CCN2 and CCN5 exerts opposing effect on fibroblast proliferation and transdifferentiation induced by TGF-β

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SUMMARY

Epidural fibrosis might occur after lumbar discectomy and contributes to failed back syndrome. Transforming growth factor (TGF)-β has been reported to influence multiple organ fibrosis, in which connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed 2 (CCN2) and CCN5 are involved. However, the effect of CCN2 and CCN5 on TGF-β induced fibrosis has not yet been elucidated. This study reports that CCN2 and CCN5 play opposing roles in cell proliferation and transdifferentiation of human skin fibroblasts or rabbit epidural scar-derived fibroblasts exposed to TGF-β. We observed that TGF-β1 induced fibroblasts proliferation and differentiation in a dose-dependent manner (from 0 μg/L to 20 μg/L). Meanwhile, CCN2 expression is up-regulated while CCN5 expression is inhibited by TGF-β1 exposure. Furthermore, it is demonstrated that CCN2 overexpression leads to promoted proliferation and elevated collagen and α-smooth muscle actin (α-SMA) expression, which are inhibited by CCN5 overexpression. Moreover, it is shown that the cysteine knot (CT) domain, present in CCN2 but absent in CCN5, plays an essential part in fibroblast proliferation and differentiation. Additionally, enhanced TGF-β and CCN2 expression but decreased CCN5 expression is found in rabbit epidural scar-derived fibroblasts. Overall, the results show the opposing effects of CCN2 and CCN5 on fibroblast proliferation and transdifferentiation induced by TGF-β.

Key words: CCN2, CCN5, epidural scar, fibroblasts, proliferation, transdifferentiation.

INTRODUCTION

Epidural fibrosis, resulting from lumbar discectomy, causes compression and/or stretching of the associated nerve root or the dura mater, contributes to post-laminectomy syndrome or failed back syndrome, and may occur in 24% of patients who undergo surgery.1–3 Meanwhile, epidural fibrosis will enhance the risk of nerve root damage, dural tears, and iatrogenic injuries and make it inconvenient for subsequent exploration of the operative field.4–6

It has been well established that epidural fibrosis formation is a complex multi-step process associated with a significant reduction in the tissue cellularity and excessive deposition of collagen, fibronectin and dermatan sulphate (DS).7 Accumulated evidence has shown that specific cytokines like interleukin (IL)-2, transforming growth factor (TGF)-β and IL-4 significantly promote the progression of fibrogenesis.8,9 In the fibrotic process, fibroblasts are activated by these cytokines, produce multiple collagens and form permanent fibrotic scars.10

The TGF-β family is widely expressed by many types of cells and contributes to matrix homeostasis and fibrosis.11 Various cellular responses, including cell proliferation, differentiation, extracellular matrix remodelling, embryonic development and apoptosis, were regulated by TGF-β.12,13 Additionally, TGF-β may bind to and bring together type I and type II receptor serine/threonine kinases on the cell surface, initiating signalling transduction.14 Then, the TβRII was engaged to phosphorylate TβRII kinase, which propagates the phosphorylation of signalling mother against decapentaplegic peptide 2 (Smad2) and Smad3 at their C-terminus and promotes their binding to Smad4 to form a heteromeric Smad complex.14 The Smad complex will accumulate in the nucleus and up-regulate the expressions of extracellular matrix proteins, including fibronectin, collagen and proteoglycans.14

Connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed 2 (CCN2) mediates multiple downstream effects of TGF-β1 leading to deposition of extracellular matrix (ECM).15 TGF-β1, as well as many other factors including hypoxia-inducible factor (HIF), angiotensin II, Smad and endothelin-1, induces CCN2 expression.16 In turn, CCN2 may modulate the signal-transducing pathways involved Smad and ERK1/2 MAPK and lead to organ fibrosis.17 Indeed, the role of CCN2 in wound healing and organ fibrosis has been reported by...
many studies. CCN2 was demonstrated to promote wound healing in a monkey model of burn-induced tissue repair.18 In a mouse model, the subcutaneous injection of either TGF-β or CCN2 alone produced only a minor transient reaction.19 However, when CCN2 and TGF-β were given together, a more persistent skin fibrotic response was induced.19

CCN5 is a special member of the CCN family of proteins lacking a cysteine-rich carboxyl-terminal repeat (CT) domain.20,21 CCN5 might act as a dominant negative regulator of other CCN family members.22 Yoon et al.23 showed that CCN2 promoted hypertrophy and fibrosis, whereas CCN5 inhibited hypertrophy and fibrosis in the heart. Furthermore, CCN5 may antagonize cardiac hypertrophy induced by CCN2.23 In vascular smooth muscle cells (VSMCs), CCN5 overexpression inhibits cell proliferation, motility, and invasiveness.24 Nevertheless, the absence of CCN5 expression results in the elevated expression of the TGF-β signalling components, favouring epithelial-mesenchymal transition (EMT) and associated cellular invasion in breast cancer cell lines.25

However, few reports have elucidated the effect of CCN2 and CCN5 on TGF-β-induced fibrosis. In the current study, with human foreskin tissue-derived fibroblasts, it was demonstrated that TGF-β exposure promoted cell viability and transdifferentiation. TGF-β exposure also up-regulated CCN2 expression, but down-regulated CCN5 expression in human skin fibroblasts. Furthermore, CCN2 overexpression led to augmented cell viability and increased collagen and α-SMA protein expression, while CCN5 overexpression resulted in the opposite effect. Additionally, it is demonstrated that the CT domain, which is absent in the CCN5 protein, plays an essential role in the induction of fibroblast differentiation. Lastly, fibroblasts from epidermal scars were proven to have an enhanced level of TGF-β and CCN2 expression, but decreased CCN5 levels. Taken together, these results show the opposing effect of CCN2 and CCN5 on fibrosis induced by TGF-β.

**RESULTS**

TGF-β1 induces proliferation and phenotype alteration of human skin fibroblasts

To investigate the effect of TGF-β1 on fibroblast, human foreskin tissue-derived fibroblasts were starved under serum-free conditions for 12 h and treated with 0, 5, 10, 15, and 20 μg/L TGF-β1 for 48 h. At the end of the treatment, cell viability of these fibroblasts was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT). Compared with the control (0 μg/L TGF-β1), cell viabilities of fibroblasts were increased in a dose-dependent manner (Fig. 1a). Furthermore, the expressions of type I, type III collagen and α-smooth muscle actin (α-SMA) were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. As the results show, type I, type III collagen and α-SMA mRNA and protein were up-regulated by TGF-β1 (Fig. 1b–h). In parallel, α-SMA expression was also analyzed in fibroblasts by immunofluorescence and an enhancement of α-SMA expression under TGF-β1 circumstances was observed (Fig. 2a). These results indicate that TGF-β1 may induce the proliferation and differentiation of human skin fibroblasts in a dose-dependent manner.

TGF-β1 promotes CCN2 expression but inhibits CCN5 expression in human skin fibroblasts

Matricellular protein CCN2 is reported to be rapidly induced by fibrogenic cytokines and highly expressed in various fibrotic conditions.26,27 Next the expression of CCN2 was examined along with a dominant negative molecule CCN5 in TGF-β1 exposed human skin fibroblasts. After treating with TGF-β1 for 48 h, CCN2 mRNA and protein levels were significantly elevated in fibroblasts compared with those treated with 0 μg/L TGF-β1 (Fig. 2b,d,f). In contrast, CCN5 mRNA and protein levels were remarkably reduced by TGF-β1 treatment (Fig. 2c,e,g). These results revealed that TGF-β1 may cause CCN2 up-regulation and CCN5 down-regulation in human skin fibroblasts.

CCN2 and CCN5 play opposite roles in modulating fibroblasts proliferation and differentiation

Next, we examined the role of CCN2 and CCN5 in fibrosis. Human skin fibroblasts were transfected with AdCCN2 or blank vector and exposed to 20 μg/L TGF-β1 for 48 h. AdCCN2 transfection led to 2.6-fold promotion of CCN2 mRNA expression in fibroblasts (Fig. 3a). Subsequently, fibroblast proliferation was evaluated at 24, 48, 72, and 96 h after the transfection and was significantly promoted by AdCCN2 transfection and TGF-β1 exposure (Fig. 3c). Furthermore, human skin fibroblasts were infected with AdCCN5 or blank vector before TGF-β1 exposure. AdCCN5 transfection caused 2.3-fold promotion of CCN5 mRNA expression (Fig. 3b) and extremely inhibited cell proliferation under TGF-β1 exposure (Fig. 3d). It was also investigated if overexpression of CCN2 might modulate CCN5 expression and if overexpression of CCN5 might modulate CCN2 expression. The results of immunoblot showed that AdCCN2 transfection did not influence the expression of CCN5 protein (Fig. 4a,c), while AdCCN5 transfection did not alter CCN2 protein expression (Fig. 4b,d) under TGF-β1 exposure. Intriguingly, when TGF-β1 was removed, AdCCN2 transfection decreased CCN5 expression at 48 h after the transfection (Fig. 4e,g) and AdCCN5 infection diminished CCN2 expression at 48 h after the transfection (Fig. 4f,h). In addition, Type I, Type III collagen and α-SMA protein were markedly up-regulated by AdCCN2 transfection (Fig. 5a–e) but were down-regulated by AdCCN5 transfection (Fig. 5f–j). These results suggest that CCN2 and CCN5 caused the opposite effect on human skin fibroblasts.

CT domain of CCN2 is essential for fibroblasts differentiation

It has been reported that cysteine knot (CT) domain, a critical part of CCN2, which is lacking from CCN5, may act as a potential proliferation module.15 To explore whether the CT domain plays a role in fibroblast differentiation, two adenoviral vectors, AdCCN2 (CT−) and AdCCN5 (CT+), were constructed and transfected into human skin fibroblasts. AdCCN2 (CT−) encodes a CCN2 protein without the CT domain (Fig. 6a) and AdCCN5 (CT+) encodes a CCN5 protein fused with the CT domain (Fig. 6f). Consequently, AdCCN2 (CT−) infection led to the inhibition of phosphorylated-smad1 (p-smad1), phosphorylated ERK (p-ERK) and α-SMA
Fig. 1 Transforming growth factor (TGF)-β1 induces proliferation and differentiation of human skin fibroblasts. (a) Evaluation of fibroblasts viabilities by MTT; (b,c,d) Analysis of type I, type III collagen and α-smooth muscle actin mRNA expression by quantitative real-time polymerase chain reaction (qRT-PCR); (e,f,g) Analysis of type I, type III collagen and α-smooth muscle actin protein levels by Western blot. G1, G2, G3, G4 indicated 5, 10, 15, or 20 μg/L TGF-β1 exposure groups. *P < 0.05 vs control.
protein expression in fibroblasts (Fig. 6b–e). In contrast, AdCCN5 (CT+) infection augmented the expressions of p-smad1, p-ERK and α-SMA protein (Fig. 6g–j). These results confirmed that the CT domain of CCN2 plays an essential role in the induction of fibroblast differentiation, in which smad1/ERK signalling was involved.

TGF-β1 and CCN2 expressions are increased while CCN5 expressions are decreased in epidural scar-derived fibroblasts

To further investigate the role of CCN2 and CCN5 in epidural fibrosis, total L5–6 laminectomy was performed on 8 rabbits to establish animal models. After the surgery for 24 weeks, obvious
epidermal scar formation was observed near the dura mater via HE staining (Fig. 7a). Twenty-four weeks after the surgery, fibroblasts were isolated from the epidural scar of these rabbit models. Total mRNA and protein were prepared and the expressions of TGF-β1, CCN2 and CCN5 were analyzed. Compared with fibroblasts derived from normal skin, TGF-β1 and CCN2 mRNA and protein were significantly enhanced in those from epidural scars while CCN5 mRNA and protein were utterly impeded (Fig. 7b–h).

DISCUSSION
The secreted cytokine TGF-β1 significantly regulates the phenotype and function of fibroblasts. Fibroblasts are activated by TGF-β stimulation and transdifferentiated into myofibroblasts, which play key roles in fibrotic states. Numerous studies have reported that multiple organs, especially the liver, kidney, lung and skin, undergo pathological fibrosis caused by TGF-β. In the current study, it was found that cell viabilities of human skin fibroblasts were promoted by TGF-β exposure in a dose-dependent manner. Meanwhile, collagen and α-SMA expressions of fibroblasts were also up-regulated by TGF-β. These results suggested that TGF-β exposure might induce phenotype transition from fibroblast to myofibroblasts. It has been reported that collagen expression triggered by TGF-β is mediated by Smad3 signalling, which along with Focal Adhesion Kinase (FAK), c-Jun N-terminal kinase (JNK), transforming growth factor–activated kinase (TAK), and PI3kinase/Akt pathways is also critical for α-SMA synthesis. Furthermore, TGF-β also suppresses the activity of matrix metalloproteinases (MMPs) and induces the synthesis of protease inhibitors like plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinases (TIMPs). Transforming growth factor-β is a potent inducer of CCN2 and promotes CCN2 expression in dermal fibroblasts via a complex network of transcriptional interactions requiring Smads, protein kinase C and Ras/mitogen-activated protein kinase (MEK)/extracellular signal-regulated protein kinase (ERK) and an Ets-1/transcription enhancer factor binding element in the CCN2 promoter. In the current study, CCN2 expressions in human skin fibroblasts were induced by TGF-β exposure while CCN5 expressions were down-regulated. Overexpressed CCN2 has been found in many fibrotic diseases of various organs, suggesting an important role of CCN2 in excessive matrix deposition. A previous study has demonstrated that transgenic mice with
CCN2-overexpressed fibroblasts would develop fibrosis in multiple organs, whereas mice lacking CCN2 expression were protected from the bleomycin-induced dermal fibrosis. Moreover, the results of Nakerakanti et al. showed that CCN2 is required for the TGF-β-induced phosphorylation of Smad1, ERK1/2 and friend leukaemia integration 1 transcription factor (Fli1) in...
Fig. 5  Collagens and α-smooth muscle actin protein expressions in fibroblasts. (a,b,c,d,e) Analysis of type I, type III collagen, α-smooth muscle actin and CCN2 protein levels by Western blot after AdCCN2 transfection; (f,g,h,i,j) Analysis of type I, type III collagen, α-smooth muscle actin and CCN5 protein levels by Western blot after AdCCN5 transfection. *P < 0.05 vs control.
Fig. 6  Cysteine knot (CT) domain plays a part in the induction of fibroblasts differentiation. (a) CCN2(CT−) expression in fibroblasts; (b,c,d,e) Analysis of phosphorylated smad1, phosphorylated ERK and α-smooth muscle actin protein levels by Western blot after AdCCN2(CT−) transfection; (f) CCN5 (CT+) protein expression in fibroblasts; (g,h,i,j) Analysis of phosphorylated smad1, phosphorylated ERK and α-smooth muscle actin protein levels by Western blot after AdCCN5(CT+) transfection. *P < 0.05 vs control.
Fig. 7  Analysis of transforming growth factor (TGF-β1, CCN2 and CCN5 expression in epidural scar-derived fibroblasts. (a) Hematoxylin & eosin (HE) staining analysis of epidural tissues in rabbits after laminectomy surgery; (b,c,d) quantitative real-time polymerase chain reaction (qRT-PCR) analysis of TGF-β1, CCN2 and CCN5 mRNA levels; (e,f,g,h) Western blot analysis of TGF-β1, CCN2 and CCN5 protein levels. EF indicates epidural fibrosis; D indicates dural mater; SC indicates spinal cord. *P < 0.05 vs normal skin.
dermal fibroblasts. However, cell proliferation and motility are inhibited by CCN5, the effect of which seems to oppose those of CCN1 and CCN2. CCN5 might suppress the pro-fibrotic effect of TGF-β via PI3K/Akt inactivation. Accordingly, our further investigation indicated that CCN2 overexpression contributed to viability promotion, collagen and α-SMA expression up-regulation in TGF-β-exposed human skin fibroblasts. In contrast, CCN5 overexpression resulted in decreased cell viability and reduced collagen and α-SMA expression induced by TGF-β. It was also observed that neither AdCCN2 transfection modulated CCN5 protein expression nor AdCCN5 transfection modulated CCN2 protein expression under TGF-β exposure (Fig. 4a–d). However, when TGF-β was removed, AdCCN2 transfection decreased CCN5 protein expression at 48 h after the transfection (Fig 4e,g). As well, AdCCN5 transfection decreased CCN2 protein expression at 48 h after the transfection (Fig 4f,h). These results might clarify that either CCN2 modulated CCN5 expression or CCN5 modulated CCN2 expression in skin fibroblasts, which were influenced by TGF-β exposure.

Notably, the present study demonstrated that the CT domain of CCN2 plays an essential role in the induction of fibroblast differentiation. CCN2 protein lacking a CT domain exerted similar differentiation. CCN2 protein lacking a CT domain exerted similar effect to TGF-β, which were augmented by CCN5(CT+). The results might suggest an involvement of smad1/ERK signalling in fibroblast differentiation modulated by CCN2 or CCN5. Emerging evidence has shown that the CT domain of CCN2 plays a key part in the regulation of multiple cell phenotypes. The CT domain of CCN2 may stimulate proliferation of normal rat kidney fibroblasts in the presence of epidermal growth factor (EGF). Moreover, overexpression of full-length CCN2 in isolated cardiomyocytes induced hypertrophic growth, whereas deletion of the C-terminal domain antagonized the hypertrophic effect.

Additionally, up-regulated TGF-β and CCN2 expression with reduced CCN5 expression were observed in epidermal scar-derived fibroblasts of rabbit models. These results further confirmed the role of CCN2 and CCN5 in epidermal fibrosis of animal models.

In conclusion, this study demonstrated that CCN2 contributed to TGF-β induced fibroblast proliferation and differentiation, while CCN5 regressed the effect of TGF-β on fibroblasts, as fibroblast proliferation and differentiation play major roles in epidermal fibrosis after laminectomy. Therefore, this study may provide evidence for the establishment of novel methods for the prevention of epidermal fibrosis.

MATERIALS AND METHODS

Cell culture

Normal foreskin samples were obtained from patients undergoing wrapping operation. Dermal portions were minced and incubated in a solution of collagenase type I (0.1 g/L) at 37°C for 3 h to separate the fibroblasts. The fibroblasts were pelleted and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 U/mL streptomycin. The cells were incubated at 37°C in a 5% (v/v) CO₂ humidified atmosphere. Fibroblasts were used in the subsequent experiments after 6–8 passages. 80% confluent fibroblasts were starved under serum-free condition for 12 h and treated with 0, 5, 10, 15, and 20 μg/mL TGF-β1 for 48 h. The present study was reviewed and approved by the Ethics Committee of the Third Affiliated Hospital, Medical College of Xi’an Jiaotong University (Xi’an, China). Human samples were obtained in accordance with the Declaration of Helsinki and informed consent was obtained from the patients.

Establishment of animal model

To establish rabbit models of laminectomy, eight rabbits weighing 2.1–2.6 kg were used in the surgery. Laminectomy model was performed according to the procedure reported previously. Briefly, all animals were anaesthetized by intraperitoneal injection of 1% pentobarbital sodium solution (4 mL/kg body weight) and fixed on a special board in prone position. A posterior midline skin incision was performed from L5 to L7 vertebrae and the lumbar fascia was opened bilaterally. The paraspinal muscles were then separated on L5–L6 spine level. The dura mater of L6 level was exposed after removing the spinous process and vertebral plate with a rongeur. Following that, the dense dorsal spinous fascia was reapproxosed using an absorbable suture, and the skin was closed with 3–0 nylon sutures. After the surgery, the animals were housed in individual cages and received free food and water consumption. After 24 weeks, five rabbits were killed, and the scar tissue from the laminectomy sites was resected for the separation of fibroblasts, which was performed as per the fibroblasts separated from foreskin samples. Fibroblasts were used in the subsequent experiments after 6–8 passages. The remaining three rabbits proceeded to histological analysis of the laminectomy sites.

Cell viability assay

Fibroblast proliferation was evaluated by MTT assay. The experiments were carried out in 96-well plates according to the manufacturer’s protocols (Roche GmbH, Mannheim, Germany). In the MTT test, tetrazolium salts were transformed by active enzymes of the cells into intracellular formazan deposits and cells were incubated for 4 h with the tetrazolium salts. After this incubation time, the purple formazan salts formed became soluble. Absorbance was determined at 570 nm at the indicated time.

Construction of AdCCN2, AdCCN5, AdCCN2(CT−) and AdCCN5(CT+)

Recombinant adenoviral vectors were constructed with the AdEasy XL Adenoviral Vector System (Stratagene, Santa Clara, CA, USA) according to a previous study. Amino-terminal hemagglutinin (HA)-tagged full-length human CCN2 and CCN5 cDNAs were subcloned into the pShuttle-IREShrGFP2 vector. A CCN2 construct lacking the CT domain, AdCCN2(CT−), and a CCN5 construct containing a CT domain, AdCCN5(CT+) were generated by PCR-mediated mutagenesis and subcloned into the shuttle vector. The linearized shuttle vectors were then recombined in Escherichia coli strain BJ5183 (Stratagene) with a
Table 1 Sequence of gene primers for quantitative real-time polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Type I Collagen</td>
<td>5'-GAAAGAAGAGCCACAGGC-3'</td>
</tr>
<tr>
<td>Type III Collagen</td>
<td>5'-CAGCAGTGTGATGCGAAAC-3'</td>
</tr>
<tr>
<td>z-SMA</td>
<td>5'-TTTACCAAGGGATGTCCTT-3'</td>
</tr>
<tr>
<td>CCN2</td>
<td>5'-TAGCAAAGCTGCGTGCT-3'</td>
</tr>
<tr>
<td>CCN5</td>
<td>5'-TTACCAAGGGATGTCCTT-3'</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5'-ACTACTAGCAGGAAGGTCAC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-TTCAGTCCATGAGTAGGATG-3'</td>
</tr>
</tbody>
</table>

CCN, connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed; SMA, smooth muscle actin; TGF, transforming growth factor.

serotype 5 first-generation adenoviral backbone, AdEasy-1 (Stratagene). Successfully recombined viral backbones were transformed into AD293 cells (Stratagene) and grown in large quantities. Adenoviral vectors were purified by standard CsCl ultracentrifugation and desalting. Viral titres were determined using plaque assay.

**Immunofluorescence staining**

Cultured fibroblasts were fixed in 4% paraformaldehyde for 10 min, and treated with 0.1% Triton X-100 in PBS for 20 min. Thereafter, the slides were blocked with 1% bovine serum albumin for 30 min. Next, the cells were incubated with a rabbit polyclonal anti-z-smooth muscle actin primary antibody (1:100 dilution; Abcam, Cambridge, UK) overnight in a humidified incubator. The slides were then rinsed and incubated with a goat anti-rabbit secondary antibody conjugated to AlexaFluor 488 (1:400 dilution, Invitrogen) for 1 h. In addition, the slides were counterstained with DAPI (Sigma-Aldrich, Shanghai, China) and analyzed with aFluoview 1000 confocal microscope.

**RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis**

The RNA was extracted from fibroblasts using TRIZol RNA-extraction reagent (Gibco) according to the manufacturer’s instructions. About 5 μg total RNA for each sample was reverse-transcribed into first strand cDNA for qRT-PCR analysis. The qRT-PCR was performed in a final volume of 10 μL, which contained 5 μL of SsoFast EvaGreen Supermix (Bio-Rad, Shanghai, China), 1 μL of cDNA (1:50 dilution), and 2 μL each of the forward and reverse primers (1 mmol/L). The steps in the qRT-PCR were performed as follows: 94°C for 2 min for initial denaturation; 94°C for 20 s, 58°C for 15 s, and 72°C for 15 s; 2 s for plate reading for 40 cycles; and a melt curve from 65 to 95°C. β-actin was used as a quantitative and qualitative control to normalize the gene expression. Data were analyzed using the formula: \( R = 2^{-[ΔC_t \text{ sample} - ΔC_t \text{ control}]} \). All of the primers used in this experiment are shown in Table 1.

**Western blot analysis**

Fibroblasts were homogenized and lysed with RIPA lysis buffer (100 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7.5, 1% TritonX-100, 1 mmol/L EDTA, 10 mmol/L b-glycerophosphate, 2 mmol/L sodium vanadate and protease inhibitor). Protein concentration was assayed using a micro-BCA protein kit (Pierce, Rockford, IL, USA). Thirty-five micrograms of protein per lane was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose (Amersham Pharmacia, Freiburg, Germany). Then, nonspecific binding was blocked by incubating with 5% nonfat milk in TBST buffer at room temperature for 1 h. Immunodetection of type I, type III collagen, z-SMA, CCN2, CCN5, TGF-β and β-actin was done using rabbit anti-human monoclonal anti-type I collagen antibody (1:1000; Santa Cruz), anti-type III collagen antibody (1:1000; Santa Cruz), anti-z-SMA antibody (1:1000; Santa Cruz), anti-p-smad1 antibody (1:1500; Santa Cruz), anti-p-ERK antibody (1:2000; Santa Cruz), anti-CCN2 antibody (1:1500; Santa Cruz), anti-CCN5 antibody (1:1500; Santa Cruz), anti-TGF-β antibody (1:1500; Santa Cruz) and anti-β-actin (1:2000; Sigma), respectively. Goat anti-rabbit IgG (1:5000; Sigma) followed by enhanced chemiluminescence (ECL; Amersham Pharmacia) was used for detection.

**Histological analysis**

After the laminectomy model was established for 24 weeks, three rabbits of these models were intracardially perfused with ice-cold saline followed by 4% paraformaldehyde. Then, the entire L6 vertebral column was totally isolated and sectioned. After decalcification and dehydration for 2 days, samples were embedded in paraffin, and the laminectomy sites were cut as 5-μm axial sections before staining with hematoxylin and eosin (HE). Epidural scar adhesion was evaluated under a light microscope.

**Statistical analysis**

Results are expressed as mean ± standard deviation (SD). Statistical significance was analyzed with one-way factorial ANOVA or Student’s two-tailed t test. A value of \( P < 0.05 \) was considered statistically significant. All analyses were conducted using SPSS software (SPSS Inc., Chicago, IL, USA).

**REFERENCES**


