# Chapter 4
# Cyclic mechanical stretch reduces myofibroblast differentiation of primary lung fibroblasts

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**Abstract**

In lung fibrosis tissue architecture and function is severely hampered by myofibroblasts due to excessive deposition of extracellular matrix and tissue contraction. Myofibroblasts differentiate from fibroblasts under the influence of transforming growth factor (TGF)$\beta_1$ but this process is also controlled mechanically by cytoskeletal tension. In healthy lungs, the cytoskeleton of fibroblasts is mechanically strained during breathing. In stiffer fibrotic lung tissue, this mechanical stimulus is reduced, which may influence fibroblast-to-myofibroblast differentiation. Therefore, we investigated the effect of cyclic mechanical stretch on fibroblast-to-myofibroblast differentiation.

Primary normal human lung fibroblasts were grown on BioFlex culture plates and stimulated to undergo myofibroblast differentiation by 10 ng/ml TGF$\beta_1$. Cells were either or not subjected to cyclic mechanical stretch (sinusoidal pattern, maximum elongation 10%, 0.2 Hz) for a period of 24 h on a Flexercell apparatus. mRNA expression was analyzed by real-time PCR.

Cyclic mechanical loading reduced the mRNA expression of the myofibroblast marker α-smooth muscle actin and the extracellular matrix proteins type I, type III, and type V collagen, and tenascin C. These outcomes indicate that fibroblast-to-myofibroblast differentiation is reduced. Cyclic mechanical loading did not change the expression of the fibronectin ED-A splice variant, but did decrease the paracrine expression of TGF$\beta_1$, thereby suggesting a possible regulation mechanism for the observed effects. The data suggest that cyclic loading experienced by healthy lung cells during breathing may prevent fibroblasts from differentiating towards myofibroblasts.

**Introduction**

Fibrosis is a process of deregulated wound healing after inflammation or other injury, resulting in the formation of excessive extracellular matrix (ECM) and tissue contraction. The disease progresses rapidly and can occur in many different tissues. Due to the destruction of tissue architecture, it results in severe tissue dysfunction and, depending on the tissue, may lead to death. One of the most fatal forms is lung fibrosis, which has an incidence of 5 to 10 per 100,000 and is often fatal within 3 to 5 years after diagnosis (Selman et al., 2004).

The exact cause of lung fibrosis is still mostly unknown but almost certainly multifactoral. So far, there is no effective treatment. Recent investigation of the cellular processes underlying the formation of fibrotic tissue has shown that, after minor damage to the lung tissue, fibroblasts inside the parenchymal tissue of the lung differentiate into myofibroblasts (Tomasek et al., 2002). These cells are specialized in wound healing and produce large amounts of extracellular matrix proteins, such as collagens, tenascin C and the ED-A splice variant of fibronectin. They also express α-smooth muscle actin (αSMA), which enables the cells to strongly contract the wound during wound healing. After tissue repair and once the balance of remodeling is restored in the repaired tissue the myofibroblasts undergo apoptosis. However, in fibrosis, myofibroblasts stay active after the wound has healed, creating excessive, strongly contracted extracellular matrix.

Fibroblast-to-myofibroblast differentiation is regulated by both chemical and mechanical factors (Tomasek et al., 2002). Chemically, transforming growth factor (TGF)$\beta_1$ is the major molecule stimulating myofibroblast differentiation. It is present in a latent complex in the ECM, from which it can be released to locally induce myofibroblast differentiation. TGF$\beta_1$ can be released either by proteases (Ge and Greenspan, 2006; Mu et al., 2002; Yu and Stamenkovic, 2000) or through mechanical tension in the tissue (Wipff et al., 2007).

Besides playing a role in latent TGF$\beta_1$ release, mechanical tension also directly influences myofibroblast differentiation: in splinted wounds, the static tension in the tissue results in more ED-A fibronectin and αSMA expression, indicating more myofibroblast differentiation (Hinz et al., 2001). However, in lung tissue, the breathing cycle will result in external loads on the lung cells of a cyclic nature. Because cells will have trouble adapting to the constantly changing tensional status of external cyclic loading (Brown et al., 1998), this cyclic loading could prevent the build-up of internal cytoskeletal tension and thus myofibroblast differentiation. Therefore, we aimed to establish whether cyclic mechanical stretch regulates the differentiation of lung fibroblasts into myofibroblasts. To study this, we investigated the effect of cyclic mechanical stretch on the myofibroblast marker αSMA, and extracellular matrix proteins such as collagens, tenascin C and the ED-A splice variant of fibronectin, which has been shown to be essential for fibrosis (Muro et al., 2008).

**Experimental procedures**

*Cell Culture*

Primary normal human lung fibroblast cells were obtained from Lonza Walkersville, Inc. (Walkersville, MD, USA). Cells were cultured in Dulbecco's minimal essential medium (D-MEM; Invitrogen, Paisley, UK) supplemented with 10% fetal clone serum (FCS; HyClone, South Logan, UT, USA) and 1% antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (PSA), Sigma-Aldrich, St. Louis, MO, USA) in an incubator set at 37 °C, 95% humidity, and 5% $CO_2$. Once grown to confluency, cells were trypsinized using 0.5% trypsin (Sigma-Aldrich) and 0.1% EDTA (Merck, Darmstadt, Germany) in PBS (Invitrogen).

For each experiment, cells from passage 6 to 10 were seeded at a density of 3,000 or 10,000 cells/cm$^2$ on BioFlex type I collagen coated six-well culture plates (BioFlex, Flexcell International Corp, McKeesport, PA, USA). After allowing the cells to attach for 24 h, medium was replaced by D-MEM with 1% FCS and 1% PSA and 24 h later myofibroblast differentiation was induced in 50% of the samples by replacing the medium with D-MEM supplemented with 1% FCS and 1% PSA containing 2-20 ng/ml recombinant human $TGF\beta_1$ (PeproTech EC, London, UK). Fibroblasts were cultured for 4 to 72 h in the presence or absence of $TGF\beta_1$.

*Cyclic Mechanical Loading*

To assess the effect of cyclic mechanical loading on the expression of fibrosis-related genes, fibroblasts cells were subjected to 24 or 48 h of cyclic mechanical strain in a Flexercell FX4000 apparatus (Flexcell International Corp) placed in an incubator set at 37°C, 95% humidity and 5% $CO_2$. Cyclic mechanical loading was applied in a sinusoidal pattern with a frequency of 0.2 Hz and a maximum elongation of 10%. Control cells not receiving cyclic mechanical loading were placed in the same incubator next to the loading station of the Flexercell apparatus. During mechanical loading, 50% of the samples were stimulated with 10 ng/ml $TGF\beta_1$. After mechanical loading, cell viability was checked with a WST-1 assay (Roche Diagnostics, Mannheim, Germany) and mRNA samples were collected.

*Quantitative Real-Time PCR*

mRNA was isolated using an RNeasy Mini Kit for RNA extraction (Qiagen, Hilden, Germany). The mRNA concentration was measured using a Nanodrop spectrophotometer (NanoDrop Technologies; Thermo-Fischer Scientific, Wilmington, DE, USA). mRNA was reverse-transcribed to complementary DNA (cDNA) using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). mRNA expression of COL1A1, COL1A2, COL3A1, COL5A2, PLOD2, αSMA, tenascin C (TNC), $TGF\beta_1$, $TGF\beta_2$, $TGF\beta_3$, fibronectin, and fibronectin ED-A and the housekeeping gene GAPDH was analyzed by Real-Time PCR performed on a 7500 Fast

Real-Time PCR system (Applied Biosystems). GAPDH mRNA expression was determined using TaqMan® Rodent GAPDH Control Reagents (Applied Biosystems). Fibronectin and its ED-A splice variant were analyzed using custom designed primers (Applied Biosystems) in combination with SYBR green (Invitrogen). All other genes were analyzed using unique TaqMan® Assays-on-Demand™ Gene Expression kits (Table 1; Applied Biosystems) specific for human.

**Table 1:   Gene product, Gene, Entrez Gene ID, primers, and Assay-on-Demand™ used in this study.**

| Gene product | Gene | Gene ID | Forward Primer | Reverse Primer |
|---|---|---|---|---|
| fibronectin | FN1 | 2335 | CAggATCACTTACggAgAgACAg | gCCAgTgACAgCATACACAgTg |
| fibronectin ED-A | FN1 | 2335 | CCCTAAAggACTggCATTCA | CATCCTCAgggCTCgAgTAg |

| Gene product | Gene | Gene ID | Assay-on-Demand™ |
|---|---|---|---|
| $\alpha_1$ chain type I collagen | COL1A1 | NM_000088.3 | Hs01076777_m1 |
| $\alpha_2$ chain type I collagen | COL1A2 | NM_000089.3 | Hs01028956_m1 |
| $\alpha_1$ chain type III collagen | COL3A1 | NM_000090.3 | Hs00164103_m1 |
| $\alpha_2$ chain type V collagen | COL5A2 | NM_000393.3 | Hs00169768_m1 |
| lysyl hydroxylase 2 | PLOD2 | NM_000935.2 | Hs01118190_m1 |
| $\alpha$-smooth muscle actin | ACTA2 | NM_001613.2 | Hs00426835_g1 |
| tenascin C | TNC | NM_002160.2 | Hs01115665_m1 |
| $TGF\beta_1$ | TGFB1 | NM_000660.4 | Hs99999918_m1 |
| $TGF\beta_2$ | TGFB2 | NM_003238.3 | Hs00234244_m1 |
| $TGF\beta_3$ | TGFB3 | NM_003239.2 | Hs00234245_m1 |

*Statistics*

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA). Groups were compared using ANOVAs with Bonferroni-adjusted t-tests as post hoc tests.

## Results

*TGFβ1 increases myofibroblast differentiation*

Culturing lung fibroblasts for 24 h with different concentrations of TGFβ1 resulted in an induction of myofibroblast differentiation at all concentrations studied (0, 2, 5, 10, and 20 ng/ml) as indicated by an increase in mRNA expression of COL1A1 and αSMA (data not shown). Time-line experiments showed that COL1A1 mRNA expression was already increased after 4 h of TGFβ1 stimulation, whereas the mRNA expression of αSMA was increased from 24 h on (Figure 1A and 1B).
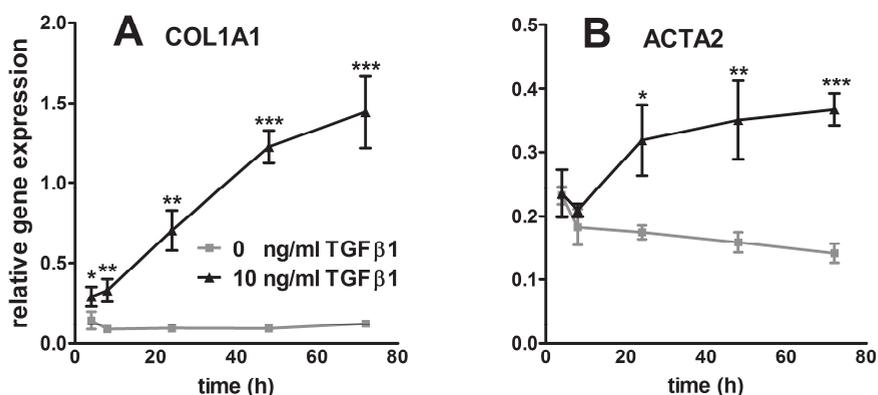


**Figure 1: TGFβ1 increases mRNA expression of the α1 chain of type I collagen and α-smooth muscle actin.**

Stimulation of normal human lung fibroblasts with application of 10 ng/ml transforming growth factor (TGF)β1 (▲) in de medium significantly increases the expression of (A) the α1 chain of type I collagen (COL1A1) and (B) α-smooth muscle actin (αSMA) at different time points (4, 8, 24, 48, and 72 h), compared to no TGFβ1 stimulation (■). Gene expression is shown relative to mRNA expression of the household gene GAPDH. Data are shown as mean ± SD (n = 3). Significant differences with 0 ng/ml TGFβ1 are indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

*Cyclic mechanical loading reduces myofibroblast differentiation*

Cyclic mechanical loading decreased mRNA expression of COL1A1, COL1A2, COL3A1, COL5A2, and tenascin C (TNC), in both TGFβ1 stimulated and non-stimulated cells (Figure 2). The effect of cyclic mechanical loading was most pronounced for COL1A1. mRNA expression of the myofibroblast marker αSMA was also lower after cyclic mechanical loading. mRNA expression of PLOD2, an enzyme involved in collagen cross-linking, was not influenced by cyclic mechanical loading. The WST-1 assay showed no cell loss after 48 h of loading (data not shown). Taken together, these data indicate that cyclic mechanical loading reduces TGFβ1-induced myofibroblast differentiation.
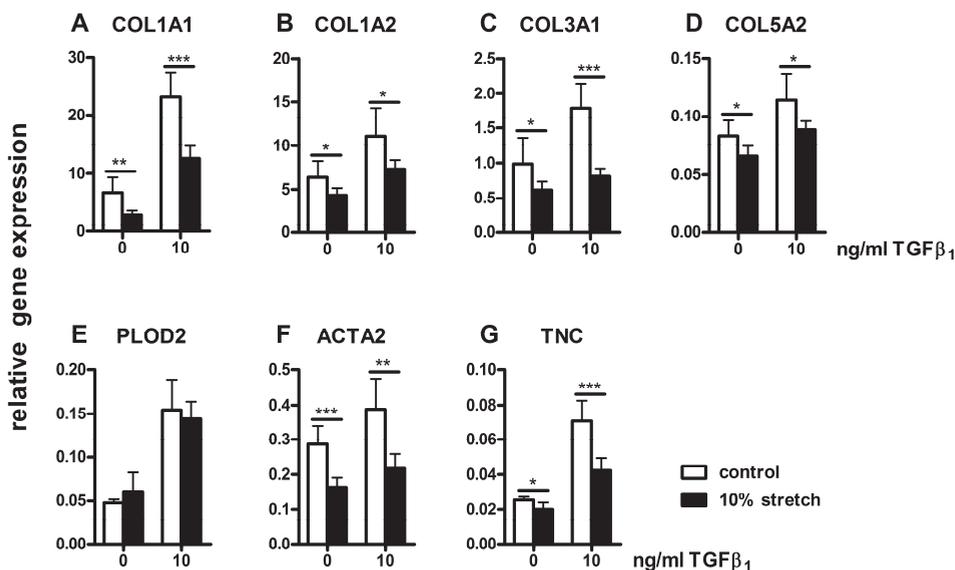
**Figure 2: Cyclic mechanical loading reduces myofibroblast differentiation**
Stimulating normal human lung fibroblasts for 48 h with cyclic mechanical loading reduces mRNA expression of COL1A1, COL1A2, COL3A1, COL5A2, α-smooth muscle actin (ACTA2) and tenascin C (TNC), independent of 10 ng/ml TGFβ$_1$ stimulation. mRNA expression of PLOD2, encoding for an enzyme important for collagen cross-linking, is not influenced by cyclic mechanical loading. Gene expression is shown relative to mRNA expression of the household gene GAPDH. Data are from one representative experiment out of three and shown as mean ± SD (n = 6). Significant differences are indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

*Cyclic mechanical loading does not influence fibronectin ED-A mRNA expression*
Both normal fibronectin (from 4 h on) and the ED-A splice variant (from 24 h on) mRNA levels were higher when cells were stimulated with TGFβ$_1$ (Figure 3A and B). However, these two fibronectin forms were not significantly reduced after 48 h of cyclic mechanical loading (Figure 3C and D).

*Cyclic mechanical loading reduces endogenous TGFβ$_1$ mRNA expression*
Since TGFβ$_1$-induced myofibroblast differentiation was repressed by cyclic mechanical loading, we examined whether this could be explained by a loading-induced change in autocrine TGFβ$_1$ stimulation. We first analyzed the mRNA expression of the different isoforms of TGFβ and the effect of exogenous TGFβ$_1$ hereupon. TGFβ$_1$, TGFβ$_2$, and TGFβ$_3$ mRNA expression was measured after 4, 8, 24, 48 or 72 h of culturing in the presence or absence of 10 ng/ml TGFβ$_1$ (Figure 4A, B, and C). Both TGFβ$_1$ and TGFβ$_2$ mRNA expression were higher after stimulation with TGFβ$_1$, whereas the expression of TGFβ$_3$ was lower in the

presence of TGFβ$_1$. Cyclic mechanical stretch lowered the expression of TGFβ$_1$ when cells were cultured with exogenous TGFβ$_1$ (Figure 4D). mRNA expression of TGFβ$_3$ decreased also under stretch conditions (Figure 4E).
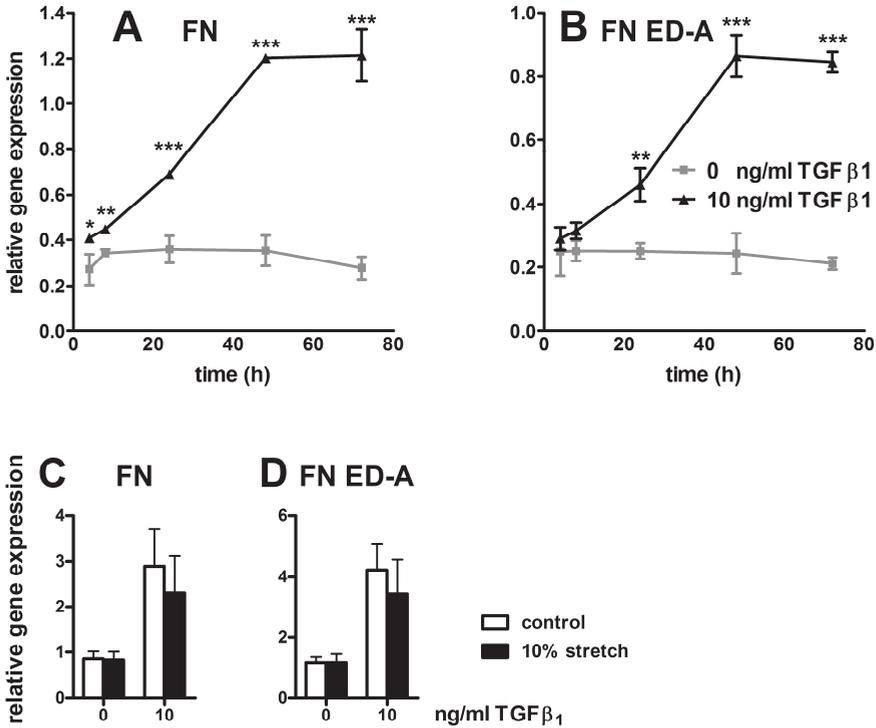


**Figure 3: TGFβ$_1$ stimulation but not cyclic mechanical loading influences the expression of fibronectin and its ED-A splice variant in fibroblasts.**

Stimulation of normal human lung fibroblasts with application of 10 ng/ml transforming growth factor (TGF)β$_1$ (▲) in de medium significantly increases the expression of (A) fibronectin (FN) and (B) the ED-A splice variant of fibronectin (FN ED-A) at different time points (4, 8, 24, 48, and 72 h), compared to no TGFβ$_1$ stimulation (■). Cyclic mechanical strain (48 h) does not change (C) fibronectin or (D) fibronectin ED-A expression. Gene expression is shown relative to mRNA expression of the household gene GAPDH. Data are from one representative experiment out of three and shown as mean ± SD (n = 3 or 6). Significant differences between 0 ng/ml and 10 ng/ml are indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
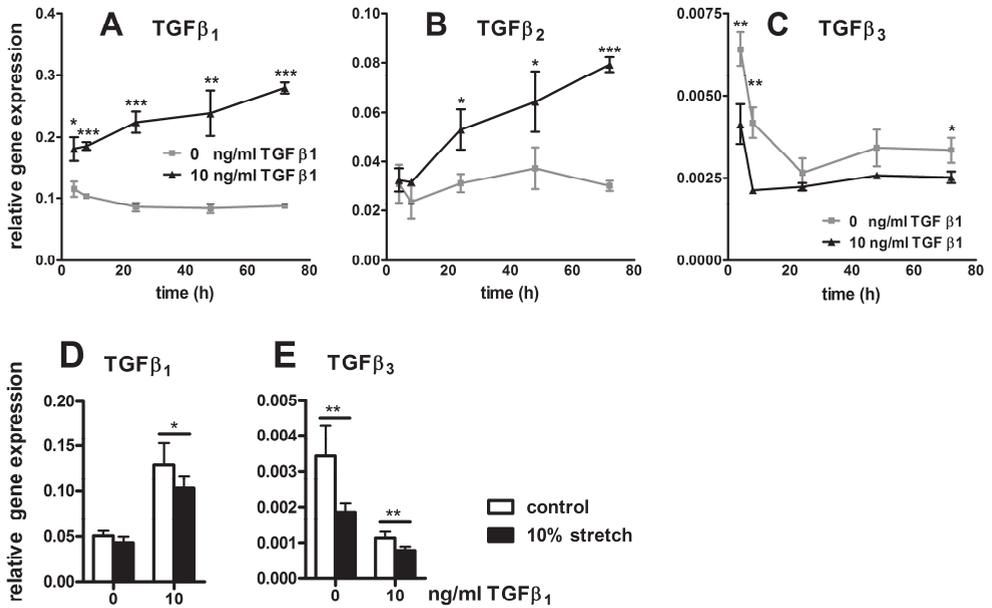
**Figure 4: TGFβ₁ stimulation and cyclic mechanical loading influence the expression of TGFβs in fibroblasts.**

Stimulation of normal human lung fibroblasts with application of 10 ng/ml transforming growth factor (TGF)β₁ (▲) in de medium significantly increases the expression of (A) TGFβ₁ and (B) TGFβ₂ and reduces the expression of (C) TGFβ₃ at different time points (4, 8, 24, 48, and 72 h), compared to no TGFβ₁ stimulation (■). Cyclic mechanical strain (48 h) reduces (D) TGFβ₁ expression only in the presence of external TGFβ₁ and only slightly. (E) TGFβ₃ expression is reduced by 48 h of cyclic mechanical strain both in the absence and presence of external TGFβ₁. Gene expression is shown relative to mRNA expression of the household gene GAPDH. Data are from one representative experiment out of three and shown as mean ± SD (n = 3 or 6). Significant differences between 0 ng/ml and 10 ng/ml are indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

**Discussion**

In this study, we aimed to establish the effect of cyclic mechanical loading on myofibroblast differentiation in lung fibroblasts. We show that application of 48 h of cyclic mechanical loading to primary human lung fibroblasts reduces the expression of fibrosis-related genes. Our findings thus indicate a reduced myofibroblast differentiation under these conditions. Although cyclic mechanical loading did not affect fibronectin ED-A mRNA expression, it did reduce endogenous expression of $TGF\beta_1$. The reduced myofibroblast differentiation may thus be the consequence of a reduced autocrine $TGF\beta_1$ stimulation.

In our experiments, lung fibroblasts were subjected to a cyclic mechanical loading regime consisting of a sinusoidal wave-form with a frequency of 0.2 Hz and amplitude of 10% elongation. The frequency was chosen to represent a normal breathing cycle that lasts 5 seconds. The chosen elongation lies within the elongation range of the epithelial basement membrane measured during breathing at 40 to 100% of the total lung capacity (Tschumperlin and Margulies, 1999). We therefore believe that this cyclic mechanical loading regime represents the loading that lung fibroblasts undergo in healthy tissue *in vivo*.

To explore a possible mechanism for the intriguing finding that myofibroblast differentiation reduced under stretching conditions, we tested whether fibronectin ED-A mRNA expression was influenced in our experiments. Since fibronectin ED-A has been shown to be essential for myofibroblast differentiation (Muro et al., 2008), a reduction of fibronectin ED-A caused by cyclic mechanical loading could be a mechanism. However, mRNA expression of fibronectin ED-A proved to be unaffected under cyclic mechanical stretch conditions (see Figure 3D), thus making it very unlikely to play a role in the reduced differentiation.

A second mechanism that could explain a reduced myofibroblast differentiation after mechanical stretch is related to the level of $TGF\beta$ in the cell culture system. Next to exogenously added growth factor the cells have the capacity to express endogenous $TGF\beta$. Here, it was found that lung fibroblasts indeed express higher levels of $TGF\beta_1$ and $TGF\beta_2$ mRNA in response to exogenous $TGF\beta_1$, suggesting a positive autocrine feedback mechanism. The expression of $TGF\beta_3$ was found to be lower after stimulation by exogenous $TGF\beta_1$, which is in line with the suggestion that $TGF\beta_3$ can act as an antagonist of $TGF\beta_1$ (Shah et al., 1995). We then explored the possibility that cyclic mechanical stretch affects this positive autocrine feedback by reducing $TGF\beta_1$ expression. After cyclic mechanical stretch, the mRNA expression of $TGF\beta_1$ was indeed found to be lower (see Figure 4D). These findings appear to provide an explanation for the reduced myofibroblast differentiation after cyclic mechanical loading. Surprisingly, the mRNA expression of $TGF\beta_3$ is also lower after cyclic mechanical loading. This contradicts the earlier mentioned alleged antagonistic effect of $TGF\beta_3$.

Although we found a consistent reduction in expression of myofibroblast-related genes after cyclic mechanical loading, others observed that expression of collagens (Howard et al., 1998; Kim et al., 2002) or tenascin C (Chiquet et al., 2004; Lutz et al., 2010; Sarasa-Renedo et al., 2006) was higher after fibroblasts were subjected to a comparable loading regime. Several differences in experimental set-up could have led to this discrepancy. In our experiments we tried to optimally mimic the *in vivo* conditions for our lung fibroblasts by culturing them on type I collagen coated membranes, an extracellular matrix protein abundantly expressed in the lung connective tissue, and applying a loading regime mimicking breathing. The other studies have used fibroblasts from different origin, a shorter loading protocol, and a different coating on the membranes. Such a different coating is recognized by different integrins (Breen, 2000; van der Flier and Sonnenberg, 2001). Since integrins are known to play an important role in mechanotransduction, this could be an important factor influencing the effect of mechanical loading on fibroblastic cells.

Our results show that cyclic mechanical loading resulted in a lower expression of myofibroblast-related genes, such as αSMA, collagens, and tenascin C. During the development of lung fibrosis *in vivo*, tissue stiffens and fibroblasts experience probably less cyclic mechanical loading during the breathing cycle. In light of our findings we hypothesize that this facilitates fibroblast-to-myofibroblast differentiation, resulting in a positive feedback loop with the devastating disease of fibrosis as the end result.

In summary, cyclic mechanical stretch reduces fibroblast-to-myofibroblast differentiation of primary lung fibroblasts. This may result from a decreased autocrine TGFβ$_1$ stimulation. Our findings suggest that tissue stiffening during fibrosis facilitates fibroblast-to-myofibroblast differentiation.

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