Characterization of Matrix Metalloprotease 1a in Tumorigenesis

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Caitlin J. Foley

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ADVISER: Athan Kuliopulos, MD, PhD

Abstract

Matrix metalloproteases (MMPs) are a family of extracellular proteases that allow cells to both sense and remodel their extracellular environment through cleavage of cell surface receptors, secreted factors and matrix proteins. While proteolytic activity of MMPs is required for normal tissue homeostasis and other physiologic processes, many pathophysiologic conditions are associated with dysregulated MMP activity. While some diversity exists between vertebrate MMPs, animal models have been extensively utilized to characterize MMP signaling in disease. However, the study of MMP1, a tumorigenic collagenase and activator of protease activated receptor 1 (PAR1), has been limited in animal models due to uncertainty as to whether mouse MMP1 is functionally equivalent to human MMP1.

In rodents, gene duplication has created to two potential *MMP1* homologues, *Mmp1a* and *Mmp1b*. *Mmp1b* lacks enzymatic activity, suggesting that *Mmp1a* is the MMP1 homologue in tumorigenesis models. Mouse-derived lung cancer and melanoma cells expressed high levels of Mmp1a. Mmp1a expression promoted PAR1-driven collagen invasion and stellate growth in three-dimensional culture. Silencing of Mmp1a reduced *in vivo* tumorigenesis, invasion, and metastasis of mouse lung cancer cells, consistent with Mmp1a acting as an MMP1-like activity in tumor models.

To better understand the functions of Mmp1a *in vivo*, *Mmp1a*-deficient animals were generated. $Mmp1a^{-/-}$ animals are healthy and fertile. Tumor growth and angiogenesis was reduced in $Mmp1a^{-/-}$ mice. This phenotype was present despite cancer cell Mmp1a expression and could be rescued by co-implantation of $Mmp1a^{+/+}$ fibroblasts, highlighting the importance of stromal Mmp1a.

Despite homologous MMP1-like functions in tumorigenesis models, Mmp1a expression was less than MMP1 in quiescent tissues. Mammalian expression systems revealed a severe defect in production of mature Mmp1a but not MMP1 protein. This defect was caused by instability of the Mmp1a prodomain, partially due to a phenylalanine to leucine prodomain substitution that interfaces with the catalytic domain. Together, these results demonstrate that Mmp1a is a relevant homologue for MMP1 in tumorigenesis models but also highlight key biochemical differences between MMP1 and Mmp1a that require further exploration for the development of relevant MMP1 mouse models of human disease.

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On a personal level, I could not have done this without my boyfriend, Daniel Herman and my parents. Daniel has put up with my bad moods when experiments don't work, the bizarre lab schedules that I keep, and the decision that adopting two dogs while doing a PhD thesis was a good idea. My parents had no idea what I was getting in to when I started an MD-PhD program but have been completely supportive of me and the fact that I won't be getting a job until I'm 35.

Table of Contents

List of Tables

List of Figures

Characterization of Matrix Metalloprotease 1a in Tumorigenesis

Chapter 1: Introduction

1.1 Matrix Metalloproteases

Matrix Metalloprotease Family and Structure:

Matrix metalloproteases (MMPs) are a family of approximately 25 zinc-dependent endopeptidases in the metzincin family of metalloproteases. In vertebrates, the metzincin family is composed of three members: adamalysins, astacins, and MMPs. The adamalysin family is composed of the ADAM (a disintegrin and metalloprotease) and ADAMTS (a disintegrin and metalloprotease with thrombospondin domain) proteins and these proteins have important functions as cellular sheddase enzymes. The astacin family is a diverse group of endopeptidases that includes enzymes such as bone morphogenic protein 1 (BMP1) (Ugalde, Ordóñez et al. 2010).

Metzincin family members are defined by the zinc-coordination motif HExxHxxGxxH/D coupled with a conserved, downstream methionine β-turn, hence the **met**zincin name (Bode, Gomis-Rüth et al. 1993). MMPs use a histidine as the third coordinating residue for the zinc (**H**Exx**H**xxGxx**H**). In this motif, the three histidines coordinate with the active site zinc moiety and the conserved glycine provides flexibility to bring the third histidine into proximity to the zinc (Bode and Maskos 2001). Peptide cleavage is catalyzed by nucleophilic attack of the carbonyl carbon on the peptide backbone. In this reaction, the catalytic site glutamate acts a general base to activate a water molecule. The charged water molecule then attacks the peptide bond, resulting in peptide cleavage (Visse and Nagase 2003).

The MMP family is categorized based on structural domains, substrate specificity, and cellular localization (Figure 1). The basic structural requirements for an MMP are an N-terminal signal peptide for secretion, a pro-domain containing a conserved PRCxxPD cysteine switch motif, and a catalytic domain with a metzincin motif. This basic structure is characteristic of the matrilysins, MMP7 and 26. The archetypal MMP domain organization contains the basic structural requirements followed by a flexible, proline rich, hinge region and hemopexin-like repeats. The collagenases (MMP1, 8, and 13), the stromelysins (MMP3 and 10), MMP12, MMP19, MMP20, and MMP27 are structurally archetypal MMPs. The membrane type (MT) MMPs are defined as having the archetypal structure plus a furin-like activation sequence in the N-terminus and a C-terminal transmembrane domain (MMP14, 15, 16, and 24) or glycophosphatidyl inositol anchor (MMP17 and 25). The gelatinases (MMP2 and 9) contain the archetypal structures but have three fibronectin type II repeats inserted into the catalytic domain. Finally, MMP23A and B are the most unique family members, containing an N-terminal transmembrane domain, a C-terminal cysteine-rich array and C-terminal immunoglobulin-like domains, in addition to the classic pro- and catalytic domains (Ugalde, Ordóñez et al. 2010).

Matrix Metalloprotease Regulation:

Given their ability to degrade the structural proteins of tissues, MMPs are tightly regulated on multiple levels, including transcription, zymogen activation, and through interactions with inhibitory proteins. Cytokines, growth factors, and hormones are potent regulators of MMP transcription, with pro-inflammatory signaling typically promoting

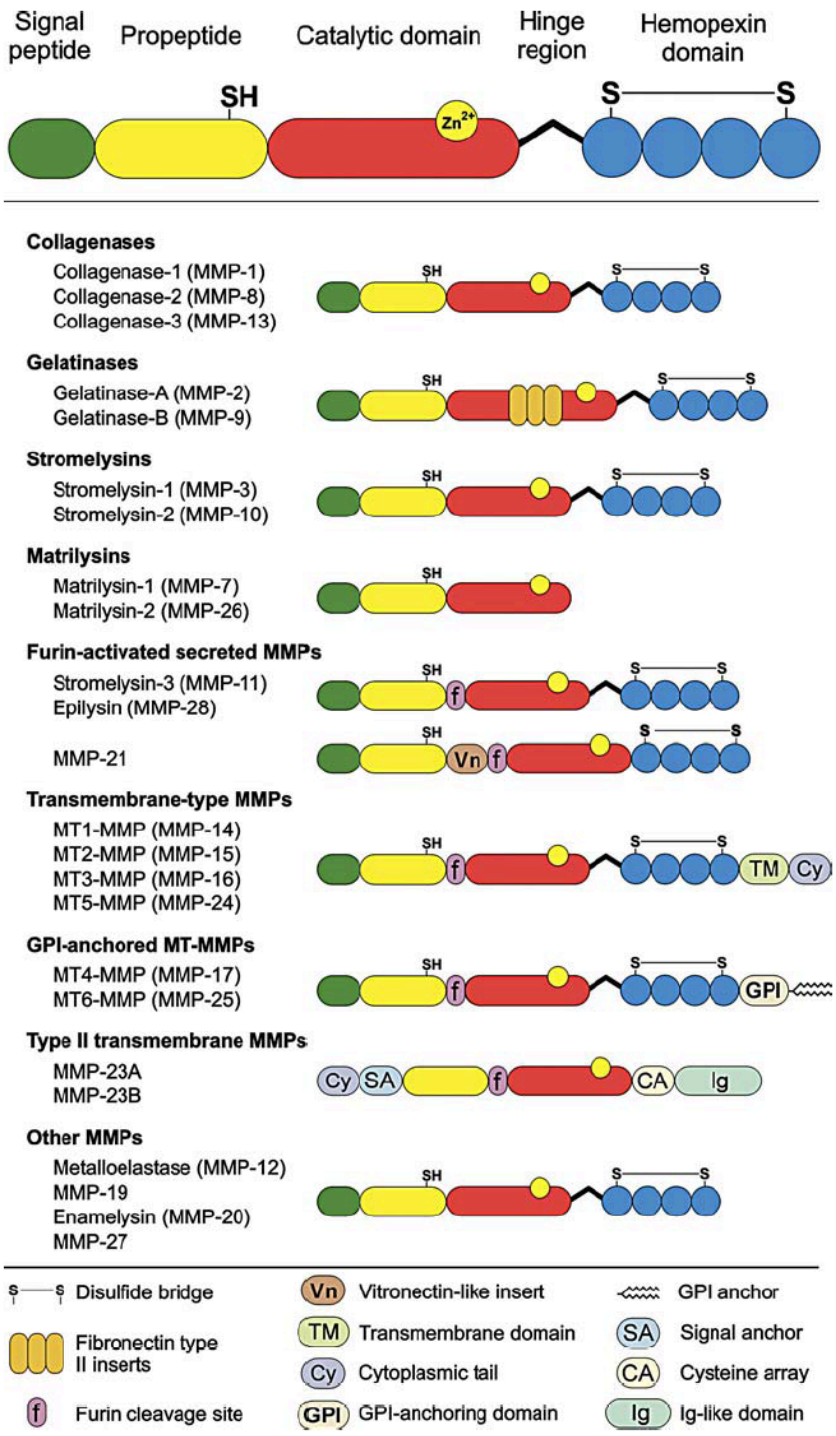

Figure 1: The MMP family member's domain composition and arrangement. Reproduced with permission from Alo-Aho, R. and Kahari, V.M., 2004.

increased MMP expression. MMP3 was originally discovered in 1985 as a cDNA that was upregulated by polyoma virus, Rous sarcoma virus, H-RAS transformation, and EGF treatment (Matrisian, Glaichenhaus et al. 1985). The vast majority of MMPs contain a functional activator protein 1 (AP-1) site in their promoter region that is believed to be the predominant regulator of MMP expression. General MMP expression can also be modulated through nuclear factor-κB (NF-κB), T-cell factor 4 (TCF4), p53 and erythroblastosis-twenty six (ETS), among other pathways (Sun, Sun et al. 1999; Vincenti and Brinckerhoff 2002; Ugalde, Ordóñez et al. 2010).

In addition to transcriptional regulation, enzyme activation is a critical level of control for MMPs. A subset of the secreted MMPs (MMP11, 21, and 28) and all of the membrane-tethered MMPs contain furin-like convertase sequences in the prodomain that lead to zymogen activation in the Golgi before transport to the cell membrane (Ala-aho and Kähäri 2005). The MMPs that lack convertase sequences are secreted as zymogens and must be activated after secretion. MMPs are maintained in the zymogen state through a conserved cysteine switch motif (PRCxxPD) in the pro-domain (Van Wart and Birkedal-Hansen 1990). In this motif, the free thiol of the cysteine acts as a fourth coordinating residue for the zinc moiety in the active site. This interaction enables the pro-domain to essentially cover the active site and prevent a water from entering the catalytic pocket (Van Wart and Birkedal-Hansen 1990). Disruption of the thiol-zinc interaction leads to zymogen activation. This disruption can occur through proteolysis of the prodomain or non-proteolytic mechanisms. Non-proteolytic activation occurs through reduction of the free thiol by oxidants, heavy metals, or alkylating agents. Once the free thiol is reduced, the pro-domain is then removed through an intramolecular,

autolytic cleavage. APMA (p-aminophenylmercuric acetate) is a non-proteolytic activator of MMPs and is frequently used in the laboratory for MMP activation (Stricklin, Jeffrey et al. 1983).

Proteolytic activation is typically a multi-step process in which a "bait" region of the pro-domain is initially cleaved. This results in intramolecular rearrangement and leads to a secondary cleavage of the remaining prodomain by the exogenous protease and/or the MMP itself. Proteolytic zymogen activation can be mediated by serine proteases, such as plasmin, mast cell chymases and trypsin, or by other MMPs. For example, membrane bound MMP14 activates MMP2 in a TIMP2-dependent manner. MMP3 is an activator of MMP1 and the cleavage sites for MMP3 in the MMP1 pro-domain have been clearly mapped (Murphy, Cockett et al. 1987). However, these activation pathways have been mostly demonstrated *in vitro* and it is not well understood how MMPs are activated *in vivo.* Studies in mouse models have been unable to show obligate evidence for these activation mechanisms *in vivo,* given that targeted knockouts of candidate activating proteases, such as plasminogen and *MMP14,* do not show significant defects in MMP activation (Ra and Parks 2007).

Once an MMP has been activated, it can be inactivated by inhibitor binding or through degradation. There are four mammalian tissue inhibitors of MMPs (TIMP) proteins. TIMPs are reversible antagonists that block MMP activity in a 1:1 stoichiometry. TIMPS are 21-28 kDa proteins that contain a variable C-terminal domain and a conserved N-terminal domain that covers the Zn^{2+} catalytic pocket to inactivate an MMP (Blavier, Henriet et al. 1999). TIMPs are potent inhibitors, with the affinity (K_i) of TIMP1 for MMP1 being 10^{-11} M *in vitro* (Gill, Kassim et al. 2010). However, TIMPs

perform many functions and are not specific to the MMPs. TIMPs also inhibit ADAM and ADAMTS family metalloproteases but have no effect on astacin family enzymes. Additionally, as noted above, TIMP2 can bind latent MMP2 and MMP14, causing MMP2 activation. Demonstrative of the complex role for TIMPs in biology, TIMPs have been shown to be tumor suppressive proteins, likely through the inhibition of MMPs. Anti-sense RNA knockdown of TIMPs leads to oncogenic transformation of 3T3 cells (Khokha, Waterhouse et al. 1989) and treatment with recombinant TIMPs can decrease tumorigenesis and experimental metastasis in animal models (Blavier, Henriet et al. 1999). However, there are also descriptions of TIMPs paradoxically promoting tumorigenesis, suggesting that tissue inhibitor of MMPs is an overly simplistic name for the TIMPs.

Several other TIMP-like MMP inhibitors have been identified. These include PCPE (type I collagen C-protease enhancer protein), the cell membrane glycoprotein RECK (reversion inducing cysteine-rich protein with Kazal motifs), and the serine protease inhibitor TFPI-2 (tissue factor pathway inhibitor 2) (Takahashi, Sheng et al. 1998; Herman, Sukhova et al. 2001; Baker, Edwards et al. 2002). Interestingly, TFPI-2 expression levels have been inversely correlated to MMP activity levels in human atherosclerotic plaques, suggesting a function for TFPI-2 inhibition of MMPs *in vivo (Herman, Sukhova et al. 2001)*.

Plasma protease inhibitors have also been shown to irreversibly inhibit MMPs. Thrombospondin 1 and 2 and alpha 2 macroglobulin (α2M) bind active MMPs and target them for degradation (Baker, Edwards et al. 2002). MMPs bind and cleave the bait region of α2M, causing the MMP to become entrapped in a complex with α2M. The

protease-α2M complex is recognized by scavenger receptors and is cleared from circulation by receptor-mediated endocytosis (Werb, Burleigh et al. 1974).

Matrix Metalloproteases in Physiology and Disease:

Given the ability of the MMP family to cleave virtually every component of the extracellular matrix, MMPs have been hypothesized to play important roles in tissue homeostasis and disease. However, generation of MMP-targeted knockout mice has demonstrated that individual MMPs are not essential for development and normal homeostasis. The vast majority of MMP knockout animals exhibit normal fertility, growth, and lifespan. The key exception to this is the *MMP14* knockout mouse, which exhibits dwarfism, osteopenia, arthritis, and death shortly after birth (Holmbeck, Bianco et al. 1999). Despite the lack of overt developmental phenotypes, numerous phenotypes for MMP transgenic mice have been described in disease models. For example, individual MMPs have been implicated in multiple carcinogenesis models, atherosclerosis, arthritis, lung disease, and aneurism formation (Fanjul-Fernández, Folgueras et al. 2010). These results make MMPs attractive therapeutic targets.

In the late 1990's/early 2000's, multiple clinical trials were conducted with broad spectrum MMP inhibitors. MMP inhibitors were tested for the treatment of advanced cancers (pancreatic, gastric, glioblastoma, lung, ovarian, etc), rheumatoid arthritis, osteoarthritis, macular degeneration, myocardial infarction, arterial restenosis, and stroke (Fingleton 2007). One MMP inhibitor, COL-3, has been shown to be well-tolerated and effective in phase II trials of AIDS-related Kaposi's sarcoma (Dezube, Krown et al. 2006). Unfortunately, all the other MMP trials were ultimately unsuccessful. Several

reasons have been suggested for the failure of MMP inhibitors in the clinic. First, in many studies, dosages had to be reduced due to musculoskeletal pain of unclear etiology. Second, many of these trials did not have defined biomarkers to determine whether the inhibitor levels were sufficient to actually inhibit target MMP activity in the patients. Third, all of the cancer trials were performed on late stage disease that may have been too advanced for MMP inhibitors to be effective (Coussens, Fingleton et al. 2002). Finally, the inhibitors used were broad spectrum inhibitors, with many targeting most MMPs as well as some ADAM family enzymes. Research in the post-MMP inhibitor clinical trial era has demonstrated that each MMP has very distinct functions, with some MMPs performing protective functions and others harmful in different disease states (Overall and Kleifeld 2006). Targeting MMPs in disease therefore requires a careful understanding of the specific functions for an individual MMP in a disease model which is why we chose to focus on the major interstitial collagenase, MMP1, in tumorigenesis for this thesis.

1.2 Matrix Metalloprotease 1

History and General Information:

MMP1 (collagenase 1, interstitial collagenase, fibroblast collagenase) was the first matrix degrading enzyme discovered. In 1962, Gross and Lapiere described an enzymatic activity responsible for collagen degradation in the resorbing tadpole tail at neutral pH. This activity was later determined to be MMP1 (Gross and Lapiere 1962). Eight years later, MMP1 enzyme was purified from human skin (Eisen, Bauer et al. 1970). *MMP1* cDNA was cloned from rabbit in 1984 (Gross, Sheldon et al. 1984) and

human *MMP1* was sequenced two years later (Goldberg, Wilhelm et al. 1986; Brinckerhoff, Ruby et al. 1987). The catalytic domain of human MMP1 in complex with a synthetic inhibitor was crystallized in 1994 (Lovejoy, Cleasby et al. 1994) and crystal structures for full length MMP1 in both the pro- and active forms have been solved (Jozic, Bourenkov et al. 2005; Iyer, Visse et al. 2006).

MMP1 is a 10 exon gene, spread over 8.2 Kb of human chromosome 11 (Pardo and Selman 2005). The *MMP1* gene is located in an *MMP*-rich cluster on chromosome 11q22 that also contains *MMP-3, -7, -8, -10, -12, -13, -20,* and *-27*. This genomic *MMP* locus suggests that the *MMPs* have arisen by duplication and subsequent diversification of an ancestral *MMP* gene (Massova, Kotra et al. 1998; Puente, Sánchez et al. 2003). *MMP1* encodes a 469 amino acid protein that has a molecular weight of 54 or 57 kDa, depending on glycosylation. MMP1 contains two potential N-linked glycosylation sites (N120 and N143) but only one site appears to be glycosylated (N120) in fibroblasts and cancer cells (Saarinen, Welgus et al. 1999).

Enzymatic Activity:

MMP1 is a secreted collagenase, which means that it has the ability to degrade fibrillar collagen (types I, II, and III) and contains the characteristic pre-, pro-, catalytic, linker, and hemopexin domain arrangement (see Figure 1). MMP8 (neutrophil collagenase, collagenase 2) and MMP13 (collagenase 3) are the other secreted collagenase family members. MMP14 and perhaps MMP16 also have collagenase activity but are membrane anchored and not secreted from cells (Sabeh, Li et al. 2009).

Collagenolysis at neutral pH is a significant enzymatic feat. Type I collagen is the most abundant structural protein in the body. It is extremely stable, with an *in vivo* stability that can span decades. Fibrillar collagen is composed of three strands with characteristic Gly-Pro-X or Gly-X-Hydroxyproline repeats that form a left-handed triple helix. Degradation of native collagen requires helicase activity to unwind the helix followed by proteolysis of each individual chain (Saffarian, Collier et al. 2004). MMP1 and the other collagenases specifically cleave fibrillar collagen between Gly-Ile or Gly-Leu 775-776 to generate characteristic ¾ and ¼ length collagen fragments (Wu, Byrne et al. 1990). Transgenic mice with type I collagen that has been mutated at this site exhibit abnormal fibrosis and uterine remodeling, suggesting that this is a physiologic cleavage (Liu, Wu et al. 1995). Cleaved ¾ and ¼ length collagen fragments are thermodynamically unstable and further unwind, allowing processing by other proteases, such as the gelatinases.

The hemopexin domain is essential for MMP1 binding of collagen. MMP1 can only bind collagen in the active state because interactions between the prodomain and hemopexin domain result in proMMP1 having a "closed" confirmation (Murphy, Allan et al. 1992; Jozic, Bourenkov et al. 2005). Surprisingly, the catalytic pocket of MMP1 is not large enough accommodate triple helical collagen. Triple helical collagen initially binds an MMP1 exosite and complex interactions between the hemopexin, linker, and catalytic domain provide helicase activity required to locally unwind the collagen strands (Williams and Olsen 2009). The unwound collagen can then be fed strand by strand into the catalytic site for progressive proteolysis (Chung, Dinakarpandian et al. 2004).

In addition to its functions as a collagenase, MMP1 cleaves many other substrates. MMP1 has proteolytic activity against fibronectin, gelatin, laminin, perlecan, and vitronectin (Ala-aho and Kähäri 2005). MMP1 modulates inflammation and cell behavior through cleavage of bioactive molecules. For example, MMP1 and several other MMPs cleave membrane bound pro-tumor necrosis factor alpha (pro-TNFα) into its active, soluble form (Gearing, Beckett et al. 1994). MMP1 degrades insulin-like growth factor binding proteins (IGFBP), creating increased bioavailability of IGF and inducing fibroblast proliferation (Fowlkes, Enghild et al. 1994). MMP1 cleavage inactivates stromal cell derived factor 1 alpha (SDF1α), leading to decreased leukocyte and hematopoietic stem cell chemotaxis (McQuibban, Butler et al. 2001). Similarly, MMP1 cleaves monocyte chemoattractant proteins 1-4 (MCP 1-4), converting a potent chemoattractant agonist into an antagonist and leading to decreased swelling and inflammation in rat paw edema models (McQuibban, Gong et al. 2002). Interleukin 1β (IL1β), which is a potent inducer of MMP transcription, is itself a target for both activation and degradation by MMP1, suggesting a regulatory mechanism by which IL1β induces MMP expression and thereby downregulates IL1β signaling once sufficient MMP levels have been reached (Ito, Mukaiyama et al. 1996; Schönbeck, Mach et al. 1998). Finally, our group has recently shown that MMP1 cleaves and activates protease activated receptor 1 (PAR1) and this signaling pathway promotes tumor progression, thrombus formation, and the pathogenesis of sepsis (see Section 1.4) (Boire, Covic et al. 2005; Trivedi, Boire et al. 2009; Tressel, Kaneider et al. 2011).

MMP1 Activation:

MMP1 is secreted as a proenzyme and must therefore be activated following secretion. The prodomain of MMP1 is a three-helix bundle. Interactions of the prodomain helices with each other and with the mature enzyme stabilize the prodomain structure (Jozic, Bourenkov et al. 2005). The cysteine switch motif, $P^{90}RCGVPD^{96}$, which is after the third helix, feeds into the catalytic cleft such that the cysteine provides the fourth coordination residue for the active site zinc moiety. As discussed in section 1.1, the *in vivo* activator of MMP1 has not been definitively determined. However, MMP1 activation has been studied extensively via *in vitro* activation by MMP3, organomercurials, trypsin, plasmin, and plasma kallikrein (Stricklin, Jeffrey et al. 1983; Murphy, Cockett et al. 1987; Suzuki, Enghild et al. 1990). The prodomain of MMP1 contains a "bait region" ($V^{51}KERRNS^{57}$) that is flexible and fully exposed (Jozic, Bourenkov et al. 2005). Activating proteases initially cleave this bait region, leading to destabilization of the helix bundle and allowing for a secondary autolytic cleavage at $T^{82}L^{83}$. This then exposes the junction of the pro- and catalytic domains so that this region can be cleaved either by exogenous protease or autolytic cleavage. Interestingly, the protease that cleaves the final region between the pro- and catalytic domains determines the new N-terminus of the mature enzyme. MMP3 activation of MMP1 generates a mature enzyme that begins with F^{100}. Activation of MMP1 by organomercurials/autolytic cleavage results in a mature enzyme starting at V^{101} or L^{102} (Suzuki, Enghild et al. 1990). MMP3-activated F^{100} aMMP1 has 10 fold more collagenase activity than V^{101} or L^{102} aMMP1, suggesting that the mechanism of activation is an additional regulator of MMP1 activity. F^{100} aMMP1 is likely more active

because in active MMP1, F^{100} forms a salt bridge with D^{251} to stabilize the catalytic domain (Li, Brick et al. 1995).

MMP1 in Disease:

MMP1 is typically expressed at very low levels in healthy human tissue but can be massively upregulated in inflammatory and disease states, suggesting a role for MMP1 in human pathobiology. MMP1 has been extensively studied in the degenerative joint diseases, rheumatoid arthritis and osteoarthritis. Elevated TNFα and IL1 levels have been shown to be major mediators of osteoarthritis and rheumatoid arthritis and TNF inhibitors are even approved for the treatment of rheumatoid arthritis. These inflammatory molecules strongly induce MMP1 expression in chondrocytes and synovial fibroblasts(Vincenti and Brinckerhoff 2002). In rheumatoid arthritis, the degree of cartilage destruction has been directly correlated to the level of MMP1 protein expression (Neidhart, Seemayer et al. 2003).

MMP1 expression is also increased in human atherosclerotic plaques (Nikkari, O'Brien et al. 1995). In fact, higher MMP1 expression is present in vulnerable atherosclerotic plaques prone to rupture and this elevated expression is also associated with increased collagenolysis (Sukhova, Schönbeck et al. 1999). However, MMP1 may also play protective roles in experimental plaque remodeling given that transgenic mice with forced overexpression of human MMP1 under a macrophage specific promoter develop smaller, less advanced atherosclerotic plaques (Lemaître, O'Byrne et al. 2001).

In addition to its roles in the pathogenesis of degenerative arthritis and cardiovascular disease, MMP1 has been shown to be an important mediator of tumor

progression and metastasis. Table 1 lists a subset of cancers with MMP1 overexpression in patient samples and any prognostications associated with MMP1 expression. As seen in Table 1, MMP1 is overexpressed by many different cancer types, including lung, breast, and melanoma, and MMP1 expression often correlates with poor patient prognosis. Additionally, an insertional polymorphism in the *MMP1* promoter that results in slightly elevated *MMP1* transcription has been associated with increased susceptibility to cancer. The insertion of a second guanosine (G) at -1607 bp in the *MMP1* promoter generates an additional Ets1 binding site and leads to higher levels of MMP1 transcription (Rutter, Mitchell et al. 1998). The frequency of the 2G/2G polymorphism is estimated to be about 30%. This polymorphism has been associated with increased risk of developing or poor prognosis for several cancer types, including melanoma, lung, and ovarian cancer (Ye, Dhillon et al. 2001; Zhu, Spitz et al. 2001; Six, Grimm et al. 2006; Sun, Gao et al. 2006; Wang, Huang et al. 2011). Meta-analysis of 35 studies with over 10,000 cancer cases found that homozygotes for the MMP1 risk allele (2G/2G) had an elevated risk of metastasis (OR 1.44) independent of the cancer type (Liu, Guo et al. 2012). Additionally, MMP1 protein levels are elevated in ovarian tumors with the 2G/2G allele, supporting the role for this polymorphism in increasing MMP1 expression *in vivo* (Kanamori, Matsushima et al. 1999).

Xenograft tumor models have also shown that inhibition of MMP1 results in decreased tumorigenesis, invasion, metastasis, and angiogenesis. Treatment of breast cancer xenografts with a broad spectrum MMP inhibitor with strong MMP1 activity,

Cancer Type	MMP1 Observation	Reference
Breast	Detected in 77% of invasive breast carcinomas (in cancer cells in 65% and stromal fibroblasts in 53%)	(Nakopoulou, Giannopoulou et al. 1999)
	Expression of MMP1 in stromal fibroblasts correlates with metastasis	(Vizoso, González et al. 2007)
Colorectal	Expression correlates with poor prognosis	(Murray, Duncan et al. 1996)
	Marker for hematogenous metastasis	(Sunami, Tsuno et al. 2000)
Esophageal	Detected in 24%, associated with decreased survival	(Murray, Duncan et al. 1998)
Lung (NSCLC)	MMP1 protein levels elevated 10 fold as compared to normal lung tissue	(Shah, Spinale et al. 2010)
	Promoter polymorphism 2G/2G genotype associated with increased NSCLC susceptibility	(Zhu, Spitz et al. 2001)
Melanoma	High expression correlates with shorter progression free survival	(Nikkola, Vihinen et al. 2002)
	Expression correlates with tumor grade	(Airola, Karonen et al. 1999)
Ovarian	Increased incidence of 2G/2G genotype in ovarian cancer cases	(Kanamori, Matsushima et al. 1999)

Table 1: Observations of MMP1 expression and clinical correlation in patient cancer samples.

FN439, results in decreased tumor growth, invasion, and angiogenesis (Boire, Covic et al. 2005). Short hairpin RNA silencing of MMP1 reduces subcutaneous tumor growth of MMP1 expressing MDA-MB-231 breast cancer cells by almost 80% in xenograft models and decreased angiogenesis and osteolysis in an *in vivo* bone metastasis model (Wyatt, Geoghegan et al. 2005; Eck, Hoopes et al. 2008). Likewise, silencing of MMP1 in melanoma xenografts reduces lung metastasis and angiogenesis, while ectopic expression of MMP1 in low-grade melanoma cells increases tumor growth, dermal invasion, and metastasis to the lungs (Blackburn, Rhodes et al. 2007; Blackburn, Liu et al. 2009). Carcinogenic treatment of transgenic mice that overexpress MMP1 in skin under the haptoglobin promoter results in increased incidence and total number of skin tumors (D'Armiento, DiColandrea et al. 1995). These results are all consistent with MMP1 promoting tumorigenesis and metastasis *in vivo*. However, the study of MMP1 in disease models has been severely limited due to uncertainty about the identity or function of the mouse homologue of MMP1.

Mouse MMP1:

The putative mouse genetic homologue for MMP1 was not described until 2001 (Balbín, Fueyo et al. 2001). Screening of mouse cDNA libraries with a full length, human MMP1 probe identified two *MMP1* homologues, *Mmp1a* and *Mmp1b*. Figure 2 shows an amino acid alignment of Mmp1a (Mcol-A), Mmp1b (Mcol-B), with human MMP1 as well as the other mouse collagenases, Mmp8 and Mmp13. These two enzymes appear to be a rodent specific duplication, having been identified in both the mouse and rat genome. *Mmp1a* and *Mmp1b* are located in the MMP-rich locus of mouse

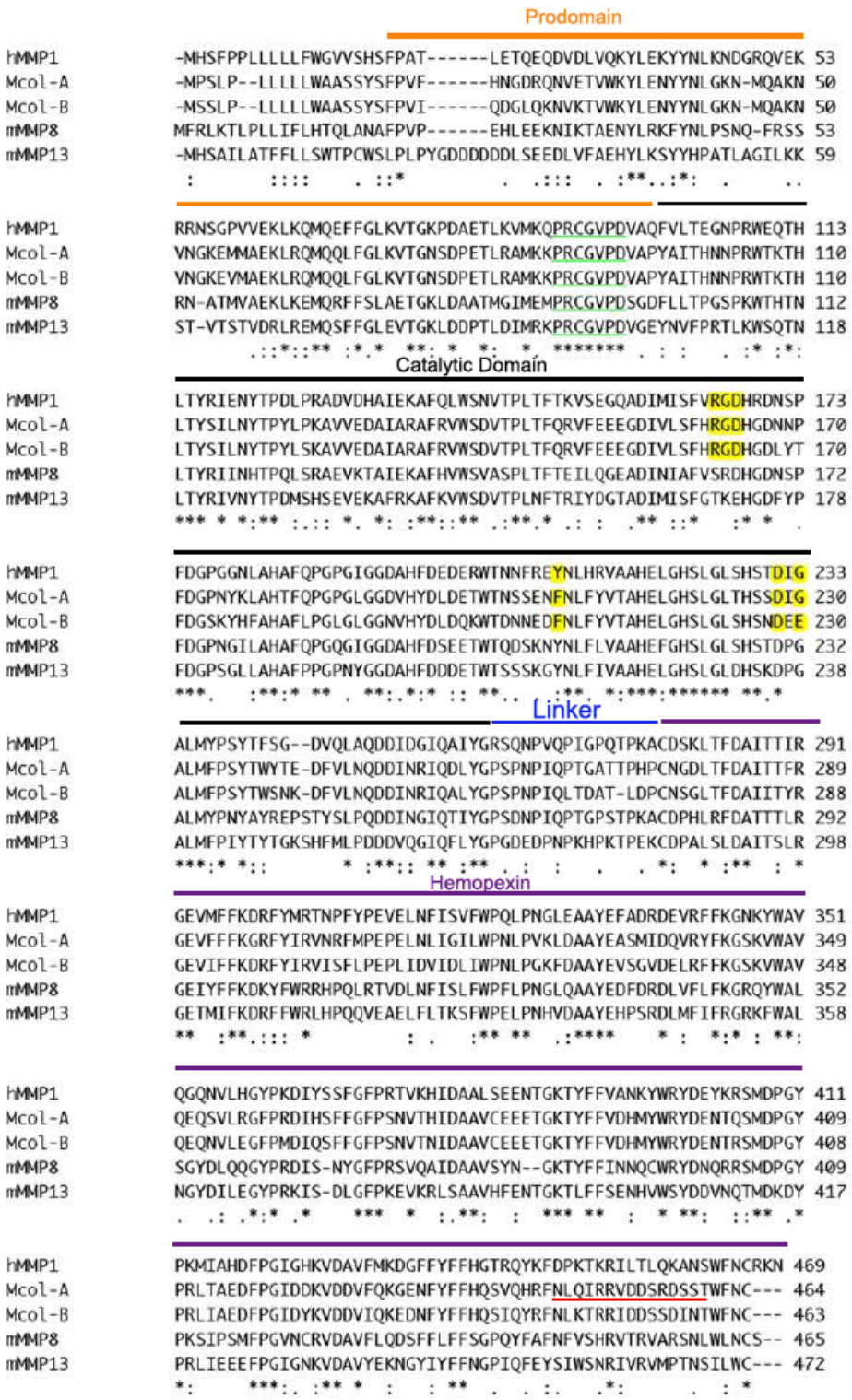

Figure 2: Clustal alignment of amino acid sequences for human MMP1 and the four mouse collagenases, Mmp1a (Mcol-A), Mmp1b (Mcol-B), Mmp8, and Mmp13. The protein domains are denoted by the following lines: orange (prodomain), black (catalytic domain), blue (linker), purple (hemopexin). The cysteine switch motif is underlined in green. MMP1 residues that are highly conserved amongst other MMP1 homologues are highlighted in yellow.

chromosome 9 and the location of *Mmp1a* is syntenic to human *MMP1*. Mmp1a and Mmp1b proteins are 82% identical to each other and most similar to human MMP1 (58% amino acid identity, 74% identical in nucleotides). Mmp1a and Mmp1b are 464 and 463 amino acid proteins, respectively, with predicted masses of 53.5 kDa. Mmp1a contains three potential N-linked glycosylation sites, while Mmp1b contains two. A conserved glycosylation site in Mmp1a and Mmp1b is shared with hMMP1. Both enzymes contain the collagenase domain arrangement (signal peptide, pro-, catalytic, linker, and hemopexin domains) and a characteristic RGD-motif in the catalytic domain that is present in all other identified MMP1 homologues. Mmp1a and Mmp1b appear to be functional metalloproteases based on amino acid sequence and predicted structure and both will undergo autolytic cleavage of their prodomain when incubated at room temperature for 24 hours. However, when expressed recombinantly, only Mmp1a surprisingly has collagenase activity, suggesting that Mmp1a, and not Mmp1b, is the true homologue of MMP1 (Balbín, Fueyo et al. 2001).

Despite *Mmp1a* clearly being the genetic homologue of human *MMP1*, there is significant controversy as to whether Mmp1a performs analogous functions in mouse physiology and disease models. Mmp1a has only 58% identity with MMP1. Figure 3 shows the percent amino acid identity based on Clustal alignment between various mammalian MMP1 homologues. Rodent Mmp1a is the least identical of the known mammalian MMP1 homologues. As a comparison, rabbit MMP1, which has been studied fairly well in disease models, is 86% identical (Yang, Kim et al. 2010).

Additionally, Mmp1a has a different expression profile from human MMP1 in quiescent tissue. Human MMP1 is believed to be ubiquitously expressed at low, but

Mus musculus (1A)	Mus musculus (1B)	Rattus norvegicus (1A)	Rattus norvegicus (1B)	Oryctolagus cuniculus	Sus scrofa	Canis lupus	Bos taurus	Equus caballus	Pan troglodytes	
57.3	55.1	56.2	54.3	86.1	84.0	79.9	83.8	85.5	98.7	*Homo sapien*
	81.6	88.1	76.3	55.6	55.8	56.5	56.5	58.0	57.1	*Mus musculus (1A)*
		76.5	81.5	54.0	54.0	51.8	53.8	54.9	54.4	*Mus musculus (1B)*
			77.6	55.4	55.8	56.5	55.2	58.2	55.6	*Rattus norvegicus (1A)*
				52.6	52.4	53.0	52.6	54.3	53.5	*Rattus norvegicus (1B)*
					82.7	78.8	83.5	84.8	85.5	*Oryctolagus cuniculus*
						80.3	86.1	86.6	83.5	*Sus scrofa*
							80.8	83.1	79.5	*Canis lupus*
								85.9	83.5	*Bos taurus*
									85.0	*Equus caballus*

Figure 3: Percent similarity of mammalian MMP1 homologues to each other. Percentage determined by Clustal sequence alignment using the Meg Align program.

detectable levels in most human tissues (Sternlicht and Werb 2001). Mmp1a, on the other hand, has only been detected in day 9.5-13.5 embryos and in the placenta, uterus, and testes of physiologically normal mice (Balbín, Fueyo et al. 2001; Nuttall, Sampieri et al. 2004).

These differences have led some to conclude that Mmp1a is not a functional MMP1 homologue and to consider the other mouse collagenases, Mmp8 and Mmp13, as functional homologues of MMP1. However, MMP8 is primarily expressed by neutrophils in both humans and mice. *Mmp8*-deficient mice exhibit increased skin carcinogenesis and prolonged inflammation during wound healing (Balbín, Fueyo et al. 2003; Gutierrez-Fernandez, Inada et al. 2007). Additionally, *MMP8* is often mutated/inactivated in human melanoma (Palavalli, Prickett et al. 2009). These data suggest that MMP8 is not a functional MMP1 homologue because MMP8 has very different pathophysiologic functions to MMP1, with MMP8 functioning as a tumor suppressor while MMP1 is an oncogene.

MMP1 and MMP13 are more pathologically similar than MMP8 and MMP1. Like MMP1, MMP13 is frequently overexpressed in inflammatory conditions and cancer and is transcriptionally regulated by many of the same factors as MMP1 (Brinckerhoff, Rutter et al. 2000; Vincenti and Brinckerhoff 2002; Johnson, Pavlovsky et al. 2007; Vizoso, González et al. 2007; Yang, Kim et al. 2010). *Mmp13*-deficient mice have decreased tumor growth and metastasis of B16 melanoma allografts and decreased growth and angiogenesis of squamous cell carcinoma allografts (Zigrino, Kuhn et al. 2009; Lederle, Hartenstein et al. 2010). While this suggests that there may be

redundancy in the functions of MMP1 and MMP13, it does not exclude specific roles for MMP1 and MMP13.

Despite the low expression of *Mmp1a* in healthy mouse tissue, *Mmp1a* upregulation has been described in multiple disease models. *Mmp1a* mRNA is elevated in the mouse stroma of human breast cancer cell xenografts (Boire, Covic et al. 2005). Elevated mRNA levels have also been described in the healing wounds of *Mmp13*-deficient mice and in pesticide-induced lung injury models. Mmp1a protein is upregulated in the plasma of mice with cecal ligation and puncture (CLP)-induced sepsis and in the joints of mice with collagen-induced arthritis (Pfaffen, Hemmerle et al. 2010; Tressel, Kaneider et al. 2011). Additionally, Mmp1a protein is expressed by the cancer cells in B16 melanoma allografts and by the mouse stroma in glioblastoma, renal, kidney, and ovarian cancer xenografts (Pfaffen, Hemmerle et al. 2010). Though Mmp1a may not be a functional homologue in tissue homeostasis, the observed upregulation of Mmp1a in disease models indicates that Mmp1a may be an important functional homologue in mouse models of human disease. Given the importance of MMP1 in human cancers and the value of highly correlative mouse models in understanding and treating disease, this thesis focuses on MMP1/Mmp1a and their role in activation of a pro-tumorigenic cell surface receptor, PAR1 in tumorigenesis models.

1.3 Protease Activated Receptor 1

The Protease Activated Receptor Family:

The protease activated receptors (PAR) are a family of four G protein-coupled receptors that share a proteolytic cleavage activation mechanism. PAR1, also known as the thrombin receptor, is the prototypical PAR and was initially discovered in 1991. *PAR1 (F2R)* was cloned by direct expression in Xenopus oocytes of cDNAs isolated from thrombin responsive cells (Vu, Hung et al. 1991). Sequencing of *PAR1* revealed in 425 amino acid protein with seven hydrophobic/transmembrane domains and a thrombin cleavage site in the N-terminus. *PAR2 (F2RL1)* was identified in 1994. PAR2 is 30% identical to PAR1 and contains an N-terminal trypsin cleavage site. *PAR3 (F2RL2)* was discovered following the observation that *PAR1* knockout mice retain their thrombin responsiveness (Connolly, Ishihara et al. 1996). PAR3 has 27% amino acid similarity to PAR1 and contains an N-terminal thrombin cleavage site (Ishihara, Connolly et al. 1997). However, a synthetic form of the PAR3 tethered ligand does not activate PAR3 and PAR3 is unable to signal alone in most reports (Kahn, Zheng et al. 1998). *PAR4* was identified in 1998. PAR4 is 33% identical to PAR1 and contains a thrombin cleavage site (Xu, Andersen et al. 1998).

PAR1, PAR3, and PAR4 are all considered thrombin receptors given the thrombin activation site in the N-terminus. PAR1 and PAR3 are high affinity thrombin receptors because they contain a charged hirudin-like motif that enhances α-thrombin binding. PAR4 is a low affinity thrombin receptor because it lacks a hirudin domain. However, α-thrombin activation of PAR4 can be enhanced through PAR1-PAR4 and

PAR3-PAR4 dimerization (Kahn, Nakanishi-Matsui et al. 1999; Nakanishi-Matsui, Zheng et al. 2000; Leger, Jacques et al. 2006).

PAR1 is located on human chromosome 5q13 (mouse chromosome 13D1) in a *PAR* gene cluster that contains *PAR3* upstream and *PAR2* downstream (Bahou, Nierman et al. 1993). *PAR4* is located on human chromosome 19p12 (mouse chromosome 8B3). The *PARs* have an interesting two exon gene structure. The first exon encodes the signal peptide followed by an intron of variable length (14 kb for *PAR1* and *PAR2*, 4.5 kb for *PAR3* and 0.25 kb for *PAR4*). The second exon is a large exon that encodes the entire mature protein (Kahn, Hammes et al. 1998). The genomic arrangement of the PARs suggests that *PAR2* arose as a recent duplication event whereas the duplication events for *PAR3* and *PAR4* were earlier.

PAR1 Signaling:

Though historically described as a thrombin receptor, multiple serine proteases and one matrix metalloprotease have been demonstrated to be PAR1 agonists, including activated protein C (APC), Factor Xa, plasmin, and MMP1 (Kuliopulos, Covic et al. 1999; Riewald, Petrovan et al. 2002; Boire, Covic et al. 2005). Serine proteases cleave the N-terminus of PAR1 between residues $R^{41}S^{42}$, revealing a previously masked tethered ligand, S^{42}FLLRN. Interestingly, MMP1 cleaves PAR1 at a slightly different site, between $D^{39}P^{40}$, resulting in the slightly longer ligand P^{40}RSFLLRN (Trivedi, Boire et al. 2009). The newly exposed tethered ligand interacts with the receptor's N-terminal domain and extracellular loop II, resulting in conformational change and activation of G protein signaling (Gerszten, Chen et al. 1994; Seeley, Covic et al. 2003). A valuable tool

in studying PAR signaling is that synthetic peptides, called activating peptides (AP), corresponding to the tethered ligand sequence (SFLLRN or TFLLRN for PAR1) are able to exogenously activate PAR signaling (Scarborough, Naughton et al. 1992).

By coupling to heterotrimer G proteins, PAR1 is able to signal through diverse pathways and exert pleiotrpic effects, including proliferation, migration/invasion, gene transcription, cell survival, granule release, and shape change (Figure 4). PAR1 couples to $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ (Hung, Wong et al. 1992; Aragay, Collins et al. 1995), likely through critical regions of the PAR1 eighth helix in concert with intracellular loop 1 and transmembrane helix 7 (Swift, Leger et al. 2006). In classical G protein signaling, $G\alpha_i$ activation inhibits adenylate cyclase, causing a decrease in cAMP accumulation. $G\alpha_q$ signals by activation of phospholipase Cβ (PLCβ). PLCβ generates diacyl glycerol and inositol 1,4,5 triphosphate (IP3) leading to release of intracellular calcium stores (Benka, Lee et al. 1995). The Gβγ subunit can also activate PLCβ (Coughlin 2000). $G\alpha_{12/13}$ is the only PAR1 effector that does not signal through second messengers; $G\alpha_{12/13}$ activates Rho guanosine exchange factors (GEFs) that lead to activation of Rho and cytoskeletal rearrangement (Nguyen, Faivre et al. 2002).

However, G protein signaling is significantly more complicated than the canonical pathways describe. The different G proteins can overlap in their signaling effectors and the downstream pathways activated by PAR1 are an integration of multiple G protein signals. For example, PAR1 stimulation leads to mitogen-activated protein kinase (MAPK) activation and proliferation. In astrocytes, proliferation and ERK1/2 activation have been shown to be mediated by a combination of both pertussis toxin

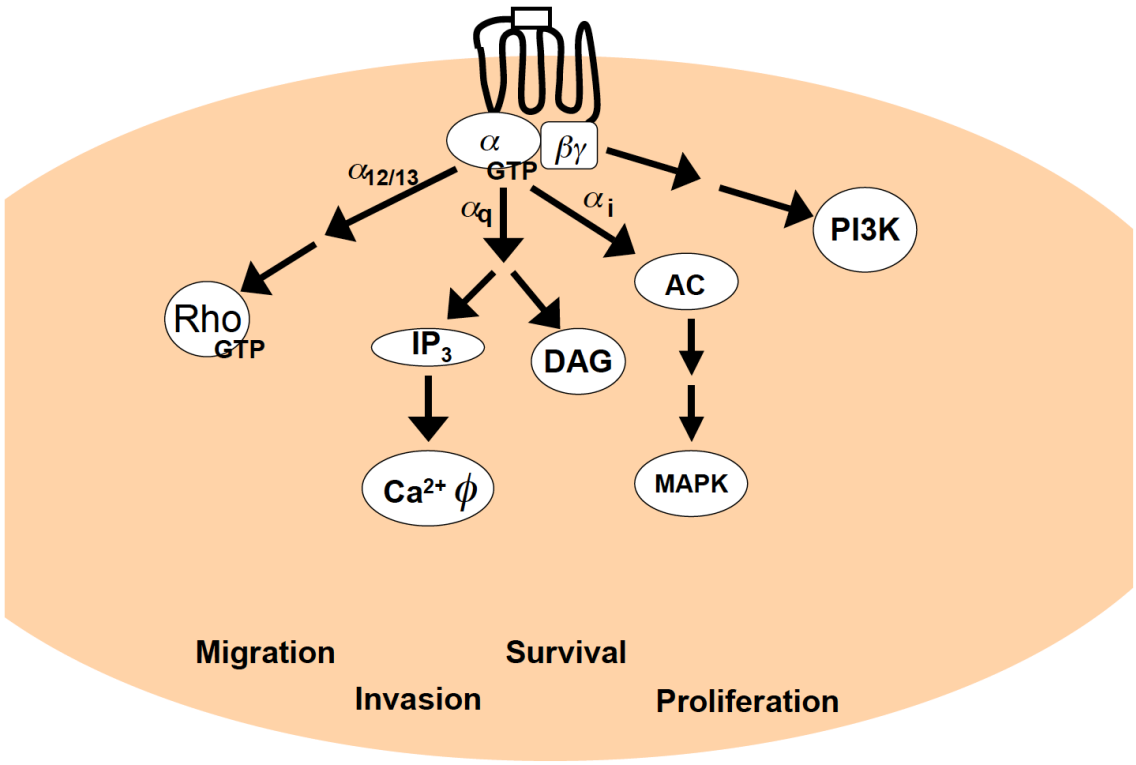

Figure 4: Highly simplified diagram depicting classical G-protein signal cascades following PAR1 activation.

(PTX) sensitive $G\alpha_i/G\beta\gamma$ pathways and PTX insensitive pathways, demonstrating the convergence of multiple downstream PAR1 pathways (Wang, Ubl et al. 2002).

Additionally, PAR1-G protein signaling activates certain major signaling pathways in mechanisms that are poorly defined. PAR1 has been shown to promote cell survival and release of inflammatory cytokines from prostate cancer cells *in vitro* through activation of NF-κB signaling (Tantivejkul, Loberg et al. 2005). In endothelial cells, PAR1-induced NF-κB signaling has been proposed to be mediated by both $G\alpha_q$ and $G\beta\gamma$ signaling through Akt activation (Rahman, True et al. 2002). However, Akt was not required in the studies involving prostate cancer cells, making it unclear how PAR1 actually activates NF-κB and whether the mechanism is cell type dependent (Tantivejkul, Loberg et al. 2005).

PAR1 has also been shown to stabilize β-catenin leading to nuclear localization in transgenic mouse mammary tissues that overexpress human PAR1 (Yin, Katz et al. 2006). This pathway appears to be independent of Wnt/Frizzled signaling and is mediated by a $G\alpha_{13}$-dishevelled interaction (Turm, Maoz et al. 2010).

Finally, PAR1 is able to transactivate EGFR family members. PAR1 transactivation of EGFR has been shown to be important for proliferation and migration/invasion of breast, colon, and renal carcinoma cells (Darmoul, Gratio et al. 2004; Bergmann, Junker et al. 2006; Arora, Cuevas et al. 2008) and PAR1-mediated Erb2 transactivation promotes breast cancer invasion (Arora, Cuevas et al. 2008). EGFR transactivation requires metalloprotease activity but is not due to liberation of HB-EGF, making it unclear how this process is actually mediated (Bergmann, Junker et al. 2006).

PAR1 Regulation:

Unlike a standard, reversible ligand-receptor interaction, activation of PAR1 is irreversible and one protease agonist can activate multiple PAR receptors. PAR1 signaling is therefore silenced by receptor inactivation and/or destruction. On the cell membrane, full-length or activated PAR1 can be inactivated by N-terminal proteolytic cleavage downstream of the tethered ligand. Multiple serine proteases, such as elastase and trypsin, have been shown to inactivate PAR1 (Adams, Ramachandran et al. 2011). A metalloprotease, potentially ADAM17, in endothelial cells has been described to constitutively shed and inactivate the N-terminal exodomain of PAR1, reducing the pool of potentially activatable receptors on the cell surface by an estimated 50% (Ludeman, Zheng et al. 2004). Interestingly, plasmin and MMP1, which are both PAR1 agonists, can also cleave the PAR1 exodomian downstream of their respective activation sites to desensitize the receptor (Kuliopulos, Covic et al. 1999; Nesi and Fragai 2007). However, the biological significance of these disarming cleavages is unclear.

PAR1 signaling can also be regulated by receptor internalization. PAR1 is internalized through a constitutive pathway and an agonist-induced pathway. The constitutive internalization pathway cycles unactivated receptors between the plasma membrane and intracellular endosomes. This pathway is mediated by an adaptor protein 2 (AP2)-clathrin interaction and provides a reservoir of PAR1 molecules that can resensitize the cell following agonist stimulation (Paing, Johnston et al. 2006).

Internalization of activated PAR1 is slightly more complicated. Following activation, PAR1 signaling is desensitized by phosphorylation of serine/threonine residues in the intracellular C-terminus and third intracellular loop by G protein receptor

kinases (GRK) (Ishii, Chen et al. 1994; Hammes, Shapiro et al. 1999). C-terminal phosphorylation leads to the recruitment of β-Arrestin which prevents PAR1 from signaling through steric hindrance of G protein interactions (Paing, Stutts et al. 2002; Chen, Paing et al. 2004). Interestingly, while β-Arrestin is required for densensitization of PAR1 signaling, it is not is necessary for internalization of activated PAR1 (Paing, Stutts et al. 2002). Activated PAR1 is endocytosed by clathrin-coated pits and interactions with sorting nexin 1 (SNX1) in a poorly understood mechanism to target activated PAR1 from the endosome to the lysosome for destruction (Hoxie, Ahuja et al. 1993; Gullapalli, Wolfe et al. 2006). Recent work has also demonstrated that the scaffold protein Bicaudal D1 (BicD1) interacts with the C-terminal of PAR1 and modulates activated receptor densitization and internalization in a manner independent of β-Arrestin and phosphorylation, suggesting multiple pathways for silencing of PAR1 signaling (Swift, Xu et al. 2010).

PAR1 in Physiology and Disease:

PAR1 signaling has been implicated in physiologic and developmental processes as well as various pathologies. This is not surprising given that PAR1 is ubiquitously expressed throughout the body. PAR1 mRNA was expressed in 41 of 41 different tissue types examined by *Regard and colleagues*, with particularly high levels of PAR1 in quiescent heart and vasculature tissues (Regard, Sato et al. 2008). PAR1 is an attractive drug target and a small molecule PAR1 antagonist, Vorapaxar, recently completed phase III clinical trials as an anti-thrombotic therapy with mixed results (Morrow, Braunwald et al. 2012; Tricoci, Huang et al. 2012).

The importance of PAR1 in development is clearly illustrated by the phenotype of *PAR1*$^{-/-}$ mice. *PAR1* deficiency is associated with 50% embryonic lethality (Connolly, Ishihara et al. 1996). *PAR1*$^{-/-}$ embryos exhibit a delay in placental development and half die between embryonic day 9 and 10 due hemorrhage. Surprisingly, this phenotype is not due to a platelet defect because unlike humans, mice do not express PAR1 on platelets. This phenotype is actually due to loss of vascular integrity caused predominantly by defective endothelial signaling. Reintroduction of PAR1 under the control of an endothelial cell-specific TIE2 promoter/enhancer reduced embryonic lethality by 65% in *Par1*$^{-/-}$ mice (Griffin, Srinivasan et al. 2001). However, it is unknown what accounts for the 50% observed penetrance.

PAR1, in combination with PAR2, has also recently been implicated in neural tube closure. Failure of the neural tube to properly close during development can cause exencephaly (brain outside the skull) and spina bifida. Over 30% of mouse embryos with combined PAR1 and PAR2 deficiency exhibit neural tube closure defects (Camerer, Barker et al. 2010). Though the exact mechanism for the failure of neural tube closure is unknown, it appears to be mediated by $G\alpha_i$ signaling through Rac in the surface ectoderm. This phenotype suggests important functions for PAR1/PAR2 in regulating epithelial development.

The best characterized physiologic function for PAR1 is perhaps in coagulation. In humans and other higher vertebrates, PAR1 is expressed at high levels on platelets and the endothelium and is an important mediator of thrombosis. Thrombin activation of PAR1 causes platelet shape change, granule release, induction of procoagulant activity and aggregation, leading to thrombus formation (Ossovskaya and Bunnett 2004).

Additionally, PAR1-PAR4 heterodimerization enhances thrombin activation of PAR4, leading to a sustained thrombin response in platelets (Leger, Jacques et al. 2006).

PAR1 is a major drug target for anti-thrombotic therapies. Bivalirudin is a direct thrombin inhibitor approved for use in humans undergoing percutaneous coronary intervention (coronary angioplasty with or without stenting). Recently, the anti-thrombotic effects of bivalirudin were shown to be a combination of inhibition of PAR1 in addition to its direct thrombin effects, demonstrating the viability of targeting PAR1 in thrombosis (Kimmelstiel, Zhang et al. 2011). However, clinical trials of daily dosing of a small molecule PAR1 inhibitor, Vorapaxar, in combination with aspirin and clopidogrel, have raised concerns about bleeding side effects of PAR1 antagonists. A Vorapaxar trial for the prevention of cardiac death in almost 13,000 patients with acute coronary syndrome did not decrease cardiac events and was associated with a more than 5-fold increase in intracranial hemorrhage when combined with Plavix and aspirin (Tricoci, Huang et al. 2012). In a second trial of over 26,000 patients with a history of myocardial infarction, peripheral artery disease, or ischemic stroke, Vorapaxar reduced the risk of cardiac death, heart attack, and stroke by 13% (20% in heart attack patient subgroup) after 30 months of treatment when used in combination with aspirin and Plavix (Morrow, Braunwald et al. 2012). Unfortunately, there was also a 1.5-fold increase in moderate to serious bleeding events in patients taking Vorapaxar and the study was discontinued in patients with a history of stroke due to a 2.6-fold increase in bleeding in these patients. Presently, it is unclear whether the adverse effects of Vorapaxar are an off-target side effect specific to the drug or are due to long term PAR1 inhibition in large populations.

Further research is needed to determine if PAR1 signaling has subtle effects on hemostasis.

Despite present uncertainty of the appropriate use of PAR1 inhibitors in the clinic, PAR1 has been identified as a therapeutic target in many disease models. PAR1 signaling promotes wound healing in rats, given that treatment with the PAR1 agonists increases wound strength and vascularization (Carney, Mann et al. 1992). This effect is potentially due to the upregulation of angiogenic factors, including CYR61 (cysteine-rich, angiogenic inducer 61) and VEGFR (vascular endothelial growth factor receptor), and through release and induction of matrix metalloproteases (Zucker, Conner et al. 1995; Tsopanoglou and Maragoudakis 1999; Pendurthi, Ngyuen et al. 2002).

PAR1 may also be important for the pathogenesis of Parkinson's disease. PAR1 is expressed by the microglia, astrocytes and certain neuronal populations, including dopaminergic neurons. *PAR1*-deficient mice exhibit dopaminergic neuroprotection in the MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) chemical model of Parkinson's disease (Hamill, Caudle et al. 2007). Additionally, α-synuclein, a major component of the Lewy bodies seen in Parkinson's pathology, has been shown to upregulate microglial MMP expression. This upregulation of MMP expression leads to increased activation of PAR1, causing increased expression of inflammatory mediators and potentially contributing to the pathogenesis of Parkinson's disease (Lee, Woo et al. 2010).

PAR1 has also been demonstrated to play multiple roles in sepsis, largely through regulation of vascular integrity. Sepsis is a severe, dysregulated inflammatory sydrome in response to systemic infection that is associated with leukocytosis/leukopenia, elevated systemic cytokines, shock, and death. Interestingly, PAR1 has both protective and

deleterious roles in sepsis pathogenesis and these effects are modulated both temporally and by the activating protease (Kaneider, Leger et al. 2007). In early stages of sepsis, PAR1 activation by MMP1 or thrombin leads to Rho activation in endothelial cells, causing endothelial cell retraction and barrier disruption (Russo, Soh et al. 2009; Tressel, Kaneider et al. 2011). Later in sepsis, PAR1-PAR2 dimerization and transactivation in endothelial cells shifts signaling from vaso-disruptive to vaso-protective through activation of Rac (O'Brien, Prevost et al. 2000; Kaneider, Leger et al. 2007). APC activation of PAR1 in sepsis is also protective. APC activation of PAR1 leads to Rac activation, maintenance of vascular integrity and transcription of anti-inflammatory factors whereas thrombin-stimulation of PAR1 has opposite effects. This is potentially due to the different kinetics of PAR1 activation, targeting of different membrane sub-populations of PAR1 receptors, and transactivation of other GPCRs, such as sphingosine 1-phosphate receptor 1 (S1P1) (Finigan, Dudek et al. 2005; Russo, Soh et al. 2009). Recombinant APC was used clinically for the treatment of severe sepsis because of its protective effects through PAR1 but has been recently withdrawn due to lack of efficacy and elevated bleeding risk (Kerschen, Fernandez et al. 2007; Mosnier, Zampolli et al. 2009).

PAR1 in Cancer:

In 1998, Even-Ram and colleagues reported the first link between PAR1 and tumor pathobiololgy (Even-Ram, Uziely et al. 1998). PAR1 is not expressed in normal breast epithelium but is upregulated in malignant breast epithelium in both patient samples and cell lines. Moreover, PAR1 expression levels directly correlate with the

metastatic potential of these samples (Even-Ram, Uziely et al. 1998). Since this initial report, PAR1 upregulation has been described in many different cancer cell lines and patient tumor types, including lung, melanoma, ovarian, and prostate (Elste and Petersen 2010). Table 2 shows a selection of reports of PAR1 expression and prognosis findings from patient samples for different tumor types. In confirmation of what was observed in breast cancer, PAR1 is often a negative prognositic marker and associated with increased invasion and metastatic potential in other cancer types.

In addition to observations from patient samples, animal models and *in vitro* cancer systems have also shown PAR1 to be an oncogenic factor. PAR1 is a bona fide oncogene, capable of inducing foci formation, as well as anchorage and serum independent growth in NIH3T3 cells (Martin, Mahon et al. 2001). Though it was observed at the time that this transformation appeared to require PAR1 activation by an endogenous protease, the autocrine PAR1 activation loop in NIH3T3 cells required for oncogenic transformation was not understood until the discovery that MMP1 is a PAR1 agonist (Boire, Covic et al. 2005).

However, PAR1 alone appears to be insufficient to induce ongenic transformation *in vivo*. Transgenic mice expressing human PAR1 under a mouse mammary tumor virus (MMTV) promoter develop "precocious hyperplasia" in the mammary gland but do not progress to carcinoma *in situ*, suggesting other factors are necessary for PAR1-dependent oncogenesis (Yin, Katz et al. 2006). Additionally, PAR1 is not required for polyoma middle T antigen (PyMT)-induced carcinogenesis, as there is

Cancer Type	PAR1 Observation	Reference
Breast	Degree of PAR1 expression correlates with tumor invasiveness	(Even-Ram, Uziely et al. 1998)
Colorectal	Expressed in 91% of colonic adenocarcinomas	(Darmoul, Gratio et al. 2003)
Lung	PAR1 expressed in 62% NSCLC, trend towards decreased 3 year survival	(Ghio, Cappia et al. 2006)
	PAR1 mRNA 10 fold higher in patient adeoncarcinomas as compared to normal lung tissue	(Jin, Fujiwara et al. 2003)
Melanoma	Correlates with tumor thickness, ulceration, and recurrence	(Depasquale and Thompson 2008)
Prostate	Overexpressed in 45%	(Black, Mize et al. 2007)
	Expression correlates to tumor stage	(Kaushal, Kohli et al. 2006)
Ovarian	Expressed in 100% of samples	(Grisaru-Granovsky, Salah et al. 2005)

Table 2: PAR1 overexpression and correlations to clinical outcome in patient tumor samples.

no change in the tumor burden or growth in PyMT-MMTV/*PAR1*$^{-/-}$ mice (Versteeg, Schaffner et al. 2008).

PAR1 has been validated as an oncogene in numerous xenograft models. Overexpression of PAR1 in the low grade breast cancer cell line, MCF-7, increases tumor growth, invasion, and metastasis (Boire, Covic et al. 2005). Pharmacologic inhibition of PAR1, using small molecule antagonists or cell penetrating lipopeptides (pepducins), decreases xenograft growth, invasion, angiogenesis, and metastasis in breast, lung, and ovarian cancers (Boire, Covic et al. 2005; Agarwal, Covic et al. 2008; Yang, Boire et al. 2009; Cisowski, O'Callaghan et al. 2011). Likewise, genetic silencing of PAR1 on breast cancer and melanoma cells, decreases xenograft growth and metastasis (Arora, Cuevas et al. 2008; Villares, Zigler et al. 2008).

Given the diverse signaling pathways that PAR1 activates (as discussed previously), PAR1 is a key player in many elements of tumorigenesis. PAR1 activation of MAPK signaling leads to cell proliferation while Akt activation and NFKB signaling promote cell survival and resistance to apoptotic stimuli (Tantivejkul, Loberg et al. 2005; Salah, Maoz et al. 2007; Kempkes, Rattenholl et al. 2012). PAR1 activation of Rho and transactivation of EGFR family members promote cellular migration and invasion (Nguyen, Faivre et al. 2002; Nguyen, De Wever et al. 2005; Arora, Cuevas et al. 2008). PAR1 signaling on both cancer cells and endothelial cells in the tumor microenvironment can promote angiogenesis. PAR1 activation leads to elevated transcription and release of various cytokines and angiogenic factors, including VEGF, CYR61, interleukin 8 (IL-8),

and monocyte chemotactic protein 1 (MCP-1) (Nguyen, Kuliopulos et al. 2006; Agarwal, Tressel et al. 2010; Cisowski, O'Callaghan et al. 2011).

There are two proposed mechanisms for aberrant PAR1 signaling in tumorigenesis: elevated transcription and dysfunctional endocytosis. Transcription appears to be the major mechanism for PAR1 upregulation in tumors given that PAR1 mRNA is elevated and PAR1 mRNA levels appear to correlate with protein levels in tumors. Additionally, there is no evidence of *PAR1* gene amplification or alterations in *PAR1* mRNA stability in tumors (Bar-Shavit, Turm et al. 2011). PAR1 transcription has been shown to be repressed by wild type p53 and activator protein-2 (AP-2) in breast epithelium and melanocytes respectively (Tellez, McCarty et al. 2003; Salah, Haupt et al. 2008). In aggressive melanoma, AP-2 expression is lost, leading to specificity protein 1 (Sp1) binding of the *PAR1* promoter and increased *PAR1* transcription (Tellez, McCarty et al. 2003). PAR1 is upregulated by the transcription factor early growth factor-1 (Egr-1) *in vitro* and PAR1 expression directly correlates with Egr-1 expression in patient prostate cancer specimens (Salah, Maoz et al. 2007).

In addition to increased transcription, prolonged signaling has been proposed to mediate the oncogenic functions of PAR1 (Arora, Ricks et al. 2007). Invasive breast cancer cells exhibit prolonged MAPK signaling and increased invasion in response to PAR1 stimulation as compared to fibroblasts or mammary epithelium ectopically expressing PAR1. This prolonged signaling is accompanied by delayed endocytosis and PAR1 degradation, suggesting that persistent PAR1 signaling due to dysregulated trafficking may promote tumorigenesis (Booden, Eckert et al. 2004). However, it has not

yet been demonstrated that this mechanism promotes tumorigenesis *in vivo* and whether it is relevant in other epithelial cancers.

1.4 MMP1-PAR1 Signaling in Tumorigenesis

Given that PAR1 is frequently overexpressed by epithelial cancers and is associated with increased tumor invasion, proliferation, and angiogenesis, understanding the activation pathways involved in the PAR1-mediated tumorigenesis offers additional potential therapeutic targets. Though thrombin is the classical PAR1 agonist, several reports suggest that thrombin is not the pathological agonist in tumorigenesis. Thrombin exerts bimodal, concentration-dependent effects on PAR1 signaling whereby low concentrations of thrombin promote PAR1-dependent migration, invasion, and proliferation but inhibits these functions at high concentrations (Zain, Huang et al. 2000; Kamath, Meydani et al. 2001). More compellingly, there was no observed survival benefit for cancer patients in clinical trials of low molecular weight heparin, an anticoagulant that inhibits the generation of active thrombin in addition to other effects (Kakkar, Levine et al. 2004).

Several reports suggest that MMP1 may be the pathological PAR1 agonist in tumorigenesis. The mouse homologue of MMP1, Mmp1a, is induced in the stroma by PAR1-driven breast cancer xenografts and pharmacologic inhibition of MMP activity decreases growth, invasion, and angiogenesis of these tumors (Boire, Covic et al. 2005). This induction of MMP1 activity in the stroma is potentially mediated directly by cancer cell PAR1 via upregulation of cancer cell Cyr61, which induces stromal MMP production (Nguyen, Kuliopulos et al. 2006). Melanoma and colon cancer derived MMP1 has also been demonstrated to activate PAR1 on endothelial cells, leading to a pro-inflammatory, pro-thrombotic, cell adhesive endothelial cell activation state that is frequently observed in the tumor microenvironment (Goerge, Barg et al. 2006). Additionally, MMP1-PAR1

signaling induces angiogenesis in *in vivo* models of blood vessel formation and PAR1 activation by MMP1 induces transcription of pro-angiogenic genes that are distinct from genes induced by thrombin-PAR1 signaling (Blackburn and Brinckerhoff 2008). In addition to activating tumor endothelium, MMP1-PAR1 signaling has been proposed to activate stromal cells and recruit mesenchymal stem cells to human gliomas (Ho, Chan et al. 2009). MMP1-PAR1 signaling has been shown to mediate the transition from low-grade radial growth phase (RGP) melanoma to invasive vertical growth phase (VGP) melanoma through the induction of growth, invasion, and metastasis programs (Blackburn, Liu et al. 2009). Finally, studies of patient samples have demonstrated that co-expression of MMP1 and PAR1 is a poor prognostic marker. MMP1-PAR1 expression is significantly associated with increased stage and tumor recurrence in hepatocellular carcinoma (Liao, Tong et al. 2011). In primary gall bladder carcinoma, patients with MMP1-PAR1 expression had significantly increased tumor invasion and lymph node metastasis, suggesting that MMP1-PAR1 is an important mediator of tumorigenesis and metastasis in human cancers (Du, Wang et al. 2011).

1.5 Conclusion

MMP1 and PAR1 are two proteins significantly upregulated in human cancers. MMP1, through cleavage of extracellular matrix and bioactive molecules, enables cells to remodel and sense their environment. PAR1, which can be activated by MMP1 and serine proteases, is a G protein coupled receptor that transduces extracellular signals to promote proliferation, migration/invasion, and transcription of pro-tumorigenic and angiogenic genes among other functions. MMP1 is suspected to be the pathological PAR1-agonist in the human tumor microenvironment. However, the study of MMP1-PAR1 in mouse models has been limited because of the uncertainty about the functionality of the mouse homologue of *MMP1*, *Mmp1a*.

This thesis seeks to characterize the expression and function of Mmp1a in tumorigenesis models. Chapter 2 describes the expression of Mmp1a in lung cancer and melanoma models and identifies an autocrine Mmp1a-PAR1 signaling loop in cancer cells that promotes tumorigenesis, invasion, and metastasis. Chapter 3 characterizes a novel *Mmp1a*-deficient mouse and demonstrates that stromal Mmp1a promotes subcutaneous tumor growth and angiogenesis. Additionally, mammalian heterologous expression systems of Mmp1a reveal that the Mmp1a prodomain limits expression levels and potentially explains why Mmp1a expression levels are lower that those of human MMP1. Chapter 4 describes the methods utilized for Chapters 2 and 3. Chapter 5 discusses the significance of these results and future avenues for research.

Chapter 2

Mmp1a-PAR1 Signaling in Tumorigenesis and Metastasis

A modified version of this chapter was originally published in The Journal of Biological Chemistry:

Foley, CJ, Luo, C, O'Callaghan, K, Hinds, PW, Covic, L, and Kuliopulos, A. (2012). "Matrix metalloprotease-1a promotes tumorigenesis and metastasis." J Biol Chem **287**(29):24330-8.

Matrix metalloprotease-1 (MMP1), a collagenase and activator of the G protein-coupled protease activated receptor-1 (PAR1), is an emerging target implicated in oncogenesis and metastasis in diverse cancers. However, the functional mouse homologue of MMP1 in cancer models has not yet been clearly defined. We report here that Mmp1a is a functional MMP1 homologue that promotes invasion and metastatic progression of mouse lung cancer and melanoma. Lewis lung carcinoma (LLC1), and primary mouse melanoma cells harboring active BRAF (V600E), express high levels of endogenous Mmp1a, which is required for invasion through collagen. Silencing of either Mmp1a or PAR1 suppressed invasive stellate growth of lung cancer cells in 3-dimensional collagen matrices. Conversely, ectopic expression of Mmp1a conferred an invasive phenotype in epithelial cells that do not express endogenous Mmp1a. Consistent with Mmp1a acting as a PAR1 agonist in an autocrine loop, inhibition or silencing of PAR1 resulted in a loss of the Mmp1a-driven invasive phenotype. Knockdown of Mmp1a on tumor cells resulted in significantly decreased tumorigenesis, invasion, and metastasis in xenograft models. Together, these data demonstrate that cancer cell-derived Mmp1a acts as robust functional homologue of MMP1 by conferring pro-tumorigenic and metastatic behavior to cells.

2.1 Introduction

Matrix metalloproteases (MMPs) are a family of 25 zinc-dependent endopeptidases that allow cells to both sense and remodel their environment through cleavage of extracellular factors and matrix proteins, such as collagen (Fanjul-Fernández, Folgueras et al. 2010; Rodríguez, Morrison et al. 2010; Ugalde, Ordóñez et al. 2010).

There are three secreted collagenases with different specificities identified in humans, namely MMP1, MMP8 and MMP13 (Minond, Lauer-Fields et al. 2006). In particular, MMP1 has been implicated in a wide range of pathophysiologic processes including arthritis, atherosclerosis, thrombosis, tumorigenesis and metastasis (Murray, Duncan et al. 1996; Murray, Duncan et al. 1998; Sukhova, Schönbeck et al. 1999; Brinckerhoff, Rutter et al. 2000; Visse and Nagase 2003; Blackburn, Rhodes et al. 2007; Trivedi, Boire et al. 2009). MMP1 overexpression is associated with many cancer types, including lung, breast, and melanoma, and often correlates with a poor clinical prognosis (Murray, Duncan et al. 1996; Airola, Karonen et al. 1999; Nakopoulou, Giannopoulou et al. 1999; Nikkola, Vihinen et al. 2002; Poola, Dewitty et al. 2005; Giricz, Lauer et al. 2010; Shah, Spinale et al. 2010). An insertional polymorphism in the human MMP1 promoter that leads to elevated MMP1 transcription has also been associated with increased risk of development and metastasis of non-small cell lung cancer (NSCLC) and with increased invasiveness in cutaneous melanoma (Rutter, Mitchell et al. 1998; Ye, Dhillon et al. 2001; Sun, Gao et al. 2006).

While MMP1 cleaves many secreted factors and matrix proteins important for tumor progression and invasion, a newly identified mechanism of tumor promotion is through non-canonical activation of protease-activated receptor-1 (PAR1) (Boire, Covic et al. 2005; Goerge, Barg et al. 2006; Blackburn, Liu et al. 2009). PAR1 is a G-protein coupled receptor that is activated by cleavage of its extracellular N-terminal domain (Vu, Hung et al. 1991). Cleavage reveals a tethered ligand that activates the receptor in an unusual intramolecular binding mode (Seeley, Covic et al. 2003) which triggers transmembrane signaling to intracellular G proteins (Swift, Sheridan et al. 2000). PAR1

signaling activates oncogenic transformation (Martin, Mahon et al. 2001) including mitogenesis, survival, gene transcription, and migration/invasion pathways (Ossovskaya and Bunnett 2004; Boire, Covic et al. 2005; Nguyen, Kuliopulos et al. 2006; Arora, Ricks et al. 2007; Yang, Boire et al. 2009).

Like MMP1, PAR1 is frequently overexpressed by variety of cancer types, including melanoma, lung, breast, and ovarian cancers (Even-Ram, Uziely et al. 1998; Grisaru-Granovsky, Salah et al. 2005; Ghio, Cappia et al. 2006; Dorsam and Gutkind 2007; Agarwal, Covic et al. 2008; Depasquale and Thompson 2008). Tumorigenesis, angiogenesis, and experimental metastasis of several cancers can be effectively inhibited by pharmacologic blockade or knockdown of PAR1 gene expression (Agarwal, Covic et al. 2008; Arora, Cuevas et al. 2008; Villares, Zigler et al. 2008; Cisowski, O'Callaghan et al. 2011). Recent work indicates that dual expression of MMP1 and PAR1 on cancer cells is significantly associated with increased tumor recurrence and stage in hepatocellular carcinoma patients, and invasion and lymph node metastasis in primary gall bladder carcinoma (Liao, Tong et al. 2011; Kempkes, Rattenholl et al. 2012).

Given the emerging importance of MMP1 and PAR1 in human cancer pathogenesis, it is useful to develop relevant mouse models to understand the complex pathobiology and potential therapeutic relevance of the MMP1-PAR1 axis in cancer. However, the functional mouse homologue of MMP1 in murine cancers has not yet been clearly defined. Mapping of the *Mmp* gene locus revealed a rodent-specific duplication of *MMP1*, resulting in *Mmp1a* (*Mcol-A*) and *Mmp1b* (*Mcol-B*) genes (Balbín, Fueyo et al. 2001). Mmp1a and Mmp1b are 74% identical to human MMP1 and 82% identical to each other. When expressed in bacteria, Mmp1a, but not Mmp1b exhibited

collagenolytic activity in vitro (Balbín, Fueyo et al. 2001). Mmp1a and Mmp1b contain a RGD-motif in their catalytic domains which is characteristic of MMP1. The location of the *Mmp1a* gene between *Mmp10* and *Mmp3* in the mouse MMP cluster on chromosome 9 is identical to human *MMP1* whereas the *Mmp1b* gene is inverted and located 73 kb further away, between the *Mmp3* and *Mmp12* genes.

The tissue expression of Mmp1a appears to be limited in healthy adult mouse tissue. However, elevated Mmp1a expression levels have been documented in multiple different disease states, including sepsis, wound healing, lung injury, and arthritis (Nuttall, Sampieri et al. 2004; Hartenstein, Dittrich et al. 2006; Tomita, Okuyama et al. 2007; Pfaffen, Hemmerle et al. 2010; Tressel, Kaneider et al. 2011). Moreover, *Mmp1a* mRNA was found to be significantly upregulated in the stroma of breast cancer xenografts driven by ectopic expression of the PAR1 oncogene (Boire, Covic et al. 2005).

To determine whether Mmp1a-PAR1 signaling is relevant in mouse cancer biology, we studied a mouse-derived lung cancer cell line, LLC1, and two primary cell lines isolated from spontaneous BRAF V600E/p19$^{ARF-/-}$ mouse melanomas. LLC1 is a clonal cell line derived from the Lewis lung carcinoma tumor widely used in C57BL/6 tumor allografts (Bertram and Janik 1980). BRAF is a serine-threonine kinase that is activated by RAS and is frequently mutated in human melanomas, with the constitutively active V600E form occurring in over 50% of patient melanomas (Davies, Bignell et al. 2002).

Here, we demonstrate that LLC1 cells express high levels of endogenous Mmp1a and PAR1 and autocrine Mmp1a-PAR1 signaling is required for LLC1 invasion. Silencing of *Mmp1a* expression results in significantly decreased invasion and decreased

tumor growth in mice. Furthermore, suppression of Mmp1a expression inhibits experimental metastasis of LLC1 cells to the lungs. This signaling pathway is also conserved in mouse melanoma. BRAF V600E/p19$^{ARF-/-}$ melanoma cells express Mmp1a and PAR1, and Mmp1a-PAR1 activity is required for melanoma cell invasion. Together, these results demonstrate that the newly described Mmp1a matrix metalloprotease has oncogenic functions in mice by enhancing both tumorigenesis and metastasis.

2.2 Results

Mmp1a and PAR1 Signaling Promotes Cancer Cell Invasion:

MMP1 and PAR1 are frequently overexpressed in human lung cancers and melanoma (Airola, Karonen et al. 1999; Depasquale and Thompson 2008; Blackburn, Liu et al. 2009; Shah, Spinale et al. 2010; Cisowski, O'Callaghan et al. 2011). Therefore, we examined whether PAR1 and Mmp1a were co-expressed using Western blot and FACS analysis in the mouse lung cancer cell line, LLC1, and the primary melanoma cell lines, 4228 and 4246, isolated from spontaneous, BRAF V600E/p19$^{ARF-/-}$ mouse melanomas. A strong protein band at 46 kDa, corresponding to active Mmp1a, was secreted from LLC1 lung carcinoma, and 4228 and 4246 melanoma cells (Figure 5A). A weaker band at 56 kDa, corresponding in size to proMMP1, was detected in 4228 and 4246 melanoma cells (Figure 5A). All three cell lines had strong surface expression of PAR1 as determined by flow cytometry (Figure 5B and Figure 6).

We next examined whether Mmp1a and PAR1 impacted invasion of LLC1 cells through extracellular matrix (Boire, Covic et al. 2005; Blackburn, Liu et al. 2009). LLC1

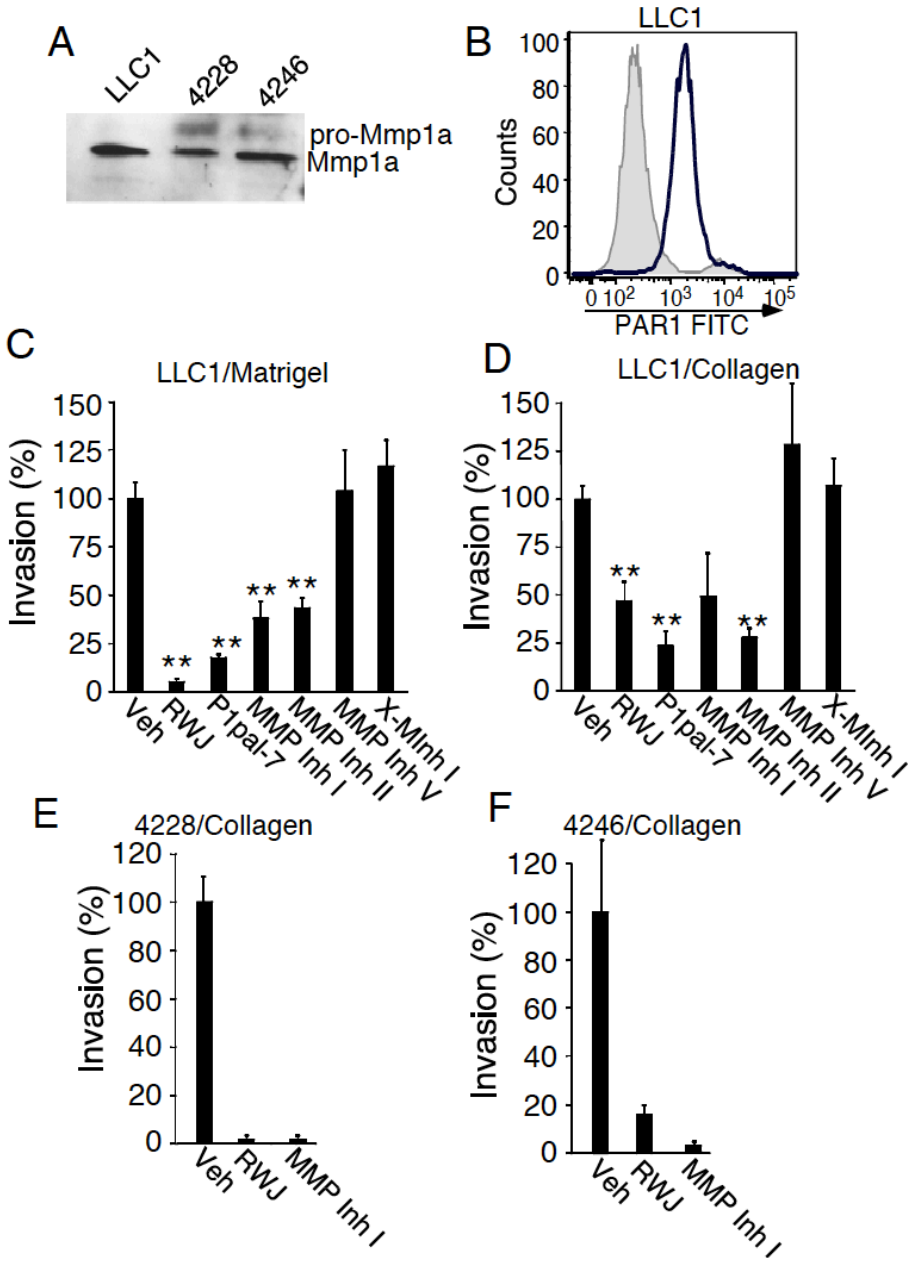

Figure 5: Endogenous Mmp1a and PAR1 regulate invasion of mouse lung cancer and melanoma cells. *A*, Western blot analysis of secreted Mmp1a (proMmp1a ~56kDa, Mmp1a ~46 kDa) in media from LLC1 lung cancer, and 4228 or 4246 melanoma cells. *B*, Flow cytometry analysis of PAR1 surface expression on LLC1 cells using S19 FITC-PAR1 Ab versus secondary alone control (grey). *C-D*, LLC1 invasion through Matrigel (*C*) or type I collagen (*D*) in the absence or presence of PAR1 inhibitors (P1pal-7 (5 μM) or RWJ-58259 (3 μM), MMP Inh I (3 μM) and MMP Inh II (5 μM), MMP Inh V (1 μM) and X-MInhI (3 μM). All data represent mean ± SE of three experiments, * $p<0.05$, ** $p<0.005$

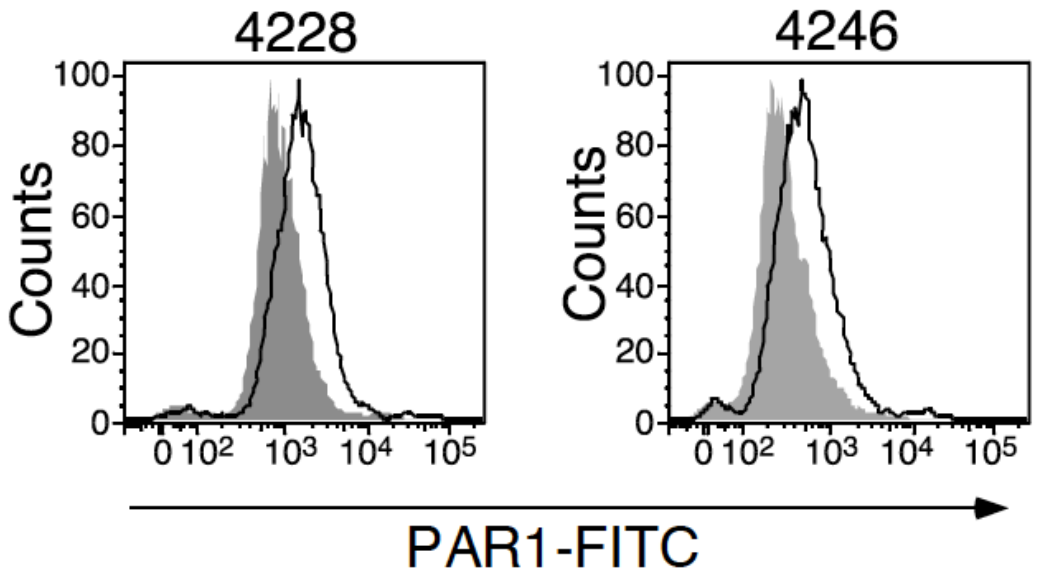

Figure 6: Primary mouse melanoma cells 4228 and 4246 express surface PAR1. Flow cytometric analysis of PAR1 surface expression on 4228 (left) and 4246 (right) melanoma cells, using S19 FITC-PAR1 Ab versus secondary alone control (grey).

cells readily invaded through Matrigel (Figure 5C) and collagen (Figure 5D). Pharmacological inhibition of PAR1 using a small molecule antagonist, RWJ-58259 (Damiano, Cheung et al. 1999), or a cell-penetrating pepducin antagonist of PAR1, P1pal-7 (Kuliopulos and Covic 2003), decreased LLC1 invasion by up to 95% and 75% for Matrigel and collagen, respectively. Furthermore, MMP Inh I and MMP Inh II, which both preferentially target MMP1, reduced invasion of LLC1 cells through Matrigel and collagen invasion by 60-70% (Figure 5C-D). In contrast, MMP Inh V, which blocks a variety of other MMPs, including MMP-2, -3, -8, -9, -12, -13 but not MMP-1, did not impact LLC1 invasion. Additionally, an inactive control MMP Inh I (X-MInh I) lacking the C-terminal hydroxamate, had no effect on LLC1 invasion. These results indicate that both Mmp1a and PAR1 are required for invasion of LLC1 cells through collagen.

We determined whether Mmp1a and PAR1 are also required for melanoma cell invasion. Collagen invasion assays were performed on the primary 4228 and 4246 melanoma cells. Inhibition of PAR1 with RWJ-58259 or Mmp1a with MMP Inh I in 4228 and 4246 melanoma cells reduced collagen invasion by 85-97% (Figure 5E-F). Together, these data suggest that lung and melanoma cells expressing Mmp1a and PAR1 are highly invasive and that Mmp1a-PAR1 signaling may lead to a highly malignant cellular phenotype.

Enzymatic Activity of Mmp1a Confers Invasive Potential through PAR1:

To directly show that Mmp1a has collagenase activity in mammalian expression systems, full-length human *MMP1, Mmp1a, and Mmp1b* were expressed in HEK293T cells. All MMP constructs contained a C-terminal Myc-tag and protein expression levels

were found to be comparable (Figure 7A, lower panel). The MMP-expressing cells were embedded into three-dimensional collagen gels, and collagenolysis assessed by the release of trapped liquid from the gels (Wyatt, Geoghegan et al. 2005). After 24 h, gels containing mouse Mmp1a-expressing cells had significantly degraded comparable amounts of Type I collagen as human MMP1-expressing cells (Figure 7A). Conversely, Mmp1b did not confer any additional collagenase activity over basal levels.

To confirm that Mmp1a catalytic activity was directly responsible for the collagenase activity observed, the critical active site glutamate of MMP1 (E219) and Mmp1a (E216) were mutated to alanine (Saffarian, Collier et al. 2004). The E219A-MMP1 and E216A-Mmp1a mutants had no significant collagenase activity, consistent with the requirement of the conserved active site glutamate for both the human and mouse homologues.

We then examined whether gain-of-exogenous expression of Mmp1a bestows an invasive phenotype on the PAR1-expressing cell line C57MG. C57MG is a mouse mammary epithelial-derived cell line that does not express Mmp1a (Figure 7B, lower panel) (Lynch, Vargo-Gogola et al. 2007). C57MG cells were stably transduced with vector, Mmp1a, or E216A-Mmp1a and tested for invasive activity. Expression of Mmp1a in C57MG cells caused a significant 4-fold increase in collagen invasion above vector control, an effect that was lost with the active-site defective Mmp1a mutant, E216A (Figure 7B). Blockade of PAR1 with RWJ-58259 caused a 75% loss of invasion in the Mmp1a-transduced cells, and the PAR1-expressing C57MG cells did not invade in the absence of Mmp1a (Vec control) (Figure 7C). Together, these data indicate that

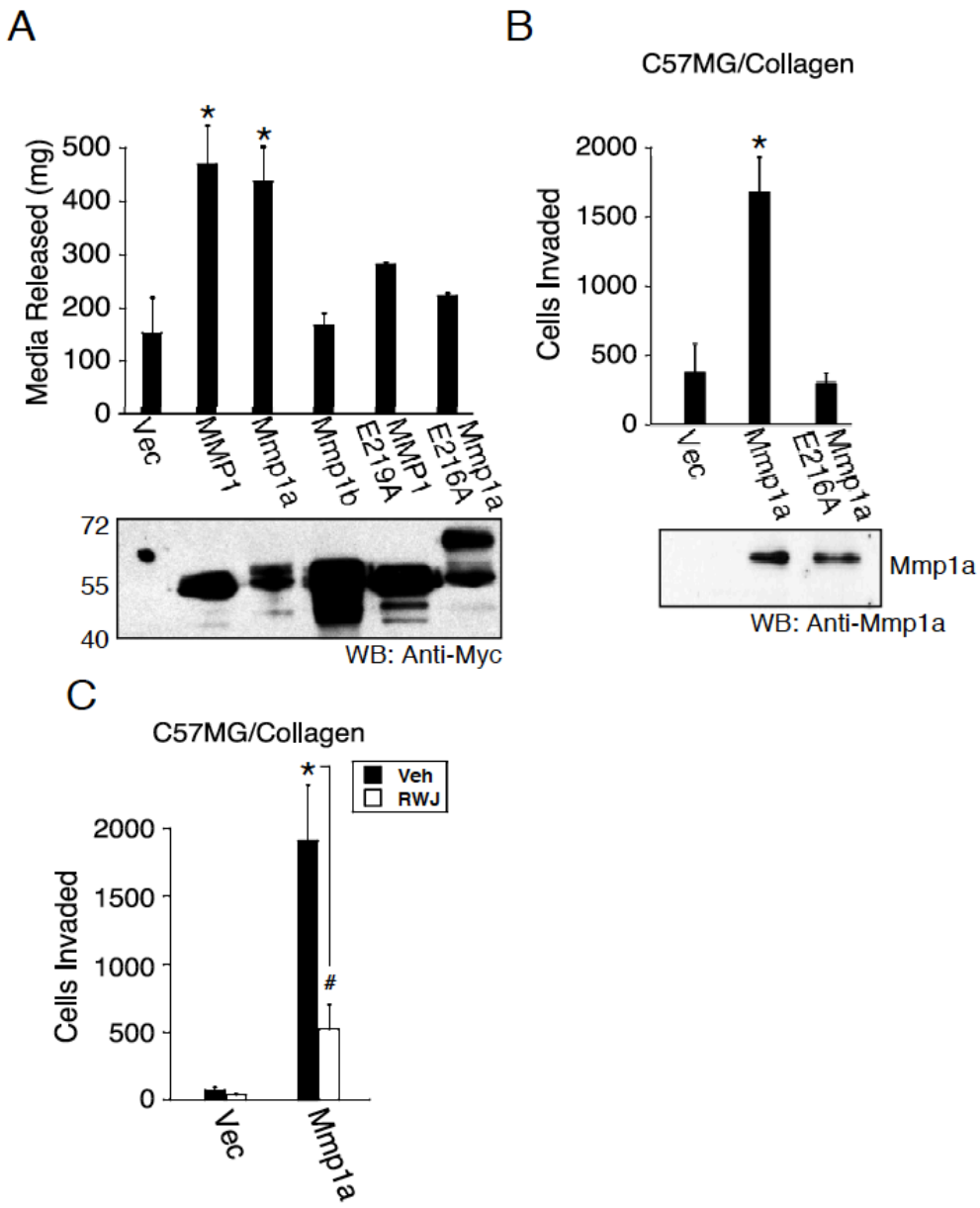

Figure 7: Mmp1a confers collagenase activity and invasive behavior through PAR1. *A*, Collagenase activity of MMP-Myc transfected HEK293T cells plated in type I collagen gels as measured by the conversion of collagen gel to liquid. Corresponding MMP protein expression in the conditioned media was determined by Western blot (lower panel). *B*, Invasion of Mmp1a-null C57MG cells ectopically expressing Mmp1a, inactive E216A Mmp1a or vector control, through type I collagen towards a gradient of 10% FBS. Corresponding MMP protein expression in the conditioned media was determined by Western blot (lower panel). *C*, Mmp1a-driven invasion of C57MG cells requires PAR1 activity. C57MG cells ectopically expressing Mmp1a or vector control, were allowed to invade through type I collagen towards a gradient of 10% FBS, in the presence or absence of the PAR1 inhibitor RWJ-58259 (5 μM). * $p<0.05$, #$p<0.1$

Mmp1a is an invasogenic factor that requires the activity of PAR1 to confer an invasive phenotype to mouse epithelial-derived cells.

Knockdown of Mmp1a and PAR1 suppresses the invasive phenotype of LLC1 lung cancer cells:

A screen of a panel of Mmp1a-targeted shRNAs identified two constructs (shMmp1a #1 and #2) which caused a >95% reduction in Mmp1a mRNA in LLC1 cells using quantitative RT-PCR analysis (Figure 8A). These shRNAs were specific and did not affect expression of the most related mRNA transcripts from Mmp1b and Mmp13 (data not shown). As shown in Figure 8B, a strong band at 46 kDa corresponding to Mmp1a was detected in shLuc-LLC1, which was reduced by 63% and 76% upon transduction with shMmp1a #1 and #2, respectively. Consistent with the previously observed effects of pharmacologic inhibition of Mmp1a, silencing of Mmp1a expression with shMmp1a #2 significantly reduced LLC1 invasion through matrigel and type I collagen by 70-85% (Figure 8C-D). Highly similar results were observed following silencing of Mmp1a in the 4228 and 4246 melanoma cell lines, with 80-90% reduction in Mmp1a mRNA expression (Figure 9) and suppression of collagen invasion by 70-75% (Figure 9B-C). Likewise, 75% knockdown of PAR1 surface expression with shPAR1 led to 70% reduction in LLC1 invasion (Figure 8E), consistent with the effects of Mmp1a-invasion being mediated through PAR1.

To more closely examine the effects of Mmp1a on the invasive behavior of the LLC1 lung cancer cells, we employed a three-dimensional Matrigel invasion assay. LLC1 cells transduced with negative control shLuc produced numerous grade 1 and 2

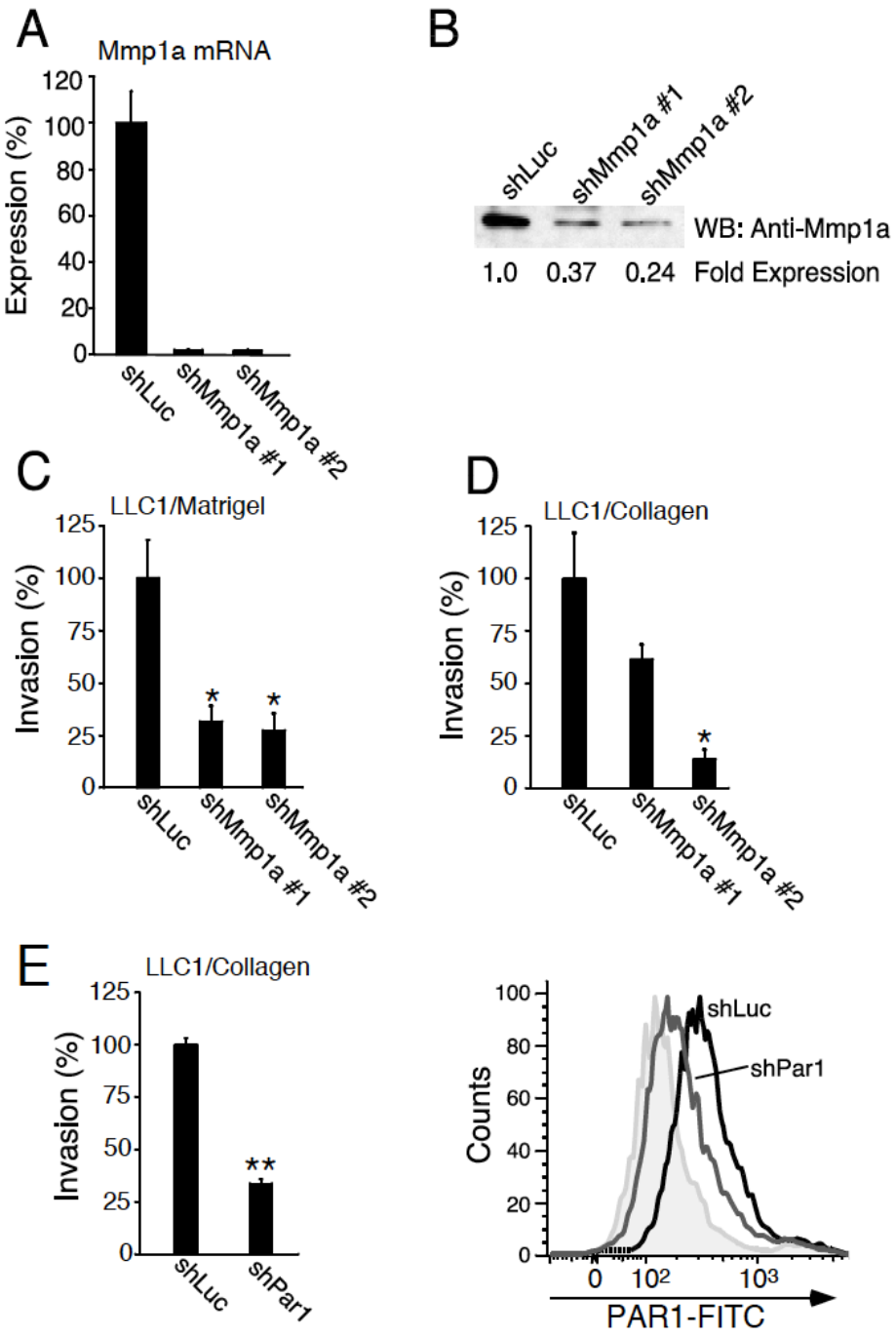

Figure 8: Knockdown of Mmp1a-PAR1 decreases invasion of LLC1 lung cancer cells. *A-B*, *Mmp1a* mRNA (*A*) and Mmp1a protein expression (*B*) following stable, lentiviral knockdown in LLC1 cells with Mmp1a-targeted shRNA (shMmp1a #1 or 2) vs shLuc (luciferase) control. *C-D*, Matrigel (*C*) and collagen (*D*) invasion of Mmp1a knockdown LLC1 cells. *E*, LLC1 cell invasion through collagen following stable transduction with a PAR1-targeted shRNA (shPar1) vs shLuc control (left panel), with FACS analysis of PAR1 surface expression (Right panel) using S19 FITC-PAR1 Ab vs control (grey).*$p<0.05$, **$p<0.005$

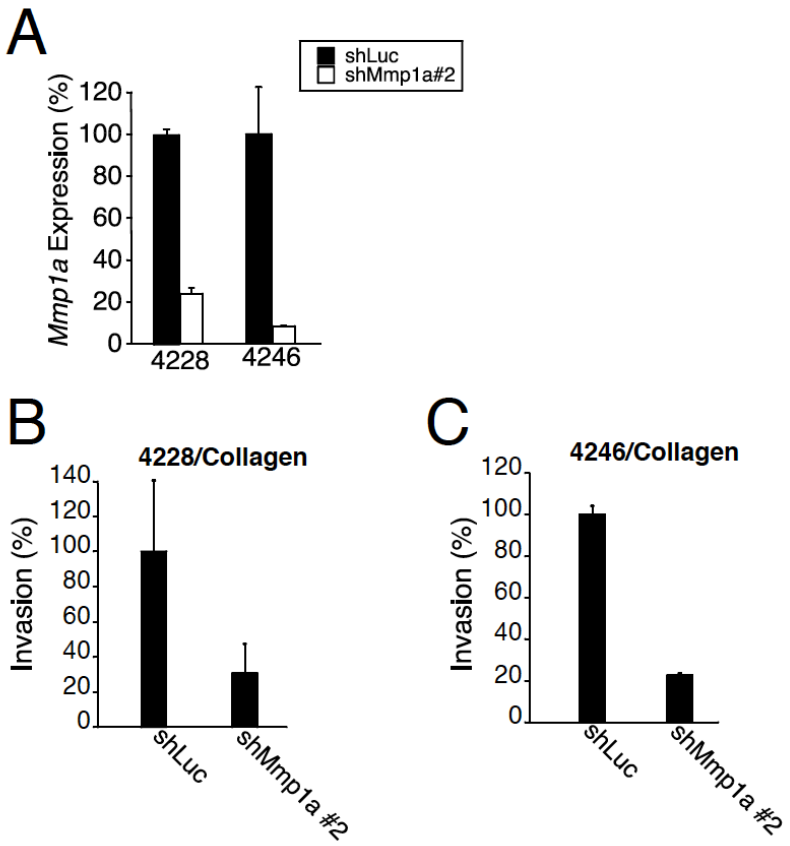

Figure 9: Knockdown of Mmp1a in 4228 and 4246 melanoma cells suppresses invasion through type I collagen. *A*, *Mmp1a* mRNA expression following lentiviral transduction of 4228 and 4246 primary mouse melanoma cells with shLuc or shMmp1a#2 as measured by real time PCR. *B*, Type I collagen invasion of shLuc or shMmp1a#2 transduced 4228 or 4246 cells.

stellate colonies with multiple, long projections invading deeply into the 3-dimensional Matrigel culture (Figure 10A-B). Silencing of Mmp1a with shMmp1a #2 caused a striking loss of invasive stellate colony growth with complete absence of grade 2 colonies (Figure 10A-B). PAR1 knockdown resulted in a similar phenotype with the appearance of predominantly non-invasive colonies with the remaining invasive colonies exhibiting truncated stellate projections (Figure 10A-B). The supression of the invasive phenotype following silencing of Mmp1a or PAR1 in the LLC1 lung cancer cells in 3-dimensional matrix is highly consistent with the results from the transwell collagen invasion assays, where shMmp1a#2 reduced invasion by 90% and shPAR1 resulted in a 68% reduction in invasion. However, silencing of PAR1 did not completely mimic the effects of Mmp1a silencing, suggesting both PAR1-dependent and PAR1-independent functions for Mmp1a, such as direct lysis of collagen.

Mmp1a Promotes Tumorigenesis and Metastasis of LLC1:

To determine whether Mmp1a plays a role in tumorigenesis and invasion of lung cancer in vivo, tumor xenograft experiments were performed with LLC1 cells. LLC1 cells were injected into the abdominal fat pads of C57BL/6 mice and tumor growth monitored over 26 days. shMmp1a #2 knockdown tumors grew significantly slower than control shLuc LLC1 tumors at all time points (Figure 11A). At the day 26 endpoint, shMmp1a #2 tumors weighed significantly less than shLuc tumors (Figure 11B). Analysis of Mmp1a mRNA levels by real time PCR in whole tumor homogenates surprisingly showed a 33-fold upregulation of Mmp1a mRNA as compared to parental shLuc LLC1 cells grown in culture. However, Mmp1a mRNA was reduced on average by 75% in

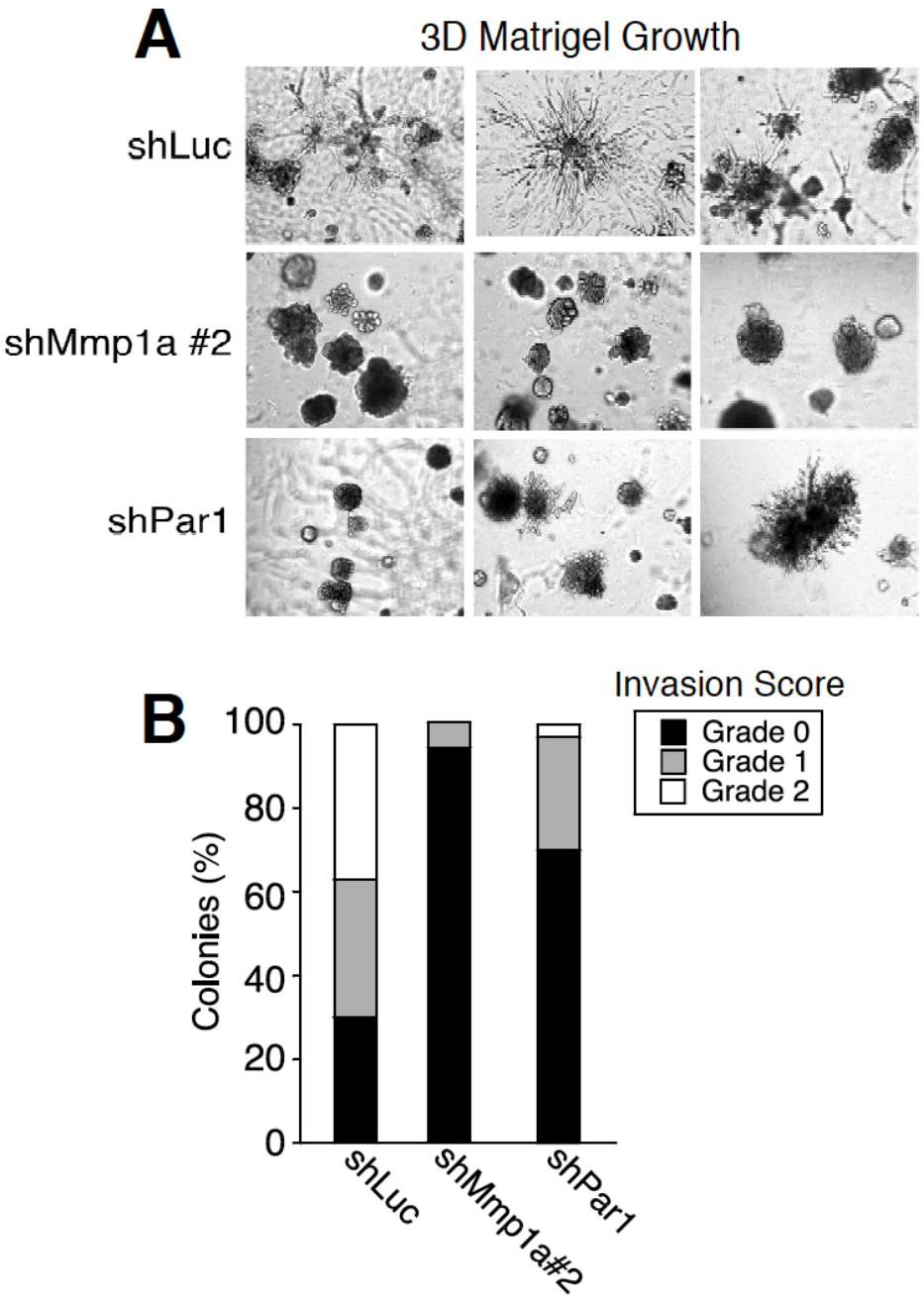

Figure 10: Mmp1a and PAR1 are required for invasive stellate colony formation of lung cancer cells in three-dimensional matrices. *A,* Invasive growth of LLC1 cells transduced with control (shLuc), Mmp1a knockdown (shMmp1a#2), or PAR1 knockdown (shPar1) after 7 days in three-dimensional, Matrigel cultures. Digital images were acquired at 6X magnification, n=3. *B,* The grade of invasiveness was measured for each colony in at least ten 6X fields, and scored (grade 0,1,2, noninvasive to invasive) as described in the methods.

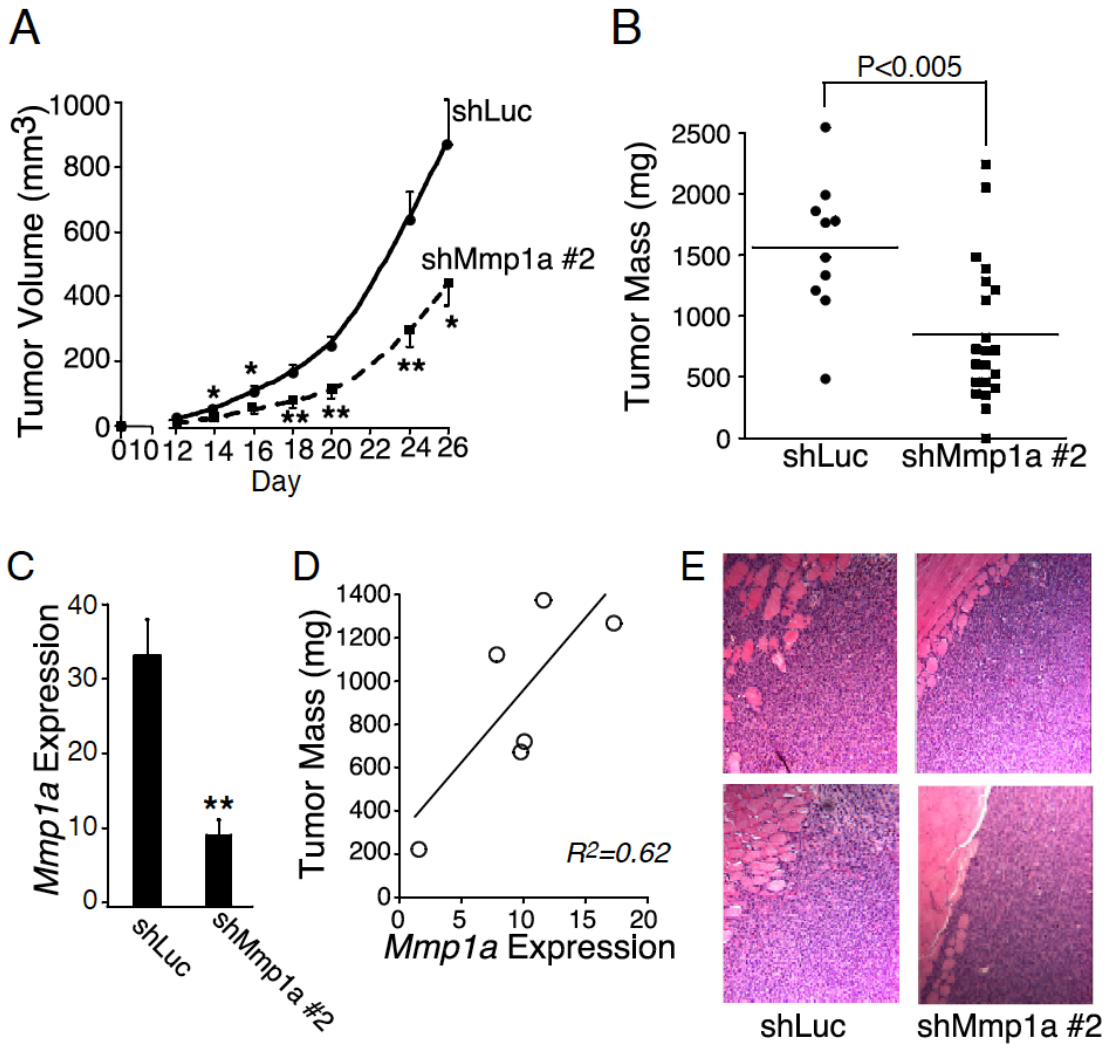

Figure 11: Silencing of Mmp1a suppresses tumor growth and invasion of LLC1 lung cancer cells in mice. *A*, Tumor growth following subcutaneous implantation of 200,000 shLuc (n=10) or shMmp1a#2 (n=21) transduced LLC1 cells in the abdominal fat pads of C57BL/6 female mice. *B*, Mass of excised LLC1 tumors at the day 26 endpoint. *C, Mmp1a* mRNA expression in whole tumor homogenates (n=5 per cohort) as determined by real time PCR and expressed relative to *Mmp1a* mRNA levels in cultured shLuc LLC1 cells (1-fold). *D*, Correlation between tumor size and *Mmp1a* mRNA expression of excised shMmp1a#2 tumors. *E*, Invasion of shLuc or shMmp1a LLC1 transduced tumors into the rectus abdominus muscle of mice as assessed by H&E stained sections from subcutaneous tumors. Values shown are mean ± SE, * $p<0.05$, ** $p<0.01$.

shMmp1a #2 knockdown tumors as compared to shLuc control tumors (Figure 11C), indicating that the shRNA maintained knockdown of Mmp1a throughout the experiment. Residual elevated Mmp1a mRNA in the tumors may be due to stromal sources. There was a correlation between Mmp1a mRNA levels and tumor size (Figure 11D), consistent with the notion that residual and/or stromal Mmp1a expression levels may be growth limiting in the shMmp1a #2 tumors. Histologic examination revealed that shMmp1a #2 tumors exhibited decreased tumor invasion into the underlying abdominal musculature (Figure 11E).

To quantify the effect of Mmp1a knockdown on tumor cell experimental metastasis in vivo, shLuc or shMmp1a #2-silenced LLC1 cells were inoculated into the venous circulation by tail vein injection. At day 28, lungs were harvested and metastatic nodules were quantified. Consistent with the hypothesis that Mmp1a promotes a malignant phenotype in epithelial cells, mice inoculated with Mmp1a-knockdown LLC1 cells had a 40% reduction in metastatic nodules (Figure 12A-B). This provides evidence that Mmp1a plays a role in the late events of invasion and metastasis in mouse lung cancer models.

2.3 Discussion

Emerging evidence suggests that the matrix metalloprotease MMP1 plays a pivotal role in the pathogenesis of multiple human diseases, however, a function for the putative mouse homologue, Mmp1a, has not yet been clearly identified. Here, we report that Mmp1a is highly expressed in mouse lung cancer and is critical for in vivo tumor growth, invasion, and metastasis. Primary melanomas isolated from BRAF V600E/

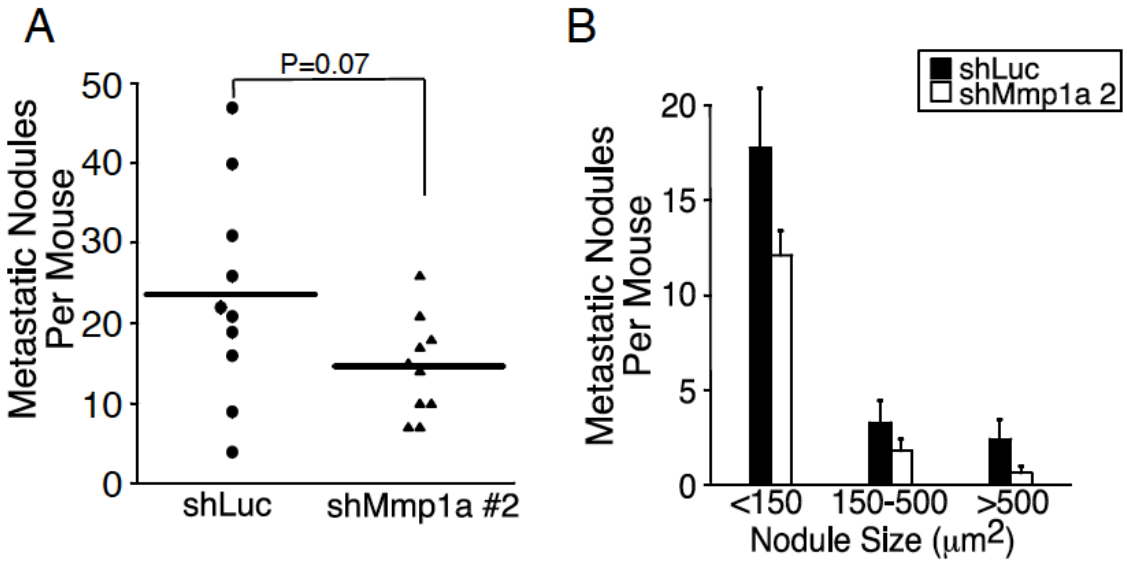

Figure 12: Silencing of Mmp1a reduces experimental metastasis of lung cancer cells in mice. *A-B*, Number (*A*) and size (*B*) of metastatic lung nodules per mouse as determined by sum of three coronal sections per animal. LLC1 cells transduced with shMmp1a#2 or shLuc (1 x 10^6) were injected into the tail vein of C57BL/6 female mice and lungs were harvested 28 days later, and analyzed for metastases by histology.

$p19^{ARF-/-}$ mice also endogenously express Mmp1a that is essential for invasion, thus providing further support for a pathophysiologic role for Mmp1a in mouse tumor biology.

Gain-of-function migratory and invasive activity of Mmp1a in mouse epithelial cells required the G protein-coupled PAR1 receptor, which had been shown to be an oncogene in human cancers (Whitehead, Zohn et al. 2001). Previous work had also shown the importance of stromal MMP1 activity as being required for PAR1-driven cancer cell growth, tumorigenesis and invasion of human breast cancer xenografts that lack endogenous MMP1 (Boire, Covic et al. 2005). However, the present study describes for the first time, an autocrine Mmp1a-PAR1 system that promotes lung cancer pathogenesis. A similar autocrine system in human melanomas has recently been reported to promote growth and invasion (Blackburn, Liu et al. 2009). Blackburn et al. provided evidence that activation of human PAR1 by MMP1 in less advanced melanomas leads to increased transcription of MMP1 (Blackburn, Liu et al. 2009). While this would promote increased cancer cell signaling, it has not yet been determined whether the action of cancer cell-derived MMP1 on the stromal component also leads to increased stromal MMP1(a) production in vivo. Stromal MMP1 has been shown to be induced by a PAR1-Cyr61-MMP1 pathway, whereby secreted Cyr61 from human breast cancer cells induces MMP1 expression in human mammary and other stromal fibroblasts in co-culture experiments (Nguyen, Kuliopulos et al. 2006). Media from human breast cancer cells can also induce MMP1 expression in human mammary fibroblasts possibly through a CXCR4-regulated mechanism (Eck, Côté et al. 2009).

Gain-of-MMP1 expression by breast cancer cells has been proposed to be a key component of the secreted protein toolbox necessary for metastasis to the lung and bone (Kang, Siegel et al. 2003; Minn, Gupta et al. 2005; Minn, Kang et al. 2005). Recently, it was shown that expression of MMP1 by stromal cells is correlated with breast cancer subtype and risk of distant metastasis in patients, suggesting that stromal MMP1 expression may also modulate tumor phenotype (Vizoso, González et al. 2007; Boström, Söderström et al. 2011). This suggests the likelihood that there are multiple sources of Mmp1a in the tumor microenvironment including the stroma (Boire, Covic et al. 2005). Additional studies are required to understand the interplay between autocrine and paracrine MMP1 activity on various tumor types and correlate these with clinical outcomes. Given the conservation of Mmp1a-PAR1 signaling in the murine tumor cells used here, we propose that mouse models may be an appropriate tool for understanding the relative contribution of stromal versus tumor-derived MMP1 in tumorigenesis.

In addition to the Mmp1a collagenase, there are two additional soluble collagenases identified in mice, namely Mmp8 and Mmp13. *Mmp8* mRNA was not present in LLC1 cells, however, *Mmp13* mRNA was detected. While we did not directly address a potential role for Mmp13 in these cells, Mmp1a activity was required for the invasive, tumorigenic and metastatic phenotype of the LLC1 lung cancer cells. Thus, collagen invasion was decreased with inhibitors that targeted MMP1 (MMP Inh I and MMP Inh II) while a potent MMP13 inhibitor (MMP Inh V) had no effect. Additionally, there were no changes in *Mmp13* mRNA levels with the *Mmp1a*-targeting shRNA (data not shown) that significantly suppressed invasion, stellate colony growth, tumorigenesis and metastasis. Similarly, *Mmp13* mRNA was also detected in the 4228 and 4246

primary melanoma cell lines, but was unaffected by the *Mmp1a*-targeting shRNA that also suppressed invasion. Together, these data point to Mmp1a as mediating the invasive phenotype in the LLC1 and melanoma cells, with little or no compensatory role for Mmp13.

Moreover, there is increasing evidence that MMP1 and MMP13 are pro-tumorigenic, whereas MMP8 collagenase may have tumor-suppressive activities (Balbín, Fueyo et al. 2003; Palavalli, Prickett et al. 2009). *Mmp8*-deficient mice exhibit increased skin tumorigenesis, and *MMP8*-inactivating somatic mutations have been identified in patient melanoma samples (Freije, Balbín et al. 2003; Palavalli, Prickett et al. 2009), in marked contrast to the frequently observed overexpression of MMP1 in melanomas (Blackburn, Rhodes et al. 2007; Giricz, Lauer et al. 2010; Ryu, Moriarty et al. 2011). This suggests that despite the apparent commonality of collagenase activity, the three secreted collagenases MMP1, MMP8 and MMP13, have distinct functions in cancer biology, and that MMP1/Mmp1a may play a specific role in the invasive and metastatic progression of melanoma and lung cancer.

Chapter 3

Characterization of *Mmp1a*-Deficiency in Tumorigenesis

A modified version of this chapter was originally published in Oncogene: Foley, CJ, Fanjul-Fernandez, M, Bohm, A, Nyugen, N, Agarwal, A, Austin, K, Koukos, G, Covic, L, Lopez-Otin, C, Kuliopulos, A. "Matrix metalloprotease 1a-deficiency suppresses tumor growth and angiogenesis." Oncogene. 2013 May 27. Epub ahead of print.

Acknowledgements: The *Mmp1a*$^{-/-}$ mouse was generated by Miriam Fanjul-Fernandez in the laboratory of Carlos Lopez-Otin. The data shown in figure 13, panels A-C were generated by Miriam Fanjul-Fernandez. Mmp1a homology modeling (Figure 18, A-B) was performed by Andrew Bohm.

Matrix metalloprotease 1 is an important mediator of tumorigenesis and metastasis through its ability to degrade critical matrix proteins, such as type I collagen, and activate oncogenic factors, such as PAR1. MMP1 is secreted as a proenzyme and can therefore be regulated in the extracellular milieu by enzyme activation and stability. Though MMP1 has been identified as a pathogenic factor in multiple human diseases, the study of MMP1 in mouse models has been hindered because little is known regarding the putative mouse homologue of MMP1, Mmp1a. We describe here the generation of *Mmp1a*-deficient mice and characterize Mmp1a expression in heterologous systems. *Mmp1a*$^{-/-}$ mice are healthy and fertile. Stromal *Mmp1a*-deficiency resulted in significantly decreased growth and angiogenesis of Lewis lung carcinoma (LLC1) allograft tumors. Co-implantation of *Mmp1a*$^{+/+}$ fibroblasts restored tumor growth in *Mmp1a*$^{-/-}$ animals, demonstrating the importance of stromal Mmp1a in promoting tumorigenesis. To better understand the differences between human and mouse MMP1, we next examined Mmp1a overexpression *in vitro*. Surprisingly, Mmp1a was poorly expressed as a soluble protein in heterologous expression systems. Exchange of the Mmp1a prodomain for that of human MMP1 or introduction of a point mutation predicted to stabilize interactions between the Mmp1a prodomain and catalytic domains markedly increased Mmp1a secretion. These results suggest that Mmp1a is upregulated in cancer in the mouse and that the prodomain of Mmp1a regulates heterologous expression and stability.

3.1 Introduction

MMP1 is a zinc-dependent endopeptidase that is frequently overexpressed in human cancers, often correlating with poor prognosis. MMP1 is defined as a collagenase due to its ability to cleave fibrillar collagens, but also cleaves a variety of other substrates, including the oncogenic receptor, PAR1 (Boire, Covic et al. 2005). Because of its ability to digest important substrates, MMP1 expression is regulated both transcriptionally and by enzyme activation. MMP1 is expressed as a zymogen. The prodomain of MMP1 contains a conserved cysteine-switch motif in which the thiol of a cysteine coordinates with the catalytic zinc to cover the active site. MMP1 activation occurs when the thiol-zinc interaction is disrupted.

MMP1 expression in patient tumor samples has been associated with metastasis and decreased progression-free survival in melanoma, colorectal and esophageal cancers (Murray, Duncan et al. 1996; Murray, Duncan et al. 1998; Nikkola, Vihinen et al. 2002). MMP1 is produced by many sources in the tumor microenvironment, including cancer cells, fibroblasts, inflammatory cells, and the endothelium, and because it is a secreted enzyme, can have both paracrine and autocrine effects in the microenvironment (Westermarck and Kähäri 1999; Tressel, Kaneider et al. 2011). In patient lung cancers with MMP1 overexpression, approximately 35% express MMP1 in the cancer cells and 71% had stromal MMP1 expression (Bolon, Gouyer et al. 1995). MMP1 expression specifically in stromal cells, has been associated with increased risk of metastasis in breast cancer patients, highlighting the importance of tumor versus stromal MMP1 production (Vizoso, González et al. 2007). Interestingly, fibroblast-derived and cancer cell-derived MMP1 have been shown to be differentially glycosylated, suggesting that

there may be functional differences in MMP1 depending on the source (Saarinen, Welgus et al. 1999).

Though MMP1 has multiple substrates, MMP1 activation of PAR1 has emerged recently as an important pathway in mediating of tumor growth, angiogenesis, invasion, and metastasis (Boire, Covic et al. 2005; Agarwal, Covic et al. 2008; Blackburn, Liu et al. 2009). PAR1 is a G protein coupled receptor that is activated by proteolytic cleavage (Vu, Hung et al. 1991). PAR1 activation has many effects on cellular phenotype, including proliferation, migration/invasion, and gene transcription (Adams, Ramachandran et al. 2011). PAR1 is overexpressed in many of the same tumors that overexpress MMP1, including melanoma, breast, lung, and ovarian cancers, and PAR1 expression is often associated with poor prognosis (Even-Ram, Uziely et al. 1998; Grisaru-Granovsky, Salah et al. 2005; Ghio, Cappia et al. 2006; Depasquale and Thompson 2008). Furthermore, co-expression of both MMP1 and PAR1 in tumor samples is associated with increased stage and recurrence rate in hepatocellular carcinoma and increased lymph node metastasis in gall bladder cancer, suggesting that MMP1-PAR1 signaling promotes tumor pathogenesis in patients (Du, Wang et al. 2011; Liao, Tong et al. 2011).

In order to effectively study MMP1-PAR1 signaling in disease models, it is important to develop a highly relevant mouse model. The mouse homologue of MMP1, Mmp1a, is 58% identical to human MMP1 but is expressed less ubiquitously in healthy mouse tissue than human (Balbín, Fueyo et al. 2001; Nuttall, Sampieri et al. 2004). Very little is known about Mmp1a activity and expression in disease models. Elevated Mmp1a expression has been described in inflammatory conditions, including wound healing,

chemical lung injury, collagen-induced arthritis, and sepsis (Hartenstein, Dittrich et al. 2006; Tomita, Okuyama et al. 2007; Pfaffen, Hemmerle et al. 2010; Tressel, Kaneider et al. 2011). Mmp1a expression is induced in the mouse stroma of human breast and renal cell carcinoma xenografts, suggesting that like MMP1, Mmp1a is upregulated in the tumor microenvironment (Boire, Covic et al. 2005; Pfaffen, Hemmerle et al. 2010). Mmp1a may also be upregulated in mouse cancer cells; it is highly expressed by mouse Lewis lung carcinoma cells (LLC1) and primary mouse melanoma cells harboring activated BRAF. Additionally, Mmp1a promotes invasion, tumorigenesis, and metastasis of LLC1 cells, consistent with its role as an MMP1 homologue in mouse tumor systems (Chapter 2).

In order to understand the role of stromal-derived MMP1 in tumorigenesis, we generated *Mmp1a*-deficient mice. *Mmp1a*$^{-/-}$ mice are grossly normal and born in Mendelian ratios. However, *Mmp1a*-deficiency caused significantly decreased LLC1 tumor growth and angiogenesis. This stromal defect was rescued by co-implantation of *Mmp1a*$^{+/+}$ fibroblasts with LLC1 cells in the Mmp1a$^{-/-}$ mice. Interestingly, biochemical analysis of Mmp1a produced in mammalian expression systems identified a secretion defect in Mmp1a expression that is dependent on Mmp1a catalytic activity and the Mmp1a prodomain. Together, these data suggest that stromal Mmp1a upregulation promotes *in vivo* tumorigenesis and that Mmp1a tissue expression may be limited because of instability of the Mmp1a prodomain.

3.2 Results

Mmp1a-Null Mice Are Viable But Exhibit Impaired Tumorigenesis:

Mmp1a$^{-/-}$ mice were generated by our collaborator Dr. Carlos Lopez-Otin (*Universidad de Oviedo, Oveido Spain*) by targeted replacement of *Mmp1a* exon 5 with a PGK-neomycin cassette (Figure 13A). Exon 5 encodes the zinc-coordination motif of the active site and is therefore essential for metalloprotease activity. Homozygotes were identified by Southern blot detection of the 3.5 kb fragment of the mutant allele (Figure 13B). *Mmp1a*$^{-/-}$ mice also had no expression of *Mmp1a* mRNA in the placenta as determined by Northern blot (Figure 13C). *Mmp1a*-null animals were born within range of the expected ratios from heterozygote parents (Figure 13D) and *Mmp1a*$^{-/-}$ males and females were healthy and fertile. This indicates that *Mmp1a* expression is dispensable in development, as has been observed with genetic ablation of the other two secreted mouse collagenases, *Mmp8* and *Mmp13* (Balbín, Fueyo et al. 2003; Stickens, Behonick et al. 2004).

Given that Mmp1a is often upregulated in the stroma of tumor xenografts, we next examined tumor growth in *Mmp1a*-deficient animals using LLC1 cells (Boire, Covic et al. 2005; Pfaffen, Hemmerle et al. 2010). LLC1 are a C57BL/6-derived lung cancer cell line and are an established model for tumor allografts (Bertram and Janik 1980). LLC1 cells ($2x10^5$) were implanted into the abdominal fat pads of *Mmp1a*$^{-/-}$ and wild type control mice. The tumors in *Mmp1a*$^{-/-}$ mice grew significantly more slowly over the course of the entire experiment (Figure 14A-B), implicating stromal Mmp1a as an important factor in promoting tumorigenesis. Immunohistochemical staining of the

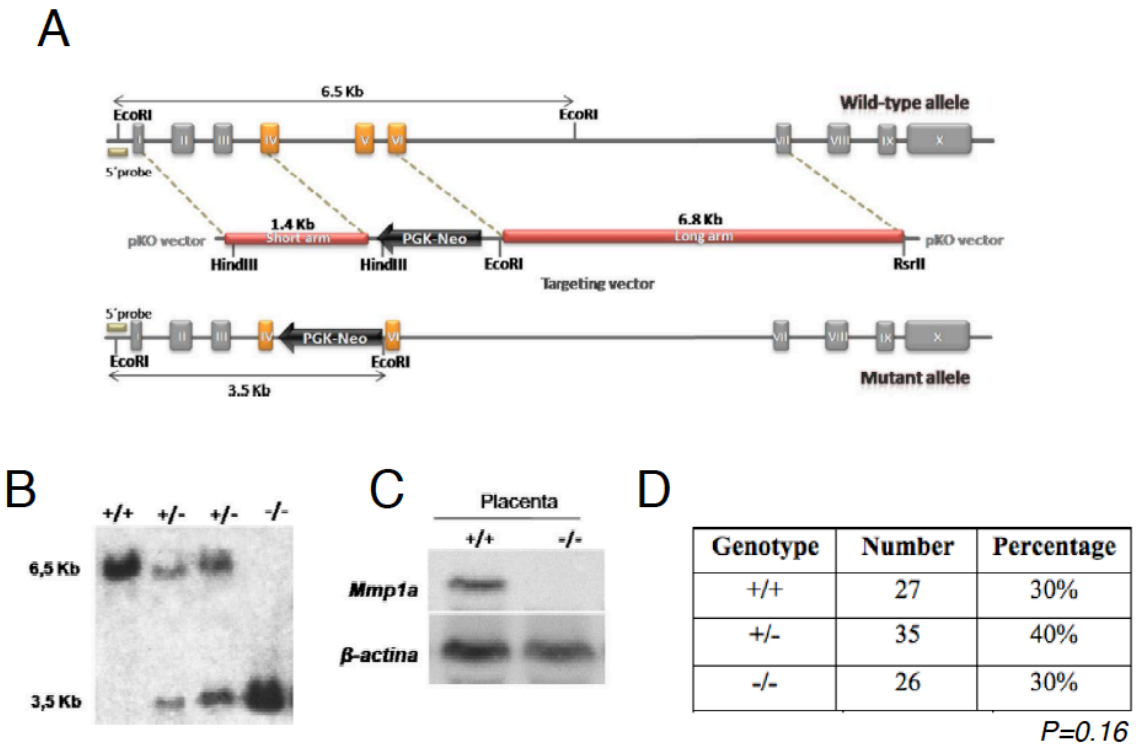

Figure 13: Generation of Mmp1a-deficient mice. *A,* Schematic depicting targeting vector for generation of *Mmp1a* exon V deleted mice *B,* Southern blot analysis of mice carrying the wild type (6.5 Kb) or *Mmp1a* mutant allele (3.5 Kb) after EcoRI digest *C,* Northern blot for *Mmp1a* mRNA in wild type and Mmp1a-deficient placenta *D,* Ratio of wild type, heterozygote, and knockout mice born from heterozygous parents (n=88).

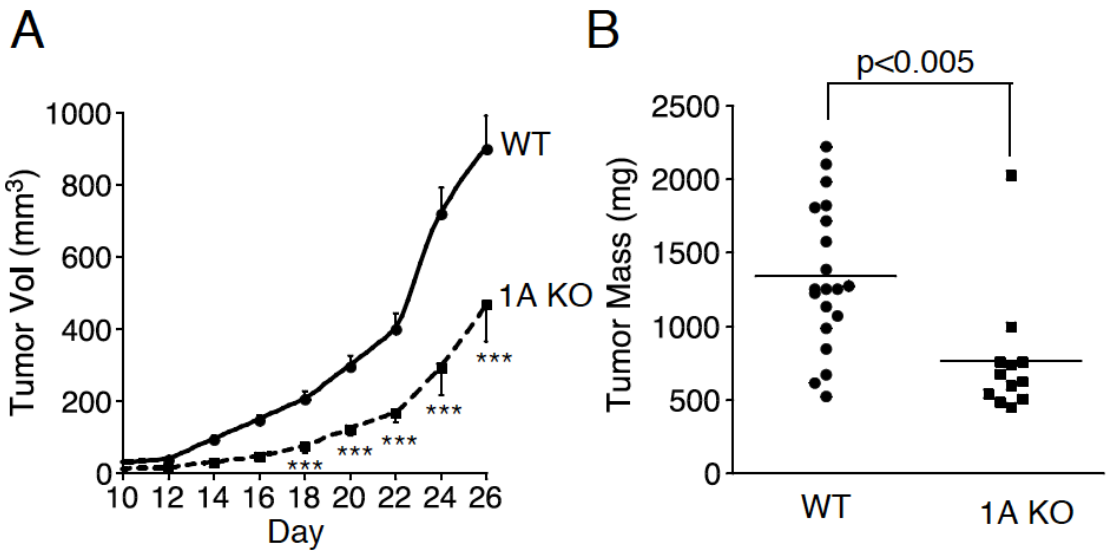

Figure 14: *Mmp1a*-deficiency attenuates subcutaneous tumor growth
A, Growth of LLC1 cells ($2x10^5$) implanted subcutaneously into the abdominal fat pad of *Mmp1a*$^{+/+}$ or *Mmp1a*$^{-/-}$ C57BL/6 female mice (*Mmp1a*$^{+/+}$ n=20, *Mmp1a*$^{-/-}$ n=12) *B,* Excised tumor mass at the experiment endpoint, day 26. ***p<0.001

tumors for Von Willebrand Factor (vWF), a marker of endothelial cells, revealed an approximately 30% decrease in angiogenesis in the *Mmp1a* knockout tumors, suggesting that defective tumorigenesis may be due in part to impaired tumor angiogenesis (Figure 15).

Restoration of Fibroblast Mmp1a Restores Tumor Formation:

Tumor stroma is a complex tissue composed of multiple cell types that could potentially produce Mmp1a, including fibroblasts, immune cells, and endothelial cells. To better understand whether fibroblasts could rescue tumorigenesis in *Mmp1a*-null stroma, we isolated mouse embryonic fibroblasts (MEFs) at embryonic day 12.5 from heterozygous crosses. Mmp1a is a potent inducer of directional migration and invasion and mitogenesis, partially through its activation of PAR1. Conditioned media from *Mmp1a* knockout MEFs resulted in approximately 55% less chemoinvasion of LLC1 towards MEF conditioned media as compared to wild type conditioned media (Figure 16A). Likewise, an almost identical reduction in the migration of A549 human lung cancer cells towards Mmp1a knockout conditioned media was observed, suggesting that MEF-derived Mmp1a is a potent chemoattractant (Figure 16B). Migration of MCF7 breast cancer cells ectopically expressing PAR1 was four fold higher towards wild type MEF conditioned media as compared to knockout conditioned media (Figure 16C). Inhibition of PAR1 using a small molecule antagonist, RWJ58259, decreased migration by roughly 50% towards wild type MEF conditioned media (Figure 16C). The decrease in migration with the PAR1 antagonist did not completely reduce migration to the level of Mmp1a knockout conditioned media, suggesting PAR1-independent effects for

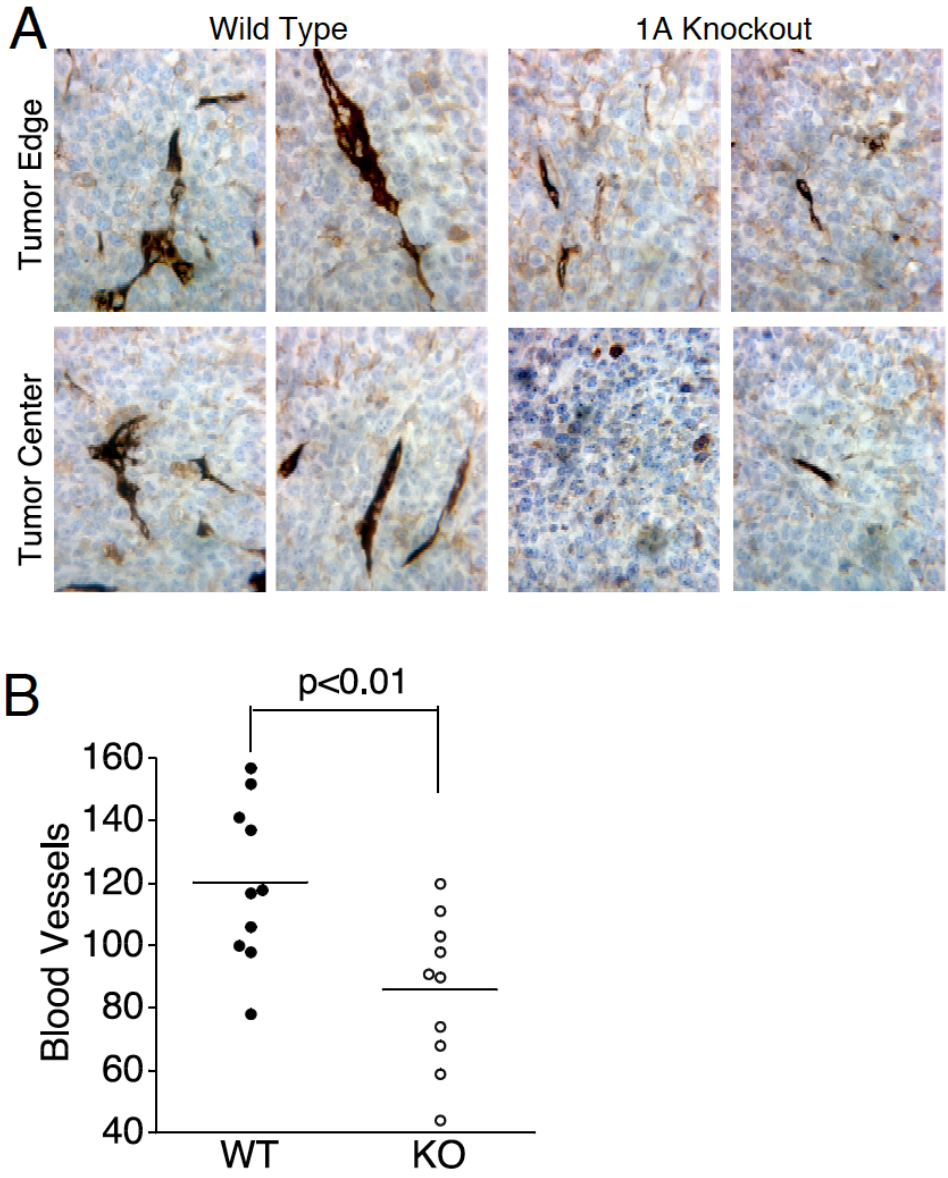

Figure 15: Tumor angiogenesis is reduced in *Mmp1a*-deficient animals *A,* Von Willebrand Factor (vWF) immunohistochemistry on LLC1 subcutaneous tumors from Figure 14 (40X magnification). *B,* Number of vWF-positive blood vessels as determined by the sum of 50 high-powered fields (40X) per tumor, n=10 per cohort.

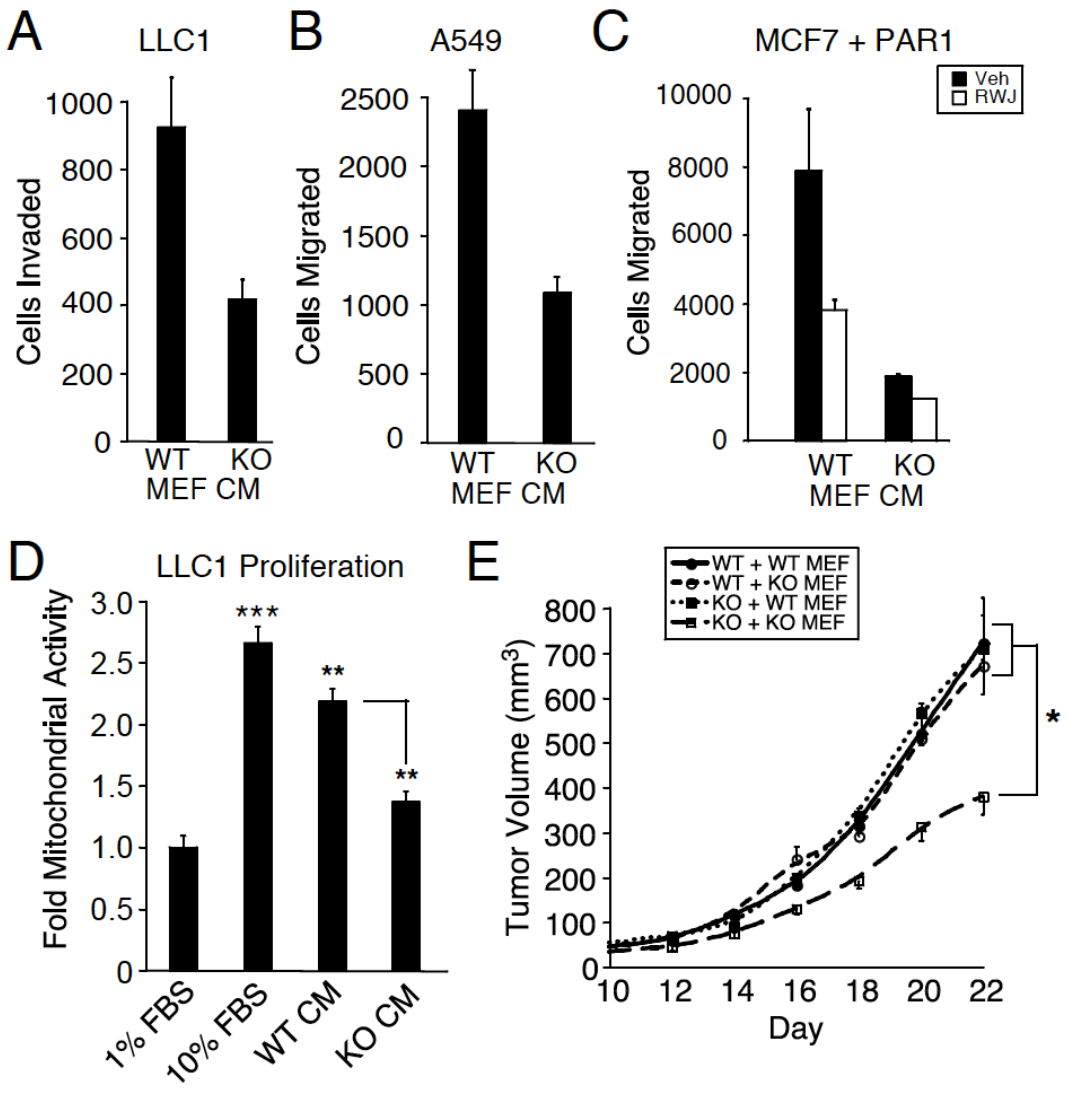

Figure 16: Mmp1a$^{+/+}$ fibroblasts restore LLC1 tumor growth in Mmp1a$^{-/-}$ animals. *A,* LLC1 chemoinvasion through type I collagen towards *Mmp1a*$^{+/+}$ (WT) or *Mmp1a*$^{-/-}$ (KO) MEF conditioned media (CM). *B,* Migration of the human lung cancer cell line A549 toward WT or KO MEF conditioned media. *C,* Migration of MCF7 breast cancer cells ectopically expression PAR1 towards MEF conditioned media in the absence (black) or presence (white) of a small molecule PAR1 antagonist, RWJ58259 (3 uM). *D,* 96 h MTT proliferation of LLC1 cells in response to 10% FBS or MEF conditioned media. *E,* Tumor growth in *Mmp1a*$^{+/+}$ (WT) or *Mmp1a*$^{-/-}$ (KO) mice co-implanted with 2x10^5 LLC1 and 1x10^5 *Mmp1a*$^{+/+}$ (WT MEF) or *Mmp1a*$^{-/-}$ (KO MEF) fibroblasts (n=12-16 per cohort). * p<0.05, **p<0.005, ***p<0.001

Mmp1a. We also observed a small decrease in migration following treatment of knockout MEF conditioned media with RWJ58259. This effect could be due to low levels of other PAR1 agonists in the conditioned media or could be due to inhibition of random chemokinesis by PAR1 antagonism.

Given the differences we observed in tumor growth *in vivo*, we also examined the ability of MEF conditioned media to induce LLC1 proliferation *in vitro* (Figure 16D). Wild type MEF conditioned media induced a significant, 2.2 fold induction in LLC1 proliferation. Knockout MEF conditioned media only induced a 1.4 fold increase in proliferation, which was a significant reduction as compared to wild type MEF conditioned media. These data demonstrate that MEFs, like cancer-associated fibroblasts, can induce cancer cell migration, invasion, and proliferation and that these tumor promoting functions are reduced in *Mmp1a*$^{-/-}$ MEFs.

To verify that the reduced tumorigenesis seen in *Mmp1a*-null mice is due to stromal defects, we next performed co-implantation experiments with LLC1 cells and MEFs. Wild type and *Mmp1a*$^{-/-}$ mice were injected in the abdominal fat pads with $2x10^5$ LLC1 cells mixed with $1x10^5$ wild type or *Mmp1a*$^{-/-}$ MEFs. LLC1/MEF tumors grew more rapidly than LLC1 tumors and MEFs implanted alone did not form tumors (Figure 16D and Figure 14A). LLC1/*Mmp1a*$^{-/-}$ MEF implants in *Mmp1a*$^{-/-}$ mice formed significantly smaller tumors. However, this phenotype was rescued by co-implantation of *Mmp1a*$^{+/+}$ MEFs with LLC1 cells in *Mmp1a*$^{-/-}$ mice, demonstrating the importance of stromal Mmp1a in promoting tumorigenesis. This observed phenotype is consistent with Mmp1a functioning as an MMP1 homologue in mouse tumor systems.

The Prodomain of Mmp1a Impairs Protein Expression:

Given the function of Mmp1a in tumorigenesis, we next sought to understand why Mmp1a is not more ubiquitously expressed in healthy adult mouse tissue as is the case for human MMP1. Recombinant Mmp1a refolded from *E. coli* inclusion bodies has been studied for collagenase activity and the ability to degrade fluorogenic substrates with no obvious defects (Balbín, Fueyo et al. 2001). However, MMPs are post-translationally modified, so we chose to utilize mammalian expression systems to obtain more correctly processed and folded protein. Three cell lines for heterologous expression, HEK293T, CHO-K1, and COS7, were transiently transfected with vector, human *MMP1*, mouse *Mmp1a*, mouse *Mmp1b*, mouse *Mmp8*, and mouse *Mmp13,* all with a C-terminal His-Myc tag. Surprisingly, when we attempted to harvest secreted Mmp1a from the conditioned media of the transiently transfected cells, Mmp1a protein levels were substantially lower than the expression levels of human MMP1 and mouse Mmp1b, Mmp8, and Mmp13 across all three cell lines (Figure 17A).

We hypothesized that perhaps a defective Mmp1a signal peptide was responsible for low expression levels. Mmp1b, which was well secreted in our expression systems, has a signal peptide identical to that of Mmp1a except at residue 2. However, mutation of the Mmp1a signal peptide to that of Mmp1b (P2S Mmp1a) had no effect on expression of soluble Mmp1a (data not shown), suggesting that decreased expression was not due to a signal peptide defect.

We next examined the prodomain of Mmp1a, given that alteration of the Mmp1a prodomain would not affect enzymatic activity of mature Mmp1a. The prodomain of

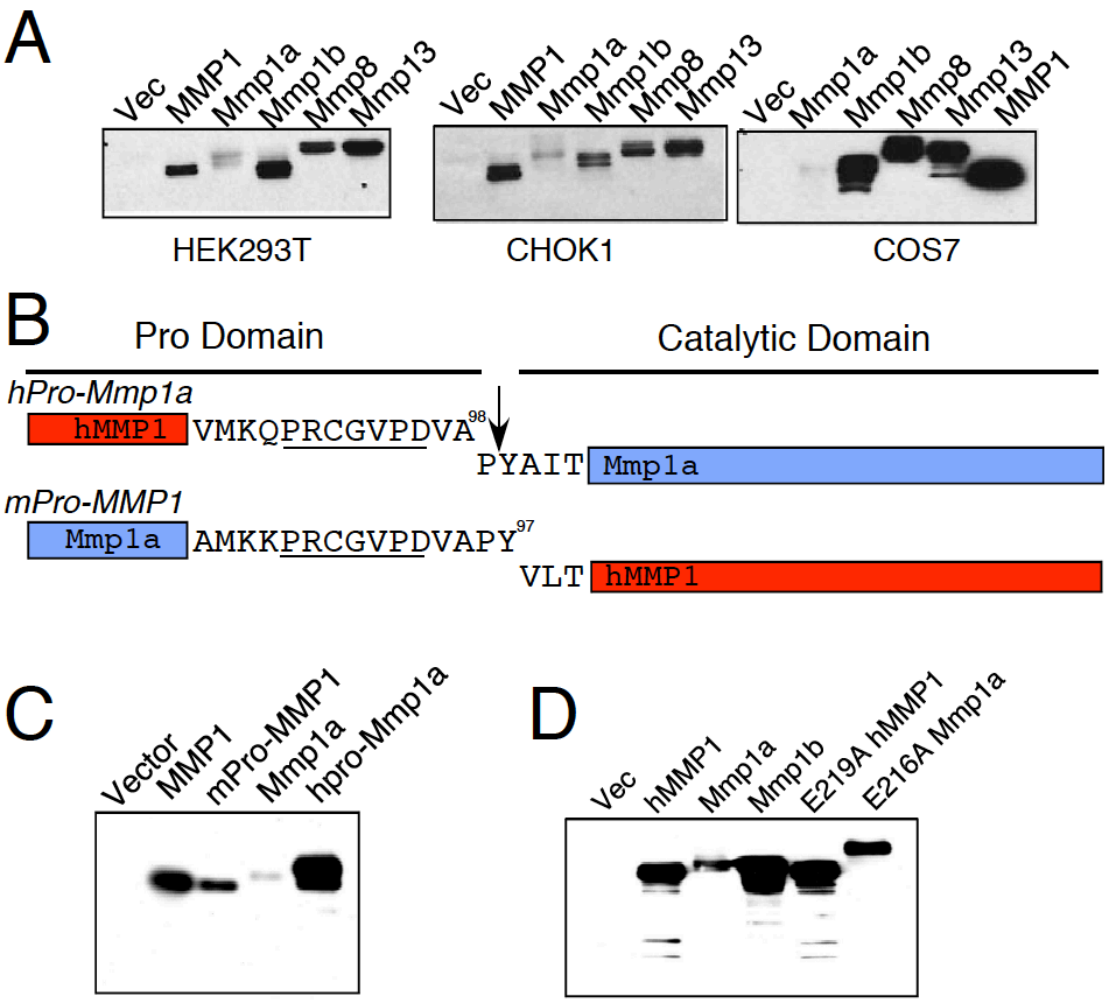

Figure 17: Mmp1a prodomain reduces Mmp1a production *A,* Western blot analysis of conditioned media (40 µl per lane) from cell lines that were transiently transfected with Myc-tagged MMPs. *B,* Schematic of the prodomain chimeras, hpro-Mmp1a and mPro-MMP1. Underlined residues are conserved cysteine switch motif. Arrow denotes cleavage site of active Mmp1a. *C,* Western blot analysis of conditioned media (40 µl) from HEK293T cells transiently transfected with Myc-tagged MMP chimera constructs. D, Western blot analysis of conditioned media (40 µl) from HEK293T cells transiently transfected with catalytically inactive Myc-tagged MMP constructs (E219A MMP1, E216A Mmp1a)

Mmp1a is 53% identical to that of human MMP1. We generated a chimera (hpro-Mmp1a) in which the prodomain of Mmp1a (residues 1-95) was exchanged with the prodomain of human MMP1 (residues 1-98) (Figure 17B). We also generated the complementary chimera for human MMP1, with residues 1-96 of Mmp1a replacing human MMP1 residues 1-99. Consistent with a defect in the prodomain of Mmp1a, replacement of the Mmp1a prodomain with that of human MMP1 resulted in massive production of soluble hpro-Mmp1a by HEK293T cells (Figure 17C). In contrast, the mouse Mmp1a prodomian reduced production of soluble human MMP1 in the mpro-MMP1 chimera.

We were surprised that the prodomain had such drastic effects on Mmp1a production. The prodomains of Mmp1a and Mmp1b are 90% identical and Mmp1b was well expressed in our systems. However, unlike Mmp1a, Mmp1b does not have any identified enzymatic activity, suggesting that perhaps the enzymatic activity of Mmp1a is required for the defect in Mmp1a expression. Consistent with this notion, catalytically inactive Mmp1a (E216A) was expressed at higher levels than active Mmp1a (Figure 17D). Additionally, the elevated expression of the inactive mutant appeared to be specific to Mmp1a given that inactive human MMP1 (E219A) was expressed at comparable levels to wild type MMP1.

Prodomain Instability Hinders Mmp1a Secretion:

Having observed that the prodomain of Mmp1a reduces secreted protein expression in mammalian systems, we hypothesized that the prodomain of Mmp1a, as compared to human MMP1, might associate less stably with the catalytic domain and

thereby be less effective in maintaining Mmp1a in an inactive zymogen state. The cysteine switch motif, in which the thiol group of a conserved cysteine acts as the fourth coordination residue for the catalytic zinc, effectively sealing off the catalytic pocket, is critical for MMP prodomain function (Van Wart and Birkedal-Hansen 1990). Consistent with this idea, the cysteine switch motif is completely conserved between human MMP1 and Mmp1a. The crystal structure of proMMP1 demonstrated that the prodomain is a three helix bundle with multiple points of interaction between the three helices and several points of interaction between the prodomain and catalytic or hemopexin domains (Jozic, Bourenkov et al. 2005). Mmp1a was modeled based on this proMMP1 structure (Figure 18A). Focusing on prodomain-catalytic domain interactions, this model revealed two prodomain residues, A^{83} (V^{86} in humans) and L^{67} (F^{70} in humans) that were suggestive of weaker catalytic domain interactions than the corresponding human counterparts (Figure 18B). In the human proMMP1 crystal structure, F^{70} interacts with $H^{228}S^{229}T^{230}$ of the catalytic domain and this phenylalanine is conserved in the other mouse collagenases and the other known mammalian homologues of MMP1 (Figure 18B).

To determine whether these residues were responsible for the low secretion levels of Mmp1a, we generated the helix 2 point mutants A58V, L67F, and A58V/L67F. The A58V substitution was generated as a negative control because it occurs in helix 2 distal to L67F and was predicted to not interact with other MMP residues. As expected, this point mutant did not have any effect on Mmp1a secretion levels alone or in combination with L67F (Figure 18C). However, the L67F point mutant exhibited increased Mmp1a

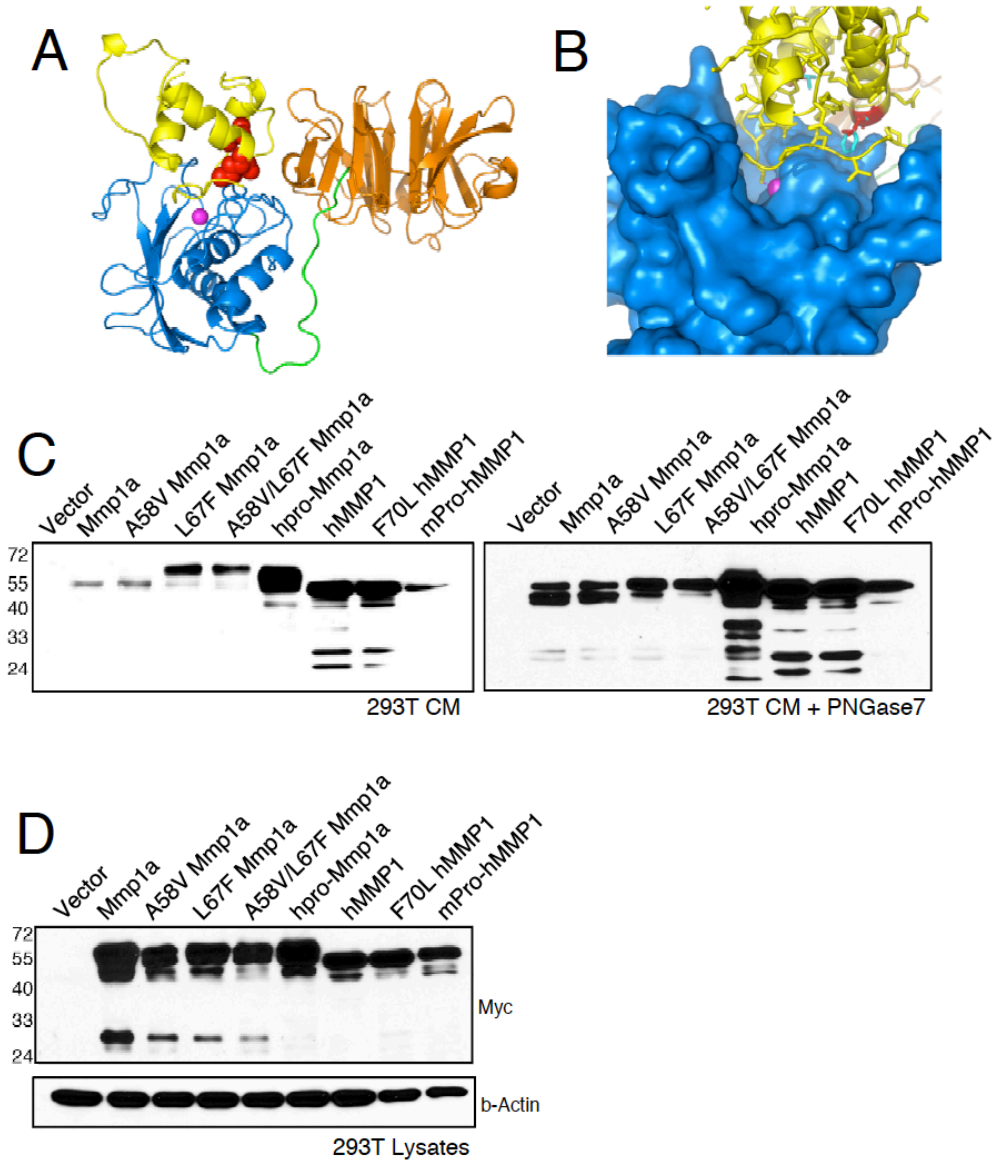

Figure 18: Destabilization of Mmp1a prodomain prevents proper secretion. *A,* 3D structure of Mmp1a as predicted by human proMMP1 homology modeling showing the prodomain (yellow), catalytic domain (blue), linker (green), and hemopexin domain (orange). Catalytic site zinc is pink and L67 residue of interest is shown as red spheres. *B,* Docking of the Mmp1a prodomain (yellow) into the active site (blue) of Mmp1a. L67 is highlighted in red while the corresponding human residues (F70) are depicted in cyan. *C,* HEK293T secretion of Mmp1a and human MMP1 prodomain point mutants into the conditioned media (40 μl) as determined by anti-Myc Western blot (left). Observed size of secreted MMPs following deglycosylation via PNGase 7 treatment (right). *D,* MMP expression levels in lysates (40 μg) of transfected HEK293T, show proMMP (56 kDa), active MMP (46 kDa), and hemopexin degradation product (26 kDa).

expression, suggesting that the L^{67} residue is destabilizing to the Mmp1a prodomain. The increased Mmp1a expression with L67F was less than that which was observed with the human prodomain chimera (hpro-Mmp1a), indicating that there are other important interactions in the human prodomain that are lacking in the mouse prodomain, such as A83V.

Interestingly, the major band observed on Western blot for L67F and hpro-Mmp1a ran at a higher observed molecular weight than wild type Mmp1a and human MMP1 (Figure 18C). This increase in size was found to be due to N-linked glycosylation, as treating the conditioned media with peptide N-glycosidase F (PNGase F) prior to Western blotting reduced the observed size to that of wild type (Figure 18C, right panel). We believe these data point to a stability defect in the prodomain that reduces proper secretion of Mmp1a in mammalian expression systems. Consistent with this hypothesis, there was significantly more Mmp1a in the lysates of transfected 293T cells as compared to MMP1. We also observed a prominent 26 kDa band corresponding the hemopexin domain in the Mmp1a lysates that was absent from the hpro-Mmp1a lysates despite comparable levels of overexpressed protein (Figure 18D). The observation of the 26 kDa hemopexin domain fragment is suggestive of Mmp1a degradation. MMP1 can autocatalyze its own the linker region between the catalytic domain and hemopexin domain. This results in enzyme that is no longer active against collagen but maintains some other enzymatic activity, including the activation of proMMP1 molecules (Clark 1989).

3.3 Discussion

We show here that Mmp1a is a functional MMP1 homologue in tumor stroma. Though *Mmp1a* was nonessential for development and fertility, there was significantly less subcutaneous allograft growth in *Mmp1a*-deficient animals and the resulting tumors had significantly less angiogenesis. Moreover, while *Mmp1a*$^{+/+}$ MEFs promoted cancer cell collagen chemoinvasion, PAR1-dependent migration, and proliferation *in vitro* and tumor growth in *Mmp1a*$^{-/-}$ mice *in vivo*, these activities were substantially reduced with *Mmp1a*$^{-/-}$ MEFs. Given the homologous role for Mmp1a in tumorigenesis, we were interested in why Mmp1a is not ubiquitously present in mouse physiology. Expression of secreted Mmp1a was deficient in three different mammalian overexpression systems. Structural comparison of Mmp1a and human MMP1 identified substitution of a key pro-catalytic domain interaction in which a phenylalanine in human MMP1 is substituted for a leucine in Mmp1a. Modification of this residue or exchanging the mouse prodomain for human resulted in increased Mmp1a protein secretion and improved stability of Mmp1a in cell lystates, suggesting that prodomain instability may discourage constitutive Mmp1a expression.

Perhaps the most unexpected finding of this work has been identification of defects in Mmp1a that impair its production. This defect would not have been observed in a prokaryotic expression system, highlighting the importance of studying theses MMPs in mammalian systems. Additionally, though extensive research has examined the prodomain of MMPs in terms of zymogen activation and substrate binding, little is known about how the prodomain affects enzyme stability, trafficking, and sequestration (Stricklin, Jeffrey et al. 1983; Suzuki, Enghild et al. 1990; Dumin, Dickeson et al. 2001).

The prodomain of MMP14, which is activated differently from MMP1 via furin-like convertases, has been shown to be essential for MMP14 activation of MMP2 in engineered systems. Interestingly, expression of the MMP14 prodomain in *trans* with a prodomain-lacking MMP14 construct is sufficient to restore MMP14 activity, suggesting independent, prodomain-specific functions (Cao, Hymowitz et al. 2000).

Our results suggest that the prodomain of Mmp1a is thermodynamically unstable and therefore unable to effectively maintain Mmp1a in a zymogen state during synthesis and secretion. One would predict that this would prime Mmp1a for activity, making it much more difficult to regulate in the tissue microenvironment. Moreover, if Mmp1a is active intracellularly, it could have effects on cytoplasmic proteins. Recent reports have demonstrated that MMPs can cleave intracellular matrix and cytoplasmic proteins (Cauwe and Opdenakker 2010). For example, MMP3 has been shown to translocate to the nucleus in multiple cell types and active MMP3 in the nucleus has been shown increase apoptosis in overexpression systems (Si-Tayeb, Monvoisin et al. 2006).

The significance of the increased glycosylation observed in the Mmp1a chimera and L67F mutant is unknown. Mmp1a contains three potential N-linked glycosylation sites, one of which is conserved with the N^{120} site of MMP1 that is selectively glycosylated by fibroblasts and fibrosarcoma cells (Saarinen, Welgus et al. 1999). Differential glycosylation has long been observed for the MMP family in many species but is typically viewed as not affecting function (Stricklin, Eisen et al. 1978). The increase in Mmp1a glycosylation could be a consequence of our heterologous expression system but glycosylation could also be an importation modulator of enzyme stability and cell secretion and requires further study.

The lack of a phenotype for the *Mmp1a*$^{-/-}$ mouse in embryonic development and fertility is somewhat surprising yet not entirely unexpected. Mmp1a is physiologically expressed in the placenta and developing embryo (Balbín, Fueyo et al. 2001; Nuttall, Sampieri et al. 2004). Though it is not known what role Mmp1a plays in placental homeostasis and embryonic development, it is reasonable to assume it plays homologous roles to human MMP1 in embryo implantation, tissue remodeling, vasculogenesis, etc. However, the majority of MMP-deficient animals, including the *Mmp8* and *Mmp13* collagenase-deficient mice, have not exhibited developmental or fertility phenotypes (Balbín, Fueyo et al. 2003; Stickens, Behonick et al. 2004). The dispensability of individual MMPs is likely due to redundancy in substrate specificity within the MMP family (Gill, Kassim et al. 2010).

The decrease in tumor growth observed in *Mmp1a*-deficient mice is consistent with observations of patient samples that stromal MMP1 is upregulated in aggressive human cancers. LLC1 cells express endogenous Mmp1a that also promotes tumorigenesis, suggesting that both stromal and tumor-derived MMP1 promote tumorigenesis. Additional studies with more sophisticated mouse models of tumorigenesis will be valuable in understanding the specific roles of MMP1 in the various stages of cancer progression and understanding the impact of various sources of MMP1 in the tumor microenvironment. Together these results demonstrate the importance of stromal Mmp1a in tumorigenesis and angiogenesis and suggest that the *Mmp1a*$^{-/-}$ mouse will be a valuable tool in further interrogating MMP1-like functions in the mouse.

Chapter 4

Materials & Methods

4.1 Reagents and Preparation of Tools

Plasmid DNA:

Human *MMP1*-pCMV6-Entry with a C-terminal Myc-DDK (FLAG) tag was purchased from Origene. Synthetic oligonucleotides were purchased from Integrated DNA technologies. All plasmid were Sanger sequenced by the Tufts University core facility. *Mmp1a*, *Mmp1b, Mmp8, and Mmp13* cDNAs were purchased from Open Biosystems and cloned via PCR into the SgfI/AsiSI and MluI sites of pCMV6-Entry (*Origene*). To generate a C-terminal His-Myc tag, a stop codon was introduced following the Myc tag in pCMV6-Entry by site directed mutagenesis (*Agilent*). The His-tag was introduced 5' to the Myc-tag by ligation of a synthetic, phosphorylated oligonucleotide into the NotI site (*Integrated DNA Technologies*). The catalytically inactive E219A MMP1 and E216A Mmp1a mutants were generated by QuikChange site-directed mutagenesis. *Mmp1a* and *E216A Mmp1a* were also subcloned into the EcoRI and SalI sites of pBabe-Puro (*Addgene*) for retrovirus generation.

The *Mmp1a* and *MMP1* prodomain chimeras were generated by utilizing naturally occurring NdeI restriction sites in *Mmp1a* at nucleotide 87 and in pCMV6Entry located 5' to the open reading frame. The prodomain of *MMP1* was generated by PCR with an exogenous NdeI site and ligated into NdeI-digested *Mmp1a* to generate *hPro-Mmp1a*. A foreign NdeI site was introduced in *MMP1* using site directed mutagenesis and *mPro-MMP1* was generated by ligation of NdeI digested *MMP1* vector and *Mmp1a* prodomain. The prodomain mutants of Mmp1a P2S, A58V, L67F, and A58V/L67F and the MMP1 prodomain mutant F70L were generated by QuickChange mutagenesis.

Mouse *Par1*/*F2R* cDNA was purchased from Open Biosystems and cloned into pCDEF3 using the BamHI and XbaI restriction sites. T7-tagged mouse *Par1* was generated by ligation of nucleotides 165-1350 into the KpnI and XbaI sites of T7-*PAR1*. This generated a mouse *Par1* construct with the human *PAR1* signal peptide and a T7 tag that is six amino acids upstream from the thrombin cleavage site.

Cell Lines and Culture:

LLC1, HEK293T, and CHO-K1 cells were obtained from ATCC. Cos7 (HyCos7) cells were a gift from Dr. Martin Beinborn (*Tufts University, Boston MA*). The human cancer cell lines, MCF7 and A549, were obtained from the National Cancer Institute Repository. The MCF7+PAR1 (N55) cell line was generated by stable transfection of PAR1 followed by clonal selection, as described previously (Boire, Covic et al. 2005). LLC1, HEK293T, and Cos7 cells were maintained in DMEM supplemented with 10% FBS, 1% Pen-Strep. CHO-K1 cells were grown in F12 media supplemented with 10% FBS, 1% Pen-Strep. MCF7, N55, and A549 were cultured in RPMI with 10% FBS, 1% Pen-Strep.

C57MG cells were a gift from Dr. Lynn Matrisian (*Vanderbilt University, Nashville TN*) and were maintained as described previously in DMEM, 10% FBS, 1% Pen-Strep (Vaidya, Lasfargues et al. 1978). The 4228 and 4246 melanoma cell lines, a gift from Chi Luo and Phillip Hinds (*Tufts University, Boston, MA*), were isolated from spontaneous cutaneous melanomas arising in transgenic mice expressing BRAF V600E under the mouse tyrosinase promoter (line 476) that were crossed to C57BL/6 $p19^{ARF}$

exon 1b knockout animals (Kamijo, Zindy et al. 1997; Goel, Ibrahim et al. 2009). 4228 and 4246 cells were isolated by collagenase I/hyaluronidase digestion of tumor fragments for 30 minutes and were cultured in RPMI supplemented with 10% FBS.

Antibodies:

Rabbit anti-Myc antibody was purchased from Cell Signaling. Goat anti-mPar1 (S19) antibody was from Santa Cruz Biotechnology. Rabbit anti-Mmp1a and Mmp1b antibodies were generated by coupling 16-mer peptides to cyanogen bromide-activated keyhole limpet hemocyanin (CnBr-KLH) via a free N-terminal cysteine residue. The peptide corresponded to the target protein C-terminal sequence N^{446}LQIRRVDDSRDSS^{459} (Mmp1a) and N^{445}LKTRRIDDSSDIN^{458} (Mmp1b). The coupled peptides were injected into two rabbits per antigen by an outside company, SDIX (*Strategic Diagnostics, Newark, DE*), using a standard protocol at days 0, 21, 35, and 49. Serum used for antibody was harvested by ear bleed at days 54 and 63 and exsanguination at day 72. Exsanguination was ordered following positive serum dot blots against the antigen peptide with reactivity present at greater than 1:20,000.

The antibodies were purified with at least two peptide affinity columns. The initial affinity column was a negative selection purification, where any antibody binding a non-specific peptide (SFLLRN) was removed. The second column was then a positive selection for antibodies that recognize the antigen peptide. To remove Mmp1b reactivity from the Mmp1a antibody, the Mmp1a antibody was negative selected against the Mmp1b antigen peptide in a third purification step. Antibodies were characterized based

on their ability to recognize Mmp1a or Mmp1b in transfected HEK293T conditioned media without cross reactivity towards Mmp8, Mmp13, and human MMP1.

Pharmacologic inhibitors:

The N-palmitoylated peptide, P1pal-7, was synthesized by the Tufts University Core Facility as described previously (Covic, Gresser et al. 2002). The MMP inhibitors **MMP Inh I** (IC_{50}= 1 μM [MMP1], 30 μM [MMP3], 1 μM [MMP8] and 150 μM [MMP9]), **MMP Inh II** (IC_{50}= 24 nM [MMP1], x 18.4 nM [MMP3], 30 nM [MMP7] and 2.7 nM [MMP9]), and **MMP Inh V** (IC_{50}= 0.73 nM [MMP2], 42 nM [MMP3], 1.1 nM [MMP8], 2.1 nM [MMP9], 0.45 nM [MMP12] and 1.1 nM [MMP13]) were from EMD Biosciences. An inactive MMP Inh I analogue (X-MInhI) in which the hydroxamic acid is missing and a L-Leu has been converted to D-Leu (4-Abz-Gly-Pro-Leu-D-Ala-NH-OH converted to 4-Abz-Gly-Pro-D-Leu-D-Ala-OH) was synthesized by the Tufts University Core Facility.

RNAi:

Mmp1a-targeted, *Par1*-targeted and control luciferase-targeted short hairpin RNAs in pLKO.1-Puro were purchased from Sigma Aldrich. Five different shRNA sequences were purchased for both *Mmp1a* and *Par1*. Each shRNA construction was tested by lentiviral transduction into LLC1 cells followed by quantitative real time PCR to assess the degree of target gene knock down. The shRNAs that resulted in the greatest mRNA reduction were selected for use. The sense strand of the shRNA target sequences that were selected are as follows:

CGCTGAGTACTTCGAAATGTC (shLuc)

CCTGGAATTGATGATAAAGTT (shMmp1a #1)

CCGTGATTCTAGTACATGGTT (shMmp1a #2)

AGGGCAGTCTACTTAAATATA (shPar1)

RNA Isolation and Quantitative Real-time PCR:

Total RNA was extracted from cell lines or flash-frozen, whole tumor homogenates using the RNeasy Mini kit and treated with on-column DNase digestion (Qiagen). RNA was reverse transcribed using a standard Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase reaction. Real-time PCR was conducted using a SYBR Green master mix (Qiagen) and a standard 40 cycle thermocycling protocol.

Primers for real time PCR were designed using NCBI Primer-BLAST unless otherwise noted. Mmp1a primers were as described previously by Nuttall, *et al.*:

CGTGGACCAACAGCAGTGAA (Mmp1a-F)

GAGTGAGCCCAACCCACTCA (Mmp1a-R)

Mouse Par1 primers (5' to 3') were:

CTCCTCAAGGAGCAGACCAC (Par1-F)

CAAGAAAGAAGATGGCGGAG (Par1-R)

Primer bank designed mouse Cyr61 primers (5' to 3') were:

TAAGGTCTGCGCTAAACAACTC (Cyr61-F)

CAGATCCCTTTCAGAGCGGT (Cyr61-R)

Primer bank designed mouse Gapdh primers (5' to 3') were:

AGGTCGGTGTGAACGGATTTG (Gapdh-F)

TGTAGACCATGTAGTTGAGGTCA (Gapdh-R)

Virus Generation:

Lentiviral particles were generated by triple transfection of pLKO.1-Puro, pMD.G, and pCMV-dR8.9 in HEK293T using the calcium phosphate method (Graham and van der Eb 1973). Retroviral particles for C57MG infection were generated by calcium phosphate transfection of Phoenix Ampho cells. Both lentiviral and retroviral supernatants (DMEM, 20% FBS, no Pen-Strep) were harvested at 24 and 48 hours after transfection. All cells were transduced overnight with serial dilutions of viral supernatant for 1:1 to 1:10,000 in the presence of 8 μg/mL polybrene. LLC1 and C57MG cells were selected with 3 μg/mL puromycin for 5 days beginning 48 hours after transfection.

Preparation of Conditioned Media:

To harvest conditioned media from cell lines for Western blot analysis of endogenous Mmp1a expression, $4x10^6$ cells were plated in complete media in a 10 cm dish. After six hours, the media was then changed to low serum (0.1% FBS). At 48 hours, media was harvested and centrifuged to remove cellular debris. To harvest conditioned media for functional assays (migration, invasion, proliferation, etc), $1x10^6$ cells were plated in a 10 cm dish in complete media. The next morning, the media was changed to moderate serum (1% FBS). Media was harvested at 48 hours and centrifuged to remove debris.

Protein Overexpression:

HEK293T cells were transfected overnight by calcium phosphate transfection, using 4 and 8 μg DNA for 10 and 15 cm plates, respectively (Graham and van der Eb 1973). Cos7 and CHOK1 cells were transfected overnight with polyethylenimine (PEI) at a ratio of 3:1 (PEI:DNA). 10 μg DNA was used per 10 cm plate and 20 μg DNA per 15 cm plate. The morning after transfection, the cells were washed once with PBS and the media changed to low serum media (0.1% FBS DMEM for HEK293T and Cos7, 0.1% FBS F12 for CHOK1).

Purification of MMPs:

Conditioned media (0.1% FBS) was harvested from HEK293T or CHOK1 cells overexpressing C-terminally tagged His-Myc collagenases after 48 hours of conditioning. Media was filtered via 0.45 μm filter to remove cellular debris. HisTrap columns (1 mL, *GE Healthcare*) were equilibrated with 5 column volumes of serum free media at a flow rate of 0.5 mL/min via peristaltic pump. 30-50 mL conditioned media (from 6-10 confluent 15 cm plates of cells) was then loaded on the column at a flow rate of 0.5 mL/min. The column was then washed with 5 column volumes with low salt wash buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 5 mM imidazole). This was followed by 5 column volumes of high salt wash buffer to remove nonspecific protein binding (1M NaCl, 50 mM NaH_2PO_4 pH 8.0, 5 mM imidazole). The salt concentration was reduced with 5 column volumes low salt wash buffer (300 mM NaCl, 50 mM NaH_2PO_4 pH 8.0, 5 mM imidazole). The bound protein was eluted with (100 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4 pH 8.0) and 0.5 mL fractions were collected. Target protein containing fractions were identified by Western blotting for the Myc tag and were dialyzed

overnight in 10 kDa tubing against four liters dialysis buffer (50 mM Tris pH 7.4, 300 mM NaCl, 5 mM $CaCl_2$, 5 μM $ZnCl_2$) with a change of buffer the next morning for an additional two hours of dialysis. Protein concentration was determined by the Bradford method (Bradford 1976).

Protein De-glycosylation:

Conditioned media was denatured for 10 minutes at 95^{o}C in 1X glycoprotein denaturing buffer (New England Biolabs). The media was then incubated with G7 reaction buffer, 1% NP-40, and 1x10^{3} units PNGase F for 90 minutes at 37^{o}C. The entire reaction was then run on a 12% SDS-PAGE gel for Western blot analysis.

Structural Modeling of Mmp1a:

Modeling of Mmp1a was performed by Dr. Andrew Bohm (*Tufts University, Boston, MA*). Mmp1a was homology modeled using SWISS-MODEL (swissmodel.expasy.org) (Schwede, Kopp et al. 2003). The human pro-MMP1 structure was used as the template structure (PDB code 1su3). Images were generated using the PyMOL software package.

4.2 Functional Assays

Boyden Chamber Invasion:

All invasion assays were performed using Boyden chambers (24 well insert size) with 8 μm pores (Costar). Cells were starved for at least six hours in low serum (1% FBS) media. For Matrigel invasion, 50 μg Matrigel (BD Biosciences) was diluted in serum-free DMEM and layered on a chilled Boyden chamber membrane. For collagen invasion, 25-50 μg of rat tail type I collagen (BD Biosciences) was cross-linked via increased pH and temperature on a Boyden membrane according to the manufacturer's protocol. For invasion assays, 10% FBS/DMEM was used as a chemoattractant in the lower chamber. For chemo-invasion assays, conditioned media from wild type or Mmp1a null MEFs was used as a chemoattractant. For LLC1 invasion, the upper chamber contained 25,000 (Matrigel invasion) or 20,000 (collagen invasion) LLC1 cells in 1% FBS DMEM. For C57MG collagen invasion, 25,000 cells were placed in the upper chamber. After 48 hours, non-invasive cells were removed and membranes were stained with the Hema-3 stain system (Fisher). Invasion was quantified by counting number of cells per nine fields (membrane diameter).

Boyden Chamber Migration:

A549 or N55 cells were starved over night in serum free media. Migration was performed in Costar 8 μm Boyden chambers. MEF conditioned media (600 μl) was placed in the bottom chamber. $5x10^4$ cells in serum free media were placed in the top chamber. Pharmacologic inhibitors were placed in the upper and lower chamber. Cells were migrated for 18 hours at 37 degrees/5% CO_2/humid air. After 18 hours, non-

migratory cells were wiped from the top of the membrane and migratory cells were stained with the Hema-3 stain system. Migration was quantified by counting the number of cells per nine fields (membrane diameter) X 9.17 to obtain the total number of cells migrated.

MTT Proliferation Assay:

LLC1 cells were plated at a density of $1x10^3$ cells per well of a 96-well plate in full media. After cells had adhered, they were starved for at least 6 hours in serum free, DMEM + 0.2% BSA. Cells were then stimulated with 100 μl conditioned media per well for 72-96 hours. At the experiment end, media was aspirated from the wells. 100 μl of MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 0.5 mg/mL in sterile PBS was added to each well and the plate was returned to the incubator for 4 hours. The MTT reagent was then carefully aspirated and crystals that had formed were dissolved in 100 μl DMSO. The absorbance was measured at 570 nm and 640 nm and the amount of reduced MTT was determined as the difference between the two absorbances.

3D Growth Assays:

For 3D growth assays, 5,000 shLuc, shMmp1a #2 or shPar1 LLC1 cells in 100 μl Matrigel were layered on top of a 24 well plate that had been pre-coated with Matrigel. Once gels had polymerized, 100 μl of complete media was added to coat the gel. 3D cultures were maintained at 37 °C/5% CO2/humid air for 7 days. Cultures were imaged under 6X magnification. The invasion score was tabulated from at least 10 representative

fields as follows: Grade 0: Colony with no invasive projections, Grade 1: Colony with invasive projections that are shorter than the radius of the central colony, Grade 2: Colony with projections that are equal to or longer than the radius of the central colony.

3D Media Release Assay:

HEK293T cells were transiently transfected with vector, Mmp1a, Mmp1b, MMP1, E219A-MMP1, or E216A-Mmp1a with a C-terminal His-Myc tag. Transfected cells (2×10^5) were embedded in 1 ml of type I rat tail collagen (BD Biosciences, high concentration type I collagen) at a final concentration of 1 mg/ml of collagen with 400 nM trypsin and overlaid with 1 ml of serum-free medium, as previously reported (Wyatt, Geoghegan et al. 2005). After 24 hr incubation at 37°C, the liquid medium was gently removed and weighed to determine the amount of collagen degradation.

4.3 Mouse Generation and *In Vivo* Experiments

$Mmp1a^{-/-}$ Mouse Generation and Genotyping:

Mmp1a deficient mice were generated by the laboratory of Dr. Carlos Lopez-Otin (Universidad de Oviedo, Oviedo, Spain). A targeting vector was generated in pKO scrambler V916 (Lexicon Genetics) that was composed of 1.4 kb short arm encompassing exons 1-4 and introns 1-3 of *Mmp1a* and 6.8 Kb long arm encompassing exons 6 and 7 and intron 6. The PGK-Neo cassette replaced most of introns 4 and 5 and exon 5. The construct was linearized and electroporated into 129/SvJ-derived RW4 embryonic stem cells. Clones were selected for G418 resistance and screened by Southern blot. Heterozygous ES cells were injected into C57BL/6 blastocysts and

transferred into the uteri of pseudo-pregnant females. Chimeric males were mated to C57BL/6 females and pups were screened by PCR and Southern blot. Heterozygotes were backcrossed for 10 generations into the C57BL/6 background. Homozygotes and wild type controls were obtained by crossing heterozygous littermates.

Southern blot was performed by overnight digestion of genomic DNA with EcoRI (New England Biolabs). The digested DNA was then run on 0.8% TAE agarose gel and hybridized overnight with a dCTP-alpha P32 (Perkin Elmer) labeled probe. The Souther blot probe is a 296 nt DNA fragment generated by PCR of *Mmp1a* cDNA and results in a 6.5 Kb recognizes a 6.5 Kb fragment in the wild type allele and a 3.5 Kb fragment in the mutant allele. The sequence is:

atacaatggatatgaataagagaagacagcagtgtaaatgctgaagatcatctctttcacacatgtgctgctgg

atagtcctggtattgctgcaccaaaccaacctaactcaactgctaaggtcaaagtgtctgatttcttctctgctaca

gaagaggatgttgtcaaccatgagtcacatagcctctggctttctagaagtacaagaagtctctatataaagatg

gaagttcctgactaggatgttggagcaggcaggaaggaggccactggtgattttgcccagagaaaagctt

Genotyping PCR was performed with a three primer strategy using genomic DNA isolated from tail fragments. The primer sequences used are:

Wild type allele primer (5'-3'): acgcattctgcctactgcaagg

Knockout allele primer (5'-3'): tgaccgcttcctcgtgcttta

Common primer (5'-3'): gcagaccatggtgacaacaacc

The wild type allele gives a 200 bp amplicon and the knockout allele gives a 470 bp amplicon.

Mouse Embryonic Fibroblast (MEF) Isolation:

Embryos were harvested from *Mmp1a* heterozygote crosses at embryonic day 12.5. Aspetic technique was used to dissect the uterus into individual embryos, which were then kept in ice cold PBS. In a sterile hood, the embryo was squeezed from the placenta and the head was discarded. Embryos were diced using sterile razor blades and digested for 15 minutes in 0.05% trypsin at 37^{o}C. The digests were resuspended in 10% FBS/1%PS/DMEM and incubated overnight. The next day, the floating debris was harvested for genotyping and the adherent cells were split. Cells were maintained in DMEM supplemented with 10% FBS, 1% Pen-Strep.

LLC1 subcutaneous xenograft models:

All animal experiments were conducted in full compliance with the Tufts Medical Center Institutional Animal Care and Use Committee. Six week old female C57BL/6 were obtained from Charles River Laboratories (Chapter 2) or were a combination of 6-8 week old mice purchased from Charles River Laboratories or bred in house from *Mmp1a* heterozygote or knockout cross (Chapter 3). For subcutaneous xenograft models, $2x10^5$ shLuc or shMmp1a #2 cells in 100 μl sterile PBS were injected into the abdominal fat pad (1-2 inoculations per mouse). Twelve days after implantation, palpable tumor growth was measured every other day and tumor volume was calculated based on the equation $(LxW^2)/2$. After 26 days, tumors were flash frozen for mRNA analysis or formalin-fixed for histology.

Experimental Metastasis:

For experimental metastasis, $1x10^6$ shLuc or shMmp1a #2 LLC1 cells in 200 μl sterile PBS were injected into the tail vein. After 28 days, lungs were harvested and formalin-fixed for histology. The number of metastases per animal was determined in a blinded fashion by counting three H&E stained coronal lung sections per mouse.

LLC1-MEF Co-implantation:

Seven week old C57BL/6 were obtained from Charles River Laboratories. Six to eight week old *Mmp1a*$^{-/-}$ mice were bred in house. Passage 5 *Mmp1a* wild type or knockout MEFs at a final concentration of $1x10^6$ cells/mL were mixed with shLuc LLC1 cells at final concentration of $2x10^6$ cells/mL in sterile PBS. 100 μl was injected into the left and right abdominal fat pad and palpable tumor growth was measured from day 12 until day 22.

Histology:

Paraffin embedding, sectioning, hematoxylin and eosin (HE) staining, and vWF immunohistochemistry on tumor specimens were performed by the Tufts Medical Center pathology department. Quantification of vWF staining was performed in a blinded fashion by counting the number of vessels per 50 fields at 40X magnification.

Statistics:

Significance was determined as corresponding to an alpha value less than 0.05. Comparisons between two groups was performed in Microsoft Excel via a two-tailed,

heteroscedastic T-test. Comparison of the means between multiple groups was performed using one way ANOVA followed by Student-Newman-Keuls T-test in KaleidaGraph software.

Chapter 5

Future Directions

This dissertation has established a role for the mouse homologue of MMP1, Mmp1a, in models of tumorigenesis. Endogenous cancer cell Mmp1a expression lead to an autocrine Mmp1a-PAR1 signaling loop. Cancer cell Mmp1a expression promoted tumor growth, invasion, and experimental metastasis, consistent with the functions observed for human MMP1 and MMP1-PAR1 signaling in xenograft experiments. Tumor allograft experiments in a novel *Mmp1a*$^{-/-}$ mouse revealed decreased tumor growth and angiogenesis in the absence of stromal *Mmp1a*, furthering supporting a homologous role for Mmp1a.

The *Mmp1a*-deficient mice should provide a useful tool for understanding MMP1-like processes in mouse models. As with any animal model to study human disease, expression of Mmp1a must first be confirmed in the system to ensure that *Mmp1a*-deficient animals are a relevant model. At least in the case of subcutaneous tumorigenesis, the *Mmp1a*$^{-/-}$ mouse appears to be a relevant model. However, our current systems are limited by a lack of C57BL/6 syngenic tumor cell lines, with LLC1 lung cancer and B16 melanoma being the most commonly used. Several options exist for future tumorigenesis studies. New C57BL/6 tumor lines could be generated by transformation of primary mouse cells of different tissue origin. This would enable genetic alteration of the cancer cells independently of the mouse stroma and would allow for the examination of tumor versus stromal contributions of Mmp1a in tumorigenesis. Alternatively, *Mmp1a*$^{-/-}$ mice could be bred into an immunocompromised strain, allowing for the study of human cancer cell lines in the mouse. The most informative use of the *Mmp1a*$^{-/-}$ mice in tumorigenesis would arguably be to examine the outcome of *Mmp1a*-deficiency on spontaneous tumor formation in transgenic models associated with

MMP1/Mmp1a upregulation. The most obvious transgenic models to explore are those that lead to activation of the canonical MAPK pathway. LLC1 are hypothesized to have upregulated Mmp1a due to unrestrained Ras activation and active Ras transgenic models could yield interesting phenotypes (Ma, Liu et al. 2010). Likewise, the V600E BRAF melanoma mice appear to upregulate Mmp1a protein expression in melanocytes expressing the active BRAF transgene, even in the absence of oncogenic transformation. This suggests that V600E BRAF directly targets Mmp1a expression, as has been described for human MMP1 in melanoma samples. Thus, a clinically informative phenotype may be obtained in studying BRAF-mediated tumorigenesis in *Mmp1a*-deficient animals.

However, mice are not humans and Mmp1a is not identical to human MMP1. Mmp1a is expressed rarely in healthy mouse tissues. Based on the evidence from overexpression systems, we suggest that the limited Mmp1a expression in physiologic situations is due to defects inherent in enzyme stability. Though there are examples of MMPs that contain furin-like convertase sequences in their prodomain and are activated prior to secretion, such as MMP11, zymogen activation is believed to be an important additional level of enzyme regulation. If the prodomain of Mmp1a is thermodynamically unstable, making it prone to spontaneous activation, expression of Mmp1a could be deleterious to cells and tissues due to constitutive activity. This would suggest that Mmp1a, once expressed, is almost immediately active and the only way to control Mmp1a activity would be through silencing of expression and enzyme inhibition/degradation via TIMPs, α2M, etc. Moreover, if Mmp1a is becoming activated prior to secretion, it could be targeting intracellular proteins and have cytoplasmic effects.

However, a major limitation of this work is that our observations are based on overexpression systems and significant tools need to be developed to understand if the instability phenomenon occurs with endogenous Mmp1a. For example, we do not currently understand how Mmp1a is being trafficked within the cell and what is happening to the unsecreted protein. Fluorescently tagged Mmp1a constructs would enable time-lapse microscopy of intact cells to monitor real time trafficking and sequestration of overexpressed protein. Highly sensitive and specific antibodies that are selective for pro- and active Mmp1a need to be developed to effectively study whether Mmp1a protein instability occurs naturally. Finally, if Mmp1a prodomain instability occurs with endogenous protein, transgenic mouse models would need to be developed to determine whether this has any functional consequences.

On first examination, a phenomenon limited to a rodent specific enzyme has little significance for human disease. However, very little is understood about regulation of MMP activation/activity *in vivo*. Given that direct inhibition of MMPs has failed in human clinical trials, indirect inhibition of MMPs, potentially by controlling activation, could offer novel therapeutic strategies. Mapping of the residues of Mmp1a that regulate prodomain stability and activation kinetics will likely provide knowledge that can be extended to human MMP1 and other MMPs. Additionally, understanding MMPs in the inactive state could potentially identify novel approaches to targeting MMPs by inhibiting or promoting MMP activation.

Another interesting area for future research is the role of Mmp1b in mice. Mmp1b is 82% identical to Mmp1a. Mmp1a and 1b appear to be coexpressed in mouse tissue and in cell lines. Despite being expressed, no enzymatic activity has been

identified yet for Mmp1b. It is unable to cleave fibrillar collagen but purified Mmp1b can weakly auto-activate itself, suggesting that it has some enzyme activity. Based on our observations with Mmp1a instability, we speculate that Mmp1b may function to stabilize Mmp1a. Preliminary experiments have suggested that overexpression of Mmp1b reduces basal HEK293T collagenase activity (data not shown). Additionally, knockdown of Mmp1b in LLC1 cells resulted in slightly increased tumor growth whereas combined knockdown of Mmp1a and Mmp1b phenocopied Mmp1a knockdown alone (data not shown). However, studying Mmp1b is extremely challenging when its enzymatic function are unknown and there are no obvious functional assays for characterizing the specificity and effectiveness of Mmp1b reagents.

Further studies are necessary to fully understand how to appropriately correlate the MMP systems in mice and humans. The initial failure of broad spectrum MMP inhibitors in clinical trials despite promising preclinical results in mouse models highlights the importance of understanding the differences between human disease and mouse models. However, based on this work, MMP1/Mmp1a appear to be promising therapeutic targets in tumorigenesis and metastasis.

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