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Conversion of mechanical force into TGF-β-mediated biochemical signals

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Abstract

Mechanical forces influence homeostasis in virtually every tissue [1–2]. Tendon, constantly exposed to variable mechanical force, is an excellent model in which to study the conversion of mechanical stimuli into a biochemical response [3–5]. Here we show in a mouse model of acute tendon injury and *in vitro* that physical forces regulate the release of active transforming growth factor (TGF)-β from the extracellular matrix (ECM). The quantity of active TGF-β detected in

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Author contributions

Takao Sakai initiated and supervised the project. Dusko Ilic and Takao Sakai conceived ideas and designed experiments. Toru Maeda, Tomoya Sakabe, Ataru Sunaga, Keiko Sakai and Alexander Rivera performed experiments. Douglas Keene carried out electron microscopy studies. Ronen Schweitzer generated *ScleraxisGFP* transgenic mice. Harihara Baskaran generated microfluidic chambers for the fluid shear stress system. Takako Sasaki generated anti-type III and anti-type I collagen antibodies. Joseph Iannotti initiated the botulinum toxin A experiments. Edward Stavnezer initiated the TGF-β receptor inhibitor SD208 experiments and provided inhibitors. Toru Maeda, Tomoya Sakabe and Takao Sakai analyzed the data. Dusko Ilic, Harihara Baskaran and Takao Sakai wrote and edited the manuscript.

tissue exposed to various levels of tensile loading correlates directly with the extent of physical forces. At physiological levels, mechanical forces maintain, through TGF-β/Smad2/3-mediated signaling, the expression of Scleraxis (Scx), a transcription factor specific for tenocytes and their progenitors. The gradual and temporary loss of tensile loading causes reversible loss of Scx expression, whereas sudden interruption, such as in transection tendon injury, destabilizes the structural organization of the ECM and leads to excessive release of active TGF-β and massive tenocyte death, which can be prevented by the TGF-β type I receptor inhibitor SD208. Our findings demonstrate a critical role for mechanical force in adult tendon homeostasis. Furthermore, this mechanism could translate physical force into biochemical signals in much broader variety of tissues or systems in the body.

Keywords

Scleraxis; Tendon; Tensile loading; Mechanical force; TGF-β; Smad2/3

Results

Tendon is a fibrous connective tissue made of specialized fibroblasts called “tenocytes” and an abundant ECM, whose physiology and pathology depends heavily on mechanical stimuli [3–5]. Tendon transfers contraction forces (tensile loading) from skeletal muscle to bone and consequently possesses high tensile stiffness and strength. Despite numerous studies, it is still largely unknown at molecular levels how transmittal forces play a role in the regeneration and maintenance of adult tendon.

During embryonic development, tenocytes originate from mesodermal compartments, as do skeletal myoblasts, chondrocytes, and osteoblasts [6]. The basic helix-loop-helix transcription factor *Scx* is found to be essential for tendon development: *Scx*-deficient mice show a complete loss of major force-transmitting and intermuscular tendons [7]. Although multipotent mesenchymal progenitors might express *Scx* upon specific lineage commitment, only tendon progenitor cells and tenocytes retain its expression, making Scx a highly specific marker of tenogenic (precursor) cells and mature differentiated tenocytes [8–9].

ScxGFP expression in tenocytes of adult tendon

To understand the role that continuous transmittal force from skeletal muscle to bone plays in adult tendon homeostasis, we utilized a transgenic mouse strain that expresses the *Scx* promoter-driven GFP marker (ScxGFP, Figure 1A and 1B)[9]. Robust ScxGFP expression in a majority of tenocytes (94.0±3.4%) clearly distinguished them from adjacent skeletal muscle cells in the myotendinous junction and from adjacent chondrocytes in cartilage (Figure 1B). ScxGFP expression did not affect the tendon ECM composition, the expression of collagen type I or type III, fibronectin, tenascin-C, small leucine-rich proteoglycan fibromodulin or cartilage oligomeric matrix protein (COMP or thrombospondin 5) (Figure S1A, data not shown). Scx expression specificity was also confirmed *in vitro*. Primary tenocytes isolated from 10-wk-old *ScxGFP* mice expressed high levels of GFP (Figure 1C), whereas primary chondrocytes and osteoblasts isolated from newborn *ScxGFP* mouse ribs and calvaria were negative (Figure 1C). As demonstrated with RT-PCR, adult tenocytes expressed *Scx* and the mature tenocyte marker *tenomodulin* [10], the major tendon ECM component *collagen type I*, and the collagen receptors *integrin alpha1* and *integrin alpha11* [11–12]. In contrast, primary dermal fibroblasts isolated from the same mice expressed neither *Scx* nor *tenomodulin*, showing phenotypic differences between tenocytes and non-tendon fibroblasts in the adult. Neither cell type expressed the chondrogenic master gene

Sox9 [6] (Figure 1D). The *ScxGFP* transgene can therefore be used to specifically identify and study adult tenocytes.

Effects of acute loss of tensile loading on ScxGFP expression and tenocytes

The complete transection model was chosen to study tendon injury because this model best mimics clinically acute tendon injuries (i.e., definite interruption of tendon continuity and immediate loss of tensile loading) (Table S1) [13–14]. Almost 70% fewer tenocytes were found at 3 d post-transection (17.5±2.5 cells/field in transected *vs*. 56.8±3.3 in sham-operated tendons [$n = 4$; field = 0.037 mm^2, $P = 0.0003$]), and only a small portion of the remaining cells (11.7%) retained low ScxGFP expression post-transection (Figure 1E). Acute loss of tensile loading correlated with the loss of tenocyte viability by as early as 0.5 h after transection. ScxGFP loss and gain of positive TUNEL gradually spread to the proximal region of the transected Achilles tendon with time (Figure 1F and 1G). Therefore a sudden loss of continuous transmittal force from skeletal muscles leads to massive death of tenocytes. This finding could explain why tendon injuries very rarely heal. No obvious massive bleeding or inflammatory response and no significant change in microvascularity for at least up to 2 h within tendon tissues after complete transection was confirmed (Figure S1B–S1D).

Gradual loss of tensile loading allows reversible Scx expression but has profound effects on the mechanical properties of adult tendons

Loss or attenuation of ScxGFP expression even in surviving tenocytes suggested that ScxGFP expression might depend on mechanical signaling. To test that, we experimentally induced a reversible gradual loss of continuous transmittal force from skeletal muscles using botulinum toxin A. This toxin blocks the release of acetylcholine specifically from presynaptic motor nerve terminals and induces a gradual but reversible skeletal muscle weakness, resulting in decreased muscle force – 25% of normal at 3 d post injection, returning to normal after 24 wks [15–19]. A single dose of botulinum toxin A (6 U kg^{-1} body weight) injected into the triceps muscle of 10- to 12-wk-old *ScxGFP* mice (Figure 2A) did not cause any cell death (TUNEL-positive cells: 2 of 206 = 0.97% at 24 h; 1 of 237 = 0.42% at 4 d). However, at 1 wk post injection the number of tenocytes expressing ScxGFP decreased by ~79% and the level of expression was significantly lower compared to that of saline-injected controls (Table S2 and Figure 2A and 2B). Immunohistochemical analysis also showed a significant decrease in the abundance of collagen type I fibrils and COMP expression (Figure 2A and 2B). Control saline-injected tendons showed no phenotypic changes (data not shown).

Since Scx regulates the expression of *pro-α1(I) collagen (Col1a1)* in tenocytes [20], the profound impact of tensile loading on tenocyte cell viability and ScxGFP expression indicated that even a gradual decrease in tensile loading could affect the mechanical properties of adult tendons. Indeed, at 1 wk post injection, tendons in toxin-injected mice showed a significant decrease in stiffness (>3-fold) and peak force (>2.5-fold) in comparison with control mice ($P < 0.05$, Figure 2C and 2D). Ultimate stress also decreased considerably at 1 wk post injection although without significance ($P = 0.08$; Figure 2D). At 2 wks post injection, tendons from toxin-injected mice began to recover: the number of ScxGFP-expressing tenocytes and the deposition of collagen type-I and COMP increased (Figure 2A and 2B), and the difference in stiffness and peak force compared to controls was diminished (Figure 2D). Transmittal force from skeletal muscles therefore has a major impact on adult tendon homeostasis.

Mechanical force regulating ScxGFP can be reversibly operated *in vitro*

To determine how mechanical force regulates tenocyte function at the molecular level, primary tenocyte cultures were isolated from adult *ScxGFP* mouse Achilles tendon (Figure 1C, upper panels). ScxGFP was initially expressed in all primary tenocytes, but expression decreased to <30% after only 48 h and <5% by day 6 (Figure S2A and S2B). To quantify the force required to retain ScxGFP expression, populations of primary cultured ScxGFP tenocytes were simultaneously exposed to different levels of mechanical force using a microfluidic chamber system composed of multiple bifurcating networks [21]. This fluid-flow device is a model system that applies shear stress to the tenocytes and mimics the response to mechanical stimuli [22–24]. The level of mechanical force depended on the location of cells in the chamber subdivisions, designated I to IV (Figure 3A). Shear stress values for these locations in the chamber were obtained by solving Navier-Stokes (N-S) equations using computational fluid dynamics software (COMSOL). The validity and applicability of equations and the usefulness of software was verified by our group in separate experiments involving microbeads [21]. There was agreement between the velocity profiles predicted by the N-S equations and experimental results using the technique of particle image velocimetry. The fluid flow regime through the device was laminar. As such, surface roughness or ECM accumulation had negligible effects on wall shear stress and overall frictional resistance. Only under turbulent flow conditions would ECM accumulation and other factors that lead to changes in surface “roughness” have a significant effect [25]. Using an inlet flow rate of 1 ml h^{-1} that resulted in an estimated shear stress range of 0–0.60 dyne cm^{-2}, it was found that a stress of 0.14 dyne cm^{-2} (area II) was optimal for retaining ScxGFP expression in the primary tenocytes (Figure 3B and 3C). To determine whether the decreased expression of ScxGFP after 6 d of culture was a reversible or an irreversible response, primary tenocytes were then exposed to mechanical strain on day 7. Tenocytes exposed to shear stress of 0.14 dyne cm^{-2} in area II indeed showed the highest induction of ScxGFP (Figure S2C).

TGF-β and mechanical force function to maintain Scx expression, and Smad2/3 plays an essential role in both mechanical and biochemical signaling pathways that regulate Scx expression

Next, we examined 11 candidate cytokines/growth factors for their role in regulating *Scx* expression in adult tenocytes [26–32]. TGF-β1, -2 and -3 were the most potent and induced similar levels of ScxGFP expression. GDF8 (myostatin, another member of the TGF-β superfamily and a negative regulator of skeletal muscle mass [33]) was ~4-fold less potent than the TGF-βs. Neither the osteoinductive cytokine BMP2 [34], used as a negative control, nor any other cytokines/growth factors examined had any effect. With each of these, the level of ScxGFP expression dropped to that of untreated controls by 7 d of culture (Figure S2D).

In TGF-β superfamily-mediated signaling, all TGF-β ligands, including TGF-β1, -2, and -3 and GDF8, bind to cell surface TGF-β type I (designated as activin receptor-like kinases [ALKs]) and type II receptors. Upon ligand-binding, both receptor types form heterotetrameric complexes, which initiate downstream Smad-signaling pathways [35–36]. ScxGFP levels in tenocytes treated with 1.0 μM of the TGF-β type I receptor inhibitor SD208 [37] were significantly lower after 7 d of culture (24.4±13.0%, $P < 0.001$) compared to untreated controls (100%) (Figure 3D), and Scx mRNA levels were downregulated to 28.0±2.6% ($P = 0.00045$, Figure 3E). Although TGF-β is co-dependent on cytoskeletal tension in some cell types such as myofibroblasts [38–39], the addition of the cytoskeleton disrupting reagents C3 transferase (an inhibitor of the Rho signaling pathway) [40] or

blebbistatin (an inhibitor of non-muscle myosin II production) [41–42] did not affect TGF-β-mediated ScxGFP expression in tenocytes (Figure S2E). These findings suggest that in tenocytes TGF-β1 regulates Scx expression independently of cytoskeletal tension, though this may need further confirmation.

Since the TGF-βs bind to TGF-β type I receptor and activate the Smad2/3 pathway [35–36], we examined the functional relationship between mechanical force- and TGF-β-mediated Scx induction. The addition of SD208 to primary tenocytes under mechanical force resulted in significantly (88.6%) decreased nuclear levels of phosphorylated Smad2 (pSmad2) in tenocytes. Phosphorylation of Smad2 was very weak and pSmad2 was distributed diffusely, whereas untreated tenocytes showed clear nuclear localization (Figure 3F). Furthermore, the Smad 3 inhibitor SIS3 [43] reduced the expression of ScxGFP and Scx mRNA levels in both TGF-β- and mechanical force-mediated systems by ~35% to ~50% (Figure S2F). Therefore both TGF-β and mechanical force have an important function in the maintenance of Scx expression, and Smad2/3 plays an essential role in both mechanical and biochemical signaling pathways that regulate Scx expression.

Transection interrupts tensile loading, destabilizes ECM structural organization, and releases an excess active TGF-β

In intact adult tissues and organs TGF-β is in a latent form associated in a noncovalent complex with the ECM. In response to injury, local latent TGF-β complexes are converted into active TGF-β [44–45]. To address how mechanical force regulates TGF-β activation and release at a molecular level, adult tenocyte cell lines were generated from Achilles tendons of mice on *p53*- and *p21*-null genetic background [46] as the yield of primary tenocytes was insufficient for analysis. These null tenocytes were morphologically indistinguishable from normal wild-type tenocytes (data not shown). When either p53- or p21-null tenocytes were exposed to mechanical force for 3 d *in vitro*, expression levels of TGF-β1 and Scx mRNAs were remarkably upregulated (12.4±4.3 and 26.9±6.1 fold [$n = 3$] respectively at 200 μl h^{-1} flow rate) compared to untreated controls. Released active TGF-β levels in the culture media corresponded to mechanical force strength (Figure 4A). On the other hand, while adding 80 pM TGF-β1 into the medium retained ScxGFP expression for up to 10 days, exposure of primary tenocytes to 160 pM TGF-β1 led to cell death *in vitro* (Figure 4B). We therefore hypothesized that the sudden interruption of continuous tensile loading by complete tendon transection might have resulted in the destabilization of the ECM's structural organization and consequent release of excessive amounts of active TGF-β, which caused the massive tenocyte death found *in vivo* (Figure 1F).

Indeed, subsequent analyses of collagen fibril ultrastructure in transected tendons revealed irregular patterns by as early as 2 h post-injury (Figure 4C). In intact tendon, the vast majority of collagen fibrils were 60–120 nm in diameter (70.4%) and only ~2% of fibrils had a diameter <60 nm. By 2 h post injury, the number of larger fibrils with diameter 60–120 nm had decreased from 70% to 45%, whereas the number of thinner fibrils (<60 nm diameter) increased 6.3-fold (12.5%). By 24 h post injury, the collagen fibrils had deteriorated even further: only ~30% of fibrils with diameter 60–120 nm remained, and the number of thinner fibrils (<60 nm diameter) had increased to 30.6%. The number of fibrils with diameter >120 nm also changed over time (16.1% in intact tendon, 30.8% at 2 h, and 20.6% at 24 h post injury). In contrast, unlike completely transected tendons, the relative distribution of fibrils of different diameter in toxin-treated tendons did not change dramatically over time (Figure 4C). Since fibronectin and fibrillin-1 associate with latent TGF-β binding proteins [44–45], their expression patterns were examined but no obvious differences between transected and intact tendon tissues were found (Figure S3). Quantitative analysis of active TGF-β demonstrated that a significantly higher (~1.5-fold)

concentration was released into tissues at 1.5 h post injury relative to uninjured tendon (P = 0.018; Figure 4D), whereas the total amount of TGF-β indicated similar levels pre- vs. post injury (631±61.1 relative light units (RLU) *vs*. 651±69.0 RLU [n = 3]). Such an excessive amount of active TGF-β was indeed related to tenocyte cell death *in vivo* after injury (Figure 4E). The expression level of Scx mRNA at 1.5 h post injury was 36.4±5.0% (n = 3) of non-treated control. The pre-treatment of tendons with 1.0 μM SD208 for 10 min before injury significantly reduced TUNEL-positive cells, whereas massive tenocyte cell death was observed in the SD208-untreated control transection ($P < 0.001$; Figure 4E and 4F). Thus the quantity of released active TGF-β from ECM depends on changes in mechanical force and demonstrates a mechanism underlying massive tenocyte death in response to complete transection of Achilles tendons.

Discussion

The present study (1) provides compelling evidence that mechanical forces actually regulate expression of Scx through activation of the TGF-β-/Smad2/3-mediated pathway, which, in turn, is required for maintenance of tendon-specific ECM; and (2) directly links an excess of active TGF-β released from the ECM to adult tendon pathology.

An earlier study has demonstrated that myofibroblast contraction activates and releases latent TGF-β from the ECM in cultured fibroblasts [47]. This supports a functional link between mechanical force and active TGF-β. In normal physiological conditions, every cell senses mechanical signals within a distinct range of magnitude [48]. After tissue injury tissue boundaries disintegrate and ECM architecture is considerably disrupted, causing dramatic mechanical imbalance to cells [39]. Indeed, the elevated TGF-β activity in response to injury causes delayed healing after bone fracture and skeletal muscle injury [49–50]. The suppression of TGF-β1 activity can promote functional recovery after corneal and spinal cord injury [49, 51]. Furthermore, after cancer radiotherapy, TGF-β1 is overexpressed at the targeting sites which develop injury-type response with cell death, excessive fibrosis and tissue atrophy in the surrounding normal tissues [52]. The alteration of mechanical integrity and the modifications that interfere with normal mechanotransduction to mechanical strain could also be implicated in a wide disease spectrum, including muscular dystrophies, arteriosclerosis, osteoporosis and cancer [53]. Thus, the mechanism demonstrated here opens new avenues for understanding how mechanical force is converted into biochemical signals and thereby determines cell fate in a variety of adult tissues or systems in the body. Since multiple mechanisms exist in mechanotransduction pathways, they will interplay as complementary pathways to maintain tissue specific phenotypes.

Tendon injuries are a serious clinical problem since damaged tendon tissues heal slowly and rarely regain normal mechanical strength [14, 30, 54]. These data imply that more extensive use of physical modalities in therapy could benefit patients and improve functional outcome. In tenocytes Scx regulates transcription of *Col1a1* through binding to tendon-specific element 2 [20] and the induction of Scx expression in injured tendon may support healing. Furthermore, the application of TGF-β receptor inhibitors to injured tendons as a part of the first-aid treatment immediately after injury could significantly improve the prognosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALK	activin receptor-like kinase
BMP	bone morphogenic protein
COMP	cartilage olgomeric matrix protein
DAPI	4′6-diamidino-2-phenylinodole
DMEM	Dulbecco’s modified Eagle’s medium
EGF	epidermal growth factor, ECM, extracellular matrix
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDF	growth differentiation factor
GFP	green fluorescent protein
IGF	insulin-like growth factor
PAI-1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
Scx	scleraxis
RT	reverse transcription
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling

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Highlights

- Physical forces regulate the release of active TGF-β from the extracellular matrix
- Mechanical forces maintain Scleraxis expression through TGF-β-mediated signaling
- An excess release of active TGF-β directly links to adult tendon pathology

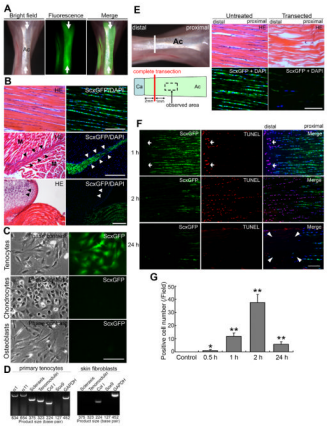

Figure 1. Loss of tensile loading causes tenocyte cell death in adult Achilles tendons

(A) – (D) Characterization of adult *ScxGFP* transgenic mice.

(A) Achilles tendons (Ac, arrows) in 10-wk-old *ScxGFP* transgenic mice express a robust ScxGFP signal (green) under fluorescence stereomicroscopy.

(B) Histological analysis of Achilles tendons in 10-wk-old *ScxGFP* transgenic mice. HE-stained sections (left panels) and the same areas with GFP/UV filters (right panels: green, ScxGFP; blue, DAPI [cell nuclei]). Upper panels: adult Achilles tendon. Note that aligned tenocytes express ScxGFP. Middle panels: Myotendinous junction at proximal Achilles tendon. ScxGFP is expressed only in tenocytes (arrow heads); myocytes (M) are completely negative for ScxGFP. Lower panels: Distal insertion of Achilles tendon. ScxGFP is expressed only in tenocytes. Chondrocytes (arrow heads) at the calcaneus are completely negative for ScxGFP. Bars = 50 μm.

(C) Only primary tenocytes from *ScxGFP* transgenic mice express ScxGFP *in vitro*. Left panels: Phase contrast micrographs of primary tenocytes, chondrocytes and osteoblasts. Right panels: Fluorescence micrographs of the same area with a GFP filter. Bar = 100 μm.

(D) RT-PCR analysis of gene expression profiles related to tendon and cartilage in primary adult wild-type mouse tenocytes and dermal skin fibroblasts. Tenocyte markers *Scx* and *tenomodulin* and chondocyte marker *Sox9* are not expressed in skin fibroblasts. α1, *α1 integrin*; α11, *α11 integrin*; Col I, *collagen type I*; GAPDH, *glyceraldehyde 3-phosphate dehydrogenase (as a control gene).*

(E) – (G) Acute tensile loading-loss model by complete transection of adult Achilles tendons.

(E) Left panels: Illustrations of the complete transection model. Achilles tendon (Ac) was completely transected at 2 mm proximal to the calcaneus (Ca) (white line in picture and red line in drawing, respectively). Middle and right panels: Histological analysis of intact (untreated) and transected tendon tissues at 3 d after operation. HE-stained sections and the same areas with GFP/UV filters (green, ScxGFP; blue, DAPI). Sections were prepared approximately 3 mm from calcaneus in both transected and intact tendons as shown in left panels. Note that very few cells retain ScxGFP expression in comparison with normal intact adult tendon. Bar = 50 μm.

(F) Analysis of cell death. Expression of ScxGFP (green; left panels), TUNEL staining (red; middle panels) and merged images (right panels) with DAPI (blue) at 1, 2, and 24 h after complete transection. Upper panels: 1 h after transection. ScxGFP expression is diminished, and TUNEL-positive cells appear at the edge of the tendon (arrows). Middle panels: 2 h after transection. ScxGFP expression is significantly diminished, and TUNEL-positive cells expand to the proximal side of the Achilles tendon. Lower panels: 24 h after transection. Extensive cell death results in the formation of an acellular region (arrowheads). Bar = 100 μm.

(G) Analysis of TUNEL-positive cells in non-transected (Control) and transected tendons. Error bars represent standard deviation ($n = 4$; field = 0.07 mm^2). *, $P < 0.05$; **, $P < 0.001$: significantly different compared to the number of positive cells in control non-transected tendons.

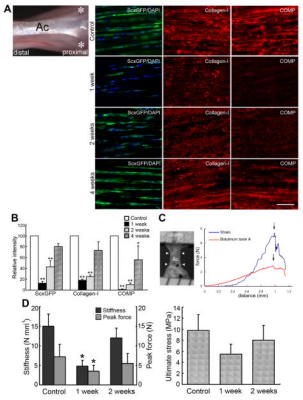

Figure 2. A gradual tensile loading-loss model by local injection of botulinum toxin A into adult Achilles tendons

(A) Left panel: Illustration of the botulinum toxin treatment. The toxin (6 U kg^{-1} weight) was injected intramuscularly into medial and lateral sides of the gastrocnemius muscle (asterisks). Ac, Achilles tendon. Right panels: Time course of fluorescence micrographs in toxin-treated Achilles tendons of *ScxGFP* mice (green, ScxGFP; blue, DAPI; red, collagen type I and COMP). Bar = 50 μm.

(B) Analysis of ScxGFP, collagen type I and COMP intensities shown in Figure 2A. Relative fluorescence intensities are shown relative to each control value of 100 (control tendon) ($n = 5$ for each group). Error bars represent standard deviation. *, $P < 0.05$; **, $P < 0.01$: significantly different compared to controls.

(C) Biomechanical analysis of toxin-treated tendon tissues. Left panel: Resected Achilles tendon (Ac, arrowheads) for biomechanical experiments. Right panel: Typical force-distance curves in toxin-treated (botulinum toxin A, red line) and saline-treated (sham, blue line) tendon tissue at 1 wk post injection. Arrows indicate peak force.

(D) Stiffness, peak force, and ultimate stress in untreated tendons (Control) and at 1 and 2 wks after toxin treatment. Error bars represent standard deviation ($n = 5$). Note that the toxin-treated Achilles tendons exhibit significantly reduced stiffness and peak force compared to untreated controls at 1 wk post treatment although ultimate stress is not significantly different. *, $P < 0.05$.

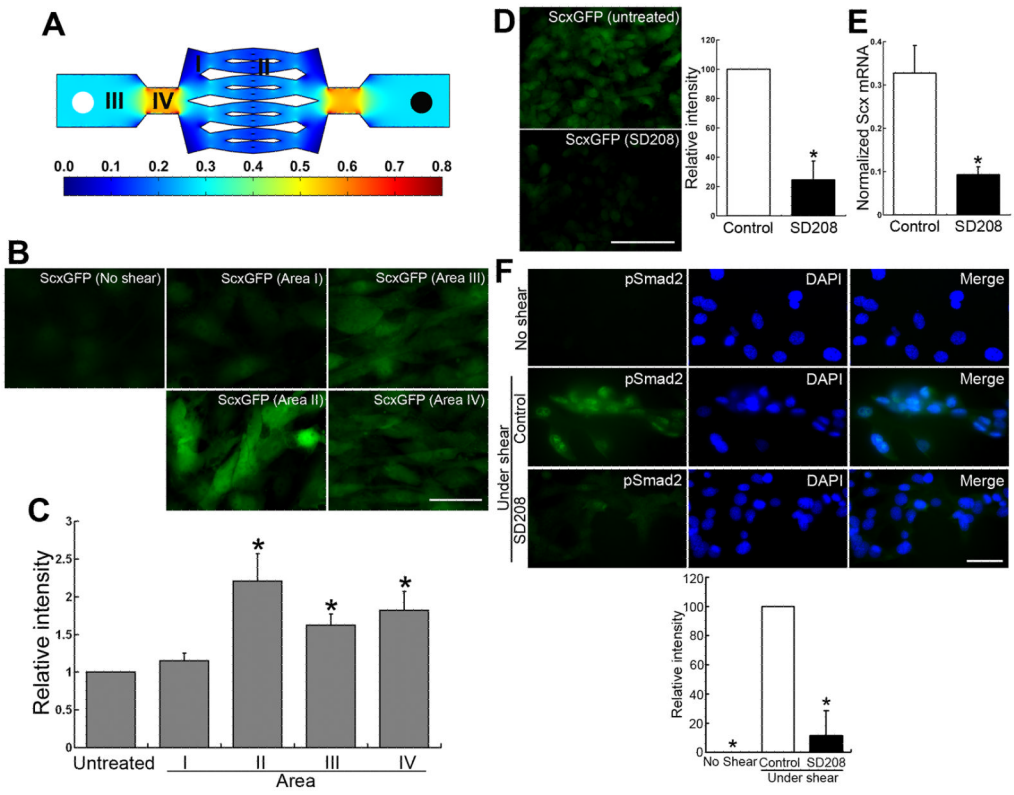

Figure 3. Mechanical force and Smad2-mediated signaling is required for maintenance of Scx expression in adult tenocytes *in vitro*

(A) Diagram of microfluidic chamber and regions. The composition is a bifurcating network of several generations with areas (I–IV) of different shear stresses (dyne cm^{-2}), as indicated by the color legend. A steady flow of cell culture medium from the input tube (left white circle) to the output tube (right black circle) was supplied by a syringe pump. For a flow rate of 1 ml h^{-1}, the wall mechanical strain in each area was about 0.1 (area I), 0.14 (area II), 0.30 (area III), and 0.60 (area IV) dyne cm^{-2}, respectively.

(B) ScxGFP expression in primary tenocytes at 7 d under different mechanical forces (flow rate, 1 ml hr^{-1}). Note that tenocytes in areas II (0.14 dyne cm^{-2}), III (0.30 dyne cm^{-2}) and IV (0.60 dyne cm^{-2}) retain ScxGFP expression, whereas tenocytes in untreated controls and area I (0.1 dyne cm^{-2}) show a marked decrease in ScxGFP levels. Bar = 50 μm.

(C) Analysis of ScxGFP intensities in primary tenocytes at 7 d under the different mechanical forces shown in Figure 3B. GFP intensity of untreated tenocytes at 7 d after culture (No shear) was set to 1.0. Relative GFP intensities of tenocytes determined in each area are shown relative to untreated control. Results are the mean of measurements for 100 cells in each area. Error bars represent standard deviation. ScxGFP levels in areas II, III and IV are significantly higher than those in untreated controls. *, $P < 0.05$.

(D) Left panels: ScxGFP in primary tenocytes at 7 d under 0.14 dyne cm^{-2} mechanical force with or without 1.0 μM TGF-β type-I receptor inhibitor SD208. Note that SD208 markedly inhibits mechanical force-mediated ScxGFP induction. Bar = 100 μm. Right panel: Analysis of ScxGFP intensities. GFP intensity of tenocytes is shown relative to the control value of 100 (percent of control). Error bars represent standard deviation. Note that SD208 significantly (75.6%) inhibits mechanical force-mediated ScxGFP induction. *, $P < 0.001$.

(E) Real-time PCR analysis of endogenous Scx mRNA expression in primary tenocytes at 7 d under 0.14 dyne cm^{-2} mechanical force with or without (Control) 1.0 μM SD208. The intensity of *Scx* signals were normalized to that of *18S rRNA* signals. Error bars represent

standard deviation obtained by error propagation ($n = 3$). Note that SD208 significantly (72.0%) downregulates mechanical force-mediated endogenous Scx mRNA expression, and this downregulation correlates with ScxGFP levels shown in Figure 3D. *, $P < 0.001$. **(F)** Effect of SD208 on pSmad2 levels in primary tenocytes from wild-type mice at 7 d under mechanical force with or without (Control) 1.0 μM SD208 or without shear stress. Upper panels: Expression of pSmad2 (green; left panels), nuclear staining with DAPI (blue; middle panels) and merged images (right panels). Bar = 25 μm. Lower panel: Analysis of pSmad2 intensities. The intensity is shown relative to the control value of 100 (Control tenocytes under shear stress). Error bars represent standard deviation. *, $P < 0.001$.

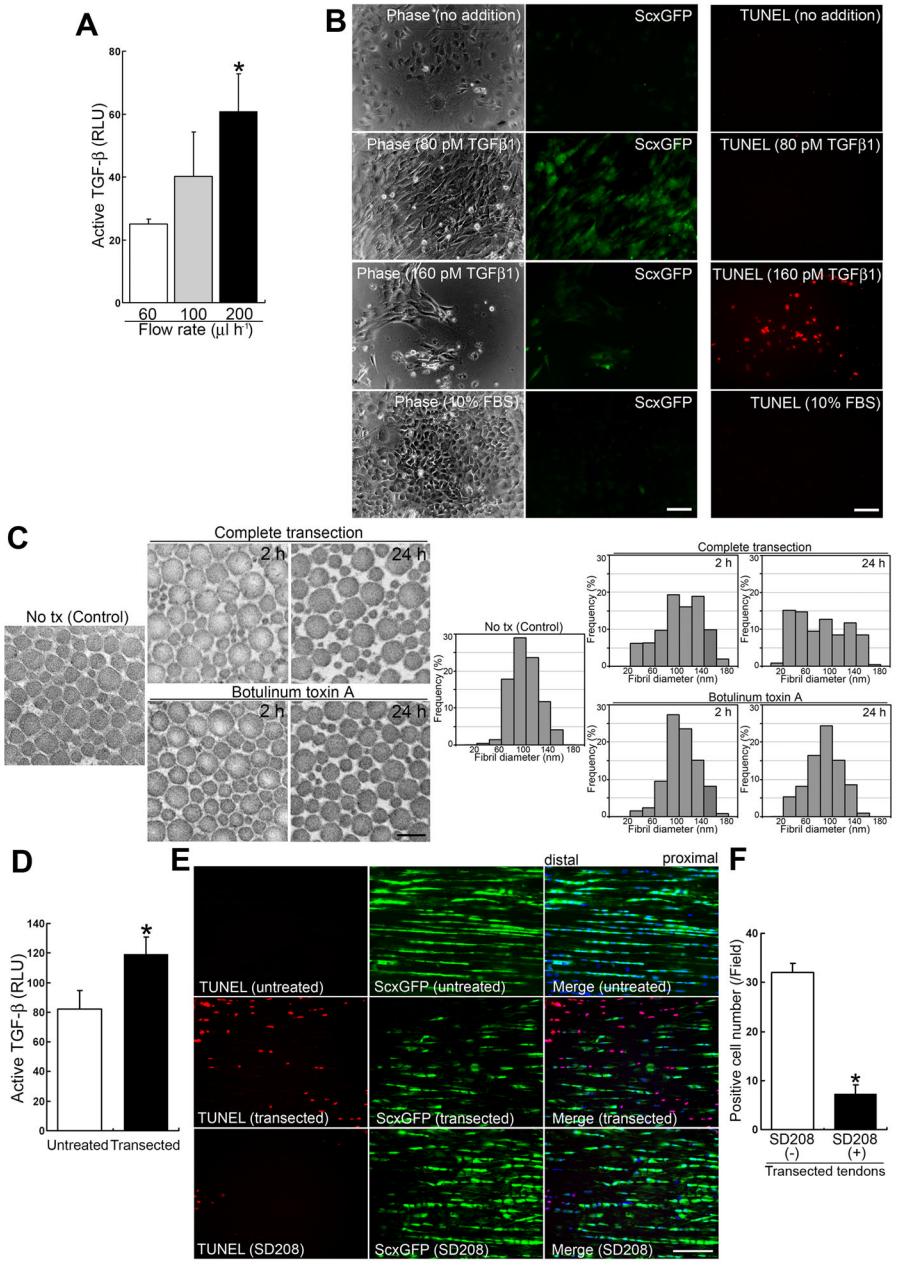

Figure 4. Sudden interruption of continuous tensile loading destabilizes the ECM's structural organization and allows consequent release of a significant amount of active TGF-β

(A) Active TGF-β bioassay in culture media from adult tenocyte cell lines cultured in a single network chamber for 5 d under different mechanical forces. Flow rate conditions for 60, 100 and 200 μl h^{-1} corresponded to 0.01, 0.015 and 0.03 dyne cm^{-2} mechanical force, respectively. Culture medium (100 μl) from each condition was used for the assay. The data show the amounts released in conditioned media for 1 h under different flow rates. Luciferase activity is presented as relative light units (RLU). Error bars represent standard deviation (n = 3). The values for active TGF-β released in the conditioned media over 1 h were 0.30 ± 0.018 pM under 60 μl h^{-1} shear stress; 0.52 ± 0.18 pM under 100 μl h^{-1} shear stress; and 1.01 ± 0.19 pM under 200 μl h^{-1} shear stress. *, significantly different (P < 0.01) compared to the amount under strain at 60 μl h^{-1} flow rate.

(B) Effects of the cytokine TGF-β1 on primary tenocytes. Left and middle panels: Phase contrast and fluorescence micrographs of the same area with a GFP filter for each condition. Right panels: The assessment of cell death by TUNEL staining (red). Primary tenocytes were cultured for 48 h, then cytokines were added for a further 7 d. Note that the addition of TGF-β1 at a low concentration (2 ng ml^{-1}; 80 pM) retains the expression of ScxGFP. In contrast to other culture conditions, only a higher concentration (160 pM) leads to tenocyte cell death (13.0 ± 2.7 cells/field [n = 4; field = 0.15 mm^2]). Retention of ScxGFP expression was seen at TGF-β1 concentrations as low as 20 pM (data not shown). Neither no addition (DMEM containing 1% FBS) nor 10% FBS maintained ScxGFP expression. Bars = 100 μm.
(C) Ultrastructural analysis of collagen fibrils. Transmission electron micrographs of transverse sections (left panels) and morphometric analysis of fibrils (right panels) in intact (Control), completely transected, and toxin-injected adult Achilles tendon tissues at 2 and 24 h post treatment. Note that collagen fibril ultrastructure in tension-collapsing tendons by complete transection reveals irregular patterns by as early as 2 h post injury. Bar in left panel = 200 nm.
(D) Active TGF-β bioassay in intact (untreated) and transected (at 1.5 h after complete transection) adult Achilles tendons. Luciferase activity is presented as relative light units (RLU). Error bars represent standard deviation (n = 3). Note that the transacted tendon tissues release significant amounts of active TGF-β. *, $P < 0.05$.
(E) Effects of SD208 on cell death *in vivo* after complete transection of adult Achilles tendon. TUNEL staining (red; left panels), expression of ScxGFP (green; middle panels) and merged images (right panels) with DAPI (blue) in intact tendons and at 1.5 h after complete transection without (untreated) or with 1.0 μM SD208 treatment. Bar = 50 μm.
(F) Analysis of TUNEL-positive cells at 1.5 h after complete transection without or with SD208 treatment. Error bars represent standard deviation (n = 4; field = 0.07 mm^2). Note that SD208 significantly attenuates tenocyte cell death. *, $P < 0.001$.