Lack of collagen VIII reduces fibrosis and promotes early mortality and cardiac dilatation in pressure overload in mice†

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Aims
In pressure overload, left ventricular (LV) dilatation is a key step in transition to heart failure (HF). We recently found that collagen VIII (colVIII), a non-fibrillar collagen and extracellular matrix constituent, was reduced in hearts of mice with HF and correlated to degree of dilatation. A reduction in colVIII might be involved in LV dilatation, and we here examined the role of reduced colVIII in pressure overload-induced remodelling using colVIII knock-out (col8KO) mice.

Methods and results
Col8KO mice exhibited increased mortality 3–9 days after aortic banding (AB) and increased LV dilatation from day one after AB, compared with wild type (WT). LV dilatation remained increased over 56 days. Forty-eight hours after AB, LV expression of main structural collagens (I and III) was three-fold increased in WT mice, but these collagens were unaltered in the LV of col8KO mice together with reduced expression of the pro-fibrotic cytokine TGF-β, SMAD2 signalling, and the myofibroblast markers Pxn, α-SMA, and SM22. Six weeks after AB, LV collagen mRNA expression and protein were increased in col8KO mice, although less pronounced than in WT. In vitro, neonatal cardiac fibroblasts from col8KO mice showed lower expression of TGF-β, Pxn, α-SMA, and SM22 and reduced migratory ability possibly due to increased RhoA activity and reduced MMP2 expression. Stimulation with recombinant colVIII increased TGF-β expression and fibroblast migration.

Conclusion
Lack of colVIII reduces myofibroblast differentiation and fibrosis and promotes early mortality and LV dilatation in response to pressure overload in mice.

Keywords
Collagen • Aortic banding • Remodelling • Extracellular matrix • Heart failure

1. Introduction
Left ventricular (LV) pressure overload, such as seen in patients with aortic stenosis, leads to concentric hypertrophy and fibrosis. If left untreated, concentric LV remodelling may progress to cardiac dilatation and heart failure (HF). Symptomatic patients with aortic stenosis have a poor prognosis unless treated with aortic valve replacement. The mechanisms involved in decompensated LV dilatation are largely unknown. LV remodelling from pressure overload is characterized by cardiomyocyte hypertrophy and quantitative and qualitative alterations in extracellular matrix (ECM) constituents such as collagens. Several collagens are structural proteins providing tissue strength by constituting a scaffold for cardiomyocytes (CM). Alterations in collagen isoforms during pressure overload are linked to compensated LV hypertrophy, overt HF, and reverse remodelling.

We recently reported reduced levels of collagen VIII (colVIII) in mice with dilated HF due to LV pressure overload, whereas we did not find any alteration in colVIII in mice with compensated hypertrophy.

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Interestingly, we identified a negative correlation between LV colVIII levels and dilatation, suggesting that colVIII might play a role in transition from concentric compensatory hypertrophy to LV dilatation. ColVIII is a non-fibrillar, short-chain collagen consisting of colVIIIa1 and colVIIIa2 chains encoded by the COL8A1 and COL8A2 genes, respectively. Mutations in colVIII are associated with corneal dystrophy, but the role of colVIII in the heart remains unknown, although its role in bridging ECM molecules predicts that it could affect tissue integrity during LV remodeling. Interestingly, colVIII increases smooth muscle cell migration and maintains their phenotype in the vasculature. This might suggest that colVIII plays a role in cardiac myofibroblast differentiation, a cell type characterized by excessive production of ECM with smooth muscle cell-like phenotype central to development of fibrosis. A recent study on vascular smooth muscle cells has shown that colVIII suppresses ras homologue gene family member A (RhoA) activation via β1 integrin receptors, thereby increasing MMP expression and cellular migration.

The aim of this study was to examine the effects of reduced colVIII on myocardial fibroblast differentiation and development of fibrosis, on survival, and on LV dilatation in the acute and more chronic phase of pressure overload. ColVIII knock-out (col8KO) and wild-type (WT) mice were subjected to banding of the ascending aorta (AB) and examined by echocardiography and molecular analyses, while effects of colVIII on cardiac fibroblast (CFB) differentiation were examined in vitro.

2. Methods
See Supplementary material online for details.

2.1 Animal experiments
Animal experiments were approved by The Norwegian Animal Research Committee and conformed to the Guide for the Care and Use of Laboratory Animals (NIH). AB was performed blinded to genotype on 7-week-old male col8KO (colVIIIa1 and colVIIIa2 deficient) and C57BL/6j BomTac WT mice (Taconic, Skensved, Denmark) as previously described.

Col8KO mice are viable, fertile, and show no major abnormalities, and they were crossed back for at least 20 generations onto C57BL/6j background by Dr Ulrike Hopfer and Dr Bjorn R. Olsen at Harvard Medical School (Boston, MA, USA). They were generously provided by Dr Hopfer (University of Basel, Switzerland) and bred by homozygous intercrosses at our institution. To reduce the possibility that different C57BL/6 substrains could contribute to different responses to pressure overload in col8KO and WT mice, both groups were on C57BL/6j background. Animals were maintained in pathogen-free facilities, with free access to water and fed with standard rodent chow. Echocardiographic examination under light sedation with isoflurane was carried out using the VEVO 2100 (Visual-Sonics, Toronto, Canada) with VEVO 2100 1.1.0 software at several time points for phenotypic characterization. For histological and molecular analyses, mice were randomized to 48 h or 6 weeks of AB, with echocardiography prior to sacrifice. Surgery, echocardiography, and echocardiography analyses were performed blinded to mouse genotype. After sacrifice under anaesthesia with isoflurane and stored at −80°C.

2.2 Non-invasive blood pressure measurements
Blood pressure was measured blinded to mouse genotype by CODA standard tail-cuff blood pressure system (Kent Scientific, CT, USA). Mice were
placed on the heated examination table under light sedation, spontaneously
breathing a mixture of 1.5% isoflurane and 98.5% oxygen on a mask. The
blood pressure cuff was placed around the root of the tail. Each animal
\((n = 10–13)\) was subjected to seven measurements, and systolic and diastolic
blood pressures were calculated as the mean of the recordings accepted by
the CODA software.

### 2.3 Histology

Left ventricles were fixed in 4% formaldehyde and embedded in paraffin.
Representative 4 μm mid-ventricular sections were stained with Picosir-
ius Red stain kit (no. 24901 Polysciences, Warrington, PA, USA) and photo-
graphed using ×20 objectives (Axiovision Rel 4.6, Carl Zeiss microscope,
GMbH, Jena, Germany). For immunohistochemistry, the sections were
incubated with colVIIx1 antibody (Bioss antibodies; no. 7529R, Bioss
Inc., Woburn, MA, USA) followed by incubation with biotinylated anti-
rabbit secondary antibody (VectaStain Elite Kit, Burlingame, CA, USA),
stained with DAB substrate, and counterstained with Sirius Red/Fast
Green solution.

### 2.4 Total LV collagen content

Quantification of hydroxyproline by HPLC-chromatography was used to
evaluate total LV collagen content as previously described.4

### 2.5 CFB culture

CFBs were isolated from 1- to 3-day-old col8KO and WT mice.22 Neonatal
pups (25–110 per isolation) were quickly decapitated and hearts immediate-
lly removed by a controlled sternotomy. Hearts were placed in cold Hanks
Balanced Salt Solution (HBSS) and the LV excised and cut into pieces.
CFBs were isolated from LV and maintained in culture essentially as previous-
ly described.23 After serum starvation for 24 h, cells were stimulated with re-
combinant colVIIx1 (5–10 μg/mL) or vehicle and subjected to a migration
(scratch) assay for 24 h.22 Briefly, the migration assay consisted of scratching
a 90% confluent cell layer using a 10 μL pipette on top of three lines marked
under the culture dish. The scratched areas were photographed immediately
after the scratching and 24 h later. Additionally, WT CFB stimulated with re-
combinant colVIIx1 were photographed at 6, 12, 16, and 20 h. Using Adobe
Photoshop, the area of ‘wound healing’ was calculated. CFB migration was
expressed in %.
CFB cultures were also prepared from adult mice. Fibroblasts were isolated by cardiac perfusion with collagenase (480 U/mL; Worthington, Lakewood, NJ, USA). After 5 days in culture, cells were starved for 24 h and stimulated with collagen VIIIα1 (10 mg/mL) or vehicle. RhoA activity was measured in cell lysates using the G-LISA RhoA Activation Assay Biochem Kit (BK124; Cytoskeleton, Denver, CO, USA), and total RhoA was quantified by the RhoA ELISA Assay (BK150, Cytoskeleton). Both assays were used according to the manufacturer’s guidelines. Adult CFB were also incubated with integrin β1 blocking antibody (ab24693, 10 mg/mL, Clone: P5D2; Abcam, Cambridge, MA, USA).

### 2.6 Immunohistochemistry of neonatal CFBs
Neonatal CFBs grown on Chambered Coverglass (Lab-Tek, Thermo Fischer Scientific Inc., Rochester, NY, USA) were fixed in 4% paraformaldehyde (no. 158127, Sigma Aldrich) and incubated with anti-α-SMA (no. A5228, Sigma Aldrich) followed by incubation with Alexa Fluor 488 secondary antibody (Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 546-phalloidin (Invitrogen). Cells were imaged using a LSM 710 confocal microscope (Carl Zeiss, GmbH).

### 2.7 In vitro collagen fibre formation assay
Recombinant collagen VIIIα1 (0.01 μg) was mixed with 10 μg collagen (BD Biosciences, Bedford, MA, USA) in 25 μL TES buffer, 2 μL 10 mM ZnSO4, and dH2O to a total volume of 50 μL. The mixture was incubated at 37°C for 24 h and content examined using an electron microscope by performing negative staining with 2% aqueous uranyl acetate directly on the grids.

### 2.8 Protein extraction and immunoblotting
Protein was extracted and immunoblotted using Criterion Tris–HCl Gel (Bio-Rad, Hercules, CA, USA), run on SDS–PAGE, blotted onto PVDF membranes (GE Healthcare Life sciences, Uppsala, Sweden), and quantitated using chemiluminescence, as previously described. GAPDH was used for loading control.

### 2.9 RNA extraction and real-time PCR
RNA was extracted, quantified, and quality-controlled as previously described. Reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad) prior to quantitative PCR analysis (qPCR).

### 2.10 Statistical analyses
Statistical analyses were performed using SigmaPlot 11 (Systat Software Inc., San Jose, CA, USA). Results are presented as mean ± SEM, and a two-tailed significance level of P ≤ 0.05 was used. Survival rates were calculated using Log-rank (Mantel–Cox) test (Figure 1A). Two-way ANOVA was used to study the effects of AB and lack of collagen VIII (Figures 2A and B, 4A, B, and D, 5A, D, and F, 7A and B). When comparing two groups (Figures 1B and D, 5B, E, and G, 6C, 7C–F), two-sided Student’s t-test or Mann–Whitney Rank Sum Test was used. One-way ANOVA was used in Figure 6B. Correction for multiple comparisons was done using the Holm–Sidak or Dunn’s method.

### 3. Results

#### 3.1 Increased early mortality after induction of LV pressure overload in col8KO mice
As depicted in Kaplan–Meier survival curves (Figure 1A), col8KO exhibited increased mortality following AB compared with WT. The increased mortality was obvious within 3 days where 30% of col8KO
mice were dead, compared with 15% of controls, and continued until 9 days after AB where 39 and 23% in col8KO and WT were dead, respectively. After the first 9 days of AB, mortality was stabilized in col8KO and WT. All mice that died within the first 9 days of AB (n = 24 col8KO, n = 11 WT) underwent autopsy. None of the deceased mice showed signs of cardiac or aortic rupture; however, autopsy revealed 1.22-fold higher lung weight in col8KO mice compared with that in WT (Figure 1B).

We did not observe mortality in sham-operated animals.

3.2 LV dilatation after induction of LV pressure overload in col8KO mice

Preoperative echocardiography revealed no differences in diastolic LV internal diameter (LVIDd) between col8KO and WT mice (Figure 1C), and preoperative measurements of blood pressure showed no difference in systolic, diastolic, or mean arterial pressure (MAP) between genotypes (Figure 1D). Interestingly, echocardiographic assessment at 1, 2, 7, 14, 21, 28, 42, and 56 days after AB revealed LV dilatation at all time points investigated in col8KO mice compared with WT (Figure 1C). Consistent with a similar increase in wall thickness in both groups and increased dilatation following AB in col8KO, the increased LV weight seen 6 weeks after AB compared with respective sham was more pronounced in col8KO mice compared with WT (Table 1). Despite increased LV dilatation in col8KO cardiac biomarkers of HF, atrial and brain natriuretic peptides (ANP and BNP, respectively) were unaltered after 48 h of AB and similarly increased in both groups following 6 weeks AB (Table 2).

3.3 Attenuated synthesis of fibrillar collagens accompanied by LV dilatation in col8KO mice in response to pressure overload

Interestingly, 48 h after AB, a time point where col8KO mice showed LV dilatation and 1 day before they exhibited increased mortality, the AB-induced approximately three-fold increased mRNA expression of fibrillar collagens I and III (col1/3) seen in WT mice was absent in col8KO mice (Figure 2A). At the same time point, total collagen content was not yet altered and was not different between groups (Figure 2A). One week post AB, total collagen was increased in WT mice while the increase in col8KO did not reach statistical significance.
Lack of collagen VIII in pressure overload

3.4 Localization of collagen VIII in the myocardium

ColVIII has been suggested to function as a bridging molecule in the ECM. 

In pressure overload, deficiency of ColVIII affects differentiation of neonatal CFBs to myofibroblasts. (A) LV mRNA of the myofibroblast marker α-SMA in both genotypes 48 h after AB (n = 8–14 per group) and (B) in CFB of both genotypes (n = 14–15 per group). (C) Immunohistochemical staining of α-SMA fibres in CFB of both genotypes (experiment repeated three times); scale bar = 50 μm. (D) LV mRNA of the myofibroblast marker SM22 in both genotypes 48 h after AB (n = 8–14 per group) and (E) in CFB of both genotypes (n = 16–18 per group). (F) LV mRNA of the myofibroblast marker Pxn in both genotypes 48 h after AB (n = 8–14 per group) and (G) in CFB of both genotypes (n = 10–11 per group). Col8KO, colVIII knock-out; WT, wild-type. Data are presented as mean ± SEM, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 AB vs. sham, §§p ≤ 0.01 KO vs. WT.

(see Supplementary material online, Figure S4). Following 6 weeks of AB, LV mRNA expression of col1 and 3 was significantly less increased in col8KO compared with WT (Figure 2B). In line with this, total LV collagen was increased to a lesser extent in col8KO mice compared with WT at 6 weeks after AB (Figure 2B). LV histology confirmed this finding, showing a lower degree of increased collagen accumulation after 6 weeks of AB in col8KO mice compared with WT (Figure 2C). Expression of the collagen cross-linking enzyme, lysyl oxidase (LOX),

promoting collagen formation. Soluble collagen will spontaneously form fibres when incubated at 37°C for 24 h. Recombinant colVIIIα1 (0.01 μg) was mixed with soluble collagen (10 μg) in a test tube. Compared with collagen alone (see Supplementary material online, Figure S5A), this increased the amount of collagen fibre formation (see Supplementary material online, Figure S5B) supporting a role for collagen VIII in facilitating ECM interactions. No difference in collagen fibre diameter was observed.

3.5 Decreased pro-fibrotic TGF-β signalling and differentiation of neonatal CFBs into myofibroblasts in col8KO mice in response to pressure overload

The altered early fibrotic response, i.e. attenuated mRNA expression of col1 and 3 in col8KO mice, suggested that colVIII affects major pro-fibrotic signalling and differentiation of neonatal CFB to myofibroblasts. Interestingly, 6 h after AB expression of the pro-fibrotic cytokine TGF-β was significantly lower in col8KO mice compared with an approximately three-fold increase in WT mice (Figure 4A). Accordingly, in isolated neonatal CFB in culture, TGF-β expression was lower in col8KO cells than in WT (Figure 4B). Interestingly, recombinant colVIIIα1 increased TGF-β expression in WT CFB 1.8-fold and rescued the reduced expression of TGF-β in CFB lacking colVIII (Figure 4B). There was no significant difference in the relative expression of TGF-β between WT and KO cells, meaning that they have equal response to exogenous colVIII. Reduced TGF-β signalling in col8KO mice was supported by reduced p-SMAD2 protein levels following AB in col8KO mice compared with WT (Figure 4C and D).
Importantly, expression of the myofibroblast marker gene \(\alpha\)-SMA was increased approximately three-fold in WT and only approximately two-fold in col8KO mice 48 h after AB (Figure 5A). Accordingly, \(\alpha\)-SMA mRNA expression was reduced in isolated neonatal CFB from col8KO mice compared with WT (Figure 5B). Immunohistochemistry also illustrated reduced staining and organization of \(\alpha\)-SMA fibres in isolated CFB from col8KO compared with WT (Figure 5C). These findings were supported by reduced expression of the myofibroblast marker genes SM22 and Pxn, which were increased in WT mice 48 h after AB, but attenuated in col8KO mice (Figure 5D and E). This was confirmed also at the protein level in a total RhoA ELISA (Figure 7B).

Finally, we investigated whether colVIII affected the migratory ability of neonatal CFB, another important aspect of their function. Results showed that in contrast to WT CFB (n = 9), which migrated into the scratched zone within 24 h (Figure 6A and B), col8KO CFB (n = 9) had a slower migration. Interestingly, stimulating col8KO CFB with recombinant colVIII increased their migratory ability in a dose-dependent manner, resembling the WT response (Figure 6A and B). Furthermore, we examined whether recombinant colVIII affected the migration of WT CFB. Interestingly, recombinant colVIII also increased the migration of WT CFB (n = 11) in a dose-dependent manner (Figure 6D).

In addition to our observation that col8KO CFB proliferate more than WT CFB in cell culture, we measured the level of proliferating cell nuclear antigen (PCNA) expression, a marker of cellular proliferation, which confirmed our observation that col8KO CFB proliferate more rapidly compared with WT CFB (Figure 6C).

### 3.6 Collagen VIII inhibits RhoA signalling in adult CFBs

RhoA regulates cell dynamics and, especially, aspects of cellular migration.\(^{27}\) RhoA activity was increased in adult CFB from col8KO mice (Figure 7A). Although stimulation with colVIII did not affect RhoA activity in either genotype significantly, RhoA protein levels were increased in col8KO CFB following stimulation with colVIII, meaning that RhoA activity per RhoA molecule was reduced (Figure 7B).

We also measured RhoA mRNA in adult CFB from col8KO mice, showing reduced expression (Figure 7C). This was confirmed also at the protein level in a total RhoA ELISA (Figure 7B). ColVIII has been shown to suppress RhoA activity through integrin \(\beta1\) in vascular smooth muscle cells.\(^{17}\) Interestingly, integrin \(\beta1\) mRNA was significantly reduced in CFB from col8KO mice (Figure 7D), and incubation of CFB from WT mice with an integrin \(\beta1\) blocking antibody increased RhoA activity.
activity (Figure 7E). RhoA has been reported to suppress migration by inhibiting MMP2 expression and activity, and consistent with this hypothesis, MMP2 mRNA was markedly reduced in CFB lacking colVIII (Figure 7F).

4. Discussion

This study demonstrates the novel findings that lack of colVIII disrupts the acute adaptation of the heart to increased load and is involved in survival and development of LV dilatation during cardiac remodelling in pressure overload. Key findings were increased early mortality and increased LV dilatation in col8KO mice in response to AB in vivo, accompanied by reduced expression of structural col1 and 3, TGF-β signalling, and myofibroblast differentiation. In vitro, neonatal CFB lacking colVIII showed reduced expression of TGF-β and myofibroblast markers α-SMA, Pxn, and SM22. Migratory ability was reduced in CFB from col8KO mice possibly due to increased RhoA activity and reduced MMP2 expression. Stimulation of fibroblasts with recombinant colVIII increased TGF-β expression and migration, suggesting that colVIII might act as a signalling molecule in the heart directly affecting CFB function.

We used an experimental mouse model of AB to induce pressure overload and simulate clinical aortic stenosis. In this model, banding induces an acute LV pressure overload, which stabilizes over time and does not have the same potential for development of collateral circulation compared with transverse aortic constriction. Pressure overload leads to compensated concentric remodelling that may progress to LV dilatation and HF. In the present study, we demonstrate that lack of colVIII in LV pressure overload leads to increased early mortality 3–9 days after AB which may suggest that lack of colVIII disrupted the acute adaption of the heart to increased load. Autopsy findings excluded cardiac or aortic rupture as a cause of death, suggesting that increased early mortality of col8KO mice may be due to a reduced ability of the heart to withstand the acute increased myocardial wall stress. Acute HF as supported by the increased lung weight in the deceased col8KO animals as well as lethal arrhythmias might be considered as possible explanations. We have previously shown that colVIII protein levels in mice subjected to LV pressure overload correlate negatively with LV dilatation. In the present study, we demonstrate that col8KO mice exhibited LV dilatation during remodelling in response to AB. Our data therefore suggested that colVIII is important for the initial survival and LV dilatation in pressure overload of the heart, although the mechanisms behind these findings are not clear.

ColVIII is a non-fibrillar collagen suggested to serve as a molecular bridge between different types of ECM molecules. Moreover, colVIII has previously been described as an important factor in tissue repair where it directs adhesion of ECM components, especially the major

Figure 7 Collagen VIII (colVIII) is a potent inhibitor of RhoA. (A) RhoA activity was increased in CFBs from adult colVIII knock-out (col8KO) mice (n = 6 per group). (B) RhoA protein levels were increased in col8KO CFB following stimulation with colVIIIa1 (n = 6 per group). (C) RhoA mRNA in CFB from col8KO mice, showing reduced expression in the absence of colVIII (wild-type (WT) n = 4, col8KO n = 6). (D) Integrin β1 mRNA was significantly reduced in CFB from col8KO mice (WT n = 4, col8KO n = 6). (E) Incubation of CFB from WT mice with an integrin β1 blocking antibody increased RhoA activity (WT n = 4, col8KO n = 6). (F) MMP2 mRNA was reduced in CFB lacking colVIII (n = 9 per group). Data presented as mean ± SEM; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 vs. WT or as indicated by lines.
Table 1  Organ weights and echocardiographic measurements of collagen VIII knock-out and wild-type mice subjected to aortic banding

<table>
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<td>LAD (mm)</td>
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<td>LAD (mm)</td>
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<td>1.1 ± 0.0*</td>
<td>0.7 ± 0.0§</td>
<td>1.1 ± 0.0*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. AB, aorta banding; sAB, sham operated; WT, wild-type; KO, knock-out; LV, left ventricular; LVWTindex, LV weight/tibia length; LWTindex, lung weight/tibia length; V<sub>max</sub> stenosis, max. flow velocity across banding; LAD, left atrial diameter; FS, fractional shortening; LVIDd/s, LV internal diameter in diastole/systole; IVSd, interventricular septum in diastole; LVPWTd, posterior LV wall thickness in diastole.

*P ≤ 0.05 AB vs. sAB.
§P ≤ 0.05 KO vs. WT.

Table 2  Cardiac mRNA expression in collagen VIII knock-out and wild-type mice subjected to aortic banding

<table>
<thead>
<tr>
<th></th>
<th>sABWT</th>
<th>ABWT</th>
<th>sABKO</th>
<th>ABKO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>48 h after AB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>14</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>ANP</td>
<td>1.00 ± 0.27</td>
<td>0.52 ± 0.21</td>
<td>0.98 ± 0.25</td>
<td>0.56 ± 0.25</td>
</tr>
<tr>
<td>BNP</td>
<td>1.00 ± 0.29</td>
<td>0.81 ± 0.21</td>
<td>1.00 ± 0.29</td>
<td>0.51 ± 0.26</td>
</tr>
<tr>
<td>LOX</td>
<td>1.00 ± 0.21</td>
<td>12.69 ± 2.78*</td>
<td>0.97 ± 0.09</td>
<td>15.22 ± 2.72*</td>
</tr>
<tr>
<td><strong>6 weeks (42 days) after AB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>10</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>ANP</td>
<td>1.00 ± 0.01</td>
<td>28.81 ± 0.21*</td>
<td>1.42 ± 0.12</td>
<td>24.16 ± 4.97*</td>
</tr>
<tr>
<td>BNP</td>
<td>1.00 ± 0.08</td>
<td>1.33 ± 0.05*</td>
<td>0.92 ± 0.04</td>
<td>1.29 ± 0.05*</td>
</tr>
<tr>
<td>LOX</td>
<td>1.00 ± 0.33</td>
<td>3.65 ± 0.48*</td>
<td>0.80 ± 0.33</td>
<td>3.93 ± 0.35*</td>
</tr>
</tbody>
</table>

qPCR data are normalized with GAPDH transcript abundance; data are presented as mean ± SEM. AB, aorta banding; sAB, sham operated; WT, wild-type; KO, knock-out; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; LOX, lysyl oxidase.

*P ≤ 0.05 AB vs. sAB.
structural coll. Thus, these effects might be reduced in col8KO mice affecting tissue integrity directly by its absence. This hypothesis is supported by our in vitro findings that colVIII modifies the structural organization of coll. Accumulation of collagens in the ECM, i.e. fibrosis, is thought to contribute to impaired cardiac function and HF, but at the same time, it increases ECM stiffness in the myocardium. In WT mice with initial concentric remodelling in response to AB, the increased collagen amounts, thus increased cardiac stiffness, reduces diastolic function, but also counteracts LV dilatation. Interestingly, in col8KO mice developing LV dilatation, the increased mRNA expression of fibrillar col1 and 3 and total collagen protein were attenuated within the first week of AB. Hence, reduced pressure overload-induced synthesis of the fibrillar collagens might potentially contribute to the LV dilatation and early mortality in col8KO mice. Effects linked to CFB function might also contribute. In the heart, CFBs are responsible for synthesis, deposition, and degradation of collagens and other ECM molecules. In the present study, we have demonstrated that colVIII is synthesized by CFB in vitro and is localized to the ECM in fibroblasts and their surrounding tissue of mouse hearts in vivo. As previously shown, colVIII was also localized to the inner vessel wall/endothelium/endothelial cells. Interestingly, recombinant colVIIIa1 seemed to increase cell fibre formation when mixed in vitro. Although this finding remains to be confirmed in vivo, it might support a role for colVIII in facilitating ECM interactions and that this loss of the colVIII isoform may impair the structural integrity of the myocardium leading to LV dilatation.

During pressure overload, CFBs differentiate into myofibroblasts, characterized by their increased collagen production and smooth muscle cell-like phenotype, leading to increased fibrosis and stiffness of the heart, counteracting cardiac dilatation. Differentiation of CFB into myofibroblasts is mainly promoted by TGF-β and experimentally defined by increased expression of marker genes α-SMA, Pxn, and SM22. Interestingly, we found that in col8KO mice with LV dilatation, cardiac expression of TGF-β and myofibroblast markers were reduced compared with WT. This suggests that lack of colVIII reduces TGF-β signalling and thereby reduces CFB differentiation into myofibroblasts, suggesting a mechanism for attenuated collagen synthesis and increased LV dilatation. This finding was supported by in vitro studies, demonstrating reduced expression of TGF-β and myofibroblast marker genes in neonatal CFB from mice lacking colVIII. Importantly, attenuated expression of TGF-β in col8KO CFB could be rescued by stimulation with recombinant colVIIIa1, suggesting a direct effect of colVIII on CFB function. The concept of colVIII acting as a signalling molecule within the heart has not been reported previously. Finally, consistent with findings in smooth muscle cells, lack of colVIII led to markedly reduced migration of CFB, another important aspect of CFB function in development of fibrosis. The reduced migratory response in col8KO CFB could be rescued by adding recombinant colVIIIa1, again suggesting that colVIII might affect the fibrotic response of the heart through activation of pro-fibrotic TGF-β signalling, myofibroblast differentiation, and migration.

One might speculate that lack of migration of col8KO CFB could be influenced by reduced proliferation compared with WT CFB. However, on the contrary, we have shown that col8KO CFB actually proliferate more than WT CFB, indicating that the reduced migration of CFB in col8KO mice is most likely not a result of reduced proliferation.

To identify a molecular mechanism for the effect of colVIII on CFB migration, we measured levels of MMP2, an ECM enzyme known to promote migration. MMP2 levels were markedly reduced in CFB from adult col8KO mice, possibly accounting for the reduced migration of these cells. MMP2 expression and activation can be suppressed by active RhoA in endothelial cells and vascular smooth muscle cells. We found increased RhoA activity in CFB from col8KO mice. Hence, it is possible that the increased RhoA activity observed in cells lacking colVIII inhibits MMP2 expression and activity also in CFB as shown for vascular smooth muscle cells and that this in turn affects migratory properties. Interestingly, integrin β1 mRNA was significantly reduced in CFB from col8KO mice, and incubation of CFB from WT mice with an integrin β1 blocking antibody increased RhoA activity, suggesting that colVIII signals through integrin β1 to suppress RhoA signalling. Thus, the increased RhoA activity of CFB from col8KO mice could be a result of reduced colVIII-integrin β signalling, as is the case in smooth muscle cells.

In the present study, we have measured comparable preoperative blood pressures in col8KO and WT mice, making preoperative hypertension in col8KO mice and its potential effects on early dilatation of the LV after AB less likely. We cannot completely rule out the possibility that different degrees of aortic constriction may contribute to different degree of LV dilatation and increased mortality in col8KO compared with WT mice. However, the banding procedure was carefully standardized and echocardiography showed comparable V̇ max, whereas cardiac output and heart rate were not significantly different. Importantly, all surgery, blood pressure measurements, echocardiography, and data analyses were performed blinded to genotype. Furthermore, animals were randomized to be sacrificed 48 h or 6 weeks after AB.

In conclusion, the present study demonstrates the novel finding that the non-fibrillar colVIII is important for the early adaptation of the heart to pressure overload affecting survival, and development of LV dilatation. Moreover, mice lacking colVIII exhibit attenuated TGF-β signalling which may disrupt the normal myofibroblast differentiation and fibrosis formation in response to pressure overload. We suggest a role for colVIII in regulation of ECM structure and fibrosis in the hearts of patients with aortic stenosis, counteracting LV dilatation.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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