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Original Paper

TGF-β1 Induces EMT in Bovine Mammary Epithelial Cells Through the TGFβ1/Smad Signaling Pathway

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Key Words
Transforming growth factor-beta 1 • Epithelial mesenchymal transition • Extracellular matrix • Mammary fibrosis • Bovine

Abstract
Background/Aims: Transforming growth factor-β1 (TGF-β1) plays a crucial role in chronic inflammation in various tissues, and is related to inflammation-caused organ fibrogenesis associated with the epithelial-mesenchymal transition (EMT) and the deposition of the extracellular matrix (ECM). However, the effect of TGF-β1 on bovine mammary epithelial cells (BMECs) with mastitis, and its mechanism, remain unknown. ***Methods:*** We analyzed the level of TGF-β1 in inflamed mammary tissues and cells using western blotting. BMECs were treated with TGF-β1, and EMT-related gene and protein expression changes were evaluated using quantitative real-time polymerase chain reaction (qPCR), western blotting, and immunofluorescence. We also inhibited the TGF/Smad signaling pathway using a receptor inhibitor, and analyzed EMT-related protein expression by western blotting. In addition, we injected TGF-β1 into mice mammary glands to investigate whether it can cause mammary fibrosis *in vivo*. ***Results:*** The TGF-β1 level was up-regulated in mammary tissues with mastitis and in inducible inflammatory BMECs. TGF-β1 treatment activated the TGF/Smad signaling pathway in BMECs during their transition to the EMT phenotype, as indicated by morphological changes from a cobblestone-like shape to a spindle-like one. TGF-β1 treatment also up-regulated the expression of α-smooth muscle actin, vimentin, and collagen I, albumin, and down-regulated the expression of E-cadherin both in mRNA level and protein level. Furthermore, TGF-β1 enhanced the gene expressions of *MMP2, MMP7*, and *fibronectin* in BMECs. TGF-β1 injection induced mice mammary infection and fibrosis. ***Conclusion:*** These findings suggested that aberrant up-regulation of TGF-β1 in bovine mastitic mammary glands might play an important role in bovine mammary fibrosis caused by unresolved inflammation.

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Introduction

Bovine mammary fibrosis is a common pathological process associated with mastitis, which is the primary clinical disease in cattle [1]. Mastitis has a high incidence and infection rate worldwide, and causes varying extents of bovine mammary fibrosis. The decreased milk production by the fibrotic breast can result in development of mammary sclerosis, which necessitates sacrificing the cow, thus leading to severe economic losses in the dairy industry [2].. Fibrosis, characterized by the excessive growth of connective tissues and the formation of scleroses and scars, is attributed mainly to the accumulation of the extracellular matrix (ECM) and increase in cytokines, such as transforming growth factor-beta (TGF-β), basic fibroblast growth factor, and platelet-derived growth factor [3].

A superfamily is a group of multifunctional proteins that play important roles in crucial biological activities. The TGF-β superfamily regulates cell growth and differentiation. At least four subtypes of TGF-β, including TGF-β1, TGF-β2, TGF-β3, and TGF-β1β2, have been found in mammals; however, TGF-β1 is the most studied and is the critical cytokine in the process of fibrosis. Previous research, reviewed by Liu et al [4]., showed that diseased tissues contain higher levels of TGF-β1, especially in the fibrosis region. Experimental animals treated with exogenous TGF-βl develop tissue and organ fibrosis, as well as excessive deposition of ECM proteins, in their cells. Furthermore, treatments for TGF-βl resistance, including the administration of neutralizing antibodies, a soluble type II receptor, antisense oligonucleotides, small molecule inhibitors, and RNA interference, inhibited the formation of fibrosis [5].

Recently, research on the role of TGF-β1 in tissue fibrosis has shown that the functional changes resulting from TGF-β1-induced phenotypic changes lead to cell proliferation, ECM deposition, and liver fibrosis [6, 7]. In addition, Liu et al [6]. showed that TGF-β1 induces the conversion of primary kidney fibroblasts into myofibroblasts, increases the expression of α-smooth muscle actin (α-SMA), activates the synthesis of fibronectin, and up-regulates the expression of collagen type I (collagen I) *in vitro*. Similar effects of TGF-β1 were reported in studies on pulmonary fibrosis [7, 8].

In addition to producing various ECM proteins, epithelial cells undergo epithelial-mesenchymal transition (EMT) in the presence of certain inflammatory cytokines, thereby promoting the occurrence and development of fibrosis [9]. During EMT, epithelial cells acquire certain characteristics of the stromal cells around them, as seen by the loss of epithelial polarity and the acquisition of stromal features, including a stromal-like appearance, and vimentin, snail, and osteopontin expression [10, 11]. Epithelial cells often change from a cobblestone-shape to a spindle-shape, the level of E-cadherin decreases, and those of vimentin, α-SMA, and fibronectin increase; after EMT, epithelial cells show functional similarity to fibroblasts.

Recent research has focused on the theory that EMT can increase cancer cell invasion and metastasis [12, 13]; however, little work has been carried out on tissue fibrosis caused by EMT. Kaimori et al. confirmed that exogenous TGF-β1 induced EMT in mouse liver cells via the Smad2/3 pathway, demonstrated mainly by the morphological change from a hexagonal cell shape to a spindle-shape [7, 14]. Vimentin and albumin expression levels also decreased significantly at the gene and protein levels [7]. In addition, Chen et al [9]. demonstrated that TGF-β1 induces EMT in human lung cancer A549 cells, as demonstrated by changes in mesenchymal morphology, increase in invasion and metastasis ability, up-regulation of the mesenchymal marker genes encoding vimentin and fibronectin, and down-regulation of the epithelial marker E-cadherin [15, 16].

A deeper understanding of the role of TGF-β1 in bovine mammary fibrosis and mastitis could lead to the development of new therapeutic methods to prevent fibrosis during the early phase of bovine mammary mastitis, and could assist in curing mammary fibrosis. Hence, we studied the effects of TGF-β1 in mammary mastitis and fibrosis by stimulating bovine mammary epithelial cells (BMECs) with exogenous TGF-β1. Morphological and functional changes were studied, in addition to investigating the possible signaling pathways involved.

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Materials and Methods

Cell culture and treatment

Primary BMECs were derived from healthy lactating bovine by our research group during previous research [17]. BMECs were recovered and cultured in DMEM/F12 with 10 % fetal bovine serum, 100 IU/mL of penicillin, and 100 μg/mL streptomycin (GibcoBRL, Grand Island, NY, USA).

BMECs were seeded in 60-mm culture dishes. Six hours after seeding, the culture medium was replaced with or without TGF-β1 (2 ng/mL, Creative BioMart, Shirley, NY, USA), according to manufacturer's instructions. Cells were cultured for 72 h and photographed every 12 h under a phase-contrast microscope, before being harvested for subsequent experiments.

To inhibit the TGF/Smad pathway, the cells were treated with LY2109761 (Selleck Chemicals, Houston, TX, USA), a TGF-β receptor type I/II inhibitor, at a final concentration of 10 μmol/mL for six hours. This was followed by treatment with TGF-β1 at the aforementioned concentration. After incubation with both LY2109761 and TGF-β1 for 72 h, the cells were harvested for subsequent experiments.

Enzyme-linked immunosorbent assay (ELISA)

TGF-β1 secreted into the culture supernatants by BMECs was measured by ELISA. BMECs were stimulated with lipoteichoic acid (LTA) (20 ng/μL) for 12 h or lipopolysaccharide (LPS) (10 ng/μL) for 3 h, based on an inflammatory cell model of bovine mastitis established in a previous publication [18]. The medium was replaced with fresh serum-free medium, harvested 24 h later, and centrifuged to remove cell debris. Culture supernatants were used to measure TGF-β1 levels using a bovine TGF-β1 analysis ELISA kit (Huzhen Biological Technology, Shanghai, China), according to manufacturer's instructions.

Total RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Cells were washed and re-suspended in ice-cold TriZol solution (TransGene, Shanghai, China). Total mRNA extraction was performed using an RNA Easy Kit (TransGene), according to the manufacturer's instructions. The concentration of mRNA was measured using a spectrophotometer (ND 2.0; NanoDrop Technologies, Wilmington, DE, USA). One microgram of total RNA from each sample was reverse-transcribed into cDNA using a TransScript II First-Strand cDNA Synthesis SuperMix (TransGene).

Real-time PCR was performed with an ABI StepOnePlus PCR system (Applied Biosystems, Foster City, CA, USA), using the TransStart Probe qPCR SuperMix (TransGene) and the reaction system recommended by the manufacturer. Specific forward and reverse PCR primers were purchased from Sangong Biotech (Sangong Biotech, Shanghai, China), and are listed in Table 1.GAPDH was used as an internal control in all reactions. The PCR conditions were as follows: activation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, extension at 60 °C for 30 s. Melt curves were generated to evaluate the specificities of the primers. The comparative cycle threshold (Ct) method was used to quantify normalized target gene expression relative to the calibrator. Data are shown as the relative gene quantity (RQ) =$2^{-\Delta\Delta Ct}$.

Western blotting analysis

Mammary tissue was obtained from dairy cows, with or without mastitis, and confirmed by macroscopic dissection, followed by microscopic examination, in our previous work [17]. The tissues were ground into powder in liquid nitrogen, and cells or tissue powders were lysed in PRO-PREP Protein Extraction Solution (iNtRON Biotechnology Inc., Gyeonggi-do, Korea). The precipitate was removed by centrifugation, and the supernatant was mixed with 6 × SDS sample buffer (TransGene) and boiled at 100 °C for 5 min. The mass of total protein was determined using the Bradford Easy Protein Quantitative Kit (TransGene). Total protein, ranging from 20 μg to 40 μg for each sample, was resolved by SDS-PAGE on a 10% Bis-Tris Gel with running buffer, and transferred to

Table 1. PCR primers used in the present study

Gene name	Forward Primers(5'-3')	Reverse Primers(5'-3')
GAPDH	GGCGTGAACCACGAGAAGTA	GGCGTGGACAGTGGTCATAA
α-SMA	GAAGCCCAGCCGAGAACTTT	TCCCACCATCACTCCCTGAT
E-cadherin	AAAGAGAGTGGAAGTGCCCG	GCAGGTGGAGAACCATTGTC
vimentin	GTCCAAGTTTGCTGACCTCTC	TAGTCCCTTTGAGCGCATCC
fibronectin	AACCCTTCCACACCCCAATC	CCGGGAAGCTGAATACCGTT
collagen I	ACTGAAACCCCCGAAAAGCC	GTGGGTCTTCAAGCAAGTGG
albumin	CCAAGGCAACAGAGGAACAAC	TCAGGTAGGCTGAGATGCCTT
MMP2	ACCAAGAACTTCCGCCTGTC	TTCGCCAGATGAATCGGTCC
MMP9	CAGACCTTTGAGGGCGAACT	TACCCATCTCCGTGCTCTCT
Smad2	TGGAGGAGAGCAGAATGGAC	CTGTAAAGGCCTGTTGTATCCC
Smad3	GCCGAGAGTTGAAGCGAAGT	CTCTTGACTGCCTTCTCGCA
Smad4	CCCCAGCCATCAGTTTGTCG	GTCGGCAATAGGCATGGTGT

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polyvinylidene difluoride membranes. The membranes were blocked with 10% non-fat milk in Tris Buffered Saline Tween (TBST) at 23–28 °C for 2 h. The blots were then probed with antibodies against TGF-β1, Smad2 (Phospho-Ser467) and Smad2 (1:500 dilution, Sangong Biotech); α-SMA (1:1000 dilution, Sigma-Aldrich, St Louis, MO, USA); vimentin and collagen type I (1:500 dilution, Beijing Biosynthesis Biotechnology, Beijing, China); albumin (1:1000 dilution, Bethyl Laboratories, Montgomery, TX, USA); E-cadherin (1:500 dilution, Sigma-Aldrich); and GAPDH (1:500 dilution, Santa Cruz Biotechnology, Dallas, TX, USA). Primary antibodies were diluted with 5% non-fat milk and incubated with the membranes at 4 °C overnight. After washing with TBST, the membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. Finally, an enhanced chemiluminescence detection kit (Beyotime, Beijing, China) was used to visualize the immunoreactive proteins.

Immunofluorescence (IF) Staining

BMECs were seeded in 48-well culture dishes and treated with TGF-β1 as mentioned above. The culture medium was discarded after the treatment and the cells were washed three times with normal saline (NS). Cells were fixed with 4% paraformaldehyde in NS for 15 min and permeabilized in 0.5 % Triton X-100/PBS for 5 min at 23–28 °C. After washing with NS, cells were blocked with immunofluorescence sealing fluid (Beyotime) for 1 h at 23–28 °C and incubated with specific primary antibodies at 4 °C overnight. The primary antibodies included anti-α-SMA (1:500; Sigma-Aldrich), anti-vimentin, anti-collagen I (1:200; Beijing Biosynthesis), and rabbit anti-Smad4 (1:200; Sangon Biotech). Finally, the cells were washed with NS and incubated with corresponding fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 2 h at room temperature. Note, for F-actin staining, the cells were only incubated in FITC Phalloidin (Yeasen, Shanghai, China) for 15 min. Hoechst 33342 (Sigma-Aldrich) was used to visualize the nuclei. IF was viewed under an immunofluorescence microscope.

Intramammary challenge with TGF-β1 in mice

Two to three-month-old female Kunming strain mice were obtained from the Laboratory Animal Center of the Fourth Military Medical University (Xi'an, Shaanxi, China) and fed carefully according to the institutional guidelines of Northwest A&F University. Animal experiments conformed to the regulations approved by the Animal Care Commission of the college of Veterinary Medicine, Northwest A&F University.

On days 7 to 10 of lactation, 12 mice were separated into two groups randomly, narcotized with pentobarbital sodium, and fastened supinely. The abdomen areas of all mice were disinfected with alcohol wipes, and the top 2–3 mm of the nipples at the 4th pair of mammary glands were removed quickly with ophthalmic scissors. Then, 50 μL of TGF-β1 (10 ng/mL), diluted in NS, was injected into the 4th left mammary gland through the milk ducts using a 1000-μL syringe with a 30-gauge blunt needle; the 4th right mammary gland was injected with 50 μL of NS as a control. After injection, mice were placed supinely in separate clean and warm cages. One group of mice were given a single injection, and the other group of mice given a multiple injections in quadruplicate with a 3-day interval. Three days after the last injection, the mice were killed by cervical dislocation, and the 4th pair of mammary glands were extracted and fixed in 4% formaldehyde overnight. Four slices of each sample were prepared for routine histopathological analysis using hematoxylin and eosin staining.

Statistical analysis

A two-tailed test was used in this study, and the data are presented as mean ± SD. Differences were considered significant if the p value was less than 0.05 (*P < 0.05; **P < 0.01). All data are representative of at least three different experiments and were analyzed using GraphPad Prism software (San Diego, CA, USA).

Results

TGF-β1 expression in bovine mammary tissue with or without mastitis and inducible inflammatory BMECs.

Analysis of TGF-β1 levels in mammary tissue from four healthy and four mastitic dairy cow mammary glands indicated that TGF-β1 levels were higher in mastitic tissue than in normal tissue (Fig. 1a).

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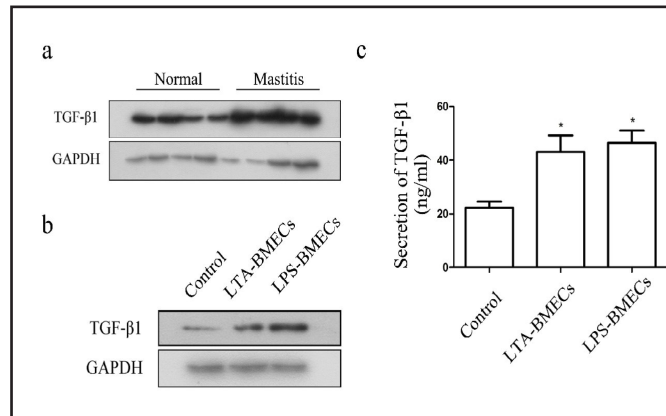

Fig. 1. TGF-β1 levels in bovine mammary tissues or BMECs. (a) TGF-β1 protein levels in four healthy and four mastitic mammary glands were measured by western blotting. GAPDH was used as an internal control. Each lane represents the tissues from a specific individual. (b) TGF-β1 protein levels in lipoteichoic acid (LTA)/lipopolysaccharide (LPS)-induced inflammatory BMECs measured by western blotting. Control, normal BMECs; LTA-BMECs, LTA-treated BMECs; LPS-BMECs, LPS-treated BMECs. (c) TGF-β1 secreted into the cell culture supernatants by LTA/LPS-induced inflammatory BMECs, as measured by ELISA. TGF-β1 secretion was promoted by LTA and LPS. Control, normal BMECs; LTA-BMECs, LTA-treated BMECs; LPS-BMECs, LPS-treated BMECs. Data were expressed as the mean ± SD *p<0.01 vs. control.

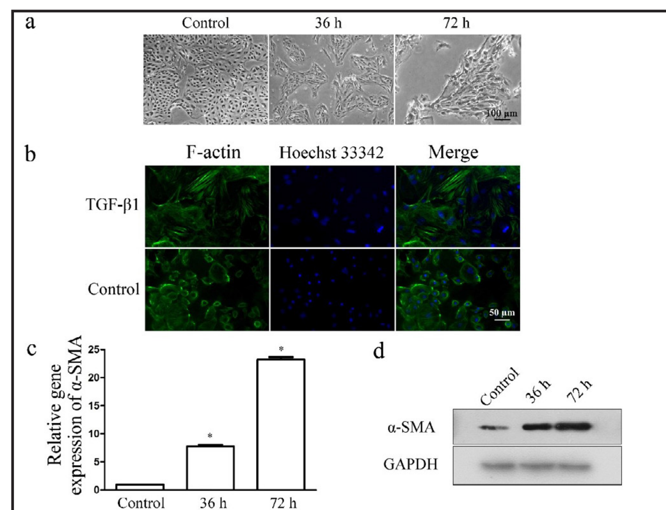

Fig. 2. TGF-β1 induced mesenchymal morphology changes in bovine mammary epithelial cells (BMECs). (a) BMECs showing obvious morphology changes during TGF-β1 treatment under a phase-contrast microscope. Control, normal BMECs; 36 h, BMECs treated with TGF-β1 for 36 h; 72 h, BMECs treated with TGF-β1 for 72 h. (b) Immunofluorescence staining for F-actin (green) in BMECs. Hoechst 33342 stained the nuclei blue. Scale bar, 50 µm. (c) α-SMA gene expression level in BMECs were measured by quantitative real-time PCR. Control, normal BMECs; 36 h, BMECs treated with TGF-β1 for 36 h; 72 h, BMECs treated with TGF-β1 for 72 h. GAPDH was used as internal control. Data were expressed as the mean ± SD *p<0.01 vs. control. (d) α-SMA protein levels in BMECs were measured using western blotting. Control, normal BMECs; 36 h, BMECs treated with TGF-β1 for 36 h; 72 h, BMECs treated with TGF-β1 for 72 h.

To confirm the correlation between TGF-β1 and inflammation, BMECs were treated with LTA or LPS, and TGF-β1 levels were evaluated. The western blotting results showed that TGF-β1 levels in LTA-BMECs and LPS-BMECs were higher than those in normal BMECs (Fig. 1b). Secretion of TGF-β1 in LTA-BMECs and LPS-BMECs was also measured using ELISA and the results were consistent with those obtained by western blotting (Fig. 1c).

TGF-β1 induced mesenchymal phenotype changes in BMECs

To determine the function of TGF-β1, normal BMECs were treated with TGF-β1 (2 ng/mL) for 72 h and photographed every 12 h under a phase-contrast microscope. The images of the control and treated samples at 36 h and 72 h are shown as typical examples. Cells began to change shape at an early stage (24 h), and by 36 h had changed from a hexagon to a spindle-shape, which is recognized as a mesenchymal phenotype. The morphological changes became increasingly obvious throughout the treatment (Fig. 2a). The presence of

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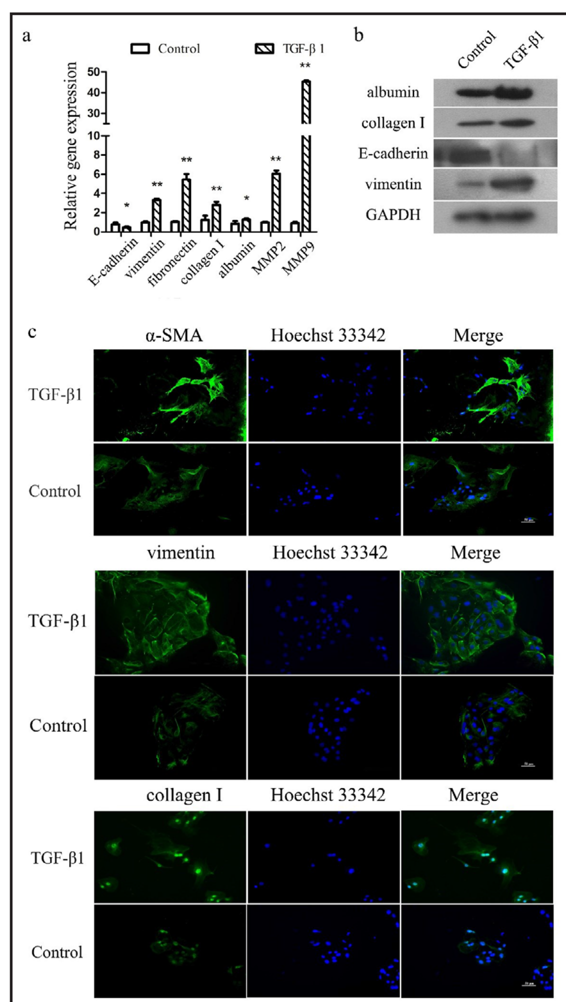

Fig. 3. TGF-β1 induced the epithelial-mesenchymal transition (EMT) and caused extracellular matrix (ECM) protein overexpression in bovine mammary epithelial cells (BMECs). (a) The mRNA levels of E-cadherin, vimentin, fibronectin, collagen I, albumin, MMP2, and MMP9 were measured by quantitative real-time PCR. Data were expressed as the mean ± SD *p<0.05, **p<0.01 vs. control. (b) Albumin, collagen I, E-cadherin, and vimentin protein levels were measured using western blotting. (c) The protein level and location of α-SMA, vimentin, and collagen I were measured using immunofluorescence. Hoechst 33342 stained the nuclei blue. Scale bar, 50 μm.

F-actin indicates stress fibers throughout the cells. The rearrangement of F-actin inside TGF-β1 treated cells was visualized by F-actin staining. The results showed that TGF-β1-treated BMECs acquired a mesenchymal morphology (Fig. 2b), which correlated with the cell shape observed under the phase-contrast microscope.

TGF-β1 promoted the expression of α-SMA in BMECs

In addition to morphological changes, the up-regulation of α-SMA, a well-known marker of EMT, was confirmed at the gene and protein levels (Fig. 2c–d). BMECs were treated with TGF-β1 (2 ng/mL) for 72 h, and harvested at 36 h and 72 h. Samples analyzed by qPCR and western blotting to determine if TGF-β1 induced α-SMA expression. The mRNA levels of *α-SMA* reached a peak at 72 h. As a result, we treated BMECs with TGF-β1 at a concentration of 2 ng/mL for 72 h as the uniform treatment method for subsequent experiments.

TGF-β1 induced an EMT state in BMECs

The significant morphological changes and up-regulation of α-SMA levels suggested that TGF-β1 induces EMT in BMECs. To confirm this, the expression levels of several genes encoding widely recognized EMT markers, including E-cadherin, vimentin, fibronectin, collagen I, albumin, matrix metalloproteinase-2 (MMP2), and matrix metalloproteinase-9 (MMP9), were examined. The results of the qPCR analysis showed that TGF-β1 down-regulated the expression of E-cadherin, whereas it up-regulated the mRNA expression of all the other EMT-related genes (Fig. 3a). This is supported by western blot analysis of albumin, collagen I, E-cadherin, and vimentin protein levels (Fig. 3b). The results were further confirmed by IF staining showing elevated levels of α-SMA, vimentin, and collagen I following TGF-β1 treatment (Fig. 3c).

TGF-β1 upregulated the expressions of Smad2 and Smad3 in BMECs

To understand how TGF-β1 causes EMT in BMECs, activation of the TGF/Smad signaling pathway, which is closely related to EMT, was examined. Gene expressions of *Smad2, Smad3*, and *Smad4,* which are key molecules in the TGF/Smad pathway, were evaluated using qPCR.

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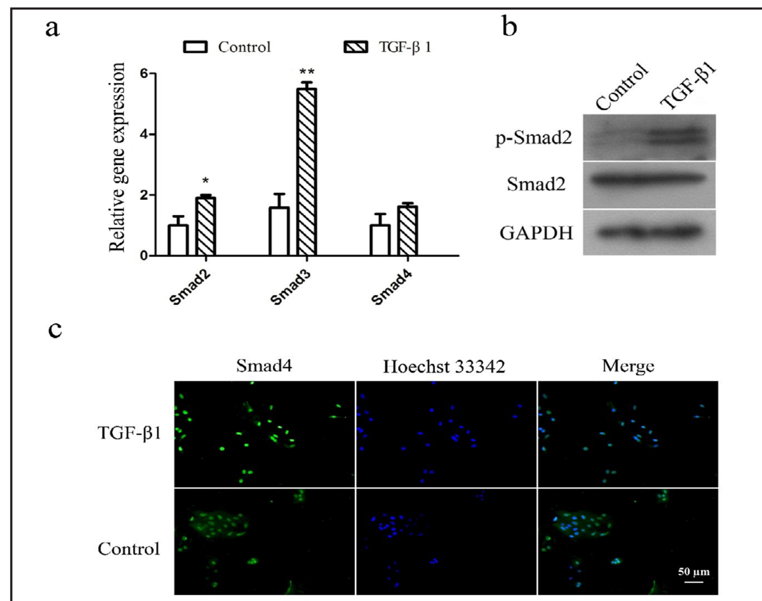

Fig. 4. TGF-β1 induced the up-regulation of Smad2 and Smad3 and caused translocation of Smad4. (a) Smad2, Smad3, and Smad4 mRNA expression as measured using quantitative real-time PCR. Data were expressed as the mean ± SD *p<0.05, **p<0.01 vs. control. (b) The amount of p-Smad2 was measured using western blotting. (c) The location of Smad4 was visualized using immunofluorescence staining.

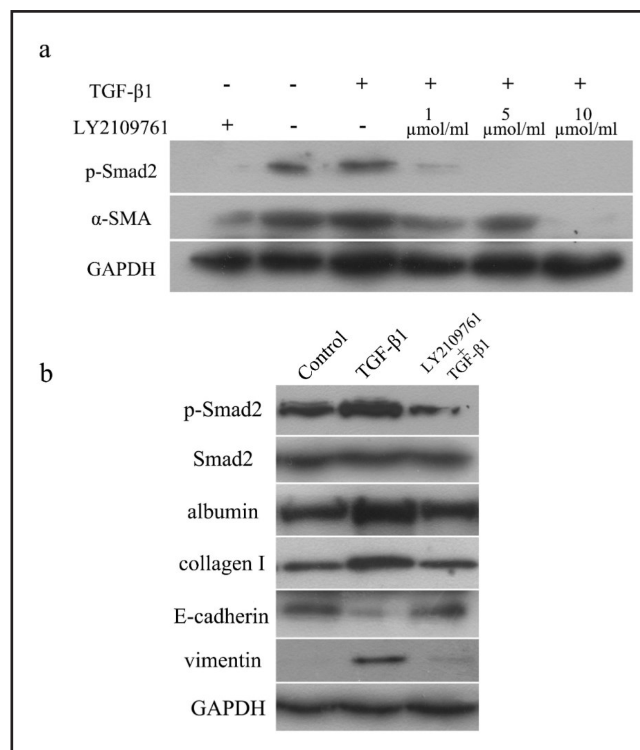

Fig. 5. LY2109761 inhibited the function of TGF-β1 in bovine mammary epithelial cells (BMECs). (a) The protein expression of p-Smad2 and α-SMA in BMECs was measured using western blotting. LY2109761 was added at the following concentrations: 1 μm/mL, 5 μm/mL, and 10 μm/mL. (b) The amounts of p-Smad2, Smad2, albumin, collagen I, E-cadherin, and vimentin in BMECs were measured using western blotting. Smad2 was used as an internal control for p-Smad2. Control, normal BMECs; TGF-β1, BMECs treated with TGF-β1; LY2109761+ TGF-β1, BMECs treated with LY2109761 and TGF-β1.

Smad2 and *Smad3* expressions were up-regulated after TGF-β1 stimulation; however, the expression of *Smad4* did not change (Fig. 4a).

Phosphorylation of Smad2 is a universally recognized marker of the activated TGF/Smad signaling pathway [19]. Using western blotting, we showed that level of p-Smad2 was elevated significantly after TGF-β1 treatment (Fig. 4b).

Smad4 is translocated into the nucleus of BMECs after treatment with TGF-β1

Previous research showed that Smad4 is transported into the nucleus when the TGF/Smad signaling pathway is activated [20]. In the present study, IF staining of Smad4 showed that in normal BMECs, Smad4 was distributed in both the cytoplasm and the nucleus. When

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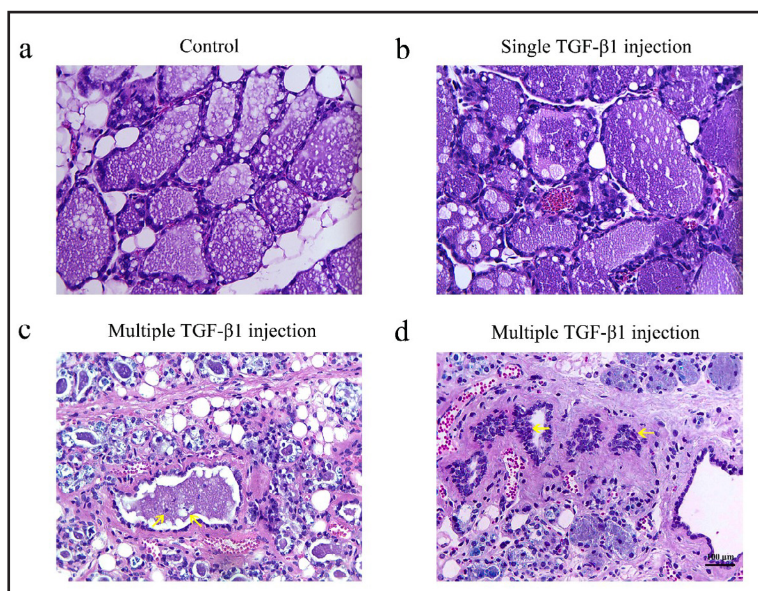

Fig. 6. TGF-β1 induced inflammation and fibrosis in mice mammary glands. Mouse mammary glands injected with TGF-β1 or normal saline (NS) (Control) were examined using hematoxylin and eosin staining. (a) The alveoli in the mammary tissue of mice in the control group exhibited a large luminal area, an integrated epithelium, and limited stromal areas with little adipose tissue. (b) No obvious pathological changes were observed in mammary tissue sections of mice that received a single TGF-β1-injection. (c) Alveoli in an area of the mammary gland of mice that received multiple TGF-β1-injections were characterized by infiltration of leukocytes in the lumen. The arrows indicate infiltrated leukocytes. (d) Damaged alveoli, a stratified epithelium, and a large number of collagen fibers around the alveoli were detected in the mammary tissue section of mice that received multiple TGF-β1-injections. The arrows indicate the change from a simple cuboidal epithelium to a stratified epithelium. Scale bar, 100 μm.

treated with TGF-β1, however, Smad4 was enriched in the nucleus and was barely detectable in the cytoplasm, which indicated that Smad4 is translocated into the nucleus after TGF-β1 stimulation (Fig. 4c).

LY2109761 suppressed SMAD2 phosphorylation and α-SMA expression

To further confirm that TGF-β1 causes EMT in BMECs via TGF/Smad signaling, TGF receptors I and II were inhibited using LY2109761, which was added under a concentration gradient. The appropriate experimental concentration was chosen based on the down-regulation of p-Smad2 and α-SMA. Western blotting analysis indicated that p-smad2 and α-SMA levels were down-regulated strongly by 10 μmol/mL LY2109761 (Fig. 5a), which indicated the successful inhibition of the TGF/Smad signaling pathway. Thus, 10 μmol/mL was chosen as the final concentration for use in subsequent experiments.

LY2109761 suppressed TGF-β1-induced protein level changes

LY2109761 was added into the culture medium 6 h before TGF-β1 treatment. Up-regulated p-Smad2, albumin, collagen I, and vimentin levels were observed in the TGF-β1 treated group, but disappeared in the LY2109761+TGF-β1 treatment group (Fig. 5b). Conversely, the E-cadherin level increased in the LY2109761+TGF-β1 treatment group compared with the TGF-β1 treatment group. The results indicated that TGF-β1's effects on BMECs are inhibited by LY2109761.

TGF-β1 caused obvious inflammation and fibrosis in a mouse model

In the present study, we demonstrated the fibrosis-inducing function of TGF-β1 in BMECs; however, to ascertain whether TGF-β1 also induces fibrosis *in vivo*, a mouse model was used. No fibrosis or inflammation was observed in the control group or the TGF-β1 treated group with a single injection. Alveoli with large luminal areas were integrated; no infiltration of leukocytes in the alveoli or hyperplasia in the connective tissue around the

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alveoli were observed in the tissue sections (Fig. 6a and b). However, significant fibrosis and inflammatory infiltration were evident in the mammary glands in those mice that received multiple injections, as indicated by damaged alveoli, stratified epithelium, and the large number of collagen fibers around the alveoli. In addition, some alveoli were filled with exfoliative epithelium (Fig. 6c).

Discussion

This study was the first to confirm that TGF-β1 can induce EMT and the accumulation of ECM proteins in BMECs. We demonstrated that aberrant up-regulation of TGF-β1 in the mammary glands of bovine with mastitis could cause a mesenchymal morphology and functional changes in BMECs via the activation of the TGF/Smad signaling pathway, thereby contributing to the fibrogenesis of bovine mammary glands. Exogenous TGF-β1 also induced mammary fibrosis in mice. Thus, our findings provide a theoretical basis for the prevention of bovine mammary fibrosis induced by mastitis.

TGF-β1 is a multifunctional cytokine that modulates diverse cellular activities, such as regulating the growth, differentiation, and function of immune and non-immune cells. Up-regulated expression of TGF-β1 has been described in the rat small intestine and mouse mammary glands, mainly in infected cells showing an inflammatory response [21]. This study represents the first demonstration that the level of TGF-β1 is increased in mammary tissue with mastitis and in LPS or LTA-induced inflammatory BMECs. Accumulating evidence suggests that TGF-β1 promotes fibrosis in many kinds of tissues [6, 7, 22, 23], and the critical role played by TGF-β1 in the initiation, development, and persistence of radiation fibrosis has been reviewed [21]. Inflammation is recognized as the earliest stage of fibrosis, and long-term inflammation is associated with the development of fibrogenesis [24, 25]. In other words, inflammation is essential for the onset of fibrosis [26]. Mastitis has a high incidence and infection rate worldwide, and causes varying extents of bovine mammary fibrosis. Thus, identifying the role of elevated TGF-β1 in mammary glands in the process of mastitis and clarifying its mechanism of action might lead to the development of new methods to resist the fibrosis-inducible function of TGF-β1 in bovine mastitis.

With this goal in mind, we developed an *in vitro* model by treating cultured BMECs with TGF-β1 to investigate whether TGF-β1 mediates the bovine mammary fibrosis caused by mastitis. EMT is associated with fibrosis in the kidney, liver, and intestines [27-29]. Up-regulation of α-SMA and vimentin and down-regulation of E-cadherin are reliable markers that characterize EMT during the development of fibrosis in a variety of organs [30-32]. As expected, our findings revealed that TGF-β1 induces EMT in BMECs, with marked molecular changes.

ECM proteins, such as fibronectin and collagen I, constitute complex grid structures that support and connect surrounding connective tissues [33]. The overexpression of ECM proteins, combined with the obstruction of their degradation, contributes to the accumulation of the ECM, eventually leading to tissue fibrosis [34-38]. Moreover, Kim et al. found that the ECM can regulate EMT during pulmonary fibrosis [39]. Using western blotting analysis, we proved that TGF-β1 caused an increase in the level of collagen I, a major ECM protein (Fig. 3b). In addition, histopathological analysis of mouse mammary glands revealed dense bundles of collagen fibers in the glands. Consequently, accumulation of the ECM stimulated the expression of *MMP2* and *MMP9*. We also observed an increase in albumin levels after TGF-β1 treatment, which contradicts a previous report [7].

When the TGF/Smad signaling pathway is activated, Smad2 is phosphorylated by a type I receptor kinase, and Smad4 is translocated into the nucleus [19, 40]. Furthermore, Smad3 is present at high levels in fibrotic tissues [41], and up-regulated *Smad2* expression compensates for *Smad3* [42] [43]. We found that the mRNA expressions of *Smad2* and *Smad3* were elevated and the amount of phosphorylated Smad2 was up-regulated conspicuously. In addition, Smad4 was translocated to the nucleus, as shown in the IF staining result.

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LY2109761 blocked the effects of TGF-β1 treatment, which indicated that TGF-β1 induces EMT and ECM-related protein accumulation via the TGF/Smad signaling pathway.

Based on these results, we assessed whether TGF-β1 induces mammary fibrosis *in vivo*. Pathological analysis showed apparent fibrosis and inflammation in mammary glands that were injected with TGF-β1 four times, but not in those treated once, suggesting that long-term TGF-β1 treatment can induce mammary infection and fibrosis in mice, which also supported the correlation between mastitis and mammary fibrosis.

Conclusion

The high level of TGF-β1 in mastitic tissues and LTA/LPS-induced inflammatory BMECs suggested that TGF-β1 is an important factor in the transition phase from mammary inflammation to fibrosis. Characteristic changes in the morphology and function of TGF-β1-treated BMECs suggested that TGF-β1 plays a significant role in EMT and in ECM protein accumulation. The inflammation and fibrosis of mouse mammary glands induced by TGF-β1 further indicated the crucial role of TGF-β1 in bovine mammary fibrosis. However, further study of the function of TGF-β1 in bovine mammary fibrosis is needed to develop new therapies to stop deterioration of mammary fibrosis at the early stage.

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Disclosure Statement

The authors declare that they have no Disclosure Statement.

References

1 Wu J, Ding Y, Bi Y, Wang Y, Zhi Y, Wang J, Wang F: Staphylococcus aureus induces TGF-β1 and bFGF expression through the activation of AP-1 and NF-κB transcription factors in bovine mammary gland fibroblasts. Microb Pathog 2016;95:7-14.

2 Ruegg PL: Investigation of mastitis problems on farms. Vet Clin North Am Food Anim Pract. 2003;19:47-73.

3 Min X, Jing C, Hong W, Meng Z, Ping X, Huang H, Peng W, Du F, Gong A, Zhang Y: Scoparone Protects Against Pancreatic Fibrosis via TGF-β/Smad Signaling in Rats. Cell Physiol Biochem 2016;40:277.

4 Liu X, Hu H, Yin JQ: Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. Liver Int 2006;26:8-22.

5 Pan B, Liu G, Jiang Z, Zheng D: Regulation of renal fibrosis by macrophage polarization. Cell Physiol Biochem 2015;35:1062-1069.

6 Kanzler S, Lohse AW, Keil A, Henninger J, Dienes HP, Schirmacher P, Rose-John S, zum Büschenfelde KH, Blessing M: TGF-beta1 in liver fibrosis: an inducible transgenic mouse model to study liver fibrogenesis. Am J Physiol 1999;276:1059-1068.

7 Kaimori A, Potter J, Kaimori J, Wang C, Mezey E, Koteish A: Transforming Growth Factor-β1 Induces an Epithelial-to-Mesenchymal Transition State in Mouse Hepatocytes *in vitro*. J Biol Chem 2007;282:22089-22101.

8 Wang J, Bao L, Yu B, Liu Z, Han W, Deng C, Guo C: Interleukin-1beta Promotes Epithelial-Derived Alveolar Elastogenesis via alphavbeta6 Integrin-Dependent TGF-beta Activation. Cell Physiol Biochem 2015;36:2198-2216.

Downloaded from http://karger.com/cpb/article-pdf/43/1/82/2444204/000480321.pdf by guest on 15 March 2025

9 Wang B, Ding W, Zhang M, Li H, Gu Y: Rapamycin Attenuates Aldosterone-Induced Tubulointerstitial Inflammation and Fibrosis. Cell Physiol Biochem 2015;35:116-125.

10 Thiery JP: Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002;2:442-454.

11 Lucena MC, Carvalho-Cruz P, Donadio JL, Oliveira IA, de Queiroz RM, Marinho-Carvalho MM, de Paula IF, Gondim KC, Mccomb ME, Costello CE: Epithelial mesenchymal transition induces aberrant glycosylation through hexosamine biosynthetic pathway activation. J Biol Chem 2016;291:jbc.M116.729236.

12 Ying X, Sun Y, He P: Bone Morphogenetic Protein-7 Inhibits EMT-Associated Genes in Breast Cancer. Cell Physiol Biochem 2015;37:1271-1278.

13 Hu Q, Tong S, Zhao X, Ding W, Gou Y, Xu K, Sun C, Xia G: Periostin Mediates TGF-β-Induced Epithelial Mesenchymal Transition in Prostate Cancer Cells. Cell Physiol Biochem 2015;36:799.

14 Zhou Q, Zheng X, Chen L, Xu B, Yang X, Jiang J, Wu C: Smad2/3/4 Pathway Contributes to TGF-β-Induced MiRNA-181b Expression to Promote Gastric Cancer Metastasis by Targeting Timp3. Cell Physiol Biochem 2016;39:453.

15 Chen XF, Zhang HJ, Wang HB, Zhu J, Zhou WY, Zhang H, Zhao MC, Su JM, Gao W, Zhang L: Transforming growth factor-β1 induces epithelial-to-mesenchymal transition in human lung cancer cells via PI3K/Akt and MEK/Erk1/2 signaling pathways. Mol Biol Rep 2012;39:3549-3556.

16 Zhang L, Li Z, He W, Xu L, Wang J, Shi J, Sheng M: Effects of Astragaloside IV Against the TGF-β1-Induced Epithelial-to-Mesenchymal Transition in Peritoneal Mesothelial Cells by Promoting Smad 7 Expression. Cell Physiol Biochem 2015;37:43-54.

17 Chen Q, He G, Zhang W, Xu T, Qi H, Li J, Zhang Y, Gao MQ: Stromal fibroblasts derived from mammary gland of bovine with mastitis display inflammation-specific changes. Sci Rep 2016;6:

18 Zhang W, Li X, Xu T, Mengru M, Zhang Y, Gao MQ: Inflammatory responses of stromal fibroblasts to inflammatory epithelial cells are involved in the pathogenesis of bovine mastitis. Exp Cell Res 2016;349:45-52.

19 Massagué J, Wotton D: Transcriptional control by TGF-β/Smad signaling. EMBO J. 2000;19:1745-1754.

20 Xu L: Regulation of Smad activities. Biochim Biophys Acta 2006;1759:503-513.

21 Martin M, Lefaix J, Delanian S: TGF-beta1 and radiation fibrosis: a master switch and a specific therapeutic target? Int J Radiat Oncol Biol Phys 2000;47:277-290.

22 Liu N, He S, Ma L, Ponnusamy M, Tang J, Tolbert E, Bayliss G, Zhao TC, Yan H, Zhuang S: Blocking the class I histone deacetylase ameliorates renal fibrosis and inhibits renal fibroblast activation via modulating TGF-beta and EGFR signaling. PLoS One 2013;8:e54001-e54001.

23 Kang HR, Cho SJ, Lee CG, Homer RJ, Elias JA: Transforming growth factor (TGF)-beta1 stimulates pulmonary fibrosis and inflammation via a Bax-dependent, bid-activated pathway that involves matrix metalloproteinase-12. J Biol Chem 2007;282:7723-7732.

24 Wick G, Backovic A, Rabensteiner E, Plank N, Schwentner C, Sgonc R: The immunology of fibrosis: innate and adaptive responses. Trends Immunol 2010;31:110-119.

25 Torres IB, Moreso F, Sarro E, Meseguer A, Seron D: The Interplay between inflammation and fibrosis in kidney transplantation. Biomed Res Int 2014;2014:750602.

26 Wick G, Grundtman C, Mayerl C, Wimpissinger TF, Feichtinger J, Zelger B, Sgonc R, Wolfram D: The immunology of fibrosis. Annu Rev Immunol 2013;31:107-135.

27 Zeisberg M, Kalluri R: The role of epithelial-to-mesenchymal transition in renal fibrosis. J Mol Med (Berl) 2004;82:175-181.

28 Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, Kalluri R: Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. J Biol Chem 2007;282:23337-23347.

29 Kalluri R, Weinberg RA: The basics of epithelial-mesenchymal transition. J Clin Invest 2009;119:1420-1428.

30 Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG: Identification and characterization of a fibroblast marker: FSP1. J Cell Biol 1995;130:393-405.

31 Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R: BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med.2003;9:964-968.

32 Sa Y, Li C, Li H, Guo H: TIMP-1 Induces α-Smooth Muscle Actin in Fibroblasts to Promote Urethral Scar Formation. Cell Physiol Biochem 2015;35:2233-2243.

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33 Beachley VZ, Wolf MT, Sadtler K, Manda SS, Jacobs H, Blatchley MR, Bader JS, Pandey A, Pardoll D, Elisseeff JH: Tissue matrix arrays for high-throughput screening and systems analysis of cell function. Nat Methods 2015;12:1197-1204.
34 Ruperez M, Lorenzo O, Blanco-Colio LM, Esteban V, Egido J, Ruiz-Ortega M: Connective tissue growth factor is a mediator of angiotensin II-induced fibrosis. Circulation 2003;108:1499-1505.
35 Pardo A, Selman M: Matrix metalloproteases in aberrant fibrotic tissue remodeling. Proc Am Thorac Soc.2006;3:383-388.
36 Thomasset N, Lochter A, Sympson CJ, Lund LR, Williams DR, Behrendtsen O, Werb Z, Bissell MJ: Expression of autoactivated stromelysin-1 in mammary glands of transgenic mice leads to a reactive stroma during early development. Am J Pathol 1998;153:457-467.
37 Sternlicht MD, Lochter A, Sympson CJ, Bing H, Rougier JP, Gray JW, Dan P, Bissell MJ, Werb Z: The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell 1999;98:137-146.
38 Ha HY, Moon HB, Nam MS, Lee JW, Ryoo ZY, Lee TH, Lee KK, So BJ, Sato H, Seiki M: Overexpression of Membrane-type Matrix Metalloproteinase-1 Gene Induces Mammary Gland Abnormalities and Adenocarcinoma in Transgenic Mice. Cancer Res 2001;61:984-990.
39 Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA: Alveolar epithelial cell mesenchymal transition develops *in vivo* during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci U S A 2006;103:13180-13185.
40 Heldin CH, Miyazono K, Ten DP: TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 1998;390:465-471.
41 Xiaodong S, Weili L, Shuyang X, Meirong W, Guohong C, Cuiping M, Changjun L: All-transretinoic acid ameliorates bleomycin-induced lung fibrosis by downregulating the TGF-β1/Smad3 signaling pathway in rats. Lab Invest 2013;93:1219–1231.
42 Flanders KC, Major CD, Arabshahi A, Aburime EE, Okada MH, Fujii M, Blalock TD, Schultz GS, Sowers A, Anzano MA: Interference with transforming growth factor-beta/ Smad3 signaling results in accelerated healing of wounds in previously irradiated skin. Am J Pathol 2003;163:2247-2257.
43 Kim MJ, Park SA, Kim CH, Park SY, Kim JS, Kim DK, Nam JS, Sheen YY: TGF-β Type I Receptor Kinase Inhibitor EW-7197 Suppresses Cholestatic Liver Fibrosis by Inhibiting HIF1α-Induced Epithelial Mesenchymal Transition. Cell Physiol Biochem 2016;38:571.

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