Roles of Fibroblast MMP2 in Breast to Lung Metastasis

By

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To my wonderful children, Rajah and Eris:

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2D	two dimensional
3D	three dimensional
ACTR	activin receptor
ALK1	serine/threonine protein kinase receptor R3
ALS	acid labile subunit
APMA	4-aminophenylmercuric acetate
BCOU	Breast Cancer Outcomes Unit
bFGF	basic fibroblast growth factor
BME	basement membrane extract
BMP	bone morphogenetic protein
BMP-1	Bone morphogenetic protein 1-like metalloproteinase
BMPR	BMP receptor
CAF	cancer associated fibroblasts
Cm	conditioned media
CTGF	connective tissue growth factor
CTL	control
CXCL12	chemokine (C-X-C motif) ligand 12
DQ	dye-quenched
ECM	Extracellular matrix
ED-A	fibronectin extra domain A
EMT	epithelial to mesenchymal transition
ER	estrogen receptor
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
FAP	fibroblast activation protein
FBS	fetal bovine serum
Fitc	fluorescein isothiocyanate
FRET	Förster resonance energy transfer
FSP1	fibroblast specific protein 1
FSP1-TK	FSP1-thymidine kinase, mouse model
Gapdh	glyceraldehyde-6-phosphate dehydrogenase
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor receptor 1
IGFBP	IGF binding protein
IL-1β	interleukin 1β
IR	insulin receptor
IRS	insulin receptor substrate
JNK	c-Jun N-terminal kinases
KD	knockdown
LAP	latency associated peptide
LLC	large latent complex
LOX	lysyl oxidase
LTBP	latent TGFβ binding protein
MAPK	mitogen activated protein kinase
MEK	mitogen-activated protein 2kinase
MF	mitotically active fibroblasts
MMP	Matrix metalloproteinase
MPI	MMP inhibitor
MSS	musculoskeletal syndrome
MT-MMP	membrane type-MMP
OPG	osteoprotegerin
PCNA	proliferating cell nuclear antigen
PDAC	pancreatic ductal adenocarcinoma
PDGFR	platelet derived growth factor receptor
PI3K	phosphoinositide 3-kinase
PMF	post-mitotic fibrocytes
PyMT	polyomavirus middle T oncogene
pyvt	murine polyomavirus middle T gene
RGD	Arginine-Glycine-Aspartate (Arg-Gly-Asp) motif
rhMMP2	recombinant human MMP2

ROCK	Rho associated protein kinase
RT-PCR	reverse transcriptase-polymerase chain reaction
SBE	Smad binding element
SDF1	stromal derived factor 1
SHC	Src homology domain containing transforming protein
Shctl	short hairpin (Sh) control
Shh	sonic hedgehog
SLC	small latent complex
Smad	mothers against decapentaplegic homologue
TBRII	TGFβ receptor II (ALK5)
TDF	tumor derived fibroblast
TGFβ-1	Transforming growth factor beta 1
TIMP	tissue inhibitor of metalloproteinase
TMA	tissue microarray
TNFα	tumor necrosis factor α
VEGF	vascular endothelial growth factor
Vim	vimentin
WAP-STR1	whey acidic protein-stromelysin 1, transgenic model
WT	wild-type
αSMA	alpha smooth muscle actin
αSMA-TK	αSMA-thymidine kinase, mouse model

CHAPTER I

Introduction

Significance

The majority of cancer patients with distant metastases will eventually succumb to their disease. In the case of breast cancer, tumor cells can metastasize to various organs-including lung, liver, brain and bone. This leads to a dramatic decrease in the five-year survival rate to less than 25%, compared to over 90% for loco-regional disease [1]. Understanding the molecular mechanisms by which cancer cells spread, and perhaps more importantly, what allows them to grow in a foreign environment, is essential to disrupting metastasis and saving lives. In 1889, English surgeon Stephen Paget proposed his famed "seed and soil hypothesis" which relates tumor metastasis to plants growing on congenial soil. In his analysis of the autopsy records of 735 women with breast cancer, he noted a non-randomized pattern of breast tumor dissemination. He concluded that tumor cells, or "seeds", preferentially metastasize to organ sites within the body, or "soil", that are most permissive to their growth [2, 3]. This hypothesis has withstood more than a century of research, and lays the foundation for studying the role of the host organ-specific microenvironments in tumor metastasis.

Role of fibroblasts in breast cancer

The host microenvironment

The host microenvironment is often termed the tumor "stroma". The stroma is important to the support and growth of the tumor. It mediates crosstalk between tumor cells and the surrounding

environment as well as provides nutrients and other factors needed for survival. The stroma consists of blood vessels, extracellular matrix (ECM), and multiple cell types. These include endothelial cells and other vascular components, adipocytes, macrophages and other inflammatory cells, and fibroblasts[4]. Crosstalk between tumor cells and the microenvironment is accomplished in a number of ways. Firstly, the release of soluble factors such as cytokines, chemokines and growth factors provide signaling cues between cells. The hematopoietic and lymphatic vasculature provide oxygen and nutrients to the tumor as well as a means of transporting tumor cells to distant sites. The ECM provides mechanical support for the tumor and also serves as a reservoir for soluble factors sequestered within it. Tumor cells can interact with a number of different cell types within the tumor microenvironment simultaneously, and many of these interactions may be interdependent. Understanding how these tumor-stroma relationships contribute to tumor progression is key to uncovering novel methods of therapeutic intervention. This work investigates how the main cellular component of the stroma, fibroblasts[5], contribute to tumor progression. The term "fibroblasts" constitutes diverse subpopulations of cells that not only have differential gene expression patterns depending upon their tissues of origin [6, 7], but also vary phenotypically and functionally within the same tissue. Because these studies focus on the lung tumor microenvironment, definitions relevant to pulmonary fibroblasts will be discussed in more detail.

Fibroblast biology and activation

Fibroblasts are spindle-shaped connective tissue cells that are derived from mesenchymal progenitors and are embedded in the fibrillar network of the extracellular matrix [8]. They are an important part of both normal homeostasis and the tumor microenvironment since they are major

producers of ECM. Because of this, fibroblasts are responsible for structural support, including the construction of the basement membrane, which supports epithelial cell layers in many tissues. Fibroblasts also secrete a number of factors that influence local inflammation, angiogenesis, and when dysregulated, fibrosis or cancer. Understanding how tumor cells exploit normal fibroblast functions as a means of growth support may provide insight into how tumors can survive and thrive in various metastatic environments and eventually become overt metastases.

Fibroblasts within the lung consist of two major populations, mitotically active fibroblasts (MF) and postmitotic fibrocytes (PMF) [9]. These fibroblast subsets are further divided based on morphological and biochemical characteristics. The MF subsets are considered progenitor fibroblasts and are subdivided into MFs I-III [10, 11]. Fibroblasts differentiate along a continuum that proceeds from the MF subsets to the PMF terminally differentiated cells. Replicative potential decreases as these cells become more differentiated. However, functionality increases, i.e., more collagen matrix is produced [11].

Fibroblasts from all tissues, including lung, classically function to remodel the ECM, which occurs in both wound healing and cancer. Upon tissue injury or the presence of cancer cells, profibrotic cytokines released by neighboring injured epithelial cells, inflammatory cells (i.e. neutrophils and macrophages) and platelets transform quiescent fibroblasts into an activated, terminally differentiated state. These modified fibroblasts were first noticed in granulation tissue from four wounding sites in Wistar rats [12], where it was concluded that they function to contract the wound. With the commencement of wound healing, these fibroblasts began to exhibit features that were similar to smooth muscle cells: 1) closely packed bundles of fibrils that

resembled attachment sites of smooth muscle cells, 2) condensed nuclei, and 3) intercellular contacts as well as cell surface adhesions [12]. Later studies found that stimulation of fibroblasts by TGFβ-1 induces expression of a specific extra domain A (ED-A) splice variant of fibronectin. This provides the extracellular mechanical tension needed to transmit signals inside the cell that lead to the recruitment of alpha smooth muscle actin (αSMA) protein to contractile fibers, forming myofibroblasts [13]. Presently, the term 'activated fibroblasts' is used to signify fully differentiated, contractile myofibroblasts. Myofibroblasts are considered to be αSMA and vimentin expressing cells that form stress fibers connected to extracellular fibronectin [14]. Importantly, myofibroblasts produce an abundance of extracellular matrix and lack many markers of smooth muscle cells, allowing the two to be distinguished.

Because of the myogenic nature of myofibroblasts and the expression of shared markers with smooth muscle cells, it was once postulated that smooth muscle cells could be a source of myofibroblasts. Even though myofibroblast and smooth muscle cell characteristics share a significant amount of overlap, such as microfilament bundles and the ability to contract, each cell type maintains distinct functions. For example, myofibroblasts depend on stress fibers for force generation [15]. Table 1 is a summary of the accepted markers for fibroblast activation, collated from several extensive review articles [13, 15, 16].

Table 1: Markers of fibroblast activation.	
Expressed	**Not Expressed**
Vimentin mesenchymal marker [17]	Desmin [17]
α-smooth muscle actin [18]	Myosin [19]
FSP1 (S100A4) [20]	n-caldesmon [21]
Neuronglial Antigen 2 (NG2) [22]	Smoothelin [23]
Chondroitin sulfate proteoglycans [22]	
PDGFR-β [22]	
FAP [24]	
Fibroblast associated antigen [25]	
Prolyl 4-hydroxylase [26]	
PDGFR-α [27]	

In normal physiology, myofibroblasts promote wound healing and contraction through traction forces on the ECM, secretion of remodeling enzymes such as matrix metalloproteinases (MMPs), production of growth factors like transforming growth factor-β 1(TGFβ-1), as well as deposition of ECM components such as collagens and fibronectin. Apoptosis of myofibroblasts, begins the completion of the healing process and allows for re-modeling of the tissue [28]. In pathological conditions such as fibrosis, this process is perturbed and myofibroblasts persist, continuing the healing process unnecessarily.

Many players are key in regulating the physiological activation of fibroblasts. These include TGFβ-1, which is necessary for the initiation of fibroblast activation. TGFβ-1 is also produced by myofibroblasts once they become activated, inducing the production of matrix collagens [29]. Alpha-smooth muscle actin expression is also induced upon fibroblast activation by TGFβ-1 and is required for the force generation needed for matrix collagen contraction [30]. Studies utilizing an amino terminal peptide of αSMA as an inhibitor, demonstrated that inhibition of αSMA also inhibited stretch mediated nuclear translocation of NFκB and subsequent downregulation of the promoter activity of connective tissue growth factor (CTGF) [31]. Contraction of the collagen matrix is mechanosensed by β1 integrins and normally downregulates the PI3K/Akt pathway leading to apoptosis of myofibroblasts. However, phosphorylation of focal adhesion kinase (FAK), in response to matrix contraction and integrin clustering, leads to activation of the PI3K/Akt pathway and protection of myofibroblasts from apoptosis [32]. These studies identify just a few regulators of fibroblast activation that work coordinately at various steps in the activation process. Uncovering how this system is deregulated in pathological settings may lead to therapeutic interventions for fibrosis and cancer. The studies described in this dissertation will

examine a novel role for a matrix metalloproteinase, MMP2, in the regulation of pulmonary fibroblast activation, and suggest how this is important during the outgrowth phase of breast cancer metastasis.

Myofibroblasts in breast cancer progression

Many of the same host responses that are initiated in wound healing are also active in tumors. In 1986, Harold Dvorak classically described tumors as "wounds that do not heal" upon observing characteristics in cancer similar to an impaired wound healing process [33]. These responses include an upregulation of cytokines and growth factors, the rapid proliferation of cells that are normally quiescent, intense remodeling of the extracellular matrix, the migration of epithelial and stromal cells, and the formation of new vasculature. The persistence of myofibroblasts is another feature common between tumors and non-healing wounds. Myofibroblasts present within and around tumors belong to a class of cancer associated fibroblasts (CAFs), which serve paradoxical functions in cancer, playing both tumor promoting and protective roles. Many functions of CAFs are exploited by tumor cells to promote tumor cell growth. Some of these functions include the production of multiple cytokines and growth factors, secretion of ECM proteins, stimulation of angiogenesis, and serving as guides for invading tumor cells to follow [8, 34–36].

Previous studies of breast cancer biology investigated the potential of conditioned media from fibroblasts to enhance tumor growth. In initial studies, unidentified fibroblast-derived soluble factors in the conditioned media were found to markedly increase tumor growth [37]. Later, stromal derived factor 1/chemokine (C-X-C motif) ligand 12 (SDF-1/CXCL12) was identified as

one of the CAF-produced factors that exhibited both endocrine and paracrine functions in breast cancer [38]. Orimo and colleagues found that SDF-1 from CAFs stimulated the mobilization of endothelial progenitor cells, leading to angiogenesis in addition to enhancing the growth of adjacent tumor cells. More recently, the expression of a clear pro-inflammatory gene signature in CAFs was described in breast cancer [39–41]. The inflammatory response incited by CAFs has been shown to be tumor-promoting, leading to angiogenesis and the recruitment of macrophages and endothelial cells in skin carcinoma, and is mediated by the NFκB pathway [42]. Clinically, the presence of abundant myofibroblasts is an indicator of poor prognosis in patients with invasive ductal carcinoma of the breast [43]. Patients with a higher content of myofibroblasts had higher grade tumors that were more proliferative with increased expression of the growth factors VEGF and bFGF.

In addition to using signaling proteins to enhance tumor growth, CAFs also regulate mechanical cues that mediate tumor expansion. CAFs generate tracks in the ECM that are followed by tumor cells as a sort of path to metastasis [44]. Crosslinking of ECM collagen by lysyl oxidase (LOX) expressed by activated fibroblasts creates a fibrotic environment that increases tumor persistence and survival, thereby enabling breast cancer metastasis [45, 46]. The desmoplastic nature of tumor ECM created by constant remodeling by CAFs can enhance tumor growth [47–49] through mechanisms that are being currently revealed. Importantly, increased CAF secreted collagen led to increased tumor cell proliferation and lung metastasis of mammary tumors [49], a finding that is of particular relevance to the studies in this dissertation.

In addition to promoting tumor growth in cancer, some studies have described a protective role for CAFs against tumors. Traditionally, quiescent fibroblasts are described to function in this

capacity[50]. Fibroblast specific protein 1 positive ($FSP1^+$) fibroblasts inhibited malignancy in a carcinogen induced fibrosarcoma model by depositing layers of collagen that encapsulated the tumors. Upon dispersal of this capsule using ganciclovir in FSP-TK transgenic mice, there was apoptosis of $FSP1^+$ fibroblasts and transformation of surrounding epithelial cells was rapidly induced, suggesting that this population of fibroblasts protected the epithelial cells from malignancy[51]. In a different study investigating pancreatic ductal adenocarcinoma (PDAC), the authors used ganciclovir treatment to ablate αSMA^+ CAFs in PKT; αSMA-TK mice ($Ptf1a^{cre/+}$/LSL-$Kras^{G12D}$/$TGF\beta^{flox/flox}$ (PKT) mice crossed to αSMA-TK mice). The PKT mouse model closely mimics the human form of PDAC, spontaneously developing pancreatic intraepithelial neoplasias and progressing to invasive PDAC. Ablation of αSMA^+ fibroblasts resulted in poorly differentiated tumors that were more invasive and necrotic, as well as suppressed immune responses. These effects collectively led to decreased survival in animals [52]. Additional pancreatic cancer studies targeting sonic hedgehog (Shh) in epithelial cells resulted in reduced stromal content. The tumors were poorly differentiated, highly proliferative, and showed increased vascularity [53]. The authors suggest that the stroma could limit tumor growth by restraining tumor vasculature.

A number of factors can account for the protective roles for CAFs noted in these studies, in opposition to the tumor-promoting roles discussed previously. Firstly, these studies occur in sites other than breast cancer, having distinct stromal microenvironments. Secondly, the protective phenotypes were a consequence of 1) CAF interactions with other, non-epithelial cell types, 2) targeting of a tumor derived factor, or 3) manipulations that originated before a tumor was present. Thirdly, in the CAF ablation studies, the heterogeneity of CAFs would subvert

most attempts to eliminate the total population. It is possible that eliminating subsets of CAFs may lead to more aggressive subsets remaining that effectively promote tumor progression. At present, functional and mechanistic roles for subsets of CAFs are still being uncovered.

Transforming growth factor beta 1 in breast cancer growth and progression

Cancer associated fibroblasts secrete multiple factors which promote tumor progression. These factors include SDF-1 [54–56], TGFβ-1 [57, 58], IGFs [59], and proteases [60, 61]. Their context dependent secretion leads to enhancement of tumor growth and survival mechanisms, or matrix degradation to enable metastasis. Because of the integral role that TGFβ-1 plays in the activation of fibroblasts, coupled with the importance of activated fibroblasts in breast cancer progression, this important cytokine will be discussed in more detail below.

TGFβ-1 signaling

Transforming growth factor beta 1 (TGFβ-1) is a pleiotropic cytokine with diverse effects in development and cancer. TGFβ-1 has been shown to regulate differentiation, migration, proliferation, and immune suppression in normal and cancerous cells of the tumor and stroma [62]. It is one of three TGFβ isoforms along with TGFβ-2 and TGFβ-3 that are a part of the larger TGFβ superfamily. TGFβ-1 signaling primarily occurs through the serine/threonine kinase receptors TGFβR1-3. Our focus is on canonical TGFβ-1 signaling and it will be briefly discussed followed by an overview of other non-canonical TGFβ signaling pathways.

Active TGFβ-1 binding to TGFβR2 results in homodimerization followed by recruitment of a TGFβR1 (ALK5) homodimer. TGFβR2 then phosphorylates TGFβR1, thus activating it and

initiating downstream signaling. The receptor complex phosphorylates regulatory Smads 2 or -3 (R-Smad2/3), mediating binding of the common Smad4. The R-Smad2/Smad 4 (or R-Smad3/Smad4) complex then translocates to the nucleus where it binds with transcriptional cofactors to the Smad binding element (SBE), initiating the transcription of target genes [63]. Additional Smad dependent signaling occurs with bone morphogenetic protein (BMP) ligands, which are dependent on Smads1/5/8 for transmission of intracellular signaling. BMPs bind a diverse set of receptor heterodimers: BMPs 9/10 bind BMP receptor 2 (BMPR-2) or activin receptor IIA (ACTRIIA) and serine/threonine protein kinase receptor 3 (ALK1); BMPs 2/4 bind BMPR-2 and ACTRI, BMPR-1A, or BMPR-1B; BMP7 binds BMPR2 or ACTRIIA and ACTRI, BMPR1A, or BMPR1B [64].

Heightened complexity is added to TGFβ superfamily signaling through non-canonical, Smad-independent pathways. TGFβ-1 activation of receptors (or activation by other superfamily members) can also regulate other intracellular pathways such as p38-MAPK, JNK, small GTPases, Erk, and PI3K-Akt [65]. These non-canonical pathways can be classified into three groups of Smad-independent proteins that 1) can modify Smad function, 2) have their function regulated by Smads and in turn signal to other pathways, or 3) directly interact with or are phosphorylated by TGFβ receptors without impacting Smad function. The functional significance of these Smad-independent pathways include cellular processes such as apoptosis, EMT, cell proliferation and differentiation, matrix regulation, and angiogenesis [66, 67].

TGFβ-1 activation

Before TGFβ-1 mediated receptor activation is initiated, the ligand itself must first be activated, as it is secreted in an inactive form. TGFβ-1 is synthesized intracellularly and released as a complex. The pro-form TGFβ-1 is bound with high affinity by the latency associated protein (LAP), collectively known as the small latent complex (SLC). The SLC is further bound by latent TGFβ binding protein (LTBP) via disulfide bonds to LAP. This SLC-LTBP complex is termed the large latent complex (LLC) [64]. Cleavage of LAP from TGFβ-1 in the Golgi by furin yields a non-covalent bond between the SLC peptides [68] (Figure 1.1; obtained from [64]). This produces the mature form LLC that is ready for secretion from the cell.

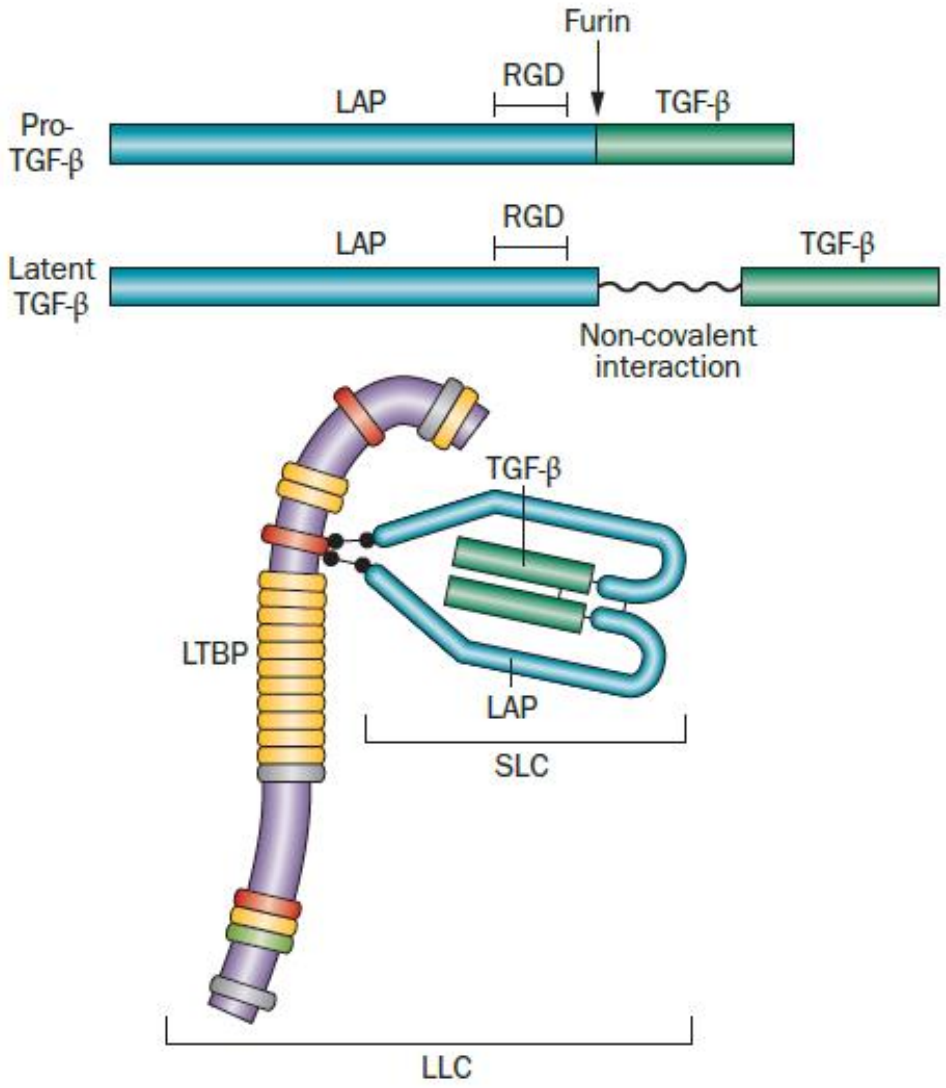

Figure 1.1: Latent TGFβ-1 ligand. TGFβ-1 is secreted from the cell in inactive form as part of a large latent complex with LTBP and LAP [64].

One of the many ways TGFβ-1 activity is regulated is through sequestration of the dimeric LLC to the ECM [69]. The LTBP fragment allows the complex to be anchored within the ECM to proteins such as fibronectin and fibrillin[70–73]. Release of TGFβ-1 dimer from the latency proteins activates the ligand so that it may initiate receptor mediated signaling. Many different sources have been identified as activators of TGFβ-1. Integrins bind the LAP of TGFβ-1 or TGFβ-3 due to the presence of the RGD integrin recognition motif [74]. The $\alpha_v\beta_6$ integrin activates both TGFβ-1 [75] and TGFβ-3 [76]. In the case of TGFβ-1, activation requires binding to fibronectin [77], and it is suggested that mechanical pulling releases TGFβ-1 from the LLC. The $\alpha_v\beta_8$ integrin also binds the LAP and is able to release active TGFβ-1 by the recruitment of MT1-MMP, which cleaves LAP [78].

Other proteases have also been implicated in the release of active TGFβ isoforms from the LLC. The docking of matrix metalloproteinase 9 (MMP9) or MMP2 by cell surface protein CD44 cleaves LLC, thereby releasing active TGFβ-2 or -3 [79]. Bone morphogenetic protein 1-like metalloproteinase (BMP-1), a non TGFβ superfamily protein, frees the LLC from the extracellular matrix by cleaving LTBP1. Subsequently, active TGFβ-1 is released by cleavage of LAP by MMP2 [80]. MMP2 was also shown to increase the bioavailability of active TGFβ-1 by the cleavage of LTBP3 [81].

Mechanical factors also have the capability to activate TGFβ-1. As discussed earlier, integrin mediated release of active TGFβ-1 involved the use of traction forces to pull apart the latency complex. Additionally, traction forces are exerted on the LLC complex from myofibroblast contraction of matrix. This results in stiffening of the matrix and subsequent release of active TGFβ-1, a mechanism that is dependent on $\alpha_v\beta_5$ integrins [82]. This particular mode of

activation can exacerbate such conditions as fibrosis and cancer where fibroblasts respond to activated TGFβ-1 by increasing ECM production and matrix contraction, leading to a stiffer matrix and potentially more activated TGFβ-1.

Opposing effects of TGFβ-1 in breast cancer

TGFβ-1 has paradoxical effects on tumor progression, acting as a tumor suppressor during early stages and a tumor promoter in later stages. The tight control of TGFβ-1 activity in the normal mammary gland coupled with the sensitive nature of normal mammary cells to TGFβ-1 signaling results in a cytostatic response of normal and early tumor cells to cancer. This was first evidenced by the involution of terminal end buds upon TGFβ-1 treatment during normal mammary development [83]. However, cancer cells evade normal responses, including those to TGFβ-1, and assume new regimens, resulting in tumor enhancement. Thus, previously antitumorigenic signals can become exploited and subsequently used as protumorigenic mechanisms.

Most of the cytostatic responses of cells to TGFβ-1 occur through canonical signaling [84] and are highly dependent on the signaling and transcriptional programs already in place within a given cell. Early studies revealed that the cytostatic program was a result of downregulation of c-Myc [85, 86] or induction of cell cycle inhibitors such as p21 or p15INK4B in epithelial cells [87, 88]. These inhibitors are also involved in G1 phase growth arrest of various other cell types such as T-cells, astrocytes, and neural progenitor cells [89]. In fibroblasts, TGFβ-1 encouraged proliferation associated with estrogen receptor (ER) induction of c-Myc [90]. Conversely,

TGFβ-1 from mammary epithelial cells acted to suppress proliferation in ER positive cells during estrus [91].

Phenotypic changes occur in epithelial cells upon the loss of normal response to TGFβ-1 signaling. Epithelial to mesenchymal transition (EMT) is induced in a number of models upon TGFβ-1 activation. TGFβ-1 signaling in epithelial cells directed single cell migration, while the loss of signaling due to TGFβR2 knockout resulted in collective cell migration [92]. Importantly, these studies also accounted for tumor cell-fibroblast crosstalk, as the tumoral effects were promoted by stromal fibroblasts. Metastatic MDA-MB-231 breast cancer cells that have activated TGFβ-1 show increased tumor cell proliferation and angiogenesis [93]. This was found to be due to Smad independent, MEK-ERK dependent induction of MMP9, which increased invasion as well as angiogenesis. Tumor survival is also enhanced by activated TGFβ-1 signaling in epithelial cells [94]. Clinically, the loss of TGFβR2 in breast cancer cells was associated with increased metastasis to lung and bone and predicts poor disease free outcome in patients [63, 95].

While it is important to understand the impact of TGFβ-1 on tumor cells themselves, it is equally important to consider the effects of TGFβ-1 on the tumor stroma. Because of the interdependency of tumor cells with the tumor microenvironment, misregulation of TGFβ-1 signaling in stromal cells can have adverse paracrine effects on tumor progression even when tumor cells themselves have lost responsiveness to TGFβ-1[96]. An earlier section previously discussed autocrine effects of TGFβ-1 regulation in fibroblasts. However, altered signaling in fibroblasts has many paracrine effects on breast tumor cells and a host of other epithelial cancers [57, 97, 98]. For example, activation of TGFβ-1 signaling in fibroblasts leads to a desmoplastic

stroma which can increase breast cancer risk [99]. However, it is important to also note that loss of TGFβ-1 signaling in fibroblasts is associated with a more invasive tumor phenotype. This invasive tumor phenotype was mostly attributed to increased inflammatory infiltration into the tumor as a result of the loss of TGFβ-1 signaling in fibroblasts, which resulted in increases of the inflammatory chemokines CXCL12 and CCL2[100].

These studies demonstrate how paradoxical modulations of TGFβ-1 signaling in fibroblasts can result in tumor progression, even if by indirect means. They stress the importance of determining key molecules secreted by fibroblasts, as well as other stromal cells, that encourage tumor growth and the mechanistic details of this process. Lastly, the studies discussed in the previous paragraphs illustrate how TGFβ-1 signaling in fibroblasts leads to increased production of other molecules that may have a direct impact on tumor growth. The next section focuses on MMP2, which is also secreted by activated fibroblasts and assists in TGFβ-1 bioavailability. Details of the roles of MMP2 in tumor progression will be addressed below.

Matrix metalloproteinases and tumor expansion

Introduction to MMPs and disease

Matrix metalloproteinases (MMPs) are a family of 24 zinc-dependent endopeptidases with multiple roles in development and pathological diseases. As the name suggests, MMPs are traditionally known for their abilities to cleave most components within the ECM (Table 2). MMPs are classified into eight groups according to their structural architecture, which allows them to share functionalities [101]. The basic structure of MMPs includes a pre-domain, a pro-

domain containing a zinc-interacting thiol group, and a catalytic domain with a zinc binding site [102]. These structural domains are important in maintaining the activity of MMPs.

TABLE 2: MATRIX METALLOPROTEINASES AND PROTEIN SUBSTRATES

COLLAGENASES	
MMP-1	Collagens I, II, III, VII, VIII, X, and XI, gelatin, Clq, entactin, tenascin, aggrecan, link protein, fibronectin, vitronectin, myelin basic protein, α_2-macroglobulin, ovostatin, α_1-proteinase inhibitor, α_1-antichymotrypsin, IL-1β, proTNF- α, IGFBP-3, casein, proMMP-2, proMMP-9
MMP-8	Collagen I, II, and III, Clq, aggrecan, α_2M, ovostatin, α_1Pl, substrate P
MMP-13	Collagen I, II, III, IV, IX, X and XIV, gelatin, collagen telopeptides, Clq, fibronectin, SPARC, aggrecan, α_2M, casein
MMP-18	Collagen l, gelatin
GELATINASES	
MMP-2	Collagen I, III, IV, V, VII and X, gelatin, fibronectin, laminin, aggrecan, link protein, elastin, vitronectin, tenascin, SPARC, decorin, myelin basic protein, α_1Pl, α_1-antichymotrypin, IL-1β, proTNF- α, IGFBP-3, substance P
MMP-9	Collagen IV, V, XI, XIV, elastin, aggrecan, link protein, decorin, laminin,entactin, SPARC, myelin basic protein, α_2M, α_1Pl, IL-1 β, proTNF-α, substrate P, casein
STROMELYSINS	
MMP-3	Collagen III, IV, V, IX, X and XI; teropeptides (collagen I and II), gelatin, aggrecan, link protein, elastin, fibronectin, vitronectin, laminin, entactin, tenascin, SPARC, decorin, myelin basic protein, α_2-macroglobulin, ovostatin, α_1-Pl, α_1-antichy- motrypsin, IL-1β, proTNF- α, IGFBP-3, substance P, T kininogen, casein, proMMP-1, proMMP-3, proMMP-8, proMMP-9
MMP-10	Collagen III, IV and V, gelatin, fibronectin, elastin, aggrecan, link protein, casein, proMMP-1, proMMP-7, proMMP-8, proMMP-9
MMP-11	Collagen IV, gelatin, fibronectin, laminin, aggrecan, α_1Pl, α_2M
MATRILYSINS	
MMP-7	Collagen IV, gelatin, aggrecan, link protein, elastin, fibronectin, vitronectin, laminin, SPARC, entactin, decorin, myelin basic protein, tenascin, fibulin-1 and α2, proTNF-α, casein, α_1-Pl, proMMP-1, proMMP-2, proMMP-9
MMP-26	Collagen IV, gelatin, fibronectin, vitronectin, Pro-α-defensin, Fas ligand, β4-integrin, E-cadherin, α_2M, α_1Pl, fibrinogen, proMMP-9
MT-MMPS	
MMP-14	proMMP-2, Collagen I, II, and III; gelatin, fibronectin, vitronectin, laminin, entactin, aggrecan, α_2M, α_1Pl, proTNF- α, decorin
MMP-15	ProMMP-2, laminin, fibronectin, tenascin, entactin, aggrecan, perlecan, proTNF- α
MMP-16	ProMMP-2
MMP-24	Gelatin, proMMP-2
OTHERS	
MMP-12	Elastin, collagen IV, gelatin, fibronectin, vitronectin, laminin, entactin, aggrecan, myelin basic protein, α_2M, α_1Pl, proTNF-α
MMP-19	Gelatin, large tenascin C, aggrecan
MMP-20	Amelogenin
MMP-22	Casein, gelatin
MMP-23	Autoproteolysis of proMMP-23, Mca-peptide
MMP-25	Collagen IV, gelatin, fibronectin, chondroitin sulphate proteoglycan, dermatan sulphate proteoglycan, fibrinogen, fibrin, α_1Pl, proMMP-2
MMP-17 AND MMP-21 HAVE UNKNOWN SUBSTRATES. REFERENCES: [102–104]**.**	

The interaction of the catalytic domain zinc ion and the thiol of the pro-domain keeps the enzyme in a locked, inactive conformation. However, cleavage of the pro-domain by furin or other proteases, activates the enzyme, enabling it to exert its proteolytic functions [105]. Because of their potential to cleave a plethora of substrates, most MMPs are secreted from the cell as zymogens with the exceptions of the membrane type, MT-MMPs. Activated MMPs are tightly regulated by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) [106]. The delicate balance between MMPs, TIMPs, and activating mechanisms prevent the excessive processing of MMP substrates.

In addition to matrix degradation, other normal functions of MMPs include wound healing, post-partum involution, bone remodeling, and release of active growth factors from the extracellular matrix [103]. Even though there are several physiological functions for MMPs, their roles in disease are more widely studied. Unmitigated MMP activity leads to tissue damage associated with fibrosis, chronic inflammation, and cancer [107, 108]. Of particular interest to the studies herein are the roles of MMPs in cancer. MMPs activate growth factors such as TGFβ1 [79, 89] which, as discussed previously, has varying effects on multiple cell types with the net effect resulting in tumor progression. MMPs help cancer cells to evade apoptosis by cleaving ligands that initiate pro-apoptotic signaling [109]. MMPs are required for tumor cells to initiate angiogenesis [110, 111] and maintenance of lymphatic vasculature [112]. In line with their classical functions, MMPs mediate migration and invasion of tumor cells into the surrounding ECM by the cleavage of matrix proteins [113, 114]. MMPs also promote cancer metastasis by establishment of a premetastatic niche [115]. In addition, MMPs play important roles as regulators of the bioactivity and bioavailability of inflammatory mediators such as TNF-α and

IL1-β [116]. Overall, MMPs have numerous roles to play in cancer progression from the very earliest stages of initiation [117] through metastasis, and new functions are still being uncovered [118]. In this work, we will focus on specific roles for MMP2 in the process of metastatic outgrowth in the lung microenvironment.

Although several MMPs have been associated with disease progression in numerous animal models, MMP inhibitors failed to reduce tumor growth in human clinical trials. This was due to a combination of many different factors, including target specificity, understanding of target biology, possible off-target effects of drugs, and dosing issues [119–121]. The trials utilized broad spectrum MMP inhibitors (MPIs) that were aimed at the activity of many different MMPs simultaneously. In retrospect, this posed multiple problems in itself. Since MMPs have both pro-tumorigenic [122, 123] and anti-tumorigenic effects [124–127] their mass inhibition was ill advised. Additionally, blockade of MMP activity does not lead to shrinkage of tumor size since MPIs are cytostatic, not cytotoxic. The treatment of late-stage patients was inappropriate given that MMPs function earlier in tumor progression. Furthermore, the debilitating side effects of MPIs, namely musculoskeletal syndrome (MSS), meant that many patients took less than optimal dosages of inhibitors. It is not known whether these reduced dosages were robust enough to be efficacious. Therefore, inadequate enzyme inhibition may have contributed to the fact that there was no significant difference between treatment and control groups. For the successful use of MPIs as a therapeutic intervention, more clearly defined roles of specific MMPs in tumor progression is warranted.

Fibroblast MMP2 implications in cancer

After the failure of broad-spectrum MMP inhibitors to reverse tumor growth in clinical trials, there was a resurgence in research focused on identifying distinct roles for MMPs with respect to cellular context. The growth of knowledge in tumor-stromal interactions perpetuating tumor growth and metastasis, helped to fuel this cell-specific approach to investigating contributions of individual MMPs. In the work presented within this dissertation, we focus on MMP2 from stromal fibroblasts, a major cell source for this enzyme, and investigate its impact on breast tumor growth.

MMP2, along with MMP9, are members of the gelatinase subfamily of MMPs, commonly named for their substrate specificity. In addition to the basic structure of MMPs, gelatinases contain three fibronectin-like repeats in the catalytic domain that mediate binding to ECM. They also contain a hemopexin-like domain connected by a hinged region that mediates anchoring to TIMPs [128]. This TIMP binding region becomes especially important in the activation of MMP2 as it involves assemblage of a protein complex involving TIMP2 and MMP14 on the cell surface [129].

MMP2 has important roles in cancer, and particularly involving fibroblasts. MMP2 was observed in the stromal cells of mammary and melanoma tumors in experimental metastasis models [130]. Studies using *in situ* hybridization revealed that MMP2 mRNA is localized to the fibroblast compartment in breast cancer tissue [131]. Co-culture of breast cancer cells and fibroblasts enhances MMP production, including active MMP2, in fibroblasts [132]. Conditioned medium from fibroblasts was able to enhance tumor growth, suggesting that a

soluble factor was involved [133]. The inhibition of MMP2 in fibroblasts abolishes pro-tumorigenic effects in nude mice [134]. Finally, global knockout of MMP2 in C57BL/6 animals resulted in a decrease in both lung metastases and angiogenesis as compared to wild type animals using Lewis lung carcinoma cells[135].

Factors secreted from fibroblasts can cooperate to enhance tumor growth. This is exemplified by MMP2 modulation of TGFβ-1 activity via cleavage of LTBP1 [136, 137] and LTPB3[81], enhancing TGFβ-1 bioavailability and supporting tumor growth. Additional studies also found that MMP2 proteolytically activates TGFβ-1 [78]. At the conclusion of this dissertation, a model is presented which depicts the potential importance of fibroblast-secreted MMP2 on the enhancement of breast tumor outgrowth (Figure 4.1A). The model outlines how tumor fibroblasts, which secrete MMP2, are activated by TGFβ-1, which requires MMP2 for its bioavailability. The activated fibroblasts then release increased matrix collagens and/or cytokines, which may both, independently or cooperatively, enhance tumor outgrowth.

Despite the multiple studies mentioned above linking MMP2 expression to breast cancer metastasis, and evidence from mouse models that the lack of MMP2 impedes metastasis, the precise mechanism involved is unclear. The goal of this work is to delineate mechanistically how MMP2 promotes breast-to-lung metastasis. Specifically, I will test the hypothesis that MMP2 dependent functions of cancer associated fibroblasts (CAFs) cause alterations in the tumor microenvironment that promote metastatic outgrowth of breast tumors in the lung.

In order to examine MMP2's roles in the appropriate context, a 3-dimensional co-culture system was first required. The establishment of such a system is described in chapter II. The findings

regarding MMP2 function are presented in chapter III. In the final chapter, implications of the work and possible future directions for these studies are discussed.

CHAPTER II

Using three-dimensional cultures to mimic the tumor microenvironment

Introduction

Like many other studies of the human body, studies of the tumor microenvironment have typically taken a reductionist approach. Reductionism is the idea that a complex system is equal to the sum of its parts [139]. With regards to the tumor microenvironment, the most simplistic application of this approach involves separating out the different components of a tumor and examining their individual functions on artificial surfaces. Incorporation of other factors and cell types enable additional layers of complexity while maintaining a relatively simple system. As a result, the simplicity and availability of two-dimensional (2D) assays has allowed elucidation of key cellular interactions and functions of tumor components. It has become increasingly evident, however, that despite its advantages, the use of 2D systems also has its limitations.

A major caveat to the use of 2D systems is that they do not physically recapitulate *in vivo* networks [140]. In stark contrast to *in vivo* systems, 2D growth consists of monolayers of cells grown on non-pliable, plastic surfaces. *In vivo*, tumors are a network of multiple cell types that constantly receive spatio-temporal cues that cannot be reproduced on flat surfaces or without signaling factors or mechanical forces. The resultant morphology of cells grown in 2D is changed and affects cellular polarity [141] as well as responses to certain signals. Cells grown in 2D have a single surface in contact with the plate forcing those cells to grow flat and have apical-basal polarity. While this polarization is appropriate for normal *in vivo* epithelial morphology, flattened cell shape is not. For example, *in vivo,* epithelial cells of the mammary gland assemble

to form an acinar structure with a central, patent lumen surrounded by luminal epithelial cells. This spheroidal structure is surrounded by myoepithelial cells and encapsulated with a basement membrane [142]. Mammary tumors *in vivo* maintain a somewhat spheroidal geometry, sometimes with finger-like projections, and polarity is lost. *In vitro,* normal acini are recapitulated when mammary gland cells are placed in ECM, reminiscent of a 3D environment [141, 143, 144]. Likewise, tumors cultured in a 3D matrix grow as a bolus of cells or cell aggregates.

Cellular adhesions are necessary for correct shaping of cell morphology. Beningo and colleagues [145] demonstrated that fibroblasts in culture assume a stellate formation, similar to their *in vivo* structures, when they are sandwiched between two layers of ECM as opposed to the elongated shape found when grown atop a single 2D surface. The two layers of ECM allow integrin-mediated adhesions to form all around the cells. Integrin-mediated adhesions have been shown to affect cell proliferation [146], differentiation [143], survival [32], and gene expression [147–149].

The non-pliable plastic or glass surfaces commonly used in 2D assays are much more rigid than what normal epithelial cells or fibroblasts *in vivo* would encounter. Like integrin-mediated adhesions, matrix rigidity regulates such cell functions as proliferation [150], migration [151], gene expression [152] and cell differentiation [153]. Tension from the ECM influences mechanical forces inside the cell to regulate these functions. Actin based contractile forces formed within the cell sense the resistance of the matrix in a process known as mechanosensing and exert forces on the ECM as traction stresses in response [154]. As a result, high matrix stiffness is sensed as increased resistance, leading to stronger traction stresses and altered cell

function [155]. The incorporation of ECM and/or synthetic gels into traditional 2D models have allowed scientists to create surfaces with variable stiffness while maintaining uniform chemical properties, in contrast to single strength glass or plastic surfaces. Using this approach, Pelham and colleagues found that fibroblasts placed on stiff surfaces take on a spread morphology with large, stable focal adhesions compared to small, dynamic adhesions and less spread morphology formed on softer gel surfaces [156]. In cancer, a stiff matrix can encourage the clustering of integrins, which then enhances ERK signaling and ROCK-dependent contractile forces within the cell [157] leading to enhanced tumor cell growth and disrupted epithelial morphogenesis.

Finally, in 2D settings, soluble factors are freely diffusible. By contrast, *in vivo* the presence of ECM creates a gradient of these factors, imposing time and physical constraints on their access to cells. The use of 3D models that include an ECM can introduce an artificial gradient that mimics these physiological settings. The gradient produced by ECM can affect the time it takes for growth factors to reach target cells as well as the level of resultant signaling [158, 159]. Additionally, presence of ECM can sequester important growth factors such as TGFβ-1 and IGF ligands. *In vivo*, TGFβ-1 was found to bind collagen IV within the basement membrane [160]. Subsequent release of TGFβ-1 from the matrix by cleavage of latency peptides allows for localized activation and increases its bioavailability [64, 161], which can lead to increased cell growth, as in cancer [89]. Similarly, intact reservoirs of IGF-1 are created by sequestration of its binding partners to the matrix, yielding context dependent pro-tumorigenic or anti-tumorigenic effects [162].

The use of 3D models can ameliorate many caveats of simple 2D cultures by providing a more physiologically relevant setting while maintaining a level of simplicity. Though not a full

recapitulation of *in vivo* physiology, the added dimension by inclusion of ECM and its associated adhesive, mechanical, and chemical features renders a reductionist approach more reflective of natural biology. Furthermore, 3D models allow for the analysis of matrix degradation through the actions of proteases as demonstrated by members of the Sloane group [163–165]. While it is important to understand the impact of single alterations in mechanotransduction, it is their concerted effects that truly mimic physiological phenomena.

In this chapter, 3D culture methodologies are used to mimic the tumor microenvironment with respect to tumor growth and proteolysis. The advantages and disadvantages of examining cell growth in 2D are explained and support for the use of 3D assays is given. These studies utilize cells derived from the MMTV-PyVT transgenic mouse model of breast cancer [166] crossed onto the FVB background. In this model, the polyomavirus middle T oncogene is expressed under control of the MMTV promoter and leads to the spontaneous development of mammary gland tumors. This is an ideal model to use since the tumors that develop closely mimic human disease and share tumor biomarkers and histological features [167] as well as metastasis genes [168]. These assays are used to investigate how tumor-stromal interactions enhance *in vitro* tumor growth rate. These studies will provide insight into the how the tumor microenvironment influences tumor growth *in vivo.*

Materials and Methods

Cells and cell culture. R221A cells are tumor cells isolated from spontaneously developing mammary gland tumors of FVB PyMT transgenic mice [138]. CAFs derived from Neu mouse mammary tumors using a similar approach to that found in Cheng et al 2005 [100] were a kind

gift from the laboratory of Dr. Harold Moses, Vanderbilt University. Cells were maintained in DMEM supplemented with 10% FBS and 10µg/ml gentamicin at 37ºC in 5% CO2.

mCherry labeling of tumor cells. Lentiviral mCherry transduction particles (5µl, LP-MCHR-LV105-0205; GeneCopoeia Rockville, MD) were used to infect parental R221A cells following manufacturer's recommendations.

3D cultures. MatTek dishes were pre-coated with basement membrane extract (BME; Cultrex Trevigen, Gaithersburg, MD). Cells were pelleted then resuspended in 300µl BME and allowed to solidify at 37ºC. Media was added to cultures after matrix fully polymerized. For DQ-substrate cultures, 100µl volumes of BME+2.5% DQ substrate were overlayed onto MatTek dishes pre-coated with the same and allowed to polymerize. A 50µl volume of cells was seeded onto each matrix for 30-45 minutes at 37ºC. Once complete seeding is achieved, the culture was overlayed with medium containing 2% of the BME/DQ-substrate mixture. Media were changed every other day.

Quantitative real time RT-PCR. RNA was isolated from fibroblasts using TRIzol reagent (Life Technologies, Grand Island, NY) for cell lysis followed by chloroform phase separation. The RNA containing aqueous phase was then used with the RNeasy mini-prep kit (Qiagen, Valencia, CA) for purification following manufacturer's instructions. Purified RNA was submitted to the Vanderbilt Functional Genomics Shared Resource (FGSR) for quantitative RT-PCR using TaqMan readily available primers.

Gelatin zymography. Conditioned media from cells was collected after 48 hours in serum free media and centrifuged to remove cellular debris. Serum was collected from WT or $MMP2^{-/-}$

mice on the FVB background. The MMP2$^{-/-}$ mice originated on a C57/Bl6 background [169] and demonstrate skeletal defects such as modest shortened body length, abnormal cranio-facial development, and reduced bone density along with a slight decrease in overall body size which persists [170, 171]. There are no other significant differences compared to their WT littermates. Upon crossing onto the FVB background, there are no overt differences between WT and MMP2$^{-/-}$ mice. Animals were maintained in the Vanderbilt Animal Housing facility and all mouse work was conducted only after review and approval by the local institutional care and use committee.

Non-reduced conditioned media and serum were loaded onto 10% SDS substrate gels containing 4% gelatin. Removal of SDS from the gels was achieved by rinsing twice with 2.5% Triton X-100 for 15 minutes. Gels were incubated in substrate buffer (50mM Tris-HCl, pH7.6; 10mM $CaCl_2$) with or without 20mM EDTA overnight at 37^{o}C. The following morning, gels were stained with 0.5% Coomassie Blue in 50% methanol/10% acetic acid until gels were a dark blue. Destaining with 50% methanol/10% acetic acid was performed followed by a secondary destain in water.

Knockdown of MMP2. Lentiviral shRNA transduction particles to MMP2 gene and control particles were obtained from Sigma (Mission TRCN0000031224, TRCN0000031226, and SHC001V PLKO.1-Puro control; St. Louis, MO), and were used to infect parental mammary tumor derived fibroblasts from mice following manufacturer's recommendations. Each shRNA particle was used independently. Successfully infected cells were selected by culturing in the presence of selection medium (DMEM/gentamicin 10µg/ml puroymcin). Knockdown was

confirmed by western blotting according to normal protocol. Multiclonal populations were used for subsequent experiments.

Statistical analysis. Linear regression was used to compare growth rates. To compare two groups, a Student's t-test was used for parametric analyses and Mann-Whitney for non-parametric analyses. Statistical significance was considered $p<0.05$.

Results

Growth of tumor spheroids embedded within a 3D matrix. Because of the different growth patterns of cells grown in 2D vs 3D, we wanted to investigate the effect of 3D conditions on tumor cells. Using a protocol adapted from Lee and colleagues [172], we compared the differences in morphology between R221A cells grown on tissue culture plastic and those embedded within reconstituted basement membrane extract (BME). Cells grown on tissue culture plastic did not assume a spheroid morphology, but rather formed a flattened geometry as a monolayer of cells (Figure 2.1B). In areas of tight confluency, the cells began to grow on top of each other while maintaining this flattened morphology. Figure 2.1A shows a schematic of the process of embedding cells within BME. When cells were grown in 3D, they formed multiple spheroids from individual cells that increased in size over time (Figure 2.1C).

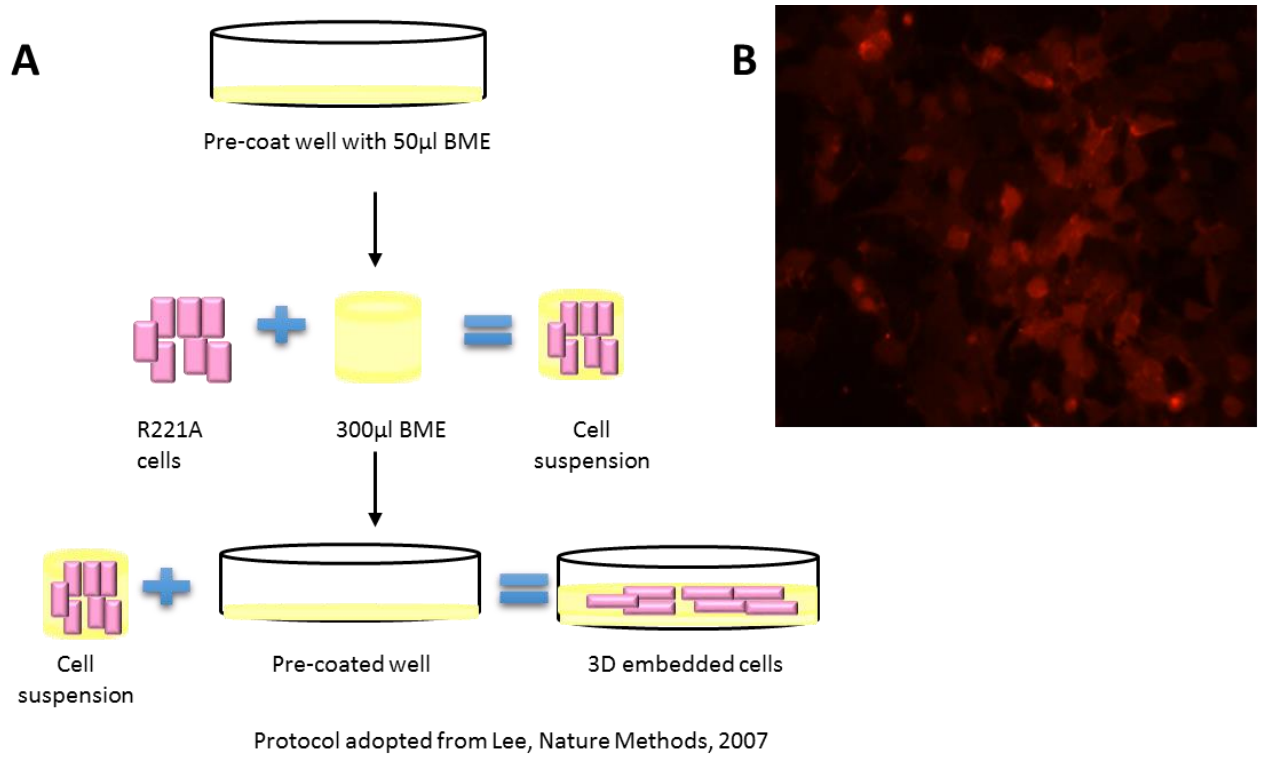

Figure 2.1: Growth of tumor spheroids embedded within a 3D matrix. (A) Schematic of 3D embedded culture of R221A cells (B) mCherry R221A cells cultured on tissue culture plastic. (200x magnification) (C) R221A cells form spheroids by Day 7 of culture when embedded within a 3D reduced growth factor matrix. Spheroids continue to grow in size through Day 14 of culture and beyond.

Many of the spheroids did not have smooth rounded surfaces, but rather developed fingerlike projections that invaded further into the matrix.

Investigation of MMPs expressed by R221A cells. We observed the invasion of R221A cells into the surrounding matrix, an event largely attributed to matrix metalloproteinases (MMPs) *in vitro* and *in vivo* [101, 173, 174]. Therefore, we next wanted to investigate the expression of MMPs by R221A cells. We found that a number of MMP gene transcripts are expressed to differing levels (Figure 2.2A). Because MMP2 and MMP9 have previously been shown to actively degrade ECM surrounding breast cancer cells in 3D [175], we wanted to investigate if our cells also express pro-and active proteins of MMP2 and MMP9. Indeed, our cells secrete both pro-MMP9 and pro-MMP2 into conditioned media (Figure 2.2B).

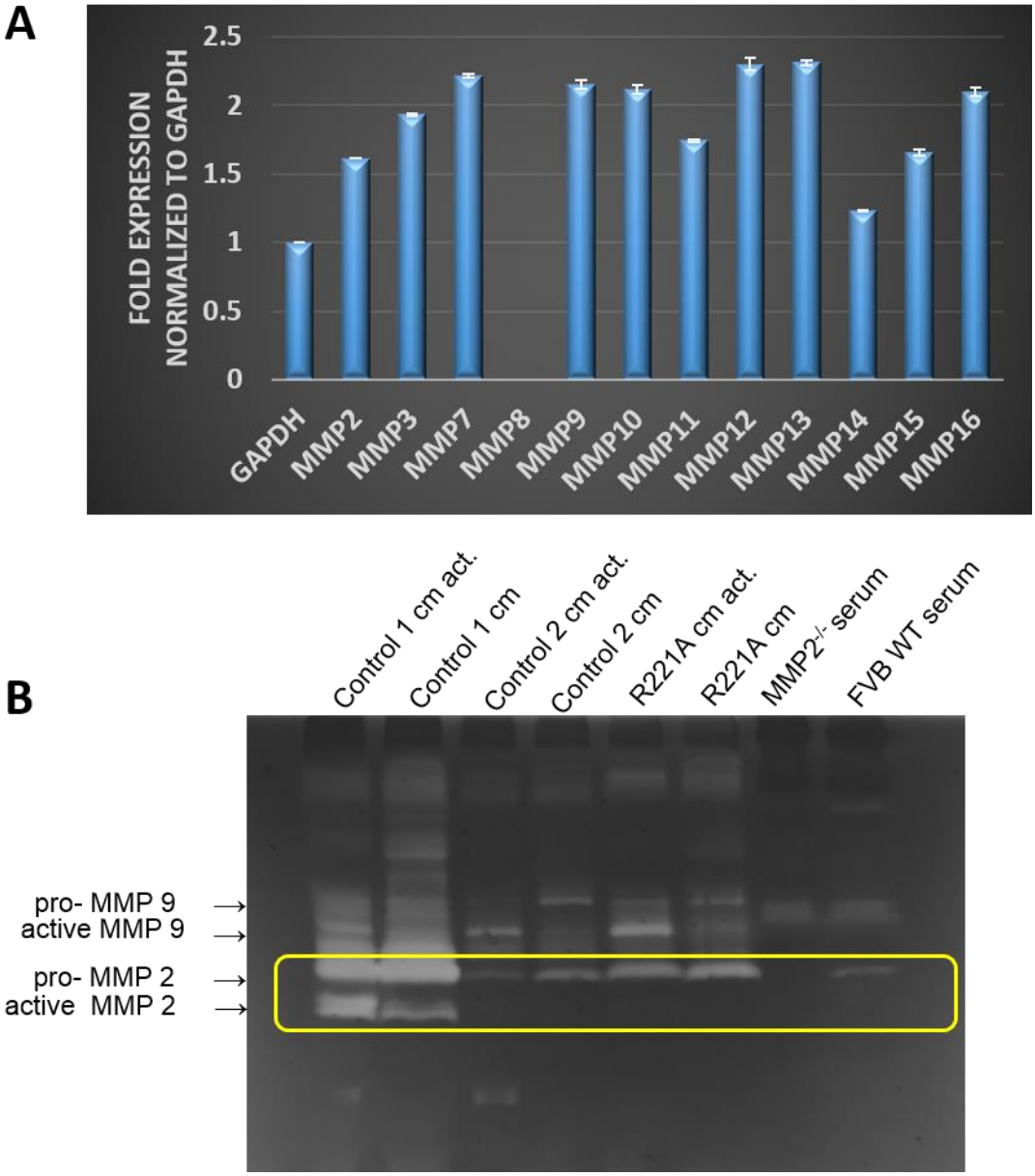

Figure 2.2: Matrix metalloproteinase expression profile of R221A cells. (A) RNA from tumor cells was extracted followed by standard cleanup. Samples were submitted to Vanderbilt Functional Genomics Shared Resource for qRT-PCR. Bars represent fold change in gene expression relative to GAPDH. (B) Zymography of conditioned media from APMA activated or naïve control or R221A tumor cell lines. Serum from FVB WT or MMP2-/- mice were used to confirm bands for pro-MMP2. act.=activated; cm=conditioned medium

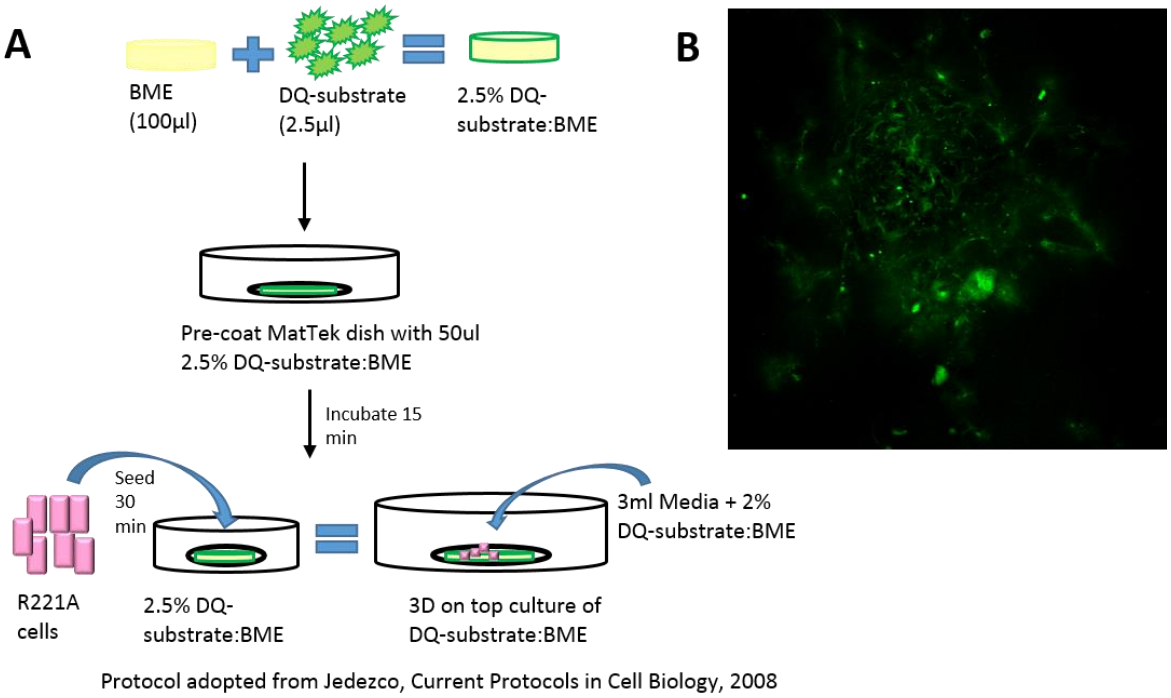

Figure 2.3: Assessment of tumor cell proteolysis using 3D cultures. (A) Schematic of 3D embedded culture of R221A cells in DQ-collagen IV (B) 3D reconstruction of an R221A spheroid embedded in BME/DQ-collagen IV. (400x magnification)

Tumor cell proteolysis in 3D. Given the apparent production of MMPs, we next utilized the dye-quenched (DQ) substrate system to test whether we could visualize active proteolysis by the tumor cells within a 3D matrix. In this system, a substrate (gelatin or collagen) is heavily conjugated to FITC fluorophores. The fluorescence signals of the fluorophores are self-quenched because of their close proximity to each other due to a Forster resonance energy transfer (FRET) effect. However, upon cleavage by a protease, the fluorophores are allowed to separate, relieving the self-quenching, and emit a green fluorescence. Using a protocol adapted from Jedezco and colleagues [176] (Figure 2.3A) with DQ-collagen IV in BME, we were able to examine active proteolysis of the ECM by our tumor cells. Figure 2.3B shows a 3D reconstruction of a spheroid z-stack with active proteolysis visible around the spheroid and along the fingerlike projections.

Contribution of fibroblasts to proteolysis. In breast tumors, fibroblasts have been suggested as the most prominent producer of MMP2 [131]. Additionally, fibroblasts make up a large part of the stromal milieu which supports tumor growth *in vivo.* We wanted to create a model more representative of a true physiology, so we began by examining murine mammary gland fibroblasts for production of MMP2 as an endogenous source of enzyme. To check the secretion of MMP2 by these fibroblasts, we used gelatin zymography. Figure 2.4A shows that fibroblasts secrete not only pro-MMP2, but also active MMP2. There was also more MMP2 produced by fibroblasts than by tumor cells. Co-culture of fibroblasts and mCherry R221A cells resulted in spheroid formation by day 7 similarly to tumor cells alone. The generation of active MMP2 by fibroblasts led us to test whether MMP2 was the dominant contributor of proteolytic activity from co-cultured spheroids, using a co-culture system set up similarly to the method in Figure 2.3A. To test the contribution specifically of MMP2, we incorporated a MMP2-nuetralizing antibody into the culture. As shown in Figure 2.4B, there was detectable fluorescence from spheroids in the presence of a control antibody. However, upon the treatment of these cultures with the neutralizing antibody to MMP2, proteolysis was significantly reduced.

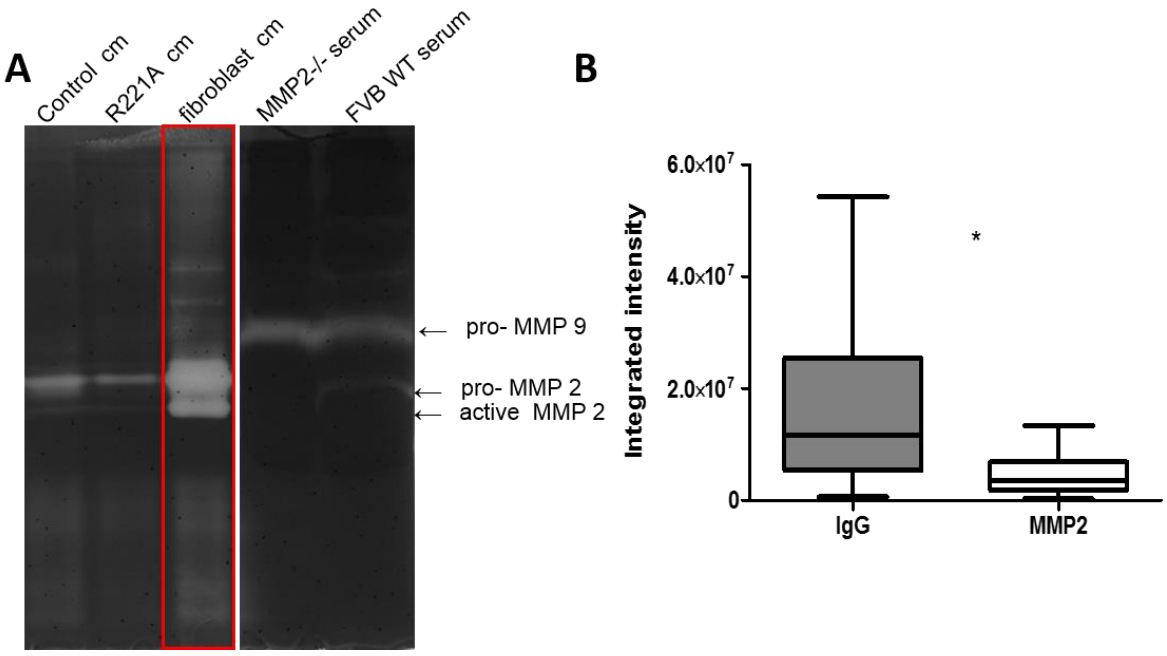

Figure 2.4: **Analysis of fibroblast MMP2 and effect on tumor associated proteolysis.** **(**A) Zymography of conditioned media from control tumor, R221A tumor, or murine mammary fibroblast cell lines. (B) Co-cultures of fibroblasts and mCherry labeled R221A spheroids treated with MMP2 neutralizing antibody or an IgG control. Proteolysis by tumor spheroids measured by analyzing integrated intensity of spheroid z-stacks. Box and whisker plot of 25th and 75th percentiles with standard deviation, p=0.014.

MMP2 is important for tumor cell proliferation in 3D but not 2D. Fibroblast MMP2 has previously been described to encourage the growth of tumor cells *in vivo* [134, 177]. Those studies employed conditioned media from fibroblasts, which also contains multiple other factors that could influence tumor growth independent of MMP2. We wanted to confirm an MMP2-dependent mitogenic phenotype *in vitro* by first testing the impact of exogenously added active MMP2 in 2D and 3D (Figure 2.5). Upon treatment of R221A cells with 20ng/ml recombinant human MMP2 (rhMMP2) in 2D and measuring the percent of cells in synthesis phase, there was no significant difference between vehicle and MMP2 treated groups (Figure 2.5A). However, when tumor cells were grown as spheroids within extracellular matrix in 3D hanging drop plates, measurement of total DNA content revealed a significant increase in tumor growth in 3D (Figure 2.5B). This suggests that MMP2 protein does enhance tumor growth, but requires a 3D environment.

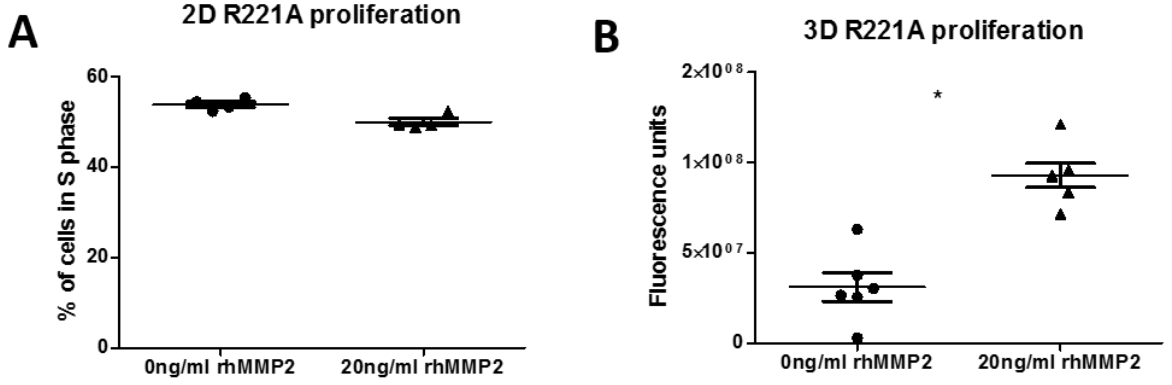

Figure 2.5: Active MMP2 enhances the proliferation of mammary cancer cells in 3D but not 2D. (A) EdU incorporation of R221A cells plated in glass chamber slides and treated with vehicle or 20ng/ml active recombinant MMP2. (p=0.057) (B) Total DNA content of R221A cells plated in hanging drop plates with 2% BME and treated with vehicle or 20ng/ml active MMP2**.** p=0.0001

Fibroblast MMP2 increases tumor growth in 3D. We have shown in Figure 2.4A that mammary derived fibroblasts express both pro and active MMP2. Therefore, we wanted to test if fibroblast derived MMP2 enhanced tumor growth *in vitro*. We stably knocked down MMP2 gene expression using short hairpins directed against MMP2 carried in lentiviral particles. Reduction in MMP2 protein was confirmed by quantitative RT-PCR and western blotting. Confirmation of reduced gene expression can be found in Figure 3.6. We then used these MMP2 knockdown or control fibroblasts in 3D co-cultures with R221A tumor cells to assess the requirement for MMP2. As shown in Figure 2.6, we used different ratios of tumor cells to either control fibroblasts or fibroblasts knocked down for MMP2. Regardless of the tumor:fibroblast ratio, tumor spheroids did not grow out when fibroblasts were knocked down for MMP2. Control fibroblasts and tumor cells alone had similar growth rates in all cultures. The exception was when tumor cells exceed fibroblasts. In this case, co-cultures with control fibroblasts had the best growth rates.

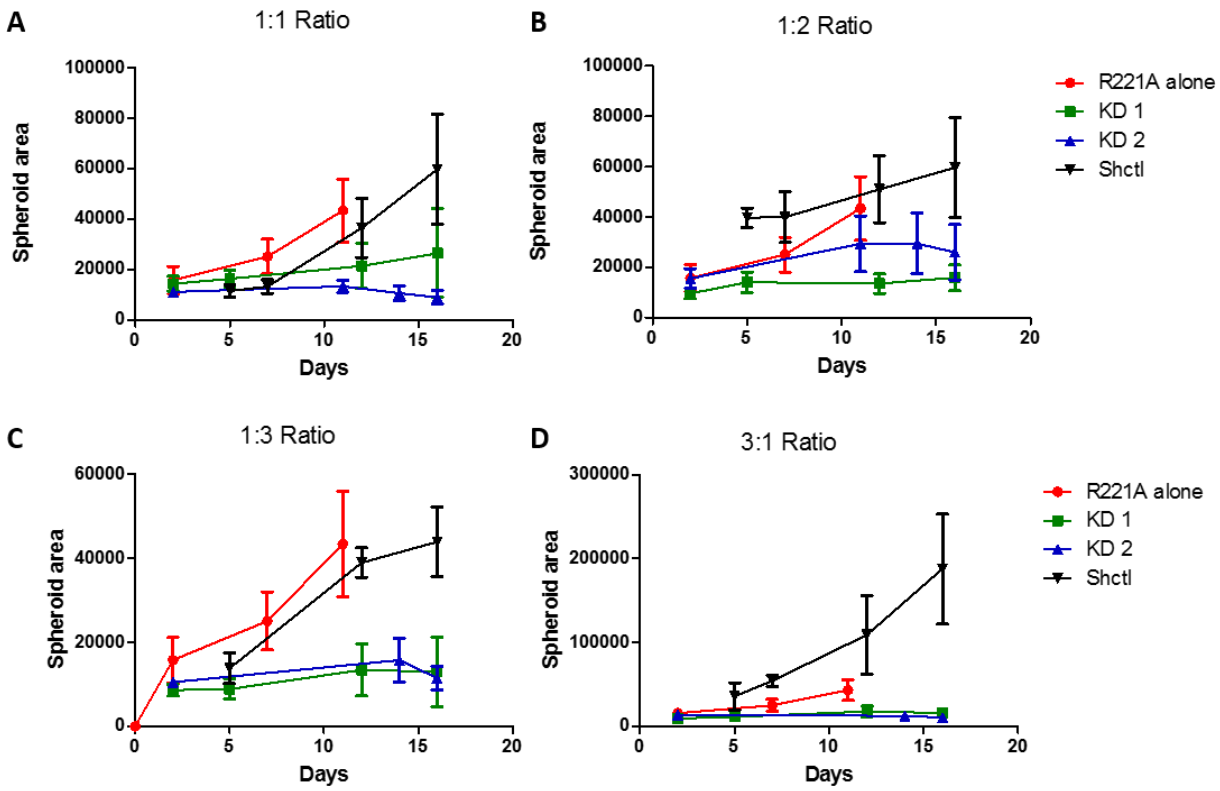

Figure 2.6: **Assessment of tumor growth in 3D upon the loss of MMP2 in fibroblasts**. mCherry R221A tumor cells and fibroblasts in ratios of (A) 1:1 (B) 1:2 (C) 1:3 and (D) 3:1 were followed over time. Areas of selected tumor spheroids were taken at specified time points using imaging analysis software.

Discussion

In this chapter, we have used a 3D environment to explore mammary tumor proliferation and proteolytic behavior and the implications for MMP2 in these processes. Tumor cell morphology more closely mirrored *in vivo* geometry in the presence of ECM. Matrix remodeling enzymes produced by tumor cells, including MMPs, resulted in invasion of the ECM via collagen cleavage. In a partial recapitulation of the tumor microenvironment, we wanted to explore the effect of MMPs in tumor-stromal crosstalk by including tumor derived fibroblasts. Fibroblasts produced MMP2 and in a higher concentration than tumor cells. Moreover, a significant amount of the fibroblast-derived MMP2 was of the processed size corresponding to activated enzyme. When placed in 3D co-culture, mammary tumor proteolysis was reduced after neutralization of MMP2, likely derived from fibroblasts. In examining if MMP2 can enhance tumor cell proliferation, exogenous active enzyme increased tumor cell proliferation in 3D but not 2D. Finally, 3D co-cultures of mammary tumor cells and control or MMP2 KD fibroblasts showed an increase in tumor growth rate when MMP2 was present compared to KD.

As shown in previous studies, tumor spheroids were formed in the presence of ECM and exhibited fingerlike projections which were not present in 2D culture. These invasive projections resembled invadopodia leading us to examine the expression of matrix remodeling enzymes. Examination of the MMP mRNA profile showed that several MMPs are expressed by these mammary tumor cells, including MMP2 and MMP9. In particular, MMP2 and MMP9 are commonly associated with basement membrane degradation due to their abilities to cleave a number of proteins within the basement membrane (see Table 2). They are often expressed at invadopodia and during invasion and metastasis [178–180]. Therefore we examined their pro-

and active protein expressions by gelatin zymography. MMP2 protein was expressed, suggesting it might play a role in the observed matrix degradation and invasion. Proteolysis by these cells was confirmed with dye quenched collagen IV, further implicating MMP2 which can cleave collagen IV.

To mimic the tumor microenvironment, fibroblasts were incorporated into 3D cultures with tumor cells. The expression of gelatinases, especially the high level of MMP2, in fibroblasts suggested that tumor-stromal crosstalk may influence tumor cell proteolysis through secretion of proteolytic enzymes. Because gelatin zymography of both tumor cells and fibroblasts showed expression of MMP2 (Figure 2.2B), and previous studies from the lab have examined the role of MMP9 [138], we chose to focus these investigations on the effect of MMP2 inhibition on proteolysis and tumor growth in 3D. Indeed upon the inhibition of MMP2 by neutralizing antibody, there was a significant reduction in proteolysis.

Active MMP2 increased tumor growth in 3D but not 2D assays. There are two likely and related explanations as to why this occurred. First, exogenous addition of active MMP2 lacks important cofactors which may be necessary to enhance tumor growth. Secondly, MMP2 mediated tumor growth requires the presence of ECM in order to enhance tumor growth. It's possible that tumor growth in this model occurs by MMP2 releasing factors from the extracellular matrix. The hanging drop 3D culture system used in this model also contained extracellular matrix. Therefore we hypothesize that MMP2 acts to release factors present in the ECM which in turn enhance tumor growth. Indeed, in co-culture with control fibroblasts, a potent source of MMP2, tumor cell proliferation was enhanced over co-culture with MMP2 KD fibroblasts. Proteolysis and proliferation decrease coincidently with MMP2 reduction. Although correlative, these

studies collectively suggest that MMP2 mediated proliferation is codependent on proteolysis of the ECM.

CHAPTER III

Stromal matrix metalloproteinase 2 regulates collagen expression and promotes the outgrowth of experimental metastases[1]

Introduction

Cancer progression is a complex interplay between tumor cells and its microenvironment. The influence of microenvironment is of critical importance for metastasis of cancer cells, a strong determinant of patient survival [181]. Breast cancer patients have an overall five-year survival rate of 99% if disease is localized, but this plummets to approximately 25% if they are diagnosed with metastases [1]. These statistics underscore the importance of understanding and ultimately defining strategies to defeat metastasis. Multiple microenvironmental as well as tumor-derived factors can contribute to metastatic progression [182–184], and key players involved in this process include matrix metalloproteinases (MMPs) [185].

MMPs are a family of 24 zinc-dependent endopeptidases associated with extracellular matrix degradation in health and disease [186]. MMPs are also implicated in the release and processing of growth factors, as well as in angiogenesis and immune surveillance [101, 187, 188]. Matrix metalloproteinase 2 (MMP2) is a 72 kDa member of the gelatinase subfamily of MMPs. MMP2 is overexpressed in a variety of malignant tissues compared to normal tissues such as cancers of the breast, colon, stomach, and lung [189–191]. Increased MMP2 has been associated with advanced stages of breast cancer [192], and decreased relative overall survival [193]. Its

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[1] [1] The contents of this chapter were published in Journal of Pathology 2014 Dec 3. doi: 10.1002/path.4493

deficiency has been linked to a favourable prognosis in node negative patients [194]. Although many MMP2 substrates have been identified [101], the exact roles that MMP2 plays in the progression of cancer are still being uncovered.

The majority of MMPs are produced in the tumor stroma [195–197]. Studies using *in situ* hybridization revealed that *MMP2* mRNA is localized to the fibroblast compartment in primary breast cancer tissue [198]. Co-culture of breast cancer cells and fibroblasts enhances MMP protein production, including active MMP2, in fibroblasts [37, 199]. Reciprocally, conditioned media from fibroblasts can enhance tumor growth [37] and the inhibition of MMP2 activity in fibroblasts abolishes pro-tumorigenic effects in nude mice [200]. Further, mice in which *Mmp2* was genetically ablated had significantly fewer lung tumor foci in experimental metastasis assays [201]. Collectively, these data point to a role for host-derived MMP2 in the metastatic progression of breast cancer. In this study, we set out to identify the mechanism by which stromal fibroblast-derived MMP2 contributes to the outgrowth of pulmonary metastases. We chose to use an experimental metastasis model for *in vivo* studies, in order to focus on contributions of MMP2 to the later stages of colonization and outgrowth.

Materials and methods

Tumor cell lines and culture: Pyvt-R221A cells (referred to as R221A cells) were generated from a mammary tumor arising in a FVB PyVT transgenic mouse [138]. E0771 cells, derived from a spontaneously developing mammary medullary adenocarcinoma in a C57Bl/6 mouse [202, 203] were purchased from CH3 Biosystems (Amherst, NY). Cells were maintained in DMEM supplemented with 10% FBS and 10µg/ml gentamycin at 37ºC in 5% CO_2.

In vivo tumor models. FVB/n and/or C57Bl/6 WT and *Mmp2*$^{-/-}$ animals were maintained in the Vanderbilt Animal Housing facility and all mouse work was conducted only after review and approval by the local institutional animal care and use committee. Six to eight week old female mice were injected with 1 million R221A-luc or E0771 cells via the tail vein. Mice were imaged using the Xenogen 200 imager at defined time points 3 minutes after retro-orbital injection of 120 mg/kg luciferin. Mice were sacrificed at 1 or 2.5 weeks post inoculation. Tumor bearing or normal lungs were perfused with sterile PBS and used for cell isolation, fresh frozen or formalin fixed for tissue analysis.

Immunohistochemistry and immunofluorescence. After formalin fixation and paraffin embedding, lungs were cut into 5 μm sections. Immunohistochemical staining was performed as previously described [204]. Sources for antibodies used were: Ki-67 (Abcam, Cambridge, MA), phospho-histone H3 (Millipore, Billerica, MA), von Willebrand factor (Dako, Carpinteria, CA), cleaved caspase 3 (Cell Signaling, Danvers, MA), and MMP2 (Abcam). Fluorescent labeling was performed on frozen sections using the following additional antibodies: vimentin (Covance, Princeton, NJ), αSMA (Sigma, Saint Louis, MO), CD31 (BD Pharmingen, San Jose, CA), CD45 (BD Pharmingen). Further details are provided in Table 3.

Isolation of fibroblasts. Tumor lung-derived fibroblasts were isolated from the tumor bearing lungs of FVB/n WT mice. Briefly, lung tissue was mechanically separated by mincing and straining through a 70μm filter followed by enzymatic digestion (collagenase and hyaluronidase, Sigma) in serum free DMEM:F12 medium (Life Technologies, Grand Island, NY). The tissue suspension was centrifuged and the pellet washed with sterile PBS containing 5% bovine serum.

Cells were then resuspended in DMEM:F12 medium with 5% serum and plated on tissue culture plastic. After 72 hours, tumor cells were separated from fibroblast cells using differential trypsinization. Tumor derived fibroblasts were cultured in DMEM (Life Technologies) with 10% FBS on tissue culture plastic. Quiescent fibroblasts were similarly isolated from the non-tumor bearing lungs of WT and *Mmp2*$^{-/-}$ mice but instead cultured in DMEM containing 1 or 2.5% FBS on collagen I coated dishes.

TABLE 3. ANTIBODY SOURCE AND CONCENTRATIONS

ANTIBODY NAME	Company	Catalog number	Concentration/Dilution
KI67	Abcam	15580	1:200
PHOSPHO-HISTONE H3	Millipore	06570	1:500
VON WILLEBRAND FACTOR	Dako	A0082	1:200
CLEAVED CASPASE 3	Cell Signaling	5A1E	1:800
MMP2	Abcam	ab37150	1:150
VIMENTIN	Covance	Pck-594	1:200
ALPHA SMOOTH MUSCLE ACTIN	Sigma	A2547-.2ml	1:200
CD31	BD Pharmingen	550274	1:50
CD45	BD Pharmingen	550539	1:20
ALEXA FLUOR 488	Life Technologies	A11039, A31620, A11006, A11007	1:2000
ALEXA FLUOR 594	Life Technologies	A11037	1:1000
PSMAD2	Cell Signaling	3108	1:500
SMAD2/3	Cell Signaling	3102	1:500
ACTIN	Santa Cruz Biotechnology	Sc-1615	1:1000
ANTI-RABBIT, HRP	Cell Signaling	7074	1:1000
ANTI-GOAT, BIOTIN	Vector	BA-9500	1:1000
STREPTAVIDIN-HRP	Jackson ImmunoResearch	016-030-084	1:15000

Knockdown of MMP2. shRNA lentiviral particles targeting *Mmp2*, and control particles were obtained from Sigma, and used to infect lung tumor derived fibroblasts from WT mice following manufacturer's recommendations. Particles encoding different shRNA sequences were used independently. Successfully transduced cells were selected by culturing in the presence of puromycin. Polyclonal populations were used for subsequent experiments. Cells transduced with the control particles are subsequently referred to as 'Shctl'.

PCR primers. PCR primers were used at 1μM concentrations and were commercially available or designed from the following sequences: mouse MMP2 (Qiagen Mm_Mmp2_1_SG), αSMA (Operon, F: ATCATGCGTCTGGACTTGG, R: AATAGCCACGCTCAGTCAGG), mouse VIM (Operon, F: CCCCCTTCCTCACTTCTTTC, R: AAGAGTGGCAGAGGACTGGA), mouse FAP (Operon, F: CCAGGAGATCCACCTTTTCA, R: GTGGCAAGCATTTCCTCTTC), FSP1 (Operon, F: GATGAGCAACTTGGACAGCA, R: ACTTCTTCCGGGGTTCCTTA), GAPDH (Qiagen Mm_Gapdh_3_SG), βACTIN (Qiagen Mm_Actb_1_SG), *Fn1* (Operon, F: GGCGTCCCCACCTCAGGACT, R: GAGTCGCCCTCCCCAGGAGG), *ColIa2*, (Invitrogen, F: GTGTTCAAGGTGGCAAAGGT, R: GACCGAATTCACCAGGAAGA), *ColIVa1* (Invitrogen, F: TGGCTCTGGCTGTGGAAAAT, R: CCAATGACACCTTGCAACCC).

Immunofluorescence. Briefly, tissue slices were fixed in acetone for 10 minutes, blocked with 10% serum and incubated with primary antibodies. Following washing, AlexaFluor secondary antibodies (Life Technologies, Grand Island, NY) were added. Sections were counterstained with bisbenzimide/Hoechst (Sigma), and mounted.

Magnetic bead-isolation of stromal populations. Lungs from tumor-bearing mice (14 days post tumor cell inoculation) were harvested and digested to single cells as described for fibroblast isolation. Following manufacturer's recommendation for cell number, volume and incubation conditions, the cells isolated from each mouse were first incubated with MACS CD45 beads (Miltenyi Biotech Inc, San Diego, CA), washed and put through a MACS column (Miltenyi Biotech) to separate cells bound by the beads from unbound. The cells bound by the beads were then released from the column and labeled as CD45+ fraction. The unbound cells were collected, incubated with MACS CD90 beads (Miltenyi Biotech) and put through MACS columns as before. The unbound fractions were labeled as CD45-/CD90-, while those released from binding to the column were CD45-/CD90+. All populations were then placed in culture with serum-free medium for 18 hours. The conditioned media were collected, centrifuged to remove dead cells and analyzed for total protein content using a BCA assay (Pierce). Equal amounts of protein for each population was loaded on to an SDS-PAGE and analyzed by western blotting for MMP2 as described previously. RNA was isolated from the cell pellets and used for real-time RT-PCR analysis.

Proliferation experiments. For media-transfer assays, R221A-luc cells were suspended in 10% Cultrex (Trevigen, Gaithersburg, MD) and added to Perfecta 3D hanging drop plates (3D Biomatrix, Ann Arbor, MI). After 48 hours, which allowed the tumor cells to aggregate and form spheroids, the spheroids were treated with control or conditioned media from sh-control (Shctl) or *Mmp2* KD cells and this was added every other day to respective wells. At endpoint, spheroids were transferred to flat bottom 96-well plates and fluorescence was measured using a CyQuant NF assay (Life Technologies). For 3D co-culture assays, co-cultures of mCherry-

labeled R221A and/or Shctl or *Mmp2* KD fibroblasts were embedded in Cultrex (Trevigen) and placed onto a MatTek dish (MatTek, Ashland, MA) pre-coated with Cultrex (Trevigen). Growth media was exchanged every other day. Spheroids were imaged with an Evos microscope (Life Technologies) at predefined intervals over 14 days. Metamorph software (Molecular Devices, Sunnyvale, CA) was used to measure area of red tumor spheroids.

Immunoblotting. Cells were lysed using RIPA buffer (0.1%SDS, 150mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 10mM Tris pH 7.4) plus protease and phosphatase inhibitors (cOmplete Mini, EDTA-free and PhosphoSTOP; Roche, Indianapolis, IN). Following SDS-PAGE, protein was transferred to nitrocellulose, blocked and incubated with primary antibodies [MMP2 (Abcam), pSmad 2 (Cell Signaling), or Actin (Santa Cruz Biotechnology, Dallas, TX)]. Secondary antibodies were directly HRP conjugated (Cell Signaling) or biotinylated (Vector, Burlingame, CA) and detected with streptavidin-HRP. Chemiluminescent detection was achieved using Western Lightning ECL reagent (PerkinElmer, Waltham, MA).

Quantitative real time RT-PCR. RNA was isolated from fibroblasts using TRIzol reagent (Life Technologies) and an RNeasy mini-prep kit (Qiagen, Valencia, CA) or the Quick-RNA mini-prep kit (Zymo Research, Irvine, CA). Reverse transcription was performed using M-MLV (Promega, Madison, WI). Real-time PCR was performed on a BioRad iQ5 instrument using Maxima SYBR green master mix (Thermo, Pittsburg, PA) according to manufacturer's instructions. Primer details are provided as supplemental information.

Analysis of microarray datasets. Publicly available microarray expression data for breast cancer stroma isolated by laser capture microdissection (gene set: GSE33692) was obtained from NCBI

GEO website. Excel files were uploaded and analyzed on Affymetrix Genespring GX 12.5. Following baseline normalization, expression values for a given gene were imported into Graphpad Prism for correlation analysis of gene expression in each sample, generation of linear trendlines, and statistical analysis as previously described [46].

Statistical analysis. One-way analysis of variance (One way ANOVA) was used for multiple group parametric comparisons using a Bonferonni post-hoc analysis. To compare two groups, a Student's t-test was used for parametric analyses and a Mann-Whitney test for non-parametric analyses. Linear regression analysis was used to calculate the growth rates. Comparison of the resultant slopes were calculated as described above. Statistical significance was considered $p<0.05$ and is indicated by an asterisk in the relevant figures. All statistical analyses were conducted using Graphpad Prism software.

Results

Host derived MMP2 potentiates the proliferation of pulmonary experimental metastases. To determine the role of host MMP2 in the outgrowth of mammary-to-lung metastases, immunocompetent FVB WT or *Mmp2*$^{-/-}$ mice were intravenously injected with syngeneic R221A-luc cells [138]. *In vivo* outgrowth was measured over time by luciferase imaging. A significant reduction in both the luminescence signal (Figure 3.1A) and the number of lung surface lesions (Figure 3.1B) were observed in *Mmp2*$^{-/-}$ mice compared to WT mice. At the 18-day endpoint, analysis of proliferation demonstrated a significant reduction of Ki67^{+} staining in tumors from *Mmp2*$^{-/-}$ animals (Figure 3.1C), with no change in apoptosis or vascularity (Figure 3.1 D-E). An independent repeat of this experiment with 5 wildtype and 4 *Mmp2*$^{-/-}$ mice gave the same results (data not shown).

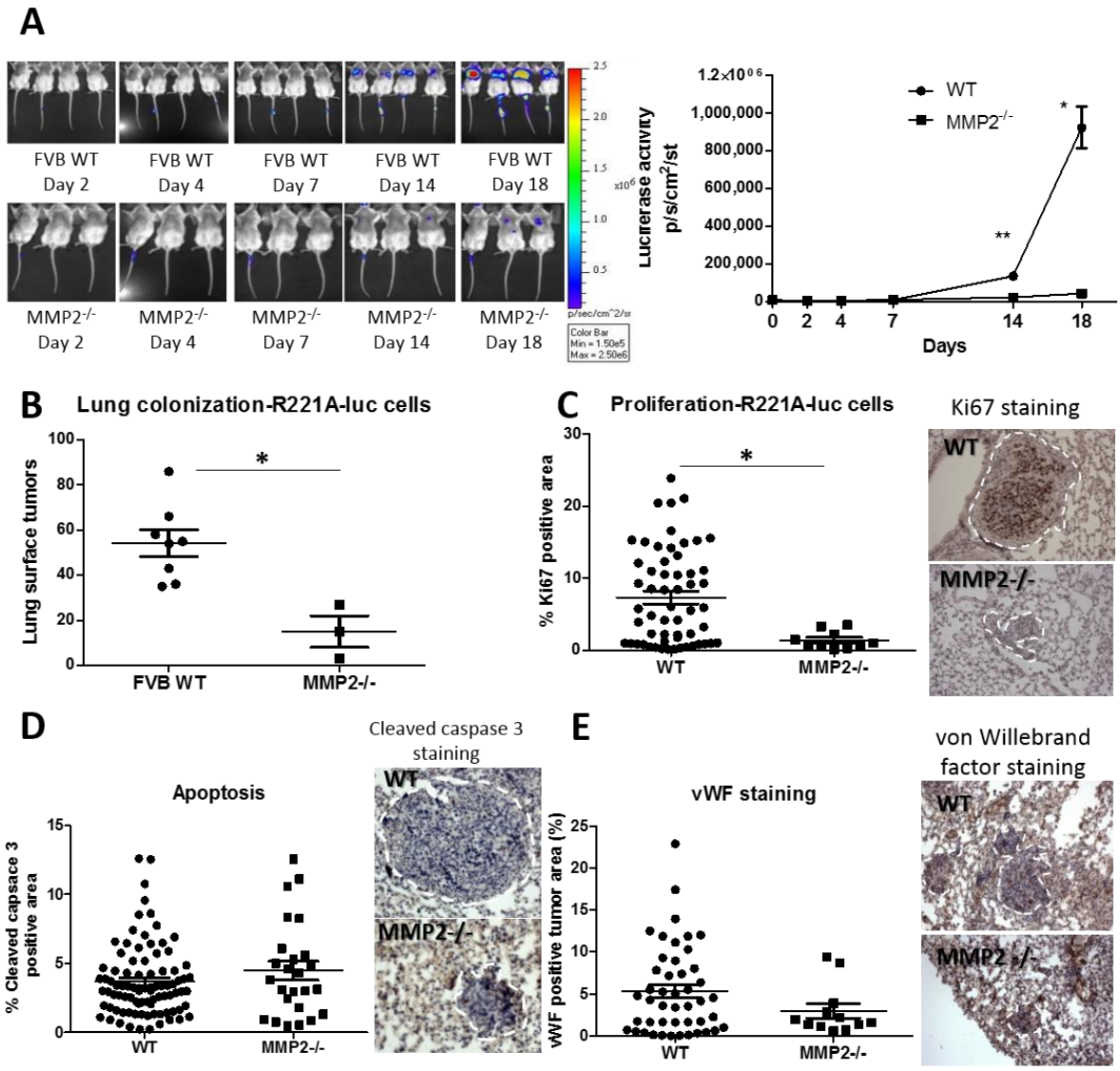

Figure 3.1: Host MMP2 contributes to the outgrowth of pulmonary metastases. (A) Luciferase activity in WT and MMP2$^{-/-}$ mice was analyzed over three weeks using IVIS imaging software and the resulting average radiance is shown for each imaging timepoint, *p=0.02, **p=0.01. (B) Visible macro-metastases on the lung surfaces for WT and MMP2$^{-/-}$ mice were manually counted and the total number obtained per mouse is shown, p=0.0055. (C) Quantitation of positive signal for Ki67 as a marker of proliferation per unit area of tumor within lung tissue sections. Examples of the staining are shown on the right with brown (diaminobenzidine) stain being a positive signal, p=0.005. (D) Quantitation of positive signal for cleaved caspase 3 as a marker of apoptosis per unit area of tumor within lung tissue sections, p=n.s. Examples of the staining are shown on the right with brown (diaminobenzidine) stain being a positive signal. The white dashed lines indicate the tumor areas analysed. Arrows point to examples of positive cells within the tumors. (E) Quantitation of positive signal for von Willebrand factor as a marker of blood vessels per unit area of tumor (indicated by white dashed line) within lung tissue sections, p=n.s. Examples of the respective stainings are shown on the right with brown (diaminobenzidine) stain being a positive signal. Histology magnifications are 200x.

Initial growth between WT and *Mmp2*$^{-/-}$ animals was similar until approximately day 8, suggesting initial seeding was similar in both genotypes. To evaluate early tumor growth, a second study was conducted where mice were sacrificed after 7 days. At this timepoint, there was no discernible difference in tumor burden or growth rate as determined by luminescent signal between WT and *Mmp2*$^{-/-}$ mice (Fig 3.2A). However immunohistochemical analysis of the tumor foci present revealed proliferation (phospho-histone H3 staining) was significantly lower in tumors growing in *Mmp*2$^{-/-}$ mice, confirming our previous finding (Fig 3.2B).

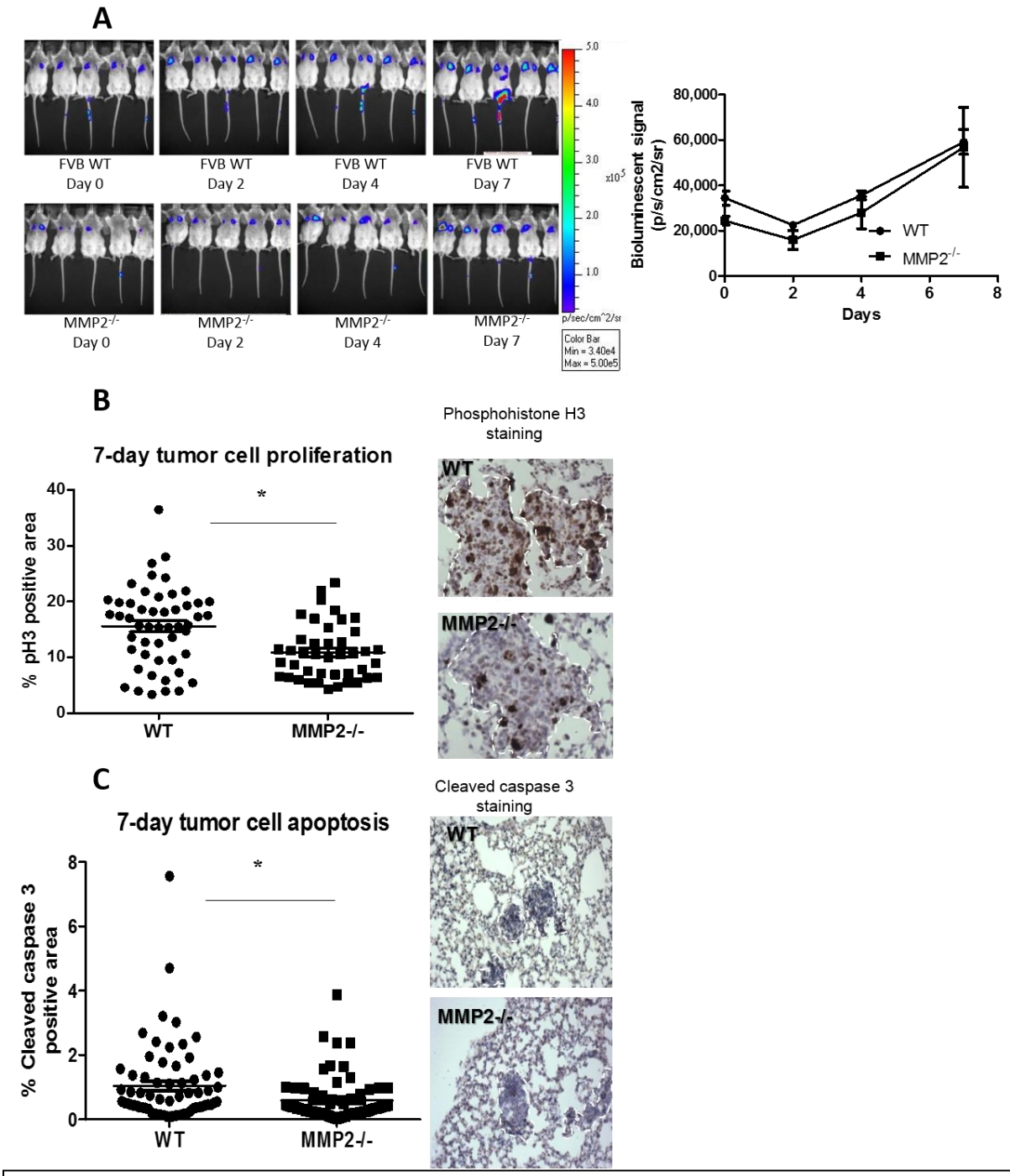

Figure 3.2: MMP2 does not affect seeding ability or early survival of tumor cells. (A) Luciferase activity and quantifications in FVB WT and MMP2$^{-/-}$ mice analyzed over seven days using IVIS, p=ns. (B-C) Immunostaining of lung tissue sections for (B) phospho-histone H3 (p=0.0008) and (C) cleaved caspase 3 (p=0.0025). Brown staining within selected tumor regions are normalized to tumor area and expressed as percentages. Magnification of histology is 200x.

Additionally, apoptosis was also reduced as measured by cleaved caspase 3 (Fig 3.2C). The reduction in proliferation was also observed in a second, slower-growing model (E0771 cells in C57Bl/6 WT and *Mmp2*$^{-/-}$ mice) suggesting that MMP2-dependent tumor cell proliferation is a general phenomenon (Fig 3.3). Together, these studies suggest that host MMP2 contributes to the outgrowth of mammary tumors in the lungs by stimulating tumor cell proliferation.

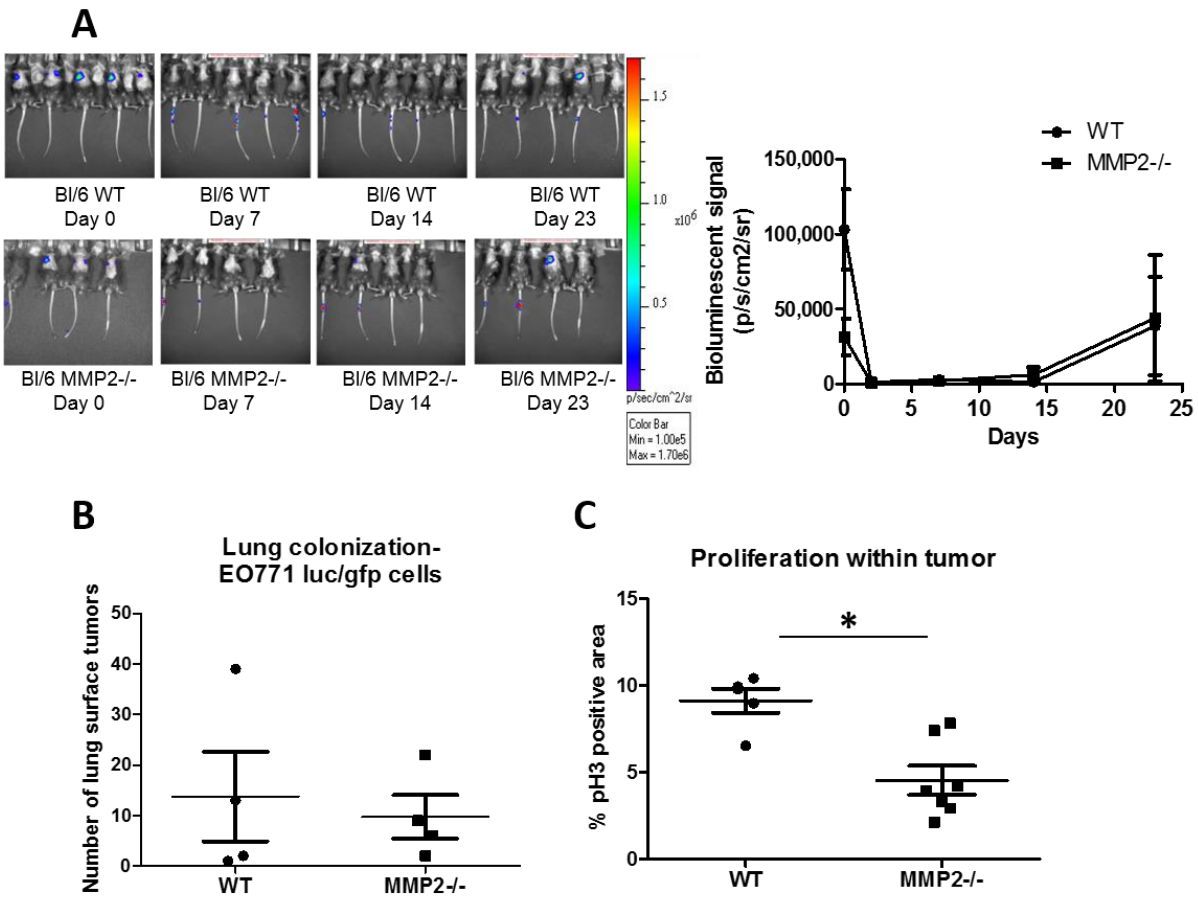

Figure 3.3: Stromal MMP2 contributes to the outgrowth of pulmonary metastases in a secondary model. (A) Luciferase activity and quantifications in C57 BL/6 WT and MMP2$^{-/-}$ mice analyzed over 26 days, p=ns (B) Total number of visible macro-metastases on the lung surfaces for WT and MMP2$^{-/-}$ mice. p=ns. (C) Immunostaining of lung tissue sections (5µm) for phospho-histone H3 and analyzed as described previously, p=0.01.

MMP2 primarily localizes to fibroblasts. Immunohistochemistry was used to localize MMP2 in tumor bearing lung sections. We observed MMP2 expression mostly in stromal cells around the perimeter of lung metastases (Figure 3.4A) and by cells between tumor cell nests. To identify the specific stromal cell types that expressed MMP2, we performed a series of dual immunofluorescence analyses where tissue sections were co-stained for MMP2 and one of a variety of stromal cell markers. Within the tumor microenvironment, MMP2 was mainly co-expressed with vimentin and α-SMA (Figure 3.4B), which are commonly accepted as markers of activated fibroblasts [205, 206]. MMP2 was sporadically co-expressed with CD31 and more frequently with CD45, representing endothelial and hematopoietic cell populations, respectively (Figure 3.4B). Indeed MMP2 expression has been associated with myeloid cells recruited to tumors previously [207, 208]. To further characterize the extent of MMP2 expression by hematopoietic versus other stromal cells, we prepared single cell suspensions from 3 tumor-bearing mice and isolated different stromal populations using magnetic beads conjugated to either anti-CD45 or anti-CD90 antibodies. Cells representing the CD45+, CD45-/CD90+, or CD45-/CD90- populations were then cultured overnight in serum-free medium, or harvested for RNA. As shown in Fig 3.4C, the MMP2 levels were lowest in the CD45+ population, and were higher and in the active form only in the CD45-/CD90- population, which for pulmonary fibroblasts, are thought to represent activated myofibroblasts [209–211].

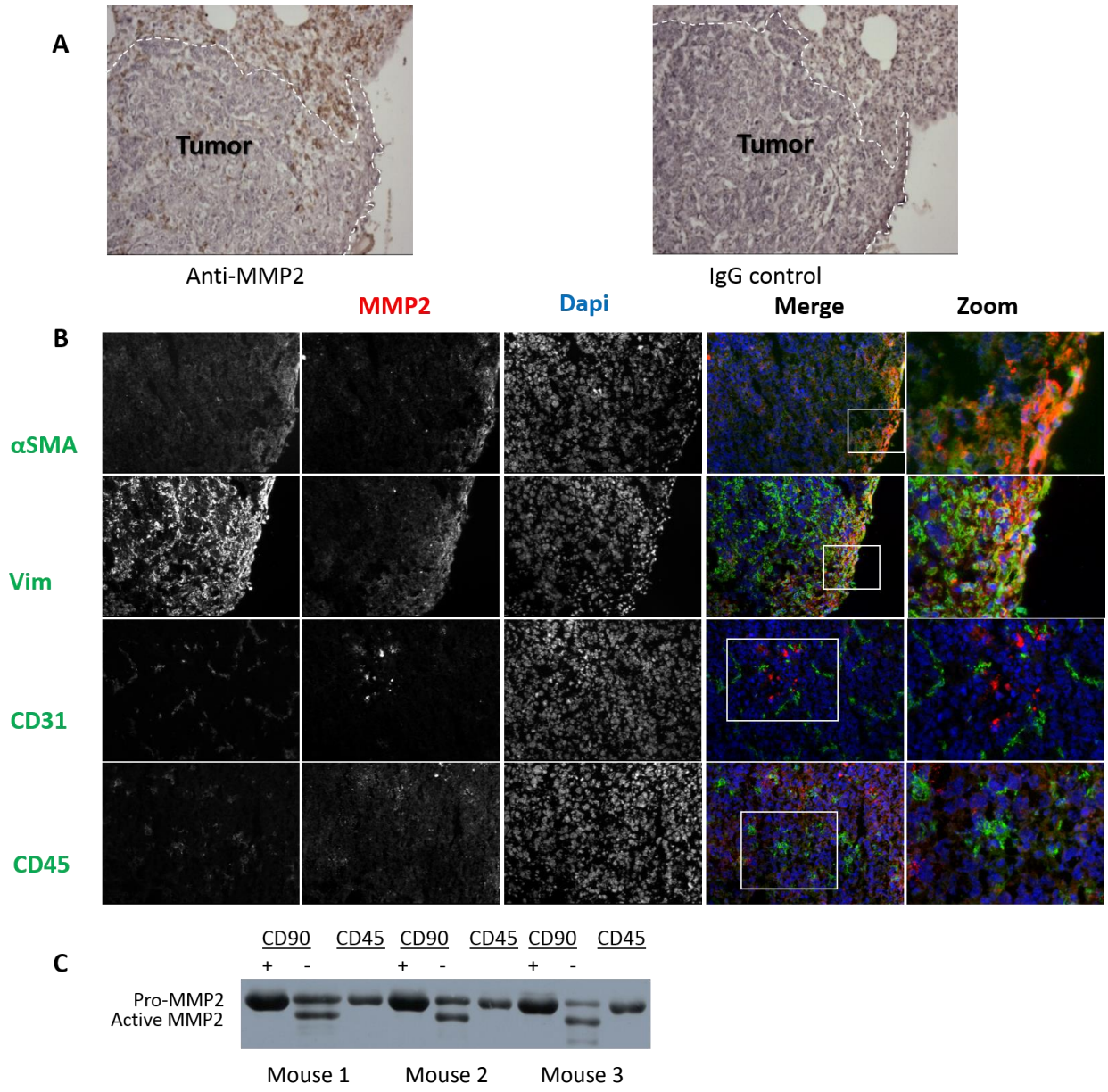

Figure 3.4: Tumor-adjacent fibroblasts are the main cellular source of MMP2. (A) Immunohistochemical staining of MMP2 in tumor bearing lung sections from FVB WT mice. Positive staining (brown) was noted in the stroma surrounding tumor metastases. Image on the right shows signal obtained when an isotype control antibody is used. (B) Co-immunofluorescent staining for MMP2 and either vimentin (Vim), alpha-smooth muscle actin (αSMA), CD31 or CD45. Nuclei were counterstained with Dapi. The signal for each individual maker is shown in greyscale in the first 3 columns with a merged image in the fourth column, where red is MMP2, blue is Dapi and green represents the specific cell-type marker. A magnified view of a portion of the merged image is shown on the right. (C) Levels of MMP2 protein (latent and active) as detected by western blotting of 24hour conditioned media normalized for protein content, from CD45-/CD90+, CD45-/CD90-, or CD45+ stromal cells isolated from lungs of tumor-bearing mice (n=3). Histology magnification is 200x.

Real-time PCR for fibroblast and tumor cell markers confirmed this concept (Fig 3.5). Levels of the transcript for the polyoma viral antigen (*pyvt*), expressed by the tumor cells, were equally negligible in the CD45- samples, while the CD90- population was higher for the fibroblast marker, fibroblast specific protein (FSP1) than for the CD90+ population (Fig 3.5A). This confirms the stromal nature of the cell populations. Furthermore, fibroblast-activation protein (FAP), a marker of activated fibroblasts, was also higher in the CD90- fraction. Taken together, these studies suggest that tumor-associated myofibroblasts are the major source of MMP2 in our

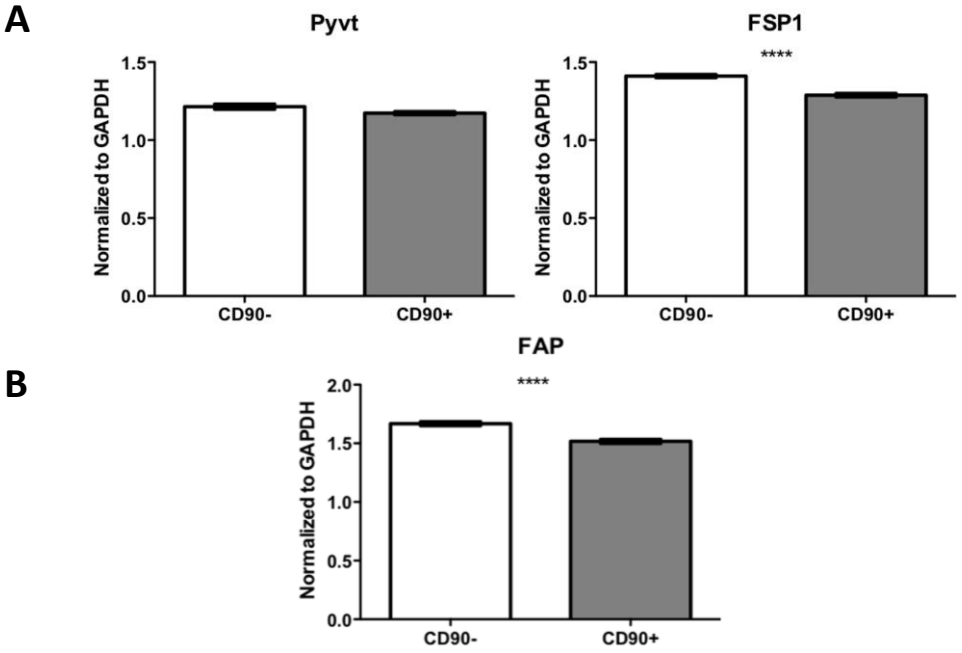

Figure 3.5: Characterization of magnetic bead-isolated fractions. (A)Transcripts for Pyvt, an indicator of tumor cell presence, were not different between the two CD45- populations and was low in both. The marker FSP1, which is expressed by activated and quiescent fibroblasts, was higher in the CD90+ population, ****$p<0.0001$. (B) The marker of activated fibroblasts, FAP, was significantly higher in the CD90- populations, ****$p<0.0001$.

model.

Fibroblast-stimulated tumor cell proliferation requires MMP2. The dominant phenotype associated with loss of stromal MMP2 was reduced proliferation, and previous studies have demonstrated that tumor cell proliferation can be stimulated by tumor derived fibroblasts both *in vivo* and *in vitro* [54]. Therefore, we next investigated whether MMP2-deficient fibroblasts could impact the growth of tumor cells *in vitro* compared to WT activated fibroblasts. We first isolated fibroblasts from the tumor bearing lungs of WT mice. The mice were injected with tumor cells 14 days prior, and thus we term these fibroblasts as 'tumor bearing lung-derived fibroblasts'. The fibroblasts were grown on tissue culture plastic in the presence of serum and resembled activated myofibroblasts. We then performed stable knockdown of *Mmp2* mRNA and protein in the WT cells by infection with lentiviral particles carrying one of two different shRNA

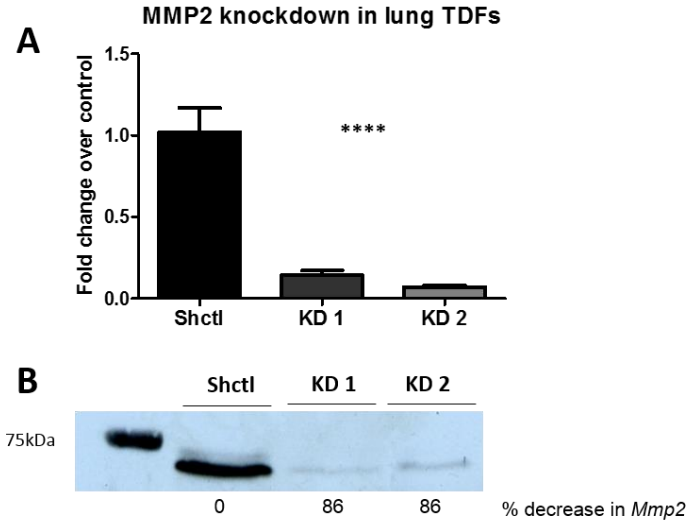

Figure 3.6: Knockdown of MMP2 in lung-tumor derived fibroblasts. (A) Quantitative real time PCR showing relative mRNA expression for *Mmp2* after knockdown with three short hairpin RNA sequences targeted to *Mmp2*, ****p<0.0001. Values are normalized to *Gapdh.* (B) Immunoblot showing Mmp2 protein levels in conditioned medium after knockdown of *Mmp2*. TDF=tumor derived fibroblast

sequences to *Mmp2* (Fig 3.6).

Because 3-dimensional (3D) conditions are more representative of the *in vivo* environment and are often required for proliferative effects *in vitro* [140], we first investigated 3D tumor growth using mCherry R221A cells in hanging drops containing BME and treating with conditioned medium from parental, Shctl, or *Mmp2* KD tumor derived fibroblasts. There was a significant decrease in proliferation, as measured by total DNA content, when tumor cells were treated with conditioned medium from *Mmp2* KD cells compared with parental or Shctl cells (Figure 3.7A). We next co-cultured tumor cells with parental, Shctl, or *Mmp2* KD fibroblasts embedded in BME. Individual spheroids from each co-culture group were followed over time and their growth rates were calculated. The growth rates between spheroid groups were then compared. Similar to conditioned media treatments, when tumor cells were co-cultured in direct contact with fibroblasts, there was also a significant reduction in the rate of spheroid growth when the co-culture contained *Mmp2* KD versus Shctl fibroblasts (Figure 3.7B). These results indicate that fibroblast MMP2 potentiates a tumor proliferation-enhancing function of activated fibroblasts.

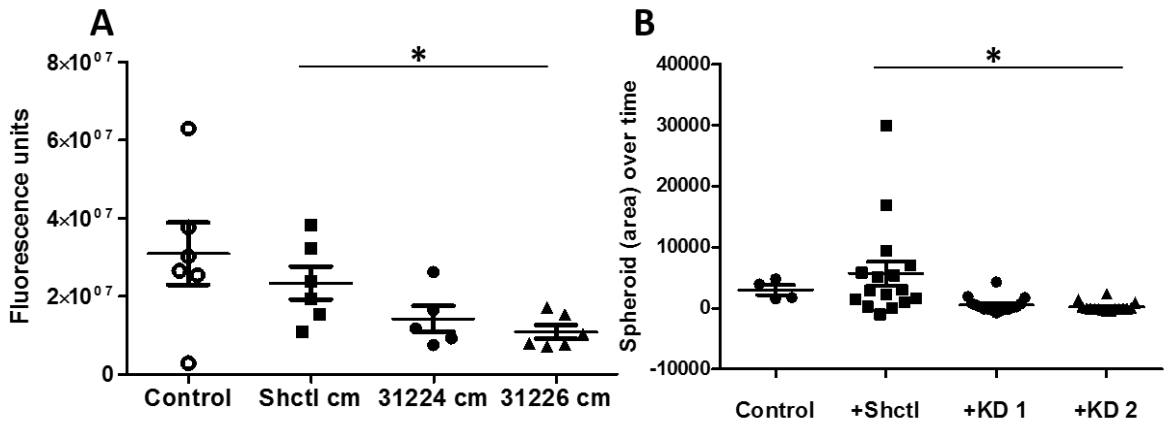

Figure 3.7: Tumor cell proliferation is enhanced by MMP2-positive, but not MMP2-negative, lung-tumor fibroblasts *in vitro*. (A) Proliferation analysis of mCherry tumor spheroids embedded within basement membrane extract in the presence or absence of control or *Mmp2* KD fibroblast conditioned media. Fluorescence units indicating DNA content as measured by Cyquant assay are shown, p=0.042. (B) Comparison of spheroid area over time in co-cultures of tumor spheroids and control or *Mmp2* KD fibroblasts following embedding in basement membrane extract, p=0.0037.

Fibroblast activation status is dependent upon MMP2 expression. We next wanted to investigate whether MMP2 altered the phenotype of fibroblasts. The change of fibroblasts from a quiescent to an activated state is associated with tumor promoting effects, and we postulated that MMP2 may contribute to this change. Quantitative RT-PCR was used to measure the levels of mRNA transcripts associated with fibroblast activation state. For these analyses, we used two fibroblast models. Firstly, we isolated RNA from activated, tumor-bearing lung derived fibroblasts described above that were either proficient (Shctl) or deficient in *Mmp2* (*Mmp2* KD). Secondly, we isolated quiescent fibroblasts from non-tumor bearing lungs of either WT or *Mmp2*$^{-/-}$ mice. In isolating these fibroblasts, we endeavored to prevent activation as much as possible by using collagen-coated dishes to prevent contact with plastic, and minimizing serum

levels [145, 212]. This was based on stiffness-associated activation of fibroblasts that is reported in the literature [213–216]. There is a wealth of evidence to suggest that mechanotransduction is a critical factor in fibroblast activation with soft matrices (represented by the non cross-linked collagen used in our experiments) linked with low activation, and rigid surfaces associated with expression of activation markers as well as permitting TGFβ-1 signaling. In activated cells, knockdown of *Mmp2* led to a significant reduction in the activation status of fibroblasts compared to control cells (Figure 3.8A). Additionally, there was a significant reduction in the mRNA levels of matrix molecules, including collagens I, -IV, and fibronectin (Figure 3.8C). The lack of MMP2 in quiescent cells appeared to make no significant difference in baseline levels of the activation marker alpha smooth muscle actin (*Acta2*) from those of WT cells (Figure 3.8B). However, *Acta2* levels in the quiescent cells, irrespective of *Mmp2* status, were significantly lower than in the Shctl fibroblasts (Fig 3.9A), as was *Mmp2* itself (Fig 3.9B). This result was expected since we strived to maintain the cultures in a quiescent state.

Similarly to activated *Mmp2* KD cells, the lack of *Mmp2* in quiescent cells was also associated with significantly lower levels of transcripts for collagens I and IV as well as fibronectin (Figure 3.8D). We also tested whether the quiescent cells could be activated by tumor cell-derived soluble factors, as might happen within a tumor microenvironment. As shown in Fig 3.9C, WT but not *Mmp2*$^{-/-}$ quiescent fibroblasts showed increases in several transcripts associated with the activated phenotype after exposure to tumor cell conditioned medium. We next investigated if the lack of *Mmp2* would impact the activation of quiescent fibroblasts. Therefore, WT or *Mmp2*$^{-/-}$ quiescent fibroblasts were grown on collagen-coated dishes for quiescent culture, and were switched to tissue culture plastic to allow for activation. This system models the stiffness-

associated activation of fibroblasts reported in the literature [5, 214, 217, 218]. When WT quiescent cells were switched to tissue culture plastic, there was a significant increase in *Acta2* and vimentin mRNA transcripts compared to when the cells were grown on collagen, suggesting that these cells could be activated. In contrast, when *Mmp2*$^{-/-}$ quiescent cells were grown on tissue culture plastic, there was no significant difference in *Acta2* or vimentin levels compared to those cultured to maintain quiescence (Figure 3.8E). In fact, the *Acta2* transcript levels of *Mmp2*$^{-/-}$ cells grown on plastic remained at a similar level to those grown on collagen. This result indicates that these cells were not transitioning to an activated state. Examination of mRNA levels of collagens I and IV in quiescent fibroblasts after 24 hours on plastic revealed no change in expression from those grown on collagen (Figure 3.8F). However, these transcripts were lower in the *Mmp2*$^{-/-}$ cells irrespective of culture surface. Our studies suggest that MMP2 expression regulates fibroblast activation status and expression of extracellular matrix transcripts.

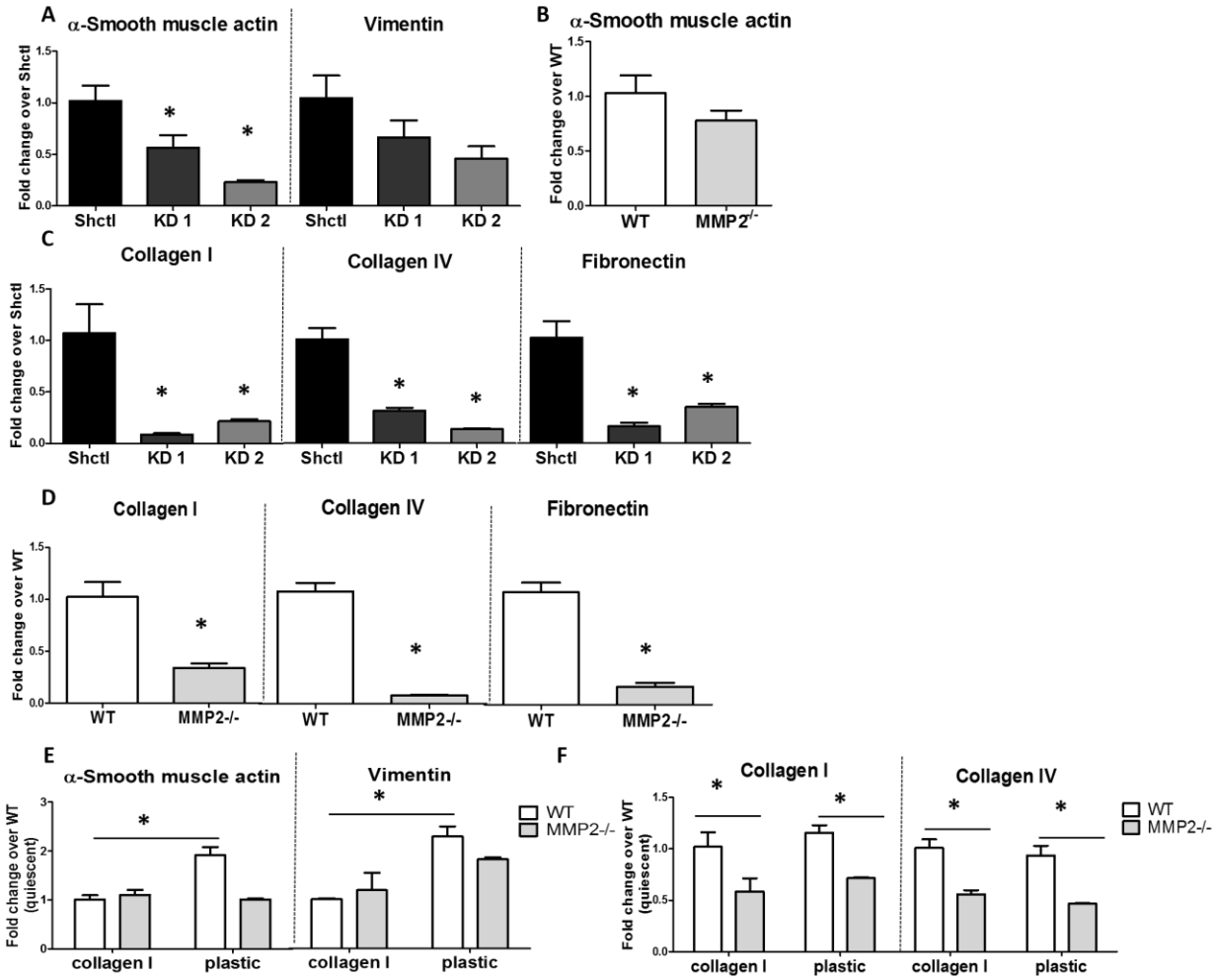

Figure 3.8: MMP2 is necessary for activation signature and matrix transcript expression in fibroblasts. (A) Expression of *Acta2* and *Vim* transcripts in *Mmp2* KD fibroblasts relative to Shctl as determined by quantitative real time PCR. Levels were normalized using *Gapdh* and analyzed using the comparative Ct method. (B) Expression of *Acta2* transcripts in quiescent *Mmp2*$^{-/-}$ fibroblasts relative to WT. p=n.s. (C-D) Expression of *ColI*, *ColIV*, and *Fn1* transcripts in (C) *Mmp2* KD fibroblasts and (D) quiescent *Mmp2*$^{-/-}$ fibroblasts relative to Shctl or WT cells, respectively. All values normalized to *Gapdh* of control cells. (D: p=0.01, p=0.0068, p=0.0008, respectively) (E-F) Expression of (E) *Acta2* and *Vim* or (F) *ColI* and *ColIV* transcripts in fibroblasts from non-tumor bearing lungs of WT or *Mmp2*$^{-/-}$ mice maintained under quiescent or activating culture conditions. Values are normalized to WT cells cultured under quiescent conditions. Asterisks indicate a significant difference to relevant control. *p< 0.05 unless otherwise noted.

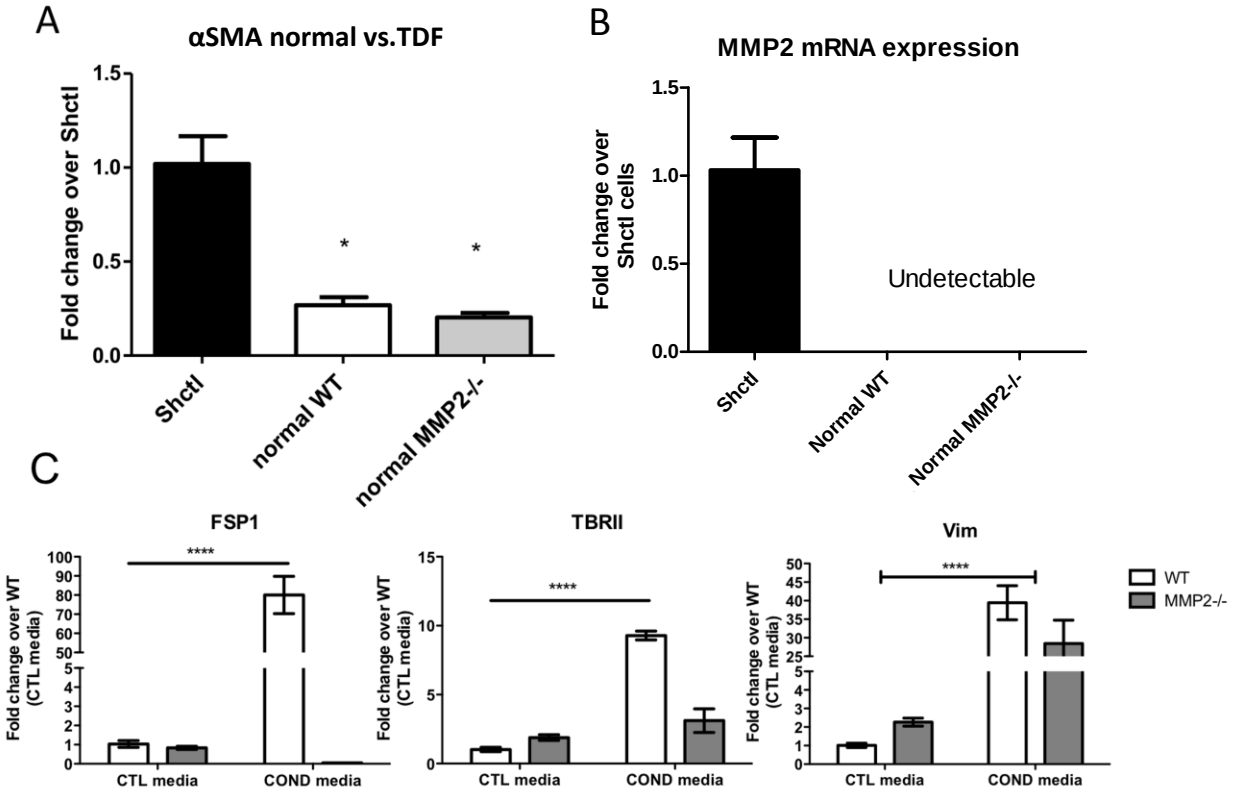

Figure 3.9: Characterization of quiescent fibroblasts for Acta2 and Mmp2 mRNA expression, and responsiveness to tumor-derived soluble factors. Quantitative real time PCR of (A) *Acta2* (p=0.0011) and (B) *Mmp2* expression in quiescent fibroblasts relative to that in activated, Shctl fibroblasts. Values are normalized to *Gapdh*. (C) Transcripts for fibroblast activation markers are increased when WT but not *Mmp2*$^{-/-}$ quiescent fibroblasts are exposed to tumor cell conditioned media, ****p<0.0001. TDF=tumor derived fibroblasts; CTL=control

MMP2-dependent fibroblast activation and collagen expression is mediated by TGFβ-1. We next investigated the underlying cause of reduced fibroblast activation. TGFβ-1 is a known activator of fibroblasts in wound healing and fibrosis [219] and is a recognized promoter of the differentiation of fibroblasts to myofibroblasts in breast cancer [220]. We first tested whether the activation of quiescent fibroblasts by culture on plastic was associated with activation of the canonical TGFβ-1 signaling pathway. Lysates from quiescent WT or *Mmp2*$^{-/-}$ fibroblasts either grown on collagen or after 24 hours on plastic in high serum were analysed for presence of pSmad2, the immediate downstream effector of TGFβ receptor 1 activation [63]. As shown in Figure 3.10A, lysates of fibroblasts cultured on plastic but not collagen demonstrated measureable levels of pSmad2. To show that this was indeed directly related to TGFβ−1, we used a TGFβ-1 neutralizing antibody (2G7) or isotype control (12CA5) in these cultures and found that the increased pSmad2 associated with growth on plastic was ameliorated. We then turned to the tumor bearing lung-derived fibroblasts to assess whether TGFβ−1 signaling was critical for their activation. Previous work from our laboratory demonstrated that MMP2 can release TGFβ-1 from its latent binding partner LTBP3, thereby allowing active TGFβ-1 to initiate signaling and exert its downstream effects [81]. We thus tested whether we could rescue the decreased matrix production phenotype in *Mmp2* KD cells by the addition of active TGFβ-1. Collagen I and IV mRNA transcripts increased in *Mmp2* KD fibroblasts in response to active TGFβ-1 (Figure 3.10B) to similar levels as shctl cells. Notably, shctl fibroblasts showed no effect suggesting that they were already maximally responsive.

To test whether addition of MMP2 to *Mmp2* KD cells could revert the phenotype in a manner dependent on TGFβ−1, we used exogenous recombinant active MMP2 in the presence of the

2G7 TGFβ-1 neutralizing antibody or isotype control antibody. In *Mmp2* KD fibroblasts, collagen expression was stimulated by rhMMP2. This MMP2-stimulated increase in collagen expression was ablated when TGFβ-1 neutralizing antibody was also included (Figure 3.10C). As expected, addition of recombinant MMP2 to shctl cells, similar to the treatment with active TGFβ-1, did not result in significantly increased collagen expression (data not shown). Since these data suggested a critical link between MMP2 and active TGFβ−1 signaling in fibroblasts, we returned to the tumor model to test *in vivo* relevance. Immunofluorescent staining of pSmad2 showed significantly higher levels associated with tumors in wildtype mice compared to *Mmp2*$^{-/-}$ mice (Figure 3.10D). Taken together these results are consistent with a model whereby MMP2 regulates the phenotype of tumor-derived fibroblasts by activating TGFβ-1.

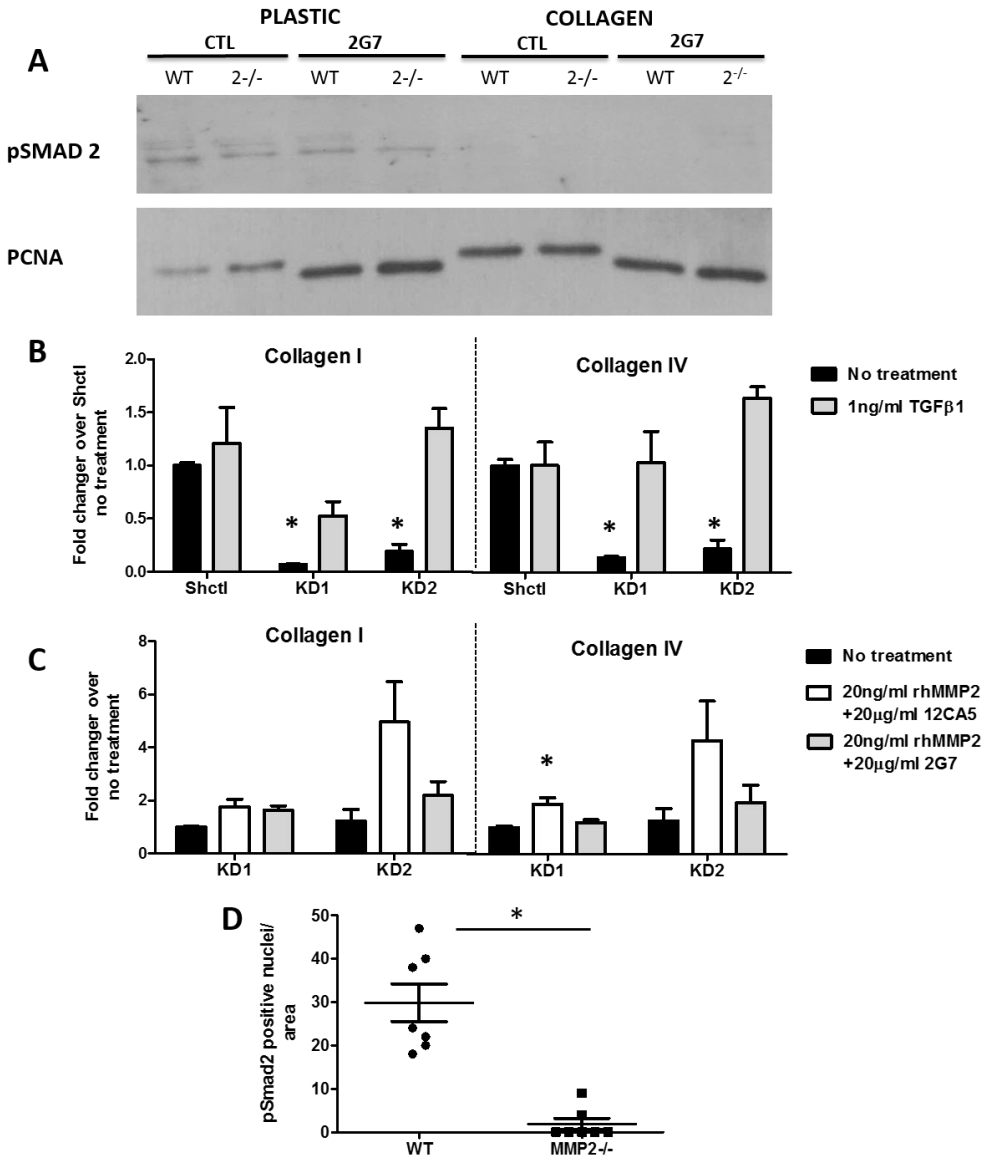

Figure 3.10: Active TGFβ-1 is sufficient to rescue the collagen expression phenotype of lung-tumor fibroblasts. (A) Immunoblot for phosphorylated Smad2 and PCNA (loading control) from quiescent WT or *Mmp2*$^{-/-}$ fibroblasts grown on collagen or after transfer to culture on plastic, in the presence of a TGFβ-1 neutralizing antibody (2G7) or isotype control (12CA5). Numbers below each lane indicate level of pSmad2 corrected for loading control. (B) Expression of *ColI* and *Col*IV transcripts in Shctl or *Mmp2* KD fibroblasts treated with 1ng/ml active mouse TGFβ-1. Values are relative to Shctl no treatment, using *Gapdh* levels for normalization, *p<0.05 (C) Expression of *ColI* and *ColIV* transcripts in *Mmp2* KD fibroblasts treated with 20ng/ml rhMmp2 and either 20µg/ml 12CA5 control IgG or 20µg/ml 2G7 TGFβ-1 neutralizing antibody. Values are relative to no treatment, using *Gapdh* levels for normalization. Asterisks indicate significant differences over control, *p<0.05 (D) Levels of pSmad2 in sections of tumor-bearing lungs from WT or *Mmp2*$^{-/-}$ mice. p =0.0017

MMP2 correlates with collagen signatures in stroma of breast cancer patients. Our data are supportive of an MMP2-dependent collagen signature in mouse models. We next confirmed the relevance of these findings to human breast cancer patients. We used a publicly available dataset, GSE33692 from Knudsen and colleagues [221], comprised of stromal tissue from 45 breast cancer patients with ductal carcinoma *in situ* or invasive ductal carcinoma to explore the relationships between expression of *MMP2* and various stromal molecules. As expected from our proposed model of MMP2-mediated TGFβ−1 activation, there was no significant correlation between *MMP2* and *TGFβ-1* expression (Figure 3.11A). There were however, significant correlations between expression of *MMP2* and the activation markers *ACTA2* and *vimentin* (Figure 3.11B-C). Importantly, *MMP2* expression correlated with collagen I expression in the stromal compartment of human breast cancers (Figure 3.11D).

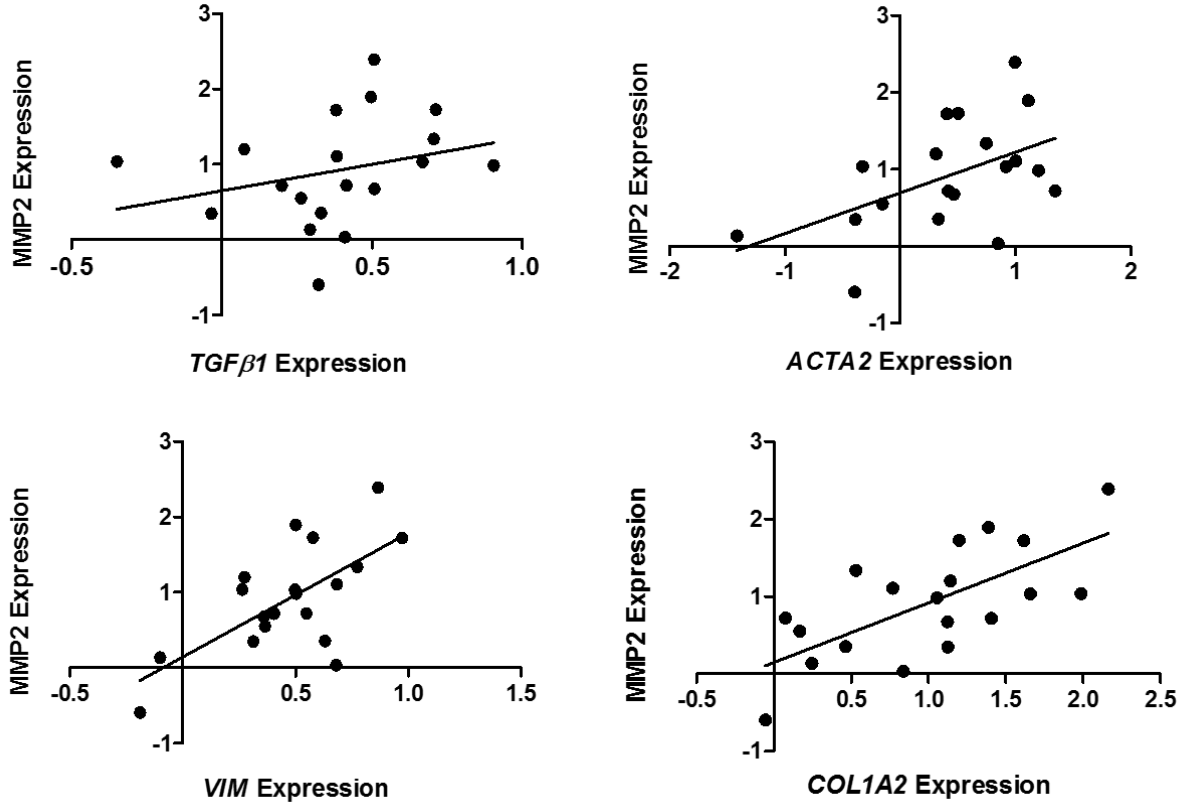

Figure 3.11. MMP2 levels correlate with expression of several fibroblast-associated transcripts in the stromal component of tumor tissue from breast cancer patients. Correlations between levels of *MMP2* expression and (A) *TGFβ1* (p=0.247), (B) *ACTA2* (p=0.025), (C) *VIM* (p=0.0019) or (D) *COLIA2* (p=0.0012) expression in breast tumor stroma from 45 patients. Data were extracted from publicly available dataset GSE33692.

Discussion

Our data show that in two different models and genetic strains (PyVT-R221A cells in FVB/n mice and EO771 cells in C57BL/6 mice) mammary pulmonary metastases proliferate less in the absence of host-derived *Mmp2*. These results echo findings from other metastasis models that demonstrated reduced tumor growth when MMP2 was absent from host cells [201]. Both spontaneous and experimental metastases of B16 melanoma and Lewis lung carcinoma cells were reduced in *Mmp2*$^{-/-}$ animals. In contrast to our findings, however, the effects were attributed to reduced angiogenesis in mutant animals. We observed no significant change in overall vascularity between WT and *Mmp2*$^{-/-}$ animals, but rather reduced tumor cell proliferation. Our assessment of vascularity was performed using tumor foci of similar sizes to allow direct comparison. The localization of MMP2 to fibroblasts is supported by previous studies conducted in primary breast cancers [198, 222], and suggests that these cells play an integral role in MMP2 dependent tumor cell proliferation.

Our *in vitro* proliferation findings show that there was a significant decrease in the growth of tumor cells treated with conditioned media from *Mmp2* KD cells in 3D compared to media from control cells. Significant decreases in tumor cell proliferation were also seen when *Mmp2* KD fibroblasts and tumor cells were in direct contact compared to control fibroblasts. Together, these results suggest a role for soluble growth factors, and perhaps also contact-mediated effects. Identification of relevant soluble factors is ongoing, however matrix molecule production by the fibroblasts may also play a role. Previous studies have shown that modulation of collagen density and architecture by fibroblasts leads to tumor cell proliferation and progression in mouse models of breast cancer [49, 223].

Many studies have shown that tumor associated fibroblasts exhibit an activated phenotype that resembles differentiation into myofibroblasts. These activated cells enhance tumor growth both *in vivo* and *in vitro* [38, 224]. Because MMP2 localized to fibroblasts *in vivo* and its loss impacted tumor growth, we investigated if reduced proliferation could be due to altered fibroblast activation. Indeed we found that in the absence of MMP2, tumor associated fibroblasts exhibited reduced activation as assessed by *Acta2* and matrix collagen expression. Additionally, quiescent fibroblasts exposed to activation-inducing conditions were unable to become activated in the absence of *Mmp2*, suggesting that *Mmp2* is required for full fibroblast activation. Independent of fibroblast activation status, however, we found that reduced *Mmp2* expression is associated with reduced collagen expression. In support of this, studies of cardiac fibrosis have found that Mmp2 stimulates collagen I expression in cardiac fibroblasts and that this occurs through FAK phosphorylation [147], which is necessary to mediate some TGFβ-1 dependent matrix remodeling [225].

TGFβ-1 is an important mediator of the transition from quiescent to a reactive stroma [226]. In fibrotic conditions, TGFβ-1 induces the differentiation of quiescent fibroblasts to myofibroblasts. These activated fibroblasts then release proteases and cytokines that induce the activity and production of TGFβ-1, creating a feed-forward loop and continual cycle of matrix remodeling. More importantly for our studies, activation of the TGFβ-1 pathway leads to the production of collagens. This coupled with previous studies from our lab [81] prompted us to investigate TGFβ-1 as the molecular mediator of *Mmp2*-dependent collagen expression. Indeed, we found that active TGFβ-1 was sufficient to restore collagen expression in the absence of Mmp2. Additionally, Mmp2-dependent increases in collagen expression could be ablated by

neutralization of TGFβ-1 and not with control antibody. Studies of fibrosis similarly found that MMP2 was essential for active TGFβ-2 induced fibrosis and matrix contraction [227].

While high mammographic density, which is associated with increased fibrillar collagen [228, 229], is a known risk factor for breast cancer development [230], there is also an association of increased collagen with aggressiveness [231] and metastatic lesions [232]. Furthermore, *COLIA1* and *COL1A2* were part of a 17-gene signature associated with decreased survival in multiple primary solid tumors [233]. Our correlative studies of MMP2 and collagen expression support a model where increased MMP2 expression is associated with collagen in the stroma of breast cancer patients. These studies shed light on a novel mechanism whereby MMP2 promotes breast tumor progression by mediating increased collagen expression. Although MMP2 expression may be protective in some disease settings [234, 235], this appears not to be true in breast cancer.

Stromal MMP2 expression and its pleiotropic effects present a possible therapeutic target for breast cancer patients. Past cancer clinical trials used broad spectrum inhibitors with debilitating side effects to target multiple MMPs [119, 120]. However, we now realize the importance of using selective inhibitors due to the detrimental effects of inhibiting protective MMPs as well as the importance of cell type specific MMP production. Our studies reveal a new role for MMP2 in breast cancer progression by enhancing tumor cell proliferation, potentially via regulating collagen in fibroblasts. Reduction of MMP2 levels in an effort to curb tumor promoting collagen expression might provide improved treatment modalities for breast cancer metastasis.

CHAPTER IV

Closing Remarks

Summary of findings

The morbidity and mortality of breast cancer patients is dependent on metastasis of tumor cells to distant organs. As hypothesized by Stephen Paget, the "soil", which is made up of multiple cell types and their secretory factors, promotes tumor cell colonization and outgrowth. These studies provided the groundwork for examining mechanisms as to how tumor stromal interactions influence tumor progression. In this body of work, we show the importance of stromal fibroblast derived MMP2, to tumor cell outgrowth *in vitro* and *in vivo*.

MMP2 derived from host fibroblasts potentiates the proliferation of pulmonary experimental metastases.

Fibroblasts are responsible for secretion of many soluble factors including remodeling enzymes like MMP2 and growth factors such as TGFβ-1. MMP2 can enhance tumor progression through inflammation, angiogenesis and degradation of the ECM. Early *in vivo* studies by our research group used MMTV-PyVT/WT or MMTV-PyVT/MMP2$^{-/-}$ mice to examine host contributions of MMP2 to tumor growth. There was no difference seen at the primary site, however spontaneous lung metastasis was significantly decreased in MMP2 null mice (unpublished data). This suggested that host MMP2 mediates site-specific metastases. This led to experimentation to understand the difference between the lung stroma of the WT and MMP2$^{-/-}$ mice. R221A mammary tumor cells were intravenously injected into WT and MMP2$^{-/-}$ mice. Loss of host MMP2 resulted in reduced proliferation of experimental lung metastases. Further, αSMA^{+}

(activated) fibroblasts in tumor bearing lungs were identified as the major source of MMP2, although expression was also noted in hematopoietic cells.

To better understand the mechanism behind fibroblast MMP2 mediated metastasis, tumor derived fibroblasts were isolated from tumor bearing lungs of WT mice. To understand the intimate crosstalk between fibroblasts and tumor cells and the role of MMP2 in this interplay, MMP2 was knocked down in fibroblasts and used in co-culture with tumor cells to mimic the tumor microenvironment. Enhancement of tumor growth was observed in both conditioned media and co-culture studies. However, this effect required growth in 3D, since no significant difference was observed in 2D studies. These studies suggested that fibroblast MMP2 enhances tumor growth by means that involve interaction with the extracellular matrix.

Fibroblast activation status is dependent on MMP2 expression.

Characterization of control and MMP2 KD fibroblasts revealed differences in activation status and ECM production. MMP2 KD fibroblasts had reduced expression of αSMA and vimentin as compared to control cells, suggesting that they were not activated. The reduction in MMP2 was also associated with decreased expression of the matrix molecules, collagen and fibronectin. A second independent experiment using quiescent cells from WT and $MMP2^{-/-}$ fibroblasts produced similar results. In addition, $MMP2^{-/-}$ quiescent fibroblasts could not achieve full activation in response to tumor cell conditioned media or by growth on plastic in the presence of serum, conditions known to artificially activate fibroblasts *in vitro.*

MMP2-dependent fibroblast activation and collagen expression is mediated by TGFβ-1.

Because TGFβ-1 is a potent activator of fibroblasts, its regulation was investigated as a potential mechanism. Quiescent cells cultured on plastic showed activation of TGFβ-1 signaling whereas cells cultured on collagen, which remained quiescent, did not suggesting that TGFβ-1 signaling was associated with the ability of the fibroblasts to undergo activation. Collagen transcripts in MMP2 KD cells were increased upon initiation of TGFβ-1 signaling by treatment with active TGFβ-1. This indicates that active TGFβ-1 is sufficient to rescue the reduced collagen phenotype. Neutralization of TGFβ-1 in activated fibroblasts reduced the MMP2 mediated increase in collagen expression, suggesting the function of MMP2 in matrix transcript expression is mediated by activated TGFβ-1 signaling. Active TGFβ-1 signaling was noted in the stroma of lung mets *in vivo* and was reduced in $MMP2^{-/-}$ mice compared to WT. Additionally, MMP2 gene expression correlated with markers of fibroblast activation and collagen I expression in breast cancer patients.

The main finding from this work was a novel autocrine effect of MMP2 on fibroblast activation and production of ECM. Although it was previously known that MMP2 could activate TGFβ−1, at least in osteoclasts, and that TGFβ−1 signaling in fibroblasts leads to activated myofibroblasts, this work is the first to show that in the setting of metastatic outgrowth, tumor cell proliferation is dependent on fibroblast activation by MMP2 in a manner associated with TGFβ−1 activation (Figure 4.1A). These novel findings increase our understanding of how an extracellular protease can profoundly influence the tumor microenvironment to support metastatic tumor growth.

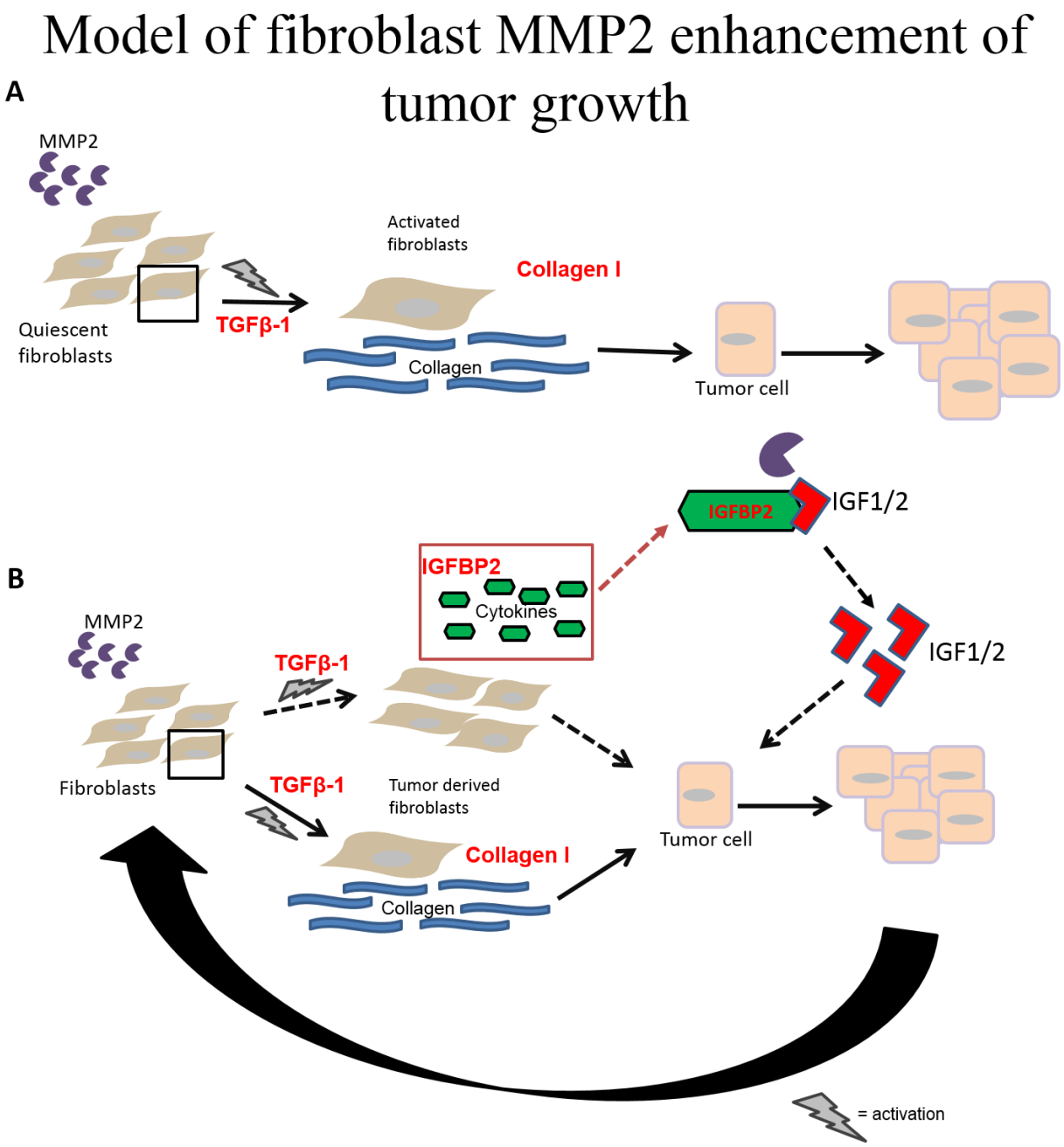

Figure 4.1 Model of fibroblast MMP2 enhancement of tumor growth. This work describes how MMP2 potentiates the outgrowth of metastatic mammary tumors in the lung by regulating fibroblast activation via TGFβ-1. (A) MMP2 activation of fibroblasts by TGFβ-1 is necessary for production of matrix proteins and subsequent tumor outgrowth. (B) MMP2 activation of fibroblasts also leads to differential production of cytokines, such as IGFBP2, which can undergo cleavage by MMP2. This potentially increases the bioavailability of IGF ligands leading to enhanced tumor outgrowth.

Unresolved questions and future directions

MMP2 is produced by a number of cell types within the tumor microenvironment. The functional significance of MMP2 varies with cellular source. Additionally, a single source may exert different effects on multiple targets. In the setting of cancer, some effects of MMP2 are pro-tumorigenic (for example, myofibroblast activation resulting in tumor cell proliferation as described previously), while others may be anti-tumorigenic (for example, tissue repair limiting astrocytoma growth.) [236]. This confounds the effectiveness of global MMP2 inhibition and suggests that in depth knowledge of specific effects of this enzyme in tumor progression are important to appreciate before proposing inhibitory strategies.

Is there a spatial requirement for MMP2-dependent tumor growth?

Despite MMP2 protein being produced by tumor cells *in vitro,* MMP2 was undetectable in tumor cells *in vivo.* It is likely that *in vitro* culture allows for spatial confinement and concentration of the secreted MMP2, allowing for detection. However *in vivo*, the soluble nature of MMP2 allows for diffusion of the enzyme throughout the ECM, resulting in concentrations that may be below detection or the levels required to enhance tumor growth. A significant amount of MMP2 was detected associated with cells in the tumor stroma; however, the lack of MMP2 from these cells was sufficient to inhibit tumor outgrowth in the lungs of MMP2 null animals. The apparent pro-tumorigenic function of stromal-derived MMP2 could also reflect that MMP2 and its substrate(s) need to be in very close proximity as might happen when they are both produced by the same cell, suggestive of a spatial requirement for MMP2 activity.

Requirements for spatial localization of MMP2 were also evidenced *in vitro* by the lack of tumor growth in 2D assays. Active MMP2 should be able to cleave, and thus activate, soluble factors suspended in serum-containing control or fibroblast conditioned media in 2D assays if MMP2 localization is irrelevant for the tumor enhancing effect. Although it's possible that serum does not contain all the necessary cofactors or their correct conformations, this caveat should be circumvented in conditioned medium from fibroblasts. Studies within this work demonstrated that neither serum with added active MMP2 (Figure 2.5A) nor conditioned medium containing active MMP2 (data not shown) resulted in mammary tumor growth in 2D. However, treatment of tumor cells with conditioned medium under 3D growth conditions resulted in significantly decreased growth in the absence of MMP2 (Figure 3.7A). Perhaps the presence of ECM serves as anchorage for MMP2, bringing the enzyme in optimal proximity to cleave soluble factors potentiating tumor growth. One simple idea to test whether ECM is sufficient to potentiate MMP2 function would be to repeat the 2D assays but with addition of both recombinant active MMP2 and matrix. This would be in contrast to the previously performed experiment where recombinant MMP2 was added either to 2D plates of cells or 3D spheroids.

It is also possible that additional proteins facilitate MMP2 enzyme activity at a particular location by forming a complex at cell membranes. The Weiss group and others have shown that MT1-MMP activity is required at invadopodia for the degradation of ECM [237, 238]. A similar mechanism could be mediating tumor growth at the tumor-stromal interface. The investigation of novel proteins that may participate in regulating the localized activity of MMP2 could be revealed with the use of yeast two-hybrid screens. Further analysis of the functional significance

of these proteins would be mediated by genetic manipulation in cells and examining their impact on tumor growth *in vivo.*

What is the functional significance of different sources of MMP2?

The studies within this work reveal a novel role for fibroblast derived MMP2 and demonstrate that MMP2 from fibroblasts potentiates fibroblast activation and subsequent metastatic tumor outgrowth. Additionally, MMP2 protein was also expressed *in vivo* and *in vitro* by immune cells and *in vitro* by tumor cells. It is not clear from our studies if these different pools of MMP2 have additional functions that would be relevant in tumor progression, although others have suggested pro-migratory roles for tumor-derived MMP2 [113], and T-cell activation roles for immune-derived MMP2 [239].

A future direction from our work would be *in vivo* assessment of the specific contribution of fibroblast MMP2. One way this could be done would be to perform subcutaneous injections of admixed tumor and WT or MMP2 KD fibroblasts into WT mice and determine if tumor outgrowth *in vivo* is diminished upon the loss of MMP2 from fibroblasts. Optimally, measuring the attenuation of metastatic tumor outgrowth attributed to fibroblast derived MMP2 only should be assessed. The use of a global MMP2 knockout mouse model presents a caveat for *in vivo* studies due to multiple sources of MMP2. A tissue-specific knockout of MMP2, such as the Cre-Lox system directed under the FSP1 promoter, would allow delineation of the contribution of fibroblast derived MMP2 from that of other sources. Although the *in vitro* studies are supportive of fibroblast MMP2 being important, MMP2 was also expressed by immune cells *in vivo*. It is possible that immune cell MMP2 may contribute to metastatic tumor outgrowth. Immune cell

specific knockouts of MMP2 under, for example LysM or CD4 promoters also using the Cre-Lox system, would enable these studies.

Does loss of MMP2 lead to alterations in the ECM and what is the functional significance?

The effect of MMP2 ablation on tumor cell proliferation could possibly be a secondary effect mediated by the extracellular matrix. The absence of MMP2 in fibroblasts could result in generation of a different matrix from that of control fibroblasts. As revealed in these studies, a reduction in MMP2 expression in fibroblasts leads to reduced collagen and fibronectin expression. Tumor cells forming in this suboptimal matrix may have reduced access to proliferative signals normally sequestered within the ECM. Studies involving tumor cells cultured on a decellularized fibroblast matrix from control or MMP2 KD fibroblasts can be employed to examine if they will differentially affect tumor growth. Preliminary studies conducted using MMP2 KD fibroblasts to produce a decellularized matrix exhibited a much thinner matrix that easily laminated off the tissue culture dish compared to that from control fibroblasts (data not shown.) If the results of tumor cells being grown on these matrices reveal that matrix from MMP2 KD fibroblasts reduces tumor cell growth, follow-up studies would be needed to test whether this was because of the differences in ECM composition or differences in ECM architecture that may be due to, for example, crosslinking mediated by LOX enzymes. Studies from the Erler group[240] demonstrate that not only is the production of collagen important for ECM, but also collagen crosslinking. Furthermore studies by Pickup and colleagues demonstrated that the activity of LOX enzyme, largely produced by activated fibroblasts, promotes metastasis of mammary carcinomas, and that TGFβ-1 signaling in fibroblasts drives LOX expression[46]. Future studies could include solubilization of the control

or MMP2 KD fibroblast produced matrix and utilizing protein arrays to look for differential protein expression followed by genetic modulation of resulting targets. To assess the amount of crosslinking that occurs in ECM produced by control or MMP2 KD fibroblasts, architectural studies of collagen crosslinking using biophysical techniques such as infrared spectroscopy could be performed.

What is the functional significance of altered fibroblast cytokine profiles on tumor growth?

Altered expression of MMP2 in fibroblasts also resulted in differences in cytokine production (Figure 4.2). Results using conditioned media derived from MMP2 KD cells showed an increase in MMP3 and IGFBP2 and a decrease in osteoprotegerin, OPG, as compared to WT conditioned media. Further validation of these potential targets using western blotting and ELISA is needed

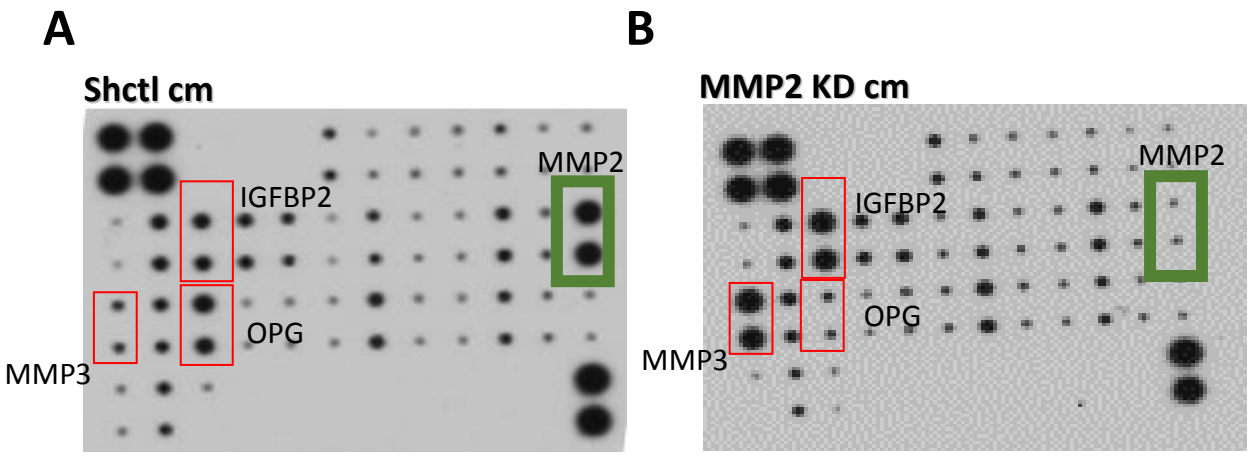

Figure 4.2: Loss of MMP2 in fibroblasts alters the cytokine profile in favor of anti-tumorigenic factors. Autoradiograph of a cytokine array using conditioned media from Shctl (A) or MMP2 KD (B) tumor derived fibroblasts. Boxes delineate factors that appear changed between two groups. cm=conditioned media; Shctl=sh-control; MMP=matrix metalloproteinase; IGFBP=insulin-like growth factor binding protein; OPG=osteoprotegerin

to confirm differential production of these cytokines upon the absence of MMP2.

Potential for MMP3 as a future target of interest

There is some support for investigating MMP3, or stromelysin 1, as a target molecule for further study. Studies have shown that MMP3 has both protumorigenic and protective roles in cancers. Sternlicht and colleagues demonstrated in a tetracycline inducible mouse model that MMP3 leads to the development of invasive tumors and promotes conversion to malignancy in the mammary tumors of MMP3 WAP-STR1 transgenic mice [241]. Recent studies have identified that the hemopexin domain of MMP3 is responsible for invasive behavior in breast cancer and mammary gland branching morphogenesis through the interaction with heat shock protein 90β which was present extracellularly [242]. In pancreatic cancer, a cohort of human PDAC tissue biopsies showed MMP3 to be highly correlated with Rac1b, an isoform of Rac1. Treatment of pancreatic cancer cells with MMP3 *in vitro* induced the expression of Rac1b. Additionally, transgenic mouse models of MMP3 and activated KRAS in pancreatic acinar cells stimulated immune infiltration and metaplasia, early signs of PDAC [243]. If MMP3 is indeed playing a protumorigenic role in our model, then it is counterintuitive as to why it is upregulated in mice that have reduced tumor growth.

In contrast to the previous studies, MMP3 null animals have an increased susceptibility to squamous cell carcinoma [126]. The overexpression of MMP3 in keratinocytes of transgenic mice resulted in a decrease in the number of tumors in response to chemicals used to induce squamous cell carcinoma. Although vascularity was increased, immune cell infiltration and tumor growth were unaffected. Overexpression of MMP3 in a human papilloma cell line

similarly demonstrated a reduced ability to form palpable tumors in immunocompromised mice, with biopsies showing non-proliferative, highly differentiated epithelial cells [244]. At this time, it is unclear why MMP3 would be upregulated in mice with reduced mammary tumor metastasis, and although interesting, leads to MMP3 as a lower priority target for investigation.

Potential for OPG as a future target of interest

OPG is a soluble decoy receptor for the cytokine Receptor Activator of NFκB (RANK) ligand, RANKL, and it is predominantly described in immunity and bone homeostasis. OPG is well known for its role in the maturation of osteoclasts, where it is a negative regulator of signaling by RANKL via preventing it from binding RANK. This subsequently inhibits the cell contact dependent signaling between osteoblasts and osteoclast precursors needed for osteoclast differentiation [245]. OPG null mice have decreased numbers of osteoclasts and, as a result, osteoporosis [246]. Conversely, overexpression of OPG in transgenic animals results in osteopetrosis, or increased bone density [247]. Recent studies more intensely investigate the usage of OPG in the prevention of breast cancer metastasis to bone, which results in painful, incurable lesions due to increased resorption by osteoclasts. Usage of OPG-Fc resulted in the inhibition of proliferation of dormant MDA-MB-231 cells within the bone, which could have led to bone metastases [248]. Although there is a very important role for OPG in breast cancer metastases, currently this involves breast to bone metastases. A role for breast to lung metastases has yet to be uncovered, therefore making OPG less than a primary target for these future studies.

Potential for IGFBP2 as an MMP2 substrate and future target of interest

There is profound support for investigating IGFBP2 as a potential target. IGFBP2 is a member of the insulin like growth factor (IGF) system consisting of growth factors (IGF1 and IGF2), IGF1 receptor and insulin receptor A (IGF1R and IR-A, respectively), and a number of IGF binding proteins (IGFBP1-6). The IGF1R transmits the majority of IGF signals and binds both IGF1 and IGF2 [249]. The structural and functional similarities between IGF1R and IR-A allow for overlap in signaling [250]. IGF1 receptors can homodimerize or heterodimerize with IR-A, which is over expressed in cancer [249, 251]. Downstream signaling of IGF1R includes phosphorylation of IRS proteins followed by activation of the PI3K/Akt or Ras/MAPK pathways [252]. Additionally, IGF2 also binds IR-A and with slightly higher affinity than with IGF1R, but does not bind IR-B [251]. Activation of IR-A by IGF2 leads to growth promotion during embryonic development [253] as well as proliferation in 3T3 fibroblast-like cells *in vitro* [254]. This is distinct from insulin stimulation of IR-A signaling which leads to glucose uptake [251]. The initiation of mitogenic signaling by IGF2 activation of IR-A is through Shc/Erk1/2, as demonstrated in leiomyosarcoma cells [255]. IGF2 can further bind the IGF2/mannose-6-phosphate receptor, though it is structurally unrelated, and acts as a means to sequester IGF2.

The bioavailability of IGFs can be tempered by their binding to the IGFBPs [256]. This could also result in an increase in half-life of IGFs. Mechanisms have been uncovered which demonstrate that IGFBPs can enhance signaling by IGFs [257–259]. Modulation of IGFBP levels have been investigated in an effort to attenuate mammary tumor growth. IGFBP2 expression was increased in breast cancer samples of archival tissue from the Breast Cancer Outcomes Unit tissue microarray (BCOU TMA) compared to a benign TMA. IGFBP2 was not

an overall prognostic indicator, but showed a strong trend to poor survival in hormone receptor negative samples. Tumor studies showed that lentiviral overexpression of IGFBP2 in MDA-MB-231 cells, which are estrogen receptor negative, resulted in increased mammary tumor growth *in vitro* and in mice *in vivo*. Treatment of these cells or MDA-MB-468 cells, which endogenously produce IGFBP2, with an antisense oligonucleotide specific for IGFBP2 decreased tumor growth and sensitized tumors to chemotherapy *in vitro* and decreased tumor growth *in vivo* [260].

Association of increased IGFBP expression with breast cancer could be attributed in part to simultaneous increases in IGF ligands that are produced by tumor cells [261]. Indeed, studies have also linked increased IGF expression with breast cancer. In a nested case-control study with pre- and postmenopausal women, there was a positive association for breast cancer risk amongst premenopausal women with the highest levels of serum IGF1 [262]. A recent phase I clinical trial with a mammary specific IGFI inhibitor demonstrated reduced growth and increased apoptosis in premalignant lesions via decreased Erk1/2 and Akt phosphorylation [263].

If IGFBP2 is validated as being increased in fibroblast conditioned media in the absence of MMP2, then additional studies modulating IGFBP2 levels *in vitro* and *in vivo* and their effect on tumor growth are warranted. IGFBP2 mostly acts to sequester IGF ligands, having a higher affinity for IGF2 than IGF1 [264]. Because IGF2 can bind both IGF1R as well as IR-A, IGFBP2 binding up free IGF2 could attenuate downstream signaling that could lead to tumor survival and proliferation. Although many studies regarding other IGFBPs have been conducted, less is known regarding the functional significance of IGFBP2, especially in breast cancer. Investigation of how fibroblast derived IGFBP2 is regulated by MMP2 and this potential to

enhance breast tumor growth may reveal more novel insights into the mechanisms of metastatic breast tumor outgrowth and provide a target for therapeutic intervention.

Characterization of IGF ligand and receptor expression in breast tumor cells should be conducted as this can vary between cell lines and degree of tumor aggressiveness. Neutralization of IGFBP2 in conditioned media from control or MMP2 KD fibroblasts and the resulting effects on tumor growth in 3D should also be investigated, with the expectation that tumor growth inhibition using MMP2 KD conditioned medium will be ablated upon IGFBP2 neutralization due to increased receptor activation by free IGF ligands. Conversely, addition of IGFBP2 to control or MMP2 KD fibroblast conditioned media should bind up free IGF ligands, resulting in reduced tumor growth of cells cultured with control cell conditioned medium. Long term goals would involve overexpression of IGFPB2 in the fibroblasts of WT and $MMP2^{-/-}$ mice and assessing metastatic outgrowth and downstream signaling. Ablation of IGFBP2 in mice results in a modest phenotype of 50% smaller spleen sizes in young adult males compared to WT, having resolved by 9 months of age, and a decreasing trend in overall size of null mice. However, these effects are concomitant with increases in the expression of other IGFBPs, which suggests functional redundancy and likely obscures any effect due to IGFBP2 [265]. Alternatively, global overexpression of IGFBP2 in mice demonstrated a reduction in the percentage of body weight gain post-weaning, and no compensatory increase in IGF ligands or IGFBPs [266]. Importantly, overexpression of IGFBP2 in kidney fibroblasts (293 cells) led to inhibition of IGF-dependent colon carcinoma cell growth as well as fibroblast proliferation [267]. It is possible that MMP2 degrades IGFBP2, as other IGFBPs have been found to be a substrate for MMP2 [268, 269]. Additional studies would involve determining if IGFBP2 is a direct substrate for MMP2 and

whether this frees IGF ligands for activation of downstream signaling *in vitro* and *in vivo.* A proposed model of how IGFBP2 may contribute to the reduced proliferation of mammary tumor cells in the lungs of MMP2-null mice is depicted in Figure 4.1B. Further insight on the mechanisms behind activation of IGF signaling in various breast cancers and how protective or protumorigenic effects are manifested is necessary.

Clinical implications

The mass inhibition of MMPs in clinical trials proved to be unsuccessful due to a number of reasons. Perhaps most important of these reasons is that the inhibition of protective MMPs undermines therapy and enhances tumor growth. Thus, knowledge on the functions of specific MMPs is necessary. The previous belief that the sole function of MMPs is matrix degradation leading to metastasis, and therefore MMPs should be inhibited, was naïve. New evidence, such as the studies presented here, demonstrate that MMPs have diverse roles in multiple tumor types and can mediate autocrine as well as paracrine signaling to enhance tumor growth.

Our studies demonstrating MMP2 inhibition in fibroblasts results in reduced matrix protein production, i.e. collagen, opens the doors for investigation of selective MMP2 inhibitors for the reduction or stasis of desmoplasia. High ECM content in the tumor microenvironment is commonly associated with enhanced tumor growth and ultimately poorer prognosis. Targeting the tumor enhancing stroma as adjuvant therapy in combination with traditional chemotherapy may slow tumor growth and cancer progression to increase five year survival. Additionally, patients suffering from fibrosis and other fibrotic diseases such as chronic kidney disease or even

cataracts, may also benefit from selective MMP2 inhibition to impede additional ECM production and further tissue damage.

Studies presented in this work demonstrate correlation of MMP2 and fibroblast activation markers in patient samples. These correlative studies can be used as the basis for larger case-control studies to investigate the potential of using a MMP2 and activation marker gene signature to identify patients at higher risk for desmoplastic tumors and poor prognosis. Early identification of those patients with poorer outlooks may lead to earlier decisions on more aggressive treatments as opposed to after the development of metastatic lesions when survival rates decrease.

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