MESENCHYMAL STEM CELL SENESCENCE AND REJUVENATION

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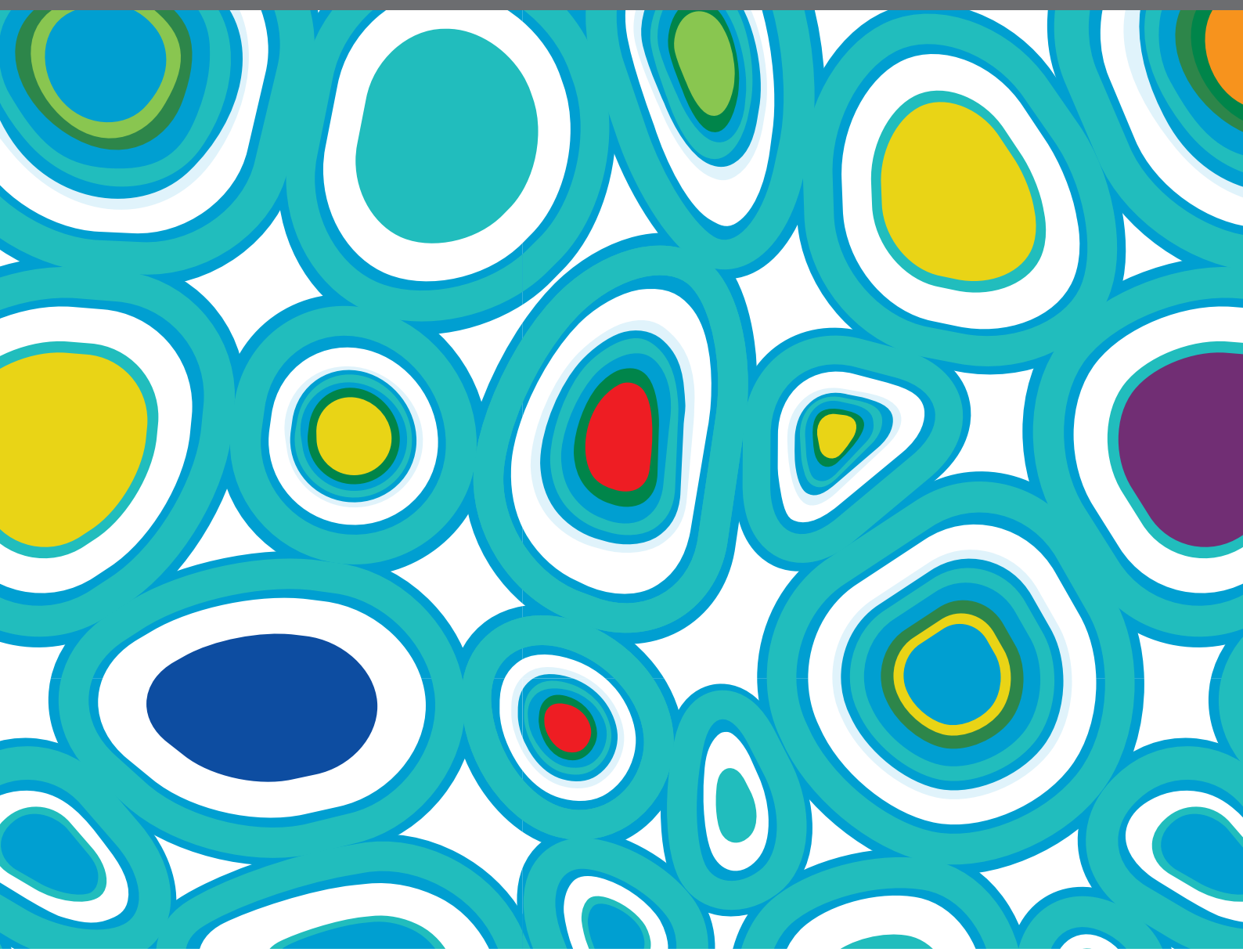

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MESENCHYMAL STEM CELL SENESCENCE AND REJUVENATION

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EDITORIAL
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Editorial: Mesenchymal Stem Cell Senescence and Rejuvenation

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Editorial on the Research Topic

Mesenchymal Stem Cell Senescence and Rejuvenation

Over the last few decades, stem cell-based therapy has become a novel strategy for a variety of disorders including cardiovascular, digestive and respiratory diseases. Among the stem cells investigated, mesenchymal stem cells (MSCs) are the ideal candidate cell source due to their many characteristics, including ease of isolation and expansion, multilineage differentiation potential and immunoregulatory function. Nevertheless MSCs derived from aged donors or patients with age-related diseases are senescent (Shi et al., 2021). In addition, prolonged *in vitro* expansion induces MSC senescence (Li et al., 2019). Although allogeneic transplantation of MSCs exerts beneficial effects in the early days post-transplantation, long-term benefits compared with those of autologous MSCs are compromised due to sustained immunorejection (Huang et al., 2010). Senescence severely affects the characteristics and function of MSCs, limiting their application in regenerative medicine. Exploring effective strategies to rejuvenate autologous senescent MSC and improve their therapeutic capacity is vital. Although multiple factors including age, oxidative stress and mitochondrial dysfunction have been reported to mediate MSC senescence, the underlying mechanisms of this senescence remain unclear.

There are twelve manuscripts in this Research Topic, highlighting the current understanding of the potential mechanisms underlying MSC senescence and novel strategies to rejuvenate the senescent MSC. Liu et al. and Zhou et al. summarize the current knowledge of phenotypic and biological properties of senescent MSCs and strategies for monitoring and rejuvenation, including gene modification and pretreatment strategies (Liu et al.; Zhou et al.). They also systematically review the molecular mechanisms underlying MSC senescence including epigenetic changes, autophagy, mitochondrial dysfunction and telomere shortening. There is accumulating evidence that autophagy acts as a positive and negative regulator of MSC senescence (Ma et al., 2018; Yang et al., 2018). Rastaldo et al. discuss these conflicting roles of autophagy in MSC senescence and potential mechanistic explanations for such an intricate liaison (Rastaldo et al.). Sirtuin 3 (SIRT3), an NAD + -dependent deacetylase, regulates a variety of physiological and pathological processes including aging and aging-related diseases. Ma et al. have shown that reduced Sirt3 expression contributes to age-related natural senescence and H_2O_2-induced premature senescence of rat bone marrow (BM)-MSCs by stimulating cellular reactive oxygen species (ROS) production and DNA injury (Ma et al.). Overexpressing Sirt3 partly reversed the senescence-associated phenotypic features of natural and premature senescent MSCs by alleviating ROS generation and upregulating SOD2 expression. Qin et al. reveal that knockout of NO synthase 2 significantly promoted the adipogenic, but not osteogenic, differentiation capacity of rat MSCs (Qin et al.). Notably, they also showed that knockout of NOS2 in MSCs resulted in significant obesity in rat fed a high-fat diet.

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It has been reported that the physiological and pathological condition of the donor plays a critical role in regulating MSC senescence (Alessio et al., 2020; Huang et al., 2019). Yin et al. summarize recent progress in understanding the impact of hyperglycemia on senescence of MSCs and strategies to suppress this senescence in a hyperglycemic environment (Yin et al.). Chen et al. discuss the current knowledge of the role of MSC senescence in the development of myelodysplastic syndromes and show that targeting senescent MSC is a potential strategy for MDS treatment (Chen et al.). Conley et al. report that MSCs isolated from obese subjects display a lower proliferative capacity than those derived from age-matched non-obese subjects. In addition, these obese-MSCs exhibited a senescent phenotype as evidenced by increased p16, p53, IL-6, and MCP-1 gene expression (Conley et al.). Furthermore, co-culture of injured HUVECs with MSCs from non-obese subjects resulted in the formation of tube-like networks but not with MSCs from obese subjects, indicating that pro-angiogenic properties were impaired. Cell source also affects the function of MSCs. Yigitbilek et al. compared the function of liver-derived MSCs (L-MSCs) and adipose tissue-derived MSCs (A-MSCs) from donors matched for gender, age, and body mass index (Yigitbilek et al.). Although both L-MSCs and A-MSCs exhibited a similar senescent phenotype manifested by similar cell cycle arrest and senescence-associated secretory phenotype genes, L-MSCs displayed an enhanced immunomodulatory capacity, while A-MSCs possessed better pro-angiogenic and vascular reparative potency.

Recently, non-coding RNA including lncRNAs and miRNA have been reported to be involved in mediating MSC senescence (Hong et al., 2020; Ren et al., 2021). Dong et al. report that elevated lncRNA lnc-CYP7A1-1 induced human BM-MSC senescence as evidenced by decreased cell proliferative ability, cell survival and migratory ability (Dong et al.). They also found that inhibition of lnc-CYP7A1-1 rejuvenated aged BM-MSCs and improved their therapeutic efficacy in a mouse model of myocardial infarction. There is emerging evidence that MSC-derived exosomes display protective effects against a variety of human diseases (Janockova et al., 2021) although these effects are much reduced in senescent MSCs. Sun. et al. isolated exosomes from young- and aged-MSCs and compared their cardioprotective activities *in vitro* and *in vivo* (Sun et al.). Compared with young-MSC-exosomes, aged-MSC-exosomes exhibited an impaired ability to promote endothelial tube formation and inhibit cardiomyocyte apoptosis *in vitro*. Transplantation of aged-MSC-exosomes also exhibited decreased cardioprotective effects in a mouse model of myocardial infarction. MicroRNA array and PCR analysis revealed dysregulation of miR-221-3p in aged-MSC-exosomes but restoration of miR-221-3p expression rescued aged-MSC-exosome reparative function.

It is well documented that clearance of senescent cells can improve tissue function and extend lifespan during aging (Dookun et al., 2020). To this end, Sharma et al. examined the effects of short-term navitoclax treatment, a chemotherapeutic drug reported to effectively clear senescent cells, on bone mass and osteoprogenitor function in aged mice (Sharma et al.). Interestingly, they found that despite clearance of senescent cells, navitoclax treatment significantly reduced the trabecular bone volume fraction in aged mice and impaired the calcified matrix production by aged BMSC-derived osteoblasts, indicating that the therapeutic effect of navitoclax on age-related bone loss was limited. Future studies including larger-scale studies in rodents and larger animal models are urgently needed to definitively assess the potential therapeutic efficacy of navitoclax in age-related diseases.

In summary, this Research Topic comprises twelve outstanding manuscripts of original research and comprehensive reviews. We summarize the most recent findings regarding the molecular mechanisms underlying MSC senescence and potential strategies for rejuvenation.

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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Human Obesity Induces Dysfunction and Early Senescence in Adipose Tissue-Derived Mesenchymal Stromal/Stem Cells

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Background: Chronic inflammatory conditions like obesity may adversely impact the biological functions underlying the regenerative potential of mesenchymal stromal/stem cells (MSC). Obesity can impair MSC function by inducing cellular senescence, a growth-arrest program that transitions cells to a pro-inflammatory state. However, the effect of obesity on adipose tissue-derived MSC in human subjects remains unclear. We tested the hypothesis that obesity induces senescence and dysfunction in human MSC.

Methods: MSC were harvested from abdominal subcutaneous fat collected from obese and age-matched non-obese subjects ($n = 40$) during bariatric or kidney donation surgeries, respectively. MSC were characterized, their migration and proliferation assessed, and cellular senescence evaluated by gene expression of cell-cycle arrest and senescence-associated secretory phenotype markers. *In vitro* studies tested MSC effect on injured human umbilical vein endothelial cells (HUVEC) function.

Results: Mean age was 59 ± 8 years, 66% were females. Obese subjects had higher body-mass index (BMI) than non-obese. MSC from obese subjects exhibited lower proliferative capacities than non-obese-MSC, suggesting decreased function, whereas their migration remained unchanged. Senescent cell burden and phenotype, manifested as *p16*, *p53*, *IL-6*, and *MCP-1* gene expression, were significantly upregulated in obese subjects' MSC. BMI correlated directly with expression of *p16*, *p21*, and *IL-6*. Furthermore, co-incubation with non-obese, but not with obese-MSC, restored VEGF expression and tube formation that were blunted in injured HUVEC.

Conclusion: Human obesity triggers an early senescence program in adipose tissue-derived MSC. Thus, obesity-induced cellular injury may alter efficacy of this endogenous repair system and hamper the feasibility of autologous transplantation in obese individuals.

Keywords: obesity, mesenchymal stem cells, cellular senescence, adipose tissue, cellular dysfunction

INTRODUCTION

As the epidemic of obesity continues to escalate globally, its prevalence is projected to dramatically increase in the United States, Mexico, and England with 47, 39, and 35% of the population being obese by 2030, respectively (Hruby and Hu, 2015; Hales et al., 2018). Currently, obesity is ranked as the leading cause of preventable disease and mortality in the United States, surpassing smoking (Hennekens and Andreotti, 2013). Furthermore, obesity is the chief contributing factor in the development and progression of comorbid complications, such as diabetes, chronic kidney disease (CKD), and cardiovascular events (Choung et al., 2019). Standard classification defines obesity as a body-mass index (BMI) greater than 30 kg/m^2, whereas a BMI $\geq$ 40 kg/m^2, or 35 kg/m^2 with the presence of weight-associated complications, is classified as morbid obesity (Heymsfield and Wadden, 2017).

Excessive fat expansion during obesity leads to a chronic inflammatory state, which may cause damage to endogenous repair systems. Mesenchymal stromal/stem cells (MSC) are endogenous, self-renewing cells capable of differentiating into mature cell lineages, and are abundantly found in subcutaneous adipose and other tissues (Eirin et al., 2014, 2016, 2017). This cell population is endowed with an ability to attenuate immune responses, decrease fibrosis, and stimulate angiogenesis through paracrine activities (Bernardo et al., 2009; Reinders et al., 2010). Studies have demonstrated promising capabilities for tissue repair when MSC are expanded *in vitro* and exogenously transplanted into injured organs (Ebrahimi et al., 2013; Eirin et al., 2015; Saad et al., 2017). Being immunomodulatory and anti-inflammatory, MSC are a choice cell type for cell-based therapy.

However, in pathological conditions such as obesity, adipose tissue-derived MSC may potentially exhibit limited regenerative capacity to repair injured tissues because of altered cellular properties and functions, including the ability to proliferate or migrate toward injury sites. Such impairments might not only interfere with the endogenous repair capacity of tissues and blood vessels in obese individuals, but could also impede the feasibility of using autologous MSC for exogenous transplantation if needed to combat organ damage. In particular, in obesity the adipose tissue microenvironment might constitute an important instigator of MSC dysfunction, because obese adipose tissue develops a pro-inflammatory profile and harbors an increased burden of senescent cells (Xu et al., 2015).

Cellular senescence is a stress response mechanism, which leads to irreversible, cell-cycle arrest mediated by the tumor suppressor proteins p53, p21, p16^{INK4A}, and p19^{ARF} (Childs et al., 2016; Sturmlechner et al., 2017). Moreover, senescent cells release diverse growth factors, cytokines, chemokines, and matrix metalloproteinases, which comprise the senescence-associated secretory phenotype (SASP) (Baker et al., 2011). Senescent and SASP factors determine the fate of cells and their neighboring *milieu* by adversely affecting the microenvironment (Baker et al., 2011; Xu et al., 2015; Schafer et al., 2017).

Our lab has demonstrated that, in addition to inducing kidney (Ma et al., 2016) and cardiac (Zhang et al., 2015) damage, obesity impairs the function of adipose tissue-derived MSC in a large animal model (Zhu et al., 2016). Other findings have demonstrated that obese mice develop cellular senescence in their pre-adipocytes (Escande et al., 2014; Palmer et al., 2019). Hence, activation of injurious pathways may be compounded by diminished repair capacity. However, the effect of obesity on cellular senescence and on the reparative potential of MSC in human subjects remains obscure. Therefore, this study was designed to test the hypothesis that obesity induces cellular senescence and decreases functionality in MSC of obese subjects. To test our hypothesis, we compared function and cellular senescence in adipose tissue-derived MSC obtained from non-obese and obese subjects.

MATERIALS AND METHODS

Subject Recruitment, Screening, and Enrollment

We examined the functional and senescent characteristics in adipose tissue-derived MSC isolated from non-obese and obese subjects. Recruitment for this study focused on non-obese and obese individuals evaluated at Mayo Clinic in Rochester, Minnesota in the Nephrology, Endocrine, and General Bariatric and Obesity clinics between October 2017 and March 2019. Eligible subjects were 18–80 years of age with a BMI $\leq$ 30 kg/m^2 (non-obese) or BMI $\geq$ 30 kg/m^2 (obese). The Mayo Clinic Institutional Review Board approved all experimental study procedures. Obese subjects scheduled for weight-reduction surgery and kidney donor candidates scheduled for nephrectomy, who gave written informed consent, underwent an adipose tissue (0.5–2.0 g) sampling at the time of their surgical procedures.

Isolation and Culture of MSC

After harvesting, MSC were isolated from tissue specimens following standard protocols (Eirin et al., 2012; Saad et al., 2017). Briefly, minced adipose tissue was aseptically processed by incubation with collagenase-H at 37°C for 45 min. After digestion with collagenase-H, serum-containing medium was added to the suspension and filtered through a 100 μm cell strainer. The cellular suspension was centrifuged for 5 min at 1000 RPM to pull-down cells, and the cellular pellet re-suspended in Advanced Minimum Essential Medium supplemented with 5% platelet lysate (PLTmax, Mill Creek Life Sciences, Rochester, MN, United States). MSC were then expanded in culture for three passages to prepare for experimentation.

MSC Phenotyping

Third-passage MSC were characterized by imaging flow cytometry (FlowSight, Amnis, Seattle, WA, United States) to confirm expression of MSC-specific surface markers (all from Abcam, San Francisco, CA, United States) CD73 (Cat.# ab106677), CD90 (Cat.# ab124527), and CD105 (Cat.# ab53321). Conversely, MSC were expected to not express CD45 (Cat.# ab51482) or CD14 (Cat.# ab82012). All antibodies were used at the manufacturer's recommended dilutions and cellular concentrations, and data analyzed using Amnis® Image

Data Exploration and Analysis Software (IDEAS version 6.2) (Aghajani Nargesi et al., 2018).

Furthermore, MSC were characterized by their ability to differentiate into adipocyte, osteocyte, and chondrocyte lineages using a Human MSC Functional Identification Kit (R&D Systems®, Minneapolis, MN, United States, Cat.# SC006) (Zhu et al., 2016). Initially, MSC were seeded according to the manufacturer's instructions, and medium changed every 3–4 days. Culture media contained specific supplements necessary to induce *trans*-differentiation into the appropriate mesenchymal lineage. Following 21 days of culture, mature cell phenotypes were detected by immunofluorescent staining using the provided antibodies: anti-mouse FABP4, anti-human Aggrecan, and anti-human Osteocalcin to distinguish adipocytes, chondrocytes, and osteocytes, respectively.

MSC Functional Analyses

Cellular proliferation was evaluated using Incucyte®, a live-cell analysis and imaging system (Satorius, Ann Arbor, MI, United States). Approximately 2.5×10^3 MSC/well were seeded in a 96-well plate and allowed to propagate in culture for 72 h. MSC migratory function was tested using a QCM™ Colorimetric Cell Assay (EMD Millipore, Burlington, MA, United States; Cat.# ECM508), performed according to the company's standard protocol.

Senescence and SASP Markers

Total RNA was isolated from 0.5 and 1×10^6 cells using mirVana™ PARIS kit (ThermoFisher Scientific, Waltham, MA, United States; Cat.# AM1556), first strand cDNA produced by SuperScript™ VILO™ cDNA synthesis kit (ThermoFisher Scientific, Cat.# 11755050), and the $\Delta\Delta$Ct method used to assess gene expression levels in MSC from non-obese and obese subjects. Adipogenic markers were assessed using the primers for CCAAT/enhancer-binding protein-α (*C/EBPα*, Cat.# HS00269972) and peroxisome proliferator-activated receptor-γ (*PPARγ*, Cat.# HS01115513). Cellular senescence was ascertained by expression of the cell-cycle arrest markers *p16* (Cat.# H00923894), *p21* (Cat.# HS00355782), and *p53* (Cat.# HS01034249), as well as the SASP markers, interleukin-6 (*IL-6*, Cat.# HS00174131), monocyte chemoattractant protein-1 (*MCP-1*, HS00234140) and galactosidase (gal)-beta-1 (*GLB1*, Cat.# HS01035168). Vascular endothelial growth factor (*VEGF*, Cat.# HS00900055) was also evaluated. All TaqMan primers were purchased from ThermoFisher Scientific and gene expression normalized to TATA-binding protein (*TBP*, Cat.# HS00427620).

The ratio of phosphorylated/total γ-H2AX expression was evaluated by Western blot to assess for DNA damage in MSC, and the activity of β-gal, a participant in cellular senescence (Dimri et al., 1995), using an assay (Enzo, Farmingdale, NY, United States; Cat.# ENZ-KIT 129).

Co-culture of MSC and Senescent Endothelial Cells

To evaluate the reparative potency of MSC from non-obese and obese subjects, co-culture experiments were performed. Briefly, commercially available human umbilical vein endothelial cells (HUVEC, Cell Applications, San Diego, CA, United States; Cat.# 200K-05f) were grown in endothelial cell growth medium (EGM™-Plus Endothelial Cell Growth Media-Plus Bulletkit™ Medium, Lonza, Cohasset, MN, United States; Cat.# CC-5035), seeded at a density of 3.5×10^5 cells/well in a transwell plate (VWR, Radnor, PA, United States, Cat.# 2944-076), and divided into four groups. Group 1 cells were cultured under normal conditions, while group 2–4 cells were co-incubated with tumor necrosis factor-α (TNF-α, 10 ng/mL) and transforming growth factor-β1 (TGF-β1, 5 ng/mL) for 3 days to induce cellular injury (Khan et al., 2017). This medium was then replaced with fresh growth medium. Groups 3 and 4 were also subsequently co-cultured with MSC from either non-obese or obese subjects, respectively (1.75×10^5 cells/well insert) for another 24 h. Afterward all cells ($n = 5$–7/group) were lysed and prepared for qPCR analyses.

Additionally, the angiogenic potential of HUVEC was tested in Groups 1–4. Following treatment and co-culture procedures HUVEC were seeded onto a Matrigel® matrix-coated plate (CORNING, Corning, NY, United States; Cat.# 354433) at a final concentration of 7×10^4 cells/500 μL, incubated overnight in a 37°C, 5% CO_2 humidified incubator. HUVEC were observed under a Zeiss Axio Observer inverted microscope for the formation of tube-like structures. The network of tubes was counted in five different fields of view for each group.

In vitro Assays

Apoptotic signal was detected in MSC from non-obese and obese subjects using a Dead-End™ Fluorimetric Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay (Promega, Madison, WI, United States; Cat.# G3250). In brief, 6.0×10^4 MSC were prepared in 4-well chamber slides, assayed using the manufacturer's directions and visualized by green fluorescence for fragmented DNA (incorporation of fluorescein-12-dUTP). The percentages of TUNEL-positive cells were quantified in 12 fields of view using the Cytation-5 Cell Imaging Reader.

Cellular oxidative stress was assessed by staining with dihydroethidium (DHE, ThermoFisher Scientific, Cat.# D11347) in MSC from non-obese and obese subjects. Fluorescence intensity was calculated using ImageJ software.

Statistical Analysis

Distribution of the data was evaluated using the Shapiro-Wilk Test. Normally-distributed data were represented as mean ± standard deviation, and non-normal data as median and interquartile range. Comparisons among groups were performed using either a two-sample *t*-test with a 5% type-I error rate or Wilcoxon Rank Sum, as appropriate. One-way analysis of variance (ANOVA) and *post hoc* pairwise testing were employed to detect differences in co-culture experiments, whereas cellular proliferation data were analyzed using repeated ANOVA. Bivariate correlation analysis was used to determine the direction of association between BMI and MSC parameters, and multivariate linear regression performed in two models among the variables with $p < 0.2$ in the bivariate analysis with

Collinearity Diagnostics. Variables with non-normal distribution were log-transformed before entering the multivariate linear regression models. All data were considered significant if $p \leq 0.05$. Statistical analysis was accomplished using JMP 14.1 Software.

RESULTS

A total of 40 subjects undergoing kidney donation or bariatric surgery donated adipose tissue for MSC isolation. Females accounted for >60% of participants, and mean age was 59 years. As expected mean BMI was significantly higher in obese subjects in comparison to non-obese (**Table 1**; $p < 0.001$). Co-morbidities such as hypertension, dyslipidemia, obstructive sleep apnea, and diabetes were also prevalent. **Table 1** summarizes the demographics, clinical characteristics, and medication use of each group.

Mesenchymal stromal/stem cells-specific cell surface markers studied in passage-3 adipose tissue-derived MSC using flow cytometry demonstrated robust expression, with 99.7, 99.5, and 97.8% of the cell population positive for CD90, CD73, and CD105, respectively, whereas expression of both CD45 and CD14 was low (**Figure 1A**). For additional characterization, MSC were cultured in specific media to induce trilineage differentiation. After 21 days, we found that MSC from non-obese and obese subjects had similar abilities to *trans*-differentiate into adipocytes, osteocytes, and chondrocytes (**Figure 1B**). Altogether, these findings confirm characteristics indicative of MSC.

Furthermore, we sought to evaluate whether obesity influences the migratory and proliferative capacities of MSC. Migration potential of MSC from obese subjects was comparable to MSC from non-obese subjects (**Figure 2A**), whereas MSC from obese subjects showed a significant reduction in proliferative activity starting at ∼32 h of incubation (**Figure 2B**). Gene expression of the adipogenic transcription factor *PPARγ* was significantly lower in MSC from obese subjects, suggesting attenuated capacity for adipogenesis, while *C/EBPα* expression levels were similar between MSC from non-obese and obese subjects (**Figure 2C**).

Senescent cells were identified by relative expression of senescent and canonical SASP factors using qPCR. As shown in **Figure 3A**, MSC from obese subjects had markedly increased expression of *p16* and *p53*, as well as inflammatory SASP factors, *IL-6* and *MCP-1*, consistent with development of senescence. Additionally, *p21* tended to be elevated in MSC from obese subjects ($p = 0.08$, **Figure 3A**). Contrarily, in our obese subject population, there was no evidence of DNA damage, reflected in unaltered H2AX protein expression (**Figure 3B**). While β-gal enzyme activity was unaltered (**Figure 3C**), *GLB1* gene expression was upregulated.

Among the entire cohort, BMI correlated directly but modestly with the expression of the cellular senescence-related genes *p16* (Spearman's correlation [r_s] 0.434; $p = 0.003$) and *p21* ($r_s = 0.398$; $p = 0.005$), and with the SASP-related gene *IL-6* ($r_s = 0.444$; $p = 0.002$), and inversely with MSC proliferation ($r_s = -0.298$; $p = 0.04$, **Figure 4**). Multivariate analysis detected *p21* as the strongest predictor of obesity (**Table 2**, Model A).

TABLE 1 | Baseline characteristics of non-obese and obese subject cohorts ($n = 40$).

Parameter	Non-obese	Obese
Demographics:		
Number of Subjects	11	29
Female Sex	64%	69%
Caucasian Race	100%	100%
Age, Years	61 ± 6	57 ± 10
Clinical:		
Body mass index, kg/m^2	25.7 ± 2.0	40.0 ± 8.5***
Hypertension	18%	41%
Dyslipidemia	27%	41%
Obstructive Sleep Apnea	0%	46%**
Asthma	0%	14%
Fatty Liver Disease	0%	10%
Glucose Intolerance/Diabetes	18%	45%
Gastro-esophageal Reflux	18%	31%
Depression	9%	48%*
Medications:		
Anti-hypertensive	18%	34%
Anticoagulation	9%	10%
Statins	18%	24%
Hypoglycemic	0%	21%
Antidepressants/Anti-Anxiety	9%	55%

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ (vs. non-obese).*

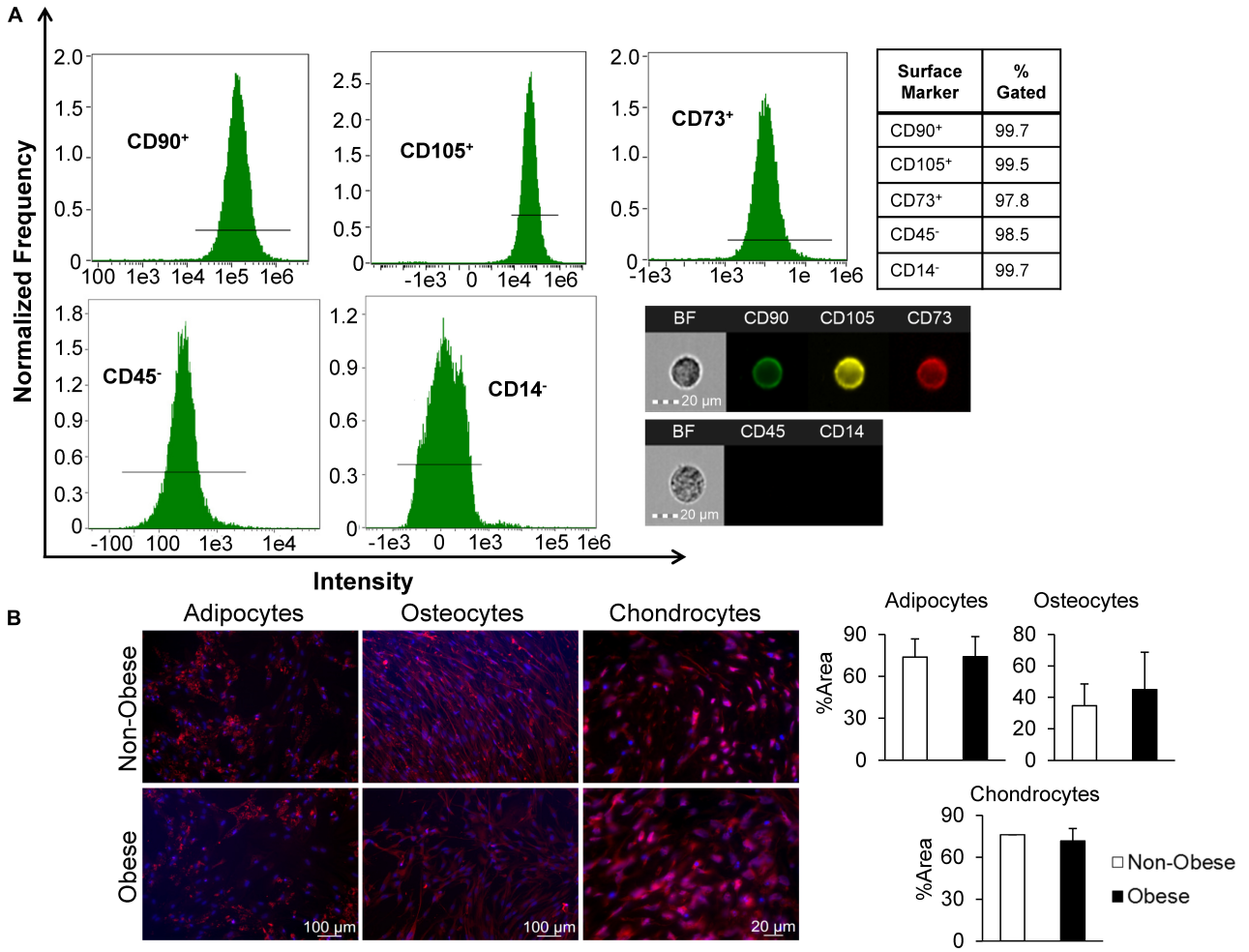

FIGURE 1 | MSC derived from human adipose tissue exhibit attributes distinct to stem cells. **(A)** Markers of MSC were measured by imaging flow cytometry, which defined MSC as CD90$^+$, CD105$^+$, CD73$^+$, and CD45,$^-$ and CD14$^-$, as shown in representative histograms and single-cell images. **(B)** MSC from non-obese and obese subjects demonstrated similar trilineage differentiation potential *in vitro*. Immunofluorescent staining (red; DAPI-blue stained nuclei) illustrates that similar expression of FABP4 (adipocytes), Osteocalcin (osteocytes), and Aggrecan (chondrocytes) in MSC from non-obese and obese subjects.

However, the presence of other predictors in Model B yielded *IL-6* as the most significant correlate of BMI.

Additionally, we examined the repair capacity of MSC from non-obese and obese subjects on cellular injury and senescence *in vitro*. Pre-incubation with TNF-α and TGF-β1 generated cell-cycle arrest in HUVEC, suggested by upregulation of *p16* and *p21* gene expression (**Figures 5A,B**). Co-culture of injured HUVEC with both MSC from non-obese and obese subjects reduced expression of *p16* (**Figure 5A**), and MSC from non-obese subjects tended to attenuate expression of *p21* as well (**Figure 5B**, $p = 0.08$ vs. MSC from obese subjects). Furthermore, *VEGF* mRNA levels were significantly decreased in injured HUVEC, and increased by co-incubation with MSC from non-obese, but not with MSC from obese subjects (**Figure 5C**), suggesting that only the former repaired HUVEC. Tube formation analysis demonstrated that co-culture of injured HUVEC with MSC from non-obese subjects formed tube-like networks (**Figure 5D**), reflecting their pro-angiogenic properties. This activity was diminished in injured HUVEC both untreated and those co-incubated with MSC from obese subjects, detected by reduced tube numbers.

Finally, TUNEL staining showed no difference in apoptotic signals between MSC from non-obese and obese subjects (**Figure 6A**), whereas MSC from obese subjects exhibited markedly higher production of superoxide (**Figure 6B**).

DISCUSSION

This study demonstrates that human obesity provokes senescence and diminishes functionality in adipose tissue-derived MSC in comparison to MSC from non-obese controls, suggested by reduced MSC proliferation, expression of the differentiation-dependent factor *PPARγ*, and pro-angiogenic efficacy. Furthermore, amplified expression of senescence-linked genes is directly associated with BMI. Taken together, these findings signify that the obesity microenvironment may

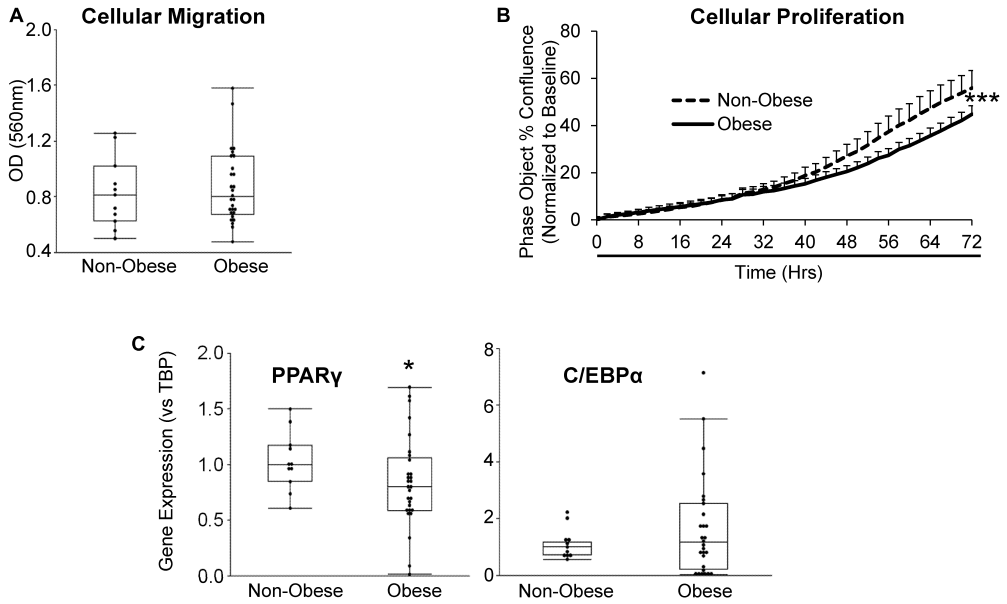

FIGURE 2 | MSC from obese subjects demonstrate functional impairments. MSC from obese subjects showed intact cellular migration **(A)**, but lower proliferation **(B)** and expression of the adipogenic marker *PPARγ*, but not *C/EBPα* **(C)** compared to MSC from non-obese subjects. $^{*}p \leq 0.05$, $^{***}p \leq 0.005$ vs. non-obese.

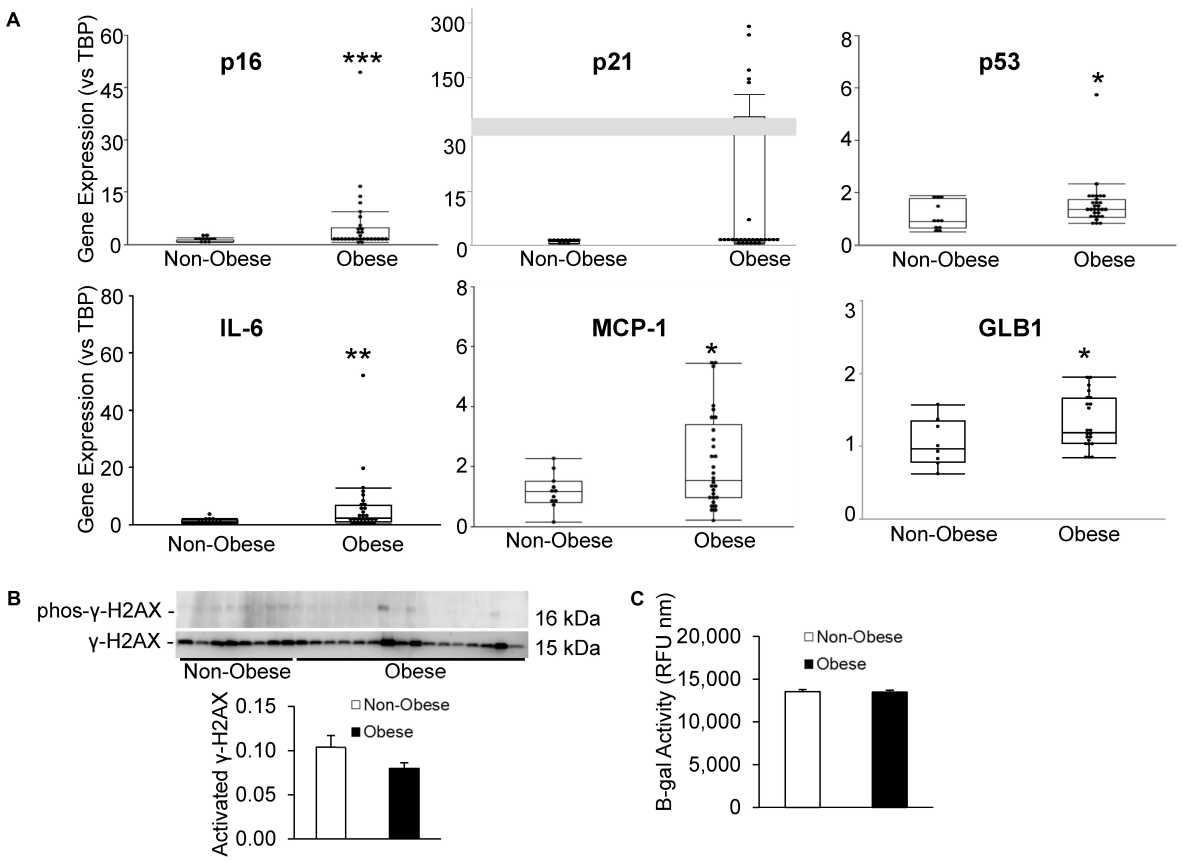

FIGURE 3 | Human obesity evokes premature cellular senescence. Expression of senescence-associated and SASP genes **(A)** was upregulated in MSC from obese subjects. **(B)** γ-H2AX protein expression and **(C)** β-gal activity were unaltered in our obese cohort, although *GLB1* gene expression was upregulated. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.005$ vs. non-obese.

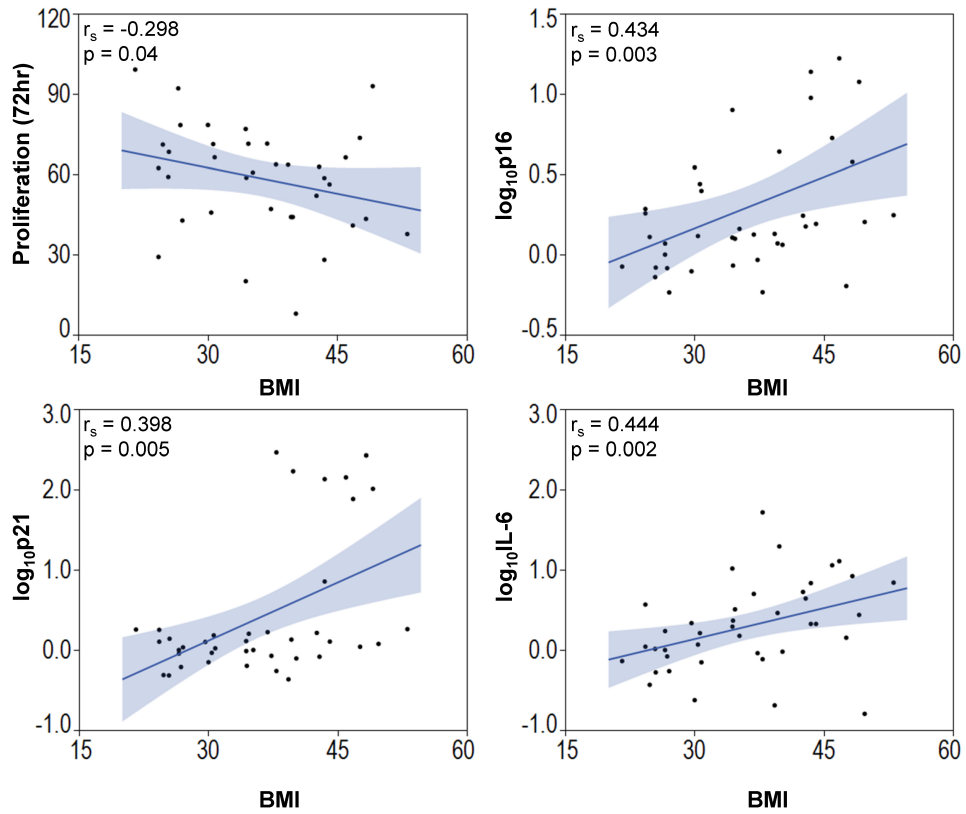

FIGURE 4 | Body mass index is associated with elevated senescence-associated gene expression and decreased MSC proliferation. BMI of subjects correlated inversely with MSC proliferation and *p16*, *p21*, and *IL-6*.

TABLE 2 | Multivariate linear regression models for predictor variables of BMI ($n = 40$).

Model A			**Model B**		
	Regression Coefficient (β)	*p*-value		Regression Coefficient (β)	*p*-value
Proliferation (72hr)	−0.205	0.164	Proliferation (72hr)	−0.148	0.310
Log-p16	0.149	0.489	Log-p16	0.241	0.132
Log-p21	**0.536**	**0.001*** **	Log-p21	0.264	0.172
Log-p53	0.159	0.311	Log-p53	0.205	0.157
PPARγ	−0.106	0.479	PPARγ	0.025	0.871
Log-MCP-1	0.125	0.396	**Log-IL-6**	**0.578**	**<0.001*** **

*** $p \leq 0.001$ Forward Selection. PPARγ, peroxisome proliferator-activated receptor-γ; MCP, macrophage chemoattractant protein-1; IL-6, interleukin-6. Values in bold font represent the strongest correlates for the model.*

limit the vitality and potency of MSC, which may account for increased propensity for tissue damage and organ injury observed in obesity.

In obesity, adipose tissue storage surpasses the given capacity and prompts disproportionate fat accumulation, leading to prolonged and uncontrolled inflammatory responses and disease progression. Dysfunction of adipose tissue is considered the main driver linked to the onset and progression of obesity-associated health problems, including insulin resistance, diabetes mellitus, hypertension, and dyslipidemia. Obesity is dramatically increasing worldwide, and if the trend continues, these health complications will also escalate (Hales et al., 2018).

Regenerative cell-based treatment strategies using adipose tissue-derived MSC are being extensively evaluated in various acute and chronic conditions. However, subject-associated comorbidities such as obesity may alter the biologic characteristics of MSC, compromising their efficacy for transplantation. We have previously shown that obesity also alters the function of adipose tissue-derived MSC and modifies the expression of genes associated with insulin signaling and mitochondrial function in an experimental pig model (Zhu et al., 2016; Conley et al., 2018; Meng et al., 2018a,b). In addition, obesity reduces the pro-angiogenic potential and "stemness" of MSC isolated from obese human subjects and their extracellular

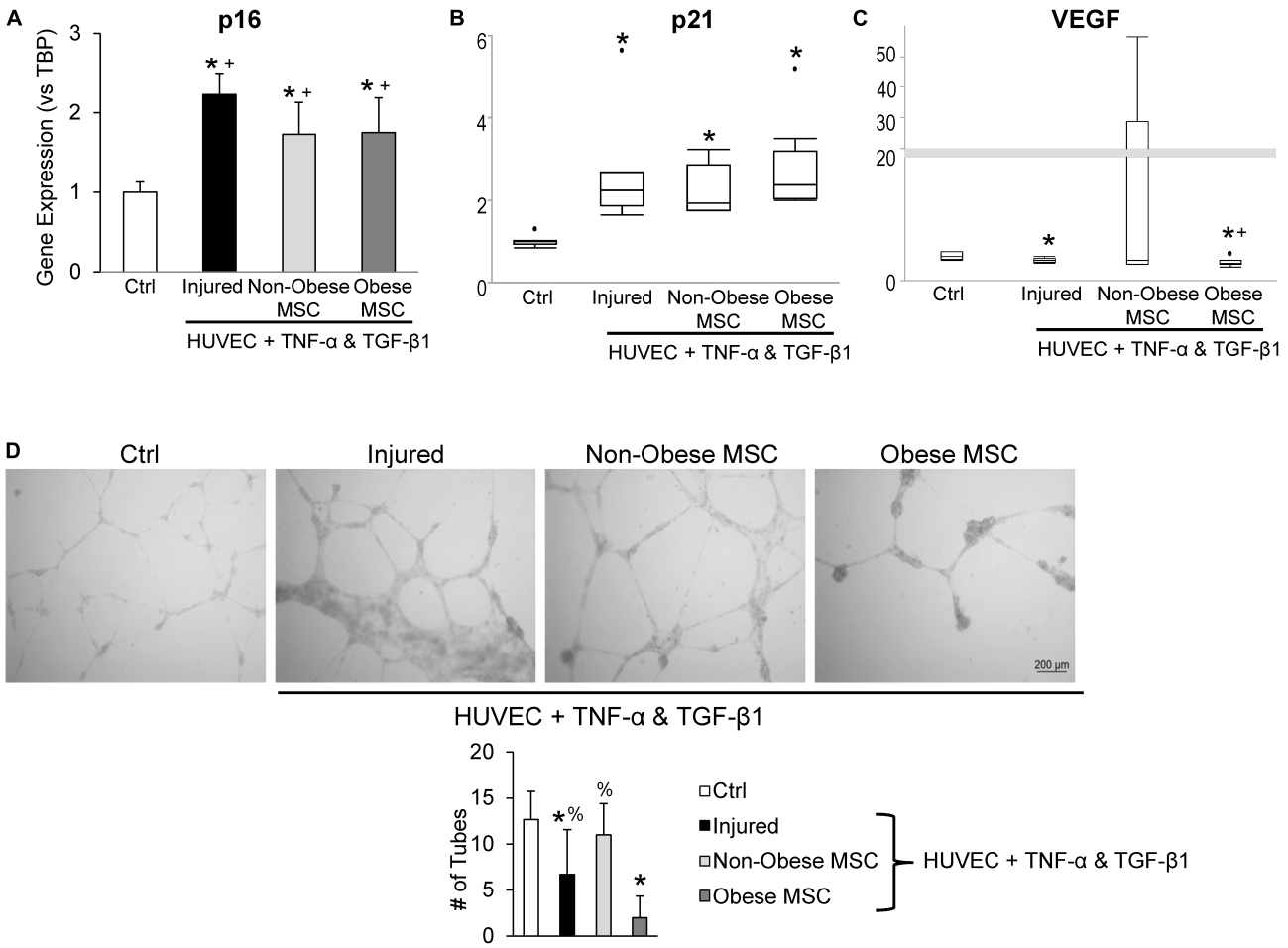

FIGURE 5 | Co-culture with MSC from obese subjects fails to restore angiogenic potential of HUVEC. Gene expression of *p16* **(A)** and *p21* **(B)** increased and *VEGF* decreased **(C)** in injured HUVEC. Co-culture of HUVEC with both types of MSC attenuated HUVEC senescence, but only MSC from non-obese subjects restored *VEGF* expression. *In vitro* tube formation analysis shows evident tube-like structures in control (Ctrl) cells and non-obese MSC, whereas MSC from obese subjects fail to repair angiogenesis, and injure further **(D)**. *vs. ctrl, + vs. injured HUVEC, % vs. obese.

vesicles (Onate et al., 2012, 2013; Togliatto et al., 2016). Our study suggests that human obesity also induces cellular senescence, SASP, and impaired proliferation in MSC, each of which is linked to the severity of obesity.

Cellular senescence is implicated in the pathogenesis of several disease states, including obesity (Palmer et al., 2019), in which accumulation of senescent cells contributes to sterile inflammation. We identified increased expression of genes associated with cell-cycle arrest (*p16* and *p53*) and SASP (*IL-6* and *MCP-1*) in MSC isolated from obese compared to non-obese subjects, potentially contributing to the impaired cellular proliferation observed in MSC from obese subjects and amplifying senescent cell accumulation *in vitro* during culture expansion. Senescent cells accumulate in diseased tissues, and disrupt their structure and function by secreting a cocktail of factors known as the SASP (Baker et al., 2011). Xu et al. (2017) demonstrated that a small number of senescent cells can produce an osteoarthritic disease phenotype. Therefore, the presence of endogenous senescent MSC in obese subjects or their exogenous transplantation may further injure neighboring cells, including healthy MSC that are meant to repair, and accelerate a forward-feeding loop of intensified cellular senescence. Indeed, both MCP-1 (Jin et al., 2016) and IL-6 (Groppo and Richter, 2011) reinforce senescence. Upregulation of the senescence-associated (SA-β-gal) enzyme, an index of increased lysosomal activity, is a common marker for cellular senescence, and its activation is dependent on *GLB1*, the gene that encodes lysosomal β-gal (Lee et al., 2006). While our cohort showed no difference in β-gal activity between MSC from non-obese and obese subjects, *GLB1* expression was significantly increased, suggesting that the involvement of SA-β-gal might be at the gene expression level rather than enzymatic activity.

*C/EBP*α and *PPAR*γ are important regulators of adipogenic metabolism and development of functional fat progenitor cells. Blunted expression of these transcriptional factors can impair adipogenesis and insulin sensitivity (Dubois et al., 2006). Matulewicz et al. (2017) demonstrated that in a young cohort of overweight and obese individuals, adipose tissue expression

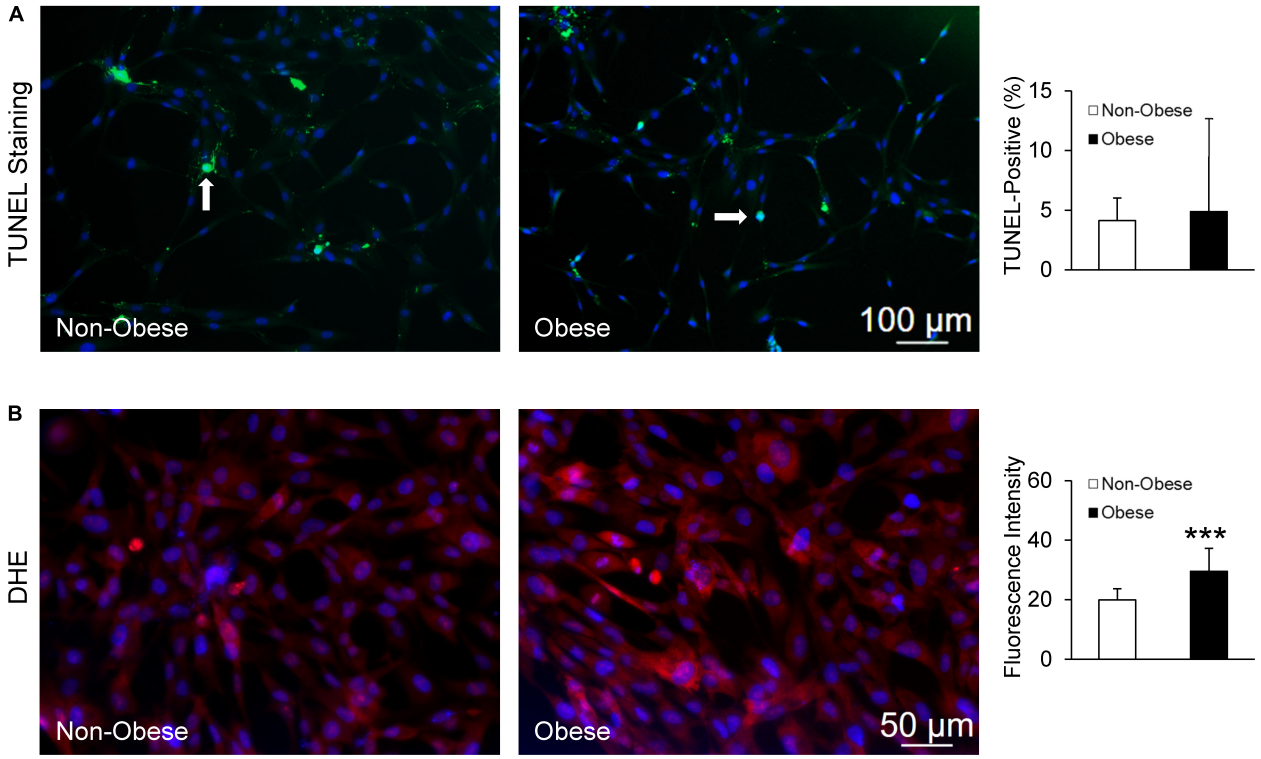

FIGURE 6 | MSC from non-obese and obese subjects displayed similarly low apoptotic signals; white arrows indicate apoptotic nuclei (green), counterstained with DAPI (blue) **(A)**. Production of superoxide anion, DHE was increased in MSC from obese subjects **(B)**.

of adipogenic markers significantly decreased in comparison to normal-weight individuals. Similarly, in our slightly older subject population we observed reduced expression of *PPARγ* in adipose tissue-derived MSC, suggesting that MSC share impairments observed in surrounding adipose tissue. Moreover, Mitterberger et al. (2014) demonstrated that adipose-derived progenitor cells show reduced adipogenic capacity as senescent cells increase. Nevertheless, the tri-lineage differentiation assay indicated intact multi-lineage potential toward adipocytes, possibly due to culture conditions and unaltered expression of *C/EBPα* in the MSC. Our cohort also exhibited no measurable differences in γ-H2AX activation, arguing against DNA damage in their MSC at this early phase of morbidity.

Previous studies have shown that co-incubation of HUVEC with TNF-α and TGF-β1 induces cellular senescence and injury (Khan et al., 2017). Indeed, in our co-culture studies, gene expression of *p16* and *p21* was upregulated. Interestingly, MSC from both obese and non-obese subjects decreased expression of *p16*, whereas *p21* remained unchanged. In addition, VEGF, an important mediator of the pro-angiogenic function of endothelial cells (Cheng et al., 2019), and its expression falls in injured HUVEC (Wang et al., 2019; Wu et al., 2019), as we observed in our injured HUVEC model. Importantly, HUVEC *VEGF* gene expression was restored to control levels by co-incubation with MSC from non-obese subjects, whereas MSC from obese subjects failed to reinstate it. Moreover, MSC from non-obese subjects were able to stimulate angiogenesis activity in injured HUVEC, illustrated by formation of tube-like networks, whereas these capacities were attenuated in MSC from obese subjects. These observations imply that human adipose tissue-derived MSC have modest capability to blunt cellular senescence *in vitro*, which is unaffected by obesity. Contrarily, obesity significantly interferes with the potency of MSC to repair injured endothelial cells.

Additionally, we observed elevated superoxide levels in MSC from obese subjects. Inflammation and lipid peroxidation in obesity may increase oxidative stress, which in turn has been shown to induce senescence through p53-specific signaling (Han et al., 2020). However, additional studies need to be done conducted to further evaluate this potential mechanism.

The present study has limitations. While the relatively old age of our subjects is pertinent to the obese population (Jura and Kozak, 2016), the entirely Caucasian composition of our study cohort limits generalizability of our findings. Additionally, given the cohort sample size, we are unable to determine the dependence of obesity-induced MSC dysfunction on specific comorbidities. Nonetheless, this multi-morbidity prevalence is characteristic of the obese population. Furthermore, future studies need to define whether the MSC phenotype can be altered to disrupt the trajectory of obesity-induced MSC dysfunction and senescence. For example, we have recently demonstrated the effectiveness of senolytic agents in reducing senescence in kidneys of obese mice (Kim et al., 2019) and adipose tissue in subjects with diabetic kidney disease (Hickson et al., 2019). The ability of anti-inflammatory approaches to transiently interrupt

the vicious cycle of the SASP and cellular senescence also requires further study.

CONCLUSION

In summary, we show that human obesity alters functional characteristics and induces senescence in adipose tissue-derived MSC. This impairment in endogenous cellular repair systems may permit development and inadequate repair of lesions in subjects with obesity, and limit the utility of exogenous autologous delivery of their MSC. Further studies are needed to identify strategies to improve MSC function in obese individuals.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Mayo Clinic Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SC, LH, TTc, JK, and LL contributed conception and design of the study. SC, LH, HT, KJ, IS, JW, BI, MA, TTc, JK, and LL collected the data, and contributed to data analysis and interpretation. LH, TK, TM, JH, TTa, JK, and LL acquired the study sample and or research materials. SC, LH, JK, and LL provided financial support. SC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Dual Role of Autophagy in Regulation of Mesenchymal Stem Cell Senescence

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During their development and overall life, mesenchymal stem cells (MSCs) encounter a plethora of internal and external stress signals and therefore, they need to put in action homeostatic changes in order to face these stresses. To this aim, similar to other mammalian cells, MSCs are endowed with two crucial biological responses, autophagy and senescence. Sharing of a number of stimuli like shrinkage of telomeres, oncogenic and oxidative stress, and DNA damage, suggest an intriguingly close relationship between autophagy and senescence. Autophagy is at first reported to suppress MSC senescence by clearing injured cytoplasmic organelles and impaired macromolecules, yet recent investigations also showed that autophagy can promote MSC senescence by inducing the production of senescence-associated secretory proteins (SASP). These apparently contrary contributions of autophagy may mirror an intricate image of autophagic regulation on MSC senescence. We here tackle the pro-senescence and anti-senescence roles of autophagy in MSCs while concentrating on some possible mechanistic explanations of such an intricate liaison. Clarifying the autophagy/senescence relationship in MSCs will help the development of more effective and safer therapeutic strategies.

Keywords: mesenchymal stem cell, senescence, general autophagy, selective autophagy, SASP

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INTRODUCTION

Mesenchymal stem cells (MSCs) are the most widely utilized adult stem cells in clinical trials (Trounson and McDonald, 2015). Thorough investigation has substantiated the capability of MSCs to differentiate into cells of the mesenchymal lineage including osteoblasts, chondrocytes and adipocytes, and to secrete several trophic components able to exert their effect at cellular level like apoptosis and differentiation, at systemic level like immune response modulation and at tissue level like angiogenesis and fibrosis; further, MSCs sustain cardiac, muscle, and neural tissue regeneration (da Silva Meirelles et al., 2009; Caplan and Correa, 2011). Cellular senescence and autophagy are stress responses essential for MSC homeostasis.

Senescence, biologically described as a cellular condition in which cells have lost the proliferative capacity, yet maintaining their metabolic activity, is a genetically based program responding to stress signals that avoids damaged cells to further proliferate, acting as a powerful tumor suppressive

Abbreviations: ATG, autophagy-related; LC3, light chain 3; MSC, mesenchymal stem cell; mTOR, mammalian target of rapamycin; OIS, oncogene induced senescence; ROS, reactive oxygen species; SASP, senescence associated secretory pathway; TASCC, TOR autophagy spatial coupling compartment.

mechanism (Kuilman et al., 2010; López-Otín et al., 2013; Muñoz-Espín and Serrano, 2014; Childs et al., 2015). As reviewed elsewhere (Turinetto et al., 2016), human MSCs react with senescence induction in response to various stress stimuli, including telomere shortening (Baxter et al., 2004), oxidative stress (Stolzing and Scutt, 2006; Burova et al., 2013; Kim et al., 2011), heat shock (Alekseenko et al., 2014), and chemotherapeutic agents (Seifrtova et al., 2013; Minieri et al., 2015; Skolekova et al., 2016). Besides demonstrating that senescence program activation is independent of the MSC tissue source, those previous contributions added several details in MSC senescence profiles and phenotypes. Specifically, similar to human fibroblasts (Rodier et al., 2009), human MSC senescence program was sustained by persistent DNA damage repair activation, evidenced by the detection of characteristic enlarged nuclear foci, containing γH2AX and 53BP1 proteins (Cmielova et al., 2012; Seifrtova et al., 2013; Minieri et al., 2015; Turinetto and Giachino, 2015). At a molecular level, the overall network is not yet completely clarified, however, so further studies are required to better comprehend the mechanisms of senescence in MSCs.

Macroautophagy (hereafter referred to as autophagy and the focus of this review) represents a crucial path for the maintenance of cellular homeostasis under both physiologic and stressful situations since upon activation it sustains cellular survival thanks to the preservation of suitable metabolic functions, bioenergetic levels and amino acid pools. It is a degradation process of cellular own elements relying on the lysosomal compartment, representing a fundamental protective answer to tough situations like nutrient deprivation, where active recycling of cellular components are needed to guarantee energy homeostasis (Kang and Avery, 2008; Kroemer et al., 2010; Choi et al., 2013). In addition to working as a straightforward means to degrade large molecules that have formed aggregates or become misfolded, it is implicated in the clearance of altered and non-functional organelles including mitochondria in order to maintain appropriate cell metabolism (He and Klionsky, 2009; Boya et al., 2013). The first step of autophagy, the autophagosome biogenesis, relies on a core machinery consisting of *ATG* (autophagy-related) genes and ATG proteins; their first identification in the yeast (Tsukada and Ohsumi, 1993) was followed by cloning of their mammalian homologs, that were found to guarantee similar functions (Mizushima et al., 2011). A macromolecular complex is involved in autophagosome nucleation, consisting in the class III phosphatidylinositol 3-kinase and Beclin 1; this complex is also implicated in the phagophore membrane positioning of several other autophagic proteins that need to be subsequently recruited (Cao and Klionsky, 2007). An elongation step at the level of the phagophore membrane follows; it is performed by two ubiquitin-like systems, comprising the ATG12–ATG5-ATG16L1 complex and MAP1LC3/LC3 (microtubule associated protein 1 light chain 3). LC3 molecule is then cleaved to produce cytosolic LC3-I through the action of ATG4. LC3-I is covalently bound to phosphatidylethanolamine through the action of ATG7, ATG3 and the ATG12–ATG5-ATG16L1 complex generating LC3-II. LC3-II represents a very useful indicator of the mature autophagosome being strictly associated with the phagophore and autophagosome membrane, so it is largely employed as a marker of autophagy (Kabeya et al., 2000, 2004). Despite the fact that autophagy process was first acknowledged as a non-selective, lysosomal degradation mechanism, referred to as general autophagy, growing evidence sustains the existence of a selective autophagy, a form of autophagy mediating the degradation of specific classes of target molecules.

The intricate relationship between autophagy induced by diverse extra- or intracellular stimuli and the molecular targets that influence MSC proliferation, differentiation, and stemness has been reviewed elsewhere (Guan et al., 2013; Sbrana et al., 2016). Briefly, in human MSCs, the detection of consistent LC3-I to LC3-II conversion rates suggests constitutive activation of the autophagic flux (Oliver et al., 2012; Salemi et al., 2012) and MSC commitment to various cell lineages relies on basal autophagy activities, more than anything towards the osteoblastic lineage. Collection of undegraded autophagosomes and reduced autophagic turnover in undifferentiated MSCs have been evidenced, while in contrast stimulation of osteogenic differentiation resulted in a steady turnover increase (Nuschke et al., 2014). Conceptually, autophagy and senescence display common features as both partake in stress cell responses that can have either cytoprotective or cytotoxic consequences. However, whether autophagy performs as a positive or negative regulator of senescence in MSCs is so far debated.

ANTI-SENESCENCE ROLE OF AUTOPHAGY IN MSCs

The main view of autophagy's impact is an efficacious anti-senescence role implicating various pathways, whose principal players are the (mammalian) target of rapamycin (mTOR), insulin-like growth factor (IGF) binding to insulin-like growth factor receptors (IGF1R), adenosine monophosphate-activated protein kinase (AMPK) and p53 (Rubinsztein et al., 2011). Indeed, prolonged autophagy impairment in primary human fibroblasts provided through knocking down ATG7 or ATG5 resulted in cellular senescence, due to mitochondrial impairment and accumulation of produced reactive oxygen species (ROS) (Kang et al., 2011). Consistent with this view, increased autophagic activity was described to be capable of extending the lifespan of aged mice and elder flies and ripristinating the self-renewal stem cell activity, providing indications that the anti-aging effect was at least in part dependent on stem cell function restoration (Simonsen et al., 2008; Harrison et al., 2009).

More recent investigations have confirmed that autophagy is requested for sustaining the stemness and differentiation properties of stem cells. Garcia-Prat et al. described a critical role for basal autophagy in the maintenance of an immature stage in satellite cells, and unsuccessful autophagic activity promoted cell senescence defined by numerical and functional decrease of these cells (García-Prat et al., 2016).

Along this line, it was found that autophagic activity of old bone marrow-derived MSCs was diminished in comparison with young MSCs (Ma et al., 2018). Authors reported that

autophagy exerted an important function in the maintenance of MSCs upon aging, and demonstrated that autophagic control could partly rescue aged MSCs' features and bone loss in mice through the regulation of ROS-p53 (Ma et al., 2018). Those evidence suggested that the autophagic activity of MSCs could at least in part regulate bone aging, allowing to speculate the diminished autophagic activity in aged MSCs as one of the mayor sources of their degenerative modifications, and bone loss caused by impaired autophagy as an inherent novel component of bone aging.

The results of premodulated autophagy on MSC senescence were explored by up- or down-regulating autophagy through the employment of rapamycin or 3-methyladenine, respectively, prior to induction of D-galactose-mediated MSC senescence (Zhang et al., 2020). These studies exemplified that the use of rapamycin for 24 h reduced MSC senescence significantly in this experimental setting, and this was accompanied by diminished ROS production. Downregulation of p-Jun N-terminal kinases (JNK) and p-38 expression could also be demonstrated in the rapamycin treated cells (Zhang et al., 2020). In addition, the protective role of rapamycin on MSC aging could be counteracted by increasing the level of ROS, and the use of p38 inhibitors could revert the senescence induction effect of H_2O_2 on MSCs (Zhang et al., 2020). Altogether, this study indicated that autophagy exerted a protecting effect on D-gal-induced MSC senescence, and ROS/JNK/p38 cascade played a relevant mediating function in autophagy-mediated delay of MSC senescence. The evidence that autophagy could protect MSCs from oxidative stress signaling (Song et al., 2014) represented another indication that autophagy exerts a preserving function during cell aging. Finally, in many cases of acute senescence the autophagy flux is seriously compromised in MSCs, further evidencing that the autophagic process counterbalances damaging paths, and its negative modulation favors a senescent state (Capasso et al., 2015).

PRO-SENESCENCE ROLE OF AUTOPHAGY IN MSCs

Conversely, autophagy markers have been observed in senescent cells and autophagy has been demonstrated to be necessary for preservation of replicative senescence of MSCs (Zheng et al., 2014). Along this line, Zheng et al. showed that autophagy increased when MSCs entered the replicative aging state, with p53 contributing a relevant function in the autophagic increment in this specific setting (Zheng et al., 2016). p53 downregulation through knockdown experiments resulted in diminished LC3-II conversion and increased mTOR expression, thus showing that it represents a crucial trigger for autophagic activation in the course of *in vitro* expansion of MSCs (Zheng et al., 2016). Indeed, in replicative senescent MSCs, up-regulation of autophagy related genes was detected, however p53 not only played a crucial role in senescence but was also essential for triggering autophagy during culture expansion of MSCs (Fafián-Labora et al., 2019).

Further experimental models showed that the senescence status was somehow dependent on a preliminary autophagy induction. As an example, diminished insulin-like growth factor 1 (IGF1) expression was found to protect senescent MSCs kept under conditions of hypoxia by means of an up-regulated autophagic flux, thus augmenting the survival of senescent MSCs after myocardial infarction transplantation (Yang et al., 2018). Also, high glucose levels were reported to induce senescence by triggering the formation of ROS and upregulating autophagy in MSCs (Chang et al., 2015). In the above work, MSCs cultivated in high glucose concentration medium exhibited premature senescence, as showed by telomeric impact and genomic instability; it was undoubtedly evidenced that autophagy upregulation, detected through increased Beclin-1, Atg 5 and 7 expression, and augmented LC3-II conversion rate, correlated with senescence induction in MSCs while, on the opposite, negative regulation of autophagy employing 3-methyladenine prevented cellular degeneration (Chang et al., 2015).

Lastly, a few reports described oncogene-induced senescence (OIS) in MSCs in conjunction with disease manifestation. In patients suffering from systemic lupus erythematosus, for example, leptin and Neutrophil-Activating Peptide 2 sustained MSC senescence through activation of the PI3K/AKT signaling (Chen et al., 2015). Another oncogene, ASPL-TFE3, was demonstrated to induce MSC senescence through p21 up-regulation in alveolar soft part sarcoma (Ishiguro and Yoshida, 2016). In this type of induced senescence, Young et al. reported for the first time in fibroblast cells a causal association between autophagy and senescence, demonstrating that autophagy was activated during OIS and, in particular, it regulated the SASP at a post-transcriptional level, leading to the interesting speculation that autophagy provided the building blocks for the SASP protein production (Young et al., 2009). When a specialized type of general autophagy known as the TOR autophagy spatial coupling compartment or TASCC was later identified to be responsible for the protein synthesis of some SASP factors, previous speculation could be corroborated (Narita et al., 2011). The field, however, awaits further research to assess whether in MSCs similar pro-senescence autophagic activities take place during OIS.

RECONCILIATION

From these previous data, it appears that autophagy acts in MSCs as either a pro-senescence or an anti-senescence process, thus if and in what manner autophagy directs MSC aging remains elusive (**Table 1**).

Although the molecular bases underpinning senescence, particularly those overlapping with autophagy, are yet poorly comprehended, reconciling these antithetic phenomena would be feasible only by speculating that autophagy may regulate a number of targets oppositely acting to modulate cellular senescence in MSCs. Interestingly, recent studies disclosed distinct functions of general versus selective autophagy in the control of senescence, partially solving seemingly conflicting evidence concerning the relation between these two fundamental homeostatic responses to stress stimuli. In model cells other than MSCs, Kwon et al. suggested the interesting possibility that

TABLE 1 | Types of autophagy and their effect on senescence in MSCs and other cell types.

Cellular model	Type of autophagy	Effect on senescence	Senescence stimulus	References
MSC	General	Anti-senescence	Replicative exhaustion	Ma et al., 2018
MSC	General	Anti-senescence	D-galactose	Zhang et al., 2020
MSC	General	Anti-senescence	Oxidative stress	Song et al., 2014
MSC	General	Anti-senescence	Oxidative stress	Capasso et al., 2015
			Doxorubicin	
			X-ray	
			Replicative exhaustion	
MSC	General	Pro-senescence	Replicative exhaustion	Zheng et al., 2014
MSC	General	Pro-senescence	Replicative exhaustion	Zheng et al., 2016
MSC	General	Pro-senescence	Replicative exhaustion	Fafián-Labora et al., 2019
MSC	General	Pro-senescence	Hypoxia	Yang et al., 2018
MSC	General	Pro-senescence	Glucose	Chang et al., 2015
MSC	Selective	Anti-senescence	Lamin accumulation	Lee et al., 2018
MSC	Selective	Pro-senescence	Lamin accumulation	Infante et al., 2014
Fibroblast	Selective	Anti-senescence	OIS	Kang et al., 2015
Fibroblast	General (TASCC)	Pro-senescence	OIS	Narita et al., 2011
Lung primary cell	Selective	Pro-senescence	OIS	Dou et al., 2015

such a dual role might be context and time dependent as well as specifically depend on the type of autophagy, general versus selective, involved (Kwon et al., 2017).

According to this model, under normal conditions, general autophagy would act as an anti-senescence process by preserving cellular homeostasis. Upon situations of induced stress, early action of general autophagy would also play the role of a homeostatic response, thus prevalently anti-senescent (with the exception of some specialized types of general autophagy like the TASCC). However, general autophagy exerted in cells that have already initiated a senescence process can become pro-senescent, in the sense that it can sustain viability of senescent cells. Indeed, senescent cells cannot dilute toxic byproducts as they do not undergo mitosis and they secrete a number of factors that can induce endoplasmic reticulum stress; in these conditions, autophagy induction could counteract the risk of proteostasis disruption and avoid cell death (Kwon et al., 2017). Accordingly, the anti-senescence autophagy roles reported above for MSCs were mainly related to general autophagy, and autophagy manipulation experiments were conducted before senescence induction. Conversely, the pro-senescence roles of general autophagy described in MSCs referred to either long-term cultured cells, with a presumably already initiated senescence pathway, or, possibly, to oncogene-induced senescence.

In the case of selective autophagy, when only a certain type of substrates is degraded, the resulting role on senescence would depend on the specific substrates and autophagic receptors involved. While looking for regulators of cellular senescence, Kang and Elledge (2016) identified GATA4 as a crucial regulator of the SASP and senescence. GATA4 is a transcription factor whose protein stability increases during cellular senescence leading to its accumulation. Intriguingly, authors showed a clear autophagic regulation of GATA4: under normal conditions, it is degraded thanks to the autophagic receptor protein SQSTM1/p62, yet when the cell encounters senescence-inducing stimuli, a steady decrease of the interaction between GATA4 and SQSTM1/p62 occurs, autophagic degradation is limited and GATA4 accumulates (Kang and Elledge, 2016). This accumulated GATA4 starts a transcriptional activity to switch on NFKB/NF-kB and in turn the SASP. Interestingly, GATA4-dependent regulation of the secretory phenotype was recently found to play an important role in human MSC aging (Lee et al., 2018). On the other hand, LC3B-lamin B1-dependent selective autophagy of nuclear lamina was found to act as a pro-senescence mechanism (Dou et al., 2015). Interestingly, a model of human aging based on MSCs with accumulated prelamin has been proposed (Infante et al., 2014). Taken altogether, these data seem to suggest that selective autophagy actively suppresses cellular senescence through the degradation of a senescence regulator, GATA4, whereas it promotes cellular senescence through the degradation of nuclear lamina and open the way for a similar dual control of autophagy over senescence also in MSCs.

So at least three elements, i.e., the context, time of action and type of autophagy involved appear to constitute the frame in which autophagy can result either pro or anti-senescence in MSCs, as depicted in **Figure 1**. However, a few intriguing open questions concerning the extent to which cell-specific features, such as cell origin, metabolic status and age might contribute the final effect will deserve further investigation.

CLINICAL IMPLICATIONS

When tissue homeostasis is disrupted due to MSC senescence the possible shortcoming is twofold: a loss of repairing capability caused by decreased self-renewal/differentiation abilities and a detrimental microenvironment modulation by senescent MSCs due to secretion of pro-inflammatory and matrix-degrading molecules contained in the SASP. Both have important clinical

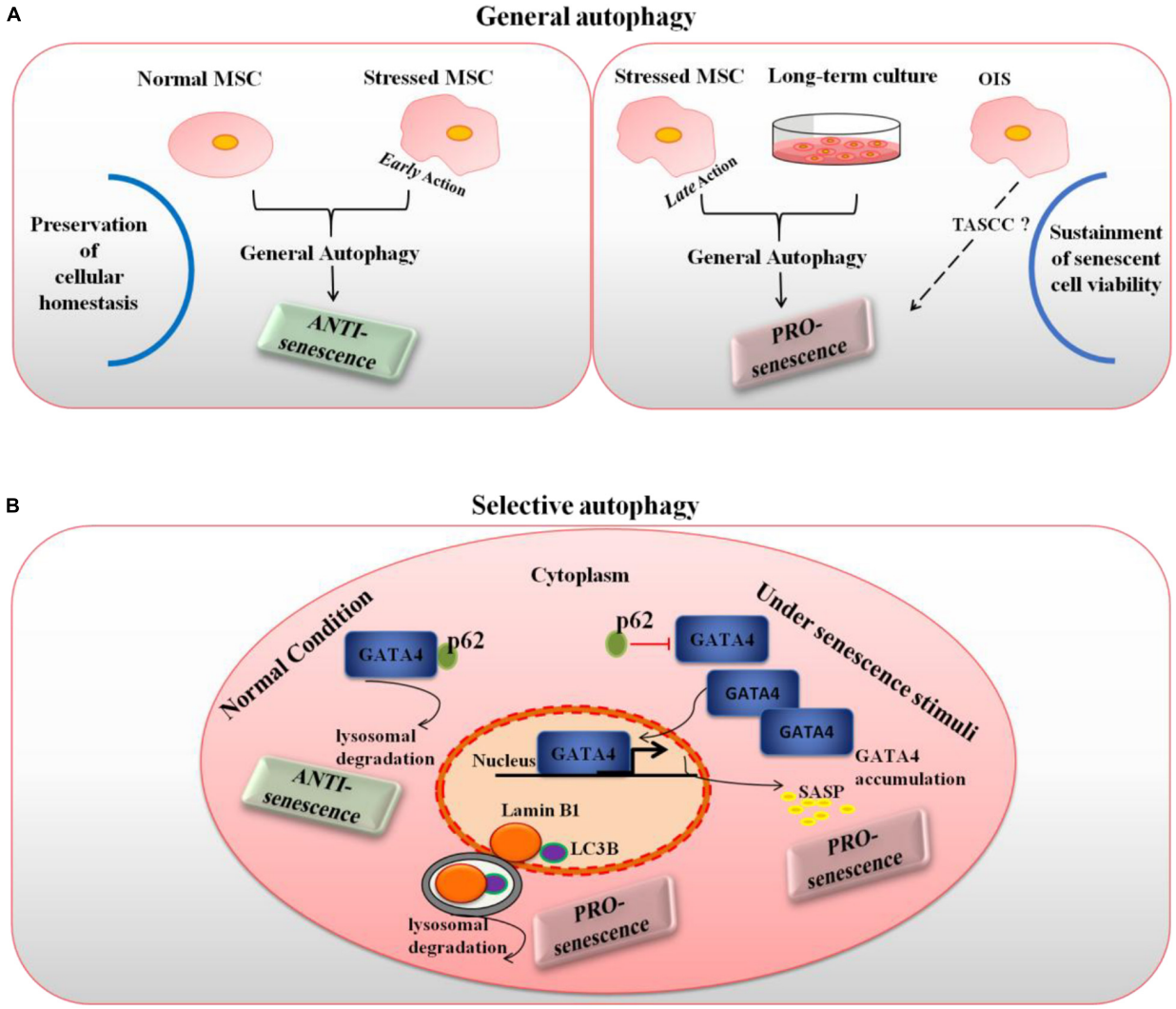

FIGURE 1 | Type of autophagy, time of action and context as the frame to explain the dual role of autophagy in MSC senescence. **(A)** General autophagy, under normal conditions and when exerting early action in stressed cells, is mainly anti-senescence as it maintains cellular homeostasis; however, in late stressed and long-term cultured MSCs (pro-senescent cells) autophagy becomes pro-senescence as it can manage several senescence-associated stresses maintaining senescent cell viability; it is also possible that, under OIS, a specialized form of general autophagy known as TASCC might occur in MSCs. **(B)** Selective autophagy is pro or anti-senescence depending on the specific substrates and context involved: in normal conditions, p62-dependent autophagy specifically degrades GATA4, a main regulator of the SASP, thus actively suppressing cellular senescence, yet when the cell encounters senescence-inducing stimuli, a steady decrease of the interaction between GATA4 and p62 occurs and accumulated GATA4 transcriptionally activates the SASP; on the other hand, LC3B-lamin B1-dependent selective autophagy of nuclear lamina acts as a pro-senescence mechanism.

implications, so clarifying the autophagy/senescence relationship in MSCs might have an impact for the development of more effective and safer therapeutic strategies.

As an example, the regulation of autophagy in MSCs exemplifies a conceivable strategy which, influencing MSC characteristics, may hit their regenerative potential, both in terms of differentiation properties and engraftment ability (Ceccariglia et al., 2020). Activation of autophagy promoted osteogenesis in bone marrow-derived MSCs isolated from osteoporotic vertebrae (Wan et al., 2017) and prevented bone loss in elderly mice, suggesting that autophagy has a crucial role in the aging of MSCs; in this setting, autophagy upregulation could partly revert this senescence process exemplifying a likely therapeutic strategy for clinically treating age-related bone loss (Ma et al., 2018). Further, evidence indicated that modulation of autophagy in MSCs prior to their transplantation enhanced survival and viability of engrafted MSCs and promoted their pro-angiogenic and immunomodulatory characteristics (Jakovljevic et al., 2018). Some organic molecules and metabolites showed a role in autophagy/senescence modulation: cholesterol retarded senescence in bone marrow-derived MSCs by modulating autophagy (Zhang et al., 2016) while kynurenine inhibited autophagy and promoted senescence in aged bone marrow-derived MSCs through the aryl hydrocarbon receptor pathway

(Kondrikov et al., 2020) which might represent a novel target to prevent or reduce age-associated bone loss and osteoporosis. Lately, to promote the efficiency of MSCs for clinical therapies, not only the intrinsic aging of these cells *in vivo* but also their aging *in vitro* upon culture expansion poses a tangible burden and should be counteracted. Among possible druggable pathways, autophagy manipulation during MSC expansion has been proposed through the employment of FDA-approved drugs like rapamycin and its derivatives (Rossi et al., 2019), which might help assessing the possibility of pharmacological extension of maximal cell lifespan while simultaneously enhancing MSC regulatory properties; further, novel small molecules known to selectively sense and react to acidic pH with high sensitivity were proved capable of promoting lysosomal acidification and inhibiting senescence in cultured MSCs through autophagy induction (Wang et al., 2018).

These and future studies on relevant targets and small molecules leading to the control and maintenance of optimal levels of autophagy might open up the way to new strategies for improving MSC transplantation.

AUTHOR CONTRIBUTIONS

RR and CG conceived and wrote the review. EV cooperated in bibliographic searches and table and figure editing.

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Down-Regulated Exosomal MicroRNA-221 – 3p Derived From Senescent Mesenchymal Stem Cells Impairs Heart Repair

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The composition and biological activity of donor cells is largely determined by the exosomes they secrete. In this study, we isolated exosomes from young (Young-Exo) and aged (Age-Exo) mesenchymal stem cells (MSCs) and compared their regeneration activity. Young Exo MSCs were more efficient than Aged-Exo at promoting the formation of endothelial tube, reducing fibrosis, and inhibiting apoptosis of cardiomyocytes *in vitro*; and improving cardiac structure and function *in vivo* in the hearts of rats following myocardial infarction (MI). MicroRNA sequencing and polymerase chain reaction (PCR) analysis revealed that miR-221-3p was significantly down-regulated in Aged-Exo. The aged MSCs were rejuvenated and their reparative cardiac ability restored when miR-221-3p was overexpressed in Aged-Exo. The protective effect was lost when miR-221-3p expression was knocked down in Young-Exo. These effects of miR-221-3p were achieved through enhancing Akt kinase activity by inhibiting phosphatase and tensin homolog (PTEN). In conclusion, exosomal miR-221-3p secreted from Aged MSCs attenuated the function of angiogenesis and promoted survival of cardiomyocytes. Up-regulation of miR-221-3p in aged MSCs improved their ability of angiogenesis, migration and proliferation, and suppressed apoptosis via the PTEN/Akt pathway.

Keywords: microRNA-221-3p, exosomes, mesenchymal stem cells, senescence, acute myocardial infarction

INTRODUCTION

Mesenchymal stem cell (MSCs) transplantation has enabled great progress in the treatment of ischemic heart diseases (Kanelidis et al., 2017). Nonetheless their cardioprotective effect declines with cell aging and this limits the application of autologous cell therapy in elderly patients with ischemic heart disease (Fisher et al., 2015; Nguyen et al., 2016). In addition, *in vitro* amplification of MSCs is necessary prior to their clinical application and inevitably leads to replicative aging process (Mathiasen et al., 2015; Guijarro et al., 2016). Accumulating evidence shows that aging affects the functions of MSCs, including differentiation, proliferation and migration, as well as angiogenic potential, and in turn reduces their clinical efficacy (Malaise et al., 2019; Zhang et al., 2019;

Wang et al., 2020). There is an urgent need to explore strategies that will enable functional recovery of elderly MSCs.

Recent studies have shown that MSCs protect the heart mainly through secretion of paracrine factors such as exosomes (Exos) (Boulanger et al., 2017; Heallen and Martin, 2018; Park et al., 2019). Exosomes originate intracellularly from a variety of cell types and transfer bioactive molecules such as miRNAs and proteins between cells (Davis, 2016). When donor cells are stimulated by the environment, the content of the exosomes will change and so also their biological effect (Boriachek et al., 2018).

miR-221-3p is a well-known miRNA that promotes cell survival and proliferation during tumorigenesis (Zhang et al., 2010; Yuan et al., 2013; Fornari et al., 2017). Circulating miR-221 has also been shown to be increased in patients with acute myocardial infarction (MI) and hypertrophic cardiomyopathy (Huang et al., 2020). Up-regulation of miR-221-3p inhibits autophagy in cardiomyocytes (Chen et al., 2016) while down-regulation enables profibrotic signaling in patients with dilated cardiomyopathy (Verjans et al., 2018). These data suggest that miR-221-3p plays a vital role in cardiovascular diseases. We performed pretest of miRNA sequencing and established that exosomal miR-221-3p was much higher in young MSCs and aged MSCs. Therefore, in this study we aimed to compare the cardiac repair effects of exosomal miR-221-3p secreted from young and aged MSCs, and to explore the possible underlying mechanism.

MATERIALS AND METHODS

Cells Culture

Human bone marrow was harvested from the posterior superior iliac spine of aged donors [70–80 years old, male (n = 3) and female (n = 3)] or young donors [20–25 years old, male (n = 3) and female (n = 3)]. All MSCs used for experiments in this study were between passages 4 and 6. Human bone marrow derived-MSCs were cultured in α-minimal essential medium (α-MEM) with 10% fetal bovine serum (FBS). An H9c2 cardiomyoblast cell line (ATCC) and human umbilical vein endothelial cells (HUVECs) were cultured in DMEM containing 10% FBS. All media and reagents for cell culture were purchased from Gibco (Carlsbad, United States). For normal culture, cells were incubated at 37°C, 21% O_2, and 5% CO_2. For hypoxia and serum deprivation (H/SD) condition, cells were cultured at 1% O_2, 5% CO_2, 94% N_2 and serum deprivation condition.

Exosome Extraction and Characterization

The supernatants of cells were collected and Exos extracted using Exosome Isolation Reagent (Ribobio, Guangzhou, China). Transmission electron microscopy (TEM) was used to observe the morphology of Exos. Briefly, Exos were fixed with 1% glutaraldehyde, then coated on a copper mesh and stained with 1% phosphotungstic acid. Samples were observed with a JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan). Nanoparticle tracking analysis (NTA) was used to evaluate the size and distribution of Exos. We recorded and tracked the Brownian motion of Exos in PBS (Invitrogen, Carlsbad, CA, United States). The Stoke-Einstein equation was used to obtain the size distribution data. A ZetaView PMX 110 system (Particle Metrix, Germany) was used for NTA.

Western Blotting

Western blotting was performed using a gel documentation system (iBrightCL1000, Invitrogen and Image Lab Software version 3.0), and a standard protocol as previously described (Zhang et al., 2019). The primary antibodies were anti-cyclin dependent kinase inhibitor 2A (P16) (80772, Cell Signaling Technology, United States), anti-cyclin dependent kinase inhibitor 1A (P21) (2947, Cell Signaling Technology), P53 (21083, Signalway Antibody, United States), PTEN (ab31392, abcam, United Kingdom), anti-phosphorylated-AKT (p-AKT) (4060, Cell Signaling Technology) and anti-AKT (4691, Cell Signaling Technology); Cleaved caspase-3 (29034, Signalway Antibody), Bcl-2 (ab196495, abcam), vascular endothelial growth factor (VEGF, ab52917, abcam), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5174, Cell Signaling Technology); TSG101 (14497, Proteintech, United States), CD63 (25682, Proteintech), CD81 (66866, Proteintech) and horseradish peroxidase-conjugated were secondary antibodies (Biosharp, China).

SA-β-Gal Staining Assay

Cell senescence was determined using a Senescence-β-Galactosidase Cell Staining Kit (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, we rinsed the plate with PBS and fixed cells in staining fixatives for 15 min at room temperature. Fixed cells were then washed twice with PBS and stained with fresh SA-β-gal staining solution overnight at 37°C. Cells that stained positive for SA-β-Gal were quantified and analyzed.

Cell Cycle Assay

Flow cytometry (Becton Dickinson, United States) was used for cell cycle assay. Briefly, 1×10^6 cells were collected and washed twice with PBS. After staining with 1 ml DNA staining solution and 10 μL permeabilization solution of Cell Cycle Staining Kit (KeyGen Biotech, Nanjing, China) for 30 min, cells were evaluated and further analyzed using FlowJo software version 10.0 (Tree Star, United States).

Cell Viability Analysis

CCK-8 Kit (Dojindo Laboratories, Kumamoto, Japan) was used for cell viability assays. Briefly, cells were seeded at a density of 5×10^3 cells/well in a 96 well plate and cultured for 24 h after which 10 μL of CCK-8 solution was added to each well. After incubation for a further 2 h, the absorbance of each well (450 nm) was determined by a microplate reader (Synergy, United States).

Identification of Surface Antigen Using Flow Cytometry

Both Aged and Young MSCs were characterized by their cell surface antigen expression and trilineage differentiation capability using flow cytometry. Antibodies used for MSC

characterization were anti-CD105-APC (17-1057-41; Thermo Fisher Scientific), anti-CD44-APC (17-0441-81; Thermo Fisher Scientific), anti-CD73-APC (17-0739-41; Thermo Fisher Scientific), anti-CD90-APC (17-0909-41; Thermo Fisher Scientific), anti-CD45-APC (17-9459-41; Thermo Fisher Scientific), and anti-CD31-APC (17-0319-41; Thermo Fisher Scientific).

Exosome Uptake by H9c2 Cells and HUVECs

To evaluate MSC-Exo uptake by H9c2 cells and HUVECs, Dil (red fluorescent dye, C1036, Beyotime, China) was used to label Exos. Then Exos and recipient cells were co-cultured for 6 and 24 h at 37°C, after which they were washed with PBS and fixed with 4% paraformaldehyde for 20 min. Nuclei were then stained with 6-diamidino-2-phenylindole (DAPI) (0.5 g/ml; Beyotime) for 10 min and observed under a confocal microscope.

Cell Apoptosis and Proliferation and Assay

Flow cytometry was used to assess cell apoptosis. H9c2 cells or HUVECs were cultured overnight at a seeding density of 1×10^5/6-well tissue culture plates and treated with Exos or PBS before H9c2 were subjected to hypoxia. To quantify the apoptotic cells, cells were washed with PBS and stained using an annexin V-FITC and propidium iodide (PI) apoptosis kit (KeyGen Biotech, China). The apoptotic cells were analyzed by Flowjo Software version 10.0 (Tree Star, United States).

Cell proliferation was assessed by Edu assay using a kFluor488-EDU Kit (KeyGen Biotech). Briefly, 1×10^5 cells were seeded in well plates and 1:1000 dilution of Edu-labeling reagent added. Cells were then fixed for 48 h and incubated with 0.1 ml 3% bovine serum albumin (BSA) in PBS. Then 0.5% Triton X-100 was added for 20 min and then Click-iT Edu reagent. The nuclei were stained with DAPI. Fluorescence microscopy was used to observe the Edu positive cells and Image J software (National Institutes of Health, NIH) was used for further analysis.

Migration Assay

HUVECs were cultured in a 6-well plate, and a fusion layer scratched using a P200 pipette tip. Cells were then washed and incubated after addition of 100 μg/well Exos. Images were taken before and 24 h after incubation and Image J software (NIH) used to determine any reduction in scratched area size.

Tube Formation Assay

Tube formation assay was performed to assess the angiogenic ability of HUVECs. Briefly, HUVECs were treated with PBS or exosomes extracted from young (Young-Exo) and aged MSCs (Aged-Exo) for 24 h. Then cells were washed with PBS and seeded (30,000 cells/well) in 96 well plates coated with growth-factor reduced Matrigel (Corning, United States). After 6 h, capillary-like tube formation was observed and photographed. Tube length and number of branches were analyzed with Image J software (NIH).

MI Model, Histological Analysis, and Immunofluorescence Staining

Sprague-Dawley (SD) rats (Male, 200–220 g weight) were anesthetized and ventilated via an orotracheal tube and rodent ventilator. Left anterior descending artery (LAD) was ligated and Exos (50 μL, 1 μg/μL) or PBS injected around the infarcted region. Echocardiography (Vevo 3100) was performed 2 and 4 weeks later to determine left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) (Zhang et al., 2019).

Rats were sacrificed 4 weeks post MI. Hematoxylin-Eosin (HE) stain was used to evaluate inflammatory cell infiltration. The fibrotic and collagen area post MI was evaluated by Masson's Trichrome stain and Sirius Red stain.

CD31 immunofluorescence staining was performed as previously described (Zhang et al., 2019). Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) staining (Roche, United States) was used to assess cell apoptosis. The primary antibodies were anti-CD31 (ab7388; Abcam, United Kingdom), and anti–α-sarcomeric actin (α-actin, a7811, Sigma-Aldrich). DAPI was used for nuclear counter staining. Images were taken using a fluorescent microscope (Zeiss, German) and Image J software (NIH) used for further analysis.

Distribution of Dil-Labeled Exosomes in Infarcted Hearts of Rats

A 50 μL (1 μg/μL) solution of Dil-labeled Exos was injected into the injured rat hearts. Rats were sacrificed 6 h after injection and the heart removed. Heart tissue was dehydrated, frozen, and sliced into 6 μm slices. Antibodies used for immunofluorescence staining were α-actin (Sigma-Aldrich), and anti-CD31 (Abcam, United Kingdom). The nuclei were stained with DAPI and cells observed under fluorescence microscopy (Zeiss, Germany).

Exosomal MicroRNA Sequencing and Real-Time RT-PCR Validation

Exosome microRNAs were sequenced in Young-Exo and Aged-Exo. Differentially expressed microRNAs were identified by $\log_2 |(\text{Fold Change})| > 1$ and $P < 0.05$ with the threshold set for up and down regulated microRNAs.

Total RNA was isolated by TRIzol (Life technologies, United States). RNA concentration was quantitated by the Nano Drop ND-2000 Spectrophotometer (Nano Drop Technologies, Wilmington, DE, United States) and cDNAs were synthesized using PrimeScript^{TM} RT reagent kit (TaKaRa, Japan). Real-time polymerase chain reaction (PCR) was performed by SYBR Premix Ex Taq qRT–PCR assays (TaKaRa, Japan) with microRNAs, Cel-mir-39, and U6 specific primers (Genscript, Nanjing, China) under 7900HT Real-Time PCR Detection System (Thermo Fisher Scientific, United States). Cel-mir-39 (exosomal) and U6 (cellular) served as external or internal standard to normalize the miRNA expression level using $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed in **Supplementary Table S1**.

Lentiviral Package and Cell Transfection

Lentiviral plasmids encoding miR-221 and its negative control were obtained from GENECHEM (Shanghai, China). Lentivirus (hU6 – MCS – Ubiquitin – EGFP – IRES – puromycin) transfected MSCs had a multiplicity of infection (MOI) of 60 for miR-221 and 80 for its negative control. Cells were selected with purinomycin (0.75 μg/ml) for 3 days. miR-221-3p mimics, inhibitor and their negative control were obtained from Ribobio (Guangzhou, China). Lipofectamine2000 (Invitrogen, United States) was used for cell transfection.

Statistical Analysis

Continuous variables and categorical variables are described as mean ± SD and percentages, respectively. Independent-Sample *T*-test was used to compare continuous variables between the two groups. One way Analysis of variance (ANOVA) followed by Tukey's correction was used for comparison of three or more groups. All statistical tests were performed using GraphPad Prism software version 8.0, and $p < 0.05$ was considered statistically significant.

RESULTS

Characterization of MSCs and Exosomes

Both young and aged MSCs were positive for MSC surface markers such as CD105, CD44, CD73, and CD90; and negative for CD45 and CD31 (**Figure 1A**). SA-β-Gal staining showed that senescence greatly increased in aged MSCs (**Figure 1B** and **Supplementary Figure S1**). Expression of senescent biomarker P21 and P53 significantly increased in aged MSCs compared with young MSCs but there was no significant difference in P16 protein expression (**Figure 1C**). The percentage of cells in G0 and G1 phase also increased significantly more in aged MSCs than young MSCs (**Figure 1D**). The aged MSCs grew more slowly than young MSCs at 3, 5, and 7 days after culturing with CCK-8 (**Figure 1E**). The characteristics of Exos derived from young and aged MSCs were detected by TEM, NTA and Western blotting. The diameter of most particles was around 110 nm and expressing surface markers included TSG101, CD81, and CD63 (**Figures 1F–H**). These results suggested that exosomes had been collected and purified successfully.

Pro-angiogenesis and Anti-apoptotic Effects of Young-Exo *in vitro*

To further determine the roles of Exos derived from young and aged MSCs *in vitro*, Dil-labeled Exos were used to assess the internalization ability of Exos. After staining, washing and centrifuging, MSC-derived Exo were obtained and cultured with H9c2 cells and HUVECs. Red fluorescence was observed in the cytoplasm of cells at 6 and 24 h after treating with Dil-labeled Exos, while no red fluorescence was observed in the control group. This indicated that Exos could be taken up by cells and located in the cytoplasm (**Figure 2A**).

To evaluate the effects of Exos derived from young and aged MSCs (Young-Exo and Aged-Exo) *in vitro*, H9c2 cells and HUVECs were incubated with PBS (Control group), Aged-Exo and Young-Exo. Cell viability of H9c2 cells under H/SD significantly increased in the Young-Exo group compared with Aged-Exo and control groups (**Figure 2B**). In addition, apoptosis reduced significantly in the Young-Exo group compared with Aged-Exo and control groups (**Supplementary Figure S2**). The angiogenic tube formation ability in HUVECs was significantly enhanced in the Young-Exo group compared with Aged-Exo and control groups (**Figure 2C**) as was the migration ability of HUVECs. Nonetheless there was no significant difference of migration ability between the Aged-Exo group and control group (**Figure 2D**). Edu assay showed a significantly higher percentage of Edu-positive (proliferating) cells in the Young-Exo group than control and Aged-Exo groups (**Figure 2E**). We also found a significant reduction in apoptosis of HUVECs under H/SD condition in the Young-Exo group compared with Aged-Exo and control groups (**Figure 2F**). These results indicated that Young-Exo cells exhibited pro-angiogenesis, proliferation and anti-apoptotic effects *in vitro*, not evident for Aged-Exo cells.

Young-Exo Cells Were Incapable of Effectively Maintaining Cardiac Function in Rats Post MI

To assess the benefits of Exos *in vivo*, PBS (AMI group), Young-Exo, and Aged-Exo cells were injected into the infarction border zone 30 min after establishing a model of MI (**Figure 3A**). The distributions of Dil-labeled-Exos in the infarcted heart were observed 6 h post MI. Dil-labeled-Exos were detected in cardiomyocytes and endothelial cells (**Figure 3B**). LVEF and LVFS were slightly enhanced in Young-Exo group compared with the AMI group but not the Aged-Exo group. Four weeks post MI, LVEF, and LVFS significantly increased in the Young-Exo group compared with the AMI and Aged-Exo groups (**Figures 3C,D**). Inflammatory cell infiltration also significantly decreased in the Young-Exo group compared with the Aged-Exo and AMI groups (**Figure 4A**). Masson staining showed that the fibrotic area was significantly reduced in the Young-Exo group (**Figure 4B**). The collagen area was also significantly and consistently more reduced in the Young-Exo group than AMI group (**Figure 4C**). In addition, the capillary density dramatically increased compared with the Aged-Exo and MI groups. TUNEL also showed less myocardial apoptosis in the Young-Exo group compared with Aged-Exo group (**Figure 4D**). These results suggest that Young-Exo cells significantly enhanced the functional recovery of infarcted hearts, while Aged-Exo showed little beneficial effects.

Exosomal miR-221-3p Level Decreased More in Aged-Exo Than Young-Exo

Increasing evidence suggests that miRNAs carried by Exos play a very important role in regulating cellular functions of recipient cells. To investigate the mechanism of Young-Exo-induced protective effects, we performed miRNA sequencing on exosomes derived from young and aged MSCs (**Figure 5A**). In total, 35 miRNAs were identified to be up-regulated in Young-Exo compared with Aged-Exo, while 52 miRNAs were identified

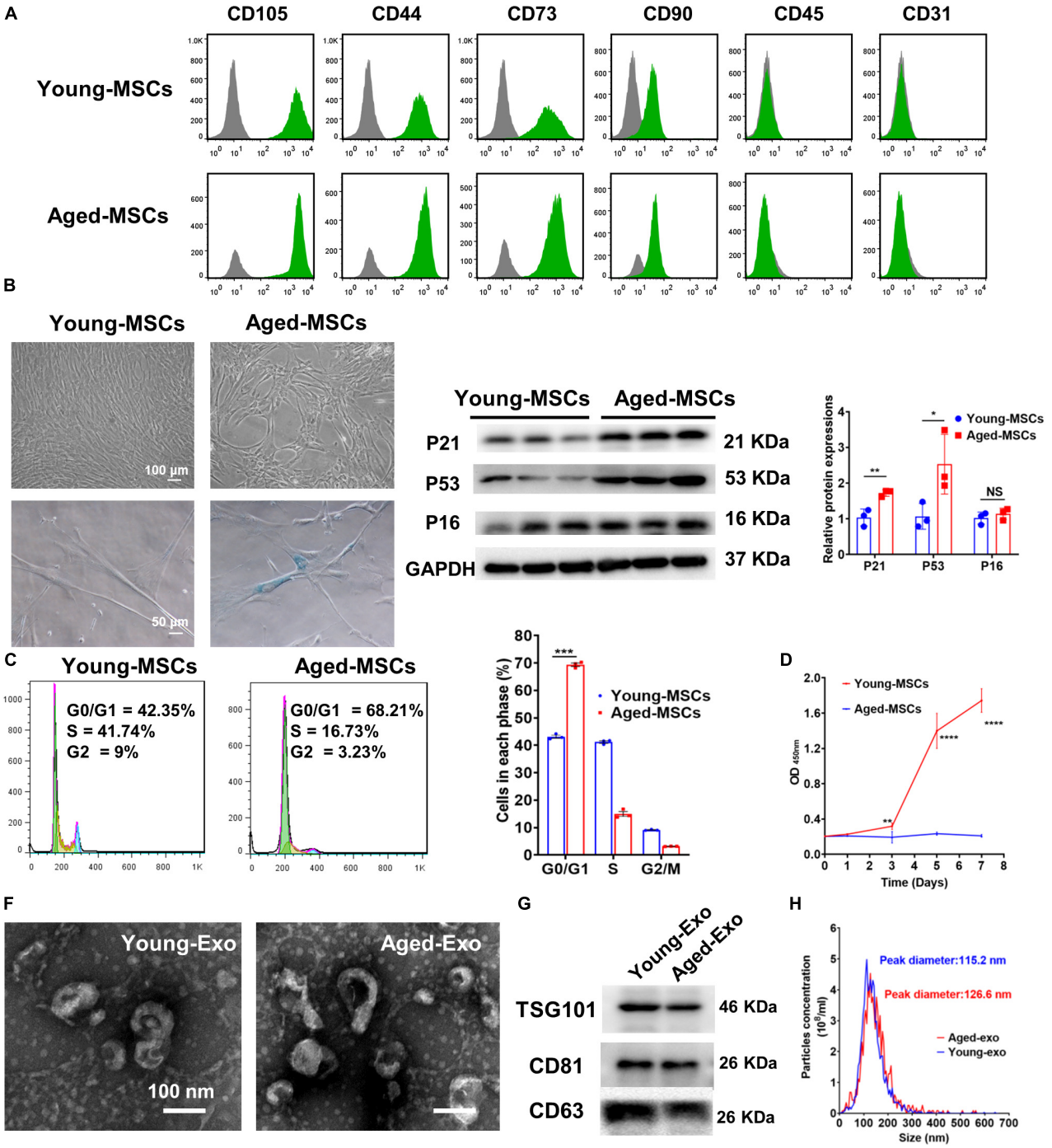

FIGURE 1 | Characterization of young and aged MSCs and exosomes. **(A)** Surface marker profiling of young-MSCs and aged-MSCs. **(B)** SA-β-Gal staining showed that senescence increased significantly in aged MSCs. **(C)** Representative immunoblot images and quantitative analysis of p21, p53, and p16 protein level in young and aged-MSCs. (n = 3). **(D)** Quantitation of cell cycle phases by propidium iodide staining. (n = 3). **(E)** The CCK-8 assay showed that aged MSCs grew more slowly than young MSCs. (n = 6). **(F)** Young and aged exosomes were observed using TEM. **(G)** The exosome surface markers were analyzed by Western blot. **(H)** Nanoparticle tracking analysis was used to analyze the particle size and concentration of Young-Exo and Aged-Exo. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; NS, not significant.

to be down-regulated (**Figure 5B**). Real time PCR analysis confirmed four up-regulated miRNAs and five down-regulated miRNAs. In particular, miR-221-3p decreased significantly in Aged-Exo (**Figure 5C**). These data suggest that exosomal miR-221-3p may play a very important role during transition of Young MSCs to Aged MSCs.

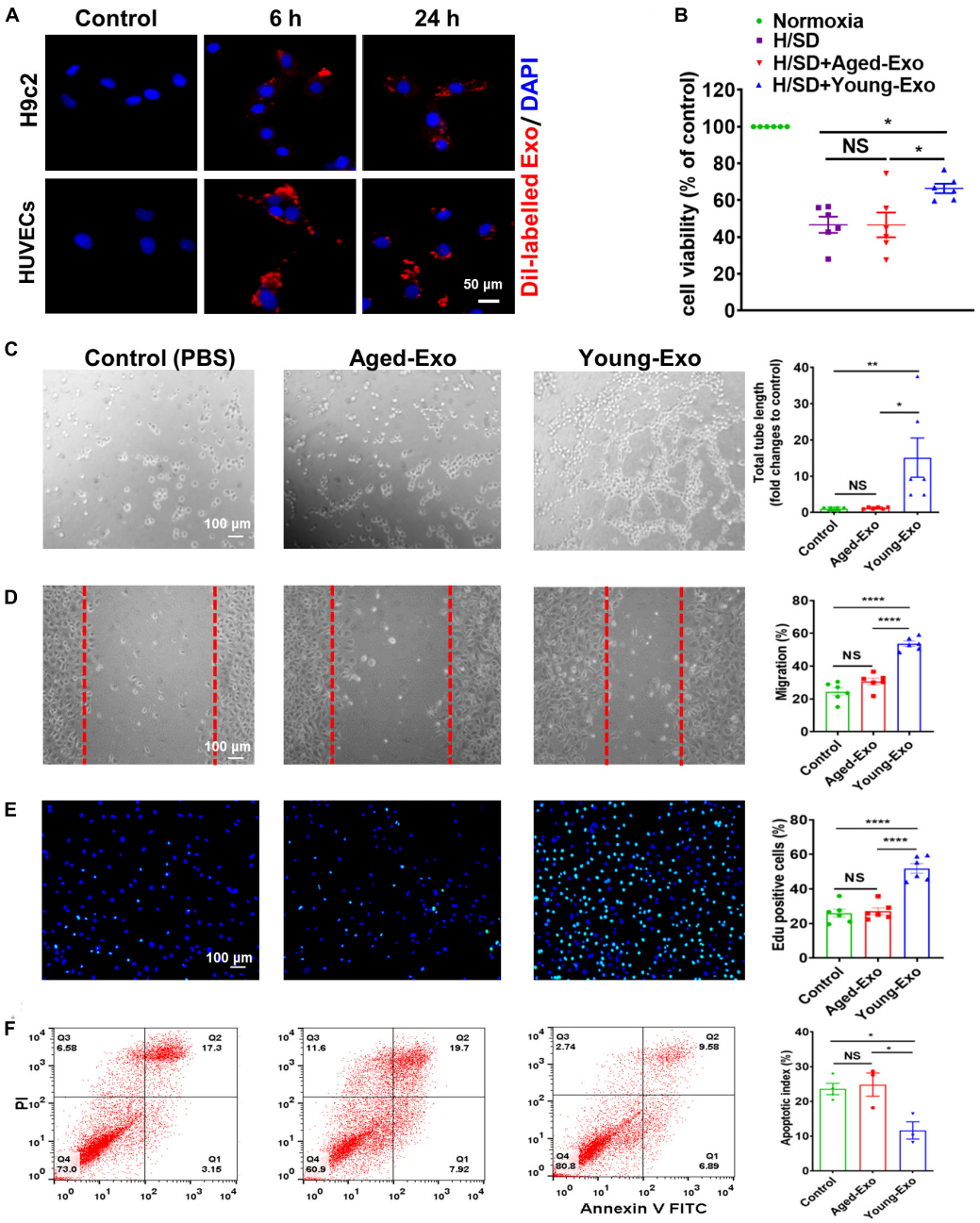

FIGURE 2 | Internalization of exosomes in H9c2 and HUVECs cells, and effects of Young-Exo and Aged-Exo on recipient cells. **(A)** Dil-labeled exosomes (red) internalized by H9c2 and HUVECs was observed by confocal microscopy. The labeled exosomes were observed in the perinuclear region of recipient cells. **(B)** Cell viability of H9c2 cells was detected among four groups. (n = 6) **(C)** The tube formation ability of HUVECs was measured in the Young-Exo group, Aged-Exo group and control group (PBS) (n = 6). **(D)** The migration ability of HUVECs was tested among three groups (n = 6). **(E)** Proliferation rate was determined by Edu staining (DAPI, blue; Edu positive cells, green). (n = 6). **(F)** Apoptotic rate was determined by Annexin V-PI staining via flow cytometry among three groups. (n = 3). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$; NS, not significant.

Exosomal miR-221-3p Played an Important Role in Angiogenesis and Cardiac Regeneration in Young-Exo

In order to confirm that exosomal miR-221-3p plays a role in the beneficial effect of angiogenesis and cardiac regeneration in Young-Exo, miR-221-3p mimics and miR-221-3p inhibitor were successfully transfected into H9c2 cells and HUVECs, respectively (**Supplementary Figure S3**). Transfection of miR-221-3p mimics could enhance the anti-apoptotic effects of Aged-Exo cultured with H9c2 cells (**Figure 6A**). Knock down of miR-221-3p could attenuate the anti-apoptotic effects of

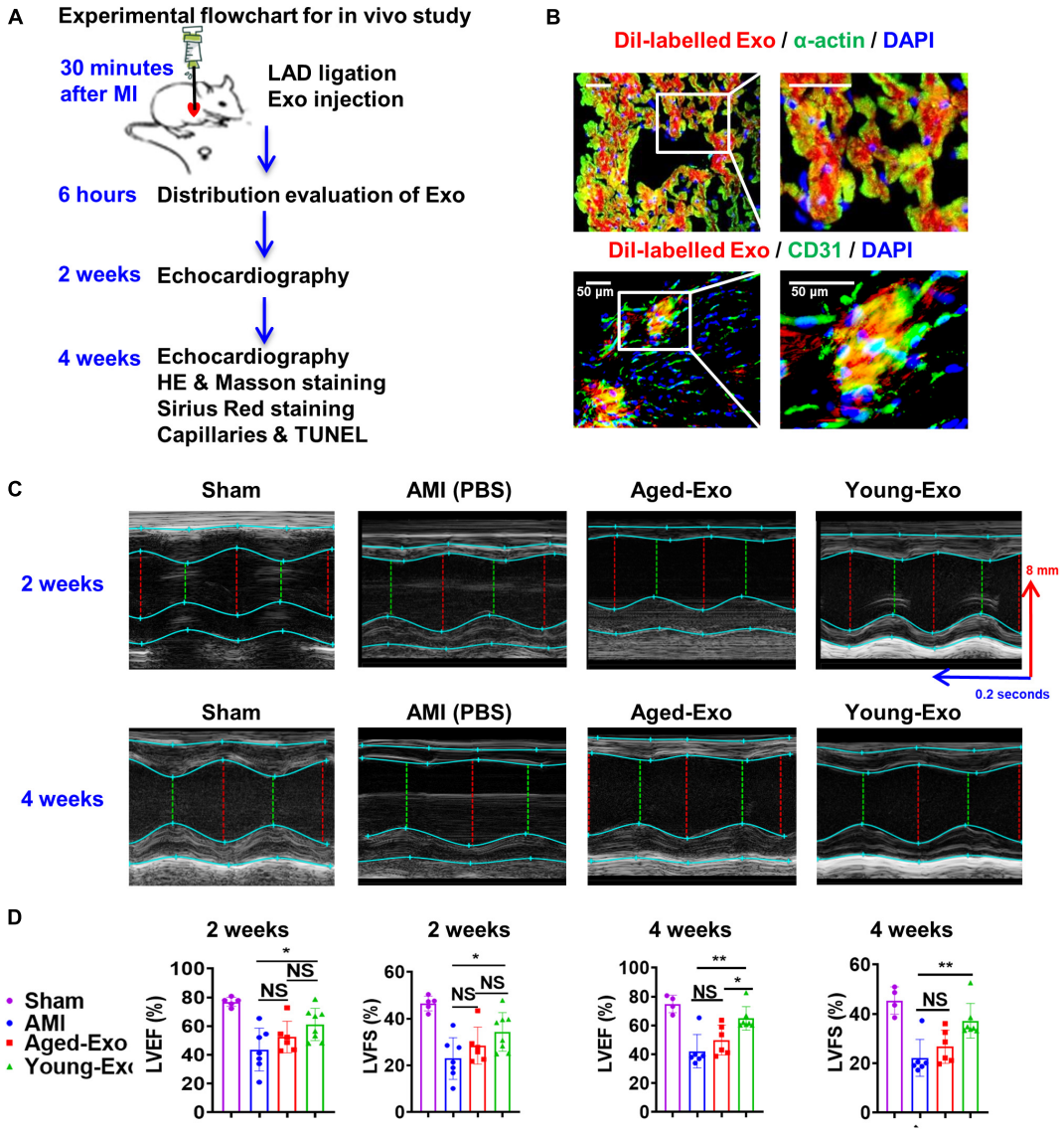

FIGURE 3 | Cardiac function in a rat model of MI was preserved effectively by Young-Exo. **(A)** Schematic of *in vivo* experiment. **(B)** Distribution of DiI-labeled exosomes in the infarcted rat hearts. **(C)** Echocardiography analysis was performed at 2 and 4 weeks after establishment of MI. **(D)** LVEF and LVFS were enhanced significantly in Young-Exo group compared with Aged-Exo and AMI groups (2 weeks post MI: n = 5–8 for each group; 4 weeks post MI: n = 4–7 for each group). $^{*}p < 0.05$; $^{**}p < 0.01$; NS, not significant.

Young-Exo incubated with H9c2 cells (**Figure 6B**). Additionally, miR-221-3p mimics could enhance angiogenesis, migration, and proliferation and also suppress apoptosis in Aged-Exo treated HUVECs (**Figures 6C,E,G,I**). In contrast, the anti-apoptotic and angiogenic effects of Young-Exo-treated HUVECs could be attenuated by transfection of miR-221-3p inhibitor (**Figures 6D,F,H,J**).

Aged MSCs were then infected with lentiviruses containing miR-221 and its negative control (**Figure 7A**). Green fluorescence was observed in both miR-221-MSCs (MSCs transfected with lentiviruses containing miR-221) and Ctrl-MSCs (MSCs transfected with lentiviruses containing miR-221 negative control) (**Figure 7B**). The cellular and exosomal expression of miR-221-3p significantly increased in the miR-221-MSC group compared with the Ctrl-MSC group (**Figure 7C**). There were fewer SA-β-Gal positive cells in the miR-221-MSC group than Ctrl-MSC group, indicating that senescence of MSCs could be alleviated by miR-221 overexpression (**Figure 7D**). We then extracted exosomes from miR-MSC and Ctrl-MSC groups. PBS, Ctrl-Exo (Exo derived from Ctrl-MSCs) and miR-221-Exo (Exo derived from miR-221-MSCs) were injected into the myocardium at the border area 30 min post MI. LVEF and LVFS significantly increased and fibrosis reduced 28 days post MI in the miR-221-Exo group compared with the Ctrl-Exo group (**Figures 7E,F**). In addition, TUNEL staining showed less myocardium apoptosis in the miR-221-Exo group compared with Ctrl-Exo and AMI

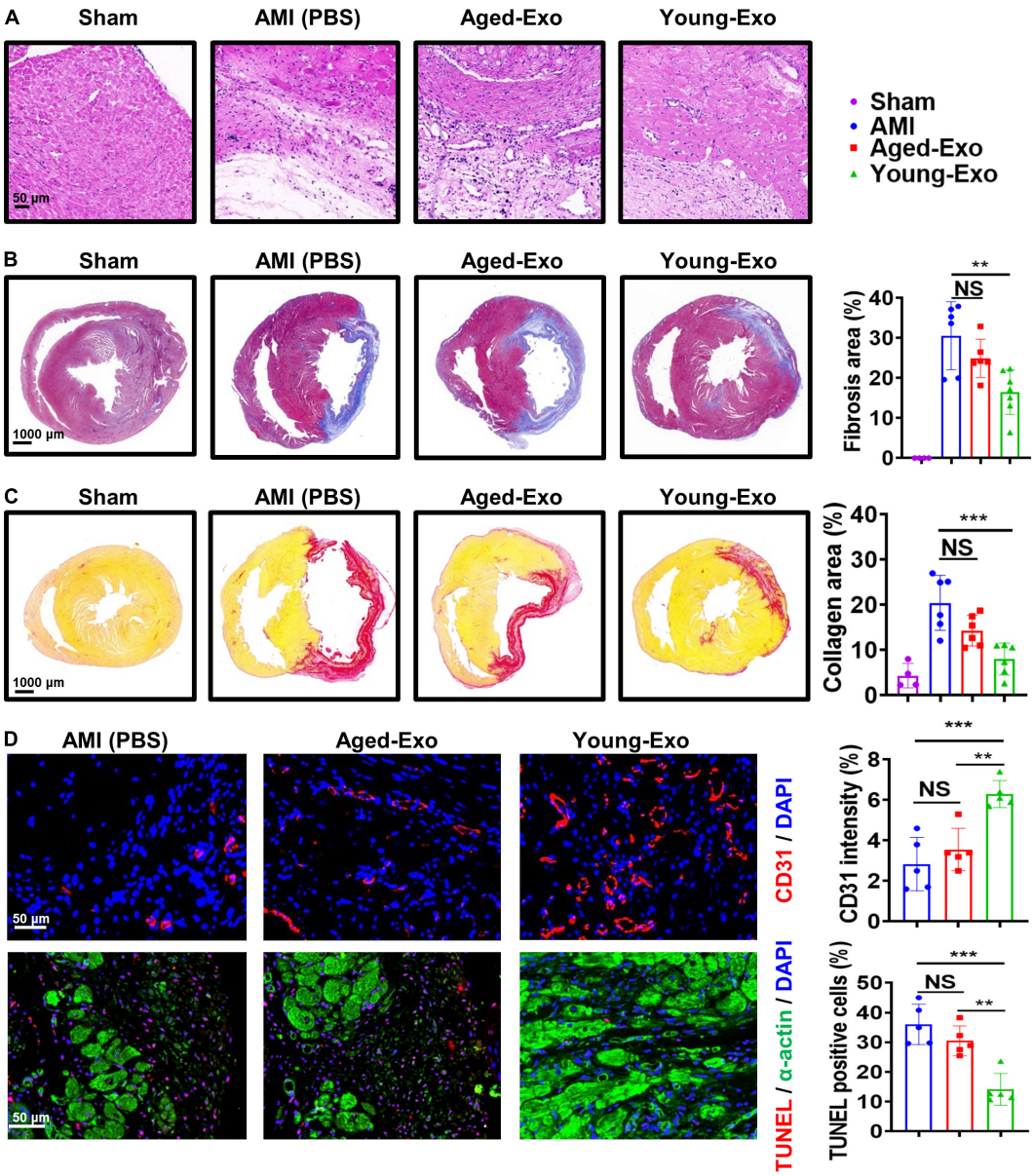

FIGURE 4 | Aged-Exo were unable to inhibiting apoptosis or promote angiogenesis *in vivo*. **(A)** HE staining images at the border zone 4 weeks after MI. **(B)** Masson staining images at 4 weeks after MI. Red, myocardium; blue, scarred fibrosis. (n = 4–7 for each group). **(C)** Sirius Red staining images 4 weeks post MI among in the four groups (n = 4–6 for each group). **(D)** Representative fluorescence images of blood vessels in the border zone of ischemic hearts stained with CD31 (red). TUNEL showed less myocardium apoptosis in the Young-Exo group compared with Aged-Exo and AMI groups. (n = 5 for each group). $^{**}p < 0.01$; $^{***}p < 0.001$; NS, not significant.

groups (**Figure 7G**). These results indicate that miR-221-3p plays an important role in Young-Exo-induced angiogenesis and cardiac repair.

miR-221-3p Decreased Pro-apoptotic and Increased Pro-angiogenic Protein Expression

miR-221-3p was overexpressed with miR-221 mimics in H9c2 cells and HUVECs. We first observed enhanced Akt phosphorylation (Ser473) with up-regulation of miR-221-3p (**Figures 8A–D**). Next, proapoptotic protein, Cl-caspase-3 was down-regulated and anti-apoptotic protein Bcl-2 was up-regulated in the miR-221 mimics group in both H9c2 cells and HUVECs. The expression of reported target gene, PTEN was also decreased in the miR-221-3p mimics group compared with its negative control in both two cell lines. Additionally, protein expression of VEGF was increased in HUVECs. Taken together, our data indicated that miR-221-3p inhibited cardiomyocyte apoptosis and promoted angiogenesis by PTEN/Akt signaling.

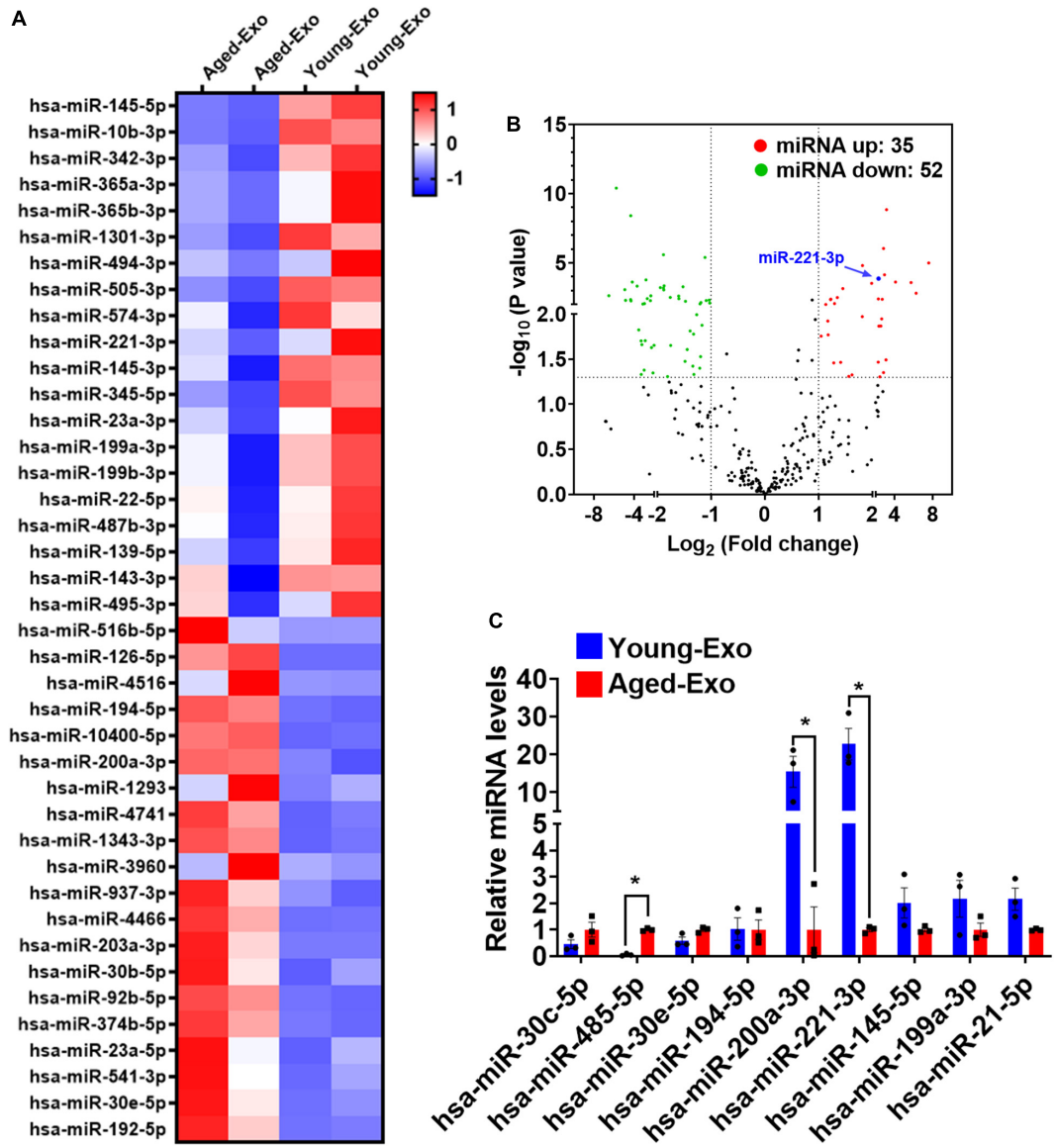

FIGURE 5 | Exosomal miRNA expression profile of Young-Exo and Aged-Exo. **(A)** Heat map based on top 20 up-regulated and down-regulated miRNA expression (red: up-regulation, blue: down-regulation). **(B)** Volcano plot showing $\log_2$ (Fold change) (Young-Exo vs. Aged-Exo) on the x-axis and $-\log_{10}$ (P value) on the y-axis. miR-221-3p (blue) was significantly decreased in Aged-Exo. **(C)** Real-time PCR of some up-regulated and down-regulated miRNAs. ($n = 3$). *$p < 0.05$.

DISCUSSION

This study revealed that Aged-Exo could not inhibit apoptosis, reduce fibrosis or promote angiogenesis and had a very weak effect on improving cardiac injury. The impairment of regenerative activities of Aged-Exo is at least partly due to down-regulation of miR-221-3p. Up-regulation of miR-221-3p in aged MSCs may be beneficial for improving their angiogenic, migration, proliferation abilities and in suppressing apoptosis through the PTEN/Akt pathway (**Figure 8E**).

Over the past decade, cell therapy has become a novel strategy for cardiac repair (Deuse et al., 2009; Zhang et al., 2011). Exosomes are important paracrine components, repairing tissue by encasing and sending RNAs and proteins (Phan et al., 2018; Shi et al., 2018). Previous study showed that exosomes from cardiac stem cells and mouse embryonic stem cells could afford the same cardioprotective effects as stem cells (Todorova et al., 2017; Liu et al., 2020). Nonetheless exosomes derive mainly from young donors and the contents of Aged-Exo cells may change and further reduce their reparative ability. In this study, Aged-Exo cells lost their proangiogenic drive of HUVECs tube formation and failed to inhibit cardiomyocyte apoptosis in injured rats' hearts. This evidence suggests a dysfunction of Aged-Exo in ischemic tissue repair. Our findings may explain the compromised therapeutic effects in clinical trials that use autologous stem cells (Mathiasen et al., 2015, 2019). Aged MSCs

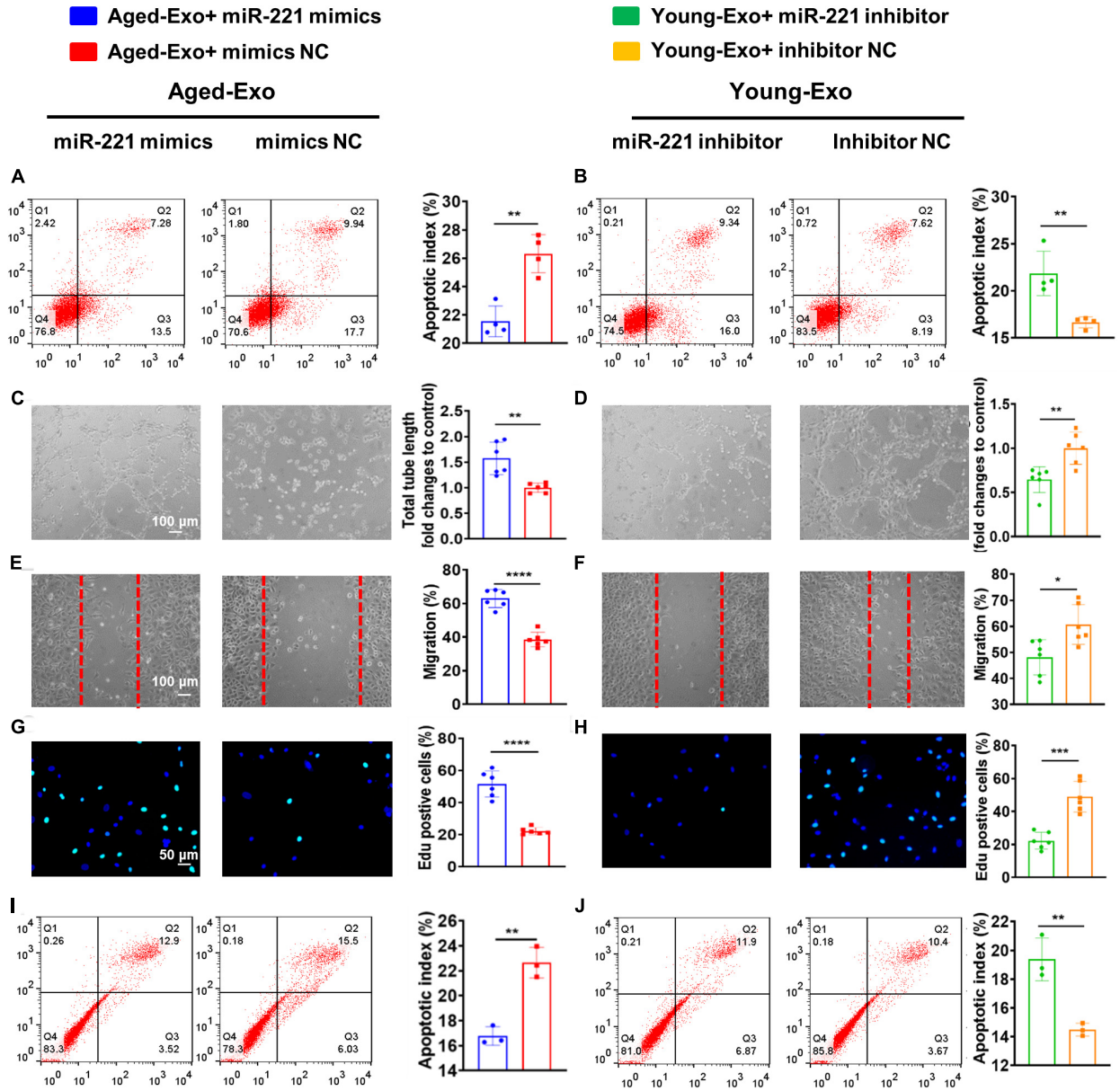

FIGURE 6 | Exosomal miR-221-3p plays a very important role in Young-Exo-induced angiogenesis and cardiac repair. **(A)** Apoptotic rate was determined by flow cytometry in Aged-Exo treated H9c2 cells between miR-221-3p mimics group and mimics negative control group. (n = 4). **(B)** Apoptotic rate was determined by flow cytometry in Young-Exo treated H9c2 cells between miR-221-3p inhibitor group and inhibitor negative control group. (n = 4). **(C)** The tube formation ability of Aged-Exo-treated HUVECs between two groups. (n = 6). **(D)** The tube formation ability of Young-Exo-treated HUVECs between two groups. (n = 6). **(E)** The migration ability of Aged-Exo-treated HUVECs. **(F)** The migration ability of Young-Exo-treated HUVECs. **(G)** Edu assay were performed in Aged-Exo-treated HUVECs. **(H)** Edu assay was performed in Young-Exo treated HUVECs. **(I,J)** Apoptotic rate among different groups in HUVECs. (n = 3). **(C–H)** n = 6. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

have altered exosomal content that renders stem cells unable to affect therapeutic repair. Thus rejuvenation of aged MSCs in the elderly (Boulanger et al., 2017) population is of great interest.

The transfer of miRNAs and proteins from specific donor cells to recipient cells is a key mechanism for exosome-mediated repair (Boulanger et al., 2017; Todorova et al., 2017). In this study, the sequence examination of exosomal miRNAs showed that miR-221-3p was much higher in Exos secreted from young MSCs than aged MSCs. When miR-221-3p was delivered to cardiomyocytes, it could reduce apoptosis and enhance angiogenesis and cardiac function in a rat model of MI. After down-regulation of miR-221-3p in Young-Exo, these benefits were attenuated while up-regulation of miR-221-3p in Aged-Exo restored their therapeutic efficacy. Coskunpinar et al. reported that circulating miR-221 has a high discriminative value and significant relation with troponin and left ventricular systolic function. It may serve

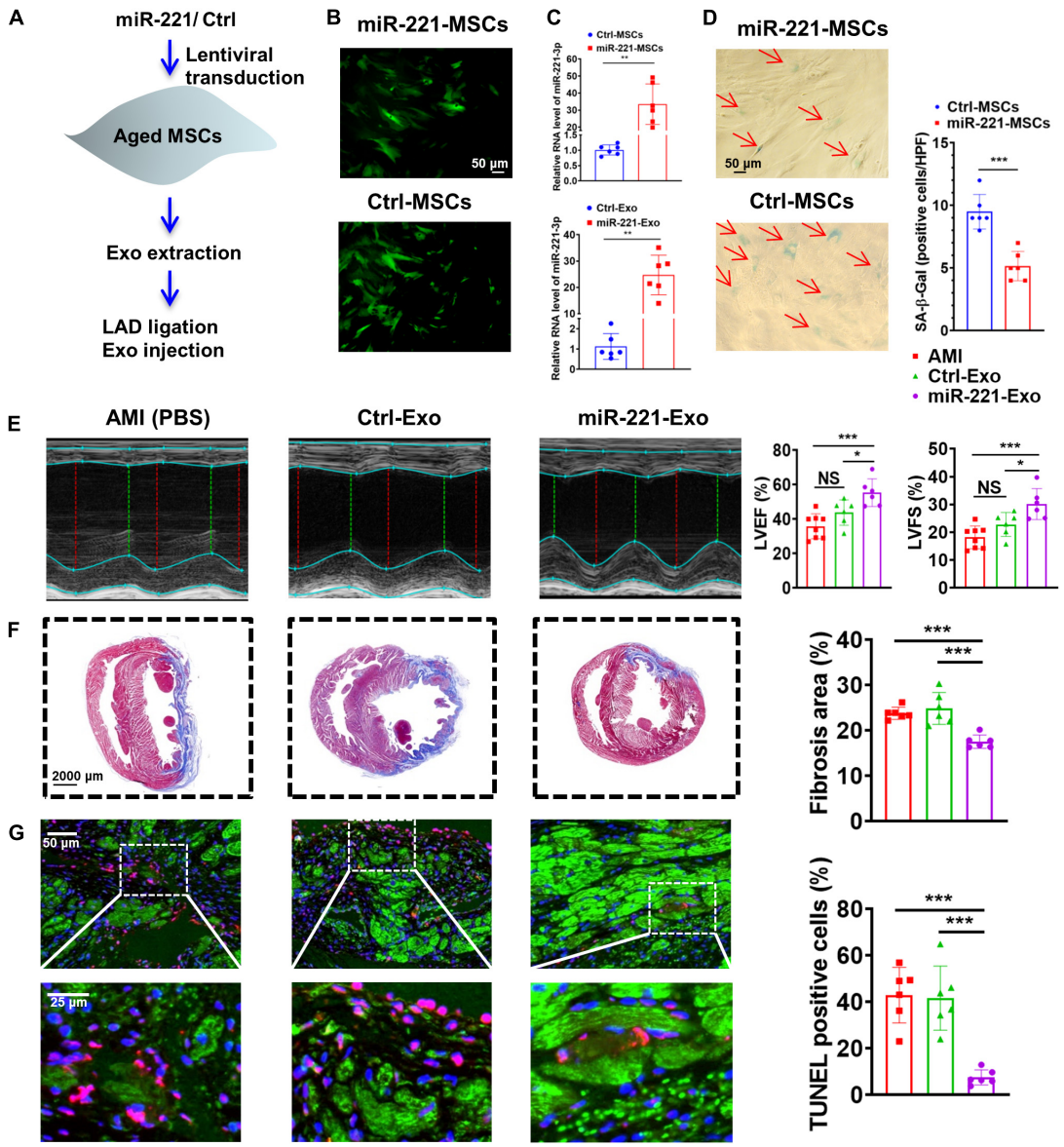

FIGURE 7 | The effect of miR-221-3p loaded exosomes in the rat after MI. **(A)** The flowchart of lentiviral transduction and *in vivo* experiments. **(B)** Successful lentiviral transduction was confirmed by positive fluorescence signal under microscope. **(C)** Cellular and Exosomal miR-221-3p expression was validated by real-time PCR. (n = 6). **(D)** There were fewer SA-β-Gal positive cells per high power field (HPF) under microscopy (×200) in the miR-221-MSCs group. (n = 6). **(E)** Echocardiography analysis was performed 4 weeks post MI, and representative images are shown. LVEF and LVFS were significantly increased in the miR-221-Exo group compared with other groups. (n = 6–8 for each group). **(F)** Masson staining 4 weeks after MI. Red, myocardium; blue, scarred fibrosis. (n = 6). **(G)** TUNEL showed less myocardium apoptosis in the miR-221-Exo group compared with Ctl-Exo and AMI groups. (n = 6). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS, not significant.

as a biomarker for early prediction of acute MI (Coskunpinar et al., 2016). Another study showed that circulating miR-221 is consistent with myocardial fibrosis and hypertrophy (Huang et al., 2020). MiR-221 also significantly reduced cardiomyocte H/R injury in association with inhibition of autophagy (Chen et al., 2016). Verjans et al. reported that the miRNA-221 level in patients with aortic stenosis correlated negatively with the extent of myocardial fibrosis and with left ventricular stiffness (Verjans et al., 2018). Wu reported that cancer cell-derived exosomal miR-221-3p promotes angiogenesis (Wu et al., 2019). These data support the important role of miR-221-3p in exosome-induced cardiac repair.

There are potential clinical implications for these findings. Unlike cell based therapeutic products, Exos provide a readily available and universal treatment option (Armstrong et al., 2017). No complicated steps are required for cryopreservation of Exos. The host immune system has good tolerance to the injection of allogeneic or even heterologous Exos (Jung et al., 2017). This study proves that compared with Young-Exo, Aged-Exo lack the ability of cardiac repair. Our findings may explain why

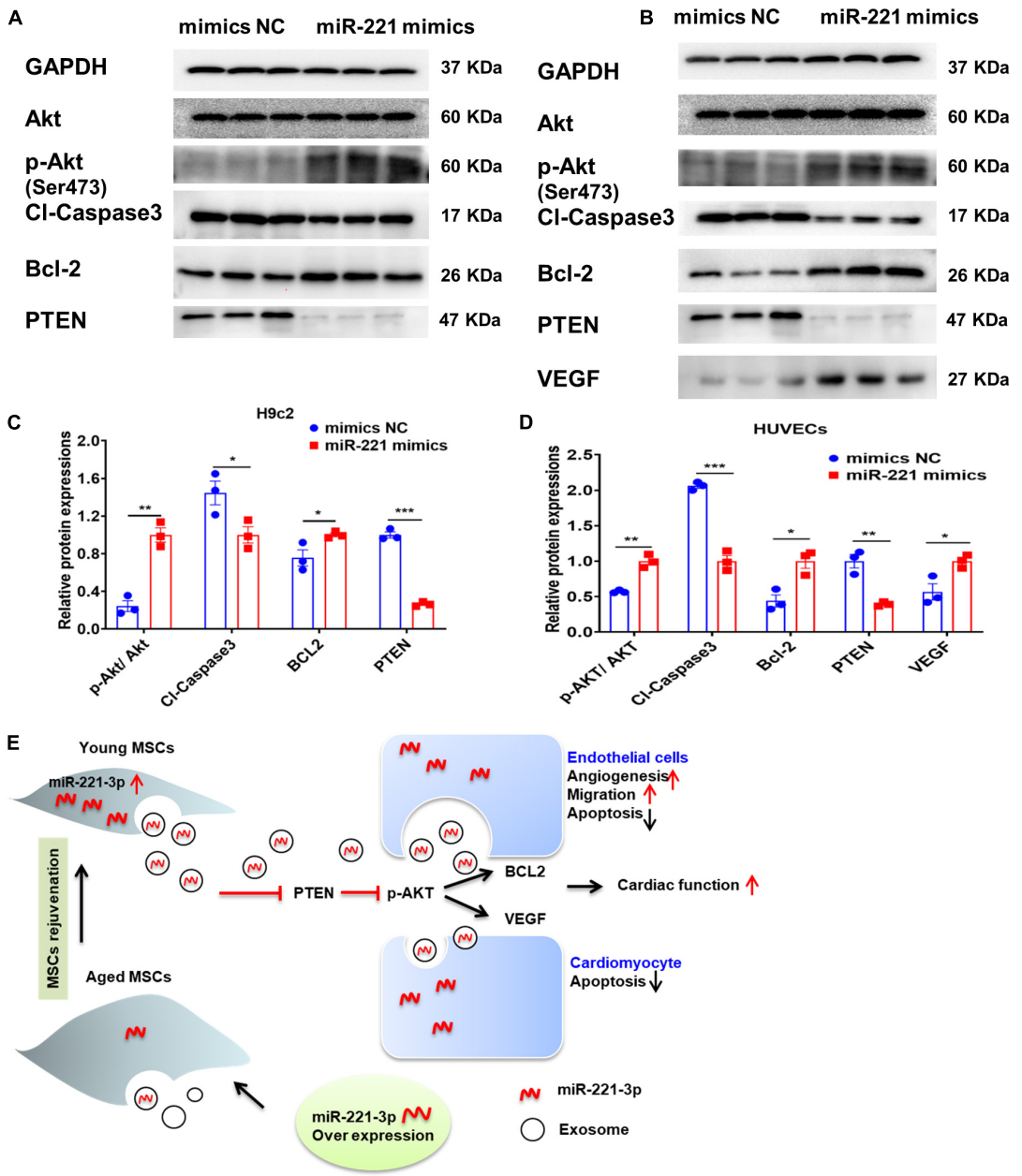

FIGURE 8 | Exosomal miR-221-3p targets the PTEN/Akt signaling pathway after myocardial injury. **(A,B)** Western blot images showed the expression of proteins associated with the PTEN/Akt pathway in H9c2 **(A)** cells and HUVECs **(B)**. **(C)** Quantitative analysis of the levels of Akt, p-Akt, Cleaved caspase-3, Bcl-2, PTEN in H9c2 cells. ($n = 3$). **(D)** Quantitative analysis of the levels of Akt, p-Akt, Cleaved caspase-3, Bcl-2, PTEN, VEGF in HUVECs. ($n = 3$). **(E)** A proposed working model of this study. All miR-221 mimic groups were normalized to the relevant mimic negative control group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

autologous cell therapy in patients has shown limited therapeutic effects in some clinical trials (Fisher et al., 2015; Nguyen et al., 2016). In addition, the data of this study suggest that the dysfunction of Aged-Exo can be partly rescued by regulating specific microRNA in Exos. This study provides insight into the therapeutic potential of Exos and strategies to treat patients with defective exosomes by modulating specific microRNA.

This study has some limitations. First, HUVECs are not a good representation compared with primary coronary endothelial cells. Second, we found that protein levels of the target gene (PTEN) were altered by up-regulation of miR-221-3p. We did not conduct dual luciferase reporter assays. Nonetheless the direct combination of miR-221-3p and PTEN has already been confirmed in other studies (Li et al., 2016; Gong et al., 2019; Han et al., 2019). Third, the rats used in this study were relatively young. Their endogenous reparative ability may differ to that of elderly rats with long-term ischemic heart disease. Finally, although this study focused on miR-221-3p, there are several

other miRNAs that are down-regulated in Aged-Exo and Young-Exo, such as miR-30c-5p, miR-485-5p and miR-200a-3p. These miRNAs may also contribute to the Young-Exo-induced cardiac protective effects post MI. Further studies are needed to clarify the function of other molecules in exosomes.

CONCLUSION

The impaired regenerative abilities of Aged-Exo are due to down-regulation of miR-221-3p. Up-regulation of miR-221-3p in aged MSCs could improve their ability of angiogenesis, migration, proliferation and suppression of apoptosis through the PTEN/Akt pathway.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was approved by the ethics committee of The First Affiliated Hospital of Nanjing Medical University. All subjects gave written informed consent in accordance with the Declaration of Helsinki. All animal experiments were approved by the Institutional Animal Care and Usage Committee of Nanjing Medical University (No. IACUC-1905024).

AUTHOR CONTRIBUTIONS

LS, WZhu, and FZ contributed to the design of the study. LS, WZhu, PZ, JZ, YLi, and WZha performed the experiments. LS, WZhu, YLu, YZ, QC, and FZ contributed to writing the manuscript. LS and WZhu contributed to the material support of the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00263/full#supplementary-material

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REVIEW
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Senescence in Mesenchymal Stem Cells: Functional Alterations, Molecular Mechanisms, and Rejuvenation Strategies

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Mesenchymal stem cells (MSCs) are multipotent cells capable of self-renewal and differentiation. There is increasing evidence of the therapeutic value of MSCs in various clinical situations, however, these cells gradually lose their regenerative potential with age, with a concomitant increase in cellular dysfunction. Stem cell aging and replicative exhaustion are considered as hallmarks of aging and functional attrition in organisms. MSCs do not proliferate infinitely but undergo only a limited number of population doublings before becoming senescent. This greatly hinders their clinical application, given that cultures must be expanded to obtain a sufficient number of cells for cell-based therapy. Here, we review the current knowledge of the phenotypic and functional characteristics of senescent MSCs, molecular mechanisms underlying MSCs aging, and strategies to rejuvenate senescent MSCs, which can broaden their range of therapeutic applications.

Keywords: aging, mesenchymal stem cells, senescence, mechanism, rejuvenation

INTRODUCTION

Mesenchymal stem cells (MSCs) were originally isolated from bone marrow (Friedenstein et al., 1968) but have since been detected in many tissues including dental pulp (Gronthos et al., 2002), adipose tissue (Zuk et al., 2002), and umbilical cord blood (Wang et al., 2004). The essential features of this heterogeneous cell population as defined by the International Society for Cellular Therapy (ISCT) in 2006 are adherence to plastic under culture conditions; expression of the cell surface markers CD44, CD90, CD105, and CD73; absence of the hematopoietic markers CD45, CD34, CD14, CD11b, CD79α, CD19, and human leukocyte antigen-DR; and multi-differentiation potential, with the capacity to generate osteoblasts, chondroblasts, and adipocytes (Dominici et al., 2006). According to the recently published ISCT position statement, although the classic set of markers still applies to *in vitro*-expanded MSCs, surface markers are evolving (Viswanathan et al., 2019). For example, while the definition of MSCs includes CD34 negativity, MSCs can be positive for this marker *in vivo* (Bellagamba et al., 2018). MSCs can differentiate into cells of ectodermal and endodermal parentage (Al-Nbaheen et al., 2013) and novel surface markers (CD165, CD276, and CD82) have been identified (Shammaa et al., 2020). Moreover, surface marker expression can

change under certain culture conditions or when stimulated by a molecule (i.e., interferon-γ) (Stagg et al., 2006). Stringent functional criteria must be met for the designation of a cell as a "stem" cell (Viswanathan et al., 2019; Nolta et al., 2020). MSCs can be safely transplanted autologously or allogeneically as they have low immunogenicity, and thus have many potential applications in cell-based therapy for various disease states (Squillaro et al., 2016). To be clinically useful, MSCs must be expanded *in vitro* over several population doublings (PDs) to obtain a sufficient number of cells for immediate administration. The age of donors is a major factor determining the lifespan and quality of MSCs (Sethe et al., 2006; Baker et al., 2015); cells from aged donors perform less well than those from young donors because of their reduced proliferative capacity and differentiation potential. For patients with age-related diseases, allogeneic MSCs from healthy young donors are clearly preferable to autologous MSCs. On the other hand, regardless of donor age or whether the cells are autologous or allogeneic, MSCs inevitably acquire a senescent phenotype after prolonged *in vitro* expansion (Dimmeler and Leri, 2008; Li et al., 2017). *In vivo* aging refers to donor age, which affects the lifespan of MSCs; *in vitro* aging is the loss of stem cell characteristics by MSCs as they enter senescence during expansion in culture; and senescence is a state where cells stop dividing, which negatively affects their immunomodulatory and differentiation capacities, leading to reduced efficacy following administration (Fan et al., 2010; Turinetto et al., 2016). Thus, for MSCs to be clinically effective, it is essential to monitor senescence and understand the molecular basis of MSC aging. In this review, we discuss changes that occur in senescent MSCs, current strategies for monitoring senescence and the molecular mechanisms involved, and interventions that can potentially slow or even reverse this process.

CURRENT STATUS OF MSC-BASED THERAPY

Mesenchymal stem cells were first used therapeutically in human patients in 1995 (Galipeau and Sensebe, 2018) and has since been applied to the treatment of a broad spectrum of diseases. As of January 2020, there were 767 MSC-based trials registered at www.ClinicalTrials.gov, most of which are at an early phase (phase I or I/II) (**Figure 1A**). Although MSCs have been obtained from a variety of human sources, those derived from bone marrow, umbilical cord, and adipose tissue are preferred for clinical applications and account for approximately 65% of MSCs being used (**Figure 1B**). Due to their multi-differentiation potential and immunomodulatory and paracrine effects, MSCs have been extensively applied in various diseases (**Figure 1C**). Interestingly, although autologous transplantation was initially favored over allogeneic MSCs, there has been a notable increase in the use of the latter over the past decade (**Figure 1D**); for example, 11 out of 19 industry-sponsored phase III clinical trials of MSCs used allogeneic transplantation (Wang et al., 2016; Galipeau and Sensebe, 2018). One reason for this popularity is their low immunogenicity—that is, allogeneic MSCs can be safely transplanted without a high risk of rejection by the recipient (Wang D. et al., 2013; Lee et al., 2016). Additionally, candidate patients for cell-based therapy usually have age-related diseases. While the regenerative capacity of MSCs declines markedly with age (Kretlow et al., 2008; Yu et al., 2011), autologous transplantation is not the best option for these patients. However, robust immunologic data from clinical trials using allogeneic MSCs are still lacking. Although MSCs are considered as immunoprivileged, their transdifferentiation into other cell types—a basic property of MSCs–can increase the risk of immunogenicity (Mukonoweshuro et al., 2014; Ryan et al., 2014). Thus, there is still much to learn and optimize in terms of *in vivo* MSC interactions in pathologic states, which can lead to a better understanding of MSC aging and improve the long-term safety and outcome of MSC engraftment.

Features of MSC Senescence

Irrespective of their source, MSCs enter a state of replicative senescence (i.e., *in vitro* aging, also known as the Hayflick limit) after repeated serial passage in culture when the cells stop dividing after a certain number of PDs (Hayflick and Moorhead, 1961). The maximum number of PDs that can be achieved by MSCs is estimated to be 30 to 40 (Banfi et al., 2002; Baxter et al., 2004). No clear information on passage number has been provided for the 15 MSC products approved to date for clinical use (**Table 1**). However, given that the differentiation potential of MSCs decreases after extended passages, low-passage cultures are recommended for clinical-scale expansion of cultures (Lechanteur et al., 2016). In the following sections, we discuss heterogeneity and biological and functional changes in MSC senescence (**Figure 2**).

Phenotypic Heterogeneity of Senescent MSCs

Despite their global features as defined by the ICST, MSCs are complex cell populations that exhibit heterogeneity depending on the donor, tissue source, and whether they are clonal populations or single cells (Phinney, 2012). MSC heterogeneity comprises proliferation rate, morphology, immunophenotype, multilineage differentiation potential, and senescence (Schellenberg et al., 2012). In symmetric cell division, a self-renewing parent cell divides into two daughter cells with comparable shape and differentiation potential. In contrast, asymmetric cell division yields a self-renewing cell and a non-dividing cell that becomes senescent in culture. These dynamics result in an initially dominant cell population being overtaken by other clonal populations after multiple passages (**Figure 3**). Heterogeneity in the proliferation potential of cultured MSCs manifests morphologically as subpopulations of small, round, rapidly proliferating cells and slowly dividing, large flattened cells (Mets and Verdonk, 1981; Colter et al., 2001). Using the limiting dilution assay at later passages, it was determined that not every cell is capable of clonal expansion and colony formation at the time of culture establishment (Schellenberg et al., 2012). More importantly, the number of colony-forming unit (CFU) fibroblasts decreased continuously during culture expansion, and were scarcely detected after >20

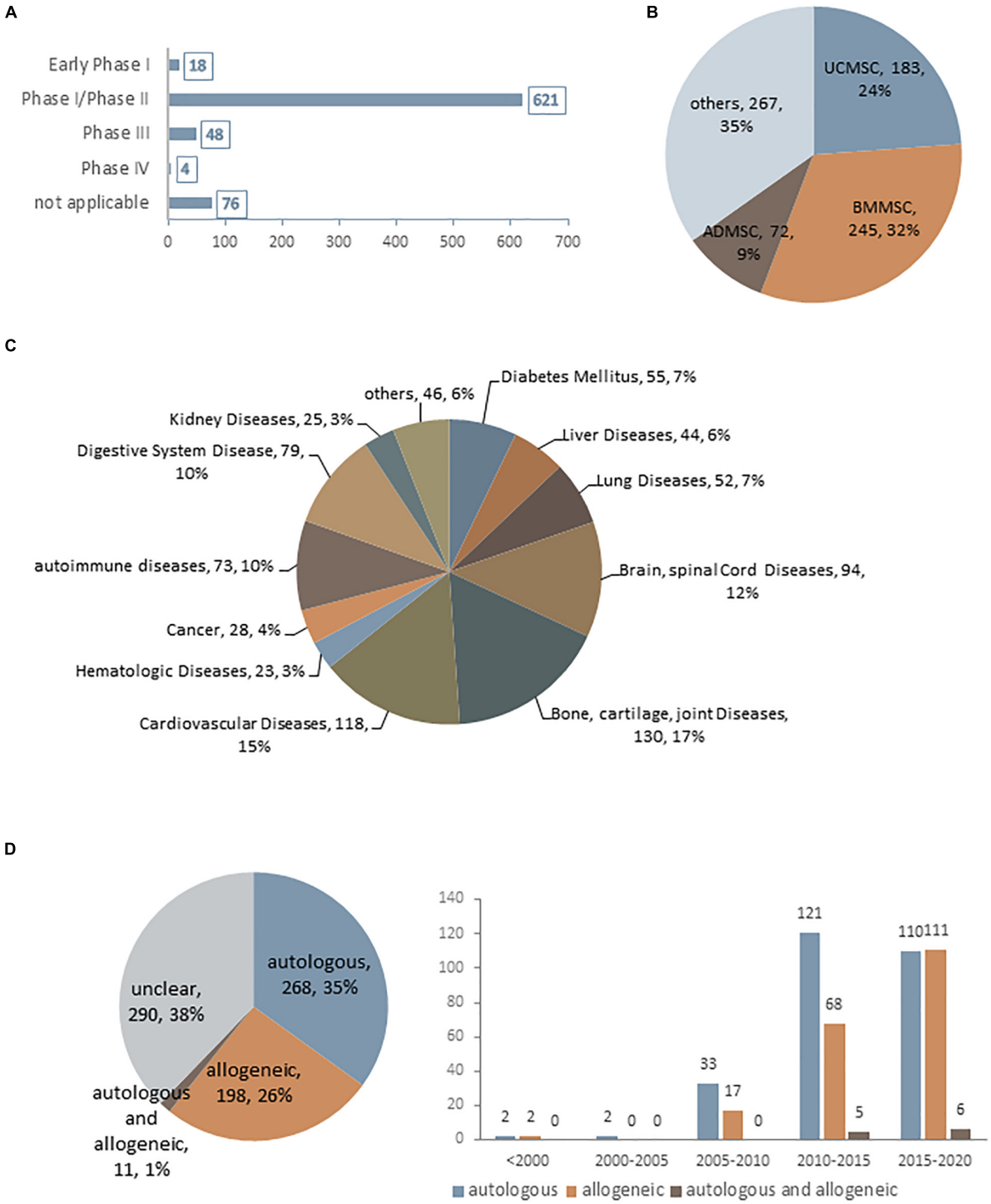

FIGURE 1 | Current statistical data for MSC-based clinical trials as of January 2020 (data accessed from ClinicalTrials.gov ~2020.1). **(A–D)** Statistics for MSC-based clinical trials in different phases **(A)**, using difference cell sources **(B)**, in different disease states **(C)**, and using autologous or allogeneic transplantation **(D)**.

passages (Schellenberg et al., 2012). Likewise, clonal analysis of single-cell–derived colonies has suggested that not every cell has trilineage (i.e., osteogenic, adipogenic, and chondrogenic) potential (Wagner et al., 2008), and subsets with high differentiation potential rapidly decline in number after a few passages (Schellenberg et al., 2012). The age-related heterogeneity of MSCs is thought to be associated with epigenetic status; subpopulations with variable expression of stem cell antigen

TABLE 1 | Approved MSC-based medicinal products.

Product name	Time of approval	MSC source	auto/allo	Country	Company	Disease
Hearticellgram-AMI	2011-07-01	BMMSCs	auto	Korea	FCB-Pharmicell	Acute myocardial infarction
Cuepistem	2012-01-18	ADMSCs	auto	Korea	Anterogen	Crohn's disease complicated with anal fistula
Cartistem	2012-01-19	UCBMSCs	allo	Korea	Medipost	Degenerative arthritis
Prochymal/ remestemcel-L	2014-05-02	BMMSCs	allo	Canada	Mesoblastinterna-tionalsar	Pediatric acute graft versus host disease (aGvHD)
Neuronata-R	2014-07-30	BMMSCs	auto	Korea	Corestem	Lateral sclerosis of spinal cord
Temcell HS	2015-09-20	BMMSCs	allo	Japan	JCR Pharmaceuticals	Acute graft versus host disease (aGvHD)
Stempeucel	2016-03	BMMSCs	allo	India	Stempeutics Research	Severe limb ischemia caused by thromboangiitis obliterans (Buerger disease)
Alofisel (darvadstrocel, Cx601)	2018-03-27	ADMSCs	allo	Japan and Belgium	Takeda Pharmaceutical Company and TiGenix NV	Complex perianal fistulas in Crohn's disease
Holoclar	2015-02-17	Limbal stem cells	auto	Italy	Chiesi Farmaceutici S.p.A	Restoration of Corneal Epithelium in Patients With Limbal Stem Cell Deficiency
MPC	2010-07	Mesenchymal precursor cell	auto	Australia	Mesoblast	Fracture healing and disc healing
ChondroCelect	2009-10-05	Cartilage cells	auto	Belgium	TiGenix NV	Osteoarthritis of the knee and repair cartilage damage of femoral condyle in adult knee joint.
Prochymal	2009-12	BMMSCs	allo	United States	Osiris Therapeutics	Diabetes mellitus type I
MultiStem	2012-07	BMMSCs	allo	United States	Athersys	Hurler's syndrome/ischemic stroke
Maci	2016-12	Cartilage cells	auto	United States	–	Osteochondral damages
Hemacord	2011-11	UCBMSCs	allo	United States	New York Blood Center	Hemorrhagic disease

Auto, autologous; allo, allogeneic; BMMSCs, bone marrow-derived mesenchymal stem cells; ADMSCs, adipose-derived mesenchymal stem cells; UCBMSCs, umbilical cord blood-derived mesenchymal stem cells.

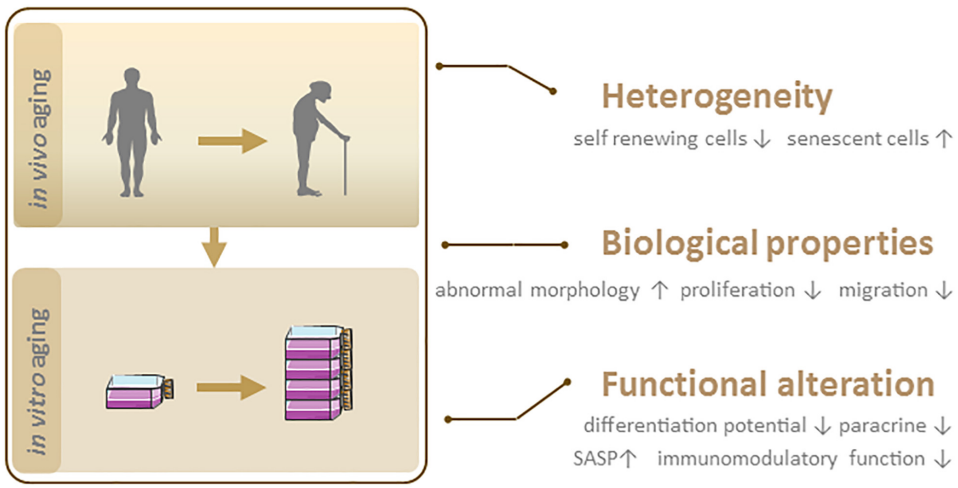

FIGURE 2 | Phenotypic features of senescent MSCs. *In vivo* and *in vitro* aging lead to MSC senescence, which is characterized by heterogeneity, biological and functional changes.

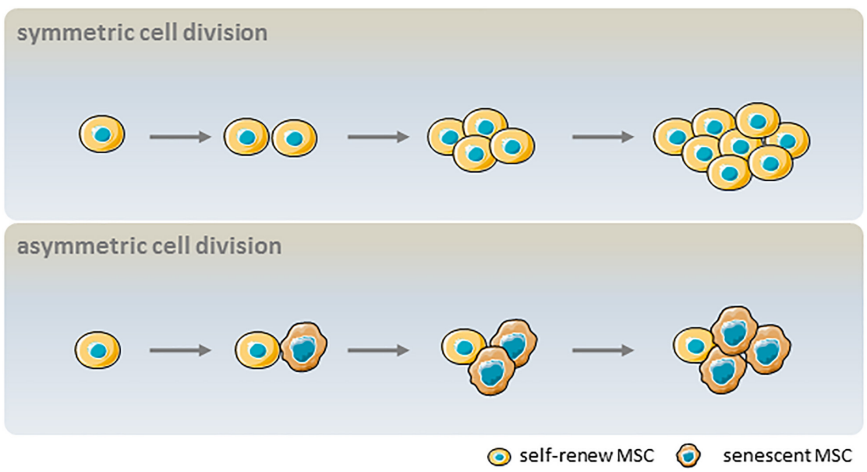

FIGURE 3 | Influence of asymmetric cell division kinetics on the heterogeneity of MSC senescence. In symmetric cell division, a parent MSC with self-renewal capacity divides into two daughter MSCs that can also self-renew. In asymmetric cell division, a parent MSC with self-renewal capacity divides into one self-renewing and one non-dividing cell that becomes senescent in culture.

(Sca)-1 regained the Sca-1 profile of the parent cell after 4–8 days of culture, which was accompanied by epigenetic changes at the lymphocyte antigen 6 complex promoter (Hamidouche et al., 2017). Given the relevance to clinical efficacy, molecular markers of aging in cultured MSCs are needed to reflect senescence-associated alterations. The current understanding of aging-related cellular changes is based primarily on homogeneous bulk cell-derived data; emerging tools for single-cell analysis can help to define the heterogeneity of MSCs.

As MSC populations with a large proportion of senescent cells are less effective when transplanted, it is critical to detect senescent MSCs during expansion. Surface marker profiling is one approach for identifying and purifying senescent cells from a culture. For example, the expression of CD146—also known as melanoma cell adhesion molecule (MCAM)–was downregulated in MSCs derived from aged donors compared to those from young donors, as well as in MSCs after prolonged *in vitro* expansion (Gnani et al., 2019). Furthermore, low but not high CD146 expression was associated with a senescent phenotype in MSCs (Jin et al., 2016), and $CD146^{+}$ MSCs showed increased migratory potential toward degenerating tissues (Wangler et al., 2019). CD264 is another surface marker of *in vitro* aging in MSCs that is unrelated to the chronologic age of the donor (Madsen et al., 2017); cells expressing this protein exhibit increased senescence-associated β-galactosidase (SA-β-gal) activity and reduced differentiation potential and colony-forming efficiency compared to $CD264^{-}$ MSCs (Madsen et al., 2020). Other surface markers that show altered expression with *in vitro*/*in vivo* aging are summarized in **Table 2**. It should be noted that although these molecules are expressed to varying degrees during the

TABLE 2 | Surface marker alteration in senescent MSCs.

Surface marker	*In vivo* aging/*In vitro* aging	Type of MSCs	PMID
Decreased expression in senescent MSCs			
CD146/ MCAM	*in vitro* aging	Human-BMMSCs	29751774
	in vitro aging	Human periapical cyst derived MSCs	27406247
	in vitro aging	Human tonsil derived MSCs	25155898
	in vitro aging	Human-UCBMSCs	26941359
	in vitro aging	Human-UCBMSCs	21144825
CD106/ VCAM-1	*in vitro* aging	Human-BMMSCs	29751774
	in vitro aging	Human-BMMSCs	30211967
	in vitro aging	Human-UCBMSCs	21144825
	in vitro aging	Human vertebral body spongiosa derived MSCs	19242838
CD90	*in vitro* aging	Human-AFMSCs	27803714
	in vitro aging	Human-UCBMSCs	21144825
CD105	*in vitro* aging	Human-AFMSCs	27803714
	in vitro aging	Human-UCBMSCs	21144825
CD44	*in vitro* aging	Human-AFMSCs	27803714
	in vitro aging	Human-UCBMSCs	21144825
CD49F	*in vitro* aging	Human-BMMSCs	26013602
CD34	*in vivo* aging	Human-BMMSCs	23197850
CD133	*in vivo* aging	Human-BMMSCs	23197850
CD166	*in vitro* aging	Human-UCBMSCs	21144825
Increased expression in senescent MSCs			
CD264	*in vivo* aging	Human-BMMSCs	28962588
	in vivo aging	Human-BMMSCs	31612990
HLA/ MHC	*in vitro* aging	Human-BMMSCs	30211967
	in vitro aging	Human-ADMSCs	22391697
CD49C	*in vitro* aging	Human-BMMSCs	30211967
CD45	*in vitro* aging	Human-ADMSCs	22391697
Controversial			
CD271/P75NTR	*in vivo* aging	Human-BMMSCs	23197850
	in vivo aging	Human-BMMSCs	31467563

BMMSCs, bone marrow-derived mesenchymal stem cells; UCBMSCs, umbilical cord blood-derived mesenchymal stem cells; AFMSCs, amniotic fluid derived-mesenchymal stem cells; ADMSCs, adipose-derived mesenchymal stem cells.

aging process and may be associated with functional changes, there is presently no consensus on whether they can serve as the gold standard for selective purification of young vs. old MSC populations.

Biological Properties of Senescent MSCs

Early-passage MSCs are small and have a fibroblast-like spindle shape but acquire a hypertrophic and flat morphology with more podia and actin stress fibers upon extended culture (Stenderup et al., 2003; Mauney et al., 2004; Stolzing and Scutt, 2006). MSCs from passages 1 to 3 have a uniform size but begin to enlarge at passage 5, such that cells at passages 6–9 are 4.8-fold larger than passage 1 cells (Oja et al., 2018). Morphologic features can predict how well cells can adapt to a given condition. Cell and nuclear morphology in MSCs in the first 3 days of osteogenic induction was found to be closely correlated with their long-term (35-day) mineralization capacity (Marklein et al., 2016). A subset of aged MSCs with a small cell size had ATP levels equivalent to those in young MSCs, whereas levels in large-sized cells were comparable to those in the aged parent MSC population (Block et al., 2017). Increased cell size and granularity were positively correlated with MSC autofluorescence; the latter has therefore been proposed as a non-invasive, real-time quantifiable marker for cellular senescence (Bertolo et al., 2019).

Animal cells sense and correct deviations in size by adjusting cell cycle length as well as growth rate (Miettinen et al., 2014), which is increased in small cells and reduced in large cells (Ginzberg et al., 2018). Loss of cell size uniformity can indicate abnormal biosynthesis or cell cycle progression. While the regulation of size homeostasis in relation to senescence is not fully understood, cell enlargement can distinguish senescent subpopulations in culture. Among aged MSCs, SA-β-gal activity is increased in large as compared to small-sized cells (Block et al., 2017). Notably, MSCs immortalized by SV40 (Negishi et al., 2000) or telomerase transfection (Kobune et al., 2003) are significantly smaller than their parent cells.

The enlargement of aging cells and their transformation to a hypertrophic morphology is accompanied by biological changes. A decline in proliferative capacity was reported in MSCs derived from old patients as compared to their healthy young counterparts (Banfi et al., 2002) and in long-term MSC cultures regardless of the cell source. MSCs from young donors

had greater mitotic activity (41 ± 10 vs. 24 ± 11 PDs), slower progression to senescence, and an increased rate of proliferation (0.09 ± 0.02 vs. 0.05 ± 0.02 PDs/day) than those from old donors (Stenderup et al., 2003). CFU is a retrospective parameter describing the clonogenic potential of a single cell; decreases in CFU and average colony size are correlated with MSC aging *in vitro* (Liu et al., 2004).

In addition to impaired proliferation, aging negatively affects MSC migration and homing ability (Liu et al., 2017). Directed migration toward stimuli by MSCs is critical for better functional outcomes in cell-based therapy. Impaired migratory capacity in senescent MSCs in response to pro-inflammatory signals was found to be closely associated with activator protein (AP)-1 pathway inhibition (Sepulveda et al., 2014). Cell migration involves the reorganization of the actin cytoskeleton (Le Clainche and Carlier, 2008). MSCs derived from old donors exhibit reduced response to biological and mechanical signals because their actin cytoskeleton is less dynamic (Kasper et al., 2009). Gene expression profiling has identified several cytokines and chemokines and their receptors important for cell migration–including stromal cell-derived factor 1 (SDF-1) and its receptor chemokine receptor type 4 (CXCR4), tumor necrosis factor receptor (TNFR), IFN-γ receptor (IFNGR), and C-C motif chemokine receptor 7 (CCR7)—that are downregulated in aged MSCs as compared to younger cells (Geissler et al., 2012; Bustos et al., 2014).

Functional Changes Associated With MSC Senescence

A basic strategy for MSC-based regeneration is to replace cells that are lost or impaired by disease with functional cells. It was previously thought that MSCs exert their therapeutic effect through *trans*-differentiation. During *in vitro* culture, MSCs progressively lose their capacity to differentiate into adipogenic and osteogenic lineages although the preferred fate is debated, with some studies suggesting that aging shifts the balance in favor of adipocytes at the expense of osteoblastogenesis (Stolzing et al., 2008), and others reporting that osteogenic activity is preserved or even increased in late passages (Wagner et al., 2008) or that both osteogenic and adipogenic potential is lost (Geissler et al., 2012). Age-associated changes in differentiation potential may be related to altered susceptibility to reactive oxidative species (ROS) and apoptosis (Bruedigam et al., 2010); MSCs undergoing osteoblast differentiation showed dose-dependent increases in apoptosis and ROS accumulation upon treatment with rosiglitazone, whereas adipogenesis was unaffected (Jiang et al., 2008). Lineage bias in differentiation is regulated by key signaling pathways, intracellular oxidative stress, and transcriptional and post-transcriptional mechanisms. Gene expression analysis has revealed an age-related downregulation of osteoblast transcription factors such as core binding factor α1 (CBFA1), runt-related transcription factor 2 (Runx2), and distal-less homeobox 5 (DIx5) as well as collagen and osteocalcin, and upregulation of adipogenic factors such as peroxisome proliferator-activated receptor-γ (PPAR-γ) and adipocyte fatty acid-binding protein (aP2) (Jiang et al., 2008). The target genes activated by PPAR-γ are related to lipid metabolism and adipocyte differentiation. Thus, the age-related increase in PPAR-γ expression shifts the fate of MSCs toward adipogenesis, and Wnt/β-catenin signaling regulates MSC differentiation by suppressing PPAR-γ and biasing differentiation toward osteoblastogenesis (Xu et al., 2016).

Aging cells acquire a senescence-associated secretory phenotype (SASP) involving the secretion of proteins that can affect the behavior of neighboring cells via autocrine/paracrine mechanisms (Borodkina et al., 2018; Campisi et al., 2019). MSCs have potent anti-inflammatory and immunosuppressive functions and thus have therapeutic potential for inflammation-related diseases. Aged MSCs have a diminished capacity for inhibiting the proliferation of allogeneic peripheral blood mononuclear cells compared to younger cells (Gnani et al., 2019). The activation of SASP factors such as interleukin 6 (IL-6), IL-8, and monocyte chemotactic protein 1 (MCP1) in the conditioned medium of aged MSCs was shown to be increased compared to young MSC cultures at early passages, an effect that was exacerbated at late passages (Gnani et al., 2019). Secretion of SASP-related chemokines/cytokines not only drives responses that reinforce senescence in a cell-autonomous manner but also acts on neighboring cells via a paracrine mechanism to accelerate senescence. For example, factors secreted by aged MSCs were shown to activate pro-inflammatory gene expression in young hematopoietic stem cells and decreased their clonogenic potential (Gnani et al., 2019).

The beneficial effects of MSC-based therapy are attributable to the action of pro-angiogenic paracrine factors. In aged MSCs, the secretion of these factors—including vascular endothelial growth factor (VEGF), placental growth factor (PGF), and hepatic growth factor (HGF)—is reduced, whereas that of anti-angiogenic factors such as thrombospondin-1 (TBS1) and plasminogen activator inhibitor-1 (PAI-1) is increased. Thus, age negatively affects angiogenesis and directly undermines the therapeutic efficacy of MSCs (Efimenko et al., 2011; Khan et al., 2011).

Strategies for Monitoring MSC Senescence

β-D-Galactosidase (β-Gal) is a eukaryotic hydrolase localized in the lysosome that is active at the optimal pH (6.0) in senescent cells but is absent in proliferating cells (Dimri et al., 1995). SA-β-gal activity is suggested as the gold standard for evaluating senescence in cells and can be detected by cytochemistry/histochemistry and fluorescence-based methods. However, when used in combination with other markers, it can yield false-positive/negative results in quiescent cells or upon stress (de Magalhaes et al., 2004; Yang and Hu, 2005). Senescence-associated lysosomal α-L-fucosidase (SA-α-Fuc) has recently been identified as a more robust biomarker in all types of cellular senescence (Hildebrand et al., 2013; Singh and Piekorz, 2013), but there is still limited evidence for its sensitivity and specificity in distinguishing senescent MSCs.

Telomeres are specialized nucleoprotein caps containing repetitive nucleotide sequences that protect chromosomes from

end-to-end fusion and prevent the loss of genetic information during DNA replication (Sahin and Depinho, 2010). Telomeres shorten with every cell division and senescence is triggered when they reach a critical length (Baird et al., 2003). As such, telomere length has been used to estimate replicative history and predict senescence in MSCs (Montpetit et al., 2014). There is increasing evidence of an association between diminished proliferative capacity and telomere shortening in MSCs. However, the exact telomere length in senescent MSCs and whether it differs according to cell source, culture conditions, and measurement method is unclear. For example, a telomere length of 10 kb was proposed as a threshold for senescence (Baxter et al., 2004), although another study reported a length of 6.8 ± 0.6 kb in senescent cells (Oja et al., 2018). In addition, a recent study described a mechanism of senescence that is independent of cell division and telomere length, involving activation of classical senescence-associated pathways and yielding a non-canonical SASP (Anderson et al., 2019). Although this phenomenon was first reported in post-mitotic cardiomyocytes, it may also occur in MSCs. Thus, telomere shortening has limitations for the measurement of senescence, and other markers may be more informative under certain conditions.

The senescent state is characterized by cell cycle arrest. Senescence-associated growth arrest is maintained by the activation of several pathways including phosphorylated inhibitor of cyclin-dependent kinase 4A ($p16^{INK4A}$)/phosphorylated retinoblastoma (pRb) and $p53/p21^{WAF1}$ signaling (Campisi, 2005). $p16^{INK4A}$ is an inhibitor of cyclin-dependent kinase (CDK) and induces premature cell senescence via telomere-dependent and -independent mechanisms (Serrano et al., 1993). $p16^{INK4A}$ level was shown to increase with chronological age or PDs of MSCs in culture, and a large proportion of the $p16^{INK4A}$-positive cells were negative for the proliferation marker Ki67 and positive for SA-β-gal. Inhibiting $p16^{INK4A}$ reduced the number of senescent MSCs and conferred cells with the ability to proliferate (Shibata et al., 2007). Similarly, overexpressing $p21^{WAF1}$—a CDK inhibitor that acts by dephosphorylating pRb–increases cellular senescence, as evidenced by elevated SA-β-gal activity and telomere shortening (Huang et al., 2004), while inhibiting $p21^{WAF1}$ in senescent cells restored their replicative capacity. However, $p21^{WAF1}$ depletion was less efficient at preventing senescence than p53 depletion, suggesting that the latter acts through $p21^{WAF1}$-independent mechanisms to exert this effect (Gire and Dulic, 2015).

Mesenchymal stem cells-derived microvesicles (MSC-MVs) that mimic the senescent state of the parent MSC have recently emerged as a potential cell-free biomarker for cellular senescence. Senescent late-passage MSCs secrete larger amounts of MSC-MVs of smaller size than those in early passages, and CD105 expression in MSC-MVs decreased with senescence in parent MSCs. RNA sequencing results suggest that most genes that are highly expressed in senescent MSC-MVs are involved in aging-related diseases (Lei et al., 2017). Functionally, senescent MSC-MVs have a lower capacity to promote osteogenesis (Lei et al., 2017) and recruit macrophages and fail to alter macrophage phenotypes (Huang et al., 2019).

Besides the abovementioned markers, new tools and approaches have been proposed to monitor MSC aging such as SiR-actin, a fluorogenic F-actin specific probe that can be used to evaluate actin turnover (Mishra et al., 2019). CyBC9 (another fluorescent probe) combined with high-throughput screening revealed accumulation of mitochondria in senescent MSCs that presumably resulted from the loss of membrane potential (Ang et al., 2019). Thus, senescence can be characterized not by a universal biomarker, but by a set of non-exclusive markers in conjunction with specific biological features.

Role of DNA Damage, ROS, and Autophagy in MSC Senescence

Senescence is a multistep process involving various mechanisms that have not been fully elucidated. One of these is irreversible cell cycle arrest—typically in response to DNA damage—in the presence of growth-promoting stimuli. DNA damage accumulates throughout the lifetime of an organism as a result of DNA replication errors and exposure to endogenous and exogenous mutagens. The DNA damage response (DDR) network can sense and initiate repair of mutations and thereby slow their accumulation. Activation of the DDR network can transiently halt cell cycle progression through stabilization of p53 and transcriptional activation of the CDK inhibitor p21. However, if DNA damage persists, $p16^{INK4A}$ is activated via p38-mitogen-activated protein kinase-mediated mitochondrial dysfunction and ROS production. This results in CDK inhibition and activation of the tumor suppressor Rb1, which induces the onset of senescence (**Figure 4**; Zhang et al., 2007; Ciccia and Elledge, 2010).

Reactive oxidative species are a group of oxygen-containing small molecules. Approximately 90% of ROS are generated endogenously by the mitochondrial electron transport machinery (Poyton et al., 2009) but extrinsic factors such as radiation, ultraviolet light, hypoxia, and low temperature can also increase their production. As a metabolic by-product, ROS induce oxidation and various cellular responses through the generation of reactive secondary metabolites. While physiologic levels of ROS are necessary for proliferation and differentiation, an excess can trigger cellular senescence, including in MSCs (Sart et al., 2015). ROS induce DNA damage and accelerates telomere erosion, both of which activate the DDR. Senescent MSCs have elevated levels of ROS compared to normal cells that cause persistent DDR activation, thereby forming a positive feedback loop in the progression of senescence (**Figure 4**; Yang et al., 2015).

Reactive oxidative species accumulate with advancing age, leading to decreased mitochondrial metabolism. Mitochondrial DNA (mtDNA) encodes 13 polypeptides of the mitochondrial oxidative phosphorylation (OXPHOS) enzyme complexes, 22 tRNAs for mitochondrial biosynthesis, and 16S and 18S rRNA for mitochondria. Because of the absence of efficient repair mechanisms, the mutation rate of mtDNA is higher than that of nuclear DNA. A point mutation in mitochondria-encoded subunit 3 of cytochrome c oxidase was found to be associated with enhanced tissue degeneration and risk of premature aging (Niemann et al., 2017). The mitochondrial free

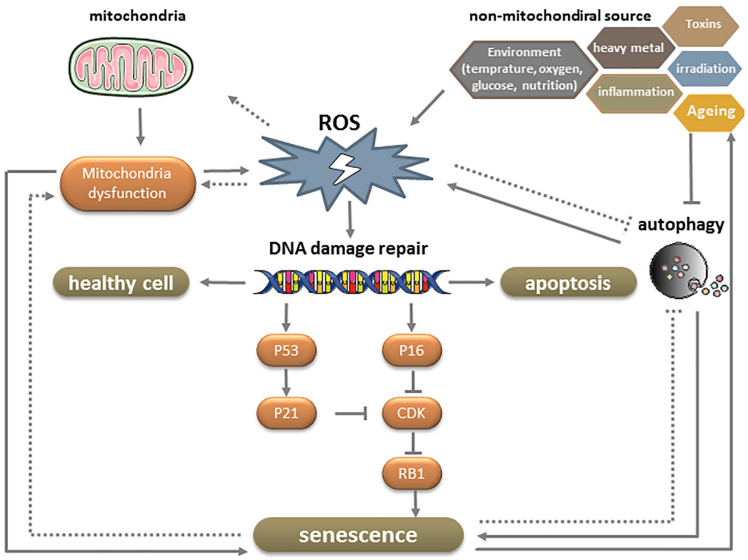

FIGURE 4 | Effect of ROS on MSC senescence.

radical theory of aging posits that age-dependent accumulation of mtDNA abnormalities, impaired OXPHOS, and altered expression of antioxidant enzymes lead to increased ROS production, which, in turn, results in progressive mitochondrial dysfunction and global cellular damage in a positive feedback loop (**Figure 4**; Wang C. H. et al., 2013). However, other studies suggest that mtDNA mutations and mitochondrial dysfunction affect aging independently of ROS production, based on the unexpected observation that genetic manipulations that increased mitochondrial ROS levels and oxidative damage did not accelerate aging in mice (Zhang et al., 2009), whereas those that impaired mitochondrial function without increasing ROS enhanced aging (Kujoth et al., 2005).

Autophagy is a critical process for maintaining cellular homeostasis under physiologic and pathologic conditions. By removing damaged cellular components including proteins and mitochondria, autophagy prevents age-related cellular injury (Wirawan et al., 2012) and allows stem cells to avoid the transformation from a reversible quiescent (G0) state to irreversible senescence (Garcia-Prat et al., 2016). Although ROS are known to stimulate autophagy, the age-related increase in ROS levels reduces autophagic capacity (Yamamoto et al., 2016). In fact, aged MSCs exhibit reduced autophagy, which is correlated with diminished self-renewal capacity and regenerative potential and replicative exhaustion. From a mechanistic standpoint, decreased autophagy results in the loss of proteostasis and increases mitochondrial activity, oxidative stress, and metabolism state in MSCs (**Figure 4**; Revuelta and Matheu, 2017).

Key aspects of the mechanisms of MSC aging remain unknown, but epigenetic changes (i.e., those occurring in the absence of DNA sequence alterations) such as DNA methylation, histone modification, and chromatin remodeling may play a role (Ozkul and Galderisi, 2016; Cakouros and Gronthos, 2019). For example, dysregulated expression of Brahma-related gene 1 (BRG1), a component of ATP-dependent chromatin remodeling complexes, was associated with senescence in MSCs via regulation of NANOG methylation status (Squillaro et al., 2015). Additionally, microenvironmental and hormonal conditions are important factors contributing to MSC aging *in vivo*. As MSCs exist in a semi-static state, replicative exhaustion is unlikely to occur (Ganguly et al., 2017). There is increasing evidence that the *in vivo* cellular aging process is caused by chronologic aging of the host and is accelerated by conditions such as obesity and systemic inflammation (Frasca et al., 2017; Franceschi et al., 2018).

Strategies for Rejuvenating Senescent MSCs

Strategies allowing the generation of large numbers of MSCs that have retained their stemness are needed for clinical applications. Here, we summarize current research efforts to prevent MSC senescence.

Induced pluripotent stem cell (iPSC)-derived MSCs (iMSCs) can be passaged more than 40 times without exhibiting features of senescence (Sabapathy and Kumar, 2016). iMSCs retain a donor-specific DNA methylation profile while tissue-specific, senescence-associated, and age-related patterns are erased during reprogramming (Frobel et al., 2014). Recent studies have demonstrated that iMSCs have superior regenerative capacity compared to tissue-derived MSCs in preclinical degenerative disease models (Lian et al., 2010; Chen et al., 2019; Wang et al., 2019). However, the generation of iMSCs from iPSCs requires a significant degree of molecular manipulation, and there are safety concerns regarding the self-renewal and pluripotency of iPSC-derived cells after *in vivo* transplantation, which have the risk of tumorigenicity and genomic instability (Hynes et al., 2013). In addition, each independent iPSC line has a unique genetic and epigenetic profile that must be characterized. The concept

TABLE 3 | Strategies for MSC rejuvenation.

	Interventing approach/medicine	Mechanism	Rejuvenation of function	Target cell	PMID
Genetic approach	miR-195 inhibition	Induced telomere relengthening	Proliferation	Human-BMMSCs	26390028
		Reduced SA-β-gal expression			
		Restored antiaging factors expression including Tert and SIRT1			
		Restored phosphorylation of AKT and FOXO1			
	ERBB4 overexpression	Inhibited PI3K/AKT and MAPK/ERK pathways	Angiogenesis	Mouse-MSCs	25996292, 30566395
			Survival		
			Mobility		
			Apoptotic resistance		
	SIRT1 overexpression	Decreased H2O2-induced oxidative stress response capabilities	Senescent phenotype	Rat-MSCs	25034794, 28258519
		Increased Ang1, bFGF expressions, decreased TBS1 expressions	Angiogenesis		
		Increased in Bcl-2/Bax ratio	Apoptosis		
	SIRT3 overexpression	Reduced ROS	Senescent phenotype	Human-BMMSCs	28717408
			Adipocytes/osteoblasts differentiation		
	TERT overexpression	Increased telomere length, prolonged population doublings	Osteoblastic differentiation	Human-BMMSCs	12042863
			Proliferation		
	$p16^{INK4A}$ Knockdown	Up-regulated TGF-β expression	Senescent phenotype	Human-BMMSCs	22820504
		Increased the percentage of Treg cells			
		Up-regulated ERK1/2 activation			
	p21 Knockdown	Increased the level of Cyclin E, cyclin-dependent kinase-2	Proliferation	Human-BMMSCs	24151513
		Increased the phosphorylation of retinoblastoma protein	Senescent phenotype		
	Silencing lincRNA-p21	Interacted with the WNT/β-catenin signaling pathway	Proliferation and paracrine function	Mouse-BMMSCs	28901439
	PTEN or p27(kip1) Knockdown	Down-regulated PTEN and p27(kip1) expression	Apoptosis, senescence phenotype	Human-BMMSCs	25649549
		Regulated protein kinase B (AKT) signaling			
		Enhanced IL-10 and TGF-β and reduced IL-17 and IL-6			
		Increased Treg/Th17 cells			
	Nampt overexpression	Up-regulated intracellular concentrations of NAD+ and SIRT1 expression and activity	Senescence phenotype	Rat-BMMSCs	28125705
	NANOG overexpression	Fortified the actin cytoskeleton and ACTA2	Myogenic differentiation	Human-hair follicle MSCs	28125933
		Restored contractile function			
	Dicer1 overexpression	Increased miR-17 family (miR-17-5p, miR-20a/b, miR-106a/b and miR-93)	Differentiation	Human-BMMSCs	25361944
		Decreased miR-93, miR-20a and p21 expression	Stemness		
	miR-10a overexpression	Repressed the KLF4-Bax/Bcl2 pathway	Apoptosis, survival, differentiation	Human-BMMSCs	29848383
		Activated AKT and stimulated the expression of angiogenic factors	Angiogenesis		

(Continued)

TABLE 3 | Continued

	Interventing approach/medicine	Mechanism	Rejunvenation of function	Target cell	PMID
	Lcn2 overexpression	Decreased senescence induced by H2O2	Proliferation, cloning	Human-BMMSCs	24452457
	FGF-21 overexpression	Decreased mitochondrial fusion and increased mitochondrial fission	Senescent phenotype	Human-BMMSCs	31178962
	NDNF overexpression	Activated the AKT signaling	Proliferation	Human-BM/ADMSCs	30062183, 31287219
			Migration		
			Angiogenesis		
	PEDF Knockdown	Induced cellular profile changes	Proliferation	Mouse-BMMSCs	21606086
			Migration		
	MIF overexpression	Activated autophagy	Cell survival after transplantation	Human-BMMSCs	31881006
			Reduced cellular senescence		
			Angiogenesis		
Phamacological approach	TMP	Inhibited NF-κB signaling	Proliferation, cell cycle	Rat-BMMSCs	31171713
		Modulated Ezh2-H3k27me3	Anti-inflammatory and angiogenesis	Mouse-BMMSCs	29488314
	RSV	Regulated SOX2	Multipotency	Rat-BM/ADMSCs	25132403, 26456654, 31440387, 27049278
		Activated SIRT1 expressison	Self-renewal		
		Decreased ERK and GSK-3β phosphorylation and β-catenin activity	Senescence phenotype		
		Promoted insulin secretion of INS-1 cells via Pim-1	Paracrine function		
	Artemisinin	Activated the c-Raf-ERK1/2-p90rsk-CREB pathway	Survival, apoptosis	Rat-BMMSCs	31655619
		Reduced the level of ROS production			
		Enhanced the levels of antioxidant enzymes including SOD, CAT and GPx			
		Increased ERK1/2 phosphorylation			
	Largazole or TSA	Affected histone H3 lysine 9/14 acetylation and histone H3 lysine 4 dimethylation	Proliferation	Human-UCMSCs	23564418
			Osteogenic differentiation		
	CASIN	Reduced Cdc42-GTP	Proliferation, differentiation	Rat-ADMSCs	29804242
		Down-graduated the levels of ROS, $p16^{INK4A}$ and F-actin			
		Inhibited the ERK1/2 and JNK signaling pathways			
	DKK1	Hyperactivated the WNT/β-catenin and the p53/p21 pathway	Senescence phenotype	Human-BMMSCs	24130040
	Melatonin	Activated Nrf2 gene through the MT1/MT2 receptor pathway	Survival, senescence phenotype	Canine-ADMSCs	30362962
		Stimulated ERAD, alleviated ERS			
		Inhibited NF-κB pathway			
	SGJ	Promoted lysosomal acidification	Senescence phenotype	Rat-BMMSCs	30526663
		Increased the concentration of H+ and the protein expression of LAMP1/2	Cell morphology		

(Continued)

TABLE 3 | Continued

	Interventing approach/medicine	Mechanism	Rejunvenation of function	Target cell	PMID
		Suppressed the expression of p21 and reduced SA-β-gal positive cells	Proliferation		
		Promoted LC3B but reduced the p62/SQSTM1 protein	Autophagy		
	ABT-263/navitoclax	Revealed a senolytic effect	Senescence phenotype	Human-MSCs	29669575
	IDB	Increased the expression of Bcl2, Nanog, octamer-binding transcription factor 4, E-cadherin	Apoptosis	Rat-BMMSCs	29393352
		Decreased the expression of N-cadherin and vimentin	Migration		
		Increased proliferating cell nuclear antigen, cyclinD1 and cyclinD3	Proliferation, cloning		
	Fucoidan	Regulated SMP30 and p21	Proliferation, cell cycle	Human-ADMSCs	29642406
		Regulated CDK2, CDK4, cyclin D1, and cyclin E proteins			
		Regulated FAK-AKT-TWIST signal transduction			
	LC	Decreased the population doubling time	Proliferation, senescence phenotype	Rat-ADMSCs	27943151
	RAPA	Improved immunoregulation	Survival, senescent phenotype	Human-BMMSCs	27048648
		Inhibition of the mTOR signaling pathway			
	Rg1	Decreased the rate of SA-β-gal positive cells	Proliferation	Human-BMMSCs	30055206
	EGCG	Activated Nrf2	Senescence phenotype	Human-BMMSCs	27498709
		Down-regulated the p53/p21 signaling pathway			
	DHJST/Ligusticum chuanxiong	Up-regulated BMP-2 and RUNX2 gene expression	Osteogenic differentiation	Human-BMMSCs	28040510
		Activated of SMAD 1/5/8 and ERK signaling	Senescence phenotype		
	Curcumin	Reduced the population doubling time	Proliferation	Rat-ADMSCs	29017189
	Apocynin	Suppressed NADPH oxidase	Senescence phenotype	Mouse-BMMSCs	26686764
		Reduced p53 expression			
	R-SFN (low doses)	Antioxidant properties	Proliferation, apoptosis, senescence phenotype	Human-BMMSCs	21465338
	1,25-VD3	Decreased systemic phosphate levels	Proliferation, apoptosis	Human-BMMSCs	22242193
Cytokine supplementation	MIF	Interacted with CD74	Self-renewal	Rat-BMMSCs	25896286
		Activated AMPK-FOXO3a signaling pathways	Senescence phenotype		
	IGF1	Activated the IGF1R/PI3K/AKT signaling pathway	Proliferation	Mouse-BMMSCs	31660081
			Stemness		
			Senescence phenotype		
	FGF/FGF-2	Regulated PI3K/AKT-MDM2 pathway	Stemness	Human-BMMSCs	21527526, 17532297
		Inhibited ROS and TGF-β	Proliferation		
	Recombinant human HSP70	Suppressed expression of p16 and p21	Proliferation	Mouse-ADMSCs	27091568
		Induced expression of superoxide dismutase and SIRT-1			
	Jagged1	Activated Notch signaling pathway	Senescence phenotype	Human-BMMSCs	28151468

of iMSCs is at its infancy and requires validation from preclinical and clinical studies before it can be clinically useful.

Aging is not a passive or random process but can be modulated through several key signaling molecules/pathways (Kenyon, 2010). Identification of age-related coordinating centers can provide novel targets for therapeutic interventions. Sirtuins (SIRTs) are a class of highly conserved nicotinamide adenine dinucleotide-dependent protein deacylases of which there are 7 (SIRT1–7) in mammals (Vassilopoulos et al., 2011). The role of SIRTs in aging is related to their regulation of energy metabolism, cell death, and circadian rhythm and maintenance of cellular and mitochondrial protein homeostasis (O'Callaghan and Vassilopoulos, 2017). Mitochondrial SIRTs (SIRT3–5) act as stress sensors and regulate protein networks to coordinate the stress response (van de Ven et al., 2017). Overexpression of SIRTs has been investigated as a potential strategy for preventing MSC aging. For instance, SIRT3 expression in MSCs decreased with prolonged culture and its overexpression in later-passage cells restored differentiation capacity and reduced aging-related senescence (Denu, 2017). SIRT1 is required for long-term growth of MSCs and SIRT1 overexpression was shown to delay senescence without loss of adipogenic or osteogenic potential (Yuan et al., 2012). Additionally, SIRT1 expression was shown to be spontaneously upregulated upon osteogenic differentiation and protected MSCs from extracellular oxidative stress (Li et al., 2018).

Genetic engineering has been used to slow MSC aging. Besides SIRTs, several molecules have been identified as potential targets for interventions to prevent senescence (**Table 3**). Ectopic expression of telomerase reverse transcriptase in MSCs extended their replicative lifespan, which preserved a normal karyotype, promoted telomere elongation, and abolished senescence without loss of differentiation potential (Simonsen et al., 2002). Introduction of Erb-B2 receptor tyrosine kinase 4 (ERBB4) in aged MSCs conferred resistance to oxidative stress-induced cell death and rescued the senescence phenotype (Liang et al., 2019). Knocking down macrophage migration inhibitory factor (MIF) in young MSCs induced senescence; conversely, its overexpression in aged MSCs rejuvenated the cells by activating autophagy (Zhang et al., 2019). However, the risk of malignant transformation remains a major barrier for the use of genetics-based approaches in clinical practice.

Pharmacologic approaches and cytokine supplementation have also shown promise for delaying senescence (**Table 3**). Inhibiting mechanistic target of rapamycin by rapamycin treatment enhanced autophagy and myogenic differentiation in aged stem cells (Takayama et al., 2017), while pretreatment with MIF rejuvenated MSCs in a state of age-induced senescence by interacting with CD74 and thereby activating 5′AMP-activated protein kinase-Forkhead box O3a signaling (Xia et al., 2015). Pharmacologic antagonism of lysophosphatidic acid, a ubiquitous metabolite in membrane phospholipid synthesis, extended the lifespan of MSCs in culture and increased their clonogenic potential while preserving their capacity for both osteogenic and adipogenic differentiation (Kanehira et al., 2012). However, as the effects of these approaches can vary–for instance, fibroblast growth factor supplementation resulted in the loss of osteogenic/adipocytic differentiation potential in long-term cultures (Gharibi and Hughes, 2012)–further research is needed to evaluate their long-term safety and efficacy.

CONCLUSION AND FUTURE DIRECTIONS

Mesenchymal stem cells senescence both *in vivo* and *in vitro* can affect MSC characteristics, which has important clinical and safety implications. MSCs must be expanded for several PDs to meet clinical dose requirements, but cellular aging significantly hinders the generation of sufficient numbers of cells. In this review, we discussed the properties of senescent MSCs and the functional changes and cellular mechanisms involved, and highlighted potential rejuvenation strategies. However, the current knowledge of senescence is mainly based on bulk-cell data. Recent technical advances such as single-cell RNA sequencing, extended time-lapse *in vivo* imaging, and genetic lineage tracing will provide a more complete understanding of the MSC aging process, making it possible to slow senescence or even rejuvenate aged MSCs. Additionally, bioinformatics-based analyses of the genome–environment interactions involved in aging can provide potential drug targets for senescence intervention. Given that functional attrition and reduced regenerative potential in stem cells are an important aspect of aging in organisms, MSC rejuvenation holds considerable promise for broadening the applications of MSC-based therapy.

Mesenchymal stem cells-based therapy has several limitations, including the invasive process of collecting the cells and their inherent immunogenicity, as well as the large numbers required to achieve a clinically relevant effect (Berebichez-Fridman and Montero-Olvera, 2018). An increasing number of preclinical trials have reported therapeutic effects exerted by MSC-MVs via paracrine mechanisms in several disease models (Fujita et al., 2018). Whether MSC-MVs are senescent and how senescent MSC-MVs can be identified are outstanding issues to be addressed in future studies.

AUTHOR CONTRIBUTIONS

JL and YD searched the literature and drafted part of the manuscript. XL and ZL designed the whole study and revised the manuscript.

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The Senolytic Drug Navitoclax (ABT-263) Causes Trabecular Bone Loss and Impaired Osteoprogenitor Function in Aged Mice

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Senescence is a cellular defense mechanism that helps cells prevent acquired damage, but chronic senescence, as in aging, can contribute to the development of age-related tissue dysfunction and disease. Previous studies clearly show that removal of senescent cells can help prevent tissue dysfunction and extend healthspan during aging. Senescence increases with age in the skeletal system, and selective depletion of senescent cells or inhibition of their senescence-associated secretory phenotype (SASP) has been reported to maintain or improve bone mass in aged mice. This suggests that promoting the selective removal of senescent cells, via the use of senolytic agents, can be beneficial in the treatment of aging-related bone loss and osteoporosis. Navitoclax (also known as ABT-263) is a chemotherapeutic drug reported to effectively clear senescent hematopoietic stem cells, muscle stem cells, and mesenchymal stromal cells in previous studies, but its *in vivo* effects on bone mass had not yet been reported. Therefore, the purpose of this study was to assess the effects of short-term navitoclax treatment on bone mass and osteoprogenitor function in old mice. Aged (24 month old) male and female mice were treated with navitoclax (50 mg/kg body mass daily) for 2 weeks. Surprisingly, despite decreasing senescent cell burden, navitoclax treatment decreased trabecular bone volume fraction in aged female and male mice (−60.1% females, −45.6% males), and BMSC-derived osteoblasts from the navitoclax treated mice were impaired in their ability to produce a mineralized matrix (−88% females, −83% males). Moreover, *in vitro* administration of navitoclax decreased BMSC colony formation and calcified matrix production by aged BMSC-derived osteoblasts, similar to effects seen with the primary BMSC from the animals treated *in vivo*. Navitoclax also significantly increased metrics of cytotoxicity in both male and female osteogenic cultures (+1.0 to +11.3 fold). Taken together, these results suggest a potentially harmful effect of navitoclax on skeletal-lineage cells that should be explored further to definitively assess navitoclax's potential (or risk) as a therapeutic agent for combatting age-related musculoskeletal dysfunction and bone loss.

Keywords: osteoblast, bone marrow stromal cell, skeleton, senescence, senolytic, osteoporosis

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INTRODUCTION

Senescence is a cellular defense mechanism that helps cells prevent acquired damage; acute senescence can be beneficial in processes related to wound healing, injury repair, and development, but chronic senescence, as in aging, can contribute to the development of age-related tissue dysfunction and disease (Van Deursen, 2014; Calcinotto et al., 2019). Characteristic aspects of senescence include proliferative arrest, changes in chromatin organization, and an altered secretome referred to as the senescence-associated secretory phenotype, or SASP (Coppe et al., 2008).

It has been clearly documented that removal of senescent cells can help prevent tissue dysfunction and extend healthspan during aging. For example, inducible elimination of $p16^{Ink4a}$-positive senescent cells delayed onset of age related pathologies and improved physical function in old (26–28 month) mice (Baker et al., 2011; Xu et al., 2018). Likewise, transplantation of senescent cells promoted physical dysfunction in young mice, whereas treatment of aged (20+ months old) mice with the senolytic drug combination of dasatinib + quercetin reduced senescent cell burden and improved physical function in aged mice (Xu et al., 2018). With specific regards to skeletal biology, expression of the $p16^{Ink4a}$ senescence marker increases with age in the skeletal niche (including bone marrow, osteoblast progenitors, osteoblasts, and osteocytes) (Farr et al., 2016). In particular, Osterix-expressing (Osx1+) osteoprogenitor cells decrease with age in mouse bone marrow, and the remaining Osx1+ cells in old mice show increased senescence (Kim et al., 2017). Critically, selective depletion of senescent cells via INK-ATTAC caspase 8 activation or inhibition of the SASP via JAK inhibitors reduced bone resorption activity and maintained trabecular bone mass in aged (20–22 month old) treated as compared to control mice (Farr et al., 2017). This suggests that promoting the selective removal of senescent cells, via the use of senolytic agents, can be beneficial in the treatment of aging-related bone loss.

Several compounds, including the dasatinib + quercetin senolytic drug combination mentioned above (Xu et al., 2018), have been tested to see the target effect on senescent cells and amelioration of aging or disease phenotypes (Grezella et al., 2018), with some even entering clinical trials (Hickson et al., 2019; Justice et al., 2019). However, many of these therapeutics have shown a high degree of cell and tissue specificity. Navitoclax (also known as ABT-263) is a chemotherapeutic drug whose role in senescent cells that was first described in 2016 (Chang et al., 2016; Zhu et al., 2016). It has been reported to target Bcl-2 family members including Bcl−2, Bcl−xl, and Bcl−w, promoting the apoptosis of senescent cells [which depend upon anti−apoptotic defenses similarly to cancer cells (Zhu et al., 2015)]. At least two reports suggest that unlike some of the more cell-type specific senolytic agents, navitoclax has a broad spectrum of activity across multiple human cell lines, although some disagreement exists (Chang et al., 2016; Zhu et al., 2016; Grezella et al., 2018). Regardless, navitoclax was previously reported to effectively clear senescent hematopoietic stem cells in the bone marrow and senescent muscle stem cells in the hindlimb of aged mice (Chang et al., 2016). In addition, navitoclax showed promise in depleting senescent cells from cultures of human and murine mesenchymal stromal cells (Kim et al., 2017; Grezella et al., 2018). However, navitoclax is also a chemotherapeutic agent, with reported toxic side effects including transient thrombocytopenia and neutropenia (Rudin et al., 2012; Kaefer et al., 2014), meaning its relative potential benefit *in vivo* as compared to risk factors was not yet known. Therefore, the purpose of this study was to assess the effects of short-term navitoclax treatment on bone mass and osteoprogenitor function in aged mice.

MATERIALS AND METHODS

Animals and *in vivo* Administration of Navitoclax

All experiments followed NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Augusta University. Male and female C57BL/6 mice (24 months of age, n = 10 females and n = 10 males) from the National Institute on Aging aged rodent colony were obtained for study. Mice were permitted water and standard rodent chow (RD: Teklad #2918) *ad libitum*, and were treated with the senolytic drug navitoclax (ABT-263, 50 mg/kg body mass, n = 5 per sex) or vehicle (10% ethanol, 30% PEG 400, 60% Phosal50, n = 5 per sex) once daily for 2 weeks by oral gavage. This dosage of navitoclax was previously shown to effectively clear senescent hematopoietic stem cells in the bone marrow and senescent muscle stem cells in the hindlimb of aged mice (Chang et al., 2016). At the conclusion of study, mice were sacrificed by carbon dioxide inhalation followed by thoracotomy. One femur per mouse was aseptically harvested for BMSC isolation as described previously (McGee-Lawrence et al., 2013). One tibia per mouse was fixed in 10% neutral buffered formalin, decalcified in 15% EDTA, paraffin embedded, histologically sectioned, and stained with hematoxylin and eosin for gross tissue visualization; one sample (female navitoclax treatment) was damaged during processing and was excluded from analysis. Trabecular bone volume fraction in the proximal tibia was quantified histologically (Dempster et al., 2013) using image analysis software (Bioquant Osteo, Nashville, TN, United States) to assess bone mass. Serial sections were treated with proteinase K and subjected to *in situ* TUNEL staining to detect apoptotic cells as previously described (McGee-Lawrence et al., 2013). Tissue sections were stained with a TUNEL detection kit (Roche #11 767 305 001, #11 767 291 910) according to the manufacturer's protocol, mounted with DAPI-containing medium (Vectashield), and imaged via confocal microscopy to assess apoptotic cells. The percentage of apoptotic (TUNEL+ nucleus) cells in the bone marrow, normalized to tissue area, was quantified at 200X total magnification for each mouse using image analysis software (Bioquant Osteo).

Isolation of Bone Marrow Stromal Cells (BMSC) From Treated Mice

Adherent bone marrow stromal cells (BMSC) from the navitoclax or vehicle-treated mice were isolated as previously

described (McGee-Lawrence et al., 2013). Briefly, bone marrow was flushed from the diaphysis, and BMSC were immediately seeded into 6-well plates (10 million cells per well) in osteogenic medium (alpha MEM + 20% FBS + 1% antibiotic/antimycotic + 50 μg/ml ascorbic acid + 10 mM beta glycerophosphate + 100 nM dexamethasone). Cells were cultured for 21 days prior to assessment of mineralized matrix production by alizarin red staining (n = 3 wells per group) as previously described (McGee-Lawrence et al., 2013). The fractional area of each well covered with alizarin red-stained calcified matrix was quantified with image analysis software (Bioquant Osteo, Nashville, TN, United States). Parallel cultures were harvested after 7 days of growth for semi-quantitative PCR-based analysis of gene expression (n = 3 wells per group; males only due to limitations in the number of cells obtained for female mice). Total RNA was extracted and purified from the cultures with TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Relative transcript levels of the early osteoblastic genes Runx2 (NM_009820, Forward 5-GGCACAGACAGAAGCTTGATGA-3′, Reverse 5-GAATGCGCCCTAAATCACTGA-3′) and Osterix/Sp7 (NM_130458, Forward 5′-GGAGGTTTCACTCCATTCCA-3′, Reverse 5′-TAGAAGGAGCAGGGGACAGA-3′) were quantified and normalized to expression of Gapdh (NM_008084, Forward 5′-GGGAAGCCCATCACCATCTT-3′, Reverse 5′-GCCTCACCCCATTTGATGTT-3′) as previously described (McGee-Lawrence et al., 2013). Reactions were performed using 37.5 ng of cDNA per 15 μl with QuantaBio Perfecta SYBR Green SuperMix (VWR) and the Bio-Rad CFX Connect Real Time PCR Detection System.

Isolation of Bone Marrow Stromal Cells (BMSC) for *in vitro* Navitoclax Treatment

To observe the direct effects of navitoclax on BMSC, primary BMSC were isolated from aged wildtype mice and treated with navitoclax (or vehicle) *in vitro*. An additional cohort of 24 month old male and female wildtype C57BL/6J mice was obtained from the NIA aged rodent colony (n = 5 per sex). In addition, for follow-up studies on TUNEL staining, a group of old female wildtype mice (27 months of age, n = 5) was culled from an in-house breeding colony [wildtype Osx1-Cre-negative Hdac3$^{fl/fl}$ mice (McGee-Lawrence et al., 2016, 2018)]. For both groups of mice, BMSC were isolated as described above and previously (McGee-Lawrence et al., 2013). Mice were sacrificed by carbon dioxide inhalation followed by thoracotomy, and long bones (femurs, tibias, humerii) were aseptically harvested. Bone marrow was flushed from the long bone diaphyses, and adherent BMSC were cultured as described in each assay below.

Colony Formation and Mineralized Matrix Production

Bone marrow stromal cells were seeded into 12 well plates (4 million cells per well) in growth medium (alpha MEM + 20% FBS + 1% antibiotic/antimycotic), or osteogenic cell culture medium (alpha MEM + 20% FBS + 1% antibiotic/antimycotic + 50 ug/mL ascorbic acid + 10 mM beta glycerophosphate) with or without dexamethasone. Dexamethasone is a common osteogenic additive which is required for osteogenic differentiation of human BMSCs (Beresford et al., 1994) and that is routinely included in osteoblast cultures from other species as well [although not strictly required for murine stromal cells (Lecka-Czernik et al., 1999)]. Dexamethasone was included in cultures to best replicate experiments conducted with BMSC isolated from the mice treated *in vivo*, but as glucocorticoids like dexamethasone can affect matrix mineralization, apoptosis, and mechanisms of lipid storage by osteoblasts (McGee-Lawrence et al., 2016; Nie et al., 2018; Deng et al., 2019), additional cultures were seeded in osteogenic medium with no dexamethasone to determine whether glucocorticoid inclusion affected the impact of navitoclax on the cells. Cells were grown for 21 days to promote colony formation as measured by crystal violet staining, or to promote formation of a mineralized extracellular matrix detectable by alizarin red staining (McGee-Lawrence et al., 2016). Navitoclax (5 μM) or vehicle (DMSO) was included in the culture medium for the entirety of the experiment. At the conclusion of studies, cells were fixed in 10% formalin and stained with crystal violet or 2% alizarin red, as described (McGee-Lawrence et al., 2013, 2016). The areal fraction of each well covered in crystal violet or alizarin red-stained calcified matrix was quantified with image analysis software (Bioquant Osteo; n = 3 wells per condition).

Senescence-Associated Beta Galactosidase (SA-β gal) Staining

Bone marrow stromal cells were seeded in 100 mm dishes (70 million cells per dish) in growth medium (alpha MEM + 20% FBS + 1% antibiotic/antimycotic) or osteogenic culture medium (growth medium + 50 ug/mL ascorbic acid + 10 mM beta glycerophosphate) with or without 100 nM dexamethasone and cultured for 10 days, after which cells were trypsinized, resuspended, and seeded into 12-well plates (100,000 cells per well). After overnight attachment, culture medium was changed to include 5 μM navitoclax or vehicle (DMSO) for 5 days. Cells were fixed in 10% formalin and stained to detect senescence-associated beta galactosidase activity with a commercial kit (Cell Signaling Technology Senescence β-Galactosidase Staining Kit #9860) as per the manufacturer's protocol to identify senescent cells (blue staining). Cells were imaged with a digital camera (Jenoptik) and inverted phase contrast microscope (Olympus IX-70), and the percentage of senescent cells normalized to total cell number for each condition was quantified from six random images per well and n = 6 wells per condition using image analysis software (Bioquant Osteo).

Cell Viability – MTT Assay

Bone marrow stromal cells were grown as described above for SA-β gal experiments and seeded into 96 well plates (20,000 cells per well). After overnight attachment, culture medium was changed to include 5 μM navitoclax or vehicle

(DMSO) for 5 days; a "no treatment" control (with no vehicle or navitoclax) was also included for calculation of relative cytotoxicity. Cellular metabolic activity was then assessed via measurement of conversion of water soluble MTT to insoluble formazan using a commercial MTT assay (abcam MTT Assay kit, #ab211091). Absorbance was measured at 590 nm in a multi-functional plate reader (Bio-Tek Cytation 5); 7 wells per group were averaged for each condition.

TUNEL Staining

Bone marrow stromal cells were grown as described above for SA-β gal experiments and seeded into 12-well plates onto sterile glass coverslips (100,000 cells per well). After overnight attachment, culture medium was changed to include 5 μM navitoclax or vehicle (DMSO) for either 24 h or 5 days. DNA damage was assessed via *in situ* TUNEL staining as previously described (McGee-Lawrence et al., 2013). Briefly, cells were fixed with 4% paraformaldehyde, lysed with PBS containing 0.1% TritonX-100 and 0.1% sodium citrate, and stained with an *in situ* TUNEL detection kit (Roche #11 767 305 001, #11 767 291 910). TUNEL stained coverslips were mounted with DAPI-containing medium (Vectashield) and examined via confocal microscopy to assess apoptotic cells. The percentage of apoptotic (TUNEL+ nucleus) cells, normalized to total cell number, for each condition was quantified from $n = 3$ random images per coverslip and $n = 2$ coverslips per condition using image analysis software (Bioquant Osteo).

Statistics

Statistical analyses were performed with JMP Pro 14.0.0 software (SAS Institute Inc., Cary, NC, United States). For qPCR data, fold changes were log transformed prior to analysis. Data were compared between groups within each experiment with Student's *t*-tests (when only two groups were compared) or 2-factor ANOVA with interaction (factor 1: sex, factor 2: drug treatment) with Tukey-Kramer HSD *post hoc* multiple comparisons tests when significant interactions were detected. A significance of $p < 0.05$ was used for all comparisons.

RESULTS

Navitoclax Administration Caused Trabecular Bone Loss *in vivo* and Impaired BMSC Function

It has been previously reported that selective removal of senescent cells was sufficient to maintain trabecular bone mass in aged mice (Farr et al., 2017). Surprisingly, 2 weeks of *in vivo* administration of navitoclax did not improve, and instead significantly decreased trabecular bone volume fraction in aged female and male mice (−60.1% females, −45.6% males) (**Figure 1A**). BMSC-derived osteoblasts from the navitoclax treated mice were impaired in their ability to produce a mineralized matrix (−88% females, −83% males), although pairwise comparison differences in females did not achieve statistical significance, likely because mineralized matrix was substantially lower overall in the BMSC cultures from females as compared to males (**Figures 1B,C**). BMSC-derived osteoblasts from the male navitoclax-treated mice demonstrated significantly lower expression levels of the osteoprogenitor gene Osterix/Sp7 (−63%), consistent with an impaired osteogenic phenotype (**Figure 1D**). TUNEL staining revealed increased TUNEL+ cells in the bone marrow of male and female navitoclax-treated mice (**Figures 1E,F**), consistent with navitoclax's previously reported pro-apoptotic effects (Zhu et al., 2016).

In vitro Treatment With Navitoclax Reduced Senescent Cell Burden, but Also Impaired Mineralized Matrix Production, Increased Cytotoxicity, and Increased Apoptosis

To test the direct effects of navitoclax on BMSC and osteoblastic function, we conducted mechanistic *in vitro* experiments. The senolytic effects of navitoclax were confirmed, as navitoclax generally reduced senescent cell burden, detected by the presence of SA-β gal staining, in BMSC and osteogenic cultures from both male (−49 to −73%) and female (−30 to −77%) cells (**Figure 2**). However, despite reducing senescent cell burden, BMSC from aged mice treated with navitoclax demonstrated a significant reduction (−89% female, −70% male) in colony formation as measured by crystal violet staining (**Figures 3A,B**). Similar to the effects seen in the primary cells from the mice treated *in vivo*, BMSC-derived osteoblasts treated with navitoclax *in vitro* were also impaired in their ability to produce a mineralized matrix under osteogenic culture conditions in the presence or absence of dexamethasone (**Figures 3C–F**), although pairwise comparisons did not reach statistical significance in females in the dexamethasone treated cultures, likely because mineralized matrix was substantially lower overall in the cultures from females as compared to males (**Figure 3F**).

With the goal of understanding why navitoclax impaired the growth and osteoblastic differentiation of BMSC and BMSC-derived osteoblasts, we conducted MTT assays to quantify relative cytotoxicity and TUNEL staining to quantify apoptosis. In MTT assays, navitoclax treatment did not significantly affect metrics of cytotoxicity in BMSC cultures ($p_{drug} = 0.110$, **Figure 4A**). In contrast, navitoclax significantly increased metrics of cytotoxicity in both male and female osteogenic cultures in the presence or absence of dexamethasone (+11.3 fold female osteogenic, +4.0 fold male osteogenic, +1.0 fold female osteogenic + dex, +3.6 fold male osteogenic + dex; **Figures 4B,C**). With regards to TUNEL staining, unfortunately, after BMSC and BMSC-derived osteoblasts were cultured on glass coverslips for 5 days in the presence of each treatment of interest, we detected no appreciable TUNEL staining in the cultures (data not shown). In a follow-up experiment, we harvested BMSC from aged female mice and subjected them to treatments of interest for the shorter timeframe of 24 h. These studies revealed that 24 h of navitoclax treatment significantly increased the fraction of TUNEL+ cells, indicative of apoptosis, in both BMSC (+117.8%) and osteogenic (+106.8%) cultures (**Figure 5**).

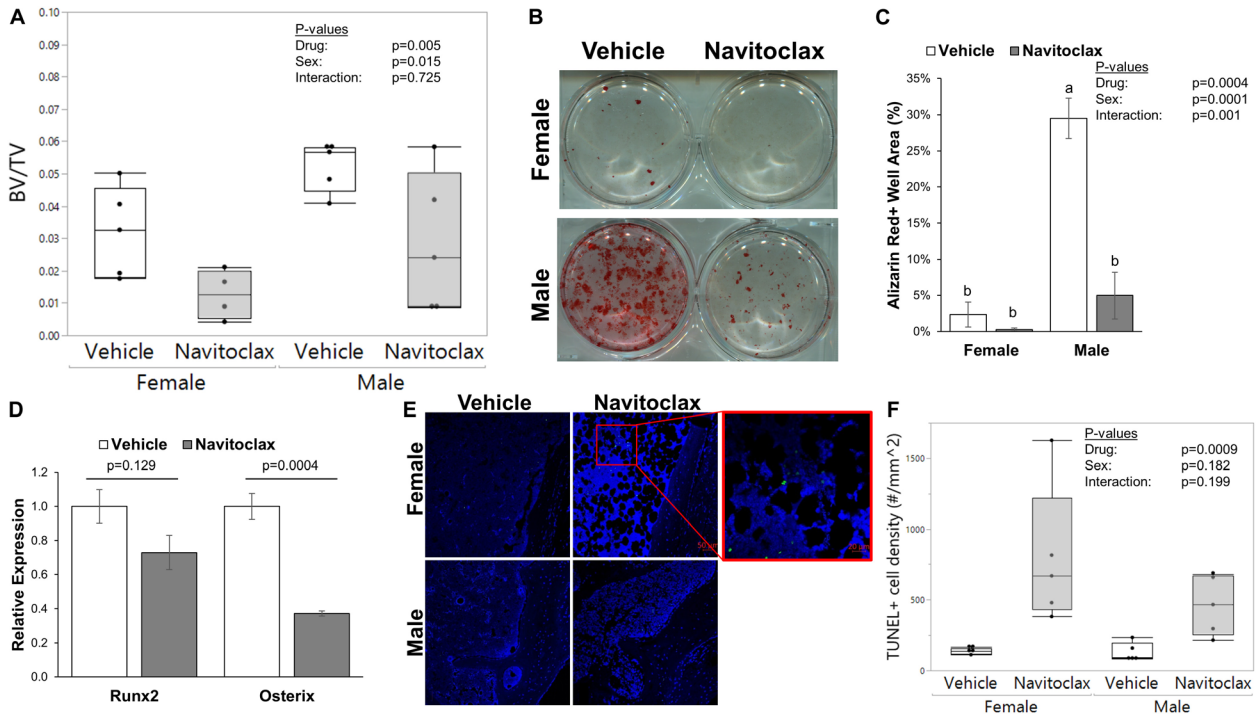

FIGURE 1 | Navitoclax administration caused trabecular bone loss and impaired osteoprogenitor differentiation. **(A)** Navitoclax (50 mg/kg body mass, once daily) was administered to 24 month-old male and female mice via oral gavage for 2 weeks. Trabecular bone volume fraction, measured histologically in the proximal tibia, was significantly decreased by navitoclax treatment. **(B,C)** Primary BMSC from the mice **(A)** were harvested at sacrifice and subjected to osteogenic culture conditions for 21 days. Cells from navitoclax-treated mice demonstrated reduced production of calcified matrix **(C)**. **(D)** BMSC were cultured under osteogenic conditions for 7 days; cells from navitoclax-treated mice demonstrated reduced expression of the osteoprogenitor gene Osterix/Sp7. **(E,F)** Frontal sections of the tibia from each mouse were stained with an *in situ* TUNEL detection kit to quantify apoptosis in the bone marrow; TUNEL+ cells were identified by green staining and normalized to tissue area. Original magnification: 200X; inset box shows the region magnified to 630X. Box plots show the median, quartiles, and outlier fences for each dataset, where outlier fences represent first quartile −1.5*(interquartile range) and third quartile +1.5*(interquartile range); each data point shown represents one mouse. *P*-values shown represent statistical comparisons between groups, *p*-value for "drug" in 2-factor ANOVAs represents the comparison between navitoclax (gray) and vehicle (white)-treated animals. A Tukey-Kramer HSD *post hoc* multiple comparisons test was performed when significant interactions were detected; bars with different superscript letters are significantly ($p < 0.05$) different from one another.

DISCUSSION

Senescent cells are not simply quiescent bystanders that accumulate during aging; rather, they instead have been described as "hyper-functional cells" (Blagosklonny, 2018) that can impair function of surrounding cell populations via release of secreted factors (SASP) and other mechanisms (Farr et al., 2016). Accordingly, senolytic therapies that target and remove these cells hold enormous promise for the treatment of age-related diseases, including osteoporosis (Baker et al., 2011; Chang et al., 2016; Farr et al., 2017; Grezella et al., 2018; Khosla et al., 2018; Geng et al., 2019; Kim and Kim, 2019). The senolytic agent navitoclax previously demonstrated efficacy in clearing senescent hematopoietic and skeletal muscle stem cells in mice, and importantly was shown to reduce senescence in both murine and human mesenchymal stromal cell populations (Chang et al., 2016; Zhu et al., 2016; Kim et al., 2017; Grezella et al., 2018). These observations suggested that navitoclax could hold promise as a treatment for age-related osteoporosis, but navitoclax's effects on bone had not yet been reported *in vivo*. This led to the current study, with the goal of determining whether navitoclax would reduce senescent cell burden in murine stromal cell populations and accordingly improve bone mass in aged mice. Surprisingly, the opposite effect was observed *in vivo*; aged (24-month-old) male and female mice treated with navitoclax for 2 weeks developed trabecular bone loss in the proximal tibia attributable at least in part to impaired osteoprogenitor function.

To understand the mechanism behind this phenomenon, we examined osteogenic differentiation patterns of BMSC harvested from the treated mice, and also conducted *in vitro* treatment studies to determine navitoclax's effects on metrics of apoptosis, cellular metabolic activity, and senescence in BMSC progenitors and in BMSC-derived osteoblasts. Osteogenic cultures were prepared with and without the addition of dexamethasone, a glucocorticoid that is commonly added to osteogenic cultures at low dosages (e.g., 100 nM) to enhance osteoblastic differentiation (Yamanouchi et al., 2001; Langenbach and Handschel, 2013; Yuasa et al., 2015), but can also affect osteoblastic cell metabolism and apoptosis particularly at higher concentrations (e.g., 1 μM) (Nie et al., 2018; Deng et al., 2019). In general, the effects of navitoclax were comparable between osteogenic cultures with and without dexamethasone (**Figures 2–4**). These

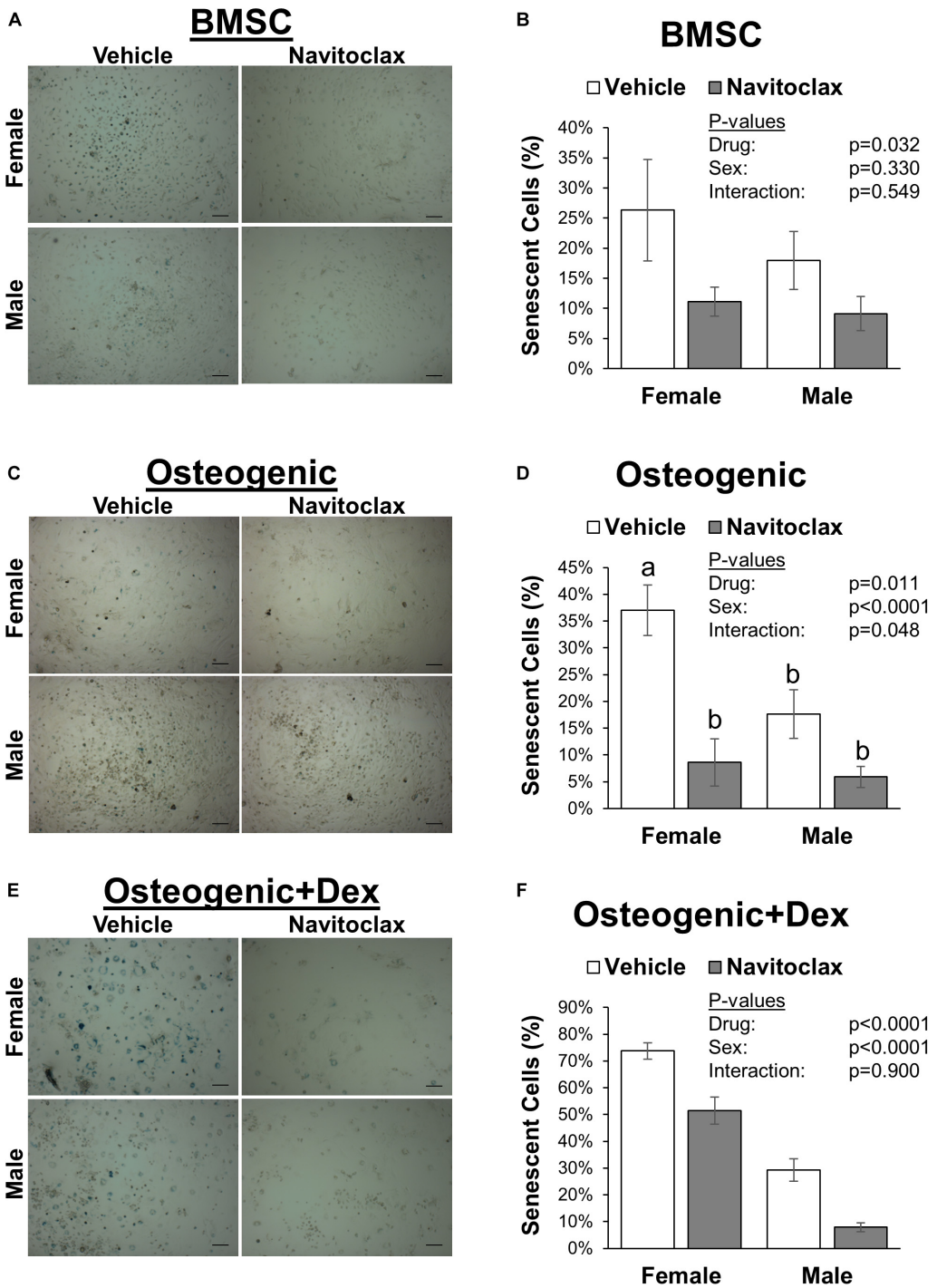

FIGURE 2 | Navitoclax decreased senescent cell burden in BMSC and osteogenic cultures. Primary BMSC were harvested from 24 month old male and female mice and treated with navitoclax (5 μM) or vehicle (DMSO) for 5 days in BMSC growth medium **(A,B)**, osteogenic medium **(C,D)**, or osteogenic medium with 100 nM dexamethasone **(E,F)**. Senescent cells were identified by blue staining and quantified relative to total cell number. Representative images for each condition are shown in **(A,C,E)**. Scale bar: 100 μm. *P*-values shown represent statistical comparisons between groups, *p*-value for "drug" in 2-factor ANOVAs represents the comparison between navitoclax (gray) and vehicle (white)-treated cultures.

in vitro studies revealed that while navitoclax was effective in targeting senescent BMSC and osteoblasts (**Figure 2**), it also drastically decreased BMSC colony formation and calcified matrix production by BMSC-derived osteoblasts (**Figure 3**), the latter of which was also observed in primary BMSC-derived osteoblast cultures from the mice that received navitoclax *in vivo*.

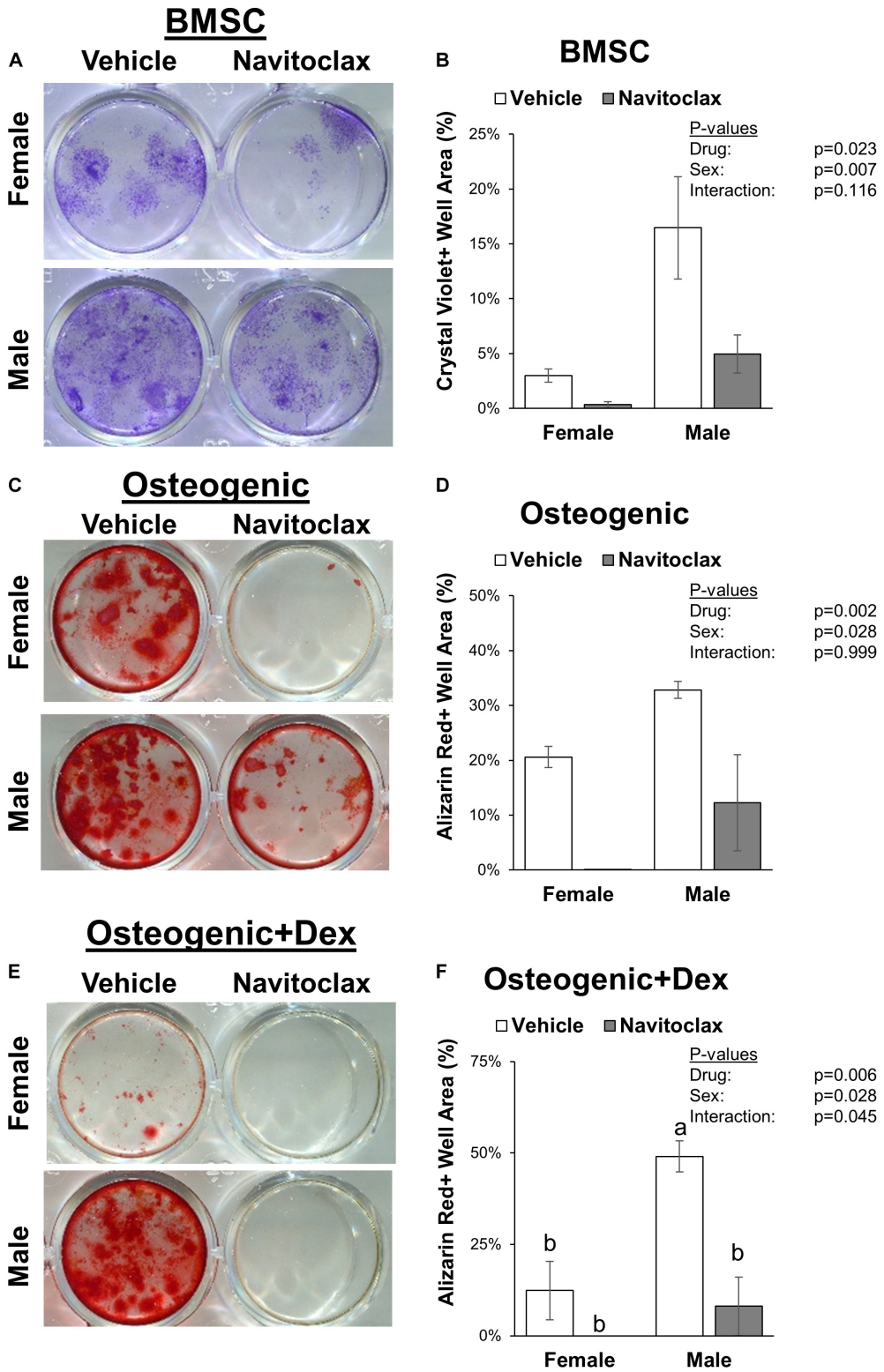

FIGURE 3 | Navitoclax impaired colony formation and mineralized matrix production. Primary BMSC were treated with navitoclax (5 μM) or vehicle (DMSO) for 21 days in BMSC growth medium **(A,B)**, osteogenic medium **(C,D)**, or osteogenic medium with 100 nM dexamethasone **(E,F)**. Colony growth and matrix production were quantified as the fractional area of the well with staining after 21 days. Representative wells for each condition are shown in **(A,C,E)**. *P*-values shown represent statistical comparisons between groups, *p*-value for "drug" in 2-factor ANOVAs represents the comparison between navitoclax (gray) and vehicle (white)-treated cultures.

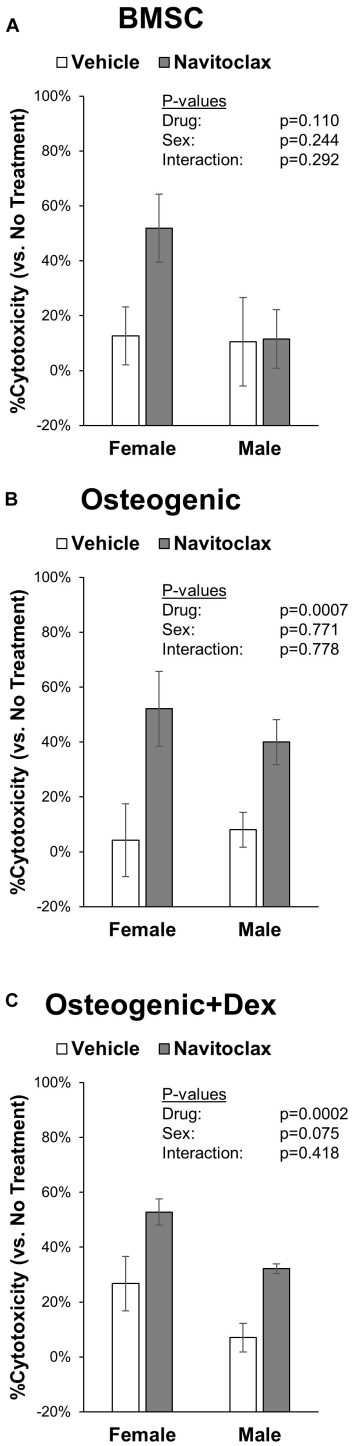

FIGURE 4 | Navitoclax has cytotoxic effects in osteogenic cultures. Primary BMSC were treated with navitoclax (5 μM) or vehicle (DMSO) in BMSC growth medium **(A)**, osteogenic medium **(B)**, or osteogenic medium with 100 nM dexamethasone **(C)**. Cellular metabolic activity was quantified in MTT assays, and cytotoxicity for each treatment was calculated relative to untreated cells for each condition *P*-values shown represent statistical comparisons between groups, *p*-value for "drug" in 2-factor ANOVAs represents the comparison between navitoclax (gray) and vehicle (white)-treated cultures.

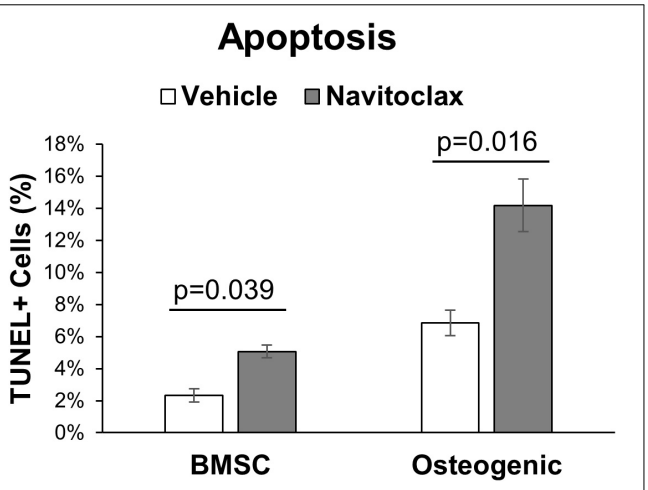

FIGURE 5 | Navitoclax promotes apoptosis in BMSC and osteogenic cultures. Primary BMSC from aged female wildtype mice (27 months old) were treated with navitoclax (5 μM) or vehicle (DMSO) for 24 h in BMSC growth medium or osteogenic medium. Cells were stained with an *in situ* TUNEL detection kit to quantify apoptosis in the cultures; TUNEL+ cells were identified by green staining and normalized to total cell number. *P*-values shown represent pairwise comparisons between navitoclax (gray) and vehicle (white)-treated cultures.

These effects are somewhat surprising, because navitoclax is an inhibitor of the Bcl-2 family, and previous studies have shown that Bcl-2-deficient mice demonstrated enhanced osteoblastic differentiation and increased trabecular bone mass at young ages (Moriishi et al., 2014), whereas increased expression of Bcl-2 in mice induced apoptosis of osteocytes and reduced osteoblast differentiation (Moriishi et al., 2011). However, it is important to note that Bcl-2 knockout mice also demonstrated impaired osteoblast proliferation (evidenced by fewer bromodeoxyuridine-positive osteoblasts *in vivo* and decreased proliferation of primary osteoblasts *in vitro*) and increased osteoblastic apoptosis (Moriishi et al., 2014), suggesting the potential for deleterious effects on bone if the mice were able to survive for longer durations. This would be consistent with a previous human report that protein and mRNA levels of Bcl-2 were reduced in osteoblasts isolated from post-menopausal women with osteoporosis as compared to controls; this same cell population from osteoporotic patients also showed decreased proliferation and increased apoptosis, consistent with trends seen in Bcl-2-deficient mice (Pang et al., 2018). As navitoclax decreased BMSC colony formation and increased TUNEL staining of BMSC and BMSC-derived osteoblasts in the current study, these potential effects on Bcl-2-mediated mechanisms warrant further consideration in future studies.

While the exact molecular mechanism behind these deleterious effects on BMSC and osteoblasts is not yet known, it is notable that navitoclax demonstrated substantial cytotoxic effects in our studies, particularly in osteoblastic cultures (**Figure 4**). The dosage of navitoclax chosen for our studies was selected because this was the dosage reported to effectively deplete senescent murine stromal cells *in vitro* (Kim et al., 2017).

Comparable doses did not appear to substantially impair survival of non-senescent IMR-90 myofibroblast cells, murine embryonic fibroblasts, or human preadipocytes (Zhu et al., 2016). Similarly, 5 μM navitoclax treatment for 72 h did not significantly affect MTT-based or trypan blue-based metrics of cell viability in non-senescent WI-38 human fibroblasts, although it did slightly but significantly decreased viability as measured by uptake of propidium iodide in the same cell line (Chang et al., 2016). Importantly, however, the *in vivo* dose selected for our studies was also chosen based on *in vivo* senolytic efficacy in a previous study with no reported side effects (Chang et al., 2016), whereas here we observed that this dose caused trabecular bone loss and impaired osteogenic differentiation of primary BMSC.

Many senolytic agents under investigation have shown a high degree of cell and tissue specificity. For example, navitoclax was previously reported to target senescent hematopoietic, mesenchymal, and muscle stem cells (Chang et al., 2016; Zhu et al., 2016; Grezella et al., 2018), but demonstrated minimal senolytic activity against primary human preadipocytes (Zhu et al., 2016). It is important to remember that senolytic agents like navitoclax are non-specific in nature; navitoclax, in particular, can act upon several different Bcl-2 family target proteins to promote apoptosis (Rudin et al., 2012; Souers et al., 2013; Zhu et al., 2016). It is possible that this promiscuity, or off-target effects, contributed to the negative effects we observed on bone *in vivo* and osteoblast cultures *in vitro*. Alternatively, there exists at least one report suggesting that the depletion of senescent cells *in vivo* could have detrimental consequences: promoting p16-driven senescence in beta cells of the pancreas improved glucose tolerance in diabetic mice, and both artificial and natural age-related increases in p16-driven senescence in pancreatic beta cells increased glucose-stimulated insulin production, suggesting that depletion of these cells (via senolytic targeting) could promote a diabetic phenotype (Helman et al., 2016; Blagosklonny, 2018). In either case, it is important to stress that the results obtained in the current study are specific to navitoclax, and should not be broadly inferred to relate to other senolytic therapeutics. In addition, we acknowledge that our endpoints in the current study were limited; for example, we did not conduct an in-depth investigation of bone mass across several skeletal sites (e.g., lumbar vertebrae, cortical bone density) in these mice, nor use more powerful techniques like micro-computed tomography to assess bone mass with high resolution. Therefore, these results should be considered preliminary, and should be confirmed by a more in-depth and extended study of the effects of navitoclax on the skeleton *in vivo*. However, the consistency observed between navitoclax's *in vivo* effects (trabecular bone loss and impaired BMSC-derived osteoblast differentiation and function) and *in vitro* effects (impaired BMSC colony formation and BMSC-derived osteoblast matrix production, impaired cellular metabolic activity in MTT assays for BMSC and BMSC-derived osteoblasts) do suggest a consistent and potentially harmful effect on skeletal-lineage cells that should be noted and explored further.

In conclusion, while navitoclax has demonstrated promise as a senolytic agent for the skeletal system via *in vitro* studies, the current work suggests that navitoclax's *in vivo* efficacy for treating age-related bone loss may be limited. It is important to emphasize that these small-scale murine studies reported here are only an initial glimpse into navitoclax's *in vivo* effects; larger-scale studies, in rodents and larger animal models, are needed to definitively assess navitoclax's potential as a therapeutic agent to combat age-related musculoskeletal dysfunction and bone loss.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at Augusta University.

AUTHOR CONTRIBUTIONS

All authors meet authorship criteria, and have read and approved the final submitted manuscript. MH and MM-L: study design. AS, RR, RB, JP, KY, and MM-L: data collection. AS and MM-L: initial manuscript preparation. AS, MH, and MM-L: final manuscript preparation.

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REVIEW
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Mesenchymal Stem Cell Senescence and Rejuvenation: Current Status and Challenges

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Over the past decades, mesenchymal stem cell (MSC)-based therapy has been intensively investigated and shown promising results in the treatment of various diseases due to their easy isolation, multiple lineage differentiation potential and immunomodulatory effects. To date, hundreds of phase I and II clinical trials using MSCs have been completed and many are ongoing. Accumulating evidence has shown that transplanted allogeneic MSCs lose their beneficial effects due to immunorejection. Nevertheless, the function of autologous MSCs is adversely affected by age, a process termed senescence, thus limiting their therapeutic potential. Despite great advances in knowledge, the potential mechanisms underlying MSC senescence are not entirely clear. Understanding the molecular mechanisms that contribute to MSC senescence is crucial when exploring novel strategies to rejuvenate senescent MSCs. In this review, we aim to provide an overview of the biological features of senescent MSCs and the recent progress made regarding the underlying mechanisms including epigenetic changes, autophagy, mitochondrial dysfunction and telomere shortening. We also summarize the current approaches to rejuvenate senescent MSCs including gene modification and pretreatment strategies. Collectively, rejuvenation of senescent MSCs is a promising strategy to enhance the efficacy of autologous MSC-based therapy, especially in elderly patients.

Keywords: mesenchymal stem cells, senescence, autophagy, mitochondrial, telomere, rejuvenation

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INTRODUCTION

Mesenchymal stem cells (MSCs) are fibroblast-like and plastic adherent with a self-renewal ability and multiple differentiation potential. Previous studies have shown that MSCs had been successfully established from bone marrow, umbilical cord blood, periosteum, and adipose tissue (Musina, 2005; Paniushin, 2006). MSCs have the property of self-renewal and differentiate into multiple cell lineages, such as bone, cartilage, adipose, muscle, tendon, stroma, and neuronal cells (Mareschi, 2006). They are widely used as seed cells for therapeutic applications in tissue engineering and regenerative medicine (Mahla, 2016). Their availability and low immunogenicity hold extensive promise for clinical application (Uccelli, 2008; Madrigal, 2014; Childs, 2018). MSCs have been

broadly applied in the treatment of various diseases, including graft-vs.-host disease (GVHD) (Landgraf, 2011), Crohn's disease (CD) (Bernardo, 2009; Zhang, 2018), diabetes mellitus (DM) (Al Demour, 2018), multiple sclerosis (MS) (Iacobaeus, 2019) and myocardial infarction (MI) (Lunde, 2006; Gyongyosi, 2015) etc. Nonetheless accumulating data have demonstrated that discrepancy in the effects of MSC-based therapy may be due to senescence-induced alterations in their function (Schimke, 2015). The neurorestorative potential of MSCs may be limited in aged patients with stroke who have a limited number of MSCs (Lee, 2010; Bang, 2016). Allogeneic MSCs, chosen as the first choice for elderly population with frailty syndrome in a phase I/II clinical trial, avoids the aging-related aberrant microenvironments of MSCs and inflamed-aging (Golpanian, 2016). The senescent features of MSCs include enlarged and more granular morphology, and a deficient capacity for proliferation (Haynesworth, 1997) and differentiation, and secretion of a variety molecules, referred to as a "senescence-associated secretory phenotype (SASP)" (Watanabe, 2017). In addition, senescence is accompanied by changes to nuclear morphology and formation of a distinct chromatin structure called senescence-associated heterochromatic foci (SAHF) (Noren, 2017). Currently, the senescent states of MSCs have been assessed by measuring senescence-associated-β-galactosidase activity, telomere length, gene expression markers, gene methylation and epigenetic markers (Jones, 2019). The mechanisms underlying MSC senescence have attracted attention since senescent MSCs hamper the rapid development of MSC grafting. Numerous studies have focused on these abnormal changes to MSC morphology and function, cells previously considered to be immortal. The mechanisms that underlie these processes remain unclear.

Senescent MSC are normally divided into different stages. After each replication cycle, the length of telomeres is shortened. Once telomeres become critically short, they trigger senescence. This is called replicative senescence (Ho, 2017). Following this, activation of oncogenes induces MSC senescence. This is termed oncogene-induced senescence (Kosar, 2011). Numerous stress stimuli also trigger senescence, known as stress-induced senescence (Kornienko, 2019). Induction of senescence can be mediated as part of the normal development process by several pathways or pluripotency genes. This is referred to as developmental senescence (Liu, 2015). The restricted therapeutic application of senescent MSCs highlights the importance of rescuing the functions of MSCs, namely rejuvenating MSCs, so they can be used for autologous transplantation. Rescuing the functions of MSCs is vital for their regeneration capacity (Block, 2017). Recent research suggests that cellular senescence is a modifiable risk factor, giving hope for autologous MSCs-based therapy (Stolzing, 2008). *In vitro* culture is essential to acquire an adequate number of MSCs for use in cell therapy. In parallel to this, targeting three intrinsic mechanisms of MSC senescence may help hinder MSC aging. In this review, we focus on the mechanisms that underlie MSC senescence including DNA damage, telomere erosion and mitochondrial dysfunction. We also summarize the current strategies being applied to rejuvenate senescent MSCs and enhance their therapeutic efficacy.

CHARACTERISTICS OF MSC SENESCENCE

Cellular senescence is defined as a state of permanent cell cycle arrest. Cell cycling is halted and cells no longer replicate and/or divide. In senescent MSCs this results in deficient proliferation and differentiation as well as changes to protein expression and chromosome structure. Senescent MSCs usually show an enlarged, more granular and flat fried egg morphology, with constrained nuclei and granular cytoplasm. They also exhibit a decreased cell colony number (CFU), one of the most convenient predictive indicators of MSC senescence (Stolzing, 2008). In addition, the cell population doubling time (CPDT) is prolonged. This may be due to a prolonged G1/G0 phase of the cell cycle and a significantly decreased S phase (Gaur, 2019).

DNA staining of senescent cells has revealed nuclei with small and distinct spots that contain heterochromatin, called senescence-associated heterochromatic foci (SAHF) (Kosar, 2011). Each spot represents condensed chromatin that is transcriptionally inactive, and expression of some transcription factors around this region have been found to be downregulated, such as E2F family members and cyclin A (Narita, 2003). SAHF can be identified by DAPI staining and the presence of heterochromatin-associated histone markers, and high levels of H3K9me3 and H3K27me3 (Koch, 2013). As inhibitory markers, an increase of H3K9me3 and H3K27me3 in gene promotor leads to decreased gene expression. Formation of SAHF is a complex process. Researchers are particularly interested in how genes are regulated and their expression affected during formation of SAHF.

Epigenetic regulation is always involved in histone modification and cellular senescence can be tracked by epigenetic modifications (Wagner, 2019). DNA methylation is the most promising marker to predict MSC senescence (Wagner, 2017). Age-associated hypomethylation occurs in heterochromatic regions of the genome, interfering with transcription factors such as repetitive elements and transposons or methylated-CpG binding proteins, and leading to silencing of the gene (Easwaran, 2019). Multiple age-related genes decrease during senescence, such as lysine specific demethylases (KDM3a-b, KDM5d, and KDM6a-b) (Gronthos and Cakouros, 2019). During the gradual process of MSC senescence, DNMT1 and DNMT3B have been shown to be downregulated with a consequent decrease in DNA methylation (Childs, 2018). These changes are not universal but occur only with specific genes and histone modifications. Senescence-associated DNA-methylation (SA-DNAm) may therefore be used to monitor cellular senescence (Koch, 2013). In addition, the expression of stemness-associated genes such as Oct4, Nanog and Tert, decreases during MSC senescence. With chromatin immunoprecipitation and whole genome sequencing (ChIP-seq), large samples can be sequenced and the epigenome scanned to map the epigenetic landscape and enable detection of cellular senescence. Multiple proteins that typically change may

serve as indicators of senescence. Such changes may be tested in blood and measures taken to prevent aging.

MSCs are known to have differentiation potential for osteogenesis and adipogenesis. This ability is altered in senescent MSCs that are more likely to differentiate toward adipogenesis (Andrzejewska, 2019). Bone-formation markers, such as the activity of alkaline phosphatase (ALP) and the expression of osteocalcin (OC), are downregulated in senescent MSCs during culture with osteogenic medium (Abuna, 2016). This change to MSC differentiation greatly limits their application. It is important to maintain their self-renewal ability and multiple differentiation potential.

Senescent cells tend to potentiate their effects to neighboring cells via paracrine mechanisms. This is known as a senescence-associated secretory phenotype (SASP) (Debacq-Chainiaux, 2009; Sikora, 2016). The SASP factors include interleukin-1 (IL-1), IL-6, IL8, matrix metalloproteinase1 (MMP1), TNF-α and vascular endothelial growth factor (VEGF) and so on (Rodier and Campisi, 2011). Senescent cells can exert certain influence on their microenvironment by their secretome. Microvesicles (MVs), is a key component of the cell secretome, can inhibit the growth of tumor and immunomodulatory regulation (Akyurekli, 2015; Xie, 2016).

MSCs accomplish their functions through the secretion of cytokines and growth factors, which exert paracrine and autocrine functions (Ranganath, 2012). MSCs-derived exosomes (MSCs-EXOs) contain biological active molecules from the MSCs, which can regulate immune responses in the body. Exosomes of MSCs contain cytokines, growth factors, various Mrna, and regulatory miRNA. But senescence greatly alters the composition of them, the micro RNAs in exosomes were largely different. Senescence greatly alters the composition of this secretome and hence impairs one of the key MSC biological functions (Özcan, 2015). A SASP is always evident in oncogene-induced senescence (OIS) and often accompanied by a global change in nuclear architecture. A broad spectrum of secretory factors produced by MSCs, such as cytokines and chemotactic and growth factors, has been studied (Alexander, 2013; Lei, 2017). They sequenced and analyzed SASP of different aging cell types and focus on 138 common canonical pathways. Putting them into four categories, extracellular matrix/cytoskeleton/cell junctions; metabolic processes; ox-redox factors; and regulators of gene expression. What should be noted is that modification of the extracellular environment is one of the main tasks of the senescence secretome and leucocyte extravasation signaling" as an overlapping network that is common among the different senescence secretome. Further research find that there are 11 proteins emerged in senescent MSCs only (Özcan, 2016). The amount of components released from SASP may partly depend on the different types of cell senescence and the microenvironment. It appears that senescent cells are prevented from becoming tumorigenic by switching on SASP (Özcan, 2016). Nonetheless SASP is thought to be partially responsible for the persistent chronic inflammation that contributes to multiple age-related phenotypes (Reitinger, 2015). Changes in functionality can lead to unexpected situations: inflammation may alter tissue microenvironments and attract immune cells, leading to tissue and organ damage and contributing to aging making treatment difficult.

Telomere shortening is believed to be a hallmark of MSC senescence, limiting long-term MSC division that is essential for tissue renewal. For this reason, telomere attrition is defined as a type of DNA damage for cells, also as a response to DNA damage that finally leads to cell cycle arrest and cell senescence. Telomerase, a type of enzyme that brings repeated TTAGGG to the chromosome end, prevents telomere attrition and induces telomere elongation. Overexpression of the enzymatic subunit of telomerase, telomerase reverse transcriptase (TERT), increases median lifespan in mice (Patel, 2016). The ability to detect telomere length may thus hold promise as a biomarker in the assessment of MSC senescence. Valid and reliable techniques to quantify telomere length have attracted much attention in the study of senescence. The use of telomere-based tests for the diagnosis and management of cellular senescence are well-established (Baxter, 2004).

DIFFERENT TYPES OF MSC SENESCENCE

Normal animal cells undergo senescence after multiple divisions *in vivo* and *in vitro*, and senescent cells will eventually die. Although MSCs have a strong ability to proliferate, they are not infinite. After multiple divisions, cells enter a state of replicating senescence with growth arrest. Studies show that MSCs isolated from elderly individuals have lower proliferation and anti-apoptosis ability than those isolated from young individuals. This is usually referred to as developmental senescence. When stimulated by oxidative stress, MSCs will begin the aging process early, that is, premature aging. This premature senescence may be classified as oncogene-induced senescence or stress-induced senescence. Based on recent published data, we briefly describe the different types of senescence (**Figure 1**).

REPLICATIVE SENESCENCE

Replicative senescence is defined as an irreversibly restricted proliferation due to telomere erosion in MSCs after a stereotypical number of cell divisions. Nonetheless replicative senescence is also intimately connected to other types of senescence including oncogene-induced (p53 and p16/Rb tumor suppressor pathways etc.), stress-induced (oxidative stress etc.) and even developmental senescence. Ultimately though they are all associated with telomere shortening and consequent replicative senescence. MSCs from elderly individuals recapitulate most parameters seen in senescent MSCs, including a flat, enlarged morphology, a great number of cells staining positive for SA-β-Gal, and lower proliferation rate. These characters have fueled the perception that replicative senescence *in vitro* may serve as a candidate model to unravel the molecular mechanisms that drive the process of body aging. Replicative senescence of MSCs is a continuous process

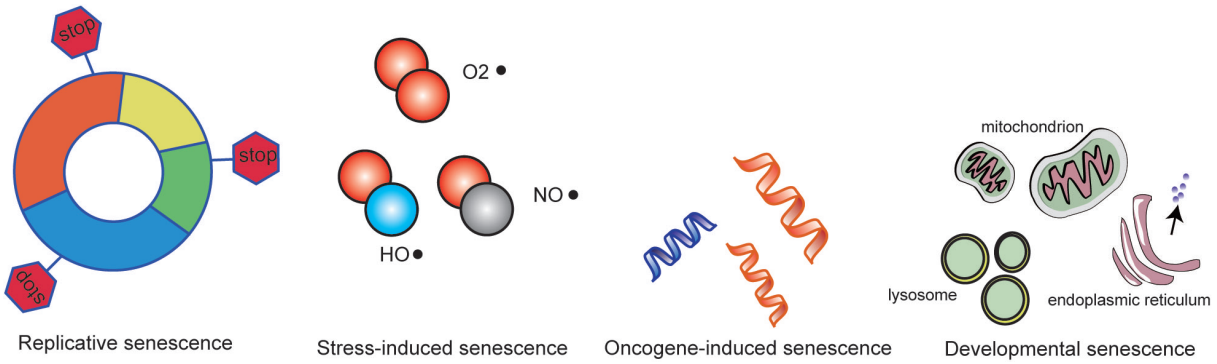

FIGURE 1 | Different types of MSCs senescence.

starting from the first passage and there is a dynamic change to senescence-related indicators. Long-term alterations to phenotype, differentiation potential, whole-map gene expression patterns and miRNA profiles are influenced by replicative senescence and all need to be considered as therapeutic targets for MSC rejuvenation (Ho, 2017).

ONCOGENE-INDUCED SENESCENCE

Oncogene-induced senescence (OIS) depends on activation and/or overexpression of oncogenes, such as cyclin E, RAF, MEK, and BRAF. Oncogenic activation has been recognized as a necessary step in tumorigenesis but may also act as a genetic stress and cause irreversible growth arrest in cultured cells. Tumor suppressor genes p16 and p21 play an important role in monitoring the normal integrity of DNA. Senescence of MSCs has been shown to be reversed by ablation of p16 or p21 (Chikenji, 2019). The protein level of p16 or p21 may indicate the parallel level of MSC senescence. For example, knockdown of p16 or p21 in senescent MSCs has been shown to increase their proliferation rate and differential potential (Mas-Bargues, 2017). Signaling pathways, not only individual genes, are always involved in senescence. The mitogen-activated protein kinase (MAPK) pathway can be activated by Ras and plays a role. Two major tumor suppressor pathways, the p14ARF-MDM2-p53 pathway and p16INK4A/pRb pathway, have been shown to be involved in the control of permanent MSC senescence (Clarke, 2004; Liu, 2015; Piccinato, 2015). OIS is often accompanied by a global change in nuclear architecture, most dramatically exemplified by the formation of SAHF. As previously mentioned, heterochromatin-associated histone markers, DNA methylation in particular, is present in SAHF. Gene expression can be regulated by DNA methylation through interference with transcription factors or methyl-CpG binding proteins (Jaenisch and Bird, 2003). Abnormal regulation of methylation will lead to the disorder of replication in cell, which resulting in DNA replication errors, thereby induce cell apoptosis.

In contrast to hyper-methylation that suppresses the translation of genes, hypomethylation enables genes to be "released" and start replication and translation. These abnormally expressed proteins trigger an intracellular response, much like hyperexpression of Ras in mammal MSCs triggers activation of tumor suppression pathways, and thus induces irreversible growth arrest (Moumtzi, 2010). In the presence of the hyperproliferative signals during the process of senescence, cells encounter a strong DNA replication stress and finally develop numerous double-stranded DNA breaks (DSBs) in fragile areas of DNA (Hladik, 2019). The damaged DNA released from the nucleus may activate inflammatory pathways and eventually lead to apoptosis. On the contrary, the accumulation of tumor suppressor gene products caused by the abnormal mitosis suggests that OIS is an anti-tumor reaction that can ensure cell proliferation within an allowable range.

STRESS-INDUCED SENESCENCE

Stress-induced premature senescence (SIPS) occurs as a result of many different stimulations including reactive oxygen species (ROS), ionizing radiation, osmotic stress, mechanical stress, hypoxia, and heat shock (Zglinicki, 2000). There are numerous cellular and molecular features that are similar for cells with SIPS and those undergoing replicative senescence although they occur at the stages of senescence. The mechanisms that underlie SIPS, especially ROS production by damaged mitochondria, involve many signaling pathways. ROS is an important factor during senescence and has been extensively studied. Indeed, our previous studies also showed that ROS plays a critical role in regulating MSC senescence (Huang, 2019; Li, 2019). An imbalance of ROS and anti-oxidants such as superoxide dismutase (SOD) in senescent MSCs initiates growth arrest, regulated by intricate networks of molecular signaling pathways (Bi, 2018). FOXO, whose subfamily (FOXO1, FOXO13a, FOXO14, and FOXO16) is the downstream target of the PI3K-AKT signaling pathway, is another molecule that regulates the ROS pathway during cellular senescence (Fukada, 2014). The p53/p21 pathways and p38MAPK pathways are responsible for the irreversible cell cycle arrest that occurs when MSCs are exposed to ROS, although inhibition of the p38MAPK pathway can restore cell proliferation. Thus controlling ROS may directly alleviate cell senescence.

As another key cause of senescence, DNA damage also plays an important role in activating the p53 pathway to cause cell cycle arrest (Pelicci, 2004). Multiple factors are involved in the repair of damaged DNA. During the replication or repair of damaged DNA, any small accidents can result in large changes: gene editing *in vitro* is one example of a means by which to alter cell phenotype. Various transcription factors including P53 are also recruited by autophagy related protein (ATG), and strengthen the autophagy when stimulated. Aging as a consequence of autophagy has been linked to cellular senescence and autophagy is recognized as a sensor of stress, similar to oxidative stress. Studies have described a decline in autophagy activity and a reduction of autophagy related genes such as Atg1, Atg5, and Atg12 in response to cellular senescence (Fafian-Labora, 2019). The autophagy response is a useful weapon for cells, but the imbalance in autophagy is a threat to their survival.

On the other hand, proteasomes are inhibited by severe oxidative stress. Damage to the proteasome leads to aberrant folding of proteins, toxic aggregation, and accumulation of damaged proteins, further promoting cell senescence. Misfolding or false modification of proteins may cause altered function that in turn leads to abnormal regulation of genes. The synthesis, modification and explanation of proteins has always been a popular subject of research. Due to the strict requirements of the physiological environment for proteins *in vitro*, it is difficult to replicate this *in vivo* synthesis and this is a major obstacle for research.

DEVELOPMENTAL SENESCENCE

Senescence can be induced by regulation of multiple pathways or pluripotent genes in non-pathological states and is a part of normal cellular development. Three signal pathways, insulin-like signaling pathway, target of rapamycin and Sirtuins/NAD +, have been shown to play a major role in MSC senescence (Severino et al., 2013; Gharibi, 2014; Oh, 2014; Chen, 2017). Interestingly, they are all intimately related to metabolism. IGF-1 and insulin signaling, named the "insulin and IGF-1 signaling pathway" (IIS signaling), is a highly conserved signaling pathway that controls aging (Chen, 2016, 2017). Current evidence indicates that IIS signaling plays a key role in regulating aging and longevity (Campisi, 2019). In mice, selective disruption of insulin receptors in adipose tissue extended longevity. Increased lifespan has also been reported in mice with deletion of insulin receptor substrate 1 (IRS1) in whole body or IRS2 only in the brain. Although dietary restriction promotes the proliferation of MSCs, the underlying mechanism may be linked to the pathway.

The mTOR pathway that comprises mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) promotes substance metabolism, participates in cell apoptosis and autophagy, and plays an important role in many diseases. The mTORC1 signaling pathway integrates at least five major intracellular and extracellular signals – growth factors, stress, energy states, oxygen supply, and amino acids – to control processes such as protein-lipid synthesis and autophagy. Studies have shown that the mTORC1 signaling pathway exhibits a pattern of diurnal oscillation. Per2, the core clock protein, can specifically bind to mTORC1 and recruit Tsc1 to mTORC1 as a scaffold protein, thus specifically inhibiting the activity of mTORC1. Activation of mTORC1 is highly associated with a calcifying phenotype of MSCs. Transition from stemness one to osteoblast remarks possibly cellular senescence in MSCs. However, reciprocal activation of mTORC2 protects MSCs from calcification to promotes protective cell fates (Gharibi, 2014; Zhang, 2017; Yang, 2018; Schaub, 2019; Wu, 2019).

Sirt1 (Sirtuin type1), a member of the Sirtuins family, is a histone deacetylase that is dependent on nicotinamide adenine dinucleotide (NAD +), and deacetylation of several transcription factors that control metabolic and endocrine signals regulates its activity *in vivo* (Yuan, 2012; Pi, 2019). It is widely involved in the regulation of mammalian cell life signals, glucose metabolism, insulin secretion and other metabolic pathways, and plays an important role in metabolic syndrome, cell apoptosis, cardiovascular diseases and neurodegenerative diseases. Sirt1 has been increasingly valued as a therapeutic target for many diseases. Reduced expression of Sirt1 impairs the adipocyte differentiation ability of MSCs (Khanh, 2018); overexpression of Sirt1 reduces the acetylation of Bmi1, which is tightly correlated with MSCs osteogenic ability (Wang, 2019).

As relatively complex and powerful signaling pathways, these three pathways play an important role in cell development. Research on them ongoing, and more mechanisms will be discovered that may extend cell life or slow cell aging.

MECHANISMS OF MSC SENESCENCE

The mechanisms involved in different types of aging are not entirely consistent, but most are interrelated and interact with each other. These four mechanisms, particularly DNA damage, mitochondria and autophagy, are closely related and play a crucial role in stem cell senescence (Li, 2017). The following is a brief discussion based on the available data.

DNA DAMAGE

DNA, as the most important genetic material of an organism, can maintain its own stability. DNA damage can accelerate cell senescence and apoptosis, and cause diseases such as cancer and tumors. MSCs are prone to DNA damage during proliferation. When DNA damage reaches a certain level, abnormal cell cycling may ensue (Hladik, 2019). Testing with antibodies that recognize the phosphorylated form of histone H2AX (γH2AX) (Kozlowski, 2015), a histone variant of the H2A protein family phosphorylated rapidly following DNA damage, has been used to assess DNA damage (Gaur, 2019).

Oxidative stress is considered the main cause of DNA damage and aging, and the occurrence of cellular senescence is closely related to reactive oxygen species (ROS). Data show that sublethal ROS and ionizing radiation can cause DNA damage to MSCs derived from human umbilical cord. The increased

intracellular ROS is an important cause of senescence of bone marrow-MSCs (BM-MSCs) that show reduced DNA synthesis and cell proliferation and consequent cell senescence (Chen, 2019). Cells cultured *in vitro* in a high oxygen environment show accumulation of ROS in cells with consequent activation of the stress signaling pathway and cell senescence due to oxidative stress (Infante and Rodríguez, 2018). ROS accumulates during normal cell metabolism and a low concentration is essential for cell proliferation and differentiation. Nonetheless a high level of ROS is produced in pathological conditions. High concentrations of ROS have a strong cytotoxic effect and induce cell damage (Kozlowski, 2015). Studies have shown that ROS is significantly increased in apoptotic cells compared with normal cells. The increase in ROS production by senescent MSCs results in excessive ROS or exogenous H_2O_2 that can impair proliferation and differentiation of MSCs (Jeong and Cho, 2015). Increased ROS can induce MSC senescence which can be partially reversed by N-acetylcysteine, an oxygen scavenger, with consequent reduction in DNA damage (Zhang, 2013). Thus, increasing the activity of DNA repair pathways may aid recovery of senescent MSCs.

TELOMERE EROSION

The telomere is a special structure located at the end of linear chromosomes in eukaryotic cells. Each time the DNA replicates, the telomere is shortened. Telomere shortening is one of the endogenous changes that occur in MSCs during aging. As MSCs passage, telomeres will gradually shorten. When telomeres are shortened such that DNA replication can no longer continue and chromosomal stability cannot be guaranteed, senescence will ensue (Montpetit, 2014; Lai, 2018). Telomere length is mainly maintained by telomerase. Inhibiting the expression of SIRT1 in liver cancer cells has been shown to decrease the expression of telomerase and cause the telomere to shorten with consequent induction of cell senescence or apoptosis (Yamashita, 2012). Disorders of telomere function can also occur in some diseases. Bone marrow mesenchymal stem cells (BMSCs) derived from congenital dyskeratosis show reduced colony formation and differentiate into adipocytes and fibroblasts spontaneously, and show signs of senescence. Gene-related telomere mutations that cause shortening of telomeres are the main cause of this disease (Nadeau, 2019). Nonetheless other studies have reported that knockout of SIRT1 in BMSCs resulted in slower cell growth and accelerated cell senescence, while overexpression of SIRT1 delayed the senescence and maintained the potential for osteogenic and lipogenic differentiation (Chen, 2014). Additionally, overexpression of human telomerase reverse transcriptase (hTERT) can activate telomerase activity and maintain telomere length. Data showed less damage due to external oxidative insult in the nuclei of hTERT-overexpressing cells compared with the control cells (Trachana, 2017).

Nonetheless the level of telomerase in cells is almost undetectable, and overexpression of telomerase can prolong telomeres. It is also unknown whether the introduction of viral plasmids will cause safety issues. Moreover, the large-scale telomere prolongation will cause some cells to lose control with a subsequent risk of tumorigenesis. Therefore, targeted regulation of telomeres in specific cells is also a prospect for future therapies, similar to CAR-T treatment in leukemia. Scientists are also trying to explore ways to reprogram *in vivo*, to ensure safer treatment.

MITOCHONDRIAL DYSFUNCTION

Mitochondria are central to cellular respiration and involved in various cellular activities such as cell matrix metabolism, apoptosis, and initiation of signal transduction pathways. Reductions in mitochondrial function and consequent respiratory chain dysfunction have been observed in senescent MSCs (Lonergan, 2006). Under normal circumstances, mitochondrial fission produces small round mitochondria and generates chain-like mitochondrial tubules. Once suffered from external serious attack, mitochondrial fission will occur and dysfunctional mitochondrion will be cleared by mitochondrial autophagy. Defects in mitochondrial function such as reduced membrane potential, open mitochondria permeability transition pore (mPTP), or increased oxidative stress will eventually lead to apoptosis or cell death. The disturbed mitochondrial dynamics that occurs in cellular senescence affects morphology of MSCs (Herranz and Gil, 2018). In replicative senescence, MSCs enter a normal senescent stage with elongated mitochondrion and damaged function. MSCs that suffer an external serious attack have discrete, fragmentary mitochondrion.

The accumulation of ROS in mitochondria is the main cause of mitochondrial dysfunction. In turn, damaged mitochondria produce more ROS. Mitochondrial oxygen consumption decreases in the later passage of MSCs indicating that cell senescence depends on the accumulation of ROS. At the same time, ROS is an important factor that affects MSC senescence (Sahin and Depinho, 2010; Ghanta, 2017). Mitochondria, as the energy centers in cells, are involved in many cell activities and a decline in mitochondrial function plays a role in aging in humans. It has been reported that a previously infertile woman gave birth following mitochondrial transplantation. Similarly, mitochondrial transplantation provides a solution for aging cells. Nonetheless many issues remain with regards mitochondrial treatment and further exploration is required.

AUTOPHAGY IMBALANCE

Autophagy is a highly conserved physiological process that is widespread in eukaryotic cells (Revuelta, 2017). It plays an important role in maintaining bio-energetic homeostasis through the control of molecular degradation and organelle turnover (Eckhart, 2019), but excessive autophagy can lead to cell death (Mortensen, 2011). When cells are exposed to internal and external stress (such as oxidative stress, hypoxia, and nutritional deficiencies), cell autophagy will be strengthened. Activated autophagy constitutes a stress adaptation pathway that promotes cell health and survival,

and prevents the accumulation of detrimental components that could result in cell damage and death (Matheu, 2017). Autophagy gradually loses its function with the growth of age, efficiency also decreases (Rubinsztein, 2011). On the contrary, enhancing autophagy function can prolong the life of organisms. Therefore, autophagy can improve protein homeostasis and mitochondrial homeostasis, delaying organ function degradation and achieving life extension (López-Otín, 2013). Previous researches showed that inhibition of autophagy can reduce cell senescence induced by proto-oncogene activation (Young, 2009). Dysfunctional proteins and damaged organelles accumulate during cellular senescence and Autophagy can removes aged or damaged organelles and ensures the normal turnover of long-living proteins.

Autophagy is also necessary for the proliferation and differentiation of MSCs. Downregulation of autophagy can limit the therapeutic actions of MSCs (Ma, 2018). Various stresses can induce autophagy of MSCs. For example, oxidative stress can induce cell apoptosis while promoting autophagy. Autophagy is closely related to senescence of MSCs with levels increased during replicative senescence or induced senescence. An increased level of autophagy has been detected in MSCs treated with glucose at high concentrations *in vitro* (Stolzing et al., 2006). The amount of ROS also increases in replicative senescence, and senescence of MSCs can be alleviated by down-regulating autophagy levels (Infante, 2014). Consistently, adipose-derived MSCs isolated from patients with abdominal aortic aneurysm exhibit senescence phenomena that increased SASP and decreased proliferation. Treatment of these MSCs with rapamycin (an autophagy activator) remarkably downregulated SASP (Oxid Med Cell Longev. 2019 Nov 25; 2019:1305049). These findings suggest that regulating the autophagy level is a novel strategy to rejuvenate senescent MSCs.

REJUVENATION OF SENESCENT MSCs

Auto-transplantation of MSCs has been shown to improve the function of MSCs extracted from patients (Sun, 2019). The same is urgently needed to improve MSC performance *in vitro*. Rejuvenation of MSCs is broadly defined as a reversal to the embryonic state or a slowing down of the aging process. In general, approaches to achieve this have been based on genetic modification, microRNA treatment and preconditioning modification (Neves, 2017; Ocansey, 2020).

MSCs REPROGRAMMING

Two types of reprogramming are involved in modifying MSCs (**Figure 2**). Fully reprogramming refers to resetting of epigenetic clocks by reprogramming within iPSCs (Galkin, 2019). Partial reprogramming is similarly regarded as epigenetic rejuvenation and involves DNA methylation and histone modification (Ocampo, 2016).

First reported in 2006, induced pluripotent stem cells (iPSCs) have been established as useful tools for regenerative medicine (Marion, 2009; Frobel, 2014). Functional MSCs have

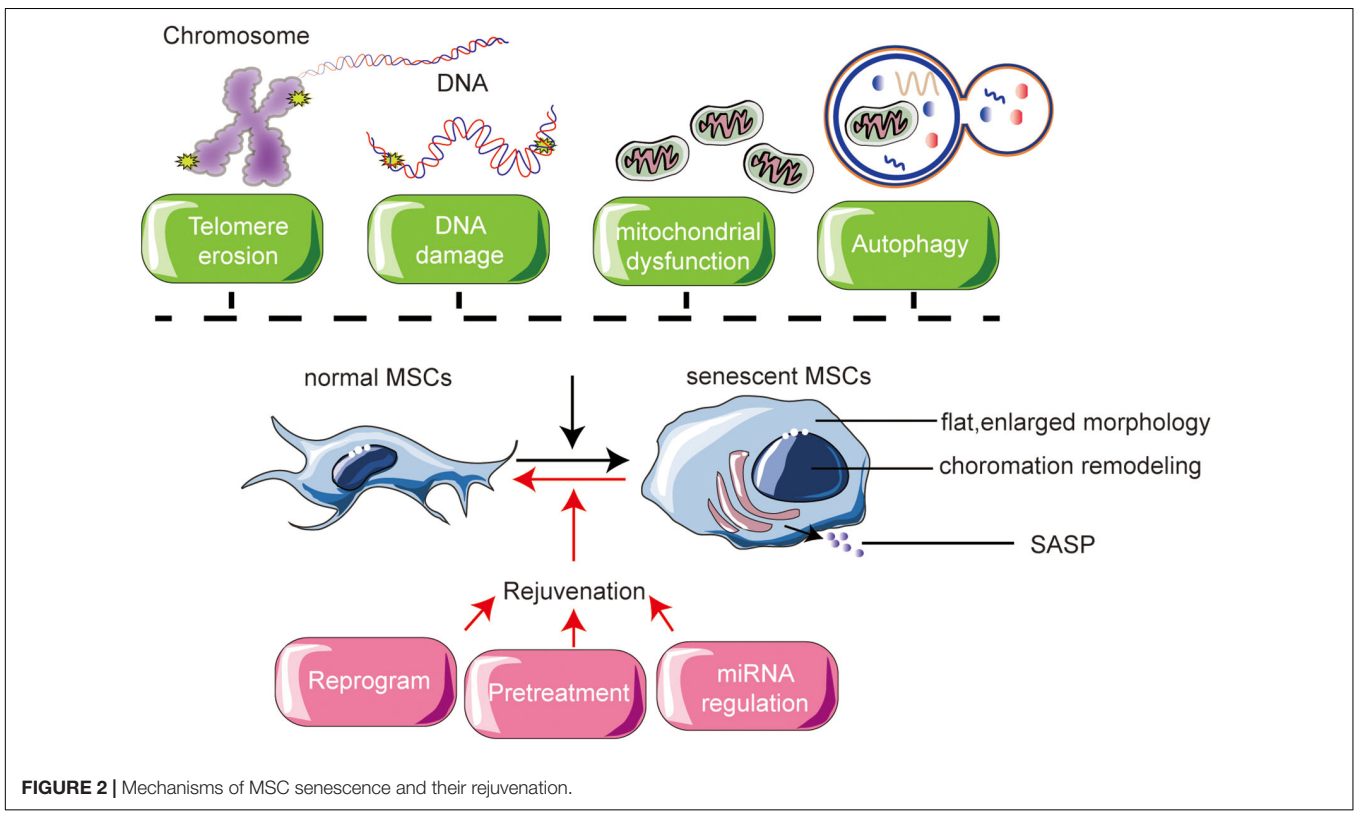

FIGURE 2 | Mechanisms of MSC senescence and their rejuvenation.

been successfully induced from iPSCs, named induced MSCs (iMSCs) (Hynes, 2013), and been shown to have improved cell vitality. iMSCs generated from iPSCs show typical characteristics of MSCs, but little epigenetic change. Compared with adult MSCs and irrespective of donor age or cell source, iMSCs show a rejuvenated profile (Spitzhorn, 2019). Nonetheless DNA methylation, related to age, was completely erased, and iMSCs reacquired senescence-associated DNA methylation during culture *in vitro*. Interestingly, overexpression of pluripotency factors without reprogramming failed to ameliorate molecular and epigenetic hallmarks of senescence (Gobel, 2018). In addition to four established factors OSKM (Oct4, Sox2, Klf4, and c-Myc), work on reprogramming with three factors, seven factors or chemical factors is ongoing. Nonetheless the efficiency is low, the number of cells available is limited, and the cost is high. There remains a large gap between the laboratory and the bedside. At present, this technology provides us with a means to study the mechanisms of aging and may at some point help prevent or treat aging.

It is proposed that partial reprogramming enables the generation of rejuvenated cells without having to go through a dedifferentiation cycle. Both hypomethylation and hypermethylation are found in aged MSCs. MSCs acquire continuous changes in gene expression and DNA methylation over subsequent passages, these senescence-associated (SA) modifications even be used as biomarker to account for the number of passages or the time of *in vitro* culture (Koch, 2012; Schellenberg, 2014; Fernandez-Rebollo, 2020). As previously noted, almost one third of the CpG sites reveal age-associated changes on DNA methylation, of which 60% become hypomethylated and 40% hypermethylated upon aging. Several researches aimed to investigate epigenetic modulation of senescent MSCs.

First, gene expression can be regulated by DNA methylation through silencing of respective promoter regions. 5-Azacytidine (5-AZA), an inhibitor of DNA methyltransferase (DNMT), reverses the aged phenotype of MSCs via reduction of reactive oxygen ROS accumulation, amelioration of superoxide dismutase activity and increased BCL-2/BAX ratio (Kornicka, 2017). The DNA methyltransferase inhibitor RG108 significantly induces expression of TERT by blocking methylation at the TERT promoter region. DNMT1 and DNMT3B, belong to DNA methyltransferases (DNMTs) which modulate the patterns of polycomb-mediated histone methylation, are significantly decreased during the replicative senescence of MSCs. In contrast, expression of DNMT3a was found to be increased during replicative senescence, participating in the new methylation associated with senescence (So, 2011). Therefore, hypomethylation is evident in aged MSCs, while demethylation at the promoter region of irreplaceable protein plays an important part in maintaining MSC phenotype, lifecycle elongation and regeneration.

Second, modification of histone has attracted attention in epigenetic modulation of senescent MSCs. It has been demonstrated that tetramethylpyrazine (TMP) significantly inhibits the cell senescent phenotype by modulating EZH2 (a histone-lysine N-methyltransferase enzyme)-H3k27me3, suggesting tri-methylation at the 27th lysine residue of the histone H3 protein (Gao, 2018). Restoring mitochondrial NAD + levels by overexpressing NNT and NMNAT3 and delaying replicative senescence can enhance reprogramming efficiency of aged MSCs (Son, 2016).

It is established that telomere shortening due to telomerase deficiency leads to progressive senescence of MSCs. Approaches to transiently enhance telomerase activity are required in order to rejuvenate MSCs. Overexpression of the catalytic subunit of the human telomerase (TERT) results in telomere extension, but does not prevent senescence-associated DNA methylation (Wagner, 2017). Previous work has shown that pretreatment with MIF improved the telomerase activity of MSCs via the PI3K-Akt signaling pathway (Xia and Hou, 2018).

MICRO RNA TREATMENT

It is becoming increasingly clear that in addition to coding genes, non-coding RNAs also regulate gene expression (Abdelmohsen, 2015). A summary of studies of miRNA and the function of microRNA in retraining MSCs from senescence is shown in **Table 1**. MicroRNA-based treatments show multidimensional targets and delayed MSC senescence. However, one or two microRNAs in the therapy of senescent MSCs appears to have little effect. Mixing several senescent-associated microRNAs together to determine the efficiency of treatments may be a new objective.

PRECONDITIONING MODIFICATION

Data show that ROS increase in aged MSCs and accumulated oxidative damage leads to abnormal proliferation and ultimately MSC senescence. Several studies have shown that MSC senescence may be reversed by modulation of ROS aggregation and oxidative metabolism. Ascorbic acid has been shown to inhibit the production of ROS due to D-galactose and activation of AKT/mTOR signaling in MSCs (Yang, 2018). Other work has revealed that lactoferrin inhibits the production of ROS induced by hydrogen peroxide, and downregulated caspase-3 and AKT activation to reduce hydrogen peroxide-induced apoptosis (Park, 2017). MSCs pretreated with Cirsium setidens, a kind of antioxidant, could inhibit production of ROS and decrease the expression of phosphorylated-p38 mitogen activated protein kinase, c-Jun N-terminal kinase and p53 (Lee, 2016). Overall, controlling ROS at a reasonable level can greatly alleviate cell aging. Nonetheless since many stimuli can increase ROS, it is unknown whether a different drug is needed for each stimulus to achieve down-regulation of ROS. Interestingly, it has been shown that when high doses of antioxidant are applied to proliferating cells to maintain physiological levels of ROS, it can also cause DNA damage and induce premature senescence (Kornienko, 2019). This suggests a need to re-evaluate unconditional anti-aging antioxidant properties.

A combination of mitochondrial biogenesis, mitochondrial dynamics and mitophagy determine mitochondrial morphology

TABLE 1 | Summary of published data on the application of microRNA retraining of MSCs from senescence.

MicroRNA	Targeted cell	Mechanism	Rejuvenation of function	References
miR-217 overexpression	BMMSCs	Targeted to DKK1	Osteogenic differentiation	Dai, 2019
Downregulation of miR-196	BMMSCs	Targeted to HOXB7	An improved osteogenesis	Candini, 2015
Downregulation of miR−195	BMMSCs	Targeted to Tert	Reactivating telomerase	Okada, 2016
Downregulation of miR-34a	BMMSCs	Targets SIRT1	Activation of the SIRT1/FOXO3a pathway, improve mitochondrial function	Zhang, 2015
Downregulation of miR-29b-3p	BMMSCs	Targets SIRT1	Regulates aging-associated insulin resistance	Su, 2019
Downregulation of miR-29c-3p	BMMSCs	Targets CNOT6	Affected the p53–p21 and the p16–pRB pathways	Shang, 2016
Downregulation of miR-27b	Ad-MSCs	Unknown	Downregulated p16 and MARP3 genes, increased MSC migration	Meng, 2018
miR-211 overexpression	BMMSCs	Targets STAT5A	Enhance migration ability	Hu, 2016
Downregulation of miR-141-3p	UCB-MSCs	Targets ZMPSTE24	Suppression of an abnormal nuclear phenotype in the HDAC-inhibitor-treated cells	Yu, 2014
Upregulation of miR-10b	Ad-MSCs	Targets SMAD2	A balancing osteogenic and adipogenic differentiation	Li, 2018
Upregulation of miR-10a	BMMSCs	Targets KLF4	Promoted implanted stem cell survival	Dong, 2018
Downregulation of miR-1292	Ad-MSCs	Targets FZD4	Delay senescence and enhance bone formation	Fan, 2018
Downregulation of miR-31	Ad-MSCs	Targets Frizzled-3	Osteogenesis	Weilner, 2016

and mitochondrial function. Deficient mitochondrial function is often regarded as a typical phenotype of senescent MSCs. Melatonin can rescue MSC senescence by enhancing mitophagy and mitochondrial function through upregulation of heat shock 70 kDa protein 1L (HSPA1L). HSPA1L binds to COX4IA, the mitochondrial complex IV protein, leading to an increase in mitochondrial membrane potential and anti-oxidant enzyme activity (Lee, 2020). The decrease in CPT1A (carnitine palmitoyltransferase1A) reverses mitochondrial dysfunction (decreased ROS and improved mitochondrial membrane potential), and reverses senescence of PD-MSCs (Seok, 2020). Our previous study showed that elevation of FGF21 could improve mitochondrial function to rejuvenate senescent MSCs by regulating mitochondrial dynamics (Li, 2019).

Therefore, optimizing the function of damaged mitochondria is a reliable way to rejuvenate senescence.

Proteostasis is protein homeostasis and involves a highly complex interconnection of pathways that determine the synthesis and degradation of protein. Maintenance of the balance of these processes within an organism is dependent on ubiquitination and autophagy. Protein synthesis is strictly regulated within the cell, and the involvement of transcription factors will affect protein synthesis. FOX is a transcription factor and FOXP1 attenuates aging by directly regulating p16INK4A transcription in MSCs. Overexpression of YAP or FOXD1 rejuvenates aged MSCs. This occurs through overexpression of YAP or FOXD1 that enhances the expression of proliferation markers and genes related to chondrocyte differentiation (Fu, 2019). Histone modification of genes also regulates their expression and thus affects protein synthesis through processes such as DNA methylation and acetylation.

Autophagy has been widely employed as an anti-aging target. Inhibition of mTORC1 with AICAR and NAM boosts autophagy and retains MSC capacity for self-renewal and differentiation, and postpones senescence-associated changes (Khorraminejad-Shirazi, 2020). Hyperactivation of mTOR can negatively regulate autophagy and cause imbalance in the proteasome, ultimately leading to cellular damage and senescence. A molecular link between age-related changes in BMMSCs and autophagy has been demonstrated: expression of p53 and ROS increased in the 3-MA (the autophagy inhibitor)-treated group and decreased in the rapamycin (the autophagy inhibitor)-treated group. AhR inhibition restored autophagy suppressed by kynurenine and increased the expression of senescence associated β-galactosidase and p21, as well as blocking aggregation of nuclear H3K9me3 (Kondrikov, 2020). HIF1α−Notch3−mediated AIMP3 regulation is a key pathway for developing antiaging interventions. Downregulation of AIMP3 (aminoacyl−tRNA synthetase−interacting multifunctional protein 3) ameliorated senescence by activating autophagy in MSCs (Kim, 2019). These results suggest that down-regulation of autophagy can indeed alleviate aging. As mentioned above, autophagy involves many proteins so its control requires the identification of specific mechanisms to enable targeted regulation.

It is well known that phosphatidylinositol 3-kinase (PI3K)/AKT is associated with premature cellular senescence (Gharibi, 2014; Liang, 2019) and scientists have devoted themselves to exploring mechanisms to rescue senescence. FGF-2 appears to maintain MSC stemness by inhibiting cellular senescence through a PI3K/AKT-MDM2 pathway (Coutu, 2011; Matsuda, 2018). Embryonic stem cell-derived extracellular vesicles (ES-EVs) can be used as a pretreatment factor to enhance the therapeutic effect of MSCs, mediated by the IGF1/PI3K/AKT signaling pathway (Zhang, 2019). Inhibition of PI3K/AKT/mTOR significantly increases the expression of some pluripotency genes like NANOG and OCT4 (Lu, 2019). NANOG has been shown effectively to reverse MSC senescence in numerous studies (Mistriotis, 2017). Various underlying mechanisms have been proposed. NANOG upregulates PBX1

(a homeodomain transcription factor) and activates the AKT signaling pathway. A feedback loop likely exists between PBX1 and AKT signaling, maintaining HF-MSCs in a highly proliferative state with differentiation potential (Liu, 2019). NANOG also restores expression of COL3 and thus stabilizes extracellular matrix synthesis (Rong, 2019).

It should be noted that the principle parts of cellular signaling pathways to rescue MSC senescence such as those of AMPK, Sirt1 and FOX, are intimately related to calorie restriction (CR) (Khorraminejad-Shirazi, 2018). CR is often recognized as a vital intervention to prevent or alleviate the severity of aging phenotypes. With CR, the function of senescent MSCs can be enhanced and repaired. CR modulates mitochondrial function and autophagy, eliminating ROS and DNA damage. The most recent research concludes that CR plays a regulatory role in the immune system (Ren, 2017).

CONCLUSION

Most cell regulatory processes are not independent events, nor are their effects (**Figure 2**). MSC senescence is a complex and comprehensive problem, so multiple different approaches are required to alleviate or prevent senescence and improve the clinical application of MSCs. A thorough understanding of the characteristics of MSC senescence, the underlying mechanisms, and different types of senescent MSCs will aid in the search for methods to rejuvenate senescent MSCs. MSCs offer great hope in regenerative medicine. To fulfill their potential, there is an urgent need to understand rejuvenation processes to optimize their application. Fully reprogram and partially reprogram of MSCs are thought to fully or partially erases the transcriptomic signatures of aging present in senescent MSCs. Preconditioning modification of MSCs also recognized as a possible source of patient-specific cells for transplantation therapies.

However, several limitations restrain the application of rejuvenated MSCs from bench to bedside. First, the proliferation arrest is continuously acquired with increasing passages *in vitro* cultivation of MSCs. Then, genetic modification of MSCs possibly end up as a secondary damage. The key pathways regulating senescence of MSCs also are the important physiological regulators of normal biological functions. Therefore, complete suppression or activation these pathways by interventions may be unacceptable. Thus, we have to weigh against the possible side effects and the therapeutic efficiency.

AUTHOR CONTRIBUTIONS

XZ and YH searched the literature and wrote the manuscript. HZ searched the literature and provided comments. XL designed the study and wrote the manuscript.

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Sirt3 Attenuates Oxidative Stress Damage and Rescues Cellular Senescence in Rat Bone Marrow Mesenchymal Stem Cells by Targeting Superoxide Dismutase 2

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Oxidative stress is one of the main causes of aging. The process of physiological aging is always accompanied by increased levels of endogenous oxidative stress. Exogenous oxidants have contributed to premature cellular senescence. As a deacetylase located in mitochondrial matrix, Sirt3 plays critical roles in mitochondrial energy metabolism, oxidative stress regulation, and cellular senescence. However, it remains unknown whether Sirt3 exerts the analogous role in cellular senescence caused by two different oxidation pathways. In this study, the function of Sirt3 was investigated in age-related natural senescence and H_2O_2-induced premature senescence of rat bone marrow mesenchymal stem cells (MSCs). Our results showed that Sirt3 expression was significantly decreased in both senescent MSCs, which was concerned with reduced cellular reactive oxygen species (ROS) and aggravated DNA injury. Sirt3 repletion could partly reverse the senescence-associated phenotypic features in natural and premature senescent MSCs. Moreover, Sirt3 replenishment led to the reduction in the levels of cellular ROS by enhancing the expression and activity of superoxide dismutase 2 (SOD2), thus maintaining the balance of intracellular oxidation and antioxidation and ameliorating oxidative stress damage. Altogether, Sirt3 inhibits MSC natural senescence and H_2O_2-induced premature senescence through alleviating ROS-induced injury and upregulating SOD2 expression and activity. Our research indicates that Sirt3 might contribute to uncovering the novel mechanisms underlying MSC senescence and provide new insights to aging and oxidative stress-related diseases.

Keywords: mesenchymal stem cells, Sirt3, oxidative stress, cellular senescence, superoxide dismutase 2

INTRODUCTION

Establishment of aging models is an important method to investigate the mechanisms of human aging and to exploit anti-aging drugs, including cellular senescence models and animal aging models (Hayflick and Moorhead, 1961; von Zglinicki et al., 1995; Mitchell et al., 2015). However, each model has its own limitations, and thus, they are utilized in different applications

(Folch et al., 2018). Natural aging model is one of the most common animal models, from which cells extracted can simulate the normal aging process and are the closest to physiological aging (Ikeda, 2011). In the aging process, the organism is inevitably exposed to various damaging factors, among which oxidative stress is one of the most common reasons of aging (Brandl et al., 2011). Our previous research found that mesenchymal stem cells obtained from aged rats generated more excessive reactive oxygen species (ROS) than cells from young rats, indicating that endogenous ROS accumulated in cells with increasing age (Ma et al., 2017). Precocious senescence is characterized by the addition of exogenous stimuli that lead to cellular premature senescence. The numerous stimulants are easy to obtain, cheap, and non-toxic, which can effectively induce cellular senescence. Accumulating studies have proved that exogenous oxidants can induce cellular premature senescence. Owing to safety, non-toxicity, and easy obtainment, hydrogen peroxide (H_2O_2) is widely used in the establishment of premature senescence model (Ko et al., 2012). The essence of individual aging is cellular senescence (López-Otín et al., 2013). The process of individual aging is accompanied by a dramatic elevation in ROS level, and multiple age-related diseases are closely associated with the alteration of oxidative stress, such as Alzheimer's disease, chronic obstructive pulmonary disease, type 2 diabetes, and age-related hearing loss (Zeng et al., 2014; Conti et al., 2015; Lemos et al., 2017; Wojsiat et al., 2018). Hence, potential targets for anti-oxidation to delay or reverse cellular senescence and individual aging are worth exploring.

In mammals, there are seven members of sirtuin family, sirt1–sirt7, with distinct subcellular localizations and functions. Sirt3 is one of the deacetylases located in the mitochondria, which can regulate the activity of multiple enzymes through deacetylation, thus affecting mitochondrial function and cellular physiological condition (Finkel et al., 2009). It has been confirmed that Sirt3 plays a critical role in the elimination of intracellular ROS and maintenance of oxygen metabolism balance (Yu et al., 2016; Denu, 2017). Growing evidences have indicated that Sirt3/superoxide dismutase 2 (SOD2) pathway is closely related to aging (Wang et al., 2014; Zhou et al., 2020). Recent studies have suggested that SOD2 is a specific target of Sirt3. Sirt3 effectively promotes SOD2 activity through the deacetylation of lysine 53, 68, and 89 (Qiu et al., 2010; Gao et al., 2018). Sirt3 over-expression in porcine fetal fibroblasts (PFF) can slow down cellular senescence by attenuating DNA damage (Xie et al., 2017). In a study of age-related hearing loss, mice lacking Sirt3 ($Sirt3^{-/-}$) significantly lose their protective role against oxidative damage compared to wild-type mice (Someya et al., 2010), manifesting that Sirt3-mediated mitochondrial oxygen metabolism may be a pivotal regulatory mechanism of aging retardation.

Mesenchymal stem cells (MSCs) are a kind of multipotent stem cells and are mainly derived from the bone marrow, fat, cord blood, and placenta (Friedenstein et al., 1970). MSCs function as precursors to a variety of cell types, including adipocytes, osteoblasts, and chondrocytes. Due to easier and safer obtainment and lower immunogenicity, MSCs have been overwhelmingly useful seed cells in tissue engineering and regenerative medicine. However, as individuals age and oxidative stress injury accumulates or removes abnormally, MSCs also exhibit senescence-like characteristics. Thereby, whether Sirt3 can rejuvenate senescent MSCs and its mechanisms merits urgent investigation. In our previous study, we revealed that ROS levels of MSCs from naturally aged rats were significantly higher than those of young individuals. In addition, we also verified an age-dependent decrease in NAD^+ content. In view of the intimate connection between Sirt3 and oxidative metabolism, as well as its biological characteristics of NAD^+ dependence, we speculate that Sirt3 might exert a certain influence on MSC senescence by manipulating the cellular oxidation levels. In order to further explore the interaction between Sirt3 and oxidative stress in MSC senescence, exogenous oxidant H_2O_2 was added to enhance cellular ROS levels and promote premature senescence. Then age-related alterations in morphology and senescence-related markers, including cell proliferation, apoptosis, senescence-associated-β-galactosidase (SA-β-gal) activity, $p16^{INK4A}$, and $p21^{WAF1/CIP1}$ expression, were detected. Moreover, we evaluated the levels of cellular ROS, the expression and activity of SOD2, malondialdehyde (MDA) contents, and DNA damage markers to identify the levels of intracellular oxidative stress in natural senescent and H_2O_2-induced senescent MSCs. In addition, senescent MSCs were transduced with lentivirus carrying Sirt3 to elucidate Sirt3 effects on cellular senescence and oxidative metabolism.

RESULTS

Senescence Induction and Age-Associated Variations in Mesenchymal Stem Cells

Premature senescence in young MSCs (Y) was established by exposure to H_2O_2 at a sub-lethal concentration as reported in a previous literature (Kumar et al., 2019). First, the optimal concentration of H_2O_2-induced premature senescence without obvious cytotoxicity was assessed by CCK-8 method (**Figure 1A**). Then, H_2O_2 at the concentration of 250 μM was selected as the exogenous oxidant to induce young MSC senescence in subsequent experiments (recorded as Y + H). Natural senescent MSCs (O) were extracted from15 to 18 months-old rats, as previously described (Ma et al., 2017). In order to eliminate the apoptotic interference, cell apoptosis was detected by flow cytometry. The results displayed that there was no significant difference among the three groups (**Figure 1B**). After treatment with H_2O_2, MSCs obtained from young rats showed senescence-like morphology with irregular shapes, flattened and enlarged cell bodies, and attenuated stereoscopic perception (**Figure 1C**), similar to the natural senescent MSCs. Statistical analysis of cell morphology revealed that the cell aspect ratios markedly decreased, whereas cell areas significantly increased both in natural senescent and premature senescent MSCs compared with young group (**Figures 1D,E**). SA-β-gal staining is considered as the classical standard for evaluation of cellular senescence (Dimri et al., 1995). The number of SA-β-gal-positive blue cells

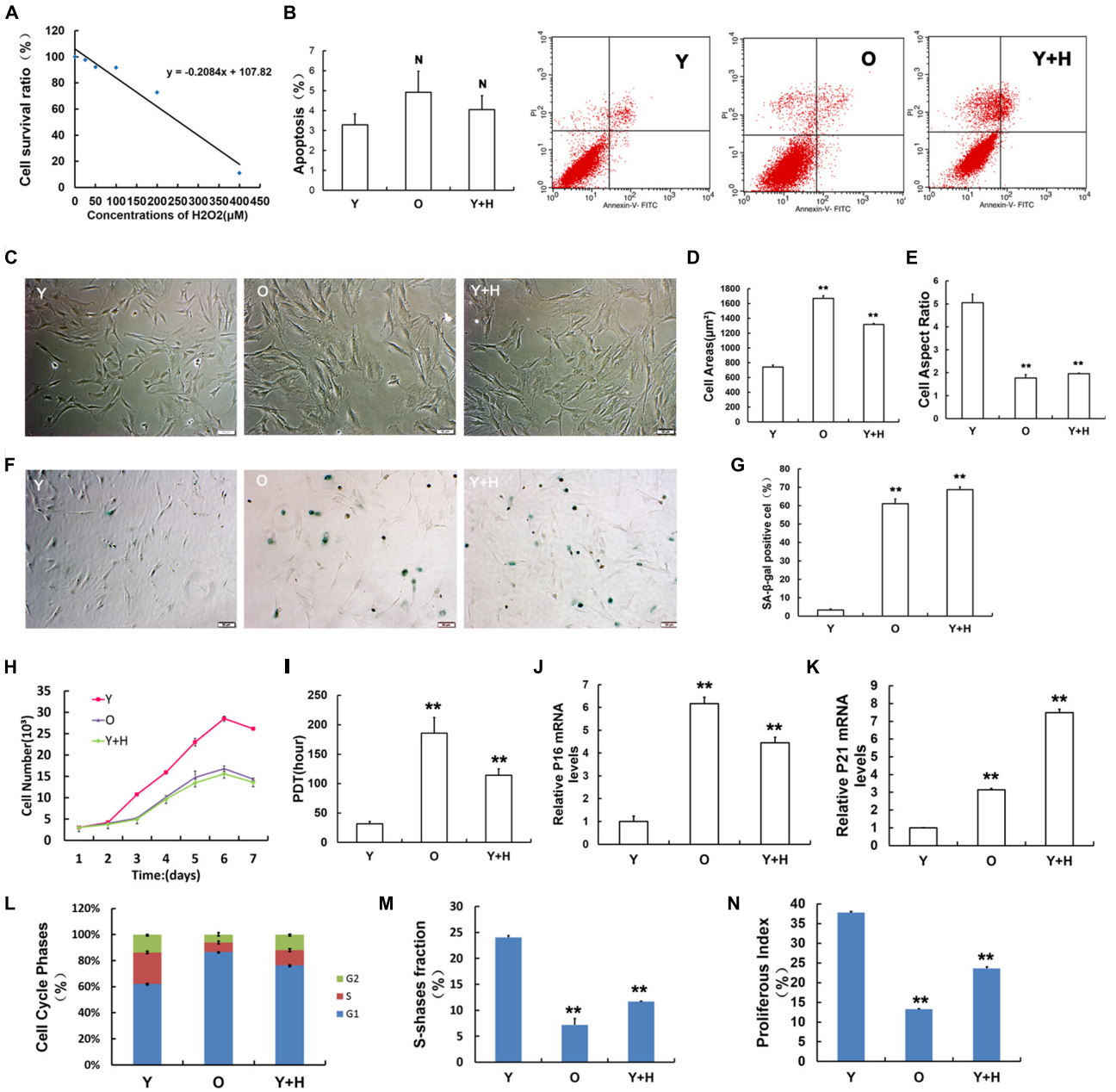

FIGURE 1 | Obtainment of senescent mesenchymal stem cells (MSCs) and identification of senescence-associated characteristics. **(A)** Cell viability of MSCs after 2 h treatment with H_2O_2 at different concentrations evaluated by CCK-8 assay. **(B)** Cell apoptosis assays in young (Y), old (O), and H_2O_2 pretreated (Y + H) MSCs. **(C)** Age-related changes in cell morphology. **(D)** Cell surface areas. **(E)** Cell aspect ratio. **(F)** Senescence-associated-β-galactosidase (SA-β-gal) staining. **(G)** Quantitative analysis of SA-β-gal staining. **(H)** Cell growth curves. **(I)** Population doubling time (PDT). mRNA expression of the senescence-related factor **(J)** $pl6^{INK4A}$ and **(K)** $p21^{WAF1/CIP1}$. **(L)** Cell cycle analysis. **(M)** Fraction of cells in S-phase (SPF). **(N)** Proliferation index (PI). Data are expressed as mean ± SD, $n = 3$. $^{**}P < 0.01$, $^{N}P > 0.05$ vs. young.

in natural senescent MSCs was extraordinarily higher than that in young MSCs. After H_2O_2 treatment, blue-stained cells in young MSCs also remarkably augmented, suggesting cellular premature senescence occurred (**Figures 1F,G**). The cell growth curves were drawn to observe cell proliferation. Data analysis results demonstrated that in young MSCs treated with H_2O_2, as well as the natural senescent MSCs, there was a decline in cell proliferation (**Figure 1H**). Moreover, the population doubling time (PDT) in both cells was prolonged to 6 and 3.6 times, respectively (**Figure 1I**). $Pl6^{INK4A}$ and $p21^{WAF1/CIP1}$, as the acknowledged biological indicators, were widely used in the assessment of cellular senescence (Choudhery et al., 2014). To evaluate senescence-associated alterations at molecular levels, mRNA expression of $pl6^{INK4A}$ and $p21^{WAF1/CIP1}$ were further

monitored. As indicated in **Figure 1J**, $pl6^{INK4A}$ expression levels were heightened in natural senescent MSCs, as well as H_2O_2-treated young MSCs. Similar trends could also be observed in the expression levels of $p21^{WAF1/CIP1}$ (**Figure 1K**). Cell cycle analysis revealed that more cells stuck in G1 phase, whether elevated endogenous oxidation in old group or addition of exogenous oxidant in H_2O_2 group (**Figure 1L**). In addition, the S-phase fraction (SPF) and proliferative index (PI) were lower in both senescent cells than those in the young group (**Figures 1M,N**).

Reduced Antioxidant Capacity and Aggravated DNA Injury in Senescent MSCs Is Associated With Down-Regulated Sirt3 Expression via Attenuated SOD2 Expression and Activity

In our previous study, we demonstrated that ROS accumulation in MSCs obtained from chronological aged rats. To further investigate the effects of oxidative stress, MSCs from young rats were treated with exogenous oxidant H_2O_2 at the concentration of 250 μM to increase the level of cellular ROS. Dihydroethidium (DHE) staining and flow cytometry were then performed to determine intracellular ROS levels. As shown in **Figure 2A**, the intensity of ROS fluorescence was much weaker in the young MSCs than that in old or H_2O_2-treated cells. The quantitative analysis results of flow cytometry illustrated that intracellular ROS level in the young group was up-regulated by approximately fivefold after H_2O_2 treatment (**Figure 2B**). ROS can trigger severe damage to cellular macromolecules, particularly making them prone to DNA damage (Cooke et al., 2003; Barzilai and Yamamoto, 2004). Therefore, a single-cell gel electrophoresis assay was further carried out to evaluate the extent of DNA breaks (**Figure 2C**). Quantitative analysis indicated the ratio of injured MSCs increased (**Figure 2D**), and the length of olive tail moment (OTM) (**Figure 2E**) prolonged in the old group and H_2O_2 group compared with the young control. Next, we also assessed intracellular MDA contents, a biomarker of lipid peroxidation in living cells (Lin et al., 2019). The data showed that intracellular MDA production was elevated along with ROS accumulation under chronologic aging conditions, as well as after the addition of exogenous oxidant (**Figure 2F**).

To explore the potential roles of Sirt3 in ROS-correlated senescence, we detected Sirt3 expression by real-time quantitative polymerase chain reaction (RT-qPCR) and western blotting in all three groups. At both mRNA (**Figure 2G**) and protein (**Figure 2H**) levels, Sirt3 expression was significantly diminished due to ROS accumulation caused by endogenous (old group) or exogenous oxidation (H_2O_2 group). Ample evidence displayed that SOD2 might be a potential specific target of Sirt3 (Chen et al., 2011; He et al., 2019). To verify whether Sirt3 was involved in the regulation of ROS-related senescence via SOD2 mediation, we examined the alterations of SOD2 expression and activity. The mRNA levels of SOD2 expression were both lower in natural senescent and premature senescent MSCs than those in the young group by 5.56- and 7.14-fold, respectively (**Figure 2I**), and similar downtrends were found in SOD2 protein expression. The expression levels of Sirt3 protein in old MSCs or H_2O_2-treated MSCs were 2.86- and 6.25-fold lower than those in the young counterparts (**Figure 2J**). In addition, various degrees of suppression in Sirt3 activity were observed in both senescent MSCs (**Figure 2K**).

Sirt3 Replenishment Accelerates Clearance of Intracellular Excessive ROS

To further explore the roles of Sirt3 in modulating oxidative stress, old and H_2O_2-treated MSCs were, respectively, transduced with lentivirus-expressing Sirt3 (LV-Sirt3) and the lentiviral vector (LV-Vector), followed by evaluating transduction efficiency though RT-qPCR and western blotting. The results confirmed that Sirt3 was successfully up-regulated at both mRNA (**Figures 3A,B**) and protein (**Figures 3C,D**) levels. Subsequently, intracellular ROS was measured in both LV-Sirt3 group and LV-Vector group. As demonstrated in **Figures 3E,F**, ROS amassing was notably diminished in Sirt3-replenished cells as compared to the control (LV-Vector), no matter in natural senescent MSCs or premature senescent MSCs. The results of DHE fluorescent staining were consistent with those of flow cytometry (**Figures 3G,H**).

Sirt3 Over-Expression Alleviates ROS-Relevant MSC Senescence

To investigate whether Sirt3 directly influenced MSC senescence, we examined the morphological characteristics and senescent biomarkers in senescent MSCs after Sirt3 over-expression. Old MSCs after Sirt3 replenishment exhibited ameliorated senescent morphology with long and fusiform shape, enhanced the stereoscopic perception, declined cell surface area, and increased cell aspect ratio (**Figure 4A**). Moreover, the number of senescence-associated blue-stained cells markedly decreased following Sirt3 over-expression (**Figure 4B**). In accordance with the old group, Sirt3 repletion improved large and flat cellular morphology caused by H_2O_2 stimulation. Statistical data displayed that the cell aspect ratio was dramatically augmented, whereas the cell surface area was diminished after Sirt3 repletion (**Figure 4C**). In addition, SA-β-gal activity in Sirt3-overexpressed MSCs was largely abated compared to that in cells transduced with the vector (**Figure 4D**). Alterations at the molecular levels that resulted from Sirt3 sufficiency have also been unraveled in natural senescent and premature senescent cells. Both $pl6^{INK4A}$ and $p21^{WAF1/CIP1}$ mRNA levels were evaluated by using RT-qPCR. Contrary to Sirt3 up-regulation, the expression of $pl6^{INK4A}$ and $p21^{WAF1/CIP1}$ was obviously reduced in the LV-Sirt3 group compared to the vector group (**Figures 4E–H**), indicating senescence-associated genetic indexes can be effectively rescued as a result of Sirt3 replenishment.

Sirt3 Attenuated MSC Senescence via Enhancing SOD2 Activation and Reducing Oxidative Stress Damage

To further explore the possible mechanisms of Sirt3-regulated MSC senescence, we then examined intracellular SOD2 level and

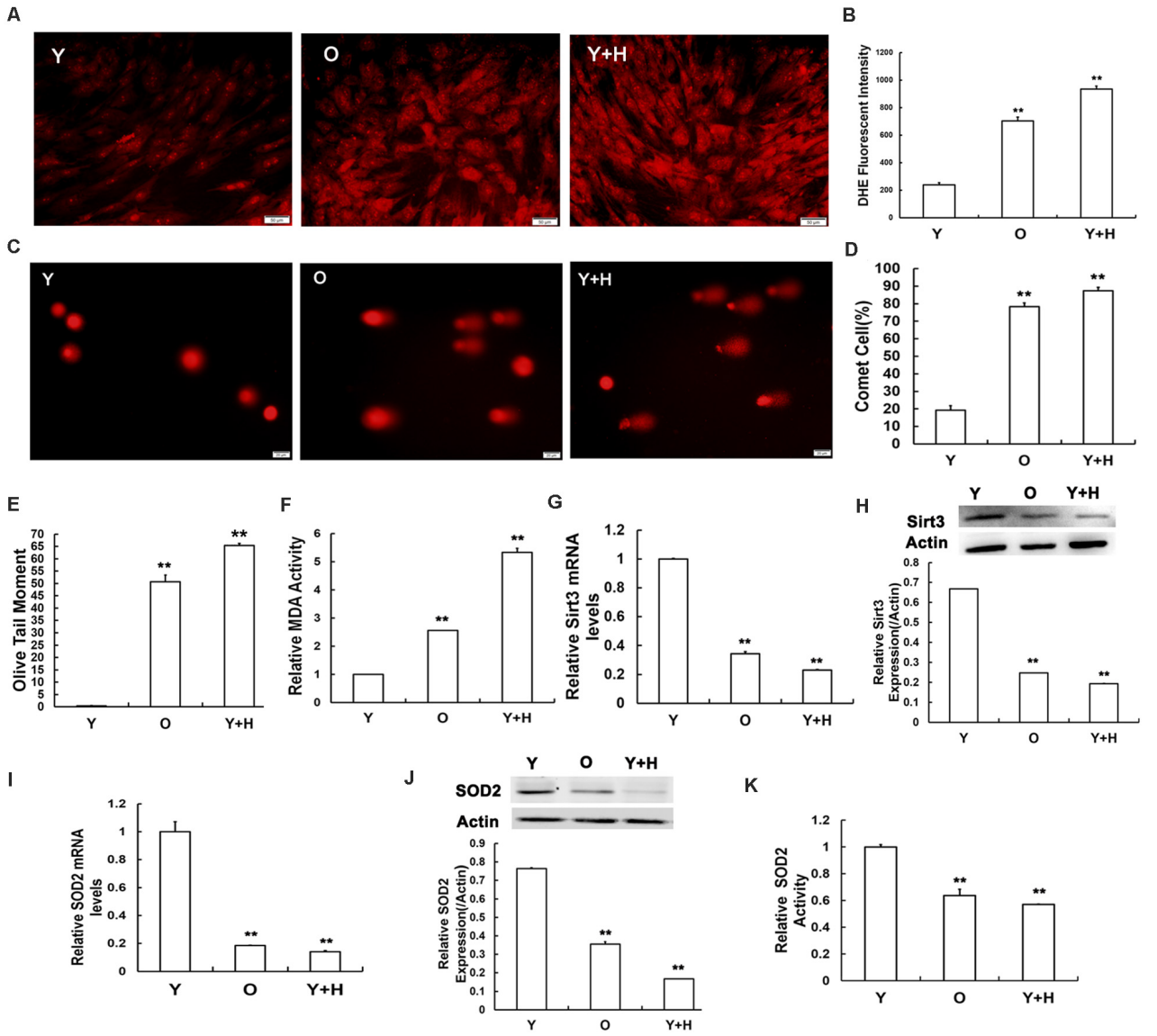

FIGURE 2 | Senescence-relevant alterations in oxidation/antioxidation capacity and measurement of Sirt3 and superoxide dismutase 2 (SOD2) expression and activity. **(A)** Intracellular reactive oxygen species (ROS) levels were determined using dihydroethidium (DHE) staining in young (Y), old (O), and H_2O_2 pretreated (Y + H) MSCs. **(B)** Quantitative analysis of DHE-fluorescent intensity. **(C–E)** DNA damage was detected by comet assays **(C)**, quantitative analysis of comet cells ratio **(D)**, and measurement of olive tail moments (OTMs) **(E)**. **(F)** Intracellular malondialdehyde (MDA) contents. **(G)** Sirt3 mRNA expression was detected by real-time quantitative polymerase chain reaction (RT-qPCR). **(H)** Sirt3 protein expression was detected by western blotting. **(I)** SOD2 mRNA expression was examined by RT-qPCR. **(J)** SOD2 protein expression was examined by western blotting. **(K)** Assessment of SOD2 activity. Data are expressed as mean ± SD, $n = 3$. $^{**}P < 0.01$ vs. young.

its activity. The results presented that SOD2 was remarkably up-regulated at the mRNA level in Sirt3-sufficient MSCs (**Figures 5A,B**). Additionally, SOD2 protein expression in Sirt3-overexpressed cells from old or H_2O_2-treated MSCs showed a semblable upward trend (**Figures 5C,D**). Furthermore, we examined whether SOD2 activity was enhanced in response to Sirt3 supplement. The data showed that Sirt3 over-expression contributed to the maintenance of SOD2 activity not only in old MSCs (**Figure 5E**) but also in H_2O_2-exposed MSCs (**Figure 5F**). The results indicated that the molecular mechanism underlying Sirt3 ameliorating MSC senescence is associated with elevated SOD2 expression and activity.

To confirm whether elevated SOD2 activation mediated by Sirt3 repletion played important roles in ROS-relevant senescence and oxidative stress injury, DNA damage and MDA levels were further examined following Sirt3 over-expression in senescent MSCs. Sirt3 abundance either in old cells or in H_2O_2-pretreated cells significantly attenuated DNA damage (**Figures 6A–F**) and intracellular MDA contents (**Figures 6G,H**) compared to that in their own LV-vector group.

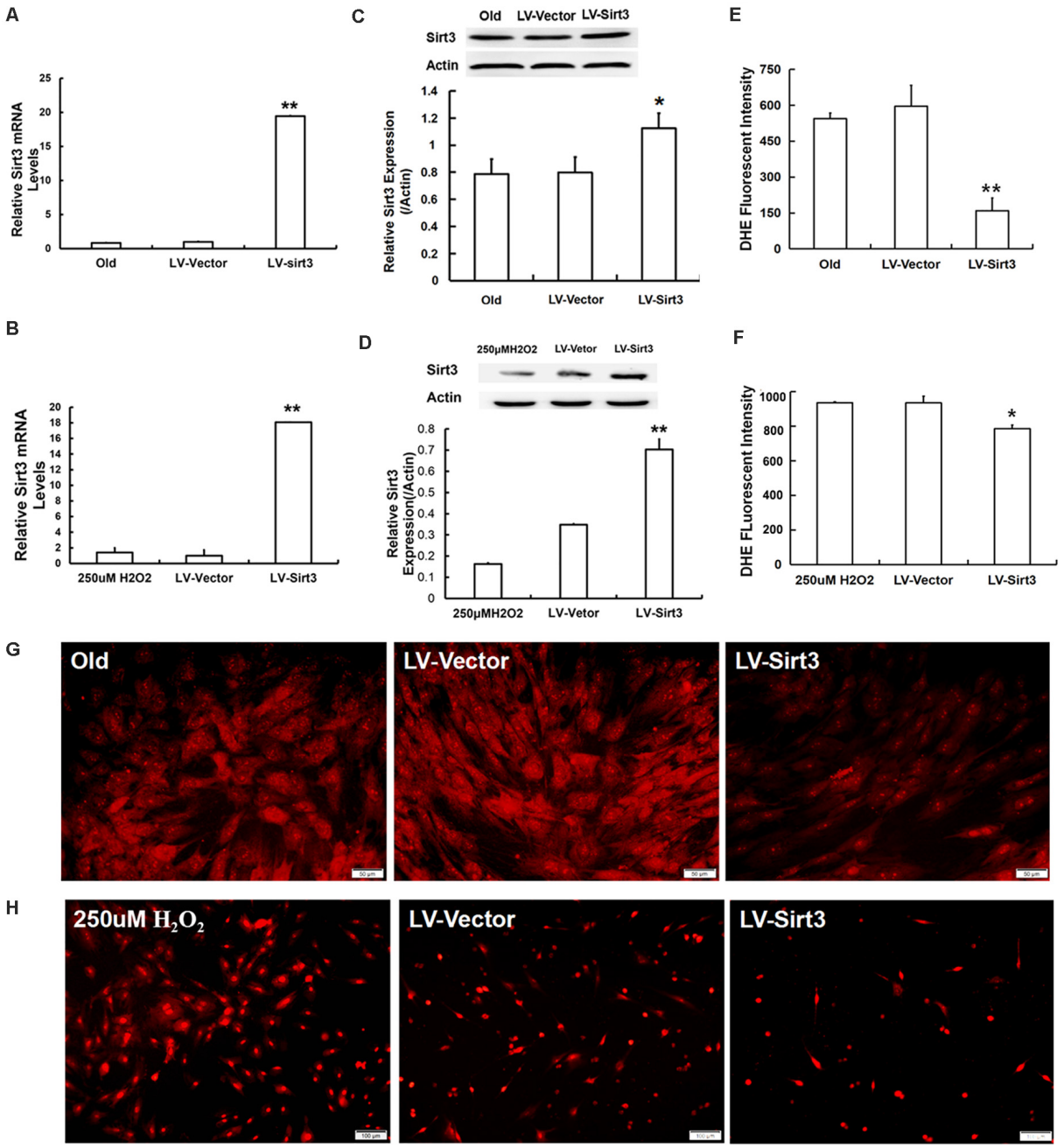

FIGURE 3 | Sirt3 replenishment improves ROS scavenging ability. **(A,B)** Sirt3 mRNA expression was determined by RT-qPCR in old MSCs **(A)** and H_2O_2-pretreated MSCs **(B)** after Sirt3 over-expression. **(C,D)** Sirt3 protein expression was tested by western blotting. **(E–H)** DHE fluorescence intensity was detected by flow cytometry and microphotography. Data are expressed as mean ± SD, $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$ vs. old or H_2O_2.

DISCUSSION

Aging is a complex phenomenon, and emerging scientific researches have brought up different theories trying to reveal its mystery. Among which, the free radical theory of aging was one of the most widely accepted theories proposed by Harman (1956). On this basis, the theory of oxidative stress aging has been developed and gradually gained considerable acceptance (Grune et al., 2005; Liguori et al., 2018). In simple terms, the cumulative damages induced by high levels of endogenous/exogenous ROS are detrimental factors for the functional maintenance of biological macromolecules such as DNA, lipids, and proteins (Schieber and Chandel, 2014). Excessive ROS mediated protein denaturation, lipid peroxidation, DNA modification, and mitochondrial dysfunction and ultimately led to cellular senescence (Chen et al., 2017). In

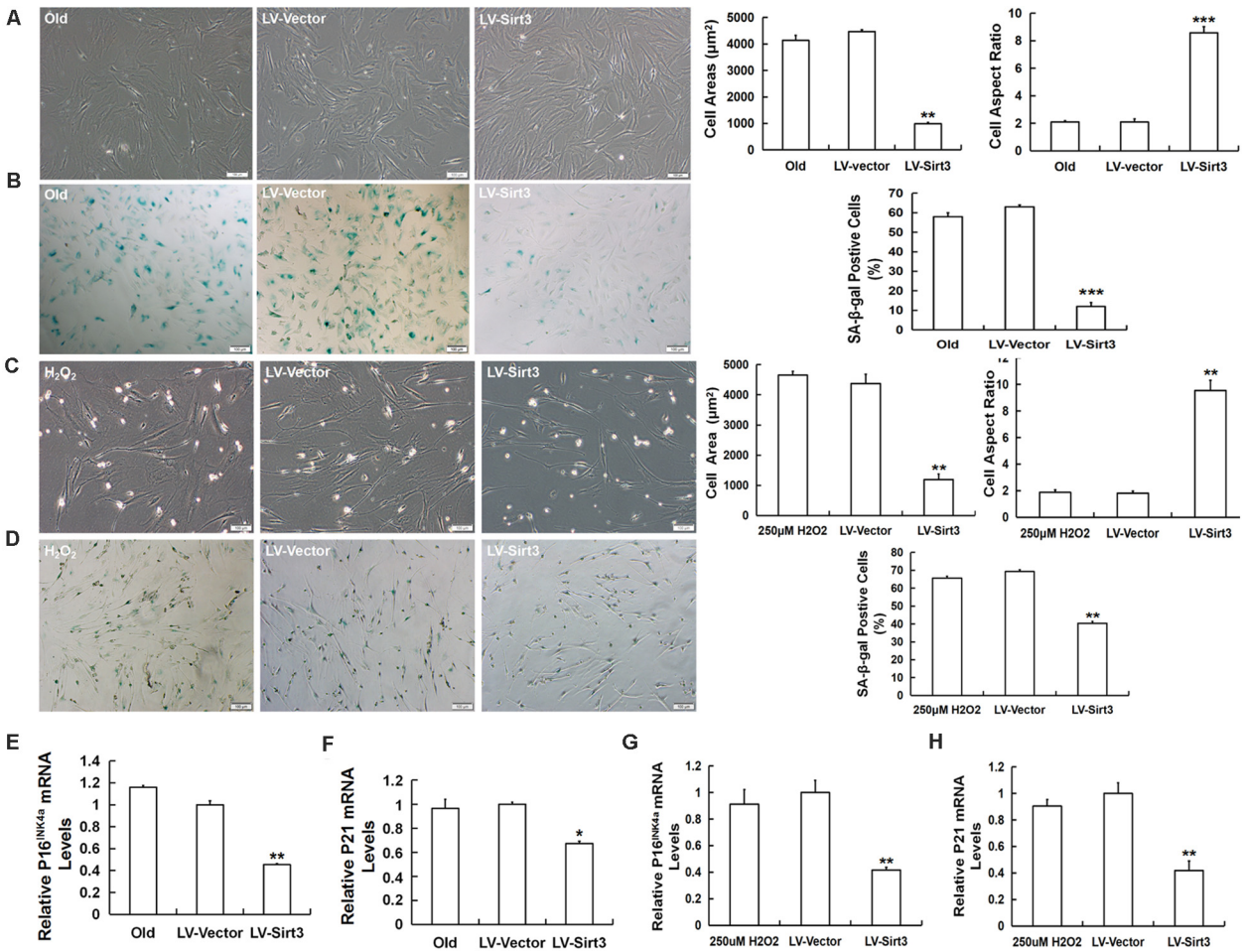

FIGURE 4 | Sirt3 over-expression mitigates senescence-associated phenotypic features in natural senescent MSCs and H_2O_2-induced premature senescent MSCs. **(A)** After Sirt3 over-expression, age-related morphological alterations were evaluated in both senescent MSCs, and the cell areas and the cell aspect ratio were quantified. **(B)** SA-β-gal staining and quantification were performed. Meanwhile, lentivirus-expressing Sirt3 (LV-Sirt3) was also transduced into H_2O_2-treated MSCs, and its morphological alterations **(C)** and the ratio of SA-β-gal-positive cells **(D)** were assessed. Gene expression of senescence-related factor $p16^{INK4A}$ **(E)** and $p21^{WAF1/CIP1}$ **(F)** in MSCs obtained from old rats after Sirt3 repletion. Gene expression of senescence-related factor $p16^{INK4A}$ **(G)** and $p21^{WAF1/CIP1}$ **(H)** in Sirt3 over-expressed MSCs derived from H_2O_2 treatment. Data are expressed as mean ± SD, $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. old or H_2O_2.

fact, when exogenous oxide (H_2O_2) was added, different types of young cells transformed into senescent phenotypes (Ben-Porath and Weinberg, 2005). In addition, endogenously formed ROS, such as superoxide (O_2^-) and the highly reactive hydroxyl radical (^-OH), also played a crucial role in the process of cellular senescence (Höhn et al., 2017). Although several key pathways are leading to oxidative stress-related stem cell senescence, our study focused on the sirtuin pathway.

The previous studies indicated that cell proliferation slowed down, the PDT increased, osteogenic and adipogenic differentiation potentials diminished, and cell cycle arrested in senescent MSCs (Ma et al., 2017; Pi et al., 2019), consistent with our present results. Importantly, MSC senescence has contributed to tissue, organ, and organism aging and age-related diseases. Macrophage migration inhibitory factor (MIF) can rejuvenate senescent MSCs by activating autophagy and enhancing their therapeutic efficacy for myocardial infarction (Zhang et al., 2019). It has been demonstrated that only young MSC-derived extracellular vesicles (MSC-EVs) are able to alleviate lipopolysaccharide (LPS)-induced acute lung injury and change macrophage phenotypes, although both old and young MSC-EVs have similar physical and phenotypical characteristics (Huang et al., 2019). Therefore, exploring the potential molecular mechanisms of MSC senescence is of great significance to the therapeutic application of MSCs in age-related diseases.

Since sirtuins were initially found to be related to the lifespan extension of yeast in 1997, the roles of sirtuin family members in cell longevity have been gradually revealed (Sinclair and Guarente, 1997). Seven members of sirtuin family are localized in different cellular compartments and highly conserved from bacteria to humans (Favero et al., 2015). As a mitochondria-localized deacetylase, Sirt3 had been linked to defense against oxidative stress in a variety of systems and their function executed depending on the content of intracellular NAD^+

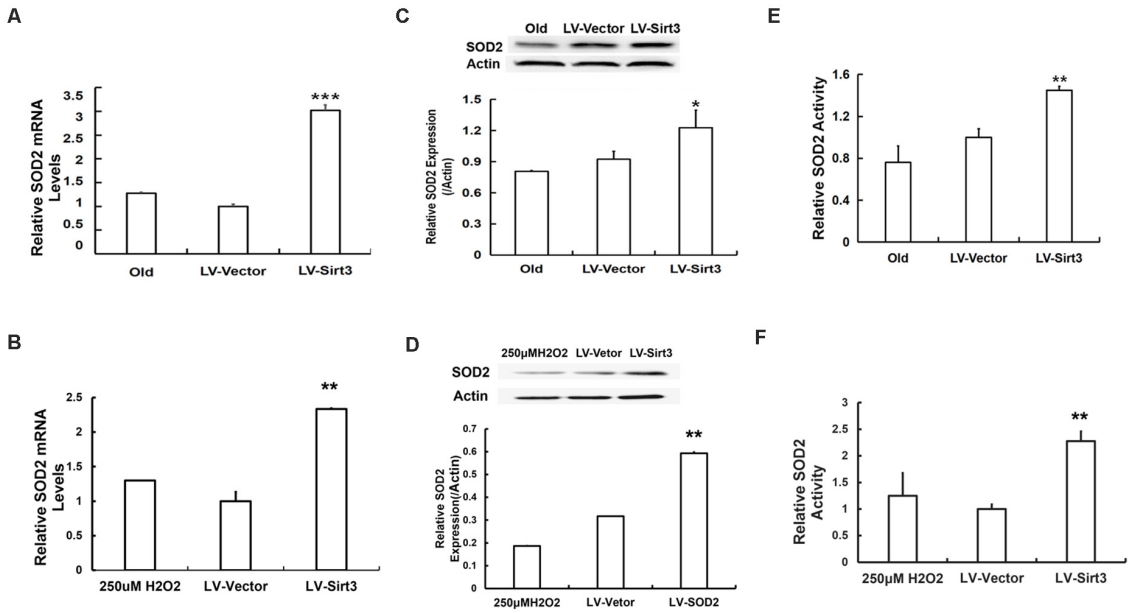

FIGURE 5 | Sirt3 repletion slows down MSC senescence associated with enhanced SOD2 expression and activity. SOD2 mRNA levels were tested in old **(A)** and H_2O_2-treated **(B)** MSCs after Sirt3 transduction. Protein levels of SOD2 **(C,D)** were measured using western blotting as well. SOD2 activity was also evaluated in old **(E)** or H_2O_2-treated **(F)** cells after Sirt3 over-expression. Data are expressed as mean ± SD, $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. old or H_2O_2.

(Michan and Sinclair, 2007). Sirt3 participated in almost every major aspect of mitochondrial biology, such as mitochondrial respiratory, ATP synthesis, ROS production, and detoxification (Bause and Haigis, 2004). In particular, there were abundant evidences that Sirt3 was closely related to age-dependent elevation in mitochondrial oxidative stress (Lee et al., 2018). In Sirt3 heart-specific knockout ($SIRT3^{-/-}$) mice, Sirt3 deficiency weakened resistance to oxidative stress, disrupted mitochondrial homeostasis, and finally resulted in obvious aging features in the myocardium, which suggested that Sirt3 might regulate age-related heart diseases by maintaining the normal biological functions of the mitochondria (Li et al., 2018). It has been reported that Sirt3 over-expression in late-passage MSCs reduced oxidative stress, enhanced their ability to differentiate, and thus ameliorated age-related senescence (Denu, 2017). Nonetheless, the mechanistic links between Sirt3 expression and oxidative stress relevant senescence are not well studied. The current study showed that Sirt3 mRNA and protein expressions were dramatically decreased in old and H_2O_2-pretreated MSCs compared to those in young MSCs, which indicated Sirt3 decline was associated with age-related elevated ROS. Despite the different triggering mechanisms, MSCs from natural aging or premature senescence models were accompanied by the accumulation of intracellular ROS. In this study, we tried to compare the role of Sirt3 under different activation mechanism of MSC senescence.

To further test the function of Sirt3 on ROS-related MSC senescence, we modulated its expression through gene manipulation. Senescent cells exhibited the changes in cellular morphology and gene levels, and SA-β-gal activity and the expression of the senescence-related factors $p16^{INK4A}$ and $p21^{WAF1/CIP}$ were commonly selected for senescence identification (Shibata et al., 2007; Wagner et al., 2008). Concomitant with elevated ROS concentration, an increased number of MSCs manifested as typically senescence-like morphological alterations, namely, increased cell body and vanishing stereoscopic sensation. Moreover, both MSCs from the old group and H_2O_2 addition group showed a high percentage of SA-β-gal-positive cells. Sirt3 supplementation in ROS-relevant senescent MSCs cannot only rejuvenate senescent appearance to a youthful phenotype but also decrease the number of SA-β-gal-positive cells. Molecular changes are also important biological indicators of cellular senescence. In Campisi and d'Adda di Fagagna (2007), summarized two classical effectors of senescence pathways, the p53-$p21^{WAF1/CIP}$ and $p16^{INK4A}$-pRB pathways. Subsequently, accumulating studies have clarified that p53-$p21^{WAF1/CIP}$ and $p16^{INK4A}$-pRB serve as significant pathways to regulate various cellular senescence (Stein et al., 1999). Although ample evidences suggest that $p16^{INK4A}$ is more closely related to stress-related senescence, and $p21^{WAF1/CIP}$ seems to be more associated with replicative senescence (Shang et al., 2016; He L. et al., 2017; He X. et al., 2017), there are still some different voices (Itahana et al., 2003; Brookes et al., 2004). Sugihara et al. (2018) demonstrated that H_2O_2 pretreatment in mesenchymal progenitor cells (MPCs) enhanced the expression of $p21^{WAF1/CIP}$ but not that of $p16^{INK4A}$, indicating that oxidative stress-related senescence can also be mediated by the up-regulation of $p21^{WAF1/CIP}$. Our previous study has similar results that in MSC replicative senescence, $p16^{INK4A}$ expression was elevated, while $p21^{WAF1/CIP}$ expression was not significantly changed (data not shown). However, our current results showed both $p16^{INK4A}$ and $p21^{WAF1/CIP}$ expressions were significantly up-regulated

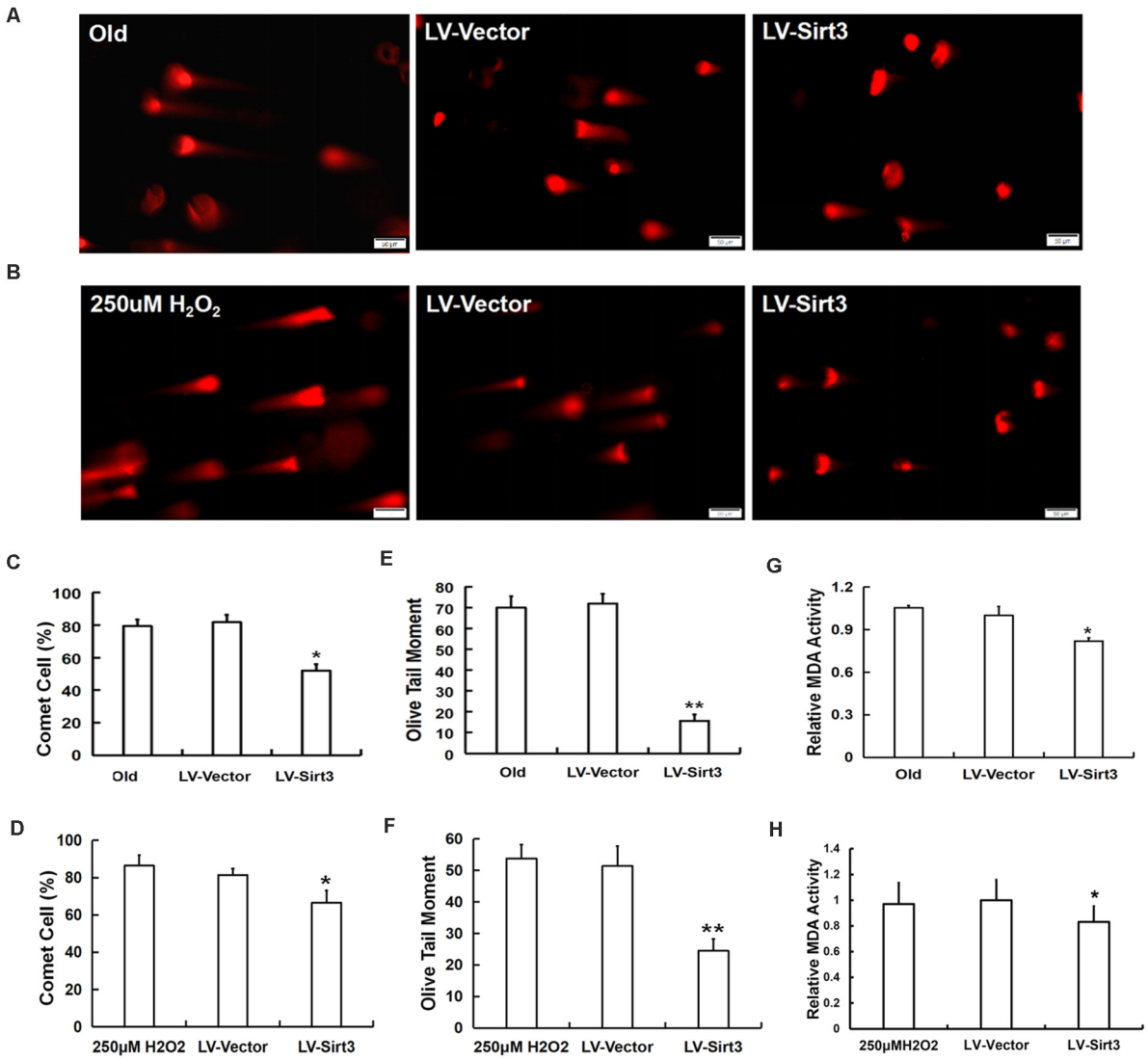

FIGURE 6 | Sirt3 alleviated MSC senescence through ameliorating DNA damage and intracellular MDA contents. DNA damage was determined by comet assay in both natural and premature senescent cells upon Sirt3 replenishment **(A,B)**. Fluorescent images and the quantitative analysis of comet-positive cells **(C,D)** and olive tail moments (OTM) **(E,F)**. **(G,H)** The levels of intracellular MDA were determined by colorimetry in the old group **(G)** and H_2O_2-treated group **(H)**. Data are expressed as mean ± SD, $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$ vs. old or H_2O_2.

in the old MSC group and H_2O_2-pretreated MSC group. We thus speculate that cell specificity might be the reason for the inconsistent expression of $p16^{INK4A}$ and $p21^{WAF1/CIP}$ in different types of senescent processes. We also analyzed the effects of Sirt3 over-expression on ROS-related senescent MSCs. Our findings showed that Sirt3 repletion in old or H_2O_2-pretreated MSCs suppressed the expression of $p21^{WAF1/CIP}$ and $p16^{INK4A}$ to some degrees, indicating that Sirt3 exerts the regulatory effects on cellular senescence in response to the elevated endogenous or exogenous oxidation levels. Additionally, we found that whether in the old or H_2O_2 group, Sirt3 supplement mediated by lentivirus markedly decreased the intracellular ROS content, further strengthening our finding that Sirt3 plays a regulatory role in oxidative stress-associated MSC senescence.

In mammals, there are three forms of SODs localized in different cellular compartments, defending cells from oxidative stress damage (Balaban et al., 2005). SOD2 is a specific SOD located in the mitochondria, which is considered as a scavenging enzyme that inhibits mitochondrial ROS (Liang et al., 2007). Ample evidences demonstrated that SOD2 might be a potential specific target of Sirt3, and enhanced Sirt3/SOD2 signaling improved endothelial reparative capacity of endothelial progenitor cells (EPCs) via suppressing mitochondrial oxidative stress (He et al., 2019). Chen et al. (2011) confirmed that Sirt3 over-expression can improve the antioxidant activity of SOD2 and enhance mitochondrial ROS-scavenging capacity. A study brought up that SOD2 might be directly activated by Sirt3 deacetylation at specific lysine residues (Qiu et al., 2010).

Sirt3 can also enhance SOD2 activity by increasing FoxO3a DNA binding at the SOD2 promoter (Jacobs et al., 2008). All previous studies mentioned suggest that SOD2 may be an important target of Sirt3 in the process of cellular senescence. To test our hypothesis, we evaluated the expression and activity of SOD2. As expected, after Sirt3 over-expression, senescent MSCs presented young phenotypes. Meanwhile, intracellular SOD2 level was markedly elevated, as well as Sirt3 activity was obviously increased. These results imply that Sirt3 replenishment attenuating ROS-relevant MSC senescence is related to the Sirt3/SOD2 signaling pathway. To further clarify the possible mechanism of Sirt3/SOD2 axis regulating ROS-related MSC senescence, DNA damage and intracellular MDA levels were assessed. Gene silence of Sirt3 increased ROS production, and an excess of ROS damaged biological macromolecules, which can be reflected by comet assay and MDA content detection (Kim et al., 2010; Groschner et al., 2012; Ma et al., 2017). Xie et al. (2017) reported that Sirt3 attenuated cellular senescence in porcine fetal fibroblasts possibly via decreased oxidative damage and enhanced the SOD2 activity. In the present study, the expression and activity of Sirt3 and SOD2 were reduced in both senescent MSCs compared with young MSCs. Simultaneously, the intracellular ROS accumulation, DNA damage, and MDA contents were all elevated in senescent MSCs. However, after Sirt3 over-expression in senescent MSCs, the expression and activity of SOD2 increased, while the intracellular ROS level, DNA damage, and MDA levels decreased. Therefore, Sirt3 might inhibit MSC natural senescence and H_2O_2-induced premature senescence through facilitating SOD2 activation and alleviating ROS-induced injury. Our results were consistent with the published data showing that Sirt3 over-expression in senescent MSCs has protective antioxidant capacity, effectively eliminating DNA damage and lipid peroxidation, so as to alleviate the cellular senescence induced by ROS accumulation.

In conclusion, the results of the current study validated that Sirt3 has regulatory effects on ROS-relevant MSC senescence. Sirt3 over-expression in natural senescent and H_2O_2-induced premature senescent MSCs inhibited intracellular ROS generation and enhanced SOD2 levels and activity, thus reducing oxidative stress damage to delay cellular senescence. Our present study may not only enrich insights into the molecular mechanisms underlying stem cell senescence but also potentially provide a novel targeted therapeutic strategy for age-associated disorders. However, the complex mechanisms of Sirt3 regulating MSC senescence and its role *in vivo* merit in-depth exploration. Thus, further investigations are underway to unravel these issues to benefit therapeutic applications of MSCs.

MATERIALS AND METHODS

Cell Culture

One to two-months-old (young group) and 15–18 months-old (old group) male Wistar rats were purchased from the Experimental Animal Center of Jilin University, Changchun, P.R. China. The whole bone marrow adherent method was used to isolate MSCs, as previously described (Ma et al., 2017). MSCs were cultured in complete medium containing 89% Dulbecco's Modified Eagle Medium with nutrient mixture F-12 (DMEM-F12, Gibco, United States) supplemented with 10% fetal bovine serum (Gibco, United States) and 1% penicillin streptomycin (HyClone, United States). Then MSCs at passage 3 (P3MSCs) obtained by serial passages were used in subsequent experiments.

H_2O_2 Treatment and Cell Proliferation Assay

MSCs (5×10^3) were seeded in each well of 24-well plates with complete medium and cultivated for 24 h. For establishment of premature senescence model, MSCs were, respectively, treated with complete medium containing 50, 100, 200, and 400 μM H_2O_2 at 37°C with 5% CO_2 for 2 h. Then, cells were washed twice with serum-free DMEM-F12 to remove residual H_2O_2 and cultured for additional 48 h to examine the sustained toxicity of H_2O_2 on cell proliferation. Cell Counting Kit-8 (CCK8, Dojindo, Japan) was used to determine cell survival according to the manufacturer's instruction. Thereafter, the absorbance was measured at 450 nm using a microplate reader (TECAN, SWIT). The survival curve was drawn according to the absorbance value, and half lethal dose of H_2O_2 was calculated (Burova et al., 2013).

Cell Growth Assay and Population Doubling Time

For cell growth assay, 5×10^3 MSCs were seeded in each well of 24-well plates with complete medium. The cells were stained by trypan blue, and cell numbers were counted every day for 7 days. To detect population doubling time (PDT), 7×10^5 MSCs were plated onto each 10 cm dish at t1, and the number of cells at this time was recorded as Nf. When cells reached 80% confluency, they were harvested and cell numbers (Ni) counted at this time (t2). PDT was calculated using the following formula: $PDT = t2 - t1/\ln (Nf/Ni)/\ln (2)$.

Cell Cycle Analysis

Cell cycle assay was performed using a Cell Cycle Detection Kit (KeyGEN BioTECH, China) according to the manufacturer's instructions. In short, 1×10^6 cells were collected and then fixed in 70% methanol overnight at 4°C. Cells were subsequently resuspended in phosphate-buffered saline (PBS) and incubated with 100 μl of RNaseA for 30 min at 37°C in the dark. Before flow cytometry analysis, 400 μl of propidium iodide (PI) was added at 4°C for at least 15 min. Cell cycle distribution was assessed using a FACS Calibur (BD Biosciences, United States) with Cell Quest software.

Senescence-Associated-β-Galactosidase Activity Assay

The percentage of senescent cells was calculated to assess MSC senescence using a senescence cell histochemical staining kit (Beyotime, China) according to the manufacturer's instructions. Briefly, cells were immobilized in fixation buffer for 15 min at room temperature, followed by washing twice with PBS. Then, 200 μl of staining solution mix was added before incubation

for 12–14 h at 37°C. Ten high-power microscopic fields were randomly selected to count the number of blue cells (β-gal-positive cells) out of at least 200 cells.

Apoptosis Assay

To measure cell apoptosis, PI-Annexin V Apoptosis Detection Kit I (BD Biosciences, United States) was used according to the manufacturer's instructions. Briefly, MSCs were collected and washed three times. Then, cell pellets were resuspended in 1 × binding buffer (100 μl) and stained with Annexin V/FITC (5 μl) and PI (10 μl) for 15 min at room temperature in the dark. Afterward, apoptotic events were detected using a flow cytometer (FACS Calibur, BD Biosciences, United States).

Measurement of Intracellular ROS

The intracellular accumulation of ROS was measured using a dihydroethidium (DHE) kit (Beyotime, China) according to the manufacturer's instructions. Briefly, 1×10^5 cells were seeded in 24-well plates and incubated with 10 μM DHE for 30 min at 37°C. Then, after removing the medium and washing the cells with serum-free culture medium, the fluorescence images were captured using fluorescence microscopy (excitation 300 nm and emission 610 nm) (OLYMPUS, Japan) and quantified with ImageJ software.

Comet Assay

To determine DNA damage, comet assay was performed using a CometAssay Kit (Trevigen, United States) in accordance with the manufacturer's instructions. Cells (4×10^3) were collected and mixed with low-melting agarose and subsequently rapidly dripped onto a slide. After coagulation, the slide was placed in lysis buffer for 2 h at 4°C. The slides were then immersed in alkaline unwinding solution (pH > 13, 300 mM NaOH, and 1 mM EDTA) for 20 min at 4°C. Next, the slides were submerged with the mixture in a pre-cooled electrophoresis buffer (pH > 13, 300 mM NaOH, and 1 mM EDTA) and subjected to electrophoresis at 300 mA for 30 min. After being washed three times with PBS, cells were quickly incubated with PI staining and viewed under a fluorescence microscope. The olive tail moment (OTM) values were measured using CASP software.

Real-Time Quantitative PCR Analysis

Total RNA was extracted from MSCs using TRIzol (Takara, China), and 1 μg of total RNA was used for cDNA synthesis using an RNA PCR Kit (AMV) Ver.3.0 (Takara, China). mRNA levels were determined by real-time quantitative polymerase chain reaction (RT-qPCR) using TransStart Top Green qPCR SuperMix (TRANS, China) in a 7300 Real-Time PCR System (ABI, United States). Relative gene expressions were normalized using β-actin mRNA as a reference and calculated using the $2^{-\Delta\Delta Ct}$ method. All the primers used in the experiment were designed and synthesized as shown in **Table 1**.

Western Blot Analysis

MSCs were collected and incubated for 30 min on ice in RIPA Lysis Buffer (Beyotime, China) for extracting total protein. The BCA Protein Assay Kit (Beyotime, China) was used for protein quantification. Protein sample lysate (30 μg of each) was resolved by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred onto PVDF membranes (Millipore, United States). Membranes were blocked at room temperature for 2 h with 5% non-fat milk in Tris-buffered saline (TBS) to avoid non-specific blots and then incubated with primary antibodies: anti-Sirt3 (1:1,000, Santa Cruz) and anti-SOD2 (1:1,000, Santa Cruz) overnight at 4°C. Membranes were washed three times to remove excessive primary antibodies and then incubated for 1 h at room temperature with anti-rabbit IgG secondary antibody at an appropriate dilution of 1:2,000. The immunoreactive protein bands were visualized on an electrochemiluminescence detection system (JENE, United Kingdom) by enhancing ECL Plus (Beyotime, China). β-Actin was used as an internal control.

TABLE 1 | The primers used in this study.

Gene name	Forward (5′–3′)	Reverse (5′–3′)
Sirt3	TGCACGGTCTGTCGAAGGTC	ATGTCAGGTTTCACA ACGCCAGT
SOD2	GAGCAAGGTCGCTTACAGA	CTCCCCAGTTGAT TACATTC
$p16^{INK4A}$	AACACTTTCGGTCGTACCC	GTCCTCGCAGT TCGAATC
$p21^{WAF1/CIP1}$	GACATCACCAGGA TCGGACAT	GCAACGCTACTAC GCAAGTAG
β-actin	GGAGATTACTGCCCT GGCTCCTA	GACTCATCGTACTCCT GCTTGCTG

Measurement of SOD2 Enzyme Activity

SOD2 enzymatic activity was assayed using superoxide dismutase (SOD) assay kit with WST-1 (Nanjing Jiancheng, China). MSCs were adjusted to 1×10^6 cell/ml after trypsinization, washed twice with PBS, and centrifuged at 1,000 rpm for 10 min. The supernatant was then removed. The precipitate obtained through centrifugation was crushed by ultrasonic wave, and the cell lysates were resuspended. According to the manufacturer's instruction, SOD2 activity was determined with a microplate reader.

Detection of Malondialdehyde Contents

The MDA was assayed according to Cell Malondialdehyde (MDA) Assay Kit-Colorimetric method (Nanjing Jiancheng, China). Briefly, the cell culture supernatant was discarded, and then, cells were scraped and transferred to the EP tube. The cell samples were prepared by addition of 0.5 ml extract and mixed for 2 min. Then, 0.1 ml of absolute ethanol and 1 ml of working solution were added and mixed. After incubation at 95°C for 40 min and centrifugation at 4,000 rpm for 10 min, the absorbance was measured by a microplate reader (Tecan, Switzerland) at 450 nm.

Lentivirus Transduction of MSCs

The cells were transduced with lentiviral particles encoding rat Sirt3 or control vector as previously described (Pi et al., 2019). Briefly, cells were plated at 1.5×10^5/well in six-well plate and incubated at 37°C for 18 h, and then, cells were transduced with lentivirus-expressing Sirt3 in the presence of 4 μg/ml polybrene (Genechem Co. Ltd., China) for 12 h. Sirt3 over-expressed MSCs were used in subsequent experiments.

Statistical Analysis

All experimental data were expressed as the mean ± standard deviation (SD). Comparisons between two groups were performed using a two-tailed Student's *t*-test. A P value < 0.05 was considered statistically significant.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

CM and YS were responsible for performing the experiments and writing the manuscript. CP, HW, HS, XY, and YS contributed to data collection, data analysis and interpretation. XH was responsible for conception, design, manuscript revision and confirmation, and financial support. All authors have read and approved the final version of the manuscript.

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Down-Regulation of Lnc-CYP7A1-1 Rejuvenates Aged Human Mesenchymal Stem Cells to Improve Their Efficacy for Heart Repair Through SYNE1

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Background: Several long non-coding RNAs (lncRNAs) have been associated with cell senescence, termed senescence-associated lncRNAs (SAL-RNAs). However, the mechanisms involved for SAL-RNAs in aging are not fully elucidated. In the present study, we investigated the effects of SAL-RNAs on aged human bone marrow-derived mesenchymal stem cells (hBM-MSCs), and the possible means to counteract such effects to improve the regenerative capacity of aged hBM-MSCs.

Methods: By comparing the lncRNAs expression of hBM-MSCs derived from young and old individuals, lnc-CYP7A1-1 was identified as being significantly increased with age. Using predictive software, the expression of Spectrin Repeat Containing Nuclear Envelope Protein 1 (SYNE1), was found to be decreased with age. Next, through lentiviral constructs, we downregulated the expression of lnc-CYP7A1-1 or SYNE1 in hBM-MSCs separately. Additionally, hBM-MSCs proliferation, survival, migration, and senescence were investigated *in vitro*. *In vivo*, lnc-CYP7A1-1 downregulated aged hBM-MSCs were implanted into infarcted mouse hearts after myocardial infarction (MI), and cardiac function was measured. Through lentivirus-mediated downregulation of lnc-CYP7A1-1 in aged hBM-MSCs, we revealed that cell senescence was decreased, whereas cell proliferation, migration, and survival were increased. On the other hand, downregulation of SYNE1, the target gene of lnc-CYP7A1-1, in young hBM-MSCs increased cell senescence, yet decreased cell proliferation, migration, and survival. Downregulation of lnc-CYP7A1-1 in aged hBM-MSCs induced cell rejuvenation, yet this effect was attenuated by repression of SYNE1. *In vivo*, transplantation of lnc-CYP7A1-1 downregulated old hBM-MSCs improved cardiac function after MI.

Conclusion: Down-regulation of lnc-CYP7A1-1 rejuvenated aged hBM-MSCs and improved cardiac function when implanted into the infarcted mouse hearts, possibly through its target gene SYNE1.

Keywords: aging, rejuvenation, lncRNAs, myocardial infarction, proliferation, mesenchymal stem cell

INTRODUCTION

Human bone marrow (hBM)-derived mesenchymal stem cells (MSCs), with their abilities of multipotent potential and promoting regenerative processes in host tissues via paracrine signaling, show great promise in tissue repairing. The potency of hBM-MSCs in the treatment of cardiovascular disease has been shown to decrease with the age of the donor (Dong et al., 2018). This decreased potency of hBM-MSCs has a significant impact on the use of autologous stem cells for treating a predominantly older cohort of patients with cardiovascular disease (Li S.-H. et al., 2013; Li et al., 2018, 2019). Part of this reduced efficacy is due to hBM-MSCs taking on a senescent phenotype and losing their proliferative capacity (Yan et al., 2017). To circumvent this deficiency of hBM-MSCs, a better understanding of the aging process, and finding means to restore the cells to a younger state, are required.

Long non-coding RNAs are non-coding RNAs greater than 200 base pairs in length. Their functions range from transcriptional to translational regulation by binding to DNA or RNA, and can also affect protein activity (Derrien et al., 2012). In humans, approximately 24% of all RNAs are lncRNA (Atianand and Fitzgerald, 2014), yet their diverse functions and incomplete/variable annotations have made them difficult to study. With respect to aging, several lncRNAs have been associated with senescence, termed senescence-associated lncRNAs (SAL-RNAs) (Abdelmohsen et al., 2013). Some examples of SAL-RNAs include ANRVIL, MALAT1, and H19 (Gomez-Verjan et al., 2018). Mechanisms for SAL-RNAs involved in aging range from transcriptional repressors/activators (Dimitrova et al., 2014; Montes et al., 2015), mRNA stability (Kumar et al., 2014), protein localization (Wu et al., 2015)/ubiquitination (Yoon et al., 2013)/translation (Abdelmohsen et al., 2014), to telomere remodeling (Cusanelli and Chartrand, 2015). For their non-coding nature and particularities, lncRNAs are emerging as potential targets for anti-aging therapies (Tan and Bird, 2016).

With age, MSCs lose proliferative potential and take on a senescent phenotype (Goodell and Rando, 2015; Ermolaeva et al., 2018). However, the role of lncRNAs in MSC aging is still an unexplored field. To better understand how lncRNAs change with aging, we compared the expression profiles of lncRNAs in young and old hBM-MSCs. Using significance analysis of lncRNA expression microarray software, we identified 12 lncRNAs, including lnc-CYP7A1-1, changed with aging. We found that lnc-CYP7A1-1 expression increased with age and postulated that it may play a role in hBM-MSC senescence. Using lentiviral knockdown of lnc-CYP7A1-1, we investigated hBM-MSC activities *in vitro*, including proliferation, survival, migration and senescence. Using predictive software, we identified a putative target gene interacting with lnc-CYP7A1-1, Spectrin Repeat Containing Nuclear Envelope Protein 1 (SYNE1), and found that its expression decreased with age. We characterized the relationship between lnc-CYP7A1-1 and SYNE1 *in vitro* using both gain and loss of function approaches. Lastly, the detrimental effect of lnc-CYP7A1-1 was investigated *in vivo*. After MI, old or lnc-CYP7A1-1 downregulated old hBM-MSCs were transplanted into the infarcted mouse hearts. Subsequently, mice heart function and scar thickness was evaluated. We found that lnc-CYP7A1-1 was increased in aged hBM-MSCs and played a role in hBM-MSC senescence. Furthermore, we proved that down-regulation of lnc-CYP7A1-1 rejuvenated aged hBM-MSCs, and improved cardiac function, when implanted into the infarcted mouse hearts, possibly through its target gene SYNE1.

Abbreviations: lncRNA, long non-coding RNA; hBM-MSCs, human bone marrow mesenchymal stem cells; CCK8, cell counting kit-8; MI, myocardial infarction; FS, fractional shortening; EF, ejection fraction; LVIDs, left ventricular internal end systolic dimension; LVIDd, left ventricular internal end-diastolic dimension.

MATERIALS AND METHODS

HBM-MSCs Culture

Human BM was collected during cardiac valve replacement surgery, all the procedures were approved by the Research Ethics Board of Guangzhou Medical University and the Hospital's Ethics Committee, and patients provided written informed consent which was approved by the Research Ethics Committee of Guangzhou Medical University. Young BM was obtained from young patients (15 females and 15 males, 23.4 $\pm$ 3.9 years), and old BM from old patients (15 females and 15 males, 72 $\pm$ 4.5 years). All patients were of the same pathological status, and received the same medical treatments, with no genetic diseases or malignancies based on the primary diagnosis were used.

The hBM-MSCs were cultured as previously described (Dong et al., 2018). Briefly, after centrifugation through a Ficoll-Paque gradient (1.077 g/mL density; GE Healthcare, Kretztechnik, Zipf, Austria), cells were separated and plated in IMDM medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). After 48 h, non-adhesive cells were removed by changing the culture medium. The adhesive cells were harvested for passage when confluence reached approximately 80%.

Cell Proliferation Assay

Human bone marrow-derived mesenchymal stem cells were cultured from different group BM and characterized as previously (Dong et al., 2018). For BrdU (5-bromo-2′-deoxyuridine, Sigma, Cat#: B5002) labeling, cells ($2 \times 10^4/cm^2$) were seeded with BrdU supplementary (10 μM/mL). After BrdU pulse chasing for 72 h in hypoxic conditions (0.1% O_2), the cells were fixed for immunofluorescent staining with BrdU antibody (Abcam, Cat#: ab6326). An MTT assay was also used to detect viable proliferating cells at 1, 3, 5, and 7 days after plating. In each well (4,000 cells/well), 50 μl of 1 mg/ml solution of MTT in PBS was added for 4 h on each of the cells. The results were measured at 560 nm test and 690 nm reference wavelength by using an automatic plate reader.

Cell Survival Evaluation

Human bone marrow-derived mesenchymal stem cells ($1 \times 10^4/cm^2$) were cultured for 72 h under hypoxic conditions.

Cell survival rate was measured by the Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan).

Cell Migration Assay

The trans-well assay was used to study hBM-MSCs migration. Briefly, cells were harvested and plated ($1 \times 10^4/cm^2$) in a trans-well cell culture insert (8-μm diameter pores). After 24 h in hypoxic conditions, cells that migrated to the other side of the membrane were fixed and stained. The wound-scratch assay was also used to study hBM-MSC migration ($2 \times 10^4/cm^2$). Scratches were created with a p200 pipette tip. After 12 h in hypoxic conditions, images were obtained using a microscope (Nikon Eclipse Ti) after washing.

SA-β-Gal Staining

Human bone marrow-derived mesenchymal stem cells ($2 \times 10^4/cm^2$) were stained with the senescence β-galactosidase staining kit (Cell Signaling, Cat#: 9860). Pictures were taken using a Nikon microscope.

Real-Time Reverse Transcription-Polymerase Chain Reaction

The expression of senescence-related genes (p16INK4a and p27Kip1) and lncRNAs were evaluated using real-time reverse transcription-polymerase chain reaction. GAPDH was used as a housekeeping gene. Real-time polymerase chain reaction was conducted using SensiFAST SYBR Green PCR Master Mix (Bioline USA Inc., Taunton, MA, United States), with the following parameters: 95°C 2 min; [95°C 5 s; 60°C 30 s for 40 cycles]. The oligonucleotide primer sequences are shown in **Supplementary Table S1**.

Western Blotting

For Western blotting, 50 μg of lysate was fractionated and transferred to a PVDF membrane. The blots were reacted with the antibodies (p16INK4a [Abcam, Cat#: ab54210], p27Kip1 [Abcam, Cat#: ab32034]) overnight at 4°C. And then were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature. For quantification, the density of the target bands were divided by the corresponding densitometry of β -tublin band.

Microarray and Computational Analysis

Total RNA was isolated from hBM-MSCs after cultured in hypoxic conditions for 72 h. 12 × 135K lncRNA Expression Microarray (Arraystar, Rockville, MD, United States) was used to detected hBM-MSCs cDNA. After hybridization, the processed slides were scanned by the Axon GenePix 4000B microarray scanner. NimbleScan software was used to extract the raw data. And NimbleScan software's implementation of RMA offered quantile normalization and background correction as previous (Jia et al., 2019). Differentially expressed genes were measured by the random variance model (Barter et al., 2017). A *p*-value was calculated by the paired *t*-test. The threshold set for up-regulated and down-regulated genes were fold change ≥ 2.0 and *p*-value ≤ 0.05. Cluster Tree-view software was used to perform hierarchical clustering, based on differentially expressed mRNAs and lncRNAs. Gene co-expression networks were used to identify interactions among genes. According to the normalized signal intensity of specific expressed genes, gene co-expression networks were built as previous (Bianchessi et al., 2015). The network adjacency was constructed between two genes, i and j, defined as a power of the Pearson correlation between the corresponding gene expression profiles xi and xj. The adjacency matrix M (i, j) was obtained and visualized as a graph, and the topological properties of this graph were measured. Only the strongest correlations (0.99 or greater) were drawn in these renderings (Li J. et al., 2013).

Lentiviral Vector Transduction

Lentiviral constructs for inhibition of lnc-CYP7A1 or SYNE1 in hBM-MSCs were ordered from OBio Co. (Shanghai, China). The sh-CYP7A1 sequence was GCAGTTCTTAGATTCCCTTTG. The sh-SYNE1 sequence was GCTGAAGTCTTGGATCATTAA.

Myocardial Infarction and Mice Heart Function Measurement

All animal procedures were approved by the Animal Care Committee of the Guangzhou Medical University. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, 8th Edition, 2011). Mice with infarct sizes between 30–35% of the left ventricular free wall were used in the following experiments ($n = 20$/group). HBM-MSCs (3×10^5 in 20 μl serum-free IMDM medium/mouse) were transplanted into three sites around the border zone immediately after MI. Serum-free IMDM medium was injected into the border zone as a negative control. Echocardiography was used to measure the mice heart function at different time points. The mice heart scar area and thickness were measured by planimetry. Cyclosporine A (5 mg/kg) was used to induce immunosuppression during the experiments as previous (Li S.-H. et al., 2013; Li et al., 2018).

Statistical Analysis

All values are expressed as mean SD. Analyses were performed using GraphPad InStat software (La Jolla, CA, United States). Student's *t*-test was used for two-group comparisons. Comparisons of parameters among three or more groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey, or two-way ANOVA with repeated measures over time, which were succeeded by Bonferroni *post hoc* tests for multiple comparisons. Differences were considered statistically significant at $P < 0.05$.

RESULTS

The Proliferative and Migratory Functions of hBM-MSCs Were Decreased With Aging

First, BrdU pulse-chasing was used to evaluate cell proliferation in old (O) and young (Y) hBM-MSCs after isolation and

culturing. When compared to Y hBM-MSCs, the percentage of $BrdU^+$ cells was significantly lower in O hBM-MSCs (**Figures 1A,B**). The same trend was found by the MTT assay, showing decreased cell proliferative activity in O hBM-MSCs (**Figure 1C**). The cell survival was decreased in O compared with Y hBM-MSCs when evaluated by CCK-8 assay (**Figure 1D**). Next, cell migration was detected by transwell and wound-scratch assays (**Figures 1E–H**), which both showed significantly lower migration rates (**Figures 1F,H**) in O compared to YhBM-MSCs. Senescence-associated beta galactosidase (SA-β-Gal) staining revealed more positive cells in O than Y hBM-MSCs (**Figures 1I,J**). Accordingly, the expression of senescence-related genes, $p16^{INK4a}$ and $p27^{Kip1}$, were significantly increased (**Figure 1K**) in O than in Y hBM-MSCs. This finding was confirmed by Western blots, showing increased protein expression of $p16^{INK4a}$ and $p27^{Kip1}$ in O relative to Y hBM-MSCs (**Figures 1L,M**). All these results pointed out the decreased proliferative and migratory abilities, and increased cell senescence, in O compared to Y hBM-MSCs.

Expression Profiles of lncRNAs in Y and O hBM-MSCs

Microarrays were carried out to profile the expression of different lncRNAs and mRNAs in Y and O hBM-MSCs. Twelve lncRNAs were identified as significantly increased in O compared with that of Y hBM-MSCs, by using significance analysis of microarray software (**Figure 2A**). Next, real-time qPCR was used to validate the microarray data. Notably, SH3TC2-DT, lnc-RBBP6-4, LINC01809, TRHDE-AS1, LINC02372, lnc-OR4F5-7, lnc-CYP7A1-1, LINC00222, LINC01366, LINC02267, TNFRSF14-AS1 and lnc-MYO10-2 were significantly increased in O compared to Y hBM-MSCs (**Figure 2B**). Furthermore, lnc-CYP7A1-1 showed the most dramatic increase with a 5.29-fold increase in O, compared to Y hBM-MSCs, suggesting it may play an active role in cell aging and senescence.

Down-Regulation of lnc-CYP7A1-1 in O hBM-MSCs Restored Cell Regenerative Functions

Next, to investigate whether down-regulation of lnc-CYP7A1-1 can restore some aspects of regenerative function in O hBM-MSCs, a lentiviral construct was produced (sh-CYP7A1) to inhibit lnc-CYP7A1-1 expression in O hMSCs (O-sh-CYP7A1, **Supplementary Figure S1**). In accordance with our expectations, cell proliferation was increased in O-sh-CYP7A1 when compared to control lentivirus-transduced O hMSCs (O-c). The percentage of $BrdU^+$ cells was much higher in O-sh-CYP7A1, compared to control O-c (**Figures 3A,B**), and the same trend was also observed by the MTT assay (**Figure 3C**). Next, the cell survival was increased in O-sh-CYP7A, compared to O-c, in the CCK-8 assay (**Figure 3D**). Cell migration, when detected by trans-well and wound-scratch assays (**Figures 3E–H**), was significantly higher in O-sh-CYP7A1 compared to O-c. SA-β-Gal staining revealed fewer positive cells in O-sh-CYP7A1 than O-c (**Figures 3I,J**). The mRNA expression of $p16^{INK4a}$ and $p27^{Kip1}$ was significantly decreased (**Figure 3K**) in O-sh-CYP7A1 than O-c, and this was confirmed by Western blots showing decreased protein expression of $p16^{INK4a}$ and $p27^{Kip1}$ in O-sh-CYP7A1, relative to O-c (**Figures 3L,M**). These results revealed that inhibition of lnc-CYP7A1-1 restored the regenerative capacity, and decreased cell senescence, in O hBM-MSCs.

The Expression of SYNE1 Was Inhibited by lnc-CYP7A1-1

Gene co-expression network was built to detect the interactions among lncRNA and genes (**Figure 4A**). SYNE1 was identified as a strong candidate to interact with lncRNA CYP7A1-1. SYNE1 (aka Nesp-1) is a component of the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex, which connects the nuclear envelope to the cytoskeleton. The expression of SYNE1 was significantly decreased in O compared to Y hBM-MSCs, in contrast to the increase of lnc-CYP7A1-1 at O hBM-MSCs (**Figure 4B**). The down-regulation of SYNE1 in O hBM-MSCs was confirmed by real-time qPCR (**Figure 4C**). On the other hand, compared to control lentiviral transduced O-c, the expression of SYNE1 was significantly increased when lnc-CYP7A1-1 was down-regulated in O-sh-CYP7A1 hBM-MSCs (**Figure 4D**), strongly suggesting SYNE1 as the underlying target gene of lnc-CYP7A1-1.

Down-Regulation of SYNE1 in Y hBM-MSCs Decreased Cell Regenerative Functions

To further explore the role of SYNE1, relative to cell regenerative capacity, a lentiviral construct was produced (sh-SYNE1) to inhibit SYNE1 expression in Y hBM-MSCs (Y-sh-SYNE1, **Supplementary Figure S2**). Cell proliferation was examined in Y-sh-SYNE1, and compared to control lentivirus transduced Y hMSCs (Y-c). The percentage of $BrdU^+$ cells was much lower in Y-sh-SYNE1, compared to control Y-c (**Figures 5A,B**), and similar result was observed by MTT assay (**Figure 5C**). Next, cell survival was decreased in Y-sh-SYNE1, compared to Y-c, in the CCK-8 assay (**Figure 5D**). Cell migration, again detected by trans-well and wound-scratch assays (**Figures 5E–H**), was significantly lower in Y-sh-SYNE1 than Y-c. SA-β-Gal staining revealed more positive cells in Y-sh-SYNE1 than Y-c (**Figures 5I,J**). The mRNA expression of $p16^{INK4a}$ and $p27^{Kip1}$ was significantly increased (**Figure 5K**) in Y-sh-SYNE1 than Y-c. Western blots confirmed increased $p16^{INK4a}$ and $p27^{Kip1}$ protein expression in Y-sh-SYNE1, relative to Y-c (**Figures 5L,M**). All these findings suggested SYNE1 as an important factor involved in cell regeneration.

Down-Regulation of SYNE1 in O-sh-CYP7A1 hBM-MSCs Reduced Cell Regenerative Ability

To confirm the causative relationship between lnc-CYP7A1-1 and SYNE1, the expression of SYNE1 was inhibited in O-sh-CYP7A1 hBM-MSCs by transduction with the sh-SYNE1 lentivirus (O-sh-CS, **Supplementary Figure S3**). Cell proliferation was assessed in O-sh-CS and compared to negative control lentivirus transfected O-sh-CYP7A1 hBM-MSCs (O-sh-CC). The percentage of $BrdU^+$ cells was lower in

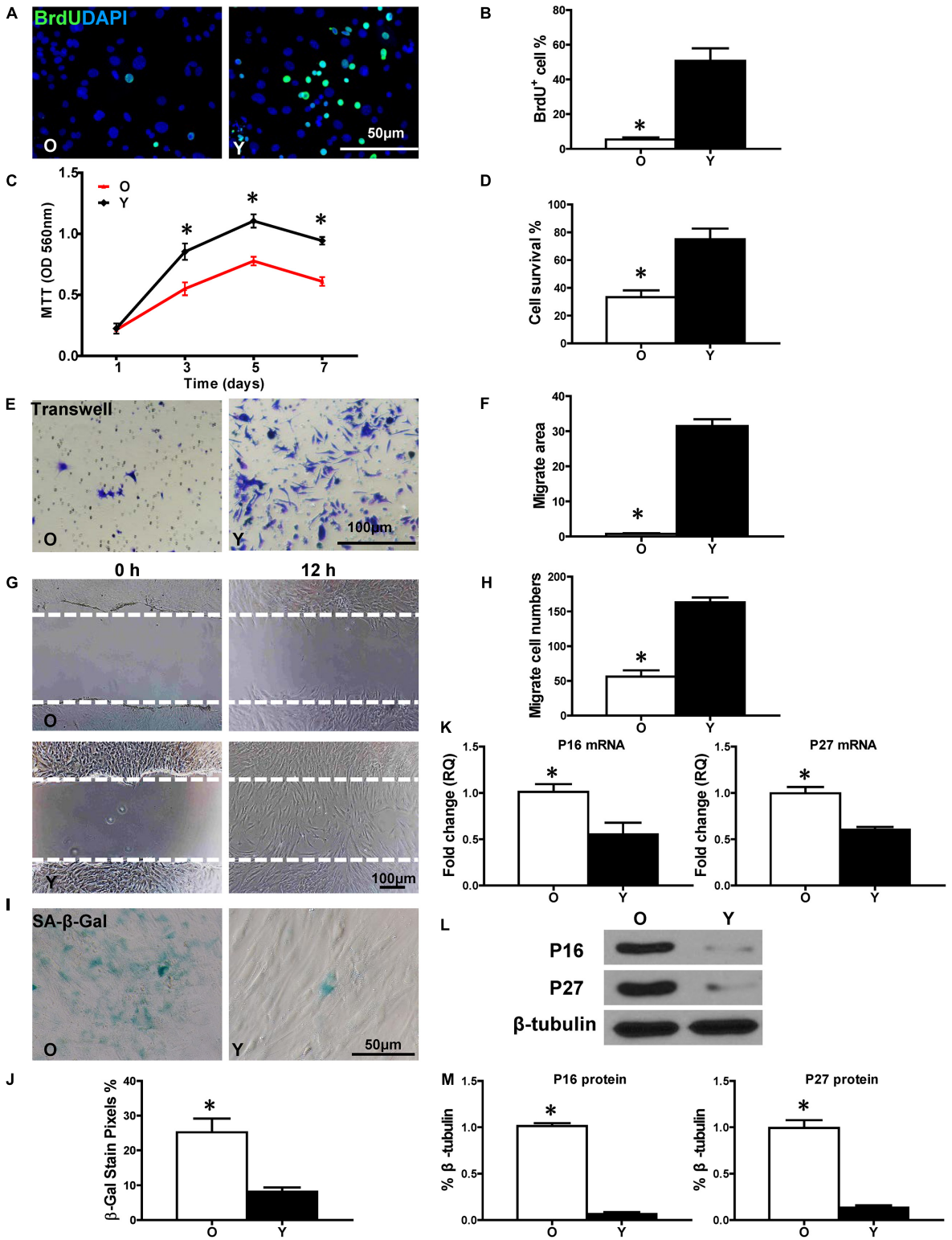

FIGURE 1 | Increased cell senescence and decreased regenerative function in aged hBM-MSCs. The cell regenerative function of old (O) and young (Y) hBM-MSCs was compared. **(A)** Immunofluorescence staining of BrdU and **(B)** quantification of $BrdU^+$ (proliferating cells) cells in the O and Y hBM-MSCs. **(C)** Cell proliferation was determined by the MTT assay. **(D)** Cell survival was evaluated in O and Y hBM-MSCs. Cell migration was evaluated by the trans-well **(E,F)** and wound-scratch **(G,H)** assays. SA-β-Gal staining and quantification of cell senescence in O and Y hBM-MSCs **(I,J)**. The expression of senescence-related genes **(K)** and proteins of $p16^{INK4a}$ and $p27^{Kip1}$ **(L,M)** in O and Y hBM-MSCs. n = 6/group for all the assays; $^*P < 0.05$ O vs. Y.

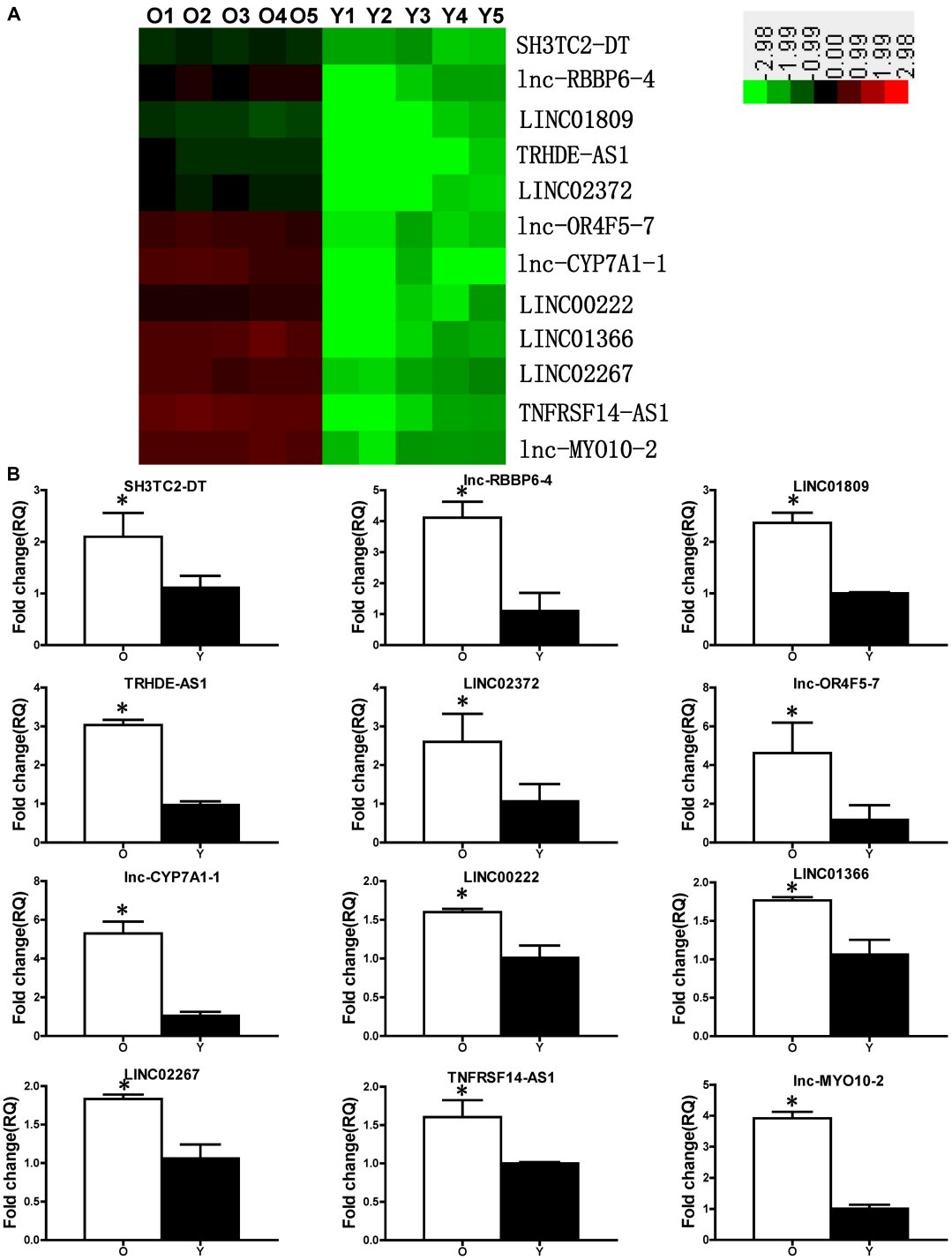

FIGURE 2 | Expression profile of lncRNAs in young and old hBM-MSCs. **(A)** lncRNA expression in old (O) and young (Y) hBM-MSCs was determined by microarray analysis. **(B)** Differential lncRNA expression was validated by real-time qPCR. n = 5/group; *P < 0.05 O vs. Y.

O-sh-CS compared to O-sh-CC (**Figures 6A,B**). This result was confirmed by the MTT assay (**Figure 6C**). Next, cell survival was decreased in O-sh-CS, compared with O-sh-CC, in the CCK-8 assay (**Figure 6D**). Cell migration was evaluated by trans-well and wound-scratch assays (**Figures 6E–H**), and was found to be significantly lower in O-sh-CS, compared to O-sh-CC. SA-β-Gal staining revealed more positive cells in O-sh-CS than in O-sh-CC (**Figures 6I,J**). The $p16^{INK4a}$ and $p27^{Kip1}$ mRNA expression was significantly increased (**Figure 6K**) in O-sh-CS than in O-sh-CC, and this result was further confirmed at the

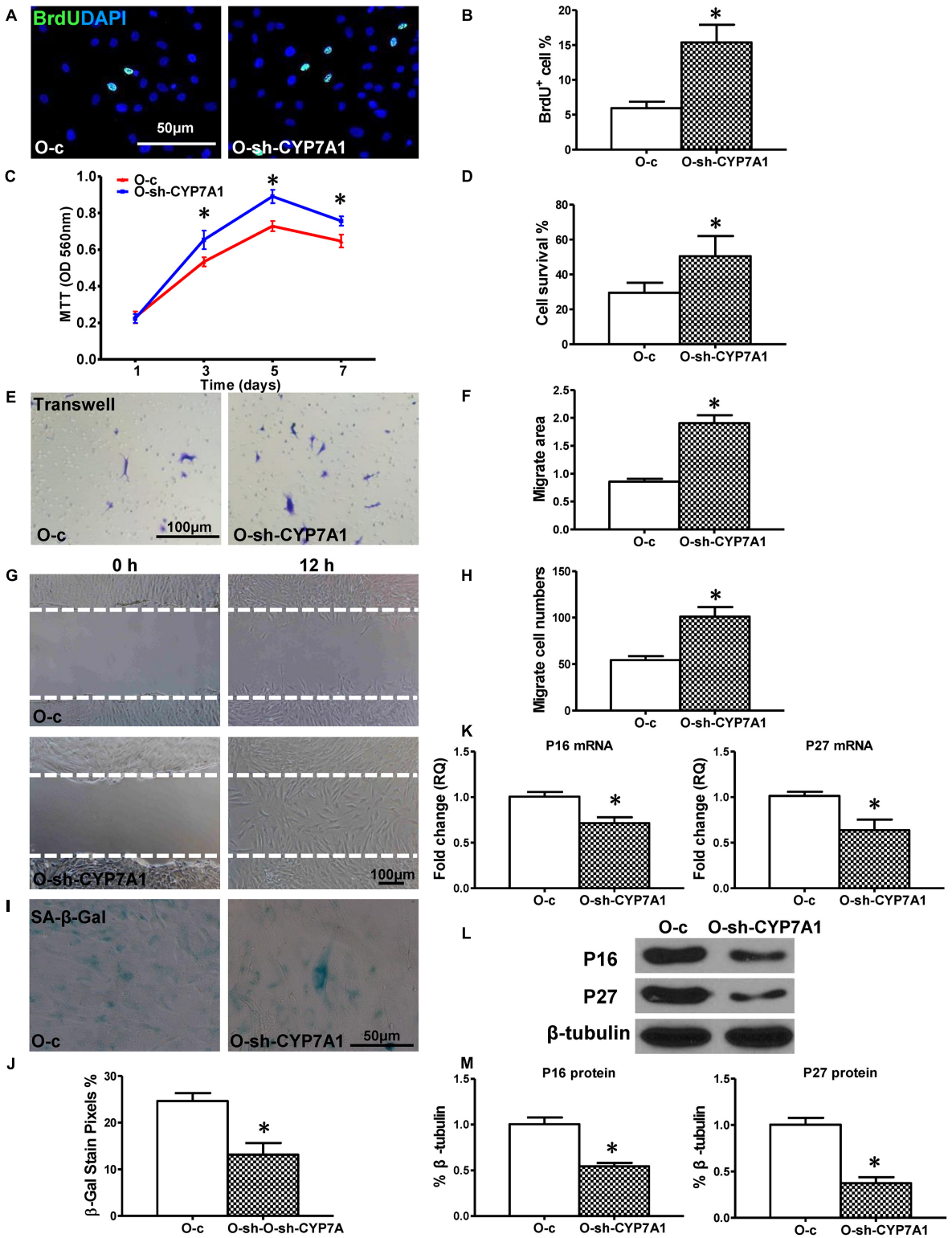

FIGURE 3 | Down-regulation of lnc-CYP7A1-1 in old hBM-MSCs restored cell regenerative function and decreased senescence. Old (O) hBM-MSCs were transfected with inhibition lentivirus (O-sh-CYP7A1) or control lentivirus (O-c), respectively. Cell regenerative function was compared. **(A)** Immunofluorescent staining of BrdU and **(B)** quantification of $BrdU^+$ (proliferating) cells in the hBM-MSCs. **(C)** Cell proliferation was determined by the MTT assay. Cell survival was evaluated in hBM-MSCs **(D)**. Cell migration was evaluated by the trans-well **(E,F)** and wound scratch **(G,H)** assays. SA-β-Gal staining and quantification of cell senescence in hBM-MSCs **(I,J)**. The expression of senescence-related genes **(K)** and proteins of $p16^{INK4a}$ and $p27^{Kip1}$ **(L,M)** in hBM-MSCs. $n = 6$/group for all the assays; $^*P < 0.05$ O-sh-CYP7A1 vs. O-c.

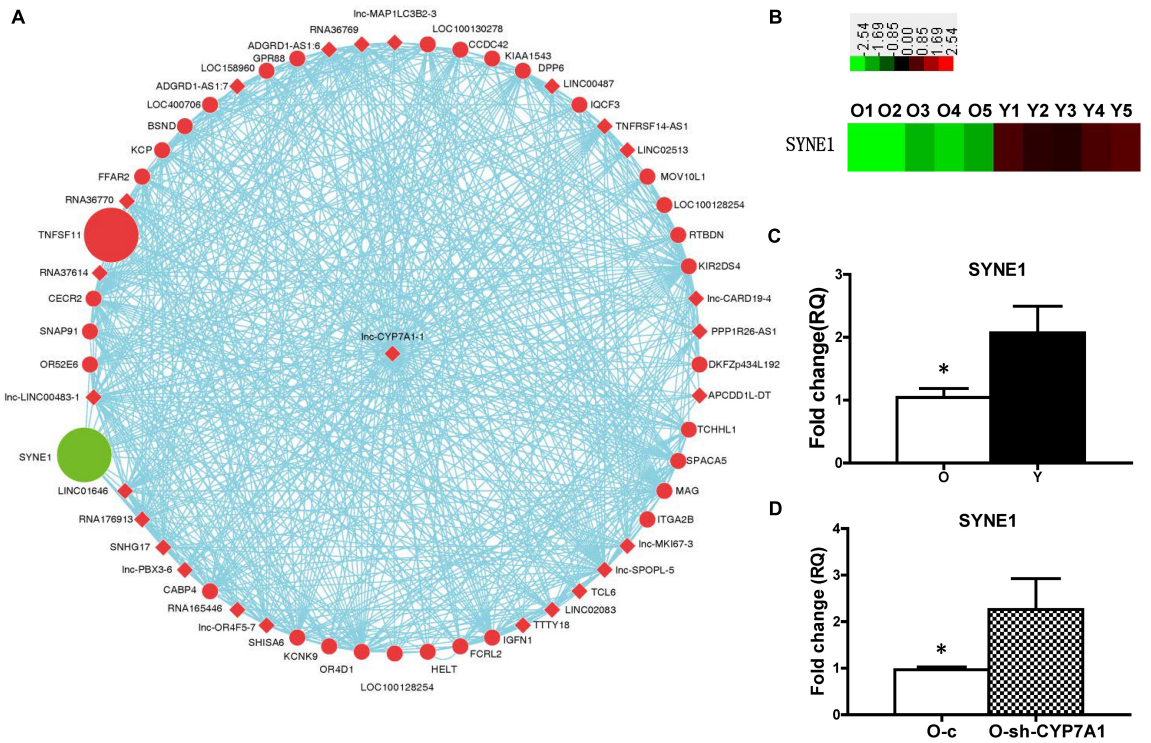

FIGURE 4 | The expression of SYNE1 was inhibited by lnc-CYP7A1-1. **(A)** Gene co-expression networks were built to detect the interactions among lncRNAs and genes. Red color indicates expression up-regulated and green color indicates expression down-regulated. The dots represents genes and the diamond represents lncRNAs. **(B)** The expression of SYNE1 in old (O) and young (Y) hBM-MSCs was determined by microarray analysis. **(C)** The expression of SYNE1 in Old (O) and young (Y) hBM-MSCs was compared by real-time qPCR. **(D)** Old (O) hBM-MSCs were transfected by inhibition lentivirus (O-sh-CYP7A1) or control lentivirus (O-c), respectively. The expression of SYNE1 was compared by real-time qPCR. n = 5/group; $^*P < 0.05$ O vs. Y, O-c vs. O-sh-CYP7A1.

protein level by Western blot (**Figures 6L,M**). All these findings strengthened our hypothesis that SYNE1 is the underlying target gene of lnc-CYP7A1-1.

Implantation of lnc-CYP7A1-1 Downregulated O hBM-MSCs Into Infarcted Mouse Hearts Improved Heart Function After MI

To evaluate whether down-regulation of lnc-CYP7A1-1 levels in O hBM-MSCs can maximize the beneficial effects of stem cell therapy, CYP7A1-1-downregulated O hBM-MSCs (O-sh-CYP7A1) were implanted into infarcted mouse hearts. Heart function was measured by echocardiography in mice which received implantation of control medium (Media), control vector-transfected O hBM-MSCs (O-c), or CYP7A1-1 downregulated O hBM-MSCs (O-sh-CYP7A1), into the border region immediately after MI. Heart function was evaluated before MI (0 days), as well as 1, 7, 14, and 28 days after MI. Representative M-mode echocardiographic images was taken 28 days post MI (**Figure 7A**). After MI, there was a significant decrease in ejection fraction (EF; **Figure 7B**) and fractional shortening (FS; **Figure 7C**), along with an increase in left ventricular internal end-diastolic dimension (LVIDd; **Figure 7D**) and left ventricular internal end-systolic dimension (LVIDs; **Figure 7E**), in all three groups. However, there was an improvement in all of these parameters in the O-sh-CYP7A1 group when compared with O-c and media groups (**Figures 7B–E**). Similarly, the infarct size at 28 days post MI was smaller (**Figures 7F,G**), and the scar thickness (**Figure 7H**) larger in O-sh-CYP7A1, when compared with the O-c and the media groups. All evidence, therefore, indicated that the down-regulation of lnc-CYP7A1-1 enhanced the therapeutic efficacy of O hBM-MSCs and effectively improved heart function. The survival of implanted lnc-CYP7A1-1-downregulated old hBM-MSCs was also evaluated through lentiviral-mediated GFP expression in the border region of the mouse hearts at 3 days post MI. In agreement with *in vitro* data, downregulation of lnc-CYP7A1-1 expression (O-sh-CYP7A1) increased implanted cell survival when compared with the group receiving O-c hBM-MSCs (**Supplementary Figure S4**).

Down-Regulation of lnc-CYP7A1-1 in O hBM-MSCs Changed the Cell Paracrine Function *in vitro*

To investigate whether down-regulation of lnc-CYP7A1-1 can change the cell paracrine function. Control vector-transfected O hBM-MSCs (O-c) or CYP7A1-1 downregulated O hBM-MSCs (O-sh-CYP7A1) were cultured in serum-free medium

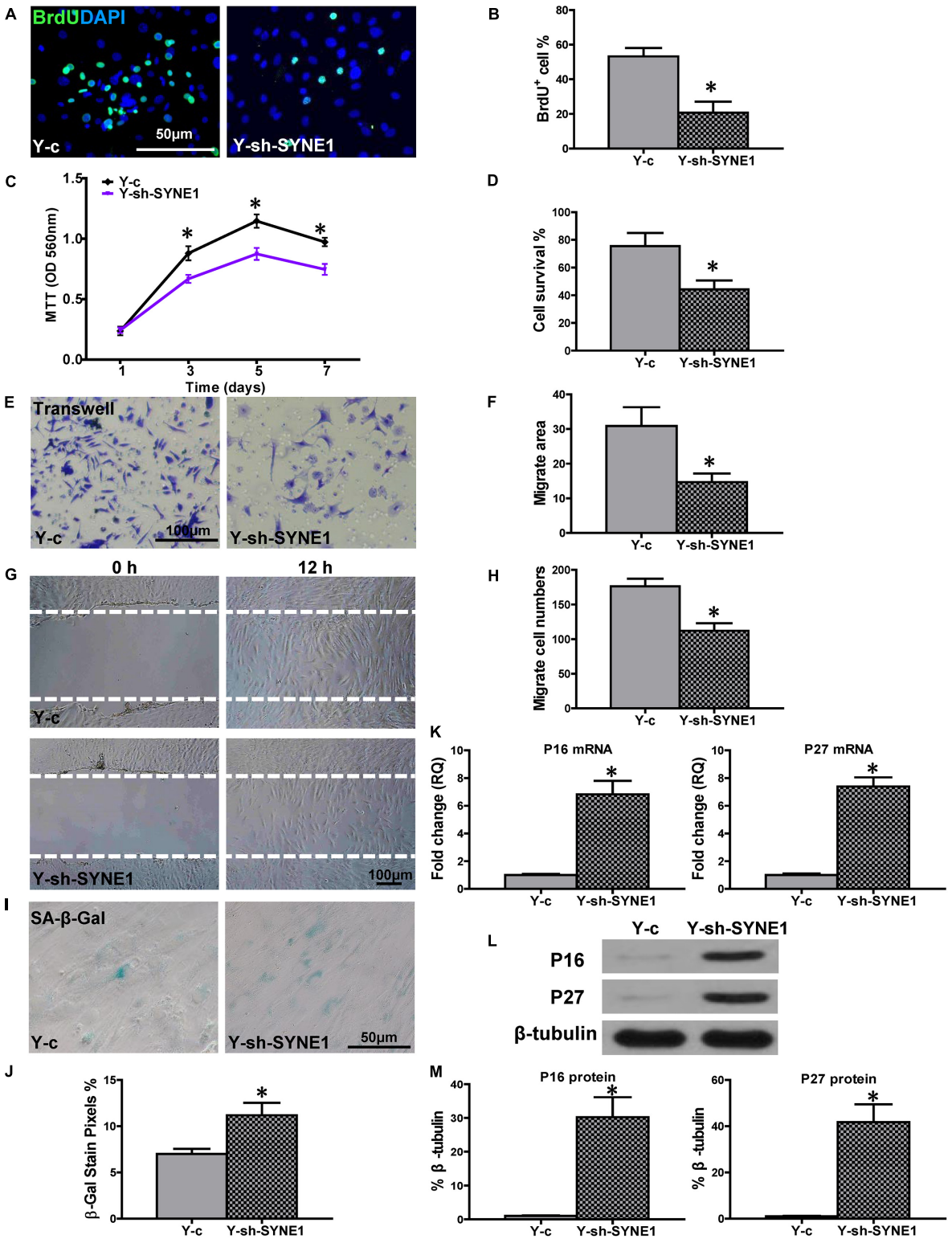

FIGURE 5 | Down-regulation of SYNE1 in Y hBM-MSCs decreased cell regenerative functions. Young (Y) hBM-MSCs were transfected by inhibition lentivirus (Y-sh-SYNE1) or control lentivirus (Y-c), respectively, and cell regenerative functions were compared. **(A)** Immunofluorescent staining of BrdU and **(B)** quantification of $BrdU^+$ (proliferating) cells in the hBM-MSCs. **(C)** Cell proliferation was determined by the MTT assay. Cell survival was evaluated in hBM-MSCs **(D)**. Cell migration was evaluated by the transwell **(E,F)** and wound-scratch **(G,H)** assays. SA-β-Gal staining and quantification of cell senescence in hBM-MSCs **(I,J)**. The expression of senescence-related genes **(K)** and proteins of $p16^{INK4a}$ and $p27^{Kip1}$ in hBM-MSCs **(L,M)**. n = 6/group for all the assays; $^*P < 0.05$ Y-sh-SYNE1 vs. Y-c.

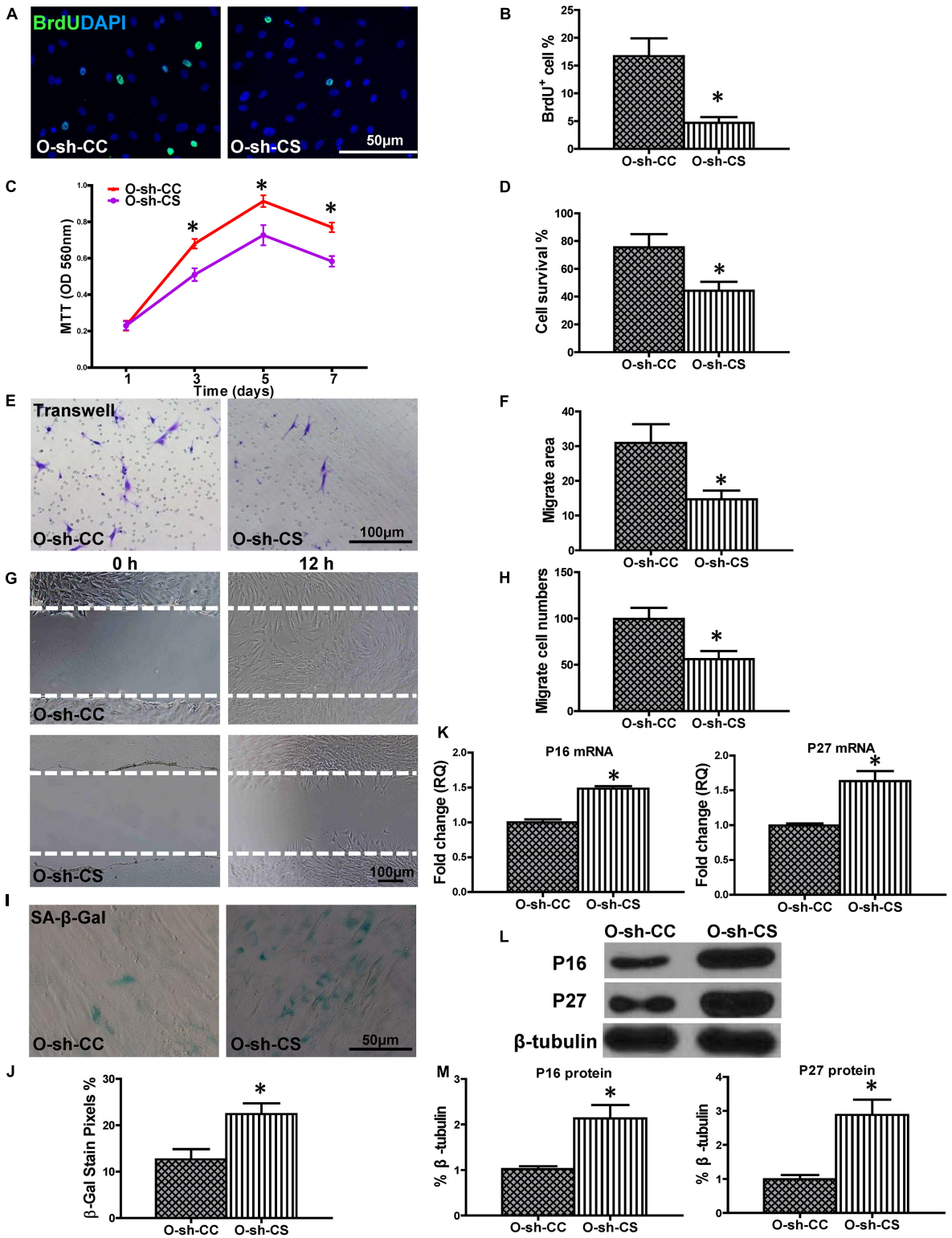

FIGURE 6 | Down-regulation of SYNE1 in O-sh-CYP7A1 hBM-MSCs reduced cell regenerative ability. O-sh-CYP7A1 hBM-MSCs were transfected by SYNE1 inhibition lentivirus (O-sh-CS) or control lentivirus (O-sh-CC), respectively, and cell regenerative functions were compared. **(A)** Immunofluorescent staining of BrdU and **(B)** quantification of $BrdU^+$ (proliferating cells) cells in the hBM-MSCs. **(C)** Cell proliferation was determined by the MTT assay. Cell survival was evaluated in the hBM-MSCs **(D)**. Cell migration was evaluated by the transwell **(E,F)** and wound scratch **(G,H)** assays. SA-β-Gal staining and quantification of cell senescence in hBM-MSCs **(I,J)**. The expression of senescence-related genes **(K)** and proteins of $p16^{INK4a}$ and $p27^{Kip1}$ in the hBM-MSCs **(L,M)**. $n = 6$/group; $^*P < 0.05$ O-sh-CS vs. O-sh-CC.

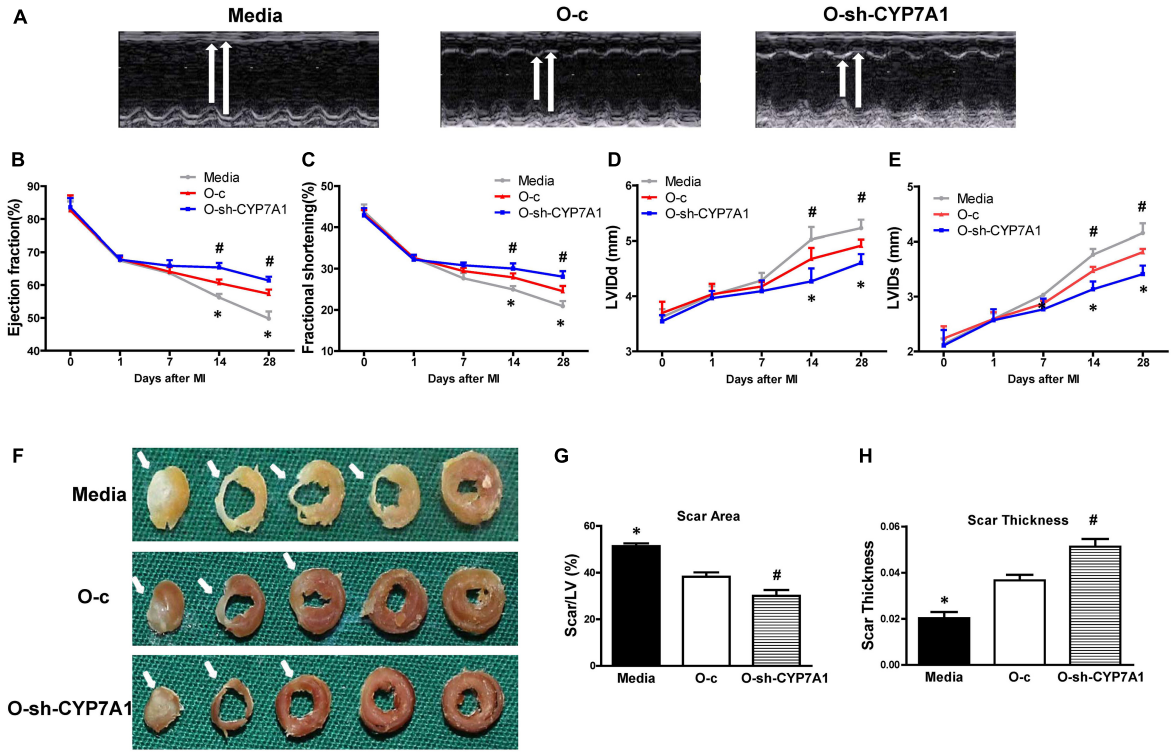

FIGURE 7 | Implantation of lnc-CYP7A1-1 downregulated old hBM-MSCs into infarcted mouse hearts improved cardiac function after MI. Cardiac function was determined by echocardiography in mice which received implantations of control medium (Media), control vector-transfected old hBM-MSCs (O-c), or lnc-CYP7A1-inhibited old hBM-MSCs (O-sh-CYP7A1) into the border region immediately following myocardial infarction (MI). Cardiac function measured by echocardiography, before (0 day), as well as 1, 7, 14, and 28 days after MI. **(A)** Representative M-mode echocardiographic images. **(B)** Ejection fraction. **(C)** Fractional shortening. **(D)** Left ventricular internal end-diastolic dimension (LVIDd). **(E)** Left ventricular internal end-systolic dimension (LVIDs). **(F)** Representative whole sectioned heart (from base to apex) at 28 days after MI to show scar areas (arrows) **(G)** and scar size thickness **(H)**. n = 6/group for all the experiments. $^{*}P < 0.05$, Media vs. other groups; $^{\#}P < 0.05$, O-sh-CYP7A1 vs. O-c.

under hypoxic conditions (0.1% O_2) for 72 h. The mRNA expression of vascular endothelial growth factor A (VEGFA), platelet derived growth factor A (PDGFA), fibroblast growth factor 2 (FGF2), insulin-like growth factor 1 (IGF1), transforming growth factor beta-1 (TGFB1), angiogenin (ANG), and C-C motif chemokine ligand 2 (CCL2) was quantified by RT-qPCR. The mRNA expression of VEGFA, PDGFA, and FGF2 was increased in O-sh-CYP7A1 when compared to O-c group (**Supplementary Figure S5**). These results revealed that inhibition of lnc-CYP7A1-1 may increase the cell paracrine function in O hBM-MSCs.

DISCUSSION

In this study, we found that lnc-CYP7A1-1 contributed to hBM-MSCs senescence, as the evidence showed that increased lnc-CYP7A1-1 expression in old hBM-MSCs was associated with decreased cell proliferative ability, survival, and migratory ability, along with increased senescence and senescence-related gene expression. In contrast, down-regulation of lnc-CYP7A1-1 improved regenerative capacities, and decreased cell senescence, in old hBM-MSCs. Based on predictive software, we characterized a putative lnc-CYP7A1-1 interacting gene, SYNE1, whose expression was decreased in old hBM-MSCs. Through inhibiting SYNE1 expression in young hBM-MSCs, using a lentiviral construct, we confirmed the role of SYNE1 as an important factor involved in cell regeneration. Furthermore, via down-regulation of SYNE1 expression in O-sh-CYP7A1 hBM-MSCs, we established the causative relationship between lnc-CYP7A1-1 and SYNE1, showing that inhibition of SYNE1 reversed the beneficial effects stemming from lnc-CYP7A1-1 down-regulation in old hBM-MSCs. *In vivo* implantation of lnc-CYP7A1-1 downregulated old hBM-MSCs into infarcted mouse hearts improved cardiac function after MI, suggesting that down-regulation of lnc-CYP7A1-1 enhanced the therapeutic efficacy of old hBM-MSCs for cardiac repair.

Long non-coding RNAs have been reported as an unexploited reservoir of potential therapeutic targets for reprogramming cell function and aging. Huang et al. (2015) found that lncRNA H19 promotes osteogenic differentiation of hMSCs, and miR-675 partially mediates lncRNA H19-induced pro-osteogenic activity. The expression of TGF-b1, HDAC4/5 and Smad3 phosphorylation was decreased, but the expression of osteogenic markers was increased by H19/miR-675 (Huang et al., 2015). LINC00707 has also been proved to directly bind to miR-370-3p and promote osteogenic differentiation in hMSCs (Jia et al.,

2019). Studies have reported that lncRNA ROCR increases during chondrogenic differentiation of hMSCs; the lncRNA is involved in inducing SOX9 gene and cartilage gene expression there (Barter et al., 2017). The role of lncRNAs has also been studied in other cell types. Compared to early passage fibroblasts (young), late-passage fibroblasts (old) have decreased expression of SAL-RNA1. Furthermore, inhibiting the expression of SAL-RNA1 in fibroblasts increases cell senescence (Abdelmohsen et al., 2013). Another study in fibroblasts showed that Zeb2-NAT lncRNA has higher expression levels in old fibroblast cells, and modulation of its expression can improve pluripotent cell reprogramming from old fibroblasts (Bernardes de Jesus et al., 2018). In aging and senescent endothelial cells, ASncmtRNA-2 accumulates at the G2/M phase of cell cycle, and causes cell aging by inducing hsa-miR-4485 and hsa-miR-1973 expression (Bianchessi et al., 2015). In agreement with our findings, all pieces of evidence point toward the important role of lncRNAs for reprogramming cell function and aging.

In our previous studies, we have demonstrated that with aging, proliferation and differentiation capacity decreased, and cell senescence increased, in hBM-MSCs (Li J. et al., 2013; Dong et al., 2018). Effective strategies to rejuvenate aged hBM-MSCs to improve their regenerative capability are required to maximize the beneficial effects of stem cell therapy, such as the small RNAs (Liu et al., 2019). By bone marrow reconstitution animal model, we also demonstrated that young bone marrow Scal-1 cells can rejuvenate age animal heart function after MI. Examination of the underlying molecular mechanisms revealed that young bone marrow Scal-1 cells secreted more growth factors, such as Tgfβ1 and Cxcl12, in order to regenerate the aged heart (Li et al., 2018, 2019). In this study, we also detected paracrine functional changes, with down-regulation of lnc-CYP7A1-1 in O hBM-MSCs. The mRNA expression of VEGFA, PDGFA, and FGF2 was increased in O-sh-CYP7A1 when compared to O-c groups. These results revealed that inhibition of lnc-CYP7A1-1 may potentiate paracrine functions in O hBM-MSCs. In the present study, we identified target lncRNAs associated with MSCs aging. These aging related lncRNAs may modulate the regenerative abilities of hBM-MSCs. Indeed, we specifically identified lncRNA CYP7A1-1, as it had the most dramatic increase in expression, and showed the most prominent effects in aged hBM-MSCs. Lnc-CYP7A1-1 is an intergenic lncRNA, located on human Chromosome 8 (hg38 chr8:58258605-58272587). We found that suppression of lnc-CYP7A1-1 expression improved proliferation, cell survival, migration, and paracrine function along with reducing senescence in old hBM-MSCs, which is consistent with the idea that lncRNA contributes to the pathological phenotypes associated with hBM-MSCs (Barter et al., 2017).

A portion of the difficulties for studying lncRNAs in a biological context is in determining their underlying mediators. Using predictive software, we found a putative interacting partner of lnc-CYP7A1-1, SYNE1 (Nesprin-1). SYNE1 is a structural protein that links the nucleus to the cytoskeleton. It plays a role in cardiomyocyte and skeletal muscle development, particularly with respect to the DNA damage response pathway (Razafsky and Hodzic, 2015). In Emery–Dreifuss muscular dystrophy and dilated cardiomyopathy patients, mutations in SYNE1 have been found, which affects nuclear morphology and impairs protein-protein interaction with lamin A/C and SUN2 (Meinke et al., 2014). Defection in myoblast differentiation and fusion are observed when expressing SYNE1 mutants in C2C12 cells (Zhou et al., 2017). SYNE1 and SYNE2 have also been reported to play key roles in neurogenesis and neuronal migration in mice (Zhang et al., 2009). In human umbilical vein endothelial cells, cell migration and endothelial loop formation capacity is decreased when either SYNE1 or SYNE2 is suppressed (King et al., 2014). The modulation of SYNE1 expression also impacted stem cell pluripotency and differentiation capacity (Smith et al., 2011; Yang et al., 2015). Furthermore, SYNE1 has been reported to participate in laminopathies and lamin-associated signaling pathways, leading to laminopathies and premature aging (Zhavoronkov et al., 2012). Most importantly, inhibiting the expression of SYNE1 in rat MSCs decreases cell proliferation and increases apoptosis (Yang et al., 2013). In the present study, we also found that SYNE1 had a beneficial effect on BM-MSC function, as its loss in young BM-MSCs reduced their proliferative capacity. Interestingly, lnc-CYP7A1-1 and SYNE1 show inverse expression patterns during aging, where SYNE1 was abundant in young BM-MSCs, while lnc-CYP7A1-1 was highly expressed in old BM-MSCs. Loss of lnc-CYP7A1-1 in old BM-MSCs increased SYNE1 expression, implying possible negative regulation of SYNE1 by lnc-CYP7A1-1. Additionally, the beneficial effects of lnc-CYP7A1-1 knockdown on old BM-MSCs were lost when SYNE1 expression was also reduced, suggesting that lnc-CYP7A1-1 and SYNE1 act on a shared signaling pathway in modulating BM-MSC function. All these findings supported our notion that SYNE1 play an important role in mediating cell function and regeneration, and may be the key downstream mediator of lnc-CYP7A1-1. Recent studies have found that, as pseudogenes, lncRNAs can act as miRNA "sponges" by sharing common microRNA recognition elements (MREs), thereby inhibiting normal miRNA activity (Yang et al., 2018). In a study related to hMSCs, Jia et al. have reported that LINC00707 effectively inhibits miR-370-3p to promote osteogenesis, in which it serves as a competing endogenous RNA for the target gene of miR-370-3p, WNT2B. By directly binding miR-370-3p, LINC00707 upregulates WNT2B expression (Jia et al., 2019). In a study to evaluate the chondrogenic differentiation of hMSCs, lncRNA ADAMTS9-AS2 has been reported to serve as a competing endogenous RNA for miR-942-5p, which is involved in regulating the expression of Scrg1, a transcription factor promoting chondrogenic gene expression. There, lncRNA ADAMTS9-AS2 controls hMSC chondrogenic differentiation (Huang et al., 2019). We postulate that lnc-CYP7A1-1 may act in a similar fashion in competing with microRNAs to regulate SYNE1 and hBM-MSCs functions. In our on-going study, via the MicroRNA Target Prediction Database, we found that miR-144, miR-597, and miR-421 may interact with lnc-CYP7A1-1, which may play a role in regulating SYNE1.

Mechanistically, based on our *in vitro* data showing increased cell proliferative and migratory abilities, we postulate that downregulation of lnc-CYP7A1-1 in old hBM-MSCs may improve cell survival, thus increasing the regenerative capacities

of old hBM-MSCs after transplantation into infarcted mouse hearts. Indeed, we found that in agreement with our *in vitro* data, downregulation of lnc-CYP7A1-1 expression (O-sh-CYP7A1) increased implanted cell survival when compared with the group receiving O-c hBM-MSCs. Furthermore, we found that downregulation of lnc-CYP7A1-1 (O-sh-CYP7A1) increased expression of VEGFA, PDGFA, and FGF2 when compared with the O-c group, suggesting greater angiogenic potentials among these cells. In the present study, we focused on the discovery of this senescence-associated lncRNA (lnc-CYP7A1-1) and the cardio-protection effects after its downregulation in old hBM-MSCs. In our future studies, we will dissect the detailed cardioprotective mechanisms associated with the downregulation of lnc-CYP7A1-1 in old hBM-MSCs *in vivo*. We will further evaluate the survival of lnc-CYP7A1-1-downregulated old hBM-MSCs and its paracrine and angiogenic potentials *in vivo*.

CONCLUSION

In summary, we have proved that the lncRNA lnc-CYP7A1-1 contributed to hBM-MSCs senescence, as the evidence showed that increased lnc-CYP7A1-1 expression in old hBM-MSCs were associated with decreased cell proliferative ability, cell survival and migratory ability, as well as increased senescence and the condition's associated gene expression. Downregulation of lnc-CYP7A1-1 improved cell regenerative capacities and decreased cell senescence in old hBM-MSCs, probably through upregulation of its target gene SYNE1. *In vivo* implantation of lnc-CYP7A1-1-downregulated old hBM-MSCs into infarcted mouse hearts improved cardiac function after MI, suggesting that down-regulation of lnc-CYP7A1-1 rejuvenated old hBM-MSCs and improved their regenerative capability for cardiac repair. Modulation of lnc-CYP7A1-1 levels may offer a useful therapeutic intervention to maximize the efficacy of stem cell therapy.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

JD, JWL, and YW contributed to analysis and interpretation of the data. ST, CZ, HZ, ZH, YF, and DZ collected and analyzed the *in vivo* data. SL, ZZ, and JL designed the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.600304/full#supplementary-material

Supplementary Figure 1 | The expression of lnc-CYP7A1-1 was inhibited by lentivirus. **(A)** To inhibit lnc-CYP7A1-1 expression in Old (O) hBM-MSCs, a lentiviral construct was produced (O-sh-CYP7A1). **(B)** The down-regulation of lnc-CYP7A1-1 was confirmed by real-time qPCR. $n = 5$/group; $^{*}P < 0.05$ O-sh-CYP7A1 vs. O-c. O-c: control lentivirus transduced O hBM-MSCs.

Supplementary Figure 2 | The expression of SYNE1 was inhibited by lentivirus in Y hBM-MSCs. **(A)** To inhibit SYNE1 expression in young (Y) hBM-MSCs, a lentiviral construct was produced (Y-sh-SYNE1). **(B)** The down-regulation of SYNE1 was confirmed by real-time qPCR. $n = 5$/group; $^{*}P < 0.05$ Y-sh-SYNE1 vs. Y-c. Y-c: control lentivirus transduced Y hBM-MSCs.

Supplementary Figure 3 | Down-regulation of SYNE1 in O-sh-CYP7A1 hBM-MSCs. O-sh-CYP7A1 hBM-MSCs were transfected by SYNE1 inhibition lentivirus (O-sh-CS) or control lentivirus (O-sh-CC), respectively. The down-regulation of SYNE1 was confirmed by real-time qPCR. $n = 5$/group; $^{*}P < 0.05$ O-sh-CC vs. O-c, $^{\#}P < 0.05$ O-sh-CS vs. O-sh-CC.

Supplementary Figure 4 | Lnc-CYP7A1-1 down-regulation increased old hBM-MSCs survival *in vivo*. The survival of the implanted cells was detected by green fluorescent protein (GFP) expression, which was carried out by the lentiviral-vector transduction prior to cell transplantation in the border region of the infarcted mouse hearts at 3 days post MI. $n = 6$/group; $^{\#}P < 0.05$ O-sh-CYP7A1 vs. O-c.

Supplementary Figure 5 | Down-regulation of lnc-CYP7A1-1 in O hBM-MSCs changed the cell paracrine function *in vitro*. Quantification of mRNA expression of VEGFA, PDGFA, FGF2, IGF1, TGFB1, ANG, and CCL2 in the O-c and O-sh-CYP7A hBM-MSCs after cultured for 72 h under hypoxia conditions by real-time qPCR. $n = 6$/group; $^{\#}P < 0.05$ O-sh-CYP7A vs. O-c.

Supplementary Table 1 | qRT-PCR primer sequences.

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REVIEW
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Senescent Mesenchymal Stem Cells in Myelodysplastic Syndrome: Functional Alterations, Molecular Mechanisms, and Therapeutic Strategies

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Myelodysplastic syndrome (MDS) is a group of clonal hematopoietic disorders related to hematopoietic stem and progenitor cell dysfunction. However, therapies that are currently used to target hematopoietic stem cells are not effective. These therapies are able to slow the evolution toward acute myeloid leukemia but cannot eradicate the disease. Mesenchymal stem cells (MSCs) have been identified as one of the main cellular components of the bone marrow microenvironment, which plays an indispensable role in normal hematopoiesis. When functional and regenerative capacities of aging MSCs are diminished, some enter replicative senescence, which promotes inflammation and disease progression. Recent studies that investigated the contribution of bone marrow microenvironment and MSCs to the initiation and progression of the disease have offered new insights into the MDS. This review presents the latest updates on the role of MSCs in the MDS and discusses potential targets for the treatment of MDS.

Keywords: mesenchymal stem cells, senescence, myelodysplastic syndrome, bone marrow microenvironment, treatment

INTRODUCTION

Myelodysplastic syndromes (MDS) lead to a clonal disease of the hematopoietic system, characterized by ineffective hematopoiesis and a high risk of transforming into acute myeloid leukemia (AML).

In most studies, MDS is considered a hematopoietic cell disorder in which disease initiation and progression are exclusively driven by hematopoietic cell-intrinsic genetic events. During the past 10 years, a large number of studies have been conducted on the genetic and molecular aspects of cloned cells in MDS, and more than 40 gene mutations associated with the prognosis of patients, as well as treatment targets, have been revealed. However, these mutations are not specific and have been found in normal people as well as patients with idiopathic cytopenia of undetermined significance (ICUS), where a certain proportion of the latter population eventually develops MDS (Glenthøj et al., 2016). Therefore, the mechanisms underlying MDS initiation and progression cannot be fully attributed to genetic and molecular changes alone. Several earlier observations have challenged this reductionist view, and a large number of studies have shown that MDS is associated with an abnormal bone marrow (BM) microenvironment.

The post-birth hematopoietic microenvironment is mainly located in the BM and comprises interstitial cells, helper cells, and sympathetic nerve cells. Bone abnormalities such as "adynamic" bone, characterized by reduced osteoblast numbers, decreased mineral apposition rates, and osteoporosis, have been noted in MDS patients in comparison with age-matched controls (Mellibovsky et al., 1996; Weidner et al., 2017). Mesenchymal stem cells (MSCs), which are a source of stromal cells in the hematopoietic environment, have subsequently been identified as key components of this disrupted architecture. *Ex vivo*-expanded MSCs display altered differentiation characteristics, transcriptional abnormalities, and a reduced ability to support hematopoietic stem/progenitor cells (HSPCs) in MDS, suggesting that MSCs may play a potential role in BM failure seen in MDS (Raaijmakers, 2012; Li and Calvi, 2017). MSCs play a crucial role in the BM microenvironment (Kfoury and Scadden, 2015; Pleyer et al., 2016). These cells display a potential for self-renewal and multidirectional differentiation and may differentiate into a variety of mesenchymal cells, such as osteoblasts, adipocytes, and chondrocytes. Precise regulation of hematopoietic stem cells (HSCs) maintains hematopoiesis for life. In addition, MSCs also display immunoregulatory functions, by maintaining the stability of the BM immune microenvironment and reducing the damage caused to HSCs by stress stimuli. Animal studies have shown that genetic abnormalities in MSCs are sufficient to induce MDS formation. Dysfunctional MSCs also play an important role in the progression of MDS and its transformation to AML.

At present, the understanding of genetics and gene expression characteristics associated with MDS-derived MSCs remains limited, and experimental results pertaining to the morphology, proliferation, differentiation, and hematopoiesis show inconsistencies. However, the association between functional changes in aging MDS-MSCs and disease progression, as well as the relevance of such changes to prognostic evaluation and treatment, is increasingly attracting the attention of researchers. Thus, a better understanding of the role played by MSCs in the pathogenesis of MDS may help strengthen knowledge regarding the complexity of MDS pathogenesis and help determine new treatment options.

This review describes current target-HSC treatments and elaborates on the role of the BM microenvironment in MDS. The importance of MSC senescence and phenotypic characteristics, as potential targets for MDS treatment, is discussed. This review may help improve existing knowledge regarding the initiation and progression of MDS and enable new targets for the treatment of MDS to be determined.

CURRENT STATUS OF MDS TREATMENT

Treatment of MDS, which is based on the WHO Prognostic Scoring System (WPSS) and the International Prognostic Scoring System (IPSS and IPSS-R) for risk stratification, involves individualized treatment measures (Ferrer et al., 2013). For the MDS treatment algorithm, all patients should receive appropriate supportive treatment. Since then, the MDS expert group proposed to initially divide patients with clinically significant cytopenias into two main risk groups: (A) lower-risk patients [including IPSS low, intermediate-1; IPSS-R very low, low, and intermediate ($\leq$3.5); and WHO-based Prognostic Scoring System for risk stratification (WPSS) very low, low, and intermediate] and (B) higher-risk patients [including IPSS intermediate-2 and high; IPSS-R intermediate (>3.5), high, and very high; and WPSS high and very high]. In addition, intermediate-risk patients with disease that does not respond to therapy for lower-risk disease would be eligible to receive therapy for higher-risk MDS. In addition, intermediate-risk patients who do not respond to treatment of lower-risk diseases are eligible for treatment of higher-risk MDS.

Patients with low-risk MDS are provided with supportive treatment using cytokines and immunomodulatory drugs, such as lenalidomide. Patients with del(5q) ($5q^-$) chromosomal abnormalities with or without one other cytogenetic abnormality, except those involving chromosome 7 and symptomatic anemia should be treated with lenalidomide. Studies have shown the relative safety of lenalidomide in these patients and the improved quality-of-life (QOL) outcomes in randomized clinical trials (Oliva et al., 2013; Revicki et al., 2013). Patients without the $5q^-$ abnormality, with or without one other cytogenetic abnormality and accompanied by symptomatic anemia, are categorized by serum erythropoietin (EPO) levels. These patients should receive erythropoietin, G-CSF, or iron repletion treatment (Hellström-Lindberg, 1995; Negrin et al., 1996; Hellström-Lindberg et al., 1997; Casadevall et al., 2004; Spiriti et al., 2005) (**Figure 1**). Patients without symptomatic anemia, with increased BM blasts or any other clinically relevant cytopenias, should be considered for treatment with decitabine, azacytidine, immunosuppressive therapy, or a clinical trial (Jabbour et al., 2017).

Patients with high-risk MDS are administered demethylating drugs, such as decitabine and azacytidine, or allogeneic HSC transplantation, which is considered the only possible cure for MDS (**Figure 1**). However, due to limitations associated with the application of transplantation technology to MDS patients, most patients are found to be unsuitable for this treatment program (Alessandrino et al., 2008; Hicks et al., 2013; Oliva et al., 2013; Revicki et al., 2013). In therapeutic trials, the panel, using the standardized International Working Group (IWG) response criteria for evaluating studies, found it important to stipulate that all MDS patients should be given relevant supportive care (Cheson et al., 2000, 2006; Hicks et al., 2013; Pfeilstöcker et al., 2016). The latest developments pertaining to MDS treatment during the past 10 years indicate that lenalidomide and decitabine may improve the hematology of low-risk and high-risk patients, respectively, and that azacytidine is the only drug that may prolong the overall survival of high-risk MDS patients (Silverman et al., 2011). However, lenalidomide does not delay the progression of patients to high-risk MDS and subsequent conversion to AML. Failure of MDS treatments to achieve a breakthrough may be due to the fact that genetic and molecular abnormalities in the cloned cells associated with MDS do not clearly relate to events leading to the onset of MDS or to disease progression.

Therefore, new drugs developed to counter different molecular mechanisms related to MDS-cloned cells have been largely ineffective. These results indicated that other mechanisms may be involved in the initiation and progression of MDS.

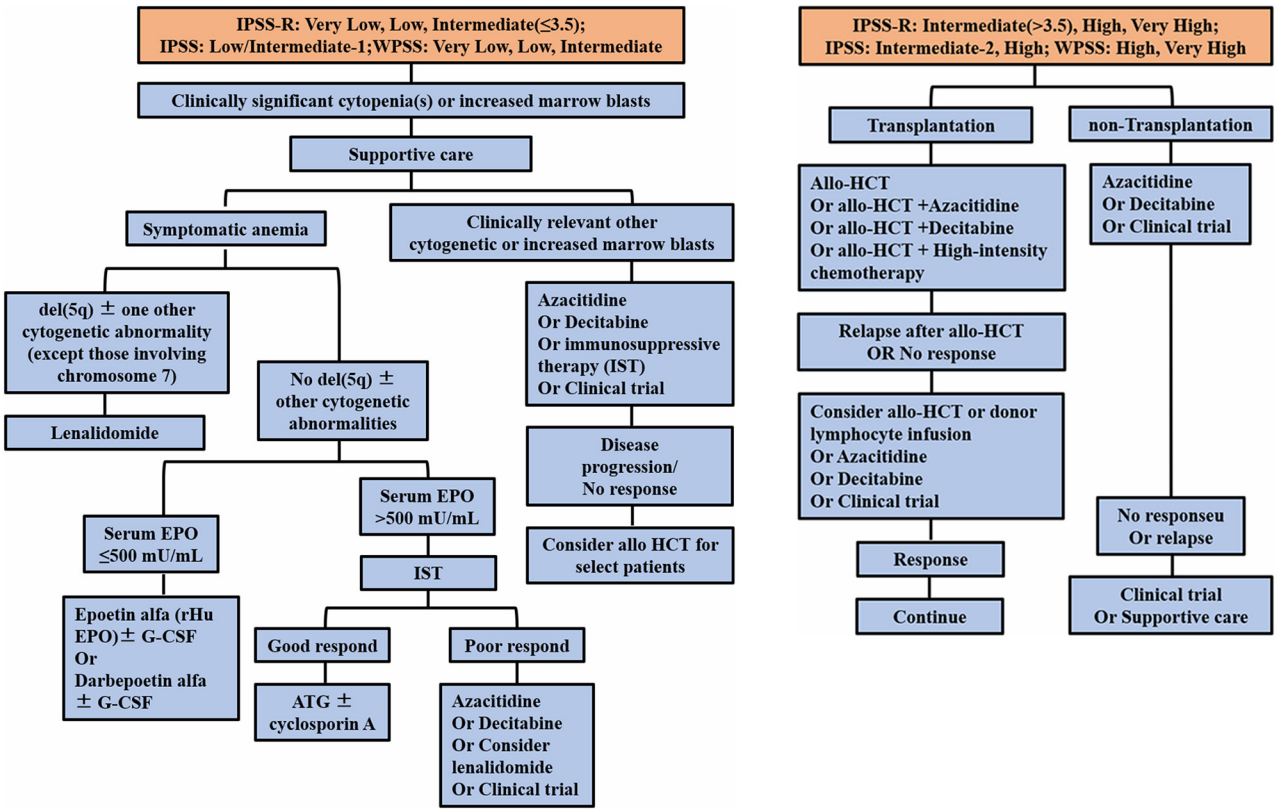

FIGURE 1 | Current treatment of MDS, based on the IPSS and revised IPSS and WHO-based Prognostic Scoring System for risk stratification (WPSS). EPO, erythropoietin; G-CSF, granulocyte colony-stimulating factor; IST, immunosuppressive therapy; HCT, hematopoietic cell transplantation.

Mouse MDS models, such as Dicer1-knockout and NHD13 mice, exhibited MSC dysfunction and MDS-like morbidity hematopoiesis, which play an important role in the progression of MDS disease (Lin et al., 2005; Balderman et al., 2016). These results indicated that the dysfunctional MSCs derived from MDS are related to the pathogenesis of MDS.

THE ROLE OF BM MICROENVIRONMENT IN MDS

It has long been considered that hematopoietic cell disorders, which are solely driven by genetic events, are capable of inducing the initiation and progression of MDS. However, several previous studies have suggested that the BM microenvironment also contributes to MDS pathogenesis (Verstovsek et al., 2002; Iwata et al., 2007; Ferrer et al., 2013; Geyh et al., 2013). The BM microenvironment plays an essential role in the maintenance and development of HSPCs. It regulates quiescence, self-renewal, proliferation, and differentiation of stem cells (Adams and Scadden, 2006; Li and Li, 2006; Moore and Lemischka, 2006). The cells in the BM microenvironment are dynamic in nature and may shape the environment to favor an abnormal population of niche residents *via* certain mechanisms. Colmone et al. reported that the interaction between normal HSPCs and the BM was influenced by a leukemia cell line (Colmone et al., 2008). More definitively, genetic changes in hematopoietic cells cause secondary changes in the cells constituting the BM microenvironment, which supports abnormal hematopoietic populations (Schepers et al., 2013). Bone abnormalities in MDS patients such as "adynamic" bone, displayed decreased osteoblast numbers and osteoporosis, compared to age-matched controls (Mellibovsky et al., 1996; Weidner et al., 2017).

Prior to hematopoietic cell transplantation, chemotherapy and radiation therapy were used to exert direct effects on HSCs and the microenvironment and markedly changed the normal supportive BM environment (Barcellos-Hoff et al., 2005; Wright, 2005). Mice that were irradiated following transplantation of growth factor-dependent syngeneic mouse BM cells developed leukemia at a faster rate and in higher numbers compared with non-irradiated recipients (Dührsen and Metcalf, 1990). Thus, in allogeneic patient BM environments, normal transplant-donor cells are transformed into hematopoietic neoplastic cells, demonstrating that the BM environment may contribute to the initiation of MDS (Flynn and Kaufman, 2007). Although infrequent, it appears that interaction between the BM microenvironment and genetically aberrant HSPCs may induce MDS pathogenesis. Thus, during the last decade, the BM microenvironment has been shown to exhibit a potentially permissive or causative role, challenging the belief that hematopoietic cell disorder is solely an element of the initiation and progression of MDS.

MSC SENESCENCE AND PHENOTYPIC CHARACTERISTICS IN MDS

MSCs, which display pluripotent and undifferentiated capacities, are key components of the BM microenvironment (Sacchetti et al., 2007). In recent years, evidence has increasingly demonstrated that MSCs in MDS are intrinsically pathological and that senescence is increased by a continuous decline in proliferation (Ferrer et al., 2013; Geyh et al., 2013). Starting from birth and proceeding into adulthood, the composition of BM stromal cells changes over time. MSCs and HSCs/HSPCs that colonize adult BM during development undergo changes in composition over time. During later stages of fetal development, skeletal stem cells (SSCs), which are characterized by the expression of *Osx* (encoding osterix), *Sox9* (encoding SRY-box 9), and *Col2a1* (encoding collagen type II α1 chain), produce bone and BM stromal cells, which persist postnatally (Maes et al., 2010; Mizoguchi et al., 2014; Ono et al., 2014b). However, as depicted in **Figure 2**, SSCs are not labeled by these markers, while stromal cells are depleted during adulthood (Maes et al., 2010; Park et al., 2012; Mizoguchi et al., 2014; Ono et al., 2014b). Neural crest-derived cells make a short-term contribution to BM stromal cells and the activity of colony-forming unit fibroblasts (CFU-Fs) during the early postnatal period, but these cells decrease in adulthood too and are replaced by nonneural crest-derived stromal cells (Takashima et al., 2007; Komada et al., 2012; Isern et al., 2014). Consistent with this, Nes-CreER^{+} (*Nes* encodes nestin) stromal cells promote bone formation in early postnatal BM but exert little or no effect on osteogenesis or CFU-F activity in adult BM (**Figure 2**) (Ono et al., 2014a; Zhou et al., 2014). By contrast, leptin receptor-expressing cells appear in the BM postnatally and exert an effect on the stromal cell population or osteogenesis. As numbers increase, these become the main source of adipocytes and osteoblasts in adults over time (**Figure 2**) (Méndez-Ferrer et al., 2010; Zhou et al., 2014).

Cellular senescence, a complex process that is usually accompanied by functional changes, is a special state of cell cycle arrest in proliferating cells under the stimulation of stress factors, wherein cells undergo a series of changes in morphology, proliferation, differentiation, secretion, and other functional abnormalities (Pleyer et al., 2016). Changes in morphology intuitively reflect the phenomenon of senescence in MDS-MSCs. MSCs in the donor group are usually slender fibers or fusiform, whereas the volume of MDS-MSC is obviously increased, showing a flat irregular polygon. A cytoskeletal morphology study showed that the morphological changes in MDS-MSCs were related to increased, as well as disorderly F-actin distribution. The expression level of β-galactosidase is significantly increased in senescent cells. Staining experiments indicated that β-galactosidase in MDS-MSCs was significantly increased, directly illustrating the aging of MSCs in MDS (Ferrer et al., 2013; Geyh et al., 2013; Fei et al., 2014; Zhao et al., 2015). Senescence of MDS-MSCs also manifests as a decline in proliferation ability. Parameters such as CFU-F, cumulative number of passages, and doubling time were significantly worse in the MDS-MSC group compared to those of control MSCs. The proliferation ability of MDS-MSCs was significantly reduced. Such a decrease in MSC proliferation capacity may be related to increases in cell telomerase length, chromosome methylation status, abnormal proliferation regulation signal pathway, cell cycle arrest, and other factors (Pavlaki et al., 2014; Falconi et al., 2016).

Decreased differentiation in aging MDS-MSCs both *in vivo* and *in vitro* was associated with the senescence and aging of MSCs (Stenderup et al., 2003; Bonab et al., 2006; Zhou et al., 2008). Alizarin Red chemical staining showed that the osteogenic differentiation potential of MDS-MSCs decreased significantly during the *in vitro* induction of differentiating MSCs into osteoblasts (Ferrer et al., 2013; Geyh et al., 2013; Fei et al., 2014; Zhao et al., 2015). The expression levels of genes involved in osteogenic differentiation accurately indicated the differences between the osteogenic differentiation potentials of cells. The transcription factors, RUNX2 and Osterix, involved in regulating early osteogenic differentiation and genes encoding osteocalcin, serve as mature osteogenic markers in MDS-MSCs. The expression levels of these were significantly lower than those of the control group, indicating that the osteogenic differentiation potential of MSCs in aging MDS had decreased (Ferrer et al., 2013; Geyh et al., 2013; Fei et al., 2014; Zhao et al., 2015).

Senescent cells still show metabolic activity, but many cytokines associated with senescence in MDS-MSCs, such as transforming growth factor β1 (TGFβ1), HGF, Jagged1, angiopoietin-1, osteopontin, CXCL-12, IL-6, TGF-β, SCF, and VEGF, are expressed abnormally. Jagged1 is a ligand involved in the Notch signaling pathway, the activation of which plays an important regulatory role in the differentiation of HSCs and the formation of myeloid tumors (Geyh et al., 2013; Pavlaki et al., 2014). The expression of *jagged-1* in MDS-MSCs was significantly increased, and 38% of MDS tumor cells showed excessive activation of the Notch signaling pathway (Geyh et al., 2013; Pavlaki et al., 2014). S100A9 promotes cellular senescence of BM stromal cells via TLR4, NLRP3 inflammasome formation, and IL-1β secretion (Shi et al., 2019). Some reports have indicated that MSCs exhibited insufficient hematopoietic support capability in MDS compared with donor cells (Zhao et al., 2012; Ferrer et al., 2013). Finally, MSCs from MDS were more prone to cellular senescence than donor MSCs, where senescent MDS-MSCs exhibited reduced differentiation potential and stem cell support capacity.

CHANGES IN THE FUNCTION OF AGING MDS-MSC TO SUPPORT HEMATOPOIESIS

As described above, *in vivo* mouse experiments have demonstrated that abnormal MSCs may affect the initiation and progression of MDS. Knocking out *Dicer1*, which encodes endonuclease III of miRNA in mouse MSCs, caused MDS-like morbid hematopoiesis in the BM, transforming a portion of the mouse population into AML (Kfoury and Scadden, 2015). Several mouse models have been developed to mimic human MDS, of which the mouse NUP98-HOXD13 (NHD13) transgenic model, wherein MSCs and osteoblast dysfunction play an important role in MDS progression, may be the most accurate (Lin et al., 2005; Balderman et al., 2016). Following

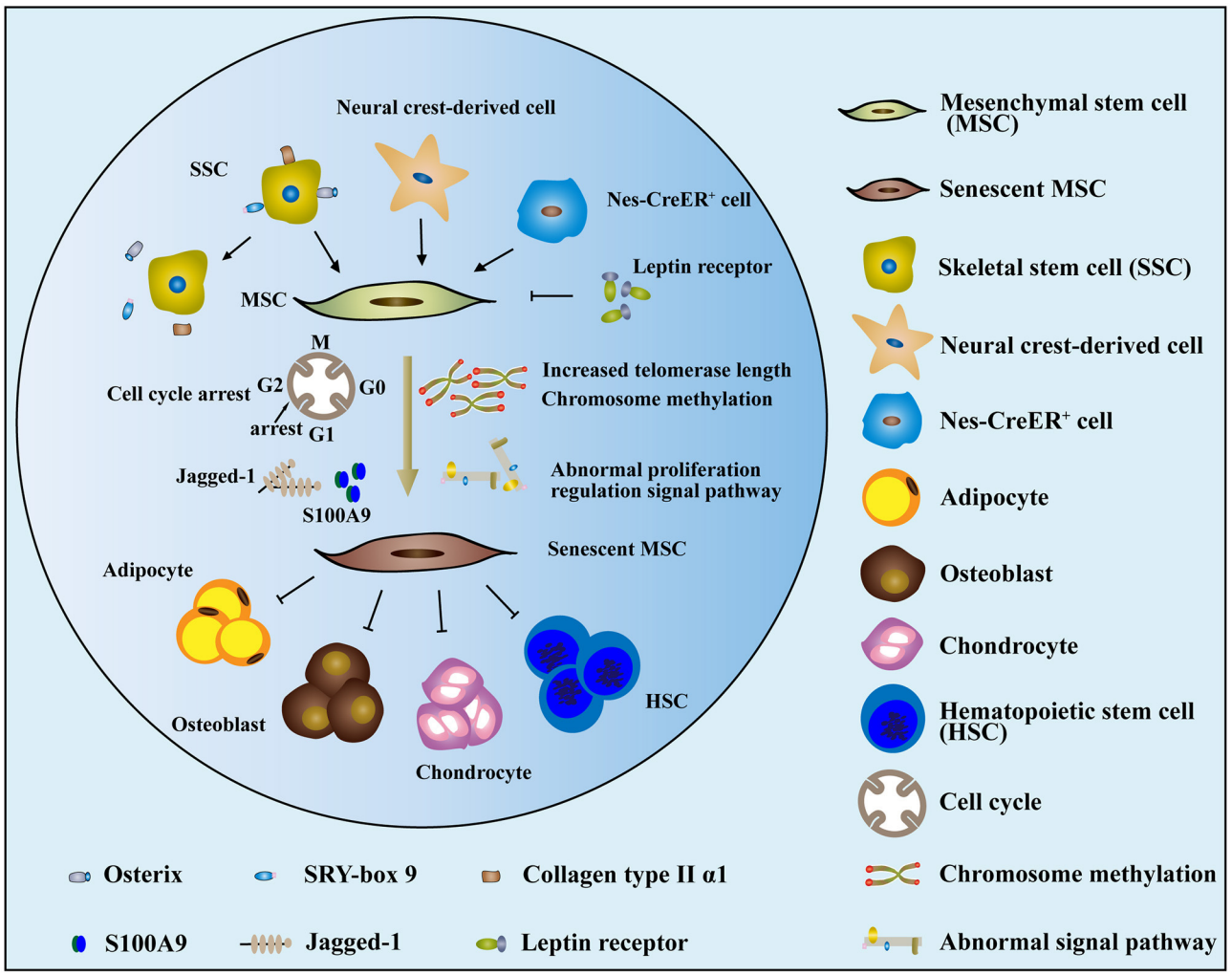

FIGURE 2 | The mechanism and dysfunction of MSC senescence in MDS. (1) Cells that contributed to MSCs, such as SSCs, neural crest-derived cells, and nes-CreER+ stromal cells, were reduced or depleted in MDS. In contrast, leptin receptors, which are detrimental to the function of MSCs, increased over time. (2) The senescence of MDS-MSCs may be related to increases in cell telomerase length, chromosome methylation status, abnormal proliferation regulation signal pathway, cell cycle arrest, and other factors. (3) The senescent MDS-MSCs exhibited reduced differentiation potential and stem cell support capacity.

transplantation of NHD13 mouse hematopoietic cells into both NHD13 and WT mice, the rates of AML transformation and mortality in NHD13 mice were found to be significantly higher than those in WT mice. By contrast, hematopoiesis of NHD13 mice transplanted with BM from WT mice showed a myeloid bias, while the hematopoietic function of WT mice was impaired due to dysfunctional MSCs (Balderman et al., 2016).

Abnormal functioning of proliferation, differentiation, and secretion of aging MDS-MSCs leads to changes in the regulation of hematopoietic function. In an *in vitro* co-culture experiment using MDS-MSCs and donor HSCs, the proportion of hematopoietic cells in the G1 phase decreased while the proportion in the G0 phase increased, in a manner which was significantly different from that in normal MSCs ($P = 0.0076$) (Geyh et al., 2013). MDS-MSCs showed a significantly altered cell cycle status and displayed a shift toward increased apoptosis compared to control MSCs. These changes may contribute to the pathogenesis of MDS (Abbas et al., 2019).

High levels of hyaluronan (HA) were detected in the BM sera of higher-risk MDS patients in comparison with those of donor controls. High levels of HA in BM serum, which enhances osteogenic differentiation of MSCs, were associated with adverse clinical outcomes and significantly shorter median survival in MDS (Fei et al., 2018).

Defective proliferation was observed in pediatric MDS-derived MSCs. Pediatric MDS-derived MSCs were more prone to cellular senescence than healthy controls and showed a decrease in the S phase (Liu et al., 2015). Iron overload (IO) reportedly promotes mitochondrial fragmentation and enhances autophagy in MSCs of MDS patients by activating the AMPK/MFF/Drp1 pathway (Zheng et al., 2018). These results indicated that dysfunctional MSCs derived from MDS were associated with MDS-associated pathogenesis.

MSCs AS A POTENTIAL TARGET FOR MDS TREATMENT

The functions of MDS-MSCs not only play an important role in the initiation and progression of the disease but also provide a protective microenvironment for tumor cells, which is a poor prognostic factor of MDS. Improving MDS-MSC function as an option for MDS treatment has gradually advanced from its theoretical basis to the research level. Co-cultivation experiments using HSCs *in vitro* have shown that MDS-MSCs inhibited erythroid hematopoiesis and promoted myeloid cell production, significantly restoring the contention that MDS-MSCs support erythroid hematopoiesis following lenalidomide treatment (Geyh et al., 2013). Demethylation drugs may simultaneously affect the functions of cloned cells and MSCs in MDS. Following transplantation of NHD13 mouse hematopoietic cells into normal mouse BM, the rates of transformation to acute leukemia and death were significantly reduced, while hemoglobin and white blood cell indicators in peripheral blood were enhanced (Balderman et al., 2016). These data indicated that improving MSC function may delay disease progression and enhance cytopenia of MDS.

However, the genetic and molecular changes occurring in MDS cloned cells do not fully clarify the initiation of MDS and disease progression. This may be an important factor affecting the treatment of hematopoietic cells, where most patients will eventually show a declining treatment response. Thus, treatment of MDS cloned cells alone may not produce a cure. However, intervention involving clonal cells and the BM microenvironment may lead to a new treatment strategy for MDS.

MDS-MSCs showed increased production of pro-inflammatory cytokines. Treatment with 5-azacytidine significantly decreased IL-6 levels in MDS-MSCs *in vitro*, compared to the IL-6 levels in MSCs from the donor. As MSCs produce much more inflammatory cytokines involved in MDS pathogenesis, these may represent a potential therapeutic target. Moreover, 5-azacytidine may exert a stromal effect, thereby modulating the immune response in MDS (Boada et al., 2020).

Following the administration of α-lipoic acid (ALA), the levels of reactive oxygen species (ROS) in MSCs were gradually decreased, intracellular iron content was reduced, and the potential and integrity of the mitochondrial membrane were restored. ALA treatment resulted in a significant decrease in autophagy, whose factor may be used against MDS (Camiolo et al., 2019). BM-MSCs showed improved proliferation activity in MDS patients. CDKN2A shows potential as a therapeutic target in regulating the BM microenvironment because early senescence is reversible *via* de-induction of CDKN2A (Choi et al., 2020).

Menatetrenone treatment of BM-MSCs enhanced CD34+ cell generation in cocultures by accelerating the cell cycle. MDS-derived cells underwent apoptosis when co-cultured with BM-MSCs, an effect that was enhanced by menatetrenone. These findings indicated that pharmacological treatment with menatetrenone bestows a unique hematopoiesis-supportive capability on BM-MSCs, which may contribute to clinical improvement of cytopenia in MDS (Fujishiro et al., 2020).

Upon exposure to TGFβ1, healthy MSCs developed functional deficits and adopted a phenotype similar to what was observed in patient-derived stromal cells. These suppressive effects of TGFβ1 on stromal cell functionality were abrogated by SD-208, an established inhibitor of TGFβ receptor signaling. Blockade of TGFβ signaling by SD-208 also restored the osteogenic differentiation capacity of patient-derived stromal cells (Geyh et al., 2018).

All such changes in MDS-MSCs may provide potential targets for the treatment of MDS.

CONCLUSION

In brief, an increasing number of studies have shown that MDS is a group of heterogeneous diseases caused by abnormalities in both hematopoietic cells and the microenvironment. Tumor hematopoietic cells alter the function of the hematopoietic cell microenvironment *via* direct contact and secretion of cytokines, causing it to be conducive to the growth of tumor cells. A change in the number and function of MSCs leads to a decline in the supporting function of normal hematopoietic cells, by providing a protective microenvironment for tumor cells, which is conducive to the proliferation of tumor cells. This confers proliferation advantages to tumor cells and reduces tumor cell sensitivity to chemotherapeutic agents and drugs. Gender may be an important reason for the widespread resistance to the disease. If large-sample clinical trials indicate that changes in the number and function of MSCs may be used as potential biomarkers for predicting patient response to treatment or recurrence following therapy, then the changes in MSCs at the time of diagnosis and during treatment will contribute to the formation of personalized therapies.

AUTHOR CONTRIBUTIONS

XC and NL searched the literature and drafted part of the manuscript. XD designed the entire study and revised the manuscript. JW revised the manuscript. All authors contributed to the article and approved the submitted version.

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Knockout of NOS2 Promotes Adipogenic Differentiation of Rat MSCs by Enhancing Activation of JAK/STAT3 Signaling

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Mesenchymal stromal cells (MSCs) are a heterogeneous population of cells that possess multilineage differentiation potential and extensive immunomodulatory properties. In mice and rats, MSCs produce nitric oxide (NO), as immunomodulatory effector molecule that exerts an antiproliferative effect on T cells, while the role of NO in differentiation was less clear. Here, we investigated the role of NO synthase 2 (NOS2) on adipogenic and osteogenic differentiation of rat MSCs. MSCs isolated from NOS2-null ($NOS2^{-/-}$) and wild type (WT) Sprague–Dawley (SD) rats exhibited homogenous fibroblast-like morphology and characteristic phenotypes. However, after induction, adipogenic differentiation was found significantly promoted in $NOS2^{-/-}$ MSCs compared to WT MSCs, but not in osteogenic differentiation. Accordingly, qRT-PCR revealed that the adipogenesis-related genes PPAR-γ, C/EBP-α, LPL and FABP4 were markedly upregulated in $NOS2^{-/-}$ MSCs, but not for osteogenic transcription factors or marker genes. Further investigations revealed that the significant enhancement of adipogenic differentiation in $NOS2^{-/-}$ MSCs was due to overactivation of the STAT3 signaling pathway. Both AG490 and S3I-201, small molecule inhibitors that selectively inhibit STAT3 activation, reversed this adipogenic effect. Furthermore, after high-fat diet (HFD) feeding, knockout of NOS2 in rat MSCs resulted in significant obesity. In summary, NOS2 is involved in the regulation of rat MSC adipogenic differentiation *via* the STAT3 signaling pathway.

Keywords: rat mesenchymal stromal cells, NOS2, adipogenesis, differentiation, JAK/STAT3 signaling

INTRODUCTION

Mesenchymal stromal cells (MSCs) are self-renewing multipotent stromal cells that can be isolated from mesenchymal tissues, such as bone marrow, adipose tissue, dental pulp, umbilical cord blood, and other tissues. MSCs are defined according to their lack of the hematopoietic and endothelial markers CD45 and CD34 and their expression of the stromal markers CD105, CD90, CD29, CD44,

and CD73 (Friedenstein et al., 1970; Zuk et al., 2001). MSCs have drawn significant attention from the research community with their ability to exert suppressive and regulatory effects on both adaptive and innate immunity, as demonstrated by recent studies (Bernardo and Fibbe, 2013; Li and Hua, 2017). The therapeutic potential of MSCs is attributed to complex cellular and molecular mechanisms of action, including regulation of immune responses *via* immunomodulation and differentiation into multiple cell lineages.

In vitro, MSCs are capable of differentiating into cartilage, bone, tendon, adipose tissue, muscle, etc. Among these tissues, MSCs are commonly progenitors of osteoblasts and adipocytes (Pittenger et al., 1999). Interestingly, the relationship between osteogenesis and adipogenesis in the bone marrow seems to be reciprocal, as stimulation of MSC osteogenesis occurs at the expense of adipogenesis (Chen et al., 2016; Li et al., 2019). Understanding the signaling pathways and regulators that govern MSC osteogenic and adipogenic differentiation may improve the appreciation of MSC functions and holds great promise for the application of cell therapy and regenerative medicine, particularly from the viewpoint of developing new therapeutic treatments for bone loss. However, the precise mechanisms that regulate the differentiation of MSCs and determine the fate of stem cells are still unclear (Pittenger et al., 1999; Discher et al., 2009; Chen et al., 2016; Li et al., 2019).

Studies on embryonic and adult stem cells suggest that many biological processes, including self-renewal, viability, migration, proliferation and differentiation, are regulated by nitric oxide (NO). Indeed, to a great extent, NO production also largely influences MSC survival, homing and even differentiation (Ren et al., 2008; Bonafè et al., 2015; Wang et al., 2015). NO is an endogenous molecule produced by NO synthases (NOSs) through a complex oxidoreductase reaction that consumes L-arginine and oxygen. There are three types of NOSs, inducible NOS (NOS2), NOS1, and NOS3. NOS1 and NOS3 are constitutively expressed in neurons and epithelial cells and produce low (nM) levels of NO in a process that is regulated by Ca^{2+} binding to calmodulin (Alderton et al., 2001). In contrast, NOS2 is only induced by immunological stimuli in a calcium-independent manner and produces large amounts of NO (μM levels) (Nathan, 1992; Moncada and Higgs, 1993).

The production of NO by NOS2 may be applied in mouse and rat MSCs to inhibit T cell proliferation (Ren et al., 2008; de Castro et al., 2019). However, the roles of NO in T cell-mediated immunity, inflammation and tumor growth and metastasis remain debated, and the lack of clarity may be due to variance in NO concentration or NO sources (endogenous or exogenous) (Vannini et al., 2015; Garcia-Ortiz and Serrador, 2018). Therefore, this debate requires an update with new data. In addition, whether and how NOS2 plays a role in MSC differentiation remains largely unknown.

In this study, using NOS2-null ($NOS2^{-/-}$) rats, we found that MSCs without NOS2 significantly shifted toward adipogenic differentiation. The clear association between NOS2 and adipogenic differentiation in bone marrow-derived MSCs (BMSCs) was mediated by the STAT3 signaling pathway. This study provides deeper insights into the regulation of MSC adipogenic differentiation and presents evidence for new roles played by NOS in this process.

MATERIALS AND METHODS

Animals

As previously described (Shen et al., 2017), wild type (WT) and $NOS2^{-/-}$ Sprague–Dawley (SD) rats were used in this study. Rats were housed in a specific pathogen-free facility and were given free access to food and water. All animal procedures were performed under the supervision of the Experimental Animal Ethics Committee of Guangzhou Medical University (Guangzhou, China).

To observe osteogenesis and fat formation, eight-week-old female WT and $NOS2^{-/-}$ rats were randomly divided into two groups and fed either a normal chow diet (NCD) or a high-fat diet (HFD) (D12492, Research Diets, New Brunswick, NJ, United States) for 8 weeks and weighed weekly until sacrifice. Bone mineral content (BMC) and bone mineral density (BMD) were measured by dual-energy X-ray absorptiometry (DXA), and adipose tissues were collected for HE staining and western blot assay.

Isolation and Cultivation of Rat MSCs

Bone marrow-derived MSCs were isolated from femurs and tibias of $NOS2^{-/-}$ and WT SD rats under aseptic conditions. Briefly, femurs and tibias were dissected and soaked in cold PBS. The bone marrow was exposed and flushed with 4–5 ml cell culture medium. BM cells, including hematopoietic stem cells and marrow stromal cells, were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, United States) containing 15% FBS at 37°C and 5% CO_2. After 3 days in culture, non-adherent cells were washed off, and adherent MSCs were cultured in medium that was replaced every 3 days for 10–14 days, at which time cells had formed homogenous fibroblast-like colonies. MSC colonies were further passaged, and passages 3–5 were used in subsequent assays. Flow cytometry analysis was used to assess MSCs and their multipotent properties.

Adipose-derived MSCs (AdMSCs) were isolated from the inguinal area of rats, washed several times with HBSS, and minced with scissors. Then, 0.1% type I collagenase in HBSS without Ca^{2+} or Mg^{2+} was added, and samples were incubated for 60 min at 37°C, followed by a wash and resuspension in HBSS. After filtering through a 70 μm cell filter and washing twice, cells were pelleted, resuspended in MSC culture medium, and then plated in a 25 cm^2 dish. MSCs were isolated, passaged and quantified as described above.

Flow Cytometry

To identify cell surface markers, a BMSC suspension (1×10^6 cells/mL) was prepared and washed twice with PBS and then incubated in the dark for 40 min at 4°C with monoclonal antibodies (mAb) against α-CD34-PE (188-10041, RayBiotech, United States), α-CD29-FITC (Clone: Hα2/5), α-CD45-FITC (Clone:OX-1), α-CD90-PE (Clone:OX-7) (BD Biosciences,

Franklin Lakes, NJ, United States) and their corresponding isotype antibodies. Stained cells were subsequently rinsed with cold PBS and immediately subjected to flow cytometry analysis (BD LSRII; BD Biosciences) to determine cell surface marker expression.

T Cell Proliferation Assays

T cell proliferation was evaluated by CellTrace CFSE (5,6-carboxyfluorescein diacetate, succinimidyl ester) cell proliferation kit (Invitrogen, Carlsbad, CA, United States). Briefly, single-cell suspensions of WT rat spleens were prepared and stained with CD3-APC (Clone: 1F4) for 40 min at 4°C. CD3 + T cells were isolated by flow cytometry sorting (BD LSRII; BD Biosciences) and were then labeled with 5 mM CFSE. The labeled T cells were cultured in replicate wells with or without previously plated BMSCs at a 20:1 ratio and were stimulated with α-CD3 (0.5 mg/mL) and α-CD28 (1 mg/mL) mAbs (EBioscience, San Diego, CA, United States) for 96 h. Cells were then stained for surface marker expression with CD4-PE (Clone: OX-38) and CD8α-PerCP (Clone: OX-8) antibodies (BD Biosciences) and incubated 40 min at 4°C. T cell proliferation was detected by flow cytometry.

Cell Survival and Proliferation Assays

MSC survival was determined by plating 1×10^5 BMSCs per well in six-well plates. After overnight incubation, media were replaced with serum-free DMEM, and cells were cultured for an additional 48 h. Cell survival was determined using an Alexa Fluor 488 Annexin V/PI Kit (Invitrogen) according to the manufacturer's instructions. The ratios of dead to cells alive were determined from five randomly selected 40x microscopic fields.

A cell proliferation assay was performed to determine the population doubling time (PDT) of NOS2$^{-/-}$ and WT BMSCs using a fluorescence-based Cell Counting Kit-8 (CCK8) cell proliferation assay (Dojindo, Kumamoto, Japan). In brief, 100 μl of BMSC suspension was plated onto a 96-well plate at 2,500 cells per well and cultured for 24 h, after which 10 μl of CCK8 solution was added to each well and the plate was incubated for 1–4 h. Cell proliferation was assessed at 450 nm using a microplate reader (Tecan Trading AG, Switzerland).

Multilineage Differentiation *in vitro*

Cells were induced to adipogenic and osteogenic differentiation in culture. All differentiation cultures received regular MSC expansion medium (Ren et al., 2008; Qin et al., 2017).

For adipogenic differentiation, MSCs derived from rats were seeded into 6-well or 12-well plates (Nest, China). When confluence reached 80–90%, cells were cultured in adipogenic differentiation medium (AM A or AM B) (Cyagen BioSciences, United States) for 12 days. AM A was made from AM B by adding 1 μM dexamethasone, 0.2 mM indomethacin and 0.5 mM isobutylmethylxanthine. AM B was composed of DMEM plus 10% FBS, 1% penicillin-streptomycin, 1% glutamine and 0.01 mg/mL insulin. BMSCs were maintained in AM A for 3 days, AM B for 1 days, AM A for 3 days, AM B for 1 days, AM A for 3 days and AM B for 1 days to promote adipocyte differentiation. Lipid content was evaluated with Oil Red O staining (Sigma, St. Louis, MO, United States) after cells were fixed in 4% PFA. Images were acquired using a phase contrast light microscope (Leica Dmi8) (Leica Microsystems, Germany).

For osteogenic induction, BMSCs were seeded into 6-well or 12-well plates. When BMSCs reached a density of 80–90%, cells were grown for 21 days in osteogenic medium (OM) (Cyagen Biosciences), which contained 10% FBS, 1% glutamine, 0.2% ascorbic acid, 1% penicillin-streptomycin, 0.01% dexamethasone, and 1% β-glycerophosphate to promote osteoblast induction. After fixation, cells were analyzed by staining with Alizarin red (Sigma-Aldrich) as previously reported (Qin et al., 2017). Experiments for each group were conducted in triplicate wells.

NO Detection

Supernatants of MSC differentiation and activated T/MSCs cocultures were collected and stored at −80°C. The NO concentration was assessed following the manufacturer's protocol (Beyotime, China).

RNA Extraction and Quantitative Real-Time PCR Analysis

Total cellular RNA was extracted using TRIzol reagent (Invitrogen). A NanoDrop spectrophotometer (Thermo Fisher Scientific) was utilized to analyze the quantity and quality of the RNA. First-strand cDNA was synthesized from 1 μg RNA according to the instructions of the TaKaRa reverse transcription kit. The mRNA levels of the indicated genes were analyzed in triplicate using QuantiTect SYBR Green PCR Master Mix (Takara, Kyoto, Japan). Reactions were run on a LightCycler480 real-time PCR instrument (Roche, Indianapolis, IN, United States). The gene expression levels were normalized to GAPDH. The relative expression of target genes was assessed by the $2^{-\Delta\Delta Ct}$ method. Specific primers for rat PPAR-γ, C/EBP-α, FABP4, LPL, ALP, RUNX2, COL1A1, and GAPDH are listed in **Supplementary Table 1**.

Western Blotting

Quantitative analysis of changes in protein expression was conducted by western blot analysis according to previous reports (Qin et al., 2017). BMSCs were inoculated into 6-well plates and differentiated when cells reached 80% confluence. After induction, cells were washed twice with precooled PBS, lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific) at 4°C for 30 min, sonicated for 30 s, and centrifuged at 12,000 g for 20 min. The resulting supernatants were collected, and protein concentrations were measured using a bicinchoninic acid protein assay kit (Sigma-Aldrich). Total protein was separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked in 5% non-fat milk (in Tris-buffered saline containing 0.1% Tween-20) for 1.5 h and then incubated with primary antibodies [α-phosphorylated (p)-STAT1 (1:1,000, #7649), α-p-STAT3 (1:1,000, #9145), α-p-STAT 5 (1:1,000, #4322), α-STAT1 (1:1,000, #14994), α-STAT3 (1:1,000, #9139), α-STAT 5 (1:1,000, #94205), α-GAPDH (1:1,000, #5174), α-p-JAK2

(1:1,000, #3776), α-JAK2 (1:1,000, #3230) from Cell Signaling Technology (Danvers, MA, United States) and α-PPAR-γ (1:500, #ab209350), α-NOS2 (1:500, #ab3523) from Abcam (Cambridge, MA, United States)] and then with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Finally, blots were digitally processed using a western blot imaging system (GE Amersham Imager 600, United States), and captured images were quantified using ImageJ software (NIH).

Dual-Energy X-Ray Absorptiometry

Body BMD was assessed by dual-energy X-ray absorptiometry (DXA) (LU43616CN, GE Healthcare, Madison, WI, United States) using the small laboratory animals scan mode. Animals were anesthetized with an i.p. injection of sodium pentobarbital prior to scanning. Whole-body DXA assays were conducted at the end of the experiment. BMC and BMD from NOS2$^{-/-}$ and WT rats were detected by DXA. All rats were coded, and the investigator was blinded to group allocation during the experiments. BMC and BMD were calculated automatically by a software package (enCore 2015; GE Healthcare).

Histological Analysis

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were obtained from subcutaneous white adipose tissue (S.C. WAT) and stained with hematoxylin-eosin (H&E). All samples were coded, and the investigator was blinded to the group allocation during the experiment.

Statistical Analysis

For *in vitro* experiments, all results presented represent data collected from at least three independent experiments. Statistical analyses were performed using paired *t*-tests (2-tailed). Differences between groups *in vivo* were tested for statistical significance using the unpaired two-tailed Student's *t*-test. Statistical tests were performed using GraphPad Prism version 7.0, and a p-value < 0.05 was considered statistically significant.

RESULTS

Characteristics of BMSCs Derived From NOS2$^{-/-}$ and WT SD Rats

Bone marrow-derived MSCs from NOS2$^{-/-}$ and WT SD rats were easily obtained by adherent culture of BM cells. Isolated BMSCs exhibited fibroblast-like cell morphology and formed homogenous colonies (**Figure 1A**). Flow cytometry analysis revealed that BMSCs from both WT and NOS2$^{-/-}$rats expressed the same panel of surface markers, including CD29 and CD90, but not the hematopoietic stem cell markers CD34 or CD45 (**Figure 1B**), indicating that NOS2 knockout may not alter the phenotype of BMSCs.

Furthermore, knockout of rat NOS2 did not alter the proliferative properties of BMSCs, which were verified by CCK8 assays ($p = 0.49$, **Figure 1C**). We additionally tested whether NOS2 knockout altered the rate of apoptosis of two types of MSCs. As shown in **Figure 1D**, culture under serum-deprived conditions for 48 h produced only a mild, non-significant increase in the death ratio that was similar to that found in NOS2-/- BMSCs (9.83 ± 0.75%) and WT BMSCs (8.72 ± 0.62%; $p = 0.35$) (**Figure 1E**). These results demonstrate that the morphology, phenotype, and proliferative and survival characteristics of rat MSCs with knockout of NOS2 showed no observable differences from those of WT rat MSCs.

Immunosuppressive Functions of BMSCs From NOS2$^{-/-}$ and WT SD Rats

The immunosuppressive effects of MSCs on T cell proliferation were evaluated by co-culture of MSCs during T cell activation, which was rescued by a specific inhibitor of NOS (e.g., N^G-monomethyl-L-arginine acetate salt, L-NMMA) (Ren et al., 2008). Thus, we cocultured BMSCs with purified T cells at graded ratios of 1:20 (MSC to T cells) for 4 days in the presence of α-CD3 and α-CD28, observing that T cell proliferation was completely blocked in the presence of WT BMSCs (**Figure 2A**). However, NOS2$^{-/-}$ BMSCs or the presence of the NOS inhibitor L-NMMA failed to inhibit T cell proliferation (NOS2$^{-/-}$ BMSCs, $p < 0.001$; L-NMMA, $p < 0.001$) (**Figure 2B**). This failure seems to be corresponding to NO production in the supernatant of BMSC-T cell cocultures. High levels of NO were detected in WT BMSCs coculture system, whereas little NO was detected in NOS2$^{-/-}$ BMSCs, and L-NMMA cocultures (NOS2$^{-/-}$ BMSCs, $p < 0.01$; L-NMMA, $p < 0.01$) (**Figure 2C**). These results strongly suggest that rat MSC-mediated immunosuppression depends on NO production or NOS2 activity.

Differentiation Potential of NOS2$^{-/-}$ and WT MSCs

To define the potential of MSCs to differentiate into adipocytes and osteoblasts, BMSCs from NOS2$^{-/-}$ and WT SD rats were cultured under their respective culture conditions (AM A/B for 12 days and OM for 21 days). After staining with Oil Red O or Alizarin Red S, we found that both sets of BMSCs had successfully differentiated into adipocytes (**Figure 3A**) and osteoblasts (**Figure 3B**). The multipotency of these cells further confirmed their identity as MSCs. However, to our surprise, quantitative analysis revealed that there was a significantly higher proportion of adipogenically differentiated BMSCs in NOS2$^{-/-}$ samples than in WT samples ($p < 0.001$, **Figure 3C**), while osteogenic differentiation was not altered between the groups ($p = 0.91$, **Figure 3D**). These findings were confirmed in AdMSCs, that is, MSCs from subcutaneous adipose tissue of NOS2$^{-/-}$ and WT SD rats, as similar characteristics were observed for these cells (**Supplementary Figure 1**). Furthermore, administration of L-NMMA (NOS inhibitor) and 1,400 W {N-[3-(aminomethyl)benzyl] acetamidine}, a highly selective NOS2 inhibitor] to adipogenic differentiation conditions resulted in decreases in both adipogenic capacity and NO production (**Figures 3E–G**). These results strongly suggest that NO

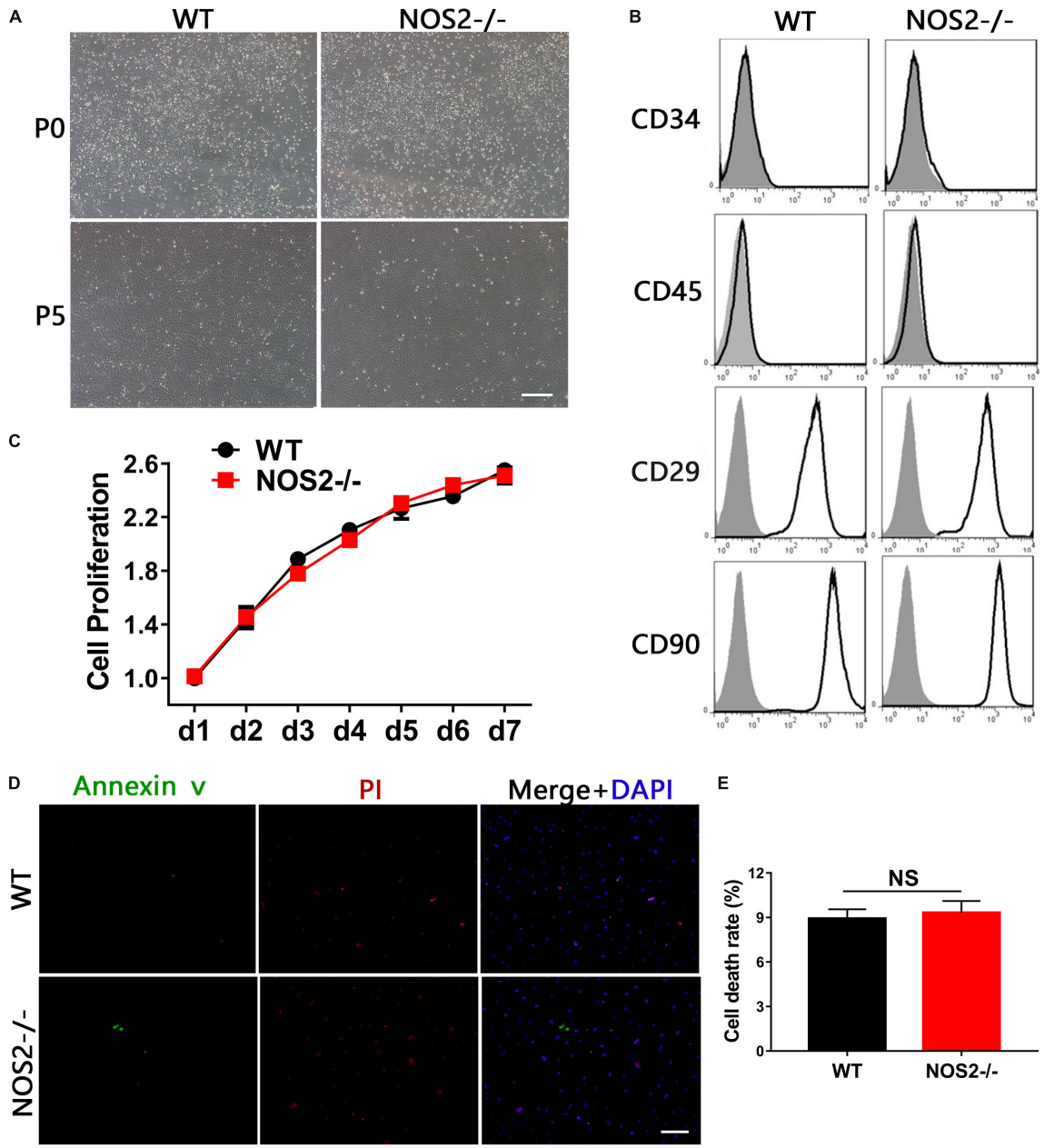

FIGURE 1 | Characteristics of BMSCs from NOS2$^{-/-}$ and WT SD rats. **(A)** The morphology of BMSCs was monitored under a microscope at passages 0 and 5; the scale bar indicates 200 μm. **(B)** The third passage of BMSCs derived from NOS2$^{-/-}$ and WT rats were subjected to flow cytometry after staining with α-CD34, α-CD45, α-CD29 and α-CD90 (black line) or their corresponding isotype (shadowed). **(C)** Cell proliferation was analyzed by CCK8 assay. **(D,E)** BMSCs derived from NOS2$^{-/-}$ and WT SD rats were cultured in serum-free medium for 48 h. Cell survival was analyzed using a LIVE/DEAD viability/cytotoxicity kit, and the scale bar indicates 100 μm. The results are expressed as the means ± SEM; N.S., not significant. $n = 4$.

from NOS2 plays an important role in the adipogenesis of rat MSCs.

NOS2 Knockout Increases Expression of Adipogenesis-Related Genes

During recent decades, a number of reports have revealed that adipogenesis is controlled by a complicated network of transcription factors, of which CCAAT-enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor gamma (PPAR-γ) play critical roles (Rosen et al., 2002; Farmer, 2006). In our study, Quantitative Real-Time PCR (qRT-PCR) analysis of the major regulators and markers of differentiation, including C/EBP-α, PPAR-γ, lipoprotein lipase (LPL), and fatty acid-binding protein 4 (FABP4), exhibited marked differences between NOS2$^{-/-}$ and WT rat BMSCs

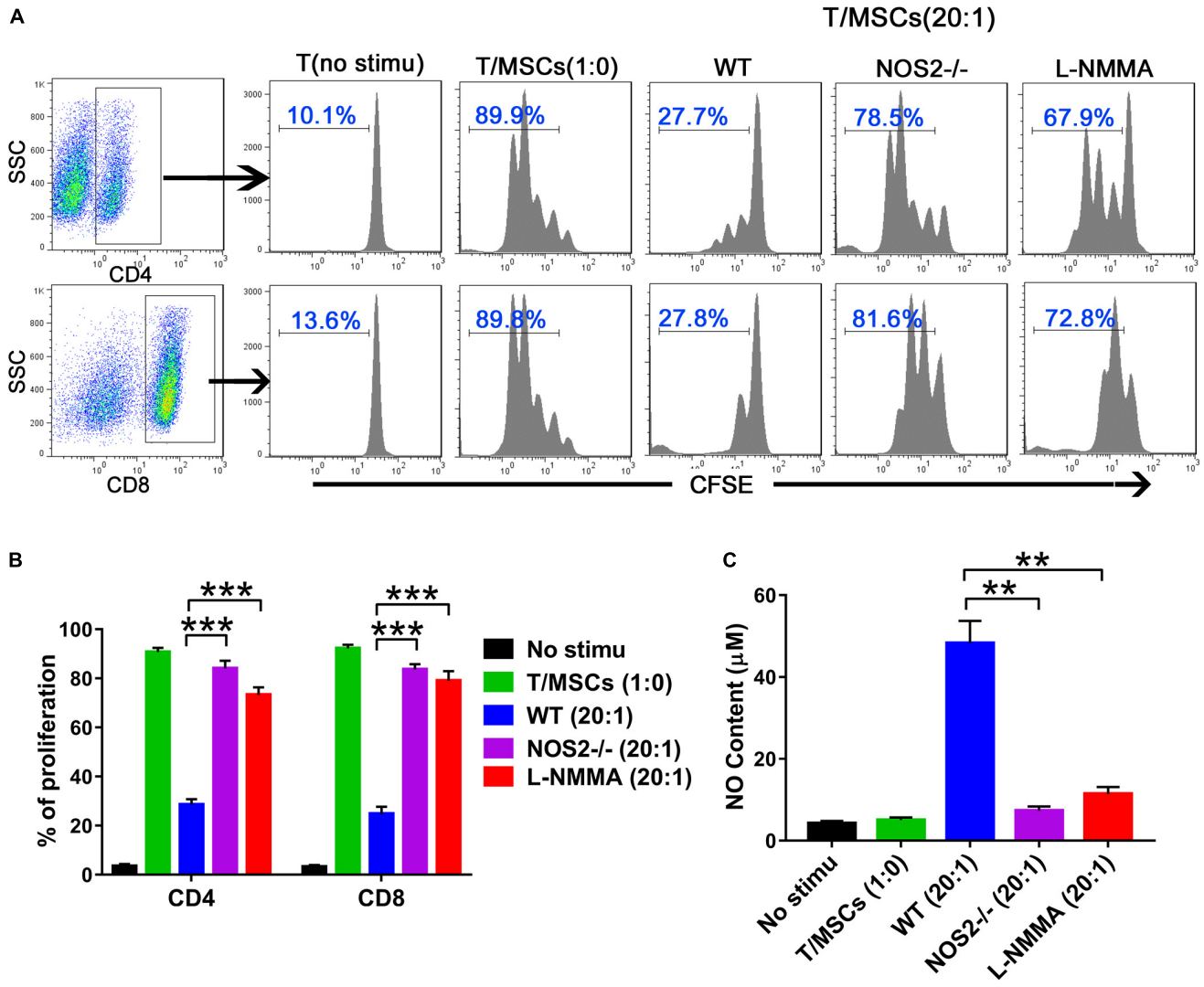

FIGURE 2 | The immunosuppressive effects of NOS2$^{-/-}$ and WT rats BMSCs on splenic T cells. Purified CD3 + T cells were stained with CFSE and cocultured with BMSCs (5.0×10^4 cells per well in 24-well plates) at a ratio of 1:20 (MSC: T cells) for 96 h in the presence of α-CD3 and α-CD28 mAbs. The NOS2 inhibitor L-NMMA (1 mM) was added into the coculture system. **(A,B)** CFSE fluorescence intensity of CD4 + and CD8 + T cells was detected by flow cytometry. **(C)** NO production in T cells and activated T/MSCs cocultures was determined by Griess reagent. All results are expressed as the means ± SEM; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 3$.

during the differentiation period of 7 days, particularly the transcription factor PPAR-γ (**Figure 4A**). Therefore, protein expression levels of PPAR-γ and NOS2 during the period of differentiation were further investigated by western blot. As shown in **Figures 4B,C**, expression of PPAR-γ were greatly increased in NOS2$^{-/-}$ BMSCs, and as expected, NOS2$^{-/-}$ BMSCs failed to express the NOS2 protein. Furthermore, we also assessed the gene expression of major regulators and markers of osteogenic differentiation, including HOXA2/runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and collagen type I alpha 1 chain (COL1A1). The results showed little difference between NOS2$^{-/-}$ and WT BMSCs during the differentiation period of 14 days (**Figure 4D**). Therefore, our findings strongly suggest that the greater adipogenic potential in NOS2$^{-/-}$ BMSCs is primarily due to the greater activation of adipogenic transcription factors than in WT BMSCs.

Genetic Deletion of NOS2 Tends to Develop HFD-Induced Obesity in Rats

Given the importance of NOS2 in the regulation of adipogenic differentiation and in disease, we investigated WT and NOS2$^{-/-}$ rats under conditions of metabolic stress. WT and NOS2$^{-/-}$ rats were fed a NCD or a HFD for 8 weeks to induce obesity. WT and NOS2$^{-/-}$ rats had comparable weights on NCDs, but NOS2$^{-/-}$ rats more easily became obese in response to the HFD (**Figure 5A**). The subcutaneous white adipose tissue (WAT) pads from NOS2$^{-/-}$ rats were bigger than those in WT rats on a HFD (**Figure 5B**), while no considerable difference was

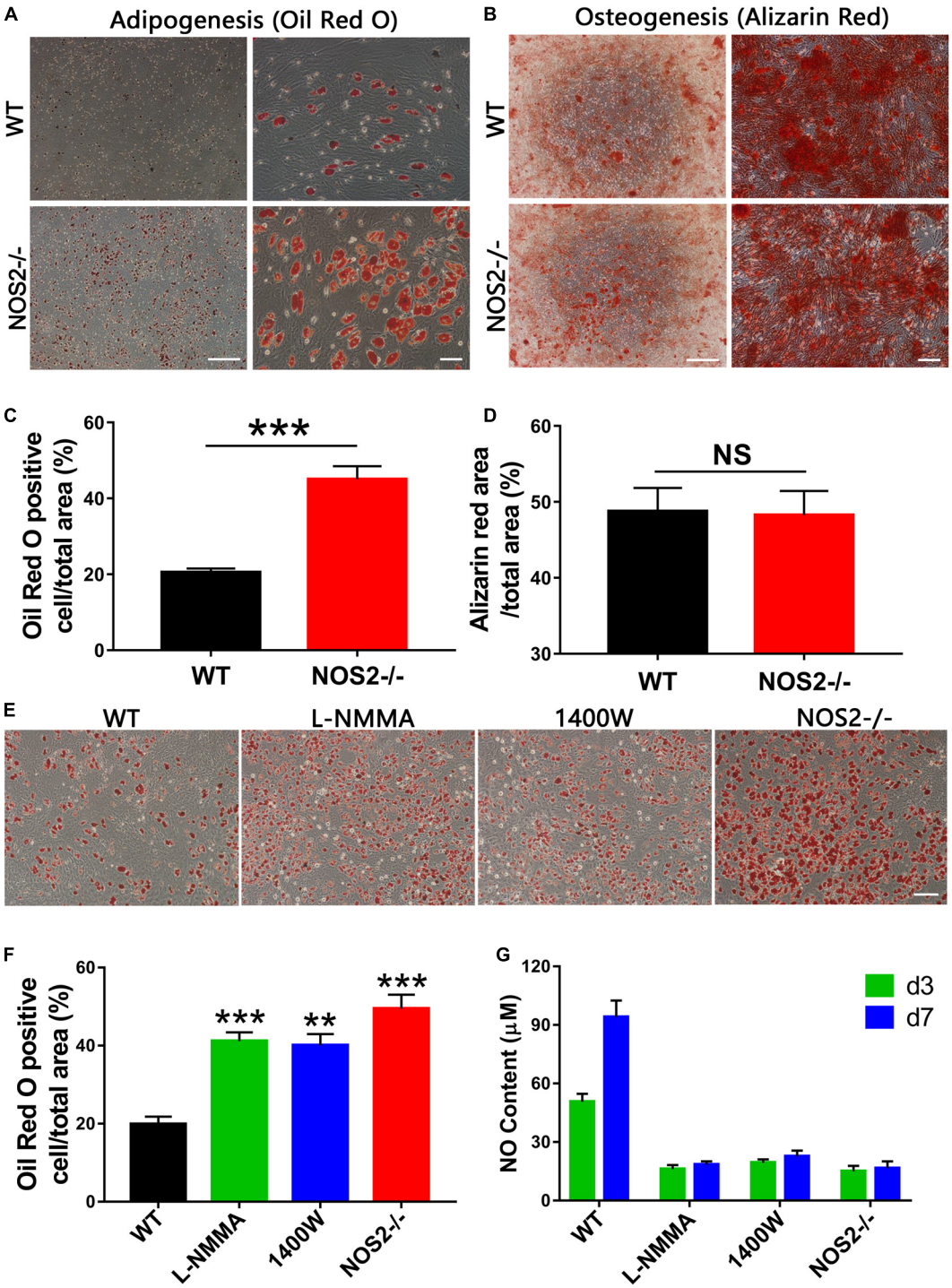

FIGURE 3 | Differentiation capacity of MSCs from NOS2$^{-/-}$ and WT SD rats. **(A)** BMSCs from NOS2$^{-/-}$ and WT rats at passage 3 were induced to differentiate into adipocytes and osteoblasts in differential medium and were detected as described in the Materials and Methods. **(A,B)** Adipocyte and osteoblast differentiation indicated by Oil Red O staining **(A)** and Alizarin red S staining **(B)**, respectively; the scale bars indicate 200 μm (left) and 50 μm (right). **(C,D)** Statistical analyses of panels **(A,B)** (n = 4), respectively. **(E)** Adipocyte differentiation indicated by Oil Red O staining. L-NMMA (1 mM) and 1400 W (100 μM) were added to adipogenic differentiation conditions of WT BMSCs; the scale bar indicates 100 μm. **(F)** Statistical analyses of panel **(E)** (n = 4). **(G)** NO content of cell supernatants in panel **(E)** was detected by Griess reagent. All results are expressed as the means ± SEM; $^{**}P < 0.01$; $^{***}P < 0.001$; N.S., not significant. n = 4.

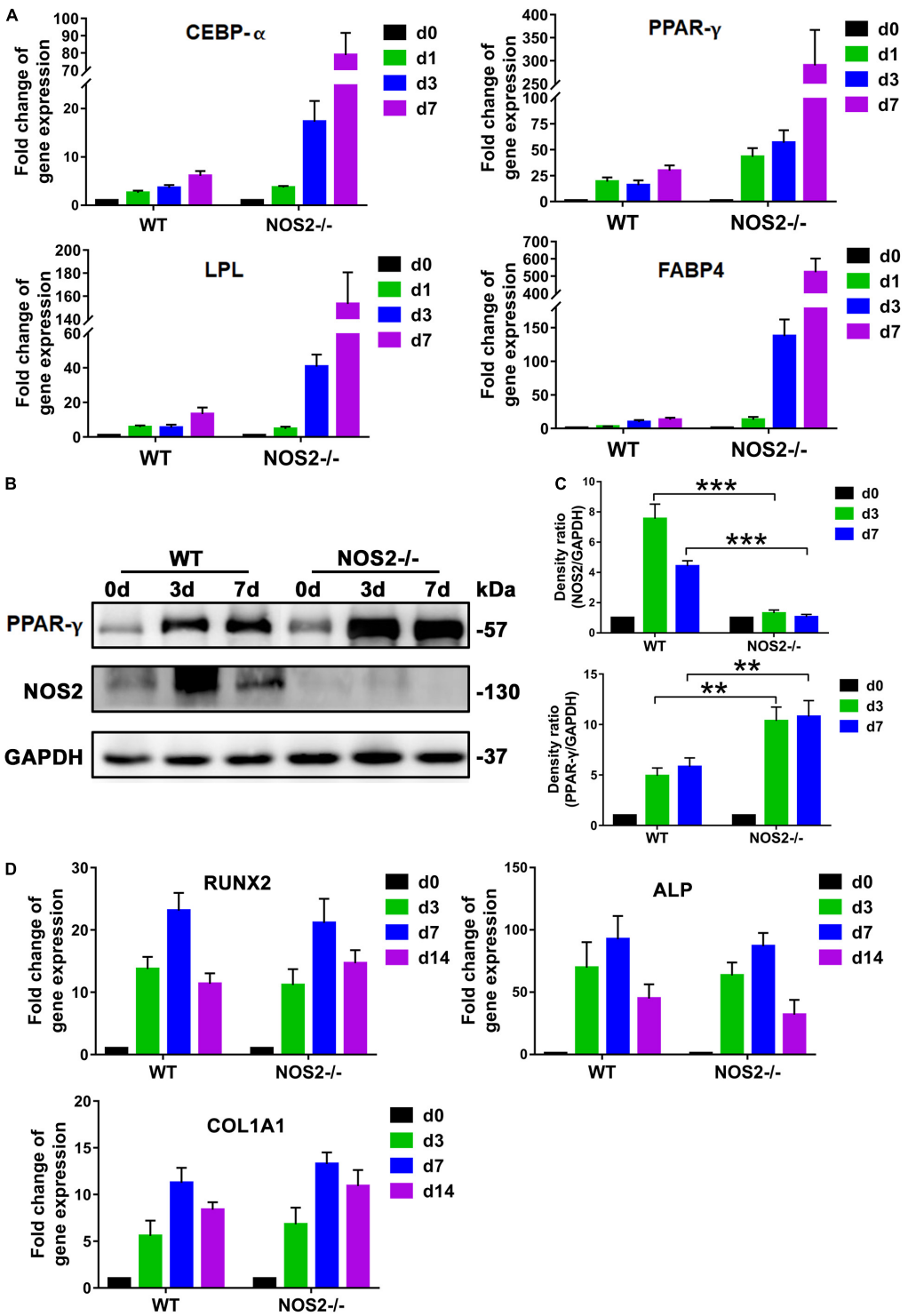

FIGURE 4 | NOS2 knockout increases the expression of adipogenesis-related genes. BMSCs from NOS2$^{-/-}$ and WT rats at passage 3 were induced to differentiate into adipocytes and osteoblasts and then collected to detect differentiation related genes. **(A)** qRT-PCR analyses of the expression of the transcription factors C/EBP-α and PPAR-γ and adipose differentiation-related genes LPL and FABP4 in MSCs from NOS2$^{-/-}$ and WT rats on d 1, 3, and 7 after differentiation **(A)** compared to those of the controls without differentiation (d 0). **(B)** BMSCs were cultured in AM for d 0, 3, and 7 days, and western blotting was performed to analyze induction of PPAR-γ. **(C)** Density ratio of PPAR-γ or NOS2 and GAPDH in panel **(B)**. **(D)** BMSCs were cultured in OM for d 0, 3, 7, and 14 days, and osteogenesis differentiation-related genes in BMSCs were detected by qRT-PCR. All results are expressed as the means ± SEM; $^{**}P < 0.01$; $^{***}P < 0.001$; N.S., not significant. $n = 4$.

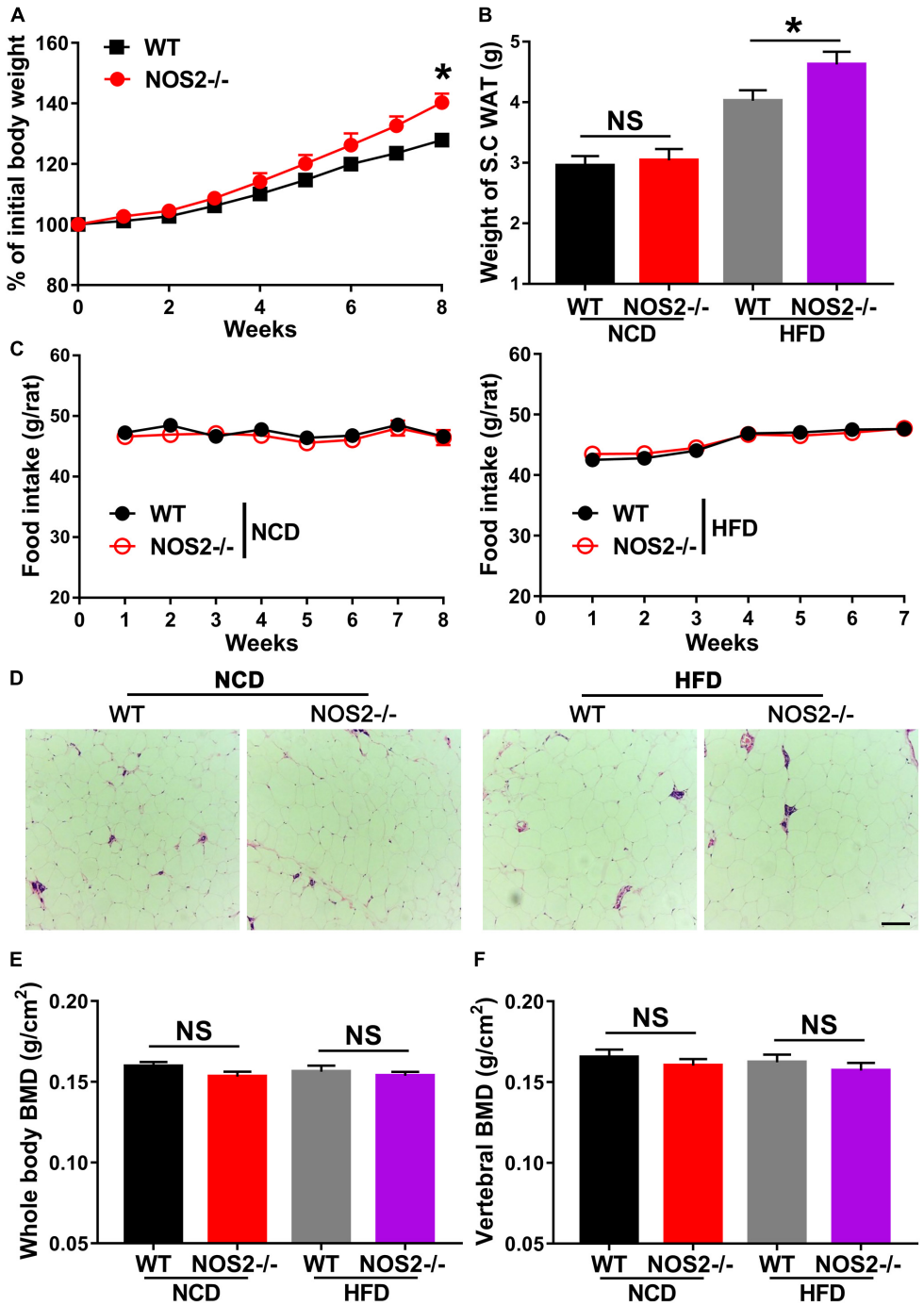

FIGURE 5 | Genetic Deletion of NOS2 promotes HFD-Induced Obesity. Eight-week-old female WT and NOS2$^{-/-}$ rats were fed either NCD or HFD for 8 weeks. **(A)** NOS2$^{-/-}$ rats gained more weight compared to WT rats on HFD (n = 10). **(B,C)** Changes in S.C. WAT pads and food intake in WT and NOS2$^{-/-}$ rats after 8 weeks of NCD or HFD feeding (n = 10). **(D)** Representative H&E staining images of S.C. WAT after 8 weeks of NCD or HFD feeding (n = 6), the scale bar indicates 100 μm. **(E,F)** Whole-body BMD **(E)** and vertebral BMD **(F)** evaluated by DXA after 8 weeks of NCD or HFD feeding (n = 6). All results are expressed as the means ± SEM; *P < 0.05; N.S., not significant.

observed in food intake between WT and NOS2$^{-/-}$rats on either NCD or HFD (**Figure 5C**). Furthermore, H&E staining of histological sections of WAT from WT and NOS2$^{-/-}$ rats showed no differences in WAT organization (**Figure 5D**).

To determine whether knockout of rat NOS2 affects osteoblast phenotype in response to a HFD, we analyzed BMD and BMC in rat whole body using DXA. As shown in **Figures 5E,F**, NOS2$^{-/-}$ rats displayed almost no changes in whole body BMD

and vertebral BMD compared to WT rats on either NCD or HFD. Whole body BMC and vertebral BMC were also unchanged in WT and NOS2$^{-/-}$ rats (**Supplementary Figure 2**). Taken together, our results clearly indicate that knockout of rat NOS2 promotes adipogenesis without affecting osteogenesis *in vivo*, consistent with our *in vitro* findings.

NOS2 Knockout Promotes Adipogenic Differentiation of Rat BMSCs Through the JAK2/STAT3 Signaling Pathway

Since the JAK/STAT signaling pathway is related to cell proliferation and differentiation (Richard and Stephens, 2014), we further investigated whether this pathway is involved in the upregulated adipogenesis in NOS2$^{-/-}$ rat MSCs. Western blot analysis showed that JAK activation (p-JAK2) was increased by 52.6 and 19.9% on days 3 and 7 after differentiation, respectively, compared to the WT BMSC group (d3, $p < 0.05$; d7, $p < 0.01$) (**Figures 6A,B**). The downstream protein p-STAT3, but not p-STAT1 or p-STAT5, was also increased by 33.4 and 62.5%, respectively (d3, $p < 0.01$; d7, $p < 0.01$) (**Figures 6A–E**).

To determine the role of STAT3 in rat MSC adipocyte differentiation, we utilized the JAK2 -specific inhibitor AG490 and the STAT3-selective inhibitor S3I-201 (NSC 74859) in NOS2$^{-/-}$ and WT BMSC adipogenesis induction. As shown in **Figures 7A–D**, both AG490 and S3I-201 inhibited STAT3 activation (**Figures 7A,B**) and NOS2$^{-/-}$ BMSC adipogenesis (for AG490, $p < 0.01$; for S3I-201, $p < 0.01$) (**Figures 7C,D**) after 12 days' differentiation. In contrast, the two inhibitors had no significant effect on WT BMSC adipogenesis (**Figures 7C,D**). Most importantly, western blotting also revealed increased expression of p-STAT3 protein in NOS2$^{-/-}$ rat WAT with HFD feeding (**Figures 7E,F**). Our findings strongly suggest that the greater adipogenic potential of NOS2$^{-/-}$ BMSCs was primarily due to greater activation of the JAK2/STAT3 signaling pathway than occurs in WT BMSCs.

DISCUSSION

In recent years, much attention has been given to NO as a key regulator of cell differentiation. The inducible synthase of NO, NOS2, was found to regulate the differentiation and function of many immune cells, including T cells (Lee et al., 2011; Niedbala et al., 2011; Jianjun et al., 2013; Obermajer et al., 2013; Garcia-Ortiz and Serrador, 2018). Here, our data confirmed that BMSCs derived from NOS2$^{-/-}$ rats showed a clear immunosuppressive effect on T cell proliferation. These findings are consistent with the specific NOS inhibitor L-NMMA (Su et al., 2014) and are further supported by studies in NOS2-knockout mice, which also exhibited abolished cytokine-induced immunosuppression in MSCs (Ren et al., 2008). In mouse, low concentrations of NO promote Th1 differentiation, whereas high concentrations of NO from NOS2 promote Th2 cell differentiation and inhibit Th17 differentiation (Lee et al., 2011; Niedbala et al., 2011; Jianjun et al., 2013). In contrast, NO produced by NOS2 in activated CD4 + T cells is necessary for the induction and stability of human Th17 cells (Obermajer et al., 2013). In a mouse muscle injury model, NO released by infiltrating NOS2-positive macrophages was necessary for the proliferation and differentiation of myogenic precursor cells (Rigamonti et al., 2013). Indeed, various studies have shown that the differentiation and functional regulation of many immune cells, including T cells, macrophages, and mature dendritic cells (mDCs), by NOS2 are mediated *via* nitration of key molecules in transcriptional or signaling pathways (Mao et al., 2013; Mishra et al., 2013; Bogdan, 2015). Our findings once again highlighting the role of NOS2 and MSCs in the regulation of immunity.

In addition to their roles in immunoregulation, MSCs are fibroblast-like multipotent cells that have the ability to differentiate into osteoblasts, adipocytes, chondrocytes, and thus a powerful tools for tissue engineering, regenerative medicine and also drug delivery (Pittenger et al., 1999; Gjorgieva et al., 2013; Ackova et al., 2016; Chen et al., 2016). As osteoblast differentiation was not altered in our NOS2$^{-/-}$ rat BMSCs, we considered that NOS2 may not play an essential role in rat MSC osteogenic differentiation. However, many studies on total NO have observed its effects on the physical activities of bone, including bone development, bone healing, and bone resorption (Collin-Osdoby et al., 1995; Chow, 2000; Zhang et al., 2016). Recently, Yang et al. (2018) reported that NO balances osteoblast and adipocyte lineage differentiation *via* the JNK/MAPK signaling pathway in periodontal ligament stem cells. The contrast in these findings may be due to the source of NO, as these results did not distinguish among the activities of NOS1, NOS2 or NOS3, any of which could be the true regulator(s) of osteogenic differentiation. A study on mouse did support this explanation, as NOS1$^{-/-}$ mice exhibited profound abnormalities in bone formation, and their osteoblasts showed significant delays in differentiation (Van'T et al., 2004). Importantly, a study on NOS2$^{-/-}$ mouse also showed decreased osteoblast growth, and the resulting osteoblasts covered a smaller culture dish area and generated fewer resorption pits (Herrera et al., 2011). Since mouse NOS2 produces significantly lower NO levels than rat NOS2 (Zhao et al., 2013), and NOS1 in both mouse and rat produces even lower levels (Li et al., 2012), we considered only an appropriate amount of NO may influence osteogenesis. On the other hand, NOS2 may only influence the growth of osteoblasts, which was not observed in our experiments. Therefore, we conclude that NOS2 may not affect osteogenic differentiation of MSCs.

A theoretical inverse relationship exists between osteogenic and adipogenic lineage commitment and differentiation such that differentiation toward adipogenesis occurs at the expense of osteogenesis. However, we found this may not be true in the present study, as NOS2$^{-/-}$ rat BMSCs showed significantly increased adipogenesis by Oil Red O staining with osteoblast differentiation remaining unaltered (**Figure 3**). Our finding on adipogenesis is supported by previous studies on the production of NO promoting adipocyte differentiation (Yan et al., 2002; Engeli et al., 2004; Hemmrich et al., 2007), although other observations have suggested that NO exerts the opposite effect on adipogenesis (Kawachi et al., 2007; Cordani et al., 2014; Yang et al., 2018). These conflicting findings may be due to the effect of a less specific inhibitor, the use of different MSCs, or the assaying

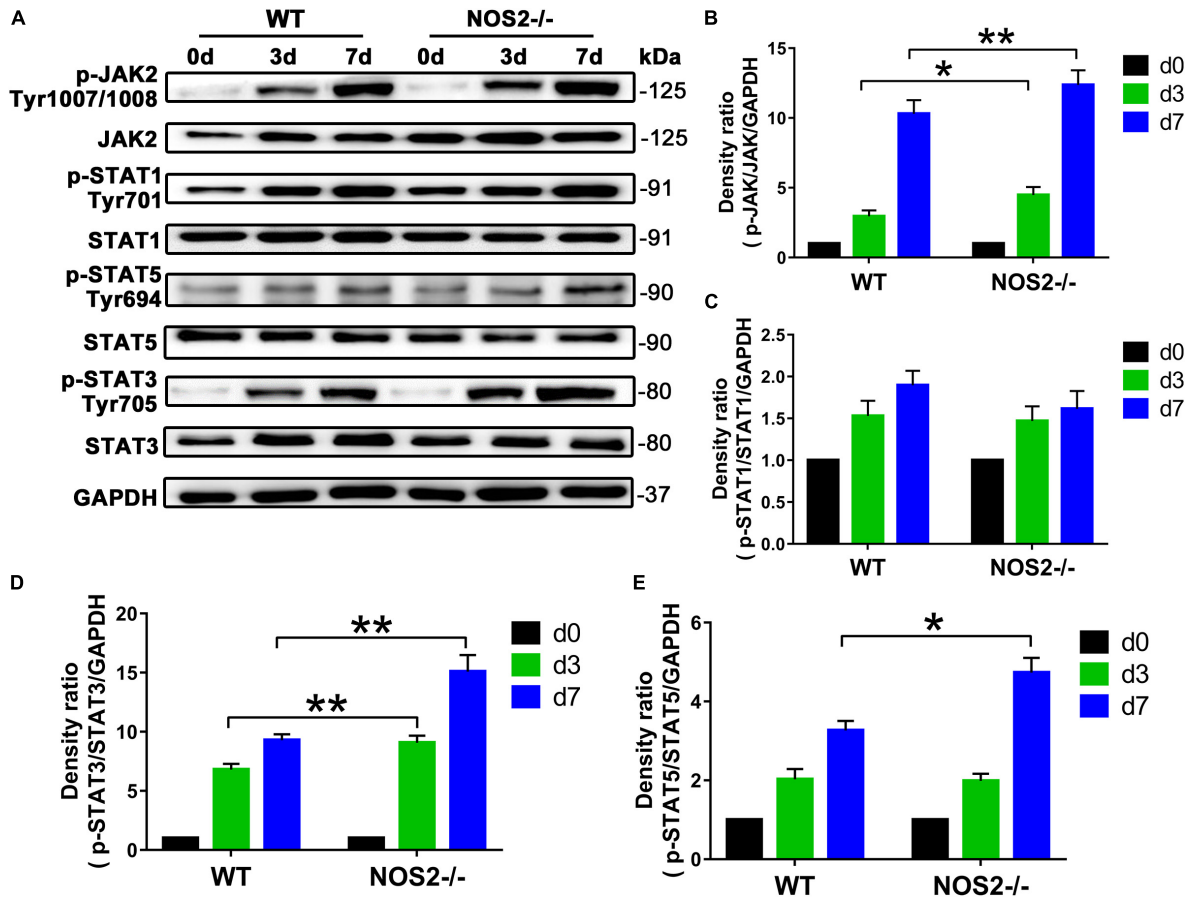

FIGURE 6 | Knockout of NOS2 promotes rat MSC adipocyte differentiation through signal transducer and activator of transcription (STAT)3 and JAK signaling. BMSCs from NOS2$^{-/-}$ and WT rats at passage 3 were induced to differentiate into adipocytes and were collected to detect the expression of proteins in the JAK/STAT signaling pathway at the indicated time points. The protein levels of p-JAK2, JAK2, p-STAT1, –3, and –5 and STAT1, –3, and –5 in NOS2$^{-/-}$ and WT rat BMSCs were assessed by western blotting. **(A)** Representative western blots are shown. **(B–E)** Density ratios of p-JAK2 and JAK2, p-STAT1 and STAT1, p-STAT3 and STAT3, and p-STAT5 and STAT5. Data are normalized to GAPDH. All results are expressed as the means ± SEM; *$P < 0.05$; **$P < 0.01$; $n = 4$.

of different NO sources (endogenous or exogenous), since NO is also generated by NOS1 and NOS3, and not just NOS2, in these cells. These specificity issues may be solved by using our model of NOS2 knockout rats. Therefore, we propose that NOS2 and its product NO are essential regulators suppressing excessive adipogenesis differentiation in rat MSCs. This is an interesting indication that perhaps when losing NOS2/NO, MSCs tends to switch their role from immunomodulation (specific inhibition of T cell proliferation) to energy storage (adipocyte differentiation).

A cascade of sequential transcriptional regulatory bursts underpins adipogenesis, including C/EBP-α and PPAR-γ, which are major late transcription factors (Rosen et al., 2002; Farmer, 2006). PPAR-γ is a ligand-activated nuclear receptor and is indispensable for adipocyte differentiation both *in vitro* and *in vivo* (Kawai and Rosen, 2010). In our study, qRT-PCR and western blot results revealed that PPAR-γ expression was significantly increased in NOS2$^{-/-}$ MSCs compared to WT MSCs, suggesting a role for PPAR-γ in connecting NOS2 depletion and adipogenesis. Such a connection is also suggested by a few reports that PPAR-γ regulates human mesenchymal lineage allocation, favoring adipocyte over osteoblast development (Yu et al., 2012). Lee et al. (2010) reported anti-adipogenesis by 6-thioinosine is mediated by downregulation of PPAR-γ through JNK-dependent upregulation of NOS2. On the other hand, our findings also suggest that PPAR-γ may not influence osteogenesis. Consistent with our data, a study in human MSCs observed that PPAR-γ RNAi had no obvious effect on osteogenesis under permissive conditions (Bruedigam et al., 2008). These similarities between human and rat models may indicate the potential for using rat models to address human questions.

In addition to PPAR-γ, STAT3 may directly regulate adipogenic differentiation of MSCs. STAT3 belongs to the STAT (signal transducers and activators of transcription) signaling pathway family, which is a common pathway for signal transduction to regulate cytokines that affect several physiological processes, including cell proliferation, differentiation, apoptosis, and interactions with other signaling pathways (Stark and Darnell, 2012). Many studies on development of adipocytes from preadipocytes have revealed that STAT-mediated gene expression

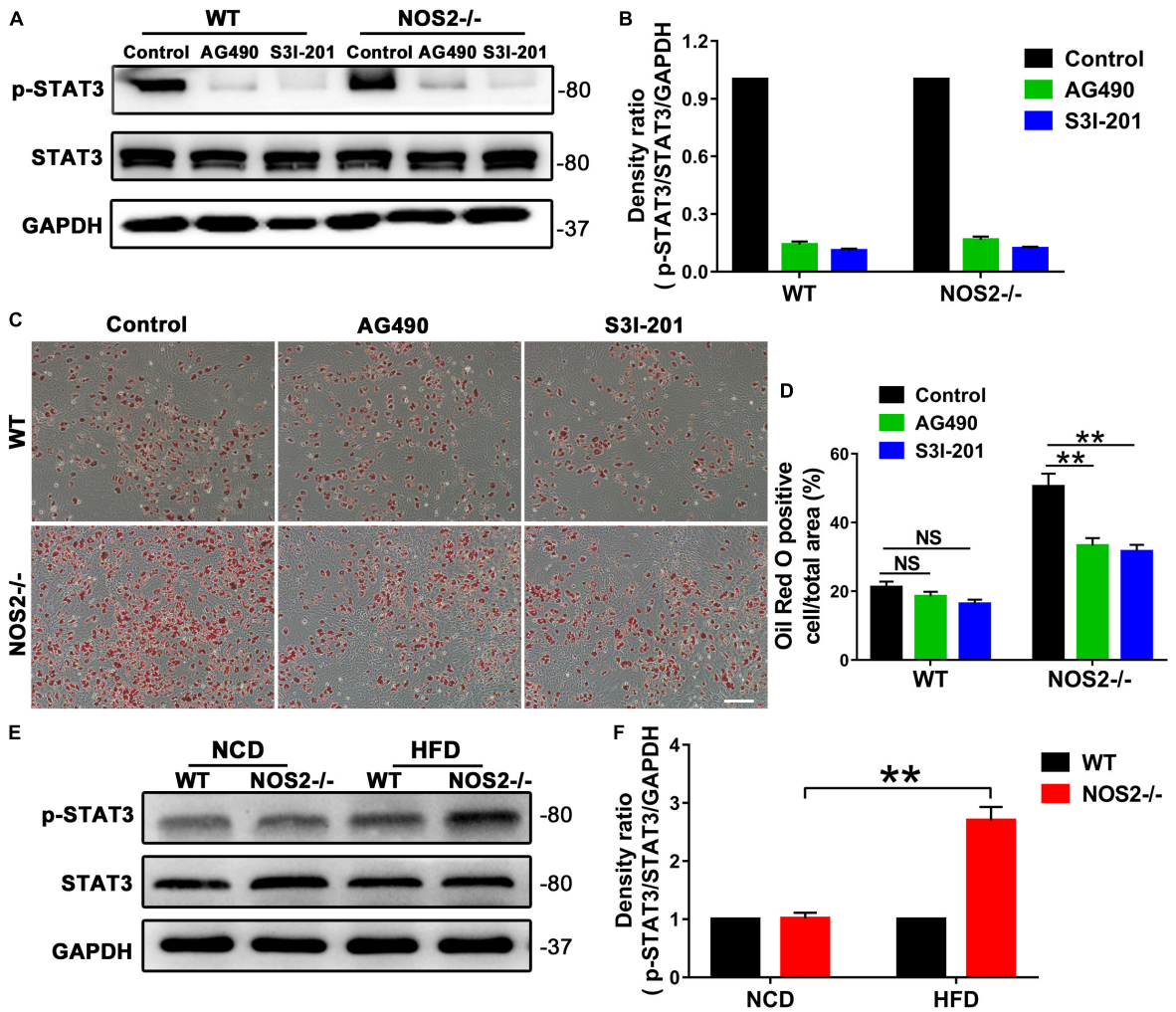

FIGURE 7 | AG490 and S3I-201 inhibit STAT3 activation and adipocyte differentiation in NOS2$^{-/-}$ rat MSCs. BMSCs from NOS2$^{-/-}$ rats at passage 3 were induced to differentiate into adipocytes in the presence or absence of AG490 (10 μM) and S3I-201 (20 μM) and were collected to detect p-STAT3 and STAT3 expression levels **(A,B)** and adipocyte differentiation **(C,D)** on d 12 after differentiation. **(E)** Representative western blots are shown in WAT from WT and NOS2$^{-/-}$ SD rats after HFD feeding. **(F)** Density ratios of p-STAT3 and STAT3. The scale bar indicates 100 μm. All results are expressed as the means ± SEM; $^{**}P < 0.01$; $n = 4$.

and modifications are cell-type specific. Transgenic knockout studies have also shown critical roles for every member of the STAT family (Richard and Stephens, 2014). STAT1, STAT3, and STAT5 are critical for adipogenesis (Zhao and Stephens, 2013). Our data demonstrated that STAT3 proteins from NOS2$^{-/-}$ BMSCs are tyrosine phosphorylated (activated form) during the initiation of adipocyte differentiation (**Figure 4**), and this activation in NOS2$^{-/-}$ BMSCs preceded the corresponding increase in WT BMSCs. Furthermore, deactivation of STAT3 by an inhibitor suppressed adipocyte differentiation in NOS2$^{-/-}$ BMSCs. The clear involvement of the STAT3 pathway in adipocyte differentiation is consistent with the knowledge of the JAK2/STAT3 pathway's involvement in early adipogenesis through regulation of C/EBP-β transcription (Zhang et al., 2011). Interestingly, a connection between STAT3 and NOS2 has been previously reported in glial proliferation and transformation (Puram et al., 2012), as well as in human macrophages in response to *Mycobacterium tuberculosis* infection (Zhou et al., 2019). Therefore, our finding that STAT3 expression was strongly upregulated during adipocyte differentiation in NOS2$^{-/-}$ BMSCs suggests that STAT3 is a key factor in adipogenic differentiation.

Another possible route by which NO favors adipogenic differentiation may be its role in regulating lipid metabolism. Pioneering studies revealed the expression and activity of NOS2 in adipose tissue long ago (Ribiere et al., 1996) and verified an NO-mediated effect on lipolysis regulation both *in vitro* and *in vivo* (Gaudiot et al., 1998; Adam et al., 1999). The inhibitory action of NO on basal lipolysis, shown with *in vitro* chemical NO donors, may function *via* the inhibition of adenylyl cyclase (AC) and protein kinase A (PKA) (Adam et al., 1999; Klatt et al., 2000). NOS2 is a negative modulator of lipolysis *via* an oxidative signaling pathway upstream of cAMP production (Penfornis and Marette, 2005). In adipose tissue, its fuel-buffering

capacity is also dependent on the physiological levels of NO (Jankovic et al., 2017) as a small redox molecule. These results indicate that physiological levels of NOS2/NO play a pivotal role in maintaining healthy metabolic function of adipose tissue. The existence of NOS2 may ensure MSC's adipogenic differentiate without excessive differentiation or no differentiation. Of course, the pathogenesis of obesity is far more complex than just lipid accumulation and involves interactions among many cell types (Choe et al., 2016). Under conditions of metabolic stress, lipid accumulation in NOS2-/- rat maybe develop into a variety of metabolic syndrome, such as insulin resistance (Jiang et al., 2011), type 2 diabetes (Kopelman, 2000), vascular pathology (O'Rourke et al., 2011) and hepatic steatosis (Festi et al., 2004). Therefore, whether and how these lipid metabolic changes due to NO depletion alter the tendency toward MSC differentiation into adipocytes would be very interesting to address in future studies.

Moreover, NO may influence cells, tissues/organs, and consequently the whole body in various ways. In subcutaneous WAT, NO produced by NOS2 in obese individuals may impair insulin-stimulated glucose uptakes or contribute to decreased lipolytic rates, contributing to increased lipid storage (Penfornis and Marette, 2005). In liver, NO decreases hepatic lipogenesis through its actions on coenzyme A, forming a metabolically inactive compound (Greif et al., 2002). In skeletal muscle, NO also decreases lipogenesis *via* the activation of AMP-activated protein kinase, which is associated with increased fatty acid oxidation during exercise (Winder and Hardie, 1996). Therefore, it is not surprising to find that NO is depleted in NOS3-knockout mice and that these mice exhibited increased abdominal fat mass, dyslipidemia, and insulin resistance (Nisoli et al., 2003). Therefore, our findings regarding the effects of NO on adipogenesis greatly highlight its roles in the physiology of adipogenesis.

In addition, NOS2 activity has been found relevant to cellular senescence in various cell types (Ropelle et al., 2013; Katsuumi et al., 2018). Aging BMSCs display a shift in differentiation ratio of less osteoblasts and more adipocytes (Qadir et al., 2020), which is interestingly a bit similar with our findings in NOS2-/- MSCs. A further clarification of both processes may reveal the potential of sharing underlying mechanisms to some extent.

In summary, knockout of NOS2 impairs the function of MSCs with respect to regulation of immunity by enhancing their capacity for adipogenesis, but not osteogenesis, *via* PPAR-γ and STAT3.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Experimental Animal Ethics Committee of Guangzhou Medical University.

AUTHOR CONTRIBUTIONS

AQ was responsible for isolating and culturing rat MSCs, participated in the design of the study and analysis of the data, and drafted the manuscript. SC and PW performed major experiments and analyzed data. XH was in charge for the detection and analysis of flow cytometry samples. YZ performed the qRT-PCR experiment. LL and L-RD were in charge of detection and analysis of osteoblast phenotype or adipocytic phenotype from rats. D-HL participated in the design of the study and drafted the manuscript. LD performed the detection and analysis of HE staining. XY and AX supervised the experiments and secured grants. All authors read and approved the final version of the manuscript.

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Comparable *in vitro* Function of Human Liver-Derived and Adipose Tissue-Derived Mesenchymal Stromal Cells: Implications for Cell-Based Therapy

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Mesenchymal stem/stromal cells (MSCs) have been investigated extensively for their immunotherapeutic and regenerative properties, which may differ by cell source. In MSCs harvested from donors matched for sex, age, and body mass index, we compared the proliferative and migration functions of liver-derived MSCs (L-MSCs) and adipose tissue-derived MSCs (A-MSCs) (n = 6 donors each). Cellular senescence was evaluated by senescence-associated beta-galactosidase enzyme activity and expression of senescence-associated secretory phenotype (SASP) factors using real-time quantitative polymerase chain and by western blot assay. The pro-angiogenic and reparative potency of MSCs was compared by co-culturing MSCs with injured human umbilical vein endothelial cells (HUVEC). The proliferation and migration properties were similar in L-MSCs and A-MSCs. Although cell cycle arrest and SASP genes were similarly expressed in both MSCs, tumor necrosis factor alpha gene and protein expression were significantly downregulated in L-MSCs. In co-cultured injured HUVEC, A-MSCs restored significantly more tubes and tube connections than L-MSCs. Therefore, despite many functional similarities between L-MSCs and A-MSCs, L-MSCs have enhanced immunomodulatory properties, while A-MSCs appear to have better pro-angiogenic and vascular reparative potency. Availability of a broad range of cellular options might enable selecting cell-based therapy appropriate for the specific underlying disease.

Keywords: Mesenchymal stroma/stem cells, liver, adipose tissue, cellular senescence, angiogenesis

INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) are non-hematopoietic multipotent cells with the inherent ability to self-renew and differentiate into tissues of mesenchymal origin (Dominici et al., 2006). They have essential roles in tissue repair and regeneration through their anti-apoptotic activity and stimulation of angiogenesis, as well as notable immunomodulatory activities (Han et al., 2019).

MSCs have been demonstrated to regulate immune reactivity through paracrine mechanisms by inhibiting both innate and adaptive responses (Zhou et al., 2019). Their impact on lymphocytes, macrophages, dendritic cells, and natural killer (NK) cells has been investigated extensively (Wang et al., 2014; Najar et al., 2016). Owing to these potent immunomodulatory properties, MSCs are actively investigated as cellular therapeutics in multiple disease processes.

Mesenchymal stem/stromal cells have been successfully isolated from several tissues, including fat, placenta, peripheral blood, and liver since their first discovery, and while MSCs from different sources have many functional similarities, they also show several differences (Mattar and Bieback, 2015; Kozlowska et al., 2019). Given the liver's unique tolerogenic microenvironment (Taner et al., 2016, 2017, 2018), we had postulated that liver MSCs (L-MSCs) are superior immunomodulators than their counterparts isolated from other tissues. In fact, we demonstrated that L-MSCs inhibited alloreactive T cell proliferation better than MSCs isolated from adipose tissue (A-MSC) and bone marrow (BM-MSC) (Taner et al., 2020). Transcriptome analysis also demonstrated that the L-MSCs have significantly upregulated expression of genes and gene sets associated with immune regulation. Therefore, L-MSCs may be better candidates for cellular therapies.

However, besides immunomodulation, other properties might determine the functional potency of MSC. Among the most beneficial functions of MSCs are their angiogenic and tissue repair properties. MSC-based cellular therapy has been beneficial in various disease conditions, including coronary artery disease and skin wound repair through regulation of angiogenesis and tissue repair (Fan et al., 2020). These MSC functions are strongly influenced by the premature aging of the cells, termed cellular senescence (Suvakov et al., 2019). Cellular senescence is defined as a state of cell cycle arrest mediated by the tumor suppressor proteins p53, p21, and p16 that alters the cellular functions. Cells respond to the senescence state by activating the senescence-associated secretory phenotype (SASP) (Krtolica and Campisi, 2002). The SASP is characterized by diverse growth factors, cytokines, chemokines, and matrix metalloproteinases and might alter the cell metabolism, influence tissue homeostasis, and compromise the immunoregulation of MSCs (Lunyak et al., 2017). Senescence characteristics of MSCs have been shown to differ based on their tissue origin (Turinetto et al., 2016). Therefore, we believe the proper use of MSCs for clinical applications requires a general understanding of the functions and senescence characteristics of MSCs from different sources.

While tissue source accounts for key differences in the immunomodulatory properties of MSCs generated *in vitro*, whether other functions of L-MSC are similarly altered remain unknown (Yoo et al., 2009; Waldner et al., 2018). This is particularly important as variations in tissue microenvironment within the same tissue also influence MSCs. For example, we have recently shown that A-MSCs obtained from obese individuals undergo early senescence compared to A-MSCs from age-matched non-obese individuals, which was linked to decreased function (Conley et al., 2020). However, whether intrinsic cellular senescence in L-MSC differs from A-MSCs remains unknown, especially given the well-known superior regenerative capacity of the liver compared to other organs. Thus, we aimed to compare different properties of L-MSCs, which might affect their potential therapeutic applications.

To that end, we have tested the proliferative capacity, migratory activity toward inflammatory signals, response to stress signals, and reparative function on blood vessels of L-MSCs in comparison to A-MSCs.

MATERIALS AND METHODS

Isolation and Culture of MSC

The study was approved by the Mayo Clinic Institutional Review Board. MSCs were isolated from liver tissue of deceased organ donors (L-MSC) and subcutaneous abdominal adipose tissue of living kidney donors and weight reduction surgery (A-MSC). These donors underwent screening and met the criteria for organ donation. All samples were processed within 8 h of procurement. Liver and adipose tissues were minced in a Petri dish and mixed with 0.075% Collagenase IV (STEMCELL Technologies, Cambridge, MA, United States) in Dulbecco's phosphate-buffered saline. After 45 min, enzyme action was stopped by adding platelet lysate (PL5%) MSC media; Advanced Minimum Essential Medium (Thermo Fisher Scientific, Waltham, MA, United States), PLTGold Human Platelet Lysate (EMD Millipore, Burlington, MA, United States), and GlutaMAX (Thermo Fisher Scientific, Waltham, MA, United States). Digested tissue was centrifuged, resuspended in fresh media, and filtered twice before plating in PL5% MSC media. Non-adherent cells were removed every 3 days thereafter. Cultures were maintained at 37°C, 5% CO_2 in a humidified incubator.

MSC Characterization

Cell morphology, trilineage differentiation, and phenotype were analyzed to characterize MSCs according to The International Society for Cellular Therapy (Dominici et al., 2006). Briefly, MSCs' differentiation ability into adipocyte, osteocyte, and chondrocyte lineages was assessed using the Human MSC Functional Identification Kit (R&D Systems, Minneapolis, MN, United States), according to the manufacturer's recommended protocol. Differentiation was tested by immunofluorescent staining using anti-mFABP4 (adipocyte), anti-human osteocalcin (osteocyte), and anti-human Aggrecan (chondrocyte).

The detailed characterization of both the L-MSC and the A-MSC used in the current study was reported previously (Conley et al., 2020; Taner et al., 2020). Both types of MSCs demonstrated plastic-adherent characteristics and spindle-shaped morphology when cultured in PL5% MSC media. Furthermore, the cells were labeled with fluorochrome-conjugated monoclonal antibodies to confirm canonical MSC markers (CD73, CD90, and CD105) expression, as well as non-expression of CD14 and CD45 by flow cytometry. Additionally, CD200 and CD274 expressions were tested for L-MSC. L-MSC and A-MSC data were analyzed using Kaluza software (Beckman Coulter, Chaska, MN, United States) and Analysis Software (IDEAS version 6.2), respectively.

MSC Migration and Proliferation

Cellular proliferation of MSCs was evaluated by a live cell analysis and imaging system (Incucyte®, Sartorius, Ann Arbor, MI, United States). Approximately 2.5×10^3 A-MSCs or L-MSCs (n = 6 each type) were seeded per well in a 96-well plate, then placed into Incucyte® SX1 Live-Cell Analysis System at 37°C in a CO_2 incubator and allowed to propagate in culture for 48 h. During incubation, MSCs' images were taken every 2 h, and proliferation was analyzed using Incucyte® Cell-by-Cell Analysis Software. Migratory functions of MSCs were tested using a QCM™ Colorimetric Cell Assay (EMD Millipore, Burlington, MA, United States) as well as a scratch assay, according to the manufacturer's instructions. Briefly, for colorimetric cell assay, 1.5×10^5 MSCs (n = 6 each type) were seeded into the insert of 24 wells transwell system, and PL5% MSC media was added to the lower chamber, then MSCs were incubated 24 h at 37°C in a CO_2 incubator. Afterward, the insert was stained, and the stain then extracted. The optical density of the extracted stain was measured at 560 nm.

The migration scratch assay was performed in two independent experiments. A 5×10^5 L-MSCs (n = 5) and A-MSCs (n = 5) were seeded on a 24-well plate as duplicates. The semi-automated BioTek AutoScratch™ Wound Making Tool has created scratches in confluent cell monolayers, and then wells were washed twice to remove detached cells before placing them on BioTek Cytation™ 5 Cell Imaging Multi-Mode Reader, which was maintained at 37°C and 5% CO_2 for 24 h. Images were taken at 2-h intervals to observe their movements which were analyzed by BioTek Scratch Assay App. The change in the scratch area was calculated every 2 h and normalized to the baseline scratch area. The migration speed was calculated individually for cells until the scratch closed, and the average migration speed was calculated by taking the mean of their individual speeds.

Chemoattractants used in the migration assay were those enriched in human platelet lysate, including stromal cell-derived factor-1 (SDF-1), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and platelet-derived growth factor-1 (PDGF-1).

Senescence and Senescence-Associated Secretory Phenotype (SASP) Marker Expression by Quantitative Polymerase Chain Reaction (qPCR)

Using the mirVana™ PARIS kit (Thermo Fisher Scientific, Waltham, MA, United States), total RNA was isolated from MSCs according to the kit protocol. RNA concentration was measured using a NanoDrop spectrophotometer and the first-strand cDNA produced by the Superscript™ VILO™ cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, United States). Relative qPCR was performed on a QuantStudio 7 Real-Time PCR system using TaqMan® assays. The fold change of gene expressions was calculated using the 2-ΔΔCT method. All TaqMan® probes were purchased from Thermo Fisher Scientific. Cellular senescence was determined by the expression of the cell cycle arrest markers p16 (Cat.# HS00923894), p21 (Cat.# HS00355782), and p53 (Cat.# HS01034249), as well as the SASP markers, activin A (INHBA, Cat.# HS01081598), monocyte chemoattractant protein-1 (MCP-1, HS00234140), plasminogen activator inhibitor-1 (PAI-1, Cat.# HS00167155), interleukin-1 alpha (IL-1α, Cat.# HS00174092), interleukin 6 (IL-6, Cat.# HS00174131), and tumor necrosis factor alpha (TNFα, Cat.# HS00174128). Gene expression was normalized to TATA-binding protein (TBP, Cat.# HS00427620).

Western Blot Assay

Mesenchymal stem/stromal cells were detached with Tryple™ (Gibco™, Invitrogen, Carlsbad, CA, United States) and centrifuged to obtain a pellet. Then pellets were lysed for 20 min on ice with cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, United States), and total proteins transferred on 4–20% SDS-PAGE gels and transferred onto PVDF membranes as duplicates. The membranes were blocked with 5% BSA for an hour and incubated with primary antibodies. After washes, the membranes were incubated with secondary antibodies for an hour at room temperature. The membranes were washed and then incubated with ECL Western Blot Substrate (Cell Signaling Technology, Inc., Danvers, MA, United States) and were visualized on ImageQuant™ LAS 4000. Anti-TNFα (Cat# ab6671) and PAI-1 (Cat# ab66705) antibodies were purchased from Abcam (Cambridge, MA, United States). GAPDH antibody was used to normalize the results. All experiments were done separately and independently for each cell line.

Senescence-Associated Beta-Galactosidase Enzyme Activity

Senescence-associated beta-galactosidase (SA-β-Gal) enzyme activity, a participant in cellular senescence (Dimri et al., 1995), was evaluated using the assay of the β-galactosidase enzyme (Enzo, Farmingdale, NY, United States) and staining (Dojindo Molecular Technologies, Inc., Rockville, MD, United States) according to manufacturer's instructions. The percentages of β-gal positive cells were quantified in 3 fields of view using the Cytation-5 Cell Imaging Reader. Galactosidase beta 1 (GLB1, Cat.# HS01035168), the encoding gene for SA-β-Gal, expression was evaluated using qPCR.

Co-culture of MSCs With Human Umbilical Vein Endothelial Cells

Co-culture experiments were performed to evaluate the pro-angiogenic and reparative potency of the MSCs, as previously described (Conley et al., 2020). In brief, human umbilical vein endothelial cells (HUVEC, Cell Applications, San Diego, CA, United States) were grown in endothelial cell growth medium (EGM™-Plus Endothelial Cell Growth Media-Plus BulletKit™ Medium, Lonza, Cohasset, MN, United States), and seeded at a density of 3.5×10^5 cells/well in the lower chamber of a transwell plate. The cells were divided into 4 groups to test the effect of MSCs. Group 1 was the control group and was cultured in normal conditions, whereas groups 2–4 were co-incubated with TNFα (10 ng/mL) and

transforming growth factor-beta 1 (TGF-β1, 5 ng/mL) for 3 days to induce cellular injury (Khan et al., 2017). After the co-incubation, this media was changed with fresh growth medium, subsequently co-cultured with either L-MSCs (group 3) or A-MSCs (group 4) (1.75×10^5 cells/well insert) for another 24 h. HUVEC were then harvested, lysed, and prepared for qPCR analyses.

Following treatment and co-culture procedures (as described above), HUVEC were plated onto a Matrigel® matrix-coated plate (CORNING, Corning, NY, United States) at a final concentration of 7×10^4 cells/500 μL and incubated overnight in a cell culture incubator. Zeiss Axio Observer microscope was used to determine the number of tube-like structures and connections in five different fields of view for each well.

Statistical Analysis

Normally distributed data were represented as mean ± standard deviation and non-normal data as median and interquartile range. Comparisons among groups were performed using the two-sample *t*-test with a 5% type-I error. One-way analysis of variance (ANOVA) was employed to detect differences in co-culture experiments, whereas cellular proliferation data were analyzed using repeated ANOVA. All data were considered significant if $p \leq 0.05$. Statistical analysis was accomplished using JMP software (SAS Institute, Cary, NC, United States).

RESULTS

MSC Culturing and Characterization

All the cells used in the current study had been isolated and characterized in our previous studies independently. The six primary L-MSC batches tested herein were generated from deceased donor liver allografts (Taner et al., 2020). **Table 1** summarizes the demographics of each group. A-MSC from six subjects matched for sex, age, and body mass index (BMI) were harvested from fat tissue (Conley et al., 2020). The mean age of the L-MSC and A-MSC donors was similar, as was their mean BMI. After harvesting and isolating, MSCs were expanded in culture for three passages to prepare for the experiments. Culture media was replaced every 3 days until MSCs reached 80% confluence throughout this study.

TABLE 1 | L-MSC from liver tissue of deceased organ donors and A-MSC from subcutaneous abdominal adipose tissue of living kidney donors and weight reduction surgery were matched for sex, age, and body mass index (n = 6 per group).

	L-MSC (n = 6)	A-MSC (n = 6)	*P*-value
Sex (M/F (ratio))	3/3 (50%)	3/3 (50%)	
Age (years ± SD)	44 ± 18	48 ± 15	0.37
Body mass index (kg/m^2 ± SD)	31.2 ± 5.9	35.1 ± 5.8	0.14

The results are presented as the mean ± standard deviation, and statistical significance was analyzed by the two-sample t-test. MSC, Mesenchymal stem/stromal cell; L-MSC, Liver MSC; A-MSC, Adipose MSC.

Migratory and Proliferative Capacities of MSC

In order to compare the proliferation capability of L-MSCs and A-MSCs, cells were incubated for 48 h and assessed with live-cell imaging. At none of the time points was there a difference in percent confluence, demonstrating similar proliferate kinetics in both cell types (p = 0.10) (**Figure 1A**). Two methods were employed to assess cellular migration. When the migration of the MSCs toward chemoattractants across a semi-permeable membrane was tested, A-MSCs were noted to have variable migratory capacity, consistent with our previous data (Conley et al., 2020). Migration kinetics of L-MSCs were more homogenous. On average, the migratory function of L-MSCs and A-MSCs was similar (p = 0.24) (**Figure 1B**). Moreover, we performed a migration scratch assay for 24 h to further characterize their migratory kinetics (**Figure 2A**). The closure of scratch was significantly faster for A-MSCs than L-MSCs for the first 14 h of the assay (**Figure 2B**). A-MSCs had reached

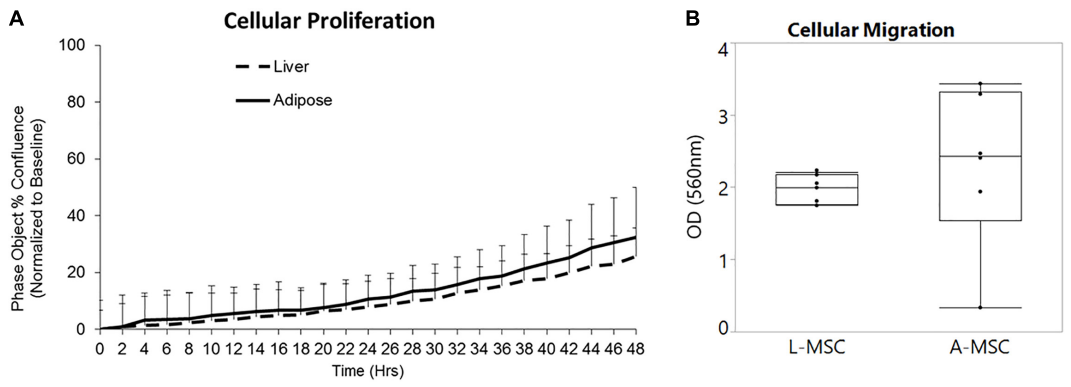

FIGURE 1 | L-MSCs and A-MSCs demonstrate similar proliferative and migration functions *in vitro*. **(A)** To compare the proliferative capability of MSCs, cells were incubated for 48 h and evaluated by a live-cell analysis and imaging system. There was no significant difference at none of the time points between MSCs (p = 0.10). **(B)** The migratory function toward chemoattractants was tested using colorimetric cell assay. The migratory function of L-MSCs and A-MSCs was similar (p = 0.24). The results are presented as the mean ± standard deviation. Statistical significance was analyzed by repeated ANOVA for cellular proliferation and the two-sample *t*-test for cellular migration. MSC, Mesenchymal stem/stromal cell; L-MSC, Liver MSC; A-MSC, Adipose MSC.

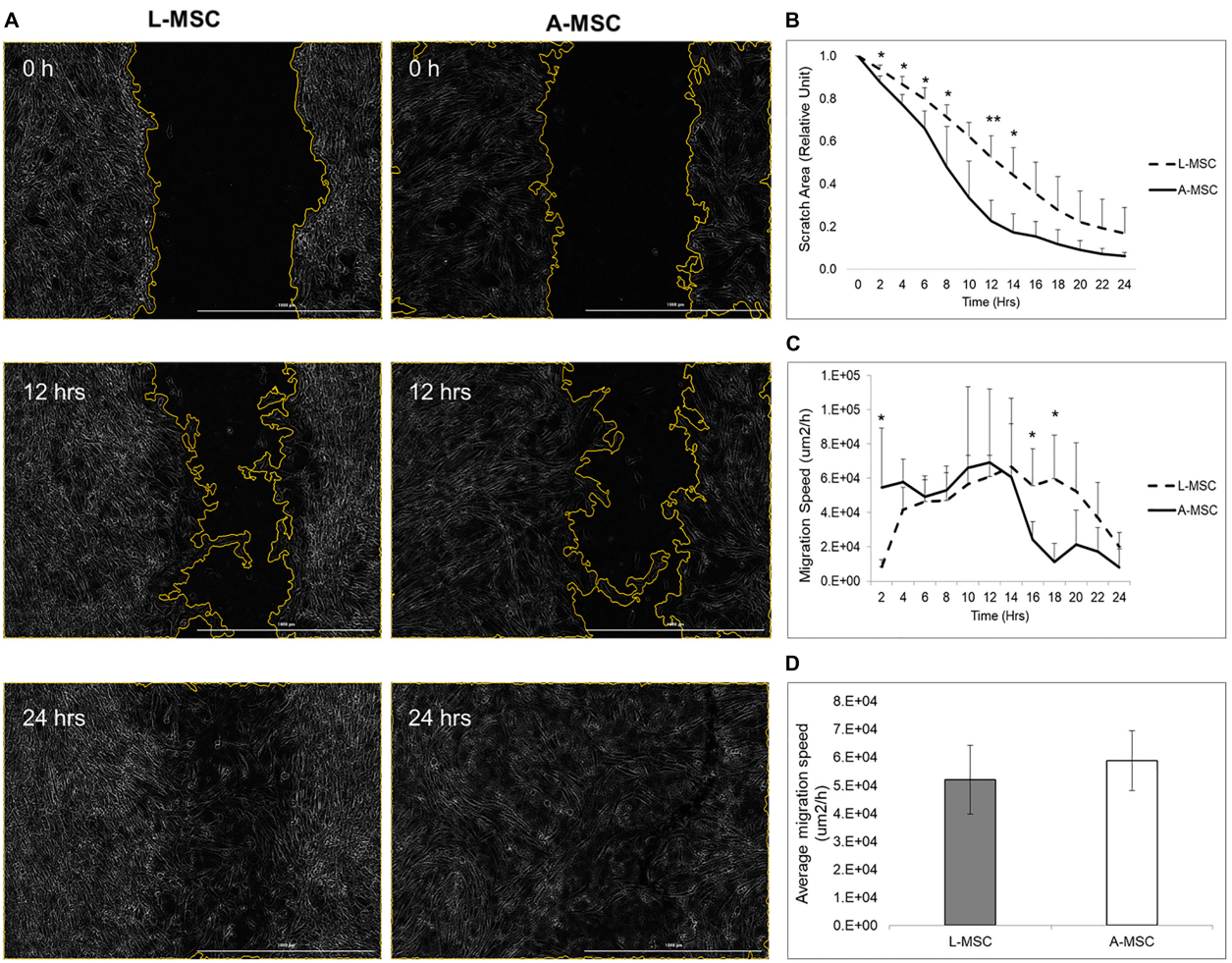

FIGURE 2 | MSC migration was assayed by BioTek AutoScratch™ Wound Making Tool, with cells incubated and observed under BioTek Cytation™ 5 Cell Imaging Multi-Mode Reader. **(A)** Representative images were obtained immediately, 12 h, and 24 h after the scratch. **(B)** Quantified scratch area closure by A-MSCs and L-MSCs. The relative unit was calculated from the time of scratch creation. **(C)** The cell migration speed, calculated using scratch areas, plotted at 2-h intervals. **(D)** The average migration speed was calculated to the point when the MSCs' reached their confluences. The data presented as mean ± standard deviation and statistical significance was analyzed by the sample *t*-test (*$p < 0.05$, **$p < 0.005$). L-MSCs: Liver MSCs, A-MSCs: Adipose MSCs.

their peak migration speed after 10–12 h, while L-MSCs reached theirs after 14–16 h. Initially, A-MSCs' migration speed was significantly higher than L-MSCs, which decreased substantially as the cells became confluent. Thus, L-MSCs' migration speed was significantly higher than A-MSCs at the 16th and 18th hours of the assay (**Figure 2C**). However, their average migration speed until they reached confluence was similar ($p = 0.45$) (**Figure 2D**).

Expressions of Senescence and SASP Markers

Cellular senescence is a stress response mechanism that leads to irreversible cell cycle arrest, and senescence in MSCs impairs their function (Turinetto et al., 2016). Senescence in L-MSCs vs. A-MSCs was compared by testing the relative expression of cell cycle arrest (p16, p21, and p53) and canonical SASP markers (activin A, MCP-1, PAI-1, IL1A, IL6, TNFα) using qPCR. There was no difference between the L-MSCs and A-MSCs in the expression of p16, p21, and p53. Yet, the L-MSCs had significantly lower expression of the pro-inflammatory TNFα gene ($p = 0.003$) compared to the A-MSCs (**Figure 3**).

The protein expression of two representative SASP markers, TNFα and PAI-1, was tested with Western blot (**Figure 4A**). Similar to the qPCR results, PAI-1 protein expression was comparable in L-MSC and A-MSC ($p = 0.7$), whereas TNFα expression was significantly lower in L-MSCs than A-MSCs ($p = 0.03$) (**Figure 4B**).

Lysosomal Activity of L-MSC

Because the cellular response to stress correlates with DNA damage and subsequent increase in lysosomal activity, next, we investigated the SA-β-Gal enzyme activity both at the enzyme (**Figure 5A**) and gene (**Figure 5B**) levels. SA-β-Gal enzyme activity and gene expression of GLB1, which encodes the SA-β-Gal enzyme, was similar in L-MSCs and A-MSCs. Likewise,

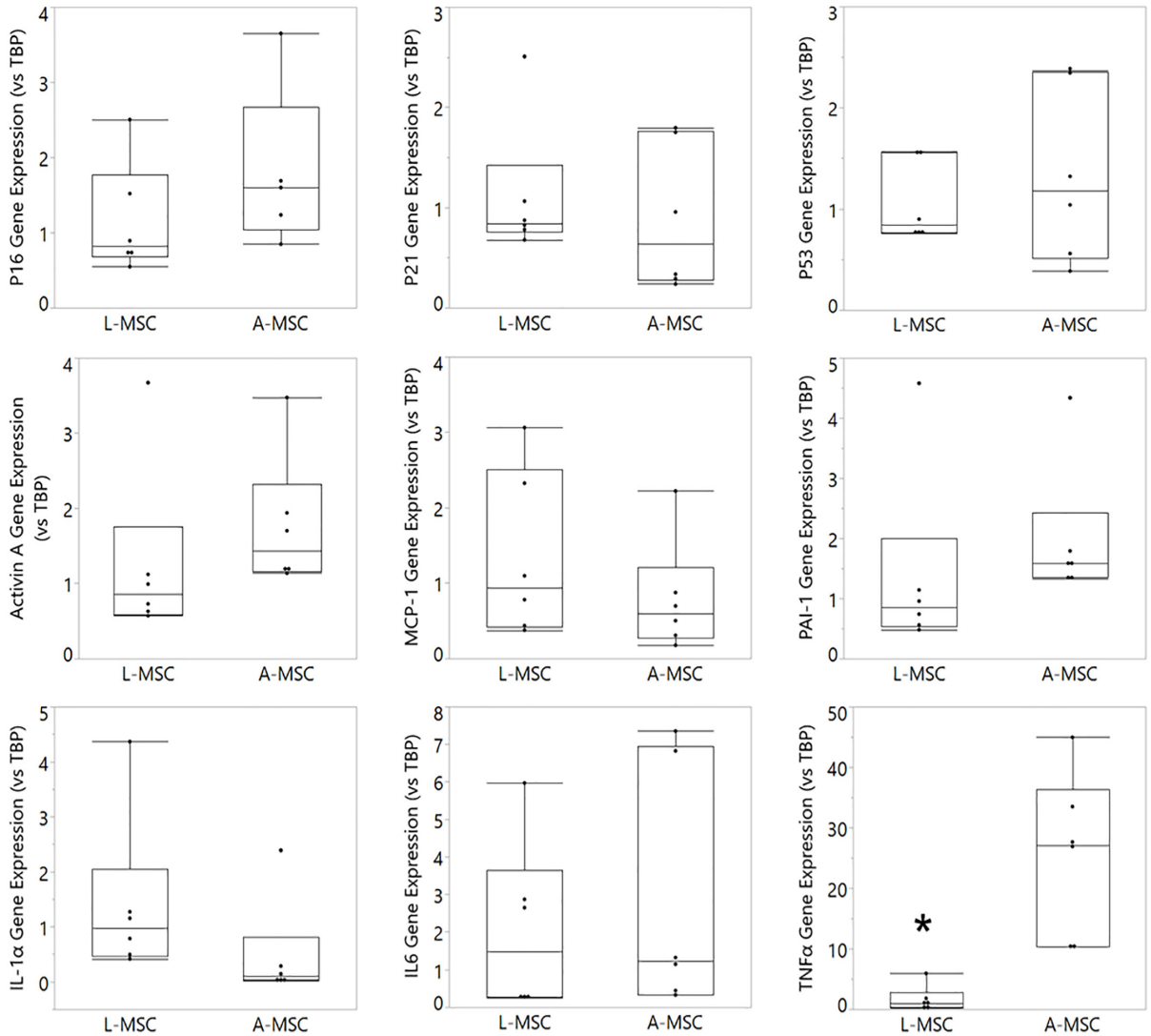

FIGURE 3 | Comparative analysis of senescence and SASP marker expression on L-MSC and A-MSC. Cellular senescence was determined by the expression of p16, p21, and p53. SASP markers were determined by the expression of activin A, MCP-1, PAI-1, IL-1α, IL-6, and TNFα. Gene expressions were normalized to TBP and calculated using the 2-ΔΔCT method. Data are presented as the mean ± standard deviation, and statistical significance was analyzed by the sample *t*-test (*$p < 0.005$ vs. A-MSC). MSC, Mesenchymal stem/stromal cell; L-MSC, Liver MSC; A-MSC, Adipose MSC; MCP-1, Monocyte chemoattractant protein-1; PAI-1, Plasminogen activator inhibitor-1; IL-1α, Interleukin-1 alpha; IL-6, Interleukin 6; TNFα, Tumor necrosis factor alpha; TBP, TATA-binding protein; SASP, Senescence-associated secretory phenotype; qPCR, Quantitative Polymerase Chain Reaction.

both MSC subjects displayed similarly β-galactosidase stained apoptotic signals (**Figure 5C**).

Characterization of L-MSC Protective and Pro-Angiogenic Properties

Next, we tested the protective and angiogenic properties of L-MSCs in comparison to A-MSCs. HUVEC were injured by incubating them with TNFα and TGF-β1 for 3 days, and the injury was confirmed by the upregulation of cell cycle markers. The expression of the cell cycle makers in injured HUVEC was reduced down to the baseline level when they were co-cultured with either L-MSCs or A-MSCs for 24 h ($p < 0.0001$) (**Figure 6**).

The pro-angiogenic capacity of MSCs was tested by tube formation capacity of injured HUVEC. The analysis demonstrated that after HUVEC were injured, their tube formation was significantly blunted; however, the co-culture of injured HUVEC with A-MSCs increased the number of tubes and tube connections more than L-MSCs (**Figure 7**), suggesting greater pro-angiogenic potency.

DISCUSSION

In attempt to identify the liver components that contribute to its tolerogenic microenvironment, we have recently isolated and

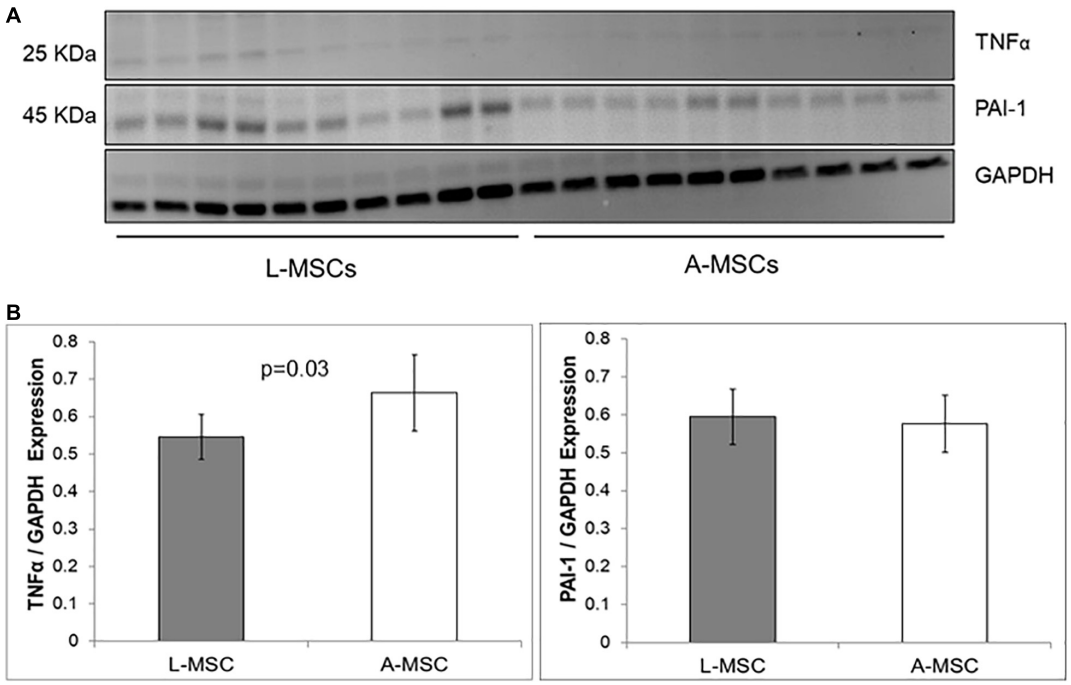

FIGURE 4 | Comparison of SASP markers protein expression by western blot assay. **(A)** The western blot gel images of TNFα and PAI-1 are shown. The gels were run under the same experimental conditions, one blot for each cell line. **(B)** TNFα and PAI-1 expressions in both MSC types. Protein expressions were quantified by GAPDH antibody. TNFα expression is significantly lower levels in L-MSCs ($p = 0.03$). Data are presented as the mean ± standard deviation and statistical significance analyzed by the sample *t*-test. L-MSCs, Liver MSCs; A-MSCs, Adipose MSCs; TNFα, Tumor necrosis factor alpha; PAI-1, Plasminogen activator inhibitor-1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

expanded human L-MSCs *in vitro*. To characterize the cellular properties of L-MSCs and identify their unique properties relative to MSCs derived from other tissues, here we compared them to the commonly investigated and well-characterized A-MSCs. Interestingly, we found that the proliferative kinetics, migratory functions, and intrinsic senescence characteristics of L-MSCs are comparable to those of A-MSCs.

The liver is a highly metabolic organ with unique immunoregulatory functions through its location at a crossroads of the portal and systemic blood circulation. This strategic position allows it to carry out its tolerogenic immune function, clearing gut-derived nutrients, antigens from old cells, and bacterial degradation (Zheng and Tian, 2019). A combination of uniquely tolerogenic liver-resident immune cells and the liver's sinusoidal microanatomy contribute to the tolerogenic microenvironment of the liver (Thomson and Knolle, 2010; Dou et al., 2018; Abrol et al., 2019). Guided by the liver's unique tolerogenic characteristics, we had previously postulated that L-MSCs would possess immunomodulatory properties superior to their counterparts isolated from other tissues (Taner et al., 2020). In fact, our earlier studies demonstrated that L-MSCs inhibit alloreactive T-cell proliferation and also suppress the frequency of IFNγ-producing alloreactive T-cells more effectively than A-MSCs. At steady state, the transcriptome of L-MSCs expanded *in vitro* is remarkably different from that of A-MSCs, in that immunomodulatory genes and genesets are enriched in L-MSCs compared to A-MSCs (Taner et al., 2020). Consistent with our previous studies, both the gene and protein expression of TNFα were significantly lower in L-MSCs. As a known proinflammatory cytokine, TNFα plays a crucial role in many chronic inflammatory diseases (Davignon et al., 2018). Therefore, our study supports the notion that L-MSCs, with their immunomodulatory properties, might be better candidates for cellular therapeutics in inflammatory conditions. MSCs from different tissue sources have been reported to have variable phenotypes, transcriptomes, secretomes, and functions (Strioga et al., 2012). For example, A-MSCs possess better proangiogenic properties than BM-MSCs (Kozlowska et al., 2019). Given that L-MSCs are conceptually more difficult to harvest than A-MSCs, it is important to compare them rigorously to A-MSCs to justify their use as cellular therapeutics and establish their proliferation, migration, and vasculoprotective properties.

Clinical applications of MSCs depend on the successful homing of the cells to the target sites. When tissue damage occurs, resident MSCs are activated, and some MSCs are released into circulation and home to the injury site (Chapel et al., 2003; Caplan, 2009). Systemic administration of MSCs, however, has not resulted in reliable engraftment of MSCs in the injury sites in multiple pre-clinical and clinical trials (Barbash et al., 2003; Devine et al., 2003). Such delivery of MSCs results in entrapment of most of the cells within the pulmonary capillaries (Barbash et al., 2003; Devine et al., 2003; Scarfe et al., 2018). However, targeted administration of MSCs into the site of inflammation can, conceptually, overcome this problem. MSCs delivered locally

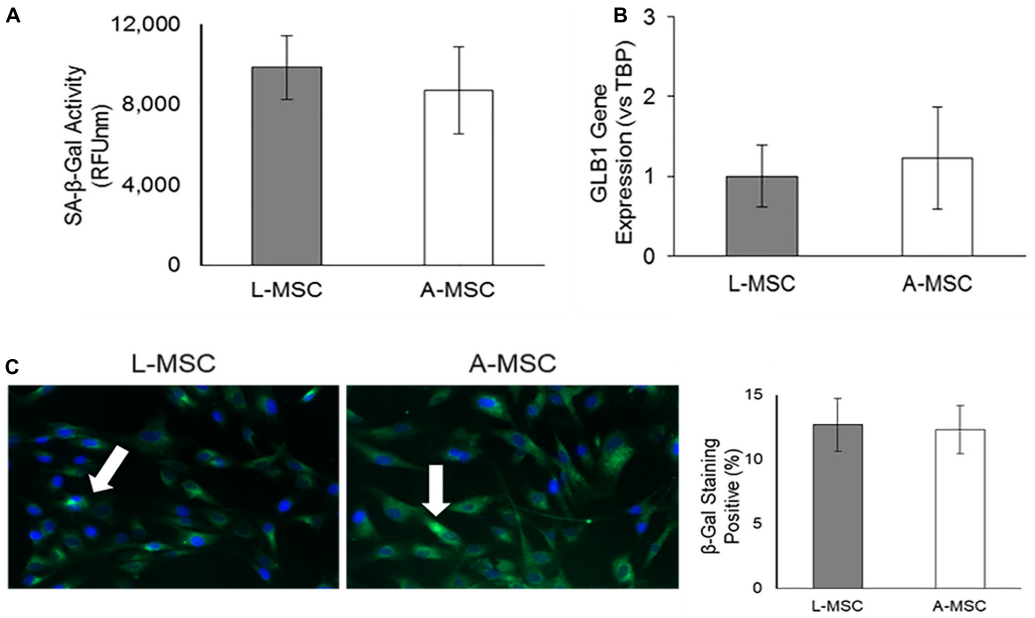

FIGURE 5 | Lysosomal activity in MSCs. SA-β-Gal is a lysosomal enzyme that participates in cellular senescence, and its activity was investigated both at the enzyme and gene levels. **(A)** L-MSCs and A-MSCs have similar enzyme activity ($p = 0.16$). **(B)** qPCR was performed on the GLB1. GLB1 gene expression was similar between L-MSCs and A-MSCs. **(C)** The percentage of β-galactosidase stained cell nuclei was not different between MSCs [white arrows indicate apoptotic nuclei (green), counterstained with DAPI (blue)]. The results are presented as the mean ± standard deviation, and statistical significance was analyzed by the two-sample *t*-test. MSC, Mesenchymal stem/stromal cell; L-MSC, Liver MSC; A-MSC, Adipose MSC; SA-β-Gal, Senescence-associated beta-galactosidase; GLB1, Galactosidase beta 1.

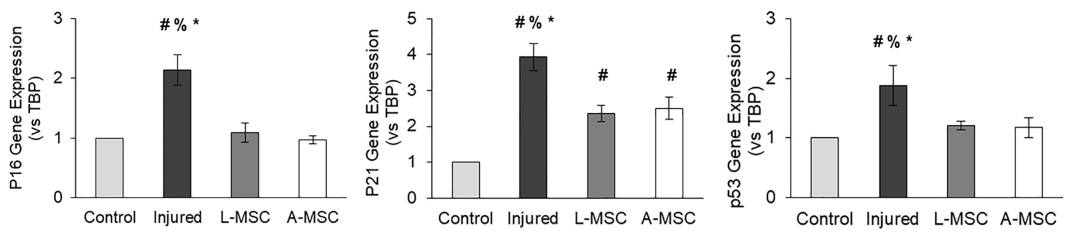

FIGURE 6 | HUVEC were incubated with TNFα and TGF-β1 for 3 days to induce injury. qPCR was performed on injured HUVEC co-cultured with MSCs to analyze cell cycle arrest. Upregulation of p16, p21, and p53 gene expression on injured HUVEC confirmed the injury. Gene expressions were normalized to TBP and calculated using the 2-ΔΔCT method. The results are presented as the mean ± standard deviation, and statistical significance was analyzed by the two-sample *t*-test ($p < 0.05$ # vs. Control, % vs. L-MSC, * vs. A-MSC). MSC, Mesenchymal stem/stromal cell; L-MSC, Liver MSC; A-MSC, Adipose MSC; TBP, TATA-binding protein.

might then migrate through the tissue via the chemoattractant gradient (Ullah et al., 2019). Mimicking this gradient *in vitro*, here we found that the L-MSCs possess similar migratory properties and kinetics as A-MSCs.

The similarity between the two types of MSCs was also notable in their proliferation kinetics. *In vitro* expansion of MSCs may gradually lead to cellular senescence and arrest of the cell cycle, mimicking responses to stress (Baxter et al., 2004). Importantly, cellular senescence involving cell-cycle arrest impairs MSC characteristics, particularly the regenerative and immunomodulatory properties of MSCs, which in turn transforms MSCs from an immunomodulatory to SASP-releasing pro-inflammatory phenotype (Lunyak et al., 2017). Gnani et al. (2019) showed that senescent BM-MSCs have a reduced inhibitory effect on the proliferation of peripheral mononuclear cells than younger BM-MSCs. Also, senescent MSCs negatively affect their niche by activating pro-inflammatory gene expression, which leads to decreased hematopoietic stem cells' clonogenic potential (Gnani et al., 2019). As such, senescence characteristics of MSC have important clinical and safety implications. We, therefore, compared the molecular changes underlying senescence and cell proliferation between A-MSCs and L-MSCs generated under the same culturing conditions and harvested at the same passage, cell cycle arrest markers were quantified. Our results indicate similar expression of genes that contribute to cell cycle arrest (p16, p21, and p53), as well as the SASP markers in both types of MSCs. Furthermore, the

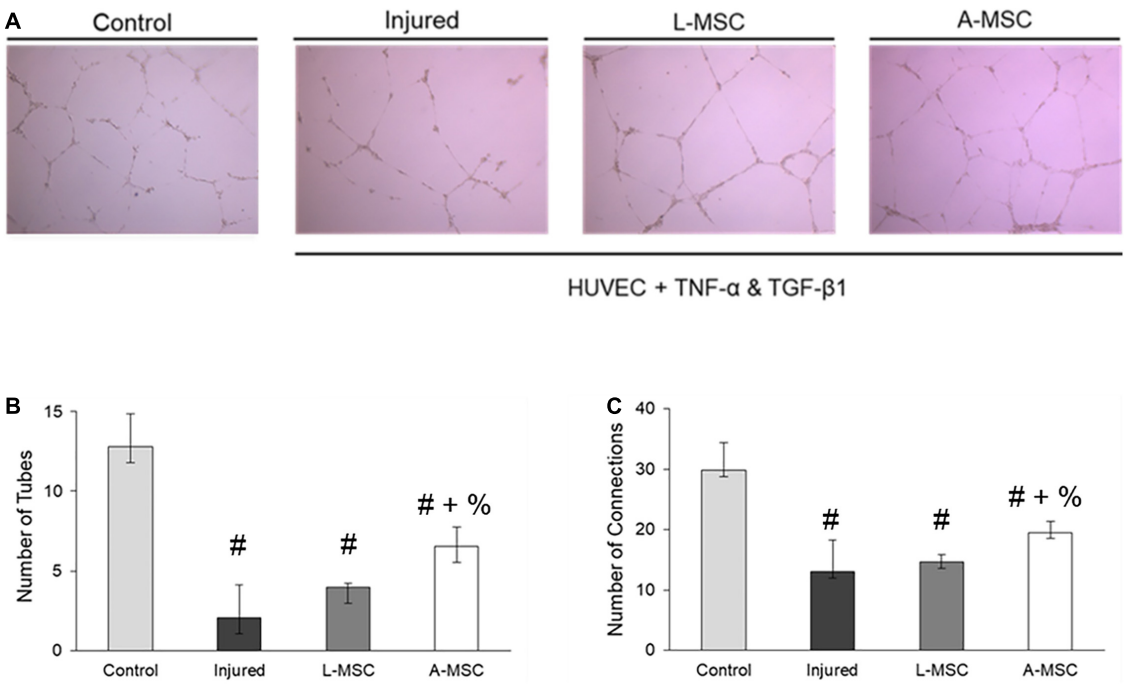

FIGURE 7 | The pro-angiogenic and reparative potency of L-MSCs and A-MSCs on HUVEC. **(A)** The representative images show tube formation under different conditions. The numbers of tubes **(B)** and tube connections **(C)** were decreased in injured HUVEC and increased after co-culture with MSCs, but more increased with A-MSCs more than L-MSCs. The results are presented as the mean ± standard deviation, and statistical significance was analyzed by the sample *t*-test ($p < 0.05$ # vs. Control, + vs. Injured, % vs. L-MSC). MSC, Mesenchymal stem/stromal cell; L-MSC, Liver MSC; A-MSC, Adipose MSC; HUVEC, Human umbilical vein endothelial cells; TNFα, Tumor necrosis factor alpha; TGF-β1, Transforming growth factor-beta 1.

protein expression of the SASP marker PAI-1 was similar. The upregulation of the SA-β-Gal enzyme, encoded by the GLB1 gene, is also a marker for cellular senescence (Lee et al., 2006). Our cohort also showed no difference in lysosomal activity at both the enzymatic and gene levels between the two MSC types.

We and others have previously shown that co-incubation of HUVEC with TNFα and TGF-β induces cellular injury, evidenced by cell cycle arrest and senescence (Khan et al., 2017). Indeed, we found that the expression of cell cycle arrest markers was upregulated in injured HUVEC. Co-culture with MSC from both tissue sources reversed the upregulation of cell cycle arrest markers injured HUVEC Additional testing of their pro-angiogenic activity using *in vitro* formation of tube-like networks (DeCicco-Skinner et al., 2014), however, demonstrated that while both MSCs types were able to partly protect the function of injured HUVEC, A-MSCs facilitated the generation of more tubes and tube-like networks.

The present study has several limitations due to the overall small sample size. However, in order to minimize the age-, sex-, and obesity-related differences in MSC, the cell donors were well-matched. Both donor groups were also in the obese range, which might have affected MSC functions (Conley et al., 2020). Furthermore, albeit not statistically significant, A-MSCs were obtained from individuals with slightly higher BMI, which could have impacted our results. Our results would need to be validated in further, larger studies.

In conclusion, we demonstrate that the L-MSCs have comparable proliferative and migratory profiles to those of A-MSCs. Similarly, senescence markers of L-MSCs do not differ from A-MSCs cultured *in vitro* under the same conditions. While L-MSCs have been shown to possess superior immunomodulatory properties, A-MSCs appear to be better in angiogenesis and repair of injured endothelial cells. Therefore, MSC-based therapy needs to be tailored to the underlying disease state. These findings provide further evidence for the safety of L-MSCs in their potential utilization as cellular therapeutics in disease processes with underlying inflammation. Further studies are needed to compare their reparative and angiogenic effects *in vivo*.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

FY, SC, LL, and TT contributed to the conception and design of the study. FY, SC, HT, IS, KJ, LL, and TT collected the data, contributed to the data analysis, and interpretation. TT and LL provided financial support. FY wrote the first draft of the

manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Role of Hyperglycemia in the Senescence of Mesenchymal Stem Cells

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The regenerative and immunomodulatory properties of mesenchymal stem cells (MSCs) have laid a sound foundation for their clinical application in various diseases. However, the clinical efficiency of MSC treatments varies depending on certain cell characteristics. Among these, the roles of cell aging or senescence cannot be excluded. Despite their stemness, evidence of senescence in MSCs has recently gained attention. Many factors may contribute to the senescence of MSCs, including MSC origin (biological niche), donor conditions (age, obesity, diseases, or unknown factors), and culture conditions *in vitro.* With the rapidly increasing prevalence of diabetes mellitus (DM) and gestational diabetes mellitus (GDM), the effects of hyperglycemia on the senescence of MSCs should be evaluated to improve the application of autologous MSCs. This review aims to present the available data on the senescence of MSCs, its relationship with hyperglycemia, and the strategies to suppress the senescence of MSCs in a hyperglycemic environment.

Keywords: mesenchymal stem cells, senescence, hyperglycemia, diabetes mettitus, mitochondrial dysfunction

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INTRODUCTION

Mesodermal mesenchymal stem cells (MSCs) have self-renewal and multi-differentiation potential and can ultimately differentiate into osteoblasts, chondrocytes, and adipocytes (Timaner et al., 2020). The regenerative and immunomodulatory properties of MSCs have attracted the attention of researchers and doctors worldwide, because of their potential for cell-based therapy (Ankrum et al., 2014; Kornicka et al., 2018). Strong evidences exist to support the effectiveness of MSC treatments in managing degenerative diseases such as diabetes mellitus, myocardial infarction, liver failure, osteoarthritis, and Alzheimer's disease (Hunsberger et al., 2016; Packer, 2018; Shi et al., 2018; Wang, 2018). However, the inconsistent results of many clinical trials have hampered their clinical application (Galipeau and Sensébé, 2018). Currently known reasons for these inconsistencies include MSC origin (biological niche), donor characteristics (age, obesity, diseases, or unknown factors), and culture conditions *in vitro* (Costa et al., 2020). However, the senescence of MSCs has often been overlooked. Autologous MSCs are considered auspicious because patient-derived cells are easily available and do not require sustained immunosuppression (Golpanian et al., 2016), but their senescence due to the personalized microenvironment should be addressed.

Hyperglycemia, oxidative stress, and altered immune reactions are prominent features of the diabetic microenvironment, all of which have been demonstrated to change bone marrow-derived MSC (BMSC) and adipose-derived MSC (AMSC) properties and functions, leading to MSC senescence (Cramer et al., 2010; Mahmoud et al., 2019). Moreover, umbilical cord-derived

MSCs (UCMSCs) extracted from obese/diabetic mothers display premature senescence and mitochondrial dysfunction (Kim et al., 2015). With the rapidly increasing prevalence of diabetes and gestational diabetes mellitus, the effects of hyperglycemia on the senescence of MSCs should be addressed for a better application of autologous MSCs. In this review, we summarize published studies on the senescence of MSCs in terms of their definition and hallmarks. Moreover, we review how hyperglycemia drives MSC senescence. Finally, we recommend strategies to suppress MSC senescence in the diabetic microenvironment.

DEFINITION OF MSC SENESCENCE

The definition of senescence can be conceptually distinguished from that of aging, with that of senescence emphasizing the cellular level (Hayflick and Moorhead, 1961). Aging is the determining risk factor for most diseases and conditions that limit health span, and seven pillars promote aging, including metabolism, macromolecular damage, epigenetics, inflammation, adaptation to stress, proteostasis and stem cell regeneration. More importantly, these seven pillars are highly intertwined and their interplay plays a crucial role in the aging process (Kennedy et al., 2014). However, "cellular senescence is a cell state triggered by stressful insults and certain physiological processes, characterized by prolonged and generally irreversible cell-cycle arrest with secretory features, macromolecular damage, and altered metabolism" (Gorgoulis et al., 2019). Cellular senescence occurs in various physiological and pathological processes, such as embryogenesis, wound healing, injury, tumor suppression, and aging (Calcinotto et al., 2019). In addition, senescent cells are associated with aging-related diseases, such as type 2 diabetes, atherosclerosis, osteoporosis, glaucoma, and neurodegeneration (He and Sharpless, 2017). The exclusion of senescent cells or compounds that block the senescence-associated secretory phenotype (SASP) has been proposed for the treatment of cancer and aging-related diseases, which might play a major role in extending the health span (Partridge et al., 2020).

Interestingly, MSCs have also been found to be senescent *in vivo* and *in vitro* (Lin et al., 2019; Banimohamad-Shotorbani et al., 2020), which might lead to altered biological function and reduced therapeutic effect. Recently, we are seeing increased interest in MSC-based therapy for improving aging-related disorders and autoimmune diseases. Thus, the precise detection of senescent MSCs and elimination or reversion of the phenotype are essential for MSC-based therapy.

TRIGGERS AND HALLMARKS OF MSC SENESCENCE

MSC senescence can be induced by many intrinsic triggers and oncogenes, as well as physiological and pathological changes in their microenvironment. Based on the nature of the triggers, MSC senescence can be categorized into various types, including replicative senescence, stress-induced senescence, oncogene-induced senescence, and developmental senescence (Zhou et al., 2020). The hallmarks (phenotypic indicators) of MSC senescence vary with the nature of the causes that drive the different types of senescence.

The classic phenotype of cellular senescence include cell-cycle arrest, SASP, macromolecular damage, and deregulated metabolic profile (Gorgoulis et al., 2019). Cell-cycle withdrawal is the nature of cellular senescence. An overwhelming amount of studies have demonstrated that SASP (Ozcan et al., 2016) can reinforce senescence and affect other cells through an autocrine and paracrine mechanism (Acosta et al., 2008; Kuilman et al., 2010). Moreover, SASP can induce a local inflammation with compound effects. Both effects are essential for tissue or organ regeneration and reconstruction (Kuilman and Peeper, 2009). This secretome is mainly composed of various extracellular growth factors, including transforming growth factor beta (TGF-β), epidermal growth factor (EGF), platelet derived growth factor, hepatocyte growth factor, and insulin-like growth factor 1-binding proteins, in addition to cytokines/chemokines, receptor decoys, receptor antagonists, and extracellular matrix remodeling proteins (Freund et al., 2010). Among macromolecular damage and deregulated metabolic profile, DNA damage (telomere shortening), chromatin remodeling, autophagy, and senescence-associated epigenetic expression changes (Sengupta and Seto, 2004; Jung et al., 2010; Li et al., 2011; Franzen et al., 2017), mitochondrial dysfunction, and lysosome dysfunction play a major role in MSC senescence.

Compared with common cellular senescence, senescent MSCs have their own specific characteristics, including multipotentiality loss, cell phenotype changes, immunomodulatory property damage, and homing and migration damage (Campisi and d'Adda di Fagagna, 2007). Many studies have shown that the osteogenic differentiation potential of MSCs declines with age (Garcia-Sanchez et al., 2019; Zhou et al., 2019). However, there is no definite conclusion regarding adipogenic differentiation, although most of the studies have demonstrated that the adipogenic differentiation potential of MSCs tends to deteriorates with successive passages under standard expantion conditions (Sugihara et al., 2018). In terms of cell phenotypes, early-passage and late-passage senescent MSCs showed similar levels of identification markers (positive for the markers CD90, CD73, and CD105, negative for the markers CD45, CD34, CD14, CD11b, CD79α, CD19, and HLA-DR) proposed by the International Society for Cellular Therapy, suggesting that their assessment may be restricted only to basic MSC characterization (Yu et al., 2014). In contrast, a number of studies found increased CD295 and CD264 (Madsen et al., 2017) and decreased Stro-1 (Bakopoulou et al., 2017; Redondo et al., 2018), CD106 (vascular cell adhesion protein 1) (Lu et al., 2019) and CD146 (melanoma cell adhesion molecule, MCAM) (Jin et al., 2016) during prolonged culture, suggesting their ability to mark senescent MSCs (Laschober et al., 2009).

DETECTION OF SENESCENT MSCS

A multi-marker, three-step workflow is recommended for identifying senescent cells: "first, assessing senescence-associated

beta-galactosidase (SA-β-gal) activity and/or lipofuscin accumulation (SBB or GL13 staining); second, co-staining with other markers frequently observed in (p16INK4A, p21WAF1/Cip1) or absent in (proliferation markers, lamin B1) senescent cells; and third, identifying factors anticipated to be altered in specific senescence contexts" (Gorgoulis et al., 2019). This workflow is applicable to all cell types. Regarding MSCs, analysis of various molecules and biological processes, including cell cycle arrest; DNA damage; and transcriptional, epigenetic, and metabolic changes, has been recommended for senescence detection. The characteristics of senescent MSCs include flattened and enlarged morphology (Kim et al., 2016; Iwasaki et al., 2019), decreased number of colony formation units (Ganguly et al., 2019), increased SA-β-gal activity (Gnani et al., 2019), altered gene and protein expression (p16, p21, p53), shortened or dysfunctional telomeres (Bonab et al., 2006; Guillot et al., 2007), increased 8-oxo-dG or γH2AZ expression (markers of DNA damage), microsatellite instability (marker of genomic instability and deficient DNA repair), and abnormal global methylation. However, specific and unequivocal markers are still lacking, and for reliability, the evaluation of MSC senescence should be based on the assessment of several markers *in vitro* (Neri and Borzí, 2020).

CHARACTERISTICS OF SENESCENT MSCS IN THE HYPERGLYCEMIC MICROENVIRONMENT

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder that affects an increasing number of people worldwide (Zheng et al., 2018). Current treatment for diabetes failed to maintain long-term blood glucose homeostasis, resulting in insulin secretion deficiency and acute and chronic diabetic complications. Therefore, new approaches for the therapy of T2DM and its chronic complications are of special interest. Currently, cellular therapies involving autologous MSCs, especially those isolated from adipose tissue, constitute a promising treatment for diabetes (Bhansali et al., 2017). Offspring of women with GDM are at high risk of T2DM in adulthood, and MSCs derived from the umbilical cord and placental membrane offer a promising source of autologous MSCs for latent diabetes treatment. However, there are still many obstacles concerning the effectiveness of autologous MSCs isolated from diabetic patients and the effect of the microenvironment that need to be overcome before MSCs can be used for this purpose. A classic feature of senescent MSCs in the hyperglycemic microenvironment is cell-cycle arrest, as confirmed by senescence marker (p16, p53, p21) mRNA and protein expression (Liu J. et al., 2020). Additionally, increased SA-β-galactosidase staining is another important identification marker. Furthermore, senescent MSCs showed decreased proliferation, differentiation alternations, cell phenotype changes and declined migration, angiogenesis, and immunomodulatory capabilities. Though SASP plays a vital role in the common cellular senescence, only few studies have focused on the SASP of senescent MSCs in the hyperglycemic microenvironment, summarized in **Figure 1**.

Proliferation

Proliferation is the most important characteristic of MSCs. Previous studies have shown that MSCs display lower proliferative capability and higher senescence in a hyperglycemic environment. The earliest study was conducted in 2010, when Cramer et al. showed that elevated glucose levels reduce the proliferative capacity of AMSCs with diabetes and AMSCs without diabetes, but the impact was more notable in AMSCs with diabetes. Similarly, AMSCs from patients with type 2 diabetes showed reduced viability and proliferative potential (Alicka et al., 2019). Intriguingly, incorporation of insulin enhances cell replication, especially in AMSCs without diabetes (Cramer et al., 2010). Another study demonstrated that diabetes mellitus was a significant determinant for forming a higher number of colony-forming units in BMSCs (Neef et al., 2012). Likewise, compared with human UCMSCs obtained from normal pregnant women, UCMSCs obtained from patients with GDM showed declined proliferative activity and increased senescence markers with a higher expression of p16 and p53 (Kim et al., 2015). In animal models, the colony formation capacity of rat adult non-adherent bone marrow MSCs (Na-BM-MSCs) was negatively influenced when cultured in high-glucose-containing medium; however, Na-BM-MSCs seemed to be relatively uninfluenced by aging, suggesting that diabetes, compared to aging, is more associated with reduced numbers and functional viability of MSCs *in vivo* (Stolzing et al., 2012). Taken together, these studies support the notion that hyperglycemia weaken the proliferation capacity of MSCs regardless of their sources both *in vitro* and *in vivo*. More importantly, hyperglycemia has a greater impact on MSC proliferation than does aging and this effect could not be completely restored by adding hypoglycemic agents.

Differentiation

MSCs are characterized by their potent multipotency and do not only have mesodermal differentiation potential but also neurogenic differentiation potential. Current studies on the differentiation potential of senescent MSCs in hyperglycemia are controversial. It has been reported that the adipogenic and osteogenic differentiation capacities of AMSCs in high glucose-treated conditions were comparable to those in low glucose-treated conditions (Cheng et al., 2016). In contrast to this study, Cramer et al showed that that high glucose concentrations reduce the osteogenic and chondrogenic potential of AMSCs (Cramer et al., 2010). Likewise, UCMSCs from patients with GDM displayed significantly lower osteogenic and chondrogenic potential (Kong et al., 2019). Regarding the adipogenic potential of MSC under hyperglycemia conditions, there are some controversial results. Cramer et al. reported that high glucose concentrations enhanced the adipogenic potential of MSCs. Further, when adult human BMSCs were incubated with sera from T2DM patients for 14 days, the expression of adipogenic genes and Oil Red O staining were greatly increased (Moseley et al., 2018). However, GDM-UCMSCs demonstrated a significantly lower adipogenic potential (Kim et al., 2015; Moseley et al., 2018).

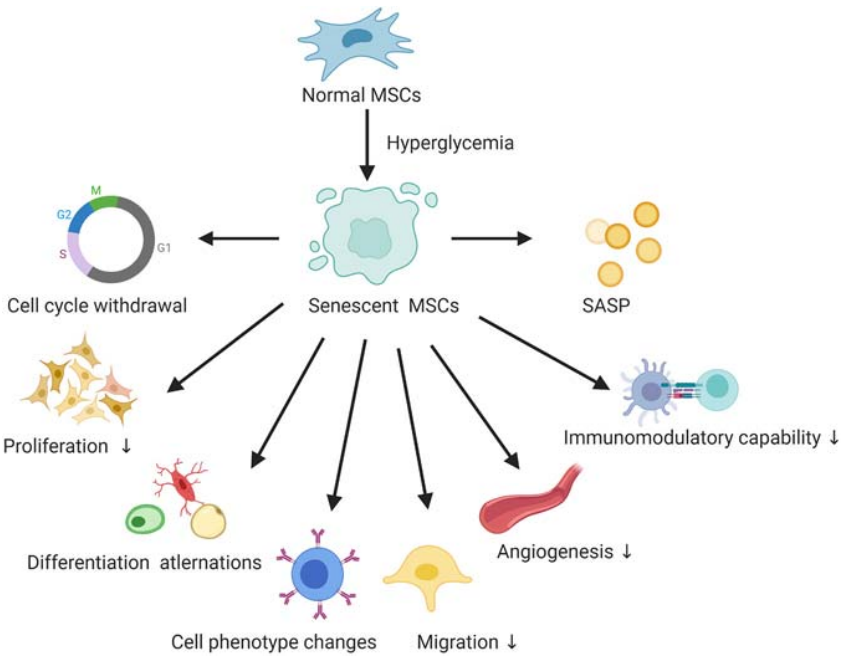

FIGURE 1 | The hallmarks of senescent MSCs in the hyperglycemic microenvironment. SASP, senescence-associated secretory phenotype.

Furthermore, another study found that BMSCs showed decreased adipogenic differentiation capability under high glucose conditions and that the mRNA expression of peroxisome proliferator activated receptor gamma, CCAAT/enhancer-binding protein alpha, leptin, and adiponectin in BMSCs decreased (Rharass and Lucas, 2019).

Interestingly, when cultured in an appropriate induction medium, both AMSCs from diabetic donors (dAMSCs) and from non-diabetic donors (nAMSCs) exhibited potential for trans-differentiation into neuron-like cells via a reactive oxygen species (ROS)-mediated mechanism (Cheng et al., 2016). Exposure to GDM may cause irreversible senescence and stress damage to endogenous amniotic MSCs; however, chorionic MSCs from GDM mothers can be efficiently reprogrammed into insulin-secreting cells and have a therapeutic potential comparable to that of chorionic MSCs from healthy mothers. Specifically, chorionic MSCs from both healthy and GDM mothers exhibited increased pancreatic transcription factor expression in parallel with retinoic acid, activin A, glucagon-like peptide-1, EGF, and other chemical components and could generate functional insulin-producing cells with betacellulin-sensitive insulin expression (Chen L. et al., 2020).

Overall, these data suggest that hyperglycemia plays a pivotal role in the differentiation process of MSCs, and this process depends on the different sources of MSC. In general, hyperglycemia weakens the osteogenic and chondrogenic differentiation capability, but interestingly, enhances trans-differentiation into neuron-like cells and insulin-secreting cells. However, there are no uncontested results regarding the adipogenic differentiation. This inconsistency may be due to the age of MSC donors, different passages of MSCs, and the extent and duration of hyperglycemia.

Cell Markers

Markers for identification and stemness are major areas of interest within the field of MSC senescence under hyperglycemic conditions. It has been reported that the expression of cell surface markers in dAMSCs and nAMSCs was similar to that of MSCs (Cheng et al., 2016). Moreover, senescent human UCMSCs, also express comparable levels of CD73, CD90, CD105, and marker proteins (Kim et al., 2015). Additionally, when cultured in high and normal glucose conditions, MSCs in the endosteal niche lining compact bone were not altered, demonstrating the presence of analogous MSC (CD73/CD105/CD90), embryonic (Slug and Snail), and multipotency (CD146) markers throughout the extended culture (Al-Qarakhli et al., 2019).

However, MSCs under hyperglycemia showed negligible pluripotency (Nanog, Oct4) and hematopoietic (CD34/CD45) markers (Al-Qarakhli et al., 2019). Recently, one study showed that stemness markers were significantly lower in insulin-controlled GDM mothers-UCMSCs and diet-controlled GDM mothers-UCMSCs than in normal UCMSCs (Kong et al., 2019). Another study using a systems biology approach identified widespread downregulation of MSC markers in the subcutaneous adipose tissue of diabetic rats (Ferrer-Lorente et al., 2014). Likewise, nucleus pulpous-derived MSCs cultured with high glucose showed decreased expression of stemness genes as well as decreased mRNA and protein expression of silent information regulator protein 1 (SIRT1), SIRT6, glucose transporter 1, and hypoxia inducible factor-1α (HIF-1α), but enhanced cell senescence, cell apoptosis, and caspase-3

expression (Liu Y. et al., 2020). Contrary to these data, Cheng et al. reported that dAMSCs or high-glucose treated nAMSCs presented higher expression of the pluripotent markers Sox-2, Oct-4, and Nanog, and this effect was related to ROS-mediated Akt attenuation. With antioxidant treatment, high glucose-treated nAMSCs exhibited enhanced cell growth but no stemness enhancement (Cheng et al., 2016). This rather contradictory result may be due to individual difference in MSC donors. Collectively, these studies indicate that hyperglycemia has a little effect on the identification markers, but decreases the stemness of MSCs in most cases.

Other (SASP, Immunomodulatory Properties, Angiogenesis, Migration, Insulin Resistance)

In addition to the above features, MSCs in hyperglycemic conditions show decreased immunomodulatory properties (Montanucci et al., 2016), angiogenesis (Xiao et al., 2020), and migration (Liu Y. et al., 2020), but increased SASP and insulin resistance. SASP is mainly composed of various extracellular growth factors, cytokines/chemokines and receptor antagonists. It is well known that the proliferation and cytokine secretion (TGF-β and VEGF) of MSCs are inhibited after long-term culture even in MSCs derived from young donors (Baek et al., 2018). One study showed that aging and metabolic changes in diabetes also modified the MSC cytokine production abilities (Kondo et al., 2015). Moreover, another study showed that AMSCs from T2DM patients exhibited lower adiponectin, VEGF, and chemokine ligand-12 secretion, but demonstrated an overproduction of leptin (Alicka et al., 2019). Interestingly, data also showed that a high glucose environment causes prominent disparities in the expression of genes involved in insulin resistance, such as resistin and adiponectin between nAMSCs and dAMSCs. Some changes in the expression of these genes were permanent in dAMSCs when treated with insulin (Cramer et al., 2010). Overall, the evidence presented in this section suggests hyperglycemia adversely affect the biological function of MSC which is critical for their therapeutic effect.

MECHANISMS OF SENESCENT MSCS IN THE HYPERGLYCEMIC MICROENVIRONMENT

Cell-cycle withdrawal is the core feature of MSC senescence. High glucose results in a significant decline in the number of cells in the G0/G1 phases and an increase in cells in the S and G2/M phases, suggesting that high glucose induces cell cycle withdrawal of MSCs. Besides, cell cycle markers (p16, p21, p27, and p53) were surprisingly upregulated in insulin-controlled GDM mothers-UCMSCs and diet-controlled GDM mothers-UCMSCs compared to normal UCMSCs (Kong et al., 2019). The increase in the expression of all these cell cycle markers results in increased levels of cyclin-dependent kinase inhibitors (CKD) which actuate entry into senescence by triggering Rb to block cell cycle progression, summarized in **Figure 2**. Apart from this classical pathway, studies have shown that phosphorylation of p38 mitogen-activated protein kinase (MAPK) in MSCs was increased under high glucose conditions without alterations of total p38 MAPK levels. The study also revealed that the p38 MAPK inhibitor SB203580 can alleviate the phosphorylation of p38 MAPK (Qi et al., 2019). In the next sections, brief discussions have been presented on the mechanisms of senescent MSCs under hyperglycemic conditions from the aspects of mitochondria dysfunction, microRNAs, telomerase and epigenetics, summarized in **Figure 2**.

Mitochondrial Dysfunction and Oxidative Stress

Mitochondrial dysfunction is a vital factor during MSC senescence in the hyperglycemic microenvironment and has been extensively investigated. A previous study showed that GDM-UCMSCs displayed significantly declined mitochondrial activity and low expression of the mitochondrial function regulatory genes COX1, PGC-1a, ND2, ND9, and TFAM (Kim et al., 2015). Similarly, another study demonstrated that the gene expression of antioxidant enzymes and that of mitochondrial function genes (NADH dehydrogenase subunit 2, mitochondrial transcription factor A (TFAM), peroxisome proliferator-activated receptor γ coactivator-1alpha, and NADH dehydrogenase (ubiquinone) 1β-subcomplex) were obviously lower in UCMSCs from GDM mothers than in UCMSCs from healthy mothers (Kong et al., 2019). Additionally, AMSCs from type 2 diabetes patients exhibit a senescence phenotype and mitochondrial dysfunction because of excessive oxidative stress (Alicka et al., 2019). Moreover, high glucose-treated nASCs exhibited declined cell migration and higher intracellular ROS than did low glucose-treated cells. This high glucose-induced biological reaction is associated with ROS-mediated Akt attenuation (Cheng et al., 2016). Consistent with previous studies, Rharass et al. showed that intracellular and extracellular ROS concentrations were significantly increased by 50% under high glucose exposure, paralleled by increased mRNA levels of the H_2O_2 generating enzyme NADPH oxidase 4 (Rharass and Lucas, 2019). All of the studies reviewed here support the notion that hyperglycemia drive mitochondrial dysfunction through the ROS pathway which is fundamental to the senescence of MSCs.

MicroRNAs and lncRNAs

MicroRNAs (miRNAs) and lncRNAs are small non-coding RNAs that regulate gene expression, playing a pivotal role in diverse biological processes, including MSC senescence. One study showed that AMSCs isolated from diabetes patients or cultured in a medium containing glycation end products (AGEs) showed decreased stem cell activity, differentiation potential, and angiogenesis effect. Moreover, the expression of miRNA-1248 was reduced, paralleled by increased expression of CBP/p300-interacting transactivator with ED-rich tail 2 (CITED2), an inhibitor of HIF-1α, which influences growth factors that promote cellular proliferation, angiogenesis, and wound healing. Thus, the authors of the study concluded that glucolipotoxicity impaired the effectiveness of AMSCs

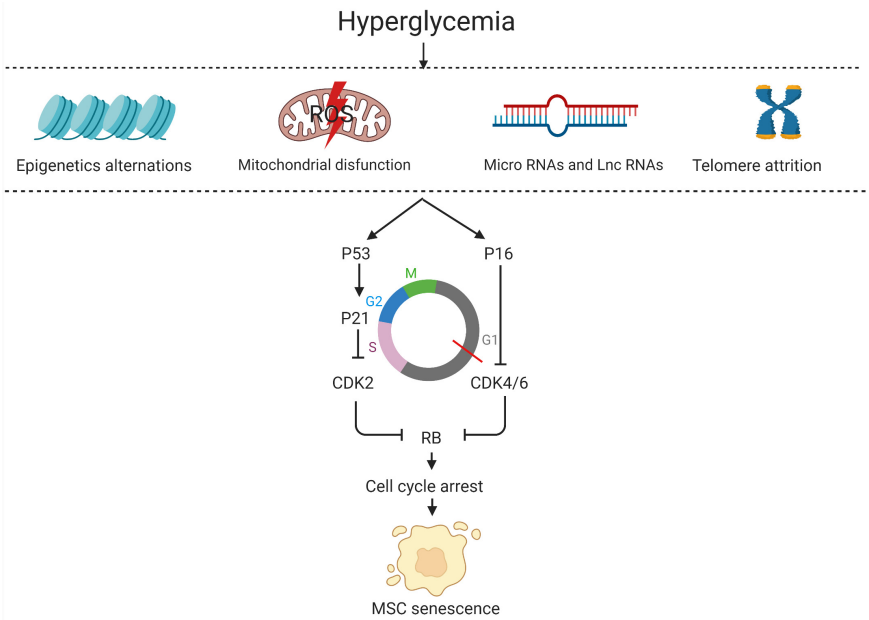

FIGURE 2 | The mechanisms of senescent MSCs in the hyperglycemic microenvironment.

through the miR-1248/CITED2/HIF-1α pathway (Xiao et al., 2020). As previously mentioned, several studies have shown that hyperglycemia reduces the osteogenic potential of MSCs. Recently, one study explored the core molecular mechanism and found that stearoyl-coenzyme A desaturase-1 (SCD1) expression was downregulated in T2DM patients, and decreased SCD1 reduced the osteogenic differentiation of BMSCs by activating the miR-203a/FOS and miR-1908/EXO1 regulatory pathways (Chen Y.S. et al., 2020). In addition, a previous study found obvious differences in the expression of miRNAs involved in cell proliferation (miR-146a-5p, miR-16-5p, and miR-145-5p), together with miRNA and genes responsible for insulin sensitivity and glucose homeostasis (miR-17-5p, miR-24-3p, 140-3p, SIRT1, TGFβ, HIF-1α, LIN28, and FOXO1). More importantly, the authors observed a similar correspondence between miR-16-5p, miR-17-5p, miR-24-3p, 140-3p, miR-145-5p, and miR-146a-5p expression in the extracellular vesicle fraction. Apart from miRNAs, lncRNAs are also involved in the MSC senescence triggered by hyperglycemia, and it has been shown that high glucose inhibits osteogenic differentiation of MSCs through the lncRNA AK028326/CXCL13 pathway, revealing new molecular mechanism of many osteogenesis-related diseases, especially in patients with diabetes mellitus (Cao et al., 2016).

Telomerase and Telomere Length

The telomere is a region of repetitive nucleotide sequences at each end of a chromatid, which protects the end of the chromosome from deterioration. Telomerase attrition is one of the hallmarks of cellular senescence. One study showed that telomerase was significantly lower in UCMSCs from GDM mothers than in UCMSCs from normal mothers (Kong et al., 2019). Moreover, the expression of the positive telomere maintenance marker (rTERT, TR) in MSCs in the endosteal niche lining compact bone was downregulated under high glucose conditions. Interestingly, telomere length is altered throughout *in vitro* expansion, with hyperglycemia markedly decreasing telomere lengths at PD50 and PD200 (Al-Qarakhli et al., 2019). Together, these studies recognize telomere attrition as one of the mechanisms of senescent MSCs in the hyperglycemic microenvironment.

Epigenetics

Epigenetics is one of the pillars of aging and MSC senescence which can be detected by epigenetic modifications. One study showed that cardiac mesenchymal cells from patients with T2DM were characterized by premature cellular senescence, reduced proliferation, decreased differentiation potential, and decreased phosphorylation at histone H3 serine 10. Global histone code profiling of cardiac MSCs rom patients with T2DM demonstrated that acetylation of histone H3 lysine 9 (H3K9Ac) and lysine 14 (H3K14Ac) was reduced, while the trimethylation of H3K9Ac and lysine 27 was greatly increased. Moreover, DNA CpG island hypermethylation was detected at the promoter of genes involved in genomic stability and cell growth control. These results reveal that epigenetic changes may be an essential factor in MSC senescence in hyperglycemia (Vecellio et al., 2014).

Other

MSCs from streptozotocin-induced diabetic rats displayed decreased proliferation and osteogenic differentiation potential, but increased senescence and apoptosis, and these effects appeared to be mediated by increased AGEs and an jincrease in the receptor for AGEs (Stolzing et al., 2010). Diabetic BMSCs showed inhibited osteogenesis and muscle ARNT-like protein 1 (BMAL1) expression, and over-expression of BMAL1 could

recover BMSC osteogenesis in T2DM partly by reducing GSK-3β expression to trigger the Wnt/β-catenin pathway (Li et al., 2017).

STRATEGIES TO SUPPRESS SENESCENCE OF MSCS IN THE HYPERGLYCEMIC MICROENVIRONMENT

Cellular senescence has been proven to be reversible. At present, the most promising drugs for this purpose include rapamycin, senolytics, and metformin (Partridge et al., 2020). For senescent MSCs, in addition to drugs, genetic modification, microRNA treatment and preconditioning modification are important approaches for rejuvenation (Neves et al., 2017; Ocansey et al., 2020). Currently, strategies to block the senescence of MSCs in hyperglycemic conditions include drugs (metformin, resveratrol, cytokines, and histone acetylase activator) and preconditioning modification (hypoxia), summarized in **Figure 3**. Besides, various *in vitro* experiments demonstrated that good glucose control could alleviate the senescence of MSCs, suggesting that blood glucose control should be advised to prevent or limit senescence of MSCs in patients with diabetes. More importantly, for MSCs from normal or diabetic donors, the appropriate glucose control in the MSC expansion process and in the transplantation process is essential for the clinical efficiency of MSC treatments (Lo et al., 2011).

Metformin is widely prescribed for T2DM; interestingly, recent studies have suggested an important role for metformin in mitigating aging. After treatment with metformin, AMSCs isolated from mice showed reduced cellular senescence and decreased ROS but increased proliferative potential and osteogenic differentiation potential and declined adipogenic differentiation (Marycz et al., 2016). Resveratrol, a small polyphenol, has emerged as a potential therapy due to its anticancer and anti-inflammatory properties. In diabetic animal models, AMSCs preconditioned with resveratrol showed increased stem cell function and better effects (Chen et al., 2019). One of the mechanisms is that resveratrol increases the expression of the survival marker p-Akt, resulting in enhanced AMSC viability (Cheng et al., 2016). It is well known that the proliferation and cytokine secretion (TGF-β and VEGF) of MSCs are inhibited after long-term culture even in MSCs when derived from young donors (Baek et al., 2018). However, this situation could be changed by the addition of substance P, suggesting that substance P could block the loss of the therapeutic potential of MSCs by preserving their proliferative and paracrine potential. Moreover, another study found that basic fibroblast growth factor promotes the proliferation and inhibits the apoptosis of AMSCs isolated from patients with T2DM by reducing cellular oxidative stress (Nawrocka et al., 2017). Similarly, when pretreated with a mixture of Stromal cell-derived factor-1 alpha and bFGF, insulin-producing cells differentiated from AMSCs showed maximally alleviated senescence, apoptosis, and cell damage. Interestingly, these AMSCs demonstrated enhanced release of insulin, increased cell proliferation and upregulation of insulin 1, insulin 2, Ngn3, Nkx6.2, and Pdx1when triggered by hyperglycemia. Epigenetics, as one of the pillars of MSC senescence, has been studied for searching targets to suppress the senescence of MSCs. For example, the histone acetylase activator pentadecylidenemalonate 1b (SPV106) has been shown to alleviate cellular senescence and recover the regenerative and multi-differentiation potential of cardiac MSCs by restoring

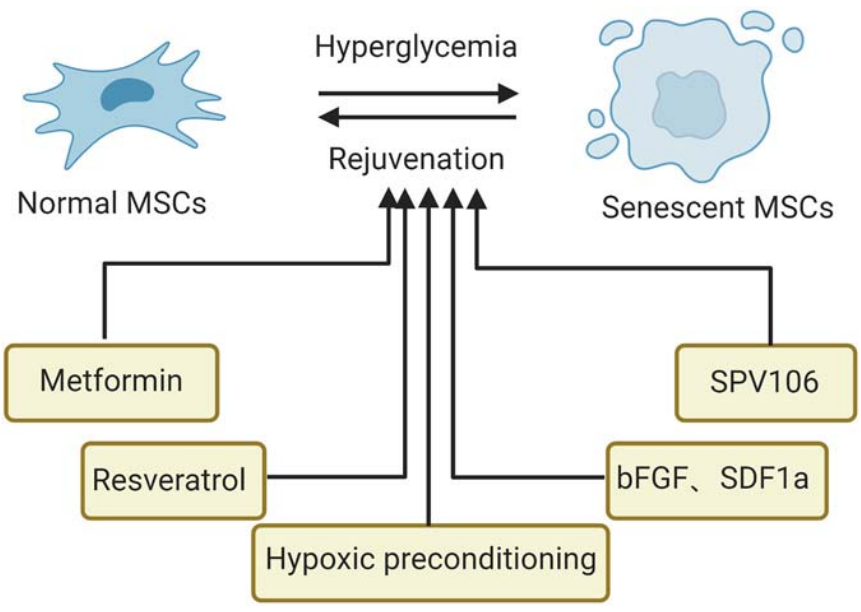

FIGURE 3 | Strategies to suppress senescence of MSCs in the hyperglycemic microenvironment. SCD1, stearoyl-coenzyme A desaturase-1; bFGF, basic fibroblast growth factor; SPV106, histone acetylase activator pentadecylidenemalonate 1b.

normal amounts of H3K9Ac and H3K14Ac and reducing DNA CpG hypermethylation (Vecellio et al., 2014). In addition, hypoxic preconditioning was able to enhance the regeneration potential of aging bone marrow MSCs into pancreaticβ-cells in streptozotocin-induced type-1 diabetic mice by altering gene expression levels of certain growth factors (Waseem et al., 2016). The underlying molecular mechanism might be related to hypoxia, which induces different regulation patterns in many cell cycle checkpoint genes such as HIF-1a, ataxia telangiectasia mutated, and ataxia telangiectasia and Rad3 related p53, p21, p27, and p21 (Sharma and Bhonde, 2015).

CONCLUSION

MSCs are promising cells for the treatment of regenerative and immunomodulatory diseases. Various studies have shown that hyperglycemia may induce MSC senescence and diminish crucial functions of MSCs, such as cell proliferation, differentiation capacity, angiogenesis and immunomodulatory capability, which remarkably restrict their therapeutic efficiency. The appropriate use of MSCs for clinical applications demands a general knowledge of the MSC senescence process. Additionally, approaches that generate large populations of MSCs *in vitro* without affecting their regenerative or immunomodulatory properties need to be established. In this review we discussed the relevant literature to date and suggested possible methods to improve therapeutic efficacy through regulating specific factors or the hyperglycemia microenvironment associated with MSC senescence.

Unlike BMSCs and AMSCs, UCMSCs have a painless collection process, faster self-renewal properties and lower immunogenicity. UCMSCs are attractive autologous or allogenic agents for the treatment of cancer and aging-related diseases (Ding et al., 2015). However, gestational diabetes is progressively prevalent and predicted to influence more than 20 million livebirths (about one in six) worldwide (Saravanan, 2020). Thus, considerably more work will need to be done to investigate the senescence of UCMSCs in the hyperglycemic environment. Besides, uniform experimental studies to elucidate the molecular basis and signaling pathways regulating senescence in MSCs in a diabetic microenvironment are urgently required, especially in the field of mitochondrial dysfunction and epigenetics. These mechanistic studies would contribute to discovering new strategy to suppress MSCs senescence in the hyperglycemic microenvironment. Furthermore, studies involving the detection of the senescence of MSCs in DM-related chronic complications such as diabetic nephropathy, diabetic neuropathy, and diabetic retinopathy will be beneficial. New specific assays to identify senescent MSCs to predict the safety and potency of autologous MSCs in DM or GDM and new strategies to suppress the senescence of MSCs should be developed. Data from these studies would facilitate accurate evaluation of the efficacy of the treatment and provide more precise inclusion and exclusion criteria for the proper selection of patient groups with diabetes that would best benefit from the treatment.

AUTHOR CONTRIBUTIONS

MY searched literature and drafted the manuscript. YZ and HY searched literature. XL provided ideas and revised the manuscript. All authors read and approved the final manuscript.

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