Pericyte-Endothelial Cell Interactions during Blood Vessel Formation and in Diabetic Scenarios

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ACADEMIC ABSTRACT

Diabetic retinopathy (DR) is an incurable, chronic disease that is the leading cause of blindness in working-age adults. A prominent characteristic of DR is the extensive dysfunction within the retina microvasculature. Specialized vascular cells known as pericytes (PCs) are lost or become dysfunctional during disease progression; a thickening of the extracellular matrix (ECM) composing the vascular basement membrane (vBM) and endothelial cell (EC) tight junction disruption are also key features of this disease and contribute to its pathogenesis. PC loss is believed to be a central cue for disease initiation. However, studies inducing PC loss and observing acute changes in the vasculature did not report severe vessel damage or vBM thickening, suggesting that the effects of PC loss occur over a longer period of time. Because the chronic effects of PC loss are more difficult to ascertain, especially in a complex condition such as DR, the mechanisms underlying microvascular defects in DR remain poorly understood.

The work presented in this dissertation focuses on pericyte-endothelial cell interactions and their interplay with the ECM/vBM during a variety of physiological and pathological conditions. First, we isolated and functionally validated a primary mouse embryonic PC cell line that we then applied to a co-culture model with ECs to better understand the dynamic interactions between these two critical components of the capillary wall. In the co-culture model, we found that primary PCs promoted EC organization into vessel-like structures and enhanced EC-EC junctions. To complement these *in vitro* studies, we analyzed animal models and human tissue for the PC-EC interactions and ECM/vBM remodeling under different conditions (physiological and pathological). Moreover, we analyzed microglia and astrocytes to enhance

our understanding of the tissue-vessel interface, bolstering our experimental results and facilitating the generation of more hypotheses for future research.

Overall, our work suggests that PC-EC interactions in diabetic scenarios play a crucial role in ECM/vBM remodeling; engagement with the ECM/vBM in turn impacted PC behaviors including migration away from the endothelium and induced EC loss of tight junctions, key changes in the onset and progression of DR.

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GENERAL AUDIENCE ABSTRACT

Diabetic retinopathy is a group of eye diseases occurring in patients suffering from diabetes and is the leading cause of adult blindness among the working-aged. About one in three people with diabetes over the age of 40 have overt signs of DR. The primary cause for this disease is long-term, high blood sugar levels that damages blood vessels systemically as well as in the eye. Current treatments for DR can prevent the condition from getting worse, but no treatment exists that results in a complete cure.

This work described in this dissertation focuses on the interactions between vascular pericytes and endothelial cells, two of the main cell types that compose capillaries (i.e. the smallest blood vessels important for oxygen delivery). The studies presented herein also focus on the response of these cells to the extracellular matrix, a scaffold of proteins that surround pericytes and endothelial cells to stabilize blood vessels. We found that extracellular matrix components dramatically increase as a result of the interactions between pericytes and endothelial cells exposed to diabetic conditions. These changes in the extracellular matrix also had important effects on pericytes and endothelial cells and their engagement with their environment and other cells. Taken together, our work suggests that pericyte-endothelial cell interactions and their crosstalk with the ECM play an important role in blood vessel formation and in the accumulation of microvascular defects that fuel diabetic retinopathy progression.

DEDICATION

I would like to dedicate this dissertation to my parents and all of my family, for all of their love and support.

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The greatest and most heartfelt appreciation goes to my advisor and Ph.D. committee chairperson, Dr. John Chappell

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ATTRIBUTIONS

The work described within this dissertation would not have been possible without help from colleagues. A list and the brief description of their contributions are included below:

All chapters:

John C. Chappell, Ph.D. is an Assistant Professor at Virginia Tech and is the corresponding author on this manuscript. He helped with the development of these projects as well as the editing of the manuscripts.

Chapter 2:

Jordan Darden (Department of Translational Biology, Medicine, and Health program) is a graduate student at the Virginia Tech Carilion, Fralin Biomedical Research Institute at Virginia Tech, and is a co-author on this manuscript. She was instrumental in the performance and analysis of the experiments. She also contributed to the writing and editing of the manuscript.

Chapter 3:

Tim Nguyen is a medical student of Virginia Tech Carilion School of Medicine at Virginia Tech and is acquiring human cadaveric eyes.

Justin Davis is an undergraduate student at Virginia Tech Department of Biochemistry. He was performance the human retinas sectioning.

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Chapter 1

Literature Review

An Overview of Diabetic Retinopathy Progression

Diabetic retinopathy (DR) is one of the more devastating and life-altering complications resulting from *diabetes mellitus* [103]. DR is the primary cause of vision loss in working-age people [105], and is clinically defined in part by retinal microvascular lesions in diabetic patients that are visible by ophthalmoscopy, also called funduscopy [104]. It is considered to be a microvascular complication of *diabetes mellitus*, affecting capillary health and homeostasis. DR can be classified into two stages by the degree and severity of retinal neovascularization, non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) [106]. Non-proliferative retinopathy encompasses the beginning, and relatively mild, stages of retinopathy, but can also be divided into mild, moderate and severe categories depending on retina condition. The causes of visual impairment include (i) retinal neovascularization that can obscure the fovea and (ii) macular edema and thickening [107-108]. The symptoms are frequently associated with the loss of vascular pericytes and/or their dysfunction, the formation of microaneurysms, increased retina vessel permeability, and microvascular closure/blockage [109-112]. These abnormalities result in large part because of localized hypoxia, which leads to abnormal and unregulated angiogenesis, one of the main symptoms of PDR [113]. PDR may also be accompanied by vitreous blood vessel contraction and hemorrhage [114-115]. In this scenario, the newly-formed vessels are leaky, leading to increased oxygen consumption in some regions, which intensifies hypoxic conditions elsewhere. The cyclic nature of these changes causes the situation to worsen as time goes on. Both Type I and Type II diabetics may develop DR during their progression with diabetes [116].

The beginning stages of DR include numerous structural changes to retinal capillaries including, but not limited to, thickening of the microvascular basement membrane, disruption of inter-endothelial junctions, and pericyte dysfunction [97-99]. Although discrete processes within

DR have been observed in animal models and in clinical samples from DR patients [4, 5], the precise mechanisms underlying how diabetic conditions disrupt microvascular cell interactions and communication are still not clear. Developing DR is closely related to a patient's diabetic progression. A large majority of patients are diagnosed with retinopathy after ~15 years from the initial onset of *diabetes mellitus* [99, 102].

The initial onset of DR due to the hyperglycemia can be difficult to determine as the development of DR symptomology can occur over a longer time course. The risk of DR can be reduced by early detection and timely control of blood glucose, blood pressure and lipid [117]. Retinal blood vessels can appear relatively normal and healthy during DR onset, but auto-regulation of blood flow may actually be lost fairly early in disease progression [117]. During these early stages, leukocytes are believed to stick on the vessel wall and begin infiltrating the retinal tissue [119-120]. The increasing thickness of the extracellular matrix (ECM) within the microvascular basement membrane may occur to compensate for this leukocyte infiltration [120]. These processes are likely to contribute to pericyte dysfunction or loss [121], and disruption of inter-endothelial tight junctions [122] and subsequent vessel permeability changes [123]. Increased capillary permeability can allow leakage of albumin and other large molecules into the retina interstitial space just outside the vessel wall, thereby raising the osmotic pressure in these regions [124]. Diabetic macular edema is thought to be a consequence of this albumin/plasma protein leakage. Elevated interstitial osmotic pressure can in turn reduce blood flow and cause adjacent tissues to become hypoxic [125]. Hypoxia-inducible Factor-1α (HIF-1α) becomes more active in these conditions, which leads to excessive Vascular Endothelial Growth Factor-A (VEGF-A) to be released, exacerbating vessel leakage and inducing angiogenic remodeling [126]. In this scenario, hyperglycemia can cause abnormal vessel formation, thereby worsening tissue hypoxia and increasing vessel permeability, which finally can ultimately cause vessels to occlude and "drop-out".

Current Diabetic Retinopathy Treatment Options

Treatment for advanced DR includes a number of options that can slow progression, but few if any can reverse the condition. Laser treatment (i.e. photo-ablation), eye injection of anti-angiogenic compounds, and even eye surgery are currently therapies provided for DR [128, 132]. For the laser treatment, the newly formed blood vessels and surrounding hypoxic tissues are burned to reduce growth factor release and help stabilize the changes in the eye capillaries in an effort to prevent vision from getting worse [128]. Photo-ablation is used to close off leaking vessels, which can in turn reduce swelling. This treatment however will not improve vision outcomes. Acute side effects from this approach include blurred vision and increased sensitivity to light in the few hours after treatment. For the long-term, side effects may include reduced night or peripheral vision, bleeding into the eye, objects floating into the vision, and a small, permanent blind spot, which can be located close to the center of vision [130].

Eye injections are used in some cases of abnormal angiogenesis and maculopathy. Specifically, the anti-VEGF-A agent ranibizumab is administered to prevent new blood vessel formation in retina and reduce vessel leakage [131]. These injections may slow and even pause the worsening of vision. Side effects however include increased risk for blood clot formation (which may cause a heart attack or stroke), increasing pressure inside the eye, retinal neuropathy, and total vision loss [134].

Eye surgery, and specifically vitrectomy surgery, can be used when laser treatment doesn't sufficiently halt disease progression [133]. For this treatment, some extra vitreous humour is removed. In this process, the lingering blood and scar tissue will be cleaned and the vitreous can then be refilled with a substitute fluid. Long-term side effects of surgical treatment include scar tissue, retinal detachment, cataract, hemorrhage, retinal detachment and glaucoma.

Potential Mechanisms underlying Diabetic Retinopathy Progression

DR is the result of hyperglycemia effects on the retinal microcirculation, triggering long-term microvascular remodeling during this pathology condition. On the cellular and molecular levels, disease progression results from disrupted pericyte-endothelial cell interactions and aberrant remodeling of the vascular ECM/BM, which can result from these defective interactions. DR progression is complex involving multiple pathways such as the polyol and Advanced Glycation End product (AGE) pathway, oxidative stress signaling and activation of protein kinase C (PKC) [134]. Increased levels of blood glucose initiates this condition, as intracellular glucose increases via activity of Glut1 (up-regulated by acute hyperglycemia) and Glut4 (an insulin-sensitive glucose transporter) [135]. This phenomenon elevates the uptake of glucose from the blood into endothelial cells and the subsequent transport of glucose to other cells nearby.

Increased glucose inside a cell enters the mitochondria, changing metabolism and activating the reactive oxygen species (ROS) pathway [136-137]. ROS activation enhances the activity of the second messenger diacylglycerol (DAG), in turn stimulating the AGE pathway. These signals activate PKC and Nuclear Factor-kB (NF-kB), causing the upregulation of VEGF-A and Insulin-like Growth Factor (IGF). The Transforming Growth Factor-β (TGFβ) pathway is also activated by NF-kB, which can up-regulate Glut1 as well as procollagen synthesis, contributing to an increased thickness of ECM. ROS can simultaneously activate the Mitogen-Activated Protein Kinase (MAPK) pathway, up-regulating the activity of matrix metalloproteinases (MMPs) that can degrade the ECM [138]. Degradation of the ECM can liberate soluble growth factors and other pro-angiogenic stimuli. Overall, the two arms of downstream ROS signaling can lead to significant changes in the composition of the vascular ECM/BM. These structural alterations induce vessel leakage and remodeling, as well as exacerbate the inflammatory response. Abnormal angiogenesis can consume considerable oxygen, while the changes in cellular metabolism and mitochondria activity also increase oxygen consumption. The sustained tissue hypoxia that ensues activates the HIF1-α signaling pathway, further increasing the synthesis of VEGF-A. In this way, the steady increase in

oxygen consumption and continuous growth of vessels incapable of resolving this hypoxia worsen the DR setting.

Vascular Pericytes

An Introduction and Basic Definitions of a Vascular Pericyte

Pericytes envelope the outer surface of microvascular endothelial cells, and are embedded within capillary basement membrane [52]. These specialized cells play key roles in microvessel homeostasis. Pericyte functionality in healthy and pathological conditions has recently gained significant attention, as numerous studies have found that pericyte dysfunction or loss is associated with or the direct cause of certain vascular-related diseases such as in DR and other neurodegenerative conditions.

Pericytes were first discovered and described as capillary adventitial cells in 1871 [1]. Two year later, researcher classified them as a contractile cell type, which wrap endothelium of microvascular and named as adventitial cells [6, 54]. Later, Zimmermann published a more detailed study of pericyte morphology and classification using light microscopy [2]. The origin of the pericytes was also described as undifferentiated mesenchymal cells [70]. At that time, pericytes were named as "Rouget cells". The name of “pericytes” now used more commonly was given to describe their location as being tightly wrapped around endothelial cells of the capillary wall. In his study, Rouget described the detailed morphologies of pericytes located across a diversity of microvessels. Pericytes were also classified as “mural cells” as well as “peri-capillary cells” in DR studies and in ultrastructure studies of cardiac tissue [55, 56]. Pericytes were then named as “peri-endothelial cells” in later publications of vascular membrane ultrastructure [57]. Pericytes have been hard to define due to heterogeneity in morphology, and shared characters and functions with other peri-vascular cells such as vascular smooth muscle cells (SMCs). Variations in pericyte and SMC morphology was classified via light microscopy in the lung capillaries [58, 59, 60]. Ultra-structural images of pericytes on retinal capillaries and in

cardiac tissue have also been presented to offer new insights into their cellular architecture [66-69]. Pericyte locations were identified by the ultra-structural analysis of terminal vascular beds, such as in the mouse heart, with pre-capillary sphincters and vascular membranes being described [61-65]. The most generally recognized definition of pericytes was proposed in 1986, where authors defined pericytes as the cells embedded with the basement membrane along with endothelial cells, drawing from evidence in electron microscopy images [3].

Nevertheless, until now, identification of pericytes has remained relatively challenging since (i) pericytes share a common lineage with other mesenchymal cells, such as vascular SMCs, fibroblasts, adipocytes, and chondrocytes [71-78], and (ii) a specific genetic marker for pericytes has yet to be identified. It remains a challenge to distinguish pericytes and other mesenchymal cells also due to their heterogeneity in morphologies across various different locations.

Diverse Functions of Pericytes

From early classification of pericytes as a vascular cell type, a number of unique functions in vascular development have been identified. Pericyte functions are wide ranging, encompassing (i) stabilization of newly formed blood vessels, (ii) maintenance of vessel integrity and barrier function (e.g. blood-brain barrier) [8, 9], (iii) contribution to the ECM and basement membrane composition [10,11], (iv) regulation of tissue regeneration [18,19], and (v) potentially modulating capillary blood flow via vasomotor capacity [12-15]. Pericyte functionality has often been interpreted from their interactions with endothelial cells, as well as from their specific location and coverage along vessels. Moreover, they may be performing unique functions depending on their specific location within a network and the conditions within their local microenvironment [85, 86].

As discussed previously, pericytes are well known to stabilize microvessels and maintain the architecture of the blood vessel wall. Pericytes play an important role in the regulation of vascular permeability, as well as in endothelial cell proliferation and vessel diameter regulation via direct contact and modulating the spatial gradient of growth factors. Pericytes extend and elongate cellular processes, contacting multiple endothelial cells during angiogenesis presumably through the existence of a growth factor gradient [32]. A number of signaling pathways have been implicated in regulating this process including VEGF-A/Flk-1/Flt-1, PDGF-B/PDGFR-β, Angiopoietin-1 (Angpt1)/Tie2, transforming growth factor-β (TGF-β), and Notch [36, 37, 40, 42]. These pathways coordinate unique aspects of pericyte recruitment, endothelial-pericyte communication and mural cell differentiation [38], which ultimately provide critical regulation of vessel diameter growth, vessel wall permeability, and endothelial cell fate during development [33-36]. These interactions stabilize the vessel wall architecture and facilitate normal vascular function during physiological conditions, but this pericyte-endothelial cell crosstalk fails during certain pathological conditions such as DR. Recent studies have implicated various signaling pathways, including VEGF-A, PDGF-B, Angpt-Tie, TGFβ, and Notch, in contributing not only to normal angiogenesis and vessel maintenance, but also to vascular dysfunction and decreased pericyte coverage on microvessels, as is reported in DR patient and animal models.

Pericytes play an important role in angiogenesis and may in fact also participate in vasculogenesis. From mesenchymal origins, newly differentiated endothelial cells assemble into primitive vascular structures, undergoing vessel elongation and subsequently sprouting. Pericytes may also be actively recruited during these processes, as live imaging data from an in vitro model of mouse embryonic stem cell differentiation suggests this may occur during vasculogenic vessel formation (Payne et al. Unpublished data from our lab). During the angiogenic phase, endothelial cells sprout, elongate nascent vessels, and form new connections to bridge two vessels. Previous work from our lab and others suggest that the

pericytes migrate along sprouting endothelial cells, presumably following a gradient of chemoattractants and/or growth factors. Numerous studies indicate that PDGF-B is one of the primary signals facilitating pericyte migration along endothelial sprouts.

Recent evidence has also suggested that ECM remodeling (i.e. assembly and degradation) by endothelial cells may also influence pericyte migration. Pericyte recruitment is a key step in the vessel formation process, as these specialized mural cells regulate vessel diameter and contribute to the unique ECM that composes the vascular basement membrane [10, 11]. Pericyte recruitment during angiogenesis occurs along vascular sprouts. Angiogenic cues induce production of specific ECM components from endothelial cells such as nidogen-1 (a growth factor “anchor” within the ECM) and laminin (a fibrous component also capable of tethering ligands to the ECM). During active vessel remodeling, cells often up-regulate their expression of corresponding integrins to match the assembly of the ECM/BM [39]. In the endothelial cell or pericyte mono-culture, corresponding changes in ECM and integrin gene expression and protein synthesis were not observed [39]. This suggests that specific interactions between pericytes and endothelial cells during angiogenesis enhance the deposition of ECM/BM and cell adhesion to the underlying substrates.

In addition to their contributions to remodeling vessel structure, pericytes have also been described as being contractile [12, 13] and potentially capable of modulating capillary blood flow [14, 15], although the exact mechanisms underlying their involvement in perfusion regulation remain an open question [15, 16]. Several studies have shown that pericyte contractility may be facilitated by tropomyosin and cyclic GMP [79, 80], and specific binding sites on these molecules have also been described in mediating pericyte contractile function [81]. Nevertheless, contradictory studies have presented observations from normal and ischemic brain suggesting pericytes are not contractile and capable of modulating blood flow [82, 83, 84]. In addition to their potential contractile function, pericytes have also been proposed to be involved in tissue regeneration by differentiating into a number of other cell types [18, 19].

These accepted and emerging pericyte functions have elicited much interest in better understanding pericyte involvement in vascular development, blood vessel homeostasis, and in a wide range of pathological conditions.

Pericytes in Pathological Conditions

Pericyte loss from the microvessel wall or their dysfunction in general can lead to the beginning stages and accelerated progression of numerous pathologies. As discussed above, proliferative diabetic retinopathy entails damage to retinal microvascular networks including pericyte "dropout." This progressive loss of pericytes within the vessel wall contributes to vascular instability and poorly regulated vessel growth [20]. Fragile vessels, as a result of inadequate pericyte investment, also exacerbate a condition in prematurely born infants known as intraventricular hemorrhage, or "brain bleeds" [21, 22]. Defects in pericyte function and coverage are also implicated in Alzheimer's disease and amyotrophic lateral sclerosis (ALS), as the chronic loss of vessel barrier function likely corrupts synaptic communication, leading to neuronal dysfunction and neuro-degeneration [23]. Lung and kidney fibrosis have also been described as entailing a component of pericyte dysfunction [24-26], and pericytes have been implicated in the limited blood flow restoration following spinal cord damage or stroke [14, 27, 28]. Impaired pericyte investment and coverage of tumor vessels plays a part in tumor progression as well as in the metastatic potential of a particular tumor type [29-31]. Pericytes coverage and per endothelial cell ratio is also reported to decrease dramatically with aging [87]. With pericytes implicated in such a broad range of clinically relevant conditions, it is imperative that we develop new tools and models to study this intriguing cell type in their interactions with endothelial cells and their contribution to vascular stability, quiescence, and function. Abnormal pericyte-endothelial cell interactions are therefore the potential cause of microvascular dysfunction and the origin of vascular-associated diseases such as diabetic retinopathy, fibrotic disease, and tumor angiogenesis. Because the interactions between pericytes and endothelial

cells play a fundamental role in the stability and development of vasculature, they may be potential targets in cell-based therapies for vessel-related diseases [154].

Vascular Components and Functions Related to Pericyte Biology

The Endothelium

Endothelial cells form the innermost layer of cells within blood vessels (and lymphatic vessels). These cells arise from hemangioblasts and subsequent angioblast precursors that originate from the mesoderm [139]. They form primitive vascular networks and remodel to ultimately maintain vessel permeability and contribute to regulating blood flow [140]. During embryonic development and wound healing, endothelial cells can become activated by mechanical and chemical stimulation that promotes their proliferation and remodeling of their associated ECM, as well as endothelial cell migration, differentiation and formation of the vessel lumen [141-144]. The endothelial cells that line the inner surface of blood vessels separate blood components from surrounding tissues. This cell layer is a selectively permeable barrier, controlling bidirectional transfer between blood and adjacent tissues via the tightly-regulated integrity of endothelial cell junctions. Trans-endothelial permeability and solute movement is controlled in part by vesicular transcytosis, cell-cell junctions, focal adhesions, and cytoskeleton [145-147].

Endothelial cells interact with a wide range of cells to accomplish the various functions of the vascular system. As discussed previously, normal pericyte functions require direct interaction with endothelial cells [88, 89]. This fact is exemplified by the observed morphologies of pericyte endothelial cell interactions, described as 'peg and socket' contact points, 'adhesion plaques', and pericyte-endothelial 'gap junctions' [90-93]. In addition, vessel diameter changes are regulated in part by dynamic modulation of vascular tone through interactions between endothelial cells and vascular smooth muscle cells. Signaling molecules that facilitate vasodilation and vasoconstriction include nitric oxide (NO), endothelin-1 (ET-1), and prostacyclin (PGI2). NO and PGI2 act as potent vasodilators, whereas ET-1 serves as a

vasoconstrictor [148-150]. Leukocyte adhesion to the vascular endothelial cell layer is a critical part of the inflammatory process. Recruitment of leukocytes requires tethering and rolling along the endothelium, followed by a strengthening and unique activation of the endothelial layer. These progressive changes in the endothelium facilitate leukocytes migration into the interstitium through adjacent endothelial cells [151-152]. Endothelial dysfunction can therefore contribute to systemic pathological states, which may be downstream of vascular-related diseases. For instance, endothelial dysfunction is directly involved in the formation of atherosclerotic plaques, as well as in disease-related oxidative stress, diabetic disease progression, hypertension, and pathological reduction in the levels of nitric oxide [153].

Vascular Signaling Pathways

A number of signaling pathways regulate the activities of endothelial cells and pericytes, independently and in their interactions with each other. Some of the most critical pathways are discussed here.

The Vascular Endothelial Growth Factor Pathway

The VEGF-A pathway is one of the most important signaling networks for vascular biology [53]. Flt-1, or VEGF Receptor-1 (VEGFR1), is a decoy receptor for VEGF-A, though it can also bind VEGF-B and Placental Growth Factor (PlGF). It exists as two receptor isoforms, a soluble version (sFlt-1) and a membrane-bound form (mFlt-1). sFlt-1 is a non-membrane-associated VEGF receptor, which can therefore distribute throughout the ECM and act as a negative regulator of VEGF-A signaling. It does so primarily by limiting the concentration of free VEGF-A and preventing its binding to Flk-1 (VEGFR2) [41]. Flt-1 is essential for regulating vessel formation, as genetic loss of *Flt-1* leads to embryonic lethality at E8.5-9. VEGF-A binding to Flk-1 (VEGFR2 or KDR) leads to activation of several signaling cascades. It activates the protein kinase C (PKC) pathway, which mediates activation of the MAPK1/ERK2, MAPK3/ERK1 and

MAP kinase signaling pathway for cell survival and proliferation. Downstream activation of Akt1 signaling pathway leads to the production of the signaling molecule nitric oxide (NO) by endothelial cells. Inducing the phosphorylation of SRC and NO in turn modulates vessel permeability [43-45]. A study by Eilken et al. recently showed that the expression of Flt-1 by pericytes may also spatially restrict VEGF signaling to regulate endothelial sprouting [46], though more work is needed to validate these intriguing findings.

The Platelet-Derived Growth Factor Pathway

The PDGF-B/PDGFRβ signaling axis is essential for vessel development and maturation. PDGF-B secreted by endothelial cell binds PDGFRβ on pericytes, up-regulating their expression of vitronectin, among other outcomes. Vitronectin deposited within the ECM leads to NFκB activation and can also up-regulate VEGF-A release. This increase in VEGF-A synthesis and production from endothelial cells can subsequently activate Flk-1 signaling [47], which indirectly modulates vessel diameter changes and permeability [43-45]. The PDGF-B gradient in peri-endothelial regions formed around endothelial cells during angiogenesis promotes pericyte recruitment [33].

The Angiopoietin/Tie and Transforming Growth Factor-β Pathways

Angiopoietin-1 (Angpt1) secreted from pericytes acts as a paracrine signal for endothelial cells. Under physiological conditions, this pathway is essential for vessel maturation and stabilization. Angpt1 from pericytes binds the Tie2 receptor on endothelial cells, initiating a signaling cascade to activate PI3K/AKT signaling, which in turn maintains vessel stabilization. However, in pathological conditions, such as sustained or chronic hypoxia, neighboring endothelial cells will express Angpt2, which is a competitive inhibitor of Angpt1 [48] and causes vessels to become less stable. TGFβ signaling also stabilizes blood vessels by directly stimulating the production of ECM components and inhibitors of matrix metalloproteinases (MMPs) that lead to ECM

degradation [49]. The TGFβ pathway can also regulate cell proliferation and differentiation by activation of ALK1-ALK5 and the downstream signaling network [49].

Extracellular Matrix and the Vascular Basement Membrane

The extracellular matrix (ECM) is the dynamic layer of structural proteins that surround cells, providing them mechanical and biochemical support [155], among other important functions. Molecules secreted by adjacent cells form the ECM. The ECM is a non-cellular tissue, which maintains tissue structure and regulates cell adhesion, intercellular communication, and segregation of tissues. The ECM has recently been implicated in playing a role in inducing cell differentiation [156]. The ECM is composed of various proteins and polysaccharides that are secreted locally and assembled into organized networks, which closely associate with cell/tissue surfaces [157].

The ECM can be roughly assigned to two main categories: basement membrane and interstitial matrix. The interstitial ECM is located between cells and contains polysaccharides and fibrous protein, which buffer mechanical stresses and help maintain tissue homeostasis. The basement membrane (BM) is a thin layer of ECM that segregates tissues, such as the vascular unit and surrounding cells including muscle cells, nerve cells, fibroblasts, etc. [158]. The BM can be considered a specialized ECM. It provides mechanical support, maintains cellular and structural integrity, and acts as a unique intercellular environment that can regulate the concentration gradients of certain molecular cues like growth factors. The vascular basement membrane (vBM) is a dense, sheet-like structure of 150-300 nm in thickness that can be visualized and identified by transmission and scanning electron microscopy. It appears similar to interstitial ECM, but it differs in molecular density and is always described as being in close association with cells [50, 51].

The BM is frequently categorized into three distinct parts: the basal lamina, attaching proteins, and the laminar reticular [158]. The basal lamina is mainly composed of the specific

ECM component laminin and its various isoforms. The BM may be further categorized into glycoproteins and fibrous proteins. Glycoproteins may be further subdivided into adhesive glycoproteins and glycosaminoglycocans (GAGs). The main fibrous proteins of the ECM are those within the collagen family, fibronectins, elastins, and laminins, all of which form a meshwork in peri-cellular spaces [159]. The glycoproteins fill most of the space within this meshwork, forming a hydrogel, which can perform various functions including buffering, hydration, signaling regulation, and maintaining the homeostasis of interstitial spaces on the cellular level [160].

The Collagen Family

Various members of the collagen family are found within the vascular basement membrane (vBM). Type IV collagen as well as collagen VIII and X, are cross-linked as they form a network for interactions with anchoring fibrils and other glycoproteins in the interstitial space [161]. Collagen IV fibers self-assemble into a polygonal or “sheet-like” matrix and form a major portion of the vBM. These structures become associated with laminins and other fibrous matrix proteins within the BM. Fibrillar collagens are another important collagen sub-type, and this class includes collagen I, II, III, V, XI, XXIV and XXVII. These contribute to the elastic properties of the vBM. Collagen III is an important component of the ECM in the blood vessels, as well as in the heart and gastrointestinal tract, facilitating tissue plasticity. Collagen I, II, and III are important in certain physiological conditions such as in the formation of the tubulo-interstitium and intima. The transmembrane collagens, including collagen XIII, XVII, XXIII, and XXV, play roles in cell adhesion and differentiation, as well as tissue development and structural integrity. A subset of collagens does not form fibers; instead, they attach to fibril-forming collagens and order collagen fibrils within the BM [162-164].

Additional ECM Components

Elastin is the ECM component that primarily conveys elasticity to the vBM. It is produced as tropoelastin in the interstitial space, and it crosslinks with other elastin molecules to form sheets and fibers [165]. Elastin in the BM provides structural support as well as elasticity, which is important for structural flexibility in blood vessels, the heart, skin and the uterus. Elastin in the BM forms cross-linked structures from random coiled molecules. Similar to elastin, laminins are critical components of the vBM. Laminins are known as a hetero-trimeric family of scaffolding proteins. The abundance of laminin within the vBM has lead to defining this layer as the basal lamina. Laminins typically contain 3 chains denoted as “a”, “b”, and “r” [166]. They are secreted and incorporated into BM. The trimeric proteins form a cross-linked structure and can bind to cell membranes and other BM molecules. The three arms of the trimeric molecules contribute to the formation of sheet-like structure, with the longest arm capable of anchoring. Of similar importance as elastin and laminin, fibronectin is a dimer protein that act as fibrils in the vBM. Fibronectin binds collagens, heparin, and other proteins to facilitate interactions with cell surface integrins [166]. Fibronectin is therefore an adhesive protein that promotes and enhances cell attachment. In the vBM, heparin sulfate proteoglycans (HSPGs) are important for the capillary BM. In the capillary BM, HSPGs, such as HSPG2 or perlecan, facilitate retention of PDGF-B for localization to the vicinity for vessel development [167]. This PDGF-B-HSPG2 interaction presumably facilitates pericyte migration along the actively remodeling ECM of sprouting endothelial cells.

Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinases

Matrix metalloproteinases (MMPs), also known as matrixins, are calcium-dependent zinc-containing endopeptidases. The functions of this class of enzymes include degrading ECM proteins and activating molecules by specific cleavage of cell surface receptors. MMPs are produced and synthesized as inactive pro-peptides. The pro-peptide domain needs to be cleaved to activate the enzyme. Additionally, tissue inhibitor of metalloproteinases inhibit MMPs

via the bidentate chelation of the zinc atom within MMPs [168]. In this way, MMP and TIMP activity achieve a necessary balance to maintain the ECM while allowing for active remodeling when necessary.

ECM Function, Regulation, and Contribution to Disease

One of the most important functions of the ECM is to maintain cell and tissue stability and integrity. The BM also segregates independent environments and tissue compartments for tissues and cells to maintain their own unique homeostasis. The BM plays a critical role as a barrier, which prevents unwanted cells from invading too deep into a specific tissue [169]. The BM has been shown to provide physical stability to tissues to modulate the input from mechanical forces, and this modulation is thought to occur downstream of TGFβ [171]. Physical forces can also be transduced from cell to cell through the ECM to affect cellular function. The ECM can buffer mechanical forces experienced by organs and tissues. Acting as a hydrogel, the BM can give provide a certain level of rigidity to respond to compressive and tensile forces. Soluble proteins such as soluble Flt-1 (sFlt-1) may also become anchored in the vBM and "filter" out excess VEGF-A, helping regulate angiogenesis [41]. Although not limiting its availability, a similar function may exist for HSPG2 in localizing PDGF-B to spatially organize its concentration and availability in specific regions [170]. Recent studies have also indicated that different components of BM may also drive cell fate and differentiation in specific directions.

The ECM also has important roles in the pathogenesis of numerous human diseases. Misregulated ECM breakdown can cause overt tissue destruction. Conversely, the excessive accumulation of collagens and other ECM components is an important feature of tissue fibrosis. ECM production is essential for wound healing, and similar processes facilitate scar formation, but in pathological conditions, fibrosis accumulation can interfere with or inhibit the normal architecture and function of the associated organ or tissue [172]. Fibrosis is often defined by ECM overproduction and abnormal stiffness of the affected tissues. These characteristics are

attributed to the excessive build-up of ECM components including collagens, fibronectin and elastin. Fibrosis is often found in scenarios involving chronic inflammation. During the tissue repair process, fibrotic connective tissues replace the normal parenchymal tissue, which results in the ECM components deposited replacing the normal tissue and forming permanent scar tissue [173]. This type of fibrosis can occur in different organs such as the lungs, liver, and kidneys as seen in pulmonary fibrosis, liver cirrhosis, and systemic sclerosis and nephritis, respectively. Tissue fibrosis may lead to organ failure and even death if not treated appropriately.

Recently, several studies have described the ECM as a dynamic niche in cancer progression, providing mechanical and biochemical cues within the tumor microenvironment. The unregulated and disorganized ECM within a tumor can affect cancer progression by promoting cellular transformation and metastasis. The ECM can also promote angiogenesis and exacerbate inflammation, leading to a more hypoxic and unstable microenvironment. Recent studies have further indicated that the increasing stiffness of the tumor ECM has profound effects on tumor growth and metastasis [174-176]. In addition to fueling metastatic spread, the ECM can play a key role in regulating tumor angiogenesis. Degradation of the ECM occurs in response to an angiogenic stimulus. This remodeling of the ECM releases bound growth factors such as VEGF-A. ECM fragments can also act as pro-inflammatory stimuli, which can in turn accelerate angiogenesis. Normal angiogenesis is well regulated by the ECM and facilitates wound healing and embryonic development under physiological conditions. Mis-regulated angiogenesis however is a hallmark of several diseases including tumor progression and diabetic retinopathy, as discussed above [177-178].

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Chapter 2

Establishment and Characterization of an Embryonic Pericyte Cell Line

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Abbreviations: NG2: Neural Glial Antigen-2. MEF: Mouse Embryonic Fibroblast. HUVEC: Human Umbilical Vein Endothelial Cell.

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ABSTRACT

Objective: Pericytes are specialized perivascular cells embedded within the basement membrane. These cells envelope the abluminal surface of endothelial cells and promote microvessel homeostasis. Recent discoveries of unique pericyte functions, particularly in neural tissues, underscore the need for overcoming existing challenges in establishing a functionally validated pericyte cell line. Here, we present methodologies for addressing these challenges as well as an embryonic pericyte cell line for use with *in vitro* and *ex vivo* experimental models.

Methods: We isolated an enriched population of Neural Glial Antigen-2 (NG2):DsRed+ pericytes from embryonic day 12.5 (E12.5) mice. This pericyte cell line was compared to mouse embryonic fibroblasts (MEFs) with respect to gene expression, cell morphology and migration, and engagement with endothelial cells during junction stabilization and angiogenesis.

Results: NG2+ pericytes displayed gene expression patterns, cell morphology, and 2D migration behaviors distinct from MEFs. In three different vessel formation models, pericytes from this line migrated to and incorporated into developing vessels. When co-cultured with human umbilical vein endothelial cells (HUVECs), these pericytes stimulated more robust VE-Cadherin junctions between HUVECs as compared to MEFs, as well as contributed to HUVEC organization into primitive vascular structures.

Conclusions: Our data support use of this pericyte cell line in a broad range of models to further understand pericyte functionality during normal and pathological conditions.

Keywords: pericytes, endothelial cells, mouse embryonic fibroblasts, vascular morphogenesis

INTRODUCTION

Pericytes were first described in the 1870s by Eberth and Rouget as cells morphologically distinct from vascular smooth muscle cells yet occupying the perivascular space adjacent to microvessels. The advent of electron microscopy allowed further characterization of these cells as being embedded within the basement membrane along endothelial cells of the microvasculature [50]. Subsequent studies have since identified a wide range of functions for these cells. It has been well established that pericytes are critical for stabilizing newly formed blood vessels, promoting their maturation while maintaining vessel integrity and barrier function [3,27]. Pericytes also contribute to the extracellular matrix (ECM) and basement membrane composition [2,45]. Pericyte contractility [15,17] and potential modulation of capillary blood flow has also been described [20,42], though the exact nature of their involvement in blood flow regulation remains an open question [23,24]. Additionally, pericytes have been implicated in tissue regeneration through differentiation into a broad range of distinct cell types [13,55]. These established and emerging pericyte functions have generated significant interest in further understanding their roles in blood vessel development, vascular homeostasis, and in various disease conditions.

Pericyte loss or dysfunction contributes to the onset and progression of numerous pathologies. For instance, diabetic retinopathy involves extensive damage of the retina microvasculature including pericyte “dropout”, which contributes to vascular instability and unchecked proliferative growth of new vessels [1]. Intraventricular hemorrhage, or “brain bleeds”, in prematurely born infants also arises in part from the inadequate investment of pericytes into the fragile and still-developing vasculature within the neonatal brain [9,62]. Alzheimer’s disease and amyotrophic lateral sclerosis (ALS) are also associated with defective pericyte function and coverage [29], as the loss of vascular barrier function contributes to the onset of neuronal dysfunction and neuro-degeneration. Pericytes have been implicated in the pathogenesis of lung and kidney fibrosis [4,32,46], as well as in limiting blood flow restoration

following stroke or spinal cord injury [18,20,31]. Tumor vessel dysmorphogenesis also arises in part from the lack of adequate pericyte coverage and investment, which likely contributes to the metastatic potential of a particular tumor type [6,36,63]. Given the involvement of pericytes in such a wide range of clinical pathologies, development of new models and tools to study this cell type during normal and disease conditions is critical.

The experimental approaches for investigating pericyte behavior and function are still being developed and refined, especially relative to the methods used to study their endothelial counterparts. A recent scientific statement from the American Heart Association indicated that major challenges in interpreting observations from *in vitro* angiogenesis assays stem from the lack of pericyte inclusion and the large variability in pericyte sources [49]. This variability is due in part to pericytes often being classified as “mural cells” alongside vascular smooth muscle cells and fibroblasts. Because these cells share some overlapping characteristics and perhaps lineages [25,57], it is critical to continue developing criteria to distinguish these distinct cell populations. For instance, He et al. recently identified enriched expression of vitronectin (*vtn*) and interferon-induced transmembrane protein 1 (*ifitm1*) as potential pericyte biomarkers [21] in addition to more established, though not exclusive, markers such as neural-glial antigen 2 (NG2, gene: *cspg4, chondroitin sulfate proteoglycan-4*) and platelet-derived growth factor receptor-□ (PDGFR□) [3]. While gene expression profiling represents one modality for refining the selection criteria for a pericyte cell line, additional methods will need to be developed to further validate pericyte cell lines as representative models of their behavior and activity.

In the current study, we combined gene transcription analysis with functional assays to validate a pericyte cell line for use with *in vitro* and *ex vivo* experimental platforms. Specifically, we isolated an enriched population of pericytes from mice at embryonic day 12.5 (E12.5). Pericytes expressed the DsRed fluorescent protein under the *ng2 (cspg4)* promoter (i.e. NG2:DsRed) [65]. The E12.5 time-point was chosen because neural oligodendrocyte progenitor cells (OPCs), a cell type that also eventually expresses NG2, are not NG2+ until after

E13.5 [56]. Pericyte gene expression, cell morphology, and 2D migration dynamics differed significantly from that of E14-15 mouse embryonic fibroblasts (MEFs). In addition, pericytes from this cell line migrated towards angiogenic vessels and incorporated into nascent vascular structures in three different models of blood vessel development. Vascular Endothelial (VE)-Cadherin junctions between endothelial cells were more prominent when co-cultured with pericytes as compared to MEFs. Pericytes also appeared to contribute to endothelial cell organization into primitive vascular structures. Taken together, our data suggest that, in addition to a distinct gene expression profile, this pericyte cell line exhibited functional characteristics consistent with their expected roles of participating in, and perhaps shaping, blood vessel formation and enhancing endothelial cell junctions.

MATERIALS AND METHODS

Embryo Collection and Pericyte Primary Cell Line Isolation

All animal experiments were conducted with review and approval from the Virginia Tech Institutional Animal Care and Use Committee (IACUC). All protocols are reviewed and approved by the IACUC Board and Virginia Tech Veterinary Staff. The Virginia Tech NIH/PHS Animal Welfare Assurance Number is A-32081-01 (Expires: 7/31/2021). Mice expressing the DsRed fluorescent protein under control of the *Ng2* promoter (i.e. *Ng2-DsRed* mice) [Tg(Cspg4-DsRed.T1)1Akik/J, JAX # 008241, The Jackson Laboratory, Bar Harbor, ME] were set up in timed matings with C57BL/6 females. On embryonic day 12.5 (E12.5), embryos were collected and placed in dissection media at 4ºC, and *Ng2-DsRed*+ embryos were visually identified. Embryonic tissues were enzymatically dissociated in Type I collagenase (2 mg/ml, Fisher) in phosphate buffer saline (PBS) with Ca^{2+} and Mg^{2+} at 37ºC for 1 hour. Following centrifugation (3 mins at 1200 rpm), supernatant was removed, and cells were re-suspended in 0.25% Trypsin-EDTA (Life Technologies, Carlsbad, CA) for 10 mins at 37ºC. Newborn calf serum was added to neutralize the Trypsin reaction. Following passage through a 70-micron pore filter and centrifugation (5 mins at 1200 rpm), cells were re-suspended in pericyte media (see Supporting Information) and plated for culture and expansion. Confluent cells were washed twice in PBS and exposed to Trypsin-EDTA for 5 mins at 37ºC, which was subsequently neutralized with serum. After centrifugation (3 mins, 2000 rpm), dissociated cells were filtered, re-suspended in a buffer suitable for Fluorescence-Activated Cell Sorting (FACS, Sony SH800, San Jose, CA), and placed on ice. FACS gates were set to (i) remove doublets, (ii) exclude *Ng2-DsRed* negative cells (based on control cell auto-fluorescence), and (iii) collect cells with the highest DsRed fluorescence intensity. Cells were imaged before and after FACS, and these images were quantified for the level of NG2+ cell enrichment (n=6 randomly chosen fields of view for each group). Collected cells were then cultured in a specific pericyte media, which was designed to promote the survival of pericytes and enrich their numbers at the expense of any

other cell types that could have been present. Enriched pericytes were utilized for subsequent experiments between passages 3-6 (p3-6). Cells were maintained under sterile conditions at all times where possible, and were not tested for the presence of mycoplasma.

Comparative Gene Expression, Cell Morphology, and Migration Behavior

Gene Expression

Mouse embryonic fibroblasts (MEFs) (Lonza, Walkersville, MD) were cultured according to manufacturer instructions. For comparative gene expression analysis, pericytes and MEFs were digested in lysis buffer (Zymo Research, Irvine, CA) at the same passage number. Messenger RNA was extracted and purified using Quick-RNA MiniPrep kit (Zymo) following manufacturer recommendations. Reverse transcription of RNA to cDNA was achieved using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) reagents and following manufacturer recommendations for PCR. Quantitative PCR was performed in triplicate (n=4 biological replicates) utilizing Taqman® Gene Expression Master Mix (Applied Biosystems) and Taqman® probes for Gene Expression including primers for TATA binding protein (*tbp*) for normalization, as well as Notch3 (*notch3*), interferon-induced transmembrane protein 1 (*ifitm1*), vitronectin (*vtn*), fibroblast specific protein-1 (*s100A4*), and fibroblast activation protein (*fap*). Samples were analyzed using standard 96 well plates on a QuantStudio 6 Flex (Applied Biosystems) with QuantStudio™ Real-Time PCR Software using comparative ΔΔT method to determine expression changes.

Cell Morphology

Morphological differences between pericytes and MEFs were evaluated by culturing each cell type individually on custom-made 6-well glass-bottom plates and by subsequently fixing with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA). Following three PBS rinses, blocking solution [3% bovine serum albumin (BSA) in PBS] was applied for 1 hour. Fixed cells were labeled for fluorescent confocal microscopy through incubation with

CytoPainter Phalloidin-iFluor 488 Reagent (Abcam, Cambridge, MA). Primary antibodies against mouse NG2 (rabbit anti-mouse NG2, EMD Millipore, Billerica, MA) were applied, followed by secondary labeling with donkey anti-rabbit AlexaFluor 568 (Invitrogen, Carlsbad, CA). Cell nuclei were labeled with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI). Immunostained cells were imaged on a Zeiss LSM 880 confocal microscope (Zeiss, Thornwood, NY) with 2-4 images acquired through the sample thickness, and these z-stack images were compressed into a single image using ImageJ/Fiji [43,47,48] or Zen software (Zeiss).

Migration Behavior

Comparison of pericyte and MEF migration dynamics was conducted by culturing each cell type individually on custom-made 6-well glass-bottom plates. Time-lapse imaging of cell migration was conducted on a Zeiss LSM 880 (Zeiss) mounted with an environmental chamber (regulated CO_2, humidity, and 37ºC temperature). Bright-field, differential interference contrast (DIC), and fluorescent (561 excitation) images were acquired every 10 mins for 24 hours. Cell migration speed was calculated by using ImageJ to measure the displacement of the cell nucleus/centroid at each time step, and these distances were divided by the time interval (n=30 cells for each cell type).

Pericyte-Endothelial Cell Co-culture Dynamics

Human umbilical vein endothelial cells (HUVECs, Lonza, Walkersville, MD) were cultured according to manufacturer instructions in Endothelial Cell Growth Media-2 (EGM2, Lonza, Walkersville, MD). Pericytes or MEFs were added to confluent HUVECs on glass-bottom plates (referred to hereafter as co-cultures) at a ratio of 1:6 (1 pericyte/MEF for every 6 HUVECs) and maintained in EGM-2 exchanged every other day. Brightfield and DIC images of co-cultures were acquired every day, and at days 0, 3, and 6 after co-culture initiation, the apparent surface area of HUVEC regions was measured and averaged for both co-culture groups (i.e. MEF:HUVEC and pericyte:HUVEC, with n=5 randomly chosen fields of view per time point).

Approximately 48 hours after adding the second cell type, live imaging of co-cultures was conducted as described above for pericyte/MEF monocultures (i.e. environmental chamber on confocal microscope, 10 min acquisition intervals for 24 hours). Co-cultures not used for live imaging experiments were fixed with 4% PFA, washed in PBS (x3), incubated in blocking solution, and labeled as described above (i.e. Phalloidin for actin cytoskeleton, anti-NG2 antibody, and DAPI). In addition, endothelial cell junctions were labeled with a primary antibody against mouse VE-Cadherin (goat anti-mouse VE-Cadherin, Santa Cruz Biotechnology, Dallas, TX), followed by secondary antibody labeling donkey anti-goat Alexa Fluor 647 (Jackson ImmunoResearch, West Grove, PA). Compressed z-stack images were acquired for each co-culture configuration as described above.

Pericyte Recruitment and Investment in Vessel Formation Assays

Tube Formation in 3D Collagen Matrix

Passage 4-6 HUVECs were cultured alone, with pericytes, or with MEFs (ratio 6:1, that is 6 HUVECs for every 1 pericyte or MEF) in $1x10^6$ cells per ml in Type I collagen (2 mg/mL, Advanced BioMatrix, Carlsbad, CA) in glass-bottom plates. Cells were cultured in EGM2 media with the addition of murine Vascular Endothelial Growth Factor- A (VEGF-A, 30 ng/mL, Peprotech, Rocky Hill, NJ). After 4-6 days, collagen-embedded cells were fixed, stained for VE-Cadherin, NG2, and DAPI, and imaged by confocal microscopy as described above.

Embryonic Stem Cell-Derived Blood Vessels

Wild-type embryonic stem cells (ESCs) [a kind gift from G.H. Fong (University of Connecticut Health Center) and V.L. Bautch (University of North Carolina at Chapel Hill)] were maintained and differentiated into primitive vascular structures as previously described [10,26,40]. "Hanging drops" of ESCs were created to initiate differentiation of embryoid body spheroids. Pericytes were added 7 days following the start of differentiation (i.e. removal from leukemia inhibitory factor, LIF). Day 10 cultures were fixed with 4% PFA and labeled for NG2 and DAPI.

In addition, platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) was detected with primary antibody labeling against mouse PECAM-1 (rat anti-PECAM-1/CD31, BD Pharmingen/BD Biosciences, San Diego, CA) and secondary antibody labeling with donkey anti-rat Alexa Fluor 488 (Jackson ImmunoResearch). Compressed z-stack images of primitive vessels with endogenous and exogenous pericytes were acquired as described above.

Embryonic Tissue Culture Assay

Culture of the remodeling vasculature within embryonic back skin was conducted as previously described [10]. Briefly, mice expressing enhanced GFP (eGFP) under control of the *Flk-1* (VEGF Receptor-2) promoter (i.e. *Flk-1-eGFP* mice) [*Kdr*$^{tm2.1Jrt}$/J, JAX #017006, The Jackson Laboratory] were set up in timed matings with C57BL/6 females.

Embryonic back skin was collected from E13.5 *Flk-1-eGFP*+ mice and embedded in fibrin within a single well of a custom-made, glass-bottom 6-well plate [38]. Pericytes (p4-6) were enzymatically dissociated into single cells and re-suspended in basic culture media: Dulbecco's Modified Eagle Medium-High Glucose (DMEM-H, Gibco/Thermo Fisher Scientific, Rockford, IL), 10% Fetal Bovine Serum (FBS, Gibco), and 1% Antibiotic-Antimycotic (Gibco). Following complete polymerization of the fibrin matrix, these cells and media were added on top of the embryonic skin cultures. After 1 hour, remodeling dermal blood vessels and exogenous pericytes were dynamically imaged by confocal microscopy (10× or 20× air objectives) at 20-25 min intervals for 18-24 hours with a Zeiss LSM 880 microscope with full incubation chamber. Z-stacks of 10-14 images were taken for each scan at 4-6 micron intervals, and compressed into a single image at each time point.

Statistics

Statistical analysis was conducted using GraphPad Prism 6 (La Jolla, CA). Where appropriate, statistics were conducted using two-way ANOVA and student t-tests. Relative quantification of qRT-PCR was analyzed by paired two-tailed t-test. P-values less than or equal to 0.05 were considered significant.

RESULTS

Embryonic Tissues Provide a Source for an Enriched Mouse Pericyte Cell Line.

Recent methodological advances have begun to address the wide-range of challenges associated with establishing vascular pericyte cell lines [19,39]. Here, we sought to build upon these approaches by incorporating a pericyte reporter, specifically DsRed expression (i.e. red fluorescence) driven by the promoter of an accepted vascular pericyte marker, NG2 [3,21,65]. Oligodendrocyte progenitor cells (OPCs) in the brain also express *Ng2/Cspg4*, but no earlier than embryonic day 13.5 (E13.5) [56]; therefore, we collected and dissociated *Ng2-DsRed*+ embryonic tissues at E12.5 and cultured these cells in pericyte-specific media (Figure 1A). Supplemental Figure 1 of tissue from an E14.5 *Flk-1-eGFP; Ng2-DsRed*+ littermate demonstrates the abundance and localization of *Ng2-DsRed*+ cells in embryonic tissues at a comparable time-

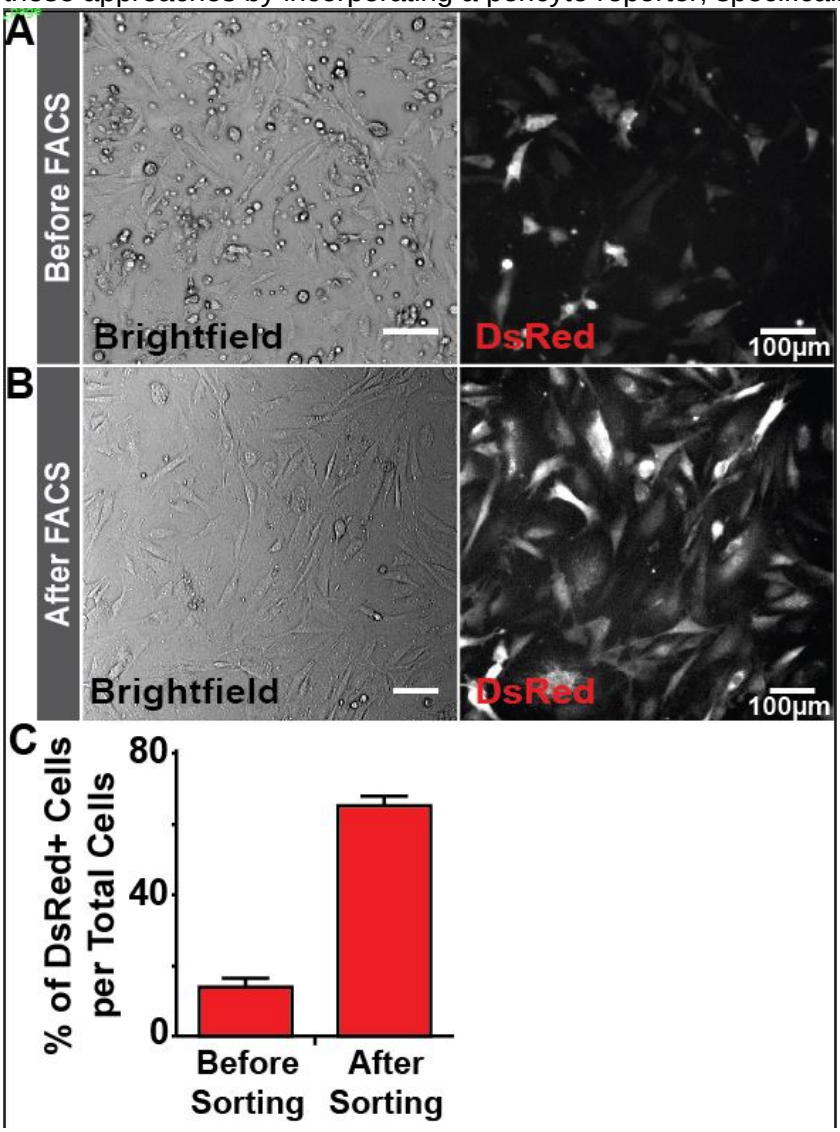


Figure 1. Embryo-derived *Ng2:DsRed*+ cell populations before and after enrichment by FACS. Brightfield (left) and fluorescence (right) images of cells acquired immediately following embryo dissociation (**A**) and after enrichment by FACS (**B**). Scale bars, 100 μm. Average percentages of *Ng2:DsRed*-positive cells per total cell number. n=6 randomly chosen fields of view for each group. Values are averages + Standard Error of the Mean (SEM) (**C**).

point. Specific media conditions have been previously shown to effectively select for distinct cell types including pericytes [39], but we sought to accelerate this process by using FACS to isolate and enrich for *Ng2-DsRed*+ vascular pericytes. Confocal images of sorted cells demonstrated a significant 4-fold enrichment in *Ng2-DsRed*+ pericytes (Figure 1B-C), such that nearly 70% or greater of the isolated cells were DsRed+, a yield comparable to that achieved with similar approaches for vascular cell isolation [30]. Thus, by combining pericyte-specific media with a genetic reporter and FACS, we were able to utilize mouse embryonic tissues as a viable source for deriving a vascular pericyte cell line for further validation.

Vascular Pericytes and Fibroblasts Exhibit Distinct *In Vitro* Cell Morphologies, Migration Dynamics, and Gene Expression Patterns.

Fibroblasts and pericytes both arise from mesenchymal origins, and although some overlap exists in their morphological features and biomarker expression, these cells perform very distinct functions in their respective tissue compartments. Fibroblasts reside in tissue/organ interstitial space and contribute to connective tissue formation. These cells rarely come in direct contact with the abluminal wall of blood vessels, nor do they become embedded within the vascular basement membrane [44]. Thus, fibroblasts served as a related but distinct cell type for evaluating characteristics of vascular pericytes. We began this comparative analysis by labeling and imaging the actin cytoskeleton (fluorescently-tagged phalloidin) in our pericyte cell line and in commercially available mouse embryonic fibroblasts (MEFs) harvested at a similar point in development, E14-15 (Figure 2A-H). Pericyte cell area appeared larger as compared to MEFs, while the signal from actin stress fiber labeling was weaker and less dense, suggesting fewer and thinner actin filaments (Figure 2A-D). Antibody labeling for NG2 displayed a relatively uniform distribution of the NG2 protein across the plasma membrane. Observations of MEFs revealed cells with stronger and more widespread actin cytoskeleton staining (Figure 2E-H),

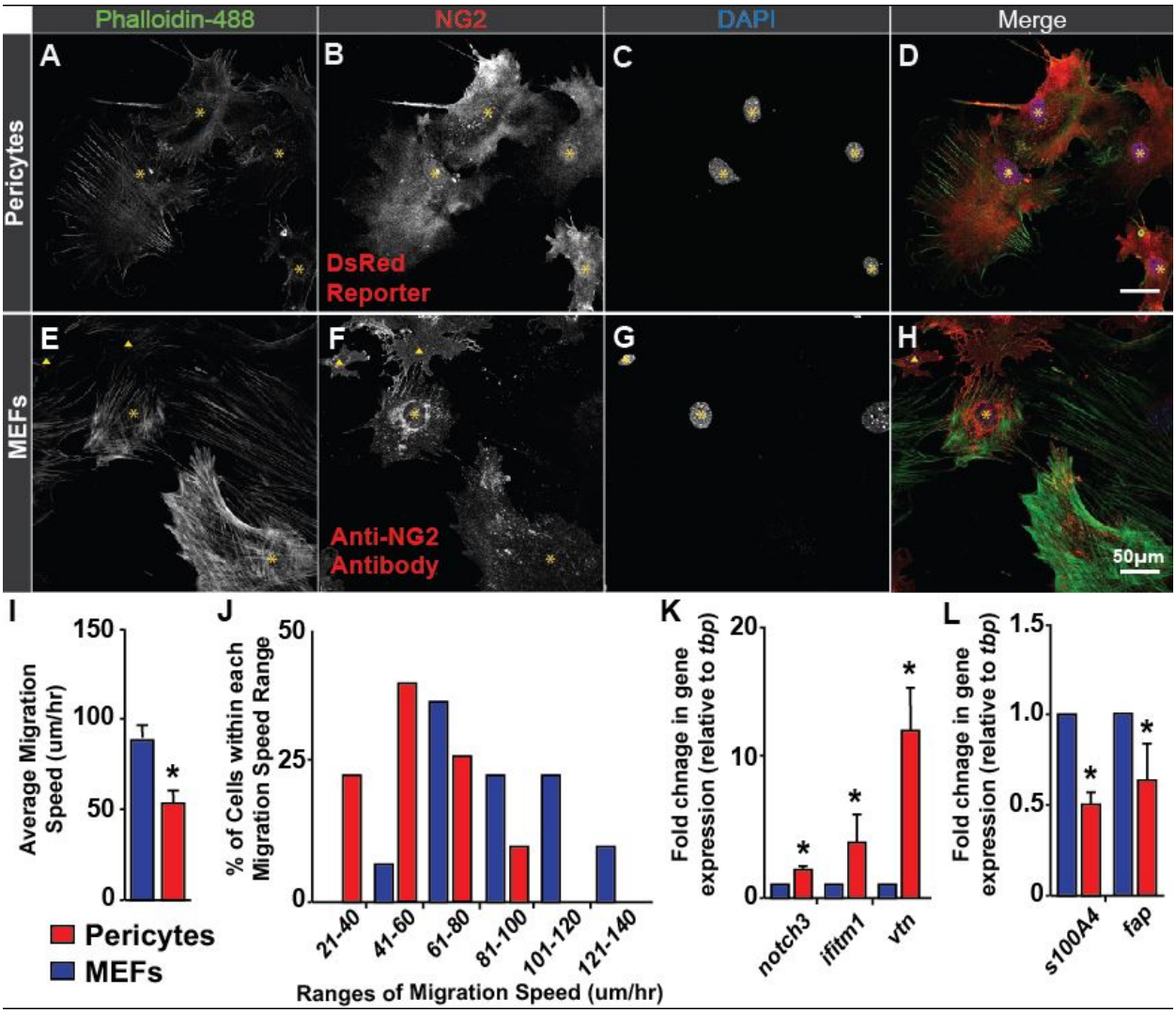


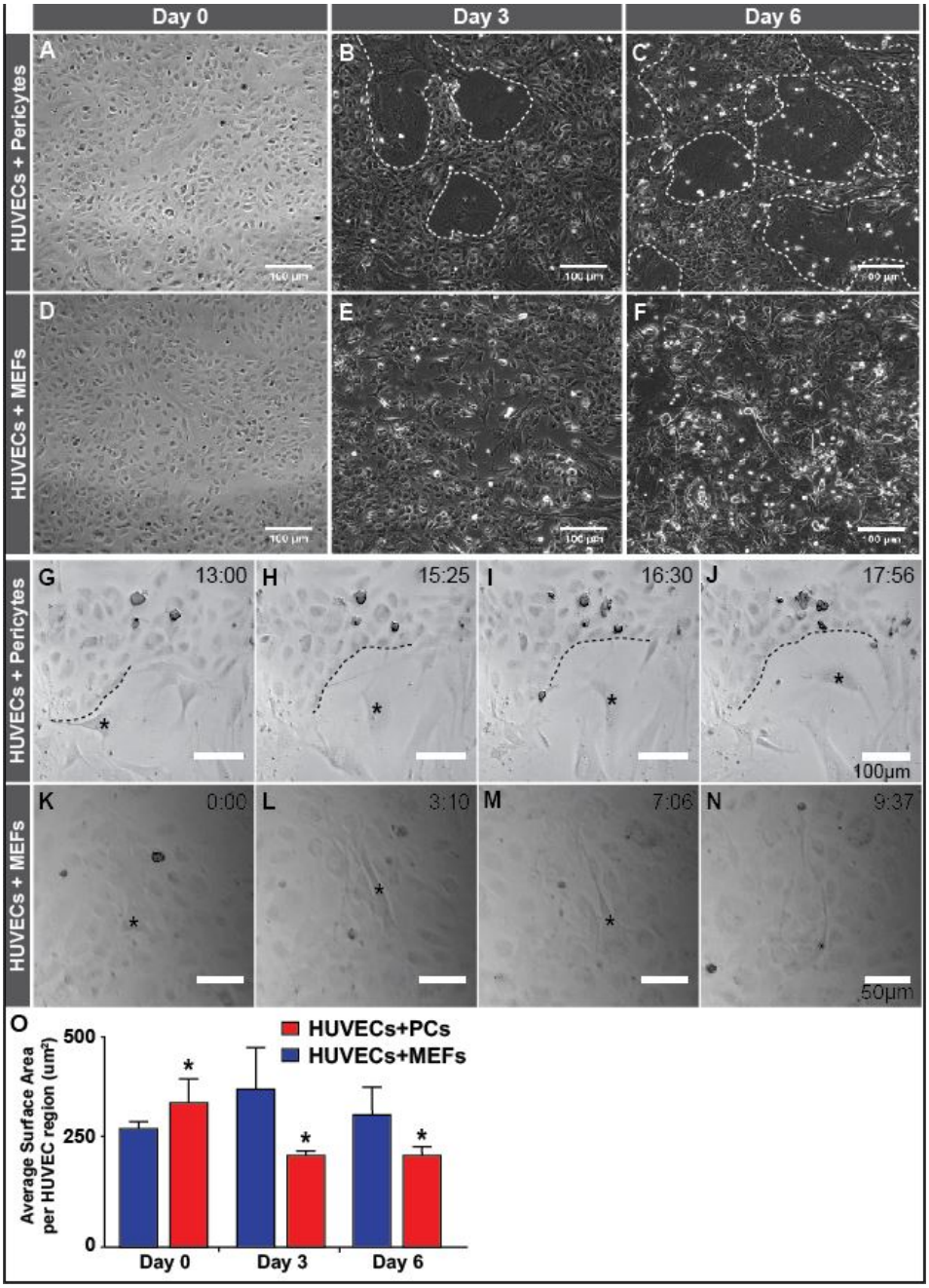
Figure 2. Mouse embryonic pericytes exhibit unique cell morphologies, migration dynamics, and gene expression compared to fibroblasts. Representative images of pericytes (**A-D**) and fibroblasts (**E-H**) labeled for actin cytoskeleton (**A & E**, Phalloidin-AlexaFluor488, green in **D & H**), NG2 expression (**B & F**, DsRed Reporter or NG2 Ab, red in **D & H**), and cell nuclei (**C & G**, DAPI, blue in **D & H**). Scale bars, 50 μm. Asterisks mark pericytes in **A-D**, while asterisks in **E-H** mark the predominant MEF population and arrowheads label a subtype of MEFs present in these cultures. (**I**) Average cell migration speeds (μm/hr) for pericytes (red bars) and MEFs (blue bars). Values are averages + SEM, n=30 cells. *P<0.05. **(J)** Distribution of pericyte and MEF migration speeds (μm/hr) across the indicated ranges. (**K**-**L**) Fold changes in gene expression between pericytes and MEFs. Values are averages + SEM, n=4 biological replicates. *P<0.05.

though some heterogeneity in cell morphology was found, consistent with manufacturer indications of high, though not complete, cell type purity. NG2 antibody labeling was detected primarily around the nucleus in these cells and not extensively along the cell membrane, with some heterogeneity in this distribution pattern.

These distinctions in actin cytoskeleton morphology between the two cell types suggested that the MEFs might be more migratory and thus requiring more robust actin dynamics, while the weaker, sparse actin signal in pericytes suggested a less migratory phenotype [37]. To test this hypothesis, we used live imaging to compare pericyte migration speed relative to that of MEFs. We found that the average pericyte migration speed was significantly lower than MEFs by about 30% (Figure 2I). In addition, approximately 10% of pericytes migrated at or above a speed of 80 microns/hour, while 50% of MEFs migrated at this speed or higher (Figure 2J).

Differences in pericyte and MEF morphology and migration dynamics suggested that these distinct cell types might also exhibit unique transcriptional profiles as well. We explored this idea by applying real-time quantitative reverse transcription PCR (qRT-PCR) to mRNA collected from each cell population. Recent transcriptome profiling studies have identified several novel candidates for exploring pericyte gene expression [21], specifically interferon-induced transmembrane protein 1 (*ifitm1)* and vitronectin (*vtn*) (Figure 2K). We measured expression of these genes as well as more established genes such as Notch3. We also evaluated genes expressed more abundantly in fibroblasts, in particular fibroblast specific protein-1 (*s100A4*), and fibroblast activation protein (*fap*) (Figure 2L). Pericyte expression of *notch3*, *ifitm1*, and *vtn* was significantly higher than MEFs, while the fibroblast-specific gene transcripts were significantly less abundant in the pericytes as compared to the MEFs. These three initial approaches in characterizing this pericyte cell line revealed important morphological and behavioral differences between these cells and fibroblasts, a closely related but distinct cell type that often confounds identification of pericytes in a range of biological contexts.

Embryonic Pericytes Promote Endothelial Cell Organization into Vessel-like Structures and Enhance VE-Cadherin Junctions.



Pericytes contribute to the blood vessel wall *in vivo* through secretion of extracellular matrix (ECM) components in the basement membrane, as well as by directly engaging the endothelium and promoting junctional stability [58]. As discussed above, fibroblasts do not become embedded in the basement membrane nor do they directly contact endothelial cells

Figure 3. Pericytes influence endothelial cell organization during long- and short-term co-culture, in contrast with MEFs, which allow greater endothelial cell spreading. Representative phase contrast images of HUVECs in co-culture with pericytes (**A-C**) and MEFs (**D-F**) at day 0 (**A & D**), 3 (**B & E**), and 6 (**C & F**). White dashed lines in **B** and **C** denote regions where pericytes have excluded HUVECs. Scale bars, 100 μm. Representative frames from short-term live imaging of HUVECs co-cultured with pericytes (**G-J**) and MEFs (**K-N**). Black dashed lines in **G-J** denote the broad leading edge of a pericyte (marked by an asterisk) contacting multiple endothelial cells, while the fibroblast marked by an asterisk in **K-N** inserts between endothelial cells. Scale bars, 100 μm in **G-J**, and 50 μm in **K-N**. Time in upper right corner, hours:minutes (hh:mm). See Supplemental Movies 1 & 2 for full time-course sequences. Average surface area of HUVEC regions at day 0, 3, and 6 of co-culture with pericytes (red bars) and MEFs (blue bars). Values are averages + SEM, n=5 randomly chosen fields of view per time point, *P<0.05.

under normal physiological conditions. We therefore characterized the unique differences between pericytes and fibroblasts in their interactions with endothelial cells *in vitro* [44]. Culturing our pericyte cell line with human umbilical vein endothelial cells (HUVECs) over several days resulted in the coordination of endothelial cells into more densely populated vessel-like structures (Figure 3A-C). In contrast, HUVECs cultured in the presence of MEFs remained more randomly distributed and spread out, with fibroblasts appearing to extend themselves in between endothelial cells but imparting no apparent organization (Figure 3D-F).

To better understand how pericytes contributed to the observed organization of endothelial cells, we used real-time imaging to visualize dynamic pericyte-endothelial interactions. In tracking pericytes over time, we found that many of these cells appeared to contact multiple endothelial cells along a broad leading edge (black dotted lines in Figure 3G-J, and see Supplemental Movie 1). This edge was maintained by endothelial cell migration and often expanded as time progressed, giving the appearance of pericytes “pushing” or “shepherding” HUVECs together to establish these denser vessel-like structures. Fibroblasts on the other hand migrated among HUVECs, extending cell processes in between endothelial cells but did not causing HUVECs to aggregate into anything resembling a primitive vascular structure (Figure 3K-N, see Supplemental Movie 2).

We assessed these changes in endothelial cell organization by quantifying the apparent surface area of individual endothelial cells. In doing so, we found that, immediately after adding pericytes to HUVEC cultures, average endothelial cell surface area was larger as compared to the area of endothelial cells cultured with MEFs (Figure 3O). However, after 3 and 6 days in culture with pericytes, HUVECs had significantly smaller individual surface areas as compared to endothelial cells in culture with MEFs. These data were consistent with the time-lapse imaging and longer time-course observations of pericytes inducing endothelial cells to coalesce into primitive vessel-like structures.

In observing endothelial cell aggregation into densely packed, vessel-like structures, we hypothesized that endothelial junctions, specifically those formed by Vascular-Endothelial (VE)-Cadherin, would be more robust in the presence of our pericyte cell line as compared to fibroblasts, perhaps via Neural (N)-Cadherin junction formation between pericytes and endothelial cells [3] or sphingosine-1-phosphate (S1P) signaling [35]. VE-Cadherin junctions between endothelial cells were in fact denser and tightly localized to cell-cell borders in the presence of pericytes, as visualized by confocal microscopy following immunolabeling (Figure 4A-E). Endothelial cells cultured with

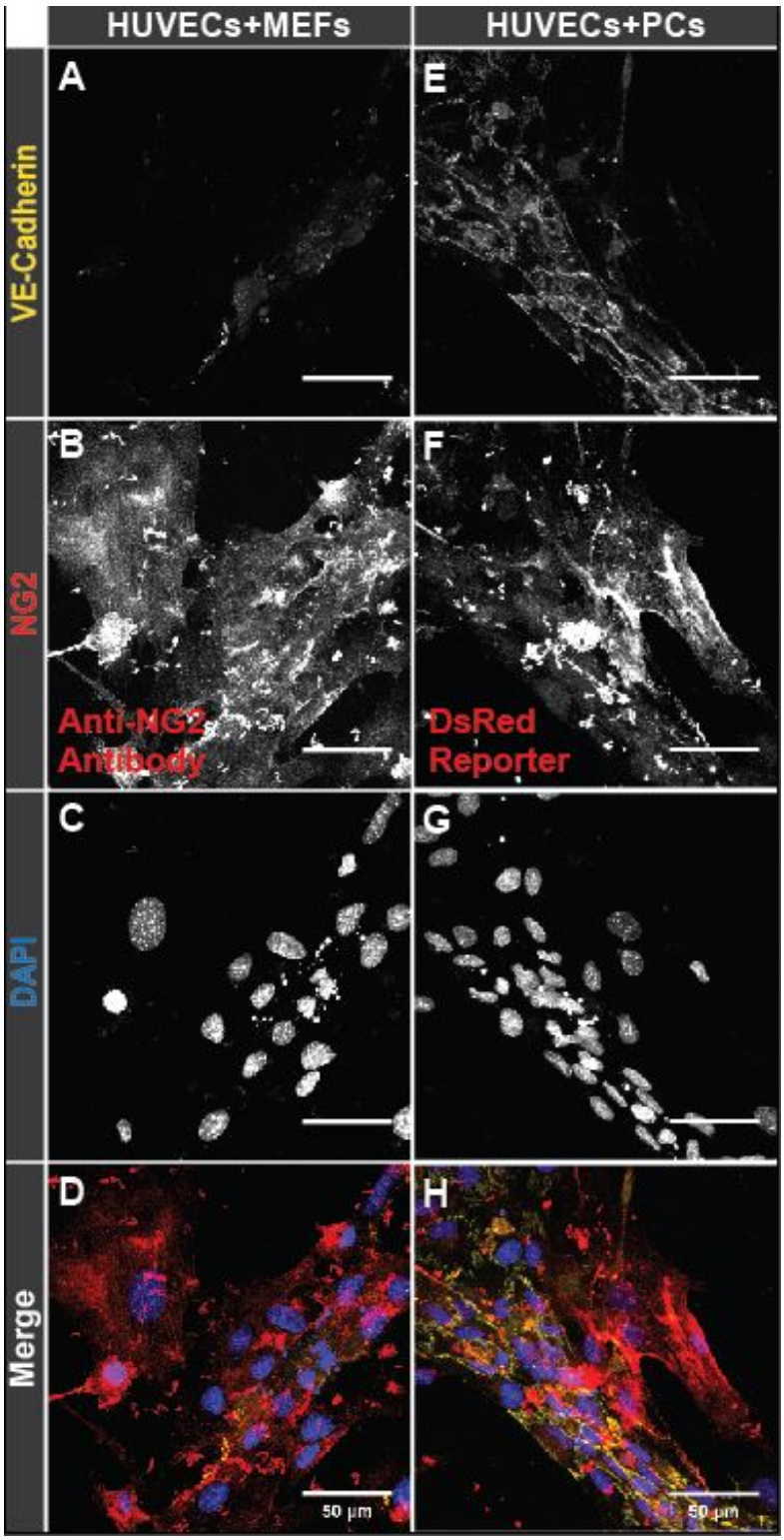


Figure 4. HUVEC junctions are more robust during co-culture with pericytes as compared to MEFs. Representative images of HUVECs co-cultured with MEFs (**A-D**) and pericytes (**E-H**). HUVEC cell-cell junctions labeled by antibodies against VE-Cadherin (**A** & **E**, yellow in **D** & **H**). Pericyte and MEF expression of NG2 detected by antibody staining (**B**, red in **D**) or by DsRed expression (**F**, red in **H**), respectively. Cell nuclei labeled by DAPI (**C** & **G**, blue in **D** & **H**). Scale bars, 50 µm.

fibroblasts however had less continuity in their VE-Cadherin junctions, with the signal being sparser and less associated with cell borders (Figure 4F-J). These observations are well aligned with the known function of pericytes in promoting endothelial cell barrier function.

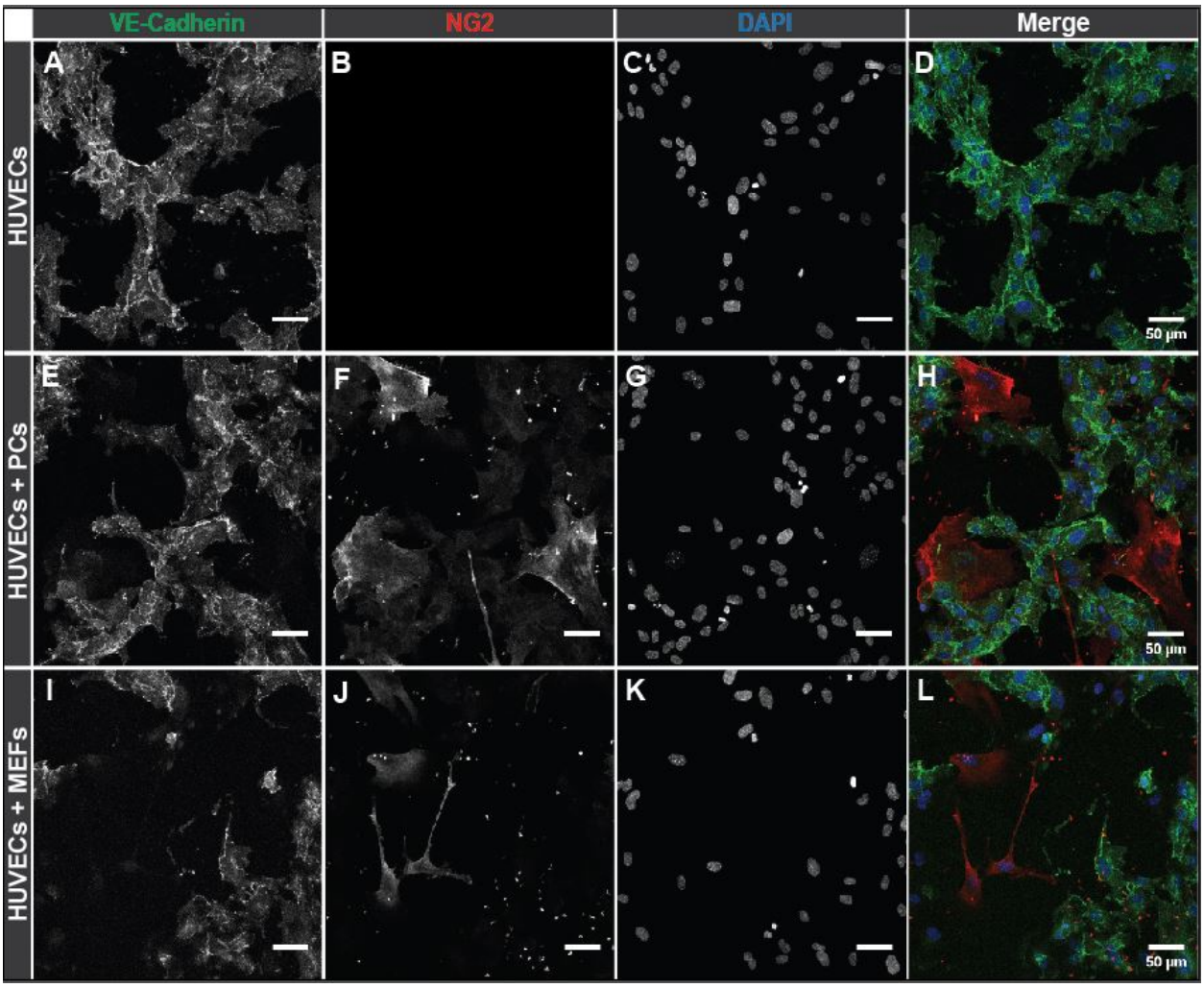


Figure 5. Pericytes engage endothelial cells forming primitive vascular structures within a type I collagen matrix *in vitro*. Representative images of HUVECs cultured in a type I collagen matrix alone (**A-D**), with pericytes (**E-H**), or with MEFs (**I-L**). VE-Cadherin junctions between HUVECs labeled by antibodies (**A, E, & I**, green in **D, H, & L**). Pericyte and MEF expression of NG2 detected by antibody staining (**B, F, & J**, red in **D, H, & L**). Cell nuclei labeled by DAPI (**C, G, & K**, blue in **D, H, & L**). Scale bars, 50 µm.

Isolated Pericytes Engage and Incorporate into Developing Blood Vessels.

Previous studies have shown that mural cell lines can incorporate into the developing vessels in various *in vitro* vasculogenesis and angiogenesis experimental platforms [16,53]. We sought to

test the ability of our pericyte cell line to incorporate into blood vessels forming within *in vitro* and *ex vivo* models. We embedded HUVECs in a Type I collagen matrix, and upon VEGF-A stimulation, these endothelial cells formed branched connections, resembling a primitive vascular network (Figure 5A-C). Pericytes added to these 3D cultures behaved similar to the 2D scenario in that individual pericytes engaged numerous endothelial cells along the perimeter of their cell membranes (Figure 5D-F). Fibroblasts cultured with endothelial cells in collagen matrix interacted with HUVECs but rarely contacted several cells along the same edge (Figure 5G-I). In addition, pericytes

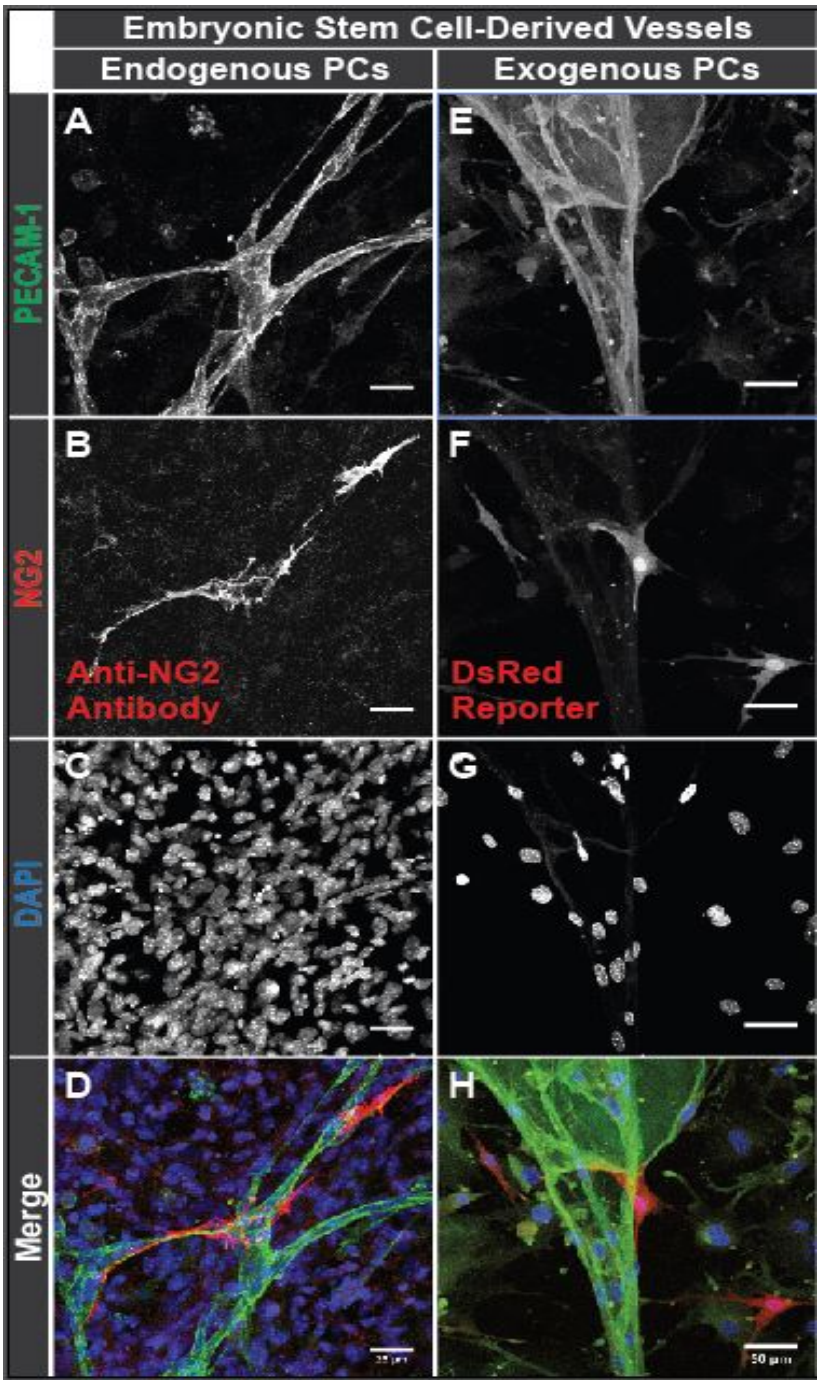
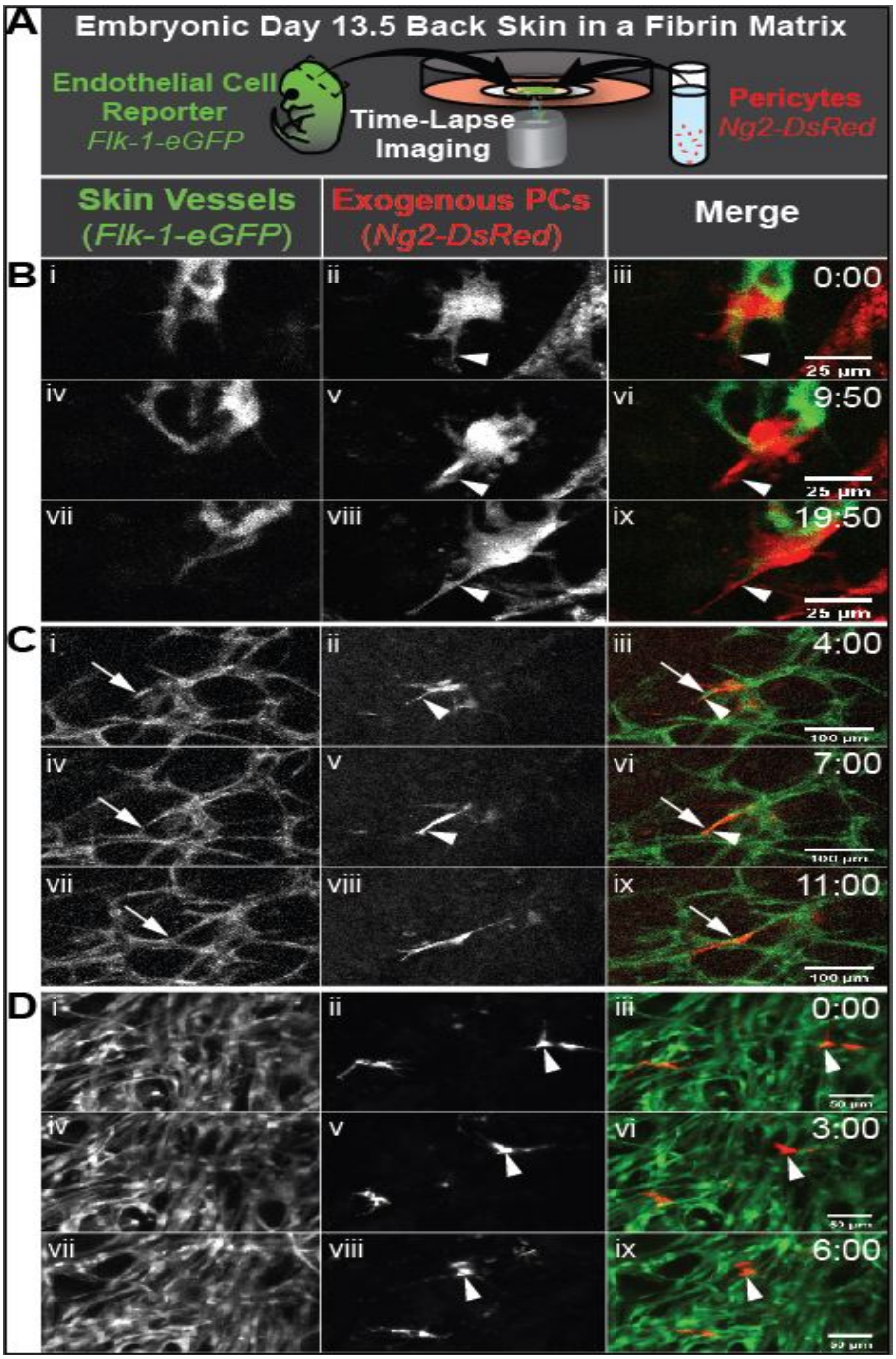


Figure 6. Exogenous pericytes interact with embryonic stem cell-derived vessels similar to endogenous pericytes. Representative images of mouse embryonic stem cell-derived vessels after 10 days of differentiation, and in association with endogenous pericytes (**A-D**) or exogenous pericytes added during vessel formation (**E-H**). Endothelial cells antibody labeled for PECAM-1 (**A & E**, green in **D & H**). Pericyte expression of NG2 detected by antibody staining (**B**, red in **D**) or by DsRed expression (**F**, red in **H**), respectively. Cell nuclei labeled by DAPI (**C & G**, blue in **D & H**). Scale bars, 25 μm in **A-D**, and 50 μm in **E-H**.



appeared to be more expansive and have larger projected areas, whereas MEFs were in general thinner and spindly.

Previous observations primarily characterized the differences between the mouse embryonic pericyte cell line and commercially available mouse embryonic fibroblasts. We next utilized a vascular development model in which we could compare the behavior of these isolated and expanded

Figure 7. Exogenous pericytes associate with distinct regions of developing blood vessels forming *ex vivo* in explanted embryonic skin. Schematic of experimental design in which live imaging captured *Ng2-DsRed*+ pericyte interactions with remodeling vessels within explanted skin of embryonic day 13.5 (E13.5) *Flk-1-eGFP*+ mice (**A**). Representative sequential images from movies of exogenous pericytes (*Ng2-DsRed*+) engaging with embryonic skin vessels (*Flk-1*-eGFP+): (i) as a tip cell sprouted from a parent vessel (**B,** arrowheads indicate pericyte extensions; see associated Supplemental Movie 3.), (ii) during endothelial sprout extension and connection (arrows) (**C,** arrowheads indicate pericyte extensions; see associated Supplemental Movie 4), and (iii) as vessels remodeled without overt sprouting (**D,** arrowheads indicate migrating pericytes; see associated Supplemental Movie 5). Scale bars, 25 μm in **B**, 100 μm in **C**, and 50 μm in **D**. Time in upper right corner, hours:minutes (hh:mm).

pericytes with that of endogenous pericytes within this vessel formation model. Specifically we released pluripotent mouse embryonic stem cells (ESCs) from an inhibitor of differentiation (i.e. exposure to LIF), and cultured these cells for 10 days, as described previously [10-12]. During this time, vasculogenic and angiogenic processes give rise to primitive blood vessels including the differentiation, recruitment and investment of vascular pericytes (Figure 6A-D). We took advantage of our pericyte cell line harboring the *Ng2-DsRed* fluorescent reporter, and added these cells to the developing vessels within differentiating ESC cultures. After 3 days, we found that exogenous pericytes had migrated to and begun investing into similar locations as endogenous pericytes, specifically localizing to branch points within the developing vessel-like networks (Figure 6E-H). The functional capacity of these embryonic pericytes was further supported by our observations of their active engagement with the ESC-derived endothelial cells in forming primitive vasculature.

Pericytes are known to migrate alongside endothelial sprouts during angiogenic remodeling [2,60]. We sought to test that functionality of our pericyte cell line by applying them to an *ex vivo* model of blood vessel formation that facilitated real-time imaging of their behaviors. Specifically, we isolated and cultured mouse embryonic skin at E13.5 from mice harboring the *Flk-1-eGFP* reporter gene in vascular endothelial cells [10]. We added *Ng2-DsRed*+ pericytes from our cell line to these cultures and observed their migration to and within remodeling vascular networks in real-time (Figure 7A). Exogenous pericytes were able to home to, and initiate contact with, emerging endothelial tip cells (Figure 7B, and see Supplemental Movie 3). In addition, added pericytes tracked along emerging tip cells as they extended from parent vessels and connected to form a new vessel branch (Figure 7C, and see Supplemental Movie 4). The most commonly observed pericyte behavior however was an engagement with the remodeling endothelium (i.e. observable and obvious association) in regions not necessarily associated with active vessel sprouting (Figure 7D, and see Supplemental Movie 5), suggesting pericyte recruitment might occur via mechanisms in addition to or in conjunction with endothelial

tip cell secretion of PDGF-BB [8]. Exogenous pericyte recruitment and migration within vascular networks forming *ex vivo* lends further support for this cell line retaining pericyte functionality during establishment and maintenance as a primary cell line.

DISCUSSION

Vascular pericytes are becoming increasingly implicated in a broad range of physiological and pathological processes, underscoring the need for advancing the tools and models for studying these cells [3,27]. To that end, we isolated a mouse embryonic pericyte cell line and applied a variety of approaches to validate the identity of these cells. Pericyte morphology, migration, and gene expression sharply contrasted with that of mouse embryonic fibroblasts, a cell type of similar mesenchymal lineage but distinct in physiological function and location. Pericyte interactions with endothelial cells were also markedly different from fibroblasts, as pericytes in 2D co-culture promoted formation of vessel-like structures and networks, and in 3D collagen matrices, they maintained contact with multiple endothelial cells simultaneously. When added to vessel formation assays, exogenous pericytes homed to endothelial tip cells as well as branch points within developing networks, and they maintained contact with sprouting endothelial cells as new anastomotic connections formed. These validation steps help address the challenges associated with pericyte source variability [49], which may arise from the current lack of a specific pericyte marker and also from the variety of tissues used for isolating pericytes. These methods may also strengthen interpretations from angiogenesis assays by including a validated pericyte cell line, and at the same time the approaches described herein are yielding new insights into pericyte biology and behaviors, such as pericyte contribution to endothelial cell organization into vessel-like structures.

Pericytes have been described to share a common lineage with a number of cell types including vascular smooth muscle cells and fibroblasts [3,32,33]. While all three of these cell types have frequently been grouped into the category of “vascular mural cells”, each cell type performs very distinct functions in their respective tissue compartments. Validated pericyte cell lines will therefore provide a means to dissect their unique gene expression profiles as well as their individual contributions to vessel structure and homeostasis. Volz et al. recently demonstrated that coronary artery smooth muscle cells arise from pericytes during embryonic

development [57]. This study, along with several others, suggests that this overlap in developmental origin may also lead to similar functionality in regulating vessel diameter and tone [20,23], though more work is needed to resolve these potential commonalities across different tissue beds. Regardless of their contractile potential, pericytes, unlike vascular smooth muscle cells, are found deep in the microcirculation, and have been well characterized in promoting vessel barrier function, particularly in the brain [64]. This function contrasts with fibroblasts, which are restricted from residing within the vessel basement membrane [44], and fibroblast-endothelial cell contact has been linked to increased vessel permeability for immune cell extravasation in the lung [59] and increased tissue fibrosis in lung and kidney pathologies [4,32,46,51]. Endothelial cells co-cultured with our pericyte cell line had enhanced VE-Cadherin junctions, while co-culture with fibroblasts led to disrupted endothelial cell junctions. Live imaging of these two co-culture scenarios was inline with these endothelial cell junction observations, as pericytes appeared to promote endothelial cell organization and association. In contrast, fibroblasts frequently inserted cellular extensions in between neighboring endothelial cells, consistent with the idea that fibroblasts support endothelial cells, and overall vascular network formation, in a paracrine manner [38,41,61] and less so through direct contact with the endothelium. Thus, an important criterion for evaluating pericyte cell lines will likely be their function in enhancing endothelial cell junctions and promoting endothelial cell aggregation, which are likely distinct functions relative to other “vascular mural cells”.

The *de novo* formation of primitive vascular networks during vasculogenesis involves endothelial cell differentiation and aggregation, and ultimately organization into basic vessel structures [14]. Pericyte involvement in this process has previously been assumed to be minimal aside from early stabilization of nascent blood vessels [5,34,52]. Our observations of pericyte-endothelial cell co-cultures suggest that pericytes may be participating in rudimentary organization of endothelial cells. Additionally, live imaging of these interactions revealed pericytes maintaining contact with multiple endothelial cells over time and seeming to “push”

them together. We have recently observed a similar phenomenon in real-time imaging of an embryonic stem cell (ESC) model in which fluorescently labeled pericytes migrate towards and initiate contact with eGFP+ endothelial cells as they begin organizing into primitive vessel-like structures (L.B. Payne, unpublished observations). Taken together, these data support the idea that pericytes, or perhaps their precursors, may play a more active role in the earliest stages of blood vessel formation, in addition to their roles in vessel stabilization and maturation at later stages.

Recent observations from our lab and others suggest that vascular pericytes are present at the leading edge of a sprouting vascular front, such as in the mouse postnatal retina, and likely engage tip cells as they emerge from existing vessels [28,60]. Sprouting endothelial tip cells are enriched for the expression of PDGF-BB [22], which is a critical recruitment factor for attracting pericytes to the developing vasculature [53]. By adding exogenous pericytes to the remodeling vessels *ex vivo*, we were able to observe the earliest events in pericyte-endothelial cell interactions as the two cell types initiate contact and begin migrating in a coordinated fashion. This coordinated migration was also observed in pericytes that tracked closely behind endothelial tip cells as they sprouted outward from existing vessels, and even as they form new anastomotic connections. Observing pericytes maintaining such close proximity to sprouting tip cells is therefore consistent with a primary function of pericytes in regulating the stability of nascent vessel branches. Incorporating this validated pericyte cell line into angiogenesis assays will facilitate identification of the mechanisms underlying this coordinated migration of sprouting endothelial cells and tip cell-associated pericytes.

Pericytes have been implicated in the onset and progression of several vascular-related diseases [7,20,64], with diabetic retinopathy being one of the clearest examples of pericyte contribution to clinical pathogenesis [1]. In diabetic retinopathy, the vascular basement membrane thickens [54] (likely in part from pericyte contribution to ECM deposition [45]), and pericytes are lost via vessel detachment and/or cell death. Interestingly, we found that

exogenous pericytes within the intact vessel networks of explanted embryonic skin were often limited in their direct engagement with the endothelium (Figure 7D), except for the tip cell interactions discussed above or at locations where the basement membrane was likely disrupted. These results, coupled with observations from the diabetic retina [54] as well as from our own experiments (H. Zhao, unpublished observations), suggest that the basement membrane, and its ECM composition in particular, limits the ability of pericytes to attach to endothelial cells. If the vascular basement membrane does indeed function as a potential "barrier" between the vessel wall and interstitial cells, this may represent an important therapeutic target for clinical strategies aimed at promoting vessel stability and maturation by restoring sufficient pericyte coverage.

While the cell line and validation approaches described in the current study address several of the existing challenges in establishing a functionally validated pericyte cell line, important limitations must also be considered. For instance, to minimize the complexity in presenting multiple cell line comparisons, we did not include commercially available pericytes in our analysis, though future studies will do so. These commercial cell lines are largely from adult specimen and from a variety of tissue types and organs [49], which would likely introduce numerous confounding factors that would require additional analysis beyond the scope of the current study. In addition, we did not exhaustively exclude the possibility that our cell line might represent, or contain small sub-populations of, other cell types including mesenchymal stem cells [13,55]. While our approaches demonstrated that the embryonic pericyte cell line exhibited behaviors consistent with a vascular pericyte identity, we cannot rule out the potential heterogeneity in cell identity within these NG2+ cells. Future work will be needed to determine if such heterogeneity in cell type exists within our cell line and, if so, the relative contributions of each subpopulation.

New roles and functions for vascular pericytes are continuing to emerge, and these cells are becoming increasingly appreciated for their importance in the onset and progression of a

wide range of pathological conditions including diabetic retinopathy, Alzheimer's disease, tumor formation and metastasis, among many others. It is therefore imperative that we develop new tools and models to better understand the basic biology of these cells as well as to elucidate how they might be therapeutically targeted during disease.

PERSPECTIVES

Vascular pericytes are an essential cell type for stabilizing growing vascular networks and maintaining blood vessel health. New tools and methodologies to investigate pericyte biology, such as the ones presented here, are therefore critical for advancing our understanding of pericyte-endothelial cell interactions during normal and pathological conditions. In addition to migration along angiogenic vessels and enhancing endothelial cell junctions, we observed unique contributions of embryonic pericytes to endothelial cell organization into primitive vascular structures, highlighting the need for further studies into the various roles that pericytes play in blood vessel formation and homeostasis.

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Disclosures.

None

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Establishment and Characterization of an Embryonic Pericyte Cell Line

SI MATERIALS AND METHODS

Cell Culture and Maintenance

Each cell line was maintained using a distinct media formulation, as detailed below:

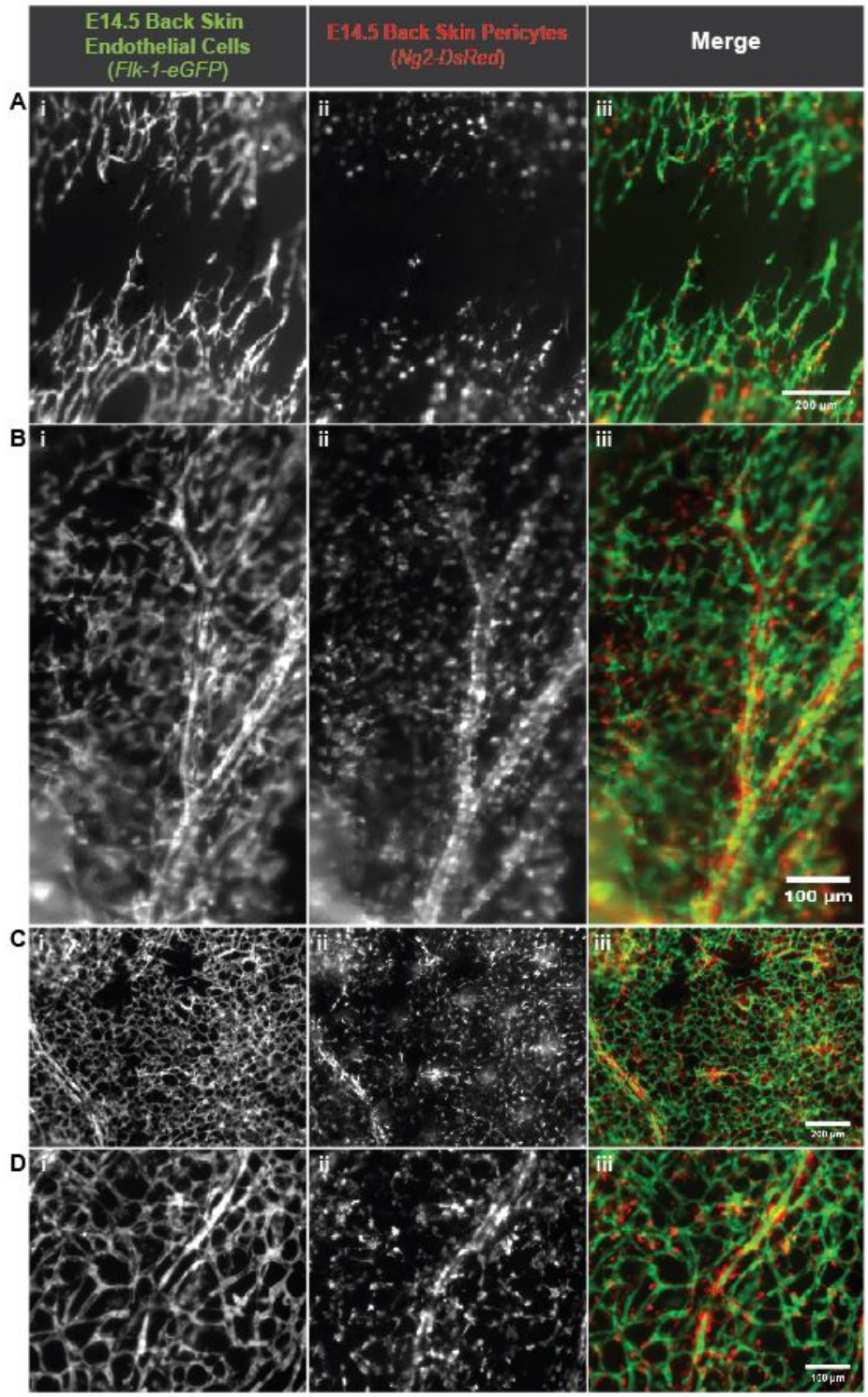
1. Human umbilical vein endothelial cells (HUVECs) – ECM Media (per 500 mL): EGM-2 kit components including antibiotics (Lonza) with 450 mL DMEM-L (low glucose at 1 g/L, Life Technologies) and 10% fetal bovine serum (FBS) (Life Technologies).
2. Pericyte (PC) cell line – PC media (per 500 mL): 450 mL DMEM-L (Life Technologies), 10% FBS (Life Technologies), 1% L-glutamine (Sigma), 1% Anti-biotic & Anti-mycotic (Life Technologies), 0.1 ng/mL Sodium Heparin (Sigma), 1 ng/mL basic Fibroblast Growth Factor (bFGF/FGF-2) (Life Technologies), 1 ng/mL Recombinant Murine VEGF165 (Peprotech).
3. Mouse Embryonic Fibroblast (MEFs) cell line – MEF media (per 500 mL): 450 mL DMEM-L (Life Technologies), 10% FBS (Life Technologies), 1% Anti-biotic & Anti-mycotic (Life Technologies).

Endothelial Cell Co-Cultures and Live Imaging

HUVECs were seeded at 2,500 cells/cm^2 on glass-bottom dishes (gel-coated) and cultured in ECM media for 6-10 days. Pericytes or MEFs were added at the ratio 1:6 (PC or MEF:HUVEC) when HUVECs reached confluence. Co-cultures were dynamically imaged as follows: confocal images (10× or 20× objectives) were acquired at 10-30 min intervals for 16-24 hours on a Zeiss LSM 880 confocal configured for live imaging (environmental chamber maintained humidity, 5% CO_2, and 37^{o}C). Image stacks of 6-8 images were acquired through the z-axis (thickness) for each scan with 4-6 microns between focal planes. After acquisition, z-stacks were compressed into a single image for each time point. Representative movie sequences shown are from non-consecutive images.

Live Imaging of Pericytes added to *ex vivo* Embryonic Skin Culture

Animal experiments were conducted with approval from the Virginia Tech Institutional Animal Care and Use Committee (IACUC). All protocols were reviewed and approved by the IACUC Board and Virginia Tech Veterinary Staff. The Virginia Tech NIH/PHS Animal Welfare Assurance Number is A-32081-01 (Expires: 7/31/2021). Culture and dynamic imaging of remodeling vasculature within embryonic back skin was conducted as previously described[1]. Briefly, mice with enhanced GFP (eGFP) expression under control of the *Flk-1* (VEGF Receptor-2) promoter (i.e. *Flk-1-eGFP* mice) [*Kdr*$^{tm2.1Jrt}$/J, JAX #017006, The Jackson Laboratory] were set up in timed matings with WT females. Back skin was collected from E13.5 *Flk-1-eGFP*+ mice and embedded in a fibrin gel within one well of a custom-made, glass-bottom 6-well plate[2]. Enzymatically dissociated pericytes (passage 4-6) were re-suspended in PC culture media (described above). Fibrin gel was composed of bovine fibrinogen (2 mg/mL, VWR), 10% Aprotinin from bovine lung (Sigma), and 1% Thrombin from bovine plasma (Sigma). Following complete polymerization of the fibrin matrix, pericytes and media were added on top of the embryonic skin cultures. After 1 hour, remodeling skin blood vessels and exogenous pericytes were dynamically imaged by confocal microscopy (10× or 20× air objectives) at 20-30 min intervals for 18-24 hours with a Zeiss LSM 880 microscope with full incubation chamber. Z-stacks of 10-14 images were taken for each scan at 4-6 micron intervals, and compressed into a single image at each time point.



Supplemental Figure 1. Tissue from an E14.5 *Flk-1-eGFP; Ng2-DsRed* mouse demonstrates the extent of vessel coverage by *Ng2-DsRed*+ pericytes. Representative images of *Flk-1-eGFP*+ endothelial cells and *NG2-dsRed*+ pericytes from E14.5 embryonic back skin (**A-D**). Scale bars, 200 µm (in **A** and **C**) and 100 µm (in **B** and **D**). *Flk-1-eGFP*+ endothelial cells (image **i** in **A-D**, and green in image **iii** of **A-D**) form extensive networks covered by vascular pericytes (image **ii** in **A-D**, and red in image **iii** of **A-D**).

MOVIE LEGENDS

Supplemental Movie 1. From *Figure 3G-J* of the main paper. Time sequence of HUVECs in co-culture with pericytes. Time in upper right corner, hh:mm (hours:minutes). Scale bar, 100 □m.

Supplemental Movie 2. From *Figure 3K-N* of the main paper. Time sequence of HUVECs in co-culture with MEFs. Time in upper right corner, hh:mm (hours:minutes). Scale bar, 50 □m.

Supplemental Movie 3. From *Figure 7B* of the main paper. Time sequence of exogenous pericytes (red, *Ng2-DsRed*+) engaging with a *Flk-1-eGFP*+ endothelial sprout (green) emerging form a parent vessel within cultured E13.5 embryonic skin. Time in upper right corner, hh:mm (hours:minutes). Scale bar, 25 □m.

Supplemental Movie 4. From *Figure 7C* of the main paper. Time sequence of exogenous pericytes (red, *Ng2-DsRed*) tracking along an *Flk-1-eGFP*+ endothelial cell sprout (green) as it extends from a parent vessel and connects to form a new vessel branch. Time in upper right corner, hh:mm (hours:minutes). Scale bar, 100 □m.

Supplemental Movie 5. From *Figure 7D* of the main paper. Time sequence of exogenous pericytes (red, *Ng2-DsRed*+) engaged the remodeling endothelium (green, *Flk-1-eGFP*+) in regions without appreciable vessel sprouting. Time in upper right corner, hh:mm (hours:minutes). Scale bar, 50 □m.

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Chapter 3

Extracellular Matrix Remodeling and Engagement by Pericytes and Endothelial Cells during Diabetic Conditions

ABSTRACT

Diabetic retinopathy (DR) is the convergence of multiple eye pathologies in diabetes mellitus patients. According to the National Eye Institute (NEI), proliferative DR is the leading cause of blindness among working-age adults. DR is an incurable, chronic disease. Current treatments such as laser ablation of the retina epithelial pigment layer or the injection of the ranibizumab (Lucentis®, anti-angiogenic agent) slow the progression of DR, but these treatments have key limitations and may actually cause deleterious side-effects such as neuron degeneration [1-3]. Prominent features of DR include retinal pericyte (PC) loss or dysfunction, thickening of the extracellular matrix (ECM) surrounding microvessels [also known as the vascular basement membrane (vBM)], and the disruption of endothelial cell (EC) tight junctions [4]. The exact mechanisms underlying DR progression as a chronic disease of the retina microcirculation are not well understood. Specifically, the contribution of PC-EC interactions with their surrounding ECM and vBM has slowed the development of curative therapies. This work furthers our understanding of potential mechanisms underlying DR as the result of the dynamic interactions between the ECM, PCs, and ECs. We also describe the utility of a PC-EC co-culture platform in the study of microvascular dysfunction associated with DR. Lastly, we present a working model for how retinal diabetic microvascular dysfunction contributes to DR, an irreversible disease that has devastating consequences for those who are affected.

INTRODUCTION

Diabetic retinopathy (DR) is a group of incurable eye conditions for which there are no uniformly effective therapies aside from laser ablation or anti-VEGF treatment, with both inducing substantial side effects [1-3]. This disease is characterized by gradual vision loss in diabetic patients due in part to dysfunction of retinal capillary pericytes (PCs), loss of endothelial cell (EC) tight junctions, and thickening of the vascular basement membrane (vBM) [4]. While the current paradigm for DR pathogenesis defines pericyte loss or dysfunction as a primary cause [5] for the observed increase in vascular permeability and for albumin leakage into the retinal interstitial space, recent studies have suggested that a thickened vBM by aberrant extracellular matrix (ECM) remodeling may also contribute to disease progression [6]. Emerging evidence implicates PC defects as predominant factors in the formation of abnormal retinal vasculature, contributing to vBM/ECM remodeling and to destabilizing EC tight junctions, as seen in the development of vascular instability in other disease states [7].

Pericytes are microvascular mural cells largely of mesenchymal origin and identified in part by their peri-endothelial location and being fully embedded within the vBM [8]. PC biomarkers that have recently emerged are neural/glial antigen-2 (NG2) (gene name: chondroitin sulfate proteoglycan 4, Cspg4) and platelet-derived growth factor receptor-β (PDGFRβ) [9]. PC dysfunction and loss of EC tight junctions in diabetic scenarios suggest a potential role of PC-EC interactions in the dynamic remodeling of vBM/ECM components during pathological conditions and in animal models aiming to recapitulate DR pathogenesis. Interestingly, PC abnormalities, EC tight junction disruption, and vBM/ECM defects can be observed when these cell types are co-cultured in simulated diabetic conditions for only a short period of time. While these acute observations provide a potential window into DR disease progression, the complete time-line for DR as a chronic condition remains poorly understood, hampering efforts to develop potential therapeutic strategies. Evidence linking PC-EC

interactions to aberrant vBM/ECM remodeling that contributes to DR progression has recently emerged, though more work is needed to clarify the exact involvement of these dynamics in this disease.

Remodeling of the retinal vBM/ECM during DR primarily involves excess collagen synthesis and deposition, mis-regulated ECM degradation by matrix metalloproteinases (MMPs), and a shift in cell-matrix binding profiles via integrin changes [10-12]. In the current study, we mainly focused on Type III collagen (col III – vBM component but also found in the interstitial ECM) and Type IV collagen (col IV – primarily found in the vBM). With respect to MMPs, we concentrated our analysis on: (i) MMP2, known to be a “collagenase” targeting col III and col IV, (ii) MMP9, which primarily degrades col IV, and (iii) MMP13, a collagenase which enzymatically targets col III. Regarding PC and EC integrin expression, we selected specific integrin subunits including integrin subunits α1 and α10, which a have higher binding affinity for col IV in the vBM, integrin subunits α2 and α11, which bind more strongly to col I and III in the interstitial ECM, and subunit β1, a corresponding subunit for each of the α subtypes.

Cell culture-based experimental models have emerged as useful tools in studying cell-ECM interactions in several pathologies [13]. Co-cultured cells contribute to ECM formation that mimics both healthy and pathological conditions, even recapitulating features of diseases like DR. However, the dynamic interplay between the ECM and PC-EC interactions remains relatively unexplored. While it is increasingly recognized that mechano-transductive signaling and biochemical communication can impact the function, morphology, and direct engagement of many cell types within a given tissue, currently used cell culture models are often unable to distinguish these various interactions and outcomes. Thus, the development of a functional model decoupling ECM remodeling and PC-EC interactions would be beneficial to the field of vascular biology. To that end, we applied our recently isolated PC cell line to a co-culture model with ECs, which was not only consistent with PC-EC interactions observed in vivo, but also

mimicked ECM remodeling dynamics. Utilizing this PC-EC co-culture model, we performed a series of experiments aimed at understanding (i) the crosstalk between ECM alterations and PC-EC interactions, and (ii) how these interactions contribute to microvascular defects observed during DR progression. To test our hypothesis that PCs and ECs remodel the vBM/ECM in DR to counteract microvessel disease progression (Figure 1), we used human umbilical vein endothelial cells (HUVECs) and a mouse primary PC cell line in our functional co-culture model. We demonstrated that PC-EC interactions had specific effects on the synthesis and degradation of the ECM (i.e. collagens and MMPs produced), and the cellular response to the altered ECM

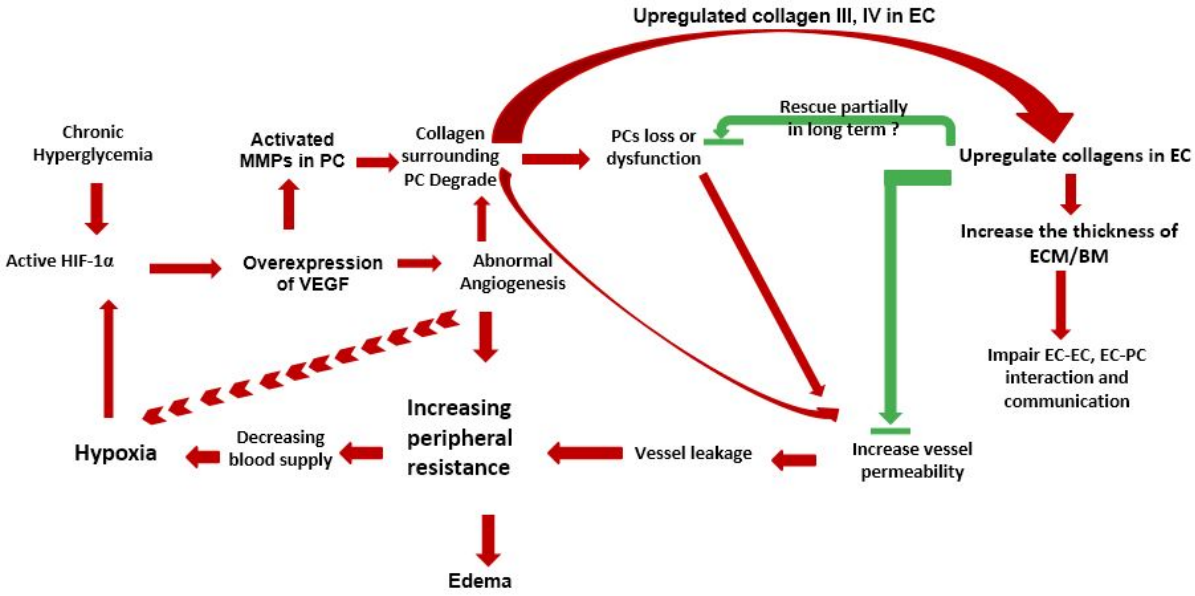

Figure 1. Hypothesis framework in DR progression as a chronic disease.

in shifting integrin expression profile. In addition, we used differentiated mouse embryonic stem cells (ESCs) into primitive vascular networks as a comparative developmental blood vessel model. In vivo studies included analysis of microvascular defects in the retinas of diabetic and obese leptin receptor-deficient mice (LepR KO mouse model), as well as human cadaveric retinas from diabetic and non-diabetic patients as a clinical model for further validation.

MATERIALS AND METHODS

Primary Pericyte Cell Line Isolation

The primary PC cell line was isolated as described in Zhao et al. Microcirculation 2018 [14]. Briefly, primary PCs, positive for NG2-DsRed expression, were collected from embryonic day 12.5 (E12.5) mice via whole-embryo dissociation, fluorescence-activated cell sorting (FACS), and culturing in a PC media composed of experimentally determined factors (see [14]). Collected cells were utilized for subsequent experiments from passages 3-6 (p3-6), and maintained under sterile conditions where possible.

Pericyte-Endothelial Cell Co-culture Dynamics

Our previously validated PC cell line was co-cultured with primary human endothelial cells (HUVECs, Lonza, Walkersville, MD), which were cultured according to manufacturer instructions in Endothelial Cell Growth Media-2 (EGM2, Lonza, Walkersville, MD). Specifically, PCs were added to confluent HUVECs cultured on glass-bottom plates (referred to hereafter as PC-EC co-cultures) at a ratio of 1:6 (1 PC to 6 HUVECs) and maintained in the corresponding EGM2, exchanged every other day. Approximately 48 hours after adding PCs, all cells were fixed with 4% PFA, washed in PBS (x3), and incubated in a blocking solution.

Culture Conditions and Media

Normal media: EGM2 (Lonza), DMEM-L (Gibco/Thermo Fisher Scientific, Rockford, IL), 10% Fetal Bovine Serum (FBS, Gibco), and 1% Antibiotic-Antimycotic (Gibco).

High glucose media (HG): EGM2 (Lonza), DMEM-L (Gibco/Thermo Fisher Scientific, Rockford, IL), 10% Fetal Bovine Serum (FBS, Gibco), and 1% Antibiotic-Antimycotic (Gibco), 30 mM D-glucose (Sigma).

High VEGF-A media (HV): EGM2 (Lonza), DMEM-L (Gibco/Thermo Fisher Scientific, Rockford, IL), 10% Fetal Bovine Serum (FBS, Gibco), and 1% Antibiotic-Antimycotic (Gibco), 10 ng/ml VEGF-A (Peprotech).

Both High glucose and VEGF-A media (BH): EGM2 (Lonza), DMEM-L (Gibco/Thermo Fisher Scientific, Rockford, IL), 10% Fetal Bovine Serum (FBS, Gibco), and 1% Antibiotic-Antimycotic (Gibco), 30 mM D-glucose (Sigma), 10 ng/ml VEGF-A (Peprotech).

Comparative Gene Expression

For comparative gene expression analysis, PCs and HUVECs in both monoculture and co-culture were digested in lysis buffer (Zymo Research, Irvine, CA) at the same passage number. Messenger RNA was isolated and purified using Quick-RNA MiniPrep kit (Zymo) following manufacturer instructions. Reverse transcription of RNA to cDNA was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) reagents and following manufacturer information for PCR. Quantitative PCR was performed (triplicate) utilizing Taqman® Gene Expression Master Mix (Applied Biosystems) and probes (mouse and human species) for Gene Expression. Probes included primers for TATA binding protein (tbp) for expression normalization, as well as collagen III (col3a1), collagen IV isoforms (col4a1 and col4a4), MMP2 (mmp2), MMP9 (mmp9), MMP13 (mmp13), integrin subunit α1 (itga1), α2 (itga2), α10 (itga10), α11 (itga11) and β1 (itgb1). Samples were analyzed in standard 96 well plates on a QuantStudio6-Flex (Applied Biosystems) with QuantStudio™ Real-Time PCR Software utilizing comparative ΔΔT method to evaluate expression changes.

Morphological Analysis of Co-culture Extracellular Matrix

Extracellular matrix (ECM) morphologies across single culture and co-culture scenarios and in different culture conditions were evaluated by culturing HUVECs and PCs in sequence on custom-made 6-well glass-bottom plates and by subsequently fixing with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA). Following three PBS rinses, blocking solution [3% bovine serum albumin (BSA) in PBS] was applied for 1 hour. Fixed cells were labeled for fluorescent confocal microscopy through incubation with primary and secondary antibodies. Primary antibodies used were against mouse Col III (mouse anti Collagen type III, Abcam, Cambridge, MA) and goat Col IV (rabbit anti Collagen type IV, BioRad, Hercules, CA), followed by secondary labeling with donkey anti-mouse AlexaFluor 488 (Invitrogen, Carlsbad, CA) and donkey anti-goat Alexa Fluor 647 (Jackson ImmunoResearch, West Grove, PA). Cell nuclei were labeled with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI). Immunostained cells were imaged on a Zeiss LSM 880 confocal microscope system (Zeiss, Thornwood, NY) with 2-4 images acquired through the sample thickness, and these z-stack images were compressed into a single image using ImageJ/Fiji [43] or Zen software (Zeiss).

Analysis of Pericyte Embedding within Extracellular Matrix

Comparison of the degree to which PCs became embedded within EC-derived collagen (i.e. PC embedded status) was conducted after HUVEC-PC co-culture individually on custom-made 6-well glass-bottom plates in different media conditions. Compressed z-stack images were acquired for each co-culture configuration as described above. PC embedded status was scored as: 1—Fully Embedded/Covered, 2—Partially Embedded (i.e. appearing able to “escape” from being fully embedded in collagen), and 3—Unembedded (i.e. PC seems to have “escaped” from being embedded in EC-derived collagen).

Differentiation of Mouse Embryonic Stem Cells into Primitive Blood Vessels and Cell Sorting

Wild-type mouse embryonic stem cells (ESCs) [a kind gift from G.H. Fong (University of Connecticut Health Center) and V.L. Bautch (University of North Carolina at Chapel Hill)] were maintained and differentiated into primitive vascular structures as previously described [16-18]. PCs and ECs were collected from mouse ESC-derived vessels via magnetic-bead sorting as described in Darden et al. *Angiogenesis* 2018 [15].

Analysis of Diabetic Mouse Retinal Vasculature

LepR$^{-/-}$ (i.e. LepR KO) and *LepR*$^{+/-}$ mice were analyzed at the ages of 4 months and 6 months. The mice were weighed and euthanized, and their blood glucose was then measured. Mice were then perfused with 0.5% PFA and 0.5% Evans blue to assess any vessel leakage. Eyes were collected and fixed in 2% PFA for 2 hours, then stored in PBS at 4^{o}C. Mouse eyes were dissected to isolate the retinal layer only and immuno-staining was accomplished via incubation in rabbit anti-collagen III (Abcam), rabbit or goat anti-collagen IV (BioRad), and PECAM-1 conjugated to Alexa Fluor 488 (Gibco/Thermo Fisher Scientific, Rockford, IL). Images were acquired by confocal microscope using 20x, 40x, and 63x objectives.

Whole-mount and Cryo-sectioned Human Retina Immunostaining, Imaging, and Analysis

Cadaveric human eyes from diabetic and non-diabetic patients were acquired from Old Dominion Eye Foundation (Roanoke, VA), fixed in 4% PFA at 4^{o}C for 3-4 days, and then stored in PBS at 4^{o}C. The retina layer was dissected from each eye, and small sections were isolated for further cryo-sectioning. Whole-mount and cryo-sections were immuno-stained for collagen III

and collagen IV as described above. Images were acquired by confocal microscope using 20x, 40x, and 63x objectives.

Statistics

Statistical analysis was performed using GraphPad Prism 6 (La Jolla, CA). Where necessary, statistics were conducted via two-way ANOVA and post hoc student t-tests. Analysis of relative gene expression levels by qRT-PCR was performed by paired two-tailed t-test. P-values that were less than or equal to 0.05 were considered to be significant.

RESULTS

Collagen expression and deposition increased in HUVEC-PC co-cultures compared to mono-cultures in the same media conditions.

Extracellular matrix (ECM) remodeling is a critical phase during the structural alteration of blood vessels in both physiological and pathological conditions, such as during wound healing, cancer progression, and diabetic microvascular dysfunction [20-22]. Because increased ECM deposition and a thickened vBM are hallmarks of DR, we hypothesized that the ECM/vBM remodels as a result of diabetic conditions. The diabetic setting is reported to stimulate ECM synthesis and thicken the vBM as well as activate and inhibit specific MMPs from PCs and ECs, all of which contribute to PC dysfunction or loss. To test this hypothesis, we established a PC-HUVEC co-culture model exposed to different culture conditions (i.e. mimicking diabetic conditions). We fixed these different cultures and immunostained for collagen III, a main component of both the interstitial ECM and within the vBM, and collagen IV, which is primarily found within the vBM, seeking to identify any shift in the ECM/vBM produced in each culture condition (Figure 2).

Although other retinal cell types were absent from these cultures, this model mimics key features of vascular cell interactions that likely occur in vivo. To observe cellular responses to certain elements of the diabetic retina microenvironment, we tested normal and elevated levels of D-glucose, specifically using 30 mM D-glucose as our “high-glucose” (HG) scenario. HG conditions were applied to mono-cultured PCs and HUVECs, as well as to PC-HUVEC co-cultures during dense vessel-like network formation yielded by PC-EC interactions. Retinal tissue in diabetic patients is known to be hypoxic and therefore contain high levels of growth factors, specifically VEGF-A, which drives aberrant vessel formation leading to blindness. Here, we replicated the same culture procedures as described above including groups exposed to media containing an additional 10 ng/mL of VEGF-A, representing our high VEGF (HV)

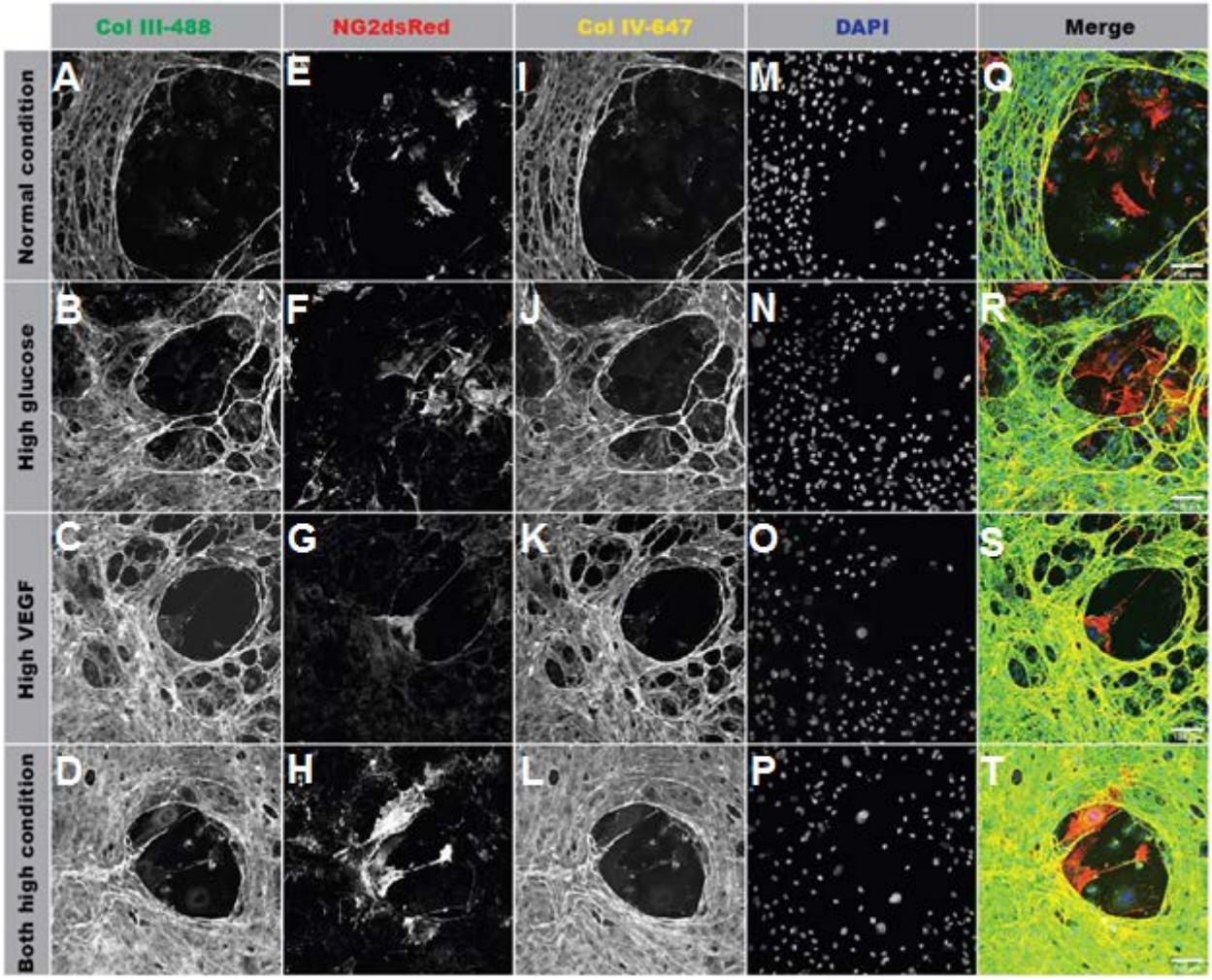

Figure 2. The extracellular matrix staining of co-culture model in four culture conditions. Representative images of co-culture (**A-T**) labeled for extracellular matrix, collagen III (**A-D**, collagen III-AlexaFluor488, green in **Q-T**), collagen IV (**I-L**, collagen IV-AlexaFluor647, yellow in **Q-T**) NG2 expression (**E-H**, DsRed Reporter or NG2 Ab, red in **Q-T**), and cell nuclei (**C & G**, DAPI, blue in **Q-T**). Scale bars, 50 μm.

condition. To test for the potentially additive effects of elevated glucose levels and excess VEGF-A, we also included media conditions with 30 mM D-glucose and an additional 10 ng/mL of VEGF-A as our “both high” (BH) condition. Interestingly, our ECM morphology analysis suggested that exposing PC-HUVEC co-cultures to the HG and HV conditions up-regulated both col III and col IV production (Figure 2). These results are consistent with the idea that multiple signaling inputs regulate ECM/vBM production from vascular cells. Moreover, in the BH condition, there appeared to be a substantial accumulation of col III and col IV compared to control, HG, and HV conditions (Figure 2). These results indicate that glucose and VEGF-A may

regulate ECM deposition independently and this regulation may be integrated, though not quite additive, when levels of both are abnormally high, as in the diabetic retina.

Exposure to Diabetes-Like Culture Conditions Shifts Pericyte and Endothelial Cell Gene Expression for Extracellular Matrix Deposition, Matrix Metalloproteinase Synthesis, and Integrin Production.

To compare ECM production, degradation, and engagement in mono- and co-cultures, we used real-time quantitative Reverse Transcription PCR (qRT-PCR or qPCR) to test gene expression levels for collagens, MMPs, and integrin subunits, respectively. Taking advantage of our PCs being derived from mice and ECs from a human tissue source, we were able to compare each cell type from both culture formats (i.e. single vs. co-culture) and across the different culture conditions. We selected TaqMan® probes that were verified to have no species cross-reactivity, and herein we denote mouse genes with an "m-" prefix and human genes preceded by "h-". We analyzed the qPCR results by focusing on: (i) specific collagen family members (*col3a1, col4a1, col4a4*), (ii) MMP sub-types (*mmp2* or "collagenase III and IV"; *mmp9* or "collagenase IV only"; and *mmp13* or "collagenase III only"), and (iii) integrin subunits (α1 and α10 – higher binding affinity for collagen IV; α2 and α11 – higher binding affinity for collagen I and III; β1 – indispensable subunit for the collagen binding of all four α subunits). Compared with PCs only in the same baseline culture conditions (i.e. no added glucose or VEGF-A), PCs from co-cultures had significant lower collagen III (col 3a1) expression and higher *mmp9* and *mmp13* (collagenase IV and III, respectively) expression. PC expression of α1 (*itga1*), α11 (*itga11*), and β1 (*itgb1*) integrin subunits was lower, while the α2 integrin subunit (*itga2*) was significantly higher (Figure 3). These results suggest that in the co-culture model under baseline conditions, PCs actively degrade collagen networks to facilitate ECM remodeling and perhaps maintain microenvironment plasticity until vessel formation is complete. The decreased gene expression

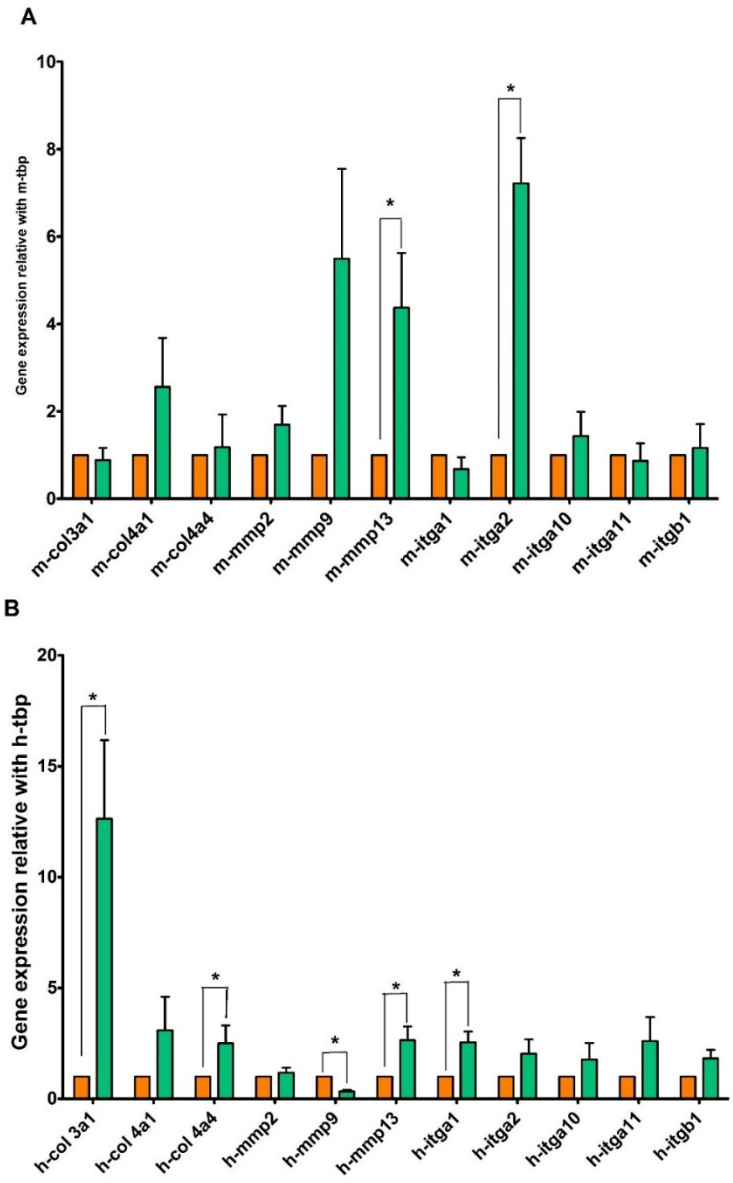

Figure 3. Mouse embryonic pericytes and HUVEC exhibit unique gene expression compared with co-culture in normal condition. (A) Fold changes in gene expression between single culture and co-culture pericytes; **(B)** Fold changes in gene expression between single culture and co-culture HUVECs; Values are averages + SEM, n=3 biological replicates. *P<0.05.

of most integrin subunits indicates a reduction in ECM binding of adjacent PCs. Interestingly, the gene expression profile of ECs was nearly the opposite of PCs. Relative to mono-culture, co-cultured ECs in baseline conditions showed a significantly higher collagen III and IV expression (*col3a1* and *col4a1*, respectively), lower expression of *mmp9* (collagenase IV), and higher transcription of almost all integrin subunits, including *itga1*, *itga2*, *itga10*, and *itgb1* (Figure 3). This result suggests ECs in the co-culture model are trying to synthesize more collagens and develop firmer adhesion to the ECM nearby. Overall, we found that PCs demonstrated a lower affinity for the ECM, which suggests that they may be less restricted and therefore capable of increased migration, as seen in their formation of multiple contact points with surrounding cells. It appears that the ECs prefer stronger adhesion with each other, and they remodel the ECM via expression of collagens.

To further investigate the response of interacting PCs and ECs in diabetes-like conditions, potentially via remodeling of ECM/vBM, we performed qPCR to test PC and EC gene expression of specific ECM components in the different culture conditions (i.e. normal, HG, HV and BH conditions). Analysis of PCs from the BH condition revealed a significant up-regulation in *col3a1*, while a down-regulation of *mmp9* (collagenase IV) and *mmp13* (collagenase III) (Figure 4). These data suggest the elevated gene expression of collagen III and IV from PCs in the high glucose and high VEGF-A condition may contribute to ECM accumulation and a thickened vBM. Integrin expression showed a similar trend; specifically, PCs up-regulate α2 subunit expression, which suggests an increased binding affinity with collagen I and III (i.e. interstitial ECM components) (Figure 4). These results are consistent with previously reports such as in idiopathic pulmonary fibrosis where PCs seem to "escape" from the vBM and migrate into the interstitial space.

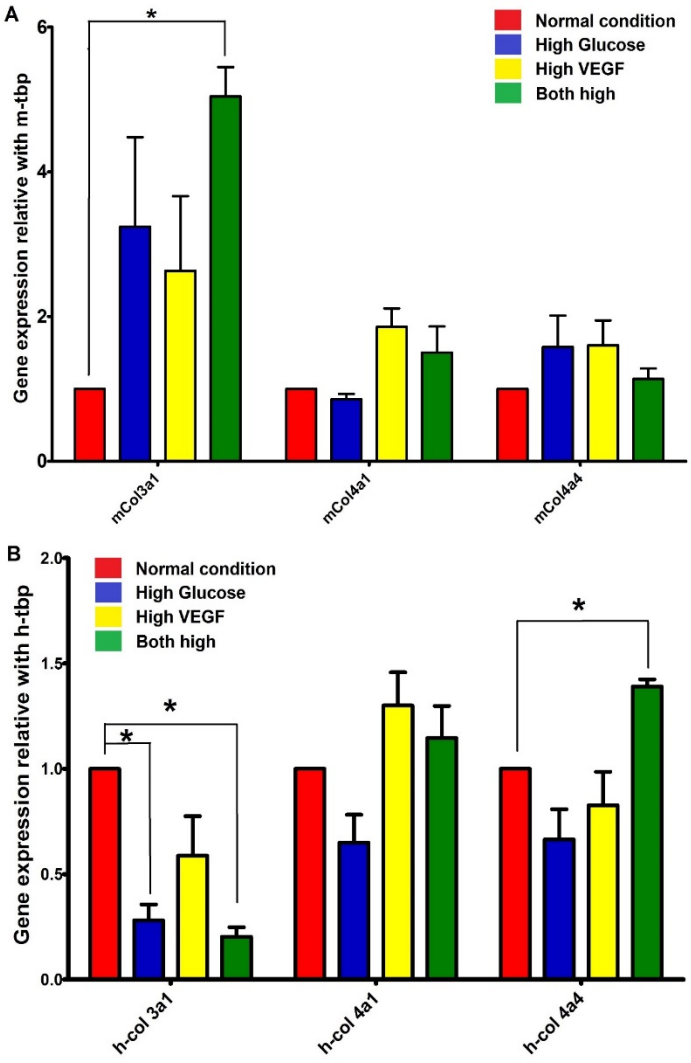

Figure 4. Mouse embryonic pericytes and HUVEC exhibit unique gene expression in co-cultue compared with diabetic condition. (A-B) Fold changes in collagen gene expression among co-culture pericytes and HUVECs in different conditions; Values are averages + SEM, n=6 biological replicates. *P<0.05.

In contrast to PCs, ECs up-regulated collagens (*col3a1* and *col4a4*), down-regulated *mmp13* (collagenase III), up-regulated integrin α10 subunits (*itga10*), and down-regulated integrin α2 (*itga2*) (Figure 4). EC increased expression of collagens found in both the IM and vBM, and enhanced their binding with collagen IV (predominantly in the vBM), while decreasing their adhesion to collagen III (i.e. an interstitial ECM component). These results suggest that the ECs shifted to a stronger binding with the vBM but not to PCs or to neighboring ECs. In the context of diabetic microvascular dysfunction, this transition may promote PC loss and increased vessel permeability (potentially related to EC tight junction formation as well).

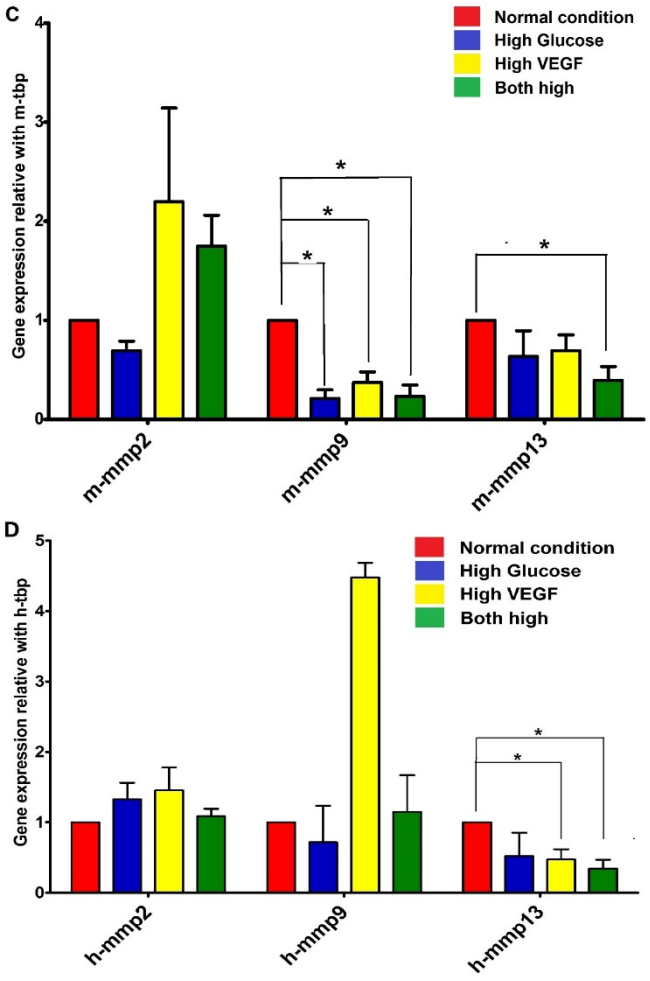

Figure 4. Mouse embryonic pericytes and HUVEC exhibit unique gene expression in co-cultue compared with diabetic condition. (CONT.) (C-D) Fold changes in MMPs gene expression among co-culture pericytes and HUVECs in different conditions; Values are averages + SEM, n=6 biological replicates. *P<0.05.

Taken together, these results indicate that co-cultured PCs and ECs exposed to diabetic-like conditions weaken the vBM surrounding PCs by down-regulating collagens and up-

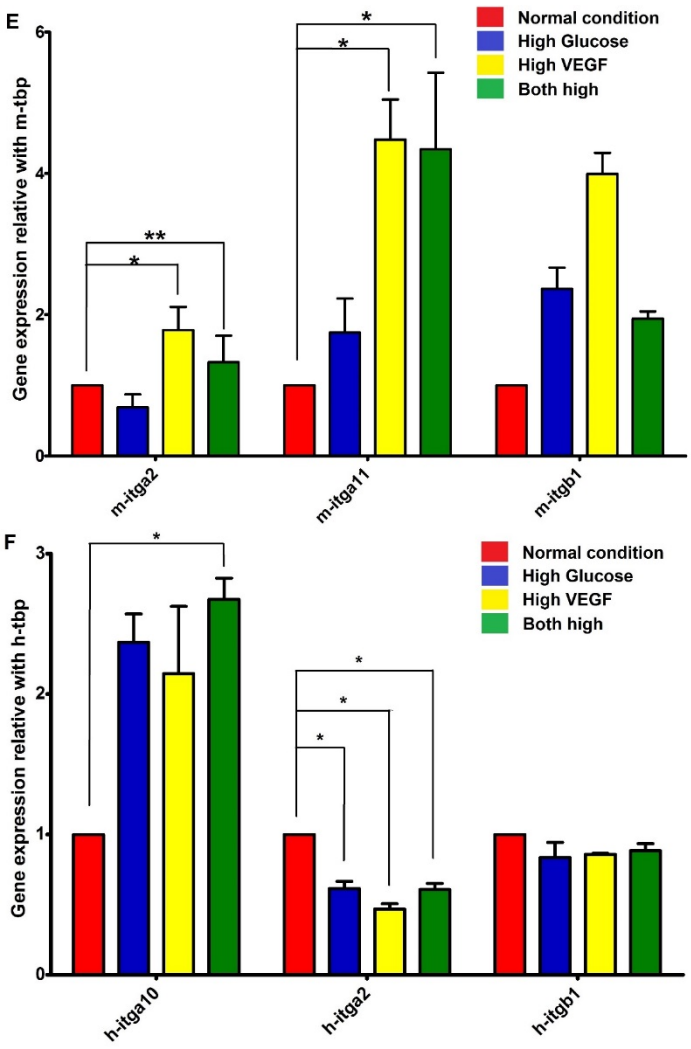

Figure 4. Mouse embryonic pericytes and HUVEC exhibit unique gene expression in co-cultue compared with diabetic condition. (CONT.) (E-F) Fold changes in integrin subunits gene expression among co-culture pericytes and HUVECs in different conditions; Values are averages + SEM, n=6 biological replicates. *P<0.05.

regulating MMPs. In addition, these conditions also promote the binding affinity of PCs with the IM by up-regulating integrin α2 and α11 subunits. For ECs, these results indicate that, in co-culture with PCs, the diabetes-like conditions induce an up-regulation of collagen III and IV gene expression, while enhancing EC binding with the vBM at the expense of binding with interstitial ECM. These data suggested three key features of vascular cells in diabetic conditions such as those in DR. Up-regulation of collagen IV, down-regulation of MMPs, and reduced PC binding to the vBM are consistent with mechanisms that would promote PC loss and vessel dysfunction in DR. The up-regulation of collagens (in both the vBM and interstitial ECM), down-regulation of MMPs, and enhanced EC binding with the vBM suggest a thickening of the vBM and the gradual loss of EC tight junctions.

Diabetes-Like Culture Conditions Shift the Degree to which Pericytes are Embedded in the Extracellular Matrix.

From our co-culture model, we not only found significant changes in ECM gene expression and protein synthesis, we also observed different configurations of PCs relative to their surrounding ECM (Figure 5). Specifically, co-cultured PCs exhibited three distinct morphologies in relation to

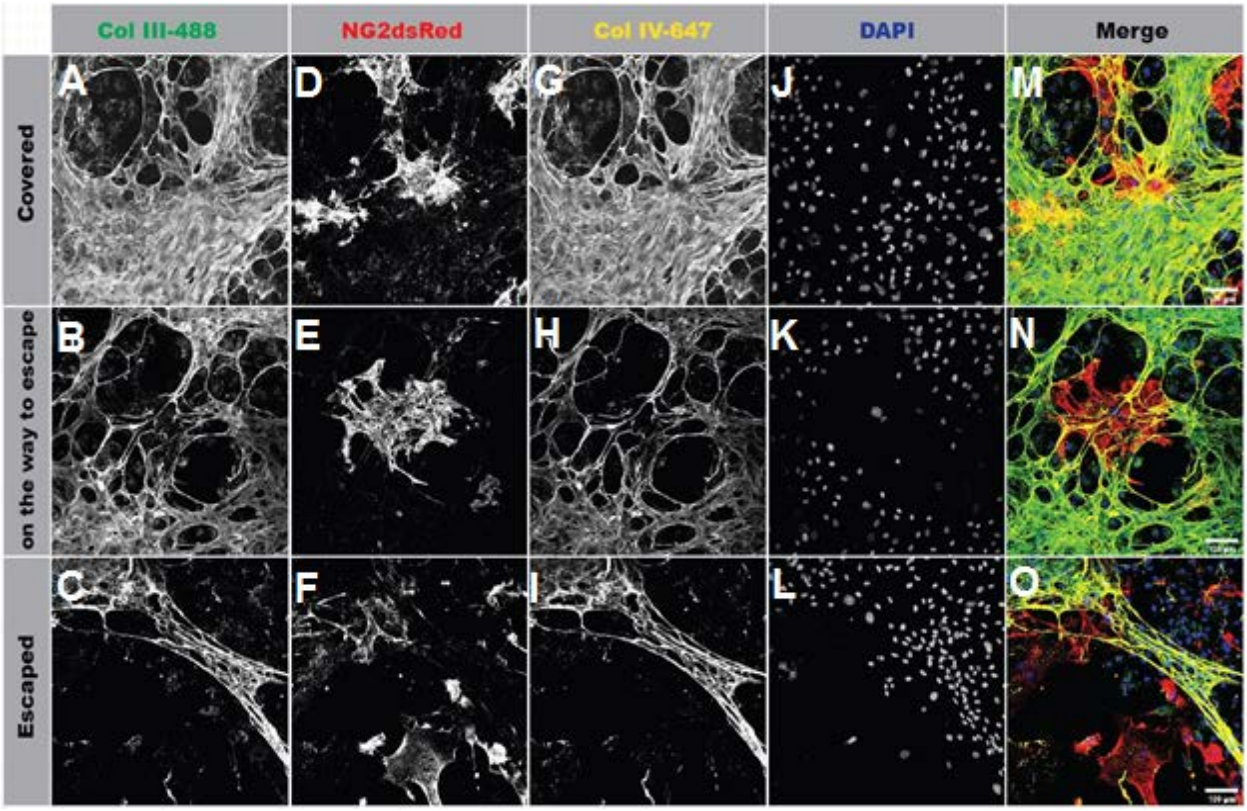

Figure 5. Mouse embryonic pericytes exhibit unique behavior patterns in the co-culture model. Representative images of pericytes (**D-F**) labeled for extracellular matrix collagen III (**A-C**, collagen III-AlexaFluor488, green in **M-O**), NG2 expression (**D- F**, DsRed Reporter or NG2 Ab, red in **D & H**), collagen IV (**G-I**, collagen IV-AlexaFluor647, yellow in **M-O**) and cell nuclei (**J-L**, DAPI, blue in **M-O**). Scale bars, 50 μm.

the ECM. In all media conditions, we found PCs are in three different configurations, that is, (i) "free" or unembedded in ECM, (ii) partially embedded or covered by collagen networks, and (iii) fully embedded or covered by collagen fibers, suggesting a lack of motility even in their cellular extensions. In a recent study from the lab of Dr. Andy Shih (Seattle Children's), we noted that in developed brain vasculature, PC somas were immobile, while their extensions moved forward and backward along vessels to contact extensions of nearby PCs [19]. Specifically, laser ablation of PCs on mouse brain vessels induced adjacent PC extensions to move forward and

contact other PCs, ultimately covering the void remaining after ablation [19]. Our lab has observed PCs capable of migrating on developing vessels to promote formation of vessel networks. Drawing from our observations and from those of the Shih lab, we hypothesize that, in fully developed vessel networks, PC soma are in the fixed locations, while their extensions may move along vessels to help maintain their function. To achieve this, the surrounding collagen network and vBM limits the migration of PC cell somas, but permits cell extensions. The three morphological states of PCs in our co-culture model may reflect PCs *in vivo* as they transition from freely mobile (i.e. unembedded) to more fully invested within the vessel wall (i.e. fully ECM embedded) (Figure 5). To validate this hypothesis, we characterized the three different morphological states of PCs relative to the ECM in our co-culture model.

In the co-culture model, PCs without any apparent binding to or coverage by the nearby collagen matrix, we defined as “free” or unembedded PCs. These PCs were considered to have broken down the collagen network and escape to interstitial matrix, consistent with the increased gene expression of MMPs and integrin subunit α2, which enhances PC binding with collagen I and III in interstitial ECM. These molecules may help them escape from the vBM and migrate into the interstitial space. We also found that some PCs were partially covered or embedded by collagen network. These PCs appeared to extend free cellular processes, indicating that these cells were able to maintain their normal functionality by retaining the mobility of their extensions. In the third state, PCs were fully covered by or embedded in the collagen network, not only the cell soma, but also the cellular extensions. In this state, PCs do not seem to be migratory or mobile and may have entirely lost their full functionality. We analyzed the ratios of PCs in different conditions and found the ratio of “functional” PCs decreased with HG and HV conditions (Figures 5 & 6), while BH conditions appeared to induce the lowest functional ratio. From this data, the HG condition seems to be a stronger stimulus for

collagen accumulation, while HV only appears to play an important role in helping rescue PC function and the excessive response caused by PC loss.

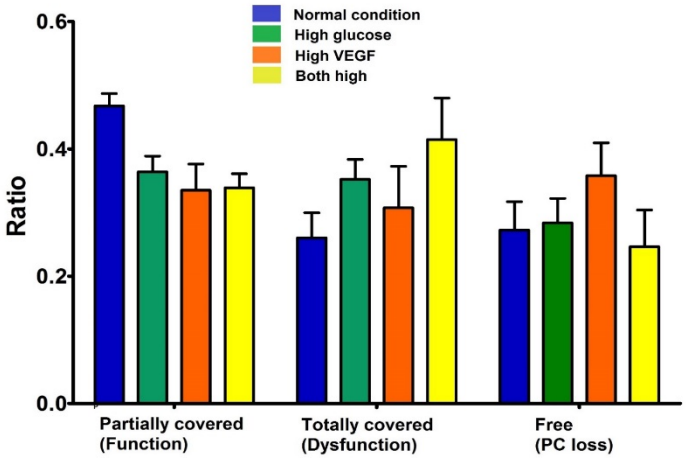

Figure 6. Mouse embryonic pericytes in co-culture exhibit different cell behavior patterns in four conditions. Average cell amount ratio for pericytes in normal condition (blue bars), high glucose condition (green bars), high VEGF condition (orange bars) and both high glucose and high VEGF condition (yellow

Pericytes and Endothelial Cells from a Developmental Model of Angiogenesis Exhibit Similar Gene Expression Profiles as Co-Cultured Cells in Baseline Conditions.

To provide a different vascular context to better understand the gene expression changes in collagens, MMPs, and integrin sub-units (which are related to PC-ECM state), we compared the observed transcriptional changes described above with EC and PC gene expression from developing mouse ESC-derived vasculature. In this developmental model, ECs are dividing, migrating, and forming new vessels, while PCs are recruited along the vessel wall, and the collagen networks are developing in this process. Based on our observations from the baseline co-culture model, we predicted that developmental PCs likely exhibit up-regulated gene expression of collagen III and IV, integrin subunits α1 and α10 for enhancing binding with vBM, and they should not have significant MMP changes; in addition, PCs down-regulating gene expression of integrin subunit α2 and α11 would indicate a shift away from binding with the interstitial ECM and enhance affinity for the developing vBM. After sorting PCs from ESC-derived primitive blood vessels, we found their up-regulation of collagen III, collagen IV, and MMP2, and down-regulation of MMP9. These signatures suggest their

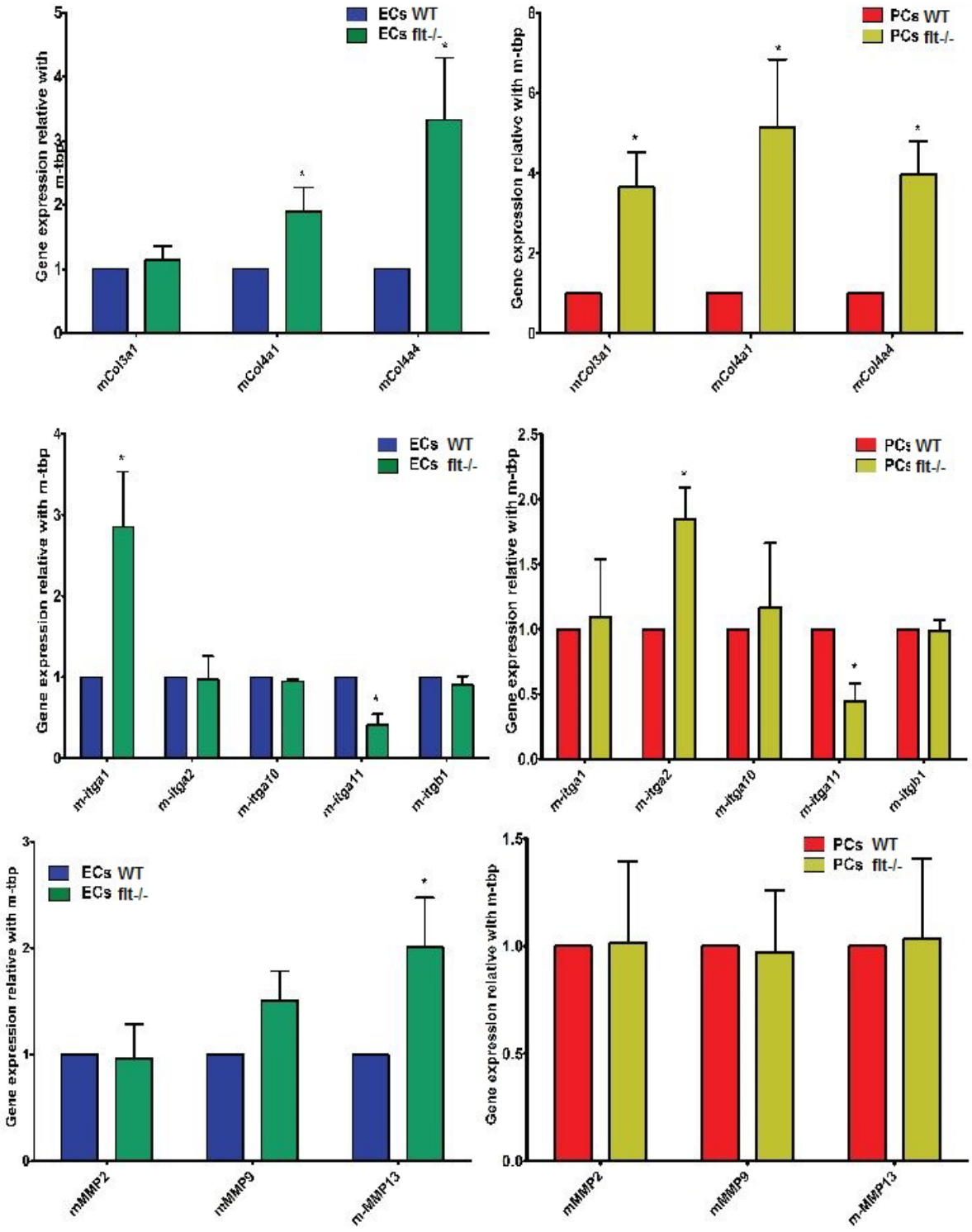

Figure 7. Gene expression of pericytes and endothelial cells sorted from embryonic stem cell-derived vessels WT compared to *flt-1*$^{-/-}$. Fold changes in gene expression between WT and *flt-1*$^{-/-}$. Values are averages + SEM, n=3 biological replicates. *P<0.05.

contribution to the formation of the vBM alongside an enhanced motility to facilitate their

recruitment during angiogenesis, ultimately helping PCs migrating along the vessel (Figure 7). Based on the results for co-cultured ECs in baseline conditions, we hypothesized that early stage angiogenic ECs up-regulate (i) collagen IV for vBM formation, (ii) MMP13 to enhance EC motility (i.e. to promote sprouting angiogenesis and cell proliferation), and (iii) integrin subunits α1 and α10 to facilitate EC binding with the vBM, alongside a down-regulation of integrin subunit α2 and α11 (i.e. decreasing affinity for the interstitial ECM). In testing for the genes described above, we found an up-regulation of collagen IV and integrin α1, again enhancing EC binding with vBM (Figure 7). We also observed EC down-regulation of integrin α11 and MMP13 (collagenase III), indicating less binding affinity with the interstitial ECM. These findings largely corresponded to the behaviors of ECs during developmental angiogenesis, specifically EC division, deposition of the vBM, and extensive vessel branching. Analysis of the PC compartment revealed an up-regulation of both collagen III and IV, while no significant changes were observed in the expression integrin subunits and MMPs. These results from a developmental model of angiogenesis revealed consistent gene expression profiles with co-cultured cells under baseline conditions, suggesting similar and consistent behaviors of ECs and PCs in physiological scenarios.

Analysis of Retinal Vasculature from Pre-Clinical and Cadaveric Models of Hyperglycemia Reveal Morphological Defects and Mis-Regulated Vascular Basement Membrane Architecture.

To analyze *in vivo* blood vessel ECs and PCs exposed to diabetic conditions, we selected an animal model in which the genetic loss of leptin receptor (i.e. LepR KO) disrupts mechanisms to signal appetite satiety, promoting a near constant level of food intake and causing an elevation in blood glucose. Specifically, leptin is a hormone that facilitates the regulation of energy balance with adipocytes [23]. The mutation of the leptin receptor causes an uncontrolled

appetite and, as a result of excessive food intake, leads to obesity and hyperglycemia [24]. Mutant mice manifest metabolic abnormalities including high circulating blood sugar and elevated body weight, which has led to their emergence as a commonly used mouse strain for diabetes and obesity research [24-25]. Under normal conditions, leptin provides an essential regulation of appetite and overall metabolism, which in turn modulates expression of several downstream target genes including Glut1 and Glut4 (i.e. glucose transporters) and HIF-1α, an important oxygen-sensing regulator [26-27].

In this mouse model, the LepR KO causes hyperglycemia and hyperinsulinemia. We hypothesized that this model shares key similarities with Type II diabetes patients, specifically in the characteristics of the retinal microvasculature. This *in vivo* diabetes model was also predicted to recapitulate certain observations from the co-culture model, namely abnormal collagen gene expression and accumulation. Although retinal vessels did not reach a level of disorganization as seen in proliferative DR patients, we did find vascular dysmorphogenesis in LepR KO mouse retinas (Figure 8). In particular, we observed (i) an apparent increase in the intensity of fluorescence signals (relative to DAPI) following immunolabeling of ECM components, (ii) dramatic changes in diameter of adjacent branches, and (iii) abnormal gene expression profiles (Figure 8 & 9). We found a significant increase in the fluorescence intensity of collagen IV in the 6-month-old LepR KO mouse retinal vessels, while both 4- and 6-month-old LepR KO displayed significant increases in the fluorescence intensity of immunolabeled collagen III. We confirmed this result with gene expression 8-month-old LepR KO mouse eyes and age-matched controls (i.e. LepR heterozygous mice, referred to as LepR-het or $LepR^{+/-}$) (Figure 10). Specifically, *col3a1* and *mmp2* were significantly elevated, while *col4a1* expression decreased in LepR KO mouse retinas. These results suggest an increasing accumulation of collagens in the vBM of LepR KO mouse retina vessels as the mouse ages and retinal vessels are chronically exposed to hyperglycemic conditions.

We also analyzed morphological features of the retinal vascular with respect to vessel width and the relative diameter changes comparing primary conduits to daughter branches. From this analysis, we found the widths of daughter branches to be abnormally smaller in LepR KO retina vasculature compared with age-matched LepR-heterozygous controls. The 6-month-old LepR KO retina vessels appeared most severely affected (Figure 8 & 9), as these retinal

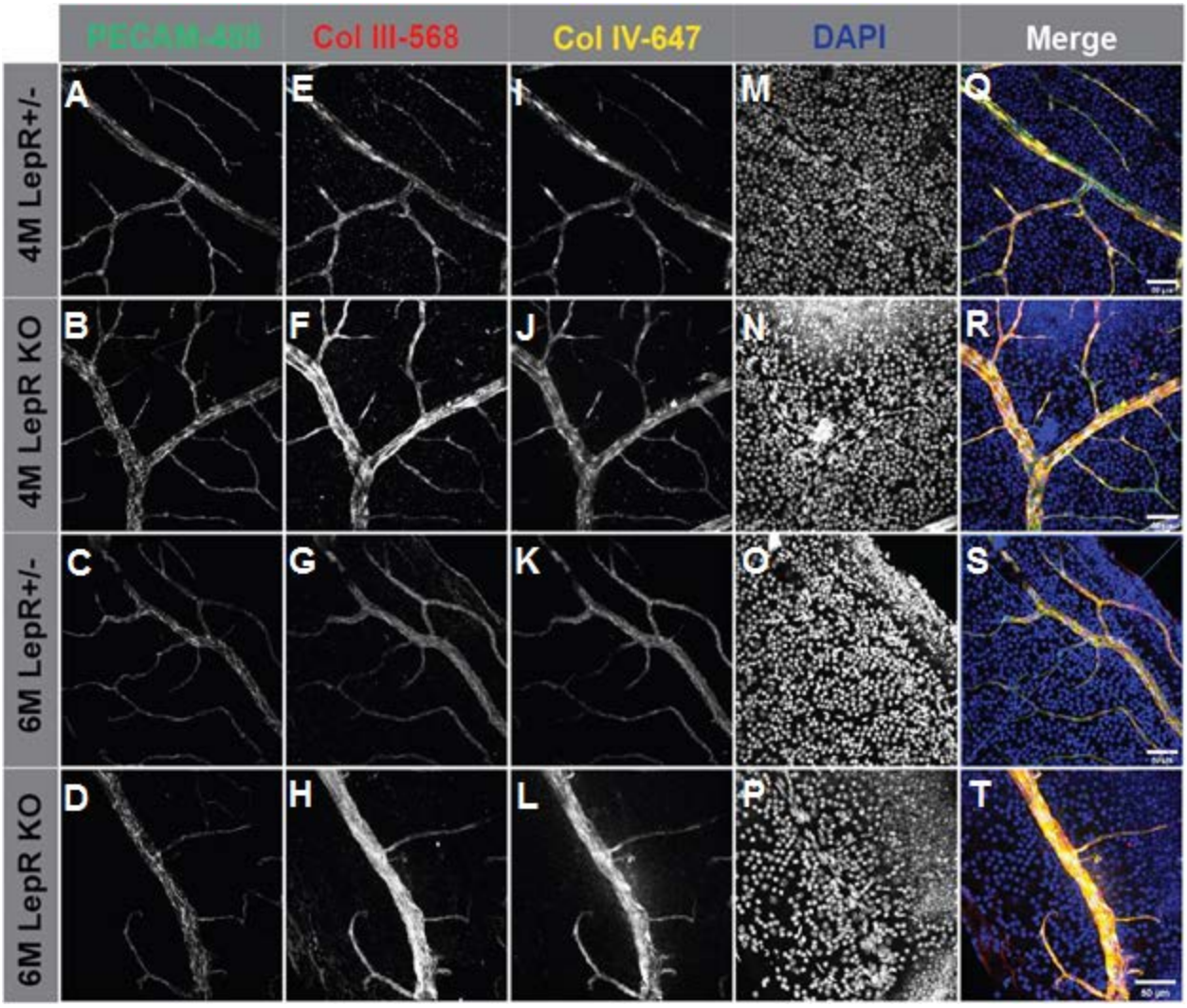

Figure 8. Extracellular matrix exhibit unique collagens deposition in mouse retina 4 month compared to 6 month, LepR$^{-/-}$ and LepR $^{+/-}$. Representative images of mouse retina (**A-T**) labeled for extracellular matrix collagen III (**E-H**, collagen III-AlexaFluor568, red in **Q-T**), PECAM expression (**A- D**, PECAM- AlexaFluor488, green in **Q-T**), and collagen IV (**I-L**, collagen IV-AlexaFluor647, yellow in **Q-T**) and cell nuclei (**M-P**, DAPI, blue in **Q-T**). Scale bars, 50 μm.

vessel diameter ratios (i.e. daughter vessel diameter divided by parent vessel diameter) sharply

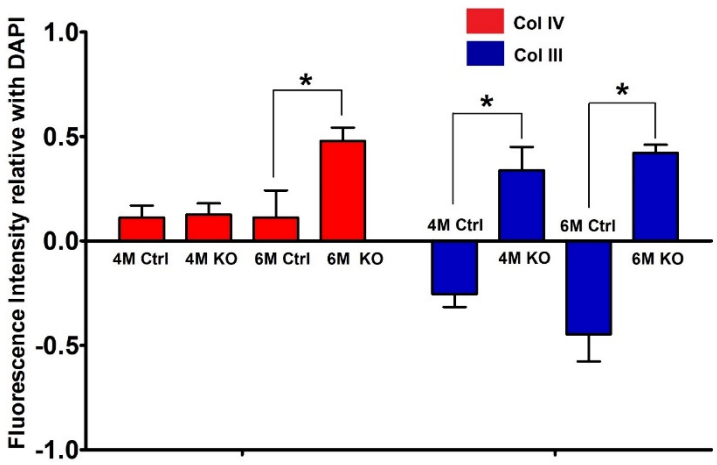

Figure 9. Fluorescence intensities of collagen III and collagen IV staining in 4 month and 6 month, *LepR*$^{-/-}$ compared to *LepR*$^{+/-}$. Average Fluorescence intensity of collagen III (blue bars), and collagen IV (red bars). Values are averages + SEM, n=4 biological replicates. *P<0.05.

decreased from 0.6 in LepR-hets to less than 0.2 in LepR KO mice (Figure 11). These morphological alterations indicate the likely dysfunction of retina vessels arising from environmental changes in the LepR KO mouse retina. Major artery diameters are largely unchanged between LepR KO and LepR-het mice, but a steep reduction in the diameter of branching arterioles was observed in the LepR KO retinal vessels, even with primary arteriole branches having the same diameter as capillaries. This result suggests that a drastic pressure drop from arterial segments to downstream capillary-sized vessels may contribute to vessel instability and albumin leakage into the interstitial space, disrupting local osmotic pressures and exacerbating tissue edema. These apparent changes in the composition of the vessel wall supported our hypothesis that an increased thickness of collagen III and IV

layers within the vBM may act as a compensatory mechanism to maintain vessel function despite this remodeling potentially giving rise to additional vessel defects.

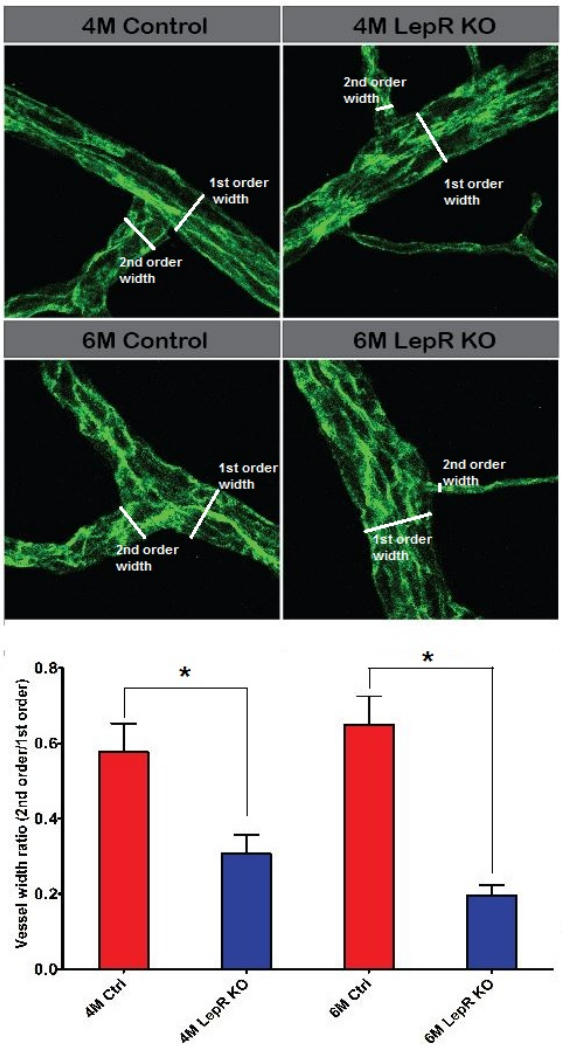

Figure 11. Mouse retinal vessel diameter ratio exhibit large difference in LepR$^{-/-}$ compared to LepR $^{+/-}$. Average vessel diameter ratios of 2nd order vessels vs. 1st order vessels in **LepR$^{-/-}$** (blue bars), and **LepR$^{+/-}$** (red bars). Values are averages + SEM, n=12 images from 4 biological replicates.

sections, we found a significant increase in the thickness of collagen layers on the capillary level (i.e. vessels with diameters less

To extend our findings from a pre-clinical model of diabetes-related vascular dysfunction, we immunostained human cadaveric retinas from patients without DM, those with Type II DM but without DR (i.e. DM II non-DR), and retinas from DM subjects also experiencing DR progression (DR) (Figure 12). From our high-power imaging analysis of these tissues, we found severe vascular abnormalities and a significant increase in the fluorescence intensity of immunolabeled collagens in DM II non-DR retinas. We also analyzed the relative thickness of the collagen III and IV layers within the vBM in non-DM conditions and in DR tissues (Figure 13) that were prepared as thin (10-30 μm) cryo-sections. In these retina

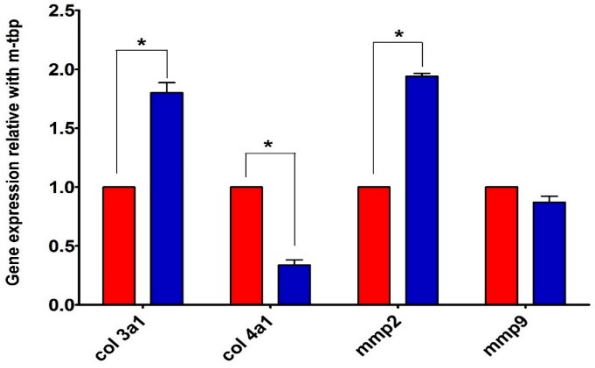

Figure 10. Gene expression of 8 Month mouse eye *LepR$^{-/-}$* compared to *LepR$^{+/-}$*. Fold changes in gene expression between *LepR+/-* (red bars) and *LepR$^{-/-}$* (blue bars) retinas. Values are averages + SEM, n=3 biological replicates. *P<0.05.

than 15 μm). Upstream arterioles or post-capillary venules did not have substantial changes in their vBM collagen layers (Figure 13).

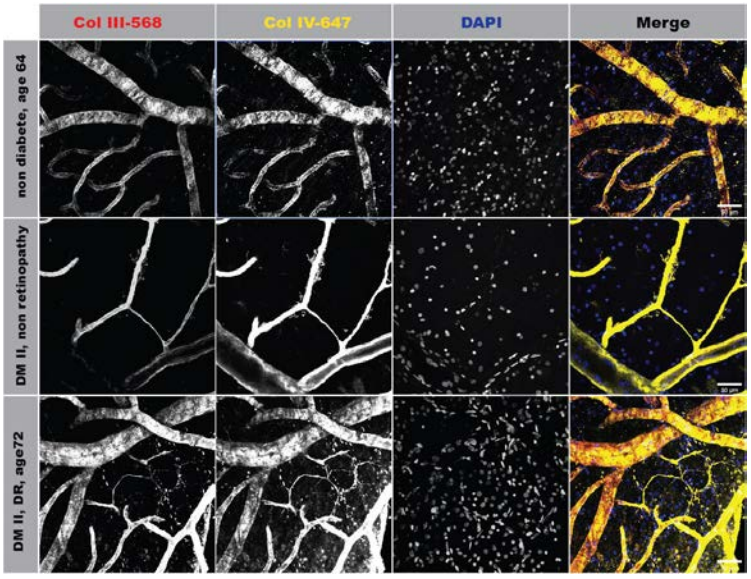

Figure 12. Human Cadaveric Retina Analysis. Representative images of human retinas from non-DM, DM non-DR, and DM+DR patients. Scale bars, 50 μm.

DISCUSSION

In the current study, we contribute several new insights into the potential mechanisms underlying DR progression as a chronic disease, providing a framework for interpreting the interactions between microvascular PCs, ECs, and their surrounding vBM/ECM during this progression. PC and EC gene expression of collagens, MMPs, and integrins within different stages of blood vessel development and in simulated pathological conditions suggest the contribution of PCs to microvascular dysfunction as well as unique ways that PCs are affected by microenvironment changes. From our *in vitro* experimental model using primary human ECs and mouse embryonic PCs, we were able to distinguish corresponding gene expression profiles for the different cell types. From these observations, we found an accumulation of collagen III and IV derived primarily from ECs responding to the diabetes-like conditions. Furthermore, our model permitted observing PCs and their behavior as a comprehensive response to interactions with ECs and the remodeling ECM in different glucose and VEGF-A culture conditions.

Moreover, this study aimed at highlighting the presence of a potential compensatory mechanism for microvessel defects that arise during DR progression. Within our interpretations, we acknowledge that the co-culture and mouse ESC-derived blood vessel experiments, while maintaining recapitulating normal condition as are found *in vivo*, were conducted over a relatively short time-frame. For example, we collected samples from the co-culture model, that were exposed to different culture conditions for less than 15 days; cell collection from the mouse

ESC-derived vessels occurred less than 10 days after initiating differentiation. The nature of these *in vitro* experiments prevent us from verifying whether or not there are other changes in collagens, MMPs and integrins over the long-term. In addition, pre-clinical animal models can only extend observations of vascular cell exposure to hyperglycemic conditions to 8 months, still short of the 12 to 15 years of elevated blood glucose the occurs in DM patients before DR symptomology is readily apparent. Nevertheless, the dynamic interactions among the vBM/ECM, PCs, and ECs may yield different effects on the vasculature as time progresses. Overall, these data support the idea that, while DR is the chronic disease, the interactions between PCs and ECs shape and remodel the surrounding vBM/ECM in a dynamic way that affects how each cell type interacts with their local microenvironment. Insight into these changes will help us understand the feedback mechanisms that may be engaged to maintain the retinal vasculature, and how these mechanisms might also compromise vessel health and eventually lead to irreversible microvascular defects, sustained retinopathy, and ultimately to diabetes-induced blindness.

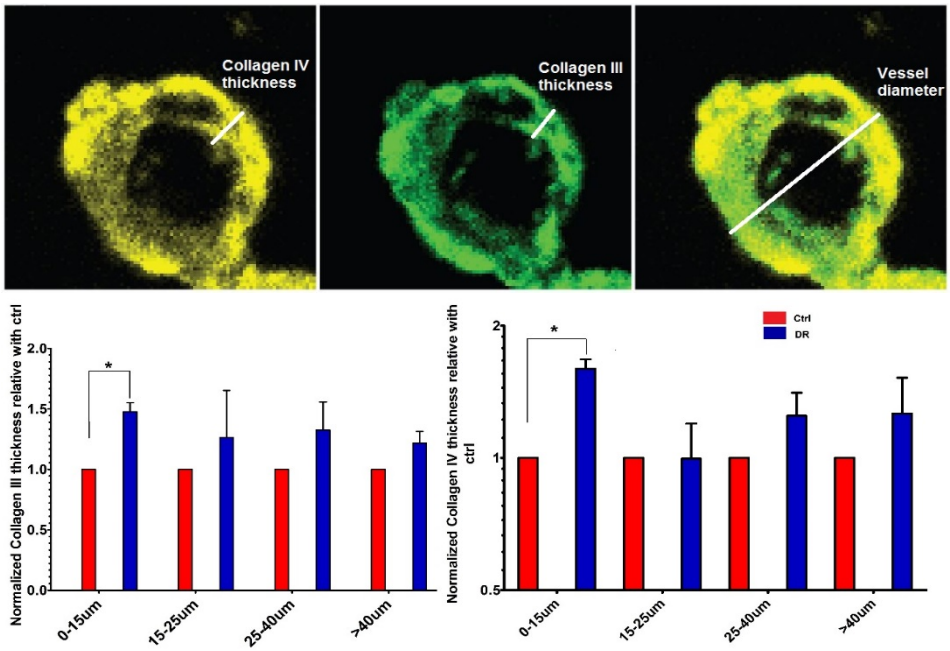

Figure 13. Human Cadaveric Retina Vessel ECM Analysis. Representative images of a human retina vessel in cross-section. Bar graphs of ECM thickness analysis.

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Chapter 4

Glial Cell Contributions to Diabetic Retinopathy Progression

INTRODUCTION

Diabetes mellitus is a metabolic disorder, characterized by hyperglycemia over a prolonged period of time [1]. Sustained hyperglycemic conditions can cause a range of associated tissue damage such as in the eye, kidney, and nervous system, each manifesting in long-term syndromes known as diabetic retinopathy (DR), diabetic nephropathy, and neuropathy, respectively [4-8]. According to the National Eye Institute (NEI) at the National Institutes of Heath (NIH), DR is the leading cause of blindness in patients of working age [5]. DR entails severe vascular complications, with the most well known abnormalities occurring within the microcirculation [6].

Diabetic retinopathy arises not only from pathological changes in the retina microvasculature, but also from significant morphological alterations in retina glial cells. Glial cell defects also occur at the earliest stages of DR and potentially play a pivotal role in disease progression [7-9]. The glial cells known to be involved in DR onset and progression include astrocytes, microglia, and Müller cells. However, the precise involvement of astrocytes and microglia in DR progression is still unclear. In this brief study, we mainly focused on astrocyte and microglia interactions with microvessels in the normal and DR retinas. This work seeks to extend the perspective of DR research to the glial-vascular interface, which likely entails the integration of multiple cell types and their respective contributions to disease progression.

MATERIALS AND METHODS

Analysis of WT and Diabetic Mouse Retinal Vasculature

LepR$^{-/-}$ (i.e. LepR KO) and *LepR*$^{+/-}$ mice were analyzed at the ages of 4 months and 6 months, and WT and *Ng2-DsRed* mice at postnatal days 7 (P7) and P21. The mice were weighed and euthanized, and their blood glucose was then measured. Mice were then perfused with 0.5% PFA and 0.5% Evans blue to assess any vessel leakage. Eyes were collected and fixed in 2% PFA for 2 hours, then stored in PBS at 4^{o}C. Mouse eyes were dissected to isolate the retinal layer only and immuno-staining was accomplished via incubation in goat anti-collagen IV (BioRad), rat anti-Glial Fibrillary Acidic Protein (GFAP, ThermoFisher), and rabbit anti-Iba1 (ThermoFisher). Secondary antibody labeling was performed with donkey anti-rat or anti-rabbit AlexaFluor 488 (Invitrogen, Carlsbad, CA), donkey anti-rabbit AlexaFluor 568 (Invitrogen), and donkey anti-goat and anti-rat AlexaFluor 647 (Jackson ImmunoResearch, West Grove, PA). Images were acquired by confocal microscope (Zeiss LSM880) using 20x, 40x, and 63x objectives.

Whole-mount Human Retina Immunostaining, Imaging, and Analysis

Cadaveric human eyes from diabetic and non-diabetic patients were acquired from Old Dominion Eye Foundation (Roanoke, VA), fixed in 4% PFA at 4^{o}C for 3-4 days, and then stored in PBS at 4^{o}C. The retina layer was dissected from each eye. Whole-mount tissues were immuno-stained for GFAP, Iba1, and collagen IV as described above. Images were acquired by confocal microscope using 20x, 40x, and 63x objectives.

RESULTS

Astrocytes and microglia in the retinal vasculature development

Astrocytes are closely associated with retinal blood vessel developmental as well as with pathological conditions associate with vascular dysfunction [10-11]. Astrocytes exhibit a similar regional distribution in the retina to the vasculature that develops [12]. Astrocyte end-feet extend along and around vessels and are an essential part of the blood-retina barrier [13]. In contrast, microglia are considered to be involved primarily in the immune response for clearing waste and unnecessary materials, as well as maintaining normal vessel functionality [14-15]. Activated microglia are also indicators of pathological conditions [16].

Recent studies have suggested that retinal astrocytes are necessary for regulating vessel development [17-19]. As seen in Figure 1, we found astrocytes to be engaged not only with newly formed vessels (e.g. at P7), but also part of a denser networks of astrocytes. At P21, when the retinal vessels are almost fully developed, the astrocyte end-feet were observed to cover nearly the entire profile of vessel structures (Figure 1). Astrocyte dense branching at this time-point was substantially reduced in comparison to P7 retinas (Figure 1). This result indicates that astrocytes are likely involved in the early stages of vessel development and potentially act as guidance structures for EC sprouting and for constructing the ECM that forms the vascular

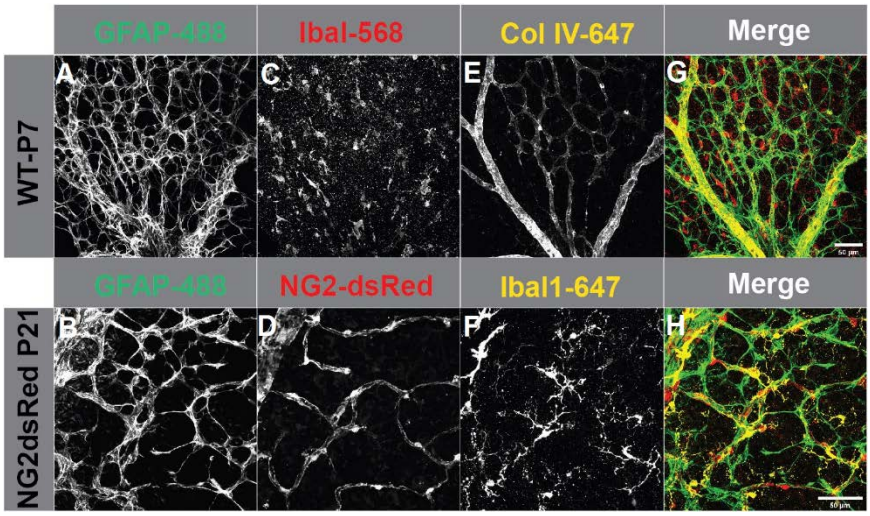

Figure 1. Mouse retinal astrocytes and microglia exhibit unique cell morphologies and distribution patterns in P7 retinas as compared to those at P21. Representative images of astrocytes (**A-B**) and microglia (**C, F**) labeled for GFAP (**A & B**, GFAP-AlexaFluor488, green in **G & H**), NG2 expression (**D**, DsRed Reporter, red in **H**), Iba1 (**C**, Iba1-AlexaFluor568, red in **G**), Iba1 (**F**, Iba1-AlexaFluor647, yellow in **H**), and collagen IV (collagen IV--AlexaFluor647, yellow in **H**). Scale bars, 50 µm.

basement membrane (vBM). Similar to astrocytes, microglia appear less dense at P21 relative to P7 retinas, but the cellular extensions radiating from microglia are more explicit and appear interconnected in a complex network (Figure 1).

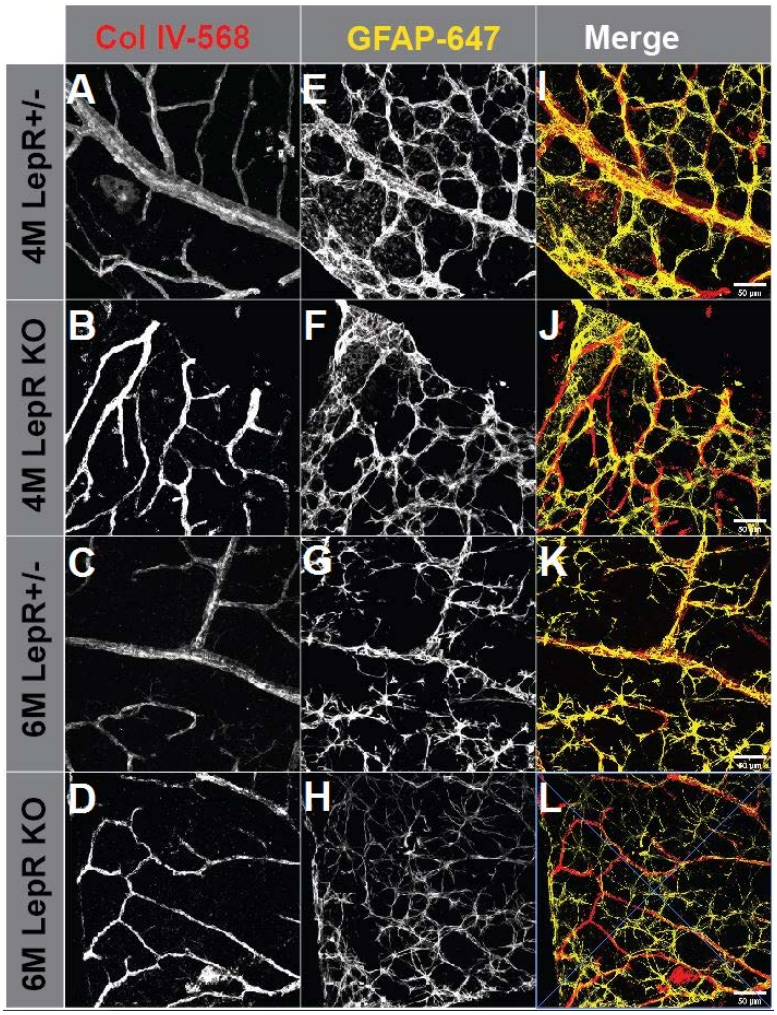

Figure 2. 4 and 6 month old mouse retinal astrocytes exhibit unique cell morphologies and distribution patterns in LepR$^{+/-}$ mice compared to LepR$^{-/-}$. Representative images of astrocytes (**E-H**), labeled for GFAP (**E-H**, GFAP-AlexaFluor647, yellow in **I-L**), and collagen IV (collagen IV-AlexaFluor568, red in **I-L**). Scale bars, 50 µm.

Glial Cells in LepR KO and LepR Heterozygous Mice at 4 and 6 months of age

Retinal astrocytes and microglia were long assumed to be abnormal in diabetic conditions. Work from Rungger–Brändle et al. revealed that the coverage of astrocyte end-feet was significantly decreased [9] as an early feature of DR, which the authors interpreted as an important factor contributing to decreased vessel permeability. The increase in microglia density and their physical clustering together in a similar location [20] are regarded as

indications of their activated immune response in DR, which is believed to further provoke the inflammatory conditions and exacerbate the deleterious nature of the diabetic microenvironment. Here, we used LepR KO and *LepR*$^{+/-}$ mice at 4 and 6 months of age to compare and test the hypothesis of chronic changes in glial cell organization during prolonged exposure to diabetic conditions (Figures 2 and 3).

In the LepR heterozygous control retinas, the astrocyte end-feet covered the outer surface of retina vessels and formed another outer layer adjacent to but not within col IV layer of the vBM (Figure 2). In contrast, analysis of retinas from the LepR KO group revealed that astrocytes were not well distributed, leaving sizeable regions of retina vessels void of astrocyte end-feet (Figure 2). The apparent astrocyte-vessel coverage ratio also seemed to decrease with increasing age, that is, with increased exposure to hyperglycemia, as we found when comparing mice at 6 months of age to mice at 4 months (Figure 2).

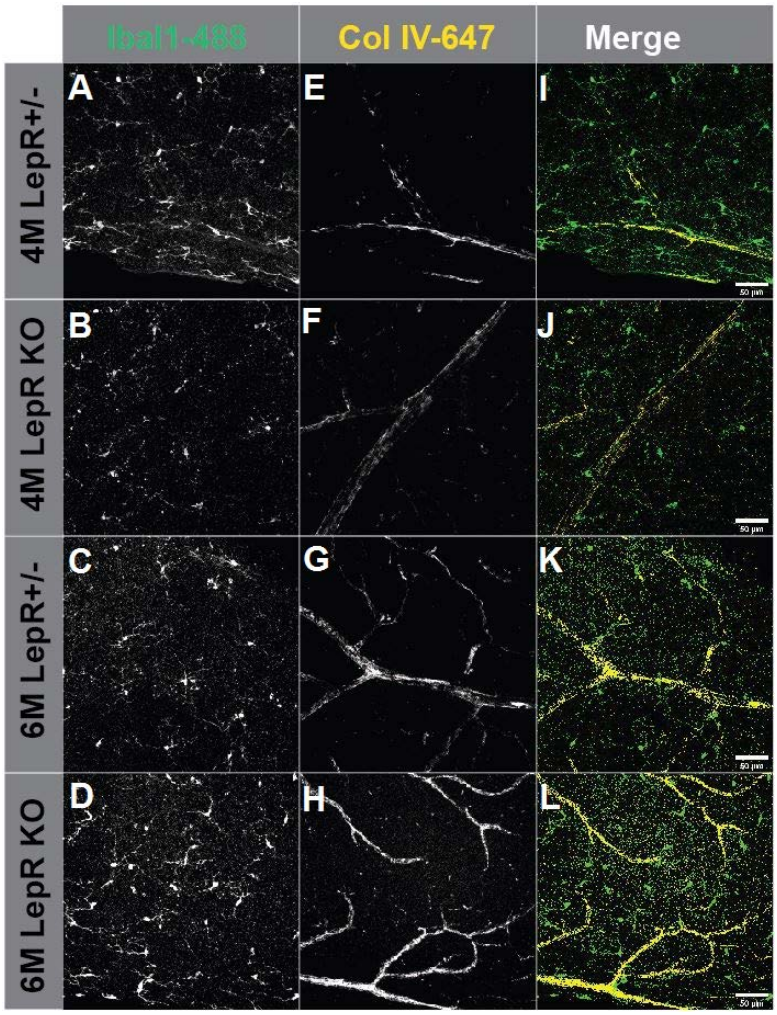

Figure 3. 4 and 6 month old mouse retinal microglia exhibit unique cell morphologies and distribution patterns in LepR$^{+/-}$ mice compared to LepR$^{-/-}$. Representative images of microglia (**A-D**), labeled for Iba1 (**E-H**, Iba1-AlexaFluor488, green in **I-L**), and collagen IV (collagen IV-AlexaFluor647, yellow in **I-L**). Scale bars, 50 µm.

With respect to microglia, there was not a significant difference in LepR het control retinas and LepR KO retinas at 4 months of age (Figure 3). However, in the LepR KO retinas, microglia displayed a tendency towards increased “crowding” around vessels (Figure 3). In the 6-month-old LepR KO mice, we found that there were many more microglia as compared to control retinas of the same age. These cells were also more associated with adjacent vessels, which indicated that these microglia were involved in the inflammatory response during the early stages of DR progression (Figure 3).

Astrocytes and Microglia in Diabetic and Non-diabetic Human Retinas

Microglia in diabetic human retinas displayed many unique morphological differences when compared to microglia in non-diabetic conditions. The microglia in the healthier retina were much less dense and appear less active than in the DR retina, suggesting a more robust inflammatory response under prolonged diabetic conditions (Figure 4). Compared with the mouse retina immunostaining for these cells, astrocytes in the diabetic retinas showed a remarkable morphological difference, which also contrasted with astrocytes in the non-diabetic conditions. In the non-diabetic retina, astrocytes appeared to form a network while their end-feet covered the vessel outer surface. In the DR retina, the astrocytes were severely overgrown, and end-feet on retinal vessels were almost non-existent. In comparing these differences with the mouse model, these observations indicate

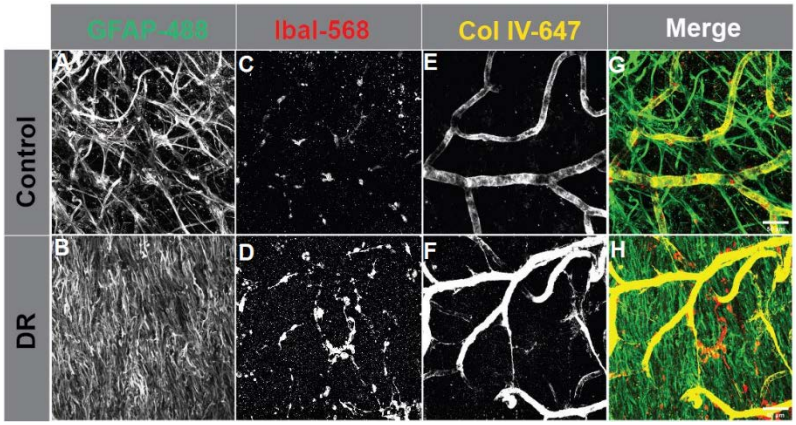

Figure 4. Human astrocytes and microglia exhibit unique cell morphologies and distribution patterns in **non-diabetic retinas compared to a retina with defined diabetic retinopathy.** Representative images of astrocytes (**A & B**), microglia (**C & D**), labeled for GFAP (**A & B**, GFAP-AlexaFluor488, green in **G & H**), Iba1 (**C & D**, Iba1-AlexaFluor568, red in **G & H**), and collagen IV (collagen IV--AlexaFluor647, yellow in **G & H**), Scale bars, 50 µm.

that astrocyte morphology undergoes extreme variations during moderate to long-term exposure to diabetic conditions. The abnormal morphology and cell density of astrocytes and microglia provided a clear indication of the overall dysfunction of the blood-retinal barrier and potential defects involved in DR progression.

DISCUSSION

The results from the current study support the idea that retinal astrocytes and microglia are likely involved in DR progression, and sustained defects in their structure and distribution may contribute to DR as a chronic disease. In addition, the interaction of the ECM/vBM with astrocytes and microglial is still unknown and should be on the list of future research.

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Chapter 5

SUMMARY, PERSPECTIVES, AND FUTURE DIRECTIONS

Microvascular abnormalities are the hallmark of numerous pathological conditions such as diabetic retinopathy (DR), diabetic nephropathy, and tumor angiogenesis. Microvessel defects include a broad range of perturbations including aberrant structural dilation of capillaries, endothelial cell (EC) activation, hyper-permeability, disorganized remodeling of the extracellular matrix (ECM) within the vascular basement membrane (vBM), and loss or dysfunction of PCs, among others [1-3]. Because these features and their effects fuel numerous pathological conditions, biomedical researchers across a number of disciplines have worked tirelessly to better understand the mechanisms behind these complications. DR is a prominent example of vascular-related pathology receiving significant attention over the past few decades because the irreversible progression of this disease results in vision loss [4].

Despite several treatments including intraocular anti-vascular endothelial growth factor-A (VEGF-A) injections being shown to slow down DR progression, the side effects of these treatments cannot be underestimated in their negative consequences for otherwise healthy tissues [1-3]. Also, fully establishing the mechanisms behind DR progression remains an elusive goal. The majority of the experiments described in this dissertation were aimed at shedding light on potential mechanisms underlying DR pathogenesis, using cell-based mono- and co-culture models, a pre-clinical animal model, and observations from diabetic and non-diabetic human retinas. Results from this portion of the dissertation (i.e. Chapter 3) are consistent with the hypothesis that the interactions amongst the ECM, ECs, and pericytes (PCs) in diabetes-like conditions (i.e. hyperglycemia and high VEGF) likely cause long-term changes to the retina capillary vBM, leading to vascular defects that accumulate and ultimately exacerbate DR progression. This study further supports the hypothesis that the chronic mechanisms underlying

DR that result from ECM and cellular interactions may slow down this progression but may also fuel its irreversibility.

This chapter will summarize how the findings from this dissertation contribute to a general characterization of the PC-HUVEC co-culture model, and how it might be applied towards increasing our understanding of PC-EC interactions and how ECM remodeling downstream may advance disease progression. It will also discuss potential questions and future directions suggested by this work and highlight goals that merit further exploration by future studies.

Characterization and validation of a primary pericyte cell line and co-culture model – Summary, conclusions and future directions.

PCs are located along capillaries and surround vascular endothelial cells to maintain normal function and homeostasis of blood vessels [5]. One main challenge in researching the interplay between ECs and PCs is the variability in identifying PCs, which is due in part to the morphological variability and overlap in the characteristics and features of other cell lineages [6, 7]. In the work presented in Chapter 2, we described our isolation of a primary mouse embryonic PC cell line and the steps taken to functionally validate this cell in using *in vitro* and *ex vivo* assays. This PC cell line was isolated from embryonic day 12.5 (E12.5) mice using *Ng2/Cspg4* expression as a biomarker (specifically via the expression of the DsRed reporter). We compared the morphology of isolated PCs with another cell line that shares a similar lineage (specifically fibroblasts). These data helped us distinguish two cell lines via their general morphologies, migration dynamics, and gene expression patterns.

To confirm that the PC cell line we isolated was indeed functional, we applied these cells to angiogenesis assays, testing their ability to promote EC organization into vessel-like structures as well as enhance EC-EC junctions. Our results strongly support the idea that PCs contribute to the blood vessel wall through secretion of ECM components into the vBM, as well

as by directly engaging with the endothelium and promoting junctional stability. These data also support a functional but straightforward co-culture model for future investigation.

Our study validated the ability of this PC cell line to incorporate into developing vessels in various *in vitro* vasculogenesis assays. We then tested this PC line for its engagement with and incorporation into *ex vivo* developing blood vessels. Exogenous PCs were able to home to and initiate contact with emerging endothelial tip cells. Added PCs tracked along endothelial tip cells as they extended from parent vessels and connected to form a new vessel branch. The overall result from the work described in Chapter 2 highlighted the successful collection of a functionally validated PC cell line, and the initial use of a co-culture model for future studies exploring EC-PC interactions *in vitro*.

A potential mechanism underlying DR in the chronic progression of this disease resulting from the interplay between ECM/vBM remodeling and interactions with ECs and PCs during diabetic conditions.

Diabetic retinopathy is an irreversible, chronic disease, and it is the leading cause of blindness of working-age adults in the United States [4]. Recent studies have identified microvascular complications as clear indicators of DR progression, including PC loss/dysfunction, increased thickness of the ECM within the vBM, and the loss of EC tight junctions, among others. Treatments that attempt to mitigate the hypoxic tissue response in the diabetic eye (i.e. laser ablation) or temporarily disrupt the abnormal angiogenesis occurring (i.e. anti-VEGF-A injections) have been shown to only pause or slow down disease progression. There is therefore no treatment capable of reversing or completely curing this disease. In addition, because DR is a chronic disease developing over many years, the mechanisms underlying pathogenesis and progression remain unclear. In Chapter 3, we hypothesized, and found evidence supporting, a potential mechanism that may explain the sustained progression of DR and may provide possible directions for designing novel therapeutics.

We formulated our hypothesis from the existing observations of microvascular dysfunction in DR, including loss or dysfunction of PCs, increased thickness of the vBM, and loss of EC tight junctions. Since PC “drop-out” and EC tight junction disruption are features of several microvascular diseases, they may not constitute the primary mechanisms driving this chronic disease; therefore, we conjectured that the increased thickness of the vBM/ECM may be a more deleterious process in the prolonged nature of this condition. Specifically, the results of collagen immunostaining and gene expression analysis from our co-culture model support our hypothesis that the ECM/vBM is modulated by PC-EC interactions during exposure to diabetic conditions. Additionally, we found three distinct morphological conditions for PCs relative to their surrounding ECM, similar to different scenarios described for PCs in DR conditions and other diseases such as idiopathic pulmonary fibrosis. To better understand PCs within these distinct situations, we analyzed the ratio of PCs in the different ECM-embedded states across culture conditions and found results consistent with our working hypothesis. In a comparative analysis experiment, we found that gene expression of PCs and ECs isolated from mouse embryonic stem cell (ESC)-derived vessels aligned with our expectation for each cell type in remodeling and engaging the vBM and interstitial ECM. Specifically, the affinity of PCs and ECs for the interstitial ECM and the vBM corresponded to their expression of collagens, MMPs and integrin subunits. These distinct profiles for both PCs and ECs are suspected to shift during diabetic conditions and impact the remodeling of the vBM and the engagement of each cell type with the vBM and interstitial ECM.

Observations of LepR KO mouse retinas and human cadaveric retina staining further support our hypothesis that the accumulation of collagens covering microvessels is dynamically regulated and ultimately increases with age and even more dramatically during diabetic conditions. Thus, the results of Chapter 3 implicate a potential mechanism underlying DR progression, which may inspire new directions for research into this devastating disease.

Glial cells may be involved in DR progression.

Diabetic retinopathy arises in part from microvascular complications, and many studies have focused on the abnormal angiogenesis and vascular changes involved in DR progression. Work has just begun in exploring the role retinal astrocytes and microglia may have in DR pathogenesis. In Chapter 4, we took a brief look at the morphological changes in retina astrocytes and microglia in the diabetic context, aiming to connect these observations with the results from our previous research and providing a more comprehensive study on the glial-vascular interactions during DR.

In Chapter 4 of this dissertation, we analyzed the differences in morphology and distribution of retinal astrocytes and microglia. We found that diabetes-induced changes in the retina glial compartment are also time-dependent, such that the longer the exposure to hyperglycemia, the more severe the outcome. Also, these results may partially support our findings in other chapters suggesting a slow but steady increase in long-term vessel permeability in DR. This information may be useful for additional studies that may focus on vessel permeability changes that result not only from PC loss and vBM/ECM remodeling, but also changes in astrocyte end-feet coverage and the extent of microglia distribution.

Significance and Perspectives

Even though the treatments for DR patients have improved to control and delay diabetic complications, research into the underlying mechanisms involved in DR remains important since this disease and its effects are irreversible. DR is considered to arise in large part from microvascular complications, and retinal PCs and ECs are strongly implicated during the onset and progression of this condition. Therefore, it remains critical to increase our understanding of the mechanisms behind PC-EC interactions that drive the degradation and synthesis of the ECM/vBM in diabetic conditions. Studies that endeavor to do this will provide insights that could be important for the development of therapies that might control and/or pause DR progress, or

therapies that may even result in a complete cure. In particular, the work presented in this dissertation has shed more light on the regulation of ECM/vBM remodeling associated with the interactions between PCs and ECs and the dynamic regulation of ECM surrounding PCs, which may impact cell communication.

While the work described in this dissertation contributes to our understanding of a possible compensatory mechanism that fuels microvessel defects arising during DR progression, many more questions have emerged for future research. I hope that my research on DR and the interplay between ECs, PCs, and ECM will help inform future work that will deepen our knowledge of DR. Indeed, a better understanding of the underlying mechanisms will lead to potential therapeutic approaches and perhaps a cure of this disease. Also, regarding general microvascular complications, these studies may inspire new directions for research into other vascular-associated diseases.

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