Role of Tissue Inhibitor of Metalloproteinase-2 in Human Cardiac Fibroblast-Mediated Extracellular Matrix Remodeling

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Role of Tissue Inhibitor of Metalloproteinase-2 in Human Cardiac Fibroblast-Mediated Extracellular Matrix Remodeling

by

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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Abstract

After a myocardial infarction, extracellular matrix (ECM) dysregulation leads to maladaptive cardiac remodeling that constitutes the basis of development of heart failure. Tissue Inhibitor of Metalloproteinase-2 (TIMP-2) is an endogenous biomolecule that is critical in the maintenance of ECM architecture. Cardiac fibroblasts are the main cell type that regulates ECM homeostasis. This study employed an innovative method of three-dimensional collagen gel assay, which mimics the natural in vivo ECM. We investigated the effect of TIMP-2 on the human cardiac fibroblast-mediated ECM remodeling. TIMP-2 induced differentiation of cardiac fibroblasts into myofibroblasts that are active in collagen synthesis. Concurrently, TIMP-2 induced an increase in the total protease activity within the collagen gel microenvironment. TIMP-2 did not promote a fibrotic response, despite its ability to activate myofibroblasts. These actions appear to be independent of its MMP-inhibitory actions. In conclusion, TIMP-2 promotes ECM homeostasis via simultaneous induction of myofibroblast activation and total protease activity.
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Lastly, I thank my faith. I believe everything happens for a beautiful purpose and all things work together for good. The challenges I have encountered in the past have shaped me into a better person. As I move on, I look forward to overcome more obstacles in life with hope.
This work is dedicated to the memory of my beloved mother
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<tbody>
<tr>
<td>2-D</td>
<td>Two-Dimensional</td>
</tr>
<tr>
<td>3-D</td>
<td>Three-Dimensional</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha-Smooth Muscle Actin</td>
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<tr>
<td>AT-11</td>
<td>Angiotensin-II</td>
</tr>
<tr>
<td>AT1-Receptor</td>
<td>Type 1 Angiotensin-II Receptor</td>
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<tr>
<td>CR</td>
<td>Cardiac Remodeling</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>HF/CHF</td>
<td>Heart Failure/Congestive Heart Failure</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LSM</td>
<td>Laser Scanning Microscope</td>
</tr>
<tr>
<td>LV</td>
<td>Left Ventricular</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>MT-MMP</td>
<td>Membrane Type-Matrix Metalloproteinase</td>
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<tr>
<td>MT1-MMP</td>
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<tr>
<td>NYHA</td>
<td>New York Heart Association</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-Angiotensin-Aldosterone System</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-Beta</td>
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<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinase</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<td>WT</td>
<td>Wild Type</td>
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Chapter One: Introduction

1.1 Significance/Background

Due to advances and innovations in treating acute myocardial infarction (MI), a significant increase in the incidence and prevalence of heart failure among the Canadian population has been observed recently (Johansen et al., 2003). The number of patients surviving an acute episode of MI has significantly increased, resulting in a growing population of patients predisposed to the development of congestive heart failure (CHF). Within 6 years of the clinical event of acute MI, 22% of male and 46% of female patients develop CHF (Rosamond et al., 2008). CHF currently affects over 500,000 people in Canada with 50,000 new diagnoses each year (Ross et al., 2006). The average annual mortality rate is 10% per year with a 50% five-year survival rate. Hospital readmission rates are high, mainly due to recurrent episodes of clinical decompensation and overt congestion (Lee et al., 2004). Contemporary treatments involve medical management for symptomatic relief only and heart transplantation is the only “cure” for end-stage heart failure. Only a small proportion of the end-stage heart failure patients receive heart transplantation due to significantly limited supplies of donor organs. The consistent rise in the incidence and prevalence of end-stage heart failure during the past two decades has raised concerns as to the fate of this expanding patient group.

After MI, the myocardium undergoes progressive changes in structure and function that can lead to clinical decompensation and CHF. Accordingly, there is a growing interest in the pathophysiological mechanisms that regulate and coordinate the functional and structural changes after MI. Understanding these mechanisms may lead to novel therapeutic strategies and important clinical targets.
Literature Review

1.1.1 Definition and Concepts of Remodeling

Remodeling is a term that indicates changes that give rise to rearrangement of normally existing structures (Swynghedauw, 1999). “Cardiac remodeling” (CR) is the process that ultimately leads to heart failure, regardless of its etiology (Udelson & Konstam, 2002; Lijnen et al., 2003). CR is defined as the changes in genome expression, molecular, cellular and interstitial that manifest clinically as changes in the size, shape and function of the heart after a myocardial injury (Cohn, Ferrari, & Sharpe, 2000). Another term, “myocardial remodeling,” is often used to describe the pathological changes in myocardium in diseased conditions, which exclude gestational and developmental aspects as well as the so-called physiological cardiac hypertrophy that follows intensive exercising (Swynghedauw, 1999).

In spite of successful early therapy, the ventricular remodeling causes progressive LV dilatation and heart failure even in post-MI patients with good ventricular functions at time of discharge (Pfeffer & Braunwald, 1990). The clinical experience from coronary artery reperfusion indicates that the LV functions do not normalize in 30% of the patients (Ferreira, 2010). The process of remodeling begins rapidly within the first few hours after an infarct and continues to progress. Numerous changes occur in post-infarct remodeling, which include inflammation and reabsorption of necrotic tissue, infarct expansion, scar formation and fibrosis, thinning of ventricular wall, reshaping and dilation of left ventricle, myocyte hypertrophy and ongoing loss (Cohn, Ferrari, & Sharpe, 2000). Clinically, the progression of heart failure is highly predictive with well-defined anatomical and functional changes to the heart. However, the fundamental
mechanisms underlying these events still remain elusive and warrant further investigation in order to develop new targeted therapy for heart failure.

1.1.2 Cardiac Fibroblasts & Myofibroblasts

The myocardium is composed of several cell types, such as cardiomyocytes, fibroblasts and endothelial cells, and vascular and neuronal networks (Van den Borne et al., 2010). Cardiac fibroblasts are the predominant cell type in myocardium (Eghbali et al., 1988). Cardiac fibroblasts outnumber cardiomyocytes by a factor of 2 to 3 (Banerjee et al., 2007), in order to account for constant ECM collagen turnover rate of approximately 5% per day (McAnulty & Laurent, 1987). Morphologically, cardiac fibroblasts are very flat and somewhat spindle shaped, with multiple processes projected from the main bodies. They are also characterized by the lack of a basement membrane (Souders, Bowers, & Baudino, 2009). Fibroblasts are present in virtually all parts of body and they demonstrate extensive phenotypic heterogeneity depending on their organ of origin. Chang and coworkers demonstrated that human fibroblasts from different anatomic sites displayed distinct and characteristic topographic differentiation and gene expression (Chang et al., 2002). Interestingly, with regard to the surrounding physiological or pathological conditions, fibroblasts of the same organ origin also exhibit different phenotypic characteristics. For instance, when compared to healthy skin fibroblasts, skin fibroblasts isolated from patients with systemic sclerosis were shown to be more resistant to Fas-mediated apoptosis and more active in collagen synthesis (Fries et al., 1994; Jelaska, Strehlow, & Korn, 1999). In myocardium, fibroblasts are the main cell type that is responsible for the regulation of ECM homeostasis through at least three mechanisms: (1) the synthesis and deposition of ECM molecules (collagen and others); (2) the regulation of ECM turnover by secreting MMPs and
TIMPs; and (3) the maintaining of mechanical tension on 3-D collagen network (Souders, Bowers, & Baudino, 2009). Cardiac fibroblasts are the main source of several cytokines critical in the process of cardiac remodeling, such as transforming growth factor-beta (TGF-β) (Chen et al., 2004), angiotensin-II (AT-II) (Sanghi et al., 2005), IL-6, IL-1β and tumor-necrosis factor-alpha (TNF-α) (Corda, Samuel, & Rappaport, 2000; Brown et al., 2005).

Quiescent fibroblasts differentiate into the invasive myofibroblast phenotype, driven by increased expression of TGF-β and other profibrotic cytokines after a myocardial injury (Brown et al., 2005). Gabbiani and colleagues first described myofibroblasts in 1971 as a unique cell type that contains ultrastructural and functional features of smooth muscle cells, as well as extensive endoplasmic reticulum of synthetically active fibroblasts to produce collagen (Gabbiani, Ryan, & Majne, 1971). In healthy myocardium, there is no evidence of myofibroblast activity (Porter & Turner, 2009) with the exception of valve leaflets. Shortly after myocardial injury, myofibroblasts begin to appear at the site of injury. The cells reach the site of injury by way of: (1) local phenotypic conversion from resident interstitial and adventitial fibroblasts (Desmoulière, Chaponnier, & Gabbiani, 2005); (2) from bone marrow-derived circulating precursors “fibrocytes” (Van Amerongen et al., 2008); (3) from cells undergoing epithelial/endothelial-to-mesenchymal transition (Kalluri & Neilson, 2003; Zeisberg et al., 2007); and (4) from “pericytes” dissociating from the walls of capillaries and other small blood vessels (Díaz-Flores et al., 2009). Figure 1-1 summarizes the origin of cardiac myofibroblasts at the site of myocardial injury (Daskalopoulos, 2012).
In response to numerous stimuli, several cell types can differentiate into myofibroblasts. These cells first differentiate into an intermediate form (proto-myofibroblast) and eventually transform into fully differentiated myofibroblasts characterized by the de novo expression of α-SMA and extra-domain A fibronectin (ED-A FN).

Figure 1-1: Origin of cardiac myofibroblasts at the site of myocardial injury (adapted from Daskalopoulos 2012)
1.1.3 Regulations of Myofibroblast Activation

The initial transition of fibroblasts to the proto-myofibroblast phenotype is mainly driven by the increased mechanical tension secondary to the disrupted ECM integrity. The primary characteristics of the proto-myofibroblasts are the increased expression of fibronectin and the presence of mature focal adhesion proteins. The central feature that discriminates proto-myofibroblasts from differentiated myofibroblasts is the absence of α-SMA expression (Meran & Steadman, 2011).

Activation of myofibroblasts can be driven by a broad spectrum of stimuli: chemical, mechanical, electrophysiological, and miscellaneous stimuli. Among all these classes, the chemical stimuli play a major role in this differentiation (and this class will be further reviewed in the following section). Cyclic mechanical stretch provokes the expression of Collagen I and III (Husse et al., 2007), proteoglycans, and elastin (Fomovsky, Thomopoulos, & Holmes, 2010), which are the components actively synthesized by myofibroblasts. The myofibroblast gap junctions are composed of connexins, mainly Cx43 and Cx45 (Zhang et al., 2008). Increased Cx43 levels are associated with suppressed myofibroblast proliferation and vice versa (Zhang et al., 2008). Other unclassified stimuli involved in activation of myofibroblasts include ED-A fibronectin (Manabe et al., 1997; Serini et al., 1998), hyarulonan (Webber et al., 2009), and osteopontin (Lenga et al., 2008; Trueblood et al., 2001). Figure 1-2 below summarizes the several classes of stimuli for differentiation of fibroblasts into myofibroblasts.
Several classes of stimuli can lead to the differentiation of fibroblasts into myofibroblasts. Chemical stimuli are the predominant mediator for this differentiation. **Abbreviations:** TGF-β, transforming growth factor-β; AT-II, angiotensin-II; ET-1, endothelin-1; PDGF, platelet-derived growth factor; CTGF, connective tissue growth factor.
1.1.3.1 Chemical Stimuli of Myofibroblast Differentiation

TGF-β is a multifunctional growth factor, critical for cell proliferation, migration, differentiation, and apoptosis (Tomasek et al., 2002) and it is the most important regulator of the post-MI fibrotic response. TGF-β exists in three isoforms (TGF-β1, TGF-β2 and TGF-β3), with TGF-β1 being the predominant form (Sporn & Roberts, 1992). There are two main mechanisms in which TGF-β induces phenotypic differentiation of fibroblasts into myofibroblasts: via the well-known Smad pathway and several mitogen-activated protein kinase (MAPK) pathways (Biernacka et al., 2011). In the Smad pathway, the downstream effectors of TGF-β are a family of transcriptional activators called Smad (Shi & Massagué, 2003). Briefly, active TGF-β binds to the TGF-β receptor type II (TβRII), which leads to the phosphorylation and recruitment of TGFβ receptor type I (TβRI) into a heterodimer receptor complex (Massagué, 2000). Binding of TGF-β to its receptor phosphorylates Smad2 and Smad3 which each binds to Smad4 and translocates into the nucleus to enhance transcription of target genes, such as α-SMA (Feng & Derynck, 2005; Massagué, Seoane, & Wotton, 2005). Inhibitory Smads serve as negative regulators by antagonizing TGF-β signalling by binding to TβRI (action of Smad7) (Monteleone et al., 2008) or by competing with activated Smads for binding to Smad4 (action of Smad6) (Imamura et al., 1997). An illustration of TGF-β/Smad signalling pathway is shown in Figure 1-3.

In addition to the activation of Smad-dependent cascades, TGF-β also transduces signals in a non-canonical fashion that involves several members of MAPK family. TGF-β can activate all three known MAPK pathways: extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun-N-terminal kinase (JNK) (Choi, 2000). Besides cellular differentiation, these mediator
proteins also regulate several other actions of TGF-β, which include proliferation, cell survival and apoptosis.

Figure 1-3: TGF-β/Smad signalling pathway (adapted from Pinzani & Marra 2001)

TGF-β binds to TβRII and this binding may be enhanced by the presence of TβRIII. After binding to TGF-β, TβRII recruits and phosphorylates TβRI and forms a heterodimer receptor complex. This complex subsequently activates Smad2 and Smad3, which each heterodimerizes with Smad4. Eventually, these heterodimers migrate into the nucleus to regulate specific gene transcription. Smad7 inhibits the binding of Smad3 and Smad4 to the phosphorylated (activated) TβRI.

Besides TGF-β, a number of chemical stimuli including vasoactive peptides and cytokines are capable of inducing the differentiation of fibroblasts into myofibroblasts. AT-II (angiotensin-II) and RAAS (renin-angiotensin-aldosterone system) have been implicated in the progression of cardiac remodeling. During the post-MI fibrotic repair, myofibroblasts actively
express ACE (angiotensin converting enzyme) and type 1 angiotenin-II receptors (AT1-receptors) (Sun & Weber, 1996). High levels of AT-II are associated with enhanced proliferation and differentiation of fibroblasts (Lijnen, Petrov, & Fagard, 2001; Rosenkranz, 2004; Sun et al., 1998). Pharmacological inhibition of AT-II signalling by ACE inhibitors and AT1-receptor antagonists suppressed TGF-β levels, indicating a direct link between AT-II and TGF-β (Sun et al., 1998; Yu et al., 2001).

Endothelin-1 (ET-1) is predominantly synthesized by endothelial cells, but also can be expressed by cardiomyocytes and fibroblasts (Shi-Wen et al., 2006). The expression of ET-1 can be induced by TGF-β (Shi-wen et al., 2007) and AT-II (Shephard et al., 2004). ET-1 is capable of inducing ECM production and differentiation of fibroblasts into myofibroblasts (Mucke, 2008). ET-1 is also a downstream mediator of fibrogenic responses of TGF-β (Shi-wen et al., 2007). Platelet-derived growth factor (PDGF) stimulates differentiation of fibroblasts into myofibroblasts in vitro (Rhee & Grinnell, 2006; Jinnin et al., 2005) and may also provoke cardiac fibrosis by elevating TGF-β levels (Tuuminen et al., 2009). Injection of a neutralizing antibody specific for PDGF-α receptors attenuated development of atrial fibrosis (Liao et al., 2010). Connective tissue growth factor (CTGF) is significantly expressed by cardiac myocytes in the process of cardiac remodeling (Daniels et al., 2009; Dziadzio et al., 2005). The CTGF is induced by TGF-β, AT-II, and ET-1, and therefore it is potentially a downstream mediator of these proteins (Leask, 2008; Schultz et al., 2002).

Several cytokines also play a role in activation of myofibroblasts. Abnormally high levels of circulating interleukin-6 (IL-6) are related to cardiac fibrosis (Dixon, 2010). The soluble IL-6 receptors induce differentiation of fibroblasts into myofibroblasts and enhance collagen deposit
by fibroblasts (Meléndez et al., 2010). Cardiotrophin-1 (CT-1), which is a member of the IL-6 cytokine family, also activates several TGF-β downstream signalling pathways, such as MAPK (Freed et al., 2003). Additionally, both IL-1β and TNF-α enhance the expression of AT1-receptors, resulting in enhanced myofibroblast activation (Gurantz et al., 2005).

1.1.4 Adverse Myocardial Fibrosis

Reparative fibrosis occurs as a replacement for necrotic myocytes in which a scar is formed (Weber & Brilla, 1992). After a myocardial injury, new ECM components are deposited; first at the borderzone between infarcted and un-infarcted areas, and later in the central area of injury (Van den Borne et al., 2010). The induction of myofibroblasts is initially adaptive in preventing myocardial rupture by increasing the stiffness of the infarct site (Van den Borne et al., 2010; Porter & Turner, 2009). A well-healed infarct scar contains a large amount of collagen fibrils and this is essential in preventing dilatation of the infarct area in order to maintain overall ventricular morphology.

On the other hand, reactive fibrosis can occur in the interstitial space and adventitia of intramyocardial coronary arteries without concurrent cell necrosis (Weber & Brilla, 1992). Most of the ECM-producing myofibroblasts are oriented parallel to the surviving adjacent cardiomyocytes (Willems et al., 1994). Excessive collagen synthesis at sites remote from the infarct area can result in myocardial stiffness and dysfunction that constitutes the basis of development of end-stage heart failure. Unlike in skin, in which myofibroblasts are relatively short-lived (Desmouliere et al., 1995), these cells persist for a long time in the myocardium with net balance in favour of ECM deposition, leading to myocardial fibrosis. Post-mortem human hearts showed persistent presence of myofibroblasts, even 17 years after MI (Willems et al.,
Ongoing myofibroblast activity in the late phase of post-MI remodeling can predict progression of heart failure by prolonging the initially adaptive remodeling, which creates in maladaptive changes over time. Several observations in the post-MI myocardium are associated with prolonged activation of myofibroblasts. The myocardial up-regulation of RAAS contributes significantly to excessive ventricular remodeling. Overexpression of AT1-receptors in the post-MI myocardium occurs in relation with adverse interstitial remodeling (Weber, Sun, & Katwa, 1997). An enhanced local production of AT-II—by activated macrophages, cardiomyocytes, and myofibroblasts—promotes myofibroblast activation by directly stimulating TGF-β1 synthesis (Sun et al., 2002). The activated myofibroblasts establish an autocrine cycle of myofibroblastic differentiation of activation (Wynn, 2008). As a result, myofibroblasts induce persistent ECM turnover via MMPs secretion (Romanic et al., 2001; Tao et al., 2004), which is followed by excessive collagen accumulation that results in myocardial fibrosis (Weber, 1997; Li, McTiernan, & Feldman, 2000). Myocardial fibrosis is characterized by increased density of type I collagen fibrils with enhanced crosslinking within the interstitial space in the remote un-infarcted area (Cleutjens et al., 1999).

1.1.5 Extracellular Matrix (ECM) of the Myocardium

Extracellular Matrix (ECM) of the myocardium is a three-dimensional scaffold that consists of a complex arrangement of fibrillar collagen (mainly type I collagen), elastin, microfibrillar proteins, proteoglycans, laminin, fibronectin, cytokines, growth factors, matrikine, and proteases (Bowers, Banerjee, & Baudino, 2010; Holmes, Borg, & Covell, 2005; Visconti & Markwald, 2006; Corda, Samuel, & Rappaport, 2000). Under normal conditions, ECM provides
structural support that facilitates cardiac function by: (1) preserving the alignment of myocytes and vessels; (2) preventing misalignment of cardiac myocytes and their muscle fibres during contraction; (3) acting as a force transducer that facilitates heart contraction during systole; and (4) calibrating diastolic myocardial stiffness and thereby protecting myocytes from overstretch (Weber et al., 1994).

In myocardium, ECM not only provides the physical scaffolding for the organization of cells, but presents a dynamic microenvironment to enmeshed cells that influences cellular behaviors. ECM responds to myocardial injury by altering its abundance, composition, and spatial organization with profound consequences on chamber structure and function (Libby & Lee, 2000). Immediately after a myocardial infarction, an inflammatory response is initiated that triggers the release of matrix metalloproteinases (MMPs). MMPs degrade the ECM components within the infarct zone where myocytes undergo necrosis, the resultant cellular debris is then removed by infiltrating macrophages. Simultaneously, new collagen fibrils will be deposited at the infarct area in the process of de novo scar formation in an effort to prevent myocardial rupture. The myocardium is subject to elevated mechanical stress including the remote non-infarcted myocardium. It is believed that mechanical stress initiates the remodeling of the myocardial ECM in an attempt to normalize the increased load. The properties of the interstitial collagen matrix determine the compliance of the myocardium, which together, influence both systolic and diastolic functions (Brower et al., 2006). To that end, any significant changes to the myocardial ECM can jeopardize the structural and mechanical integrity of the heart. Accordingly, the ECM has emerged as a key target in the search of novel therapeutic interventions to control myocardial remodeling after MI and prevent heart failure. Studies to
further our understanding of the complex myocardial microenvironment and its influence on cardiac fibroblasts in warranted.

### 1.1.6 Homeostasis of ECM

In a normal healthy heart, ECM synthesis and degradation is a continuous and tightly regulated process (Vanhoutte et al., 2006). The homeostasis and the integrity of the ECM are dependent on the balance between degradative matrix metalloproteinases (MMPs) and their highly regulated endogenous inhibitors: tissue inhibitors of matrix metalloproteinase (TIMPs) (Spinale, 2007). The post-MI myocardial expression of MMP and TIMPs is highly regulated within unique spatial and temporal windows (Vanhoutte et al., 2006).

![Figure 1-4: Post-MI infarct repair and LV remodeling](Source: Vanhoutte et al., 2006)

Post-MI ventricular remodeling begins immediately after an ischemic injury and continues for weeks and months. The expression profile of MMPs and TIMPs changes gradually as the wound healing progresses, indicating that both MMPs and TIMPs continue to interplay an important role in regulating the post-MI ECM turnover. A chronic imbalance between MMPs and TIMPs in favour of ECM degradation during the late remodeling phase leads to adverse LV remodeling.
1.1.6.1 Matrix Metalloproteinases (MMPs)

MMPs are a family of zinc-dependent enzymes that have the ability to degrade all components of ECM (Coussens, Fingleton, & Matrisian, 2002). To date, there are more than 20 known distinct MMP species and they are classified into 6 major classes on the basis of substrate specificity, sequence similarity, and domain organization. These 6 major classes include: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMP), and other MMPs (Visse & Nagase, 2003). MMPs play a dual role in regulating various body functions including morphogenesis, tissue repair, wound healing and post-injury remodeling; on the other hand, MMPs also have a detrimental role in the pathological progression of several diseases, such as cancer, arthritis, chronic tissue ulcer, and development of atheroma. Increased MMP expression and activity were indicated in failing hearts of various etiologies (Spinale et al., 1998; Tyagi, 1997; Tyagi, Ratajska, & Weber, 1993; Mann & Spinale, 1998; Spinale et al., 2000; Li & Feldman, 2001). Particularly, MMP-2 and MMP-9, both classified as gelatinases, have been indicated as potent stimuli for profound cardiac remodeling (Bergman et al., 2007; Thomas et al., 1998; Thompson & Squire, 2002; Li, McTiernan, & Feldman, 2000). The cause-effect relationship between MMPs and adverse myocardial remodeling has been implicated in several targeted gene studies in animals (Ducharme et al., 2000; Heymans et al., 1999; Lindsey et al., 2006; Matsumura et al., 2005; Matsusaka et al., 2006).

1.1.6.2 Myocardial Expression of MMPs after Myocardial Infarction

*Early wound healing: 0-7 days post-MI* - Immediately after a myocardial infarction (MI), the pre-existing pro-MMPs within the myocardium are activated. MMPs disrupt the fibrillar collagen
network to allow for infiltration of inflammatory cells into the infarct zone to remove the necrotic cells. The infiltrating inflammatory cells release additional MMPs and this may account for the early rise in MMPs during the early phase of post-MI wound healing. Within the first 24 hours, MMP-9 protein levels are increased at the infarct area, and its levels continue to rise on day 3 in all the infarct zone, border zone and the remote non-infarcted zone (Lindsey et al., 2006; Ducharme et al., 2000). At this stage, the main cellular sources for MMP-9 are neutrophils and macrophages (Lindsey et al., 2001; Tao et al., 2004). At the same time, MMP-2 expression is also increased and its levels remain elevated afterwards (Tao et al., 2004; Lindsey et al., 2002). Activated macrophages, fibroblasts, and myocytes are thought to be the main cellular sources for the increased MMP-2 levels (Heymans et al., 1999; Romanic et al., 2001; Tao et al., 2004). MMP-3 is also raised 2 days after MI and its levels remain upregulated into next phase of wound remodeling. Most MMP-3 is detected within myocytes (Romanic et al., 2001). Fibroblasts secrete MMP-1 on day 3 and its level peaks at day 7 (Cleutjens et al., 1995). The significant increase in MMP activity during the early phase of infarct healing may be adaptive and beneficial, but ongoing ECM turnover may be maladaptive over time, and excessive MMP activity early after infarction may lead to rupture. For instance, the elevation in MMP-2 and MMP-9 levels during the early phase of infarct healing is positively correlated with risk of fatally ventricular rupture (Tao et al., 2004).

**Early Remodeling Phase: 7-21 days post-MI** - After the first 7 days, the MMP-9 levels decrease, but its levels still remain significantly elevated compared to its baseline levels (Heymans et al., 1999; Tao et al., 2004). Similar to MMP-9, MMP-2 levels decrease from peak levels but remain elevated (Tao et al., 2004; Heymans et al., 1999; Lindsey et al., 2002). Increased levels of MMP-
2 at this stage are primarily the result of activated myofibroblasts (Heymans et al., 1999; Tao et al., 2004). MMP-8, which is localized to neutrophils, increases at 2 weeks post-MI and continues to remain elevated in the late phase of infarct wound healing (Peterson et al., 2000; Wilson et al., 2003). Concurrently, both MMP-3 levels (Romanic et al., 2001) and MMP-13 levels (Heymans et al., 1999) are elevated.

**Late Remodeling Phase: >21 days post-MI** - At 8 weeks post-MI, MMP-2 continues to increase substantially within the border and the infarct regions (Wilson et al., 2003). As mentioned in the previous section, MMP-8 continues to remain elevated within the border and MI regions during the late remodeling phase (Peterson et al., 2000; Wilson et al., 2003). Since MMP-8 is localized to neutrophils, it is suggested that MMP-8 is associated with chronic inflammatory response (Frangogiannis, 2012). Several MMPs emerge as healing proceeds, such as MMP-13 and MT1-MMP (Wilson et al., 2003). Nevertheless, not all MMPs remain elevated after 8 weeks post-MI. Particularly, MMP-1, MMP-7 and MMP-9 levels are reduced within the border and the infarct region, whereas MMP-3 is only reduced within the infarct region (King et al., 2003; Mukherjee et al., 2003; Wilson et al., 2003).

**1.1.6.3 Plasma Levels of MMPs as Clinical Biomarkers in Cardiac Diseases**

Many studies have focused on utilizing MMP-2 and MMP-9 as clinical biomarkers to predict prognosis of several heart diseases. For instance, plasma levels of MMP-2, MMP-3 and MMP-9 were positively correlated with circulating TNF-α, which was significantly elevated in patients with congestive heart failure (CHF). The same report also demonstrated a positive correlation between the expression of these MMPs and the severity of CHF (Wang et al., 2004).
The circulating MMP-2 has been shown to be an independent predictor of the mortality in patients with CHF (George et al., 2005) and the severity of CHF as classified according to the NYHA (New York Heart Association) functional class (Noji et al., 2004; Yamazaki et al., 2004). In addition, plasma MMP-2 levels were also associated with neurohormonal activation profile in patients with heart failure. For example, circulating noradrenaline (Banfi et al., 2005), ANP (atrial natriuretic peptides), BNP (brain natriuretic peptide), and endothelin-1 (ET-1) levels were positively correlated with plasma MMP-2 levels (Yan et al., 2008). In terms of acute heart failure, circulating MMP-2 levels decreased with improvement of clinical symptoms and a rapid decrease in plasma MMP-2 levels predicted better clinical outcomes in these patients (Shirakabe et al., 2010). Increased plasma concentrations of MMP-2 were related to adverse LV remodeling, and thus a worse prognosis in patients with hypertrophic cardiomyopathy (Kitaoka et al., 2010). Circulating MMP-2 levels were also suggested as an independent predictor of all-cause mortality after an episode of acute coronary syndrome (Dhillon et al., 2010). Interestingly, a recent study demonstrated that plasma MMP-2 concentrations were actually superior to BNP (brain natriuretic peptide) in identifying patients with heart failure with preserved ejection fraction (HF-PEF), which is challenging to diagnose in clinical practice. The study showed that at a cutoff plasma MMP-2 level of 1585 ng/mL, HF-PEF could be predicted with 91% sensitivity and 76% specificity (Martos et al., 2009).

Like MMP-2, plasma concentrations of MMP-9 were also associated with various heart diseases. Plasma MMP-9 levels increased by >150% from control even at day 1 after MI and remained elevated (Webb et al., 2006). In patients with CHF, the MMP-9/TIMP-1 ratio increased by 3 fold, whereas the MMP-9/TIMP-2 ratio increased by 16 fold (Wilson et al., 2002). In addition, increased plasma levels of pro-MMP-9 and MMP-9 in patients with CHF positively
correlated with LV volumes, inflammation, LDH (lactate dehydrogenase), AST (aspartate aminotransferase), and serum fibrinogen, indicating higher risks of cardiovascular diseases (Altieri et al., 2003). Plasma MMP-9 levels were positively correlated with LV dimension and were negatively correlated with fractional shortening (FS) (Noji et al., 2004; Yan et al., 2006). Consistent with this, several other studies also demonstrated that detectable plasma MMP-9 was associated with adverse LV remodeling and significant cardiac ECM degradation (Yan et al., 2006; Sundstrom et al., 2004; Kelly et al., 2008). Measurements of plasma levels of MMP-9, when combined with tissue Doppler measures of diastolic dysfunction, might aid in the risk stratification of patients with systolic heart failure (Buralli et al., 2010). In addition, the plasma levels of MMP-9 might hold a prognostic significance to predict development of CHF after acute myocardial infarction (Wagner et al., 2006).
1.1.6.4 Tissue Inhibitors of Metalloproteinases (TIMPs)

Tissue inhibitors of metalloproteinases (TIMPs) are known as the highly regulated endogenous inhibitors of MMPs. They have the ability to completely abolish the proteolytic activities of activated MMPs by forming 1:1 inhibitor-protease complexes that are resistant to heat denaturation and proteolytic degradation (Gomez et al., 1997). To date, four distinct TIMP species have been identified (TIMP-1, -2, -3, and -4), which are extensively characterized with respect to structure, expressions, activity and biological functions. All 4 TIMPs inhibit MMPs with affinities that vary for different inhibitor-protease pairs. Human myocardium is unique in such a way that all 4 TIMP species are expressed in normal tissue (Greene et al., 1996). During the late phase of post-MI cardiac remodeling, the abundance of TIMPs was consistently reduced to an undetectable level within the infarct region, as evidenced in several animal studies. The expression of TIMPs was reduced at the borderzone region as well (King et al., 2003; Mukherjee et al., 2003, 2006; Peterson et al., 2001, 2000; Wilson et al., 2003). Nevertheless, in spite of extensive studies, the profile of TIMPs expression during the progression of cardiac remodeling of various etiologies is largely inconsistent and somewhat confusing. For instance, Li and colleagues demonstrated that the myocardial TIMP-1 and TIMP-3 gene expression and proteins were significantly reduced in both human idiopathic dilated cardiomyopathy and human ischemic cardiomyopathy, whereas TIMP-4 was reduced only at the level of protein (Li et al., 1998). TIMP-2 was not altered at both the transcription and protein levels. In contrast, myocardial TIMP-1 and TIMP-2 levels were profoundly increased in human dilated cardiomyopathy (Thomas et al., 1998). TIMP-2 levels were also increased in myocardium of patients with aortic valvular stenosis (Fielitz et al., 2004) and with pressure-overloaded
cardiomyopathy (Heymans et al., 2005). Collectively, the profile of TIMPs expression during progression to heart failure is complex and is largely dependent on the underlying pathology.

1.1.6.5 Myocardial Expression of TIMPs after Myocardial Infarction

**TIMP-1 (29 kDa):** In normal myocardium, TIMP-1 is expressed by cardiac myocytes and fibroblasts and its expression is co-localized with MMP-1 (Lindsey et al., 2005; Romanic et al., 2001; Tyagi, Kumar, & Glover 1995). During the early phase of infarct wound healing, TIMP-1 protein is not increased until a later stage, at 2 and 16 weeks post-MI (Peterson et al., 2000). In a rabbit model, TIMP-1 protein is reduced within the first week after a MI, but its levels return to control levels by day 7 (Romanic et al., 2001). In the sheep, TIMP-1 levels are lower within the borderzone compared to the control, and its levels within the infarct area are below detection (Wilson et al., 2003). Consistently, TIMP-1 protein is also reduced in tissue obtained from human myocardium with ischemic cardiomyopathy (Li et al., 1998).

**TIMP-2 (28 kDa):** Similar to TIMP-1, TIMP-2 is also present in normal myocardium, and its main cellular source is cardiac fibroblasts (Lindsey et al., 2005; Porter & Turner 2009). In a rat MI model, TIMP-2 protein levels are not altered within the first week post-MI, but its levels show dual peaks at week 2 and week 16 after MI, then they gradually decrease (Peterson et al., 2000). In a sheep model, TIMP-2 levels are undetectable within the infarct region. However, its levels remain similar to control within the borderzone region (Wilson et al., 2003).
**TIMP-3 (24 kDa):** Comparably, at 8 weeks post-MI in a sheep model, TIMP-3 levels are significantly lower in the infarct zone, as compared to its levels either in control or non-infarcted region (Wilson et al., 2003). Similar results are observed in a pig model of MI (King et al., 2003; Mukherjee et al., 2003). TIMP-3 protein is also reduced in human failing myocardium secondary to ischemia (Li et al., 1998).

**TIMP-4 (28 kDa):** TIMP-4 is a unique member of the TIMP family because it is highly expressed in specific tissues, namely brain, skeletal muscle and ovary (Leco et al., 1997). TIMP-4 is also expressed abundantly by cardiomyocytes in normal myocardium and it once had a misnomer of cardiac-specific inhibitor of MMP (CIMP) (Cox et al., 2004; Greene et al., 1996). Similar to other TIMPs, TIMP-4 protein is reduced at 8 weeks post-MI within the infarct region (Peterson et al., 2000; Wilson et al., 2003).

### 1.1.6.6 Plasma Levels of TIMPs as Clinical Biomarkers in Heart Diseases

Differential plasma levels of TIMPs have been correlated with heart diseases of various etiologies. Identifying and mapping the profile of plasma TIMPs levels could allow the development of biomarkers to identify and to risk stratify patients with heart diseases and clinical manifestations. Interestingly, one should note that the plasma levels of TIMPs are somewhat different from the local myocardial levels of TIMPs in response to myocardial infarction. The reasons underlying these observations warrant further investigations, and there exists the possibility that the increased levels of circulating TIMPs is a compensatory mechanism in helping to ameliorate the detrimental effects of the increased MMP levels and reduced TIMP levels at the site of infarct. After an acute MI, plasma TIMP-1 increased by > 60% from control
at day 1 after MI, whereas TIMP-2 increased only at later time points. TIMP-4 decreased by 40% at day 5 after MI and remained reduced (Webb et al., 2006). Plasma TIMP-1 and MMP-9 levels correlated with echocardiographic parameters of LV remodeling and dysfunction. A plasma TIMP-1 cut-off level of at least 135 ng/mL predicted the risk of adverse prognosis, such as death and heart failure in patients following a MI (Kelly et al., 2008). Further study had indicated that elevated levels of plasma TIMP-1, TIMP-2, and TIMP-4 after MI were associated with major adverse cardiac events (Kelly et al., 2010).

In patients with hypertrophic cardiomyopathy (HCM), circulating TIMP-2 levels were elevated in patients with systolic dysfunction, but not in patients with preserved systolic functions. However, the plasma TIMP-1 levels were elevated in all HCM patients, regardless of whether the systolic function was preserved or not (Noji et al., 2004). Plasma TIMP-1 levels correlated with the markers of LV diastolic filling, and a plasma level of >500 ng/mL had a specificity of 97% and a positive predictive value of 96% in predicting diastolic dysfunction (Lindsay, Maxwell, & Dunn, 2002). Consistent with this, in patients with hypertension who had never received antihypertensive treatments, there were significant correlation between the plasma TIMP-1 levels and the LV wall thickness and hypertrophy (Timms et al., 2002). A recent study demonstrated that in patients with heart failure with normal ejection fraction and elevated filling pressure, the plasma TIMP-1 levels were significantly increased. Further investigation revealed that the collagen synthesis predominated over degradation among these patients. Therefore, the authors concluded that the relative excess of TIMP-1 might facilitate myocardial fibrosis and subsequently contribute to the elevation of LV filling pressures in these patients (Gonzalez et al., 2010). Plasma TIMP-1 is likely a disease marker and therefore may be used as a useful tool for assessing the cardiovascular risk factors, even in patients with no prior history of
heart diseases. In 2004, the famous Framingham Heart Study had performed a cross-sectional investigation on its study participants who were free of heart failure and previous myocardial infarction at the time of study. The investigation showed that the plasma total TIMP-1 levels were related to the major cardiovascular risk factors, namely male gender, increasing age, body mass index (BMI) and total/HDL-cholesterol level. Plasma TIMP-1 was directly related to smoking, diabetes and the use of anti-hypertensive treatment. Interestingly, alcohol intake was associated with lower plasma TIMP-1. When adjusted for age, sex and height, plasma TIMP-1 was also related with indices of LV hypertrophy and systolic dysfunction (Sundstrom et al., 2004). In the future, more investigations should be carried out on other TIMPs, with the possibility to exploit TIMPs, in adjunction with MMPs, as “multi-biomarkers” in assessing and risk-stratifying various cardiovascular diseases.
1.1.7 TIMP-Deletion Animal Models in Heart Diseases

The causal roles of TIMPs in the development and progression of various heart diseases have been extensively studied by exploiting genetically knockout animal models with altered expression of specific TIMP (summarized in Table 1-1).

**TIMP-1**: Deletion of the TIMP-1 gene in mice did not affect cardiac phenotype at birth. At 4 months of age, LV and end-diastolic wall stress were increased, while LV systolic pressure and ejection performance were preserved. No myocyte hypertrophy was detected, but the myocardial fibrillar collagen content was reduced (Roten et al., 2000). After an episode of MI, the LV end-diastolic volume and the LV end-diastolic pressure were significantly increased in the TIMP-1−/− mice. In addition, LV myocyte hypertrophic responses were more pronounced in these mice (Creemers et al., 2003). All these adverse LV remodeling effects were prevented with the use of exogenous MMP-inhibitors (Ikonomidis et al., 2005). These findings consistently demonstrate that TIMP-1 plays an important role in maintaining normal cardiac structure, and that its MMP-inhibitory action is critical in cardiac recovery from myocardial infarction.

**TIMP-2**: Myocardial infarction (MI) induced in the TIMP-2−/− mice led to greater infarct expansion, markedly exacerbated LV dilation and more severe inflammation compared to the wild type MI mice. However, the LV rupture rates were comparable in both groups. Further analysis of the TIMP-2−/− infarcted hearts revealed adverse ECM remodeling, evidenced by reduced density and enhanced disarray of fibrillar collagens. Enhanced collagenase activity, particularly MT1-MMP (Membrane Type 1-MMP) activity, was determined to be the main
culprit of the adverse ECM remodeling, in spite of the complete abrogation of MMP-2 activation in the TIMP-2\(^{-/-}\) mice (Kandalam et al., 2010). Consistent with this, adenoviral-mediated overexpression of TIMP-2 at the peri-infarct myocardium led to improved survival and blunted adverse cardiac remodeling in a murine model of MI (Ramani et al., 2011). When subjected to pressure overload, the TIMP-2\(^{-/-}\) mice developed exacerbated left ventricular hypertrophy, fibrosis, dilation, and cardiac dysfunction. This was because of the excessive MT-MMP (membrane type-MMP) activity and the loss of integrin \(\beta1D\), leading to non-uniform ECM remodeling and impaired myocyte-ECM interaction (Kandalam et al., 2011).

**TIMP-3:** Among all the TIMP-deficient mice, the TIMP-3\(^{-/-}\) mice have been most extensively studied. The first report was published by Fedak and co-workers, demonstrating that TIMP-3 deletion in mice led to spontaneous LV dilatation, cardiomyocyte hypertrophy, and contractile dysfunction at 21 months of age, consistent with human dilated cardiomyopathy. This severe phenotype was due to elevated MMP-9 activity, as well as activation of pro-inflammatory TNF-\(\alpha\) cytokine system (Fedak et al., 2004). At a younger age, these TIMP-3\(^{-/-}\) mice were susceptible to complications of cardiac diseases. For example, mortality rate was significantly higher in the TIMP-3\(^{-/-}\) mice after MI (Tian et al., 2007; Kandalam et al., 2010). Consistently, the gelatinase MMP activity and the TNF-\(\alpha\) levels were significantly greater in the TIMP-3\(^{-/-}\) mice than in the wild type mice at different time points after MI (Tian et al., 2007). In the pressure overload model, the TIMP-3\(^{-/-}\) mice developed early onset of heart failure and increased mortality, with exacerbated LV dilatation and dysfunction, excessive hypertrophy and fibrosis (Kassiri et al., 2005, 2009). Consistently, all these cardiac diseases involved initial rise in TNF-\(\alpha\) level,
followed by enhanced proteolytic activities (mainly MMP-2 and MT1-MMP). Accordingly, simultaneous interruption of the TNF-α activation pathway and the inhibition of MMPs significantly prevented the cardiac diseases in the TIMP-3+/−-MI mice (Kassiri et al., 2005). TIMP-3 was also indicated as a common innate regulator of two key cytokines in heart disease (i.e., TGF-β1 and TNF). The TIMP-3+/− mice demonstrated a disrupted regulation of both TGF-β1 and TNF, which led to fibrosis and early cardiac failure in response to pressure overload. Accordingly, the genetic and pharmacologic manipulations of these two cytokines abolished fibrosis and improved heart functions in the TIMP-3+/−-pressure overloaded mice (Kassiri et al., 2009).

**TIMP-4:** TIMP-4 was once referred as the “cardiac TIMP” because of its tissue-specific expression pattern in the heart (Nuttall et al., 2004). However, its deletion did not cause any developmental abnormality in the experimental mice, although these mice suffered moderately reduced cardiac function with aging. These mice showed no compensation by overexpression of TIMP-1, TIMP-2, or TIMP-3 in the heart, indicating that the presence of TIMP-4 is not essential in maintenance of normal myocardial structure and functions (Koskivirta et al., 2010). Following cardiac pressure overload by aortic banding, TIMP-4+/− mice demonstrated comparable survival rate and cardiac functions to the wild type pressure overloaded mice. In this case, increased myocardial TIMP-2 compensated TIMP-4 deficiency in these mice. Strikingly, induction of myocardial infarction in the TIMP-4+/− mice led to significantly enhanced mortality rate primarily due to LV rupture. The mortality rate was reduced by administration of synthetic MMP inhibitor and genetic deletion of MMP-2, indicating that the increased LV rupture rates in the TIMP-4+/−-
MI mice were due to increased MMP activities. Nevertheless, the survived TIMP-4+/−-MI mice showed similar extent of cardiac dysfunction compared with the control mice (Koskivirta et al., 2010). The subtle impact of TIMP-4 absence on cardiac response to disease is probably due to the fact the total myocardial level of TIMP-4, despite of its cardiac-specific expression, is lower than TIMP-2 and TIMP-3 (Nuttall et al., 2004).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cardiac Phenotype</th>
</tr>
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<tbody>
<tr>
<td>TIMP-1/−</td>
<td>Preserved systolic pressure and ejection fraction, LV dilation at 4 months of age (Roten et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Accelerated LV remodeling, increased LV dilation, and LV end-diastolic volume following MI (Creemers et al., 2003; Ikonomidis et al., 2005)</td>
</tr>
<tr>
<td>TIMP-2/−</td>
<td>Increased infarct size, LV dilation, systolic and diastolic dysfunction, but unaltered rate of LV rupture following MI (Kandalam et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Reduced fractional shortening and enhanced LV dilation, degraded integrin β1 and impaired myocyte–ECM interaction following pressure overload (Kandalam et al., 2011)</td>
</tr>
<tr>
<td>TIMP-3/−</td>
<td>Dilated cardiomyopathy at 21 months of age (Fedak et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Increased rate of LV rupture, dilation, and dysfunction following MI (Tian et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Precipitous dilated cardiomyopathy and early heart failure, hypertrophy, and fibrosis following pressure overload (Kassiri et al., 2005, 2009)</td>
</tr>
<tr>
<td>TIMP-4/−</td>
<td>Increased mortality due to enhanced rate of LV rupture, but similar cardiac function compared with WT mice following MI (Koskivirta et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>No worsening of cardiac function or structure following pressure overload (Koskivirta et al., 2010)</td>
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Table 1-1: Summary table of cardiac phenotype and myocardial responses to injury in specific TIMP-deficient animal models
1.1.8 TIMP-2 and Key Features

TIMP-2 is a 21-kDa protein that was first discovered in 1989. It consists of 12 conserved cysteine residues that form six disulphide bonds in order to maintain its functional and structural integrity. The amino-terminal cysteine plays a critical role in neutralizing active MMPs by binding directly to the active site of MMPs (Stetler-Stevenson, Krutzsch, & Liotta, 1989). The expression of TIMP-2 is constitutive in most cell types (Nuttall et al., 2004), and the proposed functional role of TIMPs in the setting of normal tissues lacking MMP activity is to maintain tissue homeostasis and cellular differentiation (Stetler-Stevenson, 2008). The main cellular source of TIMP-2 is fibroblasts, which are the most abundant cell type in the myocardium (Polyakova et al., 2004). TIMP-2 is able to inhibit essentially all types of MMPs and ADAM12 (a disintegrin and metalloproteinase) (Jacobsen et al., 2008).

1.1.8.1 TIMP-2 in Cardiac Remodeling

The critical role of TIMP-2 in cardiac remodeling has been demonstrated by its expression profile in post-MI remodeling. During the early post-MI (7-21 days), TIMP-2 protein levels are significantly increased; but the levels fall to an undetectable level during the late remodeling phase (8 weeks post-MI) (Vanhoutte et al., 2006). Fedak and colleagues demonstrated that the transplantation of vascular smooth muscle cells into the dilating LV myocardium of cardiomyopathic hamsters attenuated the LV dilatation. Immunoblotting analysis subsequently demonstrated an increased expression of TIMP-2 (as well as TIMP-3) in the cell-treated hearts, suggesting that smooth muscle cell transplantation might have attenuated the LV dilatation via TIMPs-dependent mechanism (Fedak et al., 2005; Fedak et al., 2012).
The direct role of TIMP-2 in mediating post-MI cardiac remodeling was demonstrated by assessing the cardiac response to MI in TIMP-2 knockout mice (Kandalam et al., 2010). Before this study, the role of TIMP-2 in the development and progression of cardiac remodeling was the least studied among all the other TIMPs due to the complexity of its dual functions as both inhibitor and activator of MMPs. In this study, MI was induced by ligation of left anterior descending artery (LAD) in the TIMP-2\(^{-/-}\) mice and the wild-type mice (WT). The TIMP-2\(^{-/-}\)-MI mice exhibited worse echocardiographic parameters including exacerbated LV dilation, greater wall motion abnormalities and worse systolic dysfunction. These mice also suffered more prominent cardiac disease post-MI, as indicated by elevated markers for heart failure (i.e., increased BNP [brain natriuretic peptides] and increased lung water content). As compared to the WT-MI mice, the microscopic analysis of ECM revealed that the TIMP-2\(^{-/-}\)-MI mice exhibited significantly lower collagen densities and enhanced disarray of collagen fibrils in the infarct and peri-infarct regions. The lack of TIMP-2 was associated with enhanced inflammatory response. Strikingly, the active MMP-2 was completely absent in the TIMP-2\(^{-/-}\) hearts, despite the presence of similar levels of pro-MMP-2 in both genotypes (TIMP2\(^{+/-}\) vs. WT). This was the first \textit{in vivo} evidence indicating that the presence of TIMP-2 is essential for cell surface activation of pro-MMP-2. Nevertheless, further analysis of collagenase activity revealed increased activities of several other MMPs, particularly the membrane type 1-MMP (MT1-MMP). In conclusion, for the first time, this study demonstrated that the MMP-inhibitory action of TIMP-2 is more critical than its MMP-2-activating action in mediating the cardiac recovery from MI.

Subsequently, the same TIMP-2\(^{-/-}\) mice model was exploited to explore the role of TIMP-2 in mediating cardiac response to pressure overload-induced cardiomyopathy (Kandalam et al., 2011). In the hearts of patients with aortic valve stenosis (Fielitz et al., 2004) and pressure-
overloaded cardiomyopathy (Heymans et al., 2005), TIMP-2 levels were increased and these observations raised a suspicion whether the increased expression of TIMP-2 was actually a causative agent that led to adverse myocardial remodeling, particularly in these patient populations. Transverse aortic constriction (TAC) was performed in the TIMP-2\(^{+/−}\) mice and the WT mice to induce pressure overload cardiomyopathy. Cardiac functions analysis at 2 weeks post-TAC demonstrated accelerated LV dilation and systolic dysfunction in the TIMP-2\(^{+/−}\)-TAC mice. Additionally, this study showed that the lack of TIMP-2 was also associated with more extensive fibrosis, due to increased SPARC (secreted protein acidic and rich in cysteine), which contributed to post-translational stabilization of collagen fibrils (Bradshaw & Sage, 2001).

Further investigation demonstrated that the enhanced fibrosis was not brought about by an increase in collagen production because the collagen contents were similar in both genotypes. Microscopic analysis of the ECM revealed a severe and non-uniform disorganization pattern, which was likely due to enhanced collagenase activities, particularly MT1-MMP (membrane type 1-MMP). Consistently, the elevated MT1-MMP levels and activities in the TIMP-2 knockout mice were the common causative factor that led to adverse cardiac remodeling, regardless of its etiology. In addition to the ECM disorganization, this study also demonstrated that a lack of TIMP-2 impaired the myocyte-ECM interaction via down-regulation of integrins β1D, which subsequently contributed to the enhanced LV dilation. In conclusion, this study has proven that the elevation of TIMP-2 level in pressure overload cardiomyopathy is, in fact, an adaptive attempt of the heart. Again, TIMP-2 was indicated as beneficial mediator in preventing adverse cardiac remodeling, in spite of its ability to activate pro-MMP-2.

The direct effects of myocardial TIMP-2 overexpression in post-MI cardiac remodeling were examined by injecting adenoviral particles of TIMP-2 gene directly into myocardium at the
time of MI in a murine model (MI+AdTIMP-2) (Ramani et al., 2011). The overexpression of TIMP-2 significantly improved survival rate as compared to the wild type-MI group. The major cause of mortality was LV rupture, which was positively correlated with MMP-2 levels and activities (Matsumura et al., 2005). In support of this observation, the analysis of gelatinolytic activity revealed that the MMP-2 and MMP-9 levels and activities were significantly reduced in the MI+AdTIMP-2 mice. In situ gelatinolytic activity analysis demonstrated the inhibitory effect of TIMP-2 overexpression on these gelatinolytic MMPs. As a result, the cardiac functions were generally well preserved in the MI+AdTIMP-2 group, as evidenced by decreased dilation, improved fractional shortening, and improved ejection fraction. In addition, the myocardial overexpression of TIMP-2 also blunted the post-MI infiltration of CD45+ inflammatory cells. Overall, this study provided a solid evidence to demonstrate the beneficial effects of TIMP-2 in post-MI cardiac remodeling. More importantly, this study proved that the direct application or induction of TIMP-2 at the target site might be a potential therapeutic intervention in the future to prevent adverse cardiac remodeling.

The concept that TIMPs exhibit their beneficial effects in cardiac remodeling solely via their MMP-inhibitory actions has been deemed to be premature, as TIMPs have been shown to exert various biological actions on various cell types, which the synthetic MMP inhibitors lack. Over the past decade, researchers have been focusing on developing and utilizing synthetic MMP inhibitors to limit the MMP-mediated cardiac remodeling. While the studies in animals have shown encouraging results, these agents have generally failed in clinical trials due to unanticipated off-target and adverse effects (Hutchinson et al., 1998; Tierney et al., 1999) and limited efficacy (Hudson et al., 2006) in human subjects. The explanation for this failure is largely due to our incomplete understanding of complex and diverse biological functions of
TIMPs. On the other hand, current synthetic MMP inhibitors only neutralize active MMPs, but they do not possess the entire spectrum of diverse biological actions of TIMPs.

1.1.8.2 MMP-Independent Actions of TIMP-2

TIMP-2 possesses diverse biological actions: it stimulates a proliferation of fibroblasts; suppresses endothelial cell mitogenesis and angiogenesis in response to angiogenic growth factors (i.e., vascular endothelial growth factor-A [VEGF-A] and fibroblast growth factor-2 [FGF-2]) (Murphy, Unsworth, & Stetler-Stevenson, 1993; Hoegy et al., 2001; Seo et al., 2008); inhibits endothelial cell migration in response to FGF-2 (Murphy, Unsworth, & Stetler-Stevenson, 1993); inhibits growth and promotes neurite differentiation in vitro (Perez-Martinez & Jaworski, 2005); inhibits T-cell infiltration (Woods et al., 2006); and induces apoptosis of activated human T-cells in peripheral blood (Lim, Guedez, & Stetler-Stevenson, 1999). In fact, some of these actions appear to be independent of its ability to inhibit MMPs. Several sophisticated signalling pathways of TIMP-2 mediated by cell surface receptors have been identified and extensively reviewed (Stetler-Stevenson, 2008; Vanhoutte & Heymans, 2010).

It is intriguing to delineate the direct actions of TIMP-2, which are independent of its MMP-inhibition, on cardiac fibroblasts. TIMP-2 blocks the catalytic activity of MMPs by binding its amino-terminal Cys-1 residue to the active site of MMPs (Wingfield et al., 1999). Ala+TIMP-2 is a special analogue of TIMP-2 that completely lacks MMP-inhibitory activity while maintaining its secondary and tertiary structure. Ala+TIMP-2 was named as such because an alanine (Ala) residue was appended to the amino-terminal cysteine of TIMP-2, acting to completely disrupt its MMP-inhibitory functions. Removal of the appended alanine residue from Ala+TIMP-2 by exopeptidase was able to restore its MMP-inhibitory activity (Wingfield et al.,
Interestingly, Ala+TIMP-2 still forms a hydrodynamically stable complex with MMP-2 despite of its inability to bind to the active site of MMP-2. This indicates the presence of other interaction site on the MMP-2 molecule that TIMP-2 can interact with: the carboxyl-terminal hemopexin-like domain (Olson et al., 1997). The binding of Ala+TIMP-2 to this site does not alter interaction with other substrates, and therefore the TIMP-2-MMP-2 complex still retains its proteolytic activity (Wingfield et al., 1999).

Several studies have utilized Ala+TIMP-2 to specifically study the biological actions of TIMP-2 that are independent of its MMP-inhibition. For instance, researchers have determined that TIMP-2 is able to inhibit the proliferation of endothelial cells (i.e., angiogenesis), and this effect was determined to be independent of its MMP-inhibitory actions by the means of Ala+TIMP-2. Subsequently, the corresponding downstream signalling pathways have been gradually delineated over the past decade by using Ala+TIMP-2 (Seo et al., 2003; Seo et al., 2006; Seo et al., 2008; Lee et al., 2010). Accordingly, this special analogue of TIMP-2 is an excellent biochemical tool to assist us in eliciting novel functions of TIMP-2 on cardiac fibroblasts that are independent of its MMP-inhibitory actions.

**1.1.8.3 The Unique Relationship between TIMP-2 and MMP-2**

The net proteolytic activity in the ECM is dependent on the activation of the secreted proenzymes and the interaction between active MMPs and their specific inhibitors. MMPs are secreted in a latent zymogen form, which requires further activation in order to initiate ECM remodeling by proteolytic degradation of collagens and proteoglycans (Strongin et al., 1995). Surprisingly, TIMP-2 is also essential for the surface activation of MMP-2, **via** selective interaction with membrane type-1-MMP (MT1-MMP) and pro-MMP-2, forming a trimolecular
complex, TIMP-2/pro-MMP-2/MT1-MMP (Kandalam et al., 2010; Strongin et al., 1995; Wang, Juttermann, & Soloway, 2000). MMP-2 (Gelatinase A, 72-kDa gelatinase, type IV collagenase) has been indicated as critical factor that drives ECM turnover in cardiac remodeling process (Li, McTiernan, & Feldman, 2000). Activation of pro-MMP-2 is unique in that it cannot be activated by any of the suggested physiological activators of other MMPs, including serine proteinases, plasmin, plasma kalikrein, neutrophil elastase, and cathepsin G (Nagase et al., 1991; Okada et al., 1990). Instead, several studies have shown that pro-MMP-2 can be activated by other MMPs, such as matrilysin, collagenase (Crabbe, O’Connell et al., 1994; Crabbe, Smith et al., 1994), and membrane type-MMP (MT-MMP) (Ingvarsen et al., 2008).

As mentioned earlier, TIMP-2 is able to activate pro-MMP-2 by forming a trimolecular complex of TIMP-2/pro-MMP-2/MT1-MMP. This activation process first involves an initial cleavage of pro-MMP-2 by MT1-MMP at the Asn37-Leu38 bond of pro-MMP-2 to produce an intermediate product of 64 kDa. Subsequently, this intermediate product will undergo an autocatalytic cleavage at the Asn80-Try81 bond, generating the fully active MMP-2 (Will et al., 1996). TIMP-2 functions as a co-activator of pro-MMP-2 in this activation mechanism: it facilitates the assembly of pro-MMP-2 and MT1-MMP on the cell surface. In this trimolecular complex, the amino-terminus of MT1-MMP interacts with TIMP-2, while the carboxyl-termini of pro-MMP-2 and TIMP-2 interact (Atkinson et al., 1995; Strongin et al., 1995; Zucker et al., 1998). The TIMP-2-dependent activation of latent pro-MMP-2 is essentially the most efficient mechanism to yield active MMP-2. This is evidenced by the complete abrogation of pro-MMP-2 activation in TIMP-2 knockout mice (Kandalam et al., 2010). Addition of exogenous TIMP-2 was only able to partially restore the activation of pro-MMP-2 in TIMP-2−/− cells, which suggests
that the cellular surface interaction feature of the endogenous TIMP-2 might be absent in the exogenously produced TIMP-2 (Wang, Juttermann, & Soloway, 2000).

Besides the TIMP-2-dependent activation mechanism, other MMPs, such as matrilysin and collagenase, may also activate pro-MMP-2 (Crabbe, O’Connell, et al., 1994; Crabbe, Smith, et al., 1994). TIMP-2 is a potent inhibitor to all types of MMPs, and therefore TIMP-2 is able to inhibit pro-MMP-2 activation via its inhibitory functions on other MMPs. Hence, at lower concentrations, TIMP-2 is essential to facilitate the cell surface activation of pro-MMP-2; whereas at higher concentrations, TIMP-2 might be able reduce MMP-2 activation via inhibition of other MMPs that are able to activate pro-MMP-2 (Sato & Takino, 2010). Furthermore, TIMP-2 is only active in inhibiting active MMPs when the amino-terminus of the Cys-1 residue is free (Wingfield et al., 1999).

1.1.9 Knowledge To Date

Lovelock and colleagues published an extensive study of the distinct role of each TIMP species in modulating cardiac fibroblasts (Lovelock et al., 2005). This was the first systemic report to study the direct roles of all 4 TIMPs in regulating cardiac fibroblasts. First of all, the authors induced the overexpression of TIMPs in mice cardiac fibroblasts by adding adenoviral constructs containing human recombinant TIMPs to cell culture. To make sure that infection with adenoviral constructs containing human recombinant TIMPs (AdTIMP) did not directly cause the observed effects in treated cardiac fibroblasts, the authors ran parallel experiments with conditioned media collected from the AdTIMP-infected cells (AdTIMP-conditioned media). This study showed that overexpression of each TIMP induced proliferation of the cardiac fibroblasts
respectively, especially TIMP-3. Similar result was obtained in the parallel experiments with AdTIMP-conditioned media, and these proliferative effects were completely abolished by pretreatment with a neutralizing anti-TIMP-1, -2, -3, or -4 antibody. The authors also suggested that TIMP-1, -2, -3, and -4 stimulate cardiac fibroblast proliferation through a MMP-independent mechanism, as these proliferation-promoting actions of TIMPs were not seen with the use of a synthetic MMP inhibitor. Infection with AdTIMP-3 and treatment with AdTIMP-3-conditioned media caused a significant increase in the incidence of apoptosis in cardiac fibroblasts. In terms of collagen synthesis, TIMP-2 induced a significant increase in collagen synthesis by cardiac fibroblasts although the other TIMPs did not exhibit such biological function. Again, a neutralizing anti-TIMP-2 antibody abolished this action of TIMP-2. All TIMP species stimulated an increase in \( \alpha \)-SMA expression by cardiac fibroblasts, indicating that TIMPs stimulated differentiation of fibroblasts into a more activated myofibroblast phenotype. Interestingly, TIMP-2 exhibited the most profound effect in the \( \alpha \)-SMA induction among all TIMPs.

**1.1.10 Summary**

Collectively, TIMP-2 is regarded as a key regulator in the post-MI myocardial remodeling process. However, a majority of these studies only focus on the MMP-inhibitory actions of TIMP-2 to explain the observed *in vivo* beneficial effects. As mentioned earlier, TIMPs possess a broad range of biological activities that are independent of their MMP-inhibitory actions. As of current, most of the efforts have been focused on MMP-dependent mechanisms in order to explain the observed beneficial effects of TIMP-2 in preventing adverse cardiac remodeling. However, it is unlikely that these MMP-dependent mechanisms are the only explanation for these beneficial effects, as partly indicated by the disappointing outcomes of
synthetic MMP inhibitors in clinical trials for heart failure (Hudson et al., 2006). There is emerging evidence regarding the diverse biological functions of TIMP-2 that are independent of its MMP-inhibitory actions. A complete delineation of these underlying mechanisms would allow us to optimize TIMP-2 as a potential therapeutic agent in preventing heart failure in the future. For instance, if the myofibroblast-activation by TIMP-2 is deemed to be detrimental in cardiac remodeling process, we could possibly shut down that action specifically, while maintaining the other beneficial actions of TIMP-2.

The cardiac fibroblasts are the critical cellular regulator of the integrity of myocardial ECM, which in turn determines the fate of the myocardium in response to stress and injury (Souders, Bowers, & Baudino, 2009). Currently, the understanding of the matricellular interactions of cardiac fibroblasts with ECM, and the regulation in both healthy and diseased state, is limited. The complex roles of TIMPs are less understood despite their potential key role in regulating cardiac fibroblast responses. TIMP-2 is especially intriguing given its dual ability to inhibit and stimulate MMP activity. In this study, we aimed to elucidate the role of TIMP-2 in human cardiac fibroblast-mediated regulation of ECM remodeling.
1.2 Rationale, Hypotheses and Objectives

1.2.1 Rationale

Lovelock and colleagues examined the biological functions of different TIMP species in regulating cardiac fibroblasts (Lovelock et al., 2005). The study had several limitations, which are important to highlight in the context of our work.

This particular study exploited adenovirus-mediated overexpression of TIMPs in a manner that does not necessarily represent physiological conditions. For example, the concentrations of TIMPs used were supraphysiological. Myocardial TIMP-2 protein concentrations are not clear, but the median plasma TIMP-2 level in human is estimated to be 163 ng/mL in EDTA plasma and 139 ng/mL in citrate plasma (Larsen et al., 2005). Alternatively, by utilizing exogenous TIMP-2, we would be able to control the concentrations at a range that is closer to its physiological levels. Accordingly, the concentration of exogenous TIMP-2 or Ala+TIMP-2 used in this study was standardized at 220 ng/mL (10 nM).

Lovelock and colleagues employed rodent cells for their study. The physiologic aspects in mouse and rat models are substantially different from those in humans; for example, the cardiac basal metabolism varies substantially across different species (Gibbs & Loiselle, 2001). The findings in mouse and rat species might not necessarily be applicable to human species. On that account, our experiments with human cells would ultimately maximize the translational potential of our current study.

All experiments in this study were performed in standard 2-D culture system (conventional culture dish), in which cells were grown in a monolayer manner on a rigid surface. Fibroblasts cultured in this system have a pronounced tendency to undergo phenotype switch to
myofibroblasts (Rohr, 2011). The baseline α-SMA expression in these cells is high and this would affect the cellular responses to experimental interventions. For example, when controlled stress was applied to these cells by different methods, such as magnetic beads or stretching of silicon substrates, cells with low α-SMA expression reacted with rapid up-regulation of α-SMA expression, whereas fully differentiated myofibroblasts exhibited a down-regulation of α-SMA (Wang et al., 2003; Poobalarahi, Baicu, & Bradshaw, 2006). Therefore, the use of standard culture techniques in this particular study is not suited to gain insights into the function of fibroblasts proper.

Furthermore, we are extremely fortunate to receive generous donation of Ala+TIMP-2 from Dr. William G. Stetler-Stevenson, M.D., Ph.D. (National Institute of Health, MD). With this special analogue, we are able to delineate possible novel actions of TIMP-2 on cardiac fibroblasts, which are independent of its MMP-inhibitory roles.

**1.2.2 Hypotheses**

i. TIMP-2 induces cardiac fibroblast-mediated collagen gel contraction.

ii. TIMP-2 induces phenotypic conversion of cardiac fibroblasts into cardiac myofibroblasts.

iii. TIMP-2 has no effect on cardiac fibroblast cell viability.

iv. MMP-inhibitory actions of TIMP-2 are not essential in differentiation of cardiac fibroblasts into myofibroblasts.

v. TIMP-2 induces collagen gel contraction *via* activation of MMP.

vi. Amino-terminus of TIMP-2 is not involved in formation of trimolecular complex of TIMP-2/Pro-MMP-2/MT1-MMP.
1.2.3 Objectives

To address these hypotheses, this research project employs functional 3-D collagen gel contraction assay, human cardiac fibroblasts, exogenous TIMP-2, exogenous Ala+TIMP-2 and broad spectrum MMP inhibitor (GM6001) to complete the following objectives:

i. To examine the effects of TIMP-2 on cardiac fibroblast-mediated collagen gel contraction.

ii. To differentiate the MMP-dependent and MMP-independent actions of TIMP-2 on cardiac fibroblast-mediated collagen gel contraction.

iii. To confirm that TIMP-2 induces phenotypic conversion of cardiac fibroblasts into cardiac myofibroblasts.

iv. To provide a possible mechanism underlying the TIMP-2-induced collagen gel contraction.
2.1 Human Cardiac Fibroblasts

2.1.1 Isolation of Human Cardiac Fibroblasts

Human cardiac tissues were obtained from patients undergoing coronary artery bypass graft surgeries (CABG) at the Foothills Medical Center, with consent obtained directly from patients beforehand. Samples of human right atrium were collected during cardiac surgery at the time of insertion of a cardiopulmonary bypass cannula. All experiments involving human tissue
were approved by Conjoint Health Research Ethics Board at the University of Calgary. Samples were minced and dissociated in sterile-filtered 0.2% Collagenase Type II (Worthington Biochemical Corporation) at 37°C in an Isotemp® Dry Bath (Fisher Scientific) with gentle shaking. Cell suspension was collected and remnant tissue was removed using tissue strainer of 40µm pore size (BD Falcon™). Collected cells were centrifuged at 1,500 RPM (rotations per minute) for 5 minutes at room temperature. Cell pellet was subsequently seeded in complete medium composed of Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% fetal bovine serum (Canadian Source, Invitrogen) plus 50,000 units of penicillin and 50,000 µg of streptomycin (Invitrogen). Cells were cultured in an incubator at 37°C with a 5% CO₂ atmosphere. Only cells from passage 4-8 were used for experiments.

2.1.2 Characterization of Human Cardiac Fibroblasts

Cultured cells were characterized as cardiac fibroblasts via immunohistochemistry staining. Cells were seeded on coverslips in complete medium for at least 24 hours in an incubator at 37°C with a 5% CO₂ atmosphere. Seeded cells were fixed using 4% paraformaldehyde (PFA) at room temperature for 20 minutes. The coverslips with fixed cells were blocked in blocking buffer containing 2% goat serum and 1% bovine serum albumin. Simultaneously, the fixed cells were permeabilized in 0.1% Triton® X-100. Next, coverslips were incubated in corresponding primary antibody, all at a dilution of 1:500 unless otherwise stated: mouse anti-fibronectin (Calbiochem), mouse anti-fibroblast surface protein (Sigma-Aldrich), rabbit anti-vimentin (Santa Cruz Biotechnology), rabbit anti-discoidin domain receptor 2 (Santa Cruz Biotechnology), rabbit anti-troponin I (Santa Cruz Biotechnology), rabbit anti-smooth muscle-22-α (1:200 dilution; Abcam), rabbit anti-desmin (Santa Cruz Biotechnology),
and rabbit anti-von Willibrand Factor (Santa Cruz Biotechnology). The coverslips were then incubated at 4°C overnight with gentle shaking, followed by incubation in secondary antibody for 1 hour at room temperature, either Alexa Fluor® 633 goat anti-mouse or Alexa Fluor® 633 goat anti-rabbit, at a dilution of 1:500. Lastly, the coverslips were mounted onto microscope glass slides in Prolong Gold Antifade Reagent (Invitrogen) containing diamidinophenylindole (DAPI) for counterstaining of nuclear visualization. All fluorescent images were captured using confocal laser microscopy (LSM 5, Carl Zeiss) and processed using Zen software.

2.2 3-D Collagen Gel Contraction Assay

This assay is based on several published reports (Huet et al., 2008; Lijnen, Petrov, & Fagard, 2001; Lijnen et al., 2004), with slight modification. Prior to experiment, cultured human cardiac fibroblasts were serum-starved for 24 hours to synchronize the cells. Cells were trypsinized and added to a liquid form of neutralized rat-tail type I collagen (BD Biosciences) at a final concentration of 1.8 mg/mL. The liquid gel mixture containing cells was kept on ice during the preparation. On a 24-well-plate, 400µL of the liquid gel mixture containing 2.5x10^5 cells/mL was dispensed into each well. The plate was subsequently incubated at 37°C for at least 1 hour to allow for gel polymerization. Immediately after polymerization, 500 µL of IMDM, either alone or containing TGF-β1 (10 ng/mL), MMP-2 (100 ng/mL, 200 ng/mL), TIMP-2 (10 nM), Ala+TIMP-2 (10 nM), TIMP-3 (10 nM), GM6001 (0.25 nM, 0.5 nM), TGF-β1 (10 ng/mL) + GM6001 (50 nM) or TIMP-2 (10 nM) + GM6001 (50 nM), was added to each well before incubating the plate overnight in a 37°C incubator with a 5% CO₂ atmosphere. To initiate gel contraction, the gels were released from the wall and the bottom of each well using a sterile micro-spatula (Corning®) until they floated in the treatment medium. The collagen gel images
were taken by photographing serial images up to 24 hours from the time of gel release. Image J analysis software (NIH, USA) was used to measure the area of gel contraction.

2.3 Confocal Laser Scanning Confocal Microscopic Analysis of \( \alpha \)-SMA Expression

After the 24-hr images were taken for functional gel contraction data, the collagen gels from Section 2.2 were immediately fixed in 4% PFA at room temperature for 30 minutes with no shaking. Collagen gels were then simultaneously blocked and permeabilized at room temperature for 1 hour by incubating the gels in blocking buffer (2% goat serum and 1% bovine serum albumin in 1xPBS) containing 0.1% Triton X-100. Subsequently, the collagen gels were incubated in primary antibody (mouse anti-\( \alpha \)-SMA at 1:500 dilution, Sigma-Aldrich) at 4°C for 48 hours with gentle shaking. Next, the collagen gels were washed with 1x PBS containing 0.1% Tween-20 and incubated with secondary antibody (Alexa Fluor 488 goat anti-mouse at 1:500 dilution, Invitrogen) at room temperature for 1 hour with gentle shaking. Lastly, collagen gels were mounted onto microscope glass slide in Prolong Gold Antifade Reagent (Molecular Probes) containing DAPI for counterstaining of nuclear visualization. All fluorescent images were captured using confocal laser microscopy (LSM 5, Carl Zeiss) in Z-stack module and processed using Zen software. To enable comparison between images, all microscopic settings were set identically for each image under all experimental conditions. \textit{Quantification of proportion of collagen gel volume that is positive for \( \alpha \)-SMA expression:} Z-stack images were taken from 8 random fields of a collagen gel. By using Volocity software (PerkinElmer), the acquired stack of images from each random field was used to reconstruct a 3-D image that consists of a definite volume, defined as “\textit{Total Image Volume}.” The image volume that was stained positive for \( \alpha \)-SMA was measured and defined as “\( \alpha \)-SMA-Positive Image Volume.”
\( \alpha \)-SMA expression of the cells embedded in a collagen gel was calculated using the formula below:

\[
\text{Proportion of } \alpha \text{-SMA-Positive Volume} = \frac{\alpha \text{-SMA-Positive Image Volume}}{\text{Total Image Volume}} \times 100\%
\]

2.4 Estimation of Cell Number and Cell Viability

Cells were directly retrieved from collagen gels from by incubating the gel Section 2.2 in 100 \( \mu \)L of 500 units/mL Collagenase Type II (Worthington Biochemical Corporation) in 37°C water bath with constant agitation until the gel was completely dissolved. Subsequently, the cell suspension was centrifuged at 5,000 RPM (rotations per minute) for 5 minutes. Cell pellet was resuspended in 1mL of IMDM and 0.1 mL of 0.4% trypan blue stain (GIBCO\textsuperscript{®}). 10 \( \mu \)L of cell suspension was loaded onto a hemacytometer, and then the number of total cells and the number of blue staining cells were counted. Cell viability (%) was calculated using the formula as below:

\[
\text{Cell Viability (\%)} = [1.0 - (\text{number of blue cells} \div \text{number of total cells})] \times 100
\]

2.5 Visualization of Collagen Gel Matrix Content and Architecture

To directly visualize the content and spatial architecture of the fibrillar collagen network within the collagen gels from Section 2.2, gels were stained for type I collagen and imaged using a confocal laser microscopy (LSM 5, Carl Zeiss). Similarly, collagen gels from Section 2.2 were fixed in 4% PFA and then blocked as described in Section 2.3. Collagen gels were incubated in primary antibody (rabbit anti-type I collagen, in 1:500 dilution, Abcam\textsuperscript{®}) at 4°C overnight with gentle shaking. After washing with PBS containing 0.1% Tween-20, collagen gels were
incubated in secondary antibody (Alexa Fluor® 633 goat anti-rabbit at 1:500 dilution, Invitrogen) at room temperature for 1 hour with gentle shaking. Gels were mounted onto microscope glass slides in Prolong® Gold Antifade Reagent (Molecular Probes®) prior to confocal microscopic imaging. To reconstruct a 3-D image, Z-stack images of each collagen gel were acquired at optimal intervals determined by Zen software. By using Volocity software (PerkinElmer), the reconstructed 3-D images were generated into short video clips demonstrating rotation in all three dimensions to facilitate the qualitative assessment of collagen fibril content and spatial organization within a collagen gel.

2.6 New Collagen Synthesis

To estimate the collagen synthesis activity, human cardiac fibroblasts embedded in collagen gel were stained for pro-collagen I, and Z-stack images were captured using a confocal laser microscopy (LSM 5, Carl Zeiss). Immunostaining for pro-collagen I was carried out using the same method as previously described in Section 2.3 and Section 2.5. In this experiment, the primary antibody used was mouse anti-pro-collagen I in 1:200 dilution (Developmental Studies Hybridoma Bank) and Alexa Fluor® 633 goat anti-mouse at 1:500 dilution (Invitrogen) was used as the secondary antibody for detection. Gels were mounted in Prolong® Gold Antifade Reagent (Molecular Probes®) containing DAPI. 3-D images were reconstructed using Volocity software (PerkinElmer) as previously described. To quantitatively assess the number of cells expressing pro-collagen I, the number of red objects (Alexa Fluor® 633) identified in each 3-D image was divided by the total DAPI (nuclei) in each image. Pro-collagen I-positive cells of 8 random images from each group were averaged and expressed as a percentage (%) of the total cell number.
In situ zymography, as described by several studies (Cha & Purslow, 2010; Chhabra et al., 2012), has been modified to be applied in our functional 3-D collagen gel contraction assay. The basic principle of this method involves digestion of specific substrate by activated protease present in the collagen gel, followed by detection of the liberated signal by fluorescent microscopy. DQ™ Gelatin-FITC is a special analogue of gelatin that is conjugated to quenched fluorescein and it emits high fluorescent signals as it is cleaved (Chhabra et al., 2012; Frederiks, 2004). In brief, collagen gels containing human cardiac fibroblasts were prepared as described previously (refer to Section 2.2). On top of this, DQ™ Gelatin-FITC (Molecular Probes®) was added to the liquid gel solution at a final concentration of 10 µg/mL prior to gel polymerization. The collagen gels were subsequently treated with 500 µL of IMDM either alone or containing TGF-β1 (10 ng/mL), MMP-2 (100 ng/mL & 200 ng/mL), TIMP-2 (10 nM), or Ala+TIMP-2 (10 nM). Collagen gels were fixed in 4% PFA for 30 minutes at room temperature. After fixation, gels were washed with 1x PBS three times before mounted in Prolong Gold Antifade Reagent (Invitrogen). All fluorescent images were captured using confocal laser microscopy (LSM 5, Carl Zeiss) in Z-stack module and processed using Zen software. Subsequently, Z-stack images taken from 8 random fields of a collagen gel were analyzed using Volocity software (PerkinElmer). Bright green fluorescent spots indicating proteolytic digestion in the acquired 3-D collagen gel image were identified as “green fluorescent objects” on the Volocity software.
platform and the total volume of these objects was summed as “Total Volume of Green Fluorescence.” The total protease activity in a collagen gel normalized to the corresponding gel image volume was calculated using the formula that follows:

\[
\text{Total Protease Activity} = \frac{\text{Mean Fluorescence Intensity} \times \text{Total Volume of Green Fluorescence}}{\text{Total Image Volume}}
\]

2.8 Gelatin Zymography: Semi-Quantification of MMP-2 & MMP-9

Gelatin zymography is a simple, sensitive and semi-quantifiable method to measure mainly the gelatinases: MMP-2 and MMP-9. Cell lysates were directly extracted from the collagen gels from Section 2.2 by submerging the gels in ice-cold 2x RIPA lysis buffer (Millipore; 0.5 M Tris-HCl, pH 7.4; 1.5 M NaCl, 10% NP-40, 2.5% sodium deoxycholic acid, 10 mM EDTA) containing protease inhibitor cocktail (cOmplete mini EDTA-free protease inhibitor cocktail tablets, Roche). The remnants of cell debris and collagen gel were spun down by centrifugation at 12,000 rpm for 20 minutes at 4°C. The supernatant was collected and stored at -80°C until use. Equal amounts of the collected cell lysis supernatant (15µL) from each group were incubated with 1x LDS sample buffer (NuPage®, Invitrogen) at room temperature for 10 minutes. Samples were loaded onto a 12% SDS-PAGE electrophoresis gel containing 0.1% of gelatin (1mg/mL). Odyssey® Protein Molecular Weight Marker (LI-COR Biosciences) and exogenous MMP-2 (R&D Systems) were loaded for molecular weight determination and as positive control, respectively. Gel was run in 1x Tris/Glycine/SDS buffer (Bio-Rad) at 120V until the dye molecule reached the bottom of the gel. After running, proteases within the gel were renatured by incubation in 100 mL of 1x Zymogram Renaturing Buffer (Novex®, Invitrogen) for 30
minutes at room temperature with gentle agitation. The Zymogram Renaturing Buffer was decanted and the gel was equilibrated for 30 minutes in 100 mL of 1x Zymogram Developing Buffer (Novex®, Invitrogen) at room temperature with gentle agitation. The gel was further incubated for at least 48 hours at 37°C in fresh 1x Zymogram Developing Buffer (Novex®, Invitrogen). After incubation, the gel was washed with deionized water 3 times for 5 minutes before staining the gel with IRDye® Blue Protein Dye (LI-COR Biosciences) for 1 hour at room temperature. The gel was de-stained with deionized water overnight before scanning the gel on the Odyssey® Infrared Imaging System (LI-COR Biosciences) using the 700 nm channel only. Digested areas “pale bands” on the gel were quantified using the “Average” background setting to detect a net decrease in intensity.

2.9 Statistical Analysis

The data shown in each figure are taken from a representative experiment of which each was repeated on multiple occasions. Results are presented as mean ± standard deviation (S.D.). When only two groups were compared, Student’s t-test was performed. For comparison of more than two groups, one-way ANOVA was used; if the F-ratio was significant, pairwise tests of individual group means were compared using the appropriate post hoc tests. All statistical analyses were performed using the GraphPad Prism version 6.0 statistical analysis software, with P<0.05 considered statistically significant.
3.1 Isolation and Characterization of Human Cardiac Fibroblasts

3.1.1 Introduction

Heart cells consist of cardiomyocytes and non-cardiomyocytes that include cardiac fibroblasts, endothelial cells, smooth muscle cells and nerve cells (Eghbali et al., 1988). Cardiac fibroblasts are the most abundant cell type in myocardium. Fibroblasts tend to outgrow other cell types in culture as the passage increases; therefore, isolating and culturing primary human cardiac fibroblasts is a reliable and reproducible procedure. At early passages, primary cell cultures from human heart tissues usually represent a heterogeneous population of cells. Sub-culturing the cells will increase the purity of fibroblasts in the cultures. Purification of fibroblasts from contamination of other cell types is particularly critical for ensuring a constant proportion of cardiac fibroblasts in each experiment. Following the establishment of a primary culture of fibroblasts, it is necessary to characterize the new strain of cells to ensure their purity and fibroblastic phenotype. This is done using phase-contrast microscopy to assess cell morphology and immunocytochemistry to detect the presence or absence of cell-specific surface markers.

3.1.2 Results

We first examined the morphology of the cultured cardiac fibroblasts under a phase-contrast light microscope (Figure 3-1). At early passages (Passages 1 & 2), several cellular morphologies were observed indicating the presence of a heterogenous population of cells. As the cells were further passaged (Passage 3), a majority of the cells showed a flat and spindle
shape, with a few projections from their main bodies. These are the features of cardiac fibroblasts as described by Souders and colleagues (Souders et al., 2009). When the cells reached later passages (as represented by Passage 9 in Figure 3-1), they changed from spindle shape into a rounder shape. These cells exhibited a stellate form with nuclei marked by nucleoli and featured prominent projections of stress fibres and extensive rough endoplasmic reticulum. These morphological features are consistent with differentiated cardiac myofibroblasts (Tomasek et al., 2002).

To characterize the primary human cardiac fibroblasts in culture, we performed immunocytochemistry to confirm the presence of several fibroblast-specific markers: fibronectin, vimentin, fibroblast surface protein and discoidin domain receptor 2. >95% of the cultured cells were stained positive for fibroblast markers (Figure 3-2). We also stained the cells for negative markers to rule out other major cells types that can be found in myocardium (Figure 3-3). Specifically, all cells were not stained for SM22-α (smooth muscle cells) (Li et al., 1996); Troponin I (cardiomyocytes); desmin (smooth muscle cells, skeletal muscle cells, cardiomyocytes); and von Willebrand Factor (endothelial cells).

3.1.3 Summary

In summary, the phase-contrast light microscopic assessment of cellular morphology and the confocal microscopic images of immunostained cells confirmed the success and purity of the cultured human cardiac fibroblasts in this study.
Figure 3-1: Morphology of human cardiac fibroblasts under phase contrast light microscopy

Photomicrographs obtained from different passages of cells from the same isolation: (a) Passage 1; (b) Passage 2; (c) Passage 3; (d) Passage 9. Objective: 20x. Note the changes in cellular morphology as the cell passages increased.
Figure 3-2: Immunostaining of cultured human heart cells for fibroblast markers

All cells exhibited staining for several fibroblast markers: fibronectin (top row), vimentin (second row), fibroblast surface protein (third row) and discoidin domain receptor 2 (bottom row). All these markers confirmed these cells as fibroblasts. Nuclei were stained with diamidinophenylindole (DAPI), blue. Images shown are at objective 20x.
Figure 3-3: Immunostaining of cultured human heart cells for negative markers to rule out other major cell types in myocardium

None of these cells were stained for markers of non-fibroblast cell types: SM-22-α (top row), Troponin-I (second row), desmin (third row) and von Willebrand Factor (bottom row). These negative markers minimized the possibility of other cell types and further confirmed these cells as fibroblasts. Nuclei were stained with diamidinophenylindole (DAPI), blue. Images shown are at objective 20x.
3.2 Three-Dimensional Collagen Gel Contraction Assay

3.2.1 Introduction

3-D collagen gel is an in vitro assay that mimics in vivo arrangement of collagen fibrils in the extra-cellular matrix (ECM). Embedded cardiac fibroblasts have the ability to reorganize collagen fibrils and contract collagen gel in proportion to the extent of their phenotypic differentiation into myofibroblasts (Huet et al., 2008; Lijnen, Petrov, & Fagard, 2001; Lijnen et al., 2003). Therefore, measuring the amount of collagen gel contraction can directly assess the extent of myofibroblast activation within a 3-D collagen gel.

3.2.2 Results

3.2.2.1 Stimulators of Collagen Gel Contraction

TGF-β1 (Lijnen et al., 2003; Petrov, Fagard, & Lijnen, 2002) and active MMP-2 (Margulis et al., 2009) are known as stimulators of collagen gel contraction assay. Therefore, to validate the use of a collagen gel contraction assay in our laboratory, we performed preliminary experiments with TGF-β1 and MMP-2 (Figure 3-4). The serum-free medium group served as a baseline control in every assay, respectively. The time point when collagen gel was released was defined as 0 hour, and contraction was measured up to 24 hours after the gel release. Our preliminary experiments indicated that most of the collagen gel contraction occurred within this time frame, and the contraction consistently slowed down or reached a plateau after this time point. The TGF-β1 group induced more collagen gel contraction as compared to the serum-free medium group (39.8±3.7% [TGF-β1] vs. 23.7±4.6% [serum-free medium], P<0.01). Similarly, the MMP-2 group also stimulated more collagen gel contraction assay than the serum-free
medium group (39.3±4.8% [MMP-2] vs. 23.7±4.6% [serum-free medium], \( P<0.01 \)). These data demonstrated that we were able to independently perform collagen gel contraction assay in our laboratory and to generate data that is consistent with the current literature.

### 3.2.2.2 Effects of MMP Inhibition on Baseline Collagen Gel Contraction

GM6001 (N-[((2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide, \( C_{20}H_{28}N_4O_4 \); Calbiochem) is a potent, cell-permeable, broad-spectrum hydroxamic acid inhibitor of MMPs (Grobelny, Poncz, & Galardy 1992). We examined the effects of MMP inhibition on baseline collagen gel contraction, by addition of increasing concentration of GM6001 to the collagen gel (Figure 3-5). The baseline contraction of collagen gel in the serum-free group was inhibited by GM6001 in a dose-dependent manner (30.0±3.0% [serum-free medium] vs. 23.8±1.0% [250 pM], \( P<0.05 \)) and (30.0±3.0% [serum-free medium] vs. 19.2±2.7% [500 pM], \( P<0.01 \)). A negative control compound for GM6001 (N-t-butoxycarbonyl-L-leucyl-L-tryptophan methylamide, \( C_{23}H_{34}N_4O_4 \); Calbiochem) was applied to ensure the specificity of the GM6001 actions. The negative control did not yield an effect on the baseline collagen gel contraction (30.0±3.0% [serum-free medium] vs. 27.8±1.0% [GM6001 negative control], \( P=0.29 \)).

### 3.2.2.3 Effects of MMP Inhibition on TGF-β1-Induced Collagen Gel Contraction

We then examined the effect of MMP inhibition on collagen gel contraction stimulated by TGF-β1 (Figure 3-6). TGF-β1 induced more collagen gel contraction compared to the serum-free medium group (45.3±6.2% [serum-free medium] vs. 67.1±0.7% [TGF-β1], \( P<0.05 \)).
Addition of GM6001 reduced the TGF-β1-induced collagen gel contraction (67.1±0.7% [TGF-β1] vs. 39.8±3.0% [GM6001 + TGF-β1], \( P<0.01 \)).

3.2.2.4 Effects of TIMP-2 and TIMP-3 on Collagen Gel Contraction Assay

Subsequently, we examined the effect of TIMP-2 and TIMP-3 on collagen gel contraction (Figure 3-7). Surprisingly, known as an MMP inhibitor (Stetler-Stevenson et al., 1989), 10 nM of TIMP-2 resulted in a significant increase in collagen gel contraction compared to the serum-free medium group (46.0±2.0% [TIMP-2] vs. 23.7±4.6% [serum-free medium], \( P<0.001 \)). We also determined that this effect was specific to TIMP-2 only, as the same concentration of TIMP-3 did not induce an increase in collagen gel contraction, as compared to the baseline collagen gel contraction (20.8±3.3% [TIMP-3] vs. 23.7±4.6% [serum-free medium], \( P=0.43 \)).

3.2.2.5 TIMP-2-Induced Collagen Gel Contraction: Role of MMP-Inhibitory Actions

To examine whether the TIMP-2-induced collagen gel contraction is dependent on its MMP-inhibitory actions, we utilized Ala+TIMP-2 that is completely devoid of its MMP-inhibitory activity while maintaining its tertiary structure of protein (Wingfield et al., 1999) (Figure 3-8). Ala+TIMP-2 stimulated collagen gel contraction as compared to the serum-free medium group (45.7±1.5% [Ala+TIMP-2] vs. 23.7±1.5% [serum-free medium], \( P<0.001 \)). The extent of collagen gel contraction induced by Ala+TIMP-2 was the same as that of the TIMP-2 group (45.7±1.5% [Ala+TIMP-2] vs. 46.0±2.0% [TIMP-2], \( P=0.86 \)).
3.2.2.6 Effects of MMP Inhibition on TIMP-2-Induced Collagen Gel Contraction

We also explored the effect of MMP inhibition on collagen gel contraction stimulated by TIMP-2 (Figure 3-9). TIMP-2 stimulated more gel contraction compared to the serum-free medium group (54.6±2.6% [TIMP-2] vs. 40.9±5.0% [serum-free medium], P<0.05). An addition of GM6001 reduced the TIMP-2-induced collagen gel contraction to the baseline level (54.6±2.6% [TIMP-2] vs. 37.5±5.4% [GM6001 + TIMP-2], P<0.01).

3.2.2.7 Summary

In summary, we demonstrated that TGF-β1 and MMP-2 induced collagen gel contraction. MMP inhibition by the means of GM6001 (broad spectrum synthetic MMP inhibitor) inhibited the baseline collagen gel contraction. GM6001 inhibited the TGF-β1-induced collagen gel contraction. TIMP-2, known as an endogenous MMP inhibitor, stimulated collagen gel contraction and this effect seems to be specific to TIMP-2; treatment with TIMP-3 did not generate the same observation. Ala+TIMP-2 induced collagen gel contraction to the same extent as TIMP-2. GM6001 inhibited the TIMP-2-induced collagen gel contraction. In conclusion, TIMP-2 induces cardiac fibroblast-mediated collagen gel contraction and this action likely involves a separate mechanism that is independent of its MMP-inhibitory actions.
Figure 3-4: Stimulators of collagen gel contraction

(a) Representative photographs of the collagen gels demonstrating different surface areas at 0-hr and 24-hr in several treatment groups (serum-free medium, TGF-β1, and MMP-2). (b) Percentage of collagen gel contraction (%) from initial gel surface area (measured as well surface area). Both TGF-β1 and MMP-2 significantly enhanced collagen gel contraction. Bars represent mean ± SD. **, P<0.01 vs. control (serum-free medium).
Figure 3-5: Effects of MMP inhibition on baseline collagen gel contraction

(a) Representative photographs of the collagen gels demonstrating different surface areas at 0-hr and 24-hr in several treatment groups (serum-free medium, GM6001 [250 pM], GM6001 [500 pM], and GM6001 negative control. (b) Percentage of collagen gel contraction (%) from initial gel surface area (measured as well surface area). GM6001 dose-dependently inhibited the baseline collagen gel contraction (serum-free medium). Bars represent mean ± SD. *, P<0.05; **, P<0.01.
Figure 3-6: Effects of MMP inhibition on TGF-β1-induced collagen gel contraction

(a) Representative photographs of the collagen gels demonstrating different surface areas at 0-hr and 24-hr in several treatment groups (serum-free medium, TGF-β1, and GM6001 + TGF-β1).

(b) Percentage of collagen gel contraction (%) from initial gel surface area (measured as well surface area). GM6001 inhibited the TGF-β1-induced collagen gel contraction. Bars represent mean ± SD. *, P<0.05; **, P<0.01
Figure 3-7: Effects of TIMPs on collagen gel contraction

(a) Representative photographs of the collagen gels demonstrating different surface areas at 0-hr and 24-hr in several treatment groups (serum-free medium, 10 nM TIMP-2, and 10 nM TIMP-3).

(b) Percentage of collagen gel contraction (%) from initial gel surface area (measured as well surface area). TIMP-2 significantly enhanced collagen gel contraction, whereas TIMP-3 did not induce more collagen gel contraction as compared to control (serum-free medium). Bars represent mean ± SD. ***, P<0.001 vs. serum-free medium; ns, non-significant vs. serum-free medium.
Figure 3-8: TIMP-2-induced collagen gel contraction: Role of MMP-inhibitory actions

(a) Representative photographs of the collagen gels demonstrating different surface areas at 0-hr and 24-hr in several treatment groups (serum-free medium, 10 nM TIMP-2, and 10 nM Ala+TIMP-2). (b) Percentage of collagen gel contraction (%) from initial gel surface area (measured as well surface area). Both TIMP-2 and Ala+TIMP-2 significantly enhanced collagen gel contraction as compared to control (serum-free medium). Bars represent mean ± SD. ***, P<0.001 vs. serum-free medium.
Figure 3-9: Effects of MMP inhibition on TIMP-2-induced collagen gel contraction

(a) Representative photographs of the collagen gels demonstrating different surface areas at 0-hr and 24-hr in several treatment groups (serum-free medium, 10 nM TIMP-2, and GM6001 + 10 nM TIMP-2). (b) Percentage of collagen gel contraction (%) from initial gel surface area (measured as well surface area). GM6001 inhibited the TIMP-2-induced collagen gel contraction. Bars represent mean ± SD. *, P<0.05; **, P<0.01.
3.3 Expression of Alpha-Smooth Muscle Actin (α-SMA)

3.3.1 Introduction

Cardiac fibroblasts embedded in a collagen gel have the ability to reorganize collagen fibrils within the gel into a denser arrangement, and thus act to contract the gel (Lijnen et al., 2001; Lijnen, Petrov, & Fagard, 2003). Cardiac myofibroblasts possess a higher contractile property than that of cardiac fibroblasts (Gabbiani et al., 1971), and hence they tend to induce more collagen gel contraction. Studies have confirmed that increased collagen gel contraction is directly proportional to the extent of the phenotypic differentiation of fibroblasts into myofibroblasts (Huet et al., 2008; Lijnen et al., 2001, 2003). The hallmark of differentiation is the de novo expression of alpha-smooth muscle actin [α-SMA] (Gabbiani et al., 1971; Tomasek et al., 2002). We wondered if TIMP-2 enhances collagen gel contraction by promoting differentiation of cardiac fibroblasts into cardiac myofibroblasts. Accordingly, we investigated the alteration of α-SMA expression by cardiac fibroblasts after targeted exposure to TIMP-2 and controls.

3.3.2 Results

We directly measured the α-SMA expression of the cardiac fibroblasts embedded in a collagen gel (Figure 3-10 & Figure 3-11). Cells were stained for α-SMA (green) and for nuclei (DAPI, blue). Z-stack images of collagen gel containing the cardiac fibroblasts were captured using a laser scanning confocal microscopy, and 3-D images were reconstructed. α-SMA
expression was quantified as the proportion of the reconstructed 3-D image volume that was stained positive for α-SMA (green).

TGF-β1 and MMP-2 induced the cellular expression of α-SMA within the cardiac fibroblast population (Figure 3-10). TGF-β1 yielded a 1.7-fold increase in α-SMA expression compared to the serum-free control group ($P<0.01$). Similarly, treatment with MMP-2 also increased the α-SMA expression by 1.8 fold (vs. serum-free medium, $P<0.001$). Both groups increased collagen gel contraction to same extent.

Both TIMP-2 and Ala+TIMP-2 stimulated more cardiac fibroblasts to express α-SMA. TIMP-3 did not exert such effect (Figure 3-11). TIMP-2 induced a 1.8-fold increase, whereas Ala+TIMP-2 yielded a 2.2-fold increase in the α-SMA expression as compared to that of the serum-free medium group ($P<0.001$). Both TIMP-2 and Ala+TIMP-2 exhibited the same extent of α-SMA induction ($P = 0.10$). TIMP-3 did not exert the same effect as TIMP-2/Ala+TIMP-2. Instead, it exhibited a downward trend in α-SMA induction although the difference was not statistically significant (vs. serum-free medium).

3.3.3 Summary

In summary, we determined that TGF-β1 and MMP-2 induced the α-SMA expression within a population of cardiac fibroblasts. TIMP-2 stimulated a significant increase in α-SMA expression. This effect is independent of its MMP-inhibitory actions as Ala+TIMP-2 led to the same outcome. This observation was unique to TIMP-2 because TIMP-3 did not enhance α-SMA expression. Cellular α-SMA expression was positively correlated with collagen gel contraction.
Figure 3-10: Effects of TGF-β1 and MMP-2 on cellular expression of α-SMA
(a) Representative confocal microscopic images of cardiac fibroblasts/myofibroblasts embedded in collagen gel, following targeted treatments (serum-free medium, TGF-β1, and MMP-2). Cells were stained for α-SMA (green) and for nuclei (DAPI, blue). Scale bar=80 μm. (b) Proportion of image volume that was stained positive for α-SMA (green) and values were normalized to that of the serum-free medium group (control). Both TGF-β1 and MMP-2 significantly induced the cellular expression of α-SMA. n=8 random field images per treatment group. Bars represent mean ± SD. **, P<0.01 vs. serum-free medium; ***, P<0.001 vs. serum-free medium.
Figure 3-11: Effects of TIMPs on cellular expression of α-SMA

(a) Representative confocal microscopic images of cardiac fibroblasts/myofibroblasts embedded in a collagen gel, following particular treatments (serum-free medium, TIMP-2, Ala+TIMP-2, and TIMP-3). Cells were stained for α-SMA (green) and for nuclei (DAPI, blue). Scale bar=80 μm. (b) Proportion of image volume that was stained positive for α-SMA (green) and values were normalized to that of the serum-free medium group (control). Both TIMP-2 and Ala-TIMP-2 induced the cellular expression of α-SMA to the same extent. TIMP-3 did not exert such effect. n=8 random field images per treatment group. Bars represent mean ± SD. ***, P<0.001 vs. serum-free medium; ns, non-significant vs. serum-free medium.
3.4 Measurement of New Collagen Synthesis

3.4.1 Introduction

Myofibroblasts contain extensive endoplasmic reticulum, and they are more active in collagen synthesis as compared to fibroblasts (Gabbiani et al., 1971). Type I collagen is the most abundant collagen in myocardium, and it is synthesized in cardiac fibroblasts in a precursor form, pro-collagen I. Pro-collagen I is produced within cellular cytoplasm before it is secreted into extracellular space where it undergoes further processing by specific collagen peptidases to form new collagen (Freiberger et al., 1980). Therefore, pro-collagen I abundance is an estimate of synthesis of new collagen.

3.4.2 Results

We estimated the extent of new collagen synthesis by measuring the expression of pro-collagen I in cardiac fibroblasts embedded in a collagen gel (Figure 3-12). A majority of these cells (>80%) expressed pro-collagen I. Treatment with TGF-β1 (positive control) induced a significant increase in pro-collagen I synthesis in these cells as compared to the serum-free medium group (89.7±5.8% [TGF-β1] vs. 81.8±5.6% [serum-free medium], P<0.05). TIMP-2 stimulated the highest increase in pro-collagen I synthesis among all the groups (91.9±5.5%, P<0.01 [TIMP-2] vs. serum-free medium). Devoid of MMP-inhibitory actions, Ala+TIMP-2 also promoted pro-collagen I expression in cardiac fibroblasts (87.4±4.2% [Ala+TIMP-2], P<0.05 vs. serum-free medium). This observation indicated that this action of TIMP-2 is independent of its MMP-inhibitory roles. The pro-collagen I level in the cells treated with TIMP-3 was comparable
to that of the serum-free medium group (81.4±5.1% [TIMP-3] vs. 81.8±5.6% [serum-free medium], \( P= 0.88 \)).

3.4.3 Summary

This section of results allowed us to estimate the collagen synthesis activity in cardiac fibroblasts/myofibroblasts embedded in a collagen gel, following targeted treatment. A majority of these cells (>80%) were active in new collagen synthesis. This is probably due to the fact that these cells were fibroblasts. Stimulation with TGF-\( \beta \)1 stimulated an increase in pro-collagen I expression. Treatment with both TIMP-2 and Ala+TIMP-2 enhanced the collagen synthesis activity of these cells. This effect is specific to TIMP-2 only as TIMP-3 treatment did not alter the baseline pro-collagen I expression. As cardiac myofibroblasts are relatively more active than cardiac fibroblasts in terms of collagen synthesis, these data indirectly indicate that collagen gel contraction is positively correlated with the extent of myofibroblast activation.
Figure 3-12: Expression of pro-collagen I

(a) Representative confocal microscopic images of cardiac fibroblasts/myofibroblasts embedded in collagen gel, following targeted treatments (serum-free medium, TGF-β1, 10 nM TIMP-2, 10 nM Ala+TIMP-2, and 10 nM TIMP-3). Cells were stained for pro-collagen I (red) and for nuclei (DAPI, blue). Scale bar=50μm. (b) Percentage of cells that was positive for pro-collagen I. TGF-β1, TIMP-2 and Ala+TIMP-2 stimulated more cardiac fibroblasts to express pro-collagen I. However, TIMP-3 did not exert such effect. n=8 random field images per treatment group. Bars represent mean ± SD. *, P<0.05 vs. serum-free medium; **, P<0.01 vs. serum-free medium; ns, non-significant vs. serum-free medium.
3.5 Cell Number and Cell Viability

3.5.1 Introduction

Although the main factor that drives collagen gel contraction is the proportion of myofibroblasts presents in the collagen gel, the alteration in cell number and cell viability may also play a role in regulating the gel contraction. To investigate if the cell number and the cell viability play a role in manipulating the collagen gel contraction, we harvested the cells directly from collagen gel after the exposure to targeted treatments, and subsequently performed cell count and trypan blue exclusion test for cell number and cell viability.

3.5.2 Results

In Table 3-1, we showed that cell numbers in all the treatment groups were equal: 12.4±3.7 [serum-free medium], 15.5±2.6 [TGF-β1, $P=0.09$ vs. serum-free medium] and 13.7±1.6 [TIMP-2, $P=0.43$ vs. serum-free medium]. On trypan blue exclusion test, we demonstrated that TGF-β1 (93.4±1.0%, $P=0.94$) and TIMP-2 (91.2±5.9%, $P=0.49$) did not significantly alter the cell viability as compared to the serum-free medium group (93.6±6.6%).

3.5.3 Summary

In conclusion, TGF-β1 and TIMP-2 did not significantly alter the cell number and the cell viability of the cardiac fibroblasts/myofibroblasts embedded in collagen gel. These data supported that collagen gel contraction is mainly driven by the phenotypic differentiation of fibroblasts into myofibroblasts.
Table 3-1: Cell count and cell viability

(a) Representative photomicrographs of cardiac fibroblasts/myofibroblasts harvested directly from collagen gel after the exposure to targeted treatments. Dead cells were stained blue by trypan blue stain (arrows). Objective: 20x. n=8 samples per treatment group. (b) All treatment groups (serum-free medium, TGF-β1 and TIMP-2) yielded an equal cell number and cell viability.
3.6 Collagen Gel Matrix Architecture

3.6.1 Introduction

Guidry and Grinnell suggested that the extent of collagen gel contraction has little dependence on collagen synthesis or degradation, and fibril cross-linking or enzymatic modification (Guidry & Grinnell, 1985). Therefore, it is intriguing to visualize and evaluate the matrix content and architecture directly within collagen gel. As per our protocol, the collagen gel is made up of type I collagen. By using immunostaining technique and laser scanning confocal microscopy, we reconstructed 3-D images of the type I collagen fibrils to visualize their organization and structure within the collagen gel.

3.6.2 Results

Figure 3-13 demonstrated the validity of this method. First of all, we determined that TGF-β1 treatment increased the collagen fibril density within the collagen gel. This observation was anticipated, as TGF-β1 is a potent pro-fibrotic stimulator that gives rise to new collagen production (Figure 3-13: second row). Degradation of ECM components is one of the main functions of MMPs, therefore MMP-2 notably disrupted the collagen gel matrix architecture (Figure 3-13: third row and bottom row). The extent of degradation and disruption was directly proportionate to the concentration of MMP-2 applied (100 ng/mL vs. 200 ng/mL).

We next investigated the effects of TIMP-2, Ala+TIMP-2 and TIMP-3 in regulation of collagen gel matrix organization. Interestingly, we did not see an increase in collagen fibril density as expected; instead a modest amount of collagen breakdown was observed (Figure 3-14: second row). Ala+TIMP-2 neither enhanced nor reduced collagen density within the collagen gel.
(Figure 3-14: third row). Similarly, TIMP-3 did not significantly alter the collagen gel matrix architecture (Figure 3-14: bottom row).

3.6.3 Summary

In summary, we demonstrated that TGF-β1 increased the collagen fibril density (fibrosis) within the collagen gel. MMP-2 dose-dependently degraded the gel content and significantly disrupted the organization of collagen fibrils. All TIMPs (TIMP-2, Ala+TIMP-2, and TIMP-3) did not significantly alter the collagen gel matrix organization and content as compared to the serum-free medium group. Collagen gel contraction was not dependent on the alteration in collagen gel matrix organization and content. This observation is in agreement with past publication (Guidry & Grinnell 1985).
Figure 3-13: Effects of TGF-β1 and MMP-2 on collagen gel matrix architecture and content

Type I collagen fibrils within collagen gel were stained red and 3-D images were reconstructed. Each row consists of three different views of one representative 3-D collagen gel image of each treatment group. **Top row**: serum-free medium, serving as a control. **Second row**: TGF-β1 increased the collagen fibril density in collagen gel. **Third row**: 100 ng/mL significantly disrupted the collagen gel matrix architecture. **Bottom row**: higher concentration of MMP-2 (200 ng/mL) further degraded the collagen fibrils, demonstrating a mesh-like appearance.
Figure 3-14: Effects of TIMPs on collagen gel matrix architecture and content

Type I collagen fibrils within collagen gel were stained red and 3-D images were reconstructed. Each row consists of three different views of one representative 3-D collagen gel image of each treatment group. **Top row:** serum-free medium, serving as a control. **Second row:** TIMP-2 did not increase the density of collagen fibrils, instead a modest amount of collagen degradation was revealed. **Third row:** Ala+TIMP-2 did not significantly alter the collagen gel matrix architecture. **Bottom row:** TIMP-3 neither increased nor decreased the collagen fibril density.
3.7 In Situ Zymography (Gelatin-FITC)

3.7.1 Introduction

We employed a unique technique—in situ zymography, to measure the total protease activities within a collagen gel. DQ™ Gelatin-FITC is a special analogue of gelatin that is conjugated to quenched fluorescein. It emits high fluorescent signals as it is cleaved, thereby liberating the quenched fluorescein molecule (Chhabra et al., 2012; Frederiks, 2004). To measure the emitted fluorescent signals within the collagen gel, Z-stack images were captured using a laser scanning confocal microscopy and 3-D images were analyzed. Total protease activity was quantified as total fluorescence per image volume.

3.7.2 Results

We first examined the feasibility of this method in collagen gel (Figure 3-15). We utilized activated MMP-2 as our positive control. Both concentrations of MMP-2 (100 ng/mL and 200 ng/mL) enhanced the total protease activity within collagen gel (332.5±39.5 [100 ng/mL] & 376.0±139.4 [200 ng/mL]), as compared to that of the serum-free medium group (166.0±45.3, P<0.001). A dose-dependent trend was observed.

Subsequently, we investigated the effects of TGF-β1 and TIMP-2 on the total protease activity within collagen gel (Figure 3-16). TGF-β1 stimulated the protease activity as compared to the serum-free medium group (307.6±119.6 [TGF-β1] vs. 166.0±45.3 [serum-free medium], P<0.05). 10 nM of TIMP-2 also induced a significant increase in the total protease activity than the serum-free medium group (500.3±130.4 [TIMP-2] vs. 166.0±45.3 [serum-free medium],
Surprisingly, TIMP-2 yielded a much higher protease activity than TGF-β1 (500.3±130.4 [TIMP-2] vs. 307.6±119.6 [TGF-β1], P=0.008).

We also compared the protease activity following treatment with TIMP-2 and Ala+TIMP-2 (Figure 3-17). Ala+TIMP-2 significantly increased the total protease activity from the baseline level (685.5±160.9 [Ala+TIMP-2] vs. 166.0±45.3 [serum-free medium], P<0.001), indicating that the induction of protease activity is independent of TIMP-2’s MMP-inhibitory actions. In fact, Ala+TIMP-2 yielded a higher protease activity than TIMP-2 (685.5±160.9 [Ala+TIMP-2] vs. 500.3±130.4 [TIMP-2], P<0.05), likely due to a lack of MMPs inhibition.

3.7.3 Summary

We have established the feasibility of in situ zymography in the collagen gel assay in our laboratory. The addition of activated exogenous MMP-2 resulted in a higher protease activity in a dose-dependently trend. TGF-β1 significantly enhanced the protease activity within the collagen gel. Both TIMP-2 and Ala+TIMP-2 induced a higher protease activity than TGF-β1. Ala+TIMP-2 yielded the highest protease activity, likely due to its inability to inhibit MMPs. These data indicated that TIMP-2 plays a dual role in both protease inhibition and activation. The protease activity stimulated by TIMP-2 could possibly explain the stimulation of collagen gel contraction and the net effect on collagen gel matrix modulation.
Figure 3-15: Effects of MMP-2 on the total protease activity within collagen gel

(a) Representative confocal microscopic images of the emitted fluorescent signals (green) within collagen gel, following proteolysis of the embedded DQ™ Gelatin-FITC. Scale bar=200 µm. (b) Total protease activity within collagen gel quantified as total fluorescent signals per image volume. Positive controls: both 100 ng/mL and 200 ng/mL of MMP-2 enhanced the total protease activity within the collagen gels. A dose-dependent trend was observed. n=8 random field images per treatment group. Bars represent mean ± SD. ***, P<0.001 vs. serum-free medium.
Figure 3-16: Effects of TGF-β1 and Ala+TIMP-2 on the total protease activity within collagen gel

(a) Representative confocal microscopic images of the emitted fluorescent signals (green) within collagen gel, following proteolysis of the embedded DQ™ Gelatin-FITC. Scale bar=200μm. (b) Total protease activity within collagen gel quantified as total fluorescent signals per image volume. Both TGF-β1 and TIMP-2 increased the total protease activity within collagen gel. TIMP-2 yielded a higher total protease activity than TGF-β1 ($P=0.008$). $n=8$ random field images per treatment group. Bars represent mean ± SD. *, $P<0.05$ vs. serum-free medium; ***, $P<0.001$ vs. serum-free medium.
Figure 3-17: Effects of TIMP-2 and Ala+TIMP-2 on the total protease activity within collagen gel

(a) Representative confocal microscopic images of the emitted fluorescent signals (green) within collagen gel, following proteolysis of the embedded DQ™ Gelatin-FITC. Scale bar=200 μm. (b) Total protease activity within collagen gel quantified as total fluorescent signals per image volume. TIMP-2 increased the total protease activity within the collagen gel. Ala+TIMP-2 exhibited a higher total protease activity than TIMP-2, likely due to a lack of MMP inhibition. n=8 random field images per treatment group. Bars represent mean ± SD. *, P<0.001 vs. serum-free medium; #, P<0.05 vs. 10 nM TIMP-2.
3.8 Gelatin Zymography

3.8.1 Introduction

MMPs are secreted in the latent pro-MMP form, which requires further activation by a proteolytic processing (Strongin et al., 1995). The total MMP proteolytic activity is dependent on the expression of MMP proteins and the activation of the secreted pro-MMPs. In this study, we were particularly interested in MMP-2, given that TIMP-2 is critical in the surface activation of pro-MMP-2 (Kandalam et al., 2010; Strongin et al., 1995; Wang et al., 2000). Therefore, we extended our investigation to explore the effect of particular treatment on the total MMP-2 expression level and the extent of MMP-2 activation. Gelatin zymography was performed to estimate the expression of active MMP-2 and pro-MMP-2. The total MMP-2 protein level was calculated by summing the active MMP-2 level and the pro-MMP-2 level. The extent of MMP-2 activation was presented as proportion of active MMP-2 within the total MMP-2 protein level.

3.8.2 Results

We first explored the effect of TGF-β1, GM6001, TIMP-3, TIMP-2, and Ala+TIMP-2 on the total MMP-2 protein expression level (Figure 3-18). At baseline, the serum-free medium group resulted in a total MMP-2 protein level of 6.9, and TGF-β1 increased the MMP-2 expression level to 8.7. MMP inhibition by GM6001 did not alter the total MMP-2 expression (7.0), as compared to the serum-free medium group. However, treatment with TIMP-3 reduced the MMP-2 protein level to 5.5. TIMP-2 treatment slightly increased the overall MMP-2 expression (7.5), but Ala+TIMP-2 treatment did not alter the baseline MMP-2 expression level (6.8).
We next examined the extent of MMP-2 activation by calculating the proportion of active MMP-2 within the total MMP-2 protein (Figure 3-19). 10 nM of TIMP-3 demonstrated a relatively low degree of MMP-2 activation at only 55%. Surprisingly, TIMP-2, although it belongs to the same family, exhibited a much higher extent of MMP-2 activation (79%). Ala+TIMP-2 also yielded a similar magnitude of MMP-2 activation (77%), indicating that TIMP-2 stimulated the activation of MMP-2 through a mechanism that is independent of its MMP-inhibitory actions. In fact, the extent of MMP-2 activation of TIMP-2/Ala+TIMP-2 was similar to that of the TGF-β1 group (82%).

3.8.3 Summary

In summary, TGF-β1 resulted in a remarkably higher expression of total MMP-2 protein levels as compared to the serum-free medium. MMP inhibition by GM6001 did not alter the total MMP-2 levels, but TIMP-3 remarkably reduced the protein levels. TIMP-2 slightly increased the MMP-2 levels, whereas Ala+TIMP-2 did not alter the protein levels.

Treatment with TIMP-2 resulted in a higher extent of MMP-2 activation as compared to TIMP-3. Ala+TIMP-2 treatment also stimulated MMP-2 activation to an extent that was comparable to that of the TIMP-2 group. This observation indicated that the MMP-2 activation by TIMP-2 does not involve its amino-terminus. The MMP-2 activation induced by both TIMP-2 and Ala+TIMP-2 was similar to that of the TGF-β1 group.
Figure 3-18: Measurement of total MMP-2 proteins (active MMP-2 and pro-MMP-2)

(a) An example of gelatin zymography film that comprised of all treatment groups. (b) The total MMP-2 protein levels in each group were calculated by summing up the active MMP-2 levels and the pro-MMP-2 levels. The baseline expression of MMP-2 proteins was measured in the serum-free medium group (6.9). TGF-β1 stimulated an increase in the expression of total MMP-2 proteins (8.7). GM6001 did not exert any effect on the total MMP-2 expression (7.0), whereas TIMP-3 remarkably reduced the MMP-2 protein levels (5.5). Treatment with TIMP-2 slightly increased the overall MMP-2 expression (7.5), but Ala+TIMP-2 treatment did not alter the baseline MMP-2 expression levels (6.8).
Figure 3-19: Measurement of MMP-2 activation- proportion of active MMP-2

The extent of MMP-2 activation is shown as the proportion of active MMP-2 within the total MMP-2 expressed (active MMP-2 and pro-MMP-2). Dark bars represent active MMP-2 and grey bars represent pro-MMP-2. 10 nM of TIMP-3 demonstrated a relatively low magnitude of MMP-2 activation (55%). Both TIMP-2 and Ala+TIMP-2 exhibited much higher extent of MMP-2 activation (79% and 77%, respectively), which was similar to that of the TGF-β1 group (82%).
4.1 Discussion

4.1.1 3-D Collagen Gel Culture System More Accurately Reflects the Actual in vivo Environment

In this study, we assessed the difference in the cellular morphology of cardiac fibroblasts cultured in 2 different culture systems: coverslips (2-D) vs. collagen gel (3-D). Conventional culture dish/flask and coverslip are described as a 2-D culture system, as cells cultured in this system grow in a monolayer manner and attach on a flat surface that is usually rigid. In this culture system with minimal substrate pliability, cardiac fibroblasts are subjected to high tensile force and mechanical stress through its contractile stress fibres. Mechanical tension is the most potent stimulator for differentiation of fibroblasts into myofibroblasts (Tomasek et al., 2002; Wang et al., 2003), and expression of α-SMA by cardiac fibroblasts is extremely sensitive to its culture environment (Rohr, 2011). Therefore, cardiac fibroblasts cultured in a conventional culture dish/flask high tend to express high level of α-SMA at baseline. As demonstrated in Figure 4-1, cardiac fibroblasts grown in this culture system (left top panel and left bottom panel) exhibit a stellate shape that includes extensive projections from the cell body. Virtually all fibroblasts cultured in this system expressed a high level of α-SMA at baseline, even without prior stimulation.

Correspondingly, cardiac fibroblasts cultured in a floating 3-D collagen gel culture have a much lower baseline α-SMA expression, as the pliability of collagen gel provides room for reorganization by cardiac fibroblasts. Therefore, the mechanical stress in cardiac fibroblasts
embedded in a collagen gel is much lower as compared to their counterpart in a 2-D culture system. Figure 4-1 below (right top panel and right bottom panel) showed the cellular morphology of cardiac fibroblasts grown in a 3-D collagen gel. These cells maintained their spindle and elongated shape with less projections. The baseline α-SMA expression in these cells was significantly lower, and some cells were completely negative of α-SMA expression. These observations indicate that phenotypic conversion of cardiac fibroblasts into cardiac myofibroblasts is highly dependent on cell culture environment. We also demonstrated the difference in cellular morphology and baseline α-SMA expression in two different culture systems: 2-D vs. 3-D. Our main findings in this study are well supported by several publications (Ceresa, Knox, & Johnson, 2009; Hakkinen et al. 2010; Rohr, 2011). Besides the discrepancy in cellular morphology and baseline α-SMA expression, cells cultured in 2-D and 3-D culture systems exhibited difference in terms of mechanical tension, basal proliferation rate, and collagen synthesis (as summarized in Table 4-1).

When there appear to exist discrepancies between 2-D and 3-D culture systems, questions may arise as to which culture system is preferable to produce data that better reflects the actual in vivo environment. In reality, cardiac fibroblasts in myocardium exist in a 3-D environment with extension of projections in all dimensions, rather than just one surface, as in monolayer in 2-D culture system. Cardiac fibroblasts play an important role in modulating the surrounding ECM via cell-ECM and cell-cell interactions. As outlined in Table 4-1, several cellular features and cell fate of cardiac fibroblasts are highly dependent on the surrounding environment. 3-D culture system mimics the natural in vivo ECM features and thus it is able to stimulate an in vivo tissue-like environment to the embedded cells (Carlson et al., 2008; Lee, Cuddihy, & Kotov, 2008;
Rhee & Grinnell, 2007). The morphological features of cells cultured in a 3-D culture system have been consistently reported to be similar to those of the *in vivo* resting cells (Fringer & Grinnell, 2001; Tamariz & Grinnell, 2002). In other words, a 3-D experimental model serves as an excellent *in vitro* tool to understand how cells sense and generate mechanical forces in response to their surrounding environment (Rhee & Grinnell, 2007; Pedersen & Swartz, 2005; Grinnell, 2003). This model also allows us to directly explore and delineate the complex *in vivo* tissue physiology in an *in vitro* setting (Griffith & Swartz, 2006; Grinnell, 2003; Rhee & Grinnell, 2007). Therefore, in this study, we performed all our assays and experiments in a 3-D collagen gel model, in order to obtain data that more accurately reflects the actual *in vivo* situations.
Cardiac fibroblasts cultured on coverslip (2-D) and in collagen gel (3-D) were stained for α-SMA (green) and for nuclei (DAPI-blue). *Left (Top & Bottom):* Cells cultured on coverslip exhibited a flat and stellate shape with extensive projections, featuring cardiac myofibroblasts. Virtually all cells in this culture system expressed high baseline level of α-SMA. *Right (Top & Bottom):* Cells cultured within collagen gel maintained its spindle and elongated shape. The baseline α-SMA expression was significantly lower than that of the cells cultured on coverslip. Some cells did not expression α-SMA at all.
<table>
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<tr>
<th>Cellular Morphology</th>
<th>2-D (Culture Dish/Coverslip)</th>
<th>3-D (Collagen Gel)</th>
<th>References</th>
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<td></td>
<td>Changed from an elongated shape to a stellate shape with extensive projections</td>
<td>Maintain their elongation and have less projections</td>
<td>Ceresa, Knox, &amp; Johnson 2009; Hakkinen et al., 2010; Rohr 2011; Tamariz &amp; Grinnell 2002; Tomasek et al., 2009</td>
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<tr>
<td>Baseline α-SMA Expression</td>
<td>Higher</td>
<td>Lower</td>
<td>Ceresa, Knox, &amp; Johnson 2009; Rohr 2011</td>
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<tr>
<td>Mechanical Tension</td>
<td>Higher</td>
<td>Lower in floating gel; Higher in restrained gel</td>
<td>Grinnell 2003; Pedersen &amp; Swartz 2005; Tomasek et al., 2002; Wang et al., 2003</td>
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<tr>
<td>Basal Proliferation Rate</td>
<td>Higher rate</td>
<td>Lower rate; Requires higher concentration of serum for stimulation</td>
<td>Fringer &amp; Grinnell 2001; Hadjipanayi, Mudera, &amp; Brown 2009; Mio et al., 1996; Ceresa, Knox, &amp; Johnson 2009, 2009</td>
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<tr>
<td>Collagen Synthesis</td>
<td>Higher</td>
<td>Lower</td>
<td>Burgess et al., 1994; Mauch et al., 1988</td>
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Table 4-1: A summary of differences in fibroblasts cultured in 2-D and 3-D culture systems
4.1.2 TIMP-2 Induces Phenotypic Differentiation of Cardiac Fibroblasts into Myofibroblasts

We aimed to explore the role of TIMP-2 in regulation of cardiac fibroblasts, and to determine whether this role is dependent on its MMP-inhibitory actions. We focused on experiments with collagen gel contraction assay (Section 3.2) that mimics in vivo environment to functionally assess the phenotypic conversion of cardiac fibroblasts into myofibroblasts. 3-D collagen gel is an in vitro assay that mimics in vivo arrangement of collagen fibrils in the extracellular matrix (ECM) (Ehrmann & Gey, 1956). Further modification of this method into a functional contraction assay to study fibroblast-mediated ECM remodeling in vitro was first described by Bell and colleagues 1979 (Bell, Ivarsson, & Merrill, 1979). Collagen gel contraction has shown little dependence on collagen synthesis or degradation, and fibrils cross-linking or enzymatic modification (Guidry & Grinnell, 1985). Measuring the amount of collagen gel contraction is believed to be positively associated with the extent of myofibroblast activation within a 3-D collagen gel. For example, Lijnen and colleagues demonstrated that the TGF-β1-induced collagen gel contraction is directly correlated with the TGF-β1-evoked appearance of α-SMA in collagen gel matrix (Lijnen et al., 2003). Angiotensin-II (AT-II) is a well-known stimulator of differentiation of fibroblasts into myofibroblast (Leask, 2010; Rosenkranz, 2004; Sun et al., 1998), and the addition of AT-II dose-dependently stimulated the cardiac fibroblast-mediated collagen gel contraction (Lijnen et al., 2004) without altering the DNA content and [3H]-thymidine incorporation within the collagen gel (Lijnen et al., 2005).

We confirm that the main contributor of collagen gel contraction is differentiation of cardiac fibroblasts into myofibroblasts (Section 3.3 & 3.4). The results of these sections specifically evaluated the hypotheses that TIMP-2 induces cardiac fibroblast-mediated collagen
gel contraction (*Hypothesis i*), and that TIMP-2 induces phenotypic conversion of cardiac fibroblasts into cardiac myofibroblasts (*Hypothesis ii*).

In *Section 3.2*, we demonstrate that TIMP-2 treatment significantly enhanced the collagen gel contraction (Figure 3-7). In *Section 3.3*, we show that TIMP-2 enhanced the expression of α-SMA (Figure 3-11); and *Section 3.4* shows that TIMP-2 stimulated an increase in collagen synthesis in the cardiac fibroblasts, indicating the presence of myofibroblasts within these TIMP-2-treated cell population (Figure 3-12). All these effects of TIMP-2 were not observed with TIMP-3 treatment, suggesting the specificity of these effects to TIMP-2, as well as to the diversity of biological actions of each TIMP species. Differentiated myofibroblasts are defined by the presence of their specific marker α-SMA and up-regulated collagen synthesis activity (Gabbiani, Ryan, & Majne, 1971). Therefore, we confirm that the induction of collagen gel contraction is directly proportionate to the extent of myofibroblast differentiation. Figure 4-2 shows a comparison of the results obtained from both the collagen gel contraction assay, and the measurement of cellular α-SMA expression, a positive correlation is clearly demonstrated between these two variables.

These findings are consistent with the publication by Lovelock and coworkers (Lovelock et al., 2005), which reported that TIMP-2 resulted in a significant increase in α-SMA expression within the subpopulation of α-SMA-positive cardiac fibroblasts. Furthermore, the same study also demonstrated that TIMP-2 stimulated a significant 4- to 5-fold increase in collagen synthesis.
Figure 4-2: Positive correlation between collagen gel contraction and cellular α-SMA expression

(a) A summary of the collagen gel contraction data of all targeted treatments. (b) An overview of the cellular α-SMA expression following the same treatments as above. Bars represent mean±SD. *, P<0.01 vs. serum-free medium; ns, non-significant vs. serum-free medium. Note that there is a strong correlation between collagen gel contraction and cellular α-SMA expression. (c) A positive correlation (r² = 0.932) between collagen gel contraction and cellular α-SMA expression was clearly demonstrated, indicating that collagen gel contraction is primarily dependent on the phenotypic conversion of fibroblasts into myofibroblasts.
4.1.3 TIMP-2 Does Not Alter Cardiac Fibroblast Cell Proliferation and Cell Viability Cultured in 3-D Collagen Gel Matrix

TIMP-2 has been shown to possess the ability to induce proliferation of fibroblasts and several other cell types (Hayakawa et al., 1994; Corcoran & Stetler-Stevenson, 1995; Lovelock et al., 2005). In our collagen gel contraction assay, an equal number of cells (1x10^5 cells) were seeded into each collagen gel of 400 μL just prior to treatment; these cells were subsequently cultured for another 48 hours (24 hours of initial treatment + 24 hours post-gel release). Previous studies have suggested that the proliferation rate of the cells embedded in a 3-D collagen gel culture system is low (Ceresa, Knox, & Johnson, 2009; Fringer & Grinnell, 2001; Mio et al., 1996). Therefore, the cells seeded in our collagen gel model were unlikely to proliferate significantly over this time period. The cell numbers within the collagen gel in all treatment groups should remain constant or similar before and after treatment. To confirm this, we directly harvested the cardiac fibroblasts/myofibroblasts from collagen gel and performed cell count on a hemacytometer (Section 3.5). In Table 3-1, the TIMP-2 group yielded very similar cell count as compared to the serum-free medium group, implicating that TIMP-2 does not induce the collagen gel contraction by stimulating fibroblast proliferation.

TIMP-2 is also believed to support the survival of cells cultured in the absence of serum (Nemeth & Goolsby, 1993; Matsumoto et al., 1993). Only viable cells are able to contract collagen gel, thus it is necessary to investigate whether cell viability plays a role in regulating the collagen gel contraction (Hypothesis iii). We measured the cell viability by performing trypan blue exclusion test on the cells harvested directly from the collagen gel after treatment with serum-free medium and TIMP-2 (Section 3.5). As shown in Table 3-1, TIMP-2 did not significantly alter the cell viability as compared to the serum-free medium. These data further
support the concept that TIMP-2 induces collagen gel contraction mainly via phenotypic conversion of cardiac fibroblasts into myofibroblasts.

4.1.4 MMP-Inhibitory Actions of TIMP-2 Are Not Essential in Differentiation of Cardiac Fibroblasts into Myofibroblasts

Over the past two decades, there has been emerging evidence that TIMP-2, as well as other TIMP species, possesses biological actions that are independent of its MMP-inhibitory actions (Stetler-Stevenson, 2008). Ala+TIMP-2 is a special analogue of TIMP-2 that is completely devoid of MMP-inhibitory activity, while maintaining TIMP-2’s secondary and tertiary structure of protein (Wingfield et al., 1999). Ala+TIMP-2 is named as such because a single amino acid, alanine, is appended to the amino-terminus of TIMP-2 that is involved in MMP inhibition (Wingfield et al., 1999). Ala+TIMP-2 is a useful biochemical tool to explore the novel biological activities of TIMP-2 against a background of its MMP-inhibitory actions.

Accordingly, we utilized Ala+TIMP-2 to explore the hypothesis that MMP-inhibitory actions of TIMP-2 are not essential in induction of cardiac myofibroblast activation (Hypothesis iv). The results of Section 3.3 shows that Ala+TIMP-2 also stimulated an increase in α-SMA expression that characterized the presence of differentiated cardiac myofibroblasts in the treated cell population (Figure 3-11). Similarly, in Section 3.4, we determine that Ala+TIMP-2 treatment up-regulated the collagen synthesis activity within the treated cells (Figure 3-12). The phenotypic conversion of fibroblasts into myofibroblasts induced by Ala+TIMP-2 was also reflected in the functional collagen gel contraction assay (Section 3.2), in which Ala+TIMP-2 gave rise to a collagen gel contraction of similar extent as compared to that of the TIMP-2 group (Figure 3-8). Bildt and colleagues showed that the application of synthetic MMP inhibitors
reduced collagen gel contraction and down-regulated the cellular α-SMA expression (Bildt et al., 2009). Consistently, our data also confirm that treating cardiac fibroblasts with synthetic broad-spectrum MMP inhibitor (GM6001) did not enhance the collagen gel contraction as seen with TIMP-2 treatment. Instead, GM6001 dose-dependently inhibited the collagen gel contraction (Figure 3-5). Thus, we have strong evidence that MMP-inhibitory actions of TIMP-2 are not involved in the differentiation of cardiac fibroblasts into myofibroblasts.

4.1.5 ECM Homeostasis: Balance of Matrix Degradation and Deposition

A dynamic balance of MMP degradation activity and new collagen synthesis is critical in the maintenance of ECM homeostasis (Lu et al., 2011). Dysregulation of ECM homeostasis, either with excessive degradation of matrix component or with increased matrix deposition (fibrotic response), is implicated in pathophysiology of heart failure (Brower et al., 2006; Weber et al., 1994). Consistent with previous publications (Lovelock et al., 2005), our experimental data show that TIMP-2 promotes the differentiation of cardiac fibroblasts into myofibroblasts active in new collagen synthesis (Sections 3.3 & 3.4). Nevertheless, while TGF-β1 strikingly increased the collagen fibril density in collagen gel (fibrosis), we did not observe such effect in the TIMP-2 group and the Ala+TIMP-2 group (Section 3.6). The collagen gel matrix density and organization of both the TIMP-2 and the Ala+TIMP-2 groups were similar to that of the serum-free medium group, although a moderate amount of matrix degradation was observed in the TIMP-2 group. We speculated that TIMP-2 and Ala+TIMP-2 stimulated new collagen formation and collagen degradation simultaneously. The net effect can explain the overall matrix turnover observed in these 2 groups.
In Section 3.4, we demonstrate that all TGF-β1, TIMP-2 and Ala+TIMP-2 enhanced the collagen synthesis in cardiac fibroblasts/myofibroblasts (Figure 3-12). Subsequently, in Section 3.7, we observed that although TGF-β1 stimulated an increase in the total protease activity within the collagen gel, the magnitude was significantly lower than that of the TIMP-2 group (Figure 3-16). Both TIMP-2 and Ala+TIMP-2 enhanced the total protease activity (Figure 3-17). Collectively, we are able to explain our observations in the alteration of collagen gel matrix content in Section 3.6 as follows: TGF-β1 can promote a relatively large increase in collagen synthesis as compared to the increase in total protease activity, leading to a net fibrotic response with accumulation of ECM components. On the other hand, TIMP-2 and Ala+TIMP-2 induce a similar degree of increase in both collagen synthesis and total protease activity, resulting in ECM homeostasis with no obvious alteration in the overall ECM content and architecture. A summary of our proposed explanations is illustrated as below (Figure 4-3).
Figure 4-3: *In vitro* ECM homeostasis in a 3-D collagen gel

Illustration of *in vitro* collagen gel ECM homeostasis in several groups of targeted treatments: serum-free medium (baseline control), TGF-β1 (fibrotic control), TIMP-2/Ala+TIMP-2, and TIMP-3. We assumed that the serum-free medium group exhibited a state of ECM homeostasis, in which the collagen degradation rate and the collagen synthesis rate were equal. TGF-β1 shifted the ECM balance toward a fibrotic response. TIMP-2, Ala+TIMP-2 and TIMP-3 resulted in the collagen gel matrix appearance that is similar to that of the serum-free medium group. We proposed that TIMP-2/Ala+TIMP-2 achieved a near-homeostatic state by inducing a balanced increase in both collagen degradation and collagen synthesis.
4.1.6 TIMP-2 Induces Collagen Gel Contraction via MMP Induction

In this study, what led us to the speculation that TIMP-2 might increase collagen gel contraction via MMP activation (Hypothesis v) was the observation in collagen gel matrix architecture in Section 3.6. Based on the results in the Sections 3.2-3.4, we confirm that TIMP-2 stimulated myofibroblast differentiation and promoted new collagen synthesis, to an extent that was comparable to that of the TGF-β1 group. Thus, we anticipated a similar increase in collagen gel matrix density (fibrosis) in both the TGF-β1 and the TIMP-2 groups. Surprisingly, the collagen gel containing cardiac fibroblasts treated by TIMP-2 did not display a denser collagen fibril organization; rather it exhibited a slight degradation of collagen gel matrix (Figure 3-14). This implies the possibility of simultaneous collagen synthesis and degradation in the TIMP-2-treated collagen gel.

Subsequently, we employed in situ zymography in order to measure the total protease activity within the collagen gel (Section 3.7). TIMP-2 significantly enhanced the total protease activity in the collagen gel. On top of that, Ala+TIMP-2 exhibited a much greater amount of protease activity, due to its inability to inhibit active MMPs (Figure 3-17). These data tell us that there was indeed an increase in total protease activity in the TIMP-2-treated group that could possibly explain the enhanced collagen gel contraction, however they do not reveal specifically which MMPs or other proteolytic proteins were elevated.

Up-regulated MMP levels and activities are associated with increased collagen gel contraction (Margulis et al., 2009; Lijnen, Petrov, & Fagard, 2001; Toriseva et al., 2007; Davis et al., 2001; Fredriksson et al., 2006; Mikko et al., 2008). Likewise, synthetic MMP inhibitors repress collagen gel contraction (Margulis et al., 2009; Bildt et al., 2009; Ceresa, Knox, & Johnson, 2009; Myers & Wolowacz, 1998). MMPs and synthetic MMP inhibitors do not
modulate collagen gel contraction by directly manipulating the collagen content and fibrillar architecture within the gel matrix. Rather, MMPs enhance collagen gel contraction via proteolytic activation of latent TGF-β1. TGF-β1 is known to be the most potent stimulator of collagen gel contraction (Lijnen et al., 2003; Lijnen, Petrov, & Fagard, 2003) and it is often used as a positive control in collagen gel contraction assay.

TGF-β1 is synthesized in considerable excess and the activation of TGF-β1 is the rate-limiting step in its bioavailability (Annes, 2003; Jenkins, 2008). TGF-β1 is secreted as pro-peptides that have high affinity for another TGF-β1 molecule and thus assembles into a non-covalent complex, the “small latent complex (SLC).” SLC composes of an active TGF-β1 molecule and heterodimers of the pro-peptides that are also known as “latency associated protein (LAP).” For the most part, the SLC is bound to a latent TGF-β1 binding protein (LTBP), via a disulphide bond, forming the “large latent complex (LLC).” The LTBP is covalently bound to the ECM and can therefore determine the cellular localization and tissue distribution of TGF-β1. A diagram illustrating a “large latent complex (LLC)” containing TGF-β1 is shown in Figure 4-4 (Wipff & Hinz, 2008). Several MMPs have the ability to cleave the LLC in order to activate it, especially MMP-2, MMP-9, and MMP-13 (Yu & Stamenkovic, 2000; Ge & Greenspan, 2006; Maeda et al., 2002; D’Angelo et al., 2001; Karsdal et al., 2002; Dallas et al., 2002). Figure 4-5 illustrates activation of latent TGF-β1 via interactions with MMPs and integrin (Wipff & Hinz, 2008).

To further explore the possibility that TIMP-2 induces collagen gel contraction via MMP induction, we first determined in Section 3.2 that active MMP-2 has the ability to enhance collagen gel contraction in vitro (Figure 3-4). The TIMP-2-enhanced collagen gel contraction
was completely abolished by the application of GM6001 (Figure 3-9). Therefore, we conclude that TIMP-2 induces collagen gel contraction via MMP induction and this stimulation is prevented by MMP inhibition.

**Figure 4-4: Illustration of a “large latent complex (LLC)” containing latent TGF-β1 (adapted from Wipff & Hinz 2008)**

A TGF-β1 molecule has high affinity for another TGF-β1 molecule and thus assemble themselves into a non-covalent complex that binds to a heterodimer of “latency associated protein (LAP)” to form a “small latent complex (SLC)”. Most SLC bind to the ECM-bound “latent TGF-β1 binding protein (LTBP)” via a disulphide bond, forming a “large latent complex (LLC)”.
Latent TGF-β1 activation by MMPs is mediated by integrins. MMP-2 and MMP-9 interact with αvβ3 integrin (a) and liberate active TGF-β1 by proteolytically cleaving LAP (b). Proteolytically activated TGF-β1 is freely diffusible and principally acts on a local autocrine or paracrine level due to the restriction of the activation process to the cell surface.
4.1.7 Amino-Terminus of TIMP-2 is Not Involved in the Binding of Trimolecular Complex of TIMP-2/Pro-MMP-2/MT1-MMP

Our current data shows that treatment with TIMP-3 yielded a MMP-2 activation of 55% (Section 3.8, Figure 3-19). However, treatment with TIMP-2 did not have a similar inhibitory effect; instead it induced a much higher activation of MMP-2 to an extent that is similar to that of the TGF-β1 group. Activation of pro-MMP-2 by TIMP-2 via formation of a trimolecular complex with MT1-MMP occurs by a complex mechanism that has been well studied. In this activation mechanism, MT1-MMP is usually localized on cell surface and binding of TIMP-2 to MT1-MMP stabilizes its expression on cell surface. Subsequently, this MT1-MMP/TIMP-2 complex acts as a receptor for pro-MMP-2 where the propeptide of the pro-MMP-2 will be cleaved by the adjacent active MT1-MMP yielding active MMP-2 (Sato & Takino, 2010).

Interestingly, Ala+TIMP-2 also exhibited a similar profile of MMP-2 activation as TIMP-2, despite its inability to inhibit active MMPs. There are two possible implications for this observation: (i) activation of pro-MMP-2 by TIMP-2 is independent of its amino-terminus (-NH₂); (ii) Ala+TIMP-2 possesses the ability to bind its carboxyl-terminus (-COOH) to MT1-MMP. The carboxyl-terminal domain of TIMP-2 participates in binding to the catalytic site of MT1-MMP (Sato et al., 1996; Zucker et al., 1998). Furthermore, TIMP-2 binds to the pro-MMP-2 at the carboxyl-terminal domains of each protein (Fridman et al., 1992; Kleiner et al., 1992; Willenbrock et al., 1993) and this binding appears to be independent of MMP-inhibitory activity as Ala+TIMP-2 still forms a tight binary complex with pro-MMP-2 in a highly specific manner (Wingfield et al., 1999). Therefore, the formation of a trimolecular complex of Ala+TIMP-2/Pro-MMP-2/MT1-MMP is possible and thus Ala+TIMP-2 retains its ability to activate pro-MMP-2.
Figure 4-6: Schematic diagram of a trimolecular complex of TIMP-2/Pro-MMP-2/MT1-MMP with corresponding binding sites.

The carboxyl-terminus (-COOH) of TIMP-2 binds to the catalytic domain of MT1-MMP and stabilizes its localization on cell surface. This MT1-MMP/TIMP-2 complex subsequently serves as a receptor for pro-MMP-2 through the binding between the –COOH termini of both TIMP-2 and pro-MMP-2. The propeptide of pro-MMP-2 is eventually cleaved by an adjacent active MT1-MMP to yield active MMP-2. Ala+TIMP-2 contains an Alanine (Ala) residue appended to its amino-terminus (-NH$_2$), which does not interfere its interactions with MT1-MMP and pro-MMP-2.
4.2 Summary of Data

Figure 4-7: An overview of experimental data with corresponding sections in Chapter 3

4.2.1 Comparison: The Current Study vs. Lovelock et al., 2005

Lovelock and co-workers published an extensive study on heterogenous effects of all TIMPs on cardiac fibroblasts by employing adenovirus-mediated overexpression of each TIMP species in murine cardiac fibroblasts (Lovelock et al., 2005). The particular study demonstrated that among all TIMP species, TIMP-2 stimulated the most increase in α-SMA expression and collagen synthesis, featuring characteristics of differentiated myofibroblasts. Clinical data have shown that TIMP-2 levels were increased in the hearts of patients with several cardiac pathologies with adverse myocardial fibrosis, such as aortic valve stenosis (Fielitz et al., 2004) and pressure-overloaded cardiomyopathy (Heymans et al., 2005). Collectively, these observations raised a doubt whether TIMP-2 actually mediates the pathological fibrotic response.
The present study employed a functional 3-D collagen gel contraction assay to create an environment that better represents the actual in vivo settings. This unique method allows for direct assessment of complex matricellular interactions. Initially, the data collected from this approach supported the observations published by Lovelock and colleagues that TIMP-2 induced differentiation of cardiac fibroblasts into myofibroblasts, as shown by the increase in collagen gel contraction, α-SMA and type I pro-collagen expression. However, further investigation revealed that TIMP-2 did not induce a fibrotic response in the collagen gel matrix, as was observed following the TGF-β1 treatment. TIMP-2 is known to play a critical role in activation of pro-MMP-2 via formation of a trimolecular complex of TIMP-2/Pro-MMP-2/MT1-MMP (Sato & Takino 2010). Therefore, we speculated that TIMP-2 simultaneously induced ECM degradation. This speculation was confirmed by the in situ zymography assay, which demonstrated that TIMP-2 induced the total protease activity in the collagen gel (Figure 3-17).

Lovelock and colleagues did not explore the possible alteration in protease activity caused by TIMP-2. Second, this study did not consider the influence of culture system (2-D vs. 3-D) on phenotypic characteristics and functional behavior of cardiac fibroblasts. On the other hand, our study provides an overview of the complex matricellular interactions between TIMP-2 and human cardiac fibroblasts in a 3-D culture setting. On the basis of our findings, we can conclude that TIMP-2 induces a balanced increase in both myofibroblast activation and protease activity resulting in ECM homeostasis. This differs from Lovelock and colleagues’ conclusion regarding TIMP-2’s increased profibrotic activity (Lovelock et al., 2005).
4.3 Potential Limitations & Future Directions

For the first time, we are able to provide evidence that TIMP-2 plays a complex role in regulating ECM homeostasis, based on its effects on the balance between ECM component degradation and synthesis. Our data show that TIMP-2 stimulates fibroblasts to transform into myofibroblast phenotype that can enhance collagen synthesis. Simultaneously, TIMP-2 also induces the total protease activity within the same microenvironment. As a result, TIMP-2 is unlikely to bring about a pathological fibrotic response like TGF-β1, a fibrotic cytokine implicated in detrimental progression to heart failure. Further studies are warranted to explore the entire MMP profile within the cells and the conditioned medium after treatment with TIMP-2 in order to investigate if any other MMP is accountable for the induction of total protease activity by TIMP-2. This thesis also demonstrates that TIMP-2 provokes MMP-2 activation within the human cardiac fibroblast population cultured in a 3-D collagen gel culture system. Further studies should explore whether TIMP-2 treatment alters other components involved in this MMP-2 activation pathway, for example the cell surface localization of MT1-MMP and the expression of αvβ3 integrins. Mouse model of TIMP-2 deletion has been created and widely used in several in vivo studies to study cardiac pathophysiology (Kandalam et al., 2011; Kandalam et al., 2010). Application of TIMP-2−/− cardiac fibroblasts in our current experimental model could provide further information regarding the overall ECM balance in the total absence of TIMP-2 expression. A complete delineation of these complex interactions would allow us to optimize TIMP-2 as a potential therapeutic agent, or at least as an adjunct therapy, for patients at risk of congestive heart failure. If the prolonged myofibroblast-activating action of TIMP-2 is deemed to be detrimental in cardiac remodeling process, we could possibly shut down that action specifically, while maintaining the other beneficial actions of TIMP-2.
The human cardiac fibroblasts used in this study were isolated from the left atrial appendage. Studies have indicated that atrial cardiac fibroblasts behave differently than ventricular cardiac fibroblasts over a range of \textit{in vitro} and \textit{in vivo} paradigms. Burstein and colleagues have performed an extensive study comparing both \textit{in vitro} and \textit{in vivo} behaviours of atrial fibroblasts and ventricular fibroblasts in a canine model (Burstein et al., 2008). This study demonstrated that atrial cardiac fibroblasts are consistently more reactive than ventricular fibroblasts, and exhibit distinct growth characteristics, secretory functions, proliferative responses and gene expression profile (Burstein et al., 2008). Atrial cardiac fibroblasts tend to be more responsive to several pro-fibrotic cytokines, such as TGF-\(\beta\)1 (Nakajima et al., 2000; Verheule et al., 2004) and angiotensin-converting enzyme (ACE) (Xiao et al., 2004). The difference in cellular characteristics of atrial and ventricular cardiac fibroblasts might influence the interpretation of our data and caution may be needed in extrapolating our conclusions from these data to settings in the ventricular remodeling. Nevertheless, this does not diminish the overall significance of our findings in this study. In fact, atrial fibroblasts and ventricular fibroblasts exhibit similar trend of responses to numerous stimulations, albeit the extent of response is different with atrial fibroblasts being more sensitive to certain stimulations (Burstein et al., 2008). Moreover, this study is still able to contribute novel knowledge to cardiovascular basic science field, and there are a large number of possibilities in terms of application of this knowledge. Further studies could be carried out specifically on human ventricular cardiac fibroblasts, in order to determine whether these cells respond to TIMP-2 treatment differently than their atrial counterpart.

Our \textit{in vitro} 3-D collagen gel system mimics many key properties in the actual \textit{in vivo} situation and thus provides a much-improved imitation of \textit{in vivo} microenvironment in an \textit{in vitro}
setting. Several modifications can be considered to improve this innovative method in order to provide an even better imitation of a living structure. For instance, co-culturing several cell types in this 3-D collagen gel culture system might provide a better mimicking of the actual microenvironment, as in reality there exist multiple cells types in a living organ. It should also be noted that the collagen gel system used in our lab is constituted by using mainly type I collagen. A healthy human heart is primarily composed of type I collagen (85%) and type III collagen (11%) (Weber, 1989); whereas in an end-stage failing heart, the type I/type III collagen ratio is increased (Gunja-Smith et al., 1996; Marijjanowski et al., 1995). The constituents of the 3-D collagen gel may be adjusted accordingly, in order to imitate the \textit{in vivo} pathological ECM environment. Results from these studies do, however, provide data with much improved translational significance due to their resemblance to the actual \textit{in vivo} environment.

\subsection*{4.4 Clinical Perspective}

After a myocardial injury, structural ventricular remodeling determines the clinical progression of heart failure and has emerged as a key target in the search of novel therapeutic interventions aiming at attenuation of post-MI adverse remodeling. Dysregulated ECM homeostasis in favour of either fibrosis or degradation can both have detrimental impacts on post-MI ventricular remodeling. TIMP-2 levels are remarkably reduced during the late phase of myocardial remodeling after an MI. In a rodent model, TIMP-2 deletion exacerbated infarct expansion and LV dilatation, whereas induction of myocardial TIMP-2 overexpression at the time of MI significantly improved the survival rate and limited the ventricular remodeling. We explored the effects of TIMP-2 on human cardiac fibroblast-mediated ECM regulation using a functional 3-D collagen gel contraction assay that mimics important biomechanical properties in
the actual *in vivo* microenvironment. Our data suggest that TIMP-2 preserves ECM homeostasis through simultaneous induction of myofibroblast activation and protease activity. Preserving ECM homeostasis after MI by direct application to the infarcted ventricle or induction of TIMP-2 at this target site may limit or delay the subsequent transition to mechanical and structural heart failure.
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