Chapter

In vitro Effect of TGF- β 1 on Gene Expression in Flexor Digitorum Profundus Tendon

Subhash C Juneja[1]*, Tony Chen[2] and Youssef M Farhat[3]

[1]Division of Orthopaedic Surgery, Bathurst St. Toronto Western Hospital, Canada
[2]Department of Biomechanics, Hospital for Special Surgery, USA
[3]University of Rochester Medical Center, USA

***Corresponding Author:** Subhash C Juneja, Email: sjuneja.phd@gmail.com

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Abstract

Among hand injuries, exor tendon lacerations remain a challenge for hand surgeons. There are presently no therapeutic agents available for the prevention of tendon adhesions. It is already known that TGF-1 plays a role in tendon healing as well as in adhesion formation. Anti-TGF-1 therapies are not eective in preventing adhesion formation. The goal of the present study was to identify possible genes that are aected by TGF-1 in human Flexor Digitorum Profundus (FDP) tendon cells (tenocytes) in vitro. Tenocytes were isolated from human FDP tendons and treated with TGF-1 in low-serum cell culture medium. Gene expression was assessed at 6h and 24h using RTPCR. TGF-1 caused upregulation of several genes (SERPINE1, PLAU, ACTA2, CTGF, FN1, COL1A1, COL3A1, LOX, COMP, MMP13, TIMP1, TIMP3, BGN, SCX, POSTN, SMAD7, IL6, IGF1), downregulation of MMP9, DCN and ACAN, and had no eect on MMP2 and TIMP2.

Targeting TGF-1-aected genes may be an alternative therapeutic approach in controlling adhesion formation that may lead to optimal healing of injured FDP tendon or FDP tendon graft.

Keywords

Tendon; Gene Expression; TGF-β1; Adhesion Formation; Tendon Healing

Introduction

Each year, millions of North Americans injure their hands resulting in significant morbidity and lost workdays [1,2]. In the USA, among all the injuries reported at private, state, and local government, 12.7% were hand injuries; and the average days away from work was 5 days in 2014 [2]. Among hand injuries, flexor tendon lacerations remain a challenge for hand surgeons. While flexor tendons heal with reduced mechanical strength, the most clinically relevant issue is adhesion formation, which impairs hand activity. There are presently no therapeutic agents available for the prevention of tendon adhesions [3]. In fact, the only accepted means of preventing adhesion formation in flexor tendons is physical therapy [3]. Even with physical therapy, the strength of healed tendons is markedly less than uninjured tendon, and debilitating adhesions have been reported to occur in as many as 50% of zone II flexor tendon injuries [4]. TGF-β1 is a cytokine that plays multiple roles in wound healing and is also implicated in the pathogenesis of excessive scar formation [5]. TGF-β1 stimulates chemotaxin, promotes angiogenesis, and regulates a wide spectrum of matrix proteins. It accelerates the wound-healing process in several animal models. However, this effect may proceed uncontrollably and result in pathological fibrosis, with excessive disordered collagen deposition resulting in tendon adhesions [6]. Hence, inhibitors of TGF-β1 have been tried to reduce adhesion formation.

Biologic strategies to inhibit TGF-β signaling have been reported, e.g., administration of neutralizing antibodies, application of soluble receptors, usage of antisense nucleotides, and chemically-synthesized inhibitors of the receptor serine/threonine kinases. Clinical trials have been performed with neutralizing antibodies, especially in fibrotic diseases, including TGF-β2 neutralizing antibody (lerdelimumab), which effectively decreased the amount of scarring after glaucoma surgery [7]. A TGF-β1 neutralizing antibody (CAT-192, metelimumab) has been administered intravenously to patients with systemic sclerosis, which causes scarring in skin and internal organs [8]. In rat cutaneous wound healing, exogenous addition of neutralizing antibody to TGF-β1 plus neutralizing antibody to TGF-β2 reduced the monocyte and macrophage profile, neovascularization, fibronectin, collagen III and collagen I deposition in the early stages of wound healing compared to control wounds. This reduced the scarring while the control wounds healed with scar formation [9]. In transected and repaired rabbit flexor tendons, neutralizing antibody to TGF-β1 increased range of motion [5,10]. Mannose-6-phosphate reduced TGF- β-upregulated collagen production in rabbit flexor tendon cells *in vitro* , and *in vivoapplication* of mannose-6-phosphate to transected repaired rabbit zone II flexor tendon significantly improved the range of motion [11]. Using anti-sense oligonucleotides to reduce the expression of TGF-β superfamily members has been applied in clinical trials for cancer treatment [12]. The TGFBR1 inhibitors have shown to be efficient in mouse tumor models [13]. ALK-5 inhibitor (SB-505124) blocked TGF-β1-induced CTGF expression in gingival fibroblasts [14] and suppressed the *in vivo* and *in vitro* action of TGF-β in rabbit subconjuctival fibroblasts [15]. None of the foregoing anti-TGF-β therapies addressed or resolved the prevention of adhesion formation in tendon injury or tendon graft repair. The purpose of this study is to understand the effect of TGF-β1 on the expression of multiple genes *in vitro* in human FDP tenocytes in cell culture. Instead of anti-TGF-β1 therapy to control adhesion formation, targeting individual molecules regulated by TGF-β1 could be an alternative option to control adhesion or scar formation *in vivo*.

Methods and Materials

Isolation of Tendon Cells from FDP and their Culture *in vitro*

Human FDP tendon specimens, discarded for surgical reasons, were obtained from the University of Rochester Medical Center (Rochester NY USA) from patients following a protocol that was approved by University ethics committee. The normal part of the tendon specimen was used to isolate tendon cells known as tenocytes or tendinocytes. The tissue was transported to the laboratory in sterile DPBS (ThermoFisher Scientific #14287072) at room temperature. Specimen were stripped of surrounding tissue, washed in DPBS containing 1% Penicillin-Streptomycin (10,000 U/mL; ThermoFisher Scienific#15140122), and minced into ~1-mm pieces with sterile scalpels. The minced tissue pieces were treated with trypsin (0.25% trypsin; #25200056, ThermoFisher Scientific) for 30 min at room temperature under a sterile cell culture Biosafety Cabinet. The tendon pieces were than transferred into 100-mm cell culture dishes containing cell culture medium composed of MEM-alpha (ThermoFisher Scientific #12571) supplemented with 20% heatinactivated fetal bovine serum (Sigma-Aldrich, #F6178), 1% Penicillin- Streptomycin, and 100 μM 2-mercaptoethanol (Sigma-Aldrich, #M7522). Medium was changed gently every 3 days without loosing minced tissue pieces. Tenocytes proliferated from tendon pieces by day-10, and the monolayer of cells was obtained by day-16. The cells were expanded further upto passage 3. For experimental purpose, a total of 0.6x106 cells were plated in 100 mm cell culture dishes in 10%- FBS medium for 48h. Medium was replaced by 1%-FBS medium. After serum starving for 16h, medium was replaced by fresh 1%-FBS medium (control) or 1%-FBS medium containing 5 ng/ml rhTGFb1 (Recombinant human TGF-beta 1 protein; TGF-β1; #240-B-010, R&D Systems). The cell culture experiment was terminated at 6h and 24h. We used 5 ng/ml concentration since it is in the accepted physiological range as shown in literature [16].

RNA Extraction and Real-Time Reverse Transcription Polymerase Chain Reaction

For total RNA isolation, medium was removed from the dishes. The monolayer of tenocytes was first scraped with a tissue scraper and than resuspended in 1 ml of Trizol reagent (ThermoFisher Scientific #15596018) and homogenized using a hand-driven glass homogenizer, and total RNA was isolated using manufacturer's protocol. Complementary DNA (cDNA) was prepared from 1 µg total RNA in a 20 µL of reaction mixture in 0.2-mL tubes (Bio-Rad) using MMLV Reverse Transcriptase system (ThermoFisher Scientific#28025013) and following the manufacturer's protocol. A fixed volume of 0.5 µL cDNA was used for real-time reverse transcription polymerase chain reaction (RT-PCR) using SYBR Green (Applied Biosystems#: 4309155) and specific primers for human genes (Table 1). The mRNA expression of several genes at different time-points was assessed (Figures 2-4). The amplification was monitored real time using the 96-well iCycler iQTM Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). The threshold cycle (Ct) values were related to a standard curve made with the cloned PCR products, and specificity was confirmed by melting curve analysis after amplification. The general range of Ct values were 15-30. Beta-actin gene Actb was chosen as an internal control. Data at different time-points (6h and 24h) of TGF-β1- treatment groups, in triplicates, are presented as the mean fold induction over untreated groups. Data at each time-point, in triplicates, were presented as the mean fold induction ±SD; p-value less than 0.05 to differ treatment from control, was considered as significant.

Top 5 Contributions in Orthopedics

Table 1: Human primer sequences used in RT-PCR.

Gene		Primer sequence (5'è3')	NCBI Ref. No. Sequence	Size
ACAN	F	CCCAACCAGCCTGACAACTTT	NM_013227.3	216
(aggrecan)	R	GTACCGCACCAGGGAATTGAT		
ACTA2	F	CAGGGCTGTTTTCCCATCCAT	NM_001141945.1	142
(alpha 2, smooth muscle, aorta)	R	GCCATGTTCTATCGGGTACTT		
ACTB	F	CATGTACGTTGCTATCCAGGC	NM_001101.3	250
(actin, beta)	R	CTCCTTAATGTCACGCACGAT		
BGN	F	GAACAGTGGCTTTGAACCTGG	NM_001711.5	178
(biglycan)	R	CAGCTTGGAGTAGCGAAGCA		
COLIA1	F	GCCGTGACCTCAAGATGTG	NM_000088	208
(collagen, type I, alpha 1)	R	GCCGAACCAGACATGCCTC		
COL3A1	F	GGTGCTCGGGGTAATGACG	NM_000090.3	84
(collagen, type III, alpha 1)	R	TCCAGGGAATCCGGCAGTT		
COMP	F	CGAGTCCGCTGTATCAACACC	NM_000095.2	170
(cartilage oligomeric matrix protein)	R	GAGTTGGGGACGCAGTTATGT		
CTGF	F	GGCAAAAAGTGCATCCGTACT	NM_001901.2	113
(connective tissue growth factor)	R	CCGTCGGTACATACTCCACAG		
DCN	F	AGTTGGAACGACTTTATCTGTCC	NM_133503.3	160
(decorin)	R	GTGCCCAGTTCTATGACAATCA		
FN1	F	GAAGGCTTGAACCAACCTACG	GeneBank: AB191261.1	96
(fibronectin 1)	R	TGATTCAGACATTCGTTCCCAC		
IGF1	F	GGAGCTGTGATCTAAGGAGGC	NM_001111283.1	119
(insulin-like growth factor 1)	R	GGGCTGATACTTCTGGGTCTT		
IL6	F	AAATTCGGTACATCCTCGACGG	NM_000600.3	112
(interleukin 6)	R	GGAAGGTTCAGGTTGTTTTCTGC		
LOX	F	GCCCGTCACTGGTTCCAAG	GeneBank: EF094938.1	164
(lysyl oxidase)	R	TAGGGGTTGTAAGGGTCGTCG		
MMP2	F	GCCCCAGACAGGTGATCTTG	NM_001302510.1	101
(matrix metallopeptidase 2)	R	GCTTGCGAGGGAAGAAGTTGT		
MMP9	F	TGGCAGAGATGCGTGGAGA	NM_004994.2	229
(matrix metallopeptidase 9)	R	GGCAAGTCTTCCGAGTAGTTTT		
MMP13	F	ACTGAGAGGCTCCGAGAAATG	NM_002427.3	103
(matrix metallopeptidase 13)	R	GAACCCCGCATCTTGGCTT		
PLAU	F	GTGAGCGACTCCAAAGGCA	NM_002658.3	117
(plasminogen activator, urokinase type)	R	GCAGTTGCACCAGTGAATGTT		
POSTN	F	CTTGGCTCATAGTCGTATCAGGG	NM_006475.2	69
(periostin, osteoblast specific factor)	R	CCCAAAATCTGTTGAAGGGCA		
SCX	F	GCACGCTGATCCCCACCGAG	NM_001080514.2	95
(basic helix-loop-helix transcription factor scleraxis)	R	CACGTTGCCCAGGTGCGAGA		
SERPINE1 or PAI1	F	CATCCCCCATCCTACGTGG	NM_000602.4	109
(serpent peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	R	CCCCATAGGGTGAGAAAACCA		
SMAD7	F	TTCCTCCGCTGAAACAGGG	NM_001190821.1	116
(SMAD family member 7)	R	CCTCCCAGTATGCCACCAC		
TIMP1	F	CTTCTGCAATTCCGACCTCGT	NM_003254.2	127
(TIMP metallopeptidase inhibitor 1)	R	CCCTAAGGCTTGGAACCCTTT		
TIMP2	F	AAGCGGTCAGTGAGAAGGAAG	NM_003255.4	136
(TIMP metallopeptidase inhibitor 2)	R	GGGGCCGTGTAGATAAACTCTAT		
TIMP3	F	CCCAGTGATGCTTGTGTTGAC	NM_000362.4	101
(TIMP metallopeptidase inhibitor 3)	R	GGCAGATGTTTAAGTCTTCACCA		

The experiment was repeated three times to observe the consistency of RNA data. Data were analyzed using one-way ANOVA followed by Tukey's all-pair comparisons at alpha = 0.05. A computer software KaleidaGraph was used to analyze the data and MS office Excel was used to draw graphs.

Results

A diagrammatic sketch of a human finger is shown to demonstrate the location of FDP tendon (Figure 1A). Also hematoxylin & Eosinstained paraffin section of human FDP tendon is shown (Figure 1B). Gene expression data is shown as below and in Table 2.

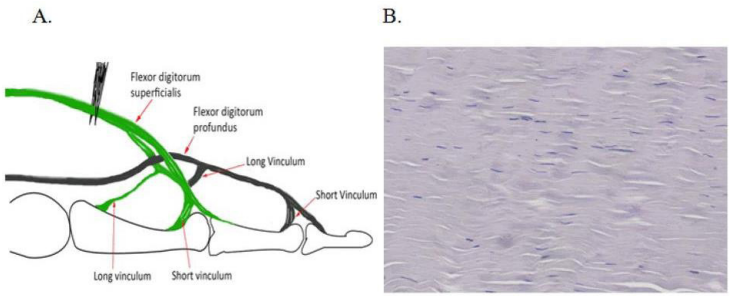

Figure 1: (A) A diagrammatic sketch of human finger (lateral view) showing the location of Flexor Digitorum Profundus (FDP) tendon and Flexor Digitorum Superficialis (FDS) tendon. (B) Hematoxylin and eosin-stained histological paraffin section (longitudinal) of FDP tendon showing collagen fibers and tenocyte nuclei (blue stained).

Table 2: Summary of fold change in gene expression in FDP tendon cells in culture in response to TGFβ1 treatment.

Gene	Expression at 6h (fold change)	Expression at 24h (fold change)
ACAN (aggrecan)	2.9*↓	3.9*↓
ACTA2 (alpha 2, smooth muscle, aorta)	NS	6.5*↑
BGN (biglycan)	NS	2.0**↑
COLIA1 (collagen, type I, alpha 1)	NS	2.5*↑
COL3A1 (collagen, type III, alpha 1)	NS	1.6**↑
COMP (cartilage oligomeric matrix protein)	3.4**↑	17.1*↑
CTGF (connective tissue growth factor)	5.2*↑	13.0*↑
DCN (decorin)	2.9*↓	2.9*↓
FN1 (fibronectin 1)	NS	2.0**↑
IGF1 (insulin-like growth factor 1)	5.3*↑	14.1*↑
IL6 (interleukin 6)	9.6*↑	12.3*↑
LOX (lysyl oxidase)	NS	2.2*↑
MMP2 (matrix metallopeptidase 2)	NS	NS
MMP9 (matrix metallopeptidase 9)	2.2*↓	2.1**↓
MMP13 (matrix metallopeptidase 13)	2.2*↑	3.0*↑
PLAU (plasminogen activator, urokinase type)	2.9**↑	4.2*↑
POSTN (periostin, osteoblast specific factor)	NS	1.8*↑
SCX (basic helix-loop-helix transcription factor scleraxis)	5.0*↑	11.3*↑
SERPINE1 or PAI1 (serpent peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	6.7*↑	17.0*↑
SMAD7 (SMAD family member 7)	1.9*↑	2.0*↑
TIMP1 (TIMP metallopeptidase inhibitor 1)	NS	1.3**↑
TIMP2 (TIMP metallopeptidase inhibitor 2)	NS	NS
TIMP3 (TIMP metallopeptidase inhibitor 3)	1.9*↑	6.6*↑

Note: Significance: *P<0.01; **P<0.05

TGF-β1 caused an Increase in *SERPINE1, PLAU, ACTA2* and *CTGF* Gene Expression

SERPINE1 gene expression increased 6.7 fold at 6h and 17.0 fold at 24h of TGF-β1 treatment to FDP tenocytes in cell culture (P<0.01,Figure 2a). *PLAU* gene expressed 2.9 fold higher at 6h (P<0.05) and 4.2 fold higher at 24h (P<0.01) respectively (Figure 2b). *ACTA2* gene expression was higher at 24h and that was 6.5 fold (P<0.01, Figure 2c). *CTGF* gene expression was 5.2 fold higher at 6h and 13.0 fold higher at 24h as compared to their respective controls (P<0.01, Figure 2d).

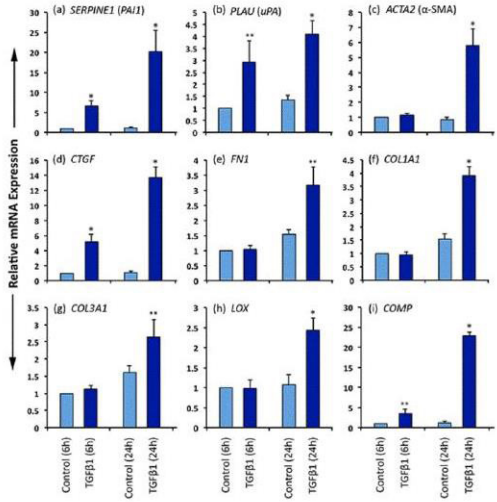

Figure 2: mRNA expression of *SERPINE1* (a), *PLAU* (b), *ACTA2* (c), *CTGF* (d), *FN1* (e), *COL1A1* (f), *COL3A1* (g), *LOX* (h), and *COMP* (i), at 6h and 24h of post-TGF-β1 treatment (5ng/mL) of human FDP tenocytes, in monolayer cell culture. ±S.D., n = 3. *P<0.01; **P<0.05.

TGF-β1 caused an Increase in *FN1, COL1A1 , COL3A1, LOX* and *COMP* Gene Expression

TGF-β1 caused 2-fold increase in *FN1* gene expression at 24h (p<0.05; Figure 2e). It caused 2.5-fold increase in *COL1A1*gene expression at 24h (p<0.01; Figure 2f), and 1.6-fold increase in *COL3A1* at 24h (P<0.05, Figure 2g). *LOX* gene expression increased 2.2 fold at 24h due to TGF-β1 treatment to FDP tenocytes in cell culture (P<0.01, Figure 2h). TGF-β1 caused a sharp increase of *COMP* gene expression. The level raised 3.4 fold at 6h (P<0.05) and 17.1 fold at 24h

(P<0.01, Figure 2i).

TGF-β1 caused Decrease in *MMP9*, Increase in *MMP13*, whereas it has no Effect on *MMP2* Gene Expression

MMP2 gene expression was not affected by TGF-β1 treatment (Figure 3a). TGF-β1 caused a 2.2- and 2.1-fold reduction in expression reduction in *MMP9* gene expression at 6h (P<0.01) and 24h (P<0.05) as compared to their respective control values (Figure 3b), respectively. On the other hand, TGF-β1 caused a 2.2 and 3.0 fold increase in expression of *MMP13* at 6h (P<0.01) and 24h (P<0.01), respectively (Figure 3c).

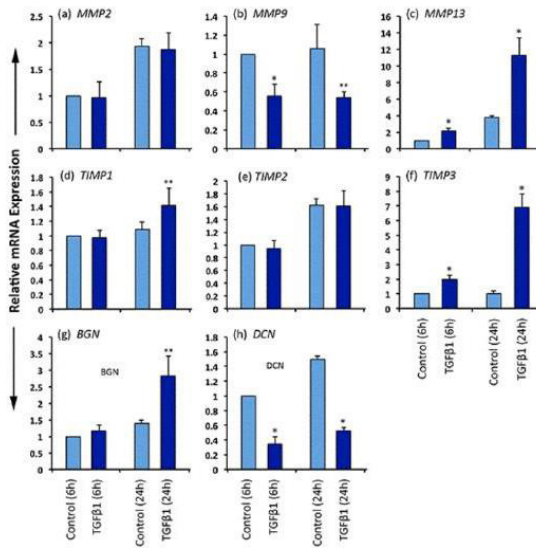

Figure 3: mRNA expression of *MMP2* (a), *MMP9* (b), *MMP13* (c), *TIMP1* (d), *TIMP2* (e), *TIMP3* (f), *BGN* (g), and *DCN* (h), at 6h and 24h of post-TGF-β1 treatment (5ng/mL) of human FDP tenocytes, in monolayer cell culture. ±S.D., n = 3. *P<0.01; **P<0.05.

TGF-β1 caused an Increase in *TIMP1* and *TIMP3* but did not Affect *TIMP2* Gene Expression

TGF-β1 caused 1.3-fold increase in *TIMP1* gene expression at 24h ($P<0.05$, Figure 3d). *TIMP3* gene expression increased 1.9 fold at 6h ($P<0.01$) and 6.6 fold at 24h ($P<0.01$) in response to TGF-β1 treatment to FDP tenocytes (Figure 3f). TIMP2 gene expression was not affected by TGF-β1 treatment to FDP tenocytes in culture (Figure 3e).

TGF-β1 caused Increased BGN and Decreased DCN Gene Expression

BGN gene expression increased 2-fold ($P<0.05$) at 24h of TGF-β1 treatment (Figure 3g), whereas, DCN gene expression decreased 2.9 fold at 6h and at 24h of TGF-β1 treatment to FDP tenocytes in cell culture ($P<0.01$, Figure 3h).

TGF-β1 caused Varied Effect on the Expression of Other Genes

TGF-β1 caused sharp increase of SCX gene expression ($P<0.01$). The level raised 5.0 fold at 6h and 11.3 fold at 24h (Figure 4a). *POSTN* gene expression increased 1.8 fold at 24h ($P<0.01$, Figure 4b). *ACAN* gene expression was lower at 6h ($P<0.01$) and 24h ($P<0.01$) due to TGF-β1 treatment. The expression was 2.9 fold and 3.9 fold lower than their respective control (Figure 4c). *SMAD7* was expressed 1.9 times higher at 6h ($P<0.01$) and 2 times higher at 24h ($P<0.01$) in response to TGF-β1 treatment (Figure 4d). Interleukin 6 (*IL6*) was expressed 9.6- fold higher at 6h ($P<0.01$) and 12.3-fold higher at 24h ($P<0.01$) due to TGF-β1 treatment (Figure 4e). TGF-β1 caused an increase in *IGF1* gene expression (Figure 4f). The increase was 5.3-fold at 6h and 14.1- fold at 24h ($P<0.01$).

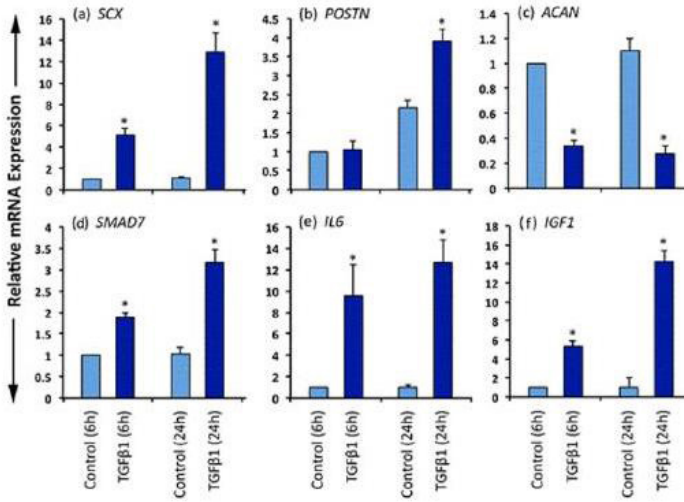

Figure 4: mRNA expression of *SCX* (a), POSTN (b), ACAN (c), SMAD7 (d), IL6 (e), and IGF1 (f), at 6h and 24h of post-TGF-β1 treatment (5 ng/mL) of human FDP tenocytes, in monolayer cell culture. ±S.D. n = 3. *P<0.01; **P<0.05.

Discussion

SERPINE1, PLAU

Plasminogen activator inhibitor 1 (PAI-1) is a single-chain glycoprotein and is encoded by SERPINE1 gene. It is present in plasma as well as synthesized by many tissues. It inhibits uPA and tPA (tissue-etype plasminogen activator) and is a regulator of plasminogen activation and plays primary role in fibrinolysis and is involved in the regulation of cell adhesion, cell migration, and invasion [17]. Skin wound healing is accelerated in PAI-1-deficient mice [18]. PAI-1 deficiency reduces hepatic fibrosis after bile duct obstruction mainly through the activation of tPA and Hepatocyte Growth Factor(HGF) [19]. Earlier we showed that during inflammatory phase of mouse Flexor Digito-

rum Longus (FDL) tendon graft healing, Serpine1 gene expression was higher along with *TGF-β1* gene expression [20]. In the current study, *TGF-β1* upregulated SERPINE1 gene expression in FDP tenocyte cell culture (Figure 2a). This may indicate that *SERPINE1* gene is regulated by TGF-β1 and may be involved in FDP tendon repair and adhesion *in vivo* .

Urokinase-type plasminogen activator (uPA; gene: PLAU) specifically cleaves the zymogen plasminogen to form the active enzyme plasmin. Specific cleavage of Arg-|-Val bond, in plasminogen to form plasmin, is inhibited by PAI-1 [21]. Urokinase-type plasminogen activator plays a vital role in early phases of wound healing by aiding fibrin dissolution, promoting migration, proliferation, and adhesion of various cells to the wound bed [17]. In wounded gingival granulation tissue, TGF-β1 caused an enhanced expression of uPA in cells expressing α-SMA indicating its role during wound healing [22]. Expression of *PLAU* mRNAs was maximal at day 4 and 7 following Achilles tendon injury [23]. In our earlier study, mouse *Plau* mRNA expression was highest at day-3 of FDL tendon graft healing as compared to other days (day-4 onwards), indicating its role in early phase of healing. Farhat et al. [24] showed that TGF-β1 (at 10 ng/ml) did not affect *Plau* gene expression signi icantly at 48h in mouse FDL tendon cells grown on collagen-coated dishes. In the present study, TGF-β1-induced a sharp increase in *PLAU* gene expression at 6h and 24h in human FDP tenocytes (Figure 2b), indicating that TGF-β1's effects on PLAU expression could be more pronounced in the irst 24h. TGF-β1-induced *PLAU* gene expression may play a role in the modulation of FDP tendon in lammation, adhesion and ibrosis *in vivo* .

ACTA2

Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells. α-actin-2 or α-SMA (gene: *Acta2* in mouse or *ACTA2* in human), though mainly expressed in muscle, has been identified in fibroblastic

cells of normal tendons, ligaments, and myofibroblasts [25]. α-actin-2 expressing cells (myofibroblasts), in injured rabbit ligament (day 3 to 12-weeks post-injury), were identified at day 3 in medial collateral ligament, and their density increased to day 21 [26,27]. In mouse FDL tendon graft healing, *Acta2* mRNA expression remained higher on all the days as compared to day 3 post-surgery indicating its role in the post-inflammatory events [20,28]. Cultured fibroblasts acquire myofibroblast phenotype in the presence of TGF-β1 [29]. Myofibroblasts are characterized by large focal adhesions, prominent stress fibers, and enhanced expression of contractile marker proteins such as α-SMA [30]. TGF-β1 (at 10 ng/ml) caused an increase in *ACTA2* gene expression in rat embryonic fibroblasts *in vitroat* 48h [31]. Subcutaneous administration of TGF-β1 to rats results in the formation of a granulation tissue in which α-SMA expressing myofibroblasts were particularly abundant [32]. A universal process in fibrosis is the formation of myofibroblasts and the subsequent collagen deposition by these cells. TGF-β1 plays a major role in the formation of myofibroblasts, e.g., by activating fibroblasts. TGF-β1 caused upregulation of *ACTA2* gene at 48h in PDL (periodontal ligament) stem/progenitor cell lines (PDLSCs) undergoing fibroblastic differentiation [33]. In the current study, we showed that TGF-β1 caused an increase in *ACTA2* gene expression at 24h in FDP tenocytes, in cell culture (Figure 2c). That suggests a role for TGF-β1-induced *ACTA2* in FDP tendon healing and fibrosis *in vivo* .

CTGF

Connective tissue growth factor (CCN2/CTGF) is a growth factor of 38–40 kDa that acts as a potent fibroblast mitogen and angiogenic factor. CTGF belongs to the CTGF, Cyr61 and Nov (CCN) family of growth factors [34]. CTGF is best known as a molecule that mediates the development of fibrotic disorders in a variety of tissues and organs [35,36]. Inflammation, wound healing and fibrosis are mutually related biological events. Inflammation occurs upon tissue injury and/or invasion of pathogenic factors, which usually causes ad-

ditional damage to the tissues. The involvement of CTGF in this phase is well represented by the fact that CTGF regulates the behavior of the mediators of inflammation and vice versa. For example, CTGF is induced by TGF-β1 and is repressed by TNF-α, whereas, this gene product induces inflammatory IL-6, monocyte chemoattractant protein-1 (MCP1) and Extracellular Matrix (ECM)-remodeling MMPs [36-38]. In fact, elevated CTGF expression is observed in the inflamed joints of patients with rheumatoid arthritis and osteoarthritis [39]. TGF-β1 stimulated the transcriptional activity of *CTGF* gene promoter in NIH/3T3 fibroblasts [40]. After exposure to TGF-β1 (5 ng/ml), the maximal level of luciferase activity reached at 12h and maintained to 24h by 2.76- and 2.20-fold, respectively. TGF-β1 stimulated CTGF expression in airway smooth muscle cells [41]. In a lung fibrosis mouse model, CTGF inhibition using an inhibitory antibody (FG-3019) resulted in less fibrosis [42]. In human gingival fibroblasts and Periodontal Ligament (PDL) cells, the expression of *CTGF* mRNA and protein was significantly increased in a dose- and time-dependent manner in the presence of TGF-β1 [43]. The current study showed thsat TGF-β1 plays a role in the modulation of *CTGF*gene expression of cultured human FDP tenocytes, suggesting that *CTGF* may play role in FDP tendon healing and fibrosis *in vivo* (Figure 2d).

FN1

Fibronectin (FN) is a high-molecular weight (~440kDa) glycoprotein of the ECM that binds to membrane-spanning receptor proteins called integrins [44]. The *FN1* gene in humans encodes it. Similar to integrins, fibronectin binds ECM components such as collagen, fibrin, and heparan sulfate proteoglycans (e.g., syndecans). Fibronectin plays a major role in cell adhesion, growth, migration, and differentiation, and it is important for processes such as wound healing and embryonic development [44]. Altered fibronectin expression, degradation, and organization are associated with a number of pathologies, including cancer and fibrosis [45]. TGF-β1 increases the expression of fibronectin. Based upon the 48-72h period required for a maximal fibroproliferative response to dermal injections of TGF-β1, human

fetal lung fibroblasts were exposed to TGF-β1 for periods up to 48h *in vitro* . A 6-fold increase in fibronectin synthesis was observed at 24h [46]. Similar observations were also reported for fibroblastic cells [47]. TGF-β1 induces fibroblast proliferation and transformation into myofibroblasts and stimulates the accumulation of matrix proteins, including laminin, collagens 1 and 3, as well as fibronectin [48]. In the current study, TGF-β1 enhanced the FN1 mRNA expression at 24h in human FDP tenocytes, in cell culture (Figure 2e). TGF-β1-induced fibronectin may play role in physiology and pathology of FDP tendon healing *in vivo* .

COL1A1, COL3A1, LOX

Collagens contribute to tensile strength to tendon and other tissues. Collagen, type I or $[\alpha1(I)]_2$ α2(I) and type III or [α1(III)]3, lays an important role in tendon healing process. During wound healing, one of the fibroblasts' dominant functions is the production of collagen. Higher gene expression of all the collagen genes (*Col1a1*, *Col1a2*, and *Col3a1*) indicated their role during maturation and remodeling phase of FDL tendon graft healing [20,28]. In rat flexor tendon healing study, *Col1a1* mRNA expression increased from day 3 to 28 with a peak at day 28, whereas Col3a1 expression increased from day 3 to the peak value at day 14 and then sharply decreased [49].

TGF-β1 at 1 ng/ml to 100 ng/ml caused significant increase in COL1A1 gene expression at 24h in FDL tendon cells in collagen gels [50]. Varga et al. [51] reported that TGF-β1 caused a marked enhancement in the production of type I and III collagen, and fibronectin by cultured normal human dermal fibroblasts. In another instance, TGF-β1 increased the expression of *COL1A1* in human dermal fibroblasts and in human lung fibroblasts [52]. TGF-β1 caused an increase in collagen I expression at 48h in two PDL stem/progenitor cells lines [33]. Lung fibroblast cultures maintained in medium containing TGF-β1 sustained an activated rate of collagen production at 5 nmol/ml/24h for at least 72h [53]. We showed that TGF-β1 enhanced *COL1A1* and *COL3A1* mRNA expression at 24h in cultured FDP teno-

cytes indicating the importance of TGF-β1-induced collagen production in FDP tendon physiology, pathology and repair (Figure 2f, 2g).

Lysyl Oxidase (LOX) is a key extracellular enzyme responsible for the post-translational modification of collagen I, and III to form mature fibrillar collagen. LOX plays a key role in the post-translational modification of collagens and elastin, catalyzing inter- and intracrosslinking reactions. Since the cross-linked ECMs are highly resistant to degradative enzymes, it is considered that the overexpression of LOX may cause severe fibrotic degeneration. Goto et al. [54] showed that TGF-β1 upregulated the production of LOX in kidney tubular epithelial cells of ICGN (The Institute of Cancer Research (ICR)-derived glomerulonephritis) mice. As a result, the highly cross-linked collagens induce an irreversible progression of chronic renal tubulointerstitial fibrosis in the kidneys of ICGN mice. Transfection studies showed that the Lox and *Col1a1*promoters may be regulated by similar negative and positive cis-acting elements, which include TGF-β response element, reported for rat *Col1a1* [55] and for mouse *Col1a2* promoters [56]. *in vitro* studies have shown that TGF-β1 caused an increase in Lox mRNA expression in murine tail tenocytes [57]. Increased expression of LOX is associated with fibrosis and cardiac dysfunction [58]. Adult cardiac fibroblasts were isolated from male rat hearts and were treated with TGF-β1. TGF-β1 treatment upregulated *LOX* mRNA, and protein expression in cardiac fibroblasts. Concomitant increases in collagen types I and III, and bone morphogenetic protein 1 expression were found in response to TGF-β1 [58]. The current study showed that TGF-β1 increased *LOX* gene expression at 24h in human FDP tendon cells in culture (Figure 2h). That indicates that TGF-β1-induced LOX gene expression may play role in FDP tendon healing *in vivo*.

COMP

Cartilage Oligomeric Matrix Protein (COMP) is a non-collagenous glycoprotein expressed in the ECM of articular cartilage, tendon and ligaments [59], and is normally produced by chondrocytes, osteoblasts and synovial fibroblasts [60]. COMP plays role in the structural integrity of cartilage via its interaction with other ECM proteins such as the collagens and fibronectin. It mediates the interaction of chondrocytes with the ECM through interaction with cell surface integrin receptors. It plays role in the pathogenesis of osteoarthritis [61,62]. In the presence of TGF-β1, human dermal fibroblasts have been shown to increase COMP production *in vitro* suggesting a correlation between TGF-β1 and COMP production [63]. COMP accumulates in Systemic Sclerosis (SSc) skin and is upregulated by TGF-β1. TGF-β1 treatment increased COMP and SMA-expressing cells. *COMP* mRNA expression in lesional skin from patients with diffuse cutaneous SSc (dSSc) correlated with TGF-β1 staining [64]. Immunohistochemical analysis revealed that COMP was expressed in dense fibrotic regions of IPF lungs and co-localized with vimentin and around pSMAD3 expressing cells. Stimulation of normal human lung fibroblasts with TGF-β1 increased *COMP* mRNA and protein expression [65]. In cultured human FDP tenocytes, we showed that TGF-β1 induced *COMP* mRNA expression 3.4 and 17.1 fold at 6h and 24h respectively indicating its possible role in FDP tendon healing and fibrosis in vivo (Figure 2i).

MMP2, MMP9, MMP13, TIMP1, TIMP2 and *TIMP3*

Matrix metalloproteinase-2 (MMP-2) protein, encoded by *MMP2* gene, is a 72kDa type IV collagenase (72kDa gelatinase or gelatinase A). It contains three-fibronectin type II repeats (FNII) in its catalytic site that allow binding of denatured type IV collagen (the major structural component of basement membrane), type V collagen and elastin. Unlike most MMP family members, activation of this protein can occur on the cell membrane. This enzyme can be activated

extracellularly by proteases, or intracellulary by its S-glutathiolation. This protein is thought to be involved in multiple pathways including roles in the nervous system, endometrial menstrual breakdown, regulation of vascularization, and metastasis. Mutations in this gene have been associated with Winchester syndrome and Nodulosis- Arthropathy-Osteolysis (NAO) syndrome [66,67]. During wound healing, fibroblasts transition from quiescence to a migratory state, then to a contractile myofibroblast state associated with wound closure. Howard et al. [31] found that the myofibroblast phenotype, characterized by the expression of high levels of contractile proteins, suppresses the expression of the pro-migratory gene, *MMP2* .Fibroblasts cultured in a 3-D collagen lattice and allowed to develop tension showed increased contractile protein expression and decreased MMP-2 levels in comparison to a stress-released lattice. In 2-D cultures, factors that promote fibroblast contractility, including serum or TGF-β1, down regulated MMP-2. The current study showed that TGF-β1 did not cause any significant effect on MMP2 gene expression in FDP tenocytes, in cell culture (Figure 3a). In a previous study, in mouse FDL tenocytes in collagen gel culture, the authors showed that *MMP2* gene expression was also not affected by TGF-β1 (at 1, 10 and 100 ng/ml) treatment at 6h until 48h post-treatment [50].

Matrix metalloproteinase-9 (MMP-9) protein, encoded by the *MMP9* gene, plays an essential role in local proteolysis of the ECM and in leukocyte migration. It plays a role in bone osteoclastic resorption. It cleaves type IV and type V collagen into large C-terminal threequarter fragments and shorter N-terminal one-quarter fragments. It degrades fibronectin [68]. The decreased degradation of ECM is a potential mechanism of renal fibrosis. Normal kidneys produce proteases responsible for the hydrolysis of ECM, among which MMPs are the most important ones, and their activity is subject to the regulation of the TIMPs (Metalloproteinase inhibitors). MMP-9 is one of the most important MMPs in the human body inducing en-

zymatic degradation of ECM molecules; and TIMP-1 is specific for the inhibition of MMP-9. The MMP-9/TIMP-1 ratio regulates the aggregation and degradation of the ECM, which are closely related to renal fibrosis [69]. A normal ratio of MMP-9 and TIMP-1 plays an important role in the regulation of ECM secretion and accumulation in glomerular mesangial cells [70]. The activation of hypoxia-inducible factor 1α (HIF-1α)-dependent HGF-signaling can promote the expression of TIMP-1 [71]. MMP-9/TIMP-1 ratio imbalance, by either reduced MMP-9 expression or increased TIMP-1 expression, promotes the progression of renal fibrosis. The mRNA and protein expression of TIMP-1 decreased when TGF-β1 was low in Human amniotic (WISH) cells, whereas those of MMP-9 elevated when TGF-β1 was low. The disruption in the ratio of TIMP-1 and MMP-9 was related to the pathology of the premature rupture of membrane [72]. The current study showed that TGF-β1 decreased *MMP9* gene expression and increased *TIMP1* gene expression in FDP tenocytes, in cell culture (Figure 3b,3d), indicating that fibrotic behavior in FDP tendon healing or repair may be modulated in vivo by TGF-β1 level.

Matrix metallopeptidase-13 (collagenase 3; MMP-13) is encoded by *MMP13* gene in humans. MMP-13 plays role in the degradation of ECM proteins including fibrillar collagen, fibronectin, TNC and ACAN. It cleaves triple helical collagens, including type I, type II and type III collagen, but has the highest activity with soluble type II collagen. It can also degrade collagen type IV, type XIV and type X and may also function by activating or degrading key regulatory proteins, such as TGF-β1 and CTGF. MMP-13 plays role in wound healing, tissue remodeling, cartilage degradation, bone development, bone mineralization and ossification. It is required for normal embryonic bone development and ossification [73-75]. TGF-β1 induced a rapid decrease in *MMP13* mRNA within first 6h post-cytokine administration and that was accompanied by a 2-fold increase in gene transcription and reached maximum values by 48h [76]. Expression of *MMP-13* by human gingival fibroblasts cultured in monolayer or

in collagen gel was induced by TGF-β1 [77]. Leivonen et al. [78] reported in Squamous Cell Carcinoma (SCC) cells of the head and neck that specifically express *MMP-13* , the expression of which correlates with their invasion capacity. TGF-β1 enhanced *MMP-13* and *MMP-1* expression and invasion of SCC cells. The current study showed that TGF-β1 enhanced *MMP13* gene expression at 6h and 24h post-TGFCitation β1 treatment in human FDP tenocytes, in cell culture. This may indicate an active role of TGF-β1-induced MMP13 in FDP tendon healing and fibrosis *in vivo* (Figure 3c).

Metalloproteinase inhibitor 2 (TIMP-2) complexes with metalloproteinases (such as collagenases) and irreversibly inactivates them by binding to their catalytic zinc cofactor. It is known to act on MMP-1, -2, -3, -7, -8, -9, -10, -13, -14, -15, -16 and -19 [79,80]. MMP-2 and TIMP-2 expression were not altered by TGF-β1 [81]. In mouse FDL tenocytes, Farhat et al. [50] showed that TGF-β1 did not affect *Timp2* gene expression upto 48h, in collagen gel culture. The current study also showed that TGF-β1 did not alter human *TIMP2* gene expression at 6h and 24h in FDP tendon cells, in culture (Figure 3e). Metalloproteinase inhibitor 3 (TIMP-3) is encoded by the gene TIMP3 and forms part of a tissue-specific acute response to remodeling stimuli. It is known to act on MMP-1, -2, -3, -7, -9, -13, -14 and -15 [82,83]. Idiopathic Pulmonary Fibrosis (IPF) is characterized by fibroblast expansion and ECM accumulation. TGF-β1 induced strong upregulation of TIMP-3 at the mRNA and protein levels. In IPF tissues, *TIMP3* gene expression was increased and the protein was localized to fibroblastic foci and ECM. Induction of TIMP-3 by TGF- β1 could be a mediator in lung fibrogenesis [84]. TGF-β1 induced expression of TIMP-3 in mouse embryonic fibroblasts. Inhibition of Smad signaling by expression of Smad7 and dominant negative Smad3 completely abolished TGF-β1-elicited expression of TIMP-3 in human fibroblasts, whereas overexpression of Smad3 enhanced it [85]. In the current study, TGF-β1 increased *TIMP3* gene expression at 6h and 24h in human

FDP tenocytes, in cell culture (Figure 3f). The study supports that FDP tendon may be affected by TGF-β1-induced *TIMP3* gene expression for inflammation, repair and fibrosis.

BGN and DCN

Biglycan and decorin belong to the SLRPs class I subfamily and are encoded by *BGN* and *DCN* gene respectively in human. Both contain 12 LRRs [86]. Biglycan has two attached GAG chains and decorin has one. Biglycan is found in several connective tissues, predominantly in articular cartilage. It is a homodimer and forms a ternary complex with microfibrillar-associated protein 2 and elastin and may be involved in collagen fiber assembly. Biglycan binds to Col I in the gap zone of the fibrils, and decorin competes for that interaction [86]. TGF-β1 differentially regulates *DCN* and *BGN*gene expression in skin and gingival fibroblasts. TGF-β1 (at 5 ng/ml) increased BGN 24-fold and inhibited *DCN* gene expression upto 70% [87]. Farhat et al. [50] also reported that TGF-β1 increased BGN and decreased DCN gene expression in mouse FDL tenocytes in collagen gels [50]. We showed that TGF-β1 increased BGN and decreased *DCN* gene expression in cultured human FDP tenocytes (Figure 3g), indicating that FDP tendon function can be modulated by TGF-β1-induced biglycan expression *in vivo* .

TGF-β1 and decorin have important interactions in tendon and other tissues. Decorin has the ability to bind to TGF-β1, which is involved in the regulation of cell proliferation, differentiation, ECM production, wound healing, and tissue repair [88]. TGF-β1 is of crucial importance in triggering excessive formation and deposition of connective tissue matrix molecules. Decorin-TGF-β1 complex formation may lead to inactivation of some cytokines and TGF-β1 itself in connective tissue [89]. To investigate, if scar formation could be prevented by controlling decorin in tenocytes, rabbit Achilles tendon cells were transfected with antisense decorin; the authors found that it suppressed TGF-β1 production. The results showed that the antisense approach is an attractive therapeutic strategy, not only for prevent-

ing decorin deposition in scar tissue, which decreases collagen fibril diameter, but also for controlling TGF-β1 production, which leads to organ fibrosis [90]. *in vitro* studies showed that TGF-β1 suppressed DCN gene expression at 6h and 24h in human FDP tenocytes, in cell culture (Figure 3h), indicating a possible modulatory role of TGF-β1 in tendon function, scarring and pathology. Baghy et al. [91] sought to take advantage of this TGF-β1-decorin relation in treating hepatic fibrosis and cirrhosis. One of the natural inhibitors of TGF-β1 is decorin, which binds with high affinity to TGF-β1 and prevents its interaction with pro-fibrotic receptors. Decorin has a protective role in liver fibrogenesis insofar as its genetic ablation in mice leads to enhanced matrix deposition, impaired matrix degradation, and "activation" of hepatic stellate cells, the main producers of fibrotic tissue. Moreover, TGF-β1 exerts a stronger effect when functional decorin is absent. Endogenous decorin prevents and retards hepatic fibrosis; and thus boosting the endogenous production of decorin, or systemic delivery of recombinant decorin could represent an additional therapeutic modality against hepatic fibrosis [91], and possibly could have a similar effect in the prevention of TGF- β1-mediated tendon adhesions.

SCX

The basic helix-loop-helix transcription factor, scleraxis, is a specific marker for all the connective tissues that mediate attachment of muscle to bone, including limb tendons, and its expression marks the progenitor cell populations for these tissues. Scleraxis is encoded by *SCX* gene in humans [92,93]. Tendon healing is a regenerative process, and tendon progenitor cells are expected to play a role in the healing process. In our earlier study, we demonstrated higher Scx gene expression during mouse FDL tendon graft healing, and that indicated that scleraxis plays a role in the healing process [20]. In a murine patellar tendon injury model, Scx mRNA expression was measured at 1-, 4- and 8-week time-points. The authors observed increased expression at 4- and 8-week time-points [94]. Zeglinski et al.

[95] showed that TGF-β1 regulates scleraxis expression in primary cardiac myofibroblasts by a Smad-independent mechanism. TGF-β1 (at 5 ng/ml) caused 6-fold higher expression at 24h [95]. TGF-β1 at 10ng/ml has been shown to cause 4-fold induction of Scx gene expression in mouse FDL tenocytes in collagen gels at 24h [50]. In the current study, we showed that TGF-β1 (at 5 ng/ml) caused an increase in SCX gene expression in human FDP tenocytes, in cell culture at 6h and 24h (Figure 4a). That suggests that scleraxis is modulated by TGF-β1 in injured FDP tendon and contributes to the healing and regeneration process.

POSTN

Periostin, a secreted cell adhesion protein, is a matricellular protein and is encoded by POSTN gene in human [86,96]. Periostin is predominantly expressed in collagen-rich fibrous connective tissues that are subjected to constant mechanical stress including heart valves, tendons, and PDL [86,96]. Periostin binds to collagen I and plays a role in collagen fibrillogenesis as evidenced by periostin knockout mice [97]. We showed earlier that periostin plays a role in healing of FDL tendon graft in mouse since Postn mRNA expression increased at day-7 and remained higher until day-28, indicating its possible role in tendon maturation, collagen fibril arrangement, and remodeling events [20]. Periostin protein is strongly expressed in the human PDL. *in vitro* , POSTN mRNA level is modulated by cyclic strain as well as TGF-β1 via FAK-dependent pathways [98]. In one study, to understand the importance of masticatory forces, Manokawinchoke et al. [99] showed that an intermittent compressive force regulates SOST / POSTN gene expression by hPDL cells via TGF-β1 signaling pathway. The current study showed that TGFβ1 increases POSTN gene expression at 24h in human FDP tenocytes, in cell culture (Figure 4b), supporting that TGFβ1 may be a regulator of periostin in FDP tendon *in vivo* .

ACAN

Aggrecan, a core protein of a proteoglycan, is a major component of cartilaginous ECM. It is encoded by gene *ACAN* in human. The major function of this proteoglycan is to resist compression in cartilage. The protein binds avidly to hyaluronic acid via an N-terminal globular region [100]. The expression of *ACAN* decreased in nucleus pulposus cells with the addition of 2.5 ng/ml TGFβ1 [101]. Aggrecan expression was increased by the presence of TGF-β1 [102] or β3 [103] during chondrogenic differentiation of mesenchymal stem cells. In human FDP tendon cells, in cell culture, TGF-β1 suppressed ACAN gene expression indicating that it has opposite effect than differentiating cartilage cells (Figure 4c). One possible explanation of this is that chondrocytes are required to impart cushionary properties to cartilage in order to absorb repeated joint shock, whereas tendon needs to be stiff and strong to tolerate the tension of daily activity.

SMAD7

Mothers against decapentaplegic homolog 7 (SMAD7), is an antagonist of signaling by TGF-β type I receptor superfamily members. It has been shown to inhibit TGF-β and activin signaling by associating with their receptors thus preventing SMAD2 access [104,105]. TGF-β1, bone morphogenetic protein 4, and oocyte-derived growth differentiation factor 9 were capable of inducing Smad7 expression, suggesting a modulatory role of SMAD7 in a negative feedback loop. Using a siRNA approach, this was further demonstrated that SMAD7 was a negative regulator of TGF-β1. SMAD7 seemed to play role during follicular development via preferentially antagonizing and/or fine-tuning essential TGF-β superfamily signaling, which is involved in the regulation of oocyte–somatic cell interaction and granulosa cell function [106]. In mouse confluent dermal fibroblast, TGF-β1 (at 12.5 ng/ml) caused an increase in *Smad7* gene expression for 24h [107]. In the current study, we showed that TGF-β1 (5 ng/ml) caused an increase in the *Smad7* gene expression in cultured FDP tenocytes,

suggesting that TGF-β1 modulates SMAD7 in FDP tendon *in vivo* (Figure 4d).

IL6

Interleukin-6 (IL-6) is an interleukin that acts both as a pro-inflammatory cytokine and an anti-inflammatory myokine. In human, it is encoded by IL6 gene [108]. IL-6 is secreted by T-cells and macrophages to stimulate immune response [109]. In addition, osteoblasts secrete IL-6 to stimulate osteoclast formation [110]. IL-6 expression is stimulated by tumor-producing TGF-β1 in human prostate cancer cells through multiple signaling pathways and enhanced expression of IL-6 contributes to the oncogenic switch of TGF-β1 role for prostate tumorigenesis [111]. TGF-β1 induced Smad2 phosphorylation, and blockade of Smad2/3 prevented both the TGF-β1 modulated IL-6 increase in asthmatic and non-asthmatic cells. Understanding the mechanism of aberrant pro-inflammatory cytokine production in asthmatic airways allows the development of alternative ways to control airway inflammation [112]. While studying the mechanism of subconjunctival fibrosis, in human Tenon's fibroblasts, TGF-β1 stimulates the expression of α-SMA protein and increase mRNA expression levels of *IL6* . The autocrine IL-6 may participate in the TGF-β1-induced trans differentiation of human Tenon's fibroblasts to myofibroblasts, which is known to be an essential step for subconjunctival fibrosis [113]. IL-6 is an essential mediator of growth factor-induced proliferation of lung fibroblasts. Eickelberg et al. [114] showed that TGF-β1 is a potent inducer of IL-6 mRNA and protein in primary human lung fibroblasts. The current study showed that TGF- β1 enhanced *IL6* gene expression multi-fold at 6h and 24h, in FDP tenocytes cell culture, indicating possible *in vivo* role of TGF-β1-induced *IL6* in FDP tendon physiology or pathology (Figure 4e).

IGF1

Insulin-like growth factor I (IGF-I) has been shown to play a role in wound healing and regeneration. Expression levels of IGF1 mRNA and IGF-I protein increased in healing rabbit medial collateral liga-

ment [115], and in canine flexor tendon after laceration [116]. Mouse *Igf1* mRNA expression upregulated, during day-7 through 35 of FDL tendon graft healing, indicating its possible role in angiogenesis and growth of cells [20]. During healing of deep flexor tendon repair in rabbit, the expression of *Igf1* mRNA was higher in tendon and sheath at all the time-points (day-6 to 42) as compared to day-3 [117]. TGF-β1 is a potent modulator of IGF-I production in mouse bone cells where it is thought to act in the local regulation of bone remodeling [118]. In liver, TGF-β1 has been postulated to play a role in fibrogenesis related to disease [119]. Voci et al. [120] showed that TGF- β1 increases IGF-I production in hepatocytes. The current study showed that TGF-β1 caused an increase in *IGF1* gene expression in FDP tenocytes, in cell culture (Figure 4f); indicating that IGF-I may play a role in tendon metabolism, regeneration or pathology, and may be modulated by TGF-β1 *in vivo* .

Summary and Conclusion

The present study investigated the effect of TGF-β1 (5 ng/ml) on the expression of several genes in FDP tenocytes in cell culture at 6h and 24h. The results showed that TGF-β1 modulates the expression of genes involved in fibrinolysis (*SERPINE1 , PLAU*), contraction (*ACTA2*), angiogenesis, inflammation and fibrosis (CTGF), cell adhesion, growth, migration, and differentiation (*FN1*), tensile strength, maturation, remodeling and healing (COL1A1, COL3A1), cross-linking in ECM fibrils (*LOX*), ECM (*ACAN*), mechanical strength and fibrosis (*COMP*), remodeling (*MMP9, MMP13, TIMP1, TIMP3*), collagen fiber assembly (*BGN*), cell proliferation, differentiation, ECM production, wound healing, and tissue repair (*DCN*), differentiation and neotendon formation (*SCX*), cell adhesion and collagen fibrillogenesis (*POSTN*), regulation and fine tuning of TGF-β signaling as a negative regulator (*SMAD7*), inflammation (*IL6*), and wound healing and regeneration (*IGF1*). The expression of *MMP2* and *TIMP2* was not affected under these conditions. Future studies are needed to identify whether the therapeutic modulation of these downstream targets of TGF-β1 can improve the results of tendon

healing. In conclusion, TGF-β1 plays a pleotropic role in human FDP tendon physiology, structure, regeneration and adhesion formation, and the therapeutic targeting of these TGF-β1 affected genes may be a novel approach to help improve FDP healing and reduce the formation of adhesions.

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