Differential role of TIMP2 and TIMP3 in cardiac hypertrophy, fibrosis, and diastolic dysfunction

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1. Introduction
Cardiac extracellular matrix (ECM) comprises fibrillar collagens (types I and III), basement membrane, and non-structural proteins.1 Tissue inhibitor of metalloproteinases (TIMPs) can mediate myocardial remodelling, hypertrophy, and fibrosis in heart disease. We investigated the impact of TIMP2 vs. TIMP3 deficiency in angiotensin II (Ang II)-induced myocardial remodelling and cardiac dysfunction.

Methods
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Aims
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Conclusion
TIMP2 and TIMP3 play fundamental and differential roles in mediating pathological remodelling, independent from their MMP-inhibitory function. TIMP2−/− and TIMP3−/− mice provide a unique opportunity to study myocardial hypertrophy and fibrosis independently, and their impact on cardiac dysfunction.

Keywords Tissue inhibitor of metalloproteinases • Hypertrophy • Fibrosis • Diastolic dysfunction • Extracellular matrix • Matricellular proteins • Cardiac fibroblast

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effective therapies. Diastolic dysfunction is often associated with myocardial hypertrophy and fibrosis which have also been associated with LV dyssynchrony in HFrEF. Since myocardial hypertrophy and fibrosis often co-exist in cardiomyopathies, differentiating between their contributions to disease progression is often challenging.

TIMPs have been shown to impact cell growth and hypertrophy. In vivo animal studies have shown that cardiac pressure overload results in severe cardiac fibrosis and hypertrophy in mice lacking TIMP3 and TIMP2, but not in TIMP4 mice. In this study, we examined the causal role of TIMP2 vs. TIMP3 in agonist-induced cardiac fibrosis and hypertrophy, and subsequently in cardiac remodelling and dysfunction. We report that, in response to angiotensin II (Ang II) infusion, TIMP2 deficiency exacerbates myocardial hypertrophy with suppressed fibrosis, whereas TIMP3 deficiency results in severe myocardial fibrosis in the absence of hypertrophy. This differential myocardial remodelling impacts different aspects of diastolic function as evident by suppressed active relaxation in TIMP2 Ang II mice compared with compromised passive compliance in TIMP3 Ang II mice.

2. Methods

2.1 Experimental animals and protocol

Wild-type (WT, C57Bl/6) and TIMP2+/− (C57Bl/L6) mice were purchased from Jackson Laboratory, TIMP3+/− mice were generated as described, and backcrossed into C57Bl/6 background 12 times. In vivo, Ang II (1.5 mg/kg/day) or saline (control) was delivered by Alzet micro-osmotic pumps (Model 1002, Durect Co.) in anaesthetized (2% isoflurane) 8- to 9-week-old male mice. After 2 weeks, mice were anaesthetized (ketamine/xylazine, 100 mg/kg; 10 mg/kg), hearts were excised and flash-frozen in liquid nitrogen, paraffin-embedded, and processed for trichrome, picrosirius red staining, and immunohistological analyses. All experiments were conducted in accordance with the guidelines of the University of Alberta Animal Care and Use Committee (ACUC) and the Canadian Council of Animal Care.

2.2 Echocardiographic analyses and in vivo haemodynamic measurements

Systolic and diastolic cardiac functions were determined non-invasively by transthoracic echocardiography in anesthetized mice (0.75% isoflurane) as before. LV pressure–volume relationship was measured invasively, using a 1.2-Fr intraventricular catheter (Sciensense, Inc.), in anesthetized mice (1.5% isoflurane) as described. End-diastolic pressure–volume relationships (EDPVRs) were obtained following the inferior vena cava (IVC) occlusion method. P–V data analyses were performed using the LabScribe software (version 2.3.47000).

2.3 Taqman RT-PCR

Total RNA was extracted using TRizol Reagent (Invitrogen), and mRNA expression levels for collagen I, collagen III, brain natriuretic peptide (BNP), β-myosin heavy chain (βMHC), α-skeletal actin, MMP-2, -9, -8, -13, membrane-type 1 (MT1)-MMP, and TIMP1–4 were detected by TaqMan RT-PCR as before. For the remaining genes, TaqMan primer/probe cocktails were purchased from Applied Biosystems, Inc., as listed in Supplementary material online, Table S1. Hypoxanthine-guanine phosphoribosyltransferase-1 (HPRT) was used as an internal control.

2.4 Histological and immunohistochemical staining and imaging

Freshly excised hearts were arrested in diastole in 1 M KCl, fixed in 10% formalin, paraffin-embedded, and processed for trichrome, picrosirius red (PSR), and wheat germ agglutinin (WGA) staining as before. Myocyte cross-sectional area was determined from the WGA-stained sections by using the Metamorph Basic software (version 7.7.0.0) as before. Paraffin sections were stained with primary antibody for Secreted Protein Acidic and Rich in Cysteine (SPARC; R&D Systems), osteopontin (OPN, Abcam), Macrophage (Mac3, Serotec AbD), and Neutrophil (R&D Systems), followed by HRP-conjugated (SPARC and Mac3), FITC-conjugated (OPN), or Cy3-conjugated (Neutrophil) secondary antibodies, and DAPI nuclear staining as before.

2.5 Hydroxyproline assay

Collagen content was determined using the hydroxyproline assay as described. Collagen content is reported as µg of hydroxyproline per mg of LV tissue.

2.6 Isolation and culture of adult cardiac fibroblasts and myocytes

Cardiac fibroblast (CFB) and cardiac myocyte (cMyo) were isolated from adult mice of different genotypes as previously described. All CFBs were used at the second passage. Fibroblasts were cultured on collagen-coated BioFlex under quiescent conditions, or subjected to cyclic mechanical stretching (5% elongation 0.33 Hz) using the Flexcell FX-500076 Tension System. CFBs were serum-deprived for 24 h prior to the experimental protocol. For cMyo–CFB co-cultures, cardiac fibroblasts were added to the cultured cMyo. Cultures and co-cultures were treated with 1 µM Ang II or saline for 24 h in FBS-free medium. At the end of the protocol, cells were harvested and RNA was extracted using Trizol as before.

2.7 Fibroblast proliferation and migration assays

Migration of CFB was assayed using in vitro scratch assay as described in serum-starved CFBs in the presence of Ang II (1 µM) or saline. Images were captured at 0, 24, and 36 h. Proliferation of CFB was assayed by determining the rate of 5-bromo-2-deoxyuridine (BrdU) incorporation in serum-starved CFBs treated with saline or Ang II. The number of positively staining cells is reported as the percentage of total number of cells in the same field.

2.8 Statistical analysis

All analyses were performed using the IBM SPSS Statistics 19 software. Averaged data are presented as mean ± SEM. Two-way ANOVA was used to compare each TIMP-deficient group with the WT. Student’s t-test was used to compare the Ang II-treated group with the saline group for each genotype. Statistical significance was recognized at P < 0.05.

3. Results

3.1 Ang II infusion enhanced hypertrophy in TIMP2−/− and fibrosis in TIMP3−/− mice

Two weeks of Ang II infusion resulted in myocardial hypertrophy in WT mice as evident by the cross-sectional images of the heart (Figure 1A), elevated heart weight-to-tibial length ratio (Figure 1B), and increased myocyte cross-sectional area (Figure 1Ci and ii). TIMP2 deficiency augmented the Ang II-induced myocardial hypertrophy resulting in a more gross enlargement of the heart, significantly greater heart weight-to-tibial length ratio (Figure 1A and B) and myocyte cross-sectional area (Figure 1G and ii). In TIMP3−/− mice, however, the hypertrophy response was completely blunted (Figure 1A–C).

Assessment of myocardial fibrosis revealed that Ang II infusion resulted in interstitial and perivascular fibrosis in WT mice (Figure 2A and B), increased hydroxyproline content (Figure 2C), and increased de novo mRNA synthesis of collagen types I and III (Figure 2D and E). TIMP2−/− Ang II mice exhibited reduced myocardial fibrosis...
consistent with unchanged hydroxyproline content (Figure 2C), although collagen type I mRNA levels were elevated (Figure 2D). TIMP3^{2/2} mice, however, showed striking interstitial and perivascular fibrotic lesions (Figure 2A and B) and elevated hydroxyproline content (Figure 2C), but no increase in de novo synthesis of collagen type I (Figure 2D), although collagen type III levels were elevated compared with the parallel saline group (Figure 1E). Therefore, collagen synthesis and protein deposition are uncoupled in TIMP2^{2/2} and TIMP3^{2/2} hearts.

Assessment of MMPs and their activity showed the absence of cleaved MMP2 in TIMP2^{2/2} hearts, consistent with the lack of TIMP2,4,35 while total collagenase activity was not significantly altered in either TIMP-deficient group compared with WT-Ang II group (see Supplementary material online, Figure S1Ai–ii). At mRNA level, expression of MMP2 and MT1-MMP were increased in all Ang II-infused groups; however, these increases were less in TIMP2^{2/2}-Ang II hearts along with a reduction in MMP9 and MMP13 expression (see Supplementary material online, Figure S1Ci, iv, and v). The increase in MMP8 reached statistical significance only in TIMP2^{2/2}-Ang II hearts (see Supplementary material online, Figure S1Ci and ii). TIMP1 expression was elevated in all Ang II-infused hearts, whereas other TIMPs were not altered by Ang II infusion (see Supplementary material online, Figure S1D). These data indicate that the observed impact of TIMP2 or TIMP3 deficiency on myocardial hypertrophy and fibrosis is likely not through their MMP-inhibitory function.

3.2 Diastolic dysfunction with distinct characteristics in TIMP2^{−/−}-Ang II vs. TIMP3^{−/−}-Ang II mice

Myocardial hypertrophy and fibrosis have been associated with diastolic dysfunction.36,37 We investigated the differential impact of enhanced hypertrophy in TIMP2^{−/−}-Ang II vs. fibrosis in TIMP3^{−/−}-Ang II on myocardial dysfunction, using non-invasive echocardiographic measurements followed by invasive haemodynamic pressure–volume measurements. All three genotypes showed preserved systolic function while TIMP2^{−/−}-Ang II and TIMP3^{−/−}-Ang II mice exhibited diastolic dysfunction compared with WT-Ang II mice (see Supplementary material online, Table S2 and Figure S3). Interestingly, different aspects of LV diastolic function were altered in TIMP2^{−/−}-Ang II following Ang II infusion. Based on echocardiography (Figure 3A and see Supplementary material online, Table S2), reduced peak E-wave (i), E’-wave (ii), E-wave deceleration rate (iii), and increased left atrium-to-body weight ratio (iv) were observed in Ang II-infused TIMP2^{−/−}- and TIMP3^{−/−}-Ang II, but not in TIMP3^{−/−}-Ang II, mice compared
with their saline and WT-Ang II groups. TIMP3\(^{-/-}\) -Ang II mice additionally exhibited elevated E/E' ratio (Figure 3Avii), a measure of LV filling pressure.

Invasive haemodynamic measurements revealed that the slope of the EDPVR, obtained by occlusion of IVC, was increased in WT-Ang II and TIMP3\(^{-/-}\) -Ang II, but not in TIMP2\(^{-/-}\) -Ang II, mice (Figure 3B). Averaged EDPVR, an indirect measure of myocardial stiffness, was markedly greater in TIMP3\(^{-/-}\) -Ang II, but not in TIMP2\(^{-/-}\) -Ang II, mice (Figure 3C). Consistent with the elevated filling pressure in TIMP3\(^{-/-}\) -Ang II mice, end-diastolic pressure (EDP) was significantly elevated in these mice, but not in TIMP2\(^{-/-}\) -Ang II mice (Figure 3D). These data collectively demonstrate that the excess hypertrophy in TIMP2\(^{-/-}\) -Ang II mice prolonged the active phase of LV relaxation, whereas fibrosis in the absence of hypertrophy in TIMP3\(^{-/-}\) -Ang II mice increased the passive LV stiffness.

3.3 Hypertrophic response in TIMP2\(^{-/-}\) and TIMP3\(^{-/-}\) cMyo \textit{in vitro}

Cardiomyocyte hypertrophy can result from mechanical stress (increased afterload) and/or direct hormonal effects. Ang II, in addition to being a hypertrophic agent, is a strong hypertensive agent which can trigger marked significant hypertension. We previously reported that
Ang II-induced hypertension in TIMP2^{−/−} mice is comparable to WT mice, but reduced in TIMP3^{−/−} mice. To examine whether the differential Ang II-induced myocardial hypertrophy in TIMP2^{−/−} vs. TIMP3^{−/−} mice is due to the different degrees of hypertension, or inherent to the cardiomyocyte of each genotype, we tested the hypertrophic response of isolated adult cMyo in vitro. When cultured alone, cMyo of neither genotype showed an increase in the expression of hypertrophy markers, βMHC, α-skeletal actin, and BNP, in response to Ang II (1 μM, Figure 4Ai–iii). Meanwhile, when co-cultured with adult cFB, Ang II treatment triggered marked elevations in expression of hypertrophy markers in WT and TIMP2^{−/−}, but not in TIMP3^{−/−}, co-cultures (Figure 4Bi–iii). These data indicate that TIMP3 deficiency suppresses the Ang II-induced hypertrophic capacity of the cardiomyocytes.

3.4 Fibrogenic properties of adult cFB lacking TIMP2 or TIMP3 in vitro

Next, we investigated if the function of TIMP2- and TIMP3-deficient cFBs in vitro could explain the differential Ang II-induced fibrogenic response in these mice. Second-passage adult cFB from each genotype was cultured under quiescent conditions or subjected to cyclic mechanical stretching, and treated with 1 μM Ang II or saline. Under quiescent conditions (Figure 5Ai–C), WT fibroblasts exhibited no increase in the expression of collagen type I, while collagen type III expression increased following 24 h of Ang II treatment (Figure 5Ai and ii); however, Ang II treatment did not alter the expression of collagen type I (i) or type III (ii) in TIMP2^{−/−} or TIMP3^{−/−} cFBs (Figure 5B and C). Similar
observations were made with different concentrations of Ang II (0.1 and 10 μM) or longer treatment of cFBs (48 h) (data not shown).

To simulate the *in vivo* condition where the cFBs are subjected to a rhythmic stretch and strain as a result of cardiac contraction and relaxation, we subjected the cultured cFB to a cyclic stretch protocol, 5% elongation at 0.33 Hz, as reported by others. This protocol of mechanical stretching was selected as it did not increase collagen production. The combination of cyclic mechanical stretch and 1 μM Ang II significantly increased the expression of collagen types I and III in WT cFB (Figure 5Di and ii) and collagen type I synthesis in TIMP2−/− cFB (Figure 5Ei and ii), whereas collagen expression was not altered in TIMP3−/− cFB (Figure 5Fi and ii). These data closely resemble the pattern of Ang II-induced expression of collagens in vivo, demonstrating that the TIMP3−/− cFBs in fact have reduced Ang II-induced de novo collagen production.

We further examined whether the more severe myocardial fibrosis in TIMP3−/−-Ang II mice could be due to enhanced migration or proliferation of the cFB. We compared the rate of migration (scratch assay) and proliferation (BrdU incorporation) of cultured cFB among genotypes. The rate of cFB migration was comparable among genotypes treated with saline or Ang II (see Supplementary material online, Figure S2A). BrdU incorporation increased with Ang II treatment in WT, but not in TIMP2−/− or TIMP3−/− cFB (see Supplementary material online, Figure S2Bi and ii). Ang II did not trigger cell death in any genotype as assessed by lactate dehydrogenase levels in the culture media (data not shown). Therefore, the exacerbated myocardial fibrosis in TIMP3−/−-Ang II hearts is not due to increased collagen production capacity, migration, or proliferation of TIMP3-deficient cFB.

### 3.5 Elevated matricellular proteins co-localize with fibrotic myocardium in TIMP3−/−-Ang II hearts

Matricellular proteins such as SPARC and OPN mediate post-translational modification, deposition, and organization of ECM proteins independent from their synthesis. Immunostaining for SPARC revealed greater elevations in Ang II-infused TIMP3−/−, but not TIMP2−/−, cFB (Figure 6Ai), whereas mRNA expression of SPARC was increased following Ang II infusion in all genotypes (Figure 6Aii). Immunostaining and fluorescent microscopy (Figure 6Bi and ii) and expression analysis for OPN (Figure 6Biii) showed increased protein and mRNA levels in WT-Ang II and to a significantly greater extent in TIMP3−/−-Ang II hearts, whereas no increase
Figure 5 Ang II-increased collagen expression in adult mouse cardiac fibroblast (cFB) in vitro. (A–C) TaqMan mRNA levels of collagen type I (i) and collagen type III (ii) in cFB isolated from WT (A), TIMP2−/− (B), and TIMP3−/− hearts (C) cultured under quiescent conditions and treated with saline or Ang II. (D–F) TaqMan mRNA levels of collagen type I (i) and collagen type III (ii) in cFB isolated from WT (A), TIMP2−/− (B), and TIMP3−/− hearts (C) cultured with cyclic mechanical stretching (5% elongation, 0.33 Hz) and treated with saline or Ang II. n = 4 hearts/genotype, 4 culture plates/group/genotype. *P < 0.05 compared with the corresponding saline.
was detected in Ang II-infused TIMP2$^{-/-}$ hearts. The elevated OPN and SPARC levels co-localized predominantly with the fibrotic regions within the myocardium highlighting their region-specific function.

To determine whether the elevated matricellular proteins in TIMP3$^{-/-}$-Ang II hearts are the direct result of TIMP3 deficiency in the fibroblasts, which have been reported to be the main source of matricellular proteins,$^{41}$ we measured the expression of SPARC and OPN in cultured cFB (quiescent and cyclically stretched). However, we found that Ang II treatment did not increase the expression of SPARC or OPN in cultured cFBs (see Supplementary material online, Figure S3). Inflammatory cells could be another source of matricellular proteins. We assessed if Ang II infusion triggered an inflammatory response in any genotype. Infiltration of macrophages and neutrophils was negligible in WT and TIMP2$^{-/-}$ hearts, but greatly elevated in TIMP3$^{-/-}$-Ang II hearts, primarily in the fibrotic areas (Figure 7A and B). Consistently, expression of inflammatory cytokine, interleukin-1β, was increased in TIMP3$^{-/-}$-Ang II hearts while the increase in interleukin-6, tumor necrosis factor, and monocyte chemoattractant protein-1 did not reach statistical significance (Figure 7C).

We further assessed if the lack of TIMP2 or TIMP3 could alter transport of pro-collagen to the extracellular space, conversion of pro-collagen to collagen and finally cross-linking of the triple helices and formation of collagen fibres. Coat protein complex II (COPII) vesicles, surrounded by a coat consisting of a number of genes including SEC23-24 and SEC13-31, mediate intracellular transport of numerous molecules. Given the large size of pro-collagens, Cul3-KLHL12 are required to make the COPII vesicles large enough to accommodate pro-collagen molecules.$^{42,43}$ Expression of the COPII-related genes (Sec23A, Sec31A, KLHL1, and Cullin 3) were not different among genotypes (see Supplementary material online, Figure S4A). Among

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**Figure 6** Up-regulation of SPARC and OPN in Ang II-infused TIMP3$^{-/-}$ hearts in vivo. (A) Representative immunostaining (i) and mRNA expression for SPARC; and (B) representative images (i), averaged quantification (fluorescent signal/field) (ii) and mRNA expression (iii) for OPN in the indicated groups after 2 weeks of Ang II or saline infusion. Scale bar = 50 μm (inset = 25 μm). $^*$p < 0.05 compared with saline; $^#p < 0.05$ compared with WT-Ang II; n = 6–8 fields per/group/genotype.
pro-collagen endopeptidase and enhancers reported to contribute to cardiac fibrosis (BMP-1, PCPE1, and PCPE2). BMP1 and PCPE1 increased in WT-Ang II, but not in TIMP2 or TIMP3 hearts (see Supplementary material online, Figure S4Bi and ii). Lysyl hydroxylase (PLOD1) and lysyl oxidase (LOX) are responsible for hydroxylation and oxidative deamination of collagens leading to maturation and stability of collagen fibres. Ang II infusion increased expression of PLOD1 in WT and TIMP3 hearts, while LOX expression increased with Ang II infusion but was lower in TIMP2 -/- -Ang II compared with other groups (see Supplementary material online, Figure S4Ci and ii).

4. Discussion

Myocardial hypertrophy and fibrosis are two characteristics of a number of cardiomyopathies which can lead to cardiac dysfunction culminating in
heart failure.\textsuperscript{10,36,37} Disruption of ECM can adversely impact the cell–
cell and ECM–cardiomyocyte interactions triggering pathological signaling
and hypertrophy,\textsuperscript{1,4–6} while excess accumulation of fibrillar ECM
proteins, primarily collagen types I and III, results in fibrosis.\textsuperscript{5,48} In this
study, we report that the lack of TIMP2 and TIMP3 distinctly and differ-
entially influences cardiac hypertrophy and fibrosis, thereby impacting
different aspects of diastolic function. Diastolic dysfunction, if left un-
treated, can lead to HFpEF which is a common and increasingly prevalent
health problem affecting up to 50% of all patients with chronic heart
failure.\textsuperscript{49–51} The pathophysiological mechanisms underlying HFpEF
are heterogeneous and complex. Diastolic dysfunction is associated
with abnormal active relaxation and elevated passive stiffness,\textsuperscript{37,51,52}
while additional factors have also been described in HFpEF\textsuperscript{53} including
impaired LA function indicated by enlarged LA size and reduced empty-
ing (A-wave).\textsuperscript{54} As cardiac hypertrophy and fibrosis are often concurrent
events in diastolic dysfunction, the TIMP2\textsuperscript{−/−} and TIMP3\textsuperscript{−/−}
mice provide valuable models in differentiating the impact of these
two aspects of myocardial remodelling on diastolic dysfunction. Diastol-
ic dysfunction can result from abnormal active relaxation, increased
passive stiffness, or both.\textsuperscript{37} We found that Ang II infusion resulted in
diastolic dysfunction with preserved ejection fraction in both TIMP-
deficient mice; however, excess hypertrophy in TIMP2\textsuperscript{−/−} mice
impacted the active relaxation phase, indicated by prolonged isovolumic
relaxation time and deceleration times, whereas severe myocardial
fibrosis in TIMP3\textsuperscript{−/−} mice markedly compromised LV passive compli-
ance. Hypophosphorylation of titin, the giant cytoskeletal protein that
serves as a spring-mediating diastolic recoil and distensibility of the
cardiomyocytes, can lead to HFpEF associated with hypertrophy as
reported in patients\textsuperscript{55,56} and in animal models.\textsuperscript{57} Titin hypophosphory-
lation could contribute to the diastolic dysfunction in TIMP2\textsuperscript{−/−}-Ang II
hearts, although the detailed mechanism of this process will require
further investigation.

Ang II is a fibrogenic and hypertrophic hormone that is elevated in
different heart diseases.\textsuperscript{58–60} In an earlier study, we reported that the
Ang II-induced hypertrophy is comparable between WT and
TIMP2\textsuperscript{−/−} mice, but reduced in TIMP3\textsuperscript{−/−} mice.\textsuperscript{38} This study further
emphasizes the importance of TIMP3 deficiency in mediating diastolic
dysfunction despite the less severe hypertension. In addition, while
hypertension can also trigger hypertrophy, the milder hypertension in
TIMP3\textsuperscript{−/−}-Ang II mice is not solely responsible for the suppressed
hypertrophy in these mice since in vitro cMyo–cFB co-culture experi-
ments also revealed blunted hypertrophy in TIMP3\textsuperscript{−/−} myocytes. This
indicates inherent resistance of these cardiomyocytes to Ang II-induced
hypertrophy, although the underlying molecular mechanism requires
further investigation. Consistent with our finding that Ang II-induced
hypertrophy was only accomplished in cMyo–cFB co-cultures and not in
cMyo cultures alone, it has been reported that the paracrine interaction
between cMyo and fibroblasts are essential for the agonist-induced hypertrophy in adult cardiomyocyte.\textsuperscript{51,62} We previously
reported that cardiac pressure overload results in myocardial hyper-
trophy and interstitial fibrosis in mice lacking TIMP2,\textsuperscript{4} and additionally
severe focal fibrotic lesions in TIMP3 mice.\textsuperscript{54,63} The increased hyper-
trophy in pressure-overloaded TIMP3\textsuperscript{−/−} mice could be the cumulative
outcome of enhanced mechanical stress concomitant with the triggered
neurohormonal factors. In addition, TIMP3\textsuperscript{−/−} mice are more prone to
inflammation,\textsuperscript{59,64} which could have also contributed to the pressure
overload-induced remodelling.

The lack of TIMP2 or TIMP3 did not trigger compensatory up-regulation of other TIMPs, while TIMP1 expression increased
similarly in all genotypes with Ang II infusion. An increase in TIMP1
levels is frequently linked to fibrosis, and therefore, its elevation in
TIMP2\textsuperscript{−/−}-Ang II hearts, which exhibited that no fibrosis is intriguing
and could be linked to the elevated collagen mRNA. We found that
the lack of myocardial fibrosis despite elevated collagen mRNA in
TIMP2\textsuperscript{−/−}-Ang II hearts, compared with the excess fibrotic lesions
in TIMP3\textsuperscript{−/−} hearts without increased de novo synthesis of collagen, is
due to an inherent response of cFB of either genotype to Ang II. More-
over, quiescently cultured cFBs did not exhibit the Ang II-induced col-
gen synthesis observed in vivo. This is not surprising since quiescently
cultured cFB has been compared with the status in a healthy heart where
the intact matrix shields cFB from mechanical stretching, sustaining their
quiescent phenotype with basal production of ECM proteins.\textsuperscript{5,48} As
such, it is plausible that fibrogenic capacity of quiescent FB is com-
promised. An earlier study by Lovelock et al.\textsuperscript{17} reported that overexpres-
sion of human TIMPs in quiescently cultured mouse cFB increased
proliferation, while TIMP2 further induced apoptosis and TIMP2
increased collagen production by these cells. These findings are different
from ours most likely due to differences in experimental settings.
Lovelock et al. utilized quiescently cultured cFB at passages 4–5, whereas we
limited our studies to cFB in passage 2 since myofibroblast characteris-
tics are detected beyond the second passage.\textsuperscript{65} Moreover, the fold
increase in TIMP levels upon overexpression is an important factor in
cell response since an increase to supra-physiological levels can trigger
variable responses.

Collagen production and formation of collagen fibre involve a number
of steps and a number of proteins and enzymes.\textsuperscript{1,44} Following assembly
into a triple helix structure, pro-collagen molecules are transported
in specialized COPII vesicles (large enough to accommodate the pro-
collagen triple helix) and are secreted into the pericellular space.\textsuperscript{42,43}
Pro-collagen is processed by endopeptidases that cleave the N- and
C-propeptide-forming collagen, which then self-assembles into fibrils.\textsuperscript{44,45,67} Finally, PLOD1 converts lysine into hydroxylysine and
LOX catalyses oxidative deamination of lysine and hydroxylysine in
the triple helix-mediating cross-linking of the collagen fibrils and forma-
tion of collagen fibres. The lower levels of LOX and lack of elevation in
PLOD1 in TIMP2\textsuperscript{−/−}-Ang II hearts could impair collagen cross-linking
and fibre formation, thereby partly contributing to the lack of fibrosis
despite elevated collagen mRNA in these hearts.

Matricellular proteins, such as OPN and SPARC, are major contribu-
tors to post-translational modification, stabilization, and deposition
of collagen proteins in the heart.\textsuperscript{5,6,68} SPARC is a collagen-binding matricel-
lar protein that is produced by cardiac fibroblasts\textsuperscript{69} and regulates the
assembly and organization of the ECM and can stabilize the collagen
molecules extracellularly resulting in their accumulation.\textsuperscript{5,39,68} SPARC
has also been linked to fibrosis in multiple organs,\textsuperscript{70–72} while SPARC
deficiency led to the formation of unstable collagen,\textsuperscript{73} morphological
defects of the fibrillar collagen, and reduced collagen deposition in
different tissues.\textsuperscript{27,74} OPN is another matricellular protein whose
expression has been associated with fibrosis in different organs.\textsuperscript{5} Ang
II-induced myocardial fibrosis was ablated in OPN\textsuperscript{−/−} mice,\textsuperscript{59,75} while
collagen accumulation was decreased in these mice following myocardial
infarction.\textsuperscript{76} It has been reported that macrophages\textsuperscript{77,78} and neutro-
philphi\textsuperscript{39} express OPN, while cytokines such as IL-1β can synergistically
enhance the Ang II-induced OPN expression by fibroblasts.\textsuperscript{41} OPN
and SPARC expression were increased in Ang II-infused hearts, but
not in cultured cFB fibroblasts. Therefore, it is plausible that the main
source of these pericellular proteins is non-fibroblasts cells, while
inflammatory cells and elevated cytokines such as IL-1β further elevated
OPN levels in TIMP3<sup>−/−</sup>–Ang II hearts. The marked up-regulation of these matricellular proteins, together with the lack of de novo expression of collagen in the TIMP3<sup>−/−</sup>–Ang II myocardium, indicates that the post-translational processing and deposition of collagen fibres underlie the severe perivascular and interstitial myocardial fibrosis in these mice.

In summary, our findings challenge the canonical concepts regarding TIMP biology by establishing that reduced TIMP levels do not invariably result in reduced fibrosis due to increased ECM-degrading function of MMPs. This highlights the complex function of TIMPs in cardiac pathology beyond their MMP-inhibitory function. TIMP1<sup>−/−</sup> and TIMP3<sup>−/−</sup> mice provide the unique opportunity to decipher the impact of myocardial hypertrophy vs. fibrosis in diastolic dysfunction. Our studies demonstrate that myocardial hypertrophy impairs active relaxation, while fibrosis enhances passive stiffness of the myocardium. Our in vitro studies further demonstrate the complexity of cellular events involved in these processes, highlighting the significance of post-translational regulation of matrix proteins, independent from their de novo synthesis, in mediating myocardial fibrosis.

5. Clinical relevance

Diastolic dysfunction can occur in patients with normal LV EF and can lead to diastolic heart failure (HFpEF).<sup>10</sup> The hospital re-admission rates of patients with HFpEF are similar to those with HFrEF, and worsening of diastolic dysfunction has been shown to be an independent predictor of mortality.<sup>80</sup> In this study, we demonstrate that, in a model of diastolic dysfunction with sustained EF, TIMP2 and TIMP3 have divergent effects on cardiac remodelling and can impact different aspects of diastolic function by differentially regulating myocardial hypertrophy and fibrosis, the two common co-morbidities of HFpEF.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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