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Extracellular Matrix Proteins: Implications For Angiogenesis

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EXTRACELLULAR MATRIX PROTEINS:

IMPLICATIONS FOR ANGIOGENESIS

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ABSTRACT

The extracellular matrix (ECM) is an essential requirement for maintaining permanent shape and rigidity in multicellular organisms. The ECM serves two main functions: scaffolding and signaling. Insoluble collagen and soluble proteoglycans, glycosaminoglycans (GAGs), and glycoproteins allow for water retention and flexibility. The signaling role of the ECM is essential for a multitude of events including vascular development and angiogenesis. Via interactions with vascular endothelial cells, proteins of the ECM can induce or repress angiogenesis.

PREFACE

The aim of this thesis is to demonstrate the importance of ECM proteins as regulators of angiogenesis in general and to highlight my research on the proteins Lumican, Clusterin, Nephronectin, SerpinE2, and Gremlin-1 as they relate to angiogenesis.

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CHAPTER 1

EXTRACELLULAR MATRIX PROTEINS: IMPLICATIONS FOR ANGIOGENESIS

What comprises a complex multicellular organism? Most would consider the classic understanding of systems composed of organs, organs composed of tissues, and tissues composed of cells. This view however, completely overlooks an essential element in the structuring and functioning of such an organism: the extracellular matrix. The extracellular matrix (ECM) is an evolutionarily conserved substrate consisting of a variety of structural and signaling molecules secreted from differentiated mesenchymal cells including chondrocytes and fibroblasts [1]. Every cellular function from development to differentiation to reproduction to death involves the ECM in one way or another.

Extracellular matrix: an evolutionary perspective

Basement membranes are some of the oldest and best conserved extracellular matrices [2]. Because such matrices are essential to complex and permanent organismal structure, some have suggested the formation of extracellular matrix provides evidence of a monophyletic origin of the animal kingdom [3]. Basement membranes consist of laminin, collagen IV, and additional proteins to provide structural support and sites for cellular anchoring. Laminins have been identified in organisms as simple as *C. elegans* [2]. The collagen IV observed in basement

membranes is arguably one of the oldest matrix proteins as it appears in simple organisms such as *C. elegans* and members of the phylum Porifera [2-3].

Extracellular matrix: form and function

The ECM consists of an aggregation of soluble and insoluble factors. In vertebrates (and some invertebrates), collagen provides a rigid, insoluble boundary for structures. Other elements in the matrix swell in water to resist compressive force and maintain structural shape. One such water soluble molecule, hyaluronic acid, is an abundant nonsulfated glycosaminoglycan (GAG) of the ECM. GAGs are charged amino sugar chains and are often found attached to a protein backbone. The resulting large branched molecules are known as proteoglycans. Glycoproteins are secreted proteins glycosylated by N-linked oligosaccharides, and represent important soluble members of the ECM [3].

The ECM serves two major functions: providing structure and regulating cellular activities via outside-in signaling. Insoluble and soluble elements both play an important role in defining structure and providing sites for cellular attachment. But it would be an error to consider the ECM a lifeless molecular skeleton. On the contrary, many of the molecules of the ECM act as matrikines to relay signals to the surrounding cells. Matrikines are ECM proteins which act as ligands for cell surface receptors [4]. The ECM affects a plethora of processes within an organism. One condition in which the ECM is intimately involved is the process of angiogenesis [5].

Angiogenesis

Angiogenesis is the development of new blood vessels from the existing host vasculature [6]. The process of angiogenesis can be thought of as several unique events. The blood vessel must be stimulated to initiate the breaching of the vessel wall. Surrounding tissue must be degraded, often by matrix metalloproteinases (MMPs), or avoided while the endothelial cells migrate and invade to the region requiring vascularization [7]. Supporting cells, such as smooth muscle cells and pericytes are recruited to reform the vascular tubule. Finally, the newly formed vessel must slow this process down and return to angiostasis. Each of these distinct stages requires interactions with the ECM.

Angiogenesis arises under a number of conditions in the healthy adult human. Examples include during exercise, prior to menstruation, and during wound healing [8]. There are other instances where angiogenesis is the result of an underlying pathology. There are over 70 human diseases associated with a disproportionate amount of angiogenesis [9]. One example of this is the increase in angiogenesis associated with tumor growth. Tumor cells, like healthy cells, require nutrients and oxygen to survive. Oxygen is only able to diffuse approximately 110 μm beyond the vasculature and therefore requires blood vessels for efficient cellular transport [10]. To maintain their rapid growth and survival, as well as to establish a pathway for metastasis, tumors encourage the growth of new vasculature. This requires the manipulation of the tumor microenvironment by the tumor. The hypoxic condition of the tumor stroma, as well as proteins secreted by the tumor cells into the microenvironment, provide cues to the invading endothelial cells to encourage migration [6]. Tumors exploit existing ECM proteins and signaling pathways to encourage angiogenesis. Identifying and characterizing the constituents of the extracellular matrix and the signaling pathways they manipulate provides new avenues for the treatment of the

many diseases associated with pathological angiogenesis. Many of the proteins I will discuss are involved in multiple pathways and these pathways may interact with one another in a positive or negative manner [11-13]. The purpose of the following review is to discuss some of the general mechanisms by which ECM proteins encourage or discourage angiogenesis. A simplified illustration of ECM-endothelial cell interactions is given in (Fig.1).

Extracellular matrix involvement in angiogenesis

Many signaling pathways are involved in the process of angiogenesis. Some are much better understood than others and possess more evidence supporting ECM involvement in angiogenesis. The pathway most unambiguously regarded as involving the ECM is that of integrin receptor binding [14]. Integrins exist as heterodimers on the cell surface, consisting of one alpha and one beta subunit. This heterodimeric arrangement has been observed in *C. elegans*, although the number of possible alpha/beta receptor combinations is considerably smaller than in more complex animals as they possess only two alpha and two beta chains [2]. When a protein ligand binds to an integrin, the subunits are ligated triggering the signaling cascade inside the cell. Many of the ligands known to be relevant to angiogenesis possess a distinct three amino acid sequence arginine-glycine-aspartic acid (RGD) that is necessary for binding to certain integrins. Several important integrins include $\alpha_5\beta_1$ (fibronectin receptor), $\alpha_6\beta_1$ (laminin receptor), and $\alpha_v\beta_3$ (vitronectin receptor) (Fig 2B). Although recognized for binding a particular matrix protein, an integrin heterodimer can bind multiple ligands. For instance, $\alpha_v\beta_3$ can bind fibronectin, thrombospondin, and osteopontin [14].

The Notch signaling pathway represents another mechanism by which ECM proteins can affect angiogenesis. Notch signaling is important for cellular development, differentiation, and

adhesion. Cells may express one or more of the four known mammalian Notch receptors (Notch 1-4). When bound by one of the five known ligands (Delta-like ligand 1, 3, 4, Jagged 1, 2) the intracellular domain of the Notch receptor is cleaved by γ-secretase. The intracellular domain is then released to the nucleus to regulate gene expression. The matrix proteins CCN3, MAGP-1, and MAGP-2 are all known to interact with Notch 1 [15-16]. Notch 1 interactions with MAGP-1 and MAGP-2 result in the shedding of the receptor's ectodomain [16]. In endothelial cells, the interaction between MAGP-2 and Notch 1 suppresses the signaling pathway resulting in a decrease in angiogenesis [17]. MAGP-2 can induce the shedding of Jagged 1 (soluble Jagged 1), and can also interact with Jagged 2, and Delta-like ligand 1 (Dll-1) [18]. Thrombospondin-1 and thrombospondin-2 both interact with the receptor Notch 3 and the ligand Jagged 1, but only thrombospondin-2 enhances the interaction between the receptor and ligand [19-20]. An illustration of these interactions is also provided (Fig 2C).

An intriguing mechanism employed by proteins of the ECM to reduce angiogenesis is the induction of endothelial cell apoptosis. There are multiple pathways utilized to induce apoptosis in endothelial cells (Fig 2A). Some ECM proteins inhibit proliferation by inducing endothelial cell cycle arrest, ultimately resulting in apoptosis. The collagen XVIII fragment endostatin induces G1 arrest while the plasminogen fragment angiostatin disrupts the transition between G2/M [21-22]. The intrinsic pathway of apoptosis, in which internal signaling cascades from the mitochondria mediate cell death, is also targeted by several ECM proteins. Angiostatin can upregulate p53 expression, resulting in endothelial cell apoptosis [23]. Endostatin and thrombospondin-1 both upregulate the pro-apoptotic protein Bax while downregulating the anti-apoptotic protein Bcl-2 [24-25]. Endostatin downregulates the anti-apoptotic protein Bcl-X_L as well [24].

The extrinsic pathway of apoptosis involves outside-in signaling via a receptor-ligand mediated pathway. One such receptor-ligand system is the Fas-Fas-L apoptosis pathway. In the extrinsic Fas-apoptosis pathway, the cell surface receptor Fas (CD95) is bound and aggregated by Fas ligand (Fas-L) existing as a membrane-bound ligand or as a multimeric soluble ligand. When activated, Fas triggers an intracellular signaling cascade by cleaving multiple caspases, ultimately leading to apoptosis. The cytoplasmic FLICE-like inhibitory protein (FLIP) can act as a competitive inhibitor to caspase-8, preventing apoptosis from occurring. The collagen IV fragment canstatin induces Fas-L expression in endothelial cells, contributing to autocrine induction of apoptosis [26]. Similarly, angiostatin upregulates mRNA expression of Fas-L while downregulating the mRNA expression of the anti-apoptotic FLICE-like inhibitory protein (FLIP) [23].

In addition to the previous examples of direct ECM-endothelial cell interaction, several other well known angiogenesis pathways (e.g. Growth factor-Receptor tyrosine kinase, Wnt-Frizzled, Hedgehog-Patched, Cytokine-Cytokine receptor, and Angiopoietin-Tie) may result in the upregulation or downregulation of ECM proteins or may interact with the pathways previously discussed. For example, TGF-β and bFGF can influence the expression of integrins and integrin-receptor tyrosine kinase (RTK) co-activation is often involved in triggering signaling cascades [11-13].

ECM protein discovery and pathway mapping are continuously illuminating the number of known ECM-endothelial cell interactions with regard to angiogenesis, yet many of these interactions remain unknown. More research is needed to address these questions if we are to treat the underlying causes of pathological angiogenesis. The second chapter presented herein elucidates a unique interaction between endothelial cells and lumican, an extracellular matrix

protein believed to have a role in cancer. I will now discuss a brief history of the protein lumican as well as the contributions of my research to the understanding of this ECM-derived regulator of angiogenesis.

Lumican

The Small Leucine Rich Proteoglycan (SLRP) family member lumican was first identified in the stroma of the cornea as a regulator of corneal transparency via its organization of collagen fibrillogenesis [27]. The 37 kDa core protein of lumican consists of an 18 amino acid signal peptide, a negatively charged N- terminus containing conserved cysteine residues, a middle region containing 11 leucine rich repeats (LRRs), and a C- terminal LRR "ear repeat" [28-31]. The protein can be glycosylated at four points with glycosaminoglycan (GAG) side chains and exists as a 55-57 kDa glycoprotein in many tissues [32]. The protein is also modified through the addition of keratan sulfate chains in the 50-100 kDa proteoglycan form seen most frequently in the cornea [32-33]. Lumican can be cleaved by several matrix proteases including MT1-MMP, MMP-12, and ADAMTS-4 [34-35]. Figure 3 illustrates many important domains and motifs of the lumican core protein (Fig.3).

Lumican is best understood as a regulator of collagen fibrillogenesis. Given the homology of lumican to other, better-described members of the SLRP family (e.g. Decorin and Fibromodulin), the protein is thought to have a banana-shaped appearance although this remains to be directly confirmed [31]. The convex side of the classic SLRP banana shape possesses the hydrophilic GAG side chains, which provide interfibrillar spacing via water retention, while the concave side is wide enough to come into contact with an individual collagen fiber [32]. The concave side of lumican shares a homologous collagen-binding domain with its closest relative

fibromodulin: this domain is on LRR5-7 [36-37]. Indeed, both *lum* -/- and *fmod* -/- knockout mice lack appropriate collagen organization and possess non-uniformly sized collagen [38-39]. Studies demonstrate that Cysteine 41 within the conserved N-terminal cysteine region is necessary for proper packing and structure of collagen fibrils [29]. Additionally, *lum* -/- knockout mice exhibit corneal opacity and skin laxity [38]. Thus, lumican is essential for proper collagen organization and distribution.

Lumican in Cancer

Though first identified in the corneal stroma, lumican is now known to be expressed in a variety of tissue types including heart, lung, intestine, bone, articular cartilage, pancreas, placenta, kidney, breast, brain, cervix, colon, liver, smooth muscle, and uterus [27-28, 33, 40-44]. Lumican's collagen organizing ability, and its relationship to decorin, created an interest in this protein's role in cancer. Immunohistochemical (IHC) analysis of lumican expression has been reported in breast, pancreatic, colorectal, cervical, neuroendocrine, and lung carcinomas. Similarly, microarray and cell culture studies have highlighted trends in *lum* mRNA expression in various stages of these and other cancer types [41, 43-55].

The expression of lumican in breast cancer has been correlated with higher tumor grade, but also with smaller tumor volume [41, 50]. Many breast tumors exhibit a reduction in lumican expression [56]. However, the role of lumican may not always be protective as expression in invasive ductal breast carcinoma is nearly fivefold greater compared to normal [51]. Such conflicting reports underscore the difficulty of establishing causation from correlative data. In pancreatic cancer, lumican commonly localizes to the alpha cells of islets as well as the lesions surrounding the cancer [45]. While the presence of lumican can be observed pancreatic stellate

cells of the tumor stroma, there is substantially less expression in the actual cancer cells. However stromal expression of lumican in patients has been correlated with shorter survival time compared to patients lacking stromal lumican [45-46]. Colorectal cancer cells and adjacent fibroblasts and epithelial cells have been known to synthesize lumican [43]. The highest levels of lumican have been identified in colorectal cells infiltrating lymph follicles and at the invasive front of the tumor [57]. Expression of lumican in colorectal cancer cells has been correlated with deep tumor invasion, lymph node metastasis, and lower patient survival [57]. In squamous cell lung carcinoma, lumican is expressed more in the cancer cells than surrounding stroma and is correlated with greater keratinization and vascularization, but in adenocarcinoma, lumican expression is greater in the stroma and is correlated with pleural invasion and larger tumor size [54]. For many forms of cancer, no immunohistochemical (IHC) data have been collected. However, microarray analysis of mRNA expression levels allows for the correlation of lumican expression with distinct phenotypes. For example, microarray analysis of prostate tumors has demonstrated a reduction in lumican expression as the cancer cells progress from more benign to more malignant phenotypes [58-59].

In spite of this substantial correlative evidence, none of these studies have directly tested a role for lumican in cancer. However, recent experimental cell biology studies have suggested several hypotheses for lumican's function in cancer. The soft agar assay is an excellent analog for the formation of tumors *in vitro*. Lumican overexpression in multiple cell types has consistently resulted in reduced soft agar colony formation [60-62]. Additionally, cellular migration and invasion are both inhibited in melanoma cells exposed to lumican and LRR9 (lumicorin) is sufficient to inhibit melanoma migration *in vitro* [30]. In a mouse model of tumor metastasis, lumican-overexpressing melanoma cells formed fewer lung nodules [63]. Lumican

also reduces the growth of subcutaneous tumors, although the underlying mechanism remains to be elucidated [60, 62].

Explanations as to how such tumor reduction occurs have mostly focused on direct cancer cell effects, such as reduction in overall proliferation and invasive potential. For instance, lumican's binding to cell surface β1 integrin has been proposed as a mechanism for inhibiting melanoma cell proliferation [47]. However, other potential mechanisms may involve lumican's interaction with other cells in the tumor stroma. One such interaction proposed is the reduction of angiogenesis to the tumor. Multiple lines of evidence support a role for lumican in the regulation of angiogenesis. Lumican is localized to the peripheral blood vessels in adult human lungs, the renal artery, and the thickened intima of the coronary artery [33, 64-65]. Functionally, endothelial cell expression of lumican increases during the resolution phase of angiogenesis in which vascularization ceases and the vessel returns to a state of angiostasis [66]. Not surprisingly, *lum* -/- *fmod* -/- knockout mice exhibit increased vascularization in the myocardium, suggesting an anti-angiogenic role for the protein [39]. Our previous data demonstrate that lumican can reverse the pro-angiogenic affects of bFGF in Matrigel plug assays, highlighting its effectiveness as anti-angiogenic molecule [66].

To study the effects of lumican on cancer cells, we created stable polyclonal lumican-overexpressing cell lines from murine fibrosarcoma (MCA102) and pancreatic cancer (Pan02) as well as empty vector control cell lines [67-68]. The cell lines were analyzed via soft agar growth assay, invasion assay, and proliferation assay. Intriguingly, while lumican overexpression in Pan02 cells increased invasiveness, decreased soft agar colony size, and increased proliferation, in MCA102 cells, lumican decreased invasiveness, increased soft agar colony size, and had no

effect on proliferation, suggesting that lumican acts in a cell-specific manner with respect to its effects on cancer cell phenotype.

We created subcutaneous tumors of our cell lines in syngenic host animals. In stark contrast to what is seen *in vitro*, the effect of lumican overexpression in MCA102 and Pan02 *in vivo* was remarkably consistent: lumican-overexpressing tumors were smaller. Closer analysis of the tumors revealed that lumican-overexpressing tumors also possessed a lower vascular density. Hence, the reduction in tumor size may involve a reduction in angiogenesis mediated by lumican. Previous studies lead us to hypothesize that lumican might inhibit angiogenesis by promoting apoptosis in invading endothelial cells.

Like several other ECM proteins, lumican may be able to induce apoptosis [62, 69-70]. Lumican preferentially binds Fas-L. Lumican -/- murine embryonic fibroblasts (MEFs) and corneal fibroblasts (CFs) express little or no Fas on their surface; a condition reversed by transfection with lumican [69-70]. MEFs from lum -/- mice express less of the pro-apoptotic p53 and more G1/S cyclins while murine melanoma cells expressing lumican have inhibited cyclin D1 expression (causing cell cycle arrest) and increased expression of the pro-apoptotic Bax [62, 70]. Hence, lumican can increase susceptibility to apoptosis in certain fibroblasts and cancer cells.

No research has directly examined the potential apoptotic effects of lumican on endothelial cells. Hence, we created a lumican-overexpressing cell line from the murine brain microvascular endothelial cell line MB114, as well as a control cell line [71]. Consistently, more MB114-Lum cells survived to produce colonies as compared to MB114-Neo cells when plated at low density. We have not previously observed any anti-proliferative effect of lumican on

endothelial cells, suggesting that this reduction in colony formation is due to decreased survivorship [66].

Endothelial cells overexpressing lumican also demonstrated a greater amount of apoptosis as measured by caspase-3 cleavage in both the presence and absence of the Fas-activating antibody Jo-2 as compared to the control cells. Lumican-overexpressing cancer cells exhibited more or less apoptosis than their controls, dependent on cell type. Thus, indiscriminate apoptosis is an unlikely mechanism for our observed reduction in tumor growth. Control endothelial cells cultured in conditioned media from the cancer cell lines in the presence or absence of Jo-2 also exhibited an increased level of caspase-3 cleavage in conditioned media from lumican-overexpressing cells.

Whole cell lysates from the cancer cell lines and the endothelial cell lines were collected and analyzed for the presence of Fas-L, Fas, and FLIP. Fas-L was detected in all cell lines and at higher levels in the cancer cell lines. This is in agreement with previous reports of tumor cells expressing Fas-L, possibly as a means of achieving immune privilege [72-77]. Surprisingly, in contrast to what has been reported in murine embryonic fibroblasts (MEFs) and corneal fibroblasts (CFs), endothelial cells overexpressing lumican do not have a greater amount of Fas receptor. However, lumican-overexpressing endothelial cells do appear to downregulate the expression of the anti-apoptotic protein FLIP. Thus our current model supports a role for lumican in cancer that involves regulating FLIP expression in the invading endothelial cells as a means for exacerbating apoptosis, reducing angiogenesis and consequently tumor growth.

More research is required to further explore many of the unanswered questions produced in our research. We have not yet demonstrated that reintroduction of FLIP into the MB114-Lum cell line would correct their susceptibility to apoptosis. Additionally, the vessels observed in the

tumor sections appear different in structure between the lumican and neo tumors. Perhaps the vessels in the lumican-overexpressing tumors have a more normal phenotype than that commonly seen in cancer. Less leaky vessels are often established via the recruitment of pericytes and smooth muscle cells. Therefore, we would like to stain for markers of pericytes and smooth muscle cells in the tumor sections. It would also be beneficial to stain for cleaved caspase-3 to determine if this co-localizes with the vasculature. Other future experiments include the creation of cell lines that express both lumican and a soluble form of Fas receptor (sFas) lacking the transmembrane region. Such cell lines will allow us to test the hypothesis that lumican triggers Fas-mediated apoptosis by binding Fas as the sFas should presumably interact with some of the secreted lumican. Finally, it should be possible to examine which region of the lumican protein actively induces apoptosis and determine if this region alone is sufficient.

Zebrafish Angiogenesis: Gene Knockdown

The third chapter takes the first steps of characterizing potential novel regulators of angiogenesis using anti-sense morpholinos in transgenic zebrafish. Zebrafish (*Danio rerio*) have been used as a model organism to study development for over fifty years [78]. Zebrafish are easy to maintain, develop rapidly in a nearly transparent system, and with nearly 80% of the *D. rerio* genome sequenced, researchers are increasingly utilizing zebrafish for genetic studies. Because they share many organs and possess similar genomes, zebrafish remain an excellent model for human conditions [79].

One can quickly screen through many gene targets utilizing a gene knockdown approach. The targeting reagent used should be relatively stable in the zebrafish embryo and specific for a particular target. One class of gene targeting reagent is anti-sense morpholino oligonucleotides

[80]. Morpholinos are similar in structure to DNA but possess morpholine rings rather than deoxyribose. Their increased stability makes them ideal for studying targeted gene knockdown within the first five days of zebrafish development. Although the morpholinos are specific for their target genes, the effects of these knockdowns may not be specific. Occasionally, observed phenotypes are the result of non-specific apoptosis. Thus co-injection of zebrafish embryos with a specific target morpholino and an anti-p53 morpholino can be utilized to determine if some of the developmental defects associated with the specific target morpholino are due to nonspecific induction of apoptosis [81].

The study of neovascularization has been pursued in the zebrafish as well. In 2003, a transgenic line was created that expresses GFP under the blood vessel-specific *fli1* promoter. These Tg(*fli1*:EGFP) zebrafish could be observed under fluorescence to study the development of the blood vasculature [82]. In that same year, another group created a transgenic line that expresses dsRed under the erythrocytic *gata1* promoter [83]. When crossed, these fish could be studied under fluorescence in the development of blood vessels (GFP) and the development of red blood cells (dsRed). Previous research supports the potential for success using this transgenic model.

Embryos are collected in the 1-2 cell stage and injected with the desired morpholino in a vehicle of phenol red. Typically 4 ng of morpholino are delivered to each embryo, although more may be required to produce an observable phenotype. No more than 12 ng are injected. If 4 ng results in an observable phenotype, then lower doses are utilized until a minimally sufficient dose can be found. However, typically no less than 0.5 ng are injected. Sham injections of equal volume of empty vehicle are used to create control fish. When a target has been shown to produce a vascular phenotype, p53 morpholino is co-injected to verify vascular specificity. Fish

are observed on the following days to compare morphological differences in the sprouting intersegmental vessels (ISV) between control and test fish.

Knockdown targets that are potential regulators of angiogenesis

Choosing the proper target for morpholino knockdown is essential for discovering likely regulators of angiogenesis. Previously, we performed microarray experiments on mRNA extracted from tubulating MB114 endothelial cells at 1hr, 5hr, 15hr, and 25hr [84]. Our results confirmed the differential regulation of known angiogenic genes. More importantly, many novel gene targets were also found to be differentially regulated. Several of these targets were assessed via reverse transcription (RT) PCR and their patterns of regulation during the different time points were found to be in agreement with the patterns observed in the microarray. Of the substantial list of targets identified, I have chosen four that are secreted matrix proteins which are potential regulators of angiogenesis. These four genes include the upregulated *clu* (clusterin), *grm* (gremlin-1), and *serpine2* (serpinE2), as well as the downregulated *npnt* (nephronectin).

Clusterin (apolipoprotein J) has been implicated in a variety of cancers, including renal cell cancer, colorectal cancer, and endometrial carcinoma [85-87]. Expression of clusterin in melanoma is correlated with increased malignancy but is only upregulated in a minority of melanomas [88-89]. In hepatocellular carcinoma, clusterin expression results in a more aggressive phenotype and might function in metastasis [90-91]. However, in non-small cell lung cancer, cytoplasmic clusterin expression may promote patient survival [92]. In prostate cancer, clusterin is downregulated and may reduce cancer cell proliferation and migration [93-94]. In many cases clusterin is thought to make cancer cells chemoresistant by acting as a pro-survival factor in its cytoplasmic form. Anti-sense oligonucleotides to clusterin have been researched as a

means to combat this effect in cancer cells. In the endothelial cell line HUVEC, anti-sense oligonucleotides to clusterin inhibit growth and angiogenesis, while promoting apoptosis [95]. However, clusterin expression is also increased in tissues affected by Fuchs' Endothelial Dystrophy (FED), a syndrome marked by increased apoptosis [96]. In the microarray experiment, clusterin was upregulated 6.6 fold over the 25 hr timecourse [66].

Gremlin-1 (Drm) is a bone morphogenic protein (BMP) antagonist overexpressed in a variety of cancers including sarcoma, ovarian, breast, colon, pancreatic, lung and cervical cancer, and is associated with increased growth stimulation and telomerase activity [97]. Hypoxia can drive the expression of gremlin-1 and it is highly expressed in the endothelial cells of lung tumor vasculature [98-99]. Additionally, gremlin-1 is believed to play a role in the aberrant angiogenesis observed in endometriosis [100]. In the microarray experiment, gremlin-1 was upregulated 3.9 fold over the 25 hr timecourse [66].

Nephronectin (POEM) was first identified as a binding partner to integrin α8β1 in the human embryonic kidney [101]. Although nephronectin has been localized to the tumor epithelium of highly metastatic breast tumors, it increases melanoma cell adhesion *in vitro* and reduces invasion and migration [102-103]. During zebrafish development nephronectin regulates the spatial expression of BMP4 required for proper heart development [104]. In the microarray experiment, nephronectin was downregulated 0.2 fold over the 25 hr timecourse [66].

SerpinE2 (Protease nexin-1 (PN-1)) is strongly expressed in oral squamous cell, pancreatic, gastric, and colorectal cancers as well as the metastatic subclone of the pancreatic cancer cell line SUIT-2 [105-106]. SerpinE2is believed to increase invasion of cancer cells by altering the production of matrix proteins and may regulate uPA-mediated cancer cell migration

and metastasis in breast cancer [105, 107]. In the microarray experiment, serpinE2 was upregulated 29.5 fold over the 25 hr timecourse [66].

Although this project is still in its infancy, we have data suggesting that most of these targets are essential for vascular development, with one exception. Increasing doses of anti-clusterin morpholino (up to 12 ng) had no apparent effect on zebrafish development: morpholino-treated fish were indistinguishable from vehicle-injected controls. Conversely, embryos treated with 4 ng anti-gremlin-1 morpholino had a less-developed vasculature compared to controls at 1 day. Treatment with 4 ng anti-serpinE2 was lethal in the majority of fish tested. The one surviving fish possessed underdeveloped vasculature at day 1 as compared to controls. Future work will involve determining the lowest effective doses of these morpholinos. Injection with as little as 1 ng anti-nephronectin morpholino resulted in delayed vascularization as compared to control fish. Co-injection of each of the three effective morpholinos with anti-p53morpholino is required to confirm vascular specificity. Validated targets will ultimately be overexpressed in a cell culture model to further characterize the effects obtained in a gain-of-function cell system.

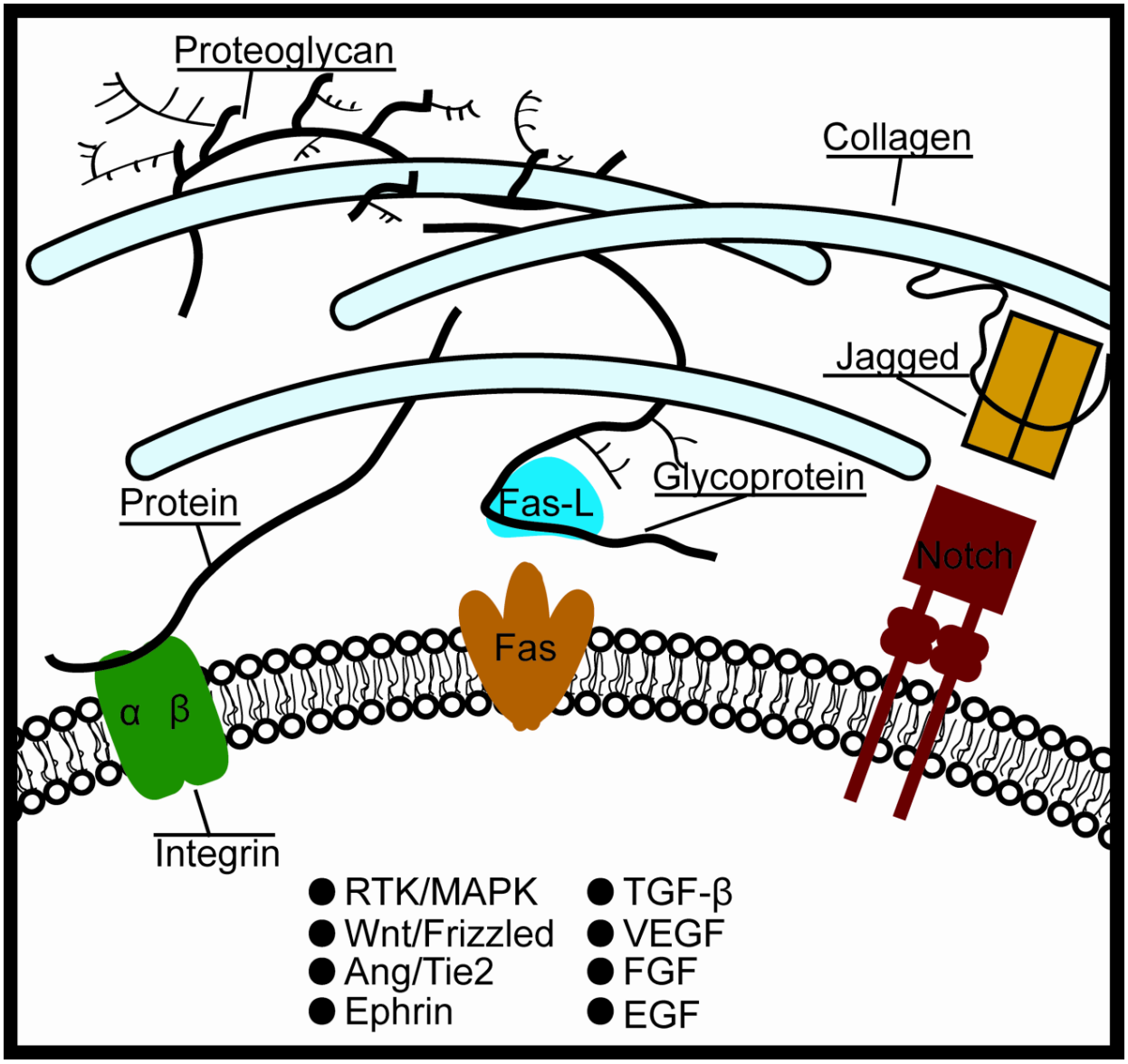

Figure 1. Common interactions between proteins of the extracellular matrix and endothelial cells

ECM proteins interact with endothelial cells via Integrin, Notch, and Apoptosis signaling pathways. Several non-matrix interactions are also listed. (RTK- Receptor tyrosine kinase, MAPK- Mitogen-activated protein kinase, Ang- Angiopoietin, TGF-β- Transforming growth factor- β, VEGF- Vascular endothelial growth factor, FGF- Fibroblast growth factor, EGF- Epidermal growth factor.)

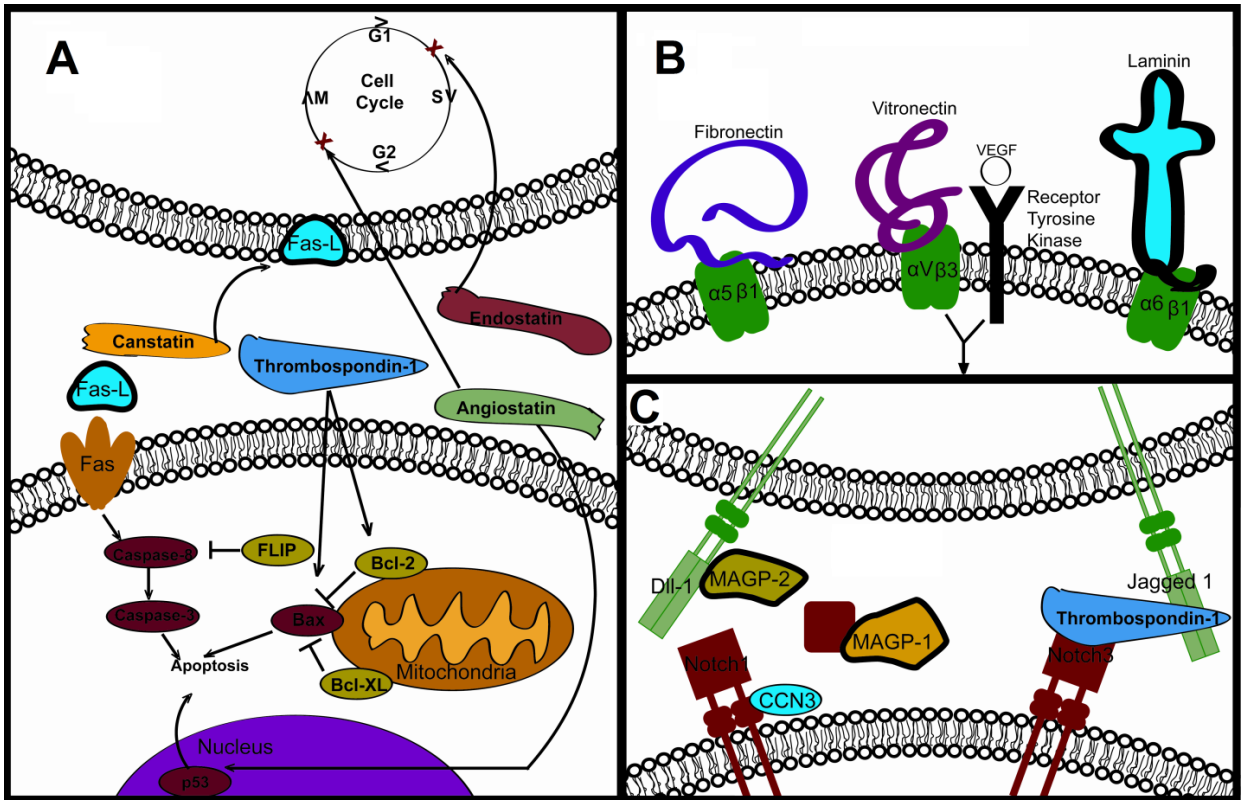

Figure 2. Specific interactions of known extracellular matrix proteins with endothelial cells

A) The extracellular matrix (ECM) proteins canstatin, angiostatin, endostatin, and thrombospondin-1 can all influence endothelial cell apoptosis. B) Many ECM proteins, such as fibronectin, laminin, and vitronectin attach to endothelial cells via integrin receptors on the cellular surface. In some cases, co-activation of receptor tyrosine kinases (RTK) also occurs. C) The ECM proteins CCN3, MAGP-1, MAGP-2, and thrombospondin-1 mediate Notch/ligand interactions between endothelial cells.

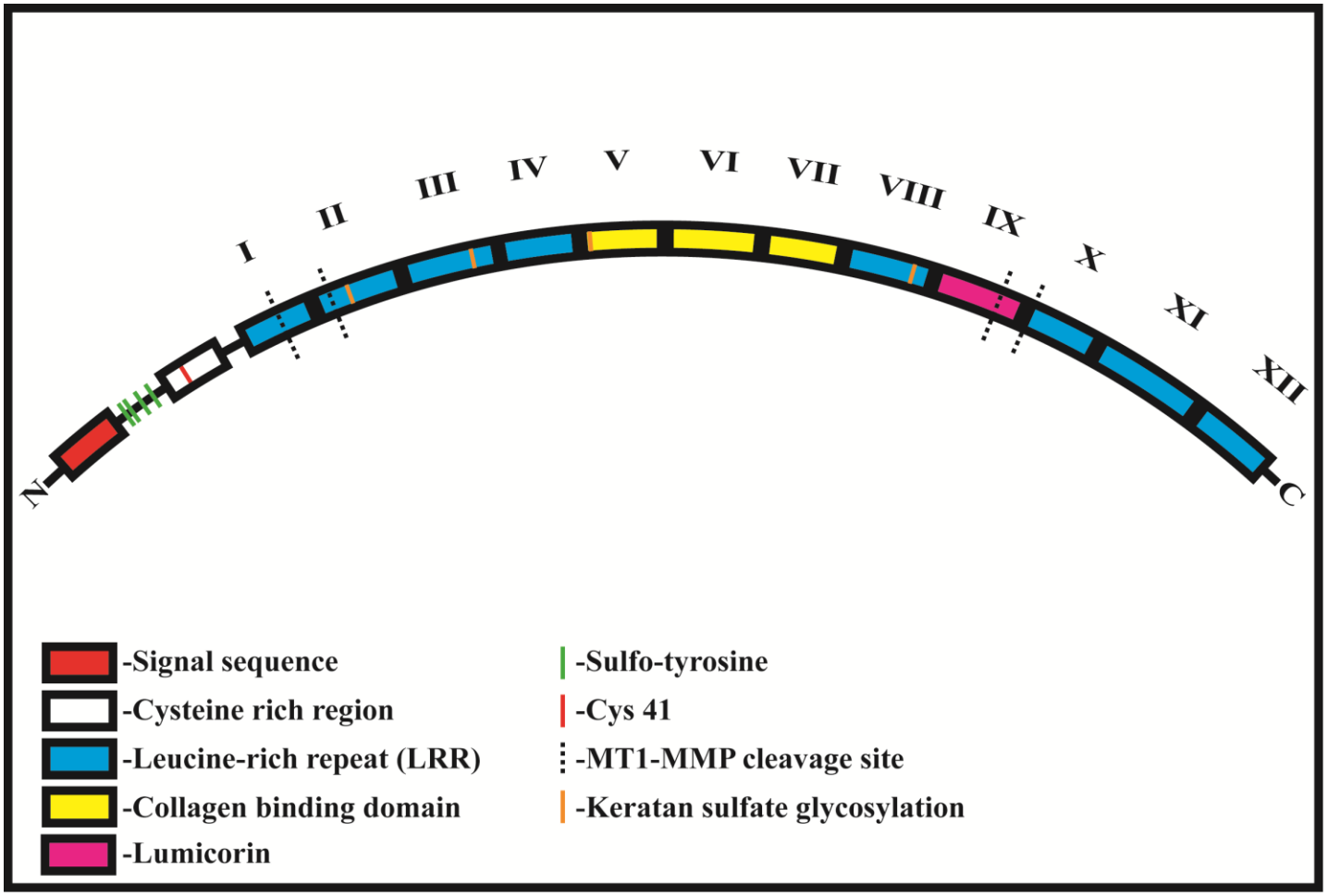

Figure 3. Lumican motifs and domains

An illustration of known motifs and domains of importance within the protein lumican.

CHAPTER 2

LUMICAN REDUCES TUMOR GROWTH VIA INDUCTION OF FAS-MEDIATED ENDOTHELIAL CELL APOPTOSIS

Abstract

To study the effects of lumican on cancer cells, we create stable polyclonal lumican-overexpressing cell lines from murine fibrosarcoma (MCA102) and pancreatic cancer (Pan02). Intriguingly, while lumican overexpression in Pan02 cells increased invasiveness, decreased soft agar colony size, and increased proliferation, in MCA102 cells, lumican decreased invasiveness, increased soft agar colony size, and had no effect on proliferation. In stark contrast to these *in vitro* results, the effect of lumican overexpression in MCA102 and Pan02 cells *in vivo* was remarkably consistent: lumican-overexpressing tumors were smaller. Closer analysis of revealed that these lumican-overexpressing tumors also possessed a lower vascular density. As is the case for several other ECM proteins, previous work suggests that lumican may induce apoptosis; hence we examined this potential role in the tumor cell lines and the murine brain microvascular endothelial cell line MB114. In MB114 endothelial cells, the presence of lumican increased the amount of apoptosis as measured by caspase-3 cleavage in both the presence and absence of the Fas-activating antibody Jo-2. Pan02 cells overexpressing lumican were also affected in this manner, however MCA102 cells were not. Thus, indiscriminate apoptosis is an unlikely mechanism for our observed reduction in tumor growth as this apoptotic effect was not

generalized. In contrast to what has been reported in murine embryonic fibroblasts and corneal fibroblasts, endothelial cells overexpressing lumican do not have more Fas expression. However, lumican-overexpressing endothelial cells had reduced anti-apoptotic FLIP (FLICE-like inhibitory protein) expression. Thus our current model supports a role of lumican in cancer of downregulating FLIP expression in the invading endothelial cells as a means for reducing angiogenesis and consequently tumor growth.

Introduction

The extracellular matrix has garnered much attention in the last two decades, due in large part to the discovery of constituent proteins that interact with both structural elements such as collagen as well as with membrane-bound receptors on the surface of cells. These "matrikines," as these matrix constituents are now called, can play important roles in regulating signaling pathways including those involved in tumorigenesis and angiogenesis [108]. Members of the small leucine-rich proteoglycan (SLRP) family have been extensively studied for both their ability to bind collagen and other matrix proteins, and their ability to perform outside-in signaling [28]. The best characterized members of the SLRP family include decorin, fibromodulin, and lumican. The latter of these proteins is our focus as it is the least understood.

Lumican is a 338 amino acid member of the SLRP family that exists as a 50-100 kDa keratan sulfate proteoglycan in the cornea but exists as a 55-57 kDa glycoprotein in most other tissues [28, 33]. The 37 kDa core protein possesses a middle region containing 11 leucine rich repeats (LRRs), and a C- terminal LRR "ear repeat" arranged in a "banana-shaped" tertiary structure [31]. Lumican was originally identified as a regulator of collagen fibrillogenesis and the concave side of lumican shares a homologous collagen-binding domain on LRR5-7 with its

closest relative fibromodulin, another collagen regulator [36]. Indeed, both *lum* -/- and *fmod* -/- knockout mice lack appropriate collagen organization [38-39, 109]. Proper collagen organization is vital to establishing corneal transparency (for which lumican derives its namesake) and *lum* -/- mice appropriately exhibit corneal opacity [27, 38]. Studies examining SLRPs and their involvement in cancer have mostly focused on decorin, but the role of the SLRP family member lumican in cancer is receiving increasing attention [41, 43-49, 110-112].

There is substantial work implicating lumican's involvement in cancer. Immunohistochemical (IHC) analysis of lumican expression has been reported in melanoma and osteosarcoma as well as in breast, pancreatic, colorectal, cervical, neuroendocrine, and lung carcinomas. Similarly, microarray studies have highlighted trends in *lum* mRNA expression in various stages of these and other cancer types [41, 43-55]. At best, these methods provide correlative data for the presence or absence of lumican and the severity of disease, but they do not elucidate the role of lumican in cancer. More information has been gleaned on the role of lumican in cancer from experimental cell biology. For example, lumican-overexpression consistently results in reduced colony formation in anchorage-independent soft agar growth assays [60-62]. Additionally, melanoma cells exhibit decreased migration, invasion, and metastasis when treated with lumican [62-63]. Lumican also results in a reduction in subcutaneous tumor volume in mouse models, possibly by reducing angiogenesis [60, 62].

Multiple lines of evidence support a role for lumican in the regulation of vascular function. For example, lumican is localized to the peripheral blood vessels in adult human lungs and to the thickened intima of the coronary artery [33, 64]. Functionally, endothelial cell expression of lumican increases during the resolution phase of angiogenesis in which vascularization ceases and the vessel returns to a state of angiostasis [66]. Similarly, lumican is

strongly expressed in the resting endothelium of the renal vein [65]. Not surprisingly, *lum* -/- *fmod* -/- knockout mice exhibit increased vascularization in the myocardium, suggesting an anti-angiogenic role for lumican [39]. Finally, our previous data demonstrate that lumican can reverse the pro-angiogenic affects of basic fibroblast growth factor (bFGF) in Matrigel plug assays, highlighting lumican's effectiveness as an anti-angiogenic molecule [66].

Our goal in the present study was to determine what effect lumican overexpression has on cancer cells *in vitro* and *in vivo*, and the potential mechanism(s) of these effects. Specifically, we test the hypothesis that lumican plays an anti-angiogenic role in the tumor microenvironment. We demonstrate that overexpression of lumican in the murine models for fibrosarcoma (MCA102) and pancreatic cancer (Pan02) resulted in pleiotropic *in vitro* effects on invasion, proliferation, and soft agar colony formation [67-68]. In a subcutaneous tumor model in syngenic mice, lumican overexpression consistently resulted in reduced tumor volume and lower blood vessel density. Furthermore, lumican increased MB114 endothelial cell susceptibility to Fas-induced apoptosis, reduced survival, and downregulated the anti-apoptotic FLICE-like inhibitory protein (FLIP) [71]. Together, these results support a model in which lumican enhances apoptosis of endothelial cells as they invade the tumor stroma during angiogenesis, possibly via regulating FLIP expression.

Materials and Methods

Cell culture, plasmids, and retroviral infections

The full length murine lumican cDNA (#5707371) less the secretory signal was cloned into pSecTag A plasmid with Myc-His_6 appended to the 3'end via the 5'BamHI and 3' NotI restriction sites (fwd 5'-GGCGGCGGATCCCAATACTACGATTATGAC-3') (rev 5'-

GGCGGCGCGGCCGCGTTAACGGTGATTTCATT-3'). The resulting Igκ-Lumican-Myc-His_6 cassette was ligated into the bicistronic retroviral vector pMSCV-Neo via the 5'HpaI and 3' BglII restriction sites (fwd 5'-CCGGCCGAATTCTTAATACGACTCACTATAGGG-3') (rev 5'-CCGGCCAGATCTCAACAGATGGCTGGCAACTAG-3'). Retroviral supernatants were produced by EcoPack2 retroviral packaging cells (Clontech, USA) and used to infect the murine fibrosarcoma cell line MCA102, murine pancreatic carcinoma cell line Pan02, and the murine brain microvascular endothelial cell line MB114 as described previously [113]. Cells were selected via addition of 400 nM Neomycin and maintained with 200 nM Neomycin.

Detection of endogenous lumican was achieved via TCA 50%/DOC .01% protein precipitation from conditioned serum free media (SFM) and confirmation of myc-his-tagged lumican overexpression was achieved via Ni-NTA Agarose (Qiagen, Valencia, CA) binding from conditioned serum free media.

In vitro cancer cell assays

The effect of lumican overexpression on MCA102 and Pan02 cellular invasion was measured using a modified Boydenchamber assay as described previously [114]. Briefly, a porous membrane (8 μm pore, 24-well format; BD Biosciences, San Jose, CA) was coated with 100 μl of a 1:50 dilution of Matrigel (BD Biosciences, San Jose, CA) which was allowed to dry overnight at room temperature. The following day, 100,000 control and lumican-overexpressing cells were cultured on dried membranes in SFM +.1% BSA. Cellular invasion was induced by adding 5% serum to the lower chamber and was allowed to proceed at 37º C for 48 h. Subsequently, Matrigel-invading cells were washed twice with ice-cold PBS and immediately fixed for 10 min with 95% ethanol. Cells remaining in the upper chamber were removed with a

cotton swab, whereas those remaining in the lower chamber were stained with crystal violet. Invasion was measured by densitometry utilizing the software ImageJ (NIH, Bethesda, MD).

The ability of lumican to alter the anchorage-independent growth of MCA102 and Pan02 cells was performed as described previously [115]. Briefly, 2 ml of a 1.2% agarose mixture in DMEM were allowed to solidify in 6-well plates. Control or lumican-overexpressing cells (50,000 cells/well) were diluted with an equal amount of DMEM-agarose mixture and allowed to solidify before placing in 37º C for 24 h. Plates were observed and 1 ml DMEM+ 10% FBS was added to each well as needed to avoid drying out. Colony areas were measured after 30 days using the software NIS-Elements D 3.00 SP1 (Build 455) (Nikon, Inc., Melville, NY).

Cell proliferation assays were conducted with WST-1 (Clontech, Mountain View, CA). Briefly, 500 cells were placed in 100 µl complete media in 12 wells of a 96-well plate and allowed to grow at 37º C for 24 h. The following day, the first three wells were replaced with 100 µl complete media containing 10 µl WST-1. Empty wells were also treated to establish a blank baseline. After four hours, wells were measured at OD 450nm to determine proliferation. This procedure was followed for each of four days to determine the proliferation rates for the control and lumican-overexpressing cells.

In vivo tumor growth studies

Control and lumican-overexpressing MCA102 and Pan02 cells were resuspended in sterile phosphate buffered saline (PBS) and injected subcutaneously at a density of 1,000,000 cells/ 100 µl injection between the shoulder blades of 10-week-old male C57BL/6 mice (three mice per condition; Jackson Laboratories, Bar Harbor, ME). Mice were monitored daily and primary tumors were measured with calipers between days 9 and 17. Tumor volumes were

calculated using the following equation: Volume = (d^2 x D)/2, where D is the long side and d is the short side. After 17 days (or if tumors became necrotic or achieved a size greater than 2000 mm^3) mice were killed and their primary tumors were excised and weighed. 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 #1-19-2008AA).

Immunohistochemistry

Excised tumors were fixed in 4% paraformaldehyde for 1 hr and placed in 70% ethanol before paraffin embedding and sectioning following standard procedure [116]. Sections were prepared via hematoxylin and eosin staining and additional sections were probed with anti-mouse CD31 in the Clarian Pathology Laboratory at Indiana University (Indianapolis, IN).

Apoptosis assays

The effect of lumican on endothelial cell apoptosis was assessed by measuring caspase-3 cleavage upon extended treatment with an apoptosis-inducing agent. Approximately 50,000 Lumican-overexpressing or control MB114 cells were plated onto 12-well culture plates. Cells were allowed to grow 24 h before washing with PBS and treating with 1 ml of serum free media (SFM) in the presence or absence of the hamster anti-mouse CD95 (Fas) agonizing antibody Jo-2 (final concentration 1 μg/ml) (554254; BD Pharmingen, San Jose, CA). To examine the effect of lumican conditioned media on endothelial cells, media isolated from lumican-overexpressing or control MCA102 or Pan02 cells was filtered and buffered with 10mM pH 7.3 HEPES buffer. MB114 control cells were grown in the presence of each conditioned media in the presence or

absence of Jo-2. After 48-72 h live and dead cells were lysed in 1x SDS-loading buffer, and assayed via western blot.

Colony forming survival assays

The survival ability of lumican-overexpressing endothelial cells was compared to controls in the following manner. Five hundred MB114-Neo and MB114-Lum cells were plated onto 6 cm plates containing 4 mL MB114 media. Cells were allowed to adhere and grow for approximately 1 week until colonies were visible by eye. Media was removed and plates were washed with 1 x PBS. Cells were fixed with 95% ethanol for five minutes and stained with crystal violet stain. Excess stain was removed with water and plates were allowed to dry before scanning and densitometry utilizing the software ImageJ.

Western Blot

Western blotting was performed as described previously [114]. Antibodies utilized in the experiments include the following: mouse anti-c-Myc (1:1000) (9E10, MMS-150R) (Covance, Inc., Princeton, NJ); mouse anti-β-actin (1:1000) (sc-47778), rabbit anti-Fas-L (1:500) (C-178, sc-6237), rabbit anti-Fas (1:500) (A-20, sc-1023) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-FLIP (1:500) (#3210), rabbit anti-caspase-3 (1:500) (#9662) (Cell Signaling Technology, Inc., Danvers, MA); rabbit anti-lumican (1:100) (kindly provided by Dr. S. Chakravarti, Johns Hopkins University, Baltimore, MA); and sheep anti-mouse (1:5000) (NA931-1ML), donkey anti-rabbit (1:5000) (NA934-1ML) (GE Healthcare, Piscataway, NJ).

Results

Lumican overexpression exhibits pleiotropic effects on MCA102 murine fibrosarcoma cells and Pan02 murine pancreatic cancer cells in vitro

The MCA102 fibrosarcoma and Pan02 pancreatic cancer cell lines were transduced with retroviral constructs encoding either murine lumican cDNA to which a C-terminal Myc-epitope had been appended or an empty vector. Neomycin selection was subsequently used to establish stable polyclonal cell lines. Conditioned media from these cell lines were precipitated with TCA/DOC. Western blotting with the anti-Myc antibody confirmed the overexpression of lumican in the stably selected cell lines (Fig. 1). Furthermore, western blotting with the anti-lumican antibody revealed an undetectable level of endogenous lumican expression in either MCA102 or Pan02 cell lines (Fig. 1). Due to the deficit of direct evidence regarding the role of lumican in cancer, we subjected the lumican-overexpressing MCA102 and Pan02 cells (MCA102-Lum and Pan02-Lum) and their control counterparts (MCA102-Neo and Pan02-Neo) to a variety of *in vitro* experiments designed to mimic several aspects of tumor behavior.

Tumor cell invasion is one of the deadliest aspects of cancer as the ability to enzymatically degrade a collagen matrix is correlated with metastatic potential [117]. Thusly, Boyden chamber invasion assays were performed to assess the ability of lumican to affect invasion through a Matrigel matrix. Lumican overexpression resulted in a 22% reduction in invasion in the MCA102 cells, but enhanced invasion in the Pan02 cells by 91% (Fig. 2A,B).

Cancer cells have the unique ability to form colonies in soft agar, as they do not require anchorage via an extracellular substrate to grow. As this is an excellent *in vitro* analog for tumor formation, we performed soft agar assays to determine if lumican overexpression affects anchorage-independent growth of cancer cells. In contrast to the results obtained in the invasion

assays, lumican overexpression increased the average colony size of MCA102 cells 38%, but in Pan02 cells the average colony size was reduced 36% (Fig. 2C,D).

A hallmark of cancer cells is their rapid rate of proliferation. To monitor the effect of lumican on cancer proliferation, we used WST-1 proliferation assays to determine if lumican overexpression influences the growth rate of cancer cells. Similar to the results of the invasion assay, lumican overexpression significantly increased the proliferation of Pan02 cells (Fig. 2F). However, lumican overexpression had no detectable effect on MCA102 cell proliferation (Fig. 2E).

Collectively, these *in vitro* data indicate that the overexpression of lumican in these tumor cell lines resulted in no consistent pattern of effects on the various tumor cell activities of invasion, proliferation, or anchorage-independent growth between the two cell lines. From this data we were unable to arrive at a conclusive effect of lumican on cancer cells, however *in vitro* data is removed from the complex microenvironment of the host organism and may not reflect the behavior of the cancer *in vivo*. In particular, our previous data suggest that lumican is an inhibitor of angiogenesis [66]. In light of this, we next sought to determine what effect lumican would have in an animal model of tumor growth.

Lumican overexpression consistently reduces MCA102 murine fibrosarcoma and Pan02 pancreatic cancer tumor volume in syngenic mice

Understanding the effect of lumican in cancer requires consideration of the microenvironment established by complex interactions between the host and the cancer cells. To determine how lumican-overexpressing cancer cells would interact with a syngenic host, we injected male C57BL/6 mice subcutaneously with equal numbers of MCA102-Lum, MCA102-

Neo, Pan02-Lum, or Pan02-Neo cells. Tumor growth curves and final tumor masses both indicate that lumican overexpression results in an approximately 60% reduction in mean tumor volume for both MCA102 and Pan02 cells (Fig. 3A,B). This is in stark contrast to the *in vitro* data which demonstrated inconsistent effects on invasion, proliferation, and soft agar colony formation. Previously, we have demonstrated lumican can reduce blood vessel growth into Matrigel plugs [66]. Such a reduction might explain the reason for the smaller average tumor volume via a reduction in angiogenesis. We therefore examined blood vessel density within the extracted tumors.

Tumors overexpressing lumican possess reduced vasculature

Vascular density in the tumors was determined using hemotoxylin and eosin staining followed by counting the number of vessels in each of 10 fields of each tumor section at 200x magnification. MCA102-Lum tumors averaged 62% fewer vessels per field compared to MCA102-Neo tumors. Similarly, Pan02-Lum tumors averaged 43% fewer vessels per field compared to Pan02-Neo tumors (Fig. 4A). In addition to hemotoxylin, tumor sections were stained for the endothelial cell marker CD31. Again, the lumican-overexpressing tumors had a lower vascular density compared to the control tumors (Fig. 4B).

Previous research suggests that lumican may mediate Fas-Fas-L interactions, contributing to induction of apoptosis; an established mechanism for the reduction of angiogenesis by extracellular matrix molecules [62-63, 69-70]. Based on these previous findings, we sought to determine what effect, if any, lumican has on the induction of apoptosis in MCA102, Pan02, and MB114 cells.

Lumican increases apoptosis in endothelial cells

To determine if lumican could facilitate apoptosis in endothelial cells, we first established lumican-overexpressing and control MB114 endothelial cell lines and confirmed overexpression via western blot (Fig. 5A). Once again, western blot analysis with the anti-lumican antibody revealed that lumican was undetectable in control cells but readily apparent in the conditioned media of MB114-Lum cells.

Consistent with a role in promoting apoptosis, 37% fewer MB114-Lum cells survived to form colonies when plated at a low density as compared to MB114-Neo cells (Fig. 5B,C). We have not previously noted an effect on proliferation associated with lumican in endothelial cells [66]. Thus, we investigated the possibility that lumican promotes endothelial cell apoptosis.

In the extrinsic Fas-apoptosis pathway, the cell surface receptor Fas (CD95) is bound and aggregated by Fas ligand (Fas-L) existing as a membrane-bound ligand or as a multimeric soluble ligand. When activated, Fas triggers an intracellular signaling cascade by cleaving multiple caspases, ultimately leading to apoptosis. The cytoplasmic FLICE-like inhibitory protein (FLIP) can act as a competitive inhibitor to caspase-8, preventing apoptosis from progressing.

MB114-Lum and MB114-Neo cells were cultured in the presence or absence of the Fas-activating antibody Jo-2 under serum free conditions. After 48-72 hr, live and dead cells were collected and Fas apoptosis was assessed via immunoblotting for cleaved caspase-3. Blots were subsequently stripped and reblotted with anti-βactin to monitor protein loading. Lumican-overexpressing MB114 cells demonstrated a greater amount of cleaved caspase-3 than control cells in both untreated conditions and upon treatment with Jo-2 (Fig. 5B). Interestingly, while lumican overexpression did appear to increase apoptosis in Pan02 cells in the presence of Jo-2, it

lacked this effect in MCA102 cells (Fig. 5B). This is particularly interesting as our *in vitro* data demonstrated consistently greater proliferation and invasion in the Pan02-Lum cell line. As no increase in apoptosis was observed in the MCA102-Lum cell line, an overall increase in susceptibility to apoptosis is an unlikely mechanism for the reduction in tumor size observed in the mice. To mimic the conditions of the animal study, MB114 control cells were treated with conditioned media from MCA102-Lum, MCA102-Neo, Pan02-Lum, or Pan02-Neo cells. Prior to applying the conditioned media, we confirmed that MCA102-Lum and Pan02-Lum media expressed lumican while their control media did not (Fig. 5C). Consistent with the overexpression results, MB114-Neo cells treated with the lumican conditioned media in the presence or absence of Jo-2 possessed more cleaved caspase-3 activity than those treated with control conditioned media (Fig. 5D).

To investigate the mechanism by which lumican promotes apoptosis, we assessed the relative amounts of proteins known to affect apoptosis within the MCA102, Pan02, and MB114 control and lumican-overexpressing cell lines. Previous reports have demonstrated that *lum* -/- murine embryonic fibroblasts and corneal fibroblasts possess little or no Fas receptor and that transfection with lumican can restore this to wild-type level [69-70]. However, our analysis of MB114, MCA102, and Pan02 cell lysates from lumican-overexpressing and control cells revealed that the level of Fas receptor remained static (Fig. 5E). Expression of Fas-L was determined to be slightly higher in MCA102 and Pan02 cells, but it was also present in MB114 cells; however, there was no difference in expression between control cells and those expressing lumican (Fig. 5E). Interestingly, expression of the anti-apoptotic protein FLIP (FLICE-like inhibitory protein) was reduced in the lumican-overexpressing MB114 cells compared to control cells. (Fig. 5E).

Discussion

Much of our current understanding of lumican in cancer is derived from immunohistochemical correlations of the relative abundance of the protein in the stroma of cancerous and noncancerous tissues. Our hypothesis-driven approach illuminates a functional role of lumican in tumor growth. In this study, we demonstrated that overexpression of the extracellular matrix protein lumican has differential effects on cancer cell proliferation, invasion, and anchorage independent growth in the fibrosarcoma cell line MCA102 and the pancreatic cancer cell line Pan02. Despite the pleiotropic *in vitro* effects, lumican overexpression consistently reduced tumor size and blood vessel density *in vivo*. Furthermore, we provide evidence that this reduction in blood vessel density is due to a pro-apoptotic effect of lumican on the endothelial cells invading the tumor stroma.

The results of our *in vitro* analyses on MCA102 and Pan02 cells overexpressing lumican demonstrate the cell-specific effects of lumican on several deadly aspects of cancer without regard to the host microenvironment. Pan02 cells overexpressing lumican were more invasive in Matrigel-coated Boyden chamber invasion assays than their control counterparts. The opposite effect was observed in MCA102, which exhibited a marked reduction in invasion. Soft agar assays were performed to assess lumican's effect on anchorage independent growth. Lumican overexpression resulted in smaller average colony size in MCA102. Past experiments on a variety of cell lines support the notion that lumican reduces the size of soft agar colonies [60-62]. However this pattern was not observed in Pan02, in which lumican overexpression resulted in increased colony size. We assessed the effect lumican overexpression has on cellular proliferation. In MCA102, lumican overexpression did not affect proliferation. In Pan02 however, lumican overexpression produced a significant increase in proliferation. This result is

in direct contrast with previous reports of lumican's effect on melanoma, osteosarcoma, murine embryonic fibroblasts, and HEK 293T [55, 62, 70, 118]. Although there is no consensus between lumican's *in vitro* effects on MCA102 and Pan02, we have demonstrated that both cell lines behave consistently *in vivo*. We found lumican overexpression in MCA102 and Pan02 reduced the growth of subcutaneous tumors in syngenic mice. Moreover, since the effect of lumican on these cell lines *in vitro* is inconsistent, the reduction in tumor size is unlikely due to direct effects on the tumor cells. The reduction in tumor size is consistent with previous reports using induced oncogenic fibroblasts and melanoma cell lines [60, 62]. The consistency of effects *in vivo* highlights the importance of the host microenvironment in cancer progression.

Hematoxylin and eosin (H&E) staining and CD-31 localization of the extracted tumor sections revealed a reduced vessel density in lumican-overexpressing MCA102 and Pan02. This finding suggests that lumican may perform an anti-angiogenic role in the tumor microenvironment. Myocardial vascularization increases in *lum* -/- *fmod* -/- knockout mice and Matrigel plug experiments have demonstrated that lumican can reduce vascularization induced by bFGF [66]. Recent reports of endothelial cells plated on lumican reveals reduced pseudotube formation [63] and previous research on tubulating endothelial cells has demonstrated expression of lumican increases during the resolution phase of angiogenesis in which vascularization ceases and the vessel returns to a state of angiostasis [66]. This angiostatic state can be observed in large resting vessels where expression of lumican is high [65]. Collectively, these results highlight the potential for lumican to function as a negative regulator of angiogenesis.

Although highly resistant to apoptosis when quiescent, endothelial cells are very susceptible to a particular form of apoptosis known as anoikis during angiogenesis [119]. Anoikis is apoptosis resulting from the loss of cell adhesion to the ECM. Several known anti-

angiogenic proteins of the ECM including angiostatin, canstatin, thrombospondin-1, and decorin promote apoptosis in the invading endothelial cells [110, 120-124]. In the living organism, lumican overexpression alone was sufficient in reducing the vascular density and size of tumors. Upon analysis of colony-forming assays as well as caspase-3 cleavage, we report that lumican consistently increases susceptibility to Fas-induced apoptosis in lumican-overexpressing endothelial cells; an effect observed in endothelial cells cultured in conditioned media from lumican-overexpressing tumor cells as well.

Previous reports have demonstrated lumican preferentially binds to soluble Fas-L and increases apoptosis. Membrane bound Fas-L is also expressed in a variety of cancer types, presumably as a means for immune evasion [72-77]. We identified the presence of Fas-L in equal abundance in MCA102 and Pan02 cells, without regard to lumican overexpression suggesting that increased endothelial cell apoptosis is not due to increased tumor cell expression of Fas-L. Although previous reports have suggested that lumican expression may drive Fas receptor expression, our analysis of MB114-Neo and MB114-Lum cell lysates revealed no significant difference in the level of Fas detected. However, the anti-apoptotic protein FLIP was found to be downregulated in the MB114-Lum cell line as compared to MB114-Neocell line. FLIP acts as a competitive inhibitor of caspases-8, effectively halting the caspase cascade and progression of apoptosis. By downregulating FLIP within the endothelial cells, lumican could increase the susceptibility to apoptosis, thereby preventing angiogenesis.

Conclusion

We have shown that lumican overexpression consistently reduces tumor growth *in vivo* regardless of its pleiotropic *in vitro* effects. Furthermore, this reduction in tumor growth is

associated with reduced vascular density. Finally, we have provided evidence to support a Fas-specific pro-apoptotic role for the SLRP lumican in the endothelium.

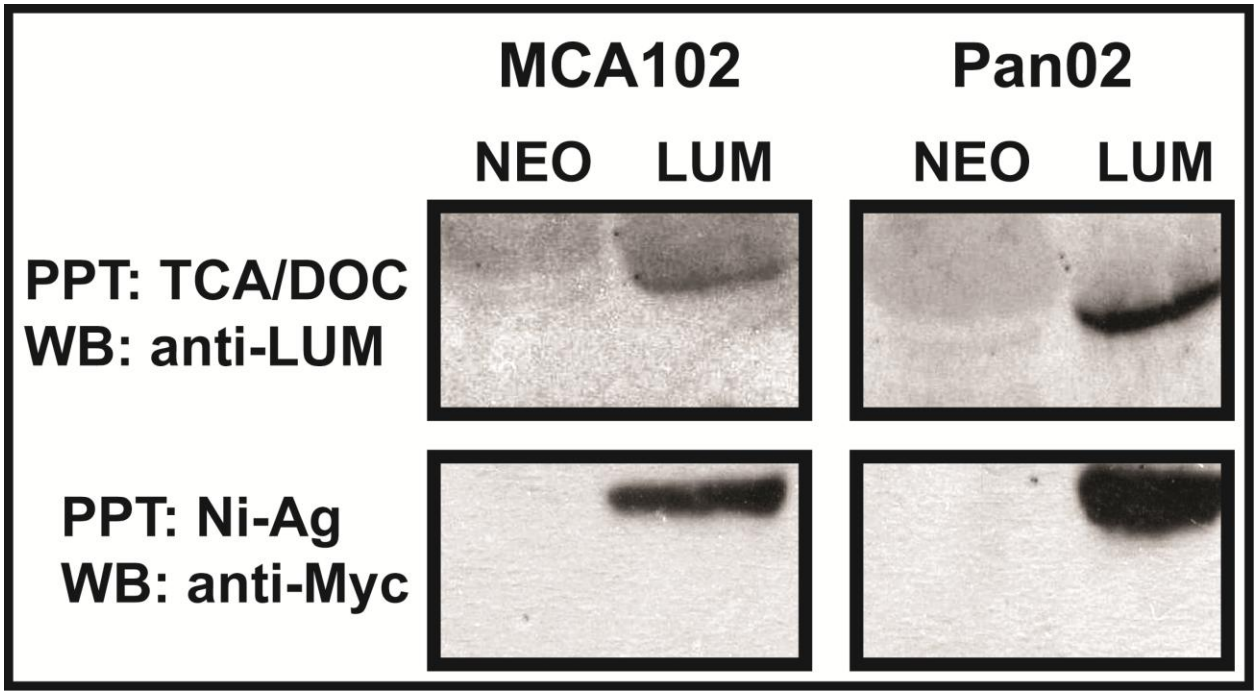

Figure 1. Confirmation of lumican overexpression

Confirmation of lumican overexpression was performed on conditioned media from the MCA102 and Pan02 cell lines. TCA/DOC precipitation and detection with rabbit-anti-lumican (1:100) revealed little or no endogenous expression. Ni-Ag precipitation and detection with mouse-anti-cMyc (1:1000) confirmed plasmid overexpression of lumican.

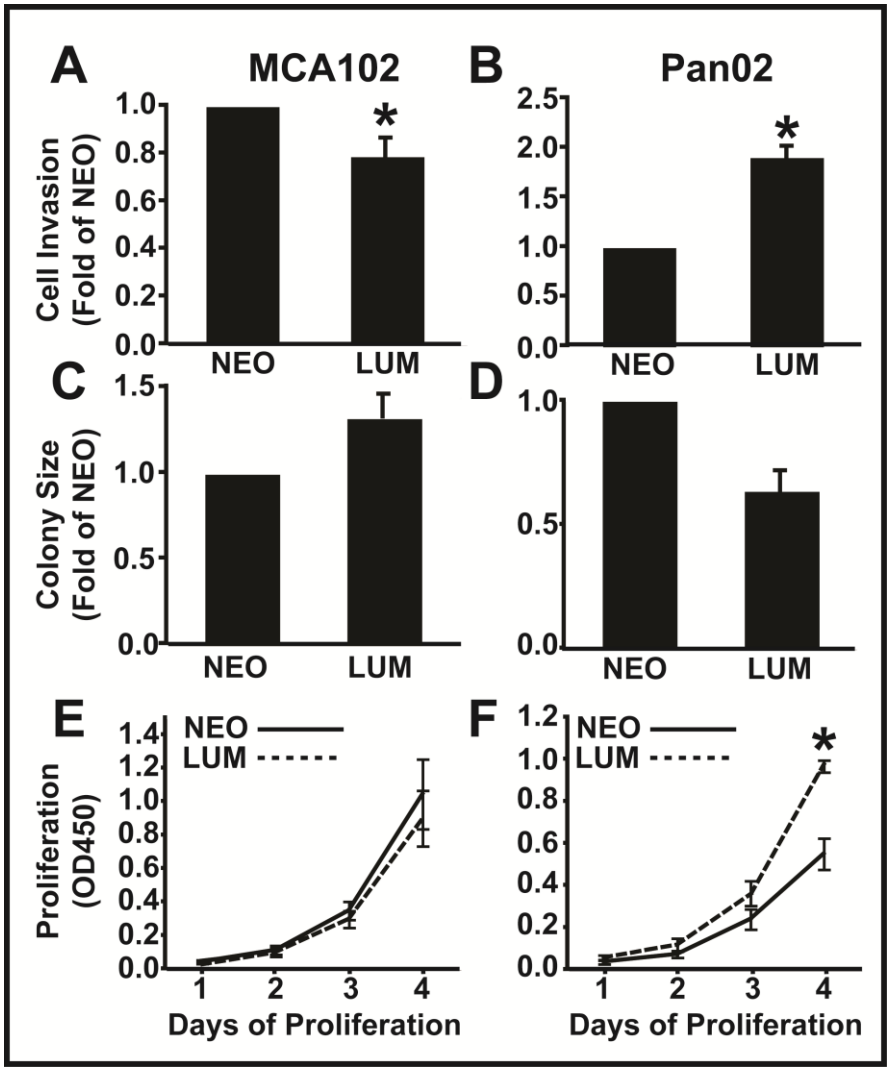

Figure 2. Lumican overexpression exhibits pleiotropic effects *in vitro*

(A, B) Lumican-overexpressing cell lines and their corresponding control cell lines were cultured in Matrigel coated Boyden chambers and induced to invade. Lumican overexpression increased invasion in PanO2 cells but decreased invasion in MCA102 cells. Data is the average +/- 1SEM of at least four independent experiments. (C, D) Lumican-overexpressing and control cells were cultured in soft-agar. The areas of the resulting colonies were calculated from two dimensional measurements of colony diameter. Lumican increased MCA102 colony size but decreased PanO2 colony size. Data is presented as the average of three independent experiments +/- 1SEM. (E, F) Lumican-overexpressing PanO2 and MCA102 cells were cultured for 1 to 4 days. Each day, the relative number of cells was measured with WST-1 cell proliferation reagent. Lumican increased PanO2 proliferation, but did not affect MCA102 proliferation. Data is presented as the average of four independent experiments +/- 1SEM. (* indicates $p<.05$, Student's T-Test)

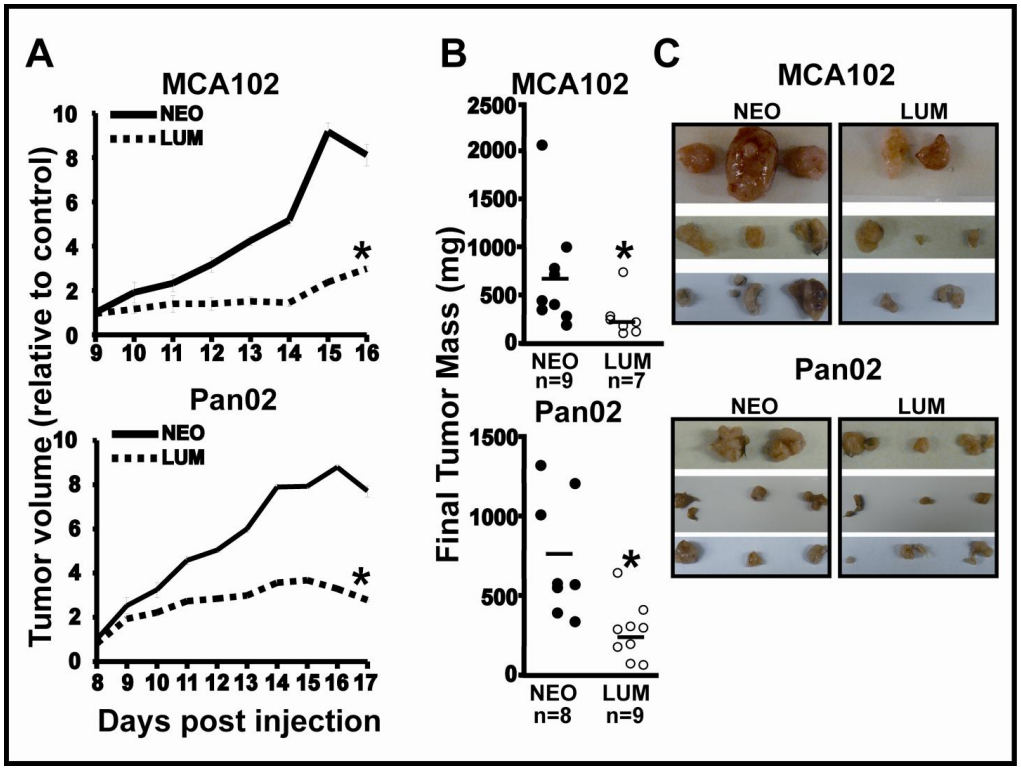

Figure 3.Lumican consistently reduces tumor growth *in vivo*

Equal numbers of lumican-overexpressing MCA102 and Pan02 cells or their corresponding control counterparts were injected in triplicate into syngenic C57BL/6 mice. (A) Tumor volumes were calculated daily and are reported as the average for each day relative to the first day after inoculation that tumors appeared. Data is the average +/-1SEM of three independent experiments. (B) Tumor masses were recorded at time of removal. Data is the final tumor mass (mg) for each tumor. Midlines represent mean mass. (C) Photos depicting the actual tumors removed for each experiment. (* indicates $p<.05$, Mann-Whitney rank sum test).

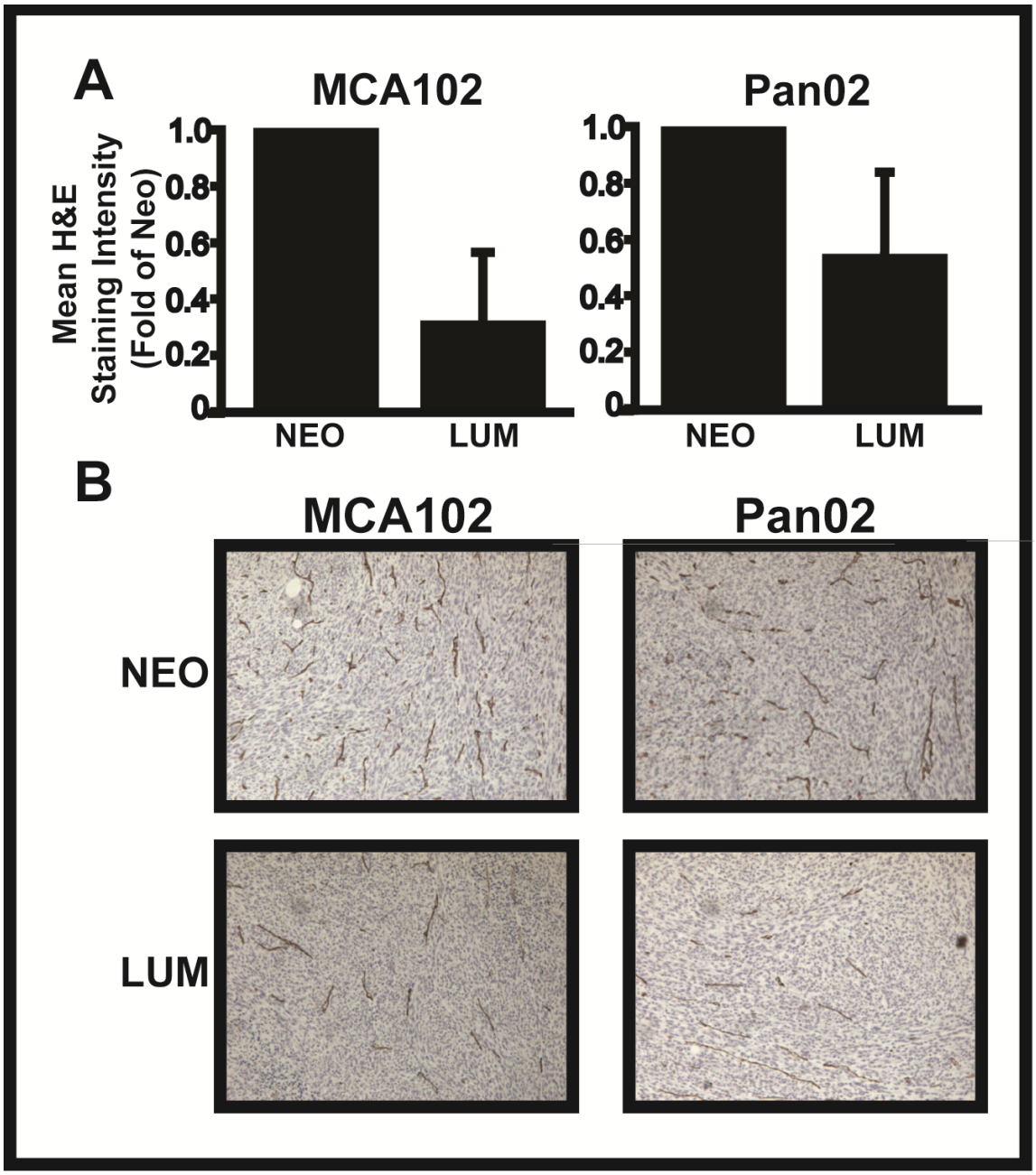

Figure 4. Lumican overexpression reduces tumor vasculature

(A) Lumican-overexpressing and control tumors were sectioned and stained for hematoxylin and eosin (H&E) and vessels were counted in each of 10 fields at 200x. Data is presented as the average number of vessels per field for each tumor type +/- 1SEM. (B) Lumican-overexpressing and control tumor sections were probed for the endothelial cell specific marker CD-31. Fewer vessels can be observed in the lumican-overexpressing tumors.

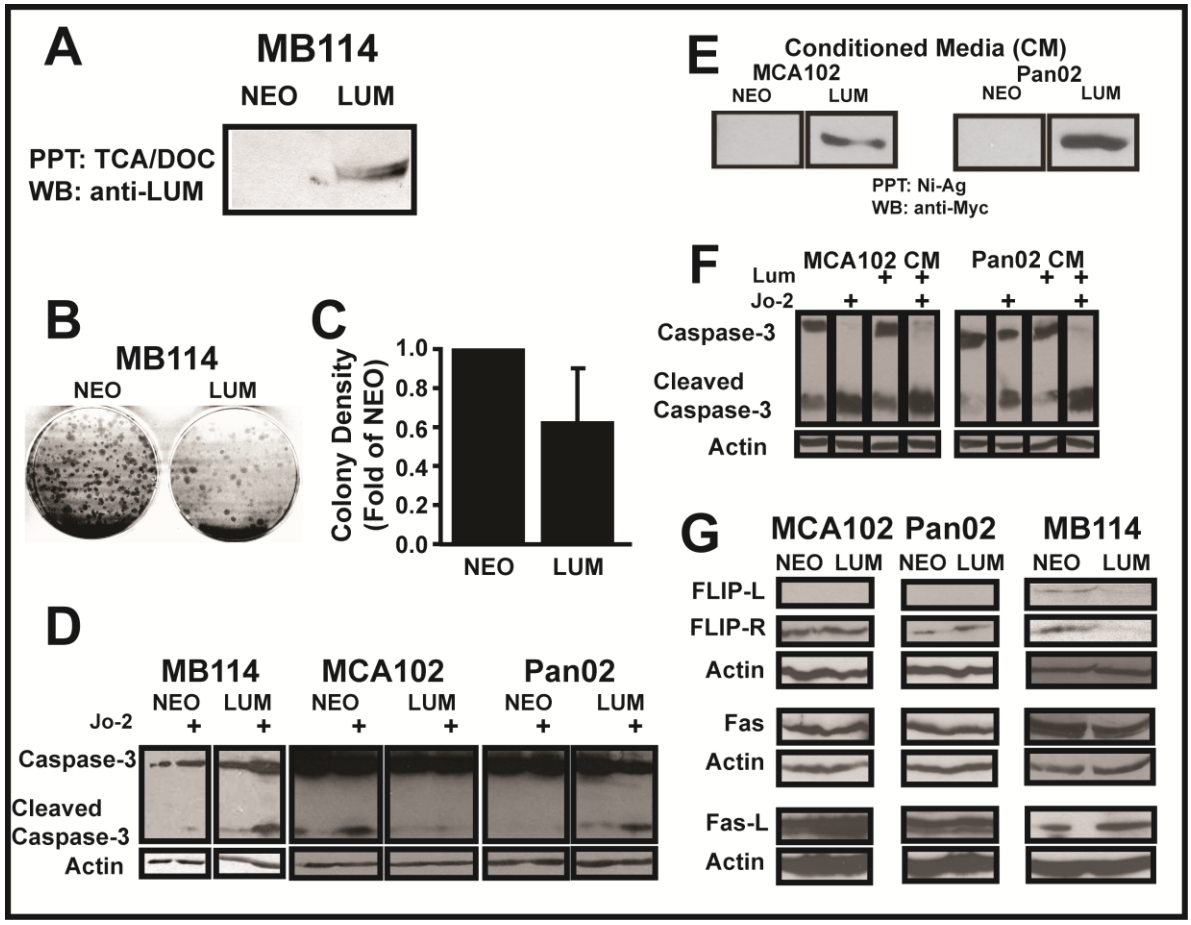

Figure 5. Lumican enhances Fas-mediated apoptosis

(A) Confirmation of lumican overexpression was performed on conditioned media from the MB114 cell line. TCA/DOC precipitation and detection with rabbit-anti-lumican (1:100) revealed little or no endogenous expression. Ni-Ag precipitation and detection with mouse-anti-cMyc (1:1000) confirmed plasmid overexpression of lumican. (B) Representative MB114 colonies formed after plating 500 cells. (C) Average amount of colony survival after plating 500 cells. Data is presented as the average fold of colonies formed by MB114-Neo +/- 1SD. (D) Control or Lumican-overexpressing cells were cultured in the presence of absence of Fas-agonizing antibody Jo-2. Whole cell lysates were probed with anti-caspase-3 antibodies to detect apoptosis. (E) Conditioned media was collected from control and lumican-overexpressing tumor cells. The presence of lumican was confirmed by western blot. (F) MB114 endothelial cells were cultured in conditioned media from control or lumican-overexpressing tumor cells in the presence or absence of Jo-2 antibodies. Apoptosis was monitored by western blot with anti-caspase-3 antibodies. (G) Expression levels of FLIP, Fas, and Fas-L was detected in whole cell lysates from control or lumican-overexpressing MB114, MCA102, and Pan02 cells by western blot analysis. Actin served as a loading control.

CHAPTER 3

ANALYSIS OF NOVEL ANGIOGENESIS TARGETS VIA TARGETED ANTI-SENSE MORPHOLINO OLIGONUCLEOTIDE KNOCKDOWN IN TRANSGENIC ZEBRAFISH

Introduction

The extracellular matrix is a dynamic environment consisting of structural proteins as well as secretory signaling proteins. The expression and secretion of such proteins is of particular importance in regulating angiogenesis. Discovering the roles played by novel extracellular matrix proteins in vascularization is a crucial step in the process of creating new treatments for pathological angiogenesis.

Our previous microarray analysis of sprouting endothelial cells has provided numerous novel targets, many of which have been confirmed by reverse transcription (RT) PCR. Over the last decade, anti-sense morpholino oligonucleotide knockdown of specific genes in early zebrafish embryos has proven to be an effective method in the pursuit of validating research targets. During this same period, transgenic zebrafish have been created which express GFP under the endothelial *fli1* promoter and dsRed under the erythrocytic *gata1* promoter. The combination of these two advances has established a novel method to assess the role specific genes play in the process of vascularization.

Our microarray data reveal that amongst many other genes, *clu* (Clusterin), *grm* (Gremlin-1), and *serpine2* (Serpin E2) increased in expression during 25 hours of sprouting on matrix in the endothelial cell line MB114. During this same time period, *npnt* (Nephronectin) was among the genes found to decrease in expression. The aim of this study was to determine what role these genes play, if any, in the vascularization during the first five days of life using morpholino knockdown in the transgenic *fli1*^{GFP}/ *gata1*^{dsRED} zebrafish. Targets that demonstrate potential may then be expressed in cell culture and further studied as novel regulators of tumor angiogenesis.

Materials and Methods

Zebrafish

Tg(*fli1*^{GFP}/ *gata1*^{dsRED}) *Danio rerio* (generously provided by Stephen Ekker, PhD, Mayo Clinic, Rochester, MN) were maintained on a 14 hour light 10 hour dark cycle at 28.5 ºC. Breeding pairs were arranged the night prior to embryo injection and egg fertilization was triggered with the beginning of the light cycle. All experiments were performed in accordance with the animal protocol procedures approved by the Institutional Animal Care and Use Committee of Indiana State University.

Confirmation of zebrafish mRNA expression

Ten zebrafish embryos were collected at 2 hr, 6 hr, 2 d, 3 d, and 4 d in Tri-Reagent for the purpose of RNA extraction (T9424, Sigma-Aldrich, St. Louis, MO). Samples were prepared as per manufacturer's instructions. Reverse transcription (RT) was performed using iScript cDNA synthesis kit (170-8891, Bio-Rad, Hercules, CA) as per manufacturer's instructions.

Oligo primers were synthesized for the zebrafish Clusterin (fwd 5'-AATCCGTCGCAATTCTTTCGGCTG-3') (rev 5'-TTCCTGTGCCACCACTTCAGAGAA-3'), Gremlin-1 (fwd 5'-ATACAGTCCAAACCAGTCGGAGCA -3') (rev 5'-TCCGTAGCAGAAGCGGTTGATGAT -3'), Nephronectin (fwd 5'-GTAAGCACCGCTGCATGAACACAT-3') (rev 5'-TGACGTACTGAAGGTCAAAGCCGT-3'), SerpinE2 (fwd 5'-TCAGATCTGGGTCTGCAGGTGTTT-3') (rev 5'-ACCACACTGGGAATCTGGCCTTTA-3'), and GAPDH (fwd 5'-AGGCTTCTCACAAACGAGGACACA-3') (rev 5'-ATCAATGACCAGTTTGCCGCCTTC-3') (Integrated DNA Technologies, Skokie, IL). PCR was performed for 40 cycles (95º→53º→72º) and amplified products were analyzed via gel electrophoresis on 2% agarose.

Morpholino design

Morpholinos were designed to prevent transcription by binding approximately 50-75 bp upstream of the target start codon. Anti-sense morpholino oligonucleotides were synthesized for the zebrafish Clusterin (5'-ACAGAGGTCAGAGACATAGTAGATC-3'), Gremlin-1 (5'-TAAATCATAGGCTATAAATATATAT -3'), Nephronectin (5'-GGTCTGTGAATGGGAATGATGATGA -3'), and SerpinE2 (5'-AGATGGAGAGCTCGTGTCTTCCGCG -3') (Gene Tools, LLC, Philomath, OR). Antisense zebrafish p53 morpholino was designed by the manufacturer previously (5'-GCGCCATTGCTTTGCAAGAATTG -3') (Gene Tools, LLC, Philomath, OR).

Morpholino injection and analysis

The PV830 pneumatic PicoPump (World Precision Instruments, Sarasota, FL) was utilized in the delivery of morpholino to the target embryos. Morpholinos were suspended in deionized water and further diluted with phenol red such that one 0.3 nl injection delivered 4 ng of morpholino to an embryo. Multiple injections were utilized to deliver up to 12 ng of morpholino. Further dilutions were required to establish a 1 ng per 0.3 nl injection solution. When co-injected, anti-p53 morpholino was utilized at 1.5 times the amount of the target morpholino as described previously [81]. Control fish were injected with phenol red alone. Approximately 10 embryos were injected with a given dose or empty vehicle. Embryos were dechorionated at 24 hr and observed under bright field and fluorescent light and photographed. Proper dosage was determined experimentally.

Results

Confirmation of target expression via RT-PCR of early embryos

To establish the temporal expression patterns of the target genes, and ensure they are indeed expressed during our period of observation, we performed reverse transcription (RT) PCR at time points ranging from 2 hours to 4 days. Analysis of the zebrafish mRNA via RT-PCR revealed that clusterin is expressed as early as day 2, and gremlin-1, nephronectin, and serpinE2 are expressed as early as 2 hours post fertilization. The housekeeping gene GAPDH was expressed at all time points as a control (Fig. 1)

Effects of morpholinos on zebrafish development

Increasing doses of anti-clusterin morpholino (up to 12 ng) had no apparent effect on zebrafish development as morpholino-treated fish were indistinguishable from vehicle-injected controls. Conversely, fish treated with 4 ng anti-gremlin-1 morpholino possessed a less developed vasculature compared to controls at 1 day. Additionally, injection with as little as 1 ng anti-nephronectin morpholino resulted in delayed vascularization as compared to control fish. Finally, treatment with 4 ng anti-serpinE2 was lethal in the majority of fish tested. The one surviving fish possessed greatly underdeveloped vasculature at day 1 as compared to controls (Fig. 2).

Discussion

Because they share many organs and possess similar genomes, zebrafish remain an excellent model for human conditions [79]. Zebrafish are a particularly useful model for the rapid screening of potential angiogenic targets. Advances in transgenic zebrafish lines, including those expressing GFP-vasculature and dsRed-erythrocytes, as well as morpholino technology have enabled researchers to pursue investigations more simply and effectively than can be done in other model systems [80, 82-83]. Morpholino screening has led to the establishment of entire databases describing the effects of this knockdown technology [120]. Researchers also have suggested approaches that combine the Tg(*fli1*^{GFP}/ *gata1*^{dsRED}) zebrafish with an *in vivo* tumor model system to study tumor angiogenesis [121].

We report the effects of anti-sense morpholino oligonucleotides against multiple targets suspected to play a role in angiogenesis during the first five days of embryonic development in zebrafish. Clusterin has been implicated in angiogenesis and in a variety of cancers and is

thought to play a role in regulating apoptosis [85-95]. However, injection of the anti-sense morpholino oligonucleotide against clusterin failed to evoke any response within the zebrafish vasculature over the course of the first five days of development, even at doses as high as 12 ng. This suggests that in spite of previous data, clusterin is not important in regulating angiogenesis during early zebrafish development. Conversely, morpholinos against the targets gremlin-1, nephronectin, and serpinE2 all inhibited or delayed vascular neogenesis to some degree, suggesting that these are important for early zebrafish angiogenesis regulation. Gremlin-1 has been implicated in angiogenesis and is strongly expressed in the endothelial cells of lung tumor vasculature [98-100]. Similarly, nephronectin has been implicated in several cancers and is a known regulator of zebrafish heart development [102-104]. SerpinE2 has not been directly implicated in angiogenesis, but its ability to alter the production of matrix proteins is thought to mediate cancer cell invasion [105-107].

Future experiments will be required to determine the minimally effective doses for these morpholinos. Additionally, co-injection with anti-p53 morpholino is required to confirm that the observed effects are vascular-specific. Targets will then be overexpressed in cell culture models to be further characterized.

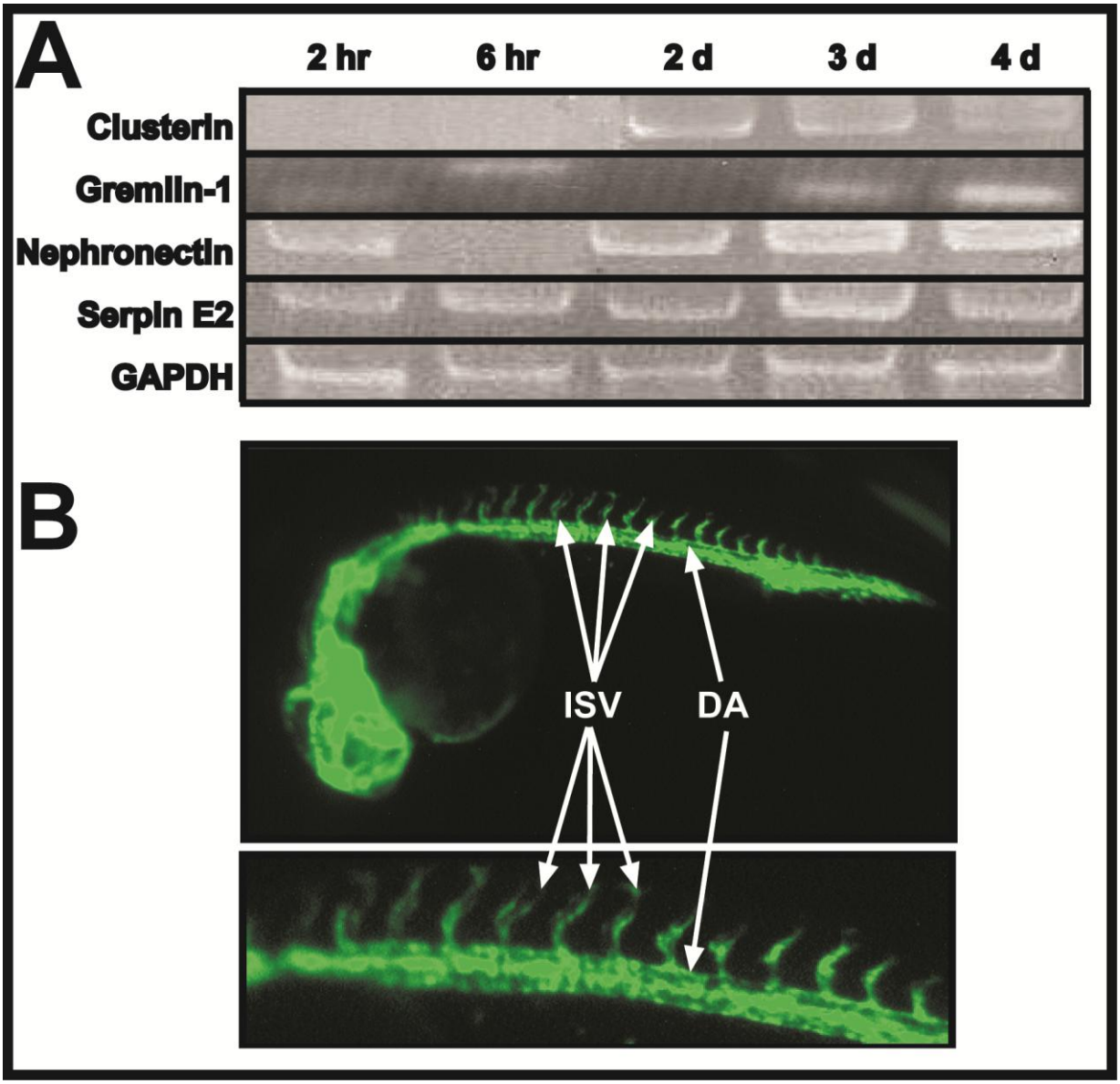

Figure 1. Confirmation of mRNA expression in early embryo and vessel anatomy

(A) Results of RT-PCR demonstrate the presence or absence of particular mRNAs at the given time points. The housekeeping gene GAPDH served as a positive control for mRNA. (B) GFP image of control fish at 24 hr time point. Intersegmental vessels (ISV) and dorsal aorta (DA) are indicated.

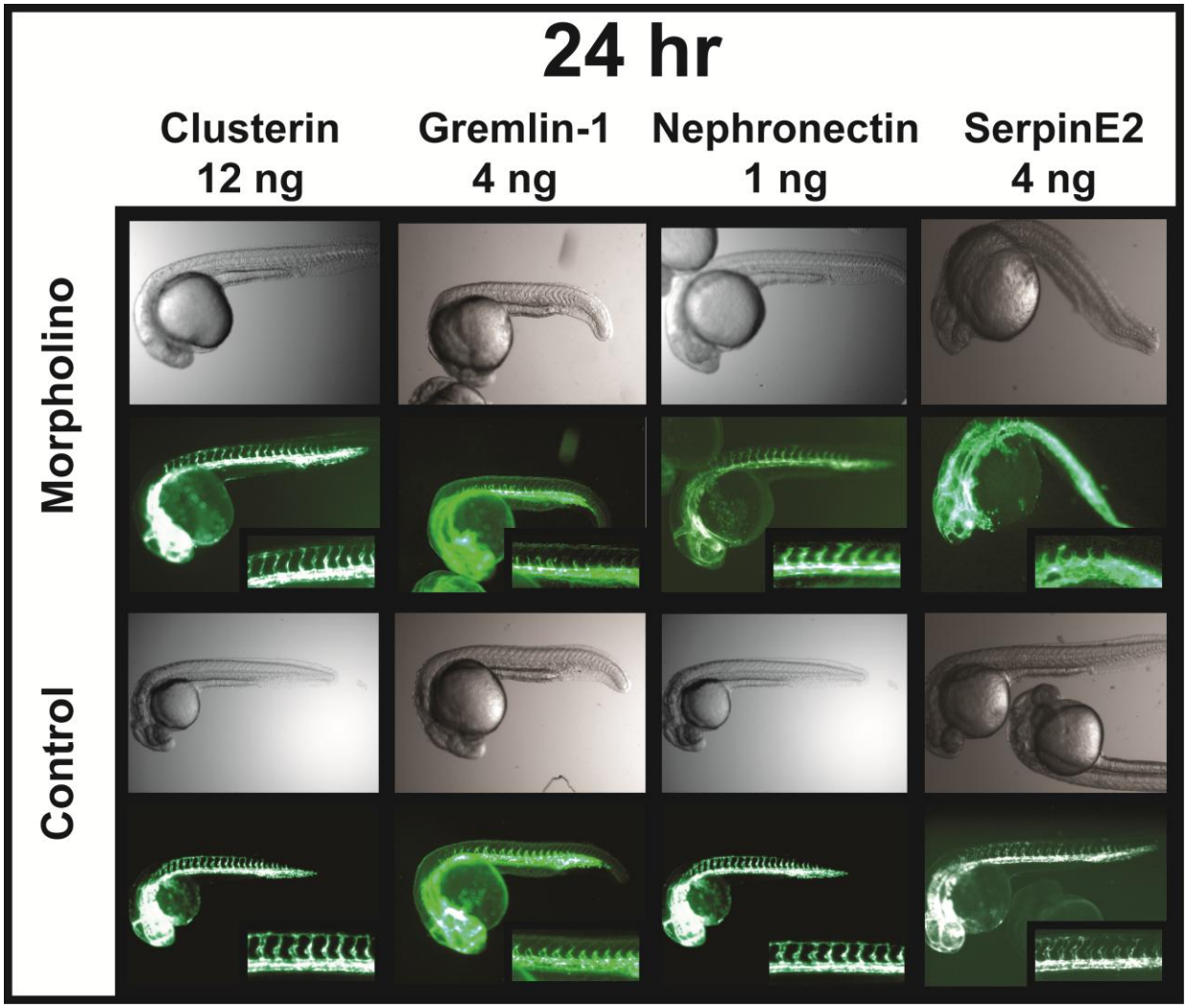

Figure 2. Observed effects of morpholino knockdowns

Bright field images demonstrate overall morphology of embryos at 24 hr. GFP images highlight the developing vasculature. Age matched control embryos are represented in the bottom two rows. Intersegmental vessels (ISV) are shown in lower left hand corner of images.

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