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# Tissue Stretch Decreases Soluble TGF-β1 and Type-1 Procollagen in Mouse Subcutaneous Connective Tissue: Evidence From Ex Vivo and In Vivo Models

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## Abstract

Transforming growth factor beta 1 (TGF-β1) plays a key role in connective tissue remodeling, scarring, and fibrosis. The effects of mechanical forces on TGF-β1 and collagen deposition are not well understood. We tested the hypothesis that brief (10 min) static tissue stretch attenuates TGF-β1-mediated new collagen deposition in response to injury. We used two different models: (1) an ex vivo model in which excised mouse subcutaneous tissue (N = 44 animals) was kept in organ culture for 4 days and either stretched (20% strain for 10 min 1 day after excision) or not stretched; culture media was assayed by ELISA for TGF-β1; (2) an in vivo model in which mice (N = 22 animals) underwent unilateral subcutaneous microsurgical injury on the back, then were randomized to stretch (20–30% strain for 10 min twice a day for 7 days) or no stretch; subcutaneous tissues of the back were immunohistochemically stained for Type-1 procollagen. In the ex vivo model, TGF-β1 protein was lower in stretched versus non-stretched tissue (repeated measures ANOVA, $P < 0.01$). In the in vivo model, microinjury resulted in a significant increase in Type-1 procollagen in the absence of stretch ($P < 0.001$), but not in the presence of stretch ($P = 0.21$). Thus, brief tissue stretch attenuated the increase in both soluble TGF-β1 (ex vivo) and Type-1 procollagen (in vivo) following tissue injury. These results have potential relevance to the mechanisms of treatments applying brief mechanical stretch to tissues (e.g., physical therapy, respiratory therapy, mechanical ventilation, massage, yoga, acupuncture).

Transforming growth factor β1 (TGF-β1) is well-established as one of the key cytokines regulating the response of fibroblasts to injury, as well as the pathological production of fibrosis (Barnard et al., 1990; Sporn and Roberts, 1990; Leask and Abraham, 2004). Tissue injury is known to cause auto-induction of TGF-β1 protein production and secretion (Van Obberghen-Schilling et al., 1988; Morgan et al., 2000). Elevated extracellular levels of TGF-β1 have a major impact on extracellular matrix composition by causing autocrine and

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paracrine activation of fibroblast cell surface receptors, leading to increased synthesis of collagens, elastin, proteoglycans, fibronectin, and tenascin (Balza et al., 1988; Bassols and Massague, 1988; Kahari et al., 1992; Cutroneo, 2003). In vivo, connective tissue remodeling is not limited to tissue injury, but also occurs in response to changing levels of tissue mechanical forces (e.g., immobilization, beginning a new exercise or occupation). Long-standing physical therapy practices also suggest that externally applied mechanical forces can be used to reduce collagen deposition during tissue repair and scar formation (Cummings and Tillman, 1992). The mechanisms underlying these effects, however, are not well understood. In this study, we have used an ex vivo mouse subcutaneous tissue explant model and an in vivo mouse microinjury model to examine the effect of applying brief (10 min), static mechanical stretch on TGF-β1 and Type-1 procollagen in response to subcutaneous tissue injury.

## Materials and Methods

The experimental protocols used in these experiments were approved the University of Vermont IACUC Committee. All mice were male C57Black6 weighing 19–21 g.

### Ex vivo mouse tissue explant study design

We first examined the time course of TGF-β1 protein (per mg tissue) and lactate dehydrogenase (LDH) (per mg tissue) concentration in the culture media at days 0, 1, and 3 post-stretch (or no stretch), with stretch occurring 1-day post-excision. The time course experiment was based on 8 mice (one sample per mouse) cultured and assayed in stretch/no-stretch pairs. Additional samples (N = 36 mice, paired for stretch/no stretch factor) were then assayed on day 3 post-stretch (or no stretch).

### Ex vivo equipment and tissue preparation

All equipment was rinsed in 70% ethanol/d$H_2O$ for 15 min (min) then air-dried. Immediately after death by decapitation, a 2 cm × 5cm flap of tissue containing dermis, subcutaneous muscle, and subcutaneous tissue was excised from the trunk of each mouse and viewed under a dissecting microscope. The subcutaneous tissue layer was dissected as a continuous sheet while applying minimal traction to the tissue. Samples were incubated for 24 h floating in separate culture wells at 37°C each containing 670 μl of 37°C Dulbecco's Modified Eagle Medium (DMEM F-12 Ham) 1:1 (containing 15 mM HEPES buffer) with penicillin/streptomycin (1–100 ml DMEM) (Invitrogen Corporation, Carlsbad, CA) and without added serum.

### Ex vivo tissue stretch

After 24 h of incubation, tissue samples were placed in grips and immersed in an incubation bath containing DMEM at 37°C with the proximal grip connected to a 4.9 Newtons (500 g) capacity load cell as previously described (Langevin et al., 2005). Tissue samples were elongated at a rate of 1 mm/sec by advancing a micrometer connected to the distal tissue grip until 19.6 milliNewtons (2 g) of preload was achieved. The tissue was then further stretched to 20% beyond preload length and incubated at that final length for 10 min. Control samples were placed in grips and incubated for 10 min without stretch. The samples were then removed from the grips, returned to the culture wells and incubated for an additional 3 days with a culture medium change on day 2 post-stretch (or no stretch). No tension was applied to the tissue during the post-stretch incubation period.

### Ex vivo TGF-β1 and LDH assays

Aliquots of 600 μl DMEM were harvested from the tissue culture wells on specified days and immediately assayed for (1) TGF-β1 protein using a human TGF-β1 ELISA assay (R&D Systems, Minneapolis, MN) including sample acidification with 1N hydrochloric acid for activation of latent TGF-β1 and (2) LDH, an indicator of cell death due to necrosis or apoptosis (Loo and Rillema, 1998), measured by reflectance spectrophotometry on an Ortho Clinical Diagnostics Instrument (Johnson & Johnson, New Brunswick, NJ).

### Ex vivo tissue viability stain

One pair of stretched and non-stretched tissue samples was incubated for 3 days post-stretch and stained as whole tissue mounts (30–50 μm thickness) with a Live (green)/Dead (red) assay kit (Molecular Probes, Eugene, OR) at 1:500 and imaged with a Bio-Rad MRC 1024 confocal microscope (Bio-Rad Microsciences, Hercules, CA) using a 20 × objective and 568 nanometer laser excitation and iris aperture of 2.7 and software package LaserSharp 2000.

### In vivo microinjury study design

Twenty-two mice first underwent unilateral microsurgical subcutaneous tissue injury on the back, and were then randomized to either stretch or no stretch (10 min twice a day for 7 days). The mice were sacrificed at day 7, subcutaneous tissues of the back were harvested and stained for Type-1 procollagen using indirect immunohistochemistry.

### Microsurgery procedure

Under isoflurane anesthesia, a 5 mm incision was performed in the middle of the back of mice at the level of the scapula. A microsurgery blade was then used to cut the subcutaneous tissue attachments between the subcutaneous (pannicular) muscle and the back muscles over a 1.5 cm × 1.5 cm area on one side of the back lateral to the midline with the other side serving as the control (Fig. 1A). One mouse in the stretch group was eliminated due to a bacterial wound infection, thus the final sample size was 21 (10 and 11 for stretch and no stretch conditions, respectively).

### In vivo tissue stretch method

In the stretch group, the mice underwent stretching of the trunk for 10 min twice a day for 7 days in the following manner: each mouse was suspended by the tail such that its paws barely touched a surface slightly inclined relative to the vertical. In response to this maneuver, the mouse spontaneously extended its front and hind limbs (Fig. 1B) with the distance between ipsilateral hip and shoulder joints becoming 20–30% greater than the resting distance. Mice in the no-stretch group were observed for 7 days without stretch. Seven days after injury, all mice were sacrificed by decapitation. The skin of the back, including subcutaneous tissues, was excised and fixed for 2 h in 3% paraformaldehyde in phosphate buffered saline (PBS).

### Type I procollagen immunohistochemistry

Following fixation, subcutaneous tissue samples (1 cm ×1 cm and 30–50 μm thickness) were dissected from the same location (centered 1 cm lateral to the spine at the level of the surgical incision) and mounted on glass slides. Immunohistochemistry was performed on the whole tissue mounts for the detection of Type-I Procollagen using indirect immunofluorescence with a primary rabbit monospecific polyclonal antibody (Shull and Cutroneo, 1983) at a dilution of 1:100 in PBS/1.0% BSA/0.1% triton and a CY3 secondary goat anti-rabbit antibody (Invitrogen Corporation) at a dilution of 1:500 in PBS/1.0% BSA/0.1%triton. Following secondary antibody staining, samples were rinsed in PBS/1.0% BSA

for 10 min and then overlaid with a glass coverslip using 50% glycerol in PBS with 1% *N*-propylgallate as a mounting medium.

### Quantitative evaluation of subcutaneous tissue Type I procollagen

Type-1 procollagen immunoreactivity was measured by quantifying Cy3 fluorescence intensity using the same detection threshold across all samples. For each immunostained sample, three fields chosen by a blinded investigator and imaged using a Zeiss LSM 510 META scanning laser confocal microscope at 63×. Fluorescence intensity was measured in projected three-dimensional stacks of images (nine serial optical sections at 0.76 μm inter-image interval) using Metamorph image analysis software (version 6.0; Universal Imaging Corporation, Downington, PA). Results are expressed as percent staining area (staining area over total imaged area).

### Statistical methods

Repeated measures analyses of variance were performed to test for differences in mean TGF-β1 and LDH concentrations between stretch and no stretch conditions and to examine temporal changes in these measurements. Because each animal assigned to stretch was paired (during incubation and ELISA assay) with a corresponding animal assigned to non-stretch, animal-pair was an additional factor in the analysis of variance (ANOVA). ANOVA also was used to analyze the effects of stretch on TGF-β1 concentrations at day 3 post-stretch, with stretch as a within-pair factor. For these analyses, TGF-β1 and LDH data were log transformed prior to analysis in order to satisfy the normality and homogeneity of variance assumptions associated with the ANOVA, which were examined based on residual plots. Means presented in the text and figures for these outcome measures are geometric means to be consistent with the log transformed analyses. For the in vivo experiment, repeated measures analysis of variance was also used to analyze the effects of stretch and injury on Type-I procollagen. For these analyses, stretch was an across-animal factor and injury was a within-animal (across-side) factor. Statistical analyses were performed using SAS statistical software (PROC MIXED).

## Results

### Effect of stretch on TGF-β1 ex vivo

In ex vivo short-term organ cultures of mouse subcutaneous tissue explants, TGF-β1 protein levels in the culture media increased significantly in both stretched and non-stretched samples during the 4-day incubation (Repeated measures ANOVA, ($F_{2,6}$ = 32.7, $P < 0.001$) (Fig. 2A). Stretching the tissue for 10 min, 24 h after excision, partially suppressed the rise in TGF-β1. In the time course experiment, tissue exposed to stretch had significantly lower overall mean TGF-β1 levels compared with non-stretched tissue ($F_{1,3}$ = 64.6, $P$ = 0.004), though there was no evidence that the time effect was different between stretch and non-stretch conditions ($F_{2,6}$ = 1.4, $P$ = 0.31) (Fig. 2A). In a larger number of samples tested at day 3, mean TGF-β1 protein levels were significantly lower in stretched samples compared with non-stretched samples ($F_{1,26}$= 11.5, $P$ = 0.002, N = 36) (Fig. 2B).

In contrast to TGF-β1, mean LDH levels decreased significantly over time from day 0 to day 3 ($F_{2,4}$ = 6.9, $P$ = 0.05) and were not significantly different in the stretched compared with the non-stretched samples ($F_{1,2}$ = 0.10, $P$ = 0.81) (Fig. 3A). This suggested that the overall amount of tissue injury was comparable between the stretched and non-stretched groups. This was further supported by cell viability staining of one pair of samples incubated with and without stretch, which showed a similar proportion of live and dead cells in both samples at day 3 (Fig. 3B,C): mean ± SE percent live cells per low power field (20×) were

54.2 ± 3.6% and 65.3 ± 3.6% in non-stretched and stretched samples, respectively (based on 500–600 cells counted for each sample).

### In vivo microinjury model

Masson trichrome staining of tissue histological sections obtained 7 days post-injury showed increased collagen density within the injured subcutaneous tissue layer (Fig. 4B) compared with the contraleral non-injured side (Fig. 4A).

Immunohistochemical staining of the subcutaneous tissue layer for Type-1 procollagen showed a uniform increase in Type-1 procollagen, a marker for newly formed collagen, throughout the fibroblast tissue population on injured side (Fig. 4D) compared with the non-injured side (Fig. 4C). Among the 21 mice randomized to stretch and no stretch (Fig. 5A), microinjury resulted in a significant increase in Type-1 procollagen in the absence of stretch ($F_{1,19} = 16.2$, $P < 0.001$). However, when injury was followed by tissue stretch, no significant difference between microinjury and no microinjury was found ($F_{1,19} = 1.65$, $P = 0.21$). Thus, tissue stretch attenuated the increase in Type-1 procollagen in response to injury (Fig. 5B,C).

## Discussion

In this study, two complementary approaches are employed to examine the effect of brief tissue stretch on TGF-β1 and connective tissue matrix remodeling. First, stretching mouse subcutaneous tissue explants by 20% for 10 min decreases soluble TGF-β1 levels measured 3 days after stretch. During the 4-day incubation, TGF-β1 levels in the culture media increase in both stretched and non-stretched samples; because some tissue trauma occurs at the time of excision, this progressive rise in TGF-β1 is consistent with an injury response. However, the increase in the level of TGF-β1 is slower in the samples that are briefly stretched for 10 min, compared with samples that are not stretched. Since TGF-β1 auto-induction is an important mechanism driving the increase in collagen synthesis following tissue injury (Cutroneo, 2003), we hypothesized that brief stretching of tissue following injury in vivo would decrease soluble TGF-β1 levels, attenuate TGF-β1 auto-induction and decrease new collagen deposition. Testing this hypothesis in a mouse subcutaneous tissue injury model showed that elongating the tissues of the trunk by 20–30% for 10 min twice a day significantly reduces the amount of subcutaneous new collagen 7 days following subcutaneous tissue injury.

The data presented in this paper support the long-standing, but poorly understood, physical therapy practice of using brief, judiciously applied stretching of tissues to treat excessive scarring, connective tissue adhesions, and contractures (Hardy, 1989; Cummings and Tillman, 1992). To date, studies of cultured cells and tissues examining the effect of mechanical forces on TGF-β1 and matrix remodeling have nearly exclusively employed prolonged (hours to days) cyclical or static stretch of low amplitude (less than 15% strain) (Stauber et al., 1996; Zhuang et al., 1996; Hirakata et al., 1997; Gutierrez and Perr, 1999; Lee et al., 1999; Hinz et al., 2001; Kessler et al., 2001; Grinnell and Ho, 2002; van Wamel et al., 2002; Atance et al., 2004; Sakata et al., 2004; Loesberg et al., 2005; Balachandran et al., 2006; Balestrini and Billiar, 2006;). Under normal physiological circumstances, some amount of static tissue tension or “prestress” is present to varying degrees in different parts of the body while prolonged, repetitive cyclical stretching of tissues occurs due breathing, arterial pulsations, and repetitive movements such as walking (Tomasek et al., 2002; Silver et al., 2003; Ingber, 2006). During wound healing or chronic pathological conditions such as fibrosis, tissue tension can slowly increase over days to weeks due to the contractile activity of myofibroblasts (Stopak and Harris, 1982; Tomasek et al., 1992; Arora et al., 1999; Grinnell, 2000; Hinz et al., 2001). It is generally accepted that, when applied over an

extended time period in the presence of TGFβ, low amplitude static or cyclical stretch causes an increase in the synthesis and deposition of collagen (Leung et al., 1976; Lambert et al., 1992; Chiquet, 1999; Kessler et al., 2001; Grinnell and Ho, 2002; Atance et al., 2004; Balestrini and Billiar, 2006). Our results, in contrast, show that brief, moderate amplitude (20–30% strain) stretching of connective tissue decreases both TGF-β1 and collagen synthesis. This effect, opposite to that of prolonged stretch, highlights the critical importance of "dose" (i.e., duration, amplitude, frequency) in mechanically induced connective tissue remodeling.

Pharmacological approaches to inhibiting excessive collagen deposition and fibrosis have been evaluated in various studies aiming to decrease collagen production either directly using double stranded oligodeoxynucleotide decoys (Cutroneo and Ehrlich, 2006), or indirectly by inhibition of TGF-β1 using antisense oligonucleotides (Han et al., 2000; Isaka et al., 2000), TGF-β1 latency-associated peptide (Bottinger et al., 1996), Smad7 (Kanamaru et al., 2001; Roberts, 2002) or thrombospondin-1 (Yevdokimova et al., 2001). However, drug or gene therapies bring a new set of challenges and potential side effects (Lahn et al., 2005). Fully understanding the dose-related effects of mechanical stretch on TGF-β1 and collagen would encourage the development of therapies based on measured amounts of stretch that may decrease fibrosis at low cost, without pharmacological side effects, or risking blocking TGF-β1 protein completely, as it is necessary for normal development and cellular function.

Several possible mechanisms may contribute to decreasing released soluble TGF-β1 following tissue stretch. Our TGF-β1 assay measures soluble (non-matrix bound) latent and active TGF-β1, but not soluble pro-TGF-β1 (since proteolytic cleavage of the bonds between the TGF-β and its latency-associated protein is necessary for acid activation) (Annes et al., 2003). In the tissue explant model, TGF-β1 levels in the culture media reflected its combined rates of (1) mRNA expression, protein synthesis and secretion, (2) extracellular sequestering via binding of large latent TGF-β binding protein to the matrix, (3) cleavage and activation of latent TGF-β complexes by tissue enzymes (metalloproteinases and plasmin), (4) binding of activated TGF-β by cell membrane receptors and (5) breakdown of free activated TGF-β (Shi and Massague, 2003; Stamenkovic, 2003; Annes et al., 2004; Rifkin, 2005). The observed effect of tissue stretch on TGF-β1 levels in this study therefore may involve one or more of these mechanisms. We recently reported that tissue stretch causes dynamic cytoskeletal reorganization in mouse subcutaneous tissue fibroblasts both ex vivo and in vivo (Langevin et al., 2005, 2006b). Because integrin-mediated TGF-β activity is cytoskeletal-dependent (Munger et al., 1999), an interesting possibility is that stretch-induced fibroblast cytoskeletal reorganization may stabilize the latent TGF-β binding protein, preventing release of soluble TGF-β from the cell's surface. Studies are currently underway to investigate this potential mechanism.

The results of this study suggest that stretch-induced decrease in TGF-β1-mediated new collagen formation may be an important natural mechanism limiting excessive scarring and fibrosis following injury (Fig. 6). Reducing scar and adhesion formation using stretch and mobilization is especially important for internal tissue injuries and inflammation involving fascia and organs, as opposed to open wounds. For open wounds (including surgical incisions) and severe internal tears (such as a ruptured ligament or tendon), wound closure and strength are critical and thus a certain amount of scarring is necessary and inevitable. In the case of minor sprains and repetitive motion injuries, however, scarring is mostly detrimental since it can contribute to maintaining the chronicity of tissue stiffness, abnormal movement patterns, and pain (Langevin and Sherman, 2007). We have proposed that therapies that briefly stretch tissues beyond the habitual range of motion (physical therapy, massage, yoga, acupuncture) locally inhibit new collagen formation for several days after

stretch and thus prevent and/or ameliorate soft tissue adhesions (Langevin et al., 2001,2002,2005,2006a,2007). Brief stretching of lung tissue beyond tidal volume during periodic sighing also may decrease fibrosis following lung injury, although to our knowledge this has not been studied. The model shown in Figure 6 can be further tested in other experimental injury models examining changes in collagen and signaling molecules downstream of TGF-β1 such as SMADS, connective tissue derived growth factor (CTGF) and plasminogen activation inhibitor (PAI-1) as well as cell and matrix constituents influenced by TGF-β1 such as α-smooth muscle actin and fibronectins.

In summary, our results show that brief, 20-30% tissue stretch can attenuate the increase in both soluble TGF-β1 (ex vivo) and Type-1 procollagen (in vivo) following tissue injury. This decreased fibrogenic response with brief, moderate amplitude stretch is in marked contrast to the well-established increase in TGF-β1 and collagen following prolonged low amplitude mechanical stimulation. The amount, timing and duration of therapeutically applied stretch therefore is likely critical to obtain beneficial anti-fibrotic effects.

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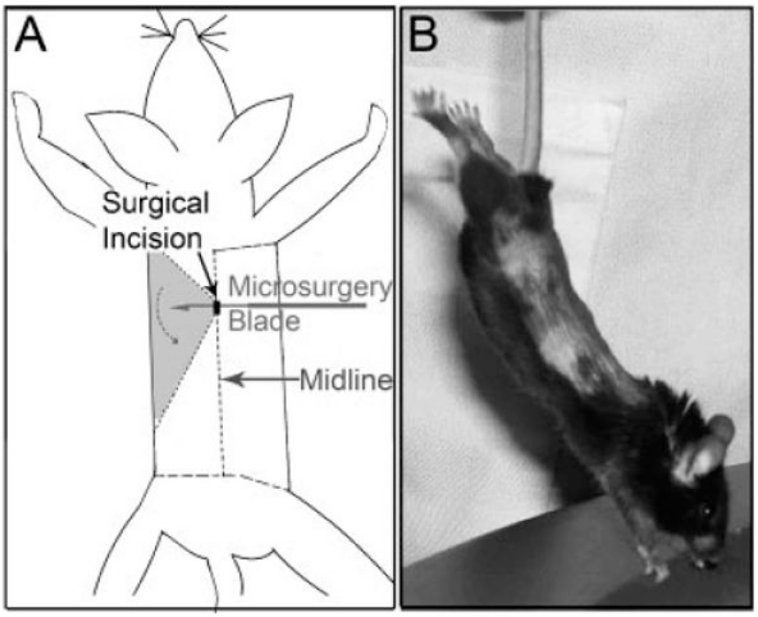


**Fig. 1.**

A: In vivo mouse microinjury procedure. A microsurgery blade is introduced subcutaneously via a 5 mm midline incision. The subcutaneous tissue layer is cut over a 1.5 cm × 1.5 cm area on one side of the back lateral to the midline with the other side serving as the control. B: Method used to induce tissue stretch in vivo. Mice are suspended by the tail such that their paws barely touch a surface slightly inclined relative to the vertical. The mice spontaneously extend their front and hind limbs, the distance between ipsilateral hip and shoulder joints becoming 20–30% greater than the resting distance.

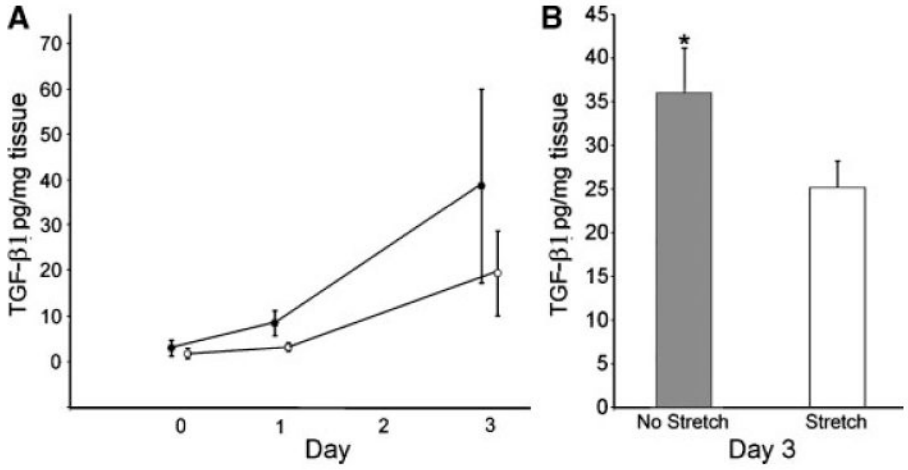


**Fig. 2.**

Effect of tissue stretch on TGF-β1 protein ex vivo. A: Time course of TGF-β1 protein levels in the culture media for non-stretched (closed circle, N = 4) and stretched (open circle, N = 4) mouse subcutaneous tissue explants on days 0, 1, and 3 post-stretch (or no stretch). All tissue samples were excised and incubated for 24h prior to day 0.B:Levels of TGF-β1 protein in the culture media at day 3 for non-stretched and stretched sbcutaneous tissue samples (N = 36). Asterisk (*) indicates significant difference from stretched ($P = 0.002$). Error bars represent standard errors.

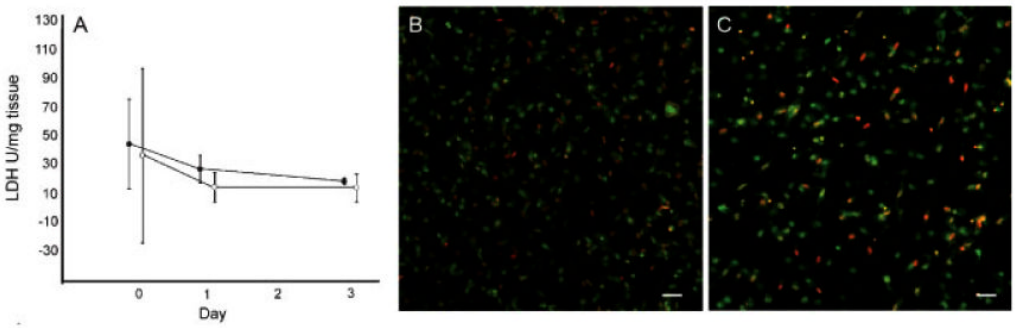


**Fig. 3.**

Ex vivo tissue injury and cell viability assessment. A: Time course of LDH concentration in the culture media (marker of cell death) for non-stretched (closed circle, n = 4) and stretched (open circle, n = 4) mouse subcutaneous tissue explants on days 0, 1, and 3 post-stretch (or no stretch). B,C: Confocal microscopy imaging of mouse subcutaneous tissue explants showing similar proportions of live (green) and dead (red) cells in non-stretched (A) versus stretched (B) tissue after 3 day incubation post-stretch (or no stretch). Images are projections of three-dimensional image stacks. Scale bars: 40 μm.

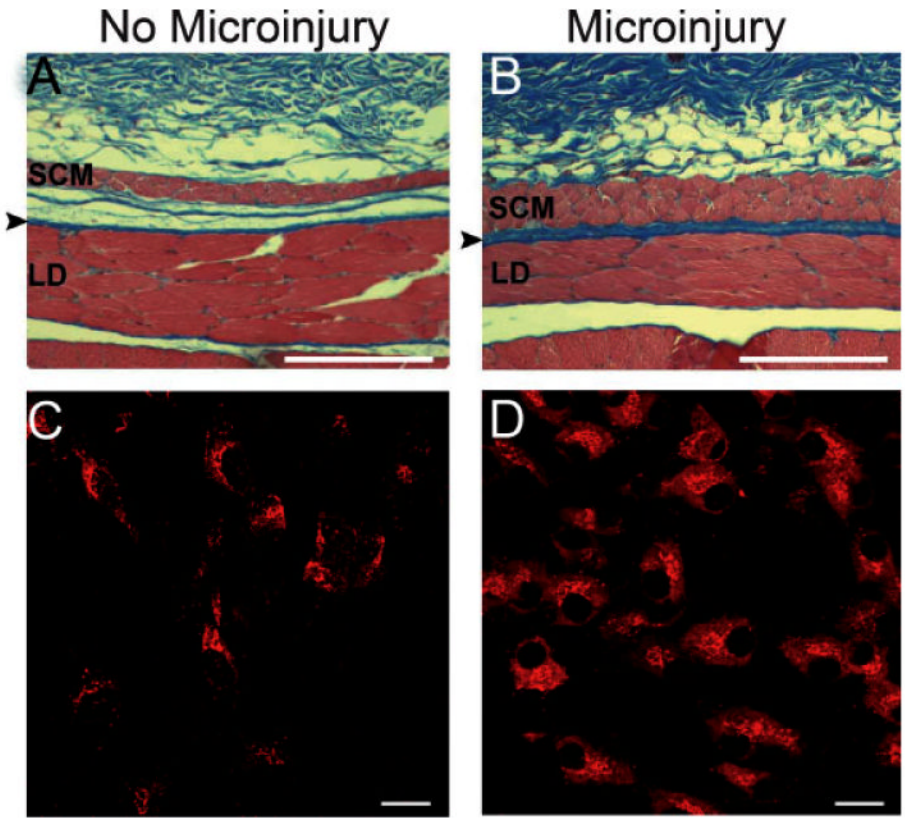


**Fig. 4.**

Mouse in vivo microinjury model. Effect of microinjury on mouse subcutaneous tissue newly formed collagen. A,B: Masson Trichrome (stains collagen blue) of paraffin-embedded histological sections cut perpendicular to the skin; arrowhead indicates subcutaneous tissue located between the subcutaneous muscle (SCM) and latissimus dorsi (LD) muscle. C,D: Immunohistochemical staining for Type-1 procollagen (marker of newly formed collagen) of dissected whole subcutaneous tissue mounts. Scale bars: 1 mm (A,B) and 40 μm (C,D).

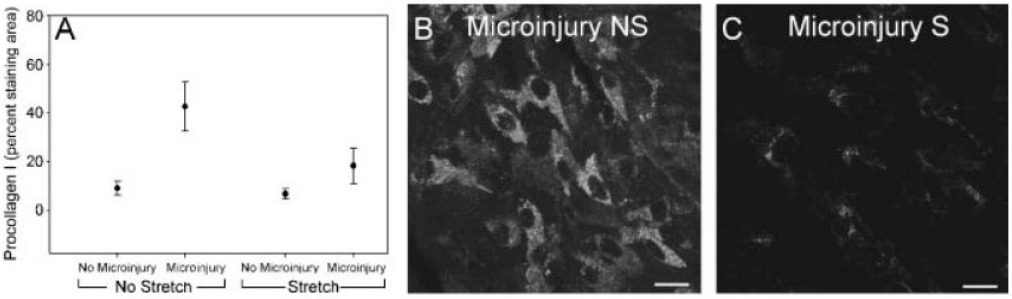


**Fig. 5.**

Effect of tissue stretch in vivo on subcutaneous tissue Type-1 procollagen in mouse microinjury model. A: Mean ± SE procollagen percent staining area in non-injured versus injured sides, without stretch (N = 11) and with stretch (N = 10); B,C: Type-1 procollagen in non-stretched and stretched tissue (both injured). Scale bars, 40 μm.

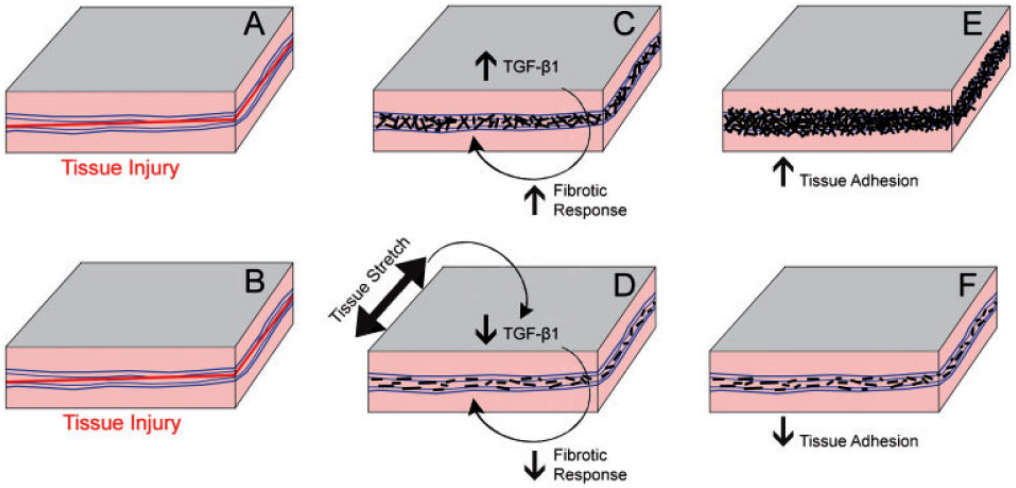


**Fig. 6.**

Proposed model for healing of connective tissue injury in the absence (A,C,E) and presence (B,D,F) of tissue stretch. In this model, brief stretching of tissue beyond the habitual range of motion reduces soluble TGF-β1 levels (D) causing a decrease in the fibrotic response, less collagen deposition, and reduced tissue adhesion (F) compared with no stretch (E). Black lines represent newly formed collagen.