

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

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## Aims

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/nuclear factor of activated T-cells (NFAT) promotes myofibroblast differentiation and collagen production upon mechanical stress. The aim of this study was to investigate whether 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<sup>-/-</sup> 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 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<sup>-/-</sup> mice, which exhibited less collagen cross-linking after AB. Expression of osteopontin (OPN), a matricellular protein able to induce LOX in cardiac fibroblasts, was up-regulated in hearts after AB, in mechanically stressed fibroblasts and in fibroblasts overexpressing syndecan-4, calcineurin, or NFAT, but down-regulated 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 signalling leading to collagen, OPN, and LOX induction in cardiac fibroblasts; the other involving the extracellular domain promoting LOX-dependent cross-linking.

## Keywords

Syndecan-4 • Collagen cross-linking • Myofibroblast • Lysyl oxidase • Myocardial stiffness

## 1. Introduction

Diastolic dysfunction is central to the development of heart failure, a common and fatal disease.<sup>1</sup> 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

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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,<sup>2</sup> changes in extracellular matrix (ECM) composition and structure are important determinants of myocardial stiffening.<sup>3</sup>

Cardiac fibroblasts are the major non-muscle cells of the ventricular myocardium and are key players in myocardial stiffening. During ventricular remodelling 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.<sup>4</sup> The activated fibroblast acquires smooth muscle-like features including expression of smooth muscle  $\alpha$ -actin (SMA) and SM22, is therefore referred to as myofibroblast,<sup>5</sup> as well as increases expression of PDGF receptor  $\beta$  (PDGFR $\beta$ ).<sup>6,7</sup>

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.<sup>8,9</sup> 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,<sup>10</sup> collagen cross-linking induced by mechanical stress seems to be largely attributed to enzymatic cross-linking by the enzyme lysyl oxidase (LOX), which is up-regulated in the pressure-overloaded heart.<sup>8</sup> Despite its potentially central role in myocardial stiffening, little is known regarding mechanisms regulating LOX in the heart. Interestingly, the matricellular protein osteopontin (OPN) was recently found to induce LOX expression and activity in cardiac fibroblasts.<sup>11</sup> Although sparsely expressed in the healthy heart, OPN is dramatically increased in mice subjected to pressure overload by aortic banding (AB).<sup>12</sup>

Recent work in our laboratory demonstrated that expression of the transmembrane heparan sulfate proteoglycan syndecan-4 increases in the left ventricle after pressure overload in mice<sup>5</sup> and man,<sup>13</sup> through inflammatory mediators such as TNF- $\alpha$  and IL-1 $\beta$ .<sup>14</sup> Also, that in response to mechanical stress, syndecan-4 engages the calcineurin/nuclear factor of activated T-cells (NFAT) signalling pathway in cardiac fibroblasts to promote ECM production and differentiation into activated myofibroblasts.<sup>5</sup> Thus, one specific aim of this study was to examine whether syndecan-4 expression and/or signalling has a direct impact on myocardial stiffness. A second aim of this study was to explore whether 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.<sup>15</sup> We therefore hypothesized that OPN might be a downstream target of syndecan-4/NFAT signalling 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<sup>-/-</sup>) mice<sup>16</sup> as previously described,<sup>13,17</sup> and mice were sacrificed by cervical dislocation after 24 h, 3 days, or 7 days after AB. Animals were handled according to the National Regulation on Animal Experimentation in accordance with an

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.<sup>18</sup> 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–polyacrylamide gels<sup>19</sup> and titin N2B and N2BA isoforms detected at 3000 and 3200 kDa, 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 EM. For quantification, five images ( $\times 440$ ) per section were analysed to determine non-cardiomyocyte fractions, and 24–30 images ( $\times 2900$ ) per section of the non-cardiomyocyte fraction to determine the areas of fibroblast nuclei (reflecting the number of fibroblasts) and blood vessels. Two to 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.<sup>20</sup> Briefly, soluble collagen assessed using colorimetric and enzymatic procedures was subtracted from total collagen determined by staining with picosirius 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 picosirius red and examined using non-polarized and circularly polarized light, respectively.

### 2.6 Fibroblast cell culture

Cardiac fibroblasts were isolated as previously described,<sup>5</sup> 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, BC, Canada) was used. The NFAT blocker A-285222 was kindly provided by Abbott Laboratories (1  $\mu$ mol/L; Abbott Park, IL, USA). Cells were stimulated with 10 ng/mL of TGF $\beta$ 1 (Merck Millipore, Darmstadt, Germany) and 250 ng/mL of 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.<sup>5</sup>

### 2.8 Immunoblotting

Immunoblotting was performed on left ventricle homogenates as previously described.<sup>21</sup> Anti-OPN (IBL, Hamburg, Germany) and anti-LOX (NB100-2527, Novus Biologicals, Littleton, CO, USA) were used as primary antibodies, and quantification achieved by normalization to loading controls.

### 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, Schnellendorf, Germany) and Alexa fluor 488-secondary anti-mouse antibodies (Invitrogen, Paisley, UK). Formalin-fixed myocardial tissue sections (4  $\mu$ m) were

stained for SMA (DAKO, Glostrup, Denmark) and PDGFR $\beta$  (Cell Signaling, Danvers, MA, USA), and visualized using secondary antibodies conjugated to horseradish peroxidase.

## 2.10 LOX 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 fibre formation assay

The extracellular domain of syndecan-4 (1–4  $\mu$ g, Sino Biological, Inc., Beijing, China) was mixed with 10  $\mu$ g collagen I with or without recombinant human LOX homologue 2 (0.1  $\mu$ g, LOXL2 with activity >2 pmol/min/ $\mu$ g; R&D Systems, Abingdon, UK). The mixture was incubated at 37°C for 24–72 h before performing turbidimetry and EM.

## 2.12 Statistical analysis

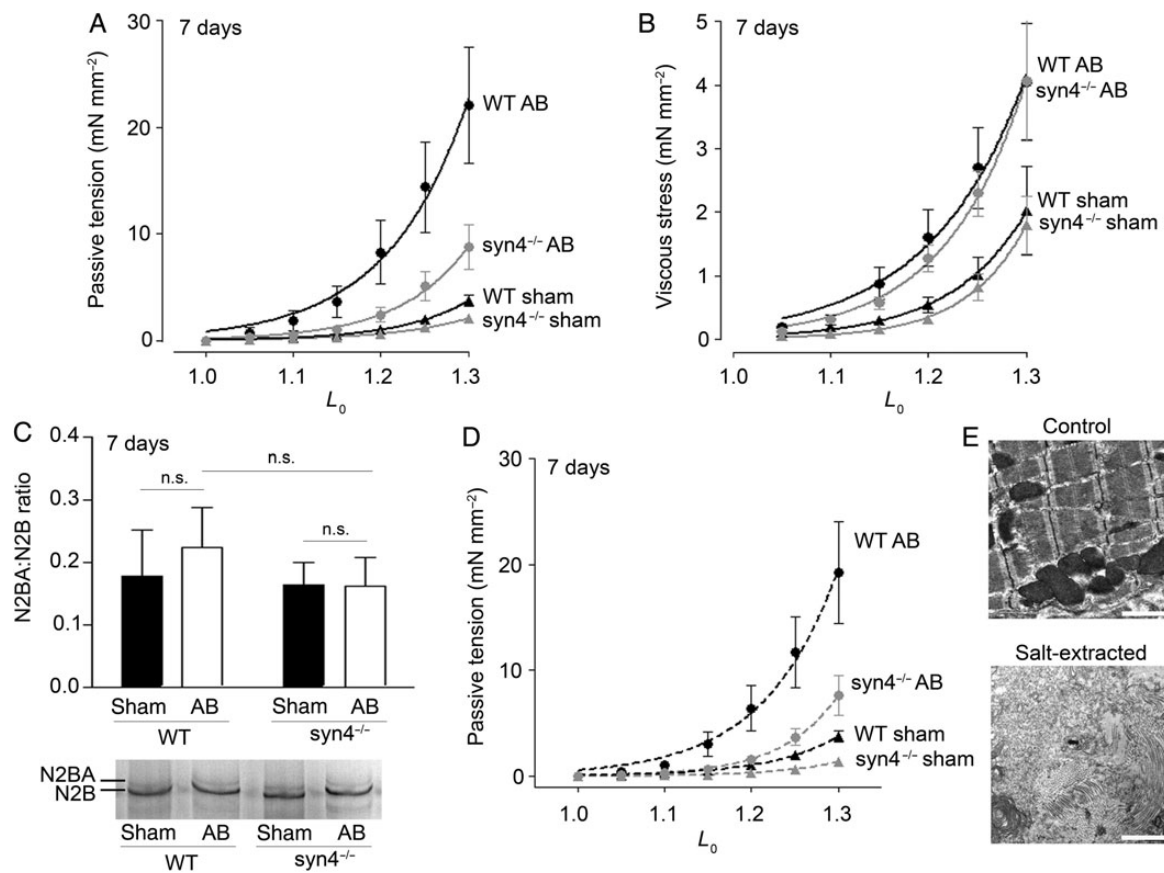
Data are expressed as means  $\pm$  SD unless otherwise specified in the figure legends. Statistical analysis was performed using the GraphPad software (Prism 5). The use of parametric or non-parametric tests was based on results from analyses of distributions. Statistical significance was determined using the Mann–Whitney test and Kruskal–Wallis test 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 remodelling as determined by echocardiography (see Supplementary material online, Table S1), which was accompanied by an increase in passive tension (Figure 1A). Along with reduced concentric hypertrophic remodelling in syndecan-4<sup>-/-</sup> mice (see Supplementary material online, Table S1), which is in accordance with our previous findings,<sup>13</sup> passive tension was significantly lower in myocardial tissue from syndecan-4<sup>-/-</sup> mice (Figure 1A). Another factor contributing to myocardial stiffness is the viscosity of the tissue. Viscous stress was increased following AB (Figure 1B), but 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.<sup>2</sup> To investigate the cardiomyocyte contribution to the observed reduction of passive tension in



**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 WT and syndecan-4<sup>-/-</sup> ( $syn4^{-/-}$ ) mice 7 days after sham operation (sham) or 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: 2  $\mu$ m. Two-way ANOVA analyses showed an effect of genotype on passive tension before and after salt extraction. No difference in N2BA : N2B ratio (C) was found by using the Kruskal–Wallis test with Dunn's multiple comparison test. Data are presented as mean  $\pm$  SEM (A, B, and D) for clearer visualization, and as mean  $\pm$  SD (C).  $N = 5-8$ .

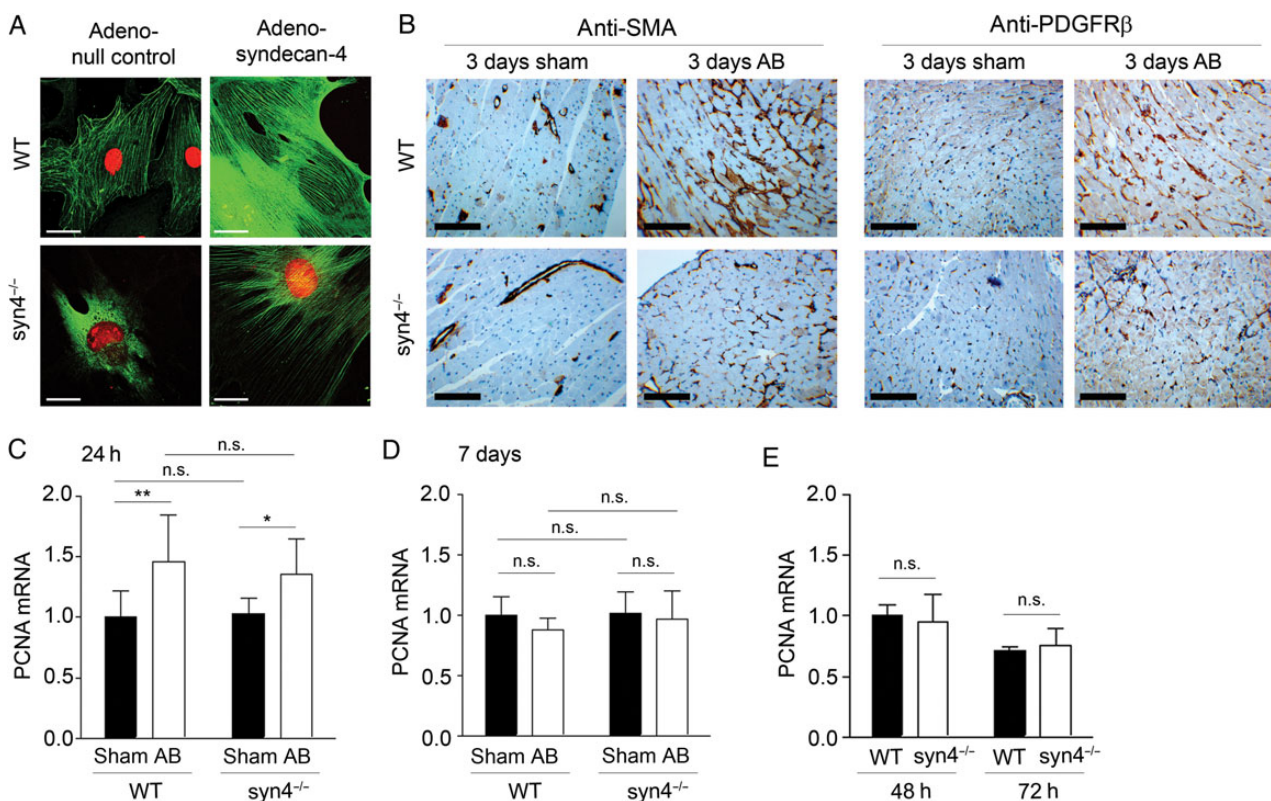
syndecan-4<sup>-/-</sup> mice after pressure overload, we measured the ratios between the longer and more compliant titin isoform N2BA and the shorter and stiffer N2B isoform. N2BA:N2B ratios were unchanged by AB or the lack of syndecan-4 (Figure 1C). To study whether the reduced passive tension in syndecan-4<sup>-/-</sup> 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.<sup>18</sup> Indeed, ECM-dependent passive tension was significantly lower in muscle strips from syndecan-4<sup>-/-</sup> mice compared with WT (Figure 1D). The complete disruption of sarcomere structure by salt extraction was verified using EM (Figure 1E). The area of myocardial tissue consisting of cardiomyocytes vs. non-cardiomyocyte (non-CM) fraction seemed unaltered by AB or the lack of syndecan-4 (see Supplementary material online, Table S2).

### 3.2 Increased expression of syndecan-4 promotes cardiac myofibroblast differentiation

As we showed in neonatal cardiac fibroblasts,<sup>5</sup> the lack of syndecan-4 had profound effects in adult cardiac fibroblasts. While adult syndecan-4-deficient cardiac fibroblasts exhibited virtually no SMA fibre formation after 48 h on fibronectin, fibroblasts from WT mice

had clear SMA fibres (Figure 2A). This impaired ability to undergo myofibroblast differentiation of cells lacking syndecan-4 was restored by adenoviral transduction of syndecan-4 (Figure 2A). Furthermore, overexpression of syndecan-4 in WT cardiac fibroblasts resulted in enhanced SMA fibre formation (Figure 2A). Myofibroblast differentiation was also impaired *in vivo* as demonstrated by reduced staining of SMA and PDGFR $\beta$  (Figure 2B). Negative and positive controls are included in Supplementary material online, Figure S1.

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<sup>-/-</sup> mice after pressure overload. However, as shown in Figure 2C and D (24 h and 7 days after AB, respectively), expression of the cell cycle S-phase marker, proliferating cell nuclear antigen (PCNA), 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<sup>22,23</sup> most of the PCNA signal could be attributed to cell proliferation in the non-CM fraction. Furthermore, lack of syndecan-4 did not affect the proliferative capacity of cardiac fibroblasts *in vitro*, as assessed after 48 or 72 h 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 (see Supplementary material online, Table S2).



**Figure 2** Increased syndecan-4 expression induces myofibroblast differentiation. (A) immunofluorescence staining of SMA (green) in cardiac fibroblasts from WT and syndecan-4<sup>-/-</sup> (syn4<sup>-/-</sup>) mice transduced with an empty adenoviral vector (adeno-null) or containing full-length syndecan-4 (adeno-syndecan-4). Scale bar: 50  $\mu$ m. (B) Immunohistochemistry showing staining for SMA and PDGFR $\beta$  in left ventricular tissue. Scale bar: 100  $\mu$ m. Relative mRNA levels of PCNA normalized to GAPDH in left ventricles 24 h (C) and 7 days (D) after AB, and cardiac fibroblasts plated on fibronectin for 48 and 72 h (E). Statistical significance was determined by two-way ANOVA with the Bonferroni *post hoc* test (C and D) and Kruskal–Wallis test with Dunn’s multiple comparison test (E). n.s., not significant. Data are presented as mean  $\pm$  SD. *N* = 8–10 (C and D) and 4 (E).



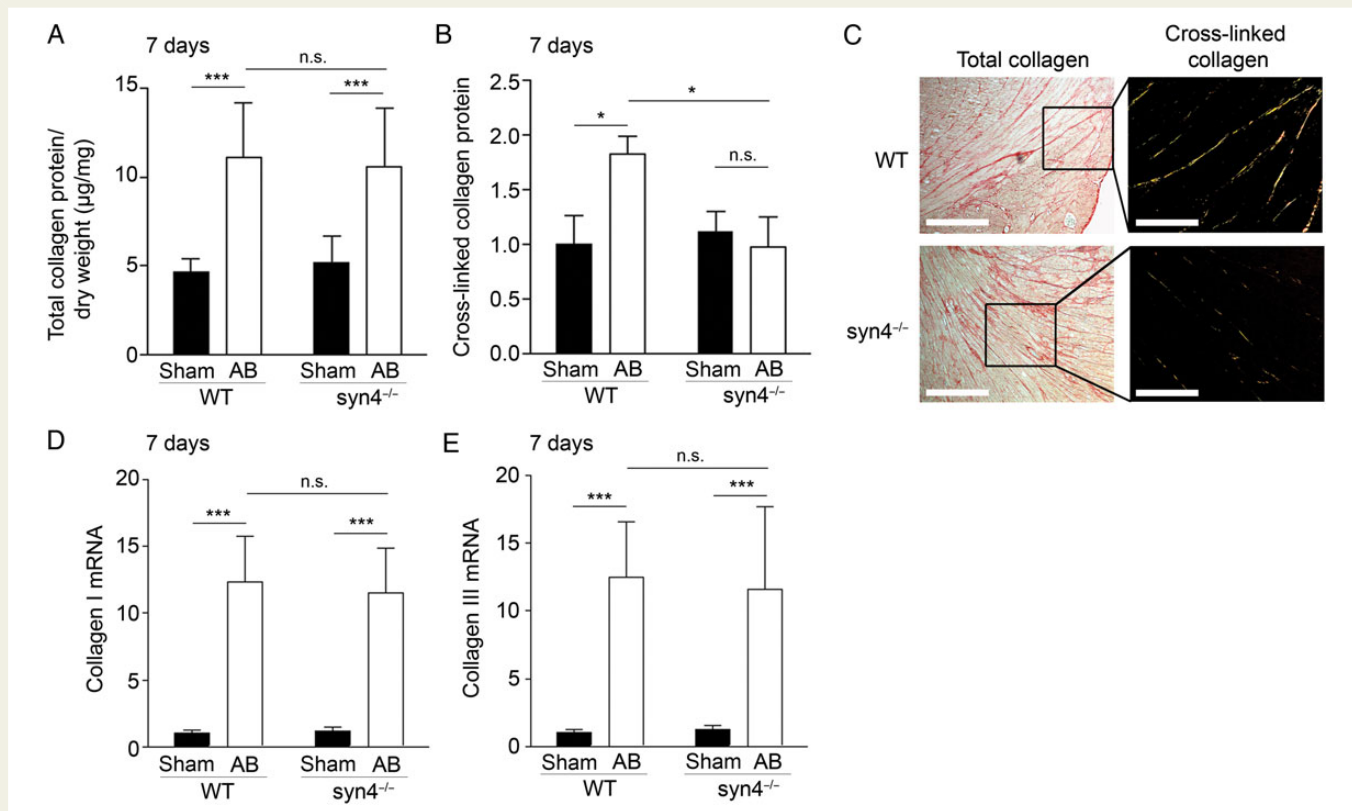
### 3.3 Collagen cross-linking is impaired in left ventricles of syndecan-4<sup>-/-</sup> mice following pressure overload

Total collagen contents as determined by HPLC were significantly increased 7 days after AB, but this response was unaffected in syndecan-4<sup>-/-</sup> mice (Figure 3A). A similar pattern was observed for the expression of collagen I and III mRNA at this time point (Figure 3D and E). This is in contrast with recent data from our group, showing that the absence of syndecan-4/Ca<sup>2+</sup>/NFAT signalling impairs collagen I and III mRNA expression 24 h after AB,<sup>5</sup> 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 (see Supplementary material online, Figure S2). 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<sup>-/-</sup> 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

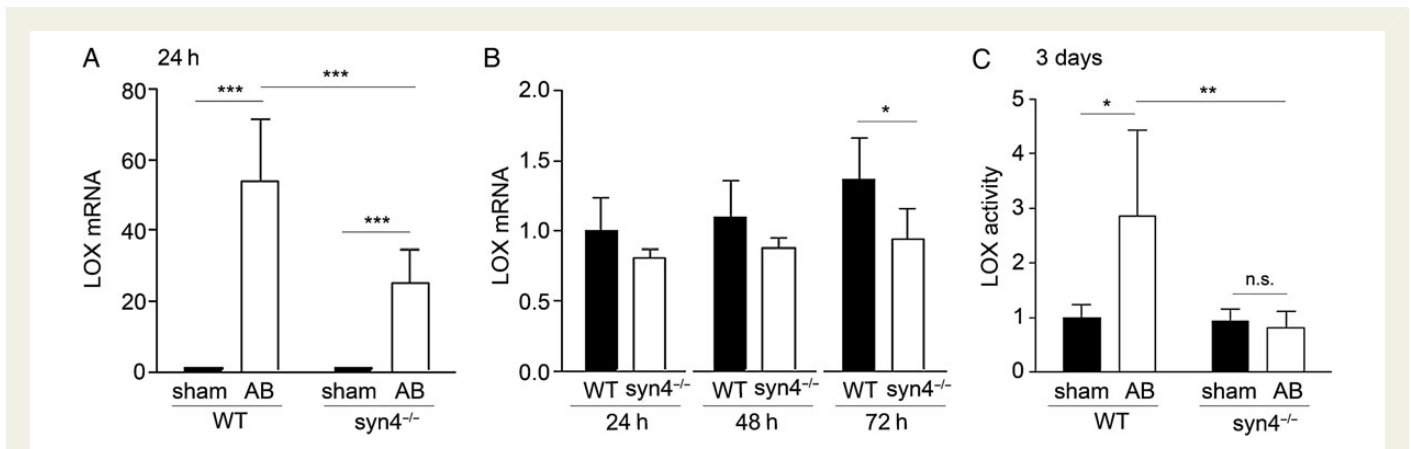
collagen after AB in WT mice, while less apparent responses in sections from syndecan-4-deficient mice (Figure 3B and D). Other structural matrix proteins may also be altered in syndecan-4<sup>-/-</sup> mice and thereby, contribute to changes in compliance, such as elastin. However, mRNA and protein levels of elastin, despite being increased after AB, were not affected by the genotype of the animals (see Supplementary material online, Figure S3A and B). Moreover, elastin levels decreased during myofibroblast differentiation (see Supplementary material online, Figure S3C).

### 3.4 LOX expression and activity are reduced in left ventricles and cardiac fibroblasts from syndecan-4<sup>-/-</sup> mice

AB resulted in significantly increased LOX mRNA levels in the left ventricles of syndecan-4-competent mice 24 h 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, whereas 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).



**Figure 3** Collagen cross-linking is reduced in the myocardium of syndecan-4<sup>-/-</sup> mice. (A) Total collagen determined by HPLC in left ventricles from WT and syndecan-4<sup>-/-</sup> (syn4<sup>-/-</sup>) mice following sham operation (sham) or 7 days 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 syn4<sup>-/-</sup> mice. Scale bar 100 µm for total collagen and 50 µm for cross-linked collagen. (D and E) Relative mRNA levels of collagens I and III normalized to GAPDH 7 days after AB. Statistical significance was determined by two-way ANOVA with Bonferroni *post hoc* tests (A, D, and E) and the Kruskal–Wallis test with Dunn's multiple comparison test (B). Data are normalized to WT sham and presented as mean ± SD. N = 5–9 (A and B) and 14–16 (D and E).



**Figure 4** LOX expression and activity is reduced in syndecan-4<sup>-/-</sup> mice. (A) Relative LOX mRNA levels normalized to GAPDH in left ventricular tissue from WT and syndecan-4<sup>-/-</sup> (syn4<sup>-/-</sup>) mice 24 h after sham operation (sham) or AB. (B) Relative LOX mRNA normalized to GAPDH in cardiac fibroblasts from WT and syn4<sup>-/-</sup> mice plated on fibronectin for 72 h to induce myofibroblast differentiation. (C) Relative LOX activity in left ventricular tissue from WT and syn4<sup>-/-</sup> mice subjected to sham operation or 3 days AB. Two-way ANOVAs with the Bonferroni *post hoc* test (A), Mann–Whitney test (B), and Kruskal–Wallis test 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  $\pm$  SD. *N* = 10 (A), 5 (B), and 7–8 (C).

### 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 24 h after AB, both at the mRNA (>600-fold) and protein (6.9-fold) level (Figure 5A and B). OPN mRNA remained significantly elevated 7 days after AB, although levels were lower than those at 24 h (Figure 5C). In mice lacking syndecan-4, the induction of OPN by pressure overload was clearly blunted at both 24 h and 7 days (Figure 5A–C). OPN mRNA levels were positively correlated with 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<sup>-/-</sup> 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,<sup>5</sup> and that NFAT activation regulates OPN expression in vascular smooth muscle cells.<sup>15</sup> 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 24 h has recently been shown to increase pro-LOX protein expression and LOX activity in cardiac human fibroblasts.<sup>11</sup> Here, we demonstrate increased LOX protein expression after OPN stimulation in mouse cardiac fibroblasts that had been cultured on fibronectin-coated plates with TGF $\beta$  (see Supplementary material online, Figure S4A and B), a protocol that promotes myofibroblast differentiation.<sup>5</sup> The lack of syndecan-4 had no impact on OPN-induced LOX (see Supplementary material online, Figure S4A and B), confirming that the effects are downstream of OPN and do not involve signalling via syndecan-4. Interestingly, myofibroblast

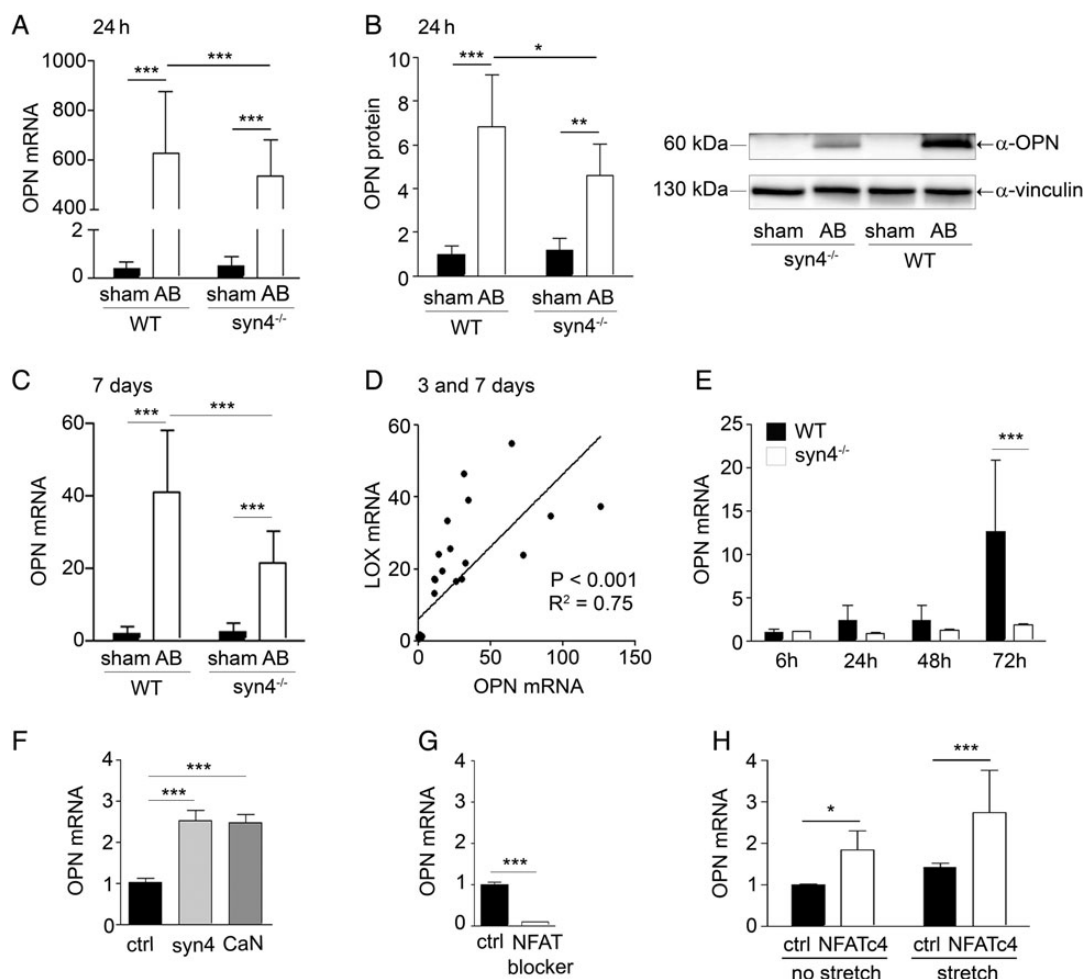
differentiation also yielded significantly increased expression of known OPN receptors including integrins  $\alpha_v$  and  $\beta_1$ , and CD44 (see Supplementary material online, Figure S4C).

### 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 fibre formation in a test tube. Incubation of collagen I at 37°C for 72 h with increasing concentrations of ECsyn4 resulted in a dose-dependent increase in turbidity measured as optical density at 340 nm (Figure 6A). Incubation of the highest concentration of ECsyn4 alone (4  $\mu$ g) failed to increase optical density. At the EM level, collagen fibrils appear similar in thickness and structure in samples containing the mixture of collagen and ECsyn4, and collagen alone (Figure 6B). However, only the samples containing the mixture 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 48 h due to collagen cross-linking and formation of extensive collagen fibre networks as observed by EM (Figure 6D). Turbidity of these samples after 24 h, 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 fibres between the thicker collagen fibres (Figure 6D; arrows). In samples containing collagen and LOX, similar dense structures to those seen in samples without LOX but with ECsyn4 were observed (Figure 6D; asterisks). Taken together, these results suggest that the extracellular domain of syndecan-4 promotes collagen fibre 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

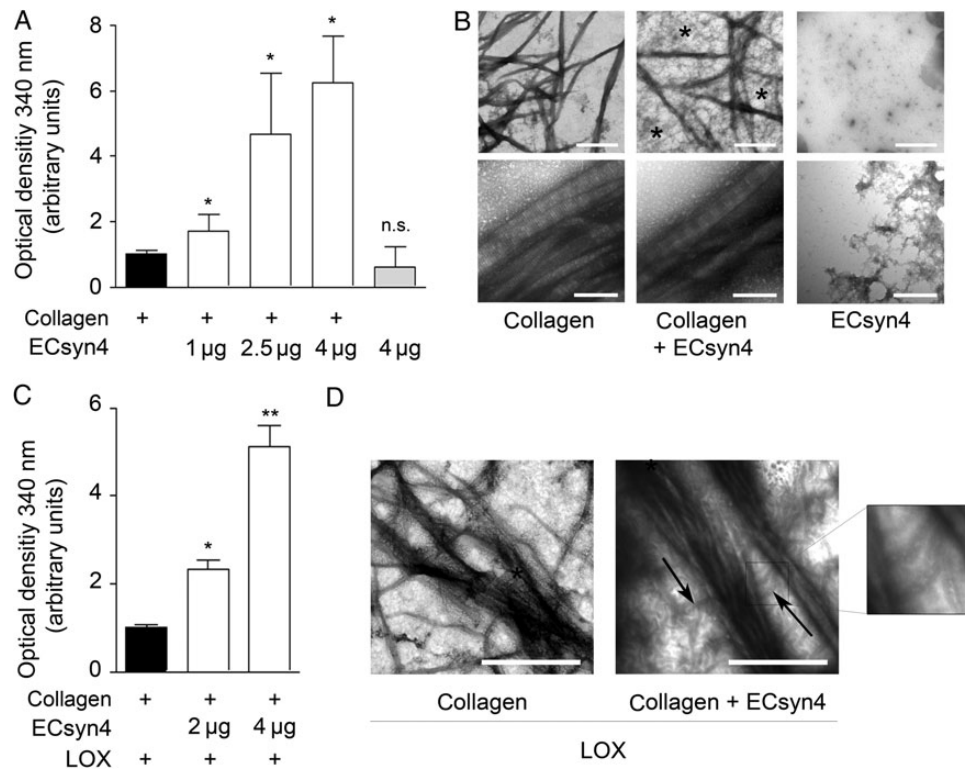


**Figure 5** OPN is regulated by syndecan-4. Relative OPN mRNA (A and C) and protein (B) in left ventricular tissue from WT and syndecan-4<sup>-/-</sup> (*syn4*<sup>-/-</sup>) mice 24 h (A and B) and 7 days (C) after sham operation (sham) or AB. (D) Correlation of cardiac LOX and OPN mRNA 3 and 7 days after sham or AB determined by Pearson regression analysis. (E) OPN mRNA levels in cardiac fibroblasts from WT and *syn4*<sup>-/-</sup> mice 6, 24, 48, and 72 h after plating on fibronectin. HT1080 fibroblast control (ctrl) and overexpressing syndecan-4 (*syn4*), calcineurin (CaN; F), and (H) NFATc4. (G) Cardiac fibroblasts treated with an NFAT blocker. mRNA data were normalized to GAPDH. Protein data were normalized to vinculin. Significant differences were determined by two-way ANOVA with the Bonferroni *post hoc* test (A–C and E), Mann–Whitney test (F and H) and Student’s paired *t*-test (G). Data are relative to WT sham (A–D), WT 6 h (E), and ctrl (F–H) and presented as mean ± SD. N = 8–10 (A–C) and 5–6 (E–H).

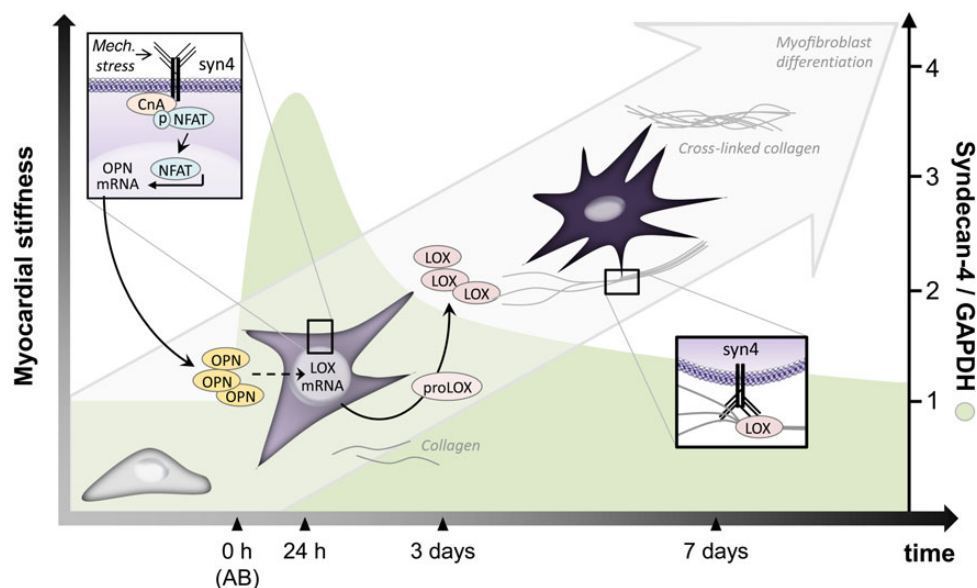
understanding the molecular mechanisms underlying myocardial stiffening as a cause of diastolic dysfunction. With this study, we demonstrate the importance of syndecan-4 in determining passive tension of the myocardium following pressure overload. Our understanding of how this is achieved in the mouse model used here is schematically presented in Figure 7: Within the first 24 h after AB, a rapid up-regulation 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 signalling pathway to promote myofibroblast differentiation.<sup>5</sup> While syndecan-4 levels will eventually return to normal levels 3 weeks after AB (this study and ref.<sup>14</sup>), a series of events triggered by mechanical stress-induced syndecan-4 signalling 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.<sup>5</sup> Here, we show that the extracellular domain of syndecan-4 seems also to be involved in determining ECM stiffness by interacting with collagen fibres 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,<sup>24</sup> and the ECM. Considering the location of syndecan-4 in the costameres overlaying the Z-disc of cardiomyocytes,<sup>25</sup> 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,<sup>26</sup> titin accounted for about one-third of the total passive tension of left



**Figure 6** Syndecin-4 facilitates collagen cross-linking by LOX. (A and C) Turbidity measured as optical density at 340 nm of solutions containing collagen, extracellular syndecin-4 (ECsyn4), and LOX. (B and D) EM 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 interfibrillar structures (D). Scale bars: 1  $\mu\text{m}$  (upper panel in B and in D) and 200 nm (lower panel in B). Comparisons are made by the Mann–Whitney test to the ctrl in A and C, and significance denoted with asterisk or n.s. for non-significant comparisons. Data are relative to collagen (A) and collagen with LOX (B), and presented as mean  $\pm$  SD.  $N = 3\text{--}6$ .



**Figure 7** Syndecin-4 regulates cardiac fibroblast phenotype and function, collagen cross-linking, and myocardial stiffness in response to mechanical stress. A 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<sup>14</sup> for 0, 24 h, 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.



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<sup>-/-</sup> mice, suggesting intact titin function in mice lacking syndecan-4, and that the observed difference in passive tension was caused by altered ECM remodelling.

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.<sup>27</sup> Although increased myocyte stiffness did not prevail in the AB mouse model used in this study (reflecting variability between species and aetiologies of disease), the molecular processes of ECM remodelling 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.<sup>27</sup> Also in hypertensive patients, collagen cross-linking correlated with elevated left ventricular filling pressures, whereas collagen amount did not.<sup>8</sup> 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-cross-linking enzyme, that is tissue transglutaminase, which exhibited decreased activity in syndecan-1<sup>-/-</sup> mice following myocardial infarction (MI)<sup>28</sup> and in syndecan-4<sup>-/-</sup> mice during kidney tissue remodelling.<sup>29</sup>

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.<sup>11</sup> 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.<sup>11,30–32</sup> OPN correlates with cross-linked collagen in hypertensive heart disease patients and with left ventricular chamber stiffness in pressure-overloaded rats. OPN-deficient mice fail to develop fibrosis in response to AngII treatment<sup>33</sup> and have reduced collagen accumulation after acute MI.<sup>32</sup> Cardiac OPN expression can be triggered by mechanical stress as demonstrated in this study, which also suggests that syndecan-4/NFAT signalling is involved in the regulation of OPN in the context of pressure overload.

Recently, syndecan-4 signalling was shown to inhibit apoptosis and regulate NFAT activity after MI,<sup>34</sup> but in this context, syndecan-4<sup>-/-</sup> mice exhibited increased NFAT activity, as opposed to the reduced activity we report in syndecan-4<sup>-/-</sup> 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 remodelling 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,<sup>35</sup> also in the context of MI,<sup>36</sup> and heart failure.<sup>14</sup>

We also demonstrated that collagen fibre 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<sup>37</sup> and fibronectin.<sup>38</sup> Other proteoglycans such as decorin and fibromodulin are known to bind to and stabilize collagen fibrils,<sup>39</sup> and have been suggested<sup>40</sup> 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,<sup>14</sup> further studies are encouraged to determine the exact details of the syndecan-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 up-regulated in the pressure-overloaded left ventricle of mice<sup>5,14</sup> and man,<sup>13</sup> and based on recent research regarding the role of syndecan-4 in ECM remodelling, we speculate that targeting syndecan-4 may be an effective anti-fibrotic approach.<sup>41,42</sup> 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,<sup>13</sup> the next challenge will be to identify differential molecular traits between syndecan-4-dependent signalling 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.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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