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Characterization of Novel Extracellular Matrix (Ecm) Proteins (Mgp and Lumican) and Their Implications in Vascular Development, Angiogenesis, and Cancer

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CHARACTERIZATION OF NOVEL EXTRACELLULAR MATRIX (ECM) PROTEINS (MGP AND LUMICAN) AND THEIR IMPLICATIONS IN VASCULAR DEVELOPMENT, ANGIOGENESIS, AND CANCER

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ABSTRACT

Extracellular matrix (ECM) constitutes a large component of our tissue structure. Primarily, ECM provides structural and adhesive support to our cells, but it also controls cellular signaling and behavior. Homeostasis of extracellular matrix composition and function is maintained by our body through a balanced synthesis, degradation and remodeling of ECM. However, under pathological conditions and genetic mutations, ECM homeostasis is disrupted due to deregulation in ECM synthesis, assembly, remodeling, and degradation. A number of diseases, including cardiovascular diseases and cancer, are found to occur due to alterations in ECM. Therefore, targeting ECM can be an attractive therapeutic approach to treat these diseases, and it requires our complete understanding of the ECM molecules and the molecular mechanism it employs in controlling cellular functions. To this end, this study is aimed at the characterization of two ECM proteins—Matrix Gla Protein (MGP) and Lumican—for their roles in vascular development, angiogenesis, and cancer. Findings from this study show that MGP is a critical ECM regulator that promotes angiogenic resolution by suppressing endothelial sprouting and stabilizing vascular lumen formation. In addition, MGP also inhibits tumor growth by inhibiting tumor angiogenesis. On the other hand, our findings show Lumican suppresses tumor growth and has anti-angiogenic activity in a context specific manner.

PREFACE

This study provides a review on ECM proteins and their importance in human health and diseases. The main focus of this study, however, is to highlight Matrix Gla Protein (MGP) and Lumican as critical ECM regulators of angiogenesis and cancer. Our findings on MGP are published in *Microvascular Research* Journal and we plan to submit our findings on Lumican to a peer-reviewed journal soon in the future.

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CHAPTER I

EXTRACELLULAR MATRIX (ECM): IMPLICATIONS FOR HUMAN HEALTH AND DISEASES

Extracellular Matrix: Structural and Functional Overview

Our tissues are not made up of only cells but also contain a significant volume of extracellular spaces. These extracellular spaces are packed with a heterogeneous fusion of macromolecules including proteins, glycoproteins, proteoglycans, and polysaccharides deposited by cells, which are collectively called the extracellular matrix (ECM) [1]. Although the identity of ECM constituents is far from complete, a subset of those that have been identified and characterized is listed in Table 1.

Primarily, there are two distinct types of ECM—the basement membrane and interstitial matrix. The basement membrane is a specialized ECM distinct from that of interstitial matrix. The basement membrane is produced by epithelial cells, endothelial cells, and stromal cells and separates epithelium or endothelium from its underlying stroma. It is more compact and less porous than interstitial matrix and is mainly composed of type IV collagen, laminin, fibronectin, perlecan, and linker proteins such as nidogen and entactin [2]. On the other hand, interstitial matrix is markedly different from the basement membrane in its composition and function. Interstitial matrix is comprised

of fibrillar collagens, proteoglycans, and glycoproteins, which are highly charged and hydrated, and thus provides great tensile strength to tissues.

ECM has distinct physical, biochemical, and biomechanical properties, which collectively impacts cell behavior [1]. For instance, physical properties of ECM include its rigidity, insolubility, porosity, and spatial arrangement that confer upon its role as scaffold to support tissue structure. Cell surface proteins such as integrins bind to various ECM molecules including collagens, glycoproteins, and proteoglycans and help cellular adhesion. Furthermore, cell-matrix interaction also creates a mechanical signal that changes intracellular cytoskeletal rearrangements and control cell motility. In addition, cell-matrix interactions define cell shape and polarity. More importantly, ECM provides cell signaling and regulates various cellular parameters including proliferation, survival and growth, apoptosis, and differentiation during development as well as in adult life of living organisms. A subset of ECM molecules, besides their structural roles, also has cell signaling and regulatory functions, and these molecules are categorized as “matricellular” molecules [3].

Collectively, ECM provides both physical support and biochemical signals to our body cells, which are requisites for cellular organization into multicellular form. Therefore, ECM is highly conserved evolutionarily across species. For instance, a number of ECM molecules such as fibrillar collagen, the basement membrane (collagen IV, laminin, Fibronectin, Perlecan), Fibrillin, Thrombospondin, Agrin, Syndecan, and Glypican are found in simple organisms such as choanoflagellates, sponges, and *c. elegans*. During the evolutionary process, higher organisms such as vertebrates innovated other components of ECM (Integrin, CCN, vitronectin, Tenascin, etc) along with cell-

matrix and matrix-matrix interacting domains [4-7]. Therefore, ECM components are vital elements that are believed to unite the whole animal kingdom into a monophyletic group of multicellular organisms [8]. The importance of cell-matrix interaction is illustrated in Figure 1.

ECM in Human Health and Diseases

Appropriate ECM composition and function is requisite during embryonic development and in the maintenance of tissue homeostasis during adult life [9]. ECM interacts with cells and controls cellular adhesion, migration, differentiation, and morphogenesis, which are critical events during embryonic development. ECM interacts with cells through a number of cell surface receptors such as integrins. To fulfill their biological need for appropriate adhesion, signaling cues, and migratory passage, cells can manipulate the (or their) ECM through a regulated cycle of synthesis, degradation, and, remodeling to create an appropriate extracellular microenvironment [1]. For instance, degradation of the basement membrane and stromal ECM by endothelial cells is a common process during physiological angiogenesis that occurs during wound healing and tissue repair. Additionally, during developmental processes, ECM remodeling and makeover are critical events in guiding proper cellular proliferation, migration, differentiation, and tissue morphogenesis [9]. In addition, infiltration and migration of immune cells during inflammatory response is another common example where the remodeling of both the basement membrane and interstitial ECM takes place for a proper recruitment of immune cells to the infected tissue [10]. Similarly, cell-matrix interactions, matrix remodeling, and matrix degradation is commonly found in the pathophysiology of number of diseases. Most importantly, ECM deregulation is commonly found in cancer

during its growth, invasion, metastasis, and angiogenesis [11]. Collectively, the changes in ECM create a favorable "microenvironment" within the tissue to foster appropriate cellular function both in physiological as well as in pathological conditions. Deregulation of ECM under pathological conditions and its consequences are illustrated in Figure 2.

Changes in ECM is a common mechanism both in physiological as well as in pathological conditions. The changes during physiological processes are temporary which can be reversed to its homeostasis. However, under pathological conditions, changes in ECM occur permanently and are beyond the control of our bodily regulations. For instance, under pathological conditions such as cancer invasion, the activity of collagenase and other ECM-degrading proteases such as matrix metalloproteases are persistently higher. Likewise, the deregulation of endothelial membrane tethered MMP (MT-MMPs) dramatically changes angiogenesis responses. Such deregulations in ECM function are commonly found among a number of human diseases including cardiovascular abnormalities and cancer. Therefore, in this chapter, I aim to highlight the importance of ECM in vascular function particularly focusing on the pathophysiology vascular diseases and cancer.

ECM and Vascular Diseases

ECM surrounds vascular tissues, and its proper functioning is critical during vascular development and stabilization of the vascular wall [12-15]. Various cell types present within vascular microenvironment including endothelial cells, mural cells (vascular smooth muscle cells and pericytes), and stromal cells such as fibroblast deposit ECM in the vascular tissues. The basement membrane is in direct contact with endothelial cells and controls endothelial cell behavior such as angiogenic responses. The

interstitial components of ECM interacts with mural cells and together build a stable and mature vascular wall [16-18]. Therefore, alterations in ECM function changes the structure of vascular tissue and ultimately its function. Therefore, a number of vascular diseases are associated with ECM dysfunctions [19-21]. For example, deregulation of collagen due to altered fibroblast function results in fibrosis of cardiac and vascular tissues. Compared to normal fibroblasts, differentiated myofibroblasts deposits significantly increased the level of collagens, which increases the stiffness of cardiac and vascular tissues and thus promote hypertensive conditions. Additionally, these changes in the ECM of the heart greatly impacts systolic and diastolic function, which is also associated with hypertensive conditions [20].

In addition, alterations in ECM due to the dysfunction of ECM degrading enzymes such as matrix metalloproteases (MMPs) are associated with cardiovascular abnormalities. For example, during post-myocardial infarction, the increased MMP activity prevents appropriate ECM replacement needed to recover the damaged tissue and worsens the condition, which can lead to heart failure [22, 23]. Conversely, decreased MMP activity resulting in an excessive accumulation of ECM is also associated with several cardiovascular defects including myocardial stiffness, cardiac hypertrophy, and hypertension. Besides structural damages, alterations in ECM also change cell-matrix interactions, which ultimately change gene expression and cellular function. Therefore, a balanced ECM composition is critical in maintaining tissue homeostasis.

ECM within the vascular tissues plays a central role in the pathogenesis of atherosclerosis [24, 25]. During the development of atherosclerotic lesions, vascular remodeling through degradation and reorganization of matrix scaffold of the vascular

wall is a primary step. For instance, in the inflammation-induced atherosclerosis, degradation of ECM promotes cell migration, proliferation, and tissue remodeling within the vascular wall that allows the development of vascular lesions and plaque build up [26, 27]. Matrix metalloproteases and their tissue inhibitors are critical players in this process since they control matrix degradation and remodeling. Therefore, deregulation of matrix metalloproteases and their tissue inhibitors (TIMPs) are commonly found in atherosclerosis [28]. In addition, Matrix Gla Protein (MGP), a component of ECM is known to have a direct role in the development of atherosclerosis and arteriosclerosis [29, 30]. MGP is a small ECM protein, which has a primary role as a calcification inhibitor. Furthermore, MGP also suppresses atherosclerosis since MGP deficiency in mice promoted atherosclerosis and its overexpression in the atherosclerotic lesion significantly reduced atherosclerotic plaque buildup [31]. In addition to these cardiovascular abnormalities, ECM also regulates angiogenesis, and consequently, the deregulation of ECM is associated with abnormal angiogenesis commonly found in a number of human diseases including cancer.

ECM and Cancer: An Overview

Cancer development involves distinct phases including initiation, growth and survival, angiogenesis, invasion, and metastasis. Initiation of cancer begins by cellular transformation following defects in the cellular program that regulates cell growth and death. In cancer, cells become self-sufficient in pro-growth and survival signals by either 1) gain-of-function mutation in pro-oncogenic genes or 2) loss-of-function mutation in tumor suppressor genes, or 3) both. Once a cell becomes cancerous, it grows as a primary tumor within the local tissue. Eventually, however, primary tumors invade tissues, get

access to the lymph nodes and blood stream, and deposit in distant tissues where they establishes as secondary tumors called metastases. Tumors that remain in local tissues are "benign tumors" and those metastasize to distant tissues are "malignant tumors" [32].

Angiogenesis, invasion, and metastasis are some of the properties of cancer that make it a deadly disease. Primary tumor require blood vessel to supply it with essential nutrients and oxygen to grow beyond certain size (2 mm) and eventually become malignant. Therefore, growing primary tumors drive angiogenesis and orchestrate other cellular and molecular programs to create a pro-tumorigenic niche called the "tumor microenvironment" that fosters the tumors' growth, invasion, and metastasis. In summary, cancer cells are able to acquire unique capabilities such as ability to overcome anti-growth and apoptotic signals to survive and grow, build their own blood vessels by angiogenesis, invade surrounding tissues, and metastasize at distant organs [32]. These unique capabilities acquired by cancer are collectively called "hallmarks of cancer."

ECM and Cancer: Cancer Development and Progression

Appropriate ECM interaction is a requisite in the maintenance of cancer stem cell niche and its fate [11, 33, 34]. Localization of cancer stem cells is critical to maintain contact with surrounding niche cells and obtain paracrine signaling to maintain their stem cell properties. In addition, stem cell interaction with ECM is also critical to maintain their cell polarity and orient mitotic spindle. Such orientation is important to undergo asymmetric cell division, an inherent property of stem cells. Therefore, availability of appropriate ECM for anchorage is a basic need for the maintenance of cancer stem cells.

ECM is a common regulator of cancer development and progression and deregulated ECM dynamics is one of the hallmarks of cancer [11, 35-37]. Cancer cells employ a number of cellular and molecular interactions to change the ECM dynamics and create a tumorigenic niche. For example, increased collagen deposition by cancer cells and cancer-associated fibroblast enhances integrin signaling and promotes cell survival and proliferation [38, 39], which is a requisite for transforming cancer cells. Increased expression of lysyl oxidase (LOX), a collagen cross-linker, is commonly found in cancers [40] where its increased expression enhances cross-linking and creates stiff ECM. Stiff ECM provides an appropriate condition for focal adhesion assembly and consequently the upregulation of cell survival PI3K/MAPK pathway [41]. In addition, a number of ECM proteins and their fragments have pro-or-anti apoptotic effects and tumors are found to selectively manipulate these molecules to evade apoptosis [42, 43]. These and a number of other interactions between cancer cell, ECM, and stromal cell selectively de-regulate ECM to create pro-survival and pro-growth environment necessary for cancer initiation and development.

Furthermore, cancer cells also interact with its stromal microenvironment to selectively alter ECM to promote angiogenesis and its invasion and metastasis [44]. For instance, cancer cells and stromal cells (cancer associated fibroblast, immune cells, endothelial cells, etc.) produce a high level of ECM degrading proteases such as collagenases and MMPs [45]. These enzymes collectively degrade the basement membrane and promote cancer invasion. In contrast, the linearization and thickening of collagen is also commonly found in invasive cancers. Such linearization of stromal collagen fibers enhances cancer cell adhesion and migration during invasion [41, 46].

Therefore, cancers employ both degradation and re-organization of ECM components on a need basis to promote their progression. Tumors also remodel vascular the basement membrane and perivascular ECM to enhance angiogenesis and metastasis. For instance, tumors and stromal cells produce MMPs and collagenases to degrade vascular the basement membrane to drive tumor angiogenesis [47]. In addition, invasive cancer cells also create a more porous and leaky endothelial the basement membrane [48] that promotes metastasis and infiltration of immune cells [49]. Recruitment of immune cells such as tumor-associated macrophages can produce angiogenic growth factors and ECM degrading proteases, which collectively enhances cancer development and progression.

In addition, tumors also secrete factors that influence endothelial cell behavior. Tumors increase integrin expression and enhance endothelial cell-matrix interactions. Cell-matrix interaction via integrins promote endothelial survival, proliferation, morphogenesis [50], and migration required for angiogenesis. Tumors also enhance angiogenesis by suppressing the release of angiostatic fragments from various ECM molecules such as endorepellin (from perlecan), endostatin (from collagen XVIII), restin (from Collagen XV), arresten, canstatin, and tumstatin (from Collagen IV) and create pro-angiogenic tumor microenvironment [51]. Moreover, tumors also produce angiogenic growth factors such as VEGF and FGF and manipulate ECM to localize these factors and create the appropriate angiogenic gradient. For instance, VEGF contains a heparin-binding domain that interacts with heparin-sulfate proteoglycans (HSPGs) [52-54]. Interestingly, tumors are shown to upregulate the expression of these HSPGs (such as perlecan) and localize VEGF gradient to promote angiogenesis. In addition, a number of proteoglycans (such as decorin and Versican) also bind to integrins and other cell surface

receptors present in the cancer and endothelial cells and modulate tumor cell growth, migration, angiogenesis, and metastasis [55-57].

Collectively, ECM dynamics is critical aspects of tumor development and progression, and tumors employ a complex system of cellular and molecular interactions within the growing tumor microenvironment to selectively manipulate its ECM to promote cancer hallmarks such as invasion, angiogenesis, and metastasis.

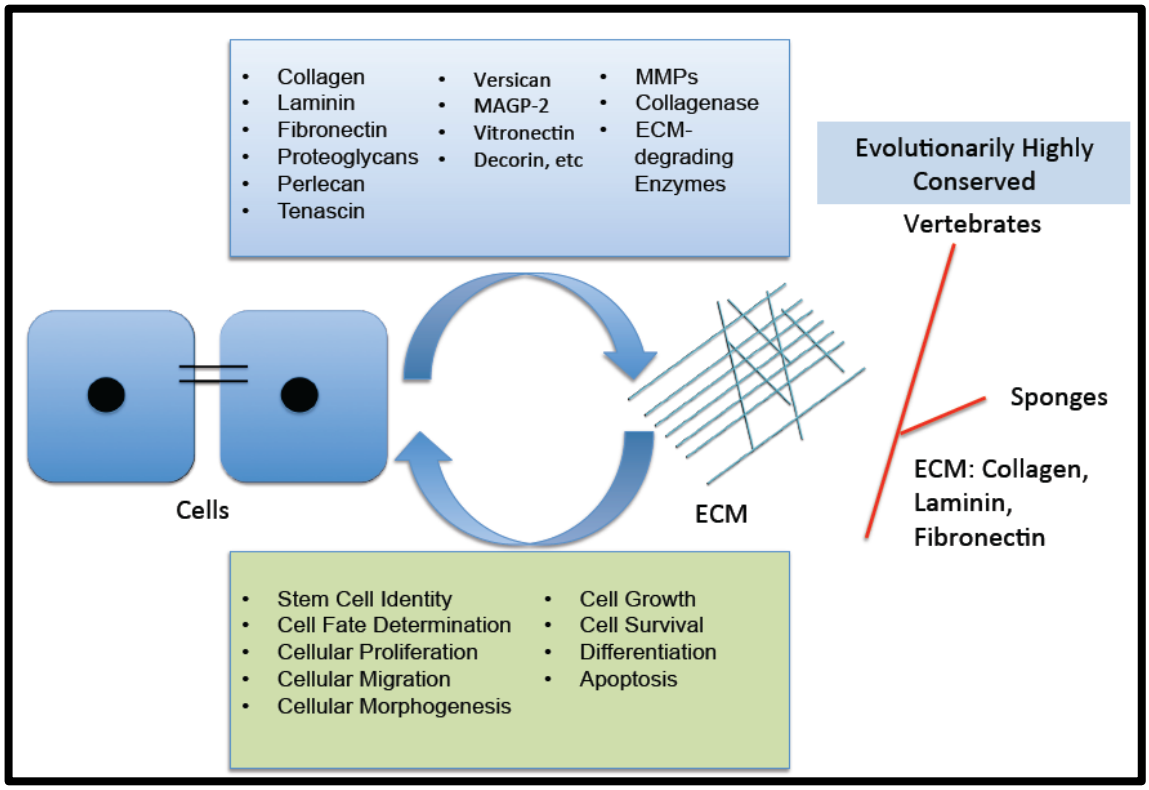


Figure 1. Appropriate cell-matrix interactions are critical in multicellular organisms.

Cells tightly regulate their extracellular matrix and deposit appropriate matrix component into their extracellular space to fulfill their physiological needs. The blue text box shows list of common ECM molecules. Besides the synthesis of ECM, cells also degrade ECM in a need basis and produces ECM-degrading proteases (displayed in the last column of the blue text box). Thus, cells deposit and remodel the ECM and in return the ECM provides essential support and signaling system to control cellular behavior. Cellular behavior commonly regulated by ECM is shown in a green box in the bottom. Thus, cells and their extracellular matrix maintain two-way communication (cells deposit ECM and ECM control cell function) to maintain multicellular function. The importance of extracellular matrix in multicellular organisms can be highlighted by the evolution of ECM in as simple organism as sponges as depicted in the evolutionary map on the right.

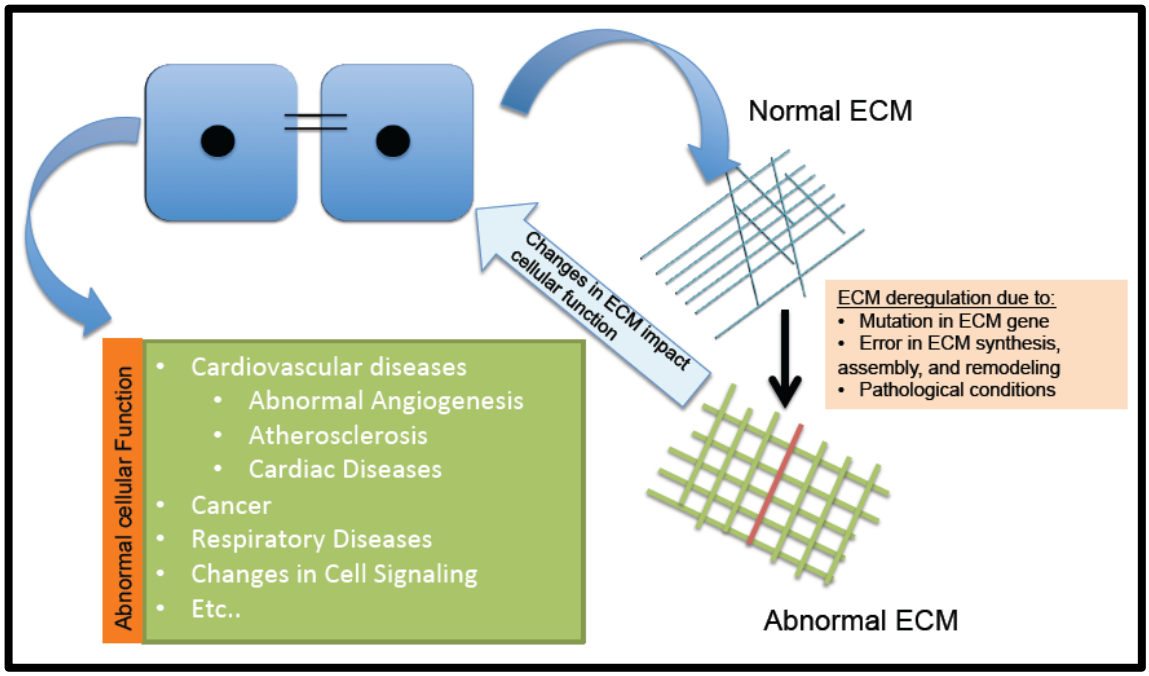
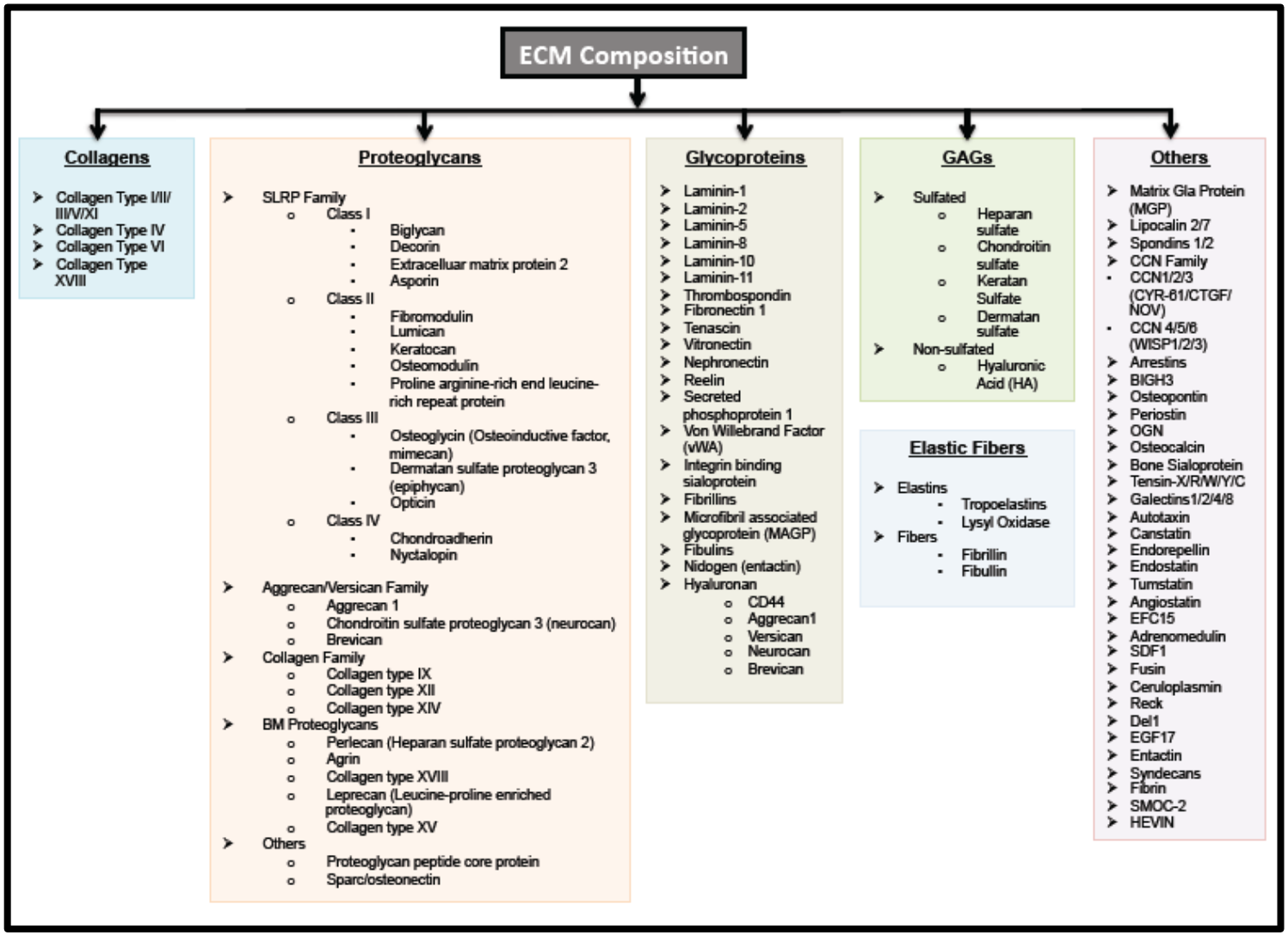


Figure 2. Deregulation of ECM is detrimental to our health.

Normally body cells deposit ECM that is requisite for their physiological functioning and it tightly controls its extracellular matrix. However, under circumstances such as mutation in the ECM encoding gene and other pathological conditions, alterations in the ECM synthesis, assembly, and remodeling is commonly found and that changes the normal ECM composition to abnormal. Abnormal ECM sends abnormal signals and manipulates cellular function. Such changes in cellular function due to abnormal ECM activity are commonly found in a number of diseases (some of them listed in the green box).

Table 1. Commonly known ECM proteins.



CHAPTER II

ANGIOGENESIS AND ITS REGULATORS: IDENTIFICATION OF NOVEL ECM-BASED REGULATORS OF ANGIOGENESIS

Introduction

In the previous chapter, I have highlighted the importance of ECM function in maintaining our health and emphasized how ECM components are critical factors in the pathogenesis of cardiovascular and cancer diseases. In the same chapter, it was also briefly discussed that ECM impacts angiogenesis, which is a requisite for cancer malignancy. Besides cancer, abnormal angiogenesis is also a common pathogenic factor in as many as seventy other human diseases [58-60]. Therefore, therapeutic manipulation of angiogenesis represents an effective approach for the treatment of those diseases that involves abnormal angiogenesis. Attempts have already been made to treat cancer diseases using anti-angiogenesis therapy, but the results are not satisfactory.

For instance, Avastin, a humanized monoclonal antibody against VEGF developed by Genentech, Inc. and approved by FDA for clinical use as anti-angiogenic drug, only showed an average increase in lifespan by only 4-6 months with very high cost at $ 100,0000/year for colorectal cancer treatment [61]. Also, the response for Avastin is variable among different cancers such as colorectal cancer, lung cancer, kidney, brain, and breast cancer. In addition, tumors are found to resist anti-VEGF therapies and restore

their growth and progression over a period of time [62]. Anti-VEGF treatment is also shown to impose serious side effects including hypertension, thrombosis, impaired wound healing, gastrointestinal perforations, fatal hemorrhages, fatigue, anorexia, diarrhea, mucositis, handfoot syndromes, and in some cases congestive heart failure [63]. Recently FDA disapproved the use of Avastin in breast cancer treatment due to no significant improvement in patient health and survival and due to very serious safety concerns (www.FDA.gov).[1] One of the rationales for the failure of anti-VEGF therapies is lack of efficacy biomarkers to validate optimal dose and resulting responses in patients with different cancer types [63]. Identification of such biomarkers and mechanism of action by anti-VEGF treatment under different criteria would allow us to determine appropriate treatment regiments unique to colorectal, lung, breast cancer, and others.

To this end, perhaps the variations in ECM components of cancer microenvironment plays a critical role in modulating anti-VEGF response to cancers, since ECMs have been shown to manipulate VEGF signaling during angiogenesis [64-67]. In addition, changes in the ECM dynamics are critical aspect in the orchestration specific cellular and molecular events required for angiogenesis [65, 68, 69]. However, the importance of ECM on angiogenesis regulation is overlooked in the design of current regiments of anti-angiogenic therapies. Therefore, identification and characterization of ECM regulators of angiogenesis is critical not only because it can be a novel therapeutic target but also to improve current therapeutics. In this context, the identity of ECM-based angiogenic molecules represents an important, yet poorly understood avenue towards potential angiogenic-based therapeutics for diseases that involves abnormal angiogenesis

[1] http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm280536.htm

such as cancer. Therefore, the overall goal of this dissertation study was to 1) identify novel ECM regulators of angiogenesis and 2) apply our findings to understand their role in cancer. To this end, the two main subjects of this dissertation study are 1) understanding the specific role for Matrix Gla Protein (MGP), an ECM protein, in the context of vascular development, angiogenesis, and cancer and 2) advancing our previous knowledge on another ECM protein Lumican concerning its role in cancer angiogenesis and metastasis are. Although the actual research study on MGP and Lumican will not be discussed until chapter III, my goal in this chapter is to introduce several topics that need to be understood to understand the research study that will be discussed in chapters III and IV.

Angiogenesis: An Overview

Vascular network in our body is formed by two distinct processes: vasculogenesis and angiogenesis [70]. During vasculogenesis, a primary capillary plexus is formed from angioblast cells that originate from mesodermal cell lineage during embryonic development. However, the new vascular networks during the later stages of embryonic development and adult life are solely formed by angiogenesis [18, 70]. Normally in the adults, existing vasculature is stable and quiescent, held in stasis by a balance between pro- and anti-angiogenic molecules present in the vascular the basement membrane and surrounding stroma [47, 71, 72]. However, during specific conditions such as tissue injury, exercise, and during pregnancy and the menstrual cycle in women, angiogenesis builds new vessels to repair injured tissue and fulfill other physiological needs [73]. Angiogenesis also occur under pathological conditions such as cancer, inflammation, age-related macular degeneration, and others [59]. In these circumstances,

increasing pro-angiogenic stimuli (such as VEGF and FGF) present in the nearby avascular microenvironment upsets angiogenic homeostasis [74, 75] and activates endothelial cells by binding to specific receptors present on the cell surfaces [76].

Upon activation, endothelial cells begin to loose their cell-to-cell adhesions, proliferate, dedifferentiate, and sprout from the inner wall of the vessels toward the avascular microenvironment [66]. In the process, endothelial cells release proteases to digest the ECM (ECM) and clear its passage while sprouting [77-79]. Two distinct types of cells, namely the tip cells and stalk cells coordinate endothelial sprouting. Tip cells are leader cells that can move along the angiogenic gradient, and stalk cells are the follower cells that are more stable and quiescent and that align adjacent to tip cells forming an angiogenic sprout. Distinct cellular and molecular interactions determine the fate of these cell types [80].

During the terminal phase of angiogenesis, endothelial cells resume differentiation and cease extraneous sprouting, organize themselves to form a tubular structure, reconstitute a the basement membrane, recruit mural cells (pericytes and vascular smooth muscle cells), re-establish cell-cell junctions, and return to cellular quiescence to form a stable vasculature [70]. A number of molecules have been identified to be positive and negative regulators of angiogenesis, and interestingly, a number of these regulators are resident within the ECM present in the vascular tissues and surrounding stroma [42, 81-83]. An overall angiogenesis process is illustrated in Figure 1.

Regulators of Angiogenesis

VEGF Signaling

Vascular endothelial growth factor (VEGF) is a key regulator of both physiological as well as pathological angiogenesis [84]. Many positive regulators of angiogenesis have been identified both in the tumors and in the normal tissues since the discovery of neovessel formation in transplanted tumors [72], but studies suggest that VEGF is one of the major limiting factors during angiogenesis [85-87]. Importantly, VEGF receptor is expressed in the early vascular cell lineages and initiates embryonic angiogenesis. VEGF (also VEGF-A) belongs to a gene family that comprises of placental growth factor (PLGF), VEGFB, VEGFC, and VEGFD [88]. VEGF receptors belong to receptor tyrosine kinase (RTK) family of receptors and comes in various types: VEGFR 1, 2, and 3 and co-receptors Neuropilin-1 and Neuropilin-2. VEGF-A stimulates angiogenesis through VEGFR1 and VEGFR2. VEGF-A binds to its co-receptor Neuropilin-1 and this binding increases VEGF-A binding affinity to VEGFR2 thereby enhancing VEGF-A stimulation of angiogenesis. Therefore, VEGFA-VEGFR1/2 has become an attractive pharmaceutical target for angiogenesis inhibition in various diseases. VEGF-A is also an embryonic stimulator of angiogenesis that promotes endothelial progenitor differentiation and endothelial cell growth, survival, migration, and tubular formation [75, 85, 88]. Mice lacking a single allele of VEGF (-/+) showed severe defects in early angiogenesis, such as failure of aortic connection to the heart and poor development of dorsal aorta [89, 90]. Despite the heterozygotic lethality being very rare among mammals, VEGF -/+ lethality in mice suggests that appropriate concentration of VEGF is essential in the development of functional circulatory system in embryos.

Under normoxic conditions, VEGF-A expression is known to be under the transcriptional control of Fos/Jun complex, nuclear factor kappa B (NFkB), and hormones such as estrogen [91-93]. However, under hypoxic condition VEGF-A gene expression is controlled by hypoxia-inducible factor complex [94]. VEGF-A gene contains hypoxia responsive element (HRE) site, which binds to HIF complex and induce VEGF-A gene expression [95]. In addition, hypoxic condition is also known to stabilize VEGF-A mRNA post-transcriptionally and enhances translation to VEGFA protein. Other forms of VEGF also regulate angiogenesis and their operation is mainly context dependent. For instance, VEGF-C/D stimulates Lymphangiogenesis through VEGFR3. VEGF-B activates VEGFR1 and is commonly expressed in tissues such as heart and skeletal muscle. To sum up, VEGF/VEGFR signaling is primary regulators of angiogenesis.

Notch Signaling

In humans, Notch signaling operates via four notch receptors, Notch 1 to 4, and five ligands, namely jagged 1-2 and Dll1, Dll3, and Dll4. Notch signaling can be initiated by either adjacent heterologous ligand-expressing cells or by the same cell (Notch autonomous signaling). Upon ligand binding, a proteolytic processing (S1, S2, S3) on the notch receptors releases an intracellular domain of Notch (NICD). NICD translocates into the nucleus and associates with the CSL family of binding proteins and become transcriptional activator, which turns on transcription of a set of target genes, including notch responsive promoters such as HRT, Hes-1, Hey-1, Hes-5. Most of the notch-target genes are transcription regulators of tissue-specific helix-loop-helix transcription factors. Notch signaling also activates its target gene via NF-kB [96].

Notch signaling is a critical regulator of angiogenesis particularly during sprouting and stabilization of vascular lumen [97, 98]. Two distinct notch ligands work in an opposing manner to control angiogenic sprouting [80]. Notch ligand DLL4 "switches off" angiogenic signal and makes endothelial cells irresponsive to VEGF signaling. As a result, DLL4 suppresses endothelial sprouting and vessel branching [99] and promotes stable vascular lumen formation [97]. In contrast, notch ligand Jagged-1 functions as angiogenic "switch on" signal that antagonizes DLL4 function and enhances endothelial sprouting [80]. This opposing effect of DLL4 and Jagged-1 is due to their ability to regulate the differentiation of endothelial cells into leading tip cells vs. stalk cell phenotype [80]. DLL4 promotes endothelial cell differentiation into more stable and quiescent stalk cell phenotype, whereas jagged-1 promotes endothelial differentiation into leading tip cell phenotype. Tip cells exist as filapodia-like projections extended and pointed toward the angiogenic stimuli and migrate in the same direction to form the extending sprouts. Therefore, jagged-1 and DLL4 activation oppose each other to maintain a stasis between leading tip cell vs. quiescent stalk cell phenotypes during angiogenic sprouting. Differential role of notch ligands DLL4 and Jagged-1 is illustrated in Figure 2.

Besides its role in sprouting angiogenesis, notch signaling also controls other aspects of vascular development including arterial/venous specification [100, 101] and vascular mural cell function [102]. During embryonic development, notch expression promotes endothelial differentiation into arterial vessels and suppresses the venous identity. Mural cells such as pericytes also provide signals to stop endothelial proliferation and sustain survival, which are requisite for vascular stability [103].

Recruitment of Mural cells by nascent vessels is heavily dependent upon notch receptor/ligand interaction between endothelial cells and mural cells [104]. Similar notch system regulates angiogenesis in tumors as well, and notch presents an attractive therapeutic target in restricting angiogenesis in tumors [102, 105, 106].

BMP Signaling

Bone morphogenetic proteins (BMPs) belong to TGFbeta superfamily of secreted proteins and BMP signaling is critical during embryogenesis, tissue morphogenesis and homeostasis. BMP signaling is similar to that of TGFbeta signaling in that it is smad dependent. Like TGFbeta, BMP binds to specific type II receptor and recruit type I receptor (ALK 1, 2, 3, and 6) and leads to phosphorylation of specific receptor smads (R-smads). Endoglin (CD105) and Betaglycan (TGFBR3) are co-receptors for BMPs. Unlike TGFbeta, BMP signaling is activated through receptor smads 1, 5, and 8. Eventually, an activated receptor smads complex with co-smad, smad 4, and the resulting heterocomplex is translocated into the nucleus where it binds to promoter of target genes and regulate transcription [36, 107].

BMP is an important regulator of vascular development and angiogenesis [36]. For instance BMP-2/4 function is critical for vascular development, since the deletion of BMP-2/4 and its receptor impaired the mesodermal differentiation into vascular lineages and severely impacted vascular development [10, 37]. In addition, mice with smad 1 and 5, key transcription factors for BMP2/4, showed defects in angiogenesis [10, 108]. Importantly, BMP-2 is expressed by a number of tumors and functions as key stimulator of angiogenesis in tumors. For instance, BMP-2 increased angiogenesis in A549 subcutaneous lung cancer tumor model [109]. In addition, matrigel implants

supplemented with BMP-2 contains more angiogenesis than the control, and the effect is neutralized by BMP-2 antagonist noggin. These data highlighted the importance of BMP-2 function both in pathological, as well as in physiological, angiogenesis. Moreover, the angiogenic effect of BMP-2 is shown to be smad 1,5, and 8 dependent, which stimulates endothelial cell proliferation.

A Cross-talk Between VEGF, Notch, and BMP Signaling

A number of studies show that Notch, VEGF, and BMP signaling crosstalk to regulate a number of vascular functions including angiogenesis [80, 110-114]. VEGF activates notch ligand DLL4 in the endothelial tip cells. During sprouting, the expression of DLL4 in the tip cells activates notch in the adjacent stalk cells and suppresses jagged-1 expression in the stalk cell. In the absence of DLL4, VEGF stimulation elevates jagged-1 expression in the stalk cells, which increases VEGF-R2 expression in the adjacent endothelial cells and initiate sprouting [80, 115]. Therefore, loss or suppressed DLL4 function elevates inappropriate angiogenic response with increased vessel branching and loss of vascular lumen formation. Therefore, two distinct notch ligands crosstalk with VEGF signaling to balance angiogenesis. Consequently, blockade of DLL4 expression in tumors produced highly branched and dysfunctional vessels and suppressed tumor growth due to non-functional angiogenesis [97, 106, 112, 116]. Interestingly, tumors are found to elevate DLL4 expression in a VEGF-dependent manner to enhance productive angiogenesis [112]. Besides its direct effect on endothelial cells, VEGF/notch crosstalk is also critical in mural cell function and stabilization of the vascular wall. Collectively, these findings emphasize the importance of notch signaling during vascular development and angiogenesis.

A crosstalk with VEGF signaling is essential for BMP in its regulation of angiogenesis and other vascular functions. For instance, BMP stimulates VEGF-A production in differentiating osteoblasts to stimulate angiogenesis during bone development [113]. BMP-9 has been shown to stimulate endothelial cell proliferation via VEGF-VEGFR2 signaling and has been shown to enhance *in-vivo* angiogenesis in a matrigel implant and in the human pancreatic xenograft tumor model [114]. Interestingly, however, other studies show BMP2 regulate angiogenesis independent of VEGF-A/VEGFR signaling pathway [3, 8]. For instance, in the zebrafish, BMP stimulates venous angiogenesis via BMP/Bmpr/2/Alk2/3/smad/Erk pathway independent of VEGF signaling. On the other hand, arterial angiogenesis from the dorsal aorta is strictly under the control of VEGF stimulation. [117]. Therefore, BMP regulates angiogenesis either with or without crosstalk with VEGF in a context-dependent manner.

Besides its interactions with VEGF signaling, BMP also involves notch signaling in its regulation of varieties of vascular function including angiogenesis. Studies show that Notch signaling being a requisite for BMP function in the induction of osteogenic differentiation and mineralization of vascular smooth muscle layer [118, 119]. Most importantly, BMP also depends on notch function for its angiogenic activity. For example, a study showed that the stalk cell phenotype of endothelial cells during angiogenesis is dependent upon the cooperative action of notch and BMP signaling [111]. Collectively, angiogenesis is a complex process and its regulation is under the control of various signaling mechanisms that work in cooperation of each another (see figure 3).

ECM and Angiogenesis

ECM is a critical regulator of vascular development. During embryonic development, ECM develops around differentiated endothelial cells. ECM proteins such as Fibronectin, Laminin, and Collagen are very prominent around endothelial layer and provide adhesive support to growing endothelial cells and help mature into a primary capillary plexus [18]. Furthermore, ECM also helps to maintain vascular homeostasis, morphogenesis, remodeling, and angiogenesis during adult life [120-122]. During angiogenesis, ECM (ECM) not only provides a scaffold to the growing endothelial cells, but it also contains distinct angiogenic signals to initiate, drive, and complete angiogenic process [50, 121]. Moreover, ECM manipulates angiogenic growth factors (such as VEGFs and FGFs) by regulating their release, binding, and activation [68, 123].

ECM binding with integrins lies central to endothelial cell-matrix interactions. ECM also controls endothelial cell behavior through its interaction with integrin [16, 50, 124, 125]. A number of ECM molecules, including Collagen, Fibronectin, Proteoglycan, and Laminin, bind to integrin receptors present on the surface of endothelial cells and control neovessel formation. ECM interactions with integrins promote focal adhesion and, in the absence of proper ECM-integrin interaction, endothelial cells lose focal adhesion and undergo apoptosis through a p53 dependent pathway [126]. As a result, a subset of ECM binding integrins is upregulated during angiogenesis. Interestingly, VEGF is also found to increase the expression of a1B1 and a1B2 integrin in microvasculature, suggesting that VEGF-induced integrin is essential during angiogenesis [127].

ECM also regulates angiogenesis through its pro-and-anti-angiogenic activities on endothelial cells [69, 75]. For instance, several ECM fragments that are derived from

collagens, including endostatin, tumstatin, canstatin, arresten, and hexastatin, have stimulatory or inhibitory function on angiogenesis [71]. Likewise, endorepellin, a derivative of perlecan, serves as anti-angiogenic agent. Furthermore, the basement membrane restricts angiogenesis, and its degradation is requisite for angiogenesis. As a result, endothelial cells increase the expression of ECM-degrading enzymes such as membrane type-matrix metalloproteinases (MT-MMP) during angiogenesis [77]. Therefore, coordinated degradation of ECM—both the basement membrane and interstitial matrix—by matrix metalloproteinases is essential in the initiation of angiogenesis [47], whereas its re-establishment is critical in the resolution phase of angiogenesis. Collectively, ECM provides a foundation to initiate, grow, and complete angiogenesis. Therefore, a more complete understanding of ECM function and its regulators is urgent in the design of better angiogenic therapeutics to treat diseases due to abnormal angiogenesis.

Tools Used To Discover Novel ECM Regulators of Angiogenesis

To successfully identify and characterize the novel ECM regulators of angiogenesis, we need appropriate tools in hand. In this study, I have utilized modern cell and molecular techniques (cell culture, transfection, western blot, RT-PCR, luciferase reporter assay etc), morpholino based gene knockdown in a transgenic fli-1^{GFP}/GATA-1^{RFP} zebrafish model for vascular development and angiogenesis, ex-vivo tissue culture model of angiogenesis (Aortic Ring Assay), specific ECM gene knockout mouse model systems, and transgenic cancer cell line models to achieve my research goals. In the following section, I will provide a review of the Microarray and Transgenic Zebrafish model used for the angiogenesis study.

Microarray: Identification of Novel ECM Regulators of Angiogenesis

Microarray experiments have led us to identify novel ECM regulators of angiogenesis. Microarray is a high-throughput system that allows us to monitor the expression of multiple genes simultaneously. A DNA microarray system contains specific sequences of DNA from various genes known as probes that are attached to the solid surface called biochips. Fluorescently labeled complementary DNAs from various samples can be hybridized to the known DNA probes in the chip to determine mRNA expression, and the expression can be quantified based on the intensity of the labeled fluorescence.

We have identified differential expression patterns for a number of genes from a microarray experiment on mRNA extracted from MB114 endothelial cells undergoing *in-vitro* angiogenesis on matrigel matrices at 1 hr, 5 hr, 15 hr, and 25 hr period [83]. Our results identified a number of genes that were previously known to be angiogenic regulators. and we also identified a number of other new genes that were differentially regulated during the *in-vitro* angiogenesis. Among these novel genes were secreted ECM proteins such as Matrix Gla Protein (MGP) and Lumican (See figure 4).

Transgenic Zebrafish Model of Angiogenesis

We have utilized transgenic zebrafish model of angiogenesis as one of the tools to study angiogenic effect of newly identified ECM molecules. The transgenic zebrafish is produced by fusing GFP with a blood vessel specific *Fli-1* promoter and fusing RFP with a blood cell specific GATA1 promoter. The double transgenic Fli-1^{GFP}/$GATA1^{RFP}$ zebrafish is obtained by breeding $Fli1^{GPF}$ with $GATA1^{RFP}$. The resultant GFP and RFP transgenic fish is a great tool to study vascular development and angiogenesis because it

allows us to visualize the blood vessels (GFP) and the blood flow (RFP) in real time under fluorescent microscope during vascular development in zebrafish.

Morpholino based gene knockdown system is utilized in these transgenic zebrafish by injecting morpholinos specific to ECM proteins to study ECM function in vascular development and angiogenesis. Morpholinos are synthetic oligonucleotides similar in structure to DNA molecules that binds to mRNA and blocks mRNA's translation to protein. Thus, morpholino injections block the expression of ECM proteins in the zebrafish. Thus, coupling morpholino based gene knockdown system and transgenic $Fli1^{GFP}$/$GATA1^{RFP}$ zebrafish makes it easier and efficient tool to screen novel ECM molecules for their roles in vascular development and angiogenesis.

Matrix Gla Protein (MGP): A Literature Review

Matrix Gla protein (MGP) was first identified in bovine bone and contains gam ma-carboxylated glutamine residues [128]. The glutamine residues are carboxylated at NH2-terminal by gamma-carboxylase in a vitamin-K dependent manner. Therefore, MGP is a vitamin-K dependent protein. MGP has a sequence homology with Bone Gla Protein (BGP), and these two proteins are believed to have evolved through gene duplication and subsequent divergent evolution [129]. Analysis from rat bone development revealed that MGP accumulation in the bone appeared much earlier than BGP and that MGP levels among newborn, juvenile, and adult rats were similar. These findings from rat bone indicate that MGP function is critical in the early bone development. Transcriptome analysis show MGP is highly expressed in the Lung, Heart, and Kidney [130].

In humans, MGP gene comprises 3.9 kilobases of chromosomal DNA and is located in the shorter arm of chromosome 12 (12p) [131]. MGP gene is comprised of four

exons separated by three introns and is present as a single copy. Besides TATA and CAT boxes, MGP gene promoter is comprised of a number of putative regulatory sequences for hormone and transcription factors including binding sites for retinoic acid and vitamin D. In rat vascular smooth muscle cells, transcription of MGP gene is found to be downregulated by retinoic acid and TGFbeta and upregulated by vitamin D3 and cyclic AMP [132]. In addition, MGP promoter also contains a binding site for RunX2, a primary transcription factor involved in bone development, and RunX2 is shown to control MGP expression in A6 cells. Furthermore, an additional exon in the 5' region containing calcium-sensitive promoter is found in *Xenopus laevis* [133], suggesting a complex transcriptional regulation of MGP gene. MGP is expressed as an 84-residue (~ 10 kD) vitamin K dependent protein. MGP protein sequence comprises transmembrane signal peptide, carboxylation site, and Gla-containing domain in the N-terminal region. MGP is secreted into ECM upon its synthesis. MGP is almost ubiquitously expressed in human tissues including vascular tissues [134].

MGP function in mediating cellular adhesion to ECM is known [135] and it promotes cellular adhesion through its Gla domain [135]. Antibodies against the non-gla domain did not affect adhesion, but the removal of Gla residues or its inhibition by synthetic peptide significantly inhibited the cellular adhesion, suggesting that Gla domain is solely responsible for its function in cellular adhesion. Purified MGP from bovine bone was shown to adhere to various cell types including chondrocytes, fibroblasts, osteosarcoma cells, and kidney mesangial cells. Interestingly, MGP is also shown to interact with vitronectin, a component of ECM, through a specific domain in its C-terminal region [136]. MGP interactions with vitronectin are prominent in embryonic

tissues, suggesting a critical role for MGP in the modulation of cell-matrix interactions during development. Besides vitronectin, MGP peptide is also shown to interact with fibronectin. MGP interactions to extracellular component is most likely very specific to vitronectin and fibronectin since it did not bind to other components of ECM including collagen, laminin, fibromodulin, heparin, Osteocalcin, and chondroitin sulfate. Sequence analysis showed no similarity in the sequences between MGP and other ECM-based cellular adhesion proteins such as fibronectin, laminin, vitronectin, fibrinogen, von Willebrand factor (vWf), entactin, thromospondin, collagen type I, and collagen type IV. These data suggest that MGP may have a unique physical and biochemical properties from other ECM component.

Although MGP has some role in cellular adhesion, its primarily known as an inhibitor of tissue mineralization [137]. MGP is heavily expressed by growth plate cartilage and inhibition of MGP cause excessive growth plate mineralization and thus growth retardation. This effect was observed in rats treated with Warfarin, a vitamin K antagonist. A similar disorder is also observed in infants whose mothers were subjected to Warfarin during pregnancy. In humans, MGP deficiency causes Keutal Syndrome, a disease syndrome collectively resulting in calcification of cartilages, pulmonary stenosis, and mid-face hypoplasia [138]. One of the ways by which MGP inhibits mineralization is via inhibition of calcification since MGP directly interacts with hydroxyapatite crystals, a form of calcium deposit in tissues [139]. MGP also inhibits BMP 2/4 signaling and suppresses BMP induced chondrogenic and osteogenic differentiation in cartilages and bone tissues respectively [140].

MGP is heavily expressed in vascular tissues [134], and it plays a critical role in maintaining vascular function. For instance, mice deficient in MGP (MGP-/-) show severe calcification of aorta and die within 2 months possibly due to damages in vascular system [141]. MGP also suppresses mineralization of vascular tissues and prevents from arteriosclerosis and atherosclerosis in arterial vessels [31]. Furthermore, MGP is also a critical regulator of artery-venous formation since MGP deficiency in mice caused arteriovenous malformation [117]. In addition, polymorphism in MGP gene is associated with a number of cardiovascular dysfunctions including coronary artery calcification and vascular damages in chronic kidney diseases [142, 143].

Although the primary function of MGP in vascular tissues is associated with its function as a calcification inhibitor, it has also been described as a critical regulator of endothelial cell function. For instance, MGP expression is significantly increased during the angiogenesis process [83] and it has been shown to regulate both physiological as well as tumor angiogenesis [144, 145]. MGP function in vascular tissues is attributed to its ability to interact with BMP 2/4 and inhibit BMP function [31, 146-149]. BMP 2/4 is previously characterized as an angiogenesis stimulator [3, 109], and its inhibition by MGP would suggest MGP to be an anti-angiogenic agent. In contrast, a number of other studies show MGP as pro-angiogenic molecule [144, 145]. Despite these conflicting data, an in-depth study to pinpoint the mechanistic details of MGP function during angiogenic process is still lacking. Therefore, one of the main objectives of this dissertation study is to evaluate the role for MGP and its underlying molecular mechanism in the context of angiogenesis both in normal as well as in tumor tissues. The findings for MGP from this study will be discussed in detail later in chapter III.

Lumican: A Literature Review

Lumican is an extracellular matrix protein belonging to a family of small leucine-rich proteoglycans (SLRPs). Lumican expression in human tissues is widespread and commonly found in cornea and dermal layer of skin. Human Lumican gene is localized in chromosome 12 (12q21.3-q22). Lumican core protein contains 338 amino acid residues and share features common to small leucine-rich proteoglycans. It contains leucine repeats in the central domain flanked by N-terminal and C-terminal domains containing highly conserved cysteine residues. The Lumican core-protein contains 4 distinct sites for N-glycosylation, and some of these sites are substituted by keratan sulfate side chains. Therefore, Lumican is commonly called a keratan sulfate proteoglycans and exist as a 55-57 kDa proteoglycan in tissues. Lumican is a substrate for a number of matrix proteases including MT1-MMP, MMP-12, and ADAMTS-4 (Ref 34-35). Lumican is homologous to other member of SLRP family proteins such as decorin and fibromodulin and has shared function with fibromodulin in the organization of collagen fibril. Details on Lumican core protein structure and Lumican gene is reviewed in [150, 151].

Primarily, Lumican is known as a regulator of collagen fibrillogenesis [152]. It is highly expressed in the cornea and is a critical regulator of corneal collagen fibrillogenesis [153]. Proper assembly of collagen is essential for corneal transparency and a study show that Lumican knockout (Lum -/-) mouse exhibits corneal opacity [154]. Lumican deficiency also causes loose skin. These data suggest that Lumican function is indispensible in proper organization of collagen in tissues and its proper function is highly critical in maintaining extracellular dynamics in tissues since collagen is one of the most abundant components of ECM.

Although initially identified in the corneal stroma, Lumican expression is ubiquitously found in various tissues including heart, lung, intestine, bone, articular cartilage, pancreas, placenta, kidney, breast, brain, cervix, colon, liver, smooth muscle, and uterus [155-161]. Therefore, a proper functioning of Lumican is critical in maintaining tissue homeostasis and consequently its defect have been implicated in a number of diseases including cancer. Lumican expression is significantly altered in various cancers. However, the description of Lumican's role in cancer is based on correlation data at the best. For instance, compiled expression data from various breast cancer patients show high level of Lumican expression correlates with higher tumor grade in breast cancer.[2] Consistently, increased level of Lumican expression in tumors is also shown to lower long-term survival in breast cancer.[3] These expression data from breast cancer suggested that Lumican promotes tumor growth and progression. In contrast, ectopic expression of Lumican in various cancer including pancreatic adenocarcinoma, fibrosarcoma, and B16 melanoma is previously shown to reduced tumor growth and angiogenesis, suggesting an anti-tumorigenic role for Lumican [42]. Most importantly, Lumican is previously shown to induce apoptosis in endothelial cells and suppressed tumor angiogenesis suggesting that anti-tumorigenic role for Lumican in some of these tumors is due to its anti-angiogenic activity. In addition, anti-angiogenic activity of Lumican has been previously described in other contexts [162] including *in-vitro* model of angiogenesis [83].

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[2] www.oncomine.org

[3] www.kmplot.com

Unfortunately, these findings on Lumican and its role in cancer and angiogenesis are solely based on either correlative expression data or the measurement of its effect due to ectopic expression. However, the anti-tumorigenic and anti-angiogenic role for endogenous Lumican has not been measured directly. In addition, previous studies have shown that Lumican promotes wound healing of corneal and skin epithelium. This finding raises another question that is whether Lumican has pro-angiogenic effect in the physiological setting. Therefore, this dissertation aimed to further elaborate on the roles of Lumican to delineate its differential function, if any, from endogenous versus exogenous expression in both physiological versus pathological context. The findings from Lumican will be discussed in detail in chapter IV.

Summary

From the review in this chapter, I highlighted angiogenesis as an important process of vascular development and defect in angiogenesis can lead to serious vascular abnormalities during development as well as in adult life. In addition, I also rationalized the need for the novel angiogenic regulators since the ones that we identified as prominent angiogenesis regulators such as VEGF could not develop into a reliable therapeutic target for angiogenesis. To this end, we needed to look for a better therapeutic target, and ECM is a prominent source for angiogenic regulators. We have also identified a number of ECM based regulators of angiogenesis. Among these newly identified ECM regulators of angiogenesis, I studied MGP and Lumican for their role in angiogenesis and cancer.

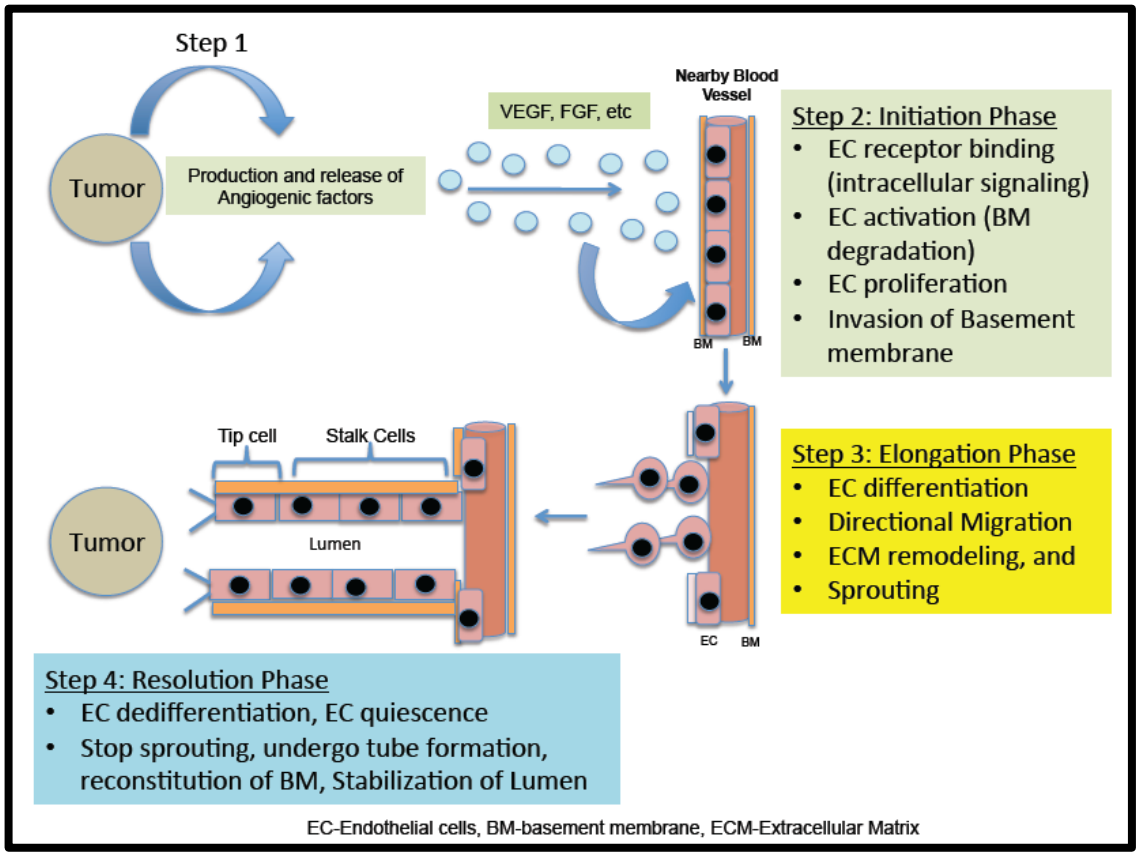

Figure 1. Angiogenesis is a complex multi-step process.

Step 1: An avascular tissue (tumor as an example shown here) when requires angiogenesis releases angiogenic factors such as VEGF, FGF, and others to its surrounding. The angiogenic factors reach to nearby blood vessels. Step 2: Angiogenic factors interact with endothelial cells lining the blood vessels through endothelial cell surface receptors specific for the angiogenic factors. Upon these interactions between endothelial cell surface receptor and corresponding angiogenic factor, endothelial cells becomes activated and initiates angiogenic process (initiation phase). Initiation phase proceeds to Step 3: Elongation phase and ultimately to step 4: resolution phase. Critical steps occurring during each steps is shown in colored text boxes.

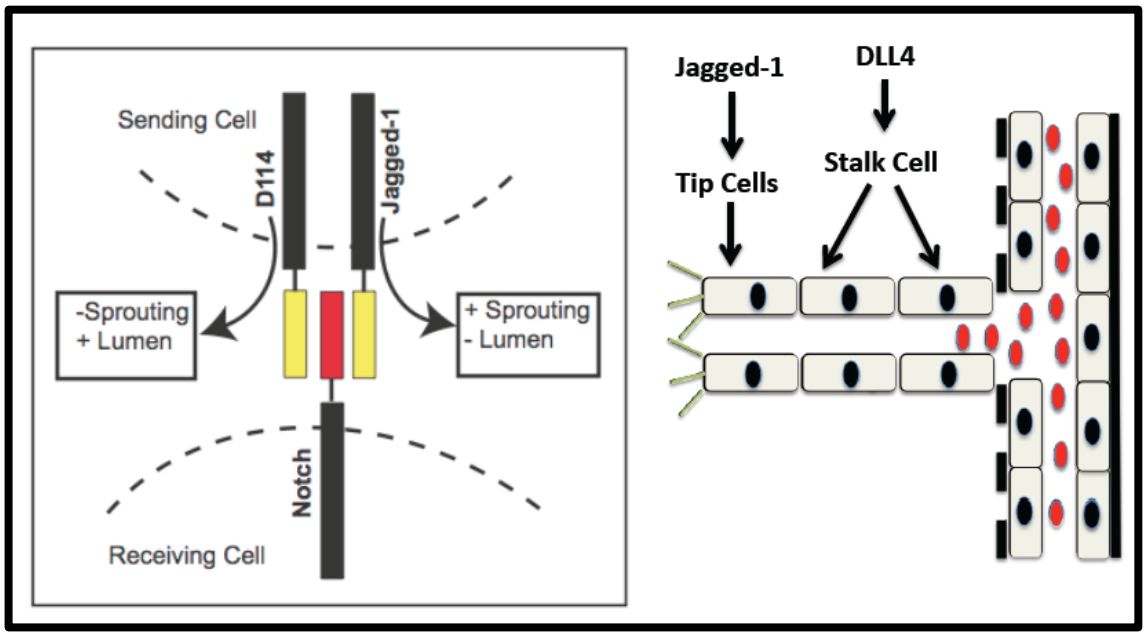

Figure 2. Differential notch signaling regulates angiogenic sprouting: DLL4 and Jagged-1 has an opposing effect.

Shown here is a representation of notch activation via two distinct notch ligands DLL4 and Jagged-1, which exert an opposing effect during sprouting angiogenesis. DLL4 suppresses endothelial sprouting and promotes endothelial quiescence and promotes lumen formation. Whereas jagged-1 promotes sprouting of endothelial cells and inhibits vessel quiescence and thus suppresses lumen formation. Shown in the figure right hand side, Jagged-1 promotes angiogenic tip cells whereas DLL4 promotes quiescent follower stalk cells. A balance between these two distinct cell types is critical during the formation of productive angiogenesis and thus the balance act of these two opposing notch ligands produce a functional new vessel sprout.

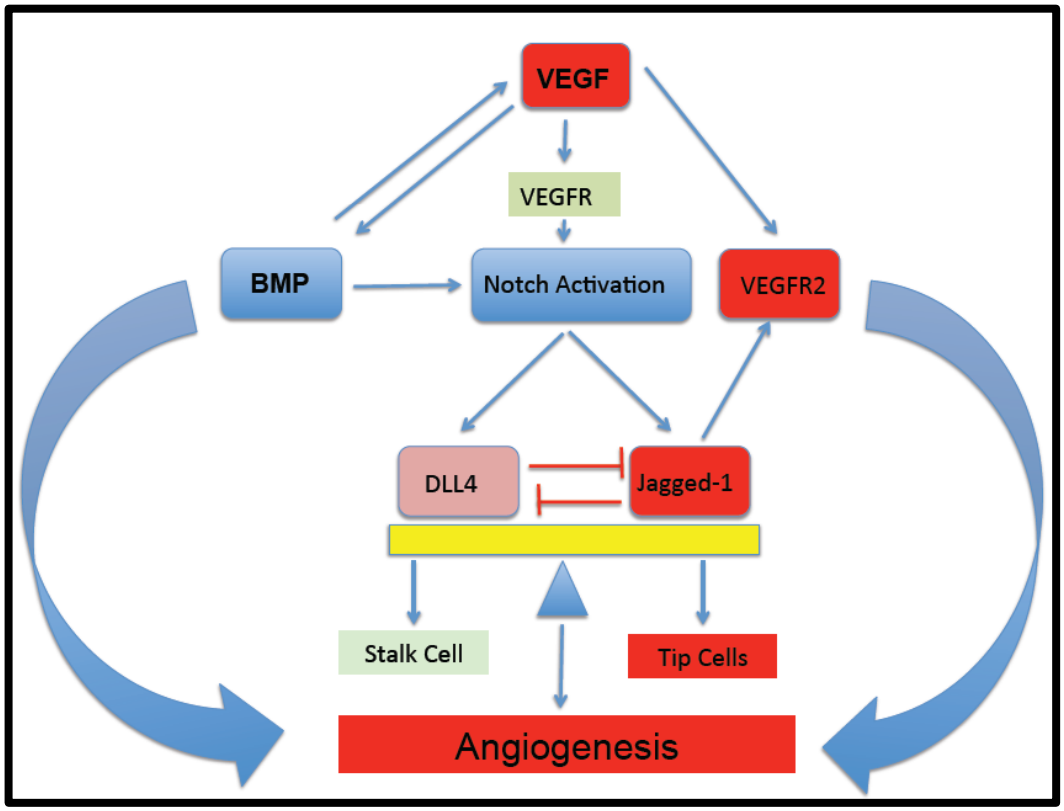

Figure 3. The crosstalk between VEGF, notch, and BMP signaling in the regulation of angiogenesis.

Shown here is a complex interaction between three distinct signaling mechanisms VEGF, notch, and BMP signaling where notch controls angiogenesis sprouting via two distinct notch ligands interaction to adjacent endothelial cells. VEGF signaling also initiates notch activation in endothelial cells. Like wise, BMP can activate both VEGF and notch signaling in the regulation of angiogenesis. BMP and VEGF can activate angiogenesis independent of each other or notch signaling as well. Therefore, angiogenesis regulation by VEGF, notch, and BMP is a collective effort from VEGF, notch, and BMP signaling and tightly controls a complex multi-step angiogenesis process.

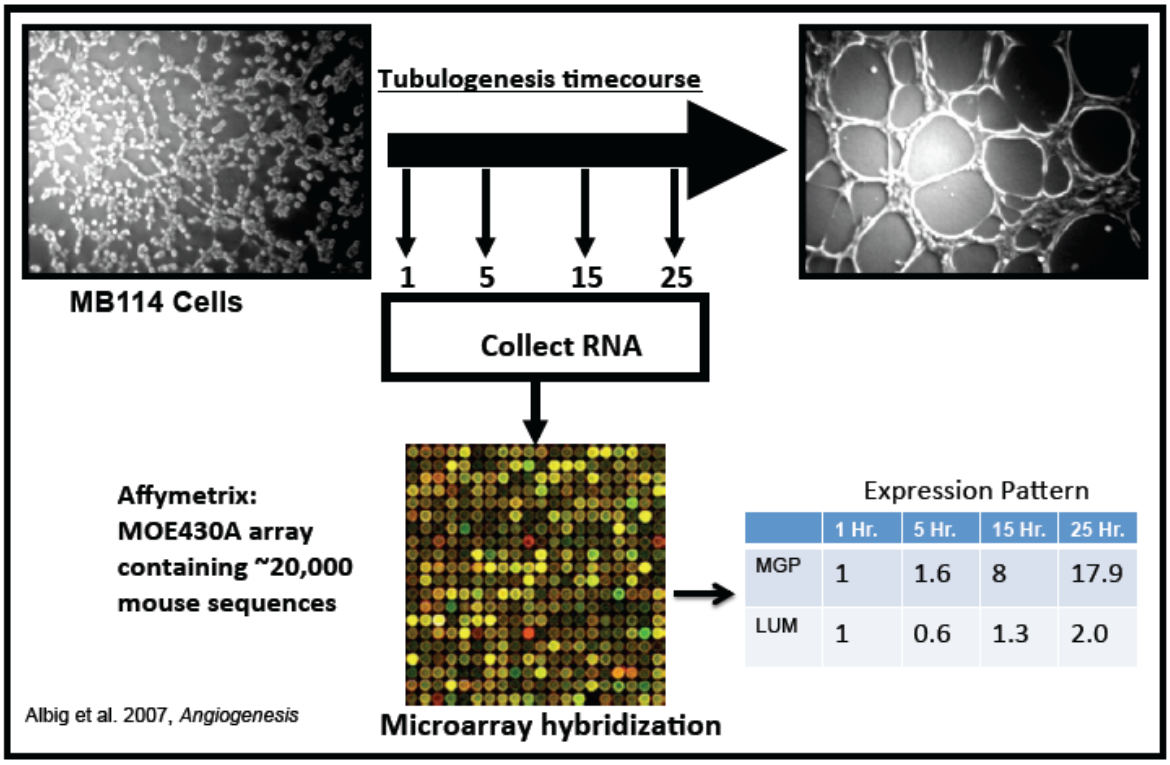

Figure 4. The microarray experiment used to identify MGP and Lumican as regulators of angiogenesis.

MB114 endothelial cells were induced to undergo tube formation (an *in-vitro* angiogenesis model). During the course of tube formation, total RNA was collected from endothelial cells at 1, 5, 15, and 25 hours. RNA collected at 1 hour (labeled green) is mixed in equal quantity with that from 5 hours, 15 hours, and 25 hours labeled red separately. Each mixture of RNA was hybridized with microarray biochip (Affymetrix MOE430A array) containing cDNA and the expressions of genes were determined based on the intensity of green (decreased expression), yellow (equal expression), and red (increased expression) fluorescence as detected by computerized detection system. Such microarray analysis of *in-vitro* model of angiogenesis (tube formation assay) revealed significant increased in the expression of MGP and Lumican and their expression levels are shown in the table to the right.

CHAPTER III

MATRIX GLA PROTEIN (MGP) REINFORCES ANGIOGENIC RESOLUTION

Abstract

Matrix Gla Protein (MGP) is an ECM molecule commonly associated with dysfunctions of large blood vessels such as arteriosclerosis and atherosclerosis. However, the exact role of MGP in the microvasculature is not clear. Utilizing a mouse MGP knockout model we found that MGP suppresses angiogenic sprouting from mouse aorta, restricts microvascular density in cardiac and skeletal muscle, and is an endogenous inhibitor of tumor angiogenesis. Similarly, morpholino based knockdown of MGP in zebrafish embryos caused a progressive loss of luminal structures in intersegmental vessels, a phenotype reminiscent of Dll4/Notch inhibition. Accordingly, MGP suppressed Notch-dependent Hes-1 promoter activity expression of Jagged1 mRNA relative to Dll4 mRNA. However, inhibition of BMP but not Notch signaling reversed the excessive angiogenic sprouting phenotype of MGP knockout aortic rings suggesting that MGP may normally suppress angiogenic sprouting by blocking BMP signaling. Collectively, these results suggest that MGP is a multi-functional inhibitor of normal and abnormal angiogenesis that may function by coordinating with both Notch and BMP signaling pathways.

Keywords: Angiogenesis/ ECM/ Matrix Gla Protein/Notch Signaling/ BMP signaling/

Introduction

Angiogenesis is the development of new capillaries from pre-existing vessels. Normally, existing vasculature is stable and quiescent, held in stasis by a balance between pro- and anti-angiogenic molecules present in the vascular basement membrane and surrounding stroma. During angiogenesis, however, increasing pro-angiogenic stimuli present in the microenvironment upsets this balance and activates angiogenesis by binding to specific receptors present on endothelial cell surfaces. Upon activation, endothelial cells begin to loose their cell-to-cell adhesions, proliferate, dedifferentiate, and sprout toward the avascular microenvironment. During the terminal phase of angiogenesis, endothelial cells re-differentiate, cease extraneous sprouting, organize themselves to form tubular structures, reconstitute a basement membrane, recruit mural cells (pericytes and vascular smooth muscle cells), re-establish cell-cell junctions, and return to cellular quiescence to form a stable vasculature [86].

Inappropriate angiogenesis is involved in the pathogenesis of several diseases including cancer [58-60, 163]. Therefore, therapeutic manipulation of angiogenesis represents an attractive approach for the treatment of these diseases. In order to improve angiogenic therapeutics however, the identification of both pro as well as anti-angiogenic molecules and characterization of their mechanism of action is important. Interestingly, ECM (ECM) not only provides a scaffold to the growing endothelial cells but also contains distinct angiogenic signals to initiate, drive, and complete angiogenic process [50, 121]. Indeed, several ECM (ECM) molecules have been identified as angiogenic regulators [16, 17, 64, 164]. Therefore, the complete characterization of ECM-based angiogenic molecules, and the molecular

mechanism by which these molecules regulate angiogenesis represents an important, yet underdeveloped avenue towards potential angiogenic-based therapeutics.

Matrix Gla Protein (MGP) is an ECM molecule commonly found near vascular tissues [31, 141, 165]. Primarily, MGP has been described as a calcification inhibitor and is crucial for the maintenance of normal vascular function [141, 146]. For example, MGP -/- mice show severe aortic calcification and uniformly die within two months of birth [141]. In addition, MGP polymorphisms have been linked to coronary artery calcification [143, 166], and MGP suppresses the formation of atherosclerotic lesions in the apolipoprotein E (APOE) knockout mouse model of human atherosclerosis [31]. Mechanistically, MGP sequesters BMP2 and BMP4, thereby blocking BMP signaling through ALK receptors [149]. However, it is not clear whether MGP suppresses vascular calcification via inhibition of BMP signaling alone or if additional mechanisms may be involved. In light of this, a recent discovery suggested that BMP alone is insufficient to promote smooth muscle calcification. Rather, BMP requires cooperation with the notch signaling pathway to promote vascular calcification [118, 119]. This observation is further supported by results showing that signaling through the BMP and Notch pathways synergistically suppress VEGF expression [110, 167].

MGP is not only physiologically important in large blood vessels, but also appears to be an important regulator of capillary function and angiogenesis. MGP suppresses excessive branching of pulmonary capillaries during vascular development in mice [146]. Furthermore, MGP is differentially expressed during angiogenesis [81, 83, 168] and is increased in tumor vasculature where it appears to promote tumor angiogenesis in glioblastoma [145]. Finally, MGP deficiency in mice has been shown to cause arteriovenous malformations in lungs and kidney [117]. Collectively, MGP has been broadly implicated in angiogenesis and vascular

biology, but its exact role in this context is unclear. Therefore, the main goal of this study is to investigate a more direct role for MGP during angiogenesis.

Here, we show that MGP suppresses excessive endothelial sprouting, maintains stable vascular luminal structures, prevents excessive microvascular densities, and reduces tumor angiogenesis. Mechanistically, we find that MGP suppresses notch signaling suggesting that MGP may mediate vascular quiescence at least in part by blocking BMP and notch signaling. Collectively, these results suggest that MGP re-inforces vascular quiescence and promote angiogenic resolution.

Materials and Methods

Ethics Statement

Animal studies were performed in accordance with the animal protocol procedures approved by the Institutional Animal Care and Use Committee of Indiana State University (protocol #1-19-2008:AA and 11-08-2007:AA).

Mouse Breeding and Genotyping

MGP -/+ mice in C57BL/6 background were generously provided by Gerard Karsenty (Columbia University Medical Center, NY). MGP -/+ mice were crossed with wild type C57BL/6 mice and the siblings were crossed to produce MGP -/- mice. Genotype was determined by PCR amplification of DNA from ear tissue using DirectPCR Lysis Reagent (Viagen Biotech, Inc., LA, CA). PCR was performed using specific primers targeting wild type vs. mutant MGP alleles. The wild type primer pairs targeted a 450 bp wild type MGP allele whereas the mutant primer pairs targeted a 1 Kb MGP mutant allele. Wild type and mutant PCR reactions were performed separately using the following conditions: 1 μl template DNA (from ear sample), 100 nM primers, 1X standard buffer, 320 μM dNTPs, and 66 U/ml Taq

polymerase (New England Bio Labs Inc.), and total volume 25 µl. Reactions were cycled according to the following conditions: 94ºC for 2 min (1X); [94ºC for 45 sec; 57ºC for 40 sec; 72ºC for 60 sec] (35X); 72ºC for 5 min (1X); and 4ºC.

Aortic Ring Angiogenesis Assay

Aortas extending from the aortic arch to the diaphragm were removed from five week old C57BL/6 wild type or MGP -/- mice. The aortic sections were washed in 1X PBS and dissected into small rings of equal sizes (~1mm) before implantation into fibrin gels. Fibrin gels were prepared by mixing 1.5 mg/ml fibrinogen with serum free EGM2 media (Lonza Inc.), and filtering through 0.22 µm sterile filters. The fibrin gel was formed by adding 0.06 U/ml Thrombin to 0.5 ml fibrinogen solution in 24-well plates into which the aortic rings were immediately implanted. Fibrin gels were allowed to form at room temperature for 20 minutes before being overlaid with 1 ml EGM2 + growth factors (Lonza Inc.). The plates were incubated in 37ºC in a 5% CO_2 incubator. Aortic rings were observed daily for signs of angiogenic sprouting. Individual sprout lengths were measured after 10 days.

MGP Morpholino Injection in Zebrafish

The MGP specific anti-sense morpholino (Genetools) (5' GAGACACACACATG ACTGCAGGAGC 3') was designed to interfere with MGP mRNA translation initiation. Morpholinos were dissolved in water and diluted 1:1 into 0.1% phenol red/water injecting solution. 12 ng of MGP morpholino was injected into 1 to 8 cells *Fli1*-GFP/*GATA1*-RFP embryos in a total volume of 0.9 nl per embryo. Embryos were sedated in tricaine and monitored for vascular phenotypes on a Nikon SMZ-1500 fluorescent dissecting microscope. Morpholinos directed against p53 were synthesized and used according to previously published work [169].

Immunohistochemistry

Excised tissues were fixed in 4% paraformaldehyde for 1 hr and stored in 70% ethanol before paraffin embedding, sectioning, and staining with CD-31 antibody to visualize vascular structures in the Clarian Pathology Laboratory at Indiana University (Indianapolis, IN). To quantitate vascular density, CD-31 staining patterns were traced onto white paper with black ink and the resulting copy was scanned to obtain total vascular area using Image J software (NCBI).

Immunobloting

The N-terminally Flag-tagged human MGP (N-Flag-hMGP) plasmid was generously provided by Kristina Bostrom (UCLA, CA). Approximately, seventy five percent confluent 293T cells in 10 cm plates were transfected with 10 μg of N-Flag-hMGP plasmid DNA using Trans-IT LT1 transfection reagents (Mirus Inc.) as per the manufacturer's recommendations. After 24 hours, plates were washed with 1X PBS and cultured in serum free media (SFM) overnight. Confirmation of MGP expression in the conditioned media was performed by precipitation with .1% DOC/TCA as described in our previous study [42] followed by western blot analysis using anti-flag M2-antibody.

RT-PCR

RT-PCR was performed to determine the expression of Delta Like-4 (DLL4), jagged-1, Hes-1, CD31, smooth muscle Actin, MGP, and GAPDH. Total RNA was isolated using Trizol reagents (Life technologies, Grand Island NY) as per the manufacturer's instructions. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio Rad Inc.). Primer sequences, target genes, and PCR product sizes for each primer set are listed in Table 1. The PCR reactions were performed under the following conditions: 10 ng cDNA; 200 nM oligos; 320 μM dNTP; 1x

standard buffer; and 66 U/ml Taq Polymerase in a total reaction volume of 25 μl. Cycling parameters used were as follows: 1 cycle at 94ºC for 2 min; 30 cycles at 94ºC for 45 sec, 57ºC for 40 sec, and 72ºC for 60 sec; 1 cycle at 72ºC for 5 min; and hold at 4ºC.

Transfections and Luciferase Assay

Transient transfection of human microvascular endothelial cells (HMEC) was performed in triplicate in 24-well plates. HMEC cells were seeded into 24-well plates at 20,000 cells/well 20-24 hrs prior to transfection. Individual wells were transfected with 200 ng of Hes-1 luciferase plasmid and DNA25 ng of CMV β-galactosidase control plasmid using Trans-IT LT-1 (Mirus Inc.) reagent according to the manufacturer's recommendations. Two days after transfection, the cells were lysed in 100 μl/well of Passive Lysis Buffer (Promega, Madison, WI), freeze-thawed, and centrifuged for 1 minute. Luminescence activities for luciferase and β-galactosidase were measured by Glomax Luminometer using Promega luciferase assay reagents according to the manufacturer's recommendations.

In-vivo Tumor Growth Studies

Pancreatic Adenocarcinoma (PanO2) cells were resuspended in sterile phosphate buffered saline (PBS) and $1x10^6$ cells per 100 µl were injected subcutaneously between the shoulder blades of approximately 10-week-old MGP -/+ and MGP +/+ C57BL/6 mice (three mice per condition; bred in-house). Mice were monitored on a daily basis and primary tumors were measured externally with calipers between days 9 and 17.

Supplementary Methods and Materials

Invasion Assay

For invasion assays, 100,000 endothelial cells suspended in control or MGP containing condition media were cultured (in triplicate) into the upper well of invasion chambers that were previously coated with matrigel. To the lower well of the invasion chamber, EGM-2 media was added and the cells were incubated overnight. Assays were fixed the following day with 95% ethanol and the invading cells were stained with 1X crystal violet. The density of invading cells was measured by densitometry of scanned membranes using ImajeJ (NCBI) software.

Proliferation Assay

For proliferation assay, 2000 endothelial cells/well were cultured in EGM2 growth media supplemented with condition medium containing either control (empty vector) or human MGP (Flag-hMGP) plasmids. Cell proliferation in triplicate wells were examined daily over the next two days by measuring the fluorescent conversion of WST1 cell proliferation reagent essentially as described in our previous publications [83].

Capillary Formation Assay

For capillary formation assay, 200,000 cells/ml were treated with conditioned (hMGP containing) or control (empty vector) media and cultured into a matrigel containing culture plate. Cells were incubated overnight at 37C incubator.

Wound Healing Assay

Confluent plates of endothelial cells were scratched uniformly and were treated with conditioned (hMGP containing) or control (empty vector) media and cultured for few days. Wound healing was determined by relative migration of endothelial cells into the scratched region.

Results

MGP Suppresses Endothelial-Sprouting In-vitro

In our previous study, MGP expression was significantly increased during angiogenesis, but the exact role of MGP in this context was not clear. Here, we used MGP deficient mice to study MGP function during angiogenesis. PCR based genotyping was used to identify MGP -/- mice and RT-PCR analysis was used to confirm the absence of MGP mRNA in MGP-/- animals (Figure S1). Aortas were isolated from ~ 5 week old MGP +/+ or MGP -/- mice, sectioned, and implanted into fibrin gels. As shown in fig 1A and B, ~ 40% of the MGP -/- rings initiated sprouting on day 3 while MGP +/+ rings did not initiate sprouting until day 4. Furthermore, 100% of the aortic rings from MGP -/- mice had sprouted by day 5 whereas 100% of the MGP +/+ rings only sprouted by day 7. Finally, after ten days in culture, angiogenic sprouts from MGP -/- rings were ~1.5 fold longer compared to angiogenic sprouts from MGP +/+ rings (Fig 1C). To determine if MGP was directly responsible for suppressing aortic sprouting, we generated control or MGP containing conditioned media by transfection of 293T cells (Figure S1) and applied these conditioned medias to MGP+/+ and MGP-/- aortic ring cultures. As shown in figure 1, compared to control conditioned media, MGP containing conditioned media delayed sprouting initiation and decreased final sprout length in both MGP+/+ and MGP-/- aortic cultures. Collectively, these results showed that MGP normally acts to suppress angiogenic sprouting and suggested that either 1.) MGP is an endogenous inhibitor of angiogenesis, or 2.) that MGP does not inhibit angiogenesis per se, but rather is important for re-inforcing angiogenic resolution.

MGP Suppresses Microvascular Density in Heart and Skeletal Muscles

Based on the increased sprouting from MGP -/- rings, we predicted that MGP -/- mice

might have increased vascular densities compared to MGP +/+ mice. To investigate this, we isolated various tissues from MGP +/+ and MGP -/- mice and used immunohistochemistry with anti-CD31 antibodies to monitor vascular density. Compared to the MGP +/+ mice, MGP -/- mice had significantly increased CD-31 staining in heart (2.8 fold increase) and skeletal muscles (1.7 fold increase) (Fig 2A), suggesting that MGP inhibits formation of excessive microvasculature in these tissues. These results suggested that MGP is crucial in the maintenance of normal vascular densities in skeletal muscle and heart and further emphasized the importance of MGP in microvascular function.

MGP Suppresses Tumor Angiogenesis

In contrast to our results showing that MGP suppresses angiogenic sprouting, MGP was previously shown to promote tumor angiogenesis [145]. These conflicting observations suggested that MGP may have differential roles in normal physiological and pathological angiogenesis and it was therefore important to distinguish between these possibilities. To accomplish this, we subcutaneously injected pancreatic adenocarcinoma (PanO2) cells into syngeneic MGP +/+ or MGP heterozygous (MGP +/-) mice and monitored tumor growth and vascular density in the resulting tumors. Performing this experiment in MGP +/- mice was necessary since MGP -/- mice typically die by five weeks of age, which prohibits long-term tumor studies whereas -/+ mice have normal life spans. As shown in figure 3, tumors grew significantly faster (Fig 3A) and at dissection were approximately 2.5 fold larger in MGP +/- compared to tumors grown in MGP +/+ mice (Fig 3B, C). Immunohistochemistry of tumor sections with anti-CD31 antibodies (Fig 3D, 3E) and RT-PCR analysis of CD31 mRNA (Fig 3F, 3G) revealed that MGP +/- tumors contained approximately 1.5 fold more blood vessels compared to tumors grown in control mice. Therefore, these results are consistent with our

previous results and do not support differential roles for MGP in normal vs. pathological angiogenesis.

MGP is Important for the Maintenance of Vascular Lumen Structures

To further dissect the role of MGP in vascular function, we examined vascular development in zebrafish embryos injected with anti-MGP morpholinos. Multiple sequence alignment of MGP from seven different species including zebrafish indicated that MGP is highly conserved among species (Figure S2) and therefore suggested that MGP is likely to serve conserved functions in vascular development and function. RT-PCR was used to confirm expression of MGP mRNA in developing zebrafish. As shown in figure S2, MGP mRNA was expressed as early as 10 hours post fertilization (HPF) and continued to be expressed throughout the observed time period. This was consistent with previous observations that showed a similar pattern of MGP expression by immunohistochemistry in zebrafish [170]. Within 24 hours after morpholino injections, MGP morphants showed a progressive developmental defect characterized by an abnormal curvature of the back. A slightly curved phenotype was observed on day 1 (Fig 4A) that became more prominent by day 3 (Fig 4B) and day 6 (Fig 4D). MGP expression in zebrafish has been found in chondrocytes and is important for cartilage mineralization during bone formation [170]. In MGP -/- mice, MGP is important for suppressing pathological calcification of ECM [141]. Therefore it is likely that the deformation of MGP morphants is related to the role of MGP in cartilage and bone development. Importantly however, despite the curved phenotype, normal body segmentation was observed (Fig 4A), which is requisite for vascular development [101]. Inspection of the vasculature revealed that MGP knockdown slightly delayed the angiogenic sprouting of intersegmental vessels (ISV) from aorta on day one (Fig 4A). However, by day 3 ISV vessels

in MGP morphants were similar to those in control fish suggesting MGP is dispensable for the initial establishment of vascular networks. This was consistent with our observations that MGP did not have an observable affect on activities operant during angiogenesis including invasion, proliferation, migration, and tube formation activity of endothelial cells (Figure S3). By day 3 however, MGP-knockdown in the developing zebrafish caused a progressive loss of vascular luminal structures in the ISVs. Day 3 MGP morphants had narrower ISV lumens compared to control fish (Fig 4C, arrows) and blood flow was partially blocked (Fig 4B). By day 6, luminal structures in MGP morphant aortas and ISVs were completely non-evident compared to the control fish (Fig D, E) and MGP morphants developed edema near the anterior side of the heart (Fig 4D, arrow). Overall, these results suggested that MGP is not required for initial vascular development but is crucial for proper maintenance of normal vascular structure.

MGP Decreases Notch Signaling in Cultured Endothelial Cells

Notch activation by DLL4 and jagged-1 has opposing effects on endothelial sprouting during angiogenesis. Jagged-1 promotes endothelial sprouting and angiogenesis whereas DLL4 suppresses sprouting and promotes vascular quiescence [115]. Interestingly, DLL4 knockdown in zebrafish [97], DLL4 -/+ mice [171], and Dll4 blockade [112] all exhibit excessive angiogenesis and loss of vascular lumen structures similar to our results with MGP -/- aortic rings and MGP morphants and suggested a link between MGP and notch signaling. To test this, we utilized a notch responsive luciferase construct featuring the Hes-1 promoter upstream of the luciferase gene to examine notch responsive transcription activity in Human Microvascular Endothelial cells (HMEC) cultured in control or MGP conditioned media. MGP containing conditioned media was prepared by transfection of MGP cDNA into 293T cells and expression was confirmed by western blot (Fig S1). Our luciferase data showed that HMECs

cultured in MGP conditioned media had decreased Hes-1 activity compared to the control HMECs cultured in control conditioned media (Fig 5A). To support this observation, we performed RT-PCR analysis to determine if MGP controls expression of different components of the notch signaling pathway. To accomplish this, 10 day old aortic ring cultures from MGP +/+ and MGP -/- mice were collected by tryptic digestion of fibrin gels, removal of the original aortic ring, and extractions of RNA from the remaining outgrowths. RT-PCR analysis was performed to compare expression levels of smooth muscle actin (smActin), endothelial cell marker (CD-31), GAPDH, the Notch target gene Hes-1, and the notch ligands jagged-1 and DLL4. RT-PCR detected expression of both smooth muscle actin (smActin) and CD-31, confirming that aortic outgrowths were co-cultures of smooth muscle and endothelial cells (Fig 5B). More importantly however, expression of jagged-1 mRNA and Hes-1mRNA in MGP -/- outgrowths was elevated compared to MGP +/+ outgrowths whereas expression of DLL4 mRNA remained unchanged (Fig 5B). Conversely, we also examined gene expression levels in HMEC cells treated with condition media collected from control or MGP transfected 293T cells (Fig 5C). Consistent with previous results, MGP significantly decreased expression of jagged-1 and Hes-1 mRNA but did not affect DLL4 mRNA expression. Collectively, this data provided a correlation between MGP function and notch signaling and suggested that MGP might inhibit angiogenic sprouting by suppressing jagged-1 expression.

MGP Suppresses Aortic Sprouting Independently of Notch and VEGF Signaling

Our results have shown that MGP suppressed angiogenic sprouting and Notch signaling in cultured aortic rings and isolated endothelial cells. Previously, MGP was shown to suppress BMP signaling [144, 147]. Interestingly, BMP and Notch signaling have been co-implicated in the development of vascular calcification [119], angiogenic sprouting [111] and BMP together

with Notch are reported to synergistically suppress VEGF expression [110, 167]. Based on these results, it was important to determine if enhanced aortic sprouting in the absence of MGP was a consequence of enhanced Notch, BMP, or VEGF signaling or a synergistic manipulation of these pathways. To address this question, we monitored sprouting from control or MGP -/- aortic rings in the presence or absence of either the BMP antagonist Dorsomorphin, the γ-secretase/Notch antagonist DAPT, or the VEGF inhibitor SU5416. As previously shown, MGP -/- aortic sections demonstrated significantly enhanced aortic sprouting compared to aortic rings from control mice (Figure 6A). Surprisingly, inhibition of Notch signaling with DAPT or inhibition of VEGF signaling with SU5416 had a minimal effect on aortic sprouting from either control or MGP knockout aortic rings indicating these signaling mechanisms play a minor role for angiogenic sprouting in this assay. However, inhibition of BMP signaling with Dorsomorphin elicited a striking blockade on aortic sprouting from both control and MGP -/- aortic sections suggesting that BMP signaling is the predominant signaling system active under these conditions. Collectively, these results show that BMP is critical for aortic sprouting however, the complete suppression of sprouting in the presence of Dorsomorphin prevents a clear conclusion about the importance of MGP mediated BMP signaling in this assay.

Discussion

Arteriosclerosis, atherosclerosis, and the inappropriate angiogenesis within tumors collectively account for some of the most common abnormalities of the vascular system. An important realization is that each of these diseases is strongly impacted by interactions between vascular cells and components of ECM (ECM) in the vascular microenvironment. In light of

this, growing interest has been shifted toward understanding the interplay between ECM and endothelial cell activity to dissect the pathophysiology of these vascular diseases.

Matrix Gla protein is an ECM protein commonly found in vascular tissues [31, 141, 165] and has been implicated in several vascular abnormalities including arteriosclerosis, atherosclerosis, arterial-venous malformations, and abnormal angiogenesis in tumors [31, 117, 141, 145]. However, the functional role and mechanistic basis by which MGP impacts these abnormalities is unclear. In this study we used a combination of mice and zebrafish MGP knockout models coupled with cell and tissue culture approaches to refine our understanding of the role of MGP in vascular formation and function.

In order to clarify the role of MGP in angiogenesis, we compared angiogenic sprouting from aortas dissected from wild type or MGP knockout mice. Angiogenesis encompasses both the activation phase wherein vascular networks are established by the proliferation, migration, and invasion of endothelial cells, and the resolution phase wherein vascular networks are stabilized and endothelial cell quiescence is established. Our results show that in the absence of MGP, aortic sprouts appeared significantly earlier and grew to greater lengths compared to control aortic rings (Figure 1). Similarly, Yao et al. showed that MGP suppresses pulmonary blood vessel branching [146]. This observation suggested that either MGP normally functions to reinforce the resolution phase of angiogenesis or that MGP actively suppresses the activation phase of angiogenesis. Our data does not support the idea that MGP actively suppresses angiogenesis since we were unable to detect any impact of MGP on endothelial cell activities operant during angiogenesis including proliferation, invasion, migration, and capillary formation (Figure S3). Furthermore, our previous findings showed that MGP expression is increased during the later stages of endothelial network formation [83], which is consistent

with a role during resolution phase. Finally, ISV sprouting in zebrafish embryos was only minimally impacted by MGP knockdown and the initial establishment of functional vascular networks was normal in MGP morphants (Figure 4B). Therefore, we hypothesize that MGP is important for the establishment and maintenance of the endothelial quiescence program. In support of this hypothesis, CD31 staining of MGP knockout adult tissues revealed increased vascular densities in heart and skeletal muscle, suggesting a failure of vascular tissues to achieve quiescence. In addition, tumors grown in MGP -/- mice contained more blood vessels than their control counterparts further illustrating the role of MGP in vascular development. Finally, the progressive loss of vascular lumens in MGP morphant zebrafish shows that MGP is indispensable for the maintenance of functional vascular lumen structures, an important aspect of quiescent vasculature. Collectively, these data suggest that MGP does not significantly impact angiogenesis activation, but rather is important during the resolution phase of angiogenesis.

In mice, MGP blocks BMP-2/4/7 signaling [117, 149]. In the absence of MGP, elevated BMP signaling has been linked to vascular defects including arteriosclerosis, atherosclerosis [31], and arterial-venous malformations [117]. However, in zebrafish embryos, it is not clear that MGP suppresses BMP since zebrafish MGP does not contain a BMP binding domain (Yao et al, 2008). Moreover, the role of BMP signaling in zebrafish intersegmental vessel formation is uncertain since the BMP responsive BRE promoter is not active in developing ISV vessels [172], and specific inhibition of BMP has been shown to have no affect on ISV formation [173]. However, BMP signaling has been implicated in angiogenic sprouting from the axial vein [8]. Taken together, it is not likely that the effect of MGP knockdown on zebrafish ISV luminal structures is strictly due to abnormal BMP signaling. Interestingly,

BMP has been shown to function in synergy with Notch signaling [110, 111] thus potentially implicating Notch with our results.

Activation of Notch signaling by Jagged-1 or DLL4 initiates opposing angiogenic activities. Although opposing, a balance between Dll4 and Jagged-1 is required to establish and maintain functional vasculature [115]. DLL4 promotes quiescent stalk cell selection whereas jagged-1 opposes Dll4 by suppressing the stalk cell phenotype and instead inducing pro-angiogenic tip cell selection in endothelial cells. Disruption of this balance by Dll4 knockdown in zebrafish allows the initial establishment of functional vasculature followed by excessive angiogenic sprouting and the eventual loss of vascular lumen structures [97]. Similarly, blockade of notch ligand DLL4 has been implicated in the development of excessive but non-lumanized vessels in tumors [106, 112]. Similar to these results, we found that MGP morphants experienced a progressive loss of vascular lumen structures and that MGP-/- aortic rings exhibited excessive angiogenic sprouting compared to their control counterparts. Based on these similarities, we hypothesized that MGP may function via the notch signaling pathway. In support of this hypothesis, our luciferase data revealed that MGP suppressed notch activation in endothelial cells. Moreover, our RT-PCR results from mouse aorta and HMEC cells showed that MGP suppressed Hes-1 and jagged-1 mRNA expression but did not alter DLL4 expression (Figure 5). Therefore, our findings suggested that MGP normally functions to maintain a balance between jagged-1 and DLL4 and that in the absence of MGP, excessive jagged-1 destabilizes stalk cell structures resulting in a failure to achieve a stable, quiescent vasculature. Despite this data, BMP inhibition with Dorsomorphin but not Notch inhibition with DAPT completely blocked angiogenic outgrowths from both MGP+/+ and MGP-/- mouse aorta (Figure 6). Therefore, while our *in vitro* gene expression data and zebrafish morpholino

data suggest that MGP functions through Notch in these systems, it is not yet clear if BMP, Notch, or an alternative signaling pathway is responsible for the enhanced sprouting observed in MGP -/- aortic ring cultures.

MGP is not only associated with suppression of arteriosclerosis and atherosclerosis, but up-regulation of MGP has also been associated with poor prognosis in breast cancer [174], gastric cancer [175] and glioblastoma [145]. MGP appears to be significantly up regulated in these cancers and is under evaluation as a potential prognostic marker. In glioblastoma, MGP appears to promote tumor growth, angiogenesis [145], and migration of glioma cells [176]. In contrast, our findings in pancreatic adenocarcinoma (PanO2) showed that tumors grew more rapidly and were more densely vascularized in MGP+/- mice compared to MGP+/+ mice (Figure 3), suggesting a pleiotropic role for MGP in various cancers. One possibility to explain these conflicting observations involves decreased MGP expression in early stage tumors to enable angiogenesis, and increased MGP expression in larger tumors to promote vascular stabilization and increase tumor perfusion. However, it is evident that additional experimentation is required to more precisely define the role of MGP in tumor growth and angiogenesis.

In summary, we have presented evidence suggesting that MGP promotes angiogenic resolution and vascular stabilization. Our findings provide a new avenue toward understanding the role of MGP in both large as well as small vessel dysfunctions. We believe these results will shed light on the pathogenesis of vascular diseases in which MGP is involved such as tumor angiogenesis, atherosclerosis, arteriosclerosis, and arterial-venous malformation.

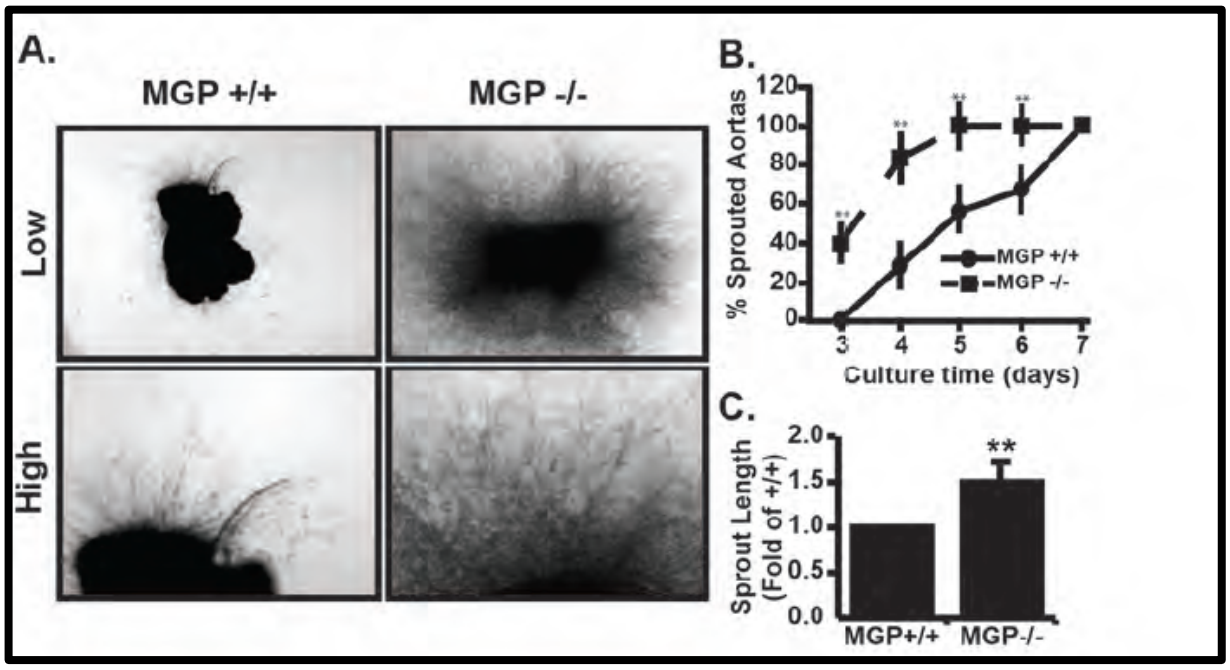

Figure 1. Matrix Gla Protein (MGP) suppresses endothelial sprouting in mouse aortic rings.

A) Aortic rings from MGP +/+ and MGP -/- mice were implanted into fibrin gels, cultured for seven days, and photographed under low and high power magnification. Shown are representative pictures from a single experiment that was performed four times in its entirety. Each experiment consisted of 6-8 aortic ring sections harvested from an individual control or MGP-/- mouse. **B**) The initiation of aortic sprouting was monitored daily and graphed as a percentage of sprouting rings vs time. Data presented are the average +/- SE of four independent experiments. **C**) Average sprout length after seven days in culture was measured using imageJ software. Data represents average +/- SE of four independent experiments. In all experiments, ** indicates $P < 0.05$, student's t-test.

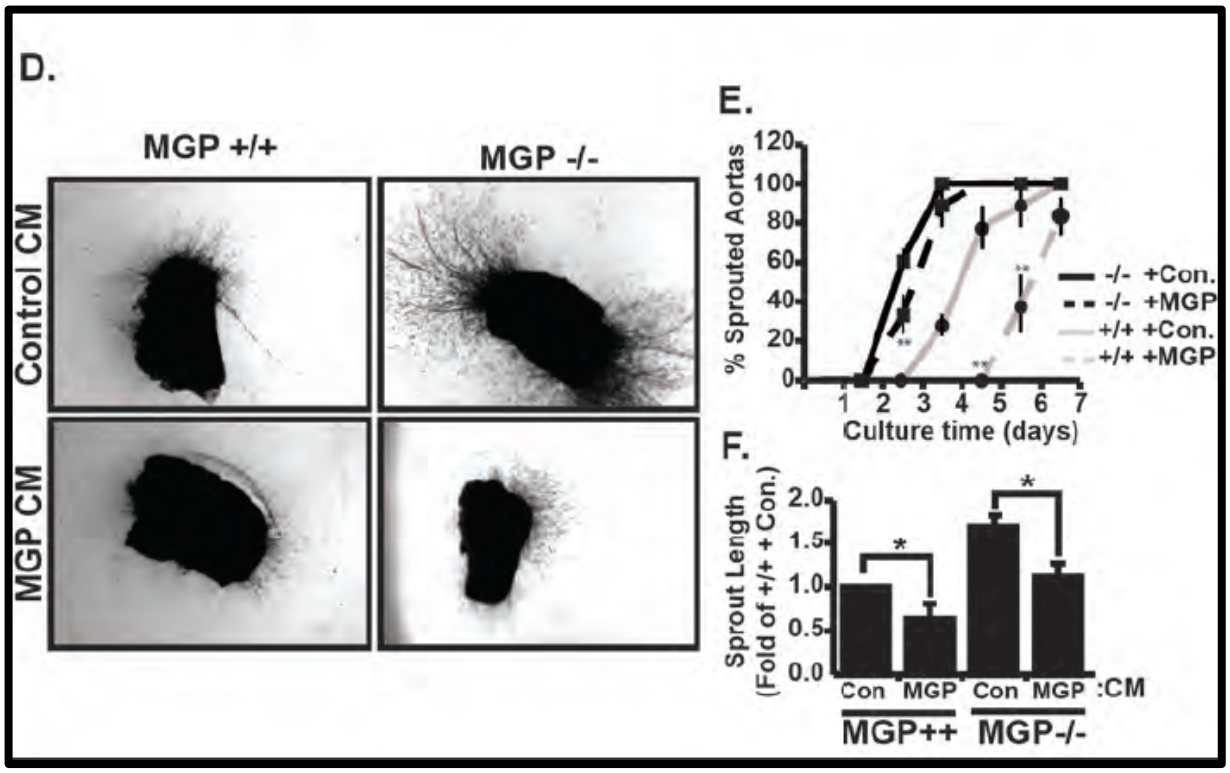

Figure 1D-F. **D**) MGP+/+ and MGP-/- aortic rings were cultures in the presence or absence of conditioned media collected 293T cells transfected with either empty vector (control) or MGP cDNA. Shown are representative images from a single experiment that was performed three times in its entirety. Each experiment consisted of 3-4 MGP+/+ or MGP-/- rings in control or MGP containing conditioned media. **E**) Sprouting from MGP+/+ and MGP-/- aortic rings was monitored daily and the resulting data is depicted as in B. **F**) Average sprout length was measured and the resulting data is presented as in C. In all experiments, ** indicates $P<0.05$, student's t-test.

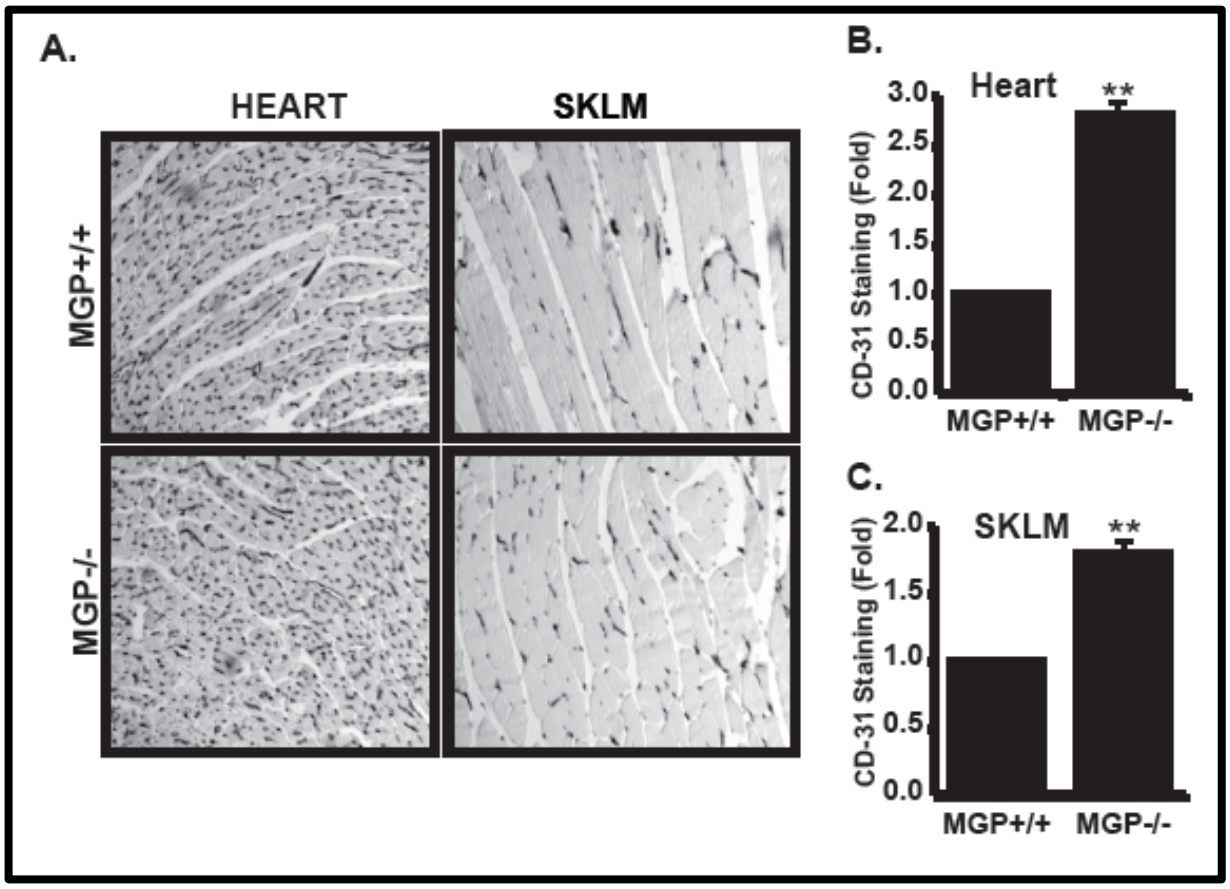

Figure 2. MGP suppresses microvascular density in heart and skeletal muscles.

A) Vascular density in heart and skeletal muscle was monitored by immunohistochemistry with anti-CD31 antibodies. Shown are representative images of a single experiment that was performed three times in its entirety. **B**) Quantification of CD-31 staining in heart tissue form MGP +/+ and MGP-/- mice was performed by imageJ analysis. Data shown represents the average +/- SE of three independent experiments and is presented as the fold change compared to MGP+/+ tissue. **C**) CD31 staining in skeletal muscle (SKLM) was quantified and presented as in B. In both B and C, ** Indicates $P<0.05$, student's-test.

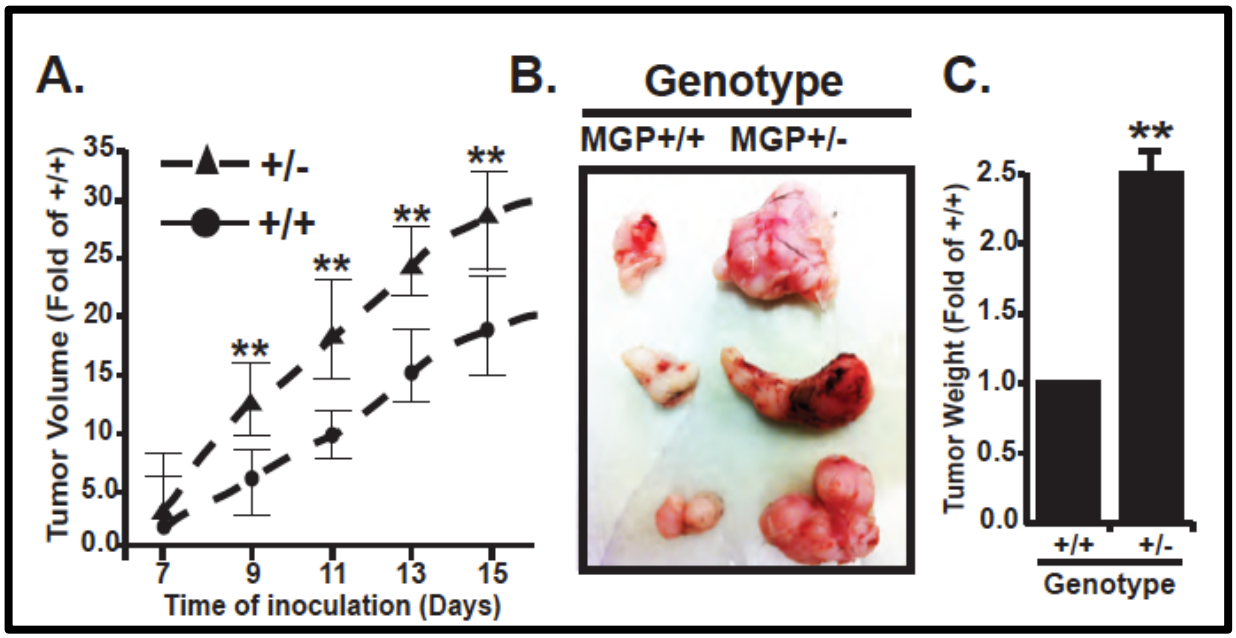

Figure 3. MGP suppresses tumor growth and vascular density.

A) Tumor growth was initiated in control (MGP+/+) or heterozygous (MGP+/-) C57BL/6 mice by subcutaneous injection (in triplicate) of $1x10^6$ Pancreatic Adenocarcinoma ($PanO_2$) cells. Tumor sizes were measured one week after injection and subsequently every other day. Data shown depicts average tumor size +/- SE compared to tumors grown in control mice. **B**) Representative set of triplicate tumors from a single experiment that was performed three times in its entirety. **C**) Tumors mass was recorded and is presented as the average fold change (+/-SE) compared to MGP+/+ tumor mass (N=3). In all experiments, ** indicates $P<0.05$, Student's t-test.

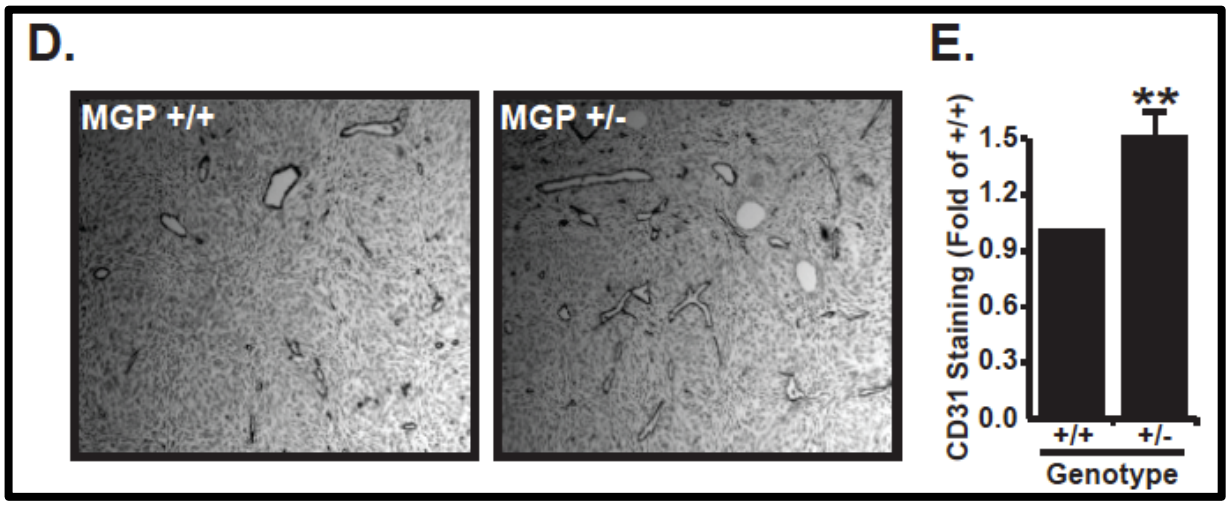

Figure 3D-E. **D**) Immunohistochemistry with anti-CD31 antibodies was used to monitor vascular densities in tumors from MGP+/+ and MGP+/- mice. **E**) Anti-CD31 staining in tumors was quantified by imageJ analysis and is presented as the average (+/- SE) compared to MGP+/+ tumors. In all experiments, ** indicates $P<0.05$, Student's t-test.

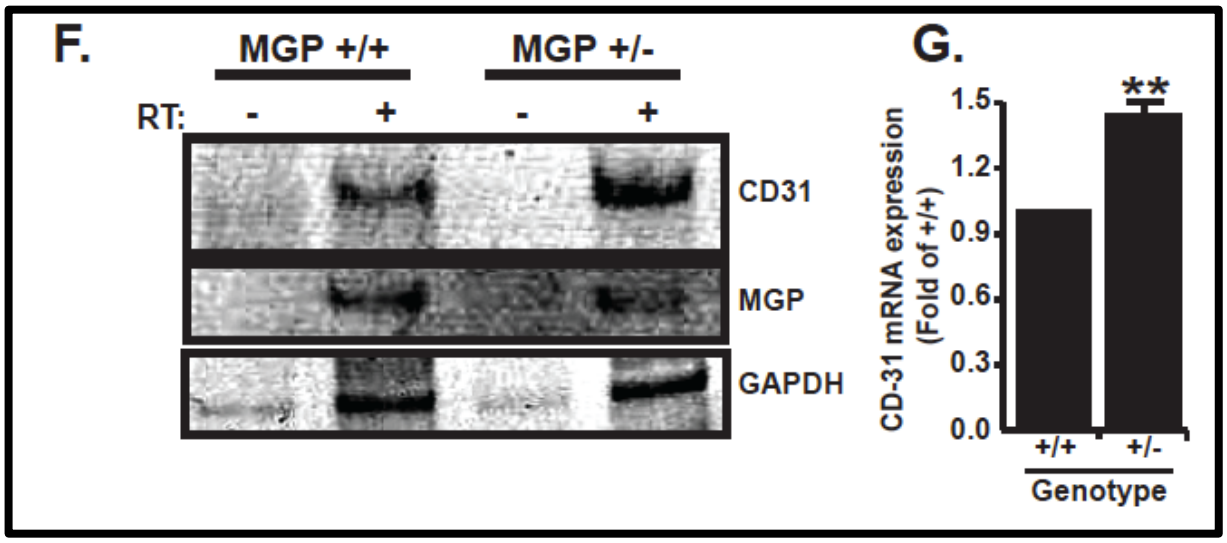

Figure 3F-G. **F)** Total RNA was extracted from MGP+/+ and MGP+/- tumors and used for RT-PCR analysis of CD31, MGP, and GAPDH mRNA expression. Shown are representative images of a single experiment that was performed three times in its entirety. **G)** CD31 RT-PCR results were quantified by imageJ analysis and normalized by GAPDH signal. Data shown depicts CD31 expression in MGP+/- compared to MGP+/+ control tumors and is the average (+/- SE) of three independent experiments. In all experiments, ** indicates $P<0.05$, Student's t-test.

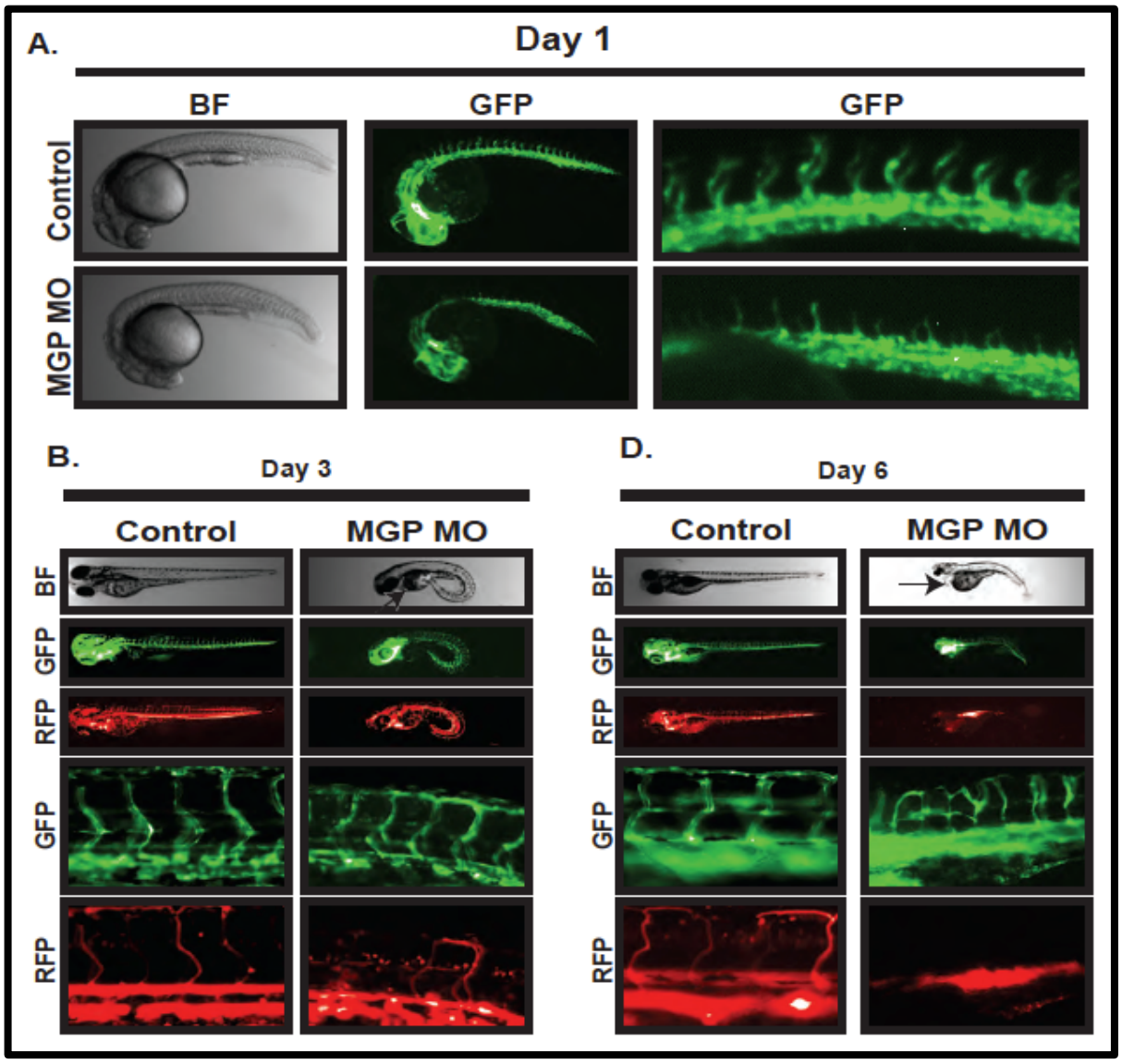

Figure 4. MGP stabilizes vascular structure in zebrafish embryos.

A) Bright field (BF) and endothelial network analysis (GFP) of ~24 hour post fertilization (hpf) embryos at low (middle panel) and high (right panel) magnification. **B**) Analysis of bright field (BF), endothelial network (GFP), and flow dynamics (RFP) in 3 day post fertilization (dpf) embryos. **D**) Analysis of bright field (BF), endothelial network (GFP), and flow dynamics (RFP) in 6 day post fertilization (dpf) embryos. Arrow indicates site of edema accumulation in MGP morphant.

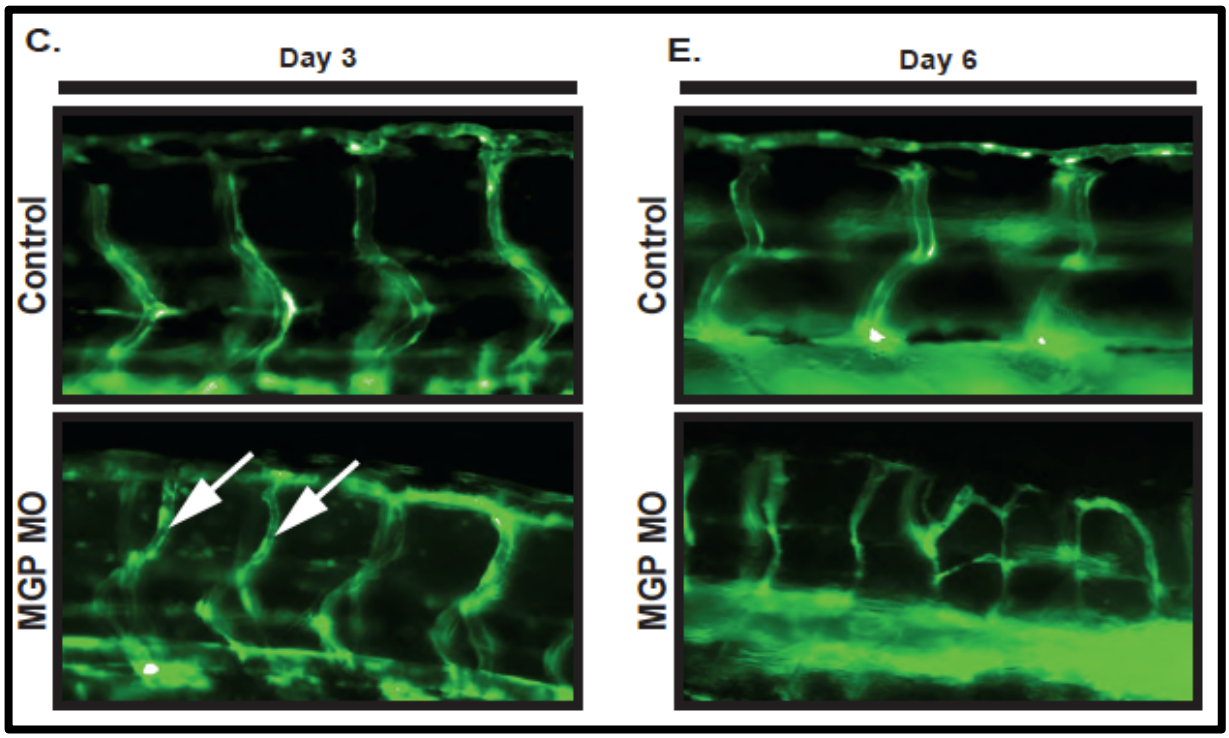

Figure 4C-E. **C**) High power imaging of endothelial networks in 3 dpf embryos injected with either vehicle or MGP morpholinos. Arrows indicate sites of endothelial lumen restriction in MGP morphant. **E**) High power imaging of endothelial networks in 6 dpf embryos injected with either vehicle or MGP morpholinos.

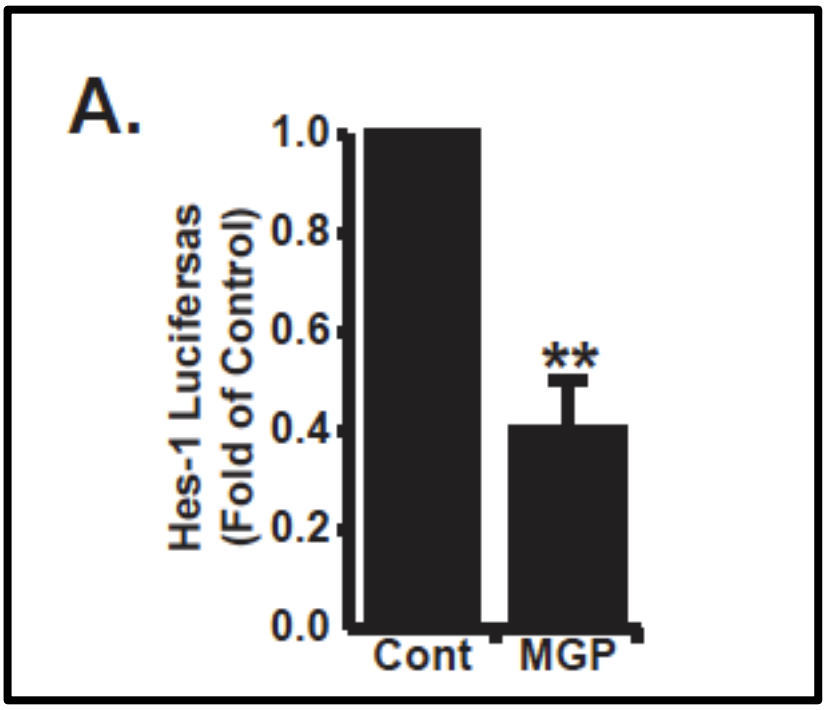

Figure 5. MGP suppresses notch signaling and blocks jagged-1 expression in sprouting endothelial cells.

A) Human microvascular endothelial cells (HMECs) were co-transfected with Hes-1 luciferase + CMV- βgal constructs were cultured either with control condition media (Cont.) or MGP containing conditioned media (MGP). Data depict the average (+/-SE) of four independent experiments and are presented as the fold change relative to HMEC cells cultured in control conditioned media. In all panels, ** indicates $P<0.05$, student's t-test.

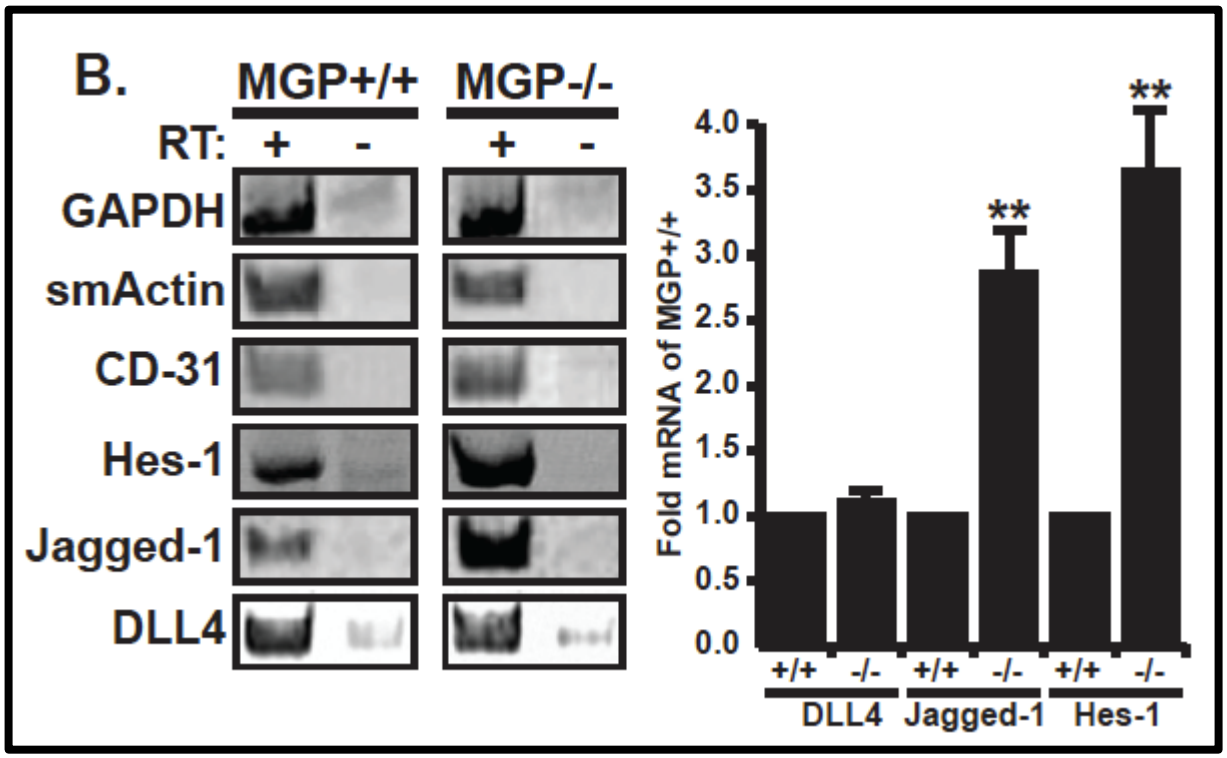

Figure 5B. MGP suppresses notch signaling and blocks jagged-1 expression in sprouting endothelial cells. Total RNA was collected from sprouted MGP+/+ or MGP-/- aortic rings and used to perform RT-PCR analysis of smooth muscle actin (smActin), endothelial cell marker (CD-31), GAPDH, Notch target Hes-1, and notch ligands jagged-1 and Delta like 4 (DLL4) genes. Non-reverse transcribed samples were included as a negative control. Shown are representative images of a single experiment that was performed four times in its entirety. ImageJ analysis was used to compare Dll4, Jagged1, and Hes-1 mRNA in MGP+/+ and MGP-/- aortic rings (right panel). Data shown is the average (+/-SE) of four experiments presented as fold change compared to MGP+/+ samples. In all panels, ** indicates $P<0.05$, student's t-test.

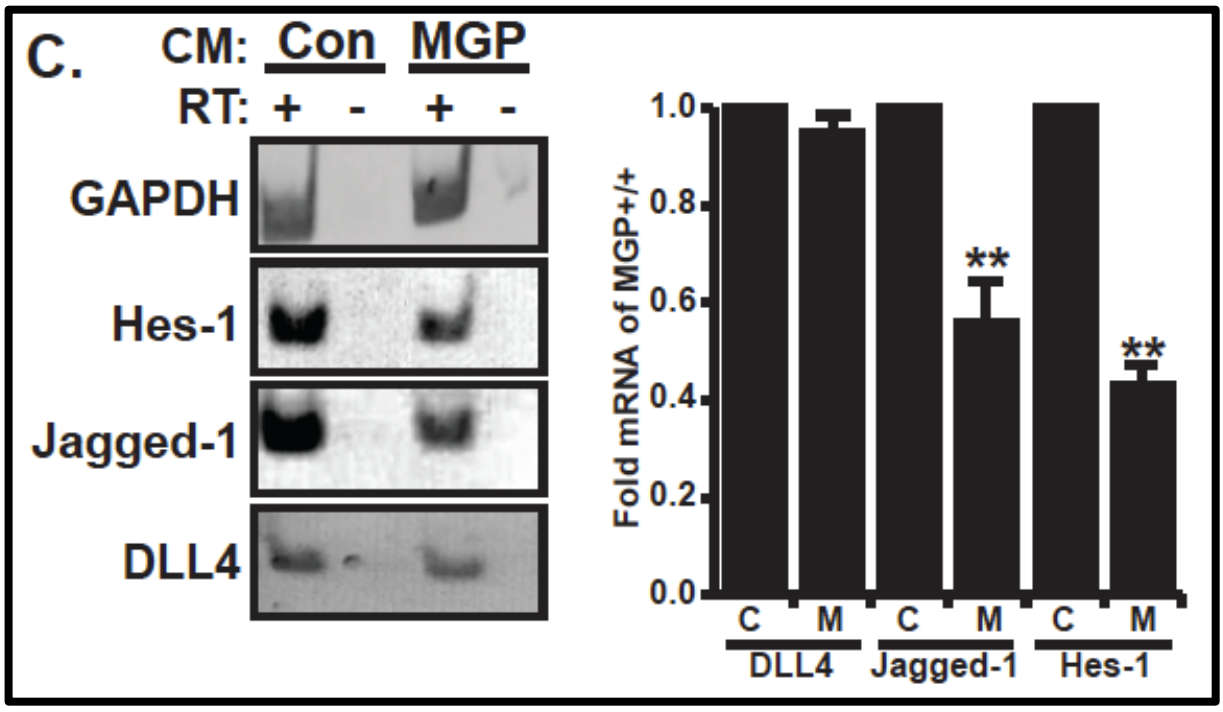

Figure 5C. MGP suppresses notch signaling and blocks jagged-1 expression in sprouting endothelial cells. Total RNA was collected from HMECs cultured in conditioned media from 293T cells transfected with empty vector (C), or MGP cDNA (M). RT-PCR analysis was to monitor the relative mRNA expression levels of Hes-1, Jagged-1, D114, and GAPDH. Non-reverse transcribed samples were included as a negative control. Shown are representative images from a single experiment that was performed three times in its entirety. ImageJ analysis was used to compare Dll4, Jagged1, and Hes-1 mRNA in the absence and presence of MGP (right panel). Data shown is the average (+/-SE) of three experiments presented as fold change compared to MGP+/+ samples. In all panels, ** indicates $P<0.05$, student's t-test.

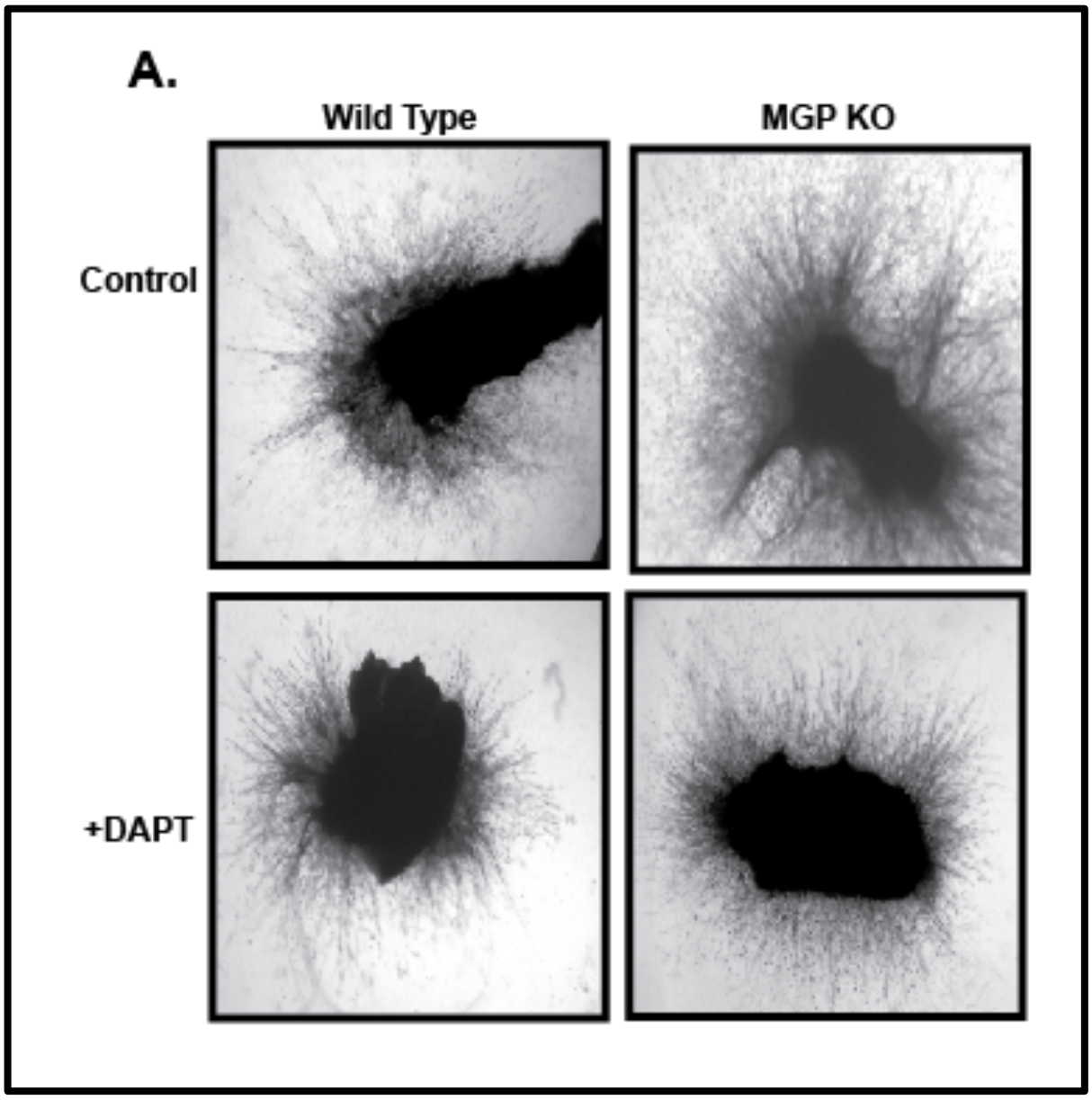

Figure 6. MGP suppresses aortic sprouting independently of Notch and VEGF Signaling.

A) Aortic rings were isolated from MGP+/+ or MGP-/- mice and embedded into fibrin gels containing vehicle (DMSO), gamma-secretase Notch inhibitor (DAPT), broad spectrum BMP inhibitor dorsomorphan (Dorso), or VEGFR2 inhibitor (SU5416). Shown are representative images were collected after 10 days in culture from a single experiment that was performed three times in it's entirety.

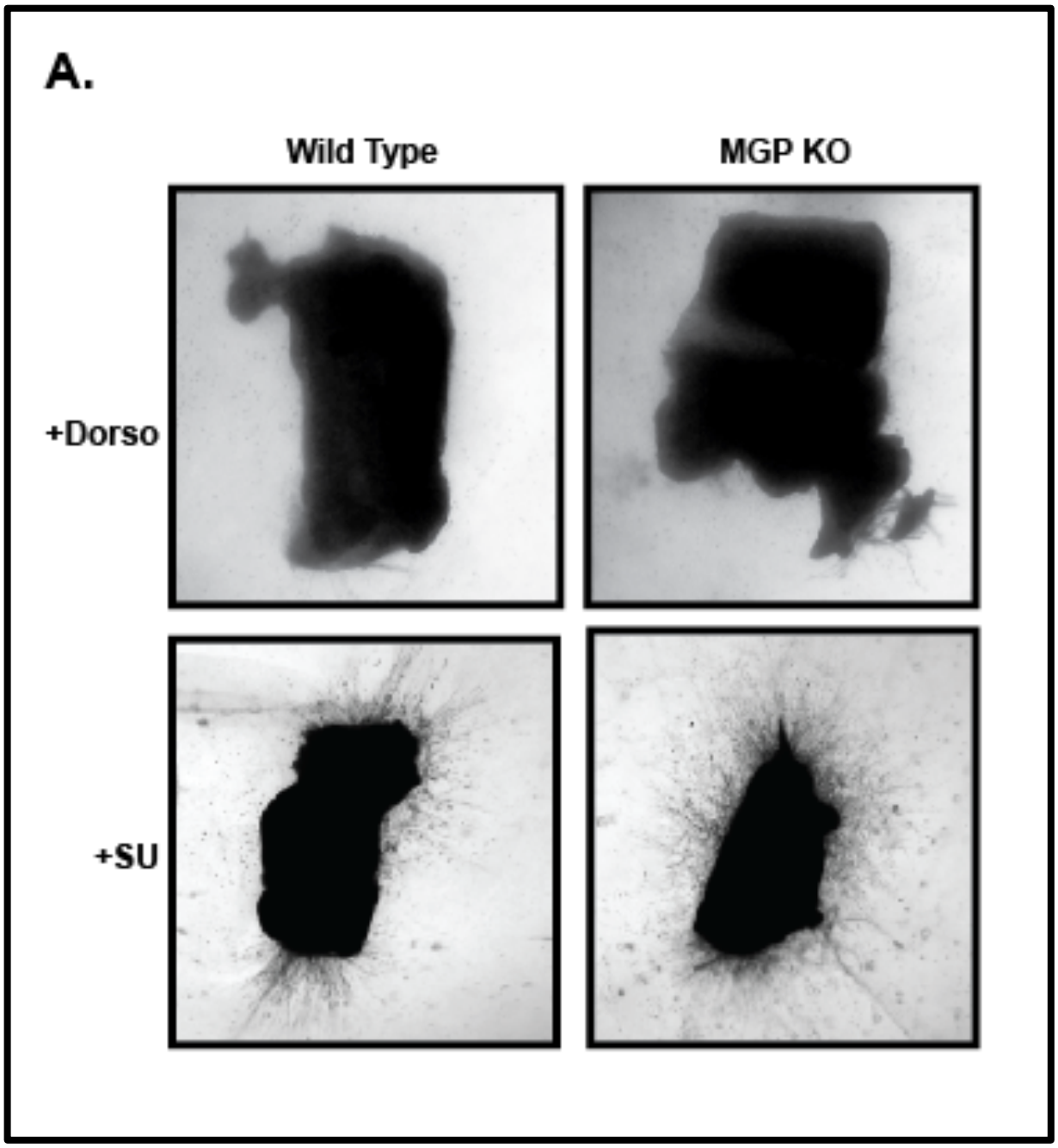

Figure 6A (contd...). Aortic rings were isolated from MGP+/+ or MGP-/- mice and embedded into fibrin gels containing broad spectrum BMP inhibitor dorsomorphan (Dorso), or VEGFR2 inhibitor (SU5416). Shown are representative images were collected after 10 days in culture from a single experiment that was performed three times in it's entirety.

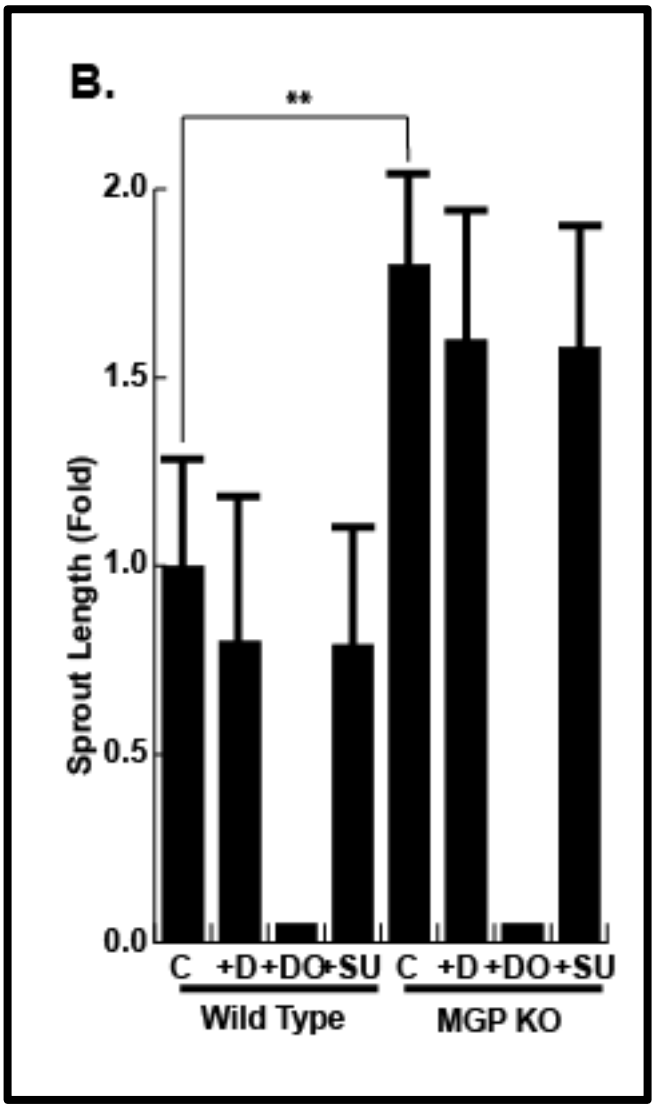

Figure 6B. Quantification of sprout lengths. Data shown is the average (+/-SE) of three experiments presented as fold change compared to MGP+/+ samples. In all panels, ** indicates $P<0.05$, student's t-test.

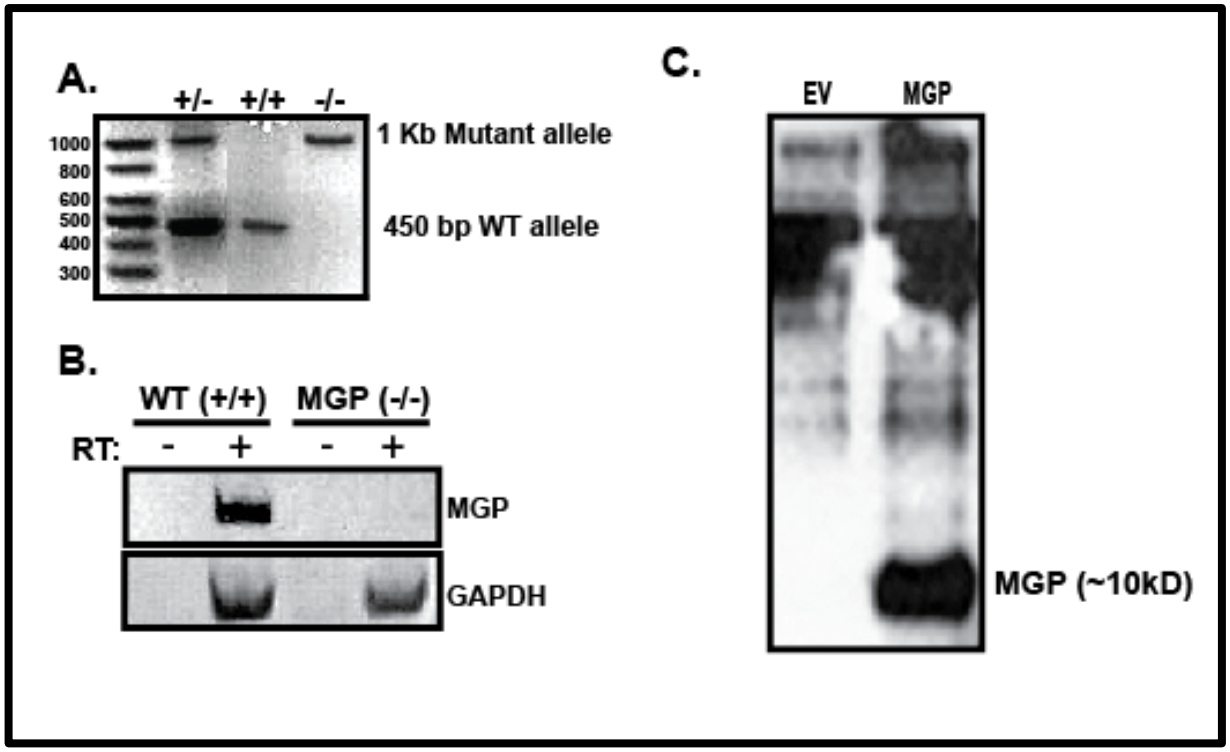

Figure S1. Confirmations of mouse genotypes and MGP protein expression in 293T cells.

A) PCR based genotyping was used to identify MGP+/+, MGP+/-, and MGP-/- mice. PCR amplified 450 bp MGP product in MGP +/+ (WT) mice and 1 Kb mutant DNA product in the MGP -/- (KO) mice. **B)** RT-PCR showing lack or MGP mRNA expression in aortic ring cultures. **C**) MGP containing conditioned media was made by transfecting 293T cells with empty vector or N-terminally flag tagged human MGP construct (N-Flag-hMGP). MGP overexpression in conditioned media was confirmed by western blot analysis of condition media using anti-flag antibody.

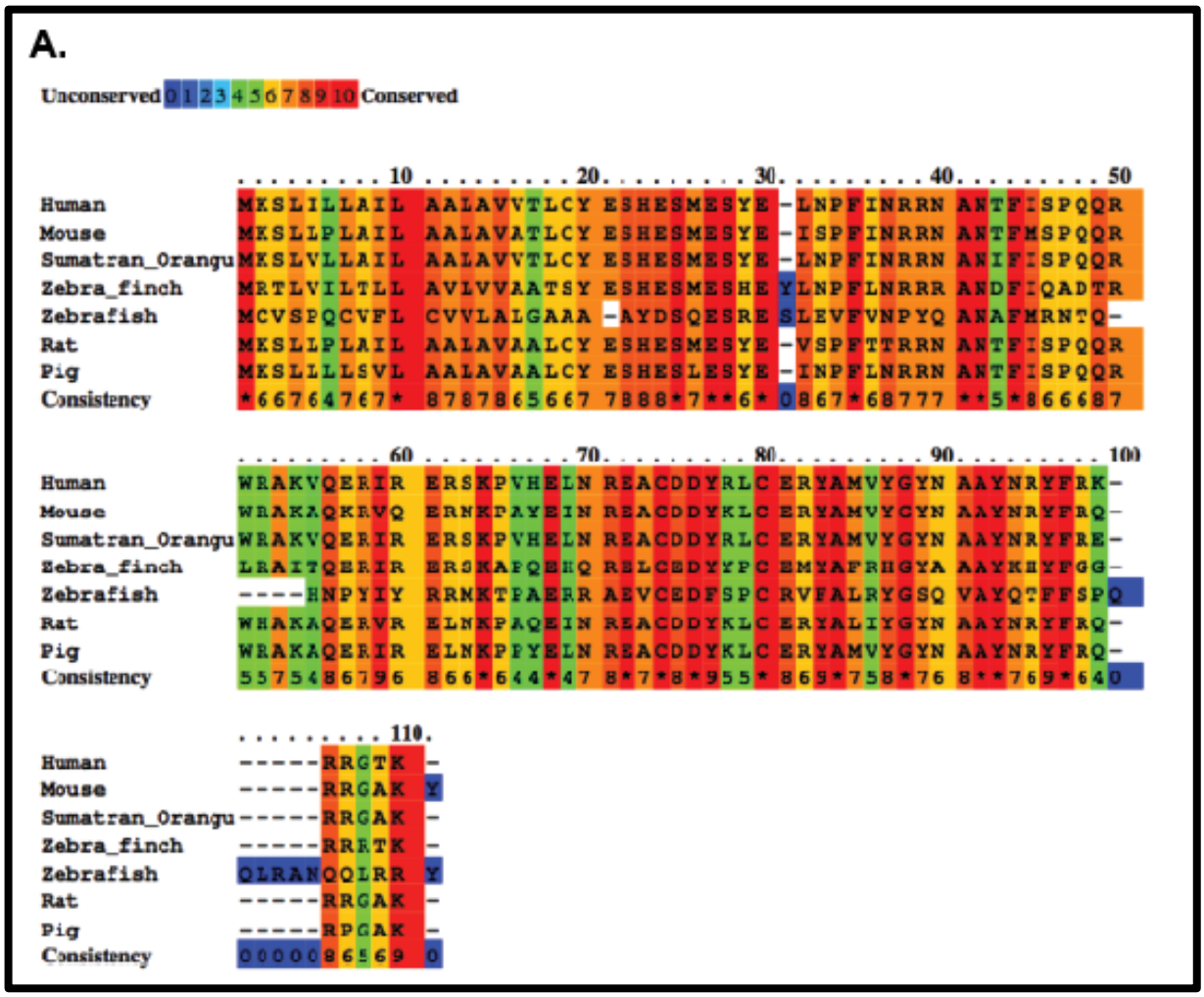

Figure S2A. MGP is evolutionarily conserved across species.

MGP protein sequences from Human, Mouse, Orangutan, Zebra finch, Zebrafish, Rat, and Pig were compared using the Praline sequence alignment tool (http://www.ibi.vu.nl/programs/pralinewww/). The degree of conservation for each amino acid is indicated by color according to the key and in the "conservation" line of the alignment where * indicates a fully conserved residue (i.e. 100% identity). Overall, the alignment showed 62% sequence identity over the entire length of the alignment.

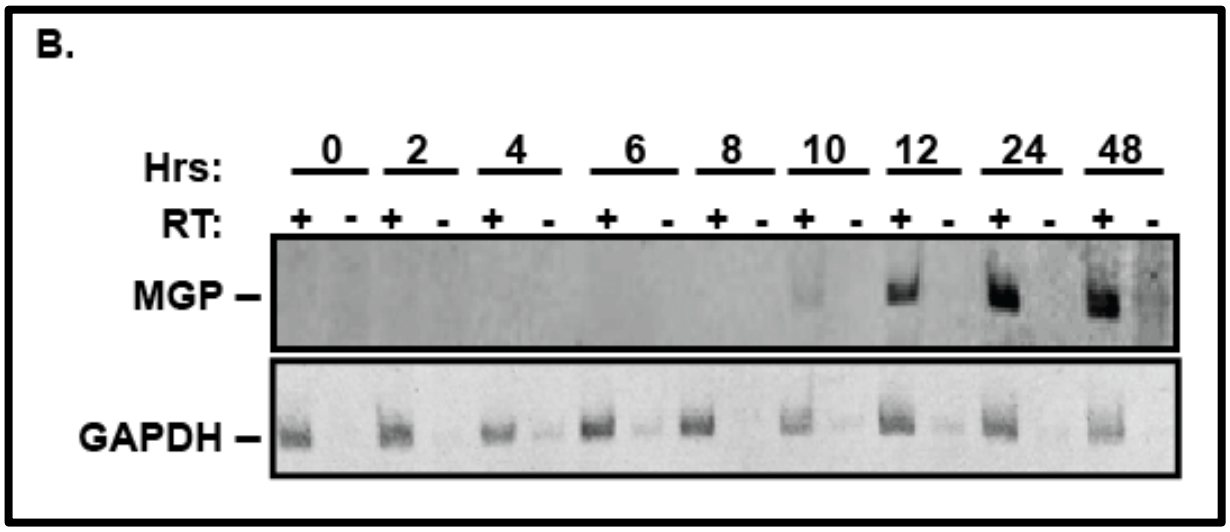

Figure S2B. MGP expression was detected in developing transgenic zebrafish (fli-1-GFP/GATA-1-RFP) embryos by RT-PCR analysis.

cDNAs were generated from embryos ranging from 2 to 48 hours and subjected to RT-PCR analysis with MGP or GAPDH specific oligonucleotides.

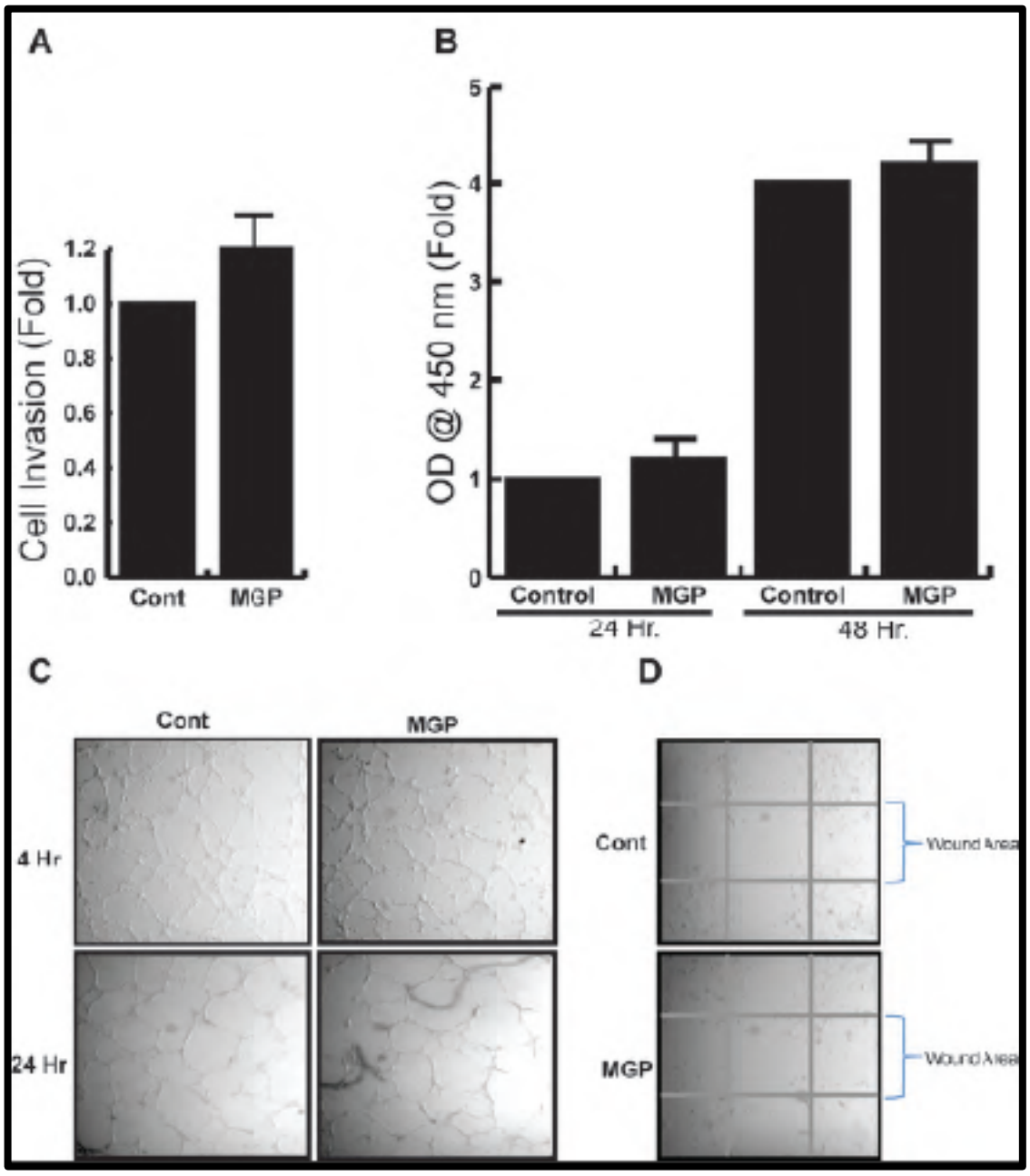

Figure S3. MGP did not affect early angiogenic events including endothelial cell invasion, proliferation, capillary formation, and migration.

Endothelial cells treated with MGP conditioned (MGP) or control (Cont.) conditioned media were subjected to A) Invasion assay, B) Proliferation assay, C) Capillary formation assay, and D) Wound healing assay.

Table 1. List of oligonucleotides.

No.	Oligonucleotide sequences	Targets	Organisms
1.	FORWARD-5' GCC ACA ATT TCT GCA TCC TGC 3' REVERSE- 5' CGG GAA AGA TGA GGA AGA AGG G 3'	MGP (WT)	Mouse
2.	FORWARD- 5' TGC CTG AAG TAG CGG TTG TA 3' REVERSE- 5' TGA ATG AAC TGC AGG ACG AGG 3'	MGP (Mutant)	Mouse
3.	FORWARD- 5' GAC AAT GAA TAC GGC TAC AGC AAC 3' REVERSE- 5' GTG CAG CGA ACT TTA TTG ATG GTA 3'	GAPDH	Mouse
4.	FORWARD- 5' TGC TGC AGC TTA TGA CTC TCA GGA 3' REVERESE- 5' AAA CAC ACG ACA CGG AGA GAA GTC 3'	MGP	Zebrafish
5.	FORWARD- 5' AGG CTT CTC ACA AAC GAG GAC ACA 3' REVERSE- 5' ATC AAT GAC CAG TTT GCC GCC TTC 3'	GAPDH	Zebrafish
6.	FORWARD- 5' TCC GTG ACA TCA AGG AGA AGC TGT 3' REVERSE- 5' GGA AGC GTT CGT TTC CAA TGG TGA 3'	Aortic smActin	Mouse
7.	FORWARD- 5' AGC TAG CAA GAA GCA GGA AGG ACA 3' REVERSE- 5' TAA GGT GGC GAT GAC CAC TCC AAT 3'	CD-31	Mouse
8.	FORWARD- 5' TAG TGA ATG TGC CCT GGT GTC CAT 3' REVERSE- 5' TGA TCC TAA GGC TGC CAT CAC CAT 3'	Jagged-1	Mouse
9.	FORWARD- 5' TCT TCC GCA TCT GCC TTA AGC ACT 3' REVERSE- 5' GCC AGG TGA AAT TGA AGG GCA ACT 3'	DLL4	Mouse
10.	FORWARD-5' TCC ATG ACA ACT TTG GTA TTC GT 3' REVERSE- 5' AGT AGA GGC AGG GAT GAT GTT 3'	GAPDH	Human
11.	FORWARD- 5' ATT GCC AGC TAC TAC TGC GAC TGT 3' REVERSE- 5' TTC TGG CAG GGA TTA GGC TCA CAA 3'	Jagged-1	Human
12.	FORWARD- 5' CCT GCA TTG TGA ACA CAG CAC CTT 3' REVERESE- 5' ACA TGA GCC CAT TCT CCA GGT CAT 3'	DLL4	Human
13.	FORWARD- 5' TGG AGA AGG CGG ACA TTC TGG AAA 3' REVERSE- 5' ATG GCA TTG ATC TGG GTC ATG CAG 3'	Hes-1	Human

CHAPTER IV

LUMICAN EXIHIBITS ANTI-ANGIOGENIC ACTIVITY IN A CONTEXT SPECIFIC MANNER

Abstract

Lumican belongs to small leucine rich proteoglycan (SLRP) family of ECM proteins. We have previously shown the anti-angiogenic effect of Lumican in various cancer models. However, these studies were primarily based on exogenously overexpressed Lumican in cancer cell lines and the results were insufficient to separate the effect of Lumican from the host. Here, we ask whether the endogenous Lumican also has anti-angiogenic properties and if Lumican's activity is similar between physiological versus pathological angiogenesis. Our PanO2 tumor study in knockout mice shows that Lumican is an endogenous inhibitor of angiogenesis in the tumor and restricts the tumor growth. However, Lumican shows a null effect in physiological angiogenesis since we did not find any difference in angiogenic response between Lumican WT and KO mouse measured by matrigel plug assay and aortic ring assay. Interestingly, however, Lumican did not have an anti-angiogenic effect in 4T1 breast cancer but suppressed 4T1 metastasis to lungs. Collectively, we show that Lumican as an anti-tumorigenic agent but its anti-angiogenic effect is context dependent.

Introduction

The cellular microenvironment provides critical input as cells attempt to achieve and maintain cellular homeostasis. In particular, extracellular matrix (ECM) proteins in the microenvironment govern a variety of processes such as proliferation, migration, apoptosis, and response to cellular signaling stimuli. While ECM provides critical input to cells and tissues, cells also remodel their local microenvironment to reinforce cellular activities and programs. Because of this interplay between cells and ECM, altered cellular microenvironments can have profound impacts on various disease states. In particular, the development and growth of cancers involves pathological remodeling of ECM into a reactive tumor stroma that supports cellular programs of malignancy and drives the growth, neovascularization, invasion, and metastasis of tumors.

While reactive tumor stroma generally promotes tumor aggressiveness, not all components of the tumor stroma work in favor of the tumor. Instead, there have also been identified several ECM molecules that possess distinct anti-tumor, anti-angiogenic, and anti-metastatic activities. Identification and characterization of these anti-cancer ECM proteins represents an important therapeutic avenue towards suppressing cancer in humans.

Lumican is a member of small leucine rich proteoglycan (SLRPs) family of extracellular matrix (ECM) proteins. The structural role of Lumican is attributed toward its ability to promote fibrillogenesis and stabilization of collagen fibers [153, 177]. Lumican is also a matricellular protein that interacts with the VEGF, FGF and Fas [42, 83] signaling mechanisms. However, in recent years, growing evidence has implicated Lumican in cancer and angiogenesis. To this end, differential expression of Lumican has been detected in cancers of the breast, colon, cervix, and pancreas [157, 159, 161, 178-182]. However, a clear

correlation between differential Lumican expression and cancer malignancy has yet to be identified. This is surprisingly given that in experimental models of tumor angiogenesis, Lumican has consistently demonstrated significant anti-angiogenic activity [42, 83, 162, 183].

The results of this study confirm the anti-angiogenic activity of Lumican, but also demonstrate that Lumican is not anti-angiogenic under all circumstances. Rather, we find that Lumican suppresses angiogenesis in some but not all cancer models and that Lumican does not suppress angiogenesis in non-pathological settings including healing wounds, cultures of aortic rings, and matrigel plugs.

Materials and Methods

Wound Healing Model

Control and Lumican knockout Male C57BL/6 mice were anaesthetized by intraperitoneal injection of ketamine hydrochloride (2 mg/g body weight) and xylazine (0.4 mg/g body weight). Shaved skin was sterilized using an alcohol swab and a biopsy punch was applied to create a circular full thickness skin wound about 6 mm in diameter below the shoulder blades. Mice were sacrificed and tissues were collected 3, 7, 14, and 21 days after wounding.

Mouse Breeding and Genotyping

Lumican -/- mice in C57BL/6 background were generously provided by. Heterozygous (Lum -/+) male and female mice were crossed produce homozygous knockout (Lum -/-) mice. PCR based genotyping was performed on DNA isolated from ear tissue using DirectPCR Lysis Reagent (Viagen Biotech, Inc, LA, CA) to identify the mouse genotype. PCR was performed using specific primers targeting wild type vs. mutant Lumican alleles. The wild type primer set targeted 190 bp wild type Lumican allele whereas the mutant primer pairs targeted a 390 bp

mutant allele. Wild type and mutant PCR reactions were performed in a single reaction using following conditions: 1 μl template DNA (extracted from ear sample), 100 nM primers, 1X standard buffer, 320 μM dNTPs, and 66 U/ml Taq Polymerase (New England Bio Labs Inc.), and total volume 25 μl. Thermocycle conditions used for each reactions are as follows: 94°C for 2 min (1X); [94°C for 45 sec; 64°C for 30 sec; 72°C for 30 sec] (35X); 72°C for 5 min (1X); and 4°C.

Immunohistochemistry

Tumors were isolated, sectioned, and fixed in 4% paraformaldehyde for 1 hour and placed in 70% ethanol before paraffin embedding, sectioning, and staining with CD-31 antibody to analyze microvascular densities in tumors in the Clarian Pathology Laboratory at Indiana University (Indianapolis, IN). Quantification of vascular density was performed by tracing CD-31 patterns onto white paper with black ink and scanning the resulting copy to obtain total vascular area using Image J software (NCBI).

In-vivo Tumor Growth and Metastasis Studies

Freshly cultured Pancreatic Adenocarcinoma (PanO2) cells were resuspended in sterile 1X PBS and $1x10^6$ cells per 100 μl were injected subcutaneously between the shoulder blades of approximately 10-week-old Lumican WT, HET, and KO C57BL/6 mice (three mice per condition). For 4T1, 4000 cells per 100 μl were injected directly into breast fat-pads of syngeneic BalbC mice. Tumors in mice were monitored on a daily basis and primary tumors were measured externally using calipers between days 7-15 in an interval of 2 days. Metastasis to lungs was monitored by dissecting both lungs from tumor bearing mice, mincing the tissue with a razor, and chemically digesting the minced lung tissue for 2 hours with collagenase

solution. Large undigested pieces of lung were allowed to settle, then 10 X 10^6 cells were plated into 10 cm dishes in the presence of 6-thioguanine to kill normal cells and select for resistant 4T1 cells present in lung. After approximately 10 days in culture, colonies of metastatic cells were stained with crystal violet and overall staining density was determined with image-J software.

In-vitro Angiogenic Outgrowth Assay (Aortic Ring Assay)

Aortas (~1 cm in length) were removed from 5 week old C57Bl/6 wild type or Lum -/+ or Lum -/- mice. The aortic sections were washed in 1X PBS and cut into small rings of equal sizes and implanted into fibrin gels. Fibrin gels were prepared by mixing 1.5 mg/ml fibrinogen with serum free EGM2 media (Lonza Inc.), and filtering through 0.22 μm sterile filters. The fibrin gel was formed by adding 0.06 U/ml Thrombin to 0.5 ml fibrinogen solution in 24-well plates into which the aortic rings was immediately implanted. After fibrin gels are formed at room temperature for 20 minutes, 1 ml EGM2 complete media (Lonza Inc.) was added from the top. The plates were incubated in 37°C in a 5% CO_2 incubator. Aortic rings were observed daily for signs of angiogenic sprouting and recorded. Individual sprout lengths were measured after 10 days of culture.

Matrigel Plug Assay

Matrigel implantation was performed on 6-week-old C57BL/6 WT, HET, or KO mice. Briefly mice were injected subcutaneously in the ventral groin area with Matrigel (700 μl/injection; BD Biosciences, Bedford, MA) supplemented with bovine bFGF (300 ng/mL; R&D Systems, Minneapolis, MN). Seven days postimplantation, the mice were sacrificed and

the plugs were harvested. Three mice were used per experimental condition and the experiments were repeated thrice in its entirety.

Results

Lumican Is an Endogenous Inhibitor of Angiogenesis

Previously, Lumican was shown to decrease tumor angiogenesis in pancreatic adenocarcinoma (PanO2) cells, MCA102 fibrosarcoma cells, and B16F10 melanoma cells [42, 184]. However, these studies relied on ectopic expression from implanted tumors to drive Lumican expression and therefore fail to account for host expression of Lumican. Therefore, we compared the growth and angiogenesis of tumors formed by injection of pancreatic adenocarcinoma (PanO2) cells in wild-type (WT), heterozygous (HET), and Lumican knockout (KO) mice. Importantly, the PanO2 cell line was previously used to illustrate the anti-angiogenic activity of overexpressed Lumican *in vivo* [42] and therefore made an ideal cell line with which to examine host Lumican contributions to tumor growth and angiogenesis. Wild-type C57BL6 mice, or HET and KO littermates were identified by PCR as demonstrated in Figure 1A. PanO2 tumor growth in Lumican KO mice was significantly accelerated compared to tumor growth in WT mice (Fig. 1B). Tumor growth in Lumican HET mice strongly tended toward accelerated growth although this increase in growth rate failed to reach statistical significance. At dissection, tumors recovered from KO and HET mice were approximately 4.4 and 2.2 fold larger respectively than tumors recovered from WT mice (Fig. 1C). To determine if host expressed Lumican also suppressed PanO2 tumor angiogenesis, tumors were dissected from WT, HET, and KO mice and processed for immunohistochemistry with anti-CD31 antibodies to detect vascular elements. As shown in figure 2, both Lumican KO and HET tumors exhibited a striking increase in vascular density. Interestingly, vessels in

Lumican KO and HET tumors tended to be of larger caliber than their WT counterparts. Densitometry with imageJ software revealed 4.0 and 2.7 fold more CD31 staining in Lumican KO and HET tumors respectively. Collectively, these results illustrated that host derived Lumican suppresses tumor growth and angiogenesis in a similar fashion to ectopically expressed Lumican and therefore indicated that Lumican is an endogenous inhibitor of PanO2 tumor angiogenesis.

Lumican Suppresses Breast Cancer Growth and Metastasis, but Not Angiogenesis

The fact that host derived Lumican suppressed angiogenesis in PanO2 tumors confirmed our previous results showing that Lumican overexpression suppresses tumor angiogenesis [42] and indicated that Lumican is an endogenous inhibitor of angiogenesis. However, the role of Lumican in many other cancers remains undefined. Therefore, to further investigate the potential anti-cancer role for Lumican, we studied the function of Lumican in breast cancer, a pathology wherein Lumican has been alternatively correlated with increased and decreased malignancy but has not been directly assayed [156, 179]. To clarify the role of Lumican in breast cancer, we created Lumican overexpressing breast cancer cell lines by transducing NMuMg normal murine mammary gland, and 4T1 breast cancer cell lines with retroviral particles encoding either Lumican or empty vector control sequences. Overexpression of Lumican in the resulting cell lines was confirmed by TCA/DOC precipitation of conditioned media followed by western blotting with either anti-Myc 9E10 (NMuMg) or anti-FLAG M2 (4T1) antibodies to detect C-terminally tagged Lumican transgene expression. As shown in figure 3A and B, transgene expression of Lumican protein was evident in overexpressing cell lines but not in empty vector control cells. To determine of Lumican had a direct affect on breast cancer cells, we compared proliferation rates and

invasive activity in control and Lumican overexpressing cells. As shown in figure 3C and D, Lumican did not significantly affect cell proliferation in either NMuMg or 4T1 cell lines but did significantly decrease cell invasion through Matrigel coated Boyden chambers in both cell lines (Fig 3E, F).

Having shown that Lumican suppresses breast cancer cell invasion *in vitro*, it was important to determine if Lumican suppresses tumor growth, angiogenesis, and invasion/metastasis *in* vivo. To accomplish this, control and 4T1-Lumican cells were orthotopically injected into the fatpad of syngenic BALB/c mice. After seven days, tumors were measurable and tumor volume was recorded every other day for fourteen days. As shown in figure 4A, Lumican overexpressing 4T1 cells formed tumors significantly slower than their control counterparts. Moreover, Lumican expression decreased the final tumor mass by approximately 70% compared to control tumors (Fig 4B). To determine if reduced tumor mass was associated with reduced tumor angiogenesis, sections of control and 4T1-Lumican tumors were sectioned and stained with anti-CD31 antibodies. Surprisingly, we were unable to detect any difference in the vascular density between control and 4T1-Lumican tumors (Fig D, E). Given our *in vitro* data showing that Lumican suppressed 4T1 and NMuMg invasion, it was important to determine if Lumican suppressed 4T1 invasion/metastasis *in vivo*. To accomplish this, lungs were collected from control or Lumican overexpressing tumors and rendered to single cell suspensions by digestion with Collagenase solution. Equal numbers of cells were subsequently cultured on tissue culture dishes in the presence of 6-thioguanine to select for 4T1 cells/colonies. As shown in figure 5, lungs from Lumican overexpressing cells generated significantly fewer 4T1 colonies compared to lungs from control animals.

Collectively, these results showed that Lumican blocks breast cancer growth and metastasis to lung independently of angiogenesis suppression. Interestingly, this was the first example wherein Lumican failed to suppress angiogenesis and suggested that Lumican may not be a universal inhibitor of angiogenesis but rather may inhibit angiogenesis only under restricted circumstances.

Endogenously Expressed Lumican Does Not Suppress Non-Pathogenic Angiogenesis

Based on our results showing that Lumican did not suppress angiogenesis in 4T1 tumors, we were interested to determine if there are other circumstances where Lumican does not suppress angiogenesis. In particular, new blood vessels are a crucial component of granulation tissue where they help drive tissue healing. Therefore we compared vascular density in healing wounds from control and Lumican KO mice. Cutaneous wounds were made by punch biopsy and the resulting healing wounds were collected 3, 7, 14, and 21 days after initial wounding. Wound samples were stained with anti-CD31 antibodies to visualize neovascularization in the healing wounds. As shown in figure 6, similar densities of CD31 staining were detected at all time points in WT and KO mice suggesting that Lumican did not significantly affect angiogenesis in healing tissue. In addition, we also compared angiogenic sprouting from WT, HET, and KO aortic rings. Aortas were isolated from WT, HET, and KO mice, embedded into fibrin gels, and monitored over a ten day period for signs of angiogenic sprouting. As shown in figure 7A, the initiation of angiogenic sprouting was indistinguishable between WT, HET, and KO rings. In addition, the final length of aortic outgrowths was not significantly longer in HET and KO cultures compared to WT outgrowths (Fig 7B). Importantly, RT-PCR confirmed the presence of Lumican in WT aortic outgrowth indicating that the failure to observe a decrease in angiogenic sprouting was not due to the absence of

Lumican expression (Fig. 7C). Finally, we also examined the affect of Lumican deletion on Matrigel plug angiogenesis. Our previous results demonstrated that recombinantly produced and purified Lumican suppressed angiogenesis in matrigel plugs [83]. Therefore, this system provided a unique opportunity to directly compare exogenous versus endogenous Lumican angiostatic activity. Solutions of matrigel containing 300 μg/ml of bFGF were subcutaneously injected into either WT, HET, or Lumican knockout mice and ten days later were dissected and compared for evidence of vascularization. As shown in figure 7E matrigel plugs dissected from WT, HET, and KO mice all contained similar amounts of blood suggesting angiogenesis was not altered in the absence of Lumican. Importantly RT-PCR detected Lumican mRNA in control matrigel plugs indicating that Lumican was present in the matrigel plugs (Fig. 7F). Collectively, the failure of Lumican deletion to affect angiogenesis in healing wounds, aortic ring assays, or matrigel plugs strongly suggested that endogenously expressed Lumican is anti-angiogenic only under certain circumstances.

Discussion

We and others have begun to unravel the function of Lumican in experimental models of cancer and angiogenesis however there remains significant questions regarding Lumican function in these processes. One persisting question has to do with the fact that although Lumican has until now been shown to consistently suppresses angiogenesis [42, 83, 183], Lumican is nonetheless commonly over expressed in cancers but does not consistently correlate with decreased malignancy as would be predicted for an angiogenesis inhibitor. Our current results help clarify this quandary by showing that Lumican's anti-angiogenic activity manifests only under restricted circumstances. In combination with our previous results [42] we have found that both overexpressed and endogenously expressed Lumican suppressed

angiogenesis in PanO2 tumors while overexpressed Lumican failed to suppress angiogenesis in 4T1 breast cancer tumors. Therefore, we believe that the failure of Lumican to consistently correlate with decreased cancer malignancy is due to the differential ability of Lumican to suppress angiogenesis in various tumor types. The mechanistic basis for this remains unexplored, but several hypotheses seem probable. For example, heterogeneous endothelium within different tissues or tumor types [185] may have differential sensitivity to Lumican. However, our data does not support this since recombinant Lumican, but not endogenously expressed Lumican suppressed angiogenesis in matrigel plugs ([83], and Fig. 7). Based on this observation, an alternative hypothesis is that non-glycosylated Lumican core protein such as recombinantly produced protein blocks angiogenesis while endogenously expressed and glycosylated Lumican is unable to suppress angiogenesis. If correct, it will be critical in future studies to compare Lumican glycosylation in various tumor cell types. Future studies will be directed at testing this hypothesis.

Despite the fact that Lumican did not suppress 4T1 tumor angiogenesis, Lumican overexpression did potently suppress 4T1 tumor growth and metastasis. This result indicates that in addition to Lumican's anti-angiogenic activity, this matricellular molecule also directly impacts tumor cell physiology. This finding is consistent with our findings that Lumican suppresses 4T1 invasion and recent findings showing that Lumican suppresses melanoma cell migration by binding to $\alpha 2\beta 1$ integrins and suppressing MMP14 activity [162]. Thus, the direct affect on tumor cell invasion and migration coupled with the anti-angiogenic activity of Lumican indicates that Lumican is a multi-functional matricellular protein that like many other matricellular molecules exhibits contextual specific activities.

Finally, we also investigated the anti-angiogenic activity of Lumican in non-tumor models of angiogenesis and were unable to detect a significant anti-angiogenic activity in wound healing assays, aortic ring assays, or matrigel plug implantations. These results are also consistent with our previous results showing that wound healing is delayed in Lumican knockout mice, not accelerated as would be expected if Lumican is anti-angiogenic in granulation tissue [186]. Moreover, as is the case for tumors, these results seem to indicate that Lumican is only anti-angiogenic under restricted circumstances. More provocatively, these results also suggest that the anti-angiogenic activity of Lumican functions only within a restricted subset of tumor microenvironments therefore raising the possibility that Lumican may represent an attractive therapeutic avenue to specifically inhibit angiogenesis in some tumors while sparing angiogenesis in other tissues such as healing wounds.

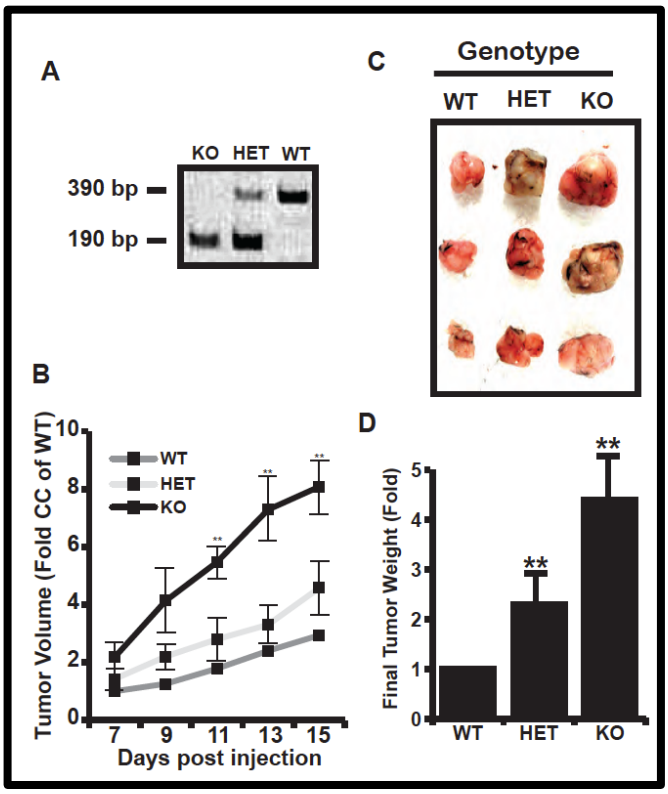

Figure 1. Lumican is an endogenous inhibitor of tumor growth.

A) PCR based genotyping. PCR amplified 190 bp Lumican product in wild type (WT) mice and 390 bp mutant DNA product in Lumican knockout (KO) mouse. PCR amplified both 190 bp Lumican product and 390 bp mutant DNA product in heterozygous (HET) mouse. Freshly cultured 1 x 10^6 Pancreatic Adenocarcinoma (PanO2) cells were injected subcutaneously into WT, HET, and KO mice and the tumors were allowed to grow in mice for 15 days. Tumor sizes were measured externally using calipers after a week post injection at an interval of 2 days. B) Graph represents a tumor growth curve. C) Representative tumors isolated from mice after 15th day. D) Bar graph representing final tumor weights from WT, HET, and KO mice. Each data point represents Mean ±SD. ** indicate $P<0.05$, Student's t-test, N = 3.

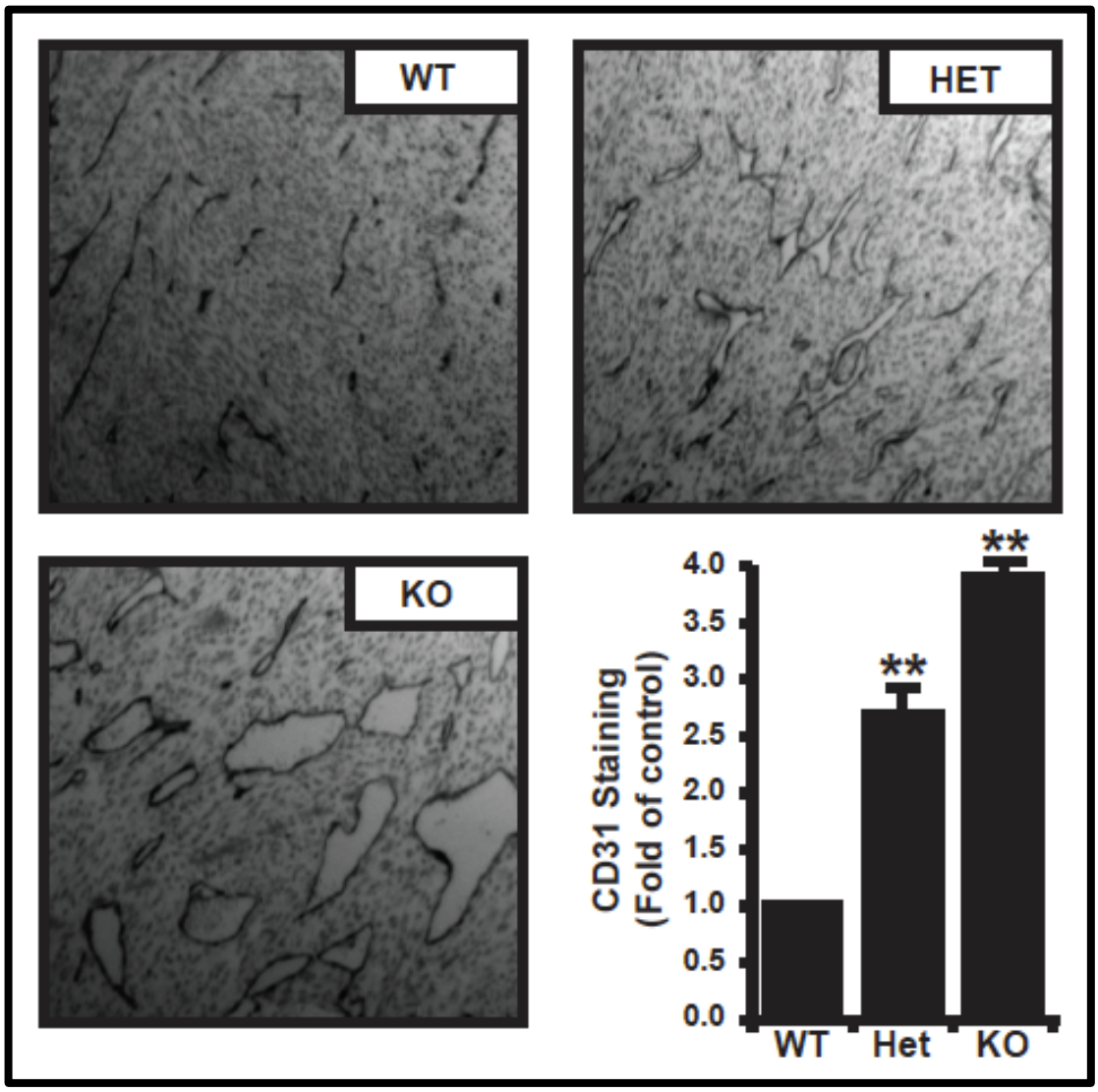

Figure 2. Endogenous Lumican has anti-angiogenic activity in Pancreatic Adenocarcinoma.

PanO2 tumor isolates from mice were sectioned, fixed, and stained with anti-CD-31 antibody to detect microvascular density in tumors. Shown are representative immunostaining results from a single experiment that was performed three times in its entirety. CD-31 staining was quantified by densitometry with image-J software and is depicted as the fold increase compared to control mice. Each data point represents the mean ±SE of three independent experiments. ** indicates $P<0.05$, Student T-test.

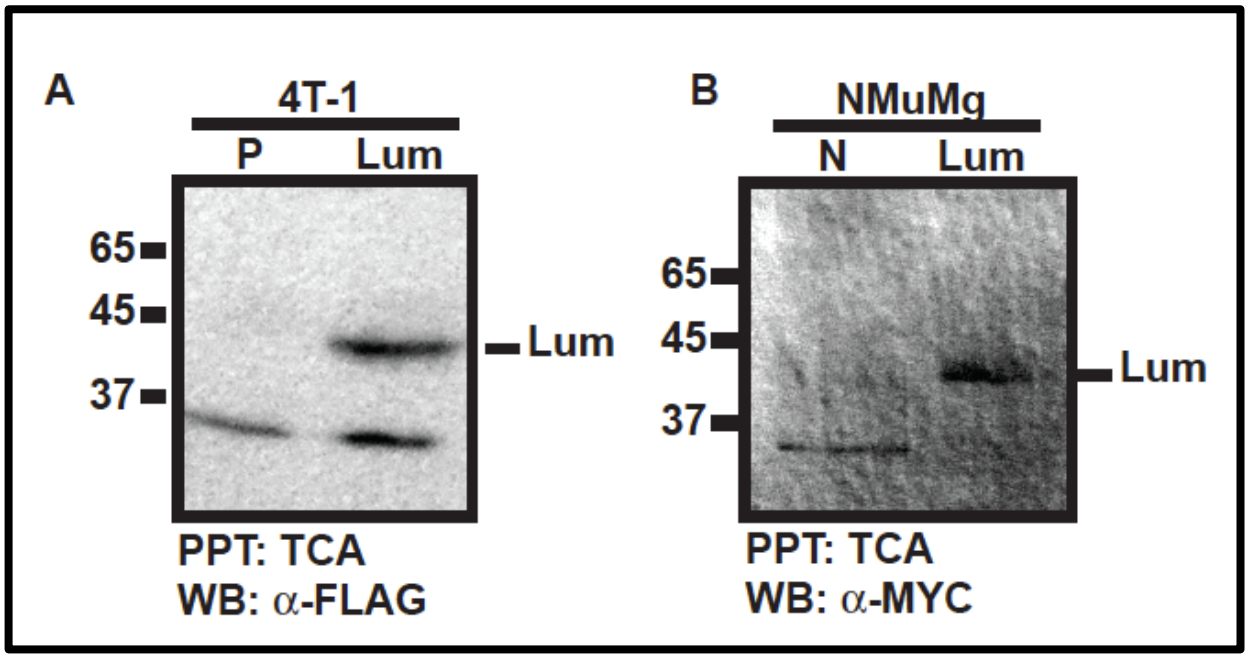

Figure 3 A-B). Confirmation of Lumican overexpression in 4T1 and NMuMG breast cancer cells.

4T1 and NMuMg breast cancer cells were transduced with either empty vector or Lumican expressing retroviral vectors. Overexpression of Lumican was confirmed by western blot analysis of TCA/DOC precipitated conditioned media.

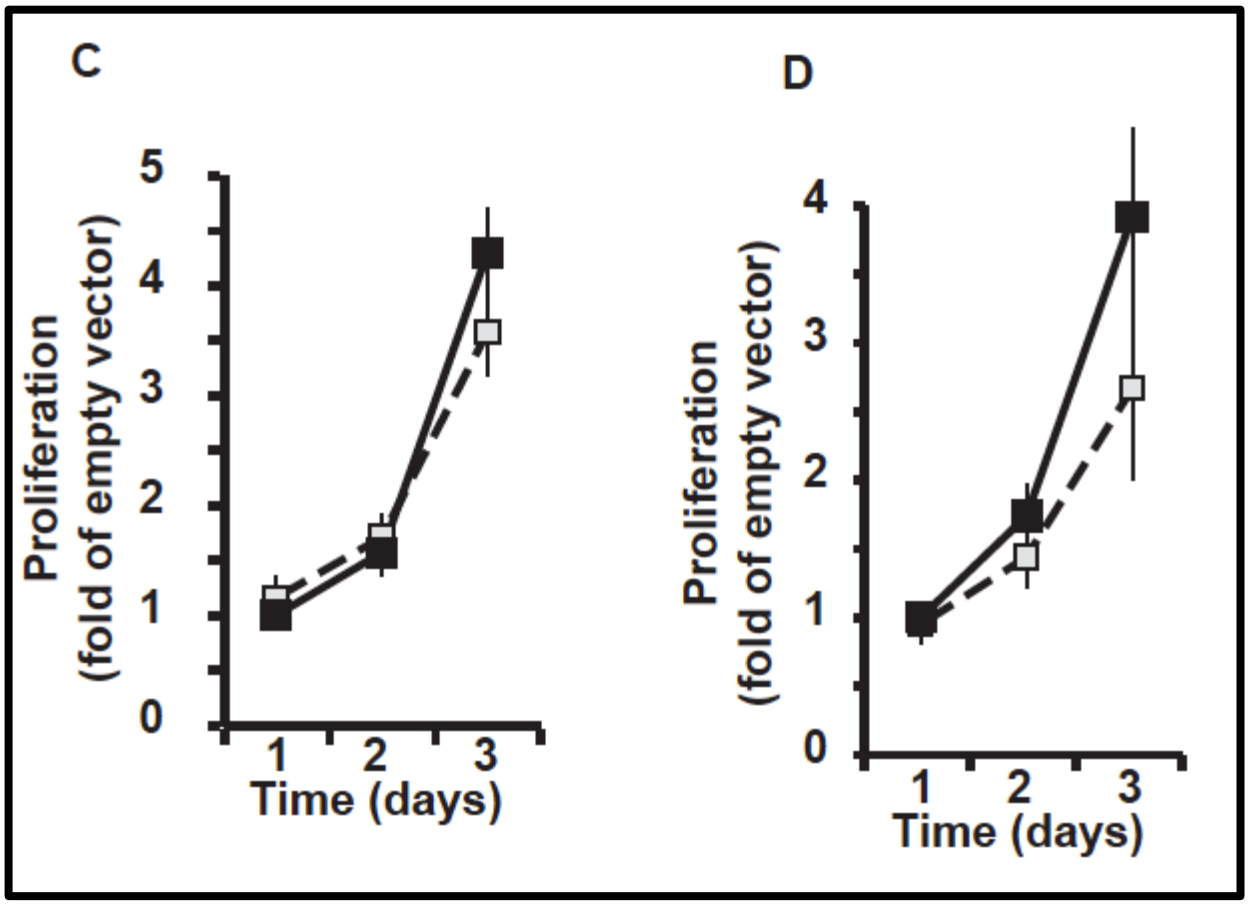

Figure 3 C-D). Effect of Lumican on the proliferation of breast cancer cells.

WST1 reagent was used to compare proliferation rates of empty vector control and Lumican overexpressing 4T1 and NMuMg cells. Proliferation was measured daily for three days. Data is depicted as average fold increase (+/- SE) of four independent experiments compared to empty vector control on day 1.

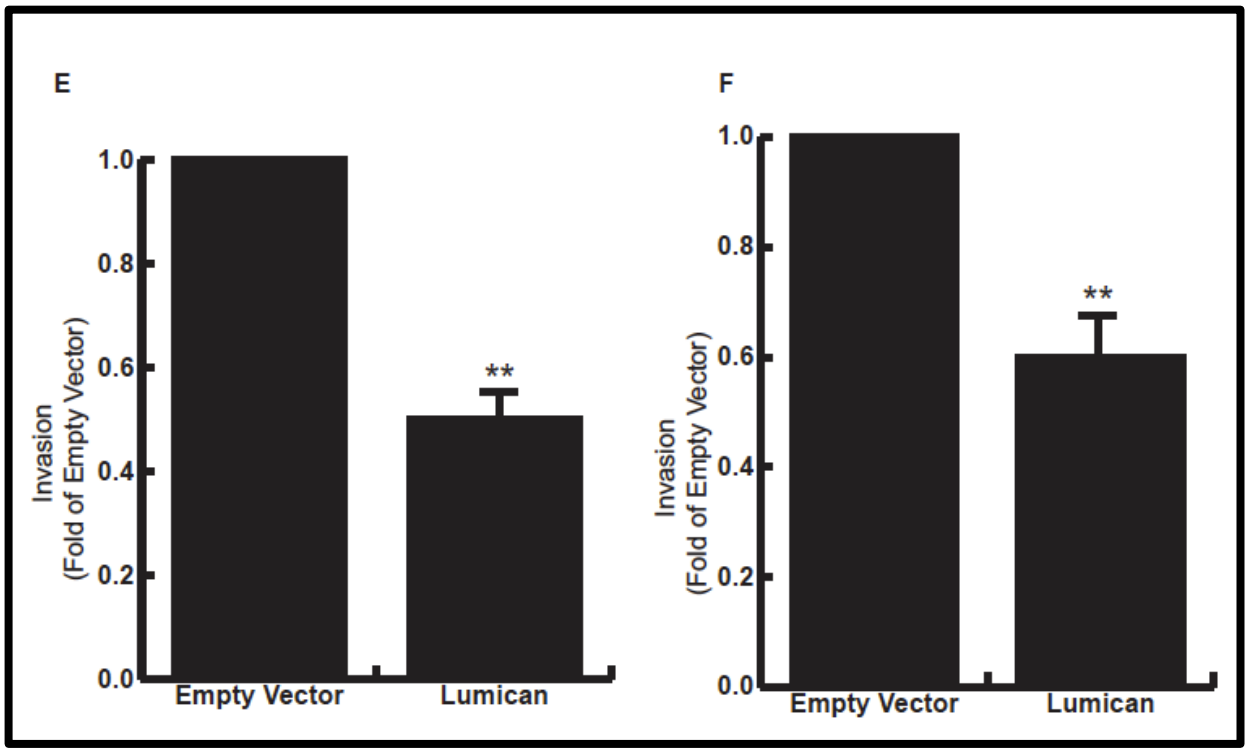

Figure 3E-F). Effect of Lumican on breast cancer cell invasion.

Empty vector control and Lumican overexpressing 4T1 or NMuMg cell lines were cultured on Matrigel coated Boyden chambers and induced to invade towards a 2% FBS gradient for 48 hours. Invading cells were stained with crystal violet and quantified by densitometry. Data presented is the average fold decrease +/- SE of four independent experiments. ** Indicates $p<0.05$, student's t-test.

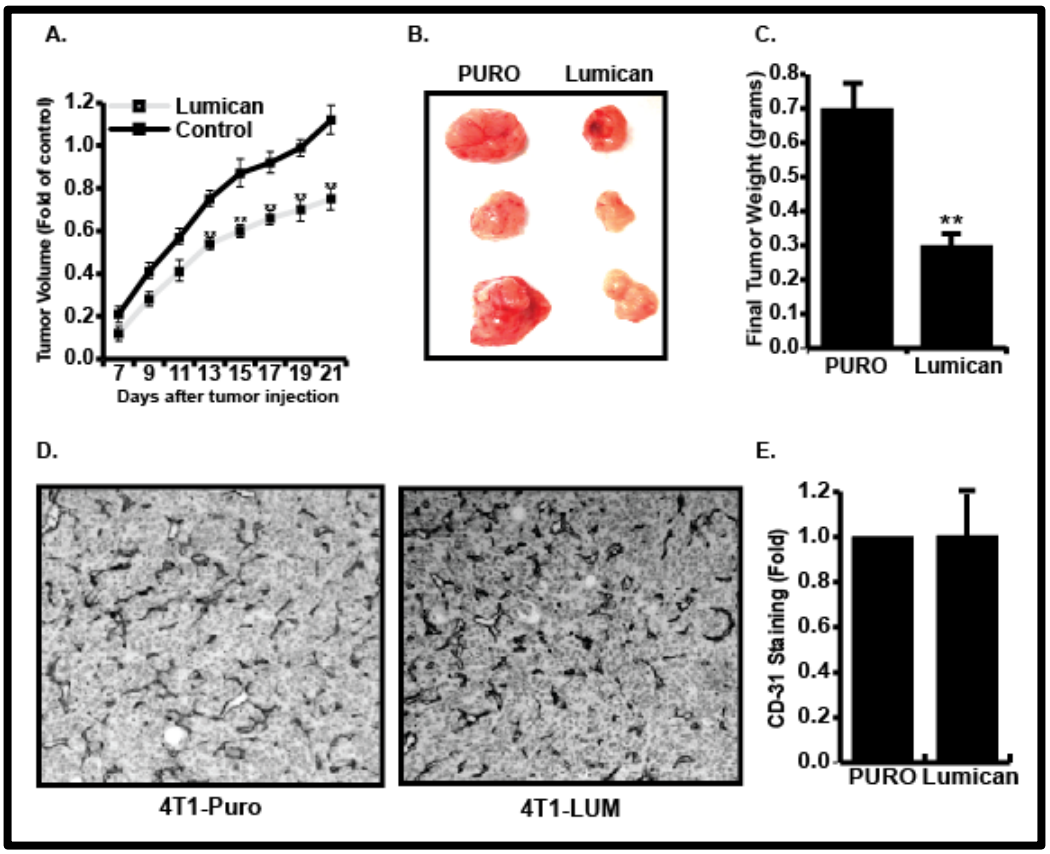

Figure 4. Lumican suppresses but not angiogenesis in 4T1 breast cancers.

A) Equal numbers of empty vector control and Lumican overexpressing 4T1 breast cancer cells were orthotopically injected in triplicate into the fat-pad of syngenic mice. Tumors were evident after 7 days and tumor volume was measured every other day for 14 days. Tumor volume is depicted as average fold increase (+/- SE) of three independent experiments compared to control tumors on day 7. **B**) Representative pictures of tumors dissected from a single experiment. **C**) Final mass of dissected tumors was compared and is depicted as the average decrease (+/- SE) of three independent experiments compared to final control tumor mass. ** Indicates $p<0.05$, student's t-test. **D**) Dissected tumors were sectioned and vascular density was monitored by immunohistochemistry with anti-CD31 antibodies. **E**) Densitometry by image-J software was used to quantify CD31 staining in tumor sections.

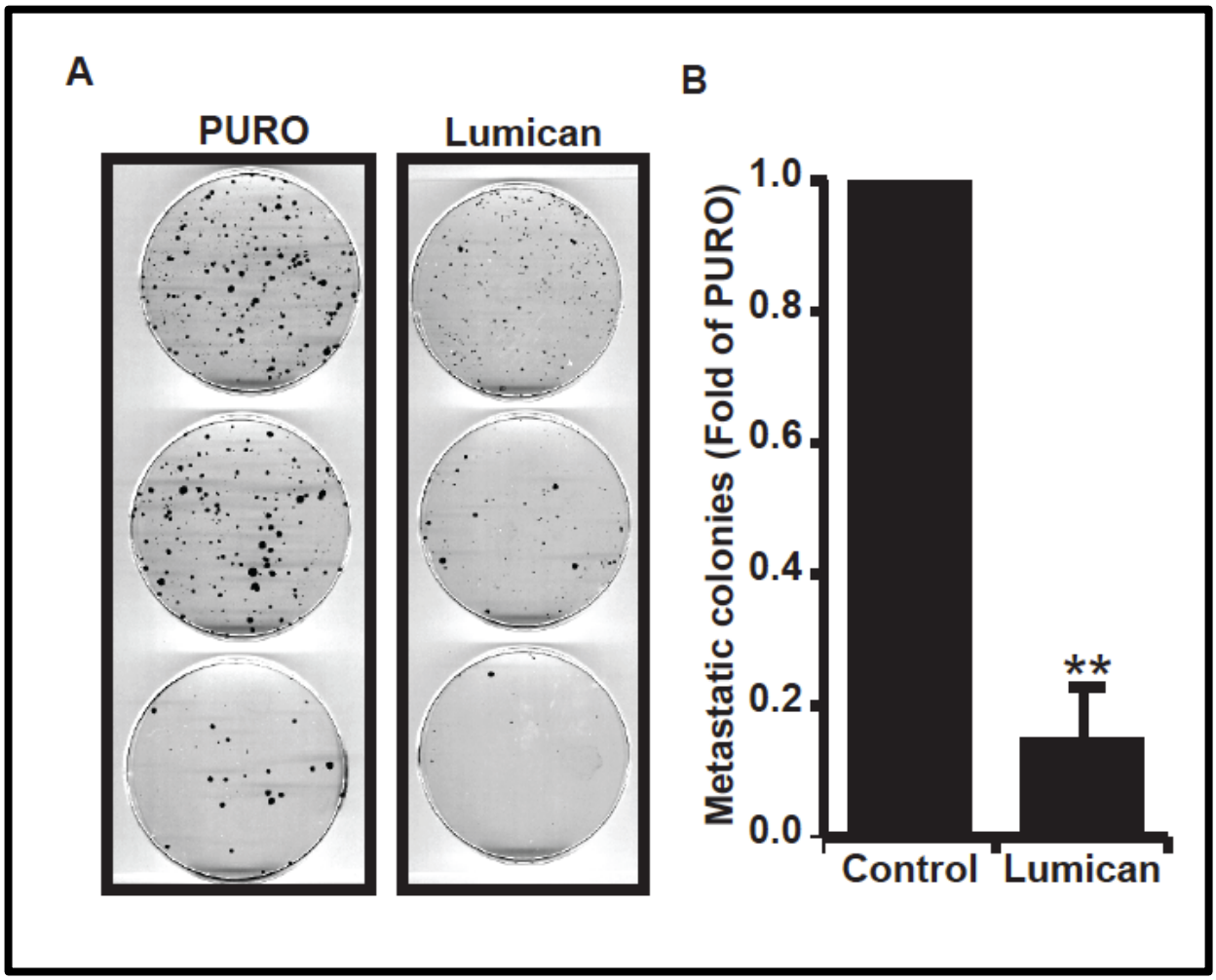

Figure 5. Lumican suppresses 4T1 breast cancer metastasis to lungs.

A) Images showing metastatic colonies of 4T1 breast cancer cells in lungs from mice injected with control or Lumican overexpressing (Lumican) 4T1 breast cancer cells. B) Represents a quantification of metastatic colonies in lungs from mice injected with either control or Lumican overexpressing (Lumican) 4T1 cancer cells. Each data point represents Mean±SD. ** indicate $P<0.05$, Student's t-test, $N = 3$.

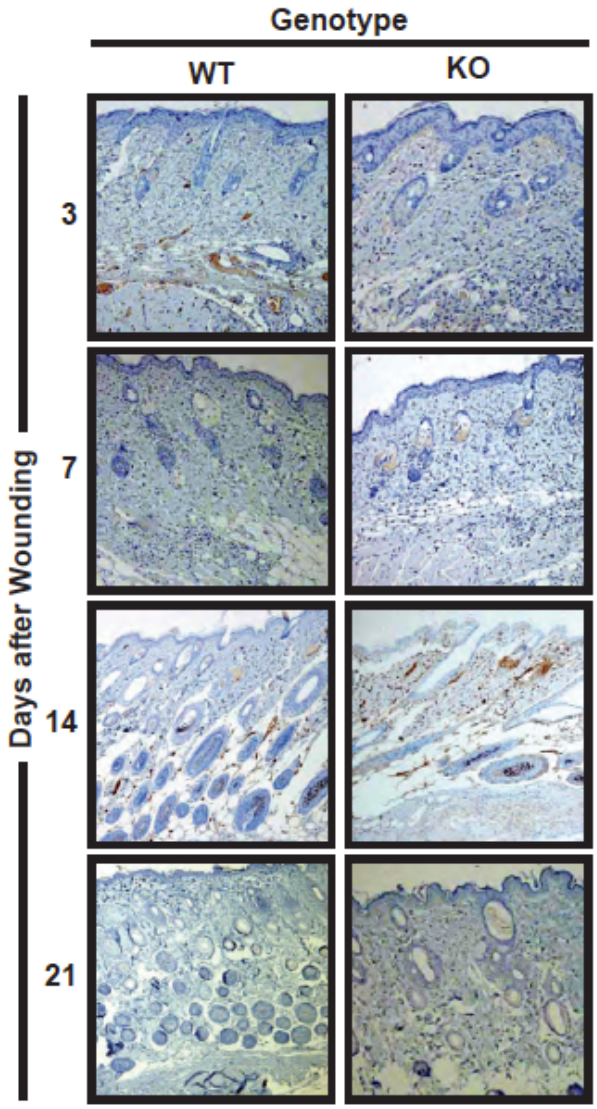

Figure 6. Endogenous Lumican does not suppress angiogenesis in healing wounds.

Skin punches from wild-type (WT) and Lumican knockout (KO) mice were removed to produce wounds. Healing wounds were collected from mice 3, 7, 14, or 21 days after wounding and vascular density was compared by immunohistochemistry with anti-CD31 antibodies.

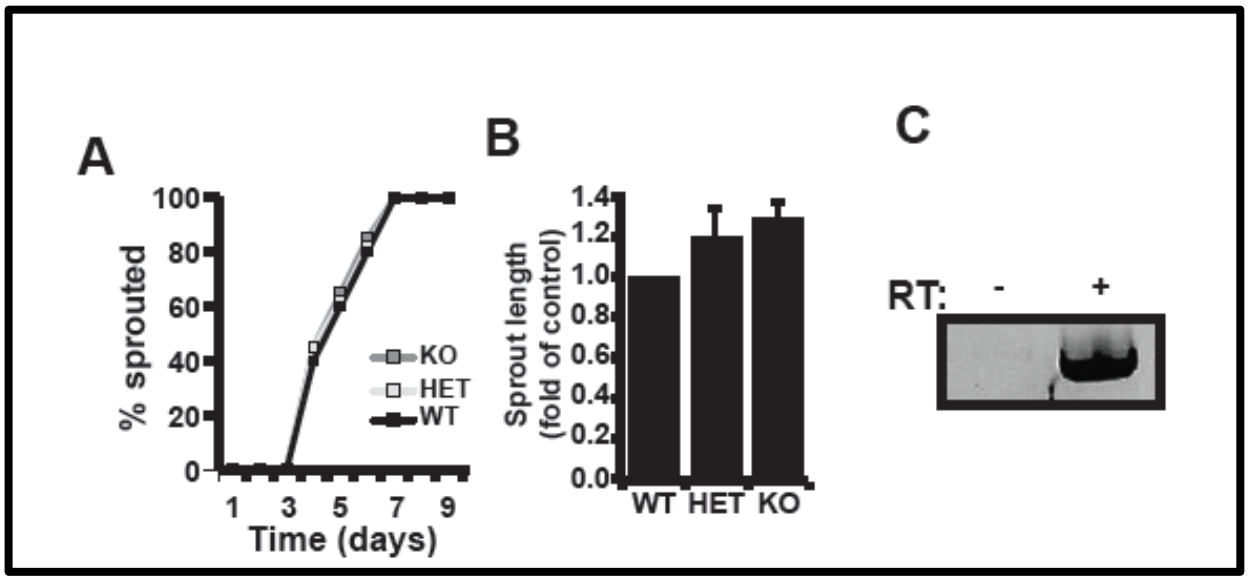

Figure 7. Endogenous Lumican does not suppress aortic sprouting or vascularization of Matrigel plugs.

A) Aortas were isolated from wild-type (WT), heterozygous Lumican knockout (Het), or homozygous Lumican knockout (KO) mice and sectioned then implanted into fibrin gels. Aortic cultures were inspected daily for angiogenic sprouting. The time required to initiate angiogenic sprouting is depicted as the percent number of sprouting rings over a 10 day window until 100% of rings had sprouted. Data presented is the average of four individual experiments each consisting of at least 5 aortic rings for each genotype. **B**) The final length of angiogenic sprouts was measured (pixel length) and average length compared to WT rings is depicted. **C**) Lumican mRNA expression was confirmed in WT culture of aortic rings. A negative reverse transcription (-RT) control was used to control for specific Lumican mRNA amplification.

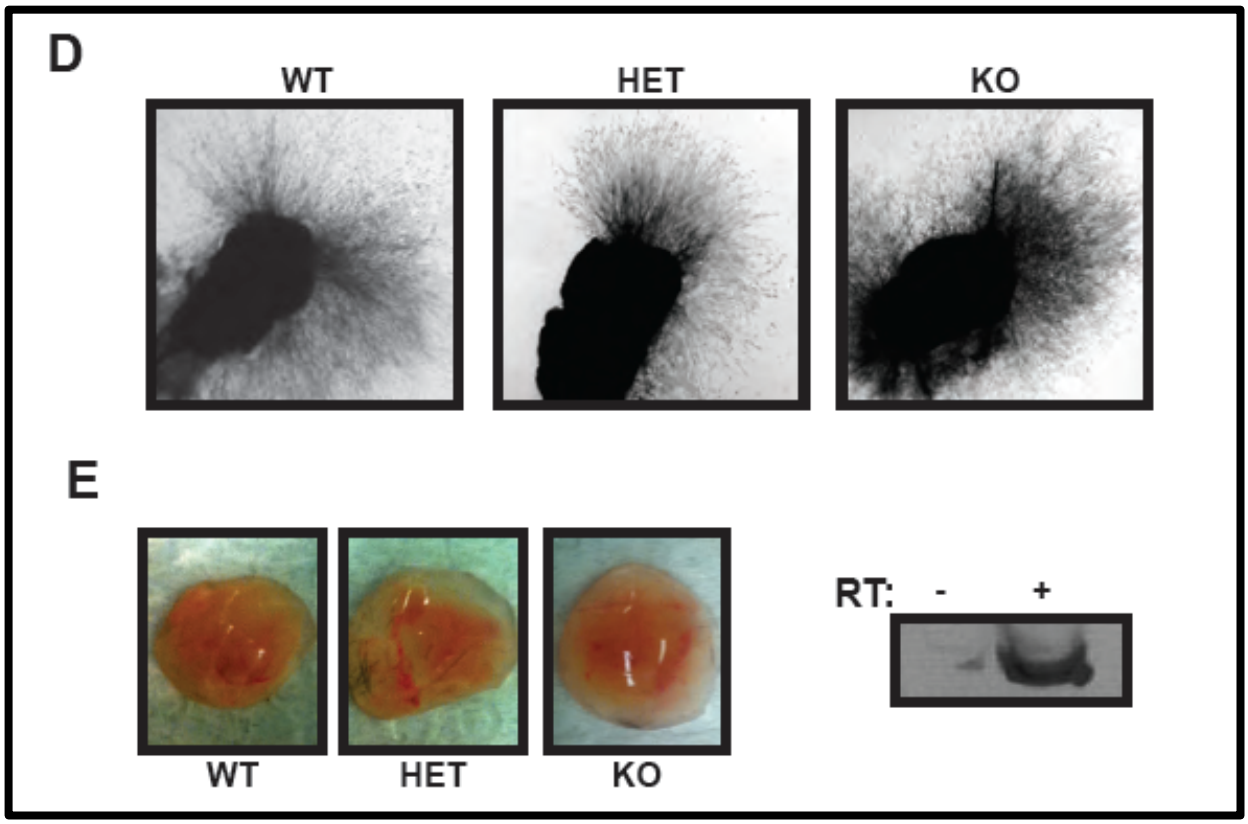

Figure 7D-E. **D**) Representative images of WT, Het, and KO aortic ring cultures after 9 days in culture. **E**) Solutions of Matrigel containing 300ng/ml of bFGF were injected subcutaneously into WT, Het, and Lumican KO mice. After 10 days, the resulting Matrigel plugs were dissected and blood content was compared as a sign of vascularization. RT-PCR was used as in C to confirm expression of Lumican in control Matrigel plugs.

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