

## Interaction of ERK1/2 and Smad2/3 signaling pathways in TGF- $\beta$ 1-induced TIMP-3 expression in rat chondrocytes <sup>☆</sup>



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### ABSTRACT

Tissue inhibitor of metalloproteinase-3 (TIMP-3) is an important natural inhibitor of matrix metalloproteinases (MMPs) and of a disintegrin and metalloproteinase with thrombospondin motif (ADAMTs), which can cleave cartilage extracellular matrix components to cause cartilage degradation. In this study, our data suggest TGF- $\beta$ 1 induces TIMP-3 expression through activations of both the ERK1/2 and Smad2/3 signaling pathways. TGF- $\beta$ 1-stimulated TIMP-3 expression was significantly inhibited by SB525334 (TGF- $\beta$  receptor I kinase inhibitor), accompanied by a reduction in ERK1/2 and Smad3 phosphorylation. We used PD98059 (MEK inhibitor) and SIS3 (inhibitor of Smad3 phosphorylation) to investigate the respective roles of ERK1/2 and Smad2/3 signaling pathways in TGF- $\beta$ 1-induced TIMP-3 expression. The results show PD98059 treatment significantly suppressed TGF- $\beta$ 1-induced ERK1/2 phosphorylation and TIMP-3 expression. Under these conditions, the degree of Smad3 phosphorylation correlated with ERK1/2 activation, which suggests that ERK1/2 may activate Smad3 phosphorylation. SIS3 significantly inhibited TGF- $\beta$ 1-induced Smad3 phosphorylation and TIMP-3 expression. ERK1/2 phosphorylation alone had no effect on TGF- $\beta$ 1-induced TIMP-3 expression, which suggests ERK1/2 via Smad3 phosphorylation regulates TGF- $\beta$ 1-induced TIMP-3 expression. Here, we demonstrate that ERK1/2 may be capable of activating the Smad2/3 signaling pathway to result in TGF- $\beta$ 1-induced TIMP-3 up-regulation.

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### Introduction

While the complete pathogenesis of osteoarthritis (OA)<sup>1</sup> is not fully understood, cartilage damage and disorders in cartilage repair are major characteristics of OA. TGF- $\beta$  has been suggested to be a protective factor for articular cartilage [1,2]. This cytokine is crucial for the maintenance of articular cartilage homeostasis, and TGF- $\beta$  has been shown to induce expression of type II collagen, aggrecan, and SOX-9 in chondrocytes [3,4]. Furthermore, TGF- $\beta$  significantly decreases the expression of proteinase-activated receptor-2 (PAR-2)

in OA chondrocytes and in human primary synovial cells [5,6]. This reduction in PAR-2 can induce synthesis of the major catabolic factors in OA cartilage, including matrix metalloproteinase-1 (MMP-1) and MMP-13. Additionally, inhibition of TGF- $\beta$  signaling triggers the development of OA in murine joint tissues [7]. TGF- $\beta$  acts to protect articular cartilage by reversing interleukin (IL)-1 $\beta$ -induced up-regulation of MMP-13 and down-regulation of COL2A1. Based on these reports, TGF- $\beta$  is generally regarded as a positive stimulus for the regeneration of articular cartilage [8–10].

Tissue inhibitor of metalloproteinase-3 (TIMP-3) is an important natural inhibitor of MMPs and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), which can cleave cartilage extracellular matrix (ECM) components to result in cartilage degradation. In vitro, TGF- $\beta$  can markedly up-regulate TIMP-1 and TIMP-3 [11,12] and prevent the expression of MMP-1, -3, -8 and -13 in chondrocytes [13]. In vivo, TIMP-3(-/-) mice are also more susceptible to age-dependent cartilage degradation [14], suggesting that TIMP-3 has a protective function against MMP-mediated damage. Taken together, these findings demonstrate that

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<sup>1</sup> Abbreviations used: OA, osteoarthritis; PAR-2, proteinase-activated receptor-2; MMP-1, matrix metalloproteinase-1; TIMP-3, Tissue inhibitor of metalloproteinase-3; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TNF, tumor necrosis factor.

TIMP-3 plays a key role in TGF- $\beta$ -mediated prevention of cartilage degeneration. Additional studies have indicated that the ERK1/2 and Smad2/3 signaling pathways are involved in the TGF- $\beta$ -induced TIMP-3 up-regulation in chondrocytes [12,15]. Cross-talk between ERK1/2 and Smad signaling pathways following stimulation from TGF- $\beta$  occurs in different cell types and the effect of ERK1/2 activation on Smad signaling is cell type-specific [16–20]. In chondrogenic ATDC5 cells, TGF- $\beta$ -induced aggrecan gene expression requires the transcriptional activation of Smad2/4, and is absolutely dependent on the activation of ERK1/2 and p38 MAPK signaling pathways [21]. Although the contribution of the individual ERK1/2 and Smad2/3 signaling pathways to TGF- $\beta$ -induced TIMP-3 in chondrocytes is well understood and the cross-talk between the ERK1/2 and Smad2/3 signaling pathways are described in other cell types, the cross-talk between the ERK1/2 and Smad2/3 signaling pathways in TGF- $\beta$ 1-induced TIMP-3 expression in chondrocytes is not. Chondrocytes are the only cell type found in normal articular cartilage. Understanding of the signaling network in TGF- $\beta$ 1-induced TIMP-3 expression in chondrocytes can improve our ability to prevent the degeneration of cartilage.

The aim of this study was, therefore, to demonstrate the contribution of the ERK1/2 and Smad2/3 signaling pathways to TGF- $\beta$ 1-induced TIMP-3 expression, as TIMP-3 is a natural inhibitor of MMPs and ADAMTS. We further defined the interaction between the ERK1/2 and Smad2/3 signaling pathways as it relates to TGF- $\beta$ 1-mediated TIMP-3 regulation. We found that TGF- $\beta$ 1 up-regulates TIMP-3 expression in rat chondrocytes and that this response involves TGF- $\beta$ 1-induced activation of both the ERK1/2 and Smad2/3 signaling pathways. Additionally, our results suggest that ERK1/2 signaling pathways act via the Smad2/3 signaling pathways to enhance the effects of TGF- $\beta$ 1-induced TIMP-3 up-regulation.

## Materials and methods

### Chondrocyte isolation and culture

Normal rat knee cartilage was obtained from the tibial platform and the femoral condyle, in accordance with protocols approved by the Shanghai Jiao Tong University School of Medicine ethics committee. Cartilage pieces were washed with phosphate-buffered saline (PBS, a buffer solution containing 137 mM/L sodium chloride, 10 mM/L sodium phosphate, 2.7 mM/L potassium chloride, and 1.8 mM/L potassium phosphate) twice and treated with 0.2 mg/ml type II collagenase (Sigma Chemical Co., Poole, UK) in serum-free Dulbecco's modified Eagle's medium (DMEM, Earle's Balanced Salts modified to contain 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate; GIBCO-BRL, San Diego, CA, USA) overnight at 37 °C. Since type II collagenases are endopeptidases that digest native type II collagen, we treated our cartilage with some to release the cells from the tissue, a procedure which often requires the action of collagenase enzymes and neutral proteases. The cells were collected by filtering through a 100  $\mu$ m mesh nylon cell strainer, centrifuging at 1000g for 5 min, and washing twice with PBS. Finally, the cells were resuspended and cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen), plus 1% penicillin and streptomycin solution (10,000 units of penicillin and 10,000  $\mu$ g of streptomycin per ml; GIBCO-BRL, San Diego, CA, USA). The culture medium was changed every second day. The chondrocytic phenotype of the cultured cells was confirmed by positive immunostaining for type II collagen and toluidine blue staining of glycosaminoglycans. First passage chondrocytes were used in all experiments.

### Chondrocyte treatment

First passage chondrocytes were removed from tissue culture flasks by treatment with 0.025% trypsin for 2–3 min at room temperature and then seeded into six-well plates ( $3 \times 10^5$  cells/well) for 24 h. Chondrocytes were maintained in serum-deficient DMEM for 24 h and subjected to the following treatments. Chondrocytes were cultured with or without 10 ng/ml of recombinant human TGF- $\beta$ 1 (Perprotech, Rocky Hill, NJ, USA), the amount published in the study by Takahashi [22], for 48 h. TIMP-3 expression was evaluated by real-time polymerase chain reaction (PCR) and Western blotting. Chondrocytes were cultured with 10 ng/ml of TGF- $\beta$ 1, and at 0, 1, 5, 10, 15, 30, 60, and 180 min time points the expression levels of phosphorylated Smad3 (p-Smad3), Smad2/3, phosphorylated ERK1/2 (p-ERK1/2) and ERK1/2 were examined by western blotting.

For inhibition studies, chondrocytes were pre-treated in serum-free medium with a TGF- $\beta$  receptor I (ALK5) kinase inhibitor (1  $\mu$ M SB525334, as used in the study by Thomas et al. [23]) (Selleckchem, Houston, TX, USA), a MEK pharmacological inhibitor (10  $\mu$ M PD98059, as used in the study by Prasad et al. [24]) (Promega, Madison, WI, USA) to inhibit ERK1/2 activation, or a Smad3 phosphorylation pharmacological inhibitor (10  $\mu$ M SIS3, as used in the study by Qureshi et al. [15]) (Santa Cruz, CA, USA) for 60 min, and then stimulated with or without 10 ng/ml of TGF- $\beta$ 1 for 48 h. TIMP-3 expression was evaluated by real-time PCR and western blotting. To examine the involvement of the ERK1/2 and Smad signaling pathways, chondrocytes were pre-treated in serum-free medium with SB525334 (1  $\mu$ M), PD98059 (10  $\mu$ M) or SIS3 (10  $\mu$ M) for 60 min, and then stimulated with 10 ng/ml of TGF- $\beta$ 1. At 0, 1, 5, 10, 15, 30, 60, and 180 min time points, the levels of p-Smad3, Smad2/3, p-ERK1/2 and ERK1/2 expression were examined by western blotting. To further examine the interaction of ERK1/2 and Smad2/3 signaling pathways, chondrocytes were pre-treated in serum-free medium with PD98059 (10  $\mu$ M) or SIS3 (10  $\mu$ M) for 60 min, and then stimulated with 10 ng/ml of TGF- $\beta$ 1 for 15 min. The levels of p-Smad3, Smad2/3, p-ERK1/2 and ERK1/2 expression were examined by western blotting.

### RNA extraction and real-time PCR

Total RNA was extracted from chondrocytes using TRIzol reagent (Invitrogen, Carlsbad, CA USA), following the manufacturer's instructions. For first-strand complementary deoxyribonucleic acid (cDNA) synthesis, 2  $\mu$ g mRNA was reverse transcribed using the reverse transcriptase M-MLV cDNA synthesis kit (Takara, Tokyo, Japan) according to the manufacturer's recommended conditions. Briefly, a 12  $\mu$ l reaction mixture containing 2  $\mu$ l of oligo d(T)18 primer (50  $\mu$ M), 2  $\mu$ g of total RNA and RNase-free dH<sub>2</sub>O was incubated at 70 °C for 10 min. Subsequently, 1  $\mu$ l of dNTP mixture (10 mM each), 4  $\mu$ l of 5X M-MLV buffer [250 mM Tris-HCl (pH 8.3 at room temperature), 26 375 mM KCl, 15 mM MgCl<sub>2</sub>], 0.5  $\mu$ l ribonuclease inhibitor (40 U/ $\mu$ l), 1  $\mu$ l of reverse transcriptase M-MLV (RNase H-free, 200 U/ $\mu$ l) and RNase-free dH<sub>2</sub>O were added to a final volume of 20  $\mu$ l and the reaction was incubated for 60 min at 42 °C. Next, the reaction was inactivated by heating at 70 °C for 15 min. 2  $\mu$ l of the 20  $\mu$ l reverse transcription reaction were in turn subjected to PCR amplification for 35 cycles (Promega, Madison, WI, USA). Amplification of GAPDH was performed to quantify PCR products and confirm the use of equal amounts of RNA. Primer sequences of rat genes were as follows: GAPDH: F: 5'-CAAGTTC AACGGCAGTCAAG-3', R: 5'-ACATACTCAGCACCAGC ATCAC-3'; TIMP-3: F: 5'-TCTGCAACTCCGACATCG-3', R: 5'-GCGTAG TGTGGACTGATAGC-3'. All the quantitative data were calculated

using the comparative  $\Delta\Delta\text{CT}$  method, as normalized against corresponding levels of the housekeeping gene GAPDH and then normalized against levels of the control group to obtain the relative fold change.

#### Protein extraction and Western blotting

Whole cell lysates were prepared using RIPA buffer [50 mM Tris–HCl (pH7.4), 150 mM NaCl, 1% (g/ml) Triton X-100, 0.1% (g/ml) sodium dodecyl sulfate (SDS), 2 mM ethylene-diaminetetraacetic acid, 2 mM PMSF] in the presence of protease and phosphatase inhibitors. The protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). For p-Smad3, Smad2/3, p-ERK1/2 and ERK1/2 protein levels, 10  $\mu\text{g}$  of protein from rat chondrocytes were then loaded on a 10% SDS–polyacrylamide gel, subjected to electrophoresis, and transferred to polyvinylidene fluoride membranes by electroblotting. For TIMP-3 protein levels, 10  $\mu\text{g}$  of total cellular extracts were separated by 15% SDS–polyacrylamide gel electrophoresis in the presence of protease and phosphatase inhibitors. Membranes were then blocked with 5% skim milk. Anti-rat TIMP-3 (Abcam, Hong Kong), anti-rat p-Smad3 (Abcam), anti-rat Smad2/3 (Novus, Littleton, CO, USA), anti-rat p-ERK1/2 (Abcam) and anti-rat ERK1/2 (Cell Signaling Technology Inc., Danvers, MA, USA) were incubated with the membranes overnight at 4 °C. The membranes were then washed and reacted with horseradish peroxidase-conjugated secondary antibodies (anti-mouse for TIMP-3 or anti-rabbit IgG for p-Smad3, Smad2/3, p-ERK1/2, and ERK1/2, (Santa Cruz, CA, USA)) at room temperature for 2 h. Finally, protein bands were visualized by chemiluminescence. Western blots were reprobed with monoclonal anti-GAPDH antibody (Cell Signaling Technology Inc., Danvers, MA, USA) as a control. Intensity levels were quantified using Total Lab 100 software (Nonlinear Dynamics, Durham, NC, USA).

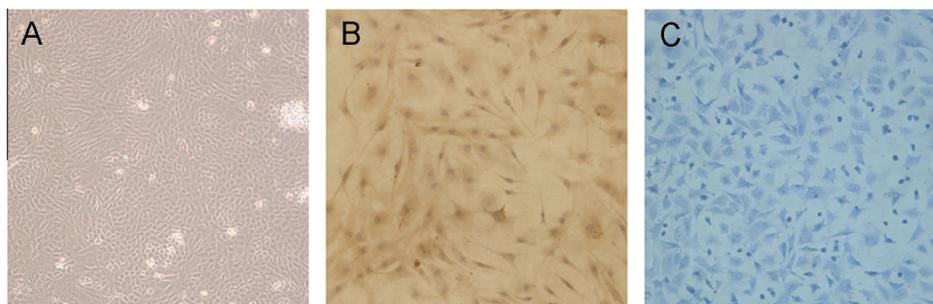
#### Statistical analysis

All the experiments were performed three times and the results are expressed as the mean  $\pm$  standard deviation. Statistical significance was determined using the Mann–Whitney *U* test or Kruskal–Wallis analysis of variance test, when appropriate, using SPSS 13.0 statistical software. A *p* value of  $<0.05$  was considered statistically significant.

## Results

#### Positive immunostaining of type II collagen and toluidine blue staining of glycosaminoglycans in the primary cells

As shown in Fig. 1, the chondrocytic phenotype of the cultured cells was confirmed by positive immunostaining for type II collagen and toluidine blue staining of glycosaminoglycans.



**Fig. 1.** Immunostaining of type II collagen and toluidine blue staining of glycosaminoglycans in primary cells. (A) The cultured cells was observed by light microscopy. (B) Immunostaining for type II collagen was observed. (C) Toluidine blue staining of glycosaminoglycans was observed (magnification = 100).

#### ERK1/2 and Smad2/3 signaling pathways mediate TGF- $\beta$ 1-stimulated TIMP-3 expression in rat chondrocytes

To confirm the effect of TGF- $\beta$ 1 on TIMP-3 expression in chondrocytes, rat chondrocytes were cultured with or without TGF- $\beta$ 1 for 48 h. Real-time PCR and Western blotting were then performed to examine the expression of TIMP-3. As shown in Fig. 2A, real-time PCR revealed significant up-regulation of TIMP-3 after the treatment with TGF- $\beta$ 1. Western blotting also indicated that TIMP-3 protein was up-regulated following TGF- $\beta$ 1 treatment (Fig. 2B). These results suggest that TGF- $\beta$ 1 stimulates TIMP-3 expression in rat chondrocytes.

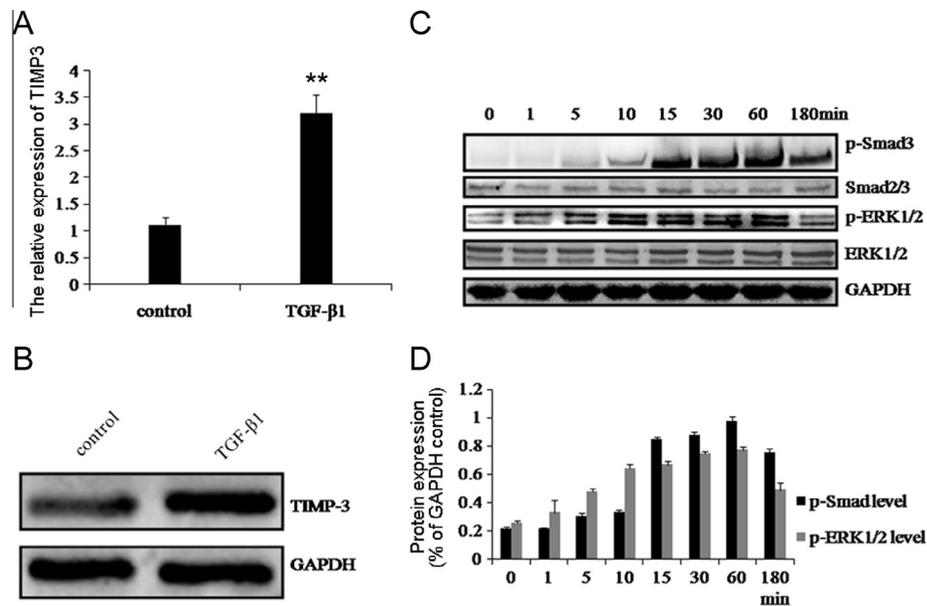
Next, we evaluated whether the ERK1/2 and Smad2/3 signaling pathways were involved in TGF- $\beta$ 1 stimulation of TIMP-3. Rat chondrocytes were cultured in the presence of TGF- $\beta$ 1 and at indicated time points, the activation of ERK1/2 and phosphorylation of Smad3 were examined by Western blotting. As shown in Fig. 2C and D, Smad3 was rapidly phosphorylated within 5 min of treatment with TGF- $\beta$ 1, with a further increase in phosphorylation after treatment for 15 min. ERK1/2 phosphorylation occurred within 10 min of treatment with TGF- $\beta$ 1. Taken together, these observations suggest that the ERK1/2 and Smad2/3 signaling pathways may play an important role in TGF- $\beta$ 1-stimulated TIMP-3 induction in rat chondrocytes.

#### Blockade of TGF- $\beta$ 1-stimulated TIMP-3 expression by inhibition of ERK1/2 and Smad2/3 signaling pathways

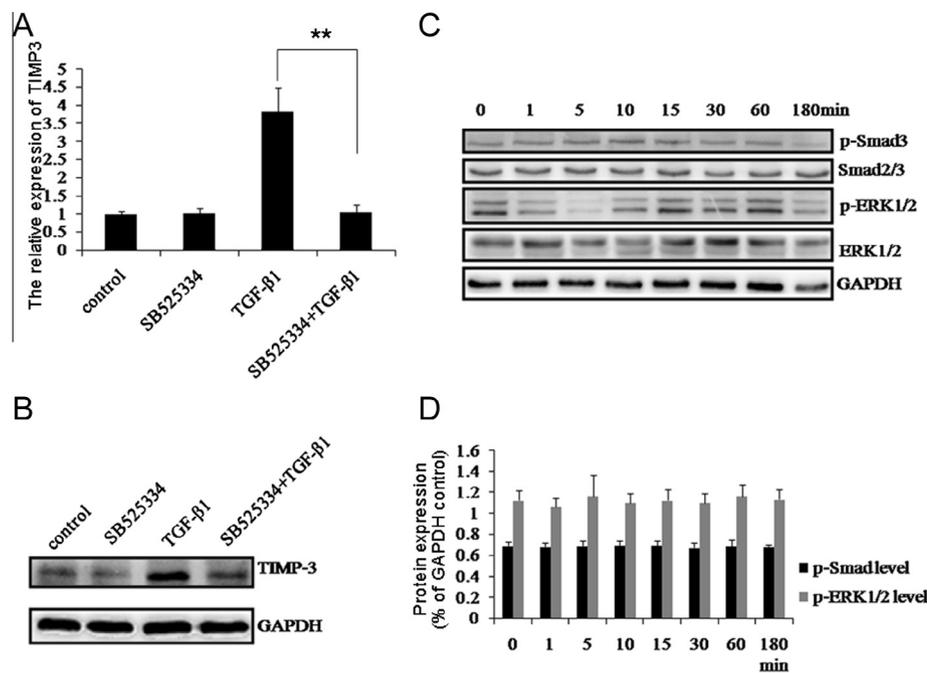
To further investigate the involvement of the ERK1/2 and Smad2/3 signaling pathways in TGF- $\beta$ 1-induced TIMP-3 expression, chondrocytes were pre-treated with TGF- $\beta$  receptor I (ALK5) kinase inhibitor, SB525334, for 60 min and then stimulated with or without 10 ng/ml of TGF- $\beta$ 1 for 48 h. Real-time PCR and western blotting confirmed that TGF- $\beta$ 1 stimulated TIMP-3 expression was inhibited by SB525334 (Fig. 3A and B). To examine the involvement of the ERK1/2 and Smad2/3 signaling pathways, chondrocytes were pre-treated with SB525334 and then stimulated with 10 ng/ml of TGF- $\beta$ 1. Western blotting results show that phosphorylation of both ERK1/2 and Smad3 were blocked by SB525334 treatment (Fig. 3C and D). These data further validate the requirement for the activation of ERK1/2 and Smad2/3 signaling pathways in the induction of TIMP-3 by TGF- $\beta$ 1.

#### Blockade of TGF- $\beta$ 1-stimulated TIMP-3 expression by inhibition of the ERK1/2 signaling pathway

We next examined the role of ERK1/2 in TIMP-3 induction by TGF- $\beta$ 1. Chondrocytes were pre-treated with the MEK inhibitor PD98059 for 60 min to inhibit ERK1/2 activation followed by culture with or without TGF- $\beta$ 1 for 48 h. Real-time PCR and Western blotting revealed TGF- $\beta$ 1-induced expression of TIMP-3



**Fig. 2.** Effect of TGF-β1 stimulation on the expression of TIMP-3, p-Smad3, Smad2/3, p-ERK1/2 and ERK1/2 in rat chondrocytes. Rat chondrocytes were cultured with or without TGF-β1 (10 ng/ml) for 48 h. (A) TIMP-3 mRNA levels, as measured by real-time PCR, are expressed as the mean ± SD (\*\* $p < 0.01$ ). (B) TIMP-3 protein levels, as evaluated by Western blotting. (C) Chondrocytes were treated with TGF-β1 for 0, 1, 5, 10, 15, 30, 60, and 180 min. At the indicated time points, Western blotting was used to examine ERK1/2, p-ERK1/2, p-Smad3 and Smad2/3 expression levels. (D) Densitometric analyses of p-ERK1/2 and p-Smad3. Data shown is from one representative experiment out of the three performed.



**Fig. 3.** The effect of ERK1/2 and Smad2/3 signaling pathway inhibition by SB525334 on the TGF-β1-stimulated expression of TIMP-3, p-Smad3, Smad2/3, p-ERK1/2 and ERK1/2. Chondrocytes were pretreated with the TGF-β1 receptor I (ALK5) kinase inhibitor SB525334 (1 μM) for 60 min followed by stimulation with or without TGF-β1 (10 ng/ml) for 48 h. (A) TIMP-3 mRNA levels, as measured by real-time PCR, are expressed as the mean ± SD (\*\* $p < 0.01$ ). (B) TIMP-3 protein levels, as evaluated by Western blotting. (C) Chondrocytes were treated with TGF-β1 for 0, 1, 5, 10, 15, 30, 60, and 180 min following pre-treatment with SB525334 for 60 min. At the indicated time points, Western blotting was used to examine ERK1/2, p-ERK1/2, p-Smad3 and Smad2/3 expression levels. (D) Densitometric analyses of p-ERK1/2 and p-Smad3. Data shown is from one representative experiment out of the three performed.

was significantly suppressed in the presence of PD98059 (Fig. 4A and B). Next, chondrocytes were pre-treated with PD98059 for 60 min followed by culture with TGF-β1 for 0, 1, 5, 10, 15, 30, 60, and 180 min. At the indicated time points, activation of ERK1/2 and Smad2/3 signaling pathways was assessed by western blotting. The results, shown in Fig. 4C and D, confirm that the ERK1/2

signal pathway was inhibited by PD98059 treatment. These data suggest that the ERK1/2 signaling pathway plays an important role in TIMP-3 induction by TGF-β1. Importantly, the Smad2/3 signaling pathway alone was not sufficient to induce TIMP-3 expression. As shown in Fig. 4C and D, TGF-β1-induced Smad3 phosphorylation was not influenced by PD98059 treatment. In the presence of

PD9859, Smad3 was rapidly phosphorylated within 5 min and reached peak levels of phosphorylation after 10–15 min.

#### *Inhibition of ERK1/2 signaling pathway reduced the quantity of Smad3 phosphorylation*

Although PD98059 treatment did not alter the ability of Smad3 to become phosphorylated following TGF- $\beta$ 1 stimulation, we wanted to investigate whether the quantity of Smad3 phosphorylation was changed by PD98059 treatment. We chose to stimulate the cells with TGF- $\beta$ 1 for 15 min in this experiment because Smad3 was significantly phosphorylated by TGF- $\beta$ 1 after 10–15 min (As shown in Fig. 2C). Therefore, chondrocytes were pre-treated with or without PD98059 and stimulated with TGF- $\beta$ 1 for 15 min. Western blotting was used to examine the ERK1/2 and Smad3 phosphorylation. As shown in Fig. 5A and B, in the presence of TGF- $\beta$ 1, the phosphorylation of ERK1/2 and Smad3 was elevated. When the ERK1/2 signaling pathway was inhibited by PD98059, the quantity of Smad3 phosphorylation was attenuated. This reduction correlated with a similar reduction of ERK1/2 phosphorylation, as compared with the levels in the uninhibited, TGF- $\beta$ 1-stimulated group. Collectively, these data suggest that the ERK1/2 signaling pathway may be responsible for the quantity of Smad3 phosphorylation. These results indicate that the ERK1/2 signaling pathway may activate the Smad2/3 signaling pathway to cause TGF- $\beta$ 1-induced TIMP-3 expression.

#### *Blockade of TGF- $\beta$ 1-stimulated TIMP-3 expression by Smad3 phosphorylation*

To determine whether ERK1/2 affected TGF- $\beta$ 1-induced TIMP-3 through activation of the Smad2/3 signaling pathway, we used a specific inhibitor of Smad3 phosphorylation (SIS3). First, chondrocytes were cultured with or without TGF- $\beta$ 1 for 48 h following pre-treatment with SIS3 for 60 min. Real-time PCR and Western blotting indicated that TGF- $\beta$ 1-induced TIMP-3 expression was significantly inhibited by SIS3 (Fig. 6A and B). Next, we examined

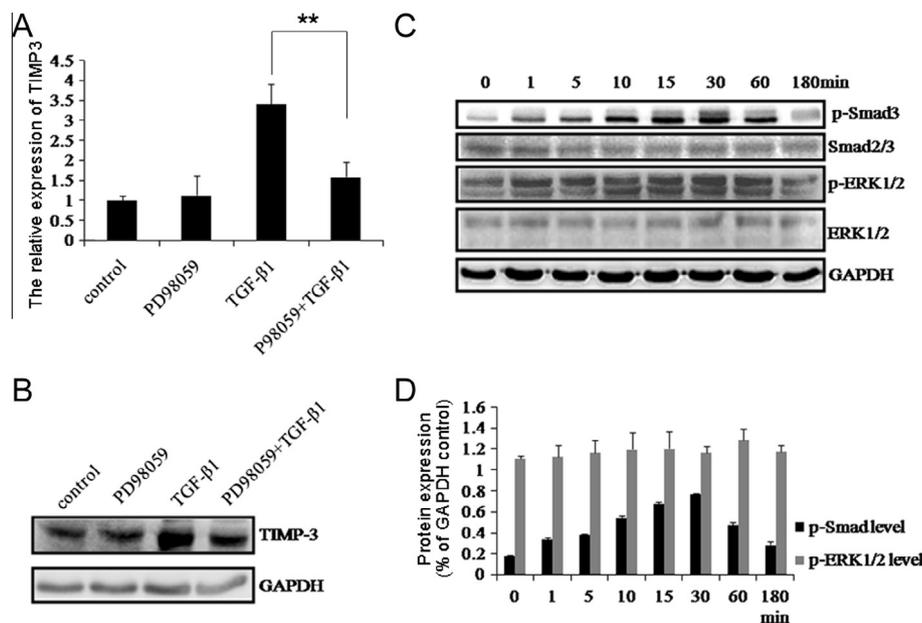
activation of the ERK1/2 and Smad2/3 signaling pathways by TGF- $\beta$ 1 in the presence of SIS3. Chondrocytes were treated with TGF- $\beta$ 1 for 0, 1, 5, 10, 15, 30, 60, and 180 min following pre-treatment with SIS3 for 60 min. At the indicated time points, Western blot results show that Smad3 phosphorylation was markedly suppressed by SIS3. However, ERK1/2 phosphorylation occurred within 10 min of treatment with TGF- $\beta$ 1, with a further increase in phosphorylation after treatment for 15 min (Fig. 6C and D). Taken together, these results demonstrate that TGF- $\beta$ 1-induced TIMP-3 expression requires activation of the Smad2/3 signaling pathway.

#### *Inhibition of Smad3 phosphorylation had no effect on the quantity of ERK1/2 activation*

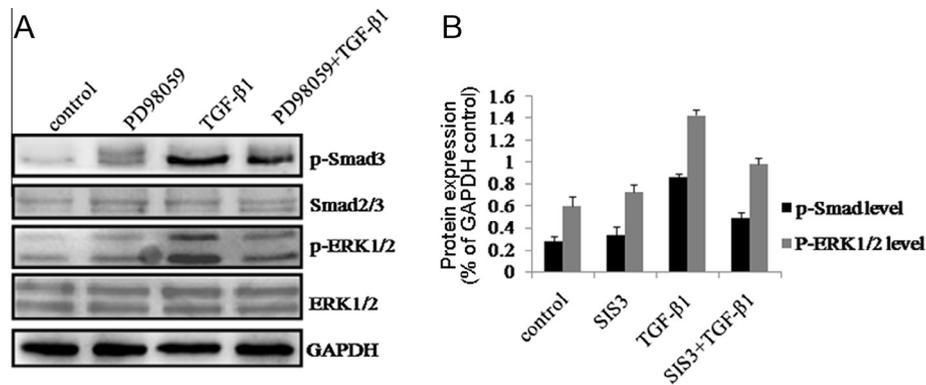
We also chose to examine the quantity of ERK1/2 activation after 15 min of TGF- $\beta$ 1 treatment when Smad3 phosphorylation was inhibited. As shown in Fig. 7A and B, in the presence of TGF- $\beta$ 1, the level of ERK1/2 activation remained unchanged when Smad3 phosphorylation was decreased by SIS3, as compared with the uninhibited, TGF- $\beta$ 1-treated group. These results demonstrate that inhibition of Smad3 phosphorylation had no effect on the quantity of ERK1/2 activation. Together, these data suggest that activation of ERK1/2 alone has no effect on TGF- $\beta$ 1-induced TIMP-3 expression when the Smad2/3 pathway is blocked. These results further suggest that ERK1/2 may mediate the level of TGF- $\beta$ 1-induced TIMP-3 through activation of the Smad2/3 signaling pathway.

## Discussion

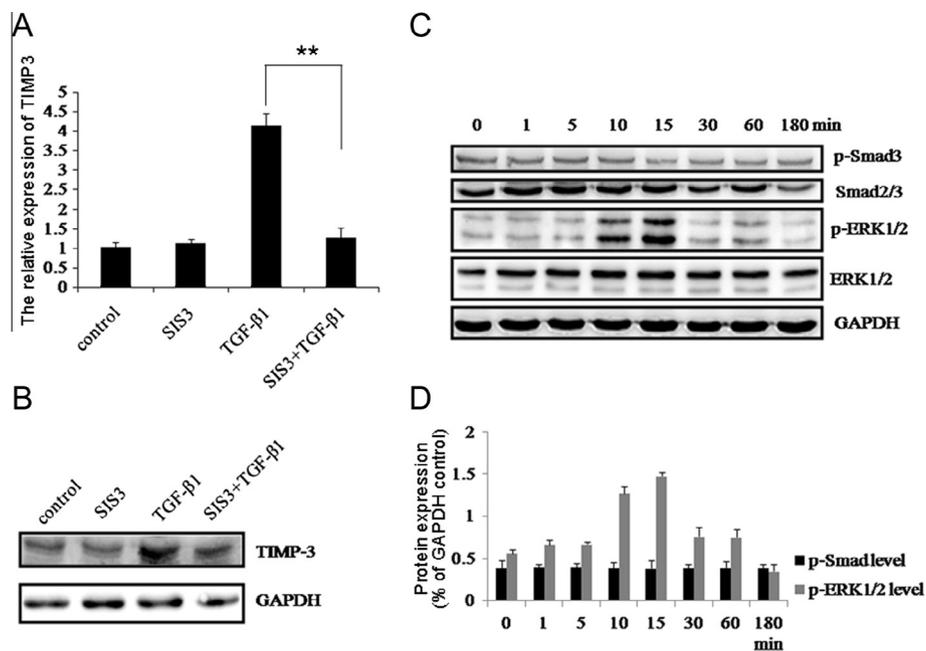
Degradation and loss of articular cartilage is a hallmark of OA. It is believed that cartilage destruction in OA is associated with unregulated production of proteinases, such as MMPs, which may be induced by inflammatory cytokines (e.g., IL-1 and tumor necrosis factor (TNF)) [25–28]. MMPs are capable of triggering resorption of cartilage ECM, while TIMPs, such as TIMP-3, are



**Fig. 4.** The effect of ERK1/2 inhibition by PD98059 on the TGF- $\beta$ 1-stimulated expression of TIMP-3, p-Smad3, Smad2/3, p-ERK1/2 and ERK1/2. Chondrocytes were pretreated with the MEK inhibitor PD98059 (10  $\mu$ M) for 60 min followed by stimulation with or without TGF- $\beta$ 1 (10 ng/ml) for 48 h. (A) TIMP-3 mRNA levels, as measured by real-time PCR, are expressed as the mean  $\pm$  SD ( $*p < 0.05$ ). (B) TIMP-3 protein levels, as evaluated by Western blotting. (C) Chondrocytes were treated with TGF- $\beta$ 1 for 0, 1, 5, 10, 15, 30, 60, and 180 min following pre-treatment with PD98059 for 60 min. At the indicated time points, Western blotting was used to examine ERK1/2, p-ERK1/2, p-Smad3 and Smad2/3 expression levels. (D) Densitometric analyses of p-ERK1/2 and p-Smad3. Data shown is from one representative experiment out of the three performed.



**Fig. 5.** The effect of ERK1/2 signaling pathway inhibition by PD98059 on the quantity of Smad3 phosphorylation. Chondrocytes were pretreated with PD98059 (10  $\mu$ M) for 60 min and then stimulated with TGF- $\beta$ 1 (10 ng/ml) for 15 min. Total protein from first passage confluent chondrocytes was extracted. (A) ERK1/2, p-ERK1/2, p-Smad3 and Smad2/3 expression levels were examined by Western blotting. (B) Densitometric analyses of p-ERK1/2 and p-Smad3. Data shown is from one representative experiment out of the three performed.

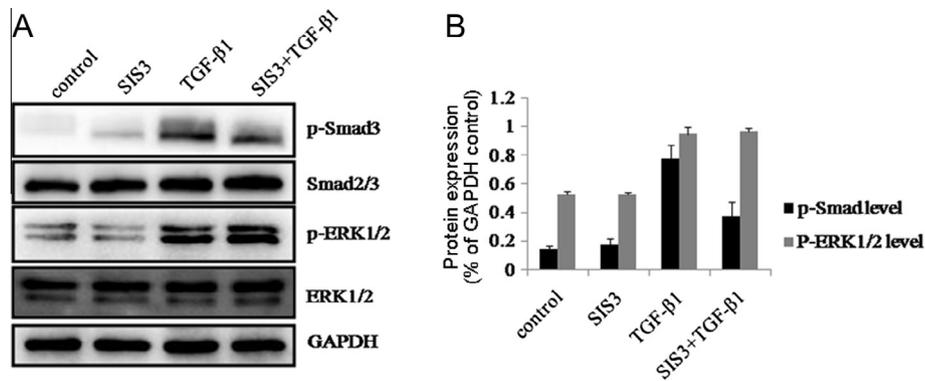


**Fig. 6.** The effect of SIS3-induced inhibition of Smad3 phosphorylation on the TGF- $\beta$ 1-stimulated expression of TIMP-3, p-Smad3, Smad2/3, p-ERK1/2 and ERK1/2. Chondrocytes were pretreated with the specific Smad3 phosphorylation inhibitor SIS3 (10  $\mu$ M) for 60 min followed by stimulation with or without TGF- $\beta$ 1 (10 ng/ml) for 48 h. (A) TIMP-3 mRNA levels, as measured by real-time PCR, are expressed as the mean  $\pm$  SD (\*\* $p$  < 0.01). (B) TIMP-3 protein levels, as evaluated by western blotting. (C) Chondrocytes were treated with TGF- $\beta$ 1 for 0, 1, 5, 10, 15, 30, 60, and 180 min following pre-treatment with SIS3 for 60 min. At the indicated time points, western blotting was used to examine ERK1/2, p-ERK1/2, p-Smad3 and Smad2/3 expression levels. (D) Densitometric analyses of p-ERK1/2 and p-Smad3. Data shown is from one representative experiment out of the three performed.

physiological protein inhibitors of MMPs. The imbalance between MMPs and TIMP-3, at least in part, causes articular cartilage homeostasis to shift toward degeneration [14,29].

A previous study suggested that TGF- $\beta$  activates ERK1/2 signaling pathways, resulting in TIMP-3 induction in primary human or bovine chondrocytes [12]. An additional study has demonstrated that TGF- $\beta$  activates Smad2/3 signaling pathways, leading to the induction of TIMP-3 expression in human chondrocytes [15]. Furthermore, a recent report has described how the activation of Smad2/3, p38 MAPK, and ERK1/2 is essential for the induction of TIMP-3 expression by TGF- $\beta$  in fibroblasts, with complex crosstalk between Smad2/3 and ERK1/2 and p38 MAPK [30]. The present study found that TIMP-3 is significantly up-regulated in rat chon-

drocytes following treatment with TGF- $\beta$ 1. It also showed that ERK1/2 is rapidly and transiently phosphorylated in these cells. Experiments using a p-Smad3 antibody that recognizes the C-terminal phosphorylation sites indicated that these sites are rapidly and transiently phosphorylated, as well. Moreover, phosphorylation of both ERK1/2 and Smad3 peaks at 15–30 min and is sustained until 60 min after TGF- $\beta$ 1 treatment. Our results also demonstrate that TGF- $\beta$ 1-induced TIMP-3 up-regulation is significantly prevented by the TGF- $\beta$  receptor I (ALK5) kinase inhibitor SB525334 via the inhibition of ERK1/2 and Smad3 phosphorylation. Taken together, these data suggest that both the ERK1/2 and Smad2/3 signaling pathways may be involved in TGF- $\beta$ -induced TIMP-3 expression in chondrocytes.



**Fig. 7.** The effect of SIS3-induced inhibition of Smad3 phosphorylation on the quantity of ERK1/2 activation. Chondrocytes were pretreated with SIS3 (10  $\mu$ M) for 60 min and then stimulated with TGF- $\beta$ 1 (10 ng/ml) for 15 min. Total protein from first passage confluent chondrocytes was extracted. (A) Western blotting was used to examine ERK1/2, p-ERK1/2, p-Smad3 and Smad2/3 expression levels. (B) Densitometric analyses of p-ERK1/2 and p-Smad3. Data shown is from one representative experiment out of the three performed.

It has been reported that TGF- $\beta$  auto-induction requires the activation of ERK1/2, p38 MAPK and Smad2/3 signaling pathways, which can occur independently in renal proximal tubular epithelial cells. These pathways regulate distinct transcription factors [31]. In human fibroblasts, activation of Smad3 and ERK1/2 cooperatively mediate the induction of connective tissue growth factor by TGF- $\beta$  [32]. Previously, the interaction between the ERK1/2 and Smad2/3 signaling pathways in TGF- $\beta$ 1-induced TIMP-3 up-regulation was unclear. We now show that inhibition of the ERK1/2 pathway by PD98059 significantly prevents TGF- $\beta$ 1-induced TIMP-3 up-regulation. PD98059 treatment had no effect on Smad3 phosphorylation, which still reached peak levels at 15–30 min and was sustained until 60 min after TGF- $\beta$ 1 treatment. Other studies suggest that cross-talk between the ERK1/2 and Smad2/3 signaling pathways enhances the effect of TGF- $\beta$ . In chondrogenic ATDC5 cells, TGF- $\beta$ -induced transcriptional activation of a specific Smad2/4-dependent response element is dependent on the cooperative activation of p38 MAPK and ERK1/2 signaling pathways [21]. In mesenchymal cells, ERK1/2 phosphorylates the linker region of nuclear localized Smads. This implies that the regulation of Smad signaling by the TGF- $\beta$ /PI3K/Pak2/Raf/MEK/ERK1/2 signaling pathways is critical for TGF- $\beta$ -induced fibroblast replication [33]. In human mesangial cells, ERK blockade inhibits TGF- $\beta$  stimulation of Smad2/3 serine phosphorylation [16]. We further speculate that the ERK1/2 pathway affects the quantity of p-Smad3 activation in TGF- $\beta$ 1-induced TIMP-3 up-regulation, rather than the ability of Smad3 to be phosphorylated. Because phosphorylation of both ERK1/2 and Smad3 was maximal at 15–30 min, we measured the effect of ERK1/2 inhibition on the phosphorylation of Smad3 following stimulation of chondrocytes with TGF- $\beta$ 1 for 15 min. Interestingly, the quantity of Smad3 C-terminal phosphorylation sites was dependent on the phosphorylation of ERK1/2. Additionally, one study indicated that the Smad3 linker region cooperates with the C-terminal domain for TGF- $\beta$ -inducible transcriptional activation [34]. These results imply that the ERK1/2 signaling pathway may operate through activation of p-Smad3, resulting in up-regulation of TGF- $\beta$ 1-induced TIMP-3 expression.

We further investigated the requirement of Smad3 phosphorylation for TGF- $\beta$ 1-induced TIMP-3 expression through ERK1/2 signaling pathways by using SIS3, a specific inhibitor of Smad3 phosphorylation. Our data suggest that TGF- $\beta$ 1-induced TIMP-3 up-regulation was significantly decreased by inhibition of Smad3 phosphorylation. We found that both the ability of ERK1/2 to become activated and the quantity of that activation were unaffected when Smad3 phosphorylation was suppressed by SIS3. A previous study suggested that SIS3 is a potent and selective inhib-

itor of Smad3 function [35]. Additionally, the present study indicates that the ERK1/2 signaling pathway elevates the quantity of Smad3 phosphorylation. These data indicate that activation of ERK1/2 alone has no effect on TGF- $\beta$ 1-induced TIMP-3 expression when Smad3 function is inhibited by pretreatment of SIS3. Taken together, these results further confirm that the ERK1/2 signaling pathway may activate the Smad2/3 signaling pathway to cause TGF- $\beta$ 1-induced TIMP-3 expression.

In summary, we have demonstrated that the activation of both the ERK1/2 and Smad2/3 signaling pathways are involved in TGF- $\beta$ 1-mediated stimulation of TIMP-3 production in rat chondrocytes. Furthermore, the ERK1/2 signaling pathway requires activation of the Smad2/3 signaling pathway for TGF- $\beta$ 1 to induce TIMP-3 expression. Overall, both the ERK1/2 and Smad2/3 signaling pathways may be crucial in the maintenance of extracellular matrix proteins induced by TGF- $\beta$ 1 and the resulting suppression of degeneration of articular cartilage.

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