Heart disease continues to be a major cause of morbidity and mortality worldwide. According to a recent report from American Heart Association, death caused by cardiovascular diseases accounts for 1 in every 3 deaths in North America. Fibrosis is a common feature of various cardiomyopathies including dilated and hypertrophic cardiomyopathy, and myocardial infarction (MI), although the type of fibrosis differs in these diseases. Myocardial fibrosis in hypertrophic and DCM is reactive fibrosis, whereas fibrosis in MI is reparative fibrosis as it replaces the lost cardiomyocytes secondary to ischemia. Reparative fibrosis in the infarcted myocardium is mediated by inflammatory influx, and transformation of fibroblasts to myofibroblast that rapidly populate the affected area, produce, and deposit extracellular matrix (ECM) and form a fibrotic scar. The mechanism underlying reactive fibrosis is less explored as it occurs in the absence of cardiomyocyte loss. Fibrosis, regardless of its initiating cause, unfavorably alters cardiac structure and function and can lead to heart failure, and as such, understanding its underlying molecular mechanism is essential in developing effective therapies for patients with heart failure.

Fibrosis is the result of adverse remodeling and excess deposition of the ECM, primarily fibrillar collagens type I and type III. Tissue inhibitor of metalloproteinases (TIMPs) maintain the homeostatic balance of myocardial ECM by inhibiting the activated matrix metalloproteinases (MMPs). Among the 4 TIMPs, TIMP2, TIMP3, and TIMP4 have been shown to contribute to myocardial fibrosis through different mechanisms and their loss often exacerbated myocardial fibrosis; however, the role of TIMP1 has been less explored. TIMP1 can inhibit many MMPs except the membrane-bound MMPs. Elevated tissue and plasma TIMP1 levels have been correlated with myocardial fibrosis and diastolic dysfunction, and TIMP1 has been used as a biomarker for fibrosis in patients and in animal models of heart disease. Despite the consistent link between TIMP1 and fibrosis, it remains unknown whether TIMP1 directly promotes tissue fibrosis or whether its role is simply a bystander alteration in this process. We investigated the role of TIMP1 in myocardial fibrosis in...
hypertrophic and dilated cardiomyopathies and whether its function is mediated through an MMP-dependent or MMP-independent mechanism.

Here, we provide evidence from 2 in vivo models of myocardial fibrosis in Timp1-deficient mice, in vitro adult mouse cardiac fibroblasts, and myocardial specimens from patients with heart failure, showing that TIMP1 is a driving force in promoting myocardial fibrosis through a mechanism independent from its MMP-inhibitory function. We demonstrate that TIMP1 activates a signaling pathway that involves the interaction of CD63 and integrin β1, nuclear translocation of phospho-Smad2/3 and phospho-β-catenin, and expression of ECM collagens.

**Methods**

**Experimental Animals and Surgical Procedures**

Wild-type (WT) and Timp1-deficient male mice (Timp1−/−) in C57BL/6 background were purchased from Jackson Laboratories and bred in-house at University of Alberta. All experiments were performed in accordance with the Canadian Council on Animal Care Guidelines and regulations of Animal Care and Use Committee at University of Alberta.

Cardiac pressure overload was induced in male WT and Timp1−/− mice (8–10 weeks of age) by transverse aortic constriction (TAC) as described previously.10,20 Male 8- to 9-week-old mice of either genotype received angiotensin II (Ang II, 1.5 mg/kg per day) or saline by Alzet micro-osmotic pump (Model 1002; Durect Co.) implanted dorsally and subcutaneously as before.13,14 MMP inhibitor (MMPi), PD166793 (30 mg/kg per day) was administered by daily gavage as before.22,23 At the indicated time points, hearts were excised, (Optimal Cutting Equilibrium) cryosectioned, or perfused with heart, showed a significant Ang II–induced rise in WT but not Timp1−/− mice (Figure S1B). In addition, total gelatinase activity increased significantly but similarly in both genotypes after Ang II infusion (Figure S1A in the online-only Data Supplement), whereas total gelatinsase activity increased significantly but similarly in both genotypes after Ang II infusion (Figure S1A in the online-only Data Supplement), whereas total gelatinsase activity increased significantly but similarly in both genotypes after Ang II infusion (Figure S1A in the online-only Data Supplement).

**Cardiac Function Assessment**

Heart structure and function were assessed in post-sham/TAC hearts noninvasively by transthoracic echocardiography under anesthesia (1% isoflurane) using Vevo 3100 high-resolution imaging system equipped with a 30-MHz transducer (Visual Sonics) as described before.13,14 Invasive hemodynamic and pressure–volume measurements were performed in anesthetized mice (2% isoflurane) post-TAC/sham using 1.2F Scisense catheter (TCP-500 Scisense Inc.).

**Human Explanted Heart Tissue**

Heart tissues from patients with heart failure were procured at the time of cardiac transplantation as part of the Human Explanted Heart Program at the Mazankowski Alberta Heart Institute (Edmonton, Alberta), and healthy control hearts were obtained through the Human Organ Procurement and Exchange program (Edmonton, Alberta) as before.25 All experiments were approved by institutional ethics committee and performed in accordance with the institutional guidelines with informed consent from all subjects.

**Histological and Immunohistochemical Staining and Imaging**

Immunohistochemical staining for trichrome, picrosirus red and wheat glutenine staining were performed on formalin-fixed hearts as before.13,14 OCT-frozen 5-μm sections were used for coimmunostaining of CD63 (R&D Mab5417; NovusNBP2-32829) and integrin β1 (Millipore Mab1900), and immunostaining for scleraxis (ab58655). Fibrosis quantification was performed on picrosiris red–stained sections as before.13,14 Proximity ligation assay was performed on formalin-fixed hearts or cultured fibroblasts using Duolink in situ fluorescence Sigma kit and proximity ligation assay probes for mouse (DUO92004) and for rabbit antibodies (DUO92002).

**RNA Extraction and Expression Analysis**

Total RNA was extracted from frozen heart tissues using TriZol reagent (Invitrogen), and mRNA expression analysis was performed by TaqMan RT-PCR (reverse transcription–polymerase chain reaction) as before, and Hprt (hypoxanthine-guanine phosphoribosyltransferase-1) as the internal control. Taqman primers and probes for procollagen I-α1 and procollagen III-α1, Timp1 (-1 to –4) were used as before,10,13,14,22 and for scleraxis (Scx, Assay ID: Mm01205675_m1) purchased from ThermoFisher Scientific.

**Protein Extraction, Activity Assay, Immunoblot, and Coimmunoprecipitation**

Total protein was extracted using Sigma extraction buffer.26 Fluorescent-based activity assays were performed as before (EnzCheck, Molecular Probes).13,14 Immunoblot analyses were performed as before.26 Coimmunoprecipitation experiments were performed to detect CD63 and integrin β1 association. Nuclear and cytosolic protein fractions were extracted using hypotonic lysis buffer, and purity of the nuclear and the cytosolic protein was assessed by blotting for a nuclear-specific protein (Histone) and cytosole-specific protein (Caspace 3), respectively.

**Adult Cardiac Fibroblast Isolation and Culture**

Adult cardiac fibroblasts were isolated from WT and Timp1−/− mice as described previously.13,14 Fibroblasts were used at second passage, and serum-deprived for 24 hours before experiments. Fibroblasts from WT and Timp1−/− mice were treated with transforming growth factor-β (10 ng/mL) or Ang II (1 μmol/L) for 24 hours.

**Statistical Analysis**

All statistical analyses were performed using IBM SPSS Statistics 19 Software. Shapiro–Wilks test for normality and homogeneity of variance was performed for all data. Comparison of groups from different genotypes was done by 2-way ANOVA. Multiple comparisons between groups within the same genotype were performed using 1-way ANOVA followed by Bonferroni or Tukey post hoc test. Averaged values represent means±SEM. Statistical significance determined at P<0.05.

**Results**

**Angiotensin II–Mediated Fibrosis Is Reduced in Timp1-Deficient Mice and Not Reversed by MMPi Treatment**

Ang II infusion triggered a significant increase in interstitial and perivascular fibrosis in WT mice, whereas this fibrotic response was markedly reduced in mice lacking TIMP1 (Figure 1Ai and ii). A broad-spectrum MMPi (PD166793) did not increase myocardial fibrosis in Timp1−/− mice, indicating that the reduced fibrosis in this group is not because of elevated MMP activities in the absence of TIMP1 (Figure 1Ai and ii).

Measurement of mRNA expression for collagen type I and collagen type III, the 2 main fibrillar collagens in the heart, showed a significant Ang II–induced rise in WT but not in Timp1−/− mice (Figure 1B). Gelatin zymography showed comparable levels of MMP2 and MMP9 in both genotypes (Figure S1A in the online-only Data Supplement), whereas total gelatinase activity increased significantly but similarly in both genotypes after Ang II infusion (Figure S1B). In addition, Ang II–induced myocardial hypertrophy were comparable between WT and Timp1−/− mice as assessed by heart weight:tibial length ratio, and expression of molecular markers of hypertrophy (atrial natriuretic peptide, brain natriuretic peptide, and α-skeletal actin; Figure S1C and S1D). Ang II infusion induced a marked increase in Timp1 mRNA in WT mice,
Figure 1. Myocardial fibrosis induced by angiotensin II (Ang II) infusion or cardiac pressure overload is markedly reduced in TIMP1 (tissue inhibitor of metalloproteinase-1)–deficient mice. A, (i) Representative trichrome and picrosirius red (PSR)–stained images from wild-type (WT) and Timp1−/− mice after 2 wk of receiving saline, Ang II, or Ang II+matrix metalloproteinase inhibitor (MMPi; PD166793). (ii) Myocardial collagen content in indicated groups (n=10–15 images per heart; 4–5 hearts per group). B, mRNA expression of procollagen Iα1 and procollagen IIIα1 in indicated groups (n=5–7 per group). C, Representative trichrome and PSR-stained images (i) and myocardial collagen content (ii) in WT and Timp1−/− mice after 2 wk of cardiac pressure overload induced by transverse aortic constriction (TAC). D, mRNA expression of procollagen Iα1 and procollagen IIIα1 in indicated groups. Averaged data represent mean±SEM. *P<0.05 compared with the corresponding saline group, §P<0.05 compared with the corresponding WT group. A.U. indicates arbitrary units; and R.E., relative expression.
whereas mRNA expression of other *Timp* was comparable between the 2 genotypes (Figure S1E). These data indicate that TIMP1 deficiency suppressed the Ang II–mediated myocardial fibrosis but not hypertrophy, perhaps not because of increased MMP activity because MMPI treatment did not alleviate the differential collagen accumulation in WT and *Timp1*−/− hearts.

We further examined whether TIMP1 deficiency could reduce proliferation or migration of cardiac fibroblasts, thereby suppressing the fibrosis response. Using the wound healing (scratch) assay and adult cardiac fibroblasts from WT and *Timp1*−/− mice, we found that the rate of wound healing (closure of the scratched area) was not significantly different between the genotypes (Figure S2A). Consistently, expression of α-smooth muscle actin, a marker of myofibroblast transformation, was not different between WT and *Timp1*−/− hearts after Ang II infusion (Figure S2B). Therefore, the reduced myocardial collagen production and deposition in *Timp1*−/− Ang II mice could not be because of suppressed cardiac fibroblast proliferation or migration in these mice.

**Loss of TIMP1 Ameliorates Myocardial Fibrosis After Biomechanical Stress by Reducing Collagen mRNA Expression**

Next, we examined whether the impact of *Timp1* deficiency on myocardial fibrosis applies to other models of heart disease. After 2 weeks of cardiac pressure overload, significant interstitial and perivascular fibrosis was detected in WT mice, whereas this response was markedly reduced in *Timp1*−/− mice (Figure 1Ci and ii). Similar to the Ang II model, mRNA expression of collagen type I and type III was markedly elevated in WT mice post-TAC but not in *Timp1*-deficient mice (Figure 2B), whereas the rise in total gelatinase activity was similar in both genotypes, and similar MMP2 and MMP9 levels were observed in both genotypes (Figure S3Ai and ii). Moreover, expression of α-smooth muscle actin was increased similarly in WT and *Timp1*−/− hearts post-TAC (Figure S2B). Post-TAC myocardial hypertrophy was comparable between WT and *Timp1*−/− mice after 2 and 5 weeks of TAC (Figure S3B and S3D). mRNA expression of *Timp1* increased in WT mice at 2 and 5 weeks post-TAC, consistent with the observed myocardial fibrosis in these mice, whereas mRNA expression of other TIMPs remained comparable among groups (Figure S3C and S3E).

**TIMP1 Mediates the Association Between CD63 and Integrin β1 in Mouse Myocardium After Pressure Overload and in Adult Cardiac Fibroblasts**

CD63 is the only known cell surface receptor for TIMP1.27 TIMP1 has been reported to mediate CD63 interaction with integrin in regulating chemotaxis in neural crest cells.28 We investigated whether TIMP1 could use a similar mechanism to modulate collagen expression in the heart. Coimmunoprecipitation experiments revealed a strong interaction between CD63 and integrin β1 in WT myocardium post-TAC; however, this interaction was significantly suppressed in *Timp1*−/− hearts (Figure 2Ai and ii). Consistently, proximity ligation assay (Figure 2B) and coimmunostaining (Figure 2C) further confirmed a significantly greater increase in colocalization of CD63 and integrin β1 after pressure overload in WT compared with TIMP1-deficient mice.

Fibroblasts are key players in ECM remodeling as they are the main cell source for production of fibrillar collagens and other ECM proteins. Therefore, we investigated whether the observed TIMP1-mediated CD63–integrin β1 interaction occurs in cardiac fibroblasts. In cultured adult cardiac fibroblasts from WT and *Timp1*−/− mice, treatment with profibrotic agents Ang II or transforming growth factor-β1 increased the interaction between CD63 and integrin β1 in WT, but not in *Timp1*−/− fibroblasts as determined by communoprecipitation (Figure 3A), proximity ligation assay (Figure 3B), and coimmunostaining for these 2 molecules (Figure 3C). These data collectively support the notion that TIMP1 is required for the CD63–integrin β1 interaction.

**TIMP1-Mediated CD63–Integrin β1 Interaction Correlates With Myocardial Fibrosis in Patients With DCM**

Because TIMP1 has been consistently linked to myocardial fibrosis in patients with DCM,15,17 we investigated whether TIMP1-mediated CD63–integrin β1 interaction is also present in fibrotic human myocardium. As anticipated, DCM hearts exhibited marked fibrosis compared with nonfailing control hearts as evident by picrosirius red and trichrome-stained sections (Figure 4Ai and ii). We performed communoprecipitation on the membrane and the cytosolic protein fractions of the human myocardial specimens and found a strikingly higher CD63–integrin β1 interaction in the DCM membrane protein fraction (but not cytosolic fraction) compared with the nonfailing control hearts (Figure 4Bi), along with greater TIMP1 protein levels in this group (Figure 4Bii).

**TIMP1 Activates a Signaling Pathway That Involves Activation and Nuclear Translocation of Smad2/3 and β-Catenin**

Next, we investigated the downstream signaling pathway that could lead to induction of mRNA expression of collagen and subsequently tissue fibrosis. Smad2/3 is a profibrotic transcription factor that is activated in response to various profibrosis stimuli, often as a part of transforming growth factor-β1 pathway activation, which is a key mechanism of fibrosis in various tissue. Consistent with the greater degree of fibrosis in WT-TAC hearts, phospho-to-total Smad 2/3 ratio was significantly higher in WT than in *Timp1*−/− hearts post-TAC (Figure 5A). It has also been reported that phospho-Smad2/3 can associate with phosphorylated β-catenin (Y654)9,29 or scleraxis30 before its nuclear localization. β-catenin is associated with cadherins and other transmembrane proteins that can interact with CD63.31,32 Immunoblotting for phospho-β-catenin and phospho-Smad2/3 on the nuclear and cytosolic protein fractions from WT and *Timp1*−/− hearts showed a markedly greater level of nuclear translocation for phospho-Smad2/3 and phospho-β-catenin after pressure overload in WT but not in *Timp1*−/− mice (Figure 5B). Together, these data show a coordinated function between the transforming growth factor-β pathway and the TIMP1-mediated CD63–β-catenin association that lead to transcriptional induction of collagen (Figure 5C).

Assessment of contribution of scleraxis showed that in myocardial specimens from patients with DCM, scleraxis
levels were markedly higher compared with nonfailing control hearts (Figure S4A). After TAC, scleraxis levels increased more in WT than in Timp1−/− hearts (Figure S4B and S4C). However, nuclear translocation of scleraxis was not altered with Timp1 deficiency (Figure S4D), suggesting that scleraxis may not be a key player in TIMP1-mediated collagen synthesis. Purity of the nuclear and non-nuclear protein fractions in these experiments was confirmed by immunoblotting for histone (nuclear) and caspase 3 (cytoplasm) proteins (Figure S5).

**Absence of TIMP1 Has Long-Term Beneficial Effects After Cardiac Pressure Overload**

We investigated whether the suppressed fibrosis in Timp1-deficient mice exerts protective (or deleterious) effects over long-term after cardiac pressure overload. The suppressed myocardial fibrosis in Timp1−/− mice persisted up to 9 weeks post-TAC (Figure 6A), whereas these mice also exhibited ameliorated cardiomyopathy compared with WT-TAC mice. At 9 weeks post-TAC, myocardial hypertrophy, measured by heart weight:tibial length (Figure 6B), myocyte cross-sectional area (Figure S6A), disease markers (α-skeletal actin, β-myosin heavy chain, and brain natriuretic peptide), and pulmonary edema, a measure of heart failure (Figure 6C and 6D) were significantly lower in Timp1−/−-TAC than in WT-TAC mice.

Assessment of cardiac structure and function by noninvasive echocardiography (Figure S6B and S6C; Table S1) showed that biomechanical stress (TAC) significantly decreased
left ventricular (LV) contractility (ejection fraction; i), resulted in LV dilation (ii, iii) and left atrial enlargement, which is a marker of LV diastolic dysfunction and heart failure (iv) in WT mice. Interestingly, Timp1 deficiency ameliorated the decrease in ejection fraction and LV dilation and improved the parameters of diastolic dysfunction, E/E' ratio and left atrial enlargement. Invasive hemodynamic measurements further demonstrated partially preserved cardiac function in Timp1−/− TAC mice as evidenced by a leftward shift in the pressure–volume loop compared with WT-TAC mice (Figure 6Ei), and the lack of elevated LV end-diastolic volume (Figure 6Eii).

Consistent with the less severe diastolic dysfunction in Timp1−/− mice, LV stiffness (end-diastolic pressure–volume slope) was enhanced in WT-TAC but not in Timp1−/−-TAC mice (Figure 6Eiii). These data collectively indicate that the absence of TIMP1 prevented decompensation and heart failure after long-term pressure overload.

Discussion

Tissue fibrosis is a key pathogenic feature of end-organ failure in various organs, including heart failure. This study reveals a new function for TIMP1 in promoting myocardial fibrosis.
fibrosis, which can be helpful in treating and managing fibrosis and heart disease. Elevated plasma TIMP1 levels have been consistently linked to myocardial fibrosis in patients and in animal models, and it has been proposed to be a reliable biomarker for tissue fibrosis. TIMP1 is best known as an inhibitor of soluble MMPs (and not of membrane-bound-MMPs), and as such it is often assumed that a rise in TIMP1 promotes fibrosis by inhibiting MMPs and their ECM-degrading function. In this study, we demonstrate that Timp1 deficiency suppresses myocardial fibrosis through an MMP-independent mechanism. Using 2 in vivo models of myocardial fibrosis, in vitro adult cardiac fibroblast cultures, and human myocardial specimens, we demonstrate that TIMP1 promotes myocardial fibrosis by mediating the interaction between CD63 and integrin β1 on fibroblasts, activation and nuclear translocation of Smad2/3 and β-catenin, and induction of de novo mRNA expression of collagens type I and III. Interestingly, the absence of other TIMPs (TIMP2, TIMP3, and TIMP4) resulted in enhanced myocardial fibrosis in different heart disease models, making TIMP1 a unique member of the TIMP family to have an inverse impact on myocardial fibrosis.

Integrin β1 in cardiac fibroblasts has been shown to contribute to heart failure, as its levels rise in response to profibrotic factors triggering collagen expression. Contribution of fibroblast integrins to fibrosis has also been reported in other organs. CD63 is a membrane protein present on the cell surface of various cell types, and although it is abundant on endosomes and lysosomes, it is well-recognized for its lysosome-independent functions. CD63 is the only known cell surface receptor for TIMP1. In stellate cells in the liver, binding of TIMP1 to CD63–integrin β1 complex promoted cell migration, whereas this interaction inhibited tumor growth through activating the FAK (focal adhesion kinase) and PI3K (phosphoinositide-3 kinase) signaling pathways and regulated chemotaxis in neural crest cells. Our study is the first to report a novel function of TIMP1 in induction of collagen synthesis and fibrosis in the heart by mediating the interaction...
between CD63 and integrin β1 in cardiac fibroblasts, leading to subsequent activation and nuclear translocation of Smad2/3 and β-catenin, and induction of collagen mRNA expression (Figure 7C). Scleraxis is another transcription factor that has been reported to work synergistically with Smad2/3 to trigger fibrosis.30,43 Although scleraxis levels were increased in the fibrotic myocardium (human and mouse), this increase was comparable between WT and TIMP1-deficient mice suggesting that Scx is not a key player in TIMP1-mediated induction of collagen synthesis and fibrosis although it could contribute to TIMP1-independent fibrosis response.

MMP-independent functions of TIMP1 and other TIMPs have been reported in different cell types. TIMP1 can promote proliferation of oligodendrocytes.44 TIMP3 deficiency led to severe myocardial fibrosis after Ang II infusion because of post-translational regulation of matricellular proteins.14 TIMP4 modulated cardiac recovery from ischemia-reperfusion by regulating the inflammatory response rather than by MMP inhibition.13

In a model of MI, TIMP1-deficient mice exhibited a greater rise in MMP activity and exacerbated cardiac dysfunction compared with parallel WT mice, and treatment with an MMPi improved the cardiac dysfunction in Timp1−/−-MI mice.45,46 Therefore, the function of TIMP1 in cardiac response to MI seems to be primarily through its MMP-inhibitory function. However, after pressure overload or Ang II infusion, lack of TIMP1 did not result in an additional rise in MMP activity, and consistently, MMPi treatment did not reverse the suppressed myocardial fibrosis in Timp1−/− mice. It is important to note that the mechanism of cardiac fibrosis after MI is distinct from that after pressure overload, as the former is reparative fibrosis3–5 and the latter is reactive fibrosis.2 After MI, MMP activity peaks shortly after the onset of MI and drives the remodeling of the infarcted myocardium,22 whereas the rise of MMP activities after pressure overload occurs gradually such that its impact on tissue remodeling may not be as stark as in post-MI. Hence, it is not surprising that TIMP1 plays distinct functions in cardiac recovery from MI compared with pressure overload. Furthermore, we reported previously that TIMP1 deficiency does not alter Ang II–induced hypertension47; therefore, the reduced fibrosis in Timp1−/−-Ang II mice is not because of suppressed hypertension. The antifibrotic effects of TIMP1 deficiency has long-term beneficial effects as it leads to ameliorated myocardial hypertrophy, LV dilation, and systolic and diastolic dysfunctions in Timp1−/− mice.
Figure 6. TIMP1 (tissue inhibitor of metalloproteinase-1) deficiency ameliorates myocardial fibrosis, hypertrophy, and left ventricular (LV) dysfunction in long-term post–transverse aortic constriction (TAC). A, Representative picrosirius red (PSR)–stained images (i) and myocardial collagen content (ii) for wild-type (WT) and Timp1−/− mice at 9 wk of sham or TAC (n=3–4 hearts per group per genotype). B, Representative transverse heart sections and heart weight:tibial length ratio (HW/TL). C, mRNA expression of markers of heart disease, α-skeletal actin, β-myosin heavy chain, and brain natriuretic peptide. D, Lung water content (edema) in WT and Timp1−/− mice postsham/TAC. E, Hemodynamic parameters showing the pressure–volume loops (i), LV end-diastolic volume (n=5–7 per group per genotype; ii), and LV myocardial stiffness or assessed by end-diastolic pressure–volume relationship (EDPVR; iii) in indicated groups (n=6–10 per group per genotype). *P<0.05 compared with corresponding sham group, §P<0.05 compared with corresponding WT group. A.U. indicates arbitrary units; and R.E., relative expression.
In conclusion, this study defines a novel function of TIMP1 in triggering fibrosis independent from its MMP-inhibitory function and through mediating an interaction between fibroblast membrane proteins, CD63 and integrin β1. This finding is supported by data from in vivo genetic mouse models, human myocardial specimens, and in vitro cell culture. Although the cause of the cardiomyopathy in patients with DCM is different from that in murine pressure overload and Ang II infusion models, the consistent observation that TIMP1 can mediate CD63–integrin β1 interaction in correlation with myocardial fibrosis further supports the diverse applicability of this pathway to different types of non-ischemic cardiomyopathies. With this new information on the role of TIMP1 in fibrosis, targeting TIMP1 could prove to be beneficial in limiting fibrosis in patients with nonischemic cardiomyopathies, which could subsequently hinder disease progression and heart failure.

Perspectives

Our study defines a novel and MMP-independent function for TIMP1 in inducing myocardial fibrosis through mediating an interaction between CD63 and integrin β1 on cardiac fibroblasts that leads to de novo expression of fibrillar collagens (I and III). Discovery of this MMP-independent function of TIMP1 in fibrosis provides new insight into developing antifibrosis therapies by targeting TIMP1.

Sources of Funding

This study was funded by Heart and Stroke Foundation (HSF) operating grant (GIA-G14-0006063) and Canadian Institute of Health Research (CIHR) (MOP-84279). A. Takawale is supported by Alberta Innovates-Health Solutions (AI-HS) graduate scholarship. V.B. Patel received support from HSF and AI-HS postdoctoral fellowships.

References


**Novelty and Significance**

**What Is New?**

- This study identifies a novel role for TIMP1 (tissue inhibitor of metalloprotease-1) in mediating cardiac fibrosis in a matrix metalloproteinase-independent manner.
- TIMP1 regulates de novo collagen synthesis by mediating an interaction between integrin β1 and CD63 in cardiac fibroblasts thereby activating nuclear translocation of p-Smad2/3 and p-β-catenin.
- Reduced myocardial fibrosis with TIMP1 deficiency has long-term beneficial effects is partially preserving systolic and diastolic cardiac functions after pressure overload.

**What Is Relevant?**

- We report that the rise in TIMP1 levels in the fibrotic myocardium is not a compensatory mechanism to reduce matrix metalloproteinase activities, but rather TIMP1 can directly induce collagen synthesis by fibroblasts.

This function of TIMP1 is particularly important in reactive fibrosis (not reparative fibrosis). Therefore, inhibiting TIMP1 could prove to be an effective approach in limiting reactive fibrosis.

**Summary**

TIMP1 is a well-known matrix metalloproteinase inhibitor, but here we report that its matrix metalloproteinase–independent function is key to induce de novo collagen synthesis and in triggering reactive fibrosis in the heart, through mediating an interaction between integrin β1 and CD63 in cardiac fibroblasts. Therefore, TIMP1 is unique among TIMPs because it is the only TIMP whose loss reduced myocardial (reparative) fibrosis, and as such anti-TIMP1 therapy could be beneficial in limiting cardiac fibrosis and progression to heart failure in patients with nonischemic cardiomyopathies.
Tissue Inhibitor of Matrix Metalloproteinase-1 Promotes Myocardial Fibrosis by Mediating CD63–Integrin β1 Interaction
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*Hypertension*. 2017;69:1092-1103; originally published online April 3, 2017; doi: 10.1161/HYPERTENSIONAHA.117.09045
*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/69/6/1092

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Tissue inhibitor of matrix metalloproteinase-1 (TIMP1) promotes myocardial fibrosis by mediating CD63-integrin β1 interaction

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Detailed Methods

Experimental Animals and surgical procedures
Wild type (WT) and TIMP1-deficient male mice (Timp1−/−) in C57BL/6 background, were purchased from Jackson Laboratories and bred in house in the Animal facility of University of Alberta. All animal experiments were carried out in accordance with Canadian Council on Animal Care Guidelines and regulations of Animal Care and Use Committee (ACUC) at University of Alberta.

Cardiac pressure overload was induced in male WT and TIMP1−/− mice (8-10 weeks age) by transverse aortic constriction as described previously.1, 2 Briefly, anesthetized and intubated mice underwent partial thoracotomy at the second rib, a blunt 27-gauge needle was placed next to the aortic arch between the left carotid artery and the brachiocephalic trunk, and a suture was then tied around the needle. The needle was quickly removed to resume blood flow through the aorta. This constriction consistently generates a pressure gradient of 55-60 mmHg. The skin was then sutured in layers and the mouse was allowed to recover on a heating pad. Sham-operated animals underwent the same procedure without the constriction of the aorta.

Male 8-9 weeks old mice of either genotype received Angiotensin II (Ang II, 1.5 mg/kg/day) or saline by Alzet micro osmotic pump (Model 1002, Durect Co.) implanted dorsally and subcutaneously (under 2% isoflurane anesthesia).3, 4 MMP inhibitor, PD166793 (30 mg/kg/day) was administered by daily gavage in parallel groups of WT and Timp1−/− throughout the 2 weeks of Ang II/saline infusion as before.5, 6

At the indicated time points, hearts were excised, frozen in OCT medium, or formalin-fixed and processed for immunohistochemical analyses. Alternatively, hearts were excised and flash-frozen for molecular analyses.

Cardiac function Assessment
Heart structure and function were assessed in post-sham/TAC hearts non-invasively by transthoracic echocardiography under anesthesia (1% isoflurane) using Vevo 3100 high resolution imaging system equipped with a 30-MHz transducer (Visual Sonics) as described before.1, 4, 7 Invasive hemodynamic and pressure-volume measurements were performed in anesthetized mice (2% isoflurane) post-TAC/sham using 1.2F Scisense catheter (TCP-500 Scisense Inc.).8 After baseline PV loop measurements, end diastolic (EDPVR) and end systolic (ESPVR) pressure volume relationship was derived by transient occlusion of inferior vena cava and infra renal aorta, respectively.

Human Explanted Heart Tissue
Heart tissues from heart failure patients were procured at the time of cardiac transplantation as part of the Human Explanted Heart Program (HELP) at the Mazankowski Alberta Heart Institute (Edmonton, AB), and healthy control hearts were obtained through the Human Organ Procurement and Exchange (HOPE) program (Edmonton, AB) as before.9 All experiments were approved by Institutional Ethics Committee and performed in accordance with the institutional guidelines with informed consent from all subjects.

Histological and Immunohistochemical Staining and Imaging
Freshly excised hearts were arrested in diastole (1M KCl) and fixed in 10% formalin, paraffin embedded and processed for trichrome, picrosirius red (PSR) and wheat glutamine staining (WGA)4, 7. Myocardial collagen deposition (PSR) and myocyte cross-sectional area (WGA-stained
sections) were quantified using Metamorph Basic software (version 7.7.0.0).\textsuperscript{4,7} OCT-frozen 5μm sections were used for co-immunostaining of CD63 (R&D Mab5417; NovusNBP2-32829) and integrin β1 (Millipore Mab1900), and immunostaining for scleraxis (ab58655).

**Proximity ligation assay**

Proximity ligation assay (PLA) detects a proximity of 40nm or less between two molecules. PLA was performed on formalin-fixed heart sections, or on cultured adult cardiac fibroblasts, to detect physical proximity of integrin β1 and CD63 using Duolink *in situ* fluorescence Sigma kit and PLA probes for mouse (DUO92004) and secondary, rabbit (DUO92002).

**RNA extraction and expression analysis**

Total RNA was extracted from frozen heart tissues using TriZol reagent (Invitrogen) and mRNA expression analysis was performed by TaqMan RT-PCR as before, and *Hprt* (hypoxanthine-guanine phosphoribosyltransferase-1) as the internal control. Taqman primers and probes for pro-collagen I-α1 and pro-collagen III-α1, *Timps* (-1 to -4) were used as before,\textsuperscript{1,3,4,7} and for scleraxis (*Scx*, Assay ID: Mm01205675_m1) purchased from ThermoFisher Scientific.

**Protein extraction, activity assay, Western blot and co-Immunoprecipitation**

Total protein was extracted using Sigma extraction buffer.\textsuperscript{10} Fluorescent based activity assays were performed using fluorescent-tagged gelatin (EnzCheck, Molecular Probes) to measure total gelatinase activity as before.\textsuperscript{4,7} Western blot analyses were performed by using the following antibodies: CD63 (Abcam, Ab193349), integrin β1 (Novus, NBP110-57123), p-Smad2/3 S465/467 & S423/425 (CST8828S), totalSMAD2/3 (CST3102S), p-β-catenin Tyr654 (SC57333), total-β-catenin (Ab79089), Histone (CST44995), Caspase3 (CST9665S) and SCX (ab58655).

For co-immunoprecipitation (Co-IP) experiments, protein extracts were prepared by homogenizing LV tissue (or 1X10\textsuperscript{6} fibroblasts) in 300μL of IP lysis buffer (Thermo Scientific). Lysates were centrifuged at 16,000 \times g for 15 minutes at 4°C. Pellet was discarded and the supernatant was precleared with Dynabeads protein G (Thermo Scientific). Anti-CD63 (Novus) or IgG isotype control (Santa Cruz) was cross-linked to Dynabeads protein G in Bissulfosuccinimidyld substrate (BS3) according to the manufacturer's directions. Precleared lysates were incubated with anti-p120 preabsorbed with Dynabeads protein G-antibody over night at 4°C under a continuous mixing. Pellets were collected after 4 washes with lysis buffer and mixed with 2×SDS buffer (0.2% bromophenol blue, 4% SDS, 100 mM Tris [pH 6.8], 200 mM DTT, 20% glycerol) in 1:1 ratio. Samples were boiled for 3 min and 30 μL was loaded onto a SDS-PAGE gel and proteins were transferred to a nitrocellulose membrane by electroblotting. Primary antibodies, anti-CD63 (Abcam) and anti-integrin β1 (Novus), were used to detect respective antigens. The labeled proteins were visualized using a chemiluminescence kit.

Nuclear and cytosolic protein fractionation was performed using hypotonic lysis buffer (10mM potassium-HEPES, 1.5mM MgCl\textsubscript{2}, 10mM KCl, 1mM Dithiothreitol (DTT) and 0.2mM Na\textsubscript{3}VO\textsubscript{4}) containing protease and phosphatase inhibitors. After centrifugation (100×g, 5 min), supernatant was collected and centrifuged again (10 min, 2000×g) to separate the nuclear protein (pellet) from the non-nuclear protein (supernatant). The pellet was suspended in 200μL hypotonic lysis buffer with 2.4M sucrose and centrifuged using ultra centrifuge 1000,000×g for 90 minutes to obtain the nuclear protein. Purity of the nuclear and the cytosolic protein were assessed by blotting for a nuclear-specific protein (Histone), and cytosole-specific protein (Caspase 3), respectively.
Adult cardiac fibroblast (cFB) Isolation and culture

Adult cFB were isolated from WT and Timp1<sup>−/−</sup> mice as described previously.<sup>4, 7</sup> Briefly, 10-11 weeks old mice were heparinized (0.05 mL i.p. injection of 1000 USP/L heparin) and anesthetized with 2% isoflurane. Heart was quickly excised and perfused with perfusion buffer at a constant flow (4 mL/minutes), after 10 minutes, perfusion buffer was replaced with digestion buffer (containing collagenase type 2, Worthington). Subsequently, LV was separated, and gently dissociated into small pieces in stopping buffer (perfusion buffer containing 10% FBS). Cardiac myocytes and fibroblasts were separated using differential centrifugation. Fibroblasts were cultured in DMEM/F12 media including 10% FBS. Fibroblasts were used at second passage, and serum-deprived for 24 hours prior to experiments. Fibroblasts from WT and Timp1<sup>−/−</sup> mice were treated with TGFβ (10 ng/mL) or Ang II (1 µm/mL) for 24 hours. Cells were then harvested and flash-frozen for co-IP experiments, or fixed in 4% paraformaldehyde (PFA) for immunostaining and PLA assay.<sup>4, 7</sup>
References


Table S1- Echocardiographic assessment of systolic and diastolic function in WT and Timp1-/- mice following sham or 2, 5 and 9 weeks of pressure overload (TAC). HR-Heart rate; E-wave= early transmitral inflow velocity; A-wave= transmitral inflow velocity due to atrial contraction; E'= Early tissue Doppler velocity; A'= Tissue Doppler velocity due to atrial contraction. E'/A'= Ratio of early Doppler velocity to the tissue Doppler velocity due to atrial contraction; E/E'=Ratio of early transmitral inflow velocity to early tissue doppler velocity, LVEF= Left ventricular ejection fraction; ET=Ejection time, LVID-D= Left ventricular internal diameter at the end of diastole; LVID-S= Left ventricular internal diameter at the end of Systole; LVPW-S= Left ventricular posterior wall thickness at the end of systole; LVPW-D= Left ventricular posterior wall thickness at the end of diastole. * p<0.05 compared to corresponding sham; § p<0.05 compared to the corresponding WT group.
Figure S1- TIMP1-deficiency did not alter MMP acivity and myocardial hypertrophy in response to Ang II

A) Gelatin Zymography

B) Gelatinase activity from WT and TIMP1-/- mice at 2 weeks post-Ang II

C) Heart weight-to-Tibial length ratio (HW/TL), D) mRNA expression of disease markers: Anp (i), Bnp (ii) and α-ske-actin (iii) between WT and Timp1-/- Sham and post-Ang II infusion (n=6-8/group).

C) mRNA expression levels for Timp1s in response to Ang II infusion in WT and Timp1-/- mice. n=5-6/group/genotype.

Data represent mean ± S.E.M. *p<0.05 compared to corresponding saline or sham.
Figure S2: A) Proliferation and migration of adult cardiac fibroblast (cFB) is not compromised by TIMP1-deficiency. Wound healing (scratch) assay on cFB treated with saline, 1 µM Ang II or 10ng/mL TGFβ1 showed a similar rate wound healing potency between WT and Timp1-/- cFBs. B) Expression of alpha-smooth muscle actin (αSMA), a myofibroblast marker, in the indicated groups. n=6-8/group/genotype. * p<0.05 compared to corresponding saline/sham. A.U= Arbitrary Units.
Figure S3: TIMP1-deficiency did not alter MMP acivity and myocardial hypertrophy myocardial hypertrophy following pressure overload. 

A) i) Gelatin Zymography ii) Gelatinase activity from WT and Timp1^-/- at 2 weeks post-TAC. 

B) Heart weight/Tibial length (HW/TL) (n= 10-12/group/genotype). 

C) mRNA expression levels for Timps in WT and Timp1^-/- mice from sham and 2 weeks post-TAC (n= 5-6/group). 

D) i) Heart weight/Tibial Length (HW/TL), ii) Pulmonary Edema (Lung wet-dry weight) (n= 10-12/group), and mRNA expression of hypertropic marker (Bnp) in WT and Timp1^-/- mice following 5 weeks of TAC or sham (n=5-6/group/genotype). 

E) mRNA expression levels for Timps in WT and Timp1^-/- mice following 5 weeks of TAC/sham (n=5-6/group/genotype). Data represent mean ± S.E.M. *p<0.05 compared to corresponding saline or sham.
Figure S4 - Expression of scleraxis (Scx) is increased in fibrotic myocardium, but not altered with TIMP1-deficiency.

A) Representative images of immunostaining for Scleraxis in myocardial specimens from patients with dilated cardiomyopathy and non-failing control hearts. 

B) mRNA expression, and immunostaining (C) for Scleraxis (Scx) in WT and Timp1^{−/−} hearts post-TAC/sham. D) Scleraxis protein levels in the nuclear and cytosolic protein fractions from WT and Timp1^{−/−} mice post-sham/TAC. Loading control is coomassie blue-stained gel of the corresponding immunoblot. A.U. = Arbitrary units. * p<0.05 vs. corresponding sham group; § p<0.5 vs. corresponding WT group.
**Figure S5**- Assessment of purity of nuclear and non-nuclear protein fractionations. Immunoblotting for a nuclear specific proteins (Histone) and a cytosol-specific protein (Caspase-3) was performed on nuclear and cytosolic protein fractions in representative post-sham and post-TAC samples from WT mice.
Figure S6- Timp1 deficiency resulted in improved heart functions, LV dilation and significantly less cardiac hypertrophy at 9 weeks post-TAC. A) Representative M-mode images from WT and Timp1⁻/⁻ hearts post-TAC and corresponding sham hearts. B) Echocardiographic parameters showing ejection fraction, left ventricular end-diastolic (LVIDd) and end-systolic diameter (LVIDs) left atrial, and diameter-to-body weight ratio (LA/BW) in WT and Timp1⁻/⁻ mice following sham (n=9) or TAC (n=10-12/group/genotype). C) Representative WGA staining images and measured cross sectional area from WT and Timp1⁻/⁻ mice at 9 weeks post-TAC.