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Original Article

Hepatocyte Growth Factor-mediated Regulation of *OCT4* in human Mesenchymal Stem Cells

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Mesenchymal stem cells (MSCs) hold great promise as a source of stem cells for therapy, but several limitations remain. We previously proposed that human embryonic stem cell-derived MSCs (hE-MSCs) expressing higher hepatocyte growth factor (HGF) levels were better alternatives, exhibiting greater expandability *in vitro* and greater therapeutic capacity *in vivo*. In this study, we aimed to examine the regulation of *OCT4* expression in stem cells and to elucidate its underlying mechanism of transcriptional regulation of *OCT4*. We detected higher expression of *OCT4*, a stemness-associated gene in hE-MSCs than in human bone marrow-derived MSCs (hBM-MSCs). To determine the underlying regulatory mechanism of *OCT4* expression in human MSCs (hMSCs), ELISA was performed using cell culture supernatants of hMSCs. Unlike fibroblast growth factor 2 or vascular endothelial growth factor, HGF was strongly expressed in hE-MSCs, also HGF treatment significantly increased *OCT4* expression in hBM-MSC. Moreover, senescence-associated heterochromatin foci were decreased in HGF-treated hBM-MSCs compared with those in the HGF non-treated group. HGF increased Rb phosphorylation, and we confirmed the increased binding of E2F1 to the *OCT4* promoter region at -233 from the transcription start point in the presence of HGF. Taken together, these results suggest that HGF-mediated regulation of *OCT4* via E2F1 can help enhance the lifespan of hBM-MSCs during *in vitro* expansion.

Key Words: Human mesenchymal stem cells, Hepatocyte growth factor, *OCT4*, pRb, E2F1

INTRODUCTION

Mesenchymal stem cells (MSCs) are a promising solution for stem cell-based therapies in regenerative medicine. They possess several characteristics such as mesodermal differentiation potentials (adipogenic, osteogenic, and chondrogenic lineages), immunomodulatory function, low immunogenicity, and low risk of tumorigenesis (Weng et al., 2022). Due to these beneficial natures along with the ability of self-renewal of MSCs, the number of registered MSC clinical trials including orthopedic and cardiovascular diseases, Crohn's disease, kidney injury, lung disease, liver regeneration, Alzheimer's and Parkinson's disease, etc. has increased rapidly to more than 1000 as of 2021 (Wright et al., 2021). However, sequential passages of MSCs to attain the required

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cell number and purity for therapeutic applications lead to replicative senescence or unpredictable differentiation, thus limiting their clinical use (Campisi and d'Adda di Fagagna, 2007; Wagner et al., 2008). Therefore, strategies to overcome these challenges are critical for improving the therapeutic efficacy of MSCs.

Senescence-associated heterochromatin foci (SAHF), specific features of some senescent cells, are characterized by highly condensed heterochromatin with epigenetic modifications and marker proteins (Narita et al., 2003; Schulz and Tyler, 2005). Functional $p16^{INK4a}$ and retinoblastoma protein (pRb) with high mobility group A protein, which mainly accumulate at promoters of E2F-responsive proliferation genes, are critically involved in SAHF arrangement during senescence (Narita et al., 2006; Campisi, 2005). Therefore, E2F-responsive genomic promoter regions were maintained in a repressed state by establishing SAHF during senescence. pRb plays a crucial role in the onset and maintenance of senescence and SAHF formation (Narita et al., 2003; Chandra et al., 2012). pRb is deprived of DNA binding sites and thus regulates cell cycle progression by binding and suppressing E2F transcription factors depending on its phosphorylation status. Upon pRb phosphorylation, E2F1 is released from the inhibitory sequestration of Rb and binds to the promoters of target genes for transactivation (Nevins, 1992; Narasimha et al., 2014; Fischer and Muller, 2017).

In stem cells, *OCT4* is considered a master regulator for the maintenance of self-renewal and undifferentiated states (Orkin, 2005; Kim et al., 2008). High *OCT4* expression and low $p16^{INK4A}$ expression could be a hallmark of *in vitro* expandability and an anti-senescence state in MSCs (Piccinato et al., 2015). In addition, the Oct4-pRb axis is a regulatory circuit that controls mESC self-renewal and differentiation (Schoeftner et al., 2013). Therefore, *OCT4*, which maintains the stemness and anti-senescence state of MSCs, could be a downstream target of the pRb-E2F1 pathway.

In this study, we aimed to examine the regulation of *OCT4* expression in stem cells and to elucidate its underlying mechanism of transcriptional regulation of *OCT4*. To investigate the mechanism regulating *OCT4* expression, we first compared HGF level between human embryonic stem cell-derived MSCs (hE-MSCs) and human bone marrow-derived MSCs (hBM-MSCs) (Lee et al., 2010). Then we tested whether pRb-E2F1 pathway is involved in HGF-mediated *OCT4* expression in stem cells.

MATERIALS AND METHODS

Human MSC culture and recombinant human hepatocyte growth factor addition

hE-MSCs were cultured in EGM-2 MV™ medium (Lonza, Basel, Switzerland), and hBM-MSCs (Lonza) were grown in MSCGM™ medium (Lonza) at 37℃ with 5% CO_2. In this study, we used hE-MSCs at passage 14 and hBM-MSCs at passage 7.

Human recombinant hepatocyte growth factor (rhHGF) (10 ng/mL; R&D Systems, Minneapolis, MN, USA) was added to hBM-MSCs (low-hepatocyte growth factor [HGF] human MSCs [hMSCs]) daily for 5 days. The medium was changed daily with the addition of rhHGF.

Real-time PCR analysis

QIAshredder and RNeasy Mini Kit (Qiagen, Germantown, MD, USA) were used to prepare the total RNA according to the manufacturer's instructions. Subsequently, 1 μg RNA was transcribed to cDNA using the PrimeScript 1st strand cDNA Synthesis kit (Takara, Tokyo, Japan). PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Real-time samples performed using an ABI PRISM 7500 sequence detection system (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control for normalization.

The real-time PCR primers were as follows: Oct4: forward, 5'-GAGGCAACCTGGAGAATTTG-3', reverse, 5'-TAGCCTGGGGTACCAAAATG-3'; GAPDH: forward, 5'-TGTGAGGAGGGGAGATTCA-3', reverse, 5'-CAACGAATTTGGCTACAGCA-3'.

Western blot analysis

All hMSCs were lysed using protein lysis buffer (50 mmol /L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, and 0.5% deoxycholate) containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Total

lysates (25 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred by electroblotting onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Membranes were incubated in primary antibody overnight at 4℃. The primary antibodies used were anti-Oct4 (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Rb (1:2,000; Abcam, Eugene, OR, USA), and anti-Rb phosphoT356 (1:2,000; Abcam). The anti-α-tubulin antibody (1:5,000; Sigma-Aldrich, St. Louis, MO, USA) was used as an internal housekeeping control. The immunoblotted membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000; Sigma-Aldrich) for 2 h at room temperature.

Enzyme-linked immunoassay

To measure the secreted HGF, vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF2), cell culture supernatants of hMSCs were analyzed using human HGF, VEGF, and FGF2 quantikine ELISA kits (R&D Systems). Results were read using an ELISA reader, the Multiskan^{TM} GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), at 450 nm.

Immunocytochemistry

Cultured hMSCs were fixed with acetone for 3 min at -20℃ and washed three times with 1× phosphate-buffered saline (PBS). Unspecific binding was blocked with 0.1% bovine serum albumin and 0.05% Triton-X-100 in Dulbecco's PBS (Thermo Fisher Scientific) for 1 h. Subsequently, hMSCs were incubated overnight at 4℃. Subsequently, hMSCs were incubated overnight anti-heterochromatin protein 1γ (HP1γ) antibody (1:200; Millipore). After three 5-min washes, the cells were incubated with secondary antibodies conjugated to Alexa Fluor fluorescent dye (1:200; Invitrogen) for 2 h at room temperature. Images were obtained using a confocal microscope (Carl Zeiss LSM710; Carl Zeiss, Jena, Germany).

Chromatin immunoprecipitation assay

The hBM-MSCs were fixed in 0.8% formaldehyde for 10 min and collected in a tube. The supernatant was discarded, a buffer (0.1% digitonin prepared in PBS containing a phosphatase inhibitor cocktail and protease inhibitor cocktail) was added, and the sample was carefully pipetted several times. After centrifugation, to shear the DNA, the fraction was sonicated four times with a BIORUPTOR (30 sec on/30 sec off per cycle). The immunoprecipitation sample was supplemented with an anti-E2F1 antibody (1 μg) and rotated overnight. Protein A/G agarose beads were added to pull down the antibodies and sequentially washed three times with washing buffers (low and high LiCl). Samples were incubated for 4 h at 65℃ for decrosslinking and recovered using a PCR purification kit (Qiagen). DNA precipitation using anti-E2F1 was analyzed using quantitative PCR.

Screening of E2F1 binding sites and construction of the E2F1 binding site vector

To assay the E2F1 binding sites (EBS), a 3-kb *OCT4* promoter sequence was analyzed with GP Miner (http://gpminer.mbc.nctu.edu.tw) to identify transcription factors on the target genes. The EBS deletion mutant construct was derived from a full-length *OCT4* construct by performing PCR with specific primers for the deletion of the EBS binding sites -233 to -228 from the transcription start site. PCR products were inserted into the pGL3 vector for luciferase analysis. Human OCT4 (hOCT4) 3-kb promoter forward, 5'-CCCGGTACCTGCCTCAACCTCCCATCAGC-3'; hOCT4 3-kb promoter reverse, 5'-TTGAGATCTTGAGGGCTTGCGAAGGGACT-3'; hOCT4 ref 3-kb promoter, 5'-ATGGCTGTTGATGCATTGAGGGATACACACACATTCAATAAATTTGAGGA-3'; hOCT4 ΔEBS1 3-kb promoter, 5'-GGGACCTGCACTGAGGTCCTGGAGGAGTTGTGTCTCCCGGTTTTCCCCTT-3'.

Luciferase assay

Luciferase analysis was performed using the GLOMAX system 20/20 (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical analyses

Statistical comparisons were performed using the GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Quantitative data were reported as the mean ± standard error of the mean, and unpaired *t*-test analysis of variance was

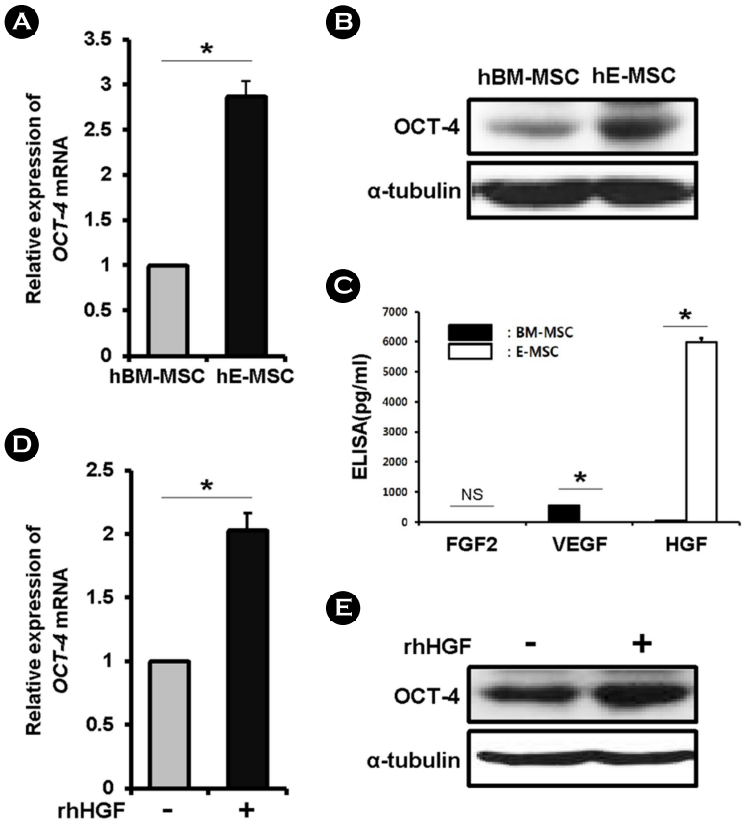

Fig. 1. Comparison of *OCT4* expression and selection of growth factor between hE-MSCs and hBM-MSCs. (A) Real-time PCR cDNA amplification and (B) western blot of *OCT4*. (C) Comparison of growth factors FGF2, VEGF, and HGF between hE-MSCs and hBM-MSCs using ELISA. (D) Real-time PCR cDNA amplification and (E) western blot of *OCT4* after rhHGF treatment (10 ng/mL) for 5 days in hBM-MSCs. NS: not significant, $*P < 0.05$ by *t*-test. FGF2, fibroblast growth factor 2; hBM-MSCs, human bone marrow-derived mesenchymal stem cells; hE-MSCs, human embryonic stem cell-derived mesenchymal stem cells; HGF, hepatocyte growth factor; rhHGF, recombinant human hepatocyte growth factor; VEGF, vascular endothelial growth factor.

used to analyze each group. *P*-values <0.05 were regarded as statistically significant.

RESULTS

HGF upregulates *OCT4* expression

To detect *OCT4* mRNA and protein expression, hE-MSCs and hBM-MSCs were prepared at passages 15 and 8, respectively. Higher *OCT4* mRNA and protein expression levels were detected in hE-MSCs than in hBM-MSCs (Fig. 1A, B). To determine the mechanism underlying such differences between the two MSCs types, we first evaluated the growth factors that were highly expressed in the supernatants of hE-MSCs. ELISA showed that HGF was increased in the hE-MSC culture supernatant, whereas VEGF and FGF2 were not (Fig. 1C). Next, to test the possibility that HGF regulates *OCT4* gene expression, we treated hBM-MSCs with rhHGF (10 ng/mL) and observed a significant increase in mRNA and protein expression of *OCT4* (Fig. 1D, E).

HGF decreases SAHF

To investigate the mechanism of regulation of *OCT4* expression by HGF, SAHF were detected. Interestingly, HP1γ, an SAHF marker, decreased according to the treatment of HGF in hBM-MSCs (Fig. 2A). Moreover, the level of phosphorylated Rb increased after HGF treatment (Fig. 2B). Based on these results, we predicted the transition of the E2F1 binding region of the *OCT4* promoter from SAHF to the open state.

E2F1 binds to the *OCT4* promoter

E2F1 is a transcription factor related to senescence, but the *OCT4* binding region of E2F1 has not yet been reported until now. Through sequential *in vitro* cultivation, hMSCs enter senescence states along with a decrease in stemness genes, including *OCT4* (Piccinato et al., 2015). Therefore, E2F1 released by phosphorylated Rb can bind to the *OCT4* promoter region, but the *OCT4* binding region of E2F1 has not yet been identified. We screened the *OCT4* promoter

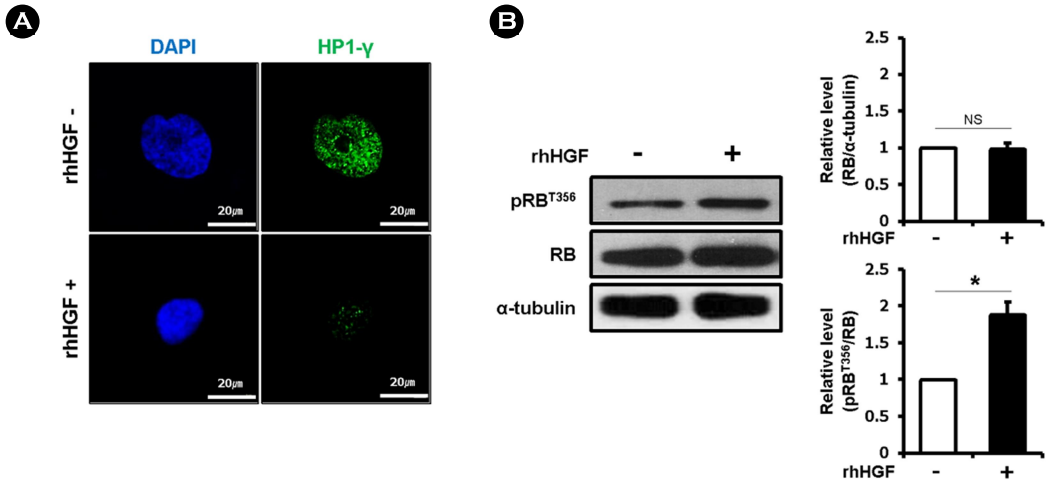

Fig. 2. SAHF is repressed in HGF-treated hBM-MSCs. (A) Immunofluorescence using HP1γ and (B) western blot of pRb-T356 and phosphorylated Rb after rhHGF treatment (10 ng/mL) for 5 days in human bone marrow-derived mesenchymal stem cells. NS: not significant, *$P < 0.05$ by *t*-test. HGF, hepatocyte growth factor; HP1γ, heterochromatin protein 1γ; pRB, retinoblastoma protein; rhHGF, recombinant human hepatocyte growth factor; SAHF, senescence-associated heterochromatin foci.

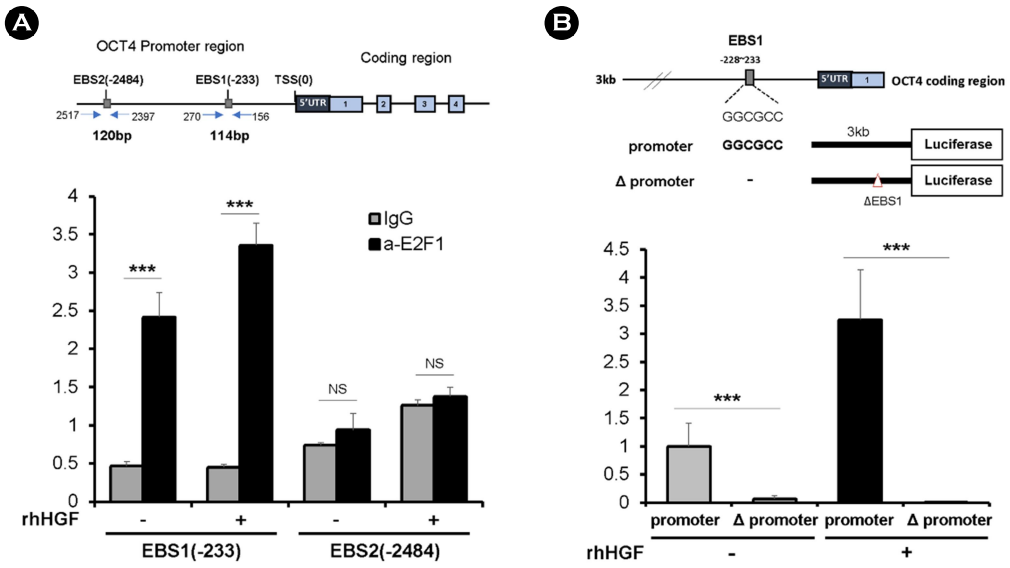

Fig. 3. E2F1 binds into the *OCT4* promoter. (A) Chromatin immunoprecipitation and quantitative RT-PCR analyses. Primers using putative E2F1 binding sites EBS1 and EBS2 were designed, and quantitative PCR was performed with an E2F1 antibody to pull down genomic DNA. Comparison of E2F1 binding after human hepatocyte growth factor treatment. IgG was used as a control. (B) Luciferase/renilla promoter assay. Promoter activity according to E2F1 binding site deletion. EBS1 was deleted within the ~3-kb *OCT4* promoter region, and its activity was compared with that of the full-length promoter under rhHGF treatment (10 ng/mL). NS: not significant, ***$P < 0.001$ by *t*-test. rhHGF, recombinant human hepatocyte growth factor.

region to find the EBS and selected two putative sites at positions -2484 (EBS2) and -233 (EBS1) from the transcription start site. To detect the binding of E2F1 to the *OCT4* promoter, we performed a chromatin immunoprecipitation (ChIP) assay with an E2F1 antibody and quantitative PCR with specific primers for EBS1 and EBS2. We observed that E2F1 binding at EBS1 and HGF treatment increased the amount of E2F1 bound to EBS1 (Fig. 3A). However, EBS2 did not bind to HGF. Next, to confirm the EBS, the promoter activities of wild-type and EBS1-deleted *OCT4* promoter

were compared, and no luciferase activity of the EBS1-deleted mutant promoter was observed, even in the HGF-treated condition (Fig. 3B).

DISCUSSION

We previously showed that hE-MSCs have greater proliferation ability than hBM-MSCs. HGF was highly expressed in hE-MSCs and could function as a rejuvenating factor by regulating telomere length and mitochondrial function in HGF-treated hBM-MSCs (Lee et al., 2018). We detected a higher expression of *OCT4* in hE-MSCs than in hBM-MSCs. Moreover, when HGF was used as an additive, we observed reduced SAHF levels and increased E2F1 binding to the *OCT4* promoter in hBM-MSCs. We further demonstrated the role of HGF in regulating *OCT4* expression via the pRb-E2F1 pathway in hBM-MSCs.

Permanent cell cycle arrest, defined as senescence, occurs through multiple triggers, including telomeric attrition, DNA damage, mitochondrial dysfunction, epigenetic alterations, and oncogenic activation (Weng et al., 2022; Di Micco et al., 2021). SAHF formation is not associated with all cellular senescence or senescence onset. However, some genes related to cell cycle progression, which are E2F target genes, are silenced by pRb-dependent reorganization of SAHFs (Narita et al., 2003; Paluvai et al., 2020). Interestingly, SAHF detected by HP1γ was decreased by HGF treatment in hBM-MSCs. Moreover, we observed an increase in phosphorylated pRb, the inactive form, following HGF treatment in hBM-MSCs (Fig. 2). Therefore, we investigated the potential interplay between the major binding partners of pRb, E2F1, and *OCT4* under HGF treatment in hBM-MSCs. We identified E2F1 binding sites and detected the binding of E2F1 to the *OCT4* promoter using ChIP and quantitative PCR. Furthermore, the promoter activities of wild-type *OCT4* were enhanced in the presence of HGF, whereas those of the E2F1 binding site deletion mutant were not (Fig. 3).

Fu et al. reported on the significant relationship between E2F1 and gastric cancer stemness, suggesting a possibility of E2F1-mediated transcription of stemness genes (Fu et al., 2021). Moreover, E2F1 binds to the promoters of *Nanog* and *BMI1* in breast cancer and neuroblastoma, respectively (Lu et al., 2018). In the present study, we demonstrated for the first time that HGF affects *OCT4* expression at the transcriptional level via E2F1 transactivation in MSCs. *OCT4* is usually expressed in cells possessing embryonic stem cell properties or in cancer tissues, but not in somatic cells (Patra, 2020). In addition, *OCT4* plays an important role in S-phase entry and is inversely correlated with MSC senescence (Piccinato et al., 2015; Lee et al., 2010). Therefore, it is plausible that the HGF-induced upregulation of the pluripotency-related transcription factor, *OCT4*, might be an efficient target for maintaining the lifespan and stem cell properties of hBM-MSCs.

Most growth factors are involved in the cell cycle regulation through Rb phosphorylation and subsequent E2F activation. HGF, for example, induces pRb hyperphosphorylation and E2F1 transactivation, leading to muscle cell proliferation (Leshem and Halevy, 2002).

HGF is a pleiotropic cytokine involved in cell development, proliferation, survival, migration, angiogenesis, and tissue regeneration (Zhao et al., 2022). Cao et al. reported that hBM-MSCs cultured in conditioned medium from human exfoliated deciduous teeth (SHED-CM) showed enhanced cell proliferation and reduced senescence during long-term *in vitro* culture (Cao et al., 2020). Moreover, senescence-associated genes (*p16* and *p21*) and pluripotent transcription factors (*Nanog* and *Oct4*) were upregulated in SHED-CM-cultured hBM-MSCs. They revealed that HGF and stem cell factor play key roles in delaying cell senescence and maintaining the stemness of hBM-MSCs, which is similar to our current results. Currently, whether HGF stimulation is directly involved in delaying or modulating senescence in hE-MSCs is unclear. Further studies are required to delineate the precise molecular mechanisms involving pRb/E2F/Oct4 in the regulation of HGF-mediated stemness and cell senescence.

Recently, Chang et al. reported that the therapeutic effects of MSCs are largely attributed to the paracrine secretion of growth factors, cytokines, miRNA, and small signaling molecules, referred to as the MSC "secretome" (Chang et al., 2021). The application of the secretome of MSCs, such as conditioned medium or MSC-derived extracellular vesicles, has been implicated in better and safer therapeutic outcomes

in the tissue repair of myocardial infarction-induced heart and lung injury models (Park et al., 2020; Aslam et al., 2009). Therefore, secretome profiling and optimized application of components essential for engraftment and survival would be a good solution to overcome the hurdles associated with the heterogeneity and variability of MSC-based stem cell therapy. In this respect, the current results suggest a potential mechanism for enhanced longevity via paracrine secretion of HGF from MSCs.

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None

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding the publication of this paper.

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