Myofibroblast Upregulators are Elevated in Joint Capsules in Posttraumatic Contractures

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Abstract

We hypothesized specific growth factors are increased in the elbow capsules of patients with post traumatic elbow contractures. A model of surgically induced joint contracture in rabbit knees was developed to study the growth factor expression in joint contractures. This study demonstrates this model mimics the human condition and analyzes how the growth factor levels decrease with time in rabbit knees with contractures. Reverse transcription polymerase chain reaction was used to measure mRNA levels of transforming growth factor- β 1, connective tissue growth factor, ED-A of fibronectin, and α -smooth muscle actin normalized to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase. In the joint capsules of patients with elbow contractures, mRNA levels were increased for transforming growth factor- β 1, connective tissue growth factor, and α -smooth muscle actin. In the joint capsules of rabbit knees with contractures, mRNA levels were increased for transforming growth factor- β 1, connective tissue growth factor, ED-A of fibronectin, and α -smooth muscle actin. The mRNA levels for transforming growth factor- β 1, connective tissue growth factor, and α -smooth muscle actin decreased with time in rabbit knees. The elevated levels of these myofibroblast up-regulators and fibrogenic growth factors could explain the previously reported increase in myofibroblasts and collagen mRNA levels. The rabbit knee model correlated well with the human post traumatic elbow contractures.

A sound anatomic basis for the pathogenesis of joint contractures is known.⁹ For elbow joint contractures, surgical release has stressed the importance of removing the joint capsule.^{5,30,34} There is an elevated number of myofibroblasts and an increase in the expression of α -smooth muscle actin (α -SMA), a myofibroblast marker, within joint capsules obtained from human elbows with chronic post traumatic contractures.¹⁸ A rabbit model of post traumatic joint contractures has shown similar changes with regard to myofibroblast numbers and α -SMA levels in capsules obtained from knees with chronic contractures.¹⁵ Thus, there is good correlation between the human condition and the animal model. The pathologic process that leads to increases in myofibroblast numbers and α -SMA levels detected in contracted joint capsules is unknown.

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Each author certifies that his or her institution has approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.

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Myofibroblasts are tissue fibroblasts that express the contractile smooth muscle protein α -SMA.^{29,32} Myofibroblasts contract fibrin gels with greater force than fibroblasts.^{31,32} Myofibroblasts have been associated with other orthopaedic conditions characterized by contracture such as frozen shoulder, Dupuytren's contracture of the hand, and clubfoot.^{7,31,38} Growth factors such as transforming growth factor-beta1 (TGF- β_1) and the ED-A domain of fibronectin (ED-A) have been shown to increase myofibroblasts and induce α -SMA expression.^{10,32,36} The exact mechanisms of the myofibroblast induction remains to be elucidated, but the ED-A segment is required for the myofibroblast formation induced by TGF- β_1 .³²

Matrix changes in the joint capsule include a profibrotic response with increases in collagen expression in animal models and human joints.^{2-4,7,8,16,26} Collagen mRNA levels are elevated in the joint capsules of patients with chronic post traumatic elbow contractures.¹⁶ Transforming TGF- β_1 and connective tissue growth factor (CTGF) have properties that increase collagen synthesis.^{20,21} In normal healing processes, TGF- β_1 appears to increase CTGF expression, which in turn leads to collagen synthesis and repair.²⁰ However, in conditions such as scleroderma with fibrosis in the skin, CTGF expression becomes unlinked to TGF- β_1 expression and CTGF levels remain elevated.¹⁹ Thus, increased levels of TGF- β_1 or CTGF may be associated with joint capsule fibrosis.

The primary purpose of this study is to measure the mRNA levels of growth factors in joint capsules harvested from patients with chronic elbow contractures. A secondary purpose is to validate an animal model of post-traumatic joint contractures. The primary hypothesis of the study is the mRNA levels of TGF- β_1 , CTGF, ED-A domain of fibronectin, and α -SMA are increased in patients with chronic, post traumatic contractures compared with postmortem organ donors free of contractures. The secondary hypothesis maintains that the mRNA levels of TGF- β_1 , CTGF, ED-A domain of fibronectin, and α -SMA would be increased in joint capsules of rabbit knees with surgically induced contractures compared with contralateral unoperated knees. The third purpose of the study is to determine the mRNA levels of TGF- β_1 , CTGF, ED-A domain of fibronectin, and α -SMA as a function of time following creation of the contracture; this addresses the hypothesis that initially high level of expression of these mRNAs would decrease with time, but remain elevated in the joint capsules of rabbit knees with contractures compared with normal knees.

MATERIALS AND METHODS

Human anterior capsules of elbows were obtained from 11 patients (9 male, 2 female). The average age was 31 ± 12 years (mean $\pm$ standard deviation [SD]; range 14–48 years) at the time of contracture release and the release was performed at an average of 15 ± 7 months (range, 5–25 months) after injury (Table 1). The average preoperative range of motion (ROM) in the flexion-extension arc was $46^\circ \pm 22^\circ$ (range, 10–85°). The original injuries were intraarticular fractures; five patients with distal humerus fractures, one patient with a radial head fracture, two patients with proximal ulna fractures, one patient with proximal radius and ulna fractures, and two patients with radial head fractures associated with elbow dislocations. We obtained control anterior capsules from nine elbows (7 males, 2 females) of postmortem organ donors (average age, 23 ± 13 years; range 15–52 years) free of

contractures (Table 1). The tissues were immediately frozen for mRNA analysis using liquid nitrogen. The tissues from the organ donors were obtained within 5 to 18 hours of death (body stored at 4°C) and prepared as above as mRNA is stable in periarticular tissues for up to 96 hours after death.²⁴ The joint capsule tissue samples were stored at -80°C until further processed for RNA isolation and assessment of specific mRNA levels.

The animal studies used 24 skeletally mature New Zealand white female rabbits (age, 12–18 months, average mass 5.5 ± 0.5 kg; Reimans Furrier, St. Agatha, ON, Canada) divided equally amongst four groups depending on the time of remobilization (Table 2). The animals were individually housed indoors and were given food and water ad libitum. Institutional Animal Review Committee approval was obtained before their use. The right knees underwent the surgical procedure while the contralateral hind limbs served as unoperated controls. Normal knee capsules ($n = 6$) were obtained from three age- and gender-matched rabbits. The normal knees were used as a second control.

The surgical procedures have been reported previously.^{14,15} Under inhalation general anesthesia (halothane 3–5%), incisions were made over the lateral thigh and anterior aspect of the mid-tibia. Five mm² cortical windows were carefully removed from the nonarticular cartilage portion of the medial and lateral femoral condyles, preserving the collateral ligaments. The mobile rabbit skin allowed the lateral thigh incision to be brought over the knee and through parapatellar incisions the cortical windows were removed. The removal of the cortical windows mimics an intraarticular fracture with bleeding into the joint.³⁵ Immobilization of the right knee was performed with an extraarticular, smooth 1.6 mm diameter Kirschner wire (k wire) (Zimmer, Mississauga, ON, Canada). The k wire was drilled through the anterior cortex of the tibia and passed posterior to the knee. The k wire was advanced posteriorly over the extraarticular side of the lateral femoral condyle and bent around the midshaft of the femur. The lateral thigh incision was used to expose the femur. The knee was placed at 150° of flexion and then the tibial portion of the wire was bent and cut below the skin. The patella was checked to ensure it was reduced. The arthrotomies were not closed. All skin incisions were sutured with 3-0 Ethilon (Ethicon, Johnson & Johnson, Peterborough, ON, Canada). Postoperatively, the rabbits were allowed free cage activity (0.1 m³). The left knee was never surgically manipulated. For the groups with remobilized knees, a second anesthetic was administered 8 weeks after the first surgery and the k wire was removed.¹⁵

The 0-week group was sacrificed after 8 weeks of immobilization with an overdose (1.5 mL) of Euthanyl (MTC, Cambridge, ON, Canada). The other three groups were sacrificed after the prescribed number of weeks of remobilization (8, 16 or 32 weeks). We removed the hind limbs immediately after sacrifice. Biomechanical measures describing the permanent joint contractures made the day of sacrifice have been published.¹⁵ We harvested the posterior joint capsules immediately after the biomechanical measures, and all tissues were stored at -80°C until further processed for mRNA levels.

The mRNA level measurements used previously published RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) methods in our laboratory.^{1,15,17,18,22,23,28} The total RNA was extracted and purified using the RNeasy Total RNA kit

(Qiagen, Chatsworth, CA). Sybr Green II reagent was used to quantify the total RNA and was compared to standards obtained from calf liver. Reverse transcription of the total RNA was performed with the Qiagen Omiscript Reverse Transcriptase Kit (Qiagen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control gene whereby each RT sample was analyzed for GAPDH cDNA level, and the volumes were normalized. This was then verified by another PCR. PCR was performed with specifically designed rabbit primers (Table 3) and human primers (Table 4) at optimized conditions. All primers cross intron–exon boundaries and have been used previously in the literature (Tables 3, 4). The PCR profile consisted of 23 to 35 cycles. All of the assays were repeated at least twice for each sample using two separate aliquots of total RNA. No RT controls were performed confirming no cDNA came from the original samples. No template controls also tested appropriately. The PCR products were mixed with loading buffer and loaded to a 2% agarose gel in which electrophoresis occurred. Immediately afterwards, gels were stained with ethidium bromide, destained with distilled water, and photographed with Polaroid film number 55 (Technicare Inc, Calgary, AB, Canada). The proper size of the PCR products was verified with the standard 1-kb DNA Ladder (Life Technologies, Gaithersburg, MD). We then used densitometric scanning of negatives (Masterscan Interpretive Densitometer; CSPI, Billerica, MA) to determine the approximate optical densities of the bands. The scanned images were analyzed with restriction fragment length polymorphism scanalytics (Scanalytics; CSPI, Billerica, MA). The integrated density values obtained from the assays performed with the human or rabbit primers were compared to an average of the normalized GAPDH values.

Statistical comparisons of the human data were completed with a Student's t test. The comparison between rabbit contracture and contralateral control limbs were made with a paired t test. We used an analysis of variance (ANOVA) with post hoc Tukey tests to evaluate the changes over time. The normal knee joint capsules were included as one group in the ANOVA. The data are presented as mean $\pm$ standard deviation with $p < 0.05$ indicating significance.

RESULTS

No rabbits died or had to be excluded from the study. All k wires were intact after 8 weeks of immobilization. Contractures developed in the immobilized knees. The range of motion measures of the contracture knees showed a loss of extension that averaged 38° in the 0-week group; 33° in the 8-week group, 19° in the 16-week group and 18° in the 32-week group. The contralateral knee motion showed a loss of extension that averaged 8°. Motion of normal rabbit knees using methods to test the contracture and contralateral knees also showed a loss of extension that averaged 12°. Myofibroblast numbers averaged 147 cells/field in the 0-week group, 141 cells/field in the 32-week group and 33 cells/field in the contralateral controls in 0- and 32-week groups.

Growth factor mRNA levels in the human anterior elbow joint capsules were elevated in the tissues from the patients with contractures compared with the organ donor control tissues for all molecules except the ED-A domain of fibronectin (Fig 1). The mRNA levels for TGF- β_1 and CTGF were greater ($p = 0.01$ and $p = 0.001$, respectively) in the contracture capsules.

The mRNA levels of α -SMA, a myofibroblast marker, were also greater ($p = 0.002$) in the contracture capsules.

Growth factor mRNA levels in the rabbit posterior knee joint capsules were elevated in the tissues from the knees with contractures compared with the contralateral control tissues for all molecules in all groups (Figs 2–5). The mRNA levels for TGF- β_1 were significantly greater ($p = 0.008$) in the contracture capsules compared with the contralateral and normal control capsules in all groups (Fig 2). The mRNA levels for CTGF were significantly greater ($p = 0.003$) in the contracture capsules compared with the contralateral and normal control capsules in all groups (Fig 3). The mRNA levels for the ED-A domain of fibronectin were significantly greater ($p = 0.003$) in the contracture capsules compared with the contralateral and normal control capsules in all groups (Fig 4). The mRNA levels of α -SMA were significantly greater ($p = 0.04$) in the contractures capsules compared with the contralateral and normal control capsules (Fig 5).

In the rabbit model the trend with time was for a decrease in growth factor expression (Figs 2–5). When comparing the contracture knees amongst the groups, the TGF- β_1 mRNA levels of the 0-week group were significantly greater than the 32-week group (Fig 2). For CTGF the mRNA levels of the 0-week group were significantly greater than the 32-week group ($p = 0.003$) while the mRNA levels of the 8-week group were significantly greater than the 32-week group ($p = 0.04$) (Fig 3). There were no differences between the contracture knees' ED-A mRNA levels (Fig 4). The α -SMA mRNA levels for the 0-week group were significantly greater than the 16- and 32-week values ($p = 0.02$ and $p = 0.003$, respectively), and the 8-week mRNA levels were also significantly greater than the 16- and 32-week values ($p = 0.04$ and $p = 0.01$, respectively) (Fig 5).

DISCUSSION

To gain insight regarding the observations that myofibroblast numbers and collagen synthesis are increased in the joint capsules of patients with post traumatic elbow joint contractures and in the joint capsules of a rabbit knee model of post traumatic joint contractures, we measured selected growth factor mRNA levels in human and rabbit joint capsules. In chronic human elbow contractures, TGF- β_1 and CTGF mRNA levels were increased, while the increased mRNA levels of the ED-A domain of fibronectin were not significant, in the capsules of the patients with post traumatic contractures compared with tissues from organ donors free of contractures. In the rabbit knee model of post traumatic joint contractures, the capsules of the knees with contractures had elevated mRNA levels for TGF- β_1 , CTGF, and ED-A domain of fibronectin when compared with the contralateral unoperated knee or with normal rabbit knee capsules at all time periods evaluated. With time the rabbit knee joint capsule mRNA levels of TGF- β_1 and CTGF decreased but remained elevated compared to the controls. The mRNA results for TGF- β_1 , CTGF, ED-A domain of fibronectin, and α -SMA in the rabbit knee model in the 0- to 32-week groups (which is up to 40 weeks after the original injury) reflect very closely the mRNA results of these molecules observed in chronic human elbow contractures. This suggests a correlation between the human condition and this animal model.^{15,18}

Certain limitations apply to this study. In this article, mRNA levels only are reported. The mRNA levels do not necessarily predict protein levels of the molecules considered. For α -SMA where we report elevated mRNA levels, work in our laboratory has determined that protein levels (Western Blot) and immunohistochemical counts of myofibroblasts using α -SMA as a marker were elevated as well in human anterior joint capsule in patients with post traumatic contractures.¹⁸ Our laboratory has also reported that the number of joint capsule myofibroblasts as measured with immunohistochemistry using α -SMA as a marker is increased in the rabbit knee model of post traumatic contractures.¹⁵ For this molecule at least measures of mRNA levels correlate with protein measures and would suggest increased protein levels of TGF- β_1 and ED-A domain of fibronectin. The second issue is that mRNA levels of all molecules were elevated in the current study. While the values are normalized to the housekeeping gene GAPDH, one possibility is that there may be a nonspecific increase in matrix and growth factor transcription. Previous work from our laboratory using the same human and rabbit samples evaluated in the current study revealed specific upregulation and/or downregulation of collagen, matrix metalloproteinase (MMP), tissue inhibitor of matrix metalloproteinase (TIMP), and small leucine-rich proteoglycan mRNA levels.^{16,17} Thus, the mRNA levels were specifically regulated in human and rabbit tissue. The final consideration is that of controls. Contralateral unoperated control capsules and control capsules obtained from rabbits not having any surgery (normal controls) were used. In both cases, the contracture knees were significantly different than either control for all groups. While the value of the statistical significance calculation differed depending on the control used, with the statistical cut off at the commonly used $p = 0.05$ the interpretation of a statistically significant difference remained the same. The term "contralateral effect" has been used to describe the differences in the normal and contralateral control and has been observed with other biomechanical and molecular measures of rabbit knees in our research centre.^{11,16}

Growth factors have been evaluated in shoulder joint capsules and the findings are similar to elbow joint capsules.^{8,26} In frozen shoulder or adhesive capsulitis, limited shoulder motion develops idiopathically, or in association with local disease (rotator cuff tendinopathy) or systemic disease (diabetes). Using immunolocalization, Rodeo et al²⁶ found TGF- β_1 staining to be greater in the joint capsule of patients with adhesive capsulitis compared with control tissues obtained from patients free of contractures. Bunker et al⁸ reported the presence of fibrogenic growth factors, such as TGF- β_1 , in the joint capsule of patients with frozen shoulder was similar to tissues obtained from Dupuytren's contracture of the hand. While the shoulder conditions were nontraumatic processes, the increases in joint capsule TGF- β_1 were similar to the human elbow joint capsules in post traumatic contractures, and was consistent with the role of TGF- β_1 in fibrotic processes.²¹

The elevated levels of the growth factors opens speculation on the mechanisms of our previous reports regarding cellular and matrix changes in post traumatic elbow contractures and the rabbit knee model of post traumatic contractures.^{13,15-18} TGF- β_1 and the ED-A domain of fibronectin induce α -SMA and assembly of α -SMA into stress fibers, increase fibronexus adhesion complexes and increase myofibroblast numbers.^{21,32} The increased number of myofibroblasts observed in the joint capsules in human elbows and rabbit knees are consistent with the current study. TGF- β_1 and CTGF are fibrogenic and increase

collagen synthesis.^{19–21} It is postulated that CTGF may mediate the effects of TGF- β_1 . Normally CTGF expression follows TGF- β_1 expression; however, in fibrotic conditions such as scleroderma, CTGF expression is elevated and appears to operate independently of TGF- β_1 .¹⁹ The elevated mRNA levels of TGF- β_1 and CTGF in this study were consistent with our previous observations of increased collagen Types I, II, III, and V mRNA levels in joint capsules from chronic human elbow and rabbit knee post traumatic contractures.^{16,17} Many authors have determined increased collagen synthesis and turnover in the joint capsule is associated with joint contractures.^{3,4,6,12,16,25,27}

The current study further validates the animal model of post traumatic contractures. Clinically post traumatic contractures are characterized by a loss of joint motion. The rabbit knee model described here replicates the change in motion over time with an initial decrease in severity of the contracture that eventually plateaus as a permanent loss of motion in chronic stages.¹⁵ The joint capsule is a critical component to human elbow contractures and our laboratory has shown that myofibroblast numbers are elevated in post traumatic contractures.^{13,18} The posterior joint capsule is a critical contributor to joint contractures in animal models including rabbits and our rabbit model also has elevated numbers of myofibroblasts.^{15,33,37} Measures of matrix molecule mRNA levels in the joint capsules of human elbows and rabbit knees revealed increases in collagen types I and III and MMP-1 and -13 with decreases in TIMP-1 and -2.^{16,17} The increased levels of TGF- β_1 and CTGF in the joint capsules in human elbows and rabbit knees further validates the similarity of the animal model to the human condition by adding more molecules changing in the same direction; these growth factor changes are consistent with the previously reported myofibroblast and matrix changes in chronic post traumatic joint contractures. The ED-A domain of fibronectin mRNA levels were increased in rabbit capsules but not in human capsules. The reason for this discrepancy is unknown although one possibility is that the ED-A expression decreases with longer times after injury since the average time from injury for the humans was 15 months while the longest time post injury for the rabbits was 40 weeks.

This study demonstrates joint capsules in chronic post traumatic contractures in human elbows and an animal model have elevated levels of the myofibroblast upregulators and/or fibrogenic growth factors TGF- β_1 , ED-A domain of fibronectin and CTGF. These findings are consistent with clinical reports of a thickened, fibrotic capsule in post traumatic contractures and previous animal models showing a modification of joint capsule collagen turnover.^{3,9} Methods to modify the growth factor contribution may lessen the severity and/or prevent the occurrence of post traumatic joint contractures, but more studies are required.

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Fig 1.

Human anterior elbow capsule mRNA values are shown. The statistical significance refers to contracture versus control comparisons.

Fig 2.

Rabbit capsule TGF- β_1 mRNA values are shown. All contracture values are significantly greater than contralateral and normal control values ($p = 0.008$). The values of the 0-week contracture group are significantly greater than the 32-week contracture group values ($p = 0.04$).

Fig 3.

Rabbit capsule CTGF mRNA values are shown. All contracture values are significantly greater than control and normal values ($p = 0.003$). The 0-week and 8-week contracture values are significantly greater than the 32-week values ($p = 0.003$ and $p = 0.04$, respectively).

Fig 4.

Rabbit capsule ED-A domain of fibronectin mRNA values are shown. All contracture values are significantly greater than contralateral and normal control values ($p < 0.003$).

Fig 5.

Rabbit capsule α -SMA mRNA values are shown. All contracture values are significantly greater than contralateral and normal control values ($p = 0.04$). The 0-week contracture values were significantly greater than the 16- and 32-week contracture values ($p = 0.02$ and $p = 0.003$, respectively) and the 8-week contracture values were significantly greater than the 16- and 32-week contracture values ($p = 0.04$ and $p = 0.01$, respectively).

TABLE 1

Data Summary of Human Subjects

Contracture Specimens	Age (years)	Chronicity (months)	Total Motion (degrees)
Male	18	20	50
Male	48	25	56
Male	32	9	60
Male	42	5	85
Male	17	22	39
Female	41	6	57
Female	14	13	15
Male	31	23	40
Male	20	9	30
Male	36	10	65
Male	45	19	10
Mean	31	15	46
Standard Deviation	12	7	22
Control Specimens			
Male	52	—	> 130
Male	35	—	> 130
Male	18	—	> 130
Male	16	—	> 130
Female	15	—	> 130
Male	16	—	> 130
Female	15	—	> 130
Male	18	—	> 130
Male	21	—	> 130
Mean	23	—	> 130
Standard Deviation	13	—	—

Chronicity = time from injury to contracture release; total motion = extension and flexion plane

TABLE 2

Rabbit Distribution (n = 6 per group)

Group	Immobilization (weeks)	Remobilization (weeks)
0-week	8	0
8-week	8	8
16-week	8	16
32-week	8	32

TABLE 3

Rabbit-specific Primers

Primer	Sequence	bp	Temperature (C)	Cycles	Source
TGF- β_1	F-CGG CAG CTG TAC ATT GAC TT R-AGC GCA CGA TCA TGT TGG AC	271	60	32	AF 000133
CTGF	F-TTG TAG CTG ATC AGT CTT TCC AC R-CAA CTA AAA AGG TGC AAA CAT GTA A	119	55	35	NM-204274
ED-A	F-GGA GAG AGT CAG CCT CTG GTT CAG R-TGT CCA CTG GGG GCT CAG GCT TGT G	374	65	35	X07718
α -SMA	F-GTG TGA GGA AGA GGA CAG CA R-TAC GTC CAG AGG CAT AGA GG	391	60	35	X60732
GAPDH	F-TCA CCA TCT TCC AGG AGC GA R-CAC AAT GCC GAA GTG GTC GT	293	55	25	L23961

F = forward; R = reverse; bp = base pairs; TGF- β_1 = transforming growth factor- β_1 ; CTGF = connective tissue growth factor; ED-A = ED-A domain of fibronectin; α -SMA = α -Smooth muscle actin; GAPDH = glyceraldehyde-3-phosphate dehydrogenase

TABLE 4

Human-specific Primers

Primer	Sequence	bp	Temperature (C)	Cycles	Source
TGF- β_1	F-GGG GAA ATT GAG GGC TTT CG R-CCA GGA CCT TGC TGT ACT GC	388	55	30	NM-000660
CTGF	F-CCT CTT CTG TGA CTT CGG CT R-CAG GAG GCG TTG TCA TTG GT	447	65	28	NM-001901
ED-A	F-GGA GAG AGT CAG CCT CTG GTT CAG R-TGT CCA CTG GGC GCT CAG GCT TGT G	374	65	35	X07718
α -SMA	F-GCT CAC GGA GGC ACC CCT GAA R-CTG ATA GGA CAT TGT TAG CAT	591	60	30	NM-001613
GAPDH	F-TCA CCA TCT TCC AGG AGC GA R-AGT GAT GGC ATG GAC TGT GG	326	65	25	BC023632

F = forward; R = reverse; bp = base pairs; TGF- β_1 = transforming growth factor- β_1 ; CTGF = connective tissue growth factor; ED-A = ED-A domain of fibronectin; α -SMA = α -Smooth muscle actin; GAPDH = glyceraldehyde-3-phosphate dehydrogenase