EDITOR'S PICK 2021: HIGHLIGHTS IN STEM CELL RESEARCH

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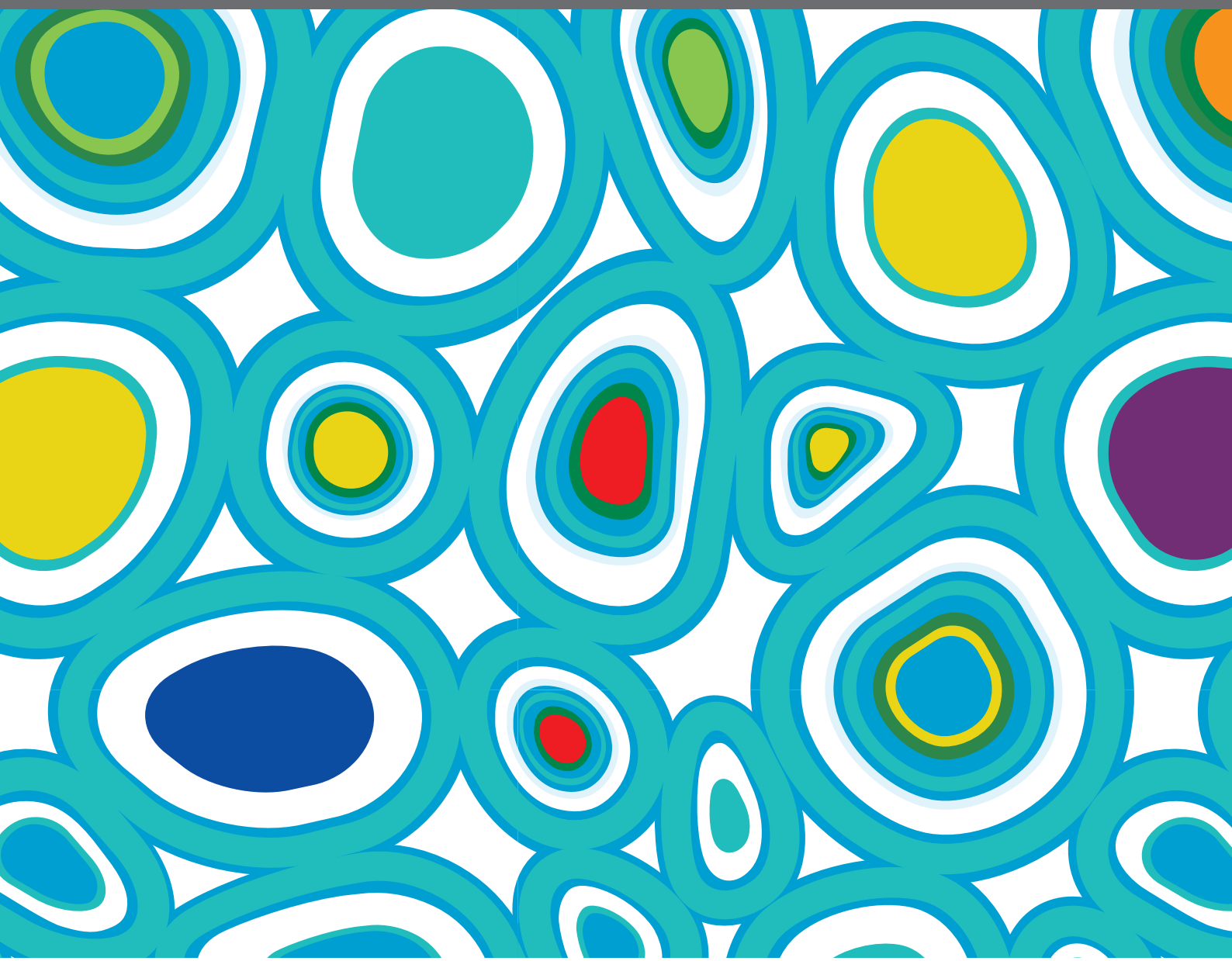

frontiers Research Topics

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EDITOR'S PICK 2021: HIGHLIGHTS IN STEM CELL RESEARCH

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EDITORIAL
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Editorial: Editor's Pick 2021: Highlights in Stem Cell Research

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Editorial on the Research Topic

Editor's Pick 2021: Highlights in Stem Cell Research

Current knowledge of stem cell biology, as well as various cell culture technologies that have been established in recent years, supports the field of developmental biology and regenerative medicine (Ikeya et al., 2021a, b). Stem cells include embryonic stem cells (Martin, 1981; Thomson et al., 1998) with pluripotent differentiation ability retained by fertilized eggs, and induced Pluripotent Stem (iPS) cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006), which have been developed by cell culture and molecular biological technologies. In addition, mesenchymal stem cells (MSCs) derived from bone marrow are known as multipotent adult stem cells (Gao et al., 2021), capable of differentiating into various mesenchymal cells and have contributed to regenerative medicine as have biological microdevices. Tissue-specific stem/progenitor cells that can be isolated from various adult tissues can contribute to the specific tissue types for physiological tissue maintenance and repair after damage (Prentice, 2019). The use of these stem/progenitor cells has been further enhanced by genetic engineering and embryological strategies (Wang et al., 2021). For example, the use of signal transduction mimicking the developmental stages of tissues to induce specific cell differentiation, the construction of target tissues by organoid culture, a three-dimensional culture method, and the use of exosomes, which play an important role in cell-cell communication, have recently attracted attention. Stem cell biology and regenerative medicine can be systematized by combining these fundamental technologies related to developmental biology and stem cells.

This Editors' pick research topic aims to highlight a few of the most noteworthy manuscripts published in the Stem Cell section of Frontier in Cell and Development al Biology over 2020 and 2021. The 12 selected manuscripts, highlighted in this topic, were not part of a research topic but have caught our attention and that of the readers by their scope, novelty and quality.

Four primary research manuscripts were chosen to be part of this Editors' pick. In the first one, Tagliaferri et al. uncovered the molecular mechanism by which retinoic acid induces the transition of embryonic stem cell to a 2-cell like state, implicating DUX and DUXbl1 in this process (Tagliaferri et al.). The second manuscript, by Gao et al. describes the use of patient specific retinal organoids to recapitulate feature of retinitis pigmentosa, establishing the first *in vitro* model system to study the mechanism implicated in the development of this disease (Gao et al.). In the third highlighted manuscript, Chen et al. investigated the role of the Mediator subunit Med23 in adult mouse hippocampal neurogenesis through loss-of-function approach, demonstrating a critical role for Med23 in the regulation of adult brain neurogenesis and function (Chen et al.). In the last primary research manuscript selected for this topic, Chen et al. explored the role of the thyroid hormone T3 on BMP9-induced osteogenesis, demonstrated that both work together to promote osteogenic differentiation via the activation of the AMPK/p38 signalling pathway (Chen et al.).

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In addition to these four primary research articles, eight reviews were chosen to complete this collection of noteworthy and recently published manuscripts in the Stem Cell section. These reviews underscore several central themes in this field of research. The therapeutic potential of MSCs was reviewed by Shammaa et al., while Xiong et al. assessed the potential of exosomes from adipose-derived stem cells in tissue regeneration (Xiong et al.). In a related topic, Rees et al. described the latest research on the regenerative properties of intestinal stem cell upon injury (Rees et al.). Two reviews discussed the role and importance of signalling pathways in stem cell maintenance and differentiation. A manuscript by Yang and Jiang describe the role of the signal transducer SMAD2/3 in the control of human embryonic stem cell pluripotency and differentiation (Yang and Jiang), while Rivetti et al. assessed our current knowledge on FGF signalling during mammary gland development, homeostasis and cancer (Rivetti et al.).

On different themes, the physiology of gastric stem cells and potential related-pathologies was reviewed by Xiao and Zhou, while Dumortier et al. summarized the current literature on the role of CFTR mutations on the commitment of induced pluripotent stem cells to bone cells (Dumortier et al.). In the last review selected as part of this topic, Nakamura et al. discussed the role of hypoxia and epigenetics in the regulation of cellular reprogramming (Nakamura et al.).

Selecting manuscripts to include in this Editor's Pick was an arduous task. This series of twelve articles, that made it to the short list, underscores the remarkable quality of manuscripts recently published in the Stem Cell Research section of Frontiers in Cell and Developmental Biology. This Editor's Pick also highlights the breadth of stem cell research and regenerative medicine and reveals a field of research moving forward at an incredible pace.

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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ORIGINAL RESEARCH
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Retinoic Acid Induces Embryonic Stem Cells (ESCs) Transition to 2 Cell-Like State Through a Coordinated Expression of *Dux* and *Duxbl1*

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Embryonic stem cells (ESCs) are derived from inner cell mass (ICM) of the blastocyst. In serum/LIF culture condition, they show variable expression of pluripotency genes that mark cell fluctuation between pluripotency and differentiation metastate. The ESCs subpopulation marked by zygotic genome activation gene (ZGA) signature, including *Zscan4*, retains a wider differentiation potency than epiblast-derived ESCs. We have recently shown that retinoic acid (RA) significantly enhances Zscan4 cell population. However, it remains unexplored how RA initiates the ESCs to 2-cell like reprogramming. Here we found that RA is decisive for ESCs to 2C-like cell transition, and reconstructed the gene network surrounding *Zscan4*. We revealed that RA regulates 2C-like population co-activating *Dux* and *Duxbl1*. We provided novel evidence that RA dependent ESCs to 2C-like cell transition is regulated by *Dux*, and antagonized by *Duxbl1*. Our suggested mechanism could shed light on the role of RA on ESC reprogramming.

Keywords: retinoic acid, metastate, ESCs, 2-cell like, pluripotency

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INTRODUCTION

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the blastocyst. When cultured in appropriate conditions, they retain their pluripotency, with the ability to differentiate into nearly all embryo cell types (Toyooka et al., 2008; Loh and Lim, 2011). *In vivo* epiblast pluripotency is a transitory state that is maintained *in vitro* through multiple metastable states that fluctuate between self-renewal and differentiation balance, and display a heterogeneous differentiation potential (Ohtsuka et al., 2012). One of these populations marked by *Zscan4* expression retains wider potency capacity, and it is marked by similar expression of 2-cell stage embryo signature, in particular the activation of MERV-L (murine endogenous retrovirus-like) endogenous retrovirus and the expression of *Zscan4* associated gene family among them Prame,

Thoc, and Tcstv (Falco et al., 2007; Zalzman et al., 2010; Macfarlan et al., 2012; Cerulo et al., 2014; Eckersley-Maslin et al., 2016). Also, *Zscan4* expressing cells show reprogramming potential and epigenetic hallmark of the early embryonic preimplantation stage (Macfarlan et al., 2012). Under standard ESCs culture conditions, about 3-5% of the whole ESCs population expresses *Zscan4* (Zscan4$^+$) which has been shown to mark the ESCs to the so-called "2C-like" transition intermediates (Rodriguez-Terrones et al., 2018).

RA, especially all-trans retinoic acid (ATRA) is the derived form of vitamin A (VitA), and it is involved in a variety of biological functions including embryogenesis, cell differentiation, and apoptosis (Kanungo, 2017). Interestingly, RA enhances Zscan4$^+$ up to about 20% of the whole ESCs population (Sharova et al., 2016; Tagliaferri et al., 2016). These effects can be observed in ESCs cultured in RA for long-term, whereby Zscan4$^+$ cells emerge within undifferentiated canonical colonies. Recently, we have shown that *Zscan4* RA-dependent activation led to the transition of ESCs to 2C-like state supported by 2-cell stage expression signature, DNA hypo-methylation and global increase of H3K27 acetylation levels (Napolitano et al., 2019).

Although it has been shown that the activation of 2C-like reprogramming is directly regulated through the transcription factor *Dux* and by its positive regulators, *Dppa2* and *Dppa4* (Eckersley-Maslin et al., 2018; De Iaco et al., 2019) the pivotal molecular driver regulation needs further investigation.

RA dependent induction of 2C-like state represents a suitable *in vitro* system to characterize the molecular mechanism orchestrating embryonic-like genome activation and the maintenance of pluripotency.

Here, we demonstrated that RA is necessary for ESCs to 2C-like transition, and reconstructed the gene network underlying 2C-like cell activation by employing reverse engineering *in silico* analysis. We found that RA induction of 2C-like is accompanied by the co-expression of two members of Dux family transcription factors, *Dux* and *Duxbl1*. We investigated the role of these two proteins revealing that such transition requires *Dux* and that Duxbl1 contrasts it. Supporting this, overexpression, chromatin immunoprecipitation (ChIP) analyses and transcription activation assays, revealed that *Dux* and *Duxbl1* coordinate regulation of 2C-like cells through a competitive promoter binding activity.

MATERIALS AND METHODS

Cell Culture

E14Tg2a.4 ES cells, derived from strain 129P2/OlaHsd were purchased from ATCC company and were cultured for two passages on gelatin-coated feeder-free plates and subsequently maintained in gelatin-coated six-well plates in complete ES medium: GMEM (Glasgow Minimum Essential Medium, Gibco), 15% FBS (HyClone), 1,000 U/ml leukemia inhibitory factor (LIF) (Millipore), 1.0 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 2.0 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol, and 500 U ml-1 penicillin/streptomycin (Invitrogen). ESCs were incubated at 37°C in 5% CO_2; medium was changed daily, and cells were split every 2 to 3 days routinely. ESCs were plated in N2B27 (VitA) or N2B27 without retinoids (VitA^{minus}) or in the medium supplemented with 1.5 μM all-trans RA(VitA^{Plus}), 50 μM citral (all from Sigma-Aldrich) and 5.0 μM BMS493 (Tocris Bioscience). The culture of ESCs with RA was also performed in the presence of 2 μg/ml protein synthesis inhibitor Cycloheximide (Sigma-Aldrich). ESCs were cultured for two passages on gelatin-coated feeder-free in 2i medium, a serum-free N2B27 medium supplemented with MEK inhibitor PD0325901 (0.5 μM) and GSK3 inhibitor CHIR99021 (3 μM) (both from Stemgent), and 1,000 U/ml LIF (Millipore). All experiments were performed at least three times. Dux-ko ES cells were a kind gift of dr. De Iaco and were cultured in 2i medium as described above. HEK293T cells were purchased from ATCC company and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Sigma-Aldrich) and 1% Pen/Strep (Sigma-Aldrich).

Plasmids

To generate pZscan4c-pcDNA3.1/CT-GFP-TOPO constructs, a putative *Zscan4c* promoter (pZscan4) corresponding to −2,400, −480, and −288 bp, respectively, from the *Zscan4c* Transcription Start Site (TSS) was amplified from BAC RP23-63I1. The *pZscan4* was amplified using primers: 5′-TTCTTAATC TGTGGTCGTCCA-3′; 5′-TGTGGTGACAATGGTGTGAA-3′; 5′-GCCAATCTTGGAATTCCTCTTC-3′; 5′-TTGCTTGTA TTTGATTCCCC-3′. Dux-HA was a kindle gift of De Iaco. Duxbl1-V5 was obtained cloning Duxbl1 coding sequence into pcDNA3-V5 His (Invitrogen). Duxbl1_CTD-Flag was obtained using the In-Fusion cloning strategy (Takara).

Site-Directed Mutagenesis

To generate mutant plasmid for *Duxbl1* binding site, QuikChange Lightning Site-Directed Mutagenesis Kit was used according to the manufactures instruction. Briefly, ML_480 pZscan4-pcDNA3.1/CT-GFP-TOPO with WT-Duxbl1 binding motif was amplified using two synthetic oligonucleotide primers, carrying the mutation for Duxbl1 motif (fw 5′-GAAAGACAT TTTTTCCTGCTGAGCCGGTCACATAAGGAATCCTAA CTCAGCTCTAGTTTTGCATCTC-3′; RV 5′- GAGATGCAA AACTAGAGCTGAGTTAGGATTCCTTATGTGACCGGC TCAGCAGGAAAAAATGTCTTTC-3′). Sequence analysis confirmed the presence of the mutation.

Flow Cytometry and Sorting of pZscan4-GFP ESCs

pZscan4-GFP cells were fed at least 2 h before harvesting by Trypsin (Gibco) and resuspended in complete ES medium containing 25 mM HEPES buffer. The cells were then FAC-sorted according to the fluorescent intensity of GFP into complete ES medium containing HEPES. Data are presented as mean % ESZscan4_GFP cells ± SEM of three independent experiments with statistical analysis performed using Student's *t*-test.

Generation of E14tg2a.4pcDNA3_pZScan4_LNGFR Stable Cell Line

For the construction of the plasmid pcDNA3_pZscan4_LNGFR, Zscan4 promoter was amplified by PCR from pZScan4-GFP vector and inserted into KpnI/EcoRV sites of pcDNA3 vector (Invitrogen). Subsequently, LNGFR fragment (874 bp) was amplified by PCR from pPRIME-CMV-LNGFR and inserted into EcoRV site of pcDNA3_pZScan4 vector. The construct was verified by sequencing. To generate the stably transfected ES cell line pcDNA3_pZScan4_LNGFR was linearized with KpnI and transfected into wild-type E14tg2a.4. 48h after transfection the cells were split and positive clones selected for Neo resistance. After 1 week of G418 (Gibco) treatment, NeoR clones were picked and propagated.

E14tg2a.4pcDNA3_pZScan4_LNGFR Culture and Magnetic Separation

The stably transfected ESCs were cultured for 3 days on gelatin-coated dishes in ES complete medium. The cells were then trypsinized and plated on gelatin-coated dishes in N2B27-VitA medium: KnockOut DMEM high glucose (Gibco) supplemented with L-Glutamine 2 mM (Gibco), Penicillin/Streptomycin 100 U-μg/ml (Gibco), B27-VitA Supplement 1x (Gibco), N2 Supplement 1x (Gibco), 2(β)Mercaptoethanol 0.1 mM (Gibco), LIF 1,000 U/ml (Millipore), G418 137,5 μg/ml, with or without 1.5 μM RA for 72 h. For magnetic labeling, single-cell suspensions were centrifuged, resuspended in PBS supplemented with 5 mM EDTA and 0.5% BSA and incubated with MACSelect (TM) LNGFR MicroBeads for 15 min on ice. Magnetically labeled cells were isolated over the AutoMACS Pro Separator (MiltenyiBiotec) with "posseld2" program according to the manufacturer's protocol. For purity assessment, aliquots of original cell population (magnetically labeled cells before separation), eluted positive (enriched target cells) and negative (untargeted cells collected in the flow-through fraction) cell populations were fluorescently stained with MACSelect Control FITC Antibody (MiltenyiBiotec) that specifically stains MACSelectMicroBead-labeled cells and analyzed by Navios Flow Cytometer (Beckman Coulter).

RNA Extraction and qPCR Analysis

For qPCR analysis of sorted cells, total RNAs were collected immediately after sorting by TRIzol (Invitrogen) according to the manufacture's instructions. One microgram of total RNA was reverse-transcribed by Quantitec reverse transcription kit (Qiagen) according to the manufacturer's instructions. qPCR analyses were performed using 10 ng cDNA per well in duplicate with the SYBR green master mix (Applied Biosystems) as previously described (Falco et al., 2006; Vivo et al., 2017). Reactions were run on QuantStudio 7 Flex system and 7900 realtime Pcr system (Applied Biosystems). Fold induction was calculated using ΔΔCt method while the normalization was performed using *Gapdh or* the mean of three housekeeping genes: *Gapdh, Actin and 18S* (16). The gene-specific primers are available in **Table S4**.

RARE Motif Analysis

The promoter sequences of Zscan4 were retrieved from assembly mm10 of the mouse genome. The known consensus sequences of RARE motifs (Bastien and Rochette-Egly, 2004) were converted into position weight matrixes using upac2meme conversion tool provided by MEME suite. Positive controls known in the literature as primary gene responsive to RA were adopted to estimate a suitable q-value threshold (Bastien and Rochette-Egly, 2004; Cunningham and Duester, 2015).

Global Regulatory Network Reconstruction

We selected a collection of 754 gene expression profiles in the context of mouse ESC where 181 ESC related transcription factors were either up-regulated or repressed (GEO Accession Numbers: GSE31374, GSE14559, GSE26520). The expression profiles were normalized with a quantile normalization approach, and each probe was associated with the corresponding gene symbol based on annotation information included of the chip NIA Mouse 44K Microarray v3.0 (Whole Genome 60-mer Oligo). We averaged all significant redundant probes for the same gene. Both control and not annotated probes were removed from the analysis obtaining an expression matrix of 24,988 genes in 754 conditions. The regulatory network was learned with a three steps procedure similar to ARACNE (Basso et al., 2005) consisting of: (i) computation of mutual information between 1,852 Transcription Factors (TFs) obtained both from a manually curated collection based on Gene Ontology and from AnimalTFDB database (Zhang et al., 2012) and 24,988 gene expression profiles in 754 conditions to determine statistical dependence between transcription factors and target genes (Basso et al., 2005); (ii) Data processing inequality to filter out indirect relationships (Sales and Romualdi, 2011); and (iii) Permutation test to keep only statistically significant relationships. In particular, for each link, we obtained the null distribution by recomputing 1,000 times the mutual information of the link with a randomly permuted expression profile of one of the two genes. We retained only links with and FDR ≤ 0.01.

Master Regulator Analysis (MRA)

MRA is widely adopted to identify Master Regulator (MR) transcription factors acting in a particular context of interest (Lefebvre et al., 2010). The enrichment, evaluated using a statistical test such as Fisher's exact test or GSEA (Subramanian et al., 2005), has the objective to place the signature genes within a regulatory context in order to identify the master regulators responsible for coordinating their activity, thus highlighting the regulatory apparatus driving the functional phenotype of interest.

We computed the enrichment of each TF with GSEA using the *fgseaMultilevel* function of fgsea R package (Sergushichev, 2016). We computed the statistical significance of the enrichment by performing 10,000 permutations, followed by multiple hypothesis testing with Benjamini Hochberg adjustment, obtaining a set of 266 candidates (FDR ≤ 0.01) (**Table S2**).

Zscan4 Promoter Analysis

To verify if a candidate MR directly regulate the expression of Zscan4, promoter analysis was performed using the Biostrings

(Pagès et al., 2019) package function matchPWM. The statistical significance of each predicted PWM match was performed using the TFMPvalue package TFMsc2pv function (Touzet and Varré, 2007). The Zscan4 promoter sequence was retrieved from assembly mm10 of the mouse genome and scanned against a list of available PWM matrices related to MRs and obtained from MEME (JASPAR 2016 CORE and Vertebrates, CIS-BP and Mouse Uniprobe PWM collections). We obtained a list of putative motifs that bind the promoter region of Zscan4 (normalized binding score ≥ 0.7 and p-value ≤ 0.0001, **Table S3**).

Chromatin Immunoprecipitation Assay

Approximately 1.5×10^7 ESCs were grown at 70-80% confluency. The cells were cross-linked by adding fresh 0.75% formaldehyde solution to the ES media for 10 min at room temperature, and they were treated with 125 mM glycine for 5 min. Cells were lysed and sonicated to solubilize chromatin and shear the cross-linked DNA. Sonications were performed at power 10 for 6 × 30 second pulses (30 s pause between pulses) at 4°C. Fifty microliters of each sonicated sample was removed and used to quantify the DNA concentration and as a control in the PCR. Fifty micrograms of DNA were incubated overnight on a rotating platform at 4°C with 20 μl of Protein A/G beads and with 10 μg of specific antibody. Immunoprecipitated chromatin was eluted, treated with 20 mg/ml Proteinase-K and was purified by phenol:chloroform:isoamyl alcohol extraction. DNA levels are quantitatively measured by qPCR. The primers used for qPCR to amplify the *Duxbl1* were 5′-TGGAATTCCTCTTCAGTGTGG-3′ and 5′-ATTCCCCCTTTTGGCATTAT-3′ resulting in a product size of 217 bp, and primers used for the negative control were 5′-ACCAACTCCAGCTAAGGGGA-3′ and 5′-GGCAGAGGTGTGTGCATACT-3′. The antibodies used for chromatin immunoprecipitation are: anti-FLAG (Sigma Aldrich), anti H3 acetyl (Upstate).

Western Blot Analysis

Total protein was extracted with cell extraction buffer using the following formulation: 100 mMTris pH 7.4, 2 mM Na3VO4, 100 mM NaCl, 1% NP40, 1 mM EDTA, 1 mMNaF, 0.5% deoxycholate, 20 mM Na4P2O7, 1 mM PMSF, and 1X Protease Inhibitor Cocktail (Sigma) as previously described (Di Martino et al., 2016; Fontana et al., 2018). Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacturer's instructions. Twenty micrograms of protein lysate were separated on SDS-PAGE and transferred onto a nitrocellulose membrane. The following primary antibodies were used: anti-FLAG (1:1,000, Sigma Aldrich), anti-GAPDH (1:1,000, Genetex) anti Zscan4 (1:5,000, Millipore) anti-Actin (1:10,000, Sigma Aldrich) (**Figure S1**). The membranes were incubated with antibodies to specific proteins followed by incubation with HRP-conjugated anti-rabbit IgG or anti-mouse IgG (1:2,500; Santa Cruz Biotechnology).

Luciferase Assay

The 1,330 bp*Zscan4c* promoter was inserted into a pGL3 vector (Clontech). An expression plasmid (pCDNA3.1/FLAG) containing the full length of mouse *Duxbl1* sequence was constructed. Dux-HA was a kind gift of dr. De Iaco. To assess *Zscan4* activation state, HEK293T cells were transfected with Dux-HA alone and with increasing concentration of Duxbl1-FLAG together with pGL3-Basic vector (Clontech) in 60 mm plates. The RSV-β-galactosidase plasmid was added to transfection mixtures to normalize the luciferase values for the efficiency of transfection. Twenty-four hours after transfection, luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI, USA).

RESULTS

Retinoic Acid (RA) Signaling Is Required for Zscan4 Metastate Activation

We have previously shown that retinoids enhance induction of the so-called 2C-like cells marked by 2-cell embryo stage signature, in particular by *Zscan4* (**Figure S1**, Tagliaferri et al., 2016). To assess how retinoids regulate the transition of ESCs to 2C-like cell population, we grew ESCs in culture medium with or without vitamin A and measured *Zscan4* expression level and the percentage of Zscan4$^+$ cells. ESCs cultured for 72 h in retinoids-free N2B27 medium (hereafter VitA^{minus}) showed a decrease of about 80% of *Zscan4* expression compared to N2B27 (which contains traces of retinoids, hereafter VitA) as measured by quantitative PCR (qPCR) (**Figure 1A**, left). Coherently, cytofluorimetric analyses on ESCs transgenic line in which the expression of *Zscan4* can be reported by GFP (ESC^{pZscan4_Em}) (Tagliaferri et al., 2016) showed that the percentage of Zscan4$^+$ cells was reduced from about 4% to about 1% from VitA to VitA$^{\text{minus}}$ cell culture media (**Figure 1A**, right). Concurrently with *Zscan4* downregulation, in VitA^{minus} condition, also *Zscan4* associated genes, including *Eif1a*, *Gm12794*, *Gm4340,* and *Tcstv1* (**Figure S2**), were significantly repressed.

We next cultured ESCs with or without citral, an inhibitor of aldehyde dehydrogenase that blocks the conversion of retinol to RA. Moreover, we treated ESCs with the RA-receptor (RAR) inhibitor, the BMS493 molecule. In the presence of either citral or BMS493, *Zscan4* expression was significantly diminished (**Figure 1B**, left) as well as the fraction of Zscan4$^+$ (**Figure 1B**, right), compared to not treated sample (NT). Collectively our results indicate that both RA biosynthesis and functional RAR signaling enhance the transition of ESCs to Zscan4$^+$ cell population.

RA signaling acts through the regulation of primary and secondary-response genes. Primary genes are induced within about few hours upon RA stimulus and do not rely on *de novo* protein synthesis, while secondary genes are transcribed at later times only once their regulators have been synthesized (Balmer and Blomhoff, 2002; Tullai et al., 2007). To investigate the mechanism of action of RA we indeed performed time-course experiments in which ESCs were treated with RA for 4, 9, 14, 36, and 48 h. The qPCR analyses showed a significant increase of *Zscan4* levels starting at 36 h, consistently with a secondary response (**Figure 1C**, left). Conversely, expression levels of a well-known RA primary response gene, *Stra8*, were significantly higher than the control levels (about 40-folds) as early as 4 h

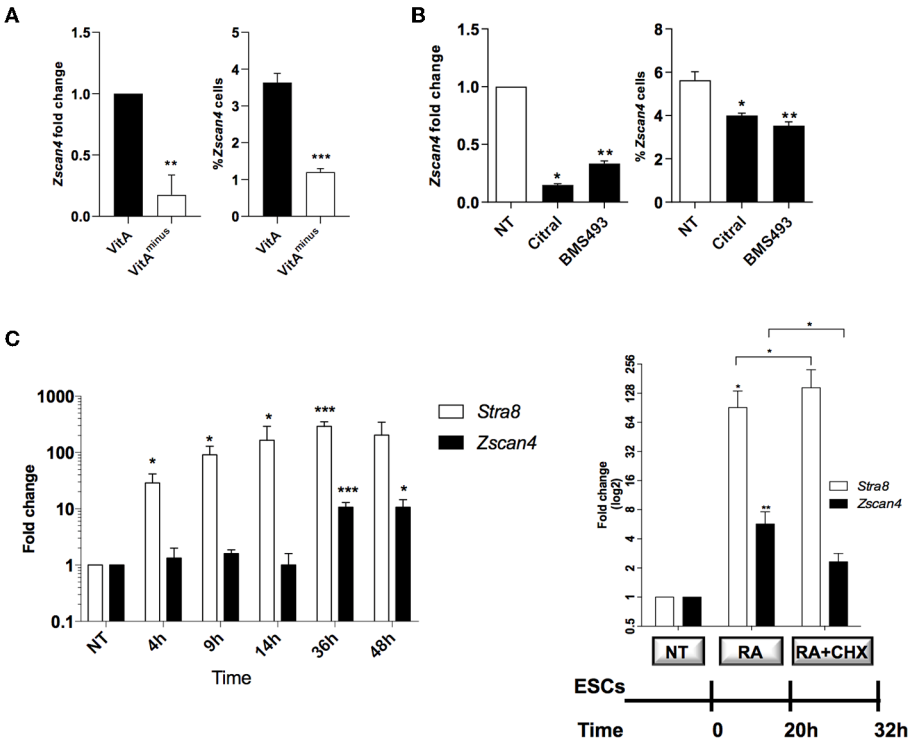

FIGURE 1 | Role of retinoids on Zscan4 cell population induction. **(A)** ESCs, cultured for 72 h in VitA and VitA^{minus} condition, were analyzed by qPCR and cytofluorimetry. The *Zscan4* expression levels were normalized to *Gapdh* expression and fold induction compared to VitA (left); the percentage of Zscan4^{+} cells was evaluated by flow cytometry analyses (right). **(B)** *Zscan4* expression levels were assessed in ESCs cultured for 72 h with Citral or with BMS493 by qPCR, normalized to *Gapdh* expression and compared to NT (left); percentage of Zscan4^{+} cells was evaluated as in **(A)** (right). **(C)** ESCs, cultured in the presence of RA for 4, 9, 14, 36, and 48 h, were analyzed for *Stra8* and *Zscan4* expression (left). ESCs were grown in the presence of 1.5 μM RA for 32 h and in the absence/presence of 2 μg/ml cycloheximide (CHX) for 12 h (right). *Stra8* and *Zscan4* expression levels were assessed by qPCR and expressed as fold change respect to NT condition. The normalization was performed using the mean of three different housekeeping genes (*Gapdh, Actin, 18S*). The average and SEM of all the experiments were performed on the duplicate samples from three independent biological experiments and are shown: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, in a Student's *t*-test.

post-RA treatment (**Figure 1C**). These observations suggest that *de novo* synthesized regulator proteins are required to induce *Zscan4*. To address this hypothesis, we cultured ESCs in the presence of RA for 20 h and then incubated with the protein synthesis inhibitor cycloheximide (CHX) or control medium for additional 12 h (**Figure 1C**, right). The addition of CHX to the culturing medium significantly impaired *Zscan4* transcription of about 50% (**Figure 1**, right) while, as expected, *Stra8* was not affected.

RA Induces the Transition of ESCs to 2C-Like Cell Through *Zscan4* Intermediates

The exit from the ESCs toward the 2-cell-like state is a multistep process by which cells encompass several intermediate states, determined by the levels of *Zscan4* expression, each characterized by a known and specific molecular signature (Rodriguez-Terrones et al., 2018). We explored the hypothesis that RA signaling could induce Zscan4 intermediate transitions. Since *Zscan4* expressing cells are scarcely abundant in the medium not supplemented with RA (**Figure 1A**), we designed a system to efficiently and quickly collect Zscan4 subpopulation. In particular, we generated a modified ESCs line harboring the extracellular portion of the human low-affinity nerve growth factor receptor gene (*LNGFR*) under the control of the *Zscan4* promoter (Napolitano et al., 2019). This strategy allowed us to efficiently separate and collect Zscan4^{+} cells (**Figure S3**), overcoming long rounds of FAC-sorting. Next, we cultured ESCs$^{Zscan4-LNGFR}$ for 72 h either in VitA$^{\text{minus}}$ or in medium supplemented with Retinoic Acid (hereafter VitA^{plus}). Subsequently, cells were isolated using a magnetically labeled anti-LNGFR antibody (**Figure 2A**). As expected the percentage of Zscan4^{+} cells in the absence of RA is extremely low (less than 1%) compared to the number of positive cells upon RA treatment (about 20%) as shown in **Figure 2B**. We next compared *Zscan4* levels in LNGFR positive cells derived from the different culture conditions by qPCR (**Figure 2C**).

Interestingly, *Zscan4* expression levels were higher in positive cells isolated from VitA$^{\text{plus}}$ than positive cells from VitA$^{\text{minus}}$ (**Figure 2C**). To investigate whether these two populations ("Zscan4$^{\text{high}}$" and "Zscan4$^{\text{low}}$") corresponded to the intermediate states arising during ESCs to 2C-like transition, we also analyzed the expression of MERV-L genes that are transcriptionally

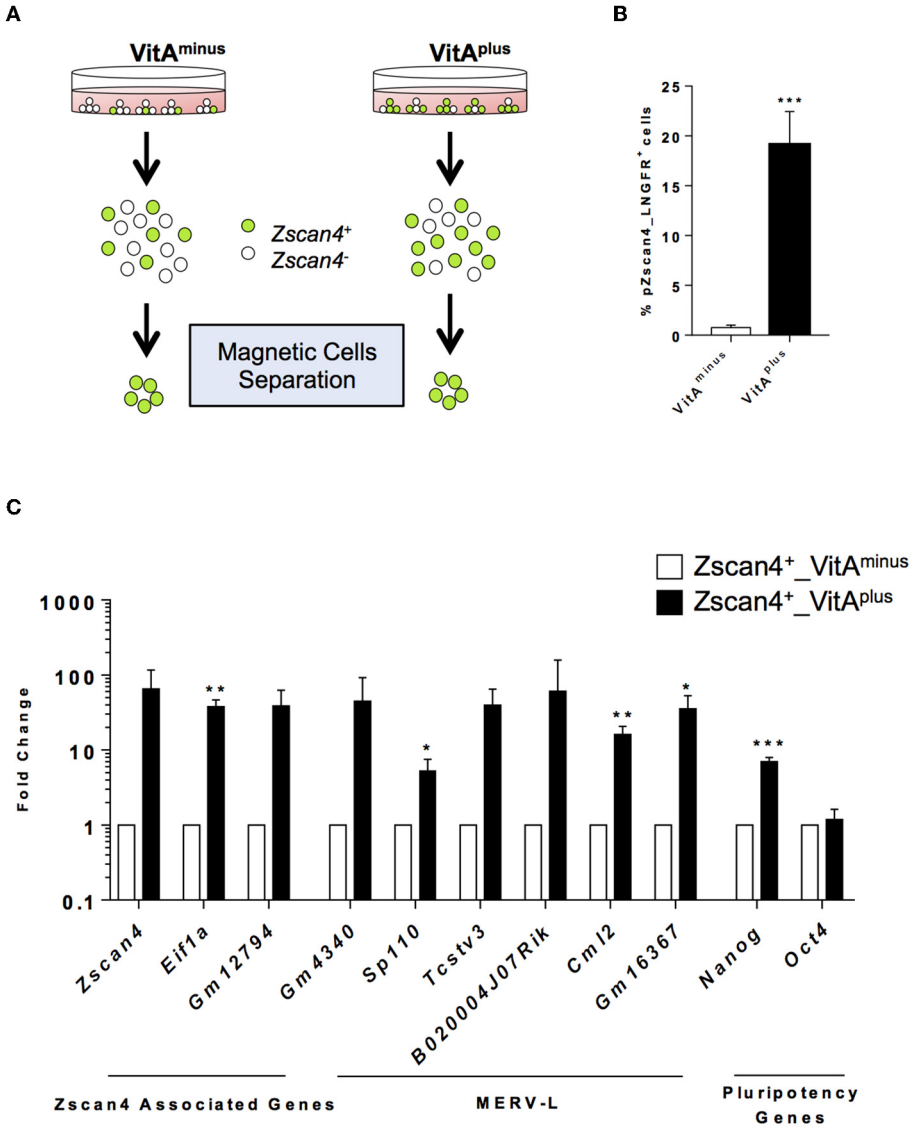

FIGURE 2 | RA effects on ESCs to 2C-like transition. **(A)** Schematic illustration of the system used for Zscan4^{+} cells magnetic separation. Zscan4 promoter drives the expression of the extracellular portion of the human cell surface receptor LNGFR. **(B)** The modified ES^{Zscan4_LNGFR} cells were cultured in VitA^{minus} and VitA^{plus} for 72 h and incubated with a LNGFR magnetically labeled antibody. Positive fractions were collected through autoMacs Separator. The histogram describes the percentage of separated cells in the different culture conditions. **(C)** Analysis of genes upregulated during the ZGA. The gene expression levels were assessed by qPCR as fold change to VitA^{minus} condition and the normalization was performed using the mean of *Gapdh, Actin* and *18S*. Statistical significance was calculated by Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

activated during ZGA (Macfarlan et al., 2012). Expression analysis revealed that MERV-L genes were either significantly upregulated or exclusively expressed in Zscan4^{high} compared to Zscan4^{low} cells (**Figure 2C**). These data suggest that RA induces 2C-like state by acting on the transition of Zscan4^{low} (less than 1% prior to RA activation) to Zscan4^{high} intermediates (**Figure 2B**, **Supplementary Video**).

Functional Genomic Analyses Reveal *Zscan4* Transcription Regulators

Given the crucial role of RA on activation of Zscan4^{low} state, we searched for intermediate regulators of *Zscan4* transcription that could shed light on the molecular networks underlying RA-dependent *Zscan4* induction. To verify whether Zscan4 is directly regulated by retinoids, we analyzed the promoter region of *Zscan4* (2,400 bp upstream the TSS) to scan the presence of retinoic acid response elements (RARE motifs). To this aim, we adopted the FIMO ("Find Individual Motif Occurrences") tool that computes a log-likelihood ratio score and a q-value for each position in a given sequence (Grant et al., 2011). In line with our previous findings, *in-silico* analysis of a 2,400 bp region upstream the Zscan4 promoter by FIMO did not result in any canonical RAR elements (RARE) identification (**Table S1**, *q*-value < 0.1) consistently with our previous findings that *Zscan4* is

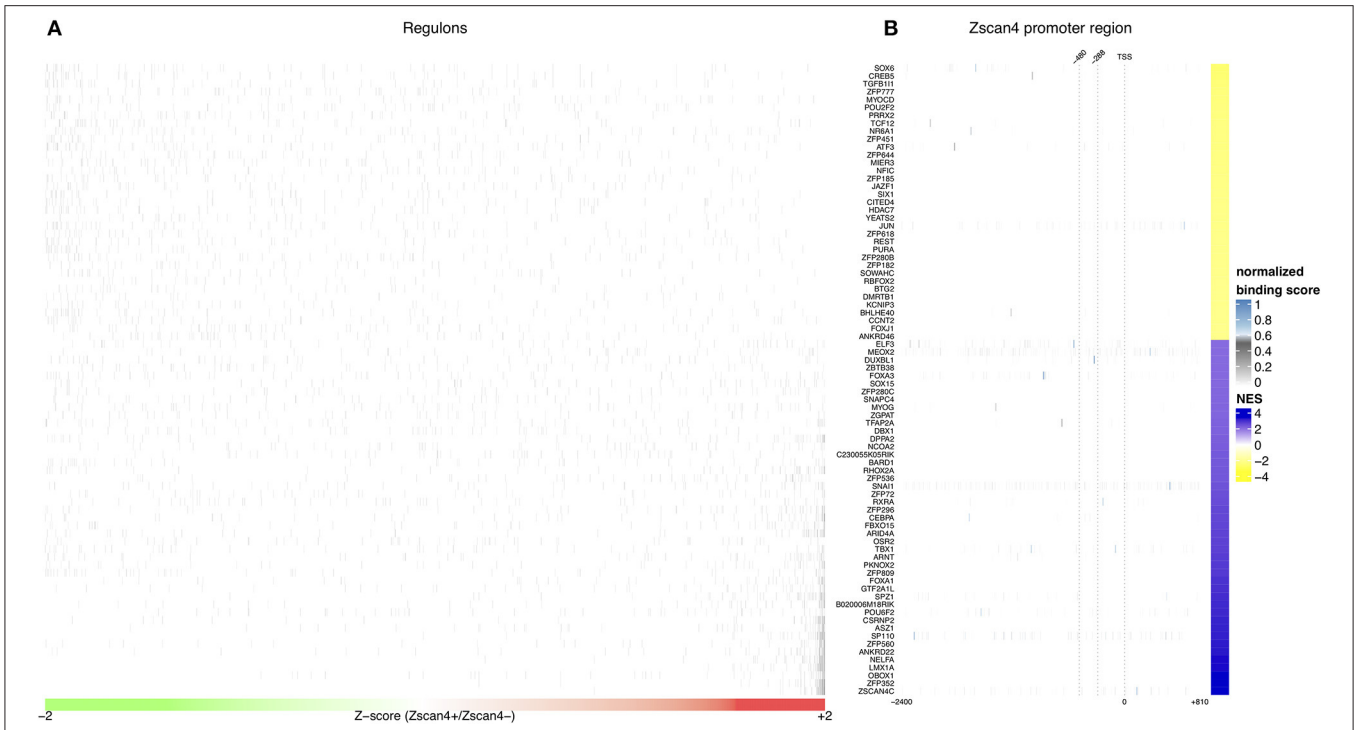

FIGURE 3 | *In silico* analysis of *Zscan4* regulatory network. **(A)** Blue and Yellow colors indicate positive vs negative regulation of Master Regulators (MRs). Overall were found 80 significant master regulators. The figure shows, for each significant master regulator, the distribution of regulons respect to RA-Zscan4^{+} vs. RA-Zscan4^{-} metastate phenotype. **(B)** The figure shows significant binding activity in the promoter region of *Zscan4c*.

a RA secondary-response gene (**Figure 1C**). To identify putative Zscan4 direct and indirect regulators, we reconstructed a global regulatory network from a wide collection of ESCs specific gene expression profiles using a consolidated reverse engineering approach (**Figure S4**) (Liu, 2015). We adopted a genome-wide reverse engineering approach that integrates both sequence (putative binding motif) and functional expression data. We used a set of 754 ESC-specific expression profiles to reconstruct a global regulatory network surrounding *Zscan4*. This network consists of 476.654 interactions among 1.852 Transcriptional Factors (TFs) and 24.988 genes (**Figure 3A**, **Figure S4**).

To better underlying the transcriptional mechanism acting for the maintenance of Zscan4^{+} metastate under RA effect, we adopted the Master Regulator Analysis (MRA) algorithm to compute the statistical significance of the overlap between the regulatory targets of each TF and the Zscan4 signature represented by the list of differentially expressed genes enriched in RA-Zscan4^{+} compared to RA-Zscan4^{-} (the dataset was previously published, Tagliaferri et al., 2016, GEO accession number: GSE75977). This analysis allowed us to identify 80 significantly enriched [p-value ≤ 0.001 and absolute Normalized Enrichment Score (NES) greater than 70th percentile] Master Regulator (MR) candidates to be regulators of the RA-mediated Zscan4^{+} metastate (**Table S2**). To identify direct regulators, we performed an additional sequence motif binding analysis to excluded MRs with no evident binding signals on *Zscan4* promoter. We identified 4 MRs that exhibited significant promoter binding activity (p-value ≤ 0.0001 and normalized binding score ≥ 0.7) (**Figure 3B**, **Table S3**): Elf3 (E74 like ETS transcription factor 3), Sox6 (Sry type HMG box 6), Foxa3 (Fork head box A3), and Duxbl1 (Double homeobox B-like 1).

Retinoids Induce *Zscan4* Expression Mediating Dux Binding Activity

To narrow down the list of MRs we characterized the minimal region of *Zscan4* promoter responsive to RA. In particular, we evaluated the promoter activity of deletion mutants upon RA treatment by mean of a GFP reporter assay. The analyses were conducted on three overlapping *Zscan4* promoter regions cloned upstream GFP: the 2,400 bp (long, L), the 480 bp (mid-length, ML) and the 288 bp (short, S) fragments (**Figure 4A**). The corresponding genetic constructs were stably transfected in ESCs. Selected clones were grown with or without RA, and GFP expression was analyzed through cytofluorimetric assay.

Interestingly, the fraction of GFP-positive cells harboring the short promoter form poorly responded to RA (**Figure 4A**). These results allowed us to map the *retinoic acid responsive region for Zscan4 activation* between −288 and −480 bp upstream the start the Transcription Start Site (TSS).

Among the subset of 4 MRs previously identified, Duxbl1 resulted the only one having a significant binding activity in this region. Consistently, canonical Dux Binding Motif (DBM), has been previously mapped on *Zscan4* promoter (Geng et al., 2012; De Iaco et al., 2017; Hendrickson et al., 2017). To

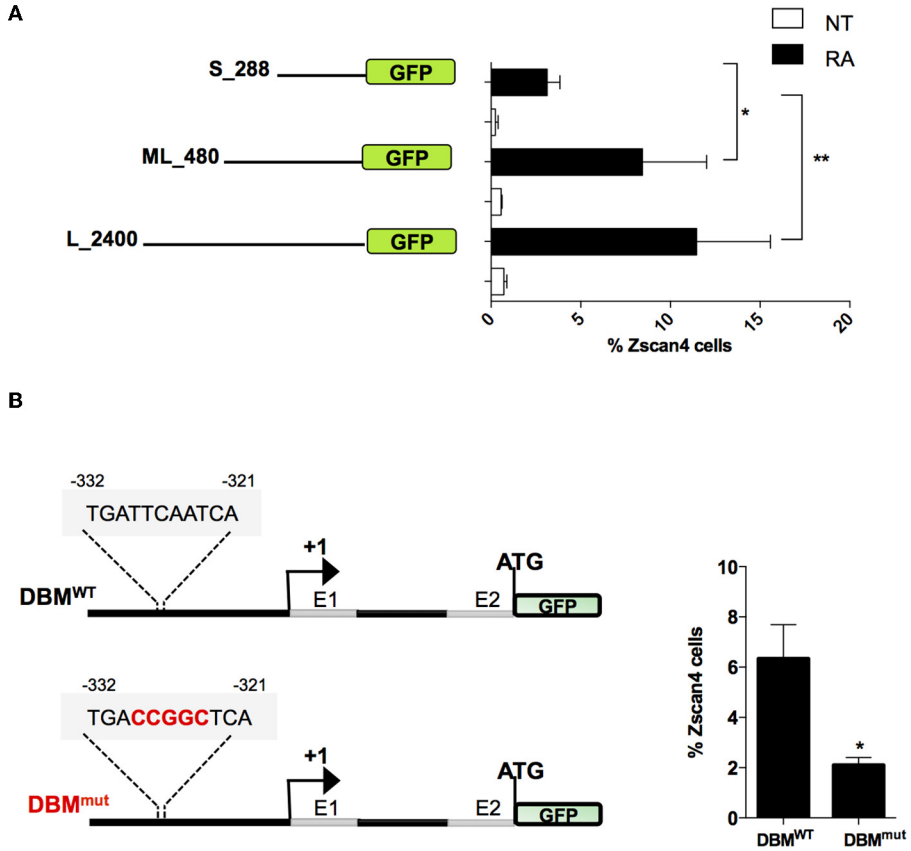

FIGURE 4 | Evaluation of RA responsive DNA element on *Zscan4* promoter. **(A)** Flow cytometry analysis of ESC^{Zscan4_GFP} lines expressing GFP under *Zscan4* promoter regions of: S_288 (short), ML_480 (Mid-length) and L_2400 (Long) starting from Transcription Start Site (TSS). ESC^{Zscan4_GFP} lines were cultured for 5 days in media with or without RA. **(B)** Schematic representation of genetic constructs of ML_480 *Zscan4* promoter that contains either wild type Dux Binding Motif (DBM^{WT}) or mutant (DBM^{mut}) at about −330 bp from the transcription start site (TSS) (left). ESCs^{DBM-WT} and ESCs$^{DBM-mut}$ were cultured in RA for 5 days, and the percentage of Zscan4^{+} cells were analyzed by cytofluorimetry assay. The average and SEM of three independent biological experiments are shown: *$p < 0.05$, **$p < 0.01$ in a Student's *t*-test.

determine whether this Dux binding motif was required for RA-dependent *Zscan4* transcriptional activation, we generated a transgenic ESCs line expressing GFP under the control of *Zscan4* promoter harboring mutated (DBM^{mut}) or wild type (DBM^{WT}) DBM sequence (**Figure 4B**, left). Following RA treatment, the percentage of fluorescent cells in the two clones was measured by citofluorimetry. The experiment showed that the fraction of Zscan4^{+} was reduced about 70% (from 6 to 2%) comparing DBM^{WT} vs. DBM^{mut} (**Figure 4B**, right). Overall, our data showed that RA activates *Zscan4* through DBM region.

Dux and Duxbl1 Directly Bind and Regulate *Zscan4* Expression

The Dux family proteins contain a double DNA binding homeodomain (HOX1 and HOX2) and a variable carboxyl-terminal domain (CT) (**Figure 5A**). The C-terminal domain tethers the histone acetyl-transferase p300 to chromatin, thereby enhancing the transcriptional activation of target genes, such as *Zscan4* (De Iaco et al., 2017). Comparing the protein structure of Dux and Duxbl1, they differ at the C-terminus as Duxbl1 is devoid of CT region (**Figure 5A**) that suggest an opposite effect on the regulation of their targets.

We validated the hypothesis that *Dux* and *Duxbl1* are part of *Zscan4* regulatory network under RA action. First, we analyzed whether *Dux* and *Duxbl1* expression in ESCs was induced by RA. Two days after RA-treatment, the qPCR analysis showed that *Dux* levels were increased 12-fold on average while *Duxbl1* increased 8-fold (**Figure 5B**, top). Second, we assessed whether their expression was preferentially induced in Zscan4^{+} cells. To this aim, we employed a transgenic ESC^{Zscan4_GFP} line to isolate Zscan4^{+} and Zscan4^{-} cell population by mean of FAC-sorting from RA medium. The experiment showed *Dux* is expressed 60 times higher in Zscan4^{+} than Zscan4^{-}, while a 20 times increase was observed for *Duxbl1* (**Figure 5B**, bottom).

Based on these results, we thus explored the hypothesis that under RA stimuli *Dux* and *Duxbl1* have opposing functions in determine *Zscan4* activation. To this aim, we generated

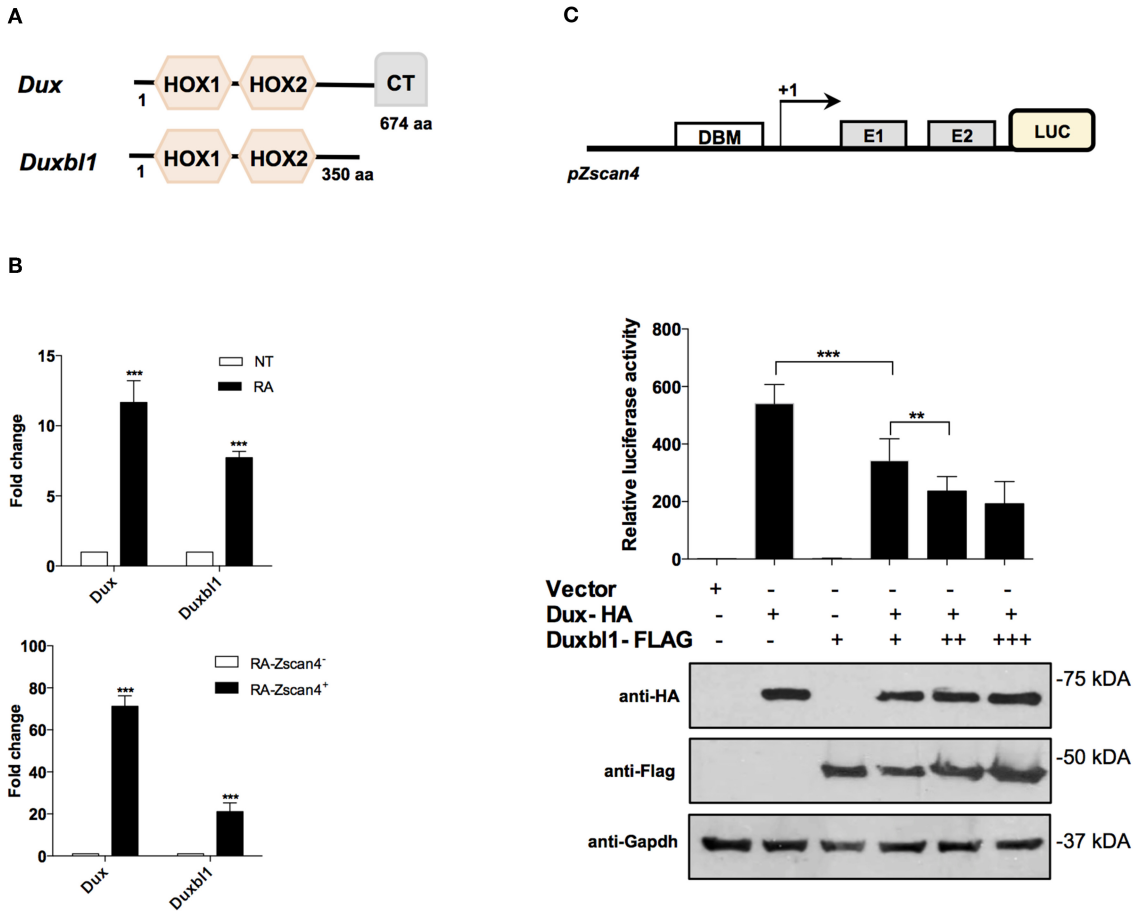

FIGURE 5 | Role of Dux *and* Duxbl1 *on Zscan4* promoter regulation. **(A)** Schematic representation of Dux and Duxbl1 protein domains. **(B)** *Dux* and *Duxbl1* expressions were significantly induced by RA. The normalization was performed using Gapdh as housekeeping gene. (top). ES^{Zscan4_Em} cells grown for 5 days with or without RA and sorted by flow cytometry. *Dux* and *Duxbl1* expressions were robustly enriched in $Zscan4^{+}$ cells (bottom). The normalization was performed using Gapdh as housekeeping gene. **(C)** HEK293T cells with Luciferase-reporter gene under Zscan4 promoter regulation (scheme) were transiently co-transfected with: control Vector, or Dux-HA, Duxbl1-Flag either alone or with Dux-HA. Data shown represent relative luciferase activity normalized against the β-galactosidase activity, in the top panel, while in the bottom panel was reported the western blot assay corresponding to luciferase assay. Dux-HA and Duxbl1-Flag were detected with anti-HA, anti-Flag antibodies while anti-Gapdh was used for proteins normalization. $^{*}p < 0.05$, $^{***}p < 0.01$, $^{***}p < 0.001$ in a Student's *t*-test.

a HEK293T reporter line in which the luciferase is under *Zscan4* promoter (**Figure 5C**). Overexpression of transfected Dux-HA significantly enhanced luciferase activity (**Figure 5C**), coherently reproducing the Dux capability to activate *Zscan4* expression (Choi et al., 2016). In contrast, Duxbl1-Flag overexpression did not induce luciferase activity. Notably, the effect of Dux on luciferase activity was significantly hampered by co-transfecting increasing amount of Duxbl1 (**Figure 5C**). In addition, in order to further demonstrate the competition between Dux and Duxbl1 for Zscan4 promoter activation, we generated a mutant of Duxbl1 carrying the Dux C-terminal transactivation domain (CTD). The luciferase assay (**Figure S5**) shows that Duxbl1 mutant acquires the capability to activate Zscan4 promoter confirming the hypothesisthat Dux and Duxbl1 parallelly binds and co-regulate *Zscan4* with contrasting effects on its expression.

Duxbl1 Hampers ESCs to 2C-Like Transition by Directly Counteracting *Zscan4* Activation

Our data collectively suggested that *Duxbl1* hampers *Zscan4* expression by competing with *Dux* activity. This prompted us to investigate whether *Dux* and *Duxbl1* could function as agonist and antagonist of ESCs transition to 2C-like cells, respectively (**Figure 6A**).

First, we determined whether Dux mediates RA dependent *Zscan4* activation. Dux wild type ($Dux^{+/+}$) and knock out ($Dux^{-/-}$) ESCs (De Iaco et al., 2017) were cultured with or without VitA. In these cell lines, RA induction of *Zscan4* is completely impaired (**Figure 6B**) confirming, as expected, that Dux is necessary for RA-dependent ESCs to 2C-like transition. Second, to further evaluate our hypothesis, we generated knock-in recombinant ESCs in which Duxbl1-Flag could be

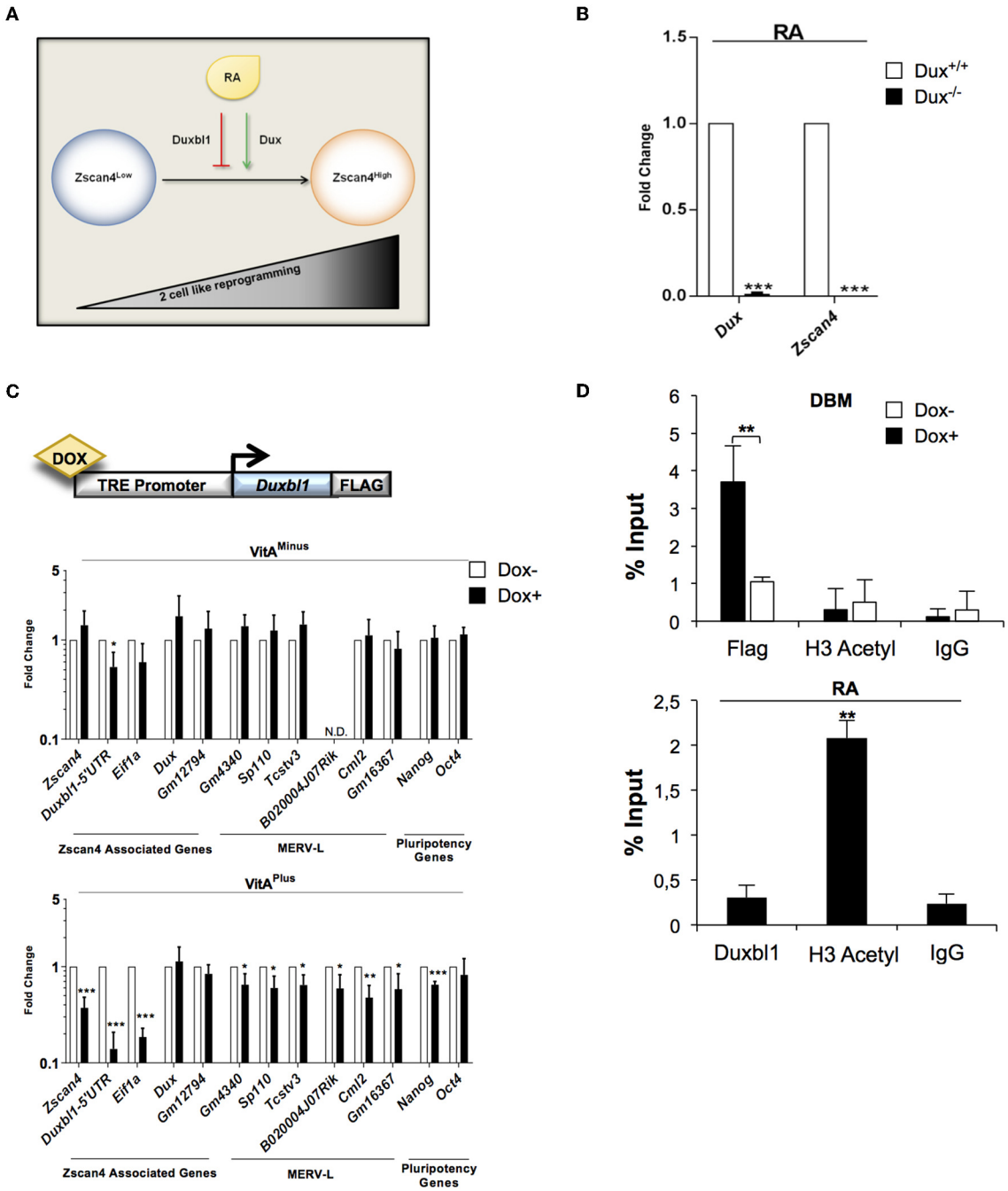

FIGURE 6 | *Dux* and *Duxbl1* effect on RA dependent ESCs to 2C-like transition. **(A)** Model of ESCs to 2C-like transition involving RA dependent Dux and Duxbl1 regulation. **(B)** $Dux^{-/-}$ ESCs were cultured in RA supplemented medium for 4 days. The *Dux* and *Zscan4* expression levels were assessed by qPCR, normalized using *Gapdh* as housekeeping gene and expressed as fold change compared to $Dux^{+/+}$ condition. The average and SEM of all the experiments were performed on three independent biological experiments and are shown: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in a Student's *t*-test. **(C)** Schematic representation of Duxbl1 tetracycline-inducible system (top). $ESC^{Duxbl1-Flag}$ transgenic lines were cultured with or without DOX (1.5 μg/ml for 72 h), in $VitA^{minus}$ and $VitA^{plus}$ conditions. qPCR analysis was performed for Zscan4 associated markers using the mean of three different housekeeping genes (*Gapdh, Actin, 18S*) (bottom). **(D)** In the top, $ESC^{Duxbl1-Flag}$ cells were cultured in presence or not of DOX and chromatin was analyzed by ChIP assay using: anti-Flag, anti-H3Acetyl and IgG antibodies. In the bottom, ESCs treated with RA for 5 days were analyzed through ChIP with: anti-Duxbl1, anti-H3Acetyl and IgG antibodies. Both ChIP assays were analyzed by qPCR for the binding to Zscan4 promoter, expressed as percentage of input.

overexpressed using by doxycycline-inducible (DOX) knock-in ESC line (Iacovino et al., 2014). The genetic construct and the strategy used for the knock-in cell line generation are reported in **Figure 6C** (top) and **Figure S6A**. The induction efficiency of Duxbl1 upon DOX treatment was assessed by western blot (**Figure S6B**). We evaluated the effects of *Duxbl1* overexpression on 2C state associated gene signature with or without retinoids. The analyses showed that the induction of *Duxbl1* without VitA leads to the reduction only of *Eif1a* and *Duxbl1;* however in the presence of VitA, *Duxbl1* ectopic expression downregulated *Zscan4* and, among others, MERV-L genes, thus reflecting a reduction of 2C-like state (**Figure 6C**, bottom). Considering *Zscan4* as ESCs to 2C-like transition bona fide marker, we checked whether *Duxbl1* inactivates *Zscan4* through direct binding to DBM by quantitative ChIP (qChIP) analysis (**Figure S7A**). Our data showed an enrichment of DBM fragments after anti-Flag immunoprecipitation in ESCs DOX$^+$ cells (**Figure 6D**, top), compared to the IgG negative control and to a furthest region from DBM on *Zscan4* promoter (**Figure S7B**). Overall, we observed that Duxbl1 binding to the *Zscan4* promoter was associated to the reduced presence of H3 acetyl (**Figure 6D**, top). Consistently, RA led decreasing of Duxbl1 binding on *Zscan4* promoter and a resultant increasing of open chromatin conformation (**Figure 6D**, bottom).

DISCUSSION

In ESCs culturing systems, RA displays mainly a dual role. On one hand, it inhibits LIF signaling, which is required for stemness condition (Tighe and Gudas, 2004; Gudas and Wagner, 2011) and drives the up-regulation of genes involved in the differentiation process (Engberg et al., 2010). On the other hand, retinoids counteract the differentiation and maintain the pluripotency, mainly through the activation of WNT signaling (Wang et al., 2008), *Nanog* (Chen et al., 2007), and Phosphoinositide 3-kinase (PI3K) (Chen and Khillan, 2010).

Recent evidence reports that RA activates ZGA molecular signature (Tagliaferri et al., 2016) that is accompanied by the transition of ESCs to a high pluripotency state so-called 2C-like cells (Napolitano et al., 2019).

Here, we showed that without retinoids *Zscan4* spontaneous population is less than 1% of the whole ESCs population. Retinoids are able to activate the mitotic division of such population that promptly transit to 2C-like state activating MERV-L genes.

To elucidate the molecular network regulating ESCs to 2C-like transitions mediated by RA, we reconstructed Zscan4 global regulatory network by employing a reverse engineering approach on combined sequence data of known binding sites with ESCs expression datasets. Integrating these inferred findings with our experimental evidence, we found that RA promotes the Zscan4$^+$ intermediates transition by co-regulating the expression of two members of Dux family: *Dux* and *Duxbl1*. Interestingly, our data showed that both *Dux* and *Duxbl1* are specifically expressed in Zscan4$^+$ while, known 2C-like regulators, among others *Dppa2*, *Dppa4* although play a crucial role in the 2C state activation, do not significantly change between Zscan4$^+$ and Zscan4$^-$ state and may act in a broad range network (data not shown).

Based on the protein domain of Dux and Duxbl1, we propose that both molecules compete for Zscan4 proximal promoter binding via their N-terminal Hox portion, but only Dux can promote *Zscan4* transcription via its C-terminal transactivation region. Notably, we show that *Dux* is required for RA dependent transition of ESCs to 2C-like state, while *Duxbl1* counteracts such transition by direct downregulation of *Zscan4* expression. It would be interesting to evaluate whether Duxbl1 directly inactivate Dux targets, among others MERV-L, suggesting a potential role in balancing Zscan4 intermediates fluctuation. Remarkably, ectopic overexpression of *Duxbl1* strongly downregulates endogenous expression of *Duxbl1* but not *Dux*. This could be a negative feedback loops act to break the balance of 2C-like transition.

It is known that retinoids are crucial during preimplantation and embryonic development (Hofmann and Eichele, 1994; Huang et al., 2003), however, their effect on early cleavage has not been examined yet. Our data could be taken in consideration to study zygotic genome activation during both *in vivo* and *in vitro* development.

Another interesting consideration about our finding derives from the similar consensus binding motifs between Dux and its human ortholog DUX4 (Wu et al., 2010; Eidahl et al., 2016). Like Dux, DUX4 is involved in the positive regulation of ZSCAN4, PRAME, TRIM, MBD3L2, RFPL1, KHDC1, and FAM90 families (Geng et al., 2012). Aberrant DUX4 up-regulation is associated with Facio-Scapulo-Humeral Dystrophy (FSHD), and it alters the muscle transcriptome, splicing and differentiation (Mitsuhashi et al., 2018). It would be interesting to investigate whether the human ortholog of Duxbl1 could counteract DUX4 in FSHD and, thus, laying the basis for targeted therapy of such disease.

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.

AUTHOR CONTRIBUTIONS

GF designed the study and performed the data analysis and the interpretation with FV, IC, and AC. Bioinformatic analysis was performed by MC, LC, and TN. The experimental procedures were performed by DT, PM, MA, VR, SR, and TA. PM and GF wrote the manuscript with input from all authors and a critical reading from MD and LD. GF supervised all the experiments.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Mesenchymal Stem Cells Beyond Regenerative Medicine

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Mesenchymal stem cells (MSCs) are competent suitors of cellular therapy due to their therapeutic impact on tissue degeneration and immune-based pathologies. Additionally, their homing and immunomodulatory properties can be exploited in cancer malignancies to transport pharmacological entities, produce anti-neoplastic agents, or induce anti-tumor immunity. Herein, we create a portfolio for MSC properties, showcasing their distinct multiple therapeutic utilities and successes/challenges thereof in both animal studies and clinical trials. We further highlight the promising potential of MSCs not only in cancer management but also in instigating tumor-specific immunity – i.e., cancer vaccination. Finally, we reflect on the possible reasons impeding the clinical advancement of MSC-based cancer vaccines to assist in contriving novel methodologies from which a therapeutic milestone might emanate.

Keywords: MSC, regeneration, autoimmunity, cancer, antigen, vaccine

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INTRODUCTION

Broadly distributed among tissues, MSCs are first generation adult stem cells of mesodermal non-hematopoietic origins. They were originally reported in bone marrow (BM) by Friedenstein et al. (1968, 1970) and later identified in adipose tissue, peripheral blood, cruciate ligaments, dental pulp, menses blood, amniotic fluid, fallopian tube, placenta, umbilical cord, and endometrial polyps (Caplan, 1991; Bianco et al., 2008; Ding et al., 2011; Sheng, 2015; Ullah et al., 2015). According to the International Society for Cellular Therapy (ISCT), MSCs are characterized by their (i) adherence to plastic, (ii) cell surface expression of CD73, CD90, and CD105 but not CD45, CD34, CD14, CD11b, CD79α, CD19, and HLA-DR (hematopoietic cell markers), and (iii) multipotency, the ability to differentiate into various mesodermal cell lineages such as osteoblasts, chondroblasts, and adipocytes (Dominici et al., 2006). However, the ISCT definition is no longer standardized

Abbreviations: ALS, amyotrophic lateral sclerosis; APC(s), antigen secreting cell(s); BM, bone marrow; CX3CL1, C-X3-C motif chemokine ligand 1; CXCR4, C-X-C Motif Chemokine Receptor 4; DC(s), dendritic cell(s); GMP, good manufacturing practice; GvHD, graft-versus-host disease; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; ISCT, International Society for Cellular Therapy; MAP, mitogen activated protein; MI, myocardial infarction; MIF, Migratory Inhibitory Factor; miRNA, microRNA; MSC(s), mesenchymal stem cell(s); NK, natural killer; NO, nitric oxide; PD-L1, programed death-ligand 1; SDF-1, stromal-derived factor-1; TLRs, toll-like receptors; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; T_{reg}, regulatory T cell; TSG6, tumor necrosis factor-inducible gene 6 protein.

as MSC identification criteria continue to change. Exemplifying this are the discovery that MSCs can also differentiate into cells of ectodermal and endodermal parentage (Wei et al., 2013) and the inclusion of novel surface markers to their identity (CD165, CD276, and CD82) (Al-Nbaheen et al., 2013). Several studies on MSC lineages have also identified distinctive molecular (Al-Nbaheen et al., 2013; Ullah et al., 2015; Wu et al., 2018), proliferation/differentiation (Kern et al., 2006), and functional properties (Keyser et al., 2007), accrediting the fact that their biology is still partially intelligible. The conventional notion, however, is that MSCs are (i) genomically stable, (ii) highly accessible, (iii) easy to isolate and expand, (iv) immune-privileged (low expression of MHC I/II and co-stimulatory molecules and – further explained in Section "Immunological Properties: A Paradigm" – immunomodulation), and – unlike other types of stem cells – (v) non-teratogenic and ethically conforming (Wei et al., 2013). Additionally, a number of reports showing that BM-MSCs from healthy donors perform better in proliferation/differentiation and secretion criteria compared to BM-MSCs from osteoarthritic (Murphy et al., 2002) and Gaucher disease patients (Campeau et al., 2009) corroborate that MSCs play a physiological role in homeostatic tissue maintenance, whereas their disturbance may foster disease pathogenesis. In this review article, we recapitulate a vast literature on MSC assets, demonstrating from preclinical and clinical perspectives how they render them fit candidates for cellular therapy. Finally, we discuss the trend of MSC utility against tumors to bridge to the highlight of this review – MSCs as cancer vaccines – pinpointing the flaws halting their clinical effectiveness while offering novel insight on how to overcome them.

MSC FITNESS FOR CELLULAR THERAPY

Regenerative Properties

Numerous studies illustrate the regenerative potential of MSCs based on their homing, engraftment, (trans)differentiation, and ability to replace apoptotic/necrotic tissue or dissipate paracrine signaling to boost injured tissue function (Prockop, 1997). *In vitro*-cultured systemically-infused MSCs home *via* their chemokine and toll-like receptors (TLRs) into several organs including BM, heart, and liver in which they can persist for prolonged periods of time (Devine et al., 2001; Allers et al., 2004; Lüttichau et al., 2005; Tomchuck et al., 2008). Factors in favor of homing are young recipient age, irradiation, decreased cell passage number, cytokines/inflammation, as well as increased chemokine receptor and TLR expression (Horwitz et al., 2002; François et al., 2006; Shi et al., 2007; Kyriakou et al., 2008; Tomchuck et al., 2008). Besides the former receptors, MSCs express a variety of adhesion molecules, endopeptidases, and growth factors in addition to their cognate receptors, which facilitate MSC tethering, endothelial rolling, and transmigration to tissues (De Becker and Van Riet, 2016). MSCs might mobilize as well under several stimuli such as growth factors (Asahara et al., 1999) and xenobiotics (Llevadot et al., 2001) before engrafting into tissues where they either (trans)differentiate to the constituent cells (Prockop et al., 2010) or secrete various humoral factors in the extracellular space such as cytokines, chemokines, and mRNA/microRNA (miRNA)-containing microvesicles to modulate tissue function (Wei et al., 2013). Factors influencing tissue engraftment efficiency are cell death, immune rejection, and first-pass lung entrapment which can be overcome by optimizing delivery methods, ameliorating target tissue receptivity, and schooling MSCs to resist tissue hostility (Kean et al., 2013; Ezquer et al., 2017).

Following adherence to plastic *in vitro* or tissue engraftment *in vivo*, MSCs form colonies and (trans)differentiate into a myriad of cell lineages (Kuznetsov et al., 1997; Li H. et al., 2006; Wang et al., 2012; Vonk et al., 2018). For this to occur, their microenvironment must contain multiple mitogenic or stimulating factors (Tontonoz et al., 1994; Sekiya et al., 2002; Lucarelli et al., 2003; Solchaga et al., 2005; Fontaine et al., 2008; Inada et al., 2008; Pavlova et al., 2012); be subjected to hypoxic conditions (Mohyeldin et al., 2010; Zhang et al., 2019); or scaffolded to closely mimic organ architecture or function (Ouyang et al., 2003; Ohgushi et al., 2005). However, a newer understanding of the regenerative abilities of MSCs *in vivo* later emerged, linking tissue regrowth not to MSC (trans)differentiation exclusively but rather to autocrine and paracrine signaling transduced through their communication with local stimuli (Crisostomo et al., 2008), growth factors (Hahn et al., 2008), and inflammatory mediators (Haynesworth et al., 1996). This creates a rich nutritive milieu to which cells in the vicinity also contribute (Caplan and Dennis, 2006). Within the trophic environment are factors dictating angiogenesis (Min et al., 2002), hindrance of apoptosis (Xu et al., 2007), inhibition of fibrosis, mitosis in local tissue (Takahashi et al., 1999), and formation of a structural niche with other resident stem cells (Méndez-Ferrer et al., 2010). In addition, MSCs secrete microvesicles and exosomes which contain pro-angiogenic growth factors and miRNA as a means to establish cell-to-cell communication (Gong et al., 2017; Phinney and Pittenger, 2017). On the other hand, multiple factors can still hamper MSC regenerative functions such as temperature, media type (Kubrova et al., 2019), interference of plastic adherence with cellular function (Mabuchi et al., 2012), chromosomal abnormalities, transformation, and tumor growth especially in MSCs of murine sources. Having said that, isolation and culture protocols recently developed for human MSCs derived from healthy subjects appear as promising endeavors to overcome those hurdles (Bernardo et al., 2007; Law and Chaudhuri, 2013; Conforti et al., 2016). For example, transformation and persistence were addressed in a protocol that uses skin tissue of patients undergoing any relevant medical intervention. To obtain MSCs, the tissues are disinfected and enzymatically digested in good manufacturing practice (GMP). Cell yields are then sorted with antibody-coupled magnetic beads, and cultured MSCs are validated according to ISCT criteria. Finally, several tests are performed to assess *in vivo* toxicity, tumorigenicity, and biodistribution/persistence (Tappenbeck et al., 2019). The data of another clinical study, which warranted its authors an "orphan designation" in Germany for graft-versus-host disease (GvHD) treatment using MSCs, authenticate the effectiveness of such

protocol. Indeed, generating the MSCs entailed the enrichment of BM aspirates of several donors using an automated cell separation unit and processing system followed by the expansion of MSCs in culture over 14 days. From this bank, clinical-grade MSCs are obtained and cultured in platelet lysate serum-free media whose utility eliminates the risks associated with the use of fetal bovine serum such as immunogenicity and pathogenicity (Kuçi et al., 2016; Bader et al., 2018).

Immunological Properties: A Paradigm

In addition to its tissue repair characteristics, the secretome of MSCs displays immunomodulatory properties. This is evident in the ability of MSCs to interfere with the cell cycle (G0/G1 phase arrest), hinder the responses of naïve and memory T cells, inhibit the activation and proliferation of effector T cells, and induce regulatory T cell (T_{reg}) function (Krampera et al., 2003; Siegel et al., 2009; Duffy et al., 2011; Haddad and Saldanha-Araujo, 2014). Such immunosuppressive activity essentially ensues in response to inflammatory signals including interferon-γ (IFN-γ), TNF-α, and interleukin-1 (IL-1). These pro-inflammatory molecules prime MSCs, such that they induce the secretion of multiple soluble immunosuppressive molecules and the upregulation of inhibitory surface co-receptors including programed death-ligand 1 (PD-L1) (Sheng et al., 2008). Those mechanisms are protective against immune cells such as natural killer (NK) cells which become cytolytic upon activation by inflammatory signals, the same signals inducing the upregulation of MHC class I/II on MSCs and subsequently their susceptibility to NK cell cytotoxicity. Interestingly, NK cells/MSCs ratio is the determinant of the inhibitory power balance. For example, lower ratios tip the suppressive balance in favor of MSCs which become capable of inducing phenotypic and secretory changes in NK cells *via* physical and paracrine interactions, thereby restricting their cytotoxicity and proliferation (Sotiropoulou et al., 2006; Jewett et al., 2010; Spaggiari and Moretta, 2012). Pro-inflammatory signals also support MSC differentiation through multiple receptors like TLRs and signaling pathways like NF-κB, p38 mitogen-activated protein (MAP) kinase, and β-catenin, ultimately inducing the transcription of lineage-specific genes (Cheng et al., 2008; Wei et al., 2013; Chen et al., 2016; Liu et al., 2018). For instance, NF-κB and MAP kinase pathways are activated by stromal cell-derived factor-1 (SDF-1), a pleotropic chemokine secreted by several cells and organs, which acts as a chemoattractant for MSCs in regenerative settings (Kucia et al., 2004). Elsewhere, however, NF-κB upregulation by pro-inflammatory cytokines was negatively correlated with MSC differentiation, particularly osteogenesis (Ansari et al., 2017). In contrast, the absence of strong inflammatory stimuli (e.g., low levels of inflammatory or anti-inflammatory cytokines) does not trigger the production of immunosuppressive factors, thus permitting a pro-inflammatory environment to takeover. This is evident in a few studies showing that *in vivo* transplantation of unchallenged allogeneic MSCs evokes cellular and humoral immune responses (Eliopoulos and Galipeau, 2002; Poncelet et al., 2007; Renner et al., 2009). Furthermore, inflammatory signals allow MSCs to govern the activity of multiple innate and adaptive immune cells including B cells, neutrophils, and macrophages through secreted soluble factors such as prostaglandins, chemokine ligands, interleukins (ILs), growth factors, and nitric oxide (NO) (Singer and Caplan, 2011). Those factors interfere with inflammatory signaling pathways (e.g., STAT3), ultimately mitigating antigen presentation and humoral immunity (Rafei et al., 2008; Loebinger and Janes, 2010). In addition to their secretome, MSCs can mitigate mixed lymphocyte reactions by physically hindering the contact of T cells with antigen presenting cells (APCs) (Krampera et al., 2003); JAG1-NOTCH interaction is shown to partake in the process (Liotta et al., 2008). Overall, immunosuppressive MSCs, later designated as MSC2, contribute to tissue healing and regeneration not only by impeding injury-driven autoimmune responses but also by educating macrophages, *via* IL-6, toward a proangiogenic M2 phenotype. M2 macrophages, therefore, tip the balance of T-cell responses in favor of immune regulation (anti-inflammatory T_{regs}) (Eggenhofer and Hoogduijn, 2012; Bernardo and Fibbe, 2013; Chung and Son, 2014).

Paradoxically, few reports have challenged the sole immunosuppressive dogma, offering a novel insight into the polarization of MSCs toward another "pro-inflammatory" type, in a similar fashion to "macrophage polarization" (Krampera, 2011). Waterman et al. designated this pro-inflammatory phenotype MSC1. Consequently, MSC2 identified its immunosuppressive counterpart (Waterman et al., 2010). The polarization into either phenotype is originally induced by TLRs and is ligand-specific. For instance, TLR3 and TLR4 priming by, respectively, poly(I:C) and lipopolysaccharide induce the MSC1 phenotype. In the process, downstream TLR signaling instigates pro-inflammatory secretome patterns (ILs, chemokine ligands, growth factors, apoptosis-inducing ligands) and impairs JAG1-NOTCH interaction between MSCs and T cells. This prevents MSC-mediated immunosuppression (Liotta et al., 2008; Romieu-Mourez et al., 2009) and permits IFN-γ-driven MSC antigen presentation to $CD4^+$ and $CD8^+$ T cells, thereby evoking immune activation (Chan et al., 2006; François et al., 2009). Similar observations are evident in co-cultures of MSCs and B cells, where the latter's proliferation, cytokine expression, and differentiation are improved (Rasmusson et al., 2007). On the other hand, immunosuppressive secretome patterns (IDO, prostaglandins) ensue downstream TLR signaling during MSC2 polarization (Waterman et al., 2010). Plus, MSC polarization is TLR type-specific. For instance, TLR4-primed MSCs polarize into MSC1, while TLR3 priming favors the immunosuppressive MSC2 profile in certain studies (Waterman et al., 2010) and MSC1 in others (Romieu-Mourez et al., 2009; Kota et al., 2014). Besides differences in TLR-ligand interactions and TLR type signaling, factors such as ligand concentrations (low concentrations license MSC1 phenotype), priming duration, microenvironment (cytokines, growth factors, stimulants), infections/diseases, tissue lesions, and MSC-T cell engagement timing are also at play in polarization licensing (Krampera, 2011; Strioga et al., 2012).

Despite its controversy, MSC polarization is thought to be part of tissue maintenance, where both distinct phenotypes homogeneously act in injury settings. To this extent, MSC1 may be important early in the process to drive chemotaxis and

subsequent reparative processes, while MSC2 may act later to resolve inflammatory tissue injury (Romieu-Mourez et al., 2009; Waterman et al., 2010). Similarly, the process can be exploited not only in regenerative medicine, which depends on inflammatory signals but also in cancer management which, as later discussed, depends on MSC inflammatory and migratory properties, both of which are induced by TLR priming (Waterman et al., 2010).

MSCs IN THERAPY: ACHIEVEMENTS AND PITFALLS

Regenerative Medicine

The regenerative and immunological assets of MSCs (see Sections "Regenerative Properties" and "Immunological Properties: A Paradigm") are widely exploited in degenerative settings. In animal models of myocardial infarction (MI), percutaneously injected allogeneic MSCs ameliorated ventricular fibrosis and scarring. Reduced infarct size, myocardial regeneration, enhanced cardiac metabolism and hemodynamics were also recorded (Amado et al., 2005; Cai et al., 2016). In *E. coli* endotoxin-injured human lungs, administration of allogeneic human MSCs reduced extravascular fluid and septal thickening, enhanced alveolar fluid transport, and restored the fluid balance of alveolar compartments (Lee et al., 2009). In rat models of retinal degeneration, the injection of MSCs into the subretinal space enhanced the viability of photoreceptor cells without replacing them (Inoue et al., 2007). In various mouse models of excisional wound healing, the application of MSC-conditioned media enriched in chemokines and cytokines increased the infiltration of macrophages and endothelial progenitor cells into the wounded area (Wu et al., 2007; Chen L. et al., 2008; Sasaki et al., 2008). Similar repair mechanisms induced by MSCs were described in the context of corneal injury (Roddy et al., 2011), colitis (Hayashi et al., 2008), neurodegenerative disorders (Tsai et al., 2019), hepatic injury (Anger et al., 2019), cardiac hypertrophy (Cai et al., 2015), and acute renal failure (Tögel et al., 2005).

A more sophisticated approach in regenerative medicine is MSC engineering on both genetic and architectural levels. In the former, MSC gene expression is altered through viral vector- or electroporation-mediated gene transfer; then their homing capacity to injured/ischemic sites is utilized for local delivery of overexpressed therapeutic genes. Examples on MSC-delivered genes are SDF-1 to ameliorate MI and ischemic brain injury (Penn and Khalil, 2008), glucagon-like peptide-1 to reduce amyloid deposition in Alzheimer's brains (Klinge et al., 2011), and IL-10 to restrain collagen-induced arthritis (Choi et al., 2008). Architectural MSC engineering involves cell culturing to obtain cellular sheets which can be further maintained in organ-specific stimulating media or assembled onto organ scaffolds to restore injured or defective tissue [e.g., bone regeneration (Yorukoglu et al., 2017) and spinal cord injury (Zeng et al., 2011) applications].

This preclinical success permitted the transit to human studies, with no records of toxicity or tumorigenicity with the use of GMP-compliant human MSCs suitable for clinical trial use (Tappenbeck et al., 2019). Up to this date, 921 clinical studies employing MSCs as the primary intervention have been registered, 704 of which date between 2011 and 2019 (U. S. National Library of Medicine, 2019). This booming, particularly in the last decade of the current century, is indicative of MSC potential to ameliorate a plethora of degenerative diseases (further elaborated in **Table 1**) (Wei et al., 2013), bearing simultaneously their major implication in physiological tissue maintenance (Murphy et al., 2002; Campeau et al., 2009).

Nevertheless, the clinical utility of MSCs faces various limitations including cell source availability (De Bari et al., 2001; Fitzsimmons et al., 2018) and specificity (De Ugarte et al., 2003; Sudres et al., 2006), clinical-grade production compliance with GMP (Sensebé, 2008), scalability (Fitzsimmons et al., 2018), administration timing (Tisato et al., 2007; Polchert et al., 2008; Le Blanc et al., 2008) and technique (Singh et al., 2016), engraftment rate (Fouillard et al., 2003; Le Blanc et al., 2008), polarization control (Polchert et al., 2008; Waterman et al., 2010), localization post-transplant (Law and Chaudhuri, 2013), and tissue persistence (Togel et al., 2005). This is explanatory of the limited number of MSC-based final stage trials and approved biopharmaceutical products. Until 2019, 50 studies have hit Phase III, with only 14 completed (NIH, 2019). Therefrom, 11 MSC-based therapies emanated (BioInformant, 2019) for the treatment of 7 degenerative and immune-based conditions including knee cartilage defects, hip joint avascular necrosis, and coronary angioplasty-reperfused acute MI (PHARMICELL, 2011; ANTEROGEN, 2012; Corestem, 2015; European Medicines Agency, 2017; MilliporeSigma, 2017; Orthocell, 2017; European Medicines Agency, 2018a; Regrow Biosciences®, 2019). However, none of these therapies are approved so far by the FDA (FDA, 2019a) which demands compelling clinical evidence from reliable well-controlled trials, stronger policy compliance, and extensive premarket reviews (Marks et al., 2017; FDA, 2019b).

Immune-Based Disorders

As discussed earlier (see section "Immunological Properties: A Paradigm"), MSCs possess immunomodulatory functions exhibited by their direct (cytokine-mediated) or indirect (T_{regs} modulation-mediated) inhibition of immune cells (Singer and Caplan, 2011; Haddad and Saldanha-Araujo, 2014). Those features are advantageous in treating immune-based disorders (Fitzsimmons et al., 2018). As such, therapies in this context exploit the immunomodulatory nature of MSC secretome which comprises NO, transforming growth factor-β, indoleamine 2,3-dioxygenase (IDO), prostaglandin E2, tumor necrosis factor-inducible gene 6 protein (TSG6), CCL-2, and PD-L1 among others. This immunomodulatory pool induces other immune cells to either modify/reprogram their response type (e.g., Th2 humoral-to-Th1 cellular immune response; dendritic cells (DCs) types 1 and 2 cytokine profile changes; and Th17-to-T_{reg} cell reprograming) (Aggarwal and Pittenger, 2005; Figueroa et al., 2012; Le Blanc and Mougiakakos, 2012) or generate immunosuppressive factors (Aggarwal and Pittenger, 2005; Han et al., 2012; Wei et al., 2013). As a result, MSCs are able to ameliorate pronounced immunity which is manifested in animal models of sepsis (Németh et al., 2009), autoimmune diseases

TABLE 1 | Clinical outcomes of MSC utility in regenerative therapy.

Clinical condition	Regenerative outcomes	References/NCT
Osteogenesis imperfecta	- Improvement of bone growth - Alleviation of fracture	Horwitz et al., 1999
Crohn's disease	Coverage of fistula	NCT01157650 (García-Olmo et al., 2005)
Deep thermal skin burns	- Restoration of wounds - Trigger of neoangiogenesis	Rasulov et al., 2005
Periodontal defects	- Reduction of pocket depth - Suppression of bleeding - Amelioration of teeth mobility	Yamada et al., 2006
Drug-resistant pulmonary tuberculosis	- Halting bacterial discharge - Resolution of tissular cavity	Erokhin et al., 2008
Liver cirrhosis	Amelioration of liver injury	NCT00420134 (Kharaziha et al., 2009) NCT00956891 (Peng et al., 2011)
Diabetic foot	Enhancement of perfusion	Lu et al., 2011
Chondral defects	- Pain alleviation - Increased activity scores - Improved histological façades	Kyriakidis et al., 2019
Maxillofacial bone defects	Increased bone cyst density	NCT01389661 (Redondo et al., 2018)

(Constantin et al., 2009; Rafei et al., 2009), neurodegenerative disorders (Ma et al., 2013), and GvHD (Polchert et al., 2008). In particular, the earliest advancement in MSC immune-based clinical applications was recorded in GvHD, a serious complication arising from MHC-mismatched allografts affecting 20–70% of transplant recipients (Lee et al., 2003; Socié and Ritz, 2014). MSC administration in this setting drew the attention of the scientific community in 2004 after the remarkable response against resistant grade IV acute GvHD of the gut and liver in a 9-year-old boy who received the first transplantation of haploidentical MSCs (Le Blanc et al., 2004). Other phase II/III clinical trials followed, reporting variable levels of effectiveness (Introna et al., 2014; Van Der Wagen et al., 2014). In 2009, an industry-led large-scale phase III study evaluated the use of allogeneic BM-derived MSCs for treating steroid-refractory GvHD (NCT00366145) which occurs after failure of first-line corticosteroid treatment and affects 30–80% of graft recipients giving patients a 10–30% chance for long time survival (Luft et al., 2011). Despite the lack of a significant difference in clinical outcomes between placebo and treatment groups, a sub-group analysis led to the conditional approval of Prochymal^{TM} for the treatment of pediatric steroid-refractory GvHD (Kurtzberg et al., 2010; Martin et al., 2010) in Canada, New Zealand (2012) (Chisholm et al., 2019), and Japan (2015) (Sipp, 2015). Although the pass of Prochymal^{TM} was considered a breakthrough for MSC-based therapies, it remained largely unattainable in Canada and New Zealand due to strict prescription regulations and high manufacturing cost (USD 200,000) (Bersenev, 2016; Chisholm et al., 2019).

Moreover, an official approval for Darvadstrocel (Alofisel), an adipose human MSC injection, was granted by the European commission for the management of complex perianal fistulas in adult patients with mildly or non-active luminal Crohn's disease (European Medicines Agency, 2018b; Panés et al., 2018). The approval emanated from a Phase III trial reporting that Darvadstrocel led to 50% combined remission, which was maintained after 1 year of treatment, in comparison with 34% in the control arm (Panés et al., 2016, 2018; Panes et al., 2017). Interestingly, several "orphan designation" approvals were granted by the European commission according to certain guidelines for the use of human MSCs in the treatment of GvHD, thromboangiitis obliterans (Buerger disease), and ALS (Yu et al., 2018; European Medicines Agency, 2019). Bader et al. (2018) the holders of one of the "orphan designations" in Germany (PEI.A.11748.01.1) for the treatment of steroid-resistant or treatment-refractory acute GvHD with their MSC preparation [MSC-Frankfurt am Main (MSC-FFM)], reported superior treatment outcomes in both adults and children as opposed to the limited efficacy of Prochymal^{TM} in children. According to their study, the effectiveness of MSC-FFM is due to donor selection in addition to strict collection and preparation processes (Bader et al., 2018), which yield adequate doses of MSCs with high batch-to-batch consistency (Elgaz et al., 2019). The distinguished data on MSC-FFM clearly elucidate the reasons behind the discrepancies (different survival rates and response levels to allogeneic MSC) and failures of other phase III clinical trials (Galipeau, 2013; Galipeau and Sensébé, 2018). In addition to the variation related to patient selection criteria (age, type, and disease clinical-grade), qualitative variabilities between MSC preparations play an important role. Lack of standardized manufacturing procedures such as donor heterogeneity, tissue origin variability (BM or adipose tissue), cell cryopreservation, culture expansion, administered dose and timing, heterogeneity of host inflammatory biomarkers, and immunogenicity are also among the variables (Galipeau, 2013; Squillaro et al., 2016; Galipeau and Sensébé, 2018). This also accords the fact that currently available MSC-based therapies for treating immune disorders – Remestemcel-L (Prochymal®) and TEMCELL® for GvHD (JCR Pharmaceuticals Co, 2015; Locatelli et al., 2017), NeuroNata-R® for ALS (Corestem, 2015), and Alofisel and

Cupistem® for Crohn's anal fistula (ANTEROGEN, 2012; MilliporeSigma, 2017; European Medicines Agency, 2018a) – are still not FDA-approved despite their worldwide regulatory approval (Bernardo and Fibbe, 2013). Henceforth, further standardization of clinical-grade MSCs will better serve future clinical trials and facilitate international clinical approval. Equally important is expanding the knowledge of MSC polarization mechanisms and fates post-delivery (Duijvestein et al., 2010; Lechanteur et al., 2016; Russell et al., 2018; Grégoire et al., 2019).

MSCs AND CANCER

Cancer Support or Suppression?

Cancer management using MSCs stems from the ability of these cells to home to tumors. Indeed, tumor tropism is a complex process involving multiple receptors and soluble factors. For example, SDF-1/C-X-C Motif Chemokine Receptor 4 (CXCR4), a chemokine/chemokine receptor axis involved in stem cell trafficking and cancer metastasis, plays a major role in MSC tumor infiltration (Phillips et al., 2003; Kucia et al., 2004). Tumor secretome induces MSC secretion of SDF-1, which activates in an autocrine fashion migratory signaling pathways (STAT3 and MAP kinase) and regulates cytoskeleton reorganization. According to certain studies, SDF-1 may also be part of tumor secretome (Gao et al., 2009; Lourenco et al., 2015). Overexpression of CXCR4 can, therefore, be considered therapeutically relevant due to its ability to augment MSC homing efficiency (Cheng et al., 2008). Macrophage Migration Inhibitory Factor (MIF), a pleotropic cytokine involved in multiple biological processes including tumor metastasis, is also implicated in MSC homing to tumors (Han et al., 2018). Like SDF-1, tumor-secreted MIF binds, among other receptors, to CXCR4 (G_i-protein coupled receptor) and activates MAP kinase signaling pathway, eventually inducing MSC migration through upregulating cell motility genes. Other cytokines/chemokine ligands secreted by tumors also act as MSC attractants and may even trigger MSC expression of CXCR4 (Lourenco et al., 2015). In a similar fashion to CXCR4 overexpression, tumor homing can be amplified by engineering MSCs to overexpress specific tumor-binding receptors (Komarova et al., 2010). The homing process can be tracked with various *in vivo* optical- and fluorescent-based imaging techniques (Reagan and Kaplan, 2011). It is important to note that a recent clinical study showed that BM-derived MSCs failed to home to prostate cancer sites, an observation linked to the absence of inflammatory signals, which usually dictate MSC migration (Schweizer et al., 2019). These data might also question the innateness of unmodified allogeneic MSCs to home to tumors without reprograming (Serakinci and Cagsin, 2019). Therefore, additional clinical studies are necessary for validating the facts.

What's more, current literature presents with data discrepancies as to whether unmodified MSCs support or suppress cancer growth. The first school reports that bearing the significant resemblance between mesenchymal tumor cells and MSCs in terms of proliferation/differentiation and pro-angiogenesis (Galiè et al., 2008), local mesenchymal progenitors or administered unmodified MSCs enhance cancer growth and metastasis, thus creating an "immunological sanctuary" in which tumor cells avoid immune surveillance (Hanahan and Weinberg, 2000; Krampera, 2011). These MSC properties of cancer support are originally licensed by tumor-infiltrating macrophages which establish a pro-inflammatory chemotactants-studded milieu (Coffelt et al., 2009; Rigo et al., 2010). This milieu evokes MSCs to (i) differentiate into highly proliferative myofibroblasts (Von Ahrens et al., 2017) and vascular cells (Peters et al., 2005), (ii) produce tumor-nurturing pro-angiogenic cytokines, miRNA, and exosomes (Roccaro et al., 2013; Zhang et al., 2013; Dong et al., 2018), (iii) secrete extracellular matrix-forming lysosomal oxidase (El-Haibi et al., 2012), (iv) provide a niche for malignant cells to thrive (Lin et al., 2019), and (v) adopt the immunomodulatory MSC2 phenotype (see section "Immunological Properties: A Paradigm") (Patel et al., 2010). As previously mentioned, MSC2 further polarizes macrophages into the M2 phenotype which is pro-tumorigenic (Rivera-Cruz et al., 2017).

Contrastingly, the other school reports that MSCs are anti-tumorigenic. This observation is upheld by studies on various tumor types which demonstrate size/metastasis reduction or inhibition of proliferation upon MSC injection (Klopp et al., 2011). In this course, MSCs home to tumor sites and reinforce their anti-neoplastic effects by interacting with cancer cells *via* cell-cell adhesive proteins (e.g., *E*-cadherin, Khakoo et al., 2006) or releasing soluble factors (Maestroni et al., 1999) [e.g., dickkopf-1, a Wnt signaling inhibitor (Qiao et al., 2008; Zhu et al., 2009)] and anti-proliferative miRNA-containing vesicles (Reza et al., 2016). Molecularly, the effects are sustained by (i) interference with pro-survival/proliferation signaling pathways [e.g., protein kinase Akt (Khakoo et al., 2006; Dasari et al., 2010a) and Wnt/β-catenin (Secchiero et al., 2010)], (ii) activation of apoptotic pathways (e.g., Smac/DIABLO) (Dasari et al., 2010b; Reza et al., 2016), and (iii) cell cycle arrest in G0/G1 phase (Lu et al., 2008; Cousin et al., 2009). The net signaling transduced favors an upregulation of cell cycle modulators (e.g., p21) and pro-apoptotic proteins (e.g., caspase 3, caspase 9, BAX) (Lu et al., 2008; Reza et al., 2016), opposed by a downregulation of anti-apoptotic mediators (e.g., XIAP, BCL2) (Dasari et al., 2010a,b; Reza et al., 2016). Besides, MSCs can inhibit neo-angiogenesis by forming gap junctions with endothelial cells and supplying them with reactive oxygen species, which induce their apoptosis (Otsu et al., 2009; Secchiero et al., 2010).

The inconsistencies between both schools are attributed to multiple factors including MSC source/preparation, administration timing/dose, polarization, and tumor variability (Klopp et al., 2011).

Therapeutic Management

Mesenchymal stem cell properties of tumor tropism and non-immunogenicity were used in antitumor research. The methodology involved transforming MSCs into a therapeutic platform able to inherently engraft in tumor architecture and genetically produce recombinant antitumor or antitumor immunity-driving molecules. Examples include tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Loebinger et al., 2009), C-X3-C motif chemokine ligand 1

(CX3CL1) (Xin et al., 2009), IFN-β (Studeny et al., 2002; Ren et al., 2008b), IFN-α (Ren et al., 2008a), IFN-γ (Li X. et al., 2006), IL-2 (Nakamura et al., 2004), and (modified) IL-12 (Chen X. et al., 2008; Seo et al., 2011). For example, a study by Li X. et al. (2006) showed that autologous MSCs derived from a leukemic patient then engineered to generate IFN-γ significantly inhibit the proliferation of leukemia cell lines and induce their apoptosis. In the same context, other genetic engineering-based methods include MSCs which express (i) replicative adenoviruses that infect cancer cells and induce oncolysis (e.g., ICOVIR5, Ad5-DNX-2401), (i) therapeutic gene-incorporating retroviral vectors, and (iii) suicidal gene-incorporating vectors. However, these efficient interventions confer toxicity and require simultaneous anti-retroviral drugs administration (Uchibori et al., 2009; Loebinger and Janes, 2010). Researchers also fostered MSC-based vehicles independent of genetic engineering. Those exploit the innateness of MSCs to uptake drugs *in vitro* allegedly through Golgi-derived vesicles (drug uptake mechanisms are insufficiently characterized and are not confined to MSCs, Girdlestone, 2016). Although their drug sensitivity varies according to cell source, MSCs rapidly internalize sufficient drug molecules, such that following MSC administration to animal models, captured drugs are slowly and sufficiently released in their original form (active or prodrug) into tumor vicinity (Pessina et al., 2011; Bonomi et al., 2013; Coccè et al., 2017). Likewise, MSCs can be loaded with prodrugs to effectively inhibit cancer growth (Levy et al., 2016). These observations led to few human cancer management studies, which are still taking their baby steps toward clinical efficacy. For example, in a phase I/II study (TREAT-ME1), autologous MSCs were isolated from patients according to GMP standards and transfected with replication-incompetent retroviral vectors to generate MSC_apceth_101, an investigational medicinal product containing a therapeutic promoter-gene construct aimed to treat advanced gastrointestinal tumors. The trial, however, did not advance to therapeutic confirmatory phase III due to adverse events and lack of disease amelioration (EudraCT Number 2012-003741-15) (Niess et al., 2015). Other challenges in MSC-based anticancer treatment are, paradoxically, cancer enhancement (Karnoub et al., 2007) even with induced anti-tumor immunity (vaccination) (Krampera et al., 2007) as well as insufficient cell homing to tumors to guarantee efficient delivery of therapeutic agents (Schweizer et al., 2019).

Cancer Vaccination

Vaccination is a robust, safe, and cost-effective preventative or therapeutic method against pathogenic diseases (Tomchuck et al., 2012). While therapeutic vaccines induce cell-mediated immunity and are used to eliminate existing pathogens/lesions or prevent their progression, preventative vaccines trigger humoral immunity (serum antibody generation) for prophylaxis of futuristic pathogens/lesions (Nayereh and Khadem, 2012).

Traditionally, vaccine development employs the attenuation or inactivation of a pathogen to create long-term immune memory and/or mount a durable immune response against intact pathogens. Although efficient against several mortal diseases (smallpox, diphtheria, polio, measles), vaccines still lack in offering protection against their ilk (HIV, malaria, common cold, tuberculosis) due to robust microbial antigen shifting or difficult intracellular pathogen accessibility which complicates the selection of target antigens. In addition to intact antigenic peptides, alternative vaccines exist, such as *in situ* antigen production or presentation using plasmid vectors (DNA) and antigen-pulsed host cells (APCs, MSCs). However, they have not yet achieved any clinical benefits, mainly due to their low immunogenicity (MacGregor et al., 1998; Tomchuck et al., 2012; Hobernik and Bros, 2018).

The notion of cancer vaccination, an increasingly active research topic, stems from the inherent role of the immune system to eliminate cancer cells and the possibility thereof to develop immune enhancing therapies to adequately eradicate tumors (Butterfield, 2015). For this purpose, synthetic neo-antigens (Ott et al., 2017) as well as DNA- and cellular-based platforms exercising foreign antigen/cytokine production or expression have been used to devise tumor epitope-specific vaccines or instigate anti-tumor T-cell reactivity *in vitro*. This strategy was efficient as an *in vivo* cancer immunotherapy, especially if the regimen involves immune-checkpoint blocking antibodies to enhance effector T cells function by blocking their inhibitory receptors (PD-1 and CTLA-4) (Schumacher and Schreiber, 2015; Wraith, 2017).

Among the best candidates for cellular-based vaccine platforms, DCs are especially efficient APCs and primers of immune responses (Guéry and Adorini, 1995; Janikashvili et al., 2010; Le et al., 2010; Palucka and Banchereau, 2013). Plus, DCs are considered natural adjuvants as they can modulate and interconnect innate adaptive immune responses through their surface molecules and secretome (Mellman and Steinman, 2001; Steinman, 2001). In clinic, Sipuleucel-T, branded as Provenge, is the first and only FDA-approved DC vaccine for the treatment of asymptomatic or minimally symptomatic metastatic and castration-resistant prostate cancer (Small et al., 2006; Higano et al., 2010; Anassi and Ndefo, 2011; Cheever and Higano, 2011). However, other attempts at DC vaccine introduction in animal and clinical studies faced more complications than anticipated, demonstrating immense variation in reported outcomes (Le et al., 2010; Robson et al., 2010; Mantia-Smaldone and Chu, 2013). Reasons for such clinical discrepancies can be attributed to DC non-standardized *ex vivo* preparation and administration protocols which entail multiple variabilities at the level of (i) DCs source/phenotype, (ii) DCs maturation stimulus used, (iii) nature/procedure for antigen loading, (iv) route of administration, and (v) dose (Nicolette et al., 2007). Besides, their high production cost, low production grade, limited effectiveness, and immunogenicity hamper their clinical acceptance and advancement (Chambers and Neumann, 2011; Bhargava et al., 2012; Datta et al., 2014; Jarosławski and Toumi, 2015; Wei et al., 2015). Therefore, the search for other cellular-based vaccines with potentially better performance in these criteria was necessary, and so MSCs came forth as a fit vaccine platform in this regard.

MSCs can elicit general and/or antigen-specific immunity, without being immunogenic themselves, depending on three assets (**Figure 1**). First, MSCs are context-specific

pro-inflammatory (see Section "Immunological Properties: A Paradigm"), a property which ultimately renders them enhancers of humoral and cellular immunity. Second, MSCs are genetically modifiable, thereby representing suitable vehicles for producing and secreting cytokines or soluble antigens which evoke robust immune responses. A report by Wei et al. (2011) follows this scenario albeit to a certain extent. In the details, the group devised a combined vaccine consisting of a fusion protein vaccine which targets E7 tumor antigen and immortalized human MSCs designed to express E7 antigens. Compared to

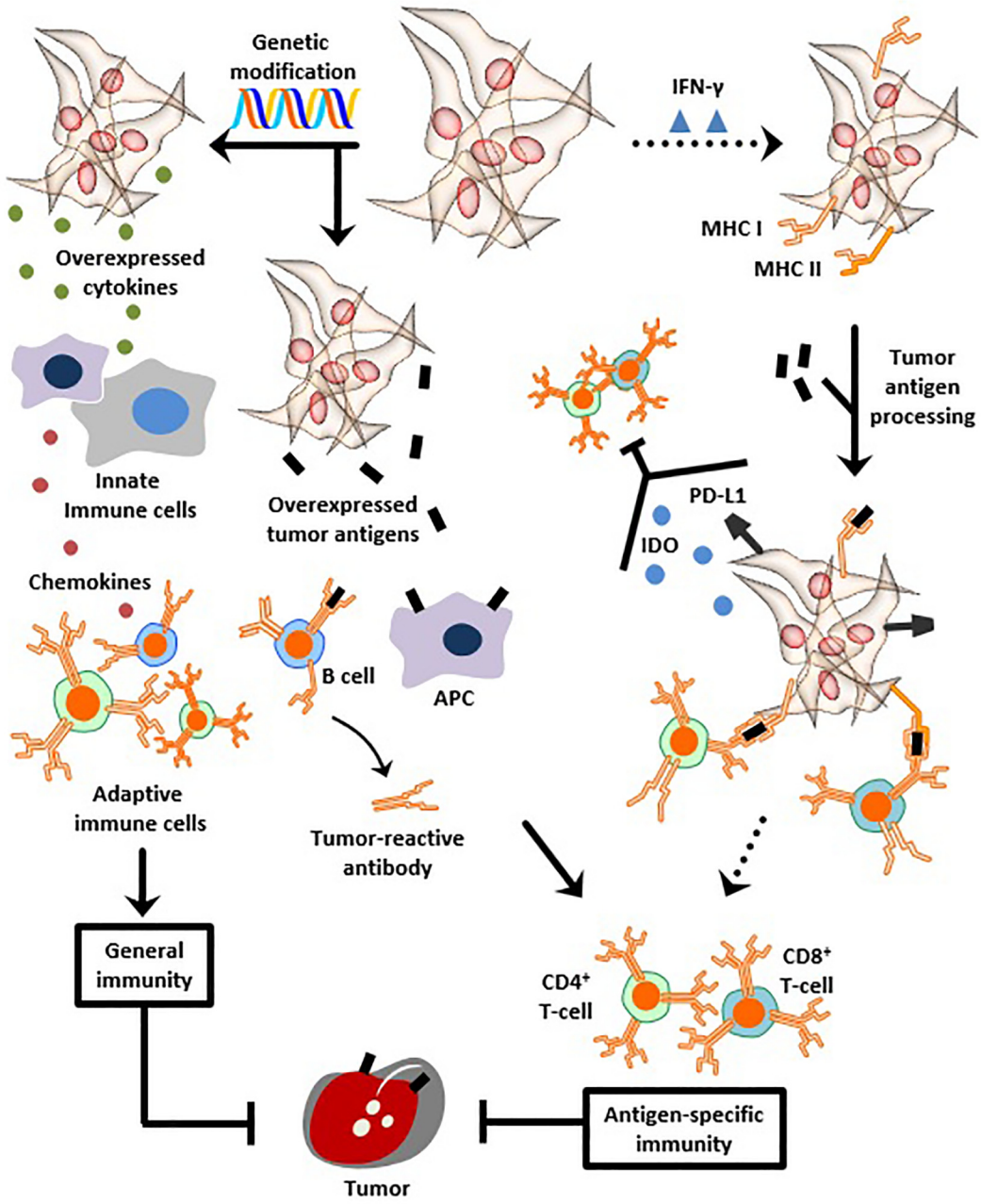

FIGURE 1 | MSCs as anti-cancer vaccines. MSCs can be genetically modified to overexpress cytokines to instigate innate and adaptive immunity, as a means to protect against neoplasms. Genetic modification can be also used to overexpress tumor antigens and instill anti-tumor humoral and cellular immunity. Likewise, dose- and time-dependent exposure to IFN-γ transforms MSCs, albeit transiently, into APCs capable of providing antigen-specific immune protection. This occurs through induction of MHC class I and II expression, followed by tumor antigen processing and MHC-mediated presentation to T-cells. Despite IFN-γ-induced antigen presentation, other observations report that MSCs simultaneously up-regulate PD-L1 and secrete IDO, both of which inhibit T-cells. Henceforth, overcoming the transient and temporary antigen presenting properties of IFN-γ-exposed MSCs is necessary to achieve vigorous stability and abundance of presented neoantigens, thus helping to create a clinically efficient anti-cancer vaccine.

the fusion protein vaccine alone, the combined vaccine elicited significantly stronger tumoricidal immunological reactions when administered to subcutaneous and lung metastasis mice models. The authors propose that those effects ensue after the tagging of tumor cells with E7 antigens released by infiltrating MSCs along with the instigation of humoral immunity by the fusion protein vaccine. The generated anti-E7 antibodies were, therefore, able to recognize tumors and eventually suppress their growth (Wei et al., 2011). Third and most importantly, MSCs can act as APCs capable of processing and presenting exogenous antigens to activate immune cells; this asset surfaces in response to IFN-γ treatment which induces MSC expression of MHCI/II molecules (Majumdar et al., 2003; Stagg et al., 2006; François et al., 2009; Tomchuck et al., 2012; van Megen et al., 2019). This property was exploited in cancer vaccination studies, which are hitherto limited. For instance, mice vaccinated with IFN-γ-licensed MSCs pulsed with ovalbumin antigen are completely protected when challenged with ovalbumin-expressing E.G7 lymphatic tumors (Stagg et al., 2006; François et al., 2009; Stagg and Galipeau, 2013). Protection against tumors using IFN-γ-treated MSCs is conferred through MHC I upregulation, MHC II induction, and, in part, through the upregulation of the antigen processing machinery responsible for translocation of processed antigens into the ER before trafficking toward the plasma membrane. Overall, this enhances antigen presentation to $CD4^+$ T-cells (MHC II-restricted) and cross-presentation to $CD8^+$ T-cells (MHC I-restricted), both of which respond by increased activation and proliferation (François et al., 2009). Another study further shows that the strong anti-tumorigenic immune responses evoked by IFN-γ-treated MSCs involve CD80 (co-stimulatory molecule) and MHC class II- but not class I-mediated antigen presentation, albeit the induction of strong $CD8^+$ T-cell responses *in vivo*. The authors argue that antigen cross-presentation which is not observed *in vitro* can develop *in vivo* not in MSCs themselves but in other host APCs which can acquire their antigens from MSCs in a process termed cross-priming (Stagg et al., 2006). Paralleling, a recent study reports that although IFN-γ-licensed human MSCs uptake and process antigens and upregulate MHC class II but not CD80, their pro-inflammatory secretome remains intact. Importantly, the study also shows that despite their IFN-γ-induced antigen presentation, MSCs inhibit autoreactive T-cells, an observation associated with PD-L1 upregulation and IDO secretion (**Figure 1**). The inhibitory effect even lasted beyond the removal of MSCs and the introduction of activation signals (antigen-pulsed DCs) (van Megen et al., 2019). However, in another report, IFN-γ-induced upregulation of PD-L1 on antigen-presenting MSCs is believed to be tied to T-cell induction rather than inhibition (Stagg et al., 2006). This discrepancy adds to the many layers of MSC character.

A side note, more prevalent is the therapeutic induction of general rather than antigen-specific anti-tumor immunity (Wei et al., 2011). This is evident in the variety of researched MSC vaccines which, as mentioned in Section "Cancer Support or Suppression?", genetically express recombinant immunostimulatory molecules (Studeny et al., 2002; Nakamura et al., 2004; Li X. et al., 2006; Chen X. et al., 2008; Ren et al., 2008a,b; Xin et al., 2009; Seo et al., 2011). Furthermore, while prophylactic MSC-based anti-cancer vaccines are more strenuous to devise compared to their therapeutic counterparts (tumor antigens have unique expression patterns), prophylactic MSC-based anti-microbial vaccines attain their purpose of triggering antigen-specific humoral and adaptive immunity against, respectively, HIV and tetanus (Tomchuck et al., 2012). In sharp contrast, the clinical knowledge available thus far on MSCs as cancer vaccines is, unfortunately, insufficient to advance further their proof of concept. **Table 2** demonstrates the only registered human studies utilizing MSC-based anti-cancer therapeutic vaccines.

CONCLUSION AND FUTURE RECOMMENDATIONS

In summary, due to their regenerative abilities, immunomodulation, tumor homing, and multiple other advantages, MSCs have demonstrated unprecedented potential in cellular therapy *in vivo*, specifically against immunological, degenerative, and cancer pathologies. Therefrom, their international clinical approval is a matter of time. Likewise, the growing notion of MSC vaccination has demonstrated promising potential for cancer prophylaxis or therapy, despite the scarcity of relevant clinical data. Reflecting on the reasons behind this, it is legit to say that MSC vaccine-based cancer research requires further understanding not only of the intervention itself but also of the multiple intricacies characterizing the interplay between MSCs and both tumors and immune cells. More specifically, an efficient MSC-based anti-cancer vaccine first needs to overcome the transient and temporary antigen presenting properties observed after IFN-γ exposure. As mentioned earlier, our current understanding of MSCs as APCs is indispensable of the dose-dependent temporary exposure to IFN-γ alongside the phenotypic responses arising therefrom (**Figure 1**) (Chan et al., 2006).

Other immunomodulatory observations upon IFN-γ licensing of antigen-pulsed MSCs are also recorded (van Megen et al., 2019). For example, IFN-γ treatment is associated

TABLE 2 | The clinical trials assessing MSC-based vaccines for cancer treatment.

NCT	Study phase	Start date	Vaccine properties	Cancer type	Results/Status
02079324	1	2014	aka GX-051, IL-12-expressing, induces IFN-γ production and subsequently cellular immunity	Head and neck	Unknown
02530047	1	2016	IFN-β-expressing, immunostimulatory	Ovarian	Completed, no disclosed results

with the upregulation of B7-H1 (PD-L1) (Krampera et al., 2006). These intricacies show that we need to understand the antigen presenting properties of MSCs beyond IFN-γ. Bypassing this conditional APC state thus warrants vigorous stability and abundance of MHCI/II-presented neo-antigens. Also, sufficient molecular knowledge of protein translation, proteasome degradation of proteins, endoplasmic reticulum transport, and affinity for MHC molecules – all in direct link to antigen presentation – is necessary (Schumacher and Schreiber, 2015). Equally important is realizing cancer complexity and the burden of tumor stromal cells in oncological settings (Brahmer et al., 2012; Joyce and Fearon, 2015). Since tumor stromal cells induce massive alterations in local metabolome and secretome profiles and are thought to ensnare $CD8^+$ T cells and other APCs (Joyce and Fearon, 2015; Hammerich et al., 2019) in the tumor microenvironment, their contribution to immune suppression, evasion, and unresponsiveness to immune-checkpoint blockers (Brahmer et al., 2012) should be investigated more in depth. Consequently, surpassing these obstacles, perhaps by instilling potent and stable antigen cross-presentation properties in properly treated MSCs, as well as ensuring that adaptive immunity is actively triggered and always one step ahead of tumor intelligence, will allow us to harness the full capacity of MSCs as robust APCs.

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Conflict of Interest: RS is the founder of IntelliStem Technologies Inc. (Toronto, ON, Canada).

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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Patient-Specific Retinal Organoids Recapitulate Disease Features of Late-Onset Retinitis Pigmentosa

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Although an increasing number of disease genes have been identified, the exact cellular mechanisms of retinitis pigmentosa (RP) remain largely unclear. Retinal organoids (ROs) derived from the induced pluripotent stem cells (iPSCs) of patients provide a potential but unvalidated platform for deciphering disease mechanisms and an advantageous tool for preclinical testing of new treatments. Notably, early-onset RP has been extensively recapitulated by patient-iPSC-derived ROs. However, it remains a challenge to model late-onset disease in a dish due to its chronicity, complexity, and instability. Here, we generated ROs from late-onset RP proband-derived iPSCs harboring a *PDE6B* mutation. Transcriptome analysis revealed a remarkably distinct gene expression profile in the patient ROs at differentiation day (D) 230. Changes in the expression genes regulating cGMP hydrolysis prompted the elevation of cGMP levels, which was verified by a cGMP enzyme-linked immunosorbent assay (ELISA) in patient ROs. Furthermore, significantly higher cGMP levels in patient ROs than in control ROs at D193 and D230 might lead to impaired formation of synaptic connections and the connecting cilium in photoreceptor cells. In this study, we established the first late-onset RP model with a consistent phenotype using an *in vitro* cell culture system and provided new insights into the PDE6B-related mechanism of RP.

Keywords: late onset, retinitis pigmentosa, iPSCs, PDE6B, retinal organoids

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INTRODUCTION

Retinitis pigmentosa (RP) has a prevalence of approximately one in 4000, affecting approximately 1.5 million individuals in total worldwide (Hartong et al., 2006). RP, a hereditary retinal degenerative disease, is characterized by irreversible loss of photoreceptor cells. At the cellular level, this syndrome correlates with the primary degeneration of rod photoreceptors. In the progressive stage of this disease, the cone photoreceptor system might be affected, which eventually causes daytime vision impairment or complete visual loss (Hartong et al., 2006). Mutations in the gene encoding the beta subunit of rod cGMP-phosphodiesterase type 6 (*PDE6B*) account for 4 to 5% of autosomal recessive RP (Danciger et al., 1995). Recently, *PDE6B* mutation was identified as disease-causing gene in 2.4% patients from a large cohort of 1095 patients with RP (Khateb et al., 2019). Prevalent mouse models harboring *Pde6b* mutations, *rd1*, and *rd10* have been widely used for deciphering pathogenesis and examining novel therapeutics for RP (Bowes et al., 1990; Barhoum et al., 2008).

Progressive loss of rod and cone photoreceptors accompanied by elevated concentration of cGMP and an influx of Ca^{2+} through the CNG channel can be observed in animal RP models, which resemble the phenotype seen in human disease. However, interspecies variation, such as differences in retinal structure and life rhythm, limits the use of animal models in mechanistic studies and especially in preclinical studies. Conflicting results have been observed in mice and canines (Frasson et al., 1999; Pearce-Kelling et al., 2001) when used for drug testing (calcium channel blockers). Recently, patient-specific induced pluripotent stem cells (iPSCs) combined with differentiation technology have provided an unlimited cell source for disease modeling and can mimic primary disease tissue after appropriate induction (Rossi et al., 2018; Ballard et al., 2019; Foltz and Clegg, 2019). Retinal organoids (ROs) have been successfully generated from human embryonic stem cells (ESCs) and iPSCs (Llonch et al., 2018; Jin et al., 2019). These ROs, generated in dishes, display proper neural retina markers (DiStefano et al., 2018), form retinal stratification with apical-basal polarity (Hasegawa et al., 2016), and even possess light responses (Zhong et al., 2014; Hallam et al., 2018). Furthermore, retinogenesis can be recapitulated in ROs derived from pluripotent stem cells and analyzed by transcriptomic analysis (Kaewkhaw et al., 2015; Voelkner et al., 2016), and single-cell RNA-seq (Collin et al., 2019; Kim et al., 2019; Mao et al., 2019). Thus, patient-specific iPSCs combined with a three-dimensional (3D) culture system can generate unlimited cell sources for personalized drug testing and organ replacement (Shimada et al., 2017; Buskin et al., 2018; Deng et al., 2018; Guo et al., 2019; Li et al., 2019).

Disease models with ROs for early-onset retinal degeneration have been established by us and others (Shimada et al., 2017; Deng et al., 2018). Defects in retinal development and ciliopathy in photoreceptors have been found in *RPGR* and *CEP290* mutant ROs. In the present study, we established a patient-iPSC-derived RO model harboring a *PDE6B* mutation. Transcriptomic analysis and morphology demonstrate relatively normal retinal development of PDE6B patient ROs compared with control ROs before differentiation day (D) 180. At D230, however, mislocated rod photoreceptors can be found in the inner layer of the patient ROs. The retention of the rod photoreceptor might be the result of cGMP accumulation, which can be found at D193 and reaches a higher level at D230. Thus, we show that patient ROs recapitulate the late-onset disease phenotype at approximately D230 and resemble the impairments in photoreceptor maturation seen in RP. This patient-specific model could provide a promising platform for a disease model that is more advantageous than animal models.

MATERIALS AND METHODS

Isolation and Expansion of Mononuclear Cells

To isolate mononuclear cells, 10 ml of peripheral blood was collected from a proband patient with a *PDE6B* mutation and an unaffected volunteer with no retinal disease and no mutation at any RP related genes following the instructions for Lymphoprep (Cat. #07851; Stemcell Technologies, Norway). Cells were maintained in StemSpan SFEM II medium (Cat. #09605; Stemcell Technologies, Canada) supplied with StemSpan Erythroid Expansion Supplement (Cat. #02692; Stemcell Technologies, Canada) for seven days for cell expansion. Both the patient and control volunteers were signed informed content, which has been approved by the Ethics Committee of the Eye Hospital of Wenzhou Medical University. The patient and control iPSCs were included in the hiPSC bank of Institute of Stem Cell Research, Wenzhou Medical University and designated as 502-PBMC-PDE6B-01 and 502-PBMC-HEALTHY-01.

Generation and Characterization of Human iPSCs

Mononuclear cells were collected and subjected to a plasmid-based reprogramming system. To generate iPSCs, Episomal iPSC Reprogramming Plasmids (Cat. #SC900A-1; System Biosciences, United States) expressing four Yamanaka factors (Oct4, Sox2, Lin28, Klf4, and L-Myc), p53shRNA, and a miR-302/367 cluster were transformed into mononuclear cells using 4D Nucleofector (LONZA). iPSC colonies appeared after approximately 25 days. In this study, three independent colonies were picked for expansion and three colonies were used for the stepwise differentiation. Genomic DNA was extracted for amplification of the *PDE6B* gene and PCR products were subjected to Sanger sequencing.

Human iPSCs were maintained on Matrigel-coated (Cat. #356231; BD Corning, United States) dishes in mTeSR-E8 medium (Cat. #05940; Stemcell Technologies, Canada) and passaged with 0.5 μM EDTA (Cat. #AM9261; Ambion, United States). For trilineage differentiation (Deng et al., 2018), iPSC aggregates (5 to 10 cells) were cultured in suspension within DMEM/F12 medium (Cat. #11320033; Gibco, United States) supplied with 20% KSR (Cat. #10828028; Gibco, United States), 0.1 mM 2-mercaptoethanol (Cat. #M7522; Sigma, United States), 0.1 mM non-essential amino acids (Cat. #M7145; Sigma, United Kingdom), 2 mM GlutaMAX (Cat. #35050061; Life Technologies, Japan), 10 mM Y-27632 (Cat. #S1049; Selleckchem, United States), 100 U/ml penicillin, and 100 mg/ml streptomycin (Cat. #15140-122; Gibco, United States) for 8 days to form embryoid bodies. For spontaneous differentiation, the embryoid bodies were transferred into DMEM/F12 medium supplied with 10% FBS (Cat. #04-002-1A; Biological Industries), 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 2 mM GlutaMAX 100 U/ml penicillin and 100 mg/ml streptomycin, and attached to glass slides coated with 0.1% gelatin (Cat. #ES-006-B; Millipore, Germany) for 10 days.

Differentiation of 3D ROs

Three colonies, from the patient and control each were subjected to ROs generation. ROs were generated from iPSCs following a published method (Nakano et al., 2012) with slight modification. Briefly, iPSCs were dissociated into single cells with TrypLE Express (Cat. #12563-011; Gibco, Denmark)

containing 0.05 mg/ml DNase I (Cat. #11284932001; Roche) and 20 μM Y-27632 resuspended in retinal differentiation medium I, and G-MEM medium (Cat. #11710-035; Gibco, United States) supplied with 20% KSR, 3 μM IWR1e (Cat. #681669; Merck Millipore, United States), 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1 mM pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin. Approximately 12,000 cells in 100 μl were added to each well and reaggregated in a V-bottom low-cell-adhesion 96-well plate (Cat. #MS-9096V; Sumitomo Bakelite, Japan). The differentiation starting day was defined as day 0; 20 μM Y-27632 was added to the retinal differentiation medium I on day 0, and half the medium was exchanged with fresh retinal differentiation medium I on day 6. From day 2 to day 18, Matrigel was added in a final proportion of 1% v/v. On day 12, the cell aggregates were transferred to petri dishes in retinal differentiation medium II, G-MEM medium supplied with 10% (v/v) FBS, 100 nM SAG (Cat. #ALX-270-426-M001; Enzo Life Sciences, United States), 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1 mM pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin. From day 18, each cell aggregate was cut into 3–5 small pieces and were maintained in neural retina culture medium, containing DMEM/F12-GlutaMAX medium (Cat. #10565-018; Gibco, United States) supplied with 10% (v/v) FBS, N2 supplement (Cat. #17502-048; Gibco, United States), 0.5 μM retinoic acid (Cat. #R2625; Sigma, United States), 12.5 μg/ml taurine (Cat. #T0625; Sigma, Japan), 100 U/ml penicillin and 100 mg/ml streptomycin. From days 18 to 30, about 50 organoids were maintained in a 100 mm Petri dish with 15–20 ml medium. After day 45, number of organoid in each dish was reduced to 30 with 15–20 ml medium. The neural retina culture medium was used from day 18 since after and changed weekly.

Immunostaining and Imaging

Cells from trilineage differentiation were stained with markers representing the endoderm, mesoderm, and ectoderm. Cryosections of the ROs were stained with neural retina cell markers. Briefly, the cells were fixed in Immunol Staining Fix Solution (Cat. # P0098; Beyotime, China) for 10 min. Then, the cells or cryosections were permeabilized with 0.2% Triton X-100 (Cat. #A600198-0500; Sangon Biotech, China) for 10 min at room temperature. After rinsing twice with PBS, the cells or cryosections were incubated in blocking buffer (PBS containing 4% BSA) for 1 h to block non-specific antibody binding. Then, the cells or cryosections were incubated in blocking buffer with a primary antibody, including OCT4 (Cat. #ab18976; Abcam), SOX2 (Cat. #sc-17319; Santa Cruz), NANOG (Cat. #ab80892; Abcam), SSEA4 (Cat. #ab16287; Abcam), GFAP (Cat. #HPA056030; Sigma), α-SMA (Cat. #A5228; Sigma), AFP (Cat. #MAB1368; R&D System), PAX6 (Cat. #PRB-278P; Covance), CRX (Cat. #H00001406-M02; Abnova), NRL (Cat. #AF2945; R&D System), Recoverin (Cat. #AB5585; Millipore), Brn3b (Cat. #sc-514474; Santa Cruz), VSX2 (Cat. #sc-21690; Santa Cruz), RHO (Cat. #O4886; Sigma), M-opsin (Cat. #AB5405; Millipore), ARL13B (Cat. #17711-1-AP; Proteintech), PKCα (Cat. #P4334; Sigma), and Synaptophysin (Cat. #MA1-213; Invitrogen) for 12 h at 4°C. After being washed twice with PBS, the cells or cryosections were stained for 1 h with a fluorescence-conjugated secondary antibody, including Alexa Fluor 488 donkey anti-rabbit IgG (H + L) (Cat. #A-21206; Invitrogen), Alexa Fluor Plus 488 goat anti-mouse IgG (H + L) (Cat. #A32723; Invitrogen), and Alexa Fluor 594 donkey anti-mouse IgG (H + L) (Cat. #A21203; Invitrogen). After washing twice with PBS, cells or cryosections were stained for 10 min with DAPI (Cat. #GD3408; Genview). Images were recorded with an inverted confocal microscope (Leica SP8; Germany).

Transcriptome Analysis and Quantitative PCR

For RNA-seq, total RNA from 3 to 5 organoids from two independent differentiations was isolated using TRIzol Reagent (Cat. #15596018; Invitrogen) and an RNeasy Plus Mini Kit (Cat. #74104; Qiagen) following the manufacturer's instructions. RNA concentration was determined with a Nanodrop 2000 (ThermoFisher Scientific, United States). RNA-seq data was analyzed using BMKCloud[1]. Briefly, sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (#E7530L, NEB, United States) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample (Annoroad Gene Technology, China). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumia) according to the manufacturer's instructions. Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. Hisat2 tools soft were used to map with reference genome.

For qPCR, total RNA from 6 to 10 organoids from two independent differentiations was isolated using TRIzol Reagent (Cat. #15596018; Invitrogen) following the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega; Cat. #M1705). The cDNA samples were used for quantitative PCR in a real-time PCR system (LightCycler 96 System; Roche, Mannheim, Germany) using a master mix (FastStart Universal SYBR Green Master [ROX]; Roche). Primer sequences are listed in **Supplementary Table S1**. The expression levels were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the deltadelta Ct method.

Enzyme-Linked Immunosorbent Assay (ELISA) of cGMP

cGMP levels in ROs were measured by ELISA using a cGMP direct immunoassay kit (Cat. #ab65356; Abcam). The ROs were homogenized in 0.1M HCl, and the supernatant was used for

[1]www.biocloud.net

cGMP examination following the manufacturer's instructions. The absorbance at 450 nm was measured using a SpectraMax M5 microplate reader (Molecular Devices). Each sample was prepared from three organoids that were selected with or cut into similar sizes. Each sample was detected in triplicate, and the results are an average of three or six independent experiments.

Statistical Analysis

Quantitative analysis of each dataset was performed by two individuals who were blinded to the samples. For the analysis of rod and cone number, images of 1.29 mm × 1.29 mm in square and 15 μm confocal Z-stacks from three organoids (six images each for rod and four images for cone) of two independent differentiations from patient and control group each were acquired. Similarly, for cilia analysis, total images of 144.72 μm × 144.72 μm in square and 15 μm confocal Z-stacks from three organoids (three images each) of two independent differentiations were performed. Cilia were defined by ARL13B positive signals. Statistical analysis was performed with GraphPad Prism version 5.0 and SPSS 16.0. A two-tailed *t*-test or one-way ANOVA was used for comparison. A statistically significant difference was defined as $P < 0.05$.

RESULTS

Generation and Characterization of Patient-Specific iPSCs Harboring a *PDE6B* Mutation

In a consanguineous family, a proband patient with night blindness was diagnosed with RP in his late 40s (**Figure 1A**). Severe loss of the photoreceptor layer was observed using optical coherence tomography (OCT) compared with the healthy contemporary control OCT (**Figure 1B**). To identify its genetic cause, targeted exon sequencing of 164 known retinal disease genes (Huang et al., 2015) was performed. A homozygous mutation within the *PDE6B* gene (c.694G > A) was identified, and direct Sanger sequencing validated the mutation (**Figure 1C**). This mutation was localized in the intervening sequence of two GAF domains arranged in tandem, causing the glutamate to change to lysine (232E-K) (**Figures 1D,E**). This amino acid is conserved across various species (**Figure 1F**).

Peripheral blood mononuclear cells isolated from the PDE6B patient and an unaffected control individual were reprogrammed with a non-integrated method (**Figure 2A**). Three iPSC lines were derived from the patient and control and tested for AP staining and pluripotency markers (**Figures 2B,C**). Trilineage differentiation demonstrated that the iPSCs from the patient and control were capable of differentiation into three germ layers (**Figure 2D**). These results demonstrated the successful generation of patient-specific iPSCs.

Generation of Long-Lived ROs for Disease Modeling

Human ROs were generated following the method described by Nakano et al. (2012) and Rossi et al. (2018). ROs derived from both patient and control iPSCs exhibited similar morphology and neural retina structure until D180 (**Figure 3A**). Structurally, the stratified architecture was clearly visible in phase-contrast imaging at D180 (**Figure 3A**). Brn3b expresses in retinal ganglion cells (RGCs) and VSX2 expresses in retinal progenitor cells (RPCs). PAX6 has a broad expression pattern, including RPCs, RGCs, amacrine and horizontal cells. Here, we chose Brn3b, PAX6 and VSX2 as RGC and RPC marker at the early-stage of retinal organoid. The expression patterns of Brn3b, PAX6 and VSX2 were similar in patient and control ROs at D45 (**Figure 3B**). By D45, neural retinal structures were formed and showed the expression of RGCs in the inner layer of the organoid.

CRX expresses in photoreceptor precursors and mature photoreceptors and NRL expresses in rod precursors and mature rods. At early-stage of ROs, CRX and NRL were identified as photoreceptor precursors and rod precursor marker. No obvious differences were found in CRX expression at D60 or in NRL expression at D120 (**Figure 3B**). The signal of the photoreceptor-specific marker CRX was spread through the inner and outer layers at D60 (**Figure 3B**) and aligned mainly at the outer layer at D105 (**Figure 3C**), indicating a stratified architecture similar to the early postnatal retina *in vivo* (Hendrickson et al., 2008). The expression of RCVRN appeared at approximately D60 and increased with the development of the ROs (**Figure 3C**). At D105, most of the CRX^+ photoreceptor cells were coexpressed with RCVRN (**Figure 3C**), consistent with previous reports (Nakano et al., 2012). Immunostaining analysis showed that the ROs recapitulated a synchronized onset and progression of retinogenesis, and no obvious differences were observed in retinal progenitors or photoreceptor precursors between the patient and control ROs before D120. As RO development progressed, however, the expression of a rod photoreceptor marker (RHO) and rod bipolar cell marker (PKCα) showed obvious differences in patient ROs compared with control ROs at D180 (**Figure 3C**). Thus, patient ROs were successfully generated and showed obvious rod photoreceptor defects.

Distinct Transcriptome of Late-Stage *PDE6B* Patient ROs

To investigate the transcriptional effects of the *PDE6B* c.694G > A mutation, comparison of bulk RNA-seq profiles were performed in patient and control ROs, which were collected from the mid-stage (D90, 120, 150, and 180) to late-stage (D230). A total of 2578 differentially expressed genes were found between patient and control ROs at D230 compared with the other tested time points (**Figure 4A**). Among these genes, 968 genes were not found at other time points (**Figure 4B**). Principal components analysis (PCA) of RNA-seq data indicated that the most variance was in PC1, which separated the patient ROs at D230 from the other samples (**Figure 4C**). This result was consistent with previous morphology and immunostaining results, indicating that a mild influence was found in mid-stage patient ROs. Furthermore, correlation analysis also confirmed the difference of patient

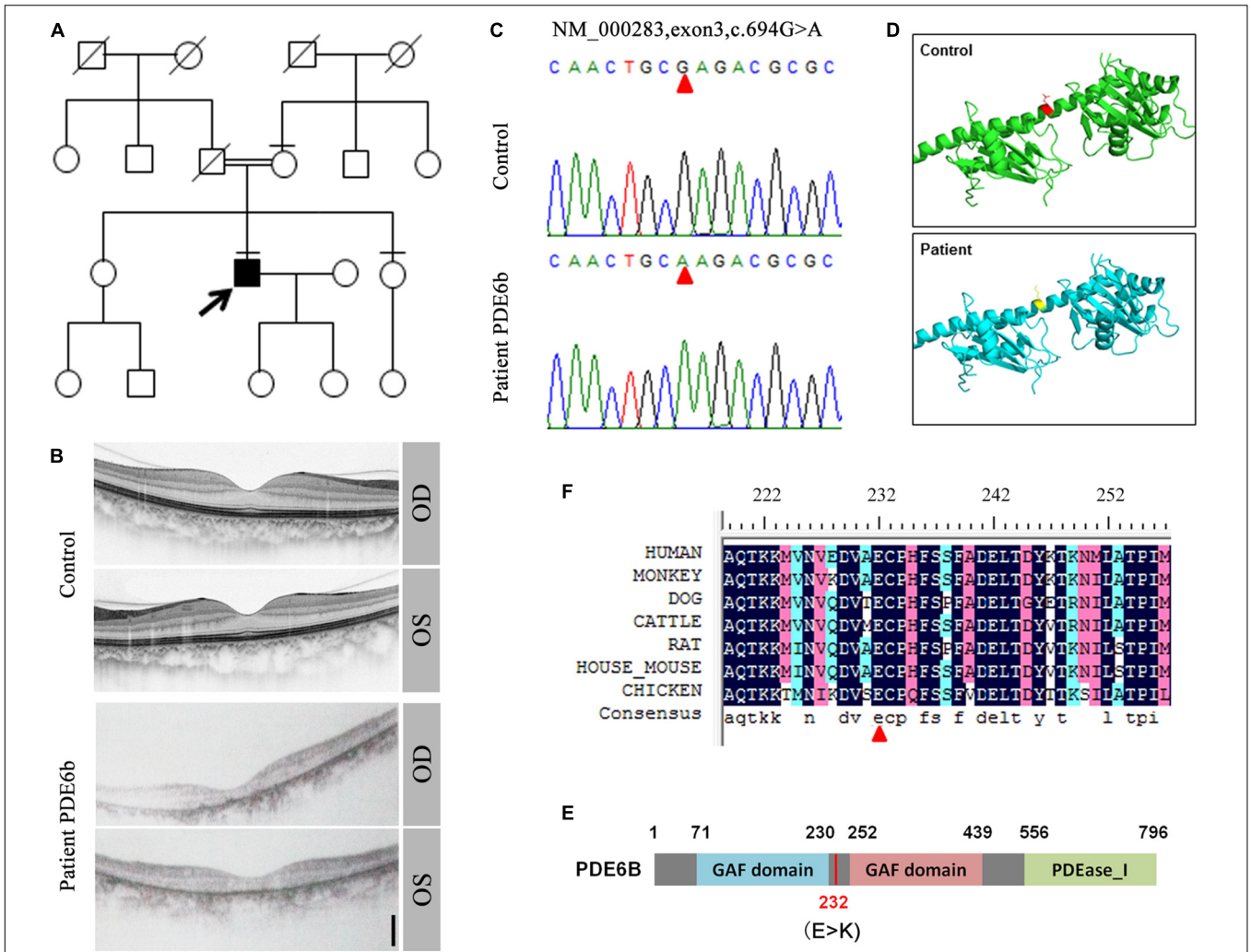

FIGURE 1 | Identification of the PDE6B mutation in the RP patient. **(A)** The pedigree of the family. **(B)** Optical coherence tomography (OCT) of the RP patient and a healthy control. **(C)** Sequencing results of the RP patient and control. A single nucleotide mutation was found in the RP patient compared with the control and is indicated with red arrows. **(D)** Predicted crystal structures of the wild-type and mutant *PDE6B*. Amino acid 232 is indicated in red and yellow. **(E)** The domain organization of human PDE6B. The *PDE6B* c.694G > A mutation is located in the intervening sequence of two GAF domains arranged in tandem. **(F)** Multiple sequence alignment of PDE6B of the indicated species showing conserved amino acid residues at the mutation site which are indicated with a red arrow.

ROs at D230 from the other ROs (**Figure 4D**). These results suggest that the *PDE6B* c.694G > A mutation may cause late-onset retinal disease, which is consistent with the latent RP clinical phenotype.

Mislocalization of Rod Photoreceptors in Late-Stage ROs From the Patient

To further decipher the defects in late-stage *PDE6B* patient ROs, gene expression profiles of different retinal cell types, including RGCs, bipolar cells, horizontal and amacrine cells, photoreceptor cells, cone cells and rod cells, were compared between the patient and control ROs (**Figure 5A**). Among these retinal cell types, the expression of genes related to photoreceptor cell genesis, especially rod cell markers, was significantly higher in patient ROs than in control ROs (**Figure 5A**). Upregulation of transcription factors or regulators that promote rod development (SAG, NR2E3 and NRL) as well as genes related to phototransduction in rods were found at the mid-stage and decreased to levels relatively similar to those of the control at the late stage (**Figure 5A**). This abnormality in gene expression indicated rod development defects in the patient ROs.

Strong RHO expression was observed in both the control and patient ROs at D230. However, the distribution of the RHO signal was very different between the control and patient ROs (**Supplementary Figure S1A**). In the control ROs, RHO^{+} rod cells and M-$opsin^{+}$ cone cells were mainly located in the outer layer with an outer-segment-like structure, indicating the mature morphology of photoreceptors. In contrast, most of the RHO and M-opsin signals appeared in the inner layer in patient ROs with immature morphology (**Figure 5B**).

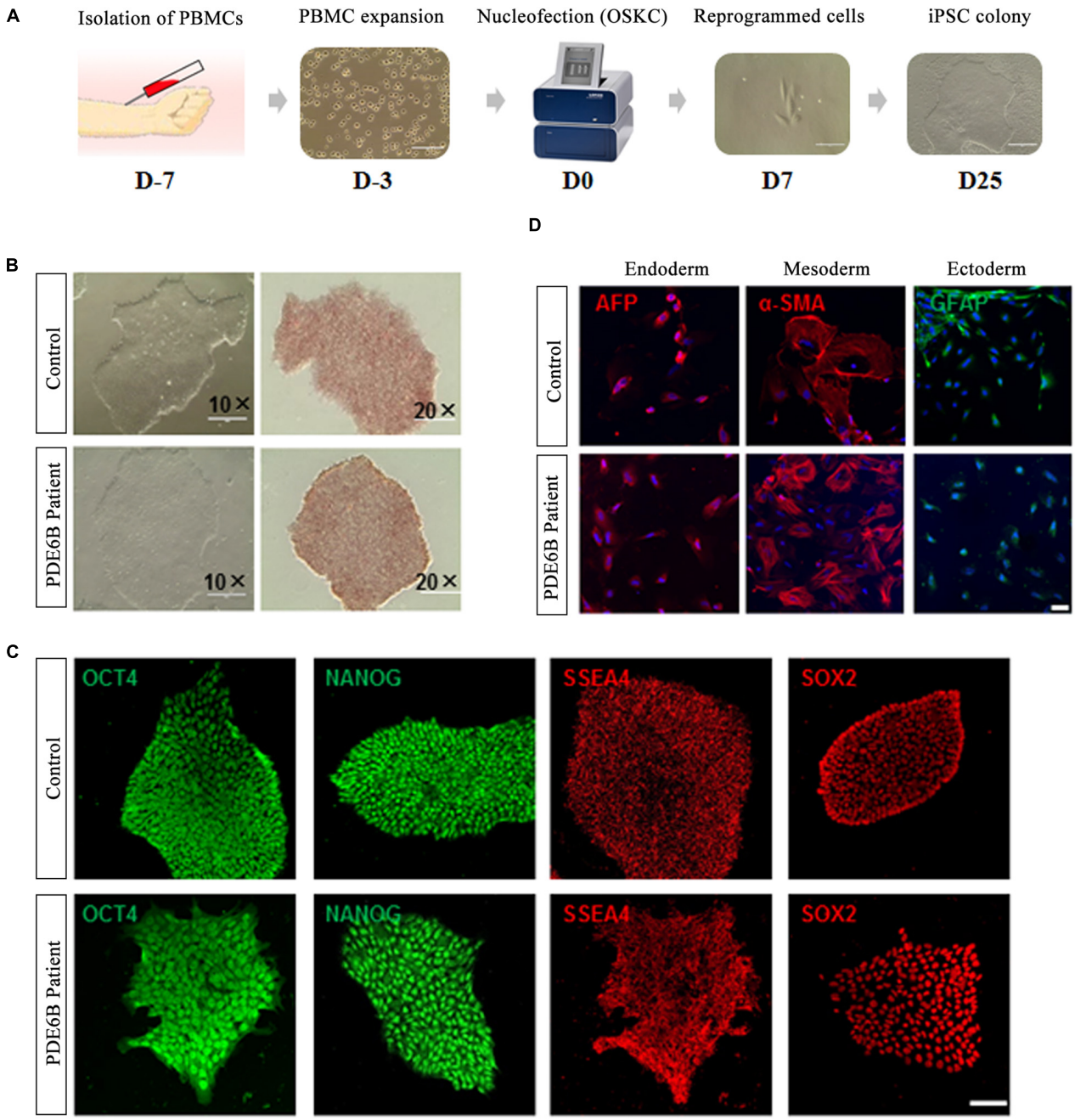

FIGURE 2 | Generation and characterization of iPSCs of RP patient and non-phenotype control. **(A)** Timeline of iPSC generation. **(B)** Phase-contrast imaging (left; bar: 400 μm) and AP staining of iPSCs (right; bar: 200 μm). **(C)** Immunostaining of pluripotent markers. Bar: 50 μm. **(D)** Trilineage differentiation of iPSCs into three germ layers, endoderm (AFP^+), mesoderm (α-SMA^+) and ectoderm ($GFAP^+$). Bar: 75 μm.

RCVRN positive cells and layered DAPI positive signal indicated outer layer clearly (**Figure 5C**). Few TUNEL positive cells were observed and no significant differences were found either at D180 (**Figure 5C** and **Supplementary Figure S1B**) or D230 (**Figure 5C**). Statistical analysis of three ROs (six images each) suggested that the ratio of rod cells in the outer and inner layers in the control ROs was significantly higher than that in the patient ROs (**Figure 5D**). The number of cones was also counted and standardized with ROs perimeter. However, no significantly differences were shown between control and mutation (**Supplementary Figure S1C**). Thus, these results implied that the *PDE6B* mutation complementarily deregulated rod cell marker expression in mid-stage ROs and specifically impaired rod cell maturation in

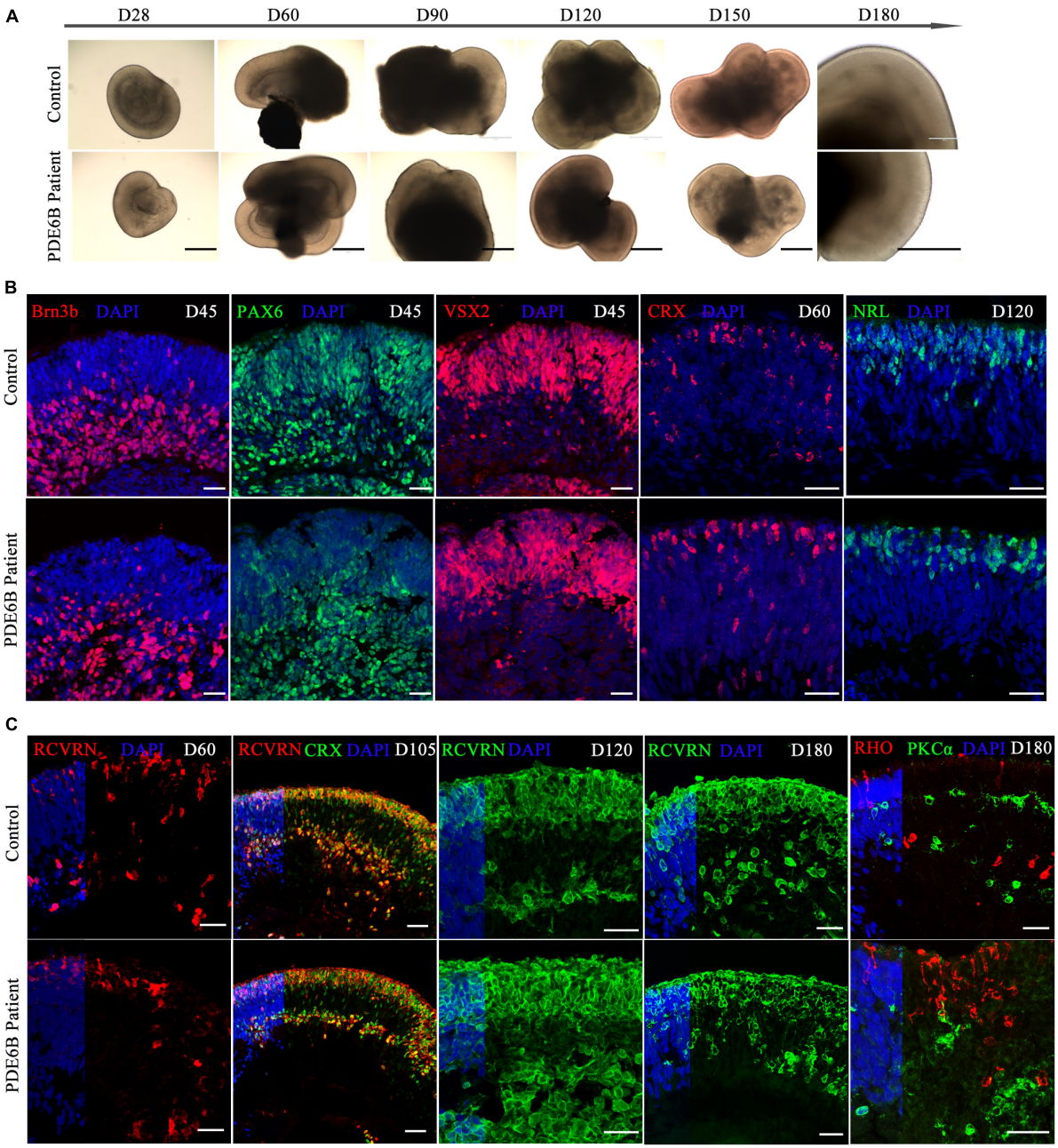

FIGURE 3 | Comparison of retinal development between patient and control iPSC-derived organoids. **(A)** Phase-contrast images of retinal organoids from the patient and control at the indicated time points. Bar: 400 μm. **(B,C)** Immune fluorescence images of retinal organoids from the patient and control at the indicated time points. Brn3b, retinal ganglion cell marker; PAX6, retinal progenitor marker; VSX2, retinal progenitor marker; CRX, photoreceptor precursor marker; NRL, rod precursor marker; RCVRN, photoreceptor and cone bipolar marker; RHO, rod photoreceptor marker; PKCα, rod bipolar marker. Bar: 25 μm.

late-stage ROs, and cell death might not be the main cause of rod mislocation.

Functional Defects of *PDE6B* Patient ROs

PDE6B plays an important role in cGMP hydrolysis, which is a key part of phototransduction. Thus, we sought to gain further insights into the functional defects caused by the *PDE6B* mutation in ROs. Gene Ontology (GO) analysis of the transcripts significantly different at D230 between the patient and control ROs indicated an enrichment of genes implicated in G-protein-coupled receptor activity, G-protein-coupled receptor signaling pathway and calcium ion binding (**Figure 6A**). Higher expression of G-protein subunit alpha transducing 1 (GNAT1), a stimulator of rhodopsin and

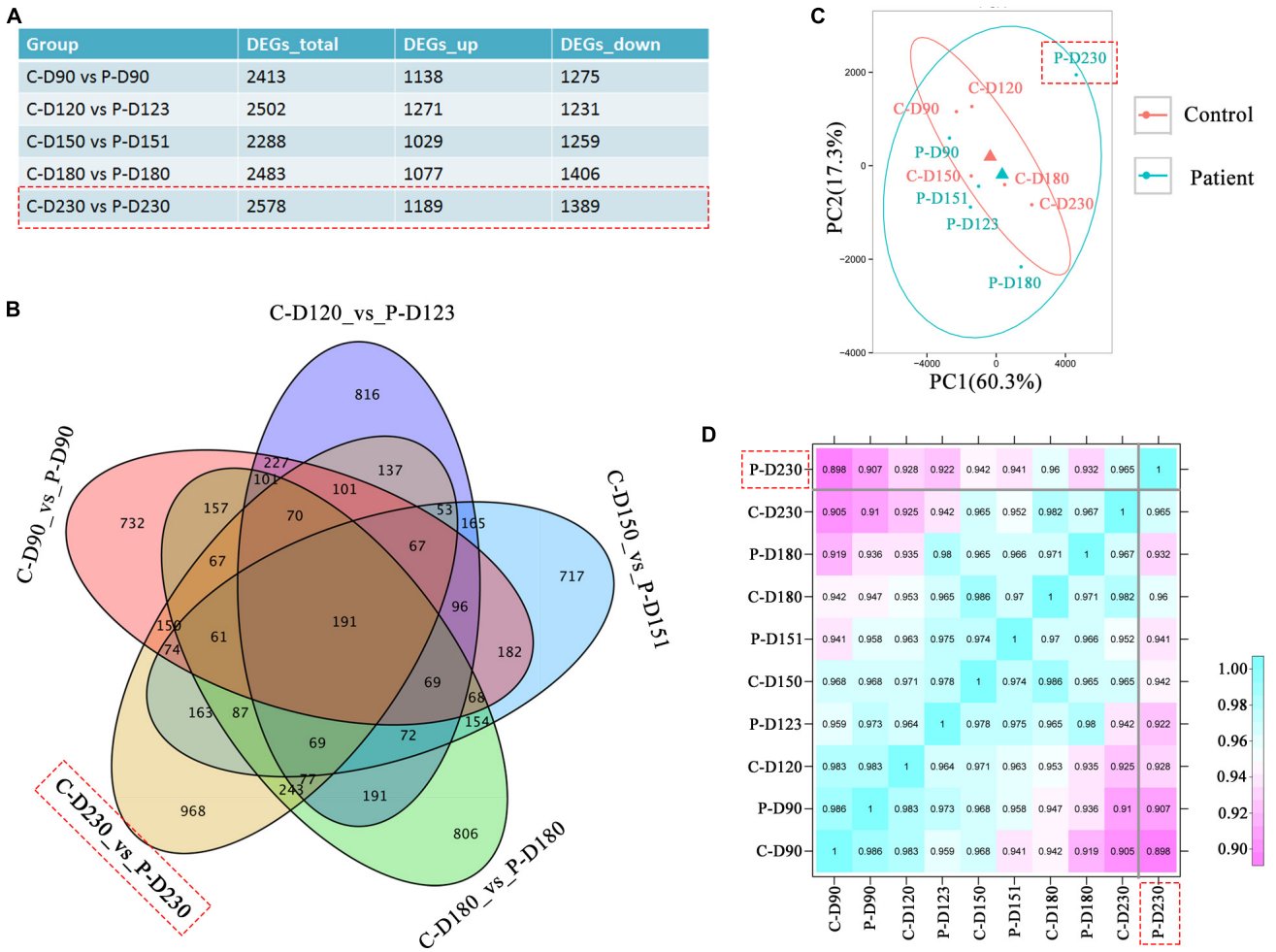

FIGURE 4 | Transcriptome analysis identified defects in patient retinal organoids at D230. **(A)** Table of differentially expressed gene numbers in the control and patient organoids at the indicated time points. Total RNA of each sample was extracted of 3–5 organoids of two independent differentiations. **(B)** Venn diagram showing the number of genes differentially expressed in the control and patient and their overlaps at the indicated time points. **(C)** PCA of RNA-seq samples. **(D)** Correlation analysis of RNA-seq samples.

cGMP-phosphodiesterase coupling, PDE6B and PDE6G, and the beta and gamma subunits of cyclic GMP-phosphodiesterase, which are key players in stimulating cGMP hydrolysis in rods during visual impulses, was identified in patient ROs at D230 (**Figure 6B**). To validate the RNA-seq results, qPCR was performed with several target genes including SAG, PDE6B, GNAT1, PDE6G, RCVRN, and ATOH7 (**Figure 6C**). The expression of PDE6G, PDE6B, GNAT1, SAG, and ATOH7 were significantly higher in the patient ROs than that in the control. The level of RCVRN expression was similar in the patient and control ROs at D230. These results were consistent with the RNA-seq. Accompanied by slightly different RCVRN expression, indicating a similar number of cells in the patient and control ROs, these extraordinary changes implied impaired cGMP hydrolysis in the patient ROs at D230 (**Figure 6B**). A prominent change in the expression of genes involved in the cGMP-PKG signaling pathway was observed, suggesting abnormal cGMP levels in the patient ROs at D230 (**Figure 6D**). Genes that promote cGMP hydrolysis, such as the essential component for rod cGMP-phosphodiesterase biosynthesis (AIPL1), were significantly deregulated in ROs at D230 (fold change > 2) (**Figure 6D**). Conversely, genes involved in the G-protein-coupled receptor signaling pathway and ion channel, such as G-protein subunit beta 1 (GNB1), regulator of G-protein signaling 9 binding protein beta, and subunit of a cyclic nucleotide-gated ion channel (CNGB3), were profoundly downregulated in ROs at D230 (fold change > 2) (**Figure 6D**). These results implied that the cGMP level in the patient ROs might be affected.

Thus, the concentrations of cGMP in the ROs were detected by ELISA at D193 and 230. At D193, a significantly higher cGMP concentration was found in patient ROs. As the ROs developed into the late stage (D230), this difference was remarkably increased, reaching almost 10 times higher in patient ROs than in control ROs (**Figure 6E**). These results suggested that the *PDE6B*

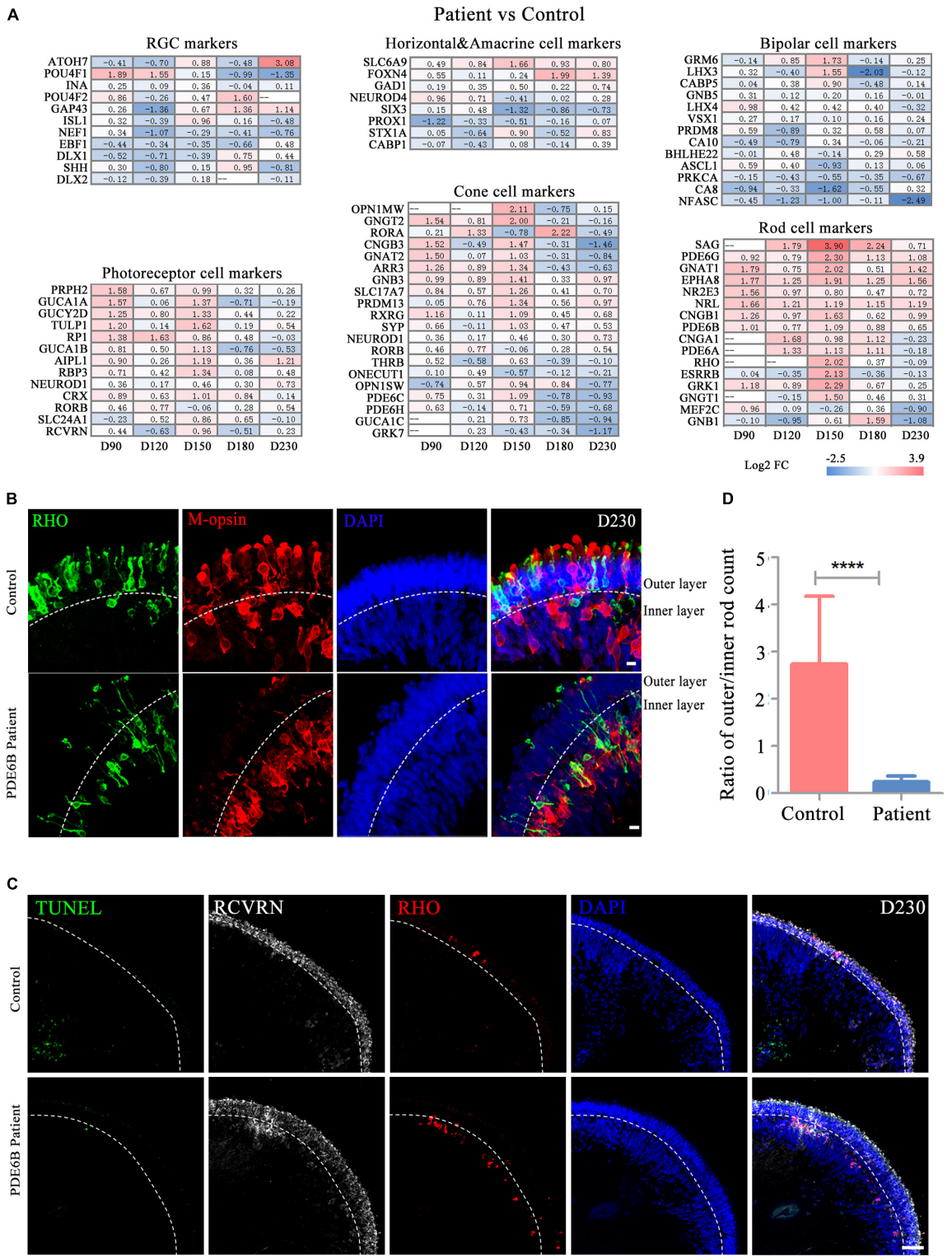

FIGURE 5 | Defects in rod cell migration in patient retinal organoids at D230. **(A)** Changes in marker gene expression of retinal cell types were compared for control versus patient organoids. **(B)** Representative pictures of immunostaining analysis of rod and cone marker gene expression in control and patient organoids. Bar: 10 μm. **(C)** Representative pictures of TUNEL staining and immunostaining of RCVRN and RHO. Bar: 50 μm. **(D)** The ratio of rod numbers in the outer layer to the inner layer indicated in **(B)**. Statistical results are the mean values ± SD of 3 organoids (six images each) (D230) of two independent differentiations from the control and patient. Two-tailed *t*-test, $^{****}P < 0.0001$.

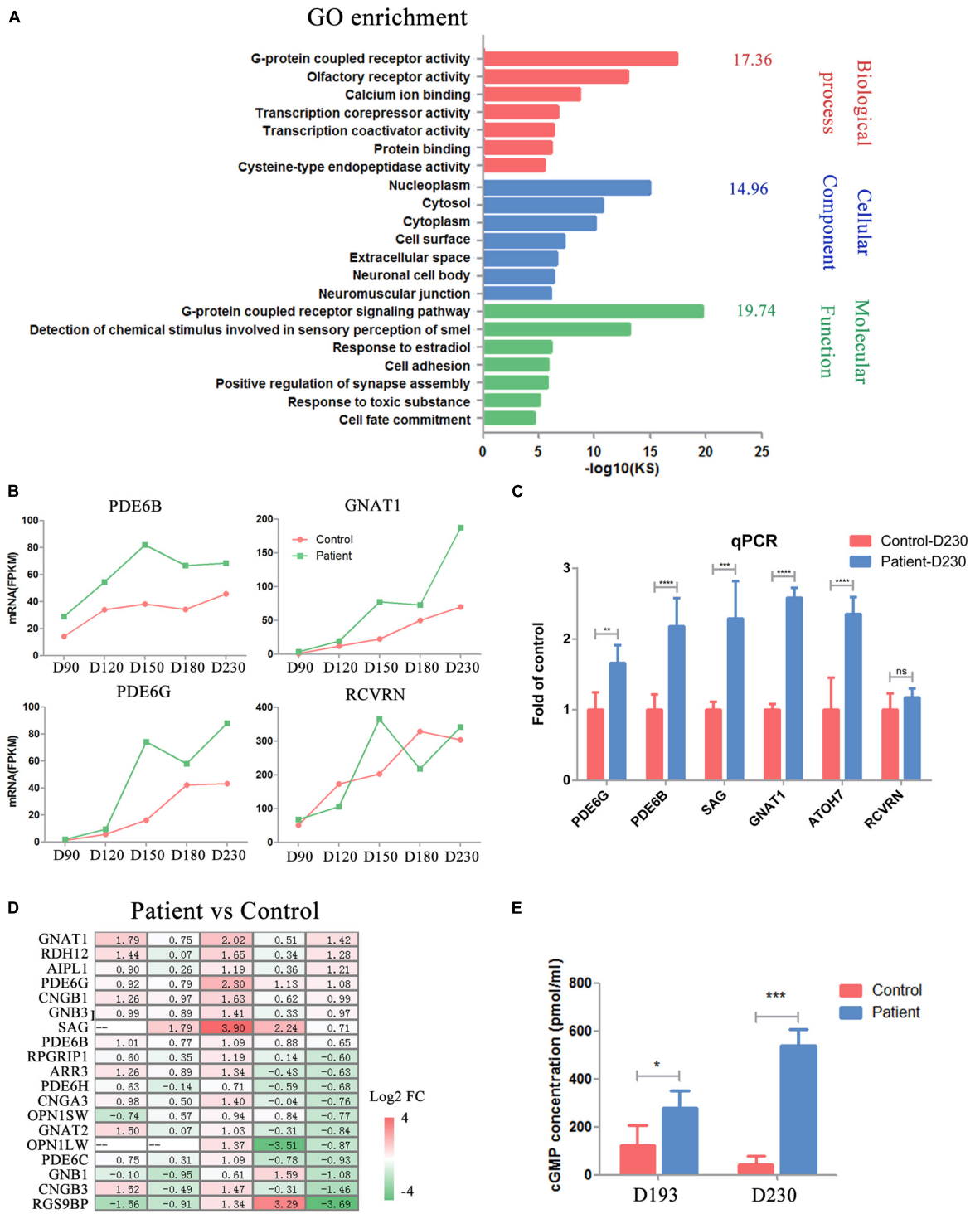

FIGURE 6 | Impaired phototransduction was found in PDE6B patient retinal organoids. **(A)** GO analysis of differentially expressed genes for control and patient organoids at five different time points indicated in RNA-seq. **(B)** Expression of key genes in phototransduction (PDE6B, GNAT1, PDE6G) and photoreceptor and cone bipolar marker (RCVRN) at different time points included in the RNA-seq analysis. Total RNA of each sample was extracted of 3–5 organoids of two independent differentiations. **(C)** Representative qPCR results of two independent experiments. The results are the mean values ± SD of three replicates of each sample with 3–5 organoids. Two-tailed *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns $P > 0.05$. **(D)** Changes in the expression of genes in the cGMP signaling pathway compared to patient versus control organoids. **(E)** Concentration of cGMP in retinal organoids. The results are the mean values ± SD of three independent experiments with three organoids in each sample of Control D193, Control D230 and Patient D230, and six independent experiments with three organoids in each sample of Patient D193. LSD test, $^{*}P < 0.5$, $^{***}P < 0.001$.

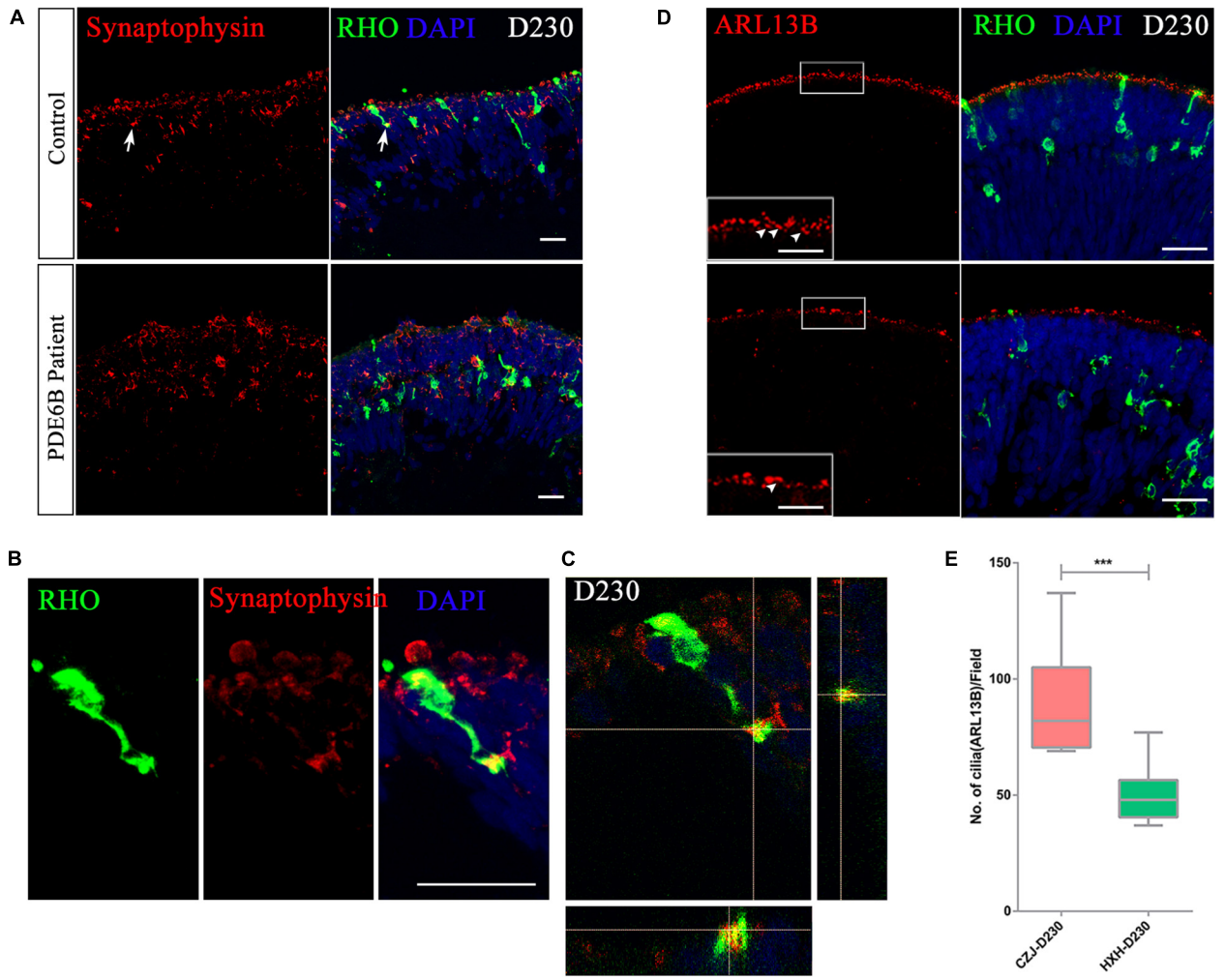

FIGURE 7 | Impaired rod maturation in PDE6B patient retinal organoids. **(A)** Representative immunostaining pictures of the presynaptic marker synaptophysin and rhodopsin (RHO) in retinal organoids. Bar: 25 μm. **(B)** Amplified presynaptic formation of rod photoreceptors indicated with arrows in **(A)**. Bar: 25 μm. **(C)** Colocalization of RHO and synaptophysin. **(D)** Representative immunostaining pictures of the cilium marker ARL13B and RHO in retinal organoids at D230. Bar: 25 μm. Boxes were amplified images. White arrows indicated definite signal of ARL13B represent cilia. Bar: 6.25 μm. **(E)** Statistic analysis of cilia number in retinal organoids at D230. Statistical results are the mean values ± SD of 3 organoids (3 images each) (D230) of two independent differentiations from the control and patient. Two-tailed *t*-test, $^{***}P < 0.001$.

mutation impaired its function in cGMP hydrolysis, leading to increased cGMP levels in ROs.

To further identify whether the accumulated cGMP affects the formation of synaptic connections, the expression of the synaptic vesicle membrane protein, synaptophysin, was analyzed at D230. A distinct expression pattern of synaptophysin was observed in the control and patient ROs (**Figure 7A**). In the control ROs, most of the synaptophysin signal formed condensed vesicles located in the two apical regions of the outer nuclear layer. However, synaptophysin was spread through the cytoplasm in the patient ROs (**Figure 7A**). In addition, the presynaptic structure was formed at the end of the rod photoreceptor cells in the control ROs (**Figures 7B,C** and **Supplementary Movie**). No similar structure was found in the patient ROs.

The connecting cilium is an important structure for material transfer between the outer segment and inner segment in photoreceptor cells. The formation of the connecting cilium can be considered a hallmark of photoreceptor cell maturation. Thus, we also examined the connecting cilium via ARL13B expression in ROs at D230 (**Figure 7D** and **Supplementary Figure S1D**). A remarkably reduced number of cilia was observed in *PDE6B* patient ROs (**Figure 7E**), which is more obvious in 3D reconstruction images (**Supplementary Figure S1D**). Overall, in the *PDE6B* mutant RO model, elevated cGMP levels are suspected to be linked to impaired rod migration from the inner layer to the outer layer and impeded formation of synapses and the connecting cilium in photoreceptor cells.

DISCUSSION

The heterogeneity of RP inevitably promotes the difficulty of the development of treatment or therapeutic methods. Although animal models have provided invaluable tools for mechanism unveiling and drug testing; large gaps in understanding gene variability and structural discrepancy remain. With the development of new technologies comes the need to obtain more precise models of the human retina to study this highly organized tissue. With the development of 3D culture of stem cell-derived ROs *in vitro*, researchers can now generate well-structured retina-like tissue and mature photoreceptors possessing the appropriate electrophysiological properties and photosensitivity. Transcriptome analysis has demonstrated that ROs are excellent resources for studying retinal development (Kaewkhaw et al., 2015; Voelkner et al., 2016). Eldred and colleagues have used hESC-derived ROs as a model for determining the mechanism that controls photoreceptor cell fate during human retinal development (Eldred et al., 2018). Combined with single-cell sequencing of cells isolated from hESC-derived ROs, critical pathways and novel genes regulating retinal progenitor cell commitment have been found (Mao et al., 2019).

Using patient-derived stem cells, patient-specific ROs can be established to better understand pathogenesis and to test new therapeutics. To compensate for the inconsistency of ROs derived from different iPSC lines with varied methodologies, Capowski et al. (2019) promoted a staging system for RO differentiation. Here, we have analyzed the disease phenotype and expression profile within a staging system, which is largely consistent with their findings. Based on the staging system, the RO disease models established to date have all been early- or mid-stage (Parfitt David et al., 2016; Shimada et al., 2017; Teotia et al., 2017; Buskin et al., 2018; Deng et al., 2018; Guo et al., 2019; Huang et al., 2019; Li et al., 2019; Quinn et al., 2019). For example, in a previous RPGR-RP model established in our lab (Deng et al., 2018), impaired gene expression was found as early as D90, and impaired photoreceptor morphology was observed at approximately D150. In addition, another PRPF31-RP11 model established by Buskin et al. (2018), impaired pre-mRNA splicing and impaired photoreceptor morphology was found at week 21 of differentiation. Notably, in the PDE6B RO model, a severe disease phenotype is observed only at D230, which is considered late-stage.

By now, 65 genes have been identified as disease-causing genes of RP[2]. Mutations in *PDE6B* cause recessive RP and dominant congenital stationary night blindness with vastly variable phenotypes. Here, we used a patient-derived RO model to demonstrate how the *PDE6B* mutation causes retinal degeneration in RP. Akin to the *rd1* and *rd10* mouse models (Bowes et al., 1990; Barhoum et al., 2008; Wang et al., 2018), this defect in PDE6B leads to an accumulation of cGMP in ROs at D193, and the accumulation increases to 10 times higher than that of control ROs as the ROs grow to D230 (**Figure 6D**). Additionally, defects in photoreceptors can be observed in the patient ROs, including mislocation and abnormal morphology at D230 (**Figure 5**), which is consistent with the photoreceptor death found in the mouse models at approximately P8 in *rd1* and P18 in *rd10* (Bowes et al., 1990; Gargini et al., 2007; Barhoum et al., 2008). Furthermore, the expression of cGMP metabolic-related genes was significantly changed in patient ROs at D230, indicating impaired photoreceptor function (**Figure 7A**). In summary, we have established a PDE6B-RP model with patient iPSCs exhibiting cardinal characteristics as predicted based on the phenotypes shown in animal models. Additionally, our findings have been drawn from three colonies of one patient and one healthy control. In future study, we will screen for more PDE6B-RP cases and perform gene correction as we reported previously (Deng et al., 2018).

Our hope is that the establishment of a late-stage RP model *in vitro* may provide a reasonable platform that will be utilized in drug screening in addition to rodent models. With a recent study demonstrating the successful infection of retinal organoids with AAV (Quinn et al., 2019) and rescue of CEP290 function with oligonucleotides in patient ROs (Dulla et al., 2018), this well-characterized patient-based system is promising for deciphering disease mechanisms, evaluating the efficacy of new drugs, and testing the efficiency of gene therapy before clinical trials.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Gene Expression Omnibus with accession number GEO: GSE141531.

SIGNIFICANCE STATEMENT

Retinitis pigmentosa (RP) is a hereditary retinal degenerative disease, and 65 disease-causing genes have been identified. Animal models have been used for pathogenesis interpretation and drug testing, but conflicting results have been found because of interspecies variation. However, human iPSCs and a retinal organoid (RO) differentiation system provide an unlimited cell source for disease modeling and drug screening. Here, we developed patient ROs with a *PDE6B* mutation, and an obvious disease phenotype was found at differentiation day 230. Moreover, elevated cGMP levels and mislocalization of rod cells were observed. This patient-based late-onset RP model can be utilized to decipher the mechanisms underlying RP and evaluate new treatments.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Eye Hospital of Wenzhou Medical University Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

[2]https://sph.uth.edu/retnet

AUTHOR CONTRIBUTIONS

M-LG conducted the data analysis and interpretation, manuscript writing, financial support, and the final approval of the manuscript. X-LL carried out the collection and assembly of data, data analysis and interpretation, and the final approval of the manuscript. FH carried out the collection and assembly of data, data analysis, manuscript revision, and final approval of manuscript. KW-H carried out the collection and assembly of data. S-QJ carried out the assembly of the data. Y-YZ carried out the data collection. Z-BJ conceptualized the design, provided the materials and the financial support for the study, and gave final approval for 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.2020.00128/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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Fgf10/Fgfr2b Signaling in Mammary Gland Development, Homeostasis, and Cancer

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Fibroblast growth factor 10 (Fgf10) is a secreted ligand acting via the Fibroblast growth factor receptor 2b (Fgfr2b). Fgf10/Fgfr2b signaling plays important roles both in the epithelium and in the mesenchyme during mammary gland development. Evidence in mice show that Fgf10 is critical for the induction of four out of five of the mammary placodes and for the formation of the white adipose tissue. Fgfr2b ligands also play important function in the maintenance of the terminal end buds, specialized structures at the tip of the ramified ducts during the postnatal phase of mammary gland development. Finally, in humans, FGF10 has been described to be expressed in 10% of the breast adenocarcinoma and activation of FGFR2b signaling correlates with a worse prognostic. Therefore, Fgf10 plays pleiotropic roles in both mammary gland development, homeostasis and cancer and elucidating its mechanism of action and cellular targets will be crucial to either enhance mammary gland development or to find innovative targets to treat aggressive breast cancer.

Keywords: Fgf10, Fgfr2b, mammary gland, development, stem cells, cancer

This review article focuses on the role of Fibroblast growth factor 10 (Fgf10) both pre- and post-mammary gland formation. We provide evidence that this dual role is conserved in the lung and limb, where Fgf10 has been shown to be important. In addition, we propose that the molecular mechanisms regulating embryogenesis are conserved during post-natal development as well as in breast cancer progression.

Fgf10 BELONGS TO THE Fgf7 SUBFAMILY OF SECRETED GROWTH FACTORS ACTING MOSTLY THROUGH Fgfr2b

The Fibroblast growth factor 7 subfamily is made of four secreted growth factors (Fgf3, Fgf7, Fgf10, and Fgf22). Initial studies took advantage of a conserved region between the first two members of this family (Fgf3 and Fgf7) to identify additional subfamily members (Fgf10 and Fgf22). In addition to sequence identity, the members of this subfamily also bind to common receptors albeit with different affinities (Ornitz and Itoh, 2001). Fgf10 in particular plays a crucial role during organogenesis. Fgf10, which is mostly expressed by mesenchymal cells, acts principally through the Fgf receptor 2b (Fgfr2b) and Fgfr1b expressed in the epithelium to control the formation of ramified structures such as the embryonic lung (Bellusci et al., 1997). Fgf10 elicits its action through chemotaxis, which involves a coordinated migration of an epithelial sheet toward a localized source

of Fgf10 (Park et al., 1998; Weaver et al., 2000; Jones et al., 2018, 2019). Consistently, *Fgf10* or *Fgfr2b* null mice display agenesis of many organs such as the lung, limb and mammary gland (Min et al., 1998; Sekine et al., 1999; Ohuchi et al., 2000; Mailleux et al., 2002).

EARLY MAMMARY GLAND DEVELOPMENT STARTS WITH THE FORMATION OF ECTODERMAL-DERIVED PLACODES

Mammary gland formation in the mouse begins around embryonic day 10 (E10) with the formation of two mammary lines, located in antero-posterior direction along each flank of the embryo (Turner and Gomez, 1933). By E11-E12, five lens-shaped structures, the mammary placodes, are detected along each mammary line, as ectodermal thickenings that in 24 h develop into epithelial buds. These buds, three thoracic and two inguinal, are located at reproducibly precise positions. This reproducibility suggests a tight spatial-temporal control of placode induction. However, the genes involved in such regulation are still unclear. Previous reports suggest that mammary placodes in rabbit are formed by the migration of ectodermal cells along a mammary line (Propper, 1978), rather than by local increase in cell proliferation (Balinsky, 1950). In agreement with these results, analysis of proliferation in mice indicated that cells that contribute to the mammary placode are proliferating less than the adjacent cells in the surface ectoderm (Lee et al., 2011). In addition to cell migration, it was also shown that these placode cells undergo hypertrophy (Lee et al., 2011). Therefore, both cell migration and cell hypertrophy contribute to the growth of the mammary placode, regardless of their thoracic or inguinal position.

Fgf10 AND *Fgfr2b* NULL MICE FAIL TO DEVELOP NORMAL MAMMARY GLANDS

Little is known about the genes that regulate the induction of the mammary placodes and the early phases of mammary gland development. However, there are several indications for the requirement of Fgf10-signaling via Fgfr2b for placode induction and development. It was previously described that the formation of the mammary line as well as the subsequent induction of four out of five mammary placodes was impaired in both *Fgf10* and *Fgfr2b* knock out embryos (Mailleux et al., 2002). Interestingly, in the *Fgfr2b* KO, the mammary placode number 4, situated inguinally progressively disappeared between E11.5 and E13.5 through decreased proliferation and increased apoptosis of the mutant epithelium. In the *Fgf10* KO, the mammary bud 4 is still present due likely to the redundant expression of Fgf7, another ligand of Fgfr2b (Mailleux et al., 2002). It has been therefore proposed that Fgfr2b signaling is important to control survival and proliferation of the mammary epithelium during the branching morphogenesis phase of mammary gland development. Interestingly, the expression of Fgfr2 is elevated after weaning and remains high in virgin mice only to decrease during pregnancy and lactation. This increase during the weaning period and in the virgin stage can be directly associated with the tremendous ramification process taking place during that time. Interestingly, compared to Fgf7 expression, Fgf10 expression is expressed 15 times higher (Pedchenko and Imagawa, 2000) suggesting that Fgf10 takes also center stage during the postnatal phases of mammary gland development.

A POSSIBLE INTERPLAY BETWEEN Fgf AND Wnt SIGNALING IS AT WORK FOR MAMMARY PLACODE FORMATION

Wnt signaling is likely also connected to Fgf10 signaling during mammary placode development as demonstrated by a mammary gland agenesis phenotype when Wnt signaling is inhibited (Andl et al., 2002). Lef1 (a transcriptional effector of Wnt signaling) is a well-described marker for mammary placode formation (Mailleux et al., 2002), and its ablation leads to an arrest in the bud phase (van Genderen et al., 1994). Moreover, transgenic overexpression of Dkk1, a secreted inhibitor of Wnt ligands leads to an arrest in mammary gland development prior to the bud stage (Andl et al., 2002).

Using the Topgal reporter, a mouse allowing to monitor the activation of β-catenin signaling via the expression of β-galactosidase (DasGupta and Fuchs, 1999), it has been shown that in the embryo at E10.5, the activation of the Wnt signaling could be detected in the ectoderm at the level of the putative mammary line (**Figure 1A**) (Veltmaat et al., 2006). In addition, Fgf10/Fgfr2b signaling controls the expression of several Wnt ligands including Wnt10b (**Figure 1B**) (Veltmaat et al., 2006). These results suggest that the Wnt pathway intersects with the Fgf10 pathway to control mammary placode formation (Veltmaat et al., 2003, 2004, 2006).

The answer to the question about how could Fgf10 and Wnt signaling intersect came from the analysis of the role of Fgf10 at early developmental stage of the mouse lung, another related organ which ramifies. This study was carried out using a double transgenic mouse allowing the doxycycline-based expression of a soluble form of Fgfr2b acting as a scavenger for all Fgfr2b ligands (Parsa et al., 2008, 2010; Jones et al., 2018). Unlike the genetic inactivation using the Cre/LoxP technology of *Fgr2b* or its ligands (which is even more challenging because of the potential redundancy between members of Fgf7 subfamily), this approach allows inhibiting efficiently Fgfr2b signaling within 1 h following intra-peritoneal injection of doxycycline to the pregnant females (Danopoulos et al., 2013). Fgf10 is the main Fgfr2b ligand expressed in the lung at early developmental stages. Fgf10 is normally expressed in the distal mesenchyme adjacent to the epithelial tips of the lung buds (Bellusci et al., 1997). These distal epithelial structures respond to Fgf10 signaling. This is illustrated by the expression at the epithelial tip of several Fgf10-downstream target genes such as *Etv4*, *Etv5*, *Shh,* and *Sox9* (Jones et al., 2018). In addition, the lung tip epithelium displays significant expression of phosphorylated (Ser-552) ß-catenin. This protein corresponds to a transcriptionally active form of ß-catenin thereby indicating

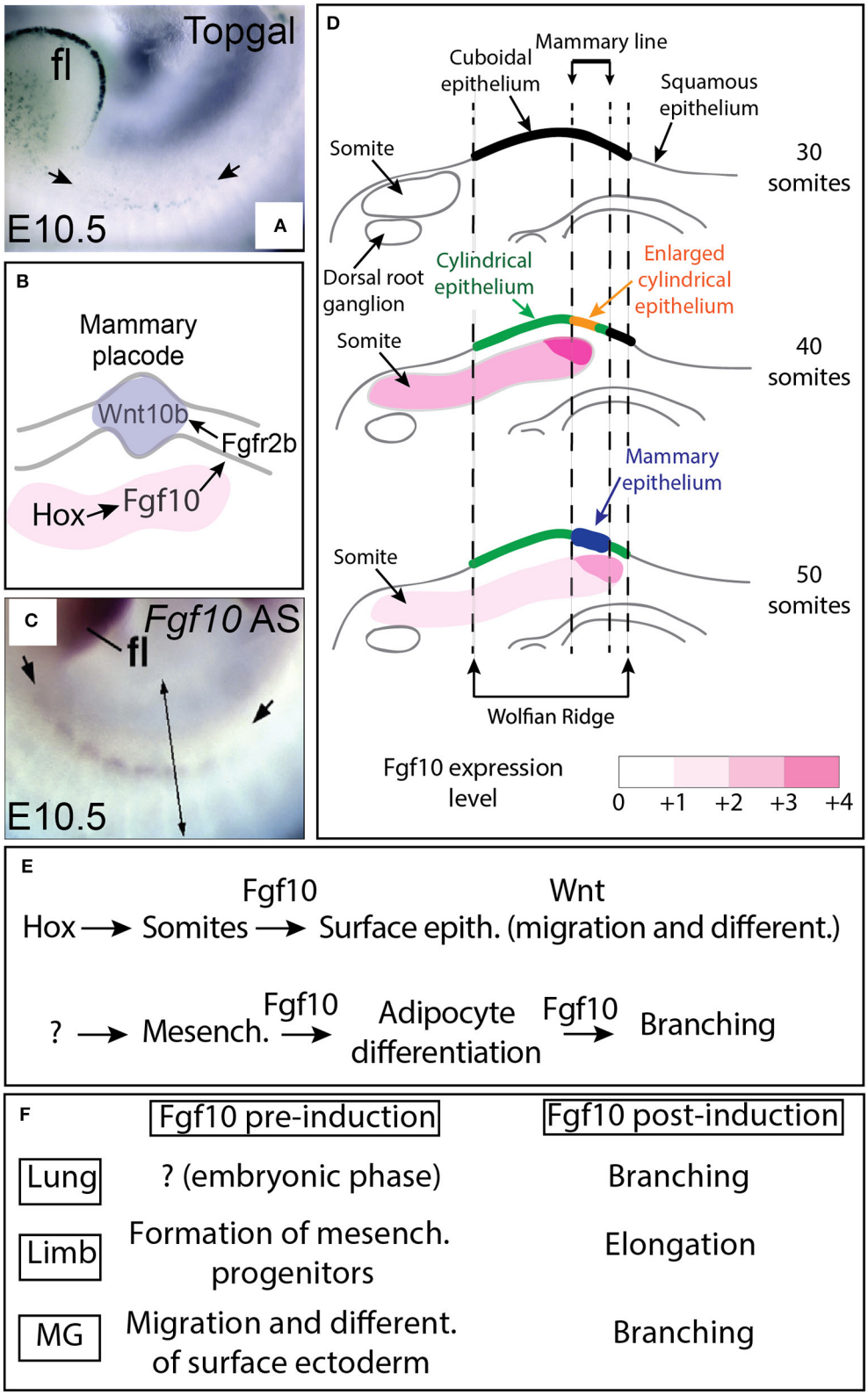

FIGURE 1 | Fgf10 expression from the somites controls placode formation from the ectodermally-derived mammary line. **(A)** β-galactosidase expression in the Wnt reporter Topgal mice at E10.5 showing Wnt-responding cells in the ectoderm where the mammary line is forming (from Veltmaat et al., 2006). **(B)** Schematic model.

(Continued)

FIGURE 1 | Fgf10 expression in the somites is regulated by *Hox* genes, Fgf10 acts via Fgfr2b expressed in the ectoderm to trigger Wnt10b expression leading to Wnt signaling activation in the epithelium of the forming mammary line/placode. **(C)** *Fgf10* mRNA expression by *in situ* hybridization at E10.5 showing *Fgf10* expression in the somites (from Veltmaat et al., 2006). **(D)** Progressive differentiation of the ectoderm-derived epithelium in the mammary line from cuboidal (30 somites stage) to cylindrical/enlarged cylindrical (40 somites stage) to form the mammary epithelium constituting the placode (at 50 somites stage) is associated with increased Fgf10 expression as the somites grow dorsally. **(E)** Summary of Fgf10 regulation and activity during embryonic mammary gland development. *Hox* genes control Fgf10 expression in somites. Fgf10 from the somites acts on the surface ectoderm to trigger migration and differentiation. Fgf10 is also induced in the fat pad precursor through still to be identified mechanisms and acts on both the epithelium (to control branching) and the mesenchyme (to control adipocyte differentiation). **(F)** Fgf10 activity pre and post-organ induction in lung, limb and mammary gland. Scale **(A,B)**: 70 μm. fl: forelimbs. Simple arrows in **(A)** indicate the forming mammary line. Double arrow in **(C)** indicates a virtual section through the somites displayed in **(D)**.

Wnt signaling activation. The presence of phosphorylated (Ser-552) ß-catenin is drastically reduced in the lung tip epithelium following Fgfr2b signaling inhibition suggesting that in this cellular compartment, Fgf10 functions upstream of Wnt/ß-catenin signaling. IQ-1, a pharmacological inhibitor of the interaction between ß-catenin and P300 (Miyabayashi et al., 2007), was shown to reproduce most of the effects at the cellular and transcriptomic level induced by blockade of Fgf10 signaling (Jones et al., 2018). How is Fgf10 regulation of ß-catenin activity achieved is still unknown. One possibility is that decreased Fgf10 signaling impacts the stability of ß-catenin *per se* leading to a decrease in the cytoplasmic level of free ß-catenin. Such regulation can be the consequence of altered phosphorylation of ß-catenin leading to its degradation in combination with decreased availability of free ß-catenin in the cytoplasm. This last aspect could be achieved through the perturbation of cell adhesion. As ß-catenin associates with the cell adhesion molecule E-Cadherin (Cdh1), such interaction regulates the level of ß-catenin available for signaling. Interestingly, catenin delta 2 (Ctnnd2) has been reported to be a downstream target of Fgf10 (Jones et al., 2018). Ctnnd2 plays a functional role in cell adhesion by destabilizing Cdh1 (Lu et al., 1999; Kim et al., 2012). In the context of active Fgf10 signaling, such destabilization could therefore be associated not only with increased cell mobility (which is one of the characteristic of the cells responding to Fgf10 signaling) but also with increased availability of free ß-catenin (arising from the pool of ß-catenin initially associated with Cdh1) in the cytoplasm that can be used for signaling upon translocation to the nucleus. In the context of blockade of Fgf10 activity, the decrease in *Ctnnd2* expression is associated with increased Cdh1 expression and reduced presence of the transcriptionally active form of ß-catenin (Jones et al., 2018).

Interestingly, a similar observation was made in the in the context of limb bud development in a structure called the apical ectoderm ridge (AER). The AER is a transient epithelial structure located at the apex of the developing limb bud (between E9.5 and E13) and involved in the elongation of the limb bud. The functionality of the epithelial cells in the AER is dependent upon the maintenance of a Fgf10/ß-catenin signaling axis (for a review on this topic see Jin et al., 2018). A very significant decrease of ß-catenin in the AER is observed as early as 1 h after blockade of Fgfr2b signaling at E11. In addition, 6 h following the inhibition, it was found that the subcellular localization of ß-catenin in the AER was abnormal and consistent with the lack of recovery of Wnt signaling in this critical epithelial structure (Danopoulos et al., 2013). Considering how quickly both the free pool of ß-catenin in the cytoplasm and the transcriptionally active form in the nucleus are affected, it is very likely that post-translational regulations, in particular phosphorylation events, are involved. Advanced phosphoproteomic studies combined with the use of transgenic tools allowing the rapid and reversible inhibition of Fgfr2b ligands activity will shed new lights on this critical interaction between Fgf10 and ß-catenin. Equally important will be the further analysis of the role of Ctnnd2 and more generally cell adhesion to mediate the interaction between Fgf10 and Wnt/ß-catenin signaling during mammary gland formation.

Fgf10 EXPRESSION IN THE SOMITES CONTROLS MAMMARY PLACODE INDUCTION

The characterization of the expression of *Fgf10* and *Fgfr2b* during early embryonic mammogenesis has been instrumental to fully understand the nature of the epithelial-mesenchymal interactions orchestrating mammary placode induction as well as the mammary phenotypes displayed by the *Fgf10* and *Fgfr2b* null mutants. As epithelial cell migration is suggested to underlie placode formation, it was expected to find *Fgfr2b* expression in the surface epithelium located in the region from which the placode will be forming and *Fgf10* in the underlying dermal mesenchyme. While *Fgfr2b* is spatio-temporally expressed according to the expectation, surprisingly, *Fgf10* is not expressed in the region of the mammary line nor in the underlying dermal mesenchyme until E15.5 (Mailleux et al., 2002). However, *Fgf10* is expressed in the nearby dermamyotome of the somites at E10.5. It has therefore been proposed that *Fgf10* expression in this structure is involved in mammary placode induction (see **Figure 1C**). In a follow up study (Veltmaat et al., 2006), it has been reported that the somites underlying the inguinal placodes 2 and 3 expressed *Fgf10*, in a gradient-like pattern across and within these somites. It was therefore therefore proposed that Fgf10 expression in the dermamyotome is required for inguinal mammary placode formation and that the Fgf10 and the Wnt pathway cooperate to control cell migration during mammary placode induction. Several mutants displaying abnormal *Fgf10* expression in the somites were monitored for the status of Wnt signaling as well as cellular changes at the level of the ectoderm as read out of both the mammary line and mammary placode formation. In $Pax3^{ILZ/ILZ}$ mutants (Relaix et al., 2003), characterized by the absence of ventral somitic buds, the position

of the mammary line is relocated dorsally and is associated with absent placode 3. Similarly, in $Gli3^{Xt-J/Xt-J}$ mutants [(Maynard et al., 2002), displaying a shortened somatic Fgf10 gradient dorsally] and in hypomorphic *Fgf10* mutants [(Kelly et al., 2001), characterized by a general decrease in Fgf10 expression], both the mammary line and the placode 3 are absent. Interestingly, exogenously applied beads soaked with recombinant Fgf10 grafted on the flank of mutant embryos, at the location of the somites where placode 3 develops, was sufficient to rescue the formation of placode 3 in both *Fgf10* null and $Gli3^{Xt-J/Xt-J}$ mutants. It is tempting to speculate that the progressive increase in somitic Fgf10 expression is causative for the progressive maturation of the surface ectoderm (**Figure 1D**). The current model proposes that Fgf10 expression in the somite, downstream of the transcription factor Gli3, is critical for the flank ectoderm to be committed toward the mammary epithelial lineage. Fgf10 gradients pattern across and within the somites, associated with the spreading of the somites ventrally, are instructive for the correct spatial positioning of the committed mammary epithelium. Interestingly, Homeobox (*Hox)* genes have been proposed to also play a critical function in the induction of the mammary placodes. In particular, *Hoxc8* expression is significantly detected at E10.5 in the surface ectoderm where the mammary line is forming and its ectopic expression using the *Wnt6-Cre* driver line both in the surface ectoderm and in the somites in the thoracic region leads to the formation of ectopic mammary placodes which were positive for *Wnt10b*. Interestingly, *Fgf10* expression was also increased in the thoracic somites (Carroll and Capecchi, 2015). It is therefore proposed that *Hox* genes both in the somites and in the surface ectoderm are important for mammary placode induction (**Figures 1B,E**).

It is important to mention at this point that Fgf10/Fgfr2b signaling is important not only for the induction of the mammary placode (**Figure 1F**) but also plays important function at later developmental stages during the process of branching of the mammary epithelial tree (see thereafter the chapter about the formation of the mammary epithelial tree). Interestingly, Fgf10 plays also multiple roles in the formation of the limb, which is another ectoderm-derived organ. Understanding the role of Fgf signaling in the limb is relevant for the understanding of mammary gland biology as the basic cellular and molecular mechanisms involved in the induction and the subsequent formation (elongation for the limb and branching for the mammary gland) of these 2 organs are likely conserved (see **Figure 1F**). Fgf10/Fgfr2b signaling was initially reported to be important in limb formation for the induction of the apical etodermal ridge (AER) and for the elongation of the limb bud along the proximal distal axis (Danopoulos et al., 2013; Jin et al., 2018). These two processes (AER induction and limb elongation) could be compared to the process of mammary placode formation and the subsequent bud formation, elongation, and branching. Interestingly, in the limb, Fgf10 has been reported to play a key role before the induction of the AER which takes places at embryonic day 9.5 and E10 for the forelimb and hindlimb, respectively. Before AER induction, Fgf10 has been proposed to control the process of epithelial to mesenchymal transition for the somatopleural epithelium. Such transition is instrumental for the generation of limb progenitors (Gros and Tabin, 2014). Fgf10 plays therefore important roles both before the induction of the AER (which was thought to be the earliest event in limb formation) as well as before mammary placode induction. It is not clear if this dual function played by Fgf10 prior and after organ induction is also conserved for other branched structures either endoderm-derived (lung, pancreas, cecum) or ectoderm-derived (teeth, tongue, palatal shelves as well as salivary and lacrimal glands for example) [For a comprehensive review on the role of Fgf10 in cranio-facial development see (Prochazkova et al., 2018)]. Interestingly, in both endoderm [especially the lung, see (Jones et al., 2018)] and ectoderm-derived organs (particularly for the cleft palate), a common feature appears to be the role of Fgf10 signaling in modulating cell adhesion. It is also clear, however, that Fgf10 signaling plays an important role in cell differentiation (Veltmaat et al., 2006; Jones et al., 2018).

THE EPITHELIUM OF THE MAMMARY GLAND 4 IN *Fgf10* KO EMBRYOS DID NOT UNDERGO BRANCHING MORPHOGENESIS- A PHENOTYPE CAUSED BY DEFECTIVE FAT PAD FORMATION

Branching morphogenesis in mammary gland development starts during embryogenesis but occurs mostly postnatally. Around E15.5, the epithelial buds elongate to invade the fat pad progenitor and subsequently ramifies at E16. At birth, the mammary rudiment displays main and accessory ramifications (Lyons, 1958; Nandi, 1958; Sakakura, 1987; Watson and Khaled, 2008). Analysis at E18.5 of the *Fgf10* KO mammary gland indicated that the mammary gland epithelium remained as a sprout failing to ramify. Close up examination of the gland indicated a very thin, underdeveloped mammary fat pad. Transplantation experiments of the mutant mammary gland epithelium from the *Fgf10* KO embryos into a cleared fat pad from wild type mice indicated that the mutant mammary epithelium was still capable of expanding and forming a complete mammary tree with well distinguishable terminal end buds similar to wild type mammary glands (Mailleux et al., 2002). These results suggested that lack of Fgf10 signaling in the mutant mammary epithelium did not lead to the loss of the stem cell capabilities of this rudimentary structure. In addition, the impaired fat pad in *Fgf10* KO mammary glands suggested that Fgf10 was important for the normal formation of the mammary fat pad, or more generally for the formation of the adipocytes. At this point, it is not clear if in addition of proper fat pad formation, Fgf10 itself is required for the mammary epithelial sprout invasion and ramification. This question is so far difficult to address during mammary gland development as Fgf10 plays a dual function. Fgf10 targets not only the epithelium, mostly via Fgfr2b but also the mesenchyme (likely via a combination of Fgfr2b and Fgfr1b, Al Alam et al., 2015) to trigger its differentiation toward the adipocyte lineage (**Figure 1E**). This conclusion about Fgf10's role in the mesenchyme, arising from

the analysis of the mammary gland derived from placode 4 in *Fgf10* KO embryos was confirmed by an elegant study showing that Fgf10 acts directly on the mesenchyme via the transcription factor C/EBPß to control the differentiation of the pre-adipocytes into adipocytes (Sakaue et al., 2002). Interestingly, a similar situation is found during lung development where from E16.5 onwards, Fgf10 acts both on the alveolar epithelial progenitors to control their differentiation toward the alveolar epithelial type 2 lineage (Chao et al., 2017) as well as on the mesenchyme to control its differentiation toward the lipogenic lineage (Al Alam et al., 2015).

ROLE OF Fgfr2b LIGANDS IN TERMINAL END BUD (TEB) MAINTENANCE

During the 2 months-period following birth, the tips of the mammary rudiments keep invading the mammary fat pad and branching through a combined process of proliferation and differentiation (Hogg et al., 1983). At the cellular level, the mammary gland is made of ducts ending distally into a structure called "terminal end buds (TEBs) (**Figures 2A,B**). The ducts are composed of luminal epithelial cells which express or not the estrogen and progesterone receptor. Basal/myoepithelial cells are located around the luminal cells in the ducts (**Figures 2C,D**). Distally, the TEBs are dilated structures which appear around 3 weeks after birth as a result of hormonal changes (Williams and Daniel, 1983; Sakakura et al., 1987; Kouros-Mehr et al., 2006). Genetic deletion of the *estrogen receptor alpha* (*ERα*) leads to arrested mammary gland development postnatally with no terminal end buds forming (Feng et al., 2007). This phenotype is very similar to the one observed upon blockade of Fgfr2b signaling postnatally (Parsa et al., 2010) suggesting that Fgfr2b expression in the epithelium could be under the control of estrogen signaling. It is worth noticing that in the prostate, Fgfr2 expression is required for androgen activity and its expression is under the control of androgens (Lin et al., 2007; Yu et al., 2013). It is therefore possible that estrogens signaling, via the regulation of Fgfr2b expression in the epithelium, controls the response of the epithelium to the abundant presence of Fgf10 in the stroma.

The TEB structures contain mammary progenitors giving rise to both luminal epithelial and myoepithelial cells. In addition, bipotent progenitor cells located in the basal layer of the mammary duct (thereby called basal stem cells) represent another source of progenitors for the luminal epithelial and myoepithelial cells (Stingl et al., 2006; Rios et al., 2014). These basal stem cells present in the ducts are characterized by the expression of CD49f or CD29 (Shackleton et al., 2006; Stingl et al., 2006). The maintenance of the lumen structure within the mature mammary duct is important for efficient milk secretion (Streuli et al., 1991). Luminal epithelial cells are cuboidal cells tightly connected with each other and with myoepithelial cells. Loss in cell adhesion, and obstruction of lumen by tumor cells is commonly observed in earlier stages of breast cancer (Cardiff, 2010; Nistico et al., 2014).

Constant mutual interactions between mammary epithelial cells and their microenvironment mediated by paracrine signals control the behavior of the mammary epithelium (Fata et al., 2007; Alcaraz et al., 2008; Simian et al., 2009; Mori et al., 2013). Interestingly, TEBs disappear upon blockade of Fgfr2b signaling only to re-appear when Fgfr2b signaling is restored. A similar phenotype was observed for the incisors in mice indicating that the survival of the stem cells responsible for the formation/maintenance of the TEBs or the incisors is not dependent on Fgfr2b signaling (Parsa et al., 2008, 2010). While deletion of *Fgfr1* in the mammary epithelium using the *K14-Cre* driver leads only to a slight delay in development without any observable defect in mammary gland function, simultaneous ablation of both *Fgfr1* and *Fgfr2* (using an adenovirus-Cre approach) leads to a significant loss of self-renewal in the basal stem cell population (Pond et al., 2013). Transplantation experiments into a cleared fat pad of FACS-isolated basal stem cells showed that inactivation in Fgfr2 expression in these cells leads to a drastic reduction in self-renewal and impaired differentiation into luminal epithelial cells as well as defective epithelial branching (Zhang et al., 2014). In addition to an important role for Fgfr2b at the level of the TEB, Fgfr2b could also play an important role at the level of the ductal epithelium. In particular, the function of Fgfr2b signaling in the maintenance of cell adhesion in the mammary duct is still unclear. Our data recently published in the context of the embryonic lung clearly demonstrate that Fgf10/Fgfr2b signaling regulates cell-cell and cell-matrix adhesion (Jones et al., 2018). Phenotypically, blockade of Fgf10 in the early developing lung results in the partial disruption of the lumen within the lung bud as well as increased density of the epithelial layer. All of these changes are likely the consequences of cell rearrangements within the epithelial layer. Interestingly, no changes in the proliferation or survival of the embryonic lung epithelium was observed upon inhibition of Fgfr2b signaling indicating that at these early stages, Fgfr2b signaling mostly impacts cell adhesion.

IN HUMANS, ECTOPIC FGF10 EXPRESSION IS ASSOCIATED WITH TUMOR PROGRESSION IN 10% OF THE BREAST CANCERS

In humans, FGF10 is expressed in both normal and breast cancer tissue, being detectable in 92% of the primary tumors (Theodorou et al., 2004). Supporting the paracrine nature of FGF10 signaling described during early mammary gland development FGF10 expression in normal and breast cancer tissue is limited to the stromal fibroblasts- Luminal epithelial cells of the normal human breast duct do not express FGF10 (Grigoriadis et al., 2006). However, in 10% of the breast carcinomas displaying a high epithelial/stroma ratio, FGF10 is ectopically expressed in the epithelium at high level (Theodorou et al., 2004). Interestingly, some breast carcinoma cell lines show high expression of FGF10 (Theodorou et al., 2004), supporting the possible role of autocrine FGF10 signaling in human breast cancer progression.

In addition, it was reported that FGFR2 expression is drastically increased in breast cancer (Grose and Dickson, 2005;

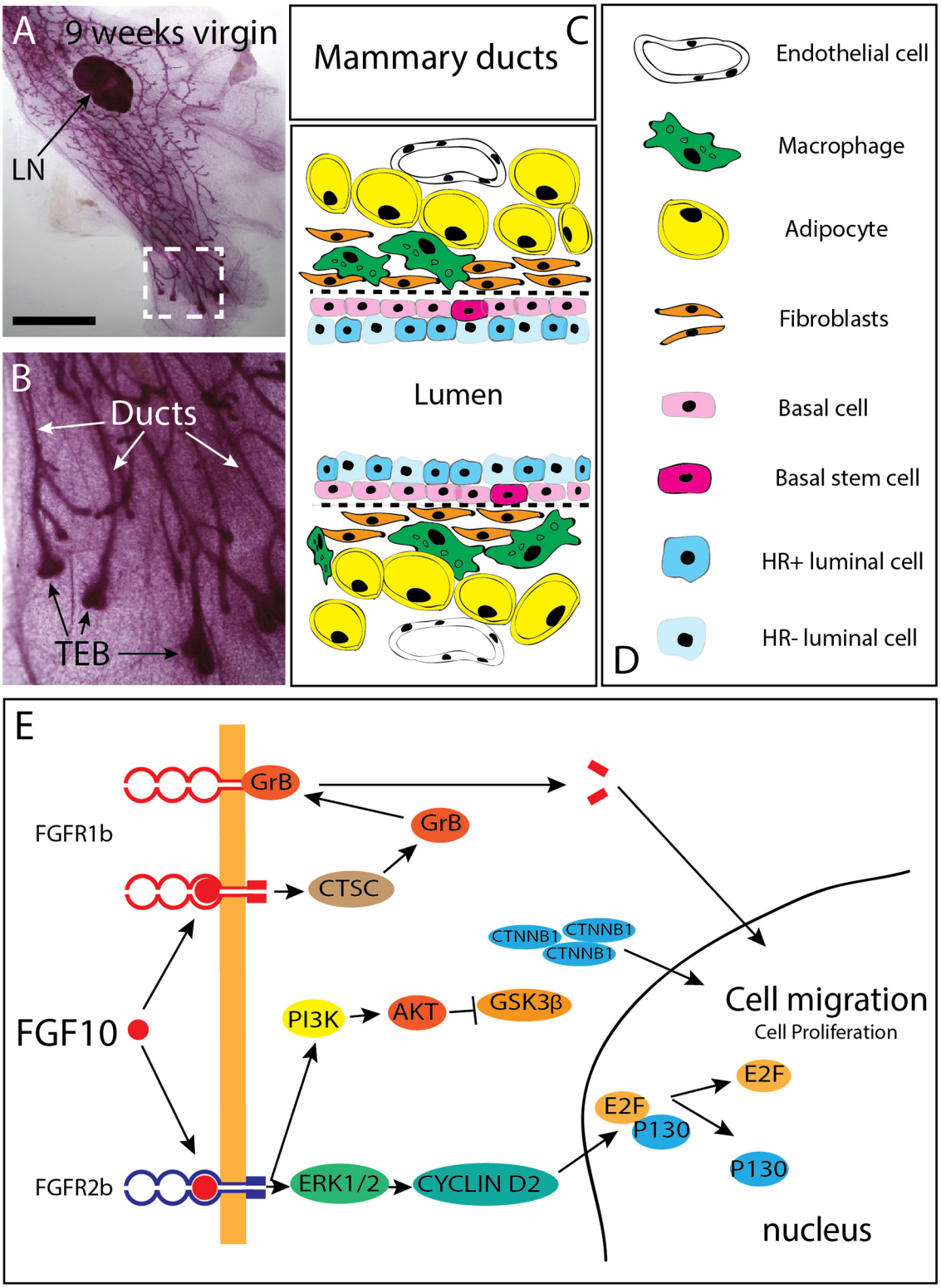

FIGURE 2 | Branching of the mammary epithelial tree starts very early during fetal development and continues post-natally. **(A)** 9 weeks-old mammary gland from a virgin female mouse showing that the mammary epithelial tree has expanded quite distally compared to the lymph node (LN). **(B)** Magnification of the box shown in C.

(Continued)

FIGURE 2 | The mammary epithelial tree is made of terminal end buds (TEB) distally and ducts proximally. **(C,D)** Schematic of the mammary ducts in the adult mammary gland. Basal stem cells are located in the basal layer. These cells differentiate into hormone receptor (HR) positive and negative luminal epithelial cells as well as basal/myoepithelial cells. Mammary fibroblasts, which secrete many cytokines (including Fgf10) are located at close proximity of the basal layer. Fgf10 is also produced by adipocytes as it is needed for their differentiation. **(E)** FGF10 binding to FGFR1 leads to cleavage of the receptor by granzyme B and the translocation of a 55 kDa fragment of FGFR1 to the nucleus, leading to increased cell migration. FGF10/FGFR2b signaling complex activates extra-cellular signal-regulated kinases (ERK)/mitogen-activated protein kinases (MAPK) and phosphoinositide-3 kinase (PI3K). ERK kinases are responsible for the activation of cyclin D2 and consequent transcription of E2F targets genes. PI3K activates protein kinase AKT with subsequent inhibition of glycogen synthase kinase 3β (GSK3β) by phosphorylation, leading to an accumulation of beta Catenin (CTNNB1) and stimulation of the transcription of WNT-dependent genes MAPK dependent phosphorylation of transcription factors allows transcription of FGF target genes. CTSC, Cathepsin C; GrB, Granzyme B; PI3K, Phosphatidylinositide 3-kinase; ERK1/2, Extracellular signal-regulated kinase ½; E2F, Transcription factor E2F; CTNNB1 Catenin beta 1; GSK3b, Glycogen synthase kinase 3 beta; AKT, Serine/threonine kinase. Scale **(C)**: 1.5 cm, **(D)** 100 μm.

Moffa and Ethier, 2007). *FGFR2* mutations are also associated with increased risk of breast cancer in women without history of breast cancer in their family (Hunter et al., 2007). It is likely that such high level of expression of the receptor leads to the significant activation of the FGF10/FGFR2b signaling pathway due to the large amount of FGFR2b ligands expressed in the adult mammary gland. Genetic variants near the *FGF10* locus have been identified through genome-wide association studies and their detection is considered as a risk factor for breast cancer formation (Stacey et al., 2008). It has also been reported that in the context of *FGFR2* genetic variants and breast cancer, lower FGFR2 expression is associated with increased response to estrogen (Campbell et al., 2016) and these FGFR2 variants are associated with poor prognosis (Castro et al., 2016). FGF10 stimulation of the breast cancer cell line MCF-7 [a cell line described as estrogen receptor-positive (ER^{pos})] drive the cells to a basal-like cancer phenotype with diminished dependency to estrogen associated with decreased sensitivity to treatments with anti-estrogen. Interestingly, ER^{pos} breast cancer cells display increased response to the anti-estrogen tamoxifen when treated with AZD4547 and PD173074, two well-known FGFR inhibitors. Therefore, it appears that inhibition of FGF10-FGFR2 signaling can be used therapeutically to bypass the resistance to anti-hormone therapy in the context of breast cancer treatments (Campbell et al., 2018). Patients with FGFR2-overexpressing breast tumors display poor survival when treated with lapatinib, a tyrosine kinase receptor inhibitor for EGFR and HER2 suggesting that FGFR2 signaling could be maintaining the self-renewal and differentiation capabilities of breast cancer stem cells (BCSCs) in the context of lapatinib treatment (Sridharan et al., 2019). Through its binding to FGFR2b, FGF10 triggers increased cell migration and proliferation. This is achieved via the activation of the ERK1/2 pathway, leading to an increased activity of Cyclin D2 and its downstream target E2F. Concomitantly, FGFR2b signaling leads to increased PI3K-AKT activity resulting in the inhibition of GSK3ß and the subsequent accumulation of ß-catenin culminating in upregulation of WNT signaling. Interaction of FGF10 with FGFR1 furthermore leads to the cleavage of the 55 kDa C-terminal fragment by granzyme B (GrB) and its translocation to the nucleus, where it promotes the transcription of target genes related to cell migration and proliferation (**Figure 2E**). Supporting this possibility that FGFR2 signaling could be maintaining the self-renewal and differentiation capabilities of breast cancer stem cells (BCSCs), FGFR2 overexpressing cells are resistant and proliferate under lapatinib selection. It has therefore been proposed that additional anti-FGFR treatment could be beneficial for breast cancer patients treated unsuccessfully with lapatinib (Azuma et al., 2011). Indeed, the use of the PI3K/mTOR inhibitor NVP-BEZ235 in association with the pan-RTK inhibitor dovitinib has been reported to be more beneficial than treatment with single inhibitors (Issa et al., 2013). In mice, efficient responses in terms of tumor growth and apoptosis are associated with the use of FGFR inhibitor in combination with either the PI3K/mTOR inhibitor or the pan-ErbB inhibitor (Issa et al., 2013). In addition, GP369 is a new therapeutic tool in our anti-FGFR arsenal. This monoclonal antibody specifically binds and inactivates the FGFR2-IIIb receptor isoform which is specifically expressed in the epithelium and which overexpression is associated with tumorigenesis (Hackenberg et al., 1991). In transplantation experiments with MFM-223 breast cancer cells (a cell line with 287 genomic copies of FGFR2), the administration of GP369 prevents tumor growth (Bai et al., 2010). Interestingly, FGF10 expression could be regulated through a non-coding antisense RNA called *FGF10-AS1*. Low level of *FGF10-AS1* is associated with triple negative breast cancer (Fan et al., 2019).

IN CONCLUSION

Developmental biology and cancer research are revealing complementary aspects of FGF signaling in both the normal biology and the pathological processes of the mammary glands. The characterization of the molecular and cellular basis of mammary placode induction allows to get a better insight in the control of proliferation, migration and differentiation. These developmental processes are often simultaneously mis-regulated during breast cancer progression and metastasis. Understanding these developmental processes may therefore offer potential novel therapeutic targets for breast cancer treatment.

AUTHOR CONTRIBUTIONS

SR and SB wrote the review and made the illustrations. ChaC and CheC contributed to the revisions and made the updated figures. All authors contributed to the article and approved the submitted version.

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The Role of SMAD2/3 in Human Embryonic Stem Cells

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Human embryonic stem cells (hESCs) possess the potential of long-term self-renewal and three primary germ layers differentiation, and thus hESCs are expected to have broad applications in cell therapy, drug screening and basic research on human early embryonic development. Many efforts have been put to dissect the regulation of pluripotency and direct differentiation of hESCs. TGFβ/Activin/Nodal signal pathway critically regulates pluripotency maintenance and cell differentiation through the main signal transducer SMAD2/3 in hESCs, but the action manners of SMAD2/3 in hESCs are sophisticated and not documented yet. Here we review and discuss the roles of SMAD2/3 in hESC pluripotency maintenance and differentiation initiation separately. We summarize that SMAD2/3 regulates pluripotency and differentiation mainly through four aspects, (1) controlling divergent transcriptional networks of pluripotency and differentiation; (2) interacting with chromatin modifiers to make the chromatin accessible or recruiting METTL3-METTL14-WTAP complex and depositing m6A to the mRNA of pluripotency genes; (3) acting as a transcription factor to activate endoderm-specific genes to thus initiate definitive endoderm differentiation, which happens as cyclin D/CDK4/6 downstream target in later G1 phase as well; (4) interacting with endoderm specific lncRNAs to promote differentiation.

Keywords: SMAD2/3, human embryonic stem cell, pluripotency, cell cycle, differentiation, lncRNAs, epigenetic modification

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INTRODUCTION

The TGF-β superfamily comprises TGF-βs, activins, nodal, growth and differentiation factors (GDFs) and bone morphogenetic proteins (BMPs). More than 60 TGF-β family members have been identified in multicellular organisms, with at least a half of proteins are encoded in human genome (Feng and Derynck, 2005). TGF-β signal originates from the binding of ligand dimers and heteromeric complex of type I (ALK1-7) and type II transmembrane serine/threonine kinase receptors. Activated type II receptors phosphorylate the type I receptors kinase domain, and then phosphorylate the intracellular SMAD proteins (Wu and Hill, 2009). There are three functional classes containing eight SMAD proteins: receptor-regulated SMADs (R-SMAD) including SMAD1/2/3/5/8, Co-mediator SMAD4 (Co-SMAD), and the inhibitory SMAD6 and 7 (I-SMAD). All SMAD proteins share Mad Homology domains MH1 and MH2: the MH1 domain contains a β-hairpin structure that mediates DNA binding, and the MH2 domain mediates SMAD oligomerization and establishes signal specificity by mediating proper type I receptor interaction

with specific DNA binding partners (Weiss and Attisano, 2013). TGF-β/Activin/Nodal signaling occurs through ALKs 4, 5, and 7 and SMAD2/3 (Massague et al., 2005). It is reported that inhibition of Activin/Nodal signaling causes hESCs differentiation (James et al., 2005; Vallier et al., 2005; Xiao et al., 2006). On the other hand, Activin/Nodal signaling, through the TGF-β receptors and its effector SMAD2/3, initiates definitive endoderm differentiation in human and mouse ESC (Tremblay et al., 2000; Kubo et al., 2004; D'Amour et al., 2005). Accumulated reports show that TGF-β/Activin/Nodal signaling and its effectors SMAD2/3 are not only necessary for hESC self-renewal but also required for germ layer differentiation. However, the mechanism that SMAD2/3 coordinates different partners to determine different cell fate decisions is not systematically elaborated. Herein, this review is focusing on the roles and mechanisms of SMAD2/3 in regulating hESC pluripotency maintenance and germ layer differentiation.

SMAD2/3 CONTROLS DIVERGENT TRANSCRIPTIONAL NETWORKS OF PLURIPOTENCY AND ENDODERM DIFFERENTIATION

Human ESCs are derived from the blastocyst with the potential of long-term self-renewal and three primary germ layers differentiation (Thomson et al., 1998) and cultured in medium with basic fibroblast growth factor (bFGF) and Activin or TGF-β. TGF-β/Activin/Nodal signaling is essential for the maintenance of hESC pluripotency and self-renewal, while FGF2 serving as a competence factor (James et al., 2005; Xiao et al., 2006). Inhibition of Activin/Nodal signaling by Follistatin or by Activin receptor inhibitor SB431542 causes hESC differentiation (James et al., 2005; Vallier et al., 2005; Xiao et al., 2006). Pluripotency is maintained by the OCT4-SOX2-NANOG transcriptional network, which forms a positive feedback loop that activates pluripotency genes and inhibits the expression of differentiation associated genes (Boyer et al., 2005; He et al., 2009). Activin A is sufficient for the maintenance of self-renewal and pluripotency and induces the expression of OCT4, NANOG and SOX2 (Xiao et al., 2006; Xu et al., 2008), suggesting that pluripotent transcription factors might be the targets of TGF-β/Activin/Nodal signaling in hESCs. Inhibition of Activin/Nodal results in a more decrease of NANOG expression than OCT4 and SOX2 in hESCs, and thus induces differentiation toward neuroectoderm. Constitutive expression of NANOG is sufficient to maintain the pluripotency of hESC in the absence of Activin/Nodal signaling (Vallier et al., 2009a,b). In addition, transfection of small interfering RNAs targeting *NANOG* in hESCs causes differentiation to extraembryonic endoderm and trophectoderm lineages (Hyslop et al., 2005). Therefore, NANOG is considered as a direct target of TGF-β/Activin/Nodal signaling, which is further demonstrated by the study that SMAD2/3 can directly bind with the NANOG proximal promoter to activate NANOG expression and repress autocrine BMP signaling (Xu et al., 2008; Vallier et al., 2009a; Brown et al., 2011; Sakaki-Yumoto et al., 2013). Meanwhile, NANOG and SMAD2/3 can bind to regulatory elements of endoderm genes with other transcription repressors to inhibit differentiation (Xu et al., 2008; Brown et al., 2011). For instance, NANOG and SMAD2/3 inhibits Smad-interacting protein 1 (SIP1) expression to block neuroectoderm differentiation (Chng et al., 2010). Like SIP1, SNON (also named as SKIL), a potent SMAD2/3 corepressor, is expressed in hESCs but rapidly down-regulated upon differentiation. In pluripotent state, SNON is transcriptionally activated by OCT4/SOX2/NANOG complex and is selectively recruited by SMAD2, and then SMAD2/SNON together binds to mesendodermal genes to suppress the expression (Tsuneyoshi et al., 2012). A recent report shows that Jun N-terminal kinase (JNK)-JUN family genes JUN co-occupies ESC-specific enhancers with OCT4, NANOG, SMAD2/3, and specifically inhibits the exit from the pluripotent state by impeding the decommissioning of ESC enhancers and inhibiting the reconfiguration of SMAD2/3 chromatin binding from ESC-specific to endoderm-specific enhancers (Li et al., 2019). Overall, SMAD2/3 is necessary for self-renewal and pluripotency maintenance: SMAD2/3 not only targets NANOG to positively regulate OCT4-SOX2-NANOG transcriptional network, but also interacts with repressors such as SIP1, SNON and JUN to inhibit lineage genes associated with primary germ layer differentiation (**Figure 1**).

However, highly activated Activin/Nodal signal results in definitive endoderm differentiation. High concentrations of Activin A induces efficient differentiation of hESCs towards definitive endoderm: 50–100 ng/ml Activin A drives endoderm differentiation (D'Amour et al., 2005), whereas 5 ng/ml Activin A is supportive to maintain pluripotency of hESCs (Xiao et al., 2006; Tsai et al., 2010; Tomizawa et al., 2013). SMAD2/3 directly binds to endodermal lineage specifiers such as *SOX17*, *FOXA2*, *GATA6,* and *GSC* to induce endoderm differentiation (Brown et al., 2011; Kim et al., 2011). In addition, the pluripotent factors OCT4, SOX2, and NANOG control EOMES expression at onset of endoderm specification, and SMAD2/3 further interacts with EOMES to initiate endodermal transcription network expression (Teo et al., 2011). Activin/Nodal signaling also interacts with other signal pathways, mainly including BMP and WNT to synergistically promote endoderm differentiation (D'Amour et al., 2005; Loh et al., 2014). Higher activity of WNT/β-catenin stimulated by high dosage of WNT3A or glycogen synthase kinase-3 (GSK-3) inhibitors is required for maximally inducing definitive endoderm differentiation together with Activin A (Gadue et al., 2006; Teo et al., 2014). PI3K/AKT signaling, stimulated by fibroblast growth factor (FGF), directly modulates the downstream of WNT pathway to maintain undifferentiated hESCs (Ding et al., 2010). PI3K/AKT can also inhibit SMAD3 phosphorylation causing blocking of nuclear translocation (Remy et al., 2004). In addition, the inhibition of PI3K activity causes decrease of the mRNA and protein levels of Nanog (Storm et al., 2007), and activation of PI3K/AKT signaling is sufficient to maintain pluripotency of primate ESCs (Watanabe et al., 2006). Furthermore, Activin/Nodal specifies definitive endoderm from hESCs only when PI3K signaling is low (McLean et al., 2007). It seems that PI3K/AKT signaling is the important switcher

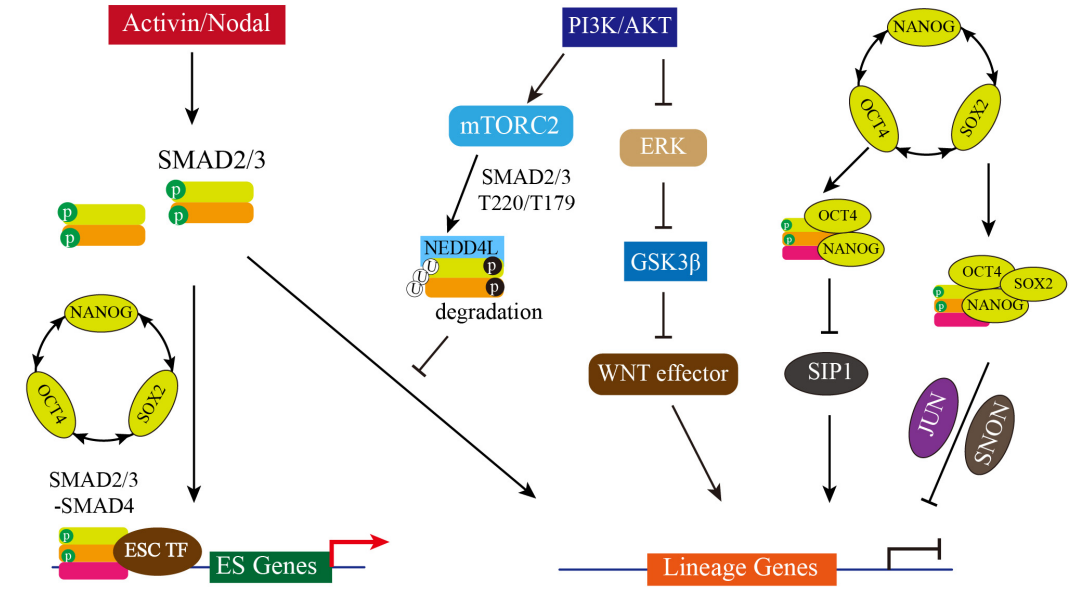

FIGURE 1 | The Multiple Roles of SMAD2/3 in Maintenance of hESCs. Activated TGF-β/Activin/Nodal signaling causes SMAD2/3 phosphorylation in distal C-terminal SXS motif (in green), which thus interacts with SMAD4 to form heterooligomeric complex and enters nucleus. Accumulated SMAD2/3/4 complex in nucleus can directly bind to the promotor regions of pluripotency marker genes, such as *NANOG* and *OCT4*, promoting the positive feedback loop of OCT4-SOX2-NANOG. In addition, SMAD2/3 can inhibit SIP1 expression to block neuroectodermal differentiation. SMAD2/3 also interacts with NANOG to repress differential genes expression, or recruits SNON activated by OCT4/SOX2/NANOG, or interacts with JUN, and then together with OCT4/SOX2/NANOG complex to suppress lineage genes expression. Meanwhile, PI3K/AKT is highly activated in hESCs. PI3K/AKT on one hand inhibits ERK and WNT signaling, allowing SMAD2/3 to activate pluripotency associated target genes rather than lineage genes; on the other hand, the downstream of PI3K/AKT mTORC2 can phosphorylate SMAD2/3 resulting the degradation mediated by NEDD4Land thus blocking the direct activation of lineage genes.

for Activin/Nodal signaling in pluripotency or differentiation. Supporting with this notion, a later study suggests that PI3K governs Activin A/SMAD2/3 to promote pluripotency or differentiation cell fate in hESCs when PI3K activity is in high or low level, respectively (Singh et al., 2012). Singh and colleagues found that PI3K/AKT inhibits RAF/MEK/ERK and canonical WNT signaling, allowing SMAD2/3 to activate pluripotency associated target genes, such as NANOG, at high level of PI3K in undifferentiated state. When PI3K/AKT signaling is absent, the ERK activates canonical WNT signaling pathways and then the WNT effectors such as β-catenin and SNAIL can permit SMAD2/3 to activate differentiation associated genes (Singh et al., 2012). However, there is a controversial: Na and colleagues observed the inhibition of MEK/ERK prevents differentiation and promotes hESCs self-renewal (Na et al., 2010) while others showed that MEK/ERK signaling positively contributes to maintain self-renewal of hESCs (Armstrong et al., 2006; Li et al., 2007). Yu and colleagues provide more details in molecular mechanism. PI3K antagonizes the Activin/Nodal signaling in definitive endoderm differentiation by activated rapamycin complex 2 (mTORC2). Activation mTORC2 induces SMAD2/3 phosphorylation at T220/T179 residues which is not dependent on AKT, CDK, or ERK activity. The phosphorylated SMAD2/3 recruits E3 ubiquitin ligase NEDD4L, which causes SMAD2/3 degradation and blocks endoderm differentiation (Yu et al., 2015).

Taken together, Activin/Nodal signaling via SMAD2/3 on one hand is involved in controlling divergent transcriptional networks by interacting with different partners to regulating pluripotency or differentiation in hESCs. On the other hand, Activin/Nodal signaling antagonizes with PI3K/AKT pathway in pluripotency and differentiation. High PI3K and low Activin activity in hESCs promotes SMAD2/3 binding and activating gene expression of pluripotent transcription factors (**Figure 1**).

SMAD2/3 INTERACTS WITH EPIGENETIC MODIFIERS

The pluripotency and differentiation of ESC is regulated not only by the core transcriptional network but also epigenetic modifiers, as epigenetic modification supports a permissive or repressive chromatin environment for transcription. In mouse ESCs, SMAD2/3 recruits the histone demethylase JMJD3 to target genes *Nodal* and *Brachyury*, thereby counteracting repression of Polycomb (Dahle et al., 2010). A follow-up study reveals that SMAD2/3 can also counteract Polycomb repression to regulate *Oct4* expression during initiation of ESC

differentiation (Dahle and Kuehn, 2013). Similarly, helix-loop-helix (HLH) proteins HEB also interacts with SMAD2/3 at distal enhancer elements and associates with PRC2 at promotors of mesendodermal genes (Yoon et al., 2015). In addition, it is reported that TGF-β/Nodal signals trigger differentiation in mouse ESC by influencing H3K9me3 modification, the hallmark of heterochromatin. The activation of Nodal signal induces the formation of TRIM33-SMAD2/3 complex. The PHD and Bromo cassette of TRIM33, respectively, recognizes K9me3 and binds an adjacent K18ac, making the chromatin accessible, which in turns allows SMAD2/3-SMAD4 to bind to the promotor of *Gsc* and *Mixl1* to finally promote stem cell differentiation (Xi et al., 2011).Whether there is similar mechanism that SMAD2/3 interacts with Polycomb complex to respond to pluripotency maintenance or differentiation initiation in hESCs is rarely reported until recent years. Wang and colleagues show that in hESCs Activin signal impairs PRC2 activity by SMAD2-mediate reduction of EZH2 protein level, which is the catalytic subunit of PRC2 (Wang et al., 2017). Further study documents that the global reduction of H3K27me3 by Activin signal causes the forkhead protein FOXH1 to recruit into open chromatin regions, which together with SMAD2 and β-catenin activates mesendodermal genes expression, such as HAS2 and ALDH3A2 (Xu et al., 2018). Except for inhibitory epigenetic modification, it is also reported that Activin/Nodal signaling is relevant with the H3K4me3 maintenance on the master regulators of both pluripotency and germ layer specification in hESCs (Bertero et al., 2015). SMAD2/3 interacts with H3K4 methyltransferases complex COMPASS and its cofactor DPY30 to maintain H3K4me3 marks on pluripotency and mesendoderm genes. Inhibiting Activin/Nodal signaling causes specifically impaired H3K4me3 level on promotor and enhancer regions. Knockdown DPY30 in hESCs reduces expression of pluripotency and endoderm markers but increases neuroectoderm genes. Further mechanistic studies show that SMAD2/3 cooperates with NANOG and DPY30 to regulate H3K4me3 deposition on pluripotency and cell fate decision associated genes (Bertero et al., 2015). Recently the same group shows SMAD2/3 interacts with the METTL3-METTL14-WTAP complex identified by genome-wide analysis of SMAD2/3 interactome, which deposits N6-methyladenosine (m6A) on RNA. The interaction between SMAD2/3 and METTL3-METTL14-WTAP complex promotes m6A deposition on specific regulators of pluripotency such as NANOG, which resulting the degradation of NANOG mRNA and thus facilitating pluripotency exit and differentiation initiation (Bertero et al., 2018). Taken together, SMAD2/3 can interact with multiple epigenetic modifiers to function in different levels, together controlling pluripotency and mesendoderm differentiation initiation (**Figure 2**).

SMAD2/3 MEDIATES hESC DIFFERENTIATION PROPENSITY IN G1 PHASE

Human ESCs in different cell cycle phases exhibit biased differentiation propensity, which involves in CDK4/6-mediated SMAD2/3 phosphorylation. Primate ESCs exhibit unusual cell cycle features which are different from mouse ESCs, including dramatically shortened G1 phase (Becker et al., 2006; Fluckiger et al., 2006). Subsequent studies show that shortened G1 phase of cell cycle in hESCs is a cause, rather than a consequence of pluripotency. A short G1 limits the "window of opportunity" for which a cell can be responsive to differentiation cues (Neganova et al., 2009; Lange and Calegari, 2010), and lengthening G1 phase of pluripotent cell promotes differentiation in mouse and human (Filipczyk et al., 2007; Koledova et al., 2010; Lange and Calegari, 2010; Sela et al., 2012). Human ESCs express all G1-specific Cyclins (D1, D2, D3, and E) and cyclin-dependent kinases (CDK) (CDK2, CDK4, and CDK6) at variable levels (Neganova et al., 2009; Lange and Calegari, 2010). Knockdown of CDK2 in hESCs results in arrest at G1 phase and differentiation to extraembryonic (Neganova et al., 2009), suggesting that CDK2 is very important for cell cycle regulation and pluripotency maintenance in hESCs. Recent reports have provided some explanations for how to determine cell fate propensity in G1 phase. Using FUCCI reporter system, which is a sensor of cell cycle, Pauklin and Vallier observed hESCs in early G1 phase can only initiate endoderm differentiation but neuroectoderm differentiation is limited in later G1 phase. Further mechanistic analysis shows that the activity of Activin/Nodal signaling is controlled by Cyclin D, which activates CDK4/6 to phosphorylate SMAD2/3 in the linker region and thus makes the phosphorylated fail to enter nucleus. Since the Cyclin D expression is low in early G1 and high in later G1, thus Cyclin D/CDK4/6 inhibits the transcriptional activity of Activin/Nodal signaling to determine cell fate propensity by controlling the cellular localization of SMAD2/3 (Pauklin and Vallier, 2013). Consistently, SMAD3 is proved to be the substrate of CDK4 in MEF, and the phosphorylated SMAD3 by CDK4 inhibits its transcriptional activity (Matsuura et al., 2004).

Singh and colleagues also reported the heterogeneity correlated with cell cycle, but their results indicate that WNT/ERK signal promotes heterogeneity in late G1 cells, including developmental regulators expression such as *GATA6*, *SOX17*, *FOXA2* (Singh et al., 2013). A later study by the same group further proved that the bivalent state is not stable in hESCs either, especially in G1 phase. H3K4me3 is transient increased at developmental genes by CDK2-dependent phosphorylation of the MLL2 histone methyl-transferase during G1, thereby opening a "window of opportunity" for lineage specification (Singh et al., 2015). In addition, a recent single-cell level study shows that hESCs exhibit high single-cell variation in absolute G1 length which is controlled by WNT/β-catenin pathway. A longer and wider distribution of G1 phase is regulated by WNT inhibition, allowing global reduction of 5-hydroxymethylcytosine (5hmC) on lineage-specific genes and thus causing biased differentiation toward neuroectoderm lineages but not affecting pluripotent genes expression. Transient decrease of G1 length by transgenic manipulation shows predominantly contributing to mesendoderm (Jang et al., 2019). Different with the previous report (Pauklin and Vallier, 2013), the single-cell analysis results show that the length of G1 has no effect on SMAD2/3 activity (Jang et al., 2019). Taken together, it is consistent that G1 phase creating a "window

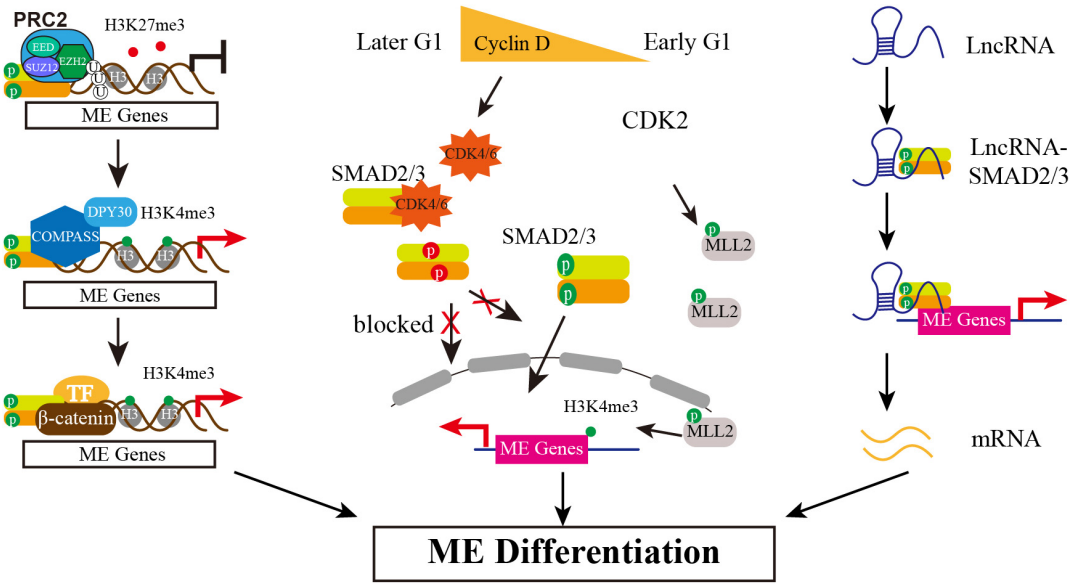

FIGURE 2 | The Multiple Mechanisms of SMAD2/3 Regulating Mesendoderm Differentiation of hESCs. Epigenetically, SMAD2/3 recruits JMJD3 to its target genes, erasing H3K27me3 marker. Then, SMAD2/3 interacts with COMPASS and its cofactor DPY30 to maintain H3K4me3 marks on these genes. In addition, differentially expressed Cyclin D in early and later G1 phase determines the biased lineage differentiation propensity. Low expression of Cyclin D in early G1 causes low level of CDK4/6, blocking the phosphorylation of SMAD2/3 in the linker region (in red) and thus allowing SMAD2/3 enter nucleus to activate mesendodermal genes expression. Meanwhile, CDK2 may phosphorylate MLL2 during G1 and causes H3K4me3 transiently increased at developmental genes, thereby opening a "window of opportunity" for lineage specification. The third action model is mediated by lncRNAs. Certain highly expressed lncRNAs interact with SMAD2/3 and recruit SMAD2/3 to and activate the expression of endodermal transcription factors such as *FOXA2*/*GSC*.

of opportunity" for mesendodermal genes expression, which endows hESC differentiation propensity to mesendoderm in early G1 phase (**Figure 2**). However, how to initiate lineage specification and whether SMAD2/3 is controlled in G1 phase are still controversial and need to be further clarified.

SMAD2/3 INTERACTS WITH lncRNA PROMOTING DIFFERENTIATION

Long non-coding RNAs (lncRNAs) are transcribed with longer than 200 nucleotides and have been found wildly expressed from mammal genome, and play an important role in diverse biological processes, including regulation of stem cell (Fatica and Bozzoni, 2014). During hESCs differentiates into endoderm, there are reported that SMAD2/3 serve as RNA-interacting protein to actively participate in endoderm differentiation by different action mechanism (Jiang et al., 2015; Daneshvar et al., 2016; Chen et al., 2020). *DEANR1* is the first reported lncRNA that regulates human definitive endoderm differentiation. *DEANR1* is highly expressed in endoderm and contributes to endoderm differentiation by positively regulating endoderm factor FOXA2. Mechanistically, *DEANR1* facilitates FOXA2 activation by interacting with SMAD2/3 and recruiting to the FOXA2 promotor (Jiang et al., 2015). *DIGIT*, an endoderm-expressing lncRNA, is regulated by a SMAD3-occupied enhancer proximal to *DIGIT*. *DIGIT* regulates the transcription of *GSC in trans*, and deletion of the SMAD3-occupied enhancer inhibits *DIGIT* and *GSC* expression and definitive endoderm differentiation (Daneshvar et al., 2016). Very recently, lncRNA *LINC00458* is reported up-regulated in hESCs upon cultured with soft substrate. Gain- and loss-of-function experiments confirm that *LINC00458* is functionally required for endodermal lineage specification from hESCs induced by soft substrates. Importantly, the endoderm-promoting function of *LINC00458* depends on the interaction with SMAD2/3 (Chen et al., 2020). These reports together suggest a new model that lncRNA may function as a new co-factor of SMAD2/3 to mediate lineage differentiation (**Figure 2**).

SUMMARY AND PROSPECT

SMAD2/3 is the major effector of TGFβ/Activin/Nodal signal, which plays different roles in undifferentiated hESCs and committed cells. Herein, we summarize the multiple action manners of SMAD2/3 in pluripotency maintenance and differentiation initiation in terms of transcription regulation, epigenetic modification, cell cycle related differentiation bias and interaction with lncRNAs. SMAD2/3 protects pluripotent state by directly targeting OCT4-SOX2-NANOG network to maintain pluripotency and interacts with other factors such as SIP1, SNON, and JUN to inhibit differentiation at high PI3K/AKT activity. When in differentiation context, SMAD2/3 is highly activated by higher Activin/Nodal signal while low PI3K/AKT activity. Accessible chromatin due to the increase of 5hmC and H3K4me3 allows SMAD2/3 binding to developmental

regulators to perform cell-fate decision function. It is very likely that Activin/Nodal and its antagonistic PI3K/AKT signal together determine SMAD2/3 functional switch in pluripotency and differentiation. In addition, WNT pathway synergies with Activin/Nodal to permit SMAD2/3 to activate differentiation associated genes. Meanwhile, the co-regulators of SMAD2/3 also are decisive for SMAD2/3 function in different contexts. However, there are controversies about the role of SMAD2/3 in cell cycle dependent differentiation initiation. In addition, how SMAD2/3 chooses different co-regulators during the pluripotency maintenance and differentiation transition is still unclear but very important to understand the underlying mechanism of cell fate determination.

Furthermore, the SMAD2/3 interactome analysis suggests SMAD2/3 interacts with many other functional complexes except for classic TGF-β signaling associated pathways, including E3 ubiquitin ligase, mRNA processing and degradation, DNA repair and apoptosis (Bertero et al., 2018). Whether or which of these interactions with SMAD2/3 are important for ESCs pluripotency maintenance or differentiation is not uncovered yet. In addition, SMAD2/3 also participates in lipid metabolism in mouse hepatocytes (Yang et al., 2013) and epithelial-mesenchymal transition and metastasis induction in breast cancer (Rios Garcia et al., 2017). Of note, there is significant metabolic difference between pluripotent state and differentiation in stem cells (Mathieu and Ruohola-Baker, 2017). Does SMAD2/3 also directly regulate metabolism related protein or signal pathways to influence pluripotency or differentiation in hESCs? In addition to the roles of SMAD2/3 reviewed in this article, there are still many unknown roles of SMAD2/3 in hESCs that need to be clarified.

AUTHOR CONTRIBUTIONS

JY conceived the project and drafted the manuscript. WJ and JY prepared the figures and finalized the manuscript. Both authors contributed to and approved the final 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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Mediator Med23 Regulates Adult Hippocampal Neurogenesis

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Mammalian Mediator (Med) is a key regulator of gene expression by linking transcription factors to RNA polymerase II (Pol II) transcription machineries. The Mediator subunit 23 (Med23) is a member of the conserved Med protein complex and plays essential roles in diverse biological processes including adipogenesis, carcinogenesis, osteoblast differentiation, and T-cell activation. However, its potential functions in the nervous system remain unknown. We report here that Med23 is required for adult hippocampal neurogenesis in mouse. Deletion of Med23 in adult hippocampal neural stem cells (NSCs) was achieved in Nestin-Cre^{ER}:Med23$^{flox/flox}$ mice by oral administration of tamoxifen. We found an increased number of proliferating NSCs shown by pulse BrdU-labeling and immunostaining of MCM2 and Ki67, which is possibly due to a reduction in cell cycle length, with unchanged GFAP^{+}/Sox2^{+} NSCs and Tbr2^{+} progenitors. On the other hand, neuroblasts and immature neurons indicated by NeuroD and DCX were decreased in number in the dentate gyrus (DG) of Med23-deficient mice. In addition, these mice also displayed defective dendritic morphogenesis, as well as a deficiency in spatial and contextual fear memory. Gene ontology (GO) analysis of hippocampal NSCs revealed an enrichment in genes involved in cell proliferation, Pol II-associated transcription, Notch signaling pathway and apoptosis. These results demonstrate that Med23 plays roles in regulating adult brain neurogenesis and functions.

Keywords: Mediator complex 23, hippocampus, proliferation, cell cycle, adult neural stem cells

INTRODUCTION

The mammalian Mediator complex is an evolutionarily conserved multi-protein complex, which functions as a key transcriptional cofactor by forming a link between sequence-specific transcription factors and the RNA polymerase II (Pol II)-associated basal transcription machinery. In this context, it may be required for the transcription of thousands of protein-coding genes (Lewis and Reinberg, 2003; Malik and Roeder, 2005; Hentges, 2011). The Mediator complex contains up to

30 proteins, and given its fundamental role in gene transcription, inactivation of individual genes may lead to global gene expression defects. However, based on data showing that mutation of different Mediator complex genes in the same organism leads to different phenotypes, individual Mediator complex proteins have unique biological functions (Bourbon et al., 2004; Malik and Roeder, 2010; Hentges, 2011).

The Mediator subunit 23 (Med23, also known as sur2) was originally identified as a genetic suppressor of activated let-60 ras mutation in *Caenorhabditis elegans* (Singh and Han, 1995). Med23 functions in the mitogen-activated protein kinase (MAPK) signaling pathway by interacting with the ternary complex factor Elk1, and data from embryonic fibroblasts have indicated that MAPK-activated interaction of Med23-Elk1 is required for hormone-induced adipogenesis through controlling transcription of immediate early gene Egr2 (Stevens et al., 2002; Wang et al., 2005, 2009). Important roles of Med23 have also been reported in other biological processes including osteoblast differentiation (Liu et al., 2016), myogenesis (Yin et al., 2012), lung carcinogenesis (Yang et al., 2012; Yao et al., 2015), glucose and lipid metabolism (Chu et al., 2014), and T-cell activation (Sun et al., 2014).

Our previous study has shown that Med23-deficient embryonic stem cells display enhanced neural differentiation (Zhu et al., 2015). However, the roles of Med23 in the nervous system remain unknown. Importantly, a missense mutation of Med23 has been reported in patients with intellectual disability and dysregulation of expression of immediate early gene JUN and FOS (Hashimoto et al., 2011; Trehan et al., 2015). In this study, we focused on adult hippocampal neurogenesis in order to explore possible roles of Med23 within the brain.

Adult neurogenesis in the mammalian brain has received increased attention in recent years, especially because of its potential roles in neurological and psychiatric disorders. The process of adult neurogenesis includes the proliferation and differentiation of neural stem cells (NSCs), neuronal survival and migration, and integration of newborn neurons into existing circuits (Zhao et al., 2008; Ming and Song, 2011). The subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus is one of the active neurogenic niches of the adult brain (Morales and Mira, 2019). Impaired SGZ neurogenesis is associated with defective spatial learning and memory, and retrieval of contextual fear memory in mice (Zhang et al., 2013, 2014; Lieberwirth et al., 2016). It has also been reported that enhanced hippocampal neurogenesis is involved in mediating the antidepressant effects of fluoxetine and cognition in Alzheimer's mouse model (Santarelli et al., 2003; Choi et al., 2018).

Here, we provide evidence that Med23 is involved in the regulation of adult hippocampal neurogenesis in mouse. Med23-deficient NSCs in the SGZ display faster self-renewal activity possibly by reducing cell cycle length, but neuroblasts, immature and mature new-born neurons are reduced. In addition, inducible Med23 conditional knockout (CKO) mice show defects in spatial and contextual fear memory. Gene ontology (GO) analysis of Med23-deficient NSCs reveals an enrichment in genes involved in cell proliferation, Pol II-associated transcription, Notch signaling pathway and apoptosis. Taken together, these results demonstrate that Med23 is an important regulator of adult brain functions.

MATERIALS AND METHODS

Animals

To investigate the role of Med23 in adult hippocampal neurogenesis, we first generated Nestin-Cre^{ER}:Med23$^{flox/+}$ mice by crossing Nestin-Cre^{ER} (Imayoshi et al., 2008) with Med23$^{flox/flox}$ mice with two LoxP sites flanking exons 5–7 of Med23 allele as described in our previous study (Chu et al., 2014). Nestin-Cre^{ER}:Med23$^{flox/+}$ mice were further crossed with Med23$^{flox/flox}$ mice to obtain Nestin-Cre^{ER}:Med23$^{flox/flox}$ (Med23 CKO) mice. Littermates with other genotypes (i.e., Med23$^{flox/+}$ and Med23$^{flox/flox}$) were used as controls, because they did not exhibit any detectable alterations in the SGZ (see below). To visualize Med23-deficient NSCs and its progeny *in vivo* using Rosa26-stop-YFP mice (Romer et al., 2011), Nestin-Cre^{ER}:Med23$^{flox/flox}$:Rosa26-stop-YFP (referred to as Med23 CKO:Rosa-YFP) mice were generated by crossing Nestin-Cre^{ER}:Rosa26-stop-YFP:Med23$^{flox/+}$ mice with Med23$^{flox/+}$ mice; in this set of experiments littermates with the genotype of Nestin-Cre^{ER}:Rosa26-YFP:Med23$^{+/+}$ were used as controls, because they did not show any detectable alterations in the SGZ either. All animal experiments were carried out under the protocols approved by the Animal Care and Use Committees of Tongji University School of Medicine, China.

Tamoxifen Administration

To activate Cre-mediated recombination, Tamoxifen (TAM; 200 mg/kg; Sigma) dissolved in corn oil solution (Sigma) was administered once daily by oral gavage for 5 consecutive days in adult (2 months) Med23 CKO mice. Littermate control mice received the same treatment. TM-treated mice were allowed to recover 3–4 weeks and then used in most experiments. In the experiment of hippocampal NSC culture, the TAM treatment were done at the age of one month.

BrdU and EdU Administration

To observe the survival of newborn neurons, mice were treated with TAM as described above, followed by intraperitoneal (i.p.) injection of 5-bromo-2′-deoxyuridine (BrdU; 100 mg/kg; Sigma) the day after, daily for 3 consecutive days, and sacrificed 21 days later. To observe proliferation of NSCs in the SGZ, mice were injected with BrdU (50 mg/kg) four times with a 2 h interval in-between injections were sacrificed 2 h after the last injection. To analyze cell cycle length of NSCs, mice received a single injection of BrdU (50 mg/kg), and were sacrificed 30 min later for double immunostaining of BrdU and Ki67 as described previously (Qu et al., 2013).

To explore the length of the S-phase of NSCs, mice were first injected with 57.5 mg/kg of 5-ethynyl-2′-deoxyuridine (EdU; Life Technologies), followed by another single injection of 42.5 mg/kg BrdU 3 h later. Mice were sacrificed 45 min later

for double staining of BrdU and EdU as described previously (Brandt et al., 2012).

Immunostaining of BrdU, EdU, and Ki67

For BrdU detection, sections were incubated in 2N HCl for 25 min at 37°C prior to neutralization with 0.01 M sodium borate (pH 8.5) for 10 min. Sections were washed in phosphate-buffered saline (PBS) and incubated with mouse anti-BrdU (1:400; Calbiochem) at 4°C overnight. Then sections were incubated with biotinylated horse anti-mouse (1:500; Vector) for 3 h and finally with Cy3-conjugated streptavidin (1:1000; Sigma) for 1 h at room temperature (RT).

For double staining of BrdU and EdU, sections were processed for detection of BrdU first as described above, followed by EdU staining as follows: incubation of sections for 3 h at RT in PBS containing 1 mM CuSO4, 50 mM ascorbic acid and 10 μM fluorescent azide 488 (Salic and Mitchison, 2008).

For double staining of BrdU and Ki67, HCl/sodium borate-pretreated sections were incubated with a mixture of rat anti-BrdU (1:1000; Accurate) and rabbit anti-Ki67 (1:400; Leica), then with biotinylated horse anti-rat IgG (1:500; Vector) for 3 h, and finally with a mixture of Cy3-conjugated streptavidin (1:1000; Sigma) and 488-donkey anti-rabbit (1:500; Invitrogen) for 1 h at RT.

Immunostaining, *in situ* Hybridization and TUNEL Staining

Under deep anesthesia with sodium pentobarbital (Merck), mice were transcardially perfused with 0.1 M PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in PBS. After cryoprotection with 30% sucrose in PBS, brains were cut into 30-μm-thick coronal sections on a cryostat, which were then treated with 0.01 M citrate buffer (pH 6.0) at 95°C for 7 min before incubating with the following primary antibodies for single or double immunostaining: rabbit anti-Ki67 (1:400; Leica), mouse anti-MCM2 (1:800; BD Pharmingen), goat anti-Sox2 (1:200; Santa Cruz), rabbit anti-glial fibrillary acidic protein (GFAP; 1:1000; DAKO), goat anti-doublecortin (DCX; 1:400; Santa Cruz), goat anti-NeuroD (1:400; Santa Cruz), rabbit anti-GFP (1:1000; Life Tech), mouse anti-NeuN (1:1000; Millipore) or rabbit anti-cleaved caspse3 (1:1000; Abcam). Sections were washed in PBS and incubated with appropriate secondary antibodies: biotinylated horse anti-goat IgG (1:500; Vector), 488-donkey anti-rabbit (1:500; Invitrogen), biotinylated goat anti-rabbit (1:500; Vector), biotinylated horse anti-mouse (1:500; Vector) or 488-donkey anti-rabbit (1:500; Invitrogen) at RT for 3 h. For biotinylated secondary antibodies, sections were washed in PBS and incubated with Cy3-conjugated streptavidin (1:1000; Sigma) for 1 h at RT. In addition, *in situ* hybridization of Tbr2 was performed in brain slice as described previously (Pruski et al., 2019), the sequence of primers for making RNA probe of Tbr2 were: Forward, 5′-TTATCAGAGGAAGATGGCAGC-3′; Reverse, 5′-AGAGCCCACTGTTAACTCAAGG-3′. TUNEL staining was performed in cultured NSCs and brain slice as described previously (Ding et al., 2003; Yang et al., 2018; Pruski et al., 2019).

Western Blotting

Hippocampal tissues were homogenized in RIPA lysis buffer as described previously (Yang et al., 2018). After SDS-PAGE and protein transfer, membranes were incubated with following primary antibodies: rabbit anti-DRIP130 (1:2000; Abcam) and mouse anti-β-actin (1:5000; Sigma) overnight at 4°C, followed by incubation with HRP-conjugated anti-rabbit or anti-mouse IgG (1:2000; Proteintech) for 1 h at RT. Antibodies were visualized using an ECL kit (Thermo Fisher Scientific).

Stereotactic Injections

After anesthetizing with 10% chloral hydrate (0.4 ml/100 g body weight), mice were stereotactically injected with 1 μl of retrovirus expressing GFP (pRov-U6-shRNA-EF1a-EGFP) into the DG. Coordinates from the Bregma were (in mm): −1.94 anterior/posterior ± 1.25 medial/lateral, and −2.0 dorsal/ventral from the dura mater. Twenty-one days later, animals were perfused as described above. Dendritic lengths and branch points of EGFP-labeled newborn neurons in the SGZ were traced with ImageJ and analyzed.

Hippocampal NSC Culture

Primary NSC cultures were prepared from the hippocampus of 2-month-old Med23 CKO and littermate control mice as described previously with some modifications (Brewer and Torricelli, 2007). Briefly, under anesthesia with chloral hydrate, the hippocampus was removed and dissected in HABG, which contained B27 (1×; Invitrogen), Glutamax (0.25×; Invitrogen), and HA (1×; GIBCO). Hippocampal tissues were digested in 0.025% Trypsin-EDTA (1×; GIBCO) for 15 min at 37°C, then DMEM + 10% FBS was added to terminate the reaction before centrifuging at 800 rpm for 15 min, and the tissue was finally dissociated into a single-cell suspension by mechanical disruption. The single-cell suspension was grown in Neurobasal-A Medium (1×; GIBCO), supplemented with B27-A (1×; Invitrogen), L-glutamine (1×; Invitrogen), FGF (20 ng/ml; Invitrogen), EGF (20 ng/ml; Invitrogen), N2 (1×; Invitrogen), and gentamicin (0.01 mg/ml; Invitrogen). The desired cells were incubated at 37°C in 5% CO_2 for 8 days until primary neurospheres were formed. Primary neurospheres were mechanically dissociated into single cells and incubated for 5–6 days to allow secondary neurosphere formation. The sizes through measuring the length of primary and secondary neurospheres were recorded under a microscope (Nikon). Finally, primary neurospheres were harvested for quantitative real-time RT-PCR (qRT-PCR) (see below).

To evaluate the proportion of dividing cells in the neurospheres, primary neurospheres were treated with 10 μM BrdU for 4 h and then subjected to immunostaining of BrdU as mentioned above.

RNA Extraction and qRT-PCR

Analyses were conducted with RNA extracts from primary neurospheres from Med23 CKO and control mice, harvested after 8 days of incubation. Total RNA was extracted as per Trizol (Life Technologies) protocol. cDNA was generated with iScript™

cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed using the ABI Prism 7500 Sequence Detector System. Primer sequences are as follows: β-actin (Forward: 5′-GGCTGTATTCCCCTC CATCG-3′; Reverse: 5′-CCAGTTGGTAACAATGCCATGT-3′), Med23 (Forward: 5′-AGGAGTGGATTACAAGGGTG-3′; Reverse: 5′-TAGGCAGGCATTTCGTTC-3′).

RNA-Sequencing and Data Analysis

An RNA-sequencing (RNA-seq) library was prepared from 8-day cultured primary neurospheres using NEBNext® Ultra™ RNA Library Prep Kit for Illumina (E7530) following the manufacturer's instructions, and all libraries were sequenced using the Illumina HiSeq platform. Raw.fastq files were analyzed using FastQC, and adapter removal was performed using cutadapt1.12. Reads were aligned to mouse genome assembly mm 10 using Tophat (Trapnell et al., 2009) with default parameters. Differential expression analysis was performed using Cuffdiff (Trapnell et al., 2012) and FPKM was calculated. GO analysis of differentially expressed genes was carried out using DAVID online tools[1].

Behavioral Tests

Adult male mice (3–4 months old) were used in behavioral tests, and in the other experiments mice with either of sex were used. The experiments were performed during 9:00 a.m. to 5:00 p.m., and animal behaviors were videotaped. Mice were habituated in the test room for over 30 min before behavioral experiments. Ethanol (75%) was used to clean arenas and objects between trials to remove excrements and odors for the following behavioral test (except forced swimming and tail suspension test). All behavioral tests were performed by a trained person who was blind to the genotypes.

Morris Water Maze

The Morris water maze was used to examine spatial memory (Vorhees and Williams, 2006). The apparatus contained two parts: a circle tank (120-cm diameter) and a hidden platform (10-cm diameter) submerged 1–2 cm below the surface of the opaque water (24–26°C). Mice movements were monitored and analyzed using EthoVision (8.0). In the spatial learning phase, mice were trained for four trials per day for 6 days at intertrial intervals of 40–50 min to discover the escape platform. The escape latencies of four trials per day were averaged for each animal. To test spatial memory, the hidden platform was removed and mice were subjected to retention tests 1 day after the spatial learning task. Memory retrieval was measured by quantifying the time spent in the target quadrant, the time taken to first cross the platform location, and the number of platform location crossings in a 1-min trial. Swim velocity was also measured during both the spatial learning and memory retrieval tasks.

Contextual Fear Conditioning

This test was used to assess contextual fear memory in mice, performed as described previously (Dai et al., 2008; Hu et al., 2014). In brief, mice freely explored the box for 10 min before the test, prior to being placed back in their home cage. About 1 h later, mice were placed in the box and given 5 foot shocks (1.2 mA, 2 s duration) at 2-min intervals to learning contextual fear memory. To study retrieval of contextual fear memory, mice were placed in the conditioned fear context for 11 min to observe the percentage of freezing without any foot shock at 30 min, on day 1 and day 7.

Novel Object Recognition Test

This test was conducted in a black sound-proof chamber, containing a Plexiglas box (25 × 25 × 25 cm) and camera. The task procedure was described previously (Wang et al., 2007; Botton et al., 2010). In brief, the procedure consisted of three phases: habituation, familiarization, and test phase. In the habituation phase, the mouse was placed into the box without any object and allowed to explore for 10 min on day 1. During familiarization, two identical sample objects (A + A) were placed into opposite corners of the box approximately 8 cm from the walls. A single mouse was placed into the box facing away from the objects and allowed to explore for 10 min on day 2. During the test phase, mouse was returned to the box which contained the location of novel (B) versus familiar object (A) about 1 h later, and allowed to explore for 5 min. Exploration was defined by directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose or forepaw. During the test phase, a recognition index for each mouse was expressed by TN/(TN + TF) ratio (TF = time spent exploring familiar object; TN = time spent exploring novel object).

Cell Count and Statistical Analysis

Positive cells were counted in every 7th sections in the DG from anterior to posterior, expressed as count per mm^3, by a trained observer who was blind to genotypes. All samples showed normal distribution examined by OriginPro9.1, and unpaired Student's *t*-test was performed using GraphPad Prism 5. Date are expressed as mean ± SEM, and p-values < 0.05 were considered to be statistically significant.

RESULTS

Increased Number of Proliferating NSCs in Med23 CKO Mice

To examine whether hippocampal NSCs are affected by the inactivation of Med23, we first performed BrdU pulse-labeling (**Figure 1A**) and quantified $BrdU^+$ cells in the SGZ as reported in our previous study (Song et al., 2016). Deletion of Med23 was confirmed by western blot of hippocampal tissues of Med23 CKO mice (**Figure 1B**), and by qRT-PCR analysis of Med23 transcript of neurospheres prepared from Med23 CKO mice (**Figure 1C**). Quantification of $BrdU^+$ cells in the DG showed a significant increase in Med23 CKO mice compared with controls (**Figure 1D**). In addition, cell cycle markers Ki67 (**Figure 1E**) and MCM2 (**Figure 1F**) were also significantly increased in the SGZ of Med23 CKO mice compared with controls. We next examined the population of $GFAP^+/Sox2^+$ cells, which represent quiescent and active NSCs, and found no significant changes between control and Med23 CKO mice

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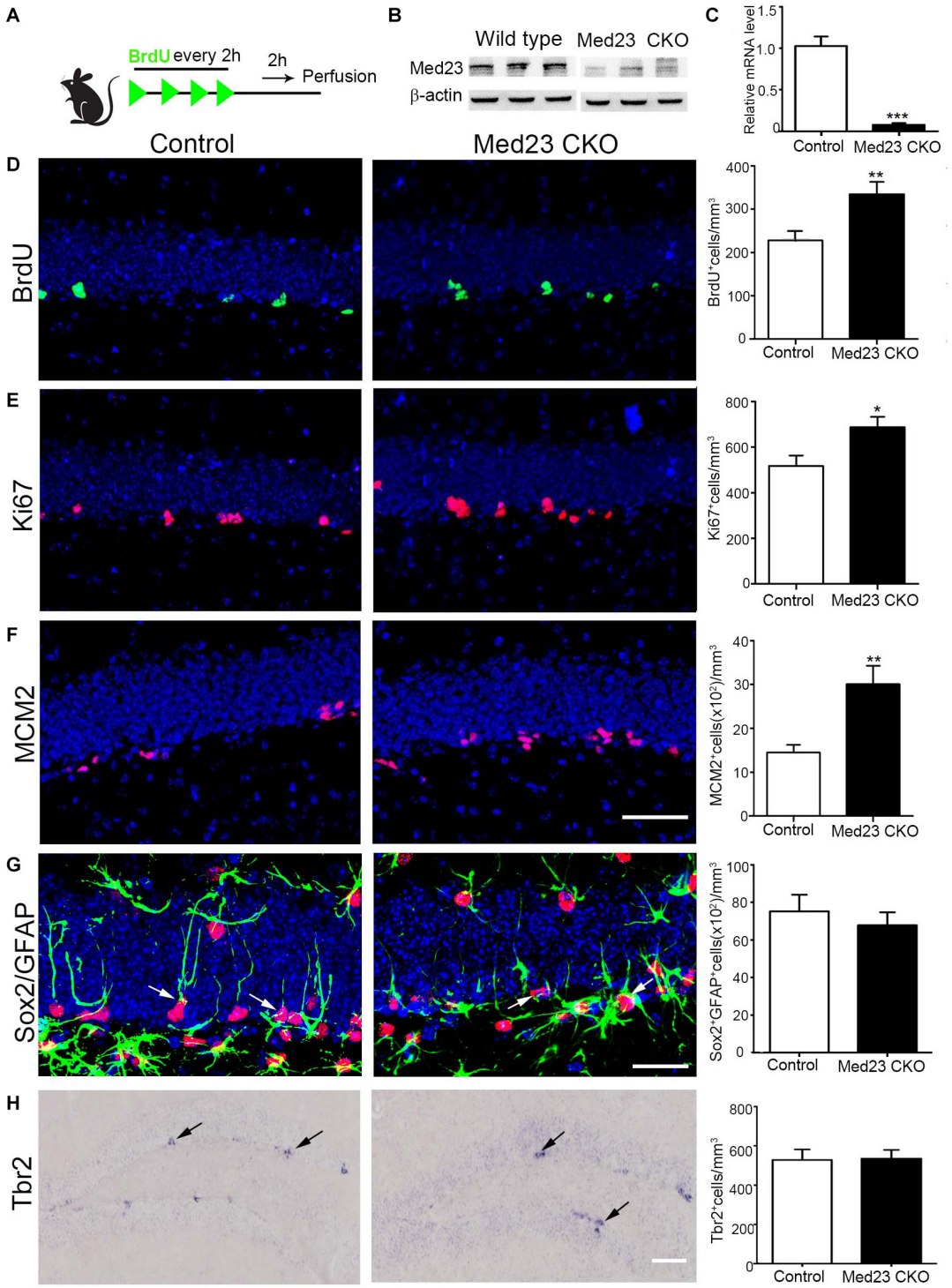

FIGURE 1 | Increased proliferation of Med23-deficient hippocampal NSCs *in vivo*. **(A)** Schematic representation of BrdU injection. **(B)** Western blot of hippocampal tissues of Med23 CKO mice relative to wild type littermates. *N* = 3 for each. **(C)** qPCR data showing the Med23 mRNA levels in the primary neurospheres of control and Med23 CKO. Neurospheres from six control and three Med23CKO mice was included. **(D)** Distribution and quantification of $BrdU^+$ cells in the SGZ of control and Med23 CKO. Seven control mice and six Med23 CKO mice were included. **(E)** Distribution and quantification of $Ki67^+$ cells in the SGZ of control and Med23 CKO mice. *N* = 8 mice in control and seven mice in Med23 CKO groups. **(F)** Distribution and quantification of $MCM2^+$ cells in the SGZ of control and Med23 CKO mice. *N* = 5 mice in each group. Scale bar = 50 μm **(D–F)**. **(G)** Distribution and quantification of $Sox2^+/GFAP^+$ cells (arrows) in the SGZ of control and Med23 CKO. *N* = 5 mice in control and six mice Med23 CKO groups. Scale bar = 25 μm. **(H)** Distribution and quantification of $Tbr2^+$ cells (arrows) in the SGZ of control and Med23 CKO mice. *N* = 4 mice in each group. Scale bar = 100 μm. Data are plotted as the mean ± SEM. Student *t*-test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(**Figure 1G**). The proliferating cells in the SGZ also include progenitors that express Tbr2 (Kalamakis et al., 2019; Morales and Mira, 2019) and there was no obvious alteration in the number of $Tbr2^+$ cells in the SGZ (**Figure 1H**). Taken together, although the whole population of NSCs is not changed the active NSCs is likely to be increased in the SGZ of adult Med23 CKO mice.

To further confirm this observation, we isolated hippocampal NSCs from Med23 CKO and control mice and cultured them for 8 days until primary neurospheres were formed. The size of primary neurospheres was significantly increased in cultures from Med23 CKO mice relative to controls (**Figure 2A**). Next, the primary neurospheres were dissociated into single cells and cultured to form secondary neurosphere. The size of secondary neurospheres was also larger in Med23-deficient NSCs than in control NSCs (**Figure 2B**). Primary neurospheres were treated with BrdU for 4 h and the proliferation of cultured NSCs was monitored by BrdU-labeling. The percentage of $BrdU^+$ cells in the total cell population was significantly increased in Med23-deficient NSCs compared with control cells (**Figure 2C**). Taken together, these results suggest that the self-renewal of hippocampal NSCs is enhanced in the absence of Med23.

Reduced Cell Cycle Length of NSCs in Med23 CKO Mice

There are several possibilities that may underlie the phenotype of increased NSC proliferation, such as enhanced cell cycle entry via recruitment of previously quiescent stem cells, reduced cell cycle exit, or modification of cell cycle length (Fischer et al., 2014). To investigate the increased NSC proliferation in more detail, we first performed double immunostaining of BrdU and Ki67 to estimate cell cycle length. Following BrdU injection (**Figure 3A**), we calculated the labeling index by the ratio of $BrdU^+/Ki67^+$ cells in the total of $Ki67^+$ cells as described previously (Chenn and Walsh, 2002; Qu et al., 2013). This labeling index provides an estimation of cell cycle length, in that a higher index represents a shorter cell cycle and vice versa. The index was 47.7 and 39.5% in Med23 CKO and control

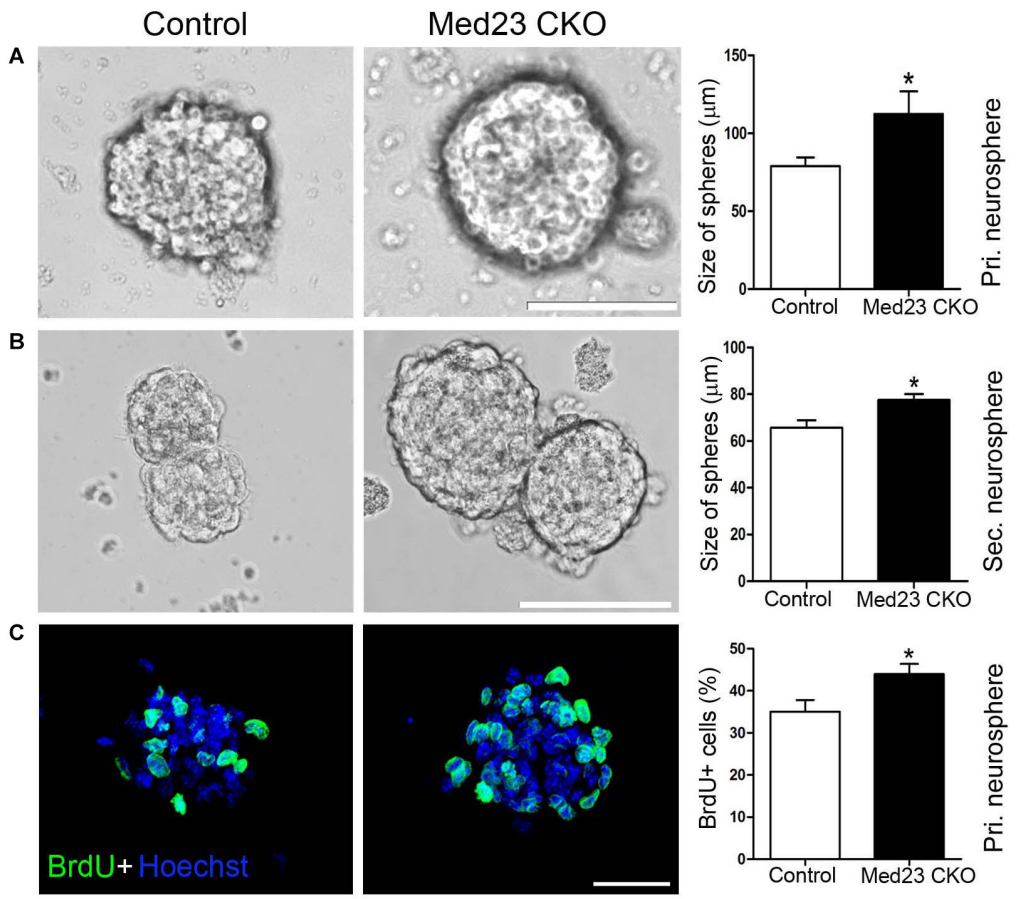

FIGURE 2 | Increased proliferation of Med23-deficient NSCs *in vitro*. **(A,B)** Primary and secondary neurospheres cultured from NSCs of control and Med23 CKO mice. About 11 primary neurospheres were obtained from each mice, and a total of eight mice were used in each group. For the analysis of secondary neurospheres, eight neurospheres were included from each mouse and eight mice were used in each group. Scale bars = 100 μm. **(C)** Immunostaining of BrdU in primary neurospheres from control and Med23 CKO mice, and comparison of percentages of $BrdU^+$ cells in the total cell population. About six neurospheres were included from each mouse, and five mice were used in each group. Scale bar = 25 μm. Data are plotted as the mean ± SEM. Student *t*-test, $^*p < 0.05$.

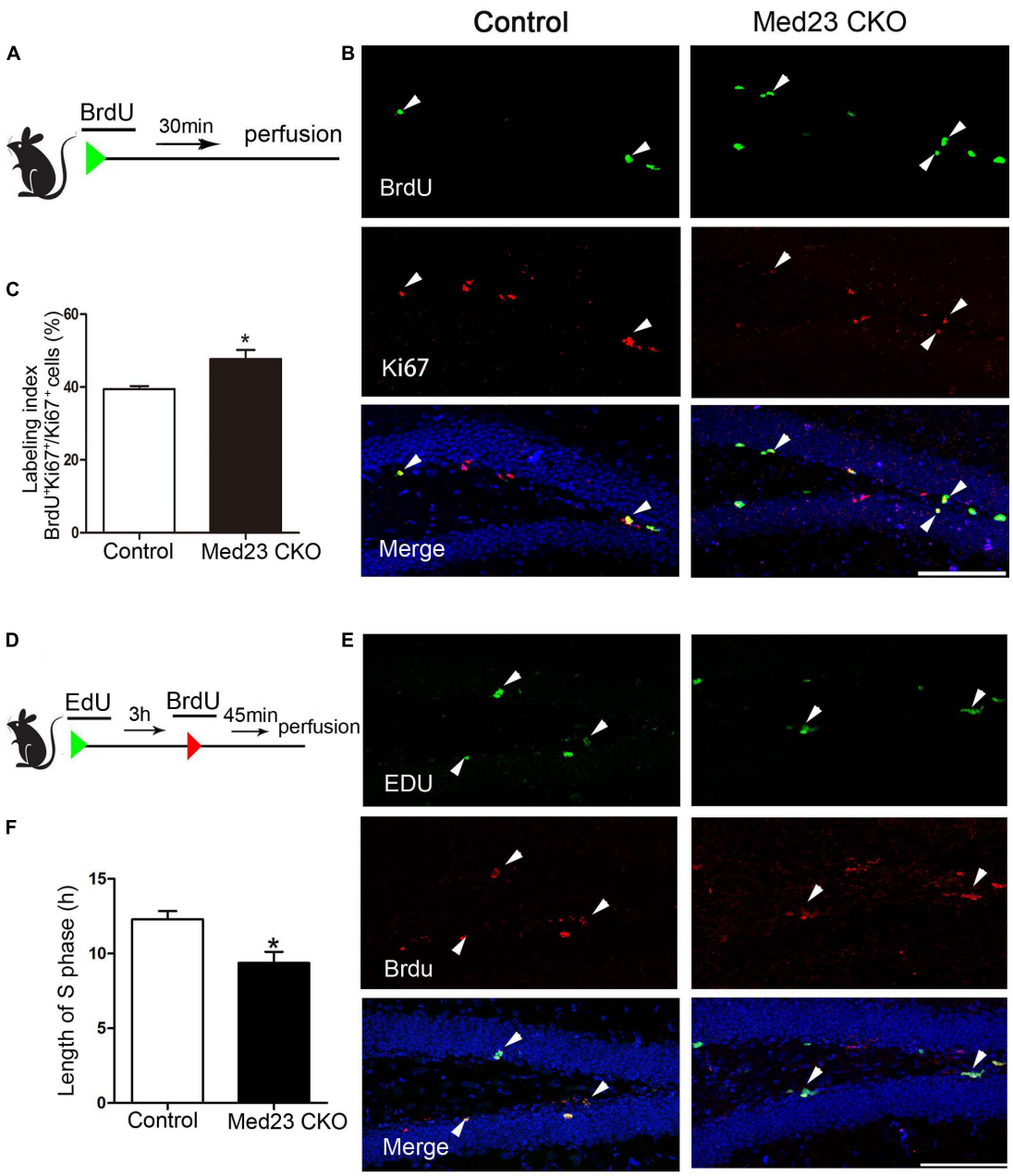

FIGURE 3 | Loss of Med23 affects cell cycle progression of NSCs in the SGZ of adult mice. **(A)** Schematic representation of BrdU injection. **(B)** Double immunostaining of BrdU (green) and Ki67 (red) in BrdU-injected mice. Triangles point to $BrdU^+/Ki67^+$ cells. Hoechst (blue) was used for counterstaining. Scale bar = 100 μm. **(C)** Percentage of $BrdU^+/Ki67^+$ cells in the total of $Ki67^+$ cells (labeling index) is significantly increased in Med23 CKO mice compared with controls. *N* = 5 mice in control and 4 in Med23 CKO groups. **(D)** Schematic representation of EdU and BrdU injection. **(E)** Double staining of EdU (green) and BrdU (red) in the SGZ. Triangles point to $BrdU^+/EdU^+$ cells. Hoechst (blue) was used for counterstaining. Scale bar = 100 μm. **(F)** The length of S-phase of NSCs is shorter in Med23 CKO than controls. *N* = 3 in control and 4 in Med23 CKO groups. Data are plotted as the mean ± SEM. Student *t*-test, $^*p < 0.05$.

mice, respectively, showing that cell cycle length of hippocampal NSCs is shortened in the absence of Med23 (**Figures 3B,C**). As the shortened cell cycle length of NSCs may be due to changes of S-phase in relation to the total cell cycle length, we next injected EdU and BrdU in a 3-h interval (**Figure 3D**) to estimate S-phase by calculation of 3(h) × EdU^+/EdU^+BrdU^- cells (Brandt et al., 2012). It showed that the length of S phase in hippocampal NSCs was 9.37 h in Med23 CKO mice, thus being significantly shorter than in controls (12.29 h) (**Figures 3E,F**). These results suggest that the reduction of cell cycle length may contribute to the increased proliferation of NSCs in the SGZ of Med23 CKO mice.

Reduction of Neuroblasts, and Immature and Mature Newborn Neurons in the Hippocampus of Med23 CKO Mice

Given our finding of the increased proliferation of Med23 CKO NSCs both *in vivo* and *in vitro*, we were prompted to examine how neurogenesis is changed in Med23 CKO mice by the examination of molecular markers expressed in different stages of hippocampal neurogenesis (Duan et al., 2008; Zhao et al., 2008; Ming and Song, 2011). TAM was administered at the age of 2 months and sacrificed 4 weeks after the first TAM treatment (**Figure 4A**). NeuroD mainly expressed by neuroblasts and there was a significant reduction in NeuroD$^+$ cells in Med23 CKO mice compared with controls (**Figure 4B**). In addition, DCX expressed by neuroblasts and immature neurons also displayed a reduction in cell number in Med23 CKO mice (**Figure 4C**). Thus, although the proliferation of hippocampal NSCs is enhanced, differentiating newborn neurons are reduced in the hippocampus of Med23 CKO mice.

To further confirm this, we generated Med23 CKO:Rosa26-YFP mice, in which YFP is expressed in Nestin$^+$ NSCs and their progeny, including differentiating newborn and mature neurons. We found that YFP$^+$ cells were significantly decreased in number in the SGZ of Med23 CKO:Rosa26-YFP mice relative to Nestin-Cre^{ER}:Rosa26-YFP mice (**Figure 4D**); the Nestin-Cre^{ER}:Rosa26-YFP mice with Med23$^{+/+}$ genotype showed no obvious changes in the number of BrdU$^+$, Ki67$^+$, MCM2$^+$, or DCX$^+$ cells relative to control mice and used as control mice here. However, the percentage of cells expressing YFP$^+$/MCM2$^+$ in YFP$^+$ cells was increased in CKO mice relative to controls (**Figure 4E**), which is consistent with the increase of NSCs as described above. Thus, the decrease in YFP$^+$ cell number is likely caused by a reduction in the number of differentiating and/or mature newborn neurons. Indeed, the percentage of YFP$^+$/DCX$^+$ cells in YFP$^+$ cells was reduced in Med23 CKO mice compared with controls (**Figure 4F**). To explore how many of the YFP$^+$ cells were differentiated neurons, we performed NeuN immunostaining. It showed that some YFP$^+$ cells expressed NeuN in both control and CKO mice, but it is percentage of YFP$^+$/NeuN$^+$ cells in YFP$^+$ cells was significantly reduced in Med23 CKO mice (**Figure 4G**). Noted that no significant differences in the proportion of YFP$^-$/MCM2$^+$ cells in the total of MCM2$^+$ or YFP$^-$/DCX$^+$ cells in the population of DCX$^+$ cells were detected between the two groups. There results demonstrate that the neurogenesis is impaired in the SGZ of Med23 CKO mice.

In addition, BrdU was administered in 2 month-old mice, and BrdU-labeled newborn neurons were examined 3 weeks later (**Figure 5A**) by double immunostaining of BrdU with NeuN (**Figure 5B**). Quantitative analysis revealed that the number of BrdU$^+$ cells (**Figure 5C**) and BrdU$^+$/NeuN$^+$ neurons (**Figure 5C**) were significantly decreased in the SGZ of Med23 CKO mice compared with controls. Consistent with the reduction of GFP$^+$/NeuN$^+$ neurons mentioned above, mature new-born neurons are reduced in the DG of Med23 CKO mice, which is likely to be a consequence of the reduction of neuroblasts and immature neurons. In support of this, TUNEL staining and cleaved caspse3 immunostaining did not detect positive signals in the DG of both control and Med23 CKO.

Defective Dendritic Arborization of Newborn Neurons in Med23 CKO Mice

We next examined dendritic morphogenesis of the surviving newborn neurons in Med23 CKO mice. To this end, we stereotactically injected GFP-expressing retrovirus infecting dividing cells into the DG, and examined dendritic tree of newborn neurons 3 weeks later. Sparse labeling of newborn neurons allowed us to clearly visualize and quantify the dendritic arborization of individual newborn neurons. Dendritic length of GFP-labeled neurons was reduced (**Figure 6A**) and dendrites were less branched in Med23 CKO mice relative to controls (**Figure 6B**). These findings are consistent with DCX immunostainings in which we observed many fewer dendrites in DCX$^+$ neuroblasts in the SGZ of Med23 CKO mice compared with controls (**Figure 4C**). Thus, the loss of Med23 leads to aberrant dendritic morphogenesis of newborn neurons in the SGZ.

Defective Spatial Memory and Contextual Fear Memory in Med23 CKO Mice

The Morris water maze was used to investigate hippocampus-dependent spatial learning and memory. Spontaneous locomotor activity was examined in the open field test first, and no differences were observed in traveled distance or velocity between the two groups (data not shown). In the training phase of Morris water maze, the learning curves shown by escape latencies in finding the platform over a 6-day training was comparable, although the latency was significantly increased in Med23 CKO mice as compared with controls on day 4 (**Figure 7A**). One day after the 6-day training, we examined the retrieval of spatial memory in the absence of the platform. Med23 CKO mice required significantly more time to locate the platform (**Figure 7B**), spent less time in the platform zone (**Figure 7C**) and had a reduced frequency of crossing the platform in the probe test compared with control mice (**Figure 7D**). It should be noted that Med 23 CKO and control mice showed similar velocity during the training and probe test. Thus, a deficiency in spatial memory was observed in Med23 CKO mice.

Adult hippocampal neurogenesis has been shown to be implicated in contextual fear memory (Antunes and Biala, 2012). To explore whether the acquisition and retrieval of the fear memory was changed in Med23 CKO mice, we conducted a contextual fear test as described in our previous studies (Dai et al., 2008; Song et al., 2016). Contextual fear conditioning was tested using foot shocks, and the freezing behavior was measured as the percentage of time spent in freezing during the conditioning and retrieval periods. Interestingly, the freezing behavior was not different during the initial four post-shock intervals, but significantly decreased after the final foot shock in Med23 CKO mice relative to controls (**Figure 7E**), suggesting a mild impairment in the acquisition of contextual fear

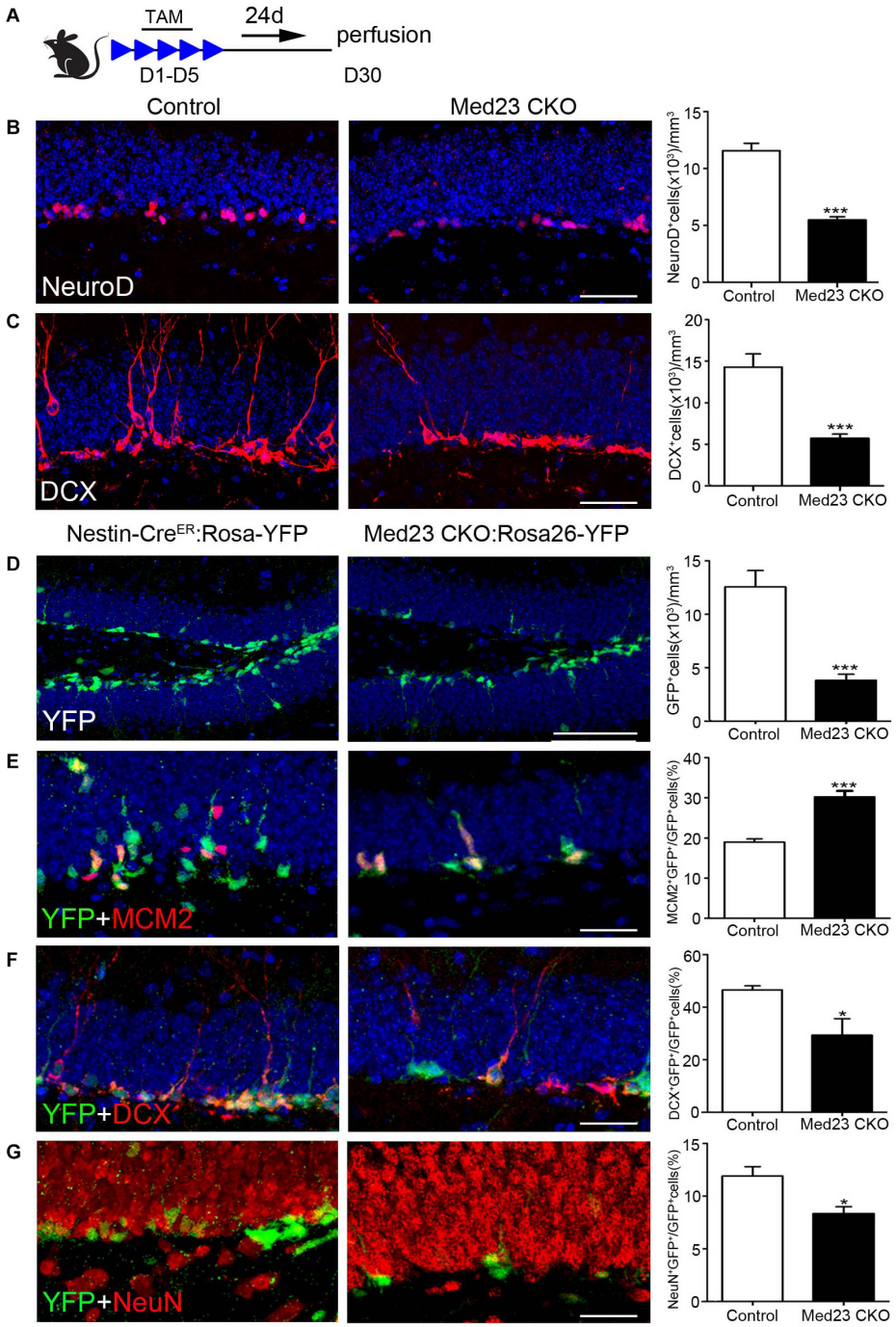

FIGURE 4 | Med23 deficiency results in a reduction of neuroblasts and immature newborn neurons in the SGZ. **(A)** Schematic representation of TAM treatment. **(B,C)** Distribution and quantification of NeuroD$^+$ and DCX$^+$ cells in the SGZ of Med23 CKO mice compared with controls. *N* = 6 in control and 4 in Med23 CKO groups **(B)** and *n* = 5 in each group **(C)**. **(D–G)** Distribution and quantification of YFP-expressing cells (green, **D**), and its combination with MCM2 (red, **E**), DCX (red, **F**), and NeuN (red, **G**) in the SGZ of Med23 CKO:Rosa26-YFP mice and control Nestin-Cre^{ER}:Rosa26-YFP with Med23$^{+/+}$ genotype. *N* = 5 mice in each group **(D)**; *n* = 4 mice in control and 3 mice in Med23 CKO groups **(E–G)**. Scale bars = 25 μm. Data are plotted as the mean ± SEM. Student *t*-test, $*p < 0.05$, $***p < 0.001$.

memory. When placed back into the conditioned surrounding in the absence of foot shocks, Med23 CKO mice showed less freezing behavior at 30 min, day 1 and day 7 (**Figure 7F**) post freezing conditioning compared with control mice. The learning and retrieval of contextual fear memory is defective in Med23 CKO mice.

As the above results reflect the possibility that spatial memory and contextual fear memory are impaired in the absence of

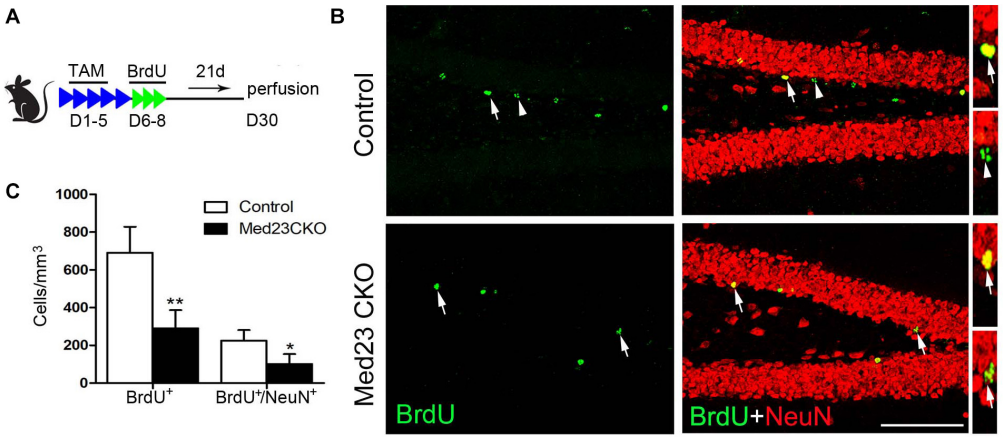

FIGURE 5 | Mature new-born neurons is also reduced in Med23 CKO mice. **(A)** Schematic representation of TAM treatment and BrdU injection protocol for the examination of newborn neuron survival. **(B)** Double immunostaining of BrdU (green) and NeuN (red). Arrows point to BrdU/NeuN-double positive cells and triangles point to cells labeled with BrdU only. Scale bar = 100 μm. **(C)** Quantification data show that the numbers of BrdU-single and BrdU/NeuN-double positive cells are significantly reduced in Med23 CKO mice compared with controls. $N = 5$ in each group. Data are plotted as the mean ± SEM. Student t-test, $^{*}p < 0.05$, $^{**}p < 0.01$.

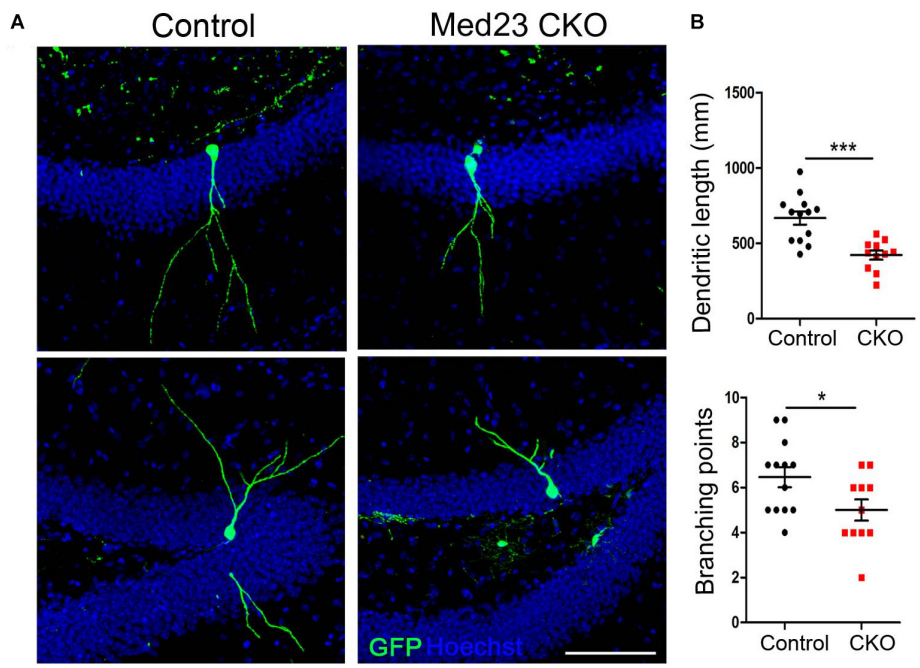

FIGURE 6 | Aberrant dendritic morphogenesis of newborn neurons in Med23 CKO mice. GFP-encoding retrovirus was injected into the dentate gyrus and analyzed 3 weeks later. **(A)** Representative confocal images showing dendrites of newborn neurons in the SGZ of Med23 CKO and control mice. Hoechst (blue) was used as counterstaining. Scale bar = 100 μm. **(B)** Quantification of the total dendritic length and total branching points of GFP^{+} newborn neurons. A total of 13 neurons from 3 control and 11 neurons from 3 Med23CKO mice were included. Data are plotted as the mean ± SEM. Student t-test, $^{*}p < 0.05$, $^{***}p < 0.001$.

Med23, we then performed a novel object recognition test to evaluate object recognition memory between two groups. Firstly, we identified no differences in the location index between Med23 CKO and control mice (**Figure 7G**). We then measured cognitive function using a preference index as described previously (Wang et al., 2007). Both mice showed a preference to the novel object, and no differences in preference index were observed between Med23 CKO and control mice (**Figure 7H**), suggesting that Med23-deficient may not affect recognitive memory.

Transcript Profiling of Med23-Deficient NSCs

To explore possible cues for future exploring mechanisms underlying the increased number of NSCs and reduced

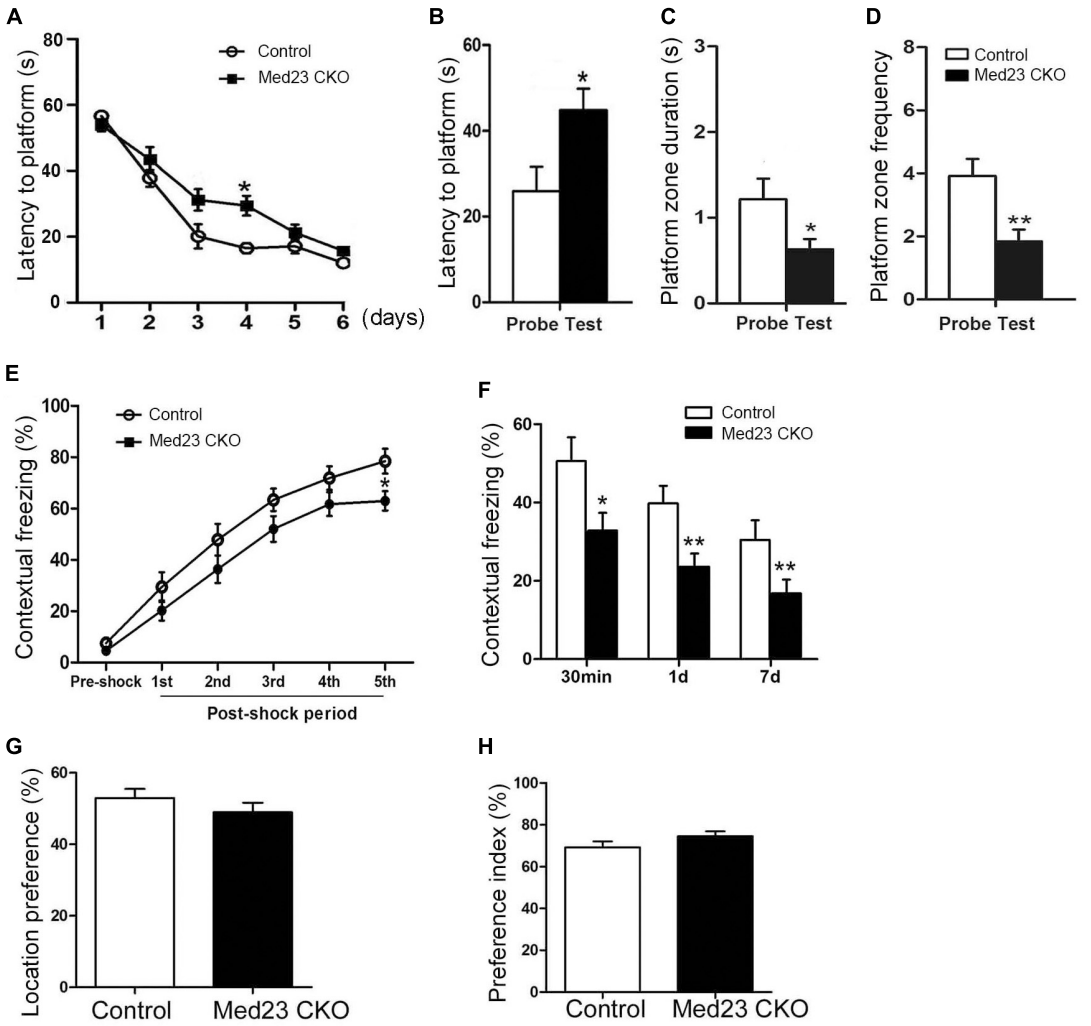

FIGURE 7 | Defective spatial memory and contextual fear memory in Med23 CKO mice. **(A–D)** Morris water maze test. Learning curve of Med23 CKO mice is similar to that of control mice in the probe training except at day 4 **(A)**, but in the probe test Med23 CKO mice show longer latency to platform **(B)**, less time spent in the platform zone **(C)**, and reduced frequency of crossing the platform **(D)** compared with controls. **(E,F)** Contextual fear memory test. Foot shocks provoke a significant decrease in freezing behavior at 5th shock in Med23 CKO mice during conditioning period **(E)**, and Med23 CKO mice also display reduced freezing behavior in the retrieval of contextual fear memory at all time points post conditioning compared with control mice **(F)**. **(G,H)** In the novel object recognition test, no differences in location index **(G)** and recognition index **(H)** are detected between Med23 CKO and control mice. *N* = 12 mice in control and 13 mice in Med23 CKO groups **(A–F)**; *n* = 7 mice in control and 8 in Med23 CKO groups **(G,H)**. Data are plotted as the mean ± SEM. Student *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$.

survival of newborn neurons in the SGZ of Med23-deficient mice, we performed transcriptome profiling and analyzed global gene expression patterns of primary neurospheres from Med23 CKO and control mice (**Figure 8A**, GSE152113). We detected genes that were upregulated (667 genes) or downregulated (196 genes) in Med23-deficient NSCs relative to controls (−log10 $p < 0.05$, **Figure 8B**). GO analysis revealed that the upregulated genes show an enrichment for cell proliferation (e.g., SAT1, TNF, IGF1, CD37, and EREG), Notch signaling pathway (e.g., DLL4, HEYL, PSEN2, TGFBR2, NOTCH4, and FOXC1) and apoptotic processes (e.g., TSPO, TNF, CYP1B1, PTGS2, MMP9, TLR4, TGFB1, PSEN2, and CASP1) (**Figure 8C**). Downregulated genes were observed to be enriched for multiple processes important for nervous system development (e.g., SFRP2, SFRP4, SFRP5, EGR1, EGR2, EGR4, DLL1, MYT1, SLITRK1, FOS, FGFR3, SHH, CDKN2A, Wnt7B, and Wnt8B), positive regulation of transcription from Pol II promoter (EGR1, EGR2, RFX4, EGR4, GRIN1, GSX1, BARHL2, NR4A1, BEX2, IGF2, DLL1, HMGA2, MYT1, MEIS1, SHH, FOS, CDKN2A, BARX2, SFRP2, BMPR1B, MYC, AGAP2, andPEG3), and transcription from Pol II (EGR1, FOS, POLR2F, EGR2, MYC, and CHD5) in Med23-deficient NSCs (**Figure 8D**). Furthermore, analysis of protein-protein interaction networks among upregulated and downregulated genes indicated dysregulations in the regulatory networks involved in cell proliferation, apoptotic processes and neural development in Med23-deficient NSCs (**Figures 8E,F**). Together, gene regulatory networks particularly in cell proliferation, apoptotic processes

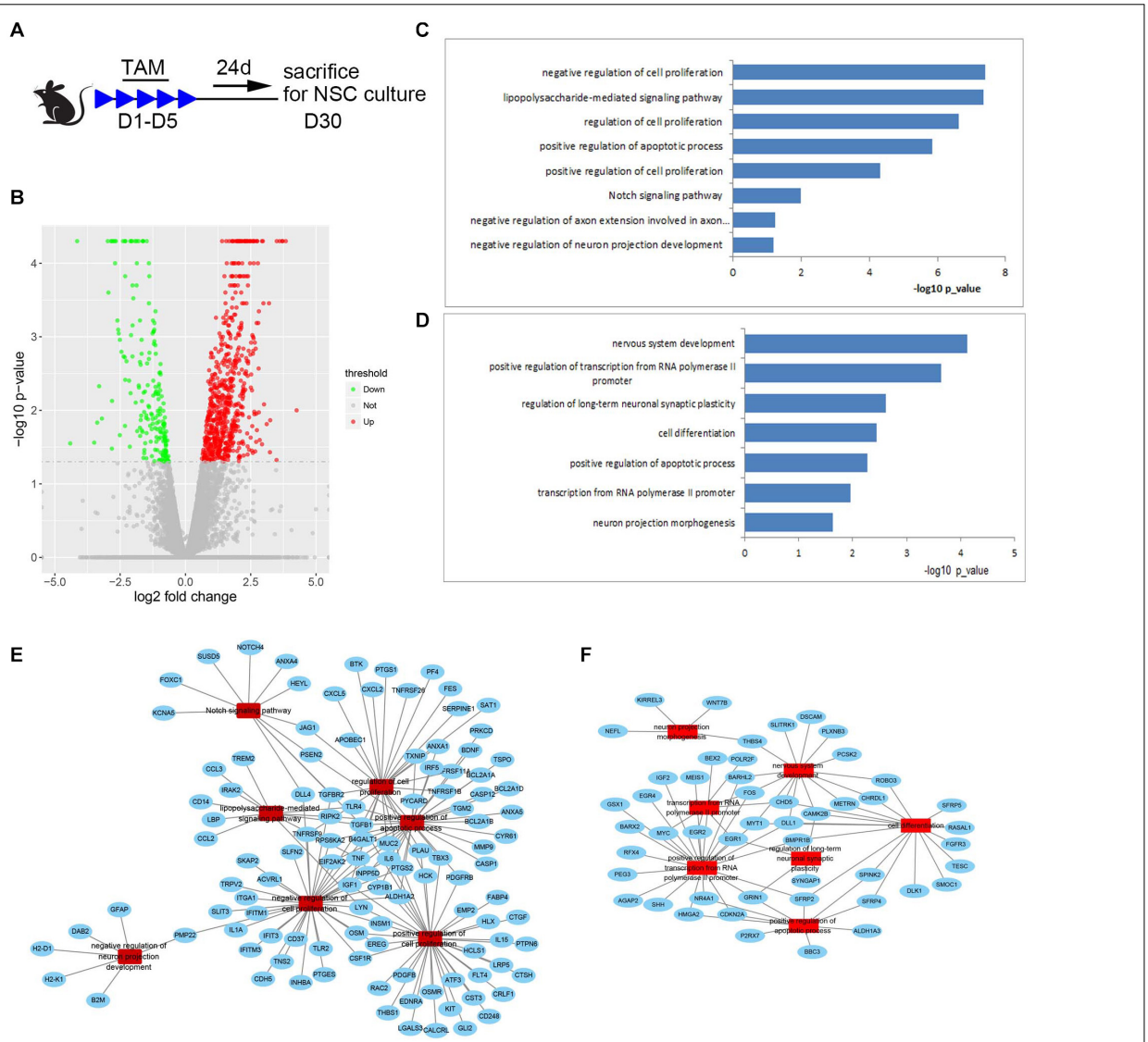

FIGURE 8 | Transcript profiling of Med23-deficient hippocampal NSCs. **(A)** Schematic representation of TAM treatment and sample collection schedule. **(B)** Volcano plots depict gene expression changes between Med23 CKO and control NSCs. Significantly differential transcripts are highlighted in color and totaled in each direction (FDR < 0.05). The cultured neurospheres prepared from individual mouse brain were collected as one sample for RNAseq analysis, and $n = 3$ in each group. **(C,D)** Gene ontology (GO) analysis of the significantly upregulated **(C)** and downregulated **(D)** genes between Med23 CKO and control NSCs. **(E,F)** Protein-protein interaction networks among upregulated **(E)** and downregulated **(F)** genes in Med23 CKO NSCs.

and neural development are disturbed in the absence of Med23 in adult hippocampal NSCs.

DISCUSSION

Whilst several physiological functions have been attributed to Med23, its role in the central nervous system has not been examined *in vivo*. In this study, we focused on adult hippocampal neurogenesis in order to explore the potential role of Med23 in the adult brain as the biological processes are well documented with multiple and reliable study tools. By crossing with Nestin-Cre^{ER} mice, Med23 in hippocampal NSCs could be inactivated by administration of tamoxifen in adult Med23 CKO mice. Conditional inactivation of Med23 in NSCs showed that the loss of this protein leads to an increase of proliferating NSCs but decreases of neuroblasts and new-born neurons in the hippocampus.

The deletion of Med23 with the help of Nestin-Cre^{ER} causes an increase of BrdU^{+}, Ki67^{+}, and MCM2^{+} cells with no changes in GFAP^{+}/Sox2^{+} and Tbr2^{+} populations, suggesting that active NSCs is selectively affected in the population of proliferating cells in the SGZ. However, the populations of neuroblasts and immature new-born neurons shown by DCX^{+} and NeuroD^{+} cells in Med23 CKO mice and YFP^{+} cells in Med23 CKO:Rosa26-YFP mice are significantly reduced. According to the data

from single-cell transcriptomics (Llorens-Bobadilla et al., 2015), Med23 is expressed in all cell types with higher levels in active NSCs and neuroblasts in the hippocampal neurogenic niche. Thus, active NSCs and immature new-born neurons require Med23 to maintain its normal biological functions in the SGZ.

In the exploration of possible cellular events leading to the increase of active NSCs, we found that the cell cycle length of proliferating cells in the SGZ is shortened as a result of reduced S-phase length. However, this does not rule out additional possibilities, such as increased cell cycle entry of quiescent NSCs or decreased cell cycle exit of proliferating NSCs toward a quiescent state or differentiation. The methods employed here is originally used in studying the cell cycle of NSCs in the ventricular zone of developing cerebral cortex where homogenous NSCs are located (Chenn and Walsh, 2002), and it was used in addressing the same question in the SGZ later (Qu et al., 2013). However, the progenitors are intermingled with NSCs in the SGZ, and they may have a different cell cycle length, which therefore contributes to the cell cycle phenotypes observed in the SGZ of Med23-CKO mice. This should be clarified in further study.

One of our previous studies has shown that Med23-deficient murine embryonic stem cells show enhanced neural differentiation through modulating BMP signaling (Zhu et al., 2015). In the present study, we show that Med23 is also required for the proliferation of adult hippocampal NSCs and the differentiation of neuroblasts and immature new-born neurons. To our knowledge, this is the first report exploring the role of Med family in the nervous system. As mentioned above, the mammalian Mediator complex is essential to the basal transcription machinery by forming a link between transcription factors and Pol II, whereby it helps regulating the transcription of a large number of protein-coding genes. Consistently, our RNA-seq data show that 667 genes are up- and 196 genes are downregulated in Med23-deficient NSCs. The downregulated portion shows an enrichment for genes involved in Pol II-mediated transcription, multiple processes involved in nervous system development and cell differentiation. In the down-regulated genes of Med23-deficient NSCs, the expression of early response genes (such as Egr1, Egr2, Egr4) that are normally induced by serum growth factor (such as SFRP) activation, are greatly attenuated. These results are consistent with our previous study that Egr1 is the most severely affected by loss of MED23 (Wang et al., 2005). However, Med23-deficient embryonic stem cells display enhanced neural differentiation via BMP signaling (Sun et al., 2014), which is not significantly altered in Med23-deficient hippocampal NSCs.

As mentioned above, Med23 is highly expressed in active NSCs and neuroblasts and the cellular phenotypes are present in the two type of cells suggesting possible cell-autonomous role of Med23 in regulating adult hippocampal neurogenesis. According to the transcript profiling data, Notch pathway is upregulated in the absence of Med23. Notch pathway is required for the maintenance of proliferation capability of NSCs and prevent neuronal differentiation (Louvi and Artavanis-Tsakonas, 2006), and the upregulation of Notch signaling pathway may contribute to the enhanced proliferation of hippocampal NSCs in the absence of Med23. However, Nestin-driven Cre expression is also present in the endothelial cells, and Dll4 and Notch4 are mostly expressed by the endothelial cells (Swift and Weinstein, 2009). This raises a question of if Notch signaling functions in Med23-involved adult neurogenesis in a non-cell-autonomous way. In addition, the upregulated genes involved in cell proliferation (e.g., SAT1, TNF, IGF1, CD37, and EREG) may be implicated in the phenotypes of abnormal neurogenesis of Med23 CKO mice as well. Further studies are needed to explore key downstream effectors that are involved in the Med23-associated transcriptional machinery and regulates adult hippocampal neurogenesis in the mouse brain.

The present findings further demonstrate that Med23 is a factor involved in the regulation of adult hippocampal neurogenesis and its associated role in mouse behavior. As mentioned above, missense mutations in Med23 have been reported in families with autosomal recessive intellectual disability. The mutation (R617Q) leads to a defective response of JUN and FOS immediate early genes to serum mitogens by altering the interaction between enhancer-bound transcription factors (TCF4 and ELK1) and Med23 (Hashimoto et al., 2011; Trehan et al., 2015). Considering the important roles of immediate early genes in brain development, plasticity, and memory formation (Herdegen and Leah, 1998; Tischmeyer and Grimm, 1999; Sanyal et al., 2002; Alberini and Kandel, 2014; Minatohara et al., 2016), it would be interesting to examine possible roles of Med23 in embryonic brain development and in other aspects of adult brain functions. Med23 CKO mice display an impaired performance in the spatial memory, which is consistent with the known functions of adult hippocampal neurogenesis (Ming and Song, 2011; Zhang et al., 2013, 2014), and may be one of factors contributing to the intellectual disability associated with the missense mutation of Med23 (Hashimoto et al., 2011; Trehan et al., 2015). It is well known that animals will learn to link the context itself (the training box) with the foot shocks in contextual fear conditioning, and there is nowadays a consensus that the hippocampus is one of key brain regions for this function (Izquierdo et al., 2016). The Med23 CKO mice show decreased freezing behavior after the last foot shock and during the retrieval period, suggesting impaired fear learning and memory, to which the impaired hippocampal neurogenesis may contribute. In addition, the performance in the object recognition test is normal in Med23 CKO mice. The object recognition can be a different type of memory, which may requires multiple brain regions rather than just the hippocampus.

CONCLUSION

In summary, we demonstrate that Med23 plays an important role in multiple steps of adult hippocampal neurogenesis including NSC self-renewal and cell cycle progression, as well as spatial and fear memory. These data provide a strong basis for future investigations about possible roles of Med23 in central nervous system development and function.

DATA AVAILABILITY STATEMENT

The raw data presented in this study can be found in the GEO, accession number GSE152113.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committees of Tongji University School of Medicine, China.

AUTHOR CONTRIBUTIONS

G-YC carried out experiments and analyzed the data. SZ, C-HL, and GW helped with RNA-sequencing experiments. Y-ZW helped with TUNEL staining and WB. C-CQ carried out *in situ* hybridization. J-YC helped with mouse work. GW, C-JS, and Y-QD conceived and planned the project. G-YC and Y-QD 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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Triiodothyronine Potentiates BMP9-Induced Osteogenesis in Mesenchymal Stem Cells Through the Activation of AMPK/p38 Signaling

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Thyroid hormone (TH), triiodothyronine (T3), and thyroxine (T4), which are released from the thyroid, control many cellular processes in various cell types. It is worth noting that TH plays a complex role in skeletal metabolic balance, and few studies have investigated whether TH exerts any effects on osteogenesis in bone mesenchymal stem cells (MSCs). We explored the effects of T3 on bone morphogenetic protein 9 (BMP9)-induced osteogenesis, which process is considered the most important in the osteogenic differentiation of C3H10T1/2 cells. *In vitro* osteogenesis was analyzed by alkaline phosphatase (ALP) activity and staining, bone mineralisation, and osteocalcin and osteopontin expression. Fetal limb explant cultures and ectopic MSC implantation further confirmed the role of T3. Finally, we examined the effect of AMPK/p38 signaling on the osteoblastic differentiation. T3 synergizes with BMP9 to enhance osteogenic marker expression induced by BMP9. Furthermore, T3 promotes BMP9-induced bone formation by fetal limb explant cultures and ectopic MSC implantation. Co-treatment with BMP9 and T3 can promote AMPK and p38 phosphorylation, and pretreatment with the AMPK inhibitor compound C and siRNA can abolish phosphorylation of p38 and BMP9+T3-induced ALP activity. Our results suggest that BMP9 and T3 promote osteogenic differentiation at least partially via the activation of the AMPK/p38 signaling pathway.

Keywords: Bone morphogenetic protein 9, triiodothyronine, osteogenesis, mesenchymal stem cells, AMPK

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INTRODUCTION

Bone is an important organ that provides support and protection for the whole body. Bone homeostasis is controlled by many types of bone cells from different lineages that are all derived from mesenchymal stem cells (MSCs), including osteoblasts, osteocytes, and chondrocytes. The commitment and differentiation of MSCs into osteogenic cells is closely related to the occurrence of bone metabolic diseases and is worthy of further study. MSCs commit to becoming osteoprogenitor cells and differentiate into pre-osteoblasts, which eventually become mature osteoblasts. This process is regulated by various signaling factors and hormones, including bone morphogenetic proteins (BMPs), Wnt, insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), and Notch. Understanding the signaling pathways that govern osteogenic differentiation has significant implications for bone metabolic diseases.

Thyroid hormones (TH), triiodothyronine (T3), and thyroxine (T4) play complex roles in skeletal development. In adulthood, hypothyroidism and hyperthyroidism induce osteoporosis and osteoporotic fractures, which lead to a substantial economic burden for society. Bone tissue

at the juvenile stage has high sensitivity to TH, and thyroid dysfunction leads to delays in bone formation and mineralization, stunted growth, or cretinism. On the other hand, childhood thyrotoxicosis accelerates bone formation, leading to craniosynostosis (Tsourdi et al., 2015). In terms of target cell types for TH action, studies showed that TH effects in various target cell types and much of them focus on chondrocytes, osteoblasts and osteoclasts, however, there is few studies investigate the effect of TH on MSCs, the source of chondrocytes and osteoblasts. Furthermore, TH has different mechanisms on various target bone cells by complex direct and indirect effects, involving many cytokines or growth factors, such as IGF-1, Wnt, PTHrP, and FGF. Thus the interactions and multiple possible points of interaction between them still need to be worked out (Kim and Mohan, 2013).

Bone morphogenetic proteins are key growth factors that have potent osteogenic capability, among which BMP9 has the most potent osteogenic activity *in vitro* and *in vivo*. BMP9 enhances osteoblast differentiation via the Smad 1/5/8-dependent and Smad1/5/8-independent pathways (Ren et al., 2016). Furthermore, a large number of factors and signaling pathways cross talk with BMPs and promote or restrict BMP9-induced osteogenic differentiation in various types of cells (Chen et al., 2016). And BMP9 has been the subject of few studies that have investigated the interaction between TH signaling and BMPs in the osteogenesis of MSCs (Nagayama et al., 2013; Zhang et al., 2016). Therefore, in this study, we aimed to analyze the effect of triiodothyronine on BMP9-induced osteogenic differentiation in C3H10T1/2 cells and to elucidate the underlying molecular mechanisms. The results showed that triiodothyronine enhanced BMP9-induced osteogenic differentiation, and the promotion of osteogenesis was partially mediated by the activation of AMPK/p38. And an increased mechanistic knowledge of TH on MSCs' osteogenesis will remarkably accelerate the in-depth knowledge of fracture repair or other bone metabolic diseases therapeutics and development bone tissue engineering.

MATERIALS AND METHODS

Cell Culture and Chemicals

The cell lines were cultured as previously described (Huang et al., 2012; Gao et al., 2013; Lindley et al., 2016). T3 (Sigma, United States) was dissolved in 1.0 N NaOH, and compound C (Skelleck, United States), and SB203580 (Skelleck, United States) were dissolved in DMSO.

Construction of Recombinant Adenoviruses Expressing BMP9 and GFP

Recombinant adenoviruses were designed and produced with the AdEasy system as described previously (Aguilar et al., 2007; Gao et al., 2013; Lindley et al., 2016). The coding region of human BMP9 was PCR amplified and cloned into an adenoviral shuttle vector. Then, HEK293 cells were used to generate a recombinant adenoviruses that was ultimately designated as AdBMP9. AdBMP9 expresses BMP9 as well as GFP, while the analogous adenovirus expresses GFP only (AdGFP).

Alkaline Phosphatase Activity and Staining

The cells were treated with AdGFP, AdBMP9, and/or T3 for different time and the Great Escape SEAP Chemiluminescence assay kit (BD Clontech, United States) was used to measure ALP activity in cells as previously described (Huang et al., 2012; Gao et al., 2013; Lindley et al., 2016). Total cellular protein levels was measured by BCA kit (Beyotime, China), and the results was the ratio of ALP activity and total cellular protein levels. After the cells were washed by PBS (GIBCO, United States) and fixed with 4% paraformaldehyde (Beyotime, China) on day 7 post-treatment, ALP staining was then performed using an ALP Staining Assay Kit (Beyotime, China).

Alizarin Red Staining

Alizarin red staining was conducted as previously described (Aguilar et al., 2007; Gao et al., 2013; Lindley et al., 2016). C3H10T1/2 cells were seeded in 6-well cell culture plates and treated as previously described (Huang et al., 2012; Gao et al., 2013; Lindley et al., 2016). Then, at the chosen time point, 21 days post-treatment, the cells were washed and fixed. The samples were then incubated with 2% alizarin red (Sigma-Aldrich, United States) for 30 min. After washing with PBS, the mineralized nodules were observed through light microscopy (Leica DMI 3000B, Germany).

Immunohistochemical Staining

Cells were permeabilized with 0.1% Triton-X (Sigma, United States) for 15 min at room temperature after fixed with 4% paraformaldehyde and washed with PBS as previously described on day 14 (Aguilar et al., 2007; Gao et al., 2013; Lindley et al., 2016). Then the cells were blocked in 5% BSA (Beyotime, China) for 60 min. Then, the cells were incubated with antibody (osteocalcin, sc30045, Santa Cruz Biotechnology; osteopontin, ab91655, Abcam) overnight at 4°C. The next day, the cells were washed and incubated with secondary antibody (Beyotime, China) according to the protocol. Before examined under a microscope, the cells were incubated with diaminobenzidine (DAB).

Real-Time RT-PCR

Total RNA was extracted from cells treated as previously described at day 7 and then the RNA was reverse transcribed into cDNA (Takara, Japan). Quantitative real time PCR was conducted as previously described (Aguilar et al., 2007; Gao et al., 2013; Lindley et al., 2016). Primers are listed as follows: For *OPN* gene, the forward primer was 5′-AGCAAGAAACTCTTCCAAGCAA-3′, the reverse primer was 5′-GTGAGATTCGTCAGATTCATCCG-3′; For *OCN* gene, the forward primer was 5′-CTGACCTCACAGATCCCAAGC-3′, and the reverse primer was 5′-TGGTCTGATAGCTCGTCACAAG-3′; For *GAPDH* gene, the forward primer was 5′-AGGTCGGTGTGAACGGATTTG-3′, and the reverse primer was 5′-TGTAGACCATGTAGTTGAGGTCA-3′.

Western Blotting Analysis

Western blotting analysis was performed according to the protocol. Protein samples were collected in RIPA lysis buffer (Beyotime, China), followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, United States). The membrane was blocked with blocking buffer (Beyotime, China) and incubated with antibodies against AMPK (2532, Cell Signaling), p-AMPK (2535, Cell Signaling), p38 (9212, Cell Signaling), p-p38 (9211, Cell Signaling), osteocalcin (sc30045, Santa Cruz Biotechnology), osteopontin (ab91655, Abcam), and GADPH (AG019, Beyotime) at 4°C overnight. Goat anti-mouse antibody and goat anti-rabbit antibody (A0216 and A0208, Beyotime) labeled with HRP were used as secondary antibodies for 1 h at room temperature.

Transient Transfection With Small Interfering RNAs

C3H10T1/2 cells were plated in six-well plates and AMPKα1/2 siRNA (Aguilar et al., 2007) or control siRNA (GenePharma, China) was transfected into the cells with Lipofectamine 2000 transfection reagent (Invitrogen, United States) according to the manufacturer's instructions. The sense strands of siRNAs for AMPKα was 5′-AAGAGAAGCAGAAGCACGACG-3′. And the sense strands control was 5′-AAGCCGGTATGCCGGT TAAGT-3′.

Fetal Limb Explant Culture

Fetal limbs were prepared from mouse embryos as described previously (Aguilar et al., 2007; Gao et al., 2013; Lindley et al., 2016) and cultured in DMEM containing 0.5% BSA, ascorbic acid (50 mg/ml), β-glycerophosphate (1 mM), and 1% penicillin and streptomycin (Sigma, United States) for 12 days after treatment ($n = 5$ per group). At day 10, calcein (100 mM, Sigma, United States) was added to the medium to trace the new bone formation. Finally, the limbs were observed under a fluorescence microscope for histological evaluation.

MSC Implantation and Micro-Computed Tomography Analysis

The subcutaneous implantation of MSCs and the induction of ectopic bone formation were performed as described previously (Aguilar et al., 2007; Gao et al., 2013; Lindley et al., 2016). Cells were treated with AdGFP or AdBMP9 alone or in combination with triiodothyronine for 7 days, and then the cells were collected for subcutaneous injection (5×10^6 cells per injection) into male athymic nude mice (5 per group, 4–6 weeks old, and Shanghai Laboratory Animal Centre, China). Then, the mice were treated with triiodothyronine (0.1 μg/g/d) or PBS subcutaneously for 5 weeks. At the chosen time point, the animals were euthanized. The implantation sites were retrieved for scanning by micro-CT (μCT 80; Scanco Medical, Zurich, Switzerland) and for histological evaluation. All experiments were performed in accordance with the guidelines for animal experimentation of the Ethics Committee of Xinhua Hospital (Approval No. XHEC-F-2018-020). All the tissues were fixed with 4% paraformaldehyde and decalcified with 10% EDTA decalcifying solution, then embedded in paraffin. Haematoxylin and eosin (H&E) and Masson's trichrome stain of serial sections were used for histological evaluation as previously described (Aguilar et al., 2007; Gao et al., 2013; Lindley et al., 2016).

Statistical Analysis

All the results are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Student's t-test or one-way analysis was used for the variable comparisons, and a P value < 0.05 was considered statistically significant.

RESULTS

Triiodothyronine Synergistically Promotes BMP9-Induced ALP Activity in MSCs

Many studies have shown previously that BMP9 is one of the most pro-osteogenic BMPs both *in vivo* and *in vitro*. T3 and TH have dual effects on the development of bone growth. To explore the influence of T3 on BMP9-induced MSCs' osteogenic differentiation, we used a BMP9-expressing adenoviral vector that can effectively transduce C3H10T1/2 cells (**Figures 1A,B**). We then tested the osteoinductivity of BMP9 in C3H10T1/2 cells in the presence or absence of T3 stimulation. We found that BMP9 induced significant ALP activity and expression, while T3 alone did not induce any ALP activity. However, ALP activity was shown to increase in the presence of T3 at days 5, 7, and 9 when the concentration of T3 was 0.1 μM. Similar results were obtained from ALP histochemical staining assays at day 7 (**Figures 1C,D**).

Triiodothyronine Potentiates the BMP9-Induced Osteogenic Markers Expression and Matrix Mineralization

Furthermore, we also analyzed the late stage osteogenic differentiation of C3H10T1/2 cells treated with AdGFP or AdBMP9 alone or in combination with T3. Alizarin red S staining showed that T3 treatment increased BMP9-induced mineral nodule formation (**Figure 2**). Protein and mRNA analysis showed that T3 significantly increased osteocalcin and osteopontin expression when cells were infected with AdBMP9 (**Figures 3A,B**). Immunohistochemical (IHC) staining also confirmed the results (**Figures 3D,E**). Collectively, these results demonstrate that BMP9 and T3 synergistically enhance osteogenesis in MSCs.

Triiodothyronine and BMP9 Act Synergistically to Promote Bone Formation in Mouse Embryo Limb Explant Cultures

We sought to analyze the effect of T3 on developing bone by using fetal limb culture assays. E18.5 mouse embryo

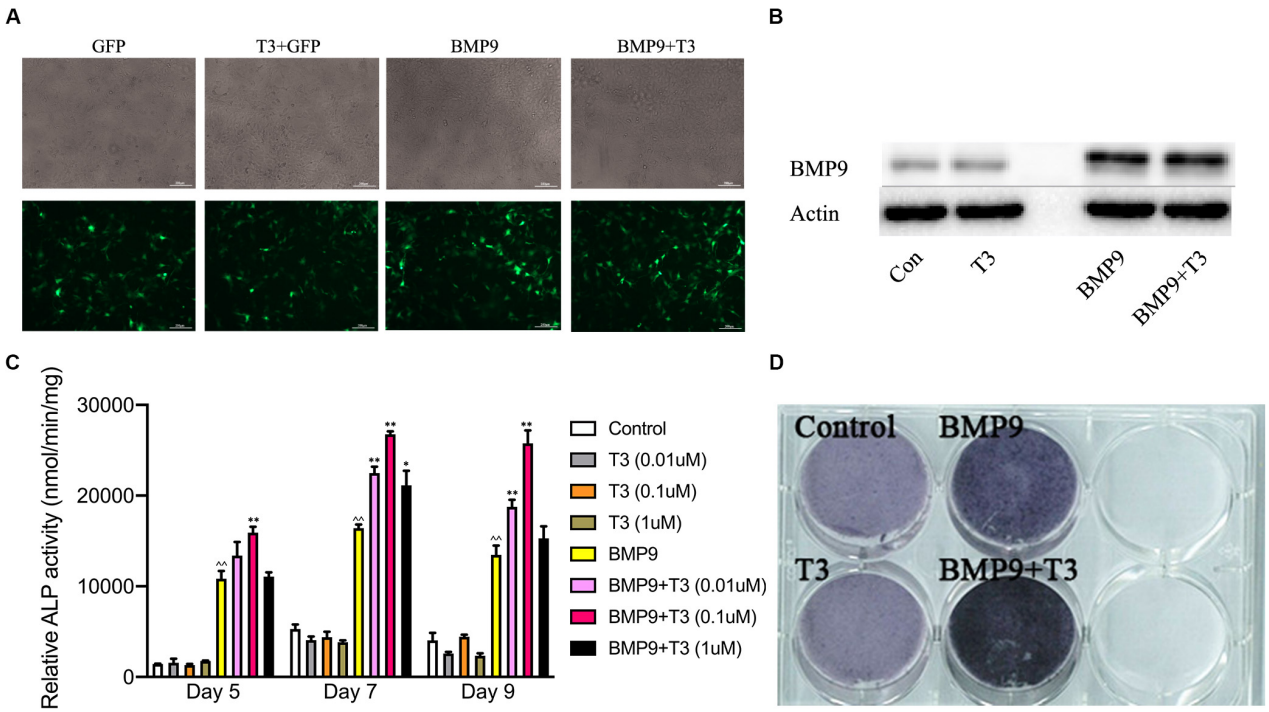

FIGURE 1 | Triiodothyronine potentiates the BMP9-induced early osteogenic marker alkaline phosphatase. **(A)** Infection of C3H10T1/2 cells with AdGFP and AdBMP9. Subconfluent C3H10T1/2 cells were infected with AdBMP9 or AdGFP for 48 h. The GFP signal was observed under a fluorescence microscope. **(B)** Western blot results showed endogenous expression of BMP9 in C3H10T1/2 cells. **(C)** Triiodothyronine potentiates BMP9-induced ALP activity. Subconfluent C3H10T1/2 cells were infected with AdBMP9 or AdGFP in the presence or absence of triiodothyronine stimulation (at different concentrations). A quantitative ALP activity assay was conducted at the indicated time points. **(D)** C3H10T1/2 cells were fixed for an ALP histochemical staining assay at day 7. $^{\wedge\wedge}p < 0.01$ compared with the control group; $^{*}p < 0.05$, and $^{**}p < 0.01$ compared with the BMP9 group.

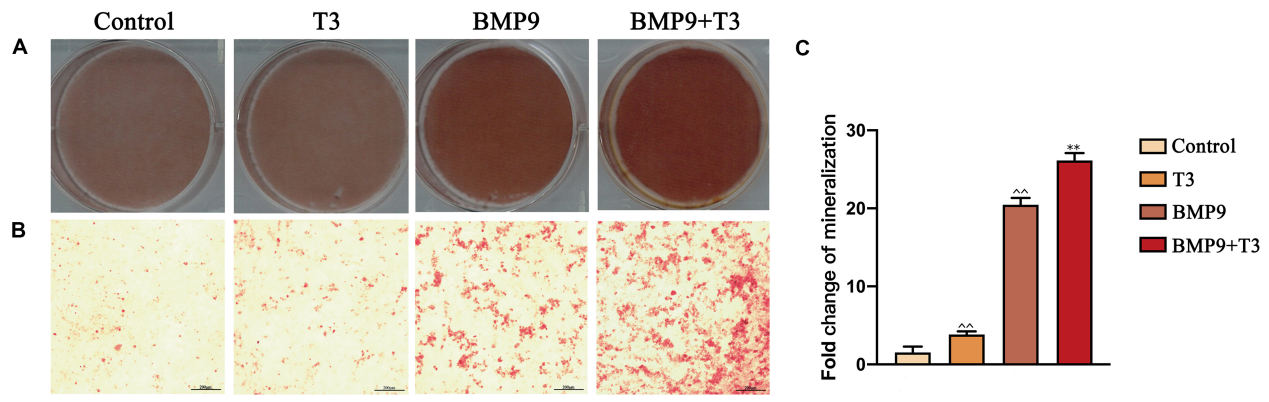

FIGURE 2 | Triiodothyronine promotes BMP9-induced mineralization in C3H10T1/2 cells. **(A,B)** Macrographic and microscopic images (200X) of alizarin red S staining after 21 days of infection. **(C)** Statistical analysis of relative area ($^{\wedge\wedge}P < 0.01$, compared with Control group; $^{**}P < 0.01$, compared with BMP9 group).

limbs were isolated (n = 5 each group) and infected with AdBMP9 and AdGFP in the presence or absence of T3 (0.1 μM). At the endpoint of each culture, the fluorescent dye calcein was used to show new bone formation. We found that treatment with BMP9+T3 induced new bone formation to the greatest extent compared with the control treatment (**Figure 4A**). Consistent with our previous reports, the histological evaluation revealed that BMP9+T3 treatment led to a significant expansion of the growth plate (**Figure 4B**).

Enhancement of BMP9-Induced Ectopic Bone Formation by Triiodothyronine

We focus on ectopic bone formation ability to further investigate the osteogenic effect of a combination of BMP9 and T3.

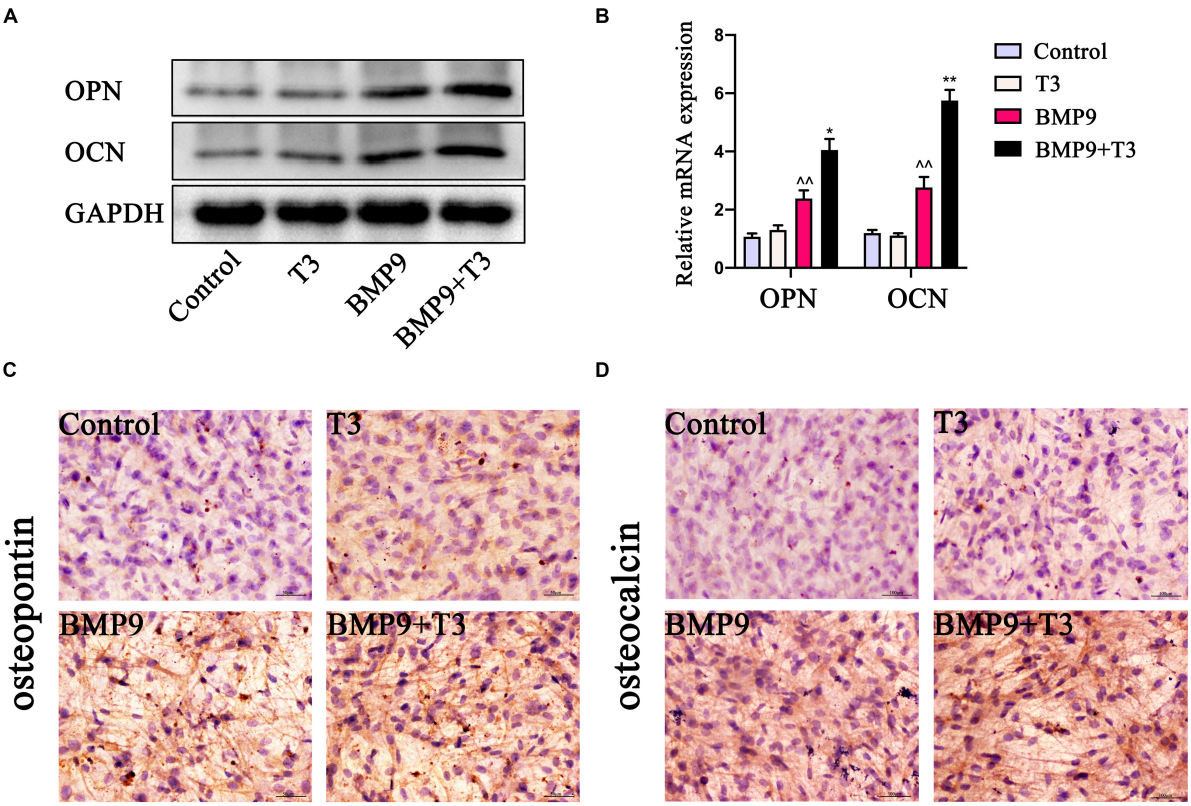

FIGURE 3 | Triiodothyronine promotes BMP9-induced osteogenic marker expression in C3H10T1/2 cells. **(A,B)** Protein and mRNA expression of OCN and OPN induced by AdBMP9 for 9 days (^^$P < 0.01$, compared with Control group; **$P < 0.01$, compared with BMP9 group). **(C,D)** Immunohistochemical staining results showed the expression of OPN and OCN in C3H10T1/2 cells for fourteen days after infection (200X).

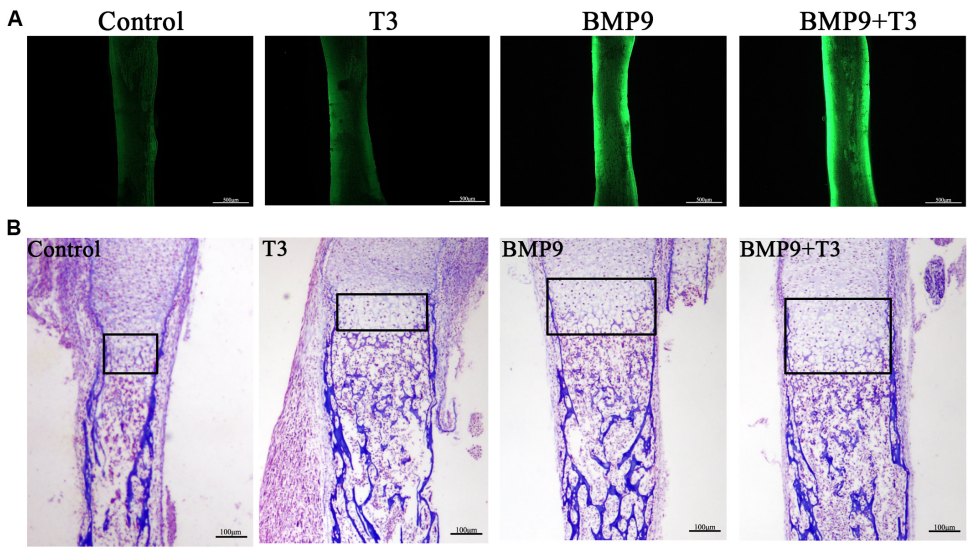

FIGURE 4 | Triiodothyronine enhances the ability of BMP9 to expand the hypertrophic chondrocyte zone. **(A)** E18.5 mouse embryo limbs were isolated and cultured in DMEM supplemented with 0.5% BSA, 50 mg/ml ascorbic acid, 1 mM beta-glycerophosphate, and 100 mg/ml penicillin-streptomycin. The embryo limbs were infected with AdBMP9 or AdGFP in the presence or absence of triiodothyronine (0.1 μM) for 14 days. On day 12, calcein (100 mM) was added to the culture medium. The harvested limbs were subjected to fluorescence microscopy. **(B)** The harvested tissues were fixed, paraffin-embedded, and subjected to Masson's trichrome staining. The boxed areas indicate the growth plate. Representative images are shown.

We pretreated C3H10T1/2 cells with AdBMP9 and/or T3, and then the collected cells were subcutaneously injected into male athymic nude mice, after which the mice were treated with T3, and/or PBS subcutaneously. After 5 weeks, all nude mice were sacrificed, and increases in bone mass were only detected in the groups treated with AdBMP9 alone and with T3 (**Figure 5A**). Micro-CT scans and histological results showed that C3H10T1/2 cells treated with AdBMP9+T3 formed slightly larger bone masses and showed more mineralization (**Figures 5B–D**) than cells in the BMP9 group. Altogether, these data confirmed that triiodothyronine can accelerate BMP9-induced bone formation.

Triiodothyronine Enhances BMP9-Induced Osteogenic Differentiation Through an AMPK-Dependent Pathway

Triiodothyronine has been found to induce osteocalcin synthesis in osteoblasts via the activation of AMPK or p38. Many studies have reported that AMPK and p38 activation can promote early during osteoblast differentiation and that their induction is required for normal bone formation *in vitro* and *in vivo*., p38 is involved in BMP9-induced osteogenic differentiation. Additionally, we examined the effects of T3 and BMP9 on AMPK and p38 MAPK activation. AMPK phosphorylation at Thr172 and p38 phosphorylation were significantly increased in the BMP9+T3 group, whereas the total protein levels remained unchanged (**Figure 6A**). However, the phosphorylation of AMPK and p38 was not affected by T3 in C3H10T1/2 cells. To further assess the significance of this change in osteoblast differentiation, inhibitors of AMPK and p38 were used. When C3H10T1/2 cells were treated with compound C, siRNA and SB203580, the ALP activity in the BMP9+T3 group was significantly inhibited (**Figures 6B,C**). Furthermore, exposure of C3H10T1/2 cells to compound C and siRNA (**Figure 6D**) inhibited the phosphorylation of p38 MAPK (**Figures 6E,F**). Collectively, these data indicate that the activation of AMPK and p38 MAPK mediate combined BMP9/T3-induced osteogenic differentiation and that AMPK may function as an upstream regulator of p38 MAPK.

DISCUSSION

We investigated whether T3 potentially has an effect on the promotion of BMP9-induced osteogenic differentiation of C3H10T1/2 cells, and we also examined the underlying molecular mechanisms. To clarify the effect, cells were treated with AdBMP9 alone or in combination with T3 and the expression of several osteogenic markers, such as ALP, OCN, and OPN were tested. Here, we found that ALP activity and OCN and OPN expression was most strongly induced in the co-treatment group. Furthermore, T3 was shown to promote BMP-9-induced

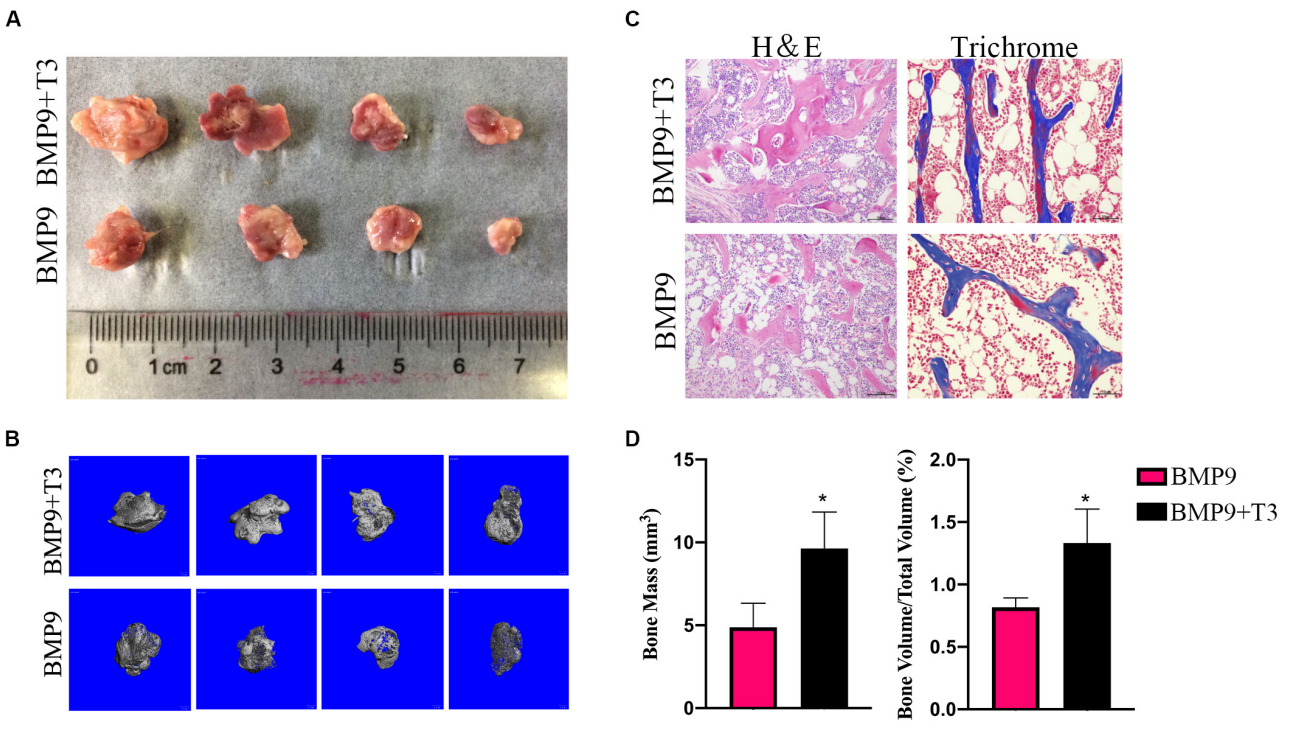

FIGURE 5 | (A) Macrographic images of ectopic bones from the BMP9 and BMP9 + triiodothyronine groups after 5 weeks. Ectopic bones did not form in the control and triiodothyronine groups. **(B)** 3D reconstruction of bone masses by micro-CT analysis. The bone masses were evaluated and analyzed with the μCT 80 system. **(C)** H&E staining and Masson's trichrome staining of serial sections of paraffin-embedded ectopic bones. **(D)** Analysis of bone mass and relative bone density. *$p < 0.05$.

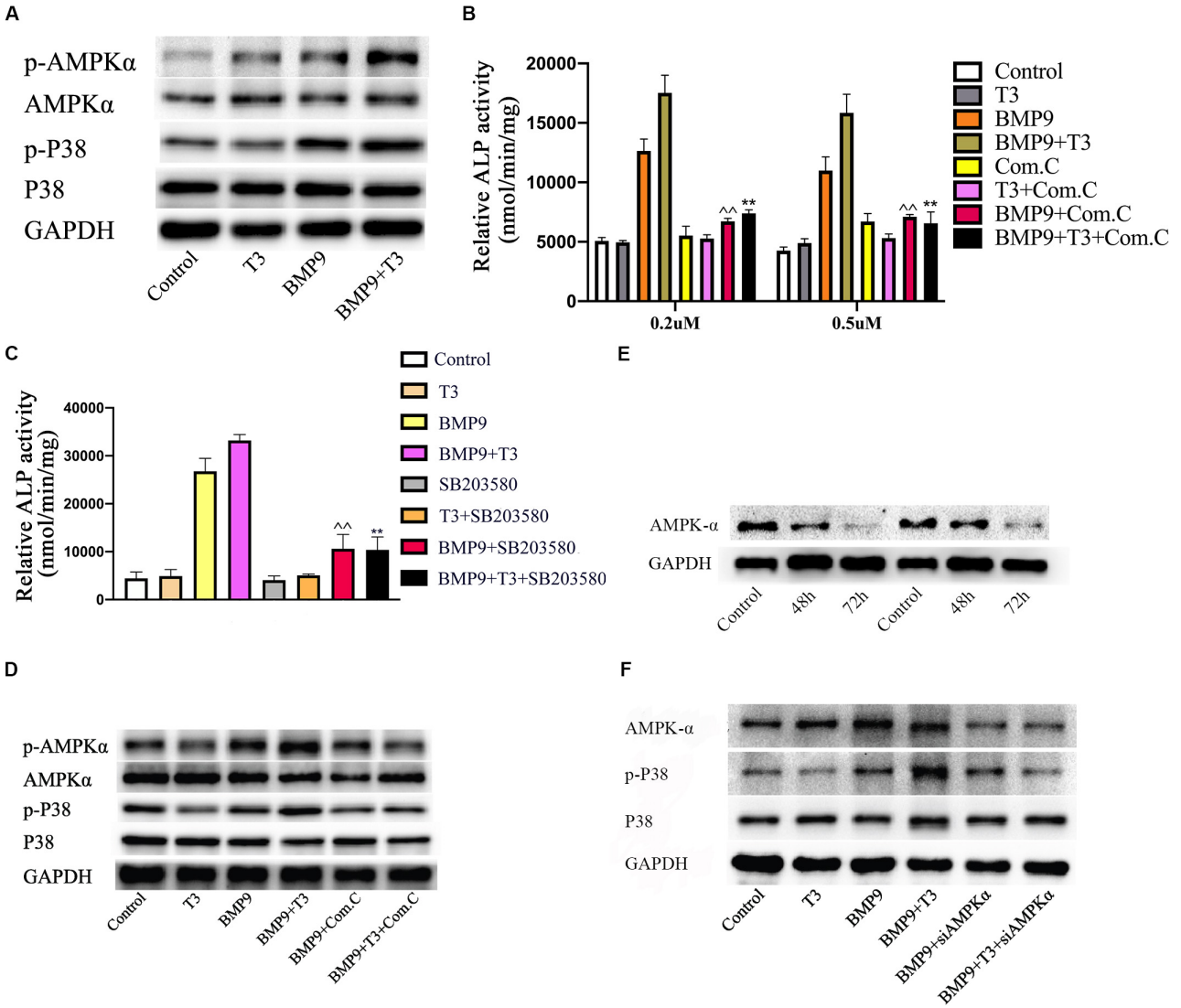

FIGURE 6 | Triiodothyronine enhances BMP9-induced osteogenesis through the AMPK/p38 pathway in MSCs. **(A)** Cells were infected with AdGFP or AdBMP9 in the presence or absence of triiodothyronine for 72 h, and then protein levels were analyzed by Western blotting. **(B,C)** The effects of compound C and at different concentrations and SB203580 (5 μM) on ALP activity in all groups on day 7. ^^$p < 0.01$ compared with BMP9 group; **$p < 0.01$ compared with the BMP9+T3 group. **(D)** C3H10T1/2 cells were pre-treated with compound C and then treated with AdBMP9 or AdGFP in the presence or absence of triiodothyronine. Protein levels were analyzed by Western blotting. **(E)** Changes in protein levels 48 and 72 h after siAMPK treatment. **(F)** Treatment with siAMPK inhibited p38 phosphorylation.

ectopic bone formation in stem cell implantation assays and embryo limb explant culture assays. T3 and BMP9 co-treatment was shown to enhance the phosphorylation of AMPK and p38. In addition, both compound C, and siAMPK abolished the promotional effect, suggesting that BMP-9 may engage in crosstalk with T3 through the AMPK signaling pathway during osteogenic differentiation in MSCs. Collectively, our results showed that T3 has a potentially effect on the promotion of BMP9-induced osteogenesis and bone formation partially by activating AMPK/p38 signaling pathway.

Thyroid hormone has received considerable attention as a regulator of bone metabolism based on its role in stimulating the expression of osteocalcin, type 1 collagen, and ALP to enhance osteoblast activity and influence the activity and formation of osteoclasts through the increased expression of receptor activator of nuclear factor κB ligand and other cytokines involved in osteoclastogenesis (Tsourdi et al., 2015). In the process of promoting osteogenesis, TH regulates a number of osteogenic-related growth factor signaling factors, including IGFs, parathyroid hormone-related protein, FGFs, and Wnt, to influence skeletal growth (Kim and Mohan, 2013). TH also has also been shown to regulate energy metabolism involving cholesterol levels, lipolysis and gluconeogenesis via activating AMPK signaling pathway, which is also important for bone

metabolism (Wojcicka et al., 2013; Mullur et al., 2014). BMP9, a member of the BMP family, is known to be the most potent BMP family osteogenic factor in MSCs. BMP9 also regulates several biological processes, such as glucose and lipid metabolism (Chen et al., 2003; Caperuto et al., 2008; Luo et al., 2017).

The potential of T3 to interactive with BMP signaling pathway has been supported by several studies. T3 has been shown to function cooperatively with BMP4 to regulate cartilage differentiation and endochondral bone formation. In the processes of chondrocyte maturation and the synthesis of collagen X, T3 treatment stimulated the expression of BMP 4, which was accompanied by the downregulated expression of the BMP inhibitor Noggin (Lassova et al., 2009). Another study also showed that T3 signaling is essential for BMP4-induced colorectal cancer cell differentiation (Catalano et al., 2016). However, we found that T3 didn't influence the mRNA level of BMP receptor (**Supplementary Figure S2** and **Supplementary Table S1**). Furthermore, the result of western blot showed that T3 treatment can slightly promote phosphorylation level of Smad1/5/8, while when co-treated with BMP9 the phosphorylation of p-Smad1/5/8 was also slightly increased (**Supplementary Figure S3**). In addition, to the best of our knowledge, several studies have demonstrated that TH is a major regulator of IGF-1 expression, β-catenin accumulation, and TCF/LEF transcriptional activity (O'Shea et al., 2005; Xing et al., 2012). IGF signaling is critical for the development and homeostasis of bone (Chen et al., 2010a, 2016). Both IGF1 and IGF2, two ligands involved in IGF signaling, have been crosstalk with BMP9 in osteogenic differentiation of MSCs evidenced by the activation of Smad signaling (Chen et al., 2010b; Li et al., 2015). Wnt/β-catenin signaling pathway is a classical osteogenic differentiation pathway and have synergistically osteogeneic effect when treated with BMP9 simultaneously (O'Shea et al., 2005). Likewise, our results suggest that T3 alone has no significant effect on ALP activity, OPN expression. In C3H10T1/2 cells infected with AdBMP9, we found that T3 enhanced BMP9-induced osteogenic marker expression, matrix mineralization, new bone formation and increased the phosphorylation of AMPK/p38. Thus, our data suggested that the augmentation of BMP9-induced osteogenesis by T3 may be partly mediated by AMPK/p38 signaling.

The effects of THs are mediated through thyroid hormone receptors (TR) that act as ligand dependent transcription factors, but we found that the mRNA expression of both TRα and TRβ showed no significant changes in our study (**Supplementary Figure S4** and **Supplementary Table S1**). Futrther, several studies reported that TH can stimulate AMPK signaling pathway through rapid, transcription-independent (non-genomic) effects in other cells and the activation of AMPK is required for osteoblast differentiation in both MC3T3E1 cells and primary murine osteoblasts (Shah et al., 2010; Xi et al., 2016; Wang et al., 2018). It is reported that triiodothyronine can induce osteocalcin synthesis in MC3T3E1 cells via the activation of AMPK or p38 (Ishisaki et al., 2004; Chen et al., 2010b; Kondo et al., 2013). In addition, T3 induced IGF-1, and IGFBP-2 expression can stimulate AMPK activation during MC3T3E1 cells osteogenesis (Xing et al., 2012; Xi et al., 2016). Our results significantly support these observations by confirming that T3 and BMP9 treatment stimulate osteogenic differentiation via the phosphorylation of AMPK. Nevertheless, we did not find that T3 can induce osteocalcin expression in C3H10T1/2 cells or induce the phosphorylation of AMPK and p38 MAPK. It is conceivable that the role of T3 may be different in different cells and cell stages. It was reported that p38 may function as a downstream signaling molecule of AMPK in several studies. AMPK was reported to interact with p38 to regulate glucose metabolism, BMP2 expression, and COX-2 expression, all of which play an important role in the processes of bone formation and remodeling (Xi et al., 2001; Hou et al., 2008; Huang et al., 2010). Additionally, COX-2 was reported to form an important regulatory loop with BMP9 and to induce osteogenic differentiation in MSCs (Wang et al., 2013). Additionally, p38 is involved in BMP9-induced osteogenic differentiation (Xu et al., 2012; Kamel et al., 2017). Then, we examined the potential role of p38 on the effects of T3 on BMP9-induced osteogenesis. Pretreatment of C3H10T1/2 cells for 60 min with SB203580 markedly attenuated ALP activity. In addition, the phosphorylation of p38 were inhibited by compound C and AMPK siRNA in our study. Therefore, we proposed that the mode of action of T3 and BMP9, which leads to the induction of osteogenesis, is partly achieved by the sequential activation of AMPK and p38 MAPK. However, several studies have also reported that the activation of p38 is not influenced by the modulation of AMPK in the control of myocardial glucose metabolism and tumourigenesis. Thus, further studies are required to clarify the mechanism involved in the AMPK/p38 pathway in MSC osteogenic differentiation.

Overall, we found that the synergistical effects of T3 on BMP9-induced MSCs osteogenesis may be mediated by increased AMPK/p38 signaling. These findings provide insights into the complex effect of T3 involved in osteogenic differentiation in MSCs.

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 Animal Ethical and Welfare Committee of Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Approval No. XHEC-F-2018-020).

AUTHOR CONTRIBUTIONS

XC and YG designed the study. XC, TJ, CX, and YH performed the experiments. XC wrote the original draft of the manuscript. YG reviewed and edited 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.2020.00725/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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REVIEW
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Exosomes From Adipose-Derived Stem Cells: The Emerging Roles and Applications in Tissue Regeneration of Plastic and Cosmetic Surgery

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Adipose-derived stem cells (ASCs) are an important stem cell type separated from adipose tissue, with the properties of multilineage differentiation, easy availability, high proliferation potential, and self-renewal. Exosomes are novel frontiers of intercellular communication regulating the biological behaviors of cells, such as angiogenesis, immune modulation, proliferation, and migration. ASC-derived exosomes (ASC-exos) are important components released by ASCs paracrine, possessing multiple biological activities. Tissue regeneration requires coordinated "vital networks" of multiple growth factors, proteases, progenitors, and immune cells producing inflammatory cytokines. Recently, as cell-to-cell messengers, ASC-exos have received much attention for the fact that they are important paracrine mediators contributing to their suitability for tissue regeneration. ASC-exos, with distinct properties by encapsulating various types of bioactive cargoes, are endowed with great application potential in tissue regeneration, mechanically via the migration and proliferation of repair cells, facilitation of the neovascularization, and other specific functions in different tissues. Here, this article elucidated the research progress of ASC-exos about tissue regeneration in plastic and cosmetic surgery, including skin anti-aging therapy, dermatitis improvement, wound healing, scar removal, flap transplantation, bone tissue repair and regeneration, obesity prevention, fat grafting, breast cancer, and breast reconstruction. Deciphering the biological properties of ASC-exos will provide further insights for exploring novel therapeutic strategies of tissue regeneration in plastic and cosmetic surgery.

Keywords: adipose-derived stem cells, exosomes, tissue regeneration, biological activity, function

INTRODUCTION

Adipose tissue provides a major energy storage depot for the body. Over the past years, adipose tissue has been considered as a multifunctional organ that controls metabolic homeostasis, immunity, and satiety (Yang et al., 2018). Adipose tissue can be broadly separated into fat-storing adipocytes and the adipose tissue stromal vascular fraction (SVF). SVF is a heterogeneous cell group that is traditionally isolated using enzymes such as collagenase. SVF is an easily accessible system comprised of various immune cells, erythrocytes, endothelial cells, and adipose-derived stem cells (ASCs), with the exception of adipocytes (Kim and Lee, 2020). ASCs are defined as a subset of mesenchymal stem cells (MSCs) isolated from the SVF within adipose tissue by enzymatic digestion

(Gentile et al., 2019b). ASCs are specifically valuable because they can be easily harvested with the properties of abundance and convenient separation. In terms of cell identification, ASCs exhibit a mesenchymal-like morphology and the expression profile of $CD34^+$, $CD44^+$, $CD31^-$, and $CD45^-$ cell surface markers (Shukla et al., 2020). ASCs are not only precursors to adipocytes but also multipotent progenitors to a variety of cells including osteoblasts, chondrocytes, myocytes, epithelial cells, and neuronal cells. Furthermore, ASCs possess several unique characteristics, including easy availability, high proliferation potential, self-renewal, and secretion of trophic factors and extracellular vesicles (EVs), thus offering a feasible and valid alternative to other sources of MSCs, such as bone marrow-MSCs (BMSCs) (Mazini et al., 2019). The secreted factors derived from ASCs, such as growth factors and cytokines, are known to exert paracrine signals responsible for chemoattractant, angiogenic, and prosurvival effects required for tissue regeneration (Bajek et al., 2016). ASCs are particularly useful as they can be easily harvested with minimal donor site morbidity and have a differentiation potential similar to other MSCs. Thus, ASCs have been successfully proposed as a prominent candidate in the development of tissue engineering products. As well, plastic and cosmetic surgery is a growing field uniquely positioned for the application of ASCs (Zuk et al., 2001).

The term "exosome" specifically defines a small subset of extracellular vesicles that range from 50 nm to 200 nm, which have been found in numerous body fluids, including blood, urine, cerebrospinal fluid, breast milk, saliva, lymph, and bile, under both healthy and pathological conditions (Wang et al., 2018). Exosomes are formed when the endosome membrane invaginates to produce a multivesicular body which upon fusing with the cell membrane to release the vesicles within as exosomes into the extracellular space (Toh et al., 2018). Consequently, the key expression biomarkers of exosomes, which are generally recognized as exosome-associated characteristics, are proteins associated with endocytosis and endosomal traffickings such as tetraspanins (CD81, CD63, CD9), ALIX, TSG101, caveolins, clathrin, and transferrin receptors, due to the different mechanisms of secretion (Hong P. et al., 2019). Exosomes are packed with cell-type-specific combinations of proteins (cytoskeletal proteins, transmembrane proteins, and heat shock proteins), nucleic acids (DNA, mRNA, miRNA, long and short non-coding RNA), lipids, and enzymes (GAPDH, ATPase, pgk1), shuttling these active cargoes between different cells involving in a complex intercellular communication system. These cargoes are wrapped in the membrane to protect from degradation and transport to the surrounding cells (Kalluri and LeBleu, 2020). Thus, exosomes have some special biological characteristics and processes and are capable of potentially modulating the specific activity of the recipient target cells.

The important aim of plastic and cosmetic field is closely associated with tissue regeneration, and to repair the morphology and function of congenital or acquired defects through many treatment methods, including medical imaging, microsurgery, composite tissue allotransplantation, nanotechnology, cell biology, and biomaterials (Naderi et al., 2017). Adipose tissue possesses important functions in immune modulation, wound healing, and tissue regeneration. Nowadays, autologous adipose tissue application is explored to be applied in improving skin quality, contour irregularities, wound repair and soft tissue regeneration in plastic and cosmetic surgery (Strong et al., 2019). Its efficacy and safety are widely accepted, but there is a lack of universally recognized mechanisms. However, given that the characteristics of adipose tissue vary dramatically depending on the donor status, the effect is of individual difference (Wang et al., 2013). Besides, significantly altering the components or biomechanical properties of the adipose tissue, such as by removing stromal cells from the adipose tissue, will subject to more complex applications. Therefore, autologous adipose tissue cannot be easily used as a drug. Thus, ASCs and ASC-exos are very important derivatives from fat tissue, capturing intensive attention. Exosomes carry specific contents of the parental ASCs, including DNAs, RNAs, lipids, cytokines, enzymes. Exosomes are capable of protecting their cargoes from degradation and are highly stable in serum and blood, thus efficiently delivering cargoes to target cells. ASC-exos and ASCs perform their functions via different mechanisms. The ASC-exos are used as tools for repairing and regenerated activation of damaged cells, and are now considered to orchestrate the events required for tissue regeneration, immune function, tissue homeostasis and development of cell fate. Hence, although without differentiation ability, ASC-exos can mimic the capacity of ASCs for innovative cell-free therapy, such as tissue regeneration and repair, reduction of injuries, and anti-inflammation. In terms of storage and delivery, unlike ASCs, exosomes are small non-living substances that can be sterile filtered and frozen without cryo-preservatives. From manufacturing and storage to delivery, there is no need to maintain cell viability and function. ASC-exos have certain advantages over ASCs in production, storage, shelf life, delivery, and potentially ready-to-use biological products (Vizoso et al., 2017). Moreover, ASC-exos are potentially safer therapeutic agents than ASCs. Compared with ASCs, ASC-exos might avoid cell therapy-associated problems, including limited cell survival, immune rejection efficacy, senescence-induced genetic instability, inactivate function, and the possibility of unfavorable differentiation (Figueroa et al., 2014). Owing to their multiple

Abbreviations: γH2AX, Phosphorylated Histone H2AX; AKT, Protein Kinase B; AP-1, Activator Protein 1; Arg-1, Arginase 1; ASC-CM, ASC-Conditioned Medium; ASC-exos, ASC-derived exosomes; ASCs, Adipose-derived Stem Cells; BAT, Brown Adipose Tissue; Bax, Bcl-2-associated X Protein; Bcl-2, B-cell lymphoma/leukemia 2; CAL, Cell-Assisted Lipotransfer; Caspase-3/9, Aspartate Proteolytic Enzyme 3/9; ECM, Extracellular Matrix; EVs, Extracellular Vesicles; H_2O_2, Hydrogen Peroxide; HaCaTs, Human Keratinocytes; HDFs, Human Dermal Fibroblasts; I/R, Ischemia-Reperfusion; IFN-γ, Interferon Gamma; IL-4/5/6/13, Interleukin 4/5/6/13; iNOS, Inducible Nitric Oxide Synthase; MAPKs, Mitogen-Activated Protein Kinases; MMP-1/9, Matrix Metalloproteinase 1/9; MSCs, Mesenchymal Stem Cells; NF-κB, Nuclear Factor Kappa B; NOX-1/4, NADPH Oxidase 1/4; Nrf2, NF-E2-related factor 2; PDGF-AA, Platelet-Derived Growth Factor-AA; PGE2, Prostaglandin E2; PI3K, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase; RANKL, Receptor Activator of Nuclear Factor Kappa B Ligand; ROS, Reactive Oxygen Species; SA-β-gal, Senescence-Associated β-galactosidase; SMP30, Senescence Marker Protein 30; SVF, Stromal Vascular Fraction; TGF-β, Transforming Growth Factor Beta; TIMP-1, Tissue Inhibitor of Metalloproteinases 1; TNF-α, Tumor Necrosis Factor Alpha; TSG-6, TNF-Alpha-Stimulated Gene/Protein 6; UCP-1, Uncoupling Protein 1; UVB, Ultraviolet B; VECs, Vascular Endothelial Cells; VEGF, Vascular Endothelial Growth Factor; WAT, White Adipose Tissue.

features, ASC-exos have shown therapeutic potential in many clinical diseases, especially in tissue regeneration, such as skin repairing, fat grafting, and various reconstruction operation. In addition, exosomes have been innovatively utilized for targeted drug delivery and as gene carriers for regenerative medicine (Mehryab et al., 2020). But actually, ASC-exos lack enough clinical trials to confirm the safety and effectiveness (**Table 1**).

In this review, we mainly summarize the latest research about the functions and investigations of ASC-exos concerned with tissue regeneration in plastic and cosmetic surgery, including skin anti-aging therapy, dermatitis improvement, wound healing, scar removal, flap transplantation, bone tissue repair and regeneration, obesity prevention, fat grafting, breast cancer, and breast reconstruction. We hope this will provide further insights into the pivotal roles and applications of ASC-exos in tissue engineering and regenerative therapies. For these goals, we searched the adipose-derived stem cells/ASCs, exosomes and the above 8 related fields in plastic and cosmetic surgery as keywords on Pubmed in the recent 5 years. These searched studies involved in cell, animal experiments, or clinical trials, especially the original articles were included according to relevance to the topic.

TABLE 1 | Characteristic comparison of ASC-exos and ASCs.

	ASC-exos	ASCs
Source	acquisition from ASCs with exosome separation methods, exist in adipose tissue and stable in serum and blood	easy acquisition and high yield from adipocyte tissues, especially from white adipose tissue
Morphology	small lipid bilayer vesicles	mesenchymal-like cells
Management	could be sterile filtered and frozen without cryo-preservatives, easily long-term storage and delivery, easily keep biological activity	should preserve cell viability and function from manufacture to storage and delivery, high storage requirements, complex cultivation
Biological properties	protect cargoes from degradation, target specificity, good tissue permeability, intercellular communication, immune function, tissue homeostasis and development of cell fate	multi-lineage differentiation, secret great kinds of growth factors by paracrine function, prosurvival effects, regulation of immune function, angiogenesis
Secretome	DNAs, RNAs, lipids, cytokines, enzymes from the parent cell	exosomes, cytokines, DNAs, RNAs, lipids, enzymes
Biosafety	limited immunogenicity, high biosafety	immunogenicity, biosafety
Applications	considered as multiple bioactive substances for tissue regeneration, could be gene modification, upload drugs as carriers, upload in other carriers such as nanomaterials	considered as ideal stem cell source for cell and tissue regeneration; could be gene modification, upload in carriers such as nanomaterials
Clinical trials	lack enough clinical trials to confirm the safety and effectiveness	security and effectiveness are verified in many diseases
Application disadvantages	relative low purity and yield, complicated components, substantial degree of heterogeneity in dosing regimens in the reported cases, lacking *in vivo* clinical trials	limited cell survival, immune rejection efficacy, senescence-induced genetic instability, inactivate function, and the possibility of unfavorable differentiation, individual differences

Abbreviations: ASCs, Adipose-derived stem cells; ASC-exos, ASC-derived exosomes.

ASC-EXOS IN SKIN AGING

The skin is subject to an unpreventable intrinsic aging process, along with the exogenous factors-induced aging state. Particularly, ultraviolet radiation results in premature skin aging, also known as extrinsic skin aging or photoaging. The most typical features of skin aging are the loss of elasticity and the generation of wrinkles, which are attributed to the structural and functional changes in skin cells and tissues (Li et al., 2019). Among skin cells, human dermal fibroblasts (HDFs) and keratinocytes (HaCaTs) are regarded as barriers to prevent skin from time aging and ultraviolet B (UVB) photoaging. Various improvement strategies, such as antioxidants, retinoids, peptides, growth factors, and autologous patient fat or collagen graft, have been used to fight against skin aging (Kim et al., 2019). Because of the poor penetration through the stratum and short-term maintenance for several months, these above strategies are now not ideal. It is hopeful to reduce skin aging pinned on ASCs and ASC-derivatives, which could regulate HDFs proliferation, migration, and collagen expression.

ASC-conditioned medium (ASC-CM) and ASC-exos, both containing key cytokines and growth factors secreted by the ASCs, could facilitate the regeneration and repair of various tissues and organs to exert influences on anti-oxidation, anti-wrinkle, and whitening skin. ASC-CM has been proved to protect HDFs from oxidative stress *in vitro* (Kim et al., 2008). Li et al. (2019) found that in UVB irradiation *in vitro* model, ASC-CM could effectively down-regulate the activation and transcription of UVB-induced signaling pathways such as mitogen-activated protein kinases (MAPKs), activator protein 1 (AP-1), and nuclear factor kappa B (NF-κB), and up-regulate the expression of antioxidant response elements such as phase II gene HO-1 and transforming growth factor-beta (TGF-β), while reducing interleukin 6 (IL-6) secretion. Thereby ASC-CM showed a positive effect on protecting HDFs and HaCaTs from UVB-induced photoaging damage. The platelet-derived growth factor AA (PDGF-AA) contained in ASC-CM also could activate the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) signal pathway, and mediate photoaging-induced HDFs proliferation, extracellular matrix (ECM) deposition and remodeling in the *in vitro* experiment, which was reported by Guo et al. (2020) group. It demonstrated that the well-prepared ASC-CM played a positive role in preventing HDFs from intrinsic and extrinsic aging damages to a certain degree. Meanwhile, the result also clarified that the PDGF-AA might contribute to better outcomes with some other components of ASC-CM. However, the ingredients in ASC-CM are rather complex to synergistically achieve the anti-aging goal. The exosomes are important components in ASC-CM, might possess a positively independent or synergistic roles. Hu et al. (2019) showed that exosomes from

three-dimensional cultured HDF spheroids (3D-HDF-exos) and BMSC-exos could both down-regulate tumor necrosis factor alpha (TNF-α) and up-regulated TGF-β expression, resulting in decreased matrix metalloproteinase 1 (MMP-1) and increased type I procollagen *in vitro* and a nude mouse photoaging model. These results indicated that the exosome-containing 3D-HDF-exos and BMSC-exos both had anti-skin-aging properties and the potential to prevent and treat cutaneous aging (**Figure 1A**).

At present, the existing research of ASC-exos on skin aging is limited. As the epidermis layer is 50–120 μm and the epidermis-dermis thickness is 2–5 mm in humans, the local treatment with exosomes can arrive at the epidermis and be absorbed on human skin (Xu et al., 2018). Exosomes derived from human stem cells, such as ASCs, are of multiple bioactive functions for skin aging treatment, deserving further research.

ASC-EXOS IN ATOPIC DERMATITIS

Atopic dermatitis (AD) is a chronic inflammatory skin disease accompanied with pruritus, erythema, edema, excoriation, and thickening of the skin, leading to decreased unaesthetic appearance of skin (Lee et al., 2018). Both defective skin barrier and abnormal immune responses are crucial factors in AD development. Therefore AD with immunologic abnormalities could be treated by different or multi-pronged approaches focused on reducing the severity and frequency of symptoms, such as dietary management, drug therapy, and ultraviolet assisted therapy (Park et al., 2019). Several studies have demonstrated that allergic progress in AD could be suppressed by BMSCs and ASCs while modulating multiple targets (Shin et al., 2017). For instance, Sah et al. (2018) found that superoxide dismutase 3-transduced MSCs ameliorated AD pathology and enhanced the efficacy of MSC therapy by controlling activated immune cells, reducing expression levels of pro-inflammatory mediators in the skin of AD mice.

Nevertheless, given that AD chronic and recurrent characteristics, the therapeutic utilization of MSCs has several drawbacks, such as poor engraftment efficiency, undesired immune responses, short half-life, and difficulties in quality control (Lou et al., 2017). Exosomes are involved in the development and prognosis of AD skin diseases, including repairing leaky skin barriers as well as suppressing skin inflammation (Alves et al., 2016). Cho et al. (2018) established

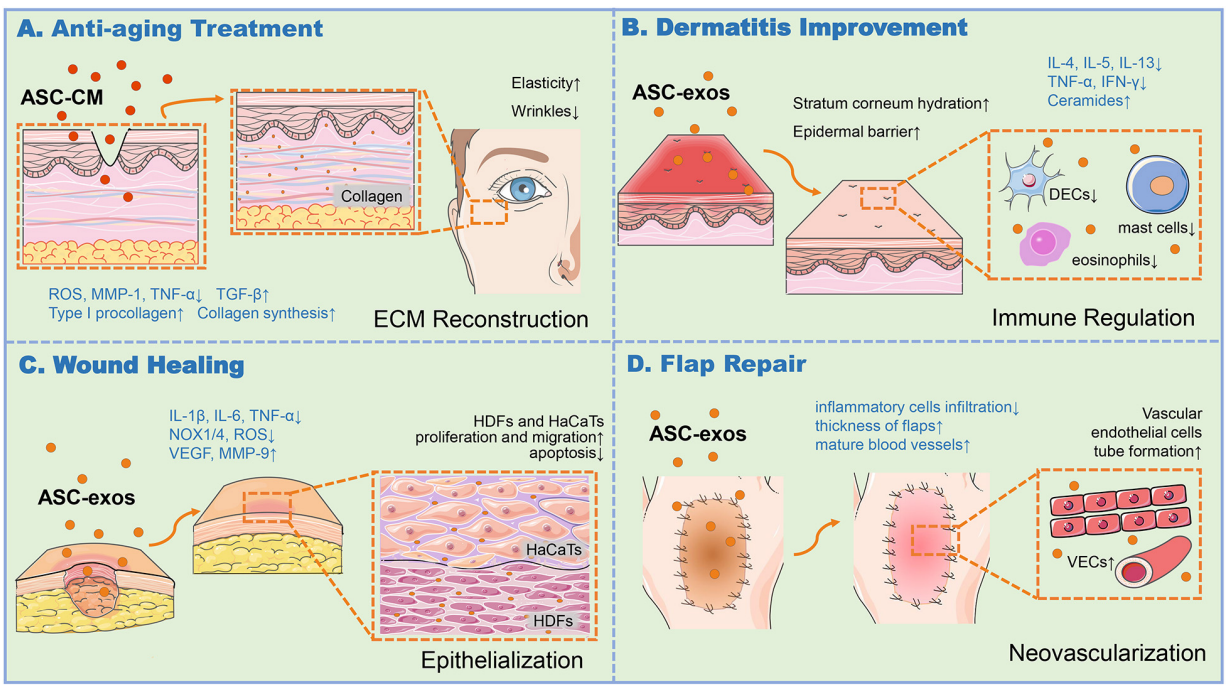

FIGURE 1 | ASC-exos function in various skin associated applications. **(A)** ASC-CM and BMSC-exos could produce ROS at a low level, downregulate TNF-α, upregulate TGF-β to increase MMP-1 and procollagen type I expression for collagen synthesis, thus enhancing the skin elasticity and ease the wrinkles for anti-aging. **(B)** ASC-exos was capable to enhance stratum corneum hydration, reduce the secretion of inflammatory cytokines such as IL-4, IL-5, IL-13, IFN-γ, and TNF-α, and alleviate the infiltration of mast cells, dendritic epidermal cells (DECs) in skin lesions and eosinophils in the blood, and produce ceramides to restore the epidermal barrier, thus relieving the dermatitis of skin. **(C)** ASC-exos reduced the production of ROS, decrease the expression of IL-6, IL-1β, TNF-α, and the oxidative stress-related proteins such as NADPH oxidase 1/4 (NOX1/4), increase MMP-9 and VEGF to ameliorate ECM reconstruction, thus fostering HDFs proliferation and migration to reinforce the re-epithelialization. **(D)** ASC-exos was conducive to promote tube formation of VECs, increase tissue thickness, and reduce the infiltration of inflammatory cells to relieve the inflammation and apoptosis for the high survival rate of the skin flap. ASCs, Adipose-derived stem cells; ASC-exos, ASC-derived exosomes; HDFs, Human Dermal Fibroblasts; HaCaTs, Human Keratinocytes; ECM, Extracellular Matrix; ROS, Reactive Oxygen Species; MMP-1/9, Matrix Metalloproteinase 1/9; IFN-γ, Interferon Gamma; TNF-α, Tumor Necrosis Factor Alpha; TGF-β, Transforming Growth Factor Beta; IL-4/5/6/13, Interleukin 4/5/6/13; NOX-1/4, NADPH Oxidase 1/4; VEGF, Vascular Endothelial Growth Factor; VECs, Vascular Endothelial Cells, VECs.

an AD model of NC/NGA mice treated with house dust mite antigens. In this mouse model, ASC-exos were found to ameliorate pathological symptoms such as the levels of serum IgE, the number of eosinophils in the blood, and the infiltration of mast cells, dendritic epidermal cells in skin lesions. The study suggested the immune regulation role of ASC-exos in AD. In the latter study, the same group also found that the subcutaneous injection of ASC-exos in an oxazolone-induced dermatitis model remarkably reduced trans-epidermal water loss, and enhanced stratum corneum hydration and markedly decreased the levels of inflammatory cytokines such as IL-4, IL-5, IL-13, TNF-α, interferon gamma (IFN-γ), IL-17 and TSLP, all in a dose-dependent manner (Shin et al., 2020). Interestingly, ASC-exos also induced the production of ceramides and dihydroceramides to promote skin barrier restoration (Shin et al., 2020). These studies suggested that the systemic administration of ASC-exos ameliorated AD-like symptoms through the regulation of inflammatory responses and the potential of effectively restoring epidermal barrier functions in AD (**Figure 1B**). ASC-exos could be a promising cell-free candidate to currently limited treatment options for AD.

ASC-EXOS IN WOUND AND SCAR

Many exposed, unsightly, or chronic wounds, such as diabetic ulcers, are difficult to heal, not only causing functional disabilities but also affecting mental health. Poor wound healing eventually leads to hypertrophic scars or keloid formation, pigmentation, prolonged healing, and ulcerative skin defects (Eming et al., 2014). There are many traditional treatment methods for skin and soft tissue trauma, such as low-intensity lasers, advanced treatment dressings, negative pressure wound treatment, hyperbaric oxygen, and skin transplantation (Bellei et al., 2018). However, as wound healing is a complicated process referring to multiple cell types, growth factors, and extracellular matrix, some traditional treatments just play an auxiliary role accompanied by undesirable healing.

ASCs and SVF can produce abundant secretome groups, leading to cell proliferation and differentiation, migration, and healing microenvironment. The migration and functions of ASCs could be enhanced via PI3K/AKT pathway activated by integrin β1, resulted in the improved chronic refractory wound (Wang J. et al., 2020). Interestingly, in the wound mouse model, Bi et al. (2019) found that both SVF and human ASCs improved the function of endotheliocytes and fibroblasts, regulated gene expression, and jointly promoted skin healing. This study showed that SVF could replace ASCs for wound healing, due to the convenience of SVF applications. In the burn wound model, only autologous ASCs, but not allogeneic ASCs, significantly improved healing in acute burn wounds of the rat (Chang et al., 2018). Fujiwara et al. (2020) also constructed an ovine burn model and proved that ASCs improved grafted burn wound healing by promoting blood flow and vascular endothelial growth factor (VEGF) expression. Besides, the localized injection of ASCs could accelerate and enhance the closure of pressure ulcers (Xiao et al., 2019). Likewise, Bukowska et al. (2020) systematically ensured the safety of human SVF when injected into a murine pressure ulcer injury model. This healing function usually depends on the trophic factors of ASCs and SVF, including cytokines, growth factors, and chemokines. Notably, exosomes containing secretome from ASCs have opened the way to a newly emerging cell-free therapy.

Studies have shown that ASC-exos played a positive role in cutaneous wound healing by means of acting on HDFs and HaCaTs and other main target cells through various signal channels (**Figure 1C**; Qiu et al., 2020). Ma et al. (2019) exposed HaCaTs to hydrogen peroxide (H_2O_2) for establishing a skin lesion model, discovering that ASC-exos could foster HaCaTs proliferation, migration, and inhibit apoptosis through Wnt/β-catenin signaling pathway. Likewise, He et al. (2020) also confirmed that MALAT1-containing ASC-exos improved wound healing by targeting miR-124 and activating Wnt/β-catenin pathway. In addition, ASC-exos might also promote and optimize collagen synthesis via upregulating PI3K/Akt pathway during cutaneous wound healing (Zhang et al., 2018). Li X. et al. (2018) found that exosomes from NF-E2-related factor 2 (Nrf2)-overexpressing ASCs significantly reduced the ulcer area in the feet of diabetic rats, by promoting the proliferation and angiogenesis of endothelial cells, improving levels of senescence marker protein 30 (SMP30) and VEGF and vascular endothelial growth factor receptor 2 (VEGFR2) phosphorylation to accelerate the wound healing, as well as inhibiting reactive oxygen species (ROS) production and inflammatory cytokine expressions, such as IL-1β, IL-6, and TNF-α. Notwithstanding this superiority, exosome-based therapy of wound healing still faces the challenges of rapid clearance rate and relatively short half-life *in vivo* (Liu et al., 2017). The sustained release and retention of exosomes in the target area is an important factor for healing. Liu et al. showed hyaluronic acid (HA) might serve as exosomes immobilizer and wound dressing for durable exosomes retention at wound sites to effectively reparative effect. ASC-exos combined with HA was able to activate the HDFs activity of the wound surface and reinforced the re-epithelialization and vascularization of the wound surface (Liu et al., 2019). Moreover, several studies have confirmed the role of ASC-exos miRNAs in skin healing. For instance, Yang et al. (2020) found that highly expressed miRNA-21 derived from ASC-exos could enhance the migration and proliferation of the HaCaTs, by increasing the matrix metalloproteinase 9 (MMP-9) expression through the PI3K/AKT pathway. The overexpressing miRNA-21 could also enhance collagen synthesis and optimize collagen deposition, significantly improve the healing effect of full-thickness skin wounds in mice (Yang et al., 2020). Shi et al. (2020) verified that mmu_circ_0000250-modified ASCs derived exosomes promoted wound healing in diabetic mice by inducing miR-128-3p/SIRT1-mediated autophagy.

Compared to the single factor therapy, MSCs application is also superior in scar removal due to the MSCs-secreted various inflammatory modulators. In the rabbit scar model, Liu et al. (2014) locally injected BMSCs to regulate inflammation and prevented hypertrophic scar formation, attributing to BMSCs secretion of an anti-inflammatory protein, TNF-α-stimulated gene/protein 6 (TSG-6). Wu et al. (2015) showed that *in vitro*

assay, the MSC-CM decreased viability, α-SMA expression, and collagen secretion of human keloid fibroblasts. Besides, in a mouse dermal fibrosis model, MSC-CM infusion induced a significant decrease in skin fibrosis due to the TGF-β3 in CM-mediated therapeutic effects on preventing collagen accumulation (Wu et al., 2015). The application of ASCs and ASC-derivatives might also provide novel scarless repair methods. In the early stage of wound healing, exogenous ASC-exos promoted the expression of type I and type III collagen to shorten the healing time, and might inhibit collagen synthesis to minimize scar formation in the later period (Hu et al., 2016). This tendency followed the histological changes observed during the natural healing of soft tissue wounds. That is, collagen deposition was more important in the early phase of healing, while in the late phase of healing, matrix reconstruction was more critical. ASC-exos ameliorated ECM reconstruction and reduced the scar formation by regulating the ratios of type III collagen/type I collagen, TGF-β3/TGF-β1, and MMP-3/tissue inhibitor of metalloproteinases 1 (TIMP-1), as well as facilitating HDFs differentiation (Wang et al., 2017). The team of Wang et al. firstly developed the FHE hydrogel and FEP hydrogel scaffold both with stimuli-responsive ASC-exos. The ASC-exos released by these two carrier materials significantly increased the regeneration of skin appendages and reduced the formation of scar tissue (Wang C. et al., 2019) (Wang M. et al., 2019). The sustained release of bioactive exosomes helps to achieve better wound healing and scar removal.

ASC-EXOS IN SKIN FLAPS INJURY

Flap transplantation is an essential method to repair refractory trauma and organ reconstruction, including the alar rim, external ear, and fingertip defects. The insufficient neovascularization and ischemia-reperfusion (I/R) injury are responsible for poor flap healing outcomes (Sorkin et al., 2020). ASC-based therapy has become an applicable method to prevent I/R injury for assisting flap transplantation. For instance, ASCs are able to enhance angiogenesis to increase the viability of chondrocutaneous composite grafts, for the application of defects in the nose, ear scales, and skin (Yucel et al., 2016).

Recent studies have shown that ASC-exos were hopeful to improve the survival status of skin flaps. By bioinformatics analysis, Xiong et al. pointed out that the miRNA-760 upregulation and miRNA-423-3p downregulation in ASC-exos could regulate the expression of ITGA5 and HDAC5 genes, respectively, consequently promoted the vascularization of the skin flap. In a leg wound model of rat, the microvascular angiography of 28 days post-flap transplantation revealed that the ASC-exos treated groups exhibited better vascularization degrees of the artificial dermis prefabricated flaps over control groups (Xiong et al., 2020). Undeniably, many differentially expressed miRNAs in ASC-exos are associated with the vascularization of flaps. The study by Pu et al. (2017) showed that IL-6-rich human ASC-exos promoted flap angiogenesis and flap repair after I/R injury in mice. In this case, the employment of ASC-exos to deliver IL-6 is beneficial for patient safety because it does not require the use of viral vectors. Bai et al. (2018) also showed that low-dose H_2O_2-stimulated ASC-exos could increase the neovascularization of the flap and relieve the inflammation and apoptosis after I/R injury, thus increasing the survival rate of the flap *in vivo*. In summary, ASC-exos play an important role in the vascularization of skin flaps, and thereby resolve the problem of insufficient neovascularization of the flaps, thus expanding the application of flap transplantation (**Figure 1D**).

ASC-EXOS IN BONE TISSUE DAMAGE

Fractures, tumor bone surgery, deformity, revision of the prosthesis, and osteomyelitis can be fully identified as segmental loss of bone structure. Particularly, bone regeneration is the main emphasis involving both surgery and aesthetics in craniofacial surgery. Exosomes, as nanoscale extracellular vesicles with an intercellular communication function, provide an excellent medium for the packaging and transportation of RNAs and proteins, benefiting for broad application in bone tissue engineering (Paduano et al., 2017). Besides, ASC-exos, with significant osteogenic induction ability, can effectively regulate the microenvironment of bone tissue by transporting a variety of bioactive molecules.

The bone remodeling cycle is composed of consequential phases: resorption, reversal, and formation. Studies have found that osteocytes in bone tissues equip with many functions of coordinating the bone remodeling of osteoclasts and osteoblasts, which maintain the bone homeostasis (Borciani et al., 2020). An *in vitro* study confirmed that ASC-exos could antagonize osteocyte apoptosis triggered by ischemia and hypoxia, and decrease osteocyte-mediated osteoclastogenesis, which was attributed to the decrease in receptor activator of nuclear factor kappa b ligand (RANKL) expression (Ren et al., 2019). RANKL interacts with its receptor RANK, which is highly expressed by osteoclasts or their precursors and is essential for osteoclast activation (**Figure 2A**). ASC-exos can be used in tissue engineering combined with efficiently biocompatible efficient carriers to improve osteogenesis efficiency. According to a recent study, ASC-exos could be immobilized on the polydopamine-coating PLGA scaffolds. This cell-free nano-sized carrier enhanced bone regeneration significantly, at least partially through its osteoinductive effects and capacities of promoting MSCs migration and homing in the newly formed bone tissue (Li W. et al., 2018). It was definitely established that an ideal scaffold for exosomes loading would be biocompatible, biodegradable, and capable of controlled releasing exosomes. Chen S. et al. (2019) showed that the exosomes derived from miR-375-overexpressing ASCs incorporated with hydrogel possessed the ability to enhance bone regeneration in a rat model of calvarial defect. More effective and convenient loading strategies should be developed. Furthermore, appropriate changes in the culture conditions of ASCs will facilitate the production of customized ASC-exos. By using hypoxia/serum deprivation (H/SD) induced osteocyte apoptosis model with murine long bone osteocyte Y4 (MLO-Y4), Zhu et al. (2017) demonstrated

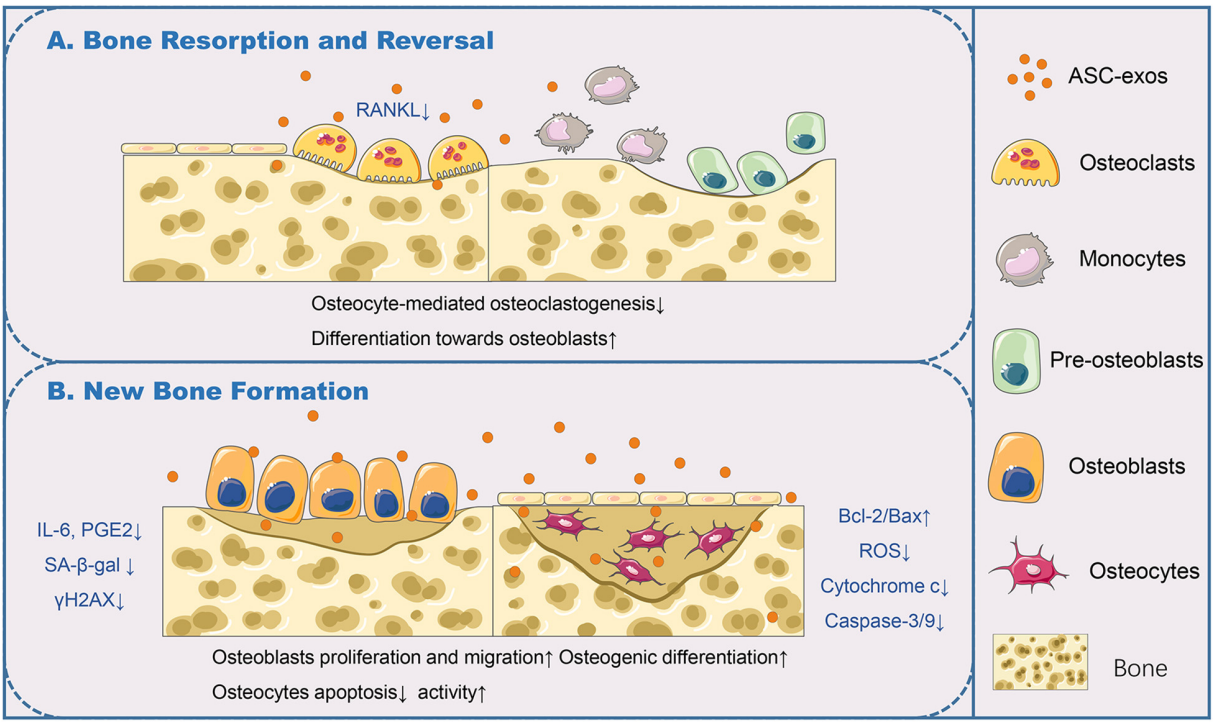

FIGURE 2 | ASC-exos function in the bone remodeling cycle. **(A)** In bone resorption and reversal, ASC-exos could decrease the expression of RANKL, which was highly expressed by osteoclasts or their precursors for osteoclast activation, to antagonize osteocyte-mediated osteoclastogenesis. **(B)** In bone formation, ASC-exos possessed the ability of lowering the production of IL-6 and PGE2, downregulating SA-β-gal activity and reducing the accumulation of γH2AX in osteoblasts. Additionally, ASC-exos could upregulate the radio of Bcl-2/Bax, diminish the production of ROS and cytochrome c, and subsequent activation of caspase-3/9 in osteocytes. ASCs, Adipose-derived stem cells; ASC-exos, ASC-derived exosomes; RANKL, Receptor Activator of Nuclear Factor Kappa B Ligand; IL-6, Interleukin 6; PGE2, Prostaglandin E2; SA-β-gal, Senescence-Associated β-galactosidase; γH2AX, Phosphorylated Histone H2AX; Bcl-2, B-cell lymphoma/leukemia 2; Bax, Bcl-2-associated X protein; Caspase-3/9, Aspartate Proteolytic Enzyme 3/9.

ASC-exos could efficiently antagonize osteocyte apoptosis and osteocyte-mediated osteoclastogenesis, via upregulated radio of B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax), diminished production of ROS and cytochrome c, and subsequent activation of aspartate proteolytic enzyme 9 (caspase-9) and caspase-3. This result also provided the *in vitro* evidence of ASC-exos application in age-related bone disease. Lu et al. (2017) demonstrated that ASC-exos, especially primed by TNF-α pre-conditioned ASCs, could promote the proliferation and differentiation of human osteoblasts through Wnt signaling pathway. Therefore, the methods for producing specific ASC-exos, offer a promising approach to replace direct stem cell transplantation, further widening the application of exosomes in bone regeneration (**Figure 2B**).

Osteoarthritis (OA) is a common degenerative joint disease characterized by cartilage degeneration, synovitis, subchondral bone sclerosis, and osteophyte formation (Henrotin et al., 2012). Current treatments are basically symptomatic to handle pain and swelling, and mainly rely on antalgics and anti-inflammatory drugs. Articular cartilage has a limited potential to repair, with progressively more clinicians emphasizing cellular therapy. Multiple adipose tissue-associated components and extractions are promising to be applied in OA therapy, including SVF, ASCs, ASCs-exos, ASC-CM, and microfragmented adipose tissue (MFAT). In a clinical trial, Spasovski pointed out that the OA patients treated with single injection of ASCs showed significant cartilage restoration (Spasovski et al., 2018). Similarly, in another trial of OA patients received with ASCs, the ASCs might cause an immediate local response due to released paracrine factors and cytokines for OA amelioration (Pers et al., 2018). Tran et al. (2019) employed SVF to regenerate damaged knee cartilage of OA patients, revealing a trend toward a better efficacy of SVF with the microfracture method for OA treatment over a period of two years. Hong Z. et al. (2019) also suggested that intra-articular SVF injection was a safe treatment of OA, and could effectively relieve pain, improve function, and repair cartilage defects in patients with bilateral symptomatic knee osteoarthritis. MFAT could reduce the phase of cell manipulation without expansion or enzymatic treatment in a short period (Paolella et al., 2019). In an inflammatory cell model of OA synoviocytes, MFAT reduced typical macrophages markers and its potentiality to induce an anti−inflammatory effect to address OA (Mautner et al., 2019).

As the cytoprotective and anti-inflammatory properties of ASCs in human chondrocytes and experimental OA may be mediated by paracrine effects, the paracrine mediators ASC-exos are attractive for alternative therapies of OA. ASC-exos might be safer, cheaper, and more effective OA therapy (Pers et al., 2015). ASC-exos are able to downregulate inflammation and oxidative

stress, which might successfully mediate antisenescence in OA (Wang Q. et al., 2020). The intra-articular injection of ASC-exos could inhibit cartilage and subchondral bone degradation, decrease osteophyte formation, and anti-synovial inflammation, thus slowing the progression of OA (Zhang R. et al., 2019). Tofiño-Vian et al. (2017) showed that both ASC-CM and ASC-exos lowered the production of IL-1β-stimulated inflammatory mediators IL-6 and prostaglandin E2 (PGE2), down-regulated senescence-associated β-galactosidase (SA-β-gal) activity, and reduced the accumulation of phosphorylated histone H2AX (γH2AX), in OA osteoblasts. Furthermore, in the next study, they confirmed that microvesicles and exosomes secreted from ASCs could affect the metabolism of OA chondrocytes by modulating inflammatory and degradative pathways associated with joint destruction (Tofiño-Vian et al., 2018). Zhao et al. (2020) separated the patient ASC-exos, which exerted a strong stimulatory effect on chondrocyte migration and proliferation with the upregulation of miR-145 and miR-221 in the model of the inflammation-inflicted oxidative stress. Woo et al. also confirmed that human ASC-EVs could potentially protect cartilage from degeneration and could delay cartilage degeneration in OA rat and mouse models. The mechanism was probably that human ASC-EVs suppressed IL-1β up-regulated catabolic molecules and enhanced type II collagen expression in human OA chondrocyte (Woo et al., 2020). Ragni established an *in vitro* model of human fibroblast-like synoviocytes (FLSs) from OA patients, showing that ASC-EVs possessed the immunoregulatory properties for OA regulation and that hyaluronan was involved in ASC-EVs internalization in FLSs (Ragni et al., 2019).

These pioneering results reinforced the great prospects for ASC-exos and ASC-EVs as a novel therapeutic option for OA. However, the number of studies is small. In the context of OA, although the ASCs and SVF have been confirmed their clinically therapeutic efficacy and safety, the ASC-exos therapies have not yet been used in clinical trials. There still needs to execute a detailed exploration in large cohorts to investigate that the functions and mechanisms of ASC-exos are necessary. In pre-clinical studies, the optimized conditions and obtainments for ASC-exos *in vitro* and the mechanisms of ASC-exos *in vivo* require further studies. In clinical trials, the ASC-exos based therapy should set optimal criteria, including exosome concentration and dose, injection times and intervals. In addition, the comprehensive immune impact following the ASC-exos administration should be performed to determine the immune response of the recipient. Totally, ASC-exos may represent the effective clinical strategy of OA once trials have been fully controlled and their benefits and safety have been fully assessed.

ASC-EXOS IN OBESITY

Obesity is a growing health pandemic whose global prevalence has increased dramatically over the last few decades. In addition to bringing the physical changes, obesity also causes considerable obesity-related inflammation and metabolic disorders, including dysfunction of adipose tissue and insulin resistance in key metabolic organs and insufficient secretion of insulin by the pancreas (Zhang B. et al., 2019). Apart from the energy storage function, the adipose tissue is also an important endocrine tissue and a great source of ASC-exos. ASCs play critical roles in controlling obesity-associated inflammation and metabolic disorders. Thus, the secretion quantity and function of ASC-exos are hypothetically shaped by obesity. Zhao et al. (2018) found that ASC-exos could transfer into macrophages to induce anti-inflammatory M2 phenotypes through the transactivation of arginase 1 (Arg-1) and IL-10 by exosome-carried active STAT3, and increase the expression of uncoupling protein 1 (UCP-1) to promote white adipose tissue (WAT) beiging, thereby improving obesity-related inflammation and metabolism (**Figure 3**). Mechanistically, this study delineated a novel exosome-mediated ASC-macrophage cross-talk that facilitated immune and metabolic homeostasis in WAT, thus providing a potential therapy for obesity and diabetes.

Extracellular vesicles belong to a heterogeneity system, including exosomes, apoptotic bodies, microvesicles (Perez-Hernandez et al., 2017). It is worth noticing on ASC-EVs peculiarity in different physiological or pathological contexts. The concrete use of ASC-EVs in cell-based therapy in the obese setting should be taken into account. Some studies suggested that obesity or diabetes could impair the capacity of ASCs for anti-inflammation and wound healing, as well as influence the production and bioactivities of ASCs-exos, thus increasing the risk for immune or metabolic disorders (Strong et al., 2016). Togliatto et al. (2016) investigated the therapeutic impact of ASC-EVs recovered from obese subjects visceral and subcutaneous tissues. Compared with ASC-EVs from non-obese subjects, ASC-EVs from obese subjects showed impaired angiogenic potential *in vitro* because of the decrease of EVs cargoes including VEGF, MMP-2, and miRNA-126 (Togliatto et al., 2016). Obesity impacts on ASC-EVs and ASC-exos pro-angiogenic potential and may raise more concerns about these crucial tissue repair mediators.

ASC-EXOS IN FAT GRAFTING

Fat grafting has been gaining large attention in tissue augmentation over the past decades for hemifacial atrophy, lipodystrophy, and breast reconstruction (Tan and Loh, 2017). Both ASCs and fat graft can exert a wrinkle-reducing effect and synergistically affect collagen synthesis and neovascularization (Kim et al., 2019). However, the survival rate of fat grafts remains unsatisfied due to the devascularization and ischemic injury of adipose tissues made by liposuction, injection, and long-term fat absorption. Several technical modifications have been described to enhance fat graft survival with more complete adipose tissue structure. Cell-assisted lipotransfer (CAL) is an efficient technique that mixes ASCs-rich SVF with lipoaspirate, for reinforcing adipogenesis and angiogenesis to augment fat graft reliability (Yoshimura et al., 2008). Importantly, Kølle et al. (2020) conducted a randomized controlled clinical trial comparing fat grafts enriched with *ex vivo*-expanded autologous ASCs to non-enriched fat grafts in breast augmentation,

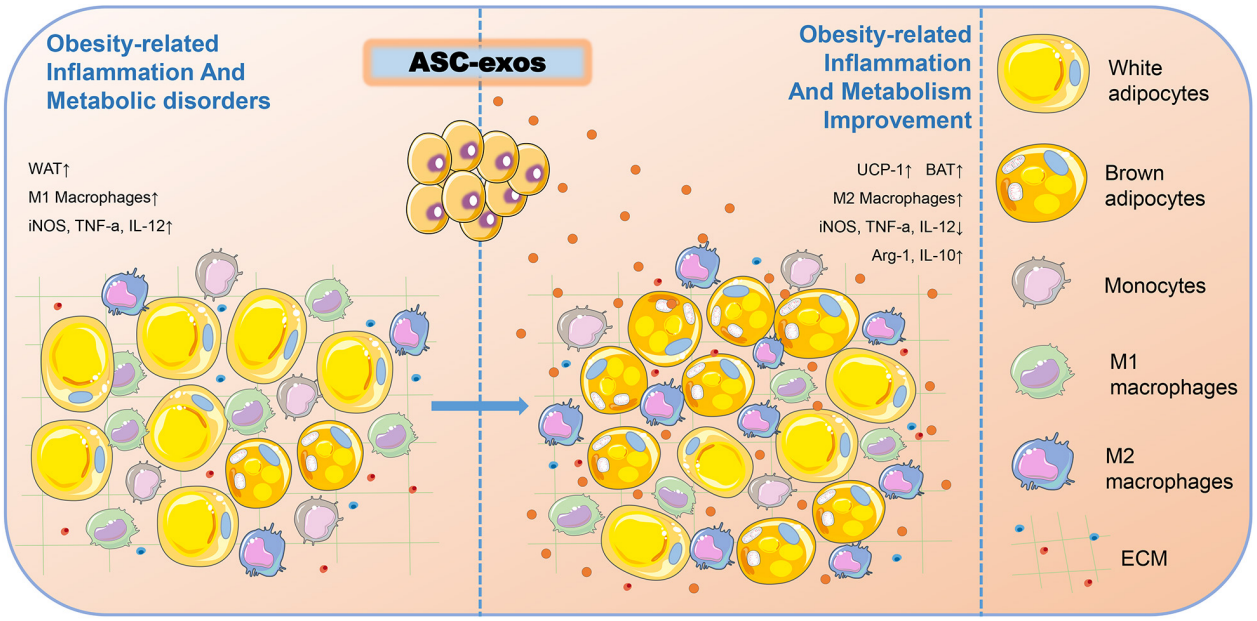

FIGURE 3 | ASC-exos could improve obesity-related inflammation and metabolism. In the obesity microenvironment of mice, ASC-exos was able to reduce the secretion of iNOS, TNF-a, and IL-12, and increase the secretion of Arg-1 and IL-10 to activate M2 macrophage polarization, thus ameliorating WAT inflammation. ASC-exos also increased the expression of UCP-1 to promote WAT beiging, thereby improving obesity-related inflammation and metabolism. ASCs, Adipose-derived stem cells; ASC-exos, ASC-derived exosomes; WAT, White Adipose Tissue; BAT, Brown Adipose Tissue; iNOS, Inducible Nitric Oxide Synthase; TNF-α, Tumor Necrosis Factor Alpha; IL-12, Interleukin 12; Arg-1, Arginase 1; IL-10, Interleukin 10; UCP-1, Uncoupling Protein 1.

demonstrating that ASCs significantly improved the volume retention of fat grafts compared with conventional fat grafting and no adverse effects were observed. This result further confirmed the significance of CAL in both reconstructive and cosmetic volume restoration. However, limitations of cell-based therapies have constrained their use, including uncommitted differentiation, unwanted side effects, immune rejection, and regulatory hurdles (O'Halloran et al., 2018). ASC-exos have been identified to motivate functional recovery in fat grafting and filling.

Recently, Han et al. (2018) discovered that co-transplantation of ASC-exos and hypoxia-treated ASC-exos in nude mouse models of subcutaneous fat grafting both could participate in neovascularization and attenuate inflammation in the grafts. In the subsequent study, they further investigated the molecular mechanism of hypoxia-enhancing promoting the effect of ASC-exos in fat grafting, raising that the hypoxia-treated ASC-exos significantly enhance the angiogenesis of the ischemic adipose tissue by regulating VEGF/VEGF-R signaling pathway (Han et al., 2019). As the functional nanovesicles secreted by ASCs, ASC-exos possess more advantages in improving the volume retention rate of fat grafts than ASCs. Chen B. et al. (2019) have shown that ASCs-exos were equivalent to ASCs in improving the survival of fat grafts by up-regulating early inflammation and enhancing angiogenesis in mice. Whereas during the mid to late stages of fat grafting, ASC-exos exerted a pro-adipogenic effect and also increased collagen synthesis level, similarly, to their source of ASCs (Chen B. et al., 2019). Zhu et al. (2020) found that in an *in vivo* mouse model of autologous fat grafting, grafts treated by ASC-EVs significantly exhibited survival with more neovascularization, increased fat retention, and decreased fibrosis and necrosis. The ASC-EVs uptake by macrophages promoted M2 type polarization and catecholamine secretion, thus the M2 macrophages-CM could enhance browning adipose differentiation with enhanced energy expenditure (Zhu et al., 2020). These results suggest that, as a cell-free strategy, ASC-exos could be an effective and appealing path to heighten graft survival in lipotransfer (**Figure 4**). However, a major drawback of fat grafting is the unpredictability of the clinical outcome since high volume absorption rates are common. It is not yet clear how long this effect lasts or whether ASC-exos from both healthy individuals and diseased patients are equally effective. Further experimental and clinical studies are required to determine the optimal concentration and source of ASC-exos enrichment required to improve fat graft without side effects.

ASC-EXOS IN BREAST CANCER AND BREAST RECONSTRUCTION

Adipocytes are the abundant cellular components in the breast cancer microenvironment. The invasion of breast cancer cells leads to the proximity of cancer cells and adipocytes, which have been referred to as cancer-associated adipocytes (CAAs) (Choi et al., 2018; De Lope et al., 2018). CAAs exhibit a fibroblastic-like morphology and overexpressed ECM proteins. CAAs can interact with cancer cells via several ways to trigger breast cancer initiation, metastasis, angiogenesis, and cachexia. Studies have

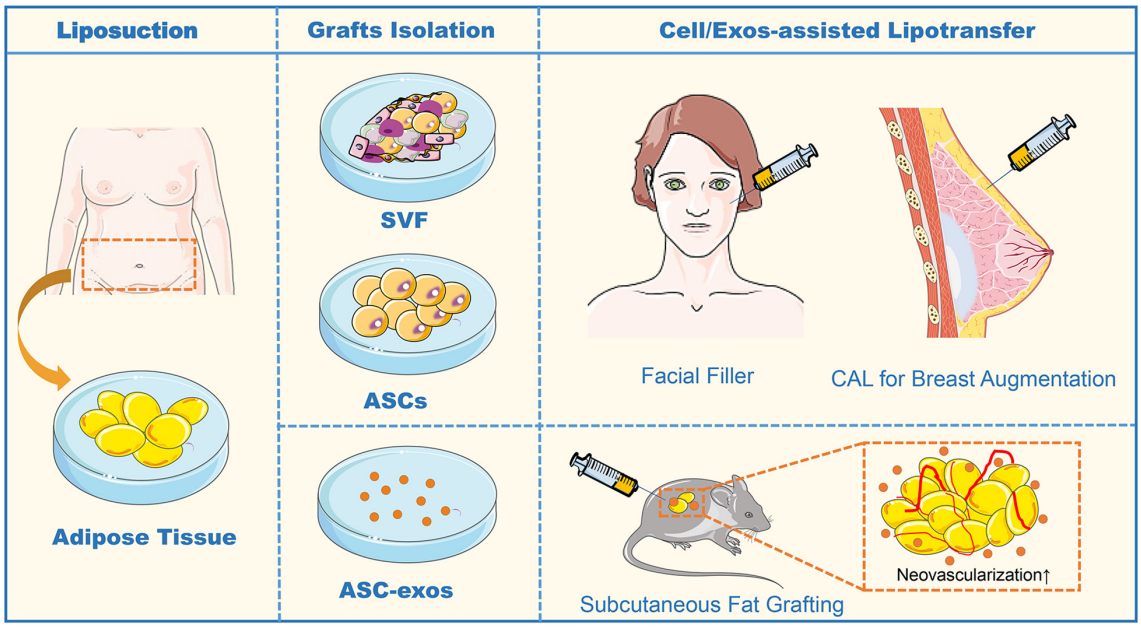

FIGURE 4 | The sequence flow diagram of fat grafting. In the clinical application of fat grafting for facial filler and breast augmentation, sterile adipose tissue is collected through liposuction. After enzyme digestion and centrifugation of the collected adipose tissue, the obtained heterogeneous mixture of endothelial cells, smooth muscle cells, fibroblast, pericytes, mast cells, and preadipocytes is named SVF. In CAL, half the volume of the aspirated fat is processed for isolation of the SVF containing ASCs, and the other half of the aspirated fat is prepared for grafting. Finally, the SVF-supplemented fat is injected into the target sites of grafting. In animal studies of the subcutaneous fat grafting, co-transplantation of ASC-exos with adipocytes can effectively promote the neovascularization to enhance survival in the fat grafts. ASCs, Adipose-derived stem cells; ASC-exos, ASC-derived exosomes; SVF, Stromal Vascular Fraction; CAL, Cell-Assisted Lipotransfer, CAL.

demonstrated the survival rate, growth, and invasiveness of tumors after interacting with ASCs, which are CAAs components. By excreting a variety of bioactive factors including visfatin, adipsin, CCL5, IGF, HGF, VEGF, IL-8, and TGF-β, ASCs can exert biological influences on proliferation, epithelial-mesenchymal transition, and metastasis (Huang et al., 2019; Goto et al., 2019). ASCs could differentiate into cancer-associated fibroblasts in breast cancer environment, especially with a more pronounced effect on obese patients (Cho et al., 2012; Ecker et al., 2019).

The exosomes, released from the adipocytes and ASCs, are emerging as a new type in the crosstalk between breast tumors and adipocytes (Sauter et al., 2019; Teufelsbauer et al., 2019). Previously, Lin et al. (2013) investigated the effect of ASC-exos on breast cancer MCF7 cells, showing that ASC-exos activated the Wnt and Hh signaling pathways to strengthen tumor cell migration. On the other hand, the breast cancer-derived exosomes could reciprocally shape the function of adipocytes. A recent study confirmed that miRNA-144 and miRNA-126-secreting exosomes from breast cancer cells co-cultured with adipocytes could promote cancer metastasis by inducing beige/brown differentiation and reprogramming the metabolism in surrounding adipocytes (Wu et al., 2019). Interestingly, Lee et al. (2019) emphasized the effect of cancer stem cells (CSCs) treated with exosomes derived from osteogenic differentiating human ASCs. The results showed that the expression of drug-resistance genes (ATP binding cassette transporter), the breast cancer gene family (BCRA1 and BCRA2), and the ErbB gene family were all significantly decreased in CSCs (Lee et al., 2019). The osteoinductive ASCs-exos could be a biochemical cue for CSCs reprogramming into non-tumorigenic cells and contribute to overcoming therapeutic resistance. He et al. (2018) noted that MSC-CM could also suppress the growth of breast cancer cells by inhibiting the STAT3 signaling pathway and MSC-CM combined with radiotherapy significantly delayed xenograft tumor growth in mice.

For patients with breast cancer, breast reconstruction requires the unification of tumor safety and aesthetics. Fat grafting using mammaplasty might complicate breast imaging and breast cancer surveillance due to the varying levels of nodule formation and calcifications in breast tissue (Bayram et al., 2019). The ASCs assisted fat transplantation is a common technique for CAL which makes great improvements in the survival rate of transplanted fat with less fat resorption and necrosis for the favorable aesthetic outcome of breast augmentation. Nevertheless, tumor safety in breast reconstruction is the primary consideration (O'Halloran et al., 2017). Silva et al. (2019) suggested that fat transplantation did not promote tumor proliferation and metastasis in mouse models of residual breast cancer. Krastev et al. carried out a long-term follow-up trail of 587 breast cancer patients who underwent autologous fat transplantation and traditional breast reconstruction, respectively. The results showed that after 5 years the local recurrence rate of breast cancer had no significant difference in these two methods (Krastev et al., 2019). Similarly, Gentile et al. (2019a) also conducted a 3-year follow-up of 121 patients with breast cancer who underwent

adipose stromal cell-enhanced engineering fat transplantation, which also showed satisfactory safety and effectiveness. Thus, these clinical studies using ASCs report safety data in breast reconstruction.

To some extent, exosomes originate from the tumor-adipocytes interaction in a complex metabolic network favoring malignant progression. However, at this stage, there are limited studies on ASC-exos used for breast reconstruction. In clinical trials and basic experiments, it is worth noting that the role of ASC-exos in breast reconstruction after breast cancer surgery is not entirely clear. To avoid the tumorigenic potential risk, the mechanisms of ASCs and ASCs-exos in breast cancer and breast construction remain to be carefully elucidated. Additionally, it is important to conduct randomized trials for illuminating the safety and efficacy of transplanted ASCs or ASC-exos, in comparison to commonly applying with conventional techniques.

CONCLUSION AND PERSPECTIVE

Adipose-derived stem cells are stem cell populations within the adipose stromal compartment that have multiple differentiation potentials, easy acquisition, and the high yield, making ASCs attractive for tissue engineering and cell therapy as an ideal stem cell source. ASC-exos, containing important paracrine mediators, have received much attention recently for functioning in intercellular communication. As cell-to-cell messengers, ASC-exos are valuable supplement with the regenerative and reconstructive strategies, and is particularly successful and safely applied to chronic wounds, scars, bone injuries, and cosmetic surgery. Both ASCs and ASC-exos possess huge application potential on the tissue regenerative field in plastic and cosmetic surgery.

In terms of source and production, ASCs boast the benefits that the stem cell yield from fat is much greater than that obtained from other sources. ASC-exos possess advantages of huge sources and high availability, indicating that ASC-exos can be an alternative when exosomes from other sources have difficulties to extract or are not suitable for therapy (Ha et al., 2020). ASC-exos and other MSC-exos have similar characteristics, such as morphology and cell surface markers, but some important biological differences have been found in proliferation, gene expression, differentiation ability, and immunosuppressive pathways. For exosomes from different sources of MSCs, they partly contain similar proteins. Interestingly, Villatoro et al. (2019) showed that canine ASCs had advantages of proliferative capacity, whereas canine BMSCs showed a significantly higher secretory production of some soluble factors. Therefore, when selecting the source of MSCs, those biological differences should be considered for cell implantation or the secretome is directly used for specific clinical applications. In addition, it is obvious that those of MSC-exos vary according to the origin of MSCs. However, comparative studies of MSC-exos by their tissue origin are still limited, and only a few reports have compared different exosomes within the same study. For example, compared with human BMSC-exos, ASC-exos exhibited a higher activity of neprilysin, which was an amyloid β peptide degrading enzyme, suggesting the therapeutic relevance of ASC-exos in Alzheimer disease (Katsuda et al., 2013). ASCs might be safer and more effective than that from BMSCs, including lack of major histocompatibility complex (MHC) class II on ASCs, induction of higher levels of anti-inflammatory macrophages and pro-lymphangiogenic activity (Maguire, 2019).

Furthermore, compared with ASCs, ASC-exos offer distinct advantages that uniquely position them as highly effective bioactive constituents. The proposed several main reasons are as follows based on previous reports: (1) Biosafety: exosomes, including ASCs-exos, are naturally occurring secreted membrane vesicles from the releasing cells with lower immunogenicity, posing the favorable biosafety of ASC-exos. (2) Biological activity: although without similar self-differentiation function as ASCs, ASCs-exos contain a broad repertoire of cargoes, including nucleic acid, proteins, and enzymes for modulating multiple cellular activities, acting in both immediate and remote areas in a paracrine manner. (3) Stability: the ASC-exos are comprised of naturally bimolecular phospholipid structure, providing sufficient stability to avoid biodegradation. Thus, ASC-exos are very well tolerated in biological fluids along with the ubiquitous presence. (4) Carrier features: ASCs-exos function as a carrier for itself, also can be used as a component uploaded in well-designed biomedical materials. Due to the intrinsic homing capacity or artificially modified targeting ability, ASC-exos can serve as stable and effective carriers to load specific proteins, lipids, and genetic material, and preferentially transport it to targeted tissues or organs. Exosome-based delivery systems may be of precedence in the treatment of diseases attributing to their endogenous origin, which minimizes the immunogenicity and toxicity and exerts the optimal effect. The development of multifunctional bioactive biomaterials with long-term ASC-exosomes release is also very important to synergistically enhance tissue regeneration and therapy. With the above desirable properties, ASC-exos hold clinically promising potential in the novel cell-free therapeutic strategies. Therefore, as mentioned in this review, ASC-exos are expectantly recognized as new candidates for the skin anti-aging therapy, skin inflammation treatment, wound and scar repair, flap grafting, bone tissue repair, obesity prevention, fat transplantation, breast cancer, and breast reconstruction (**Table 2**). Collectively, these findings reinforce the significance of ASC-exos-participated cell communication and applications in plastic and cosmetic surgery. Though lacking adequate application in clinical practice, ASC-exos are playing an increasingly greater role especially in maximizing the therapeutic effect of dermopathic features and tissue reconstruction.

Nevertheless, there are still some challenges in the development of ASC-exos application. Firstly, obtaining ASCs continues to be an inconvenience. The sources of ASCs, as well as the separation and cultivation methods, medium composition and dosage, cell passage, cell fusion and viability, mycoplasma, and other microbial contamination, all should be tightly controlled to maintain reliable biological efficacy and ASC-exos with high quality. Secondly, the extracted ASC-exos might have low purity and yield in the lab. Nowadays ASC-exos separation methods include ultracentrifugation,

TABLE 2 | The mechanisms and functions of ASC-exos in tissue regeneration.

Disease	Source	Model	Function	Mechanism	References
Skin aging	Human ASC-CM	Photoaging-induced HDFs and HaCaTs	Photoaging prevention	Downregulate the activation and transcription of UVB-induced signaling pathways and upregulate the expression of antioxidant response elements	Li et al., 2019
	Human ASC-CM	Photoaging-induced HDFs	Photoaging prevention	PDGF-AA in ASC-CM promoted HDFs proliferation and activated PI3K/Akt signal pathway to facilitate ECM deposition and remodeling	Guo et al., 2020
	Human BMSC-exos	Photoaging-induced HDFs and mice	Photoaging prevention	Produce ROS at a low level, downregulate TNF-α, upregulate TGF-β to increase MMP-1 and procollagen type I expression for collagen synthesis	Hu et al., 2019
Atopic dermatitis	Human ASC-exos	AD model of NC/NGA mice	Dermatitis improvement	Decrease the levels of inflammatory cytokines and reduce the number of eosinophils in the blood, and the infiltration of mast cells, dendritic epidermal cells	Cho et al., 2018
	Human ASC-exos	AD model of SKH-1 mice	Epidermal Barrier Repair	Reduce trans-epidermal water loss and enhance epidermal lamellar bodies and form lamellar layer at the interface of the SC and stratum granulosum.	Shin et al., 2020
Skin wound	Human ASC-exos	Skin lesion model of HaCaTs	HaCaTs viability enhancement	Foster HaCaTs proliferation, migration, and inhibit apoptosis through Wnt/β-catenin signaling pathway	Ma et al., 2019
	Human ASC-exos	Skin lesion model of HaCaTs and HDFs	HaCaTs and HDFs viability enhancement	ASC-exos containing MALAT1 could mediate H2O2-induced wound healing via targeting miR-124 through activating the Wnt/β-catenin pathway	He et al., 2020
	Human ASC-exos	full-thickness skin wound of mice	Wound healing	Promote fibroblasts proliferation and migration and optimize collagen deposition via the PI3K/Akt signaling pathway to accelerate wound healing.	Zhang et al., 2018
	Human ASC-exos	Diabetic foot ulcer of rat	Wound healing	ASC-exos overexpressing-Nrf2 promoted the proliferation and angiogenesis of endothelial cells, and increased the expression of wound growth factor, decreased the levels of inflammation and oxidative stress-related proteins.	Li X. et al., 2018
	Human ASC-exos	Full layer skin wound of mice	Wound healing	ASC-exos overexpressing miRNA-21 enhanced the migration and proliferation of the HaCaTs by increasing the MMP-9 expression through the PI3K/AKT pathway	Yang et al., 2020
	ASC-exos	Skin wound of diabetic mice	Wound healing	mmu_circ_0000250 enhanced the therapeutic effect of ASCs-exosomes to promote wound healing in diabetes by absorption of miR-128-3p and upregulation of SIRT1	Shi et al., 2020
Scar formation	Human ASC-exos	Skin wound of mice	Scar removal	Inhibit collagen expression to reduce scar formation in the late stage of wound healing	Hu et al., 2016
	Human ASC-exos	Skin wound of mice	Scar removal	Regulate the ratios of type III collagen/type I collagen, TGF-β3/TGF-β1, and MMP-3/TIMP-1, as well as facilitating HDFs differentiation	Wang et al., 2017
Skin flap injury	Human ASC-exos	Artificial dermis prefabricated flap and leg wound of rat	Flap vascularization	Upregulation of miRNA-760 and downregulation of miRNA-423-3p in ASC-exos could regulate the expression of ITGA5 and HDAC5 genes, respectively, to promote the vascularization of the skin flap	Xiong et al., 2020
Skin flap I/R injury	Human ASC-exos	Skin flap I/R injury of mice	Flap repair	IL-6 highly contained in ASC-exos could enhance skin flap recovery and angiogenesis after I/R injury	Pu et al., 2017
	Human ASC-exos	Skin flap I/R injury of mice	Flap repair	H_2O_2-treated ASC-exos increased the neovascularization and relieve the inflammation and apoptosis of the flap after I/R injury	Bai et al., 2018
Bone defect	Human ASC-exos	Hypoxic-ischemic osteocyte	Osteogenesis	Ameliorate osteocyte apoptosis and osteocyte-mediated osteoclastogenesis by lowering the expression of RANKL	Ren et al., 2019
	Human ASC-exos	Calvarial defects of rats	Bone formation	ASC-exos overexpressing miRNA-375 were absorbed by hBMSCs, and inhibit the expression of IGFBP3 to exert osteogenic effects	Chen S. et al., 2019
	Human ASC-exos	Human primary osteoblastic cells	Bone formation	TNF-α-preconditioned ASC-exos promoted the proliferation and differentiation of human osteoblasts through the Wnt signaling pathway	Lu et al., 2017
Osteoarthritis	Human ASC-exos	OA model of osteoblasts	Inflammation improvement	Downregulate SA-β-gal activity and the accumulation of γH2AX	Tofiño-Vian et al., 2017
	Human ASC-exos	Chondrocytes stimulated with H_2O_2	Chondrogenesis	Downregulated the pro-inflammatory markers IL-6, NF-κB and TNF-α, while they upregulated the anti-inflammatory cytokine IL-10 when co-cultured with activated synovial fibroblasts, promoted chondrogenesis in periosteal cells and increased collagen type II and β-catenin	Zhao et al., 2020

(Continued)

TABLE 2 | Continued

Disease	Source	Model	Function	Mechanism	References
Obesity	Mouse ASC-exos	Obese mice	Obesity prevention	Activate M2-type macrophage polarization, improve inflammation, and promote the browning of white adipose tissue	Zhao et al., 2018
Fat grafting	Human ASC-exos	Mice	Fat grafts survival promotion	Hypoxia-treated ASC-exos enhanced the angiogenesis by regulating the VEGF/VEGF-R signaling pathway	Han et al., 2019
	Mouse ASC-exos	Mice	Fat grafts survival promotion	Promote angiogenesis and up-regulate early inflammation, exert proadipogenic effect and increase collagen synthesis during the mid to late stages	Chen S. et al., 2019
Breast cancer	Human ASC-exos	Breast cancer MCF-7 cells	Tumor progression	Activate the Wnt and Hh signaling pathways to strengthen the growth of MCF-7 cells	Lin et al., 2013

ASCs, Adipose-derived stem cells; ASC-exos, ASC-derived exosomes; HDFs, Human Dermal Fibroblasts; HaCaTs, Human Keratinocytes; UVB, Ultraviolet B; ASC-CM, ASC-Conditioned Medium; ECM, Extracellular Matrix; H_2O_2, Hydrogen Peroxide; PDGF-AA, Platelet-Derived Growth Factor-AA; ROS, Reactive Oxygen Species; MMP-1/9, Matrix Metalloproteinase 1/9; TNF-α, Tumor Necrosis Factor Alpha; TGF-β, Transforming Growth Factor Beta; IL-4/5/6/13, Interleukin 4/5/6/13; VEGF, Vascular Endothelial Growth Factor; Nrf2, NF-E2-related factor 2; TIMP-1, Tissue Inhibitor of Metalloproteinases-1; I/R, Ischemia-Reperfusion; RANKL, Receptor Activator of Nuclear Factor Kappa B Ligand; SA-β-gal, Senescence-Associated β-galactosidase; γH2AX, Phosphorylated Histone H2AX; Bcl-2, B-cell lymphoma/leukemia 2; Bax, Bcl-2-associated X protein.

exclusion, ultrafiltration, two-aqueous system, immunoaffinity, and polymer precipitation. Although the ultracentrifugation is the most common method for largely separating exosome, but accompanied by the shortcomings of time-consuming, labor-intensive, costly instrumentation, and multiple overnight centrifugation steps. As it is overly idealistic to completely isolate ASC-exos from other components, the efficient, appropriate, and affordable techniques should be thoughtful to acquire exosomes. These technologies still need to achieve equilibrium in improving the yield and purity of ASC-exos.

Thirdly, given that plastic surgery frequently performs liposuction and autologous fat transplantation, plastic surgeons and researchers have unique advantages in obtaining ASCs and ASCs-exos. However, in the application of ASC-exos in tissue regeneration of plastic and cosmetic surgery, there are many aspects worth noting. Emerging studies have reported that ASC-exos can be utilized in plastic and cosmetic surgery, but almost all these studies are limited in cellular and animal assays, without the large scale exploration of clinical trials like ASCs. In addition, exosomes could be absorbed on human skin, but even if in the existing studies, there is still lack of enough reports in skin aging, atopic dermatitis and skin flaps injury, which are important and intractable skin disease. Therefore, the actual clinical prospects of ASC-exos are almost a blank, also meaning that there exist vast space for development. Another important point is that auxiliary therapy of ASC-exos. Clinically, for plastic and cosmetic surgery-related diseases, laser therapy, drug therapy, tissue filling and surgical operation are common treatments and achieve good clinical effects. Nevertheless, to a certain extent, ASC-exos can only be used as a supplementary treatment, rather than a sole therapy in the beginning. Most of the existing studies focus on ASC-exos as the main or sole treatment. Hence, whether ASC-exos possess synergistic effects or inhibitory effects on the above-mentioned common treatments needs further research.

According to the above considerable insights into the ASC-exos applications and limitations, it needs to carry out more comprehensive researches in the following aspects. (1) Quality control. There exists a substantial degree of heterogeneity in dosing regimens in the reported cases. For better outcomes of plastic and cosmetic surgery, ASC-exos utilization details, including the storage conditions, effective doses, concentration, and period of treatment, are all the important points. It is necessary to further explore suitable microenvironmental conditions or genetic engineering techniques to ascertain the efficiency of ASC-exos treatment. (2) Components and functions. The ASC-exos are comprised of multiple bioactive components. The complex multi-component substances in ASC-exos may produce diverse biological characteristics when finally used in practice. The propensity for some controversial effects of ASC-exos is contingent upon the type and state of the host cells, the type and state of the recipient cells, and the interacting microenvironments. For possibly utilizing ASC-exos in clinical application, a deep understanding of ASC-exos and their components is the priority. Identifying the key components and reforming ASC-exos to overexpress these components might maximize the therapeutic effect while reducing the side or off-target effects. In the subsequent studies, ongoing advances in the analysis of the function of ASC-exos will probably unravel the ASC-exos characteristics, allowing deepening the understanding of their role in pathogenesis and regeneration properties. (3) Carrier peculiarity exploration. ASC-exos is of carrier peculiarity due to the intricate structure of exosomes. It means that exosomes are the ideal therapeutic delivery system. ASC-exos are effective tools for cargo transportation of effective therapeutic agents with lower immunogenicity and toxicity. On the other hand, ASC-exos could also be uploaded in the specific nanomaterials or hydrogel materials to promote skin repair. Engineering ASC-exos to be effective and safe requires a comprehensive understanding of their necessary components, including but not limited to membrane stability, architecture, and packaging of the interior components. (4) Large clinical trials. At present, excavations on ASC-exos studies belong to basic research or animal level. These experiments can not clearly and actually reflect the ASC-exos usages and their physiological levels *in vivo*. In fat transplantation, it is of great value to clinically explore whether the exogenous ASC-exos could be used

for cell transplantation with safety and effectiveness. Especially, clinical implementation of any operation must be based on safety. However, the roles of ASC-exos in some diseases remain controversial. The oncological safety of ASC-exos in breast cancer and breast reconstruction is worthy of extreme attention. Moreover, the ASC-exos impacts on the common treatment of plastic and cosmetic surgery are urgent clinical problems. In the end, larger prospective, blinded, randomized clinical trials are in urgent need to further establish the long-term effectiveness, safety and dose of ASC-exos in humans. Collectively, ASC-exos are promising candidates for cell-free therapy strategy and deserve intense investigation to accelerate ASC-exos applications in tissue regeneration of plastic and cosmetic surgery.

AUTHOR CONTRIBUTIONS

MX and QZ performed the literature search and wrote the manuscript. MW and YW conceived the project and revised the manuscript. WH, CZ, WL, and YY edited the manuscript. All authors reviewed the manuscript and approved the final version.

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REVIEW
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Gastric Stem Cells: Physiological and Pathological Perspectives

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Gastric epithelium operates in a hazardous environment that curtails the lifespan of the constituent cells, imposing a requirement for continuous epithelial renewal. Stem cells that reside in the stomach are thus essential for regulating physiological tissue renewal and injury repair because of their self-renewal, high proliferation capacity and multiple differentiation potentials. Recent investigations using lineage tracing models have identified diverse populations of gastric stem cells and even fully differentiated cells that can regain stem cell capacity, so enriching our understanding on the identity and plasticity of gastric stem cells. These cell populations include the Villin promotor, Lgr5$^+$, CCKR2$^+$, Axin2$^+$ and AQP5$^+$ stem cells in the antrum, TFF2 mRNA, Mist1$^+$ cells and Troy$^+$ mature chief cells in the corpus, as well as Sox2, eR1, Lrig1, Bmi1-marked cell in both the antrum and the corpus. Establishment of gastric organoids derived from primary gastric tissues and pluripotent stem cells or embryonic stem cells characterizes niche factors required by the gastric stem cell populations, and further provides new insights into stomach development, host-Helicobacter pylori interactions and malignant transformation. Furthermore, focus on the gastric stem cells and their niches uncovers the initiation of stomach precancerous lesions and origin of gastric cancer, providing options for cancer prevention and intervention. In summary, with the development of stem cell research, gastric stem cells give us more opportunities to prevent and treat stomach diseases.

Keywords: gastric stem cells, gastric mucosa homeostasis, stem cell niche, gastric organoid, Helicobacter pylori, stomach neoplasms

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INTRODUCTION

The stomach epithelium is a hazardous environment that curtails the lifespan of constituent cells, imposing a requirement for continuous renewal of epithelium. Stems cells in our gut are, thus, essential for epithelial regeneration and damage repair owing to their ability for self-renewal, high proliferation capacity and potential for multiple differentiation. Gastric stem cells, a group of adult stem cells residing in the stomach, play a key role in maintaining the dynamic homeostasis of the gastric epithelium (Mills and Shivdasani, 2011). In recent years, advances have been made in the investigation of molecular markers identifying gastric stem cells. In addition, *in vitro* gastric stem cell models have been established, revealing the role of these cells in physiology and pathology. Although some sporadic reviews on this topic have been published in past years (Bartfeld and Koo, 2017; Hata et al., 2018), this present review aim to provide fresh and profound insights into stomach stem cells from physiological and pathological perspectives.

PROPERTIES OF GASTRIC STEM CELLS

Stems cells are a group of cells defined by their ability of self-renewal and multi-potency, which can be divided into embryonic stem cells and adult stem cells in terms of their development stage. Tissue-resident adult stem cells are a small population of adult stem cells, these specialized cells are particular important in the epithelium lining of the alimentary tracts and skin that require constant dynamic replacement of the epithelial population (Barker et al., 2010a). More importantly, given their ability of directional differentiation, tissue-resident stem cells are responsible for tissue homeostasis, injury repair, and even cancer development.

Gastric stem cells represent an adult stem cell population residing in the stomach tissues with high proliferative potential, which enables efficient stomach epithelium regeneration and repair. Following the comprehensive investigation of intestinal stems cells, the identity of gastric stem cells is being explored. In comparison with intestinal stems cells, gastric stem cells share many properties, but they differ in fundamental aspects regarding location, molecular cell markers and their specific growth niches.

IDENTITY OF GASTRIC STEM CELLS

Location of Gastric Stem Cells

The mucosa in all parts of the human stomach is lined by a simple columnar epithelium that has numerous tubular invaginations in its lamina propria. These invaginations, termed gastric units, consists of a pit, isthmus, neck and the base regardless of different anatomical zone, although their cellular composition varies with the region of the stomach in which they are located (Lee et al., 1982; Choi et al., 2014). Five types of differentiated mature cells, namely surface mucus cells, mucus neck cells, parietal cells, chief cells, enteroendocrine cells (including G cells, D cells, and ECL cells) and tuft cells, make up gastric glands. However, the mesenchymal compartment surrounding the glands is less studied and little understood. A schematic diagram depicting the structure and cell type of gastric glands in different regions is presented in **Figure 1**. Under physiological conditions, gastric epithelial cells undergo continuous dynamic renewal within as little as 3 days (Karam and Leblond, 1993b). Consequently, gastric epithelial stem cells are essential for the regeneration of lost or damaged cells in stomach mucosa. An understanding of the location of adult stem cells in the stomach is, therefore, important to explore their function.

Previous studies, using nucleotide incorporation assays and ultrastructural analysis, have demonstrated that the isthmus might be the pool of stem-like cells in an adult stomach (Leblond et al., 1948; Corpron, 1966; Karam and Leblond, 1993a). This group of cells produces descendants that undergo a complex bi-directional migration toward pit and base. However, direct evidence, describing their route of differentiation and migration, remains elusive. Then, Bjerknes and Cheng (2002), for the first time, used transgenic mice expressing a bacterial gene for β-galactosidase (lacZ) under a Rosa26 promotor (for visualization) and random chemical mutagenesis to demonstrate the existence of long-lived committed progenitors or stem cells in gastric epithelium. Cells at the base of the gastric glands were further identified as a second stem cell pool and were capable of self-renewing and differentiating. In addition, the emergence of lineage tracing models has made it possible for us to define the direction of differentiation of stomach stem cells in specific anatomic regions under normal or abnormal conditions.

Gastric Stem Cell Marker Candidates

In early studies, the identity of potential stem cells in the stomach was mainly based on their presumed morphological characteristics and cell proliferation kinetics. With the application of lineage tracing approaches combined with *in situ* hybridization or immunohistostaining, an array of biomarkers is being proposed for characterizing the population of adult stem cells in different anatomic regions of the stomach (**Figure 1**).

In the antral and pyloric glands, several markers are identified to be specific to this zone. Villin promotor is an epithelial cell-specific, calcium-regulated actin-binding protein that modulates reorganization of microvillar actin filaments. Qiao et al. showed that Villin promotor-marked cells represent a highly quiescent stem cell that only becomes apparent upon stimulation by interferon γ (Qiao et al., 2007). This group of stem cells is mainly located in the third part of the glands, with lineage tracing showing that they can differentiate into all types of cells in the antral glands. Lgr5 (leucine-rich repeat-containing G-protein coupled receptor 5) is a widely-accepted marker of stem cells in the gastrointestinal tract (Koo and Clevers, 2014). Baker et al. demonstrated that $Lgr5^+$ cells at the very base of pyloric glands were long-term, self-renewing and multipotent stem cells, responsible for maintaining gastric epithelial homeostasis (Barker et al., 2010b). They could differentiate into surface mucus cells, enteroendocrine cells and parietal cells. Lgr5 expression was also found in a subpopulation of chief cells at the antral base, where these $Lgr5^+$ chief cells functioned as damage-induced stem cells for gland regeneration following injury (Leushacke et al., 2017). In this same zone, a group of cells is $Axin2^+/Lgr5^-$ stem cells that can give rise to $Lgr5^+$ cells (Sigal et al., 2017). They are characterized by a high proliferation rate in the dependence of Wnt agonist-R spondin. Cholecystokinin type-B receptor (CCK2R)-positive cells are another set of stem cells which does not overlap with $Lgr5^+$ cell population at the antral gastric units, and treatment with progastrin interconverts $CCKR2^+$ $Lgr5^{neg\ or\ low}$ antral stem cells into typical antral $Lgr5^{high}$ cells that expand the active stem cells in the antrum (Hayakawa et al., 2015b). More recently, AQP5 expressing populations are identified to overlap with $Lgr5^+$ stem cells at the base of pyloric gland, where this population of stems cells comprises the pyloric lineages expressing Muc5AC, gastric intrinsic factor, gastrin and chromogranin A (Tan et al., 2020).

In contrast to the antral glands, TFF2 (trefoil factor family 2) transcript, Mist1 and Troy (tumor necrosis factor receptor superfamily, member 19) may be as the candidate of the adult stem cell markers in the oxygenic mucosa. Lineage tracing demonstrated that TFF2 mRNA-expressing cells above the neck region are the progenitors for mucus neck, parietal and chief cells but not for pit or ECL cell lineages in the corpus

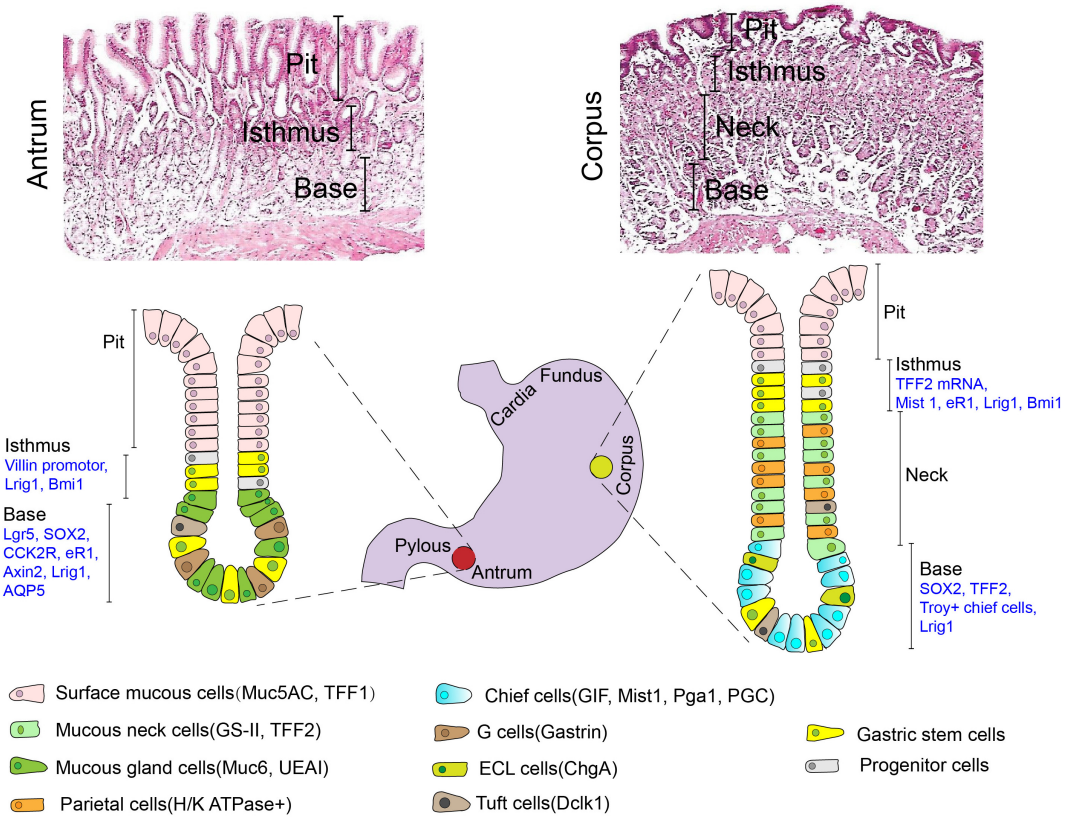

FIGURE 1 | Typical H&E histology and a schematic depicting the structure and cell types (including mature cells with their specific molecular markers and candidate stem cells) of gastric glands in different anatomical regions (Antrum vs. Corpus). Muc5AC mucin 5AC, TFF1 trefoil factor 1, GS-II griffonia simplicifolia II, TFF2 trefoil factor 2, Muc6 mucin 6, UEAI ulex europaeus agglutinin I, GIF gastric intrinsic factor, Pga1 pepsinogen 1, PGC pepsinogen C, ChgA chromogranin A, Dclk1 double-cortin-like kinase 1.

(Quante et al., 2010), while TFF2 protein is mainly expressed and secreted by mucus neck cells and deep antral gland mucus cells under normal condition (Farrell et al., 2002). Within the corpus isthmus, Mist1^{+}/Lgr5^{-} cells are also capable of giving rise to surface mucus cells, mucus neck cells, parietal cells, tuft cells and ECL cells (Hayakawa et al., 2015a; Nienhüser et al., 2020). *In vitro*, Mist1^{+} isthmus cells can form corpus organoids, but Mist1^{+} chief cells remain single (Hayakawa et al., 2015a). This evidence suggests that isthmus Mist1^{+} cells play a critical role in differentiation of corpus glands. In contrast to those with the properties of stemness and self-renewal, a subgroup of fully differentiated cells has been found capable of regaining stem cell capacity. Troy^{+} differentiated chief cells at the base of gastric corpus units are considered reserve quiescent stem cells that display plasticity in that they are capable of re-entering the cell cycle to give rise to all gastric units (Stange et al., 2013). This result provides the evidence that differentiated cells in the stomach also participate in maintaining epithelial renewal and homeostasis through dedifferentiation or transdifferentiation.

Some biomarkers are shared among gastric stem cells in both pyloric and corpus glands. Sox2 (Sex-determining region Y box protein 2)-positive cells are found in both the pylorus and corpus of the glandular stomach in mice, although no apparent overlap exists with Lgr5^{+} cells in the pylorus (Arnold et al., 2011). Cells labeled by eR1 (a Runx1 enhancer element) in the isthmus of corpus and the base of pyloric gland are reported to be involved in tissue regeneration and continuously give rise to mature cells that maintain gastric units (Matsuo et al., 2017). Lrig1 (Leucine-rich repeats and immunoglobulin-like domain 1)-marked cells are known to give rise to daughter cells, and Lrig1-expressing isthmal cells can contribute to the regeneration of parietal cells following acute gastric injury (Choi et al., 2018). Bmi1-expressing cells in the isthmus of gastric antrum and corpus also provide progeny bipolarly toward luminal and basal sides, although it is not clear whether they are colocalized with other reported stem cells (Yoshioka et al., 2019). These candidate markers mentioned above and their relevant studies are concisely summarized in **Table 1**.

Current available studies reveal a varying pattern in locations of gastric stem cells across different anatomical regions, indicating the complicated nature of gastric gland organization (**Table 2**). A recent study using lineage-tracing assays confirmed that gastric corpus gland is compartmentalized, with isthmus and base zones supported by two separate independent groups of stem cell, suggesting their specific molecular identity and functional behavior (Han et al., 2019). Future studies will be

TABLE 1 | Gastric stems cells labeled by candidate markers.

Markers	Location pattern	Differentiation by lineage tracing	Physiological characteristics	Organoid formation
Villin promotor (Qiao et al., 2007)	At or below the isthmus in the bottom third of pyloric glands	Mucus neck cells, mucus gland cells, parietal cells, enteroendocrine cells	Do not contribute to epithelial renewal under normal homeostatic conditions; respond to interferon γ	No
Lgr5 (Barker et al., 2010b; Stange et al., 2013; Leushacke et al., 2017)	Base of pyloric glands, a subpopulation of chief cells	Surface mucous cells, parietal cells, enteroendocrine cells	Maintain epithelial renewal in pyloric regions under normal homeostatic conditions; $Lgr5^+$ adult stem cells originate from fetal $Lgr5^+$ progenitors; neonate $Lgr5^+$ cells contribute to the development of mature gastric epithelium in both pylorus and corpus regions; respond to Wnt agonist R-spondin; Be activated by acetylcholine-producing tuft cells through muscarinic receptor subtype 3	Yes
TFF2 mRNA (Quante et al., 2010)	Isthmus of corpus gland	Mucous neck cells, chief cells, parietal cells	Amplified by DMP-777 (chemicals inducing acute parietal cells loss); not the cell of origin for SPEM	No
Sox2 (Arnold et al., 2011)	Base of pyloric and corpus gland	Surface mucous cells, chief cells, parietal cells, enteroendocrine cells	Half of the $Sox2^+$ cells is quiescent under homeostatic conditions; early $Sox2^+$ fetal progenitor are the precursors for $Sox2^+$ adult stem cells; no overlap with $Lgr5^+$ cells in the pylorus	No
Troy (Stange et al., 2013)	Differentiated chief cells at the base of corpus gland	Surface mucous cells, mucous neck cells, chief cells, parietal cells, enteroendocrine cells	A reserve stem cell population; Wnt-driven stem cells	Yes
Mist1 (Hayakawa et al., 2015a; Nienhüser et al., 2020)	Isthmus of corpus gland	Surface mucous cells, mucous neck cells, parietal cells, enteroendocrine cells, tuft cells	Most $Mist1^+$ stem cells in isthmus are quiescent under normal condition; kras mutation promotes $Mist1^+$ stem cells proliferation and division; give rise to the entire gland in the response to gastric injury caused by acute damage and chronic inflammation	Yes
CCK2R (Hayakawa et al., 2015b)	Above the $Lgr5^+$ cells (+ 4) at the base of antral gland	Surface mucus cells, G cells, D cells, tuft cells	No overlap with $Lgr5^+$ stem cells; more rapid cycling than $Lgr5^+$ cells; $CCK2R^+Lgr5^{negorlow}$ cells can convert to $Lgr5^{high}$ cells following progastrin treatment; gastrin secreted from antral G cells regulates $CCK2R^+$ stem cell function in a paracrine manner	Yes
eR1 (Matsuo et al., 2017)	Isthmus in the corpus gland, base of antral gland	Surface mucous cells, mucous neck cells, chief cells, parietal cells	Maintain the integrity of gastric units; play a role in tissue regeneration following tamoxifen treatment	Yes
Axin2 (Sigal et al., 2017)	Base and lower isthmus of antral gland	Surface mucous cells, mucous neck cells, enteroendocrine cells, tuft cells	Overlap with $Lgr5^+$ stem cells; repopulation time from $Axin2^+$ cells are rapid than Lgr5 + cells; $Axin2^+Lgr5^-$ cells can give rise to $Lgr5^+$ cells; high proliferation rate of $Axin2^+$ cells depend on Wnt agonist R spondin	Yes
Lrig1 (Choi et al., 2018)	Isthmus and base in the corpus and antral gland	Surface mucous cells, mucous neck cells, chief cells, parietal cells, G cells, tuft cells	Early $Lrig1^+$ fetal progenitor are the precursors for $Lrig1^+$ adult stem cells; reconstitutes gastric epithelium after acute oxyntic atrophy Do not give rise to metaplasia lineage	No
Bmi1 (Yoshioka et al., 2019)	Isthmus of pyloric and corpus gland	Surface mucous cells, mucous neck cells, chief cells, enteroendocrine cells, tuft cells	No overlap with $Lgr5^+$ or $eR1^+$ cells; provide progeny bidirectionally toward both the luminal and basal sides in the antrum and corpus; be required for the homeostasis and regeneration of gastric epithelium	Yes
AQP5 (Tan et al., 2020)	Base of pyloric gland	Surface mucous cells, parietal cells, enteroendocrine cells	Overlap with $Lgr5^+$ populations	Yes

Lgr5 leucine-rich repeat-containing G-protein coupled receptor 5, TFF2 trefoil factor 2, Sox2 sex-determining region Y box protein 2, CCK2R cholecystokinin type-B receptor, Lrig1 leucine-rich repeats and immunoglobulin-like domains 1, AQP5 aquaporin 5.

warranted to define the true identity and origin of stem cells in different regions, and unveil their specific roles in gastric mucosa homeostasis.

Gastric Stem Cells and Their Niche

A specific microenvironment is the key determinant to regulate biological behavior of stem cells. The niches consist of stem, stroma, and immune cells, as well as various growth signals, and extracellular matrices. Interactions between these components, therefore, contribute to proliferation of stem cells and directional differentiation. In the setting of gastric stem cell niche (**Figure 3**), little is known because of the complexity of gastric glands in different regions.

Some of the major regulators of gastric stem cell proliferation and differentiation include sonic hedgehog (Shh) and bone morphogenetic proteins (BMPs) signaling pathways. These have been found to exert important effects on gastrointestinal development and differentiation (Lees et al., 2005; Todisco, 2017). In the human stomach, Shh expression is restricted to the parietal cells and the glandular portion (van den Brink et al., 2001), where it controls epithelial cell proliferation and serves as a polarizing signal for fundic gland differentiation

TABLE 2 | Comparison of gastric stem cells in the antrum and corpus.

	Pyloric gland	Corpus gland
Cell location	Isthmus, Gland base	Isthmus, Gland base, certain differentiated cells (chief cells)
Morphology	No evidence	No evidence
Direction of differentiation	Stem cells derived clones expand bidirectionally for isthmus stem cells. For stem cells at the gland base, they migrating upward from the basal zone	Stem cells derived clones expand bidirectionally for isthmus stem cells. For stem cells at the gland base, they migrating upward from the basal zone
Molecular markers	Isthmus: Villin promotor, Lrig1, Bmi1 Gland base: Lgr5, SOX2, CCK2R, eR1, Axin2, AQP5	Isthmus: TFF2 mRNA, Mist1, eR1, Lrig1, Bmi1 Gland base: SOX2, TFF2, Lrig1, Troy$^+$ chief cells
Offspring	Surface mucous cells, Mucous gland cells, G cells, Tuft cells	Surface mucous cells, Mucous neck cells, parietal cells, ECL cells
Known niche factors	Wnt, Notch, Gastrin, Ach, EGF, FGF10	Wnt, BMPs, Shh, innate lymphoid cells, endothelial cells, EGF, FGF10

Lgr5 leucine-rich repeat-containing G-protein coupled receptor 5, TFF2 trefoil factor 2, Sox2 sex-determining region Y box protein 2, CCK2R cholecystokinin type-B receptor, Lrig1 leucine-rich repeats and immunoglobulin-like domains 1, AQP5 aquaporin 5, Ach acetylcholine, EGF epidermal growth factor, FGF10 fibroblast growth factor 10, BMPs bone morphogenetic proteins, Shh sonic hedgehog.

partially through autocrine (van den Brink et al., 2001, 2002). BMPs are regulatory peptides that are mainly secreted from interstitial myofibroblast-like cells (van den Brink et al., 2001; Takabayashi et al., 2014), the isthmus and neck of the glands are the targeted location receiving BMPs-generated signals (Takabayashi et al., 2014). A series studies have indicated that dysregulation of BMP signaling in the stomach can lead to perturbations of normal homeostatic mechanisms of the gastric mucosa, resulting in developing metaplasia, dysplasia and even neoplasia (Todisco, 2017). Notch signaling is another important signal in this microenvironment (Kim and Shivdasani, 2011). Activation of this pathway directly regulates proliferation of Lgr5$^+$ antral stem cells via NOTCH1 and NOTCH2 receptors, but results in a decrease in antral cellular differentiation (Demitrack et al., 2017; Gifford et al., 2017). In the gastric antrum, therefore, Notch might affect the balance between stem cell proliferation and differentiation and maintain homeostasis of gastric epithelium. Thus, abnormal activation of Notch signals can promote the transformation of gastric stem cells to cancer stem cells (Demitrack and Samuelson, 2016). Additionally, growth factors, such as Wnt, fibroblast growth factor 10 (FGF10) (Lv et al., 2019), and epidermal growth factor (EGF), also play an important role in regulation of self-renewal of gastric stem cells. The Wnt signaling pathway is essential in maintaining the undifferentiated state of progenitor cells in the isthmus region of the gastric corpus, but its activation increases the number of progenitor cells in antrum (Oshima et al., 2006). Antral Lgr5$^+$ stem cells are supported and activated by Wnt signaling via at least in part a Frizzled-7 receptor (Flanagan et al., 2017), corpus Mist1$^+$ isthmus stem cells can also be activated in part through Wnt 5a pathway (Nienhüser et al., 2020). Wnt pathway activation in gastric stem cells have reported to be associated with gastric carcinogenesis in mice (Oshima et al., 2006; Radulescu et al., 2013).

Though the mesenchymal compartment surrounding the glands is less studied and little understood, immune or stroma cells residing in gastric epithelium also provide an additional environment for gastric stem cells. Cxcl12$^+$ endothelial cells and Cxcr4$^+$ innate lymphoid cells contributed to the corpus stem cell niche partly through production of Wnt5a from innate lymphoid cells (Hayakawa et al., 2015a). Furthermore, as described above, R-spondin 3 secreted from myofibroblasts is an important component for the antral stem cell niches, and thus predominantly activating Axin2$^+$/Lgr5$^-$ stem cells (Sigal et al., 2017). Gastrointestinal hormones, such as gastrin and acetylcholine (Ach), may also play unique roles in the antral stem cell niche. Gastrin is secreted from G cells residing near the antral isthmus region, and CCK2R is the receptor of gastrin and its precursor form-progastrin under normal condition. As aforementioned, CCK2R$^+$ stem cells have been discovered in this same zone, and progastrin can stimulate the proliferation of CCK2R-expressing stem cells but gastrin dose not (Hayakawa et al., 2015b). Ach generated from tuft cells in gastric epithelium regulates gastric epithelial proliferation and regeneration, as well as the clonal expansion of Lgr5$^+$ stem cells via the muscarinic receptor subtype 3 (M3R) (Zhao et al., 2014; Hayakawa et al., 2017b).

Despite the importance of these growth factors and cell components in gastric stem cell niches, the identity of cells providing this niche factors and their interactions remain elusive. Meanwhile, knowledge about the niches surrounding the base of corpus gland is also not clear. Further efforts are needed to characterize the various components involved in the gastric stem cells niche and thus in gastric stem cell biology, in order to elucidate their roles in the regulation of gastric epithelium homeostasis.

IN VITRO MODELS OF GASTRIC STEM CELLS

Regarding the importance of these niche factors, *in vitro* reconstitution of this specific niche has led to the development of gastric stem cell culture. Some simplified techniques have been developed to build *in vitro* models to investigate stomach stem cells. For instance, Yang et al. built a gastric epithelial cell clone (named KMU-GI2) with sustained growth in a low-calcium medium supplemented with N-acetyl-L-cysteine and L-ascorbic acid 2-phosphate (Yang et al., 2007). These clones were characterized by high proliferation and differentiation potential, ability of anchorage-independent growth, gap junctional intercellular communication as well as Oct4 expression (one of the key markers of adult stem cells). Recently, Garcia et al. (2017) performed *in vitro* culture of adult gastric mucosal cells within a medium rich in growth factors such as EGF, FGF, and hepatocyte growth factor (HGF). They observed that gastric spherical clones on the surface of gastric fibroblasts grow in a single layer. Those spherical cells harbored molecular markers capable of spontaneously differentiating and were

unique to gastric stem cells. Nonetheless, these above models cannot achieve pure and long-lived culture *in vitro* after a number of cells divisions, and whether the identity of stem cells would be changed or not in this culture system is not well determined.

With the deepening understanding of the signals that govern stem cell self-renewal, proliferation and differentiation, tissue-specific organoid models were established successfully, allowing for extensive experimentation with stem cells and their niches (Clevers, 2016). In 2010, Barker et al. (2010b), firstly, found that gastric Lgr5^{+} cell isolated from the gastric glands of Lgr5-EGFP-ires-CreERT2 mice were capable of generating a single-layered epithelial structure, that was then called the gastric organoid. This *in vitro* culture system partially recapitulates the microenvironment of stomach stem cells in the body, and contains all necessary niche factors, including EGF, Noggin, R-spondin, Wnt3A, FGF10, and gastrin (ENRWFG). Specifically, these growth factors supplied in this system act different effects. EGF sustains the continuous self-renewal and proliferation of stem cells and long-term culture (Johnson and Guthrie, 1980; Dembinski and Johnson, 1985). Noggin, an antagonist of BMPs, inhibits differentiation and induces an expansion of bud-domain. R-spondin and Wnt3A are the activators of Wnt pathway that promote growth and inhibits differentiation. FGF10 drives budding event and expansion of multiunit organoids. Gastrin has a mitogenic effect on gastric cells. The same group, further, developed a long-term culture system of human gastric organoids using surgical samples from gastric corpus (**Figure 2A**; Bartfeld et al., 2015). Cultures from different anatomical regions of the stomach maintain the specific molecular characteristics of their site of origin, and the model conserves stable genetic characteristics and biological behaviors of stem cells during long-term culture. In addition to those growth factors we mentioned above (ENRWFG), nicotinamide (also named vitamin B3) and TGF-β inhibitor (A83-01) are necessary in culture human gastric organoids (ENRWFGNiTi), due to its role in promoting initial organoid formation and extending the life span of organoid, respectively (Bartfeld et al., 2015). Besides, ROCK inhibitor Y-27632 is an optional supplement that can avoid anoikis in the early culture time (Schlaermann et al., 2016). Further, gastric organoids described above can be directed into pit-type organoids with the withdrawal of Wnt activator (Bartfeld et al., 2015). Pit-type gastric organoids undergo changes in morphology (become more cystic with less bud domain) and molecular markers (increased Muc5AC, decreased PGC and MUC6). Applying this culture system, subsequent studies from different research groups have successfully established different gastric organoid models

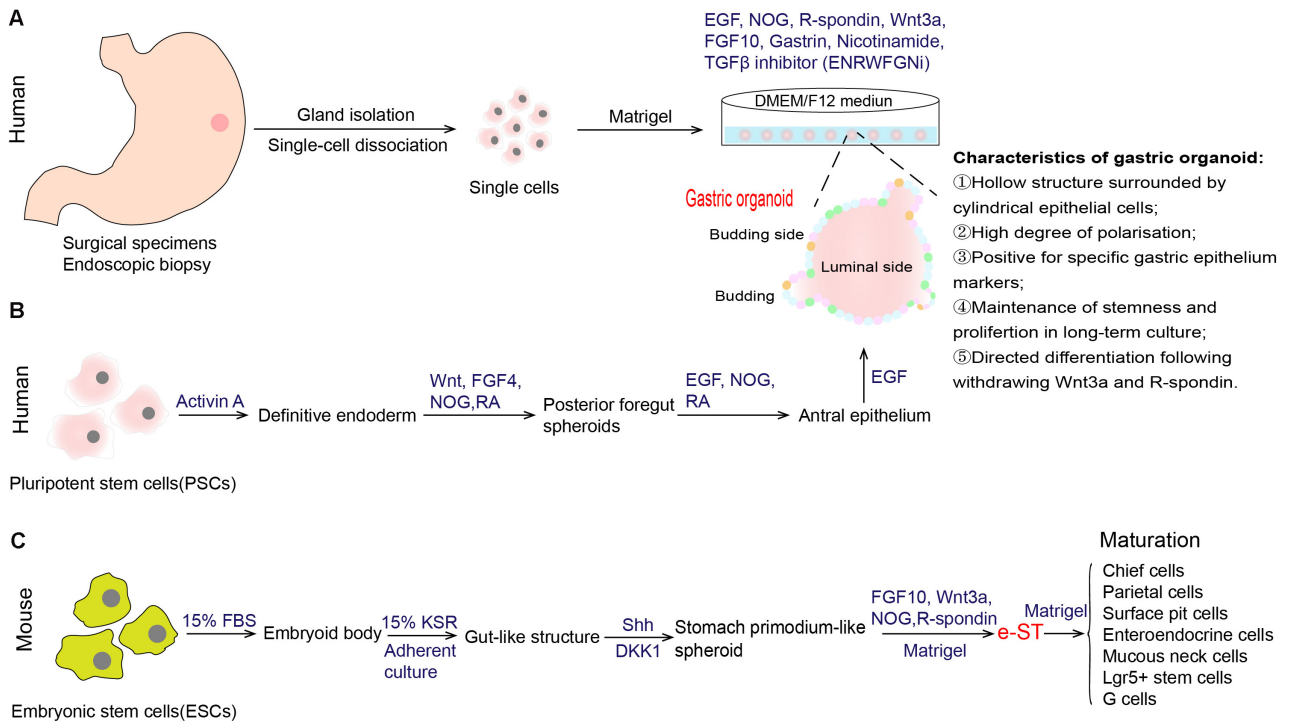

FIGURE 2 | Establishment of *in vitro* culture system of gastric stem cells. **(A)** Generation of human gastric organoid from gastric glands that are derived from surgical specimens or endoscopic biopsy (Bartfeld et al., 2015). This organoid model could achieve long-term *in vitro* culture. **(B)** Generation of gastric organoid from human pluripotent stem cells (PSCs) (McCracken et al., 2014; Broda et al., 2019). **(C)** Generation of stomach primordium-like spheroids (named e-ST) from mouse embryonic stem cells (ESCs) (Noguchi et al., 2015). e-ST could differentiate into mature stomach tissue cells in Matrigel-based culture supplemented with FGF10, Wnt3a, NOG, and R-spondin. EGF epidermal growth factor, NOG Noggin, FGF 10 fibroblast growth factor 10, TGF-β transforming growth factor beta, FGF 4 fibroblast growth factor 4, RA retinoic acid, FBS fetal bovine serum, KSR knockout serum replacement, Shh sonic hedgehog.

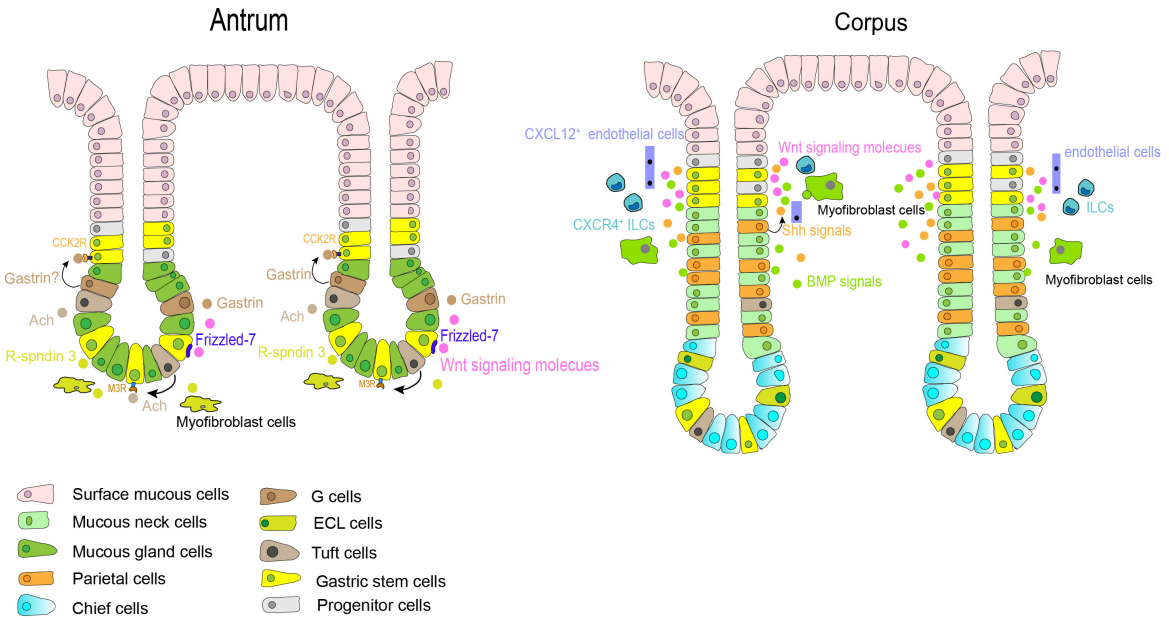

FIGURE 3 | Gastric stem cells in different anatomic sites and their niche factors. In the antrum, Notch signals affect the balance between stem cell proliferation and differentiation. Gastrin and acetylcholine (Ach) also contribute to niche factors in this region. In corpus, Shh and BMPs signals are restricted to the isthmus and neck of fundic gland. Stromal cells, such as Cxcl12 + endothelial cells and Cxcr4 + innate lymphoid cells, also contributes to the corpus stem cell niche partly through production of Wnt5a. Other growth factors, such as Wnt, EGF, and FGF10, play an important role in regulation of self-renewal and differentiation of gastric stem cells in the base gland of antrum and the isthmus of corpus.

using various marker-labeled gastric stem cells as mentioned above (Troy$^+$, Mist1$^+$, CCK2R$^+$, eR1$^+$, Axin2$^+$, Bmi1$^+$, and AQP5$^+$ stem cells) (**Table 1**). Key points that can be applied to confirm the formation of gastric organoid are summarized in **Table 3**.

In contrast to gastric organoid directly derived from stomach tissues, McCracken reported *de novo* generation of organoids from directed differentiation of human pluripotent stem cells (PSCs), through manipulation of stem cell niches targeting the FGF, Wnt, BMP, RA (retinoic acid) and EGF pathways (**Figure 2B**; McCracken et al., 2014). A detailed protocol for the generation of human antral and fundic gastric organoids from PSCs can refer to Broda TR's method (Broda et al., 2019). Another research group established a method for generating stomach primordium-like spheroids (named e-ST) from embryonic stem cells (ESCs) (**Figure 2C**; Noguchi et al., 2015). These spheroids were found to differentiate into mature stomach tissue cells in both the corpus and antrum in a three-dimensional culture system. Gastric organoids generated from PSCs or ESCs are, therefore, a valuable adjunct to *in vitro* studies of human stomach development.

Organoid culture system is a powerful method, but it is important to note its limitation. Current gastric organoids are unable to fully model the *in vivo* environment due to the lack of other cell components (such as mesenchymal cells and immune cells) which also exert an important role in stem cell niches, though coculture organoids with other cells type is a feasible and alternative approach. Considering the high cost of cultivation maintenance, it has become more common to culture organoids in medium with niche factors produced by various cell lines than to use commercial products; thus, experimental variation between scientific groups is inevitable.

ROLE OF GASTRIC STEM CELLS IN STOMACH DISEASES

The stomach is the most important organ within the gastrointestinal tract, it not only initiates the digestive process but also as a first line of defense against food-borne microbes (Hunt et al., 2015). Stomach diseases, such as gastritis, peptic ulcer, Helicobacter pylori (*H. pylori*) infection and gastric cancer, bothers a lot of people in the world. Gastritis is defined as any histologically confirmed inflammation of the gastric mucosa, and its epidemiology overlaps that of *H. pylori* infection. Longstanding mucosal inflammation leads to the loss of resident gland and the replacement of normal gland by inappropriate glandular unites, which terms as atrophy and metaplasia, respectively. Gastric atrophy and metaplasia are widely recognized as the precursor change for gastric carcinogenesis (Correa et al., 2010; Annibale et al., 2020). With regard to gastric cancer, it is the third leading cause of cancer mortality in the world and remains a major health threat in Asia-Pacific regions (Bray et al., 2018; GBD 2017 Stomach Cancer Collaborators, 2020). However, the mechanism behind gastric carcinogens is still undetermined. Thus, it is clinically important to understand the role of stem cells in stomach diseases.

TABLE 3 | Key points to confirm the formation of gastric organoids.

Index	Description	Method
Morphology	A sealed glandular lumen	Inverted microscope
	Gland-domain buds surrounding a central lumen	Inverted microscope
	Single layer epithelial structure	E-cadherin staining/ confocal microscope
Self-renewal capacity	Detection of proliferating cells located at the base of the gland-like domain	EdU staining/fluorescence microscope
	Positive for stem cell markers: Lgr5, CD44, OLFM4	Immunofluorescence/ confocal microscope/PCR
Expression of gastric-specific markers	Positive: Organoid form Antrum: Muc5AC (surface mucous cell),TFF1 (surface mucous cell), PAS (surface mucous cell), Muc6 (mucus gland cell), SST (endocrine cell), Gastrin, PDX1 Organoid from Corpus: Muc5AC (surface mucous cell),TFF1 (surface mucous cell), PAS (surface mucous cell), GIF (parietal cell), PGC (chief cell), Muc6 (mucus gland cell), TFF2 (mucous neck cell), SST (endocrine cell) Negative for intestinal markers: Muc2, CDX1/2	PCR, immunofluorescence

EdU 5-ethynyl-2′-deoxyuridine, Lgr5 leucine-rich repeat containing G protein-coupled receptor, OLFM4 Olfactomedin 4, Muc 5AC mucin 5AC, PAS Periodic acid-Schiff, GIF gastric intrinsic factor, PGC pepsinogen C, Muc 6 mucin 6, TFF1 trefoil factor 1, TFF2 trefoil factor 2, SST somatostatin, PDX1 pancreatic and duodenal homeobox1, Muc2 mucin 2, CDX1/2 caudal-type homeobox 1/2, PCR polymerase chain reaction.

Currently, relevant studies mainly focus on *H. pylori*-associated gastritis and gastric cancer (**Figure 4**).

H. pylori Infection

Long-term infection of *H. pylori* is one of the key causes of stomach cancer. This might be attributed to persistent mucosal inflammation, epigenetic modification of oncogenes or antioncogenes, and activation of oncogenic signaling pathways following *H. pylori* infection (Conteduca et al., 2013; Amieva and Peek, 2016). In addition to its known damage to differentiated gastric epithelium, available studies have reported that *H. pylori* infection also disturbed homeostasis in the gastric stem cells niche, leading to malignant transformation.

In both mice model and human specimens infected with *H. pylori*, bacteria microcolonies could be visualized in the neck region and base of gastric antral glands. Gland-colonized bacteria directly accelerated $Lgr5^+$ gastric stem cell-derived gland turnover at the early stage after infection in the dependence of CagA island (Sigal et al., 2015). The same group further revealed that in addition to directly acting on gastric stem cells, *H. pylori* infection increased expression of gastric myofibroblasts-derived R-spondin 3 expression and expanded the $Axin2^+/Lgr5^-$ cell pool to cause hyperproliferation and gland hyperplasia (Sigal et al., 2017). However, it should be noted that an increase in R-spondin 3 from myofibroblasts was also important to restrict bacteria colonization and promote mucosal regeneration (Sigal et al., 2019). Additionally, apoptotic suppression induced by *H. pylori* CagA via MEK/ERK pathway also contributed to impaired self-renewal of the gastric epithelium (Mimuro et al., 2007). On the other hand, it is well established that long-term infection of *H. pylori* induces a variety of histological changes in gastric mucosa, including oxyntic atrophy, metaplasia and dysplasia. Spasmolytic polypeptide-expressing metaplasia (SPEM) is proposed to encompass TFF2 (trefoil factor family 2)-expressing metaplasia, which is linked to mucosal injury associated with parietal and chief cell lose. Recent study documented that during chronic inflammation induced by this bacterium, some chief cells arising from $Lrig1^+$ populations might contribute to SPEM development (Wroblewski et al., 2019). Collectively, the imbalance between gastric epithelial turnover and stem cells differentiation triggers the development of *H. pylori*-associated diseases.

In vitro assays with gastric organoid, microinjection of *H. pylori* was used for fundamental studies of *H. pylori* pathogenesis. Upregulation of NF-κB-driven inflammatory response was observed in *H. pylori* infected-organoids (McCracken et al., 2014; Bartfeld et al., 2015; Schumacher et al., 2015), which validated previous results from immortalized gastric cancer cell lines. Meanwhile, in this gastroid system, Wroblewski reported that activated Wnt/β-catenin signaling pathway in a *H. pylori* CagA-dependent manner contributed to mis-localization of tight junction proteins (Claudin-7), leading to disrupted barrier function (Wroblewski et al., 2015). Additionally, application of gastric organoid/immune cell (cytotoxic T lymphocytes and dendritic cells) co-culture system further demonstrated the protective role of programmed death ligand (PD-L1) expression in gastric epithelium in response to *H. pylori*-induced immune injury (Holokai et al., 2019). Instead of 3D culture system, organoid-derived primary gastric epithelial cells cultured in collagen-coated dish (containing EGF, nicotinamide, Y-27632 and A83-01) were more easier to handle *H. pylori* infection without the usage of microinjection (Schlaermann et al., 2016). Therefore, these organoid models greatly simulate the interactions between *H. pylori* and the gastric epithelium or microenvironment, and provide opportunity to uncover mechanisms of malignant transformation induced by this bacterium.

Gastric Carcinogenesis

Gastric carcinogenesis is a multi-step process, which is correlated to host factors, environmental factors and some specific microbiotas (Tan and Yeoh, 2015; Schulz et al., 2019). Understanding the cells of origin involved in gastric cancer is of clinical significance for guiding development of effective strategies to prevent and treat this disease.

Gastric cancer is classified into intestinal, diffused and mixed types (Lauren classification) based on histological morphology of tumors (Lauren, 1965). Intestinal-type carcinoma undergoes non-atrophic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and eventually forms cancer (known as Correa cascade) (Correa, 1992). Metaplasia of the stomach, including spasmolytic polypeptide-expressing metaplasia (SPEM) and intestinal metaplasia (IM), is the tissue injury adaptation and a precursor to the dysplasia (Giroux and Rustgi, 2017).

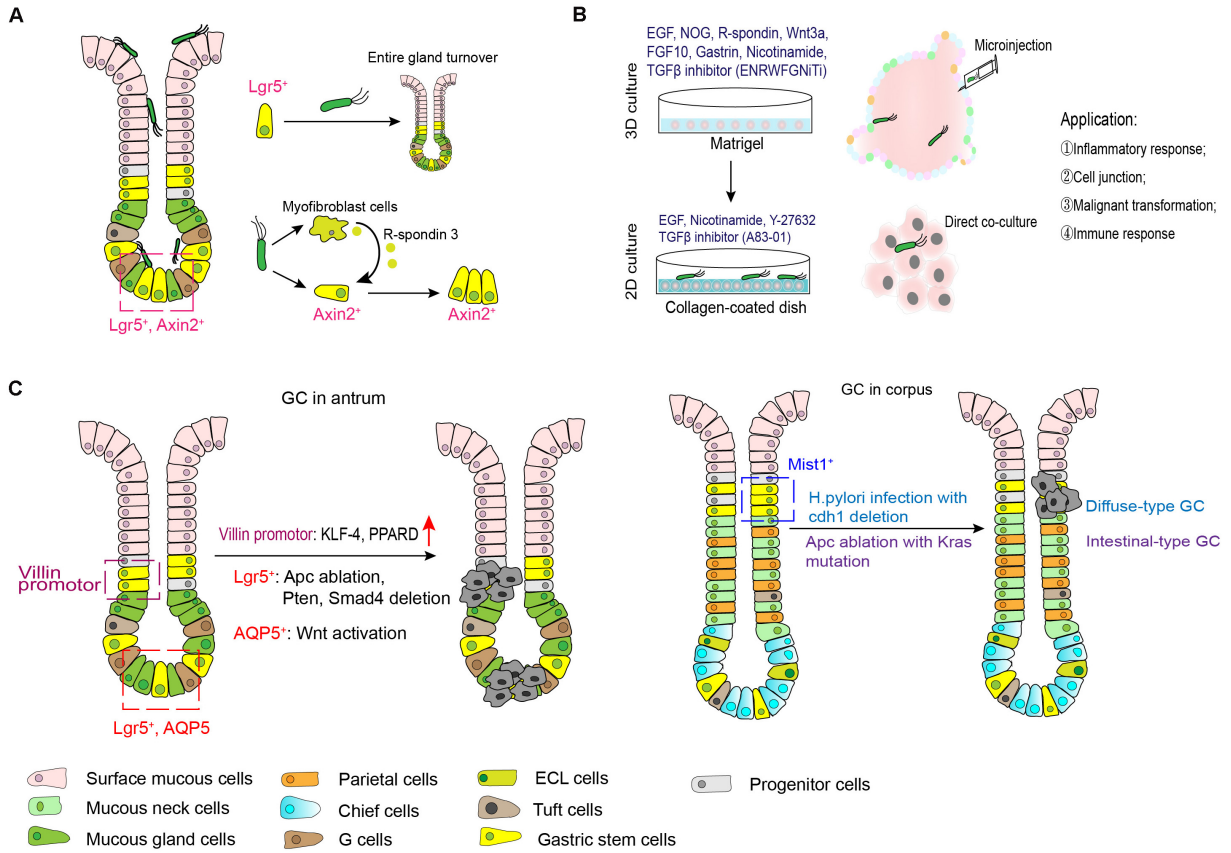

FIGURE 4 | Role of gastric stem cells in *H. pylori* infection and gastric carcinogenesis. **(A)** *H. pylori* can colonize in gastric surface cells, neck regions and even the base of antral glands. Gland-colonized bacteria directly or indirectly expands the stem cell pools. **(B)** Microinjection of *H. pylori* with gastric organoid can mimic the model of *H. pylori* infection *in vitro*, while co-culture model of *H. pylori* and organoid-derived primary gastric epithelial cells are more easier to investigate the pathogenesis of this bacterium. **(C)** The candidate stem cell origin and possible mechanism behind gastric oncogenesis.

Cell populations contributing to SPEM formation is partially understood. Some evidence indicate a link between SPEM development and mucus neck cells, chief cell transdifferentiation and even the isthmus stem cells in the corpus (Hayakawa et al., 2017a; Mills and Goldenring, 2017; Burclaff et al., 2020). During IM, expression of stem cell marker-Sox2 is reduced while intestinal marker (Cdx1 and Cdx2) emerges ectopically (Tsukamoto et al., 2004); and lineage tracing by following mutations in mitochondria DNA demonstrates the clonal expansion of IM by fission (McDonald et al., 2008). These findings raise the possibility that IM might be the consequence of abnormal differentiation in stem cells that can produce both gastric- and intestinal-type cells. Additionally, the stability and durability of metaplasia also propose that it might be maintained by a self-renewing stem cell. Regarding dysplastic lesions, more recent studies have shown that they were genetically related to metaplastic glands, indicating the clonal origin of dysplasia from metaplasia by field cancerization (Gutierrez-Gonzalez et al., 2011). For diffuse-type gastric cancer, histopathological analyses also have shown that early hereditary diffuse gastric cancer (HDGC) (with *CDH1* mutation) seems to lie within the upper neck of the gastric epithelium (Humar et al., 2007). These available evidence supports the implicit assumption that gastric cancer might arise from gastric stem cells that have potential for multi-directional differentiation potential.

Subsequent studies are identifying some candidate cells from which stomach cancer might originate. As earlier described, Lgr5$^+$ gastric stem cells at the base of the antrum in lesser curvature that is a frequent anatomical site for human gastric cancer give rise to all types of epithelial cells. Thus, gastric stem cells in this region might be the potential origin of stomach cancer. Results from a TCGA (The Cancer Genome Atlas) database analysis and immunohistologic staining revealed that intestinal adenocarcinomas of the gastric antrum and gastroesophageal junction were accompanied by the expansion of Lgr5 (Uehara et al., 2013). Ablation of *Apc* (adenomatous polyposis coli) gene in Lgr5-expressing cells leads to macroscopic adenomas and intramucosal well-differentiated carcinoma (Barker et al., 2010b). In line with this, antral stem cells expressing Sox2 or Mist1 may be among the gastric cancer origin cells in the context of *Apc* loss (Sarkar et al., 2016; Sakitani et al., 2017). Subsequent research identified that progression from microadenoma and macroscopic adenoma to invasive intestinal-type gastric cancer in Lgr5$^+$ gastric stem cells were accompanied

with the deletion of Smad4 and *Pten* in the gastric antrum, while *Smad4* and *Pten* deletions in differentiated cells (including antral pit, parietal and corpus Lgr5$^+$ chief cells) failed to initiate tumor growth (Li et al., 2016). However, whether Lgr5 expressing cells in acid-secreting corpus region are cancer cell origin is to be debatable. One study indicated that Lgr5$^+$ cells were not the origin for SPEM following the treatment with DMP-777 or L-635 (two chemicals inducing acute loss of parietal cells) (Nam et al., 2012). But, inflammation and inhibition of BMP signaling induced activation of Lgr5$^+$ cell in this anatomic region can lead to the development of metaplastic and dysplastic epithelial cell lineages (Ye et al., 2018). Leushacke et al. also found that a subpopulation of chief cells expressing Lgr5 functioned as damage-inducible stems cells effecting gland regeneration following injury and as a source of SPEM under the constitutive activation of Kras (Leushacke et al., 2017). Besides Lgr5$^+$ cells, *Klf-4* (Kruppel-like factor 4) disruption or *PPARD* (peroxisome proliferator-activated receptor delta) overexpression in Villin promoter-expressing stem cells spontaneously result in gastric carcinogenesis (Li et al., 2012; Zuo et al., 2019), CCK2R$^+$ cells potentially explains the gastrin-mediated effects on gastric cancer progression (Hayakawa et al., 2015b). More recently, AQP5$^+$ stem cells are identified as a source of Wnt-driven gastric cancer, and AQP5$^+$ tumor cells could reproducibly generate organoids in the absence of exogenous growth factors, indicating the stem potential of this cell populations (Tan et al., 2020). For gastric cancer occurred in the corpus, Mist1$^+$ isthmus stem cells can serve as an origin. In combination with Helicobacter species infection, deletion of *Cdh1* gene in Mist1$^+$ isthmus stem cells is able to generate diffuse-type gastric cancer; whereas intestinal-type adenocarcinoma can be induced by ablation of *Aps* along with Kras mutation in this group of cells (Hayakawa et al., 2015a). Another two groups of stem cells labeled by *TFF2* mRNA and Lrig1, respectively, do not give rise to metaplasia linage in the corpus (Quante et al., 2010; Choi et al., 2018).

Regarding the development of neuroendocrine tumors in the stomach, enteroendocrine cells, especially the ECL cells, are generally regarded as the cell origin (Waldum and Fossmark, 2018). As stated before, some stem cells contribute to the production of enteroendocrine cells in the gastric epithelium (**Table 1**), but whether these stem cells can directly give rise to neuroendocrine tumors is not well determined. For example, results from animal models indicated that Lgr5$^+$ cells may not be the origin cells for pyloric neuroendocrine carcinomas, though this subtype stem cells give rise to endocrine cells in this region (Vetter et al., 2016). Besides, evidence for the interaction between enteroendocrine cells and stem cells is limited. In small intestine, the enterochromaffin cell that is closely similar to the ECL cell in the stomach has been shown to participate in stem cell dynamics (Sei et al., 2018). For antral stem cells expressed the gastrin receptor CCK2R, they can be stimulated by progastrin but not by amidated gastrin, and this activation resulted in carcinogenesis (Hayakawa et al., 2015b).

On the other hand, cancer initiation is generally thought to commence after mutation in oncogenes or tumor suppressor genes. Renewal of the gastric epithelium is so rapid and frequent that differentiated epithelial cells would not survive for many decades required to achieve the mutational threshold for malignant transformation. In line with this notion, long-lived stem cells appear to the ideal cellular targets for the accumulation of mutations under the action of external environments (such as continuous inflammation and carcinogen stimulation) (Visvader, 2011; Blokzijl et al., 2016). In gastric epithelium, study in mice have documented that Lgr5$^+$ epithelial stem cell pool in the antrum was more susceptible to DNA damage than Lgr5$^-$ cells (Uehara et al., 2013). Thus, once DNA repair is impaired, increased mutagenesis of genome is possible to initiate carcinogenesis. Another evidence also showed that a deletion mutant of *Apc* in differentiated cells did not give rise to antral tumors, whereas *Apc* deletion in Lgr5$^+$ cell did (Barker et al., 2010b). Nevertheless, exactly how gastric cancer is initiated remains unclear, but adult stem cells residing in the gastric epithelium might be the direct source of cancer, given their fundamental properties of multi-potentiality and longevity.

IMPLICATIONS FOR MANAGEMENT OF STOMACH DISEASES

Since gastric stem cells in adult tissues can regenerate the resident cell types within a lineage, it might be introduced to harness their potential for regeneration of lost or damaged gastric mucosa. As aforementioned above, gastric stem cells can respond to mucosal injury caused by acute damage and chronic inflammation in animal models (Arnold et al., 2011; Choi et al., 2018; Nienhüser et al., 2020). But currently, there is no data assessing the dynamic changes of stem cells from clinical specimens. Besides, uncovering the effect of gastric mucosal protectants on gastric stem cells is also clinically practical. More importantly, gastric atrophy or intestinal metaplasia that is caused by chronic inflammation is commonly regarded as a point of no return (Annibale et al., 2020), targeted regulation of the differentiation of gastric stem cells might be an effective approach to reversing the damaged mucosa, and even reducing the risk of gastric carcinogenesis. In contrast to benign diseases in the stomach, treatment of patients with malignant poses greater challenges. Aberrant differentiation of gastric stem cells occur during tumorigenesis, development of new drugs (such as small molecule compounds) to inhibit aberrant differentiation might be an effective way to prevent gastric cancer. Additionally, understanding the mechanism behind the transformation from gastric stem cells to cancer stem cells may provide a new insight to solve the problem of tumor recurrence.

CONCLUSION AND FUTURE DIRECTION

Gastric stem cells play a key role in the dynamic renewal of gastric mucosal epithelium. Linage tracing approach has made breakthroughs that enable understanding of the physiological characteristics of gastric stem cells in their niches, but further efforts are warranted for defining the various components involved in gastric stem cell niches. Establishment of gastric organoid models overcomes the defects associated with multiple-passaged cells lines derived from cancer specimens,

thereby laying a foundation for further in-depth investigations into host-bacterial interactions and gastric carcinogenesis. Hyperproliferation of stomach stem cells, induced by *H. pylori* infection, might be one of the mechanisms of oncogenic transformation resulting from this bacterium. Furthermore, disturbance of gastric stem cell pool is involved in the multiple steps of gastric carcinogenesis. In the future, it is clinically significant to uncover the contribution of gastric stem cells to gastric mucosa homeostasis and the development of intestinal metaplasia, dysplasia and final gastric cancer. Further in-depth research is required to reveal the roles of gastric stem cells in stomach diseases and provide new insights for designing strategies to prevent gastric cancer.

AUTHOR CONTRIBUTIONS

SX researched data for this manuscript, made a substantial contribution to discussion of content designed the figures, and wrote the manuscript. LZ made a substantial contribution to discussion of content, and reviewed and edited the manuscript. Both authors have read and approved the final manuscript submitted.

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REVIEW
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Regenerative Intestinal Stem Cells Induced by Acute and Chronic Injury: The Saving Grace of the Epithelium?

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The intestinal epithelium is replenished every 3–4 days through an orderly process that maintains important secretory and absorptive functions while preserving a continuous mucosal barrier. Intestinal epithelial cells (IECs) derive from a stable population of intestinal stem cells (ISCs) that reside in the basal crypts. When intestinal injury reaches the crypts and damages IECs, a mechanism to replace them is needed. Recent research has highlighted the existence of distinct populations of acute and chronic damage-associated ISCs and their roles in maintaining homeostasis in several intestinal perturbation models. What remains unknown is how the damage-associated regenerative ISC population functions in the setting of chronic inflammation, as opposed to acute injury. What long-term consequences result from persistent inflammation and other cellular insults to the ISC niche? What particular "regenerative" cell types provide the most efficacious restorative properties? Which differentiated IECs maintain the ability to de-differentiate and restore the ISC niche? This review will cover the latest research on damage-associated regenerative ISCs and epigenetic factors that determine ISC fate, as well as provide opinions on future studies that need to be undertaken to understand the repercussions of the emergence of these cells, their contribution to relapses in inflammatory bowel disease, and their potential use in therapeutics for chronic intestinal diseases.

Keywords: intestinal epithelium, inflammatory bowel disease, enteroids, Wnt, intestinal stem cells (ISCs)

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Abbreviations: AA, arachidonic acid; ChIP, chromatin immunoprecipitation; CBCs, crypt-base columnar cells; DARSCs, damage associated regenerative stem cells; DCs, dendritic cells; DSS, dextran sodium sulfate; DTR, diptheria toxin receptor; EC, enterochromaffin; EC, enteroendocrine; EGF, epidermal growth factor; ER, endoplasmic reticulum; HNF4α, hepatocyte nuclear factor 4 alpha; IBD, inflammatory bowel disease; IECs, intestinal epithelial cells; IF, immunofluorescence; ILC3, group 3 innate lymphoid cells; iPSCs, induced pluripotent stem cells; LP, lamina propria; MSCs, mesenchymal stem cells; revSCs, revival crypt cell populations; RSCs, reserve stem cells; SI, small intestine; TA, transit amplifying cells; TAM, tamoxifen; TSS, transcriptional start sites; UPR, unfolded protein response; IECs, intestinal epithelial cells; ISCs, intestinal stem cells; ChIP, chromatin immunoprecipitation; DCs, dendritic cells; LP, lamina propria; EGF, epidermal growth factor; ER, endoplasmic reticulum; UPR, unfolded protein response; IBD, inflammatory bowel diseases; HNF4a, hepatocyte nuclear factor 4 alpha; CBCs, crypt-base columnar cells; RSCs, reserve stem cells; TA, transit amplifying cells; TSS, transcriptional start sites.

INTRODUCTION

The intestinal epithelium is composed of a variety of intestinal epithelial cells (IECs), each having specific contributions to maintaining normal intestinal function. In addition to critical roles in nutrient absorption and fluid secretion, IECs also provide structural support and a chemical and physical barrier that protects the cells of the underlying lamina propria (LP) from food antigens, commensal microbes, and other cellular insults. In order to maintain this barrier function, IECs undergo constant self-renewal, replenished from a self-sustaining intestinal stem cell (ISC) niche every 3–4 days (Reynolds et al., 2014). It is important that the signaling pathways maintaining this normal turnover remain uncompromised, efficient, and resilient in order to maintain homeostasis.

The IEC renewal cycle can be perturbed by injury, and the epithelium needs mechanisms to repair itself when this occurs. There are a number of ways that a normal intestinal epithelium can be injured, including infection, radiation, ischemia, physical trauma, or immune-mediated injury (e.g., graft-vs.-host disease). If injury is limited to the superficial, differentiated compartment, ISCs can readily repair the damage through their normal regenerative cycling. However, in severe or transmural injury, ISCs can be lost (Tao et al., 2017) and must be replenished with other stem cells that migrate to the site of injury, ultimately helping aid in the maintenance and restoration of the damaged epithelium (Feil et al., 1989; Seno et al., 2009; Miyoshi et al., 2012).

It is essential that these regenerative stem cells successfully replace their damaged counterparts and have similar efficiency in providing barrier integrity and protection against the luminal contents and other cellular insults. While these damage-associated regenerative stem cells (DARSCs) are critical to resolve acute injury, it is not known if they are beneficial or harmful in the setting of chronic intestinal inflammation, such as in inflammatory bowel disease (IBD). Recent studies have demonstrated altered gene expression and innate immune responses in primary enteroids derived from patients with IBD (Kuwabara et al., 2018; Martin et al., 2019; Smillie et al., 2019; Rees et al., 2020b), suggesting the likelihood of long-lasting changes in ISCs. Could this be the result of chronic inflammation in the ISC environment? Moreover, do these chronically damaged ISCs respond to inflammatory signals (which have been demonstrated to maintain homeostasis and affect lineage commitment in the IEC niche) similar to their parental cells? Do DARSCs accumulate epigenetic changes that ultimately contribute to the chronic, relapsing nature of IBD? To address these important questions, we will be highlighting recent literature that has characterized damage-associated ISCs, as well as the signaling factors that drive maturation of ISCs, and examine the potential exploitation of these cells using autologous organoid transplantation as a potential therapeutic tool for diseases of the intestinal epithelium.

INTESTINAL EPITHELIAL CELLS—THE CONVEYOR BELT THAT RUNS THE GUT

The intestinal epithelium is composed of a single cell layer of columnar cells that form the luminal surface of both the small and large intestine. The large intestine (colon), has a relatively flat luminal surface with crypts or glands that penetrate away from the lumen. It is covered by two distinct mucus layers that reduce direct contact with food antigens, commensal microbes, and other molecules that may invoke an immune response (Johansson et al., 2011). The small intestine (SI), in contrast, contains villi that protrude into the lumen to increase the surface area for digestion of food, as well as movement of nutrients, and has one layer of mucus, which also protects against cellular insults.

The intestinal epithelium can be thought of as a conveyor belt, where cells originate from dividing ISCs at the crypt base and differentiate as they move up the crypt/villus axis, before being sloughed into the lumen. This process takes on average 3–4 days. Situated between ISCs in the SI are Paneth cells, which, unlike other IECs, move downward toward the ISC niche as they differentiate, and provide important signaling molecules such as WNT, epidermal growth factor (EGF), and Notch ligands (DLL1, DLL4) to aid in the maturation of ISCs to absorptive and secretory cell lineages (reviewed in Gehart and Clevers, 2019). In the colon, which largely lacks Paneth cells, mesenchymal cells such as fibroblasts and myofibroblasts instead provide these factors. These signaling molecules can push the ISC progeny to two types of progenitor cells: (1) Secretory progenitors, which can differentiate into tuft, goblet, Paneth, or enteroendocrine (EE) cells; and (2) Absorptive progenitors, which can differentiate into enterocytes or M cells (**Figure 1**). It is important that these signaling processes remain intact and efficient in order to maintain the proper ratio of absorptive to secretory cells, thus providing proper epithelial function. If these pathways are disturbed, inflammation can ensue and may contribute to a variety of gastrointestinal perturbations such as penetrating Crohn's disease (Ortiz-Masià et al., 2020) and colorectal cancer (Meng et al., 2020).

Historically, two distinct types of ISCs have been distinguished by their position in the crypts and their expression of distinct markers: Leucine-rich repeat-containing G protein-coupled receptor 5 ($Lgr5^+$) stem cells, which are fast cycling ISCs; and slow-cycling +4 stem cells (so named because of their position as the fourth cell from the crypt base), which are radioresistant and considered to have ISC potential. These cells express the markers homeodomain-only protein homeobox (Hopx) (Takeda et al., 2011), telomerase reverse transcriptase (Tert) (Breault et al., 2008; Montgomery et al., 2011), polycomb complex protein Bmi-1 (Bmi1) (Sangiorgi and Capecchi, 2008), and leucine-rich glioma inactivated 1 (Lrgi1) (Powell et al., 2012), which are robustly expressed by $Lgr5^+$ CBCs (Muñoz et al., 2012) (**Figure 1**). It should be noted that +4 cells have reduced labeling efficiency of daughter cells during long-term lineage tracing studies, compared to $Lgr5^+$ cells. Thus, the notion of +4 cells being deemed stem cells has been challenged recently. Moreover, the +4-position terminology restricts the nomenclature of regenerative cells and discounts the roles of

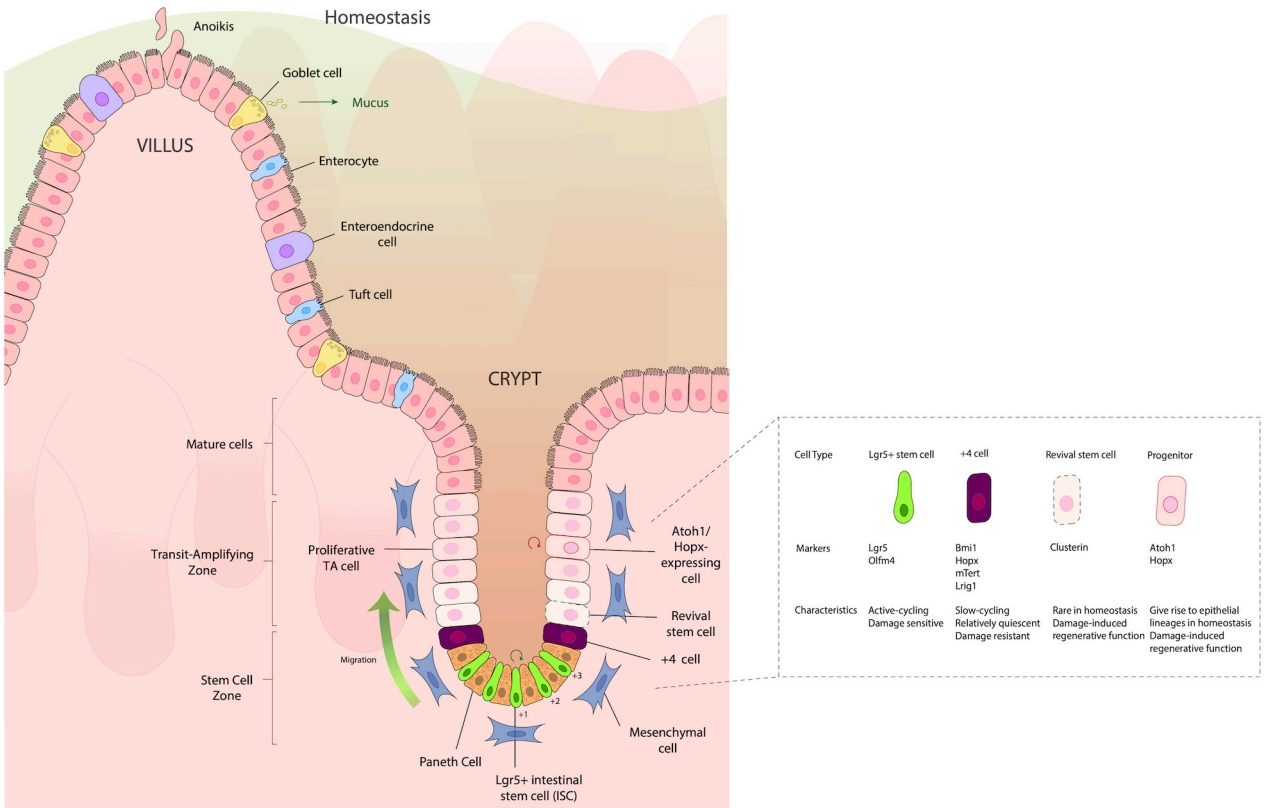

FIGURE 1 | Structure of the intestinal epithelium. The intestinal epithelium is replenished every 3–4 days by rapidly dividing Lgr5$^+$ intestinal stem cells (ISCs) in the crypt base that differentiate as they move up the crypt villus axis, until they eventually slough into the lumen. Situated between the small intestinal ISCs are Paneth cells, which are important for providing signaling molecules such as WNT, epidermal growth factor (EGF) and Notch ligands (DLL1, DLL4), which aid in the maturation and differentiation of ISCs to either absorptive (enterocytes), secretory [Goblet cell, enteroendocrine (EE)], or Tuft cell lineages. Just above the IECs, frequently in the "+4" position, are slow cycling, damage resistant cells that express the markers Bmi1, Hopx, mTert, and Lrig1. Located in the transit amplifying zone are Atoh1 and Hopx-expressing mature cell precursors, which give rise to different epithelial lineages during homeostasis, but during damage, have a regenerative function, helping aid in re-establishing damaged epithelia.

regenerative stem cells in other crypt locations. Because of the rapidly evolving understanding of what these cells are, in this review we will refer to the +4 position regenerative cells using the designations by which they were used by the authors of the respective studies that refer to them: i.e., +4 cells, +4 stem cells, regenerative cells, damaged associated, cells, etc.

Recent literature has highlighted the potential roles of both Lgr5$^+$ IECs, "+4 cells," and other cells derived by de-differentiation of more mature cells as the DARSC population, as they all have been shown to regenerate during the healing process and provide protection. The following sections will highlight recent literature on these distinct cell types, their differences and similarities, and how they might function in the setting of chronic inflammation.

LGR5$^+$ ISCs: THE INFANTRY AND THE RESERVE?

While Lgr5$^+$ crypt base columnar cells (CBCs) drive turnover and differentiation into mature epithelial cells (Barker et al., 2007) the intestinal epithelium is able to recover following injury to these CBCs (Metcalfe et al., 2014). This suggests that there are reserve stem cells (RSCs) that replenish abolished Lgr5$^+$ stem cells. Cells in the +4 position, discussed in more detail below, have generally been considered the main RSCs (Montgomery et al., 2011; Takeda et al., 2011; Powell et al., 2012; Yan et al., 2012). However, recent literature suggests that Lgr5$^+$ cells can emerge from de-differentiated epithelial cells during injury to provide epithelial restitution, thus raising the idea that +4 position cells do not migrate to the site of injury to rescue their damaged counterparts (Murata et al., 2020) but rather the damaged epithelium is replenished by newly emerged proliferative cells that express Lgr5 (**Figure 2**).

Recent evidence for the ability of Lgr5$^+$ cells to act as DARSCs following radiation injury was shown by Ayyaz et al. (2019). They found that irradiation induces a distinct cell type marked by high expression of *Clu*, which encodes stress-response genes that are relevant in cell survival (Zhang et al., 2014). To understand the role of *Clu*$^+$ stem cells during homeostatic turnover, they used a tamoxifen (TAM)-inducible label system to show that *Clu*$^+$ cells give rise to both Lgr5$^+$ CBCs and differentiated progeny

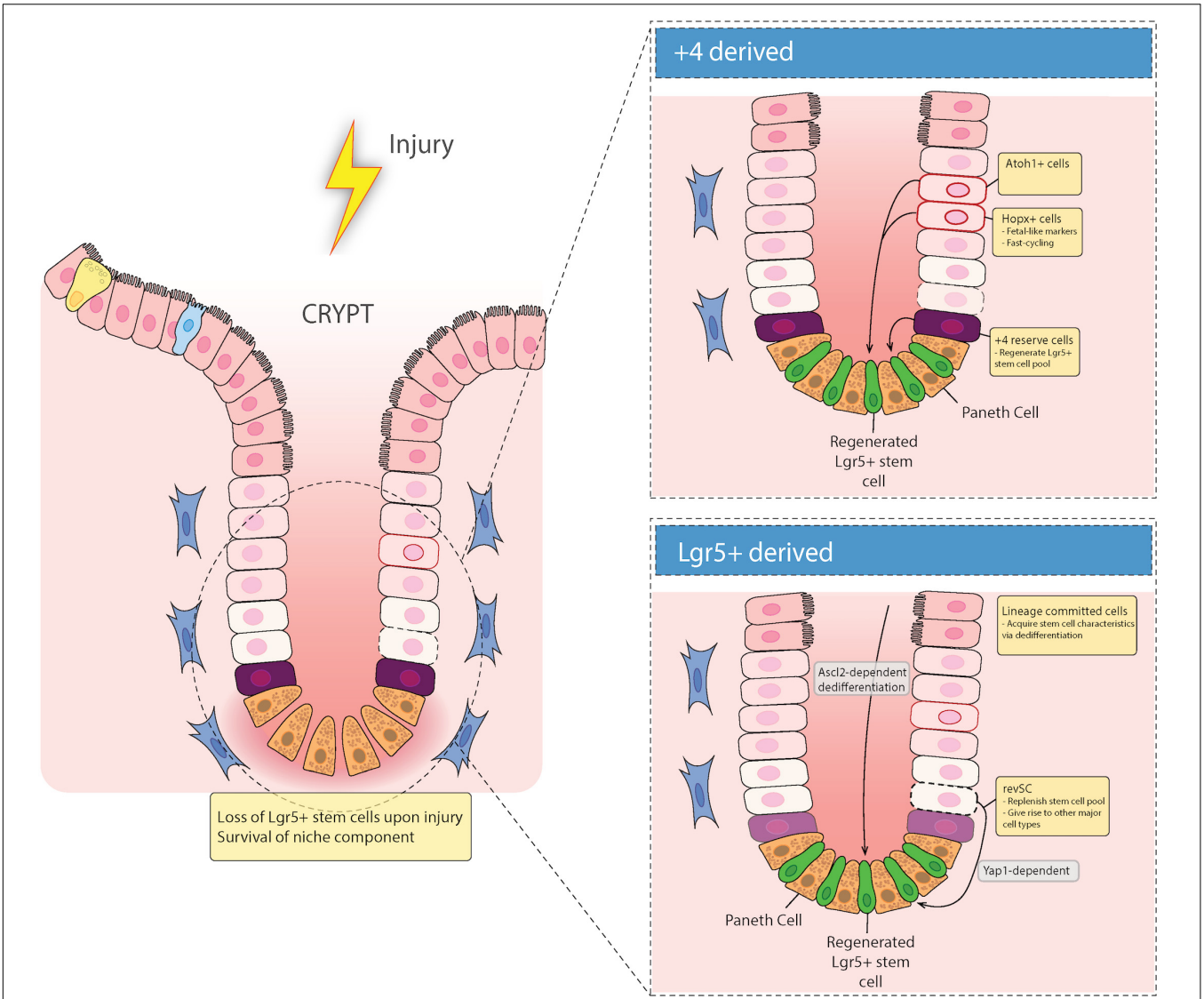

FIGURE 2 | Epithelial repair response to injury. Recent evidence has suggested that there are two types of regenerative cells that fill in the damaged epithelium after injury. When the intestinal epithelium is damaged, either acutely (e.g., via irradiation), or chronically (e.g., in inflammatory bowel diseases), there can be loss of Lgr5^{+} stem cells in the crypt base. These gaps in the epithelium are filled in with either Lgr5^{+}-derived reserve stem cells that can de-differentiate downward, or are +4 cell position-derived, including Hopx and/or Atoh1 expressing mature cell precursors that de-differentiate and migrate down from the TA zone to regenerate the stem cell niche.

cells. Moreover, they found that *Clu*$^{+}$ single cell cluster 2 (SSC2) cells were able to reconstitute the damaged Lgr5^{+} ISC niche to revival crypt cell populations (revSCs), in both acute and chronic dextran sodium sulfate (DSS) colitis mouse models. Most importantly, they found that diphtheria toxin receptor (DTR)-mediated selective depletion of *Clu*$^{+}$ cells resulted in failure of intestinal regeneration after DSS colitis, in a Yap1 dependent manner (Ayyaz et al., 2019) (**Figure 2**). These data refute previous reports that suggests DARSCs as being exclusively +4 position derived, and supports the idea that in a state of damage, reserve RSCs are derived from Lgr5^{+} cells. However, since 25% of these *Clu*$^{+}$ revSCs are positioned in the +4 position, this distinction remains uncertain.

While this study proved that DARSCs can be derived from Lgr5^{+} cells, Murata and colleagues set out to understand if Lgr5^{+}-derived progeny cells can de-differentiate to replenish the damaged Lgr5^{+} ISC niche (Murata et al., 2020) (**Figure 2**). *Ascl2*, a basic-helix-loop-helix transcription factor gene, is a transcriptional target of the Wnt signaling pathway, and is restricted to basal crypt cells in mice (Jubb et al., 2006). With this in mind, Murata and colleagues utilized a TAM-inducible CRE system to permanently GFP label Lgr5^{+} cells and their progeny, and also to selectively deplete *Ascl2*$^{+}$ cells through expression of DTR. They also employed *Ascl2* KO organoids. Using these methods, they discovered that *Ascl2* expression is essential for crypt cell de-differentiation after ISC injury

(Murata et al., 2020). Moreover, after complete depletion of Lgr5$^+$ ISCs using γ-irradiation, they demonstrated that DARSCs originate from Lgr5$^+$ (GFP$^+$) progeny, via de-differentiation. This implies that there is no recruitment from cells in the +4 position to the damaged epithelium. These two studies together indicate that Lgr5$^+$ cells can act as DARSCs; although they conflict in terms of whether this is primarily due to preservation of a small population of cells, or de-differentiation of their progeny. Additional studies may be required to resolve this discrepancy.

While there is controversy regarding the origins of DARSCs, even less is known about inflammatory signals that direct them. Previously, IL-11 derived from myofibroblasts was shown to be necessary for regeneration in the intestinal mucosa (Bamba et al., 2003). However, this cytokine has never been studied in the context of which specific cells in the crypt respond to IL-11. Murata et al. (2020) showed that healthy ISCs express little *Il11ra1*, whereas *Ascl2*- expressing regenerative cells have increased levels of *Il11ra1*. IL-11ra1, an IL-11 receptor, and recombinant IL-11, both Ascl2 target genes, enhance crypt regeneration potential. A more recent study found that type I interferons impair mouse recovery from DSS colitis and the ability to form enteroids *in vitro* (Minamide et al., 2020). Epithelial-specific deletion of interferon-regulatory factor 2 (Irf2), which downregulates type I IFN signaling, led to loss of Lgr5$^+$ ISCs and increased proliferation, suggesting a mechanism for this susceptibility, and also indicating another potential effect of inflammatory injury on the gut. Additional studies will be needed to understand the contribution of IL-11, interferons, and other inflammatory signals to DARSC development and maintenance.

THE ROLE OF +4 POSITION CELLS AND SECRETORY PRECURSORS IN REPAIRING THE DAMAGED INTESTINAL EPITHELIUM

Situated just above the last Paneth cell in the crypt are the +4 position cells, which have a unique transcriptional profile including expression of Hopx (Takeda et al., 2011), Tert (Breault et al., 2008; Montgomery et al., 2011), Bmi1 (Sangiorgi and Capecchi, 2008), and Lrig1 (Powell et al., 2012) (**Figure 2**). These cells have previously been deemed the RSC population, which reconstitutes Lgr5$^+$ CBCs during a state of injury (Breault et al., 2008; Sangiorgi and Capecchi, 2008; Montgomery et al., 2011; Powell et al., 2012), and have been previously shown to be radiation resistant (Tao et al., 2017; Montenegro-Miranda et al., 2020; Sheng et al., 2020), suggesting that these cells can survive acute injury in the crypt and fill in for their damaged counterparts. However, recent literature has underscored the complexity of characterizing these cells and called into question their "stemness." This section will discuss the current literature on the different subsets of LGR5$^-$ reserve stem cells (including "+4 cells") and their ability to restore intestinal epithelial homeostasis post DNA damage and injury.

Importance of Hopx and Atoh in Regenerative Cell Function and ISC Renewal

The homeodomain-only protein homeobox (Hopx) is a non-DNA-binding homeobox protein expressed in various tissue stem cell populations, including in the intestinal crypts. Takeda et al. demonstrated that the majority of the so-called label retaining cells in the intestinal crypt following irradiation injury (those retaining BrdU, indicating ongoing proliferative capacity) reside in the +4 position and express Hopx (Takeda et al., 2011). Moreover, they exhibited a bi-directional lineage relationship: Hopx-expressing cells can propagate to generate Lgr5$^+$ ISCs and all mature epithelial lineages, while Lgr5$^+$ cells can give rise to +4 position Hopx$^+$ cells. This suggests they have an important role as RSCs. However, other recent studies have identified a distinct colitis-associated regenerative stem cell (CARSC) population that expresses Hopx in mouse models of colitis (Wang et al., 2019). Utilizing monolayer cultures in an air-liquid interface (ALI), which leads to maturation of the monolayers, epithelial injury was induced by submerging the monolayer in media mimicking a hypoxic and ER-stress mediated damaging environment. These damaged monolayers contained fast cycling Hopx$^+$ regenerative stem cells characterized by the expression of fetal-like marker Tacstd2 (Trop2), thus distinguishing them from both Lgr5$^+$ cells and slow-cycling Hopx$^+$ +4 position cells (Wang et al., 2019). It is quite possible that these Hopx$^+$ expressing cells could be derived from other secretory derived lineages, because Hopx$^+$ regenerative stem cells co-express goblet and EE cell signatures. However, most notably, they also express the secretory IEC lineage marker Atonal homolog-1 (Atoh, also known as Math1 in mice), which is the master transcription factor for secretory IECs (Yang et al., 2001). Atoh has recently been shown to be necessary for plasticity of secretory progenitors and tissue regeneration (Tomic et al., 2018), and these cells have the ability to repair the epithelium during DSS-induced colitis (Ishibashi et al., 2018; Tomic et al., 2018; Castillo-Azofeifa et al., 2019). Thus, Atoh$^+$ secretory progenitor cells may contribute to the development of Hopx$^+$ regenerative cells during colitis (**Figure 2**).

Atoh1$^+$ cells are essential for secretory cell differentiation, as Atoh1 deletion in mice leads to development of intestines with a grossly normal crypt-villus architecture, but that are entirely populated by enterocytes (van Es et al., 2010). Atoh1 expression in populations of +4 position cells marks them as secretory lineage precursors, but recent evidence suggests that these Atoh1$^+$ progenitors have the capacity to self-renew and give rise to multiple lineages with high frequency during homeostasis, and are able to contribute to colonic regeneration after γ-irradiation or DSS-induced injury (Buczacki et al., 2013; Ishibashi et al., 2018; Tomic et al., 2018; Yu et al., 2018), thus highlighting their importance in the differentiation process during the repair stage after intestinal damage, in the large intestine. However, Atoh is dispensable for regeneration in the SI (Durand et al., 2012), suggesting that the mechanism of regeneration involving Atoh1 may be tissue specific. The exact mechanisms of epithelial regeneration were studied by Tomic et al. (2018), who found that multi-site phosphorylation of Atoh1 results in the inhibited

ability to self-renew, thus leading to blunted ability to regenerate proper clones during damage.

To understand whether Atoh1$^+$ cells are dependent on *Lgr5*$^+$ stem cells for epithelial repair, Castillo-Azofeifa et al. (2019) performed genetic fate mapping using *Lgr5*$^{GFP-IRES-CreERT2}$; *ROSA26*^{tdTomato} mice undergoing DSS-induced colitis. They discovered that after DSS-induced injury, Lgr5$^-$ Atoh1$^+$ secretory progenitors, not Notch1$^+$ absorptive progenitors, provided epithelial repair. These data suggest that Atoh1$^+$ cells are essential in maintaining ISC function after injury and can differentiate into distinct mature cells types that protect the epithelium after acute damage.

Bmi1^{hi} and *Tert*$^+$ populations, which contain mostly EE cells (Yan et al., 2012, p. 1), and rare "reserve" ISCs, as well as both secretory and enterocyte progenitors (Tetteh et al., 2016; van Es et al., 2019), and even Paneth cells (Schmitt et al., 2018; Yu et al., 2018; Jones et al., 2019), have been shown to aid in Lgr5$^+$ recovery of the epithelium. With this in mind, these studies did not look at the regenerative efficiencies of secretory and EE cells, and whether or not their ability to restore the damage epithelium is distinct in terms of (1) The speed of transit to the site of inflammation, and (2) their overall restorative capacity. This would be an interesting observation, as Yan et al. (2017) demonstrated that *Prox1*$^+$ EE lineage cells during homeostasis can propagate into reserve injury-inducible ISCs.

Harnack et al. (2019) studied the role of the Lgr5 ligand R-spondin 3 (Rspo3), which potentiates Wnt signaling, in establishing the ISC niche during homeostasis or colonic injury. Lacking Paneth cells, colonic crypts rely on mesenchymal cell production of Wnt-supporting ligands like R-spondin. In this study, myofibroblast-derived Rspo3 was found to be necessary and sufficient for maintaining Lgr5$^+$ cells during homeostasis. However, in the context of injury, myofibroblasts do not maintain Lgr5$^+$ cells, and instead interact with Lgr4 expressing cells, to generate new crypts. Mice lacking Rspo3 have a blunted ability to repair the damaged epithelium. It is important to note that these Axis inhibition protein 2 (Axin2$^+$) cells express high amounts of *Atoh1*, thus raising the possibility that Axin2$^+$ Lgr5$^-$ regenerative stem cells are derived from secretory precursors. Moreover, deletion of Rspo3 completely prevented crypt regeneration during DSS colitis. Interestingly, this was shown not to require Lgr5$^+$ cells or "reserve" stem cells (in this case, marked via expression of Axin2 and Atoh), both of which were depleted by DSS. Instead, Rspo3 was needed to support a population of differentiated Krt20$^+$ enterocytes that expressed the alternative Rspo receptor Lgr4, allowing them to de-differentiate (Harnack et al., 2019). This study provides additional evidence that differentiated IECs can repopulate a damaged ISC niche, and possibly offer intestinal restorative properties.

These data collectively suggest that Atoh1$^+$ cells have the characteristics of (1) Multipotency in self renewal capacity and (2) The ability to promote colonic repair and epithelial regeneration during colitis. Hence, they could be targeted for future therapies to resolve colonic injury. Having the ability to resect tissues from patients with acute or chronic inflammatory intestinal diseases, grow ISCs in a dish as enteroids, and genetically modify them to express *Atoh1*, or to sort Atoh1$^+$ cells to proliferate as enteroids, could allow for clinicians to engraft these ISCs back into the damaged areas of the epithelium to promote expansion of cells that will aid in a more robust colonic repair to resolve chronic injury. One limitation to this hypothesis is that overexpression of Atoh1 has been shown to increase secretory cell differentiation, thus reducing their regenerative potential. However, using a Tryptophan hydroxlayse 1 (*Tph1*)-CreERT2 mouse model, Sei et al. (2018) recently showed that EECs, specifically enterochromaffin cells (EC), have demonstrated stem cell potential and have the ability to dedifferentiate and act as a RSC during irradiation. Hence, Atoh1-mediated secretory differentiation does not preclude subsequent de-differentiation to repair ISC injury.

These studies also support the hypothesis that Atoh expressing cells can be targeted for colonic inflammatory diseases. Hopx expressing cells can possibly be used in a setting of chronic inflammatory diseases mediated by hypoxia or endoplasmic reticulum (ER) stress, such as IBD (Giatromanolaki et al., 2003; Kaser et al., 2008; Shah et al., 2008; Vanhove et al., 2018; Rees et al., 2020b), as the authors were able to show that these cells rapidly proliferate to provide protection in response to hypoxic injury, which upregulates the ER stress cellular protection pathway known as the unfolded protein response (UPR). The UPR has been shown to be important in maintaining ISC proliferation (Heijmans et al., 2013), and can be an important pathway to target for the repair of IECs in chronic intestinal diseases.

Msi1 and TIGAR: Boosting the Potential of DARSCs

One concern with "+4 cells" and possibly other DARSCs is that their slow cycling in a homeostatic state means that they need to receive signals to increase their proliferative potential at a rate fast enough to preserve the intestinal barrier following severe injury, such as following lethal irradiation. Two recent factors that may contribute to this are Musashi homolog 1 (Msi1) and TP53-induced glycolysis and apoptosis regulator (TIGAR).

Msi1 has previously been identified as a marker of ISCs (CBCs, and +4 position cells) (Kayahara et al., 2003; Li et al., 2015). Sheng et al. (2020) set out to understand if Msi1 can rapidly cycle to restore a damaged intestinal epithelium. This was investigated by generating an Msi1CreERT2 allele for lineage tracing analysis and this led to the discovery that Msi1$^+$ cells are indeed positioned in the +4 position in the intestinal crypt and are resistant to DNA damage via y-irradiation. Using ssRNA-seq, they demonstrate that Msi1$^+$ cells have low-to-negative Lgr5 expression, and repopulate the damaged intestinal epithelium before Lgr5^{high} emergence, suggesting that Msi1$^+$ cells are more rapidly cycling than Lgr5^{high} cells. This stands in contrast to the current hypothesis that +4 position cells function as RSCs that restore depleted Lgr5^{high} CBCs first, thus allowing CBCs to rapidly divide to repair the damaged intestinal epithelium. Interestingly, Msi1+ cells move both upwards and downwards along the crypt villus axis, post TAM induction, which suggests that Msi1$^+$ cells can differentiate into CBCs, Paneth cells, and villus defining cells. These data suggest, and further support earlier work, that cells of the secretory lineage are capable of

restoring a damaged intestinal epithelium. This work supports previous work by Carroll and colleagues that demonstrates while most $Lgr5^+$ cells are thought to be continually proliferative, residing in a licensed state, ∼20% of $Lgr5^+$ cells remain in an unlicensed G_1 cell cycle phase (Carroll et al., 2018).

One possible signal for $Msi1^+$ to initiate repair is arachidonic acid (AA). Previous work showed that AA may contribute to proliferation of ISCs (Hiraide et al., 2016). More recently, Wang and colleagues investigated the role of AA on intestinal regeneration post γ-irradiation. They discovered that AA promotes the proliferation of SI epithelial cells post irradiation in an *Ascl2I* and WNT signaling manner. Most notably, AA's regenerative effect was mediated via the regulation of Msi1+ radiation resistant cells, not $Lgr5^+$ cells, but the exact mechanism is not known (Wang et al., 2020).

TIGAR is a protein induced in mouse intestinal crypts by c-Myc. Under homeostatic conditions, suppression of the β-catenin/c-Myc axis within +4 position slow cycling ISCs leads to limited regenerative responses to restore intestinal integrity after injury. Chen et al. recently showed that restricted overexpression of TIGAR in $Bmi1^+$ cells, but not $Lgr5^+$ cells, was able to reverse lethal irradiation injury in mouse SI, by activating AP-1 signaling to induce proliferation (Chen F. et al., 2020).

Together, these data suggest that +4 position cells can play an important role in the regeneration of damaged small IECs, where they can provide several advantages compared to $Lgr5^+$ cells that de-differentiate to rescue the damaged epithelium. (1) $Msi1^+$ cells positioned in the +4 position cycle faster than $Lgr5^+$ cells during DNA damage, migrating both downward toward the crypt and apically toward the villus region; (2) $Msi1^+$ cells facilitate rapid tissue repair via regulation by AA, a fatty acid released by phospholipase A2 and the critical precursor for prostaglandins, both heavily involved in IEC inflammatory signaling; and (3) Induction of TIGAR in $Lgr5^-$ DARSCs can reverse their slow proliferative phenotype, facilitating rapid division and restoration of the intestinal barrier. Future studies in human cells or enteroids will be needed to determine if these observations can be generalized to human diseases.

Hypoxia and ER Stress as Drivers of Epithelial Repair and Differentiation

The process of cellular differentiation and proliferation in the intestinal crypt involves an abundance of secretory protein processing in the ER, which puts a considerable metabolic burden on the cells. These proteins can be improperly processed, misfolded, or inadequately glycosylated, thus leading to ER stress. The UPR, which is activated during ER stress to restore the cell to homeostasis, is also essential in determining stem cell fate and differentiation (Heijmans et al., 2013). The mechanisms of how hypoxic injury and ER stress affect ISC function and intestinal repair following injury are an area of active investigation.

Hepatocyte nuclear factor 4 alpha (HNF4α) is expressed in intestinal villi (Stegmann et al., 2006) and has been identified as a transcription factor that regulates the expression of genes during intestinal cellular differentiation during endoderm development (Yao et al., 2016). It was recently discovered that HNF4α is involved in the activation of ER stress during intestinal epithelial differentiation (Tunçer et al., 2019), demonstrated by the upregulation of XBP1 and ATF6 (two ER stress mediators) downstream of HNFα activity. This study is important because it ties together the importance of ER stress in maintaining proper ISC proliferation and differentiation (Heijmans et al., 2013) with the fact that HNF4α is expressed in the intestinal villi (Stegmann et al., 2006), which suggests that these two signaling pathways are essential for ISC fate and fitness.

In support of this, our group recently published that ER stress in colon-derived enteroid monolayers drives inappropriate TLR5 responses leading to the expression of unidentified factors that mature dendritic cells (DCs) to become pro-inflammatory—describing a novel pathway that may lead to the inappropriate anti-commensal inflammatory responses seen in IBD (Rees et al., 2020b). Arguably the most important finding of this study was that IBD derived enteroids have blunted cytokine responses to the TLR5 agonist flagellin (FliC), and this was found to be driven by dysregulated ER stress pathways in the IBD enteroids, but not TLR5 expression levels, compared to healthy controls. This raises the hypothesis that prolonged periods of ER stress can alter the ISC niche, permanently changing their phenotype and perpetuating inflammation and disease.

Most recently, Montenegro-Miranda et al. (2020) established an *in vitro* enteroid damage-repair model that identified HNF4α is an essential regulator of intestinal epithelial repair following γ-irradiation damage. They produced VillinCre X $Hnf4^{fl/fl}$ or Hnf4-KO mice enteroids, and assessed their ability to grow and proliferate. They found that HNF4α KO enteroids were unable to propagate and grow *in vitro* compared to their littermate control-derived enteroids. Most notably, they found that HNF4α is important for epithelial regeneration *in vivo* and HNF4α-KO mice have an increase in the secretory differentiation lineage. The blunted ability to regenerate the epithelium was linked to the inability to keep up with the metabolic demands of regenerating the ISC niche after γ-irradiation (Montenegro-Miranda et al., 2020). This could be due to effects on ISC metabolism, as Hnf4α and the related protein Hnf4γ were recently shown to be required for fatty acid oxidation in mouse ISCs, and conditional double knock-outs lost the ability to renew the ISC compartment (Chen L. et al., 2020).

These studies have particular significance in the field of IBD since it was discovered that *HNF4α* is a risk allele in ulcerative colitis, with the disease allele associated with reduced function (UK IBD Genetics Consortium et al., 2009; Marcil et al., 2012). With this in mind, it is appropriate to speculate that in a chronic setting of inflammation, as seen in UC, HNF4α is essential in maintaining barrier integrity during homeostasis and acute injury, although it is not essential for the expression or maintenance of $Lgr5^+$ cells (Montenegro-Miranda et al., 2020).

Studying the ISC niche during homeostasis and disease (acute or chronic) will be an important topic for future researchers to better understand how inflammation and dysregulated signaling pathways may led to the development of DARSCs, and whether or not these cells are beneficial in the chronic setting. Although it

has been shown in an acute ER stress-driven setting that Hopx expressing RSCs can provide protection, it remains to be seen if these cells, whether derived from the +4 position or not, can provide protection in a chronic setting. One would postulate that chronic inflammation would lead to the prolonged development of either Atoh1$^+$, Hopx$^+$, or Hnf4α$^+$ cells in the crypt, thus providing short term epithelial maintenance. However, how long can these cells or their progeny remain in the crypt? Does this ultimately not allow for the expansion of healthy Lgr5$^+$ cells once inflammation has resolved? These questions need to be addressed in order to better understand the nature of a true homeostatic ISC niche post inflammatory disease.

In summary, these studies suggest that there are several redundant layers of protection against permanent injury following acute intestinal crypt injury. Lgr5$^+$ cells, "+4 cells" and Lgr5$^+$ progeny that de-differentiate from secretory or absorptive lineages can all help maintain epithelial barrier integrity during a state of acute inflammation. Future studies should focus on which specific secretory or absorptive cells are responsible/most efficient in replenishing the damaged epithelium in acute models, as well as in a chronic setting. One important question that remains to be addressed is whether RSCs are just as efficient in providing barrier integrity, proper paracrine signaling, and appropriate innate and adaptive immune cross-talk during infection and other inflammatory states, compared to their parental counterparts. This information can lead to future therapeutics to alter cell signaling pathways that allow for the development of distinctive cell populations in order to restore barrier integrity and intestinal epithelial homeostasis. One key to these therapeutics will be to better understand regulation of IEC differentiation and plasticity. The following section will cover the genetics behind differentiation in ISCs to highlight some future directions in the field of IEC plasticity.

HOW DIFFERENTIATED IS TOO MUCH DIFFERENTIATION—IS THERE ANY GOING BACK?

As mentioned previously, there is emerging evidence that transit amplifying (TA) cells and even fully differentiated IECs may be able to de-differentiate and replenish the damaged ISC niche. There are important questions that need to be addressed about these cells: (1) Are precursors of differentiated cells (goblet cells, Paneth cells, EE, and absorptive enterocytes), innately destined (based on their location) to differentiate into their mature forms?; and (2) How do epigenetic changes result from the environmental signals that drive their maturation? Key signaling molecules, *Wnt*, *Bmp*, and *Notch*, which are produced by Paneth cells and myofibroblasts (Kosinski et al., 2007; Hughes et al., 2011; Sato et al., 2011) regulate ISC fate and function (Rees et al., 2020a). Wnt expression has been shown to be regulated in part by histone variant functions, so it is of importance to study possible epigenetic factors, as well as chromatin remodeling factors that determine the fate of the Lgr5$^+$ ISC niche. The following sections will discuss emerging literature on permissive chromatin and IEC transcription factors that may underlie IEC plasticity and repair following injury.

Chromatin Structure and IEC Plasticity

Chromatin structural modifications lead to repression or activation of certain genes that are necessary for self-renewal and differentiation (Hager et al., 2009; Skene and Henikoff, 2013). An open chromatin structure may also facilitate epigenetic modifications allowing for de-differentiation. Kim et al. (2014) demonstrated that mouse small intestinal ISCs have unusually open or "permissive" chromatin structures that allow lineage-defining transcription factors (e.g., Atoh1) to lead to differentiation, while removal of these transcription factors in the setting of permissive chromatin can reverse this; this may underlie the plasticity of IECs in response to injury. This notion was supported more recently by Jadhav et al. (2017), who used *Bmi1*^{GFP} mice to demonstrate that preterminal EE cells, as well as a goblet cell precursor population defined by the markers CD69$^+$CD274$^+$, have the ability to de-differentiate into Lgr5$^+$ cells upon ISC ablation via their dynamic chromatin accessibility, which remains open during injury.

One particular histone variant, H2A.Z, has been shown to remodel chromatin structures, leading to gene expression changes. The function of H2A.Z in ISC differentiation and renewal has recently been reported. SNF-2 related CBP activator protein complex (SRCAP) components YL1 and Znhit1 have been shown to regulate the incorporation of H2A.Z into the chromosome (Cai et al., 2005; Latrick et al., 2016; Liang et al., 2016). However, the exact mechanisms behind the roles of Znhit1 and YL1 in the SRCAP complex and how they modulate H2A.Z function was unknown. To address these questions, Zhao et al. (2019) established a *Znhit1* KO mouse model to study its role in intestinal epithelial establishment and maintenance. They found that Znhit1 regulates the expression of *Lgr5*, *Tgfb1*, and *Tgb12*, via the incorporation of H2A.Z into the transcriptional start site (TSS) regions of the aforementioned genes. Znhit1 deficiency leads to a downregulation of Lgr5 and an activation of TGFβ signaling, which mediates self-renewal capacity and drives differentiation of Lgr5$^+$ ISCs. It is important to note that Znhit1 deletion altered only *Lgr5* expression without affecting Wnt signaling, as there were no genetic expression changes in *Ascl2*, a Wnt-targeted master transcription factor for activating the transcription of *Lgr5* (van der Flier et al., 2009), or *Axin2*, which is an activity indicator of Wnt signaling (Lustig et al., 2002). These data suggest that Znhit1 is essential for the differentiation outcomes of Lgr5$^+$ ISCs, and raises the question that targeting Znhit1 or the SRCAP as a whole could restore intestinal homeostasis in diseases that perturb the ISC niche. This thought could be further supported by a recent study that demonstrates that SCRAP promotes self-renewal of mouse ISCs (Ye et al., 2020).

Several additional chromatin-modifying proteins and transcription factors have recently been identified as helping to control ISC fate and IEC differentiation. One recent study showed that the transcription factor Id3 is likely responsible for maintaining ISC fate via repression of open chromatin regions (Raab et al., 2020). Transcriptional co-repressors

MTG8 and MTG16, were recently been shown to be involved in regulating ISC fate into secretory lineages by indirect Notch signaling suppression, via ATOH (Baulies et al., 2020). These two chromatin modulators are expressed by early progenitor cells that are located in the +4/5 position along the crypt villus axis. Polycomb-repressive complexes (PRCs) repress transcription via compacting of chromatin and limiting DNA accessibility. There are two major types of PRCs, PRC1 and PRC2. PRC 1 was shown to be crucial for maintaining stem cell identity via the preservation of Wnt/β-catenin activity (Chiacchiera et al., 2016a). PRC1 is essential for maintaining stem cell self-renewal, but in contrast, PRC2 was shown to be dispensable for intestinal regeneration (Chiacchiera et al., 2016b), yet is sufficient for maintaining cellular plasticity and epithelial regeneration in the crypt bottom after radiation-induced damage.

In conclusion, ISC plasticity is tightly regulated via multiple tissue-specific transcription factors and chromatin modifiers. Studies of chromatin accessibility in ISCs during various forms of intestinal injury, including chronic diseases like IBD, could lead to important interventions to help maintain normal ISC function following chronic injury.

Control of IEC Fate After Injury by CDX2 and the Hippo Pathway

The process of migration of stem cell progeny cells from the crypt into the villus, thus leading to their differentiation into specific secretory or absorptive lineages, is controlled by transcription factors, such as members of the CDX homeobox gene family (Xu et al., 1999; Beck, 2004), namely, CDX1 and CDX2, which are essential for intestinal maintenance and compartment-specific differentiation (Hryniuk et al., 2012). p400 ATPase participates in the incorporation of H2A.Z (Kobor et al., 2004; Gévry et al., 2007), and Rispal et al. set out to understand if there was a link between p400 induction of Wnt (via CDX2) and H2A.Z dynamics. They discovered that H2A.Z modulates the expression of intestinal progenitor differentiation by preventing terminal differentiation, thus acting as a negative regulator of intestinal differentiation (Rispal et al., 2019). This was demonstrated using a *H2A.Z* KO mouse model, which led to the inhibition of mRNAs of differentiation markers of enterocytes (*sucrase-isomaltase*), and goblet cells (*Muc2* and *Muc4*). Moreover, the ability of CDX2 to repress transcription, via binding to target gene promoters, was shown to be blunted by H2A.Z incorporation into the chromatin. Taken together, these data show that H2A.Z may be an important link between Wnt signaling and IEC differentiation, acting through CDX2.

One hypothetical role of CDX2 in signaling and IEC differentiation could be a direct interaction with HNF4α. CDX2 has recently been shown to interact with transcription factors such as GATA4 and HNF1α, which regulate the expression of differentiation markers sucrase-isomaltase, lactase-phlorizin hydrolase, and μ-protocadherin (Boudreau et al., 2002; van Wering et al., 2004; Hinkel et al., 2012). A recent study using chromatin immunoprecipitation (ChIP) showed that half of DNA binding sites of HNF1 are shared with HNF4α also, suggesting that similar gene regulation may occur for both transcription factors (Yang et al., 2016). This may be particularly relevant given that HNF4α has a role in determining the fate of IECs, mainly, enterocyte differentiation and identity (Chen et al., 2019), and evokes the UPR during ER stress, which is an essential pathway in maintaining proliferation of the ISCs and differentiation of their progeny (Heijmans et al., 2013). Moreover, CDX2 has been shown to regulate the expression of Wnt inhibitors *APC* and *AXIN2* (Olsen et al., 2013). Studying CDX homeobox gene family members may therefore be essential in targeting transcription factors to induce the differentiation of specific ISCs to provide barrier maintenance.

The studies discussed above highlight the possibility that if one targets the transcriptional machinery of secretory or absorptive derived IECs, no matter how differentiated they may be, they may be able to de-differentiate and migrate toward the crypt to provide epithelial maintenance. Future research should study the efficiency of targeting the transcriptional machinery of the aforementioned cells to help repair injury *in vivo*, or potentially to generate robust, therapeutically efficient clones of cells for autologous organoid transplantation.

Another important set of proteins essential in regulating cell-specific proliferation, survival and fate determination in the gut is known as the Hippo pathway. It was first described in *Drosophila* as being required for controlling organ growth, and is implicated in cancer development. Most notably for this review, the mammalian Hippo pathway is also involved in maintenance of intestinal structure and prevention of carcinogenesis, and more recent studies have implicated it in injury repair.

The Hippo pathway in mammalian cells is composed of MST and LATS kinases, which are responsible for inactivating two cellular proliferation, survival and fate transcriptional regulators, YAP and TAZ. Yap in mice has previously been shown to be involved in regenerative cell signaling via repression of Wnt target and ISC genes (Axin 2, Lgr5, Olfm4) as well as Paneth cell differentiation suppression (Lyz, Kit, Wnt3, Math1) (Barry et al., 2013; Gregorieff et al., 2015). Notch signaling drives the proliferation and differentiation of secretory cells into Paneth cells (VanDussen et al., 2012) and it has recently been shown that there is cross-talk between Hippo and Notch signaling pathways. Serra et al. (2019) recently showed that Yap1 activation initiates Notch/DLL1 lateral inhibition, thus driving Paneth cell differentiation and crypt formation from nascent mouse enteroids. More recently, Li et al. (2020) produced an IEC-restricted Lats1/2 knockout mouse, and showed that it led to loss of ISCs coupled with Wnt-uncoupled crypt expansion, in a mechanism dependent on Yap/Taz. These data collectively suggest that Hippo signaling is essential for fate determination of ISC progeny and may be a significant target for regenerative stem cell therapy, yet these exact mechanisms need to be further investigated.

It was previously demonstrated that Yap and Taz are dispensable for Wnt signaling under homeostatic conditions (Azzolin et al., 2014; Gregorieff et al., 2015), suggesting that the Hippo pathway is not a major signaling pathway involved in cellular plasticity, but it is nonetheless important in determination of cellular fate. Hippo and Yap cross-talk

pathways have been shown to converge with Wnt, BMP, TGF-β, Notch, and EGF (reviewed in Hansen et al., 2015). As we know, BMPs promote the differentiation of ISCs toward mature IEC phenotypes, and mature differentiated epithelial cells can also produce BMPs (Haramis et al., 2004; Batts et al., 2006; Qi et al., 2017). Wnt ligands are mainly expressed by Paneth cells and mesenchymal cells surrounding the crypts (Farin et al., 2012; Kabiri et al., 2014) and recently IEC monolayers were shown to be able to control their own growth and organization through a WNT and BMP feedback loop (Thorne et al., 2018).

These data prompted the study of the Hippo pathway's involvement in suppressing Wnt activity via BMP/TGF-β signaling. Bae et al. (2018) showed that IEC-specific deletion of MOB1A/B, an important kinase involved in activation of Lats kinase and subsequent phosphorylation of Yap, led to lethal intestinal degeneration and loss of ISCs via BMP and TGFβ-induced suppression of Wnt activity, showing a requirement for Yap in mouse intestinal homeostasis.

Collectively, these studies suggest the importance of the Hippo pathway in influencing IEC homeostasis by Wnt suppression, ultimately leading to cellular plasticity modulation. Relevant for this review, there has also been recent work examining the roles of LATS and YAP/TAZ in recovery from intestinal injury. The first such study was by Gregorieff et al. (2015), who found that recovery from radiation injury in mice required Yap activity to suppress Wnt signaling and activate Egf (epidermal growth factor) pathways to induce ISC survival and division. At the same time, this Yap/Egf signaling was also required for tumor development in $Apc^{-/-}$ mice; this suggests a molecular mechanism whereby the repeated cycles of injury and repair in IBD can promote carcinogenesis. A similar proliferative effect of Yap when Apc is deleted was shown in mouse enteroids by Azzolin et al. (2014). More recently, it was shown that Lats2 and Yap1 positively regulate each other's expression during *H. pylori* infection, and that Lats2 activity restricts intestinal metaplasia, again supporting the idea that Yap1 may be involved in inflammation- or injury-induced carcinogenesis (Molina-Castro et al., 2019).

Four other recent studies have examined the role of the Hippo pathway on recovery from chemical-induced intestinal injury in mice. Yui et al. (2018) found that during recovery from DSS colitis, the colonic epithelium goes through a "primitive" or fetal-like state on its way to recovery, and that this transition requires Yap/Taz activity. Romera-Hernández et al. (2020) induced intestinal injury using methotrexate, and found that group 3 innate lymphoid cells (ILC3s) drive intestinal epithelial repair in a Yap1 dependent manner, but independent of intestinal regenerative IL-22. Xu et al. (2020) examined the role of the stromal cell protein ISLR [immunoglobulin (Ig)-like domain and five leucine-rich repeat (LRR) domains], in recovery from DSS and TNBS injury in mice. Islr was previously shown to maintain an undifferentiated state of stromal cells (Maeda et al., 2016). In this study, Xu et al. (2020) found that Islr inhibits Lats, leading to increased Yap activity. They then used a stromal cell conditional KO mouse model targeting Islr, in order to understand its role in modulating the Hippo signaling pathway during homeostasis and damage. They found that Islr is upregulated following DSS injury, and that stromal cell-specific deletion of Islr did not lead to a phenotype in uninjured mice, but impaired recovery following DSS or TNBS injury. Importantly, they also showed that ISLR is overexpressed in human tissues from patients with IBD and colorectal cancers, suggesting that inflammation drives the expression of ISLR. Finally, Lukonin et al. (2020) used a single cell imaging platform to identify a novel role of the orphan retinoid X receptor RXR in IEC proliferation and differentiation. They found that RXR activation using all-*trans* retinoic acid or a chemical activator led to maturation and differentiation of enteroids, associated with Yap cytosolic localization. In contrast, treatment with an RXR inhibitor let to reduced CDX activity and Yap nuclear localization. Moreover, RXR inhibition improved recovery from DSS colitis in mice. This study opens the door to pharmacologic treatments that can modify the Yap-mediated commitment to proliferation or repair, potentially blocking cycles of injury-mediated chronic damage.

Together, these recent studies underscore the importance of LATS/YAP/TAZ signaling not only in maintenance of intestinal homeostasis, but in repair from acute injury. Additional studies will be required to further assess how these pathways are affected in chronic states of inflammation such as in IBD. This could lead to approached to target mediators of the Hippo pathway in order to enhance restoration and repair of the intestinal epithelium, in such a way as to avoid overactivation of YAP and subsequent carcinogenesis. They could also open the door to new ways to generate reparative epithelia safely, which we discuss in the following section.

AUTOLOGOUS ORGANOID TRANSPLANTATION—CAN WE RESCUE THE DAMAGED INTESTINAL EPITHELIUM?

Despite recent advances in IBD treatment, the diseases remain incurable, and many patients fail to achieve remission even with biologic anti-cytokine therapies. Recent work suggests that there may be permanent changes in ISC function in IBD that underlie its chronic, relapsing nature. Specifically, it has been recently been shown via RNA sequencing and enteroid model experiments that there are distinct differences in gene expression profiles, inflammatory cytokine milieus, and epithelial cell phenotypes in healthy tissue compared to tissue from people with IBD (Obita et al., 2003; Mitsuhashi et al., 2005; Ito et al., 2013; Dotti et al., 2017; Ragland and Criss, 2017; Suzuki et al., 2018; Martin et al., 2019; Smillie et al., 2019; Rees et al., 2020b). With this in mind, it is imperative that researchers look for new methods/strategies to normalize and repair ISC function in IBD. After the rapid growth in technology to grow and maintain intestinal epithelial derived $Lgr5^{+}$ stem cells as enteroids, studies of autologous organoid transplantation to re-populate the ISC niche have just started to take place. This type of methodology has been demonstrated using cancer models and has been extensively reviewed for use in the SI (Qi et al., 2020), and the possibility of it being used in IBD (Okamoto et al., 2020), although the

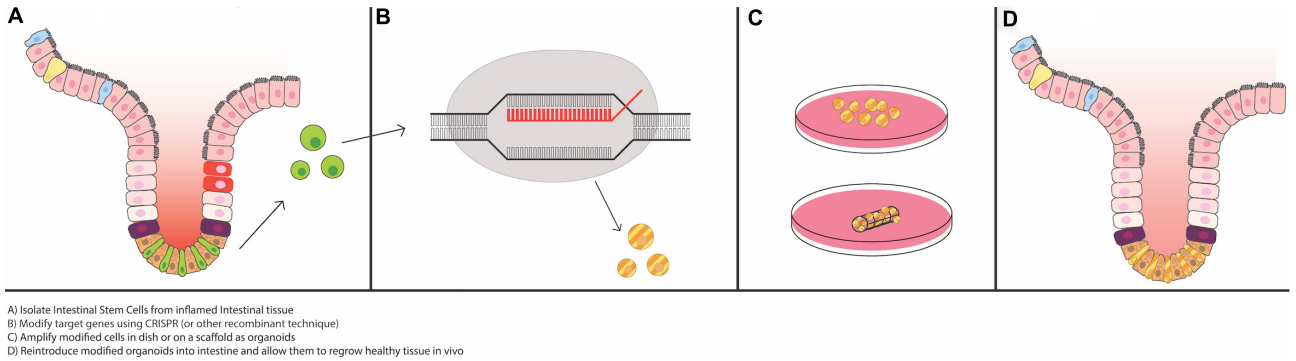

FIGURE 3 | Potential for use of intestinal organoids as "grafts" for the damaged epithelium. Recent advances in epithelial regeneration via organoid transplantation have shown that it could be possible to recover a damaged intestine or colon with autologous organoid transplantation. One approach to improve efficacy in engraftment and recovery post damage would be to isolate intestinal stem cells (ISCs) from the inflamed tissue, or if possible, non-inflamed adjacent tissue, modify target genes via CRISPR or equivalent recombinant techniques, expand the modified ISCs *in vitro*, and engraft back into the damaged area to recover the injured area. Tissue scaffolds, either synthetic or derived from human intestinal resections, could facilitate growth and proper cellular alignment.

major focus so far has been with use of organoids derived from induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs). We will highlight the current literature on ISC enteroid transplantation in the context of replacing or aiding in the development of damage associated ISCs, and **Figure 3** will illustrate methodologies to allow for successful enteroid transplantation into damaged intestinal epithelia (**Figure 3**).

To date, all of the studies that have performed ISC transplantation have used mouse models that allow for engraftment without rejection because of MHC histocompatibility issues. However, recently a model was published using xenographic human colon stem cell transplantation into NOD.cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}/Jic (NOG) mouse crypts (Sugimoto et al., 2018). Sugimoto et al. (2018) performed lineage tracing using CRISPR-Cas9 to engineer an Lgr5-CreER knock-in allele for engraftment into the NOG mice. To induce epithelial injury that allows for successful engraftment of the donor cells, they treated the recipient colons with EDTA, followed by scraping one side of the colonic epithelium, prior to engraftment with GFP-labeled human colon organoids. They were able to demonstrate successful engraftment of these donor organoids through endoscopic monitoring for up to 6 months (Sugimoto et al., 2018). Most notably, the authors determined that the Lgr5^{+} cells were located at the bottom of the crypts of the xenograft, not the mouse crypts. These cells had the ability to migrate up the crypt villus axis 10 days after TAM-induced labeling, and on day 28, the Lgr5^{+} cell progeny cells formed ribbon-like structures (indicative of proliferation up the crypt-villus axis), which indicates that these cells were xenograft derived and had the ability to self-renew. The xenografted Lgr5^{+} cells were able to differentiate into goblet cells, enterocytes, Tuft cells, and EE cells (Sugimoto et al., 2018). In conclusion, these cells have the ability to self-renew and differentiate into fully functioning mature cells of either the secretory or absorptive lineage.

This study was followed by Khalil et al. (2019) looking to replace the damaged murine SI epithelium with either mouse- or human-derived intestinal crypts. The authors injured the jejunal epithelium using EDTA and dithiothreitol (DTT) injection through a 25-gauge needle, followed by gentle scraping of the epithelium. Following injury, GFP^{+} lentiviral-transduced enteroids or spheroids (depending on the shape prior to implantation), were infected into the site. Two distinct surgical models were used: (1) An in-continuity model, which was an implantation of the jejunal segment that was in continuity of the SI, or (2) A bypassed jejunal segment model, which did not receive a regular stream of bowel contents. The authors found that the "in-continuity" model had no engraftments of GFP^{+} labeled murine cells post implant day 7. However, in the bypass model, GFP-expressing enteroids and spheroids were able to successfully engraft 75% (3 of 4 recipients), and demonstrated the ability to fully differentiate, as shown by expression of lysozyme (Paneth cell), Muc2 (goblet cell), synaptophysin (EE cell), and CD10 (small intestinal brush border marker) via immunofluorescence (IF), with engraftment lasting up to 4 weeks. Intestinal transplantation with human cells demonstrated similar differentiation patterns as the aforementioned murine cells, displaying *E*-cadherin, lysozyme, chromogranin A (Paneth cell), and Muc2; however, spheroids showed no expression of differentiation markers, but were Lgr5^{+}. In contrast, using human derived organoids, there was limited success of engraftment using the bypass model, which demonstrated a 36% engraftment success of surviving mice (61% survival).

This study provides some evidence of organoid transplantation success, but fails to provide evidence that this system is sustainable for long term engraftment. The reason behind the moderate engraftment rates and low survivability of the mice could be from the DTT treatment, not the EDTA treatment, as Sugimoto and colleagues did not see low survivability rates (Sugimoto et al., 2018). DTT treatment may indeed be altering the epithelial mesenchymal network past the point of successful of engraftment. In order to have successful engraftment, the epithelium cannot be too damaged—it likely

needs to have proper signaling, blood flow, and tissue scaffolding to remain intact in order to have successful engraftment into the crypt. These needs were demonstrated recently by Meran et al. (2020), who used intestinal crypts from biopsies from children with intestinal failure/short bowel, to produce functional organoids. Growing these organoids on decellularized human extracellular matrix scaffolds (obtained from separate pediatric intestinal resection tissue) led to jejunal grafts that were able to survive and partially differentiate when implanted into mice.

These studies provide a basis for future strategies using organoid transplantation to ameliorate inflammatory intestinal diseases. By CRISPR-Cas9 gene knock-in or knock-out strategies, one could simultaneously improve efficacy in anti-cytokine therapies by maximizing anti-cytokine targeted receptors, while re-establishing the damaged epithelium with healthy $Lgr5^{+}$ stem cells. Combining cytokine therapy and autologous organoid transplantation could also help reduce the inflammatory environment in the recipient, thus aiding in the integration of organoids into the damaged intestinal epithelium. Moreover, in a chronic setting, one could obtain mucosal biopsies and actively select for a regenerative stem cell population that has the ability to rescue the inflamed epithelium. Further studies could look into the type of growth factors and extracellular matrix that facilitate growth of regenerative cells to help aide in this mechanism. There will be difficulties, however, to obtain and successfully expand autologous ISC-derived enteroids, as well as safely and efficiently clearing the damaged intestinal ISC populations from the patient, to ensure successful engraftment for long term ISC replacement. Future studies will need to focus on how to successfully engraft organoids into the intestinal epithelium, but most importantly, be able to maintain proper signaling to ensure appropriate self-renewal and differentiation into mature epithelial cells, without creating tumorigenic potential.

CONCLUSION

The process of self-renewal and rapid turnover that evolved in the intestinal epithelium functions extremely well in most circumstances, rendering most enteric infections and epithelial injuries non-lethal. Even when damage is profound enough to destroy crypts and their CBCs, other cells are able to step in and repair the injury, helping preserve barrier, absorptive, and secretory functions and reducing the potential for long-term harm to the organisms. However, the down side of this process may be propagation of long-lived cellular and epigenetic changes that impair intestinal homeostasis and predispose to IBD, cancer, and other diseases. Recent studies into the cellular processes that underlie crypt repair, including defining specific epithelial and mesenchymal cell types that participate, have great potential to allow for manipulation of these processes to prevent and treat these diseases.

AUTHOR CONTRIBUTIONS

WR conceived and wrote majority of the manuscript. RT and EY assisted with research and produced the figures. NZ provided important scientific content and contributed to editing. TS provided financial support, wrote portions of the manuscript, and edited the manuscript and figures. All the authors contributed to the article and approved the submitted version.

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Hypoxia in Cell Reprogramming and the Epigenetic Regulations

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Cellular reprogramming is a fundamental topic in the research of stem cells and molecular biology. It is widely investigated and its understanding is crucial for learning about different aspects of development such as cell proliferation, determination of cell fate and stem cell renewal. Other factors involved during development include hypoxia and epigenetics, which play major roles in the development of tissues and organs. This review will discuss the involvement of hypoxia and epigenetics in the regulation of cellular reprogramming and how interplay between each factor can contribute to different cellular functions as well as tissue regeneration.

Keywords: hypoxia, epigenetic, muscle, cellular reprogramming, stem cells

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INTRODUCTION

When Takahashi and Yamanaka were able to reprogram a fully differentiated skin cell into an induced pluripotent stem cell (iPSC), the promising potential of cellular reprogramming became evident. Through activation of critical transcription factors Oct4, Sox2, Klf4, and c-Myc (Yamanaka factors), any somatic cell could be reprogrammed into a pluripotent stem cell (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). The implications of this discovery are already applied to the development of therapies targeting macular degeneration, spinal cord injuries, acute myeloid leukemia, and more (Chichagova et al., 2018; Maali et al., 2018; Nagoshi et al., 2019). However, the initial method of cellular reprogramming because <1% of the cells committed to reprogramming actually gained pluripotency (Takahashi et al., 2007). This inefficiency necessitates that better methods of cellular reprogramming be developed for practical use.

One such method is the culturing of cells in hypoxic conditions. In early development, before the formation of the placenta, the developing embryo is exposed to an environment that varies between 1 and 5% O_2 a concentration much lower than the physiologically normal 20–21% O_2 (Yoshida et al., 2009). When the developing embryo reaches the blastocyst stage, embryonic stem cells (ESCs) are extracted from an inner cell mass (ICM) contained within an outer layer of cells (Thomson et al., 1998; Schrode et al., 2013). ESCs are pluripotent and display high expression of the original Yamanaka factors as well as other transcription factors: Nanog and Lin28A (Moss and Tang, 2003; Pan and Thomson, 2007; Yu et al., 2007).

In an experiment done to test the effect of hypoxia on the production of induced pluripotent stem cells (iPSCs), both mouse embryonic fibroblasts and human dermal fibroblasts were transduced with Yamanaka factors and cultured under normoxia (21% O_2) or hypoxia (5% O_2). Not only did the hypoxia treated cells grow faster, production of iPSCs significantly increased when cells were cultured under 5% O_2 at all periods of induction (Yoshida et al., 2009). In another research, a low oxygen tension (5% O_2) preferentially resulted in the maintenance of a highly

proliferative, pluripotent population of human ESCs; Moreover, the culture at atmospheric 20% O2 levels expressed significantly less OCT4, SOX2, and NANOG than those maintained at 5% O2 in the hESCs (Forristal et al., 2010).

HYPOXIA AND ITS ROLE IN GENE TRANSCRIPTION

The initiation of hypoxia results in an upregulation of transcription factors called Hypoxia-Inducible Factors (HIFs). HIFs are activated in response certain environmental conditions, including a low oxygen environment and in inflammation (Lee et al., 2020). HIFs are composed of multiple transcription factors: HIF-1, HIF-2, and HIF-3.

HIF-1α is the main driver of the cellular response in low oxygen environments. At normal oxygen levels (20–21% O_2), enzymes called prolyl-hydroxylases (PHDs) hydroxylate HIF-1α at proline residues 402 and 564. This modification tags HIF-1α for ubiquitination by the E3 ubiquitin ligase complex, which in turn tags it for degradation by the proteasome (Koivunen and Kietzmann, 2018). During hypoxia, the oxygen that is required for hydroxylation, the initial step of degradation, is not present and therefore HIF-1α is able to remain intact and functional. HIF-1α accumulates within the cell and dimerizes with the HIF-1β subunit forming a heterodimeric HIF-1α/HIF-1β complex. Afterwards, the complex translocates to the nucleus, where it binds to hypoxia response elements (HREs) and upregulates the expression of its target genes (Sadaghianloo et al., 2020). HIF-1α has been implicated in the preservation and adjustment of the initial physiological response to hypoxia and plays a vital role in the body's viability in situations where the oxygen level is low. Examples of such adjustments include increasing angiogenesis and glycolysis while decreasing cell proliferation (Majmundar et al., 2010). The HIF-2 mechanism of action works similarly to HIF-1. HIF-2α is unstable at physiologically normal (20%) oxygen levels because of hydroxylation at their proline residues. During hypoxia, HIF-2α dimerizes with HIF-1β and translocates to target genes in the nucleus and upregulates the expression of target genes. The difference between HIF-1 and HIF-2 lies in their target genes (Carroll and Ashcroft, 2006). HIF-2α binds to HREs upstream of cellular reprogramming genes Oct4, Sox2, Nanog, and significantly increases their expression (Covello et al., 2006; Forristal et al., 2010). HIF-2α has also been shown to upregulate c-Myc expression by modulating its interactions with cell cycle proteins and is associated with the upregulation of Klf4 (Gordan et al., 2007).

There are many studies indicating that hypoxia regulates gene transcription and expression mostly through the HIF signaling pathways. Hypoxia stimulates the transcription of genes that help to restore oxygen levels or energy productions, in which HIF responds and regulates gene transcription. Hypoxia as an environmental factor can increase muscle stem cells (MuSCs) renewal through its transcription factor HIF-1α and the Notch pathways (Liu et al., 2012; Yang et al., 2017). There is increasing evidence that hypoxia causes reprogramming of other cells, such as dendritic cells, neural cells, and muscle cells (Bosco and Varesio, 2012; Mathieu et al., 2013; Vojnits et al., 2015). The HIF1α pathway is discovered as an enabling regulator of cellular reprogramming through early glycolytic shift and upregulation of PDK1-3 and PKM2. In fact, HIF1 controls the transcription of many target genes to initiate metabolic changes in the early stage and maintains glycolytic metabolism in the later phase of the iPSC reprogramming. Hypoxia is known to enhance the efficiency of reprogramming in the development of iPSCs by upregulating the expression of transcription factors associated with cellular reprogramming and changing the topography of chromatin (Yoshida et al., 2009; Bosco and Varesio, 2012; Mimura et al., 2012; Mathieu et al., 2014; Wang et al., 2016; Alderman et al., 2019).

HYPOXIA AND ITS ROLE IN DEVELOPMENT

There is a large amount of evidence that hypoxia and HIF play a role in pre-embryonic development (Dunwoodie, 2009). In the developing placenta, the knockdown of HIF-1α and HIF-2α results in failure of its formation. Trophoblast invasion, one of essential stages for placentation and pregnancy outcome, likely occurs in a hypoxic environment. Studies have indicated the hypoxia is able to induce 10–11 translocation methylcytosine dioxygenase 1 (TET1) expression that facilitates trophoblast cell migration and invasion through HIF-1α signaling pathways in the early pregnancy (Koklanaris et al., 2006; Zhu et al., 2017).

More specifically, knockdown of HIF-1α prevents the fusion of the chorion and the allantois which normally fuse together to form the placenta. Since the chorion and the allantois are not harmed by the knockdown of HIFs, this suggests that HIF plays a role in the integrins that regulate this formation. When exposed to hypoxia, the expression of some of these integrins increased (Dunwoodie, 2009). Additional research suggests that the lack of oxygen and the subsequent upregulation of HIFs plays a vital role in branching morphogenesis, the process that lays down the foundation for the development of key organs such as the nervous system, the respiratory system, the kidney, the salivary glands and the mammary glands (Dunwoodie, 2009; Tsuji et al., 2014). When HIF is knocked down, vascularization of the labyrinthine layer of the placenta decreases, preventing nutrients from the maternal circulatory system to reach the fetal one (Dunwoodie, 2009).

Further along in placental development, HIF is also involved in the formation of the trophoblast (Dunwoodie, 2009). The trophoblast is the outer layer of the developing blastocyst and plays a key role in nourishing the embryo with nutrients. During pre-embryonic development, the trophoblast is attached to the uterine lining and trophoblast cells physically bordering the lining express high amounts of HIF-1α and HIF-2α. When both subunits are knocked out, the formation of trophoblast subgroups within the trophoblast are inhibited, suggesting that HIF plays a vital role in trophoblast proliferation. As the embryo starts to form into a fetus, hypoxia again plays a major role in the development of major organs. Recent research suggests that HIF also interacts with the Notch signaling pathway. During

hypoxia, the HIF complex can attach itself to a Notch receptor and induce notch signaling (Hu et al., 2014). Notch signaling is implicated in the determination of cell fate and HIF induced notch signaling has proven to be involved in determining blood cell fate as well as determining neural cell fate. Lastly, hypoxia and its corresponding HIFs have shown to play a major role in bone formation, chondrogenesis, heart formation, angiogenesis and formation of the neural crest (Rankin et al., 2011; Lee et al., 2013; Muz et al., 2015; Scully et al., 2016). Overall, HIFs play a significant part in promoting cell proliferation and differentiation of multiple cells during the development of organisms.

HYPOXIA AND ITS ROLE IN DISEASE

Other than development, hypoxia may play a role in other physiological events characterized by rapid proliferation and differentiation. Many have theorized of a link between the regulation of ESCs and the regulation of cancer stem cells (CSCs). In cancers, the rapid outgrowth of cells and their consumption of oxygen overruns the limited supply of oxygen and creates a hypoxic environment in many areas of the tumor (Muz et al., 2015). The resulting increase in HIFs plays an integral role in cancer pathogenesis by upregulating various transcription factors involved in angiogenesis and cell proliferation (Muz et al., 2015). Some state that this hypoxic environment is also favorable for the cellular reprogramming of non-stem like cancer cells into cancer-stem cells. Heddleston et al. transduced HIF-2α into non-stem like glioma cells and discovered that the expression of Oct4, Nanog, and c-Myc increased in HIF-2α transduced cells (Heddleston et al., 2009). The subsequent injection of these cells into immunocompromised mice created significantly larger tumors compared to the control. Further research supports the notion that the chemoresistance of gliomas is also a result of hypoxia induced cellular reprogramming. In tests comparing the sensitivity of glioma associated cells to chemotherapy, adult non-stem like glioma cells showed sensitivity to treatment while glioma stem cells (GSCs) remained unaffected (Wang et al., 2017a). The origin of the GSCs have been debated as some believed that GSCs were derived through contamination from cells already positive for CD133 (GSC marker) while others believed they were a result of hypoxia induced reprogramming of already present $CD133^-$ glioma cells. In experiments conducted by Wang et al., they provide evidence it is the latter (Wang et al., 2017b). When $CD133^-$ glioma cells were cultured under hypoxia, expression of CD133 increased and signs of GSC behavior such as the ability to asymmetrically divide and the ability to form neurospheres appeared. Hypoxia cultured cells also showed increased expression of ABCG2 and MGMT, proteins associated with increased chemoresistance.

The presence of cells with stem-like properties after hypoxia is not confined to gliomas. Stem-like cells induced from hypoxia have been discovered in cells associated with lung cancer and liver cancer. In non-oncologic fields, increasing amounts of evidence suggest that hypoxia induces the reprogramming of resident muscle cells after injury. Novel multipotent cells have been discovered in the tibialis anterior (TA) muscle in mice after a laceration injury disrupted the vascular structure (Mu et al., 2011). These cells were termed as injury-induced muscle-derived stem cells or iMuSCs and have shown the ability to differentiate into cells from all three germ layers and form neurospheres (Vojnits et al., 2015, 2017). Similar cells, termed ischemia-induced multipotent stem cells or iSCs, have also been discovered in the brain of elderly patients after an ischemic stroke (Tatebayashi et al., 2017). The multipotent or pluripotent nature of iMuSCs and iSCs and their presence in tissues after injury point to the therapeutic potential of hypoxia induced cellular reprogramming in muscle and neural regeneration. However, like the aforementioned GSCs, some may argue that the presence of iMuSCs and iSCs can be the result of contamination from circulating cells. *In vitro* studies that can replicate the formation of such cells through hypoxia or studies identifying the mechanism behind their reprogramming will be useful in clarifying their roles.

Epigenetics in SCNT and iPSCs Reprogramming

Another major factor involved in the development of stem cells is epigenetics. Epigenetics refers to the cellular machinery that controls gene expression and occurs through the addition or deletion of epigenetic modifications (Handy et al., 2011). Epigenetic modifications refer to any heritable modifications that are made to the DNA or histones. The most basic of these are DNA and histone methylation, but other forms of modification can occur in histones such as acetylation, phosphorylation, ubiquitination, sumoylation etc. These modifications alter the accessibility of chromatin to transcription factors and polymerases that play a role in the transcription of DNA and expression of genes (Handy et al., 2011). Epigenetic modifications play major roles in development and in the development of disease (Portela and Esteller, 2010).

DNA methylation in general exists as a suppression mechanism for many genes. As organisms develop from the embryo into a fetus, areas in the genome with large amounts of CG dinucleotide repeats, known as CpG islands, experience methylation on the cytosine residue (Portela and Esteller, 2010; Handy et al., 2011). This hypothetically serves to suppress pluripotency at critical genes as pluripotent embryonic stem cells differentiate into their preferred cells and organ systems. The methyl groups block transcription by blocking the attachment of transcription factors onto the DNA segment. Interestingly, methyl groups have also shown to block HIF-1 from attaching to its HRE to regulate erythropoietin transcription (Handy et al., 2011). In addition to blocking transcription factors and polymerases, methyl groups are targeted by MeCP2 proteins, which recruit histone deacetylases (HDACs) to condense the chromatin and prevent transcription (Portela and Esteller, 2010; Handy et al., 2011).

Histones modifications have wide-ranging effects. Histones serve as molecular chaperones and organize DNA into structures called nucleosomes, which consist of 5 subunits, H1, H2A, H2B, H3, and H4. The subunits H2A, H2B, H3, and H4 consist of the core proteins and are bound together to form "beads" in

which DNA strands wrap around. H1 serves as the support that keeps the DNA strands and the core histones in place. The number of histone subunits relates directly to the diversity of epigenetic modifications found in histones. Each core histone subunit has a multitude of modifications the most common of which are found in H3 (Handy et al., 2011). H3 modifications are widely studied and have a significant impact on the activation and repression of genes. The nomenclature of these modifications proceeds in the following order: the subunit of the histone, the amino acid affected, the position of the amino acid, and the type of modification applied. For instance, H3K27me3 indicates a trimethylation at H3 on Lysine 27. Some commonly observed modifications on H3 are H3K4me1, H4K4me3, H3K36me3, H3K79me2, H3K9Ac, H3K27Ac, all of which are associated with the opening up of the chromatin structure and gene activation by transcription factors and polymerase. In contract, H3K9me3 and H3K27me3 are associated with the condensation of chromatin and the blocking of gene expression. It has been found that H3K27me3 imprinting defects impede post-implantation development (Matoba et al., 2018). H3K9me3 and H3K4me3 might also affect transcriptional reprogramming and thus impair the developmental potential of SCNT embryos (Matoba and Zhang, 2018). H3K9me3 also showed to be implicated in the majority of barriers to the Somatic Cell Nuclear Transfer (SCNT) and iPSCs reprogramming. Those data suggest the essential, diverse set of roles of epigenetics in cellular reprogramming (Wang et al., 2018).

Epigenetic Modifications in Stem Cell Potency

Histone modifications are involved in development to set up genes for activation during lineage commitment by H3K4me3 and to repress lineage control genes to maintain pluripotency by H3K27me3 (Shipony et al., 2014). The balance and interaction between these pathways are essential for stem cell homeostasis and are directly linked to cellular behaviors. H3K27me3 and PRC2 each contribute to epigenetically transmitting the memory of repression across generations and during development (Juan et al., 2011; Stojic et al., 2011). H3K27me3 and H3K4me3 promoter bivalency are observed in stem cells and their differentiation, which include embryonic and iPSCs (Liu et al., 2013, 2016; Leschik et al., 2015). Bivalency has a prominent role in post-implantation embryonic development or post-natal organ growth. However, the role of epigenetic regulation in the function of adult tissue stem cells is less well-understood. It has been discovered H3K27ac (acetylation) regulates cell reprogramming and mouse ES cell differentiation as well as MyoD expression (Mattout et al., 2011; Khilji et al., 2018; Martire et al., 2019). The effects of epigenetic dysregulation on adult stem cell function depend on the tissue type and the epigenetic regulator affected. Recently studies have increased our understanding of the epigenetic regulations during MuSCs maintenance, activation, differentiation, and homeostasis (**Figure 1**) (Liu et al., 2013; Kosan et al., 2018).

In the studies of bone marrow mesenchymal stem cells (MSCs), results indicate that hypoxia could induce senescence of MSCs via altered gut microbiota (Xing et al., 2018). However, most reports suggested that hypoxia inhibits senescence of MSCs and maintains their properties (Tsai et al., 2011; Kwon et al., 2017; Korski et al., 2019). In the cardiovascular system, hypoxia involves resident cell senescence either via promotion or prevention of the processes, suggesting the signaling pathways are complex (Korski et al., 2019; Lewinska et al., 2020). It also suggests that the level of O2 may determine the final role of hypoxic environment. Epigenetic events are essential to establish and maintain the distinct cell lineages and have been shown to be crucial players controlling the fate and function of MSCs and MuSCs(Nakade et al., 2017; Zhang et al., 2019). More recently, a study showed MuSCs forced to express Msx1 exhibited increased proliferation index as well as promoted SSEA1 and Pax7 expression, but restricted MyoD expression and accessed osteogenic genes (Ding et al., 2017).

HYPOXIA IMPACTS HISTONE MODIFICATION AND EPIGENETIC REGULATION

The Polycomb repressive complex 2 or PRC2 is a histone methyltransferase complex and functions as a transcriptional corepressor (Lee et al., 2006). PRC2 consists of three subunits, SUZ12, EED, and EZH2. EZH2 is the catalytic subunit of PRC2 that can mono-, di-, and trimethylate H3K27 (Conway et al., 2015). In ESCs, PRC2 has been shown to occupy genes that are essential for differentiation and development, and represses those genes by depositing the repressive H3K27me3 mark. When cells undergo differentiation, these genes exhibit a loss of PRC2 occupancy and a loss H3K27 trimethylation. Depletion of PRC2 or its associated subunits in ESCs or mice results in the disappearance of pluripotency, the displacement of H3K27me3, and premature differentiation during early development (Koppens et al., 2016; Shan et al., 2017). PRC2 KO mice are non-viable and die early in development (Pasini et al., 2004). These results suggest that PRC2 plays a crucial role during early development by repressing select developmental genes and maintaining ESC pluripotency.

PRC2 subunits are frequently mutated in cancers (Lee et al., 2006; Conway et al., 2015; Laugesen et al., 2016; Veneti et al., 2017). These mutations are observed in a variety of cancers such as breast cancer, colorectal cancer, hepatocellular carcinoma and glioblastoma. Most of the PRC2 mutations are inactivation mutations, leading to downregulation of H3K27me3 levels. While H3K27me3 is downregulated, it is never fully eliminated and in fact may appear in greater concentrations in select areas of the genome. In some other cancers, such as lymphomas, EZH2 has hot-spot gain of function mutations. In this case, levels of H3K27me3 actually increased and differentiation is blocked. This points to the idea that oncogenic PRC2 mutations redistribute H3K27me3 from genes that cause cancer and redistribute them toward genes that control differentiation (**Figure 2**) (Conway et al., 2015).

Another histone methylation notable for its involvement in pluripotency is H3K4me3. H3K4me3 is an active mark

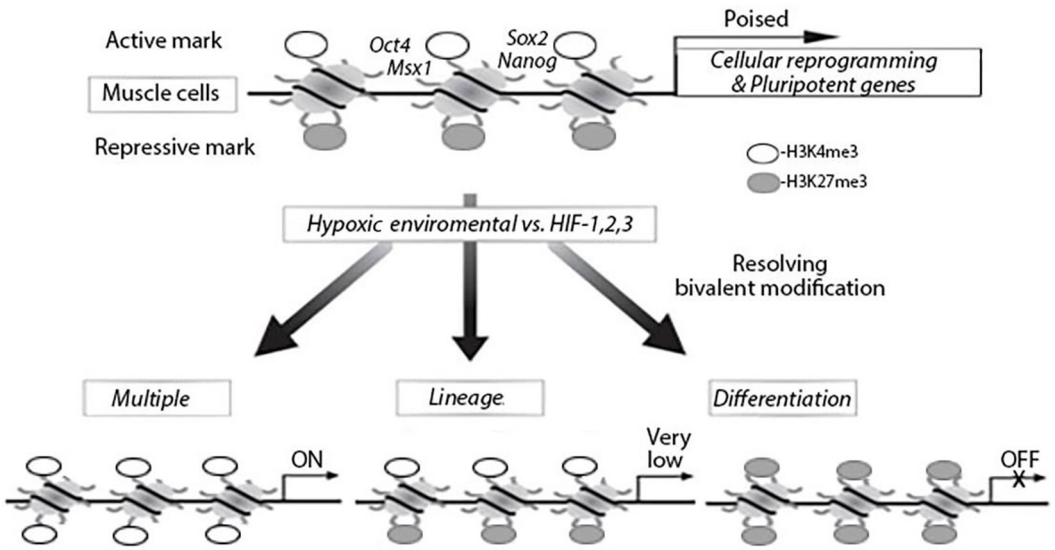

FIGURE 1 | Bivalent modification status of genes in muscle cells in hypoxia environment, myogenetic differentiation and cellular reprogramming. In muscle cells, cellular reprograming genes and pluripotent genes are epigenetically modified with bivalent modifications (H3K4me3 and H3K27me3) and are poised for expression. Under hypoxia environment, the bivalent modifications are resolved; genes with H3K27me3 remained are silenced and genes with H3K4me3 remained are actively expressed.

enriched at gene promoters. There is a set of genes that contain both H3K4me3 and H3K27me3 on their promoters, known as bivalent domains (Bernstein et al., 2006). The presence of both H3K4me3 and H3K37me3 at the developmental regulator genes puts these genes under bivalent epigenetic control. When ESCs undergo differentiation, H3K27me3 disappear from the region and is increasingly concentrated with the activating H3K4me3. On a fully differentiated gene, its promoter is only populated with H3K4me3. Downregulation of H3K27me3 via EZH2 knockout in ESCs or muscle stem cells forces the cell to undergo differentiation (Yu et al., 2018; Wang et al., 2019). These findings suggest that bivalent chromatin plays a critical role in the maintenance of stem cell pluripotency and regulation of stem cell differentiation. There are multiple interactions of epigenetic regulations during cellular reprogramming of muscle stem cells in a hypoxia environment. Hypoxia vs. active HIF-1α can increase genome-wide bivalent epigenetic making that could be an initial stimulator in cellular reprogramming. These epigenetic modifications and chromatin states may not only regulate cellular reprogramming but also normalize the multiple differentiation in a different way to support muscle regeneration (Liu et al., 2013; Faralli et al., 2016).

Hypoxia Influences Epigenetics Modifications in Cellular Reprogramming

Recent studies have shown that hypoxia can affect the level of epigenetic modifications in cells. In breast epithelial adenocarcinoma MCF7 cells, hypoxia increased the levels of H3K27me3 globally and increased the number of genes under bivalent epigenetic control by increasing levels of H3K4me3 in H3K27me3 concentrated areas (Prickaerts et al., 2016). When reoxygenated, many of the genes retained the bivalency and conferred an epigenetic profile similar to those of embryonic stem cells. Activity of the H3K27me3 demethylase KDM6B/JMJD3 decreased as well, indicating hypoxic control of H3K27me3 demethylation (Prickaerts et al., 2016). In HeLa cells and human fibroblasts, hypoxia increases the levels of H3K4me3 in enhancers of genes associated with cell division and oxidative phosphorylation (Batie et al., 2019). The increase in histone marks at select genes associated with cell proliferation and differentiation matches favorably with the increase in transcription factors, Oct4, Sox2, and Nanog associated with cellular reprogramming. In fact, the addition of Oct4, Sox2, and Nanog to mesenchymal stem cells results in an increase in cell proliferation (Han et al., 2014; Park et al., 2019). A similar increase in cell proliferation was seen when treating mesenchymal stem cells under hypoxia (Kwon et al., 2017).

The co-occupancy of transcription factors suggest the existence of a relationship between hypoxia and epigenetics when regulating cellular reprogramming (**Figure 3**). Several studies have studied this relationship. One example is the role of hypoxia in Epithelial-mesenchymal transition (EMT). EMT is the process that occurs when epithelial cells undergo reprogramming to become mesenchymal stem cells (Larue and Bellacosa, 2005). Mesenchymal stem cells are multipotent and play crucial roles in early embryonic development and the development of organs. Under hypoxia, promoters of epithelial genes e-cadherin and plakoglobin display decreased levels of H3K4 acetylation (H3K4ac) and increased levels of H3K27me3 (Wu et al., 2011). In contrast, promoters of mesenchymal genes

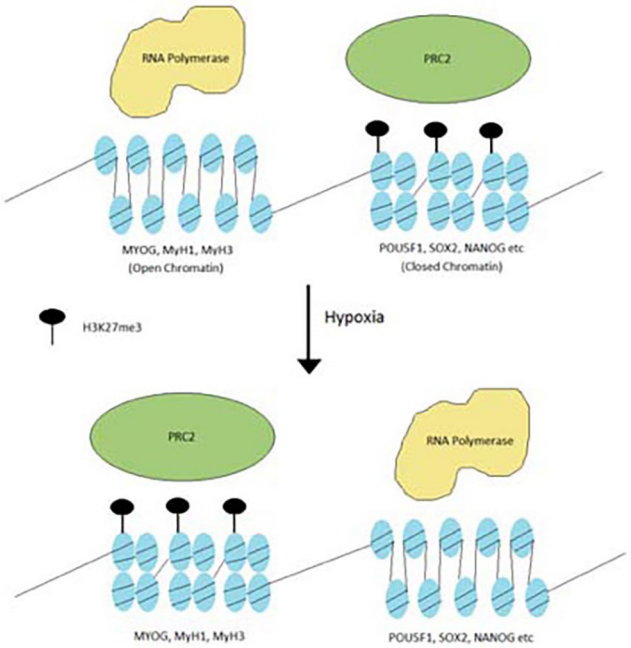

FIGURE 2 | Redistribution of H3K27me3 under hypoxia. Hypoxia stimulates the PRC2 complex to redistribute H3K27me3 from oncogenic genes to differentiation genes.

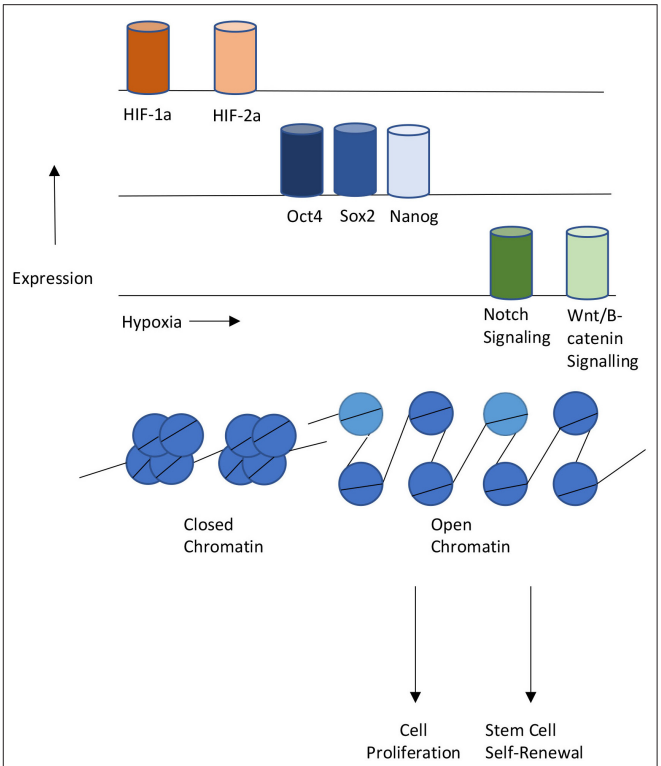

FIGURE 3 | Interplays between Hypoxia, Epigenetics, and Cellular Reprogramming. Hypoxia or HIF-1,2 activation stimulates pluripotent gene expression and the Notch and Wnt signaling pathways, Changes in chromatin states also contribute to gene expression changes that lead to proliferation or self-renewal of stem cells.

N-cadherin and vimentin showed decreased levels of H3K27me3. In the reprogramming of cancer stem cells such as GSCs, hypoxia has shown to induce the activity of TET1 and TET3 (Prasad et al., 2017). TET1 and TET3 bind to Oct4 and Nanog regulatory regions and increase the activity of each transcription factor, thereby promoting the reprogramming of glioma cells to glioma stem cells.

Another promising area of research is the impact of hypoxia on the regulation of telomerase and the telomere. Telomeres are linear guanine-rich DNA structures at the ends of chromosomes, and their length determines the behaviors and life in many cells such as germ cells, cancer cells, and pluripotent stem cells. Telomeric DNA is synthesized by way of the ribonucleoprotein called telomerase, which contains a reverse transcriptase (TERT) subunit and an RNA component. Telomeres and TERT are essentially in iPSCs induction and maintenance (Teichroeb et al., 2016). TERT is highly conserved across species and ubiquitously present in pluripotent cells. Moreover, the TERT transcription can be enhanced by DNA methylation at the TERT-DMR via binding to nuclear lamina during cellular reprogramming (Takasawa et al., 2018). Hypoxia can promote telomerase TERT expression and HIF-1α mediates upregulation of TERT (Yu et al., 2006; Song et al., 2020). This has a significant impact on cellular reprogramming from somatic cells into pluripotent stem cells.

Other Epigenetic Factors in Hypoxia

While the great majority of this review is focused on histone methylation, the potential role of histone acetylation and cellular reprogramming should not be ignored. An example of the impact of acetylation is the rapid acetylation that occurs after fertilization with the somatic nucleus. Before conception, the histones of mouse oocytes do not display any acetylation (Kim et al., 2003). However, if the same oocyte is implanted with a nucleus from a somatic cell, the histones become rapidly acetylated. This procedure is known as SCNT and through this process a permanently differentiated somatic cell undergoes cellular reprogramming to become a totipotent stem cell (Wilmut et al., 1997, 2002). This rapid acetylation that occurs after fertilization with the somatic nucleus indicates that acetylation plays a role in cellular reprogramming. More recently, a report suggests that reprogramming of H3K9ac is important for optimal SCNT efficiency and identifies Dux as a crucial transcription factor in this process (Yang et al., 2020). Despite the rapid acetylation, many of the fertilized oocytes reverted back to their previous unacetylated state 1–3 h after fertilization and the reprogramming is inefficient. In a study done by Rybouchkin et al. only 34% of the oocytes reached the blastocyst stage. However, when a histone decacetylase inhibitor (Trichostatin) was introduced during fertilization, a staggering 81% of the treated oocytes were able to reach the blastocyst stage compared to 41% of the non-treated oocytes (Rybouchkin et al., 2006). Another histone deacetylase inhibitor, valproic acid, found similar results (Huangfu et al., 2008). These studies

indicate that inhibition of histone deacetylation can enhance cellular reprogramming.

Hypoxia has been proven to modulate histone acetylation by increasing histone acetylation at H3 and regulating the downstream processes of many genes. In addition, the initiation of hypoxia and induction of HIF-1α can increase the acetylation of H3 and H4 in neuroblastoma cells and increases the aggressiveness of neuroblastomas (Poljakova et al., 2014). A potential, exciting future area of research would be investigation to see if histone acetylation can play a role in cellular reprogramming of cancer cells to cancer stem cells.

LOOKING AHEAD

Hypoxia stimulation greatly affects gene expression and is considered to play an essential role in early embryo development, cell differentiation, and cellular reprogramming. Meanwhile, new techniques have been introduced to this field, such as the development of next-generation sequencing, single cell RNA sequencing, and micro-omics technologies. Recent studies have clearly depicted certain epigenetic changes, including DNA methylation, hydroxymethylation, histone modifications, organoids and 3D structure formation during early embryo development, iPSCs, and somatic cell reprogramming. There is potential for the use of the interplay of hypoxia and epigenetics in regenerative medicine. The existence of reprogrammed cells after hypoxia has been reported in multiple different types of tissue and organs (Shyh-Chang et al., 2013; Mosteiro et al., 2016). These conditional ischemic events via low oxygen stimulation are an intriguing prospect for future investigation. They suggest that a low oxygen environment may contribute or regulate the processes of tissue regeneration (Fang et al., 2018; Huels and Medema, 2018; Yui et al., 2018). There is undeniable involvement of HIF signaling and epigenetics during events that determine cell fate and cause rapid proliferation and differentiation. There is also evidence of epigenetic modifications at play in muscle regeneration and during differentiation of primary myogenic stem cells (Liu et al., 2013; Faralli et al., 2016). These factors and their association with tissue regeneration is potentially a new field for further study. Additionally, epigenetics have played a significant role in recent drug design (Harrison, 2012). Obviously, there will be a large benefit from the use of these epigenetic drugs or peptides to directly modulate the epigenome and normalize tissue regeneration.

AUTHOR CONTRIBUTIONS

NN drafted the manuscript and created the figures. XS discussed and edited the manuscript. RD revised and edited the manuscript. YL searched literature, structured, modified figures, and wrote and edited the manuscript. All author approved the final version of the manuscript.

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REVIEW
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Bone Cells Differentiation: How CFTR Mutations May Rule the Game of Stem Cells Commitment?

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Cystic fibrosis (CF)-related bone disease has emerged as a significant comorbidity of CF and is characterized by decreased bone formation and increased bone resorption. Both osteoblast and osteoclast differentiations are impacted by cystic fibrosis transmembrane conductance regulator (CFTR) mutations. The defect of CFTR chloride channel or the loss of CFTR's ability to interact with other proteins affect several signaling pathways involved in stem cell differentiation and the commitment of these cells toward bone lineages. Specifically, TGF-β, nuclear factor-kappa B (NF-κB), PI3K/AKT, and MAPK/ERK signaling are disturbed by CFTR mutations, thus perturbing stem cell differentiation. High inflammation in patients changes myeloid lineage secretion, affecting both myeloid and mesenchymal differentiation. In osteoblast, Wnt signaling is impacted, resulting in consequences for both bone formation and resorption. Finally, CFTR could also have a direct role in osteoclast's resorptive function. In this review, we summarize the existing literature on the role of CFTR mutations on the commitment of induced pluripotent stem cells to bone cells.

Keywords: cystic fibrosis, iPSCs, bone differentiation, osteoblast, osteoclast

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INTRODUCTION

Cystic fibrosis (CF) is the most common autosomal recessive genetic disorder in Caucasians, affecting 75,000 patients world-wide and occurring in approximately 1 in 3,500 newborns in the United States (Farrell et al., 2008). This disease is caused by a mutation of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. To date, more than 2,000 mutations have been identified, resulting in the absence or dysfunction of CFTR protein: a transmembrane chloride channel, mainly known to be involved in maintaining the proper composition and amount of fluid covering different mucosal membranes in the body (Castellani et al., 2008). CFTR mutations are organized by classes based on the mechanism affected: defect of synthesis, defect of traffic through the membrane, defect of protein folding, impairment of gating, defect of conductance or defect of stability (Rowe et al., 2005). The most common mutation, accounting for approximately 80% of CF cases, is the F508del, which is a class II mutation where the protein is misfolded and destroyed before reaching the membrane. The second most common mutation, representing around 5% of total CF mutations, is the pre-mature stop mutation G542X, causing early termination of translation (Du et al., 2002). This is followed in frequency by the G551D mutation, which causes a gating defect in the CFTR channel, accounting for less than 5% of CF cases. The remaining mutations, although numerous (more than 2000), represent less than 2% of the CF mutations.

The most common and lethal manifestation of CF disease is respiratory impairment resulting from defective mucociliary clearance, bacterial infection, airway inflammation, mucus accumulation and airflow obstruction (Stoltz et al., 2015). Due to improved therapies, optimization of nutrition, and early established healthcare for children, the life expectancy of CF patients has improved significantly, thus resulting in the emergence of new comorbidities associated with the pathology: pancreatic disease and cystic fibrosis-related diabetes (CFRD), hepatobiliary disease, gastrointestinal tract, kidney disease, genitourinary disease, cystic fibrosis-related bone disease (CFBD), and coronary artery disease (Elborn et al., 2016; Ronan et al., 2017).

Cystic fibrosis-related bone disease occurs in 20–35% of adults with CF, in which patients present with low bone density and osteoporosis. Prior to the evidence that CFTR dysfunction influences bone cell activity (Dif et al., 2004; Stalvey et al., 2013; Velard et al., 2014; Le Henaff et al., 2015), many of these bone defects were believed to be a result of malnutrition, sedentary lifestyle, endocrine disease, pancreatic insufficiency, delayed puberty, vitamin D and K insufficiency, calcium malabsorption, and/or use of exogenous glucocorticoids (Plant et al., 2013; Jacquot et al., 2016; Putman et al., 2019). Although it has now been established that absence or abnormal CFTR protein plays a role in bone disease, the pathways underlying the onset of CFBD remain elusive. The role of CFTR in bone cells have been determined using animal models (mice, rats, and sheep) or *in vitro* culture models using cells derived from patient biopsies. However, knowledge gained from these models remain limited due to model relevance (animal vs. human) as well as the limited accessibility to human bone biopsies from CF patients. Moreover, bone formation is initiated *in utero* and take several months to years to become a fully mature bone structure (Katsimbri, 2017). Thus, disorders resulting from CFTR absence or dysfunction may occur during embryogenesis, perturbing stem cells commitment toward bone cells. Therefore, the use of induced pluripotent stem cells (iPSCs) might represent great promise and a readily available alternative to study the effects of CFTR mutations on bone cell development. Furthermore, CF-iPSCs and "healthy" iPSCs, *via* CRISPR/Cas9-mediated correction of the *CFTR* gene, can be generated and compared from the same patient, providing controls with identical genetic background.

Induced pluripotent stem cells provide an opportunity to develop any cell type from an easily accessible somatic cell source. Human iPSCs can be generated from a wide spectrum of somatic cells, including fibroblasts, keratinocytes, mesenchymal stem cells (MSCs) or peripheral blood mononuclear cells (Abdal Dayem et al., 2019). Pluripotency is induced with a combination of reprogramming factors: OCT3/4, SOX2, KLF4, L-MYC, LIN28, and shRNA for TP53 (Okita et al., 2013). iPSCs have normal karyotypes, maintain telomerase activity, express characteristic cell surface markers and genes of human embryonic stem cells (ESCs), possess high self-renewal capacity and maintain the developmental potential to differentiate into mature cells of all three primary germ layers (Yu et al., 2007).

This review explores the main pathways involved in osteoblast and osteoclast differentiation from iPSCs and summarizes which pathways are known to be impacted by CFTR absence or malfunction.

iPSCs COMMITMENT TOWARD MESENCHYMAL STEM CELLS AND HEMATOPOIETIC LINEAGE

Bone cells derive from hematopoietic and mesenchymal precursor cells. From the undifferentiated pluripotent stage until terminal differentiation forming osteoblasts, osteocytes and osteoclasts, the two lineages are related. Sacchetti et al. (2007) proved the self-renewal potential of osteoprogenitors in bone marrow sinusoids by showing their capacity to organize the hematopoietic microenvironment, suggesting the link between MSCs and myeloid ones. MSCs express angiogenic proteins, allowing the formation of cartilage, which in turn induce a better angiogenesis. On the other hand, monocytes or osteoclast precursors can differentiate into mature osteoclasts. Osteoclast resorption along with matrix mineralization promote osteogenic differentiation, which in turn regulate the osteoclast commitment and bone resorption (LoGuidice et al., 2016). Myeloid and lymphoid cells circulating in blood and MSCs in bone marrow secrete factors essential for stem cell renewal or differentiation, osteoblasts and osteoclasts differentiation thus regulating bone formation or resorption. Finally, the mature bone cells also secrete components of the different pathways allowing bone modeling and remodeling.

Generation and Characterization of MSCs and Hematopoietic Lineage From iPSCs

Mesenchymal stem cells are pluripotent cells able to differentiate into a variety of mature cell types: adipocytes, myocytes, chondrocytes, and osteoblasts. The differentiation potential of iPSCs into functional MSCs can be achieved using different methods: use of growth factors [basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF-α/β)] in combination with CD24−/CD105+ sorting, repeated passage with trypsinization, culture in hypoxic condition with growth factors, embryoid bodies (EBs) formation, biomimetic, fibrillar, type I collagen coatings, and use of small molecule inhibitors [such as transforming growth factor-β (TGF-β) pathway inhibitor)] (Abdal Dayem et al., 2019). However, each method presents with its own set of advantages and disadvantages such as time-consuming laborious techniques, efficacy of differentiation and tumorigenicity of iPSC-induced cells (Abdal Dayem et al., 2019). The International Society for Cellular Therapy (ISCT) proposed the following basic criteria to characterize MSCs (Dominici et al., 2006): exhibition of typical fibroblastic cell morphology, expression of MSC surface markers CD44, CD73, and CD105, and the potential to differentiate into the three different cell lineages: osteoblasts, chondrocytes and adipocytes.

Different protocols have been used to differentiate iPSCs into myeloid cells, pre-requisite step to osteoclast commitment.

Basically, three differentiation methods are described: co-culturing iPSCs with stromal cells, EBs formation, and monolayer cultures of iPSCs on extracellular matrix protein coated plates, such as collagen IV (Chen, 2014). The different protocols with their advantages and disadvantages were reviewed by Chen (2014).

Early mesoderm formation is indicated by the expression of *Brachyury* (*TBXT*), *MIXL1*, and *GSC* (Herrmann, 1991) in conjunction with pluripotency genes silencing. In primitive hematopoietic precursors, co-expression of *MIXL1* and *PDGFRA* genes is highly enriched (Davis et al., 2008). The final characterization of myeloid progenitors is represented by Lin-CD34+CD43+CD45+ cell population (Choi et al., 2009).

Different transcription factors are required for hematopoietic commitment. Hemangioblasts are the first differentiated mesodermal derivatives, displaying both hematopoietic and endothelial potential. Runt-related transcription factor 1 (RUNX1) is important for the hemangioblast stage and erythroid lineage commitment is RUNX1-dependent (Lacaud, 2002). In the absence of stem cell leukemia/T-cell acute lymphoblastic leukemia 1 (SCL/tal-1) transcription factor, hematopoiesis is undetectable (Porcher et al., 1996). However, SCL/tal-1 expression may be induced by the addition of bone morphogenetic protein 4 (BMP4) and VEGF. Furthermore, VEGF-R2, which is already detected in human iPSCs, is increased during mesoderm to hematopoietic lineage transition (Kennedy et al., 2007). Finally, GATA1 and GATA2 transcription factors have also been shown to be involved in hematopoietic commitment (Iwasaki et al., 2003).

The TGF-β Signaling Pathway

Members of the TGF-β superfamily, which includes TGF-β, bone morphogenetic proteins (BMPs), activins, and growth and differentiation factors (GDFs), are secreted proteins that have important roles in directing mesenchymal cell fate. By binding to transmembrane receptors with serine/threonine kinase activity (type I and type II also called activin receptor-like kinases, ALKs), the TGF-β family members initiate intracellular signaling through phosphorylation of specific SMAD proteins, which in turn translocate from the cytoplasm to the nucleus and control the transcription of target genes (Roelen and ten Dijke, 2003).

Transforming growth factor-β family has been shown to preserve cell morphology of undifferentiated ESCs by maintaining *POU5f1*, *NANOG*, *TRA-1-60*, and *SSEA4* expression, through increased phosphorylation of SMADs 2/3 (Hannan et al., 2009). Conversely, inhibition of TGF-β reduces SMADs 2/3 phosphorylation in ESCs resulting in the loss of ESC phenotype and pluripotency (Sánchez et al., 2011). TGF-β signaling (through SMAD-2/3) negatively regulates MSC generation from human ESCs. Alternatively BMP signaling promotes ESC differentiation by activating SMADs 1/5/8 (Xu et al., 2002; **Figure 1**).

Interestingly, inhibiting FGF receptor 1 (FGFR1) increases MSC differentiation without affecting cell number, apoptosis or cell cycle status, suggesting that FGF signaling plays a role in ESCs maintenance (Bendall et al., 2007). Fibroblast growth factor 2 (FGF2) is known to promote self-renewal of human ESCs by modulating the expression of TGF-β ligands: TGF-β1, GREM1 (a BMP antagonist), and BMP4 (Greber et al., 2007). Furthermore, FGF signaling interacts with the TGF-β pathway to synergistically inhibit BMP signaling, directly by repressing SMAD1/5/8 phosphorylation or indirectly by promoting SMAD2/3 phosphorylation, allowing for the maintained expression of pluripotency genes (NANOG, OCT4, and SOX2) and promoting long-term undifferentiated proliferation of human ESCs (Xu et al., 2008). In summary, self-renewal of human ESCs is promoted by TGF-β signaling, whereas differentiation is promoted by TGF-β inhibition or BMP signaling.

However, iPSCs and ESCs differ in their commitment toward MSCs, whereby iPSCs pose a greater challenge due to their resistance to SMADs 2/3 inhibition (Sánchez et al., 2011). To overcome this challenge, a TGF-β pathway inhibitor SB-431542 is added to serum-free medium, resulting in MSC differentiation from human iPSCs (Chen Y. S. et al., 2012). After 10 days, iPSCs showed downregulation of pluripotency genes and upregulation of mesodermal genes (*MSX2*, *NCAM*, and *HOXA2*), thus proving that TGF-β pathway inhibition is an efficient method for the commitment of iPSCs toward MSCs.

Other Signaling Pathways Known to Maintain Pluripotency

Other pathways such as MAPK/ERK, PI3K/AKT, and NF-κB are necessary to maintain the pluripotent and undifferentiated state of ESCs (Armstrong et al., 2006).

The RAS-Mitogen Activated Protein Kinase (MAPK) pathway transduces signals from cytokines and growth factors through Receptor Tyrosine Kinases (RTK), causing ERK1/2 to translocate to the nucleus and activate JUN and FOS transcription factors. This pathway could be activated by FGF ligands, also involved in the TGF-β pathway. MAPK/ERK pathway is active in undifferentiated human ESCs and upon differentiation, several components of this pathway are downregulated such as RASAL2, SOS1, RAF, MAP2K6, or KRAS (Armstrong et al., 2006).

The PI3K/AKT pathway is activated by cytokines and growth factors, but also endogenously by Ras family protein Eras (ES cell-expressed Ras) (Dreesen and Brivanlou, 2007). Phosphoinositide 3-kinase (PI3K) phosphorylates PIP2 (phosphatidylinositol-4,5-bisphosphate), converting it to PIP3 (phosphatidylinositol-3,4,5-trisphosphate). This can be reversed by the Phosphatase and tensin homologue (PTEN). One major downstream mediator of PIP3 is AKT which is activated by Pyruvate Dehydrogenase Kinase 1 (PDK1). Phosphorylated AKT then regulates a number of downstream targets. PI3K is important for the maintenance of undifferentiated murine ESCs and promotes short-term self-renewal (Paling et al., 2004). In fact, it has been reported that tumor suppressor p53 promoted differentiation of murine ESCs by suppressing *NANOG* expression. However, this is dependent on the phosphorylation of Ser315 of p53 which is a residue substrate of Glycogen Synthase Kinase 3 Beta (GSK3β). And GSK3 is negatively regulated by PI3K and AKT (Lin et al., 2005).

The NF-κB transcription factor family consists of p50/p105, p52/p100, c-Rel, RelA (also known as p65), and RelB, which altogether regulate the expression of hundreds of target genes. In the absence of signaling, these factors are inactivated by

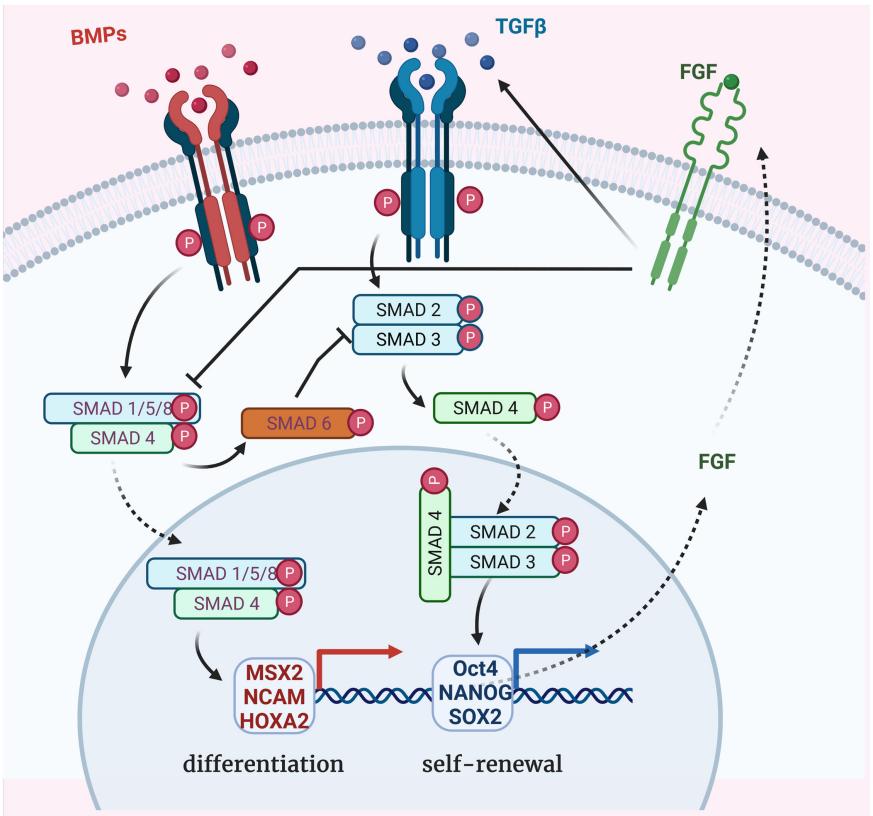

FIGURE 1 | Bone morphogenetic protein (BMP), TGF-β, and FGF-dependent SMADs pathways for regulation of stem cell differentiation. TGF-β super-family members binding to the receptors which propagate phosphorylation signal to receptor-regulated SMAD proteins (R-SMADs in blue). BMP signaling occurs through SMADs 1/5/8 phosphorylation and TGF-β signaling through SMADs 2/3 phosphorylation. Once activated, their binding with the common partner (Co-SMAD in green) SMAD 4 results in complexes which translocate to the nucleus to activate other transcription factors and regulate specific genes expression. MSX2, NCAM, and HOXA2 expression leads to the differentiation of ESCs whereas Oct4, NANOG, and SOX2 expression contributes to the undifferentiated proliferation. BMP signaling negatively regulates TGF-β signaling via the expression of the inhibitory SMAD 6 (I-SMAD in orange). FGF signaling promotes TGF-β receptor (TGFBR1) activation, resulting in self-renewal transcription factors activation and, in turn, FGF expression. FGF signaling also directly represses SMAD1/5/8 phosphorylation, inhibiting the BMP pathway.

the interaction with IκB inhibitory protein. NF-κB pathway is activated by a variety of extracellular factors such as tumor necrosis factor alpha (TNFα), interleukin 1 (IL1), growth factors, bacterial or viral infections or oxidative stress. In response to such stimuli, IκB is phosphorylated, ubiquitinated and degraded, allowing the NF-κB factors to freely translocate to the nucleus. A number of NF-κB components have been shown to decrease upon cell differentiation, such as LCK, a lymphocyte-specific tyrosine kinase, and PELLINO1 (both required for NF-κB activation) as well as TNFSF11/RANKL, the receptor activator of NF-κB ligands (Armstrong et al., 2006). Moreover, RelA is only present in the nucleus of undifferentiated ESCs, indicating active NF-κB pathway. When miRNA was used to target p65/RelA, ESC pluripotency was lost, resulting in epithelial to mesenchymal transition (Lüningschrör et al., 2012). Finally, inhibition of NF-κB signaling enhances the differentiation of human ESCs into MSCs by diminishing expression of pluripotent markers and increasing the expression of MSC surface markers. The depletion of p65 led to a 3-fold increase of CD73+CD90+CD146+CD45− MSCs (Deng et al., 2016).

Crosstalk amongst these pathways have been described in numerous cell processes. The receptor activator of NF-κB ligand (RANKL/TNFS11) has been shown to be translationally regulated by PDK1 of the PI3K/AKT pathway (Tanaka et al., 2005). PDK1 activates NF-κB signaling by phosphorylating and degrading IκB, thus allowing p65 to enter the nucleus. **Figure 2** shows the interaction between these different pathways to maintain the ESCs in an undifferentiated state.

To summarize, the different protocols for iPSC commitment to MSCs are based on the main pathways described above. Various growth factors or small molecule inhibitors can be used to either activate or inhibit of a specific pathways, which in turn may activate or inhibit others.

OSTEOGENIC COMMITMENT AND BONE FORMATION

Mesenchymal stem cells express *RUNX2* (transcription factor required for osteoblast cell fate), *SOX 9* (chondrocyte-specific

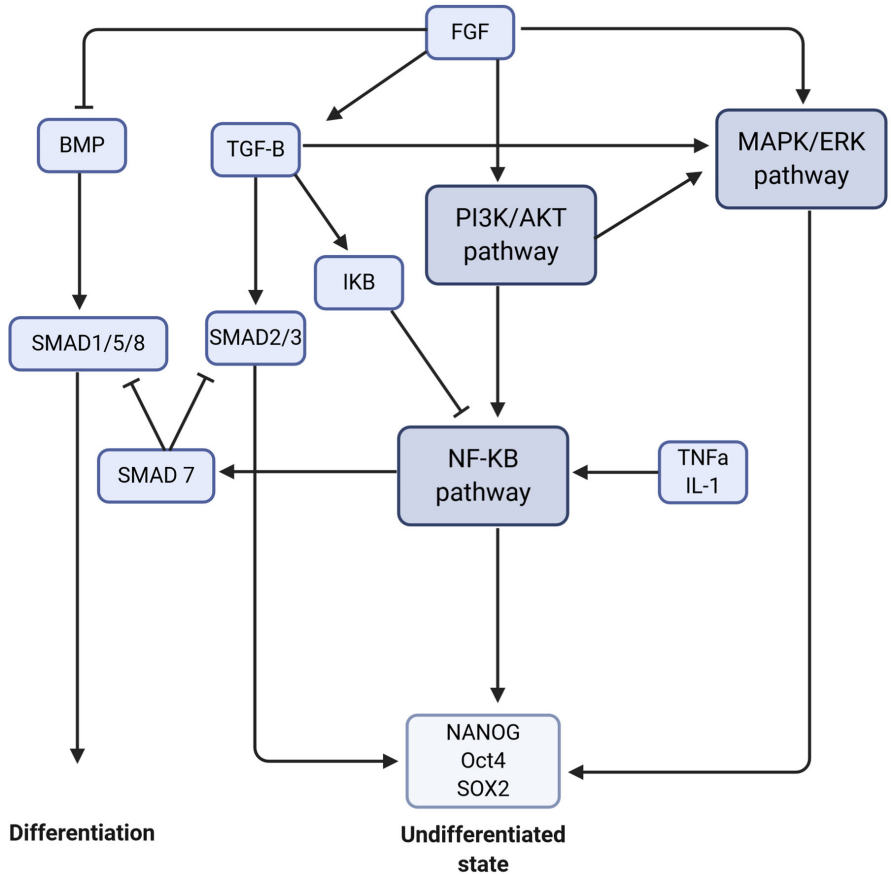

FIGURE 2 | Crosstalk between signaling pathways to maintain stem cells in an undifferentiated state or to trigger the differentiation. The signaling pathways are in dark purple, the molecules/proteins are represented in blue, and the transcription factors in light blue. The arrows mean activation and the flat arrows inhibition.

transcription factor), and *PPARG* (adipocyte-specific transcription factor) and have the ability to differentiate into one of three cell-types: osteoblasts, chondrocytes, or adipocytes. This is determined by the different genetic pathways expressed in MSCs. For osteogenic differentiation, cells must go through three different stages: (1) the immature osteoblasts with *COL1A1*, *ALPL*, and *SSP1* differentiation markers (respectively collagen 1, alkaline phosphatase, and osteopontin genes), (2) the mature osteoblasts which express *BGLAP* (osteocalcin gene), and (3) terminal differentiation into osteocytes with *SOST* (sclerostin gene) and Dentin matrix acidic phosphoprotein 1 (*DMP-1*) expression (Ducy et al., 1997).

Osteoblast differentiation from MSCs is primarily dependent on the BMPs, parathyroid hormone (PTH) and Wnt pathways.

The Bone Morphogenetic Proteins Pathway

Bone morphogenetic proteins are known to activate both the SMAD and MAPK pathways. In the SMAD pathway, both type I and type II BMP receptors are needed for signal transduction. Upon the activation of these receptors by BMP ligands such as BMP-2 and BMP-4, the intracellular SMADs 1, 5, and 8 become phosphorylated and form a complex with SMAD 4. This complex translocates into the nucleus and participates in gene transcription (**Figure 1**). SMADs 1 and 5 directly interact with the bone-specific transcription factor RUNX2 and activate the transcription of target genes such as *COX-2* and *COL10A1* in osteoblasts and chondrocytes (Zhao et al., 2003). SMAD1 directly interacts with HOWC8 protein to promote osteopontin production (Yang et al., 2000). Moreover, both SMAD1 and RUNX2 undergo ubiquitin-proteasome-mediated degradation. SMURF1 (SMAD Specific E3 Ubiquitin Protein Ligase 1), a member of the HECT family of E3 ubiquitin ligase, has been found to interact with SMADs 1 and 5, thereby triggering their ubiquitination and degradation. Inhibition of SMURF1 and proteasome degradation lead to increased osteoblast function and bone formation (Zhao et al., 2003).

For the MAPK pathway, BMP-2 has been shown to activate ERK1/2, p38 and JNK in human osteoblastic cells, inducing osteoblast differentiation with the increased expression of alkaline phosphatase (ALP) and osteocalcin (Guicheux et al., 2003). In addition, BMP-2-activated-ERK1/2 inhibits collagen X expression in osteoblasts, resulting in increased phosphorylation of RUNX2, which in turn upregulates ALP expression (Reilly et al., 2005).

Influence of Parathyroid Hormone (PTH) on Osteoblasts

Parathyroid hormone is the primary calcium metabolism regulating hormone. Osteoblasts are rich in PTH receptors and PTH-related protein receptors (Gardinier et al., 2019). Intermittent PTH injections in rat promotes osteoblast differentiation and bone formation, whereas continuous PTH injection inhibits osteogenesis (Frolik et al., 2003). It has been explained that continued exposure to PTH *in vitro* causes a desensitization of the adenylate cyclase and phospholipase C responses as well as receptor downregulation (Frolik et al., 2003). It was further confirmed in human that levels of PTH expression and PTH receptors influence osteoblast formation (Osagie-Clouard et al., 2017). This can be explained by several molecular pathways.

First, PTH promotes ubiquitinylation, ultimately stimulating proteasome activities resulting in the degradation of osteoblast protein substrates (Murray et al., 1998). Moreover, the anabolic effect of PTH is RUNX2-dependent (Krishnan et al., 2003). The binding of PTH to PTH receptor 1 (PTH1R) stimulates production of cAMP and activation of protein kinase A (PKA). PKA subsequently phosphorylates transcription factors such as RUNX2 and c-AMP-response element-binding protein (CREB) thus promoting intracellular free Ca^{2+} which in turn regulates TGF-β1 expression (Wu et al., 2009).

Parathyroid hormone is also involved in the Wnt pathway (**Figure 3**). Mice expressing constitutively active PTH1R in osteocytes present increased Wnt signaling and bone mass, whereas deletion of the co-receptor LRP5 suppresses this bone gain (O'Brien et al., 2008). Conversely, mice lacking PTH1R in osteocytes demonstrate osteopenia associated with increased *SOST* expression and decreased canonical Wnt signaling (Powell et al., 2011). Furthermore, PTH1R has been shown to activate the Wnt pathway in the absence of Wnt ligands by forming a complex with LRP5/6 after PTH binding. This leads to the phosphorylation of LRP6 which allows for the recruitment of axin and β-catenin stabilization (Wan et al., 2008). PTH also represses expression of several secreted Wnt antagonists, such as Sost, DKK1, and Wif1 (Li et al., 2007; Guo et al., 2010).

Finally, PTH has been shown to activate PI3K/AKT signaling by phosphorylating AKT (Yamamoto et al., 2007). AKT phosphorylation then promotes Signal transducer and activator of transcription 5 (STAT5) phosphorylation resulting in increased receptor activator of nuclear factor kappa-B ligand (RANKL) expression (Tsubaki et al., 2007). In turn, RANKL upregulation stimulates osteoclast precursor commitment into mature osteoclasts.

In summary, PTH is involved in several molecular pathways that regulate osteoblast differentiation, bone formation, and/or bone resorption.

The Wnt Pathway

The Canonical Pathway

Osteoblasts are located close to the bone marrow, which serves as a major source for MSCs and hematopoietic stem cells (Spradling et al., 2001). Osteoblasts and peripheral blood mononuclear cells (PBMCs) are involved in the regulation of hematopoietic stem cells by releasing many factors that impact differentiation, such as Wnt family proteins. Some Wnt secreted glycoproteins, like Wnt3a and Wnt10b, can bind to a Frizzled receptor (FZD) and recruit the Low-density lipoprotein receptor-related proteins 5/6 (LRP5/6) co-receptors, thus activating the canonical signaling pathway (**Figure 3**). The proteolysis GSK-3 complex is inhibited by activating Dishevelled (Dsh) protein, resulting in β-catenin stabilization and translocation into the nucleus. This then regulates the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcriptional activity which in turn activates downstream genes. Canonical Wnt signaling can be inhibited by osteocyte secreted factors such as sclerostine, Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1) or Secreted Frizzled Related Protein 1 (SFRP1), which bind to LRP5/6 causing its inactivation. In result, GSK-3 is no longer inhibited and can target β-catenin to be ubiquitinated and degraded (Logan and Nusse, 2004).

Canonical Wnt activity is essential for the development and differentiation of multiple organ systems including bone. Canonical Wnt signaling enhances ossification and suppresses chondrocyte formation, whereas inhibition of β-catenin in MSCs results in chondrocyte differentiation (Day et al., 2005). Furthermore, Wnt10b, which is expressed in bone marrow by osteoblast progenitors (Andrade et al., 2008) and T lymphocytes (Terauchi et al., 2009), stimulates osteoblastogenesis and inhibits adipogenesis of mesenchymal precursors (Bennett et al., 2005). Osteogenic commitment was confirmed by upregulation of osteoblastogenic transcription factors RUNX2, DLX5, and OSTERIX, whereas inhibited adipocyte differentiation was validated by suppression of the adipogenic transcription factors C/EBPA and PPARG. In contrast, deficiency in the canonical Wnt inhibitor DKK1 was associated with increased bone formation in mice and humans whereas its presence in mesenchymal progenitor cells lead to adipocyte commitment (Pinzone et al., 2009). Using RNAi to knock-down LRP5 and β-catenin expression, it was shown that only *ALPL* seemed to be positively regulated by the canonical Wnt pathway, whereas the other osteogenic markers were negatively affected (Ilmer et al., 2009).

Conversely, other studies showed opposing effects where Wnt3a inhibits *in vitro* MSC osteogenic differentiation, with decreased matrix mineralization and reduced ALP mRNA levels and activity (Boland et al., 2004). This was corroborated by other reports demonstrating that Wnt3a and LRP5 overexpression inhibited osteogenic differentiation (Baksh et al., 2007), and that β-catenin inhibits osteoblast differentiation of human MSCs *in vitro* in osteogenic medium (Zhou, 2011). This controversy into the role of canonical Wnt signaling on osteogenic differentiation from MSCs was resolved by Liu et al. (2009) who argued that under conditions permissive for binary lineage differentiation, Wnt signaling could shift the commitment from adipocytes toward osteoblasts, whereas osteoblast differentiation remains inhibited in osteogenic conditions. Others reported that Wnt10b induction of osteogenesis in mouse progenitors was due to inhibition of *PPARG* and *C/EBPA* activity (Kang et al., 2007).

The common osteoblast differentiation medium contains dexamethasone, ascorbic acid and β-glycerophosphate. These three compounds play a critical role in the different pathways

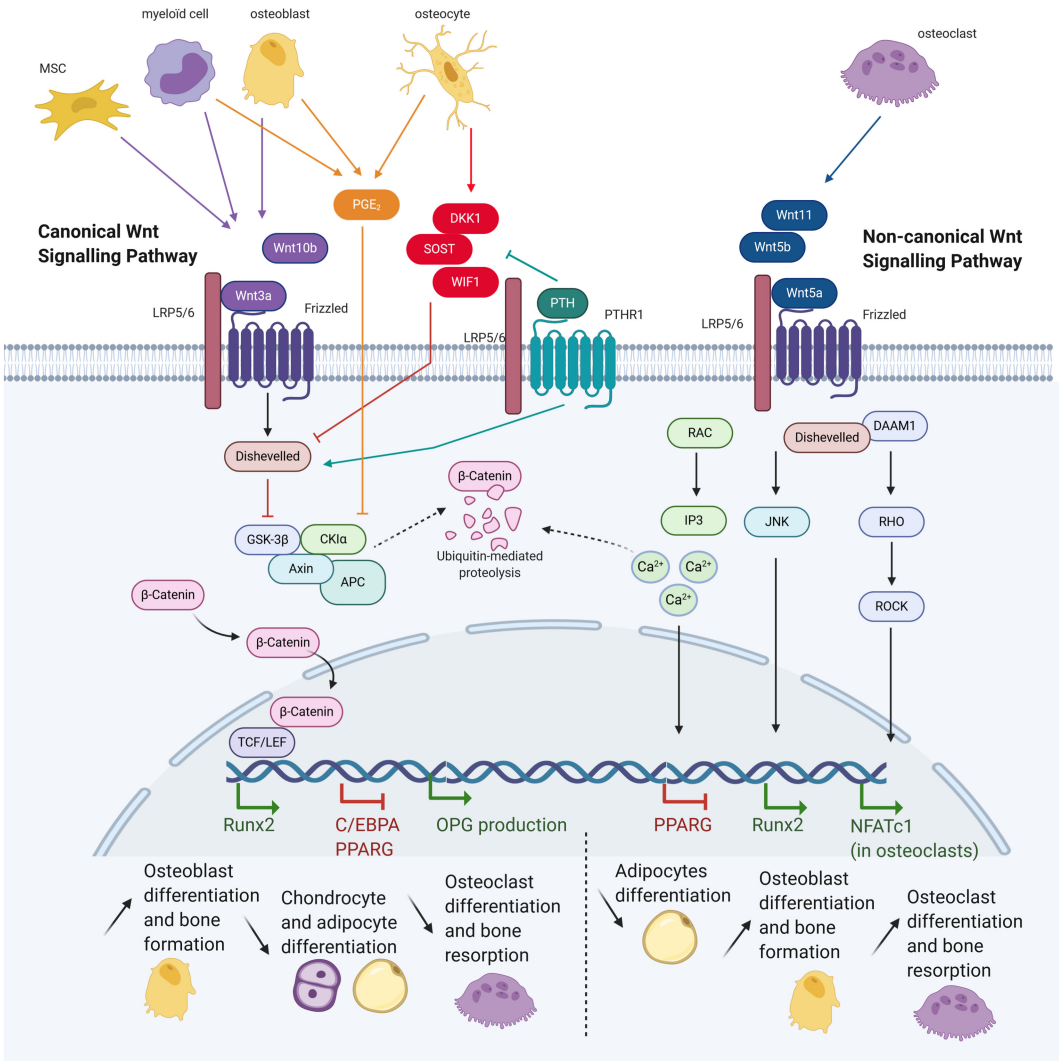

FIGURE 3 | Wnt pathway in osteoblast and consequences on bone cells differentiation. Mesenchymal stem cells (MSCs), myeloid cells, osteoblasts, osteoclasts, and osteocytes express different molecules, triggering or inhibiting the canonical or non-canonical Wnt signalings. Both signalings allow translation of different genes, having consequences for cells commitment and bone activity (Adapted from Baron and Kneissel, 2013).

required for osteoblast differentiation (Langenbach and Handschel, 2013). Dexamethasone activates Wnt/β-catenin signaling by upregulating FHL2, a LIM-domain protein which in the presence of Wnt3a potentiates β-catenin transport to the nucleus (Hamidouche et al., 2008). The addition of Ascorbic acid and β-glycerophosphate facilitates osteogenic differentiation by enhancing Runx2 activity via the MAPK signaling pathway (Xiao et al., 2002).

Canonical Wnt signaling also indirectly represses osteoclast differentiation and bone resorption through the increased secretion of osteoprotegerin (OPG), a major inhibitor of osteoclast differentiation. Its expression is regulated by β-catenin and TCF proteins (Glass et al., 2005). In addition, Wnt3a also inhibits murine osteoclast differentiation (Santiago et al., 2012).

Other pathways that regulate GSK-3 may also modulate the canonical Wnt pathway. Activation of AKT or integrin-like kinases (ILK) has been shown to upregulate β-catenin level by inhibiting GSK-3 through phosphorylation (Topol et al., 2003). Coordination between TGF-β and canonical Wnt signaling was shown to promote chondroblast differentiation at the expense of adipocytes and osteoblasts (Zhou, 2011). TGF-β1 activates β-catenin signaling pathway *via* ALK-5, SMAD3 [which prevents β-catenin degradation and facilitates its nuclear translocation (Jian et al., 2006)], SMAD4 [which interacts with β-catenin and TCF/LEF1 (Nishita et al., 2000)], PKA and PI3K pathways and requires ALK5, PKA, and JNK interaction to inhibit osteoblastogenesis in human MSCs.

The Non-canonical Pathway

The non-canonical Wnt pathway is activated by other Wnt proteins such as Wnt-5a, -5b, and -11, and is mediated *via* Rho-GTPase-proteins, calcium fluxes and/or c-Jun N-terminal kinases

(JNK) (**Figure 3**). Non-canonical Wnt ligands bind to FZD and recruit Dsh protein to form the DAAM1 complex. This then triggers activation of the small G protein RHO, in turn activating RHO-associated kinase (ROCK), leading to the inhibition of NFATC1 transcription factor in osteoclasts. Alternatively, Dsh may also form a complex with RAC, resulting in JNK activity and the activation of *RUNX2*. Finally, the Wnt-Ca^{2+} pathway is activated by osteoblast-expressed Wnt-5a binding to FZD and to the osteoclast-expressed co-receptor ROR2. Intracellular calcium concentrations increase, resulting in dystoglycan 1 (DAG) and inositol 1,4,5-triphosphate, type 3 (IP3) generation. cGMP amount decreases, causing the inhibition of *PPARG* and pro-osteogenic commitment (Bilkovski et al., 2010).

The osteoclast differentiation was also achieved by this Wnt-5a-Ror2 non-canonical pathway (Maeda et al., 2012) and in turn osteoclasts release Wnt ligands to trigger osteoblastogenesis. However, releasing intracellular calcium may also activate protein kinase C which can antagonize the canonical pathway by promoting degradation of β-catenin (Topol et al., 2003). Lastly, the non-canonical Wnt4 protein improves osteogenic differentiation *in vitro* and promotes bone regeneration and repair *in vivo* (Chang et al., 2007).

In summary, the canonical Wnt pathway allows osteoblast commitment of MSCs by inhibiting adipocyte and chondrocyte differentiation, promotes bone formation and represses bone resorption. In contrast, the non-canonical Wnt pathway induces both bone formation and bone resorption *via* osteoclastogenesis.

Effect of CFTR Mutations on Canonical Wnt Pathway

Cystic fibrosis transmembrane conductance regulator is an ATP-binding cassette (ABC) transmembrane chloride channel that belongs to the ABC transporters family. It is composed of two repeated motifs: six hydrophobic membrane helices and a cytoplasmic hydrophilic region for ATP binding. These motifs are linked by a cytoplasmic regulatory domain that possesses many phosphorylation sites (Riordan et al., 1989). The terminal tails, located in the cytoplasm, mediate several interactions with binding proteins. The carboxyl terminus contains a PDZ-binding domain (PDZBD), which can bind to other proteins with PDZ domain (Li and Naren, 2011). CFTR is expressed in osteoblasts and osteoclasts, but in lower amount than in epithelial cells (Le Henaff et al., 2016).

Loss of CFTR protein or/and its chloride channel function has a detrimental impact on bone cells. Recent studies in CFTR-deficient new-born pigs showed high porosity of cortical bones and altered chemical composition of the trabecular bones (Braux et al., 2020). *Cftr−/−* mice display severe osteopenia in both trabecular and cortical bone (Dif et al., 2004). They demonstrate drastic reduction in bone formation accompanied by increased bone resorption. Delayed osteoblast differentiation, reduced ALP expression and increased proliferative rate were shown in F508del CFTR bone marrow stromal cells (Velard et al., 2014; Le Henaff et al., 2015). The main outcomes resulting from CFTR mutations are its diminished ability to interact with other proteins (**Figure 4**) and faulty chloride channel function (**Figure 5**), both ultimately influencing bone cell differentiation.

Canonical Wnt signaling is impacted by these two outcomes. Dsh protein contains a PDZ domain. In zebrafish, it has been shown that CFTR interacts with Dsh *via* PDZBD regardless of its channel function (Sun et al., 2018). The use of CFTR PDZBD deletion suggested the importance of CFTR in stabilizing Dsh through a direct protein-protein interaction. Moreover, CFTR deficiency resulted in accelerated Dpr1-induced lysosomal degradation of Dsh by preventing the Dpr1-Dsh interaction. The impaired Wnt signaling was rescued by overexpression of G551D CFTR mutant, which causes a chloride channel defect but keeps the PDZBD domain intact. Moreover, the lack of CFTR channel function was also directly involved since Wnt pathway is pH- and charge-dependent (Simons et al., 2009). The change in pH induced by defective CFTR, resulting in increased extracellular acidification (Massey et al., 2021), perturbs the recruitment of Dsh by FZD, which does not possess any specific binding site but uses some polybasic amino acid to interact with acidic and negatively charged lipids of the plasma membrane (Wong et al., 2000). Furthermore, studies have shown that CFTR directly interacts with β-catenin to regulate the differentiation of ESCs into mesoderm, a critical step toward bone lineage differentiation (Gadue et al., 2006; Liu et al., 2017). Such direct interaction of CFTR with β-catenin might therefore play an important role in bone lineage commitment and differentiation.

Finally, it has been shown that Cftr−/− mice have increased level of PTH (Dif et al., 2004). As previously described, PTH has an ambivalent role in bone development, being an activator of the canonical Wnt signaling pathway and permitting bone formation, as well as inhibiting osteogenesis. Therefore, it is plausible that CFTR mutations alter bone differentiation through the interference with the canonical Wnt pathway and PTH.

OSTEOCLAST DIFFERENTIATION AND BONE RESORPTION

The RANKL/GM-CSF/MCP-1/RANTES Pathway

Osteoclasts are multinucleated cells that express tartrate-resistant acid phosphatase (TRAP) and have a bone resorption function. They are differentiated from mononuclear precursor cells of the monocyte macrophage lineage. RANKL and macrophage colony-stimulating factor (M-CSF) are crucial cytokines for osteoclast differentiation (Yoshida et al., 1990). RANKL, expressed at the surface of osteocytes and osteoblasts, interacts with RANK on osteoclast precursors, resulting in a cascade of gene expression controlled by transcription factors including NF-κB and NFATC1. The differentiation depends on signaling through c-fms (the receptor for M-CSF) in mononuclear precursor cells which in turn up-regulate RANK expression (**Figure 6**). Proximity between osteoblastic lineage and hematopoietic cells is therefore required to form osteoclasts. The two factors induce expression of osteoclast marker genes such as *ACP5*, *CTSK*, *CALCR*, *TRAP*, and *ITGB3* (Boyle et al., 2003).

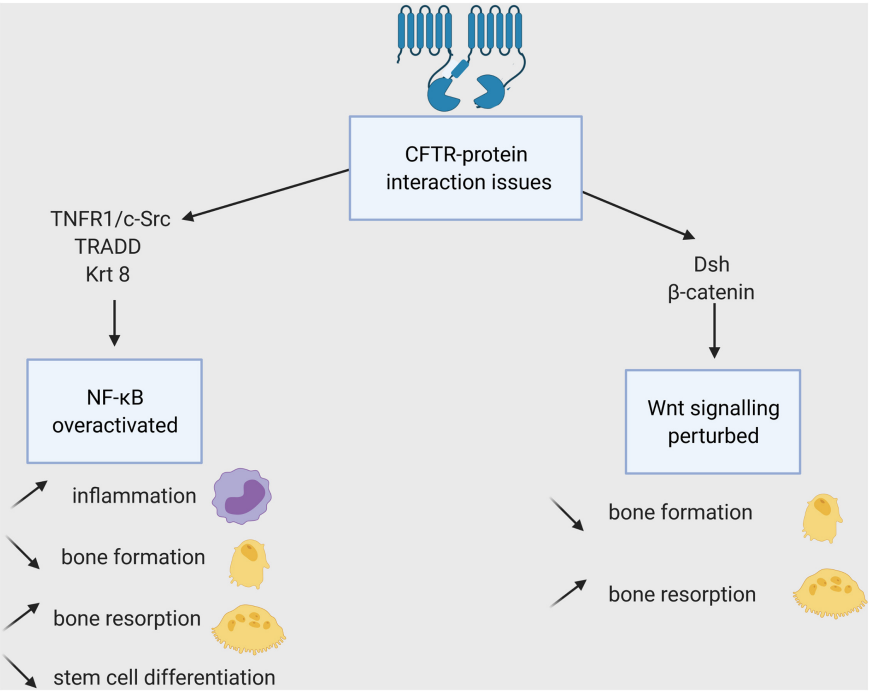

FIGURE 4 | Impacts of the CFTR-proteins interactions loss on bone cells. WT-CFTR, because of its PDZBD interacts with c-Src, TNFR1 and TRADD (shown in epithelial cells), Krt8 (in osteoblasts), and Dsh (in osteoblasts, osteoclasts, and osteocytes). The lack of these interactions leads to NF-KB overexpression and Wnt signaling disruption and to several consequences for bone cells and their progenitors.

Osteoclast differentiation is regulated by OPG (a soluble antagonist of RANKL also secreted by the osteocytes), which binds to RANKL with high affinity and inhibits its action. The OPG/RANKL ratio determines the degree of osteoclast differentiation (Hofbauer and Heufelder, 2001). T and B cells produce several cytokines to regulate osteoclast differentiation. Granulocyte macrophage colony-stimulating factor (GM-CSF), secreted by T cells, has shown both inhibitory and stimulatory effects on osteoclast formation. It has been reported that short-term treatment triggers osteoclast differentiation, whereas long-term exposure suppresses it (Hodge et al., 2003). Monocyte chemotactic protein-1 (MCP-1), a cytokine expressed by mature osteoclasts, is regulated by NF-κB (Donadelli et al., 2000). Furthermore, RANKL-induced NFATC1 signaling has been demonstrated to increase expression of MCP-1 and RANTES chemokine (Regulated upon Activation Normal T cell Expressed and Secreted), promoting the formation of TRAP-positive multinuclear and bone-resorptive cells (Kim et al., 2005). The addition of exogenous MCP-1 reverses the GM-CSF mediated suppression of osteoclast formation, permitting bone resorption. Consequently, it has been suggested that pathologies associated with high levels of MCP-1 will result in increased osteoclast differentiation and bone resorption.

Regulation of Resorptive Function of Osteoclasts by Other Cells

There are a number of signaling pathways that influence bone resorption. Downregulation of NF-κB transcription factors results in diminished osteoclastogenesis which causes osteoporosis (Franzoso et al., 1997). As previously described, TGF-β inhibits the NF-κB pathway by increasing IκB inhibitors, whereas TNFα, IL1, or FGF through PI3K/AKT activate it.

The canonical Wnt pathway also influences bone resorption *via* PTH or prostaglandin E2 (PGE2). PGE2, secreted by osteoblasts and osteocytes (Rochefort et al., 2010), is essential for Wnt signaling in stem cells, promoting the differentiation of several lineages by interacting with the β-catenin destruction complex (Goessling et al., 2009). PGE2 is also expressed in cells of the hematopoietic lineage (Hackett et al., 2006), directly affecting osteoclastic cells. Interestingly, whereas PGE2 increases RANKL-stimulated osteoclast differentiation in murine cells (Kobayashi et al., 2005), it inhibits differentiation in cultured human PBMCs (Take et al., 2005).

Furthermore, a relationship has been shown between NF-κB and Wnt pathway. Because NF-κB is activated during inflammation, and chondrocyte DKK1 expression was found to correlate with IL1β and TNFα levels (Weng et al., 2009), NF-κB could also down-regulate β-catenin by inducing DKK1 (Chang et al., 2013). It has also been demonstrated that canonical Wnt signaling stimulates OPG expression in mature osteoblasts leading to the suppression of osteoclast formation (Glass et al., 2005). Finally, it has been indicated that β-catenin interacts with p65 of the NF-κB signaling pathway, resulting in its activation (Liu et al., 2016).

These properties were originally noted by Yasuda et al. (1998) who made osteoclast-like cells from spleen cells in presence of

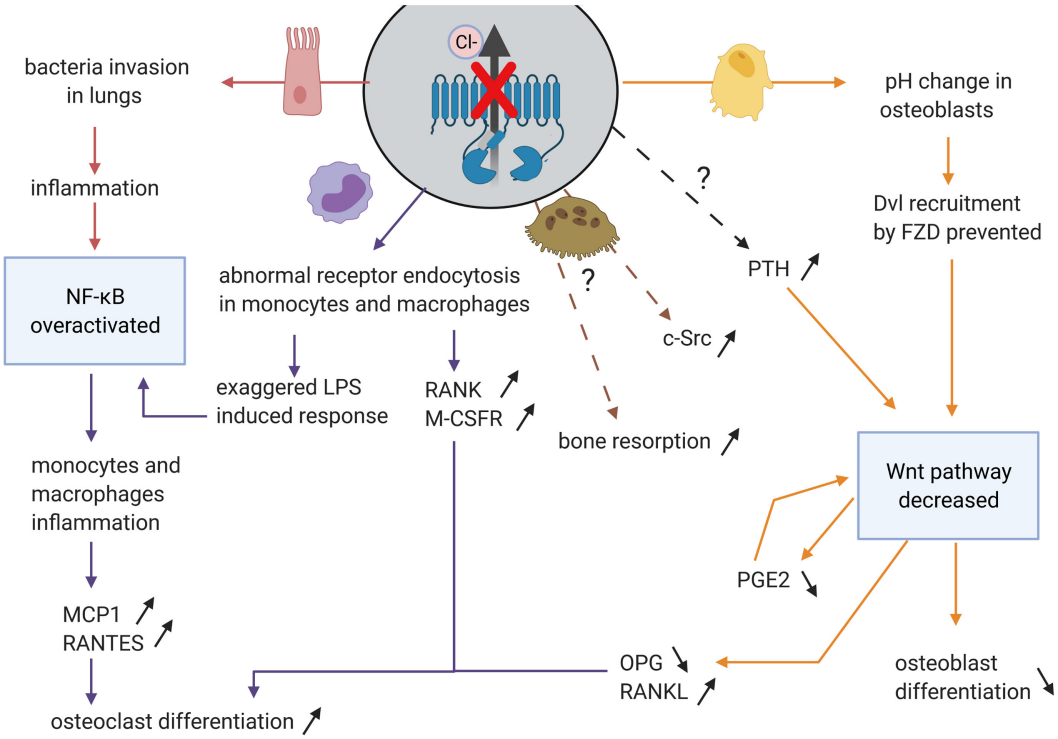

FIGURE 5 | Impact on disruption of chloride channel function of CFTR in the different organs and the consequences for osteoblasts and osteoclasts differentiation and activity. CFTR channel function defect impacts airway epithelial cells (in pink), monocytes and macrophages (in purple), possibly directly osteoclasts (in brown), and osteoblasts (in yellow). In these cells, some pathways (in blue) are disturbed leading to increased osteoclast differentiation and bone resorption and decreased osteoblast differentiation and bone formation.

osteoblasts with IL6, IL11, PTH, and PGE2. However, it is now better understood how Wnt and NF-κB pathways are involved in osteoblasts promoting osteoclast differentiation.

Effect of CFTR on NF-κB Pathway

More TRAP+ osteoclasts were observed in CFTR KO mice bone marrow leading to increased osteoclastic bone resorption (Stalvey et al., 2013). CFTR was shown to mediate RANTES expression in airway epithelial cells directly by inserting into the membrane, without its chloride transport function, allowing the activation of NF-κB pathway (Estell et al., 2003).

Some direct interactions with CFTR and proteins involved in NF-κB signaling have been shown in human bronchial epithelial cells but not yet in osteoclasts. TNFR1 expression is strongly associated with CF due to its interaction with the PDZBD domain of CFTR (Dudez et al., 2008). TNFα triggers the translocation of CFTR, TNFR1, and c-Src into the lipid rafts but the recruitment of CFTR and TNFR1 is dependent on protein tyrosine kinase activity and an intact C-terminus domain of CFTR. Intact CFTR contributes to the formation of a TNFR1/c-Src (a proto-oncogene tyrosine-protein kinase) complex in the lipid raft of epithelial cells. This complex stimulates the regulation of gap junction, intracellular communication and IL8 secretion in intact CFTR cells. C-Src is also highly expressed in the ruffled border of osteoclasts (Tanaka et al., 1992) and proven necessary for bone resorption (Roodman, 1999). Its interactions with CFTR has not been shown in osteoclast yet but could explain the decreased resorptive activity of CF osteoclasts (Jourdain et al., 2021).

Cystic fibrosis transmembrane conductance regulator binds also to another compound connecting TNFα and NF-κB signaling: the TNF receptor-associated death domain protein TRADD (Wang et al., 2016; **Figure 4**). TRADD binds to SODD, a domain released from TNFR1 throughout TNFα binding, and phosphorylates inhibitory protein IκB, allowing NF-κB to translocate to the nucleus (Pobezinskaya and Liu, 2012). As predicted, TRADD binds to WT-CFTR and G551D CFTR but not to TNR, a variant that has chloride channel function but lacks PDZ binding. Such binding of functional CFTR promotes TRADD degradation and inhibits the ability of TNFα to stimulate NF-κB activity in human bronchial epithelial cells.

An interaction between CFTR and Krt8 protein has been described in mice (Le Henaff et al., 2016; **Figure 4**). Genetic deletion of Krt8 in mice expressing F508-del-Cftr resulted in a rescue of the bone phenotype seen in F508-del-Cftr (decreased markers of bone formation and bone mass) in part through the modulation of NF-k-B and Wnt-β-catenin pathways (Le Henaff et al., 2016). Krt8 interacts with p62, a regulator of NF-κB signaling (Janig et al., 2005) and influences osteoblast differentiation by decreasing *RUNX2* and *COL1A1* expression (Le Henaff et al., 2016). However, the study used the F508del mutant, which produces minimal viable CFTR protein, making it inconclusive whether the CFTR-Krt8 interaction is PDZBD- or

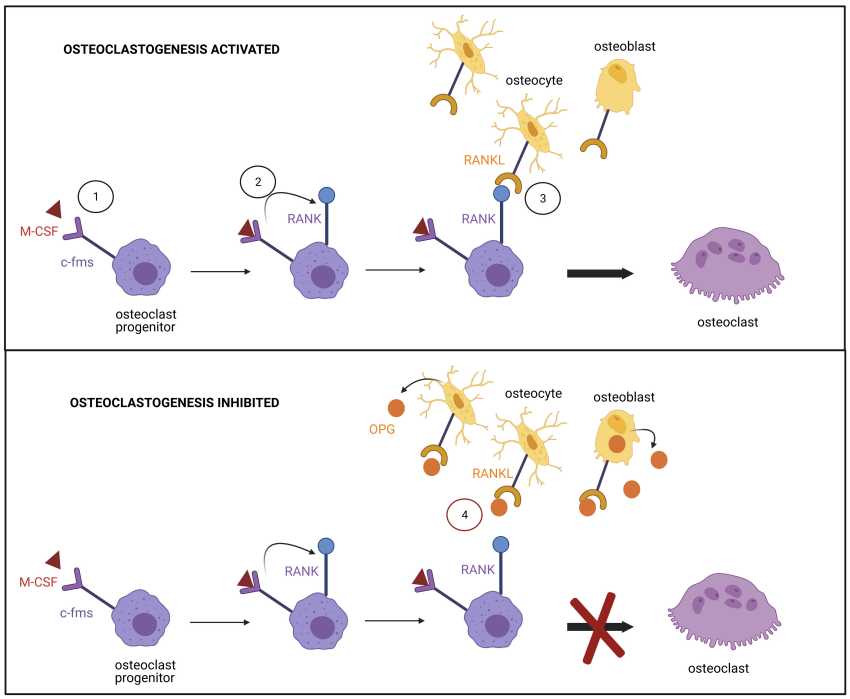

FIGURE 6 | Regulation of the osteoclast differentiation. (1) Binding of M-CSF to its receptor c-fms at the surface of osteoclast progenitor promotes (2) RANK expression. (3) Signaling induced by the binding of RANK to RANKL, expressed by osteocytes and osteoblasts, triggers transcription factor activation and osteoclast differentiation. (4) The differentiation is prevented by the high affinity association of osteoprotegerin (OPG), produced by osteoblasts, to RANKL.

channel function-dependent. Controversial data of the effect of Inh-172, an inhibitor of CFTR function, has been presented in mouse and humans. In mice, Inh-172 had no significant effect on osteoblast gene expression; whereas, it enhanced RANKL/OPG ratio in human osteoblast (Delion et al., 2016). Therefore, further studies are needed to determine whether CFTR has a direct role on osteoblast differentiation.

Although it has not been shown that NF-κB signaling is increased in osteoclasts, other organs, such as lung and pancreas, are affected by the absence of CFTR chloride channel (Al Alam et al., 2010; Cavestro et al., 2010; Gambari et al., 2012; **Figure 5**), and this, in turn, impacts monocytes and macrophages secretion, leading to the dysregulation of osteoclast differentiation.

Increased MCP-1 levels result in increased osteoclast differentiation and bone resorption. RANKL-induced NFATC1 signaling causes increased expression of MCP-1 cytokine and RANTES chemokine (regulated on activation normal T cell expressed and secreted) which promotes the formation of TRAP-positive multinuclear and bone-resorptive cells (Kim et al., 2005). Studies in other organ systems (lung and pancreas) demonstrated increased MCP-1 and IL-8 secretion as a direct result of CFTR gene mutations (Augarten et al., 2004; Cavestro et al., 2010). This increase in cytokines secretions can be attributed to high levels of NF-κB and low levels of IκB factor (Tabary et al., 1999). Zaman et al. (2004) proved that a single allelic CFTR mutation was sufficient to increase IL-8 secretion in peripheral blood monocytes in response to lipopolysaccharides (LPS): bacterial endotoxins mainly recognized by the Toll-Like Receptor 4 (TLR4). The reduction of CFTR expression resulted in increased LPS induced cytokine secretion, increased phosphorylation of NF-κB, which in turn is a positive regulator of IL-8 expression and decreased IκBα expression. The mechanism was specified in both CF mice and humans (F508del) macrophage studies (Bruscia et al., 2011). Naïve macrophages lacking CFTR had an abnormal TLR4 subcellular localization and trafficking which increased LPS-induced activation of NF-κB, MAPK, and IRF-3 pathway. It was also shown that TLR4 was not well degraded in CF macrophages, maybe due to acidification induced by CFTR defect (Shah et al., 2016; Murase et al., 2018).

Endocytosis of certain receptors including TLR4 is disrupted in CF macrophages, due to the acidification generated by a compromised CFTR channel. Velard et al. (2018) showed overexpression of RANK and M-CSFR in monocytes of G551D CF patients, which was partially restored upon treatment with Ivacaftor, a potentiator which increases CFTR-G551D channel opening. This proved a role of the chloride channel function in RANK and M-CSFR expression. Moreover, a recent study reported defective differentiation of CF-F508del human monocytes (PBMC) into osteoclasts. These defects were characterized by a decrease in the number of mature CF-osteoclasts derived from CF PBMCs compared to non-CF; and a higher expression of sphingosine-1-phosphate (S1P) in CF osteoclasts, an important factor in bone formation and density (Jourdain et al., 2021).

To conclude, CFTR mutations resulting in compromised channel function, induces inflammation in both lung and

pancreas, which influences osteoclast differentiation (**Figure 5**). Aberrant chloride channel function influences endocytosis of certain monocyte and macrophage receptors, which may lead to impaired NF-κB pathway. Since monocytes and macrophages are progenitors of osteoclasts, impairment in the NF-κB signaling may affect osteoclast differentiation and bone resorption.

Effect of CFTR on Wnt Signaling Through NF-κB Pathway and PGE2 Production

Nuclear factor-kappa B signaling is known to inhibit osteogenic differentiation in part by promoting β-catenin degradation. F508del mice have been shown to present defective osteoblast differentiation due to increased NF-κB signaling and reduced Wnt signaling (Le Henaff et al., 2015). This result was corroborated by a study showing decreased β-catenin level in the F508del mouse intestine (Liu et al., 2016).

Cystic fibrosis is associated with prostanoids overproduction, such as PGE2, PGF2, PGF1, and thromboxane B2 in the saliva and urine of CF patients (Jabr et al., 2013). Cyclooxygenase (COX) enzymes are required for the conversion of arachidonic acid into prostaglandins (PGs). COX-2 was described as highly inducible at inflammatory sites, in particular by IL1α, and is considered as the main target for NF-κB activation (Maier et al., 1990). In lungs, a positive feedback loop exists where PGE2 upregulates both COX-2 expression and p38 MAPK activity during inflammation through cAMP/AMPK signaling (Faour et al., 2008). Both PGE2 and cAMP activator induce COX-2 transcription, by increasing CREB phosphorylation *via* the PKA/p-CREB pathway, with CFTR being a negative regulator (Chen J. et al., 2012). In addition, basal CFTR gene transcription is regulated by intracellular cAMP. Therefore, PGE2 is also responsible of CFTR transcription through the cAMP pathway. Chen J. et al. (2012) suggested that only intact CFTR could be upregulated by PGE2, in turn switching off the PGE2-mediated feedback loop and reducing the inflammatory response. Therefore, it is obvious that a relationship exists between CFTR and PGE2 in lung inflammation.

However, the production of PGE2 by F508del osteoblasts was significantly reduced (Velard et al., 2014). Chikazu et al. (2002) showed that COX2 mRNA and PG production were induced by BMP-2 in osteoblasts *via RUNX2* binding. Although there is presently no direct link described between CFTR and PGE2 in bone cells, an indirect cause may be assumed since *RUNX2* is regulated by Wnt, TGF-β- and BMPs-SMAD pathways, which are all downregulated by the NF-κB pathway. Low PGE2 production primarily influences Wnt signaling (Goessling et al., 2009) which in turn has an effect on bone formation, stem cell differentiation, inhibition of OPG expression, and indirectly bone resorption *via* upregulation of RANKL in osteoblastic cells (Stalvey et al., 2013; Velard et al., 2014; Delion et al., 2016). The non-canonical Wnt pathway could also be affected by NF-κB overexpression in monocytes. Wnt5b has been shown to repress myeloid differentiation (osteoclast progenitors) in the presence of IL-3, and increase it in the presence of GM-CSF, leading to premature progenitor cell exhaustion (de Rezende et al., 2020). The increase of cytokine secretion induced by a defective CFTR could, in association with Wnt5b, over-activate the non-canonical Wnt pathway and dysregulate osteoclast differentiation from myeloid progenitors. Altogether, this suggests that impaired Wnt signaling alters osteoblast and osteoclast differentiation.

Possible Impacts of CFTR on Bone Resorption

Bone resorption is achieved when osteoclasts attach to the bone matrix and form a bone-resorbing acid compartment by exocytosis of lysosomes. A low pH is necessary for the solubilization of the alkaline salts in bone mineral and the digestion of organic bone matrix (Schlesinger et al., 1997). Acidification is mediated in osteoclast ruffled border by the combined activity of a V-type H+-ATPase pump, which provides the proton force needed to generate a pH gradient, and a chloride channel, that allows a passive chloride transport (Schaller et al., 2004). Vesicles from mature osteoclasts have both pump and channel allowing the resorption, whereas vesicles from osteoclast progenitors (bone marrow cells at different steps of differentiation) have limited acidification with minimal anion permeability. It is only upon exposure to bone that the cells are able to sufficiently express both pump and channel to support acidification (Schlesinger et al., 1997). Although, CFTR has been shown to be expressed both at the membrane and in cytoplasm of osteoclasts and their myeloid progenitors (Shead et al., 2007; Le Henaff et al., 2016), there are currently no studies proving a direct role of CFTR in bone resorption. A new study revealed that a loss of CFTR chloride activity in human osteoclasts, differentiated *in vitro* from human CF-F508del PBMCs, led to a reduced trench-resorption mode (Jourdain et al., 2021). However, the role of CFTR in bone resorption remains poorly understood and needs further investigation.

CONCLUSION

Cystic fibrosis transmembrane conductance regulator mutations affecting chloride channel functionality or its possibility to interact with other proteins, impact different signaling pathways: NF-κB, Wnt/β-catenin, MAPK/ERK, or TGF-β. These pathways are interrelated, meaning dysregulation of one has the ability to impact the others. In CF, increased NF-κB signaling enhances inflammation *via* monocytes and macrophages, which are both precursors of osteoclasts, which in turn disrupts osteoblast differentiation. Moreover, NF-κB prevents Wnt signaling, also resulting in decreased bone formation and increased bone resorption.

The impact of CF on bone resorption has yet to be determined. However, some CF patients develop clinically significant anemia, suggesting that CFTR may regulate hematopoiesis. Furthermore, the hematopoietic system is clearly related to bone as myeloid cells are osteoclast progenitors and allow bone formation through osteoblast differentiation and chondrocyte colonization. Additionally, CFTR plays an important role in chondrocytes, which need chloride ions. Since cartilage formation is the first part of bone formation, the impacts of CFTR mutations on chondrocytes may ultimately affect bone formation, osteoblast differentiation and their reciprocal regulation.

Cystic fibrosis transmembrane conductance regulator mutations perturb many pathways necessary for stem cell differentiation into bone cells. To date, studies have demonstrated the impact of CFTR on mature cells *in vitro* or *in vivo* on mice, fish, rat models. However, nothing has demonstrated the impact of CF on the differentiation process from stem cells to bone cells. iPSCs would be a great means by which to perform these studies, assessing different mutations carried by CF patients. Yet, to be successful, an efficient generation and differentiation method is necessary in order to prevent issues of tumorigenicity and preferential commitment due to the cell's epigenetic memory.

AUTHOR CONTRIBUTIONS

All authors wrote and edited the manuscript, read and approved the final manuscript.

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