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Review article

TIMPs and cardiac remodeling: 'Embracing the MMP-independent-side of the family'

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ABSTRACT

Unraveling the biological role of tissue inhibitors of metalloproteinases (TIMPs) during cardiac remodeling and the progression of heart failure has proven to be an enormous challenge. Remodeling of the cardiac extracellular matrix (ECM), regulated by matrix metalloproteinases (MMPs) and their endogenous inhibitors, TIMPs, is a well-established paradigm in cardiac health and disease. Originally, TIMPs were thought to function exclusively as endogenous inhibitors of MMP activity, thereby fine-tuning MMP-mediated ECM degradation and numerous related processes. However, during the last two decades, the concept of MMP-independent TIMP-mediated receptor signaling and regulation of cell fate has emerged. Although our current knowledge is still limited, in this review, we highlight some of the novel data, illustrating the MMP-independent biological properties of the four TIMP family members. Moreover, we discuss how these cell-specific insights may contribute to the process of cardiac remodeling, disease and failure. Finally, we identify where additional research is needed that will codetermine the possible future of TIMPs as therapeutic targets.

Contents

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1. Introduction

Myocardial extracellular matrix (ECM) remodeling is known to play an important role in the development of ventricular dilatation and heart failure [1,2]. Among many proteases present in the cardiac ECM, matrix metalloproteinases are the driving force behind myocardial matrix degradation during cardiac remodeling (Table 1). All cell types present in the myocardium, either under basal conditions or in response to an inflammatory stimulus, express one or more types of MMP-species [1–4]. MMPs are secreted as latent zymogens termed pro-MMPs, which must be activated by proteolytic cleavage of the amino terminal propeptide domain, thereby exposing the Zn^{2+}-binding site in the catalytic domain, termed the "cysteine switch" (reviewed in [1] and [2]). Besides matrix degradation, the established roles for MMPs include the release of growth factors from the ECM, cleavage of growth factor receptors from the cell surface, shedding of cell adhesion molecules, and activation of other MMPs, and thereby modulating numerous molecular and cellular processes that are crucial during cardiac remodeling in health and disease (Table 1). Increased MMP activity may thus result in fibrillar collagen degradation, an increased inflammatory response, ECM remodeling and progressive ventricular dilatation and dysfunction. To date, 25 members of the MMP-family have been identified [1], however, it is important to mention that only the MMPs that were evaluated in the heart are listed in Table 1.

Because MMPs degrade various components of the ECM, a tight regulation of their activity is essential to maintain a normal structure and function of the heart. A group of four endogenous proteins, tissue inhibitors of matrix metalloproteinases (TIMP-1, -2, -3 and -4), firmly regulate MMP activity (Table 2) [1,2,5]. TIMPs bind to the active site of the MMPs in a stoichiometric 1:1 molar ratio, thereby blocking access to their extracellular substrates. The low level of sequence homology between the genes encoding the four TIMP-species, indicates a potentially distinct and unique biological role for each TIMP (Table 2) [5]. TIMP-2 is a unique member of the TIMP family as, in addition to inhibiting MMPs, it selectively interacts with membrane type 1-MMP (MT1-MMP) at the cell surface, which ultimately leads to the activation of the bound pro-MMP-2 molecule. And while TIMP-1, -2 and -4 remain soluble and diffusible, TIMP-3 is unique in that it interacts with the ECM [6], suggesting that TIMP-3's pericellular distribution, bioavailability and effects may be more localized, potent and prolonged than its counterparts. By comparison, relatively little is known about the last member of the TIMP family, TIMP-4, also known as cardiac inhibitor of MMPs [5].

The discovery of the TIMP family has been an enormous breakthrough in the field of ECM metabolism and cardiac remodeling [1,2]. As shown in Tables 1 and 2, several genetic studies clearly demonstrated that any deviation from the delicate balance between MMP and TIMPs influences the pathogenesis of cardiovascular diseases, including myocardial infarction[7–12], viral myocarditis [13,14], dilated cardiomopathy [15,16] and pressure-overload induced heart failure [17–19]. It has long been presumed that the regulatory *in vitro* and *in vivo* effects of TIMPs were solely mediated by their MMP-inhibitory activity. However, research over the last two decades has shown that all four members of the TIMP family not only function to inhibit MMPs, but also display 'cytokine-like' properties that are just beginning to be fully characterized (Fig. 1). Therefore, in this review, we highlight some of the novel data regarding the non-MMP-inhibitory functions of TIMPs and discuss how these insights will codetermine the successful application of TIMP-based therapeutics during the process of cardiac remodeling, disease and failure. In addition we discuss a number of areas in which knowledge is lacking and further research is needed.

2. TIMPs as MMP-independent mediators of cellular behavior in the heart

TIMPs have a much broader spectrum of targets than originally believed, which have misled our understanding of TIMPs in cardiac remodeling. It has become increasingly clear that MMP-dependent and -independent mechanisms of TIMPs co-exist, adding to the complexity of their biological role. Mounting evidence points towards an authentic signaling capacity for TIMPs distinct from their MMP-inhibitory activity, that seem to play an important role in the regulation of apoptosis, cell survival, growth, migration, differentiation, angiogenesis, inflammation and overall ECM remodeling, and may play a central role in the process of cardiac remodeling (Fig. 1).

Physiological and pathological myocardial remodeling, induced by physical stress, ischemia or infection, is a phenomenon resulting from complex but highly regulated interactions between fibroblasts, smooth muscle cells, endothelial cells, infiltrating inflammatory cells and cardiomyocytes, all contributing to the composition and architecture of the cardiac interstitium [1,2]. Therefore, the remainder of this review will primarily focus on the MMP-independent biological functions of TIMPs and their effects on the main cell types contributing to the remodeling of the adult heart (Fig. 1). In addition, we identify where further study is necessary to advance our basic

Table 1
Matrix matalloproteinases and cardiac remodeling.

MMPs	Cellular source	Mouse model	Cardiac phenotype	Ref.
Collagenases				
MMP-1	Fibroblasts, endothelial cells, VSMC	MMP-1-TG	Spontaneous LV hypertrophy at 6 months and dilatation at 12 months of age	[16]
MMP-8	Neutrophils, VSMC	MMP-8$^{-/-}$, VM	No phenotype observed	[13]
MMP-13	Fibroblasts	ND	ND	[2]
Gelatinases				
MMP-2	Macrophages, T- and B-lymphocytes fibroblasts, VSMC, cardiomyocytes	MMP-2$^{-/-}$, MI	Decreased inflammatory response, slower wound healing, decreased LV dilatation and cardiac rupture	[7]
MMP-9	Neutrophils, macrophages, T-and B-lymphocytes fibroblasts, VSMC, cardiomyocytes	MMP-9$^{-/-}$, MI	Decreased inflammatory response and collagen deposition, increased angiogenesis, decreased LV dilatation and cardiac rupture	[8,9]
		MMP-9$^{-/-}$, TAC	Reduced hypertrophic response, fibrosis and preserved cardiac function	[17]
		MMP-9$^{-/-}$, VM	Increased inflammatory response, fibrosis and preserved cardiac output	[13]
Stromelysins				
MMP-3	Fibroblasts, VSMC, cardiomyocytes	MMP-3$^{-/-}$, MI	No phenotype observed	[8]
MMP-7	Macrophages, cardiomyocytes	MMP-7$^{-/-}$, MI	Decreased connexin 43 processing, decreased arrhythmia, and improved survival	[10]
Membrane-type MMPs				
MT1-MMP	Fibroblasts, VSMC and cardiomyocytes	ND	ND	[2]

MMP, matrix metalloproteinase; VSMC, vascular smooth muscle cells; TG, transgene mouse (transgene MMP-1 expression in mouse); MI, myocardial infarction; LV, left ventricle; VM, viral myocarditis; TAC, trans aortic constriction; ND, not done.

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Table 2
Tissue inhibitors of matrix matalloproteinases and cardiac remodeling.

TIMPs	Cellular source	Mouse models	MMP-target/Cardiac phenotype	Ref.
TIMP-1	Fibroblasts, macrophages, endothelial cells, VSMC and cardiomyocytes		Inhibits all MMPs, except MMP-2 and MT1-MMP	
		TIMP-1$^{-/-}$, MI	Increased hypertrophic response, reduced fibrillar collagen content and adverse LV remodeling	[11]
		AdTIMP-1, TAC	Reduced hypertrophic response, fibrosis, dilatation and dysfunction	[18]
		AdTIMP-1, VM	Decreased cardiac inflammation, necrosis, fibrosis, LV dilatation and dysfunction	[14]
TIMP-2	Fibroblasts, macrophages, endothelial cells, VSMC, cardiomyocytes		Inhibits all MMPs, except MMP-9; activates pro-MMP-2	[2]
		ND	ND	
TIMP-3	Fibroblasts, VSMC, cardiomyocytes		Inhibits MMP-1, -2, -3, -9 and -13	
		TIMP-3$^{-/-}$	Spontaneous hypertrophic response, LV dilatation and contractile dysfunction	[15]
		TIMP-3$^{-/-}$, MI	Reduced fibrosis, increased proliferation and apoptosis, angiogenesis, LV dilatation and mortality	[12]
		TIMP-3$^{-/-}$, TAC	LV dilatation and dilated cardiomyopathy	[19]
TIMP-4	Fibroblasts, VSMC and cardiomyocytes		Inhibits MMP-1, -3, -7 and -9	[2]
		ND	ND	

TIMP, tissue inhibitor of matrix metalloproteinases; VSMC, vascular smooth muscle cell; AdTIMP, adenoviral mediated TIMP overexpression; MI, myocardial infarction; LV, left ventricle; VM, viral myocarditis; TAC, trans aortic constriction; ND, not done.

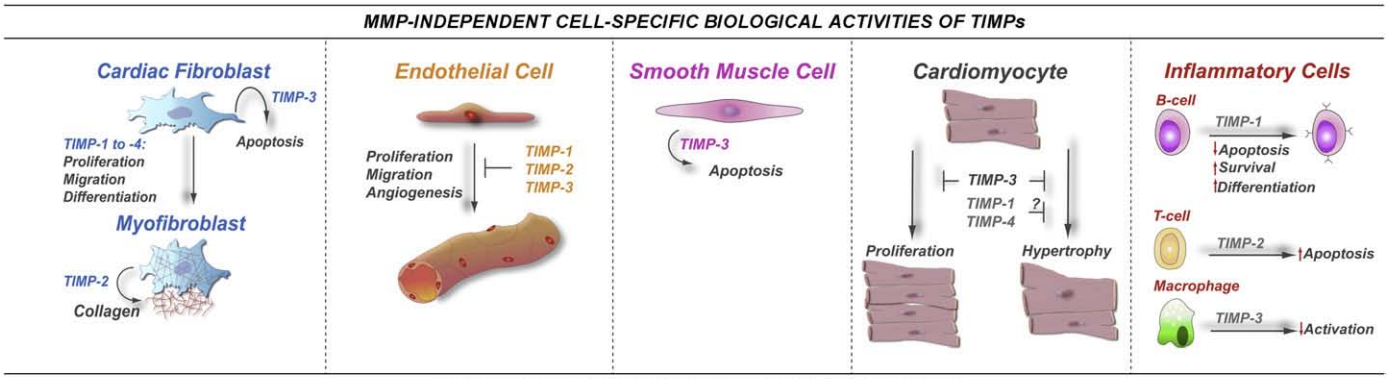
knowledge of TIMPs in cardiac remodeling and provide novel insights into possible therapies.

2.1. TIMPs modulators of cardiac fibroblast phenotype and fibrosis

Fibroblasts play a key role in the structural and functional remodeling of the myocardium [4]. After myocardial infarction, normally quiescent fibroblasts transform into a proliferative and invasive myofibroblast phenotype that has the ability to deposit a new collagen matrix [2,4,20]. This results in the formation of a qualitative and mature scar that is imperative for the structural and functional recovery of the infarcted left ventricle. Intriguingly, Lovelock et al. [21] recently demonstrated a distinct role for each TIMP, as modulators of the cardiac fibroblast phenotype independent of their ability to influence MMP activity (Fig. 1).

Adenoviral overexpression of TIMP-1, -2, -3, and -4 in cultured cardiac fibroblast revealed that all TIMPs induce fibroblast proliferation and provoke a switch to a more activated myofibroblast phenotype. In addition, TIMP-2 was capable of increasing collagen synthesis (Fig. 1) [21], while conversely TIMP-3 also increased fibroblast apoptosis. The use of a broad-spectrum MMP inhibitor (RS-130830) had no effect on fibroblast growth, indicating that the effects of TIMP-1, -2, -3, and -4 were MMP-independent and probably receptor-mediated [21]. The specific TIMP receptor and downstream targets modulating the fibroblast phenotype are currently still a point of discussion. However, given the extent of the effects of TIMPs on cardiac fibroblasts, it is likely that these molecules engage a variety of different fibroblast receptors.

The results described above clearly demonstrate that all four TIMPs might play a key role in cardiac repair and hence might influence wound healing, independent from their MMP-inhibitory actions. TIMPs may stimulate cardiac fibroblast proliferation and phenotypic differentiation into myofibroblasts at the site of tissue injury, thereby contributing to the formation of a qualitative and



IDENTIFIED RECEPTOR-BINDING / SIGNALING-PATHWAY					
TIMP-1	Unknown	Receptor unknown, ↑PTEN-signaling	Unknown	Unknown	Unknown
TIMP-2	Unknown	↑ α3β1-Integrin / Shp1-signaling	Unknown	Unknown	Unknown
TIMP-3	Unknown	⊥ VEGF-receptor-2 Angiotensin-II-type-2 receptor	Unknown	⊥ EGF-receptor-signaling ⊥ Tumor necrosis factor-α converting enzyme Angiotensin-II-type-2 receptor signaling	⊥ Tumor necrosis factor-α converting enzyme
TIMP-4	Unknown	Unknown	Unknown	Unknown	Unknown

Fig. 1. TIMPs as MMP-independent mediators of cellular behavior in the heart. All four tissue inhibitors of matrix metalloproteinases (TIMPs) have recently been shown to exhibit MMP-independent effects on (i) cardiac fibroblasts, (ii) endothelial cells, (iii) smooth muscle cells, (iv) cardiomyocytes and (v) inflammatory cells; all involved in cardiac remodeling and disease. The MMP-independent cell-specific biological activities of TIMPs and related TIMP-binding partners/signaling pathways are indicated. ⊥, Inhibits or antagonizes; ↑ increases; ANP, atrial natriuretic peptide; EGF, epidermal growth factor; PTEN, phosphatase and tensin homolog; Shp1, protein tyrosine phospatase; VEGF, vascular endothelial growth factor.

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mature collagen matrix [2,20]. Furthermore, increased expression of TIMP-2 could contribute to cardiac wound healing by directly stimulating collagen synthesis within the microenvironment of the injured heart. Finally, the opposing observations that were reported with TIMP-3, influencing both fibroblast proliferation and apoptosis, indicate that TIMP-3 could exert different *in vivo* biological functions depending on the time and localization of its expression after tissue injury. Early after cardiac injury, increased expression of TIMP-3 might directly stimulate the proliferation and differentiation of resident fibroblasts to preserve the mechanical stability of the wound. In contrast, in a later stage, TIMP-3 may act as an "off switch" that prevents and/or limits the excessive presence of myofibroblasts at the site of wound healing, thereby preventing exaggerated fibrosis and subsequent myocardial stiffness. However, further examination will be necessary to fully understand the exact *in vivo* relevance of TIMPs on the fibroblast phenotype after cardiac injury.

2.2. TIMPs, endothelial cells and angiogenesis

Both cardiac size and function are dependent on angiogenesis [22,23]. Disruption of coordinated tissue growth and angiogenesis is a major contributing factor to the progression from adaptive cardiac hypertrophy to congestive heart failure [22,23]. The dogma that TIMPs functions as endogenous inhibitors of angiogenesis only by blocking MMP activity has recently been challenged by the finding that two synthetic MMP inhibitors, batimastat [24] and CP471,474 [25], do not impair endothelial cell growth or inhibit angiogenesis. Furthermore, TIMP-1 to -3 were reported to inhibit endothelial cell migration and/or angiogenesis in response to vascular endothelial growth factor (VEGF) stimulation in an MMP-independent manner [26–29] (Figs. 1 and 2). An elegant study by Akahane et al. [26], showed that TIMP-1 causes MMP-independent stimulation of phosphatase and tensin homolog (PTEN) with subsequent dephosphorylation of focal adhesion kinase and cytoskeletal remodeling, leading to inhibition of endothelial cell migration.

To date, TIMP-2 and its inhibition of angiogenesis are probably one of the best-described examples, suggesting that TIMPs function to regulate cellular responses to growth factors. TIMP-2 blocks endothelial cell migration and proliferation via several distinct mechanisms. TIMP-2 interacts with α3β1-integrin on the cell surface of endothelial cells, initiating a signaling cascade through the protein tyrosine phosphatase Shp-1. As illustrated in detail in Fig. 2, activation of this signaling cascade results in at least three distinct mechanisms that inhibit endothelial cell 'activation' and preserve endothelial cell homeostasis. First, Seo et al. [29] demonstrated that TIMP-2 has the ability to silence two important angiogenic growth factor receptors – vascular endothelial growth factor receptor-2 (VEGFR-2) and fibroblast growth factor-receptor-1 (FGFR-1) – via Shp-1. Briefly, TIMP-2 binds to α3β1-integrin on endothelial cells causing the protein tyrosine phosphatase Shp-1 to move from the integrin to the neighboring VEGFR-2 or FGFR-1, resulting in a decreased activation and phosphorylation of both tyrosine kinase growth factor receptors (Fig. 2, pathway 1) [29]. The cells are thereby rendered resistant to VEGF- and FGF2-induced endothelial cell proliferation, migration, and hence angiogenesis. This inhibitory effect was not observed using a pro-MMP-2–TIMP-2 complex [24], suggesting that only free TIMP-2 was capable of inhibiting endothelial cell growth in response to FGF-2 stimulation. Second, TIMP-2 mediated activation of Shp-1 can also propagate its signal through inhibition of Src tyrosine kinase activity, dephosphorylation of paxillin Tyr31/118, leading to the switch of Rac to Rap1 signaling (two small GTPases, Fig. 2, pathway 2) [30]. Rac controls actin cytoskeletal dynamics and integrin adhesion that are essential for lamellipodium extension during cell migration, whereas Rap1 acts as a negative regulator of cell migration [31,32]. This pathway requires the transcription, synthesis, and cell surface localization of the *reversion-inducing-cysteine-rich protein with kazal motifs* (*RECK*)*-gene* product, a membrane-bound inhibitor of MMP activity, and leads to the suppression of endothelial cell migration (Fig. 2, pathway 2) [33]. Finally, subsequent studies have demonstrated that TIMP-2 or mutant Ala+TIMP-2 (which does not inhibit MMP-2 or MT1-MMP activity or mediate MT1 activation of pro-MMP2) binding with α3β1 results in G1 growth arrest and enhanced the *de novo* synthesis of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ in human endothelial cells, an important inhibitor of cell proliferation (Fig. 2, pathway 3) [34]. Collectively, these findings suggest that TIMP-2 is a key negative regulator of angiogenesis by promoting endothelial cell differentiation into a quiescent state to maintain vascular homeostasis in the cardiac microenvironment, and that the central mediator of these pathways, protein tyrosine phosphatase Shp-1, could represent a novel potential therapeutic target, that warrants further investigation.

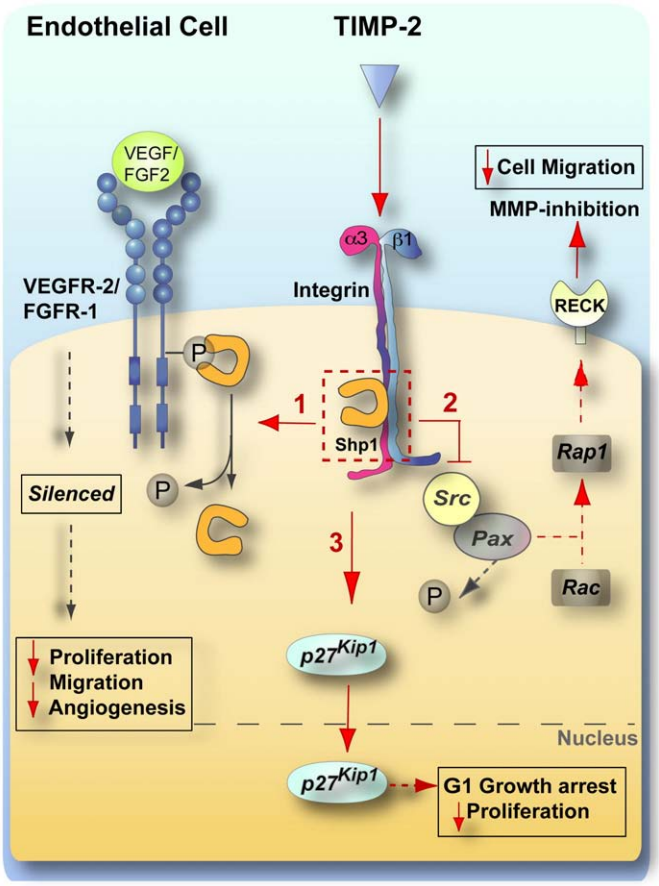

Fig. 2. TIMP-2, endothelial cells and angiogenesis. TIMP-2's inhibition of endothelial cell proliferation and migration and angiogenesis is, to date, the best-described example of MMP-independent TIMP-mediated receptor signaling and regulation of cell fate. Here, the binding of TIMP-2 with α3β1-integrin has the ability to inhibit angiogenesis via at least three distinct pathways mediated by protein tyrosine phophatase Shp-1. For details, see text. FGF-2, fibroblast growth factor-2; FGFR-1, FGF-receptor-1; P, phosphor; $p27^{Kip1}$, cyclin-dependent kinase inhibitor $p27^{Kip1}$; Pax, paxillin; Rac and Rap1, two small GTPases; RECK, membrane anchored MMP-inhibitor; Shp-1, protein tyrosine phophatase Shp-1; Src, Src tyrosine kinase; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2.

TIMP-3 has the ability to inhibit capillary morphogenesis *in vivo* and endothelial cell migration *in vitro*, through its interaction with VEGFR-2 and directly antagonizing the binding of VEGF, but not FGF-2, and thereby blocking angiogenesis (Fig. 1) [27]. Besides VEGFR-2, human angiotensin-II-type-2-receptor was recently identified as a novel TIMP-3 interacting partner [28]. Subsequent *in vitro* analysis revealed that TIMP-3 and angiotensin-II-type-2-receptor additively inhibits VEGF-induced human umbilical vascular endothelial cell proliferation and angiogenesis, in comparison to TIMP-3 or angiotensin-II-type-2-

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receptor alone. Moreover, overexpression of TIMP-3 and angiotensin-II-type-2-receptor completely inhibits VEGF expression in human umbilical vascular endothelial cells [28]. These experiments indicate that TIMP-3 and angiotensin-II-type-2-receptor inhibit the autocrine effect of VEGF in endothelial cells and thereby exhibit an anti-angiogenic effect. Previous studies have shown that increased neovascularization/angiogenesis in a permanent infarct model helps to "rescue" the ischemic cardiomyocytes in the infarct border, thereby reducing the extent of the infarct scar and improving overall cardiac performance [2,35]. At this point, one may wonder to what extent the absence of TIMP-3's ability to directly inhibit angiogenesis contributed to the increased angiogenic response and accelerated infarct healing observed in TIMP-3-deficient mice (Table 2) [12].

Of equal interest, is the observation that TIMP-3 could be implicated in the progression of vascular diseases, independently from its ability to inhibit MMPs (Fig. 1). In this regard, *in vitro* and *in vivo* adenoviral overexpression of TIMP-3, but not TIMP-1 or -2, promoted apoptosis in vascular smooth muscle cells, an effect that – at least in the *in vivo* experiments – was not achieved by the use of a synthetic MMP inhibitor [36,37].

These studies indisputably indicate that TIMPs function as MMP-independent endogenous inhibitors of angiogenesis. However, these results do evoke some intriguing questions. If the 'angiogenic switch' needs to be turned on to facilitate coronary angiogenesis during cardiac wound healing, the question remains what the physiologic state of this switch is in the healthy adult heart and to what extent TIMPs play a part in this tightly regulated process. 'Is it turned "off", with inhibitors such as TIMPs, outweighing the stimulators? Or do the TIMPs contribute to maintaining the "balance", keeping the pro-angiogenic properties of many factors in check?' Angiogenesis is a process limited to adult tissues responding to a pathologic stimulus [22,23], yet whether this TIMP-mediated modulation of angiogenesis is counteracting the angiogenic response during adaptive remodeling or actually contributes to maladaptive cardiac remodeling, still remains to be elucidated.

2.3. TIMPs, cardiomyocytes and hypertrophic remodeling

In contrast to most adult cardiomyocytes, fetal cardiomyocytes do proliferate [38,39]. After a brief period of neonatal proliferation, adult mammalian cardiomyocytes become binucleated, downregulate cell-cycle activators, upregulate cell-cycle inhibitors and withdraw from the cell-cycle. Further growth of the hearts occurs only by cardiomyocyte hypertrophy [39]. In response to a number of disease stimuli, including hypertension and myocardial infarction, individual cardiomyocytes respond to stress by growing in length and/or width through increased protein synthesis or decreased protein degradation, as a means to increase cardiac pump function and decrease the ventricle wall tension [39].

2.3.1. TIMP-3 and cardiomyocyte proliferation

The discovery that TIMP-3 is able to modulate neonatal cardiomyocyte proliferation, provided a link between TIMPs and cardiomyocytes [15,40,41]. As illustrated in Figs. 1 and 3, it was recently demonstrated that neonatal cardiomyocytes proliferation was increased in TIMP-3-deficient hearts [15], whereas treatment with recombinant TIMP-3 inhibited proliferation [40]. Although the exact mechanism is still unclear, a recent study by Hammoud et al. [41] revealed that TIMP-3 inhibits neonatal mouse cardiomyocyte proliferation via the epidermal growth factor receptor signaling pathway (Fig. 3). Here, TIMP-3 seems to mediate the inhibition of the epidermal growth factor receptor phosphorylation, thereby decreasing Jun N-terminal kinase and specific protein-1 phosphorylation. Decreased phosphorylation of the transcription factor specific protein-1 consequently releases the inhibition on cell cycle dependent kinase inhibitor p27, an important inhibitor of cell proliferation.

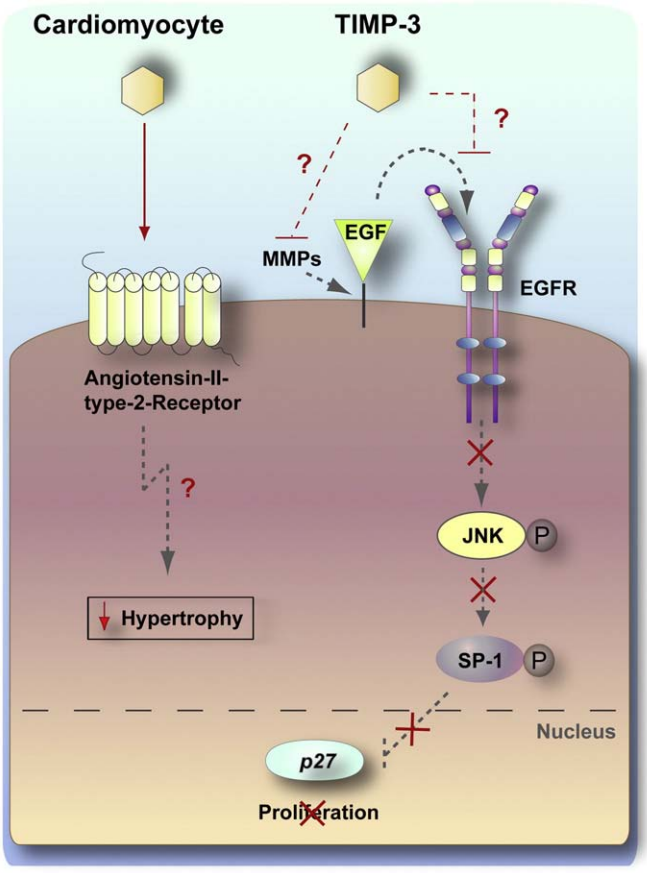

Fig. 3. TIMP-3 in cardiomyocyte proliferation and hypertrophy. Proposed signaling pathways by which TIMP-3 influences the cardiomyocyte. TIMP-3 seems to mediate the inhibition of the 'epidermal growth factor'-(EGF)- receptor pathway, thereby restraining neonatal cardiomyocyte proliferation. Next, although further investigations are mandatory, the identification of angiotensin-II-type-2-receptor as a binding-partner for TIMP-3 led to the hypothesis that TIMP-3 might play a direct role in cardiomyocyte hypertrophy by influencing the renin–angiotensin-system. For details, see text. EGF, epidermal growth factor; EGFR, EGF-receptor; JNK, jun N-terminal kinase (mitogen-activated protein kinase); P, phosphor; P27, cyclin-dependent kinase inhibitor P27; SP-1, specific protein-1 (transcription factor); ?, pathway unknown.

Interestingly, p27 mRNA levels were also increased in endothelial nitric oxide synthase (eNOS)-deficient cardiomyocytes while both p21 and p27 mRNA levels were decreased in eNOS-transgenic and TIMP-3-deficient cardiomyocytes [40]. These data suggest that nitric oxide produced by eNOS (an important signaling molecule involved in cardiomyogenesis, angiogenesis and cell survival) promotes neonatal cardiomyocyte proliferation by inhibiting TIMP-3 expression [40,41]. Hammoud and colleagues suggested that the inhibitory effect of TIMP-3 on epidermal growth factor receptor-activation is probably established by the inhibition of MMP-mediated shedding of epidermal growth factor from the cell surface (Fig. 3) [40,41]. However, other possible mechanisms, such as a direct epidermal growth factor receptor-antagonizing interaction of TIMP-3, have not been excluded (Fig. 3). Since the present studies were conducted on postnatal day 1 hearts, we need to keep in mind that the effects and mechanisms identified herein may not be applicable to adult cardiomyocytes, which have very limited ability to proliferate [39].

2.3.2. TIMPs and hypertrophic remodeling

Whether TIMPs are able to influence cardiomyocyte hypertrophy and/or hypertrophic remodeling of the heart; and whether this influence is MMP-dependent and/or -independent, are captivating questions that have yet to be addressed. However, several lines of evidence allude to such a role for TIMPs. TIMP-1 and -2 are markedly

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increased in chronic pressure overloaded human hearts and their expression correlates with the degree of interstitial fibrosis [18] that, at least in case of TIMP-1, was confirmed in a rat model during the early phase of hypertension [42]. Second, as presented in Table 2, TIMP-1-gene overexpression significantly reduced hypertrophic growth of cardiomyocytes and prevented cardiac dilatation during acute left ventricle pressure overload [17]. TIMP-1-gene deficiency also resulted in increased cross sectional areas of left ventricle myocytes 2 weeks after MI [11] In addition, TIMP-3-gene deficiency spontaneously led to cardiomyocyte hypertrophy, left ventricle dilatation and cardiac dysfunction at 21 months of age, a phenotype that is consistent with human dilated cardiomyopathy [15]. Finally, expression of TIMP-4 showed an early increase in spontaneous hypertensive rats exhibiting compensatory hypertrophy, suggesting a protective role for TIMP-4 in the compensatory hypertrophic phase during cardiac remodeling [43].

To date, very little TIMP-cardiomyocyte-specific research has been conducted, allowing a limited analysis on possible pathway(s) responsible for the TIMP-mediated protection against hypertrophic remodeling of the heart. However, analysis revealed that TIMP-3 uniquely inhibits 'tumor necrosis factor-α converting enzyme' (TACE, ADAM-17) via its N-terminal domain [44]. TACE primarily activates tumor necrosis factor-α (TNF-α), which is a key inflammatory mediator during cardiac remodeling and the development of congenital heart disease [19]. Double mutant TIMP-3$^{-/-}$TNF-α$^{-/-}$ mice were protected from cardiac hypertrophy in response to aortic constriction-induced pressure overload [19]. The latter indicates that TIMP-3/TNF-α is a critical axis leading to pressure-induced hypertrophic cardiac remodeling (Fig. 1). Meier et al. [45] recently demonstrated that norepinephrine induced hypertrophic remodeling in mice was accompanied by a time dependent elevation of TIMP-1 in cardiomyocytes. This increase of TIMP-1 was shown to be necessary for the induction of 'atrial natriuretic peptide', as a neutralizing TIMP-1 antibody suppressed the latter (Fig. 1). Interestingly, Bubikat et al. [46] produced evidence that 'atrial natriuretic peptide', which is part of the 'fetal gene program' – a hallmark of cardiac hypertrophy [39] – functions as a local anti-hypertrophic cardiac factor. Although together, these experiments suggest that TIMP-1 may mediate atrial natriuretic peptide-induced delay of norepinephrine-induced hypertrophy; 'how' TIMP-1 induces atrial natriuretic peptide stimulation and whether this is MMP-related is still unknown.

2.3.3. A link between TIMPs and the renin–angiotensin-system?

As previously discussed, Kang and colleagues identified human angiotensin-II-type-2-receptor as a novel TIMP-3 interacting partner (Figs. 1 and 3) [28], linking TIMP-3 with the renin–angiotensin-system. Although the physiopathological roles and signaling mechanisms of angiotensin-II-type-2-receptor are still largely unknown, it was shown to be increased during hypertrophy and ischemic heart disease [47,48]. In addition, angiotensin-II-type-2-receptor-gene deleted mice lost the ability to develop cardiac hypertrophy in response to pressure overload or chronic angiotensin-II infusion [48]. Consequently, a cardiac selective pathway involving angiotensin-II-type-2-receptor and phosphatidylinositol-3-OH kinase, which is strongly involved in the cardiac hypertrophic response, was identified [39,47]. As a result, TIMP-3 could be a modulator of angiotensin-II/angiotensin-II-type-2-receptor-mediated induction of cardiac hypertrophy. Nevertheless, direct proof that TIMP-3 plays a critical and direct role in the hypertrophic response of the heart by influencing the renin–angiotensin-system still needs to be given.

2.4. MMP-independent effects of TIMPs and cardiac inflammation?

A central role for inflammation in cardiac remodeling has been widely acknowledged [1,2]. As indicated in Tables 1 and 2, inflammatory cells are important cellular sources of MMPs and TIMPs. MMPs regulate numerous aspects of inflammation, including chemokine and cytokine activity, the establishment of chemotactic gradients, and leukocyte infiltration into the injured heart; so it is undisputable that TIMPs alter the inflammatory response due to their MMP-inhibitory activity [1,2]. Several genetic studies have demonstrated that any shift in the balance between MMPs and TIMPs is pivotal for the infiltration of inflammatory cells into the injured heart after cardiac injury, stress or infection (Tables 1 and 2). Although the data are still very limited and are mainly emerging from research conducted outside the cardiovascular field, there are few lines of evidence indicating that TIMPs might influence immune cells through MMP-independent pathways in the heart (Fig. 1).

As previously discussed, TIMP-3 is one of the main inhibitors of the enzyme known as 'TNF-α converting enzyme' (TACE), primarily responsible for the bioactivation of the pro-inflammatory cytokine TNF-α, a key inflammatory mediator involved in cardiac remodeling and heart failure [19,44] (Fig. 1). Challenging TIMP-3-deficient mice with lipopolysaccharide, a strong inducer of septic shock, resulted in an uncontrolled systemic inflammation leading to animal morbidity due to the dysregulated TNF-α-signaling [49]. Moreover, shedding of TNF-α and its receptor from TIMP-3-deficient macrophages was also augmented following lipopolysaccharide-stimulation. Thus, TIMP-3 seems to have a fundamental role in controlling inflammation through the regulation of TNF-α-signaling. To find out whether TIMP-3 is also implicated in cardiac inflammation, injury and repair, further cardiac studies in TIMP-3-deficient mice are needed. Unfortunately, at this moment, it is difficult to speculate on a possible role for TIMP-3 in cardiac inflammation, as detailed analysis of the inflammatory response is lacking in the cardiac phenotypes observed in the TIMP-3-deficient hearts (Table 2).

TIMP-3, however, is not the only metalloproteinase inhibitor that may function during cardiac inflammation (Fig. 1). *In vitro* analysis by Guedez and colleagues recently demonstrated that TIMP-1 directly protects B-cells from apoptosis through a non-MMP-inhibitory pathway and suggests that this protein may play a pivotal role in the maintenance of B-cell homeostasis [50]. The latter was further supported by the observation that TIMP-1 inhibited apoptosis in normal tonsillar B-cells, but not in T-cells [51], while others reported that TIMP-1 functions as a differentiating and survival factor of germinal center B-cells [52]. Conversely, recombinant TIMP-2 increased apoptosis in activated human peripheral blood T-cells, whereas unstimulated T-cells were not susceptible. This effect was specific to TIMP-2 and was not observed for TIMP-1 [53]. Nevertheless, additional experiments revealed that the MMP-inhibitory function of TIMP-2 seems to be important in this process, as a TIMP-2 peptide lacking the N-terminal domain, which is critical for MMP-inhibition, did not induce apoptosis.

Although these data are promising, one needs to realize that the results described above are suggestive, yet, not conclusive on an MMP-independent role for TIMPs in the regulation of inflammation during cardiac disease and remodeling. Moreover, future cardiac-specific research will be essential in order to predict the physiological relevance of the non-MMP-inhibitory functions of the four TIMP-species and their involvement in cardiac inflammation.

3. TIMPs and cardiac remodeling; 'Building their future from the ground up'

It is undisputable that many TIMP-mediated effects in the heart are due to their ability to inhibit MMPs [1,2]. However, in light of the data discussed above, intriguing questions are towards the therapeutic potential of these MMP-independent biological actions of TIMPs in the major cellular and molecular processes involved in cardiac remodeling (Figs. 1-3). It is very likely that the TIMP-mediated mechanisms responsible for the cardiac phenotypes seen in genetically engineered organisms are the result of a fine balance between MMP-dependent

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Table 3
Key-questions that remain to be answered.

What is the time- and space- -dependent expression pattern of each TIMP family member during the various stages of cardiac remodeling? What is the complete catalogue of their cellular sources and how is their expression regulated?
What is the complete catalogue of MMP-dependent and –independent effects of each TIMP family member on the various cell types involved in cardiac remodeling in health and disease? What are the defined structural regions of each TIMP responsible for their MMP-independent functions? What is the *in vivo* relevance of the MMP-independent functions of TIMPs in the normal and diseased heart?
What is the complete catalogue of TIMP-binding partners and subsequent signaling cascades in the myocardial microenvironment contributing to cardiac remodeling and the progression to hear failure?
Do TIMP-based therapies still have a future? Could we rule out the undesired side effects of MMP-inhibition by uncoupling the MMP-dependent and -independent functions of TIMPs? Could agents that influence specific pathways downstream of the TIMP receptors represent novel therapeutic targets to prevent adverse cardiac remodeling? Could a more localized and temporal therapeutic setup provide a clinically relevant strategy for TIMPs in cardiac disease?

and -independent biological actions (Table 2). The knowledge that we currently obtained should therefore be used as an initial starting point to encourage further research that completely dissects each of the four TIMP-species and rebuilds their future from the ground up (Table 3).

3.1. TIMP-expression and cardiac remodeling, clues for the future

As shown in Table 2, numerous animal studies have revealed that TIMPs are direct contributors to the left ventricle remodeling process. Nevertheless, our knowledge regarding the expression profiles and cardiac distribution of each member of the TIMP family in the myocardium remains largely inconsistent and incomplete. In addition, human studies have been limited to end-stage cardiac tissue so that a comprehensive analysis of TIMPs during left ventricle remodeling has yet to be carried out. For instance, whereas several independent studies have shown a strong reduction of cardiac TIMP-1 and -3 expression in human and experimental failing heart, our group recently reported that cardiac TIMP-1 and -2 are increased two-fold in patients with long-term pressure overload without systolic dysfunction, and are related to the degree of fibrosis (extensively reviewed by Spinale [1]). Concordantly, Thomas et al. [54] revealed that TIMP-1 and -2 proteins were profoundly increased in end-stage human dilated cardiomyopathy. Unfortunately, the other members of the TIMP family were not assessed in these studies. Another comprehensive analysis of the complete TIMP profile in the end-stage failing human heart revealed that TIMP-1, -3 and -4 protein expression was reduced in patients with end-stage ischemic cardiomyopathy, while TIMP-2 was not altered [55].

Collectively these data, extensively reviewed elsewhere [1,2], support a causative role for 'TIMP-deficiency' as an underlying mechanism in cardiac remodeling. However, a comprehensive analysis of TIMP-1, -2, -3 and -4 during the various cardiac pathologies and stages of cardiac remodeling is needed. In fact, this first step could provide us valuable indications on the specific role that each TIMP represents in the normal and the failing heart.

3.2. Creating a 'functional map' for each TIMP family member

Although TIMP-1 and -3 and TIMP-2 and -4 share sequence homologies [5], the exact reasons for a number of important functional differences are still unknown. In order to better understand the structure–function relationships of these molecules, it will be of great value to outline a 'functional map' of each TIMP and further uncouple their MMP-dependent and -independent biological functions. This could be accomplished through the engineering and production of specific TIMP-regions and subsequently exploring their *in vitro* and *in vivo* biological relevance on specific cellular and molecular processes involved in cardiac healing and remodeling. Alternatively the generation and *in vivo* administration of specific TIMP-domain blocking antibodies could provide a more detailed insight into their structure–function relationships. Another interesting approach would be the systemic or cardiac-specific overexpression of genetically engineered TIMP-mutants that are unable to inhibit MMPs, as it will be important not to interfere with the endogenous MMP-inhibitory activity of the TIMPs in order to explore their MMP-independent biological functions. The latter is important to avoid the adverse effect of increased MMP-inhibitory activity or the deleterious consequences of excessive MMP activity on the cardiac microenvironment [1,2].

A very elegant example that demonstrates the uncoupling of the MMP-inhibitory and anti-angiogenic activities of TIMP-2 was recently provided by Fernandez and colleagues [56]. Here, the authors used the *Pichia pastoris* expression system to engineer and produce the N-terminal and C-terminal domains of TIMP-2. Although both domains inhibited angiogenesis in the embryonic chorioallantoic membrane assay, the C-terminal disulfide loop (loop 6) and wild-type TIMP-2 were more effective inhibitors of angiogenesis in the mouse corneal pocket assay as compared to the N-terminal TIMP-2 domain [56]. Furthermore, the inhibitory ability of the N-terminal domain was dependent on its MMP-inhibitory activity, because blocking of the N-terminus of TIMP-2 by appending glutamic acid and alanine residues, reversed the MMP-inhibitory activity and the *in vivo* angiogenic activity of this TIMP-2 domain.

Obtaining a more detailed and defined view on the various MMP-dependent and -independent mechanisms of TIMPs in the heart and, second, getting a clear view on their *in vivo* significance, may allow us to predict the outcome of novel therapies based on peptide fragments or analogues of TIMPs. In this way, we would be able to fine-tune the TIMP-related biological functions and selectively manipulate the TIMPs important for cardiac disease, while limiting any undesirable side effects that have been encountered in the past [1,2].

3.3. Exploring receptor-mediated pathways and processes in cardiac health and disease

The identification of TIMP-interacting proteins on the cell surface is only the beginning of our understanding of the MMP-independent, 'cytokine-like' properties of the TIMPs. Potential TIMP-binding partners remain to be discovered and the complex mechanisms of TIMP-signaling in the context of the myocardial microenvironment, remodeling and progression to heart failure need to be dissected in more detail (Fig. 1). Our current knowledge related to MMP-independent TIMP-mediated receptor signaling is in its early stages and the *in vivo* relevance of these findings is sometimes unclear.

For starters, several signaling pathways have been reported that link individual TIMP-species with promotion of cell growth, anti-apoptotic or growth inhibitory activities (reviewed by Stetler-Stevenson [57]), however, in the majority of the cases the specific receptor molecules that are involved remain unknown. For instance, as discussed previously, TIMP-1 seems to cause MMP-independent stimulation of phosphatase and tensin homolog (PTEN), leading to the inhibition of endothelial cell migration [26], yet the TIMP-1 binding partner responsible for the activation of PTEN is yet to be identified (Fig. 1). Next, several specific TIMP-binding partners have been reported but their biological significance remains an intriguing point of discussion. For example, low-density lipoprotein receptor-related protein (LRP) is a versatile scavenger and signaling receptor that binds multiple unrelated ligands and influences a wide variety of biological processes (reviewed by Lillis et al. [58]). Since TIMP1 and -2, either alone or complexed with proMMP-2 or -9, respectively, can interact with LRP, it will be of particular interest to investigate whether LRP represents (i) a 'mitogenic' receptor for TIMPs; as it is the 'motogenic' receptor for plasminogen activator inhibitor-1 [59], and/

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or (ii) whether LRP solely acts as a scavenger receptor to regulate the extracellular TIMP levels, and/or (iii) whether LRP-TIMP binding could antagonize or promote other ligand-LRP mediated signaling pathways. Consistent with this third alternative, recent studies have pointed towards an LRP-mediated cross-talk between matricellular proteins and the MMP/TIMP-system [58,60,61]. LRP is able to bind thrombospondin-1 and -2, two matricellular proteins that seem to be crucial regulators of the cardiac integrity necessary for the myocardium to cope with increased loading or ischemic stress [60,61]. Interestingly, the proMMP-2/TIMP-2/thrombospondin-triplex can be scavenged via LRP-mediated endocytosis, regulating the levels of both MMP-2 and TIMP-2 in the extracellular cardiac matrix. Although here the authors suggested that the major aim of this internalization is to regulate the MMP2 levels present in the ECM, the question remains whether TIMPs could also be involved in competing with thrombospondin–LRP-mediated signaling and, for example, the thrombospondin–LRP stimulation of cell migration [58]. To our knowledge, such experiments have not yet been reported.

Finally, several other MMP-independent TIMP-mediated cellular responses have been identified yet, the specific receptor-mediated signaling cascade resulting in these cellular responses are sometimes completely lacking, as illustrated by the various effects of TIMP-1, -2, -3 and -4 observed in cardiac fibroblasts (Fig. 1) [21]. Although these results suggest that the effects of each TIMP-species are receptor-mediated, so far, no attempts have been made to determine which fibroblast receptors could influence their fibroblast proliferation, apoptosis, differentiation and collagen synthesis. In addition, certain questions remain; are the effects of TIMPs on cellular behavior mediated by cell-specific pathways? Could downstream targets of TIMP-mediated receptor signaling represent novel targets for therapeutic interventions?

3.4. What about TIMPs, cardiac remodeling and therapeutics?

Although the importance of modifying cardiac ECM remodeling by the use of MMP inhibitors has been established, clinically relevant strategies have been difficult to implement [1,2]. The findings discussed above indicate several important non-MMP-inhibitory functions of TIMPs that can no longer be neglected and may help to explain why synthetic MMP inhibitors have not lived up to their expectations. The time is emerging to exploit novel, clinically relevant strategies for TIMPs in cardiac remodeling. Besides the approaches that we discussed previously in this review, a more localized and temporal therapeutic setup is necessary and could provide the answer. As such, 'cell-based gene therapy' could represent a clinically relevant method to prevent heart failure by permitting a temporal and spatial regulated release of a gene product. Proof-of-concept for such an approach was provided by Angoulvant et al. [62]. By injecting bone marrow stromal cells overexpressing TIMP-3, shortly after the induction of myocardial infarction in TIMP-3-deficient mice, the authors were able to prevent progressive cardiac dysfunction, preserve the remote myocardial collagen content, and reduce border zone apoptosis for at least 28 days after implantation, whereas only a transient (6 days) elevation of TIMP-3-mRNA was observed in the transfected cells. Thus, a limited and localized amplification of the TIMP response may confer long-term benefits in patients progressing to heart failure.

4. Conclusions

It is undisputable that many TIMP-mediated effects in the heart are due to their ability to inhibit MMPs. Though, given their complex nature, the role of each TIMP during cardiac remodeling is likely to be far more complex than a simple imbalance of MMP activity in the heart. Through their pleiotropic activities, TIMPs may regulate a wide range of cellular responses critical to cardiac remodeling.

Research of the last two decades revealed that the TIMP family members depend upon subtle interactions with other ECM components, as well as direct interactions with cell-binding partners and are thereby influencing cardiac fibroblasts, endothelial cells, smooth muscle cells, cardiomyocytes and inflammatory cells. Unfortunately, the complex and often opposing biological properties of TIMPs have hampered their complete understanding and therapeutic potential during cardiac disease.

It is clear that a complete and in-depth understanding of the MMP-independent TIMP-mediated processes and their modulation during cardiac health and disease is mandatory to design innovative TIMP-based therapeutic strategies to prevent cardiac remodeling and the progression to heart failure.

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