Expression of human telomerase reverse transcriptase mediates the senescence of mesenchymal stem cells through the PI3K/AKT signaling pathway

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Abstract. Multipotent mesenchymal stem cells (MSCs) are widely used as seed cells in studies of tissue engineering and regenerative medicine; however, their clinical application is limited due to replicative senescence. It has been demonstrated that telomerase expression extends the lifespan and maintains the bone-forming ability of MSCs; however, the detailed role and the underlying molecular mechanisms in MSCs remain largely unknown. In the present study, we found that senescence was associated with human telomerase reverse transcriptase (hTERT) expression, and telomere length and telomerase activity. We established a short interfering RNA (siRNA) targeting hTERT and a gene expression vector carrying hTERT and transfected these into the MSCs to investigate the detailed role and the underlying molecular mechanisms of action of hTERT in MSCs. We found that the downregulation of hTERT by siRNA markedly decreased telomere length and telomerase activity in the MSCs, whereas the overexpression of hTERT increased telomere length and telomerase activity in the MSCs. The downregulation of hTERT inhibited cell proliferation and promoted the senescence and apoptosis of MSCs, whereas the upregulation of hTERT increased cell proliferation and decreased the senescence and apoptosis of MSCs. Of note, we also found that the activation of the PI3K/AKT signaling pathway was mediated by hTERT and that blocking this pathway using LY294002 inhibited hTERT expression, induced senescence and decreased the proliferation of MSCs. These findings reveal a previously unknown regulatory mechanism of hTERT, indicating that hTERT mediates the senescence of MSCs through the PI3K/AKT signaling pathway.

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Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells derived from the mesoderm, and possess potent proliferative potential, and the capacity for self-renewal and multilineage differentiation (1-3). MSCs grown *in vitro* are capable of differentiating into a number of cell types including osteoblasts, chondrocytes and adipocytes (4,5). MSCs are of great interest to researchers for their potential applications in the field of regenerative medicine and tissue engineering (6). Prolonged passaging of the *in vitro* culture environment is a prerequisite for acquiring a suitable number of MSCs for use in cell therapy. The process may, however, lead to adverse effects on the physiological properties of MSCs, such as stemness, proliferation and differentiation potency (6). In addition, during long-term *in vitro* culture, MSCs easily develop cellular senescence, consequently further limiting the number of cell doublings (7). It is therefore important to understand the mechanisms of the senescence of MSCs to reverse or prevent the aging processes in these cells.

In normal somatic cells, each cell division is associated with the shortening of telomeric DNA at the end of each chromosome, which leads to the cessation of replication, and the eventual arrest of cell growth and proliferation (8-10). The activation of telomerase is responsible for extending telomere length at the end of chromosomes, which helps prevent telomere erosion and inhibit replicative senescence *in vitro* (11,12). Human telomerase reverse transcriptase (hTERT) is a catalytic subunit of human telomerase (13,14), which provides the reverse transcriptase activity needed to maintain the length of the telomere (15). It has been shown that overexpression of hTERT increases the cell lifespan of ameloblastoma cells (16), human fibroblasts (17), adipose-derived stem cells (18) and endothelial cells (19) *in vitro*. Several studies have demonstrated that the modification of human MSCs with the hTERT gene generates cells with an improved ability for proliferation and cell renewal that retain their potential to differentiate into osteocytes, adipocytes,

chondrocytes and gingival epithelial cell lines (20-25). However, the detailed role and the underlying molecular mechanisms of action of hTERT in MSCs remain largely unknown. Therefore, in the present study, we examined the effects of hTERT expression on the proliferation, apoptosis and senescence of rat MSCs, as well as the underlying molecular mechanisms.

Materials and methods

Isolation and culture of MSCs. The MSCs used in the present study were obtained from 17- to 18-month old male Sprague Dawley rats (Tonghua Laboratory Animal Center, Beijing, China). The isolation and culture of the MSCs were performed as previously described (26,27). In brief, the MSCs were isolated from the bone marrow of the femurs and tibias of SD rats by inserting a 21-gauge needle into the shaft of the bone and flushing it with α-modified Eagle's medium (α-MEM; Invitrogen, Carlsbad, CA, USA) and cultured for 1-2 days. Non-adherent cells were then removed, and adherent cells representing MSCs were washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA). The cells were then incubated for 7-10 days in DMEM medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) to reach confluence and were extensively propagated for further experiments. The culture medium was replaced every 3-4 days and the cells were passaged at 70-80% confluence. The morphology of MSCs at passages 3, 7, 9 and 12 was observed under an IX51 inverted microscope (Olympus Corp., Tokyo, Japan). MSCs at passage 3 were harvested and resuspended in culture medium at a density of $1x10^6$ cells/ml. The surface markers of MCS were examined using flow cytometry and 4 antibodies against rat surface antigens (CD29, CD45, CD71 and CD90; BD Biosciences, San Jose, CA, USA).

Senescence-associated β-galactosidase (SA-β-gal) staining. The cells ($4x10^4$) were seeded into 6-well plates. 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 provided by the β-Galactosidase Reporter Gene Staining kit (Sigma-Aldrich) for 12 h at 37˚C, and the percentage of β-galactosidase-positive cells was then determined by randomly counting 5 fields on a phase contrast microscope (Olympus Corp.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the MSCs using TRIzol reagent (Invitrogen). The RNA was reverse-transcribed into cDNA using a PrimeScript™ RT Reagent kit according to the manufacturer's instructions (Takara Bio, Dalian, China). Quantitative PCR (qPCR) was carried out using SYBR-Green Real-Time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) and qPCR amplification equipment. The PCR reactions were prepared in duplicate and heated to 95˚C for 5 min followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 54˚C for 30 sec, and extension at 72˚C for 30 sec. Standard curves (cycle threshold values vs. template concentration) were prepared for each target gene and the endogenous reference (GAPDH) in each sample. The quantification of the samples was carried out using LightCycler software version 3.5 (Roche, Mannheim, Germany) with the $2^{-\Delta\Delta CT}$ method. The sequences of the hTERT and GAPDH primers were consistent with those of our previous study (28): hTERT forward, 5'-GGAGCAAGTTGCAAAGCATTG-3' and reverse, 5'-TCCCACGACGTAGTACATGTT-3'; GAPDH forward, 5'-TG TGGGCATCAATGGATTTGG-3' and reverse, 5'-ACACCATGTATTCCGGGTCAAT-3'.

Detection of telomerase activity and measurement of telomere length. Telomerase activity was determined using the telomeric repeat amplification protocol (TRAP) with the TeloTAGGG PCR enzyme-linked immunosorbent assay (ELISA) kit≈(Roche) according to the manufacturer's instructions.

Genomic DNA from the cultured cells was isolated using the High Pure PCR Template Preparation kit (Roche) and telomere length was estimated using the TeloTAGGG Telomere Length assay kit (Roche) as previously described (28).

Cell proliferation assay. The MSCs were seeded in a 96-well plate at a density of $1x0^4$ cells/well. Cell proliferation was determined at the indicated time points using the Cell Counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was read using an ELISA plate reader (Thermo Labsystems, Vantaa, Finland) at 450 nm.

Plasmid construction and transfection. The plasmid, pGCsilencer-siRNA-hTERT (pSi-hTERT), encoding siRNA specific to hTERT was constructed as previously described (28). The plasmid pCI-neo-hTERT (phTERT) containing the hTERT coding region was kindly provided by Professor Ximin Guo (Academy of Military Medical Sciences, Beijing, China). The plasmids, pSi-hTERT and phTERT, were transiently transfected into the MSCs at passage 9 (late passage) using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. The transfection efficiency was evaluated by RT-qPCR and western blot analysis following transfection with various plasmids. Untransfected cells were used as controls in this experiment and the following experiments.

Western blot analysis. The cells were harvested by centrifugation, and the cell pellet was resuspended in lysis buffer (Sigma-Aldrich) containing proteinase inhibitors and incubated on ice for 30 min. Following centrifugation at 14,000 x g for 30 min at 4˚C, the supernatant containing total cell extract was collected, and the concentrations of total cellular protein were determined using the Bradford protein assay (Bio-Rad Laboratories, Marnes-la-Coquette, France). Equal amounts of protein (20 μg) were separated by 10% gradient sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, CA, USA) using the Trans-Blot Turbo Transfer system (Bio-Rad Laboratories). The membranes were incubated in blocking buffer (TBST containing 5% skim milk) for 1 h at room temperature to block non-specific protein binding and then incubated with the following primary antibodies overnight at -4˚C: anti-hTERT (1:2,000; Cat. no. Sc-7204; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-GAPDH (1:5,000; Cat. no. 3683), anti-PI3K (1:2,000; Cat. no. 4249), anti-phosphorylated (p)-PI3K (Tyr458, 1:1,500; Cat. no. 4228), anti-AKT (1:1,000; Cat. no. 2920) and anti-p-AKT (Ser473;

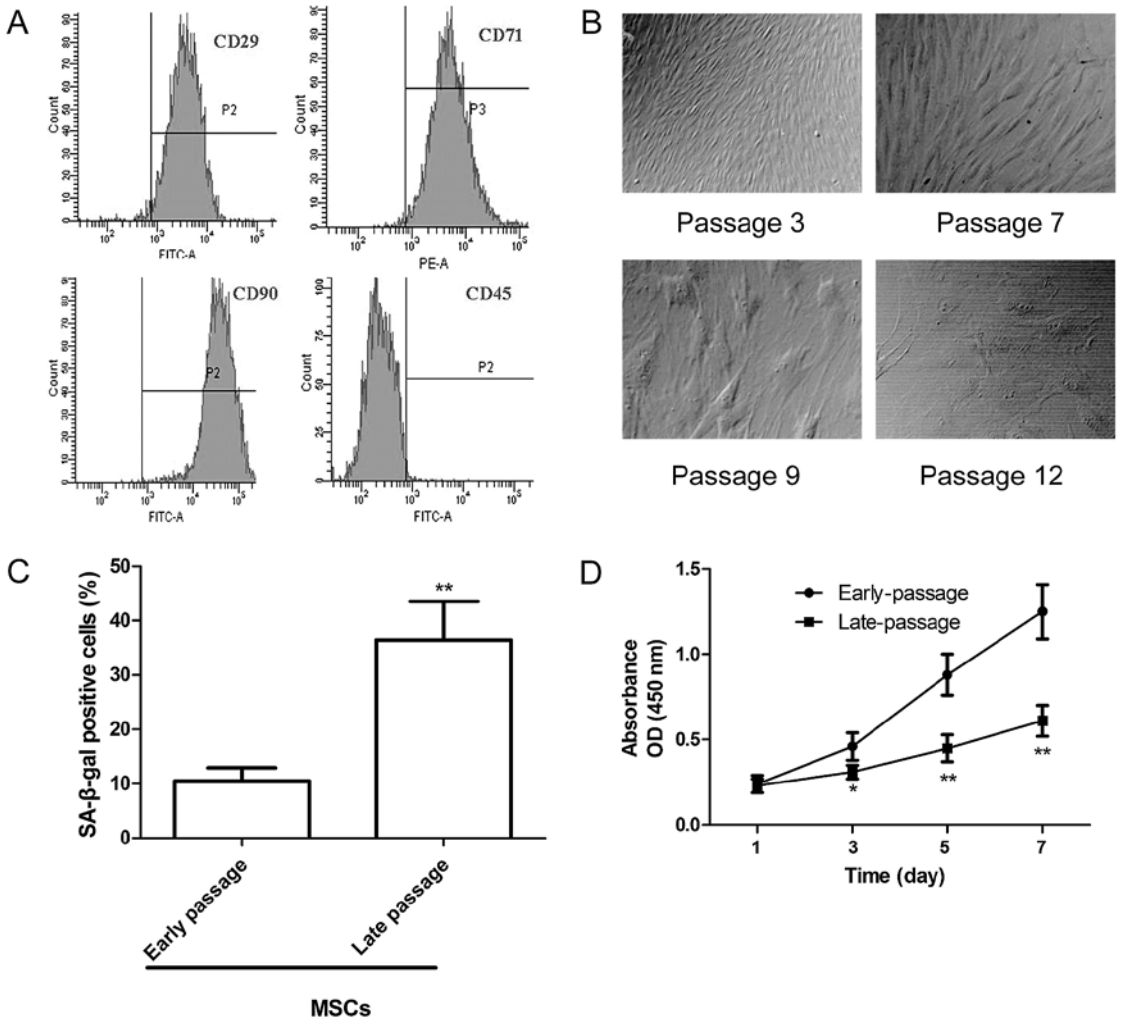

Figure 1. Characterization of mesenchymal stem cells (MSCs) at different passages. (A) Immunophenotypic characterization of MSCs by flow cytometry. The results indicated that the MSCs were positive for CD29, CD71 and CD90 but negative for CD45. (B) Morphology of MSCs at passages 3, 7, 9 and 12. (C) Early- and late-passage MSCs were stained with senescence-associated β-galactosidase (SA-β-gal) and the percentage of SA-β-gal positive cells was counted. (D) A cell proliferation assay (CCK-8) was performed on the early- and late-passage MSCs. *P<0.05, **P<0.01 vs. early-passage MSCs.

1:500; Cat. no. 4000; all from Cell Signaling Technology - New England Biolabs, Hitchin, UK). Following 3 washes with TBST, the membranes were incubated with goat anti-mouse IgG (1:5,000; Cat. no. Sc-2005) or goat anti-rabbit (1:5,000; Cat. no. Sc-2004; both from Santa Cruz Biotechnology, Inc.) horseradish peroxidase (HRP) diluted in blocking buffer for 1 h. Antibody binding was visualized using an enhanced chemiluminescence (ECL) western blotting detection system (Amersham Biosciences, Piscataway, NJ, USA). GAPDH was used for the normalization of protein loading and protein expression was measured by quantifying the density of immunoblots adjusted to GAPDH using image analysis software 3.1 Image J (Bio-Rad Laboratories).

Cell cycle analysis. The cells ($1x10^5$ cells/10-cm dish in diameter) were harvested by trypsinization, fixed with a fluorescence-activated cell sorting (FACS) lysing solution (BD Biosciences) and permeabilized with a FACS permeabilization solution (BD Biosciences). After being washed with PBS, the cells were resuspended in staining solution [containing 200 μg/ml RNase A and 20 μg/ml propidium iodide staining solution (Merck, Whitehouse Station, NJ, USA)], incubated for 30 min, and analyzed for their DNA content using a FACScan flow cytometer (BD Biosciences).

Apoptosis assay. The detection of apoptotic cells was performed on cytospin preparations using the TUNEL assay according to the manufacturer's instructions (*In Situ* Cell Death Detection kit, AP; Roche Molecular Biochemicals, Mannheim, Germany) after the MSCs at passage 3 were treated with the indicated plasmid. The number of apoptotic cells was counted under a IX51 inverted microscope (Olympus Corp.) and averaged from 3 visual fields.

Treatment with LY294002. LY294002, a PI3K inhibitor, was obtained from Sigma-Aldrich. MSCs at passage 9 (late passage; $5x10^4$ cells/well) were seeded into each well of a 24-well plate. Subsequently, 0.6 μM LY294002 was added and the cells were cultured for 48 h in DMEM (Invitrogen) supplemented with FBS at 37˚C in a humidified atmosphere containing 5% CO_2. PI3K, p-PI3K, AKT and p-AKT protein expression was then determined by western blot analysis.

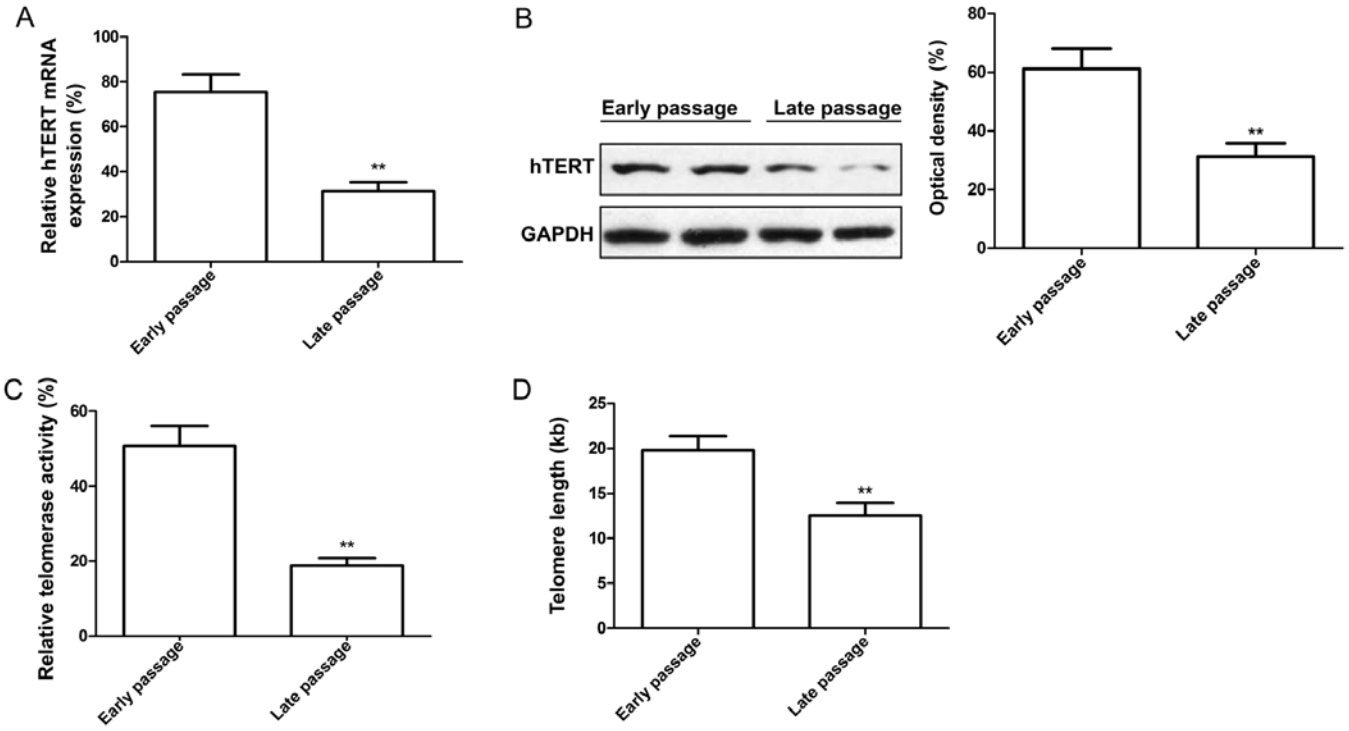

Figure 2. Human telomerase reverse transcriptase (hTERT) expression and telomerase activity and telomere length in early- and late-passage mesenchymal stem cells (MSCs). (A) mRNA expression levels and (B) protein expression levels were determined in early- and late-passage MSCs by RT-qPCR and western blot analysis, respectively. (C) Telomerase activity and (D) telomere length in early- and late-passage MSCs were measured. *P<0.05, **P<0.01 vs. early-passage MSCs.

Statistical analysis. Data are presented as the means ± standard deviation (SD). Comparisons between 2 groups were made using the Student's t-test. Statistical differences among more than 2 groups were assessed by one-way analysis of variance (ANOVA). GraphPad Prism software version 6.01 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Characterization of MSCs at different passages. High-purity MSCs were isolated and obtained by density gradient centrifugation, adherence selection and the monoclonal culture system. The immunophenotype of the MSCs obtained from the Sprague-Dawley rats was assessed by flow cytometry using specific cell surface antigens. The MSCs were positive for the expression of the MSCs markers CD29, CD71 and CD90 but negative for CD45 expression (Fig. 1A). In addition, the morphological characteristics of the MSCs at different passages were observed under an inverted microscope. As shown in Fig. 1B, at passage 3, the cells had a whirlpool or fish-like shape. At passage 7, the MSCs became larger in size with an enlarged rough endoplasmic reticulum, microvilli desquamate, with many secondary lysosomes, suggesting that the MSCs entered senescence. At passage 9, large parts of the microvilli disappeared and the enlargement of the rough endoplasmic reticulum was more obvious. At passage 12, the MSCs had lost their normal shape, and karyopyknosis and heterochromatin were observed. To ascertain alterations in the senescence of the MSCs at different passages, SA-β-gal activity was analyzed. Compared with the early-passage MSCs (passage 3), the percentage of SA-β-gal-positive cells and the staining intensity increased significantly in the late-passage MSCs (passage 9; Fig. 1C). In addition, we assessed the proliferation of MSCs at different passages by CCK-8 assay. We found that the proliferation of the late-passage MSCs decreased significantly compared with that of the early-passage MSCs (Fig. 1D). These data indicate an increase in the senescene of MSCs at a late passage.

Senescence-associated alterations in hTERT expression, and in telomerase activity and telomere length in MSCs. To determine whether hTERT expression is altered in MSCs at different passages, we measured the hTERT expression levels by RT-qPCR and western blot analysis. Our results revealed that not only was the mRNA expression level of hTERT in the late-passage MSCs lower than that in the early-passage MSCs (Fig. 2A), but the protein expression level of hTERT was also significantly decreased in the late-passage MSCs compared to the early-passage MSCs (Fig. 2B). In addition, we evaluated telomerase activity and telomere length in the early- and late-passage MSCs. We discovered that telomerase activity (Fig. 2C) and telomere length (Fig. 2D) were significantly decreased in the late-passage MSCs compared with the early-passage MSCs.

hTERT modulates telomere length and telomerase activity in MSCs. It has been demonstrated that telomere attrition triggers telomere dysfunction, induces DNA damage and, consequently, cellular senescence (29). We wished to determine whether hTERT expression regulates telomerase activity. For this purpose, the MSCs at passage 9 were transfected with the plasmids, pSi-hTERT (for the downregulation of hTERT), and

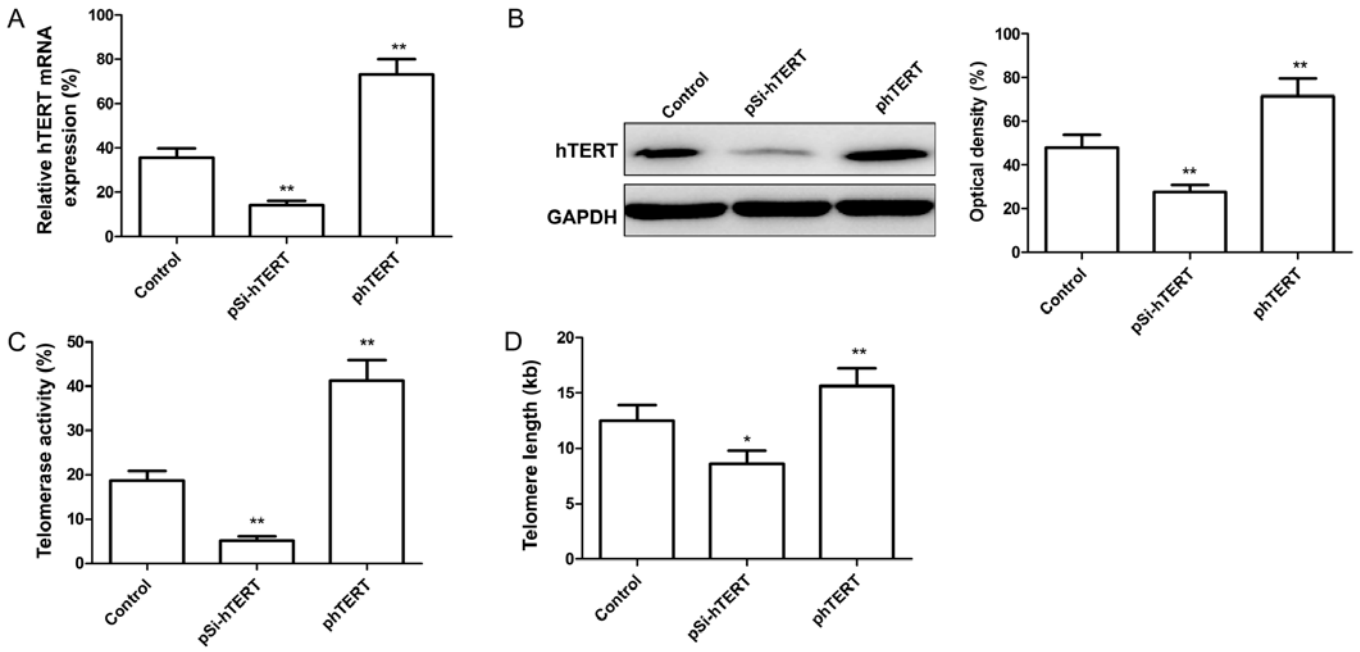

Figure 3. Human telomerase reverse transcriptase (hTERT) modulates telomerase length and telomere activity in mesenchymal stem cells (MSCs). (A) RT-qPCR analysis of hTERT mRNA levels and (B) western blot analysis of hTERT protein levels in MSCs at passage 9 following transfection with the plasmids, pSi-hTERT or phTERT. (C) Telomerase activity and (D) telomere length were determined in the MSCs at passage 9 following transfection with the plasmids, pSi-hTERT or phTERT. *P<0.05, **P<0.01 vs. control (untransfected cells were used as controls).

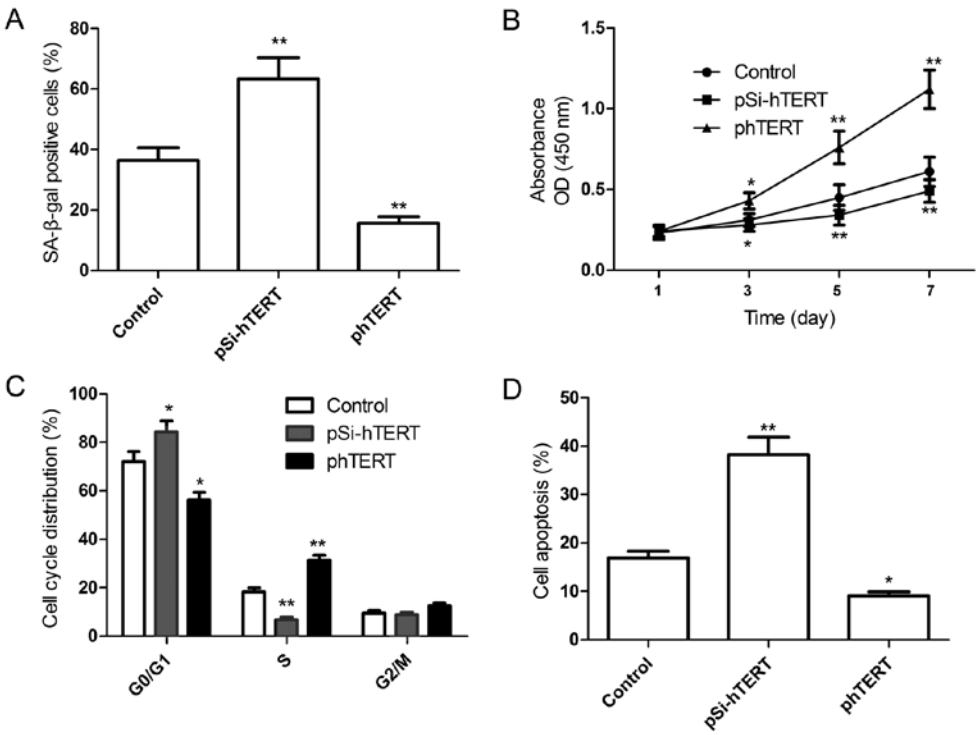

Figure 4. Human telomerase reverse transcriptase (hTERT) modulates the senescence, proliferation, cell cycle and the apoptosis of mesenchymal stem cells (MSCs). (A) Senescence, (B) cell proliferation, (C) cell cycle and (D) apoptosis were determined in the MSCs at passage 9 following transfection with the plasmids, pSi-hTERT or phTERT. *P<0.05, **P<0.01 vs. control (untransfected cells were used as controls).

phTERT (for the upregulation of hTERT). The hTERT mRNA and protein expression levels were then measured by RT-qPCR and western blot analysis, respectively. Our results revealed that the hTERT mRNA and protein expression levels decreased significantly following transfection with pSi-hTERT (Fig. 3A and B), whereas these levels significantly increased folloiwng transfection with phTERT (Fig. 3A and B). In addition, telomerase activity and telomere length in the MSCs were determined. We found that the downregulation of hTERT significantly reduced telomerase activity and telomere length in

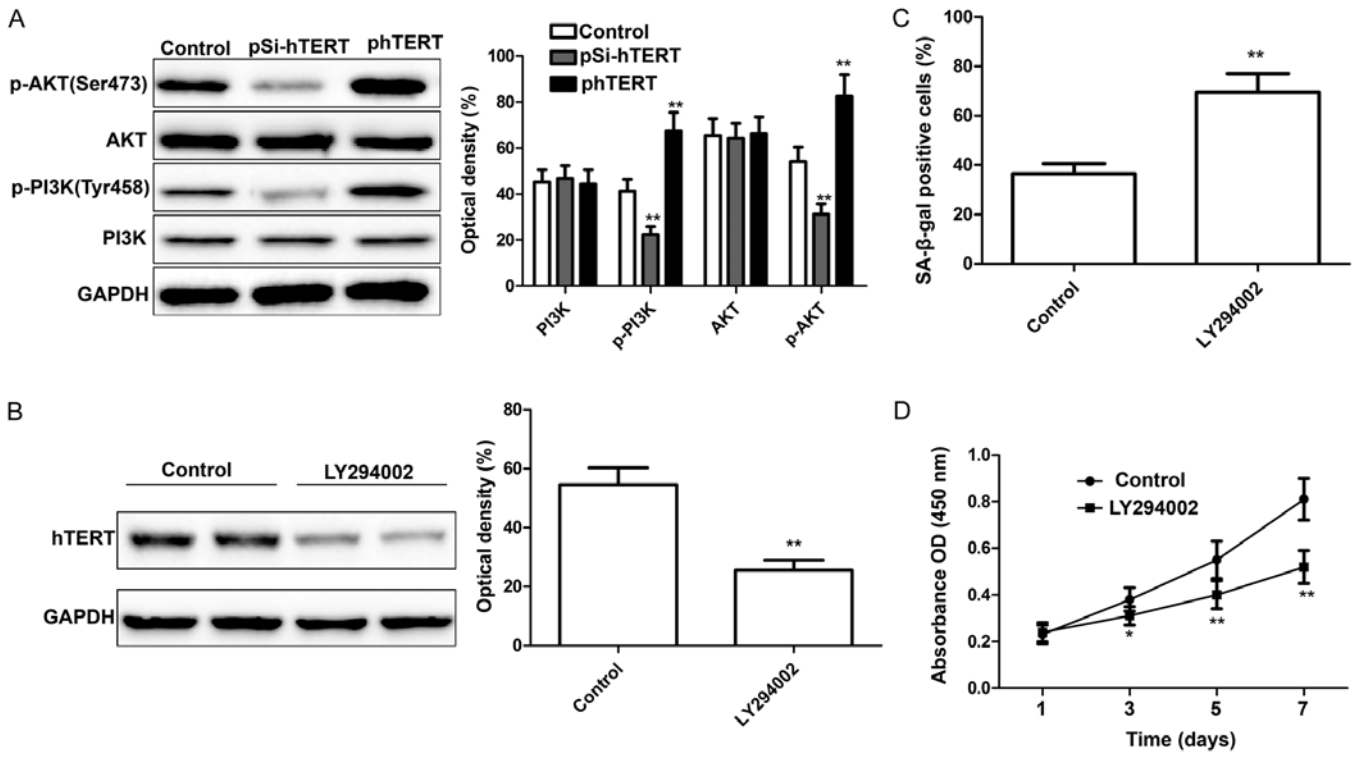

Figure 5. Human telomerase reverse transcriptase (hTERT) modulates the activatoin of the PI3K/AKT signal pathway in mesenchymal stem cells (MSCs). (A) PI3K, p-PI3K, AKT and p-AKT protein expression levels were determined in MSCs at passage 9 following transfection with the plasmids, pSi-hTERT or phTERT. (B) hTERT protein expression was determined in MSCs following treatment with LY294002. (C) Senescence and (D) proliferation of MSCs were determined following treatment with LY294002. *P<0.05, **P<0.01 vs. control (untransfected cells were used as controls).

the MSCs (Fig. 3C and D), whereas the upregulation of hTERT significantly increased telomerase activity and telomere length in the MSCs (Fig. 3C and D).

hTERT modulates the senescence, proliferation, cell cycle and apoptosis of MSCs. To further determine the role of hTERT in regulating the senescence, proliferation, cell cycle and apoptosis of MSCs, the plasmids, pSi-hTERT or phTERT, were transfected into the MSCs at passage 9 (late passage), and the senescence, proliferation, cell cycle and apoptosis of the MSCs were then determined. Compared with the control group, the downregulation of hTERT induced cellular senescence (Fig. 4A; shown by an increase in the number of SA-β-gal-positive cells), decreased cell proliferation (Fig. 4B) and the percentage of cells in the S phase (Fig. 4C) and increased the percentage of apoptotic MSCs (Fig. 4D). However, the upregulation of hTERT significantly decreased the number of senescent cells (Fig. 4A), increased cell proliferation (Fig. 4B) and the percentage of cells in the S phase (Fig. 4C) and decreased the percentage of apoptotic late-passage MSCs (Fig. 4D). Taken together, these results suggest that hTERT overexpression prevents the replicative senescence of MSCs.

hTERT modulates the activation of the PI3K/AKT signaling pathway in MSCs. It has been demonstrated that the PI3K/AKT pathway plays a role in stem cell self-renewal, maintenance and differentiation (30). In a recent study of ours, we demonstrated that the downregulation of hTERT inhibited the activation of the PI3K/AKT pathway (28). In the present study, we wished to determine whether hTERT modulates the activation of the PI3K/AKT pathway in MSCs. At 24 h following transfection with pSi-hTERT or phTERT, the expression levels of PI3K, p-PI3K, AKT and p-AKT were measured by western blot analysis. We found that the downregulation of hTERT significantly decreased p-PI3K and p-AKT expression in the MSCs, whereas the upregulation of hTERT significantly increased p-PI3K and p-AKT expression in the MSCs; no changes were observed in the levels of total PI3K and AKT in either group (Fig. 5A). To further determine whether the actiation of the PI3K/AKT signaling pathway is associated with the hTERT expression level in MSCs, the MSCs were treated with LY294002 (Sigma-Aldrich), a PI3K inhibitor, and the hTERT expression levels were then measured by western blot analysis. The results revealed that LY294002 significantly inhibited hTERT expression (Fig. 5B). In addition, the proliferation and senescence of the MSCs were determined following treatment with LY294002. The results revealed that treatment with LY294002 led to a significant increase in cellular senescence (Fig. 5C; as shown by an increase in the number of SA-β-gal-positive cells) and a decreased in the proliferation of MSCs (Fig. 5D). These findings suggest that hTERT mediates the senescence of MSCs through the PI3K/AKT signaling pathway.

Discussion

MSCs are one of the most promising resources for cell and gene therapy for osteogenesis imperfecta, the tissue engineering of cartilage and bone and post-transplant immune reconstitution due to their versatile plasticity *in vitro* and *in vivo* (32).

However, their clinical application and basic research is limited as primary MSCs have a limited lifespan. It is crucial to understand the mechanisms regulating cellular senescence, so that ways can be found to extend the lifespan of MSCs.

Cellular senescence is a complex process that, thus far, remains largely unknown. Previous studies have demonstrated that telomere attrition triggers telomere dysfunction, and induces DNA damage, leading to cellular senescence (29,33). Telomere attrition may thus be closely related to impaired telomerase activity and telomere length as the course of the telomeric DNA elongation is dependent on telomerase catalysis and may results in a reduction in telomerase activity in MSC (34). It has previously been demonstrated that if telomere shortening is not balanced by elongation, it leads to cell death, cellular senescence or abnormal cell proliferation (35). In the present study, we found that the long-term *in vitro* culture of MSCs led to cellular senescence, and telomerase activity and telomere length were both decreased in the late-passage MSCs compared with the early-passage MSCs. These findings suggested that telomeres play a key role in the senescence of long-term cultured MSCs.

It has been demonstrated that the ectopic expression of hTERT, the catalytic component of telomerase, leads to telomere elongation and extends the lifespan of a number of cell types (11-13). The upregulation of hTERT in MSCs has been shown to enhance their stem-like properties without affecting their potential to differentiate into osteocytes, adipocytes and chondrocytes (20-25). Consistent with these results, in the present study, we found that the downregulation of hTERT by siRNA markedly decreased telomere length and telomerase activity in the MSCs, whereas the overexpression of hTERT increased telomere length and telomerase activity in the MSCs. The downregulation of hTERT led to a decrease in the proliferation and an increase in the number of senescent and apoptotic MSCs, whereas the upregulation of hTERT led to an increase in cell proliferation and a decrease in the number of senescent and apoptotic MSCs.

It was well known that AKT (also known as PKB), a serine/threonine protein kinase, plays a central role in regulating cell survival, metabolism and protein synthesis through the phosphorylation of its numerous substrates (36). Previous studies have shown that AKT plays a role in the self-renewal, maintenance and differentiation of several types of stem cells, including pluripotent stem cells (31), neuronal stem cells (37) and epithelial stem cells (38). In addition, AKT is able to phosphorylate hTERT and activate telomerase activity (39). Two putative AKT phosphorylation sites within hTERT (serine residues at 227 and 824) have been identified. It has been reported that AKT enhances telomerase activity through the phosphorylation and nuclear translocation of hTERT (40,41). In the present study, we found that the downregulation of hTERT significantly inhibited p-PI3K and p-AKT expression in the MSCs, whereas the upregulation of hTERT significantly increased p-PI3K and p-AKT expression in the MSCs. This pathway was blocked with LY294002 (PI3K inhibitor), which led to a decrease in hTERT expression, increase in cellular senescence and a decrease in the proliferation of MSCs. Our findings reveal a previously unknown regulatory mechanism of hTERT; namely that hTERT mediates the senescence of MSC through the PI3K/AKT signaling pathway.

In conclusion, in the present study, we demonstrated that hTERT expression, telomerase activity and telomere length were decreased in late-passage MSCs compared to early-passage MSCs. The upregulation of hTERT in the late-passage MSCs prevented cellular senescence and apoptosis, increased cell proliferation, and increased telomerase activity and telomere length. We also demonstrated that the upregulation of hTERT activates the PI3K/AKT signaling pathway. and that the inhibition of the activation of the PI3K/AKT signaling pathway with LY294002 led to a decrease in hTERT expression, an increase in cellular senescence and a decrease in the proliferation of MSCs. These findings suggest that hTERT mediates the senescence of MSCs through the PI3K/AKT signaling pathway.

Acknowledgements

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