N	p Value
(a) Amplitude (ΔF represents the change in fluorescence intensity)		NormoK	0.861	0.707	1.16	12	***
		hypoK	1.37	1.28	1.5	13	< 0.001
(b) CaT Duration (CaT _{Dx}) (Time duration in millisecond from the peak fluorescence intensity to the corresponding fluorescence decay intervals)	x						
	30%	NormoK	141	129	153	11	**
		hypoK	161	150	170	10	0.001
	50%	NormoK	223	213	235	11	**
		hypoK	242	232	252	10	0.003
	70%	NormoK	292	285	311	11	*
		hypoK	318	306	330	10	= 0.009
	90%	NormoK	379	358	392	11	**
		hypoK	403	393	416	10	0.002
(c) Time-to-peak/Activation Latency (Time duration in millisecond from the start of the rise of the fluorescence intensity to the peak fluorescence intensity)		NormoK	141	129	150	12	*
		hypoK	153	143	158	11	= 0.045
(d) Upstroke Velocity (ΔF /millisecond; ΔF represents the change in fluorescence intensity)		NormoK	0.00498	0.00456	0.00569	11	***
		hypoK	0.00801	0.0073	0.00923	10	< 0.001

NormoK, normokalaemia; hypoK, hypokalaemia.

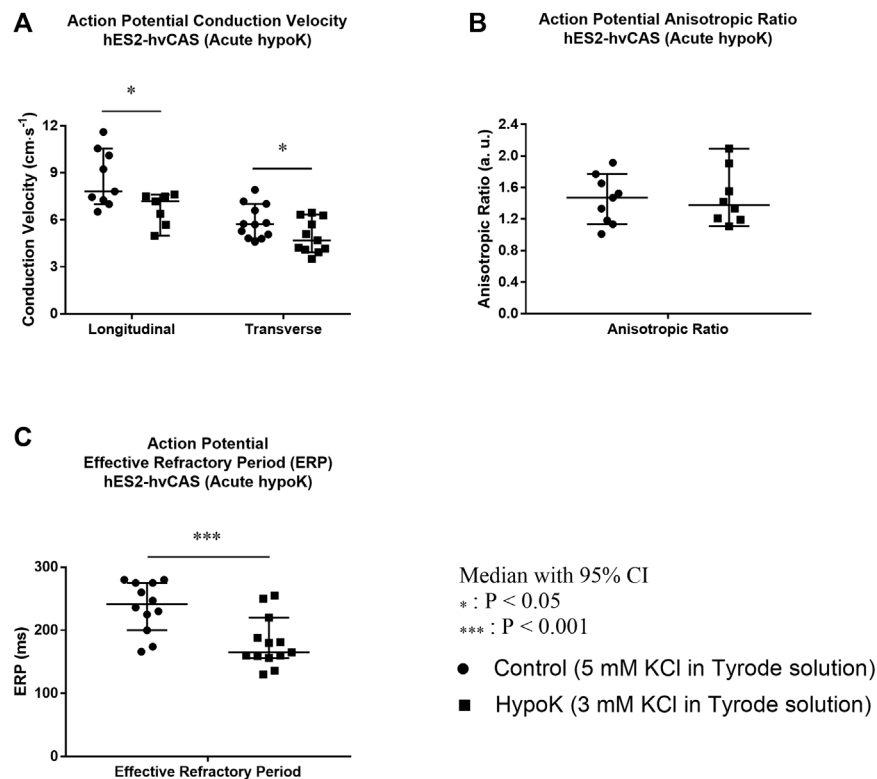


FIGURE 5 | Human embryonic stem cell (hES2)-derived human ventricular cardiac anisotropic sheet (hvCAS) paced at 1.5 Hz during acute hypokalaemic (hypoK) experiment where the treatment was for 15 min before data acquisition. Number of samples is represented by n where the first number refers to control (5 mM KCl in Tyrode solution) samples and the second number refers to hypoK (3 mM KCl in Tyrode solution) samples. **(A)** Action Potential (AP) Longitudinal Conduction Velocity (LCV) ($n = 9, 7$) and Transverse Conduction Velocity (TCV) ($n = 12, 11$). The velocity was measured by calculating the speed of AP propagation from one point to the other. **(B)** AP Anisotropic Ratio (AR) which is the ratio of LCV to TCV. ($n = 9, 8$). **(C)** AP Effective Refractory Period (ERP). ($n = 12, 13$) Conduction Velocity decreased in hypokalaemic condition (asterisk, $p < 0.05$ for both LCV and TCV). ERP of hvCAS paced at 1.5 Hz (8 trains) followed by an extra premature stimulus which was gradually shortened to find ERP was decreased in hypokalaemic condition (asterisk, $p < 0.001$). AR is between 1.4 and 1.8, resembling more closer to the native human ventricle (AR = 3) in normokalaemia as well as in hypokalaemia.

Acute Hypokalaemia Prolongs AP and CaT Time-To-Peak, Increases AP and CaT Upstroke Velocities

The AP Time-to-peak was significantly increased during hypokalaemia ($p < 0.001$; **Figure 4A**). CaT Time-to-peak was also significantly altered in hypokalaemia ($p = 0.045$; **Figure 4B**).

Hypokalaemia increased AP upstroke velocity ($p = 0.023$; **Figure 4C**) and CaT upstroke velocity ($p < 0.001$; **Figure 4D**).

The AP and CaT values are summarized in **Tables 1, 2** respectively.

Hypokalaemia Decreases Both Longitudinal and Transverse CVs of AP Without Altering the AR and Decreases ERPs

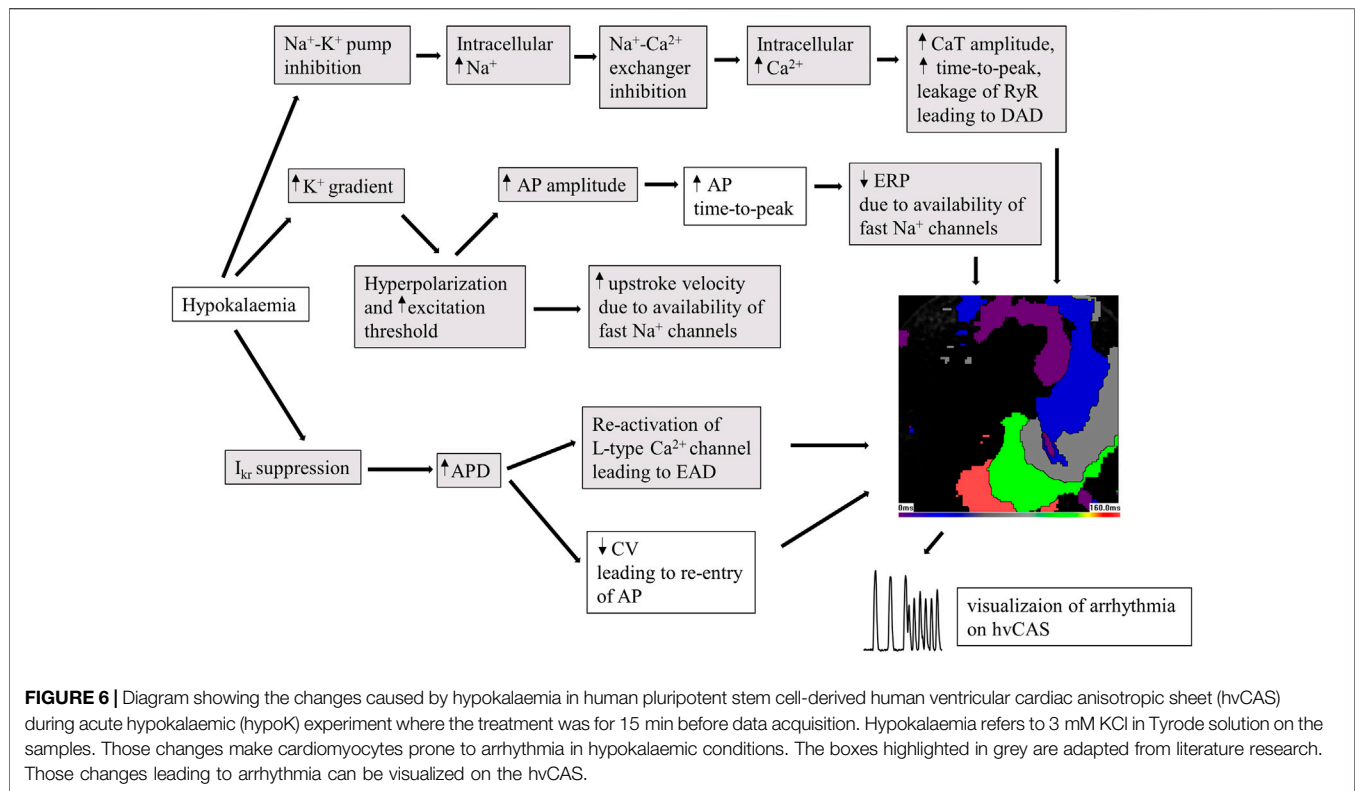
There was a significant lowering of longitudinal CV of AP in hypokalaemia versus normokalaemia from $7.81 (7.01, 10.6)$ ($n = 9$) to $7.18 (4.99, 7.62)$ $\text{cm}\cdot\text{s}^{-1}$ (centimetre per second, $n = 7$; asterisk, $p = 0.036$; **Figure 5A**) and transverse CV of AP from $5.72 (4.83, 7.02)$ ($n = 12$) to $4.69 (3.93, 6.34)$ $\text{cm}\cdot\text{s}^{-1}$ ($n = 11$; asterisk,

$p = 0.030$). However, the AR was not altered (normokalaemia: $1.47 (1.14, 1.77)$ ($n = 9$) vs. hypokalaemia: $1.38 (1.11, 2.09)$ a. u. ($n = 8$; ns, $p = 0.444$; **Figure 5B**). By contrast, ERPs were shortened from $242 (200, 275)$ ($n = 12$) to $165 (156, 220)$ ms significantly ($n = 13$; asterisks, $p < 0.001$; **Figure 5C**). Similar results were observed in chronic hypokalaemia for hES2-hvCAS as well as in hvCAS derived from N-iPSC (healthy control iPSC cells) in both acute and chronic hypokalaemia except slight deviation. These results are summarized in **Supplementary Tables SI–SVI**.

Together all of the findings associate increased re-entrant arrhythmias observed in the form of spiral waves with depolarization, repolarization, and calcium handling abnormalities in the hypokalaemia model using our stem cell-derived bioengineered platform.

DISCUSSION

The main findings of this study are that hypokalaemia can induce arrhythmogenesis, which is associated with depolarization, repolarization, and calcium handling abnormalities in stem cell-derived bioengineered platforms. These confirm that



hvCAS preparations can be used as a model system for studying human arrhythmic syndromes, providing opportunities for efficient drug screening programs for pro-arrhythmic effects.

Low extracellular K^+ levels lead to a steeper transmembrane voltage gradient and membrane hyperpolarization (Tse et al., 2021). This low level also reduces the repolarization reserve by suppressing the rapid delayed rectifier (I_{Kr}) channel, producing APD prolongation (Osadchii, 2010; Pezhouman et al., 2015). APD prolongation can in turn lead to the re-activation of L-type Ca^{2+} channels, causing EAD phenomena and triggering activity (Killeen et al., 2007). Moreover, it can predispose patients to re-entry through reduced CV, reduced tissue refractoriness, increased transmural dispersion of repolarization, and increased steepness of electrical restitution that can generate electrical alternans (Surawicz, 1985; Sabir et al., 2008; Tse et al., 2016a; Tse et al., 2016b). Reduction in CV is more likely due to APD prolongation. The membrane hyperpolarization also causes an increase in the availability of fast Na^+ channels that leads to an increase in upstroke velocity (Osadchii, 2010). Despite the higher upstroke velocity, time-to-peak is prolonged, which is possibly due to higher amplitude caused by hyperpolarization. In our study, greater AP amplitudes, reduced CV, and longer APDs were observed in hvCAS. Moreover, both longitudinal and transverse conduction velocities were decreased to similar extents, leading to no significant alteration in the AR. This took a mean value between 1.4 and 1.8 in both under normokalaemic and hypokalaemic conditions, which is fairly close to the AR value for the human ventricle of 3 (Camelliti et al., 2011).

The amplitude and time-to-peak of CaTs were higher under hypokalaemic conditions. This can be explained by the suppression

of sodium-calcium (Na^+-Ca^{2+}) exchanger and sodium-potassium pump during hypokalaemia, which leads to the accumulation of Ca^{2+} and Na^+ in the cytoplasm (Pezhouman et al., 2015). Together with increased Ca^{2+} influx owing to L-type Ca^{2+} channel re-activation, this can lead to secondary depolarization events if the activation threshold is reached, an extra premature AP. The suppression of Na^+-Ca^{2+} exchanger leads to the Ca^{2+} overload in the cytoplasm which in turn prolongs the time taken for Ca^{2+} to be removed from the cytosol via the following routes: Na^+-Ca^{2+} exchanger to the extracellular space, sarco/endoplasmic reticulum Ca^{2+} -ATPase, into the sarcoplasmic reticulum.

The different electrophysiological mechanisms at play induced by hypokalaemia are summarized in **Figure 6**.

LIMITATIONS OF THE STUDY

Although the cardiomyocytes (CMs) that we produce through directed cardiac differentiation are mostly ventricular CMs, there are still a small proportion of other subtypes of CMs (Weng et al., 2014). Additionally, stem cell-derived CMs show a relatively immature phenotype as reported in previous studies (Karakikes et al., 2015). By performing optical mapping on the hvCAS, relative values of voltage change and CaT can be obtained from the fluorescence intensity of the fast-responding voltage sensitive and Ca^{2+} dyes respectively. However, the fluorescence signal is not calibrated and absolute resting membrane potential cannot be determined. Furthermore, hvCAS contains a stack of cells at each pixel and the data presented here are from those

multiple cells. This makes the AP upstroke phase appear slow compared to single-cell actual AP measured by patch-clamp or microelectrode array in other studies. Nevertheless, we can see the differences between normokalaemia and hypokalaemia hvCASs, and most importantly, arrhythmia can be visualized as spiral waves by using optical mapping, which is not possible by other techniques.

CONCLUSION

The pro-arrhythmic effects of hypokalaemia are associated with depolarization, repolarization, and calcium handling abnormalities in a human multicellular monolayer model using a stem cell-derived bioengineered platform. This platform can be used as a model system for cardiac arrhythmic risk assessment where arrhythmia can be visualized as spiral waves.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The University of Hong Kong/Hospital Authority

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AUTHOR CONTRIBUTIONS

BG designed, performed the experiments and wrote the manuscript. GT, WK, RL, and WW supervised the experiments and performed critical manuscript editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.681665/full#supplementary-material>

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