

Modulation of TGF- β activity by latent TGF- β -binding protein 1 in human osteoarthritis fibroblast-like synoviocytes

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Abstract. Osteoarthritis (OA) is a common degenerative joint disease; however, its underlying pathogenesis remains to be elucidated. Previous studies have demonstrated that the transforming growth factor- β (TGF- β) signaling pathway has a role in the initiation and development of OA. Additionally, latent TGF- β -binding protein-1 (LTBP-1) modulates the activity of the TGF- β -mothers against decapentaplegic (Smad) signaling pathway in numerous diseases, including malignant glioma. The present study demonstrated that expression of LTBP-1 is increased in OA synovial tissues compared with normal synovial tissues. The effect of TGF- β was identified to be mediated by phosphorylated(p)-(Smad)2/3, which may activate activin-like kinase (ALK)5 receptor, and by p-Smad1/5/8, which may induce ALK1, thereby stimulating expression of matrix metalloproteinase-(MMP)-13 in OA fibroblast-like synoviocytes (FLS). Compared with normal FLS, OA FLS demonstrated an increased p-Smad1/5/8:p-Smad2 ratio, which led to elevated MMP-13 expression and aggravation of OA. Furthermore, knockdown of the LTBP-1 gene by siRNA transfection in OA FLS reduced p-Smad1/5/8 expression without affecting TGF- β mRNA levels, although p-Smad2 expression increased. It was also demonstrated that OA FLS exhibited increased proliferation compared with normal FLS *in vitro*. Furthermore, siRNA-mediated downregulation of LTBP-1 reduced proliferation of OA FLS. In conclusion, the present study demonstrated that an alteration in the p-Smad1/5/8:p-Smad2 ratio as well as association between p-Smad1/5/8 and MMP-13 expression in human OA FLS, may contribute to the development of OA. The results of the

present study suggested that LTBP-1 is a modulator of the TGF- β signaling pathway in human OA FLS, which may aid in elucidating the mechanism underlying the pathology of OA.

Introduction

Osteoarthritis (OA) is a common degenerative joint disease that affects the entire joint in patients worldwide (1). Although the primary characteristic of OA is the destruction of articular cartilage, additional characteristics of this disease include synovial inflammation, osteophyte formation and sclerosis of the subchondral bone (2). The etiology of OA is multifactorial and includes joint injury, obesity, aging and hereditary factors (3), but the underlying mechanism of pathogenesis of OA remains to be elucidated. There may be a number of initiating events of OA, as several different factors may lead to a common pathway that leads to the same disease (4). Previous studies of OA primarily focused on cartilage alterations; however, this focus has shifted, and OA is currently considered a pathological condition of the entire joint, which includes alterations in the articular cartilage, subchondral bone, ligaments, capsule and synovial membrane, ultimately leading to joint failure (5). OA remains a worldwide medical challenge and its definition, risk factors and pathogenesis remain to be elucidated.

The transforming growth factor- β (TGF- β) family consists of >35 members, including TGF- β s, activins and bone morphogenetic proteins (BMPs) (6). A total of three isoforms of TGF- β have been identified in mammals, termed TGF- β 1, - β 2 and - β 3, which are secreted as inactive complexes of a TGF- β dimer, pro-peptide latency associated peptide (LAP) and latent TGF- β binding proteins (LTBPs) (7). Normal TGF- β signaling has a role in homeostasis of articular cartilage and excessive activation of TGF- β in joint tissues, which leads to osteophyte formation, synovial fibrosis and joint pain in animal models (8). A polymorphism in TGF- β 1 at position 29 (T to C, amino acid 10) in the signal peptide sequence is associated with increased prevalence of spinal osteophytosis and ossification of the posterior longitudinal ligament (9). The above TGF- β polymorphism has also been associated with bone mineral density and fracture risk in postmenopausal Chinese women (10). However, the polymorphism may reduce morbidity of osteoporosis in Japanese women (11). A previous study indicated that TGF- β signaling is mediated by both activin-like kinase (ALK)5 and ALK1 in chondrocytes (12). Further studies have demonstrated that overexpression of ALK1 increases matrix

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metalloproteinase (MMP)-13 expression *in vitro* and its inhibition reduces MMP-13 expression (12,13).

A total of four LTBP isoforms have been identified and LTBP-1, -3, and part of LTBP-4 covalently associates with the LAP of TGF- β via the cysteine-rich domain (14). The large latent complex is secreted to the extracellular matrix (ECM), where it is targeted, stabilized and activated (15). Latent TGF- β cannot be activated unless the mature peptide is released from LAP and the mechanism underlying this process varies between cell types and environments (16). A previous study has indicated that BMP-1 may regulate TGF- β activation by cleaving LTBP-1 (17). Another study demonstrated that membrane type-1 (MT1)-MMP-mediated proteolytic processing of ECM-bound LTBP-1 was a mechanism to release latent TGF- β from the subendothelial matrix (18). When active TGF- β is released from the ECM, it binds to TGF- β receptor I and II heterodimers and induces them to directly activate receptor-regulated Smads (R-Smads) by phosphorylation. R-Smads in turn form transcriptional complexes with their common partner Smads to control target gene expression (19). Therefore, based on the aforementioned results, the authors of the present study hypothesized that the TGF- β pathway may have a role in human OA fibroblast-like synoviocytes (FLS) and that LTBP-1 may be a regulator modulating TGF- β activity.

Materials and methods

Cells. Primary OA FLS were extracted from freshly resected synovial tissues of 31 patients with OA undergoing total knee arthroplasty and primary normal FLS were obtained from freshly resected synovial tissues of 5 trauma patients undergoing lower limb amputation. Tissues were carefully minced, digested with 1% collagenase I (Worthington Biochemical Corporation, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM) (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) for 6 h at 37°C, filtered through a 200-mesh sieve, and subsequently cultured in DMEM with 10% fetal bovine serum (Hyclone, GE Healthcare Life Sciences). Cells were cultured to ~90% confluence and split at a 1:3 ratio for the next passage. All FLS were used during the third to fifth passage. FLS were analyzed by flow cytometry using Vimentin monoclonal antibody (cat. no. MA5-11883; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as a specific marker for FLS and the purity of all FLS was >95%, which met the requirement of the subsequent experiments.

Reagents. A total of three selected siRNA duplexes targeting LTBP-1 with a modification pattern that accounted for off-target effects caused by both strands and the non-targeting control pool [the non-siRNA group siRNA(-)] were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and used at 100 nM. The following sense sequences of the siRNA pool targeting LTBP-1 were used in the present study: 5'-GCC AUCUCCAUGUAUGAATT-3', 5'-GCCUAAACUUA UCAGCAUTT-3' and 5'-GCCAAUCCCAAGUCUCGU ATT-3'. Transient lipophilic transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Plasmin from human plasma (cat. no. P1867) was purchased from Sigma-Aldrich (Merck

KGaA, Darmstadt, Germany). Polyclonal LTBP-1 antibody (cat. no. BS60783) for western blot analysis was purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA), and monoclonal LTBP-1 antibody (cat. no. sc-271140) for immunofluorescence and immunohistochemistry was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). TGF- β 1 polyclonal antibody (cat. no. 18978-1-AP), TGF- β 2 polyclonal antibody (cat. no. 19999-1-AP), and TGF- β 3 polyclonal antibody (cat. no. 18942-1-AP) were purchased from Wuhan Sanying Biotechnology (Wuhan, China). The antibody against MMP-13 (cat. no. BS1231) was obtained from Bioworld Technology, Inc. The phosphorylated (p)-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad8 (Ser465/467) (cat. no. 9511) and p-Smad2 (Ser465) (cat. no. 8828) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against β -tubulin (cat. no. ab6046) and GAPDH (cat. no. ab8245) were obtained from Abcam (Cambridge, UK).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total FLSs RNA was prepared using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was prepared using the All-In-One RT Master Mix (Applied Biological Materials, Inc., Richmond, BC, Canada) with a Bio-Rad MyCycler system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as follows: 37°C for 15 min, then 85°C for 5 sec and cooling to 4°C. Gene expression was measured in a Rotor-Gene Q 2 plex System (Qiagen GmbH, Hilden, Germany) at 470 nm with Rotor-GenQ Series software version 2.1.0. The following primer sequences were used for the qPCR: LTBP-1 long (L)+ short (S) forward: 5'-GCTTCCGTCCAGATACAT CAG-3' (NM001166266, nt499-519), reverse: 5'-CTTGGT ACGAGACTTGGGATTG-3' (NM001166266, nt595-574); LTBP-1 L forward: 5'-GGTGCACCAAACCTAGCTGTG-3' (NM206943.1, nt554-574), reverse: 5'-ACAGGCTTTGCCCTT GG-3' (NM206943.1, nt636-654); TGF- β 1 forward: 5'-GCC CTGGACACCAACTATTG-3' (NM000660.3, nt1702-1727), reverse: 5'-CGTGTCCAGGCTCCAAATG-3' (NM 000660.3, nt1851-1869), TGF- β 2 forward: 5'-AAGCTTACACTGTCC CTGCTGC-3' (NM003238.1, nt847-868), reverse: 5'-TGT GGAGGTGCCATCAATACCT-3' (NM003228.1, nt934-955), TGF- β 3 forward: 5'-GGAAAACACCGAGTCGGAATAC-3' (NM003239, nt279-300), reverse: 5'-GCGGAAAACCTT GGAGGTAAT-3' (NM003239, nt399-379), GAPDH forward: 5'-GGAAAACACCGAGTCGGAATAC-3' (NM003238.1, nt847-868), reverse: 5'-GCGGAAAACCTTGGAGGTAAT-3' (NM003228.1, nt934-955). Thermocycling conditions were: Denaturation step at 95°C for 10 min, then 40 cycles at 95°C for 15 sec, 65°C for 10 sec and 72°C for 15 sec. Standard curves were obtained, and relative quantification of gene expression was assessed compared with threshold values. All results were normalized to GAPDH, and calculated using the $2^{-\Delta\Delta C_q}$ method for relative quantification (20).

Western blotting. Proteins from synovial tissues from OA and trauma patients and the conditioned media of FLSs were combined with plasmin to solubilize the LTBP-1-containing high-molecular-weight complexes. For detection of proteins from cell lysates, cells were lysed using

radioimmunoprecipitation assay (RIPA) buffer (Beijing ComWin Biotech Co., Ltd., Beijing, China) supplemented with a Protease Inhibitor Cocktail (100X; Beijing ComWin Biotech Co., Ltd.) and a phosphatase inhibitor cocktail (cat. no. P1260, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Centrplus centrifugal filter device YM-3 (3 kDa cut-off; Merck KGaA) was used to concentrate proteins from conditioned media. Protein levels were analyzed by western blotting using 20 μ g protein/lane mixed with SDS-PAGE Loading Buffer (Beijing ComWin Biotech Co., Ltd.). The extracted proteins were loaded onto a 10% SDS-PAGE gel and electrophoresed for 30 min at 80 V and then another 40 min at 120 V. Subsequently, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked in 5% skimmed milk in 1X Tris-buffered 0.05% saline Tween (TBST) for 1 h at room temperature, washed with TBST, and subsequently incubated for 24 h at 4°C with primary antibodies against LTBP-1, TGF- β s, MMP-13, p-Smad1/5/8, p-Smad2 and GAPDH at the following working concentrations: Anti-LTBP-1 antibody, 1:200; anti-TGF- β 1 antibody, 1:500; anti-TGF- β 2 antibody, 1:500; anti-TGF- β 3 antibody, 1:500; anti-MMP-13 antibody, 1:300; anti-p-Smad1/5/8 antibody, 1:800; anti-p-Smad2 antibody, 1:800; anti- β -Tubulin antibody, 1:5,000; and anti-GAPDH antibody, 1:10,000. The PVDF membranes were subsequently washed in TBST and incubated at room temperature overnight with horseradish peroxidase-conjugated secondary antibodies at the following working concentrations: Goat anti-mouse IgG (ab6789; 1:3,000; Abcam, Cambridge, UK) and Goat Anti-Rabbit IgG (ab6721; 1:3,000; Abcam). Protein bands were detected with Immobilon Western Chemiluminescent HRP Substrate (WBKLS0050; EMD Millipore, Billerica, MA, USA) using Bio-Rad chemiluminescence imaging system (ChemiDoc XRS; Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Densitometry was performed using ImageJ software (version 1.43; National Institutes of Health, Bethesda, USA).

Human tissue specimens. A total of 31 OA and 5 normal synovial tissues obtained from patients including 20 women and 16 men aged between 48 and 69 years old who underwent surgical treatment between May 2016 and January 2017 in the Department of Orthopedics of Tangdu Hospital (Xi'an, China) were investigated. The diagnoses were confirmed by a minimum of 2 senior pathologists. Each specimen was divided into three parts. The first part was fixed in 4% formalin at room temperature for 24 h (pH 7.4), embedded in paraffin, sectioned (\sim 4 μ m) with a microtome and placed on Super Frost Plus slides (Microm International GmbH, Walldorf, Germany) and representative tissue samples were prepared for tissue immunohistochemistry. The second part was ground for western blotting using a tissue grinder (Shanghai Jingxiang Industrial Company Ltd., Shanghai, China). The third part was used for primary cell culture by the enzyme digestion method (21) as follows: Tissues were minced and digested with 0.2% collagenase I (Worthington Biochemical Corporation) in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences) for 4-6 h at 37°C, filtered through a 200-mesh sieve, and then cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life

Sciences), 100 units penicillin and 100 μ g/ml streptomycin. Finally, the cells were cultured up to 90% confluence and then split in a 1/3 ratio up to passage 3-6.

All patients signed written informed consent. All human material used in the study was handled in accordance with the policies of the local institutional review board, and all operations were accepted by the patients and approved by the Ethics Committee of Tangdu Hospital (Xi'an, China).

Immunohistochemistry. Immunohistochemistry was performed with a monoclonal LTBP-1 antibody at a pretested dilution. Tissue slides, prepared as aforementioned, were deparaffinized, rehydrated and incubated in 3% H₂O₂ at room temperature for 5 min to eliminate endogenous peroxidase activity. Subsequently, the slides were washed with distilled water for 5 min, soaked in bovine serum albumin (BSA; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 10 min, incubated overnight with LTBP-1 antibody at 1:200 at 4°C, and washed with PBS 3 times for 5 min. Secondary antibody was added at room temperature for 30 min at the following working concentrations: Horseradish peroxidase-conjugated goat anti-mouse IgG (ab6789; 1:1,000; Abcam) and horseradish peroxidase-conjugated goat anti-rabbit IgG (ab6721; 1:1,000; Abcam). The samples were washed again with PBS 3 times for 5 min. Sections were incubated with 3,3'-Diaminobenzidine (Beijing Solarbio Science & Technology Co., Ltd.) for 4 min at room temperature, washed and counterstained with hematoxylin for 5 sec at room temperature. Evaluation of the immunohistochemical staining and performed using an Olympus BX51 light microscope at magnifications of 200 and x400 (Olympus Corporation, Tokyo, Japan).

Immunofluorescence. Immunofluorescence analysis of FLS was performed with a monoclonal LTBP-1 antibody at 1:200 dilution. Both OA and normal FLS were seeded in 6-well culture plates, which were about 10⁴ FLSs per well. Following 24 h of culture, cells reached \sim 50% confluence and were washed with PBS 2 times for 3 min and mixed with 4% paraformaldehyde at room temperature. Following a 30 min incubation at room temperature, the samples were washed again with PBS 2 times for 3 min and mixed with 0.5% Triton X-100 at room temperature for 30 min. Samples were washed with 0.01 M PBS 2 times for 3 min. For antibody blocking, 5% BSA was added at room temperature for 30-60 min. LTBP-1 antibody was added at a pretested dilution at 4°C overnight. Samples were washed with PBST 3 times for 3 min, and secondary fluorescent antibody was added at room temperature for 1 h. Samples were washed with 0.01 M PBS Tween-20 (PBST) 3 times for 3 min. Subsequently, cells were incubated with DAPI for 10 min at room temperature, washed 2 times with 0.01 M PBS for 5 min and examined using Olympus IX71 fluorescent microscope (Olympus Corporation).

Cell proliferation assay. To evaluate cell proliferation by Cell Counting Kit-8 (CCK-8) assays (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), OA and normal FLS were plated in 96-well flat-bottomed plates for 24 h. The normal FLS were untreated for 24 h and termed normal control (NC). OA FLS were divided into 3 groups, one group of OA FLSs

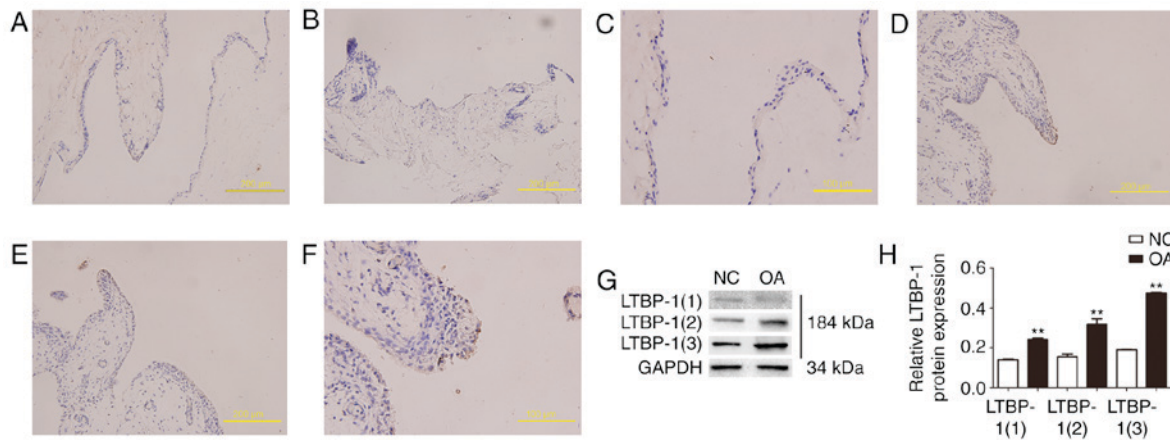


Figure 1. LTBP-1 expression is higher in synovial tissue in patients with OA compared with NC. (A-C) Three representative donor synovial tissues from NC. (D-F) Three representative donor synovial tissues from patients with OA. (G) Expression levels of LTBP-1 in synovial tissues solubilized by plasmin, measured by western blotting. (H) Quantification of relative expression of LTBP-1 to GAPDH. Magnification A-E, $\times 200$; C and F, $\times 400$. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. the NC group. NC, normal control; TGF- β , transforming growth factor- β ; LTBP-1, latent TGF- β -binding protein-1; OA, osteoarthritis.

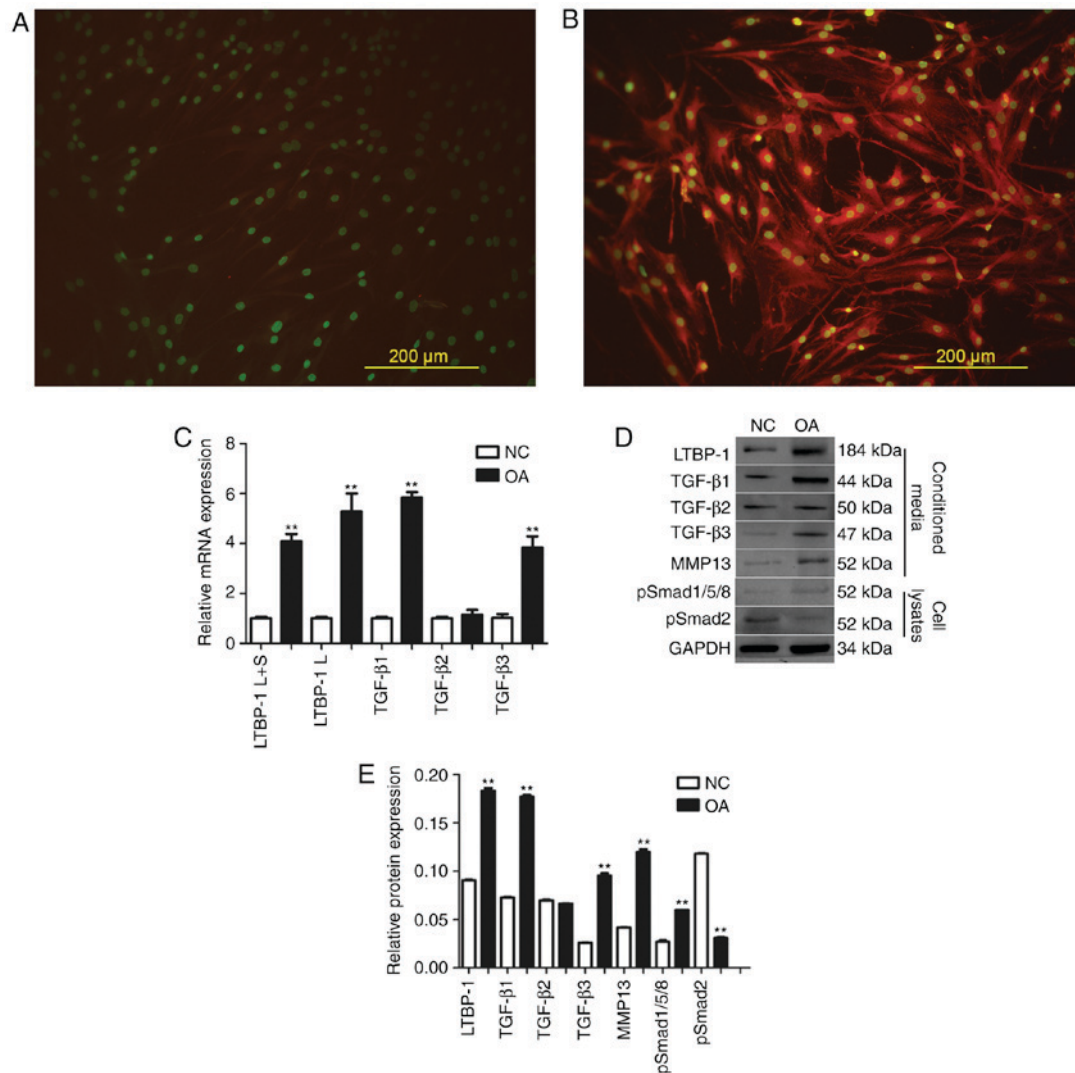


Figure 2. LTBP-1 expression is higher in OA FLS *in vitro* and LTBP-1 influences TGF- β signaling through p-Smad1/5/8, compared with NC. LTBP-1 expression in FLS in (A) NC and (B) patients with OA. (C) mRNA expression levels of LTBP-1 and TGF- β mRNA in FLS of OA and NC groups. (D) LTBP-1, TGF- β 1/2/3, p-Smad1/5/8, p-Smad2 and MMP-13 of OA and NC groups were measured by western blotting and (E) analyzed to determine relative expression levels. OA donors, $n=31$. NC donors, $n=5$. Scale bar, $200 \mu\text{m}$. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. the NC group. NC, normal control; TGF- β , transforming growth factor- β ; LTBP-1, latent TGF- β -binding protein-1; OA, osteoarthritis; MMP, matrix metalloproteinase; p, phosphorylated; Smad, mothers against decapentaplegic; L, long isoform; S, short isoform; FLS, fibroblast-like synoviocytes.

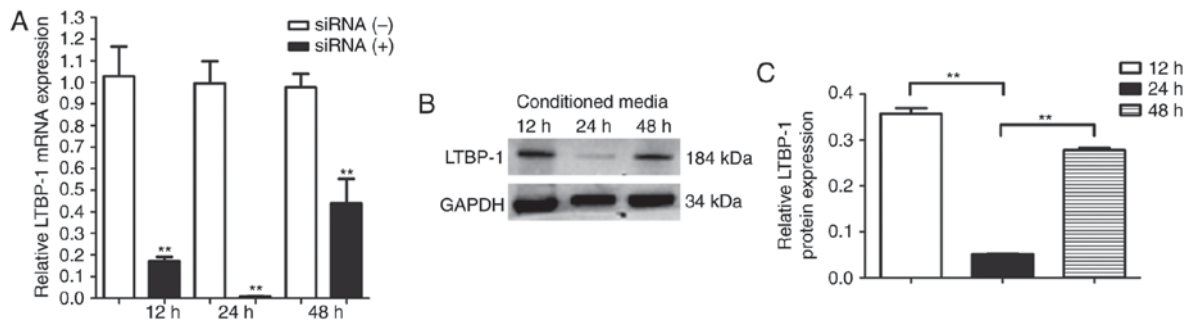


Figure 3. LTBP-1 expression in FLS of OA is lowest 24 h after siRNA transfection. (A) LTBP-1 mRNA levels were determined by reverse transcription-quantitative polymerase chain reaction 12, 24 and 48 h after siRNA transfection. ** $P < 0.01$ vs. the NC group. LTBP-1 protein levels were determined by (B) western blotting at 12, 24, and 48 h after siRNA transfection and (C) quantified. Data are presented as the mean \pm standard deviation of three independent experiments. OA donors $n = 31$. NC donors, $n = 5$. ** $P < 0.01$. LTBP-1, latent transforming growth factor- β -binding protein-1; siRNA, short interfering RNA; OA, osteoarthritis; FLS, fibroblast-like synoviocytes; NC, normal controls.

was treated with siRNA for 24 h, termed siRNA(+), another group of OA FLSs was treated with non-targeting siRNA for 24 h, termed siRNA(-), and the third group was untreated for 24 h, termed OA. The number of cells in each well was $\sim 10^2$, at ~ 70 -80% confluence. FLSs of the four groups were mixed with 10 μ l CCK-8 in each well, and after 1-4 h analyzed using the Infinite M200 Pro Multifunctional microplate reader at 450 nm (Tecan Group, Ltd., Mannedorf, Switzerland) according to the manufacturer's protocol.

Statistical analysis. Experiments were repeated 3 times and data are presented as the mean \pm standard deviation. One-way analysis of variance test followed by Tukey's post hoc test was used to analyze differences among 3 groups. Two-tailed Student's t-tests was used for the analysis between two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Human OA synovial tissues express increased LTBP-1 levels compared with normal synovial tissues *in vivo*. LTBP-1 expression was analyzed in human OA and normal synovial tissues *in vivo* by immunohistochemistry. Paraffin-embedded tissue sections were immunostained with LTBP-1 antibody. The present findings demonstrated that LTBP-1 expression in human OA synovial tissues increased compared with normal synovial tissues (Fig. 1A-F). Diagnoses were confirmed by a minimum of 2 senior pathologists. LTBP-1 expression was also analyzed in human OA and normal synovial tissues *in vivo* by western blotting (Fig. 1G and H). To determine whether LTBP-1 is associated with ECM in synovial tissues, plasmin was used to solubilize proteins from the ECM. LTBP-1 was identified in synovial ECM and there was a 2-fold increase in LTBP-1 expression when human OA synovial tissues were compared with normal synovial tissues.

Expression levels of LTBP-1, TGF- β s, p-Smad1/5/8, and MMP-13 are elevated in human OA FLS compared with normal FLS *in vitro*. To confirm that OA FLS express LTBP-1, LTBP-1 protein levels were detected by immunofluorescence. It was determined that LTBP-1 expression in OA increased compared with normal FLS (Fig. 2A and B).

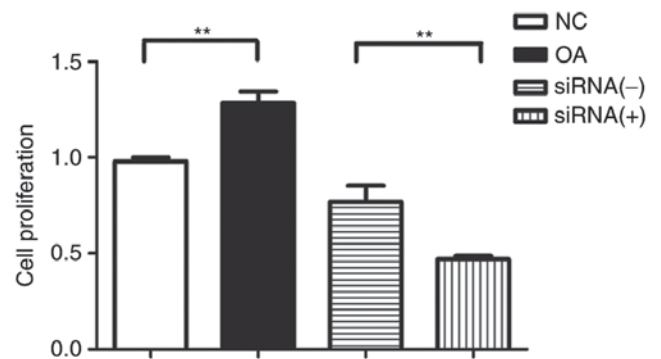


Figure 4. FLS of OA demonstrated increased proliferation compared with normal FLS *in vitro*, and siRNA-mediated downregulation of LTBP-1 reduced the proliferation of OA FLS. Data are presented as the mean \pm standard deviation of three independent experiments. OA donors, $n = 31$. NC donors, $n = 5$. ** $P < 0.01$. OA, osteoarthritis; siRNA, short interfering RNA; FLS, fibroblast-like synoviocytes; NC, normal control; LTBP-1, latent transforming growth factor- β -binding protein-1.

Subsequently, OA and normal FLS were analyzed for LTBP-1 and TGF- β mRNA expression levels by RT-qPCR (Fig. 2C). Two LTBP-1 forms, L and S, and 3 TGF- β isoforms, TGF- β 1, TGF- β 2, TGF- β 3 were analyzed. It was determined that LTBP-1 L+S and LTBP-1 SmRNAs were expressed in FLS, and LTBP-1 L+S and LTBP-1 S mRNA expression levels in OA FLS increased ≥ 4 -fold compared with normal FLS. TGF- β 1 and TGF- β 3 levels also increased in OA FLS compared with normal FLS. However, there was no significant difference identified in the expression levels of TGF- β 2 between OA and normal FLS.

Expression levels of LTBP-1 and TGF- β signaling pathway proteins, including TGF- β 1, TGF- β 2, TGF- β 3, p-Smad1/5/8, p-Smad2 and MMP-13 were analyzed by western blotting (Fig. 2D and E). LTBP-1, TGF- β 1, TGF- β 3, p-Smad1/5/8 and MMP-13 levels in OA FLS increased compared with normal FLS. Expression of TGF- β 2 was similar between OA and normal FLS. Expression of p-Smad2 in OA FLS decreased compared with normal FLS.

LTBP-1 expression in OA FLS is lowest 24 h after siRNA transfection. LTBP-1 mRNA expression levels were analyzed at 12, 24, and 48 h after siRNA-mediated downregulation of

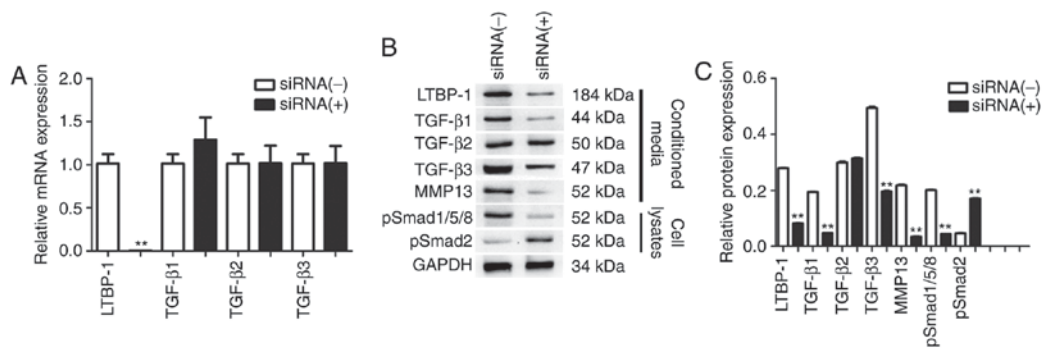


Figure 5. siRNA-mediated downregulation of LTBP-1 led to expression of TGF- β 1-p-Smad1/5/8-MMP-13 in FLS of OA. (A) LTBP-1 and TGF- β mRNA levels. Expression of LTBP-1, TGF- β s, p-Smad1/5/8, p-Smad2 and MMP-13 in OA and NC were determined using (B) western blotting and were (C) quantified. Data are presented as the mean \pm standard deviation of three independent experiments. OA donors, n=31. NC donors, n=5. **P<0.01 vs. the siRNA(-)group. siRNA, short interfering RNA; LTBP-1, latent transforming growth factor- β -binding protein-1; OA, osteoarthritis; Smad, mothers against decapentaplegic; MMP, matrix metalloproteinase; p, phosphorylated; NC, normal controls.

LTBP-1 in OA FLS. It was determined that LTBP-1 expression was lowest at 24 h (Fig. 3A). LTBP-1 protein levels were determined by western blotting, and LTBP-1 protein levels were lowest 24 h following siRNA transfection (Fig. 3B and C).

OA FLS demonstrate increased proliferation compared with normal FLS in vitro and siRNA-mediated downregulation of LTBP-1 reduces proliferation of OA FLS. Proliferation of OA and normal FLS was determined 3 h following addition of CCK-8. Proliferation of OA FLS significantly increased compared with normal FLS *in vitro* (Fig. 4). However, siRNA-mediated downregulation of LTBP-1 significantly reduced the proliferation of OA FLS (Fig. 4).

siRNA-mediated downregulation of LTBP-1 reduces expression levels of TGF- β s and MMP-13 in OA FLS. To determine whether downregulation of LTBP-1 may modulate TGF- β mRNA levels in OA FLS, mRNA levels of TGF- β 1, TGF- β 2 and TGF- β 3 were quantified using RT-qPCR 24 h following siRNA-mediated downregulation of LTBP-1. No significant difference in the expression of TGF- β s was identified (Fig. 5A).

Subsequently, it was determined whether downregulation of LTBP-1 may alter protein levels of TGF- β s and MMP-13 in OA FLS. The results demonstrated that following transfection, LTBP-1, TGF- β 1, TGF- β 3 and MMP-13 protein levels were significantly reduced, but TGF- β 2 expression levels were not affected. siRNA-mediated downregulation of LTBP-1 reduced p-Smad1/5/8 and increased p-Smad2 in OA FLS (Fig. 5B and C).

p-Smad1/5/8 and p-Smad2 expression levels were determined in cell lysates by western blotting 24 h following siRNA-mediated downregulation of LTBP-1 in OA FLS. Following transfection, p-Smad1/5/8 protein levels significantly decreased in the transfected group compared with the siRNA(-)OA FLS group. p-Smad2 protein levels increased 4-fold compared with siRNA(-)OA FLS (Fig. 5B and C).

Discussion

The present study demonstrated that in human OA FLS, TGF- β signals via p-Smad2 and p-Smad1/5/8. Furthermore, it was demonstrated that LTBP-1 may modulate the activity

of TGF- β in human OA FLS. The results of the present study contribute to the efforts to elucidate the mechanism underlying the development and pathology of OA.

TGF- β signaling is a pathway which contributes to the development of OA (22). The association of TGF- β and OA varies with the stage of OA (3). TGF- β has been identified in cartilage and synovial fluid of patients with OA at elevated levels compared with healthy control patients (4,23). Therefore, the authors of the present study hypothesized that FLS, components of an articulation, may participate in the TGF- β signaling pathway. The results of the present study demonstrated that the TGF- β signaling pathway has an important role in OA FLS. Similar to other types of cells (12), TGF- β signals not only via p-Smad2 but also via p-Smad1/5/8 in OA FLS. Previous studies demonstrated that the TGF- β signaling pathway has a role in stimulation of chondrocytes to renew the ECM in the cartilage of young animals (24-30). This process is considered a function of the TGF- β signaling pathway in cartilage. However, previous studies have suggested that the TGF- β signaling pathway may signal not only via ALK5 but also via ALK1, to phosphorylate Smad1/5/8, which may additionally stimulate the expression of MMP-13 and aggravate OA (31,32). Other studies have demonstrated that among elderly patients and patients with OA, TGF- β signaling via ALK5 is reduced in cartilage, as demonstrated by reduced Smad2 phosphorylation. The switch from ALK5 to ALK1 precedes cartilage degradation and may be necessary for OA development (33,34). In other cell types, including endothelial cells, the ratio of ALK5 to ALK1 may affect the response to TGF- β (29). Since TGF- β expression levels in synovial fluid and chondrocytes are elevated in patients with OA (23), the authors of the present study hypothesized that TGF- β secreted from FLS may degrade articular cartilage primarily via ALK1 using autocrine and paracrine mechanisms. Therefore, the alteration in the balance between ALK1 and ALK5 in chondrocytes, and between p-Smad2 and p-Smad1/5/8 in FLS may have a role in the TGF- β signaling pathway in the development of OA.

The present study demonstrated an increase in TGF- β and LTBP-1 in the conditioned media solubilized by plasmin of human OA FLS. Tumor studies have revealed that LTBP-1 associates with TGF- β in complex with its pro-peptide and

targets TGF- β to specific extracellular structures (16,35). In epithelial cells, LTBP-1 needs to be matrix-bound for $\alpha_v\beta_6$ -integrin-mediated activation (36). Protease-mediated activation leads to secretion of active TGF- β from the ECM. Subsequently, a latent complex is released from the ECM, with cleavage of LTBP-1 at protease-sensitive sites, which results in truncated LTBP-1 associating with SLC (37,38). The latent TGF- β releases active TGF- β from its noncovalent complex, which may be regulated by an interaction with proteases, thrombospondin or integrins in a cell type-specific manner (16,37). The present study investigated the role of LTBP-1 derived from OA FLS in the process of autocrine and paracrine TGF- β activation. Previous studies demonstrated that in glioma cells, FLPs were not only involved in the processing of TGF- β but also in the proteolytic truncation of LTBP-1, and human glioblastoma cells secreted different forms of TGF- β associated with LTBP-1 (39,40). Therefore, the authors of the present study hypothesized that in OA FLS, LTBP-1 may participate in TGF- β activation. The present study reported the expression of LTBP-1 in OA FLS and normal FLS. The expression of LTBP-1 markedly increased in human OA FLS compared with normal FLS. *In vitro* LTBP-1 protein levels were elevated in synovial tissues. The present study demonstrated that levels of free TGF- β in the conditioned media were associated with the LTBP-1 level and this association may involve a positive feedback loop. When LTBP-1 was knocked down, TGF- β signaling was altered via downregulation of TGF- β s, p-Smad1/5/8 and MMP-13 and upregulation of p-Smad2. LTBP-1 depletion in OA FLS led to alterations in TGF- β protein levels. Despite similar TGF- β 1, - β 2 and - β 3 mRNA levels compared with the control group, OA FLS with LTBP-1 knockdown secreted fewer TGF- β s in the conditioned medium solubilized by plasmin. Furthermore, LTBP-1 depletion significantly reduced Smad1/5/8 phosphorylation levels. Therefore, LTBP-1 knockdown in OA FLS altered the paracrine and autocrine TGF- β signaling. The aforementioned data are consistent with a hypothesis that there is positive feedback loop between LTBP-1 expression and TGF- β bioavailability in OA FLS.

The present study also demonstrated that proliferation of OA FLS was enhanced compared with normal FLS. Previous studies demonstrated that TGF- β signaling via ALK5 represses chondrocyte hypertrophic differentiation and promotes cell survival (12,26). However, the role TGF- β signaling via ALK1 was demonstrated to aggravate the formation of osteophytes and synovial fibrosis, which may enhance the proliferation of OA FLS (24,29-41). In addition, OA FLS with LTBP-1 knockdown demonstrated decreased proliferation compared with the control group, indicating that reduced TGF- β signaling via p-Smad1/5/8 may inhibit proliferation of OA FLS.

Although LTBP1s are secreted proteins and cell confluency affects LTBP secretion and ECM deposition, prior to seeding cells in 6-well plates, the cells were counted and it was ensured that cell numbers in both groups were similar. Therefore, cell confluency should not have affected LTBP secretion or ECM deposition in the present study. Although, there may be a potential influence of Lipofectamine® 2000, both groups were treated with it; therefore, both groups would have been affected in the same way. Further studies on the mechanism underlying TGF- β activation should aid in identification of therapeutic treatments for OA.

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