

Small concentrations of TGF- β 1 promote proliferation of bone marrow-derived mesenchymal stem cells via activation of Wnt/ β -catenin pathway

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Bone marrow-derived mesenchymal stem cells (BMSCs) are the most promising seed cells for cell transplant. The proliferation of BMSCs is one of the most important determinants of the efficiency of MSC-based transplant therapy. It has been reported that transforming growth factor- β 1 (TGF- β 1) activates Wnt/ β -catenin signaling and regulates cell proliferation. In this study, we investigated the effect of low concentrations of TGF- β 1 on proliferation of BMSCs and the related mechanisms. BMSCs were treated with 0, 1, 5 and 10 ng/L recombinant mouse TGF- β 1 for 12 h. Cell proliferation was measured by cell counting and MTT assay, and the proliferation-related signals p53, Mdm2, Akt1, Wnt3, phospho-Akt and β -catenin were measured by quantitative polymerase chain reaction (qPCR) and/or Western blot. Our results showed that TGF- β 1 at low concentrations induced BMSC proliferation and expression of Mdm2, Akt1, phospho-Akt, Wnt3 and β -catenin, and inhibited p53 expression in dose dependent manner. Importantly, β -catenin siRNA significantly inhibited TGF- β 1-induced BMSC proliferation. These findings suggest that low concentrations of TGF- β 1 can stimulate proliferation of BMSCs, which is at least partially dependent on the activation of Wnt/ β -catenin pathway.

Keywords: BMSC proliferation, p53 expression, Stem cells, Transforming growth factor- β 1

Bone marrow-derived mesenchymal stem cells (BMSCs) are a kind of multipotent cells capable of differentiating into many cell lineages, including osteocytes, adipocytes, cardiomyocytes, endothelial cells and neurons¹. Currently, they are widely used in regenerative medicine and tissue engineering. It has been reported that transplantation of BMSCs could treat many diseases such as neurodegenerative diseases, nervous system injury, stroke and heart failure²⁻⁵. Cell proliferative ability determines the efficiency MSC-based transplant therapy⁶.

Transforming growth factor- β (TGF- β) is a large family of cell regulatory proteins that control cell proliferation, differentiation, migration, apoptosis and other functions in many cell types⁷. TGF- β superfamily has more than 40 members including TGF- β , activins, inhibins, growth differentiation factors and other isoforms⁸. Three main isoforms are TGF- β 1, TGF- β 2 and TGF- β 3⁸. TGF- β 1, the firstly identified isoform of the TGF- β family, is the most

important one in regulation of cell growth and differentiation⁹. Several lines of evidence suggest that TGF- β 1 can inhibit proliferation of epithelial cells, cardiac fibroblasts, hematopoietic cells and cancer cells, but promotes proliferation of vascular smooth muscle cells, skin fibroblasts, endothelial cells, certain mesenchymal cells and other special cells¹⁰⁻¹². A recent study from our group showed that high concentrations of TGF- β 1 (1000~10,000ng/L) can induce senescence of BMSCs¹³.

Wnt/ β -catenin signaling pathway, a branch of a functional network, plays an important role in cell proliferation^{14,15}. Previous studies have demonstrated that the activation of Wnt/ β -catenin pathway stimulates proliferation of most cell types such as endothelial cells, cardiomyocytes, Sertoli cells, chondrocytes, cancer and other cells¹⁵⁻²⁰. Recent studies showed that TGF- β 1 can activate Wnt/ β -catenin signaling pathway in BMSCs and stimulate their differentiation^{21,22}. Recently, we demonstrated that β -catenin plays an important role in regulating proliferation of BMSCs²³. Here, we explored the hypothesis that low concentrations of TGF- β 1 may promote proliferation of BMSCs via activation of Wnt/ β -catenin pathway.

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Materials and Methods

Materials—Recombinant mouse transforming growth factor β 1 (mTGF- β 1) was purchased from R & D Systems (Minneapolis, MN, USA). RNeasy Mini-Kit, DNase I Kit, SuperScript II 1st Strand DNA Synthesis Kit and Lipofectamine®2000 Transfection Kit were obtained from Invitrogen (Carlsbad, CA, USA). The GoTaq SYBR Green Kit was purchased from Promega Corporation (Madison, WI, USA). FITC-conjugated CD90, PE-conjugated C44, phospho-Akt and β -actin antibodies were purchased from Abcam (Cambridge, MA, USA). Mdm2 and β -catenin antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). SignalSilence® β -Catenin siRNA II Kit was purchased from Cell Signaling Technology (Danvers, MA, USA).

Culture of BMSCs—BMSCs were obtained and cultured as previously described²⁴. In brief, BMSCs were isolated from mouse tibia and femoral and cultured in DMEM supplemented with 15% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 ng/mL streptomycin. A purified population of BMSCs was obtained after 3 wk culture. The third passage BMSCs were used in the following experiments.

Immunostaining—BMSCs were cultured in 24-well plates with 10 mm round cover slips for 24 h. The cells were fixed with 4% buffered paraformaldehyde for 15 min and incubated with 0.1% Triton X-100 in PBS for 10 min at room temperature (21–25°C). Then, the cells were blocked with 10% goat serum/1% BSA for 30 min for 1 h, and darkly incubated with PE-conjugated CD44 antibody for 1 h at room temperature. After washing with PBS, the cells were incubated with FITC-conjugated CD90 antibody for 1 h at room temperature in the dark. Cell fluorescence was observed under a fluorescence microscope.

Cell counting and MTT assay—Cell proliferation was measured by cell counting and MTT assay. For cell counting, BMSCs (2×10^3 /well) were plated in 24-well plates for 12 h, and then were treated with 0, 1, 5 and 10 ng/L TGF- β 1 for additional 12 h. After that, cell number was determined by cell counting. Similarly for MTT assay, BMSCs (1×10^3 /well) were plated into 96-well plates and cultured for 12 h, and then were treated with 0, 1, 5 and 10 ng/L TGF- β 1 for additional 12 h. After that, 10 μ L MTT reagent was added into each well and the plates were incubated for 2 h at 37°C; and then, 100 μ L detergent was added to each well and the plates were incubated for additional 2 h at room temperature in the dark. Absorbance was measured using a plate reader at 570 nm.

Quantitative polymerase chain reaction (qPCR)

The total RNA was isolated from the treated cells using a RNeasy Mini-Kit and cDNA was synthesized using a SuperScript II 1st Strand DNA Synthesis Kit according to the kits' instructions. Quantitative PCR (qPCR) was performed with a Fast 7500HT Real-Time PCR Instrument (Applied Biosystems, Foster, USA) using a GoTaq SYBR Green kit. Data was analyzed using the MX3000P software, and the relative mRNA expression was quantified using the comparative threshold cycle (Ct) method. The primers used for PCR reactions are shown in Table 1.

Western blot—BMSCs were treated with 0, 1, 5 and 10 ng/L TGF- β 1 for 12 h. Proteins were extracted from the treated cells and separated by 12% SDS-PAGE. After electrophoresis, proteins were transferred to the PVDF membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween-20 (TBS-T), and then incubated with Mdm2, phospho-Akt, β -catenin and β -actin primary antibodies (1:2000) at 4°C overnight. Then, the membranes were incubated with HRP-conjugated second antibodies (1:10000) for 1 h at room temperature. The immunoreactive bands were visualized by enhanced chemiluminescence.

Statistical analysis—Statistical analysis was performed with SPSS11.5 software. Data were presented as the mean $\pm$ standard deviation (SD). Univariate comparisons of means were evaluated using one-way ANOVA with Tukey's post-hoc adjustment for multiple comparisons or student's *t* test for two groups of comparison. Difference was considered statistically significant at $P < 0.05$.

Results

Morphology and identification of BMSCs—The first passage BMSCs assumed spindle and triangular shapes (Fig. 1A), and the third passage BMSCs

Table 1—Primers for qPCR

Primers	Sense	Sequence
Mdm2	Sense	5'-CGTGAAAGAGTTGAAGGAGG-3'
	Antisense	5'-GCATTTCAGGGAGAAGCTAGA-3'
Akt1	Sense	5'-AGCTCTTCTTCCACCTGTCT-3'
	Antisense	5'-GACACAATCTCCGCACCATA-3'
p53	Sense	5'-CACGTACTCTCTCCCTCAAT-3'
	Antisense	5'-AACTGCACAGGGCAGTCTT-3'
Wnt3	Sense	5'-AAGGACAAGTACGACAGTGC-3'
	Antisense	5'-CAAAAGTTGGGGGAGTTCTC-3'
GADPH	Sense	5'-AGGTCGGTGTGAACGGATTG-3'
	Antisense	5'-TGTAGACCATGTAGTTGAGGTCA-3'

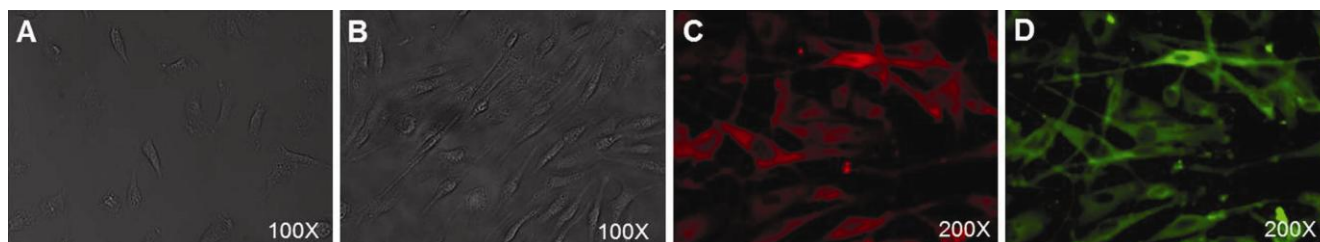


Fig. 1—Identification of mouse BMSCs. (A) Morphology of the first passage BMSCs; (B) Morphology of the third passage BMSCs; BMSCs were positive for (C) CD44 (red fluorescence); and (D) CD90 (green fluorescence).

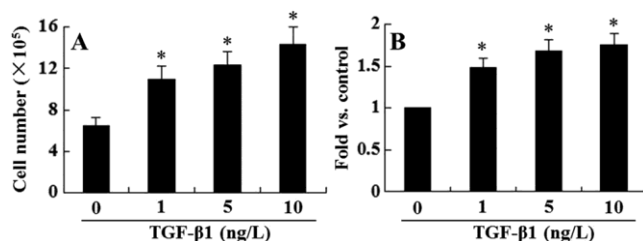


Fig. 2—Proliferation of BMSCs after exposure to different concentrations of TGF-β1 for 12 h. (A) Cell counting shows the cell numbers of BMSCs after exposure to 0, 1, 5 and 10 ng/L TGF-β1 for 12 h; (B) MTT assay shows cell viability of BMSCs after exposure to 0, 1, 5 and 10 ng/L TGF-β1 for 12 h. [Bar graphs represent mean ± SD (n = 5 per group). **P* < 0.05 vs. control.]

represented long-spindle and typical fibroblast-like shapes (Fig. 1B). Immunostaining showed that BMSCs were positive for CD44 (Fig. 1C) and CD90 (Fig. 1D). These data are consistent with previous reports by other groups²⁵.

Effect of TGF-β1 on proliferation of BMSCs—Cell counting showed that the cell numbers of BMSCs were significantly increased by treatments with 1, 5 and 10 ng/L TGF-β1 in a dose dependent manner (*P* < 0.05; Fig. 2A). This data was further confirmed by MTT assay, which also showed a marked increase of viability of BMSCs exposed to different concentrations TGF-β1 (*P* < 0.05; Fig. 2B).

TGF-β1 enhances expression of Mdm2 and Akt and represses p53 expression—The expression of cell proliferation-related genes P53, Mdm2 and Akt1 was measured by qPCR. As shown in Fig. 3, treatments with TGF-β1 (1-10 ng/L) markedly increased Mdm2 and Akt1 mRNA expression, as well as inhibited p53 expression in a dose dependent manner (*P* < 0.05). Western blot assay also showed that treatments with TGF-β1 markedly increased Mdm2 and phospho-Akt expression in BMSCs in a dose dependent manner (*P* < 0.05; Fig. 4).

TGF-β1 increases Wnt3 and β-catenin expression—Wnt-3 is an important member of Wnt/β-catenin signaling that stimulates cell proliferation²⁶.

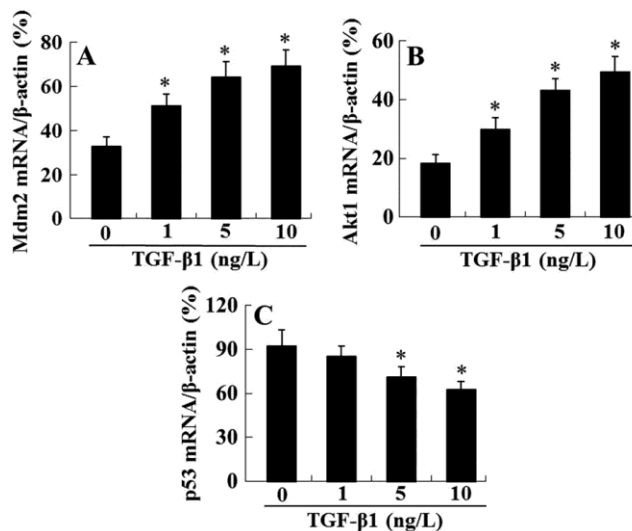


Fig. 3—Mdm2, Akt1 and p53 mRNA expression in BMSCs after exposure to 0, 1, 5 and 10 ng/L TGF-β1 for 12 h. (A) Mdm2 mRNA expression; (B) Akt1 mRNA expression; (C) p53 mRNA expression. [Bar graphs represent mean ± SD (n = 4 per group). **P* < 0.05 vs. control.]

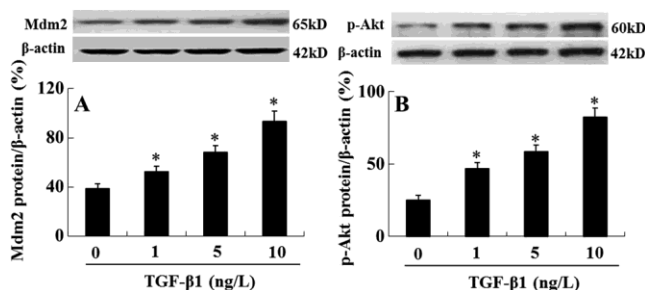


Fig. 4—Mdm2 and phosphor-Akt (p-Akt) protein expression in BMSCs after exposure to 0, 1, 5 and 10 ng/L TGF-β1 for 12 h. (A) Mdm2 protein expression; (B) phosphor-Akt protein expression. [Bar graphs represent mean ± SD (n = 4 per group). **P* < 0.05 vs. control.]

As shown in Fig. 5A, qPCR assay showed that treatments with TGF-β1 (1-10 ng/L) markedly increased mRNA expression of Wnt3 in a dose dependent manner (*P* < 0.05). Simultaneously, Western blot assay also demonstrated that TGF-β1 increased β-catenin protein expression (Fig. 5B).

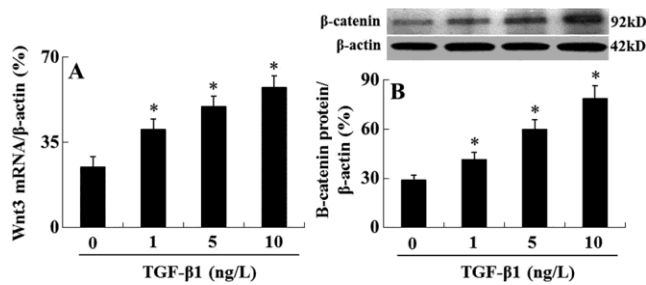


Fig. 5—Wnt3 and β -catenin expression in BMSCs after exposure to 0, 1, 5 and 10 ng/L TGF- β 1 for 12 h. (A) Wnt3 mRNA expression; (B) β -Catenin protein expression. [Bar graphs represent mean $\pm$ SD (n = 4 per group). * P < 0.05 vs. control.]

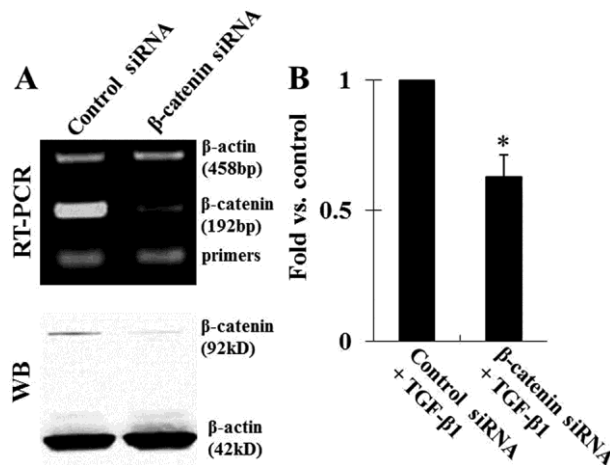


Fig. 6—Knockdown of β -catenin inhibits TGF- β 1-induced BMSC proliferation. (A) PCR and western blot assays show β -catenin mRNA (upper panel) and protein (lower panel) expression in BMSCs transfected with SignalSilence® β -Catenin siRNA (β -Cat-RNAi) or SignalSilence® Control siRNA (Con-RNAi); (B) MTT assay shows cell viability of BMSCs when the cells were exposed to 10 ng/L TGF- β 1 and transfected with β -catenin siRNA (β -Catenin siRNA + TGF- β 1) or control siRNA (Control siRNA + TGF- β 1). Bar graphs represent mean $\pm$ SD (n = 4 per group). * P < 0.05 vs. Control siRNA + TGF- β 1.

β -Catenin siRNA inhibits TGF- β 1-induced BMSC proliferation—To further confirm the role of Wnt/ β -catenin signaling in TGF- β 1-induced BMSC proliferation, we performed β -catenin siRNA. As shown in Fig. 6A, the levels of β -catenin mRNA and protein were much lower in BMSCs transfected with SignalSilence® β -Catenin siRNA as compared with the cells transfected with SignalSilence® Control siRNA (Fig. 6A). More importantly, the knockdown of β -catenin markedly inhibited TGF- β 1-induced proliferation of BMSCs (Fig. 6B).

Discussion

In this study, we found that low concentrations of TGF- β 1 has the potential to induce proliferation

of BMSCs. Furthermore, we also found that the pro-proliferative effect of TGF- β 1 on BMSCs is involved in the activation of Wnt/ β -catenin signaling pathway. However, knockdown of β -catenin markedly inhibited TGF- β 1-induced proliferation of BMSCs (Fig. 6).

BMSCs have been widely used in tissue regenerative medicine. TGF- β 1, as a growth factor, regulates proliferation of many cell types, though, its action differs among the cell types. Previous studies showed that TGF- β 1 can inhibit proliferation of epithelial cells, cardiac fibroblasts, hematopoietic cells, T-cells and a certain kinds of cancer cells, but promote proliferation vascular smooth muscle cells, skin fibroblasts, endothelial cells, mesenchymal cells and other special cells^{10-12,27}. In this study, we found that low concentrations of TGF- β 1 (1-10 ng/L) promote proliferation of BMSCs in a dose dependent manner (Fig. 2).

Akt is a key molecule for cell survival and participates in a series of cellular processes including cell metabolism, proliferation, differentiation, migration and apoptosis²⁸. Our results showed that Akt1 mRNA and phospho-Akt were both upregulated in BMSCs after exposure to 1-10 ng/L TGF- β 1 (Fig. 4).

p53, also known as cellular tumor antigen p53 or p53 tumor suppressor, is the most commonly mutated gene in human cancer. The expression of p53 negatively regulates cell proliferation by preventing progression of cells from G1 phase to S phase and causes an arrest of cell cycle in the G1 phase²⁹. The murine double minute 2 (Mdm2) serves as a negative regulator for p53 tumor suppressor activity²⁸. The Mdm2 binds p53 and block its expression and activation, and p53 gene expression also reversely inhibits Mdm2 expression³⁰. In this study, we found that expression of Mmd2 was markedly increased and p53 was decreased in BMSCs after exposure to TGF- β 1 @ 1-10 ng/L (Fig. 3).

Wnt/ β -catenin signaling pathway is also critical for cell proliferation^{14,15}. Akt is one of upstream signals of β -catenin, and its expression participates in regulation of β -catenin expression and activation^{31,32}. The Akt/Wnt/ β -catenin pathways play central roles in cell growth³³. Previous studies demonstrated that TGF- β 1 can initiate Wnt/ β -catenin signals via activation of PI3K/Akt pathway and regulate differentiation of BMSCs^{21,22}. In this study, we found that treatments with TGF- β 1 significantly

increase Wnt3 and β -catenin expression (Fig. 5). Knockdown of β -catenin markedly inhibited TGF- β 1-induced BMSC proliferation (Fig. 6). These data indicate that Wnt3/ β -catenin signals are involved in TGF- β -induced proliferation of BMSCs.

In summary, the present study clearly demonstrated that low concentrations of TGF- β 1 promote proliferation of BMSCs, which is involved in the activation of Wnt/ β -catenin signaling pathway. Our findings suggest that TGF- β 1 at low concentrations may serve as a potential reagent to improve BMSC survival prior to transplantation.

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