Studies on the Molecular Mechanisms of Fibulin-5 as a Negative Regulator of Angiogenesis

Wilson Ventura Chan

Department of Anatomy and Cell Biology
McGill University, Montreal
August 2010

A thesis submitted to McGill University in partial fulfillment
of the requirements of the degree of Master of Science

ABSTRACT

Angiogenesis is a tightly regulated process by which new blood vessels are formed from pre-existing blood vessels. It is a complex process modulated by a multitude of pro- and anti-angiogenic factors, some of which naturally include members of the extracellular matrix (ECM) due to the high degree of tissue remodeling necessary in angiogenesis. Fibulin-5 (DANCE, EVEC) is an integrin- and elastin-binding ECM protein that is highly expressed in blood vessels during development, is up-regulated upon vascular injury and is involved in the regulation of the transition of vascular SMCs from a quiescent to proliferative state. Recently, *Fbln5*$^{-/-}$ mice have been shown to have an increase in cutaneous blood vessels, increased migration and proliferation of endothelial cells (ECs) and a 30-fold increase in angiopoietin-1 (Ang-1) expression in vascular SMCs, which is a potent promoter of angiogenesis. In the present study, we sought to investigate the molecular mechanism(s) by which fibulin-5 might act as a negative regulator of angiogenesis, with a focus on the modulation of Ang-1 activity in ECs. We used solid-phase binding assays to determine the direct physical interactions that fibulin-5 might have with Ang-1 and its receptor Tie-2. We used solid-phase binding assays to determine the cell surface molecules that fibulin-5 might bind and confirmed an RGD dependency of fibulin-5 for integrin binding and showed a high affinity for heparin, suggesting a potential interaction with cell surface heparan-sulfate proteoglycans. We also sought to understand the downstream signaling mechanism of fibulin-5 and established a novel Akt-dependent pathway by which fibulin-5 can act as an anti-angiogenic molecule.

RÉSUMÉ

L'angiogenèse est un processus finement régulé par lequel de nouveaux vaisseaux sanguins se forment dans les vaisseaux sanguins préexistants. Il s'agit d'un processus complexe modulé par une multitude de facteurs pro-et anti-angiogéniques, qui incluent naturellement les membres de la matrice extracellulaire (MEC) en raison du degré élevé de remodelage tissulaire nécessaire dans l'angiogenèse. La Fibulin-5 (DANCE, EVEC) est une protéine de la MEC liant les intégrines et l'élastine. Elle est fortement exprimée dans les vaisseaux sanguins au cours du développement, est régulée à la hausse lors de lésion vasculaire et est impliquée dans la régulation de la transition des cellules musculaires lisses (CML) vasculaires d'un état de repos à un état de prolifération. Récemment, les souris *Fbln5 -/-* ont montré une augmentation de vaisseaux sanguins cutanés, une augmentation de la migration et de la prolifération des cellules endothéliales (CEs) et une augmentation de 30 fois de l'expression de l'angiopoïétine-1 (Ang-1) dans les CML vasculaires, un promoteur puissant de l'angiogenèse. Dans la présente étude, nous avons étudié le(s) mécanisme(s) moléculaire(s), par le(s)quel(s) la fibuline-5 pourrait agir comme un régulateur négatif de l'angiogenèse, plus précisément, sur la modulation de l'activité Ang-1 dans les CEs. Nous avons utilisé des essais de liaison en phase solide afin de déterminer les interactions physiques directes que la fibuline-5 pourrait avoir avec l'Ang-1 et son récepteur Tie-2. Nous avons également utilisé des essais de liaison en phase solide pour enquêter sur les molécules de surface cellulaire pouvant lier la fibuline-5 et nous avons confirmé que le motif RGD de la fibuline-5 sert à la liaison aux intégrines. De plus, la fibuline-5 a une grande

affinité pour l'héparine, suggérant une interaction potentielle avec la surface cellulaire par les protéoglycanes héparane-sulfate. Nous avons également cherché à comprendre le mécanisme de signalisation en aval de la fibuline-5 et nous avons établi une nouvelle voie dépendante d'Akt dans laquelle la fibuline-5 agit comme une molécule anti-angiogénique.

Ao Senhor,

mainha,

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e Elaine.

ACKNOWLEDGEMENTS

I would like to thank Dr. Elaine Davis for all her guidance, patience, understanding as well as her support, both academically and financially through her grant. I am proud to have Dr. Davis as my graduate mentor and I want to thank her for acting as such in every sense the word. Last but not least, I would like to thank her for the chance to be part of such a harmonious and vibrant lab. I would also like to thank Dr. Sabah Hussain and his team for providing the antibodies for the PI3K/Akt pathway and Angiopoietin-1, as well as for their advice and guidance in the signaling pathways of endothelial cells. I would like to thank the laboratory of Dr. Dieter Reinhardt for all their advice and help with many of the techniques involving protein purification and solid-phase binding assays, which were an integral component to my project. Specifically, I would like to thank Laetitia Sabatier of the Reinhardt laboratory for all the time she took to teach me how to do solid-phase binding assays, the long process of protein purification, and above all being a friend to me in the Department. I also want to thank Dr. Ling Li, our Research Associate for being such a wonderful source of knowledge when solving problems, for making sure the laboratory runs so efficiently and being a "smiling mother" to us all at the laboratory. I would also like to extend my gratitude to all the members of the Davis Laboratory past and present –Nihar, Kinsey, Amy, Sarah, Joseph, Tania, Andreas and Katie. Thank you for your help, delicious treats you brought the laboratory, friendship and great conversations in our student room. Thank you Vilayphone Luangrath of the Lamarche-Vane Laboratory for translating my abstract to French, it was beautifully done.

On a personal note, I would like to thank my family who has always been there for me, in highs, lows and mediums. I would like to thank my friends who along the way have made, and continue to make, my life more entertaining. I would like to thank the Marosy family for accepting me into my “Vermonter family”, you are wonderful. Finally, I want to thank the Universe for giving me such a winning hand, I feel blessed.

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LIST OF ABBREVIATIONS

Ang-1	Angiopoietin-1
ARMD	Age-Related Macular Degeneration
BM	Basement Membrane
BSA	Bovine Serum Albumin
cbEGF	Calcium-Binding Endothelial Growth Factor-Like Motif
CHO	Chinese Hamster Ovary Cells
DTT	Dithiothreitol
EC	Endothelial Cells
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ECM	Extra-cellular Matrix
EM	Electron Microscopy
FA	Focal Adhesions
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
Fbln5$^{-/-}$	Fibulin-5 Knockout Mouse
HEK	Human Embryonic Kidney Cells
HSPG	Heparan-Sulfate Proteoglycan
Hep-BSA	BSA-conjugated Heparin
HUVEC	Human Umbilical Vein Endothelial Cells
LOX	Lysis Oxidase
LOXL	Lysis Oxidase-Like
MAPK	Mitogen-Activate Protein Kinase
MMP	Matrix Metalloproteinase
NEAA	Non-Essential Amino Acids
NEM	N-ethylmaleimide
p-Akt	Phosphorylated Akt
P/S/G	Penicillin/Streptomycin/L-Glutamine
PBS	Phosphate Buffered Saline
PDGF	Platelet Dervived Growth Factor

p-Erk	Phosphorylated Erk
PECAM	Platelet Endothelial Cell Adhesion Molecule
PI3K	Phosphatidylinositol 3-Kinase
PMSF	Phenylmethanesulfonylfluoride
RGD	Arg-Gly-Asp Motif
RGE	Arg-Gly-Glu Motif
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SMC	Smooth Muscle Cell
SMF	Serum-Free Media
SPBA	Solid-Phase Binding Assay
TBS	Tris-Buffered Saline
TGF-β	Transforming Growth Factor β
VEGF	Vascular Endothelial Growth Factor
ε. AcA	ε-Aminocaproic Acid

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LITERATURE REVIEW

Blood Vessel Function and Formation

Blood vessels provide the body with a circulation system which supplies the needs of the tissues, such as providing nutrients, waste product removal, conducting hormones and in general, maintenance of an appropriate environment for optimal cell survival and function (1). Blood vessels are the first system to form in the embryo and constitutes the largest network structure in our body (2). Its importance is noted in the seriousness of the many pathologies which disturb the circulation system, such as atherosclerosis, diabetic retinopathies, rheumatoid arthritis or embolisms leading to tissue ischemia and necrosis (3). Blood vessels play important roles not only in the normal homeostasis of tissues, but they can also be central in the pathogenesis of diseases such as cancer. Indeed, tumours are only able to grow up to 1-2 mm^3 before their metabolic demands are restricted to the point where they switch to an angiogenic phenotype and draw sprouts from the surrounding blood vessels for nutrient supply (4). Blood vessel formation is a tightly regulated process. Once formed, the adult vasculature is for the most part quiescent, except for instances of menstrual cycling, wound healing and in cancer. Blood vessels generally form via two processes, vasculogenesis and angiogenesis.

Vasculogenesis

In vasculogenesis, individual endothelial progenitor cells called “angioblasts" arise *de novo* from embryonic tissue and assemble into chords of attached vascular endothelial cells (ECs) that go through many maturation steps before eventually

forming a young blood vessel (5). Vasculogenesis has been thought to be mainly restricted to early development, however, there is evidence suggesting vasculogenesis has been adapted in the adult where a small population of endothelial progenitor cells from the bone marrow can be brought into the circulation and incorporated into new blood vessels (6). Once established, the adult vasculature can remodel by angiogenesis, which is the formation of new vessels from pre-existing blood vessels.

Angiogenesis

Angiogenesis occurs in both early development and in adulthood. It is a tightly regulated and multi-factorial process influenced by the microenvironment and modulated by a multitude of pro- and anti-angiogenic factors (7). Angiogenesis, or the lack there of, occurs as the additive result of activating and inhibitory messages; a tipping balance first referred to by Judah Folkman over three decades ago as the "angiogenic switch" (8).

Angiogenic sprouting of blood vessels occurs in a series of steps, which can be generally categorized into EC activation and destabilization, proliferation and migration, and maturation/stabilization (Figure 1). Upon activation by a stimuli, ECs loosen their contacts with one another, their basement membranes (BM) and their supporting cells leading to destabilization and increasing vessel permeability (7). Increase in permeability is mediated by the formation of fenestrations and the redistribution of platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)−cadherin, a process which involves Src kinases (9). In this

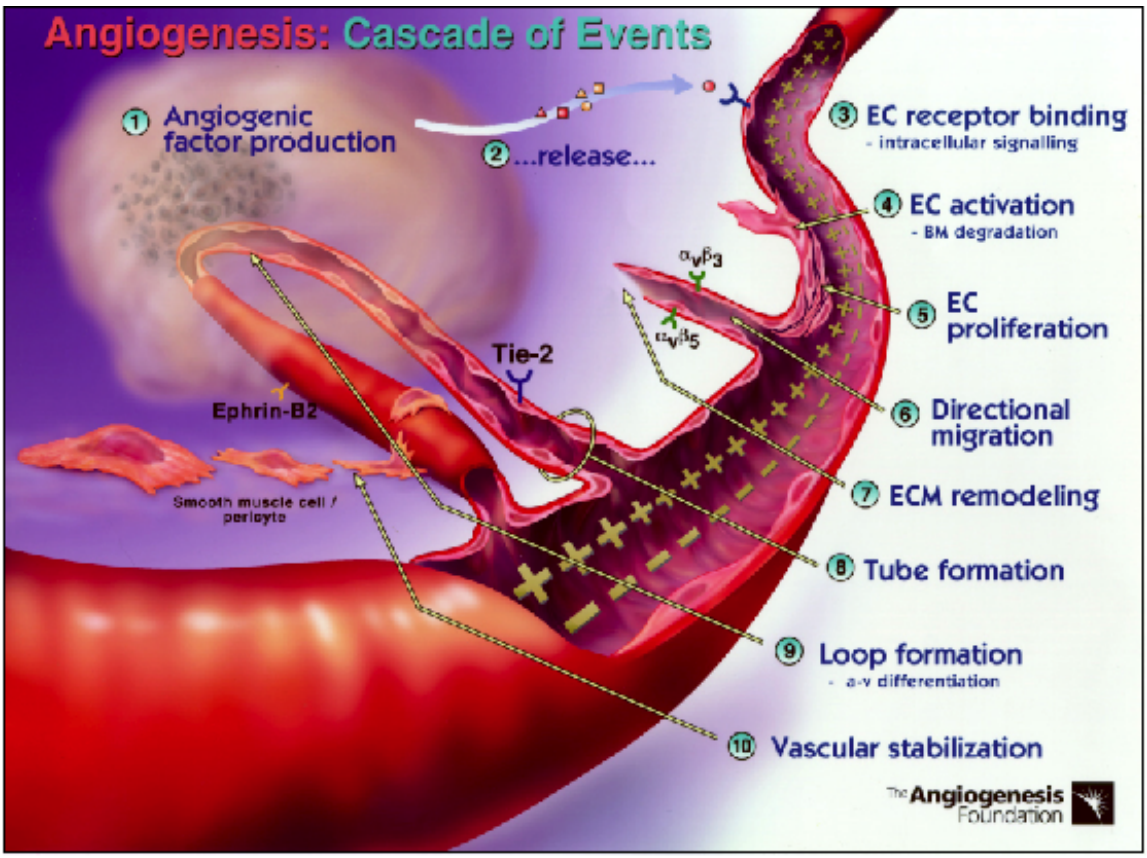


Figure 1: Angiogenesis: Cascade of Events. A simplistic scheme of the stages required for angiogenesis in 10 main steps. Following activation, ECs proliferate, migrate and form tubes which are stabilized by supporting cells such as smooth muscle cells/pericytes, which envelop the exterior of the vessels.

destabilized state, ECs are also more susceptive to angiogenic factors since they are able to diffuse with more ease into leaky vessels. Enzymes such as the matrix metalloproteinase (MMP), chymase or heparanase families also influence angiogenesis by degrading the surrounding matrix and activating or liberating growth factors, such as bFGF, VEGF, and IGF-1, that are sequestered within the extracellular matrix (ECM) (10). Activated ECs then migrate into the underlying ECM towards their angiogenic stimulus and proliferate. They then form a capillary lumen by aligning themselves in tandem (7). Once formed, the stabilization and maturation process of the vessel begins as EC proliferation and migration ceases and a new BM is assembled. Junctional complexes between ECs, their BM, and supporting smooth muscle cells (SMCs)/pericytes are also formed. This stage is referred to as arteriogenesis (11). In arteriogenesis, vessels become covered by SMCs, providing a muscular coating which allows vessels to accommodate the changing needs in tissue perfusion (11).

To date, an exhaustive number of inhibitors and activators of angiogenesis have been indentified. They vary in nature, some are diffusible cellular products others are structural components of the ECM. They also have effects on different or multiple steps of the angiogenic cascade of events. The following are a few examples of pro- and anti-angiogenic factors, however, a comprehensive discussion of these is beyond the scope of this review.

Inhibitors of Angiogenesis

Interestingly, a number of inhibitors of angiogenesis have been found to be proteolytic fragments of other molecules. Angiostatin for, example, is a proteolytic

fragment of plasminogen. It directly binds to ATP synthase on the surface of ECs which subsequently results in an intracellular pH drop, triggering apoptosis (12). Collagens are the main component in the ECM and the most abundant proteins in animals (13). Type IV collagen gives rise to canstatin and endostatin, whereas type XVIII collagen gives rise to tumstatin. The anti-angiogenic activity of canstatin has been shown to be mediated through inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, increasing apoptosis (14). Endostatin can block downstream signaling of $\alpha5\beta1$ integrin (15) and can bind cell surface heparan-sulfate proteoglycans (HSPGs), which are involved in growth factor signaling (16). Finally, tumstatin binds $\alpha v\beta3$ integrins on ECs, inhibiting proliferation and promoting apoptosis (17).

Promoters of Angiogenesis

Vascular Endothelial Growth Factor (VEGF) is to date the most well characterized and potent promoter of angiogenesis. It is a 34-46 kDa secreted glycoprotein and there are at least eight different isoforms resulting from alternative RNA splicing. It is thought to play an essential role in angiogenesis, and is alone able to induce EC proliferation, migration and survival both *in vitro* and *in vivo* without other factors (18-20). VEGF mediates its effects by binding the VEGF1, VEGF2 and VEGF3 receptors, which contrary to what was first thought, are not exclusively present on the EC surface but also on SMCs, monocytes, stem cells and bone-marrow derived hematopoietic cells (reviewed in (2)). Indeed, the best-known anti-angiogenic agents are VEGF inhibitors, such as Avastin, a VEGF analog which works to block the VEGF receptor (21).

Albeit a critical component, many molecules other than VEGF also have been identified as having important pro-angiogenic attributes, for example, fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), MMPs and the angiopoietins (22). FGF is a strong mitogen for many different cell types other than ECs and fibroblasts. FGF strongly binds to components of the ECM such as HSPGs, and is released during heparin or ECM breakdown (7). EGF is secreted by platelets, macrophages and monocytes, and although it has no direct effect on the endothelium, it is involved in tumour vascularization (7). PDGF is secreted by a variety of cell types including ECs, and results in cellular proliferation and migration by working in concert with transforming growth factor β (TGF-β) and EGF (7). The MMPs digest the surrounding ECM providing a more permissive environment for cellular migration and releasing growth factors sequestered within the ECM.

Angiopoietins

Angiopoietins are the ligands for the endothelial tyrosine kinase receptor Tie-2, reported to be found exclusively on ECs (23). Although Tie-1 is a close homologue of Tie-2, it is an orphan receptor with its *in vivo* ligand(s) yet to be identified (24). Angiopoietins are not produced by ECs, but rather are made by periendothelial-supporting cells such as SMCs and activate Tie-2 receptors on the neighbouring ECs in a paracrine fashion (25). They are comprised of Angiopoietin (Ang-1), Ang-2 and the mouse/human orthologs Ang-3/Ang-4. The angiopoietins contain a N–terminal sequence followed by a stretch of 50 amino acids termed the

N domain, a coil-coil domain that is that is responsible for multimerisation, and a C-terminal fibrinogen-like domain that is responsible for binding and activation of the Tie-2 receptor (7). The potential to activate the Tie-2 receptor lies within the fibrinogen-like domain and differences in this domain have been shown to account for the differences in Tie-2 activation between Ang-1 and Ang-2 (26). Mice deficient in Ang-1, Ang-2 or Tie-2 have similar, and severe vascular defects in development. These mice demonstrate embryonic lethal hemorrhaging as a result of leaky vessels from poor interaction between ECs and their supporting SMCs or pericytes (27). The function of Ang-3 and Ang-4 are far less characterized than those of Ang-1 and Ang-2.

Angiopoietin-1 vs Angiopoietin-2

Ang-2 has a similar binding affinity to Tie-2, but unlike Ang-1, it does not induce receptor activation, and thus acts as a natural antagonist to Ang-1 (24). Although Ang-2 has generally been thought of as an anti-angiogenic molecule due to its ability to antagonize Ang-1-induced phosphorylation of Tie-2, there are reports which suggest the contrary. Indeed, a 24-hour pre-treatment of Ang-2, followed by a short re-exposure, elicited phosphorylation levels of Tie-2 in ECs comparable to those by Ang-1 (28). Furthermore, similar to Ang-1, systemic over-expression of Ang-2 has been shown to positively affect TNF-α-induced corneal angiogenesis suggesting Ang-2 also promotes EC survival (29).

Ang-1 is widely expressed in the adult, especially in highly vascularized tissue, but not in tissues with low vascularization (30). Ang-2 on the other hand is only expressed in organs with constant blood vessel remodeling such as the ovary,

placenta and uterus (24). Ang-2 expression can be upregulated by VEGF, FGF and hypoxia and can be downregulated by Ang-1 and TGF-β (31). Ang-1 is secreted into, and binds to, the ECM via the fibrinogen-like and coil-coil domains. In this bound state, Ang-1 cannot bind the Tie-2 receptor (7). Ang-1 is released from its ECM stores and binds to Tie-2 once it comes into contact with the EC surface (32). Ang-2, on the other hand, does not bind nor localize to the ECM.

Anngiopoietin-1: Promoting Angiogenesis

Ang-1 acts as a pro-angiogenic molecule by promoting EC migration and organization, inhibiting apoptosis, and also by stabilizing blood vessels once they have formed. The exact pathways by which Ang-1 has these effects are yet to be fully elucidated, however, Ang-1 has been shown to activate two major pathways in ECs, the phosphatidylinositol (PI) 3-kinase/Akt and ERK/MAPK pathways (25). It is important to note that only tetrameric, or higher order forms of multimers can activate Tie-2 (26).

Ang-1 enhances EC migration on fibronectin and collagen in a Tie-2 dependent way; an effect which can be inhibited by its natural antagonist, Ang-2 (33). Ang-1 can also induce EC adhesion, spreading and migration in a Tie-2-independent manner through the integrins (34). There is much evidence for the importance of integrins in the Tie-2-independent activation pathways of Ang-1. Not only has the α5β1 integrin been shown to be constitutively associated with the Tie-2 receptor, demonstrating Tie2/α5β1 cross-talk, but Ang-1 activation specific to the α5β1 integrin appears to be essential for Ang-1-induced angiogenesis *in vivo* (35). Another essential feature of Ang-1 is its ability to promote EC organization into

more complex structures. This is supported by observations that Ang-1 triggers capillary-like tube formation of bovine aortic ECs cultured in collagen gels (36).

In general, Ang-1 has been considered to be unable to induce EC proliferation, but is able to protect ECs in culture from apoptosis induced by growth factor deprivation (37). However, some studies *in vivo* suggest otherwise. For example, mice treated with a recombinant form of Ang-1demosntrated vessel enlargement due to EC proliferation (38-40). In adults, constitutive Ang-1/Tie-2 signaling is thought to maintain ECs quiescence and thus prevents active growth (41).

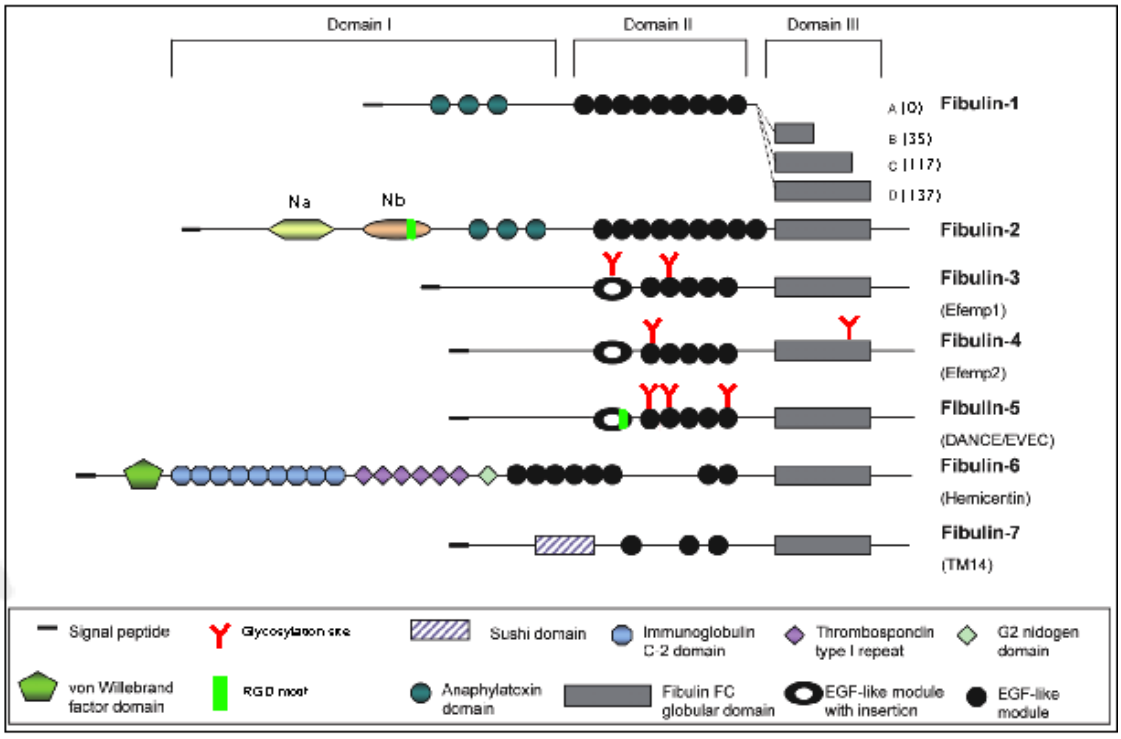
One of the most biologically important roles of Ang-1 is its ability to stabilize and mature newly formed blood vessels by inducing tighter junctions between ECs so they are not leaky, and by recruiting SMC, which envelop the EC layer. It has been shown that blocking the PDGF β receptor leads to vessels that lack supporting SMCs, remodel poorly and are leaky, however, administration of Ang-1 is able to rescue the vasculature from this leaky phenotype (27). It has also been suggested that the anti-permeability effect of Ang-1 may be partly mediated by up-regulation of expression of Krüppel-like factor 2 in ECs, a transcription factor involved in vascular quiescence (42). Other mechanisms also include the inhibition of endothelial nitric oxide synthetase, an enzyme that catalyzes the production of the vasodilator nitric oxide from L-arginine (43). Ang-1 also promotes the formation of cell-cell contacts, specifically through the tight junction protein zonula occludens protein-2 (44).

Angiopoietins and VEGF: Tipping the Angiogenic Balance

Ang-1 and Ang-2 are not able to induce new blood vessel formation alone, rather they tip the angiogenic balance by working in concert with growth factors, such as VEGF (45). Depending on the context of VEGF, Ang-2 can function as an antagonist or agonist of angiogenesis. When Ang-2 is administered in the presence of VEGF to the pupillary membrane (a transitional microvessel network which occupies the pupil in the eye during development), Ang-2 acts as pro-angiogenic molecule and promotes a rapid increase in capillary diameter, remodeling of the basal lamina, and new vessel growth (46). However, when VEGF activity is blocked addition of Ang-2 leads to EC death and vessel regression. This observation supports a model where Ang-1 promotes angiogenesis inducing vessel stability and maturation, whereby, Ang-2 can act either as a pro- or anti-angiogenic molecule depending on the presence of VEGF. The context-dependency of Ang-2 fits with the angiogenic process in general, which is complex and multi-factorial and depends on spatial and temporal context of many factors.

The Fibulin Family

The fibulins are a family of secreted ECM glycoproteins characterized by a tandem array of calcium binding EGF-like domains and a C-terminal common to all the fibulins. Since the discovery of the first fibulin in 1989, fibulin-1, the family has grown to include seven members shown schematically in Figure 2 (47). The fibulins are widely expressed ancient proteins that are highly conserved in evolutionary divergent species. They have a promiscuous range of binding partners and thus, are



(Modified from Vega et al., Cell. Mol. Life Sci., 66:1890-1902, 2009)

Figure 2: Schematic diagram of the fibulin protein family. Domain structures of the seven fibulins are indicated according to the legend at the bottom. Note that only fibulin-2 and fibulin-5 possess integrin binding RGD domains.

thought to act as intramolecular bridges which stabilize larger ECM structures (48). In fact, fibulins are named after their wide range binding ability with its name originating from the Latin word, *fibula*, which means clasp or buckle (48). The fibulins bind to one another and many additional molecules of the ECM found in basement membranes, elastic fibers, and other connective tissues [thoroughly reviewed in ref (49)].

Protein Structure

Fibulins contain modules generally grouped into domains I, II and III indicated in Figure 2. Domain I is the N-terminus and varies among the members with each fibulin having specific modules in this region. Domain II represents the central portion of the protein and contains a variable number of EGF-like domains that contain motifs which bind calcium, and thus are known as calcium binding EGF-like domains (cbEGF) (49). Domain III is the C terminal domain which is common to all fibulins. It is also specific to the fibrillin family of ECM proteins, and is termed the fibulin-type module (50).

Splicing Variability

Fibulin-1 has four gene products as a result of splicing variability in the C-terminal/domain III rendering it anywhere from 90-100 kDa (51). Fibulin-1C and -1D are the two major splice variants and are present in relatively equal amounts in most tissues (51). Variants A and B of fibulin-1 exist in humans, however, at very low levels (52-54). Fibulin-2 is the largest member in the family at 200 kDa due to an additional N-terminal region that is approximately 400 amino acids long and is not found in any other fibulin (51). The third cbEGF domain of fibulin-2 can be

absent or present due to alternative splicing, but differences in function between these variants have yet to be identified (54). Alternative splicing also gives rise to five fibulin-3 variants which may contain a portion of, or a complete lack of domain I (55). A variant of fibulin-4 lacking the signal sequence, a short peptide chain that targets to the rough endoplasmic reticulum in the secretory pathway, has also been reported (56). Fibulin-5 has alternatively spliced exons 1a and 1b in mouse and human, however, these variants only differ in the signal sequence and the mature protein remains the same (Dr. Hiromi Yanagisawa, University of Texas Southwestern Medical Center, personal communication).

Fibulin Subgroups: Large vs Short

The fibulins can be divided into two general subgroups based on their size and function, the large (fibulin-1, -2 and -6) and the short fibulins (fibulin -3, -4, -5 and -7) (57). The large fibulins are larger due to a higher number of cbEGF domains in their central portions and the presence of additional motifs, such as the anaphylatoxin modules in domain I of fibulin-1 and -2, which are involved in inflammation and parasitic defense (49). In the second group, fibulins -3, -4 and-5 are small (50-70 kDa) and similar in size, and are highly homologous to one another (51). Only fibulin-2 and fibulin-5 possess an Arg-Gly-Asp (RGD) motif that can bind integrin receptors on the cell surface. These are located on the N-terminus in fibulin-2 and on the 1st cbEGF domain in fibulin-5. Fibulin-7 has a Sushi domain in its N-terminal. This domain contains two disulfide bonds that are essential for maintenance of the tertiary structure of the protein. It is a common motif in protein-protein interactions and is also involved in blood coagulation (49, 58).

Unlike fibulin-2, which was found by comparative sequence analysis of mouse fibulin-1, the short fibulins were first discovered during attempts to discover new genes or implicated in already established disease processes (49). Fibulin-5 was discovered by subtraction hybridization in order to identify genes which modulate vascular SMCs from a quiescent, contractile state to a more primitive, proliferative phenotype (59). Fibulin-3 was first found to be overexpressed in senescent fibroblasts isolated from a Werner syndrome patient with premature aging (55), and fibulin-4 was found to be involved in stress response, cell proliferation and oncogenic activity (50, 60, 61). As a result, the short fibulins were given different and mostly unrelated acronyms at time of their discovery. Only later, once they were found to contain the fibulin C-terminal motif and a tandem array of cbEGF-like motifs, were they then recognized as fibulins (50, 59, 62). Fibulin-3 was first known as S1-5 or Efemp1 (55), Fibulin-4 as Efemp2 (50), Fibulin-5 as EVEC or DANCE (59, 63), Fibulin-6 as Hemicentin-1 (64), and finally, Fibulin-7 was also known as TM14 (62).

Fibulin-5

Fibulin-5 was first discovered in 1999 as a gene that is expressed in large blood vessels during development, and is up-regulated upon vascular injury. It also is involved in the regulation of the transition of vascular SMCs from a quiescent to proliferative state (59, 63). As one of the short fibulins, it is a 66 kDa glycroprotein that is secreted by various types of cells such as vSMCs, ECs and fibroblasts (57). Fibulin-5 is also known as developing arteries and neural crest EGF-like (DANCE) or

embryonic vascular EGF-like repeat-containing protein (EVEC) due its expression patterns when it was first discovered. *In situ* hybridization in mouse embryos revealed fibulin-5 to be expressed in developing arteries, in neural crest cell derivatives, cardiac tissue and a variety of other mesenchymal tissue (63).

Fibulin-5 seems to be an important protein that is involved in vascular regulation for it is expressed in the embryo and adult. In the adult, fibulin-5 is for the most part downregulated, except in the uterus where there is constant remodeling and angiogenesis occurring due to menstrual cycles (57). It is also localized in the heart, ovaries and colon as well as in the kidneys, pancreas, testis and lungs (49). Furthermore, fibulin-5 becomes reactivated during vascular injury such as in the neointima following balloon withdrawal and in atherosclerotic plaques in a mouse model for hypercholesterolemia (59, 63). In the vasculature, fibulin-5 is expressed in both large and small blood vessels and specifically colocalizes to the elastic lamina in the tunica media layer of the vessel wall (65). These observations suggest that fibulin-5 might be an important player involved in vascular regulation during vasculogenesis and/or angiogenesis.

Elastic Fibers

Elastic fibers are a major component of connective tissues in the body. They provide a scaffold for organs to form and are also an intimate part of the skin, lungs, blood vessels and other organs. They give these structures stretch and recoil abilities to sustain mechanical stress. There are two main components of elastic fibers, a core of amorphous insoluble elastin and a network of fibrillin-containing

microfibrils that surrounds it (66-68). In development, the microfibrils appear to form first, providing a scaffold on which the secreted 70 kDa tropoelastin monomers are deposited and assembled (66). Tropoelastin is secreted by a myriad of elastogenic cells including fibroblasts, lung alveolar cells, chondrocytes and vascular SMCs (65). Once secreted, these tropoelastin monomers coacervate or self-assemble into aggregates, which subsequently become integrated into the microfibril scaffold where they are cross-linked into an insoluble elastin polymer by the extracellular enzymes lysis oxidase (LOX) and lysyl oxidade like-1 (LOXL-1) (69, 70). Fibulin-5 localizes to the surface of elastic fiber *in vivo* at the interface between elastin and microfibrils (71).

Fibulin-5 Knockout Mice (*Fbln5*$^{-/-}$)

Although fibulin-5 gene knockout (*Fbln5*$^{-/-}$) mice live to adulthood, they demonstrate an elastinopathy with aberrant elastic fiber formation resulting in loose skin, emphysematous lungs and tortuous blood vessels (72, 73). The elastic fibers in these mice display an aberrant phenotype as seen by electron microscopy (EM) in which they demonstrate elastin globules located outside the microfibril network instead of being incorporated into the scaffold (71). Despite the aberrant elastic fibers, there is no indication of aneurysms, and other organs such as the kidney, intestine, colon, pancreas, liver, uterus, ovary, testis and skeleton are normal (73). However, 91% of *Fbln5*$^{-/-}$null female mice develop pelvic organ prolapse, usually anal in nature, by 6 months of age (74, 75).

Fibulin-5 and Elastic Fibers

Fbln5$^{-/-}$ mice show a 16% decrease in desmosine, an elastin-specific crosslink, when compared to wildtype animals (73). In addition to the fact that fibulin-5 preferentially binds tropoelastin, but not polymerized elastin *in vitro*, evidence suggests that fibulin-5 plays role in the crosslinking of tropoelastin bundles into insoluble aggregates (76). Recombinant fibulin-5 has also been shown to inhibit the maturation process of tropoleastin coacervates thereby preventing the formation of aggregates (77, 78). This suggests that the large elastic globules adjacent to the microfibrils in the *Fbln5*$^{-/-}$ mouse might be the result of excessive maturation which is otherwise regulated by fibulin-5 (65). The binding of fibulin-5 to tropoelastin monomers can be completely inhibited with 10 mM EDTA, suggesting that this binding is mediated via the cbEGF domains and/or that the binding of Ca^{2+} to this region provides a conformation which is conducive to binding tropoelastin (73). Fibulin-5 has an additional elastin-binding domain in its C-terminal; however, this domain is unable to bind elastin alone *in vitro* but rather cooperates with the cbEGF domains to bind tropoelastin (76).

In addition to binding tropoelastin (73), fibulin-5 also binds to other key components involved in elastic fiber formation such as fibrillin-1 (51) and LOXL-1 (77, 79). By binding fibrillin-1, it is perhaps able to act as a bridge anchoring tropoelastin aggregates onto the fibrillin-microfibril network. Indeed it is located in the right location on the elastic fiber, at the interface between elastin and microfibrils (71). The elastin aggregates formed beside the microfibril networks in the *Fbln5*$^{-/-}$ mice is perhaps a result of the lack of fibulin-5 acting as a bridging

molecule between the two. Fibulin-5 is able to bind LOXL-1 via its C-terminal indicating the possibility that fibulin-5 might play a role in regulating the cross-linking activity of the enzyme (65). Indeed elastic fibers in skin of *Fbln5*$^{-/-}$ mice displayed abundant immunostaining using an antibody specific to the pro-peptide of LOXL-1, which needs to be cleaved in order to activate the enzyme, whereas the wildtype animals show none (71). Furthermore, the binding of LOXL-1 to the C-terminal of fibulin-5 has been shown to be required for the tethering of the enzyme to elastic fibers in the skin (76). Thus by binding tropoelastin and the fibrillin-microfibril network, fibulin-5 could facilitate the cross-linking of tropoelastin by LOXL-1 during the initial fiber assembly (76).

The loose skin found in the *Fbln5*$^{-/-}$ mice resembles cutis laxa syndrome in humans, in which the skin is inelastic and hangs loosely in folds. Fibulin-5, along with fibulin-2, are the only fibulins which can bind integrins on the cell surface through an RGD motif. Fibulin-5 binds $\alpha5\beta1$, $\alpha4\beta1$, $\alpha v\beta3$, $\alpha v\beta5$ and $\alpha9\beta1$ integrins on ECs and SMCs in an RGD-dependent manner (72, 80). Because fibulin-5 also interacts with elastic fibers, it has been proposed that it may function to stabilize the interaction of elastic fibers with cells in the skin, lung and vasculature (49). On the other hand, fibulin-5 RGE mutant mice have been generated in which the Aspartic Acid (D) in the RGD motif of the protein has been replaced by Glutamate (E). This prevents the protein from binding the cell surface integrins (65). These mice demonstrated no abnormal elastic fiber phenotype suggesting that the RGD binding motif of fibulin-5 might not be necessary for the organization and assembly of elastic fibers *in vivo* (65).

Angiogenesis in the Fbln5$^{-/-}$ mouse

In addition to the elastinopathies observed in the *Fbln5*$^{-/-}$ mice, knockout animals also have an excess of cutaneous blood vessels and vascular sprouting from the larger systemic vessels (72, 73). This indicates that fibulin-5 not only plays a role in ECM assembly and elastic fiber development, but also functions as a negative regulator of angiogenesis. This anti-angiogenic function of fibulin-5 is further supported by previous observations *in vitro* where mouse brain ECs expressing fibulin-5 showed reduced tubulogenesis, proliferation and migration compared to control cells (81). In addition, *Fbln5*$^{-/-}$ mice showed an exaggerated vascular remodeling response to vascular injury induced by carotid artery ligation, with severe neointima formation and thickening of the adventitia (82). Furthermore, when polyvinyl alcohol (PVA) sponges were implanted subcutaneously in mice to assess fibrovascular invasion, EC invasion into sponges of *Fbln5*$^{-/-}$ was higher than in wildtype animals (83). In this same study, in order to investigate possible downstream mediators of the observed anti-angiogenic activity of fibulin-5, Ang-1 was localized within and around sponges by immunofluorescence. Sponges removed from *Fbln5*$^{-/-}$ animals demonstrated a much higher immunoreactivity for Ang-1 when compared to wildtype animals indicating that in the absence of fibulin-5, Ang-1 (a potent promoter of angiogenesis) is upregulated. Furthermore, vascular SMCs isolated from wildtype and *Fbln5*$^{-/-}$ aorta were cultivated and analyzed for expression of Ang-1 gene and other pro-angiogenic factor such as VEGF, FGF-1 and PDGF-A by qRT-PCR (83). Consistent with the immunostaining results, *Fbln5*$^{-/-}$ SMCs showed a 30-fold increase in Ang-1 gene expression when compared to

wildtype animals, whereas expression of the other pro-angiogenic factor were unchanged. This evidence suggests that fibulin-5 acts as an endogenous inhibitor of angiogenesis and possibly mediates this effect by modulating the activity and/or expression of Ang-1.

Fibulin-5 in Disease

As ECM proteins, the fibulins are involved in many pathologies arising from connective tissue disorganization. As mentioned above, fibulin-5 is essential for elastic fiber assembly and organization, with *Fbln5*$^{-/-}$ mice displaying a loose skin phenotype similar to the human cutis laxa syndrome. Fibulin-5 has also been implicated in age-related macular degeneration (ARMD), a leading cause of irreversible vision loss affecting approximately 25 million people worldwide (84). The loss of vision in ARMD is progressive and occurs due to degeneration and excessive neovascularization within the retina at the macula. Deposits of protein and lipid develop beneath the retinal pigmented epithelium and an elastin containing structure called Bruch's membrane. Abnormalities in this membrane are a key player in the pathogenesis of ARMD. Although ARMD is a multi-factorial disease with many genetic and environmental inputs, 1.7% of ARMD patients were found to have missense mutations in fibulin-5 equating to many hundreds of thousands of ARMD patients worldwide (85). Fibulin-5 fits appropriately in the model of the ARMD disease process as it has been proven to cause elastic and vascular abnormalities. *Fbln5*$^{-/-}$ mice also exhibit an elevated pulse pressure as well as decrease in the extensibility of the aorta compared to wildtype animals,

indicating that the compromised elastic fibers leads to stiff vessels that are less compliant in the homeostasis of blood pressure (57).

Fibulin-5 also appears to play an important role in tumourigenesis, although results are variable and context specific. Fibulin-5 expression is downregulated in the majority of metastatic cancers, especially those from the kidney, breast, ovary and colon (86). However, it is important to note that fibulin-5 expression has largely been based on whole tissue analyses with no distinction between tumor-specific and host-derived fibulin-5 (57). Nevertheless, studies have revealed fibulin-5 to suppress tumour growth and angiogenesis when overexpressed in HT1080 tumour cells injected subcutaneously into mice (87). However, in direct contradiction to these observations, overexpression of fibulin-5 in human HT1080 fibrosarcoma cells resulted in elevated tumourigenicity by increasing DNA synthesis, migration toward fibronectin, and invasion through synthetic basement membranes (86). These contradictory observations are perhaps a result of variability due to experimental differences (i.e. *in vivo* vs *in vitro*) or cell and context-specific differences in fibulin-5 activity, which will be discussed later in this review.

Effects of Fibulin-5 on Cell Processes

Fibulin-5 has been shown to have a myriad of effects on different cell types, most of which seem to be anti-angiogenic in nature. Overexpression or treatment of ECs with recombinant fibulin-5 was able to inhibit their angiogenic sprouting ability by inhibiting proliferation and migration into Matrigel matrices (81). This is further supported by the observation that fibulin-5 expression is downregulated during EC

tubulogenesis, further implicating it is a negative regulator of angiogenesis (81). Fibulin-5 treatment was also able to induce expression of thrombospondin-1, an antiangiogenic factor (81).

Fibulin-5 also has effects on ECs in concert with other molecules. Fibulin-5 has been shown to be a downstream gene target of TGF-β with its expression being upregulated in ECs following treatment with TGF-β (81). However, one must practice caution when interpreting results involving TGF-β for it has been shown to be either pro- or anti-angiogenic depending on the concentration at which it is administered (88, 89). Nevertheless, TGF-β-induced expression of fibulin-5 in ECs was inhibited by coadministration of VEGF, a very potent promoter of angiogenesis (81). Furthermore, fibulin-5 was able to inhibit the mitogen-activated protein kinase (MAPK) activity of VEGF, specifically that of p38 and Erk1/2 (81). The MAPK activity downstream of VEGF has been shown to be important for EC migration and invasion (90-92). Additionally, exogenous fibulin-5 incorporated into matrigel plugs can diminish the ability of bovine FGF-mediated vascular invasion when implanted into wildtype mice (93). Finally, through experiments implementing mutant RGE fibulin-5, it was shown that the ability of fibulin-5 to modulate EC response was independent of its integrin binding RGD motif as both the RGE mutant and full-length proteins were equally effective in inhibiting VEGF-mediated EC invasion and p38 MAPK phosphorylation (81).

Fibulin-5 has been shown to provide attachment for other cell types, such as SMCs via the $\alpha5\beta1$ and $\alpha4\beta1$ integrin receptors in an RGD dependent-manner (80). In spite of this, fibulin-5 was deemed unable to activate these integrins since it did

not induce stress fiber formation in SMCs, unlike fibronectin which binds and activates these integrins (80). Similar to ECs, expression of fibulin-5 in some cases can be upregulated as a result of TGF-β treatment (86). In addition, fibulin-5 overexpression in fibroblasts resulted in an increase in DNA synthesis indicating that it is a positive regulator of fibroblast growth (86). Fibulin-5 was also able to induce the MAPK activity of p38 and Erk1/2 activation in fibroblasts alone and enhance that of TGF-β when coadministered (86). Results suggest that fibulin-5 has a differential affect on migration and proliferation of ECs and fibroblasts, being a negative and positive regulator of these processes depending on the cell type. This is further supported by evidence that *Fbln5*$^{-/-}$ mice which demonstrate a normal rate of wound healing despite increased vascularization of the wound bed (94).

Exposing Fibulin-5: An Issue of Context Specificity?

In addition to the variable effects of fibulin-5 on tumour development, the wound healing model is another instance where the observed effects of fibulin-5 can be contradictory. As previously mentioned, *Fbln5*$^{-/-}$ show normal rates of wound healing although there is an apparent increase of angiogenesis in the wound bed when compared to wildtype animals (94). In this study, fibulin-5 was found in its native state, properly assembled into the ECM of the wildtype animals. In direct contrast to these results, fibulin-5 was found to promote wound healing *in vivo* (95). However, in this study the wounds were administered an exogenous preparation of fibulin-5 (95). These observations suggest that the natural role of fibulin-5 in tissues may be different than that of added exogenous or overexpressed fibulin-5 (83).

Although many differences in experimental conditions, such as those technical in nature, can account for this variation, one in particular must be considered when studying ECM molecules; exogenous exposure to the isolated protein or endogenous exposure to the protein as part of a properly assembled matrix as found in nature.

INTRODUCTION

The fibulins are a family of ECM proteins characterized by a tandem array of calcium binding EGF-like domains and a C-terminal domain common to all the fibulins, called the fibulin-type module (50). Since the discovery of fibulin-1 over three decades ago, several other fibulins have been identified expanding the family to seven members (47, 49). The fibulins are widely expressed, have been implicated in many processes such as tissue organogenesis, vasculogenesis, and tumourigenesis, and have a wide array of binding partners, most of which are also members of the ECM [reviewed in (49)]. The fibulins can be divided into two general subgroups based on their size and function, the large and short fibulins (57). The short fibulins, which comprise fibulin-3, -4 and -5, are highly homologous to one another and have been implicated in a variety of phenotypes related to elastic fiber malformation (65).

Fibulin-5, also known as EVEC or DANCE, was first identified in 1999 as a gene involved in the regulation of the transition of vascular SMCs from a quiescent to a proliferative state. It is strongly expressed in large blood vessels during development and up-regulated in vascular injury (59, 63). Fibulin-2 and fibulin-5 are the only fibulins which can bind integrins via an RGD motif (63). Although viable, *Fbln-5*$^{-/-}$ mice have aberrant elastic fiber formation and elastin aggregates instead of normal elastic fibers where elastin molecules are properly organized onto a microfibril network (72, 73). As a result, *Fbln-5*$^{-/-}$ mice display phenotypes of loose skin resembling the human cutis laxa disorder, tortuous vessels and emphysematous lungs (72, 73).

Interestingly, the *Fbln-5*$^{-/-}$ mouse also displayed an increase in cutaneous blood vessels (83). This was further confirmed by the observation that *Fbln-5*$^{-/-}$ mice displayed a higher number of small, tortuous branches off the long thoracic artery when compared to wildtype animals (83). Furthermore, PVA sponges implanted subcutaneously in *Fbln-5*$^{-/-}$ mice to assess fibrovascular invasion demonstrate a higher number of invading sprouts from surrounding vessels as well as a significant increase of vascular invasion into sponges when compared to wildtype mice (83). *Fbln-5*$^{-/-}$ skin also demonstrate higher fluorescence staining for Ang-1 and an approximately 30-fold increase in Ang-1 gene expression in cultured *Fbln-5*$^{-/-}$ SMC when compared to wildtype animals (83).

Ang-1 is a potent pro-angiogenic molecule which promotes EC migration and organization, inhibits apoptosis and promotes blood vessel stability. Thus, we hypothesized that fibulin-5 functions as an endogenous inhibitor of angiogenesis by modulating the activity and/or expression of Ang-1. We sought to characterize the direct physical interactions fibulin-5 might have with molecules in the Ang-1/Tie-2 signal transduction pathway. We also characterized the downstream signaling pathways activated by fibulin-5 and Ang-1 in human umbilical vein endothelial cells (HUVECs) and discovered the novel finding that fibulin-5 blocks Ang-1 mediated Akt phosphorylation. Finally, we used recombinant fibulin-5 mutant protein to confirm the RGD dependent cell binding properties of fibulin-5 and identified a strong affinity of fibulin-5 to heparin, which suggests a possible interaction between fibulin-5 and cell surface HSPG.

MATERIALS AND METHODS

1. Protein Purification of Recombinant Fibulin-5 Full Length and RGE mutant

1.1 Cell Expansion and Media Collection

Vectors containing a construct of rat fibulin-5 full length or RGE mutant fibulin-5 (generously provided by Dr. Hiromi Yanagisawa, UT Southwestern), were stably transfected by previous lab members into HEK 293 cells or CHO cells, respectively. The transfected cells were expanded and plated onto 8 triple-layer flasks in media containing 10% fetal bovine serum (FBS) (Invitrogen, Montreal, QC), 1% non-essential amino acids (NEAA) (Wisent, St. Bruno, QC), and 1% Penicillin, Streptomycin and L-Glutamine (P/S/G) (Wisent). Cells were kept under selection with either 100ug/mL Neomycin G418 (Invitrogen) in the case of full-length fibulin-5, or with 250ug/mL Hygromycin B (Invitrogen) for RGE mutant fibulin-5. HEK 293 cells were incubated with Dulbecco's Modified Eagle's Medium (Invitrogen) and CHO cells were incubated in HAM's F-12 (Wisent) media. Once confluent, each triple layer flask was washed twice with 150 mM NaCl in 20mM Hepes buffer [pH 7.4] to remove serum before starting collections. A total of 4L of serum-free media (SFM) with 1% NEAA, 1% P/S/G was collected in increments of 500 mL every second day for 3 weeks. Following each collection, the conditioned media was centrifuged at 6,000 *g* for 15 min at 4°C to remove cellular debris. The supernatant was collected and treated with 500 μL of 0.1 M PMSF to prevent protein degradation and frozen at -80°C.

1.2 Protein Concentration, Dialysis and Purification

Verification of the expression and secretion of the recombinant fibulin-5 proteins was done by Western blot analysis (see below). For protein purification, the SFM was filtered using a 5 μm membrane (Millipore, Bedford, MA, Cat. No. SMWP04700) under vacuum and concentrated by pressurized ultra-filtration using a 10,000 MWCO membrane (Millipore, Cat. No. PLA09005) to ~50 mL using Amicon stirred ultrafiltration cells (series 8000 system, Millipore) at 4°C. The concentrate was dialyzed using a Spectra/Por membrane MWCO 12-14,000 (Spectrum, Rancho Dominguez, CA, Cat. No. 132680) against 500 mM NaCl in 20 mM Hepes Buffer [pH 7.2], twice over a period of 24 hrs at 4°C. The dialyzed concentrate was centrifuged at 11,000g for 16 min at 4°C and the supernatant was passed through a 1mL chelating HisTrap affinity column (GE Healthcare, Baie d'Urfe QC) using an Äkta purification system (Amersham Biosciences, Baie d'Urfé, QC). The bound protein was eluted with a linear gradient of 500mM Imidazole, 500mM NaCl in 20 mM Hepes [pH 7.2]. Samples (20 μL) from the fractions were separated by SDS-PAGE followed by Coomassie Blue staining to determine the presence of recombinant fibulin-5 protein. Fractions containing pure recombinant protein were then pooled and dialyzed using a Spectro/Por membrane MWCO 12-14,000 (Spectrum, Cat. No. 132676) against 2 mM EDTA in TBS [pH 7.4], twice over a period of 24 hours at 4°C. The dialyzed pooled fractions were verified by SDS-PAGE, followed by Coomassie Blue staining and Western blot analysis (see below). Protein concentration was determined using a BCA assay kit (Pierce, Rockford, IL) and a micro-ELISA plate reader (Beckman Coulter, Mississauga, Ontario). Pooled fractions were aliquoted

and stored at -80°C. These were the stores of recombinant full-length and RGE fibulin-5 used for ELISAs, Solid-Phase Binding Assays, and Cell Signaling Experiments.

1.3 Verification of Protein Production by Western Blot

To verify that the protein present in the fractions was purified recombinant fubulin-5 and to detect if any degradation products were present, a Western blot was performed. SFM media (200 μL) was collected from the triple layer flasks and precipitated in 1 mL cold acetone at -20°C for 20 min. The solution was centrifuged at 13,200 *g* for 10 min at 4°C. The supernatant was then discarded and the pellet was allowed to dry for 10 min under the fume hood. The pellet was then solubilized in 30 μL of 0.1 M DTT in Laemmli buffer, heated for 95°C for 5 min and then separated, transferred and probed with an IgG purified fibulin-5 antibody as described below (see below).

2. Rapid Fibulin-5 Antibody IgG Purification

Protein A/G Plus-Agarose in PBS (75 μL) (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 250 μL of anti-sera raised against full-length rat fibulin-5 protein and rotated overnight at 4°C. The following day, the agarose beads were washed 3 x with 500 μL of cold PBS and centrifuged at 2000 *g* for 5 min between each wash. The supernatant was discarded and 75 μL of 0.1 M glycine [pH 3.0] was added. The supernatant was then rotated for 20 min at 4°C, followed by centrifugation at 2000 *g* for 5 min. Finally, the supernatant was transferred to a fresh Eppendorf tube and 7.5 μL of 1 M Tris [pH 7.4] was added. This IgG purified

antibody was used for Western Blotting, solid-phase binding assays, ELISA and cell-binding assays.

3. Western Blotting

3.1 Sample Preparation

Confluent 6-well cell culture plates were scraped on ice with 70 μL of cold lysis buffer per well. TETN 250 lysis buffer consisted of 25 mM Tris-HCl [pH 7.5], 5mM EDTA, 250 mM NaCl, 1% Triton-X 100, 5mM PMSF, 10 mM epsilon-aminocaproic acid (ε.AcA), 5 mM EDTA, 5 mM NEM, 200 μM Sodium Orthovanadate, 2 μL/mL Leupeptin and 1 μL/mL Pepstatin. Cell lysates were frozen at -80°C and then thawed at 4°C to increase cellular lysis. The lysates were then centrifuged at 13,200 *g* for 10 min at 4°C to remove cell debris and the supernatant was collected. Protein concentration was determined as mentioned previously and samples were heated to 95°C for 5 min in Laemmli sample buffer containing 0.1 M DTT.

3.2 Protein Separation and Transfer

Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membrane (BioRad, Mississauga, Ontario) using 20 mM Tris, 150 mM glycine and 20% methanol for 1 hr at 80 V. Non-specific binding sites were blocked with 5% dry non-fat milk in TBS for 1 hr at RT.

3.3 Probing, Stripping, and Antibodies

Following blocking, membranes were washed 1 x 5 min with 0.2% Tween-20 in TBS and incubated with primary antibody (see table) in 5% BSA in TBS containing 0.1% sodium azide overnight at 4°C. The following day, the membrane was washed 3 x 10 min with 0.2% Tween-20, incubated with peroxidase-conjugated

Affini Pure® goat anti-rabbit IgG (Jackson Immune Research, West Grove, PA, USA) secondary antibody diluted in 0.2% Tween-20 in TBS for 1 hr at RT, washed 3 x 10 min with 0.2% Tween-20 and incubated with Supersignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) before exposure to film. Membranes were stripped using Re-Blot Plus Mild Solution 10X (Millipore) at 1x dilution in distilled water with vigorous shaking at room temperature (RT) for 10 min. The blot was then washed 2 x 5 min with 0.2% Tween-20 in TBS, re-blocked, and re-probed as described above.

Table 1: Summary of Antibodies used for Western Blots.

Antibody	Source	Isotype	Application & Dilution
Phospho-Erk 1/2 (Thr202/Tyr204)	Cell Signalling, Pickering, ON *Cat. No. 9101*	Rabbit IgG	WB = 1:5,000
Erk	Cell Signalling, Pickering, ON *Cat. No. 4695*	Rabbit IgG	WB = 1:5,000
Phospho Akt (Ser 473)*	Cell Signalling, Pickering, ON *Cat. No. 5171*	Rabbit IgG	WB = 1:1,000
Phospho Akt (Thr 308)*	Cell Signalling, Pickering, ON *Cat. No. 5056*	Rabbit IgG	WB = 1:1,000
Akt*	Cell Signalling, Pickering, ON *Cat. No. 9272*	Rabbit IgG	WB = 1:1,000
Fibulin-5	Davis Lab, McGill University, QC (raised to full-length protein)	Rabbit IgG	WB = 1:10,000 SPBA = 1:1,000
GAPDH	Cell Signalling, Pickering, ON *Cat. No. 2118*	Rabbit IgG	WB = 1:1,000
Tie-2	Calbiochem, Darmstadt, Germany *Cat. No. 610205*	Mouse IgG	ELISA = SD
Ang-1*	R&D Systems, Minnepolis, MN *Cat. No. 171718*	Mouse IgG	ELISA = SD SPBA = 1:300

WB = Western Blot SPBA = Solid-Phase Binding Assay SD = Serial Dilution

* Generous gifts from Dr. Sabah SN Hussain, McGill University, Montréal, QC.

4. Cells, Reagents and Cell Signaling Experiments

HUVECs obtained from Clonetics (San Diego, CA) were provided by Dr. Hussain (McGill University) and cultured in MCDB*131 medium (Wisent) with 20% FBS, 30 μg/mL Endothelial Mitogen (Biomedical Technologies, Stoughton, MA, Cat. No. BT-203), 1% NEAA's, 1% P/S/G, 100 μg/mL Heparin (Sigma-Aldrich, Oakville, Ontario) and 500 ng/mL of anti-fungal Amphotericin B (MP Biomedicals, Solon, OH). After reaching confluency, HUVECs were trypsinized (0.025% trypsin and EDTA 0.01%) and plated at a density of 10,000 cells/cm^2 (100,000 cells/well) onto 6-well tissue culture plates. HUVECs used for experiments were no older than passage 6. Once confluent (within 2-3 days), cells were not washed before beginning 6 hr SFM starvation as outlined in Figure 3. The 20% FBS media was removed and 2 mL of SFM MCDB*131 were placed onto each well. This allowed cells to be in starvation mode, but just enough serum present to prevent Erk mediated stress response in HUVECs (data not shown). At the 5 hr starvation point, the SFM was replaced with 1 mL of SFM containing recombinant full-length fibulin-5 protein. Control wells and wells to be treated with recombinant Ang-1 (R&D Systems, Minneapolis, MN Cat. No. 923-AN-025) had the 2 mL of SFM replaced with 1mL of fresh SFM only. Finally, at the 5 hr and 45 min time point, designated wells were treated with recombinant Ang-1 for the last 15 min at concentrations as indicated in the results. At 6 hr, the experimental end point was reached and wells were rapidly washed with 2 x cold PBS and the whole plated was frozen at -80°C until ready for lysing, collection, and sample preparation (see above).

5. Cross-Reactivity ELISA

ELISAs were performed prior to solid-phase binding assays to ensure saturated protein coating onto the plates and to test for antibody cross-reactivity. Full-length recombinant fibulin-5 protein, recombinant human Tie-2/Fc chimera (R&D, Cat. No. 313-TI-100), and recombinant Ang-1 (R&D, Cat. No. 923-AN/CF) were coated overnight at 4°C onto 96-well plates (Nalge Nunc International, Rochester, NY) in 100 μL of 4 mM $CaCl_2$ in TBS at a concentration of 10 μg/mL, 4.0 μg/mL and 100 ng/mL, respectively. The following day, plates were washed with 1 x 5 min washing buffer (20mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM $CaCl_2$ and 0.05% Tween-20) and blocked for 1 hr at RT with 100 μL of 5% non-fat milk in TBS. After blocking, plates were washed 3 x 5 min with washing buffer, and incubated for 2 hr at RT with 50 μL/well of 1:2 serial dilutions of primary antibodies in 2% non-fat milk in TBS. Primary antibodies consisted of the polyclonal rabbit IgG fibulin-5 (Davis Lab), and monoclonal mouse IgG antibodies for Tie-2 (Calbiochem, Darmstadt, Germany) and Ang-1 (R&D) (see table 1). Plates were then washed 3 x 5 min with washing buffer and incubated with 50 μL/well of 1:800 dilution of secondary antibody, either peroxidase-conjugated Affini Pure® goat anti-rabbit IgG or Goat Anti-Mouse IgG (Jackson Immune Research) in 2% non-fat milk in TBS for 1.5 hr at RT. Plates were then washed 3 x 5 min with washing buffer and colour development was performed with 100 μL/well of 1 mg/mL 5-aminosalicylic acid in 20 mM phosphate buffer [pH 6.8], including 0.045% (v/v) H_2O_2 for 3-5 min and stopped by adding 100 μL/well of 2 M NaOH. Colour absorption measurements were taken at 490 nm using a micro-ELISA plate reader (Beckman Coulter).

6. Solid-Phase Binding Assay

Recombinant human Tie-2/Fc chimera, recombinant Ang-1, Hep-BSA, BSA, tropoelastin (generous gift from Dr. Robert P. Mecham, Washington University School of Medicine, St. Louis, MO) and fibronectin (Sigma-Aldrich, Cat. No. F1141) were coated in triplicates overnight at 4°C onto 96-well plates in 100 μL of 4 mM $CaCl_2$ in TBS at a concentration of 4.0 μg/mL, 200 ng/mL, 10 μg/mL, 10 μg/mL, 10 μg/mL, and 10 μg/mL, respectively. The following day, plates were washed with 1 x 5 min washing buffer (20mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM $CaCl_2$ and 0.05% Tween-20) and blocked for 1 hr at RT with 100 μL of 5% non-fat milk in TBS. After blocking, plates were washed 3 x 5 min with washing buffer, and incubated for 1 hr at RT with 50 μL/well of 1:2 serial dilutions of recombinant fibulin-5 protein in 2% non-fat milk in TBS. For competitive inhibition assays with heparin, soluble recombinant fibulin-5 dilutions were kept at the same concentration of 20 μg/mL in 2% non-fat milk in TBS, however, they were pre-mixed with increasing amounts of soluble heparin. Plates were then washed 3 x 5 min with washing buffer and incubated with 50 μL/well of 1:1,000 dilution of fibulin-5 IgG purified antibody in 2% non-fat milk in TBS for 1.5 hr at RT. Plates were then washed 3 x 5 min with washing buffer and incubated with 50 μL/well of 1:800 dilution of secondary antibody peroxidase-conjugated Affini Pure® goat anti-rabbit IgG (Jackson Immune Research) in 2% non-fat milk in TBS for 1.5 hr at RT. Plates were again washed 3 x 5 min with washing buffer and colour development was performed with 100 μL/well of 1 mg/mL 5-aminosalicylic acid in 20 mM phosphate buffer [pH 6.8], including 0.045% (v/v) H_2O_2 for 3-5 min and stopped by adding 100 μL/well of 2 M

NaOH. Colour absorption measurements were taken at 490 nm using a micro-ELISA plate reader (Beckman Coulter).

7. HUVEC Cell Binding Assay

7.1 ELISA

An ELISA was performed on 4-well chamber slides (Nalge Nunc International) for both recombinant RGE mutant fibulin-5 and the full-length protein as described above. This was done in order to ensure that the proteins were saturated on the plate surface.

7.2 Protein Coating and Cell-Binding Assay

Recombinant RGE mutant fibulin-5, full-length fibulin-5, and fibronectin (Sigma-Aldrich) were coated in duplicates overnight at 4°C onto 4-well chamber slides (Nalge Nunc International) in 500 μL of 4 mM $CaCl_2$ in TBS, all at a concentration of 10 μg/mL. The following day, the chamber slides were washed with 1 x 5 min washing buffer (20mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM $CaCl_2$ and 0.05% Tween-20) and blocked for 1 hr at RT with 500 μL of 5% BSA in PBS. Chamber slides were then washed 1 x washing buffer, followed by 2 x Hepes wash (20 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$ [pH 7.4]). HUVECs no older than passage 6 were trypsinized (0.025% trypsin and EDTA 0.01%) for 2-3 min, inhibited with 20% FBS medium, and centrifuged at 1.1 *g* for 6 min at RT. Cells were then re-suspended at a density of 150,000 cell/mL in an Adhesion Buffer (1% BSA, 2.2 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 10 mM Hepes in Hank's Buffered Salt Solution (Invitrogen) [pH 7.4]) (63). Adhesion Buffer was prepared, buffered back to pH 7.4, then filter-sterilized before usage for cell-binding experiments. 500 μL/well of the cell

suspension was placed onto chamber slides and cells were allowed to bind for 1 hr in a 37°C incubator.

7.3 Immunofluorescence

Unbound cells were removed and the wells were washed 4 x PBS. Cells were then fixed by completely covering the cell layer with 5% paraformaldehyde in PBS for 30 min at RT. Cells were then rinsed twice with 1% BSA and 0.1% saponin in PBS (PBS-BS) and allowed to further permeabilize in 2 x 15 min washes with PBS-BS. Cells were stained in the dark for 3 minutes with DAPI (Invitrogen) at a 1:2,000 dilution in PBS-BS. Cells were finally washed 2 x 5 min PBS in the dark and coverslips were mounted onto slides using Vectashield® mounting medium (Vector Laboratories, Burlingame, CA) and sealed with ordinary clear nail polish. Slides were kept in the dark at 4°C until fluorescent imaging.

7.4 Quantification of HUVEC Binding

Images were taken with digital camera AxioCam MRc (Zeiss, Toronto, ON) using Axioskop 2 fluorescence microscope (Zeiss). Six pictures per well chamber, using the 20X objective, were randomly selected from the central region of the well to avoid artifacts of excess cell adhesion near the edges. Images were imported into NIH ImageJ software for quantification and converted into a binary version. The binary version of the data could then be used by the program to estimate the cell number per field (Figure 4). The data was then evaluated by the Origin software program (OriginLab Corporation, Northampton, MA) and examined for significance by a two sample *t*-test with significance defined as $p<0.05$.

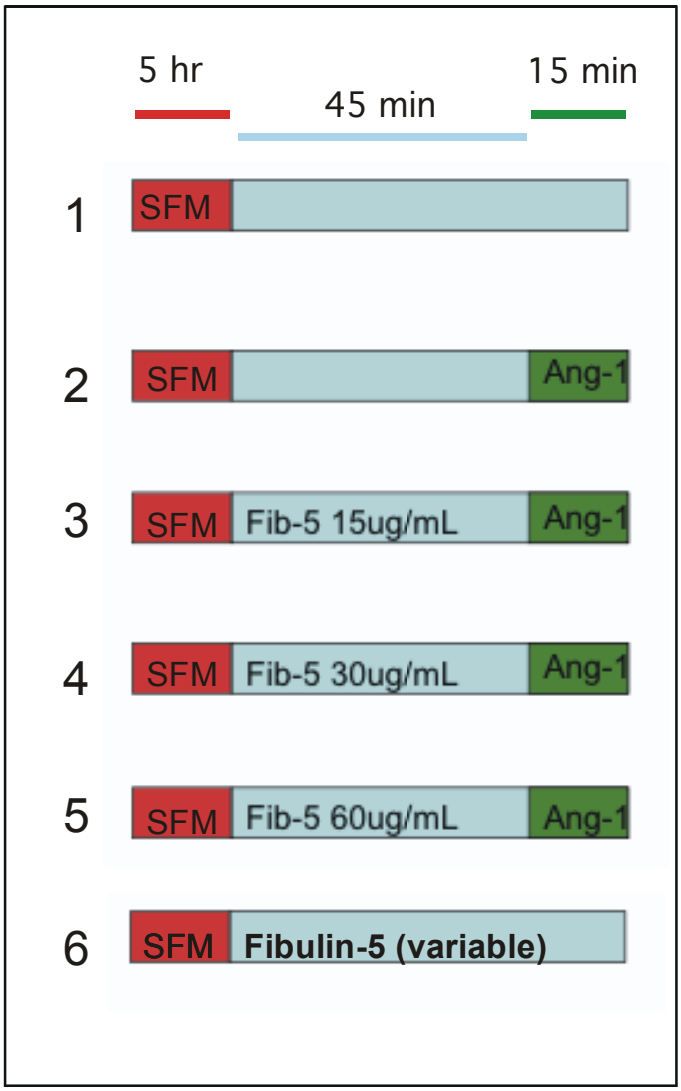


Figure 3: Schematic diagram of the experimental layout for signaling experiments in HUVECs. **1.** Control HUVECs were serum-starved for 6 hr. **2.** HUVECs treated with Ang-1 for the last 15 min of 6 hr serum-starvation (SFM). **3-5.** HUVECs treated with Ang-1 for 15 min following 45 min fibulin-5 pre-incubation in increasing concentrations. **6**. HUVECs treated with fibulin-5 alone.

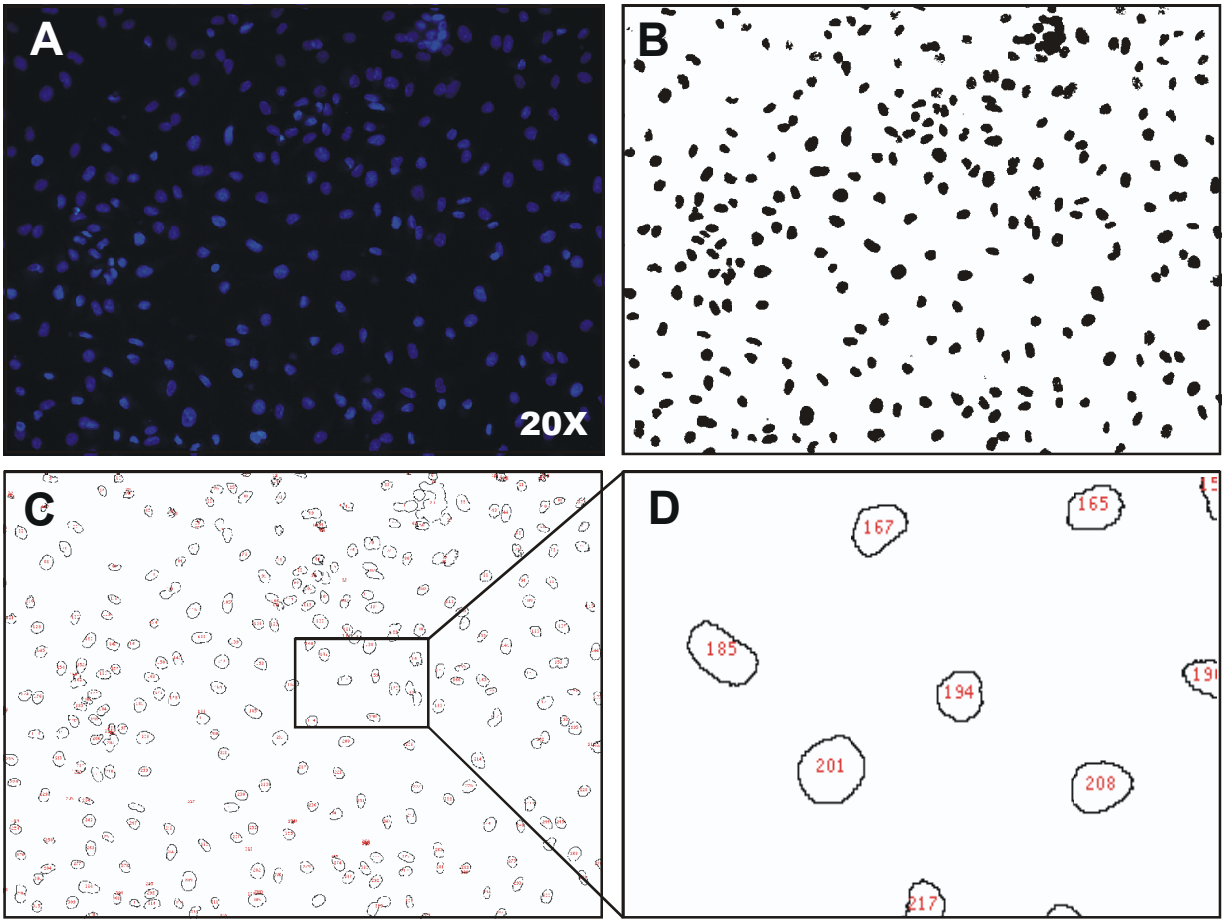


Figure 4: Quantification Method of HUVECs in Cell-Binding Assays A. HUVECs bound to a fibronectin-coated 4-well chamber slide with nuclei stained with DAPI (blue) and imaged on fluorescence microscope. **B.** Binary black and white images generated from original by ImageJ program (NIH). **C.** Computerized cell outline profile determined from binary image and used for quantification by ImageJ program. **D.** A portion of (C) in higher magnification demonstrating the quantification by ImageJ.

RESULTS

1. Recombinant Fibulin-5 Full-length and RGE mutant Protein Production

To investigate the molecular mechanisms by which fibulin-5 might act as an anti-angiogenic molecule, it was first necessary to generate both recombinant fibulin-5 full-length and an RGE mutant protein. These proteins were necessary to carry out ELISAs, solid-phase binding assays and cell signaling experiments.

As previously described in the material and methods, secreted recombinant fibulin-5 was purified from SFM conditioned by stably transfected HEK 293 cells, in the case of full-length fibulin-5, or by CHO cells, in the case of the RGE mutant fibulin-5. The SFM media was concentrated, dialyzed and loaded onto a chelating HisTrap affinity column. The recombinant fibulin-5 protein contains a histidine tag, which binds the Nickel sepharose beads in the HisTrap column with a high micromolar affinity, thus becoming trapped. A 0.5 M imidazole buffer was then used to elute the tagged protein bound to Nickel ions attached to the surface of beads in the chromatography column and fractions of the eluate were collected in 1 mL increments. Imidazole acts as a mild denaturant disrupting protein interactions and thereby causing the release of recombinant fibulin-5 bound to Nickel on the HisTrap column. Unspecifically or loosely bound proteins are the first to come off the column and as the imidazole gradient increases, the more tightly bound proteins are eluted (Figure 5A). As previously mentioned, the ability of the histidine tag to bind so strongly to the Nickel on the HisTrap column allows fibulin-5 to be eluted further away from the unwanted proteins (asterisk on Figure 5A).

To verify which fractions contain the recombinant fibulin-5, samples from the collected 1 mL fractions, starting material and flow-through were separated by SDS-PAGE followed by Coomassie blue staining (Figure 5B). Recombinant fibulin-5 was found in fractions 13 through 19, with protein in fractions 17-19 being the cleanest and purest. Fractions were pooled and dialyzed in 2 mM EDTA in TBS according to their purity. EDTA prevents the precipitation of fibulin-5 from solution by acting as a chelating agent sequestering ions such as Ca^{2+} from binding the cbEGF motifs on fibulin-5. Fractions 14 and 15, fractions 16 and 17, and fractions 18 and 19, were pooled to generate 3 preparations. The recombinant fibulin-5 in the pooled fractions was once again confirmed by SDS-PAGE followed by Coomassie blue staining demonstrating the purity of the fractions (Figure 6A). The identity of the protein was confirmed by Western blot analysis using an anti-fibulin-5 antibody (Figure 4B). There was only a single band observed on the Western blot at the expected size for fibulin-5 (66 kDa) indicating that no degradation products were present. Purification and analysis of RGE mutant fibulin-5 produced similar results (data not shown). All pooled fractions of recombinant fibulin-5 full-length and RGE mutant protein are listed in Table 2.

2. Physical Interactions of Fibulin-5

To investigate the possible mechanism of fibulin-5 activity as an anti-angiogenic molecule via the modulation of Ang-1 activity, it was important to determine whether any physical protein-protein interactions between fibulin-5 and candidate molecules in the Ang-1/Tie-2 (ligand/receptor) signaling pathway exist.

2.1 Fibulin-5 Binding to Ang-1

A solid-phase binding assay was performed to reveal the ability fibulin-5 to bind Ang-1 *in vitro*. First, an ELISA was performed to ensure that the Ang-1 antibody did not cross-react with fibulin-5 protein and to ensure that Ang-1 indeed bound to the 96-well plates being used for the solid-phase binding assays (Figure 7A). Results show that the Ang-1 antibody detected Ang-1 in a concentration dependent-manner and that it did not detect fibulin-5 protein, even at high antibody concentrations. Full-length fibulin-5 protein along with soluble Tie-2 receptor (positive control) were coated onto 96-well plates. Increasing concentrations of soluble Ang-1 were then incubated with the bound proteins and detected with an Ang-1 antibody and a colour reaction. From figure 7B, the results show a strong binding affinity of Ang-1 to its Tie-2 receptor in a concentration-dependent manner. However, no sizeable binding can be observed to fibulin-5, even at high Ang-1 concentrations. These results indicate that fibulin-5 is unlikely to sequester Ang-1 in the matrix, thereby preventing it from binding its receptor Tie-2.

2.2 Fibulin-5 Binding to Tie-2

To determine the ability of fibulin-5 to bind the Ang-1 receptor, Tie-2, a second solid-phase binding assay was performed. As before, an ELISA was performed to ensure that the fibulin-5 antibody did not cross-react with the Tie-2 protein and to ensure that both fibulin-5 and Tie-2 indeed bound to the 96-well plates being used for the solid-phase binding assays (Figure 8A). Results show that the fibulin-5 antibody detected fibulin-5 in a concentration dependent-manner and did not detect Tie-2, even at high antibody conentrations. The reverse was true of

the Tie-2 antibody for the Tie-2 protein. Therefore, both proteins coated onto the plates well and their antibodies did not cross-react. Soluble Tie-2 receptor and tropoelastin (positive control) were then coated onto 96-well plates. Increasing concentrations of soluble full-length fibulin-5 were then incubated with the bound proteins and detected with a fibulin-5 antibody and a colour reaction. From figure 8B, the results show a strong binding affinity of fibulin-5 for tropoelastin in a concentration-dependent manner. However, no binding can be observed to Tie-2, even at high soluble fibulin-5 concentrations. These results show fibulin-5 does not bind Tie-2, thereby indicating that fibulin-5 does not function to obstruct Ang-1 from binding the Tie-2 receptor in its action as an antagonist.

3. Fibulin-5 Binding to the Endothelial Cell Surface

Cells have been shown to selectively bind and recognize fibulin-5 in a context-specific manner (86). Since fibulin-5 is a secreted molecule in the ECM, the molecules which recognize fibulin-5 are most likely receptors on the cell surface, for example the integrins, which have been previously shown to interact with fibulin-5 (80). Given that we did not find any direct protein-protein interaction between fibulin-5 and molecules in the Ang-1/Tie-2 signaling pathway through the solid-phase binding assays, we wanted to investigate the interactions between fibulin-5 and candidate cell surface receptors such as the integrins and the cell surface HSPGs.

3.1 Integrin-Mediated Binding: The Role of the RGD Motif in Fibulin-5

Cells have been shown to recognize and bind fibulin-5 (96), therefore we sought to study the role of the RGD motif in fibulin-5 using an RGE mutant fibulin-5. The RGE protein has a glutamic acid (E) replacing the aspartic acid (D) in the RGD sequence of the full-length protein, ablating any RGD-dependent integrin binding to the cell surface (97). Through cell binding assays, we were able to demonstrate the binding affinity of HUVECs to full-length fibulin-5, RGE mutant fibulin-5, fibronectin (positive control) (86) and to TBS/calcium (negative control) (Figure 9). These results demonstrate that HUVECs have a significantly higher binding ability to full-length fibulin-5 than to the RGE mutant protein. HUVEC binding to fibronectin was significantly stronger than the other groups and HUVEC binding to the TBS/calcium blank treated wells was comparable to the level seen using the RGE mutant fibulin-5, with no significant difference. Figure 9 demonstrates the result of one representative experiment, however, the experiment was repeated two other times with similar results (data not shown).

To ensure that both the full-length and RGE mutant fibulin-5 proteins were able to coat the slide at saturation levels, an ELISA was performed on the 4-well chamber slides used for the cell-binding assays. No colour reaction was observed in the TBS/calcium negative control well, indicating there is no unspecific or background colour reaction to the tissue culture plastic or TBS/calcium buffer in which proteins are suspended for coating (Figure 10A). Colour reaction quantification data indicates that both proteins indeed coated the slide surface equally well (Figure 10B). Furthermore, doubling the full-length fibulin-5

concentration to 20 μg/mL did not yield a higher colour absorbance, thus indicating that the 10 μg/mL protein concentration used to coat the slides was enough to completely cover the available binding surface. These ELISA results along with the fact that HUVEC binding to RGE fibulin-5 was diminished but not completely ablated compared to full-length fibulin-5 (Figure 9), suggest the possibility that there might be other cell surface molecules other than integrins which recognize and bind different motifs other than the RGD on the fibulin-5 protein.

3.2 Fibulin-5 Binding to Heparin

Other candidate molecules which could bind fibulin-5 are the cell surface HSPGs. Since heparin molecules are very closely related in structure to heparan-sulfate, heparin was used in a solid-phase binding with full-length fibulin-5 (Figure 11). BSA-conjugated heparin molecules (Hep-BSA) were coated onto 96-well plates. BSA was used as a carrier because heparin cannot bind the plate surface alone. Wells coated with BSA alone were used as a control to account for any non-specific binding of full-length fibulin-5 directly to the BSA. Fibronectin coated wells were also used as a positive control. Increasing concentrations of soluble full-length fibulin-5 were then incubated with the coated proteins and detected with a fibulin-5 antibody and a colour reaction. Figure 11 shows that there was a very strong binding affinity of fibulin-5 to Hep-BSA in a concentration-dependent manner. However, no sizeable binding was observed to BSA-only coated wells, even at high soluble fibulin-5 concentrations. This verifies that the observed binding to Hep-BSA was specific to heparin and not the BSA carrier molecule. We also observed that the

binding affinity of fibulin-5 to fibronectin was concentration-dependent, but to a much lesser extent than to Hep-BSA.

To further determine the specificity of this very strong affinity of fibulin-5 for heparin, a competitive solid-phase binding assay with soluble heparin was performed (Figure 12). Similar to the above solid-phase binding assay, Hep-BSA, BSA and fibronectin were coated onto 96-well plates. Soluble fibulin-5 was then incubated with the coated proteins, however, for this experiment, a constant concentration of fibulin-5 was used with increasing concentrations of soluble heparin. As seen in figure 12, soluble heparin can noticeably decrease the binding affinity of fibulin-5 to Hep-BSA in a concentration-dependent manner, whereas it had no effect on the ability of fibulin-5 to bind fibronectin or BSA coated wells. These results demonstrate that the ability of fibulin-5 to bind heparin is very strong and also specific since this interaction can be competitively inhibited by increasing concentrations of soluble heparin.

4. Downstream Signaling of Fibulin-5 and Ang-1 in HUVECs

In the preceding experiments, we did not find any physical interactions whereby fibulin-5 could directly affect the Ang-1/Tie-2 (ligand/receptor) pathway. On the other hand, we found evidence that HUVECs bind full-length fibulin-5 more strongly than the RGE fibulin-5 mutant, further supporting a role for integrins in cellular recognition of fibulin-5. Furthermore, we demonstrated a very high binding affinity of fibulin-5 to heparin, strongly suggesting cell surface HSPGs could also play a role in binding and recognizing fibulin-5. Next, we wanted to change our focus to

intracellular molecules. Specifically, we wanted to characterize the intracellular signaling pathway(s) of fibulin-5. All signaling experiments done in HUVECs followed the same protocol consisting of a total of 6 hours of starvation with various treatment regiments (Figure 3). Ang-1 treatment was implemented in the last 15 min of the starvation period, whereas fibulin-5 treatment occurred over the last hour of the starvation period whether followed by Ang-1 treatment or not.

4.1 Erk Signaling

4.1.1 Effects of Fibullin-5 on Ang-1 Mediated Activation of Erk

Ang-1 has been shown to activate the Erk pathway in HUVECs by increasing Erk phosphorylation (98). To determine if fibulin-5 alters Ang-1 mediated Erk activation, HUVECs were pre-incubated with increasing concentrations of fibulin-5 prior to the 15 minutes of Ang-1 treatment (Figure 13). Figure 13A demonstrates that there was a considerable increase of phosphorylated Erk (p-Erk) in response to treatment with 300 ng/mL of Ang-1 compared to the control groups. Although some variation can be seen in the control groups, the level of p-Erk was considerably lower than in Ang-1 treated cells. The levels of p-Erk following Ang-1 treatment appear to increase with fibulin-5 pre-incubation in a concentration-dependent manner. Levels of total Erk and GAPDH (loading control) were relatively constant between treatment groups when compared to levels of phosphorylated Erk. These observations are further supported by the densitometry data shown in figure 13B, which demonstrates values of p-Erk normalized to total levels of the Erk and GAPDH. These results suggest that fibulin-5 potentiates the ability of Ang-1 to phosphorylate Erk in HUVECs.

4.1.2 Ang-1 and Fibulin-5 Individual Treatment

To determine if the observed ability of fibulin-5 to potentiate Ang-1 mediated Erk phosphorylation was an additive effect of two different upstream parallel signaling pathways which converge at Erk or truly the effect of both proteins on a single pathway, it was necessary to characterize the ability of Ang-1 and fibulin-5 to phosphorylate Erk individually. In figure 14A, HUVECs were incubated for 15 min with increasing concentrations of Ang-1 and we can observe a clear increase of p-Erk in a concentration-dependent manner, confirming what has been previously shown by other groups (98). The levels of p-Erk following Ang-1 treatment appear to increase with increasing amounts of Ang-1 treatment while levels of total Erk and GAPDH look to be relatively constant between treatment groups. These observations are further supported by the densitometry data shown in figure 14B, which shows values of p-Erk normalized to total levels of the Erk and GAPDH.

In figure 15A, HUVECs were incubated for 1 hr with increasing concentrations of fibulin-5 and we can observe an increase of p-Erk in a concentration-dependent manner, although not as pronounced as Ang-1 mediated p-Erk. When comparing quantified densitometry data we can see that at the highest concentration of Ang-1 treatment (300ng/mL) p-Erk levels are at 70,000% higher than control groups, whereas 1 hr of fibulin-5 treatment at its highest concentration (60 μg/mL) is only 339% higher than its control group (Figures 14B and 15B). From these results, we see that both Ang-1 and fibulin-5 have the ability to individually induce p-Erk, however, fibulin-5 is much weaker in doing so. This suggests that the observed ability of fibulin-5 to potentiate Ang-1 induced Erk phosphorylation might

be an additive effect of two different parallel signaling pathways, which converge at Erk, rather than the composite effect of both proteins on a single pathway.

4.2 Akt Signaling

4.2.1 Ang-1 Treatment with Increasing Concentrations of Fibulin-5 Pre-incubation

To investigate protein kinase cascades other than the Erk MAPK cascade, we wanted to look at Akt activation. We were interested in this pathway because Akt has a major outcome toward cell survival and cell growth and Ang-1 mediated cell survival has been shown to crucially rely on the Akt pathway (99). To see how fibulin-5 might affect Ang-1 mediated Akt activation, increasing concentrations of fibulin-5 were pre-incubated prior to 15 min of Ang-1 treatment. Figure 16 demonstrates a considerable increase in phosphorylated Akt (p-Akt) at two different epitopes (Thr 308 and Ser 473) in response to treatment of 300 ng/mL of Ang-1 compared to the control HUVECs. However, the level of p-Akt (at both Thr 308 and Ser 473) in response to Ang-1 treatment appears to decrease inversely with fibulin-5 pre-incubation concentration levels. Levels of total Akt and GAPDH (loading control) look to be relatively constant between treatment groups when compared to levels of p-Akt. Therefore, fibulin-5 can inhibit Ang-1-mediated Akt phosphorylation at two different epitopes in a concentration-dependent manner.

4.2.2 Fibulin-5 Individual Treatment

To determine if the observed ability of fibulin-5 to inhibit Ang-1 mediated Akt phosphorylation was an additive effect of two different upstream parallel signaling pathways which converge at Akt, or truly the effect of both proteins on a single pathway, it was necessary to characterize the ability of fibulin-5 to

phosphorylate Akt individually. In figure 17, HUVECs were incubated for 1 hr with increasing concentrations of fibulin-5 and we can observe a decrease of p-Akt (Thr 308) in a concentration-dependent manner. Note that the varying levels of p-Akt in the control samples between figure 16 and 17 are due to differences in film exposure times. These results suggest that fibulin-5 treatment of HUVECs downregulates the level of p-Akt, an effect opposite to ability of Ang-1 to induce Akt phosphorylation. Furthermore, since fibulin-5 is able to decrease the level of p-Akt alone as well as when pre-incubated with Ang-1, we can infer that fibulin-5 has the ability to override Ang-1 mediated Akt phosphorylation.

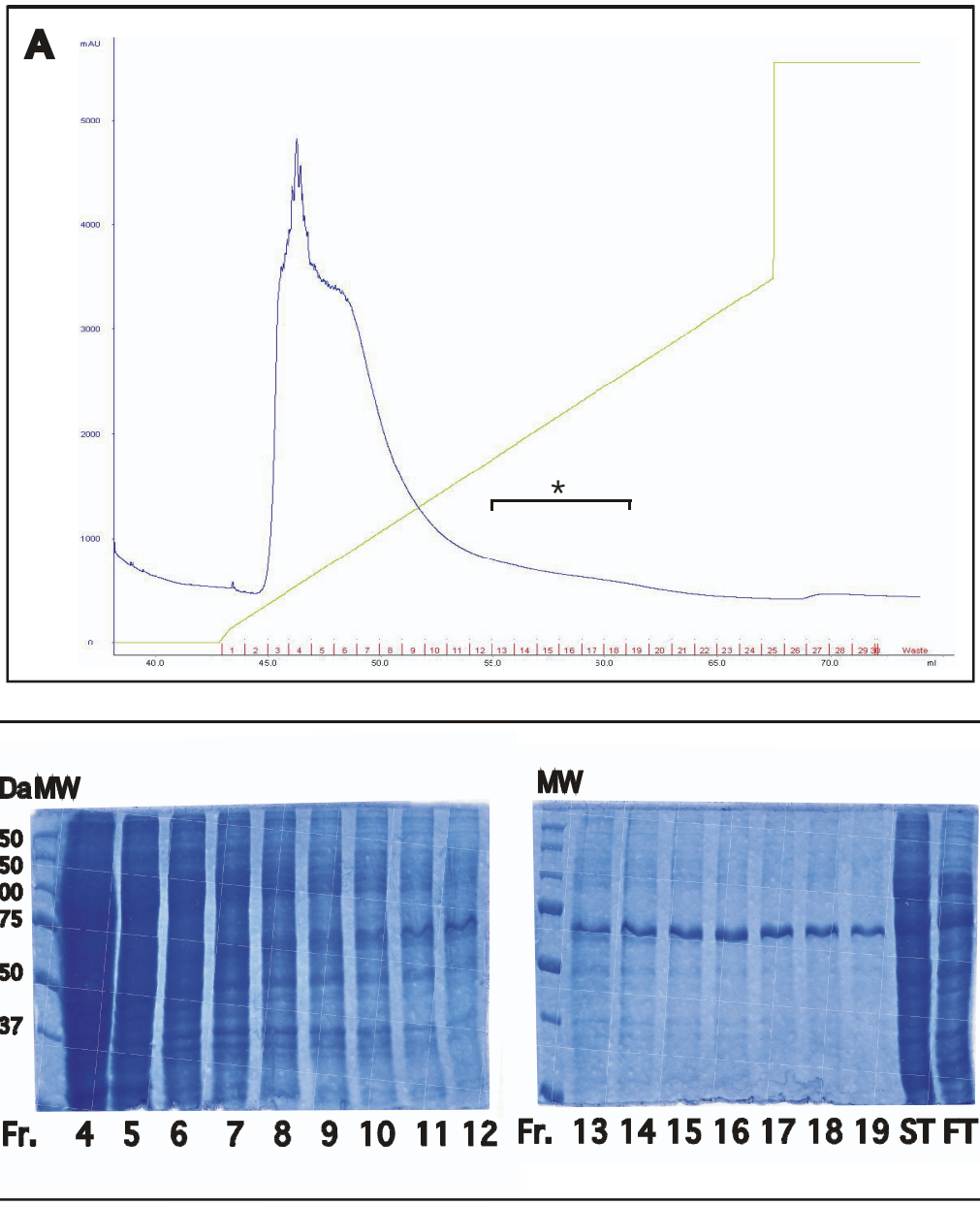


Figure 5: Purification of Recombinant Full-length Fibulin-5 Protein. **A.** Chromatogram of the purification of full-length recombinant fibulin-5 protein. The asterisk indicates the absorbance region corresponding to full-length recombinant fibulin-5 protein. The blue line on the chromatogram represents the absorbance of protein, the yellow line corresponds to the imidazole concentration gradient. Fractions of 1 mL were collected and their numbers are indicated on the horizontal axis. **B.** To verify the purity of samples, SDS-PAGE of fractions followed by Coomassie staining was done. The starting material (ST) and flow-through (FT) were also tested.

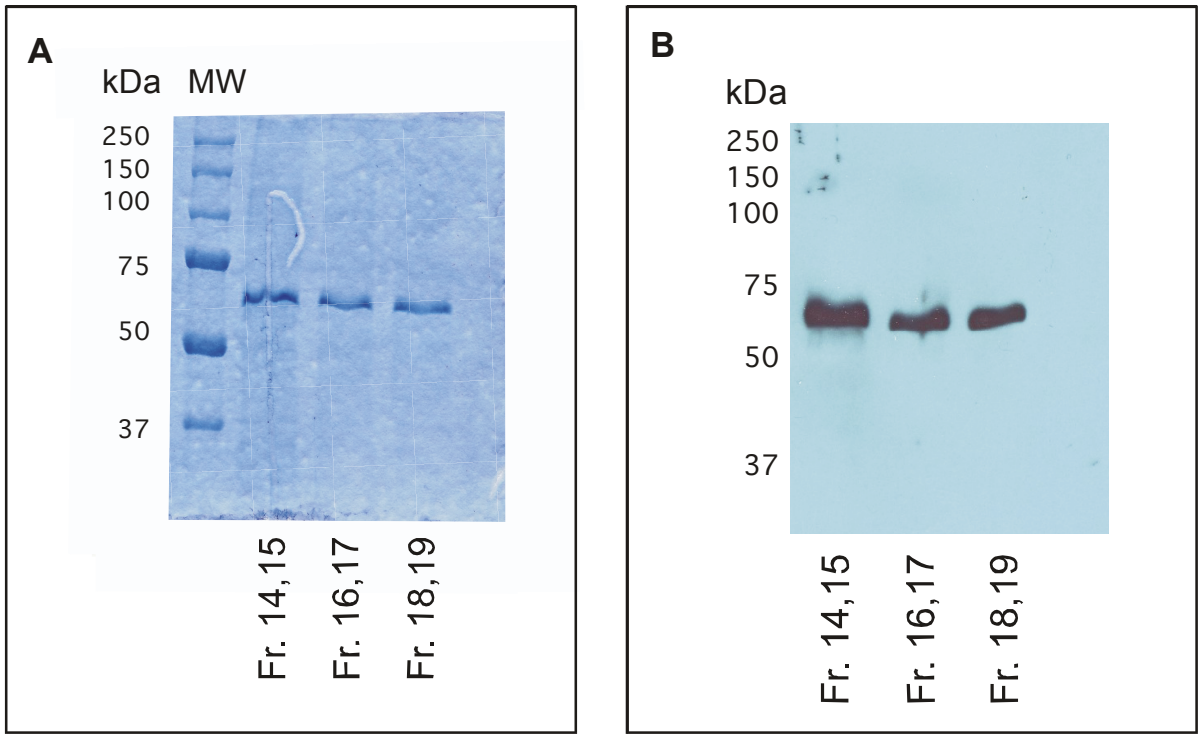


Figure 6: Purification of Recombinant Full-length Fibulin-5 Protein. A. After final dialysis of the pooled fractions containing the full-length fibulin-5 recombinant protein, SDS-PAGE was done, followed by Coomassie staining to further verify purity of fractions. **B.** Western blot of the pooled fractions using an IgG purified fibulin-5 antibody to ensure no degradation fragments are present. The fibulin-5 antibody was polyclonal and raised against the intact, full-length protein.

Table 2: Full-length and RGE Mutant Fibulin-5 Recombinant Protein.

ID	Fractions	Concentration (μg/mL)	Date	Volume	Fibulin-5 Construct
A	9, 10	431.60 **μg/mL**	28/11/08	2mL	Full-length
B	11, 12	299.60 **μg/mL**	28/11/08	2mL	Full-length
C	13, 14, 15	105.60 **μg/mL**	28/11/08	3 mL	Full-length
D	22, 23	244.67 **μg/mL**	27/04/09	2mL	RGE
E	41, 42, 43	178.00 **μg/mL**	27/04/09	3 mL	RGE
F	11	866.88 **μg/mL**	07/12/09	1 mL	Full-length
G	10, 12	303.33 **μg/mL**	07/12/09	2mL	Full-length
H	16, 17, 18	255.75 **μg/mL**	22/12/09	3 mL	Full-length
I	14, 15	317.00 **μg/mL**	22/12/09	2mL	Full-length
J	13	399.50 **μg/mL**	22/12/09	1 mL	Full-length
K	14, 15	385.10 **μg/mL**	07/05/10	2mL	Full-length
L	16, 17	318.10 **μg/mL**	07/05/10	2mL	Full-length
M	18, 19	267.60 **μg/mL**	07/05/10	2mL	Full-length

**All proteins were dialyzed in 2 mM EDTA in TBS*

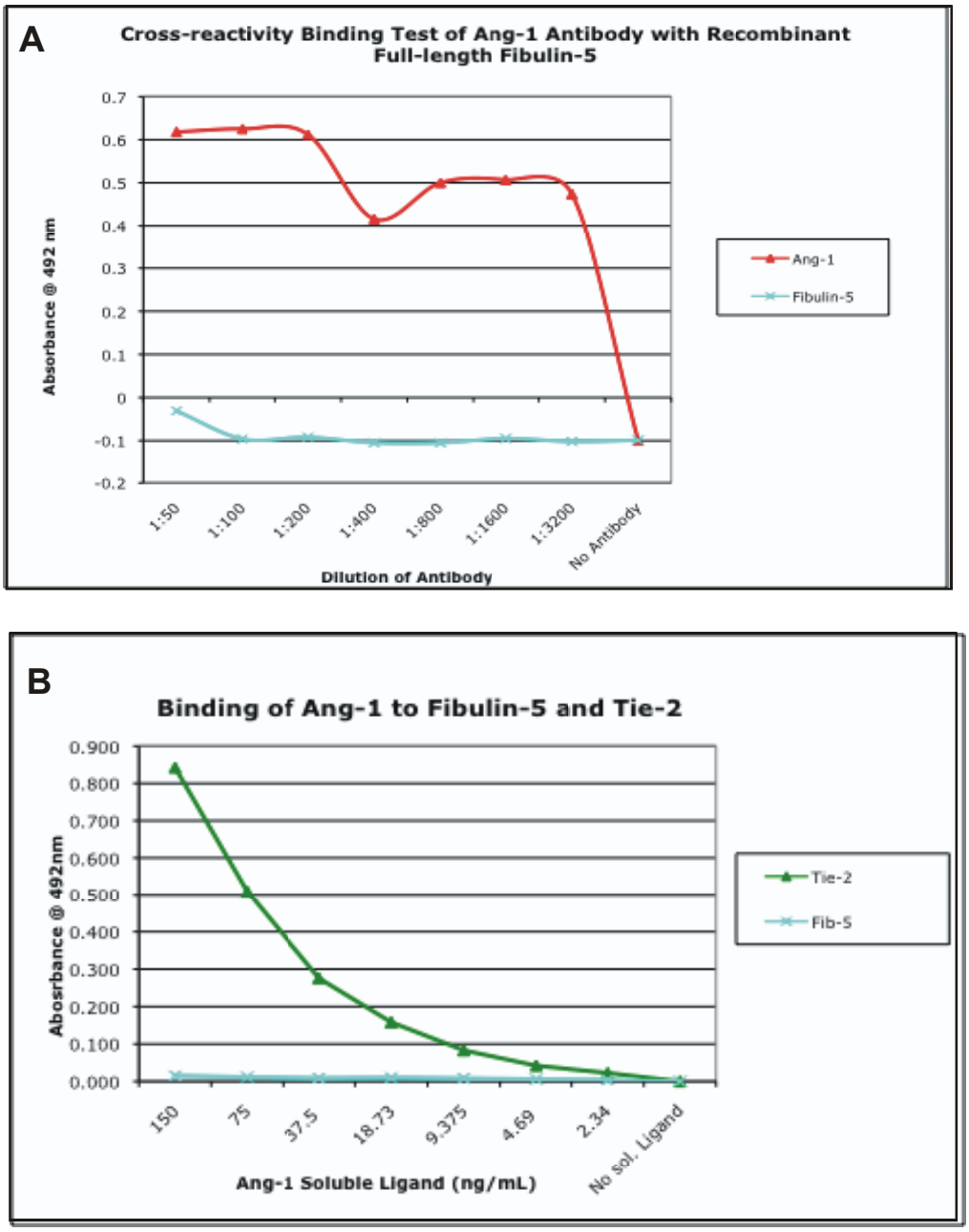


Figure 7: Binding of Fibulin-5 to Ang-1 A. ELISA testing for cross-reactivity binding between fibulin-5 protein and Ang-1 antibody. Ang-1 and fibulin-5 were immobilized onto different plates. The Ang-1 antibody demonstrated no significant reactivity to fibulin-5, whereas strongly reacting to Ang-1. **B.** Solid-Phase Binding Assay showing the affinity between Ang-1 and fibulin-5. Fibulin-5 and Tie-2 (positive control) were used as the immobilized ligands and Ang-1 was used as the soluble ligand. Ang-1 showed a strong affinity to Tie-2 as expected, and no significant binding was seen to fibulin-5 even as the soluble Ang-1 concentration increased.

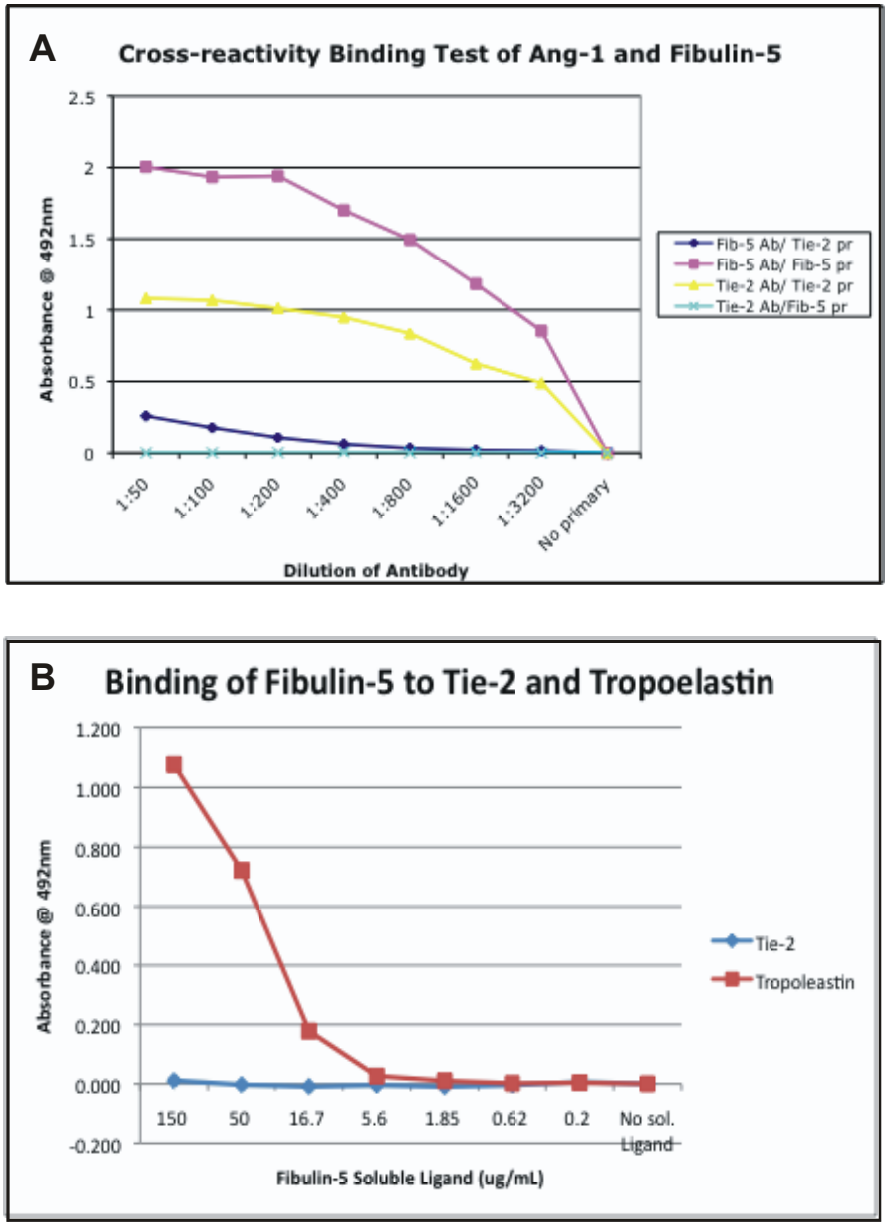


Figure 8: Binding of Fibulin-5 to Tie-2 A. ELISA testing for cross-reactivity binding between fibulin-5 and Tie-2 proteins to fibulin-5 and Tie-2 antibodies. Tie-2 and fibulin-5 were immobilized onto different plates. The fibulin-5 antibody demonstrated no significant reactivity to Tie-2 protein, whereas strongly reacting to fibulin-5 protein. The reverse was observed for the Tie-2 antibody. **B.** Solid-Phase Binding Assay showing the affinity between Tie-2 and fibulin-5. Tie-2 and tropoelastin (positive control) were used as the immobilized ligands and fibulin-5 was used as the soluble ligand. Fibulin-5 showed a strong affinity for tropoelastin as expected, and no significant binding was seen to Tie-2 even as the soluble fibulin-5 concentration increased.

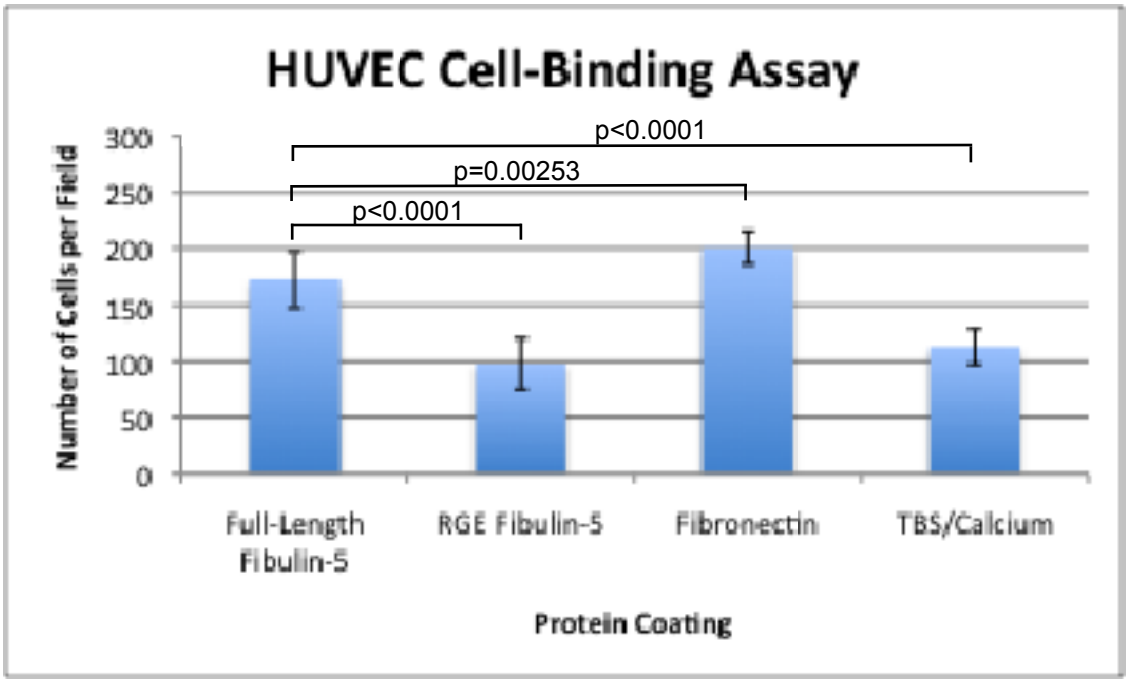


Figure 9: HUVEC Cell-Binding Assay. HUVECs were allowed to bind onto 4-well chamber slides coated with the proteins listed above or in 4 mM calcium in TBS (buffer in which the proteins were solubilized). Values are represented as the mean number of cells for the coated protein indicated. Results show HUVECs possess a significantly higher binding ability to full-length fibulin-5 protein than its RGE mutant counterpart. HUVECs bind with high affinity to fibronectin (positive control) and the binding to TBS/Calcium (negative control) shows no significant difference than to that of the mutant fibulin-5 RGE protein. $p<0.05$.

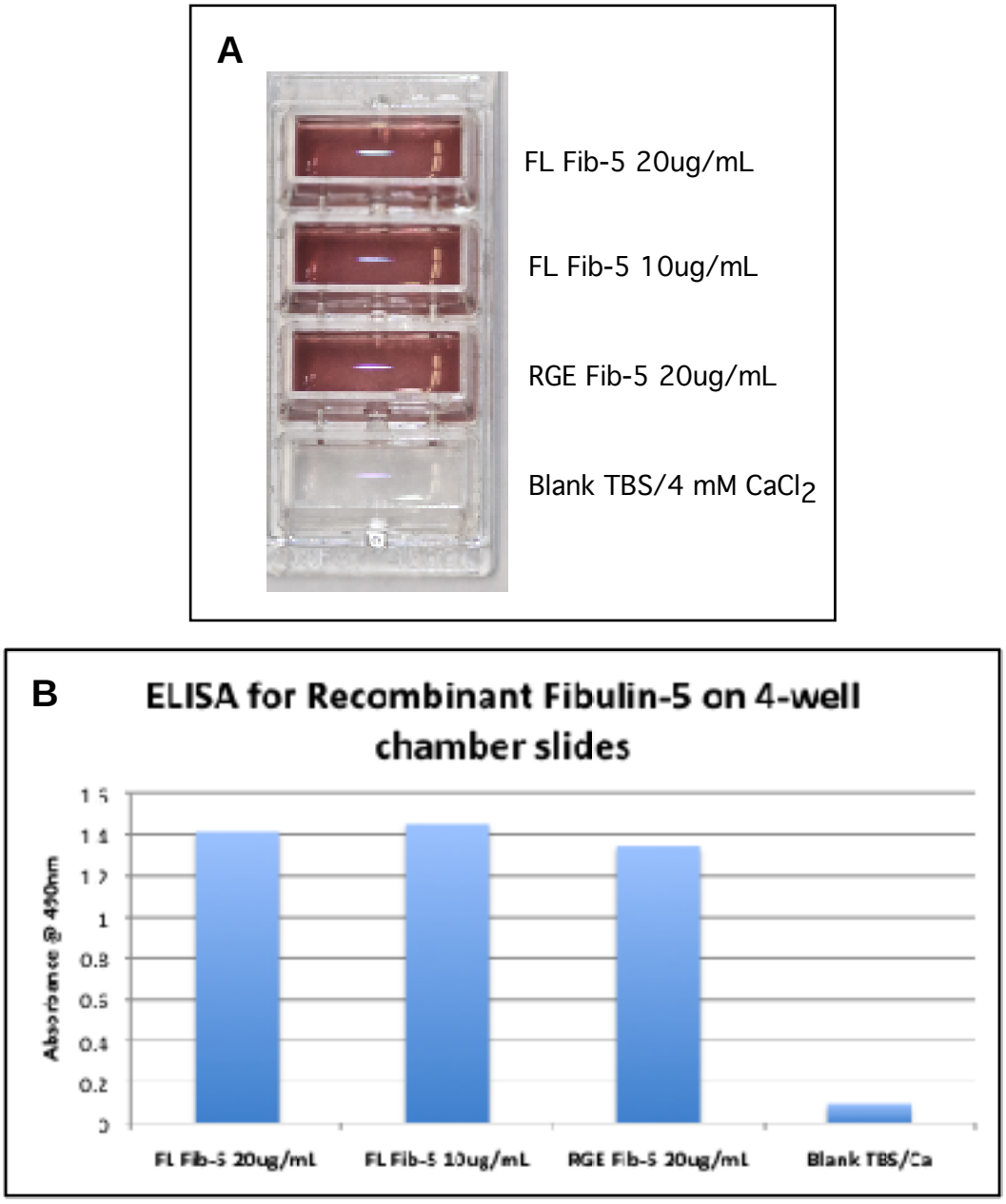


Figure 10: ELISA for Recombinant Fibulin-5 Full-length and RGE Mutant Proteins on 4-well Chamber Slides Utilized for Cell-Binding Assays. A. Photograph showing the colour development for the ELISA of recombinant fibulin-5 full-length, RGE mutant and 4 mM Calcium in TBS blank (same buffer used to coat fibulin-5 proteins). **B.** Graphic representation of the absorbance value for the colour development for the ELISA seen in panel A. Plates appear to have reached a saturation point for protein absorption at 10 ug/mL of fibulin-5 since doubling the concentration to 20 ug/mL does not increase the absorption values.

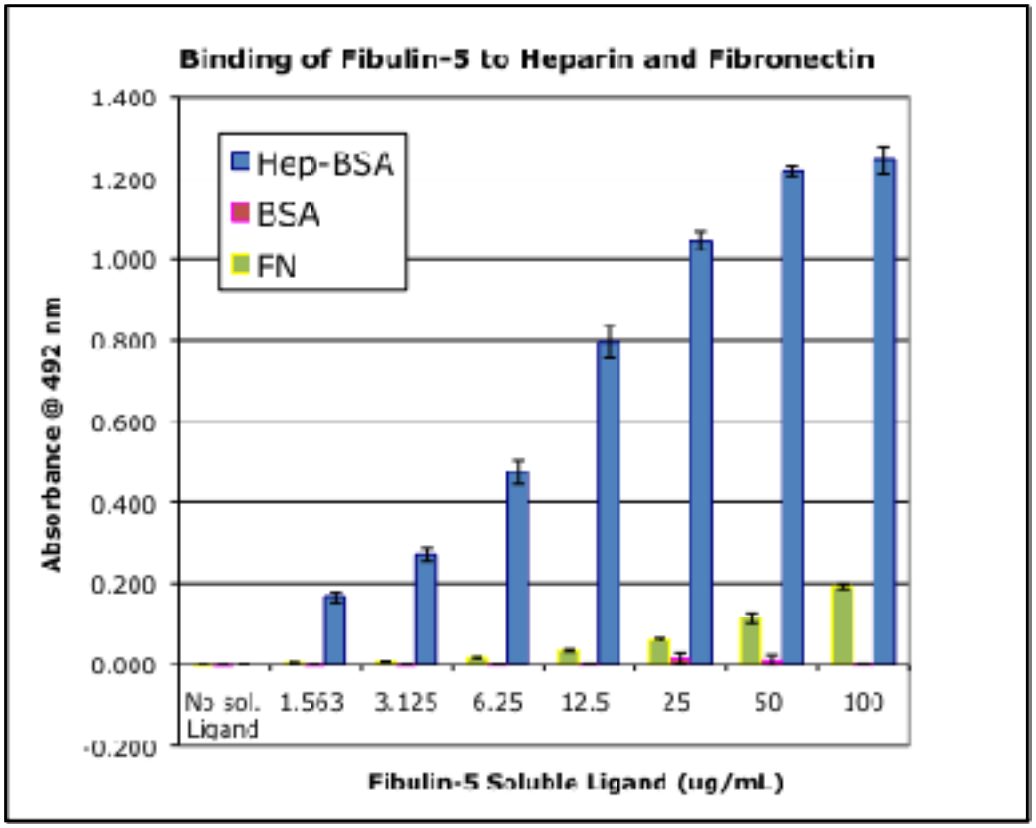


Figure 11: Solid-Phase Binding Assay of Fibulin-5 with Heparin-BSA and Fibronectin. Heparin-BSA, fibronectin (positive control), and BSA (control for unspecific binding) were used as the immobilized ligands and fibulin-5 was used as the soluble ligand. Fibulin-5 shows an affinity for Heparin-BSA that is much stronger than the positive control, fibronectin. This affinity was specific to the heparin portion of the conjugated Heparin-BSA molecule since no significant binding to BSA was seen.

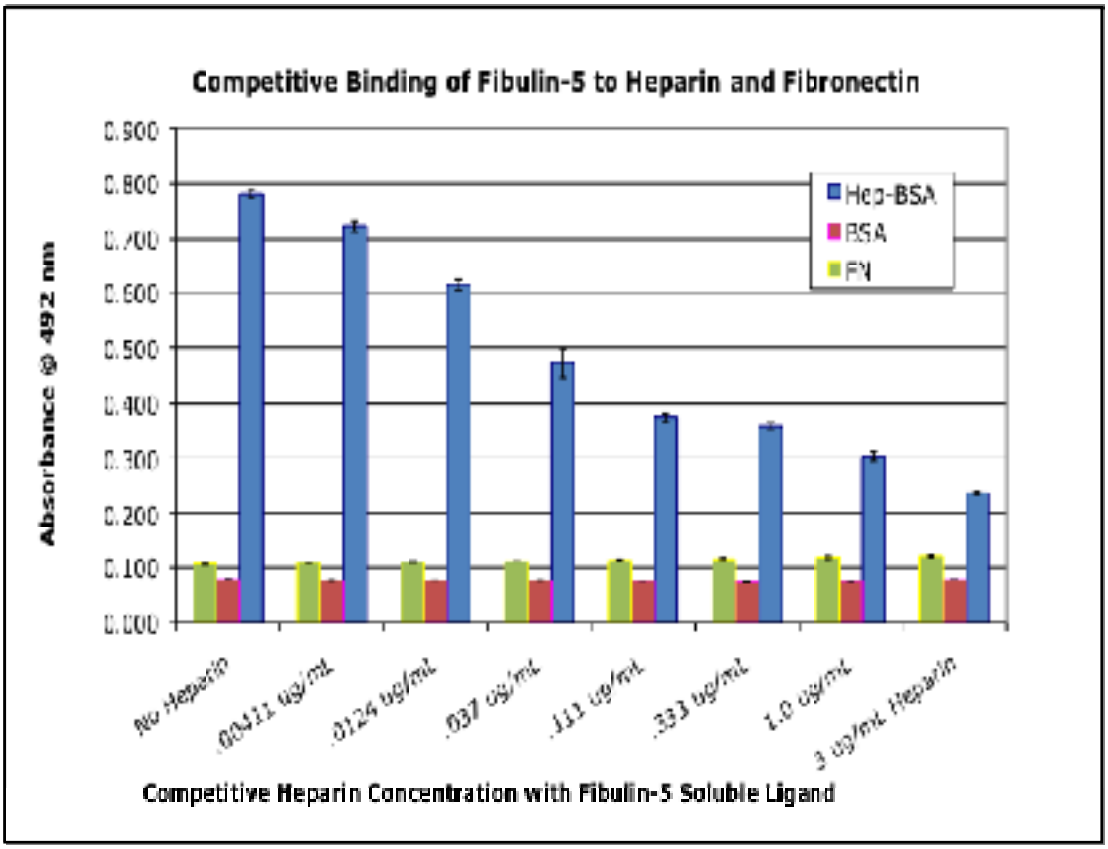
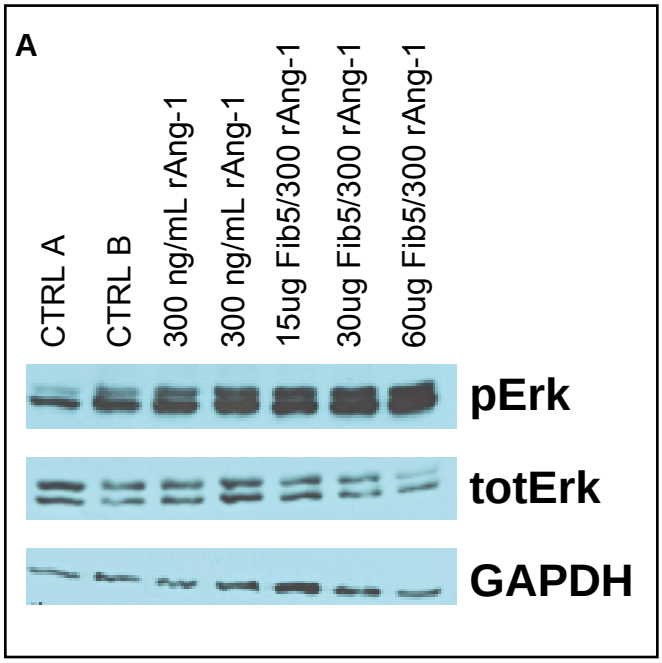


Figure 12: Solid-Phase Binding Assay of Fibulin-5 to Heparin-BSA and Fibronectin with Competing Heparin. Heparin-BSA, fibronectin (positive control) and BSA (control for unspecific binding) were used as the immobilized ligands and a constant concentration fibulin-5 was used as the soluble ligand. The ability of fibulin-5 to bind Heparin-BSA can be specifically inhibited by soluble heparin present in solution in a concentration-dependent manner. This inhibition is specific to Heparin-BSA since no inhibitory effect of fibulin-5 binding to fibronectin or BSA was seen.



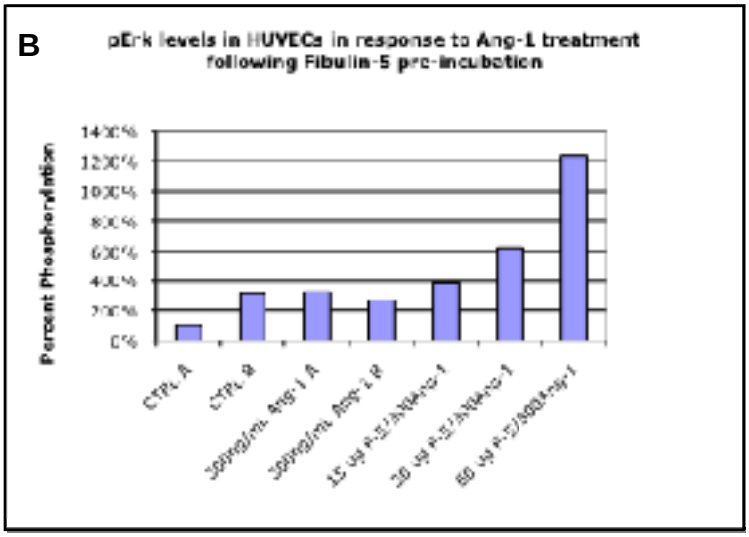
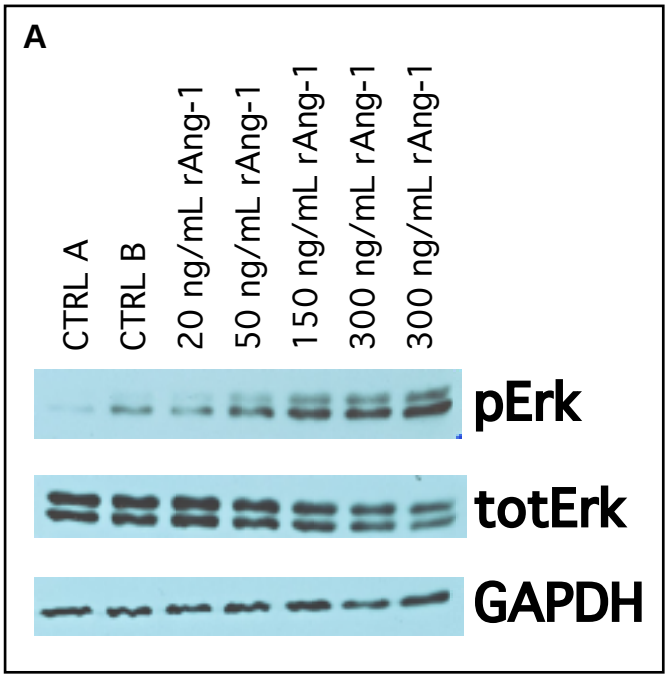


Figure 13: Phosphorylation Levels of Erk in HUVECs in Response to Ang-1 Treatment Following Fibulin-5 Pre-incubation. A. Western blots demonstrating an increase of Erk phosphorylation in response to 15 min Ang-1 treatment and fibulin-5 pre-incubation in a concentration-dependent manner. **B.** Densitometry quantifications of Erk phosphorylation normalized to levels of total Erk and GAPDH from Western blots above.



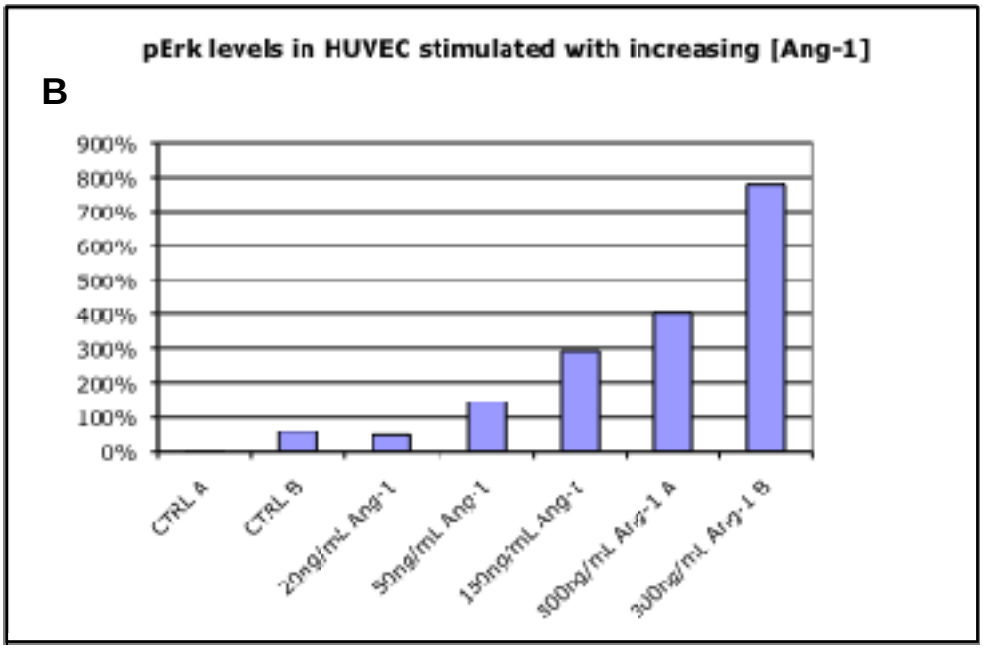
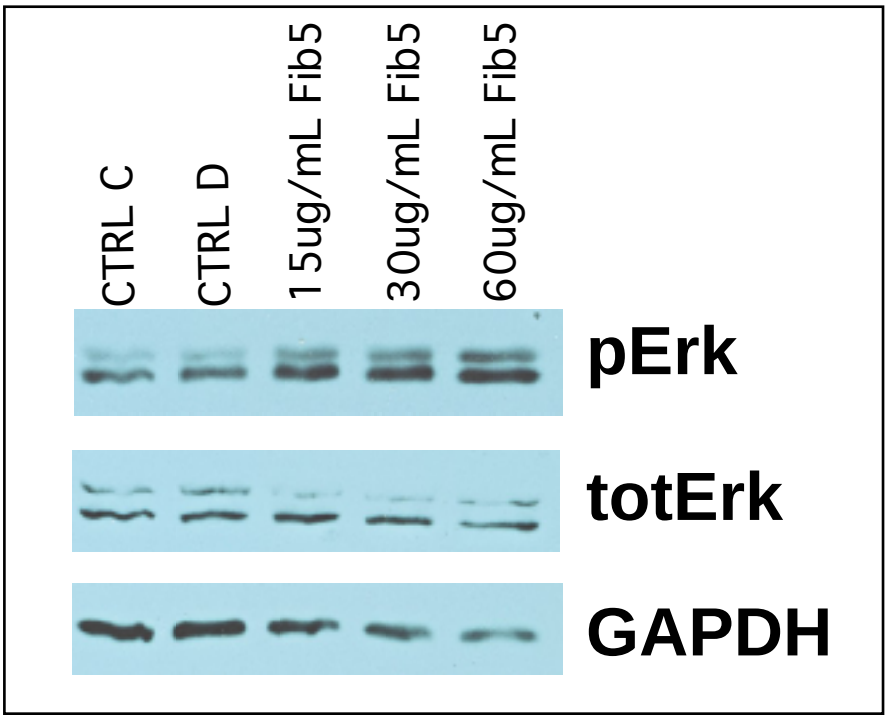


Figure 14: Analysis of Phosphorylation Levels of Erk in HUVECs Stimulated with Ang-1. A. Western blots demonstrating a considerable increase of Erk phosphorylation in HUVECs in response to 15 min of Ang-1 treatment in a concentration-dependent manner. **B.** Densitometry quantifications of Erk phosphorylation normalized to levels of total Erk and GAPDH from Western blots above.



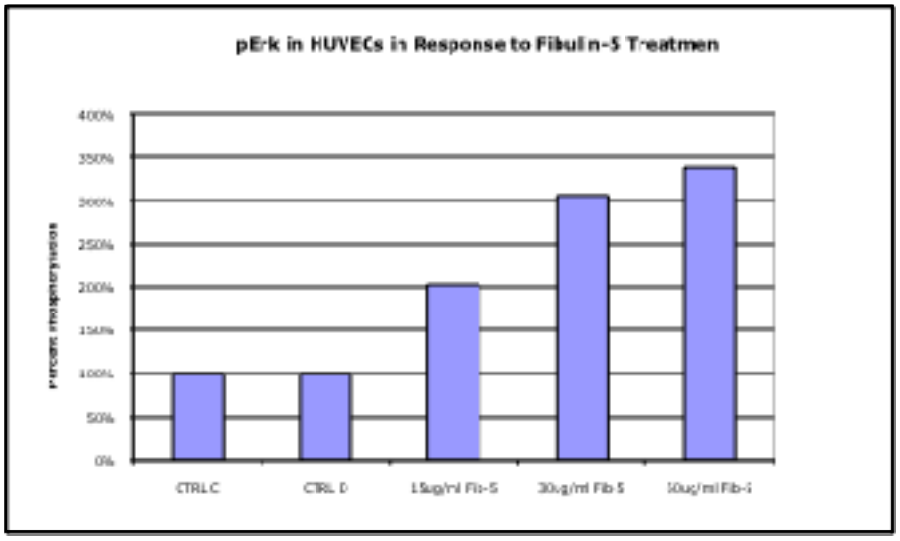


Figure 15: Phosphorylation Levels of Erk in Response to Fibulin-5 Treatment. A. Western blots demonstrating an increase in Erk phosphorylation in HUVECs in response to fibulin-5 treatment in a concentration-dependent manner. **B.** Densitometry quantifications of Erk phosphorylation normalized to levels of total Erk and GAPDH from Western blots above.

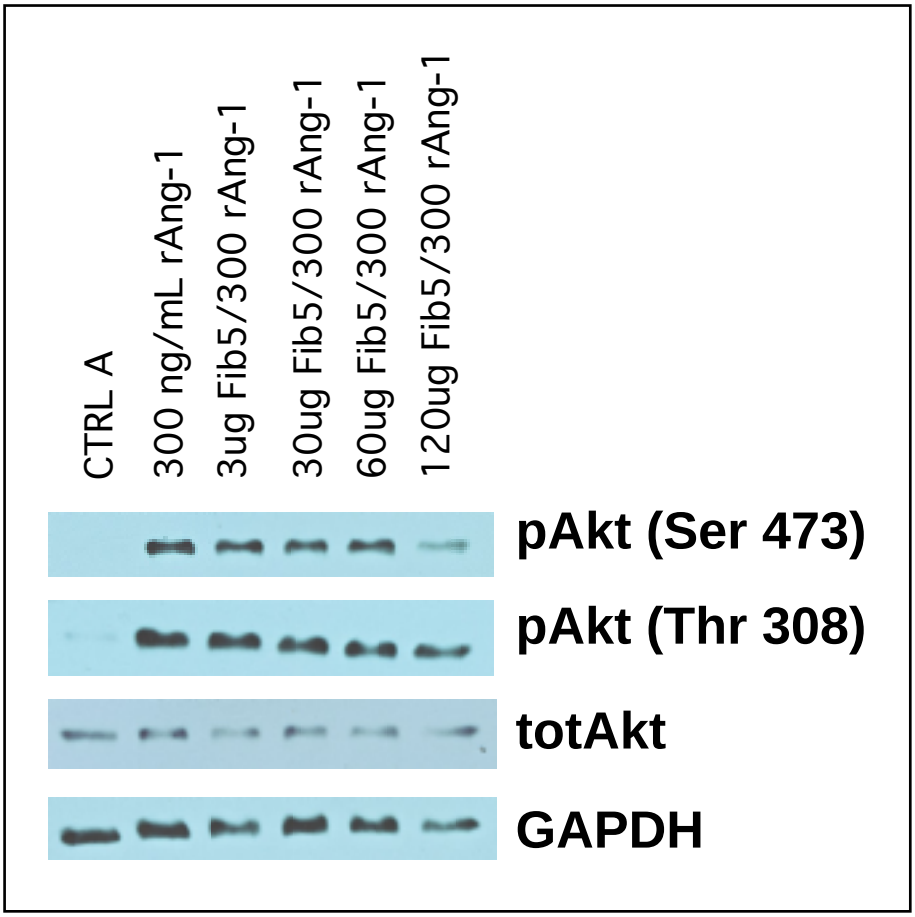


Figure 16: Phosphorylation Levels of Akt in HUVECs in Response to Ang-1 Treatment Following Fibulin-5 Pre-incubation. Western blots demonstrating Akt phosphorylation at two different epitopes (Ser 473 and Thr 308) in HUVECs. Fibulin-5 pre-incubation considerably inhibits Ang-1 induced Akt phosphorylation in a concentration-dependent manner.

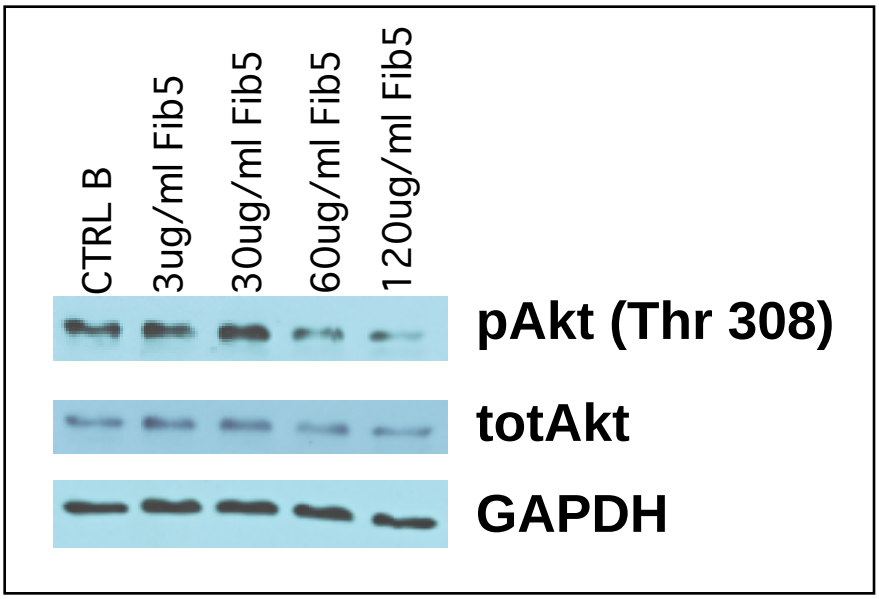


Figure 17: Phosphorylation Levels of Akt in HUVECs in Response to Fibulin-5 Treatment. Western blots demonstrating Akt phosphorylation at two different epitopes (Ser 473 and Thr 308) in HUVECs in response to 1 hr Fibulin-5 treatment. Fibulin-5 considerably inhibits basal levels of Akt phosphorylation in a concentration-dependent manner.

DISCUSSION

Fibulin-5 has been implicated as an important player in elastic fiber formation with *Fbln5*$^{-/-}$ mice displaying emphysematous lungs, loose skin and tortuous vessels (72, 73). Additionally, these mice show an increase in vascular sprouting (83), which suggests that fibulin-5 acts in the angiogenic process as an endogenous inhibitor and keeps vessels quiescent. Research in our laboratory has confirmed that pro-angiogenic molecules such as Ang-1 are indeed expressed 30-fold higher in SMCs isolated from *Fbln5*$^{-/-}$ mice when compared to wildtype animals (83). However, it is unknown if the increase in Ang-1 expression is simply an unrelated consequence of the lack of fibulin-5 in *Fbln5*$^{-/-}$ mice or that fibulin-5 acts as an inhibitor of Ang-1 expression. This notion that fibulin-5 acts as an anti-angiogenic is a relatively new concept and for the most part uncharacterized. In the present study we attempted to probe possible mechanisms by which fibulin-5 could act as an endogenous inhibitor of angiogenesis through candidate cell surface molecules such as the integrins and HSPGs, as well as by overriding the pro-angiogenic activity of Ang-1 via inhibition of Ang-1 mediated p-Akt levels.

Contrary to what has been previously reported (100), we have shown that fibulin-5 indeed has a very high affinity for heparin using solid-phase binding assays. This interaction is specific for it can be inhibited in the presence of competing soluble heparin in a concentration-dependent manner. Discrepancies in our results compared to previous reports could be due to protein folding or coating differences of fibulin-5 in the binding assays used in each study. In the study by Lomas and colleagues, where fibulin-5 was not found to bind heparin, a Biacore

3000 kinetic analysis system was used to determine binding, whereas used solid-phase binding assays (80). In our study, fibulin-5 molecules were allowed to assemble into polymers whereas the fibulin-5 used in the Biacore assay was in monomeric form which perhaps affected its binding affinity. Nevertheless, our finding that fibulin-5 binds heparin was strong and specific. This result leads to the possibility that fibulin-5 may be able to bind the cell surface HSPGs, such as syndecans and glypicans.

Interestingly, HPSGs themselves have also been implicated in the angiogenic process. Endostatin, an anti-angiogenic molecule, has been shown to block FBF- and VEGF-mediated angiogenesis only through its heparin-binding domain (101). Deletion of these domains in endostatin, which are comprised of different Arginine residues, results in decreased FGF- and VEGF-induced angiogenesis in chicken chorioallantoic membrane, decreased FGF- and VEGF-induced chemotaxis of primary endothelial cells, and reduced tumor vascularization *in vivo* (101). Fibulin-5 could potentially act as an anti-angiogenic molecule by binding cell surface HSPGs in a similar fashion to endostatin. In future studies, the heparin binding domain(s) of fibulin-5 could be mapped, mutated and the potential of the domains to decrease anti-angiogenic activity assessed.

Focal adhesions (FA) are mechanical linkages of cells to the ECM which are able to transmit regulatory signals. They usually involve cell surface integrin receptors, however, other molecules, such as the cell surface HSPG syndecan-4 have also been shown to induce FA formation (102, 103). Furthermore, it has been reported that integrins and HSPGs work in concert to promote cell attachment to

ECM molecules via motifs found on ECM proteins, referred to as synergy sites (104). These sites are composed of integrin- and heparin-binding motifs that work in concert to promote binding to the cell surface. Although not confirmed, a synergy site(s) is also likely to be found on fibulin-5. Indeed we have shown that fibulin-5 binds to heparin and it has already been shown to bind integrins via an RGD motif in its 1st cbEGF domain. Thus it has the necessary components for a synergy site(s) (63, 72). Future studies should first assess the specificity of the binding affinity of fibulin-5 to heparan-sulfate proteoglycans specifically and not just to heparin, as well as the ability of the binding motif along with the RGD motif to work in concert to promote cell surface binding.

Our findings demonstrate that recombinant fibulin-5 RGE mutant protein results in significantly lower levels of EC attachment when compared to the full-length protein. This confirms the previously reported RGD dependency of fibulin-5 for integrin binding as demonstrated by Nakamura et al. (1999) using competing RGD peptides instead of recombinant mutant protein (63). Although others have shown that binding of the RGD motif in fibulin-5 to integrins can be completely inhibited by increasing concentrations of soluble RGD peptides (63), our results did not support this observation. The RGE protein was still able to induce a considerable level of EC attachment, albeit at a significantly lower amount than full-length fibulin-5. This suggests that there is an alternate binding site(s) on fibulin-5 besides the RGD motif which can also bind molecules on the cell surface. However, it is important to note that the TBS/calcium negative controls showed no significant difference in EC attachment from that of fibulin-5 RGE protein. This is likely an

artifact of the inherent ability of the tissue culture plastic from the 4-well slides used in our cell binding assays to induce very high levels of cell attachment in general, thus promoting comparable EC attachment levels to that of the RGE protein. The binding elicited by the RGE protein is presumably not background, but specific as we performed an ELISA to certify that the plate surface was indeed saturated with the coated protein. Therefore, we can construe that in the TBS/calcium control, ECs recognized the cell culture plastic surface to which they have a high and unspecific binding affinity, whereas the ECs which bound to the RGE protein were exposed to a completely saturated surface of RGE protein and specifically bound to the epitopes encountered on this coated protein and not those of the tissue culture plastic.

Interestingly, fibulin-5 has been shown to be unable to induce any activation downstream of the integrin receptor (80). Fibulin-5 binds human SMCs through $\alpha5\beta1$ and $\alpha4\beta1$ integrins, but is unable to support their activation as assessed by the lack of FA formation in these cells (80). This suggests that fibulin-5 may act as a receptor antagonist by blocking or dampening any integrin-mediated response. In contrast, fibronectin binds the same integrins as fibulin-5 on SMCs and induces integrin activation as assessed by abundant FA (80). Our findings showing that coated fibronectin induced much higher EC cell attachment levels than those on coated full-length fibulin-5 corroborates this data. Fibronectin induced a higher number of EC cell attachment and cells also looked healthier and more well-spread as seen by the light microscope when compared to fibulin-5 (data not shown).

Given that fibronectin is also an ECM protein, it is possible that fibulin-5 not only acts as an integrin antagonist, but does so while competing with the agonistic

fibronectin molecule for the same site on the integrin. Thus, fibulin-5 could acts as an anti-angiogenic molecule by competing with the integrin-dependent pro-angiogenic effects mediated by fibronectin. Fibronectin indeed has been found to be a promoter of angiogenesis increasing by VEGF expression, endothelial proliferation and tube formation (105). Furthermore, fibronectin has also been implicated in the Ang-1/Tie-2 pathway, where it sensitizes the Tie-2 receptor to Ang-1 activation via the $\alpha5\beta1$ integrin (35). $\alpha5\beta1$ integrin is constitutively associated with the Tie-2 receptor in ECs (35). Fibronectin, by binding to the $\alpha5\beta1$ integrin, can increase its association with the Tie-2 receptors on the EC surface, thus clustering the receptor and potentiating its ability to respond to lower doses of Ang-1 as assessed by levels of phosphorylated Tie-2 and EC survival (35). In such a model, fibronectin and fibulin-5 could act as competiting integrin binding molecules sensitizing or dampening the ability of ECs to respond to the pro-angiogenic stimulus of Ang-1 via the Tie-2 receptor, respectively.

We show that Ang-1/Tie-2-mediated Akt phosphorylation is reduced with fibulin-5 pre-incubation in a concentration-dependent manner This could be possibly due to displacement of the fibronectin from the integrin by fibulin-5. This competitive hypothesis with fibronectin is further supported by our solid-phase binding assay results which show that fibulin-5 does not act as an anti-angiogenic molecule by sequestering Ang-1 in the matrix and preventing it from binding Tie-2 or by binding and blocking the Tie-2 receptor from Ang-1 stimulation. Given that no specific cell surface receptor other than the integrins and possibly the HSPGs have been shown to bind fibulin-5, the integrins are likely to be involved in mediating

downstream signaling of fibulin-5. However further studies assessing the individual affinities of fibronectin and fibulin-5 to the integrin receptor and their ability to displace each other from the integrin binding site would be necessary before arriving at such conclusions. Additionally, studies measuring binding affinities as the dissociation constant (K_d) *in vitro* run into a global problem that arises when studying ECM proteins; that is that soluble recombinant proteins likely act much differently than their insoluble forms assembled into complex matrix scaffolds as found in the native ECM.

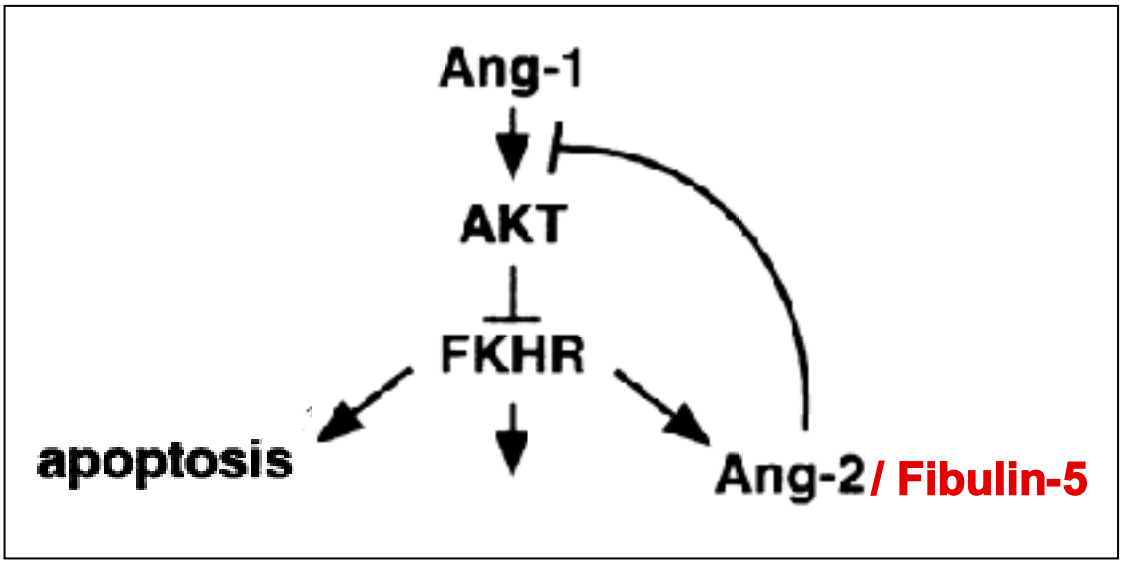
Akt is a major promoter of cell survival and cell growth. Ang-1 mediated cell survival has been shown to be dependent on the Akt pathway (99). The potent pro-angiogenic effects of VEGF are also partly induced via the PI3K/Akt pathway (106). In our experiments, we have been able to confirm induction of Ang-1-mediated Akt phosphorylation in ECs. Conversely, we have also shown that fibulin-5 alone can inhibit or decrease basal levels of p-Akt in ECs, in a concentration-dependent manner. More importantly, phosphorylation of Akt was decreased in ECs stimulated with Ang-1 following fibulin-5 pre-incubation in a concentration-dependent manner. Moreover, those levels were comparable with p-Akt levels in ECs treated with fibulin-5 alone. This suggests that fibulin-5 was not only able to inhibit p-Akt levels in ECs, but that the effect was dominant over Ang-1 and prevented Ang-1-mediated Akt phosphorylation.

Although Ang-1-dependent EC survival has been shown to be mediated by the PI3K/Akt pathway, not many of the actual downstream targets of Akt have been identified (107). A study attempting to indentify such targets was able to

demonstrate that Ang-1, via the Akt pathway is a potent inhibitor of the forkhead transcription factor FKHR (FOXO1) (107). FOXO proteins are thought to play roles in the cell stress response functioning to mediate an adaptive response to that stress, which can sometimes include apoptosis given a severe enough stress (108, 109). ECs treated with Ang-1 showed a potent ability to activate the Akt pathway, more so than VEGF or FGF. Moreover, Ang-1-mediated Akt activation in ECs was accompanied by a large increase in the phosphorylation of FOXO1 (107). Akt-mediated phosphorylation of FOXO1 inhibits its function as a transcription factor by promoting its nuclear export, as confirmed by Western blots of nuclear and cytoplasm fractions of phosphorylated FOXO1, and by preventing it from going into the nucleus and activating target genes (107).

A very exciting part of the study by Daly and colleagues was found in the microarray data used to identify target genes for FOXO1 in ECs. Interestingly, FOXO1 not only strongly induced the expression of Ang-2, an Ang-1 antagonist and inhibitor of angiogenesis, but it also up-regulated many genes involved ECM remodeling, of which one was none other than fibulin-5. FOXO1 also suppressed pro-angiogenic genes such as Survivin, which has been shown to promote EC survival by inhibiting apoptosis (110). Thus, FOXO1 appears to act as a transcription factor that not only upregulates anti-angiogenic genes, but also suppresses pro-angiogenic ones. This fits well with the model that Ang-1-mediated Akt activation consequently phosphorylates FOXO1 and ultimately sequesters it in the cytoplasm, preventing FOXO1 from activating or suppressing its array of target genes in the nucleus, which would otherwise lead to an anti-angiogenic outcome.

FOXO1 up-regulation of Ang-2 expression provides a model by which Ang-1, via Akt phosphorylation can suppress both the activity of FOXO and its many anti-angiogenic effects as well as preventing the resulting expression of its antagonist, Ang-2. As an Ang-1 antagonist, Ang-2 binds but does not stimulate the Tie-2 receptor thus preventing the downstream activation of Akt, allowing FOXO1 to localize to the nucleus and carry out its anti-angiogenic actions. Ang-2 is not only one of the genes up-regulated by FOXO but it is also able to feed into this pathway upstream of Akt activation by competing with Ang-1. In this model, Ang-2 forms a positive feedback loop whereby its expression can inhibit Ang-1-mediated Akt phosphorylation, which will consecutively result in allowing FOXO to enter the nucleus, and promote even more Ang-2 expression. Interestingly, fibulin-5, like Ang-2, is also up-regulated by FOXO1 and since we have shown that it is able to block Ang-1-mediated Akt phosphorylation, it fits perfectly into this model just as Ang-2 does (Figure 18). Thus, we have identified a novel Akt-dependent mechanism by which fibulin-5 could act as an anti-angiogenic molecule. In future studies, we will confirm this by model by localizing phosphorylated FOXO1 between nuclear and cytoplasmic fractions in ECs stimulated with fibulin-5. It is likely that we will see an effect of fibulin-5 on FOXO1 nuclear and cytoplasmic trafficking that is congruent to this current model because both Ang-1 treatment and myristoylated Akt (constitutively active Akt) were able to block FOXO target gene expression as assessed by luciferase expression which was controlled by a promoter containing FKHR-binding sites (111). Thus, Akt activity alone and anything which affects it, for example fibulin-5, is sufficient to affect FOXO localization, activity and its downstream effects.



(Modified from Daly et al.,Genes Dev.18(9):1060-71, 2004.)

Figure 18: Diagram Depicting Novel Findings for Fibulin-5 in the Ang-1/Akt Signaling Pathway. Angiopoietin-1 regulates endothelial cell gene expression via FKHR (Forkhead Transcription Factor). Ang-1, via activation of the Akt pathway, inhibits FKHR from entering the nucleus thus preventing the resulting changes in the expression of genes that regulate vascular remodeling and lead to endothelial cell apoptosis. Expression of fibulin-5, likeAng-2, is induced by FKHR. Work from this thesis has shown that fibulin-5 is able to inhibit Ang-1-mediated Akt phosphorylation upstream in this pathway. The above model not only illustrates a positivefeedback loop whereby Ang-2 and fibulin-5 stimulation leads to further expression of the proteins but more importantly, provides a novel mechanism by which fibulin-5 could act as an anti-angiogenic molecule via an Akt-dependent pathway.

CONCLUSIONS

Angiogenesis is a complex event. Understanding the multiple factors which are involved in this process is of importance not only for developmental biology but also for clinical intervention of a variety of diseases, such as cancer and age-related macular degeneration, which have vascular remodeling as part of their pathogenesis. Fibulin-5 has been previously implicated in angiogenesis and in this study, we have further characterized the molecular mechanism by which fibulin-5 might act as an inhibitor of angiogenesis. Through solid-phase binding assays, we have been able to show that fibulin-5 does not modulate Ang-1 activity in ECs via direct physical interactions with Ang-1 or Tie-2. We showed fibulin-5 does not bind to Tie-2 and therefore cannot block the receptor, nor does it bind Ang-1, which could sequester it into the matrix. Also, through solid-phase binding assays, we were able to show a high affinity of fibulin-5 for heparin suggesting it may bind cell surface heparan-sulfate proteoglycans molecules, which have also been implicated in the angiogenic pathway. Most importantly, we discovered a novel Akt-dependent mechanism by which fibulin-5 can inhibit the pro-angiogenic activity of Ang-1 in ECs. Furthermore, based on a recent publication, we can now propose a model by which fibulin-5 inhibition of Akt phosphorylation feeds into a positive feedback loop involving the forkhead transcription factor FKHR (FOXO1). This pathway leads to further production of fibulin-5 and maintenance of the anti-angiogenic activity.

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