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Syndecan-4 Signaling via NFAT Regulates Extracellular Matrix Production and Cardiac Myofibroblast Differentiation in Response to Mechanical Stress

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Abstract

Pressure overload activates cardiac fibroblasts leading to excessive production of extracellular matrix which may contribute to compromised heart function. The activated fibroblast acquires smooth muscle-like features such as expression of smooth muscle α -actin (SMA) and SM22 and is therefore referred to as myofibroblast. The molecular mechanisms underlying mechanical stress-induced myofibroblast differentiation are poorly defined. The objective of this study was to examine the potential roles of the transmembrane proteoglycan syndecan-4 and the calcineurin-dependent transcription factor nuclear factor of activated T-cells (NFAT) in myofibroblast differentiation. Aortic banding resulted in elevated collagen I and III, fibronectin, SMA and SM22 mRNA in the left ventricles of wild-type mice, whereas this response was markedly reduced in syndecan-4^{-/-} mice. Myofibroblast differentiation *in vitro* was associated with increased SMA, collagen I and III expression and NFAT-luciferase activity, all of which were reduced in fibroblasts from syndecan-4^{-/-} mice or after treatment with calcineurin/NFAT blockers. Following cyclic stretch, NFATc4 was activated in cardiac fibroblasts in a syndecan-4- and calcineurin-dependent manner. Syndecan-4 and calcineurin co-localized and mechanical stress resulted in dephosphorylation of serine179 of syndecan-4, an intracellular residue critical for calcineurin interaction. Over-expression of NFATc4 up-regulated collagen III, MRTF-A (a transcriptional regulator of SMA) and the NFAT-target regulator of calcineurin 1.4 (RCAN1.4). Our data demonstrate that syndecan-4 is important for the differentiation of cardiac fibroblasts into myofibroblasts in the pressure-overloaded heart and that the calcineurin/NFAT pathway is engaged upon mechanical stress in a syndecan-4-dependent manner, playing an active role in myofibroblast differentiation and extracellular matrix production.

Keywords: mechanical stress; cardiac fibroblast; extracellular matrix; myofibroblast; syndecan-4; NFAT

1. Introduction

Extracellular matrix (ECM) remodeling is a key process in the development of heart failure following sustained periods of pressure overload. Cardiac fibroblasts are the main producers and regulators of ECM and are therefore important for maintaining the correct passive tension and elastic properties of the myocardium. In the pressure-overloaded heart, fibroblasts experience increased mechanical stress and start producing excessive amounts of ECM which may ultimately lead to fibrosis and reduced diastolic compliance [1]. These activated fibroblasts also acquire contractile features enabling them to “pull” on the ECM and thereby increase matrix tension. Hence, they are smooth muscle cell-like in terms of expressing markers defining smooth muscle cells, such as smooth muscle α -actin (SMA) [2] and SM22, but at the same time retain typical fibroblast features such as the ability to produce large amounts of ECM. Cells with this phenotype are therefore referred to as myofibroblasts. Even though, the fibroblast-myofibroblast phenotypes reflect a continuum of functional statuses and not one single cell type, we will throughout the paper use the term myofibroblast differentiation for the process involving up-regulation of ECM genes and *de novo* expression and organization of SMA fibers in cardiac fibroblasts. Originally described in the granulation tissue of skin wounds, differentiation of relatively quiescent fibroblasts to a more proliferative and contractile cell, the myofibroblasts, was found to be a necessary step in wound healing as myofibroblasts contribute to mechanical stability of the wound by forming a scar. Mechanical stress and several signaling molecules have been found to induce myofibroblast differentiation, the most well-known being TGF β [3]. It has also been established that mechanical stress induces the differentiation of cardiac fibroblasts into myofibroblasts [4]. However, the underlying mechanisms are largely unknown.

The existence of mechanosensing molecules in the plasma membrane of cardiac cells has long been subject to great interest. Such a mechanosensor is likely attached to the ECM as well as to the cytoskeleton intracellularly where it also interacts with downstream signaling molecules. One protein that has received considerable attention lately is syndecan-4 [5] due to its localization in focal adhesions which are the contact points between the cell surface and ECM, and recognized sites of

mechanotransduction and mechanosensing [6, 7]. Similarly to integrins, syndecan-4 binds to fibronectin and collagens via its glucosaminoglycan (GAG) chains and interact, either alone or in concert with integrins, with several intracellular signaling molecules such as focal adhesion kinase (FAK), phosphatidylinositol 4,5-biphosphate (PIP2), PKC α , CASK and RhoA [8]. These interactions are important for the assembly of focal adhesions, cell attachment and spreading [9]. Whereas, expression of syndecan-4 has been shown to be increased in the hypertrophic myocardium of mice after myocardial infarction [10] and in human aortic stenosis [11], lack of syndecan-4 in mice has been shown to disturb and delay wound healing [12]. Hence, syndecan-4 is a good candidate for being a mechanosensor in cardiac fibroblasts.

NFAT signaling is established as a pathological signaling pathway in the development of cardiac hypertrophy [13-16]. However, the role of syndecan-4 and NFAT signaling in cardiac fibroblasts has not been studied. Thus, the specific objective of this study was to examine if syndecan-4 signals via calcineurin/NFAT to regulate ECM production and myofibroblast differentiation of cardiac fibroblasts in response to mechanical stress.

2. Methods

A detailed methods section is included in the Online Supplement.

2.1 Mouse Model of Pressure Overload

Animals were handled according to the National Regulation on Animal Experimentation in accordance with the Norwegian Animal Welfare Act. The protocol was approved by the Norwegian National Animal Research Committee (permit of approval no. 2845). Initially, animals were anesthetized in a gas chamber containing 5% isoflurane and 95% oxygen where after the animals were intubated and ventilated with 2% isoflurane and 98% oxygen. Aortic banding (AB) was performed on eight week old male C57BL/6JBomTac mice (wild-type (WT); N=20) and syndecan-4^{-/-} mice [12] on a C57BL/6J background (N=20) by placing a ligature distally around the ascending aorta as previously described [11]. Sham-operated animals underwent the same procedure without tightening the suture around the aorta. For post-operative analgesia, buprenorphine was injected subcutaneously. Animals with an aortic flow velocity >4 m/s, corresponding to a pressure gradient of more than 8.5 kPa, 24 hrs after AB as determined with echocardiography were included in the study. Animals were anesthetized (gas chamber with 5% isoflurane and 95% oxygen), decapitated and lungs and hearts dissected and weighed. In a separate set of mice (N=10), water content of the hearts was determined by drying the hearts at 60°C for 24 hours and presented as relative amount of water ((wet weight-dry weight)/wet weight). Under non-stressed conditions, no phenotypic differences were observed between syndecan-4^{-/-} mice and WT mice.

2.2 Fibroblast Cell Culture

Neonatal (1-3 day old mice) mice were sacrificed by decapitation and left ventricles excised and cut into small pieces. Cardiac fibroblasts were freshly isolated as previously described [11]. Primary cardiac fibroblasts from neonatal NFAT-luciferase [17] and syndecan-4^{-/-}-NFAT-luciferase reporter mice [11] were used to determine NFAT activation and luminescence quantified according to the Luciferase Assay System protocol (E1500, Promega, Madison, WI). The human fibrosarcoma cell

line HT1080 was purchased from ATCC (CCL-121, Manassas, VA). Cyclic stretch was applied to HT1080 or primary cardiac fibroblast cultures on collagen I-coated 6-well bioflex culture dishes at 1 Hz for 24 hrs using the FlexCell Tension System FX-4000 (Dunn Labortechnik, Asbach, Germany). Cardiac and HT1080 fibroblasts were transfected with syndecan-4 or enhanced green fluorescent protein (EGFP)-fused NFATc1-4 using lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Cyclosporine A (CsA, 1 μ mol/L; 586107, SandImmune Neoral, Novartis, Basel, Switzerland) was used for calcineurin inhibition. The NFAT blocker A-285222 was kindly provided by Abbott Laboratories (1 μ mol/L; Abbott Park, IL).

2.5 Gene Expression Analysis

mRNA was extracted from left ventricles and cell cultures using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of mRNA into cDNA was performed using the iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA) and real-time PCR performed using predesigned TaqMan assays (Applied Biosystems, Foster City, CA). Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.6 Immunoblotting

Immunoblotting was performed as described previously [18]. Protein from cell cultures and from homogenized left ventricles was harvested in a PBS-buffer containing 1% Triton X-100 (Sigma, Schnelldorf, Germany), 0.1% Tween 20 (Sigma) and protease inhibitors. SDS-PAGE and immunoblotting was performed essentially as described in the Criterion protocol (BIO-RAD).

2.7 Proximity Ligation Assay

Proximity ligation assays were performed using the Duolink kit from Olink Bioscience (Uppsala, Sweden) and primary antibodies toward syndecan-4 (1:50, custom made, Genscript, and 3644, BioVision, Milpitas, CA) and calcineurin A (1:50, sc-17808 and sc-6124, Santa Cruz Biotechnology). Incubation with syndecan-4 blocking peptide (3644BP, BioVision) was used as a

negative control. Cells were fixed, permeabilized and incubated with the two primary antibodies overnight at 4°C and the proximity ligation assay performed according to the manufacturer's protocol and visualized using fluorescence microscopy.

2.8 Immunocytochemistry

Cells grown on fibronectin-coated glass cover slips were fixed and incubated with primary antibodies followed by incubation with alexa fluor 488- or 546-conjugated secondary antibodies (Invitrogen), alexa fluor 546-phalloidin (Invitrogen) or sytox orange (Invitrogen). Presence of well-organized SMA fibers was determined for each cell. Mean intensity of SMA was determined using the analysis software from Zeiss (LSM 710) and normalized to the number of cells in each micrograph.

2.9 Statistics

Data are expressed as means \pm S.E.M. Statistical analyses were performed using GraphPad software (Prism 5.04) and significance determined with one-way ANOVA for experiments with one intervention and two-way ANOVA with Bonferroni post-hoc testing for experiments with intervention in both WT and syndecan-4^{-/-} groups. Unpaired Student's *t*-test was used to test differences between two groups. *** $P < 0.005$, ** $P < 0.01$, * $P < 0.05$, n.s. non significant.

3. Results

3.1 Syndecan-4 is Important for the Differentiation of Cardiac Fibroblasts into Myofibroblasts in the Pressure-Overloaded Heart

Cardiac myofibroblasts exhibit enhanced production of ECM proteins when compared to fibroblasts [1]. Here we found that mRNA levels of collagen I, collagen III and fibronectin were significantly increased in the left ventricles of wild-type (WT) mice 24 hrs after AB, and that this up-regulation was completely prevented for collagen I and III, and reduced for fibronectin in syndecan-4^{-/-} mice (Figure 1A, B and C, respectively). mRNA levels of the myofibroblast-associated genes SMA and SM22 were also increased in the left ventricle of WT mice after AB, and this up-regulation was blunted in syndecan-4^{-/-} mice (Figure 1D and E). We also examined the expression of another smooth muscle cell marker gene, smoothelin, which is generally not found in myofibroblasts [19, 20]. Smoothelin mRNA was also increased after AB but this was not dependent on signaling through syndecan-4, since the same effects were observed in WT and syndecan-4^{-/-} mice (Figure 1F). AB resulted in a 3.6-fold up-regulation of syndecan-4 mRNA in WT left ventricular tissue (Figure 1G). No changes in plasma levels of syndecan-4 were observed 24 hrs after AB. Syndecan-4 was equally expressed in neonatal cardiomyocytes and cardiac fibroblasts (Figure 1H). No significant differences were observed for any of the analyzed genes between sham WT and sham syndecan-4^{-/-} mice (Figure 1). Body, heart and lung weight values for the studied animals (Supplemental Table S1) confirmed an effect of AB which resulted in increased left ventricular and lung weights, experimental signs of cardiac hypertrophy and congestive heart failure, respectively. The relative amount of water was unchanged following AB (sham: 75.6±0.1%; AB: 75.3±0.2%; n.s.). Taken together, our data suggests that syndecan-4 may be engaged in the stress response following pressure overload and plays a role in the regulation of ECM and myofibroblast marker genes.

3.2 Formation of Smooth Muscle α -Actin Fibers is Reduced in Syndecan-4^{-/-} and Cyclosporine A-Treated Wild-Type Cardiac Fibroblasts *In Vitro*

Fibroblasts will spontaneously differentiate into myofibroblasts when cultured on a fibronectin-coated rigid substrate due to effects of fibronectin on focal adhesion assembly [21] and increased mechanical tension compared to the *in vivo* situation [22]. We took advantage of this property and compared the expression of SMA and the percentage of cells exhibiting organized SMA fibers in WT and syndecan-4^{-/-} cardiac fibroblasts after 24 and 48 hrs of culture on fibronectin-coated glass cover slips (Figure 2A and B). SMA immunofluorescence was significantly lower in fibroblasts from syndecan-4^{-/-} mice compared to those from WT mice 48 hrs after plating the cells. Already after 24 hrs of culture, a small fraction of the WT fibroblasts had organized SMA fibers whereas this was not true for the syndecan-4^{-/-} fibroblasts. At 48 hrs, approximately 50% of the fibroblasts from WT and only 10% of the fibroblasts from syndecan-4^{-/-} mice showed SMA fibers in the cytosol. Thus, fibroblasts from syndecan-4^{-/-} mice were less prone to differentiate into myofibroblasts. In agreement with this, expression levels of SMA and SM22 mRNA were reduced in cardiac fibroblasts from syndecan-4^{-/-} mice whereas they were increased in WT cardiac fibroblasts over-expressing syndecan-4 (Figure 2C). No compensatory changes in the expression of other syndecan isoforms (1-3) were observed in cardiac fibroblasts from the syndecan-4^{-/-} mice (Supplemental Figure S1). To explore a potential involvement of the calcineurin signaling pathway in myofibroblast differentiation, we tested the effect of the calcineurin blocker cyclosporine A (CsA) on SMA expression and organization (Figure 2D). After 48 hrs on fibronectin-coated glass cover slips, both protein expression and percentage of cells exhibiting organized SMA fibers were significantly lower in the CsA-treated cells. Fibroblasts stimulated with transforming growth factor β (TGF β), a well-known inducer of SMA expression and myofibroblast differentiation, were used as a positive control, whereas exclusion of primary antibodies from the staining protocol was used as a negative control (Figure 2E).

3.3 Syndecan-4 and Calcineurin/NFAT Signaling are Involved in the Regulation of Collagen I and III Expression.

Increased expression of collagen I and III is characteristic of myofibroblast differentiation and central for the development of cardiac fibrosis. Along with the time-dependent changes in SMA expression and organization described above, cardiac fibroblasts from WT mice showed increasing levels of collagen I (Figure 3A) and III (Figure 3C) mRNA upon culture on fibronectin. In fibroblasts from syndecan-4^{-/-} mice, the expression of these collagens was lower and the effect of culture blunted. Both CsA and the NFAT blocker A-285222 effectively prevented the induction of collagen I and III at 48 hrs indicating involvement of calcineurin and NFAT in the regulation of these genes (Figure 3B and D). A parallel time-dependent increase in NFAT-dependent transcriptional activity was observed in cardiac fibroblasts from NFAT-luciferase reporter mice during culture, whereas NFAT activation was attenuated in fibroblasts from syndecan-4^{-/-}-NFAT-luciferase reporter mice (Figure 3E). Incubation with CsA or A-285222 significantly decreased NFAT-luciferase activity at 48 hrs (Figure 3F) showing the specificity of these blockers in cultured cardiac fibroblasts. Hence, our data demonstrate that signaling via syndecan-4, calcineurin and NFAT is involved in the regulation of collagen I and III during myofibroblast differentiation. The reduced myofibroblast differentiation observed in syndecan-4^{-/-} cardiac fibroblasts did not appear to be due to reduced TGFβ expression, since TGFβ mRNA levels were similar in WT and syndecan-4^{-/-} at all time points (Supplemental Figure S2).

3.4 NFATc4 is Activated in a Syndecan-4-Dependent Manner in Response to Mechanical Stress

The NFAT-luciferase reporter system does not discriminate between NFATc isoforms. We therefore used NFATc1-c4-EGFP fusion proteins to determine isoform-specific activation. For sufficient transfection, we used HT1080 fibroblasts for these experiments. As this cell line did not spontaneously differentiate into myofibroblasts on fibronectin after 48 hrs, we subjected the cells to mechanical stress by applying cyclic stretch for 24 hrs. This significantly increased the amount of the well-established early myofibroblast marker, extracellular domain A (EDA) of fibronectin [23] (Figure 4A)

indicating myofibroblast differentiation in this model. TGF β -stimulation was used as positive control for myofibroblast differentiation (Figure 4A). We then quantified nuclear accumulation of NFAT fusion proteins as a measure for activation following mechanical stress. NFATc1-EGFP and NFATc3-EGFP were already predominantly nuclear under control non-stimulated conditions. NFATc2-EGFP showed a tendency to translocate to the nucleus following stretch, whereas NFATc4-EGFP was predominantly cytosolic under control conditions and clearly accumulated in the nucleus after 24 hrs of cyclic stretch (Figure 4B). Despite lower transfection efficiency, corresponding experiments were carried out in primary cardiac fibroblasts, demonstrating significantly increased NFATc4-EGFP nuclear accumulation following cyclic stretch (Figure 4B, right panel).

To investigate if stretch could activate endogenous NFATc4 in primary cardiac fibroblasts and whether activation required syndecan-4, we compared the levels of inactive phosphorylated NFATc4 in WT and syndecan-4^{-/-} fibroblasts following 24 hrs of cyclic stretch. To avoid the effect of fibronectin on fibroblast phenotype and isolate the effect of stretch *per se*, membranes were coated with collagen I in these experiments. NFATc4 phosphorylation was reduced following stretch in cardiac fibroblasts from WT mice reflecting increased activation of NFATc4, whereas phosphorylation levels were not significantly affected in fibroblasts from syndecan-4^{-/-} mice (Figure 4C), suggesting involvement of syndecan-4 in stretch-induced NFATc4 activation. Furthermore, the stretch-induced dephosphorylation of NFATc4 was prevented by CsA (Figure 4D) indicating calcineurin-dependence in this response. Expression levels of NFATc4 mRNA were unchanged in cardiac fibroblasts from WT mice (ctrl 1.0 \pm 0.3, ms 0.7 \pm 0.2) and syndecan-4^{-/-} mice (ctrl 1.0 \pm 0.1, ms 0.8 \pm 0.2) following stretch (N=10). Hence, the observed changes in the levels of phosphorylated NFATc4 proteins are less likely due to changes in total NFATc4 expression. Over-expression of syndecan-4 in HT1080 fibroblasts lead to dephosphorylation of NFATc4 (Figure 4E), again supporting a role for syndecan-4 in NFATc4 activation.

Regulator of calcineurin 1.4 (RCAN1.4) is a well-established NFAT-target in the heart [24, 25] as well as in various vascular tissues [26]. Indeed, RCAN1.4 was up-regulated upon over-expression of calcineurin or NFATc4 (Supplemental Figure S3A and B), showing that RCAN1.4 can

function as a readout for calcineurin-NFAT activation in fibroblasts. We also found that over-expressing syndecan-4, which readily activates NFATc4 (see previous paragraph and Figure 4E), resulted in significantly up-regulated RCAN1.4 expression both in HT1080 fibroblasts and in primary cardiac fibroblasts (Supplemental Figure S3C and D). Altogether these data support that a transcriptionally functional pathway is in place in these cells and that it engages syndecan-4, calcineurin and NFATc4.

3.5 Syndecan-4 and Calcineurin Co-localize in Cardiac Fibroblasts

Based on the results showing that syndecan-4 and calcineurin regulate stretch-induced activation of NFATc4, we wished to determine whether syndecan-4 and calcineurin located close enough to each other to physically interact in cardiac fibroblasts. For these experiments we used syndecan-4 antibodies directed towards epitopes of the intracellular domain of the protein [11]. Syndecan-4 and calcineurin were indeed found to co-localize in cardiac fibroblasts (<40 nm apart) as determined by a proximity ligation assay (Figure 5A), substantiating a potential interaction between these proteins. No signal was observed in assays performed with syndecan-4 blocking peptide or on cardiac fibroblasts from syndecan-4^{-/-} mice. In agreement with these results, syndecan-4 has been found by us to co-immunoprecipitate with calcineurin in mouse left ventricles *in vivo*, and to bind directly to calcineurin *in vitro* [11]. In that study, the binding region was located to the autoinhibitory domain of calcineurin, suggesting that calcineurin may be activated upon binding to syndecan-4. Hence, syndecan-4 and calcineurin could comprise a mechanosignaling complex at focal adhesions in cardiac fibroblasts causing NFATc4 activation following mechanical stress. In line with previous work [21, 27-29], vinculin confocal immunofluorescence revealed fewer and smaller focal adhesions in fibroblasts from syndecan-4^{-/-} mice when compared to those from WT mice (Supplemental Figure S4) which may imply impaired focal adhesion signaling in cardiac fibroblasts from syndecan-4^{-/-} mice.

3.6 Syndecan-4 Phosphorylation at Serine179 is Reduced in Cardiac Fibroblasts Following Mechanical Stress

We next searched for a mechanism that could explain syndecan-4-mediated activation of NFAT in response to mechanical stress. A previous study in our group had indicated that phosphorylation of serine179 (pS179) in the cytoplasmic part of syndecan-4 negatively regulated the syndecan-4-calcineurin interaction in mouse left ventricles [11]. Thus, we used HT1080 fibroblasts over-expressing syndecan-4 to investigate whether mechanical stress regulated pS179. Firstly, co-localization of calcineurin and syndecan-4 was also observed in this cell system (Figure 5B). Secondly, 24 hrs of stretch caused a 44% reduction in pS179 (Figure 5C), suggesting that syndecan-4 dephosphorylation might be a stretch-induced molecular switch causing NFATc4 activation via calcineurin in fibroblasts.

3.7 Over-expression of NFATc4 Enhances the Expression of Collagen III and Myocardin-Related Transcription Factor A

To determine whether increased expression of NFATc4 *per se* could affect the expression of myofibroblast-associated genes, we examined the effect of NFATc4 over-expression. Due to low transfection efficiency of primary cardiac fibroblasts, HT1080 fibroblasts were used for this purpose. Expression levels of NFATc4 were increased 48 hrs after transfection at which point collagen I mRNA was unchanged (Figure 6A), but collagen III was markedly up-regulated (Figure 6B) supporting a role for NFATc4 in the regulation of this gene. No difference was found in SMA mRNA (Figure 6C) but interestingly, myocardin-related transcription factor A (MRTF-A) mRNA levels were significantly increased when compared to control cells (Figure 6D). MRTF-A is a well-known regulator of SMA gene expression [30] and has recently been associated with myofibroblast differentiation [31].

4. Discussion

This study demonstrates that syndecan-4 is important for extracellular matrix production and differentiation of cardiac fibroblasts into myofibroblasts in the pressure-overloaded heart *in vivo* and in response to mechanical stress *in vitro*. We also show that the calcineurin/NFATc4 signaling pathway is engaged upon mechanical stress in a syndecan-4-dependent manner and that once activated, it plays a role in myofibroblast differentiation and the production of extracellular matrix. This is based on the following findings: 1) Myofibroblast differentiation was impaired in syndecan-4^{-/-} both *in vivo* and *in vitro*, as determined by reduced expression of ECM and myofibroblast marker genes; 2) over-expressing syndecan-4 promoted myofibroblast differentiation in cardiac fibroblasts; 3) mechanical stress effectively activated NFATc4 in fibroblasts, as shown by increased NFATc4 nuclear accumulation, decreased NFATc4 phosphorylation and increased NFAT-dependent transcriptional activity, and these responses were reduced in cardiac fibroblasts from syndecan-4^{-/-} mice and fibroblasts treated with blockers of calcineurin/NFAT signaling; 4) over-expressing NFATc4 resulted in up-regulation of collagen III and MRTF-A, a transcription factor well known to regulate SMA gene expression; 5) syndecan-4 and calcineurin co-localize at focal adhesions possibly enabling formation of a mechanosensitive signaling complex in cardiac fibroblasts that might be activated by dephosphorylation of syndecan-4 in response to mechanical stress.

Fibroblasts are the most abundant cells in the myocardium and display considerable plasticity allowing them to adjust to extracellular cues such as mechanical stress. Even though *in vivo* data from tissue homogenates presented in this study do not allow a complete distinction of the cell types contributing to the pressure-induced responses, it is generally accepted that fibroblasts are responsible for ECM production and hence cause the increase in collagen I and collagen III observed after AB. This effect was clearly altered in the syndecan-4^{-/-} animals and would be predicted to translate into reduced fibrosis following long-term pressure overload in these mice. It is important to highlight though that pressure overload likely affects multiple pathways simultaneously and that the complete lack of syndecan-4 may have an impact on these pathways, hampering the interpretation of the *in vivo* results. However, the *in vitro* results from the study are in line with the *in vivo* data. The *in vitro*

conditions used here expose cells to increased mechanical tension compared to the *in vivo* situation in a healthy heart, and thus recreate several aspects of myofibroblast differentiation as it occurs *in vivo* after AB, not only by increasing the expression of ECM gene transcripts, but also by induction of contractile proteins and formation of organized SMA fibers.

Myofibroblast differentiation in response to mechanical stress was found to be dependent on signaling via syndecan-4. Previous work from our group demonstrated increased expression of syndecan-4 in the hypertrophic myocardium of mice after myocardial infarction [10] and in human aortic stenosis [11]. Here we show that the same is true in the pressure-overloaded heart of mice after AB. Along these lines, serum syndecan-4 has recently been proposed as a novel diagnostic and prognostic biomarker for patients with heart failure [32]. Plasma syndecan-4 levels were not changed after AB in our study, but it may take longer than 24 hours for the changes to occur. Expression levels of syndecan-4 were similar in cardiomyocytes and fibroblasts, suggesting that both cell types may be contributing to the expression levels measured in heart homogenates. While our previous study clearly established that syndecan-4 in cardiac myocytes is essential for compensatory hypertrophy in the pressure-overloaded heart [11], we here show that this transmembrane proteoglycan also plays a role in a parallel process that is taking place in cardiac fibroblasts in response to the increased pressure. Findings by Matsui et al. also support a role for syndecan-4 in this process, since they found impaired myofibroblast differentiation in the infarcted region after coronary ligation in syndecan-4^{-/-} mice [33]. However, the signaling mechanism was not examined.

In our study, myofibroblast differentiation was found to be dependent on calcineurin signaling. Vast evidence substantiates the role of calcineurin/NFAT in ventricular cardiomyocytes in hypertrophy [14, 34], and *in vivo* studies using genetically modified mice demonstrated a necessary role for NFATc2 [16], NFATc3 [15] and NFATc4 [14] as downstream effectors of calcineurin. However, the role of calcineurin/NFAT signaling in cardiac fibroblasts is poorly defined. Calcineurin has been indirectly implicated in the development of cardiac fibrosis [14, 34, 35], and transgenic mice expressing constitutively active calcineurin in cardiomyocytes not only developed cardiac hypertrophy, but a concomitant accumulation of collagen deposits surrounding the degenerating

cardiomyocytes [14, 34]. Also, *in vivo* treatment with pharmacological inhibitors of calcineurin could limit the pressure overload-induced cardiac fibrosis [34]. We now provide direct evidence for a role of calcineurin/NFAT signaling in collagen production in cardiac fibroblasts. Our data show that similarly to cardiomyocytes [18], NFATc4 is engaged in response to mechanical stress in cardiac fibroblasts. NFATc1 and NFATc3 were not sensitive to applied mechanical stress, but at the moment we cannot rule out a possible contribution of NFATc2 to myofibroblast differentiation, given the observed tendency to increased activation. Reduced fibrosis has indeed been described in pressure-overloaded left ventricles of NFATc2 knockout mice when compared to those of WT mice [16]. Whether this was due to lack of NFATc2 in cardiomyocytes or in fibroblasts or in both remained unresolved.

The increased NFAT-dependent transcriptional activity measured during myofibroblast differentiation lead to increased collagen I and III mRNA. NFAT involvement in the regulation of collagen I has been shown in osteoblasts and in tendon fibroblasts [36, 37] but, to our knowledge, the here suggested role for NFAT as regulator of collagen III is new. In our hands, NFATc4 overexpression *per se* did not increase collagen I mRNA levels. One possible interpretation of these results is that collagen I expression could be driven instead by another NFAT isoform, such as NFATc2. Alternatively, collagen I transcription may require cooperation with additional transcription factors (an important feature of NFAT transcription factors) that are either not expressed in the HT1080 fibroblasts or not activated under these experimental conditions (where syndecan-4 and mechanical stress is bypassed). The exact identity of these partners remains to be elucidated, but exciting work recently published from the Molkenin's lab describes a direct and functional interaction between NFAT and NFκB, critical for cardiac hypertrophy and remodeling [38]. Even though most of the data supporting this interaction is from cardiomyocytes, interaction was also observed in mouse embryonic fibroblasts.

Several intracellular signaling pathways and molecules have previously been implicated in myofibroblast differentiation, one of the most important being the transcription partners serum response factor (SRF) and myocardin-related transcription factors A and B (MRTF-A and B) [39]. These bind to specific elements in the SMA promoter and other myofibroblast marker genes thereby

regulating transcription [30]. The means whereby mechanical stress induces myofibroblast differentiation are still unknown. One possibility is the engagement of MRTF-A which translocates to the nucleus in response to mechanical stress in a Rho kinase-dependent manner [39, 40] and has also been shown to cause myofibroblast marker gene expression in cardiac fibroblasts [41]. Interestingly, RhoA activity (which in turn activates Rho kinase) is reduced in cardiac fibroblasts from syndecan-4^{-/-} mice [33]. Furthermore, NFATc4 over-expression resulted in increased MRTF-A mRNA, which may in turn promote myofibroblast differentiation [31]. Hence, syndecan-4 may influence myofibroblast differentiation by regulating not only the expression of MRTF-A (via NFATc4) but also the activation of MRTF-A (via RhoA).

Cardiac fibrosis is a characteristic of several heart diseases including myocardial infarction and hypertrophy, and involves excessive synthesis of ECM proteins such as the collagens [42]. This increases myocardial stiffness and contributes to ventricular diastolic dysfunction which has lately been found to account for a larger proportion of heart failure patients than previously suspected. To date, there is no effective treatment for cardiac fibrosis, which remains a challenging area in clinical practice. There have been attempts to interfere with myofibroblast formation by targeting TGF β -signaling in fibrotic tissues [43, 44]. TGF β is a potent inducer of myofibroblast differentiation, but it has a wide range of effects on cardiomyocytes, mesenchymal and immune cells [45]. Drugs targeting other circulating hormones and growth factors implicated in this pathology (i.e. endothelin-1, angiotensin II, connective tissue growth factor, platelet-derived growth factor) are currently under consideration [46]. However, due to their pleiotropic and multifunctional effects total blockade of these signaling molecules may not be without side-effects. A need for more specific targets is clear. We found that syndecan-4 is involved in myofibroblast differentiation of cardiac fibroblasts in the left ventricle in response to pressure overload and in mechanically stressed cardiac fibroblasts *in vitro*. Syndecan-4 engages the calcineurin/NFAT signaling pathway in cardiac fibroblasts subjected to mechanical stress, leading to increased expression of collagen I and collagen III mRNA levels, as well as increased SMA protein expression and fiber organization. Targeting the intracellular domain of syndecan-4 to block interactions with calcineurin and hence prevent downstream activation of NFAT-

induced gene transcription may be an attractive therapeutic approach to prevent cardiac fibrosis in the context of pressure overload.

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Disclosures

None.

Reference List

- [1] MacKenna D, Summerour SR, Villarreal FJ. Role of mechanical factors in modulating cardiac fibroblast function and extracellular matrix synthesis. *Cardiovasc Res* 2000;46:257-63.
- [2] Darby I, Skalli O, Gabbiani G. Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest* 1990;63:21-9.
- [3] Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993;122:103-11.
- [4] Hinz B, Mastrangelo D, Iselin CE, Chaponnier C, Gabbiani G. Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. *Am J Pathol* 2001;159:1009-20.
- [5] Bellin RM, Kubicek JD, Frigault MJ, Kamien AJ, Steward RL, Jr., Barnes HM et al. Defining the role of syndecan-4 in mechanotransduction using surface-modification approaches. *Proc Natl Acad Sci U S A* 2009;106:22102-7.
- [6] Byron A, Morgan MR, Humphries MJ. Adhesion signalling complexes. *Curr Biol* 2010;20:R1063-R1067.
- [7] Geiger B, Bershadsky A. Exploring the neighborhood: adhesion-coupled cell mechanosensors. *Cell* 2002;110:139-42.
- [8] Couchman JR. Transmembrane signaling proteoglycans. *Annu Rev Cell Dev Biol* 2010;26:89-114.
- [9] Wilcox-Adelman SA, Denhez F, Goetinck PF. Syndecan-4 modulates focal adhesion kinase phosphorylation. *J Biol Chem* 2002;277:32970-7.
- [10] Finsen AV, Woldbaek PR, Li J, Wu J, Lyberg T, Tønnessen T et al. Increased syndecan expression following myocardial infarction indicates a role in cardiac remodeling. *Physiol Genomics* 2004;16:301-8.
- [11] Finsen AV, Lunde IG, Sjaastad I, Østli EK, Lyngra M, Jarstadmarken HO et al. Syndecan-4 is essential for development of concentric myocardial hypertrophy via stretch-induced activation of the calcineurin-NFAT pathway. *PLoS One* 2011;6:e28302.
- [12] Echtermeyer F, Streit M, Wilcox-Adelman S, Saoncella S, Denhez F, Detmar M et al. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. *J Clin Invest* 2001;107:R9-R14.
- [13] Schulz RA, Yutzey KE. Calcineurin signaling and NFAT activation in cardiovascular and skeletal muscle development. *Dev Biol* 2004;266:1-16.
- [14] Molkenstein JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 1998;93:215-28.
- [15] Wilkins BJ, De Windt LJ, Bueno OF, Braz JC, Glascock BJ, Kimball TF et al. Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth. *Mol Cell Biol* 2002;22:7603-13.

- [16] Bourajjaj M, Armand AS, da Costa Martins PA, Weijts B, van der Nagel R, Heeneman S et al. NFATc2 is a necessary mediator of calcineurin-dependent cardiac hypertrophy and heart failure. *J Biol Chem* 2008;283:22295-303.
- [17] Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, Plank DM et al. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circ Res* 2004;94:110-8.
- [18] Lunde IG, Kvaløy H, Austbø B, Christensen G, Carlson CR. Angiotensin II and norepinephrine activate specific calcineurin-dependent NFAT transcription factor isoforms in cardiomyocytes. *J Appl Physiol* 2011;111:1278-89.
- [19] van der Loop FT, Schaart G, Timmer ED, Ramaekers FC, van Eys GJ. Smoothelin, a novel cytoskeletal protein specific for smooth muscle cells. *J Cell Biol* 1996;134:401-11.
- [20] Coco DP, Hirsch MS, Hornick JL. Smoothelin is a specific marker for smooth muscle neoplasms of the gastrointestinal tract. *Am J Surg Pathol* 2009;33:1795-801.
- [21] Ishiguro K, Kadomatsu K, Kojima T, Muramatsu H, Tsuzuki S, Nakamura E et al. Syndecan-4 deficiency impairs focal adhesion formation only under restricted conditions. *J Biol Chem* 2000;275:5249-52.
- [22] Chan MW, Hinz B, McCulloch CA. Mechanical induction of gene expression in connective tissue cells. *Methods Cell Biol* 2010;98:178-205.
- [23] Serini G, Bochaton-Piallat ML, Ropraz P, Geinoz A, Borsi L, Zardi L et al. The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. *J Cell Biol* 1998;142:873-81.
- [24] Lange AW, Molkentin JD, Yutzey KE. DSCR1 gene expression is dependent on NFATc1 during cardiac valve formation and colocalizes with anomalous organ development in trisomy 16 mice. *Dev Biol* 2004;266:346-60.
- [25] Rothermel BA, Vega RB, Williams RS. The role of modulatory calcineurin-interacting proteins in calcineurin signaling. *Trends Cardiovasc Med* 2003;13:15-21.
- [26] Lee MY, Garvey SM, Baras AS, Lemmon JA, Gomez MF, Schoppee Bortz PD et al. Integrative genomics identifies DSCR1 (RCAN1) as a novel NFAT-dependent mediator of phenotypic modulation in vascular smooth muscle cells. *Hum Mol Genet* 2010;19:468-79.
- [27] Gopal S, Bober A, Whiteford JR, Mulhaupt HA, Yoneda A, Couchman JR. Heparan sulfate chain valency controls syndecan-4 function in cell adhesion. *J Biol Chem* 2010;285:14247-58.
- [28] Woods A, Longley RL, Tumova S, Couchman JR. Syndecan-4 binding to the high affinity heparin-binding domain of fibronectin drives focal adhesion formation in fibroblasts. *Arch Biochem Biophys* 2000;374:66-72.
- [29] Chen Y, Shi-Wen X, van BJ, Kennedy L, McLeod M, Renzoni EA et al. Matrix contraction by dermal fibroblasts requires transforming growth factor-beta/activin-linked kinase 5, heparan sulfate-containing proteoglycans, and MEK/ERK: insights into pathological scarring in chronic fibrotic disease. *Am J Pathol* 2005;167:1699-711.
- [30] Chen J, Kitchen CM, Streb JW, Miano JM. Myocardin: a component of a molecular switch for smooth muscle differentiation. *J Mol Cell Cardiol* 2002;34:1345-56.

- [31] Small EM, Thatcher JE, Sutherland LB, Kinoshita H, Gerard RD, Richardson JA et al. Myocardin-related transcription factor-a controls myofibroblast activation and fibrosis in response to myocardial infarction. *Circ Res* 2010;107:294-304.
- [32] Takahashi R, Negishi K, Watanabe A, Arai M, Naganuma F, Ohyama Y et al. Serum syndecan-4 is a novel biomarker for patients with chronic heart failure. *J Cardiol* 2011;57:325-32.
- [33] Matsui Y, Ikesue M, Danzaki K, Morimoto J, Sato M, Tanaka S et al. Syndecan-4 prevents cardiac rupture and dysfunction after myocardial infarction. *Circ Res* 2011;108:1328-39.
- [34] Berry JM, Le V, Rotter D, Battiprolu PK, Grinsfelder B, Tannous P et al. Reversibility of adverse, calcineurin-dependent cardiac remodeling. *Circ Res* 2011;109:407-17.
- [35] Shimoyama M, Hayashi D, Takimoto E, Zou Y, Oka T, Uozumi H et al. Calcineurin plays a critical role in pressure overload-induced cardiac hypertrophy. *Circulation* 1999;100:2449-54.
- [36] Lejard V, Brideau G, Blais F, Salingcarboriboon R, Wagner G, Roehrl MH et al. Scleraxis and NFATc regulate the expression of the pro- $\alpha 1(I)$ collagen gene in tendon fibroblasts. *J Biol Chem* 2007;282:17665-75.
- [37] Koga T, Matsui Y, Asagiri M, Kodama T, de CB, Nakashima K et al. NFAT and Osterix cooperatively regulate bone formation. *Nat Med* 2005;11:880-5.
- [38] Liu Q, Chen Y, Auger-Messier M, Molkentin JD. Interaction between NF κ B and NFAT coordinates cardiac hypertrophy and pathological remodeling. *Circ Res* 2012;110:1077-86.
- [39] Huang X, Yang N, Fiore VF, Barker TH, Sun Y, Morris SW et al. Matrix stiffness-induced myofibroblast differentiation is mediated by intrinsic mechanotransduction. *Am J Respir Cell Mol Biol* 2012.
- [40] Miralles F, Posern G, Zaromytidou AI, Treisman R. Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* 2003;113:329-42.
- [41] Zhao XH, Laschinger C, Arora P, Szaszi K, Kapus A, McCulloch CA. Force activates smooth muscle α -actin promoter activity through the Rho signaling pathway. *J Cell Sci* 2007;120:1801-9.
- [42] Swynghedauw B. Molecular mechanisms of myocardial remodeling. *Physiol Rev* 1999;79:215-62.
- [43] Meier K, Nanney LB. Emerging new drugs for scar reduction. *Expert Opin Emerg Drugs* 2006;11:39-47.
- [44] Liu X, Hu H, Yin JQ. Therapeutic strategies against TGF- β signaling pathway in hepatic fibrosis. *Liver Int* 2006;26:8-22.
- [45] Dobaczewski M, Chen W, Frangogiannis NG. Transforming growth factor (TGF)- β signaling in cardiac remodeling. *J Mol Cell Cardiol* 2011;51:600-6.
- [46] Lucas JA, Zhang Y, Li P, Gong K, Miller AP, Hassan E et al. Inhibition of transforming growth factor- β signaling induces left ventricular dilation and dysfunction in the pressure-overloaded heart. *Am J Physiol Heart Circ Physiol* 2010;298:H424-H432.

Figure 1. Aortic banding increases mRNA levels of collagen I, collagen III and fibronectin, and myofibroblast markers smooth muscle α -actin and SM22 in wild-type but not in syndecan-4^{-/-} left ventricles. Relative expression of collagen I (A), collagen III (B), fibronectin (C), smooth muscle α -actin (SMA; D), SM22 (E), smoothelin (F) and syndecan-4 (G and H) measured by real-time PCR in left ventricles of wild-type (WT) and syndecan-4^{-/-} (syn4^{-/-}) mice 24 hrs after sham operation (sham) or aortic banding (AB), and in cardiomyocytes (CM) and cardiac fibroblasts (FB). N=10 mice/group (A-G), N=4 (H). Data are related to WT sham (A-G) and CM (H).

Figure 2. Smooth muscle α -actin expression and fiber formation is dependent on syndecan-4 and calcineurin signaling in cardiac fibroblasts. Representative confocal immunofluorescence images showing smooth muscle α -actin (SMA; green) expression in cardiac fibroblasts from wild-type (WT) and syndecan-4^{-/-} (syn4^{-/-}) mice after 24 and 48 hrs in culture on fibronectin-coated glass cover slips (A). Nuclei were stained with Sytox orange (red). Relative SMA fluorescence intensity per cell and fraction of cells with organized SMA fibers (B). N=4-6 cover slips/condition. SMA and SM22 mRNA levels in cardiac fibroblasts from WT and syn4^{-/-} mice and in WT cardiac fibroblasts over-expressing syndecan-4 (syn4; C). N=5. SMA fiber formation and expression were analyzed in cardiac fibroblasts from WT mice after culture for 48 hrs with or without cyclosporine A (CsA, 1 μ mol/L; D). N=8 micrographs/condition. Transforming growth factor β (TGF β , 1 μ mol/L) was used as a positive control and omission of primary antibody as negative control (E).

Figure 3. The increased expression of collagen I and III during myofibroblast differentiation is dependent on syndecan-4 and calcineurin/NFAT signaling in cardiac fibroblasts. Collagen I (A) and III (C) mRNA levels and NFAT-luciferase activity (E) were measured in cardiac fibroblasts from wild-type (WT) and syndecan-4^{-/-} (syn4^{-/-}), or NFAT-luciferase (NFATluc) and syndecan-4^{-/-}-NFAT-luciferase (syn4^{-/-}-NFATluc) reporter mice, cultured for 24, 48 or 72 hrs on fibronectin-coated plates. The same parameters were measured in cardiac fibroblasts cultured for 48 hrs with or without

blockers of calcineurin (cyclosporine A (CsA), 1 μ mol/L) or NFAT (A-285222, 1 μ mol/L; B, D and F). N=4-6/group.

Figure 4. NFATc4 is activated by mechanical stress in a syndecan-4- and calcineurin-dependent manner. Extradomain A (EDA) protein levels in HT1080 fibroblasts after 24 hrs of mechanical stress (ms; A). TGF β was used as a positive control. Representative confocal images showing localization of NFATc1-c4-EGFP fusion proteins in control (ctrl) and ms-stimulated fibroblasts. Data are presented as the percentage of cells that had nuclear NFAT-EGFP and as mean \pm S.E.M. for cellular localization of NFATc4 in cardiac fibroblasts following mechanical stress (B, right panel). N=13-59 cells/condition. Representative immunoblots and summarized data from western blot experiments showing relative amount of phosphorylated NFATc4 (pNFATc4) in fibroblasts from wild-type (WT) and syndecan-4^{-/-} (syn4^{-/-}) mice in ctrl and following 24 hrs of ms (C) with or without cyclosporine A (CsA, 1 μ mol/L; D) and in HT1080 fibroblasts over-expressing syndecan-4 (syn4; E). N=4 (A), 9-10 (C), 6 (D and E).

Figure 5. Syndecan-4 and calcineurin co-localize in cardiac fibroblasts and syndecan-4 is dephosphorylated at serine179 upon mechanical stress. Co-localization of syndecan-4 and calcineurin (green) as determined with a proximity ligation assay in cardiac fibroblasts (A). Nuclei are stained with sytox orange (red). Omission of primary antibodies, incubation with syndecan-4 blocking peptide and cardiac fibroblasts from syndecan-4^{-/-} mice were used as negative controls. Proximity ligation assay in HT1080 fibroblasts demonstrating co-localization of syndecan-4 and calcineurin (red). Nuclei are stained with DAPI (blue; B). Large image in panel B shows a larger magnification of the proximity ligation signal. Immunoblots and summarized data from western blot experiments showing phosphorylated serine179 (pS179) levels in control (ctrl) and mechanically stressed (ms) HT1080 fibroblasts over-expressing syndecan-4 (C). Data were normalized to total syndecan-4 and related to ctrl. N=12.

Figure 6. Over-expression of NFATc4 induces collagen III and MRTF-A expression in fibroblasts. Relative mRNA levels of collagen I (A), collagen III (B), smooth muscle α -actin (SMA; C) and myocardin-related transcription factor A (MRTF-A; D) in control fibroblasts (ctrl) and in fibroblasts over-expressing NFATc4 (c4). N=6-7/group.

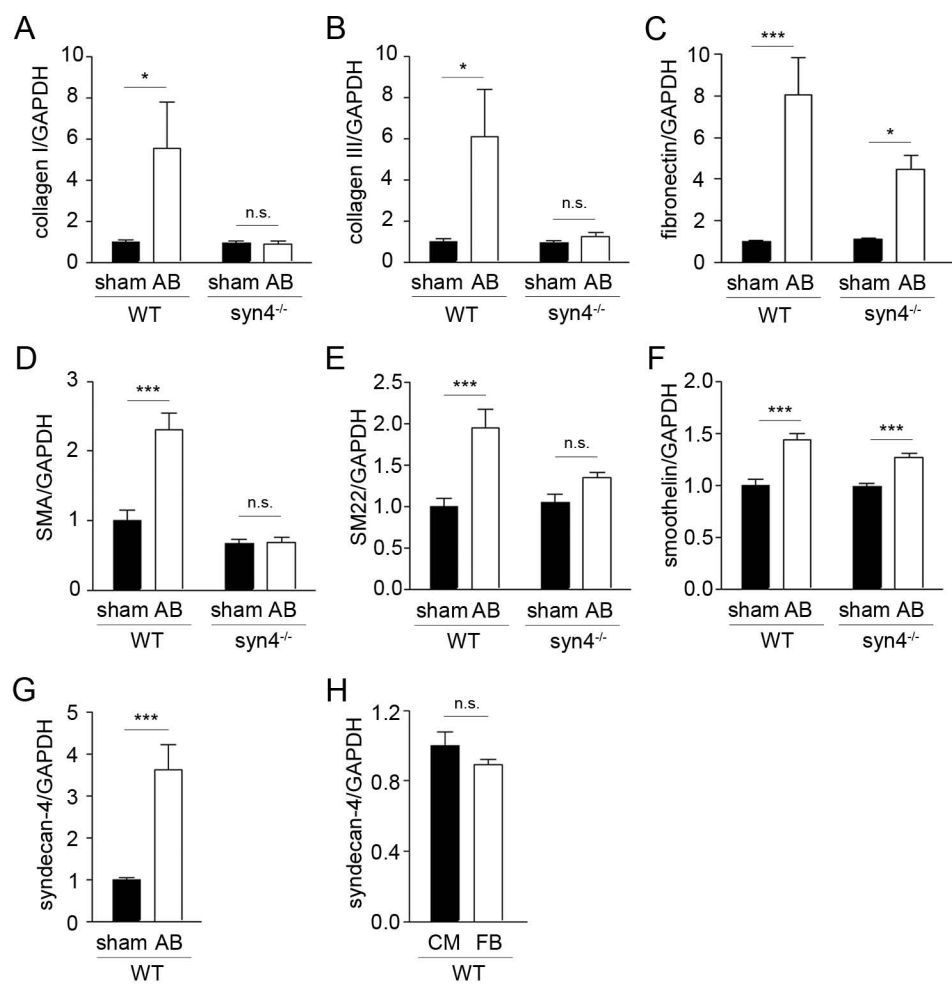


Figure 1

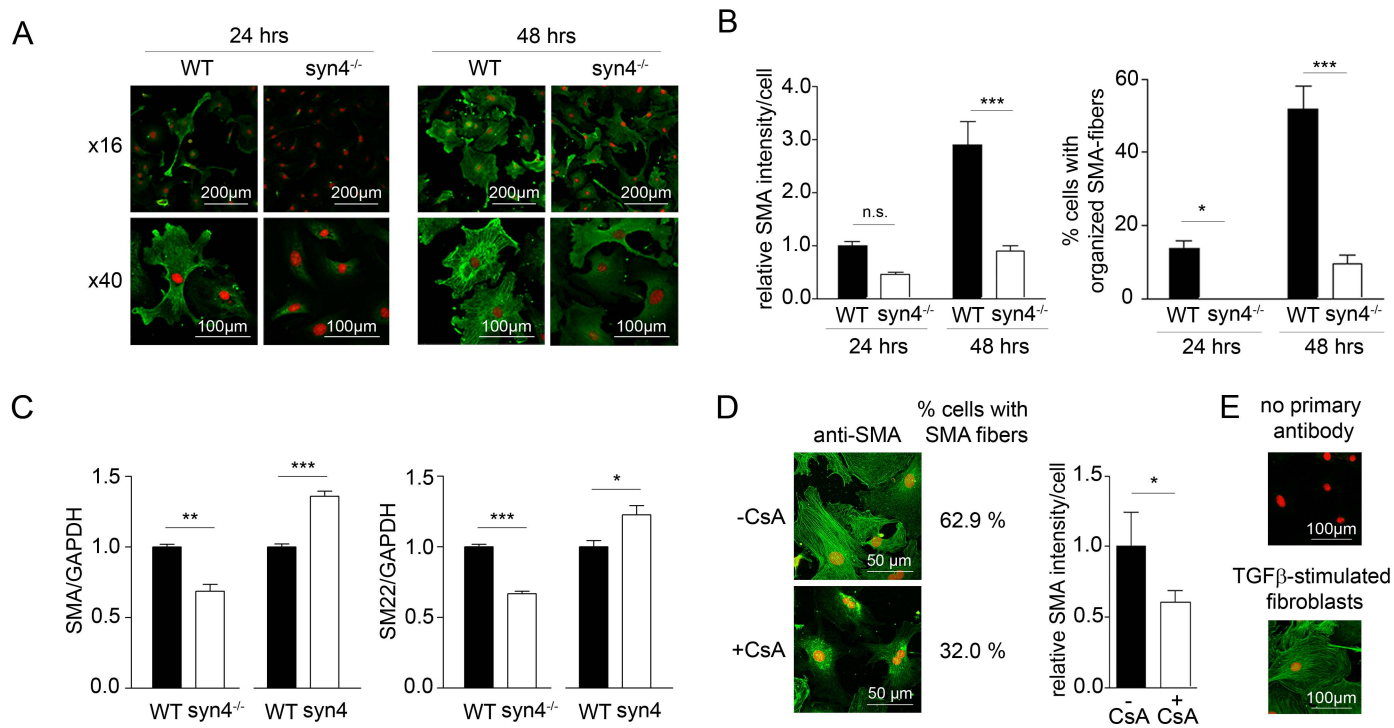


Figure 2

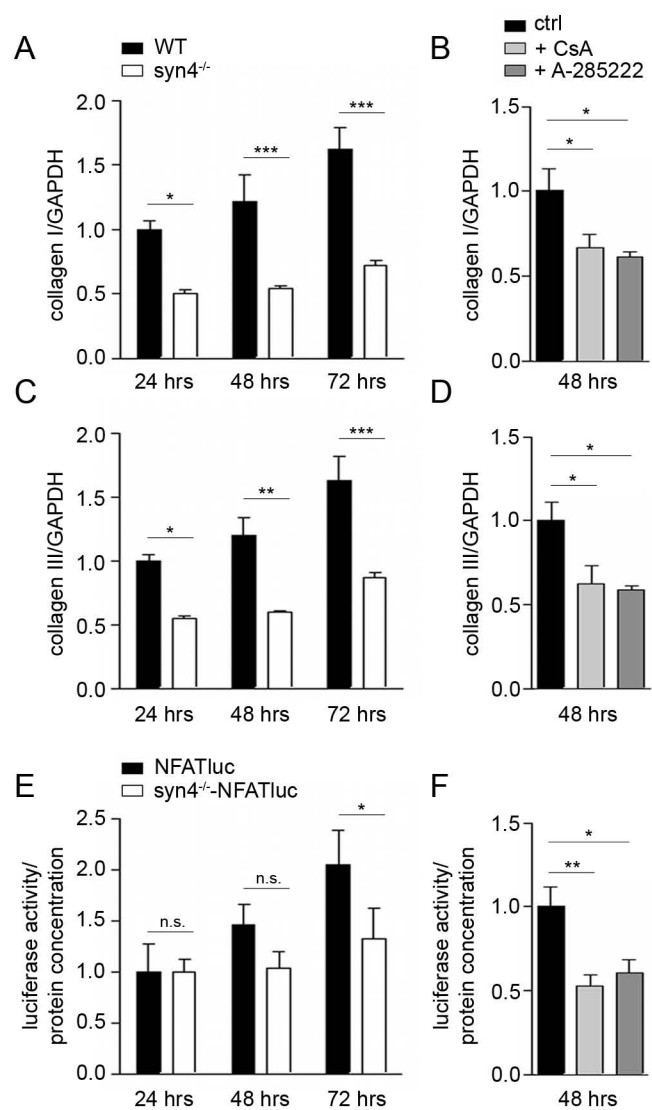


Figure 3

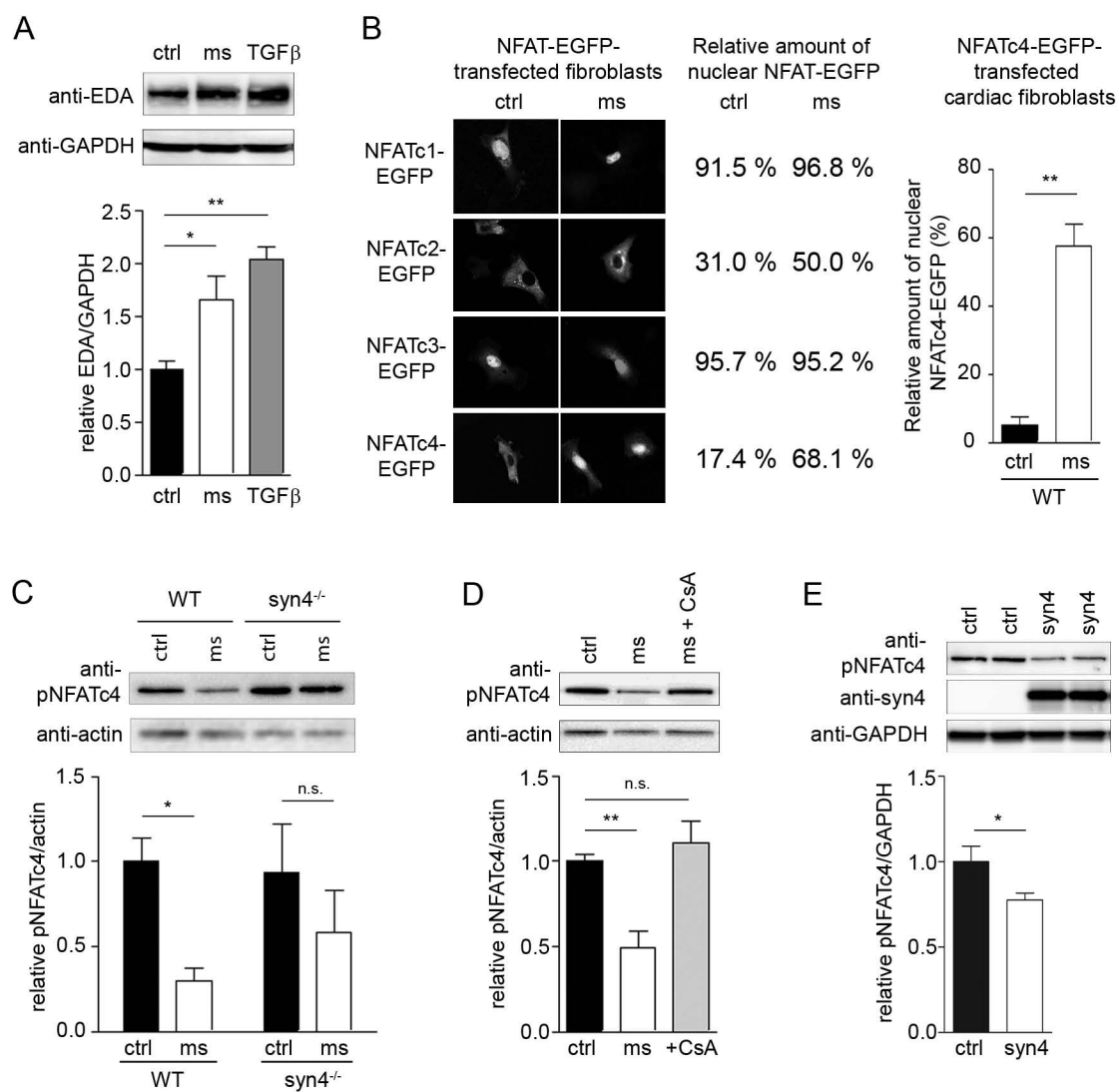


Figure 4

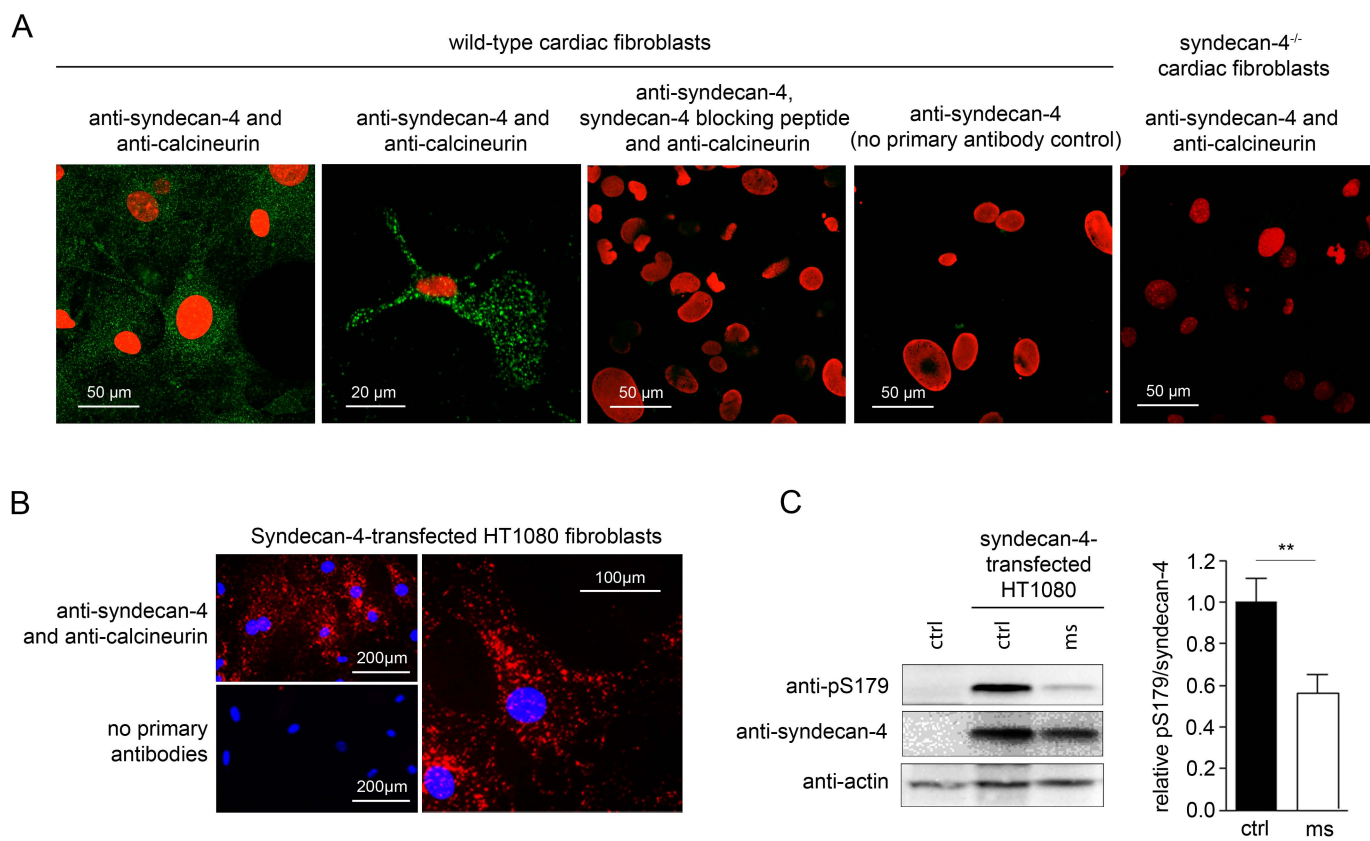


Figure 5

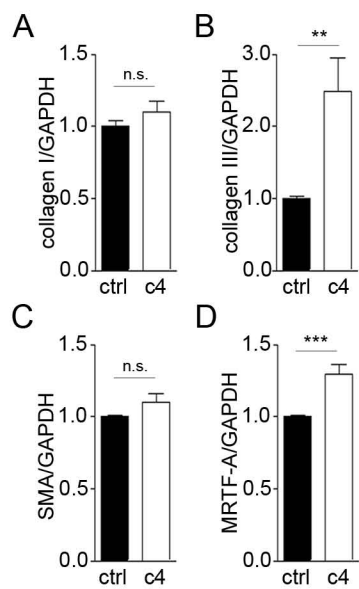


Figure 6

SUPPLEMENTAL MATERIAL

Detailed Methods

Mouse Model of Pressure Overload

Initially, animals were anesthetized in a gas chamber containing 5% isoflurane and 95% oxygen where after the animals were intubated and ventilated with 2% isoflurane and 98% oxygen. Aortic banding (AB) or sham operation was performed on eight week old male C57BL/6JBomTac mice (N=20; Taconic, Skensved, Denmark) and syndecan-4^{-/-} mice [1] on a C57Bl/6J background (N=20; 000664, The Jackson Laboratory, Bar Harbor, ME) as previously described [2]. Sham-operated animals underwent the same procedure without tightening the suture around the aorta. For post-operative analgesia, buprenorphine was injected subcutaneously. To ensure successful AB and rule out total occlusion, flow velocity across the aorta was determined with echocardiography 24 hrs after AB. Animals with an aortic flow velocity >4 m/s after AB were included in the study. Animals were anesthetized (gas chamber with 5% isoflurane and 95% oxygen), decapitated and lungs and hearts dissected and weighed.

Syndecan-4 ELISA

Blood was withdrawn and separated into serum and plasma. Plasma syndecan-4 was determined by ELISA (E03S0065, Blue Gene Biotech, Shanghai, China).

Fibroblast Cell Culture

Cardiac fibroblasts from 1-3 day old mice were freshly isolated as previously described.[2] Mice were sacrificed by decapitation and left ventricles excised and cut into small pieces. After digestion with collagenase, the suspension was transferred to 150cm² flasks and incubated at 37°C in 5% CO₂ so that cardiac fibroblasts attached to the flask whereas the suspension containing cardiomyocytes could be removed. The second passage of cells was used to minimize *in vitro* effects on fibroblast phenotype. The human fibrosarcoma cell line HT1080 was purchased from ATCC (CCL-121,

Manassas, VA). Cells were cultured in Dulbecco's Modified Eagles Medium (41965 GIBCO-BRL, Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (14-701E, Bio-Whittaker, Lonza, Basel, Switzerland) and penicillin/streptomycin (G6784, Sigma, St. Louis, MO).

Cell Migration

Cell migration was assessed using a scratch assay. Cells were serum starved overnight and a scratch was made with a pipet tip in the fibroblast monolayer. Cells were then washed to remove cell debris. After washing, images from several areas in the culture dish were obtained. Images of the same areas were taken after 24 hours and these areas were measured using Adobe Photoshop CS5. The scratch areas at 0 hours and 24 hours were used to calculate cell migration.

NFAT-luciferase Assay

Primary cardiac fibroblasts from neonatal NFAT-luciferase reporter mice[3] and syndecan-4^{-/-}-NFAT-luciferase reporter mice were isolated as described.[2] Cells were harvested according to the Luciferase Assay System protocol (E1500, Promega, Madison, WI) and luminescence quantified with a Victor 3 1420 Multilabel Counter (PerkinElmer, Boston, MA). Cyclosporine A (CsA, 1 μ mol/L; 586107, SandImmune Neoral, Novartis, Basel, Switzerland) was used for calcineurin inhibition. The NFAT blocker A-285222 was kindly provided by Abbott Laboratories (1 μ mol/L; Abbott Park, IL).

Over-expression of NFAT-EGFP and Syndecan-4

Fibroblasts were transfected with HA-tagged (N-terminal HA-tag in pcDNA3.1) mouse syndecan-4 (NP_035651.1) or EGFP-fused (C-terminal EGFP-tag in pEGFP-N1 (Clontech)) mouse NFATc1-4 (NP_940821, NP_035029, NP_035031 and NP_076188, respectively; custom made by Genscript, Piscataway, NJ) using lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's protocol. NFATc4-EGFP over-expression caused a 40,000-fold increase in NFATc4 mRNA as determined with real-time PCR.

Gene Expression Analysis

mRNA was extracted from left ventricles and cell cultures using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality was validated using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and samples with an RNA integrity number (RIN) > 8 were accepted for further analyses. RNA quantity was measured using the Nanodrop ND-1000 (ND-1000 Saveen Werner, Malmö, Sweden). Reverse transcription of mRNA into cDNA was performed using the iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA). Quantitative real-time PCR with predesigned TaqMan assays (Applied Biosystems, Foster City, CA) was used to assess the expression of syndecan-1 (Mm00448918_m1), syndecan-2 (Mm00484718_m1), syndecan-3 (Mm01179831_m1) syndecan-4 (Mm00488527_m1; Hs00161617_m1), SMA (Mm01546133_m1, Hs00909449_m1), SM22 (Mm00441660_m1), collagen I (Mm00483888_m1, Hs00164099_m1), collagen III (Mm00802331_m1, Hs00943809_m1), fibronectin (Mm01256744_m1), RCAN1.4 (Mm01213406_m1, Hs01120954_m1), MRTF-A (Mm00461840_m1, Hs01120954_m1), NFATc4 (Mm00452375_m1, Hs00190037_m1), MMP2 (Mm00439506_m1), MMP9 (Mm00442991_m1), MMP13 (Mm01168713_m1), TIMP1 (Mm00441818_m1), TIMP2 (Mm00441825_m1) and GAPDH (Mm03302249_g1, Hs99999905_m1). The results were detected on a 7900HT Fast Real Time PCR System (Applied Biosystems), and the data analyzed by using the Sequence Detection Software 2.3 from Applied Biosystems. Data were normalized to GAPDH.

Immunoblotting

Proteins from cell cultures and from homogenized left ventricles were harvested in a PBS-buffer containing 1% Triton X-100 (Sigma, Schnelldorf, Germany), 0.1% Tween 20 (Sigma) and protease inhibitors (Complete EDTA-free tablets, Roche Diagnostics, Mannheim, Germany). Protein concentration was determined with BCA Protein Assay Kit (Thermo Scientific, Barrington, IL). SDS-PAGE and immunoblotting was performed essentially as described in the Criterion protocol (BIO-RAD). PVDF Hybond membranes were blocked in 8% non-fat dry-milk or 1% casein. Antibodies were diluted in 2% non-fat dry-milk or 1% casein. The following antibodies were used: anti-SMA

(1:10,000, A5228, Sigma), anti-EDA (1:400, F6140, Sigma), pNFATc4 (1:400, sc-32630, Santa Cruz Biotechnology, Santa Cruz, CA),[4] pS179 (1:500, custom made, Genscript), syndecan-4 (1:500, custom made, Genscript), actin (1:500, sc-8432, Santa Cruz Biotechnology) and GAPDH (1:500, Sc-20357, Santa Cruz Biotechnology). Secondary horseradish peroxidase (HRP)-conjugated antibodies used were anti-mouse and anti-rabbit from GE Healthcare (Amersham, UK) and anti-goat from Southern Biotechnology (Birmingham, AL), diluted 1:2000. Blots were developed using the ECL Western blotting detection kit (GE Healthcare). Membranes were reprobed after stripping using the Restore Western Blot Stripping buffer (21059, Thermo Scientific). Densities of protein bands were measured using Image Quant TL v2003.03 and blots were processed in Adobe Photoshop. Total actin was not affected by cyclic stretch when probed against GAPDH and was therefore used to control for equal protein loading of gels.

Immunocytochemistry

Cells grown on fibronectin-coated glass cover slips were fixed in 4% paraformaldehyde (158127, Sigma) and incubated with mouse-anti-paxillin (1:500, Sigma), mouse-anti-vinculin (1:200, Sigma) or mouse-anti-SMA (1:300, Sigma). Alexa fluor 488- or 546-conjugated secondary anti-mouse antibodies (Invitrogen) were used and slides mounted with citifluor mounting medium (Cityfluor Ltd., Leicester, UK) before visualized with a LSM 710 confocal microscope (Zeiss GmbH, Jena, Germany). Sytox orange (Invitrogen) was used as nuclear stain and alexa fluor 546-phalloidin (Invitrogen) was used to stain F-actin. 5-6 micrographs were taken from 2 glass cover slips in each group. The total number of cells counted in each group was 152-169. Presence of well-organized SMA fibers was determined for each cell. Mean intensity of SMA was determined using the analysis software from Zeiss (LSM 710) and normalized to the number of cells in each micrograph. Staining without primary antibodies was used as negative controls. Fibroblasts stimulated with transforming growth factor β (TGF β , 1 μ mol/L; Sigma), were used as a positive control for SMA staining.

Supplemental Results

Expression of ECM Remodeling Proteins and Migration of Cardiac Fibroblasts.

The pattern of expression of several proteins involved in ECM remodeling was changed in parallel to myofibroblast differentiation and differentially affected by the genotype of the mice (Supplemental Figure S5A). Expression levels of matrix metalloproteinase (MMP) 13 and tissue inhibitor of metalloproteinase 1 (TIMP1) were higher in cardiac fibroblasts from syndecan-4^{-/-} mice when compared to fibroblasts from WT mice, whereas MMP9 levels were lower (Supplemental Figure S5A). MMP13 and TIMP1 are known to enhance migration of several cell types [5-7], whereas MMP9 has the opposite effect [7]. In agreement with this, we found increased migration in fibroblasts from syndecan-4^{-/-} mice when compared to those from WT mice (Supplemental Figure S5B). As demonstrated in Supplemental Figure S4 and in line with previous work [8-11], vinculin confocal immunofluorescence revealed fewer and smaller focal adhesions in fibroblasts from syndecan-4^{-/-} mice. This may also enhance migration in syndecan-4^{-/-} cardiac fibroblasts as binding strength to the substrate will be reduced.

Supplemental Figure Legends

Figure S1. Expression of syndecan isoforms in wild-type and syndecan-4^{-/-} cardiac fibroblasts. mRNA levels of syndecan (syn) -1, -2, -3 and -4 in wild-type (WT) and syndecan-4^{-/-} (syn4^{-/-}) cardiac fibroblasts. Significance was determined by unpaired Student's *t*-test.

Figure S2. Transforming growth factor β (TGF β) is equally expressed in wild-type and syndecan-4^{-/-} cardiac fibroblasts. TGF β mRNA levels in cardiac fibroblasts from wild-type (WT) and syndecan-4^{-/-} (syn4^{-/-}) mice at 24, 48 and 72 hrs after plating on fibronectin. N=3-5. Significance was determined by two-way ANOVA with Bonferroni post-hoc test..

Figure S3. The NFAT-target gene regulator of calcineurin 1.4 is up-regulated by over-expression of syndecan-4, calcineurin or NFATc4. Regulator of calcineurin 1.4 (RCAN1.4) mRNA levels in HT1080 fibroblasts over-expressing calcineurin (CaN; A), NFATc4 (c4; B) and syndecan-4 (syn4; C), and in primary cardiac fibroblasts over-expressing syndecan-4 (syn4; D). Significance was determined by unpaired Student's *t*-test.

Figure S4. Focal adhesions are fewer and smaller in syndecan-4^{-/-} cardiac fibroblasts.

Micrographs showing vinculin staining (green) and F-actin stained with phalloidin (red) in cardiac fibroblasts from wild-type (WT) and syndecan-4^{-/-} (syn4^{-/-}) mice.

Figure S5. Expression of ECM remodeling proteins and migration of cardiac fibroblasts.

mRNA levels of matrix metalloproteinase (MMP)2, MMP9, MMP13, tissue inhibitor of metalloproteinase (TIMP)1 and TIMP2 in wild-type (WT) and syndecan-4^{-/-} (syn4^{-/-}) mice at 24 and 48 hrs after plating on fibronectin (A). N=4. Significance was determined by two-way ANOVA with Bonferroni post-hoc test. Scratch assay showing migration of WT and syn4^{-/-} cardiac fibroblasts after 24 hrs (B). Significance was determined by unpaired Student's *t*-test.

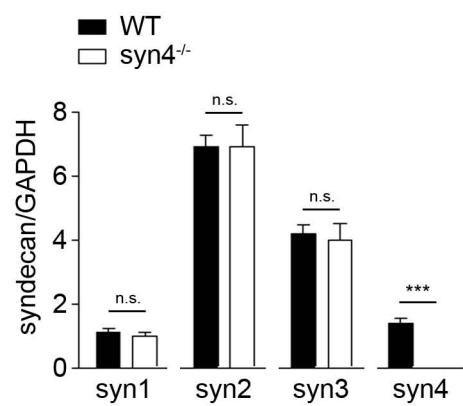


Figure S1

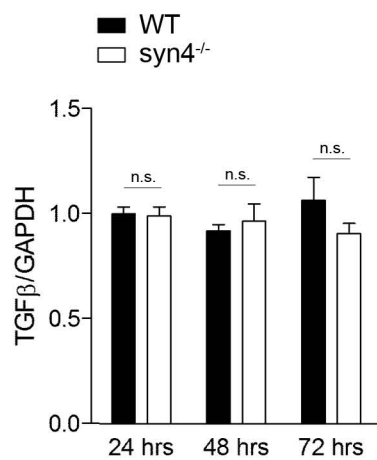


Figure S2

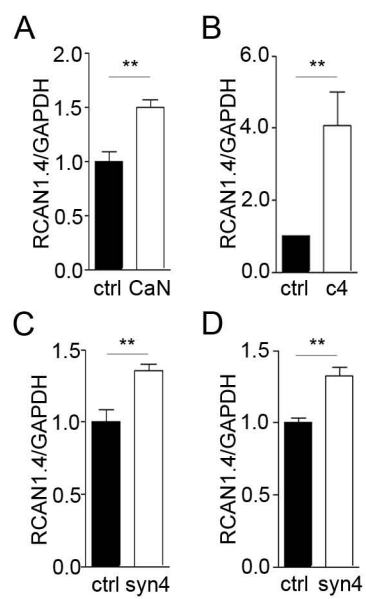


Figure S3

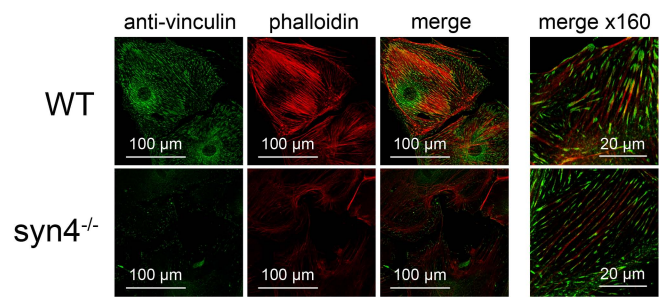


Figure S4

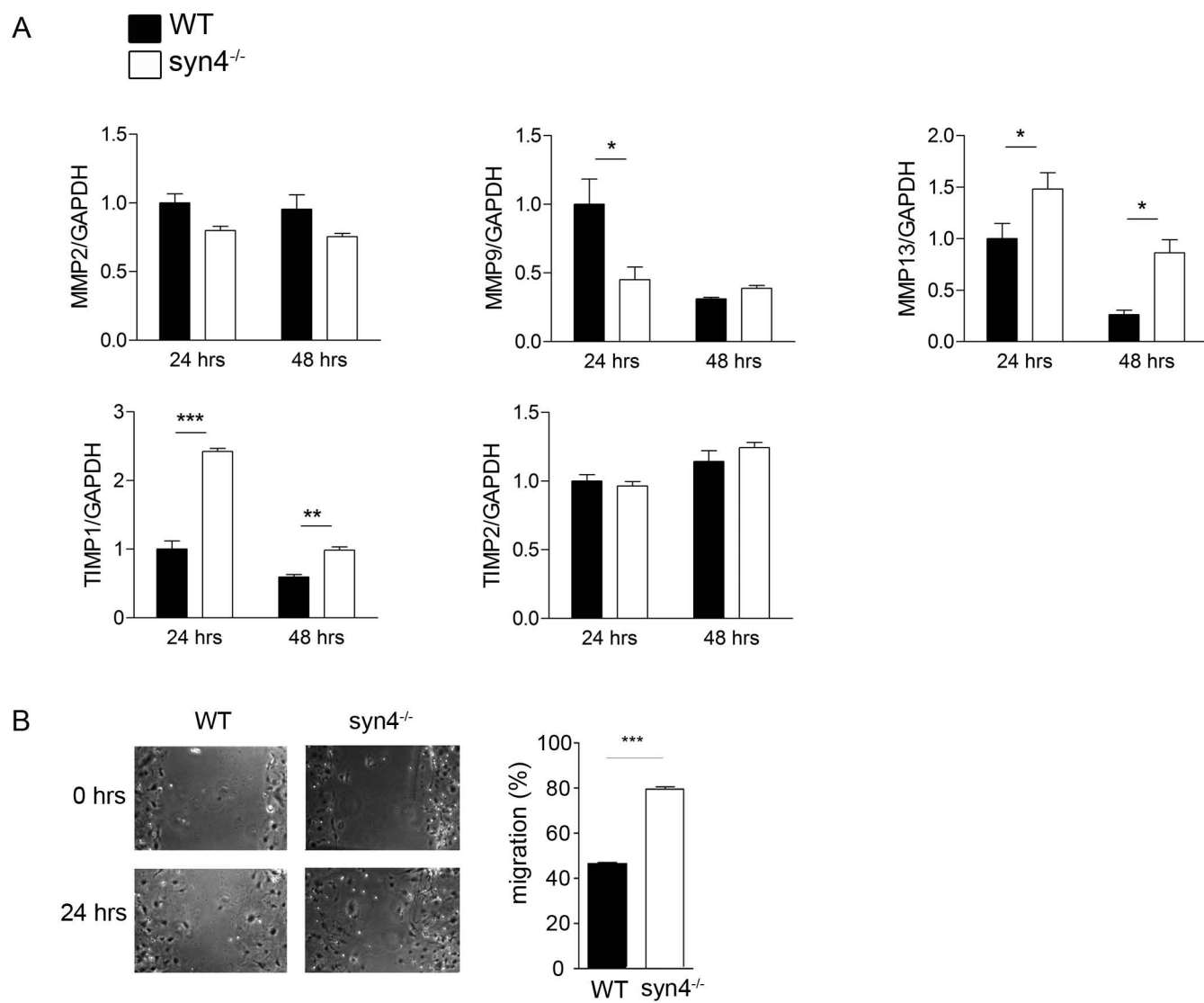


Figure S5

Supplemental Table Legends

Table S1. Characteristics of wild-type and syndecan-4^{-/-} mice after aortic banding. Characteristics of wild-type (WT) and syndecan-4^{-/-} (syn4^{-/-}) mice 24 hrs after sham operation (sham) or aortic banding (AB). BW, body weight; TL, tibia length; LVW, relative left ventricular weight; LW, relative lung weight. Two-way ANOVA (for the effects of AB and genotype) revealed significant effect of AB for BW (P<0.05), LVW/TL (P<0.01) and LW/TL (P<0.0005). Bonferroni post-hoc test yielded significant differences between sham and AB (*) as indicated in the table.

	sham		AB	
	WT	syn4 ^{-/-}	WT	syn4 ^{-/-}
N	10	10	10	10
BW, g	24.99 ± 0.91	24.90 ± 0.75	23.51 ± 0.37	23.49 ± 0.53
TL, mm	17.43 ± 0.14	17.40 ± 0.09	17.49 ± 0.07	17.45 ± 0.07
LVW/TL	1.00 ± 0.03	1.02 ± 0.03	1.20 ± 0.09*	1.15 ± 0.03
LW/TL	1.00 ± 0.03	0.96 ± 0.03	1.50 ± 0.13*	1.22 ± 0.07*

Reference List

- [1] Echtermeyer F, Streit M, Wilcox-Adelman S, Saoncella S, Denhez F, Detmar M et al. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. *J Clin Invest* 2001;107:R9-R14.
- [2] Finsen AV, Lunde IG, Sjaastad I, Østli EK, Lyngra M, Jarstadmarken HO et al. Syndecan-4 is essential for development of concentric myocardial hypertrophy via stretch-induced activation of the calcineurin-NFAT pathway. *PLoS One* 2011;6:e28302.
- [3] Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, Plank DM et al. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circ Res* 2004;94:110-8.
- [4] Lunde IG, Kvaløy H, Austbø B, Christensen G, Carlson CR. Angiotensin II and norepinephrine activate specific calcineurin-dependent NFAT transcription factor isoforms in cardiomyocytes. *J Appl Physiol* 2011;111:1278-89.
- [5] Shi ZD, Wang H, Tarbell JM. Heparan sulfate proteoglycans mediate interstitial flow mechanotransduction regulating MMP-13 expression and cell motility via FAK-ERK in 3D collagen. *PLoS One* 2011;6:e15956.
- [6] Hattori N, Mochizuki S, Kishi K, Nakajima T, Takaishi H, D'Armiento J et al. MMP-13 plays a role in keratinocyte migration, angiogenesis, and contraction in mouse skin wound healing. *Am J Pathol* 2009;175:533-46.
- [7] Xue SN, Lei J, Yang C, Lin DZ, Yan L. The biological behaviors of rat dermal fibroblasts can be inhibited by high levels of MMP9. *Exp Diabetes Res* 2012;2012:494579.
- [8] Gopal S, Bober A, Whiteford JR, Multhaupt HA, Yoneda A, Couchman JR. Heparan sulfate chain valency controls syndecan-4 function in cell adhesion. *J Biol Chem* 2010;285:14247-58.
- [9] Woods A, Longley RL, Tumova S, Couchman JR. Syndecan-4 binding to the high affinity heparin-binding domain of fibronectin drives focal adhesion formation in fibroblasts. *Arch Biochem Biophys* 2000;374:66-72.
- [10] Ishiguro K, Kadomatsu K, Kojima T, Muramatsu H, Tsuzuki S, Nakamura E et al. Syndecan-4 deficiency impairs focal adhesion formation only under restricted conditions. *J Biol Chem* 2000;275:5249-52.
- [11] Chen Y, Shi-Wen X, van BJ, Kennedy L, McLeod M, Renzoni EA et al. Matrix contraction by dermal fibroblasts requires transforming growth factor-beta/activin-linked kinase 5, heparan sulfate-containing proteoglycans, and MEK/ERK: insights into pathological scarring in chronic fibrotic disease. *Am J Pathol* 2005;167:1699-711.