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QUANTIFYING THE EFFECTS OF HYDROSTATIC PRESSURE ON FIBROBLAST GROWTH FACTOR-2 BINDING BY THE HUMAN ENDOTHELIUM

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
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By

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ABSTRACT OF THESIS

QUANTIFYING THE EFFECTS OF HYDROSTATIC PRESSURE ON FIBROBLAST GROWTH FACTOR-2 BINDING BY THE HUMAN ENDOTHELIUM

Fluid pressures regulate endothelial cell (EC) tubulogenic activity involving fibroblast growth factor 2 (FGF-2) and its receptor, FGF receptor 2 (FGFR2). Our lab has recently shown that sustained 20 mmHg hydrostatic pressure (HP) upregulates EC sprout formation in a FGF2-dependent fashion. This upregulation of sprout formation may be due to enhanced FGF-2 / FGFR2 interactions in the presence of 20 mmHg HP. We hypothesize that exposure of ECs to 20 mmHg sustained HP enhances FGF-2 binding kinetics. We used a custom hydrostatic pressure system, immunofluorescence, and FACS to quantify FGF-2 binding by ECs in the absence or presence of a range of HPs for 30 minutes. Relative to cells maintained under control pressure, ECs exposed to 20, but neither 5 nor 40 mmHg, displayed a significant increase in binding affinity to FGF-2. EC binding of VEGF-A, another angiogenic growth factor, was unaffected by similar pressure stimuli. Additional studies showed that pressure-selective FGF-2 binding was independent of FGFR2 surface expression. These results implicate the FGF-2 axis in the pressure-sensitive, magnitude-dependent angiogenic processes which we have previously described. The present study provides novel insight regarding the involvement of FGF-2 signaling and interstitial pressure changes in various microvascular physiological and pathobiological processes.

KEYWORDS: endothelial cell, mechanobiology, pressure sensitive growth factor binding, fibroblast growth factor-2, angiogenesis

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11 July 2017

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Thank you to my parents, Amy and Pat, and to my brother, Bruce, who have given me endless patience and love, not only during graduate school but in all my endeavors. And to my friends and fellow lab members – thanks for hanging out with me.

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INTRODUCTION

1.1 Cellular Mechanobiology

Mechanobiology and mechanotransduction

Cellular mechanobiology is the study of how physiological processes are influenced by the local mechanical environment – the mechanoenvironment – of cells in tissues [1]. The mechanoenvironment of a cell depends on contractile state of the inherent tissue (muscle, wound tissue, etc.), loading conditions due to forces which originate outside the body, extracellular matrix structure and geometry, and interstitial/extracellular fluid dynamics [1]. Each of these have been reported to, in some way, influence cellular processes such as proliferation, differentiation, cellular turnover, and morphology in organisms varying in complexity from single-cell bacteria to mammals [2, 3].

A central theme of cellular mechanobiology is mechanotransduction – the processes by which forces that originate outside of a cell are gathered, interpreted, and acted upon by a cell [1]. The body itself can be considered the force transduction network that transmits external mechanical stimuli from the macroscale (i.e., organs and tissues) to the nanoscale, where force-cell interactions elicit chemical changes at the molecular level [4]. From macroscale to nanoscale, this transduction occurs through deformation of the extracellular matrix (ECM) by influencing physically coupled ECM proteins and cell-cell adhesion sites [5]. Force-ECM interactions also result in the movement of fluid and nutrients over cells and proteins. Propagation of mechanical signals through the ECM and extracellular spaces ultimately converges at the surface of the mechanosensitive cell [6].

Mechanosensing

The cell membrane is the location at which transmission of the mechanical stimulus occurs through a variety of mechanosensing mechanisms. These mechanisms roughly fall into three categories: (1) enhancement of normal processes due to secondary effects of mechanical stimulation (such as enhanced nutrient distribution in articular cartilage upon movement of the joint [7]) as well as (2) mechanosensitive protein complexes, such as ion channels, and (3) mechanosensitive macromolecules (i.e., proteins), which presumably undergo deformation or changes in conformational activity and, in doing so, alter downstream cellular activity.

Mechanosensitive channels are membrane-associated pores into the cell which can essentially be considered "interpreters of membrane tension" [8]. Some channels rely on bending and stretching of the lipid bilayer for activation while other channels may rely on ligand-receptor interactions that affect the open-close state [9]. Physical perturbations of the membrane presumably force MS channels to adopt distinct conformations, and each channel conformation results in specific cellular responses. [8].

Mechanosensitive macromolecules are typically proteins associated with cell-ECM focal adhesion complexes, such as integrins, or mechanosensitive enzymes and receptors [10]. External loads applied to integral membrane proteins are transmitted across the cell membrane and accommodated intracellularly by actin cytoskeletal components, their associated adaptor proteins, enzymes, and organelles [2, 6, 10]. Some membrane-associated enzymes, such as phospholipase A_2 and C, have shown altered enzymatic activity in the presence of a mechanical stimulus [11]. Additionally, a number of G protein-coupled receptors, GPCRs, have been recognized to undergo stretch-induced receptor

activation in the absence of endogenous chemical factors [11, 12]. The mechanosensitive aspects of such receptors are thought to be due to variation in receptor conformation upon application of mechanical stress. These conformational changes result in differential activation of the downstream signaling effects of the receptors [11]. A final consideration for mechanosensitive ligand receptors are growth factor receptors such as vascular endothelial growth factor receptor-2 [13]. When subjected to mechanical forces, VEGFR-2-expressing endothelial cells spatially modulated expression of VEGFR-2 and, in a separate study, stretch-induced secretion of VEGF from human chondrocytes was observed [13, 14]. Collectively, these observations implicate stretch and other mechanical stimuli in the modulation of numerous biological systems.

1.2 Anatomy and physiology of the vasculature

Cardiovascular system

The cardiovascular system is responsible for the unidirectional transport of nutrients, dissolved gases, and waste products throughout the body via a complex, closed system of blood vessels and valves which vary in size and structure based on their function [15]. Arteries, which receive blood exiting the heart, have thick, multi-layered elastic walls of muscle and connective tissue to accommodate the pulsatile pressures and blood volume associated with the pumping actions of the heart. These large vessels transport and slow the blood before emptying into progressively smaller-diameter, thinner-walled arterioles, which ultimately branch out into a network of capillary vessels, i.e., the microcirculation. To complete the circulatory system, capillaries coalesce into progressively larger venules

and veins, which form the venous system. These veins carry metabolic byproduct-rich blood to the lungs and kidneys via the heart [15].

The microvasculature and the microvascular endothelium

At the smallest capillary diameter, vessel walls are composed solely of an endothelial monolayer [15]. It is this anatomy which allows for efficient exchange of nutrients and oxygen from the blood to the tissues and waste products from the tissues to the blood [16]. Additionally, the first stages of the immune response are mediated at the vascular endothelium as leukocytes roll over, migrate along, and eventually transmigrate across, the endothelial layer – the inflammatory process that is vital in the body's ability to fight off infection and tissue damage [17]. Blood-endothelium interactions are facilitated at the surface of the endothelial cell by membrane-bound receptors, adhesion sites, and proteins which bind a wide variety of substances including growth factors, immune response components, hormones, and plasma molecules [18].

To meet the ever-changing demands of tissues, the microcirculation undergoes continuous vascular remodeling in the form of angiogenesis and lymphangiogenesis, the creation of new vessels through the branching of existing blood and lymphatic vessels, respectively. Endothelial cells are critical for initiating and controlling microvessel growth (i.e., blood or lymphatic capillaries) and microvascular remodeling. The role of ECs in these tubulogenic processes is due, in part, to the endothelial cell being both a source and/or target of growth factors such as FGF and VEGF, both which are essential to the aforementioned tubulogenic processes [17].

ECs are consistently subjected to mechanical forces in the form of fluid shear stress, fluid pressure, and stretch. Fluid shear stress is a product of the drag created by blood or tissue fluid on endothelial cells as blood flows through the vasculature or as they migrate into the interstitium during microvascular remodeling, respectively. Fluid pressure and stretch, exacerbated by the pumping actions of the heart causing circumferential distortion of the vessels, subject ECs on the interior of blood vessels to physical stretch and hydrostatic pressure increase with increased blood volume. Similarly, pressure fluctuations and matrix deformations arise in extracellular matrices, where endothelial cells migrate during angiogenesis, of tissues undergoing mechanical loading, contractile activity, or changes in hydrostatic pressures (e.g., postural changes). The magnitude of these forces varies based on both vessel or tissue structure and location as well as the presence of pathobiology (e.g., circulatory or interstitial hypertension). In the circulation, shear stress on arterial walls, ranging from 10 to 70 dynes/cm^2, is much higher than in the venous system, where shear stress ranges from 1 to 6 dynes/cm^2 [19]. In the resident tissues of endothelial cells, healthy interstitial pressure will vary within the range of -1 to -3 mmHg where the capillary pressure will be in the magnitude of 20 mmHg, encouraging the exchange of fluids across the endothelial boundary [20].

Endothelial dysfunction is characteristic in most forms of cardiovascular disease such as hypertension, atherosclerosis, coronary artery disease, and microvascular disease [21]. For example, there is a well-established relationship between hypertension and endothelial dysfunction, though it is controversial if hypertension causes endothelial dysfunction or if patients with dysfunctional endothelial cells are more susceptible to hypertension [22]. Studies that found data to support the former include reports that

elevated systolic pressure in adolescents predicts impaired endothelial function in adulthood [23] and that significant, acute increases in blood pressure impair endothelial function temporarily [24]. The findings from these studies suggest that an irregular pressure environment may be a contributing factor to a decline in EC health in pathologies with symptomatically high fluid pressure such as hypertension. Furthermore, endothelial dysfunction due to changes in local mechanoenvironmental factors may play a role not only in the vasculature, but also in tissue environments where tubulogenic processes such as angiogenesis occurs.

Lymphatic system

Another component of the circulatory system is the network of lymphatic vessels which are distributed throughout tissues to regulate interstitial fluid volume for the purpose of preventing edema and supporting the immune system [25]. The lymphatic system is similar in branched structure to the vasculature but, instead of transporting their contents to the surrounding tissues like capillaries, they absorb excess fluid from the tissues. Small lymphatic capillaries, which are close in proximity to vascular capillaries, absorb blood plasma fluid which leaves the microcirculation. Lymphatic capillaries merge to form larger lymphatic vessels which carry the absorbed fluid, now called lymph, to lymph nodes where it can be filtered of pathogens [26]. From the lymph nodes, efferent lymphatic vessels carry lymph from different regions of the body to lymphatic trunks which merge into lymphatic ducts [26]. At the lymphatic ducts, filtered lymph is reintroduced into the blood steam. The main function of the lymphatics is to regulate blood volume and pressure, modulate

interstitial fluid volume and pressure, and also filter tissue fluid returning to the circulation [26].

Lymphatic vessels have a luminal (lymph-contacting) surface composed of endothelial cells which differ phenotypically from vascular endothelial cells [27]. However, improper function of the lymphatic system is evident in some of the same disease states as is endothelial dysfunction – for example, in patients with hypertension [28]. A recent study reported that, in patients with high salt diet associated hypertension, the anatomy of the lymphatic vessels displays increased density and hyperplasia [29]. Hyperplastic lymphatic vessels, those which are enlarged, partially compressed, and nonfunctional, can be observed in early stages of carcinogenesis alongside blood vasculature that displays altered permeability [30]. Furthermore, in developed tumors, abnormal lymphatic anatomy (e.g., increased permeability, abnormal vessel density) leads not only to decline in tissue health within the tumor but to the spread of cancerous cells throughout the body as the lymphatic vessels act as a conduit for metastasis [28, 31]. Interestingly, cancerous growth to advanced stages, including metastasis, has been associated with increased interstitial pressures in the tumor tissues [32, 33].

1.3 Tubulogenesis and tubulogenic growth factors

The formation and function of blood and lymphatic vessels of the microcirculation is essential to tissue health. Tubulogenesis, the formation of vessels, refers to the collection of biological processes by which cells form tubes stabilized by the ECM for the purpose of transporting gases, molecules, and liquids throughout the body [34]. Endothelial cells mediate three tubulogenic processes: vasculogenesis, angiogenesis, and

lymphangiogenesis [35]. Vasculogenesis is the *de novo* formation of a blood vessel network by endothelial progenitor cells, angioblasts, which occurs exclusively in embryonic development [36]. Angiogenesis is the process by which new capillary vessels sprout from existing blood vessels and lymphangiogenesis is a process similar to angiogenesis though it refers to the sprouting of new lymphatic capillaries from existing lymphatic microvessels [16, 37].

Overview of tubulogenesis in adults

Angiogenesis in healthy adults occurs during microvascular remodeling triggered by such activities as exercise training, tissue replacement during wound healing, and tissue formation during ovulation and pregnancy [16]. Angiogenesis is mediated by number of pro- and anti-angiogenic factors including tissue oxygen concentration (i.e., hypoxia) and growth factor signaling (e.g., basic fibroblast growth factor, vascular endothelial growth factor) [38]. Lymphangiogenesis is closely related to angiogenesis. During wound healing, formation of lymphatic vessels lags angiogenesis by about 3 to 5 days [39, 40]. Lymphangiogenesis is mediated primarily by vascular endothelial growth factor receptor 3 (VEGFR-3) signaling which is activated by two VEGF subtypes – VEGF-C and VEGF-D [38]. Evidence supporting a fundamental relationship between angiogenesis and lymphangiogenesis includes reports that lymphangiogenic VEGF-C can stimulate angiogenesis [40], and that two angiogenic growth factors, FGF-2 and VEGF-A, have also been shown to stimulate lymphangiogenesis [38].

Angiogenesis

Current thinking suggests that angiogenesis can occur in two different ways: sprouting angiogenesis or intussusceptive angiogenesis. Sprouting angiogenesis occurs when endothelial cells of existing vessels sprout off of a mother vessel in response to an angiogenic stimulus (e.g., growth factors) and extend into tissues devoid of blood vessels [41]. Intussusceptive angiogenesis involves the increased vascularization of an already vascularized area by a process in which an existing vessel splits into two [41]. The subtype of angiogenesis of interest in this study sprouting angiogenesis consisting of six steps (*Figure 1*).

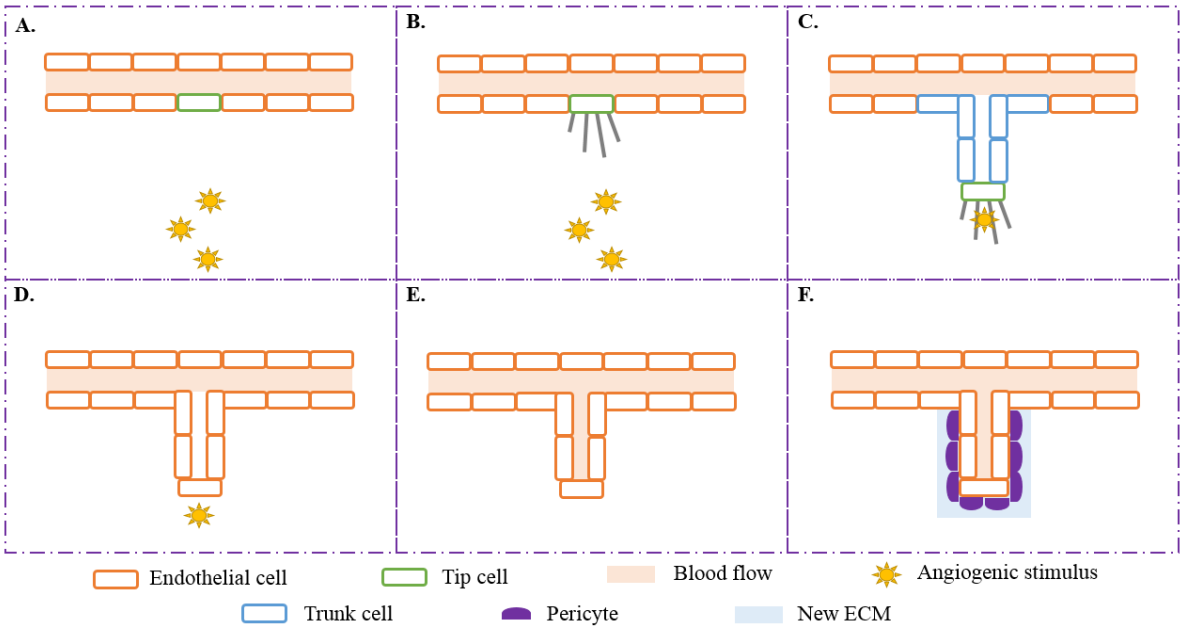

Figure 1: Process of sprouting angiogenesis. (A) Endothelial cell most strongly influenced by angiogenic stimulus differentiates into "tip cell"; (B) Tip cell extends filopodia into surrounding tissue; (C) Trunk cells extent sprout into tissue guided by tip cell; (D) Lumen of capillary forms; (E) Blood flows into new capillary; (F) Pericytes and ECM stabilize new vessel. Created with reference to [41].

First, microvascular endothelial cells sense an angiogenic stimulus, such as the presence of FGF-2 signaling. The cells closest to the stimulus will become the tip cells –

those, which “lead” the sprouting of the new vessel. These cells release matrix metalloproteinases to dissolve the basement membrane on which the ECs sit. The dissolution of the membrane allows for the tip cell to extend filopodia into the surrounding tissue to guide the growth of the vessel towards the angiogenic stimulus. Next, endothelial “trunk” cells proliferate behind the tip cell to form the length of the sprout until the full length of the new capillary is reached, at which time the “trunk” and “tip” cells differentiate and mature, and the lumen of the capillary forms. Finally, pericytes and new ECM surround and stabilize the new vessel [41].

Fibroblast growth factor 2

The fibroblast growth factor (FGF) family is comprised of a diverse group of growth factors that modulate a variety of cellular processes throughout the life of an organism from embryonic development to wound healing via a complex network of signaling pathways [42]. FGFs are secreted glycoproteins which are released to the extracellular matrix where they are sequestered by binding to heparan sulfate proteoglycans [43]. FGFs are liberated in response to chemical factors such as heparinases or proteases when their activity is required (e.g., during wound healing) [43]. The FGF family is made up of eighteen secreted proteins (e.g., FGF-1, FGF-2, etc.) that selectively interact with four unique tyrosine kinase receptor subtypes (e.g., FGFR-1, FGFR2, etc.) [44]. The function of each FGF is controlled by interactions with cofactors that mediate binding to the target FGFR [44]. FGF signaling mediates a diversity of processes ranging embryonic limb development (FGF-4), brain and eye development (FGF-8), and phosphate homeostasis in bone (FGF-23) [45].

Particularly relevant to the present study is FGF-2, also called basic fibroblast growth factor (bFGF). FGF-2 plays a role in the regulation of vascular tone and blood pressure and is a pro-angiogenic stimulus [45]. FGF-2 stimulates endothelial cell proliferation and migration and has anti-apoptotic effects on endothelial cells, all of which contribute to tubulogenesis both *in vivo* and *in vitro* [16, 45]. FGF-2 interacts with fibroblast growth factor receptor 2 (FGFR2), which, like all FGFRs, is a homodimeric transmembrane tyrosine kinase receptor. The receptor has three extracellular immunoglobulin domains (D1-D3) and one intracellular tyrosine kinase domain per subunit [46]. The specificity of the receptor is determined by D2-D3 and D1 acts as the auto-inhibitory domain [45]. The accepted mechanism of receptor activation follows a ligand-dependent dimerization model resulting in a conformational change of the intracellular tail allowing for transphosphorylation of the kinase domain of each subunit, as shown in *Figure 2* [16, 43].

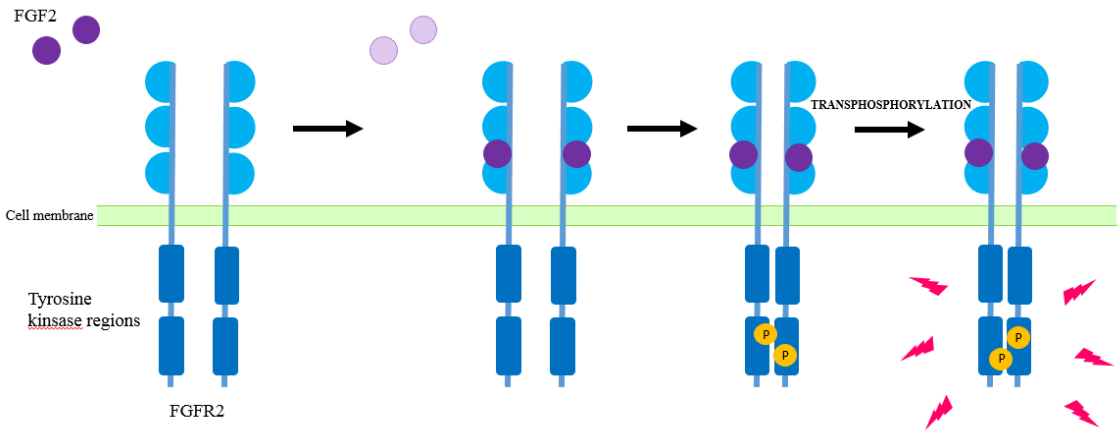

Figure 2: Schematic of accepted FGF-2/FGFR2 binding mechanism. The subunits of FGFR2 under ligand dependent dimerization and subsequent activation of the enzyme region of the receptor.

Phosphorylated intracellular kinase domains act as active docking locations for adaptor proteins, nucleotide exchange factors, and associated intracellular enzymes which initiate cytosolic signaling cascades that ultimately result in cellular actions [43]. Some of these intracellular signaling enzymes include, but are not limited to, RAS GTPase of the RAS-MAPK pathway (anti-apoptotic) the PI3K and Akt enzymes of the PI3K-Akt pathway (cell proliferation) [44].

While the aforementioned ligand-dependent dimerization model for receptor activation is widely accepted, it is important to note that some studies report discrete conformations of FGFR2 exhibiting some capacity to dimerize and transphosphorylate even in the absence of ligand [47]. Receptor activation due to ligand binding, however, does result in a higher degree of FGFR2 phosphorylation than dimerization in the absence of a ligand [47]. Furthermore, FGFR2 is capable of interacting with multiple FGFs and the FGF, which does bind (e.g., FGF-1/FGFR2 vs. FGF-2/FGFR2) dictates the degree of phosphorylation of the intercellular domain. This suggests the existence of multiple active ligand-bound and non-ligand-bound states of the FGFR which can contribute to FGF signaling and pathologies related to improper function of these pathways [47].

Vascular endothelial growth factor

The vascular endothelial growth factor (VEGF) family is composed of eleven secreted proteins which regulate angiogenesis, lymphangiogenesis, and vascular structure in vertebrates by acting on endothelial cells [48]. Similar to FGFs, VEGFs interact with transmembrane tyrosine kinase receptors containing an extracellular region for ligand binding and an intracellular enzymatic domain [49]. VEGF-A, the most abundant form,

stimulates vascular formation during embryonic development and is an important regulator of vessel formation in both healthy and diseased states [48]. VEGF-C and VEGF-D are implicated in the formation and maintenance of lymphatic vessels and have also been shown to be angiogenic [48].

1.4 The effects of mechanical stress on tubulogenic processes

Mechanosensory mechanisms fall into two categories – active and passive. Active mechanosensing refers to the process by which cells use intracellular actin-myosin stress fibers in conjunction with their focal adhesions (i.e., cell-ECM junctions) to "pull" on the surrounding ECM. In doing so, it is believed that the cell probes the topography and rigidity of the surrounding environment or substrate. Probing the environment in this way allows the cell to change shape, orientation, adhesion characteristics, and rate of proliferation to optimize function and health [2]. Passive mechanosensing refers to cells' ability to interpret forces, which originate outside the cell and are exerted on the cell or its components as these forces occur. Mechanical exertion on the cell may occur in many forms, including fluid shear stress, substrate stretch, or hydrostatic pressure. The present master's thesis studies the outcomes of passive mechanosensing by microvascular endothelial cells, which are exposed to sustained hydrostatic pressure.

Mechanosensitive traits of endothelial cells

Human endothelial cells have been well characterized with regard to their response to a variety of mechanical stresses [50-52]. Fluid shear stress across a monolayer of endothelial cells, i.e., the typical configuration in blood vessels, is known to induce

polarization of the cell population parallel to the direction of fluid flow [50]. Cyclic stretching of the substrate of endothelial cell cultures simulate diameter changes of vessels due to pulsatile blood flow and also cause morphological changes in endothelial cells – in this case, elongating with an axis perpendicular to the line of force of the stretching [51]. Finally, endothelial cells subjected to various fluid pressures have been shown to exhibit cell elongation but with orientations that are somewhat random, i.e. without a distinct preferential direction [52].

Notably, a variety of tubulogenic responses have been observed in response to mechanical stresses [41, 53, 54]. Threshold levels of fluid shear stress affect the density and architecture of the capillary bed by triggering angiogenic sprouting [41, 53]. Mechanical stretch, described above, has also been shown to enable vascular maintenance via effects on endothelial cell proliferation and tube formation as well as regulation of vascular tone and remodeling [54]. Additionally, our lab has shown that hydrostatic pressure has been shown to induce proliferation of bovine aortic endothelial cells in a pressure sensitive fashion. Specifically, exposure to a 20 mmHg pressure stimulus resulted in a more significant degree of EC proliferation than a 40 mmHg, with p-values of 0.018 and 0.047, respectively [55].

Dysregulated tubulogenesis in disease states

In many disease states associated with dysregulated tubulogenesis, there also seems to be an incidence of abnormal pressure levels in the environment of the endothelial cell. Blood vessel remodeling is a canonical feature in patients with pulmonary hypertension. This remodeling, which effects not only the microcirculation but also causes the formation

of intimal lesions in larger vessels, appears to involve misguided angiogenesis in these high-pressure locations [56, 57]. Interstitial fluid pressure in the environment of a tumor is well-characterized as abnormally elevated, in some cases reaching pressures as high as 60 mmHg [20]. Conceivably, these pressures may somehow be connected to the observed dysregulated angiogenic and lymphangiogenic behavior that is associated with patients with metastatic cancers [32]. Additionally, some patients with glaucoma experience severe neovascularization resulting in blindness as a result of chronic ocular hypertension (i.e., intraocular pressure greater than 21 mmHg) [58, 59]. In conjunction, these observations raise the question of the relationship between the pressures in the environment of angiogenic processes and the biological structures which result in angiogenesis, such as growth factor receptors.

STUDY RATIONALE

The occurrence of dysregulated tubulogenic processes in disease states with characteristically high interstitial pressures calls into question the relationship between pathogenic interstitial pressure and abnormal endothelial cell (EC) activity. While many studies have documented the effects of pressure on ECs, the mechanisms by which ECs adjust their activity level in response to changes in extracellular pressure levels has not been investigated. The present Master's thesis research was designed to elucidate additional insight related to changes in EC tubulogenic activity in response to exposure to elevations in local hydrostatic pressures. Previous studies from our lab documented the involvement of FGF in the tubulogenic responses of ECs to pressure [55, 60]. The present study attempted to extend our knowledge in this regard by focusing on revealing insight into the dependency of FGF-2 binding kinetics on the surrounding hydrostatic pressure levels of human endothelial cells.

Drawing from the findings of previous experiments by our lab, we hypothesized that select hydrostatic pressures facilitate FGF-2 / FGFR2 binding. For this purpose, a series of FGF-2 and FGFR2 binding assays were carried out to detect and quantify changes in the binding affinity of ECs to FGF-2 in response to discrete magnitudes of hydrostatic pressure. Human microvascular endothelial cell (HMVEC) cultures were exposed to select hydrostatic pressure levels between 0 and 40 mmHg using a custom hydrostatic pressure system. HMVECs were our culture model of choice based on their role in tubulogenic processes in the microcirculation and their interactions with the growth factors of interest. The present study tested the stated hypothesis according to the following aims:

1. demonstrate that FGF-2 / FGFR2 cellular binding kinetics depends on the applied hydrostatic pressure magnitude;
2. show that pressure-sensitive EC-growth factor interactions is growth factor dependent.

MATERIALS AND METHODS

3.1 Cell culture protocols

Substrates

Substrates used for cell culture were 75 cm^2 (T75) sterile tissue culture treated polystyrene cell culture flasks from BD Falcon. Substrates used for experiments with adherent cells were 60 x 15 mm sterile tissue cultured treated polystyrene culture dishes (Corning®). Experiments with non-adherent (i.e., suspension) cultures were conducted in 60 x 15 mm sterile untreated polystyrene culture dishes from (Corning®).

Cells and Culture Conditions

Human microvascular endothelial cells (HMVECs) were purchased from Lifeline Cell Technology® and cultured in Lifeline® VascuLife® Endothelial Medium (EM) supplemented with heat inactivated fetal bovine serum (FBS; HyClone™; GE Life Sciences). Cells were grown under standard cell culture incubator conditions defined as a humidified, 5% CO_2 / 95% air environment maintained at a temperature of 37 °C. During routine cell culture, the media over the cells was replaced every 48 to 72 hours.

Cell passaging

Upon reaching confluence (i.e., cell populations occupied >95% of the culture substrate surface area), HMVEC monolayers were first rinsed with 10 mL of PBS per flask for 3 to 5 minutes. The PBS was then aspirated and replaced with 2 mL of 0.5% trypsin / 1 mM EDTA solution (Sigma) per flask. The cells were incubated in the trypsin solution

for 1 to 2 minutes followed by gentle agitation to induce detachment of cells from the substrate and each other. Once cell detachment was confirmed through microscopic examination, the detached cells in trypsin solution were combined with 4 mL Medium 199 (M199), supplemented with 10% FBS (HyClone™) and 1% penicillin/streptomycin/L-glutamine solution (PSG) (HyClone™), and transferred to a 15 mL centrifuge tube (Fisher). The cell suspensions were then pelleted by centrifugation at 200xG for 5 minutes at 25 °C. Afterwards, the supernatants were aspirated and the cell pellets were suspended in a sufficient amount of VascuLife® EM to be divided between two or four new flasks (e.g., 1:2 or 1:4 split, respectively at 2 mL EM per new flask). This solution was divided between the desired number of new T75 flasks and additional EM was added for total culture volume of 10 mL. Cells of passage 3 to 11 were used in all experiments in the present study.

Cryogenic Cell storage

When needed, frozen stocks of HMVECs were prepared and preserved for use in later experiments. Cells were washed with 10 mL of PBS per flask for 3 to 5 minutes and then lifted with trypsin solution for 1 to 2 minutes. The resulting cell solution was then mixed with 4 mL of M199, containing 10% FBS and 1% PSG, and the cells were counted using a standard hemocytometer and subsequently pelleted by centrifugation at 200xG for 5 minutes at 25 °C. The supernatant was aspirated, and the cell pellet was resuspended to a concentration of 10^6 cells/mL in FrostaLife™ Xeno-Free Cryopreservation Solution (Lifeline®). Aliquots (1.5 mL) of this suspension were transferred to 2 mL sterile

cryogenic vials (BD Falcon) and stored at -80 °C overnight before being transferred to liquid nitrogen for long term storage.

As needed, frozen cell suspension stocks were rapidly thawed in a 37 °C water bath with gentle agitation. Thawed suspensions were mixed with 10 mL of VascuLife® EM and seeded into a T75 flask. To remove FrostaLife™ solution, cell culture medium was replaced within 12 – 16 hours after initial seeding.

3.2 Preparation of endothelial cell suspension cultures

Near-confluent HMVEC monolayers were rinsed with 10 mL PBS per flask for 5 minutes. Cells were then incubated with 0.05% trypsin for 1 to 2 minutes and then suspended in M199 containing 10% FBS and 1% PSG. These cell solutions were transferred to a 15 mL centrifuge tube and centrifuged at 200 G for 5 minutes. The resultant cell pellet was resuspended in 10 mL M199 supplemented with 10% FBS and 1% PSG. The suspensions were then transferred to sterile, non-tissue culture treated petri dishes to prevent cell attachment. Petri dishes were placed on a rocker within the cell culture incubator. The rocker was set to provide a low-speed, gentle agitation to the cell suspension to prevent cell attachment. Samples were incubated on the rocker for 6 hours to allow for regeneration of surface proteins, which may have been cleaved due to exposure to trypsin.

After the incubation period, the samples were transferred to 15 mL centrifuge tubes and centrifuged at 200xG for 5 minutes. The supernatant was aspirated, and the cell pellets were resuspended and subjected to two washes in 5 mL of PBS. Cells were counted with a standard hemocytometer and suspended in fresh M199 containing 10% FBS and 1% PSG to desired cell concentrations for binding and surface expression experiments. Trypan blue

was used to measure the cell viability of the HMVEC suspensions. After ensuring >90% viability, 20-μL aliquots of cell suspensions were transferred to 0.6 mL microcentrifuge tubes in preparation for pressure experiments.

3.3 Hydrostatic pressure stimulation

HMVEC suspensions were exposed to 0 (control), 5, 20, or 40 mmHg hydrostatic pressures above atmospheric using a custom-developed culture system (*Figure 3*). This hydrostatic pressure system consisted of a temperature-controlled incubator oven, a compressed 5% CO_2/95% air gas tank, a hydrostatic fluid column, an air tight chamber with inlet and exit ports, a manometer, and the interconnecting tubing.

The pressure chamber (*Figure 3.4*) consisted of a stainless-steel container with a lid that provided an air-tight seal through an O-ring. A humid environment was maintained in the chamber by adding a small amount of water to the bottom of the container. Within the chamber was a raised stage on which cell suspensions in tubes were placed to prevent contact with the water. Lids were loosely placed on the tubes containing, while in the chamber, to allow both gas exchange and proper pressurization of the cell suspensions.

During experiments, the air-tight chamber was situated in an oven to maintain it at 37 °C. The input to the chamber was downstream of a compressed gas air tank and a hydrostatic fluid column, which was used to control the chamber pressure. Specifically, the airflow path originated from the compressed gas tank through rigid polypropylene tubing that terminated at a three-way T-junction. One of the outlets to the T-junction was connected to tubing with its trailing end submerged into the water column. A second tube originating from the remaining port of the T-junction was connected to the inlet of the

stainless-steel pressure chamber. The outlet of the chamber led to a second T-junction with one branch connected to resistance tubing which was intended to create the desired back pressure in the pressure chamber. The other outlet of the final junction led to a water manometer, which was used to monitor chamber pressures.

Pressures in the chamber were created by first adjusting the outlet pressure of the gas tank to provide a slow gas flow into the water column at a pressure below the desired magnitude in the sealed pressure chamber. When a sufficient gas flow stream was achieved, the chamber pressure was fine-tuned by adjusting the depth to which the tubing in the water column was submerged (e.g., submerging tube father into water column increases chamber pressure). The pressure in the chamber was expected to be equal to the depth to which the tubing in the water column was submerged.

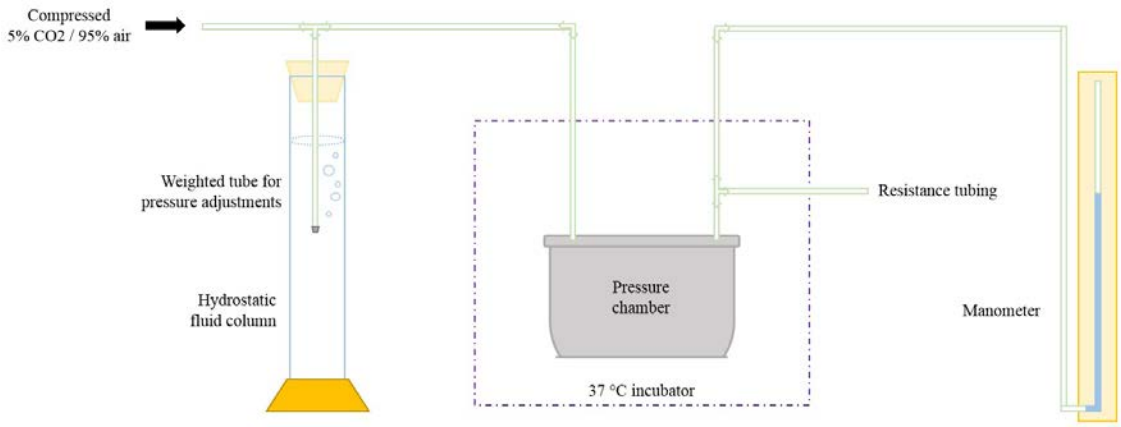

Figure 3: Schematic of custom hydrostatic pressure system.

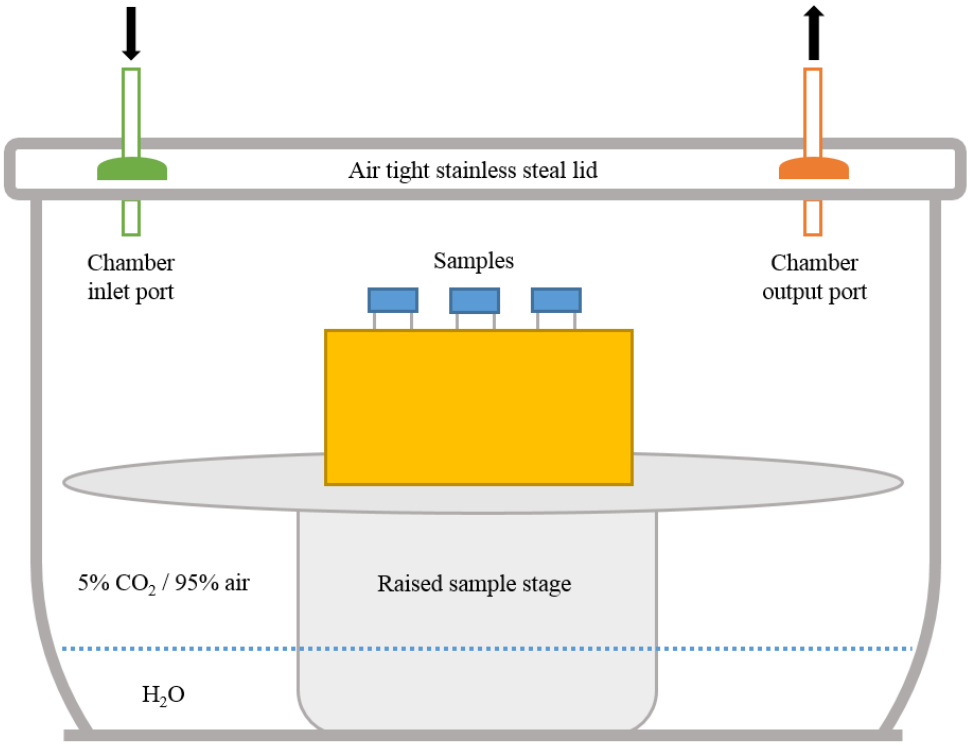

Figure 4: Schematic of pressure chamber interior.

The water manometer was used to monitor the hydrostatic pressure generated in the pressure chamber over the course of the 30-minute experiments. Centimeters of water were converted to millimeters of mercury using the conversion relationship of 1 mmHg = 1.359 cmH_2O to generate pressure of 5, 20, and 40 mmHg above atmospheric pressure (Figure 5).

Figure 5: Output monitoring of the pressure system.

During experiments, the pressure readout of the manometer was checked frequently to ensure stable exposure of cell preparations to the hydrostatic pressures of interest to the present study. Finally, for the experiments described in the present dissertation, controls were cells maintained under atmospheric pressure, but otherwise similar, experimental conditions in a standard cell culture incubator.

3.4 Growth factor binding assays

Growth factor-cell interactions was quantified using immunofluorescence, flow cytometry, and fluorescently labeled growth factors of interest. The assay used is a two-stage process, which exploits the high affinity of two naturally occurring proteins - biotin and avidin. The biotin and avidin conjugates were commercially available. The biotinylated growth factor, used as the first stain, binds to the growth factor receptor through innate binding interactions. The avidin-conjugated fluorescent marker, used as the second stain, utilizes the affinity of biotin for avidin to assign a fluorescence to bound growth factor, as shown in Figure 6.

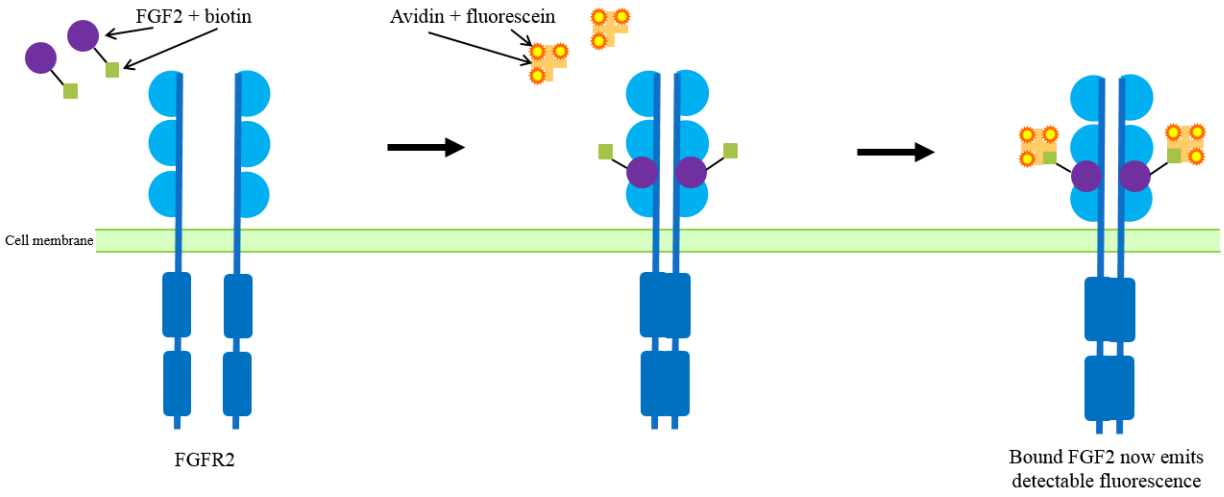

Figure 6: Schematic of biotin-conjugated FGF-2 binding assay. FGF-2 was fluorescently labeled through a two-step antibody treatment process exploiting the naturally high affinity of biotin for avidin.

For the present study, cells suspensions (5 x 10^5 HMVECs/mL) were removed from the pressure system after pressure exposure (or the cell culture incubator for controls) and immediately placed into a Nalgene® Labtop cooler to prevent conformational activity and/or prevent internalization of membrane surface proteins. The growth factor binding

assay was conducted on ice following the manufacturer's instructions (R&D Systems). Briefly, cell suspensions were incubated at 4 °C with 10 μL of commercially-available biotin-conjugated FGF-2 (R&D systems) or biotin-conjugated soybean trypsin inhibitor (negative binding control) for 30 minutes. At that time, avidin-FITC (R&D Systems) was immediately added to the cell samples and incubated for an additional 30 minutes at 4 °C in the dark.

The cells in the suspension samples were then transferred to flow cytometry tubes and washed twice with 2 mL of cold 1X wash buffer (R&D Systems). The cells were suspended in 400 μL of the same wash buffer prior to flow cytometric analysis. A Becton-Dickinson (BD) LSR II flow cytometer equipped with at 488 nm blue laser was employed to interrogate FITC-labeled samples. Briefly, the BD LSR II is composed of three systems which work in tandem to quantify fluorescence of inputted samples – a fluidics system, an optical system, and an electronic system [61]. The fluidics system uptakes sample from prepared tubes and carries these samples to the optical system. A 488 nm laser, employed for this experiment due to the excitation spectrum for fluorescein, is shone through the sample stream. The fluorescently labeled antibodies used during sample preparation absorb the 488 nm photons and emit photons with a characteristic wavelength of the fluorescent marker. The emitted photons are collected and interpreted by the electronics system of the flow cytometer [61]. In this way, mean fluorescence intensity (MFI) of a sample is indicative of the amount of growth factor bound per cell averaged over all cells analyzed. BD FacsDIVA software was used to record MFI of each sample group for analysis and comparison.

The same procedure was conducted to test the effects of pressure on HMVEC binding affinity to biotin-conjugated VEGF-A (R&D Systems).

3.5 FGFR2 surface expression quantification assay

HMVEC suspensions were prepared following the protocol detailed in **Section 3.2** to a final cell concentration of 5 x 10^5 cells / mL. After exposure to either 0, 5, 20, or 40 mmHg hydrostatic pressure for 30 minutes (**Section 3.3**) the HMVECs suspensions were placed immediately on ice to prevent receptor conformational activity. The cells were pelleted at 200xG for 5 minutes at 4 °C and subsequently fixed with 1 mL of 2% paraformaldehyde with gentle agitation for 30 minutes at room temperature. The cells were then washed three times with 1% BSA in PBS, suspended in 1 mL of 1% BSA in PBS and incubated with allophycocyanin (APC)-conjugated antibody to human FGFR2 or APC-conjugated mouse IgG, as a negative binding control, in the dark for 60 minutes at room temperature.

Afterwards, the cells in the suspension samples were washed three additional times with 1% BSA in PBS, suspended in 300 μL of PBS, and subjected to flow cytometric analysis using a BD Accuri C6 flow cytometer equipped with a 640 nm red laser. The BD Accuri C6 flow cytometer interrogates samples similarly to the BD LSR II described in **Section 3.4** with the exception of employing a light source capable of generating a higher wavelength excitation wavelength to accommodate the excitation spectrum of the APC fluorophore. For this assay, MFI values represented FGFR2 surface expression. BD Accuri C6 Plus software was used to record fluorescence intensity of each sample group for analysis and comparison.

3.6 Protein extraction

For these studies, we examined the effects of pressure on the total cellular amount of protein in HMVECs. HMVECs were seeded onto tissue-culture treated petri dishes at a density of 10^5 cells/mL and cultured under VascuLife® EM for 48 hours. At that time, the culture media was aspirated from petri dishes, and the cells were washed with 10 mL of PBS. After this wash step, the cells were submerged under 10 mL of M199 with or without 5 ng/mL FGF-2 and immediately exposed to 20 mmHg hydrostatic pressure for 30 minutes. Controls were cells prepared in similar fashion but maintained under atmospheric pressure conditions in a standard cell culture incubator.

After experiments, the tissue culture plates containing adherent HMVECs were immediately placed on ice after removal from the pressure system or the incubator, washed with 10 mL ice cold PBS per petri dish, and subsequently incubated in 0.5 mL of ice cold cell lysis buffer (Cell Signaling Technology) for 5 minutes. The lysed cell layers were then scraped into 1.6 mL micro centrifuge tubes, sonicated on ice for 3-30 second bursts in a VWR Galaxy 16 sonicator. Sonicated samples were microcentrifuged at 14,000 rpm for 10 minutes at 4 °C to pellet insoluble cellular debris. Single use aliquots of the supernatant were transferred to 0.6 mL centrifuge tubes and stored at -20 °C.

3.7 Total protein quantification

A Pierce™ Detergent Compatible Bradford assay (Thermo Fisher Scientific) for total protein quantification was used following the manufacturer's instructions. A standard curve was prepared using bovine serum albumin (BSA). Frozen cell lysates were thawed at room temperature. Aliquots (10 μL) of BSA standards at known concentrations or

experimental samples were deposited in triplicate into individual wells of a 96 well plate. Then, 300 μL of Bradford assay reagent was added to the samples, mixed well, and incubated at room temperature for 10 minutes. The bottom of the wells were gently wiped with a Kimwipe, and the plate was placed into a BioTek μQuant spectrophotometer. Colorimetric emission was determined under 595 nm illumination. The recorded transmittance of test each sample is indirectly proportional to the protein concentration.

3.8 Statistical analysis

Experimental data were expressed as mean ± standard error of the mean. For all FACS experiments, control fluorescence levels were established by including samples that were completely unstained and samples that were incubated in primary and secondary stains individually. Mean fluorescent intensity for each sample, then, was the difference between the measured fluorescence and the measured control fluorescence. For each replicate, sample means of pressurized cells were compared to controls using a paired, two sample t-test where a $p < 0.05$ indicated a significant difference. Bound FGF-2 was expressed as a fold change (fluorescent intensity of sample / fluorescent intensity of control) and fold changes were compared using a two-way analysis of variance.

RESULTS

4.1 Effect of hydrostatic pressure on FGF-2 binding by HMVECs

The present study examined the influence of hydrostatic pressure on FGF-2 binding by HMVECs. Under all conditions tested (*Figures 7-9*), HMVECs exhibited higher levels of binding affinity to FGF-2 than to soybean trypsin inhibitor (our negative binding control protein). Exposure to 5 mmHg hydrostatic pressure had no effect on FGF-2 binding compared to cells maintained under control (i.e., atmospheric) pressure conditions. In contrast, HMVEC binding affinity to FGF-2 but not soybean trypsin inhibitor was significantly ($p < 0.05$) increased under a hydrostatic pressure of 20 mmHg relative to cells under control pressures ($p < 0.05$). Notably, HMVECs incubated in the presence of FGF-2 under a 40 mmHg hydrostatic pressure exhibited binding affinities to FGF-2 and soybean trypsin inhibitor that were similar to those of controls. The pressure treatments of 0, 5, 20, and 40 mmHg were compared with a two-way analysis of variance. The results of this ANOVA show that the fold change (fluorescent intensity of sample / fluorescent intensity of control) between 0 and 20 mmHg was significantly different, but the changes within the treatments of 5, 20, and 40 mmHg were not significantly different from one another.

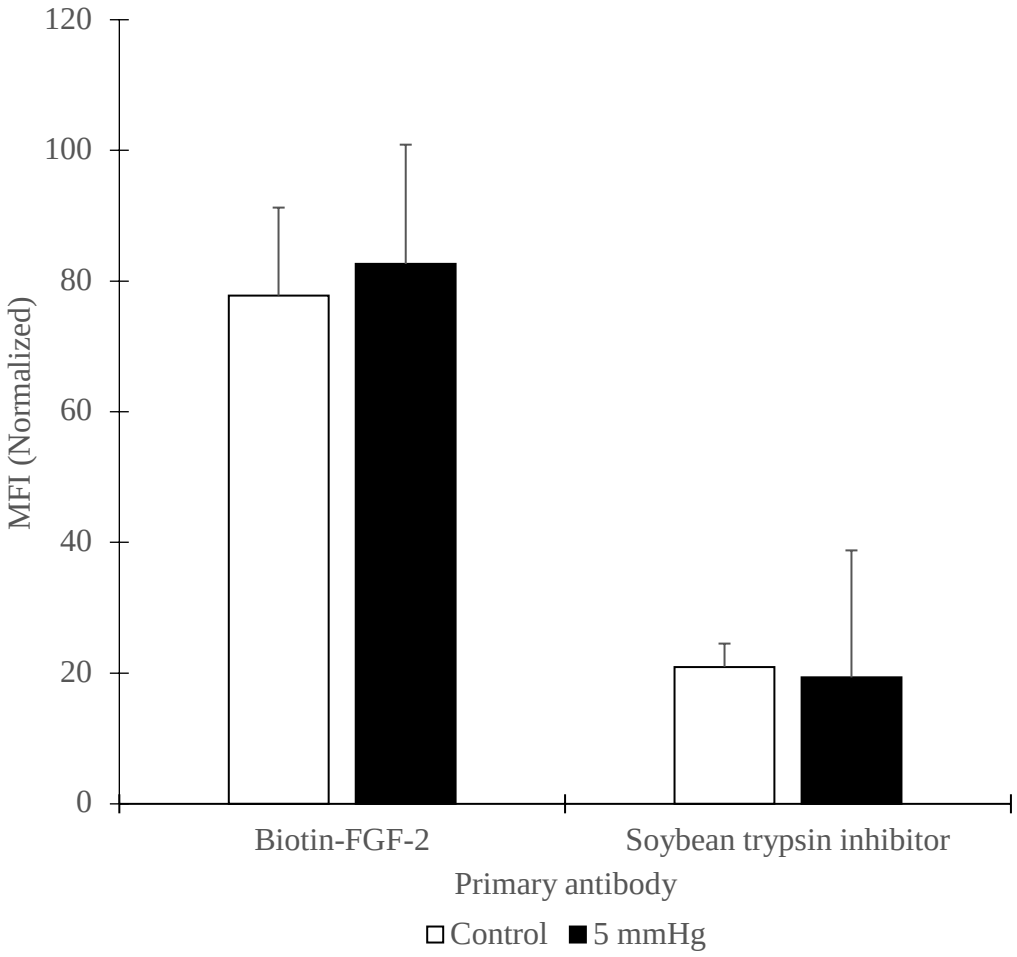

Figure 7: Exposure to 5 mmHg for 30 minutes had no effect on HMVEC-FGF-2 binding. Bars signify mean ± SEM, HMVECs of passage 4 through 11 are represented in this data, n=6.

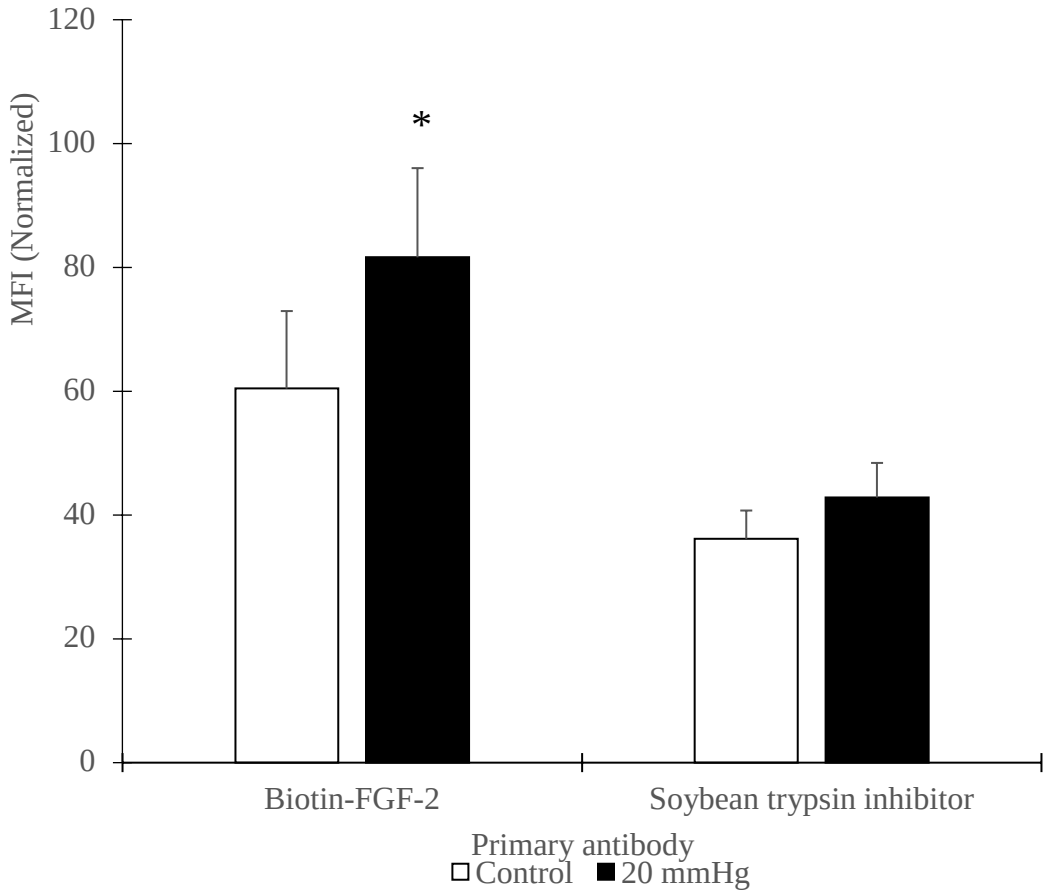

Figure 8: HMVECs exhibit significantly increased binding of FGF-2 after exposure to 20 mmHg hydrostatic pressure for 30 minutes. Compared to samples held at atmospheric pressure, samples exposed to 20 mmHg for 30 minutes exhibited significantlyincreased binding of FGF-2. Bars signify mean ± SEM (* = $p < 0.05$). HMVECs of passage 3 through 11 are represented in this data, n=7.

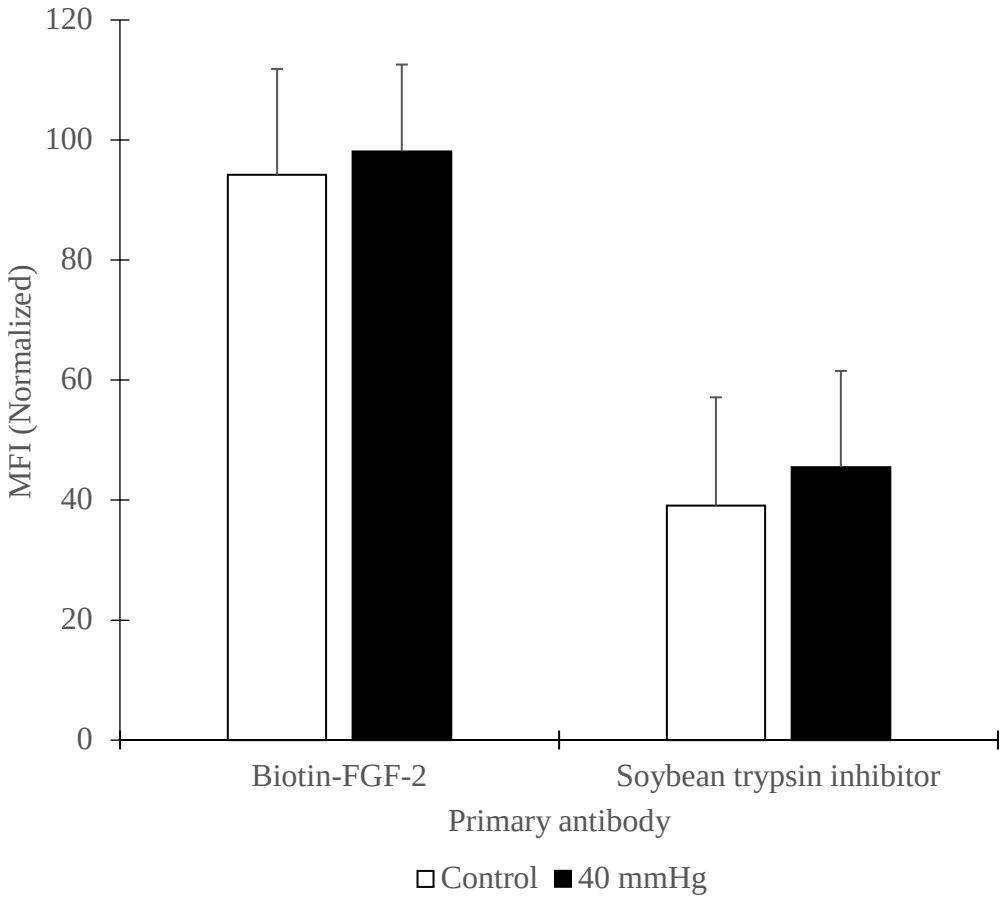

Figure 9: Exposure to 40 mmHg for 30 minutes had no effect on HMVEC-FGF-2 binding. Bars signify mean ± SEM, HMVECs of passage 4 through 11 are represented in this data, n=6.

4.2 Effect of hydrostatic pressure on VEGF-A binding by HMVECs

To evaluate the growth factor selectivity of pressure-sensitive FGF-2 binding, the present study assessed whether exposure to 20 mmHg altered HMVEC binding affinity to VEGF-A like it did for FGF-2. HMVECs displayed binding affinity to VEGF-A at higher levels than to that of soybean trypsin inhibitor under all pressure conditions tested. Exposure to 20 mmHg for 30 minutes did not enhance VEGF binding by endothelial cell cultures relative to similarly prepared cells maintained under atmospheric pressure conditions.

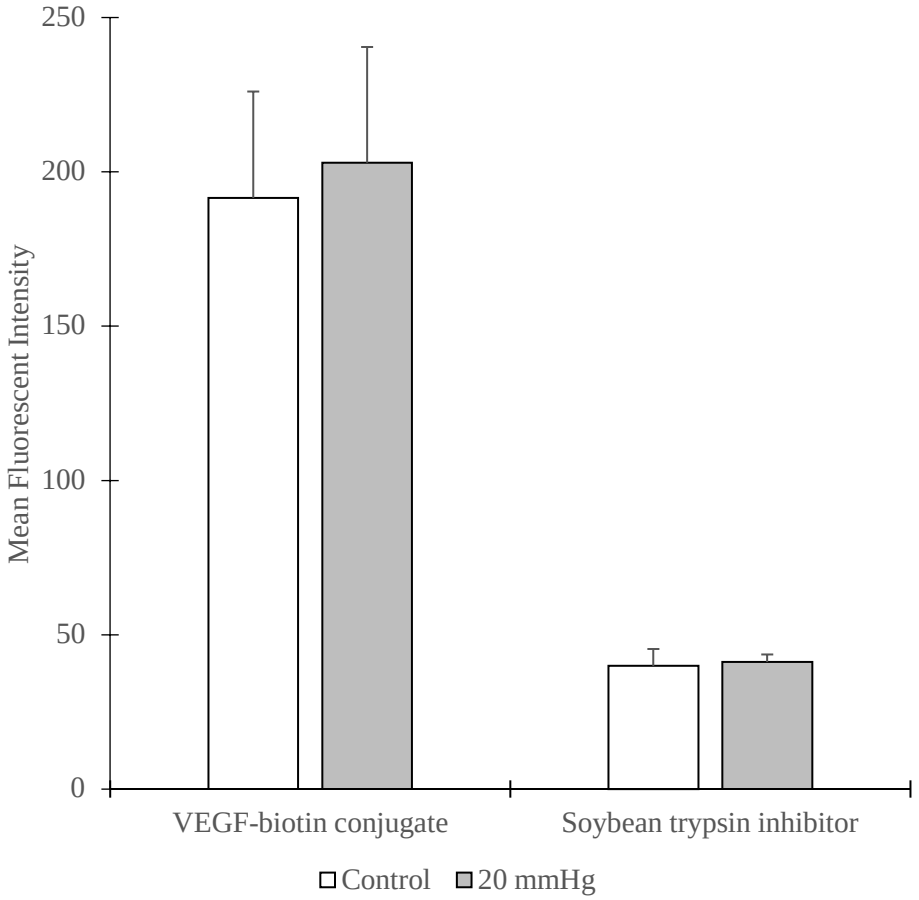

Figure 10: Observed pressure-sensitive binding is growth factor-selective. Bars signify mean ± SEM, HMVECs of passage 7 through 8 are represented in this data, n=4.

4.3 Effects of pressure on surface expression of FGFR2

The present study next examined the possibility that enhanced binding of HMVECs to FGF-2 under the 20 mmHg pressure stimulus resulted from an upregulation in the expression of its high-affinity receptor, FGFR2. Exposure to 20 mmHg hydrostatic pressure for 30 minutes had no detectable effects on HMVEC surface expression of FGFR2. Mouse IgG, which was used as a negative antibody control to quantify nonspecific binding events, also did not show detectable variations in binding due to pressure exposure. Thus, pressure-sensitive increase in binding affinity for FGF-2 was independent of FGFR2 surface expression levels.

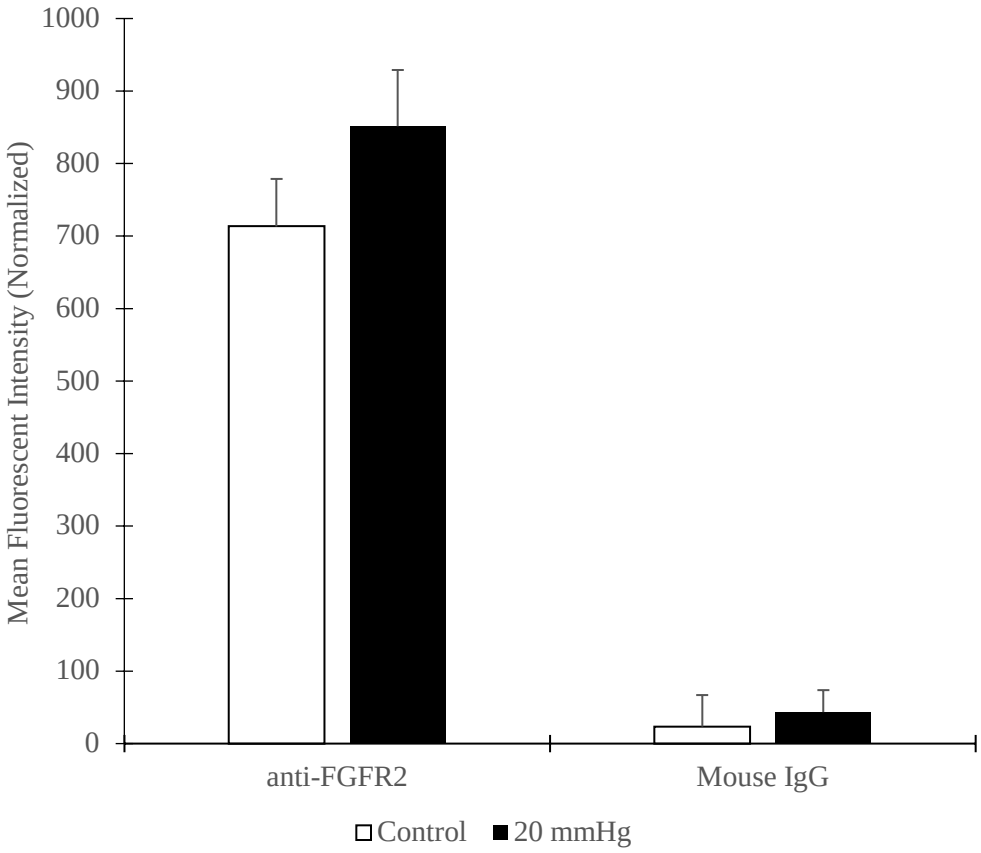

Figure 11: Pressure exposure did not significantly increase FGFR2 surface expression. HMVECs exposed to pressure and stained with APC-conjugated anti-Human FGFR2 were analyzed. Bars signify mean ± SEM, HMVECs of passage 9 and 10 are represented in this data, n=6.

4.4 Effect of pressure on total protein

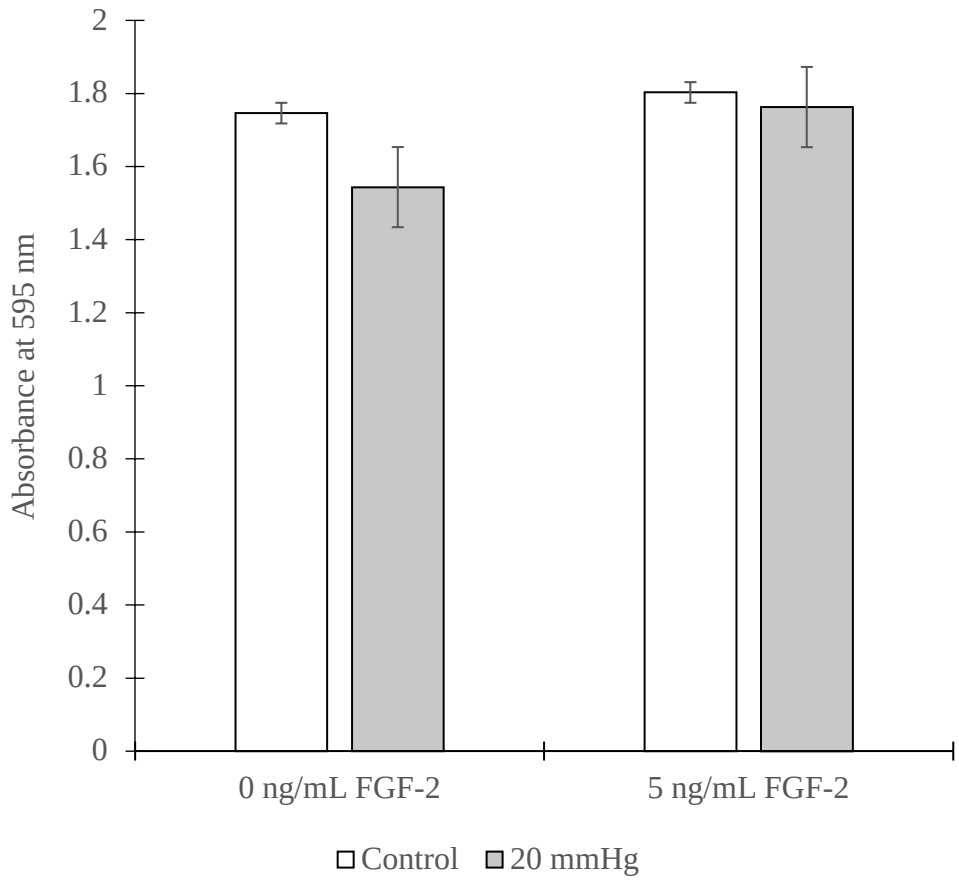

Figure 12: Total protein quantification of HMVECs showed pressure did not alter protein expression. Bars are mean ± SEM, HMVECs of passage 8 through 10 are represented in this data, n=3.

The results from this experiment showed that, within 30 minutes, the total amount of protein in HMVEC monolayers exposed to 20 mmHg hydrostatic pressure did not change. This data demonstrated that pressure had no effect on HMVECs protein expression levels at magnitudes that we observed to enhance FGF-2 binding. The effects of the pressure on FGF-2 affinity appeared to be due to enhanced binding to the cell surface layer.

DISCUSSION

The present thesis-related research was a pilot study to explore, for the first time, the growth factor binding properties of human microvascular endothelial cells (HMVEC) under the influence of hydrostatic pressure. The basic approach of the study was to expose HMVEC cultures to physiologically relevant pressure levels and immediately assess their binding affinity to FGF-2 as an endpoint assessment using immunochemistry. Overall, the present study revealed novel evidence supporting hydrostatic pressure as a magnitude dependent modulator of FGF-2 binding by ECs.

5.1 Selection of cell type

All endothelial cells line the luminal boundary of all blood vessels. Despite this commonality, the endothelial cell phenotype differs depending on anatomical location. In the microcirculation (i.e., capillaries, precapillary arterioles, post capillary venules), microvascular endothelial cells display attributes that are morphologically and phenotypically distinct from large vessel-derived endothelial cells (i.e., arterial and venous sites). Such distinctions are present even in large- and small-vessel derived cells taken from the same organ [62]. Of particular relevance to this study is that the microvasculature are the typical sites where angiogenesis primarily occurs and thus the endothelium at these sites play the primary role in capillary formation upon stimulation by growth factors [63]. For these reasons, human microvascular endothelial cell (HMVEC) cultures *ex vivo* have been used for many studies on the tubulogeneic activity of the endothelium and as the principal cell culture model for the present study [64].

5.2 Selection of pressure magnitudes and exposure durations

Pressure within the blood vessels and therefore on the endothelial cells varies greatly depending on the type and location of the vessel. Large vessels immediately downstream of the heart, on average and in healthy individuals, experience high, pulsatile pressures of 120/80 mmHg. Pressures in blood vessels decrease as blood travels away from the heart due to the branching nature of the vasculature and to blood vessel compliance. The elasticity of blood vessels in conjunction with the pressure wave created by the pumping action of the heart causes vessels to comply to a certain degree allowing for a circumferential increase with increased blood flow [65]. Furthermore, as one vessel diverges into two, conservation of momentum dictates that velocity and associated pressure in each vessel is lower than in the origin vessel.

As the blood reaches the capillaries of healthy individuals, the pulsatile nature of the flow is eliminated and the pressure exerted on the endothelial cells by the blood drops to between 18 and 38 mmHg (*Figure 13*). Based on this information, 20 mmHg and 40 mmHg pressure levels were the chosen for this study as physiologically relevant. Similar pressure levels have been used in other studies on the effects of hydrostatic pressure on EC activity [52, 55]. The upper pressure limit, 40 mmHg, is slightly higher than a healthy, average blood pressure. However, 40 mmHg consistent with pressures that may be experienced by endothelial cells in a hypertensive environment. The 20 and 40 mmHg pressures may also mimic interstitial pressures in the extracellular matrix undergoing wound scenario during which the interstitial pressure is increased around a capillary due to increases in microvascular permeability. A 5 mmHg pressure was also used as this pressure is consistent with physiological interstitial pressures in some tissues, which is relevant

considering the endothelial cells responsible for initiating and sustaining tubulogenic activity typically do so while migrating into the extracellular matrix. The interstitial pressure is a combination of hydrostatic and tissue oncotic pressure contributions, which are equal to about 0 mmHg and 5 mmHg, respectively [66].

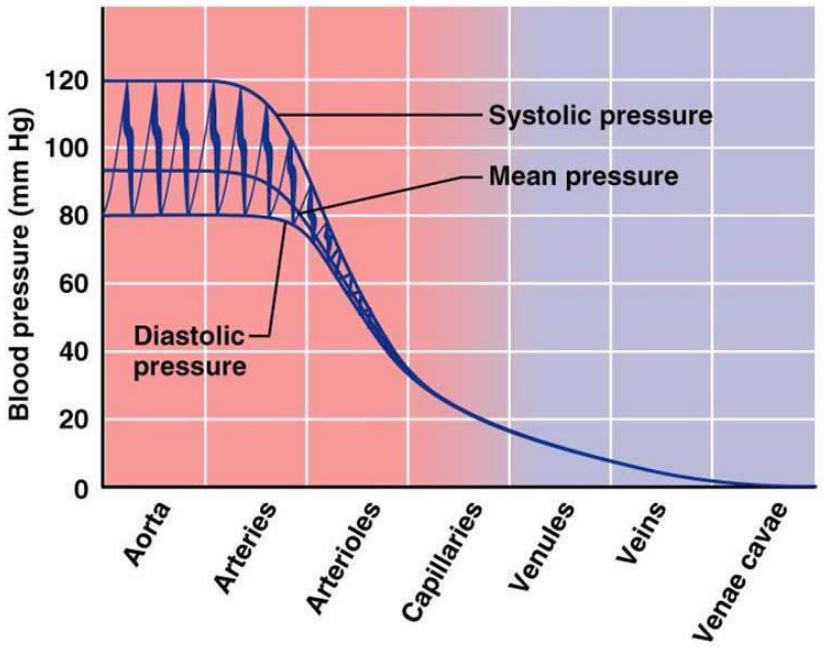

Figure 13: Standard physiological pressures within cardiovascular system. Available at http://classes.midlandstech.edu/carterp/Courses/bio211/chap19/chap19.html

Previous studies in our lab have shown that exposure to sustained hydrostatic pressures of 20 mmHg for 72 hours stimulates tubulogenic sprouting by endothelial cells *ex vivo* [55]. Interestingly, Shin et al (2004) showed that phosphorylation of the FGFR2 occurs within 15 minutes of exposure to 60/20 mmHg cyclic pressure and appears to play a role in stimulating EC proliferation though the underlying mechanisms are not fully understood. The work of Shin et al. (2004) provided the first clue that pressure may stimulate endothelial tubulogenic activity by promoting rapid upregulation of FGFR activity. The

present study leveraged the results of this earlier finding to explore the possibility that the reported increase in tubulogenic sprouting was due to a pressure dependent effect on FGFR.

5.3 Experimental setup for pressure experiments

The pressure system used in the present study was adapted from as described in *Shin et al.* (2012) [52, 60, 67]. The system was custom designed to expose cells to physiologically relevant pressure regimes, i.e. hydrostatic pressures similar to those generated in the vasculature and interstitial matrices of tissues where the microcirculation resides. The system was designed to accommodate cells grown as adherent populations in traditional cell culture vessels/plates, or as suspension cultures in culture tubes, during application of pressure levels (either 5, 20, or 40 mmHg) above atmospheric pressure (760 mmHg).

Within samples, the only variation in pressure is caused by the location of the cells within the culture. For adherent cells this variation was considered negligible. Samples that were suspended were 30 μL in total (i.e., < 2.5 mm of fluid in a 1.5 mL microcentrifuge tube), so this height difference was also taken to have a negligible contribution to the total pressure experienced by the cells. Cell populations maintained under control (i.e., atmospheric) pressures were placed in a cell culture incubator under standard conditions.

5.4 Endothelial binding affinity for FGF-2 depends on applied pressure level

FGF-2 was chosen as the primary growth factor of interest for this study due to previous studies linking hydrostatic pressure levels to FGF-2-mediated morphological and

proliferative activity of endothelial cells. Specifically, *Acevedo et. al.* (1993) reported that increased proliferation, elongation, and random polarization of endothelial cells in response to sustained hydrostatic pressure elevations involves FGF-2 release [52]. *Shin et al.* (2004) reported that exposure to 60/20 mmHg enhances the proliferation of endothelial cells via a mechanism that appears to involve FGF-2 and its receptor, FGFR2. In fact, exposure to 60/20 mmHg for as little as 15 minutes upregulates tyrosine phosphorylation (e.g., activation) of FGFR2, although the mechanism linking pressure and enhanced activation of FGFR2s was not elucidated [68].

The present study explored the possibility that upregulation of FGFR2 phosphorylation resulted from enhanced FGFR2 binding affinity of microvascular endothelial cells to the FGF-2 ligand upon exposure to pressure. Interestingly, the binding affinity of endothelial cells to FGF-2 was dependent upon local hydrostatic pressure levels within the range of 0 to 40 mmHg above atmospheric pressure. Specifically, exposure to 20, but not 5 or 40 mmHg, resulted in significant increases in the amounts of FGF-2 binding by HMVECs relative to similar cell preparations maintained under control conditions. This result is supportive of a potential relationship between hydrostatic pressure and the FGF-2 signaling pathways as suggested by *Acevedo et. al* (1993) and *Shin* (2004). Moreover, the FGF2 binding results of the present study suggest that pressure-sensitive FGF2-related binding is connected to the enhanced tubulogenic sprouting rates by endothelial cells exposed to 20 mmHg specifically, not 5 or 40 mmHg [55, 69].

The underlying mechanism for pressure-sensitive endothelial binding affinity to FGF2, however, is not clear. It is possible that hydrostatic pressure influences cell membrane dynamics, which then affects the conformational activity of FGFRs themselves. In this

regard, distinct pressures favor may different FGFR conformations that, in turn, influence binding affinity for the ligand. In the case of FGFR2, it is possible that the most favorable isoform for FGF-2 binding occurs in an environment where local hydrostatic pressures are 20 mmHg. This would be a reasonable conclusion as previous studies have proven the existence of a number of isoforms of FGFR2 each with an associated level of activation [47].

The observed overall binding increase may also be possible due to a variety of other cellular responses to pressure. For example, pressure may trigger the redistribution of stored FGF-2 receptors from intracellular locations to the cell surface membrane. A similar phenomenon happens with other proteins that are stored in the endoplasmic reticulum of the cell and modified through a protein cleavage which relocates the protein to the plasma membrane when the signaling pathway is activated [70]. In addition, it is possible that membrane-associated FGF-2 receptors experience a direct change in affinity in response to pressure. But this is not likely considering the compressive pressures used in the present study are likely to be orders of magnitude lower than those required to overcome the inter- and intra- molecular forces needed to deform the proteins. Finally, there is a chance that pressure effects another signaling pathway entirely whose effects manifest themselves in the increased binding of FGF-2. This is a conceivable given that a number of cell signaling pathways are mechanosensitive and act in parallel or concert upon mechanical stimulation [52, 71].

5.5 Endothelial binding affinity pressure response is growth factor selective

Results from previous studies have provided evidence that mechanical stimulation activates the angiogenic VEGF-A/VEGFR2 pathway in the absence of a ligand [72]. Interestingly, our lab has conducted experiments that show that after 72 hours of pressure exposure, samples that had been treated with FGF-2, but not those treated with VEGF-A, exhibit a significant increase in sprout formation of endothelial cell microbead cultures [69]. This would suggest the possibility that the FGF-2 binding, but not the VEGF-A binding, is sensitive to distinct pressure regimes as an angiogenic stimulus.

To determine if this observation is due to an effect of pressure on receptor / ligand interactions, a similar growth factor binding experiment as described above with biotin conjugated FGF-2 was performed with biotin conjugated VEGF-A in place of FGF-2. The results of this experiment showed that pressure exposure does not have an effect on the binding of VEGF-A and further supports the possibility that enhanced FGF-2 binding in the presence of pressure plays a role in pressure-sensitive endothelial sprouting. Furthermore, the results of the present study provide evidence that binding efficiency of FGF-2, an upstream event of sprouting angiogenesis, is depends on the local hydrostatic pressure level.

5.6 FGFR surface expression does not increase with pressure exposure

Endothelial cells have been shown to modulate angiogenic growth factor-related activity through endocytic processes [73, 74]. Both FGF- and VEGF-family receptors can be trafficked to and from the endothelial cell membrane to control signal transduction by varying the number of receptors available for activation on the surface [73, 74].

Furthermore, receptor expression at the surface can vary quickly – in as a short a time period as 5 minutes [73]. Therefore, a possible explanation for increased FGF-2 binding after pressure exposure is that a 20-mmHg pressure stimulus is sufficient to induce endosomal trafficking of FGFR2 from the interior of the cell to the cell membrane. This possibility was tested using an APC-conjugated antibody for human FGFR2 in parallel with a mouse IgG as a negative control. The results of this experiment showed that cells exposed to 20 mmHg sustained hydrostatic pressure for 30 minutes did not exhibit increased FGFR2 surface expression compared to cells held at atmospheric pressure but otherwise similar experimental conditions.

Additionally, an analysis of total protein was performed on adherent HMVECs to definitively rule out the synthesis of new FGFRs as a potential cause of the increased binding. It would be unlikely that this was the cause, as translation and transcription of new proteins in eukaryotes occur at approximately 5 nucleotides per second and 10 amino acids per second, respectively [75]. The human FGFR2 gene is about 156K nucleotides long – a sequence which would take well over 30 minutes to undergo transcription alone [76]. Total protein quantification experiments were carried out with the same pressure treatments from the previous experiments – 0, 5, 20, and 40 mmHg above atmospheric pressure – and either addition or exclusion of 5 ng / mL exogenous FGF-2. The cells were lysed and the lysate was tested for protein concentration using a colorimetric Bradford assay. The results of these experiments confirmed that the overall protein amounts for pressure and control samples were similar. Based on these results, an increase in FGFR2 surface expression or increased FGFR2 synthesis cannot be considered the cause of increased FGF-2 binding.

This further supports the conclusion that hydrostatic pressure affected the affinity of existing membrane-bound FGFR2 for the ligand.

5.7 Increased FGF-2 binding is not due to increased FGF-2 secretion by HMVECs

Finally, it is important to note that the observed increase in FGF-2 binding by HMVECs is not a product of increased secretion of FGF-2 by the endothelial cells in response to a pressure stimulus. *Acevedo et al.* (1993) showed that sustained hydrostatic pressure induces the release of nuclear and cytoplasmic stores of FGF-2 from bovine aortic endothelial cells (BAECs). This study states that it is this FGF-2 which causes the morphological and proliferative responses of BAECs to hydrostatic pressure, i.e., autocrine signaling [52]. This information calls into question the possibility that the pressure stimuli simply induce the release of FGF-2 from HMVECs in a magnitude-dependent fashion. The secreted FGF-2 would create a higher FGF-2 concentration close to the membrane, resulting in more efficient binding and receptor activation. However, human endothelial cells require exogeneous FGF-2 in order to exhibit enhanced proliferative effects in response to a pressure stimulus [68].

CONSIDERATIONS FOR FUTURE INVESTIGATION

The results outlined in the preceding document provide novel insight into a pressure-mediated model for fibroblast growth factor-2 binding. These results raise numerous additional questions about the nature of this model and of mechanosensation which would be best addressed in a series of future experiments. Such experiments should primarily address the phosphorylated characteristics of the FGFR2 after pressure exposure, the inclusion of this knowledge *in vivo* experiments, and, ultimately, defining the mechanism by which pressure influences FGF-2 binding.

Quantifying phosphorylation of FGFR-2

Previous studies in our lab have shown that a cyclic pressure stimulus upregulates the phosphorylation of FGFR-2 [68]. It would be necessary to know if this result is validated in the presence of a hydrostatic (i.e., non-cyclic) pressure stimulus. Many biochemical assays exist which could be utilized for this purpose – most effective would likely be the use of a Western Blot, as in *Shin et al (2004)*, or commercially available ELISA kits for FGFR2 and phospho-FGFR2. Results of these experiments that would support the findings of the present research would be an increase in phosphorylation of the receptor after exposure to distinct regimes of hydrostatic pressure.

Considerations for future ex vivo and in vivo experiments

The *ex vivo* nature of this research is a persistent consideration. Validation of these results *in vivo* would be essential to quantifying them as clinically relevant. Perhaps a

reasonable starting point for the next steps of these studies is in the study of the rat mesentery. The rat mesentery, a thin tissue which attaches the digestive organs to the wall of the abdomen, has long-been been used to study angiogenesis [77-79]. The mesentery is highly vascularized and easily accessible tissue which can be exteriorized and manipulated in a nonlethal surgery for studying angiogenesis as it relates to mechanobiology [80]. This tissue, in addition to hypertension-inducing drugs and labelled growth factors could potentially be used as a starting point for *in vivo* studies.

Determining the mechanism of pressure-mediated growth factor binding

The experiments included in this thesis accomplished the quantification of FGF-2 to its receptor under atmospheric and above atmospheric pressure conditions. We determined that this was not due to an increase in receptor expression at the surface of the endothelial cell or an increase is receptor synthesis. The question that follows these results is – what is the mechanism by which pressure influences the binding of FGF-2 to its receptor? The answer to this question will ultimately be determined by consideration of a more complete set of experiments, some of which have been described above. Additionally, inspection of the crystals structure of the FGFR2 after pressure exposure, both in the presence and absence of a ligand, would be beneficial.

CONCLUDING REMARKS

This Master's thesis-related study is an extension of previous work from the Shin laboratory that elucidates a putative, receptor-level molecular mechanism associated with pressure-sensitive tubulogenic processes. In this regard, the present study provided evidence that hydrostatic pressures promote FGF-2 binding to human endothelial cells in a pressure magnitude-dependent fashion. This pressure-selective response was independent of FGFR2 trafficking to the cell membrane or increased protein synthesis. In this regard, this study shed novel mechanistic insight into pressure-sensitive FGF-2/FGFR2 binding kinetics. This information may provide the foundation for a new perspective that may be used to investigate the pathobiology of lethal or highly-debilitating diseases, such as metastatic cancer, pulmonary hypertension, glaucoma, and pathological wound healing, that involve FGF-2 signaling and pressure changes in the local milieu of the microvascular endothelium.

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