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Developmental characterization of tissue inhibitor of metalloproteinase domain functions in Xenopus laevis

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Abstract

During development the extracellular matrix is cleaved and remodeled to facilitate the large-scale cell rearrangements that are necessary for processes like gastrulation, neurulation, angiogenesis and organogenesis. ECM remodeling occurs primarily through secreted enzymes called matrix metalloproteinases (MMPs). Regulation of MMP activity is achieved through the tissue inhibitor of metalloproteinases (TIMPs), a small family of secreted proteins that bind MMPs in a 1:1 manner to inhibit their activity. Although TIMPs were originally characterized based on their MMP-inhibitory activities, in vitro studies have revealed that TIMPs are multifunctional proteins, with structurally and functionally distinct N- and C-terminal domains. TIMP N-terminal domains bind to and inhibit MMPs, whereas their C-terminal domains have cell signaling activity in apoptosis, cell migration and cell proliferation pathways. Using Xenopus laevis as a model organism, this study examined the unique roles of the TIMP N- and C-terminal domains during development to investigate the balance between TIMP MMP-inhibitory activity and signaling activity in vivo. Microinjection was used to overexpress the three X. laevis TIMPs or their individual domains in early stage embryos. This research demonstrated that the balance between MMPs and inhibitors in the ECM is very important, and that the ECM functions as an intricate network. Here I showed that the TIMP-1 and -2 C-terminal domains downregulated MMP expression/activity within this network independent of MMP-inhibition. Additionally the TIMP-1 and -2 C-domains altered signaling markers including caspase-3 and RECK, which was not observed with N-domain constructs, and resulted in severe developmental defects. In contrast, the TIMP-3 C-terminal domain performed no independent role in development.
This research is the first comprehensive comparison of TIMP domain function \textit{in vivo}, and demonstrates for the first time a role for TIMP signaling during development.
Keywords

Tissue inhibitor of metalloproteinase; matrix metalloproteinase; RECK; extracellular matrix; embryo; development; *Xenopus laevis*; cell signaling; cell proliferation; apoptosis; cell migration; overexpression
Epigraph

“Live as if you were to die tomorrow.

Learn as if you were to live forever.”

-Mahatma Gandhi
Dedication

to my parents, who have always supported me
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First and foremost I would like to thank my supervisor, Dr. Sashko Damjanovski for making my time as a graduate student such an enjoyable experience. Sash, thank you for creating an environment that that not only fosters hard work, but also cultivates friendships. I am very grateful that I have had the opportunity to work in a lab where it is okay to make mistakes, learn from them, and still have fun while doing it. You have been a great mentor to me Sash, not only in academics, but I have also learned many important life lessons from you that will not be forgotten. You have been much more than a supervisor, thank you Sash for also being a great friend.

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<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>ALA+TIMP-2</td>
<td>TIMP-2 mutant with alanine appended onto amino terminal</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchonininc acid assay</td>
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<tr>
<td>BM</td>
<td>basement membrane</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CONT</td>
<td>control</td>
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<tr>
<td>CT</td>
<td>cut-off threshold</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>Ef-1α</td>
<td>elongation factor-1 alpha</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>GAG</td>
<td>glycosaminoglycan</td>
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<td>Description</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<tr>
<td>HPX</td>
<td>hemopexin domain</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>INTG Beta 1</td>
<td>β1 integrin</td>
</tr>
<tr>
<td>KDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEGA</td>
<td>molecular evolutionary genetic analysis</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated/ extracellular signal-regulated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MMR</td>
<td>Marc’s Modified Ringers</td>
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<tr>
<td>MT-MMP</td>
<td>membrane-type matrix metalloproteinase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PBST</td>
<td>phosphate buffered saline + tween 20</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PH3</td>
<td>phospho-histone-3</td>
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PRO  propeptide domain
PVDF  polyvinylidene difluoride
RECK  REversion-inducing Cysteine-rich protein with Kazal motifs
RNA  ribonucleic acid
mRNA  messenger RNA
RTK  receptor tyrosine kinase
RT-PCR  reverse-transcriptase polymerase chain reaction
SDS  sodium dodecyl sulfate
SE  standard error
T1C  TIMP-1 C-terminal domain construct
T1FL  TIMP-1 full-length construct
T1N  TIMP-1 N-terminal domain construct
T2C  TIMP-2 C-terminal domain construct
T2FL  TIMP-2 full-length construct
T2N  TIMP-2 N-terminal domain construct
T3C  TIMP-3 C-terminal domain construct
T3FL  TIMP-3 full-length construct
T3N  TIMP-3 N-terminal domain construct
TACE  TNF-α converting enzyme
TBST  tris-buffered saline + tween 20
<table>
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<tr>
<th>Abbreviation</th>
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<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>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAIL-R1</td>
<td>TNF-related apoptosis inducing ligand receptor 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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CHAPTER 1

GLOBAL INTRODUCTION AND LITERATURE REVIEW
1.1 The Extracellular Matrix

This work will examine the roles played by secreted enzymes and inhibitors that regulate protein dynamics outside of cells. When one considers the myriad of functions performed by the many tissues and organs within the body, these functions are often attributed solely to cells. The tissues found in multicellular organisms, however, are not composed exclusively of cells; rather, cells are embedded in an extracellular matrix (ECM) that is essential for tissue and organ function. The ECM is a dynamic, complex network of interacting macromolecules that both surrounds and structurally supports cells. As such, the ECM is necessary for preserving tissue integrity in multicellular organisms. Additionally, the bidirectional interactions that exist between cells and the ECM are important for coordinating cellular functions. Communication with the ECM allows cells to perceive their changing environment and adapt in an appropriate manner (Bissell, Hall et al. 1982). The nature, regulation, and complexity of the exchanges between cells and the ECM are becoming increasingly significant in the study of both development and disease. In this chapter, I will begin by discussing the structural components of the ECM, and the roles of secreted enzymes and their inhibitors in remodeling of the ECM. I will subsequently discuss the contributions of these ECM remodeling proteins to the regulation of cancer progression, and most pertinent to my research, embryonic development.

1.1.1. General structure of the ECM

One of the primary functions of the ECM is to provide the histoarchitecture necessary for organ function and homeostasis (Aumailley and Gayraud 1998). Accordingly, the ECM is composed predominantly of large structural proteins. The proteins that form the ECM are secreted by cells embedded within the matrix, and are subsequently assembled into a
precisely organized network that associates with cellular surfaces (Hays 1991). There are two main structural classes of proteins that make up the ECM: proteoglycans and large fibrous proteins. Proteoglycans are large hydrophilic molecules that consist of a protein core linked to polysaccharide chains termed glycosaminoglycans (GAGs). Large fibrous proteins such as collagen, elastin, fibronectin, and laminin are mainly structural in nature, although these proteins also have crucial non-structural functions such as facilitating cell migration and cell signaling. Variations in the arrangement and relative amounts of different ECM macromolecules govern, and can also alter the character of, the ECM (Frantz, Stewart et al. 2010). In animal tissues, the ECM is found in two main forms: basement membrane (BM) and stromal matrix. Basement membranes are thin layers of defined ECM molecules that underlie epithelial cell sheets. The BM anchors the epithelium, serves as a barrier separating epithelial cells from the underlying connective tissue, polarizes cells, and regulates cell behavior (Nelson and Bissell 2006). In contrast, the stromal matrix contains abundant ECM with comparatively few embedded cells, and is associated with connective tissues including cartilage and bone. Stromal matrix is rich with proteoglycans, which form a hydrated gel-like mesh due to their hydrophilic nature. Within this gel-like matrix are fibrous proteins such as collagen and elastin, which provide strength to the stroma of tissues including tendons, cartilage, skin, and artery walls (Frantz, Stewart et al. 2010).

It was originally thought that the ECM plays a passive role in mediating tissue and organ function, acting only as a framework to provide structural support for tissues. We now know that the ECM is exceedingly active, and interactions between cells and the ECM are now recognized as important factors in the regulation of cell behavior. In all tissues, cells are stably linked to the ECM. Although the composition of the ECM may vary from mainly collagen II in cartilage and bone, to laminin and collagen IV in the BM of epithelia, cells are
capable of sensing and responding to changes in the ECM. If the BM is removed from epithelia, for example, the cells will either migrate or undergo apoptosis, depending on their developmental fate. Furthermore, the organization of the ECM is both highly ordered and varied, such that the ECM structure and composition is specifically altered and adapted to serve the functional requirements of different tissues (Frantz, Stewart et al. 2010). For instance, the ECM found in joints is important in maintaining hydration, in tendons the ECM provides strength, in kidneys the ECM is involved in filtration, and in the embryo, the ECM facilitates migration (reviewed in Frantz et al., 2010).

1.1.2. The ECM facilitates cell migration via integrins and focal adhesion structures

Cell migration in tissues is dependent upon cell contact with the ECM. Cells cannot migrate freely through extracellular space; rather, motility depends on the ability of a cell to form adhesions with specific ECM substrates like fibronectin or laminin, which serve functionally like roads for cell movement. Cells bind to ECM components through transmembrane receptors on their plasma membranes. In animal cells, integrins are an important family of ECM receptors, which are necessary for cell adhesion during migration. Integrins are heterodimeric transmembrane proteins formed from association of α and β polypeptide chains (Berrier and Yamada 2007). There are many different combinations of possible α and β heterodimers, which impart binding specificity for different components of the ECM (Luo, Carman et al. 2007). For instance, α5β1 binds fibronectin whereas α6β1 binds laminin. When migrating cells adhere to the ECM, integrins link the cell cytoskeleton to specific ECM substrates, forming a structure called a focal adhesion. Specific integrin-ECM binding and focal adhesion formation initiate appropriate cellular signaling pathways to facilitate cell motility. As the cell moves along its substrate, new focal adhesions are
continuously being formed at the leading edge of the cell and released from the back of the cell (Ridley, Schwartz et al. 2003). Therefore, the cell may recognize different ECM components as suitable adhesive substrates, and the appropriate cell migration signaling cascades are then initiated. This process is essential during development when large-scale cell movements occur in a highly coordinated manner (Ridley, Schwartz et al. 2003).

1.1.3. The ECM as a regulator of cell signaling

In addition to its roles in mediating cell motility and providing structural integrity for tissues, the ECM is also an important regulator of cell signaling in multicellular organisms. Heparan sulfate, a component of many proteoglycans, can bind to secreted growth factors in the ECM, thereby transforming the matrix into a basin for biologically active molecules. The association of growth factors with the ECM is important during embryogenesis, as growth factors can be used to establish morphogenic gradients which pattern developmental events (Hynes 2009). ECM proteoglycans bind to a variety of signaling molecules and growth factors including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β) (Shi and Massague 2003; Mohammadi, Olsen et al. 2005). Cleavage of ECM proteins or of the GAGs associated with ECM proteoglycans releases these signaling factors (discussed further in sections 1.6 and 1.7), which can subsequently affect cell behavior, leading to changes in migration, proliferation, and survival (Hynes 2009).
1.2 Matrix Metalloproteinases

1.2.1. Structure and function of MMPs

Matrix metalloproteinases (MMPs) are a family of 25 secreted zinc-dependent endopeptidases. The primary function of MMPs is to facilitate cleavage and remodeling of the ECM through selective degradation of ECM components (Vu and Werb 2000). Traditionally, MMPs have been classified into four groups on the basis of their substrate specificity for particular ECM components: collagenases, gelatinases, stromelysins and matrilysins. As the list of MMP substrates has become longer and more complex, however, it is now more common to classify MMPs into two distinct groups based on their structure and localization: membrane-type MMPs (MT-MMPs), which are anchored to the cell surface, and secreted MMPs, which are soluble in the ECM (Egeblad and Werb 2002).

Although MMP localization and substrate specificity may vary, most MMPs contain several conserved domains, which are essential for their structure and function. Generally, MMPs contain a cleaved N-terminal signal sequence used for secretion, an N-terminal propeptide (PRO) domain that regulates activity, a catalytic Zn$^{2+}$-binding domain that contains the MMP catalytic site, a linker domain (also called the hinge region), and a hemopexin (HPX) domain that mediates interactions with target proteins (Nagase, Visse et al. 2006). All MMPs are synthesized as inactive proenzymes, and become activated after the removal of their PRO domain. The PRO domain inhibits MMP activity through interaction of a cysteine residue in the PRO domain with the zinc moiety in the MMP catalytic site (Nagase, Visse et al. 2006). Most secreted MMPs are activated in the ECM by other active MMPs or serine proteases; whereas, the MT-MMPs are cleaved intracellularly in the Golgi by furin-like serine proteases and inserted into the plasma membrane in their active form (Sternlicht and Werb 2001).
1.2.2. Membrane-type MMPs

Membrane-type MMPs are a particularly unique class of metalloproteinase. Unlike secreted MMPs which are activated extracellularly and may diffuse freely throughout the ECM, MT-MMPs are activated intracellularly and immobilized in the plasma membrane, thus, the activities of the MT-MMPs are limited to the surface of the cells that produced them (Seiki 2002). There are six MT-MMPs that have been identified: MT4- and MT6-MMP are anchored to the cell surface via a glycosylphosphatidylinositol (GPI) anchor; whereas, MT1-, MT2-, MT3- and MT5-MMP are type I transmembrane proteins, containing a single transmembrane domain and short cytosolic C-terminal domain (Seiki 2002). The presence of a C-terminal domain is distinctive to this subset of MT-MMPs and plays an important role in regulating cellular localization and turnover of these proteins (Wang, Ma et al. 2004).

1.2.3. MT1-MMP

Of the six MT-MMPs, MT1-MMP (also known as MMP-14) was the first identified, and is also the best characterized. MT1-MMP can digest a number of cell surface components (CD44, cadherin) and ECM proteins, including type IV collagen, and is a key regulator of cellular invasion in development and disease. The cytosolic C-terminal tail of MT1-MMP is important in regulation of invasiveness, as deletion of this domain decreased the invasion-promoting activity of MT1-MMP in human melanoma cells (Lehti, Valtanen et al. 2000). Additionally, the C-terminal domain of MT1-MMP has been linked to enhanced cell migration by facilitating localization of MT1-MMP to invadopodia, and has also been linked to internalization and turnover of the enzyme with respect to its presence on the membrane, highlighting the importance of this domain in regulating MT1-MMP function (Nakahara, Howard et al. 1997; Jiang, Lehti et al. 2001; Uekita, Itoh et al. 2001). As well as
cleaving known ECM substrates, MT1-MMP is involved in the activation of pro-MMP-2 (see section 1.2.4.), which further augments the migratory potential of MT1-MMP expressing cells (Itoh, Takamura et al. 2001). Upregulation of MT1-MMP is often associated with pathological conditions including cancer, partly due to its direct role in facilitating cell migration and invasion, and partly due to its role in activating pro-MMP-2, which is a very robust ECM protease. Not surprisingly, MT1-MMP also plays an important role in development. Of the many MMP knockout mouse models, only the MT1-MMP null mutant is lethal, resulting in death by 3 weeks of age due to severe skeletal and vascularization defects (Zhou, Apte et al. 2000).

### 1.2.4. The gelatinases

The gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are considered two potent ECM proteases primarily because of their ability to cleave type IV collagen, which is an abundant component of BMs (Khasigov, Podobed et al. 2003). MMP-2 and -9 have important functional roles in vivo, particularly during development, as cleavage of BM components is important in facilitating cell migration and differentiation (Werb, Ashkenas et al. 1996; Carinato, Walter et al. 2000). During mouse development, MMP-9 expression is detected in the liver, the endochondral plates of developing bone, neural cells, and developing respiratory structures including the bronchial epithelium of the lungs and the developing alveoli (Canete-Soler, Gui et al. 1995). In contrast, MMP-2 expression is detected mainly in mesenchymal tissues and organs such as kidney and lung (Reponen, Sahlberg et al. 1992). Further studies using avian embryos have shown that MMP-2 expression is required in neural crest cells to facilitate an epithelial to mesenchymal transition (EMT), where the neural crest cells detach from the neural epithelium and migrate away from the neural tube.
As mentioned above, MT1-MMP is involved in direct activation of pro-MMP-2. Active MMP-2 can in turn cleave and activate pro-MMP-9, thus, the gelatinases function as part of a powerful axis that controls degradation of ECM components (Toth, Chvyrkova et al. 2003). As such, upregulation in the activities of these MMPs are frequently associated with disease. MMP-2 and -9 play a significant role in cancer metastasis and facilitate EMT through cleavage of the BM, which leads to cell invasion (Khasigov, Podobed et al. 2003). Accordingly, MMP activity must be tightly regulated in a controlled manner to maintain normal tissue homeostasis both during development and in adults.

1.3 Tissue Inhibitors of Metalloproteinases

The tissue inhibitors of metalloproteinases (TIMPs) are endogenous MMP inhibitors, which play important roles in regulating MMP activity in vivo. In vertebrates, four TIMPs have been identified (TIMP 1-4). TIMP orthologs are found in many species throughout the animal kingdom including worms and insects, indicating the importance of this ancient protein family (Murphy 2011). Together, the four vertebrate TIMPs have the ability to inhibit all known MMPs (Visse and Nagase 2003). As TIMPs exhibit functional redundancy, orthologs of each TIMP are not expressed in all vertebrates; however, mammals express all four TIMPs (Murphy 2011).

TIMP genes are regulated at the transcriptional level through cytokine and growth factor signaling, and the expression patterns of the individual TIMPs are variable. While TIMP-2 tends to be constitutively expressed, the other TIMPs show more tissue-specific expression patterns (Murphy 2011). Most notably, TIMP-1 is highly expressed in reproductive organs and the central nervous system, particularly in regions of neuronal plasticity such as the hippocampus and cerebellum. In contrast, TIMP-3 expression is often
associated with the BMs of the eye and kidney (Murphy 2011). Although there is functional redundancy in many of the MMP-inhibitory activities of the four TIMPs, there are subtle differences in their inhibitory abilities. For instance, TIMP-3 is unique with respect to the other TIMPs due to its ability to strongly inhibit members of the ADAM family proteases, including ADAM-17 (also known as TACE; Tumor necrosis factor-α converting enzyme) (Amour, Slocombe et al. 1998).

1.3.1. TIMP domain structure and function

TIMPs are small secreted proteins found within the ECM, where after removal of an N-terminal signal sequence, the mature TIMP proteins generally consist of between 184 – 194 amino acids. The individual TIMP family members each have similar tertiary structure, and can be divided into distinct N- and C-terminal domains (Nagase, Visse et al. 2006). As a group, the mammalian TIMPs share 35-50% sequence similarity at the amino acid level, and there are specific structural features that are highly conserved amongst all TIMPs. Most importantly, all TIMPs contain 12 conserved cysteine resides which form 6 intramolecular disulfide bonds (Murate and Hayakawa 1999). The positions of the 12 cysteine residues and the subsequent formation of the disulfide bonds are crucial for the proper folding of all TIMP proteins into a looped structure, which delineates and stabilizes the conformations of the N- and C-terminal domains. The first 6 cysteine residues form 3 loops that comprise the TIMP N-terminal domains. Likewise, the latter 6 cysteine residues form 3 C-terminal domain loops (Figure 1.1) (Murate and Hayakawa 1999). The two TIMP domains are structurally and functionally distinct, and have been shown to fold and function independently in vitro (Brew and Nagase 2010; Bahudhanapati, Zhang et al. 2011).
The primary role of TIMPs is to inhibit MMP enzymatic activity, thereby regulating cleavage and degradation of ECM components. The TIMP N-terminal domains are responsible for direct inhibition of MMP activity via binding to the MMP active site in a 1:1 manner, chelating the Zn\(^{2+}\) ion in the MMP active site, and effectively blocking MMP catalytic activity (Nagase, Visse et al. 2006). Although it was originally believed that the sole function of TIMPs was to attenuate the action of MMPs, it is now known that the TIMP C-terminal domains have MMP-independent functions and can influence cell behavior through direct regulation of a number of cell signaling pathways (Chirco, Liu et al. 2006). The C-terminal domains of the various TIMPs have been linked to cell growth, migration, and apoptosis pathways (Guedez, Stetler-Stevenson et al. 1998; Wang, Yamashita et al. 2002; Oh, Seo et al. 2004). To date, however, much of the work characterizing TIMP domain function has been carried out in vitro. Although some cell surface binding partners and signaling events associated with the TIMP C-terminal domains have been identified, activation of specific signaling pathways in vitro varies with TIMP and cell type (discussed further in section 1.6.2.) (Bourboulia and Stetler-Stevenson 2010). Currently, comparatively little research has been done characterizing TIMP domain function in vivo.
Figure 1.1. General schematic secondary structure of TIMP proteins. Mature TIMP proteins contain 12 conserved cysteines (numbered in blue). The 6 N-terminal cysteine residues (red) form 3 disulfide bonds, which result in 3 loops that comprise the N-terminal domains (dashed line; loop 1-3). The 6 C-terminal cysteine residues (purple) form 3 disulfide bonds, which result in 3 loops that comprise the C-terminal domains (dotted line; loop 4-6). The arrow indicates the short boundary region between N- and C-terminal domains. Based on (Murate and Hayakawa 1999).
1.4 Reversion-inducing Cysteine-rich Protein with Kazal Motifs

In addition to TIMPs, REversion-inducing Cysteine-rich protein with Kazal motifs (RECK) is another important regulator of MMP activity in both development and disease. RECK is a cell surface MMP inhibitor that contains three serine protease inhibitor-like domains (termed Kazal motifs) and is bound to the plasma membrane via a GPI anchor at its carboxy terminus (Takahashi, Sheng et al. 1998). The RECK gene was first isolated from a ras-transformed mouse fibroblast cell line (NIH3T3 cells). Due to its MMP-inhibitory activity, RECK has since been identified as an important tumor suppressor protein with anti-invasive properties (Takahashi, Sheng et al. 1998). While RECK is expressed in many normal adult tissues, its expression is often undetectable in tumor cell lines. Moreover, RECK has been shown to be down-regulated in response to various oncogenes, including ras (Takahashi, Sheng et al. 1998; Sasahara, Takahashi et al. 1999).

In addition to its role as a tumor suppressor, RECK is also important in regulating developmental events. During development, RECK expression is high in cells that make up the developing blood vessels and low in the surrounding tissues (Oh, Takahashi et al. 2001). Studies using mice have shown that RECK-deficient embryos have impaired formation of the vasculature (Oh, Takahashi et al. 2001). Vascular development is highly reliant on cell migration and ECM remodeling mediated by active MMPs, however, excessive MMP activity can also be detrimental during this process, and thus, must be tightly controlled. As RECK is known to be a good inhibitor of potent MMPs including MMP-2, MMP-9 and MT1-MMP, inhibition of MMP activity by RECK is essential during vascularization (Takahashi, Sheng et al. 1998; Oh, Takahashi et al. 2001). Taken together, the action of MMP inhibitors like RECK and TIMPs balanced with the action of active MMPs is extremely important in regulating ECM remodeling events in development and in disease.
1.5 Regulation of the ECM Proteolytic Network

To achieve and maintain homeostasis, the activities of MMPs, TIMPs, and RECK must be tightly controlled and constantly adjusted. In combination with the balance between secreted MMP and TIMP levels in the ECM, regulation of cell surface proteases and inhibitors like MT1-MMP and RECK, together play important roles in controlling the function, migration, and invasiveness of cells (Bourbouia and Stetler-Stevenson 2010). In addition to maintaining the appropriate balance between the activities of ECM proteases and inhibitors, the individual contributions of the TIMP N- and C-terminal domains must also be balanced with respect to the relative abundance of their appropriate binding partners in the ECM and on the cell surface. Whereas the TIMP N-terminal domains bind to MMPs in the ECM, the C-terminal domains can also influence cell behavior by signaling through cell surface receptors. As we learn more about the ECM and the proteins within it, it is evident that regulation of the ECM proteolytic network is exceedingly complex. As a result, increasing research has focused on the relative levels of, and interactions between, extracellular and cell surface proteins. It is now clear that MMPs and TIMPs are not only important in regulating ECM turnover, but that MMP non-proteolytic and TIMP non-inhibitory domains also play important roles in regulating ECM dynamics and cell behavior. In this section I will discuss some of the complex interactions between MMPs and TIMPs, and the ways in which TIMPs can regulate ECM dynamics, aside from direct inhibition of MMP activity.

1.5.1. TIMP-2 mediated activation of pro-MMP-2

TIMPs are important multifunctional proteins that can influence cell behavior, through several different mechanisms. As previously mentioned, TIMP N-terminal domains
are known to inhibit MMP activity, and TIMP C-terminal domains have been associated with various cell surface receptors and signaling pathways, however, TIMPs can also interact with cell surface MMPs or receptors to modulate the expression and activity of other proteins. An important example of such an interaction is the association between MT1-MMP, TIMP-2, and pro-MMP-2 (Seiki 2002). Active MT1-MMP on the cell surface (primarily on migratory cells), can bind to pro-MMP-2 and TIMP-2 in a complex that ultimately results in the activation of pro-MMP-2. In this complex, two molecules of MT1-MMP are required in close proximity on the cell surface (Itoh, Takamura et al. 2001). One molecule of MT1-MMP serves as a receptor for TIMP-2 and pro-MMP-2. The C-terminal domain of TIMP-2 binds to the HPX domain of pro-MMP-2, while its N-terminal domain binds to and inhibits the catalytic domain of MT1-MMP. Thus, TIMP-2 is required for the appropriate positioning of pro-MMP-2, and serves as an adaptor between MT1-MMP and pro-MMP-2 in this complex. A second MT1-MMP then cleaves the PRO domain of the appropriately positioned pro-MMP-2 resulting in its activation (Figure 1.2) (Itoh, Takamura et al. 2001). Relative stoichiometry between the levels of MMPs, TIMPs, and cell surface receptors may have an important impact on the formation of this complex. For instance, low levels of TIMP-2 facilitate activation of pro-MMP-2, however, higher levels of TIMP-2 saturate and inhibit available MT1-MMP receptors, thereby preventing pro-MMP-2 activation (Romi, Helgeland et al. 2012). As MT1-MMP/TIMP-2 mediated activation of pro-MMP-2 is known to dramatically increase the migratory and invasive potential of cells, the stoichiometry needed for the proper formation of this complex is important during both development and cancer progression (Itoh, Takamura et al. 2001).
1.5.2. Regulation of pro-MMP-9 in the ECM

Activation of pro-MMP-9 is another important determinant in regulating cell migration and invasion. Similar to pro-MMP-2 activation, extracellular cleavage of pro-MMP-9 involves complex interactions between a variety of ECM and cell surface proteins. In cancer cells, pro-MMP-9 activation contributes to an invasive phenotype, and pro-MMP-9 is activated by MMP-2. The cell surface protein CD44 acts as a binding partner to position pro-MMP-9 for activation. CD44 and pro-MMP-9 co-localize to invadopodia where active MMP-2 can subsequently cleave and activate pro-MMP-9 (Figure 1.2) (Bourguignon, Gunja-Smith et al. 1998; Murphy, Stanton et al. 1999). As MT1-MMP activates pro-MMP-2, MT1-MMP, therefore, also plays an important role in facilitating the downstream activation of pro-MMP-9.

Additionally, pro-MMP-9 is known to form a complex with TIMP-1 in the ECM (Goldberg, Strongin et al. 1992). This interaction occurs through the C-terminal domain of TIMP-1, where the N-terminal domain remains available for MMP inhibition (Itoh and Nagase 1995). Intracellular co-localization of TIMP-1 with pro-MMP-9 has also been detected in the Golgi, and many cells secrete pro-MMP-9 and TIMP-1 together (Roderfeld, Graf et al. 2007). Although the functional significance of this complex remains unknown, it has been suggested that association of pro-MMP-9 with TIMP-1 may be required for stabilization of the zymogen (inactive pro-enzyme) and regulation of proteolytic activity. Collectively, these studies with MMP-2 and -9 highlight the complexity of the interactions between various ECM molecules as well as the importance of the non-inhibitory TIMP domains in regulating the ECM proteolytic network.
1.5.3. **TIMP-2 mediated upregulation of RECK**

In addition to their direct roles in regulating the activities of ECM proteins, TIMPs may also influence the proteolytic network indirectly through C-terminal domain-mediated activation of cell signaling pathways. TIMP-2 is perhaps the most well studied member of the TIMP family, and its MMP-independent functions have been well characterized, at least *in vitro*. In human endothelial cells, TIMP-2 has been linked to a specific signaling pathway, which ultimately results in upregulation in RECK expression and decreased cell migration (Oh, Seo et al. 2004). A series of elegant experiments have demonstrated that TIMP-2-mediated upregulation in RECK expression is independent of its N-terminal MMP-inhibitory domain, and occurs through binding of the TIMP-2 C-terminal domain to α3β1 integrin receptors on the cell surface (Seo, Li et al. 2003; Seo, Li et al. 2006). Along with co-immunoprecipitation of α3β1 integrins using TIMP-2 antibodies, competitive binding studies using antibodies that block the α3 and β1 integrin subunits have shown that TIMP-2 binding is specific to α3β1 integrin receptors on human microvascular endothelial cells (Seo, Li et al. 2003). By the addition of a single alanine residue to the N-terminal domain of TIMP-2 (ALA+TIMP-2), the N-terminal domain MMP-inhibitory activity is disrupted, leaving only a functional C-terminal domain (Wingfield, Sax et al. 1999). Both TIMP-2 and ALA+TIMP-2 were shown to bind to α3β1 integrins leading to upregulation in *RECK* RNA and RECK protein (Figure 1.2) (Oh, Diaz et al. 2006; Seo, Li et al. 2006). As RECK is a cell surface inhibitor of MT1-MMP, MMP-2, and MMP-9, increased RECK expression resulted in decreased endothelial cell migration (Oh, Seo et al. 2004; Oh, Diaz et al. 2006).

Thus, in addition to direct inhibition of MMP activity by its N-terminal domain, TIMP-2 may function to indirectly inhibit MMP activity through C-terminal domain-mediated upregulation of RECK. With the added contradictory ability of TIMP-2 to activate
pro-MMP-2 in association with MT1-MMP, regulation of this important ECM molecule is essential for maintaining homeostasis in the ECM. Given the complexity involved in the regulation of TIMP-2 activities alone, when examined in concert with the varied activities of the other TIMPs and MMPs, it is evident that disruptions to the intricate dynamics of the ECM proteolytic network may have negative consequences for the organism as a whole.
Figure 1.2. Cell surface regulation of the ECM proteolytic network. From left to right, pro-MMP-9 binds CD44 on the cell membrane where it is activated by MMP-2. TIMP-2 complexes with MT1-MMP and pro-MMP-2 to mediate cleavage and activation of pro-MMP-2 by a second molecule of MT1-MMP. The TIMP-2 C-terminal domain binds to α3β1 integrins on the cell surface leading to upregulation in the cell surface MMP inhibitor, RECK. RECK subsequently inhibits the activity of MT1-MMP, MMP-2, and MMP-9.
1.6 Functions of the ECM Proteolytic Network in Cancer

1.6.1. The role of MMPs in cancer and metastasis

For many years it has been known that upregulation of MMP levels and activities are detrimental to normal tissue function. Although MMPs are highly active during development when many cell migration and ECM remodeling events occur, their activities must still be tightly regulated at this time. In contrast, MMP expression is limited in most adult tissues and elevated MMP activity is associated mainly with controlled remodeling events such as wound healing (Ma, Tarnuzzer et al. 1999). In adult tissues, increases in MMP activity are associated with pathological conditions, most commonly cancer.

MMPs have been implicated as positive regulators of cancer progression from the early stages of tumor growth to later stages of invasion and metastasis (Mandal, Mandal et al. 2003). In early stages, cleavage of the ECM or BM components by MMPs contributes to the formation of a microenvironment that promotes tumorigenesis. The tumor microenvironment is a combination of tumor cells, stromal cells, growth factors and ECM constituents. Within the past decade, the tumor microenvironment has become recognized as an important regulator of tumor behavior (Bhowmick, Neilson et al. 2004; Littlepage, Egeblad et al. 2005). MMPs are secreted by tumor cells and tumor-associated fibroblasts, and the roles of active MMPs within the microenvironment are varied. Through direct cleavage of the structural ECM molecules collagen IV and laminin 5, MMPs expose cryptic sites on these proteins that foster tumor cell growth and angiogenesis (Xu, Rodriguez et al. 2001). Additionally, many cytokines and growth factors are sequestered within the ECM. Degradation of the ECM may facilitate release of growth factors, and further direct cleavage of latent growth factors by MMPs may facilitate their activation. Release of and/or activation of growth factors in the ECM significantly contributes to tumor progression by enhancing tumor cell growth,
angiogenesis, and tumor cell migration (Bourboulia and Stetler-Stevenson 2010). For instance, MMP-9 can release VEGF from the ECM, leading to increased angiogenesis (Bergers, Brekken et al. 2000). Similarly, insulin-like growth factors (IGFs) and TGF-β are important targets of MMP activity in tumors. MMPs cleave IGF binding proteins, permitting release of IGFs and binding of IGFs to their receptors, which results in enhanced tumor cell growth (Bergers, Brekken et al. 2000). Additionally, MMP-2 and -9 specifically, can release and activate fibronectin-bound TGF-β, which induces EMT and promotes tumor cell proliferation (Yu and Stamenkovic 2000).

As well as triggering tumorigenesis, MMPs also contribute to later stages of tumor progression by facilitating cell migration and invasion, which are important factors that contribute to metastasis. This can occur through targeted cleavage of BM or ECM components, or by cleavage of cell-cell adhesion molecules. For instance, degradation of the structural ECM molecule laminin 5 by MT1-MMP leads directly to tumor cell migration; whereas, cleavage of membrane-bound E-cadherin by MMP-3 (stromelysin-1) and MMP-7 (matrilysin) results in disruption of cell contacts leading to cell dissociation, which promotes invasion (Koshikawa, Giannelli et al. 2000; Noe, Fingleton et al. 2001).

In summary, MMPs can contribute to cancer progression through many mechanisms, such as cytokine release or activation, which are not necessarily strictly related to proteolytic cleavage of ECM structural molecules. Additionally, MMPs positively regulate processes including tumor cell growth, angiogenesis, migration, and invasion, demonstrating the significance of these molecules in regulating the tumor microenvironment. Given the many roles of MMPs in cancer, it is evident that regulation of MMP activity is critical for prevention of disease and for maintenance of normal tissue integrity.
1.6.2. The role of TIMPs in cancer

Taking into consideration the importance of MMP activity in tumor progression, some anti-cancer therapies have focused on sequestering MMP activity as a means of treatment. As all four TIMPs are naturally occurring MMP inhibitors, it was originally thought that TIMPs might act as an intrinsic guard against tumor progression (Stetler-Stevenson 2008). Accordingly, synthetic MMP inhibitors were designed to mimic the N-terminal MMP-inhibitory activities of TIMPs, however, these drugs failed as anti-cancer therapies in clinical trials (Brown 1998; Coussens, Fingleton et al. 2002). It is now broadly acknowledged that while TIMPs do have some anti-tumorigenic properties, TIMPs play a dual role in the regulation of tumorigenesis. Though TIMPs were originally characterized based on their ability to inhibit MMP activity, the importance of the additional biological activities mediated by the TIMP C-terminal domains is now widely recognized. Through their C-terminal domains, TIMPs can influence a variety of cell signaling pathways including cell growth, migration, and apoptosis. These MMP-independent functions may have significant implications for the progression and treatment of disease (Stetler-Stevenson 2008).

Both TIMP-1 and -2 have been shown to have cell growth promoting activity in a variety of cancer cell lines (Stetler-Stevenson, 2008). The cell growth promoting abilities of TIMP-1 and -2 were demonstrated through their ability to induce increased uptake of \[^{3}\text{H}]\) thymidine in several tumorigenic cell lines. This activity is specific to the TIMP C-terminal domains, as reductive alkylation, which renders TIMP N-terminal domains devoid of MMP-inhibitory activity, did not abolish the growth promoting abilities of TIMP-1 and -2 (Hayakawa, Yamashita et al. 1992; Hayakawa, Yamashita et al. 1994). It has been suggested that TIMP-1 and -2 may enhance cell growth through ras-dependent pathways, as research
using an osteosarcoma cell line showed that both TIMP-1 and -2 increased the levels of GTP-bound (active) ras in a dose-dependent manner (Wang, Yamashita et al. 2002). A more recent study showed that TIMP-2 binding to MT1-MMP on the surface of MCF-7 breast cancer cells might stimulate tumor cell growth through activation of the mitogen-activated protein kinase (MAPK) pathway (D'Alessio, Ferrari et al. 2008). This study also demonstrated that the TIMP-2 cell growth promoting activities are dependent on its C-terminal cell signaling domain, as both TIMP-2 and ALA+TIMP-2 were able to activate MAPK (Figure 1.3). Moreover, this work implicates an important role for the C-terminal domain of MT1-MMP in cell signaling, as MT1-MMP mutants devoid of a cytosolic domain were unable to transmit the signal (D'Alessio, Ferrari et al. 2008).

Although specific cell growth promoting pathways for TIMP-1 have not been as well characterized, TIMP-1 has also been linked to inhibition of apoptosis in various cancer cell lines. In Burkitt’s lymphoma cell lines, both TIMP-1 and alkylated TIMP-1 devoid of MMP-inhibitory activity suppressed apoptosis. TIMP-1 was associated with reduced activity of caspase-3 as well as an increase in the survival factor Bcl-X<sub>L</sub> (Guedez, Stetler-Stevenson et al. 1998). In MCF10A human breast epithelial cells, TIMP-1 has also been shown to suppress anoikis, which is a form of programmed cell death initiated by loss of cell adhesion (Li, Fridman et al. 1999). More recent research has identified CD63, a member of the tetraspanin family, as a cell surface binding partner for TIMP-1 in MCF10A cells. TIMP-1 has been shown to form a complex with CD63 and β1 integrin on the cell surface (Figure 1.3). Formation of this complex mediates the anti-apoptotic properties of TIMP-1, as down-regulation of CD63 using short-hairpin RNA restored the cell’s ability to undergo apoptosis (Berditchevski and Odintsova 1999; Jung, Liu et al. 2006). TIMP-3 has also been associated with apoptosis, and has been linked to both induction and inhibition of apoptosis, depending on
the model system used. Unlike TIMP-1, however, there is currently limited data to support
the notion that TIMP-3 may directly participate in cell signaling. Rather, it has been
suggested that TIMP-3 may indirectly influence cell behavior through modulation of MMP
and ADAM activity, which in turn can alter the availability of signaling molecules within the
ECM (reviewed in Stetler-Stevenson, 2008). Both the cell growth promoting and anti-
apoptotic activities of TIMPs demonstrate that TIMPs are multifunctional proteins that may
contribute to cancer progression through many different mechanisms.

In contrast to TIMP-1, which is upregulated in many cancers and has generally been
associated with negative prognosis, TIMP-2 has varying effects on tumor progression and
may also exhibit anti-tumorigenic properties through its C-terminal cell signaling domain in
addition to its MMP-inhibitory activities (reviewed in Stetler-Stevenson, 2008). This is
highlighted through research by Oh et al. (2004), which showed that TIMP-2 and
ALA+TIMP-2 binding to α3β1 integrins on the surface of human microvascular endothelial
cells initiated a signaling cascade that resulted in increased expression of RECK and
subsequently decreased cell migration (Figure 1.3, and discussed in section 1.5.3.).
Interestingly, association of TIMP-2 with α3β1 integrins also resulted in cell growth arrest in
G1 phase of the cell cycle through increased de novo expression of the cyclin-dependent
kinase inhibitor p27^Kip1 (Figure 1.3) (Seo, Li et al. 2006). Thus, TIMP-2 may act to either
promote or inhibit cell growth in different cell lines in vitro.

Taken together, these studies highlight the fact that TIMP activities are both cell type
and context dependent. Added to the complexity, TIMP function also depends on the relative
stoichiometry of matrix and signaling molecules within the ECM and on the cell surface. For
instance, the presence of MMP-9 may alter the signaling activity of TIMP-1 through CD63
receptors. MMP-9 can compete with CD63 for binding of TIMP-1, thus reducing or
abolishing the anti-apoptotic activity of TIMP-1 in complex with CD63 on the cell surface (Chirco, Liu et al. 2006). As most of the research characterizing TIMP domain function has been carried out in vitro under controlled conditions, where many ECM interactions are limited, the roles of the TIMP C-terminal domains in cell signaling seem to vary with cell type. Accordingly, there is a need to further examine the roles of TIMPs in vivo, where the complexity of the ECM in the whole organism may influence TIMP function, particularly with respect to the availability of the TIMP C-terminal domains to participate in cell signaling events.
Figure 1.3. Regulation of cell signaling by TIMP-1 and TIMP-2 at the cell surface. From left to right, TIMP-1 binds to β1 integrin and CD63 through its C-terminal domain to initiate a signaling cascade that results in inhibition of apoptosis. The C-terminal domain of TIMP-2 binds to MT1-MMP to initiate MAPK signaling. The TIMP-2 C-terminal domain binds to α3β1 integrins leading to p27Kip1-mediated inhibition of cell growth and upregulation of the membrane bound MMP inhibitor RECK.
1.7 The role of the ECM Proteolytic Network in Regulating Development

While the MMP/TIMP balance must be tightly regulated in normal adult tissues to prevent tumorigenesis, both MMPs and their inhibitors are highly expressed during development to permit ECM proteolysis and remodeling. Indeed, many of the same processes that are upregulated during tumor progression are also required for the progression of normal development. Cell migration, cell invasion, and angiogenesis are important developmental processes required for tissue morphogenesis and organ formation. Consequently, the action of MMPs and their inhibitors, TIMPs and RECK, are also important regulators of development.

1.7.1. Cell migration and tissue remodeling in development

Cell migration is vital during embryonic development and permits the cellular rearrangements and cell signaling processes that are required for many important developmental events, the earliest of which are gastrulation and germ layer induction. Migration is largely mediated by the activity of MMPs, which cleave ECM components, releasing cytokines and clearing a space in the ECM through which cells can move. For example, one process that is absolutely dependent on cell motility is the migration of the neural crest cells away from the neural tube to form the sensory ganglia (Prendergast, Linbo et al. 2012). Although MMPs are required during this process, MMP activity must also be tightly regulated to prevent aberrant development. RECK has been implicated as an important regulator of neural crest cell migration as studies examining early zebrafish development showed that mutation in the RECK gene inhibited proper formation of the dorsal root gangia (Prendergast, Linbo et al. 2012).
Cell migration is also essential during long bone development, at which time osteoclasts must invade the cartilage primordium in order to begin the process of endochondral ossification. Osteoclast invasiveness has been shown to rely on high expression and activity of MMP-9, where cell migration into the cartilage matrix is limited by MMP inhibitors (Blavier and Delaisse 1995). MT1-MMP may also play an important role in regulating bone development as *MT1-MMP* knockout mice display severe skeletal defects including craniofacial abnormalities and dwarfism (Holmbeck, Bianco et al. 1999). Additionally, the *MT1-MMP* null mouse has more acute developmental defects compared to other *MMP* knockout mouse models, indicating the significance of this multifunctional protein in regulating developmental events (Zhou, Apte et al. 2000).

Proper development of the central nervous system (CNS) is another process that is highly reliant on cell migration and invasion. During CNS development, neurons must extend long axonal extensions to form synaptic connections, and MMP-9 has been shown to be an important regulator of neuronal outgrowth (Chambaut-Guerin, Herigault et al. 2000). Aberrant regulation of MMP-9 during neural development can lead to CNS defects and improper synapse formation; therefore, it is not surprising that TIMPs have been associated with tight regulation of MMP activity during this time. *TIMP-1* knockout mice exhibit significant neuronal impairment, and have difficulty forming and retaining reward associations compared with wild-type mice (Chaillan, Rivera et al. 2006). Similarly, *TIMP-2* null mice exhibit neurological abnormalities and motor dysfunction, highlighting the importance of maintaining the balance between MMP and TIMP activity during development (Jaworski, Soloway et al. 2006).
1.7.2. ECM-dependent regulation of angiogenesis

One of the most well studied developmental processes is angiogenesis, the formation and branching of blood vessels that allows for complete vascularization of the animal. The requirement for proper blood vessel formation during embryonic development, coupled with the role of this process in facilitating tumor progression, has made angiogenesis a hot topic of research in the last several decades. Angiogenesis is highly dependent on the action of MMPs and their inhibitors, TIMPs and RECK. During angiogenesis, cells must first migrate into the surrounding ECM. Branching morphogenesis follows, which is an organized process that is very reliant on MMP activity. MMPs are needed to clear space in the ECM, which permits a column of endothelial cells to advance forward and migrate into the extracellular space, forming a new blood vessel and eventually leading to vascularization of the tissue (Stetler-Stevenson 1999). In addition to cell motility, the formation of tubular structures and branch points during angiogenesis also requires changes in cell shape, proliferation, and the formation of new cell contacts (Vu and Werb 2000). Following proliferation and migration into the ECM, endothelial cells tightly adhere to one another and to the BM, and form a lumen for the new blood vessel (Stetler-Stevenson 1999). This process is largely driven by cell interactions with the ECM and requires tightly regulated ECM remodeling events.

In particular, MMP-2, MMP-9 and MT1-MMP have been associated with angiogenesis. Although MMP activity is required for blood vessel development, the action of TIMPs and RECK must be precisely regulated during this process as well (Handsley and Edwards 2005). While the N-terminal domain of TIMP-2 directly inhibits MMPs, the TIMP-2 C-terminal domain also has demonstrated anti-angiogenic activity in vivo. As previously mentioned, the carboxy terminus of TIMP-2 can bind α3β1 integrins leading to reduced proliferation of endothelial cells (Feldman, Stetler-Stevenson et al. 2004). Additionally,
association of TIMP-2 with $\alpha_3\beta_1$ integrins led to enhanced expression of RECK, which subsequently decreased endothelial cell migration through inhibition of MMP-2, -9, and MT1-MMP (Oh, Seo et al. 2004).

Just as excessive inhibition of MMP activity can have negative effects on angiogenesis, too little inhibition of MMP activity is also associated with defects in vascularization. RECK deficient embryos die at embryonic day 10.5 as a result of arrested vascular development (Oh, Takahashi et al. 2001). Histological examination of these embryos showed that the blood vessels were abnormally large and deformed, demonstrating the requirement for MMP inhibitors in mediating MMP activity during angiogenesis (Chandana, Maeda et al. 2010). Thus, these studies again emphasize the requirement for balance between MMP, TIMP and RECK activity in the ECM.

1.7.3. ECM-dependent regulation of cell signaling in development

In addition to the direct roles of MMPs and their inhibitors in regulating ECM remodeling during development, the activities of MMPs, TIMPs and RECK can also contribute to important ECM associated signaling events, which may help to pattern development. MMP-mediated release of growth factors from the ECM may be important in initiating developmental processes. MMP-1 and MMP-3 are known to release bound FGF from the ECM, whereas MMP-2, -3 and -7 are involved in the release of TGF-$\beta$ (Whitelock, Murdoch et al. 1996; Imai, Hiramatsu et al. 1997). As FGF plays important roles in chondrocyte differentiation and proliferation, its release from the ECM may contribute to cartilage development. Similarly, TGF-$\beta$ signaling is involved in branching morphogenesis; therefore, MMPs may indirectly play a role in regulating these processes through release of growth factors from the ECM (Vu and Werb 2000).
Given the multiple roles of the TIMP C-terminal domains in regulating cell motility, proliferation, and apoptosis pathways \textit{in vitro}, it is evident that TIMPs may also have important functions in development aside from their direct roles in mediating ECM turnover. Although there is limited research that has investigated the precise roles of TIMPs in mediating cell signaling \textit{in vivo}, we can infer the significance of these molecules in regulating developmental signaling events based on the numerous \textit{in vitro} studies that have been performed. Currently, a large amount of our knowledge regarding MMP/TIMP function \textit{in vivo} is based on correlation with their expression patterns in development, as well as their known activities during pathogenesis (Vu and Werb 2000). In particular, further research is needed to investigate TIMP domain function \textit{in vivo}. Our understanding of these proteins as MMP inhibitors has been confounded by the more variable roles of their C-terminal domains in cell signaling, which currently have only been examined \textit{in vitro}. As MMPs and TIMPs are both expressed and tightly regulated during development, when ECM remodeling is high, a good \textit{in vivo} approach is to use a developmental model system to examine MMP/TIMP function.

\subsection*{1.8 Animal Model}

The organism used as an animal model in this research is \textit{Xenopus laevis}, commonly known as the African clawed frog. \textit{Xenopus laevis} has been an important model organism in the field of developmental biology since the 1950’s and remains a prominent model organism in the developmental field due to its numerous advantages. A single \textit{X. laevis} female can produce hundreds of eggs in a controlled manner by injecting the hormone gonadotropin. \textit{Xenopus laevis} eggs are fertilized externally and embryos are easily maintained and reared in simple salt solution at room temperature. Developmental processes are easily observed in \textit{X.}
*laevis* embryos due to the large size of the embryos and their blastomeres. Development occurs rapidly and has been thoroughly studied in *X. laevis* embryos. Cell movements and detailed fate maps have also been well characterized. *Xenopus laevis* embryos have easily recognizable developmental stages, which are well described. Gastrulation occurs at stage 10; neurulation begins at stage 14 and continues through to stage 20 (during which time neural crest cell migration is beginning), ending with closure of the neural tube; and organogenesis occurs from stage 28 to stage 40 (during which time angiogenesis is beginning). Each stage has recognizable structures and is associated with specific signaling pathways that can be utilized to examine developmental events, such as ECM proteolysis. For instance, it has previously been shown that disrupting the ECM during *X. laevis* development has detrimental effects. Ectopic expression of MT3-MMP and TIMP-3 have been demonstrated to lead to embryonic lethality through unknown mechanisms (Pickard and Damjanovski 2004; Walsh, Cooper et al. 2007). Thus, this model organism can be used to further examine the importance of these ECM remodeling events, and to investigate more precisely how disruption of proper ECM remodeling may affect development.

In addition, *X. laevis* embryos are robust, largely resilient to experimental manipulation, and may be grafted or injected. Embryos are able to withstand extensive surgical micromanipulation *in vitro* and heal readily. One drawback of using *X. laevis* experimentally is that embryos are not ideally suited for transgenic manipulation due to their pseudotetraploid genome and long generation time to reach sexual maturity (2 years). However, *X. laevis* embryos are extremely resistant to microinjection, and are able to translate synthetic mRNA. This technique can be used for overexpression studies, and can be extremely useful in understanding developmental signaling events.
A great deal of our current knowledge regarding early embryonic development comes from studies performed using *X. laevis* embryos. *Xenopus laevis* remains an important model organism in the study of developmental biology. For these reasons I chose to use *X. laevis* as my model organism for studying ECM remodeling events and cell signaling events associated with TIMP N- and C-terminal domains.

1.9 Research Questions

1.9.1. Summary

Remodeling of the ECM is required for the development and maintenance of multicellular organisms. Extracellular matrix remodeling occurs primarily through the action of MMPs, which cleave ECM components, and their endogenous inhibitors, TIMPs and RECK. The delicate balance between TIMPs, RECK, and MMPs are required to mediate developmental processes where large-scale tissue remodeling occurs, including angiogenesis, neural development, bone formation, and organogenesis. Disruption in the expression or activities of these molecules within the ECM proteolytic network can lead to developmental abnormalities and death.

TIMPs are multifunctional proteins that have additional functions aside from MMP inhibition. TIMPs are involved in regulating a number of cell signaling pathways, including cell growth, migration, and apoptosis, and this activity is mediated through the C-terminus of TIMP proteins, independent of their MMP-inhibitory activity (which occurs exclusively at the N-terminus). There is little information regarding TIMP domain function *in vivo*, however, the relative levels of MMPs and TIMPs plays a role in regulating the availability of TIMPs at the cell surface, and subsequently, their ability to participate in signaling events. To date, much of the work characterizing TIMP signaling activity has been carried out *in vitro*,...
and TIMP roles in cell signaling seem to vary with the cell line examined. Accordingly, there is a need to examine TIMP function (particularly in cell signaling) \textit{in vivo}. My research characterizes TIMP function \textit{in vivo}, using \textit{X. laevis} as a model to examine the role of the TIMP domains during development. Specifically, this research investigates the balance between TIMP MMP-inhibitory functions and TIMP roles in cell signaling during development, and is the first comprehensive comparison of the roles of the TIMP N- and C-terminal domains \textit{in vivo}.

1.9.2. \textit{Hypotheses}

1) The N-terminal domain sequences of each TIMP will be more highly conserved than the C-terminal domains, as TIMP MMP-inhibitory activity is the common function of these proteins.

2) Overexpression of TIMP N-terminal domains will all produce similar developmental defects resulting from increased inhibition of MMP activity, due to the fact that TIMPs are largely redundant in their MMP-inhibitory abilities.

3) Overexpression of TIMP-1 and -2 C-terminal domains will produce unique developmental defects, resulting from specific changes in cell signaling pathways; whereas, the TIMP-3 C-terminal domain will have limited influence on development, as there is currently little research supporting a direct role for the TIMP-3 C-terminal domain in cell signaling.

4) Overexpression of TIMP N-terminal domains will result in similar alterations in expression and activity of proteolytic molecules; whereas, overexpression of the C-
terminal domains will have varying effects on developmental regulation of these molecules that is dependent (presumably) on their roles in cell signaling.

1.9.3. Objectives

The goal of this research was to compare the unique functions of the N- and C-terminal domains with those of the full-length molecule for all three known *X. laevis* TIMPs during development. *TIMP*-2 and -3 had previously been cloned when I began this research. Through database searches and analysis I have cloned TIMP-1, however, there does not appear to be a TIMP-4 (discussed in Chapter 5) in *X. laevis*. This research had the following objectives:

1) Identify and characterize *Xenopus laevis* TIMP-1 (Chapter 3).

2) Perform a comparison of amino acid sequence identity for the N- and C-terminal domains of each TIMP to determine their evolutionary conservation (Chapter 2 and 3).

3) Use *X. laevis* TIMP-1, -2 and -3 sequences to generate *TIMP*-1, -2 and -3 full-length, N-terminal, and C-terminal domain mRNA constructs (Chapter 2 and 3).

4) Overexpress each TIMP construct in *X. laevis* embryos using mRNA microinjection. Examine embryos for resultant morphological changes and developmental abnormalities, to determine how each TIMP domain may contribute to large-scale patterning of embryonic development (Chapter 2 and 3).

5) Examine alterations in mRNA levels of proteolytic genes, as well as changes in MMP activity, following overexpression of each *TIMP* construct to elucidate how the
individual TIMP domains may contribute to the regulation of the ECM proteolytic network (Chapter 2 and 3).

6) Examine embryos injected with each TIMP construct to investigate changes in levels of key indicators of cell signaling pathways associated with overexpression of each TIMP domain (Chapter 4).
1.10 References


CHAPTER 2

DOMAIN SPECIFIC OVEREXPRESSION OF TIMP-2 AND TIMP-3 REVEALS MMP-INDEPENDENT FUNCTIONS OF TIMPS DURING *XENOPUS LAEVIS* DEVELOPMENT

This work has previously been published as “Nieuwesteeg, M. A., Walsh L.A., Fox, M.A., and Damjanovski, S. (2012). Domain specific overexpression of TIMP-2 and TIMP-3 reveals MMP-independent functions of TIMPs during *Xenopus laevis* development. Biochem Cell Biol 90(4): 585-595”. NRC research press Copyright Clearance Center permits reuse of figures, tables and excerpts from any NRC Research Press article, for any purpose that respects the moral rights of authors without permission. The text has been modified from the original manuscript to adhere to formatting guidelines for this thesis. Experiments were designed and carried out by M.A.N., L.A.W. assisted with embryo rearing, M.A.F. assisted with Western blotting, S.D. provided funding, resources, and intellectual contributions.
2.1 Introduction

2.1.1. TIMP overview

ECM remodelling carried out by MMPs and TIMPs is important in regulating development in multicellular organisms, and mediates processes involving large-scale cell movements and rearrangements, including gastrulation, organogenesis and angiogenesis (Blavier and DeClerck 1997; Gomez, Alonso et al. 1997; Damjanovski, Amano et al. 2001; Zhang, Bai et al. 2003; Nuttall, Sampieri et al. 2004; Myers, Applegate et al. 2011). As discussed in Chapter 1, although TIMPs were originally characterized as endogenous MMP inhibitors, it is now known that TIMPs contain structurally and functionally distinct N- and C-terminal domains, and that the TIMP C-terminal domains may be involved in direct regulation of a number of signaling pathways (Murphy, Unsworth et al. 1993; Guedez, Stetler-Stevenson et al. 1998; Ahonen, Poukkula et al. 2003; Chirco, Liu et al. 2006; Vanhoutte and Heymans 2010; Kallio, Hopkins-Donaldson et al. 2011). The two TIMP domains are thought to have evolved separately, and both domains have the ability to fold and function independently (Brew and Nagase 2010). This has been demonstrated through expression of TIMP N-terminal domains in heterologous systems, where the N-terminal TIMP domains still maintain a stable native structure, and can inhibit MMPs (Bahudhanapati, Zhang et al. 2011).

The four mammalian TIMPs all have a similar structure and are highly conserved at the amino acid level (Bode, Fernandez-Catalan et al. 1999; Murate and Hayakawa 1999). The highest degree of sequence similarity occurs within TIMP N-terminal domains, whereas the C-terminal domains are more variable (Pavloff, Staskus et al. 1992; Apte, Hayashi et al. 1994; Bodden, Harber et al. 1994; Leco, Khokha et al. 1994; Fernandez, Butterfield et al. 2003; Brew and Nagase 2010). As discussed in section 1.5.3., TIMP-2 is the most well
characterized, and in human endothelial cells has been demonstrated to bind α3β1 integrins through its C-terminus, leading to an upregulation in RECK, which subsequently downregulates MMP activity and decreases cell migration (Oh, Seo et al. 2004; Shakibaei, Csaki et al. 2008). While the C-terminal domains can act MMP-independently, the N-terminal domains of all TIMPs are involved in direct binding and inhibition of the bound MMP's catalytic activity.

2.1.2. TIMPs and MMPs in development

To date, most research characterizing TIMP domain function has been carried out in vitro, where the activation of a given cell signaling pathway is dependent on both the specific TIMP expressed, and the cell type it is expressed in (Stetler-Stevenson 2008; Bourboulia and Stetler-Stevenson 2010). Although maintaining the correct MMP/TIMP balance is essential for proper development, little is known regarding the relationship between TIMPs and MMPs during embryogenesis. TIMP-1, -2 and -3 knockout mice are viable (reviewed in Brew and Nagase 2010), suggesting overlapping redundancies in their functions. In contrast, while many MMP knockouts have been generated, only the MMP-14 (MT1-MMP) null mouse shows severe consequences (Zhou, Apte et al. 2000). MMP overexpression during X. laevis development results in both axial defects and death shortly after gastrulation, although specific mechanisms are not known (Hasebe, Hartman et al. 2007; Walsh, Cooper et al. 2007). Additionally, TIMP-3 overexpression is lethal to X. laevis during development; however, it is unclear whether this occurs as a result of aberrantly activated cell signaling pathways, or as a result of improper ECM remodelling (Pickard and Damjanovski 2004).
2.1.3. Summary and research questions

At the time this research was published, only TIMP-2 and -3 had been cloned in *X. laevis* (Yang and Kurkinen 1998; Klein, Strausberg et al. 2002). Mammalian TIMP-2 has been well characterized *in vitro* and is known to be localized pericellulary and associated with a number of cell signaling pathways (Stetler-Stevenson 2008). In contrast, TIMP-3 interacts with sulphated GAGs and is sequestered in the ECM, away from the cell surface (Silbiger, Jacobsen et al. 1994; Yu, Yu et al. 2000). To elucidate the roles of TIMPs and their domains during development, I used mRNA microinjection to disrupt endogenous TIMP levels and overexpressed TIMP-2 and -3, or their individual N- or C-terminal domains in *X. laevis* embryos. Ectopic expression of TIMP-2 and -3 as well as their N- and C-terminal domains led to distinct developmental disruptions, suggesting MMP-dependent and independent functions. Additionally, zymography and PCR analysis showed that the TIMP N- and C-terminal domains led to differential changes in MMP activity, and the expression of marker genes, indicating that each domain has unique downstream effects.

2.2 Materials and Methods

2.2.1. Sequence analysis of vertebrate and invertebrate TIMP-2 and -3 N- and C-terminal domains

Sequence alignments and amino acid sequence identity were performed using ClustalW2 analysis software at the European Bioinformatics Institute site at http://www.ebi.ac.uk/Tools/clustalw2/index.html. Phylogenetic comparisons were performed using Molecular Evolutionary Genetic Analysis (MEGA) 5.2.1. software to construct a neighbour-joining tree with a bootstrap of 1000.
2.2.2. Animals

Adult *X. laevis* were purchased from *Xenopus* I Inc (Dexter, MI). Embryos were fertilized and reared in accordance with standard protocols (Sive H.L. 2000) and staged according to Nieuwkoop and Faber (Nieuwkoop P.D. 1956). Animals were housed and treated according to UWO and CCAC guidelines.

2.2.3. Cloning of *X. laevis* TIMP-2 and -3

*X. laevis* TIMP-2 and TIMP-3 sequences were previously identified (AF042493 and MGC84288, respectively). I cloned *X. laevis* TIMP-2 and -3 from total adult liver cDNA using SuperScript™ Reverse Transcriptase (Invitrogen) with Platinum® Taq DNA Polymerase High Fidelity (Invitrogen). The coding regions of both TIMP-2 and -3 were cloned using specific primers based on the above sequences. The full-length TIMP-2 and -3 amplicons were cloned into the pCR®II-TOPO vector (Invitrogen), and sequences were confirmed at the Robarts Research Institute DNA Sequencing Facility at the University of Western Ontario.

2.2.4. Generation of TIMP-2 and -3 mRNA constructs for microinjection

PCR was used to generate full-length, N-terminal or C-terminal TIMP-2 and -3 (hemagglutinin) HA-tagged constructs using the full-length clones as templates. Briefly, all TIMP constructs (T2FL, T2N, T2C, T3FL, T3N, T3C) were HA-tagged on their C-terminal ends. In addition, the two C-terminal domain constructs (T2C and T3C) were engineered to include their appropriate secretory signal sequence through a two-step PCR process. Following PCR amplification, the sequences of all amplicons were verified. All TIMP-2 and -3 constructs were subsequently ligated into the BglII/SpeI restriction sites of the T7TS
plasmid. mMessage mMachne (Ambion) was used with T7 RNA polymerase (Ambion) to synthesize stable capped, poly(A)-tailed mRNA transcripts that were dissolved in filtered water. mRNA was quantified using Nanovue spectrophotometer (GE), and its integrity was assessed using 1% agarose gel electrophoresis. *Green fluorescent protein (GFP)* mRNA was generated and quantified in a similar way from a proven T7TS construct plasmid (Walsh, Cooper et al. 2007).

### 2.2.5. mRNA microinjection

Prior to injection, fertilized *X. laevis* embryos at the 1 cell stage were transferred from 0.1X Marc’s modified ringers (MMR) into 1X MMR containing 4% Ficoll. Embryos were injected at the 1 cell stage with *TIMP*-2 or -3 full-length, N-terminal, or C-terminal constructs using 10 µm diameter glass needles. Embryos were injected with 4 ng of mRNA construct in a volume of 2.3 nl. Embryos were maintained in 1X MMR with Ficoll for 5 hr following injection, then transferred to 0.1X MMR solution for rearing. Embryos that were dead or abnormal 2 hr post-injection were removed, and the remaining embryos (representing the 100% being monitored) were examined for phenotypic abnormalities. The percent of normal embryos present (no clear visible defects) were quantified for two days following injection. Mean values were obtained from 3 independent sets of experiments.

### 2.2.6. Protein preparations and Western blotting

Stage 30 embryos injected with the various mRNA constructs, or uninjected controls, were subject to protein extraction. Briefly, 10 embryos were lysed and sonicated in 100 µl lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1% NP-40 protease
inhibitor cocktail (Roche Applied Science, Indianapolis, IN)). Lysates were centrifuged at 15,000 g for 15 min at 4°C, the supernatant was removed, and protein was quantified using BCA protein assay kit (Thermo Scientific) in accordance with the manufacturer’s instructions. Equal amounts of protein (25 µg) were electrophoresed on a 10% SDS gel and transferred to PVDF membrane (Bio-Rad). Membranes were washed three times with TBST (Tris-buffered saline containing 0.5% Tween 20), blocked using 5% skim milk in TBST for 30 min at room temperature, and incubated overnight at 4°C with primary antibody. Primary antibodies used were rabbit anti-HA, 1/1000 dilution (Invitrogen) or mouse anti-β-actin, 1/1000 dilution (Invitrogen). After washing 3 times in TBST, membranes were incubated with secondary antibody (goat anti-rabbit HRP, 1/5000 dilution; Invitrogen, or goat anti-mouse HRP, 1/5000 dilution; Invitrogen) for 1 hr at room temperature. Membranes were washed 3 times in TBST and peroxidase activity was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and visualized, standardized to actin levels, and photographed using Bio-Rad Quantity One 4.4.0 software.

2.2.7. Zymography

Briefly, 12.5 µg of protein from injected embryos (as described above) was diluted 1:1 with 2X SDS-loading buffer (0.5 M Tris-HCl (pH 6.8), 10% SDS, 2.5 % glycerol, 1% bromophenol Blue), and then analyzed by gelatin zymography. Protein samples were loaded into a 10% gelatin zymogram gel (Bio-Rad), and electrophoresed in 1X zymogram running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS), followed by in-gel renaturation for 30 min at room temperature in renaturing solution (2.5% Triton X-100). Substrate cleavage was carried out by incubating gels in zymogram developing buffer (pH 7.5, 50 mM Tris, 200 mM NaCl, 5 mM CaCl _2_ (anhydrous), 0.02% Brij-35) for 48 hr at 37°C. The gelatinolytic activity
was visualized as a clear band in the blue background after staining with 0.5% Coomassie blue. Gels were visualized and photographed, and densitometry was performed using Bio-Rad Quantity One 4.4.0 software.

2.2.8. Real-Time PCR of marker genes

To investigate changes in the expression of embryonic genes following injection of the various TIMP constructs, real-time qPCR was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad) in a two-step procedure using PerfeCTa® SYBR® Green SuperMix (Quanta Biosciences). Amplification of the Ef-1α gene was performed to standardize the amount of sample cDNA. Primers, based on the X. laevis sequences, were as follows: Ef-1α 5’CTACAAATGTGGTGCGCATCG and 5’GCTCTGCGCTCCAGTTTGTCC; RECK 5’AGTGAAGGATGCACCAAACC and 5’GCAGTTTCAGCACCACATCAAGA; β1 integrin 5’CTGACGCGAGTTCCATT and 5’GTGACATGTCTCCGTGCAG; MMP-2 5’AGGAGAGGCAAAGTTTGTGA and 5’CTGAGCAGCAGAAATCGT; MMP-9 5’CTGGGACTGAAGGAGACTGG and 5’CCCCCTCAAATGTTGTAAT. All reactions were performed in a 96 well plate using the following cycling conditions: 40 cycles of 95 °C for 15 sec, 60 °C for 30 sec, and 72°C for 1 min. Using the ΔΔCT (delta-delta CT) method, the value of each control sample was set at 1 and used to calculate the fold change of target genes.

2.3 Results and Discussion

Understanding of TIMP function has been confounded by the realization that TIMPs may also bind to a variety of cell surface receptors through their C-terminal domains and mediate a number of cell signaling pathways in an MMP-independent manner (Lambert,
Dasse et al. 2004). There is currently little information regarding how TIMP proteins function in vivo, particularly as their domains may act through both MMP-dependent and independent mechanisms (Stetler-Stevenson 2008). In this study I examined the individual domains of TIMP-2 and -3 in early stage X. laevis embryos in order to gain an understanding of how the individual N- and C-terminal domains may regulate key developmental events, as there is evidence that TIMP N- and C-terminal domains may have unique properties, function independently as individual domains, and maintain their native topology (Bahudhanapati, Zhang et al.; Chirco, Liu et al. 2006; Brew and Nagase 2010; Bahudhanapati, Zhang et al. 2011; Wu, Wei et al. 2011).

2.3.1. The N- and C-terminal domains of X. laevis TIMP-2 differ in their sequence conservation

A direct comparison of amino acid sequence identities of the individual X. laevis TIMP-2 N- and C-terminal domains with other vertebrate and invertebrate species is of particular interest, as I investigated in vivo whether TIMP N- and C-terminal domains could function independently of one another, and how these roles may be reflected in their evolutionary conservation. My analysis indicated that for TIMP-2, the N-terminal MMP-inhibitory domains were more highly conserved across most vertebrate species analyzed, including both mammals and non-mammals, than the C-terminal domains (Fig. 2.1 A), a finding that has been previously reported (Brew and Nagase 2010). This may reflect the fact that the N-terminal domain has only one primary role, to bind to and inhibit MMP activity. As the catalytic domains of MMPs are conserved across species (Van Wart and Birkedal-Hansen 1990), this TIMP domain is also conserved. Interestingly, Caenorhabditis elegans TIMP-2 does not contain the region corresponding to the vertebrate C-terminal domain,
suggesting the possibility that the TIMP-2 C-terminal domain signaling mechanism evolved only in higher vertebrates. In contrast, the TIMP-2 C-terminal domains were slightly more variable (Fig. 2.1 A), suggesting that their role, presumably in cell signaling, may vary between species. The C-terminal domain may have evolved concomitant with changes in receptor and cell signaling pathways. However, phylogenetic analysis comparing the *X. laevis* TIMP-2 N- or C-terminal domains with other species showed that both the N- and C-terminal domains had a similar branching patterns in their phylogenetic trees, but diverged at different rates (Fig. 2.1 B vs. C).
Figure 2.1. Evolutionary conservation of TIMP-2 N- and C-terminal domains. (A) Sequence analysis comparing amino acid sequence identity of *X. laevis* TIMP-2 N- and C-terminal domains among several vertebrates and invertebrates. Light grey boxes represent species whose N-terminal domains were more highly conserved with *X. laevis* TIMP-2 than their C-terminal domains. Dark grey boxes represent species whose C-terminal domains were more highly conserved with *X. laevis* TIMP-2 than their N-terminal domains. (B) Phylogenetic tree showing sequence divergence among TIMP-2 N-terminal domains as generated by MEGA 5.2.1. software. (C) Phylogenetic tree showing sequence divergence among TIMP-2 C-terminal domains as generated by MEGA 5.2.1. software. The *C. elegans* sequence was removed from phylogenetic analysis as the C-terminal domain was missing. Accession numbers are as follows: Xenopus (*Xenopus laevis*) AF042493, Human (*Homo sapiens*) AAB19474.1, Mouse (*Mus musculus*) AAA40446.1, Chicken (*Gallus gallus*) NP_989629.1, Zebrafish (*Danio rerio*) NP_878294.1, Fugu (*Takifugu rubripes*) BAE06264.1, Elegans (*Caenorhabditis elegans*) AAA96174.
**A  TIMP-2 Full-length comparison**

<table>
<thead>
<tr>
<th>TIMP-2</th>
<th>Xenopus</th>
<th>Human</th>
<th>Mouse</th>
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**B  TIMP-2 N-terminal domains**

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  Human
   Mouse
     Xenopus
     Chicken
       Zebrafish
          Fugu
```

0.02

**C  TIMP-2 C-terminal domains**

```
  Human
   Mouse
     Xenopus
     Chicken
       Zebrafish
          Fugu
```

0.05
2.3.2. The C-terminal domain of *X. laevis* TIMP-3 is conserved

In contrast to TIMP-2, when I examined the individual domains of TIMP-3 I found the opposite trend; the TIMP-3 C-terminal domains were more conserved with *X. laevis* TIMP-3 than their N-terminal domains across all vertebrate species examined (Fig. 2.2 A). However, this relationship was not true for invertebrates, as the N-terminal domain of sea urchin TIMP-3 was more conserved. TIMP-3 is unique in that it is the only TIMP that binds directly to the ECM, and studies have shown that the C-terminal domain of TIMP-3 is important for facilitating this binding (Yu, Yu et al. 2000). Langton et al. (1998) expressed both full-length and truncated forms of TIMP-3 in Cos-7 cells, and demonstrated that full-length and C-terminal TIMP-3 were able to bind to the ECM, whereas N-terminal TIMP-3 could not (Langton, Barker et al. 1998). Therefore, the C-terminal domain of TIMP-3 may have a conserved role in mediating its attachment to the ECM, at least among vertebrate species, while its N-terminal domain functions to inhibit MMP activity. Phylogenetic analysis comparing *X. laevis* TIMP-3 N- and C-terminal domains with other species showed that, unlike TIMP-2, the two domains followed a slightly different evolutionary trend, suggesting that the individual domains in TIMP-3 evolved under different selective pressures (Fig. 2.2 B and C).
Figure 2.2. Evolutionary conservation of TIMP-3 N- and C-terminal domains. (A) Sequence analysis comparing amino acid sequence identity of *X. laevis* TIMP-3 N- and C-terminal domains among several vertebrates and invertebrates. Light grey boxes represent species whose N-terminal domains were more highly conserved with *X. laevis* TIMP-3 than their C-terminal domains. Dark grey boxes represent species whose C-terminal domains were more highly conserved with *X. laevis* TIMP-3 than their N-terminal domains. (B) Phylogenetic tree showing sequence divergence among TIMP-3 N-terminal domains as generated by MEGA 5.2.1 software. (C) Phylogenetic tree showing sequence divergence among TIMP-3 C-terminal domains as generated by MEGA 5.2.1 software. Accession numbers are as follows: Xenopus (*Xenopus laevis*) MGC84288, Human (*Homo sapiens*) AAB60373.1, Mouse (*Mus musculus*) AAA40447.1, Cow (*Bos taurus*) NP_776898.2, Fugu (*Takifugu rubripes*) BAE06261.1, Salmon (*salmo salar*) ACN10551.1, Sea Urchin (*Strongylocentrotus purpuratus*) XP_781027.1.
A  TIMP-3 Full-length comparison

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B  TIMP-3 N-terminal domains

C  TIMP-3 C-terminal domains
2.3.3. Overexpression of full-length TIMP-2 and -3 resulted in different developmental phenotypes

To assess the roles of TIMP-2 and -3 during *X. laevis* development, I generated and overexpressed full-length *TIMP-2* or -3 mRNA constructs in newly fertilized *X. laevis* embryos. Embryos were injected at the 1 cell stage and analyzed for gross morphological changes in development until stage 30 (approximately 2 days following injection). Real-time PCR analysis was used to confirm upregulation of *TIMP-2* or -3 mRNA levels following injection of each construct. This verified that injected embryos all contained relatively equal, but elevated levels of *TIMP* mRNA compared with uninjected embryos. Additionally, to compare relative protein levels following injection, Western blotting against HA-tags was used. This confirmed that all proteins produced were present in equal amounts (Fig. 2.3). The percentage of normal embryos (embryos that were alive and morphologically normal) was determined and quantified at stage 15 (one day post-injection) and stage 30 (two days post-injection). Over 90% of control uninjected embryos and embryos injected with *GFP* mRNA developed normally during this 48 hr period (Fig. 2.4 A and B). In comparison, injection of embryos with full-length *TIMP-2* or -3 (T2FL and T3FL, respectively) mRNA resulted in serious developmental defects, with T3FL being more detrimental than T2FL (Fig. 2.4 A and B). By stage 30, 55% of T2FL injected embryos developed normally, whereas only approximately 10% of T3FL injected embryos developed normally (Fig. 2.4 A and B). Phenotypically, T2FL injected embryos displayed axial defects (truncated, curved axes), and neural tube closure failure (Fig. 2.5 A). In contrast, while overexpression of T3FL also resulted in axis defects (truncated, curved axes), these embryos did not display neural tube closure defects.
Figure 2.3. Microinjection of 4 ng of TIMP-2 or -3 mRNA constructs produces equal amounts of HA-tagged protein in *X. laevis* embryos. Following the injection of equal amounts of mRNA (4 ng) at the 1 cell stage, protein was isolated from stage 30 embryos. Equal amounts of protein were electrophoresed on a 10% SDS gel. TIMP constructs were detected using an anti-HA antibody. Images are of representative bands after normalization to β-actin levels. The left panel shows bands corresponding to TIMP-2 full-length (T2FL), N-terminal (T2N), and C-terminal (T2C) constructs. TIMP-3 full-length (T3FL), N-terminal (T3N), and C-terminal (T3C) constructs are shown in the right panel.
2.3.4. Overexpression of TIMP-2 N- or C-terminal domain constructs resulted in more developmental defects than overexpression of full-length TIMP-2

Overexpression of the individual N- or C-terminal domains of TIMP-2 resulted in distinctive survival rates as well as developmental defects. Following injection of either domain of TIMP-2 (T2N and T2C constructs), embryos displayed morphological defects similar to T2FL injected embryos (truncated, curved axes, and neural tube closure failure; Fig. 2.5 A); however, the effects were more severe as less than 30% of T2N and T2C injected embryos developed normally, compared to 55% normal development with the T2FL construct (Fig. 2.4 A). The two domains also manifested their effects at different rates, as by stage 15 only 30% of T2N injected embryos developed normally, versus 50% normal at this time point with the T2C construct. Thus, the T2N construct was more detrimental at this time point (Fig. 2.4 A). By stage 30, T2N and T2C injected embryos displayed similar scores with only 21% and 26% normal development, respectively, both of which were more detrimental than the T2FL injections (Fig. 2.4 A). Overexpression of T2FL, T2N, and T2C constructs all produced large-scale morphological defects including head defects, curved, truncated axes, and failure of the neural tube to close (Fig. 2.5 A; see appendix C for additional images). Since overexpression of all three constructs disrupted the proper formation of the neural tube, this suggests that a combination of both TIMP-2 cell signaling (via the C-terminal end) and MMP-inhibitory activity (via the N-terminal end) may be required for normal development of the neural tube.
2.3.5. **Overexpression of full-length TIMP-3 or its N-terminal domain construct resulted in more developmental defects than overexpression of the TIMP-3 C-terminal domain construct**

The consequences of injecting full-length TIMP-3 and its N- or C-terminal domain did not follow the same pattern as TIMP-2. T3FL injections caused a variety of defects that resulted in only 15% normal embryos by stage 30 (Fig. 2.4 B). A similar rate of defects was also displayed in T3N injected embryos (Fig. 2.4 B), and both T3FL and T3N injected embryos also had truncated, curved axes and head and eye malformation (Fig. 2.5 B). These defects and phenotypes were in contrast to the T3C injected embryos, which had over 70% normal development at stage 30 (Fig. 2.4 B). Indeed, most surviving T3C embryos displayed little to no morphological defects (Fig. 2.5 B; see Appendix C for additional images). Thus, I observed that T3FL and T3N constructs were approximately 5-fold more toxic to X. laevis embryos than T3C (15% vs 70% normal at stage 30; Fig. 2.4 B). In contrast, the T2N and T2C constructs were more than twice as toxic as T2FL (Fig. 2.4 A). The observation that T3FL and T3N overexpression resulted in developmental defects including curved, truncated axes, as well as anterior malformations, was consistent with *in situ* hybridization studies that showed TIMP-3 expression in the head and eye (Pickard and Damjanovski 2004). Additionally, T3FL and T3N constructs resulted in high levels of lethality compared with T3C (Fig. 2.4 B), and T3C overexpression resulted in embryos that did not display any serious development defects (Fig. 2.5 B). This indicates that it is the N-terminal domain of TIMP-3 that is involved in mediating developmental events, and this likely occurs through direct inhibition of MMP activity. The C-terminal domain of TIMP-3 on its own appears to play no major role in development at this time.
Figure 2.4. Overexpression of TIMP-2 and -3 led to abnormal development. Following the injection of mRNA constructs at the 1 cell stage, embryos were scored for a normal phenotype at stage 15 and stage 30 in development. The percent of normal embryos following the injection of each construct was monitored and graphed. (A) Injections consisted of 4 ng of mRNA encoding either TIMP-2 full-length (T2FL, red circle), N-terminal domain (T2N, purple triangle), or C-terminal domain (T2C, blue square) constructs, or GFP (green circle). X represents uninjected controls. (B) Injections consisted of 4 ng of mRNA encoding TIMP-3 full-length (T3FL, red circle), N-terminal domain (T3N, purple triangle), or C-terminal domain (T3C, blue square) constructs, or GFP (green circle). X represents uninjected controls. Results are based on the average of 3 independent sets of experiments, where a minimum of 100 embryos were injected and counted for each replicate. Bars indicate standard error (SE).
Fig. 2.5 Overexpression of TIMP-2 and -3 resulted in specific morphological defects.

Four nanograms of mRNA encoding either GFP, or full-length, N-terminal or C-terminal domain TIMP-2 or -3 were injected into X. laevis embryos at the 1 cell stage. Following injection, photographs were taken of representative embryos at stage 30. Anterior is to the left. GFP injected embryos were phenotypically normal. (A) Overexpression of TIMP-2 full-length (T2FL), N-terminal (T2N) or C-terminal (T2C) constructs all caused similar early developmental defects, and neural tube closure failure. (B) TIMP-3 full-length (T3FL) and N-terminal (T3N) constructs resulted in axial defects and curvature. The C-terminal domain TIMP-3 (T3C) construct did not produce severe malformations. Images are of representative embryos where results were consistent between 3 independent sets of experiments, with a minimum of 100 embryos injected per construct for each experiment. See appendix C for additional images.
A

GFP

500 µm

B

T2FL

500 µm

T2N

500 µm

T2C

500 µm

C

T3FL

500 µm

T3N

500 µm

T3C

500 µm
2.3.6. Overexpression of TIMP constructs resulted in differential changes in MMP-2 and -9 activity

Gelatin zymography was used to determine whether overexpression of TIMP-2 or -3, or their individual domains altered the levels of active MMPs in developing embryos. I looked specifically at the activities of MMP-2 and -9, as these are two potent ECM remodelling proteins that have been shown to play important roles during development (Carinato, Walter et al. 2000; Zhang, Bai et al. 2003). Interestingly, overexpression of all TIMP constructs resulted in increased levels of active MMP-9, which was relatively low in control embryos, whereas TIMP-mediated changes in active MMP-2 (which was comparatively higher in control embryos) were much more subtle (Fig. 2.6). While it is counterintuitive that none of the TIMP constructs resulted in decreased MMP-9 activity relative to control embryos, (particularly the N-terminal constructs), the process of gelatin zymography separates TIMP-MMP complexes, and as such, is a measure of the amount of active MMP present in the embryo, as opposed to a direct measure of in vivo TIMP inhibition of MMP activity (Toth and Fridman 2001). The observed increases in MMP-9 seen here could have resulted from compensation in the embryos, whereby increasing the amount of active MMP was a response to the attenuation of nascent MMP activity due to the injected TIMP construct. Accordingly, the highest levels of active MMP-9 were observed in embryos injected with T2N and T3FL constructs (Fig. 2.6), both of which would function mainly in MMP inhibition. Additionally, this supports my embryo viability data, as these constructs resulted in the most severe developmental phenotypes, with only 21% and 15% normal development, respectively. With TIMP-3, the T3N construct also resulted in increased levels of active MMP-9, however, not to the same extent as observed in the T3FL injected embryos (Fig. 2.6). This was unexpected as both constructs should be involved predominantly in
MMP inhibition, and the embryo viability data for both constructs was remarkably similar, resulting in only approximately 15% survival by stage 30. This further highlights the distinct functions seen between the N-terminal domains of TIMP-2 and -3, as reflected by their distinct sequence conservation patterns between species.

The T3C construct caused very minor changes in MMP activity relative to control embryos (Fig. 2.6), which corroborated my embryo viability data as injection with this construct also resulted in very minor phenotypic abnormalities and 70% normal development (Fig. 2.5 B). This provided further support that the TIMP-3 C-terminal domain plays no independent role in early *X. laevis* development. Interestingly, the T2C construct did result in increased levels of MMP-9 relative to control embryos. This increase, however, was not as dramatic as that observed in T2N or even T2FL injected embryos (Fig. 2.6), even though injection with the T2C construct was comparable with the T2N construct in terms of lethality in the embryos (Fig. 2.5 A). This supports the idea that the C-terminal domain of TIMP-2 may work through a different mechanism to regulate MMPs during development.
Figure 2.6. Overexpression of TIMP-2 or -3 and their individual domains altered MMP-2 and -9 activity in *X. laevis* embryos. Gelatin zymography was used to measure changes in MMP-2 and MMP-9 activity resulting from overexpression of 4 ng of *TIMP-2* full-length (T2FL), N-terminal (T2N), C-terminal (T2C), or *TIMP-3* full-length (T3FL), N-terminal (T3N), or C-terminal (T3C) mRNA constructs. Proteins were extracted from embryos at developmental stage 30, following injection of each TIMP construct. Active MMP-9 (84 KDa) and MMP-2 (63 KDa) are visualized as bright bands against a dark background. Densitometry readings were used to quantify and graph changes in MMP-9 (dark grey) and MMP-2 (light grey) activities relative to control (uninjected) embryos. Graphed data is from one representative experiment, where trends and relative levels were observed consistently in at least 3 trials.
2.3.7. Real-time PCR analysis of marker genes in post-TIMP construct injected embryos

To better understand the phenotypes I observed following overexpression of TIMP-2 or -3 or their individual domains, I performed quantitative real-time PCR on injected embryos to look for changes in key genes associated with TIMP-2 and -3 activity. Specifically, I assayed for changes in MMP-2 and -9 expression as well as for changes in RECK and β1 integrin expression (Fig. 2.7 A and B). My results showed that none of the TIMP constructs altered RECK or β1 integrin expression levels substantially. It is noteworthy that neither T2C nor T2FL constructs resulted in substantial decreases in RECK expression levels (though the trend was towards a decrease), as this relationship has previously been demonstrated in mouse and human cell lines (Oh, Seo et al. 2004). However, previous studies were carried out with homogenous cell populations in vitro, whereas embryos contain many types of cells, only some of which may be able to bind to TIMPs and modulate RECK expression. A more careful analysis must be carried out to examine the possibility of localized changes in RECK or β1 integrin levels in response to TIMP signaling.

Although RECK expression remained largely unaltered, my results indicated that T2C may have a role in mediating other important signaling events during X. laevis development. Real-time PCR showed that the T2C construct resulted in dramatic decreases in MMP-2 and -9 expression (decreased 14 and 15 fold, respectively) compared with controls (Fig. 2.7 A). As my zymography data indicated that MMP activity is upregulated at the time, this decrease in transcript is puzzling. However, while increased MMP activity in vivo could be achieved through the activation of existing pro-MMPs, the change in transcript levels may reflect a cell signaling function of the T2C construct that is independent of the function of a native whole TIMP molecule. As such, the embryo is responding to signals mediated by a protein domain that is no longer linked to the usual activities of the entire molecule. Consequently,
genes are regulated in a fashion that is not normal and thus, detrimental. The T2N construct also decreased MMP-9 expression, however, only by about 4-fold, and T2FL showed comparable results. The less marked changes in expression following injection with T2FL and T2N constructs may be the result of an MMP-dependent regulatory feedback mechanism, as injection of T3FL and T3N constructs resulted in comparable changes in MMP-2 and -9 expression (Fig. 2.7 B). Since the T2C construct does not have any MMP-inhibitory activity, the observed decreases in MMP-2 and -9 expression are likely due to an MMP-independent TIMP-2 mediated signaling cascade. Indeed, recent research has shown that the TIMP-2 C-terminal domain may be involved in a variety of cell signaling cascades, including receptor tyrosine kinase (RTK), NF-κB, and cell proliferation pathways (Murphy, Unsworth et al. 1993; Seo, Li et al. 2003; Sun and Stetler-Stevenson 2009). In addition, I have unpublished data which showed that the T2C construct can bind to the surface of X. laevis A6 cells, indicating that there is at least one potential cell surface binding partner for the C-terminal domain of TIMP-2 to signal through in X. laevis. Interestingly, the unique TIMP-2 C-terminal signaling cascade could be species specific as sequence analysis suggests that this domain is less conserved than its N-terminal counterpart (Fig. 2.1; see appendix D for table of PCR CT values).

In contrast, the T3C construct did not result in considerable changes in gene expression (Fig. 2.7 B). This is consistent with the fact that I did not observe any large-scale morphological changes in T3C injected embryos, and supports the conclusion that the C-terminal domain of TIMP-3 is likely only involved in mediating binding of TIMP-3 to components of the ECM.
Figure 2.7. Effect of full-length TIMP-2 or -3 and their N- and C-terminal domains on transcript levels. Real-time PCR was used to measure the changes in transcript levels following the injection of 4 ng of (A) TIMP-2 full-length (T2FL), N-terminal (T2N), or C-terminal (T2C), or (B) TIMP-3 full-length (T3FL), N-terminal (T3N), or C-terminal (T3C) mRNA constructs into X. laevis embryos at the one cell stage. Changes in transcript levels were measured relative to Ef-1α and normalized to control (uninjected) embryos. INTG Beta 1 represents β1 integrin. Results are based on the average of 3 independent sets of experiments. Bars indicate SE (see appendix D for table of PCR CT values).
2.4 Conclusions

Taken together, I observed that while the N- and C-terminal domains of TIMP-2 were similar in their patterns of evolutionary conservation, the two domains differed in their abilities to alter gene expression and MMP activity. The misregulation of either domain had severe consequences on development, more so than the overexpression of full-length TIMP-2. This suggested that both domains of TIMP-2 serve a unique function, one MMP-dependent, one MMP-independent, where their function is mediated and moderated when they are part of the whole TIMP-2 molecule. In contrast, the TIMP-3 domains differed in their patterns of divergence, and the C-terminal domain had little effect on gene expression or MMP activity. With TIMP-3, however, misregulation of the full-length molecule was most detrimental, suggesting that the molecule works as a whole, and that there is little or no MMP-independent function, at least at the developmental stages examined in this study.
2.5 References


CHAPTER THREE

FUNCTIONAL CHARACTERIZATION OF TISSUE INHIBITOR OF METALLOPROTEINASE -1 (TIMP-1) N- AND C-TERMINAL DOMAINS DURING XENOPUS LAEVIS DEVELOPMENT

This work has been submitted as “Nieuwesteeg, M. A., Willson, J.A., Cepeda, M., Fox, M.A. and Damjanovski, S. (2013). Functional characterization of tissue inhibitor of metalloproteinase-1 (TIMP-1) N- and C-terminal domains during Xenopus laevis development. PlosOne manuscript ID: PONE-D-13-19519.” The text has been modified from the original manuscript to adhere to formatting guidelines for this thesis. Experiments were designed and carried out by M.A.N., J.A.W. assisted with embryo rearing, M.C. assisted with reverse zymography, M.A.F. assisted with Western blotting, S.D. provided funding, resources, and intellectual contributions.
3.1 Introduction

3.1.1. ECM dynamics in development

MMPs are important regulators of development due to their ability to cleave structural components of the ECM, as well as secreted signaling molecules within the ECM, leading to changes in both cell movement and cell signaling (Vu and Werb 2000). Controlled inhibition of MMP activity is needed to prevent excessive ECM degradation, and is largely carried out by TIMPs, as well as the cell surface MMP inhibitor, RECK (Nagase, Visse et al. 2006). Maintaining the proper balance between MMP activity and inhibition is necessary during embryogenesis and facilitates developmental events including organogenesis and angiogenesis (Liotta, Steeg et al. 1991; Blelloch and Kimble 1999), and disrupting this balance can have deleterious effects (Zhang, Bai et al. 2003; Pickard and Damjanovski 2004). Inhibition of MMP-2 or -9, two potent ECM proteases, can lead to axial defects and incomplete neural crest cell migration, respectively, while knockout of membrane-bound MMP-14 (MT1-MMP) during mouse development is embryonic lethal (Zhou, Apte et al. 2000; Hasebe, Hartman et al. 2007; Monsonego-Ornan, Kosonovsky et al. 2012). Knockout of RECK during development is also lethal due to defects in angiogenesis (Oh, Takahashi et al. 2001). Additionally, my research has shown that overexpression of TIMP-2 and -3 during Xenopus laevis development leads to axial and neural tube defects (Chapter 2) (Nieuwesteeg, Walsh et al. 2012). Thus, disruption of activity levels in this proteolytic ECM remodeling network is detrimental during development.

3.1.2. Structure and function of TIMPs

Although TIMPs are a family of only 4 secreted proteins (TIMP 1-4), together they can inhibit all known MMPs by binding to MMPs in a 1:1 manner to block their proteolytic
activity (Murphy 2011). As discussed in section 1.3.1., the four mammalian TIMPs share similar domain structure and roughly 40% amino acid sequence similarity, including 12 conserved cysteine residues which result in the formation of 6 disulfide bonds (Nagase, Meng et al. 1999). The structurally and functionally distinct N- and C-terminal domains of TIMP proteins are each stabilized by 3 disulfide bonds (Nagase, Meng et al. 1999). The N- and C-terminal domain boundary is found between cysteines 6 and 7, in between which there are only one or two amino acids (see Fig. 1.1, section 1.3.1). The N-terminal domain of a TIMP is both necessary and sufficient for MMP inhibition, and sequesters MMP activity by binding to the zinc active site found in the catalytic domain of all MMPs (Gomez, Alonso et al. 1997). In contrast, the smaller C-terminal domain of TIMPs may influence cell behavior in an MMP-independent manner through direct regulation of a number of cell signaling pathways (Chirco, Liu et al. 2006). As previously discussed, characterization of cell surface binding partners and specific signaling events involving TIMP C-terminal domains has been carried out in vitro, where activation of a resultant signaling pathway varies with the specific TIMP expressed, as well as the cell type each TIMP is expressed in (Bourboulia and Stetler-Stevenson 2010).

3.1.3. TIMP-1 overview

TIMP-1 was first identified for its erythroid potentiating activity, and as an inhibitor of metalloproteinases (Docherty, Lyons et al. 1985; Gasson, Golde et al. 1985). Both the N- and C-terminal domains of TIMP-1 have now been well characterized in vitro. The two domains of TIMP-1, as well as of the other TIMPs, have the ability to fold and function autonomously in vitro (Gomez, Alonso et al. 1997; Bahudhanapati, Zhang et al. 2011; Nieuwesteeg, Walsh et al. 2012). TIMP-1 can inhibit MMP-2 and -9, which are two powerful
ECM proteases that cleave abundant ECM components (Nagase, Visse et al. 2006; Shah, Shukla et al. 2009). Through its C-terminal domain, TIMP-1 has been linked to regulation of specific cell signaling pathways. TIMP-1 has been shown to promote cell proliferation (Hayakawa, Yamashita et al. 1992), although the receptor-mediated events involved in this pathway remain unidentified. More recently, TIMP-1 has been associated with inhibition of apoptosis in several human cell lines (Guedez, Stetler-Stevenson et al. 1998; Li, Fridman et al. 1999). TIMP-1 may also increase the abundance of cellular survival and differentiation factors, and this activity has been linked to association of TIMP-1 with cell-surface CD63 and β1 integrin (discussed in section 1.6.2.) (Guedez, Stetler-Stevenson et al. 1998; Berditchevski and Odintsova 1999; Guedez, Mansoor et al. 2001; Jung, Liu et al. 2006). To date, however, the unique activities of the individual TIMP-1 domains have not been well characterized in vivo, and the specific roles of the two domains as they pertain to development remain unknown.

3.1.4. Summary and experimental approach

I have previously preformed a series of domain specific overexpression experiments, which suggested that the TIMP-2 and -3 N- and C-terminal domains may have specific MMP-dependent and independent functions, respectively, during embryogenesis in X. laevis (Chapter 2) (Nieuwesteeg, Walsh et al. 2012). In the present study, I used a similar approach to characterize the unique functions of the TIMP-1 N- and C-terminal domains during early X. laevis development. Here I show that the TIMP-1 C-terminal domain can act autonomously to alter gene expression and MMP levels in X. laevis embryos, and that axial and head defects resulting from C-terminal domain overexpression are different than those observed with the N-terminal domain.
3.2 Materials and Methods

3.2.1. Cloning of X. laevis TIMP-1

*X. laevis* TIMP-1 was cloned using primers designed from unannotated *X. laevis* clone AAI41767.1. Primers used were Forward: 5’-GACAGAAGGACTGCCCAGCC-3’ and Reverse: 5’-CAAAAACACTTCTCCTCGAG-3’. *Xenopus laevis* TIMP-1 was isolated from total cDNA from stage 35 embryos using SuperScript™ Reverse Transcriptase (Life Technologies) with Platinum® Taq DNA Polymerase High Fidelity (Life Technologies). The full-length TIMP-1 amplicon was cloned into the pCR®II-TOPO vector (Life Technologies), and the sequence of full-length *X. laevis* TIMP-1 was confirmed at the Robarts Research Institute DNA Sequencing Facility at the University of Western Ontario, and submitted to Genbank as: KF018236 (see Appendix A for coding sequence).

3.2.2. Comparison of amino acid identity between vertebrate TIMP-1 N- and C-terminal domains

Sequence alignments and amino acid sequence identity were performed using ClustalW2 analysis software from the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/clustalw2/index.html), using default settings. Phylogenetic comparisons were performed using MEGA 5.2.1. software to construct a neighbour-joining tree with a bootstrap of 1000. Accession numbers of known full-length TIMP-1 proteins were as follows: Human (*Homo sapiens*) NP_003245.1; Mouse (*Mus musculus*) AAH51260.1; Rat (*Rattus norvegicus*) EDL97726.1; Cow (*Bos taurus*) AAP44413.1; Horse (*Equus caballus*) NP_001075984.1; Rabbit (*Oryctolagus cuniculus*) AAW79053.1; Shark (*Callorhinchus milii*) AFK11387.1; Newt (*Notophthalmus viridescens*) ABB88702.1.
3.2.3. Animals

Adult *X. laevis* were purchased from Ward’s Natural Science (Rochester, NY), and fertilized, reared and housed as described in section 2.2.2.

3.2.4. Generation of TIMP-1 mRNA constructs for microinjection

PCR was used to generate full-length (T1FL), N-terminal (T1N) or C-terminal (T1C) TIMP-1 HA-tagged constructs using the full-length clone as a template. Briefly, all TIMP-1 constructs (T1FL, T1N, T1C) were HA-tagged on their C-terminal end. The C-terminal domain construct (T1C) was engineered to include its appropriate secretory signal sequence through a two-step PCR process. Primers were as follows: T1FL forward 5’-AGATCTATGTTGTACCTTGTGGTTGTG -3’ and T1FL reverse 5’-CAGTCTGCTGCCACAACACAATAACCCATACGATGTCCAGATTACGCTACTAGT -3’; TIN forward 5’- AGATCTATGTTGTACCTTGTGGTTGTG and T1N reverse GTGTATCGCAAAGCCTGTTCCTACCCATACGATGTCCAGATTACGCTACTAGT-3’; T1C signal sequence forward 5’-AGATCTATGTTGTACCTTGTGGTTGTG-3’ and T1C signal sequence reverse link 5’-CTCAGCCAGAGGTGTGGGTTGCAACATCGTCCCCTGCTAT-3’, and C-terminal domain forward link 5’-TGCAACATCGTCCCCTGCTAT-3’ and C-terminal domain reverse 5’-CAGTCTGCTGCCACAACACAATAACCCATACGATGTCCAGATTACGCTACTAGT-3’. Following PCR amplification, the sequence of all amplicons was verified. All TIMP-1 constructs were subsequently ligated into the EcoRV/SpeI restriction sites of the T7TS plasmid. An mMessage mMachine *in vitro* transcription kit was used with T7 RNA polymerase (Ambion) to synthesize stable capped, poly(A)-tailed mRNA transcripts that
were dissolved in filtered water. mRNA was quantified using Nanovue spectrophotometer (GE), and its integrity was assessed using 1% agarose gel electrophoresis. GFP mRNA was generated and quantified in a similar manner from a proven T7TS-GFP construct plasmid (Walsh, Cooper et al. 2007).

3.2.5. mRNA microinjection

Embryos at the 1 cell stage were injected with 4 ng of TIMP-1 full-length, N-terminal, or C-terminal (T1FL, T1N, T1C) mRNA constructs in a volume of 2.3 nl as described in section 2.2.5. Embryos that were dead or abnormal 2 hr post-injection were removed, and the remaining embryos (representing the 100% being monitored) were observed for phenotypic abnormalities. The percent of normal embryos (no visible defects) was quantified for two days following injection (until stage 30 in development). Mean values were obtained from 3 independent sets of experiments.

3.2.6. Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR analysis was used to examine changes in proteolytic gene expression patterns, or to confirm upregulation of TIMP-1 following injection with T1FL, T1N, or T1C constructs compared to control embryos. Total RNA was extracted from injected and control embryos using RNeasy Mini Kit (Qiagen) and 1 µg RNA was reverse transcribed using qScript™ cDNA Supermix (Quanta Biosciences). PCR was carried out using KAPA Taq PCR Kit (KAPA Biosystems) and Eppendorf Thermal Cycler. The cycling conditions were as follows: initial denaturation, 95°C for 2 min, followed by 28 cycles of the following: denature 95°C 30 sec, anneal 55°C 30 sec, extend 72°C 30 sec. Primers, based on the X. laevis sequences, were as follows: Ef-1α 5’-TGTTGGCAGAGTGAGACTG-3’ and
3.2.7. Protein preparations and Western blotting

Stage 30 embryos injected with T1FL, T1N or T1C mRNA constructs, or uninjected controls, were subject to protein extraction. Briefly, 10 embryos were lysed and sonicated in 100 µl modified RIPA buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10µl/ml Halt™ Protease Inhibitor Cocktail (Thermo Scientific) and 10 µL/ml Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific)) and lysates were centrifuged at 15,000 g for 25 min at 4°C. The supernatant was removed and protein was quantified using BCA protein assay kit (Thermo Scientific) according to manufacturer’s instructions. Electrophoresis and Western blotting was performed as previously described in section 2.2.6. Primary antibodies used were rabbit anti-HA antibody (1:1000 dilution; Santa Cruz) or mouse anti-β-actin antibody (1:1000 dilution; Santa Cruz). Secondary antibodies used were goat anti-rabbit HRP (1:5000 dilution; Life Technologies) or goat anti-mouse HRP (1:5000 dilution; Bio-Rad). Western blots were visualized and photographed using Bio-Rad Quantity One 4.4.0 software.
3.2.8. Zymography and reverse zymography

12.5 µg of protein (as extracted in section 3.2.7.) from T1FL, T1N or T1C injected embryos, or uninjected control embryos was diluted 1:1 with 2X SDS-loading buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 2.5 % glycerol, 1% Bromophenol Blue). For zymography, protein samples were electrophoresed on a 1% gelatin, 10% polyacrylamide gel. For reverse zymography, protein was electrophoresed on a 1% gelatin, 15% polyacrylamide gel co-polymerized with MMP-conditioned media from Hs578t cells according to Hawkes et al. (2010). Hs578t cells were chosen for their ability to secrete high levels of MMPs, particularly MMP-2 and -9. For both zymography and reverse zymography, gels were electrophoresed in 1X zymogram running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS), followed by in-gel renaturation for 30 min at room temperature in renaturing solution (2.5%Triton X-100). Substrate cleavage was carried out by incubating gels in zymogram developing buffer (pH 7.5, 50 mM Tris, 200 mM NaCl, 5 mM CaCl₂ (anhydrous), 0.02% Brij-35) for 48 hr at 37°C. For zymography, MMP activity was visualized as clear bands against blue background after staining with 0.5% Coomassie blue (Bio-Rad). For reverse zymography, TIMP activity was visualized as dark bands against light background after staining with 0.5% Coomassie blue (Bio-Rad). Gels were visualized and photographed, and densitometry was performed using Bio-Rad Quantity One 4.4.0 software.

3.2.9. Statistical analysis

All statistical analysis was performed using the IBM SPSS Statistic 19 program. Results were presented as mean ± SE. Statistical significance was determined using one-way
ANOVA variance analysis and Dunnett’s multiple comparisons tests. Differences were considered statistically significant when \( p < 0.05 \).

### 3.3 Results

#### 3.3.1. Amino acid sequence conservation of vertebrate TIMP-1 N- and C-terminal domains

A direct amino acid sequence comparison of the individual TIMP-1 N- and C-terminal domains between species has not previously been reported. Current full-length TIMP-1 protein sequences were identified, which thus far have only been confirmed in vertebrates. To investigate whether the unique functions of these domains may be concurrent with their patterns of evolutionary conservation, I cloned *X. laevis* TIMP-1 (GenBank: KF018236) and compared its N- and C-terminal domains with known mammalian and non-mammalian TIMP-1 sequences. The N-terminal domain of *X. laevis* TIMP-1 was more highly conserved than its C-terminal domain with 7 of 9 vertebrate species analyzed (Fig. 3.1 A). In general, the N-terminal domains of all species were more highly conserved than the C-terminal domains, with the exception of horse and cow, where the C-terminal domains were more highly conserved between species (Fig. 3.1 A). Phylogenetic analysis of TIMP-1 N- and C-terminal domains with other vertebrate species showed the two domains follow a different evolutionary trend, as the branching patterns of the two trees differed, indicating that both domains evolved under different selective pressures (Fig. 3.1 B and C).
Figure 3.1. Evolutionary conservation of TIMP-1 N- and C-terminal domains differed. (A) Sequence analysis comparing amino acid sequence identity of *X. laevis* TIMP-1 N- and C-terminal domains versus known vertebrate species. Light purple boxes represent species whose N-terminal domains were more highly conserved with *X. laevis* TIMP-1 than their C-terminal domains. Light pink boxes represent species whose C-terminal domains were more highly conserved with *X. laevis* TIMP-1 than their N-terminal domains. Blue boxes represent species with equal conservation with both *X. laevis* TIMP-1 N- and C-terminal domains. (B) Phylogram representation of sequence divergence among TIMP-1 N-terminal domains as generated by MEGA 5.2.1. software. (C) Phylogram representation of sequence divergence among TIMP-1 C-terminal domains as generated by MEGA 5.2.1. software.
A  TIMP-1  Xenopus  Human  Mouse  Rat  Cow  Rabbit  Horse  Shark  Newt

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B  TIMP-1 N-terminal domains

C  TIMP-1 C-terminal domains
3.3.2. TIMP-1 mRNA and protein was upregulated in X. laevis embryos following ectopic expression of TIMP-1 constructs

To characterize the unique roles of the TIMP-1 N- and C-terminal domains during development, I have performed a series of domain specific overexpression experiments. For this purpose, I generated full-length, N-terminal and C-terminal HA-tagged TIMP-1 mRNA constructs (T1FL, T1N and T1C, respectively), which were injected into newly fertilized X. laevis embryos (1 cell stage). Total RNA was collected from injected and control embryos 1 day post-fertilization (stage 15), and RT-PCR analysis was used to verify levels of TIMP-1 mRNA transcripts in injected embryos compared to controls (Fig. 3.2 A). Uninjected control embryos contained much lower levels of TIMP-1 mRNA compared to T1FL, T1N or T1C injected embryos, which showed relatively equal but elevated levels of TIMP-1 relative to Ef-1α (loading control; Fig. 3.2 A). By two days post-fertilization (stage 30), each HA-tagged TIMP-1 construct was still present and detectable on Western blot via HA antibody in injected embryos (Fig. 3.2 B), indicating that all constructs produced stable protein products.
Figure 3.2. Confirmation of full-length, N-terminal or C-terminal TIMP-1 constructs overexpressed in X. laevis embryos. 4 ng of mRNA coding for full-length (T1FL), N-terminal (T1N) or C-terminal (T1C) TIMP-1 constructs was microinjected into X. laevis embryos at the 1 cell stage. (A) Overexpression of TIMP-1 mRNA as shown by RT-PCR analysis. mRNA was isolated from stage 15 embryos. Control (uninjected) embryos expressed TIMP-1 at very low levels. Primers specific to each construct were used to confirm mRNA levels of T1FL (678 bp), T1N (453 bp) and T1C (252 bp). Ef-1α loading control is shown below (479 bp). (B) Levels of TIMP-1 constructs as detected by Western blot analysis. All constructs are HA-tagged. Protein was isolated from stage 30 embryos. Anti-HA antibodies were used to confirm expression of each construct at the protein level (T1FL = 26KDa, T1N = 18KDa, T1C = 12 KDa). No HA was detected in control uninjected embryos. β-actin was used as protein loading control.
A

(\text{Ef-1}\alpha)

B

CONT T1FL T1N T1C

26 KDa
18 KDa
12 KDa

Actin (42 KDa)
3.3.3. Overexpression of full-length, N-terminal and C-terminal TIMP-1 constructs produced unique developmental phenotypes

Following injection of each TIMP-1 construct, embryos were monitored for gross morphological changes in development for 2 days post-fertilization (until developmental stage 30). Uninjected control embryos were morphologically normal (Fig. 3.3 A). By stage 30 in development, embryos overexpressing full-length (T1FL) or N-terminal (T1N) TIMP-1 displayed relatively normal anterior (head) development with posterior axis defects. T1FL and T1N injected embryos did, however, display a bent axis phenotype along with truncated anterior/posterior axes compared to controls (Fig. 3.3 B and C, respectively). Embryos overexpressing C-terminal TIMP-1 (T1C) also had truncated axis defects compared to controls, as well as other developmental abnormalities. Chiefly, T1C injections resulted in head defects, and failure of the neural tube to close (Fig. 3.3 D; see appendix C for additional images). Generally, overexpression of T1C resulted in more severe phenotypic consequences than overexpression of T1FL or T1N constructs.
Figure 3.3. Phenotypic effects of overexpression of full-length, N-terminal or C-terminal TIMP-1 constructs. Following injection, photographs were taken of representative embryos at stage 30. (A) Control (uninjected) embryos were phenotypically normal. Microinjection of T1FL (B) and T1N (C) constructs both resulted in normal anterior (head) development but truncated posterior axes. (D) Microinjection of T1C constructs resulted in lack of head structures, other head defects, and neural tube closure failure. Panel A magnification is lower than in B C and D. Actual size of all embryos is 1 mm in diameter. Images are of representative embryos where results were consistent in at least 3 independent sets of experiments, and a minimum of 100 embryos were injected and monitored for each replicate. See appendix C for additional images.
3.3.4. Overexpression of full-length and C-terminal TIMP-1 resulted in increased embryonic lethality compared to overexpression of N-terminal TIMP-1

To compare and measure the effects of overexpressing full-length vs. N-terminal or C-terminal domain TIMP-1 during X. laevis development, T1FL, T1N and T1C injected embryos were monitored for 48 hours following fertilization, and the percent of normal embryos (embryos that were alive and morphologically normal) was determined and quantified at stage 15 (1 day post-fertilization) and stage 30 (2 days post-fertilization). More than 90% of control embryos, or embryos injected with GFP mRNA, developed normally during this time period (Fig. 3.4). Overexpression of all TIMP-1 constructs resulted in increased lethality compared to controls. By stage 15, all constructs resulted in an approximate 20% decrease in normal embryo numbers compared to controls (Fig. 3.4). By stage 30, 66% of T1N injected embryos developed normally, whereas T1FL and T1C constructs were slightly more detrimental, with only 58% and 55% normal development, respectively (Fig. 3.4).
Figure 3.4. Overexpression of all three TIMP-1 constructs led to abnormal development and death. Following injection of mRNA constructs at the 1 cell stage, embryos were scored for a normal (no visible defect) phenotype at stage 15 and 30. Dead and abnormal embryos were counted as containing morphological defects. The graph shows the percentage of normal embryos following injection of GFP mRNA (GFP), or full-length (T1FL), N-terminal (T1N) or C-terminal (T1C) TIMP-1 mRNA constructs at the given stages. Control embryos are uninjected. Results are based on 3 independent sets of experiments, bars indicate SE.
3.3.5. Overexpression of full-length, N-terminal or C-terminal TIMP-1 constructs in X. laevis embryos altered expression of proteolytic genes

To examine the roles of full-length, N-terminal or C-terminal TIMP-1 in regulating ECM remodeling in vivo, I investigated the expression patterns of hallmark genes involved in regulating ECM proteolysis following microinjection with T1FL, T1N or T1C constructs into X. laevis embryos. mRNA was collected from embryos at stage 30 in development, as I had observed specific and unique morphological defects at this time point following injection of each construct (see Fig. 3.3). Semi-quantitative RT-PCR analysis, was used to assay for changes in mRNA levels of the MMP inhibitors TIMP-2, TIMP-3 and RECK, as well as the MMPs MMP-2, -9 and MT1-MMP. All of these genes have previously been shown to be important regulators of ECM remodeling during development (Hasebe, Hartman et al. 2007; Chandana, Maeda et al. 2010; Monsonego-Ornan, Kosonovsky et al. 2012; Nieuwesteeg, Walsh et al. 2012). mRNA levels were normalized to Ef-1α and compared to control (uninjected) embryos.

Overexpression of T1FL and T1C significantly decreased TIMP-2 mRNA relative to controls (Fig. 3.5 A, p < 0.05). This decrease was most significant following overexpression of T1C (p < 0.01); whereas, T1N did not significantly alter TIMP-2 compared to control embryos. Levels of TIMP-3 mRNA were not significantly altered compared to control embryos following treatment with of any of the TIMP-1 constructs (Fig. 3.5 B). RECK expression was significantly decreased relative to controls following injection of T1FL (p < 0.05) and T1C (p < 0.01, Fig. 3.5 C) constructs.

MMP-2 mRNA levels were significantly decreased compared to controls following overexpression of all constructs (T1FL, T1N and T1C; Fig. 3.6 A, p < 0.01). Following injection of T1C, MMP-9 mRNA was significantly decreased (Fig. 3.6 B, p < 0.05). No
significant change in MMP-9 mRNA levels was observed in embryos injected with T1FL or T1N constructs (Fig. 3.6). Similarly, levels of MT1-MMP mRNA were not altered relative to control embryos following overexpression of any TIMP-1 constructs (Fig. 3.6 C).
Figure 3.5. Effect of overexpression of full-length, N-terminal or C-terminal TIMP-1 on mRNA levels of MMP inhibitors. Semi-quantitative RT-PCR analysis was used to measure changes in mRNA levels of (A) TIMP-2, (B) TIMP-3 and (C) RECK at stage 30, following microinjection of 4 ng of TIMP-1 full-length (T1FL), N-terminal (T1N), or C-terminal (T1C) constructs into X. laevis embryos at the 1 cell stage. In each case mRNA levels were measured relative to Ef-1α. The results are presented as mean ± SE from 3 independent experiments. Asterisks represent a significant difference of * $p < 0.05$ and ** $p < 0.01$, all versus control (CONT; uninjected) embryos, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test.
Figure 3.6. Effect of overexpression of full-length, N-terminal or C-terminal TIMP-1 on mRNA levels of MMPs. Semi-quantitative RT-PCR analysis was used to measure changes in mRNA levels of (A) MMP-2, (B) MMP-9 and (C) MT1-MMP at stage 30, following microinjection of 4 ng of TIMP-1 full-length (T1FL), N-terminal (T1N), or C-terminal (T1C) constructs into X. laevis embryos at the 1 cell stage. In each case mRNA levels were measured relative to Ef-1α. The results are presented as mean ± SE from 3 independent experiments. Asterisks represent a significant difference of * p < 0.05 and ** p < 0.01, all versus control (CONT; uninjected) embryos, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test.
**A**

Expression relative to EF-1α

- **MMP-2**
  - CONT: 30
  - T1FL: 5
  - T1N: 5
  - T1C: 5

**B**

Expression relative to EF-1α

- **MMP-9**
  - CONT: 40
  - T1FL: 10
  - T1N: 10
  - T1C: 10

**C**

Expression relative to EF-1α

- **MT1-MMP**
  - CONT: 50
  - T1FL: 50
  - T1N: 50
  - T1C: 50
3.3.6. Overexpression of all TIMP-1 constructs decreased the amount of active MMP-2 and -9

To further understand how the individual TIMP-1 domains affect the proteolytic network *in vivo*, gelatin zymography was used to measure changes in the amounts of active MMP-2 and -9 in T1FL, T1N or T1C injected embryos relative to control (uninjected) embryos. Embryos injected with T1FL, T1N and T1C constructs showed significantly decreased levels of active MMP-2 relative to control embryos (Fig. 3.7 A, *p* < 0.01). Injection of all three TIMP constructs also resulted in significantly decreased levels of active MMP-9 relative to controls (Fig. 3.7 B, *p* < 0.01); however, interestingly, this trend was most pronounced following injection of the T1C construct.
Figure 3.7. Zymography demonstrated altered levels of active MMP-2 and MMP-9 following overexpression of TIMP-1 constructs. Embryos were injected at the 1 cell stage with 4 ng of mRNA coding for full-length (T1FL), N-terminal (T1N) or C-terminal (T1C) constructs, and protein was isolated from stage 30 embryos. Gelatin zymography was used to measure changes in (A) MMP-2 and (B) MMP-9 activity. Zymogram (top) is representative of one experiment. Graphs represent quantification zymograms, and data is presented as mean ± SE from 3 independent experiments. ** p < 0.01, all versus control (CONT; uninjected) embryos, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test.
A

MMP-2 (63 KDa)

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<th>T1N 30</th>
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MMP activity relative to controls

B

MMP-9 (84 KDa)

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MMP activity relative to controls

**
3.3.7. *Only full-length and N-terminal TIMP-1 constructs directly inhibited MMP activity*

To validate the MMP-inhibitory activity of the full-length and N-terminal TIMP-1 constructs, and also to confirm that the C-terminal TIMP-1 construct could not directly inhibit MMPs, I performed reverse zymography. Reverse zymography is a modification of traditional zymography by incorporating gelatin into the gel matrix as well as conditioned media from cancer cells as a source of MMP-2 and -9 activity. Subsequently, the proteins of interest are separated within the gel by molecular weight. The gelatin is degraded by MMPs in the matrix, except for where inhibited by active TIMPs. TIMP activity is visualized as a dark band after staining with Coomassie Blue (Hawkes, Li et al. 2010), representing a protected band corresponding to the molecular weight of the active construct. Protein was isolated from injected stage 30 embryos, and analyzed for MMP-inhibitory activity corresponding to T1FL, T1N and T1C construct sizes. T1FL and T1N constructs showed MMP-inhibitory activity at the expected sizes of 26 KDa and 18 KDa, respectively (Fig. 3.8, white arrows), whereas the T1C construct was not able to inhibit MMPs at the expected size of 12 KDa (Fig. 3.8, black arrow). Control embryos showed a faint band corresponding to endogenous TIMP-1 (26 KDa), which was upregulated in T1FL injected embryos (Fig. 3.8). Higher molecular weight bands are not indicative of TIMP activity, but represent proteins that are present at sufficiently high concentrations to be stained by Coomassie Blue, due to the large amount of yolk present in *X. laevis* embryos (Hawkes, Li et al. 2010).
Figure 3.8. Reverse zymography demonstrated full-length and N-terminal, but not C-terminal TIMP-1, can directly inhibit MMP activity. Embryos were injected at the 1 cell stage with 4 ng of mRNA coding for full-length (T1FL), N-terminal (T1N) or C-terminal (T1C) TIMP-1 constructs, and protein was isolated from stage 30 embryos. MMP-inhibitory activity is represented by dark bands. White arrows indicate the location of the expected inhibitory bands corresponding to the T1FL (26 KDa) and T1N (18 KDa) constructs, respectively. A black arrow represents the position of the T1C (12 KDa) construct, which cannot inhibit MMP activity as demonstrated by the presence of a light band. Higher non-specific molecular weight bands at the top of the gel help demonstrate equal protein loading. Such bands are not indicative of TIMP activity but are the result of high concentrations of proteins that stain deeply with Coomassie Blue. This is seen with many complex tissues (Van Wart and Birkedal-Hansen 1990) and is due to the very abundant yolk and other storage proteins that are found in the embryo.
3.4 Discussion

ECM remodeling is a key process involved in regulating tissue growth and morphogenesis during development. Maintaining the appropriate balance between MMPs and their inhibitors (TIMPs and RECK) is absolutely essential for normal development, and disruption of this balance leads to defects in neurulation, organogenesis and angiogenesis (Liotta, Steeg et al. 1991; Blelloch and Kimble 1999; Monsonego-Ornan, Kosonovsky et al. 2012). Our understanding of the regulation of this proteolytic network has been convoluted by the discovery that TIMP proteins have both MMP-inhibitory activity as well as MMP-independent cell signaling activity (Lambert, Dasse et al. 2004). With recent research on TIMP C-terminal domains demonstrating their roles in apoptosis, cell proliferation, and cell migration pathways, the functions of the TIMP C-terminal domains are becoming increasingly studied in vitro (Hayakawa, Yamashita et al. 1992; Gudez, Stetler-Stevenson et al. 1998; Li, Fridman et al. 1999; Oh, Seo et al. 2004). At the present time there has been comparatively little research characterizing the roles of the N- and C-terminal TIMP domains in vivo, particularly as they pertain to development. My research has previously examined the specific functions of the N- and C-terminal domains of TIMP-2 and -3 during X. laevis development, and found that the two domains have unique functions when overexpressed individually in X. laevis embryos (Chapter 2) (Nieuwesteeg, Walsh et al. 2012). To date, there has been no comparison of the unique functions of TIMP-1 N- and C-terminal domains in a developmental context. Here I used mRNA overexpression to examine the role of full-length TIMP-1 versus the roles of its individual N-terminal and C-terminal domains during early X. laevis development.
3.4.1. **TIMP-1 N-terminal domains were more highly conserved with other vertebrates than their C-terminal domains**

To examine whether the distinct functions of the two TIMP-1 domains are reflected in their evolutionary conservation, I identified and compared amino acid sequence identities of the *X. laevis* TIMP-1 N- and C-terminal domains to other known vertebrate TIMP-1 sequences. I found that the TIMP-1 N-terminal domains were more highly conserved than their C-terminal domains among most species analyzed, with the exception of the large mammals, horse and cow, in which the C-terminal domains were more highly conserved (Fig. 3.1 A). Since the catalytic domains of MMPs are highly conserved between species, it is not surprising that TIMP-1 N-terminal MMP-inhibitory domains are also well conserved (Van Wart and Birkedal-Hansen 1990). I have previously shown that the TIMP-2 N-terminal domains were also more highly conserved across species than their C-terminal domains, whereas for TIMP-3, I found the C-terminal domains were more highly conserved (Chapter 2, sections 2.3.1. and 2.3.2.) (Nieuwesteeg, Walsh et al. 2012). My findings suggest TIMP-1 may behave more like TIMP-2 than TIMP-3 with respect to MMP inhibition. Indeed, TIMP-3 is unique in that it is a good inhibitor of the ADAM family of proteases (Rapti, Atkinson et al. 2008). Additionally, TIMP-3 is sequestered away from the cell surface, whereas TIMP-1 and -2 function in a pericellular manner (Yu, Yu et al. 2000). While the TIMP-1 N-terminal domain is more conserved than the C-terminal domain, phylogenetic analysis of sequence divergence between vertebrate N- and C-terminal domains showed that the evolutionary patterns are different, suggesting that both domains evolved under unique selective pressures (Fig. 3.1 B and C). This suggests that functional roles of each domain are not the same.
3.4.2. Overexpression of the TIMP-1 C-terminal domain resulted in detrimental developmental defects

To examine differences in the in vivo roles of the TIMP-1 N- and C-terminal domains I generated and overexpressed full-length, N- and C-terminal TIMP-1 mRNA constructs in X. laevis embryos (T1Fl, T1N an T1C, respectively). Injection of embryos with T1C resulted in more embryonic death than injection with T1FL or T1N (Fig. 3.4). Consistent with this observation, overexpression of T1C also resulted in more severe developmental defects in embryos at stage 30 in development, with these embryos showing head and neural tube defects, as well as truncated axes. In comparison, T1FL and T1N injected embryos showed normal head development but truncated anterior-posterior axes (Fig. 3.3), indicating that the TIMP-1 C-terminal domain may contribute to unique functions in development separate from the N-terminal domain.

3.4.3. The TIMP-1 C-terminal domain altered the ECM proteolytic network independent of MMP inhibition

In order to further investigate the roles of the two TIMP-1 domains, RT-PCR analysis was used to examine changes in mRNA levels of associated genes known to be involved in the regulation of ECM remodeling. No changes were detected in levels of TIMP-3 or MT1-MMP mRNA relative to control embryos, following injection with full-length, N-terminal or C-terminal TIMP-1. This is consistent with previous work, which showed that association of TIMP-1 with MT1-MMP is exceptionally poor (Hamze, Wei et al. 2007). In contrast, TIMP-2 and RECK mRNA decreased compared to controls following injection of embryos with T1FL or T1C; however, these decreases were most significant with T1C (Fig. 3.5). This supports the concept that the C-terminal domain has the ability to regulate developmental
events independent from the N-terminal domain, presumably through cell surface receptor-mediated signaling pathways.

Similarly, overexpression of T1C resulted in the most marked decreases in amounts of \textit{MMP}-2 and -9 mRNA compared to control embryos; although, in the case of \textit{MMP}-2, ectopic expression of all three constructs significantly decreased \textit{MMP}-2 mRNA (Fig. 3.6). These results were consistent with the zymography data, which showed the amounts of active MMP-2 and -9 proteins followed the same trends as the RT-PCR analysis (Fig. 3.7). Decreases in MMP-2 and -9 protein and mRNA may be partially due to a regulatory feedback mechanism in the proteolytic network which occurs from direct catalytic inhibition of MMPs by the N-terminal TIMP-1 domain, as T1N overexpression significantly decreased \textit{MMP}-2 mRNA and MMP-2 protein compared to control embryos. My results suggest, however, that the C-terminal domain of TIMP-1 has the ability to regulate MMP mRNA and protein levels independent from the N-terminal domain. Reverse zymography using MMP-conditioned media from Hs578t cells showed that TIC constructs cannot directly inhibit MMP activity (Fig. 3.8), yet overexpression of T1C resulted in the most significant decrease in MMP-9 mRNA as well as a pronounced decrease in MMP-9 protein compared to control embryos (Fig. 3.6 and 3.7). Although the mechanism of action of the TIMP-1 C-terminal domain is still unknown, TIMP-1 has previously been shown to bind to cell surface receptors on both fibroblasts and MCF-7 breast cancer cells, which resulted in translocation of TIMP-1 into the cell (Zhao, Li et al. 1998; Hamze, Wei et al. 2007). Taken together with the emerging discoveries of new cell surface binding partners for TIMP-1, and its MMP-independent roles in cell signaling (Stetler-Stevenson 2008), it is possible that the C-terminal domain of TIMP-1 may function to regulate expression of proteolytic genes through a signaling mechanism that is separate from the N-terminal domain MMP-inhibitory activity.
3.4.4. Concluding remarks

In summary, this research characterized *in vivo* the unique role of the TIMP-1 C-terminal domain. *In vitro* studies have demonstrated new roles for the TIMP C-terminal domains in cell signaling. Additionally, disease models have highlighted the emerging significance of the C-terminal TIMP domains in regulating the proteolytic network, as synthetic MMP inhibitors designed to mimic TIMP N-terminal domains failed as an anti-cancer therapy to block metastasis associated with upregulated MMP activity (Brown 1998). Here I have shown for the first time *in vivo* that the TIMP-1 C-terminal domain has an independent role in regulating the proteolytic network during development, at a time when this network is both complex and tightly regulated. I have no direct evidence as to the mechanisms through which the individual ectopic domains are manifesting developmental anomalies; however, there is evidence that feedback is involved as disruption of the proteolytic network was compensated for in the embryo by changes in the levels of key genes. Further, while there is no evidence that nascent TIMP-1 is cleaved into its individual domains, my research suggests that the functions of the TIMP-1 N- and C-terminal domains depend on the affinities of the individual domains for their binding partners, as well the stoichiometric levels of TIMP-1 relative to secreted MMPs and cell surface receptors. Further *in vivo* studies are needed to fully investigate whether there is preferential binding of either domain in the context of abundant active MMPs and/or cell surface receptors.
3.5 References


CHAPTER 4

ANALYSIS OF THE EFFECTS OF TIMP-1, -2 AND -3 N- AND C-TERMINAL DOMAINS ON SIGNALING MARKERS DURING *X. LAEVIS* DEVELOPMENT
4.1 Introduction

Although TIMPs were originally identified and characterized based on their abilities to bind to and inhibit MMPs, it is now acknowledged that TIMPs are multifunctional proteins, which can have pleiotropic effects on ECM remodeling and cell behavior. While the TIMP N-terminal domains have been shown to impede MMP activity in many cell types and model organisms, their C-terminal domains have demonstrated cell signaling activity in specific cell lines in vitro. Characterization of TIMP C-terminal domain function has been difficult as their roles in cell signaling vary, not only between the different TIMPs, but also depending on the cell line under study (Stetler-Stevenson 2008). The seemingly contradictory roles of TIMPs in up-regulating or down-regulating apoptosis and cell proliferation pathways indicates that TIMPs may have cell or tissue-specific roles, where TIMP function may depend on both the availability of cell surface receptors and the stoichiometry of free TIMPs and MMPs within the ECM.

4.1.1. TIMPs in cell signaling

As discussed in previous chapters, in vitro studies have linked TIMPs to induction or inhibition of apoptosis and cell proliferation pathways, as well as to upregulation of the cell surface MMP inhibitor, RECK. TIMP-1 in particular has been shown to inhibit apoptosis in Burkitt’s lymphoma cell lines, and this inhibition was demonstrated to occur through the TIMP-1 C-terminal domain binding to the CD63-β1-integrin complex in MCF10A cells (section 1.6.2) (Guedez, Stetler-Stevenson et al. 1998; Berditchevski and Odintsova 1999; Liu, Taube et al. 2005; Jung, Liu et al. 2006). TIMP-1 has also been shown to have both cell growth promoting and cell growth inhibiting activities in various cell lines, which are independent from N-terminal domain-mediated inhibition of MMPs (Bertaux, Hornebeck et
Likewise, there are several conflicting reports in the literature regarding the role of TIMP-2 in mediating cell growth and apoptosis pathways. In the MCF-7 breast cancer cell line TIMP-2 had demonstrated cell growth promoting activity, which occurred through association of the TIMP-2 C-terminal domain with MT1-MMP on the cell surface to activate the MAPK pathway (section 1.6.2.) (D'Alessio, Ferrari et al. 2008). In contrast, TIMP-2 inhibited cell growth through binding to α3β1 integrin receptors, leading to upregulation of the cyclin dependent kinase inhibitor p27kip1 in cultured human endothelial cells. Interestingly, this association also led to increased expression of RECK, and subsequently decreased cell migration (section 1.6.2.) (Oh, Seo et al. 2004; Seo, Li et al. 2006). The role of TIMP-2 in regulating apoptosis is also unclear, and varies between different cell types. Whereas TIMP-2 has been shown to suppress apoptosis in melanoma cells, in human T-lymphocytes TIMP-2 promoted apoptosis (Valente, Fassina et al. 1998; Lim, Guedez et al. 1999).

In contrast to TIMP-1 and -2, TIMP-3 is sequestered in the ECM and consequently, has limited direct ability to participate in cell surface signaling events (discussed in section 1.6.2.) (Stetler-Stevenson 2008). However, as previously mentioned, TIMP-3 is a good inhibitor of ADAM family proteases, particularly ADAM-17 (also known as TACE (TNF-α converting enzyme)). ADAM-17 facilitates the shedding of transforming growth factor-α (TNF-α) as well as various cell surface receptors including FAS and TNF-related apoptosis inducing ligand receptor 1 (TRAIL-R1) (Pavloff, Staskus et al. 1992; Black 2002). Thus, although TIMP-3 has been associated with some cellular signaling pathways, particularly with changes in apoptosis, this activity is thought to occur indirectly as a result of ADAM inhibition, rather than as a result of direct signaling activity (Stetler-Stevenson 2008).
4.1.2. TIMPs in vivo

*In vivo*, TIMP knockout mouse models display developmental deficiencies, demonstrating the importance of TIMPs in regulating normal development. In particular, TIMP-1 and -2 specifically, have been associated with defects in brain and neural development. *TIMP-1* null mice had diminished neuronal development and impaired learning and memory (Jourquin, Tremblay et al. 2005; Chaillan, Rivera et al. 2006), while *TIMP-2* null mice showed decreased neurite outgrowth, delayed neuronal differentiation and considerable motor dysfunction (Perez-Martinez and Jaworski 2005; Jaworski, Soloway et al. 2006), though the precise mechanisms through which these defects manifested remain unclear. In contrast, *TIMP-3* null mice predominantly showed increased apoptosis in cells of the mammary glands, and defective alveolar and lung development (Fata, Leco et al. 2001; Leco, Waterhouse et al. 2001), both of which have been attributed to enhanced MMP activity (Brew and Nagase 2010). In *X. laevis*, ectopic expression of *TIMP-3* has been associated with perturbation of head and neural structures (Pickard and Damjanovski 2004), a finding that I have corroborated using full-length and N-terminal domain TIMP-3 constructs (but not C-terminal TIMP-3), also indicating that the role of TIMP-3 in development is dependent on its MMP-inhibitory activities (Chapter 2) (Nieuwesteeg, Walsh et al. 2012).

4.1.3. Experimental approach

Due to the conflicting roles of the TIMP C-terminal domains in different cell lines *in vitro*, there is a need to further examine TIMP domain function *in vivo* to determine whether TIMP-mediated signaling activities are conserved between whole organisms and cell culture models. There is also a need to improve our understanding of how the activities of the two TIMP domains are balanced within the complex ECM proteolytic network *in vivo*. I have
previously shown that during development, the *X. laevis* TIMP-1 and -2 C-terminal domains can regulate MMP amounts and activity levels independent of N-terminal domain MMP-inhibitory activity (Chapters 2 and 3), indicating a unique role for the TIMP C-terminal domains in regulating the stoichiometry of molecules within the ECM proteolytic network. TIMPs have been associated with apoptosis, cell proliferation, and migration (RECK) pathways *in vitro*, and as these pathways are all important regulators of developmental events, I wanted to investigate whether the TIMP C-terminal domains also have the ability to alter these pathways during development. As *TIMP-1*, -2 and -3 have all been linked to neural development in other *in vivo* studies, and my previous research has shown that overexpression of many of my TIMP constructs disrupted normal head and neural development (see sections 2.3.4., 2.3.5., and 3.3.3.), in this chapter I have specifically examined head histological sections of embryos injected with either full-length, N-terminal, or C-terminal domain *TIMP-1*, -2 or -3 constructs. Due to the severe nature of the developmental defects in many of the TIMP injected embryos, it was difficult to identify specific head structures. Accordingly, anterior sections from embryos at developmental stage 30 (organogenesis) were examined using immunohistochemistry (IHC). Using IHC I assayed for changes in markers of cell proliferation and apoptosis, as well as for changes in RECK protein levels. While analysis of changes in anterior expression could be used to confirm the known important neural and head roles played by TIMPs, a comparison of relative levels of signaling markers in specific tissues could not be compared between these deformed embryos. Therefore, I also collected whole embryo protein lysates from injected embryos and used Western blot analysis to quantify and compare changes in levels of proliferation and apoptosis markers, as well as in RECK protein levels. The purpose of this research was to compare the effects of the TIMP C-terminal domain constructs to their respective N-terminal
domains, and to demonstrate whether the TIMP C-terminal domains have any unique abilities to alter these signaling pathways in vivo. Specifically, changes in apoptosis were assayed using active caspase-3 antibody, proliferation via a phosho-histone-3 (PH3) antibody, and RECK protein levels using a RECK antibody.

4.2 Materials and Methods

4.2.1. Animals

Adult X. laevis were purchased from Xenopus I Inc (Dexter, MI), and fertilized, reared and housed as described in section 2.2.2.

4.2.2. Generation of TIMP constructs for microinjection

Briefly, PCR was used to produce HA-tagged full-length, N-terminal and C-terminal domain constructs for TIMP-1, -2 and -3. Details regarding TIMP construct generation can be found in section 2.2.4. for TIMP-2 and -3, and section 3.2.4. for TIMP-1.

4.2.3. Microinjection of TIMP-1, -2 and -3 mRNA constructs in X. laevis embryos

Fertilized embryos at the 1 cell stage were microinjected with TIMP-1, -2 or -3 full-length, N-terminal or C-terminal domain mRNA constructs. Microinjections were preformed as previously described in sections 2.2.5. and 3.2.5. Embryos were monitored until developmental stage 30 (2 days post-fertilization).

4.2.4. Protein preparations and Western blotting

Protein was extracted from stage 30 embryos injected with the various TIMP constructs, or uninjected control embryos. Protein extraction, quantification and Western
blotting were performed from 10 pooled embryos from 3 independent experiments, as previously described in section 3.2.7. Primary antibodies used were RECK (H-300) (rabbit polyclonal, 1:200 dilution; Santa Cruz), p-Histone H3 (ser-10) (rabbit polyclonal, 1:200 dilution; Santa Cruz), anti-active caspase-3 (rabbit polyclonal, 1:500 dilution; Abcam), and anti-β-actin (C4) (mouse monoclonal, 1:1000 dilution; Santa Cruz). Secondary antibodies used were goat anti-rabbit HRP (1:5000 dilution; Life Technologies) or goat anti-mouse HRP (1:5000 dilution; Bio-Rad). Western blots were visualized and photographed using Bio-Rad Quantity One 4.4.0 software. Densitometry was performed using ImageJ software, where levels of PH3, RECK or caspase-3 were standardized to β-actin, and plotted as mean ± SE based on 3 independent experiments.

4.2.5. Immunohistochemistry and fluorescence microscopy

Stage 30 embryos injected with TIMP-1, -2 or -3 full-length, N-terminal or C-terminal mRNA constructs, or control (uninjected) embryos were fixed for 2 hr at room temperature in 3.7% paraformaldehyde in 1X phosphate buffered saline (PBS). Fixed embryos were dehydrated in 70% ethanol and sectioned at the Molecular Pathology Core Facility in Robarts Research Institute (London, ON). Embryos were embedded in paraffin, sectioned at a thickness of 6 µm, and mounted on glass slides. Slides were washed in xylene for 15 min to deparaffinize the sections, and then gradually rehydrated through a series of 5 min washes in 100% ethanol, 90% ethanol + 10% PBST (1x phosphate-buffered saline + 0.1% Triton-X (Sigma)), and 80% ethanol + 20% PBST, followed by two 5 min washes in 100% PBST. Sections were blocked with 10% goat serum in 1X PBST for 45 min at room temperature. Note: For sections labeled with RECK antibody, 1X PBS was used in place of 1X PBST for all washes described above. Immunostaining was performed by incubating
sections with primary antibodies against RECK (H-300) (rabbit polyclonal; Santa Cruz), p-Histone H3 (ser-10) (rabbit polyclonal; Santa Cruz), or anti-active caspase-3 (rabbit polyclonal; Abcam). All primary antibodies were used at a 1:50 dilution and incubated overnight at 4 °C. Caspase-3 and PH3 antibodies were diluted in 2% BSA in PBST, and RECK antibody was diluted in 2% BSA in PBS. Slides were washed 3 times for 5 min in PBST (PBS for RECK labeled sections), and incubated for with secondary antibody at a 1:200 dilution for 2 hr at room temperature (Alexa Fluor® 488 Goat Anti-Rabbit IgG; Life Technologies). Secondary antibodies were diluted in 2% BSA in PBST for caspase-3 and PH3 labeled sections, and in 2% BSA in PBS for RECK labeled sections. Sections were washed 3 times for 5 min in PBST (PBS for RECK labeled sections), followed by one 5 min wash in double-distilled H2O. All sections were mounted and counterstained with ProLong® Gold antifade reagent containing 4', 6-diamidino-2-phenyindole (DAPI) (Life Technologies). Anterior head portions of embryos in a 200X field of view were visualized and photographed using a Leica DMI600 B microscope and Leica MM AF 1.4.0 software. ImageJ software was used to measure PH3, caspase-3 or RECK signal relative to DAPI (indicative of total number of cells) in anterior head sections of injected embryos all the same magnification (200X). Immunohistochemistry and quantification of fluorescence are representative measurements based on 1 image, with 4 consistent technical repeats for each embryo imaged. Results were consistent and observed in no less than 2 embryos.

4.2.6. Statistical Analysis

All statistical analysis was performed using the IBM SPSS Statistic 19 program. Results were presented as mean ± SE. Western blot data were log transformed (Log10) to achieve normal distribution. Statistical significance was determined using One Way ANOVA
variance analysis followed Dunnett’s multiple comparisons test. Differences were considered statistically significant when $p < 0.05$.

4.3 Results

4.3.1. Overexpression of full-length, N-terminal and C-terminal TIMP constructs did not alter PH3 levels in X. laevis embryo sections or protein lysates

PH3 was used as an indicator of cell proliferation in order to characterize the roles of the TIMP-1, -2 and -3 N- and C-terminal domains in regulating this pathway during development. Immunostaining of anterior head sections from stage 30 embryos showed that overexpression of full-length, N-terminal or C-terminal domain TIMP-1 resulted in relatively equal levels of PH3 compared to control embryos (Fig. 4.1 A). Western blot analysis from whole embryo protein lysates confirmed this trend, as changes in PH3 levels were not significantly different from control embryos following injection of any of the TIMP-1 constructs (Fig. 4.1 B). Similarly, overexpression of TIMP-2 full-length, N-terminal or C-terminal domain constructs did not alter levels of PH3 relative to control embryos. Although the T2N construct resulted in slightly decreased levels of PH3 as examined by IHC (Fig. 4.2 A), this decrease was statistically insignificant when quantified via Western blot (Fig. 4.2 B, $p > 0.05$). Additionally, none of the TIMP-3 constructs altered PH3 levels compared to control (uninjected) embryos (Fig. 4.3 A and B), suggesting that TIMPs may not significantly impact proliferation in X. laevis embryos.
Figure 4.1. TIMP-1 N- or C-terminal domains did not alter PH3 levels in X. laevis embryos. Embryos were injected at the 1 cell stage with 4 ng of either TIMP-1 full-length (T1FL), N-terminal domain (T1N) or C-terminal domain (T1C) mRNA constructs. (A) Anterior sections from stage 30 embryos (200X magnification) were analyzed for changes in PH3 (green) as an indicator of cell proliferation. The level of PH3 relative to DAPI (blue) fluorescence levels was measured for each section (ratio is Y-axis) and depicted graphically (black bars). (B) Western blot analysis was performed to measure changes in PH3 levels from whole embryo protein lysates. PH3 levels were measured relative to β-actin. Results are displayed as mean ± SE from 3 independent experiments. No significant changes in PH3 levels relative to control uninjected (CONT) embryos were observed following overexpression of any TIMP-1 constructs, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test ($p > 0.05$).
A.

Expression relative to actin

B.

Expression relative to actin

CONT  T2FL  T2N  T2C
Figure 4.2. TIMP-2 N- or C-terminal domains did not alter PH3 levels in X. laevis embryos. Embryos were injected at the 1 cell stage with 4 ng of either TIMP-2 full-length (T2FL), N-terminal domain (T2N) or C-terminal domain (T2C) mRNA constructs. (A) Anterior sections from stage 30 embryos (200X magnification) were analyzed for changes in PH3 (green) as an indicator of cell proliferation. The level of PH3 relative to DAPI (blue) fluorescence levels was measured for each section (ratio is Y-axis) and depicted graphically (black bars). (B) Western blot analysis measured changes in PH3 levels from whole embryo protein lysates. PH3 levels were measured relative to β-actin. Results are displayed as mean ± SE from 3 independent experiments. No significant changes in PH3 levels relative to control uninjected (CONT) embryos were observed following overexpression of any TIMP-2 constructs, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test ($p > 0.05$).
A. 

expression relative to actin

B. 

Expression relative to actin

CONT  T1FL  T1N  T1C
Figure 4.3. TIMP-3 N- or C-terminal domains did not alter PH3 levels in X. laevis embryos. Embryos were injected at the 1 cell stage with 4 ng of either TIMP-3 full-length (T3FL), N-terminal domain (T3N) or C-terminal domain (T3C) mRNA constructs. (A) Anterior sections from stage 30 embryos (200X magnification) were analyzed for changes in PH3 (green) as an indicator of cell proliferation. The level of PH3 relative to DAPI (blue) fluorescence levels was measured for each section (ratio is Y-axis) and depicted graphically (black bars). (B) Western blot analysis measured changes in PH3 levels from whole embryo protein lysates. PH3 levels were measured relative to β-actin. Results are displayed as mean ± SE form 3 independent experiments. No significant changes in PH3 relative to control uninjected (CONT) embryos were observed following overexpression of any TIMP-3 constructs, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test (p > 0.05).
4.3.2. Overexpression of full-length and C-terminal TIMP-1 constructs increased active caspase-3 levels in X. laevis embryos

Active caspase-3 was used as an indicator of apoptosis to measure differences in the abilities of TIMP-1, -2 or -3 full-length, N- or C-terminal domain constructs to alter this pathway during development. Immunohistochemical examination of anterior head sections from stage 30 embryos demonstrated increased caspase-3 levels following overexpression of T1FL and T1C constructs (Fig. 4.4 A). These increases were confirmed as significant through Western blot analysis from whole embryo protein lysates at the same developmental stage (Fig. 4.4 B, \( p < 0.05 \)). In contrast, quantification of caspase-3 levels via Western blot analysis demonstrated that none of the TIMP-2 constructs significantly altered caspase-3 levels relative to control embryos (Fig. 4.5 B); however, the trend was toward the increase following overexpression of T2FL and T2C constructs in both IHC and Western blot analyses (Fig. 4.5 A and B). Similarly, overexpression of TIMP-3 constructs did not result in significantly altered caspase-3 levels compared to control embryos (Fig. 4.6 A and B).
Figure 4.4. Overexpression of TIMP-1 full-length and C-terminal domain constructs increased active caspase-3 levels in *X. laevis* embryos. Embryos were injected at the 1 cell stage with 4 ng of either *TIMP-1* full-length (T1FL), N-terminal domain (T1N) or C-terminal domain (T1C) mRNA constructs. (A) Anterior sections from stage 30 embryos (200X magnification) were analyzed for changes in active caspase-3 (green) as an indicator of cell death. The level of caspase-3 relative to DAPI (blue) fluorescence levels was measured for each section (ratio is Y-axis) and depicted graphically (black bars). (B) Western blot analysis measured changes in caspase-3 levels from whole embryo protein lysates. Caspase-3 levels were measured relative to β-actin. Results are displayed as mean ± SE from 3 independent experiments. T1FL and T1C constructs significantly increased caspase-3 levels relative to control uninjected (CONT) embryos, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test *(p < 0.05).*
Figure 4.5. Overexpression of TIMP-2 constructs did not alter active caspase-3 levels in *X. laevis* embryos. Embryos were injected at the 1 cell stage with 4 ng of either *TIMP*-2 full-length (T2FL), N-terminal domain (T2N) or C-terminal domain (T2C) mRNA constructs. (A) Anterior sections from stage 30 embryos (200X magnification) were analyzed for changes in active caspase-3 (green) as an indicator of cell death. The level of caspase-3 relative to DAPI (blue) fluorescence levels was measured for each section (ratio is Y-axis) and depicted graphically (black bars). (B) Western blot analysis measured changes in caspase-3 levels from whole embryo protein lysates. Caspase-3 levels were measured relative to β-actin. Results are displayed as mean ± SE from 3 independent experiments. TIMP-2 constructs did not significantly alter caspase-3 levels relative to control uninjected (CONT) embryos, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test (*p* > 0.05).
A. Expression relative to actin

B. Expression relative to actin
Figure 4.6. Overexpression of TIMP-3 constructs did not alter active caspase-3 levels in *X. laevis* embryos. Embryos were injected at the 1 cell stage with 4 ng of either *TIMP-3* full-length (T3FL), N-terminal domain (T3N) or C-terminal domain (T3C) mRNA constructs. (A) Anterior sections from stage 30 embryos (200X magnification) were analyzed for changes in active caspase-3 (green) as an indicator of cell death. The level of caspase-3 relative to DAPI (blue) fluorescence levels was measured for each section (ratio is Y-axis) and depicted graphically (black bars). (B) Western blot analysis measured changes in caspase-3 levels from whole embryo protein lysates. Caspase-3 levels were measured relative to β-actin. Results are displayed as mean ± SE from 3 independent experiments. TIMP-3 constructs did not significantly alter caspase-3 levels relative to control uninjected (CONT) embryos, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test (p > 0.05).
4.3.3. Overexpression TIMP constructs altered RECK levels in X. laevis embryos

To examine whether any of the TIMP domains had the ability to regulate RECK protein levels during development, changes in RECK levels were analyzed following overexpression of full-length, N-, and C-terminal domain constructs in X. laevis embryos. All changes were confirmed at both the IHC and Western blot level. Western blot analysis from stage 30 embryos showed that RECK expression was significantly increased following overexpression of TIFL and T1C constructs (Fig. 4.7 B, \(p < 0.05\)). In contrast, whereas overexpression of full-length and N-terminal domain TIMP-2 did not significantly alter RECK levels, the T2C construct resulted in decreased levels of RECK relative to control embryos that were detectable by IHC (Fig. 4.8 A) and confirmed as significant by Western blot (Fig. 4.8 B, \(p < 0.05\)). Additionally, while the TIMP-3 full-length and C-terminal domain constructs did not result in changes in RECK compared to controls, the T3N construct significantly decreased RECK levels compared to controls. Again, this decrease was detected by both IHC and Western blot analysis (Fig. 4.9 A and B, \(p < 0.05\)).
Figure 4.7. Overexpression of TIMP-1 full-length and C-terminal domain constructs increased RECK in *X. laevis* embryos. Embryos were injected at the 1 cell stage with 4 ng of either *TIMP-1* full-length (T1FL), N-terminal domain (T1N) or C-terminal domain (T1C) mRNA constructs. (A) Anterior sections from stage 30 embryos (200X magnification) were analyzed for changes in RECK expression (green). The level of RECK relative to DAPI (blue) fluorescence levels was measured for each section (ratio is Y-axis) and depicted graphically (black bars). (B) Western blot analysis measured changes in RECK levels from whole embryo protein lysates. RECK levels were measured relative to β-actin. Results are displayed as mean ± SE from 3 independent experiments. T1FL and T1C constructs significantly increased RECK relative to control uninjected (CONT) embryos, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test *(p < 0.05).*
A. 

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B. 

Expression relative to actin

- CONT
- T1FL
- T1N
- T1C

*P < 0.05
Figure 4.8. Overexpression of the TIMP-2 C-terminal domain construct decreased RECK in *X. laevis* embryos. Embryos were injected at the 1 cell stage with 4 ng of either TIMP-2 full-length (T2FL), N-terminal domain (T2N) or C-terminal domain (T2C) mRNA constructs. (A) Anterior sections from stage 30 embryos (200X magnification) were analyzed for changes in RECK expression (green). The level of RECK relative to DAPI (blue) fluorescence levels was measured for each section (ratio is Y-axis) and depicted graphically (black bars). (B) Western blot analysis measured changes in RECK levels from whole embryo protein lysates. RECK levels were measured relative to β-actin. Results are displayed as mean ± SE from 3 independent experiments. The T2C construct significantly decreased RECK levels relative to control uninjected (CONT) embryos, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test *(p < 0.05).*
Figure 4.9. Overexpression of the TIMP-3 N-terminal domain construct decreased RECK in *X. laevis* embryos. Embryos were injected at the 1 cell stage with 4 ng of either *TIMP*-3 full-length (T3FL), N-terminal domain (T3N) or C-terminal domain (T3C) mRNA constructs. (A) Anterior sections from stage 30 embryos (200X magnification) were analyzed for changes in RECK expression (green). The level of RECK relative to DAPI (blue) fluorescence levels was measured for each section (ratio is Y-axis) and depicted graphically (black bars). (B) Western blot analysis measured changes in RECK levels from whole embryo protein lysates. RECK levels were measured relative to β-actin. Results are displayed as mean ± SE from 3 independent experiments. The T3N construct significantly decreased RECK levels relative to control uninjected (CONT) embryos, as analyzed by one-way ANOVA and Dunnett’s multiple comparisons test *(p < 0.05).*
A. DAPI  RECK

CONT

T3FL

T3N

T3C

B.

Expression relative to actin

CONT  T3FL  T3N  T3C
4.4 Discussion

I have previously shown that overexpression of all TIMP constructs, with the exception of the TIMP-3 C-terminal domain, resulted in severe developmental defects, including head, neural tube and axis defects, and that the TIMP-1 and -2 C-terminal domains altered MMP levels independent of MMP inhibition (see Chapter 2 and 3). As the TIMP-1 and -2 C-terminal domains have been shown to regulate cell proliferation and apoptosis pathways \textit{in vitro}, I wanted to investigate whether TIMPs may also contribute to cell signaling \textit{in vivo}. In order to understand whether the TIMP C-terminal domains also have unique abilities to contribute to regulation of signaling pathways during development, I overexpressed TIMP-1, -2 or -3 full-length, N-terminal and C-terminal domain constructs in \textit{X. laevis} embryos, and examined changes in levels of PH3 as an indicator of cell proliferation and active caspase-3 as a indicator of apoptosis. Additionally, I examined changes in RECK levels, as the TIMP-2 C-terminal domain has previously been shown to increase RECK expression in cultured human endothelial cells (Oh, Seo et al. 2004). PH3, caspase-3 and RECK were examined using IHC and Western blot. Due to the nature of the severe developmental defects resulting from overexpression of TIMP constructs, developmental landmarks were difficult to identify, and changes in localization could not be observed by IHC. Accordingly, the levels of PH3, caspase-3 and RECK were examined in anterior head sections from injected and control embryos and measured relative to DAPI. Western blot analysis using whole embryo protein lysates was used to corroborate trends observed in IHC and also quantify changes in the levels PH3, caspase-3 and RECK in stage 30 embryos.
4.4.1. Ectopic expression of TIMP constructs did not alter cell proliferation in X. laevis embryos

Overexpression of TIMP-1, -2 or -3 full-length, N-terminal or C-terminal domain constructs did not result in significantly altered cell proliferation in X. laevis embryos, as indicated by changes in the levels of PH3. Although modest decreases in the levels of PH3 relative to DAPI were observed in some embryo sections using IHC, these decreases were deemed insignificant by Western blot analysis from whole embryo protein lysates (Fig. 4.1, 4.2 and 4.3). Interestingly, even the C-terminal domain constructs of TIMP-1 and -2 did not significantly alter proliferation relative to control embryos, indicating that there may be no direct or indirect roles for TIMPs in regulating this pathway during X. laevis development. Both the TIMP-1 and -2 C-terminal domains have been demonstrated in various cancer and fibroblast cell lines to either promote or inhibit cell proliferation (reviewed in Stetler-Stevenson 2008), however, these experiments were performed using homogenous populations of differentiated cells. Although TIMPs may impact cell proliferation pathways through C-terminal domain-mediated cell signaling events in differentiated cell lines in culture, this mechanism may not be maintained in undifferentiated cells in embryos. Pluripotent or multipotent cells in the embryo are subject to distinct regulatory parameters, where TIMPs may not have the ability to alter proliferation. Although it is possible that small populations of cells in the embryo may be sensitive to TIMP-mediated changes in proliferation, these changes were not reflected in the whole embryo, at least as demonstrated by PH3 assays. A more careful analysis of localized changes in proliferation must be carried out to conclusively rule out a role for TIMPs regulating proliferation pathways during development.
4.4.2. The TIMP-1 C-terminal domain increased active caspase-3 in X. laevis embryos

To investigate whether any of the *X. laevis* TIMPs had the ability to alter cell death during development (particularly through their C-terminal domains), I examined changes in active caspase-3 as an indicator of apoptosis, following overexpression of each of my TIMP constructs. The TIMP-1 full-length and C-terminal domain constructs significantly increased caspase-3 levels relative to control embryos (Fig. 4.4.). As the C-terminal domain construct has no MMP-inhibitory activity (see section 3.3.7.), this data suggests that changes in apoptosis are mediated by a C-terminal domain-specific mechanism, presumably cell signaling. While the TIMP-1 C-terminal domain has been linked to changes in apoptosis in both human lymphoma and mammary epithelia cell lines, these *in vitro* studies are associated with suppression of apoptosis, rather than the increase in apoptosis observed here (Guedez, Stetler-Stevenson et al. 1998; Berditchevski and Odintsova 1999; Li, Fridman et al. 1999; Guedez, Mansoor et al. 2001; Jung, Liu et al. 2006). Although the importance of the TIMP-1 C-terminal domain in regulating caspase-3 levels seems to be maintained in *X. laevis*, the effects of this protein may be different during development. This may be due to the fact that the embryo consists of a heterogeneous population of differentiating cells, where cell fate decisions are mediated in a highly complex manner, in contrast to the homogeneous populations of differentiated cells used in *in vitro* studies. Nevertheless, the role of TIMP-1 in regulating amount of active caspase-3 during *X. laevis* development seems to be specific to the TIMP-1 C-terminal domain, as only the T1C and T1FL constructs (which contain a functional C-terminus) increased apoptosis. Furthermore, none of the TIMP-2 or -3 constructs resulted in significantly altered levels of caspase-3 relative to control embryos, demonstrating that this effect is unique to TIMP-1.
Though a similar trend was observed following overexpression of T2FL and T2C constructs (Fig. 4.5), the resultant increases in caspase-3 were not significant compared to controls, indicating the TIMP-2 C-terminal domain may not play an important role in regulating this pathway during development. With only two conflicting reports in the literature regarding the effect of TIMP-2 on apoptosis (Valente, Fassina et al. 1998; Lim, Guedez et al. 1999), it is possible that TIMP-2 is simply not an important regulatory factor for this pathway. Additionally, while overexpression of the TIMP-3 constructs did not significantly alter caspase-3 levels relative to control embryos, modest increases in levels of caspase-3 were detected following overexpression of T3FL and T3N (but not T3C) constructs by IHC analysis, and these trends were mirrored by Western blot analysis (Fig. 4.6). This is consistent with reports in the literature, which indicate that TIMP-3 may indirectly alter apoptosis through regulation of MMP and ADAM activity (Stetler-Stevenson 2008). The TIMP-3 C-domain, which has no inherent MMP-inhibitory (or signaling) activity, did not follow this trend.

### 4.4.3. The TIMP-1 and -2 C-terminal domains had opposing effects on RECK protein levels in *X. laevis* embryos

RECK is a potent cell surface inhibitor of MMPs, thus, alterations in RECK expression may have considerable effects on cell migration during embryogenesis. As TIMP-2 has previously been demonstrated to increase RECK expression *in vitro* (Oh, Seo et al. 2004), here I investigated whether the *X. laevis* TIMPs or their individual domains may contribute to regulation of RECK expression *in vivo*, during development. Intriguingly, the TIMP-1 full-length and C-terminal domain constructs resulted in significantly increased levels of RECK protein when quantified using Western blot analysis (Fig. 4.7 B). The T1N
construct, which only functions in inhibition of MMP activity, did not result in changes in
RECK expression relative to control embryos. Thus, this data suggests that the observed
increases in RECK following overexpression of T1FL and T1C constructs are due to a C-
terminal domain specific mechanism. No previous reports have linked the TIMP-1 C-
terminal domain to a particular cell signaling pathway involved in the regulation of RECK
expression; however, to date there has been limited research investigating TIMP-1-mediated
regulation of RECK and other components of the ECM proteolytic network.

Following overexpression of TIMP-2 full-length, N-, and C-terminal domain
constructs, I found that only the T2C construct significantly decreased RECK expression
relative to controls, whereas the T2FL and T2N constructs did not (Fig. 4.8). This finding is
in contrast to the report by Oh et al. (2004), which showed that the TIMP-2 C-terminal
domain increased RECK expression through binding to α3β1 integrin receptors on the
surface of human microvascular endothelial cells. As the effect of TIMP-2 on regulation of
RECK expression has currently only been examined using cultured human endothelial cells,
it is possible that TIMP-2 may have varying effects on RECK expression, or may alter RECK
expression through different pathways, depending on the requirements of different tissues
and organisms. My research, however, demonstrated that TIMP-2 has the ability to alter
RECK through its C-terminal domain, independent of MMP inhibition, during X. laevis
development.

In contrast, overexpression of full-length and C-terminal domain TIMP-3 did not
alter RECK relative to control embryos, indicating that the TIMP-3 C-terminal domain does
not participate in signaling events that regulate RECK expression in X. laevis embryos. The
T3N construct, however, did result in significantly decreased RECK levels relative to control
embryos when analyzed by Western blot. As the TIMP N-terminal domains are involved in
MMP inhibition, and have not been associated with direct signaling activity, it is unlikely that the T3N construct decreased RECK expression through a direct signaling mechanism. Rather, the explanation may be simply due to the stoichiometry of MMP inhibitors within the ECM. Under normal conditions, full-length TIMP-3 is sequestered in the ECM away from the cell surface, a function that is mediated by its C-terminal domain (Yu, Yu et al. 2000). Thus, whereas the T3FL and T3C constructs may remain bound to the ECM, this ability would be impaired with the T3N construct. The T3N construct is free to act pericellularly, and therefore, may result in feedback to the cell to downregulate expression of other MMP inhibitors, like RECK.

**4.4.4. Concluding remarks**

This research is the first detailed comparison of the unique functions of the TIMP N- and C-terminal domains in vivo. Although a more thorough analysis is needed to identify binding partners and receptors for the TIMP C-terminal domains in vivo, I have shown for the first time that the TIMP-1 and -2 C-terminal domains have the ability to influence cell signaling pathways in *X. laevis* embryos. This research indicates that TIMPs may be important not only for mediating MMP activity during development, but also for regulating cell signaling pathways that influence apoptosis and cell migration, through RECK.
4.5 References


CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS
5.1 General Overview

5.1.1. Context and significance of this research

In recent years, the complexity underlying ECM dynamics has become apparent, as research has demonstrated the importance of maintaining the appropriate balance between ECM proteases and inhibitors in both development and disease. Not only is the balance between MMPs and TIMPs important for facilitating cell migration during development and metastasis (Liotta, Steeg et al. 1991; Blelloch and Kimble 1999), but TIMP-mediated inhibition of MMPs can also indirectly influence cell behavior by altering the availability and activity of secreted signaling molecules within the ECM (Brew and Nagase 2010). It has also become evident that TIMPs themselves may be important determinants of cell fate, as in vitro studies have shown that TIMP initiated cell signaling cascades can have significant consequences on apoptosis, cell migration, and cell proliferation pathways (Stetler-Stevenson 2008; Brew and Nagase 2010). Due to the multifaceted roles of both MMPs and TIMPs, analysis of the functions of, and interactions between, ECM molecules has become exceedingly complex, particularly in vivo, where TIMP and MMP activities may be altered by the presence and relative abundance of different ECM components. To my knowledge, there have been no previous studies that have examined and compared the unique roles of the different TIMP N-terminal MMP-inhibitory and C-terminal cell-signaling domains in vivo. The research presented in this thesis is the first comprehensive comparison of the individual TIMP domains in vivo, as well as the first examination of TIMP domain functions as they pertain to development.
5.1.2. **Research summary and general conclusions**

The overall goal of this research was to investigate in isolation the N- and C-terminal domains of all three *X. laevis* TIMPs during development, and to compare the effects of overexpression of the individual domains between the different TIMPs, as well as with their full-length counterparts. By using a series of domain-specific overexpression experiments and assays for: 1) gross morphological changes in development, 2) changes in the mRNA levels of ECM genes, 3) changes in the levels of active ECM proteases, and 4) changes in indicators of cell signaling pathways, I have performed a comprehensive comparison of TIMP domain function during *X. laevis* development. In general, my hypothesis was that overexpression of the TIMP N-terminal domains would have more similar effects on development, as inhibition of MMPs is a function common to all TIMPs; whereas, overexpression of the TIMP C-terminal domains would have varying effects on development, depending on their distinct abilities to participate in cell surface signaling events. Indeed, I have demonstrated that the TIMP-1 and -2 C-terminal domains have unique abilities to contribute to development that are mirrored following overexpression of the full-length construct. This research used C-terminal domain TIMP constructs to demonstrate *in vivo* the MMP-independent effects of TIMPs (that can be attributed to direct cell signaling functions) vs. the changes in development that arise due to TIMP inhibition of target proteases (through their N-terminal domains).

5.2 **Contributions to the Current Knowledge of ECM Dynamics During Development**

5.2.1. **Identification and characterization of *X. laevis* TIMPs**

At the time this research was started, only *X. laevis* TIMP-2 and -3 genes had been identified. One of the firsts tasks I was faced with when beginning this project was to identify
and clone TIMP-1 and/or TIMP-4. As mentioned in section 1.3, while four TIMPs are present in mammals, many vertebrates do not express orthologs of all four TIMPs (Murphy 2011). Using database searches, I identified an unannotated X. laevis clone (AAI41767.1), which was highly homologous with other known TIMP-1 sequences at the amino acid level. Presently, there is no TIMP-4 in X. laevis based on available sequence data. It is possible that a TIMP-4 sequence may still be discovered as the sequencing of the X. laevis genome is still underway. However, Xenopus tropicalis, a closely related species to X. laevis, has been fully sequenced. Although a TIMP-1 ortholog has been identified, no TIMP-4 sequence has been discovered in X. tropicalis. In mammals, TIMP-4 has the most selective and restricted pattern of localization, with expression primarily in brain and heart (Baker, Edwards et al. 2002; Nuttall, Sampieri et al. 2004). Structurally, human TIMP-4 is most similar to TIMP-2 (Melendez-Zajgla, Del Pozo et al. 2008), though studies suggest that the signaling capabilities of TIMP-4 are most similar to those of TIMP-1 (Stetler-Stevenson 2008). It is possible that the functions of TIMP-4 may be redundant with those of the other TIMPs, thus, some lower vertebrates such as frog may not contain TIMP-4 along with TIMP-1 or -2. Indeed, the fish Takifugu rubripes expresses TIMP-2, -3 and -4 but not TIMP-1, whereas insects such as Drosophila melanogaster express only one TIMP (Brew, Dinakarpandian et al. 2000; Tsukamoto, Yokoyama et al. 2006; Tsukamoto, Yokoyama et al. 2007; Brew and Nagase 2010).

Amino acid sequence comparison of the three X. laevis TIMPs compared with those from other known full-length vertebrate and invertebrate species revealed that the N-terminal domains of TIMP-1 and -2 were more highly conserved than their C-terminal domains. This was expected, as the N-terminal domains are involved in the similar function of MMP inhibition, whereas the C-terminal domains of TIMP-1 and -2 have variable roles in cell
signaling that depend on cell and tissue type (Stetler-Stevenson 2008). The signaling functions of the C-terminal domains likely also vary between species; however, to date TIMP C-terminal domain function has only been examined in human, mouse and rat cell lines. Phylogenetic analysis of TIMP-1, -2 and -3 N- and C-terminal domains with those of other known species showed that for TIMP-1 and -3, the N- and C-terminal domains diverged under different selective pressures, whereas for TIMP-2 the two domains evolved in a concomitant manner. This study, however, was limited by the number of available annotated TIMP sequences, particularly from non-mammalian and invertebrate species. A broader study with more diverse species, as well as additional analysis of TIMP receptors is needed to truly determine if TIMP C-terminal domains evolved in parallel with changes in specific receptors and/or signaling pathways.

5.2.2. TIMP C-terminal domains contribute to regulation of development in an MMP-independent manner

TIMPs have long been acknowledged for their roles in MMP inhibition that are needed for regulation of developmental events involving cell migration. With the emerging signaling properties of TIMPs in cell migration, proliferation and apoptosis pathways, it was important to re-examine TIMP function in vivo, as these cell signaling pathways are not only important in regulation of cancer progression, but also in the regulation of development. The stoichiometry between MMPs, TIMPs, secreted signaling molecules, and cell surface receptors are important determinants of TIMP function. By disrupting this stoichiometry through overexpression of my TIMP constructs in early stage embryos, I have revealed for the first time in vivo that the TIMP C-terminal domains can contribute to development using an MMP-independent mechanism.
Specifically, I have shown that overexpression of the TIMP-1 and -2 C-terminal domains led to distinct developmental defects and large amounts of embryonic death. These developmental defects were linked to changes in MMP expression and activity levels, as well as to specific changes in signaling markers that were also observed following overexpression of the full-length constructs, but not with the N-terminal domain constructs. As the TIMP-1 and -2 C-terminal domains alone cannot inhibit MMP activity, these results suggest that the observed development defects occurred through a signaling mechanism. In contrast, the TIMP-3 C-terminal domain did not disrupt development, or alter any signaling markers relative to control embryos. This was consistent with my original hypothesis, which stated that the TIMP-3 C-terminal domain would not alter development on its own (only as part of the full-length molecule), and was also consistent with evidence from previous *in vitro* experiments which reported that TIMP-3 is sequestered in the ECM through its C-terminal domain, and therefore, does not participate in pericellular signaling events (Langton, Barker et al. 1998; Yu, Yu et al. 2000; Stetler-Stevenson 2008).

When comparing data in Chapter 2 (TIMP-2 and -3) and Chapter 3 (TIMP-1), one noticeable result was the ability of both the TIMP-1 and -2 C-terminal domains to alter MMP-2 and -9 expression at both the mRNA and protein levels. The full-length and N-terminal domains also had varying abilities to alter MMP expression and activity, which may have occurred as an indirect result of MMP inhibition or due to a regulatory feedback mechanism within the ECM proteolytic network. However, the fact that the C-terminal domains also altered MMP expression and activity levels is suggestive of a unique feedback mechanism, whereby the cell can alter relative levels of ECM proteins in direct response to TIMP signaling. Surprisingly, TIMP-1 and -2 differed in their abilities to regulate active MMP-9 protein. TIMP-2 resulted in increased levels of active MMP-9 relative to control
embryos, whereas TIMP-1 decreased active MMP-9 relative to controls, and this decrease was most pronounced following overexpression of the C-terminal domain. Though the precise mechanisms through which the TIMP-1 and -2 C-domains may regulate MMP-9 remain unknown, previous studies have also indicated that a special relationship may exist between TIMP-1 and pro-MMP-9 (discussed in section 1.5.2.). TIMP-1 and pro-MMP-9 have been demonstrated to complex together in the ECM, a function that is mediated by the C-terminal domain of TIMP-1 rather than the N-terminal MMP-inhibitory domain (Goldberg, Strongin et al. 1992; Itoh and Nagase 1995). More recently, in vitro experiments using human cell lines have shown that TIMP-1 and pro-MMP-9 co-localize within the cell and are secreted together. This complex has been suggested to be involved in regulation of MMP-9 activity and stabilization of the pro-enzyme, and may be found in close proximity to the cell surface (Itoh and Nagase 1995; Roderfeld, Graf et al. 2007). My research suggests that TIMP-1 may associate with pro-MMP-9 in vivo to regulate MMP-9 activity during development.

Another intriguing aspect of this study was the ability of the TIMP-1 and -2 (but not TIMP-3) C-terminal domains to alter specific cell signaling markers in X. laevis embryos. In chapter 4, I demonstrated that both the TIMP-1 and -2 C-terminal domains altered RECK protein levels relative to controls, and that these trends were mirrored with overexpression of the full-length but not N-terminal domain constructs. Again, TIMP-1 and -2 had differing effects on RECK expression, suggesting that these two TIMPs may regulate expression and activity within the ECM proteolytic network through different mechanisms (Fig. 5.1). Although the decrease in RECK protein observed following overexpression of the T2C construct was in contrast to current literature, which reported an increase in RECK in human endothelial cell lines (Oh, Seo et al. 2004), this has not been recapitulated in any other cell
lines or model organisms to date, and could likely vary between species (discussed in detail in section 4.4.3.). The decrease in RECK protein observed in Western blot and IHC studies in chapter 4, however, was in agreement with the slight decrease (2-fold decrease relative to control embryos) in RECK mRNA observed in chapter 2, which provides further support for the idea that the TIMP-2 C-terminal domain can alter RECK expression through an MMP-independent mechanism. In opposition, the TIMP-1 C-terminal domain resulted in increased RECK protein (Fig. 5.1). Although a direct signaling mechanism for TIMP-1 mediated regulation of RECK has not previously been reported, TIMP-1 is unique with respect to the other TIMPs as in vitro experiments have shown that TIMP-1 can translocate into the nucleus (Ritter, Garfield et al. 1999). While the nuclear functions of TIMP-1 remain unknown, to my knowledge, nuclear localization has never been demonstrated for any of the other TIMPs. In addition, several studies have reported that increased RECK is associated with decreases MMP-9 mRNA levels as well as secretion of MMP-9 protein (Takahashi, Sheng et al. 1998; Chang, Hung et al. 2008; Takagi, Simizu et al. 2009). This is in agreement with my data, as I have shown that while RECK protein is elevated, MMP-9 mRNA and protein are downregulated at the same developmental stage. These trends are most pronounced following overexpression of the TIMP-1 C-terminal domain. Taken together, these studies highlight the complexity of the ECM proteolytic network in vivo, and indicate that TIMP-1 may be an important regulator of this network during development, and may have important regulatory functions that have yet to be discovered.
**Fig. 5.1. Model of TIMP signaling during *X. laevis* development.** TIMP-1 binds unknown receptors through its C-terminal domain to increase active caspase-3 levels and/or RECK protein levels. TIMP-2 binds to unknown receptors through its C-terminal domain to decrease RECK protein levels. TIMP-3 remains sequestered in the ECM and has no independent signaling mechanism during *X. laevis* development. TIMP C-terminal domain signaling ability is dependent on relative levels of MMPs and other binding partners in the ECM, which sequester TIMPs away from the cell surface.
In addition, the TIMP-1 full-length and C-terminal domains were demonstrated to significantly increase caspase-3 activity in *X. laevis* embryos, and this association was not demonstrated with the TIMP-1 N-terminal domains or with any of the other TIMP constructs (Fig. 5.1). Caspase-3 was used as an indicator of apoptosis, as active caspase-3 is normally considered an important effector in apoptotic pathways. My results were in contrast to reports in the literature, which have demonstrated using several different cell lines that TIMP-1 suppressed rather than promoted apoptosis (Guedez, Stetler-Stevenson et al. 1998; Berditchevski and Odintsova 1999; Li, Fridman et al. 1999; Guedez, Mansoor et al. 2001; Jung, Liu et al. 2006). Remarkably, however, a recent study using hematopoietic cells in culture has reported that TIMP-1 can indeed lead to increased activation of caspase-3, and that this activation is not associated with increased apoptotic cell death (Dasse, Bridoux et al. 2007). Instead, in this model, increased activation of caspase-3 resulted in cell differentiation through activation of MEK1, MEK6, and p38α (Dasse, Bridoux et al. 2007). It remains unclear whether the increase in caspase-3 activation that I observed in association with overexpression of the TIMP-1 C-terminal domain is indicative of enhanced apoptosis or cell differentiation. While further studies are needed to investigate additional markers of both pathways, my research has emphasized that the C-terminal domain, particularly of TIMP-1, may potentially play intricate roles in the regulation of development.

### 5.3 Limitations of this Research and Future Studies

The major limitation of this study was the lack of mechanistic data regarding how TIMP C-terminal domain signaling altered proteolytic gene expression and activity, or cell signaling indicators. Though my research is highly suggestive of a C-terminal signaling function for TIMP-1 and -2, I did not investigate the specific receptors in *X. laevis* embryos
associated with the observed changes in caspase-3 or proteolytic markers. While in vitro studies have identified several cell surface binding partners for TIMP-1 and -2 C-terminal domains, receptors and pathways vary with cell type. Indeed, there may be several cell surface binding partners for TIMPs in X. laevis, which may vary not only between the TIMPs, but also in different developing tissues and/or at different developmental stages.

As the research presented in this thesis was the first study of TIMP domain function during development, I focused mainly on global changes within the whole embryo. I also focused solely on organogenesis (stage 30 in development), at which time many differentiation and remodeling events occur. A consequence of this approach, however, was that I was only able to observe large-scale changes at one particular developmental time point. A broader examination of TIMP domain function across other developmental stages, such as neurulation and gastrulation, would improve our understanding of TIMP signaling, as well as our understanding of how changes in gene expression may be correlated with changes in protein levels and activities throughout development. Furthermore, in order to better comprehend the mechanisms through which the N- and C-terminal TIMP domains manifest their effects, a more detailed analysis of changes in the expression patterns of TIMPs, MMPs, and various signaling markers across different tissue types throughout development is warranted. This analysis would also provide an indication of whether different tissues have distinct abilities to respond to TIMP C-terminal domain signals.

Future studies may use morpholinos to observe the consequences of TIMP knockdown during X. laevis development, compared with overexpression of the full-length constructs. Rescue experiments, whereby co-injecting MMP(s) along with TIMP N-terminal domains would be useful in attempting to attenuate the effects of N-terminal domain overexpression vs. C-terminal domain overexpression. Finally, co-localization and co-
immunoprecipitation experiments should be used to identify cell surface binding partners for
the TIMP C-terminal domains. Examination of changes in additional signaling markers may
help link the individual TIMP C-terminal domains with specific changes in signaling
pathways in order to tease apart the mechanisms behind the opposing effects of T1C vs. T2C
overexpression.

5.4 Conclusions

Much of the current understanding regarding TIMP function during development has
been inferred from in vitro cell culture experiments using controlled, and therefore artificial
conditions. While the significance of these studies cannot be devalued as they have
considerably progressed the current understanding of ECM dynamics, studies focusing on in
vivo regulation of these molecules have been very limited in comparison. Although MMPs
have been fairly well characterized during development, the discovery of the C-terminal
domain functions of TIMPs has made understanding the interactions between these proteins
much more complicated (even in cell culture). The fact that TIMPs are now known to have
both pro- and anti-tumorigenic properties, has made TIMPs a hot topic for cancer research.
Consequently, investigation of TIMP C-terminal domain function has been carried out almost
exclusively using transformed cancer cell lines. The signaling pathways, however, that
TIMPs have been associated with in cancer progression are also important in the regulation
of development. The research presented in this thesis has been the first comprehensive
comparison of TIMP domain function in vivo. Additionally, I have provided the first
evidence that TIMPs have MMP-independent functions during development. Given the ever-
increasing importance of the TIMP C-terminal domains in disease, I believe that these
proteins will also prove to be important regulators of developmental events as new and interesting roles for the C-terminal domains are discovered.
5.5. References


Appendix A:

*Xenopus laevis* TIMP-1 coding sequence (Accession number: KF018236)

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1  atgttgtaccttgtggttgttgtgctggtttctagggtgcctcagccag
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91  tactgcagtgcagattingttttattcgaggagattcattggaaag
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586  tcccagaagcccacagctcatcgtcgtgtaggaataaagaggcag
631  tctgctgccacacaacataaa 651
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Fig. B.1. Schematic *X. laevis* embryo at developmental stage 30. Adapted from Nieuwkoop and Faber, 1956.
Appendix C:

Fig. C.1. Phenotypic effects of overexpression of TIMP C-terminal constructs. Following injection, photographs were taken of representative embryos at stage 30. Microinjection of TIMP-1 (A) and TIMP-2 (B) C-terminal domain constructs resulted in head and neural tube defects. Overexpression of the TIMP-3 (C) C-terminal domain produced phenotypically normal embryos. Actual size of all embryos is 1 mm in diameter. Images are of representative embryos where results were consistent in at least 3 independent sets of experiments, and a minimum of 100 embryos were injected and monitored for each replicate.
Appendix D:

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</tbody>
</table>

Table D.1. Real-time PCR CT values for embryos injected with TIMP-2 and -3 constructs. Real-time PCR was used to measure the changes in transcript levels following the injection of 4 ng of TIMP-2 full-length (T2FL), N-terminal (T2N), or C-terminal (T2C), or TIMP-3 full-length (T3FL), N-terminal (T3N), or C-terminal (T3C) mRNA constructs into X. laevis embryos at the one cell stage. The CT value represents the PCR cycle at which the gene of interest passed a defined threshold. Changes in transcript levels were measured relative to Ef-1α and normalized to CONT (control, uninjected) embryos. INTG Beta 1 represents β1 integrin. CT values are representative of one experiment.
Appendix E:

June 21, 2011

Dear Dr. Damjanovski:

Please note your biosafety approval number listed above. This number is very useful to you as a researcher working with biohazards. It is a requirement for your research grants, purchasing of biohazardous materials and Level 2 inspections.

Research Grants:
- This number is required information for any research grants involving biohazards. Please provide this number to Research Services when requested.

Purchasing Materials:
- This number must be included on purchase orders for Level 1 or Level 2 biohazards. When you order biohazardous material, use the on-line purchase ordering system (www.uwo.ca/finance/people/). In the “Comments to Purchasing” tab, include your name as the Researcher and your biosafety approval number.

Annual Inspections:
- If you have a Level 2 laboratory on campus, you are inspected every year. This is your permit number to allow you to work with Level 2 biohazards.

To maintain your Biosafety Approval, you need to:

- Ensure that you update your Biohazardous Agents Registry Form at least every three years, or when there are changes to the biohazards you are working with.
- Ensure that the people working in your laboratory are trained in Biosafety.
- Ensure that your laboratory follows the University of Western Ontario Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories.
- For more information, please see: www.uwo.ca/humanresources/biosafety.

Please let me know if you have questions or comments.

Regards,
2009-044::3:

**AUP Number:** 2009-044  
**AUP Title:** MMP Activation During Xenopus Development

**Approval Date:** 05/29/2009

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2009-044 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than those through this system must be cleared through the ACVS office. Health certificates will be required.

**REQUIREMENTS/COMMENTS**

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D  
on behalf of the Animal Use Subcommittee
Appendix F:

Curriculum vitae

Dr. Michelle A. Nieuwesteeg

Education

2008-2013 Ph.D., Department of Biology, Graduate Program in Developmental Biology, Western University, London, ON (reclassified from MSc in 2009)

2004-2008 Bachelor of Science, Honors Specialization in Cell Biology Western University, London, ON

Honours and Awards

2013 Graduate Thesis Research Award ($1500), Western University, London, ON

2012 Michael Locke Graduate Travel Bursary ($500), Western University, London, ON

2012 Graduate Thesis Research Award, ($1000), Western University, London, ON

2010 Michael Locke Graduate Travel Bursary ($500), Western University, London, ON

2010 Nominated for Graduate Student Teaching Award

2009 Graduate Program in Developmental Biology Entrance Award ($1250)

Research Experience

2008-Current Graduate Research, Department of Biology, Western University, London, ON
  • Ph.D. Thesis: TIMP regulation of cell signaling and cell migration

2007-2008 Undergraduate Research Thesis, Department of Biology, Western University, London, ON
  • 4999E Thesis: Generating tagged human MT1-MMP construct
Research Interests

• ECM remodeling
• Xenopus Development
• TIMP domain function in vitro as well as in vivo
• Relationship between MMPs and TIMPs
• Peroxisome biogenesis and reactive oxygen species regulation
• Redox sensitive Wnt cell signaling

Contributions to Research

Peer Reviewed Publications:


Papers submitted and under review:


Papers in preparation for submission:


Poster Presentations:


Teaching Experience

2008-2013  Bio4999 mentor in lab. Involved in training 8 Bio4999 fourth year honours thesis students and 2 summer NSERC USRAs

2008-2013  Bio2382b Cell Biology Teaching Assistant. One of only 2 TAs for a class of 1200 students. Ran weekly tutorial sections and was responsible for review presentations of lecture material. Each review session was attended by over 600 people.

2008-2012  Bio3338a Developmental Biology Teaching Assistant. Ran student presentation of course related material (2 tutorials of 30 students per week).

Bio4999E 4th year Honours Research Thesis Advisory Committee Member:
2010-2011  Sun Yung Park
2011-2012  Bryan Jenkins
2012-2013  Dilan Sulevani
2012-2013  Alexandra Chaber

Volunteer Experience

2011  Western Biology Fall Openhouse
2012  Western Biology Spring Openhouse
2012  Western Biology Fall Openhouse