FUNCTIONAL DIFFERENCES BETWEEN PERICYTES AND VASCULAR SMOOTH MUSCLE CELLS DURING VASCULAR MORPHOGENESIS

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

FUNCTIONAL DIFFERENCES BETWEEN PERICYTES AND VASCULAR SMOOTH MUSCLE CELLS DURING VASCULAR MORPHOGENESIS

Presented by Amy E. Schwindt

A candidate for the degree of Master of Science

And hereby certify that, in their opinion, it is worthy of acceptance.

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Professor Charlotte Phillips

First of all I would like to thank my mom for being my biggest supporter during my graduate studies. Thank you for listening to me talk about my research everyday, both good and bad, even if you still don’t really understand what I am talking about. I know that when I talk about my research it is like listening to a foreign language, but it gives me great joy to hear you use words like pericyte and vascular smooth muscle cell. I don’t know of too many parents that care enough to listen to this odd language, much less actually learn some of it. Thank you.

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FUNCTIONAL DIFFERENCES BETWEEN PERICYTES AND VASCULAR SMOOTH MUSCLE CELLS DURING VASCULAR MORPHOGENESIS

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ABSTRACT

During vascular morphogenic events, mural cells are recruited to developing endothelial tubes to aid in stabilization and maturation of the new vessels. There are two known types of mural cells, pericytes and vascular smooth muscle cells (VSMCs). Their different locations within the vasculature (capillaries versus larger vessels, respectively) suggest that different signals may be responsible for recruiting each cell type. Platelet-derived growth factor (PDGF) is released from endothelial cells (ECs) during morphogenic events and has been shown to influence mural cell functions. In this study, I investigated the ability of PDGF isoforms to regulate pericyte and VSMC invasiveness and subsequent effects on EC monolayer stability and tube sprouting. Using novel cell-invasion systems developed during this research, I show that PDGF isoforms that bind to PDGFRβ induce pericyte, but not VSMC invasion. Coculture studies revealed that the invasive behavior of pericytes is critical to both EC stability and sprouting. When ECs were seeded as a monolayer on top of 3D collagen gels containing pericytes, monolayer stability was enhanced compared to EC monolayers seeded alone or with VSMCs. Coculture studies also revealed that EC sprouting was sustained when pericytes were present compared to EC only cultures or EC/VSMC cocultures. Results from this work define functional differences between pericytes and VSMCs during vascular morphogenesis.

1. Background

Blood vessel growth is crucial for normal development, wound healing, and many other physiological processes[1-3]. Vessels are created through the processes of vasculogenesis and angiogenesis where an endothelial tube is formed followed by the recruitment of support cells, also known as mural cells, leading to vessel maturity and stabilization[1,4,5]. Decreased or lack of mural cell recruitment is associated with several disease states including diabetic retinopathy[6-11] and preeclampsia[3]. Increased recruitment, though abnormal, has been implicated in progression of tumor growth[12-14] and lymphedema distichiasis[46]. Much of the information about blood vessel development and pathology has come primarily from the study of endothelial cells while the study of mural cells has lagged. In the past several years, however, interest in the role that mural cells play in the growth and stability of the vasculature has greatly increased. Still overlooked are the function and behavior of the individual mural cell types during developmental and angiogenic events.

Mural cells consist of cell types along the vascular smooth muscle cell lineage: pericytes and vascular smooth muscle cells (VSMCs)[5,7,11, 15-17]. This common background means that these cells express many of the same markers (e.g. nidogen, α-smooth muscle actin, RGS5, and platelet-derived growth factor receptor beta (PDGFRβ))[2,7,11,16,18], which makes them difficult to distinguish. Currently pericytes and VSMCs are defined by their morphology and location within the vasculature (capillaries versus the larger vessels, respectively) and density around the vessel[7,16,18,19]. Mural cells

are also thought to be functionally similar. Once recruited to their respective vessels, both pericytes and VSMCs are thought to help stabilize the tubes by decreasing EC profliferation, enhancing endothelial-endothelial cell contacts, controlling vascular branching, and enhancing basement membrane deposition[4,6,17,20]. The exact mechanisms of how mural cells are recruited to the EC tubes is still being studied. Of all the known growth factors that could be responsible for the recruitment of mural cells to the developing tubes, the primary focus has been on the family of platelet-derived growth factors.

There are five known platelet-derived growth factor (PDGF) isoforms that exist as dimers: PDGF-AA, AB, BB, CC, DD[21,22]. All isoforms are commonly found within the human body except for the heterodimeric PDGF-AB isoform. PDGF-AA and PDGF-BB have similar characteristics while PDGF-CC and PDGF-DD are comparable. The PDGF-AA and PDGF-BB isoforms are activated intracellularly, and upon secretion, are capable of binding to the extracellular matrix (ECM). PDGF-CC and PDGF-DD are structurally different from the previous isoforms in that they have two CUB domains that must be cleaved extracellularly in order for the isoforms to be activated. If only one of the domains is removed, the isoform is able to act as a partial antagonist. Also, unlike PDGF-AA and BB, PDGF-CC and DD lack the ability to bind to the ECM. Pericytes are responsible for the production of PDGF-AA, VSMCs create PDGF-CC and the endothelial cells are the source of both PDGF-BB and PDGF-DD.

The PDGF isoforms bind to two receptors named PDGF receptor alpha (PDGFRα) and PDGF receptor beta (PDGFRβ)[8,21,22]. Within the vasculature, both receptor types are

found mainly on the mural cells. A considerable amount of the information known about PDGF binding has been gained through *in vitro* experiments, although, *in vivo* findings have replicated much of what has been observed in cell culture. Binding studies show that PDGFRα is able to bind PDGF-AA, AB, BB and CC with strong affinity. PDGFRβ primarily binds PDGF-BB and PDGF-DD, but can also weakly bind PDGF-AB when PDGFRα is present. Once the PDGF isoform has bound to its receptor, the receptors are activated through dimerization. The PDGF receptors are able to form hetero and homodimers but the roles of the different receptor dimers are still incompletely defined.

In the context of mural cell recruitment, much of the current knowledge has come from studies of PDGF-BB and PDGFRβ mutations and knockout mice. Studies utilizing whole animal knockouts of PDGF-BB or PDGFRβ demonstrate increased death prenatally due to aneurysm formation and hemorrhaging in the microvasculature[5,19,23,24]. These mice and those that survive show a decrease in pericyte recruitment to the smaller vessels and an increased vessel width compared to wild-type animals. There is no change in the vessel length or the number of branch points. Interestingly, there is no significant difference in the number of PDGFRβ positive cells surrounding the arteries of wild-type and whole animal PDGF-BB or PDGFRβ knockout mice[19]. Studies focusing on the knockdown of PDGF-BB exclusively in endothelial cells provide a greater understanding of the mural cell recruitment process.

There are sources of PDGF-BB other than endothelial cells, including platelets, monocytes and some neuronal cells[25]. Studies performed by Bjarnegard (2004) and Enge

(2002) helped to define EC produced PDGF-BB as an important component in mural cell recruitment. PDGF-BB knockout of ECs only produces mice that are more viable than whole animal knockouts and are able to survive into adulthood. However, these mice replicate the lack of pericytes, increased vessel diameter and the microhemorrhaging seen in whole animal knockouts, suggesting that endothelial-derived PDGF-BB is critical for pericyte recruitment and that it may act in a paracrine manner. This theory was tested by using PDGF-BB retention motif knockout mice. The retention motif knockout blocks the ability of PDGF-BB to bind to the heparin sulfate proteoglycan on the endothelial cell surface or the extracellular matrix without affecting the receptor binding or biological activity. As with the other PDGF-BB knockouts, there is a decrease in pericyte recruitment to the endothelial tubes and an irregular vascular plexus with a reduced number of sprouts[13,26,27]. Pericytes that have recruited or migrated to the vessels are more likely to detach from the vessel compared to pericytes from wild-type animals. Once again, there is no noticeable difference in the number of VSMCs found around the arteries when the two animal groups are compared, although there are fewer layers[27].

Due to the distinctions seen in mural cell recruitment to small vessels versus the larger ones in PDGF-BB or PDGFRβ knockouts, I hypothesized that different factors recruit pericytes and VSMCs. The goals of my research were 1) to determine if there are differences in the signals that drive pericyte and VSMC recruitment and 2) to determine the impact of those differences on EC function. This was done through the use of 3D cell culture systems I developed during my graduate study. These systems utilize collagen

type I and allow for the examination of one cell type in response to one or more growth factors, antibodies, or Fcs or the interaction of two cells in response to growth factors produced by the cells in culture.

2. Materials and Methods

Materials

Recombinant PDGF-AA (221-AA), PDGF-AB (222-AB), PDGF-BB (220-BB), PDGF-CC (1687-CC/CF), PDGF-DD (1159-SB/CF), HB-EGF (249-HE/CF), Neuregulin (377-HB/CF), BDNF (248-BD/CF), SCF (255-SC/CF), SDF-1α (350-NS/CF), IL-3 (203-IL/CF) and antibodies to BMP-4 (MAB757), BMP-9 (AF3209), HB-EGF (AF-259-NA), IL-6 (AB-206-NA), PDGF-BB (AB-220-NA), PDGFRα (AF-307-NA), PDGFRβ (AF385), TGFβ (MAB240), VEGF (AF-293-NA), VEGFR2 (AF357) and soluble receptor traps to PDGFRβ (385-PR/CF) and TrkB (688-TK) were purchased from R&D Systems. Lysophosphatidic Acid (LPA; 857130) and sphinogosine 1 phosphate (S1P; 860492) were from Avanti Polar Lipids. Total PDGFRβ antibody was purchased from Epitomics (1469-1). Phospho-PDGFRα (Tyr1018, 4547) and phospho-PDGFRβ (Tyr751, 3161) were from Cell Signaling Technology. Alpha tubulin (T5168), fibronectin (F0916), electron microscopy grade glutaraldehyde solution (G5882), and ascorbic acid (A4403) were from Sigma-Aldrich. PECAM/CD31 was from Dako (M0823) and collagen IV from Millipore (AB769). Basic FGF (233-FB/CF) was

purchased from R&D Systems, RSII from Upstate Biotechnology and GM6001 (364205) from EMD4Biosciences.

Methods

Cell Culture

Human brain vascular pericytes (HBVPs) purchased from ScienCell were grown on gelatin-coated flasks containing Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal bovine serum (FBS). HBVPs were used from passages 4-10.

Human aortic vascular smooth muscle cells (HASMCs), human coronary artery vascular smooth muscle cells (CASMCs) and human umbilical vein endothelial cells (HUVECs) were obtained from Lonza. HASMCs were used from passages 2-6, CASMCs from passages 3-6 and HUVECs from passages 4-6. All cell types were cultured on gelatin-coated flasks. The HASMCs and CASMCs were grown in DMEM containing 20% FBS while HUVECs were cultured in supermedia (M199 with bovine hypothalamus extract and 20% FBS).

Bovine retinal pericytes (BRP4s) were isolated by Saunders et al. (2006) using methods previously described by Nayak et al. (1988). These cells were cultured in the same manner as the HBVPs and used during passages 4-8. All cultures were maintained in a 37°C incubator at 5% CO_2 until confluence.

GFP Lentiviral Transfection of Cells

Green fluorescent protein (GFP)-labeled HBVPs and HASMCs used in these experiments were produced using lentiviral transfection. The lentivirus was amplified using 293FT cells grown in 1X M199 with 10% FBS and 50 mg/mL G418. The night before

use, the media was removed and fresh 1X M199 and 10% FBS was added. The next day a vira power mix was created by adding 5.76 μg of pLP-1, 5.4 μg pLP-2, and 3.96 μg pLP/VSVG (all from Invitrogen) to a 15 mL tube and placing on ice. In a separate 15 mL tube, 1.5 mL of Optimem (Gibco) and 36 μL of Lipofectamine 2000 (Invitrogen) were added and allowed to incubate at room temperature for 5 minutes. During the five minutes, 1.5 mL of Optimem, 4 μg of the GFP pLenti, and 15.12 μg of the vira power mix made previously were added to another 15 mL tube. Once the five minute incubation period was complete, the contents of the tube containing Optimem and Lipofectamine 2000 was added to the tube containing the GFP lentivirus. This mixture was then incubated for 20 minutes at room temperature. Before the addition of the lentivirus, the 293FT cells were rinsed twice with 10 mL of Optimem before the addition of 5 mL of Optimem with 10% FBS. The incubated lentiviral mixture was then added to the cells and the cells placed back into the incubator at 37°C overnight. The following day, the lentiviral mixture and media was removed and the cells were fed 10% FBS in 1X M199. The virus was amplified in the cells over the next three days before the supernatant was removed and filtered to eliminate any dead cells. In order to transfect the HBVPs or HASMCs, 6 μg/mL of polybrene was made up in sterile milliQ water. The polybrene was then directly added to the cells followed by the addition of 5 mL of lentivirus (supernatant from above) and 3 mL of DMEM with the appropriate amount of FBS added for each cell type. The next day, 7 more milliliters of DMEM with FBS were added to the flask of cells. After three days, the production of fluorescence was checked, the media removed and new media containing blasticidin was added in order to begin the

selection process. The fluorescent label carries a resistance gene to blasticidin which also them to grow in its presence. Cells without this gene will parish in the presence of blasticidin leaving only the fluorescent cells growing in the flasks and available for use in culture.

Assay Systems

Downward Invasion System

Collagen type I gels were made at 2.5 mg/mL and the different growth factors to be tested were added at 200 ng/mL. Gels were polymerized in a 37°C incubator. HBVPs were resuspended at 4.5×10^5 in DMEM containing reduced serum supplement (RSII) and basic fibroblast growth factor (bFGF) at 40 ng/mL and ascorbic acid (AA) at 50 µg/mL then added to the gels. Cultures were placed in the incubator and fixed with 3% glutaraldehyde in PBS after 48 hours. After fixation, cultures were stained with 0.1% toluidine blue in 30% methanol for visualization and analysis.

Upward Invasion System

HBVPs or HASMCs were resuspended at a concentration of 1×10^5 cells/mL into 2.5 mg/mL type I collagen gels containing RSII and bFGF at 40 ng/mL. The collagen gels were incubated at 37°C and allowed to polymerize before the addition of DMEM with RSII, bFGF, and the different growth factors, all at 40 ng/mL, bioactive lipids at 1 μM and AA at 50 µg/mL. The cultures were placed back into the incubator and the cells allowed to invade up towards the concentration gradient for 72 hours before fixation with 3% glutaraldehyde in PBS. Gels were fixed for at least 30 minutes before staining with a

mixture containing 0.5% toluidine blue and 1% sodium borate in deionized water for visualization of the cells on the gel surface.

Angiogenic Sprouting System

Collagen type I gels (2.5 mg/mL) containing HBVPs or HASMCs at 1 x 10^5 cells/mL were prepared and incubated at 37°C for polymerization. Media containing HUVECs at 2 x 10^6, stem cell factor (SCF), stromal cell-derived factor (SDF)-1α, interleukin-3 (IL-3), RSII and bFGF at 40 ng/mL and AA at 50 μg/mL was added to each gel. Cultures were placed back in the incubator for 1, 3, 5, or 7 days. Media for the 5 and 7 day cultures was replaced with new media containing SCF, SDF, IL-3, RSII, FGF-2, and AA on days 3 and 5. The cultures were fixed with either 3% glutaraldehyde (electron microscopy grade) for electron microscopy or 2% paraformaldehyde for immunofluorescence staining.

Immunofluorescence Staining

Cultures were fixed in 2% paraformaldehyde for a minimum of one hour before the addition of triton X-100 to permeablize the gel for CD31 staining or a blocking solution comprised of detergent-free phosphate-buffered solution (PBS) with 1% bovine serum albumin (BSA) to stain for basement membrane components. Primary antibodies were added to the blocking solution and the gels stored at 4°C overnight. The gels were then washed with Tween 20 before the addition of more blocking solution and the secondary antibodies. Cultures were incubated for two more hours at 4°C washed and analyzed using immunofluorescence microscopy.

Electron Microscopy

Gels were fixed in 3% glutaraldehyde (electron microscopy grade) and prepared for transmission electron microscopy by the University of Missouri Electron Microscopy Core Facility. Images were captured using a JOEL 1400 transmission electron microscope.

Imaging and Data Collection

Images for the invasion assays were taken on an Olympus CKX41 scope with an Olympus DP70 camera. For the downward invasion system, pictures were taken at three different planes within each gel. The number of cells was counted with the aid of MetaMorph Software (Molecular Devices). To analyze the upward invasion system, a photograph of each gel surface was taken and the cells counted. Images of the sprouting system were taken on a Leica DMI 6000-B scope with a Hamamatsu Orca-ER camera utilizing IP Lab and MetaMorph Software. Tube area of the sprouting assay was calculated by taking pictures of three different planes per well and tracing the tubes in the images using MetaMorph. Monolayer integrity was examined by taking a single picture in the center of the monolayer of each gel and using MetaMorph to find the total black area. This area was then divided against the total area and multiplied to get the percentage of the monolayer that had regressed and the percentage still intact. The average counts, tube area, and percentage of the monolayer intact were calculated and graphed using Microsoft Excel.

Statistical Analysis

Student t-tests were performed using Microsoft Excel to compare two different treatment groups. The standard for significance was set at $p \leq 0.05$.

3. Results

Pericytes invade from a monolayer into 3D collagen matrices in response to PDGF-BB

Utilizing a completely defined (serum-free) cell culture system, the invasive behavior of human brain vascular pericytes (HBVPs) was examined in response to select growth factors known to be produced by the endothelium. In this downward invasion model system, HBVPs were seeded on top of 3D collagen type I gels containing either no growth factors (control), platelet-derived growth factor-BB (PDGF-BB), heparin-binding EGF-like growth factor (HB-EGF), neuregulin 1 (NRG), or brain-derived neurotrophic factor (BDNF) and allowed to invade towards the growth factor for 48 hours (Figure 1A). Of the growth factors tested, only PDGF-BB was able to significantly increase pericyte invasion from the monolayer into the 3D matrix (Figure 1B). The pericyte invasive responses to HB-EGF, NRG, and BDNF were similar to the invasion observed in the control condition as was evident from cross-sectional cuts of representative gels from each of the conditions. To further test whether PDGF-BB is truly responsible for the invasive behavior of the pericytes, blocking antibodies to PDGF-BB, HB-EGF, bone morphogenic protein 4 (BMP-4), and interleukin-6 (IL-6, added as a control) were added to gels containing PDGF-BB. It was found that the blocking antibody to PDGF-BB was

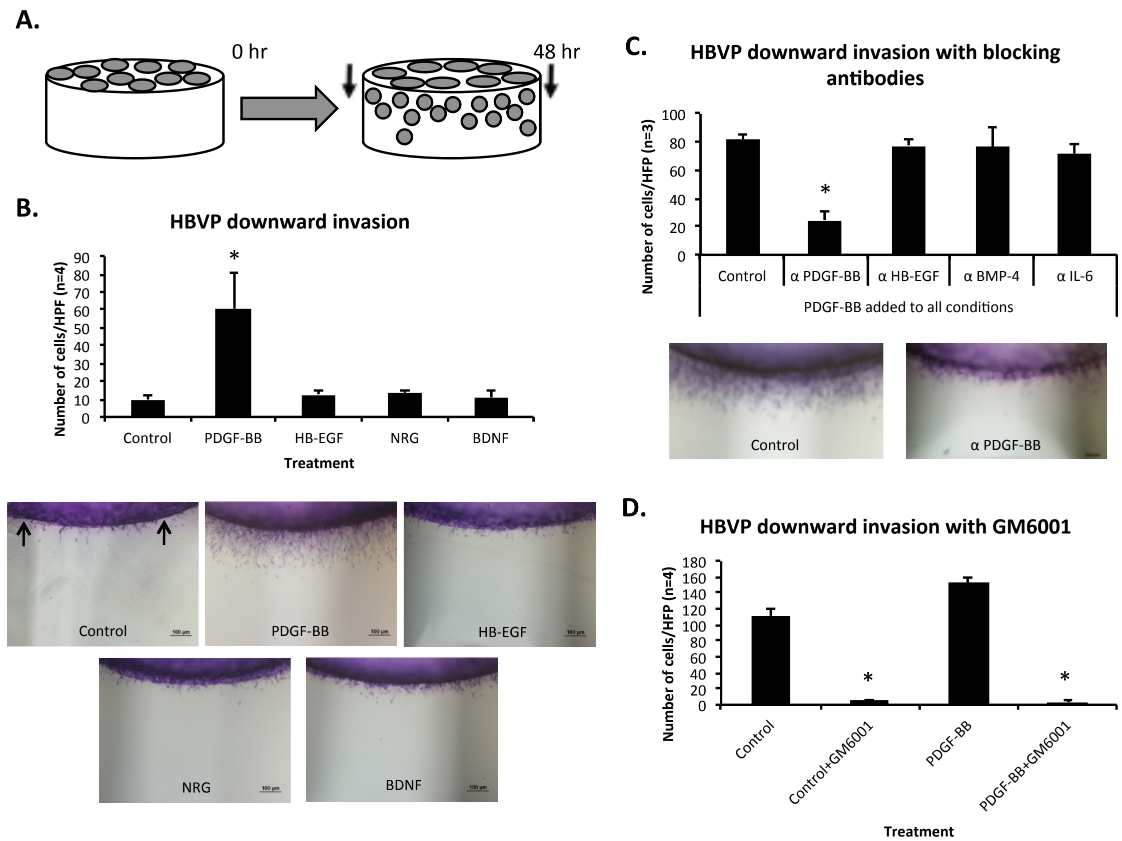

Figure 1 - PDGF-BB drives pericyte invasion into 3D collagen matrices from a monolayer

(A) Model of the downward invasion system, in which, HBVPs are seeded on the surface of a collagen type I gel containing different growth factors and are allowed to invade into the matrix for 48 hours. (B) Cell counts and cross-sections from a representative downward invasion assay testing known endothelial produced factors show that PDGF-BB is the only growth factor that is able to significantly increase HBVP invasion. Arrows point to the monolayer. (C) Blocking antibodies were added in combination with PDGF-BB to the invasion system. Other than the antibody to PDGF-BB, none of the antibodies tested were able to block invasion. (D) The addition of the pan matrix metalloproteinase inhibitor GM6001 blocked HBVP invasion in both the control and PDGF-BB conditions and demonstrates a role for MMPs in this invasive behavior. * $p \leq 0.01$, bar equals 100μm, HPF- high powered field.

able to significantly reduce HBVP invasion from the monolayer while the other antibodies had little effect (Figure 1C). Cross-sections from gels containing only PDGF-BB (control) and PDGF-BB with its blocking antibody show the differences in the invasive response of the pericytes. To confirm that the HBVPs were invading and not just migrating through the meshwork of the collagen gels, a pan-matrix metalloproteinase inhibitor, GM6001, was added at 2 μg/mL to control gels and gels containing PDGF-BB. The addition of GM6001 was able to inhibit pericyte invasion in both the control and PDGF-BB conditions (Figure 1D) suggesting that the HBVPs are using a matrix metalloproteinase to degrade the matrix and invade, although at this time it is unknown which matrix metalloproteinase they utilize.

PDGF-BB has a greater influence on pericyte invasion than on vascular smooth muscle cell invasion in 3D

Due to the contractile properties of vascular smooth muscle cells (VSMCs), the experiments above were not able to be performed using human aortic vascular smooth muscle cells (HASMCs). When placed on the gel surface the HASMCs contracted and prevented any invasion from occurring. To investigate the invasive responses of HASMCs a new upward invasion model system was developed. Again, this is a serum-free model system that allows for the study of one cell type in response to one or more defined growth factors. However, in this system, the cells are suspended within the collagen matrix and the growth factors added to the media to form a concentration gradient (Figure 2A). This prevents the suspended cells from interacting and contracting and allows for invasion up to the surface of the gel and subsequent formation of a

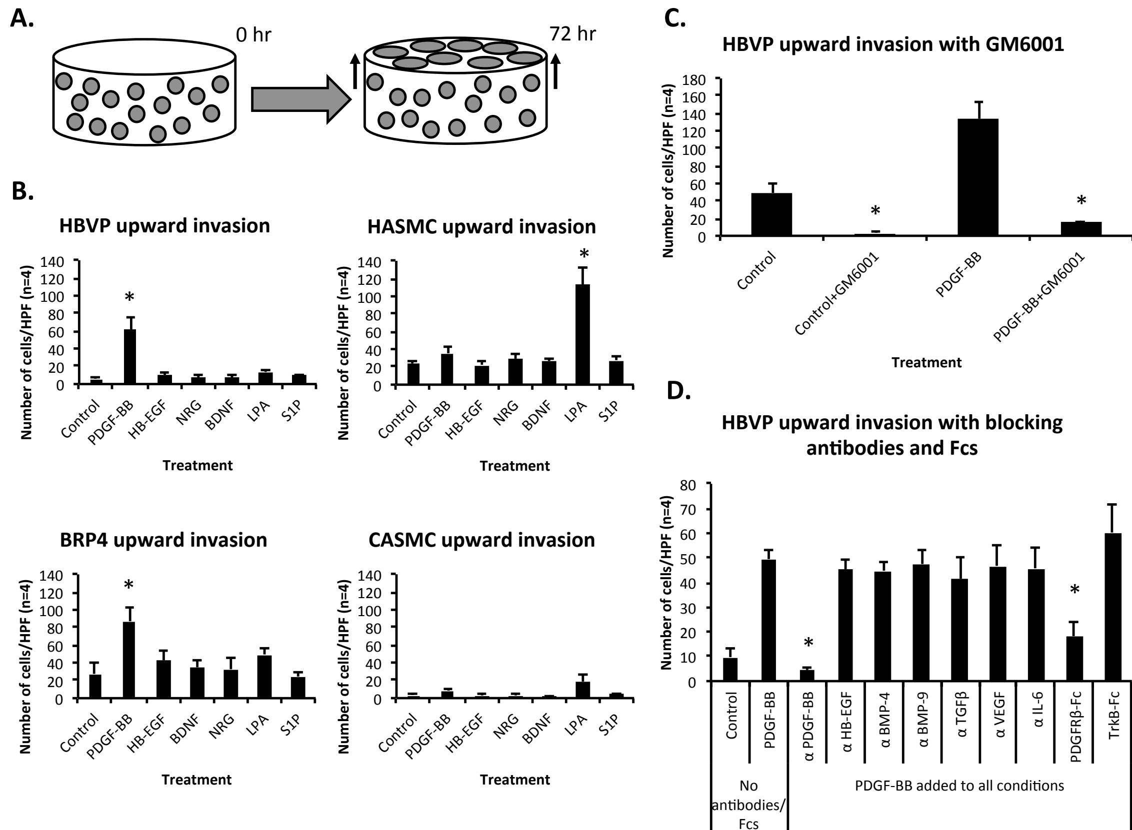

Figure 2 - Pericytes, not vascular smooth muscle cells, invade in response to PDGF-BB

(A) Schematic of the upward invasion system. In this system cells are suspended in a 3D collagen type I matrix and assessed for invasion up towards growth factors or bioactive lipids in the media after 72 hours in culture. (B) PDGF-BB stimulates HBVP invasion upward through the matrix while having little influence on HASMC invasive behavior. LPA is shown to be a driving force for HASMC invasion but has no effect on HBVPs. Similar trends were found when PDGF-BB was added to either BRP4s (pericytes) or CASMCs. PDGF-BB addition was able to increase BRP4 invasion upward yet had no effect on CASMCs. * $p \leq 0.01$. (C) Adding GM6001 to the upward invasion system containing HBVPs was able to inhibit invasion in the presence and absence of PDGF-BB. * $p \leq 0.01$. (D) The addition of blocking antibodies and soluble receptor traps to HBVP cultures containing PDGF-BB demonstrates that preventing PDGF-BB from binding to its receptor blocks HBVP invasion upward. * $p \leq 0.01$ compared to PDGF-BB, HPF- high powered field.

monolayer. This model system was tested using HBVPs and the same growth factors used in the prior experiments, with the addition of the bioactive lipids lysophosphatidic acid (LPA) and sphingosine 1 phosphate (S1P). Results show that PDGF-BB was the only factor added that was able to significantly enhance the invasive behavior of pericytes supporting the results found using the downward invasion model system (Figure 2B). The HASMCs were then assessed for invasion toward the monolayer using the same factors as in the HBVP experiment. Surprisingly, there was not an increase in invasion with the addition of PDGF-BB or any of the other growth factors. However, these cells were capable of invading as observed with the use of LPA. When comparing this result with the HBVPs, I found that LPA alone was not able to increase invasion of the pericytes. To study whether this pattern of invasion towards PDGF would hold true with different pericyte and VSMC lines, bovine retinal pericytes (BRP4s) and human coronary artery smooth muscle cells (CASMCs) were suspended in the upward invasion system and allowed to invade in response to the factors used previously. Similar to the HBVPs, the BRP4s invasive behavior was increased in response to PDGF-BB while the invasion of CASMCs toward PDGF-BB was not significant compared to control. Both BRP4s and CASMCs did not show a significant increase in invasion toward LPA although there is an upward trend with the CASMC invasion when compared to the other growth factors. Together, these data demonstrate that pericytes and vascular smooth muscle cells invade in response to different factors and cannot be considered similar when it comes to invasion during vascular morphogenesis. As in the downward system, invasion of HBVPs (the more invasive cell type) was examined in the presence and absence of

GM6001. Invasion was blocked with the addition of GM6001, once again showing that cells are invading, not simply migrating, through the collagen matrices (Figure 2C). HBVP invasion in response to PDGF-BB in the upward invasion system was further studied by utilizing the appropriate blocking antibody and/or soluble receptor Fcs. Soluble receptor Fcs (traps) contain the ligand-binding portion of appropriate receptor. The ligand binds to this soluble receptor making it unavailable to the actual receptor on the cell surface. Since the trap contains only the ligand-binding portion of the receptor, there is no activity or signal transduction due to binding of the ligand. Blocking antibodies to PDGF-BB, HB-EGF, BMP-4, bone morphogenic protein 9 (BMP-9), transforming growth factor beta (TGFβ), vascular endothelial growth factor (VEGF) and IL-6 and soluble receptor Fcs to PDGFRβ and TrkB (as a control) were added to media containing PDGF-BB. HBVP invasion was inhibited in the presence of the blocking antibody to PDGF-BB and the PDGFRβ trap. The other media conditions showed little change in pericyte invasive behavior compared to PDGF-BB alone (Figure 2D). These results suggest a role for PDGF-BB in pericyte invasion through the binding to PDGFRβ.

PDGF isoforms that bind to PDGFRβ have the greatest influence on pericyte invasion

Currently there are five known PDGF isoforms (PDGF-AA, AB, BB, CC, and DD). The role that these different isoforms play in morphogenesis is still largely unknown. A role for PDGF-BB has been demonstrated above, but it is important to test whether the other PDGF isoforms have the same effect on invasive behavior and if any of the isoforms influence vascular smooth muscle cell invasion. All PDGF isoforms were added to the upward invasion system at 40, 100, or 200 ng/mL to study whether there was an effect

of concentration on cell invasion. All of the isoforms tested, with the exception of PDGF-CC, were able to significantly influence HBVP invasion in a concentration dependent manner (Figure 3A). Upon further examination, I discovered that the isoforms that had the greatest influence on invasive behavior were those that have a higher affinity for PDGF receptor β (PDGFRβ): PDGF-BB, DD, and AB, when the PDGF receptor α (PDGFRα) is present. The same PDGFRβ binding isoforms were able to increase HASMC invasion, but only at certain concentrations. Isoforms that primarily bind to PDGFRα (PDGF-AA and PDGF-CC) had no affect on HASMC invasive behavior.

Pericytes express more PDGFRβ than vascular smooth muscle cells

To test why PDGF-BB and other PDGFRβ binding isoforms are able to significantly stimulate HBVP invasion while having little influence on HASMCs, Western blot analysis was performed to look at total PDGF receptor expression on both cell types. The varying PDGF isoforms were also added to examine receptor phosphorylation over time (Figure 3B). It was found that total PDGFRα expression was similar between the HBVPs and HASMCs. When the PDGF isoforms were added to each cell type, little PDGFRα phosphorylation was observed. Alpha tubulin was blotted as a loading control. Total PDGFRβ, on the other hand, showed a greater expression difference between the two cell types. The amount of PDGFRβ found on the pericytes was significantly enhanced compared to that of the HASMCs. HASMCs do have PDGFRβ, as can be seen in the longer exposures, but the levels are much lower than those of the pericytes. The addition of PDGF-BB and PDGF-DD (the two isoforms that bind PDGFRβ) led to increased phosphorylation of PDGFRβ in both cell types, although the effect was amplified in the

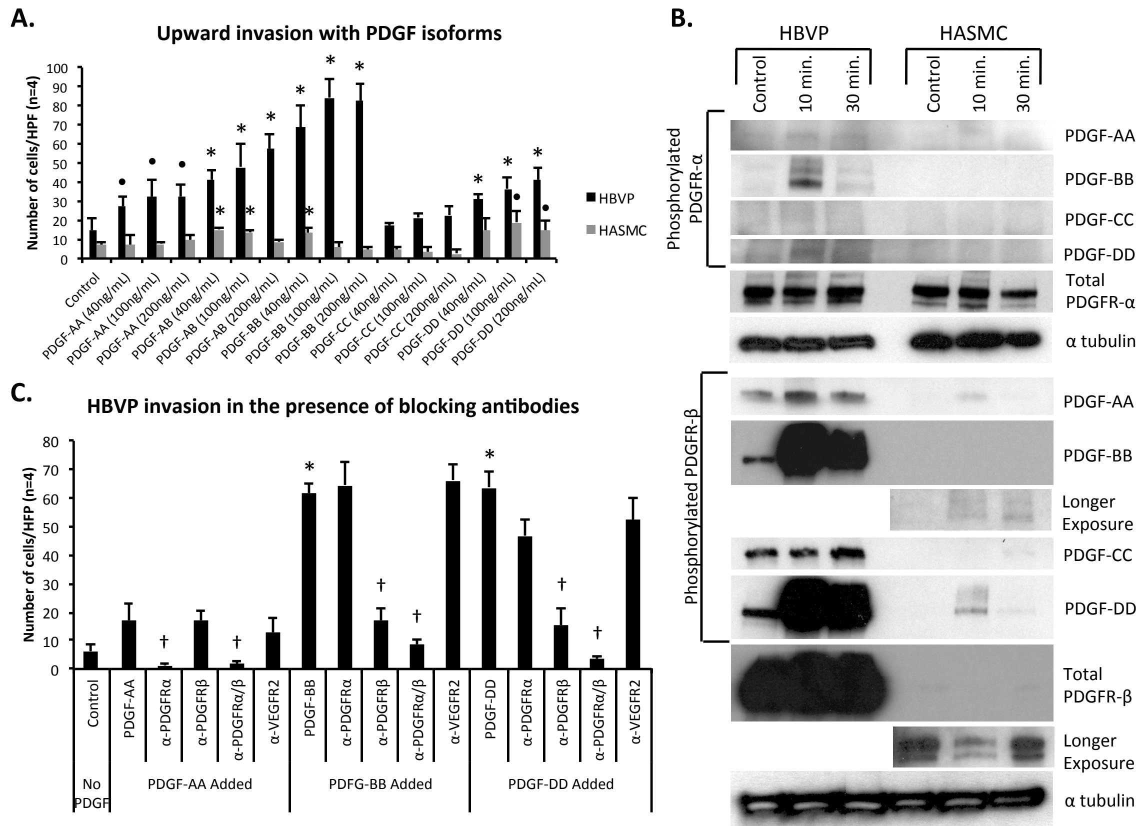

Figure 3 - HBVPs and HASMCs show differential expression of PDGFRβ and invasive behavior in response to PDGF isoforms

(A) The five different PDGF isoforms were added at different concentrations to the upward invasion assay system containing HBVPs or HASMCs. All isoforms, except PDGF-CC, are able to significantly increase HBVP invasion. PDGF isoforms that have a greater affinity for PDGFRβ increase invasion more than those that bind to PDGFRα. HASMCs show increased invasion with the addition of PDGF-AB and PDGF-BB but only at certain concentrations. * $p \leq 0.01$, • $p \leq 0.05$. (B) Western blot analysis was used to examine total PDGF receptor expression and receptor phosphorylation in HBVPs compared to HASMCs. Total PDGFRα expression was similar between the two cell types and shows little phosphorylation in response to the PDGF isoforms. Blotting revealed that PDGFRβ expression in HBVPs is greater than that of HASMCs and both PDGF-BB and PDGF-DD are able to greatly enhance receptor phosphorylation. (C) Antibodies to the different PDGF receptors were able to significantly decrease HBVP invasion in response to the PDGF isoforms that preferentially bind them and demonstrate a major role for PDGFRβ in HBVP invasive behavior. The addition of anti-VEGFR2 caused no significant changes in invasion and served as a control for antibody treatment. * $p \leq 0.01$ compared to control, † $p \leq 0.01$ compared to PDGF isoform alone, HPF- high powered field.

pericytes. These findings demonstrate why, in the upward invasion model system, PDGF-BB and other PDGFRβ binding isoforms are able to drive the invasion of HBVPs to a greater extent than HASMCs.

Pericyte invasion is primarily due to the activation of PDGFRβ

To further examine the role of PDGFRβ and to test whether PDGFRα has any influence on pericyte invasion, blocking antibodies to both receptors were added to media containing PDGF-AA, PDGF-BB, or PDGF-DD (Figure 3C). There was a slight increase in the invasion that occurred in the presence of PDGF-AA, although this was not significant. When the blocking antibody to PDGFRα was added to the media, there was a decrease in HBVP invasion. This decrease was also observed with the combination of PDGFRα/β antibodies but not with the addition of anti-PDGRβ alone, showing that PDGF-AA primarily works through PDGFRα and that these antibodies are selective for their respective receptor type. HBVP invasive response to PDGF-BB was inhibited in the presence of the blocking antibody to PDGFRβ alone or in combination with PDGFRα. PDGFRα alone had no affect on the amount of invasion that occurred in the presence of PDGF-BB. Invasion due to PDGF-DD showed a similar response to the antibodies as was seen in the PDGF-BB condition, which is explained by the preferential binding to PDGFRβ of these two PDGF isoforms. Antibodies to VEGF receptor 2 (VEGFR2) were also added as a control for each of the PDGF isoforms. In all cases, this antibody had no significant influence on HBVP invasive behavior.

Endothelial-produced PDGF-BB drives pericyte recruitment under an EC monolayer, leading to enhanced monolayer integrity

In vivo both endothelial cells (ECs) and mural cells must interact to form a stable and mature vessel. In order to simulate a more *in vivo* type of environment, a coculture system was developed that works in a similar manner to the upward invasion system except that ECs were seeded as a monolayer on top of gels containing either pericytes or VSMCs (Figure 4A). This endothelial monolayer mimics a larger vessel wall. The mural cells are seeded in the gel and allowed to recruit under the monolayer. This is similar to mural cells recruiting to the outer side of the vessel. As with all the systems used above, no FBS is added to the culture. In addition, no growth factors are added to the media of this system so any mural cell invasion is due to endogenous factors produced by the endothelial cells.

Staining the EC junctions with CD31 allows for the visualization of the monolayer and gives us the ability to quantify monolayer integrity in the presence or absence of the two mural cell types. In cultures with ECs seeded as a monolayer on top of collagen matrices containing no mural cells, the EC monolayer begins to contract by day 3, leaving large holes, and continues to regress to around 14 percent confluence by day 7 (Figure 4B). In contrast, when ECs are seeded on top of gels containing GFP-labeled HBVPs, the monolayer remains intact over several days. The same experiment was performed using HASMCs in the gel instead of pericytes. The monolayers of those gels are comparable to the EC-only conditions. Monolayers of EC/HASMC cocultures begin to

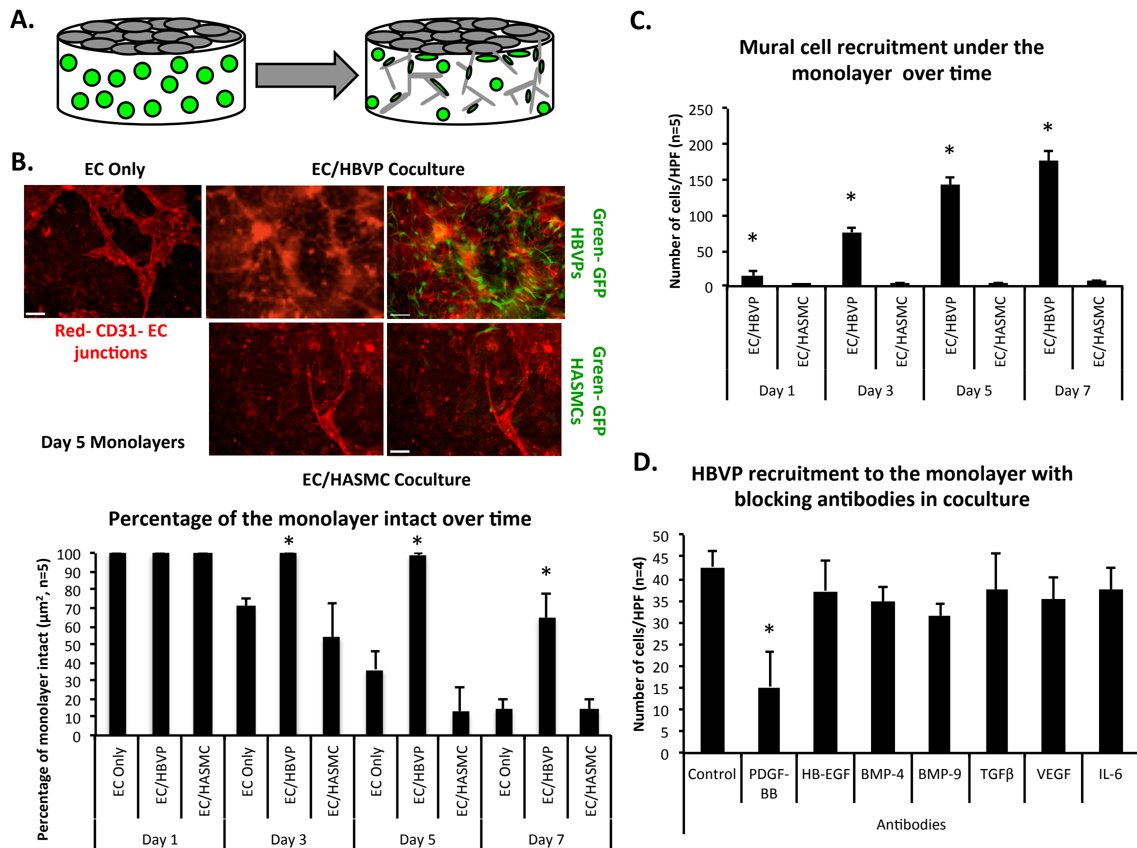

Figure 4 - EC produced PDGF-BB recruits pericytes under the monolayer which improves monolayer stability

(A) GFP labeled HBVPs or HASMCs were seeded in a 3D collagen I matrix and ECs were added to the gel surface to form a monolayer. (B) The percentage of the intact monolayer was examined over 7 days. Monolayers of EC only and EC/HASMC cultures show regression over time. Monolayers in the presence of HBVPs are stable up to 5 days of culture. Images show the different culture monolayers and mural cell recruitment at day 5. * $p \leq 0.01$ compared to EC only on the same day, bar equals 100 μm. (C) Mural cell recruitment was studied in conjunction with monolayer stability. While pericyte recruitment under the monolayer increases over time, there is no change in HASMC recruitment between days 1 and 7. * $p \leq 0.01$ compared to HASMC. (D) HBVP recruitment under the monolayer was decreased when EC produced PDGF-BB was prevented from acting on the pericytes with the addition of a ligand blocking antibody. None of the other antibodies tested had any effect on recruitment. * $p \leq 0.01$, HPF- high powered field.

contract by day 3 and become progressively worse over time. At day one 100 percent of the monolayer is intact compared to 14 percent by day 7.

Recruitment of pericytes and vascular smooth muscle cells under the monolayer in response to the endothelial-produced factors was also studied. After one day in culture, HBVPs are already beginning to recruit whereas there is no recruitment seen in the EC/HASMC cocultures (Figure 4C). HBVP recruitment towards the EC monolayer increases during the seven-day period- from around 15 cells at day 1 to about 175 cells at day 7. The recruitment of HASMCs also increases with time, although this recruitment is minimal and not significant when compared to the HBVPs. The differences in mural cell recruitment under the monolayer at day 5 of culture can be observed in Figure 4B.

Above, I provide evidence that HBVP invasion is driven by PDGF-BB. In order to confirm that this is true in the coculture system, blocking antibodies were added and HBVP recruitment under the EC monolayer examined. The same antibodies that were used in the upward invasion system were utilized in this model system. Again, the blocking antibody to PDGF-BB was able to reduce HBVP invasion towards the monolayer, signifying that endothelial produced PDGF-BB is sufficient for the invasive behavior of the pericytes in coculture and further supporting the single cell culture findings (Figure 4D).

Endothelial cells and pericytes interact at the monolayer to induce basement membrane deposition

It is hypothesized that the enhancement of the EC monolayer observed when HBVPs are present under the surface is partially a result of basement membrane deposition. A

combination of electron microscopy and basement membrane staining was used to test this hypothesis. An electron microscopy image from an EC/HBVP coculture reveals a pericyte that has been recruited under the EC monolayer. The interaction between the EC and the HBVP enhances basement membrane deposition (Figure 5A). Immunostaining cross-sections from EC/HBVP coculture gels for CD31 and collagen IV show a confluent EC monolayer and basement membrane deposition. In these cultures, GFP-labeled pericytes have been recruited below the EC surface (Figure 5B).

Pericytes are able to induce and sustain endothelial cell sprouting from the monolayer

In addition to studying the stability of the monolayer, the coculture system also allows for the analysis of EC sprouting and maturation in the presence of the different mural cells. As in the monolayer experiments, EC-only cultures were compared to EC/HBVP and EC/HASMC cocultures and average tube area analyzed. Movies were also captured during days 3 through 5 of culture to reveal the changes in EC sprouting that occur with and without pericytes or vascular smooth muscle.

All cultures have several small sprouts protruding the matrix 24 hours after set up (Figure 6A). By day 3 the EC/HBVP cocultures have significantly more sprouts than both the EC only and EC/HASMC cultures. The difference in tube area between the EC/HBVP culture and other two cultures is further defined by day 5. While the tube area in EC/HBVP cultures increases, it is substantially reduced in the EC-only and EC/HASMC cultures. EC-only movies reveal a potential cause for the diminishing tube area. By day three numerous EC tip cells can be found moving through the collagen matrix (Supplemental Movie 1). These cells have no clear directional movement and are not

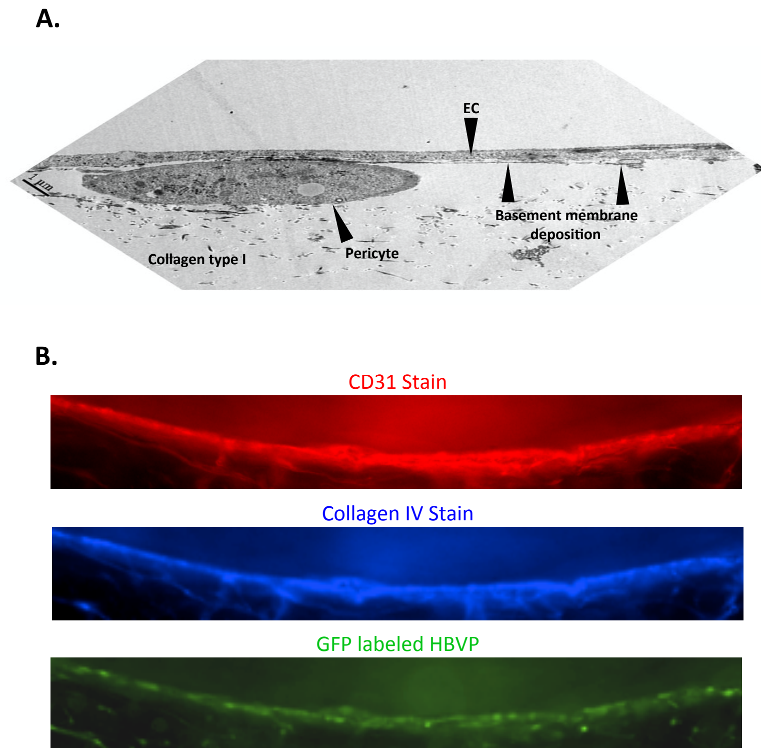

Figure 5 - Recruitment of pericytes under the EC monolayer enhances basement membrane deposition

(A) Electron microscopy shows a pericyte in the collagen type I matrix recruited under the EC monolayer and a thin line of basement membrane deposition between the two cells. (B) Cross-sections from EC/HBVP cultures display an intact monolayer through staining of the endothelial junction with CD31 (red). Basement membrane deposition was visualized by staining for collagen IV (blue). GFP labeled HBVPs (green) were utilized to show their recruitment under the EC monolayer.

followed by any tube formation. In fact, the tip cells located deeper in the gel appear to be coming from the sprouts that were present early on. Tip cells can be seen breaking away from the existing tube. These tip cells continue to invade while the vessel regresses back towards the monolayer (Supplemental Movie 2). Similar regression of the EC tube and breaking away of the tip cell is observed in cultures where HASMCs are present. Again, EC sprouts have invaded the matrix but the tip cell breaks apart from the EC tube and the tube begins to regress (Supplemental Movie 3). HASMCs are moving around in the gel but the movement does not appear purposeful as none of the HASMCs are recruited to any of the EC sprouts (Supplemental Movie 4). In contrast to the EC-only and EC/HASMC cultures, the EC/HBVP not only help to induce the EC sprouting but are also able sustain the sprouts (Supplement Movie 5). Little to no individual EC tip cells are found in the matrix of these cultures. When pericytes are present, the EC sprouts join together to form a more connected vascular network and instead of regressing the vessels become wider and more mature (Supplemental Movie 6). By day seven there are almost no surviving EC sprouts in the EC and EC/HASMC cultures. Tube area in the EC/HBVP cultures is beginning to decrease by this time, although not to the extent seen in the other two conditions (Figure 6A). Even with regression occurring there are still vessels present at the bottom of the gels in the EC/HBVP condition, which does not occur with EC alone or in combination with HASMCs. The distance that EC sprouts invade in EC/HBVP cultures compared to the sprouts of EC-only cultures can be noted in cross-sectional cuts of gels from representative cultures at days 3, 5, and 7 (Figure 6B).

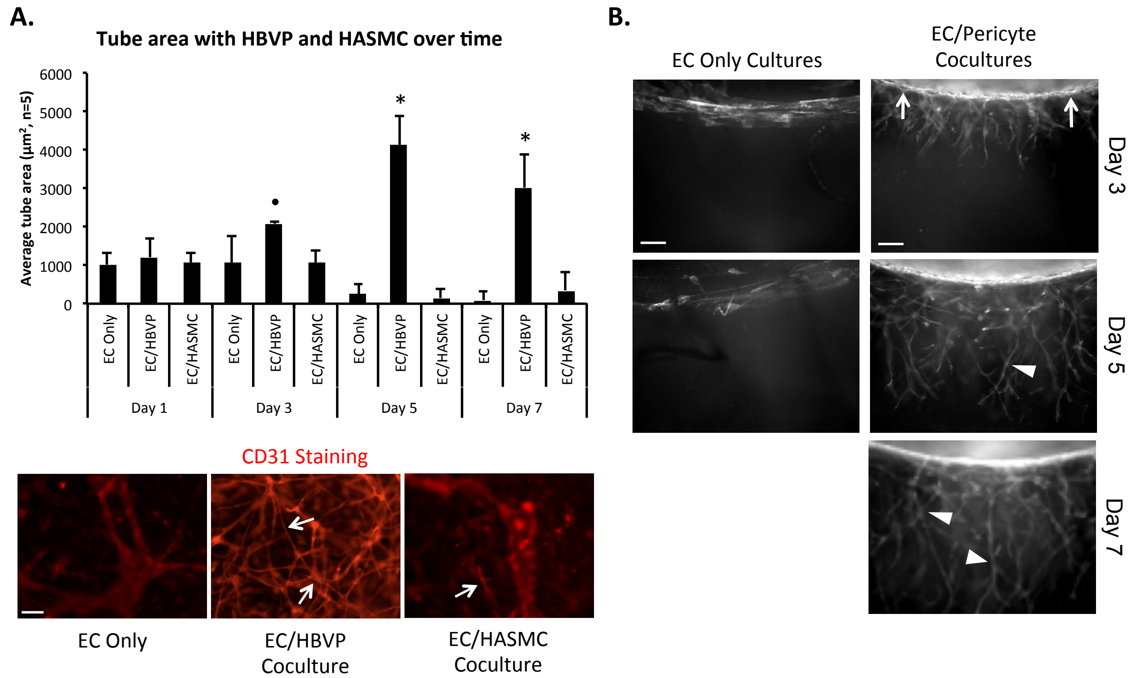

Figure 6 - Pericytes are able to induce EC sprouting from the monolayer and stabilize the EC tubes over time

(A) Cultures containing ECs only, EC/HBVPs, or EC/HASMCs were assayed for sprouting over time. EC sprouting was more abundant in the EC/HBVP culture than in the EC only or EC/HASMC cultures, which were similar in tube area. This trend was first observed at day 3 and continued through day 7. Images were taken 50µm under the monolayer using day 7 cultures. Arrows point to tubes within the matrix. * $p \leq 0.01$, • $p \leq 0.05$. (B) Cross-sections from EC only cultures show no sprouts from the monolayer (shown by the arrow). When HBVPs were present EC sprouts (arrow heads) were present at day 3 and continued to progress to the bottom of the gel through days 5 and 7 of culture.

Recruitment of pericytes to EC tubes stabilizes the vessels through basement membrane deposition

As mentioned above, it is believed that the pericytes are helping to sustain the sprouting ECs by deposition of basement membrane. In order to do this the pericytes must recruit to the forming EC tube. It is demonstrated that this does occur through both movie snapshots (Figure 7A) of an EC/HBVP culture and the use of GFP labeled pericytes and immunostaining (Figure 7B, C). Once the pericytes have recruited to the vessel, the ECs and pericytes are able to interact and form basement membrane. EC/HBVP cultures were stained with either collagen IV or fibronectin to visualize any basement membrane deposition along the EC tubes in conjunction with pericyte recruitment (Figure 7B). In these cultures, GFP labeled HBVPs are clearly recruited to the vessels and the different basement membrane components are found coated around the EC tube. Double staining the cultures with CD31 and collagen IV allows closer examination of how the EC tube and basement membrane correspond with each other and pericyte recruitment (Figure 7C). When the images of the CD31 and collagen IV stains are overlaid upon one another, the stains match up and this occurs in the presence of a pericyte that has been recruited to the tube structure.

4. Discussion

The investigation of mural cell activity during blood vessel development has been conducted primarily *in vivo*. Such studies have focused on the knockout of

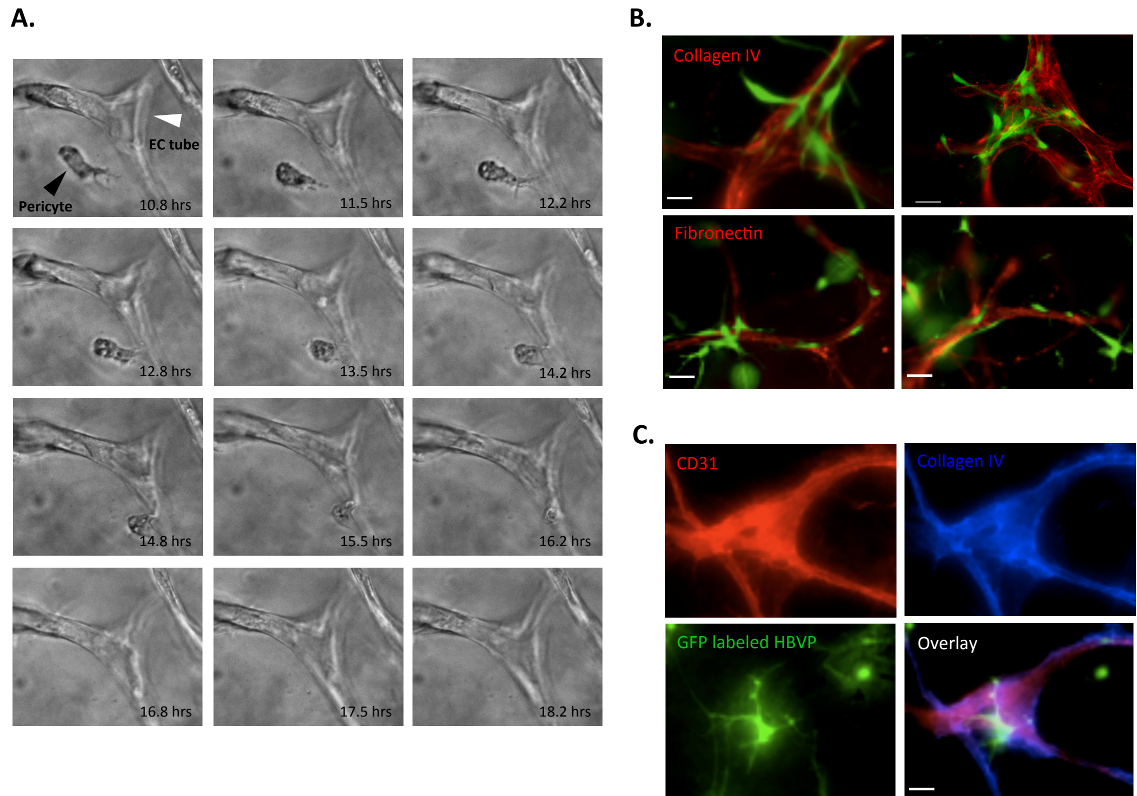

Figure 7 - Pericytes recruit to the EC tubes and help stabilize them through basement membrane deposition

(A) Time course of an EC/HBVP coculture. A cell believed to be a pericyte (black arrow head) can be seen invading the matrix toward an EC tube (white arrow head). The pericyte first makes contact with the tube in the 12.2 hrs panel and is in complete contact with the tube in the 15.5 hrs panel. From here the pericyte precedes to quickly move along the tube. (B) Detergent-free staining for collagen IV and fibronectin (red) show that both extracellular basement membrane components are deposited when pericytes (GFP labeled- green) are recruited to the vessels. (C) Images of an EC tube stained with CD31 (red), collagen IV (blue), and a GFP labeled HBVP (green). Overlaying these images shows that pericyte recruitment and basement membrane staining correspond to the EC vessel. Bar equals 100 μm.

PDGF-BB and PDGFRβ and demonstrate a role of these PDGF family members in mural cell recruitment during vasculogenesis and angiogenesis[5,9,13,19,23-27]. Knockout mice show a lack of pericyte recruitment to the microvasculature leading to hemorrhaging and an increased vessel width. In addition, the pericytes that are recruited to the vessels are more likely to detach in the knockout mice compared to controls. Of particular interest to my research is the finding that there is no significant difference in the number of vascular smooth muscle cells surrounding the larger vessels between the knockout and control mice. The difference between the mural cell recruitment to the larger versus smaller vessels led to my hypothesis that different factors are responsible for the recruitment of pericytes and vascular smooth muscle cells.

In the experiments performed for this thesis work, the two mural cell types were directly compared, utilizing a variety of serum-free model systems, which reduced the influence of confounding factors (undefined growth factors, lipids, cytokines, etc. found in serum) that would significantly affect the results. The examination of single factors in the upward invasion system revealed that PDGF-BB was able to drive pericyte invasion towards the surface of the matrix while having no affect on VSMC invasion. Western blot analysis performed on each of the mural cell types showed that an increased expression of PDGFRβ on the pericytes compared to VSMCs was likely responsible for the difference in invasive behavior. Tallquist (2003) demonstrates that expression levels of PDGFRβ is correlated to the amount of receptor signaling activity with higher expression leading to increased activity. Greater expression of PDGFRβ on the pericytes is likely to lead to stronger stimulation of downstream signaling pathways and the

increased invasion observed in my experiments while the lower expression levels of PDGFRβ on VSMCs may explain the weaker invasive response to both PDGF-BB and PDGF-DD. To my knowledge, my experiments are the first to directly compare the two cell types and, therefore, the first to note a difference in PDGFRβ expression between pericytes and vascular smooth muscle cells. The cause of this difference remains unclear but it may be due to the varying origins of the cell types. The similarities between pericytes and VSMCs has made it difficult to distinguish them during early development so there is some debate as to where each cell type arises; however, it is thought that pericytes are derived from the mesenchyme while the cardiac neural crest, mesenchyme and circulating stem cells are implicated for VSMC development[7,16,9,31-33]. Another possible explanation for the dissimilarity in receptor expression between the pericytes and VSMCs could be a result of cell differentiation[7, 16,11,10]. This theory suggests that pericytes are undifferentiated VSMCs and, as the pericytes begin to differentiate, they decrease the expression of certain genes such as PDGFRβ while increasing the expression of others. However, the evidence that this occurs is not substantial.

It has been shown that endothelial production of PDGF-BB is limited to tubes without a regular lumen[34-36]. The common belief is that mural cells migrate or spread along vessels from areas of greater mural cell density towards the new developing vessels[9,16,19,24]. In this thesis, I propose that mural cells are able to actually invade the matrix in which they are situated and not simply migrate through the mesh network. Utilizing both the upward and downward assay systems (with pericytes), the pan-matrix metalloproteinase inhibitor, GM6001, was added along with the primary invasion

stimulus for each of the cell types (PDGF-BB for pericytes and LPA for VSMCs). When GM6001 was added in combination with either PDGF-BB or LPA (see the LPA data in the Appendix), it was found that invasion was almost non-existent. Since invasion requires the use of matrix metalloproteinases and migration does not, the lack of movement towards the gel surface in response to either PDGF-BB or LPA with the addition of GM6001 provides evidence for the invasive behavior of the mural cells.

Utilizing the single cell assay systems above, I was able to directly compare pericytes and VSMCs and elucidate differences in invasive behavior that previously have not been demonstrated. Based on these findings, the second aim of my research was to discover if the differences in mural cell behavior had any impact on EC function. In the sprouting assay system, ECs are seeded as a monolayer on top of a 3D collagen gel to emulate the wall of a large vessel. Fluorescently labeled pericytes or VSMCs are resuspended within the gels and are recruited under the monolayer surface in response to endothelial-produced factors only. Pericyte recruitment under the EC monolayer increases with time, as does VSMC recruitment, although VSMC invasion is minimal compared to the invasive behavior of pericytes. Once under the monolayer, the pericytes are believed to interact with the ECs to produce basement membrane components, which are thought to promote vessel stability[7,10,17]. This appears to be true when the monolayers from EC-only and EC/HASMC cultures are compared to EC/HBVP cultures. In the EC/HBVP cultures, where recruitment is significant, the monolayer is sustained longer than in EC-only and EC/HASMC cultures where there is little or no mural cell recruitment. However, my results show that even the monolayers from

EC/HBVP cultures begin to regress by day 5 and continue to deteriorate by day 7 despite basement membrane deposition. There are several possible reasons why this occurs. First, the large number of sprouts emerging from the monolayer in EC/HBVP cocultures may generate tension on the EC junctions. As the sprouts continue to progress the strain on these junctions might become too great, leading to weakening or breaking of connections and then monolayer regression. A second reason is that flow across the ECs may be needed to enhance the monolayer stability after a certain amount of time in culture. It is well established that blood flow has an effect on EC gene expression, signal transduction, and cytoskeletal structure[37-39]. After 5 days in culture, pericyte recruitment and basement membrane deposition are not completed. Flow may be needed to enhance EC junctions[40] and transmit further signals to the pericytes in order to stabilize the monolayer and prevent the regression seen at later time points in culture. One set of signals may be transmitted from pericytes to ECs to stabilize monolayers early during recruitment and then another set may be transmitted once flow begins to further enhance stability (see Appendix).

As mentioned above, the presence of pericytes in the collagen gels induces EC sprouting from the monolayer. While sprouting is observed in both EC-only and EC/HASMC cultures, these sprouts either break away from, or regress back towards, the monolayer beginning between days 3 and 5 of culture. In the EC/HBVP cultures, the sprouts stay intact and continue to progress down toward the bottom of the gels until day 7 where regression was first observed. I hypothesize that the regression seen at this time point is likely due to a lack of flow through the vessels. During vessel development,

vessels in the forming plexus that are not considered critical or used may be pruned. The lack of flow may cause the vessels to close due to lack of use and make them difficult to discern during data analysis by day 7. However, electron microscopy has shown that the tubes formed in this system have open lumens (Figure 8), so it is likely that the lack of flow through the tubes by day 7 leads to pruning or regression of the existing vessels. The

effect of flow on tube regression or stability could be examined with the creation of a flow system (see Appendix).

To my knowledge, these experiments are the first to show functional differences between the pericytes and vascular smooth muscle in regards to recruitment and EC monolayer and tube stability. The findings also provide a way to define the mural cell types other than by their location in the vasculature, morphology or density on the vessel. I have shown that the pericytes are the more invasive mural cell in response to PDGF-BB and that they are important in maintaining the EC monolayer and tube stability. However, many questions remain to be answered about mural cell invasion and the roles that it plays in angiogenesis and vasculogenesis. At this time, the exact molecular mechanisms that the pericytes use to invade the 3D matrix remain unknown. These mechanisms could be studied through the use of siRNA treatment of the cells (see Appendix). Also of importance would be to further study the role of VSMC invasion to see whether differentiated VSMCs, such as those used in my experiments, are capable of invading under normal physiological conditions. As shown in the upward invasion studies, VSMCs invade in response to LPA, which is commonly found in wound healing

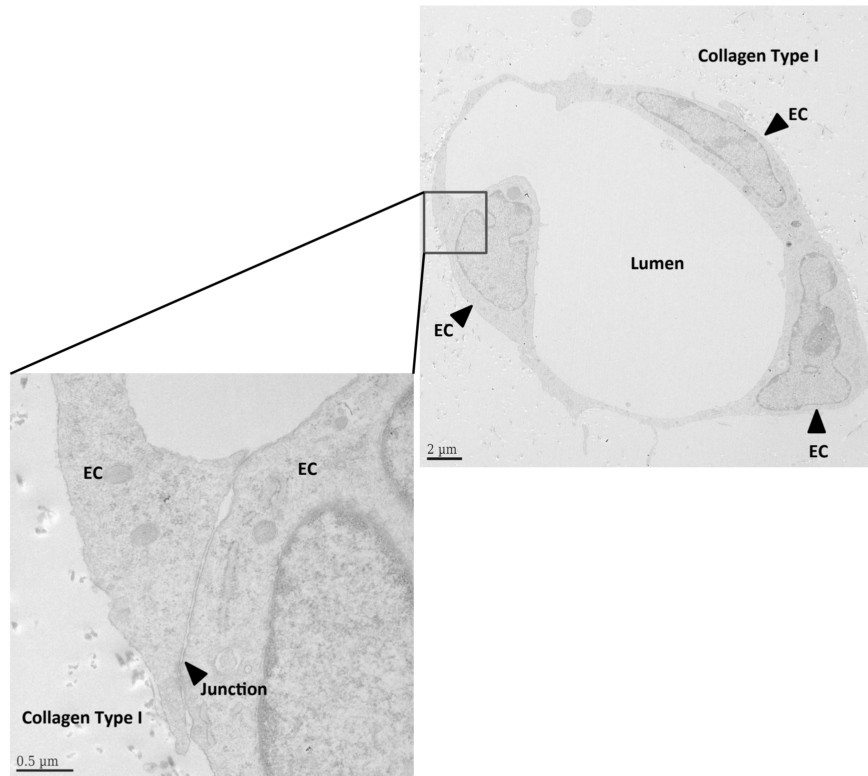

Figure 8 - Electron microscopy of an EC tube in the angiogenic sprouting system
Electron microscopy was performed on a day 5 EC/HBVP angiogenic sprouting system coculture. This particular tube is formed by the joining of three endothelial cells (EC). The boxed in area was magnified to demonstrate one of the EC-EC junctions of the tube.

and in pathological conditions such as atherosclerosis[41,42]. It is unclear if there is another factor found during normal development that would drive VSMC invasion. Finally, it is important to look into the role of differentiation and dedifferentiation of mural cells, i.e., whether a pericyte can become a VSMC or vice versa. If this transformation does occur, it would shed a great deal of understanding into the invasive behavior of mural cells and provide much needed information on how to treat numerous diseases caused by abnormal mural cell recruitment.

Appendix

Aortic versus vein ECs

My work above demonstrates that VSMCs do not invade in response to the endothelial-produced factors that I tested and do not recruit to EC monolayers or developing tubes. In the experiments presented, human umbilical vein ECs were used in the cultures whereas the VSMCs tested were obtained from coronary arteries and aorta. To rule out the possibility that vein ECs produce different factors than artery ECs and that this difference could be influencing the results, human aortic endothelial cells were purchased for testing. In these experiments, fluorescently labeled HBVPs and HASMCs were added to the sprouting assay containing either human umbilical or human aortic ECs. The results revealed that the EC type has no influence on mural cell invasion or recruitment. Pericytes recruited to both EC types while HASMCs showed little

recruitment. Based on these results, it can be concluded that the type of EC used in the presented experiments had no influence on VSMC invasive behavior.

LPA activity

As mentioned in the results, PDGF was able to stimulate pericyte invasion while lysophosphatidic acid (LPA) drove VSMC invasion through the 3D matrix. The addition of the pan-matrix metalloproteinase inhibitor GM6001 was able to inhibit pericyte invasive behavior, ruling out that the upward movement of the cells was due to migration through the matrix. It is important to mention that the upward movement of VSMCs towards the gel surface is, in fact, a result of invasive behavior. As with the pericytes, GM6001 was added to the upward assay containing HASMCs in combination with LPA. The addition of GM6001 to these cultures prevented the invasive response of HASMCs in response to LPA, showing that these cells are not merely migrating through the mesh network of the matrix (Figure 9A).

The experiments presented in the results focused on pericyte and VSMC activity in response to single growth factors and bioactive lipids. In order to fully understand pericyte and VSMC invasion, studies utilizing the upward invasion assays were conducted using multiple combinations of the growth factors and bioactive lipids to examine whether there were any synergistic effects on the invasive behavior of either mural cell. None of the combinations tested had any effect on HASMC invasion. However, when examining pericyte invasive activity, it was found that when PDGF-BB (40 ng/mL) and LPA (1 μM) were added together there was a synergistic effect that

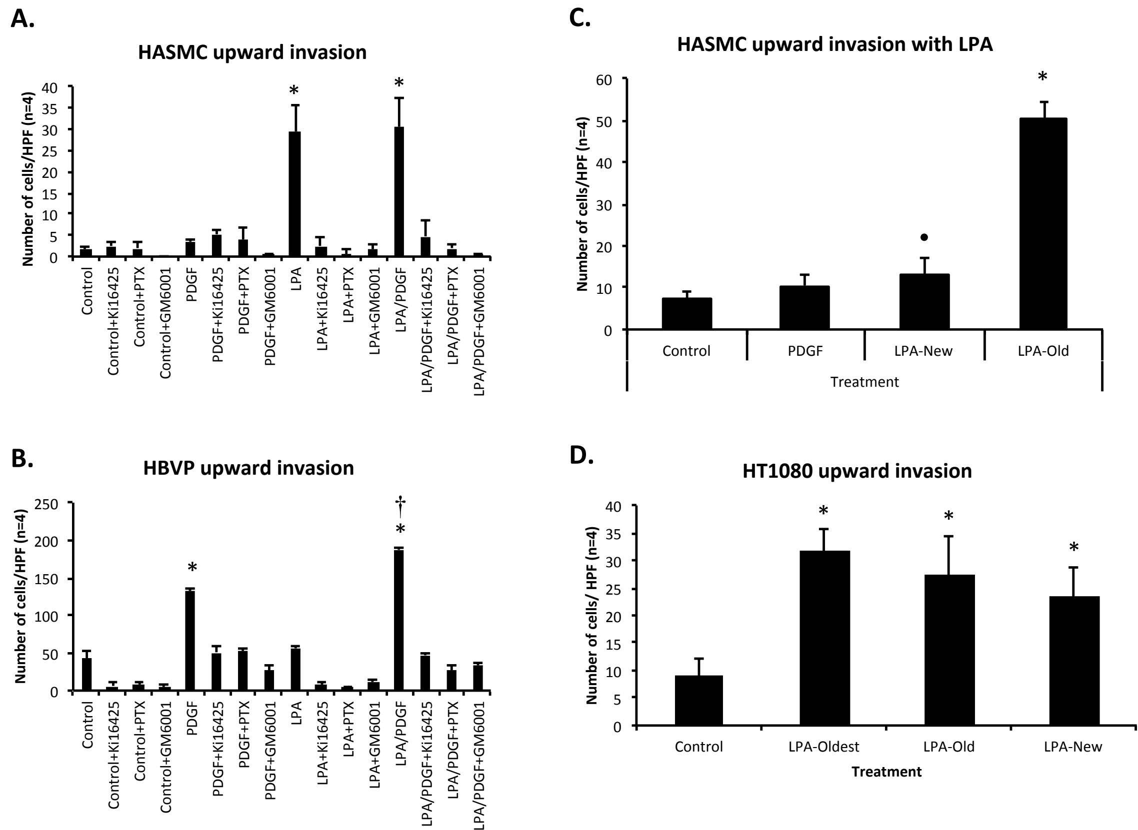

Figure 9- LPA influences mural cell invasion through synergism and age

(A) PDGF-BB, LPA, or a combination of PDGF-BB/LPA were added to the upward invasion assay system containing HASMCs in addition to GM6001 or the LPA inhibitors pertussis toxin (PTX) and Ki16425. HASMC invasion was stimulated by LPA and blocked by all three inhibitors. * $p \leq 0.01$. (B) The same factors and inhibitors tested with HASMCs were examined with HBVPs. Synergism between PDGF-BB and LPA led to a significant increase in invasion of HBVPs compared to PDGF-BB alone. This invasion was decreased to control levels with the addition of the inhibitors. * $p \leq 0.01$ compared to control, † $p \leq 0.05$ compared to PDGF. (C) HASMC invasion was studied in response to different aged LPA samples. The old LPA sample had the greatest influence on invasion while the new LPA had a less significant effect. * $p \leq 0.01$, • $p \leq 0.05$. (D) HT1080 colon cancer cells were used to further examine the age of LPA and its impact on invasion. All LPA samples increased invasion with a trend of increased invasion with age. * $p \leq 0.01$, HPF- high powered field.

enhanced pericyte invasion significantly compared to PDGF-BB alone. This invasion and invasion of the HASMCs in response to LPA could be blocked with the use of GM6001, pertussis toxin, or the LPA inhibitor Ki16425 demonstrating a role for LPA in both VSMC and pericyte invasive behavior (Figure 9A,B).

The above findings lead me to further examine the role of LPA in the invasive behavior of mural cells. LPA can be found within the plasma, is released by activated platelets[41,42] and is likely an important part of wound healing. However, outside of the healing process, it is unknown whether LPA is part of the normal VSMC recruitment process or if it is more relevant to pathological conditions. LPA is often associated with lipoproteins, especially oxidized low-density lipoproteins and has been implicated in atherosclerosis, where it is found within plaques[43,44]. A study performed by Damirin et al. (2007) showed that LPA in association with LDL was able to increase the migration of coronary artery smooth muscle cells, matching the HASMC invasion observed with LPA treatment. It is reasonable to guess that by having a factor, such as LPA, that is less abundant in normal conditions as the main stimulus for invasion, VSMCs are less likely to invade or move away from where they are needed around the larger vessels. It would also explain why VSMC invasion is sometimes observed in atherosclerosis. But why would pericytes show enhanced invasive behavior in response to LPA in the presence of PDGF-BB? This synergism may allow the pericytes to be recruited more quickly to a wound due to the release of LPA by platelets and PDGF-BB by injured endothelial cells. Invasion that requires the combination of PDGF-BB and LPA may help to prevent pathological invasion due to LPA alone.

Throughout my study of the influence of LPA influence on mural cell invasion, I discovered that the age of the LPA influenced cell activity. When identical concentrations of freshly made LPA were added to assays with VSMCs, the invasion was reduced from what had been observed in previous assays using older LPA samples. To test whether the age of the LPA was indeed responsible for the difference in the invasive behavior, I set up an upward invasion assay using HASMCs and two different LPA samples. The oldest sample greatly stimulated HASMC invasion while the newer sample showed some invasion but was reduced compared to the older sample (Figure 9C). The different samples were then tested on HT1080s, which are cells from a colon cancer cell line that have been shown to massively invade in response to LPA[45]. As with the HASMCs, HT1080 invasion was greatest when the cells were exposed to the oldest LPA sample (Figure 9D). Invasion decreased in response to a newer LPA sample and further declined in the presence of freshly made LPA, although invasion was still significantly increased compared to control. I theorize that opening the containers of LPA leads to the LPA being oxidized. This oxidation might then change the conformation of the LPA making it more potent to the mural cells. For this theory to be tested, mass spectrometry could be run on both an older and a freshly made sample of LPA to look for changes between the two samples.

Flow system

I initiated the development of a flow system to study the question of whether pericytes could differentiate into vascular smooth muscle cells. Due to the commonalities of the two cells types with respect to gene expression and the

differences in recruitment, it was thought that pericytes would recruit to the developing tubes and, once flow was started, the pericytes would begin to wrap around the vessels and differentiate into VSMCs. The creation of the angiogenic assay system, mentioned earlier, allows for the possibility of a flow system. In the angiogenic system, EC tubes invade into the matrix from an EC monolayer on top of the gel. The tubes continue to extend toward the bottom of the gel with time. This provides open tubes from the monolayer throughout the width of the gel through which media could flow. Other assay systems lack direct access to the vessel lumens, making the ability to study the influence of flow difficult.

The flow system started out in plastic cups with a porous bottom that could be placed within 24 well plates. Plastic wrap was stretched over the bottom of each well and secured with an orthodontic rubber band to prevent the gels and media leaking out of the bottom of the cups before the tubes had formed. The first several attempts at the flow system in the cups were unsuccessful due to the contraction of the gels away from the sides of the cups. This issue was resolved by scratching the inside of the cups and increasing the collagen concentration to 3.75 mg/mL instead of the normal 2.5 mg/mL. Unfortunately, due to the amount of gel that had to be added to fill the cup, it took over 10 days to get tubes to extend from the monolayer to the bottom of the cups. Because the viability of the EC tubes extends only to about 5 or 6 days before regression (Figure 6A), the next goal was to create a system that would use less collagen so that the tubes could more quickly reach the bottom of the gel.

The second flow system was attempted using 1 mm square glass tubes approximately 8 mm long. The benefit of using glass tubes is it would allow for visualization of the progress of tube invasion and also imaging of flow through the tubes if the system could be successfully developed. Fifty or 25 μL gels (3.75 mg/mL) containing pericytes were added to the middle of each tube and the EC monolayer added to one side of the gel with 1X M199 added to the other end. Again, the gels contracted away from the sides after only a few of days in culture, which did not provide enough time for the EC tubes to reach the bottom. To prevent contraction the glass tubes were coated with lysine, collagen, avidin and biotinylated collagen, or plastic. None of these options were able to stop the contraction of the gels. I discovered that the contraction of the gels began at the EC monolayer and worsened as the tubes invaded further into the matrix.

Repeated studies using the angiogenic assay in 96 well, half-area plates demonstrates that this would be the ideal set up for development of a flow system. In order to prepare the 96 well plates for the flow system, the bottoms of the wells were drilled out and a membrane used for Boyden chambers was glued to the bottom of the plate. This membrane would provide the system with a base on which to solidify the gels but would allow for flow when the cultures were ready. Plastic wrap was stretched across the bottom and a gasket, with holes cut out so that the progress of development could be observed, was placed against the bottom of the plate to prevent the leaking of media from the wells. A plastic base was then added to the bottom and bolted in place to keep all the components of the system sandwiched together. Holes were drilled in

the top of the lid to allow the bolts to pass through and the lid to sit on the plate in its normal orientation (Figure 10). This set up also made removal of the lid easier for feeding the cultures when needed. Overall, this system is the closest to the normal angiogenic set up compared to all of the other systems created. In this system, contraction was not an issue but survival of the cells became a critical limiting factor. The 96 well plate and the Boyden chamber membrane are the only components in contact with the cultures and both are commonly used in cell culture experiments. Therefore, it was concluded that the glue being used was toxic to the cells. Four different types of adhesives: super glue, Dow Corning RTV, Dow Corning Sylgard and a tissue adhesive applied to wounds were used to attach the Boyden chamber membrane but none were found to help the survival of the cells. If an adhesive that was non-toxic to the cells could be found or the system manufactured with a porous bottom already attached, it is likely that this flow system will be successful and the influence of flow on mural cell recruitment and differentiation tested.

Pericytes and the EC monolayer

Much of the other work in this field suggests that pericytes must be recruited in order to stabilize the EC tubes and monolayers[4,6,17]. This step may be important later in vessel development, but during the early stages it is possible that the presence of pericytes within the matrix may be enough to start stabilizing the forming tubes. The angiogenic assay was set up and different ligand neutralizing antibodies were added to look at the effect that lack of pericyte recruitment would have on the stability of the monolayer. As expected, the addition of anti-PDGF-BB significantly blocked pericyte

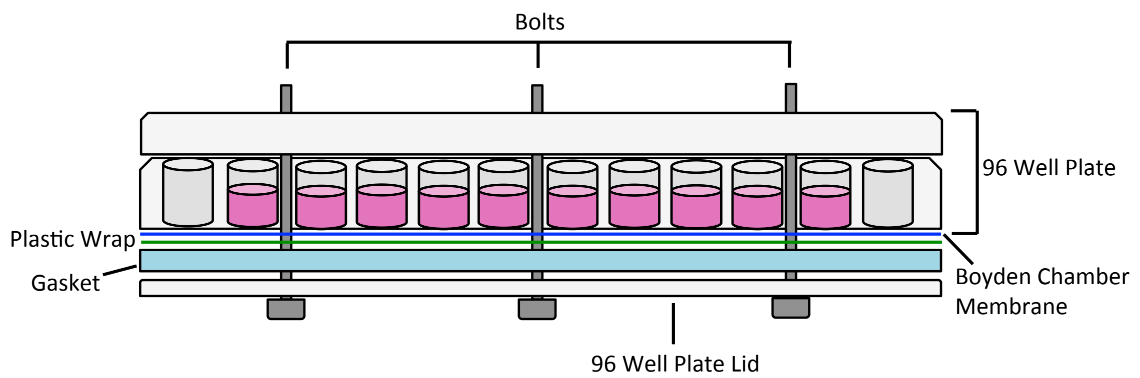

Figure 10 - Flow model system
The flow model system was created using a 96 well half-area plate. The bottoms of the wells were drilled out and a Boyden chamber membrane attached to the base of the plate to provide a porous bottom for flow. To prevent leaking of the gel or media from the wells during tube development, plastic wrap was stretched across the base and a gasket placed against the bottom of the plate. These components were held together using a lid to a 96 well plate with the edges removed and bolts going through all pieces. The lid of the culture plate had holes drilled out for the bolts to come through so the lid would sit on the plate as it would in the normal systems. This set up would also allow for the lid to be easily removed for changing culture media when needed.

recruitment to the monolayer (Figure 11A). However, the percentage of the monolayers intact after five days in the presence of the PDGF-BB neutralizing antibody were not different than the control condition or the cultures in the presence of other antibodies that had no impact on pericyte recruitment (Figure 11B). By day 7 the cultures containing the PDGF-BB neutralizing antibody had monolayers that were significantly more intact than all other conditions. The same result was found when PDGF receptor antibodies were added to the cultures. There was no change in recruitment seen with the addition of the PDGFRα blocking antibody but recruitment in the presence of the blocking antibody to PDGFRβ was significantly reduced (Figure 11C). However, this reduction in pericyte recruitment in response to the PDGFRβ blocking antibody had no effect on monolayer stability in terms of inducing regression (Figure 11D). Due to the unexpected results from these experiments, it was important to further study this phenomenon. In the upward and downward invasion assays, I found that pericytes use MMPs to invade through the 3D matrix and that this invasion could be prevented by adding GM6001 to the cultures. To more thoroughly examine whether pericytes needed to be recruited for monolayer stabilization, I utilized GM6001 to prevent their movement. EC-only and EC/HBVP angiogenic cultures were set up for 2, 3, 5 and 7 days with and without the addition of GM6001. Without the addition of GM6001 there was an increase in pericyte recruitment under the monolayer with time and this recruitment was inhibited with GM6001 (Figure 12A). The addition of GM6001 also blocked the ability of ECs to sprout into the matrix as expected (Figure 12B). Analysis of the monolayers from each of these conditions revealed that the EC-only

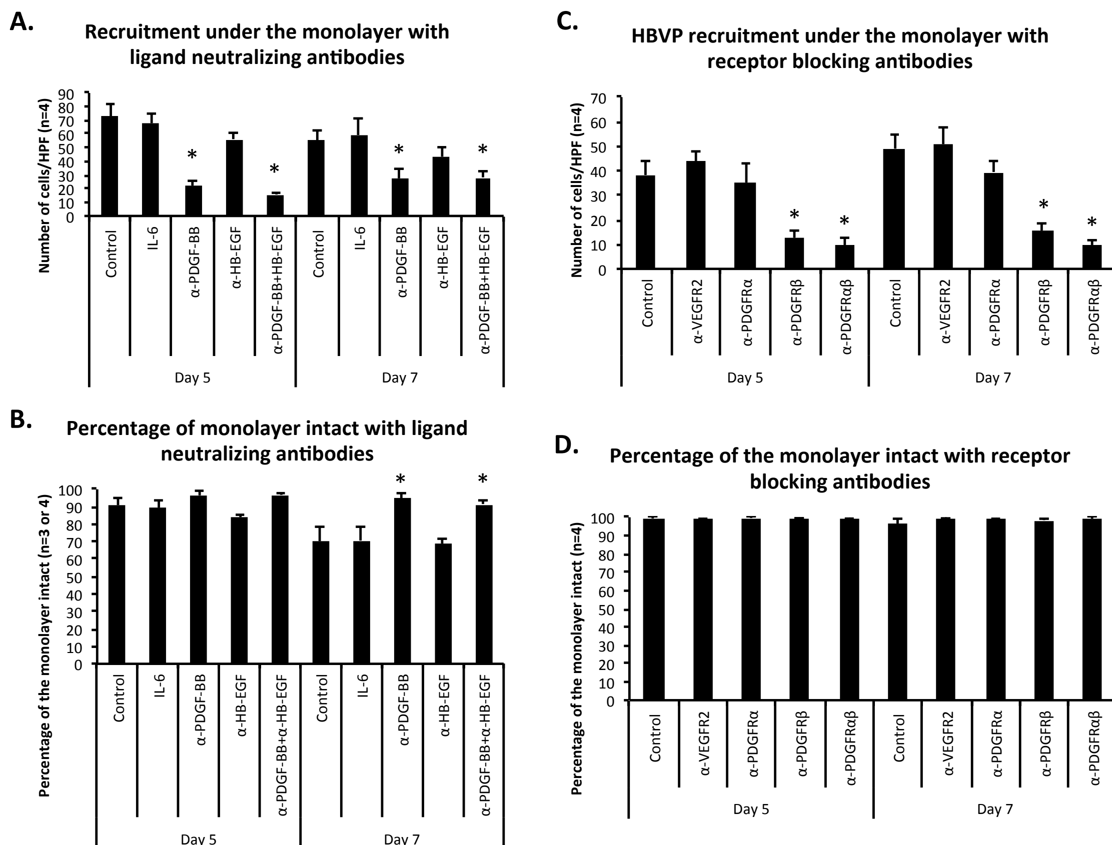

Figure 11 - The EC monolayer stays intact over time despite lack of pericyte recruitment
(A) The addition of PDGF-BB neutralizing antibody prevents the recruitment of HBVPs under the EC monolayer in the angiogenic sprouting system in response to endothelial-produced PDGF-BB. (B) The monolayers of EC/HBVP cultures were analyzed at days 5 and 7 for regression. By day 7 all monolayers had regressed but those of cultures containing the PDGF-BB neutralizing antibody remained significantly more intact than the other monolayers even though pericyte recruitment was blocked. (C) PDGF receptor antibodies were added to EC/HBVP cultures. When the PDGFRβ antibody was present, pericyte recruitment to the EC monolayer was significantly blocked. (D) The monolayers of EC/HBVP cultures were examined in the presence of receptor antibodies. There was minimal regression of the monolayers up to seven days in culture despite decreased pericyte recruitment.
* $p \leq 0.01$, HPF- high powered field.

monolayers began to regress by day 5 and further retreated by day 7 (Figure 12C). There was a concern that the use of GM6001 itself would prevent contraction of the monolayers but the findings from day 7 EC-only cultures with GM6001 shows that this is not the case (Figure 12C). The addition of GM6001 to EC-only cultures delayed the regression of the monolayer, but by day 7 the monolayer looked similar to those in the EC-only cultures. As expected, monolayers from the EC/HBVP cocultures remained mostly intact up to day 7. The regression seen by day 7 is common for these cultures and is explained in the Discussion. In the presence of GM6001, as with the addition of the PDGF-BB neutralizing antibody, the monolayers of EC/HBVP cultures are more intact at day 7 than the controls. By day 7 there is almost no regression. In fact, another surprising discovery from these studies was that the ECs of the monolayer seemed to start aligning themselves as if flow had been introduced. There is currently no known explanation for this event, but maybe the GM6001 acts like a natural matrix metalloproteinase inhibitor that would be produced *in vivo* in response to flow. This may have caused the ECs to change their gene expression as they would in the presence of flow and cause the alignment.

Focusing on the monolayer stability findings, I proposed that pericytes might not need to be recruited –they only have to be present- in order to begin stabilizing the tubes and monolayers. I hypothesized that the pericytes release a soluble factor that begins to stabilize the EC tubes and monolayers until the pericytes have had time to invade the matrix and reach them. Once there, pericytes are able to further stabilize the EC structures by basement membrane deposition.

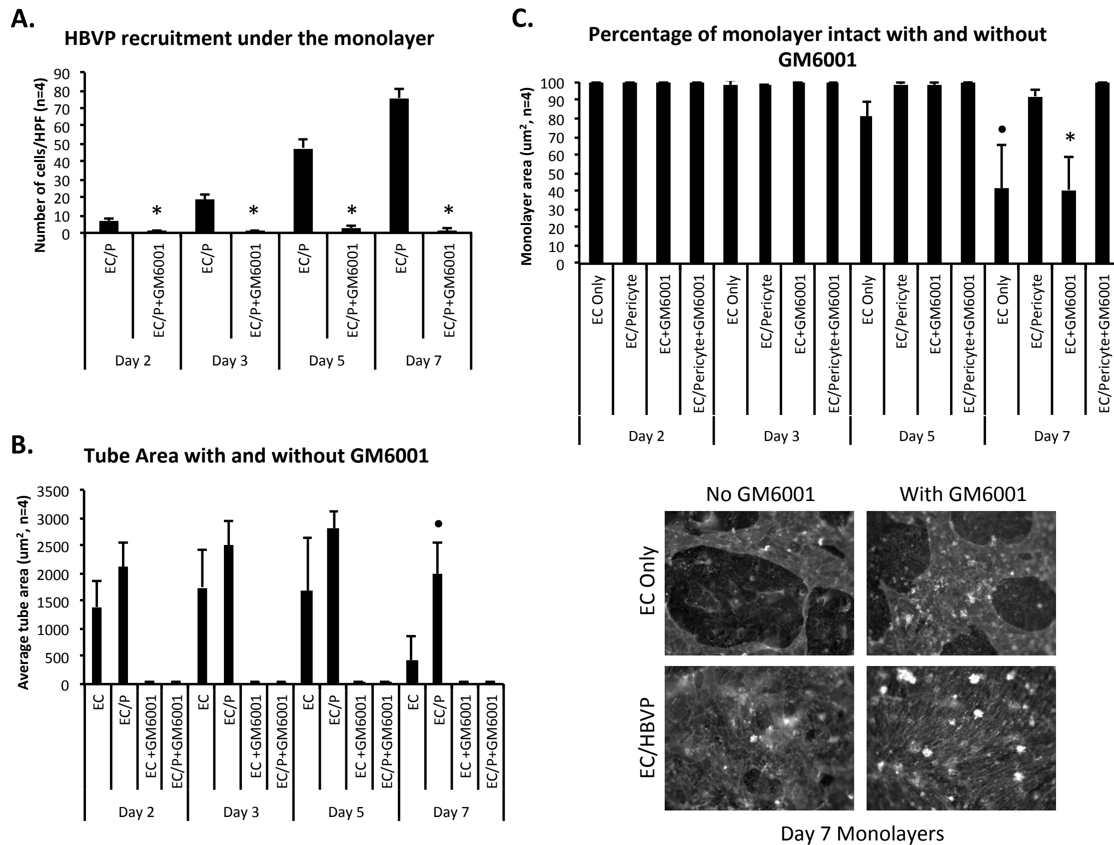

Figure 12 - Pericyte presence within the matrix helps to enhance the monolayer
(A) Recruitment under the monolayer in EC/HBVP cultures was examined with and without GM6001. Pericyte recruitment increases over time in control conditions and is blocked with the addition of GM6001. * $p \leq 0.01$. (B) Adding GM6001 to EC-only and EC/HBVP cultures was able to prevent the sprouting seen without GM6001. By day 7 there was a decrease in tube area in both EC-only and EC/HBVP cultures although there are significantly more tubes in the EC/HBVP cultures at this time point. • $p \leq 0.05$ compared to EC at day 7. (C) The percentage of the monolayer intact was analyzed on days 2, 3, 5 and 7. The monolayer of EC-only cultures begins to regress by day 5 and worsens by day 7. Regression of monolayers in EC/GM6001 cultures was delayed but significant by day 7. EC/HBVP monolayers started to show slight regression by day 7. This regression was blocked in EC/HBVP cultures with GM6001. The ECs in these monolayers also appear to align with time. The monolayers were visualized by immunostaining with CD31. * $p \leq 0.01$, • $p \leq 0.05$, HPF-high powered field.

To test this soluble factor hypothesis, cultures containing ECs only, HBVPs only, EC/HBVPs, or no cells were set up for 1, 3, 5, and 7 days. After the respective number of days in culture, the media was collected from each of the cultures and spun down to remove the dead cells. The media was then added to angiogenic sprouting assays containing an EC-only monolayer to test if the monolayers would regress. The monolayer was checked after 3 days. Upon inspection, all of the monolayers had begun showing signs of regression. While disappointing, this finding does not rule out the possibility of a soluble factor being released from the pericytes. I hypothesize that there is, in fact, a soluble factor but that it is being trapped within the matrix upon release. A way in which to remove the soluble factor from the matrix is not known and as a result made the testing for such a factor nearly impossible. This idea could be tested by blocking several different factors known to be produced by pericytes, but due to the time required to test each of factors, the project was put on hold.

siRNA knockdown of pericyte signaling molecules

To fully understand the invasive behavior of pericytes, it is important to know what molecular mechanism the pericytes are using to invade the matrix. HBVPs were treated with siRNAs, using Lipofectamine 2000 (Invitrogen) for transfection, to different molecules which may have a role in this invasive behavior (Table 1). Early siRNA treatments showed that there was some inconsistency with the control siRNA (luciferase) being used in the experiments and as a result the cell counts obtained were not reliable. New siRNA controls were purchased from Ambion and used throughout the remainder of the siRNA experiments after results showed them to be more consistent

Table 1- siRNA knockdown of pericyte signaling molecules

ADAM9	EGFR	JamC	MT2-MMP	Par6β	PKCζ
ADAM10	ErbB4	Lyn	MT3-MMP	Par6γ	Rac1
ADAM12	Fyn	MDia1	N-Cadherin	PDGFRα	RhoA
ADAM15	HIF1α	MDia2	Pak2	PDGFRβ	ROCK1
ADAM17	IQ GAP	MRCKα	Pak4	PKCα	ROCK2
CDC42	JamA	MRCKβ	PAR3	PKCδ	Src
CEP5	JamB	MT1-MMP	Par6α	PKCε	Yes

than the luciferase siRNA. However, repeated experiments using the same sets of siRNAs revealed variations between each of the experiments. After looking at the gels from the siRNA upward invasion experiments and comparing them with gels from normal upward invasion assays, I discovered that there was increased cell death when the pericytes were treated with siRNAs. This is a finding is not observed in previous experiments from the lab due to the presence of ECs in the other assay systems. I hypothesize that ECs provide the pericytes with growth factors that help to keep them alive after the harsh siRNA treatment with Lipofectamine 2000. In the upward invasion assays performed and analyzed in these experiments, there are no ECs present, therefore, no growth factors to aid their survival in the cultures.

When ECs are treated with siRNAs, they are transfected using siPort Amine (Ambion) because it is gentler on cells than Lipofectamine 2000. I decided that the HBVPs would be treated with two different concentration of siPort Amine to see if the gentler transfection agent would make a difference in pericyte survival in the upward assay and if the concentration used would make any difference in blocking ability. Treatment with siPort Amine greatly increased pericyte survival, but neither concentration significantly blocked the molecules being tested. As mentioned above, Lipofectamine 2000 treatment was used in prior siRNA experiments. Western blots examining knockdown in the experiments show there is a decrease in expression of the molecules tested. During siRNA treatment on pericytes, it would be ideal to use Lipofectamine 2000, but different concentrations would have to be tested to see which concentration would provide the greatest knockdown while maintaining the highest

level of survival. Only then will the molecular mechanisms for pericyte invasion be able to be studied definitively using the siRNA approach.

Aggregates versus single cells

Pericytes are currently viewed as the mural cell type found around small vessels such as the capillaries while VSMCs are located around the larger vessels. I wanted to test whether the size of a vessel would influence which cells were recruited to the surface of an EC tube. Cultures containing single ECs or aggregated ECs were set up with fluorescently labeled HBVPs (green), HASMCs (red), or both (Figure 13). I expected that pericytes would be recruited to the tubes formed in the single EC cultures and VSMCs would be recruited in cultures containing aggregated ECs. However, I found that pericytes recruited to the tubes formed in both cultures. The HASMCs, once again, demonstrated little to no recruitment in either set up. This finding suggests that vessel size, under these conditions, does not play a role in determining which mural cell type is recruited. Why, then, are pericytes and VSMCs always found in the same location within the vasculature? I believe that this may be explained by a combination of flow and differentiation. As stated in the discussion, the induction of flow and variations in pressure cause changes in EC gene expression and signal transduction. If it were possible to generate flow through the vessels, then there may be a difference in EC signaling between the small and large vessels and VSMCs may be recruited to the larger tubes. If introducing flow to the system is unable to change the recruitment of VSMCs it may provide more evidence for differentiation of pericytes into VSMCs. Pericytes may be the mural cell that recruits to all vessels but changes in EC signaling due to flow may

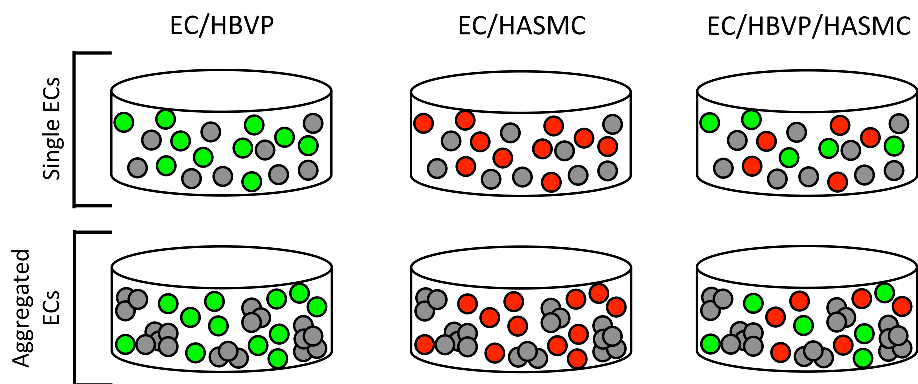

Figure 13 - Aggregate versus single cell assay set up
The aggregate versus single cell experiment was used to examine whether vessel size influences which mural cell invades towards the tube. Cultures containing single cell ECs (gray dots, top row) or aggregated ECs (bottom row) were suspended in culture with fluorescently labeled HBVPs (green dots), HASMCs (red dots) or a combination of both for a number of days before recruitment was examined.

cause the pericytes surrounding the EC tubes to change their gene expression and differentiate into a less invasive VSMC. This would allow mural cells (pericytes) to be recruited to the vessel and assist in maturation but, once recruited, prevent the cell from pathological invasion towards the lumen or from moving from its current location. VSMCs would not be able to effectively perform their function if they were constantly moving past each other or up and down the vessel.

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