COL1A1 expression induced by overexpression of both a 15-amino acid peptide from the fibrinogen domain of tenascin-X and integrin α11 in LX-2 cells

KEN-ICHI MATSUMOTO[1], KOHEI KAWAKAMI[2], KAZUO YAMADA[1,3] and HARUO TAKESHITA[3]

Departments of [1]Biosignaling and Radioisotope Experiment, and [2]Experimental Animals, Interdisciplinary Center for Science Research, Head Office for Research and Academic Information; [3]Department of Legal Medicine, Faculty of Medicine, Shimane University, Izumo, Shimane 693-8501, Japan

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Abstract. Extracellular matrix tenascin-X (TNX) is the largest member of the tenascin family. Our previous study demonstrated that TNX was involved in hepatic dysfunction, including fibrosis, in mice that were administered a high-fat and high-cholesterol diet with high levels of phosphorus and calcium. The present study investigated whether overexpression of both the fibrinogen domain of TNX (TNX-FG) and integrin α11, one of the TNX cell surface receptors, induces *in vitro* fibrosis in LX-2 human hepatic stellate cells. Overexpression of both a 15-amino acid peptide (hTNX-FGFFFF) derived from the TNX-FG domain and integrin α11 induced the expression of type I collagen α1 chain (COL1A1). Treatment with verteporfin [YAP (Yes-associated protein) inhibitor] attenuated the elevated *COL1A1* expression elicited by overexpression of both hTNX-FGFFFF and integrin α11. In addition, small interfering RNA-mediated knockdown of YAP1 resulted in a decrease in *COL1A1* expression induced by overexpression of both hTNX-FGFFFF and integrin α11. These results indicated that overexpression of both hTNX-FGFFFF and integrin α11 induced *COL1A1* expression via the YAP signaling pathway.

Introduction

The extracellular matrix (ECM) not only provides structural supports and tissue organization but is also important for regulation of vital processes, including cell proliferation, migration, differentiation and apoptosis, through specific receptor-mediated interactions (1). The constitution and property of the ECM constantly change under normal conditions such as development and aging and also under pathological conditions such as cancer, wound healing, and fibrosis (2).

Tenascins are a family of glycoproteins that act as modifiers of cell adhesiveness, and the family of tenascins comprises four members, tenascin-C (TNC), tenascin-R (TNR), tenascin-X (TNX) and tenascin-W (TNW), in vertebrates (3). Tenascins have a characteristic domain organization with heptad repeats, epidermal growth factor (EGF)-like repeats, fibronectin type III (FNIII)-like repeats, and a fibrinogen (FG)-related domain from the N-terminal to C-terminal regions.

TNX is the largest member of the tenascin family and is ubiquitously expressed, but it is expressed prominently in muscle and loose connective tissue (4). Intriguingly, TNX is mainly downregulated during cancer progression, and a high TNX expression level is associated with a good prognosis (5). As a physiological function of TNX, TNX regulates the fibril spacing between collagen fibrils through direct binding to collagen or through indirect binding to collagen-associated proteins including type XII collagen and decorin (6). Functions of TNX in collagen fibrillogenesis (7,8), stiffness of collagen (9), and elastic fiber remodeling (10) have also been suggested. Taken together, results of previous studies indicate that TNX contributes to collagen deposition, collagen stability, and the mechanical property of collagen. The absence of TNX causes a form of Ehlers-Danlos Syndrome (EDS) termed classical-like EDS (clEDS) (11-13). clEDS is inherited in an autosomal recessive pattern and is characterized by hypermobile joints, hyperextensible skin, and easy bruising without atrophic scarring (12).

Additional roles of TNX in pain (14), behavior (15), blood vessel formation and neovascularization (16-18), triglyceride synthesis (19), bone homeostasis (20), and tumor suppression (21,22) have also been proposed (23).

Importantly, our group has shown that TNX plays a role in hepatic fibrosis that develops in mice fed a high-fat and high-cholesterol diet with high levels of phosphorus and calcium (HFCD) (24). In that study, livers in HFCD-fed wild-type (WT) mice showed greater dysfunction, more type I collagen deposition, and greater inflammatory response than those in TNX-deficient mice (24).

Correspondence to: Professor Ken-Ichi Matsumoto, Department of Biosignaling and Radioisotope Experiment, Interdisciplinary Center for Science Research, Head Office for Research and Academic Information, Shimane University, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan
E-mail: matumoto@med.shimane-u.ac.jp

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Several signaling pathways including transforming growth factor-β (TGF-β) and Wingless/Int (WNT) have been identified as key mediators that are linked to fibrosis progression (25). Alcaraz *et al* (26) demonstrated that the FG-related domain of TNX activates latent TGF-β1 via integrin α11β1 as a novel receptor of TNX, thereafter leading to activation of the TGF-β/Smad signaling pathway followed by epithelial-mesenchymal transition (EMT) in epithelial cells (6). Therefore, it is reasonable to assume that the TGF-β signaling pathway is also involved in TNX-elicited hepatic fibrosis in HFCD-fed WT mice. Recently, the Yes-associated protein 1 (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) signaling pathway has been revealed to be another important signaling cascade during the development of liver fibrosis (27). YAP/TAZ acts as a downstream effector of the Hippo pathway (28). The YAP/TAZ signaling pathway is activated during the progression of matrix stiffness and then transformation of hepatic stellate cells (HSCs) into myofibroblasts occurs with augmentation of collagen synthesis and deposition (29). Furthermore, integrin α11β1 preferentially binds to type I collagen (30) and mediates pro-fibrotic signals from the collagen matrix via YAP1 and P21-activated kinase (PAK) in liver fibrosis (31). Notably, Romaine *et al* (32) reported that overexpression of integrin α11 in mice resulted in left ventricular hypertrophy and cardiac fibrosis.

In the present study, we investigated whether the TNX-FG domain can induce type I collagen α1 chain (*COL1A1*) expression in LX-2 human hepatic stellate cells. It was revealed that overexpression of both a 15-amino acid (aa) peptide derived from the TNX-FG domain (hTNX-FGFFFF) and integrin α11 (ITGA11) induces the expression of *COL1A1* through the YAP signaling pathway.

Materials and methods

Cell culture. The human hepatic stellate cell line LX-2 (Merck) was grown at 37˚C in a 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 4.5 g/l D-glucose, L-glutamine, 2% fetal bovine serum (Gibco), 50 U/ml penicillin, and 50 μg/ml streptomycin.

Plasmid construction. A full-length human integrin α 11 cDNA (pBJ1-ITGA11) (Fig. S1) cloned into the pBJ-1 vector provided by Dr Ning Lu (University of Bergen, Norway) was used to express integrin α11 in LX-2 cells (33). An empty vector, pBJΔ plasmid, was constructed by complete removal of ITGA11 cDNA region from pBJ1-ITGA11 plasmid (Fig. S1). Overexpression of ITGA11 was confirmed by western blot analysis (Fig. S2).

Expression plasmids encoding various regions derived from the fibrinogen domain of human TNX (TNX-FG) were constructed as follows. The coding regions of the clones are shown in Fig. 1. The 5'-upstream FLAG-tagged pSecF vector (16) derived from the pSecTag2/Hygro B vector (Thermo Fisher Scientific, Inc.) served as the vector backbone for the expression constructs. A PCR-generated fragment with *Eco*RI and *Xho*I sites at each end was inserted into the *Eco*RI-*Xho*I sites of the pSecF vector. The DNA templates and primer pairs for PCR used for the construction of expression plasmids, pSecF-hTNX-FG2, pSecF-hTNX-FGF-2, pSec-hTNX-FGL-2, pSecF-hTNX-FGFF-3, pSecF-hTNX-FGFL-1, pSecF-hTNX-FGFFF-2, pSecF-hTNX-FGFFL-3, pSecF-hTNX-FGFFFF-8, pSecF-hTNX-FGFFFM-1 and pSecF-hTNX-FGFFFL-5, are shown in Table I. pSecF-hTNX-FGpeptide2-5 was constructed by the primer annealing method. The forward fhTNX-FGpeptide2 primer and the reverse rhTNX-FGpeptide2 primer (Table I) were phosphorylated at the 5' terminal region and annealed. Then the annealed sample was cloned into the pSecF vector. Each primer was synthesized by Hokkaido System Science. PCR was done using 100 ng of a DNA template and 0.2 mM of each primer with TaKaRa Ex Taq® according to the instruction manual (Takara). The following thermocycling conditions were used for PCR: initial denaturation at 94˚C for 1 min and then 30 cycles of 98˚C for 20 sec and 68˚C for 5 min, and 72˚C for 10 min.

Transfection with expression plasmid DNA and/or small interfering RNA (siRNA). Five x10^5 LX-2 cells were seeded on 6-well plates in DMEM with 2% FBS, followed by culturing overnight. Then the medium was changed to DMEM with 0.5% FBS. One μg of each expression plasmid DNA was transfected using Lipofectamine® LTX with Plus reagent according to the instruction manual (Thermo Fisher Scientific, Inc.). The transfected cells were cultured for 48 h and then subjected to RNA extraction.

On the other hand, for the co-transfection of siRNA and expression plasmid DNA, 5x10^5 LX-2 cells were cultured overnight on 6-well plates in DMEM with 2% FBS. Then the medium was changed to DMEM with 0.5% FBS. Fifty nM YAP1 siRNA (sc-38637; Santa Cruz Biotechnology, Inc.), which is a pool of 3 target-specific siRNAs (sc-38637A, sc-38637B, and sc-38637C), or control siRNA (firefly luciferase GL3, Nippon Gene, Tokyo, Japan) was transfected using Lipofectamine® RNAiMAX reagent according to the instruction manual (Thermo Fisher Scientific, Inc.). The siRNA-transfected cells were cultured for 24 h. Then 1 μg of expression plasmid DNA was further transfected using Lipofectamine® LTX with Plus reagent. The co-transfected cells were further cultured for 48 h and then subjected to RNA extraction. The sequences of siRNAs used in this study are shown in Table II.

RNA extraction and RT-qPCR. Total RNA was extracted from cells using Isogen (Nippon Gene) and RNA was treated with the Turbo DNA-free™ kit according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). Thereafter, 1 μg of RNA was used for the synthesis of cDNA with the PrimeScript™ RT reagent kit (Perfect Real Time) (Takara) by iCycler 170-8720JA (Bio-Rad Laboratories). The following temperature protocol was used for RT: 37˚C for 15 min and 85˚C for 5 sec. Subsequently, qPCR analysis was performed with Thermal Cycler Dicer Real Time System TP860 (Takara) and TB Green Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara). The reaction and operation of the apparatus were performed according to the instruction manuals (Takara). The following thermocycling conditions were used for qPCR: initial denaturation at 95˚C for 30 sec, then 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec, and 95˚C for 15 sec, 60˚C for 30 sec, and 95˚C for 15 sec. Gene expression levels were normalized by the expression level of glyceraldehyde-3-phosphate dehydrogenase

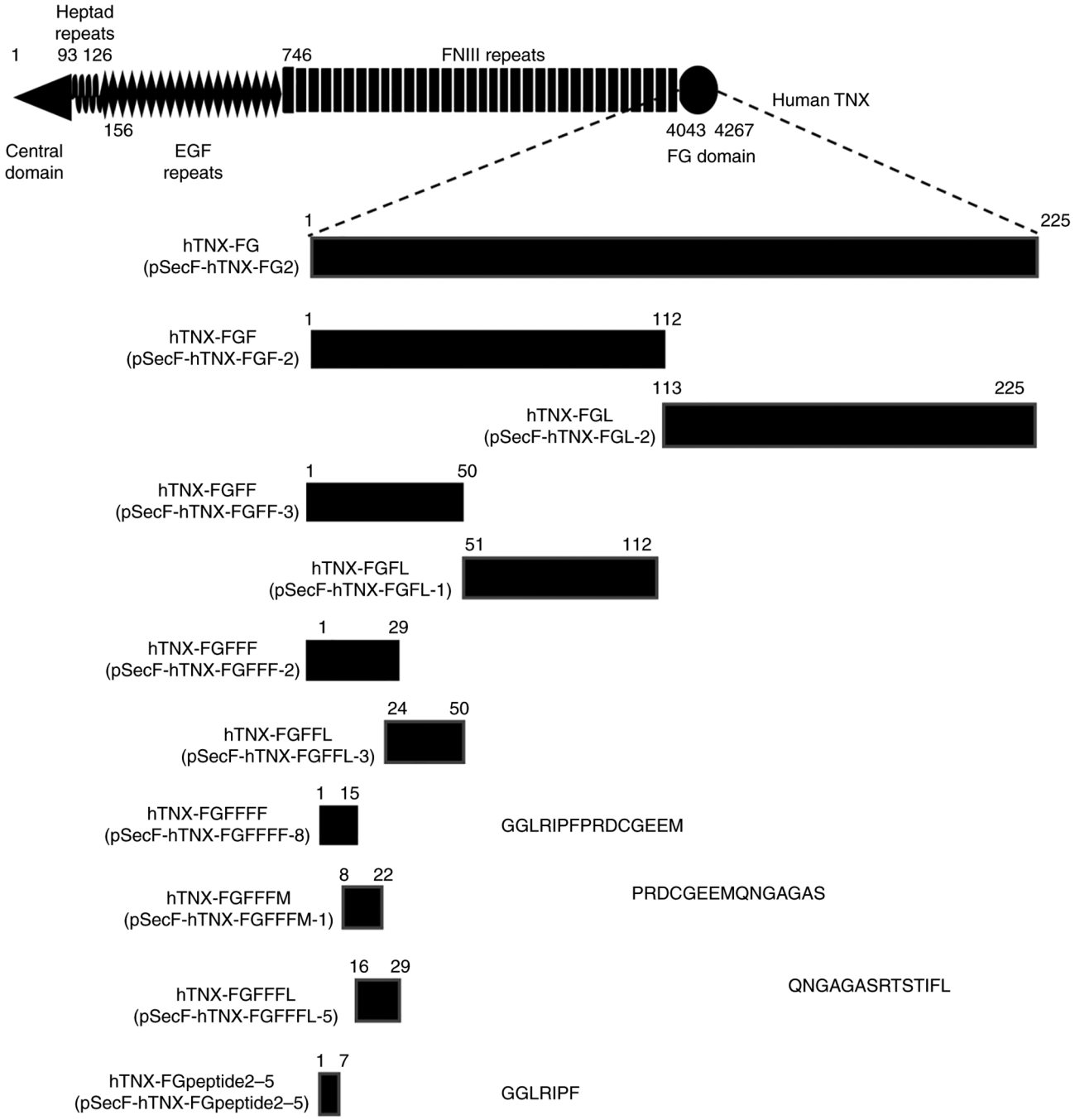


Figure 1. Encompassed TNX-FG region of constructed expression plasmid clones. A schematic diagram of the domain structure of human TNX based on our previous study (50) from the N-terminus to C-terminus showing the central domain (aa no. 1-92), heptad repeats (aa no. 93-126), 18.5 EGF-like repeats (aa no. 156-745), 33 FNIII-like repeats (aa no. 746-4,042) and an FG-related domain (aa no. 4,043-4,267). The horizontal black boxes under the diagram indicate the encompassed regions of the expression plasmids. Their plasmid names (e.g., pSecF-hTNX-FG2) are shown in parentheses. The names of expressed proteins or peptides (e.g., hTNX-FG) are also shown. The number above each black box sets aa number 4,043 (starting position of the FG-related domain) and aa number 4,267 (last position of the FG-related domain) in the full-length human TNX diagram to aa numbers 1 and 225, respectively, and then it indicates the aa numbers of the start position and last position in the TNX-FG domain. The aa sequences of hTNX-FGFFFF, hTNX-FGFFFM, hTNX-FGFFFL and hTNX-FGpeptide2-5 are also shown. EGF, epidermal growth factor; FNIII, fibronectin type III repeat; FG, fibrinogen; TNX, tenascin-X; hTNX-FG, fibrinogen-related domain of human tenascin-X; hTNX-FGF, first half of hTNX-FG; hTNX-FGL, latter half of hTNX-FG; hTNX-FGFF, first half of hTNX-FGF; hTNX-FGFL, latter half of hTNX-FGF; hTNX-FGFFF, first half of hTNX-FGFF; hTNX-FGFFL, latter half of hTNX-FGFF; hTNX-FGFFFF, GGLRIPFPRDCGEEM peptide from hTNX-FG; hTNX-FGFFFM, PRDCGEEMQNGAGAS peptide from hTNX-FG; hTNX-FGFFFL, QNGAGASRTSTIFL peptide from hTNX-FG; hTNX-FGpeptide2-5, GGLRIPF peptide from hTNX-FG.

(*GAPDH*). The sequences of primers to amplify the target genes, human α-smooth muscle actin (*ACTA2*), human type I collagen α1 chain (*COL1A1*), human tenascin-X (*TNXB*), human integrin β1 (*ITGB1*), human integrin α11 (*ITGA11*), human TGF-β1 (*TGFB1*), human YAP1 (*YAP1*) and human GAPDH (*GAPDH*), are shown in Table III. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method with normalization to *GAPDH* (34).

Furthermore, we confirmed the successful overexpression of both hTNX-FGFFFF and ITGA11 in LX-2 cells in co-transfection

Table I. Constructed expression plasmids with DNA templates and primer pairs for PCR.

Protein or peptide (expression plasmid)	DNA template (Refs.)	Primer name	Primer sequence (5'-3')	Plasmid backbone (Refs.)
hTNX-FG (pSecF-hTNX-FG2)	pcDNA3-F-XB-S (51)	fEcohTNXFG-1	F: GGGGAATTCGGTGGGCTGCGGATC	pSecF (16)
		rXhohTNXFG-1	R: GGGCTCGAGTCAGCCTCCCCCCGC	
hTNX-FGF (pSecF-hTNX-FGF-2)	pcDNA3-F-XB-S (51)	fEcohTNXFG-1	F: GGGGAATTCGGTGGGCTGCGGATC	pSecF (16)
		rXhohTNXFG-2	R: GGGCTCGAGTCAGGCGAACACAGCCTC	
hTNX-FGL (pSec-hTNX-FGL-2)	pcDNA3-F-XB-S (51)	fEcohTNXFG-2	F: GGGGAATTCCAGTACGACTCCTTC	pSecF (16)
		rXhohTNXFG-1	R: GGGCTCGAGTCAGCCTCCCCCCGC	
hTNX-FGFF (pSecF-hTNX-FGFF-3)	pcDNA3-F-XB-S (51)	fEcohTNXFG-1	F: GGGGAATTCGGTGGGCTGCGGATC	pSecF (16)
		rXhohTNXFGFF-1	R: GGGCTCGAGTCACCAGCCGCCCCCATC	
hTNX-FGFL (pSecF-hTNX-FGFL-1)	pcDNA3-F-XB-S (51)	fEcohTNXFGFL-1	F: GGGGAATTCCTGGTGTTCCAGCGC	pSecF (16)
		rXhohTNXFG-2	R: GGGCTCGAGTCAGGCGAACACAGCCTC	
hTNX-FGFFF (pSecF-hTNX-FGFFF-2)	pcDNA3-F-XB-S (51)	fEcohTNXFG-1	F: GGGGAATTCGGTGGGCTGCGGATC	pSecF (16)
		rXhohTNXFGFFF-1	R: GGGCTCGAGTCAGAGGAAGATGGTGC	
hTNX-FGFFL (pSecF-hTNX-FGFFL-3)	pcDNA3-F-XB-S (51)	fEcohTNXFGFFL-1	F: GGGGAATTCACCAGCACCATCTTC	pSecF (16)
		rXhohTNXFGFF-1	R: GGGCTCGAGTCACCAGCCGCCCCCATC	
hTNX-FGFFFF (pSecF-hTNX-FGFFFF-8)	pSecF-hTNX-FGFFF-2 (prepared in the present study)	fEcohTNXFG-1	F: GGGGAATTCGGTGGGCTGCGGATC	pSecF (16)
		rXhohTNXFGFFFF-1	R: GGGCTCGAGTCACATCTCCTCCCCGC	
hTNX-FGFFFM (pSecF-hTNX-FGFFFM-1)	pSecF-hTNX-FGFFF-2 (prepared in the present study)	fEcohTNXFGFFFM-1	F: GGGGAATTCCCCAGGGACTGCGGG	pSecF (16)
		rXhohTNXFGFFFM-1	R: GGGCTCGAGTCAGGAGGCACCGGCTCC	
hTNX-FGFFFL (pSecF-hTNX-FGFFFL-5)	pSecF-hTNX-FGFFF-2 (prepared in the present study)	fEcohTNXFGFFFL-1	F: GGGGAATTCCAGAACGGAGCCGGT	pSecF (16)
		rXhohTNXFGFFF-1	R: GGGCTCGAGTCAGAGGAAGATGGTGC	
hTNX-FGpeptide2-5 (pSecF-hTNX-FGpeptide2-5)	Primer annealing (prepared in the present study)	fhTNX-FGpeptide2	F: *AATTC*GGTGGGCTGCGGATCCCCTTCTGAC	pSecF (16)
		rhTNX-FGpeptide2	R: *TCGAG*TCAGAAGGGGATCCGCAGCCCACCG	

pcDNA3-F-XB-S (51) codes from nucleotide numbers 10,783 to 12,801 of hTNX (50). This region encodes from the middle of the FNIII-like repeat hu29 to the C-terminal FG domain of hTNX (hTNX-FG) (50). Underlined sequences in primers indicate *Eco*R I sites, whereas double underlined sequences indicate *Xho* I sites. However, in fhTNX-FGpeptide2 primer, the initial 'G' for the *Eco*R I site is lacking because this 'G' is derived from the pSecF vector after ligation. Similarly, in rhTNX-FGpeptide2 primer, the initial 'C' for the *Xho* I site is lacking because this 'C' is derived from the pSecF vector after ligation. The 5'-upstream FLAG-tagged pSecF vector (16) is derived from the pSecTag2/Hygro B vector. hTNX, human tenascin-X; FNIII, fibronectin type III; C-, carboxy-; FG, fibrinogen; F, forward primer; R, reverse primer; hTNX-FG, fibrinogen-related domain of human tenascin-X; hTNX-FGF, first half of hTNX-FG; hTNX-FGL, latter half of hTNX-FG; hTNX-FGFF, first half of hTNX-FGF; hTNX-FGFL, latter half of hTNX-FGF; hTNX-FGFFF, first half of hTNX-FGFF; hTNX-FGFFL, latter half of hTNX-FGFF; hTNX-FGFFFF, GGLRIPFPRDCGEEM peptide from hTNX-FG; hTNX-FGFFFM, PRDCGEEMQNGAGAS peptide from hTNX-FG; hTNX-FGFFFL, QNGAGASRTSTIFL peptide from hTNX-FG; hTNX-FGpeptide2-5, GGLRIPF peptide from hTNX-FG.

Table II. siRNAs used in the present study.

Gene	Cat. no. (manufacturer)	siRNA sequence (5'-3')
Human *YAP1*	sc-38637A (Santa Cruz Biotechnology, Inc.)	Sense CCACCAAGCUAGAUAAAGAdTdT
		Antisense UCUUUAUCUAGCUUGGUGGdTdT
Human *YAP1*	sc-38637B (Santa Cruz Biotechnology, Inc.)	Sense GCAUGAGACAAUUUCCAUAdTdT
		Antisense UAUGGAAAUUGUCUCAUGCdTdT
Human *YAP1*	sc-38637C (Santa Cruz Biotechnology, Inc.)	Sense GGGUGUGCCUAUCAUAACAdTdT
		Antisense UGUUAUGAUAGGCACACCCdTdT
Firefly Luciferase GL3	Control siRNA duplex, 318-05931 (Nippon Gene Co., Ltd.)	Sense CUUACGCUGAGUACUUCGAdTdT
		Antisense UCGAAGUACUCAGCGUAAGdTdT

siRNA, small interfering RNA; YAP, Yes-associated protein.

with siRNA by RT-qPCR analyses. qPCR for hTNX-FGFFFF was done with specific primers, forward fhTNX-FGFFF primer (5'-GGTGGGCTGCGGATCCCCTTC-3') and reverse rhTNX-FGFFFF primer (5'-CATCTCCTCCCCGC-3').

Confirmation of successful overexpression of constructed expression plasmids in LX-2 cells. As for FLAG-tagged pSecF-hTNX-FG2, pSecF-hTNX-FGF-2, pSec-hTNX-FGL-2, pSecF-hTNX-FGFF-3 and pSecF-hTNX-FGFL-1, after transfection in LX-2 cells followed by culture as described above, protein extracts from cells were prepared with Pierce™ RIPA buffer (Thermo Fisher Scientific, Inc.) with protease inhibitor Complete (Roche Diagnostics). The samples were sonicated and then incubated at 4˚C for 2 h and centrifuged, and the supernatant was collected in new tubes. The protein concentration was measured with the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Inc.). Then the protein extract after transfection of pSecF-hTNX-FG2, pSecF-hTNX-FGF-2, pSec-hTNX-FGL-2, pSecF-hTNX-FGFF-3 and pSecF-hTNX-FGFL-1 was subjected to 15% SDS-PAGE under a reducing. After electrophoresis, the proteins after transfection of pSecF-hTNX-FG2, pSecF-hTNX-FGF-2, pSec-hTNX-FGL-2, pSecF-hTNX-FGFF-3 and pSecF-hTNX-FGFL-1 were transferred to Immobilon-P^{SQ} membrane (Merck Millipore). The membrane was blocked with 5% skim milk (Snow Brand Milk Products) and incubated with primary antibody overnight at 4˚C followed by incubation with a secondary antibody. The primary antibody used was anti-FLAG M2 mouse monoclonal antibody (1:1,500, #F3165, Sigma-Aldrich). Horseradish peroxidase (HRP)-conjugated anti-mouse goat IgG (H+L chain) (1:25,000, #330, Medical and Biological Laboratories) were used as a secondary antibody. The blot was washed in TBST [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20]. HRP reaction was performed with ECL Prime Western Blotting Detection Reagents (Cytiva, Marlborough, MA, USA), and the developed chemiluminescent intensity was detected by Amersham™ ImageQuant™ 800 (Cytiva). Successful overexpression of FLAG-tagged proteins including hTNX-FG, hTNX-FGF, hTNX-FGL, hTNX-FGFF and hTNX-FGFL was confirmed by western blot analyses (data not shown).

As for pSecF-hTNX-FGFFF-2, pSecF-hTNX-FGFFL-3, pSec-hTNX-FGFFFF-8, pSecF-hTNX-FGFFFM-1 and pSecF-hTNX-FGFFFL-5 and pSecF-hTNX-FGpeptide2-5, we verified their successful overexpression by RT-qPCR analyses with suitable primer pairs for each transcript, since we failed to detect their proteins by Western blot analyses due to the products with small molecular weights of less than 10 kDa (data not shown).

Confirmation of successful overexpression of pBJ1-ITGA11 plasmid in LX-2 cells. The protein extracts from non-transfected LX-2 cells, pBJΔ (empty vector)-transfected LX-2 cells and pBJ1-ITGA11-transfected LX-2 cells were prepared with Pierce™ RIPA buffer with protease inhibitor complete. Subsequently, 60 μg of the protein extracts was subjected to 10% SDS-PAGE under non-reducing conditions. After electrophoresis, the proteins were transferred to Amersham™ Protran™ nitrocellulose membrane (Cytiva). The membrane was blocked with 5% skim milk and incubated with primary antibody for 2 h followed by incubation with a secondary antibody. The primary antibody used was anti-integrin α11 rat monoclonal antibody (1:1,000; #396214; R&D Systems, Inc.). HRP-conjugated anti-rat goat IgG (H+L chain) (1:25,000; #31470; Thermo Fisher Scientific, Inc.) was used as a secondary antibody. The blot was washed in TBST. The HRP reaction was performed with ECL Prime Western Blotting Detection Reagents, and the developed chemiluminescent intensity was detected using the Amersham™ ImageQuant™ 800.

Experiments with inhibitors. To investigate the possible signaling pathway involved in the induction of *COL1A1* expression by overexpression of both hTNX-FGFFFF and integrin α11 in LX-2 cells, a TGF-β receptor type 1 (TGFBRI) inhibitor (SB525334) (MedChemExpress) and a YAP inhibitor (verteporfin) (Cayman Chemical) were used. Each of the

Table III. Primers used for quantitative PCR analyses.

Gene	Primer sequence (5'-3')	DDBJ/Genbank accession number
ACTA2	F: ACTGCCGCATCCTCATCC	X13839
	R: ATGCTGTTGTAGGTGGTTTCAT	
COL1A1	F: CCCCTGGAAAGAATGGAGAT	Z74615
	R: AATCCTCGAGCACCCTGA	
TNXB	F: GTGGTCCAGTATGAGGACACG	BC130037
	R: CTGGTGGTCACGTACGTCAC	
ITGB1	F: GAAAACAGCGCATATCTGGAAAT	X07979
	R: CAGCCAATCAGTGATCCACAA	
ITGA11	F: GACGGGAGACGTGTACAAGTGTC	AF137378
	R: CCGAGGCGCATGTTGTC	
TGFB1	F: ACATTGACTTCCGCAAGGAC	X02812
	R: GTCCAGGCTCCAAATGTAGG	
YAP1	F: GAACTCGGCTTCAGGTCCTC	NM_001195045
	R: AGGGTCAAGCCTTGGGTCTA	
GAPDH	F: ACAACTTTGGTATCGTGGAAGG	X01677
	R: GCCATCACGCCACAGTTTC	

DDBJ, DNA Data Bank of Japan; F, forward primer; R, reverse primer; *ACTA2*, α-smooth muscle actin; *COL1A1*, type I collagen α1 chain; *TNXB*, tenascin-X; *ITGB1*, integrin β1; *ITGA11*, integrin α11; *YAP1*, Yes-associated protein 1.

inhibitors was dissolved in dimethyl sulfoxide (DMSO). After transfection as described above, LX-2 cells were cultured in DMEM with 0.5% FBS for 24 h, and then the culture was further continued with 2 μM verteporfin for 19 h or with 10 μM SB525334 for 42 h. The inhibitor-treated LX-2 cells were collected and then RNA was extracted from the cells.

Enzyme-linked immunosorbent assay (ELISA). ELISA was carried out to determine the concentration of TGF-β1 in the conditioned medium of LX-2 cells transfected with the expression plasmids by using the Quantikine ELISA human TGF-β1 immunoassay (R&D Systems) according to the manufacturer's manual. To activate latent TGF-β1 to the immunoreactive form, samples were treated with HCl for 10 min prior to the assay.

Statistical analysis and software. Data are expressed as means ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA with the Tukey-Kramer, Dunnett or Bonferroni post hoc test for the comparison of multiple groups in Mac ToukeiKaiseki Ver. 3.0 (ESUMI Co., Ltd., Nakano, Tokyo, Japan) to define statistical differences for the groups. P<0.05 was considered to indicate a statistically significant difference. The maps of pBJ1-ITGA11 and pBJΔ plasmids were prepared using the SnapGene Viewer software (version 5.1.3) (https://www.snapgene.com/snapgene-viewer).

Results

Endogenous expression levels of TNXB and ITGA11 are very low compared with that of ITGB in LX-2 cells. First, we examined the endogenous expression levels of genes relevant to this study, including *TNXB*, *ITGA11*, *ITGB1* and *TGFB1*, in LX-2 cells by RT-qPCR. As shown in Fig. 2, *ITGB1* and *TGFB1* expression levels were 2-times and 0.15-times higher, respectively, than the expression level of control *GAPDH*. However, the expression levels of *TNXB* and *ITGA11* were very low, and they were only 0.002-times and 0.017-times higher, respectively, than the expression level of *ITGB1*. Generally, integrin α11 forms a heterodimer with integrin β1, which is endogenously abundant in cells (35). It has been

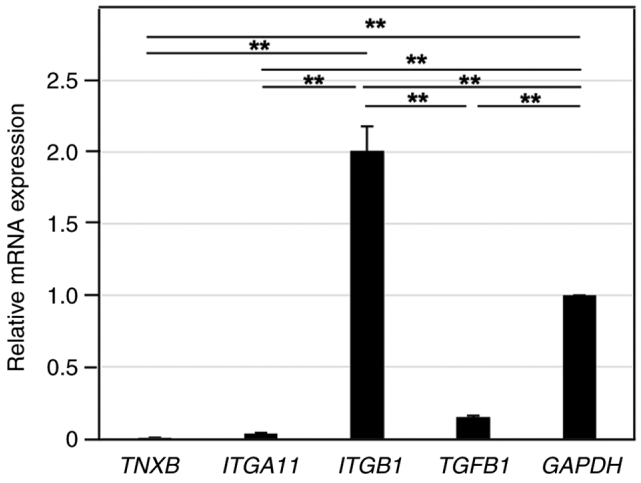


Figure 2. Endogenous expression levels of *TNXB* and *ITGA11*. Endogenous expression levels of *TNXB*, *ITGA11*, *ITGB1* and *TGFB1* in LX-2 cells were examined by reverse transcription-quantitative PCR. The expression level of *GAPDH* was set to 1, and the relative expression level of each gene compared with that of *GAPDH* is shown (n=3). Data are presented as the mean ± SD. **P<0.01, one-way ANOVA with the Tukey-Kramer post hoc test. *TNXB*, tenascin-X; *ITGA11*, integrin β11; *ITGB1*, integrin β1.

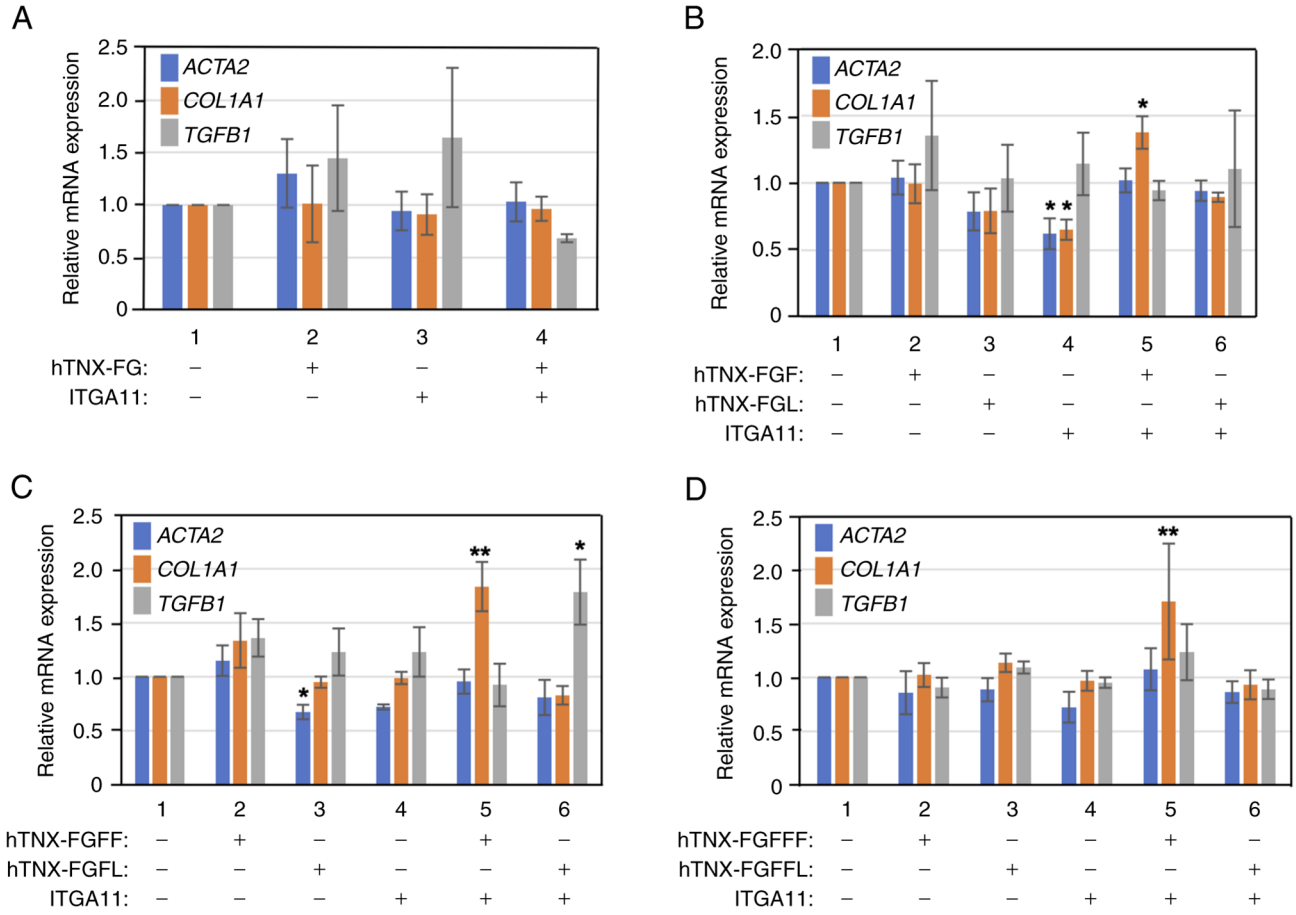


Figure 3. Narrowing down of the domain involved in the induction of *COL1A1* expression in LX-2 cells. (A) Overexpression of both full-length fibrinogen domain of TNX [TNX-FG] and ITGA11 failed to induce the expression of fibrosis marker genes, including *ACTA2*, *COL1A1* and *TGFB1*. LX-2 cells were transfected with expression vectors for hTNX-FG (lane 2), ITGA11 (lane 3) and hTNX-FG and ITGA11 (lane 4) in DMEM/0.5% FBS. (B) Induction of *COL1A1* expression by overexpression of both hTNX-FGF and ITGA11. LX-2 cells were transfected with expression vectors for hTNX-FGF (lane 2), hTNX-FGL (lane 3), ITGA11 (lane 4), hTNX-FGF and ITGA11 (lane 5) and hTNX-FGL and ITGA11 (lane 6) in DMEM/0.5% FBS. (C) Induction of *COL1A1* expression by overexpression of both hTNX-FGFF and ITGA11. LX-2 cells were transfected with expression vectors for hTNX-FGFF (lane 2), hTNX-FGFL (lane 3), ITGA11 (lane 4), hTNX-FGFF and ITGA11 (lane 5) and hTNX-FGFL and ITGA11 (lane 6) in DMEM/0.5% FBS. (D) Induction of *COL1A1* expression by overexpression of both hTNX-FGFFF and ITGA11. LX-2 cells were transfected with expression vectors for hTNX-FGFFF (lane 2), hTNX-FGFFL (lane 3), ITGA11 (lane 4), hTNX-FGFFF and ITGA11 (lane 5) and hTNX-FGFFL and ITGA11 (lane 6) in DMEM/0.5% FBS. (A-D) As a control, RNA from the cells without transfection (lane 1) was used. The cell lysate was prepared 48 h after transfection and then RNA was purified. Subsequently, the expression levels of *ACTA2*, *COL1A1* and *TGFB1* were examined by reverse transcription-quantitative PCR. The expression level of each gene in the control was set to 1.0, and the relative expression level of each gene compared with that of the control is shown (n=3). Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. control (lane 1), one-way ANOVA with Dunnett's post hoc test. *COL1A1*, type I collagen α1 chain; TNX, tenascin-X; ITGA11, integrin α11; *ACTA2*, α-smooth muscle actin; hTNX-FG, fibrinogen-related domain of human tenascin-X; hTNX-FGF, first half of hTNX-FG; hTNX-FGL, latter half of hTNX-FG; hTNX-FGFF, first half of hTNX-FGF; hTNX-FGFL, latter half of hTNX-FGF; hTNX-FGFFF, first half of hTNX-FGFF; hTNX-FGFFL, latter half of hTNX-FGFF.

reported that overexpressed integrin α11 in cells functionally interacts with endogenous integrin β1, forming integrin α11β1 (36). Therefore, we attempted to overexpress both integrin α11 and the fibrinogen domain of TNX in LX-2 cells for the sake of further analysis. The overexpression of an expression plasmid, pBJ1-ITGA11 (Fig. S1), for ITGA11 in LX-2 cells was confirmed (Fig. S2). On the other hand, we could not observe any specific expression of ITGA11 in non-transfected cells and an empty vector, pBJΔ plasmid-transfected cells (Fig. S2). In this study, we used a conditioned medium with 0.5% FBS that contained TGF-β1, eventually resulting in the concentration of TGF-β1 in the cell condition medium being 502.4 nM as determined by ELISA for TGF-β1 (data not shown). Therefore, since we considered that the amount of TGF-β1 in the cell conditioned medium is sufficient for this study, TGF-β1 was not overexpressed in LX-2 cells anymore.

Identification of the region in TNX-FG involved in the induction of COL1A1 expression in LX-2 cells. In order to determine whether overexpression of both the TNX-FG domain and integrin α11β1 is able to provoke induction of *COL1A1* expression in LX-2 cells, both hTNX-FG (Fig. 1) and integrin α11 were overexpressed and then the expression levels of fibrosis marker genes including *ACTA2*, *COL1A1* and *TGFB1* were investigated by RT-qPCR (Fig. 3A). Contrary to our expectation, induction of the expression of these genes in LX-2 cells was not observed by overexpression of both hTNX-FG and integrin α11 compared with that in LX-2 cells alone (Fig. 3A). Next, considering the possibility that TNX-FG coding a 225-aa protein contains not only a positive region(s) involved in induced expression but also a negative region(s) involved in decreased expression of fibrosis maker genes, TNX-FG was divided into the first half (referred to as hTNX-FGF) and the latter half (hTNX-FGL) (Fig. 1), and

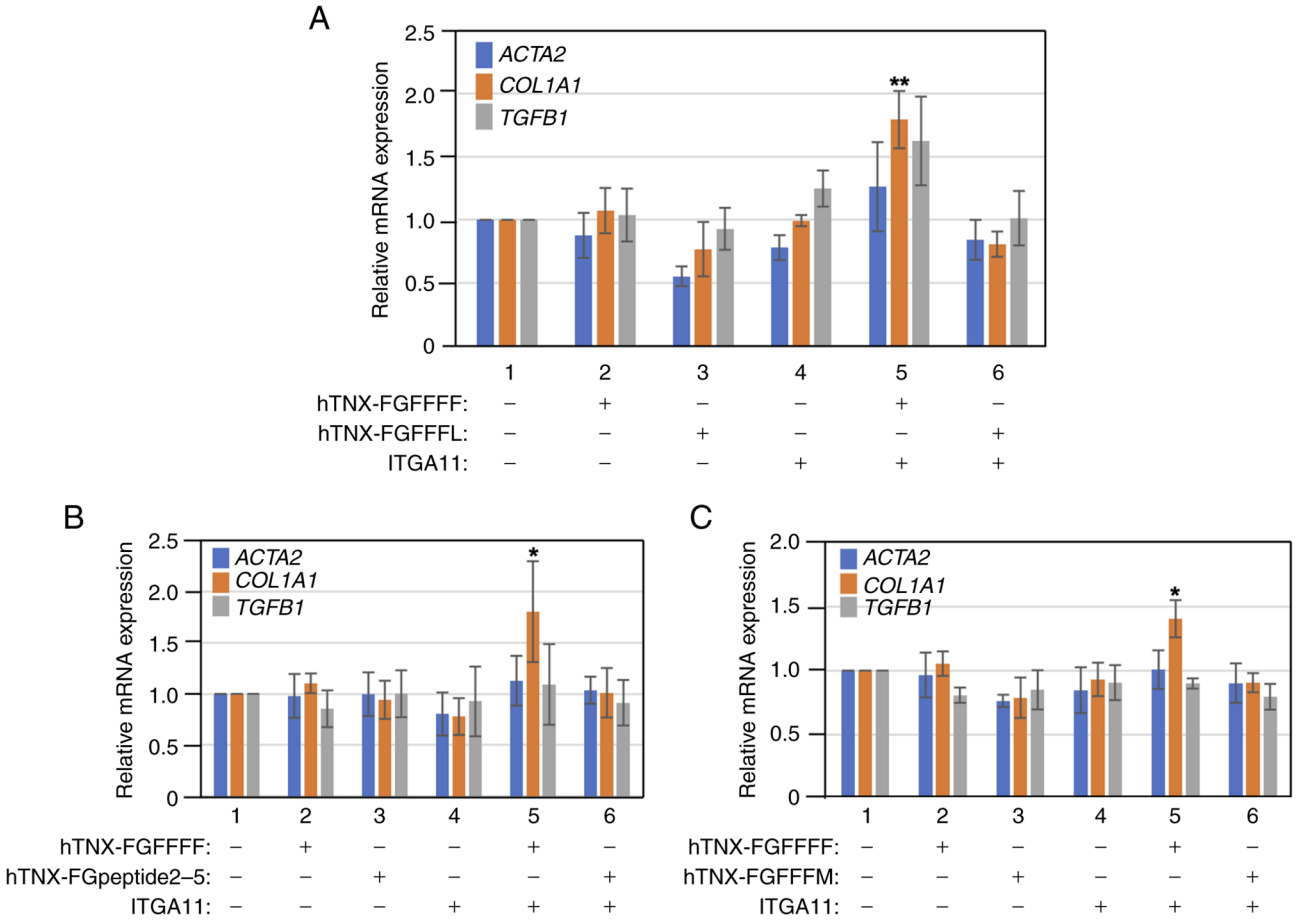


Figure 4. Identification of the minimal sequence responsible for induction of *COL1A1* expression in LX-2 cells. (A) Induction of *COL1A1* expression by overexpression of both hTNX-FGFFFF and ITGA11. LX-2 cells were transfected with expression vectors for hTNX-FGFFFF (lane 2), hTNX-FGFFFL (lane 3), ITGA11 (lane 4), hTNX-FGFFFF and ITGA11 (lane 5) and hTNX-FGFFFL and ITGA11 (lane 6) in DMEM/0.5% FBS. (B) Overexpression of both hTNX-FGpeptide2-5 and ITGA11 did not cause induction of *COL1A1* expression. LX-2 cells were transfected with expression vectors for hTNX-FGFFFF (lane 2), hTNX-FGpeptide2-5 (lane 3), ITGA11 (lane 4), hTNX-FGFFFF and ITGA11 (lane 5) and hTNX-FGpeptide2-5 and ITGA11 (lane 6) in DMEM/0.5% FBS. (C) Overexpression of both hTNX-FGFFFM and ITGA11 did not cause induction of *COL1A1* expression. LX-2 cells were transfected with expression vectors for hTNX-FGFFFF (lane 2), hTNX-FGFFFM (lane 3), ITGA11 (lane 4), hTNX-FGFFFF and ITGA11 (lane 5) and hTNX-FGFFFM and ITGA11 (lane 6) in DMEM/0.5% FBS. After transfection followed by cell culture, cell lysate extraction and RNA purification, the expression levels of *ACTA2*, *COL1A1* and *TGFB1* were examined by reverse transcription-quantitative PCR. (A-C) The expression level of each gene in the control (lane 1, RNA from cells without transfection) was set to 1.0, and the relative expression level of each gene compared with that of the control is shown (n=3). Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. control (lane 1), one-way ANOVA with Dunnett's post hoc test. *COL1A1*, type I collagen α1 chain; TNX, tenascin-X; ITGA11, integrin α11; *ACTA2*, α-smooth muscle actin; hTNX-FGFFFF, GGLRIPFPRDCGEEM peptide from fibrinogen-related domain of human tenascin-X (hTNX-FG); hTNX-FGFFFM, PRDCGEEMQNGAGAS peptide from hTNX-FG; hTNX-FGFFFL, QNGAGASRTSTIFL peptide from hTNX-FG; hTNX-FGpeptide2-5, GGLRIPF peptide from hTNX-FG.

then these regions were assessed for their activity to induce expression of fibrosis marker genes (Fig. 3B). As expected, significant induction of *COL1A1* expression was detected when both hTNX-FGF and integrin α11 were overexpressed in LX-2 cells compared with that in control cells without transfection (1.37-fold, P<0.05 vs. control), but induction of *ACTA2* and *TGFB1* expression was not observed (Fig. 3B). On the other hand, in the case of overexpression of both hTNX-FGL and integrin α11, induction of *COL1A1* expression as well as *ACTA2* and *TGFB1* expression was not observed.

In order to narrow down the region in hTNX-FGF that is responsible for the induction of expression of *COL1A1*, we constructed expression plasmids for hTNX-FGFF (first half of hTNX-FGF) and hTNX-FGFL (latter half of hTNX-FGF) (Fig. 1), and then we examined the activity of hTNX-FGFF and hTNX-FGFL for induction of the expression of fibrosis marker genes (Fig. 3C). As shown in Fig. 3C, overexpression of both hTNX-FGFL and integrin α11 was not effective for induction of *ACTA2* and *COL1A1* expression other than *TGFB1* expression (1.79-fold, P<0.05 vs. control), but overexpression of both hTNX-FGFF and integrin α11 triggered the induction of *COL1A1* expression (1.84-fold, P<0.01 vs. control). Subsequently, the hTNX-FGFF region was divided into the first half (hTNX-FGFFF) and latter half (hTNX-FGFFL) (Fig. 1). When analyzed in the same way, it was found out that overexpression of both hTNX-FGFFF and integrin α11 evoked the induction of *COL1A1* expression (1.67-fold, P<0.01 vs. control) (Fig. 3D).

Ultimately, the hTNX-FGFFF region was divided into the first half (hTNX-FGFFFF) and latter half (hTNX-FGFFFL) (Fig. 1). After similar analysis, it was revealed that overexpression of both hTNX-FGFFFF and integrin α11 significantly evoked the induction of *COL1A1* expression (1.79-fold, P<0.01 vs. control) (Fig. 4A). hTNX-FGFFFF codes GGLRIPFPRDCGEEM from

the N-terminal to C-terminal regions with a length of 15-aa. In order to determine further narrowed sequences involved in the induction of *COL1A1* expression, we prepared an expression plasmid, pSecF-hTNX-FGpeptide2-5 (Fig. 1), coding GGLRIPF within hTNX-FGFFFF and an expression plasmid, pSecF-hTNX-FGFFFM-1 (Fig. 1), coding the 15-aa sequence partially overlapped with the hTNX-FGFFFF sequence. However, neither of the expression plasmids could significantly induce expression of the three fibrosis marker genes (Fig. 4B and C). These results indicated that the minimal sequence in the TNX-FG domain expressed with integrin α11 for induction of *COL1A1* expression in LX-2 cells is GGLRIPFPRDCGEEM.

Furthermore, we synthesized the 15-aa peptide (hTNX-FGFFFF peptide) and then examined whether the addition of hTNX-FGFFFF peptide in the medium with co-transfection of integrin α11 can induce *COL1A1* expression in LX-2 cells. However, we failed to obtain conclusive results showing that *COL1A1* expression is induced by the addition of the hTNX-FGFFFF peptide (data not shown). Our results indicate that addition of synthetic 15-amino acid peptide in the medium is not sufficient for induction of *COL1A1* expression and that expression of the 15-amino acid peptide in cells is necessary for its induction.

YAP inhibitor verteporfin strongly suppresses COL1A1 expression triggered by overexpression of both hTNX-FGFFFF and integrin α11. First, to reveal whether the TGF-β signaling pathway is involved in the induction of *COL1A1* expression when both hTNX-FGFFFF and integrin α11 were overexpressed, a TGFBRI inhibitor (SB525334) was added to the culture medium after the transfection of expression plasmids for both hTNX-FGFFFF and integrin α11 in LX-2 cells. After incubation, the cells were collected and then the expression levels of *ACTA2*, *COL1A1* and *TGFB1* were analyzed by RT-qPCR. As shown in Fig. 5A the expression levels of *COL1A1* and *TGFB1* were significantly suppressed to 52.9 and 54.1%, respectively, by the addition of DMSO and SB525334 (lane 3) compared with that of DMSO alone (lane 2) (P<0.01). Next, we investigated the involvement of the YAP signaling pathway by the addition of a YAP inhibitor (verteporfin). As shown in Fig. 5B, verteporfin (lane 3) significantly suppressed *YAP1* expression to 41.6% (P<0.01) and *ACTA2* expression to 66.7% (P<0.01) and strongly suppressed *COL1A1* expression to 11.8% (P<0.01) compared with that of DMSO (lane 2). These results indicated that the TGF-β1 signaling pathway is partly involved in the induction of *COL1A1* expression but that the YAP signaling pathway is greatly involved in the induction of *COL1A1* expression by overexpression of both hTNX-FGFFFF and integrin α11.

YAP1 knockdown with YAP1 siRNA suppresses the induction of COL1A1expression by overexpression of both hTNX-FGFFFF and integrin α11. Next, YAP1 was knocked down with YAP1 siRNA prior to overexpression of both hTNX-FGFFFF and integrin α11, and then the expression levels of *ACTA2*, *COL1A1* and *YAP1* were analyzed by RT-qPCR in LX-2 cells. As shown in Fig. 6, knockdown of YAP1 with siRNA (lane 3) significantly suppressed *YAP1* expression to 45.4% (P<0.01) and *COL1A1* expression to 72.4% (P<0.05) compared to that with control siRNA treatment (lane 2). The results of

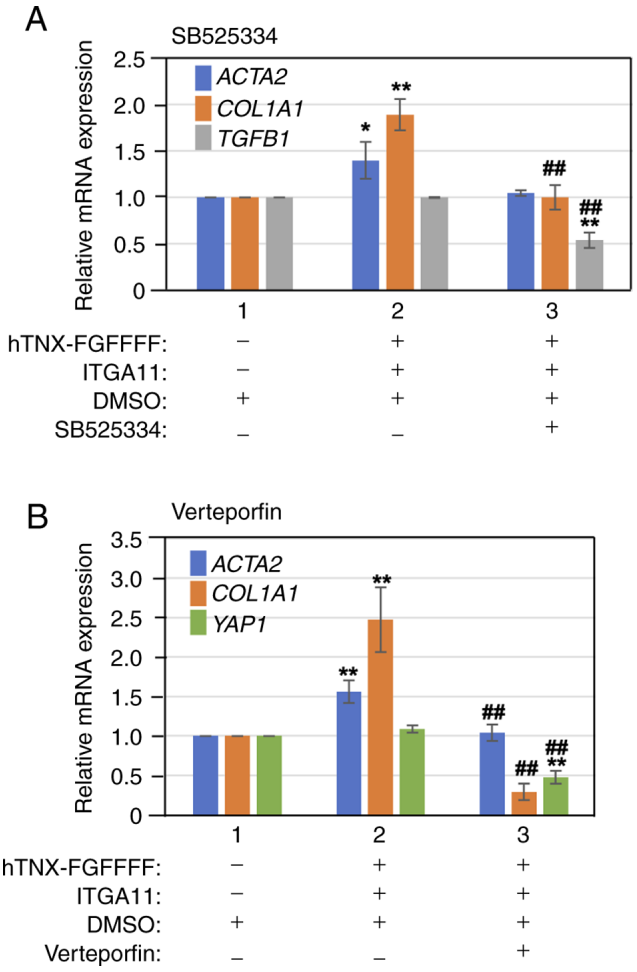
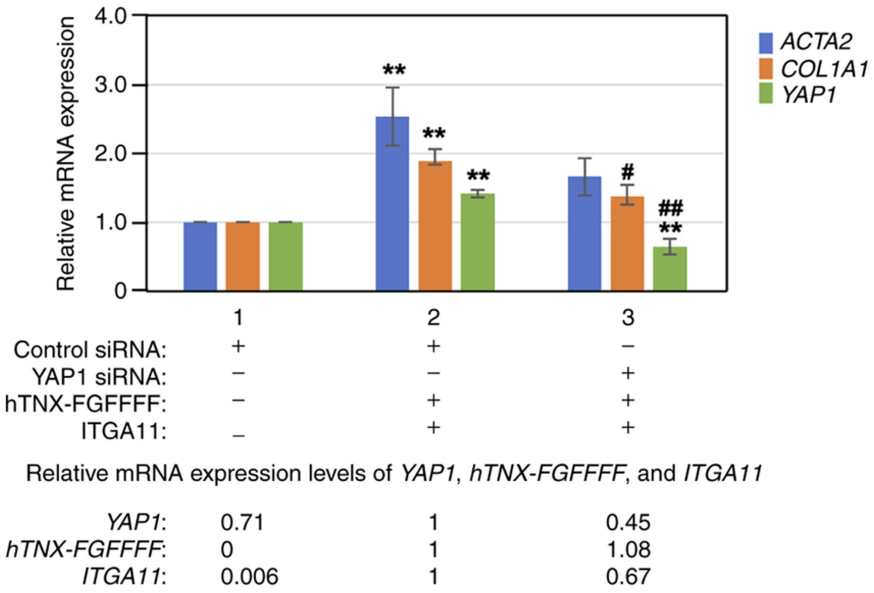


Figure 5. Induction of *COL1A1* expression by overexpression of both hTNX-FGFFFF and ITGA11 in addition to inhibitors. (A) Induction of *COL1A1* expression by overexpression of both hTNX-FGFFFF and ITGA11 with a TGFBRI inhibitor (SB525334). DMSO (lanes 1, 2 and 3) and SB525334 (lane 3) were added to the culture medium (DMEM/0.5% FBS) after the transfection of expression plasmids for both hTNX-FGFFFF and ITGA11 in LX-2 cells (lanes 2 and 3). (B) Induction of *COL1A1* expression by overexpression of both hTNX-FGFFFF and ITGA11 with a YAP inhibitor (verteporfin). DMSO (lanes 1, 2 and 3) and vertepofin (lane 3) were added to the culture medium (DMEM/0.5% FBS) after the transfection of expression plasmids for both hTNX-FGFFFF and ITGA11 in LX-2 cells (lanes 2 and 3). Subsequently, the cells were cultured followed by cell lysate extraction, RNA purification and reverse transcription-quantitative PCR. (A and B) The expression level of each gene [*ACTA2*, *COL1A1* and *TGFB1* for (A) and *ACTA2*, *COL1A1* and *YAP1* for (B)] in the control (lane 1) was set to 1.0, and the relative expression level of each gene compared with that of the control (lane 1) is shown (n=3). Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. control (lane 1); $^{\#\#}$P<0.01 vs. lane 2, one-way ANOVA with the Bonferroni post hoc test. *COL1A1*, type I collagen α1 chain; TNX, tenascin-X; ITGA11, integrin α11; *ACTA2*, α-smooth muscle actin; YAP, Yes-associated protein; hTNX-FGFFFF, GGLRIPFPRDCGEEM peptide from fibrinogen-related domain of human tenascin-X (hTNX-FG).

experiments with the YAP inhibitor verteporfin as well as with YAP1 siRNA indicated that the YAP signaling pathway is mainly involved in the induction of *COL1A1* expression by overexpression of both hTNX-FGFFFF and integrin α11.

Discussion

In this study, we revealed that overexpression of both a 15-aa peptide (hTNX-FGFFFF) derived from the TNX-FG domain



Relative mRNA expression levels of *YAP1*, *hTNX-FGFFFF*, and *ITGA11*

YAP1:	0.71	1	0.45
hTNX-FGFFFF:	0	1	1.08
ITGA11:	0.006	1	0.67

Figure 6. Effect of YAP1 knockdown on induction of *COL1A1* expression. YAP1 was knocked down with YAP1 siRNA prior to overexpression of both hTNX-FGFFFF and ITGA11, and then the expression levels of *ACTA2* and *COL1A1* were analyzed by reverse transcription-quantitative PCR in LX-2 cells. RNA from cells treated with transfection of control siRNA only (lane 1), transfection of control siRNA followed by co-transfection with both hTNX-FGFFFF and ITGA11 expression plasmids (lane 2) and transfection of YAP1 siRNA followed by co-transfection with both hTNX-FGFFFF and ITGA11 expression plasmids (lane 3) was used. The expression level of each gene (*ACTA2*, *COL1A1* and *YAP1*) in the control (control siRNA only) (lane 1) was set to 1.0, and the relative expression level of each gene compared with that of the control (control siRNA) is shown (n=3). Data are presented as the mean ± SD. **P<0.01 vs. control (lane 1); $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. lane 2, one-way ANOVA with the Bonferroni post hoc test. At the bottom of the figure, the relative expression levels of *YAP1*, *hTNX-FGFFFF* and *ITGA11* are also shown, setting lane 2 to 1.0, since *hTNX-FGFFFF* expression was not detected in lane 1. *COL1A1*, type I collagen α1 chain; TNX, tenascin-X; ITGA11, integrin α11; *ACTA2*, α-smooth muscle actin; YAP1, Yes-associated protein 1; siRNA, small interfering RNA; hTNX-FGFFFF, GGLRIPFPRDCGEEM peptide from fibrinogen-related domain of human tenascin-X (hTNX-FG).

and integrin α11 induces the expression of type I collagen α1 chain (*COL1A1*) via mainly the YAP signaling pathway and partly by the TGF-β1 signaling pathway in LX-2 cells. It is known that overexpressed integrin α11 functionally interacts with endogenous integrin β1, resulting in the formation of integrin α11β1 (36). Therefore, the expression of *COL1A1* induced by hTNX-FGFFFF would be caused via the YAP signaling pathway through integrin α11β1.

It has been shown that TNC, the most well-characterized member of the tenascin family, can promote fibrosis (37) in not only the liver (38) but also the heart (39) and lung (40). In mice with immune-mediated chronic hepatitis, El-Karef *et al* (38) showed that TNC accelerates liver fibrosis by an increased inflammatory response through recruitment of activated hepatic stellate cells (HSCs) and upregulation of TGF-β expression. However, the region in TNC that is responsible for the pathogenesis of fibrosis remains to be determined. Eponymous fibrinogen is involved in the pathogenesis of fibrosis in addition to the formation of a provisional fibrin matrix for creating a hemostatic blood clot (41). Craciun *et al* (42) showed that the expression of fibrinogen in the kidney is induced after the appearance of a renal lesion such as unilateral ureteral obstruction due to the induction of fibroblast proliferation and activation of the TGF-β canonical signaling pathway, leading to renal fibrosis. However, also in fibrinogen, the region involved in the pathogenesis of fibrosis has not yet been identified.

On the other hand, Aubert *et al* (43) recently showed that the FG domain of each tenascin family member, which is highly conserved among the four members, is associated with latent TGF-β1 and that the latent TGF-β1 is activated to its mature form to transmit TGF-β1 signals into cells. Molecular modeling and dynamic approaches have revealed that some residues mainly located in loop 9 of each FG domain, a region located at the C-terminal part of the domain, interact with some residues located in helix α1 of latency-associated peptide (LAP) and some residues in the mature TGF-β1 moiety (43). In the present study, we identified a 15-aa peptide (referred to as hTNX-FGFFFF with an aa sequence of GGLRIPFPRDCGEEM) located at the N-terminal part of the TNX-FG domain as the minimum region responsible for the induction of *COL1A1* expression. Interestingly, this minimum region in the TNX-FG domain was not overlapped with some residues in loop 9 in the TNX-FG domain reported by Aubert *et al* (43), indicating that the 15-aa peptide would fail to interact directly with LAP and mature TGF-β1. However, the fact that SB525334 weakly suppresses the induction of *COL1A1* expression with overexpression of hTNX-FGFFFF and integrin α11β1 as shown in Fig. 5A indicates that the TGF-β1 signaling pathway has only a small contribution to the induction of *COL1A1* expression. The reason why the TGF-β1 signaling pathway is weakly activated by overexpression of hTNX-FGFFFF and integrin α11β1 remains unclear, but we speculate that overexpression of hTNX-FGFFFF and integrin α11β1 weakly leads to the activation of TGF-β1 signaling following an unidentified interaction of hTNX-FGFFFF and integrin α11β1 with, for example, latent TGF-β binding protein (LTBP), a reservoir of latent TGF-β into the matrix (44). It is important to explore the possibility of tripartite interactions among hTNX-FGFFFF, integrin α11β1, and LTBP.

It has not been determined whether hTNX-FGFFFF (GGLRIPFPRDCGEEM sequence) interacts with integrin α11β1 directly or indirectly. Integrin α11β1 recognizes the GFOGER sequence (where O represents hydroxyproline) in fibrillar collagens such as type I collagen (COL1) and type II collagen (COL2) via its I domain (45). A comparison of the hTNX-FGFFFF peptide sequence and the GFOGER sequence shows that there is almost no homology between the two sequences. Therefore, we speculate that hTNX-FGFFFF peptide fails to interact with integrin α11 directly. In contrast, it is possible that hTNX-FGFFFF peptide indirectly interacts with integrin α11 via COL1A1. Human COL1A1 (UniProtKB: P02452) has a GFOGER sequence, and integrin α11 can thus interact with COL1A1. In addition, hTNX-FGFFFF peptide might interact with COL1A1 since the TNX-FG domain is known to be involved in the interaction of TNX with COL1 (46). With the interactions of hTNX-FGFFFF peptide-COL1A1-integrin α11, the stimuli from hTNX-FGFFFF peptide might be transmitted to YAP via integrin α11β1, leading to the induction of *COL1A1* expression, because it has been reported that YAP signaling downstream of integrin α11β1 promotes the fibrotic phenotype in myofibroblasts (31). As a future plan, we are considering to examine the interaction of hTNX-FGFFFF and COL1A1 by biochemical analyses.

Both *COL1A1* and *ACTA2* are well known as fibrosis marker genes in myofibroblasts. However, it is thought that stimulation of YAP signaling sometimes leads to induction of *ACTA2* expression (47) and sometimes does not (48) in myofibroblasts. It has also been reported that only some collagen-producing fibroblasts co-express *ACTA2* in the fibrotic lung and kidney (49). Therefore, the results of the present study showing that overexpression of both hTNX-FGFFFF and integrin α11 causes induction of *COL1A1* expression but not induction of *ACTA2* expression also indicate distinct regulation of the gene expression of *COL1A1* and *ACTA2* in LX-2 cells.

Previously, our group showed that HFCD-fed wild-type (WT) mice display liver dysfunction including type I collagen deposition and inflammatory response compared with those in TNX-deficient mice, indicating that TNX promotes liver fibrosis *in vivo* (24). Therefore, the results obtained in the present *in vitro* study showing induction of *COL1A1* expression by a 15-aa peptide located in the TNX-FG domain in LX-2 cells may be related to the promotion of liver fibrosis observed in HFCD-fed wild-type (WT) mice *in vivo*. As described in the Results section, addition of the synthetic 15-amino acid peptide in the conditioned medium with co-transfection of integrin α11 was not sufficient for induction of *COL1A1* expression in LX-2 cells. We speculated that expression of the 15-amino acid peptide in cells is necessary for its induction. Thereby, we speculate that mere administration of the synthetic 15-amino acid peptide to mice would not induce *COL1A1* expression *in vivo* as well. At present, this causes the lack of animal experiments as a limitation of this study.

In conclusion, the present *in vitro* study showed that a 15-aa peptide located in the N-terminal part of the TNX-FG domain induces the expression of *COL1A1* via YAP signaling downstream of integrin α11β1. Therefore, the suppression of TNX expression may be a novel therapeutic target for improving fibrosis in the liver.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KIM designed the experiments, performed the experiments and wrote the manuscript. KIM, KK and KY analyzed the data. HT contributed to the conception and experimental design on the construction of expression plasmids, and critically revised the manuscript for intellectual content. KIM and KY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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