Cardiac-restricted overexpression or deletion of tissue inhibitor of matrix metalloproteinase-4: differential effects on left ventricular structure and function following pressure overload-induced hypertrophy


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Yarbrough WM, Baicu C, Mukherjee R, Van Laer A, Rivers WT, McKinney RA, Prescott CB, Stroud RE, Freels PD, Zellars KN, Zile MR, Spinale FG. Cardiac-restricted overexpression or deletion of tissue inhibitor of matrix metalloproteinase-4: differential effects on left ventricular structure and function following pressure overload-induced hypertrophy. Am J Physiol Heart Circ Physiol 307: H752–H761, 2014. First published July 3, 2014; doi:10.1152/ajpheart.00063.2014.—Historically, the tissue inhibitors of matrix metalloproteinases (TIMPs) were considered monochromatic in function. However, differential TIMP profiles more recently observed with left ventricular (LV) dysfunction and matrix remodeling suggest more diverse biological roles for individual TIMPs. This study tested the hypothesis that cardiac-specific overexpression (TIMP-4OE) or deletion (knockout; TIMP-4KO) would differentially affect LV function and structure following pressure overload (LVPO). LVPO (transverse aortic constriction) was induced in mice (3.5 ± 0.1 mmHg, equal sex distribution) with TIMP-4OE (n = 38), TIMP-4KO (n = 24), as well as age/strain-matched wild type (WT, n = 25), whereby indexes of LV remodeling and function such as LV mass and ejection fraction (LVEF) were determined at 28 days following LVPO. Following LVPO, both early (7 days) and late (28 days) survival was ~25% lower in the TIMP-4KO group (P < 0.05). While LVPO increased LV mass in all groups, the relative hypertrophic response was attenuated with TIMP-4OE. With LVPO, LVEF was similar between WT and TIMP-4KO (48 ± 2% and 45 ± 3%, respectively) but was higher with TIMP-4OE (57 ± 2%, P < 0.05). With LVPO, LV myocardial collagen expression (type I, III) increased by threefold in all groups (P < 0.05), but surprisingly this response was most robust in the TIMP-4KO group. These unique findings suggest that increased myocardial TIMP-4 in the context of a LVPO stimulus may actually provide protective effects with respect to survival, LV function, and extracellular matrix (ECM) remodeling. These findings challenge the canonical belief that increased levels of specific myocardial TIMPs, such as TIMP-4 in and of themselves, contribute to adverse ECM accumulation following a pathological stimulus, such as LVPO.

left ventricular (LV) pressure overload (LVPO), which occurs secondary to aortic valve stenosis and/or systemic hypertension, causes myocyte hypertrophy and abnormal extracellular matrix (ECM) accumulation. While both of these cellular and extracellular processes represent crucial events in the progression of LVPO, past studies have demonstrated that the progressive changes in both the content and structure of the ECM secondary to LVPO directly affect LV function and clinical outcomes (2, 3, 8, 18, 22, 31). One pathway that contributes to ECM content and turnover is the expression and activation of the matrix metalloproteinases (MMPs), and the relationship to the endogenous tissue inhibitors of MMPs (TIMPs) (5–7, 27). TIMPs were initially identified to bind to active MMPs, and hence these small-molecular-weight proteins were considered to function primarily through inhibiting ECM degradation (6). In clinical studies of LVPO, increased TIMP levels have been associated with reduced ECM turnover and the degree of LV dysfunction (2, 31). However, there are four known TIMPs, and there is emerging evidence that TIMPs are multifunctional proteins, each with unique characteristics (5–7, 16, 17, 19, 27). For example, past studies have identified that different TIMPs impart differential effects on myocardial fibroblast growth, viability, and phenotype in vitro (19). Of potential relevance in the context of LVPO is that of TIMP-4, which has been shown to be increased in patients secondary to LVPO and developing LV failure (2, 37). However, whether the relative increase in TIMP-4 levels, which has been observed in these past clinical studies as well as animal models of LVPO (34), can be considered a compensatory response or an adverse molecular event remains unknown. Accordingly, the guiding hypothesis of the present study was that TIMP-4 overexpression or deletion would impart differential effects on LV structure and function with LVPO.

METHODS

This study employed a chronic murine model of LVPO achieved through permanent transverse aortic constriction, a technique previously demonstrated to achieve peak aortic gradients approximating 90 mmHg and significant LV hypertrophy (14, 36). LVPO was applied to three groups of mice: 1) wild-type (WT) mice (n = 25), 2) transgenic mice that overexpressed the human TIMP-4 gene in a cardiac-restricted fashion (n = 38) (TIMP-4OE), and 3) mice with transgenic deletion of TIMP-4 (TIMP-4KO) (n = 24). All of these mice were of the same background strain (FVB) and of equivalent age (3.5 ± 0.1 mo) and sex distribution at study entry. The main response variables were survival and LV geometry and function at 28 days post-LVPO induction. Secondary measurements were surrogates of ECM remodeling, including collagen volume fraction, collagen expression, MMP/TIMP mRNA profiles, and immunohistochemistry. All animals were
were obtained from these mouse preparations (26, 36). RNA (1 μg) was reverse transcribed (iScript cDNA Synthesis Kit, Bio-Rad). Real-time PCR was performed using specific primer/probe (TaqMan Universal PCR Master Mix, Applied Biosystems, Foster City, CA) sets as follows: murine TIMP-4 (cat. no. Mm001184414_m1), human TIMP-4 (cat. no. Hs00162784_m1), murine TIMP-1 (cat. no. Mm00441818_m1), murine TIMP-2 (cat. no. Mm00441825_m1), murine TIMP-3 (Mm00418277_m1), murine Collagen IA1 (cat. no. Mm00801666_g1), mouse Collagen IIIA1 (cat. no. Mm01254476_m1), murine MMP-2 (cat. no. Mm00439498_m1), murine MMP-9 (cat. no. Mm00442991_m1), murine MMP-13 (cat. no. Mm00439491_m1), and murine MMP-14 (cat. no. Mm00485054_m1), and 18s RNA (cat. no. 4333760F). Gene expression levels were determined using the ΔCt method with normalization to the 18s Ct values.

Data analysis. Survival data were analyzed using a standard Kaplan-Meier analysis (log-rank test). Values pertaining to LV geometry and function, collagen content, and mRNA expression were compared using ANOVA whereby pairwise analyses were performed with a Bonferroni adjusted t-test. Statistical analyses were performed with statistical software programs (STATA, version 8, Intercooled; College Station, TX). Data are presented as means ± SE, and values of $P < 0.05$ were considered statistically significant.

RESULTS

Survival. The majority of animal attrition occurred within 7 days following induction of LVPO (Fig. 1), whereby attrition was greatest in the TIMP-4KO group compared with either WT or TIMP-4OE. The long-term (28 days) survival was also lower in the TIMP-4KO group compared with the TIMP-4OE group. While the sex was equivalent at study entry in all strains, significant differences in sex distribution were observed in those mice surviving LVPO (Table 1). Specifically, a lower relative percentage of female mice survived LVPO in the WT and TIMP-4KO groups, whereas a higher percentage of female mice survived LVPO in the TIMP-4OE group.

LV geometry and function. LV function and geometry under ambient referent control conditions for WT, TIMP-4KO, and TIMP-4OE are summarized in Table 1, which revealed no phenotypic differences between groups. With LVPO, significant LV hypertrophy as measured by LV wall thickness and mass (normalized to either body weight or tibial length) increased in all groups (Table 1). However, specific differences were observed in the TIMP-4KO and TIMP-4OE groups.

Fig. 1. Induction of left ventricular (LV) pressure overload (LVPO) caused an early (<7 day) attrition in all groups but was lower in the tissue inhibitor of matrix metalloprotease (TIMP)-4 knockout (TIMP-4KO) group compared with wild-type (WT) or the TIMP-4 overexpression (TIMP-4OE) group (log-rank survivor function, $P = 0.002$). Longer term survival, defined as 28 days, was also reduced in the TIMP-4KO group compared with TIMP-4OE (Kaplan-Meier analysis, $P = 0.023$).
Specifically, the relative degree of hypertrophy was greatest in the TIMP-4KO group and lowest in the TIMP-4OE group. At the myocyte level, the cross-sectional area increased in all groups following LVPO but was highest in the TIMP-4KO group (Table 1). In the TIMP-4OE group, heart rate was lower and LV ejection fraction fraction higher following LVPO. While LV end-diastolic volume remained unchanged from respective baseline values in the WT and TIMP-4OE groups following LVPO, this parameter increased in the TIMP-4KO group.

Fibrillar collagen content. Representative LV sections stained for fibrillar collagen are shown in Fig. 2A and quantitative measurements in Fig. 2B. No differences in relative collagen content were observed under referent control, baseline conditions, but a robust increase occurred in all groups following LVPO. However, following LVPO, total fibrillar collagen was lower in the TIMP-4OE group compared with respective WT values. Representative photomicrographs for α-smooth muscle actin (SMA) staining in control conditions and following LVPO are shown in Fig. 3A. Punctate staining, particularly within the interstitial space, was readily identifiable following LVPO in the WT and TIMP-4KO groups but appeared reduced in the TIMP-4OE group. SMA quantification of positive stained cells revealed a significant increase following LVPO in the WT and TIMP-4KO group. In marked contrast, SMA staining was lower following LVPO in the TIMP-4OE group (Fig. 3B). TIMP-4 immunohistochemistry revealed a strong immunoreactive signal in the TIMP-4OE sections in control and LVPO conditions (Fig. 3A). This staining was most pronounced in cardiac myocytes but could be also identified along the sarcolemma surface. Immunostaining in the TIMP-4KO group was identical to negative control sections, i.e., devoid of a positive immunoreactive TIMP-4 signal.

PCR measurements. With LVPO, relative collagen type I and III mRNA expression increased in all groups following LVPO (Fig. 2C), lowered in the TIMP-4OE group compared with WT values, and were higher in the TIMP-4KO group. Human TIMP-4 mRNA levels were detected only in the TIMP-4OE group, consistent with this transgenic construct (Fig. 4A). Mouse specific TIMP-1 (mTIMP-1) and TIMP-2 (mTIMP-2) increased in the WT group following LVPO (Fig. 4B). However, mTIMP-1 levels were lower in the TIMP-4OE and higher in the TIMP-4KO groups compared with respective WT values. In the TIMP-4OE group, respective mTIMP-2 levels were lower than WT values following LVPO. Under control conditions, mTIMP-3 levels were lower in the TIMP-4OE group and higher in the TIMP-4KO group. Following LVPO, no directional changes in mTIMP-3 levels were observed. In marked contrast, mTIMP-4 levels fell by ~50% in the WT group following LVPO (Fig. 4B). In the TIMP-4OE group, mTIMP-4 levels were lower than respective WT values.
and as expected, nondetectable in the TIMP4-KO groups. MMP mRNA levels for representative MMP types from the gelatinase (MMP-2, MMP-9), collagenase (MMP-13), and membrane type (MMP-14) subfamilies were examined (Fig. 5). LVPO increased MMP-2 in all groups but was highest in the TIMP-4OE group. MMP-9 levels fell in the WT and TIMP-4OE groups following LVPO. In the TIMP-4KO group, MMP-9 levels were reduced under control, baseline conditions and were unchanged from these values following LVPO. MMP-13 fell in the WT and TIMP-4OE groups following LVPO, and remained unchanged from baseline values in the TIMP-4KO group. MMP-14 levels increased in all groups following LVPO.

DISCUSSION

Changes in ECM structure and function have been recognized as important events in the progression of LV remodeling and dysfunction in a number of cardiovascular disease states, such as that of ischemia, LVPO, and idiopathic underpinnings (8, 16, 17, 24, 27, 29, 34, 36, 37). For example, in ischemia/infarction an imbalance between MMPs and TIMPs has been identified, which would favor ECM proteolysis and thereby contribute to changes in myocardial geometry and function (23, 27). Conversely, in both experimental and clinical observations of LVPO, increased TIMP levels have been identified, which in turn have been postulated to favor a reduction in ECM turnover, contributing to myocardial fibrosis and LV dysfunction (2, 31, 34, 37). These observations have resulted in a somewhat monochromatic view of the functionality of myocardial TIMPs in that low levels will result in myocardial ECM degradation/instability, whereas increased TIMPs would favor ECM accumulation/fibrosis. However, the four known mammalian TIMPs, which are the products of distinct genes with very different sequences, are likely to display unique functionality beyond MMP inhibition (5, 6). For example, comparative studies in vitro have identified that TIMP-4 can affect fibro-
blast transdifferentiation and vascular smooth muscle cell apoptosis independent of MMP inhibitory effects (13, 19). However, much less is known about the in vivo effects of TIMP-4 in terms of a pathological stimulus, such as LVPO. Accordingly, LVPO was induced in mice with an identical background strain, whereby gene deletion of TIMP-4 (TIMP-4KO) and cardiac-restricted overexpression of TIMP-4 (TIMP-4OE) were utilized. The key findings of this study were twofold. First, in TIMP-4KO mice, LVPO caused a reduction in early survival, LV dilation, and a more robust fibrotic response. Second, in TIMP-4OE mice, survival and function were improved following LVPO and were accompanied by a more muted fibrotic response. These unique findings suggest that the induction of TIMP-4 with a LVPO stimulus may actually be beneficial and provide further evidence for the disparate effects of different TIMP types in terms of LV myocardial remodeling.

**Differential effects of TIMP-4 on survival and function with LVPO.** The induction of an identical LVPO stimulus resulted in differential effects on survival whereby TIMP-4KO reduced short-term survival and TIMP-4OE yielded similar survival to wild-type mice. Moreover, LV dilation was more profound in the TIMP-4KO group compared with TIMP-4OE or wild-type mice. In a previous study by Koskivirta and colleagues (17), LVPO as well as myocardial infarction were performed in TIMP-KO mice, whereby the most pronounced effects of TIMP-4 gene deletion was following myocardial infarction. While not the primary focus of this past study, there was a relative reduction in early survival following LVPO in the TIMP-4KO mice, not dissimilar to that observed in the present study.

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**Fig. 3.** **A, left:** representative photomicrographs for alpha smooth muscle actin (SMA) staining in control conditions and following LVPO. Punctate staining (arrows), particularly within the interstitial space, was readily identifiable following LVPO in the WT and TIMP-4KO groups but appeared reduced in the TIMP-4OE group. **A, right:** TIMP-4 immunohistochemistry revealed a strong immunoreactive signal in the TIMP-4OE sections in control and LVPO conditions. This staining was most pronounced in cardiac myocytes but could be also identified along the sarcolema surface (arrows; far right panels). Immunostaining in the TIMP-4KO group was identical to negative control sections, i.e., devoid of a positive immunoreactive TIMP-4 signal. **B:** SMA quantification of positive stained cells revealed a significant increase following LVPO in the WT and TIMP-4KO group. In marked contrast, SMA staining was lower following LVPO in the TIMP-4OE group. #P < 0.05 vs. no LVPO, same strain; *P < 0.05 vs. WT, same condition; ++P < 0.05 vs. TIMP-4OE, same condition.
In the TIMP-4OE group, relative mTIMP-4 levels were lower than respective WT values at baseline and with LVPO. BD, below threshold. In directional contrast to the other TIMP types, mTIMP-4 levels fell in the WT group following LVPO. Under baseline conditions, mTIMP-3 levels were reduced in the TIMP-4KO group and were increased in the TIMP-4KO group from respective LVPO, whereby the increase in these TIMP types was blunted in the TIMP-4OE group. In contrast, mTIMP-1 levels were higher in the TIMP-4KO group.

B: mouse specific TIMP (mTIMP) mRNA levels were measured for all TIMPs. LVPO caused an increase in mTIMP1 and mTIMP-2 in WT mice following LVPO, whereas this effect appeared reversed in the TIMP-4OE group following LVPO. Under baseline conditions, mTIMP-3 levels were reduced in the TIMP-4KO group and were increased in the TIMP-4KO group from respective WT values under control conditions and following LVPO. In directional contrast to the other TIMP types, mTIMP-4 levels fell in the WT group following LVPO. In the TIMP-4OE group, relative mTIMP-4 levels were lower than respective WT values at baseline and with LVPO. BD, below threshold. #P < 0.05 vs. no LVPO, same strain; *P < 0.05 vs. WT, same condition; +P < 0.05 vs. TIMP-4OE, same condition.

Fig. 4. A: human TIMP-4 mRNA levels were detected only in the TIMP-4OE group, consistent with this transgenic construct, which fell following LVPO. B: mouse specific TIMP (mTIMP) mRNA levels were measured for all TIMPs. LVPO caused an increase in mTIMP1 and mTIMP-2 in WT mice following LVPO, whereby the increase in these TIMP types was blunted in the TIMP-4OE group. In contrast, mTIMP-1 levels were higher in the TIMP-4KO group following LVPO. Under baseline conditions, mTIMP-3 levels were reduced in the TIMP-4KO group and were increased in the TIMP-4KO group from respective WT values under control conditions and following LVPO. In directional contrast to the other TIMP types, mTIMP-4 levels fell in the WT group following LVPO. In the TIMP-4OE group, relative mTIMP-4 levels were lower than respective WT values at baseline and with LVPO. BD, below threshold. #P < 0.05 vs. no LVPO, same strain; *P < 0.05 vs. WT, same condition; +P < 0.05 vs. TIMP-4OE, same condition.

study. However, this past study was performed on a different background strain and with a different magnitude of PO stimulus, which make direct comparisons on the effects of LVPO difficult. For example, the pressure gradient in the study by Koskivirta and colleagues by aortic constriction was 60–70 mmHg (17), whereas the target pressure gradient of the present study was 90 mmHg. Nevertheless, the present study extended the work from this past report by examining the comparative effects of TIMP-4 deletion as well as overexpression. Another finding from the present study was the sex-specific differences in survival following LVPO. In the WT group and TIMP-4KO groups, a lower relative survival was observed for female mice following LVPO, whereas this sex effect appeared reversed in the TIMP-4OE mice. Past studies have identified that sex-specific differences in myocardial growth and expression of matrix-related proteins occur following LVPO in mice (9, 25, 32). For example, a higher relative expression of TIMP-2 has been reported previously in male mice following LVPO (32). In the present study, relative mRNA levels for fibrillar collagens, TIMP-1, and TIMP-2 were the highest in the WT and TIMP-4OE groups following LVPO, which was associated with increased survival in male mice. In contrast, these determinants of matrix remodeling were lower in the TIMP-4OE mice following LVPO and in turn associated with higher relative survival in female mice. While the experimental design of the present study was balanced for age and sex at the time of LVPO, it was not designed to address the effects of sex as a confounding effect. Thus, while the present study further underscores potential sex-dependent differences with LVPO and possible interactions with transgenic modification of TIMP-4, these findings remain associative and the contributory mechanisms for these sex-dependent effects remain to be established. While the specific mechanism(s) for the reduction in survival in the TIMP-4KO mice following LVPO remains to be established, it is possible that the loss of TIMP-4 following the immediate induction of LVPO would have favored increased ECM proteolysis via a relative reduction in MMP activity. Despite a uniform LVPO stimulus, the magnitude of the hypertrophic response appeared greatest in the TIMP-4KO group, which was accompanied by the greatest degree of LV dilation and systolic dysfunction. In contrast, TIMP-4OE caused a reduction in LV hypertrophy, increased systolic function, and a preservation of LV end-diastolic volume. There are likely mechanical and biochemical factors that contributed to these observations. First, the loss of TIMP-4 and resultant LV dilatation would in turn cause increased LV wall stress, a potent stimulus for myocardial growth. In contrast, the lower LV volumes in the TIMP-4OE mice with LVPO would reduce
this mechanical stimulus and hence compensatory myocardial growth. Second, different TIMPs affect cell growth and viability in unique ways (5, 7, 13, 19, 21), and the present study identified that modifying TIMP-4 expression in turn affected expression of other TIMPs. For example, a marked increase in TIMP-1 expression was observed following LVPO in the TIMP-4KO mice, whereby TIMP-1 has been shown to accelerate growth in several cell lines and systems as well as inhibit apoptosis (5, 7, 19, 30). Thus TIMP-4KO may have amplified the growth effects of TIMP-1 in the context of LVPO.

Differential effects of TIMP-4 on TIMP, MMP, and fibrillar collagen expression. As expected, the induction of LVPO was associated with a robust increase in total fibrillar collagen content. What was not expected is the effects on this index of ECM remodeling with respect to modulating TIMP-4 with LVPO. Specifically, fibrillar collagen content and in particular mRNA levels were actually lower with TIMP-4OE following LVPO. In marked contrast, fibrillar collagen mRNA levels were highest following LVPO in the TIMP-4KO group. Past studies have identified that TIMP-4, unlike other TIMPs such as TIMP-2, do not provoke a robust increase in ECM synthesis in isolated cardiac fibroblast cultures (19). While not specific, a common approach for measuring activated fibroblasts, commonly termed myofibroblasts, is by examining α-smooth muscle actin (SMA) (11, 20). In the present study, LVPO caused an increase in SMA positive cells in both the WT and TIMP-4KO groups but was blunted in the TIMP-4OE group. These findings coupled with the past in vitro findings support the concept that TIMP-4 may actually attenuate the profibrotic response, a structural hallmark for adverse ECM remodeling in the context of LVPO.

The present study identified that transgenic modification of TIMP-4 levels did not occur in isolation and was associated with changes in the expression levels of other TIMPs. Specifically, TIMP-4 deletion caused an increase in other respective TIMPs with a profound increase in TIMP-1 expression following LVPO. TIMP-1 inhibits a number of MMPs, and increased TIMP-1 levels have been identified in the myocardium as well as in plasma of patients with LVPO and has been associated with poor prognosis (2, 3, 30, 37). Thus the compensatory increase in TIMP-1 levels in the TIMP-4KO mice may have contributed to the LV phenotype following LVPO. In support of this postulate, the present study demonstrated that TIMP-4OE caused a relative reduction in TIMP-1 expression, which in turn was associated with improved survival and myocardial remodeling. While the pathways responsible for the feedback effects of TIMP type expression with TIMP-4 remains to be established, the findings of the present study suggest that myocardial expression of TIMP-4 may regulate other TIMP types relevant to ECM remodeling in the context of LVPO. Another important observation from the TIMP expression studies was that relative native (murine) TIMP-4 expression fell in the WT mice with LVPO, which was in contradistinction to any of the other TIMP types. While not statistically significant, a similar trend for a reduction in murine TIMP-4 mRNA levels was observed in the TIMP-OE group following LVPO. What
was not expected was the relative reduction in human TIMP-4 levels following LVPO in the TIMP-4OE group. Since TIMP-4OE was based upon the cardiac myosin promoter, a relative reduction in the activity of this promoter at the later stages of LVPO may have occurred which in turn would reduce relative human TIMP-4 mRNA levels. Nevertheless, a robust induction at the mRNA level for human TIMP-4 as well as the protein level (histochemistry) occurred in the TIMP-4OE group, which in turn altered the natural history of LVPO in terms of survival, LV geometry, and determinants of matrix remodeling. While remaining associative, these observations would suggest that TIMP-4 expression may actually play a positive role in the context of LVPO and myocardial remodeling.

Overall matrix proteolytic activity is dependent upon a number of transcriptional, posttranscriptional, and posttranslational factors (5–7, 27). The present study focused primarily on transcriptional changes in terms of steady-state levels of TIMPs and MMPs, and therefore the interpretation of these results to that of overall matrix remodeling following LVPO must be tempered. In the present study, relative MMP-2 mRNA levels increased in all groups following LVPO, and this directional change is similar to that of plasma MMP-2 protein levels in patients with hypertensive heart disease (37). Following LVPO, the highest MMP-2 mRNA levels were in the TIMP-4OE group, but whether and to what degree this contributed to the net reduction in total fibrillar collagen content remains to be established. With respect to MMP-9 mRNA levels, the net reduction following LVPO in the WT and TIMP-4OE groups is not without precedent. Specifically, in animals with LVPO and in patients with hypertension and heart failure, relative MMP-9 levels either remained unchanged or were reduced from referent control values (2, 24, 37). However, other studies have identified a robust increase in MMP-9 levels following LVPO in mice (10, 15). Since MMP-9 is most associated with an inflammatory response, and an important product of inflammatory cells such as macrophages (33), then the time point of measurement as well as the relative magnitude of the LVPO stimulus are likely to contribute to these changes in relative MMP-9 levels. With respect to MMP-13 mRNA levels, a relative reduction was observed in the WT and TIMP-4OE groups following LVPO. A past report in patients with hypertensive heart disease reported a similar directional fall in plasma MMP-13 protein levels relative to referent control values (2). However, the relative changes in MMP-9 and MMP-13 mRNA levels that were observed following LVPO in the present study did not occur in the TIMP-4KO mice. Specifically, MMP-9 mRNA levels were reduced under baseline conditions in the TIMP-3KO group and did not change from these values with LVPO. MMP-13 mRNA levels remained unchanged from baseline values in the TIMP-4KO group following LVPO. While specific effects of these differential changes in MMP-9 and MMP-13 levels may have played upon overall myocardial matrix remodeling, these findings further support the postulate that significant cross talk occurs between specific patterns of TIMP expression and subsequent MMP induction.

Finally, MMP-14 mRNA levels were uniformly increased following LVPO in all groups, suggestive that the regulation of this MMP type may not be influenced by changes in TIMP-4 expression. However, unlike other TIMPs such as TIMP-1, which is a poor inhibitor of the MMP-14 (4, 21), TIMP-4 is a potent inhibitor of this MMP type. A recent study by this laboratory demonstrated that MMP-14 overexpression reduced survival in a murine LVPO model (36). Since MMP-14 is a potent proteolytic enzyme and activates other MMP types (5, 27), and MMP-14 mRNA levels were increased in all groups following LVPO, the unopposed effects of this MMP type may have caused enhanced ECM degradation and instability early following LVPO in the TIMP-4KO mice, resulting in early mortality and LV dilation. However, past studies from this laboratory have also identified that in addition to facilitating ECM degradation, MMP-14 can facilitate the activation of profibrotic signaling molecules, such as transforming growth factor with LVPO, and hence can amplify myocardial matrix accumulation (26, 36). Since TIMP-4 is a potent inhibitor of MMP-14 (4, 5, 6, 21), the relative MMP-14/TIMP-4 mRNA stoichiometry in the TIMP-4OE mice may have favored an overall reduction in MMP-14 activity, and hence the profibrotic pathways induced by this MMP type. However, the relationship between MMP-14 induction and the potential interaction with TIMP-4 in the context of LVPO remains associative, and future studies are necessary.

Study limitations and conclusions. The present study utilized transgenic models to modify TIMP-4 and as such holds inherent limitations. Specifically, TIMP-4 gene deletion was a global deletion, and while this study and past studies have failed to identify any developmental/phenotype abnormalities in this construct in the absence of a pathological stimulus (17), this approach lacks temporal and spatial specificity relative to the LVPO stimulus. In addition, this study examined specific aspects of LV remodeling and MMP/TIMP at 1 mo following LVPO. Thus, to what degree early interactions with key inflammatory mediators, such as cytokines and macrophages to that of TIMP-4 genetic modulation, may have influenced the changes in survival following the induction of LVPO remains to be established. In terms of the cardiac-restricted TIMP-4 construct, since this is driven on cardiac muscle specific promoter, increased expression in other relevant myocardial cell types such as fibroblasts was unlikely to occur. Our immunohistochemistry identified robust levels of TIMP-4 along the sarcolemma of myocytes and henceforth likely release into the interstitial space. As with other TIMPs, TIMP-4 is synthesized and released into the ECM, and thus it is likely that nonmyocyte cell types were exposed to high levels of TIMP-4. However, future studies that drive TIMP-4 expression specifically in fibroblasts with the superimposition of LVPO would be an important future direction.

Both experimental and clinical studies have identified that ECM remodeling, most notably increased fibrillar collagen (i.e., fibrosis), represents a maladaptive structural milestone in the development of heart failure secondary to LVPO (8, 31, 34, 36). Moreover, a recent clinical investigation demonstrated that the increasing degrees of myocardial fibrosis that occur secondary to LVPO were associated with reduced survival despite elimination of the pressure overload state (3). However, it is likely that TIMPs affect a number of processes over and above that of fibrillar collagen degradation/accumulation (5–7, 19, 27). For example, TIMP-3 alters key metalloproteases associated with cytokine processing and signaling (6, 16). Thus, based upon the findings from the present study, future studies that expand the scope of the potential effects of TIMP-4 in terms of cytokine signaling and fibroblast phenotype and the
 relation to ECM remodeling would be appropriate. Nevertheless, the results from the present study challenge the canonical concept that TIMPs regulate ECM remodeling in a predictable fashion in terms of stoichiometric balance to MMPs. The present study puts forth the unique concept that the induction of certain TIMPs, such as TIMP-4, may actually be beneficial in modulating adverse ECM remodeling in the context of LVPO.

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AUTHOR CONTRIBUTIONS
Author contributions: W.M.Y. and F.G.S. conception and design of research; W.M.Y. analyzed data; W.M.Y. and F.G.S. interpreted results of experiments; W.M.Y. and F.G.S. drafted manuscript; W.M.Y., C.F.B., R.M., W.M.Y. and F.G.S. interpreted results of the Department of Veterans Affairs.

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