The Cytoplasmic Domain of Membrane-type 1 Matrix Metalloproteinase is Required for its Survival-Promoting, but not its Migration-Promoting Function in MCF-7 Breast Cancer Cells

Jacob JH Pelling
The University of Western Ontario

Supervisor
Dr. Sashko Damjanovski
The University of Western Ontario

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Abstract

Membrane-type 1 matrix metalloproteinase (MT1-MMP) is a multifunctional protease that degrades proteins during cell migration, and influences cell survival. Both the protein localization and signal transduction capabilities of MT1-MMP depend on its cytoplasmic domain (CD), indicative of a diverse regulatory function. The effects of CD mutations on cell migration and survival were examined by ectopically expressing MT1-MMP variants in MCF-7 cells. CD alteration by substitution or deletion did not abolish the migration-promoting effects of MT1-MMP, but did decrease cell survival and increase apoptosis. Expression of CD-altered MT1-MMP resulted in a protrusive cell morphology in 3D culture that was lost upon serum starvation. MT1-MMP expression in a chicken embryo tumour model resulted in vascularization of MCF-7 tumours; a phenotype that was partially maintained following expression of MT1-MMP CD variants. These results suggest that the CD regulates MT1-MMP localization in a manner required for cell survival, but is dispensable for cell migration.

Keywords

Matrix metalloproteinases, MT1-MMP, cytoplasmic domain, extracellular matrix, cell migration, cell invasion, metastasis, cell survival, 3D cell culture
Acknowledgments

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<th>Description</th>
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<tbody>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ARP2/3</td>
<td>Actin related proteins 2 and 3</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BIK</td>
<td>Bcl-2-interacting killer</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>CD</td>
<td>Cytoplasmic domain</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned media</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF-1α</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GF-AFC</td>
<td>glycylphenylalanyl-aminofluorocoumarin</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GRASP-55</td>
<td>Golgi reassembly and stacking protein 55</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor 1 alpha</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Michigan Cancer Foundation 7</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>MD Anderson Metastatic Breast 231</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane-type matrix metalloproteinase</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neural Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>pH3</td>
<td>Phospho-histone 3</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative (real-time) polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>ZsGreen</td>
<td>Zoanthus species green</td>
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Chapter 1

1 Introduction

1.1 The Extracellular Matrix

The functions of cells in tissues are influenced by the composition and structure of the extracellular matrix (ECM), a dynamic physical and chemical network of secreted proteins and macromolecules that includes collagens, laminins, fibronectins and proteoglycans. Interacting reciprocally, epithelial and stromal cells locally produce and secrete ECM molecules, which assemble and in turn regulate the shape, growth and function of the embedded cells (Kim, Turnbull, & Guimond, 2011). Importantly, the composition of proteins within the ECM can differ both temporally and spatially, allowing for cell-ECM interactions to be adaptable and unique during specific cellular and developmental processes and in tissue structures (Daley, Peters, & Larsen, 2007).

The most basic function that the ECM provides is a physical scaffold on which cells grow, organize, and adhere to one another, thereby allowing for the maintenance of tissue integrity. However, current understanding of the role and function of the ECM has expanded, and it is now recognized that the ECM has an important role in regulating and directing cell behaviour (Anilkumar et al., 2005).

As the substrate upon which cells grow, the ECM acts to inhibit cell movement by functioning as a physical barrier. Indeed, invasive cell types must adopt distinct morphologies to circumvent the barrier of the ECM (Sahai & Marshall, 2003), and ECM degradation is an essential step in the progression of epithelial metastases (Rowe & Weiss, 2009). However, the proteins of the ECM also serve as the track on which cells migrate, as they provide the substrate to which focal adhesions form during the progression of cell migration (Lu, Weaver, & Werb, 2012). In this way, the ECM provides dynamic directional cues to migrating cells as its protein composition changes to be more or less permissive to the corresponding cell surface integrins. These mechanisms allow the ECM to both positively and negatively regulate the initiation and continuation of cell movement.
As an indirect effector of cell behaviour the ECM can serve as a reservoir of secreted signaling molecules, as charged components of the network attract and sequester ligands away from the cell surface. Thus, the ECM both limits the diffusive range of molecules and temporally influences the activation of cell signaling, as these sequestered factors can be liberated upon the cleavage of ECM components (Rahman et al., 2005). This retention is particularly influential during development, where molecules such as fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs) are sequestered and released along gradients that develop from secretory cells. While ECM proteins are passively involved in this process, they can also directly influence signaling, such as the specific requirement of the ECM molecule heparin to present the FGF ligand to its receptor on the cell surface (Hynes, 2009). By modulating cell signaling, the ECM serves important roles distinct from its physical properties.

Structurally, the ECM is subdivided into two distinct layers or forms: the interstitial matrix and the basement membrane. The interstitial matrix is comprised mainly of fibrous collagens and proteoglycans, and is found mostly in connective tissue. The basement membrane, which directly underlies epithelia, is a dense barrier made of primarily collagen IV and serves to support and inhibit the movement of cells (Pöschl et al., 2004). Structurally, the pattern of collagen crosslinks within the basement membrane results in a thickness of 100-300 nm and creates holes between fibers of 50 nm in size, far smaller than the permissive size of 2 µm required for cell migration (Rowe & Weiss, 2009). For cells to breach this basement membrane it must be remodeled, and indeed, the ECM differs structurally and compositionally in response to changes in normal physiology or pathology.

This plasticity of the ECM is evidenced by the many processes which underlie organismal development, including the wide-scale cell movement observed during gastrulation, where epithelial cells degrade and transverse the basement membrane during epithelial to mesenchymal transition (Rowe & Weiss, 2009). However, these same mechanisms are co-opted during pathologies such as fibrosis and cancer metastasis, where the degradation or composition of the ECM is dysregulated to result in malignant cell behaviour (Lu et al., 2011). For example, ECM modifications associated with breast
cancer involve remodeling that not only degrades components of the ECM, but also paradoxically results in the remaining ECM to become stiffer, a quality recognized by cells which can induce and increase their migration (Gilkes, Semenza, & Wirtz, 2014). In addition to altered mechanics, as the ECM experiences degradation, porosity increases and facilitates the amoeboid invasion of epithelial cells through it (Wolf et al., 2003). Thus the ECM has a great influence on the integrity of tissues and the regulation of the cells within by either promoting or restricting cell movement by both physical and chemical means.

1.2 The Matrix Metalloproteinases

In order to regulate the diverse functions of the ECM and maintain homeostasis, constituent proteins must be subject to renewal or degradation. A number of enzymes function to degrade ECM proteins, including *disintegrins* and *metalloproteinases* (ADAMs), numerous serine proteases, and *matrix metalloproteinases* (MMPs), which all proteolytically cleave the polymers of the ECM to change its composition (Lu et al., 2011; Seals & Courtneidge, 2003). Of these, the most diverse and widely-studied are the MMPs, which comprise 24 identified members in humans that collectively are able to degrade all known protein components of the ECM (Löffek, Schilling, & Franzke, 2011). MMPs are distinguished by the conserved histidine motif HEXXHXXGXX(H/D) within the catalytic domain, which chelates the zinc atom necessary for catalysis (Stocker & Bode, 1995).

MMPs are described and classified by two independent attributes: substrate specificity and cellular location. The classes of collagenases, gelatinases, stromelysins and matrilysins are distinguished by differences in the catalytic and hemopexin domains which impart specificities to these groups of ECM substrates. However, MMPs are more commonly classified based on their functional location, as 18 are secreted and function as soluble mediators of degradation, while the six membrane-type (MT) MMPs function at the cell surface (Bourboulia & Stetler-Stevenson, 2010). All MMPs share a basic domain structure which includes an N-terminal signal sequence, an autoinhibitory pro-peptide, and a catalytic domain. With the exception of MMP-7 and MMP-21, all MMPs also possess a hemopexin-like structural domain that mediates protein binding. Membrane-
type MMPs 1, 2, 3 and 5 also contain a single-pass transmembrane domain and cytoplasmic domain, while MT4-MMP and MT6-MMP are membrane localized by a GPI anchor (Cauwe, Van den Steen, & Opdenakker, 2007).

Due to their potency and the importance that ECM composition has on cell function, the activity of MMPs is tightly regulated post-translationally by both latency and the activity of the endogenous MMP inhibitor protein family, the tissue inhibitors of metalloproteinases (TIMPs). Nascent MMPs are zymogens, as their N-terminal pro-domain functions to physically bind and block the active site within the catalytic domain. The pro-domain is proteolytically cleaved and removed to allow for enzymatic activity. The manner in which this activation occurs differs depending on the structural classification of MMP, as membrane-type MMPs have the pro-domain cleaved in the secretory pathway by pro-protein convertases, primarily furin, while secreted MMPs are released to the extracellular milieu as zymogens and become activated extracellularly by other proteases, such as MT-MMPs already present at the cell surface (Remacle et al., 2006). As MT-MMPs are membrane-inserted in an active form, they are immediately in a position to be inhibited by TIMP family members, some of which are ubiquitously expressed. The mammalian TIMP family consists of TIMP-1, -2, -3 and -4, all of which are secreted and function in the extracellular space. Each TIMP family member is capable of inhibiting every MMP, though with varying efficiencies. TIMP proteins contain two functional domains, an N-terminal MMP-inhibitory domain and a C-terminal protein binding domain which is capable of binding a number of cell surface proteins (Moore & Crocker, 2012). While the primary identified role of TIMPs is to inhibit MMPs, they also have functions independent of this activity, including the regulation of cell survival (Stetler-Stevenson, 2008). Additionally, TIMPs can positively regulate the activation of MMPs, as will be described below (Itoh et al., 2001).

As many MMPs have overlapping specificities to substrates, individual protease function is often dictated by their differential expression patterns (Sternlicht & Werb, 2001). This differential expression is accomplished as their transcription is controlled by a complex orchestration of cytokines, including interleukins, growth factors, and tumour necrosis factor-α, which also restrict their expression in adult tissues to very low levels (Moore &
Crocker, 2012; Sternlicht & Werb, 2001). Additionally, depending on the time, location, or the expression of TIMP proteins, certain MMPs may be more or less capable of catalysis. As such, identifying the specific, divergent functions of individual MMPs is necessary in order to understand their many roles in the regulation of ECM degradation and cell behaviour.

1.3 Membrane-type 1 Matrix Metalloproteinase

The prototypical and most extensively studied member of the membrane-type MMPs is membrane-type 1 MMP (MT1-MMP). While its expression is heavily restricted in adult tissues, MT1-MMP is essential during embryonic development, as MT1-MMP knockout mice die shortly after birth (Holmbeck et al., 1999). MT1-MMP is classified as a collagenase, and its principle ECM substrates include collagen types I, II and III, fibronectin, and laminins-1 and -5 (Ueda et al., 2003). However, MT1-MMP is also capable of cleaving non-ECM proteins, including cell surface receptors such as CD44 and E-cadherin. This diverse repertoire of substrates means that MT1-MMP can greatly influence cell-ECM interactions and corresponding cell behaviour.

In addition to cleaving ECM protein substrates and cell surface molecules, MT1-MMP is a principal activator of the gelatinase MMP-2. As MMP-2 is one of the few enzymes capable of cleaving type-IV collagen, this effectively makes MT1-MMP activity capable of mediating the degradation of all major types of collagen, poising it as a potent organizer of invasion (Zeng, Cohen, & Guillem, 1999). Importantly, the mechanism by which MT1-MMP activates MMP-2 is well described, and highlights the importance of the domain structures of MMP-2, MT1-MMP, and its inhibitor, TIMP-2. In the extracellular space, the N-terminal of TIMPs typically bind MMPs at the catalytic site to inhibit their enzymatic activity. However, since access to the catalytic domain of pro-MMP-2 is sterically blocked due to the binding of its pro-domain, the C-terminal of TIMP-2 alternatively binds pro-MMP-2 at its hemopexin-like domain. At the cell surface, the exposed, inhibitory N-terminal of TIMP-2, now attached to pro-MMP-2 at its C-terminus, is free to bind and inhibit an MT1-MMP protein at its catalytic site. This trimeric association positions pro-MMP-2 to be cleaved by an adjacent, uninhibited MT1-MMP protein, and the active MMP-2 is subsequently released into the extracellular space.
(Itoh et al., 2001). While MT1-MMP can activate MMP-2 without the presence of TIMP-2, the paradox of the MMP-inhibiting TIMP-2 protein enhancing the activation of its substrate suggests that the regulation of ECM remodeling proteins is more complex than originally thought.

Importantly, MT1-MMP has recently been recognized to have both proteolytic and non-proteolytic functions which result in the enhancement of cell motility. Indeed, while MT1-MMP plays an integral role during development, especially throughout bone maturation (Holmbeck et al., 1999), its dysregulation promotes metastasis in many types of cancer. MT1-MMP has been found to promote or exacerbate five of the six defined “hallmarks of cancer”, which include proliferation independent of growth signals, insensitivity to growth reduction signals, angiogenesis, tissue invasion, and evasion of apoptosis. Only the heightened replicative ability of cancer cells remains unrelated to the functions of MT1-MMP (Maquoi et al., 2012). The duality of its essential function during development and its malignancy-promotion during cancer has raised intense interest in the research community to understand how cell motility and survival are regulated by MT1-MMP.

1.4 MT1-MMP and cell motility

MT1-MMP is frequently described as being pro-invasive and pro-migratory, and its increased expression is associated with invasive cell types including immune cells and cancers (Figueira et al., 2009). The process of cell migration has been extensively described, and is initiated by the binding of chemoattractants or other pro-migratory ligands to their cell surface receptors. This induces a cell signaling cascade which influences, among many things, gene transcription and cytoskeletal dynamics. Actin polymerizes at the leading edge of the cell, due to the local activity of multiple actin binding proteins including WASP and ARP2/3. This polymerization pushes membrane extensions from the cell surface, called filopodia or lamellipodia, which contact the ECM and bind to it using specific integrins to form focal adhesions. Actin and myosin can contract to move the cell body forward, while the trailing edge of cells detaches from the ECM (Friedl & Wolf, 2003).
An additional step during cell migration occurs if cells must move through the barrier of the ECM, as it must be degraded. It is well-documented that MT1-MMP localizes to the leading edge of the cells, where it is presumed to promote matrix cleavage directly at the cell-ECM interphase allowing for cell migration through it (Mori et al., 2002). Indeed, the proteolytic activity of MT1-MMP is frequently implicated in processes wherein MT1-MMP promotes both migration and invasion. The cleavage of laminin-5 specifically by MT1-MMP in a number of cell types has been shown to trigger the migration of cells (Koshikawa et al., 2000), while a soluble mutant form of MT1-MMP is incapable of promoting cell migration (Hotary et al., 2000), suggesting that membrane localized degradation is crucial for this function. In addition, CD44, a hyaluronan receptor, is notable for its involvement in cell migration and is also a substrate of MT1-MMP (Ueda et al., 2003). When the two proteins co-localize during their recruitment to the lamellipodia of migrating cells, the shedding of CD44 by MT1-MMP-mediated cleavage promotes the migration of cells over ECM (Itoh, 2006; Koshikawa et al., 2000). A proposed mechanism for the induction of cell migration suggests that this shedding disrupts the weak initial interactions between the substrate and CD44, allowing for stronger integrin interactions to form in their place (Cauwe et al., 2007).

However, a well-defined mechanism for MT1-MMP-mediated cell migration remains elusive, as a number of reports show that the expression of constitutively inactive forms of MT1-MMP still result in increased cell migration (Bonnans, Chou, & Werb, 2008; Hara et al., 2011). These reports posit that MT1-MMP may influence the ability of cells to adaptively use amoeboid-like migration mechanisms, where cells passively move through existing spaces in the ECM rather than rely on degradation. This discrepancy is supported by the recent focus on the non-proteolytic mechanism of MT1-MMP-mediated cell migration, as a transducer of the extracellular signal-regulated protein kinase (ERK) cascade. The activity of ERK, a kinase which influences the transcription of a number of genes, results the promotion of cell migration. Many reports have shown that the binding of TIMP-2 to MT1-MMP induces the activation of ERK in a dose and time-dependent manner to result in increased cell migration (D’Alessio et al., 2008; Sounni et al., 2010). However, the specific requirements for this signaling mechanism remain poorly understood, as some reports show that MT1-MMP must be in its active form to transduce
this signal, while others maintain that ERK activation occurs independently of MT1-MMP proteolytic activity.

In contrast to cell migration upon ECM protein substrates, MT1-MMP is directly involved in breaching the dense basement membrane of the ECM during cell invasion. Specific degradative cellular structures rich in MT1-MMP form in invasive cells. While structurally similar, these are called podosomes in normal cells and invadopodia if formed in cancerous cells (Om Alblazi & Siar, 2015). These actin-rich cellular protrusions form on the basal side of cells and actively recruit MT1-MMP, which functions as the principle proteolytic component to cleave ECM and activate MMP-2, which can degrade the underlying type-IV collagen (Poincloux, Lizárraga, & Chavrier, 2009). The formation of invadopodia has been described as an orchestrated, step-wise process where a membrane protrusion is created by actin polymerization, followed by MT1-MMP being shuttled to the distal end (Artym, 2006). Importantly, this formation relies on hundreds of individual proteins that regulate the initiation and regulation of actin dynamics, vesicular transport, and cellular adhesion (Alblazi & Siar, 2015). While the basal machinery of invadopodia still functions in the absence of MT1-MMP, the resulting structures are non-functional and do not degrade substrate (Buccione et al., 2009; Itoh et al., 2006).

While invadopodia and podosomes are distinct invasive cellular processes, comparisons can be drawn between the recruitment of MT1-MMP to actin structures of lamellopodia during cell migration, and suggests that MT1-MMP cell surface localization and cytoskeletal arrangements function together in multiple facets of cell motility. However, the role of MT1-MMP in promoting cell invasion through degradative structure is much more defined and understood, while the mechanisms by which it initiates and continues cell migration are less so (Artym, 2006). Indeed, while it is accepted that MT1-MMP promotes cell migration, the specific contributions of each of the diverse activities of MT1-MMP including catalytic activity, binding partners or localization are far from understood.
1.5 MT1-MMP and cell survival

The pro-invasive cell phenotype conferred by MT1-MMP extends beyond increased migration and ECM degradation, and includes its ability to promote cell survival through multiple mechanisms. Although MT1-MMP has long been described solely as a protease, a number of these non-proteolytic functions of MT1-MMP that impact cell behaviour are beginning to emerge (Hara et al., 2011; Pahwa, Stawikowski, & Fields, 2014).

Invading cells encounter new extracellular environments as they move from their point of origin, and for invasive epithelial cells which have breached the basement membrane this is typically the type-I collagen-rich interstitial matrix. Collagen-I is resistant to cleavage but to a few enzymes (including MT1-MMP), limits cell proliferation and induces apoptosis through BIK upregulation due to Src kinase activity (Assent et al., 2015). When cells expressing MT1-MMP were seeded into a collagen-1 matrix, they were able to evade collagen-induced apoptosis through an MT1-MMP-dependent mechanism (Maquoi et al., 2012). Yet this protection that MT1-MMP provides to cells against apoptosis is not necessarily dependent on proteolysis, as independent reports have shown that TIMP-2 binding to MT1-MMP results in the activation of Akt. The PI3-K/Akt cell signaling pathway is known for its involvement in the regulation of cell survival and proliferation (Grandage et al., 2005), and MT1-MMP-mediated Akt activation, which is responsive to the dosage and time of TIMP-2 exposure, results in the protection of cells against serum starvation-induced apoptosis. Yet TIMP-2, as an inhibitor to MT1-MMP, decreased cells’ resistance to collagen-I-induced apoptosis, suggesting that this regulation of cell survival is adaptive and dependent on environmental cues (Valacca, Tassone, & Mignatti, 2015).

MT1-MMP’s role in cell survival can also be mediated through its ability to alter cellular metabolism by increasing glycolysis. Elevated glycolysis, even in the presence of adequate oxygen, is termed the Warburg Effect. Activation of the Warburg effect by MT1-MMP is accomplished in part by the increased transcriptional activity of HIF-1α (hypoxia-inducing factor), regardless of oxygen conditions. This phenomenon involves multiple protein interactions and begins during processing and activation of MT1-MMP in the Golgi. At this cellular location, the cytoplasmic domain of MT1-MMP interacts with the adaptor protein Mint3, resulting in the recruitment and inhibition of Factor-
Inhibiting HIF-1α (FIH). HIF-1α – a transcription factor which mediates cells’ responses to hypoxia – is stabilized by the removal of this negative regulation and can alter expression of multiple genes, leading to elevated expression of glycolytic enzymes. Importantly, FIH typically only experiences negative regulation during hypoxic conditions, ensuring that a glycolytic shift in metabolism only occurs during times of low oxygen. MT1-MMP-mediated induction of the Warburg effect allows for an increase in substrate-level phosphorylation even in normoxia (Sakamoto & Seiki, 2010). This process has been found to be a key metabolic event in both macrophages and cancer cells, and increases cell survival. Specifically in macrophages, cells which had MT1-MMP expression knocked down exhibited less glycolysis and an impairment of cell migration (Sakamoto, Niiya, & Seiki, 2011), illustrating how the diverse functions of MT1-MMP combine to influence cell behaviour.

1.6 The cytoplasmic domain of MT1-MMP

Recent focus has turned to the cytoplasmic domain of MT1-MMP and its influence on protein function. Interestingly, this 20 amino acid sequence has been implicated in many of the diverse proteolytic and non-proteolytic functions of MT1-MMP, both directly and indirectly. The cytoplasmic domain is responsible for the membrane localization and internalization dynamics of MT1-MMP at the cell surface. The localization of MT1-MMP to the leading edge of migrating cells is disrupted upon cytoplasmic domain deletion (Lehti et al., 2000), as is its inclusion in lipid raft membrane compartments, distinct sections of the cell membrane concentrated with signaling molecules and cell surface receptors (Rozanov et al., 2003). In addition, both the clathrin- and caveolin-mediated mechanisms for MT1-MMP internalization are impaired when mutation are introduced within the cytoplasmic domain. During clathrin endocytosis, the LLY573 sequence within the cytoplasmic domain binds to the μ2 subunit of AP-2 to facilitate this internalization of MT1-MMP. The importance of this sequence for MT1-MMP internalization was revealed when alanine substitutions within LLY573 resulted in lack of binding of MT1-MMP to AP-2 and co-localization with clathrin, ultimately leading to marked decreases in MT1-MMP internalization (Uekita et al., 2001). The cytoplasmic domain is also necessary for the
localization of MT1-MMP to caveolae, special areas of the cell membrane which invaginate, as deletion of this domain abrogated the interaction of MT1-MMP with phosphorylated caveolin-1 at the cell surface (Labrecque et al., 2004). In addition, the recycling of MT1-MMP from internalized vesicles back to the cell surface has been found to depend on the cytoplasmic domain. The DKV^582 sequence in the cytoplasmic domain is necessary for regular re-insertion of internalized MT1-MMP-positive vesicles to the cell membrane (Wang et al., 2004).

The cytoplasmic domain also has a putative role in the activation of MT1-MMP during protein maturation, as it is a binding site for the adaptor protein Golgi Reassembly Stacking Protein 55 (GRASP55). GRASP55 associates with the Golgi complex and is involved in maintaining the structure of cisternae and the intracellular transport of both secreted and membrane proteins. In addition, GRASP55 has been identified as a binding partner of both MT1-MMP and furin, the pro-protein convertase responsible for MT1-MMP protein activation. The LLY^573 sequence within the cytoplasmic domain of MT1-MMP was identified as essential to mediate the binding of GRASP55 to MT1-MMP in MT1-MMP and Furin-positive locations in the Golgi, illustrating a role for the cytoplasmic domain in proper MT1-MMP maturation (Roghi et al., 2010). Once at the cell surface, the cytoplasmic domain has a further role in the MT1-MMP-mediated activation of pro-MMP-2, as the association of the trimeric pro-MMP-2, TIMP-2 and MT1-MMP complex with a second MT1-MMP protein fails to occur (Rozanov et al., 2001).

As previously described, the cytoplasmic domain of MT1-MMP is essential to MT1-MMP-mediated activation of the Warburg effect by binding Mint3 to sequester Factor-Inhibiting HIF-1α. Experiments which employ a mutant of MT1-MMP without a cytoplasmic domain show that the increase in glucose metabolism shown in MT1-MMP-expressing cells is abolished when the cytoplasmic domain is deleted (Sakamoto & Seiki, 2009). Similarly, other signaling events that are mediated by MT1-MMP, including the initiation of the ERK and Akt activation pathways, require the cytoplasmic domain (D’Alessio et al., 2008; Gingras et al., 2001). While the specific role that the cytoplasmic domain plays during these signaling events is not understood, both its phosphorylation,
which is accomplished by Src (Nyalendo et al., 2007) or PKC (Williams & Coppolino, 2011), or its involvement in the binding to other signaling proteins (Pratt et al., 2015; Uekita et al., 2004) have been implicated.

In these diverse ways, this short cytoplasmic domain has disproportionate regulatory influence over the diverse functions of MT1-MMP. Further elucidating the role of the cytoplasmic domain on MT1-MMP functions presents as a promising opportunity to examine the proteolytic and non-proteolytic contributions of MT1-MMP to cell invasion, migration and survival.

1.7 Hypothesis and objectives

MT1-MMP has multiple functions within cells that impart a pro-migratory phenotype which contributes to developmental and pathological processes. Importantly, these functions are both proteolytic and non-proteolytic in nature, suggesting that the mechanisms which regulate MT1-MMP are diverse. The recurring role of the cytoplasmic domain in MT1-MMP processes makes it an attractive target of examination to understand the regulation of the invasion and survival-promoting effects of MT1-MMP.

I hypothesize that the cytoplasmic domain of MT1-MMP is necessary for its promotion of cell migration and survival in MCF-7 breast cancer cells.

Using MT1-MMP-deficient MCF-7 human breast cancer cells, and three MT1-MMP expression constructs possessing different cytoplasmic domain mutations, a series of overexpression experiments were performed to assess changes in MT1-MMP function and cell behaviour. Expression of MT1-MMP with altered cytoplasmic domains in MCF-7 cells will help build a comprehensive model in which the role of its cytoplasmic domain in cell migration and survival is more clearly defined.
2 Materials and Methods

2.1 Acquisition of MT1-MMP cytoplasmic domain mutant constructs

Experimental cDNA constructs of human MT1-MMP with altered cytoplasmic domains were generated using PCR. All constructs were generated by PhD candidate Mario Cepeda, Western University as follows: full length human MT1-MMP and MT2-MMP cDNA were purchased (Origene, SC116190 and SC118648) and subjected to primer-directed mutagenesis to create two constructs. The cytoplasmic domain of MT1-MMP within the MT2CD MT1-MMP construct was replaced with the sequence for the MT2-MMP cytoplasmic domain. A stop codon was introduced into the ΔCD MT1-MMP construct to generate a truncated MT1-MMP protein possessing a transmembrane domain but lacking its cytoplasmic domain. The wild-type human sequence of MT1-MMP is referred to as full-length (FL) MT1-MMP. The DNA sequences of these generated clones were confirmed at Robarts Research Institute (London, ON) and subsequently subcloned into the pcDNA 3.3 expression vector (Origene). The domain structure and amino acid sequences of the cytoplasmic domain of the protein products of each clone is illustrated in Figure 1.

2.2 Cell culture conditions

Human adenocarcinoma breast cancer cell lines MDA-MB-231 and MCF-7 were a gift of Dr. Lynne-Marie Postovit, University of Alberta. All cells were cultured in Dulbecco’s Modified Eagles (DMEM)/F-12 medium (Thermo Fisher) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin and incubated at 37°C in a humidified incubator with 5% CO2. Serum-free conditions describe DMEM/F-12 media and 1% streptomycin/penicillin only. Cells were maintained below 80% confluency and passaged accordingly using 0.25% Trypsin-EDTA (Thermo Fisher).
Figure 1. Schematic representation of the human MT1-MMP protein domains and modifications made to cytoplasmic domain mutant constructs.

Multiple functional domains are present in MT1-MMP, including an N-terminal signal sequence (SS); an auto-inhibitory pro-domain; a catalytic domain, which contains the zinc-dependent active site; a flexible hinge region; a hemopexin-like domain which modulates protein interactions; a transmembrane domain (TM); and a cytoplasmic domain 20 amino acids in length. The three experimental constructs of MT1-MMP differ only in the sequences of their cytoplasmic domains (CD), the amino acid sequences of which are shown. FL (full-length) MT1-MMP possesses the wild-type human MT1-MMP cytoplasmic domain sequence, MT2CD MT1-MMP has the cytoplasmic domain sequence from MT2-MMP substituted, and ∆CD MT1-MMP contains a premature stop codon following Phe^{562} resulting in the termination of translation before the cytoplasmic domain. The site-for-site amino acid identity between the MT1-MMP and MT2-MMP cytoplasmic domains is indicated.
15

MT1-MMP

Pro-domain | Hinge | Hemopexin-like | Cytoplasmic

SS | Catalytic | Phe^{562}

FL MT1-MMP: \text{R}^{563}\text{RHTPRRLYYCQRSLLDKV}

MT2CD MT1-MMP: \text{M}^{563}\text{QRKAPRVLVCKRSLQEWV}

ΔCD MT1-MMP: –
2.3 Transfection and generation of stable cell lines

For transient transfection, MCF-7 and MDA-MB-231 cells were seeded at a density of 5x10^5 cells/mL and incubated for 24 hours. Following incubation, cells were transfected with 2.5 µg of FL MT1-MMP, MT2CD MT1-MMP, ΔCD MT1-MMP or GFP cDNA (all in pcDNA 3.3 TOPO, Thermo Fisher) using Lipofectamine 2000 (Thermo Fisher) and OPTI-MEM reduced serum media (Thermo Fisher) as per manufacturers’ instructions. For experiments utilizing transient transfection, cells were incubated for 24 hours following transfection prior to further use.

The pcDNA 3.3 vector used contained a neomycin mammalian selection marker. Stable MCF-7 cell lines, referred to as MT2CD MT1-MMP<sup>st</sup> and ΔCD MT1-MMP<sup>st</sup>, were generated by transfecting cells with each of the above constructs by the protocol outlined previously. Twenty four hours after transfection, these cells were seeded at 1:1000 dilution in DMEM/F-12 media containing 1 mg/mL neomycin (G148, TekNova) and maintained under selection conditions for four weeks in order to remove non-resistant cells that had not experience genomic insertion of the plasmid. Individual colonies representing a homogenous population arising from a single transfected cell were identified, isolated, expanded and assayed for MT1-MMP expression using qPCR. The additional stable cell lines MT1-MMP<sup>st</sup> and MT1-MMP<sup>H</sup> (high) were generated previously by PhD candidate Mario Cepeda by an identical protocol.

Parental MCF-7, and the stable cell lines MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup> and ΔCD MT1-MMP<sup>st</sup> were further subject to viral transfected with a cDNA encoding ZsGreen, a cytoplasmic localized fluorescent protein, to generate the respective stably expressing ZsGreen lines ZsMCF-7, ZsMT1-MMP<sup>st</sup>, ZsMT2CD MT1-MMP<sup>st</sup> and ZsΔCD MT1-MMP<sup>st</sup>. This procedure was generously performed by personnel in the laboratory of Dr. Hon Leong, Western University. This protocol involved treating cells with a lentivirus containing ZsGreen in polybrene, a reagent which aids in viral transfection. One day post transfection cells were treated with puromycin (2 µL/mL), which was replenished for three days. Successful stable transfection was determined using fluorescence microscopy. These cell lines were used exclusively in the avian chicken embryo assay.
2.4 Antibodies and reagents

For immunoblot analysis and immunofluorescence imaging, the following primary antibodies were used: rabbit anti-MT1-MMP (1:1000, AB6004, Millipore), mouse anti-MT1-MMP (1:200, SC-377097, Santa Cruz), mouse anti-β-Actin (1:1000, C4, Santa Cruz), and rabbit anti-phospho-histone-3 (pH3; 1:5000, SC-8656-R, Santa Cruz). Both MT1-MMP antibodies recognize the extracellular hinge region of MT1-MMP, and therefore are effective at identifying cytoplasmic-domain mutant proteins. Goat anti-mouse IgG (H+L) (Bio-Rad) and goat anti-rabbit IgG (H+L) (Thermo Fisher) HRP conjugates were used as secondary antibodies for immunoblot analysis (1:10000). For immunofluorescence, rabbit anti-MT1-MMP (1:200) was used in combination with one of anti-rabbit-IgG-Alexa488 or Alexa594 (Thermo Fisher) as the secondary antibodies (1:400). The MMP inhibitory reagent BB-94 (Batimastat) was supplied by Santa Cruz Biotechnology, while the ECM analog substance Matrigel was purchased from BD Biosciences.

2.5 Protein collection and immunoblot analysis

Transiently or stably transfected cells, seeded at a density of 5 x 10^5 cells/mL, were washed twice in cold PBS (pH 7.2) and lysed using cell lysis buffer (150 mM NaCl, 1% NP-40, 0.5% NaDC, 0.1% SDS, 50 mM Tris pH 8.0) supplemented with protease and phosphatase inhibitors (Thermo Scientific). Lysates were shaken on ice for 20 minutes and homogenized using the Misonix Ultrasonic Liquid Processors XL-2000 Series sonicator. Protein content of cell lysate was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher) and 15 µg aliquots were subject to SDS-PAGE. For analysis of MT1-MMP protein, 15% acrylamide gels were used, while for phospho-histone 3 protein analysis, 10% acrylamide gels were used. Following electrophoresis, proteins were transferred to PVDF membranes.

Blots were blocked using either 0.5% or 5% BSA in TBST, as directed by the primary antibody manufacturer, and probed using the appropriate primary antibodies. Incubation with primary antibodies occurred overnight at 4°C, and secondary antibodies for one hour
at room temperature. Blots were analyzed using QuantityOne software and Molecular Imager® ChemiDoc™ XRS System (BioRad).

2.6 Immunoprecipitation

Immunoprecipitation was performed using Protein A/G PLUS-Agarose immunoprecipitation reagent (Santa Cruz) as per manufacturer’s instructions, to detect MT1-MMP protein in the stable cell lines MT2CD MT1-MMPst and ΔCD MT1-MMPst. In brief, 2.5 million cells were seeded in a 60 mm cell culture dish (Corning) and incubated for 24 hours, lysed in lysis buffer, and homogenized using a 20G syringe. Lysates were cleared of non-specific binding proteins through incubation with a normal rabbit IgG (1 µg, Santa Cruz, SC-2027) and Protein A/G PLUS-Agarose (20 µL) by rotation for one hour at 4°C. Lysates were centrifuged, protein content of the supernatant was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher), and aliquots containing 250 µg of protein were incubated with 1 µg of either normal rabbit IgG or rabbit anti-MT1-MMP (Millipore) at 4°C overnight. Lysates were again centrifuged, the supernatant discarded, and the pellet washed and suspended in lysis buffer for use in SDS-PAGE. In order to avoid detection of the anti-MT1-MMP antibody on the final western blots, MT1-MMP in cell lysates was precipitated from lysates using rabbit anti-MT1-MMP (Millipore) and the blots were probed using mouse anti-MT1-MMP (Santa Cruz) with goat anti-mouse IgG HRP secondary antibodies.

2.7 Gelatin zymography

In order to assess the in vitro activation of pro-MMP-2 and examine the levels of MMP-2 protein isoforms in the media of transfected MCF-7 cells, gelatin zymography was performed as previously described (Toth & Fridman, 2013). As MCF-7 cells express very low levels of MMP-2 and TIMP-2 (Figueira et al., 2009), media was collected from MCF-7 cells transfected with MMP-2 or TIMP-2 cDNA. This conditioned media (CM), containing high levels of these secreted proteins, was used at a dilution of 30 µL pro-MMP-2 CM/mL incubation media or 10 µL TIMP-2 CM/mL incubation media to the incubation media of cells transfected with the MT1-MMP constructs. Conditioned media
was a gift of Mario Cepeda, Western University (Cepeda, 2012). All conditioned and incubation media lacked serum supplementation.

In brief, for zymography, 1.5 x 10^5 cells/mL were seeded in a 12-well culture dish (Corning) and transfected with GFP, FL MT1-MMP, MT2CD MT1-MMP or ΔCD MT1-MMP cDNA. Twenty four hours post-transfection, cells were incubated for 12 hours in serum-free media supplemented with the conditioned media containing pro-MMP-2, TIMP-2, both, or pro-MMP-2 with 20 μM BB-94. Following incubation, media was collected from transfected cells and 15 μL aliquots were subject to SDS-PAGE using 10% polyacrylamide gels containing 0.1% porcine gelatin (Sigma, G2500). Gels were then washed in protein renaturing buffer (2.5% Triton X-100), and incubated for two days at 37°C in a developing buffer (0.005 M CaCl2 in Tris buffered saline, pH 7.5) to allow the renatured MMPs to cleave the embedded gelatin. The gelatin was stained with a 0.5% Coomassie Brilliant Blue (BioRad) solution and progressively rinsed with a destaining buffer (50% methanol, 10% acetic acid) to reveal clear bands within the stained gel corresponding to the location of active MMPs. Gels were imaged using QuantityOne software and Molecular Imager® ChemiDoc™ XRS System (BioRad).

2.8 RNA extraction and qPCR

To verify the overexpression of *MT1-MMP* in prospective stable MT2CD MT1-MMP and ΔCD MT1-MMP cell lines, mRNA was extracted using the RNAeasy mini kit (Qiagen) following the manufacturer’s instructions. 1 μg of RNA was used to synthesize complementary DNA (cDNA) using qScript cDNA SuperMix (Quanta Biosciences). cDNA was then used for qPCR analysis to examine the levels of *MT1-MMP* and *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH), used as an internal control. qPCR was performed using SensiFAST SYBR No-ROX kit (FroggaBio) and the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Primers are described in Table 1. The reaction schedule was as follows: 10 s, 95°C; 30 s, 60°C; 30 s, 72°C for 40 cycles. The level of mRNA was calculated using the ΔΔCT method and shown as fold-change relative to MCF-7 cells. Statistical analysis was performed using the Cq values, not the calculated fold-change.
Table 1. Primer sequences used for qPCR.

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MT1-MMP</em></td>
<td>5’-GCAGAAGTTTTACGGCTTGCA-3’</td>
<td>5’-TCGAACATTGGCCTTGATCTC-3’</td>
</tr>
<tr>
<td><em>GAPDH</em></td>
<td>5’-ACCCACTCCTCCACCTTTGA-3’</td>
<td>5’-CTGTGGCTGTAGCCAAATTGT-3’</td>
</tr>
</tbody>
</table>
2.9 Immunofluorescence microscopy and MT1-MMP signal area quantification

MCF-7 cells were transfected with either FL MT1-MMP, MT2CD MT1-MMP or ΔCD MT1-MMP cDNA and incubated for 24 hours. Transfected cells were then seeded onto poly-L-lysine-coated glass coverslips (No. 0) and incubated for a further 24 hours. Cells were then fixed in 10% formaldehyde and blocked using 1% BSA in PBS (pH 7.2), the latter to prevent non-specific antibody binding. For immunofluorescence analysis cells were incubated for one hour at room temperature using rabbit anti-MT1-MMP (1:200, AB6004, Millipore), followed by one hour incubation with the secondary antibody anti-rabbit-IgG-Alexa488 (Thermo Fisher, 1:400). F-actin was stained with Alexafluor633-phalloidin (Thermo Fisher; 1:40 dilution) and nuclei stained with 4’,6-diamidino-2-phenylindole (DAPI; 1 µL/mL, BioShop Canada). Coverslips were washed, mounted using ProLong Gold Antifade Mountant (Thermo Fisher), and imaged using a Nikon A1R+ confocal microscope using NIS Elements software (Nikon).

Image analysis was performed using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA). For signal area analysis, individual colour channels were isolated, converted to binary, and the area of isolated single cells measured using the software. The area of green channels, corresponding to MT1-MMP, was then divided by the signal area taken of the same cell on the red channel, representing F-actin.

2.10 Fluorescent gelatin degradation assay

To examine the proteolytic abilities of cells to degrade ECM substrates, cells were cultured on coverslips coated with Oregon Green 488 conjugated to porcine gelatin (Thermo Fisher). These were generated by the following protocol: coverslips were sterilized with 0.1 M sodium hydroxide, coated in poly-L-lysine, and fixed using 0.5% glutaraldehyde. Oregon green gelatin was diluted 1:8 in 0.2% untagged porcine gelatin (Sigma) in PBS. Droplets of this gelatin suspension were placed onto parafilm, and coverslips flipped and incubated on top of them for 10 minutes. These coated coverslips were washed in PBS, and stored in cold DMEM/F12 media with 10X non-essential
amino acids (Thermo Fisher). These amino acids quenched reactive oxygen species generated by glutaraldehyde to protect the integrity of the gelatin.

MCF-7 cells were transfected with either GFP, MT1-MMP, MT2CD MT1-MMP or ΔCD MT1-MMP cDNA, and 25 000 cells were seeded onto gelatin slips. These were incubated for 24 hours, fixed in 10% formalin and permeabilized with 0.4% Triton X-100. Slips were then prepared for immunofluorescence microscopy using DAPI (1 μL/mL, BioShop Canada), Alexafluor633-phalloidin (Thermo Fisher, 1:40 dilution), and rabbit anti-MT1-MMP (Millipore, 1:200) with goat anti-rabbit-IgG-Alexafluor594 (Thermo Fisher, 1:400). Coverslips were washed, mounted using ProLong Gold Antifade Mountant (Thermo Fisher), and imaged using a Nikon A1R+ confocal microscope using NIS Elements software (Nikon).

2.11 Transwell cell motility assays

Cell motility of both transiently and stably transfected MCF-7 cells was assessed using 24-well 8 μm pore transwell inserts (Corning Costar) using the method described by Marshall, 2011. Cells were seeded (20 000 cells/well) in the upper chamber of transwells in serum-free media and induced to migrate towards the lower chamber containing DMEM/F-12 media supplemented with 10% FBS. Cells were incubated for 48 hours. Migration assays were performed using uncoated transwell inserts, while invasion assays were performed by coating the upper chamber of transwells with 20% Matrigel (BD Biosciences). To quantify cell motility, cells were fixed in 100% methanol, and non-motile cells remaining in the upper surface of the transwell removed with a damp cotton swab. Cells on the bottom of transwells were stained using a 0.5% solution of crystal violet in distilled water. These stained transwells were then individually destained in 33% acetic acid, and the absorbance of this solution at 490 nm was quantified using the Microplate Reader Model 3550-UV (BioRad) as a correlative measurement of cell number.
2.12 Wound closure migration assay

The two dimensional motility of parental MCF-7 and the stable cell lines MT1-MMPst, MT2CD MT1-MMPst, and ∆CD MT1-MMPst was assessed using a wound closure assay. MT1-MMP-expressing stable cell lines or MCF-7 cells were seeded on a 35 mm cell culture dish (Corning) at confluency (5x10^5 cells/mL) and incubated for 24 hours. Using a 100 µL pipette tip, an area through the monolayer approximately 5 mm in width was cleared to create a “wound”, whose re-population and closure was subsequently monitored using bright-field microscopy (Leica DM16000 B microscope) for 72 hours. Wound closure was assessed using ImageJ software by measuring the width between the wound edges at each time point in captured images, and subtracting the initial wound size to calculate distance migrated.

2.13 Measurements of cell proliferation

Cell proliferation under serum-supplemented and serum-free conditions was assessed using CellTiter® 96 AQueous One Solution (Promega) as per manufacturer’s instructions. The CellTiter® protocol utilizes the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt], which is reduced by metabolically active cells to a coloured quantifiable product, formazan. Parental MCF-7 cells and MT1-MMPst, MT2CD MT1-MMPst, ∆CD MT1-MMPst cell lines were seeded in triplicate in a 96 well culture plate at a density of 5x10^4 cells/mL in either serum-free media or media supplemented with 10% FBS and incubated for nine days. Immediately after seeding and at three day intervals, CellTiter® reagent was added to culture wells and incubated at 37°C for 2 hours. Absorbance at 490 nm was then measured using Microplate Reader Model 3550-UV (BioRad), and was used a measurement of the number of living cells in culture.

In addition, phospho-histone 3 protein was collected from cells incubated for six days in either serum-supplemented or serum-free media. 1.6 x 10^5 cells were seeded onto a 6-well culture dish for protein collection. As many cells remained loosely attached after six days of serum-starvation, cells were immediately treated with lysis buffer rather than initially washed with PBS.
2.14 Cell viability and apoptosis
Parental MCF-7 cells and MT1-MMP\textsuperscript{st}, MT2CD MT1-MMP\textsuperscript{st}, ΔCD MT1-MMP\textsuperscript{st} cell lines were seeded in triplicate in a 96 well culture plate at a density of 5x10\textsuperscript{4} cells/mL in either serum-free media or media supplemented with 10% FBS and incubated for six days, and their viability and caspases activity were assessed using the ApoTox-Glow Triplex Assay (Promega) as per manufacturer’s instructions. To assess cell viability, cells were treated with the cell-permeable GF-AFC fluorescent substrate (5.0 nM) and incubated at 37°C for 1 hour, and fluorescence measured using Tecan Infinite M1000 with Magellan 7 software (400\textsubscript{Ex}/505\textsubscript{Em}). Cells were then immediately assayed for apoptosis via treatment with a luminogenic substrate containing the DEVD sequence susceptible to cleavage by caspases 3 and 7. After one hour of incubation at 37°C, luminescence was measured using Modulus™ II Microplate Multimode Reader and GloMax® - Multi Detection System with Instinct® software as a metric of apoptosis.

2.15 Three-dimensional (3D) cell culture
MCF-7 cells and MT1-MMP-expressing stable cell lines MT1-MMP\textsuperscript{st}, MT2CD MT1-MMP\textsuperscript{st} and ΔCD MT1-MMP\textsuperscript{st} were cultured in a 3D Matrigel matrix to assess morphological features as described by Cvetković, Goertzen, & Bhattacharya, 2014. Glass-bottom cell culture dishes (MatTek) were prepared by coating the glass with 50% Matrigel (BD Biosciences) in serum-free media, allowing the matrix to solidify, and seeding 2.5x10\textsuperscript{4} cells in 50% Matrigel in serum-free media above this layer to result in cells completely suspended in matrix. Cultures were incubated in either serum-supplemented (10% FBS) or serum-free conditions. Cell colony morphology was monitored for five days using bright-field microscopy (Leica DM16000 B microscope with Leica DFC425 camera) at 10X magnification, and representative 50 µm z-stack images (2 µm interval) taken at random locations across the matrix were captured using Leica MMAF software (Metamorph®). After five days, cells within the matrix were fixed using 20% acetone, 80% methanol and prepared for immunofluorescence using Alexafluor633 phalloidin (Thermo Fisher, 1:100) and DAPI (1 µL/mL, BioShop Canada) to examine F-actin and nuclei, respectively. Prepared samples were imaged using a Nikon
A1R+ confocal microscope and NIS Elements software (Nikon), capturing Z-stacks of approximately 100 µm.

To quantify the morphology of cell colonies, images were analyzed blindly using ImageJ software. Images containing fewer than 8 colonies were excluded from analysis. Cellular protrusions were defined as discrete, unbranched extensions of a given length that emanated from a round cell colony. The number of protrusions on all cell colonies in an image were counted, with longer protrusions counted as multiples of the average protrusion length seen in that image to adjust for the heterogeneity in size observed. The score, representing the total number of protrusions, was divided by the total number of colonies to arrive at the final measurement of protrusions per colony. In serum-free conditions, the additional measurement of fractured colony morphology was counted. Fractured colonies were defined as having multiple small vesicles surrounding a central colony. The number of fracturing colonies is reported as a percentage of total colonies.

2.16 Firefly luciferase transcriptional activity analysis

MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup>, ∆CD MT1-MMP<sup>st</sup> and MCF-7 cells were seeded at a density of 3000 cells/well in a 96-well culture plate (Corning) and incubated for 24 hours. Following the method previously described, cells were transfected with 0.2 µg of mammalian 3xAP1pGL3 (addgene, Plasmid #40342) or p1242 3xKB-L (addgene, Plasmid #26699) luciferase expression plasmids to detect the transcriptional activity of AP-1 and NF-κB transcription factors, respectively. Twenty four hours post transfection, cells were lysed and treated with Firefly Luciferase Glow Assay reagents (Thermo Fisher) according to manufacturer’s instructions. Luminescence was detected using Modulus™ II Microplate Multimode Reader and GloMax® - Multi Detection System with Instinct® software.

2.17 Avian embryo tumour vascularization assay

The vascularization of artificial tumours of ZsGreen-tagged ZsMCF-7, ZsMT1-MMP<sup>st</sup>, ZsMT2CD MT1-MMP<sup>st</sup> and Zs∆CD MT1-MMP<sup>st</sup> cells in Matrigel was assessed in avian embryos following the technique developed by Dr. Hon Leong, Western University.
Embryos, materials and facilities were generously provided by Dr. Hon Leong, Western University. Chick embryos were incubated in culture boats for nine days at 37°C in a humidified chamber. A superficial wound was introduced to the vasculature of the chorioallantoic membrane (CAM) of embryos and 10 µL of Matrigel containing ~500 000 cells was injected into the wound. Embryos were incubated for a further eight days, and tumours were examined using fluorescent stereomicroscopy to assess if vasculature had developed at the tumour site. Vascularization was determined to have occurred if the wound site showed capillaries associated with a tumour which had successfully grown beneath the CAM layer. This was accomplished by comparing observed vasculature using bright field microscopy to areas of contrast observed under fluorescent conditions. Vascularization was quantified as a binary measurement, indicated by the presence or absence of tumour-localized vasculature in individual embryos.

2.18 Immunoblot densitometry analysis

Quantitative densitometric analysis of immunoblots was performed using QuantityOne software (Bio-Rad). Band intensity was calculated for MT1-MMP, phospho-Histone 3 and β-actin using three independent biological samples. Intensity is shown as a ratio of MT1-MMP signal standardized to the β-actin signal and then compared to control conditions.

2.19 Statistics

Statistical analysis and graphing was performed using GraphPad Prism version 6.0 (GraphPad software, La Jolla, CA USA). Data is presented as mean ± SEM. Means were compared using one-way ANOVA followed by Tukey’s post-hoc test, with levels of statistical significance identified as follows: ****, p ≤ 0.0001, ***, p ≤ 0.001, **, p ≤ 0.01, *, p ≤ 0.05.
Chapter 3

3 Results

3.1 Cytoplasmic domain deletion or substitution altered the activation profile of MT1-MMP protein in transiently transfected breast cancer cells.

To determine the role of the cytoplasmic domain in MT1-MMP protein activation, MCF-7 cells were transiently transfected with FL MT1-MMP, MT2CD MT1-MMP, or ∆CD MT1-MMP cDNA, or GFP cDNA as a control, lysed, and whole-cell lysate subject to SDS-PAGE and western blot analysis. The activation profile, a description of the levels of pro-, active, and degraded isoforms of MT1-MMP, was assessed for each construct. MCF-7 cells, which are deficient in MT1-MMP (Figueira et al., 2009), showed no detectable levels of MT1-MMP protein (Figure 2 a). MCF-7 cells expressing FL MT1-MMP showed an activation profile of MT1-MMP protein which included the pro- (63 kDa) and active (60 kDa) forms, as well as a 44 kDa degradation product (Cho et al., 2008). Transfection with MT2CD MT1-MMP resulted in levels of pro-MT1-MMP protein that were not significantly different from FL MT1-MMP expressing cells; however there was significantly less active MT1-MMP ($p < 0.05$) and no degradation product was observed (Figure 2 b). Transfecting MCF-7 cells with ∆CD MT1-MMP resulted in significantly higher levels of a lower molecular weight (59 kDa) truncated form of pro-MT1-MMP, as compared to FL MT1-MMP transfection ($p < 0.01$). In addition, ∆CD MT1-MMP transfection also resulted in the generation of a smaller active form of MT1-MMP (56 kDa), and no observed degradation product.

The difference in the activation profiles of MT1-MMP following transfection with MT2CD MT1-MMP and ∆CD MT1-MMP cDNAs - which despite both having altered cytoplasmic domains showed an absence or presence of active MT1-MMP, respectively - was further examined by considering alternative methods of protein activation aside from the well-documented furin-mediated cleavage of pro-MT1-MMP. As MMP members are capable of auto-activation (Kang, Nagase, & Pei, 2002), MMP-mediated activation was explored as a possible reason for the activation of MT1-MMP protein following transient
Figure 2. Cytoplasmic domain-altered MT1-MMP proteins showed different activation profiles and were activated differently in transiently transfected breast cancer cells.

(a) Western blot analysis was performed to examine the activation profiles and protein levels of MT1-MMP in MCF-7 cells transfected with FL MT1-MMP, MT2CD MT1-MMP, or ΔCD MT1-MMP constructs, or a control GFP plasmid. FL MT1-MMP transfection resulted in the generation of the 63 kDa pro- and 60 kDa active forms of the protein. MT2CD MT1-MMP transfects showed bands of active MT1-MMP with significantly less intensity ($p < 0.05$) than FL MT1-MMP. ΔCD MT1-MMP transfection resulted in significantly higher levels of a truncated pro-MT1-MMP protein (59 kDa) as compared to FL MT1-MMP ($p < 0.01$). Only cells transfected with FL MT1-MMP showed the 44 kDa MT1-MMP degradation product. β-actin is shown as a loading control. (b) Three independent protein samples were examined by western blot and subject to densitometry analysis. MT1-MMP protein band densities were standardized to β-actin, and control cells. Means are presented ± SEM, and were compared using one-way ANOVA; different letters of same case signify differences at $p < 0.05$. (c) Western blot analysis of MCF-7 cells treated with the pan-MMP inhibitor BB-94 and subsequently transfected with FL MT1-MMP or ΔCD MT1-MMP cDNA. With increasing concentration of BB-94, the levels of active ΔCD MT1-MMP protein decreased, and were significantly lower than DMSO treated cells after 30 µM treatment ($p < 0.05$). BB-94 treatment had no effect on levels of active MT1-MMP protein following FL MT1-MMP transfection. (d) Three independent protein samples were examined by western blot for levels of active MT1-MMP protein and subject to densitometry analysis. Pro and active-MT1-MMP band densities were normalized to β-actin, and DMSO treated cells. Means are presented ± SEM, and were compared using one-way ANOVA; different letters of same case signify differences at $p < 0.05$. (e) Western blot analysis of MT1-MMP protein from MDA-MB-231 breast cancer cells transfected with MT1-MMP cytoplasmic domain-altered constructs or GFP (control). MDA-MB-231 cells constitutively express MT1-MMP, mostly in the active form. FL MT1-MMP transfection resulted in an increase in the levels of both the pro- and active form of the protein, while transfection with MT2CD MT1-MMP resulted only in an increase of pro-MT1-MMP.
ΔCD MT1-MMP showed a significant increase in the level of pro-MT1-MMP as compared to control or FL MT1-MMP transfected cells ($p < 0.001$). (f) Three independent protein samples were examined by western blot for levels of pro- and active MT1-MMP protein and subject to densitometry analysis. Band densities were normalized to β-actin, and control cells. Means are presented ± SEM, and were compared using one-way ANOVA; different letters of same case signify differences at $p < 0.001$. 
transfection of ∆CD MT1-MMP in MCF-7 cells. MCF-7 cells were transiently transfected with either FL MT1-MMP or ∆CD MT1-MMP and treated with BB-94, a pan-MMP inhibitor molecule. Western blot analysis of lysate from these cells show that BB-94 treatment significantly decreased by approximately 360% the level of active MT1-MMP in ∆CD MT1-MMP transfected cells ($p < 0.05$), while the level of active MT1-MMP in FL MT1-MMP cells was not altered by the treatment (Figure 2 c, d).

Considering the abundance of distinct breast cancer cell lines, which correspondingly show variation in MT1-MMP expression levels and protein activation profiles, MT1-MMP constructs were also transfected into MDA-MB-231 cells (Figure 2 e, d). These human breast cancer cells, which naturally express high levels of MT1-MMP, were used to confirm that observations were not cell-line specific. Transfection with a control GFP plasmid demonstrated that MDA-MB-231 cells endogenously produce MT1-MMP protein, most of which present in its active form. Transfection with FL MT1-MMP increased the levels of both pro- and active MT1-MMP protein relative to GFP transfection. Transfection with MT2CD MT1-MMP resulted only in the increase of pro-MT1-MMP protein levels, a result similar to what was observed in MCF-7 cells. ∆CD MT1-MMP transfection resulted in a significant increase in the level of pro-MT1-MMP protein relative to all other constructs ($p < 0.001$).

3.2 Deletion or substitution of the cytoplasmic domain of MT1-MMP affected its cell surface localization in transiently transfected MCF-7 cells.

To assess the cell surface protein localization of MT1-MMP and cytoplasmic domain mutants of MT1-MMP, MCF-7 cells were transiently transfected and assessed by immunofluorescence microscopy. The cell surface distribution of MT1-MMP protein showed distinct and different patterns following transfection with either FL MT1-MMP, MT2CD MT1-MMP or ∆CD MT1-MMP (Figure 3 a). In mock transfected cells, very little MT1-MMP protein was observed, consistent with the low endogenous expression in this cell line. Transfection with FL MT1-MMP resulted in a distribution of MT1-MMP protein that localized to the periphery of cells in a pattern similar to F-actin. In contrast,
Figure 3. Alteration to the cytoplasmic domain of MT1-MMP disrupted its cell surface localization in transiently transfected MCF-7 breast cancer cells.

(a) Confocal microscopy images of cells transfected with MT1-MMP constructs and stained with MT1-MMP specific antibodies. Confocal microscopy images are displayed as merged images of MT1-MMP protein (green), F-actin (red), and nuclei (blue) (top panels) and as isolated MT1-MMP signal (middle panels). Mock-transfected cells showed little MT1-MMP protein, while transfected cells showed a cell surface distribution of MT1-MMP protein that differs depending on the nature of the cytoplasmic domain mutation. FL MT1-MMP transfection resulted in widespread distribution of MT1-MMP at the cell surface, while MT2CD MT1-MMP transfection showed a filamentous distribution pattern. ∆CD MT1-MMP transfection resulted in dense puncta of MT1-MMP signal at the surface of cells. In addition, neither MT2CD MT1-MMP nor ∆CD MT1-MMP transfection resulted in cell surface MT1-MMP protein localized to actin-containing protrusions, while MT1-MMP in FL MT1-MMP cells did localize to these peripheral structures. This phenomenon is highlighted in the inset images, where white arrows indicate distal, actin-rich cell surfaces (A1) and the closest MT1-MMP signal (A2). (b) The degree of co-localization of MT1-MMP with F-actin in individual transfected cells was quantified to assess the variation in patterns of MT1-MMP localization observed. Using ImageJ software, the signal area of MT1-MMP in an entire cell was divided by that of F-actin in individual cells to calculate a co-localization ratio. Both MT2CD MT1-MMP ($p < 0.01$) and ∆CD MT1-MMP ($p < 0.0001$) transfected cells show significantly less co-localization of MT1-MMP signal area compared to F-actin than do FL MT1-MMP transfected cells. Means are presented as relative signal area + SEM and were compared using one-way ANOVA; *** $p \leq 0.001$. Scale bars = 50 µm.
neither MT2CD MT1-MMP nor ∆CD MT1-MMP transfection resulted in peripheral MT1-MMP protein localization. Notably, MT1-MMP protein was not observed at the distal ends of actin-rich cell extensions in cells transfected with MT2CD MT1-MMP or ∆CD MT1-MMP, while this was observed following FL MT1-MMP transfection. Additionally, while the pattern of labelled MT1-MMP protein had relatively uniform intensity following FL MT1-MMP transfection, this patterning was more diffuse with MT2CD MT1-MMP and punctate following ∆CD MT1-MMP transfection. The patterns of MT1-MMP protein distribution observed were confirmed to be cell surface as separate experiments using Triton X-100 to permeabilize cell membranes showed different fluorescence patterns; notably distinct fluorescent puncta in all conditions. Additionally, secondary antibody-only treatment showed no fluorescent signal (data not shown).

This cortical localization of MT1-MMP protein was quantified by measuring and comparing the fluorescence signal area of MT1-MMP in captured images to the fluorescence signal area of F-actin, which was taken to represent the total cell surface area, to generate the relative signal area of MT1-MMP (Figure 3 b). Following FL MT1-MMP transfection, the degree of MT1-MMP and F-actin co-localization was significantly greater than that observed following MT2CD MT1-MMP (p < 0.01) and ∆CD MT1-MMP (p < 0.0001) transfection, representative of the disruption in protein localization that was observed.

3.3 Alteration to the cytoplasmic domain by deletion or substitution abolished MT1-MMP-mediated pro-MMP-2 activation and gelatin degradation.

Having observed irregular protein activation profiles upon cytoplasmic domain alteration to MT1-MMP, the proteolytic activity of the MT1-MMP protein generated from these constructs was assessed. First, the ability of MT1-MMP to activate pro-MMP-2 was examined using gelatin zymography. MCF-7 cells, which are deficient in MMPs and TIMPs, were transfected with FL MT1-MMP, MT2CD MT1-MMP, ∆CD MT1-MMP, or GFP, and incubated with control media or conditioned media containing pro-MMP-2 for 12 hours. Subsequent examination of culture media by gelatin zymography showed that upon incubation with pro-MMP-2, only the media from cells transfected with FL MT1-
MMP contained the intermediate and active forms of MMP-2 (Figure 4). As the process of MT1-MMP-mediated activation of pro-MMP-2 is enhanced by the addition of low levels of TIMP-2 (Itoh et al., 2001), TIMP-2 conditioned media was also added. Incubation with pro-MMP-2 and TIMP-2 did increase the levels of intermediate and active MMP-2 in FL MT1-MMP-transfected cells as expected, but did not enhance pro-MMP-2 protein activation in cells transfected with MT2CD MT1-MMP or ΔCD MT1-MMP. The addition of the MMP inhibitor BB-94 during incubation effectively attenuated pro-MMP-2 activation in FL MT1-MMP transfected cells, illustrating that this process required both the catalytic activity and the cytoplasmic domain of MT1-MMP.

The ability of cells expressing MT1-MMP or cytoplasmic domain-altered forms of MT1-MMP to degrade ECM substrates was further examined by assessing the degradation of a fluorescent gelatin substrate. MCF-7 cells were transiently transfected with MT1-MMP constructs and seeded onto glass coverslips that had been coated with fluorescent gelatin, incubated for 48 hours, and prepared for fluorescence confocal microscopy. The ability of cells to degrade this substrate could be assessed by observing black holes within the green fluorescent gelatin, representing an absence of fluorescent signal. While MT1-MMP protein (red) was observed to co-localize with actin puncta (purple) in cells following transfection with either FL MT1-MMP, MT2CD MT1-MMP or ΔCD MT1-MMP, only cells transfected with FL MT1-MMP exhibited degradation of the underlying gelatin matrix (Figure 5).

3.4 Deletion or substitution of the cytoplasmic domain of MT1-MMP increased cell migration and invasion in transiently transfected MCF-7 cells

As transfection with the different MT1-MMP constructs resulted in aberrant protein localization and different abilities of MT1-MMP to degrade ECM, the migratory and invasive capabilities of transfected cells was investigated. Migration was defined in this assay as unobstructed cell movement, while invasion involved cell movement through the biological barrier of Matrigel. MCF-7 cells were transiently transfected with GFP, FL MT1-MMP, MT2CD MT1-MMP or ΔCD MT1-MMP and the migratory and invasive abilities of cells assessed using a transwell assay (Figure 6). Significantly more cells
Figure 4. Substitution or deletion of the cytoplasmic domain of MT1-MMP disrupted MT1-MMP-mediated activation of pro-MMP-2 in transiently transfected MCF-7 cells.

Cells were transfected with MT1-MMP constructs, incubated for 24 hours, and subject to 12 hour treatment with serum-free conditioned media (CM) in order to examine the activation of pro-MMP-2 using gelatin zymography. Bands of degraded gelatin show the location of renatured MMPs. Cells transfected with FL MT1-MMP show low levels of active MMP-2 protein following incubation with control media (Control, top). After incubation with exogenous pro-MMP-2 conditioned media, FL MT1-MMP transfection resulted in high levels of the intermediate and active forms of MMP-2 (second from top). Addition of TIMP-2 during incubation did not enhance the activation of pro-MMP-2 in MT2CD MT1-MMP or ΔCD MT1-MMP transfected cells, but did increase the levels of intermediate and active MMP-2 proteins in FL MT1-MMP transfected cells (second from bottom). Treatment with BB-94, an MMP inhibitor, during incubation effectively attenuated MMP-2 activation in FL MT1-MMP transfected cells (bottom).
Figure 5. Substitution or deletion to the cytoplasmic domain of MT1-MMP disrupted MT1-MMP-mediated gelatin degradation.

Confocal microscopy images of transfected MCF-7 cells (FL MT1-MMP, MT2CD MT1-MMP, ∆CD MT1-MMP or mock) seeded and incubated on an Oregon Green-tagged gelatin substrate. MT1-MMP puncta co-localized with actin puncta in all transfected cells (merged images, pink). However, only FL MT1-MMP-expressing cells showed gelatin degradation at sites corresponding to the location of transfected cells (black holes within green substrate). White arrows indicating the location of MT1-MMP are transposed across all images. Images taken at 60x magnification, scale bars = 50 µm.
**Figure 6.** Substitution or deletion of the cytoplasmic domain of MT1-MMP increased the migration and invasion of transfected MCF-7 breast cancer cells.

(a) To examine migration, cells were transfected with GFP (control), FL MT1-MMP, MT2CD MT1-MMP, or ΔCD MT1-MMP cDNA, incubated for 24 hours, and seeded into the upper chamber of a transwell insert in serum-free media. Cells were induced to migrate towards the serum-supplemented media in the lower chamber. After 48 hours, successfully migrated cells were quantified and compared to control cells. Significantly more MT2CD MT1-MMP and ΔCD MT1-MMP transfected cells migrated through the transwell pores than did control cells ($p < 0.05$). FL MT1-MMP transfection resulted in no significant difference in migration compared to GFP cells ($p > 0.05$). The results of three biological replicates are presented as mean relative absorbance + SEM, and were compared using one-way ANOVA; different letters signify differences at $p < 0.05$.

(b) To examine invasion, a similar protocol was employed as above with the modification that the upper surface of transwells were coated in 20% Matrigel to provide a physical barrier to motility. Significantly more cells transfected with MT2CD MT1-MMP or ΔCD MT1-MMP invaded through the Matrigel and transwell pores than did control cells ($p < 0.001$). Cells transfected with FL MT1-MMP showed no significant difference in invasion compared to GFP cells. The results of three biological replicates are presented as mean relative absorbance + SEM, and were compared using one-way ANOVA; different letters signify differences at $p < 0.001$. 

migrated following transfection with either MT2CD MT1-MMP (~24% increase) or ΔCD MT1-MMP (~18% increase) compared to control or FL MT1-MMP transfected cells ($p < 0.05$; Figure 6a). Similarly, transfection with MT2CD MT1-MMP significantly increased the number of invaded cells by ~104%, while ΔCD MT1-MMP increased this number by 84%, as compared to control transfected cells ($p < 0.001$; Figure 6b), while FL MT1-MMP transfected cells showed a 14% increase in cell invasion compared to control cells.

### 3.5 Stable expression of MT2CD MT1-MMP or ΔCD MT1-MMP in MCF-7 cells did not result in gelatin degradation but increased cell migration

Due to the potentially confounding effects of artificially high and brief MT1-MMP expression in transiently transfected cells, MCF-7 cell lines were generated which stably expressed FL MT1-MMP, MT2CD MT1-MMP and ΔCD MT1-MMP to observe protein activity in a more regulated setting. These cell lines, called MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup> and ΔCD MT1-MMP<sup>st</sup> were confirmed to have both increased expression of *MT1-MMP* and increased amount of MT1-MMP protein as compared to parental cells, assessed by qPCR and western blot respectively (Figure 7a, b).

These stable cell lines were seeded on fluorescent gelatin-coated coverslips and incubated for 48 hours to detect matrix degradation. As was observed following transient transfection, only MT1-MMP<sup>st</sup> cells exhibited gelatin degradation (Figure 7c). In addition, MT1-MMP protein in all of MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup> and ΔCD MT1-MMP<sup>st</sup> co-localized with actin puncta in cells, a pattern consistent with results from transient transfection.

The migratory capability of stable cell lines was also assessed to compare with the results obtained following transient transfection. First, a wound closure assay was conducted whereby cells were allowed to grow to confluence and a wound was introduced to disrupt the monolayer. The closure of the wound was monitored over three days and used as a metric of cell migration. All of MT1-MMP<sup>st</sup> (~42% increase), MT2CD MT1-MMP<sup>st</sup> (~43% increase) and ΔCD MT1-MMP<sup>st</sup> (~54% increase) cells migrated significantly further into the wound at all time points than did MCF-7 parental cells ($p < 0.001$),
Figure 7. MCF-7 cell lines stably expressing FL MT1-MMP, MT2CD MT1-MMP and ΔCD MT1-MMP showed different gelatin degradation capabilities.

MCF-7 cells were transfected and selected for stable plasmid integration and increased expression of *MT1-MMP*. Successful individual clones were isolated and called MT1-MMP\textsuperscript{st}, MT2CD MT1-MMP\textsuperscript{st}, ΔCD MT1-MMP\textsuperscript{st}. (a) qPCR analysis of stable cell lines showed that MT1-MMP\textsuperscript{st} (*p* < 0.05) MT2CD MT1-MMP\textsuperscript{st} (*p* < 0.0001) and ΔCD MT1-MMP\textsuperscript{st} (*p* < 0.0001) expressed *MT1-MMP* at significantly higher levels than parental MCF-7 cells. The results of three biological replicates are shown as mean fold-change ± SEM, and differences assessed using one-way ANOVA; different letters signify differences at *p* < 0.05. (b) Stable cell lysates were collected and subject to immunoprecipitation to detect MT1-MMP protein levels. Both MT2CD MT1-MMP\textsuperscript{st} and ΔCD MT1-MMP\textsuperscript{st} showed increased levels of MT1-MMP protein compared to parental cells. The high-expressing MT1-MMP\textsuperscript{H} cell line was used as a positive control. Pulldown (PD) immunoprecipitation with a control, normal rabbit species IgG showed no MT1-MMP protein when subsequently probed. (c) Stable cell lines MT1-MMP\textsuperscript{st}, MT2CD MT1-MMP\textsuperscript{st}, ΔCD MT1-MMP\textsuperscript{st} and MCF-7 cells were seeded on Oregon Green-tagged gelatin and prepared for immunofluorescence using confocal microscopy. Merged images show MT1-MMP (red), F-actin (purple) and nuclei (blue; top panels). All of MT1-MMP\textsuperscript{st}, MT2CD MT1-MMP\textsuperscript{st}, ΔCD MT1-MMP\textsuperscript{st} showed a punctate pattern of MT1-MMP protein, which co-localizes with actin puncta (pink signal, top panels). Only MT1-MMP\textsuperscript{st} cells showed degradation of gelatin substrate, indicated by black holes that correspond to cells expressing MT1-MMP. White arrows indicating the location of MT1-MMP are transposed across all images. Images taken at 60x magnification, scale bars = 50 µm.
though they were not significantly different from each other (Figure 8 a, b). To corroborate this result, a transwell migration assay was also performed. After 48 hours of incubation, significantly more MT1-MMPst (~19% increase, \( p < 0.01 \)), MT2CD MT1-MMPst (~14% increase) and ΔCD MT1-MMPst (~15% increase; \( p < 0.05 \)) cells migrated than parental MCF-7 cells (Figure 8 c). Stable MT1-MMP expressing MCF-7 cell line data thus demonstrated that while the cytoplasmic domain of MT1-MMP was required for substrate proteolysis, it was dispensable in mediating cell migration.

3.6 \textit{MT1-MMP} expression induced a protrusive phenotype in MCF-7 cell lines grown in 3D culture regardless of mutations to the cytoplasmic domain

To examine how cell morphology may contribute to the increased cell migration capacity observed in MCF-7 cell lines stably expressing cytoplasmic domain-altered forms MT1-MMP, cells were incubated within a 3D matrix composed of Matrigel. Over five days, cell colonies embedded in this matrix were imaged and the morphology of cells was assessed. A recurring phenotype observed were cellular protrusions extending from individual cells within colonies (Figure 9 a). In all cell lines, the number of protrusions per colony increased with incubation time (Figure 9 b). However, MT2CD MT1-MMPst and ΔCD MT1-MMPst cells showed significantly more protrusions per colony, approximately an 3.8-fold increase than MCF-7 cells on day two of incubation \( (p < 0.05) \), while MT1-MMPst cells were not significantly different than parental cells at this time point. By day five MT2CD MT1-MMPst \( (p < 0.001) \) and ΔCD MT1-MMPst \( (p < 0.01) \) cells continued to show significantly more protrusions per colony than parental MCF-7 cells, at ~3-fold and ~2.3 increases respectively, and while not significant MT1-MMPst cells showed approximately twice as many protrusions per colony than MCF-7 colonies.

After five days of incubation, samples were fixed and prepared for immunofluorescence microscopy to examine nuclei and F-actin structures within cells and colonies. Confocal microscopy images showed that the protrusions observed in 3D culture were rich in F-actin, and were clearly distinguished in colonies of MT1-MMPst, MT2CD MT1-MMPst and ΔCD MT1-MMPst cells (Figure 9 c).
Figure 8. Deletion or substitution of the cytoplasmic domain of MT1-MMP did not impair MT1-MMP-mediated cell migration in stably expressing MCF-7 cell lines.

(a) Bright-field microscopy images of MCF-7 and MT1-MMP\textsuperscript{st}, MT2CD MT1-MMP\textsuperscript{st}, ∆CD MT1-MMP\textsuperscript{st} cell lines immediately following and 72 hours after monolayer disruption in a wound closure assay. Cells were seeded at confluence, incubated for 24 hours, and the monolayer disrupted. The closure of this wound was observed for 72 hours using bright-field microscopy. Representative images of the wound are shown, where thin lines mark initial wound edges and thick lines indicate the final wound front. Scale bars = 200 µm. (b) Images were taken along the length of the wound immediately after wounding and at 24, 48 and 72 hours. The distance between the wound edges at each time point was measured and subtracted from the initial wound size to calculate distance migrated. After 72 hours, the distance migrated by MT1-MMP\textsuperscript{st}, MT2CD MT1-MMP\textsuperscript{st}, and ∆CD MT1-MMP\textsuperscript{st} cells was not significantly different, though they all migrated significantly more ($p < 0.0001$) than parental MCF-7 cells. The results of four biological replicates are presented as mean distance ± SEM, and differences measured using one-way ANOVA; **** $p \leq 0.0001$. (c) The migration of MT1-MMP stable cell lines was assessed using a transwell migration assay. MT1-MMP\textsuperscript{st}, MT2CD MT1-MMP\textsuperscript{st}, ∆CD MT1-MMP\textsuperscript{st} and parental cells were seeded and induced to migrate along a vertical serum gradient. While there was no significant difference in the number of migrated cells among MT1-MMP\textsuperscript{st}, MT2CD MT1-MMP\textsuperscript{st}, and ∆CD MT1-MMP\textsuperscript{st}, significantly more MT1-MMP\textsuperscript{st} ($p < 0.01$), MT2CD MT1-MMP\textsuperscript{st} ($p < 0.05$), and ∆CD MT1-MMP\textsuperscript{st} ($p < 0.05$) cells migrated through the transwell pores than MCF-7 cells. The results of three independent experiments are presented as mean relative absorbance ± SEM, and differences measured using one-way ANOVA; different letters signify differences at $p < 0.05$ according to Tukey’s B test.
Figure 9. Alteration to the cytoplasmic domain of MT1-MMP exacerbated a protrusive cell morphology in 3D culture.

(a) Bright field microscopy images of MCF-7 and MT1-MMPst, MT2CD MT1-MMPst, and ΔCD MT1-MMPst cells seeded in 3D Matrigel after five days of incubation. MT1-MMPst, MT2CD MT1-MMPst, and ΔCD MT1-MMPst cell colonies all displayed discrete cellular protrusions of diverse length and number (inset, indicated by white arrows), while MCF-7 cell colonies remained mostly round. (b) These cellular protrusions were quantified and standardized to cell number, shown as protrusions per colony. In all cell lines, the number of protrusions per colony increased from day one to day five. As early as day two, both MT2CD MT1-MMPst and ΔCD MT1-MMPst cell colonies showed significantly more protrusions per colony than did MT1-MMPst or MCF-7 cells \( (p < 0.05) \). On day five, MT2CD MT1-MMPst \( (p < 0.001) \) and ΔCD MT1-MMPst \( (p < 0.01) \) colonies remained significantly more protrusive than MCF-7 cells. While not significant, the number of cell protrusions per colony in MT1-MMPst cells was higher than MCF-7 cells. Different letters of the same case signify differences at \( p < 0.05 \) according to Tukey’s B test. (c) 3D reconstruction images of z-stacks captured using confocal microscopy on cells grown in 3D culture. MCF-7 and MT1-MMPst, MT2CD MT1-MMPst, and ΔCD MT1-MMPst cells were cultured in Matrigel for five days and prepared for immunofluorescence to identify F-actin (red) and nuclei (blue). Maximum signal intensity projections of captured images showed that MT1-MMPst, MT2CD MT1-MMPst, and ΔCD MT1-MMPst cell colonies display actin-rich cellular extensions similar to those observed under light microscopy (white arrows).
3.7 Alteration to the MT1-MMP cytoplasmic domain impaired MT1-MMP-mediated cell proliferation and viability, and abolished the anti-apoptotic effects of MT1-MMP upon serum starvation in 2D and 3D cell culture

The effect of serum starvation as a cell stress condition was assessed in stable cell lines MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup> and ∆CD MT1-MMP<sup>st</sup> to gain an understanding of the non-proteolytic functions of MT1-MMP. First, cell proliferation was examined using the CellTiter® reagent under both serum-supplemented (10% FBS) and serum-free conditions for nine days. Growth in serum supplemented conditions resulted in no significant differences in measured cell proliferation among cell lines at day six, a time point in which peak proliferation occurred in all cell lines (Figure 10 a). To corroborate this result, protein was collected from cells grown for six days in serum-supplemented conditions and subject to western blot analysis to examine levels of phospho-histone 3, a marker of cell proliferation. Levels of phospho-histone 3 protein were not significantly different among cell lines at this time point (Figure 10 c, f). Interestingly, by day nine only MT1-MMP<sup>st</sup> cells were viable and showed continued significantly elevated levels of proliferation (p < 0.001).

In contrast to the similarity in proliferation observed between cell lines cultured with serum, in serum-free conditions MT1-MMP<sup>st</sup> cells exhibited significantly more proliferation at levels ~2.9 fold greater than MCF-7 cells after three days in culture, and continued to be significantly more proliferative than all other cell lines at every time point (p < 0.0001) (Figure 10 b). Additionally, at day six, MT2CD MT1-MMP<sup>st</sup> cells proliferated significantly more than MCF-7 or ∆CD MT1-MMP<sup>st</sup> cells (p < 0.0001), though still significantly less proliferative than MT1-MMP<sup>st</sup> cells (p < 0.0001). ∆CD MT1-MMP<sup>st</sup> cells showed no significant difference in proliferation compared to parental MCF-7 cells at all time points. Corroborating these observations, levels of phospho-histone 3 protein after six days of serum-free incubation were significantly greater by ~2 fold in MT1-MMP<sup>st</sup> cells (p < 0.01) and lower in ∆CD MT1-MMP cells by ~1.67 fold than in MCF-7 cells (Figure 10 d, e).
Figure 10. Mutation to the cytoplasmic domain of MT1-MMP in stably expressing MCF-7 cells decreased cell proliferation in serum-free stress conditions.

(a, b) MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup>, ΔCD MT1-MMP<sup>st</sup>, or parental cells were seeded at 5000 cell/well onto a 96-well culture dish in either serum-free or serum-supplemented media conditions. The proliferation of cells was measured in both conditions using CellTiter<sup>®</sup>96 Aqueous One Solution (Promega) reagent for nine days. (a) In serum-supplemented conditions, the proliferation of all cell lines was not significantly different at day six, the day of peak proliferation. Nine days post-seeding, MT1-MMP<sup>st</sup> cells showed significantly more proliferation than any other cell lines (p < 0.001). (b) In serum-free conditions, MT1-MMP<sup>st</sup> cells showed significantly more proliferation than all other cell lines at all time points after day zero (p < 0.0001). MT2CD MT1-MMP<sup>st</sup> cells showed significantly higher levels of proliferation (p < 0.0001) at day six than MCF-7 and ΔCD MT1-MMP<sup>st</sup>, which were not significantly different from each other. The results of three independent experiments are presented as mean ± SEM, and were analyzed using one-way ANOVA; *** p ≤ 0.001; **** p ≤ 0.0001. (c, d, e, f) Stably expressing MCF-7 cell lines were seeded in either serum-free or serum supplemented media and incubated for six days. Cells lysates were subjected to immunoblot analysis to examine levels of phospho-histone 3 as a measure of cell proliferation. (c) In serum-supplemented conditions, the levels of phospho-histone 3 protein was not significantly different among cell lines. (d) In serum-free conditions, MT1-MMP<sup>st</sup> cells had significantly higher levels of phospho-histone 3 protein than MCF-7 (p < 0.05) and ΔCD MT1-MMP<sup>st</sup> cells (p < 0.01), while the levels in MT2CD MT1-MMP were not significantly different than in MCF-7 cells. (e, f) Densitometry analysis was performed on phospho-histone 3 protein levels in serum or serum-free culture conditions. Three independent samples of protein isolated from cell lines was analyzed and results are presented as mean relative optical density ± SEM, and were analyzed using one-way ANOVA; different letters signify differences at p < 0.05 according to Tukey’s B test. β-actin was used as a loading control.
A similar experimental design was used to assess viability and apoptosis in stable cell lines in serum-free stress. Cells were cultured in either serum-supplemented or serum-free conditions for six days, and then viability and apoptosis measured using a fluorescent or luminescent substrate of relevant cell proteases, respectively. Relative to their corresponding levels in serum-supplemented conditions, MT1-MMP<sup>st</sup> cells showed a significant increase of ~19% in cell viability ($p < 0.05$) and showed significantly lowered caspase activity ($p < 0.01$) by ~11% than MCF-7 cells (Figure 11 a, b). ∆CD MT1-MMP<sup>st</sup> showed a significant ~40% decrease in viability as compared to control parental cell lines ($p < 0.0001$), while both MT2CD MT1-MMP<sup>st</sup> and ∆CD MT1-MMP<sup>st</sup> showed a ~16% significant increase in apoptosis ($p < 0.001$) in serum-free conditions than MCF-7 cells.

Upon observing the changes in cell survival that occur in MT1-MMP stable cell lines upon serum starvation, the effect of serum starvation on cell morphology in 3D culture was examined. MCF-7, MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup> or ∆CD MT1-MMP<sup>st</sup> were seeded in Matrigel and incubated in serum-free media conditions for five days. On day one, the morphology of cell colonies was predominantly round, though MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup> or ∆CD MT1-MMP<sup>st</sup> cells showed more cellular protrusions than parental cells (Figure 12 a). By day five, colonies of all cell lines began showing a unique phenotype where cells within colonies lost cohesion, which was described as a “fractured” morphology distinct from round or protrusive cell colonies. This fracturing phenotype was observed ~14% less frequently in MT1-MMP<sup>st</sup> cells than MCF-7 cells, but was significantly more frequent ($p < 0.01$) by ~52% and ~57% respectively in MT2CD MT1-MMP and ∆CD MT1-MMP<sup>st</sup> cell colonies (Figure 12 b). No protrusions were observed on day five in any cell type in this serum-free condition.

3.8 Changes to cell migration and survival are correlated to changes in AP-1 and NF-κB transcriptional activity

To gain insight into the possible mechanisms of the observed increase in migratory capability and concomitant decrease in survivability in cells expressing MT1-MMP with altered cytoplasmic domains, a firefly luciferase gene reporter assay was conducted to assess the transcriptional activity of AP-1 and NF-κB transcription factors. Both AP-1 and NF-κB have been implicated in changes in cell migration and survival
Figure 11. Mutation to the cytoplasmic domain of MT1-MMP impaired cell viability and increased apoptosis in MCF-7 cells following serum deprivation.

MT1-MMPst, MT2CD MT1-MMPst, and ΔCD MT1-MMPst, or parental MCF-7 cells were seeded in either serum-free or serum-supplemented media and incubated for six days. A fluorescent viable cell enzyme substrate and a luminescent caspase substrate were added to cultures and the fluorescence and luminescence of cells measured as a metric of viability and apoptosis, respectively. Data are presented as viability or apoptosis levels in serum-free conditions relative to serum conditions for each cell line, and normalized to control MCF-7 cells. (a) After six days of incubation, MT1-MMPst cells were significantly more viable in serum-free stress conditions than parental cells ($p < 0.05$), while ΔCD MT1-MMPst cells were significantly less viable than MCF-7 cells ($p < 0.0001$). (b) After six days of incubation, MT1-MMPst cells had significantly less caspase activity than did MCF-7 cells in serum-free stress ($p < 0.01$), while both MT2CD MT1-MMPst and ΔCD MT1-MMPst showed significantly more ($p < 0.001$) caspase activity than the parental cell line. The results of three independent experiments are presented as mean relative fluorescence or luminescence ± SEM, and were analyzed using one-way ANOVA; different letters signify differences at $p < 0.05$ according to Tukey’s B test.
Figure 12. Mutation to the cytoplasmic domain of MT1-MMP induced colony fracturing in 3D serum-free stress conditions.

(a) Bright field microscopy images of MCF-7 and MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup>, and ∆CD MT1-MMP<sup>st</sup> cells in 3D culture upon serum starvation. Cells were seeded in Matrigel and the morphology of cell colonies within the matrix was observed at day one and day five. On day one, colonies were mostly round, though MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup>, and ∆CD MT1-MMP<sup>st</sup> cells showed more cellular protrusions than MCF-7 cells. On day five, some cell colonies of MCF-7, MT2CD MT1-MMP<sup>st</sup> and ∆CD MT1-MMP<sup>st</sup> developed a fractured phenotype where colonies lost internal cohesion. Representative morphologies are shown in inset images. (b) The morphology of cell colonies was assessed on days one and five as either round, protrusive or fractured. At day one, colonies of all cell lines were predominantly round, with no fractured colonies observed. MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup>, and ∆CD MT1-MMP<sup>st</sup> cell colonies showed more protrusions than MCF-7 cells. At day five, MT2CD MT1-MMP<sup>st</sup> and ∆CD MT1-MMP<sup>st</sup> cell colonies showed significantly more fracturing than MCF-7 cells ($p < 0.01$). MT1-MMP<sup>st</sup> cell colonies remained mostly round, and had fewer fractured cell colonies than MCF-7 cells. The results of three biological replications are shown as mean proportion of total cell colonies ± SEM, and were analyzed using one-way ANOVA; different letters signify differences at $p < 0.05$ according to Tukey’s B test.
(Bakiri et al., 2015; Grandage et al., 2005). AP-1-regulated gene transcription was significantly increased by ~4.15-fold in MT1-MMPst ($p < 0.0001$), ~5-fold in MT2CD MT1-MMPst ($p < 0.0001$) and ~2.4-fold ∆CD MT1-MMPst cells ($p < 0.05$) compared to MCF-7 cells, although to different degrees (Figure 13 a). MT1-MMPst cells were the only cell lines to display significantly increased levels of NF-κB transcriptional activity, by ~12.6-fold ($p < 0.0001$), as neither MT2CD MT1-MMPst and ∆CD MT1-MMPst cells showed significant differences in luminescence as compared to MCF-7 cells (Figure 13 b).

3.9 Substitution or deletion of the cytoplasmic domain of MT1-MMP impaired, but did not abolish, MT1-MMP-mediated vascularization of artificial tumours embedded onto avian embryo CAM

The increase in migration and development of cellular protrusions in cells imparted by MT1-MMP expression, as well as the increase in survivability metrics seen in vitro could both contribute to tumour growth. To assess the role that the cytoplasmic domain has in the overall tumourigenicity of MT1-MMP, artificial Matrigel tumours composed of ZsMCF-7, ZsMT1-MMPst, ZsMT2CD MT1-MMPst or Zs∆CD MT1-MMPst cells were embedded into a wound within the CAM vasculature of a nine day old chicken embryo and incubated for eight days. Following incubation the local vasculature surrounding the tumour site was observed and was determined to be either vascularized or non-vascularized, depending on the presence or absence of capillaries above the embedded tumour (Figure 14 a). All control ZsMCF-7 cell tumours failed to grow beneath the CAM layer, the wound site failed to heal and there was no vascularization at the injection site (N = 13). Conversely, all but one (14/15) ZsMT1-MMPst cell tumours showed observable vascularization and growth beneath the CAM (Figure 14 b). Both ZsMT2CD MT1-MMPst (9/16) and Zs∆CD MT1-MMPst (7/14) showed intermediate incidences of vascularized tumours between ZsMCF-7 cells and ZsMT1-MMPst cell tumours.
Figure 13. Altered transcriptional activity of AP-1 and NF-κB in cells expressing 
*MT1-MMP* was differentially dependent on the integrity of the cytoplasmic domain.

MCF-7 and MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup>, and ΔCD MT1-MMP<sup>st</sup> cells were 
transfected with firefly luciferase under the regulation of AP-1 or NF-κB promoters.

Cells were lyed and incubated with luciferin and the luminescence of lysate measured.

All of MT1-MMP<sup>st</sup> (*p* < 0.0001), MT2CD MT1-MMP<sup>st</sup> (*p* < 0.0001), and ΔCD MT1-
MMP<sup>st</sup> (*p* < 0.05) showed significantly higher luminescence than MCF-7 cells following 
transfection with AP-1-luciferase. Only MT1-MMP<sup>st</sup> (*p* < 0.0001) cells showed 
significantly more luminescence than parental cells following transfection with NF-κB-
luciferase. The results of three biological replications are presented as mean 
luminescence ± SEM, and were analyzed using one-way ANOVA; different letters 
signify differences at *p* < 0.05 according to Tukey’s B test.
Figure 14. MCF-7 cell lines stably expressing cytoplasmic domain altered MT1-MMP showed fewer instances of tumour vascularization in avian CAM.

Stably expressing, ZsGreen-tagged cell lines ZsMT1-MMPst, ZsMT2CD MT1-MMPst, and ZsΔCD MT1-MMPst, as well as ZsMCF-7 cells were embedded in Matrigel and injected into a wound made to the vasculature of the CAM of a nine-day-old chicken embryo. Embryos were incubated for eight days, and the tumour site examined by bright field and fluorescence stereomicroscopy. The binary measurement of Tumour Vascularization was determined by identifying capillaries above and around tumours exhibiting growth beneath the CAM as present (A1, white arrows) or absent. The edge of the initial wound is also indicated (A2, white arrows) Scale bars = 1 mm (a) Representative bright field and fluorescence microscopy images of Matrigel tumours at day nine. Only ZsMT1-MMPst displayed healing and closure of the initial wound (A2, white arrows indicate scarring) as well as complete submersion and growth beneath the CAM, though some ZsMT2CD MT1-MMPst and ZsΔCD MT1-MMPst tumours did show partial growth beneath the CAM layer (middle, bottom panels). No ZsMCF-7 cell tumours exhibited growth beneath the CAM or vascularization at the tumour site. Vasculature was present at the tumour site in some tumours of ZsMT1-MMPst, ZsMT2CD MT1-MMPst, and ZsΔCD MT1-MMPst cells. (b) Multiple embryos were injected and the tumour sites were determined to be either vascularized or non-vascularized depending on the presence or absence of vasculature near the tumour (N values shown above bars). While none of the ZsMCF-7 Matrigel tumours showed vascularization, all but one ZsMT1-MMPst tumours were vascularized. Both ZsMT2CD MT1-MMPst and ZsΔCD MT1-MMPst showed instances of vascularization in between those observed in ZsMCF-7 and ZsMT1-MMPst. A chi-squared test of independence was performed to examine the relationship between cell line and vascularization, and was found to be significant; $\chi^2 (4, \ N = 58) = 24.48, \ p < 0.001$. (c) Schematic illustrating the relative positions of the tumour, CAM and vasculature in chicken embryos. Shown are cross-sections perpendicular to the images shown in (a), along the depth of the CAM. In ZsMCF-7 cells, the tumour sits above the CAM and the wound was unhealed (i), while in ZsMT1-MMPst, the tumour is beneath the CAM with near complete wound closure (ii).
Both ZsMT2CD MT1-MMP<sup>st</sup>, and ZsΔCD MT1-MMP<sup>st</sup> cells show partial growth, where part of the tumour is beneath the vascularized CAM (iii).
a. ZsMCF-7  ZsMT1-MMP<sup>+</sup>  ZsMT2CD MT1-MMP<sup>+</sup>  ZsACD MT1-MMP<sup>+</sup>

Brightfield

ZsGreen

Inset

b. 0/13  14/15  9/16  7/14

% Vascularized

MCF-7  MT1-MMP<sup>+</sup>  MT2CD  MT1-MMP<sup>+</sup>  ΔCD MT1-MMP<sup>+</sup>

c.

I. Tumour

II. Wound

III. Vascularization

CAM
Chapter 4

4 Discussion

This work aimed to examine the effects of cytoplasmic domain mutation on the migration and survival-promoting functions of MT1-MMP. Using MCF-7 breast cancer cells, which are deficient in MT1-MMP, multiple overexpression experiments were conducted using cDNAs encoding either full-length MT1-MMP, MT1-MMP possessing the substituted cytoplasmic domain of MT2-MMP, or MT1-MMP with no cytoplasmic domain. I hypothesized that swapping or deleting the cytoplasmic domain would disrupt the functions of MT1-MMP in cell migration and cell survival. However, the results presented here indicated that though the cytoplasmic domain is necessary for the promotion of cell survival, it was dispensable for MT1-MMP-mediated cell migration.

4.1 The cytoplasmic domain of MT1-MMP regulates its activation

Substitution or deletion to the cytoplasmic domain of MT1-MMP differently altered the protein activation profiles observed in transiently transfected MCF-7 cells (Figure 2 a). The lack of active MT1-MMP protein observed upon substitution of its cytoplasmic domain with that of MT2-MMP (MT2CD MT1-MMP) suggests that even with the high level of sequence similarity between these proteins at this domain, substitution interrupts the successful cleavage of MT1-MMP by its activator furin. This result seems at odds with reports that furin mediates the activation of other membrane-type MMPs possessing cytoplasmic domains, notably MT3-MMP (Kang et al., 2002), and that MT2-MMP contains a furin recognition sequence at its pro-domain (Butler et al., 1997). However, the presence of such a recognition site is not necessarily predictive of furin activity, and MT-MMPs including MT1-MMP have been found to be activated through furin-independent mechanisms (Rozanov et al., 2001; Sato et al., 1999), primarily by MMPs. In addition, the possibility that the MT2-MMP cytoplasmic domain does not interact with furin is supported by the lack of identified adaptor proteins to mediate this interaction, while a putative adaptor protein has been described for the association of the MT1-MMP cytoplasmic domain with furin (Roghi et al., 2010).
The high levels of active MT1-MMP protein observed in cells following transfection with ∆CD MT1-MMP prompted further investigation, as it was expected that cytoplasmic domain deletion would have similar negative effects on protein activation as domain substitution. Indeed, the activation of MT1-MMP protein lacking the cytoplasmic domain appeared to be mediated not by furin, but through an MMP-dependent mechanism (Figure 2 c). This MMP-mediated activation did not occur in cells transfected with FL MT1-MMP, suggesting that this activation occurs via a non-canonical process in these transfection and culture conditions. It is proposed here that this activation of MT1-MMP protein without the cytoplasmic domain occurred by auto-activation in trans through cleavage of the pro-peptide at the cell surface. Two observations support this: the high levels of pro-MT1-MMP generated following ∆CD MT1-MMP transfection, as well as the cell surface localization of the resultant protein. The distribution of cell surface MT1-MMP lacking the cytoplasmic domain appeared as dense clusters at the cell surface, and the high levels of MT1-MMP protein detected by western blot analysis suggest that these clusters are highly concentrated in MT1-MMP protein. As sequences in the cytoplasmic domain promote the internalization of this protein (Uekita et al., 2001; Williams & Coppolino, 2011), these high levels appear to be a result of a deficiency in protein internalization and degradation. This accumulation of many, densely packed MT1-MMP monomers that cannot be regulated by internalization may allow for low, atypical proteolytic activity of pro-MT1-MMP to cleave and activate nearby MT1-MMPs, which would spread within protein clusters. This hypothesis is plausible given multiple observations regarding MT1-MMP activation. MT1-MMP is known to experience autocatalysis in trans, although at the catalytic domain rather than pro-domain, as it generates the 44 kDa autocatalytic degradation product (Cho et al., 2008; Osenkowski, Toth, & Fridman, 2004). There is also evidence that MT1-MMP may undergo a two-step activation process, including an autocatalysis step (Strongin, 2010). In addition, MMP family members have been shown capable of cleaving and activating other MMPs, as observed during MT1-MMP activating MMP-2 (Itoh et al. 2001) or MMP-2 activation MMP-9 (Fridman et al., 1995).

In MDA-MB-231 cells, the activation profiles of cytoplasmic-domain altered MT1-MMP
proteins was mostly consistent with those observed in MCF-7 cells. Notable differences were the lack of a 44 kDa degradation product following FL MT1-MMP transfection, and lack of an active MT1-MMP protein band following ∆CD MT1-MMP transfection. Both of these differences can be explained by the inhibitory activity of TIMP-2, which is expressed at much higher levels in MD-AMB-231 cells than MCF-7 (Figueira et al., 2009). As both the autocatalysis and posited auto-activation of MT1-MMP are mediated by MT1-MMP proteolysis, the inhibition of MT1-MMP upon TIMP-2 exposure explains the absence of these protein forms.

4.2 Cytoplasmic domain-dependent cell surface localization of MT1-MMP determines its ability to cleave substrate

MCF-7 cells transfected with FL MT1-MMP showed a cell surface distribution of MT1-MMP protein that is consistent with patterns observed in other studies examining unstimulated, transfected cells (Remacle, Murphy, & Roghi, 2003; Wang et al., 2004). FL MT1-MMP appeared uniform across the entire cell surface, in a pattern congruent with the actin cytoskeleton (Figure 3). In contrast, neither MT2CD MT1-MMP nor ∆CD MT1-MMP transfection resulted in this surface distribution, as these altered MT1-MMP proteins failed to localize along the cell edge or to the distal end of protrusive actin cell structures. The filamentous cell surface pattern of MT2CD MT1-MMP was consistently observed, but an explanation for such a pattern cannot currently be suggested, as MT2-MMP is not as rigorously studied as MT1-MMP. Should the distribution of MT2-MMP be similar to that of MT2CD MT1-MMP, it would suggest a consistent, prominent role of the cytoplasmic domain for the localization of MT-MMPs. The clustering of MT1-MMP protein upon cytoplasmic domain deletion may be a result of non-targeted insertion and maintenance at the cell membrane, or aberrant binding of MT1-MMP proteins via the hemopexin domain arising from a lack of regulatory internalization signals normally provided by the cytoplasmic domain.

While cells transfected with either FL MT1-MMP or ∆CD MT1-MMP showed levels of active MT1-MMP, only transfection with FL MT1-MMP resulted in the activation of pro-MMP-2 and the degradation of gelatin. This suggests that while active MT1-MMP is required for this process, its cell surface localization is an important factor for proteolysis
to occur. As MT1-MMP proteins dimerize during pro-MMP-2 activation, a cell surface distribution pattern that does not permit appropriate dimerization may impact this activity and other functions. Indeed, the cytoplasmic domain mediates disulphide bonding of MT1-MMP monomers during MMP-2 activation, and its deletion abrogates this dimerization (Lehti et al., 2002). This suggests a requirement of both the proteolytic ability to cleave MMP substrates and a permissive spatial distribution required of this interaction.

However, some reports have shown MT1-MMP lacking its cytoplasmic domain is capable of activating pro-MMP-2 in zymography analysis (Itoh et al. 2008; Li et al. 2008). As these reports show that ∆CD proteins appear in an active form by western blot, this observation is plausible. The inconsistency of previous studies with the data presented here (Figure 4) may be due to the use of different cell lines, which include COS7, COS-1, and HT1080, or differences in expression vectors used, which could result in lower proteins levels that are more permissive to proper protein interactions.

The degradation of gelatin is accomplished primarily by the activity of MMP-2 (Toth & Fridman, 2013). Therefore, the presence of active MMP-2 as shown by zymography is consistent with only FL MT1-MMP-transfected cells being capable of gelatin degradation (Figures 4, 5). However, a further observation regarding MT1-MMP cytoplasmic domain mutation in cells seeded upon gelatin was the appearance of MT1-MMP that co-localized with actin puncta. While these punctate actin structures were not positively identified as invadopodia, their morphology is consistent with these basal actin protrusions. To confirm the formation of invadopodia additional protein markers are required, such as cortactin or Tsk5 (Artym, 2006; Clark et al., 2007; Leong et al., 2014). However, if these invadopodia-like structures are representative of true invadopodia, this suggests that the cytoplasmic domain is dispensable during the recruitment of MT1-MMP to invadopodia. This contrasts with other observations made in this study that show MT1-MMP protein is improperly localized at the cell surface (Figure 3), and may mean that MT1-MMP recruitment to invadopodia can occur passively through the trafficking of other proteins, such as integrins (Artym, 2006). As MT1-MMP has been shown to bind integrins
(Gálvez et al., 2002) during their translocation, MT1-MMP may be recruited with integrins through binding at the extracellular hemopexin-like domain of MT1-MMP.

4.3 The cytoplasmic domain of MT1-MMP is not required for cell migration and negatively regulates the development of invasive cell protrusions

In both transiently transfected and stably expressing MCF-7 cell lines, cytoplasmic domain substitution (MT2CD MT1-MMP) or deletion (∆CD MT1-MMP) within MT1-MMP increased the migration of cells relative to control conditions (Figures 6, 8). This was observed across multiple migration assays, as cells containing these altered forms of MT1-MMP migrated further into a wound space, and more often through the small pores of transwell chambers than did parental MCF-7 cells. As these mutants of MT1-MMP show little proteolytic activity, which has frequently been implicated in MT1-MMP-mediated cell migration, the observed migration ability is likely due to non-proteolytic mechanisms.

A number of observations regarding the migration-promoting activity of MT1-MMP directly refer to mechanisms that do not depend on proteolysis. These include sequences in the catalytic domain which do not impact enzymatic activity (Woskowicz et al., 2013), and blades I and IV of the hemopexin domain, which allow for homo- or heterodimers of MT1-MMP to form and result in ERK activation (Zarrabi et al., 2011). Indeed, some studies show that upon cytoplasmic domain deletion the invasive capability of cells is maintained (Li et al., 2008). In addition, the inhibition of all MMP proteolytic activity in invasive human breast cancer cell lines has been shown to result in the promotion of an amoeboid migration phenotype which relies on changes in cell morphology without accompanying ECM degradation (Wolf et al., 2003). Indeed, a non-proteolytic mechanism of increased cell migration involving cytoskeletal arrangements is also supported by the increased levels of cell invasion observed in this study (Figure 6). As the Matrigel barrier employed in the transwell invasion assays used was diluted, the corresponding increase in size between ECM proteins within it may have been permissive to an amoeboid method of cell invasion, rather than one dependent on proteolysis, and
may explain why cells transfected with MT2CD MT1-MMP and ∆CD MT1-MMP invaded more than FL MT1-MMP or GFP.

This sort of distinct change in cell morphology was observed in this study upon cytoplasmic domain mutation to MT1-MMP, as cells developed protrusive cell extensions in MT1-MMP stable cell lines cultured in a 3D Matrigel matrix (Figure 9). Using immunofluorescence, it was determined that these protrusions were rich in F-actin, suggesting that they may be of similar form and function as filopodia or invadopodia and could promote the migration or invasion of cells. Uncharacterized invasive protrusions have previously been reported to form in MT1-MMP-expressing breast cancer cells cultured in 3D matrices. The growth of these actin-rich formations was regulated by the actin nucleation-promoting protein N-WASP (Neural Wiskott-Aldrich syndrome protein), which was shown to have a specific role in both the trafficking of MT1-MMP to these structures, and tethering and stabilizing the protease at this location via the MT1-MMP cytoplasmic domain (Yu et al., 2012). It is then plausible that in MT2CD MT1-MMP and ∆CD MT1-MMP cells, which possess mutated MT1-MMP cytoplasmic domains, this N-WASP-mediated stabilization does not occur, and the development and growth of these protrusions lack an organized resolution provided by MT1-MMP stability. This is well-supported by the observation that MT2CD MT1-MMP and ∆CD MT1-MMP cells showed significantly more cellular protrusions at a much earlier time than MT1-MMP, and cells remained more protrusive for the duration of experiments (Figure 9).

Assessing the protrusive morphology of breast cancer cells in 3D culture has been widely utilized as it strongly correlates with invasive and migratory ability (Kenny et al., 2007; Lee et al., 2010). In particular, “stellate” cell colonies arise from invasive cell types, such as MD-AMB-231 breast cancer cells, and are characterized by long cellular extensions. In contrast, low-invasive cell lines such as MCF-7 remain predominantly round. The protrusions observed in MT1-MMP, MT2CD MT1-MMP and ∆CD MT1-MMP cells may be indicative of an acquired invasiveness, as their protrusive morphology aligns with that of stellate colonies.
The results of these migration assays and observations made in 3D culture suggest that MT1-MMP may be capable of promoting cell migration and invasion independent of proteolysis by influencing the organization of the actin cytoskeleton, priming cells to adopt features such as cell protrusions that facilitate migration. Rather than impede this mechanism, the cytoplasmic domain may be a negative regulator in the non-proteolytic mechanism of MT1-MMP-induced cell migration.

Notably, all of the described migration-promoting mechanisms of MT1-MMP are diverse and lack unification. This is in contrast to the extensive and specific mechanisms which exist to describe MT1-MMP in the activation of pro-MMP-2 (Itoh et al., 2001), the switch to glycolytic cell metabolism (Sakamoto & Seiki, 2010), or degradation at invadopodia (Artym, 2006). In addition, and consistent with the breadth of migration-promoting mechanisms ascribed to MT1-MMP, it should be noted that while the stable expression of wild-type MT1-MMP resulted in increased cell migration, expression of the same cDNA following transient transfection was incapable of changing cell migration. This difference, which did not occur with the cytoplasmic domain-altered forms of MT1-MMP, is not easily explained. However, the work presented here regarding cytoplasmic domain mutation resulting in increased cell migration adds a layer of complexity to this already poorly understood function of MT1-MMP, and suggests that the traditional view of induction via proteolytic cleavage is far from a complete model.

4.4 The cytoplasmic domain of MT1-MMP is indispensable for its ability to promote cell survival and protect cells from apoptosis

MT1-MMP wild-type, which expressed wild-type MT1-MMP, showed significantly more proliferation and increased viability than MCF-7 cells in serum-free stress conditions, while MT2CD MT1-MMP and ∆CD MT1-MMP showed less proliferation and more apoptosis than MT1-MMP (Figures 10, 11). Multiple mechanisms exist by which MT1-MMP could induce such a cell survival response. The first of these involves the ability of MT1-MMP to induce the Warburg Effect, a shift in cell metabolism to favour glycolysis. During maturation in the Golgi, MT1-MMP binds to the trafficking protein Mint3 thereby promoting the local recruitment of Factor Inhibiting HIF-1α. This sequestration
relieves the transcription factor HIF-1α of inhibition, allowing it to induce the hypoxic response through the activation of glycolytic gene expression. Observed in both lymphocytes and breast cancer cells this increase in glycolysis directly increases cell survival and does not depend on oxygen levels. Importantly, the binding of Mint3 to MT1-MMP depends on its cytoplasmic domain, explaining the observation that only MT1-MMPst cells showed increased cell survival (Sakamoto et al., 2011; Sakamoto & Seiki, 2010). In addition, MT1-MMP protects cells from apoptosis by activating the Akt pathway. Akt is a serine/threonine kinase and a central mediator of the PI3-K signaling pathway, which affects apoptosis, cell survival and cell growth (Grandage et al., 2005). Specifically, TIMP-2 binding to MT1-MMP caused the activation of Akt, which reduced breast cancer cell apoptosis induced by serum starvation. MCF-7 cells express low but not negligible levels of TIMP-2, and thus an Akt-dependent mechanism is possible in the explaining the observed low apoptosis in MT1-MMPst. In addition, this Akt activation was dependent on the activation of the cytoplasmic membrane-associated protein Ras. (Valacca et al., 2015). Therefore, both Warburg effect induction and the activation of Akt depend on distinct protein interactions. This may suggest that the loss of cell survival observed in MT2CD MT1-MMPst and ΔCD MT1-MMPst may be due to improper cell localization which does not permit proper protein interactions.

While the absence of signal transduction-capable MT1-MMP may explain a loss of protection from apoptosis, MT2CD MT1-MMPst and ΔCD MT1-MMPst showed more apoptosis than parental cells, suggesting that these mutations are actually cytotoxic. A proposed reason for this toxicity may relate to the morphology of these cells as observed in 3D cell culture. The extensive actin-rich protrusions observed in these cells is suggestive of a dysregulation in actin polymerization dynamics. It has been shown that cytoskeletal deformation caused by actin instability can result in apoptosis, and specifically excessive F-actin accumulation has been shown to instigate apoptotic cell death (Gourlay & Ayscough, 2005; Kulms et al, 2002). The unregulated protrusive growth in MT2CD MT1-MMPst and ΔCD MT1-MMPst may bring about apoptosis due to these numerous actin structures.
Most of the results collected across multiple cell behaviour assays showed a similar pattern whether cells were expressing MT2CD MT1-MMP or ΔCD MT1-MMP, a trend which was expected due to both constructs experiencing cytoplasmic domain mutation. However, the stable cell lines MT2CD MT1-MMPst and ΔCD MT1-MMPst showed differences in cell viability, whereby the former displayed no difference in viability compared to parental MCF-7 cells and the latter showed significantly lower viability. To account for this difference in viability observed, the catalytic ability of each protein product must be considered. Notably, the other difference observed between the behaviour of these two constructs was in MT1-MMP protein activation. MT2CD MT1-MMP showed very low levels of active MT1-MMP protein, while cytoplasmic domain deletion results in a protein product that showed an active form (Figure 2a). Importantly, the ΔCD MT1-MMP is presumed to lack regulation of its proteolytic activity through internalization. This persistent catalytic activity at the cell surface may result in the cleavage of cell surface receptors necessary for cell survival, or trigger other apoptotic events.

The mechanisms of these anti-apoptotic and pro-migratory effects of MT1-MMP may be related to changes in transcription in each stable cell line. Indeed, it was shown that MT1-MMP influenced the transcriptional activity of other genes in a manner that depended on the integrity of its cytoplasmic domain. Observed in this study were changes in the transcription activity of nuclear factor κB (NF-κB) and adaptor protein-1 (AP-1).

NF-κB belongs to a family of related transcription factors that were characterized during immune cell development, where they play an essential role. However, it also regulates diverse functions including cell survival, notably protecting cells from apoptosis by mechanisms such as the inhibition of PTEN (Grandage et al., 2005; Vasudevan, Gurumurthy, & Rangnekar, 2004). Importantly, NF-κB is activated through the Akt pathway, which can be induced by MT1-MMP (Valacca et al., 2015). As only MT1-MMPst cells showed increased levels of NF-κB activity, and were also the only cells to show reduced levels of apoptosis, the notion that MT1-MMP mediates apoptosis evasion through Akt/NF-κB is reasonable. This also suggests that the stimulation of NF-κB depends on the cytoplasmic domain of MT1-MMP. However, as NF-κB promotors are
present upstream of MT1-MMP, it is possible that the increased transcriptional activity is a feedback loop resulting from increased MT1-MMP expression.

Also examined was the activity of AP-1, a group of transcription factors which form from the dimerization of Fos and Jun family members, and regulate diverse cell functions including cell growth and cell death (Bakiri et al., 2015). AP-1 transcription factors are also implicated in epithelial-to-mesenchymal transitions in human breast cancer, through increasing cell migration and invasion (Bakiri et al., 2015). AP-1 transcriptional activity was observed to be elevated in all of MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup> and ∆CD MT1-MMP<sup>st</sup> cells compared to parental MCF-7 (Figure 13). Though the cause of the increase in AP-1 transcription activity upon MT1-MMP expression or activity are not known, its activity is consistent with the increase in cell motility observed across all MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup> and ∆CD MT1-MMP<sup>st</sup> cell lines, and suggests that the cytoplasmic domain is not needed for MT1-MMP-mediated AP-1 transcriptional activity. In addition, while no evidence exists that MT1-MMP directly increased AP-1 transcription, AP-1 binding sites are located in promotor regions of MMPs -1, -3, -7, -9, -10, -12 and -13, and AP-1 increases the transcription of MMP-2 (Bergman et al., 2003). It is absent in the promotor region of MT1-MMP. Therefore, a feedback system may be in place whereby migratory cells, partially induced by the function of MT1-MMP, organize transcriptional machinery to further increase MMP gene expression.

4.5 MT1-MMP-mediated cell migration and cell survival both contribute to the development of tumourigenic phenotypes <i>ex vivo</i> and <i>in vivo</i>.

Having observed changes to cell migration and cell survival, assays were performed which examined the concurrent activity of these cellular processes <i>ex vivo</i> and <i>in vivo</i>. The <i>ex vivo</i> 3D culture technique discussed earlier used incubation media supplemented with serum, which resulted in a protrusive, invasive cell morphology (Figure 9). This 3D culture model was adapted, and cultures were serum starved to introduce a stress condition which contrasted the established migration-promoting effects of embedment in Matrigel. While cellular protrusions were present after one day of incubation in MT1-MMP<sup>st</sup>, MT2CD MT1-MMP<sup>st</sup> and ∆CD MT1-MMP<sup>st</sup> cells, this phenotype was
completely absent in all cell lines after five days of serum starvation (Figure 12). This is in contrast to the dramatic protrusions observed on day five in serum-supplemented conditions, and may be a result of cells shifting from a migratory phenotype to one focused on cell survival. Indeed, aside from round cell colonies, the morphological feature observed in serum-free 3D culture was a fracturing of cell colonies as individual cells appear to lose cohesion to each other. This occurred less in MT1-MMPst cells and more frequently in MT2CD MT1-MMPst and ΔCD MT1-MMPst cells compared to MCF-7. This fracturing morphology of cells upon the loss of colony cohesion appears to be consistent with cell death, likely by apoptosis. Interestingly, the pattern of high proportion of fracturing cell colonies in MT2CD MT1-MMPst and ΔCD MT1-MMPst cells was strongly representative of the one observed for apoptosis, as both of these cell lines had increased caspase activity when serum-starved (Figure 11). This suggests that MT2CD MT1-MMPst and ΔCD MT1-MMPst cells, despite showing high migratory capability, lack longitudinal invasive potential as they are unable to survive in stress conditions.

These 3D serum-free culture results (Figure 12) were corroborated *in vivo* with the use of Matrigel tumours injected into the CAM of avian embryos (Figure 14). The CAM environment provides a balance of pro-migratory signals, as cells are induced to migrate along ECM proteins beneath and within the CAM, while also being faced with as a stress environment created by being encased in a healing chick tissue that initially lacked organized blood flow, as well as recognition by the developing avian immune system (Ribatti, 2010). Thus, CAM insertion is an excellent model to examine the contributions of cell migration and cell survival as they relate to the ability of a tumour to thrive *in vivo*, and it is frequently used to examine tumour growth, metastasis and intravasation (Deryugina & Quigley, 2009). Upon incubation in the CAM, MT1-MMPst cell tumours grew beneath the CAM layer, which healed well above the tumour, and became surrounded by vasculature, evidence that these cells were highly tumourigenic (Figure 14). This may be a result of the increased levels of migration and survival in stress conditions observed in MT1-MMPst cells. In contrast, MCF-7 tumours did not move beneath the CAM and there was little healing of the wound around them, and no MCF-7 cell tumours showed vascularization at the wound site. Interestingly, both MT2CD MT1-
MMP\textsuperscript{st} and \(\Delta CD\) MT1-MMP\textsuperscript{st} cell tumours showed an intermediate invasive phenotype, as some of the tumour has expanded beneath the CAM and became vascularized, while half of the tumour remained exposed and the wound site was not healed. In addition, incidences of vascularization occurred approximately 50% as frequently in MT2CD MT1-MMP\textsuperscript{st} and \(\Delta CD\) MT1-MMP\textsuperscript{st} cell tumours as with MT1-MMP\textsuperscript{st}. While a simplification, this pattern illustrates a relationship between cell migration and cell survival as they contribute to tumourigenicity: where MT1-MMP\textsuperscript{st} cells show increased levels of both of these aspects, MT2CD MT1-MMP\textsuperscript{st} and \(\Delta CD\) MT1-MMP\textsuperscript{st} cells lack the ability to increase cell survival, and therefore show decreased, but not abolished, levels of tumour vascularization. Thus mutations within the cytoplasmic domain of MT1-MMP that promote increased cell motility and decreased cell survival indicates that this domain has a physiological effect on breast cancer cells.

4.6 A role for the cytoplasmic domain as a regulator of protein binding and localization

Collectively, the results presented here suggest that the MT1-MMP cytoplasmic domain serves to mediate protein activation and localization, two aspects that have wide-reaching influence on the function of MT1-MMP and consequently cell behaviour. The cytoplasmic domain is necessary for the presumed furin-mediated activation of MT1-MMP protein, and for its peripheral localization at the cell surface. Disturbances to this cortical localization, which may coincide with an inability to bind N-WASP and stabilize the growth of extensive membrane protrusions, may contribute to increased cell motility. Indeed, this work suggests that non-proteolytic mechanisms of MT1-MMP-mediated cell migration exist due to cytoskeletal rearrangements, and that the cytoplasmic domain may be a negative regulator of this phenotype. Cytoplasmic domain mutation resulted in an inability to promote cell survival, which may be due to a lack of specific binding of proteins to the cytoplasmic domain or dysregulation of cell surface localization to areas lacking in necessary partner proteins. Dysregulation of the cytoskeleton upon cytoplasmic domain mutation may also contribute to apoptosis, while the negative regulation of proteolysis provided by cytoplasmic domain-mediated MT1-MMP
internalization may protect cells from toxicity induced by extended MT1-MMP proteolysis.

This work also challenges the sweeping claims regarding the role of the cytoplasmic domain in MT1-MMP function, particularly in the mediation of Akt and ERK signaling. While it may be true that these processes depend on the cytoplasmic domain, it is likely that this dependence is indirect, perhaps as a consequence of mislocalization. Indeed, there may exist a “domino effect” of altered protein behaviour that occurs upon cytoplasmic domain mutation. It is shown here that the cytoplasmic domain is required for MT1-MMP activation by furin and protein localization, but any downstream affects, such as that on cell signaling, may be more caused by mislocalization rather than a direct result of cytoplasmic domain mutation. Nonetheless, the dysregulation of cell behaviour observed upon cytoplasmic domain mutation to MT1-MMP illustrates the diverse and profound functions of MT1-MMP.
Figure 15. A model of the regulation of protein function and cell behaviour by the cytoplasmic domain of MT1-MMP.

Model outlining the proposed changes to MT1-MMP protein function upon disruption to the cytoplasmic domain. This model developed from data collected from this work, and presumptive functions described in the MT1-MMP literature. Overall, the functions of MT1-MMP promote a cell phenotype capable of surviving stress and migrating through tissues, promoting tumourigenicity (Figure 14). (a) During protein maturation in the Golgi, the cytoplasmic domain is required for furin-mediated protein activation. Immunoblot analysis of cells transfected with MT2-MMP cytoplasmic domain substitution and domain deletion constructs interrupted this activation in two cell lines (Figure 2 a - d). (b) High levels of ∆CD MT1-MMP protein as compared to FL MT1-MMP and MT2CD MT1-MMP may be due to accumulation at the cell surface as a result of disrupted internalization (Figure 2 a, e). In addition, while FL MT1-MMP localized along the cell periphery, MT2CD and ∆CD MT1-MMP showed diffuse and clustered patterns, respectively (Figure 3). Only FL MT1-MMP localized at the cell surface in a pattern permissive to pro-MMP-2 activation (Figure 4), a distribution pattern altered upon cytoplasmic domain alteration. High levels of ∆CD MT1-MMP may cluster and auto-activate, resulting in low levels of unregulated catalysis at the cell surface (Figure 2 c). (c) The cytoplasmic domain was dispensable for the recruitment of MT1-MMP to actin puncta in stimulated cells grown on gelatin substrate (Figure 5). This suggests other proteins may mediate its localization to this site through binding of extracellular domain. Only FL MT1-MMP resulted in degradation at this site, partially due to its successful activation of MMP-2. (d) The stable expression of MT2CD and ∆CD MT1-MMP caused extensive actin protrusions in cells (Figure 9), as well as increased rates of migration comparable to stable expression of FL MT1-MMP (Figure 8). The development of protrusions instigated by MT1-MMP may be negatively regulated by its cytoplasmic domain, as FL MT1-MMP still caused these structures to form albeit to a lesser degree. This regulation may occur due to the activity of N-WASP docking MT1-MMP to actin. The cytoskeletal dysregulation as evidenced by these extensive protrusions may also activate apoptosis in cells expressing MT2CD and ∆CD MT1-MMP (Figure 11). (e) Only MT1-MMP with its appropriate cytoplasmic domain showed an ability to enhance cell
viability and protect cells from apoptosis (Figure 11), presumably through the activation of Akt. This could occur directly through the cytoplasmic domain, or through interactions with binding partners at appropriate cellular locations. MT2CD MT1-MMP did not affect cell viability, while ΔCD MT1-MMP decreased cell viability, perhaps a result of unregulated cell surface receptor cleavage. (f) The activity of transcription factors AP-1 was increased in cells regardless of the integrity of the cytoplasmic domain of the expressed MT1-MMP, while increased NF-κB activity only occurred in cells expressing full-length unaltered MT1-MMP (Figure 13). The resultant gene transcription from AP-1 activity may increase cell migration, while increased NF-κB may protect cells from apoptosis. Presumably, as a result of the MT1-MMP cytoplasmic domain recruiting FIH, the HIF-1α hypoxic response is induced to enhance cell survival.
Chapter 5

5 Conclusion

MT1-MMP is a multifunctional protease strongly implicated in the instigation and progression of cancer by promoting cell motility and survival. As shown here, these functions are differentially regulated by the cytoplasmic domain. Mutation of the cytoplasmic domain by substitution or deletion interrupted the furin-mediated activation of MT1-MMP and altered its cell surface distribution. MT1-MMP was capable of promoting cell motility regardless of the status of its cytoplasmic domain. In contrast, the survival-promoting functions of MT1-MMP through increased cell proliferation and decreased apoptosis specifically depended on the cytoplasmic domain. It was hypothesized that all MT1-MMP functions would depend on the cytoplasmic domain, and that the expression of cytoplasmic-domain altered forms would impair cell migration and cell survival. However, it appears that these two aspects of MT1-MMP functionality are independent with respect to the role of the cytoplasmic domain. Both the pro-migratory and pro-survival functions of MT1-MMP contribute to cell tumourigenicity, as cytoplasmic domain alteration impaired tumour vascularization. However, tumour growth was not abolished, suggesting that cell migration is an important component to this phenotype. By expanding the understanding of both the cytoplasmic domain of MT1-MMP, as well as how multiple functions of MT1-MMP contribute to cell invasiveness, this work provides a comprehensive model regarding the roles of MT1-MMP in invasive cells.
References


# Curriculum Vitae

**Name:** Jacob Pelling

**Post-secondary Education and Degrees:**

- **The University of Western Ontario**
  - **London, Ontario, Canada**
  - **2014-2016 M.Sc.**

**Honours and Awards:**

- **Ontario Graduate Scholarship, 2015-2016**
- **NSERC Canada Graduate Scholarship, 2014-2015**
- **Ontario Graduate Scholarship *declined acceptance*, 2014-2015**
- **Helen Battle Medal in Zoology, 2014**
- **Helen I. Battle Scholarship, 2013**
- **Edward Barrow & Ida B Hodgins Scholarship, 2012-2013**
- **Andrew & Sarah Hamilton Memorial Scholarship, 2011-2014**

**Related Work Experience:**

- **Teaching Assistant, Cell Biology 2382B**
  - **The University of Western Ontario**
  - **2015, 2016**

- **Undergraduate Researcher**
  - **The University of Western Ontario**
  - **Supervisor: Dr. Percival-Smith, 2012**
  - **Supervisor: Dr. Damjanovski, 2014**

**Publications:**