A Decline in Wnt3a Signaling is Necessary for Mesenchymal Stem Cells to Proceed to Replicative Senescence

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Umbilical cord blood-derived mesenchymal stem cells are a promising source of cells for regeneration therapy due to their multipotency, high proliferative capacity, relatively noninvasive collection, and ready availability. However, extended cell culture inevitably triggers cellular senescence—the irreversible arrest of cell division—thereby limiting the proliferative lifespan of adult stem cells. Wnt/ β -catenin signaling plays a functional role as a key regulator of self-renewal and differentiation in mesenchymal stem cells (MSCs), and thus Wnt/ β -catenin signaling and cellular senescence might be closely connected. Here, we show that the expression levels of canonical Wnt families decrease as MSCs age during subculture. Activation of the Wnt pathway by treatment with Wnt3a-conditioned medium or glycogen synthase kinase 3 β inhibitors, such as SB-216763 and 6-bromoindirubin-3'-oxime, delays the progression of cellular senescence as shown by the decrease in the senescence effectors p53 and pRb, lowered senescence-associated β -galactosidase activity, and increased telomerase activity. In contrast, suppression of the Wnt pathway by treatment with dickkopf-1 (an antagonist of the Wnt coreceptor) and β -catenin siRNA transfection promotes senescence in MSCs. Interestingly, the magnitude of the response to enhanced Wnt3a/ β -catenin signaling appears to depend on the senescent state during extended culture, particularly after multiple passages. These results suggest that Wnt3a signaling might be a predominant factor that could be used to overcome senescence in long-term cultured MSCs by directly intervening in the proliferative capacity and MSC senescence. The functional role of Wnt3a/ β -catenin signaling in hedging cellular senescence may allow the development of new approaches for stem cell-based therapies.

Introduction

ADULT MESENCHYMAL STEM CELLS (MSCs) can be easily isolated, self-renew, and differentiate into a range of mesenchymal tissues both in vivo and in vitro [1–3]. Therefore, they are considered an attractive candidate for the development of cell-based therapeutics [4]. Prolonged passaging of the in vitro culture environment is a prerequisite for acquiring a suitable number of MSCs for cell therapeutics. However, the process may adversely affect the physiological properties of MSCs, such as stemness, proliferation, and differentiation potency. MSCs that are maintained in vitro for a long periods of time develop cellular senescence, which consequently limits the number of cell doublings [5]. Al-

though cellular senescence is caused by various factors, senescent cells display a number of common characteristics that make them easily distinguishable from growing cells [6]. Generally, senescence triggers morphological transformations that result in enlarged, flattened, and multinucleated cells. Given the role of tumor suppressors and cell cycle regulators in cancer cells, p53-p16-Rb signaling is used as a biomarker to identify senescent cells [7,8]. In particular, p53 mainly mediates prosenescence signals that are derived via oncogene activation, telomere dysfunction, DNA damage, and reactive oxygen species [9]. Another commonly used indicator is senescence-associated β -galactosidase (SA- β -gal) activity [10]. Increasing lysosomal biogenesis in senescent cells is accompanied by the upregulation of SA- β -gal activity at pH 6.0 [11].

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Several studies report that canonical Wnt signaling keeps stem cells in a self-renewing and undifferentiated state [12–14], although the intensity of Wnt signaling can lead to different or even opposite biological functions [15,16]. MSCs express a number of Wnt ligands (including Wnt2, Wnt4, Wnt5a, Wnt11, and Wnt16), Wnt receptors, and Wnt inhibitors [17]. Because senescence is thought to limit the proliferative lifespan of many human stem cell populations, Wnt signaling and senescence in MSCs should be coordinated with each other [18]. Some studies suggest that canonical Wnt signals delay senescence through the coordinated and combined effects of multiple pathways, including β -catenin activation, c-myc expression, and the inactivation of the senescence-associated heterochromatin foci (SAHF) assembly pathway [12,19,20].

Although efforts to explore the interrelationship between Wnt signaling and cellular senescence are underway, to what extent Wnt/ β -catenin signaling contributes to the development of senescence in MSCs remains largely unknown. In this study, we investigate the feasibility of Wnt/ β -catenin signaling as regulator of the progression of senescence during extended MSC culturing.

Materials and Methods

Cell culture

The MSCs used in this study were donated by MEDI-POST Co., Ltd. (Seongnam, Korea). Umbilical cord blood was collected from the umbilical vein after neonatal delivery and obtaining informed consent from all mothers. Umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) were separated, as previously described [21], and maintained in α -minimum essential media (α -MEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 100 μ g/mL penicillin/streptomycin. UCB-MSCs were plated at 2×10^3 cells/cm² in T-175 culture flasks and split at ~70% confluency every 5 days. We used two UCB-MSC lines that were isolated from blood samples obtained from different donors (the mother age of 34 to UCB-MSC-1 and 31 to UCB-MSC-2), which were marked as MSC-1 and MSC-2, respectively, in this study. The LacZ-L929 and Wnt3a-L929 cell lines (provided by the University of Seoul, Korea) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% FBS and G418. Media were renewed every 3 days. All cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Wnt3a-conditioned medium and reagents

Wnt3a was prepared in the conditioned culture medium using a stable, secreted Wnt3a-producing, transfected L929 cell line (Wnt3a-CM). Conditioned medium from L929 cells containing an empty vector (LacZ-CM) was used as the experimental control. Briefly, to obtain conditioned medium, cells were seeded into T-75 culture flasks in DMEM without G418 and allowed to adhere overnight. The culture medium was then replaced with fresh α -MEM for MSCs and the cells were incubated for an additional 2 days. After harvesting, the medium was filtered and freshly applied to MSCs with dilution in complete α -MEM. 6-Bromindirubin-3'-oxime (BIO), SB-216763, and dickkopf-1 (Dkk-1)

were purchased from Sigma and used to regulate the Wnt/ β -catenin signaling pathway.

Cell proliferation and viability

Cell proliferation was measured using 5-bromo-2-deoxyuridine (BrdU) cell proliferation ELISA (Roche Applied Science), which quantifies the incorporation of BrdU, a thymidine analog, during DNA synthesis. Cell viability was determined using a cell counting kit-8 (Dojindo Molecular Technology). Absorbance was measured at 450 nm using an ELISA reader. Cell proliferation and viability, expressed as percentages, were calculated relative to the control cells.

RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was isolated using TRIzol[®] reagent (Ambion) and converted to cDNA using an oligo (dT) primer and SuperScript III Reverse Transcriptase (Invitrogen). Polymerase chain reaction (PCR) was performed using the Blend Taq-plus polymerase (Toyobo). The PCR cycling conditions consisted of 95°C for 5 min, 35 cycles of 95°C for 30 s, 56°C for 40 s, and 72°C for 40 s each, followed by final extension at 72°C for 10 min. The amplified products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized under ultraviolet light. The intensity of each band was measured using ImageJ software (National Institutes of Health) and normalized to β -actin. Gene-specific primer sets were designed. The following primers were used to amplify the target mRNA: *Wnt2* (F) 5'-CCAGAGCC CTGATGAATCTT-3' and (R) 5'-TCGGTCCCTGATACA GTAG-3'; *Wnt3a* (F) 5'-CATGAACCGCCACAACAAC-3' and (R) 5'-TCGCAGAAGTTGGGCGAG-3'; *Wnt5b* (F) 5'-CTGCTGCTGCTGTTTACG-3' and (R) 5'-ATGACTCTCC CAAAGACAGA-3'; *Wnt7a* (F) 5'-CTTCGGGAAGGAG CTCAAA-3' and (R) 5'-TGCCTCGTTGTTGTGCAAG-3'; *β -actin* (F) 5'-CTTCGGGAAGGAGCTCAAA-3' and (R) 5'-TGCCTCGTTGTTGTGCAAG-3'.

SA- β -gal activity

Cells (4×10^4) were seeded onto six-well plates and treated with inhibitors or Wnt3a-CM. After 48 h, the cells were washed twice with PBS and fixed for 10 min. After removing the fixative, the cells were washed twice with PBS and stained with the staining solution prepared as indicated by the β -galactosidase reporter gene staining kit (Sigma). Cells were incubated at 37°C for an additional 12 h, and then the percentage of β -galactosidase-positive cells was determined by counting five fields on a phase contrast microscope (Carl Zeiss).

Telomerase activity

About 2×10^5 cells were harvested for each reaction and centrifuged at 3,000g for 10 min at 4°C. The cells were lysed to determine telomerase activity, and sequential reaction steps were performed using the TeloTAGGG Telomerase PCR ELISA kit (Roche Applied Science) in accordance with the manufacturer's instructions. The absorbance of the final product was measured within 30 min at 450 nm using an ELISA reader.

siRNA transfection

The β -catenin gene was silenced using siRNAs against β -catenin (Invitrogen) and a nontargeting siRNA was used as control (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen). Briefly, MSCs were transfected with each siRNA (30 nM final concentration). The medium was replaced with fresh culture medium after 6 h, and the cells were incubated for an additional 48 h. The β -catenin protein level was confirmed in all experiments using western blotting analysis.

Western blotting

Cells were harvested and lysed in PRO-PREP lysis buffer (iNtRON Biotechnology) containing Halt protease inhibitor cocktail (Thermo Scientific) for 30 min on ice. After clearing the samples by centrifugation at 13,000 rpm for 10 min, the protein concentrations of the cell lysates were measured using a bicinchoninic acid protein assay kit (Thermo Scientific). Equal amounts of protein (20 μ g) were resolved using SDS-PAGE and transferred onto a nitrocellulose membrane, which was blocked in 0.1% Tris buffered saline with Tween 20 (TBST) containing 5% bovine serum albumin and probed with the indicated antibodies: phospho-p53 (Cell Signaling), p53 (Cell Signaling), p21 (Cell Signaling), p27 (Cell Signaling), phospho-pRb (Cell Signaling), pRb (Cell Signaling), β -catenin (Cell Signaling), and β -actin (Sigma). After rinsing with TBST solution, the membranes were incubated for 1 h at room temperature in blocking solution containing peroxidase-conjugated secondary antibody. The membranes were then processed for analysis using the SuperSignal West Pico Chemiluminescence Detection Kit (Thermo Scientific). Band intensity was quantified using ImageJ software (National Institutes of Health).

Flow cytometry

To determine relative cell size and granularity, the harvested cells were fixed in 70% ice-cold ethanol, treated with RNase, and stained with propidium iodide for 30 min at 37°C. The cell volume and inner complexity of the particles were determined by analyzing forward scattering (FSC) and side scattering (SSC) using a FACSCalibur flow cytometer equipped with CellQuest Pro software (BD).

Immunofluorescence

To determine the localization of β -catenin in early- and late-stage MSCs, the MSCs were incubated with β -catenin antibody (1:100 dilution). Then, the cells were washed and incubated with FITC-conjugated antibody (1:300 dilution; Dako). 4', 6-Diamidino-2-phenylindole (DAPI) was used to visualize nuclei. After washing and mounting, the cells were examined under a fluorescence microscope.

Statistics

Statistical analysis was performed using unpaired, two-tailed student *t*-tests. In this study, $P < 0.05$ was considered statistically significant. Values were expressed as the mean $\pm$ standard deviation.

Results

Long-term in vitro UCB-MSCs cultivation results in cellular senescence

UCB-MSCs were continuously passaged in T-175 culture flasks at regular intervals until they lost their ability to divide. An additional passage (P) number was assigned once cells underwent subculturing. During this process, the number of population doublings (PD), doubling times (DT), and the total culture period were monitored. The UCB-MSC-1 (MSC-1) and UCB-MSC-2 (MSC-2) cells that were isolated from the cord blood of the two different donors demonstrated different cell morphologies and growth rates depending on the P number. Under in vitro culture conditions, MSC-1 and MSC-2 cells lost their proliferation capacity when passaged to 17 (P17, PD61) or 13 (P13, PD35), respectively.

Based on the growth rate, inflammatory chemokine and cytokine production levels (eg, IL-6, IL-8, and MCP-1), and the expression of senescence markers (eg, pRb, p53, p21, and p27) we classified the senescent process in MSCs into three stages: early, intermediate, or late (Jin et al., unpublished data). Early cells showed a fast growth rate and narrow-shaped morphology, whereas late cells no longer showed growth but instead demonstrated high SA- β -gal activity and typical senescent phenotypes in terms of morphological changes and an increase in intracellular vacuoles. Cells in the intermediate stage appeared to be beginning to enter early senescence. This definition of UCB-MSC passage is consistent with the recent results of Kim et al., which describe the detailed process of cellular senescence and unique features during replicative senescence in human diploid fibroblasts [22]. The two MSC lines used in this study demonstrated different P numbers that corresponded to the senescent stage: MSC-1 cells were defined as early cells at P7 and below (through PD37), intermediate cells between P8–P12 (through PD52), and late cells at P13 and above (through PD61). In the MSC-2 cell line, early cells were at P7 and below (through PD27), intermediate cells between P8–P10 (through PD33), and late cells at P11 and above (through PD35). We selected MSCs at the intermediate stage of senescence unless otherwise noted because we thought the degree of senescence could be modulated using signal regulators.

As shown in Figure 1A, late MSCs (PD55 for MSC-1; PD30 for MSC-2) were larger, flatter, and more intensely stained for SA- β -gal than early cells (PD29 for MSC-1; PD21 for MSC-2; Fig. 1A, left). The number of SA- β -gal-positive cells increased with the P number by about 5.5- and 6-fold in MSC-1 and MSC-2 cells, respectively (Fig. 1A, right), implying that senescence considerably progressed during the later passages. Given the fact that replicative senescence stems from shortened telomere length, telomerase activity was also significantly decreased in late cells (Fig. 1B). As expected, MSC-1 cells that were passaged to P15 (PD58) showed a dramatic decrease in phosphorylated pRb and an increase in phosphorylated p53, p21, and p27 proteins (Fig. 1C), indicating the activation of the senescence regulators p53 and pRb in senescent MSCs. Similar results were found in MSC-2 cells (data not shown). The senescence phenotypes might cause an irreversible growth arrest, as shown by the cumulative PD curve for

FIG. 1. Umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) undergo cellular senescence during culture expansion. **(A)** UCB-MSC-1 and UCB-MSC-2 cells obtained from two different donors were cultured as low or high population doubling (PD) groups: PD29 (Passage, P5) and PD55 (P13) for MSC-1; PD21 (P5) and PD30 (P8) for MSC-2. The cells were stained with senescence-associated β -galactosidase (SA- β -gal), and the percentage of SA- β -gal-positive cells is shown (* P < 0.05; ** P < 0.01). **(B)** Telomerase activity was measured using the TeloTAGGG Telomerase PCR ELISA kit. The value is presented as the fold change relative to early-stage cells. The positive control (PC) consisted of 293 cell extracts that were provided with the kit, and the negative control (NC) was the lysis reagent without any extracts. **(C)** The expression levels of the senescence markers (pRb, p53, p21, and p27) were measured in PD29, PD51, and PD58 MSC-1 cells (P5, P11, and P15, respectively) using by immunoblotting analysis. β -Actin was used as the loading control. **(D)** MSC-1 and MSC-2 cells were maintained under regular culture conditions, as described in the Materials and Methods section, in which the split cells were passaged to P5 and cumulative PD was recorded through P17 or P13 for MSC-1 and MSC-2, respectively. All data were obtained using three independent experiments (* P < 0.05; *** P < 0.005).

MSC cells (Fig. 1D). Together, these results demonstrate that UCB-MSCs develop cellular senescence during extended in vitro cultivation, in which a subset of the cell population demonstrates distinguishable senescent phenotypes and begins to lose division capacity.

Wnt signaling is repressed in senescent UCB-MSCs

Wnt signaling frequently maintains the proliferation of tissue stem cells by stimulating cell division and inhibiting differentiation and apoptosis [23]. A previous study reported that repressing Wnt2 expression and downstream signaling occurs in senescent cells, regardless that senescence is induced by extended culturing or activated Ras [20]. Based on these findings, we first examined the *Wnt2* mRNA levels in sequentially passaged MSC-1 cells. Actively growing cells showed high *Wnt2* mRNA levels, whereas the expression of *Wnt2* rapidly decreased as the P number increased (Sup-

plementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). Furthermore, the mRNAs of most other studied canonical (Wnt3a and Wnt5b) and noncanonical (Wnt7a) Wnt proteins also decreased in a passage-dependent manner (Fig. 2A). Notably, Wnt3a was markedly reduced in late senescent cells in comparison with the other observed Wnts, and the degradation of β -catenin, a major mediator of Wnt3a signaling, was clearly observed. In addition, in early stage cells, β -catenin was primarily distributed within the cytoplasm and intensively localized in the plasma membrane within the cell to cell contact region, whereas late cells with fattened morphology showed reduced levels and cytoplasm localization of β -catenin, indicating inactive canonical Wnt signaling upon passaging (Fig. 2B). Taken together, the activity of the Wnt pathway—in particular the canonical Wnt3a/ β -catenin pathway—was downregulated in MSCs as senescence progressed, which supports coordination between senescence and Wnt/ β -catenin signaling.

FIG. 2. UCB-MSCs show decreased Wnt expression during the development of senescence. MSC-1 cells were cultured in expansion medium under regular culture conditions and harvested at the indicated PDs. **(A)** The mRNA levels of the Wnt family members (Wnt2, Wnt5b, Wnt3a, and Wnt7a) were determined using RT-PCR. The expression levels were quantified and normalized to β -actin, which are presented as the fold change relative to the lowest PD of the cells (* $P < 0.05$; ** $P < 0.01$). **(B)** Intracellular distribution and the level of β -catenin were determined by immunofluorescent staining (green) in sequential PDs. Nuclei were counterstained with DAPI (blue). Representative microscopic fields for each passage are shown. Scale bar = 50 μ m.

Exogenous Wnt3a delays the progression of senescence in UCB-MSCs

To determine whether regulating Wnt3a expression modulates the development of replicative senescence, we used Wnt3a-containing conditioned medium (Wnt3a-CM) that was harvested from Wnt3a-overexpressing L929 cells. MSCs were grown in Wnt3a-CM diluted either 1:1 or 1:2 with fresh growth medium. After 48 h, we observed a dose-dependent increase in β -catenin protein in the cell lysates, indicating the presence of the active form of Wnt3a protein in the conditioned medium (data not shown). Namely, Wnt3a-CM was diluted 1:1 with fresh growth medium and used to treat MSC-1 cells that correspond to intermediate- or late-stage senescence. With Wnt3a-CM supplementation, the cells appeared narrower and more spindle-like, similar to the morphology of early-stage cells. The number of SA- β -gal-positive cells decreased to 60% in intermediate cells and to 70% in late cells in comparison with control cells that were cultured in growth media (Fig. 3A). Cell viability and proliferation increased 1.5-fold for both stages in Wnt3a-CM-treated cells (Fig. 3B, C), and telomerase activity increased about 1.4-fold (Fig. 3D). Interestingly, late-stage

cells showed clearer changes in SA- β -gal activity, cell viability, and proliferation in comparison with intermediate cells, implying the senescence-delaying potential of Wnt3a in late-stage senescent cells. In addition, treatment with Wnt3a-CM induced an increase in phosphorylated pRb and a decrease in phosphorylated p53, p21, and p27 (Fig. 3E), which indicates that rescue from cell cycle arrest retards progression toward senescence.

Next, we continuously subcultured MSCs for P8 (PD41) to P13 (PD55) in Wnt3a-CM and recorded their PDs for 24 days. With Wnt3a-CM supplementation, the MSC cells improved their proliferation capacity in comparison with LacZ-CM and control cells, resulting in an increase in the cumulative PDs (Fig. 3F). These results demonstrate that exogenous Wnt3a regulates cell proliferation and the progression of senescence in senescent MSCs and cells that are becoming senescent.

Wnt inhibitor promotes senescence in UCB-MSCs

Wnt/ β -catenin signaling is inhibited by several groups of negative regulators that interfere with either receptor-ligand binding or intracellular signaling. Secreted frizzled-related peptides and Wnt inhibitory factor 1 compete with frizzled (FZD) for binding Wnt ligand [24,25]. Dkks and sclerostin antagonize canonical Wnt signaling by binding to LRP5/6 co-receptors and disrupting the formation of the FZD-LRP complex [26,27]. In our study, treatment with Dkk-1 induced flat and enlarged morphology in MSCs (Fig. 4A, left). The percentage of SA- β -gal-positive cells increased about 2.5-fold, showing a Dkk-1 dose-dependent response (Fig. 4A, right), whereas cell viability and proliferation gradually decreased depending on the Dkk-1 concentration (Fig. 4B, C). In particular, high-dose Dkk-1 treatment (100 μ M) reduced telomerase activity to 30% of the level of the control cells (Fig. 4D). This onset of senescence corresponded with the activation of the p53-p21 and pRb pathways (Fig. 4E). However, late cells appeared to be less affected by the suppression of Wnt signaling than intermediate cells (Supplementary Fig. S4). These results confirm previous findings that blocking Wnt/ β -catenin signaling accelerates cellular senescence with the degradation of β -catenin within MSCs.

β -catenin is an important factor in senescence progression in UCB-MSCs

We observed that regulating Wnt/ β -catenin signaling at the receptor level modulates the onset of senescence in MSCs. Next, we wondered whether the progression of senescence could be retarded by increasing the level of endogenous β -catenins. Activation of Wnt/ β -catenin signaling inhibits the glycogen synthase kinase 3 β (GSK-3 β)-mediated phosphorylation of β -catenin and prevents its subsequent degradation in the proteasome complex, allowing the stabilization of β -catenin and its translocation from the cytoplasm to the nucleus to drive the expression of Wnt target genes.

As shown in Figure 5A and Supplementary Figure S2A, MSCs that were treated with the GSK-3 β inhibitory compounds, SB-216763 and BIO, appeared narrow and spindle-shaped like the cells in the early stages of senescence. The number of cells showing positive SA- β -gal staining

FIG. 3. Wnt3a-conditioned medium delays senescence in MSCs. (A) Each PD of the MSC-1 cells was treated with control conditioned medium (LacZ-CM) or Wnt3a-conditioned medium (Wnt3a-CM), which was diluted 1:1 with fresh α -MEM medium. After 48 h, the cells were stained with SA- β -gal, and SA- β -gal activity was determined by counting SA- β -gal-positive cells (* P < 0.05; ** P < 0.01). (B–D) After 48 h of Wnt3a-CM treatment, cells were assessed for cell viability (B), cell proliferation (C), and telomerase activity (D) using the cell counting kit (CCK) assay, 5-bromo-2-deoxyuridine (BrdU) incorporation assay, or TeloTAGGG Telomerase PCR ELISA kit, respectively. All results are presented as the fold change relative to the control cells (* P < 0.05; ** P < 0.01). (E) The expression levels of β -catenin and senescence markers (pRb, p53, p21, and p27) were measured in MSC-1 cells (PD54) using immunoblotting analysis. β -Actin was used as the loading control. (F) MSC-1 cells were continuously passaged (P8–P13) in the presence of LacZ-CM or Wnt3a-CM, or in expansion medium (Ctrl). Cumulative population doubling was recorded as cells passaged.

decreased to about 63% at 1 μ M and 80% at 2.5 μ M in SB-treated cells and to about 25% at 1 μ M and 40% at 2.5 μ M in BIO-treated cells. In addition, both inhibitors improved viability and the proliferation potential of MSCs in a dose-dependent manner (Fig. 5B, C and Supplementary Fig. S2B, C). Following the stabilization of cytosolic β -catenin, this rescue from senescent status was consistent with the levels of pRb, p53-p21, and p27 expression that were subsequently observed (Fig. 5D and Supplementary Fig. S2D).

To verify the role of β -catenin in the progression of senescence, we downregulated β -catenin using siRNA during the intermediate stage of senescence. Compared with non-targeting siRNA-transfected cells, cells with siRNA against β -catenin were flatter (Fig. 6A) and showed increased size (FSC) and cell granularity (SSC) (Supplementary Fig. S3A). On the other hand, cell viability and proliferation were significantly reduced in β -catenin silenced cells (Fig. 6B, C), showing a corresponding change in pRb and p53 expression (Fig. 6D). However, we were unable to reproduc-

ibly detect a comparable increase in SA- β -gal activity in β -catenin siRNA-treated cells, although a dramatic increase was observed in the molecular characteristics of senescence, including SAHF that represses the expression of proliferation-promoting genes and the expression of SAHF marker H3K9 (dimet) (Supplementary Fig. S3B, C). Thus, we conclude that Wnt/ β -catenin is an important signal for senescence progression in MSCs. In the appropriate senescent state, modulating Wnt signaling can prevent the cell from succumbing to cellular senescence.

Discussion

The MSCs that are found in many adult tissues are an attractive source of stem cells with possible clinical therapeutic potential. One critical problem to overcome is how to obtain a sufficient number of cells with appropriate stemness to attenuate cellular senescence following extended ex vivo cultivation. Thus, we are trying to better understand the

FIG. 4. Inhibition of Wnt signaling promotes senescence in MSCs. **(A)** MSC-1 cells (PD48) were treated with the Wnt inhibitor dickkopf-1 (Dkk-1) (50 or 100 μM). After 48 h, cells were stained with SA-β-gal, and activity was determined by counting SA-β-gal-positive cells. **(B–D)** At 48 h after Dkk-1 treatment, MSC-1 cells were assessed for cell viability **(B)**, cell proliferation **(C)**, and telomerase activity **(D)** using the CCK assay, BrdU incorporation assay, or TeloTAGGG Telomerase PCR ELISA kit, respectively. Data are presented as the fold change relative to control cells (* $P < 0.05$; ** $P < 0.01$). **(E)** Immunoblotting analysis was used to detect β-catenin and senescence markers (pRb, p53, p21, and p27) in cells (PD33) that were treated with Dkk-1. β-Actin was used as the loading control.

practical feasibility of controlling the signals involved in the proliferation of undifferentiated MSCs. Studies report that active canonical Wnt signaling delays the senescence of primary fibroblasts and epithelial cells by stimulating cell division and inhibiting cell differentiation and apoptosis [23,28], although of the controversial report that persistent Wnt/β-catenin signaling contributes to MSCs aging through the DNA damage response and p53/p21 pathway [16]. Here, we described the recoverable potential of senescence in UCB-MSCs via Wnt3a/β-catenin signaling. Applying the Wnt3a ligand to the cells toward senescence retards the process and improves viability and proliferative potential. Likewise, stabilizing endogenous β-catenin by treatment with GSK-3β inhibitors also demonstrates similar effects with Wnt3a treatment, indicating that Wnt3a signaling expands the pool of MSCs capable of overcoming replicative senescence due to long-term cultivation. Conversely, blocking Wnt signaling with Wnt coreceptor antagonists or siRNA against β-catenin arrests growth and triggers the effectors of senescence and the expression of senescent-like hallmark such as the formation of heterochromatin foci. These results imply the

significance of Wnt3a/β-catenin signaling in coordinating senescence.

Interestingly, we found that the magnitude of senescence that is modulated by Wnt signaling is closely related to the senescent state of MSCs. In other words, delaying the progression of senescence by activating the Wnt3a pathway is more effective in late-stage MSCs with a high P number than in intermediate-stage cells (Fig. 3A–C). On the other hand, when the Wnt pathway is inhibited to promote the onset of senescence, early passage tends to accelerate senescent phenotypes in comparison with cells in later passages (Supplementary Fig. S4). Indeed, senescent MSCs express the others of secreted Wnts and signaling mediators that affect self-renewal and differentiation [13,29]. It has been reported that elevated Wnt5a expression in aged hematopoietic stem cells causes a shift from canonical to noncanonical Wnt signaling, thereby resulting in stem cell aging [30]. However, and more importantly, our current findings indicate that enforced or abundant Wnt3a/β-catenin in cells subjected to senescence is enough of a signal to overcome replicative senescence, presumably by directly

FIG. 5. Glycogen synthase kinase 3 β (GSK-3 β) inhibitor retards the progression of senescence in MSCs. (A–C) MSC-1 cells (PD48) were treated with the GSK-3 β inhibitor, SB-216763, at different concentrations (1 or 2.5 μ M). After treatment for 48 h, cells were stained with SA- β -gal, and the activity level was determined by counting SA- β -gal-positive cells (A, $*P < 0.05$). In parallel, cell viability (B) and cell proliferation (C) were determined by using a CCK assay or BrdU incorporation assay, respectively. Data are presented as the fold change relative to the control cells ($**P < 0.01$). (D) The expression levels of β -catenin and senescence markers (pRb, p53, p21, and p27) were measured over time using immunoblotting analysis. β -Actin was used as the loading control.

intervening in senescence programming in MSC cells. In line with our results, the recent observation of reduced nuclear β -catenin bioavailability in MSCs obtained from aged patients suggests that the abnormality are potentially recoverable, providing the clinical utility in aged patients [31]. In terms of autologous MSCs therapy, this evidence shows that Wnt3a/ β -catenin signaling is promising target for restoring the function of aged stem cells.

Previous mechanistic studies support our results linking canonical Wnt signaling to the emergence of cellular senescence. Canonical Wnt2 is repressed early in senescence as a signal for driving the formation of SAHF and the relocalization of the histone chaperone HIRA to PML (acute promyelocytic leukemia) nuclear bodies [20]. In addition, β -catenin directly regulates the expression of the telomerase subunit telomerase reverse transcriptase (TERT) and

FIG. 6. Silencing β -catenin triggers cellular senescence in MSCs. (A–C) MSC-1 cells (PD48) were transfected with nontargeting siRNA or β -catenin siRNA for 48 h. The morphology of the transfected cells was examined using microscopy (A). In parallel, cell viability (B) and proliferation (C) were measured using the CCK and BrdU incorporation assays, respectively ($*P < 0.05$; $**P < 0.01$). (D) The expression levels of β -catenin and the indicated senescence markers were measured using immunoblotting analysis. β -Actin was used as the loading control.

telomere length, which controls replicative senescence in MSCs [32]. Another study reported the unanticipated role of telomerase as a transcriptional modulator of the Wnt/ β -catenin signaling pathway, in addition to supporting stem cell self-renewal and survival by maintaining telomeres [33].

Although senescence programming is an unavoidable biological consequence of the ex vivo expansion of MSCs, we propose that this process can be modulated by the signaling factors involved in the Wnt3a/ β -catenin pathway. However, it is necessary to understand whether the senescence delayed by enforcing Wnt/ β -catenin signaling compromises either stemness or the subsequent differentiation of MSCs and to determine the mechanism by which the Wnt signaling pathway crosstalks with the pathways that govern cellular senescence in MSCs.

Indeed, adult stem cells can reside within hypoxic niches, in which the cells respond to low O₂ signals differently with normoxic culture condition [34]. Recently, we found that physiological hypoxic condition (3% O₂) could offset the benefited effects from Wnt3a-CM supplement on UCB-MSCs as evidenced by a reduction of cell proliferation and a decreased level of Wnt target genes and senescence regulators (p53 and pRb, unpublished data). Considering that ex vivo expansion of MSCs at low oxygen tension will be generally adopted in the development of cell therapies for future clinical use, our perspective on the practical effect of Wnt signaling to protect expanded MSCs from being senescent should be cautiously reinterpreted with respect to low oxygen tension.

In conclusion, our current findings suggest that secreted Wnt3a and its downstream pathway are promising therapeutic targets for modulating replicative senescence in MSCs under large-scale expansion culturing. Given its exquisite control of senescence in MSCs, the Wnt3a/ β -catenin signal system could be a rational approach for attenuating cellular senescence and thereby maintaining the function of undifferentiated stem cell populations.

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Author Disclosure Statement

The authors have no potential conflicts of interest to disclose.

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