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# Regulation of MMP and TIMP expression in synovial fibroblasts from knee osteoarthritis with flexion contracture using adenovirus-mediated relaxin gene therapy

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ABSTRACT

*Purpose:* The aim of this study was to investigate the effects of relaxin (RLN) expression on fibrosis inhibition in synovial fibroblasts.
*Materials and methods:* Tissue cells from patients with knee osteoarthritis and >30° flexion contractures were utilised. Synovial fibroblasts were activated by TGF-β1 (two nanograms per millilitre) and then exposed to Ad-RLN as a therapeutic gene, adenovirus-lacZ construct as a marker gene, and SB505124 as an inhibitor for TGF-β1 signal for 48 h. The mRNA expression levels of collagens and MMPs were analysed by reverse transcription-polymerase chain reaction. Also, fibronectin, phosphorylation of Smad2 and ERK1/2, alpha smooth muscle actin, TIMP-1, TIMP-2, MMP-1 and MMP-13 levels were estimated using western blotting, and the total collagen synthesis was assayed.
*Results:* Ad-RLN-transduced synovial fibroblasts demonstrated 17%, 13%, and 48% reduction in collagen I, III and IV mRNA expression levels, respectively, and a 40% decrease in MMP-3, MMP-8, 20% decrease in MMP-9, MMP-13 mRNA expression, compared to non-Ad-RLN-transduced cells. In protein expression, Ad-RLN-transduced synovial fibroblasts demonstrated 46% increase in MMP-1, 5% decrease in MMP-2, 51% increase in MMP-9, and 22% increase in MMP-13, compared to non-Ad-RLN-transduced cells. Ad-RLN-transduced synovial fibroblasts showed a 25% decrease in TIMP-1 and 65% decrease in TIMP-2 protein expression at 48 h, compared to non-Ad-RLN-transduced cells. Ad-RLN-transduced synovial fibroblasts demonstrated a 45% inhibition of fibronectin in protein expression level and 38% decrease in total collagen synthesis at 48 h, compared to non-Ad-RLN-transduced cells.
*Conclusion:* Relaxin expression exerted anti-fibrogenic effects on synovial fibroblasts from patients with knee osteoarthritis and flexion contractures. Therefore, relaxin could be an alternative therapeutic agent during the initial stage of osteoarthritis with flexion contracture by exerting its anti-fibrogenic effects.



## 1. Introduction

A knee joint contracture is characterised by a restriction in joint full range of motion (ROM) and occurs secondary to shortening of periarticular connective tissues and muscles [1,2]. Joint contractures restrict mobility, have a negative impact on quality of life,

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limit an individual's productivity and earning potential, and can prevent basic activities of daily living [3,4]. Knee osteoarthritis (OA) is the most common joint disorder. Knee OA affects 11–15% of the United States population aged ≥65 years, is a leading cause of chronic disability, and often is treated with total knee arthroplasty (TKA) at end stage [5]. Ritter et al. reported that more than one third of patients with OA (5228 knees) who presented for TKA had knee flexion contractures [6,7]. This proportion represents a significant number, considering that 719,000 knee replacements were performed in the United States in 2010. Recently, knee OA with flexion contracture was found, through biomechanical study, histological studies and imaging studies with magnetic resonance imaging (MRI), to be closely related to the pathological changes of synovial tissue. Several recent papers have begun to suggest which biochemical and structural processes may underlie the increase in connective tissue. Studies in this area are difficult because knee OA with flexion contracture is a dynamic process with pathological and biochemical findings that evolve as the disease progresses in severity [8–10].

Joint stiffness with arthrofibrosis is a complication that inhibits postoperative ROM [11]. Since patients with diabetes mellitus and poor compliance to exercise therapy are prone to high contracture possibilities, such patients need treatment for arthrofibrosis as soon as possible [12]. Also, treatment for arthrofibrosis is costly and difficult, and the patients suffer. It is reported that joint stiffness results from intra-articular adhesion formation and capsular contracture by inflammation. The inflammation induces adhesion and contracture, which makes thick fibrotic scar tissue and causes immobilisation, and gradually restricts mobility [13]. Inflammatory cells infiltrate into the synovium and capsular tissue during joint inflammation. The invaded cells release various cytokines and other inflammatory signalling molecules. One of the representative cytokines is transforming growth factor beta 1 (TGF-β1) [13,14]. The secretion of TGF-β1 leads to arthrofibrosis with flexion contracture. The fibroblasts in this fibrotic tissue express abnormal alpha smooth muscle actin (α-SMA) and differentiate into contractile myofibroblasts [13,15,16]. In Dupuytren's contracture, for example, TGF-β1 with other inflammatory signal molecules plays an important role in this differentiation [15]. Large amounts of extracellular matrix (ECM) are accumulated by overexpression of collagen type I and fibronectin in the tissue containing the myofibroblasts, which make dense contractile connective tissue with maturation.

One important aspect of fibrosis is the increase in collagen synthesis by TGF-β1 or connective tissue growth factor (CTGF) [17–19]. In the normal healing process, TGF-β1 stimulates CTGF and then CTGF increases collagen synthesis [20]. However, in fibrosis with the scar-based joint construct, TGF-β1 does not stimulate CTGF – TGF-β1 directly stimulates collagen synthesis or CTGF stimulates collagen synthesis alone [21,22].

Matrix metalloproteinases (MMPs) directly degrade ECM in the normal scar or indirectly degrade ECM by transforming cells into proteolytic phenotypes [23]. In other words, although MMPs are primarily associated with ECM degradation, the function of MMPs is not the turnover or clearance of matrix proteins at all.

Tissue inhibitor of metallopeptidases (TIMPs) inhibit the expression of MMPs with disintegrin and metalloproteinases (ADAMs) and ADAMs with thrombospondin motifs (ADAMTSs), and control ECM proteolysis through direct inhibition of MMPs-dependent ECM proteolysis [24–26]. It is known that TIMPs generally induce ECM accumulation and the loss of TIMP expression results in matrix proteolysis [25–27]. The balance between MMPs and TIMPs is important for ECM remodelling rather than ECM proteolysis [28,29]. MMPs produced biologically active proteins such as cytokines, chemokines, and cell surface proteins in knock-out mice experiments with MMPs or TIMPs [30,31]. Therefore, they indirectly affect matrix turnover and the regulation of biologically active proteins by these MMPs/TIMPs.

Relaxin is one of the uterine contraction hormones and is involved in pregnancy maintenance and contraction or relaxation of the pelvic ligaments. In addition to the reproductive hormone function, it acts on neoangiogenesis and vasodilation for antagonism, wound healing, and the infarct of context under the fibrosis [32]. Also, relaxin is an insulin superfamily, blocks the influx of inflammatory cells through activation of G-protein-coupled receptor (GPCR), and inhibits amelioration on profibrotic factors such as TGF-β1 [33,34]. Recently, relaxin has been investigated in relation to ECM turnover. It has been known that a recombinant relaxin (rhRLN), which acts via relaxin family peptide receptor 1, induces cardiac fibroblast proliferation and prevents myofibroblast-mediated aberrant collagen deposition by avert differentiation into myofibroblasts [35,36]. Pleiotropic properties by rhRLN activate renal fibrosis and wound healing by indirectly stimulating nitric oxide production. In the lung, relaxin is also known to have a strong anti-fibrotic effect [37]. In particular, relaxin down-regulates TGF-β1-mediated collagen production, and digests aberrant collagen accumulation by up-regulating gelatinases (MMPs and MMP-9) [35,38]. Also, relaxin controls transcriptional regulation, which requires AP-1 and PEA-3 binding motif, in MMPs [39,40]. It has been known that relaxin plays an essential role in matrix turnover by regulating the expression of MMPs in the uterus, pubic symphysis and fibrocartilage of synovial joints [41,42].

Therefore, the aim of this study was to investigate the anti-fibrotic effect of relaxin in synovial fibroblasts from patients with knee OA and flexion contractures.

## 2. Methods

### *2.1. Study design*

To test the anti-fibrotic effect of adenovirus-relaxin construct (Ad-RLN) on synovial fibroblasts in vitro, tissue cells from five patients (four females, one male) with knee OA and with >30° flexion contracture were utilised. Synovial fibroblasts were activated by TGF-β1 (two nanograms per millilitre) and then exposed to Ad-RLN as a therapeutic gene, adenovirus-lacZ construct (Ad-LacZ) as a marker gene, and SB505124 as an inhibitor for TGF-β1 signal for 48 h. Synovial fibroblast cultures without adenoviral exposure served as saline control. The mRNA expression levels of collagens and MMPs were analysed by reverse

transcription-polymerase chain reaction (RT-PCR). Also, fibronectin, phosphorylation of Smad2 and Extracellular Signal-regulated Kinase (ERK1/2), alpha smooth muscle actin (α-SMA), TIMP-1, TIMP-2, MMP-1 and MMP-13 levels were estimated using western blotting, and total collagen synthesis was assayed.

### 2.2. Isolation and culture of subsynovial fibroblasts

Subsynovial tissue isolated from patients with knee OA and severe flexion contractures was minced with a scalpel and the tissues were then digested for two hours at 37 °C under gentle agitation in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL®, NY, USA) containing collagenase type IV (250 unit/ml, Sigma, MO, USA). Cells were cultured for two to three weeks in DMEM containing 10% FBS, Antibiotic-Antimycotic (Gibco-BRL®, NY, USA) in a 37 °C incubator with 5% $CO_2$ and humidity.

### 2.3. Relaxin constructs and transfection

Two different adenoviral constructs were prepared for this study: Ad-LacZ(dE1/lacZ) expressing the lacZ gene as a viral control and Ad-RLN(dE1/RLN) expressing the human relaxin gene [43]. Each recombinant adenoviral vector was amplified in human embryonic kidney 293 cells and purified by Vivapure Adenopack 100 (Satorius Stedim biotech, Germany). Titers were determined by optical density at 260 nm ($OD_{260}$). Also, one absorbance unit is equivalent to $10^{12}$ viral particle $ml^{-1}$. At confluence, the subsynovial fibroblastic cells isolated from tissues of patients with knee OA were rinsed with Hanks' Balanced Salt Solution (HBSS) and exposed to culture medium containing TGF-β1 (two nanograms per millilitre) for 10 min. 10 μl of Ad-RLN and Ad-LacZ construct (i.e. $1 \times 10^9$ viral particles) in culture medium were added. All cells were incubated in a 37 °C incubator with 5% $CO_2$ for 48 h.

### 2.4. Reverse-transcription polymerase chain reaction analysis for collagens

For mRNA expression of collagens, total RNA was isolated from synovial fibroblasts that were transfected by Ad-RLN Ad-LacZ for 48 h using the QIAGEN RNeasy® mini kit (QIAGEN) and cDNA was generated with QuantiTect® reverse transcription Kit (QIAGEN, CA, USA) following the manufacturer's instructions. The collagen type I, III and IV genes were amplified. Relative expression levels were normalised to the average beta-actin level. Data were analysed using the Image J analyser ver. 1.6 software (National Institutes of Health, MD, USA).

### 2.5. Protein extraction and western analysis

The protein expression of MMP-1, MMP-13 (Ab frontier, Korea), ERK1/2, phosphor-ERK1/2 (Cell Signalling Technology, Inc., MA, USA), beta galactosidase, relaxin, α-SMA, fibronectin, TIMP-1, and TIMP-2 (Abcam®, England) were analysed with western blot. Cells were transfected by Ad-RLN and Ad-LacZ for 48 h at a density of $2 \times 10^5$ cells per well, and were lysed in RIPA lysis buffer (ATTO Corp., Japan) and protease inhibitor (Pierce™ Mini Tablets, IL, USA). Immunoreactivity was detected with a western blot detection system (WESTSAVE™, Abfrontier, Korea). Blots were stripped of bound antibodies, and reprobed using antibodies to actin (Abcam®, England) to verify loaded protein amounts.

### 2.6. Zymograms of matrix metalloproteinase-2 and -9

Samples of medium were harvested from the cells cultured in 60-mm culture plates at a density of $2 \times 10^5$ cells per well for 48 h after transfection by Ad-RLN and Ad-LaccZ. MMP-2 and MMP-9 in the samples of cell-conditioned medium were monitored by gelatin substrate zymograms. In brief: 20 μl of the culture medium was mixed with 2× sample buffer and electrophoresed on a 10% zymogram gel (Novex®Zymogram gel, Invitrogen, CA, USA).

### 2.7. Total collagen content assay

Subsynovial fibroblasts transfected with Ad-RLN were grown at a density of $4 \times 10^4$ cells per well in in 60-mm culture plates for 48 h in DMEM medium. Collagen was harvested from these cells after lysing in a buffer containing 0.5 M acetic acid and protease inhibitor. The collagen samples were concentrated using a collagen isolation and concentration protocol, and measured at a 555-nm wavelength as a manufacture instruments (Sircol™, Biocolor Ltd., County Antrim, UK). The amounts of collagen were calculated based on a standard curve of soluble collagen provided by the collagen assay kit. The results were normalised to the total protein content.

### 2.8. Statistical analysis

All experiments were executed with independent triplicate separate cultures using the synovial fibroblasts isolated from five donors. Data were expressed as mean ± standard deviation (SD) from the results of three independent experiments. A two-tailed Student's *t*-test was used to compare the results of the two groups. A value of $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Beta galactosidase and relaxin expression on synovial fibroblasts

Synovial fibroblasts transfected with Ad-LacZ demonstrated beta galactosidase expression by western blot analysis, compared to those cells with Ad-RLN and saline control, indicating a highly efficient transduction rate of adenoviral marker gene construct. Synovial fibroblasts transfected with Ad-RLN confirmed relaxin protein expression on western blot analysis, compared to cultures with Ad-lacZ and saline control (Figure 1).

### 3.2. Collagen mRNA expression

Expression of collagen type I, III and IV mRNAs of synovial fibroblasts and transfected with Ad-RLN showed a 17%, 13% and 22% decrease at 48 h, compared to control culture, respectively (TGF-β1+, $P = 0.016$, $P = 0.017$, $P = 0.026$, respectively). However, there was no significant difference in collagen type V expression at mRNA level ($P = 0.121$) (Figure 2).

### 3.3. Matrix metalloproteinase mRNA expression

Synovial fibroblasts transfected with Ad-RLN showed a 40% increase in MMP-3 mRNA expression compared to those cultures without Ad-RLN ($P = 0.980$). However, synovial fibroblasts transfected with Ad-RLN showed a 40% decrease in MMP-8 mRNA expression at 48 h compared to those cultures without Ad-RLN ($P = 0.001$). Also, the synovial fibroblasts transfected with Ad-RLN showed a 50% reduction in MMP-9 ($P = 0.017$), and a 20% reduction in MMP-13 mRNA expression at 48 h, compared to those cultures without Ad-RLN ($P = 0.015$) (Figure 3).

### 3.4. Phosphor-ERK1/2 expression

Synovial fibroblasts with Ad-RLN showed 70% increase in levels of phospho-ERK1/2 protein expression at 48 h, compared to cells cultured without Ad-RLN ($P = 0.002$) (Figure 4).

### 3.5. TIMPs and MMPs expression

Synovial fibroblasts with Ad-RLN showed 25% decreases in the levels of TIMP-1 ($P = 0.002$) and 65% decrease at 48 h in TIMP-2 ($P = 0.002$) protein expression, compared to those cultures without Ad-RLN. However, synovial fibroblasts with Ad-RLN showed 50% increase at 48 h in MMP-13 protein expression ($P = 0.023$), compared to those cultures without Ad-RLN. There was no significant difference in the MMP-1 expression at the protein level ($P = 0.054$) (Figure 5).

### 3.6. Zymograms of matrix metalloproteinase-2 and -9

Synovial fibroblasts transfected with Ad-RLN showed no difference in MMP-2 and MMP-9 expression at 48 h, compared to those cultures without Ad-RLN (Figure 6).

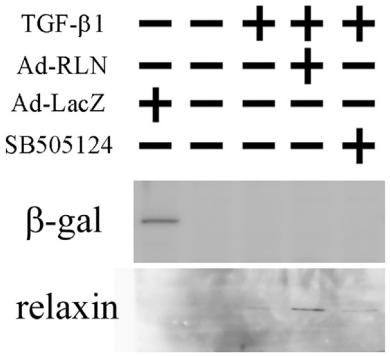


**Figure 1.** Synovial fibroblasts from patients with knee OA with severe flexion contractures and transfected with Ad-LacZ demonstrated beta galactosidase expression by western blot analysis, compared to those cells with Ad-RLN and saline control, indicating a highly efficient transduction rate of adenoviral marker gene construct. Ad-RLN, adenovirus relaxin gene construct; Ad-LacZ, adenovirus LacZ gene construct; SB505124, TGF-β1 inhibitor.

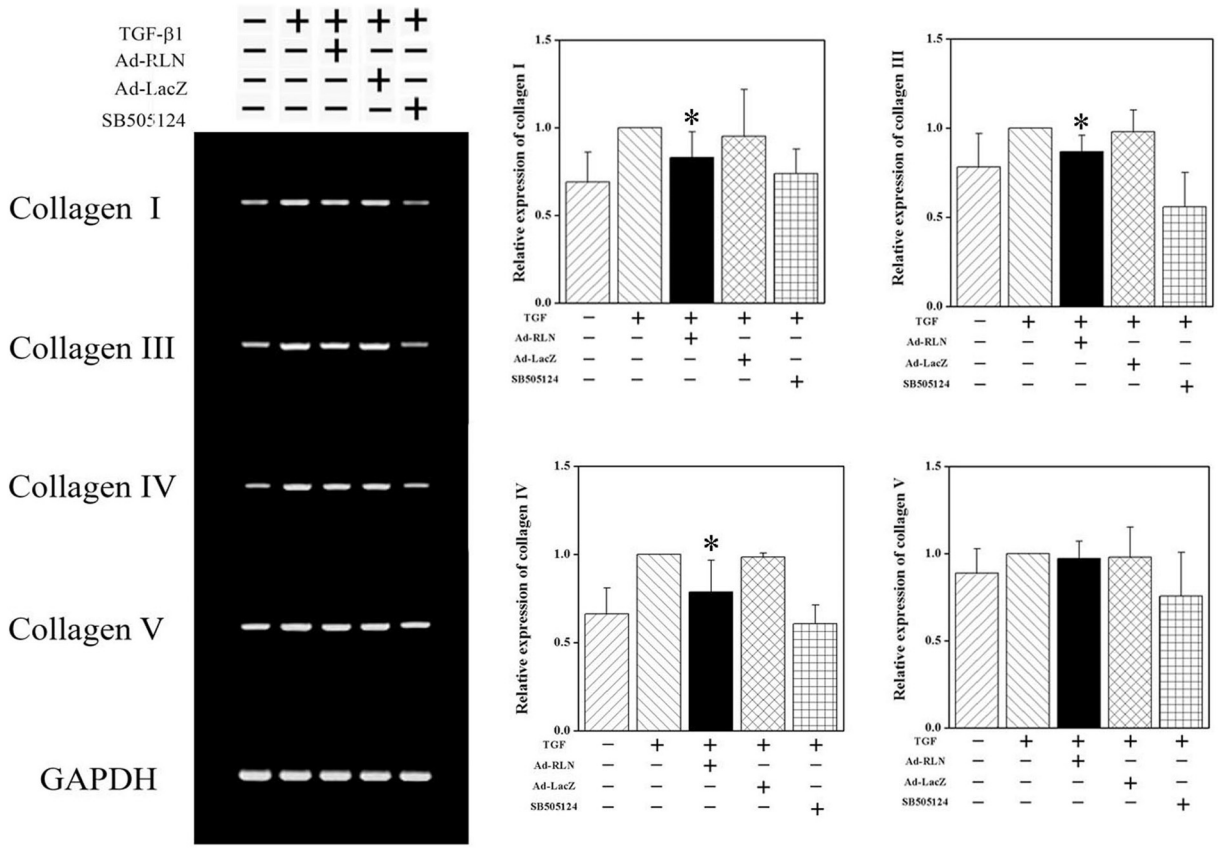


**Figure 2.** Expression of collagen type I, III and IV mRNAs of synovial fibroblasts from patients with knee OA with severe flexion contractures and transfected with Ad-RLN showed a 17%, 13% and 22% decrease at 48 h, compared to control culture (TGF-β1 +, $P = 0.016$, $P = 0.017$, $P = 0.026$), respectively. However, there was no significant difference in the collagen type V expression at the mRNA level ($P = 0.121$). Ad-RLN, adenovirus relaxin gene construct; Ad-LacZ, adenovirus LacZ gene construct; SB505124, TGF-β1 inhibitor; GAPDH,

### *3.7. Alpha smooth muscle actin and fibronectin expression*

Synovial fibroblasts with Ad-RLN showed 70% decreases in levels of α-SMA expression ($P = 0.031$) at 48 h, compared to cells cultured without Ad-RLN. There were also 45% decreases in fibronectin expression ($P = 0.002$) at protein level (Figure 7).

### *3.8. Total collagen content*

Synovial fibroblasts transfected with Ad-RLN showed a 38% decrease in total collagen protein expression at 48 h, compared to those cultures without Ad-RLN ($P = 0.003$) (Figure 8).

## 4. Discussion

The cause of stiffness after TKA has been believed to be inflammatory arthropathy and prior knee surgery with poor preoperative ROM and osteoarthritis [44]. Intraoperative factors that may cause stiff TKA are improper balancing from overstuffing in the patellofemoral joint, inadequate sizing of the component, bone resection in osteophytes, failure of normal tibial slope, and contracted capsule [45–47]. In addition, after operation, patients may lead to secondary stiffness due to factors such as infection, arthrofibrosis, heterotopic ossification, inadequate rehabilitation, and complex regional pain syndrome.

Primary arthrofibrosis results in painful and reduced joint ROM during fibrotic tissue remodelling after joint trauma or surgery. It differs from secondary arthrofibrosis by inaccurate implant positioning [48]. Diagnosis of arthrofibrosis depends on histopathological findings, as clinical symptoms such as loss of ROM are inaccurate diagnostic markers [49].

The reparative inflammatory mechanism in arthrofibrosis remains poorly understood. It may be similar to those in other fibrotic disorders [48]. In general, fibrosis is formed by profibrotic molecules (i.e. TGF-β1) being secreted, which in turn induce differentiation of fibroblasts into myofibroblasts, followed by matrix accumulation resulting in stiffening. The activated myofibroblasts in fibrosis do not undergo apoptosis after wound healing but instead cause pathologic scarring with continuous

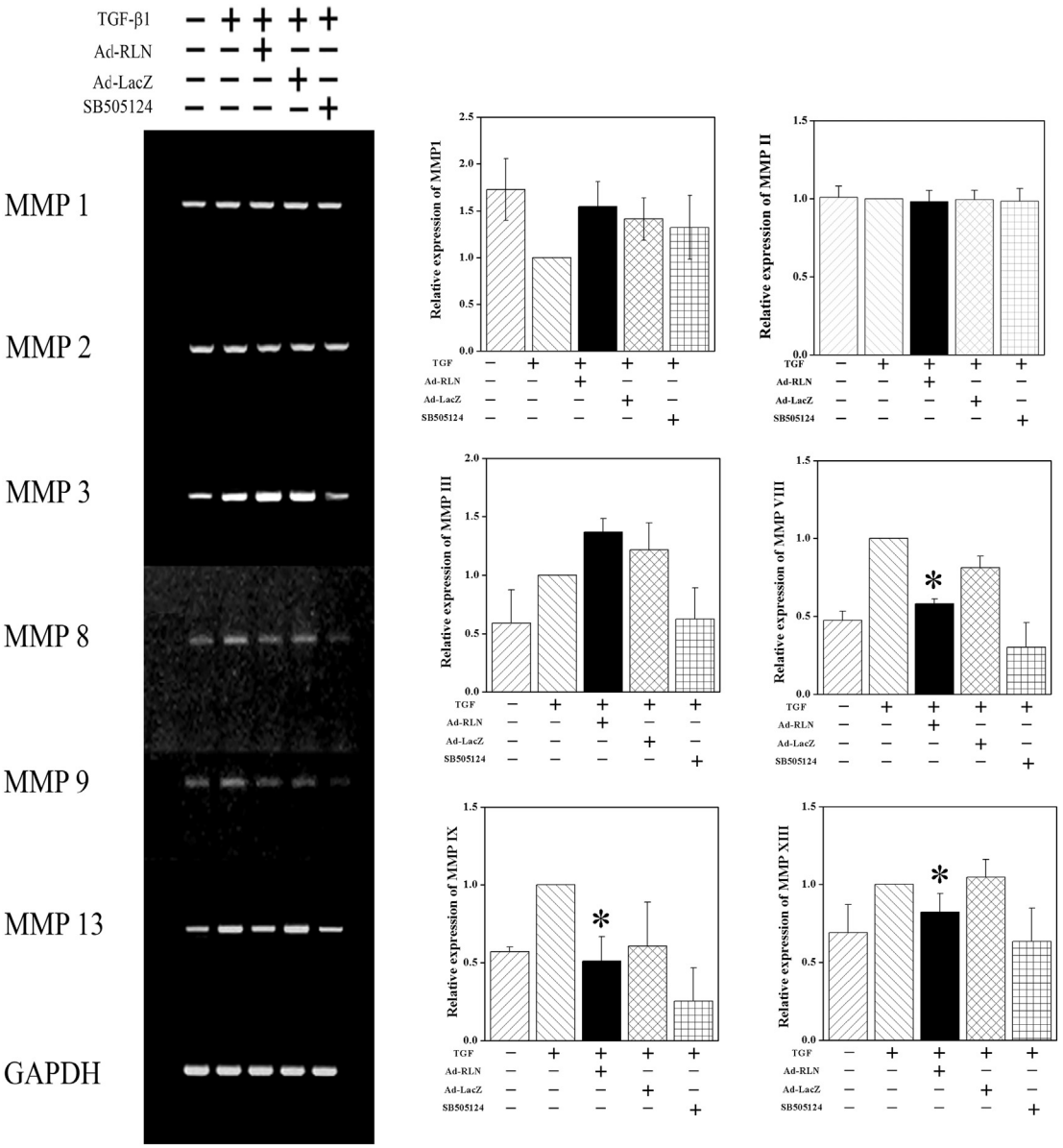


**Figure 3.** Synovial fibroblasts from patients with knee OA with severe flexion contractures transfected with Ad-RLN showed a 40% increase in MMP-3 mRNA expression compared to those cultures without Ad-RLN ($P = 0.980$). However, synovial fibroblasts from patients with knee OA with severe flexion contractures transfected with Ad-RLN showed a 40% decrease in MMP-8 mRNA expression at 48 h, compared to those cultures without Ad-RLN ($P = 0.001$). Also, the synovial fibroblasts transfected with Ad-RLN showed a 50% reduction in MMP-9 ($P = 0.017$), and a 20% reduction in MMP-13 mRNA expression at 48 h, compared to those cultures without Ad-RLN ($P = 0.015$). Ad-RLN, adenovirus relaxin gene construct; Ad-LacZ, adenovirus LacZ gene construct; SB505124, TGF-β1 inhibitor; GAPDH,

ECM production. Unbalanced synthesis and degradation of ECM traps molecules such as collagens and proteoglycans in the intercellular space [50,51].

TGF-β1 is a representative profibrotic molecule with connective tissue growth factor (CTGF) and a major factor in liver and kidney fibrosis. It has been reported that the capsule in an immobilised knee model can potentially control TGF-β1 or CTGF and may prevent contracture in the joint by blocking the fibrotic process [52]. Therefore, the current study investigated the proteins that are involved in the accumulation and degradation of ECM in the knee subsynovial fibroblasts transformed by TGF-β1. It was identified that relaxin expression transduced by adenovirus may regulate molecules that control ECM components such as MMPs and TIMPS.

The expression of collagen IV mRNA was significantly decreased in the Ad-RLN-transduced cells treated with TGF-β1, compared to cells without Ad-RLN. However, the expression levels of collagen I and III mRNA were not significantly decreased. Protein expression of MMP-1 and MMP-13 was increased on the cell with Ad-RLN and TGF-β1, compared to cells without Ad-RLN in protein level. However, protein expression of TIMP-1 and TIMP-2 was significantly reduced in Ad-RLN-transduced

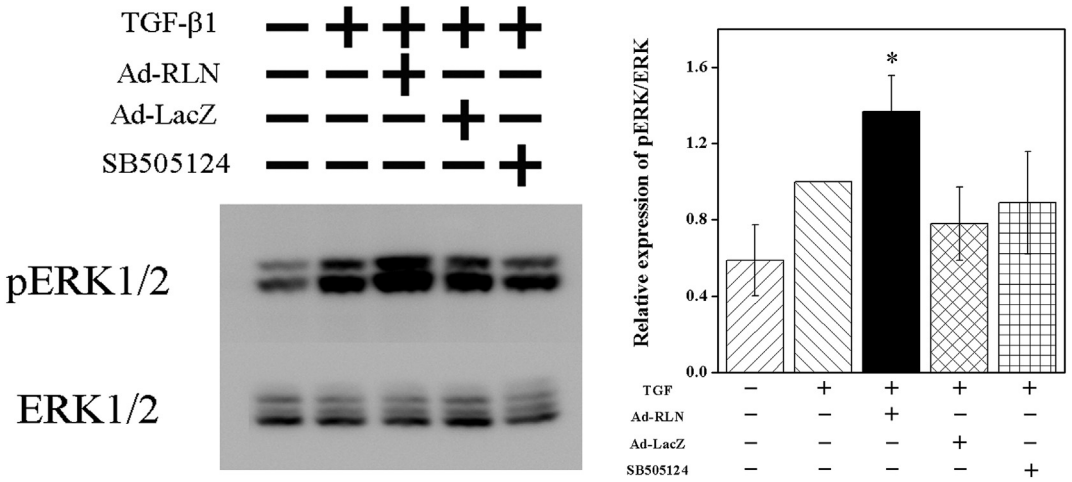


**Figure 4.** Synovial fibroblasts from patients with knee OA with severe flexion contractures with Ad-RLN showed 70% increases in levels of phospho-ERK1/2 protein expression at 48 h, compared to cells cultured without Ad-RLN ($P = 0.002$). Ad-RLN, adenovirus relaxin gene construct; Ad-LacZ, adenovirus LacZ gene construct; SB505124, TGF-β1 inhibitor.

cells treated with TGF-β1, compared to cells without Ad-RLN. Protein expression of α-SMA and fibronectin was remarkably reduced in the cell with Ad-RLN and TGF-β1, compared to cells without Ad-RLN.

In many cases of tissue-induced fibrosis, the expression of MMPs decreased whilst that of TIMPs increased. It has been demonstrated that the expression of MMP-1, MMP-13, and/or MMP-2, MMP-9 is increased in vivo and ex vivo using rhRLN [36,53–55]. This study confirmed that MMP-13 expression increased as an anti-fibrotic effect. Relaxin plays an important role in musculoskeletal system remodelling. Relaxin alters cartilage and tendon stiffness activating collagenases, is involved in the bone remodelling process, and acts on healing injured ligaments and skeletal muscles [41,51,56]. The soft tissue healing cascade consists of three phases – inflammation, regeneration and fibrosis – and relaxin acts as a regulator of inflammation and fibrosis [56,57]. Also, relaxin is an anti-fibrotic agent involved in skeletal muscle healing, against muscle fibrosis, and a factor in muscle regeneration to promote regrowth of myofiber. In the bone remodelling process, relaxin regulates the cytokine system, which is the receptor activator of nuclear factor kB ligand (RANKL), RANK, and osteoprotegerin (OPG), with TGF-β1 and oestrogen [58]. Relaxin is one of the osteoclast-activating factors that increase bone resorption [58]. In osteoclastogenesis, relaxin has been reported to regulate bone metabolism and proliferation [58,59]. During pregnancy, increased relaxin and oestrogen levels attenuate inflammation in rheumatoid arthritis patients and relaxin itself exerts an anti-inflammatory effect to downregulate neutrophil function through adhesion and migration of leukocytes [60–62]. From this information, it is believed that relaxin has a potential beneficial effect in the treatment of synovial disease.

Also, relaxin alters ligament mechanics through collagenolytic effect by discharging MMPs, collagenases, and plasminogen activators [63–65]. Relaxin activates the collagenolytic system, which increases collagenase synthesis and degrades extracellular matrix composition [66,67]. This implies that relaxin regulates inflammation, tissue remodelling, and fibrosis in the skeletal muscle healing process [34,68]. It has been reported that relaxin modulates the length of tendon growth by increasing tendon laxity through activation of collagenases, and acts on tendon metabolism thus reducing tendon stiffness [56,69]. Relaxin enervates knee articular cartilage stiffness through induction of collagenase-1, MMP-1, and MMP-3 in fibrocartilaginous cells [70,71]. However, the potential benefits and remodelling of relaxin are still unknown.

Relaxin stimulates the signalling pathway in fibroblasts from various tissues that cause fibrosis. It has been reported that relaxin up-regulated MMPs expression via the RXFP1-pERK-nNOS-NO-cGMP-dependent pathway in the renal myofibroblasts [35,36]. It also increased ERK1/2 mitogen-activated protein kinase signalling in Dupuytren's fibroblasts, which had a high induction of TGF-β1. Relaxin was also found to increase dose-dependent phosphorylation of ERK1/2 [72]. Moreover, it was demonstrated that relaxin stimulated phosphorylation of ERK1/2 in a null mouse [36]. Also, in this study, relaxin increased phosphorylation of ERK1/2. Moreover, it has been considered that RLN makes fibrotic protein expression, which regulates cell phenotypes; abate to respond to change plasticity and microenvironment by TGF-β1 [73]. Reduction of the molecules is through phosphorylation of the ERK1/2.

These findings suggest that RLN acts on the process that suppresses MMPs and TIMPs expression by inhibiting collagen synthesis. Furthermore, the decreased expression of extracellular matrix is a positive feedback system, which results in increased production of collagenases and reduced inhibition of collagenases. Therefore, the overall sum of fibrosis is reduced.

Several limitations of this study should be noted: First, interpretation of the results was limited by the small cohort of patients. In addition, whether relaxin is directly involved in the expression of MMPs and TIMPs, and its exact mechanisms in reducing fibrosis were not investigated. Further evaluation with a larger sample size is recommended. Second, the gender distribution of the study should be considered when comparing findings to those of other research groups. In the study, all five patients were

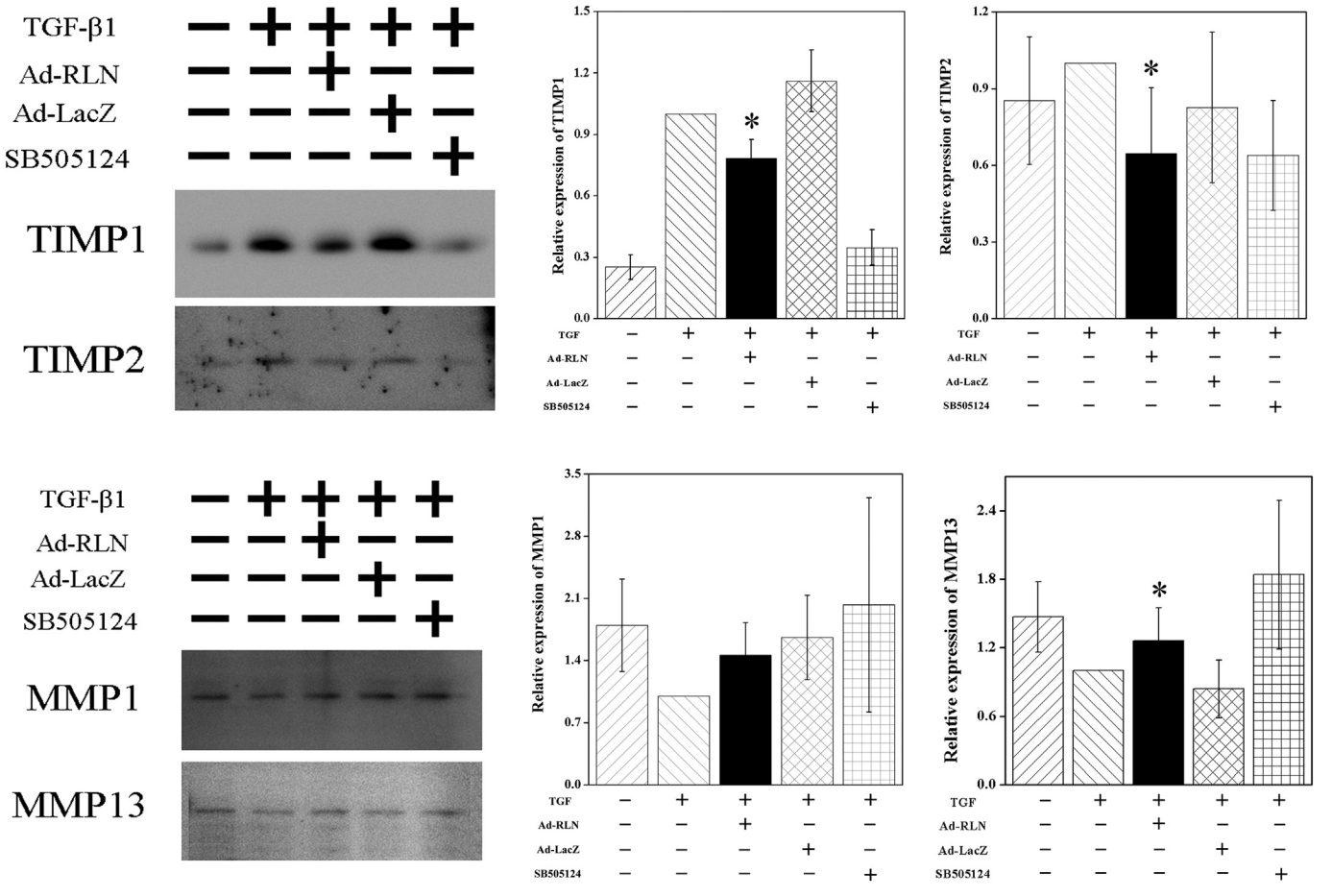


**Figure 5.** Synovial fibroblasts from patients with knee OA with severe flexion contractures with Ad-RLN showed 25% decreases in the levels of TIMP-1 ($P = 0.002$) and 65% decrease at 48 h in TIMP-2 ($P = 0.002$) protein expression, compared to those cultures without Ad-RLN. However, synovial fibroblasts from patients with knee OA with severe flexion contractures with Ad-RLN showed 50% increase at 48 h in MMP-13 protein expression, compared to those cultures without Ad-RLN ($P = 0.023$). There was no significant difference in the MMP-1 expression at the protein level ($P = 0.054$). Ad-RLN, adenovirus relaxin gene construct; Ad-LacZ, adenovirus LacZ gene construct; SB505124, TGF-β1inhibitor.

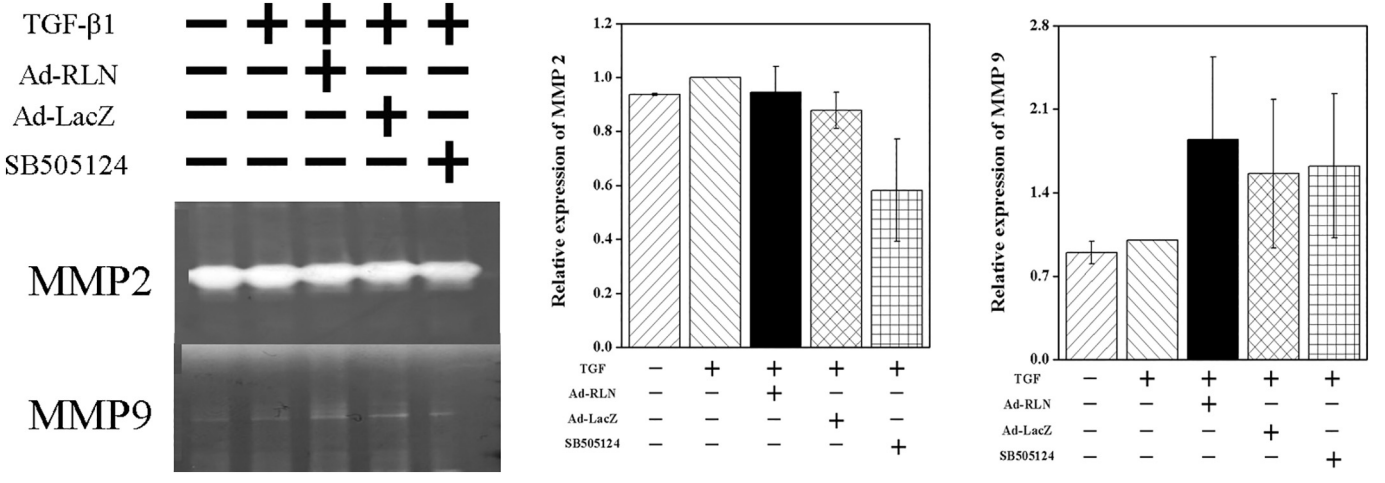


**Figure 6.** Synovial fibroblasts from patients with knee OA with severe flexion contractures transfected with Ad-RLN showed no different in MMP-2 and MMP-9 expression at 48 h, compared to those cultures without Ad-RLN. Ad-RLN, adenovirus relaxin gene construct; Ad-LacZ, adenovirus LacZ gene construct; SB505124, TGF-β1 inhibitor.

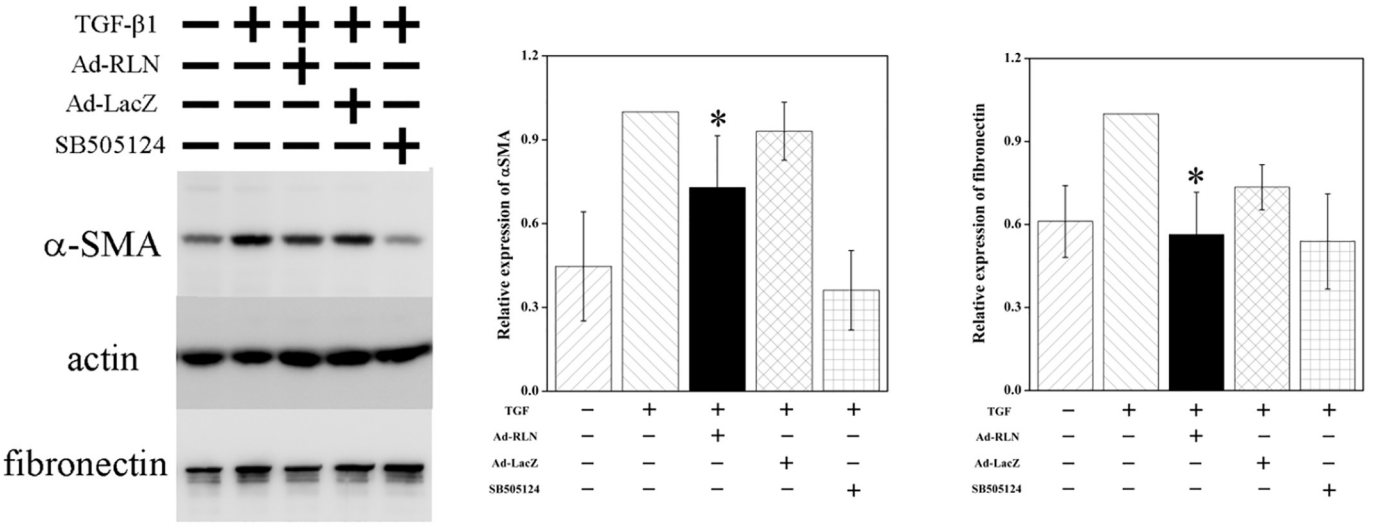


**Figure 7.** Synovial fibroblasts from patients with knee OA with severe flexion contractures with Ad-RLN showed 70% decreases in levels of alpha smooth muscle actin (α-SMA) expression at 48 h, compared to cells cultured without Ad-RLN ($P = 0.031$). Also, there were 45% decreases in the fibronectin expression at the protein level ($P = 0.002$). Ad-RLN, adenovirus relaxin gene construct; Ad-LacZ, adenovirus LacZ gene construct; SB505124, TGF-β1 inhibitor.

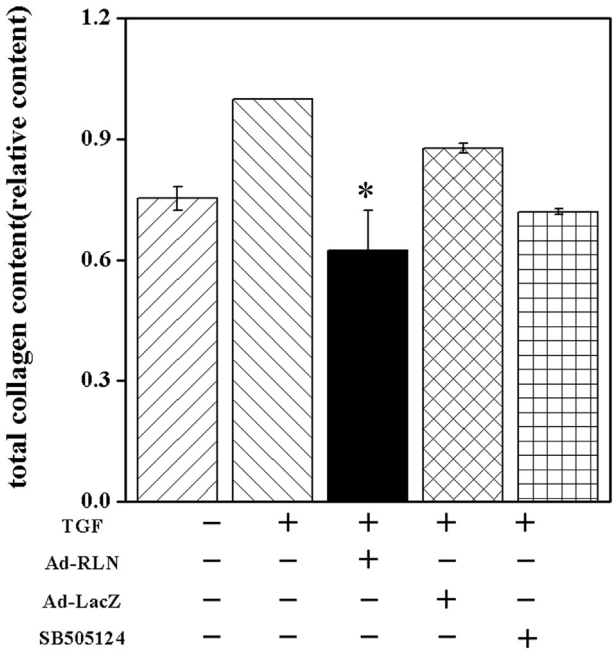


**Figure 8.** Synovial fibroblasts from patients with knee OA with severe flexion contractures transfected with Ad-RLN showed a 38% decrease in total collagen protein expression at 48 h, compared to those cultures without Ad-RLN ($P = 0.003$). Ad-RLN, adenovirus relaxin gene construct; Ad-LacZ, adenovirus LacZ gene construct; SB505124, TGF-β1 inhibitor.

women. Female gender predominance has been noted in many published reports and, for reasons unknown, this female predominance is even more notable in Korean patients undergoing total knee arthroplasties [74–77].

RLN can promote matrix remodelling by increasing cell proliferation, reducing α-SMA expression, and decreasing collagen synthesis in renal fibroblasts [78]. RhRLN treatment can alter the connective tissue phenotype of human lung fibroblasts, decrease overexpression of procollagen type I and III induced by TGF-β1, and reduce synthesis and secretion of MMP-1. In addition, RLN has been found to control excessive collagen deposition by blocking bleomycin-induced pulmonary fibrosis in human lung fibroblasts [79]. Furthermore, human dermal fibroblasts exposed to RLN have been found to modulate secretion of MMPs, collagenase inhibitors, and expression of TIMPs [80]. Moreover, it has been reported that relaxin can regulate the expression of collagens and MMPs in myofibroblasts isolated from Dupuytren's contracture [81]. The current study demonstrated that relaxin may also control the synthesis and degradation of ECM in myofibroblasts transformed by TGF-β1. The antifibrogenic effect of RLN opens a new arena for prevention of postoperative adhesions and contractures in the musculoskeletal system.

Expression of the RLN gene exerts anti-fibrogenic effects on synovial fibroblasts from patients with OA with flexion contractures via direct inhibition of collagen synthesis and through the collagenolytic pathways such as MMP-1, MMP-13, TIMP-1, and TIMP-2. Therefore, relaxin can be an alternative therapeutic agent in the initial stages of OA with flexion contracture by exerting its anti-fibrogenic effects. In summary, this is the first report to evaluate the anti-fibrotic effect of RLN in fibroblasts isolated from patients with arthrofibrosis.

## Conflict of interest statement

None to declare.

## Ethical review committee statement

This study was approved by the Institutional Review Board at the authors' institute (4–2015-0302).

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