

Low levels of TGF- β 1 enhance human umbilical cord-derived mesenchymal stem cell fibronectin production and extend survival time in a rat model of lipopolysaccharide-induced acute lung injury

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Abstract. Mesenchymal stem cells (MSCs) are an attractive cellular source for cell-based therapy, tissue engineering and regenerative medicine. However, the use of MSCs is limited by their low incorporation rate in the graft environment. The majority of cells are lost from the graft within 1 month, due to reduced microenvironment or local inflammation at the graft site. The extracellular matrix (ECM) may assist the survival and expansion of MSCs. The present study aimed to identify an effective approach to increase ECM expression levels by MSCs in order to enhance the therapeutic effect and survival rate of MSCs at the injury site. The concentration-dependent effect of transforming growth factor (TGF)- β 1 on human umbilical cord (hUC)-MSC proliferation and expression of ECM genes was investigated. MSCs were successfully isolated,

cultured and expanded from hUC. A low concentration of TGF- β 1 (0.1 ng/ml) exhibited the optimal effect on hUC-MSC proliferation and markedly stimulated the expression of ECM genes, particularly fibronectin (FN). Furthermore, treatment with TGF- β 1 caused no alteration in the immunophenotype and differentiation capacity of MSCs. *In vivo* experiments in rats demonstrated that intravenous injection of control UC-MSCs or TGF- β 1-pre-treated UC-MSCs reduced the severity of lipopolysaccharide-induced lung injury, assessed using histology, measurements of the wet-dry lung weight ratio, and neutrophil count and protein concentration in bronchoalveolar lavage fluid. However, the short-term (48 h) therapeutic effects of untreated and TGF- β 1-pre-treated UC-MSCs were similar. The survival of MSCs in damaged lungs, determined by Sry gene expression levels, were significantly increased in MSCs pre-treated with TGF- β 1. In conclusion, pre-treatment of MSCs with a low concentration of TGF- β 1 enhanced the expression of ECM components, particularly FN, thus, improving the survival and potential therapeutic benefits of MSCs. Pre-treatment of MSCs with TGF- β 1 may prolong the effective therapy time and represent an efficient therapeutic approach for tissue repair.

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Abbreviations: ALI, acute lung injury; ALP, alkaline phosphatase; BALF, bronchoalveolar lavage fluid; BM, bone marrow; Col-1, collagen I; Col-IV, collagen IV; ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hUC-MSC, human umbilical cord mesenchymal stem cell; LN, laminin; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; SMC, smooth muscle cell; Sry, sex-determining region Y; TGF- β 1, transforming growth factor beta-1; TIMP, tissue inhibitor of metalloproteinase; UC, umbilical cord; VEGF, vascular endothelial growth factor

Key words: mesenchymal stem cells, transforming growth factor- β 1, extracellular matrix, fibronectin, acute lung injury

Introduction

Mesenchymal stem cells (MSCs) are an important resource for tissue repair and regeneration. The multilineage differentiation and immunomodulatory capabilities of MSCs make them highly useful for cardiac repair (1), improving marrow engraftment (2), treating graft-versus-host disease (3,4) and generating connective tissue elements (5-9).

MSCs also secrete various cytokines and angiogenic mediators that repair damaged tissues (10). A previous comparative study has indicated that the umbilical cord (UC) is an excellent alternative to bone marrow (BM) as a source of MSCs for cell therapy (11). UC-MSCs share numerous characteristics with BM-MSCs, including morphology, immunophenotype, cell cycle status, potential to differentiate and hematopoiesis-supporting functions (11-13).

Experiments in animal models have previously demonstrated that MSCs tend not to persist in the graft environment, they either do not incorporate well into the host tissue, or if incorporation occurs, the majority of the cells are lost within a month of adoptive transfer (1,14,15). The low incorporation rate of MSCs may be due to decreased cell viability caused by ischemia and loss of a suitable microenvironment, or local inflammation at the graft site (16). The extracellular matrix (ECM) may aid MSCs to survive and expand. Furthermore, chemotaxis and homing of MSCs are closely associated with ECM components. A previous study demonstrated that three ECM proteins, fibronectin (FN), vitronectin and collagen I (Col-I), induce significant mitogenic activity in MSCs (17). The ECM also stimulates neuronal differentiation of MSCs and serves as a nerve-regenerative scaffold (18,19). However, despite its importance, the regulation of the functional activities of the ECM remains to be completely understood.

Transforming growth factor (TGF)- β 1 stimulates ECM synthesis in cultured cells, including renal, vascular smooth muscle and type-II pulmonary epithelial cells (20-22). TGF- β 1 is also involved in MSC proliferation (23). In BM-derived adult human MSCs, SMAD family member 3 (SMAD3)-dependent nuclear translocation of β -catenin is required for TGF- β 1-induced proliferation (24). Previous studies suggested that TGF- β 1 induces increased expression levels of vascular smooth muscle cell (SMC)-like ion channels and the differentiation of human adipose tissue-derived MSCs into contractile vascular SMCs (25).

The present study investigated the effect of TGF- β 1 on the proliferation of MSCs from human (h)UC-MSCs and the expression levels of ECM genes, including Col-I, Col-IV, FN, laminin (LN), integrin and tenascin-C. The effects of TGF- β 1 on the differentiation and cell phenotype of MSCs were also examined. An effective approach to increase ECM expression by MSCs was determined. A lipopolysaccharide (LPS)-induced rat model of acute lung injury (ALI) was selected to assess the survival and therapeutic benefit of TGF- β 1-treated MSCs.

Materials and methods

Generation and culture of UC-MSCs. UCs (n=10, male newborns, clinically normal pregnancies) were dissected and the blood vessels were removed. The remaining tissues were cut into small pieces (1-2 mm³) and placed in plates with α -minimum essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) 2 ng/ml vascular endothelial growth factor (VEGF; R&D Systems, Inc., Minneapolis, MN, USA), 2 ng/ml epidermal growth factor (EGF; R&D Systems, Inc.) and 2 ng/ml fibroblast growth factor (FGF; R&D Systems, Inc.). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. The culture medium was changed every 3-4 days. Adherent cells proliferated from individual explanted tissues 7-12 days after incubation. These cells were subsequently trypsinized using 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) and cultured at a density of 1x10⁴ cells/cm² in the medium described. The cells

were used at ≤ 5 passages. The present study was approved by the Human Research Ethics Committee of Qilu Hospital (Jinan, China). Informed consent was obtained from all donors.

Proliferation of MSCs in response to TGF- β 1. The proliferation of UC-MSCs at passage 4 was measured. The cells (6,000 cells/cm²) were seeded into 24-well culture plates and allowed to adhere. Different concentrations (0.1, 0.5, 1, 5, 10 and 20 ng/ml) of TGF- β 1 (R&D Systems, Inc.) were then added to the plates. The plates were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 24, 48 or 72 h. Proliferation was measured after these incubations using a Cell Counting kit-8 assay (Beyotime Institute of Biotechnology, Haimen, China). The average optical density values of triplicate measurements were calculated and used for comparisons.

Measuring mRNA expression levels of ECM and ECM-associated genes following TGF- β 1 treatment. MSCs were cultured in 6-well plates at a density of 3x10⁵ cells/well in 2 ml culture medium. Following cell adherence, the cells were incubated for 24 h in different concentrations of TGF- β 1 (0.1, 1 or 10 ng/ml). The total RNA was extracted from each sample using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to analyze the expression levels of ECM genes, including Col-I, Col-IV, FN, LN, integrin and tenascin-C, and ECM-associated genes, including matrix metalloproteinase (MMP)-1, MMP-2 and MMP-9, and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. cDNA was generated from the total RNA via RT using an Omniscript cDNA Synthesis kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Typically, 0.2 μ g total RNA was reverse transcribed in a 20 μ l final reaction volume containing the following components: 1X RT buffer, deoxynucleotide triphosphate mix (5 mM), RNase inhibitor (RNaseOUT; 10 U/ μ l; Invitrogen; Thermo Fisher Scientific, Inc.), oligo dT primers and 4 units Omniscript RT. The samples were incubated at 37°C for 60 min, and the resulting cDNA was stored at -80°C prior to RT-qPCR analysis using an ABI 500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR green I dye (Toyobo Co., Ltd., Osaka, Japan). The primers used are listed in Table I. All reagents and primers were obtained from Bioasi Co., Ltd. (Shanghai, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The expression of each gene was determined using the 2^{- $\Delta\Delta$ C_q} method. The qPCR conditions were as follows: 1 cycle at 95°C for 4 min; 1 cycle at 94°C for 15 sec; and 1 cycle at 60°C for 1 min; for a total of 40 cycles.

The data were analyzed using Sequence Detection software (version 1.4; Applied Biosystems; Thermo Fisher Scientific, Inc.). The data are presented as the mean \pm standard deviation of ≥ 3 independent experiments. mRNA expression is presented as the fold difference with respect to the untreated control groups and the control group values are set at a fold change equal a value of 1.

Cell surface antigen phenotyping. Based on the above experimental results, 0.1 ng/ml was the concentration of

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction.

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Product (bp)
Human GAPDH	GACAACTTTGGTATCGTGGA	AGGCAGGGATGATGTTCTGG	114
Col-I	TGACCGAGACGTGTGGAAC	GTCTCGTCACAGATCACGTCATC	90
Col-IV	GCTGTGGATCGGCTACTCTT	AAGCGTTTGCGTAGTAATTGC	158
Fibronectin	AAATTCTGTAGGCCGTTGGAA	CTTCTTGGTGGCCGTAAGTC	131
Laminin	CTTTCAAGACATTCCGTCCA	AACGAGGCCTCACAGTCATAG	106
Integrin	TGCCCTCCAGATGACATAGAAA	CCTTTGCTACGGTTGGTTACATT	80
Tenascin-C	CTGACATAACTCCCGAGAGC	CTGAGATATGGGCAGTTCGTT	150
MMP-1	TGATGGACCTGGAGGAAATCTT	AAAATGAGCATCCCCTCCAA	70
MMP-2	ACTGTGACGCCACGTGACA	CGTATACCGCATCAATCTTTTCC	88
MMP-9	TGCGTGGAGAGTCGAAATCTC	GTCTCGGGCAGGGACAGTT	70
TIMP-1	GCTGACATCCGGTTCGTCTAC	GGTTGTGGGACCTGTGGAAGT	70
TIMP-2	TGGATGGACTGGGTCACAGA	TTCTCTTGATGCAGGCGAAGA	70
Sry	GCGAAGTGCAACTGGACAAC	GCCTAGCTGGTGCTCCATTC	80
Rat GAPDH	GTTACCAGGGCTGCCTTCTC	GCCTAGCTGGTGCTCCATTC	152

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Col, collagen; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; Sry, sex-determining region Y.

TGF- β 1 most effective at promoting the proliferation of MSCs and ECM gene transcription in MSCs. Thus, all subsequent experiments assessing MSC gene expression used TGF- β 1 at a concentration of 0.1 ng/ml.

The cells were collected and treated with 0.25% trypsin. The cells were blocked with phosphate-buffered saline (PBS) containing 1% FBS for 10 min at room temperature in the dark. Subsequently, the cells were incubated with either fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies (single labeling experiments) in 100 μ l phosphate buffer, for either 15 min at room temperature or 30 min at 4°C, as recommended by the manufacturers. Antibodies against human cluster of differentiation (CD)45 (cat. no. 10894), CD31 (cat. no. 16284), CD34 (cat. no. 28906), CD29 (cat. no. 05556), CD44 (cat. no. 16070), CD73 (cat. no. 21589), CD90 (cat. no. 15421) and CD105 (cat. no. 17813) were purchased from AbD SeroTec (Raleigh, NC, USA) and used at a 1:10 dilution. Immunofluorescence was detected using flow cytometry (Guava easyCyte 6HT; EMD Millipore, Billerica, MA, USA) and the data were analyzed using Guava Incyte (version 2.8; EMD Millipore). Positive cells were counted and compared with the signal of corresponding immunoglobulin isotypes.

Differentiation of MSCs in response to TGF- β 1. To investigate the differentiation potential of MSCs in response to administration of TGF- β 1, MSCs at passage 4 were treated with 0.1 ng/ml TGF- β 1 for 24 h prior to inducing the differentiation of each lineage. The cell density was adjusted to 2×10^4 cells/cm², and the inducing medium was changed every 3-4 days for differentiation assays. The osteogenic differentiation medium consisted of low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 0.1 μ M dexamethasone, 50 mM β -glycerol phosphate and 0.2 mM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA).

Adipogenic differentiation medium consisted of high-glucose DMEM supplemented with 0.25 mM 3-isobutyl-1-methyl-xanthine, 0.1 μ M dexamethasone, 0.1 mM indomethacin (Sigma-Aldrich), 6.25 μ g/ml insulin (PeproTech EC Ltd., London, UK) and 10% FBS. Cells were maintained in regular culture medium served as a control.

Mineralized matrix was evaluated by fixing the cells with 4% formaldehyde and staining with 1% alizarin-red S (Sigma-Aldrich) solution in water for 10 min. Alkaline phosphatase (ALP) staining was performed using an ALP staining kit (Tuo Yang Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's recommendations. Briefly, the cells were fixed in 4% paraformaldehyde and incubated with a substrate buffer at room temperature for 30 min. For oil red O staining, the cells were fixed with 4% formaldehyde, stained with oil red O (Sigma-Aldrich) for 10 min, and then counterstained with Mayer's hematoxylin (Sigma-Aldrich) for 1 min. Then, images were obtained with a light microscope (Olympus BX53; Olympus Corporation, Tokyo, Japan) fitted with a digital camera (Olympus cellSens Standard; Olympus Corporation).

Immunocytochemical analysis of ECM expression levels following treatment of MSCs with TGF- β 1. For immunocytochemical analyses, the cells were cultured in 6-well plates at a density of 3×10^5 cells/well in 2 ml culture medium. Following cell adherence, the cells were incubated for 24 h with 0.1 ng/ml TGF- β 1. Subsequently, the cells were fixed in 4% paraformaldehyde and washed in 0.01 M PBS (pH 7.2-7.4). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in absolute methanol for 30 min. Prior to the immunocytochemical procedure, proteolytic treatment was applied using 0.4% pepsin in 0.01 M HCl for 30 min at 37°C. Non-specific binding was blocked using 5% goat serum (Beijing Kang Century Biotechnology Co., Ltd., Beijing, China). Primary

anti-Col-I (cat. no. ab34710), -Col-IV (cat. no. ab6586), -FN (cat. no. ab45688) and -integrin (cat. no. ab15459) antibodies (all obtained from Abcam, Cambridge, UK) were added to two wells of each group at 1:300 dilution, and allowed to bind for 1 h at 37°C. Peroxidase-conjugated secondary antibody (cat. no. CW0220S; Beijing Kang Century Biotechnology Co., Ltd.) were added for 30 min at room temperature. The cells were washed three times in PBS, incubated with diaminobenzidine chromogen (Biocare Medical, LLC., Concord, CA, USA) for 10 min at room temperature, and then counterstained with hematoxylin.

Western blot analysis. Following stimulation with 0.1 ng/ml TGF- β 1 for 24 h, the cells were washed twice with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 8.0); 150 mM NaCl; 1% Nonidet P-40; 0.5% deoxycholate and 0.1% sodium dodecyl sulphate (SDS)], containing protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentrations in cleared cell lysates were determined using a Bradford assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein extracts (3 μ g/ μ l) were subjected to 7% SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membranes (EMD Millipore). The membranes were blocked with 5% milk in Tris-buffered saline Tween-20 [20 mM Tris-HCl (pH 7.6); 137 mM NaCl and 0.1% Tween-20] at room temperature for 1 h. Subsequently, the membranes were incubated with the following primary antibodies in blocking buffer (1:1,000) at 4°C for 16 h: Monoclonal rabbit anti-RhoA (clone 67B9; cat. no. 2117), anti-SMAD3 (clone C69H7; cat. no. 9523), monoclonal rabbit anti-phospho-SMAD3 (Ser423/425; clone C25A9; cat. no. 9520) and anti-GAPDH (clone D16H11; cat. no. 5174), all from Cell Signaling Technology, Inc. Danvers, MA, USA. Following incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. CW0103S; Beijing Kang Century Biotechnology Co., Ltd.), immunoreactive bands were detected by electrochemiluminescence (Beyotime Institute of Biotechnology) and quantified using a C-DiGit blot scanner (model 3600; Li-cor, Lincoln, NE, USA) and Image Studio software (version 4.0; Li-cor).

Creation of the rat model of ALI and MSC transplantation. Female Sprague-Dawley rats (weighing 240–280 g) obtained from Shandong University Experimental Animal Centre (Jinan, China) were randomly divided into four groups (n=16/group) as follows: i) Control; ii) ALI/PBS; iii) ALI/MS; iv) ALI/MS + TGF- β 1. The animals were maintained on a standard 12-h light/dark cycle, at a constant temperature (22 \pm 2°C) and a relative humidity of 55 \pm 10% with free access to water *ad libitum* and standard laboratory rodent food. ALI was induced by intraperitoneal injection of LPS (10 mg/kg) from *Escherichia coli* O111:B4 (Sigma-Aldrich). After 1 h, the rats were administered with either MSCs (MSCs or MSC + TGF- β 1; 5 \times 10⁵ cells in 300 μ l PBS) or 300 μ l PBS (as the control) via injection into the tail vein. For the MSC + TGF- β 1 group, MSCs were stimulated with 0.1 ng/ml TGF- β 1 for 24 h prior to injection. At each time point (6, 24 and 48 h post-injection of LPS), three rats from each group were anesthetized by intraperitoneal

injection of sodium pentobarbital (50 mg/kg) and sacrificed to assess lung injury. All procedures were approved by the Animal Care and Use Committee of Shandong University (Jinan, China).

Collection of bronchoalveolar lavage fluid (BALF) and tissue samples. The rats were sacrificed, the trachea was isolated and the right bronchial tube was ligated. BALF was obtained by placing a 20-gauge catheter into the trachea, through which 3 ml cold PBS was flushed back and forth three times. BALF was centrifuged at 2,400 \times g for 20 min at 4°C. A counter (Beckman Coulter, Inc., Brea, CA, USA) was used to determine the total cell count in the resulting cell pellet. The cells were smeared with Wright-Giemsa stain to confirm the neutrophil percentage. The protein concentration of cell-free BALF from all groups was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories), and was used to indicate endothelial and epithelial permeabilities.

The right upper lung lobes were used for wet-dry analysis to quantify the magnitude of pulmonary edema. The lungs were placed into previously weighed microcentrifuge tubes and were subsequently weighed. Subsequently, tissues were desiccated under a vacuum overnight at 80°C and weighed again. The wet lung mass was divided by the dry lung mass to obtain the wet-dry ratio. The right middle lung lobes were stored in TRIzol at -20°C to detect male UC-MSC survival *in vivo*. The right lower lobes were used for histological evaluation. Paraffin-embedded lungs were cut into 5 μ m thick sections and were subsequently stained with hematoxylin and eosin for histological analysis.

Measurement of MSC survival in vivo. To measure male MSC survival *in vivo*, 27 female Sprague-Dawley rats were randomly divided into three groups (n=9/group) as follows: i) ALI/PBS, ii) ALI/MS; iii) ALI/MS + TGF- β 1. ALI was induced as described, and the rats were administered with either male MSCs (MSC or MSC + TGF- β 1; 5 \times 10⁵ cells in 300 μ l PBS) or 300 μ l PBS via injection into the tail vein. At each time point (days 1, 7 and 14 post-injection of LPS), three rats from each group were anesthetized and sacrificed, as previously, to assess MSC survival. The survival of male donor human MSCs, which expressed the sex-determining region Y (Sry) gene, in the female recipient rat lung was calculated using RT-qPCR. Different numbers of male MSCs (100, 50, 25, 10, 5, 1 and 0 \times 10³ MSCs/10 mg lung tissue) were added to female DNA (from rat lung tissue) as standards, to allow the construction of a calibration curve. Sry expression levels were quantified by normalizing the values relative to the rat housekeeping gene, GAPDH. The number of stem cells was calculated according to the calibration curve, which plotted the cycle threshold (Cq) of Sry expression against the number of serially diluted MSCs (26).

Statistical analysis. The data were analyzed using SPSS software (version 14.0; SPSS Inc., Chicago, IL, USA). Quantitative data are presented as the mean \pm standard deviation. Analysis of variance with Fisher's protected least significant difference as a post hoc analysis was used for multigroup comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Biological characteristics of UC-MSCs. Adherent cells with fibroblastic morphology were observed as early as 48 h using the tissue-explant adherent method (Fig. 1A). The cells formed a monolayer of homogeneous bipolar spindle-like cells with a whirlpool-like array within 2 weeks. Following several passages, adherent cells from the UC formed a monolayer of typical fibroblastic cells (Fig. 1B).

Effect of TGF- β 1 on UC-MSC proliferation. The concentration-dependent effects of TGF- β 1 on UC-MSC proliferation were further investigated (Fig. 1C). The results suggested that exposure to 0.1 ng/ml TGF- β 1 was optimal for enhancing UC-MSC proliferation from 24 h after treatment, with a significant effect on proliferation after treatment for 72 h compared with control ($P=0.0241$). Higher concentrations of TGF- β 1 demonstrated minimal effects on UC-MSC proliferation. Thus, all subsequent experiments investigating the effects of TGF- β 1 on MSCs utilized a TGF- β 1 concentration of 0.1 ng/ml, and the TGF- β 1 treatment group was denoted as MSC + TGF- β 1.

TGF- β 1 causes no effect on the UC-MSC phenotype. Flow cytometry results (Fig. 2) demonstrated that the UC-derived cells exhibited a similar immunophenotype to MSCs, including positive expression of stromal markers (CD29, CD44, CD73, CD90 and CD105), and negative expression of endothelial (CD31) and hematopoietic (CD34 and CD45) markers. Treatment with 0.1 ng/ml TGF- β 1 demonstrated no significant effect on the percentage of cells expressing each marker (Table II).

Osteogenic and adipogenic differentiation capacities of TGF- β 1-treated UC-MSCs. MSCs treated with TGF- β 1 retained the ability to differentiate into osteoblasts and adipocytes. When induced to differentiate under osteogenic conditions, the TGF- β 1-treated MSCs began to grow in clusters with increasing induction time, and the cells formed a mineralized matrix, confirmed by alizarin red staining (Fig. 3A). The majority of the cells became alkaline phosphatase-positive by day 14 (Fig. 3B). No mineralized matrix was observed in cells maintained in regular growth medium (Fig. 3E). The spindle shape of TGF- β 1-treated MSCs flattened and broadened after 1 week of adipogenic induction (Fig. 3C). Small oil droplets gradually appeared in the cytoplasm, and after 2 weeks, almost all cells contained numerous oil red O-positive lipid droplets (Fig. 3D). The observed morphological changes and staining characteristics indicated that treatment with TGF- β 1 caused no affect on the ability of UC-MSCs to undergo osteogenic and adipogenic differentiation.

Influence of TGF- β 1 on the mRNA and protein expression levels of ECM components and ECM-related molecules. The effect of TGF- β 1 on the mRNA expression levels of ECM components and ECM-associated molecules in cultured UC-MSCs was also investigated. RT-qPCR assays were performed following treatment of UC-MSCs with various concentrations of TGF- β 1 for 24 h. A low concentration of

Table II. Effect of TGF- β 1 on the immunophenotype of umbilical cord-derived cells.

Phenotype	MSC	MSC + TGF- β 1
CD29	97.31 \pm 3.04	97.55 \pm 2.43
CD31	2.79 \pm 0.75	2.12 \pm 1.50
CD34	0.69 \pm 0.47	0.72 \pm 0.52
CD44	79.10 \pm 4.70	89.96 \pm 7.80
CD45	3.19 \pm 2.01	3.62 \pm 0.87
CD73	94.96 \pm 6.11	95.09 \pm 2.42
CD90	90.20 \pm 2.83	94.24 \pm 2.32
CD105	66.45 \pm 5.68	62.70 \pm 7.39

Data represent the percentage of cells (mean \pm standard deviation) expressing each marker ($n=20$). MSCs were treated with 0.1 ng/ml TGF- β 1. TGF, transforming growth factor; MSC, umbilical chord-derived mesenchymal stem cell; CD, cluster of differentiation.

TGF- β 1 (0.1 ng/ml) significantly increased the expression level of five ECM genes (Col-I, Col-IV, FN, integrin and tenascin-C; $P=0.0387$, 0.0219, 0.0274, 0.0402 and 0.0336, respectively), whereas TGF- β 1 at 1 ng/ml significantly increased only Col-I expression ($P=0.0057$; Fig. 4A). Furthermore, higher concentrations of TGF- β 1 (1 and 10 ng/ml) significantly inhibited LN ($P=0.0004$ and 0.0023, respectively) and integrin ($P=0.0011$ and 0.0048, respectively) expression levels (Fig. 4A). Regarding the ECM-associated genes, including MMP-1, MMP-2 and MMP-9, the mRNA expression levels were maximally increased by TGF- β 1 at a concentration of 1 ng/ml (Fig. 4A). Treatment with 0.1 ng/ml TGF- β 1 significantly inhibited MMP-1 expression in UC-MSCs ($P=0.0012$). The mRNA expression levels of TIMP-1 and -2 were not significantly altered by TGF- β 1 at any concentration (Fig. 4A). Notably, high concentrations of TGF- β 1 (10 ng/ml) did not increase the mRNA expression levels of various ECM components and ECM-associated molecules, except MMPs.

Based on these data, a TGF- β 1 concentration of 0.1 ng/ml was selected to examine the effects of TGF- β 1 on ECM protein expression. The expression levels of four ECM components (Col-I, Col-IV, FN and integrin) were examined by immunocytochemistry prior to and following treatment with TGF- β 1 (Fig. 4B). UC-MSCs in the TGF- β 1 group maintained their morphology compared with the control group. MSCs in the untreated control group demonstrate no immunoreactivity toward Col-I, Col-IV or integrin. Furthermore, 0.1 ng/ml TGF- β 1 increased the protein expression levels of Col-I, Col-IV and, in particular, FN compared with the untreated control. However, TGF- β 1 failed to observably increase the protein expression of integrin.

TGF- β 1 upregulates RhoA expression and activates phosphorylation of SMAD3 in UC-MSCs. To further investigate the mechanisms underlying the effects of TGF- β 1 described above, the expression levels of RhoA and SMAD3, which are important genes in FN synthesis, were analyzed. Western blot analysis demonstrated that 0.1 ng/ml TGF- β 1 significantly

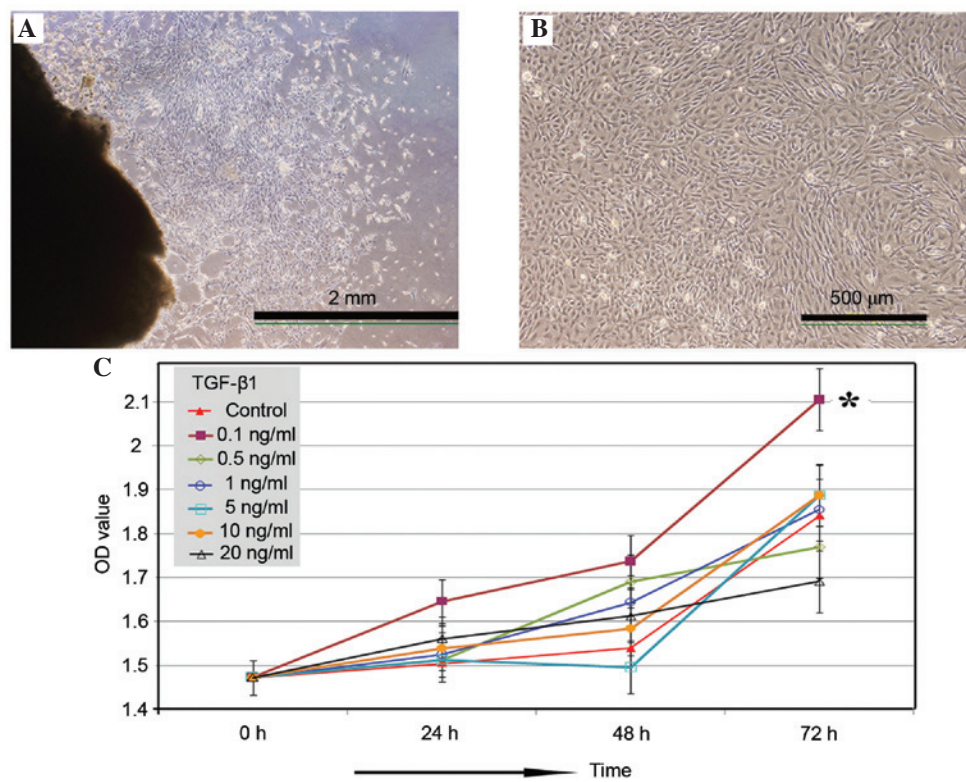


Figure 1. Morphology of hUC-MSC and the effects of TGF- β 1 on hUC-MSC proliferation. (A) From day 2, the cells began to grow out from the umbilical cord explant (Scale bar, 2 mm). (B) Umbilical cord-derived cells exhibited a bipolar spindle-shaped morphology and grew as a monolayer (Scale bar, 500 μ m). (C) The effects of TGF- β 1 at different concentrations on the proliferation of hUC-MSC. Growth was measured based on formazan dye production using a cell counting kit-8 assay, following incubation for 24, 48 and 72 h. The data are presented as the mean \pm standard deviation of three independent experiments (* P <0.05 vs. control). TGF; transforming growth factor; OD, optical density; hUC-MSC, human umbilical cord mesenchymal stem cells.

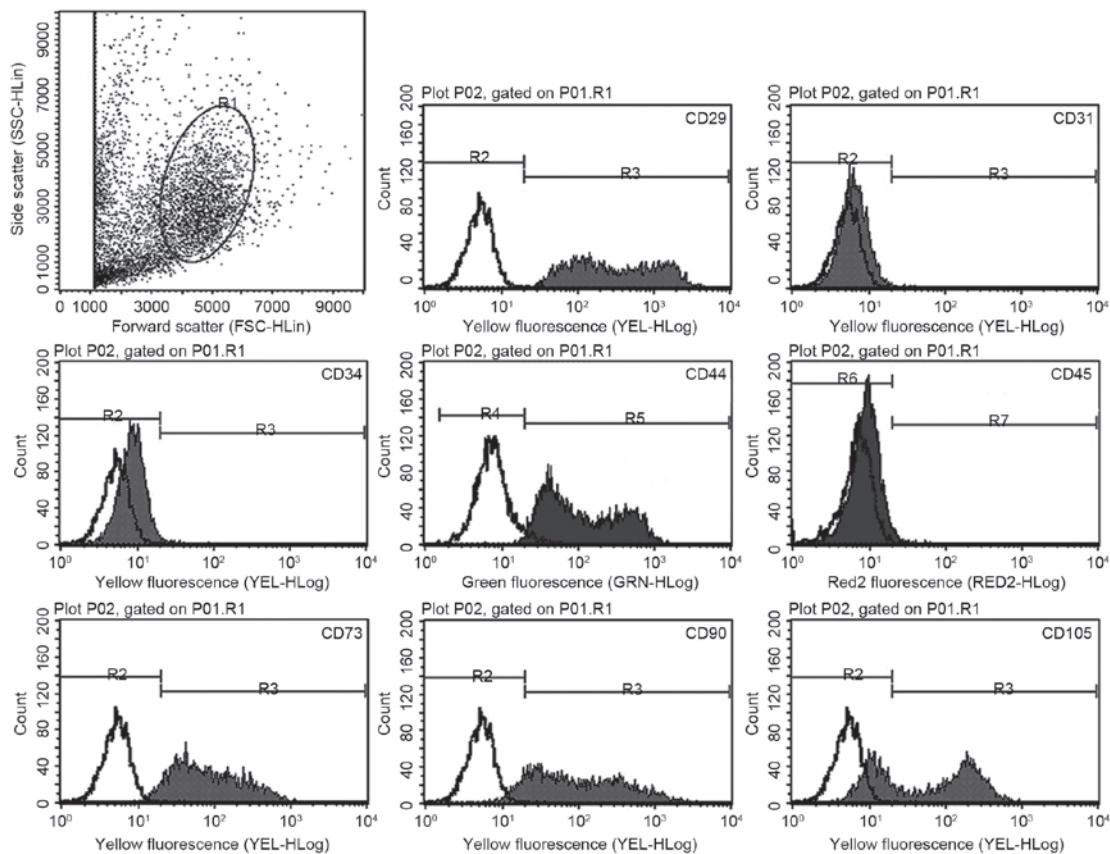


Figure 2. Immunophenotyping of umbilical cord-derived cells at the fifth passage by flow cytometry. The cells expressed CD29, 44, 73, 90 and 105, but not CD31, 34 and 45. CD, cluster of differentiation.

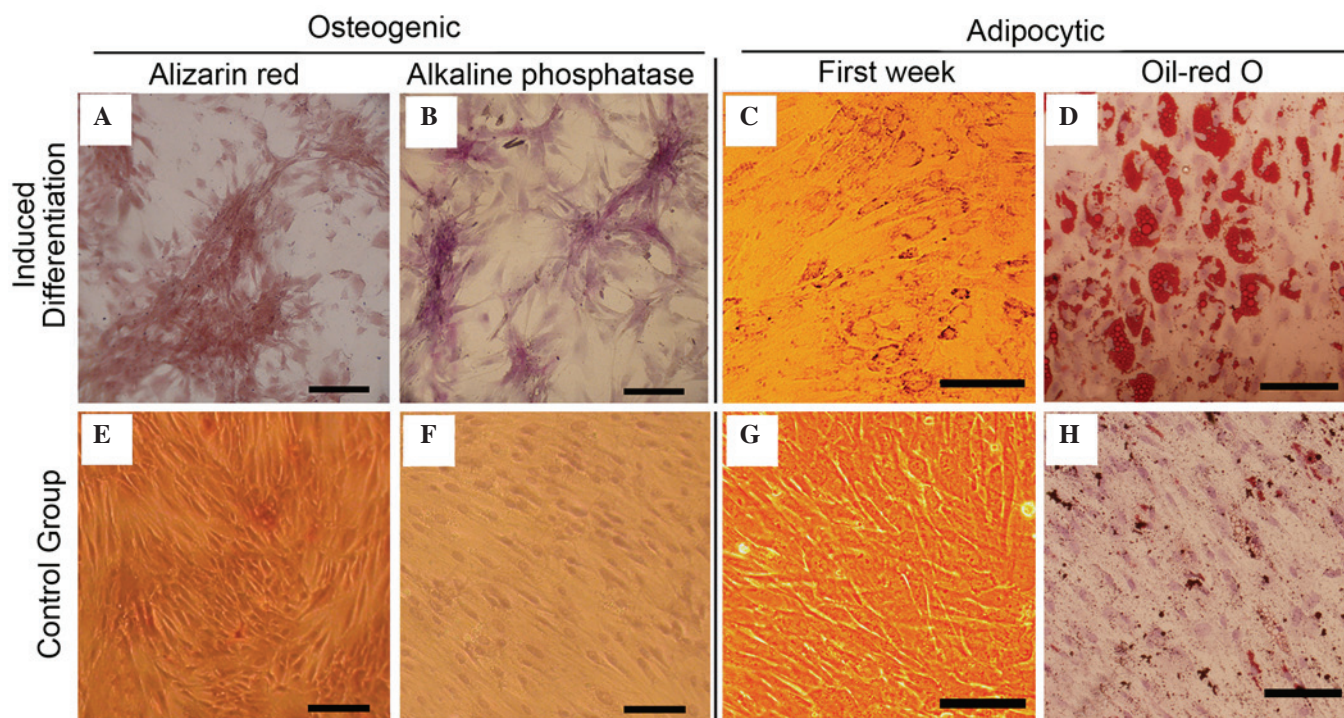


Figure 3. Osteogenic and adipogenic differentiation of umbilical cord mesenchymal stem cells treated with 0.1 ng/ml transforming growth factor β 1. Osteogenic differentiation was demonstrated by the formation of mineralized matrix shown by (A) alizarin red and (B) alkaline phosphatase staining (magnification, $\times 200$). Adipocytic differentiation was demonstrated by the formation of lipid vacuoles visible in (C) phase-contrast micrographs and (D) oil-red O staining. The corresponding controls for (E) alizarin red, (F) alkaline phosphatase, (G) phase-contrast imaging and (H) oil-red O staining are also presented (magnification, $\times 200$). All scale bars, 100 μ m.

increased the expression level of RhoA compared with control MSCs ($P=0.0215$), whereas the total expression level of SMAD3 was not significantly altered. However, the levels of phosphorylated SMAD3 were significantly higher in TGF- β 1-treated MSCs compared with the control MSCs ($P=0.0240$; Fig. 5).

Treatment with UC-MSCs attenuates LPS-induced systemic injury in rats. Animals receiving LPS exhibited physical signs of systemic illness, including lethargy, piloerection and diarrhea. Following intraperitoneal injection of LPS, the capillaries in the lung tissue expanded and became congested by a significant increase in neutrophils compared with untreated rats (Fig. 6A and B). Histological assessment of the lungs of animals injected with LPS demonstrated various pathological changes associated with ALI, including lung tissue hyperemia, hemorrhage, infiltration of inflammatory cells and neutrophil accumulation. These results indicated that the rat model of ALI was successfully developed. Additionally, the lung septa were observably thickened, and exhibited no improvement at the 48 h time-point (Fig. 6A and B). The MSC + LPS and MSC + TGF- β 1 + LPS groups also exhibited signs of moderate injury, however, the severity of the injury was observably reduced compared with the LPS alone group at the same time points (Fig. 6C and D).

The lung wet-dry weight ratio was significantly elevated at 24 and 48 h after LPS administration compared with the normal group ($P<0.05$), with the ratio marginally lower at 48 h compared with 24 h. However, the administration of MSC or MSC + TGF- β 1 significantly attenuated these increases

in lung wet-dry weight ratio at 24 h compared with the ALI group ($P=0.0148$ and 0.0102 , respectively), while at 48 h only MSC + TGF- β 1 administration significantly attenuated the wet-dry weight ratio compared with the ALI group ($P=0.0144$; Fig. 6E). LPS injection also resulted in significant increases in the BALF neutrophil count ($P=0.0207$, 0.0153 and 0.0119 for 6h, 24h and 48h, respectively; Fig. 6F) and protein concentration (an indicator of endothelial and epithelial permeability; $P=0.0298$, 0.0131 and 0.0243 for 6h, 24h and 48h, respectively; Fig. 6G) compared with the normal group, both of which peaked at 24 h. The increase in BALF neutrophil count at 24 h was significantly reduced in the MSC-treated group compared with the ALI group ($P<0.05$), and although the count was also numerically lower in the MSC + TGF- β 1-treated group, this did not reach statistical significance (Fig. 6F). Furthermore, BALF protein concentration at 24 h was significantly lower in the MSC-treated and MSC + TGF- β 1-treated groups compared with the ALI group ($P=0.0155$ and 0.0130 , respectively; Fig. 6G).

MSC survival in damaged lungs. To characterize the survival of transplanted MSCs in damaged lungs, a calibration curve was established to calculate the number of MSCs in the recipient lung compared with Sry gene expression (Fig. 7A). Cq values of the Sry gene were plotted against a semi-logarithmic scale of MSC number to obtain a balanced contribution of all reference dilutions. Subsequently, lungs (3/group) were harvested from rats at 1, 7 and 14 days after their respective treatment. The number of MSCs was calculated from the above calibration curve. The expression of the Sry gene

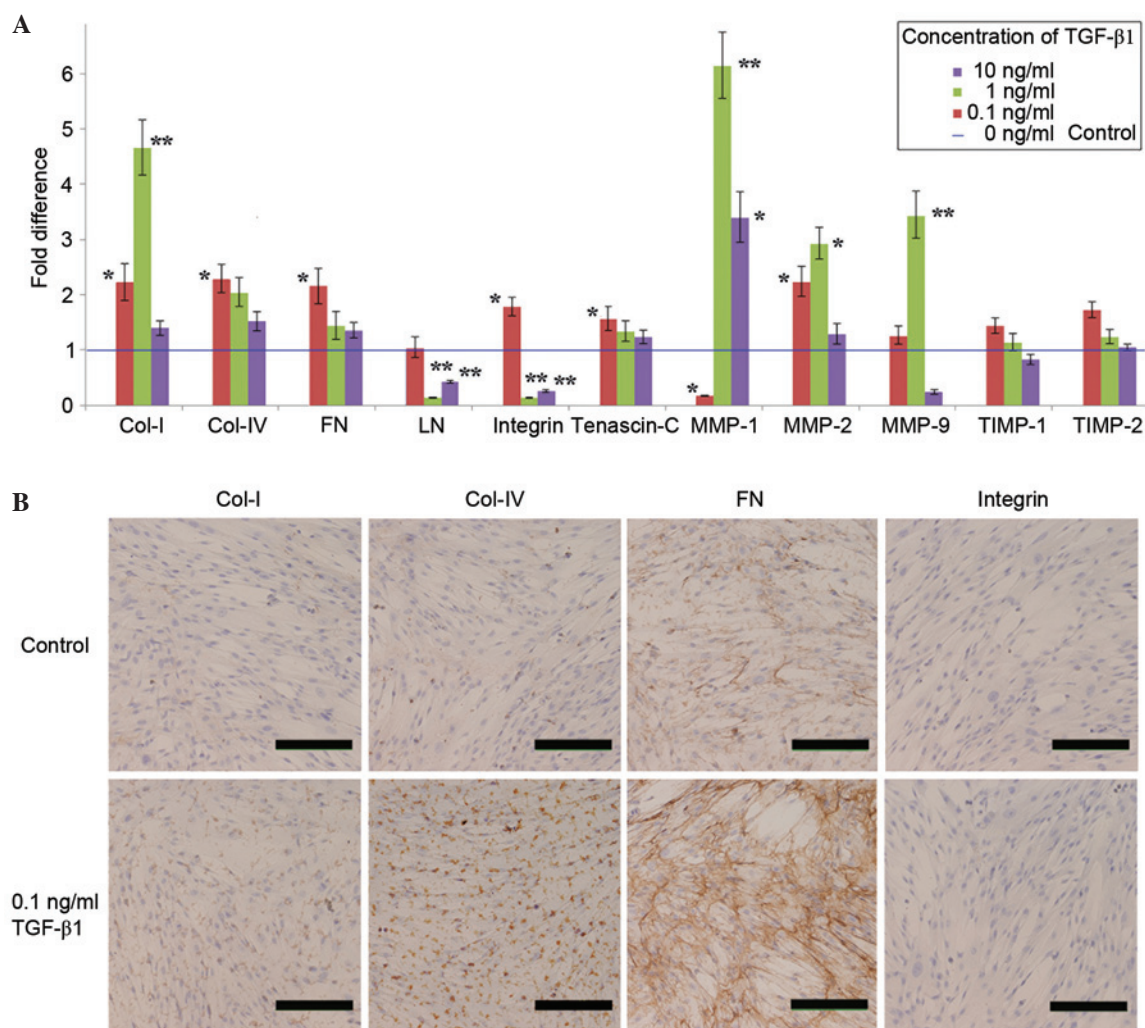


Figure 4. Effect of TGF- β 1 (0.1 ng/ml) on the expression levels of ECM components and ECM-associated molecules. (A) The mRNA expression is presented as the fold difference with respect to the control group, with the control values set at fold change=1 (blue line). The data are presented as the mean \pm standard deviation of ≥ 3 independent experiments (* $P < 0.05$, ** $P < 0.01$ vs. control). (B) The effect of TGF- β 1 (0.1 ng/ml) on the protein expression levels of Col-I, Col-IV, FN and integrin. As demonstrated by the increased brown-coloured staining, 0.1 ng/ml TGF- β 1 promoted the expression of Col-I, Col-IV, and particularly FN (scale bar, 200 μ m). TGF, transforming growth factor; Col, collagen I; FN, fibronectin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ECM, extracellular matrix.

in the lungs at each time-point was significantly higher in the MSC + TGF- β 1 group compared with the MSC group. TGF- β 1 pre-treatment (0.1 ng/ml) significantly increased MSC survival in damaged lungs at day 14 compared with untreated MSCs ($P = 0.0326$; Fig. 7B). Sry expression was not detected in the control animals that were administered with medium without MSCs.

Discussion

Numerous pre-clinical studies have demonstrated the potential of using MSCs for tissue repair in type-1 diabetes (27), ALI (28), radiation-induced injury (29) and nephropathy (30). However, the use of MSCs is limited by their rare occurrence in BM, with MSCs constituting only 0.001-0.01% of the BM population (31). Thus, MSCs require expansion prior to their use in tissue regeneration. A variety of growth factors, including hepatocyte growth factor, VEGF, platelet-derived growth factor, EGF and FGF, have previously been used to amplify MSCs (32). The present study successfully isolated,

cultured and expanded MSCs from hUC using three cytokines (EGF, VEGF and FGF). It was additionally demonstrated that 0.1 ng/ml TGF- β 1 promoted UC-MSC proliferation. This finding is in accordance with a previous study that reported that low levels of TGF- β 1 promotes MSC proliferation in the presence of fetal calf serum (33). TGF- β 1 inhibits the proliferation of the majority of cell types, including epithelial cells, endothelial cells, embryonic fibroblasts and hematopoietic cells (34). By contrast, TGF- β 1 stimulates the proliferation of mesenchymal cells (35). However, the mechanisms responsible for the effects of low TGF- β 1 concentrations on MSC proliferation remain to be fully elucidated. The results of the present study demonstrate that higher TGF- β 1 concentrations do not promote MSC proliferation. Thus, it is speculated that TGF- β 1 may stimulate different signaling pathways in MSCs, in a manner that is tightly regulated and concentration-dependent.

Subsequent experiments of the present study demonstrated that low doses of TGF- β 1 cause no effect on the phenotype of the UC-MSCs, with no significant changes demonstrated

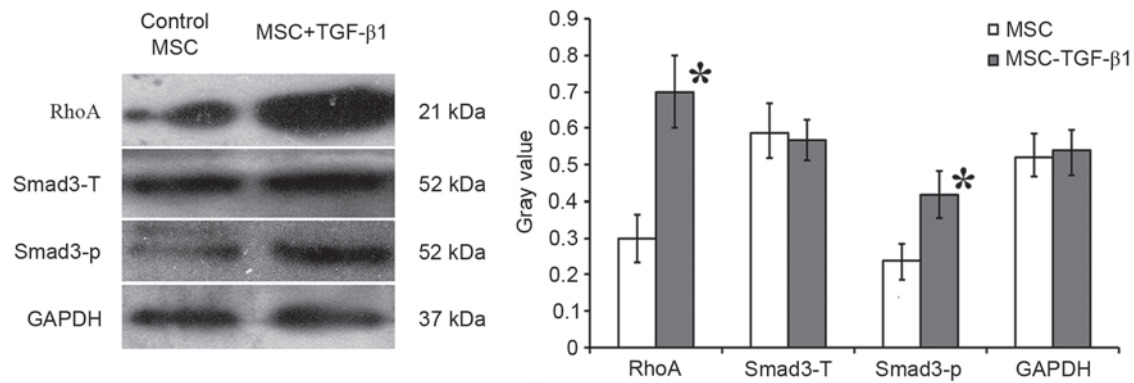


Figure 5. Treatment with TGF-β1 (0.1 ng/ml) upregulated the expression of RhoA and activated the phosphorylation of SMAD3 in umbilical cord mesenchymal stem cells. Upregulation of RhoA and phosphorylation of SMAD3 were demonstrated by western blot analysis (*P<0.05 vs. untreated MSC). No significant change in the expression of Smad3-T (Smad3-p plus unphosphorylated) was observed. MSC, mesenchymal stem cell; TGF-β1; transforming growth factor-β1; RhoA, ras homolog family member A; Smad3-T, total Smad family member 3; Smad3-p, phosphorylated Smad3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

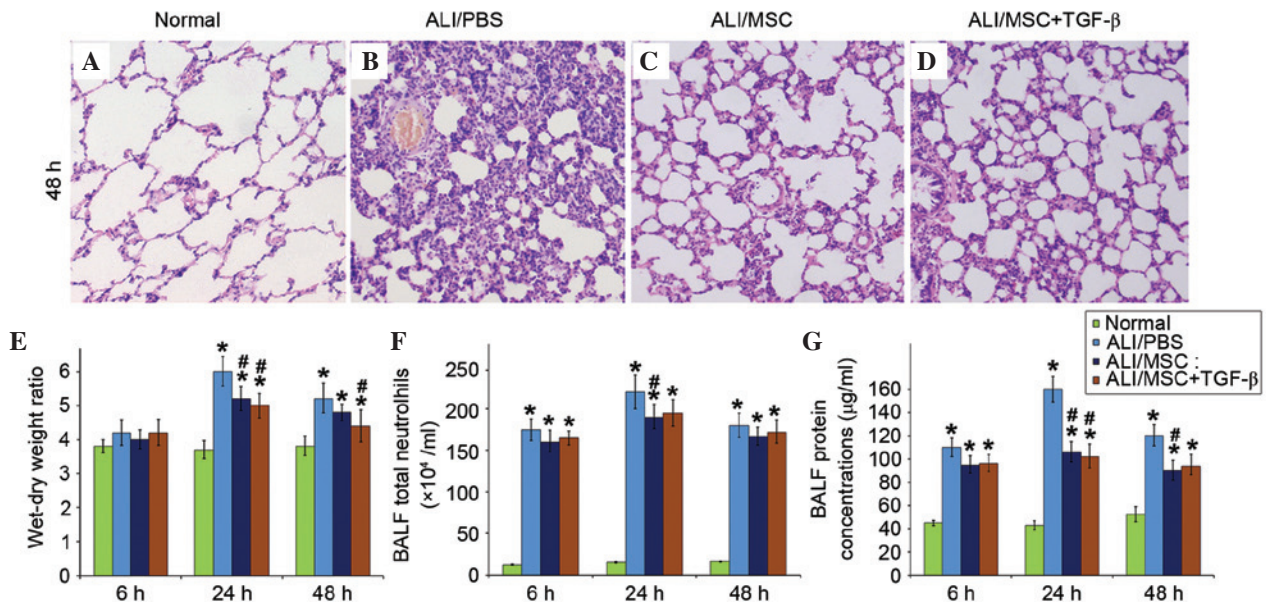


Figure 6. Injection of untreated MSC or MSC pre-treated with 0.1 ng/ml TGF-β1 attenuated LPS-induced lung injury in rats. The severity of lung injury was determined from assessments of lung oedema, lung wet/dry weight ratio, BALF neutrophil count and BALF protein concentration. Histological analysis of (A) normal, (B) ALI/PBS, (C) ALI/MSC and (D) ALI/MSC + TGF-β indicated that injection of LPS caused pulmonary capillary expansion and congestion, as well as neutrophil infiltration into the lung tissue (ALI/PBS group) at 48 h. In addition, the lung septa were noticeably thickened. Administration of MSC or MSC ± TGF-β reduced the severity of the lung injury at 48 h. (E) Injection of MSC or MSC ± TGF-β reduced pulmonary oedema induced by LPS. Pulmonary oedema was measured as the wet-dry weight ratio. (F) Neutrophil counts were higher in the LPS group compared with the control group. Treatment with MSC significantly reduced the LPS-induced increase in BALF neutrophil count at 24 h. The effects of MSC + TGF-β were not statistically significant, although the neutrophil count at 24 h was lower compared with that in the ALI/PBS group. (G) BALF protein concentration increased significantly after LPS injection, peaking at 24 h. Administration of MSC or MSC ± TGF-β significantly attenuated the LPS-induced increase in BALF protein concentration at 24 h. (*P<0.05 vs. normal healthy control group; #P<0.05 vs. ALI/PBS group). ALI, acute lung injury; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; MSC, mesenchymal stem cells; TGF, transforming growth factor; BALF, bronchoalveolar lavage fluid.

in any of the markers assessed. Similarly, the *in vitro* differentiation capacity of UC-MSCs was not affected by a low concentration of TGF-β1, with TGF-β1-treated UC-MSCs retaining adipogenic and osteogenic differentiation potential. Additionally, low-concentration TGF-β1-treated UC-MSCs were demonstrated to perform a function in cell therapy.

Further analyses in the present study demonstrated that a low concentration of TGF-β1 increases the mRNA expression level of several ECM components and their regulatory elements, including MMPs and TIMPs, in UC-MSCs. The

ECM is an intricate network of proteins surrounding cells and performs a central function in establishing the stem cell phenotype. The composition and mechanical properties of the ECM can affect cell shape and stem cell differentiation (36). The predominant fibrillar components of the ECM can be divided into two groups: Collagens and cell-adhesive glycoproteins (e.g. FN) (34). The PCR and immunocytochemical analyses of the present study demonstrated that the expression levels of Col-I, Col-IV and FN were enhanced by 0.1 ng/ml TGF-β1. Notably, FN exhibited the greatest upregulation in response to

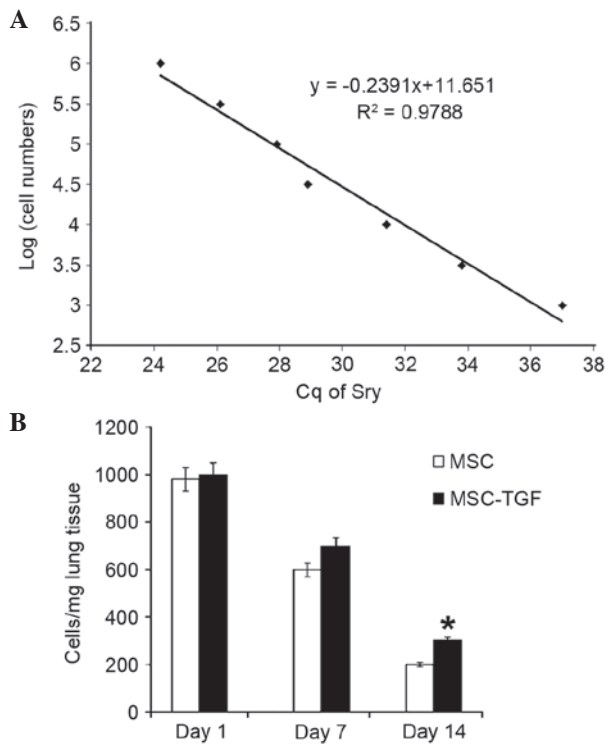


Figure 7. Survival time of transplanted MSCs in a rat model of lipopolysaccharide-induced ALI. (A) Calibration curve showing the association between Cq of the Sry gene and MSC number. (B) MSC or MSC \pm TGF- β 1 (pre-treated with 0.1 ng/ml TGF- β 1) number in the lungs of rats with ALI. (* $P < 0.05$ vs. normal MSC group). Cq, cycle quantification; Sry, sex determining region Y; ALI, acute lung injury; MSC, mesenchymal stem cell; TGF- β 1, transforming growth factor- β 1.

the low concentration of TGF- β 1. FN is a multidomain protein that contains binding sites for integrins, col and other ECM proteins (37,38). The multidomain structure of these proteins provides a mechanism for connecting cells to the ECM network. FN primarily supports mesenchymal cell migration during embryonic development (39). Additionally, FN signals support cell survival and growth (40), and may contribute to cell viability during differentiation. For example, hematopoietic stem cells express $\alpha 4 \beta 1$ integrin, which binds to FN and receptors on neighboring cells (41). Thus, this receptor can tether stem cells to the ECM and support cells within the BM microenvironment. Tenascin-C, a multimeric ECM protein, is also a stem cell marker present in the BM (42). Tenascin-C binds to FN and modulates cell adhesion (43). Notably, the PCR results of the present study also demonstrated increased expression levels of tenascin-C following treatment with 0.1 ng/ml TGF- β 1.

SMAD signaling is a major pathway stimulated by TGF- β 1 in the regulation of the ECM. In the signaling cascade, activated TGF- β receptor 1 induces the phosphorylation and activation of SMAD2 and 3. The activated SMAD2/3 complex forms oligomers with SMAD4, which translocate into the nucleus to regulate the expression levels of target ECM genes (44). Additionally, the RhoA/Rho-kinase pathway is also involved in TGF- β 1-induced synthesis of FN and Col-I (45). The western blot results of the present study demonstrated that the expression levels of RhoA and phosphorylated SMAD3 were increased in the TGF- β 1-treated

UC-MSCs. This data suggested that these signaling pathways may be involved in the regulation of MSCs by the ECM.

To investigate whether pre-treatment of MSCs with 0.1 ng/ml TGF- β 1 demonstrated any benefit when the cells were used as a therapeutic intervention *in vivo*, a rat model of endotoxemia was induced. This model was generated using intraperitoneal injection of LPS to simulate sepsis-associated lung injury, and the effects of TGF- β 1-treated and untreated UC-MSCs on ALI were observed. As previously demonstrated in several animal studies, MSCs can alleviate LPS-induced ALI and improve survival by restoring lung function through anti-inflammatory, antiapoptotic and immune regulatory actions (46-48). Histological analysis from the present study demonstrated that intravenous injection of control UC-MSCs or TGF- β 1-pre-treated UC-MSCs 1 h after endotoxin injury ameliorates LPS-induced lung injury. Furthermore, rats administered with TGF- β 1 or untreated UC-MSCs demonstrated less prominent elevations in BALF neutrophil count and protein content (indicative of epithelial permeability) in response to LPS. Additionally, measurements of lung wet-dry weight ratio indicated that therapy with TGF- β 1 or untreated UC-MSCs resulted in reduced pulmonary edema. However, no clear differences were demonstrated in the therapeutic benefits of TGF- β 1-treated and untreated UC-MSCs, indicating that pre-treatment of UC-MSCs with a low concentration of TGF- β 1 provided no additional benefits in this model of ALI over the short-term (48 h). However, it cannot be excluded that TGF- β 1-treated UC-MSCs provided longer-term advantages due to improved survival.

Transplanted MSCs can be identified in the damaged tissue area through the use of an eGFP marker or 4',6-diamidino-2-phenylindole staining. However, given the autofluorescence of pulmonary vascular endothelial cells, comparing differences in survival between various MSCs is challenging. A previous study has evaluated stem cell survival by observing Sry gene expression in the heart (26). Since the Sry gene is located on the Y-chromosome, gene-positive stem cells can be inferred to be of male origin. In the present study, qPCR results demonstrated that pre-treatment with TGF- β 1 significantly increased the number of UC-MSCs in the lung 2 weeks after transplantation. This novel finding indicated that pre-treatment with 0.1 ng/ml TGF- β 1 enhances MSC survival *in vivo*. Taken together with the other observations of the present study, it is speculated that enhanced expression levels of ECM components in UC-MSCs by 0.1 ng/ml TGF- β 1, particularly the upregulation of FN, is favorable for their survival *in vivo*, and thus may augment their longer term therapeutic benefit when used for tissue repair.

In conclusion, pre-treatment with 0.1 ng/ml TGF- β 1 resulted in the upregulation of FN and other ECM components in MSCs, and improved the survival of engrafted MSCs in a rat model of LPS-induced ALI. Furthermore, as TGF- β 1-treated MSCs retain multilineage differentiation and tissue repair functions, these cells may demonstrate promise in regenerative medicine and adoptive stem cell therapy.

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