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# Myocardial matrix metalloproteinase-2: inside out and upside down

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## Abstract

Since their inaugural discovery in the early 1960s, matrix metalloproteinases (MMPs) have been shown to mediate multiple physiological and pathological processes. In addition to their canonical function in extracellular matrix (ECM) remodeling, research in the last decade has highlighted new MMP functions, including proteolysis of novel substrates beyond ECM proteins, MMP localization to subcellular organelles, and proteolysis of susceptible intracellular proteins in those subcellular compartments. This review will provide a comparison of the extracellular and intracellular roles of MMPs, illustrating that MMPs are far more interesting than the one-dimensional view originally taken. We focus on the roles of MMP-2 in cardiac injury and repair, as this is one of the most studied MMPs in the cardiovascular field. We will highlight how understanding all dimensions, such as localization of activity and timing of interventions, will increase the translational potential of research findings. Building upon old ideas and turning them inside out and upside down will help us to better understand how to move the MMP field forward.

### Keywords

MMP-2; TIPTOP; Myocardial infarction; Heart failure; Cardiovascular disease

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## Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases responsible for cleaving protein substrates, with the most commonly identified substrates being extracellular matrix (ECM) proteins.[1] MMPs are involved in both physiological processes, such embryogenesis and organogenesis during development, and in pathological processes such as wound healing, metastasis, and tissue remodeling.[2–4] The general structure of an MMP includes an inhibitory pro-peptide domain, a zinc-containing catalytic domain, a linker peptide, and a hemopexin domain.[2] The majority of MMPs are synthesized in an inactive pro-MMP form, and the MMP can be activated by the removal of the approximately 10 kDa pro-peptide, which exposes the Zinc-binding catalytic domain. While this cleavage of the pro-domain is generally required for MMP activation, recent studies have reported that post-translational modifications of MMPs, in the absence of the removal of the pro-domain, can also result in MMP activation.[3] (See Figs. 1 and 2.)

The broad family of MMPs can be further classified into collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), matrilysins (MMP-7 and MMP-26), and membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24 and MMP-25), a classification system that is loosely based on initial ECM substrate screens. An original criteria to being classified as an MMP was the ability of the enzyme to proteolytically process at least one ECM protein.[2,5] The first non-ECM substrates to be identified were cytokines, and one of the first to make the list was interleukin-1β which is cleaved into its active form by several MMPs, including MMP-2 and -9.[6,7] A multitude of cytokines, chemokines, and growth factors are now known to be substrates of extracellular MMPs.

Approximately a decade ago, the Schulz laboratory first showed that MMP-2 targeted intracellular proteins, and proteolysis of these intracellular proteins mediated myocardial ischemia-reperfusion (I/R) injury.[8] Specifically, they found that proteolytic degradation of the thin filament protein troponin I (TnI) by MMP-2 was directly involved in the acute contractile defect of isolated rat hearts exposed to I/R. This article initiated a cascade of research focused on intracellular roles for MMPs. Subsequently, degradomics experiments used the power of proteomics and bioinformatics approaches to reveal a wide variety of potential intracellular MMP substrates.[9–11]

Myocardial ischemia is defined as the sustained loss of oxygen, resulting from an obstruction in coronary blood flow. When ischemia lasts beyond a critical window of 20–30 minutes, myocyte necrosis ensues. Irreversible ischemic damage resulting from ischemia for longer periods of time is defined as myocardial infarction (MI).[12] Optimal therapy for MI includes reperfusion strategies (whether mechanical or thrombolytic) to restore blood flow to the ischemic region. Reperfusion, while beneficial to the patient, also initiates a robust inflammatory response that can extend myocardial damage.[13]

In the myocardium, MMPs regulate both physiological and pathophysiological processes. MMP-2 is one of the most studied MMPs identified, as it is found in almost all cardiac cells and, in part, due to its ease of evaluation. This review will focus on MMP-2 roles in cardiac

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pathology (namely myocardial infarction and I/R injury), which will serve as a template for other MMPs known to be elevated in these same models.

## Known ECM substrates and novel non-ECM extracellular substrates

MMP substrates identified to date can be grouped into extracellular and intracellular proteins (Table 1). The MEROPS database provides a resource for information about many proteinases, including MMPs, as well as the proteins that inhibit them (http://merops.sanger.ac.uk/). Analysis of the cleavage site sequences across substrates provides information on consensus sequences. For example, the consensus sequence of the MMP-2 cleavage site is XPXX↓(L/I)XXX (where X is any amino acid), based on evaluation of 3413 cleavages. Note that the consensus cleavage sites are based primarily on early data gathered on the premise that MMPs target only ECM proteins. While they are useful for *in silico* identification of putative substrates, using only these sequences will generate many false positive and negative hits. A small set of predicted substrates for MMP-2, including both intracellular and extracellular substrates, have been derived from *in vitro* and *in silico* cleavage assays.[14,15] Of note, several of these substrates are processed by MMP-2, as well as other MMPs, suggesting that there is some redundancy built into this system. Redundancy, therefore, should be a consideration in the design of selective MMP-2 inhibitors. In some cases, a better inhibition strategy may be to target the substrate rather than the protease.

In terms of cellular localization of MMPs and their substrates, insight can be gathered by knowing which cells produce MMPs and which cells have a particular substrate inside or surrounding them. Table 2 highlights production of MMP-2 by individual myocardial cell types.

## Intracellular targets

In isolated rat hearts subjected to global ischemia, a burst of peroxynitrite biosynthesis peaks within 30 seconds of reperfusion. However, if peroxynitrite production is blocked, contractile function post-ischemia is improved.[16] Enhanced biosynthesis of peroxynitrite was also found to contribute to pro-inflammatory cytokine-induced contractile failure of hearts.[17] Because pharmacologically targeting peroxynitrite pharmacologically is difficult, compounds that catalyze peroxynitrite decomposition are useful research tools but have major limitations for in vivo use, including undesirable side-effects.[18]

The limited in vivo applications of peroxynitrite prompted investigation of immediate downstream effectors of peroxynitrite action. Mechanistic studies by Maeda's group showed that several MMPs (namely, -1, -8, and -9) could be directly activated by low micromolar concentrations of peroxynitrite in a reaction requiring cellular glutathione. This reaction results in the S-glutathiolation of $Cys^{102}$ in the autoinhibitory propeptide domain and allows substrate access to the catalytic side (the so-called cysteine-switch).[19] This mechanism also holds true for MMP-2, with maximal activation of 72 kDa MMP-2 with 0.3–1.0 μM peroxynitrite.[20] Note that the S-glutathiolation of MMP-2 at $Cys^{102}$ results in a molecular weight difference of only 305 Dalton, and a catalytically active full length enzyme which is capable of cleaving TnI, and gelatin, as well as artificial fluorogenic substrates such as

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OmniMMP peptide. This difference is too small to resolve in SDS-PAGE, so the older notation of MMPs implying MMP activity (namely, pro-MMP vs. active MMP) based solely on the assumption that proteolytic removal of the peptide domain of MMPs is an absolute requirement for their catalytic activity (eg. finding a several kDa lower molecular weight cleaved form from the zymogen form) is strongly discouraged. This mechanism of MMP-2 activation has allowed for several insights: 1) A protease-independent mechanism is responsible for MMP-2 activation inside or outside cells, even under physiological levels of peroxynitrite; 2) Major structural differences between 72 kDa S-glutathiolated MMP-2 and 64 kDa MMP-2 are predicted to result in different substrate cohorts and/or affinities between these forms; [21] and 3) Drug design for an effective MMP-2 inhibitor, which plays a role in oxidative stress-induced injury, suggests a very specific and unique target (72 kDa S-glutathiolated MMP-2 vs. 64 kDa MMP-2), the latter which may not need to be targeted, depending upon the pathological condition.

Using the isolated rat heart model of I/R, MMP-2 activation in the heart was found to peak within 2–3 minutes of reperfusion following ischemia. During this time, stunning injury occurs without cellular necrosis and without detectable changes in the ECM. Importantly, intervention with MMP inhibitors reduces stunning injury.[22] MMP-2 activation followed the peak of peroxynitrite biosynthesis and preceded the development of stunning [16], and blocking MMP proteolytic activity protected the contractile function of the heart.[22] Combined, our thoughts about reversibility of injury to the stunned myocardium, oxidative stress-induced activation of MMP-2, and the high susceptibility of sarcomeric proteins such as TnI to proteolysis in myocardial I/R injury led us to naively hypothesize that the effects of MMP-2 may occur via the proteolysis of TnI. This idea was met with great resistance, particularly from MMP biologists who thought that MMPs only function extracellularly.

Wang et al. showed that, indeed, TnI is highly susceptible to proteolysis by MMP-2 *in vitro*, and that loss of myocardial TnI in I/R hearts is blocked by MMP inhibitors.[8] Immunogold electron microscopy clearly demonstrated that both MMP-2 and TIMP-4 colocalize with sarcomeric proteins in the normal heart.[8,23] Biochemical preparations of highly purified thin myofilaments, immunoprecipitation assays, and confocal microscopy evidence verified that MMP-2 and TnI are colocalized within cardiomyocytes.[8] During acute I/R injury, TIMP-4 is lost from the sarcomere so that MMP-2 is uninhibited and there is a net positive gelatinolytic activity in heart tissue.[23] Moreover, TIMP-4 can also be directly inactivated by peroxynitrite.[24] That MMP-2, but not calpain, is responsible for TnI proteolysis in stunned myocardium is corroborated by transgenic mice studies (both myocardial specific, alpha-myosin heavy chain promoter driven transgene models) which show that transgenic MMP-2 but not transgenic mu-calpain mouse hearts show reduced TnI levels.[25,26] Collectively, these studies suggest that following I/R injury, MMP-2 degrades intracellular proteins within the injured myocyte.

Several other sarcomeric and cytoskeletal proteins undergo proteolysis as a result of stunning injury. Therefore, we looked beyond TnI for more possible MMP-2 substrates. Using an unbiased pharmacoproteomics approach, myosin-light chain-1 was shown to be an MMP-2 substrate during I/R injury, with MMP-2 colocalizing with the thick myofilament fraction of the heart.[11] Alpha-actinin and desmin were also susceptible to MMP-2

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proteolysis *in vitro*, and rat hearts subjected to peroxynitrite-induced contractile dysfunction showed reduced levels of alpha-actinin.[27] Both the contractile dysfunction and loss of alpha-actinin were prevented by an MMP inhibitor. Taken together, these studies show that MMP-2 targets several myocardial proteins, leading to cardiac dysfunction.

Titin is a multifunctional giant protein (2–3 megaDalton), with two molecules spanning the cardiac sarcomere. This protein determines both systolic and diastolic function,[28,29] and its degradation was noted in several human cardiac pathologies, including I/R injury.[28, 29] Given the presence of MMP-2 in the sarcomere, we investigated whether titin is a target of MMP-2. In both human and rat hearts, we found that MMP-2 localizes to the Z-disc but not M-band region of titin. Titin was able to bind MMP-2 and was also susceptible to MMP-2 proteolysis both *in vitro* and *in situ*. Rat hearts subjected to *in vitro* I/R injury showed titin fragmentation, which was reduced by the MMP inhibitor ONO-4817. In vivo, I/R injury in mouse hearts also caused titin hydrolysis which did not occur in MMP-2 knockout mice.[28]

Glycogen synthase kinase-3 beta (GSK-3β) is a ubiquitous kinase that is found in heart muscle. This kinase is activated by dephosphorylation of its regulatory Ser9 residue, which inhibits its activity,[30,31] or by proteolytic removal of this phosphorylated domain, thought to occur by action of calpain.[32] Phosphatidylinositol 3-kinase (PI3K)/Akt signaling results in Ser-9 phosphorylation, and, therefore, inhibits GSK-3β activity. Inhibitors of GSK-3β are cardioprotective, decrease apoptosis in the ischemic heart and reduce infarct size in hearts subjected to I/R.[33,34] We showed that GSK-3β colocalized with MMP-2 in H9c2 cardiomyoblasts, and that MMP-2 proteolysis of recombinant GSK-3β enhanced its activity by removal of the N-terminal domain. $H_2O_2$ challenge of H9c2 cells significantly increased the activity and abundance of MMP-2, reduced GSK-3β levels, and significantly increased GSK-3β kinase activity. Activation of GSK-3β in hearts undergoing oxidative stress may be another downstream consequence of the intracellular actions of MMP-2.[35]

## MMP-2 localized in or near mitochondria

MMP-2 is not only localized to the subcellular fraction represented by the sarcomere in cardiomyocytes, but is also found in other subcellular locales, including the mitochondria. [8] Several groups have shown that a constitutively active, myocardial-specific MMP-2 transgene or transient oxidative stress increased mitochondrial MMP-2 levels, specifically by the appearance of a unique MMP-$2_{NTT\text{-}76}$ isoform.[36] This isoform is an N-terminal truncated MMP-2 that is not present under physiological conditions, but is generated by oxidative stress-induced activation of a latent promoter in the first intron of the MMP-2 gene and is localized to both the cytosol and the mitochondria.[36] However, these studies used only mitochondrial-enriched preparations which would contain a portion of the endoplasmic reticulum that is proteinaceously bound to mitochondria, known as the mitochondria-associated membrane (MAM). The MAM is a highly specialized and dynamically regulated subdomain of the ER, which transmits $Ca^{2+}$ signals between the ER and mitochondria, and therefore plays important roles in mitochondrial energy production, ER oxidative protein folding, mitochondrial fission and lipid metabolism.[37] When highly purified mitochondria and MAM fractions were isolated from normal rat hearts, MMP-2 (72 kDa) is predominantly localized to the MAM in comparison to mitochondria. Further, the ability of

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this isoform to cleave calreticulin, an important regulator of ER-MAM $Ca^{2+}$ homeostasis, has been demonstrated.[38]

In summary, MMP-2 is found in both mitochondria and MAM in normal hearts, with much greater levels of MMP-2 in the mitochondria compared to the MAM. Additionally, the MMP-$2_{NTT\text{-}76}$ isoform is only expressed following the induction of oxidative stress, and appears to target the cytosolic and crude mitochondrial fractions.[36] Given the key role of both mitochondria and MAM in I/R injury of the heart, the discovery of the precise targets and biological functions of MMP-2 at these locales awaits further confirmation.

## Nuclear MMP-2

By immunogold electron microscopy, MMP-2 protein was found located within the nuclei of the cardiac myocyte.[39] Purified nuclear extracts from both human hearts and rat liver showed MMP-2 protein and activity. Analysis of the MMP-2 amino acid sequence suggests that this protease possesses a nuclear localization sequence in its C-terminal domain. Gelatinolytic activity of 72 kDa MMP-2 was found in anti poly-ADP-ribose polymerase (PARP) immunoprecipitates from human heart nuclear extracts. Moreover, PARP was susceptible to cleavage by MMP-2 *in vitro*. This led to speculation that MMP-2 cleavage of PARP in the nucleus could either be cytoprotective, by reducing PARP activity (as oxidative stress results in excessive PARP activation and energy depletion of the cell), or detrimental, by preventing PARP's ability to repair DNA strand breaks.[39] However, the biological role of MMP-2 in the nucleus remains unclear at this time.

## Caveolae-bound MMP-2

In addition to mitochondrial and nuclear localization, MMP-2 is found in the caveolae of cardiomyocytes. Studies in isolated working hearts demonstrated that MMP-2 is present in lipid raft enriched fractions prepared from the hearts of wild type but not caveolin-1 null mice.[40] Sequence analysis of human MMP-2 reveals seven caveolin binding motifs. The caveolin scaffolding domain of caveolin-1 reduces MMP-2 activity *in vitro*. Thus, it is possible that caveolae-bound MMP-2 may be a further means to reduce intracellular activity by maintaining a fraction of total cellular MMP-2 in a membrane-associated and inhibited state. Indeed, the level of MMP-2 activity was increased in whole heart extracts from caveolin-1 knockout mice compared to wild type controls.[41] However, contractile function was not impaired and no changes in alpha-actinin or TnI levels were demonstrated in the null mice, leaving the role of caveolae-associated MMP-2 unclear.

## Intracellular MMP-2 isoforms

Despite biochemical and imaging evidence for intracellular MMP-2, a great deal of skepticism towards intracellular MMP-2 biology exists. MMP-2 possesses a signal sequence domain that allows a newly formed protein to enter the lumen of the ER for entry into the secretory pathway, yet, paradoxically, at least two intracellular MMP-2 isoforms have been described.[42] Importantly, using a chimeric protein approach, the signal sequence of canonical 72 kDa MMP-2 was found to inefficiently target MMP-2 to the ER, resulting in about half of nascent MMP-2 remaining in the cytosolic fraction. In addition to 72 kDa

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MMP-2, splice variant of MMP-2 with an N-terminal truncation (MMP-$2_{NTT-50}$) is exclusively present in the cytosolic fraction of both adult human and neonatal rat cardiomyocytes. Interestingly, as the level of mRNA for MMP-$2_{NTT-50}$ is only about one tenth that of canonical MMP-2, it appears that the latter is likely the major intracellular MMP-2 moiety in normal cardiomyocytes.[42]

Beyond these two isoforms, the MMP-$2_{NTT-76}$ variant discussed above only appears following oxidative stress,[36] making a total of at least 3 possible intracellular MMP-2 isoforms. The MMP-$2_{NTT-76}$ isoform lacks both the signal sequence and two of the three alpha-helices of the prodomain, which function to maintain MMP-2 in its latent form. This MMP-$2_{NTT-76}$ isoform is therefore predicted to be proteolytically active.[36] Transgenic mice with myocardial specific overexpression of MMP-$2_{NTT-76}$ display mitochondrial dysfunction, inflammation, hypertrophy and systolic dysfunction.[43] Canonical MMP-2 was shown to be (and the MMP-$2_{NTT-50}$ variant should also be) activated by low levels of peroxynitrite predicted to be found under both physiological and pathological conditions.[20] Little is known about the comparative substrate profiles and specificities, TIMP binding capacities, or MMP inhibitor profiles amongst the three intracellular MMP-2 moieties. The roles of these isoforms in cardiac physiology and pathology are an exciting area of future research.

## Targeting intracellular versus extracellular MMP-2 or other proteases

MMP-2 is an important intracellular protease effector of the acute consequences of oxidative stress in the heart and other organs. Prolonged periods of ischemia, during which myocyte necrosis occurs, results in MI.[12] While reperfusion of the infarcted area is beneficial to the patient, this procedure also induces oxidative and inflammatory responses in the heart that can lead to further organ damage, a phenomenon known as reperfusion injury.[13] Intracellular activation of MMP-2 by post-translational modifications, such as glutathiolation and/or dephosphorylation, contributes to an amplified inflammatory response and subsequent cellular damage that can induce cardiac dysfunction.[44]

The post-translational modifications of the intracellular isoforms of MMP-2 by both peroxynitrite and phosphorylation have consequences on its three dimensional structure and activity.[21] This provides further clues as to how MMP inhibitors may influence its biological effects. Human and rat MMP-2 are phosphorylated at serine, threonine and tyrosine residues, and proteomic analysis confirmed at least 5 phosphorylated residues on the surface of MMP-2, some of which are in the collagen-binding domain or adjacent to the catalytic cleft.[44,45] Protein kinase C treatment of MMP-2 results in reduced MMP-2 activity, whereas dephosphorylation of MMP-2 (with alkaline phosphatase) increases its activity. The combined effects of both peroxynitrite and phosphorylation on MMP-2 activity *in vitro* showed that 72 kDa MMP-2 activity was primarily affected by its phosphorylation status, yet it could still be activated by as little as 0.1 μM peroxynitrite in the presence of glutathione. The secondary structure of MMP-2 is also affected by its phosphorylation status.[21] The substrate specificities of intracellular vs. extracellular MMP-2 isoforms are likely to be quite different. Therefore, an important consideration is to find a preferential substrate of intracellular MMP-2 activity (in contrast to gelatin and commercially available

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substrates such as OmniMMP). TnI has been used as a model substrate of intracellular MMP-2 activity. Additionally, substrate post-translational modifications, such as phosphorylation, can change sub-cellular localization of the substrate or the binding and susceptibility to cleavage by MMP-2. For example, phosphorylation of myosin light chain-1 increases as a consequence of I/R, which subsequently enhances its affinity to proteolysis by MMP-2 both *in vivo* and *in vitro* in isolated rat hearts.[46] This area involving the effects of substrate modification on MMP efficacy requires further exploration.

Because phosphorylation regulates MMP-2 activity, the phosphorylation status of MMP-2 in isolated rat hearts subjected to I/R injury was investigated. The serine/threonine phosphatase inhibitor, okadaic acid, at a concentration which specifically inhibited protein phosphatase 2A but not protein phosphatase 1 activity (100 nM), increased the phosphorylation of myocardial MMP-2, reduced TnI loss in the hearts, and improved recovery of mechanical function post-I/R. This highlights that maintaining MMP-2 in a highly phosphorylated (and thus, less active) state may be another means to prevent its detrimental effects during I/R injury.[45]

Other intracellular proteases, such as calpains and caspases, are also implicated in myocardial I/R injury.[47,48] However, much of this work was done prior to the discovery of intracellular MMP-2, so the possible role of these other proteases was often predicted using enzyme inhibitors thought to be specific towards these individual proteases. When caspase or calpain inhibitors were tested for their specificity against MMP-2, it was found that several widely employed calpain and caspase inhibitors also block MMP-2 activity at commonly used concentrations in cell and isolated organ studies.[49,50] Thus, it is likely to emerge that several effects previously ascribed to the intracellular biological activities of either caspases or calpains, especially in the setting of oxidative stress injury such as I/R, may be due to the activation of MMP-2.

The direct activation of MMP-2 very early during oxidative stress injury is likely to influence long-term processes, which may tip the balance from reversible to irreversible injury in the myocyte. As a result of irreversible cellular damage, an induced inflammatory state in the heart contributes to the progression to heart failure. Indeed, the formation of MMP-$2_{NTT\text{-}76}$ in response to oxidative stress is another example of the initiation of an innate immune response via NF-κB and NFAT stress signaling to cause progressive myocardial contractile dysfunction.[36] Indeed, MMP-$2_{NTT\text{-}76}$ transgenic mice develop progressive cardiomyopathy, ventricular hypertrophy, and eventually systolic heart failure.[43] In addition, proteolytic fragments of MMP-2 intracellular targets, no longer recognized as endogenous proteins, may also trigger auto-immune responses, inflammation, and chronic disease.[8] While the relationship between MMP-2 and inflammation has been established, the complex cascades of protease activation, as well as exactly where MMP-2 lies in these cascades, are not clear. It is likely that oxidative stress-induced MMP-2 activation results in degradation of cellular and tissue components in the heart leading to inflammation, but the exact sequence and targets of proteases in the inflammatory cascade remain unknown.

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## MMP translational studies: from animal models to humans

The involvement of MMPs in normal and pathological myocardial remodeling of the heart is well documented.[51,52] With few exceptions, the relationships between myocardial disease evolution, changes in MMP levels and activation state, and MMP-mediated ECM substrate processing have been demonstrated in numerous preclinical models of myocardial disease, as well as in patients.[53]

Animal models have been useful in defining the temporal changes that occur in MMP expression and activity following ischemic injury.[54,55] For example, pigs subjected to MI had increased MMP-2 activity detected over the course of several hours following MI.[56] Pigs subjected to MI were examined by non-invasive imaging methods and revealed increases in MMP-targeted tracer uptake within the infarct zone 1 week post-MI and maximal uptake of the tracer two weeks post-MI.[57] Assessment of MMP activity (MMP-2/9) and protein content (MMP-7, MT1-MMP) also showed early robust increases within the infarcted region 1 week post-MI, and these elevations were sustained four weeks post-MI compared to controls.

With pathological cardiac remodeling, such as that observed with LV pressure and volume overload, stress on the myocardium evokes adverse changes in ventricular microstructure that leads to cardiac hypertrophy and altered LV wall anatomy.[58] Studies using pressure overload in the dog, as well as volume overload models, revealed differences in MMP activation profiles that were time and model dependent.[59] Notable differences in MMP-1 changes were observed between models, whereas MMP-2 levels activity tracked to both early and later time points (i.e., 6 hours vs. 10 days). In the setting of congestive heart failure-associated cardiac hypertrophy, pacing tachycardia in pigs showed time-dependent MMP activity profiles associated with declining ventricular function.[60] Further, early increases in several LV tissue MMPs (MMP-1, MMP-2, MMP-3) occurred in parallel with the early evolution and progression of LV remodeling and dysfunction.

The translational merits of these and other preclinical studies, which aim to better understand the role of MMPs in human myocardial diseases, cannot be overstated. MMP-2 activity has been analyzed in myocardial and blood samples taken from patients with varying degrees of cardiovascular disease.[61] In patients with end-stage dilated cardiomyopathy, activity of several MMPs, including MMP-2, was shown to increase in the LV.[62,63] Moreover, examination of MMP activity profiles from failing human myocardium showed increased tissue gelatinase activities that were associated with reductions in fibrillar collagen cross-links.[63,64] Changes in circulating MMP levels have been used as surrogate indicators of MMP activity in the diseased myocardium. Several studies in post-MI patients have shown that plasma MMP levels rise early after MI and that these changes can predict the propensity for adverse LV dilation.[65] In the setting of pathological myocardial fibrosis, such as that observed in patients with heart failure with preserved ejection fraction (HFpEF), excessive collagen deposition is not only a consequence of increased collagen synthesis, but also suggests attenuated collagen degradation.[66] Indeed, patients in hypertensive HFpEF demonstrated decreased matrix degradation, which was associated with attenuated MMP/gelatinase activity and increased

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TIMP gene expression, suggesting a role for MMPs in cardiac disease.[66] Together, animal and human studies have demonstrated the potential for targeting MMPs therapeutically in myocardial disease.

## MMPs and clinical trials

Recognizing the important roles that MMPs play in mediating injury to the myocardium in the setting of ischemic events, multiple investigators implemented pre-clinical studies to examine the capacity of various MMP inhibitor compounds to protect the myocardium.[67]. MMP inhibitors, which are predominantly hydroxamate based, were originally developed by several drug companies with the intent of using these agents to inhibit cancer metastasis. While pre-clinical results from cancer studies were mostly encouraging, they failed to translate into the clinic due to issues related to unexpected adverse effects.[67] Only one clinical trial used a hydroxamate MMP inhibitor (PG116800) to assess for protective effects on heart structure and function following myocardial infarction.[68] However, results failed to show a significant positive effect on the primary endpoints, including LV diastolic or systolic volume, ejection fraction, sphericity index, or the incidence of reinfarction or death in these patients. Almost simultaneously, a promising area of MMP inhibition emerged with tetracyclines. Tetracyclines, are a class of broad spectrum antibiotics discovered in the 1940's from screening of soil samples for antibiotic organisms (a recent review on tetracycline pleiotropy can be found in [69]). Members of this class of antibiotics include oxytetracycline, chlortetracycline, minocycyline and doxycycline amongst others. The antibiotic mechanism of action of tetracyclines is thought to be related to the inhibition of protein synthesis. However, many other tissue protective properties have been ascribed to this class of compounds, including anti-oxidant, anti-inflammatory, anti-apoptotic and protease inhibitor properties. The most potent tetracycline MMP inhibitor, doxycycline, is best known for its anti-MMP activity, which was first reported by Golub and colleagues in the 1980's.[70] Because of the MMP inhibitory effect of doxycycline, studies began to investigate its efficacy in treating cardiac injury.

Mimicking what occurs in patients undergoing reperfusion during coronary bypass surgery where an early release of MMPs is observed, Cheung et al. demonstrated that after 20 min of global no-flow ischemia in isolated rat hearts, there was a marked increase in MMP-2 in the coronary effluent that peaked within the first minute of reperfusion.[71] This group also demonstrated that the release of MMP-2 into the effluent during reperfusion was enhanced with increasing duration of ischemia, and this MMP-2 release correlated negatively with the recovery of mechanical function during reperfusion.[72] Doxycycline improved mechanical function during reperfusion, demonstrating for the first time its cardioprotective potential. Multiple other studies evidenced the capacity of doxycycline to act as an MMP inhibitor. Most convincingly, the molecular mapping of doxycycline binding to MMP-7 yielded evidence for the mechanistic means by which this compound acts as an effective MMP inhibitor.[73] As MMPs play a critical role in mediating periodontitis, doxycycline was extensively examined for this application and eventually gained FDA approval, becoming the first FDA approved small molecule MMP inhibitor.[67] Interestingly, the anti-MMP effect of doxycycline occurs at sub-antimicrobial plasma levels (at about 1/5 the level

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necessary for antimicrobial activity), and a sub-antimicrobial formulation of doxycycline is currently the only MMP inhibitor clinically approved for treatment of any disease.

Villarreal and colleagues were the first to demonstrate *in vivo* that early, short term treatment of rats subjected to MI with doxycycline (one dose 48 hours before and one dose 36 hours after coronary occlusion) significantly decreased cardiac hypertrophy, myocyte cross-sectional area, and internal LV diameter, while preserving infarcted wall thickness 14 days after MI.[74] Doxycycline yielded parallel left shifts in LV pressure-volume relationships and epicardial scar area strain patterns, similar to normal myocardium. The assessment of global MMP and MMP-2 and -9 activities in the LV 1 hour post-MI also evidenced significant differences with doxycycline. Subsequent studies further substantiated these observations in pigs, and also expanded on the anti-remodeling properties of doxycycline when given from 2–7 days post-MI.[75,76] As a result of these studies, a clinical trial was implemented and the results were recently reported.[77] The Early Short-term Doxycycline Therapy In Patients with Acute Myocardial Infarction and Left Ventricular Dysfunction to Prevent The Ominous Progression to Adverse Remodeling (TIPTOP) trial evaluated the efficacy of antimicrobial doses of doxycycline (100 mg twice daily for 7 days started immediately post-percutaneous intervention, n = 110) on post-MI remodeling observed up to 6 months post-treatment. Results yielded significant decreases in LV end-diastolic volumes, infarct size and severity in the doxycycline treated patients. Thus, emerging clinical evidence supports a possible role for an MMP inhibitor in ameliorating adverse cardiac remodeling. However, larger double-blinded, multi-site clinical trials will need to be implemented to validate the use of this class of compounds as a means to limit adverse cardiac remodeling. Trials including non-antimicrobial variants, specifically, chemically modified tetracyclines that have demonstrated an apparent safe clinical profile, would be of particular interest.[78]

Important lessons have emerged from studies involving MMPs in inflammation and cardiac injury. Early in the setting of ischemic injury (and in particular, with reperfusion) activation of MMP-2 contributes to the development of myocardial injury. As much as activated MMP-2 mediates injury to the myocyte contractile apparatus, it and other MMPs act on the structural proteins that comprise both the intracellular and extracellular matrix lattices, which both provide structural support. Early protection of these matrices would thus be essential for preventing myocardial contractile dysfunction and infarct wall rupture, and for limiting infarct expansion, which contributes to latter progressive adverse remodeling. It is also increasingly recognized that in the latter phases of injury and dysfunction, MMP activation may be necessary for proper healing and scarring to occur.[79] Thus, the timing and duration of MMP inhibitor treatment is likely to be critical for future clinical studies to succeed, as evidenced by the TIPTOP trial. Currently, it appears that providing MMP inhibition just prior to reperfusion and for a maximum of 7 days post-infarction may be a good strategy. It is important to recognize the potential for implementing strategies that provide monitoring of MMP activity levels in plasma early after infarction in a manner akin to the monitoring for creatine kinase, lactate dehydrogenase, or troponin. Currently, there is an ample catalog of well-validated tests, such as ELISA kits and radioimmunoassays, which detect MMP-2 in plasma, the coupling status with TIMPs, and MMP-2 proteolytic targets.

Their practical validation in the clinical setting will facilitate the best optimization of treatment and monitoring of outcomes.

## Future directions and conclusions

While a tremendous amount of work has been done to uncover new intracellular substrates processed by MMP-2, a number of areas of investigation need to be performed. For one, greater understanding of the connection between intracellular and extracellular MMP-2 is needed. While the myocardial ECM is an interstitial transport system, the tracks leading from the ECM continue intracellularly to provide a continuum from the nucleus of one cell to the nucleus of another.[80] Another area of investigation involves the different intracellular MMP-2 isoforms, the effects of their post-translational modifications, and their unique roles in cardiac pathologies. The development of MMP-2 specific inhibitors, particularly for intracellular isoforms, is highly encouraged. At the same time, doxycycline, a well understood, safe, and effective MMP inhibitor, requires further clinical studies in ischemic injury. Variation in the structure of other MMPs, as well as their activity, remains to be evaluated. Understanding how MMPs, and MMP-2 in particular, regulate intracellular functions will provide greater insight into our understanding of myocardial ischemic injury.

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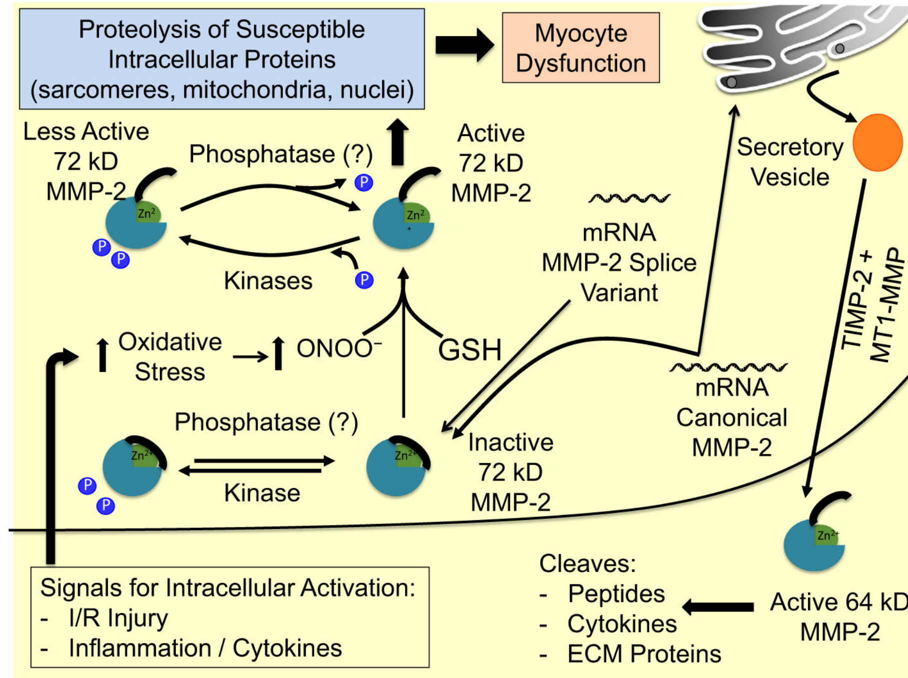


**Fig. 1.**

MMP-2 targets to the cytosol of the myocyte, where it undergoes post-translational modifications. The 72 kDa form of MMP-2 can be activated extracellularly by proteases (e.g., plasminogen or MMP-14) to yield a 64 kDa active form. The 72 kDa form of MMP-2 can also be activated intracellularly by S-glutathiolation, in the presence of peroxynitrite induced by ischemia/reperfusion or pro-inflammatory cytokines. This activation occurs in the presence of glutathione and does not require proteolytic removal of the pro-domain. This active 72 kDa MMP-2 form targets susceptible intracellular proteins within the cardiomyocyte to induce contractile dysfunction. MMP-2 targets to the cytosol by at least two mechanisms: 1) An MMP-2 splice variant in cardiomyocytes lacks the secretory signal sequence, resulting in its intracellular retention. 2) The canonical MMP-2 signal sequence is inefficient, yielding MMP-2 that targets to both the endoplasmic reticulum for secretion as well as MMP-2 that targets to the cytosol. In addition, MMP-2 has been shown to be phosphorylated at several sites, which modulates its activity. GSH, glutathione; $ONOO^-$, peroxynitrite; PKC, protein kinase C.

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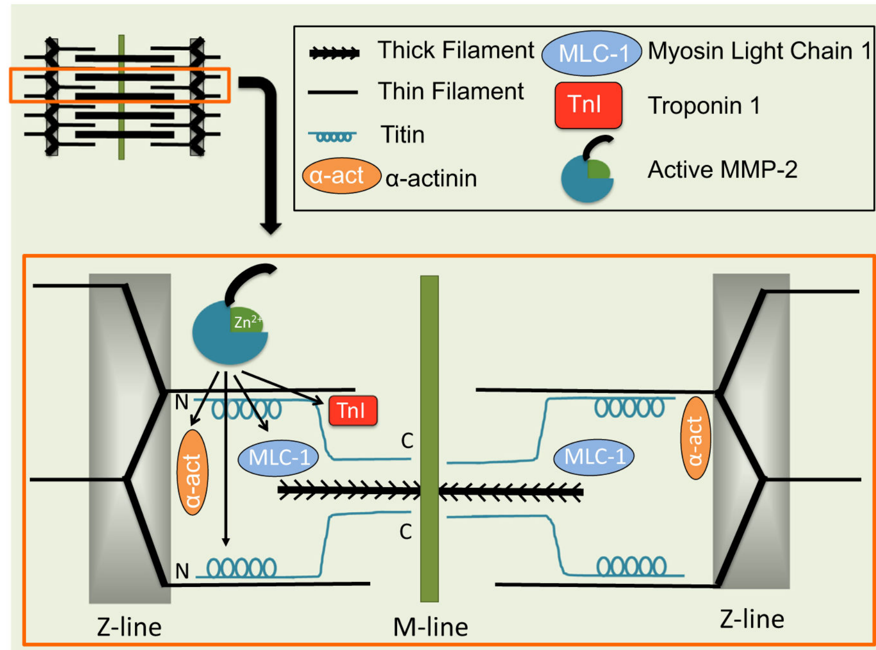


**Fig. 2.**

MMP-2 intracellular substrates in the cardiac sarcomere. MMP-2 co-localizes with and proteolytically processes multiple sarcomeric proteins during ischemia/reperfusion. Included in the list of MMP-2 targets are troponin I (a thin myofilament component), myosin light chain-1 (a thick myofilament component), titin, α-actinin (a cytoskeletal protein found in the Z-disc). Titin is the largest mammalian protein, with one molecule spanning half the length of the sarcomere, from Z-disc to the M-line. C, titin C-terminus; N, titin N-terminus.

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**Table 1**

MMP-2 substrates.

| | References |
|---|---|
| *Extracellular matrix substrates* | |
| α-1-antichymotrypsin | [81] |
| A beta peptides | [82,83] |
| Aggregan | [84] |
| Chondroitin sulfate proteoglycans | [85] |
| Collagen I, III, IV, VI (α-chain), IX, X, XII, V/XI | [82,84,86–91] |
| Beta-1 integrin | [92] |
| Big endothelin-1 | [93] |
| Elastin | [87,94] |
| Fibroblast growth factor receptor 1 | [95] |
| Fibronectin | [96] |
| Galectin-3 | [97] |
| Gelatin | [97] |
| Insulin-like growth factor binding protein | [98,99] |
| Interleukin-1-beta | [6,7] |
| Laminin-5 | [100,101] |
| Latent transforming growth factor-β | [102] |
| Monocyte chemoattractant protein-3 | [103] |
| Myelin basic protein | [104] |
| Pro-lysyl oxidase | [105] |
| Pro-MMP-1, -2, -9, -13 | [99,106–108] |
| Proteoglycan link protein | [109] |
| Secreted protein acidic and rich in cysteine | [110] |
| Substance P | [111] |
| Tenascin | [112,113] |
| Tumor necrosis factor precursor | [114] |
| Tumor necrosis factor-related apoptosis inducing ligand | [115] |
| Vitronectin | [94] |
| *Intracellular protein substrates* | |
| α-actinin | [27] |
| Calponin-1 | [49] |
| Desmin | [27] |
| Glycogen synthase kinase-3β | [35] |
| Myosin light chain-1 | [11] |
| Poly-ADP ribose polymerase | [39] |
| Troponin-I | [8] |
| Titin | [28] |

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**Table 2**

Myocardial cells expressing MMP-2.

| Cell | Extracellular | Intracellular | Intracellular NTT-76 MMP-2 |
|---|---|---|---|
| Myocytes | Yes | Yes | Yes* |
| Endothelial cells | Yes | Yes | Unknown |
| Fibroblasts | No | Yes | Unknown |
| Leukocytes | Yes | No | No |

* no expression under basal conditions, but only in response to oxidative stress