Institute for Experimental Medical Research
Oslo University Hospital and University of Oslo

and

KG Jebsen Cardiac Research Centre and
Center for Heart Failure Research, University of Oslo

Syndecan-4 in Cardiac Fibroblasts

Role in Regulation of Extracellular Matrix and Myocardial Stiffness

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A love of what you do is one of the highest forms of success.
- Bear Grylls

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Abbreviations

ECM	extracellular matrix
syn4$^{-/-}$	syndecan-4 knockout
SMA	smooth muscle α-actin
AB	aortic banding
CsA	cyclosporine A
NFAT	nuclear factor of activated T cells
ED-A	extradomain A of fibronectin
EGFP	enhanced green fluorescent protein
MRTF-A	myocardin-related transcription factor A
RCAN1.4	regulator of calcineurin 1 isoform 4
AS	aortic stenosis
ADAMTS	a disintegrin and metalloproteinase domain with thrombospondin motifs
DAMP	damage-associated molecular pattern
HSPG	heparan sulfate proteoglycan
IL	interleukin
LPS	lipopolysaccharide
LV	left ventricle
MMP	matrix metalloproteinase
NF-κB	nuclear factor kappa B
PAMP	pathogen-associated molecular pattern
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
LOX	lysyl oxidase
GAG	glucosaminoglycan

List of papers in thesis

1. **Syndecan-4 signalling via NFAT regulates extracellular matrix production and cardiac myofibroblast differentiation in response to mechanical stress.**
   Herum KM, Lunde IG, Skrbic B, Florholmen G, Behmen D, Sjaastad I, Cathrine R. Carlson, Maria F. Gomez, Geir Christensen.
   J Mol Cell Cardiol. 2013;54:73-81.
   Commented on in Editorial.[1]

2. **Syndecan-4 is a key determinant of collagen cross-linking and passive myocardial stiffness in the pressure-overloaded heart.**
   Herum KM, Lunde IG, Skrbic B, Louch WE, Hasic A, Boye S, Unger A, Brorson SH, Sjaastad I, Tønnessen T, Linke WA, Gomez MF*, Christensen G*.
   *In revision. *These authors contributed equally.*

3. **Innate immune signalling induces expression and shedding of the heparan sulfate proteoglycan syndecan-4 in cardiac fibroblasts and myocytes, affecting inflammation in the pressure-overloaded heart.**
   Strand ME, Herum KM, Rana ZA, Skrbic B, Askevold ET, Dahl CP, Vistnes M, Hasic A, Kvaløy H, Sjaastad I, Carlson CR, Tønnessen T, Gullestad L, Christensen G and Lunde IG.
   FEBS J. 2013;280:2228-2247.

Introduction

Heart failure, diastolic dysfunction and myocardial stiffness

The pumping action of the heart generates the pressure needed to transport blood throughout the circulation, allowing exchange of nutrients for metabolic waste products. It is no surprise that dysfunction of this vital organ has serious consequences for physical performance and well-being. Regardless the reason, severe dysfunction of the heart results in heart failure, a condition defined by failure of the heart to pump blood at a rate commensurate with the requirements of the metabolizing tissues.[2] Despite advanced medical treatment, the 5-year survival rate is merely 40-60%[3,4] and although prevalence of heart failure is high, it is projected to further increase with the growing elderly population.

Heart failure is a result of dysfunction during systole and/or diastole. Whereas systolic dysfunction, involving compromised contraction of the heart, has been heavily studied, diastolic dysfunction has only recently become recognised as a major cause of heart failure[5,6,7,8] and has thus gained increasing amount of attention in the cardiovascular research community. Defined by abnormal relaxation and/or decreased compliance of the ventricles,[9] diastolic dysfunction results in impaired filling of the heart in diastole. In healthy individuals, the major risk factor for diastolic dysfunction is age. However, any condition that leads to stiffening of the ventricles can result in diastolic dysfunction, e.g. pressure overload as in hypertensive and aortic stenosis patients. Myocardial stiffness is largely determined by the structure and composition of the extracellular matrix (ECM), and diastolic dysfunction is often a result of myocardial fibrosis. *There is currently no effective medical treatment for myocardial fibrosis, reflecting the need for a better understanding of its molecular basis. This comprises the background for the overall motivation of the work summarised in this thesis.*

All cells in all tissues are constantly affected by the mechanical properties of the extracellular environment, while these same cells are generating forces that regulate the extracellular environment. In this way, equilibrium is achieved thereby maintaining tissue structure and function and enabling physiological adaption to signals that result in normal growth and development. In the heart, the extracellular environment is mainly regulated by cardiac fibroblasts. These are major producers and organisers of ECM and are highly plastic cells that adjust their phenotype and function to meet the challenges of the

mechanical load that is constantly inflicted upon the heart. When the balance between mechanical stress and cellular response is disturbed, pathological remodelling may occur compromising cardiac function and ultimately causing heart failure. *In this thesis, we have investigated the role of the transmembrane proteoglycan syndecan-4 in transducing mechanical stress into cellular changes in cardiac fibroblast function and phenotype, and studied the consequence of these changes on ECM remodelling and myocardial stiffness.*

Cardiac fibroblasts and myofibroblasts

Cardiac fibroblasts comprise more than 50% of the total cell population in the human heart[10,11] and up to 30% of the total cells in the murine heart.[12] These plastic cells are responsible for a balanced production and turnover of ECM. In response to pressure overload of the heart, cardiac fibroblasts proliferate, boost ECM synthesis and develop contractile forces that act on the freshly produced ECM thereby remodelling tissue structure to meet the increased mechanical burden.[13] Hence, they are smooth muscle cell-like in terms of expressing markers defining smooth muscle cells such as smooth muscle α-actin (SMA)[14] 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.[15]

Of particular relevance for myocardial stiffness is the high expression of fibrillar collagen I that is maturated into large collagen fibers through extensive cross-linking mediated by the enzyme lysyl oxidase, providing tensile strength to the ECM. In the normal adaptive response, myofibroblasts will undergo apoptosis[16] as ECM tension is restored and again takes over the mechanical load. However in situations where mechanical load continues to be elevated such as in patients with chronic hypertension, persistent myofibroblast activity and development of myocardial fibrosis is observed.[17] Also of interest and in agreement with these results, a recent report suggests that cardiac myofibroblasts may dedifferentiate back into fibroblasts when mechanical tension is decreased or in the presence of a transforming growth factor β (TGFβ)-receptor-I kinase blocker.[18] Whether one or both of these interventions were needed to accomplish dedifferentiation, depended on the proliferative status of the myofibroblasts indicating the presence of multiple degrees of differentiation. It will be important to determine whether these findings also apply to myofibroblasts *in vivo*.

The fibroblast-myofibroblast phenotypes do not reflect one single cell type but rather a continuum of functional statuses, and to date no markers exclusively expressed in myofibroblast have been identified. Myofibroblasts are most commonly defined by the high expression of collagen I and *de novo* expression of SMA and the extradomain A (ED-A) splice variant of fibronectin.[19] Also, platelet-derived growth factor receptor β (PDGFRβ) has recently arisen as a promising myofibroblast marker in several tissues.[20,21] The heterogeneity of myofibroblasts may also reflect their many potential cellular origins. This issue has been debated over several years and has been deluded by the observation that many cell types will adopt a myofibroblastic phenotype *in vitro* when stimulated with TGFβ. The traditional view is that myofibroblasts in the heart derive from resident cardiac fibroblasts, but endothelial cells,[22] bone-marrow-derived circulating progenitor cells[23,24] and pericytes[25] surrounding blood vessels may also contribute.

TGFβ is a potent inducer of cardiac myofibroblast differentiation.[26,27] A requirement for TGFβ-induced myofibroblast differentiation is the expression of ED-A[19] and mechanical stress.[28] This may indicate that TGFβ is secondary to mechanical stress-induced changes, emphasizing the importance of studying mechanosensing of cardiac fibroblasts. Although it is well-established that mechanical stress induces differentiation of cardiac fibroblasts into myofibroblasts,[29] the underlying mechanisms are largely unknown.

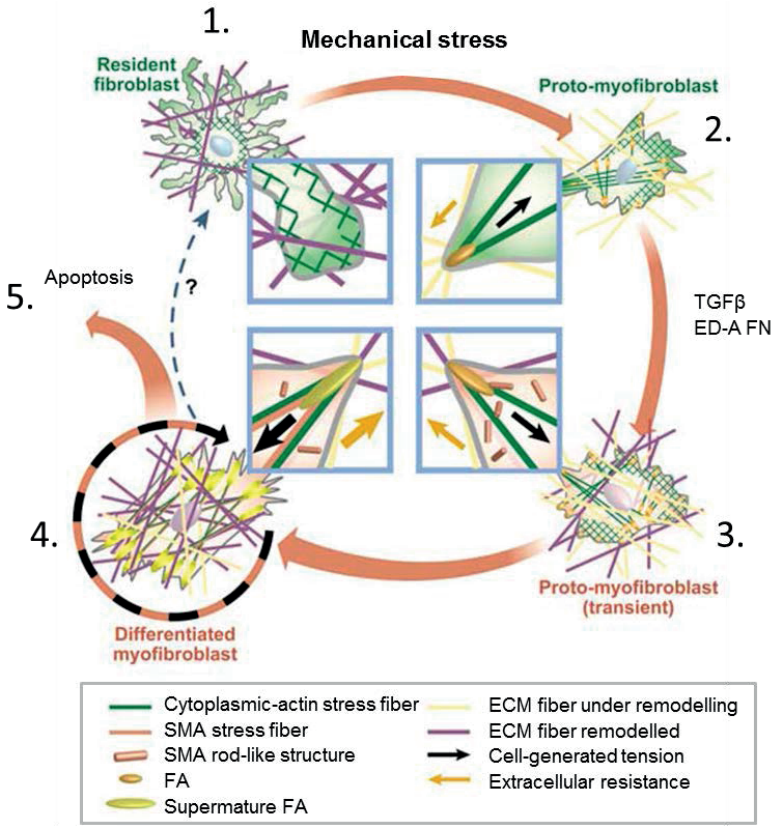

Figure 1. The mechanical loop of cardiac myofibroblast differentiation.

1) Quiescent fibroblasts residing in the cardiac tissue have low extracellular matrix (ECM) producing activity and regulate the basic turnover of ECM. 2) In response to mechanical stress, such as in the pressure-overloaded heart, cardiac fibroblasts start producing ECM and forming actin stress fibers and focal adhesions (FA) that apply traction forces on the ECM thereby increasing matrix stiffness. 3) This further promotes myofibroblast differentiation increasing FA size and initiating expression of ED-A splice variant of fibronectin (FN). Transforming growth factor β (TGFβ) is produced or released from the ECM[20,30] and works in a paracrine and autocrine fashion to induce expression of α-smooth muscle actin (SMA) and increase ECM production. Again, all these properties promote ECM stiffening. 4) FAs will keep growing in size and become "super mature", and SMA molecules will incorporate into functional SMA stress fibers with high contraction force, hence marking the completion of the myofibroblast phenotype. Myofibroblasts persist as long as mechanical stress continues to be elevated, producing more and more ECM in a viscous cycle until cardiac fibrosis finally compromises cardiac function to a degree where transition to heart failure is unavoidable. 5) Alternatively, myofibroblasts may ultimately undergo apoptosis when the ECM maturates and takes over the mechanical load. Whether myofibroblasts may dedifferentiate back into quiescent resting cardiac fibroblast when mechanical stress in alleviated *in vivo*, is not entirely clear. Modified with permission from Hinz, 2007.[31]

Syndecan-4 in cardiac fibroblasts: one protein, multiple roles

Syndecans are a family of evolutionary ancient transmembrane heperan sulfate proteoglycans which consists of four family members (syndecan-1-4) in mammals.[32] The core proteins of syndecans have a cytoplasmic part, a transmembrane region and an extracellular domain (ectodomain). The intracellular cytoplasmic part consists of two constant regions (C1 and C2) that are conserved among the syndecan family, and a variable (V) region that is unique to each syndecan.[33] Multiple interaction partners including cytoskeletal proteins, adaptor proteins and signalling molecules have been identified as being essential for syndecan structure and function.[34,32] In conjunction with the cytoplasmic domain of syndecan-4, the transmembrane domain is responsible for syndecan-4 dimerization[35,36] by forming strong twisted clamp dimers as shown by NMR structural studies.[37] These dimers may occur during synthesis, implying that the dimeric state of syndecans is the native one. The ectodomains of syndecan-1,[38] -2 and -4[39] have been found to promote integrin-mediated adhesion of mesenchymal cells although the mode of interaction is not known. The extracellular domains of syndecans display little homology but have in common that they are all substituted with glycosaminoglycan (GAG) chains, mainly heparan sulfate (HS). These GAG chains may vary in length, charge, sugar composition and degree of sulfation and may therefore also display a wide array of functions. HS can interact with numerous and different types of ligands including growth factors,[40] chemokines[41] and matricellular proteins,[42,43] and thereby possibly act as co-receptors for other cell surface receptors.[44,45,46] HS GAG chains also interact with extracellular structural proteins such as collagen I[47] and fibronectin[48] and are highly hygroscopic due to their negative charge.

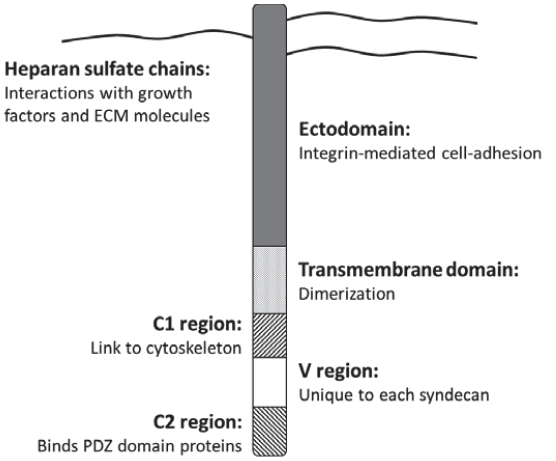

Figure 2. Summary of syndecan structure and interactions.

All four syndecans share this basic structure containing an extracellular domain (ectodomain), transmembrane domain, and a cytoplasmic domain consisting of constant regions and a variable region specific for each syndecan. Heparan sulfate GAG chains are attached to the ectodomain. The domains have different functions and interaction partners enabling syndecans to hold multiple roles in cellular function and phenotype.

Since the first description of syndecans in 1988[49] the importance of syndecans in health and disease has been a riddle. Although profound effects of syndecan-4 activation or inhibition on cell behavior are observed, mice lacking syndecan-1, -3 or -4 have only mild defects upon initial observation, suggesting some degree of redundancy among syndecans or HS-proteoglycans in general. On the other hand, syndecan-specific effects are clear when syndecan knockout mice are subjected to injury or disease.[50,51,52] The first hint that syndecans played a role in connective tissue remodelling and fibroblast function was the detection of syndecan-1 expression in granulation tissue around growing capillaries.[53] Both syndecan-1 and syndecan-4 knockout mice have since confirmed this initial observation, having delayed wound healing in skin[50] and adverse matrix remodelling after myocardial infarction increasing susceptibility to cardiac rupture.[54,55] The myocardial ECM defects of syndecan-4 knockout (syndecan-$4^{-/-}$) mice were largely attributed impaired fibroblast responses; however the exact mechanisms were not determined.[54]

Mechanical stress sensor

The existence of mechanosensing molecules in the plasma membrane of cardiac cells has long been a focus of attention. Such a mechanosensor is likely attached to the ECM as well as to the cytoskeleton, forming a physical link between extra- and intracellular structures. Transmembrane proteins with such properties are found in focal adhesions (FAs). FAs are points in the cell membrane that constitute the binding sites to the ECM and are likely to

be involved in mechanosensing and mechanotransduction.[56,57] FAs consist of cell binding receptors such as integrins, cytoskeletal proteins, and signalling molecules, many of which are known to interact with syndecan-4. These include syntenin,[58] CASK[59], synectin,[60] focal adhesion kinase (FAK)[61] and protein kinase C (PKC).[62,36] During myofibroblast differentiation FA size and strength increase, developing into "super mature" FAs[63] which may affect downstream signalling molecules.

Located in FAs, and being attached both extracellularly to fibronectin[64] and intracellularly to the cytoskeleton through α-actinin,[65,66] syndecan-4 is an attractive candidate for a mechanosensor.[67] In a previous study by our group,[51] syndecan-4 was found to play such a role in cardiomyocytes inducing concentric hypertrophic remodelling of the left ventricle in response to pressure overload. The underlying mechanism for this effect involved activation of the transcription factor nuclear factor of activated T cells (NFAT) which is a well-known inducer of hypertrophy in the pressure-overloaded heart.[68,69] NFAT is heavily phosphorylated in its inactive state and changes conformation following dephosphorylation thereby revealing a nuclear localisation signal causing nuclear translocation and transcription of target genes.[70,71] Although NFAT has been extensively studied in cardiomyocytes, the role of NFAT in cardiac fibroblasts is highly elusive. There are four calcium-regulated NFAT isoforms (NFATc1-4), all expressed in cardiac fibroblasts at the mRNA level, NFATc3 having the highest expression, followed by NFATc1, NFATc4 and NFATc2.

Dephosphorylation of NFAT is performed by the Ca^{2+}-sensitive phosphatase calcineurin.[72] The intracellular part of syndecan-4 was found to bind to the autoinhibitory domain of calcineurin when syndecan-4 was dephosphorylated at serine179, and experiments assessing NFAT activation suggested that this binding activated calcineurin and subsequently NFAT. Indeed, activation of this syndecan-4/calcineurin/NFAT signalling pathway was present in the pressure-overloaded hypertrophic heart and in mechanically stressed cardiomyocytes,[51] but its presence in cardiac fibroblasts was not examined. *In Paper 1 we examined a possible mechanosensing role for syndecan-4/calcineurin/NFAT in cardiac fibroblasts, inducing myofibroblast differentiation and ECM production. Since these are important for regulating mechanical properties of the heart, we examined the consequence of syndecan-4 deletion on myocardial stiffness in Paper 2.*

Organiser of cytoskeleton and extracellular matrix assembly

Syndecan-4 plays a central role in cytoskeletal organization and focal adhesion assembly and turnover,[44,73] having profound effects on migration,[74] adhesion[75] and cell spreading.[64] The mechanism for this involves PKCα activation[76] in response to a conformational change of syndecan-4 that occurs upon binding of phosphatidylinositol 4,5-bisphosphate (PIP2) to the cytoplasmic domain of syndecan-4. This also leads to clustering of syndecan-4, coordinated by PDZ domain-containing scaffold proteins, and interaction of syndecan-4 with the cytoskeleton via α-actinin.[77] PKCα activates the small GTPase RhoA[73] and its kinases leading to focal adhesion assembly and stress fiber formation through their phosphorylation of myosin phosphatase and myosin II.[78] Fibroblasts lacking syndecan-4 have smaller focal adhesions and defect cytoskeleton substantiating the role of syndecan-4 in organizing these structures.

Many ECM structural proteins are assembled into fibers at the cell surface after secretion where cell surface receptors bind and initiate fibrillogenesis. Although focal adhesions are thought to be important matrix assembly points, the exact role of syndecan-4 in matrix assembly is not known. The heparin-binding domains of fibronectin are required for fibrillognesis[79] and studies manipulating syndecan-2 structure or expression reveal impaired assembly of laminin and fibronectin at the cell surface.[80,81] Syndecan-4 may also interact with collagen I through its GAG chains,[47] although the effect of this interaction of collagen fibrillogenesis is unknown. *In Paper 2 we extend our findings on the regulatory role of syndecan-4 cytoplasmic domain, to explore the role of the extracellular domain of syndecan-4 in regulating collagen fibril assembly and cross-linking.*

Expression and shedding

Cardiac syndecan-4 expression has been shown to be increased in the hypertrophic myocardium of mice after myocardial infarction[82] and in human aortic stenosis.[51] Reports from other cell types indicate a role for mechanical stress,[83] tumor necrosis factor α (TNFα)[84,85,86] and interleukin 1β (IL-1β)[85] in regulating syndecan-4 expression. TNFα and IL-1β are cytokines associated with the innate immune system which is activated upon tissue injury and exposure to pathogens.[87] TNFα and IL-1β were initially discovered in cells of the immune system[88,89] but have since proven to be present in a wide variety of cell types, including cardiac cells.[90,91,92,93] Importantly, TNFα and IL-1β are rapidly (within 2h)

upregulated after inducing left ventricular pressure overload by aortic banding (AB; unpublished results).

The ectodomain of syndecan-4 can be shed by proteolytic cleavage. This is a highly regulated process involving the direct action of "sheddase" enzymes.[94] In the heart, syndecan-4 shedding is elevated in response to myocardial injury and disease as indicated by increased plasma levels after myocardial infarction in humans[95] and in serum from patients with chronic heart failure.[96] Syndecan-4 shedding may play several roles in the cardiac response to mechanical stress such as decrease mechanosensing by syndecan-4 and disrupt cellular attachment to ECM thus abrogating down-stream signalling of syndecan-4 and promoting migration, respectively. In addition, the shed ectodomain may itself act as a soluble effector or antagonist,[94] or create a chemotactic gradient for leukocyte infiltration of the myocardium.[97]

The question whether syndecan-4 shedding is good or bad for cardiac function remains to be answered. In studies of myocardial infarction, overexpression of the extracellular shed part of syndecan-4 increased mortality,[54] whereas overexpression of full-length syndecan-4, showed reduced mortality and improvement of cardiac function.[98] The role of shedding in the pressure-overloaded heart is not known. However, syndecan-4 shedding was induced by stretch of vascular smooth muscle cells,[99] indicating regulation in response to mechanical stress. *In Paper 3 we investigate the regulation of syndecan-4 expression and shedding, and examine its effect on inflammation and focal adhesion assembly.*

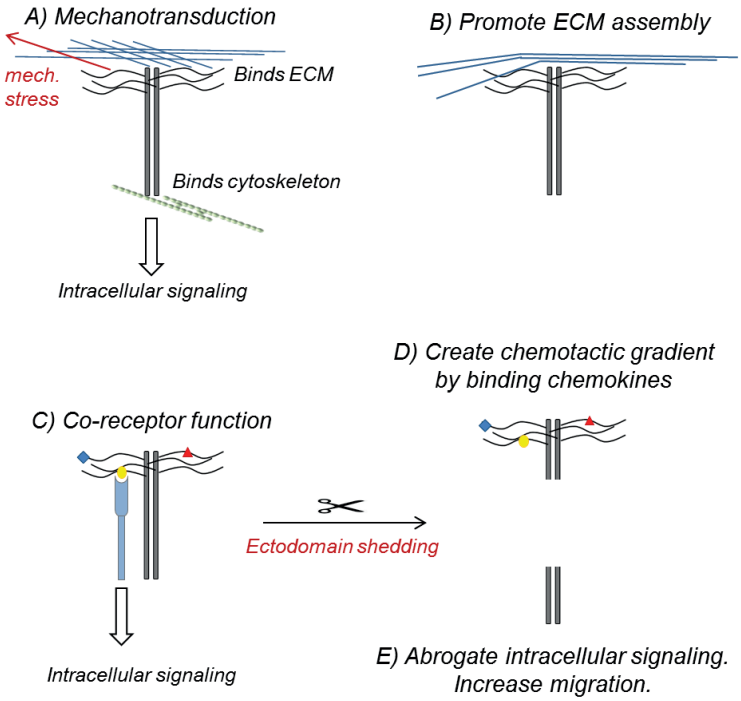

Figure 3. Multiple proposed roles for syndecan-4.

The location of syndecan-4 in focal adhesions (FAs) suggests that it plays a role in sensing and transducing mechanical stress. B) Heparan sulfate chains of syndecan-4 interact with extracellular matrix (ECM) molecules promoting assembly into higher order ECM structures. C) Syndecan-4 acts as a co-receptor enhancing signalling by soluble factors such as growth factors. D) Enzymatic shedding of syndecan-4 ectodomain may enable the creation of a chemotactic gradient by binding and thereby increasing the concentration of chemokines in the ECM. E) The deprived attachment to ECM after shedding abrogates intracellular signalling and may enhance cell migration.

Aims of the thesis

The overall aim of this thesis was to investigate the role of syndecan-4 in cardiac fibroblast function and phenotype, ECM remodelling and myocardial stiffening of the pressure-overloaded heart.

The specific aims of the separate studies were:

1. To study whether syndecan-4 signals via calcineurin/NFAT to regulate myofibroblast differentiation and ECM production in response to mechanical stress

2. To examine the role of syndecan-4 in regulating myocardial stiffness and explore if syndecan-4 may be important for collagen cross-linking

3. To investigate the signals and mechanisms that induce cardiac syndecan-4 expression and shedding

Methodological considerations

To study the role of syndecan-4 in mechanical stress-induced changes we use model systems of mechanical stress *in vivo* and *in vitro,* as well as "gain and loss of function" approaches where we manipulate the expression of syndecan-4 in mice and in fibroblast cultures. This enables us to study the consequence of lacking syndecan-4 on the mechanical stress response of the heart and study molecular mechanisms by use of cell biology techniques such as syndecan-4 overexpression.

Models of mechanical stress

Animal model of left ventricular pressure overload

Mechanical stress is elevated in patients with pressure overload of the left ventricle, i.e. hypertension or aortic stenosis. To mimic aortic stenosis, AB of the ascending aorta was performed. As controls, sham-operated animals underwent an identical operation but without tightening the suture around the aorta. To ensure successful AB, flow velocity across the aortic stenosis was determined with echocardiography 24h after AB. Animals with an aortic flow velocity > 4 m/s after AB were included in the study. After 24h or 7 days, echocardiography was performed as previously described,[51,100] obtaining left ventricular dimensions and parameters of cardiac function. Animals were sacrificed 24h, 3 days or 7 days after AB, and lung and left ventricular weight were obtained as an indication of congestive heart failure and hypertrophy, respectively. Left ventricular tissue was snap frozen or fixed for further analysis. Although using mouse models to study human disease have obvious limitations such as body size, heart rhythm, mode of left ventricular pressure overload onset (immediate pressure overload in AB mouse model v.s. gradual increase in humans) and time frame for development of disease (days and weeks in AB mouse model v.s. several years in humans), the AB mouse model of left ventricular pressure overload is considered a valid model of concentric hypertrophy of the heart.[101]

Mechanical stress in vitro

To study mechanisms involved in mechanotransduction and cardiac myofibroblast differentiation *in vitro*, we isolated primary cardiac fibroblasts from left ventricles of neonatal and adult mice.[51] The mechanical tension of the culturing conditions will itself induce myofibroblast differentiation of primary cardiac fibroblasts.[28] In addition,

fibronectin enhances attachment of cardiac fibroblasts and drives myofibroblast differentiation.[102] Although this makes studies on cardiac fibroblasts challenging, we took advantage of these two properties and studied the process of myofibroblast differentiation by examining cardiac fibroblasts at different time points after culturing them on fibronectin. Although SMA protein amount reflects myofibroblast phenotype, it is not always coherent with fiber formation. Thus, myofibroblast differentiation was determined by the appearance of SMA stress fibers by immunocytochemistry. The limitations of this myofibroblast differentiation model are mainly associated with the type of mechanical stress induced by the culturing conditions which is unlike the cyclic mechanical stretch that cardiac fibroblasts experience *in vivo*. Also, the ECM *in vivo* is composed of a wide variety of proteins and not just fibronectin as studied here. That said, highly controlled culturing conditions are needed to get reliable and consistent results *in vitro*, since the cardiac fibroblast exhibit high plasticity. The myofibroblast differentiation model used here was simple, consistent and, in our opinion, appropriate for the purpose.

Although immortalised cell lines such as the fibrosarcoma human cell line HT1080 are cultured for long periods of time and for several passages, they have fewer features of myofibroblasts than primary cardiac fibroblasts in culture. To induce myofibroblast differentiation of this cell line, we subjected them to 10% cyclic (1 Hz) mechanical stress by using the FlexCell tension system, thereby mimicking the mechanical stress present in the heart.[103] Since FlexCell stretch membranes are unsuitable for immunocytochemistry, myofibroblast differentiation was determined by upregulation of the early myofibroblast marker ED-A FN.[19]

Gain and loss of function studies

Genetically modified mice

Genetically modified mouse models have enabled huge progress in understanding the importance of specific proteins *in vivo*, and are widely used in basic research. We have used syndecan-4$^{-/-}$ mice to study the role of syndecan-4 in the pressure-overloaded heart. To study the regulation of NFAT transcriptional activity, we utilised NFAT-luciferase reporter mice[69] that express the luciferase enzyme when NFAT is activated. Luciferase enzymatic activity can then be measured as luminescence when luciferin (the substrate of luciferase) is added to the protein lysate of homogenised tissue or cell cultures. To study

the role of syndecan-4 in NFAT activation, syndecan-4$^{-/-}$-NFAT-luciferase reporter mice (generated in our laboratory) were used.

The syndecan-4$^{-/-}$ mouse is a complete knockout, meaning that syndecan-4 protein is lacking in all cells of the body which may complicate interpretation of *in vivo* data regarding cell-specific functions of syndecan-4.[50] Also, syndecan-4 protein will be absent from the time of birth. Thus, compensatory mechanisms may have emerged or developmental processes may have been disturbed, thereby affecting the phenotype of the adult mouse. Regardless, "loss of function" mouse models comprise powerful tools in medical research.

Manipulating gene expression in vitro

The use of primary cells in culture enables discrimination of responses in cardiac fibroblasts and cardiomyocytes, which are both present in tissue homogenates harvested directly from the left ventricle. In the studies included in this thesis, primary neonatal cardiomyocytes and cardiac fibroblasts from neonatal and adult syndecan-4$^{-/-}$ mice have been isolated to examine changes in cellular responses when syndecan-4 is lacking. Although cardiac cell types are in constant interaction within the heart, cell-specific *in vitro* studies provide valuable insight into cell function, phenotype and molecular signalling mechanisms.

"Loss of function" studies in the primary cells from syndecan-4$^{-/-}$ mice can be complimented by "gain of function" experiments. Plasmids containing full-length syndecan-4 cDNA under the control of a strong promoter were transfected into fibroblasts and thereby caused syndecan-4 overexpression. Since primary cell lines are difficult to transfect, we used a fibroblast cell line for this purpose. Often such "gain of function" studies strengthen findings from "loss of function" experiments showing opposite effects, but sometimes results are conflicting and interpretation becomes a challenge. The biology of living cells harbors a plethora of regulatory mechanisms that fine tune cell behavior and activity. Thus, the amount of protein does not always translate into effect. Transfection with plasmids also enables introduction of genes encoding modified proteins, e.g. carrying fluorescent tags such as enhanced green fluorescent protein (EGFP). In Paper 1, we utilised EGFP-NFAT fusion proteins which allowed us to determine cellular localization of EGFP-NFATc1-4 and thereby examine which NFAT isoforms were activated (determined by nuclear translocation) in response to mechanical stress.

Myocardial stiffness

The combined passive mechanical properties of the myocardium such as viscosity and elasticity constitute the myocardial stiffness. Viscous stress reflects the stretch velocity sensitive component of the muscle, and is highly determined by the amount of water present in the tissue. On the other hand, passive tension is dependent on the amplitude of stretch and reflects the elastic properties of the muscle. In the heart, passive tension is largely determined by the cytoskeletal spring-like protein of cardiomyocytes called titin, and the composition and structure of ECM. In response to changes in mechanical load such as in the pressure-overloaded heart, modifications in all these properties may take place and thereby increase myocardial stiffness.

In Paper 2 we determined the passive tension of myocardial tissue strips isolated from the left ventricular wall. The strips, about 2 mm in length and 0.5 mm in width, were dissected parallel to muscle fiber bundle orientation. To eliminate the active component of myocardial stiffness, skinning of the preparations was performed using 0.5% triton in calcium free solution. To eliminate the contribution of titin to passive tension, salt extraction of actin and myosin filaments was performed by treatment with 0.6 M KCl for 20 min and 1 M KI for 20 min.[104]

Passive tension was measured by mounting the strips between two stainless steel clips attached to a micromotor and force transducer. It is essential for accuracy to standardise the initial length from where stretching is started (L_0). Slack length was used as L_0 in this study and strips were stretched in equal steps to 30% of L_0. Ideally L_0 is set by measuring sarcomere length of the cardiomyocytes in the preparation. By using laser diffraction[105,106] we were able to verify in three muscle strips that the applied range of stretch corresponded to a sarcomere length of 1.8 – 2.3 μm. Passive tension was obtained by dividing force with the cross-sectional area at the thinnest point of the strip. Disruption of sarcomere structure was verified by subsequent examination of muscle strips with electron microscopy. Viscous stress was determined as peak force upon stretching minus steady-state force, divided by cross-sectional area.

Collagen expression and maturation

Fibrous collagens are macromolecules that have a high content of hydroxyproline. Hence, a standard procedure for quantifying total collagen is to measure hydroxyproline content with high-performance liquid chromatography (HPLC). However, not only the amount of protein but also the degree of collagen cross-linking is important for tensile strength of the collagen matrix.

Collagen cross-linking can be assessed both quantitatively and qualitatively by staining with picrosirius red. Picrosirius red stains all collagen a deep red colour and can be visualised in tissue sections of the heart using standard light microscopy. Also, this linear molecule binds parallel to the long axes of collagen fibers, thereby enhancing birefringence of collagen that can be visualised when the sample is illuminated with polarised light.[107] To quantify collagen cross-linking, we stained thick (20 μm) tissue sections of the left ventricle with two different dyes: Picrosirius red to stain total collagen and fast green to stain total non-collagen protein. These dyes are readily extracted from the stained tissue and the amount of collagen relative to total protein can be calculated based on absorbance at λ540 (for picrosirius) and λ620 (for fast green). To assess the amount of soluble collagen (not cross-linked), tissue sections were incubated with pepsin overnight. The amount of pepsin-solubilised collagen was determined by a colorimetric assay that is also based on picrosirius red staining. Finally, cross-linked collagen could be determined by subtracting soluble collagen from total collagen. This protocol for quantitating cross-linked collagen was adapted from the Diez group in Pamplona[108] after a research visit by members of our group.

To study the role of the extracellular domain of syndecan-4 in collagen fiber assembly *in vitro*, recombinant syndecan-4 consisting of the extracellular part of syndecan-4 fused with the Fc region of human IgG_1 was mixed with collagen I in a test tube. The effect of adding recombinant human lysyl oxidase homolog 2 was also examined. Turbidity of the solution served as a measure of collagen fibrillogenesis and samples were further examined with electron microscopy after negative staining with 2% aqueous uranyl. Although this is a highly artificial and non-physiological method, it does shed light on the ability of the ectodomain of syndecan-4 to facilitate collagen I fiber formation and how it may play a role in matrix assembly *in vivo*.

Molecular biology techniques

Additional molecular biology techniques used in this thesis include: quantitative real-time PCR, LOX activity assay, migration scratch assay, ELISA for syndecan-4 in plasma, adenoviral transduction of primary cardiac fibroblasts, western blot, proximity ligation assay and immunocytochemistry. A challenge with several of these methods is the dependency on antibodies, where specificity will always be a question. To increase the soundness of our antibody-based results we have complemented with other methods, used antibodies with verified specificity (such as pNFATc4/NFATc4[109]), and used blocking peptides (for the proximity ligation assay) and multiple negative controls when applicable.

Summary of Results

Paper 1

In this study we demonstrated the importance of syndecan-4 in differentiation of cardiac fibroblasts into myofibroblasts. Mechanically-stressed cardiac fibroblasts from syndecan-$4^{-/-}$ mice had impaired SMA production and fiber formation *in vitro*, and markers of myofibroblasts were reduced *in vivo* following AB. Furthermore, mRNA of central ECM genes such as collagen I, III and fibronectin were upregulated in a syndecan-4-dependent manner in response to pressure overload. We also showed that NFAT is activated in cardiac fibroblasts in response to mechanical stress. This activation was dependent on syndecan-4 and calcineurin, demonstrated by reduced NFAT activity in cardiac fibroblasts from syndecan-$4^{-/-}$ mice and in the presence of the calcineurin inhibitor cyclosporine, respectively. By utilizing EGFP-NFAT fusion proteins, NFATc4 was found to be the NFAT isoform that was activated by mechanical stress. Accordingly, over-expression of NFATc4 upregulated collagen III and myocardin-related transcription factor A (MRTF-A), the latter being a transcriptional regulator of SMA. Syndecan-4 and calcineurin were localised closely together suggesting interaction between the two molecules. This possible interaction may be promoted when syndecan-4 is dephosphorylated at serine179, which has previously been shown to be critical for interaction between calcineurin and syndecan-4 cytoplasmic domain in response to mechanical stress.[51] We concluded from this study that syndecan-4 engages the calcineurin/NFAT pathway to induce differentiation of cardiac fibroblasts into myofibroblasts and ECM production in response to mechanical stress.

Paper 2

In this study we found that passive tension increased in response to AB (7 days AB), but the response was blunted in syndecan-$4^{-/-}$ mice. Disruption of titin anchoring by salt extraction of actin and myosin filaments revealed that the effect of syndecan-4 on passive tension was due to ECM remodelling. Expression and activity of the cross-linking enzyme lysyl oxidase (LOX) increased with mechanical stress and was lower in left ventricles and cardiac fibroblasts from syndecan-$4^{-/-}$ mice, which exhibited less collagen cross-linking after 7 days AB. Expression of osteopontin (OPN), a matricellular protein able to induce

LOX protein in cardiac fibroblasts, was upregulated in hearts after AB, mechanically-stressed fibroblasts and fibroblasts overexpressing syndecan-4, calcineurin or NFAT, but downregulated in fibroblasts lacking syndecan-4 or after NFAT inhibition. Interestingly, the extracellular domain of syndecan-4 facilitated LOX-mediated collagen cross-linking. Hence, syndecan-4 appears to exert a dual role in collagen cross-linking, one involving its cytosolic domain and NFAT signalling leading to collagen, OPN and LOX induction in cardiac fibroblasts; the other involving the extracellular domain promoting LOX-dependent cross-linking.

Paper 3

In vivo, syndecan-4 mRNA expression was upregulated by 24h and 1 week AB-induced pressure overload. *In vitro*, TNFα, IL-1β, and lipopolysaccharide (LPS), induced syndecan-4 mRNA in both cardiac myocytes and fibroblasts. Bioinformatical and mutational analyses in human embryonic kidney 293 (HEK293) cells identified a functional site for the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB) in the syndecan-4 promoter and indeed, NF-κB regulated syndecan-4 mRNA in cardiac cells. Interestingly, TNFα, IL-1β and LPS also induced NF-κB-dependent shedding of the syndecan-4 ectodomain from cardiac cells. To examine the specific cleavage site of syndecan-4, overexpression experiments of syndecan-4 with mutated enzyme-interacting domains were performed and suggested enzymes dependent on binding to heparan sulfate to regulate shedding. LPS-induced shedding reduced FA size of cardiac fibroblasts, suggesting that inflammation-induced shedding affects cardiac fibroblast function. After AB, a time-dependent cardiac recruitment of T lymphocytes was observed by measuring CD3, CD4 and CD8 mRNA, which was reduced in syndecan-$4^{-/-}$ hearts. Finally, syndecan-4 mRNA and shedding were upregulated in failing human hearts. Conclusively, our data suggest that syndecan-4 plays an important role in the immune response of the heart to increased pressure, influencing cardiac remodelling and failure progression.

Discussion

Mechanosensing by syndecan-4 in cardiac fibroblasts induces myofibroblast differentiation

Syndecan-4 is located in focal adhesions, sites known to be important for mechanotransduction in adherent cells.[110] Here, transmembrane proteins enable bi-directional signalling between the cytoskeleton and ECM. In response to pressure overload of the left ventricle, syndecan-4 is rapidly upregulated as demonstrated in Paper 1 and 3. Similar to vascular smooth muscle cells,[83] mechanical stress induced syndecan-4 expression in cardiomyocytes, but the *in vitro* mechanical stress applied for these experiments (10% stretch, 1 Hz, 24h) had no effect on syndecan-4 expression in cardiac fibroblasts. The proinflammatory cytokines IL-1β and TNFα caused upregulation syndecan-4 in both cardiomyocytes and cardiac fibroblasts through activation of NF-κB. Thus, upregulation of syndecan-4 in cardiac fibroblasts seems to be triggered by autocrine and paracrine signalling induced by pressure overload more than mechanical stress *per se*. Left ventricular syndecan-4 expression peaks at 6h post AB (unpublished data) and declines back to initial levels 3 weeks after AB. In light of the rapid upregulation in the pressure-overloaded heart, it seemed likely that syndecan-4 might play a pivotal role in the immediate response to mechanical stress and possibly trigger other signalling pathways that take over at later time points. Based on previous findings by others[67] and us[51,82] and the work presented in Paper 1 and 2, we propose syndecan-4 to be a transducer of mechanical stress in cardiac fibroblasts that is active in initial stages of the pressure overload response.

Bellin and collegues were the first to show a direct role for syndecan-4 in mechanotransduction.[67] By using antibodies directed toward the ectodomain of syndecan-4 they were able to specifically study extracellular binding properties of syndecan-4. Application of mechanical stress exclusively to syndecan-4 caused activation of specific mechanosensitive signalling pathways in NIH 3T3 fibroblasts.[111] This mechanotransduction was dependent on intact actin filaments. In Paper 1 we found impaired SMA fiber formation and others have reported reduced RhoA activity in cardiac fibroblasts from syndecan-4$^{-/-}$ mice,[54] suggesting altered cytoskeletal dynamics in cells lacking syndecan-4, thus contributing to the blunted fibroblast response to mechanical stress.

In Paper 1 and 2 we demonstrated impaired cardiac myofibroblast differentiation in response to mechanical stress *in vitro* (SMA fiber formation) and *in vivo* (immunohistochemistry for SMA and PDGFRβ) when syndecan-4 was lacking. Results from Paper 1 indicated that the mechanism for syndecan-4–dependent myofibroblast differentiation involved dephosphorylation of serine179 in the cytoplasmic domain of syndecan-4 thereby possibly functioning as a mechanical stress-sensitive switch for activation of the calcineurin/NFAT signalling pathway. Overexpression of NFATc4 enhanced the expression of collagen III, MRTF-A (Paper 1) and OPN (Paper 2). Apart from a few studies showing that NFAT can regulate collagen I,[112,113] SMA[114] and OPN[115] expression in other tissues, syndecan-4/calcineurin/NFAT regulation of myofibroblast differentiation and ECM production was a novel finding in this study.

Although little is known regarding the role of NFAT in cardiac fibroblasts, studies manipulating cardiac calcineurin and NFAT activity *in vivo* have indicated a role for NFAT in cardiac fibrosis,[116,117,118,119] and blocking NFAT *in vivo* and *in vitro* reduced fibrosis and expression of matricellular proteins, respectively, in response to mechanical stress.[120] Furthermore, recent publications have examined a potential role for NFAT in regulating cardiac fibroblast phenotype. In primary cardiac fibroblasts isolated from the left atria of dogs subjected to atrial fibrillation, NFAT activity was increased leading to proliferation of cardiac fibroblasts and upregulation of ECM gene expression. The mechanism was attributed upregulation of transient receptor potential canonical (TRPC) channel 3, a cation channel permissive to calcium.[121] TRPC channels are known to activate calcineurin/NFAT signalling in the heart[122] and in a study by the Molkentin group[123] mice lacking TRPC6 had impaired myofibroblast differentiation and cardiac wound healing after injury due to reduced calcineurin/NFAT signalling. In contrast, NFAT activity in response to endothelin-1 (ET-1)-induced upregulation of TRPC6 was found to suppress myofibroblast differentiation in rat neonatal cardiac fibroblasts[124] despite ET-1 being a profibrotic ligand. In these studies, TRPC6 was upregulated by TGFβ and ET-1, respectively, suggesting that calcineurin/NFAT signalling is secondary to these mediators. In Paper 1 we suggest a direct activation of calcineurin/NFAT via syndecan-4 by mechanical stress, that is independent of TGFβ and ET-1 signalling.

Results from Paper 1 suggested that syndecan-4 could regulate MRTF-A expression by activating NFAT. Being an essential cofactor for the transcription factor serum response factor (SRF), MRTF-A is well-established from studies in vascular smooth muscle cells to

be central for SMA expression by binding to CArG boxes in the promoter region of the SMA gene (ACTA2),[125,126] and was also recently found to be necessary for myofibroblast differentiation.[127] The collagen 1a2 promoter has been identified as a target of MRTF-A/SRF, and MRTF-A knockout mice have diminished fibrosis and scar formation following MI or angiotensin II treatment.[128] Interestingly, MRTF-A activity is known to be regulated by actin polymerization which is involved in its nuclear translocation.[127,129] Since syndecan-4 was found to regulate both transcription of MRTF-A through NFAT and actin polymerization, it seems likely that this signalling pathway also will be affected by loss of syndecan-4 and may in part be responsible for the impaired myofibroblast differentiation of cardiac fibroblasts lacking syndecan-4.

OPN expression was regulated by syndecan-4 and NFAT (Paper 2). Although OPN was traditionally considered an extracellular protein that plays a role in structure and function of the extracellular matrix as well as inflammation,[130] it may also exist as an intracellular variant (iOPN) derived from alternative translation.[131] It is worth noting that iOPN has been shown to be essential for TGFβ-induced myofibroblast differention.[132] Thus the impaired myofibroblast differentiation of cardiac fibroblasts from syndecan-$4^{-/-}$ mice could be suspected to result from reduced expression of iOPN. However, iOPN did not seem necessary for mechanical stress-induced myofibroblast differentiation[132] nor have we or others[54] detected any effect on TGFβ-induced myofibroblast differentiation by the presence or absence of syndecan-4.

Together with mechanical stress, TGFβ is the most potent inducer of cardiac myofibroblast differentiation and fibrosis.[27,133,26] Interestingly, TGFβ harbors a heparin-binding motif and has been shown to bind syndecan-4 on macrophages.[134] However to our knowledge, no interaction between syndecan-4 and TGFβ has so far been shown in cardiac fibroblasts. It has previously been suggested[135] and was recently confirmed[20] that TGFβ acts as a mechanosensitive factor. TGFβ is sequestered in the ECM in a biologically inactive complex, the large latent complex (LLC). Integrins $\alpha_v\beta_8$ and $\alpha_v\beta_3$ exert traction forces on the ECM and LLC thereby releasing active TGFβ. In Paper 1, there was no difference in TGFβ expression by cardiac fibroblasts from WT and syndecan-$4^{-/-}$ mice. Hence, it is unlikely that impaired myofibroblast differentiation of cardiac fibroblasts lacking syndecan-4 was caused by diminished production of TGFβ. However, if TGFβ is "stored" in the ECM as proposed[20,136] there may be a blunted release/activation of TGFβ *in vivo* simply because traction forces are lower (due to impaired myofibroblast

differentiation) when syndecan-4 is lacking. Also, syndecan-4 has been found to promote adhesion properties of some integrins, which might affect the liberation of active TGFβ.[39] Whether this mechanism takes place in our *in vitro* models of myofibroblast differentiation is not known.

There are several pathways independent of syndecan-4 that are important for myofibroblast differentiation,[27,137,128,132,138] and myofibroblast differentiation will eventually occur even in the absence of syndecan-4. However, syndecan-4 may be one of the initial mediators of a mechanical stress-induced response enabling rapid adaptation to the surrounding environment.

Syndecan-4 regulates collagen production and maturation

Since myofibroblasts are efficient collagen producing cells, impaired cardiac myofibroblast differentiation will likely affect collagen amount and composition. Tensile strength and structure of the ECM is mainly provided by fibrillar collagens. In the heart, the main fibrillar collagen is collagen I (comprising up to 85% of the total amount of collagen) followed by collagen III. Indeed, whereas collagen I and III mRNA levels were markedly increased in left ventricles of WT mice following 24h AB, this response was absent in syndecan-$4^{-/-}$ mice (Paper 1). Thus, we considered it likely that this would translate into altered fibrosis at later time points. However, to our surprise total collagen protein amount and collagen I and III mRNA levels were similar in WT and syndecan-$4^{-/-}$ mice 7 days after AB, suggesting that other pro-fibrotic signaling pathways had taken over collagen production in syndecan-$4^{-/-}$ mice.

Traditionally, the increase in myocardial stiffness in the pressure overloaded heart has alone been attributed to accumulation of collagen I causing myocardial fibrosis. Also, a shift in the ratio of collagen I and III was thought to influence myocardial stiffness.[139,140] This view has since been challenged by reports suggesting that the amount of total collagen and the collagen I:III ratio does not necessarily translate into changes in myocardial stiffness.[141] Even though collagen amounts are similar, collagen quality and structure may be altered and accumulating data suggest collagen cross-linking to be a determining factor for myocardial stiffness.[142,143] Cross-linked collagen was in fact reduced in pressure-overloaded ventricles of mice lacking syndecan-4, indicating impaired maturation of newly synthesised collagen.

Collagen cross-linking can occur either enzymatically or non-enzymatically. While non-enzymatic cross-linking (i.e. of glycated lysine and hydroxylysine collagen residues) is generally associated with myocardial stiffening due to age or diabetes,[144] collagen cross-linking induced by mechanical stress seems to be largely attributed enzymatic cross-linking by the enzyme lysyl oxidase (LOX). LOX is upregulated in the pressure-overloaded heart,[142] and pharmacologically inhibiting LOX decreases collagen cross-linking and myocardial stiffness.[145] Cross-linked collagen is less susceptible to degradation by ECM proteases thereby reducing overall collagen turnover. Thus, although no difference in MMP2, 9 and 13 mRNA was detected in ventricles from syndecan-4$^{-/-}$ and WT mice, collagen degradation may be altered in syndecan-4$^{-/-}$ mice due to less collagen cross-linking. This may also partly constitute a possible explanation for the premature left ventricular dilatation observed in syndecan-4$^{-/-}$ mice 3 weeks after AB.[51]

Despite its potentially central role in myocardial stiffening and diastolic dysfunction, there are few studies on the mechanisms regulating LOX in the heart. Among the identified LOX-inducing factors are TGFβ[146] and angiotensin II.[147] Also of interest, another proteoglycan called lumican was recently found to upregulate LOX mRNA in cardiac fibroblasts *in vitro*.[148] In Paper 2, LOX mRNA and activity was reduced in left ventricles from syndecan-4$^{-/-}$ mice 24h and 3 days after AB, respectively, indicating a role for syndecan-4 in regulating this enzyme. Syndecan-4$^{-/-}$ mice had substantially reduced production of OPN which was recently found to induce LOX expression and activity in human cardiac fibroblasts.[149] In agreement, we demonstrated in Paper 2 that incubation of mouse cardiac fibroblasts with exogenous OPN increased LOX protein. Interestingly, the effects of OPN on LOX protein expression were only evident in highly differentiated myofibroblasts, suggesting differential effects of OPN depending on cardiac fibroblast/myofibroblast phenotype. Although OPN is sparsely expressed in the healthy heart, it is rapidly and markedly upregulated in the pressure overloaded heart[150] with a peak in mRNA levels 24h after AB (Paper 2). At this time point myofibroblast differentiation is taking place, but highly differentiated myofibroblasts are most likely not present at this early time point. Thus OPN may have other effects on cardiac fibroblasts in the initial phase (such as proliferation and survival[130]) whereas induce LOX expression in later stages of the pressure overload response when highly differentiated myofibroblasts are present.

Our data indicate that collagen cross-linking was enhanced *in vitro* in the presence of the extracellular domain of syndecan-4. Indeed, proteoglycans of the small leucine-rich proteoglycan (SLRP) family such as decorin and biglycan are known to bind to and stabilise collagen fibrils, and lack of these proteoglycans leads to deranged collagen matrices in skin, tendon and elsewhere in the musculoskeletal system.[151,152,153,154,155] Collagen cross-linking by LOX was also promoted in the presence of the extracellular domain of syndecan-4. In agreement with these results, it has previously been suggested that SLRPs[156] and cellular fibronectin[157] may act as co-receptors for LOX, thereby facilitating collagen cross-linking. Moreover, syndecan-4 has been found to regulate the cell surface trafficking, localization and activity of another cross-linking enzyme called tissue transglutaminase (TG2)[158] and the beneficial effect of syndecan-4 deletion on kidney fibrosis has been attributed altered function of this enzyme.[159]

Myocardial stiffness is determined by syndecan-4

In Paper 2 we determined the passive tension of the myocardium. An important question is how this relates to diastolic function. Although intuitively rather simple, diastolic dysfunction is a multi-facetted concept. In general, it can be divided into an active and a passive component. The active component (Figure 4, orange box) is associated with relaxation of cardiomyocytes and involves removal of calcium from the cytoplasm into the sarcoplasmic reticulum and extracellular space. This process is carried out mainly by energy consuming ion pumps in the sarcoplasmic reticulum membrane and plasma membrane, hence the term active phase or component. The passive component (Figure 4, green box) manifests itself in later phases of diastole and is determined by mechanical properties of the myocardium such as viscosity (viscous stress) and elasticity (passive tension) and constitutes the myocardial stiffness. In the heart, viscosity is mainly determined by the amount of water present in the myocardium, whereas passive tension is largely determined by the cytoskeletal spring-like protein of cardiomyocytes called titin,[160] and the composition and structure of ECM.

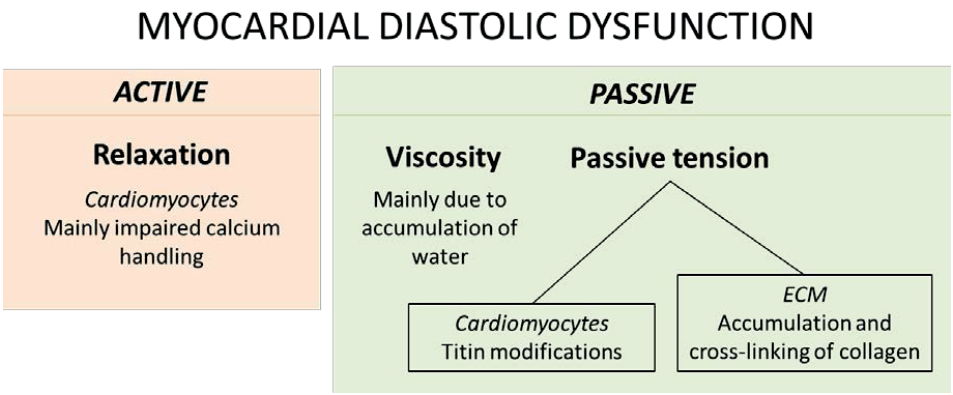

Figure 4. Factors contributing to myocardial diastolic dysfunction.

Diastolic dysfunction involves changes in both the active process i.e. relaxation, and passive forces (myocardial stiffness) including viscous stress and passive tension of the myocardium. Increased passive tension can mainly be attributed modifications of the spring-like protein titin of cardiomyocytes and altered amount and composition of the extracellular matrix (ECM), particularly fibrillar collagen.

Diastolic function can be determined from pressure-volume loops (P-V loops). P-V loops are difficult to acquire from the AB hearts as the aortic constriction is too tight to allow for retrograde insertion of the catheter. Doppler echocardiography also provides information about diastolic function *in vivo*. In Paper 2 we measured early mitral inflow velocity (E) / early mitral annular velocity (E´) as an indication of early diastolic function. However, E/E´ reflects mainly the active relaxation of cardiomyocytes and may thereby mask or prevent an accurate determination of passive myocardial stiffness.[161] Thus, even though E/E´ was increased (indicating impaired relaxation) in both genotypes following AB, no significant differences were observed between WT and syndecan-4$^{-/-}$ mice.

An important finding in Paper 2 was that passive tension, reflecting intrinsic myocardial stiffness, was reduced in myocardial strips from mice lacking syndecan-4. This was mainly attributed altered ECM stiffness. In contrast, increased passive myocardial stiffness of patients with diastolic dysfunction can often also be attributed modifications of the giant spring-like cardiomyocyte protein, titin.[162] In the AB mouse model used in this study, we did not observe changes in titin isoforms and no major effect of eliminating titin function by salt extraction on passive tension, possibly reflecting variability between species and etiologies of disease. Moreover, since calcium handling was never addressed in these mice and is central for determining the active component of diastole, we cannot draw

final conclusions regarding *in vivo* diastolic function in syndecan-4$^{-/-}$ mice. Regardless, our results on intrinsic myocardial passive tension theoretically imply that interfering with syndecan-4 function in cardiac fibroblasts would be beneficial with respect to ECM stiffness.

Apart from regulating collagen, syndecan-4 might also affect myocardial stiffness through intrinsic properties of the syndecan-4 molecule. The GAG chains of proteoglycans have high affinity for water and may thus affect viscoelasticity.[163,164] This is especially important in cartilage where shock-absorbing properties can be assigned large proteoglycans of the hyalectin family. Although we observed significantly increased viscous stress of muscle strips from ventricles subjected to 7 days AB (Paper 2) along with increased water content (unpublished data), there was no difference in these properties between WT and syndecan-4$^{-/-}$ mice, supporting instead a regulatory role for syndecan-4 in determining ECM structure and composition.

Syndecan-4 shedding and inflammation in the pressure-overloaded heart

Enzymatic shedding of syndecan-4 has been implicated as part of the innate immune response which is involved in heart failure development.[165,166] The innate immune system is the first line of defense against pathogens and becomes activated when cell surface toll-like receptors (TLRs) recognise unspecific highly conserved structural motifs of pathogens called pathogen-associated molecular patterns (PAMPs; eg. LPS) and thereby induce an inflammatory response. An innate immune response is also triggered during tissue injury by specific danger-associated molecular patterns (DAMPs), often comprising fragments of ECM components (e.g. hyaluronic acid) that are released from the host.[167] In Paper 3, triggering innate immunity by stimulation with LPS upregulated syndecan-4 in a TLR4-dependent manner both cardiomyocytes and fibroblasts and enhanced syndecan-4 shedding. Interestingly, the two cytokines TNFα and IL-1β that are rapidly increased by pressure overload had a similar effect on syndecan-4 *in vitro* to that of LPS, suggesting activation of signalling pathways of the innate immune system. Thus, although LPS is not present in the pressure-overloaded heart, it serves well as a model system when studying the innate immune response.

As demonstrated in Paper 3, left ventricular pressure overload caused T cell infiltration 1-3 weeks after AB in WT mice indicating that T cell recruitment is a part of

the late, rather than the early response to pressure overload. At these time points, T cell infiltration was reduced in mice lacking syndecan-4 indicating a defective innate immune response in the absence of syndecan-4. In agreement, syndecan-4$^{-/-}$ mice have higher mortality to LPS when compared to WT mice.[134] Considering that syndecan-4 is widely expressed,[168,169] there are several possible explanations for reduced T cell infiltration in syndecan-4-deficient mice: lack of the chemotactic gradient provided by shed syndecan-4 in the myocardial tissue; lack of full-length syndecan-4 on endothelial cells thus reducing immune cell adherence and infiltration, or lack of full-length syndecan-4 on T cells thus affecting migration. Regardless, results obtained from the syndecan-4$^{-/-}$ mouse provide evidence that syndecan-4 expression and shedding are involved the immune response of the pressure-overloaded heart.

The regulation of syndecan-4 shedding involves extracellular proteases, many of which are produced by cardiac fibroblasts and myofibroblasts and are upregulated in the pressure-overloaded heart.[170] These include matrix metalloproteases (MMPs) and ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs).[94] Syndecan-4 shedding was to some degree dependent on intact HS GAG attachment sites, indicating that syndecan-4 shedding is primarily regulated by enzymes dependent on interacting with HS GAG chains. Of interest, activation of pro-MMP2 by thrombin has been found to depend on heparan sulfate[171] and as shown in Paper 2, MMP2 was upregulated in response to pressure overload. In Paper 3, cardiac fibroblasts treated with a NF-κB blocker had reduced expression and shedding of syndecan-4. This might suggest that proteases that shed syndecan-4 are transcribed by NF-κB. In vascular smooth muscle cells syndecan-4 shedding has been found to be regulated by MAP kinases (ERK1/2 and JNK).[99] However, the responsible proteases were not identified.

Syndecan-4 ectodomain is not found in normal plasma but appears in fluids surrounding injured tissue, suggesting a role in the wound healing response.[172] Furthermore, it has been detected in plasma of patients after myocardial infarction[95] and heart failure.[96] Due to the increased syndecan-4 shedding in the patients with end-stage heart failure studied in Paper 3, we suspect that the increased syndecan-4 ectodomain plasma levels could originate from the heart. However, the source of this shedding might also be endothelial cells considering that plasmin and thrombin (proteases involved in coagulation and fibrinolysis during tissue injury) are also known syndecan-4 "sheddases".[173] At least during early stages of left ventricular pressure overload (24h,

Paper 1), shed syndecan-4 was not detected in blood. In Paper 3, syndecan-4 shedding was increased in the myocardium 1 week after AB, and further enhanced 3 weeks after AB, indicating that syndecan-4 shedding is a process taking place at progressed stages of cardiac remodelling where also inflammation is more prominent, compared to syndecan-4 expression which is a rapid response occurring within the first 24h of pressure overload.

Interestingly, T helper lymphocytes have recently been implicated in the regulation of ECM remodelling of the pressure-overloaded heart, inducing collagen cross-linking and diastolic dysfunction[174,175] through activation of lysyl oxidase.[176] Accompanying the increased myocardial stiffness, was an increase in T helper cell marker CD4 7 days after AB. Hence, the reduced collagen cross-linking and LOX activity observed in Paper 2 might also partly be explained by less T helper cell infiltration. Similar to what has been found for the cross-linking enzyme TG2, syndecan-4 shedding might also promote collagen cross-linking by transporting LOX into the ECM in response to injury or mechanical stress.[177,178,179]

In Paper 3 we show that LPS-induced shedding of syndecan-4 reduced focal-adhesion size. Based on our results in Paper 1 and 2, this will likely interfere with downstream signalling of syndecan-4 hence reducing LOX expression via OPN. Reduced focal adhesion size and stability will most likely also affect migration properties and reduce myofibroblast differentiation of cardiac fibroblasts which is somewhat contradictory to the role of full-length syndecan-4.

The role of syndecan-4 during the sequence of events occurring in response to left ventricular pressure overload.

In attempt to clarify the role of syndecan-4 in the pressure-overloaded heart and based on the results obtained in this thesis, the following section will deal with placing syndecan-4 and its multiple roles in a timely context following induction of pressure overload (Figure 5), with particular focus on cardiac fibroblasts and ECM remodelling.

Pressure overload of the left ventricle due to aortic stenosis or hypertension, is mimicked by aortic banding (AB). This triggers a rapid increase in IL-1β and TNFα mRNA (2h post AB, unpublished data), cytokines involved in a so-called “sterile” immune response of the innate immune system. Although the source of this cytokine production is not entirely clear, it is likely that both cardiomyocytes and cardiac fibroblasts contribute.

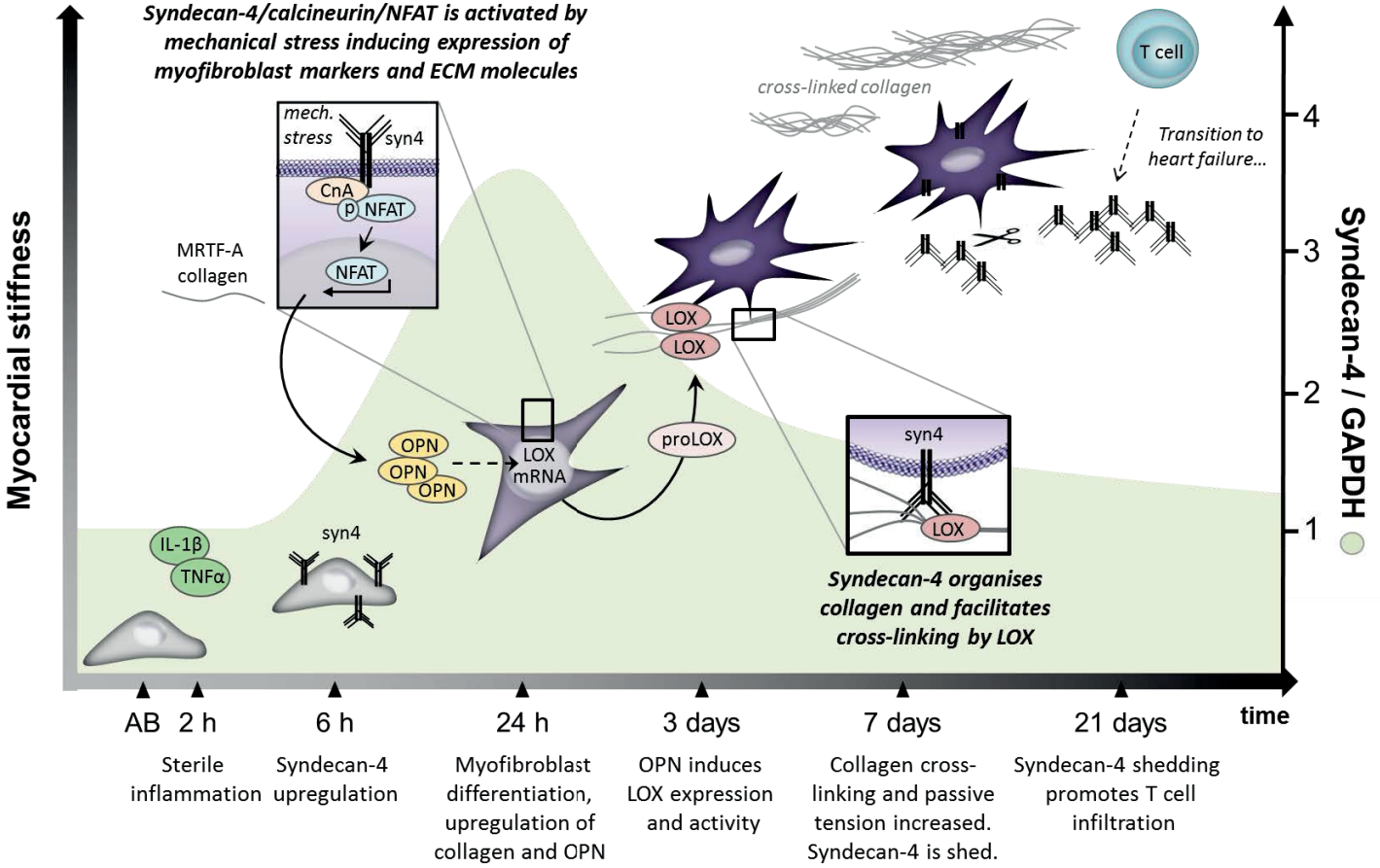
Syndecan-4/calcineurin/NFAT is activated by mechanical stress inducing expression of myofibroblast markers and ECM molecules
mech. stress
syn4
CnA
p NFAT
NFAT
MRTF-A
collagen
OPN
OPN
OPN
LOX mRNA
proLOX
LOX
LOX
cross-linked collagen
T cell
Transition to heart failure...
syn4
LOX
Syndecan-4 organises collagen and facilitates cross-linking by LOX
IL-1β
TNFα
syn4
Myocardial stiffness
Syndecan-4 / GAPDH
1
2
3
4
AB
2 h
6 h
24 h
3 days
7 days
21 days
time
Sterile inflammation
Syndecan-4 upregulation
Myofibroblast differentiation, upregulation of collagen and OPN
OPN induces LOX expression and activity
Collagen cross-linking and passive tension increased. Syndecan-4 is shed.
Syndecan-4 shedding promotes T cell infiltration

Figure 5. The multiple roles of syndecan-4 in the context of left ventricular pressure overload.

Mechanical stress is induced by aortic banding (AB). This leads to a rapid increase in IL-1β and TNFα as part of a sterile inflammation. IL-1β and TNFα upregulate syndecan-4 in proliferating cardiac fibroblasts, thereby enhancing mechanosensing by syndecan-4. This activates calcineurin/NFAT signaling causing expression of the myofibroblast marker gene, myocardin-related transcription factor A (MRTF-A) and extracellular matrix (ECM) molecules including collagen. MRTF-A is known to be central for transcription of smooth muscle α-actin (SMA) which defines the myofibroblast phenotype. NFAT activation also increases the expression of osteopontin (OPN) which induces expression of the collagen cross-linking enzyme lysyl oxidase (LOX) in cardiac myofibroblasts. LOX is activated 3 days following AB and collagen cross-linking is present 7days after AB. Moreover, the extracellular domain of syndecan-4 interacts with collagen and possibly LOX to assist collagen fiber assembly and cross-linking at the cell surface. Collagen cross-linking has a massive effect on passive tension which is highly elevated at this time point. Shedding of the ectodomain of syndecan-4 also appears 7 days after AB and is further increased 21 days after AB. The shed ectodomain may reside in the myocardial tissue and binds chemokines thereby creating a chemotactic gradient for T cell infiltration. Finally, multiple processess beyond the scope of this thesis, involving the immune system, cardiac cell necrosis and apoptosis as well as matrix degradation drives the transition into heart failure.

Cardiac fibroblasts start proliferating (proliferating cell nuclear antigen mRNA upregulated 6h post AB, unpublished results) and increase syndecan-4 expression (6h post AB, unpublished data) in response to stimulation with IL-1β and TNFα.

The presence of more syndecan-4 in cardiac fibroblasts increases attachment to the ECM, thereby enhancing the overall mechanosensing by syndecan-4. Mechanical stress leads to dephosphorylation of the cytoplasmic domain of syndecan-4 thereby enabling binding and activation of calcineurin. Calcineurin dephosphorylates NFAT which can then translocate to the nucleus and transcribe target genes involved in myofibroblast differentiation such as MRTF-A, and production of ECM molecules including collagen III and OPN.

OPN is markedly increased in the pressure-overloaded heart of WT mice and was found to induce LOX production in cardiac myofibroblasts which are prevalent in the myocardium 3 days after AB. At this time point myocardial LOX activity was increased, hence inducing collagen cross-linking which was clear 7 days after AB. *In vitro* experiments with the extracellular domain of syndecan-4 suggest a role in collagen fiber assembly and cross-linking by LOX at the cell surface. Hence syndecan-4 may have a dual role in regulating collagen structure and tensile strength. Importantly myocardial passive tension of the ECM is markedly increased after 7 days with pressure overload. This increased myocardial stiffness (and the accompanied hypertrophic remodelling of cardiomyocytes) enables the heart to perform under conditions of increased myocardial pressure but will eventually compromise diastolic function.

At this stage, syndecan-4 shedding is initiated. This may serve to abrogate syndecan-4-induced myofibroblast differentiation, causing reduction in focal adhesion size and loss of syndecan-4 mechanosensing activity. However, the shed ectodomain of syndecan-4 seems also to have activities of its own, possibly creating a chemotactic gradient enhancing the T cell infiltration of the myocardium observed 21 days after AB which may further affect ECM remodelling.[175]

This is a coarse and somewhat speculative summary of our results in order to simplify the confusing (and to some extent contradicting) roles of syndecan-4. Syndecan-4 is here put in the center of events leading to heart failure. However, it is clear that numerous factors are involved and supplement the proposed model.

Syndecan-4 as a therapeutic target

The novel finding of Paper 2 that syndecan-4 has profound effects on myocardial stiffness renders it an attractive drug target for treatment of diastolic dysfunction. However, as with most signalling pathways, syndecan-4 signalling does not seem to work as a simple "on-off" switch. As such, complete deletion of syndecan-4 may inhibit myocardial stiffening, but also accelerates heart failure development and left ventricular dilatation.[51] This clearly underscores the need for more knowledge regarding syndecan-4 in the heart, including identification of binding partners, monitoring spatial and temporal dynamics of syndecan-4 signalling and understanding the role of syndecan-4 shedding. Another future challenge will be to identify differential traits between syndecan-4-dependent signalling in cardiac fibroblasts and cardiomyocytes to specifically target processes leading to changes in passive tension and limit an exacerbated fibrosis while keeping the critical adaptive hypertrophic response of cardiomyocytes to pressure overload intact. Thus, although syndecan-4 is merging as an attractive target in anti-fibrotic[180] and cardiac therapy,[1] further studies are necessary to determine when and where to intervene to achieve beneficial outcome of a potential future treatment.

Conclusions

The results presented in this thesis demonstrate a role for syndecan-4 in determining cardiac fibroblast function and phenotype, ECM remodelling and myocardial stiffening of the pressure overloaded heart.

In Paper 1 we show that syndecan-4 regulates myofibroblast differentiation of cardiac fibroblasts in response to mechanical stress, as seen by impaired organisation of SMA stress fibers and reduced FA size. The cytoplasmic domain of syndecan-4 was dephosphorylated in response to mechanical stress leading to activation of calcineurin/NFAT signalling and upregulation of genes defining the myofibroblast phenotype such as MRTF-A, and involved in ECM remodelling such as collagen.

Using syndecan-$4^{-/-}$ mice, we show in Paper 2 that ECM production is blunted and collagen cross-linking reduced when syndecan-4 is lacking. Our data suggested a dual role for syndecan-4 in collagen cross-linking: promoting lysyl oxidase expression by upregulating OPN in response to mechanical stress, and by direct interaction of syndecan-4 ectodomain with collagen I, thereby facilitating LOX cross-linking activity. Importantly, these effects translated into reduced myocardial passive stiffness in syndecan-$4^{-/-}$ mice which may have consequences for diastolic function of the heart.

Finally, in Paper 3 we show that IL-1β and TNFα, which are rapidly increased in the pressure-overloaded heart, regulated syndecan-4 expression via NF-κB signaling. At more advanced stages of cardiac remodelling, syndecan-4 shedding occurred, possibly affecting myofibroblast function and T cell infiltration of the pressure-overloaded heart. Importantly, syndecan-4 shedding was elevated in failing human hearts, supporting syndecan-4 as a potential target for treatment of heart failure patients.

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Appendix: Papers 1-3

1

2

Syndecan-4 is a key determinant of collagen cross-linking and passive myocardial stiffness in the pressure-overloaded heart

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Abstract

Aim: Diastolic dysfunction is central to the development of heart failure. To date, there is no effective treatment and only limited understanding of its molecular basis. Recently we showed that the transmembrane proteoglycan syndecan-4 increases in the left ventricle after pressure overload in mice and man; and that syndecan-4 via calcineurin/NFAT promotes myofibroblast differentiation and collagen production upon mechanical stress. The aim of this study was to investigate if syndecan-4 affects collagen cross-linking and myocardial stiffening in the pressure-overloaded heart.

Methods and Results: Aortic banding (AB) caused concentric hypertrophy and increased passive tension of left ventricular muscle strips, responses that were blunted in syndecan-4$^{-/-}$ mice. Disruption of titin anchoring by salt extraction of actin and myosin filaments revealed that the effect of syndecan-4 on passive tension was due to extracellular matrix remodeling. Expression and activity of the cross-linking enzyme lysyl oxidase (LOX) increased with mechanical stress and was lower in left ventricles and cardiac fibroblasts from syndecan-4$^{-/-}$ mice, which exhibited less collagen cross-linking after AB. Expression of osteopontin (OPN), a matricellular protein able to induce LOX in cardiac fibroblasts, was upregulated in hearts after AB, in mechanically-stressed fibroblasts and in fibroblasts overexpressing syndecan-4, calcineurin or NFAT, but downregulated in fibroblasts lacking syndecan-4 or after NFAT inhibition. Interestingly, the extracellular domain of syndecan-4 facilitated LOX-mediated collagen cross-linking.

Conclusions: Syndecan-4 exerts a dual role in collagen cross-linking, one involving its cytosolic domain and NFAT signaling leading to collagen, OPN and LOX induction in cardiac fibroblasts; the other involving the extracellular domain promoting LOX-dependent cross-linking.

1. Introduction

Diastolic dysfunction is central to the development of heart failure, a common and fatal disease.[1] Defined by abnormal relaxation and/or decreased compliance of the ventricles, diastolic dysfunction results in impaired filling of the heart. While, in healthy individuals, the major risk factor for diastolic dysfunction is age, any condition that leads to stiffening of the ventricles can result in diastolic dysfunction, e.g. pressure overload as in hypertensive and aortic stenosis patients, or diabetes. Despite improved medical options for cardiovascular disease, there is currently no effective treatment for diastolic dysfunction, reflecting the need for a better understanding of its molecular basis. Next to modifications of the elastic titin springs of cardiomyocytes[2], changes in extracellular matrix (ECM) composition and structure are important determinants of myocardial stiffening[3].

Cardiac fibroblasts are the major non-muscle cells of the ventricular myocardium and are key players in myocardial stiffening. During ventricular remodeling following sustained periods of pressure overload, cardiac fibroblasts become activated and start producing excessive amounts of ECM proteins, such as fibrillar collagens, eventually resulting in increased myocardial fibrosis.[4] The activated fibroblast acquires smooth muscle-like features including expression of smooth muscle α-actin (SMA) and SM22 and is therefore referred to as myofibroblast,[5] as well as increases expression of platelet-derived growth factor receptor β (PDGFRβ)[6,7]

Recent clinical studies have questioned whether overexpression of collagen can alone explain the increased myocardial stiffness, and collagen cross-linking has been suggested as an additional contributing factor.[8,9] While non-enzymatic cross-linking (i.e. of glycated lysine and hydroxylysine collagen residues) is generally associated with myocardial stiffening due to age or diabetes,[10] collagen cross-linking induced by mechanical stress seems to be largely attributed to enzymatic cross-linking by the enzyme lysyl oxidase (LOX), which is upregulated in the pressure-overloaded heart.[8] Despite its potentially central role in myocardial stiffening, little is known regarding mechanisms regulating LOX in the heart. Interestingly, the matricellular protein OPN was recently found to induce LOX expression and activity in cardiac fibroblasts.[11] Although sparsely expressed in the healthy heart, OPN is dramatically increased in mice subjected to pressure overload by aortic banding (AB).[12]

Recent work in our laboratory demonstrated that expression of the transmembrane heparan sulphate proteoglycan syndecan-4 increases in the left ventricle after pressure overload in mice[5] and man,[13] through inflammatory mediators such as tumor necrosis factor (TNF) α and interleukin (IL)-1β.[14] Also, that in response to mechanical stress, syndecan-4 engages the calcineurin/NFAT (Nuclear Factor of Activated T-cells) signaling pathway in cardiac fibroblasts to promote ECM production and differentiation into activated myofibroblasts.[5] Thus, one specific aim of this study was to examine whether syndecan-4 expression and/or signaling has a direct impact on myocardial stiffness. A second aim of this study was to explore if syndecan-4 may be important for collagen cross-linking. Previous work has shown that NFAT activation leads to the induction of OPN expression in vascular smooth muscle cells.[15] We therefore hypothesized that OPN might be a down-stream target of syndecan-4/NFAT signaling in cardiac fibroblasts and that enhanced OPN expression after pressure overload, by virtue of an increase in LOX expression and activity, may promote collagen cross-linking and myocardial stiffening of the heart.

2. Methods

A detailed description of the methods is provided in supplementary material online.

2.1 Mouse Model of Pressure Overload

AB or sham operations followed by echocardiography were performed on adult wild-type (WT) and syndecan-4 knockout (syndecan-$4^{-/-}$) mice[16] as previously described[13,17] and mice were sacrificed by cervical dislocation after 24h, 3 days or 7 days after AB. Animals were handled according to the National Regulation on Animal Experimentation in accordance with approved protocol (ID#2845) and the Norwegian Animal Welfare Act, and conform the NIH guidelines (2011).

2.2 Mechanical measurements

Left ventricular muscle strips were skinned and passive tension, reflecting the elastic components of the muscle, was determined by stretching from slack length (L_0) to 30% L_0 while measuring force. Salt extraction was performed as previously described.[18] Viscous stress, reflecting the stretch velocity sensitive component of the muscle, was determined as peak force upon stretching minus steady-state force, divided with cross-sectional area.

2.3 Titin gels

Samples prepared from snap frozen left ventricular tissue were loaded on agarose-strengthened 2% SDS-polyacrylamid gels[19] and titin N2B and N2BA isoforms detected at 3000kDa and 3200kDa, respectively, by coomassie brilliant-blue staining.

2.4 Electron microscopy

Mouse hearts were fixed in 3.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.2; Sigma) by perfusion and small pieces of the left ventricular wall were prepared for electron microscopy. For quantification, 5 images (x440) per section were analyzed to determine non-cardiomyocyte fractions, and 24-30 images (x2900) per section of the non-cardiomyocyte fraction to determine the areas of fibroblast nuclei (reflecting number of fibroblasts) and blood vessels. Two-three sections per animal were inspected.

2.5 Collagen cross-linking and quantification

Total collagen was determined by hydroxyproline content measured by HPLC. Insoluble cross-linked collagen was quantified as previously described.[20] Briefly, soluble collagen assessed using colorimetric and enzymatic procedures was subtracted from total collagen determined by staining with picrosirius red and normalized to total protein content determined by fast green staining. For visualization of total and cross-linked collagen, sections from formalin-fixed left ventricles were stained with picrosirius red and examined using non-polarized and circularly polarized light, respectively.

2.6 Fibroblast Cell Culture

Cardiac fibroblasts were isolated as previously described[5] and used at passage 2 to limit *in vitro* effects on fibroblast phenotype. For overexpression, an adenoviral vector containing mouse syndecan-4 (Applied Biological Materials Inc, Richmond, Canada) was used. The NFAT blocker A-285222 was kindly provided by Abbott Laboratories (1μmol/L; Abbott Park, IL). Cells were stimulated with 10 ng/ml TGFβ1 (Merck Millipore, Darmstadt, Germany) and 250 ng/ml OPN (Cat. no.120-35; Peprotech, Hamburg, Germany).

2.7 Gene Expression Analysis

RNA was extracted, cDNA synthesized and real-time performed as previously described.[5]

2.8 Immunoblotting
Immunoblotting was performed on left ventricle homogenates as previously described.[21] Anti-osteopontin (IBL, Hamburg, Germany) and anti-LOX (NB100-2527, Novus Biologicals, Littleton, CO) were used as primary antibodies.

2.9 Immunocytochemistry and immunohistochemistry
Cells grown on fibronectin-coated glass cover slips were fixed in 4% paraformaldehyde and stained using mouse-anti-SMA (Sigma, Schnelldorf, Germany) and alexa fluor 488-secondary anti-mouse antibodies (Invitrogen, Paisley, UK). Formalin-fixed myocardial tissue sections (4 μm) were stained for SMA (DAKO, Glostrup, Denmark) and platelet-derived growth factor receptor β (PDGFRβ; Cell Signaling, Danvers, MA) and visualized using secondary antibodies conjugated to horseradish peroxidase (HRP).

2.10 Lysyl oxidase activity assay
LOX activity was measured in left ventricular tissue and cardiac fibroblasts with a fluorometric LOX activity assay (Abcam, Cambridge, UK) according to the manufacturer's instructions.

2.11 *In vitro* collagen fiber formation assay
The extracellular domain of syndecan-4 (1-4μg, Sino Biological Inc., Beijing, China) was mixed with 10μg collagen I with or without recombinant human lysyl oxidase homolog 2 (0.1μg, LOXL2 with activity > 2pmol/min/μg, R&D systems, Abingdon, UK). The mixture was incubated at 37ºC for 24-72h before performing turbidimetry and electron microscopy.

2.12 Statistics
Data are expressed as means ± standard deviation (S.D.) unless otherwise specified in the figure legends. Statistical analysis was performed using GraphPad software (Prism 5). The use of parametric or non-parametric tests was based on results from analyses of distributions. Statistical significance was determined using Mann-Whitney test and Kruskal-Wallis followed by Dunn's multiple comparison test for non-parametric data; or Student's *t*-test and two-way ANOVA followed by Bonferroni post hoc tests for normally distributed data. Pearson's test was used for correlation analyses. ***$P<0.005$, **$P<0.01$, *$P<0.05$.

3. Results

3.1 Myocardial stiffness is reduced in mice lacking syndecan-4

AB caused hypertrophic remodeling as determined by echocardiography (Supplementary Table 1) which was accompanied by an increase in passive tension (Figure 1A). Along with reduced concentric hypertrophic remodeling in syndecan-4$^{-/-}$ mice (Supplementary Table 1), which is in accordance with our previous findings,[13] passive tension was significantly lower in myocardial tissue from syndecan-4$^{-/-}$ mice (Figure 1A). Another factor contributing to myocardial stiffness is the viscosity of the tissue. Viscous stress was increased following AB (Figure 1B) but it was not affected by the genotype of the mice. Cardiomyocytes and ECM both contribute to passive tension. In cardiomyocytes, passive tension is mainly determined by the sarcomeric "spring-like protein" titin.[2] To investigate the cardiomyocyte contribution to the observed reduction of passive tension in syndecan-4$^{-/-}$ mice after pressure overload, we measured the ratios between the longer and more compliant titin isoform N2BA and the shorter and stiffer N2B isoform. N2BA:N2B ratios were unchanged by AB or lack of syndecan-4 (Figure 1C). To study whether the reduced passive tension in syndecan-4$^{-/-}$ mice could be due to changes in the ECM, we performed salt extraction of actin and myosin filaments, a treatment that destroys the anchoring points of titin leaving the ECM to account for the remaining passive tension.[18] Indeed, ECM-dependent passive tension was significantly lower in muscle strips from syndecan-4$^{-/-}$ mice compared to WT (Figure 1D). The complete disruption of sarcomere structure by salt extraction was verified using electron microscopy (Figure 1E). The area of myocardial tissue consisting of cardiomyocytes vs. non-cardiomyocyte (non-CM) fraction seemed unaltered by AB or lack of syndecan-4 (Supplementary Table 2).

3.2 Increased expression of syndecan-4 promotes cardiac myofibroblast differentiation

As we showed in neonatale cardiac fibroblasts,[5] lack of syndecan-4 had profound effects in adult cardiac fibroblasts. While adult syndecan-4 deficient cardiac fibroblasts exhibited virtually no SMA fiber formation after 48h on fibronectin, fibroblasts from WT mice had clear SMA fibers (Figure 2A). This impaired ability to undergo myofibroblast differentiation of cells lacking syndecan-4 was restored by adenoviral-transduction of syndecan-4 (Figure 2A). Further, overexpression of syndecan-4 in WT cardiac fibroblasts resulted in enhanced SMA fiber formation (Figure 2A). Myofibroblast differentiation was also impaired *in vivo* as demonstrated by reduced staining of SMA and PDGFRβ (Figure 2B). Negative and positive controls are included in Supplementary Figure 1.

We also examined whether changes in fibroblast proliferative capacity and consequently a larger number of fibroblasts in the non-CM fraction could account for the reduced myocardial passive tension observed in syndecan-4$^{-/-}$ mice after pressure overload. However, as shown in figures 2C and D (24h and 7 days after AB, respectively), expression of the cell cycle S-phase marker PCNA (proliferating cell nuclear antigen) was not different in cardiac homogenates from WT and syndecan-4 deficient mice. Given the limited capacity of cardiomyocytes to re-enter the cell-cycle[22,23] most of the PCNA signal could be attributed to cell proliferation in the non-CM fraction. Further, lack of syndecan-4 did not affect the proliferative capacity of cardiac fibroblasts *in vitro*, as assessed after 48h or 72h after culture on fibronectin coated plates (Figure 2E). This was consistent with EM data suggesting no differences in the number of cardiac fibroblasts in the non-CM fraction between genotypes (Supplementary Table 2).

3.3 Collagen cross-linking is impaired in left ventricles of syndecan-4$^{-/-}$ mice following pressure overload

Total collagen contents as determined by HPLC were significantly increased 7 days after AB, but this response was unaffected in syndecan-4$^{-/-}$ mice (Figure 3A). A similar pattern was observed for the expression of collagen I and III mRNA at this time-point (Figure 3D-E). This is in contrast with recent data from our group showing that absence of syndecan-4/CaN/NFAT signaling impairs collagen I and III mRNA expression 24h after AB,[5] suggesting a delayed induction of collagen mRNA in the syndecan-4 null mice, which is no longer apparent at later time-points. While AB caused dramatic changes in the expression of several of the main enzymes that regulate collagen degradation (MMP2, MMP13, TIMP1 and TIMP2), these and also levels of MMP9 were not affected by the genotype of the mice (Supplementary Figure 1). To investigate if altered collagen cross-linking could contribute to the observed differences in passive tension, we measured soluble collagen content and calculated the amount of insoluble cross-linked collagen. Indeed, insoluble collagen was increased in left ventricles after AB, but this response was blunted in syndecan-4$^{-/-}$ mice (Figure 3C). For visualization purposes, left ventricular tissue sections 7 days after AB were stained with picrosirius red and examined using polarized light. Images show increased total and cross-linked collagen after AB in WT mice, while less apparent responses in sections from syndecan-4 deficient mice (Figure 3B and D). Other structural matrix proteins may also be altered in syndecan-4$^{-/-}$ mice and thereby, contribute to changes in compliance, such as elastin. However, mRNA and protein levels of elastin, despite being increased after AB, they were not affected by the genotype of the animals (Supplementary Figure 3A-B). Moreover, elastin levels decreased during myofibroblast differentiation (Supplementary Figure 3C).

3.4 Lysyl oxidase expression and activity are reduced in left ventricles and cardiac fibroblasts from syndecan-4$^{-/-}$ mice

AB resulted in significantly increased LOX mRNA levels in the left ventricles of syndecan-4 competent mice 24h after surgery (Figure 4A). This response was blunted in the ventricles of syndecan-4 deficient mice. Culture of cardiac fibroblasts from WT mice on fibronectin-coated plates also resulted in a time-dependent increase in LOX mRNA expression, while culture of fibroblasts from syndecan-4 deficient mice failed to induce any changes in LOX mRNA expression (Figure 4B). Myocardial LOX activity was also increased after AB in WT mice, but the same was not observed in tissue from syndecan-4 deficient mice (Figure 4C).

3.5 Pressure overload and mechanical stress induce OPN expression in a syndecan-4/calcineurin/NFAT-dependent manner

Pressure overload resulted in a rapid and dramatic induction of OPN expression 24h after AB, both at the mRNA (>600-fold) and protein (6.9-fold) level (Figure 5A-B). OPN mRNA remained significantly elevated 7 days after AB, although levels were lower than those at 24h (Figure 5C). In mice lacking syndecan-4, the induction of OPN by pressure-overload was clearly blunted both at 24h and 7 days (Figure 5A-C). OPN mRNA levels were positively correlated to LOX mRNA levels in left ventricular tissue of WT mice (Figure 5D). A similar induction of OPN was observed when cardiac fibroblasts from WT mice were mechanically stressed by culture on fibronectin coated plates, while this response was absent in cardiac fibroblasts from syndecan-4$^{-/-}$ mice (Figure 5E). Conversely, overexpression of syndecan-4 in HT1080 fibroblasts increased OPN mRNA levels (Figure 5F). We have previously demonstrated that upon mechanical stress, syndecan-4 engages the calcineurin-dependent transcription factor NFAT[5] and that NFAT activation regulates OPN expression in vascular smooth muscle cells.[15] Here we found that OPN mRNA levels were elevated in HT1080 fibroblasts overexpressing calcineurin (Figure 5F), but reduced in cardiac fibroblasts treated

with the NFAT blocker A-285222 (Figure 5G). Furthermore, overexpression of NFATc4, clearly increased OPN mRNA expression in HT1080 fibroblasts and this effect was more pronounced after mechanical stress (Figure 5H).

Stimulation with exogenous OPN for 24h has recently been shown to increase pro-LOX protein expression and LOX activity in cardiac human fibroblasts.[11] Here we demonstrate increased LOX protein expression after OPN stimulation in mouse cardiac fibroblasts that had been cultured on fibronectin coated plates with TGFβ (Supplementary Figure 4A-B), a protocol that promotes myofibroblast differentiation.[5] Lack of syndecan-4 had no impact on OPN-induced LOX (Supplementary Figure 4A-B), confirming that the effects are downstream of OPN and do not involve signaling via syndecan-4. Interestingly, myofibroblast differentiation also yielded significantly increased expression of known OPN receptors including integrins α_v and β_1, and CD44 (Supplementary Figure 4C).

3.6 The extracellular domain of syndecan-4 interacts with collagen fibrils and promotes collagen cross-linking by LOX

We next examined the effect of the extracellular domain of syndecan-4 (ECsyn4) on collagen I fiber formation in a test tube. Incubation of collagen I at 37ºC for 72h with increasing concentrations of ECsyn4 resulted in a dose-dependent increase in turbidity measured as optical density at 340nm (Figure 6A). Incubation of the highest concentration of ECsyn4 alone (4μg) failed to increase optical density. At the electron microscopy level, collagen fibrils appear similar in thickness and structure in samples containing the mix of collagen and ECsyn4, and collagen alone (Figure 6B). However, only the samples containing the mix of collagen and ECsyn4 exhibited extensive dense networks surrounding the collagen fibrils (marked with asterisks in Figure 4B). Addition of LOX to samples containing collagen caused formation of large insoluble protein aggregates after 48h due to collagen cross-linking and formation of extensive collagen fiber networks as observed by electron microscopy (Figure 6D). Turbidity of these samples after 24h, when the collagen was still in solution, further increased with the addition of ECsyn4 (Figure 6C) and resulted in the appearance of structures resembling small collagen fibers between the thicker collagen fibers (Figure 6D; arrows). In samples containing collagen and LOX, similar dense structures to those seen in samples without LOX but with ECsyn4, where observed (Figure 6D; asterisks). Taken together, these results suggest that the extracellular domain of syndecan-4 promotes collagen fiber formation, possibly facilitating collagen cross-linking by LOX.

4. Discussion

Since the recognition of diastolic dysfunction as a common and prominent feature of the failing heart, there has been an increased focus on understanding the molecular mechanisms underlying myocardial stiffening as a cause of diastolic dysfunction. With this study we demonstrate the importance of syndecan-4 in determining passive tension of the myocardium following pressure overload. Our understanding of how this is achieved in the mouse model used here is schematically presented in Figure 7: Within the first 24h after AB, a rapid upregulation of syndecan-4 takes place in the left ventricle of the heart. In this context of abundant syndecan-4 expression, cardiac fibroblasts will more readily sense the increased mechanical stress and engage the calcineurin/NFAT signaling pathway to promote myofibroblast differentiation.[5] While syndecan-4 levels will eventually return to normal levels 3 weeks after AB (this study and [14]), a series of events triggered by mechanical stress-induced syndecan-4 signaling will contribute to increased myocardial stiffness of the left ventricle. Activated myofibroblasts will produce excessive amounts of ECM proteins, including collagen I and III, and OPN. A concomitant increase in the expression and activity of LOX will result in increased collagen cross-linking and hence, increased passive tension (stiffness) 7 days after AB. In our previous work, we demonstrated that phosphorylation of serine 179 in the cytoplasmic part of syndecan-4 was required for calcineurin/NFAT activation.[5] Here we show that the extracellular domain of syndecan-4 seems also to be involved in determining ECM stiffness by interacting with collagen fibers and facilitating collagen cross-linking by LOX.

Deletion of syndecan-4 had a striking effect on myocardial stiffness, with passive tension being clearly lower in myocardial muscle strips from mice lacking syndecan-4 after AB. There are two main contributors to passive tension in the left ventricle: the giant "spring-like" protein of the cardiomyocyte sarcomere, titin,[24] and the ECM. Considering the location of syndecan-4 in the costameres overlaying the Z-disc of cardiomyocytes,[25] a site to which titin is attached, we might expect an effect of syndecan-4 deletion on titin function. To examine this, we eliminated the contribution of titin to passive tension by performing myofilament extraction of muscle strips. In agreement with a previous report[26] titin accounted for about one third of the total passive tension of left ventricular tissue from sham-operated mice. In contrast, passive tension of pressure-overloaded left ventricles was almost entirely accounted for by the ECM. Importantly, the relative decrease in passive tension following titin disruption was similar in muscle strips from WT and syndecan-4$^{-/-}$ mice, suggesting intact titin function in mice lacking syndecan-4, and that the observed difference in passive tension was caused by altered ECM remodeling.

Although results gained *in vitro* from muscle strips do not translate directly into the mechanical properties of the whole heart, they strongly support an important role for the ECM in myocardial stiffening in response to elevated pressure. In patients with congenital heart disease, elevated end-diastolic pressure is accounted for by higher stiffness of both myocytes and ECM.[27] Although increased myocyte stiffness did not prevail in the AB mouse model used in this study (reflecting variability between species and etiologies of disease), the molecular processes of ECM remodeling investigated here are likely to apply also in humans with pressure-overloaded ventricles.

One exciting finding in this study was the effect of syndecan-4 deletion on collagen cross-linking. In addition to collagen amount, the structure of collagen has a major impact on the tensile strength of the ECM and passive tension of the left ventricle. This is demonstrated in muscle strips from pressure-overloaded and volume-overloaded congenital heart failure patients where, despite equal levels of collagen, ECM stiffness was higher in patients with pressure-overloaded ventricles due to differences in collagen cross-linking.[27] Also in

hypertensive patients, collagen cross-linking correlated with elevated left ventricular filling pressures, whereas collagen amount did not.[8] We here found that mice lacking syndecan-4 have reduced LOX expression and activity *in vivo* and lower degree of collagen cross-linking, suggesting a regulatory role for syndecan-4 of this enzyme. A similar link has been found between syndecan and another ECM-crosslinking enzyme, tissue transglutaminase, which exhibited decreased activity in syndecan-1$^{-/-}$ mice following myocardial infarction[28] and in syndecan-4$^{-/-}$ mice during kidney tissue remodeling.[29]

Despite its apparently critical role in myocardial stiffening little is known regarding mechanisms regulating LOX in the heart. Recently, OPN was found to induce LOX expression and activity in human cardiac fibroblasts.[11] Here we demonstrate that OPN induces LOX also in mouse cardiac fibroblasts and that the effects are potentiated under conditions leading to myofibroblast differentiation (i.e. culture on fibronectin coated plates or pre-conditioned with TGFβ). Increasing evidence also suggest an important role for OPN in the development of cardiac fibrosis.[30,31,11,32] OPN correlates to cross-linked collagen in hypertensive heart disease patients and to left ventricular chamber stiffness in pressure-overloaded rats. OPN-deficient mice fail to develop fibrosis in response to AngII treatment[33] and have reduced collagen accumulation after acute myocardial infarction.[32] Cardiac OPN expression can be triggered by mechanical stress as demonstrated in this study, which also suggests that syndecan-4/NFAT signaling is involved in the regulation of OPN in the context of pressure overload.

Recently, syndecan-4 signaling was shown to inhibit apoptosis and regulate NFAT activity after myocardial infarction,[34] but in this context, syndecan-4$^{-/-}$ mice exhibited increased NFAT activity, as opposed to the reduced activity we report in syndecan-4$^{-/-}$ mice after pressure overload. This discrepancy is intriguing and may be at least in part be explained by the different nature of the experimental models. While the cardiac remodeling process is predominantly triggered by inflammation and apoptosis in MI, it is in the pressure-overloaded heart initiated by mechanical stress. It is possible that syndecan-4 may act as a negative regulator of inflammation, which is in line with previous work[35] also in the context of MI[36] and heart failure.[14]

We also demonstrated that collagen fiber formation was enhanced *in vitro* in the presence of ECsyn4, suggesting a novel role for syndecan-4 in facilitating collagen cross-linking. Coincidentally, heparan sulfate GAG chains have been shown to interact with extracellular structural proteins such as collagen I[37] and fibronectin.[38] Other proteoglycans such as decorin and fibromodulin are known to bind to and stabilize collagen fibrils,[39] and have been suggested[40] to act as co-receptors for LOX. Indeed, collagen cross-linking by LOX appeared to be promoted in the presence of ECsyn4. Taken together, our results suggest a dual role for syndecan-4 in collagen cross-linking, inducing collagen and LOX expression by cardiac fibroblasts, and facilitating LOX cross-linking. In light of recent work reporting shedding of the extracellular domain of syndecan-4 in the failing human heart[14] further studies are encouraged to determine the exact details of the syndecam-4-collagen interaction and its physiological significance.

The limited number of therapeutic targets in cardiac fibrosis is a major problem for heart failure treatment. Syndecan-4 is upregulated in the pressure-overloaded left ventricle of mice[14,5] and man,[13] and based on recent research regarding the role of syndecan-4 in ECM remodeling we speculate that targeting syndecan-4 may be an effective anti-fibrotic approach.[41,42] Supporting this, we here demonstrate for the first time that syndecan-4 regulates passive tension in the pressure-overloaded myocardium by regulation of OPN and LOX expression, LOX activity and collagen cross-linking. Given that syndecan-4 is not only expressed in cardiac fibroblasts but also in cardiomyocytes, where it is necessary for the

development of concentric left ventricular hypertrophy in response to pressure overload,[13] the next challenge will be to identify differential molecular traits between syndecan-4-dependent signaling in these two cell types (i.e. using cell specific knockouts of syndecan-4). This will allow to specifically target processes leading to changes in passive tension and to limit exacerbated fibrosis while maintaining intact the critical adaptive hypertrophic response of cardiomyocytes to pressure overload.

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Disclosures
None declared.

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Figure Legends

Figure 1. Myocardial passive tension is lower in mice lacking syndecan-4 due to altered ECM. Passive tension (A) and viscous stress (B) at 1.0- to 1.3-fold slack length (L_0) of left ventricular muscle strips from wild-type (WT) and syndecan-$4^{-/-}$ (syn$4^{-/-}$) mice 7 days after sham operation (sham) or aortic banding (AB). C, representative titin protein gel and summarized data showing left ventricular N2BA:N2B ratio. D, passive tension after salt extraction. E, electron microscope images of untreated (control) and salt extracted muscle strips. Scale bar 2µm. Two-way ANOVA analyses showed effect of genotype on passive tension before and after salt extraction. No difference in N2BA:N2B ratio (C) was found by using Kruskal-Wallis with Dunn's multiple comparison test. Data are presented as mean ± S.E.M. (A, B and D) for clearer visualization, and as mean ± S.D. (C). N=5-8.

Figure 2. Increased syndecan-4 expression induces myofibroblast differentiation. A, immunofluorescence staining of smooth muscle α-actin (green) in cardiac fibroblasts from wild-type (WT) and syndecan-$4^{-/-}$ (syn$4^{-/-}$) mice transduced with an empty adenoviral vector (adeno-null) or containing full-length syndecan-4 (adeno-syndecan-4). Scale bar 50µm. B, Immunohistochemistry showing staining for smooth muscle α-actin (SMA) and platelet-derived growth factor receptor β (PDGFRβ) in left ventricular tissue. Scale bar 100µm. Relative mRNA levels of proliferating cell nuclear antigen (PCNA) normalized to GAPDH in left ventricles 24h (C) and 7 days (D) after AB, and cardiac fibroblasts plated on fibronectin for 48h and 72h (E). Statistical significance was determined by two-way ANOVA with Bonferroni post-hoc test (C-D) and Kruskal-Wallis with Dunn's multiple comparison test (E). n.s., not significant. Data are presented as mean ± S.D. N=8-10 (C-D); 4 (E).

Figure 3. Collagen cross-linking is reduced in the myocardium of syndecan-$4^{-/-}$ mice. A, total collagen determined by HPLC in left ventricles from wild-type (WT) and syndecan-$4^{-/-}$ (syn$4^{-/-}$) mice following sham operation (sham) or 7 days aortic banding (AB); B, cross-linked collagen content in left ventricles from mice as in A; C, visualization of total collagen and cross-linked collagen using unpolarized and circularly polarized light, respectively, after picrosirius red staining of left ventricular sections from WT and syn$4^{-/-}$ mice; Scale bar 100µm for total collagen and 50µm for cross-linked collagen. D and E, mRNA levels of collagen I and collagen III 7 days after AB. Statistical significance was determined by two-way ANOVA with Bonferroni post-hoc tests (A, D and E) and Kruskal-Wallis with Dunn's multiple comparison test (B). Data are normalized to WT sham and presented as mean ± S.D. N=5-9 (A and B), 14-16 (D and E).

Figure 4. Lysyl oxidase expression and activity is reduced in syndecan-$4^{-/-}$ mice. A, lysyl oxidase (LOX) mRNA levels in left ventricular tissue from wild-type (WT) and syndecan-$4^{-/-}$ (syn$4^{-/-}$) mice 24h after sham operation (sham) or aortic banding (AB). B, LOX mRNA in cardiac fibroblasts from WT and syn$4^{-/-}$ mice plated on fibronectin for 72h to induce myofibroblast differentiation. C, LOX activity in left ventricular tissue from WT and syn$4^{-/-}$ mice subjected to sham operation or 3 days AB. Two-way ANOVA with Bonferroni post-hoc test (A), Mann-Whitney test (B) and Kruskal-Wallis with Dunn's multiple comparison test (C) were applied. Data are relative to WT sham (A and C) or WT (B) and presented as mean ± S.D. N=10 (A), 5 (B), 7-8 (C).

Figure 5. Osteopontin is regulated by syndecan-4. Osteopontin (OPN) mRNA (A and C) and protein (B) in left ventricular tissue from wild-type (WT) and syndecan-$4^{-/-}$ (syn$4^{-/-}$) mice 24h (A and B) and 7 days (C) after sham operation (sham) or aortic banding (AB). D,

correlation of cardiac lysyl oxidase (LOX) and OPN mRNA 3 and 7 days after sham or AB determined by Pearson regression analysis. E, OPN mRNA levels in cardiac fibroblasts from WT and syn4$^{-/-}$ mice 6, 24, 48 and 72h after plating on fibronectin. HT1080 fibroblasts control (ctrl) and overexpressing syndecan-4 (syn4) and calcineurin (CaN; F) and H, NFATc4. G, cardiac fibroblasts treated with an NFAT blocker. Significant differences were determined by two-way ANOVA with Bonferroni post-hoc test (A-C, E), Mann-Whitney test (F and H) and Student's paired *t*-test (G). Data are relative to WT sham (A-D), WT 6h (E) and ctrl (F-H) and presented as mean ± S.D. N=8-10 (A-C), 5-6 (E-H)

Figure 6. Syndecan-4 facilitates collagen cross-linking by LOX. A and C, turbidity measured as optical density at 340nm of solutions containing collagen, extracellular syndecan-4 (ECsyn4) and lysyl oxidase (LOX). B and D, electron microscopy images of collagen alone or mixed with ECsyn4 with and without LOX, or ECsyn4 alone. Asterisks denote structures only present in solutions containing both collagen and ECsyn4 (B) or collagen and LOX (D). Arrows indicate inter-fibrillar structures (D). Scale bars: 1µm (upper panel in B, and in D) and 200nm (lower panel in B). Comparisons are made by Mann-Whitney to the ctrl in A and C and significance denoted with * or n.s. for non significant comparisons. Data are relative to collagen (A) and collagen with LOX (B) and presented as mean ± S.D. N=3-6.

Figure 7. Syndecan-4 regulates cardiac fibroblast phenotype and function, collagen cross-linking and myocardial stiffness in response to mechanical stress. Schematic model showing syndecan-4 expression (green) during the sequence of events taking place in response to left ventricular pressure overload in mice. Syndecan-4 expression is based on previously published data[14] for 0, 24h, 7 and 21 days; as well as data for 0 and 3 days in this study. AB, aortic banding; mech. stress, mechanical stress; syn4, syndecan-4; CaN, calcineurin.

Figure 1

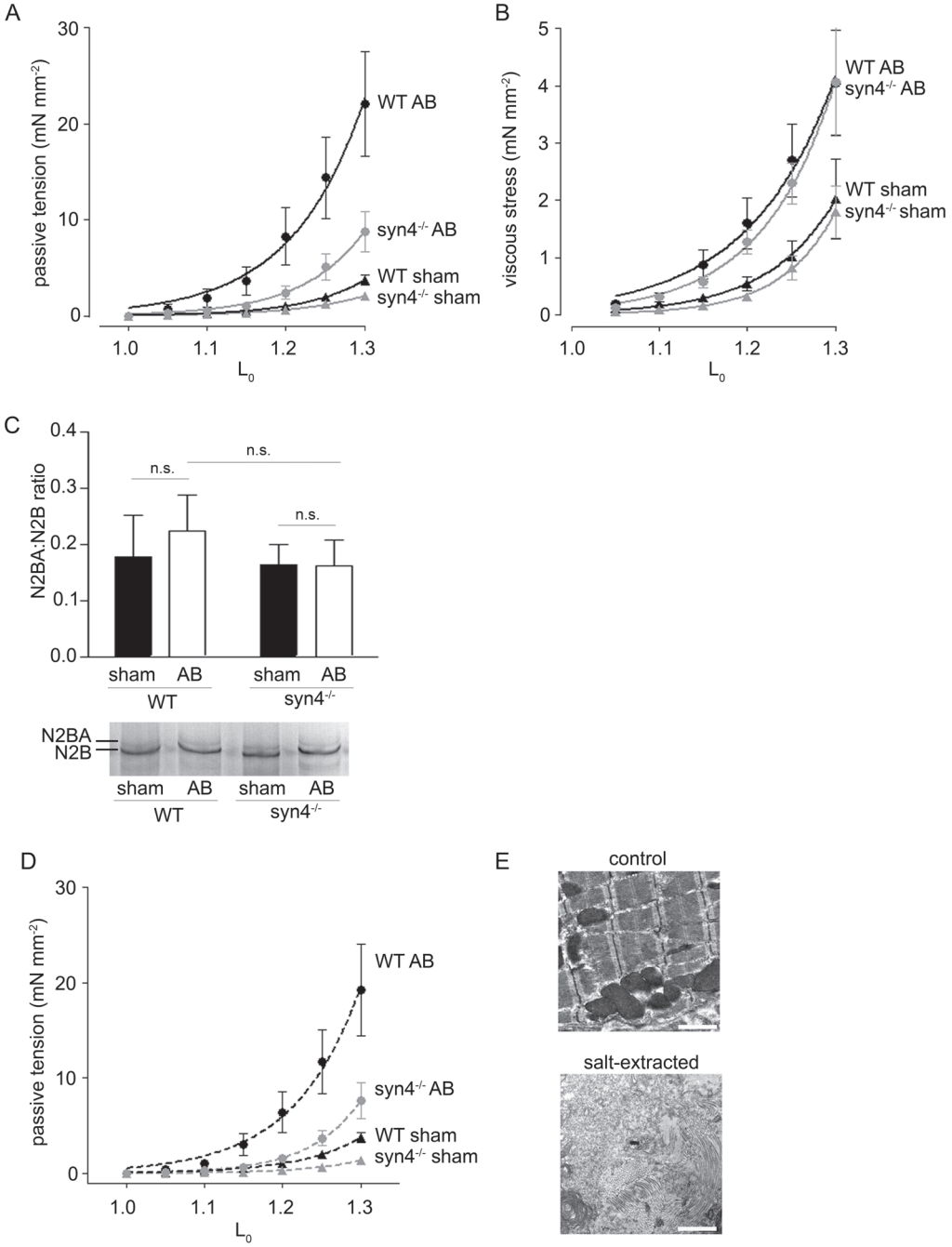
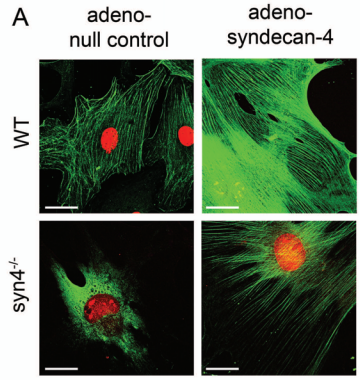
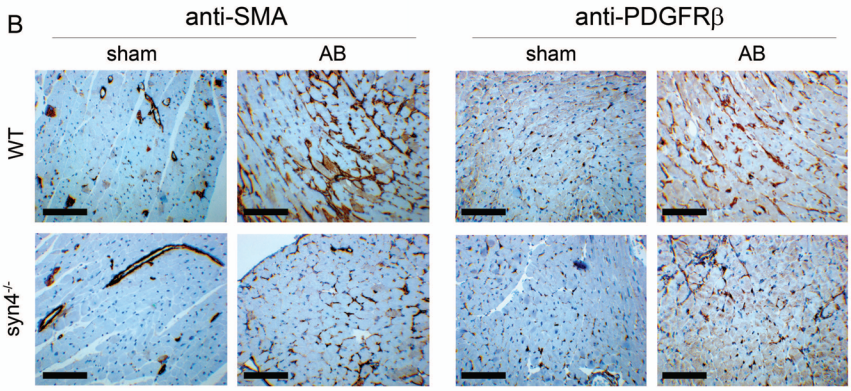
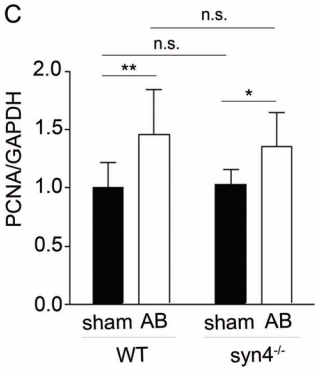
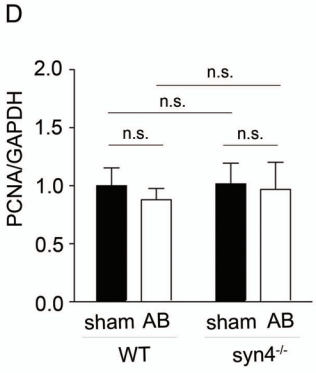

A
passive tension (mN mm-2)
30
20
10
0
1.0
1.1
1.2
1.3
L0
WT AB
syn4-/- AB
WT sham
syn4-/- sham
B
viscous stress (mN mm-2)
5
4
3
2
1
0
1.0
1.1
1.2
1.3
L0
WT AB
syn4-/- AB
WT sham
syn4-/- sham
C
N2BA:N2B ratio
0.4
0.3
0.2
0.1
0.0
n.s.
n.s.
n.s.
sham
AB
sham
AB
WT
syn4-/-
N2BA
N2B
sham
AB
sham
AB
WT
syn4-/-
D
passive tension (mN mm-2)
30
20
10
0
1.0
1.1
1.2
1.3
L0
WT AB
syn4-/- AB
WT sham
syn4-/- sham
E
control
salt-extracted

Figure 2


A
adeno-
null control
adeno-
syndecan-4
WT
syn4-/-


B
anti-SMA
anti-PDGFRβ
sham
AB
sham
AB
WT
syn4-/-


C
n.s.
n.s.
**
*
PCNA/GAPDH
2.0
1.5
1.0
0.5
0.0
sham AB
sham AB
WT
syn4-/-


D
n.s.
n.s.
n.s.
n.s.
PCNA/GAPDH
2.0
1.5
1.0
0.5
0.0
sham AB
sham AB
WT
syn4-/-

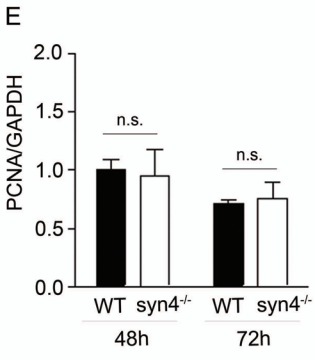

E
n.s.
n.s.
PCNA/GAPDH
2.0
1.5
1.0
0.5
0.0
WT syn4-/-
WT syn4-/-
48h
72h

Figure 3

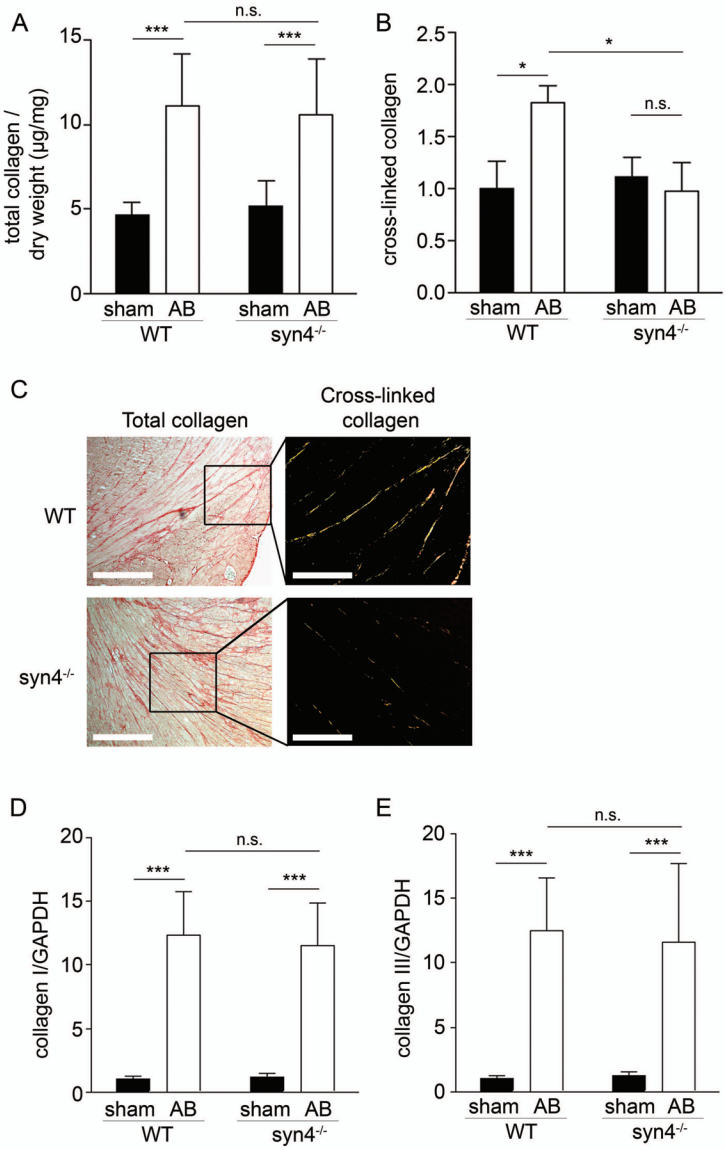

A
total collagen / dry weight (µg/mg)
15
10
5
0
***
n.s.
***
sham AB
WT
sham AB
syn4-/-
B
cross-linked collagen
2.5
2.0
1.5
1.0
0.5
0.0
*
*
n.s.
sham AB
WT
sham AB
syn4-/-
C
Total collagen
Cross-linked collagen
WT
syn4-/-
D
collagen I/GAPDH
20
15
10
5
0
***
n.s.
***
sham AB
WT
sham AB
syn4-/-
E
collagen III/GAPDH
20
15
10
5
0
***
n.s.
***
sham AB
WT
sham AB
syn4-/-

Figure 4

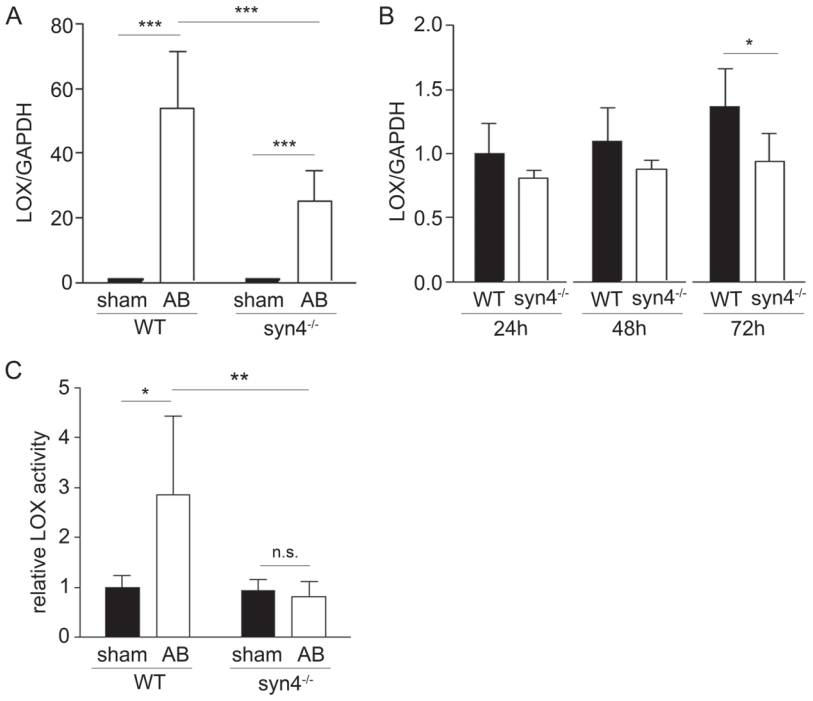

A
LOX/GAPDH
80
60
40
20
0
***
***
***
sham AB
WT
sham AB
syn4-/-
B
LOX/GAPDH
2.0
1.5
1.0
0.5
0.0
*
WT syn4-/-
24h
WT syn4-/-
48h
WT syn4-/-
72h
C
relative LOX activity
5
4
3
2
1
0
*
**
n.s.
sham AB
WT
sham AB
syn4-/-

Figure 5

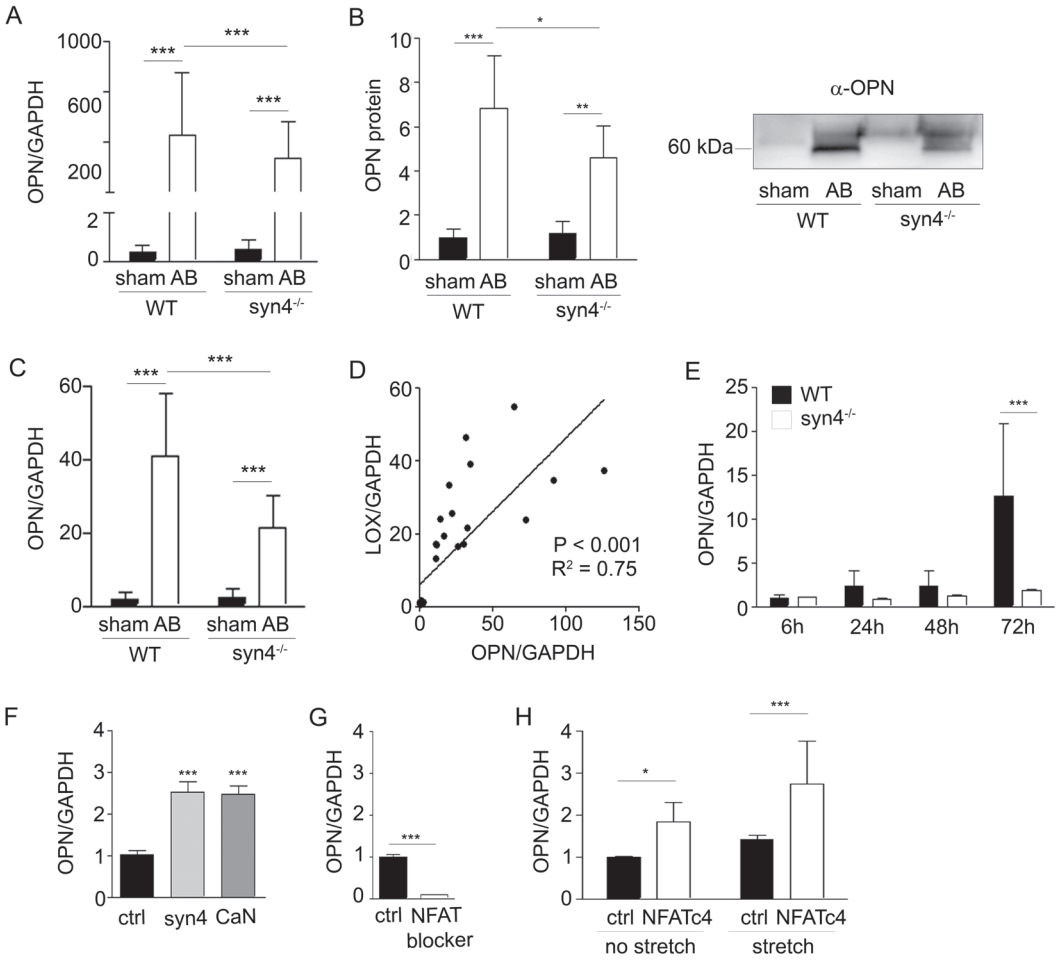

A
OPN/GAPDH
1000
600
200
2
0
***
***
***
sham AB
sham AB
WT
syn4-/-
B
OPN protein
10
8
6
4
2
0
***
*
**
sham AB
sham AB
WT
syn4-/-
α-OPN
60 kDa
sham AB sham AB
WT
syn4-/-
C
OPN/GAPDH
60
40
20
0
***
***
***
sham AB
sham AB
WT
syn4-/-
D
LOX/GAPDH
60
40
20
0
P < 0.001
R2 = 0.75
0
50
100
150
OPN/GAPDH
E
OPN/GAPDH
25
20
15
10
5
0
WT
syn4-/-
***
6h
24h
48h
72h
F
OPN/GAPDH
4
3
2
1
0
***
***
ctrl
syn4
CaN
G
OPN/GAPDH
4
3
2
1
0
***
ctrl
NFAT blocker
H
OPN/GAPDH
4
3
2
1
0
*
***
ctrl
NFATc4
ctrl
NFATc4
no stretch
stretch

Figure 6

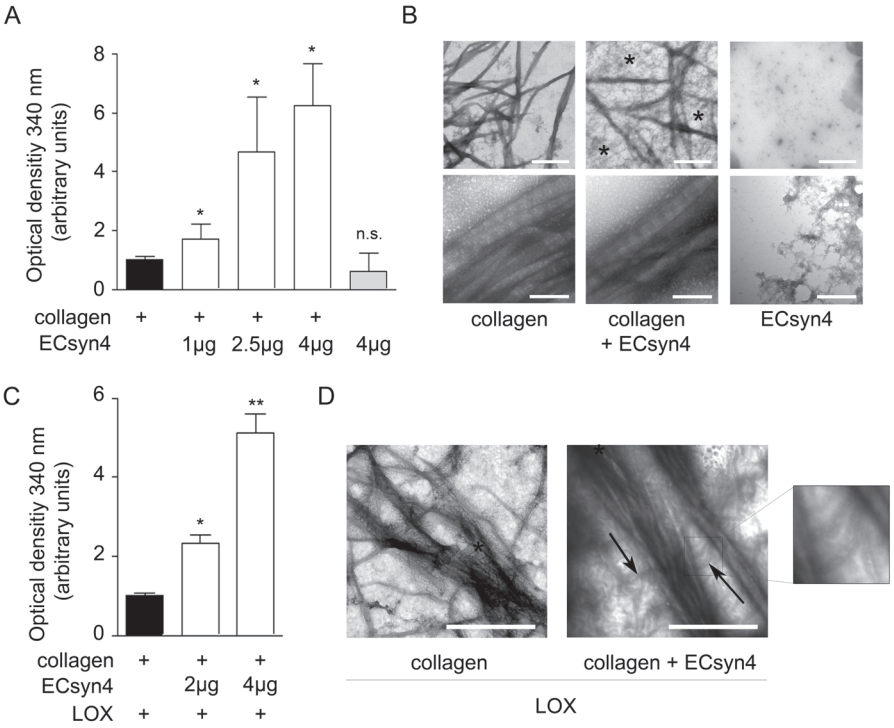
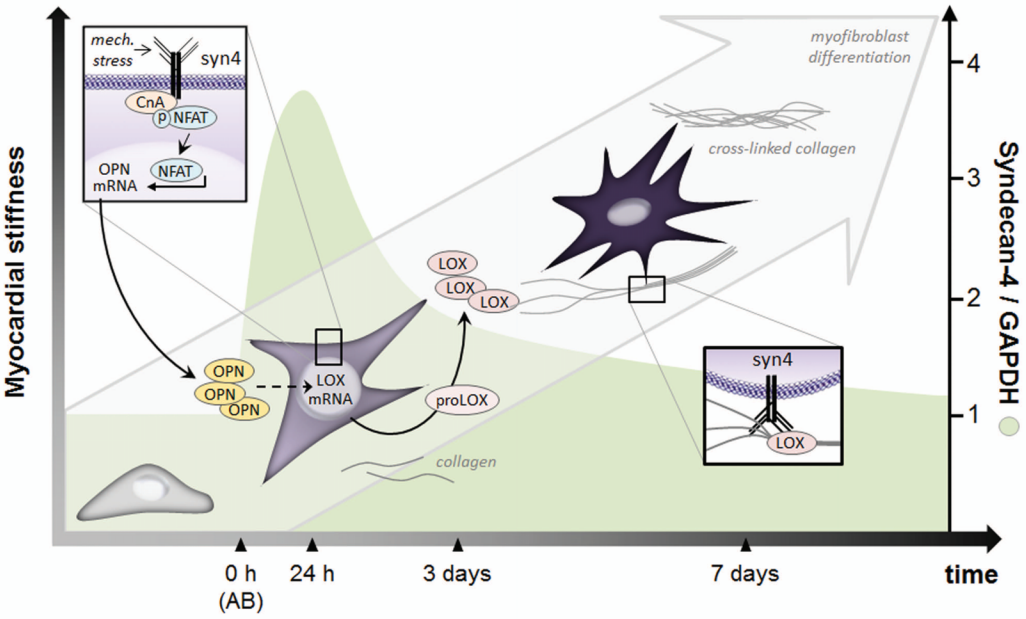

A
Optical densitiy 340 nm (arbitrary units)
0
2
4
6
8
*
*
*
n.s.
collagen + + + + +
ECsyn4 1µg 2.5µg 4µg 4µg
B
collagen
collagen + ECsyn4
ECsyn4
C
Optical densitiy 340 nm (arbitrary units)
0
2
4
6
*
**
collagen + + +
ECsyn4 2µg 4µg
LOX + + +
D
collagen
collagen + ECsyn4
LOX

Figure 7


Myocardial stiffness
Syndecan-4 / GAPDH
4
3
2
1
time
0 h
(AB)
24 h
3 days
7 days
mech.
stress
syn4
CnA
P
NFAT
OPN
mRNA
NFAT
OPN
OPN
OPN
LOX
mRNA
proLOX
LOX
LOX
LOX
myofibroblast
differentiation
cross-linked collagen
collagen
syn4
LOX

Supplementary Material

Detailed Methods

Mouse Model of Pressure Overload

AB of the ascending aorta or sham operations were performed on 8-12 week old male C57Bl/6JBomTac wild-type (WT) and syndecan-4 knockout (syndecan-$4^{-/-}$) mice[1] on C57BL/6J background (000664, The Jackson Laboratory, Bar Harbor, ME) as previously described.[2] Mice were initially anesthetized with 5% isoflurane and 95% oxygen in a chamber. Subsequently, mice were intubated and ventilated with 3% isoflurane and 97% oxygen. For post-operative analgesia, animals were given 0.02 ml buprenorphine (0.3 mg/ml) subcutaneously and allowed to recover at 35ºC. Animals with an aortic flow velocity > 4 m/s, as determined by echocardiography were included in the study. Before euthanizing the mice by cervical dislocation 24h, 3 days or 7 days after AB, echocardiography was performed as previously described.[2,3]

Mechanical measurements

Muscle strips from snap frozen left ventricular tissue were skinned in 0.5% Triton X-100 in relaxing solution containing (in mM): ATP 7.8; creatine phosphate 20; imidazole 20; EGTA 4; Mg-propionate 12; K-propionate 97.6 (pH 7.1), and passive force was measured at room temperature after mounting the strips between two stainless steel clips attached to a micromotor and a force transducer (Scientific Instruments, Heidelberg, Germany). L_0 was set by determining slack length, verified in three muscle strips by laser diffraction.[4,5] to correspond to a sarcomere length of 1.8 µm. Muscle strips were stretched in several equal steps, each followed by a 1-minute hold period,to a maximum of 30 % L_0, corresponding to a sarcomere length of ~2.3 µm. Quasi steady-state passive force at the end of the hold period was determined as a measure of the elastic component of the muscle strip, and was related to the cross-sectional area at the thinnest point of the strip at slack length, to obtain passive tension. Viscous stress, reflecting the stretch velocity sensitive component of the muscle, was determined as peak force upon stretching minus steady-state force, divided by cross-sectional area. Salt extraction of actin and myosin filaments was performed by treatment with 0.6 M KCl (Sigma, Schnelldorf, Germany) for 20 min, followed by 1 M KI (Sigma) for 20 min.[6] After passive tension measurements, muscle strips were fixed and disruption of sarcomere structure verified by electron microscopy. Mean passive tension/viscous stress values were plotted against L_0 and best fits to mean data points were made using exponential growth equations.

Collagen cross-linking and quantification

For visualization of cross-linked collagen, left ventricles were fixed in 4 % neutral buffered formalin, dehydrated, cleared and embedded in paraffin. Transverse sections (4 µm) were stained with picrosirius red and examined by light microscopy with polarized light. For collagen quantification, thick transverse sections (20 µm) were stained with picrosirius red (Sigma) and normalized to total protein content determined by fast green (Sigma) staining.[7] The amount of soluble collagen was assessed using colorimetric and enzymatic procedures as previously described[7,8] and by using Sircol Collagen Assay from Biocolor (Carrickfergus, UK).

Fibroblast Cell Culture

Neonatal mice were sacrificed by decapitation and cardiac fibroblasts isolated as previously described[9] and used at passage 2 to limit *in vitro* effects on fibroblast phenotype. Cells were seeded on fibronectin-coated plates to induce myofibroblast differentiation, as previously shown to mimic the phenotypic modulation that takes place *in vivo* during pressure overload

of the left ventricle.[9] LOX expression was measured in cardiac fibroblasts from neonatal WT and syndecan-4$^{-/-}$ after 24, 48 and 72h on fibronectin. Also, cardiac fibroblasts from 6-12 week old mice (sacrificed by decapitation after anesthesia with isoflurane in a chamber) were isolated using collagenase[2] and transduced with adenoviral vector containing mouse syndecan-4 (196608A; Applied Biological Materials Inc, Richmond, Canada). Cardiac fibroblasts were stimulated with 10 ng/ml TGFβ1 (GF111, Merck Millipore, Darmstadt, Germany) and 250 ng/ml OPN (Cat. no.120-35; Peprotech, Hamburg, Germany). Human fibrosarcoma HT1080 fibroblasts were transfected with mouse syndecan-4 (NP_035651.1; HA-tagged), mouse calcineurin (A000420; His-tagged) NFATc3 (NP 035031; GFP-tagged) or NFATc4 (NP_076188; GFP-tagged) custom made by Genscript (Piscataway, NJ) using lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Successful transfection was determined by real-time PCR on syndecan-4 (Mm00488527_m1), calcineurin (Mm01317678_m1) and NFATc4 (Mm00452373_g1). 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). The NFAT blocker A-285222 was kindly provided by Abbott Laboratories (1μmol/L; Abbott Park, IL).

Gene Expression Analysis

RNA was extracted from frozen left ventricles and cell cultures using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Only samples with RNA integrity number (RIN) > 6, assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) were accepted. cDNA synthesis 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 proliferating cell nuclear antigen (PCNA; Mm00448100_m1) collagen I (Mm00483888_m1) and collagen III (Mm00802331_m1), OPN (Mm00436767_m1, Hs00959010_m1), LOX (Mm00495386_m1) and glyceraldehydes 3-phosphate dehydrogenase (GAPDH; Mm03302249_g1, Hs99999905_m1), MMP2 (Mm00439506_m1), MMP9 (Mm00442991_m1), MMP13 (Mm00439491_m1), TIMP1 (Mm00441818_m1), TIMP2 (Mm00441825_m1), fibronectin (Mm01256744_m1), elastin (Mm00514670_m1), CD44 (Mm01277163_m1), $Itga_v$ (Mm00434486_m1) and $Itgb_1$ (Mm01253230_m1). Data were normalized to GAPDH.

Immunoblotting

Immunoblotting was performed on left ventricle homogenates as previously described.[10] Anti-osteopontin (IBL, Hamburg, Germany) and anti-LOX (NB100-2527, Novus Biologicals, Littleton, CO) were used as primary antibodies.

Immunocytochemistry

Cells grown on fibronectin-coated glass cover slips were fixed in 4% paraformaldehyde (Sigma) and stained using mouse-anti-SMA (1:300, Sigma) and alexa fluor 488- secondary anti-mouse antibodies (Invitrogen). Images were obtained a LSM 710 confocal microscope (Zeiss). Nuclei were stained with sytox orange (Invitrogen). Staining with IgG2a or omitting primary antibodies served as negative controls.

Immunohistochemistry

Formalin-fixed myocardial tissue sections (4 μm) were stained for SMA (1:250, M0851, DAKO, Glostrup, Denmark) and platelet-derived growth factor receptor β (PDGFRβ; 1:100, #4564, Cell Signaling, Danvers, MA) and visualized by the use of secondary antibodies conjugated to horseradish peroxidase (HRP). Positive controls were obtained by staining tissue from appendix and lung cancer for SMA and PDGFR, respectively.

In vitro collagen fiber formation assay
A recombinant protein (1-4 µg) consisting of the extracellular domain of syndecan-4 fused with the Fc region of human IgG_1 (syndecan-4 homodimer; Sino Biological Inc., Beijing, China) was mixed with 10 µg collagen I (BD Biosciences, Bedford, MA) in 25 µl TES buffer, 2 µL 10 mM $ZnSO_4$ and distilled water to a total volume of 50 µl, with or without recombinant human lysyl oxidase homolog 2 (0,1µg, LOXL2 with activity > 2 pmol/min/µg, R&D systems, Abingdon, UK). The mixture was incubated at 37 ºC for 24-72 h. After mixing vigorously, turbidity of the solution was measured as optical density at 340 nm and content examined with electron microscopy by performing negative staining with 2% aqueous uranyl acetate directly on the grids.

References

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3. Finsen AV, Christensen G, Sjaastad I. Echocardiographic parameters discriminating myocardial infarction with pulmonary congestion from myocardial infarction without congestion in the mouse. *J Appl Physiol* 2005;**98**:680-689.

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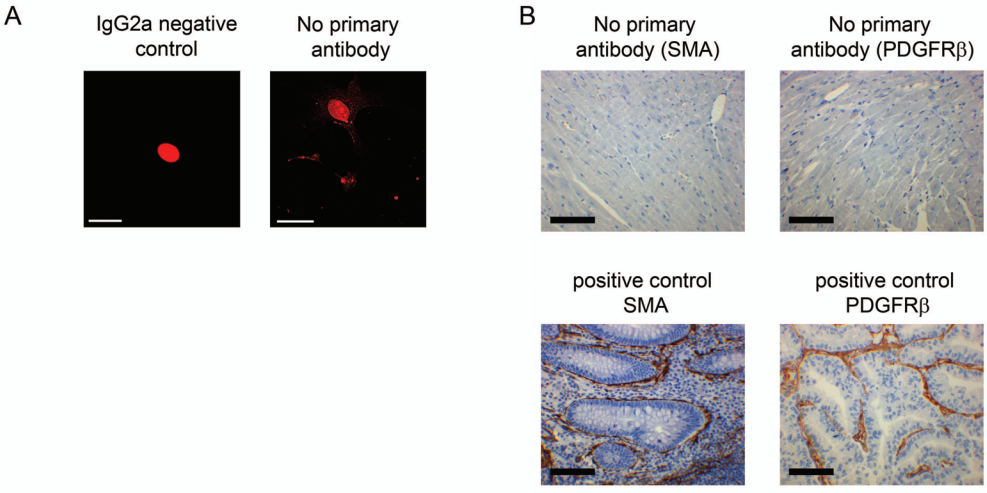
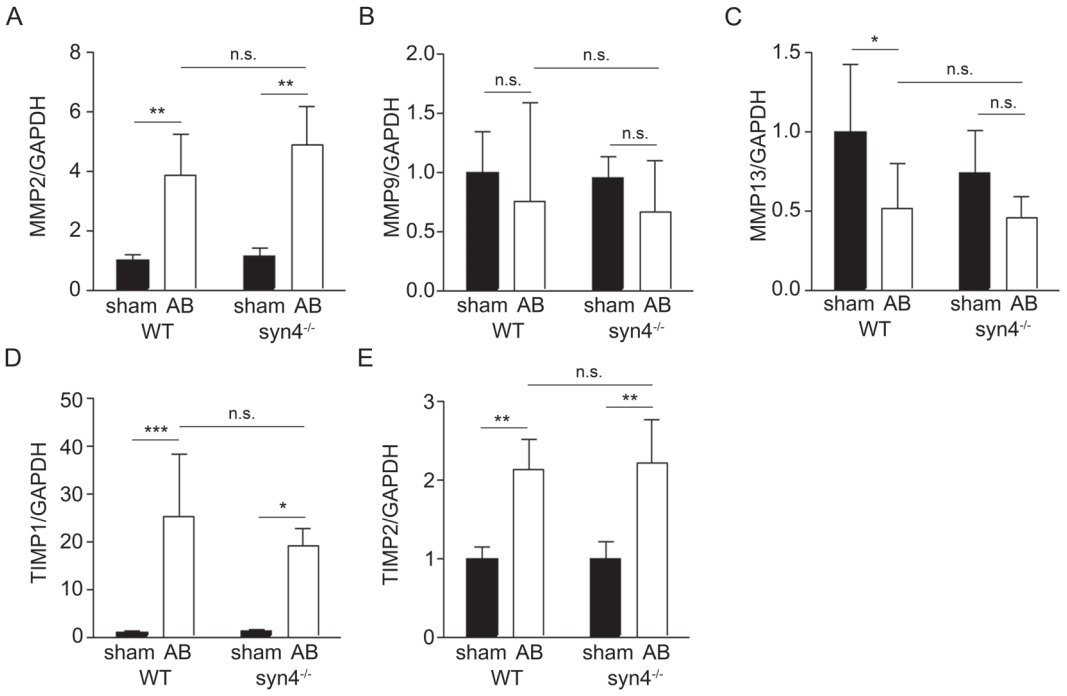
Supplementary Figure 1



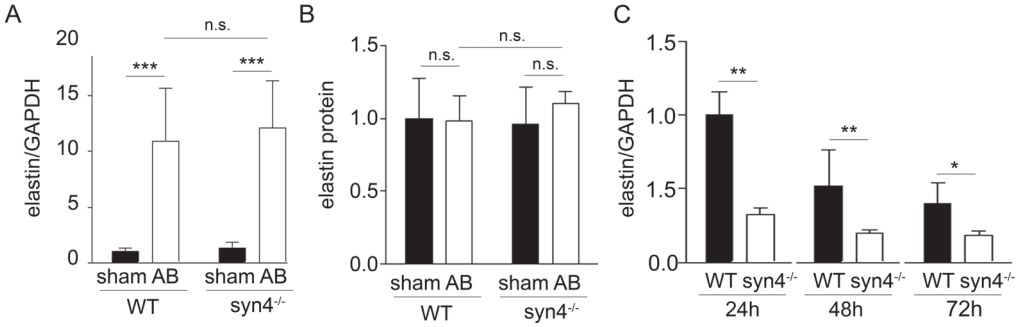
Supplementary Figure 1. Negative controls for smooth muscle α-actin and platelet-derived growth factor receptor β immunostaining. A, Nuclei are stained red and staining with IgG2a or omitting primary antibody was used as negative control for smooth muscle α-actin (SMA) immunocytochemistry (see Figure 2A). Scale bar 50μm. B, omitting primary antibodies was used as negative controls for SMA and platelet-derived growth factor receptor β (PDGFRβ) immunohistochemistry (see Figure 2B). Tissue from appendix and lung cancer were used as positive controls for SMA and PDGFRβ, respectively. Scale bar 100μm.

Supplementary Figure 2



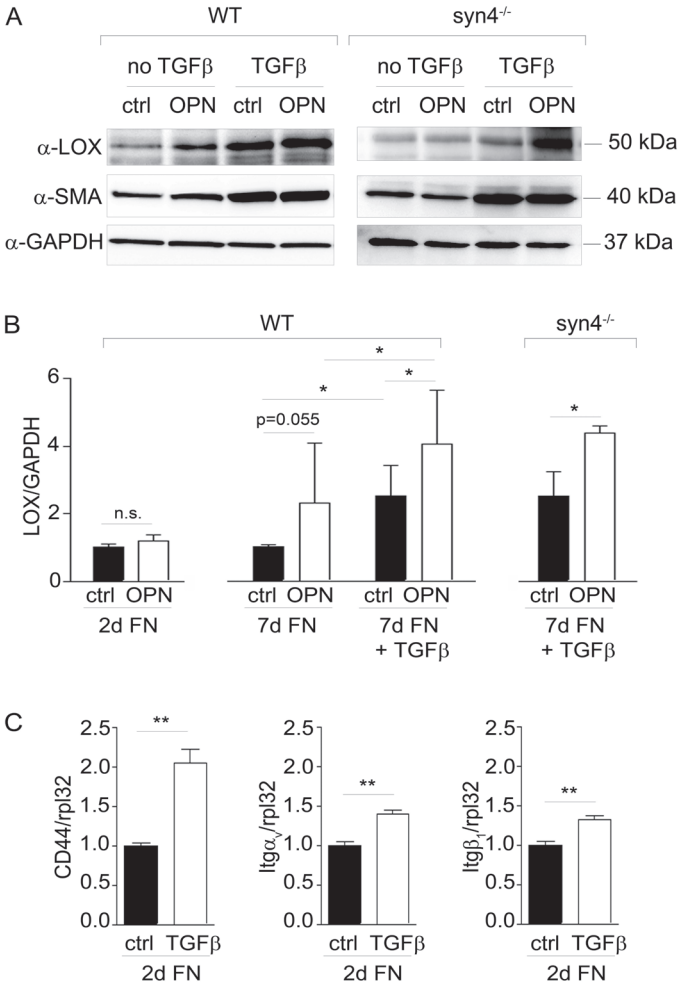
Supplementary Figure 2. Cardiac expression of matrix metalloproteinases and their inhibitors. Relative mRNA levels of A, matrix metalloproteinase (MMP) 2; B, MMP9; C, MMP13; D, tissue inhibitor of metalloproteinase (TIMP) 1; E, TIMP2 in wild-type (WT) and syndecan-4$^{-/-}$ (syn4$^{-/-}$) subjected to sham operation (sham) or 7 days aortic banding (AB). Significant differences are indicated with * and were determined by Kruskal-Wallis with Dunn's multiple comparison test. n.s., non significant. Data are normalized to GAPDH and presented as mean ± S.D. N=10.

Supplementary Figure 3



Supplementary Figure 3. Expression of ECM genes in response to mechanical stress. Relative mRNA (A) and protein (B) levels of elastin in left ventricular tissue from wild-type (WT) and syndecan-4$^{-/-}$ (syn4$^{-/-}$) sham operated animals (sham) and animals subjected to 7 days aortic banding (AB). Elastin mRNA in cardiac fibroblasts plated on fibronectin for 24, 48 and 72h. Two-way ANOVA revealed effect of genotype (A), and effect of AB (B). Bonferroni post hoc testing is indicated with * or n.s. for non significant results. Mann-Whitney test was used in C for each time point. Data are presented as mean ± S.D. N=12-15 (A), 7-10 (B), 5 (C).

Supplementary Figure 4



Supplementary Figure 4. Osteopontin induces LOX expression in cardiac myofibroblasts. A, representative immunoblots for lysyl oxidase (LOX), smooth muscle α-actin (SMA) and GAPDH in wild-type (WT) and syndecan-4$^{-/-}$ (syn4$^{-/-}$) cardiac fibroblasts plated on fibronectin for 7 days. B, LOX protein relative to GAPDH in cardiac fibroblasts plated on fibronectin for 2 days, 7 days and after stimulation with transforming growth factor β (TGFβ), with and without osteopontin (OPN) in WT and syn4$^{-/-}$ cardiac fibroblasts. C, relative mRNA levels of CD44, integrin alpha v (Itgα_v) and integrin beta 1 (Itgβ_1) normalized to ribosomal protein L32 (RPL32) mRNA. Significance was determined by Mann-Whitney test (B-C) and two-way ANOVA with Bonferroni post-hoc test (B). Data are relative to ctrl and presented as mean ± S.D. N=6.

Supplementary Table 1. Mice lacking syndecan-4 have reduced left ventricular concentric hypertrophy after 7 days of pressure overload. Echocardiography revealed significant differences between wild-type (WT) and syndecan-4$^{-/-}$ (syn4$^{-/-}$) mice subjected to aortic banding (AB) as indicated with * and significant difference between sham operated animals (sham) and those subjected to 7 days AB as denoted with $^{\#}$ as determined by two-way ANOVA with Bonferroni post-hoc test. LV, left ventricular; LAD, left atrial diameter; IVSd, end diastolic interventricular septum thickness; LVPWd, end diastolic left ventricular posterior wall thickness; LVIDd, end diastolic left ventricular internal diameter; LVFS, left ventricular fractional shortening; E, early mitral inflow velocity; E´, early mitral annular velocity**.** Data are presented as mean ± S.D.

	Sham		AB	
	WT	syn4$^{-/-}$	WT	syn4$^{-/-}$
N	14-16	14-15	15-16	13-17
Body weight, g	26.41 ± 1.72	25.30 ± 2.45	25.83 ± 1.52	23.70 ± 2.38*
Tibia length (TL), mm	17.69 ± 0.62	17.17 ± 0.47	17.54 ± 0.53	17.52 ± 0.30
LV weight/TL, mg/mm	4.99 ± 0.26	5.07 ± 0.69	8.28 ± 0.73$^{\#\#\#}$	8.15 ± 1.04$^{\#\#\#}$
Lung weight/TL, mg/mm	8.51 ± 0.81	8.58 ± 0.92	17.47 ± 4.99$^{\#\#\#}$	14.82 ± 3.52$^{\#\#\#}$
LAD, mm	1.80 ± 0.10	1.76 ± 0.13	3.14 ± 0.35$^{\#\#\#}$	3.16 ± 0.36$^{\#\#\#}$
IVSd, mm	0.53 ± 0.06	0.53 ± 0.08	1.03 ± 0.18$^{\#\#\#}$	0.81 ± 0.17***$^{\#\#\#}$
LVPWd, mm	0.52 ± 0.08	0.58 ± 0.09	1.15 ± 0.19$^{\#\#\#}$	0.90 ± 0.12***$^{\#\#\#}$
LVIDd, mm	4.33 ± 0.29	4.18 ± 0.34	3.91 ± 0.40$^{\#\#}$	4.24 ± 0.38*
LVFS, %	19.46 ± 3.51	17.03 ± 4.28	11.59 ± 4.67$^{\#\#\#}$	11.29 ± 3.66$^{\#\#\#}$
E/E´	37.58 ± 9.65	34.34 ± 5.90	50.48 ± 12.54$^{\#\#}$	47.82 ± 11.92$^{\#\#}$
(IVSd + LVPWd) / LVID	0.25 ± 0.04	0.27 ± 0.04	0.57 ± 0.12$^{\#\#\#}$	0.41 ± 0.06***$^{\#\#\#}$
LV weight/LVID (mg/mm)	20.71 ± 1.32	20.39 ± 1.95	37.33 ± 3.72$^{\#\#\#}$	33.90 ± 5.11*$^{\#\#\#}$

Supplementary Table 2. Non-cardiomyocyte, blood vessel and fibroblast fraction in left ventricular tissue. Quantitative analysis of electron micrographs of left ventricular tissue sections. Comparisons between sham operated (sham) and aortic banded (AB) animals were made using Mann-Whitney test as indicated with $^{\#}$. No significant differences were found between wild-type (WT) and syndecan-4$^{-/-}$ (syn4$^{-/-}$) mice. Non-CM, non-cardiomyocyte; WT, wild-type; syn4$^{-/-}$, syndecan-4$^{-/-}$; sham. Data are presented as mean ± S.D. N=6-10 tissue sections.

	Sham		7 days after AB	
	WT	syn4$^{-/-}$	WT	syn4$^{-/-}$
N	6	10	6	8
Non-CM fraction, %	29.6 ± 4.0	31.5 ± 8.2	26.3 ± 6.1	24.6 ± 0.9
Area occupied by fibroblast nuclei in non-CM fraction, %	2.52 ± 0.8	2.65 ± 2.5	6.73 ± 2.1$^{\#\#}$	5.28 ± 2.2$^{\#}$
Area occupied by blood vessels in non-CM fraction, %	46.2 ± 15.2	29.1 ± 8.8	35.8 ± 11.4	35.7 ± 7.4

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