Paxillin-Dependent Control of Tumor Angiogenesis

by

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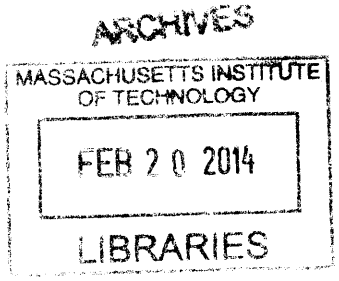

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ABSTRACT

Angiogenesis- the growth of new capillaries from existing vessels- is required for tumor growth; however, tumor vessels exhibit abnormal structure and function, which impairs the targeted delivery of anti-cancer agents. While directional migration of capillary endothelial cells is critical for normal angiogenesis, the mechanism by which oriented capillary cell migration is controlled or how it is deregulated during tumorigenesis is unknown. Recently our lab reported that the focal adhesion protein, paxillin, is required for directional migration of fibroblasts. Endothelial cells also express paxillin and localize it in their focal adhesions. Thus, I set out to analyze whether paxillin influences directional migration of endothelial cells. When the expression of paxillin is knocked down in endothelial cells, this enhances their migration but decreases their directional persistence *in vitro* and *in vivo* in migration, angiogenesis and developmental assays. Having confirmed that paxillin plays a central role in controlling oriented capillary cell migration, I then studied the mechanism by which it contributes to normal microvessel network formation and tumor angiogenesis.

I found that paxillin knockdown increases microvessel density but causes loss of sprout orientation. These characteristics resemble those of tumor vasculature, and, in fact, studies revealed that tumors inhibit paxillin expression in endothelial cells *in vitro* and *in vivo* by secreting soluble factors, such as the potent angiogenic factor VEGF. Mechanistically, paxillin knockdown decreases expression of the VEGF receptor neuropilin 2 (NRP2) but not VEGF receptor 2, and this is mediated by the transcription factor GATA2. Direct knockdown of NRP2 also increases endothelial cell migration and vessel density *in vitro* and *in vivo* and these effects are rescued by over expressing paxillin.

In summary, these studies have led to the discovery of a new mechanism for control of directional endothelial cell migration during angiogenesis that is mediated by paxillin-NRP2 signaling. Importantly, this previously unknown mechanism is deregulated in tumor angiogenesis, which may contribute to the enhanced, disorganized microvasculature that is hallmark of cancer. These findings also revealed a new function for the focal adhesion protein, paxillin, as a mediator of tumor angiogenesis, and elucidated a novel mechanism for control of the expression of NRP2.

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Ever a cell migration enthusiast, my self-proclaimed second biggest fan was always ready to belt out my thesis theme song, "Don't stop, Paxillin". From the stranger in Fluid Mechanics, to my TA, to my travel companion, volleyball instructor, concert partner, martini maker, collaborator and cheerleader, Bill is the perfect complement to this crazy life. The fun we have makes all the work worth it.

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LIST OF ABBREVIATIONS

AMD- age-related macular degeneration

CE- capillary endothelial

ConA- concanavalinA

ECM- extracellular matrix

FA- focal adhesion

FAK- focal adhesion kinase

H&E- hematoxylin and eosin

HUVE- human endothelial vein endothelial

kd- knockdown

LLC- Lewis lung carcinoma

NRP2- neuropilin 2

Pax kd- paxillin knockdown

PaxC- paxillin C-terminus truncation mutant

PaxN- paxillin N-terminus truncation mutant

PDMS- poly-dimethylsiloxane

PI3K- phosphatidylinositol-3 kinase

ROCK- Rho associated protein kinase

Sema3F- Semaphorin 3F

VEGF- Vascular Endothelial Growth Factor

CHAPTER 1: INTRODUCTION

1.1 ANGIOGENESIS IN DEVELOPMENT AND CANCER

Angiogenesis- the growth of new capillaries from existing vessels- is important for both normal and pathological tissue development (Adamis et al., 1994; Aiello et al., 1994; Alon et al., 1995; Jager et al., 2008). Physiological angiogenesis is commonly stimulated by the presence of soluble angiogenic factors, such as Vascular Endothelial Growth Factor (VEGF), fibroblast growth factor (FGF) or many other molecules that are potent stimulators of capillary endothelial (CE) cell migration and growth (Ferrara et al., 2003; Lamalice et al., 2007).

The construction of new microvessels involves multiple steps occurring concurrently. The first step involves local degradation of the basement membrane surrounding the vessel, which activates the underlying CE cells and permits them to extend processes into the surrounding stroma (Fig. 1.1A). Hypoxic conditions that characterize most angiogenic microenvironments activate the hypoxia-sensitive transcription factor, HIF1α, in endothelial cells, which stimulates them to produce VEGF as well as its surface receptor VEGFR2 (Ferrara et al., 2003; Tang et al., 2004). The binding of angiogenic factors such as VEGF to receptors on the surfaces of CE cells within preexisting vessels leads to the establishment of a tip cell at the leading edge of the extending cord of endothelial cells that migrates up the tissue-produced VEGF gradient in a directional manner, and it induces trailing stalk cells to proliferate (Lamalice et al., 2007; Li et al., 2005; Rousseau et al., 1997) thereby forming a new sprout (Gerhardt et al., 2003a; Ruhrberg et al., 2002). As the sprout extends, endothelial cells continue to release proteolytic enzymes, such as matrix metalloproteinases (MMPs), that continually degrade the basement membrane (Hughes, 2008), while simultaneously depositing new ECM proteins that form a dynamic scaffold for CE cell movement and growth (Ingber et al., 1986; Ingber and Folkman, 1988). MMPs and matrix degradation are also required for vessel maturation

and lumen formation (Iruela-Arispe and Davis, 2009; Whelan and Senger, 2003). The pro-angiogenic signals, such as VEGF, are counterbalanced by binding of the VEGF-induced delta-like ligand 4 (DLL4) to notch 1 and 4 receptors that prevent excess angiogenesis and promote orderly vessel formation. Angiogenesis concludes with inhibition of MMPs by tissue inhibitor of metalloproteinases (TIMPs) (Saunders et al., 2006), establishment of cell-cell junctions, production of new basement membrane, stabilization of new vessels by pericytes and vascular smooth muscle cells (VCSMCs), and initiation of tissue perfusion.

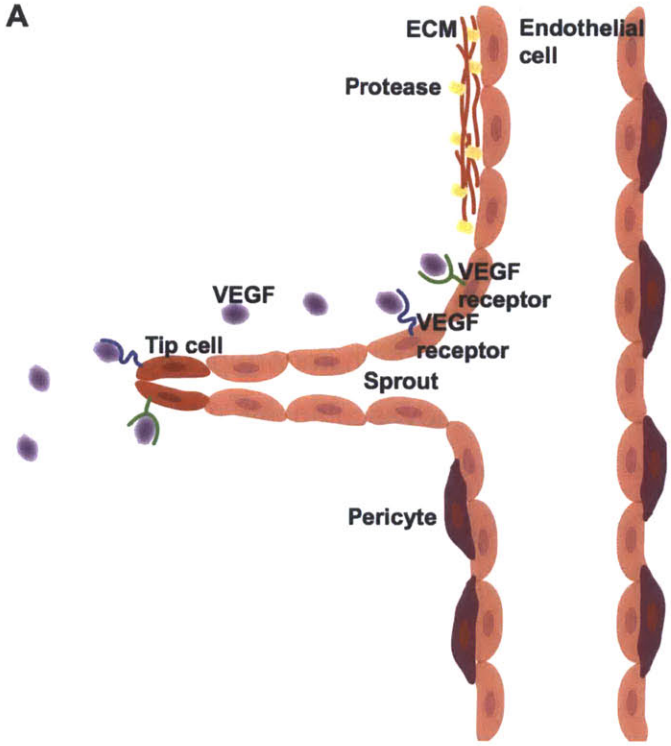

Figure 1.1A Schematic of angiogenic sprouting.

Cancerous tumors also require the growth of new blood capillaries from preexisting vessels to support their continued growth and expansion (Folkman, 1971). Tumor angiogenesis is similar in many ways to physiological neovascularization that is critical for growth and development of normal organs in that both are mediated by directional migration and growth of CE cells in response to increased demand for oxygen and nutrients. In tumors, however,

cancer cell proliferation can overcome the ingrowth of capillaries, and as tumor size surpasses the diffusion the limit of oxygen, hypoxia is generated at the tumor core. Hypoxia activates HIF1α-dependent expression of VEGF and VEGFR2 on endothelial cells during tumor angiogenesis (Chung and Ferrara, 2011). In addition, HIF1α can be activated or synthesized by tumor cells or tumor stromal cells (e.g. fibroblasts, monocytes, platelets) to further increase VEGF production (Chung and Ferrara, 2011; Kut et al., 2007).

Importantly, over-expression of VEGF creates a proangiogenic environment causing the microvasculature of solid cancers to display a characteristic loss of normal microvessel morphology (Ausprunk and Folkman, 1977; Folkman, 1986; Hahnfeldt et al., 1999) with the tumor vessels appearing tortuous and disorganized (Fig. 1.1B) (Konerding et al., 1995). Loss of vessel stabilization by pericytes and VSMCs also leads to leakiness and fragility (Nagy et al., 2007). The disorganized structure of tumor microvessels is important clinically because it reduces delivery of chemotherapeutic agents to the tumor. For these reasons, angiogenesis has become an important target for cancer therapy with the goal of either inhibiting neovascularization to deprive the tumor of oxygen or to normalize the vasculature to improve chemotherapy delivery (Carmeliet and Jain, 2000; Dvorak, 2002; Ferrara et al., 2004; Folkman, 1971; Jain, 2005; Kerbel and Folkman, 2002).

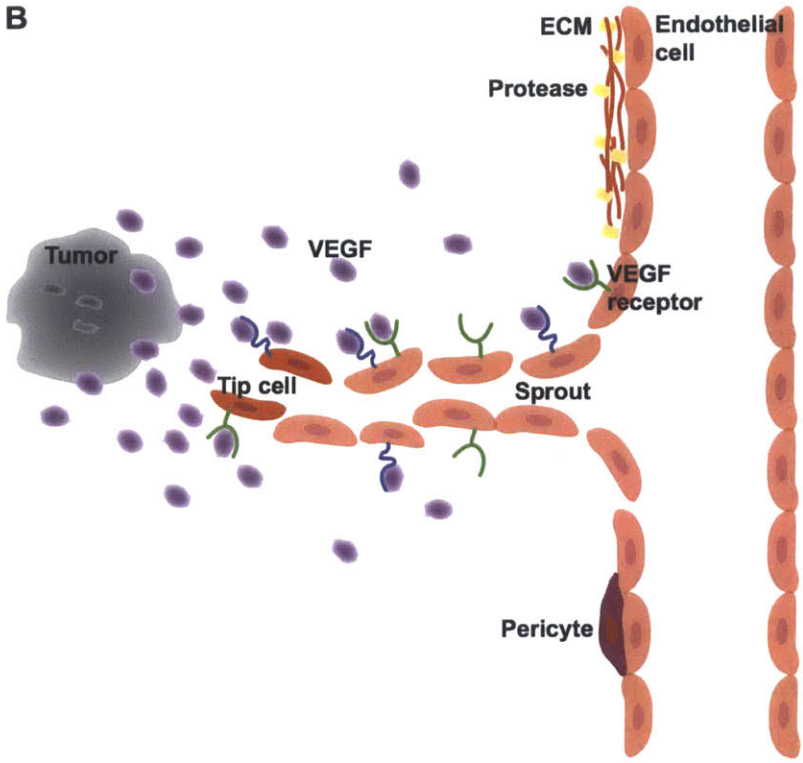

Figure 1.1B Schematic of tumor angiogenesis.

Although the cause of the malformed microvessels of tumors is not fully understood, one potential cause is deregulation of the highly oriented, directional CE cell movements that mediate normal capillary ingrowth during physiological angiogenesis (Lamalice et al., 2007; Li et al., 2005; Rousseau et al., 1997). Most past approaches to the development of therapeutic modulators of angiogenesis have focused on suppressing VEGF activity, VEGF receptor activity and CE cell proliferation (Carmeliet and Jain, 2000; Dvorak, 2002; Ferrara et al., 2004; Ferrara and Kerbel, 2005; Folkman, 1971; Jain, 2005; Kerbel and Folkman, 2002; Kim et al., 1993). While this effort has led to new FDA-approved cancer therapeutics, drug tolerance and side effects have been observed, which has led to recognition of the need to identify new angiogenesis pathways as alternative targets for drug intervention (Chung and Ferrara, 2011). Thus, in this dissertation, I focus on how directional migration of endothelial cells is controlled, and explore whether it is deregulated in tumor angiogenesis.

1.2 CONTROL OF DIRECTIONAL CELL MIGRATION

Directional motility of CE cell migration during angiogenesis is regulated by three types of cues: soluble factors, ECM components and physical forces (Bhowmick et al., 2004; Ingber, 2002; Kalluri and Zeisberg, 2006; Petrie et al., 2009; Tlsty and Coussens, 2006). Each of these factors has been shown to independently modulate cell migration speed, direction and persistence in *in vitro* assays (Ingber, 2002).

Soluble motility factors

Most of the work in this field has focused on analyzing the role of soluble factors in directing CE cell migration. VEGF, basic FGF (b-FGF), platelet-derived endothelial cell growth factor (PDGF), transforming growth factor-b (TGFb) have all been shown to stimulate CE cell migration (Lamalice et al., 2007).

The ubiquitous angiogenic factor, VEGF, is also one of the most potent motogens and it plays a key role in controlling CE cell migration (Chung and Ferrara, 2011; Koch et al., 2011; Lohela et al., 2009). VEGF and other growth factor gradients drive endothelial cell migration in a process known as chemotaxis (Lamalice et al., 2007). VEGF is secreted by endothelial cells, tumor cells and stromal cells (e.g. fibroblasts, monocytes and platelets) (Dong et al., 2004; Hlatky et al., 1994; Kut et al., 2007; Pietras et al., 2008) in the tumor microenvironment, and tumor-associated endothelial cells over-express VEGF receptors (Chung and Ferrara, 2011). VEGF and b-FGF produced by migrating cells also can feed back to increase the expression and activation of several integrins involved in angiogenesis (Byzova et al., 2000).

VEGF and its receptors are predominant in the tumor environment, whereas there is a balance between multiple types of angiogenic modulators during physiological angiogenesis (Jain, 2003). As a result, the tumor microenvironment presents altered CE cell migratory stimuli, which lead to microvessel abnormalities including increased tortuosity, fragility, lack of

stabilizing pericytes, propensity for leaks, and high interstitial pressure (Nagy et al., 2009; Nagy et al., 2007).

VEGFR2 is the key receptor that VEGF utilizes to stimulate CE cell migration (Chung and Ferrara, 2011; Koch et al., 2011; Lamalice et al., 2007; Lohela et al., 2009). Upon VEGF binding to VEGFR2, VEGFR2 tyrosine kinase activity conveys migratory signals (Lamalice et al., 2007) and mitogenic, chemotactic and pro-survival cell activities (Ferrara et al., 2003), which are mediated by multiple downstream signaling molecules including FAK (focal adhesion kinase), Cdc42, phosphatidylinositol-3 kinase (PI3K), p38, Rho, Rac and ROCK (Lamalice et al., 2007). In particular, Cdc42 is involved in the formation of filopodia, which are migratory and sensory bodies that guide microvessel sprouts in the retina (Gerhardt et al., 2003b) and Rac is involved in the formation of migratory protrusions called lamellipodia. RhoA stimulates rho-associated kinase (ROCK) and myosin-dependent tension generation in the actin cytoskeleton, which regulates physical interactions between cells and the ECM by promoting stress fiber formation, stimulating FA assembly and increasing traction forces that cells apply to their ECM adhesions (Brock and Ingber, 2005; Gardel et al., 2010; Ridley, 2011). RhoA also mediates stimulation of VEGFR2 phosphorylation and activates PI3K, which regulates actin-binding proteins such as profilin, cofilin and α-actinin (Lamalice et al., 2007).

Transcriptional control of VEGFR2 is complex (Mammoto et al., 2009) and it is over-expressed by many tumors (Chung and Ferrara, 2011) thereby contributing to tumor angiogenesis (Chung and Ferrara, 2011). For example, stress-induced changes in the capillary cell cytoskeleton *in vitro* (Mammoto et al., 2007) and ECM stiffness *in vivo* control the activity of the RhoA regulator, p190RhoGAP, which regulates VEGFR2 expression by tipping the balance between two opposing transcription factors, GATA2 and TFII-I (Mammoto et al., 2009), that control its expression (Jackson et al., 2005; Minami et al., 2004; Minami et al., 2001; Roy, 2001).

While most work in the VEGF signaling field has focused on the role of VEGFR2, VEGF also binds to cell surface Neuropilin (NRP) 1 & 2 receptors. Binding of VEGF to neuropilins alters the activities of intracellular signaling molecules that are central to migratory control, including Cdc42, Rho, ROCK, FAK, PI3K, and p38 MAP kinase (Chung and Ferrara, 2011; Koch and Claesson-Welsh, 2012; Lohela et al., 2009; Rousseau et al., 1997). Through this mechanism, the neuropilins are able to promote endothelial cell survival and migration in response to VEGF (Favier et al., 2006; Takashima et al., 2002).

However, the neuropilins also bind to semaphorin 3 (SEMA3), a chemorepulsive cytokine family that normally suppresses motile process formation and inhibits endothelial cell movement (Bielenberg et al., 2004; Bielenberg et al., 2006; Carmeliet, 2005; Geretti et al., 2008). In fact, VEGF-A and SEMA3F competitively bind to NRP2 thereby eliciting antagonistic effects on endothelial cell migration (Geretti et al., 2008). NRP2-mediated chemorepulsion of CE cells through SEMA3F is mediated by inactivation of RhoA, which is necessary for actin stress fiber formation and cytoskeletal tension generation (Shimizu et al., 2008). Interestingly, this is disrupted during tumor progression (Bielenberg et al., 2004) and is accompanied by increased ingrowth of malformed capillaries. Decreased NRP2 expression also suppresses lymphatic vessel development (Yuan et al., 2002), embryonic angiogenesis (Takashima et al., 2002) and retinal neovascularization in the presence of ischemia (Shen et al., 2004). Also, NRP2 mediates neuronal chemorepulsion (Chen et al., 2000; Ellis, 2006; Gaur et al., 2009; Giger et al., 2000; Guttmann-Raviv et al., 2006; Klagsbrun and Eichmann, 2005; Takashima et al., 2002). Therefore, in this dissertation, I explore whether VEGF, VEGFR2 and NRP2 contribute to control of directed motility of CE cells.

Control of directional motility by insoluble adhesive cues

Similar to chemotactic cytokines, ECM components such as collagen, laminin and fibrin also can drive endothelial cell migration (Davis and Senger, 2005) both in the presence or absence of soluble chemoattractants. Matrix metalloproteases secreted by CE cells during angiogenesis initiate motogenic signals from either proteolytic matrix fragments or the release of embedded angiogenic stimuli such as VEGF and b-FGF (Wang et al., 2005).

When encountered by CE cells during sprouting angiogenesis, gradients of ECM components, such as interstitial collagen or fibronectin also can guide cell migration (Smith et al., 2006; Smith et al., 2004) in a process known has haptotaxis in which cells tend to move toward regions coated with higher molecular densities of the insoluble ECM ligand (Jiang et al., 2006; Lamalice et al., 2007). Activation of integrins may be triggered by binding to high densities of ECM molecules, which stimulates FA formation and activates Rac and Cdc42 to induce actin remodeling. FAs formation is important for motility control because it physically couples ECM components to the intracellular contractile actin cytoskeleton through cell surface integrin receptors (Giancotti, 2000; Klemke et al., 1997) that bind to actin-associated molecules, such as FAK, paxillin and vinculin (Gardel et al., 2010; Kanchanawong et al., 2010). Many of the molecules (e.g., small and large G proteins, protein kinases, inositol lipid kinases, ion channels, etc.) that mediate both integrin and growth factor signaling physically associate with the cytoskeletal backbone of the FA (MA et al., 2004). Thus, these anchoring complexes represent sites where these signaling pathways converge and integrate (Plopper et al., 1995) to control behaviors, such as cell movement, and as a result, both angiogenic signal transduction and directional migration can be mediated through changes in FA position or assembly.

Mechanical control of directional motility

Directional cell motility also can be modulated by altering physical

interactions between cells and the extracellular matrix (ECM) to which they adhere, and this mechanical form of motility regulation plays an important role during normal development (Korff and Augustin, 1999; Mammoto et al., 2012; Schaper and Scholz, 2003) as well as tumor formation (Ingber et al., 1981; Lamalice et al., 2007; Paszek et al., 2005). For example, durotaxis is a migratory process in which cells move up or down a gradient of ECM stiffness, which represents variations in the substrates ability to resist cell traction forces (Gray et al., 2003; Lo et al., 2000). Durotaxis is important for normal tissue development, epithelial-to-mesenchymal transitions, cancer metastasis (Paszek et al., 2005) and angiogenesis (Ingber and Folkman, 1989). Rigidity mechanosensing is also critical for integrin-dependent processes such as proliferation and differentiation (Ingber and Folkman, 1989), FA formation, contractility and cell polarization (Jiang et al., 2006). It also might be important for angiogesis; for example, CE cells in a region of basement membrane thinning will experience increased tension in their FAs, and this could induce them to extend migratory processes and sprouts in the direction of the pull (Ingber, 2002). Importantly, studies on durotaxis in other cell types have revealed that knockdown or phosphorylation mutations of the FA protein paxillin abrogates durotaxis without interfering with chemotaxis (Plotnikov et al., 2012; Polacheck et al., 2014; Sero et al., 2011).

Work from the Ingber laboratory where I performed my dissertation research showed that directional cell motility is controlled by altering mechanical forces that are balanced across cell surface integrin receptors (Parker et al., 2002; Xia et al., 2008). Because of this molecular bridge, forces applied to the ECM are transmitted to the cytoskeleton, and cytoskeletal traction forces are exerted on the ECM. For this reason, changes in the ability of the ECM to physically resist cell contractile forces will result in changes in cytoskeletal tension and alterations of cell shape. This is important because changes of endothelial cell shape produced by altering physical interactions between cells and ECM alters cell sensitivity to soluble growth factors, and switch cells between different fates (growth, differentiation, and apoptosis) *in vitro* (Chen et

al., 1997; Ingber, 1990; Ingber and Folkman, 1989); it also controls both expression of VEGFR2 and the level of angiogenesis produced *in vivo* (Mammoto et al., 2009).

To examine the role of physical interactions between cells and ECM in regulating cell migration, the Ingber laboratory developed a microengineering technique to microcontact print single cell-sized, ECM islands on which individual cells could be cultured, surrounded by non-adhesive regions. By altering the geometry of the island (e.g., circular versus square), cells can be induced to take on the shapes of the different islands, and this results in local stress concentrations in the corners of the square cells (Parker et al., 2002). FA formation has been shown to be sensitive to the level of force applied to integrins (Balaban et al., 2001), and for this reason, FAs form preferentially in the corner regions of square cells, whereas there is no bias in round cells. Importantly, when fibroblasts adherent to the square islands were stimulated with motility factors (e.g., PDGF), they preferentially extended new motile processes (lamellipodia and filipodia) from their corners, whereas again there was no detectable orientation in round cells (Brock and Ingber, 2005; Parker et al., 2002).

Subsequent work demonstrated that oriented cell spreading and migration occur through stress-dependent control of FA formation at specific locations, and spatial localization of Rac signaling at sites of new FA formation (Xia et al., 2008). Most pertinent for this dissertation is that reduction in expression of the FA protein, paxillin, prevented directional protrusion of new motile processes in fibroblast cells cultured on square islands, suggesting that the spatial coupling of cytoskeletal stress and motile process formation that drives directional cell migration is mediated by paxillin (Sero et al., 2011).

1.3 PAXILLIN AND MOTILITY

Paxillin is a multifunctional, multidomain FA protein that is phosphorylated by Src and FAK (Tachibana et al., 1995; Turner et al., 1990), and associates with many other FA proteins and signaling molecules (Brown and Turner, 2004; Hagel et al., 2002; Ishibe et al., 2004; Turner et al., 1990). Paxillin also can be found in ruffling edges (lamellipodia) of migrating cells as well as in the cytosol. Paxillin can transport to the nucleus in complex with other proteins to regulate cell functions (Sen et al., 2012) and it controls mRNA localization to lamellipodia (Woods et al., 2002). Knockdown of paxillin causes defects in embryonic stem cell spreading (Wade et al., 2002) and fibroblast adhesion (Webb et al., 2004), while it enhances HeLa cell migration (Yano et al., 2004) and paxillin knockout mice are embryonic lethal because of developmental defects in the mesoderm (Hagel et al., 2002). Expression of paxillin is regulated by growth factors such as heregulin (HRG) in breast cancer cells (Vadlamudi et al., 1999) and TGF-β in endothelial cells (Walsh JE and Young MR, 2010). In general, it is largely unknown what growth factors, other molecules or transcription factors control paxillin expression.

As described above, paxillin was found to be a critical regulator of directional movement in fibroblasts (Sero et al., 2011). These studies revealed that paxillin knockout fibroblasts do not exhibit spatial coupling between FA formation and lamellipodia extensions on square ECM islands (Sero et al., 2011). Paxillin exerts control of protrusion localization by regulating Rho and Rac recruitment to FAs and thereby modulating actin polymerization at these sites (Brock and Ingber, 2005). Specifically, paxillin controls RhoA activity by its association with p190RhoGAP (Tsubouchi et al., 2002). Also, paxillin knockdown decreased the persistence of directional migration in two-dimensional (2D) cultures and increased migration in a 3D *in vitro* assay in studies with fibroblasts (Sero et al., 2011). In fact, paxillin can positively and negatively regulate cell migration depending on whether it is located at the leading edge (Nayal et al., 2006) or tail end (Nishiya et al., 2005) of the cell.

Paxillin also has been shown to mediate protrusion formation in response to applied ECM stresses (e.g. ECM stresses generated by interstitial fluid pressure gradients) in 3D cultures of breast adenoma cells and fibroblasts (Polacheck et al., 2014; Sero et al., 2012; Sero et al., 2011). This is important because protrusion formation is a critical step in directed cell migration (Plotnikov et al., 2012), and high interstitial pressure is present in the tumor environment as a result of leakiness of microvessels (Jain, 1996). Also, tumors exhibit different mechanical properties relative to normal tissue, including increased ECM stiffness (Levental et al., 2009; Mammoto et al., 2013; Polacheck et al., 2013). Tumor endothelial cells, therefore, are exposed to both chemokines and ECM stresses, and changes in paxillin expression could be critical in integrating these cues to determine the directionality of endothelial cell migration.

1.4 PAXILLIN AND CANCER

Paxillin is mutated and misexpressed in many cancers (Deakin et al., 2012) including breast (Madan et al., 2006; Short et al., 2007), lung (Jagadeeswaran et al., 2008; Salgia et al., 1999) and melanoma (Eskandarpour et al., 2009; Velasco-Velázquez et al., 2008), and it is a target of oncogenes such as v-Src, c-MET, papilloma virus E6 oncoprotein and BCR-Abl (Ma et al., 2003; Salgia et al., 1995; Tong and Howley, 1997; Wade et al., 2008). Paxillin levels are decreased in lung, breast and colorectal tumors, among others (Deakin et al., 2012; Salgia et al., 1999; Yang et al., 2010). For example, paxillin expression and activation are inversely correlated with positive lymph nodes and lymphatic invasion in breast carcinoma (Madan et al., 2006). Also, decreased paxillin expression is correlated with worse clinical outcome in *HER2* amplified breast cancers, where the opposite was found in *HER2* non-amplified tumors (Short et al., 2007). This seems to be controversial as others studies show that paxillin expression is elevated in lung, prostate, breast, cervical and other tumors (Deakin et al., 2012; Jagadeeswaran et al., 2008; Mackinnon et al., 2011; Salgia et al., 1999; Sen et

al., 2012). Similarly, down regulation of paxillin expression is associated with decreased invasion (Eskandarpour et al., 2009) and metastasis (Velasco-Velázquez et al., 2008). Mutations in the paxillin gene in cancer cells have also been reported (Jagadeeswaran et al., 2008). In addition to tumor cells, where paxillin expression is shown to be up- or down-regulated (Deakin et al., 2012), the essential roles of tumor stromal cells, including fibroblasts, immune cells and endothelial cells could be controlled by paxillin as well. Given these complexities, the precise role of paxillin expression and/or activity in disease is unclear. It is possible, however, that paxillin's role may be cell type-specific and biological context-specific.

1.5 THESIS AIMS

Past studies from my mentor's (Donald Ingber's) laboratory, and the studies reviewed above, suggest that paxillin plays a critical role in integrating soluble and physical cues to elicit directed cell motility in fibroblasts. Paxillin is also expressed in CE cells, it closely associates with actin stress fibers under shear stress (Hu and Chien, 2007), and it is phosphorylated in response to VEGF treatment over short time periods (Abedi and Zachary, 1997). These observations led to my **working hypothesis** that paxillin plays a central role in control of directional CE cell motility, and that it might be deregulated in tumor angiogenesis where tight control of these processes is lost. As the mechanism by which paxillin might exert control over CE cell migration has never been explored in the context of normal or tumor angiogenesis, pursuing this mechanism formed the basis of my dissertation research.

Thus, in Chapter 2, I explore whether paxillin is required for directional migration of CE cells. I present results of studies utilizing siRNA transfection to modulate the expression of paxillin in human umbilical vein endothelial (HUVE) cells in scratch wound healing assays and Transwell migration studies carried

out in the presence of soluble angiogenic factors (e.g. VEGF, β-FGF, PDGF) *in vitro*. Studies involving quantification of directional migration using fluorescence microscopy and computerized image analysis are also described, as well as the results of knocking down paxillin *in vivo* in Matrigel plugs implanted subcutaneously in C57BL/6 mice (Mammoto et al., 2009) and in a neonatal mouse retinal angiogenesis assay. In these studies, immunohistochemistry (IHC) and computerized image analysis are used to quantify directional capillary ingrowth and microvessel network formation. These studies revealed that suppression of paxillin expression results in enhanced endothelial cell invasion *in vitro* and *in vivo*, and increases microvessel density *in vivo* in Matrigel plugs and in neonatal retina during development. Furthermore, paxillin knockdown decreased directional persistence of endothelial cells *in vitro* and disrupted sprout orientation in growing microvessel networks in the neonatal retina.

In Chapter 3, I carry out additional studies to analyze the mechanism by which paxillin controls CE cell migration. The effects of modulating paxillin expression using siRNA or DNA transfection on expression of VEGF receptors (VEGFR2, NRP2) in cultured HUVE cells or in CE cells in Matrigel plugs are measured by immunoblotting, RT-qPCR and IHC image analysis. The VEGF receptor NRP2 is shown to mediate the effects of altering paxillin expression on endothelial cell migration and invasion *in vitro* and *in vivo*, and results are presented that suggest changes in the intracellular localization of the angiogenesis transcription factor GATA2 mediate these effects.

Then in Chapter 4, I explore whether this paxillin-dependent motility control mechanism contributes to tumor angiogenesis=. I show that addition of either soluble factors produced by lung tumor cells or purified VEGF inhibit expression of paxillin by both cultured endothelial cells and ingrowing CE cells within Matrigel plugs *in vivo.* Moreover, these tumor-derived angiogenic factors also decrease expression of NRP2, and this is accompanied by acceleration of CE cell migration and enhanced microvessel network formation. Importantly, all of these effects can be reversed by over-expressing paxillin.

Finally, in Chapter 5, I discuss the implications of these findings and research directions that might be pursued in the future, including studies that might bring these findings closer to a therapeutic context. I also discuss the generality of the paxillin-NRP2 pathway beyond angiogenesis and tumor angiogenesis.

CHAPTER 2: PAXILLIN CONTROLS ENDOTHELIAL CELL MIGRATION AND ANGIOGENESIS

INTRODUCTION

Our recent finding that paxillin regulates directional cell migration in fibroblasts (Sero et al., 2011) led us to examine whether paxillin also controls oriented migration of endothelial cells, which is required for capillary network formation during angiogenesis. In fact, in CE cells, paxillin becomes rapidly phosphorylated in response to VEGF stimulation (Abedi and Zachary, 1997) and closely associates with actin stress fibers under shear stress (Hu and Chien, 2007). Here we utilize a variety of assays *in vitro* and *in vivo* to probe endothelial cell migration, invasion, directional persistence and speed. Additionally, we utilize assays *in vitro* and *in vivo* to determine the role of paxillin in endothelial cell chord formation, developmental angiogenesis and microvessel network formation.

RESULTS

2.1 Paxillin controls endothelial cell migration in vitro

Human umbilical vein endothelial (HUVE) cell migration in response to paxillin gene suppression was analyzed in Transwell migration chambers using EBM2 supplemented with bFGF, VEGF, IGF, EGF and 5% serum. Treatment with two distinct siRNA sequences against paxillin resulted in knockdown of paxillin expression by ~90% as confirmed by immunoblotting (Fig. 2.1 A). Furthermore, quantitative real time-PCR (Fig. 2.1 B, $p<0.01$) confirmed that siRNA against paxillin reduced paxillin mRNA levels. Paxillin knockdown (pax kd) cells exhibited about a 2-fold increase in migration after 24 hours when compared with control cells transfected with scrambled siRNA (Fig. 2.1 C, $p<0.05$).

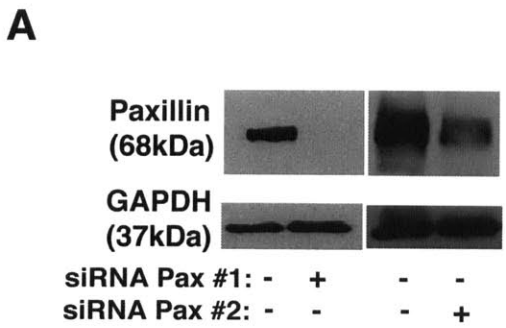

Figure 2.1A Immunoblots showing paxillin and GAPDH protein levels in HUVE cells transfected with control or paxillin-targeted siRNA.

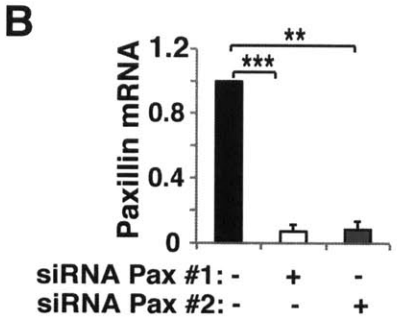

Figure 2.1B Graph showing mRNA level of paxillin in HUVE cells treated with control or paxillin siRNA (**, p<0.01,***, p<0.001). Data are represented as mean +/- s.e.m.

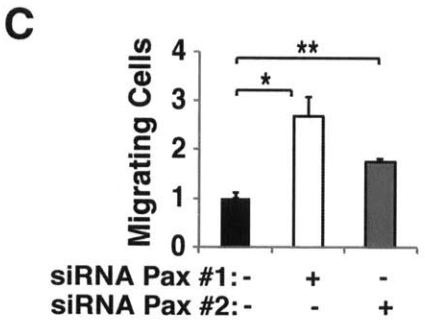

Figure 2.2C Graph showing the number of migrating HUVE cells transfected with control or paxillin siRNA normalized by control siRNA transfected cells in Transwell migration assay (*, p<0.05, **, p<0.01). The migratory stimulus is 5% serum EGM2. Data are represented as mean +/- s.e.m.

When the Transwell chambers were coated with Matrigel to create a 3D ECM invasion assay (Sero et al., 2011), the pax kd cells again displayed about a

2-fold increase in the average distance they invaded into gel relative to control cells (Fig. 2.1 D, $p<0.05$), which is consistent with increases in directional cell migration produced by knocking out paxillin in mouse embryonic fibroblasts (Sero et al., 2011). These data suggest that paxillin expression may be required for control of the normal processes of directional endothelial cell migration and invasion that underlie capillary network formation.

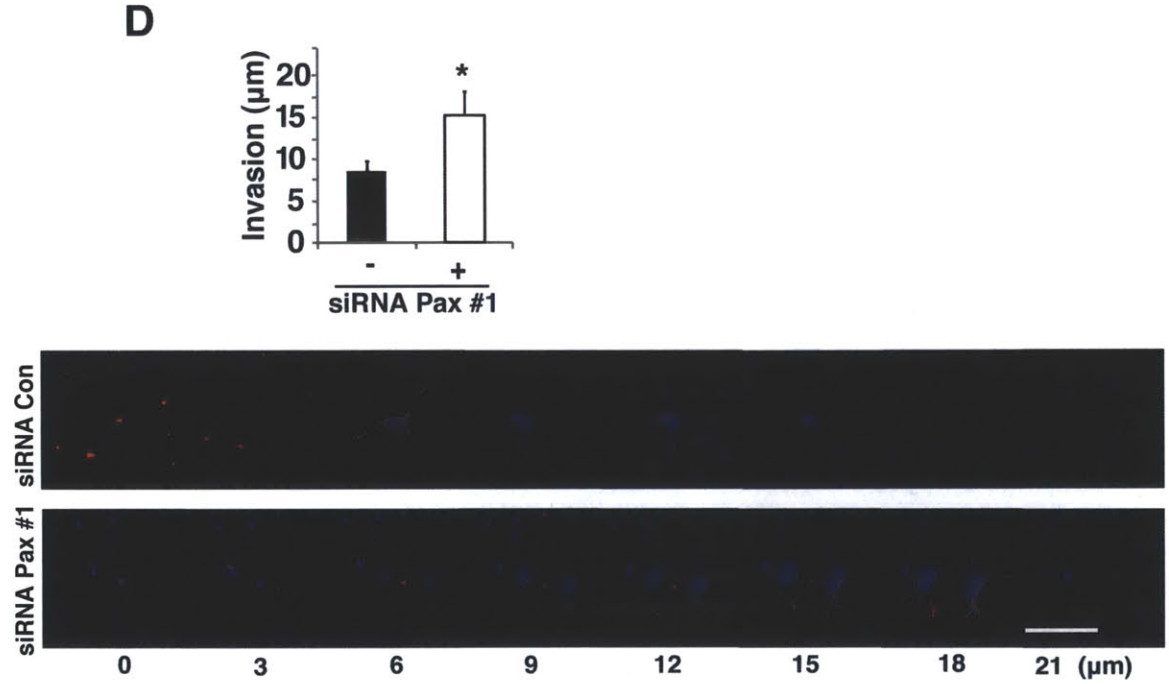

Figure 2.1D Graph showing the distance of invasion of HUVE cells transfected with control or paxillin siRNA in Transwell invasion assay (*, $p<0.05$). The migratory stimulus is 5% serum EGM2. Confocal micrographs showing HUVE cell invasion into Matrigel transfected with control or paxillin siRNA with 5% EGM2 as chemoattractant. Scale bar; 50 µm. Data are represented as mean +/- s.e.m.

2.2 Paxillin controls endothelial cell migration speed and persistence

Oriented HUVE cell migration was further analyzed using a scratch wound healing assay where confluent endothelial cells are tracked as they migrate to close a scratch wound over 16 hours. I found that pax kd did not change the percentage of wound closure relative to scramble siRNA control (Fig. 2.2 A). Additionally, pax kd cells did not display altered displacement distances into the

wound (Fig. 2.2 B). However, when cell populations were classified as leading edge cells (within 50 μm of the leading edge) or cells within the surrounding monolayer (>50 μm from edge), I found that pax kd decreased displacement of the cells of the monolayer into the wound by 35% (Fig. 2.2 B, $p<0.01$).

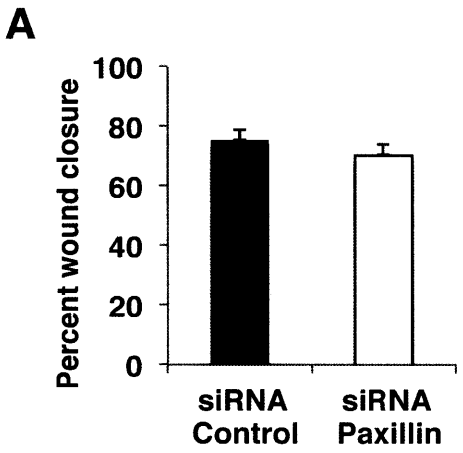

Figure 2.2A Graph showing percentage of HUVE cell wound closure 16 hours after 100 μm scratch wound induction in control versus paxillin-targeted siRNA cell populations. Data are represented as mean +/- s.e.m.

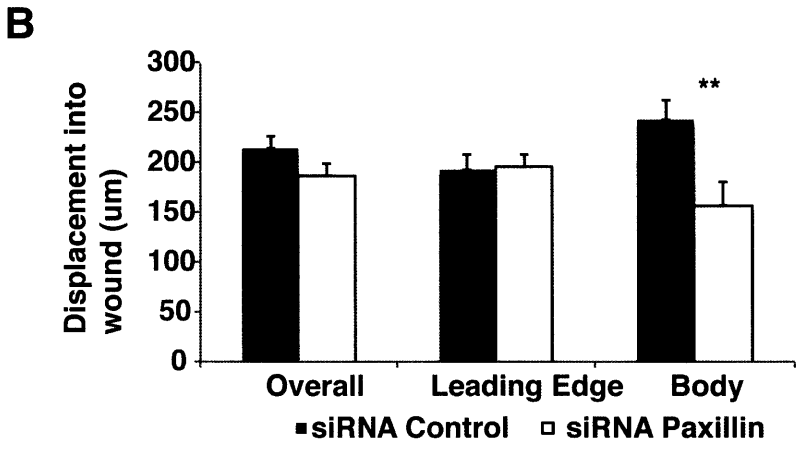

Figure 2.2B Graph showing displacement of HUVE cells after 16 hours of migration into 100 μm scratch wound in control versus paxillin-targeted siRNA cell populations. Cell populations are classified as all cells, leading edge cells (within 50 μm of wound edge) or body cells (> 50 μm from wound edge). **, $p < 0.01$. Data are represented as mean +/- s.e.m.

This indicates that pax kd can alter the migration profile of endothelial cells, and so I looked further at persistence time and migration speed.

Persistence time is a measure of directionality, and can be thought of as the time interval over which a cell's displacement vector is no longer correlated to the previous time interval displacement vector. In tracking individual cells (Fig. 2.2 C) I saw that pax kd cells displayed about a 60% decrease in persistence time (Fig. 2.2 C, $p<0.01$). This was true for both leading edge cells and body cells. Furthermore, pax kd cells displayed a 50% increase in migration speed (Fig. 2.2 D, $p<0.01$). Thus, while pax kd does not globally affect wound healing, it changes the migration properties of endothelial cells in terms of their persistence and migration speed.

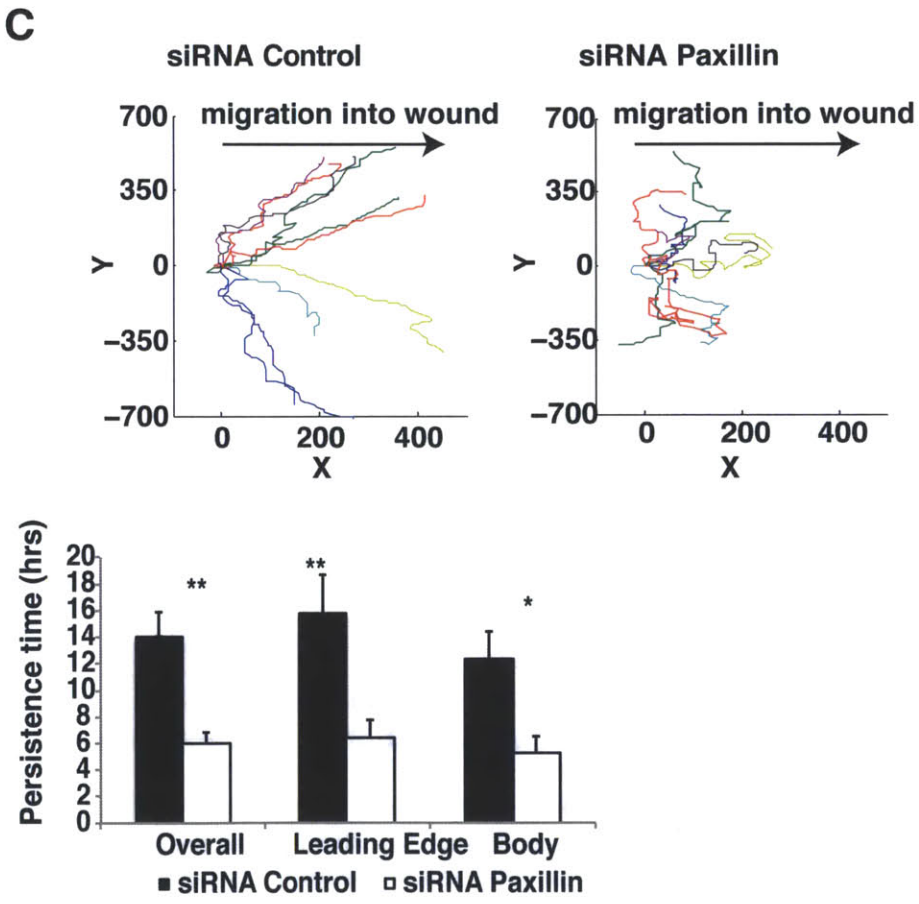

Figure 2.2C Graphs showing representative displacement tracks of HUVE cells migrating for 16 hours into 100 µm scratch wound in control versus paxillin-targeted siRNA cell populations. Bar graph showing persistence time (a measure of directionality) in control- or paxillin-targeted siRNA cells. Cell populations are classified as all cells, leading edge cells (within 50 µm of wound edge) or body cells (> 50 µm from wound edge). *, $p < 0.05$, **, $p < 0.01$. Data are represented as mean +/- s.e.m.

D

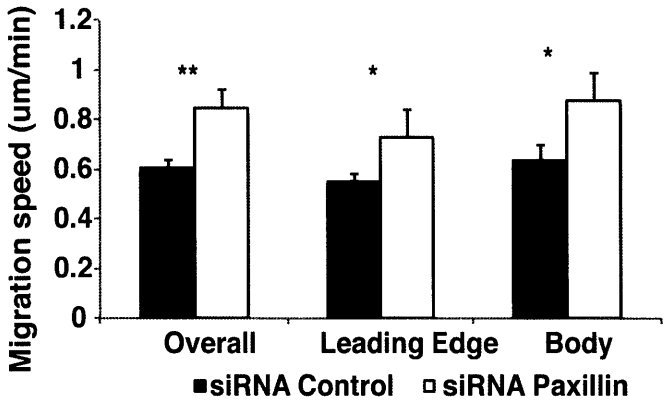

Figure 2.2D Bar graph showing migration speed in control- or paxillin-targeted siRNA cells. Cell populations are classified as all cells, leading edge cells (within 50 μm of wound edge) or body cells (> 50 μm from wound edge). *, $p < 0.05$, **, $p < 0.01$. Data are represented as mean +/- s.e.m.

Next, I assessed the role of paxillin in CE cell differentiation in an *in vitro* tube assay, since CE cell migration is a precursor step for capillary tube formation (Arnaoutova and Kleinman, 2010). Paxillin knockdown severely disrupted histodifferentiation of HUVE cells and decreased the total cord length and number by 40% and 60%, respectively (Fig. 2.2E, $p<0.01$). Taken together, these data indicate that paxillin expression is required for the control of cell migration and invasion, and for the formation of a two dimensional, interconnected EC network *in vitro*.

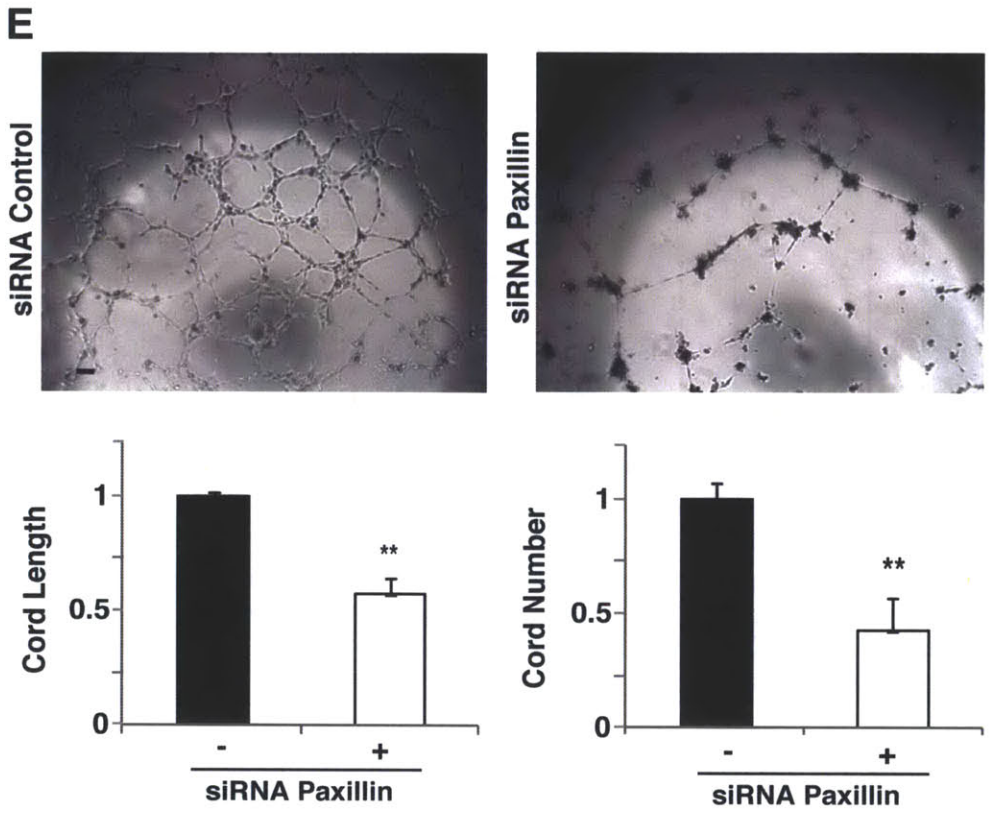

Figure 2.2E Micrographs showing HUVE cell tube and chord formation on Matrigel. Bar graph showing normalized chord length and chord number in control- and paxillin-targeted siRNA cells. **, $p < 0.01$. Scale bar; 50 μm. Data are represented as mean +/- s.e.m.

2.3 Paxillin controls angiogenesis in a Matrigel implantation assay in vivo

One limitation of the in vitro capillary tube formation assay is that it involves reorganization of cords of cells rather than coherent directional cell movement of new sprouts. I therefore explored whether paxillin knockdown also alters CE cell migration, invasion and capillary tube formation *in vivo*. Matrigel plugs were pre-cast in poly-dimethylsiloxane (PDMS) polymer molds and implanted subcutaneously in a mouse (Fig. 2.3A), as previously described (Mammoto et al., 2009).

This implantation model provides multiple advantages. First, the geometric shape of the Matrigel is maintained constant because it is cast in a pre-formed PDMS mold, and this effectively provides a fiducial marker such that

cell migration direction and distance can be assessed after implant harvesting. Second, mRNA from cells that have migrated into the Matrigel can be directly harvested for gene expression determination. Third, the implant can be modified by addition of a second chamber housing a cell suspension. Separation of the chambers by a permeable membrane allows diffusion of growth factors and other chemoattractants, but not migration of implanted cells. The second chamber acts as a soluble factor source, and stimulant for host cell migration. Finally, the stiffness of the Matrigel can be modulated by addition of a crosslinker such as transglutaminase (Mammoto et al., 2009).

In my studies, I injected paxillin or control siRNA (10 μg) into the Matrigel plug after 3 days, and vascular ingrowth was analyzed 4 days later (Fig. 2.3 A). Treating implants with paxillin siRNA decreased paxillin expression in infiltrating CD31+ endothelial cells by 60% as measured by immunofluorescence staining (Figs. 2.3 B, $p<0.05$), and by about 50% in all cells when analyzed by qRT-PCR (Fig. 2.3 C, $p<0.05$).

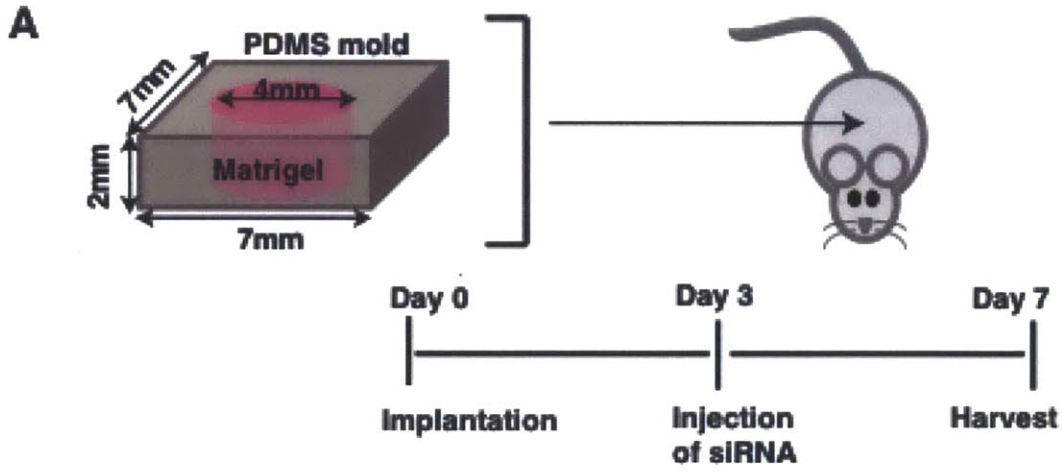

Fig. 2.3A Schematic of Matrigel plug implant and experimental timeline.

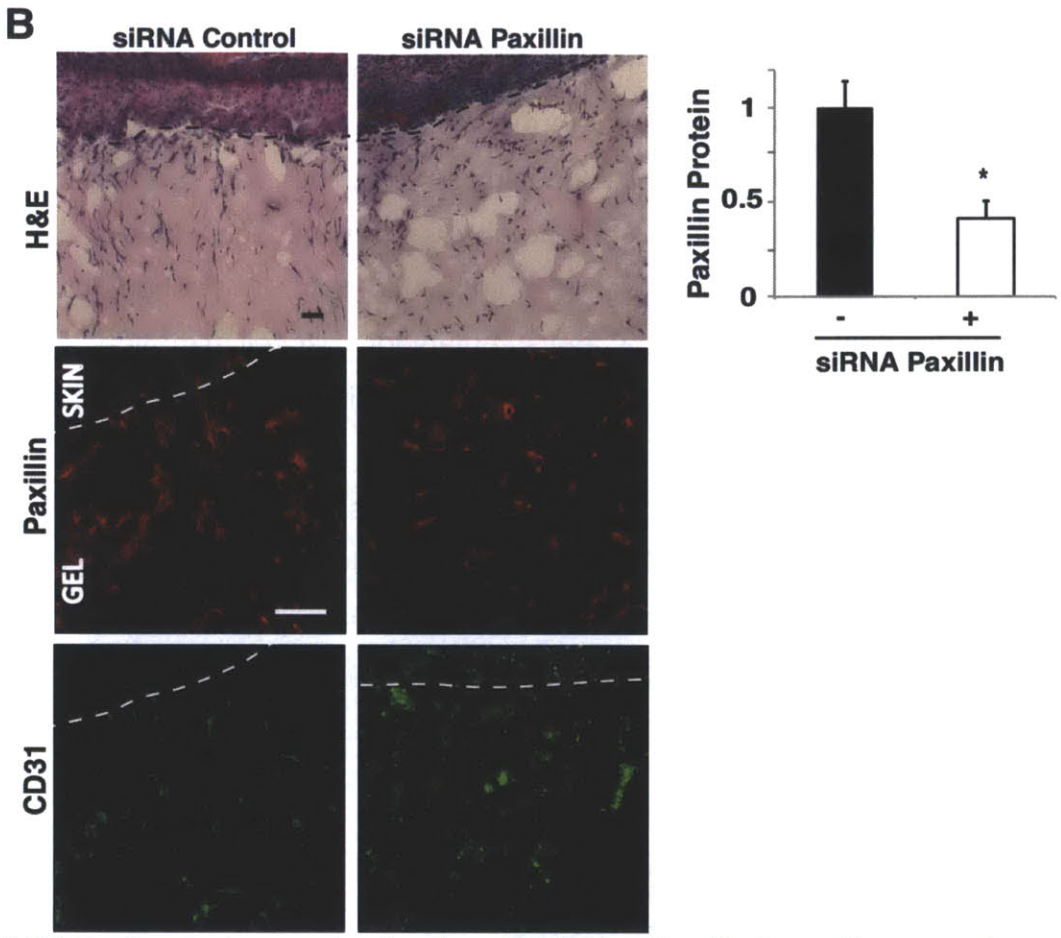

Figure 2.3B Confocal micrographs showing all cell migration (*top*), paxillin expression (*middle*) and CD31 expression (*bottom*) in the Matrigel implanted subcutaneously on the back of C57BL/6 mice treated with control or paxillin siRNA. The region of skin is shown above the dotted line and Matrigel with invading cells is below the dotted line. Scale bars; 50 µm. Graph showing paxillin protein levels quantified via immunohistochemical image analysis where paxillin colocalized to CD31+ cells was measured in 3 random 50x50 µm fields per gel (n=5, *, $p<0.05$). Data are represented as mean +/- s.e.m.

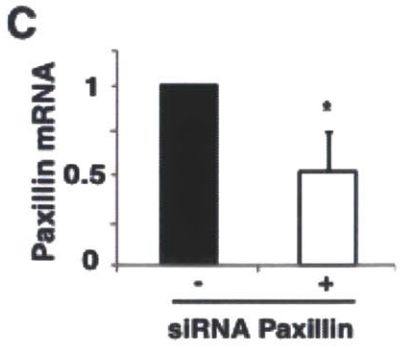

Figure 2.3C Graph showing mRNA level of paxillin in the infiltrated cells in the implanted Matrigel (n=8, *, $p<0.05$). Data are represented as mean +/- s.e.m.

2.4 Paxillin controls capillary endothelial cell migration in vivo

Interestingly, suppression of paxillin expression resulted in approximately a 1.5 fold increase in the number of CD31+ CE cells that invaded into the Matrigel plugs (Fig. 2.4 A, $p<0.05$). Quantification of the number of CE cells at increasing depths through the gel revealed that there were 2- to 8-fold increases in the number of CE cells throughout the depth of gels containing paxillin siRNA compared to control gels (Fig. 2.4 B, $p<0.05$). This increased ingrowth of capillary microvessels was confirmed by perfusing the entire mouse vasculature with fluoresceinated-concanavalin A (conA) via injection into the retro-orbital vein just prior to harvesting the implants, and generating 3D reconstructions (Fig. 2.4 C, $p<0.01$). Thus, knocking down paxillin expression not only increased CE cell ingrowth, it also stimulated formation of new functional microvessels *in vivo*.

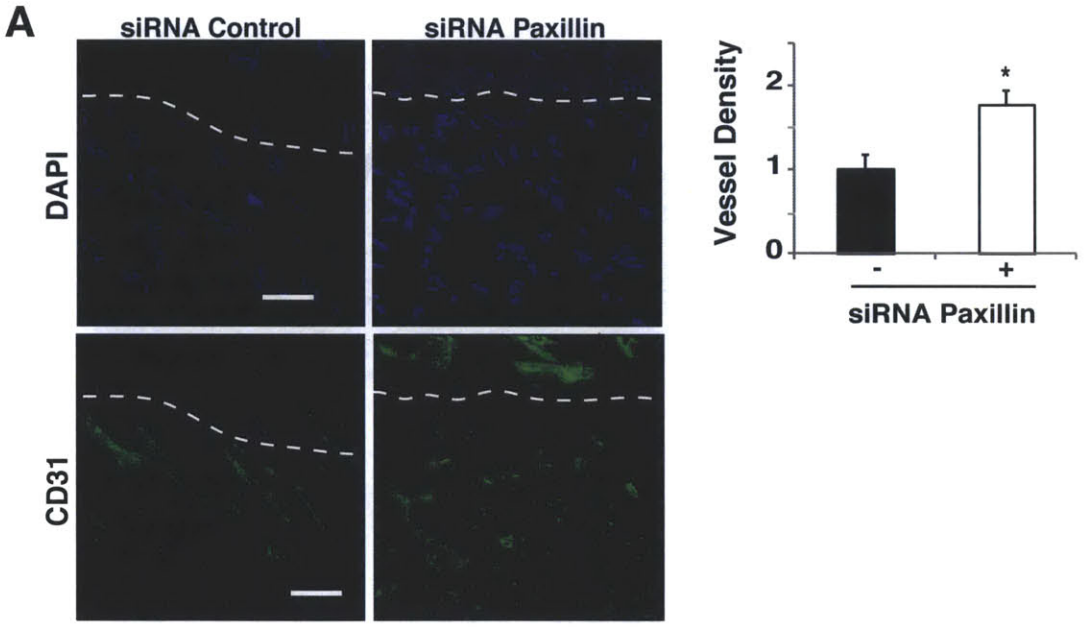

Figure 2.4A DAPI stained micrographs showing host cell migration from C57BL/6 mouse skin into implanted Matrigel treated with or without paxillin siRNA (*top*). Confocal micrographs showing CD31-stained endothelial cells in the Matrigel treated with control or paxillin siRNA (*bottom*). Scale bars; 50 µm. Quantification of endothelial cell density in the implanted Matrigel with or without paxillin siRNA via immunohistochemical CD31 image analysis (n=7, *, $p<0.05$). Data are represented as mean +/- s.e.m.

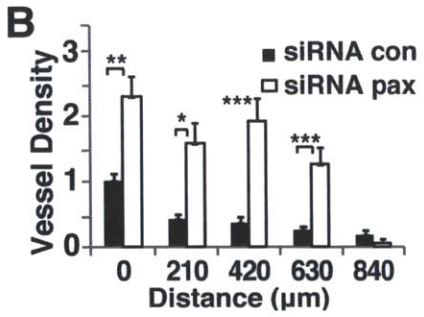

Figure 2.4B Graph showing migration distance of CD31-stained endothelial cells from the skin into Matrigel implant treated with control or paxillin siRNA (n=6, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$). Data are represented as mean +/- s.e.m.

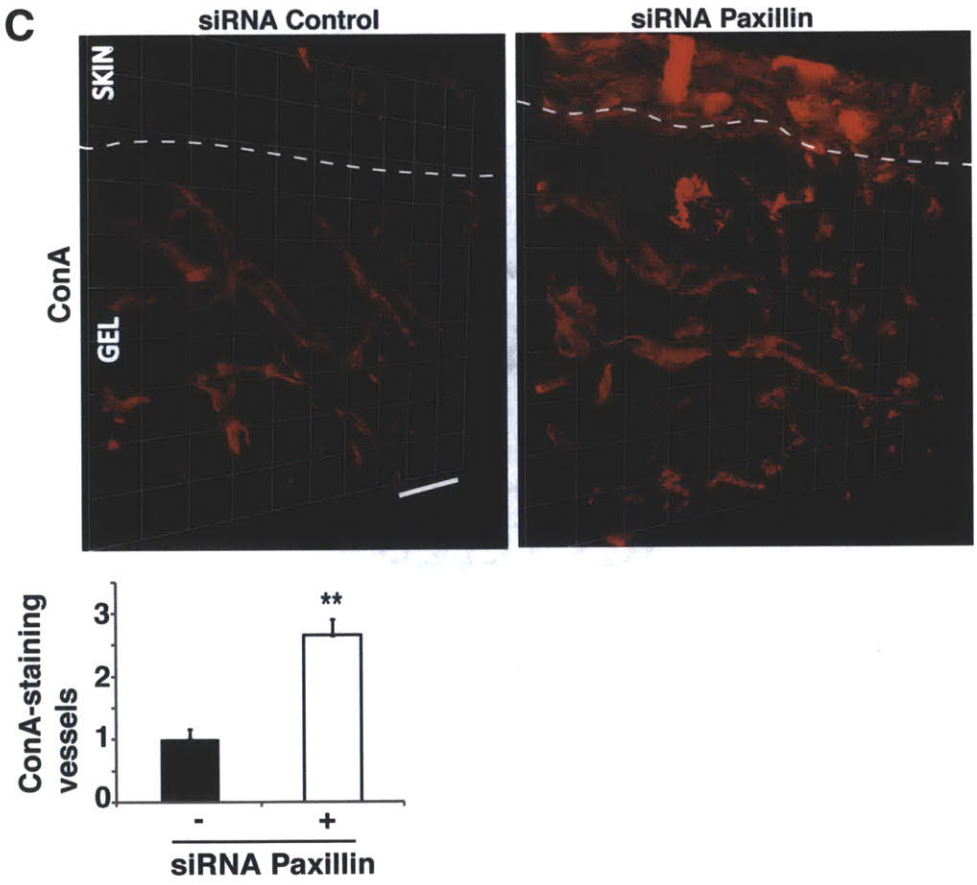

Figure 2.4C 3D reconstructed confocal micrographs of Matrigel implant treated with control or paxillin siRNA perfused with fluorescein-conA. Scale bar; 50 μm. Graph showing endothelial cell migration into Matrigel implants treated with control or paxillin siRNA analyzed using immunohistochemical fluorescein-conA image analysis as measured in 3 random 50x50 μm fields per gel (n=4, **, $p<0.01$). Data are represented as mean +/- s.e.m.

The total number of cells (e.g., immune cells, fibroblasts and CE cells) migrating into the Matrigel plug did not vary significantly between control and paxillin siRNA-treated implants (Fig. 2.4 D). Suppression of paxillin also did not alter the number of CD45+ immune cells that invaded the gels (Fig. 2.4 E); however, it significantly decreased the number of invading fibroblasts (Fig. 2.4. F, $p<0.05$). Thus, it appears that paxillin suppression has different effects on the migration of different cell types *in vivo*.

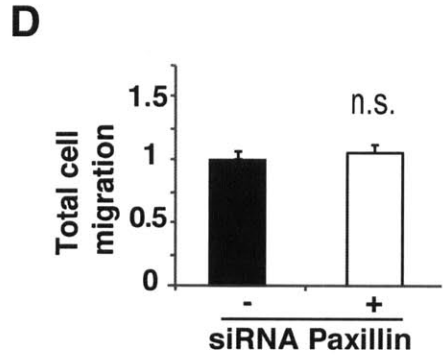

Figure 2.4D Graph showing quantification of total cell migration into Matrigel implant treated with control or paxillin siRNA (n=7). Data are represented as mean +/- s.e.m.

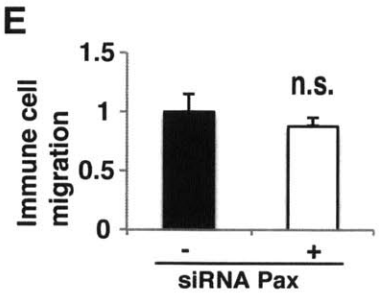

Figure 2.4E Graph showing quantification of immune cell migration into Matrigel implant treated with control or paxillin siRNA. Data are represented as mean +/- s.e.m.

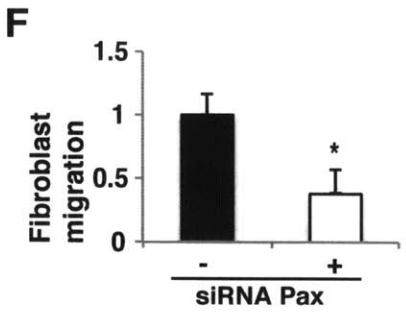

Figure 2.4F Graph showing quantification of fibroblast migration into Matrigel implant treated with control or paxillin siRNA (n=4, *, $p<0.05$). Data are represented as mean +/- s.e.m.

2.5 Paxillin controls developmental angiogenesis

I then examined whether paxillin is important for control of normal developmental angiogenesis by analyzing the effects of paxillin knockdown during mouse neonatal retinal angiogenesis *in vivo*. This is a relevant model system because directional cell migration is required for new microvessel formation in the growing retina (Gerhardt et al., 2003a; Wacker and Gerhardt, 2011). Specifically, as the retina develops, astrocytes and neuronal precursors spread and migrate radially away from existing blood vessels where they then encounter hypoxia. Subsequent production of VEGF, and an associated VEGF gradient, stimulates growth of new vessels toward the astrocytes by promoting oriented migration of endothelial cells. Decreased hypoxia leads to decreased VEGF production and an eventual stabilization of the vessels (Neufeld et al., 1999).

Paxillin siRNA (0.5 µg) was injected directly into the vitreous humor of a postnatal day 4 (P4) mouse eye, the eye was harvested 2 days later and the vessels were visualized by staining with fluorescent isolectin. Knockdown of paxillin resulted in increased sprout formation throughout the forming vascular network (Fig. 2.5 A, $p<0.001$). To determine if pax kd has other effects on the histodifferentiation of the vascular network, we quantified sprout length and found no change between control siRNA treated eyes and paxillin siRNA treated eyes (Fig. 2.5 B). However, when we measured the sprout orientation relative to the

edge of the retina, we found that pax kd significantly ($p<0.001$) reduced the percentage of sprouts oriented within 20 degrees of the line perpendicular to the retinal edge (Fig. 2.5 C) while there was no change in the sprout angle relative to the parent vessel (Fig. 2.5 D).

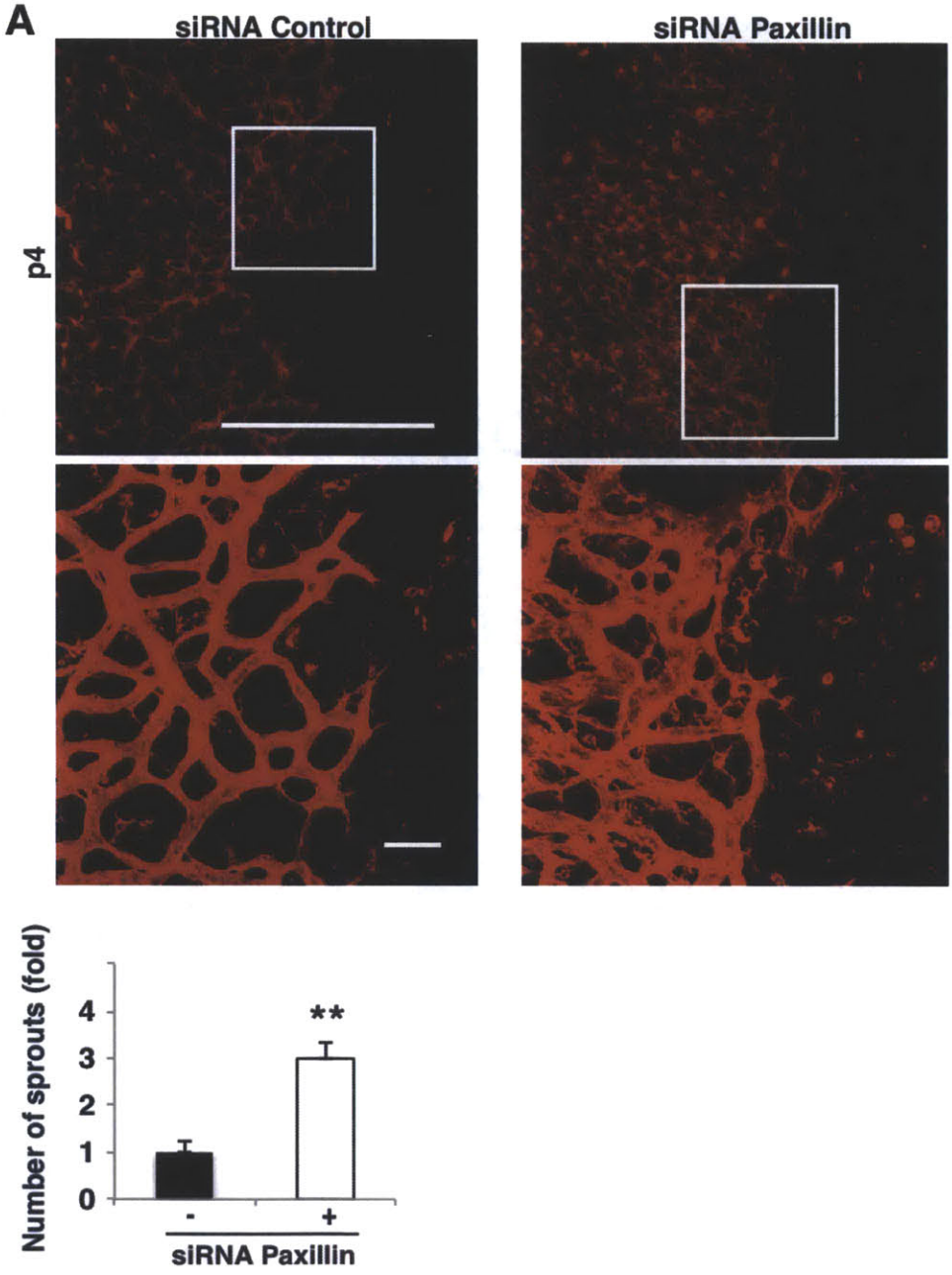

Figure 2.5A Confocal images of isolectin-stained retina from P6 neonatal mouse 2 days post intravitreous injection of control or paxillin siRNA. Scale bar *(top)*; 0.5 mm, scale bar; *(bottom)* 50 µm. Normalized average number of sprouts per 400x200 µm retinal area with control or paxillin siRNA (n=3, **, $p < 0.001$). Data are represented as mean +/- s.e.m.

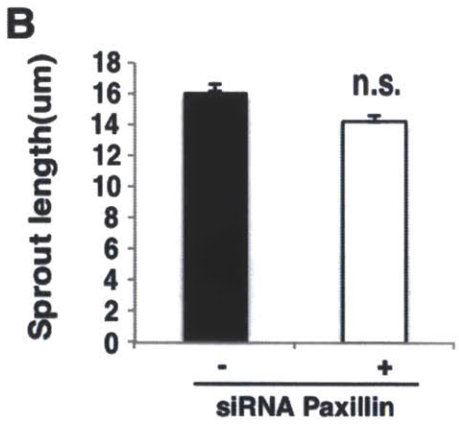

Figure 2.5B Average neonatal retina sprout length with control or paxillin siRNA (n=3). Data are represented as mean +/- s.e.m.

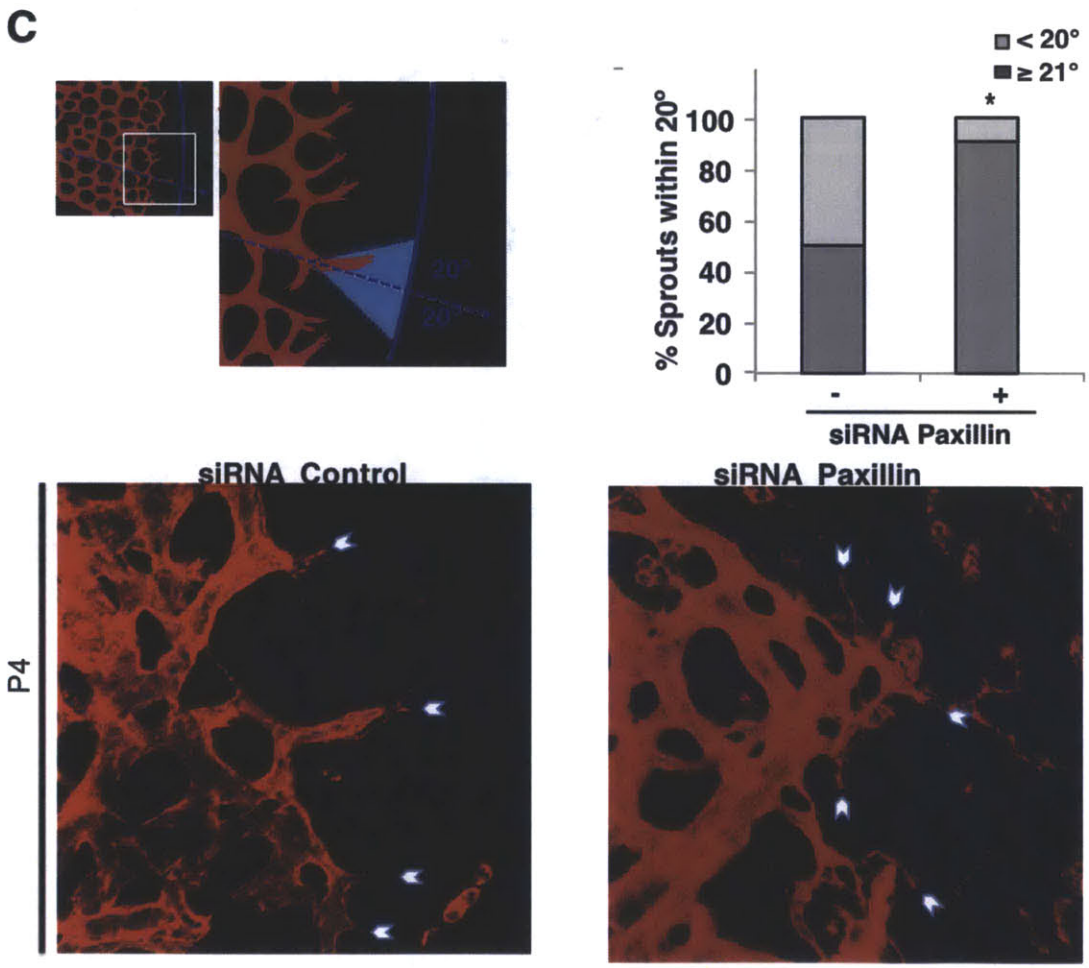

Figure 2.5C Diagram of sprout orientation quantification. Dark blue lines represent retina edge arc and vector perpendicular to this arc. Graph is percentage of sprouts within 20 degrees of a line perpendicular to the retina edge with control or paxillin siRNA (n=3, *, $p<0.001$). Confocal images of isolectin-stained retina from P6 neonatal mouse 2 days post intravitreous injection of control or paxillin siRNA. White arrowheads demark sprouts and sprout orientation.

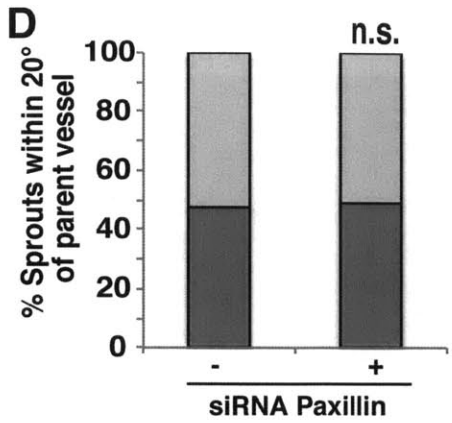

Figure 2.5D Graph showing percentage of neonatal retina sprouts oriented within 20 degrees of parent vessel with control or paxillin siRNA (n=3).

We also studied the retinal vascular network at P8 when most of the vessels reach the edge of the retina. Knockdown of paxillin resulted in increased formation of tortuous microvessels, as indicated by a 1.5-fold increase in vessel density compared to control siRNA treated eyes (Fig. 2.5 E, $p<0.05$). Thus, suppression of paxillin expression appears to result in enhanced migration *in vitro* as well as increased but less oriented microvascular ingrowth *in vivo* during normal vessel development.

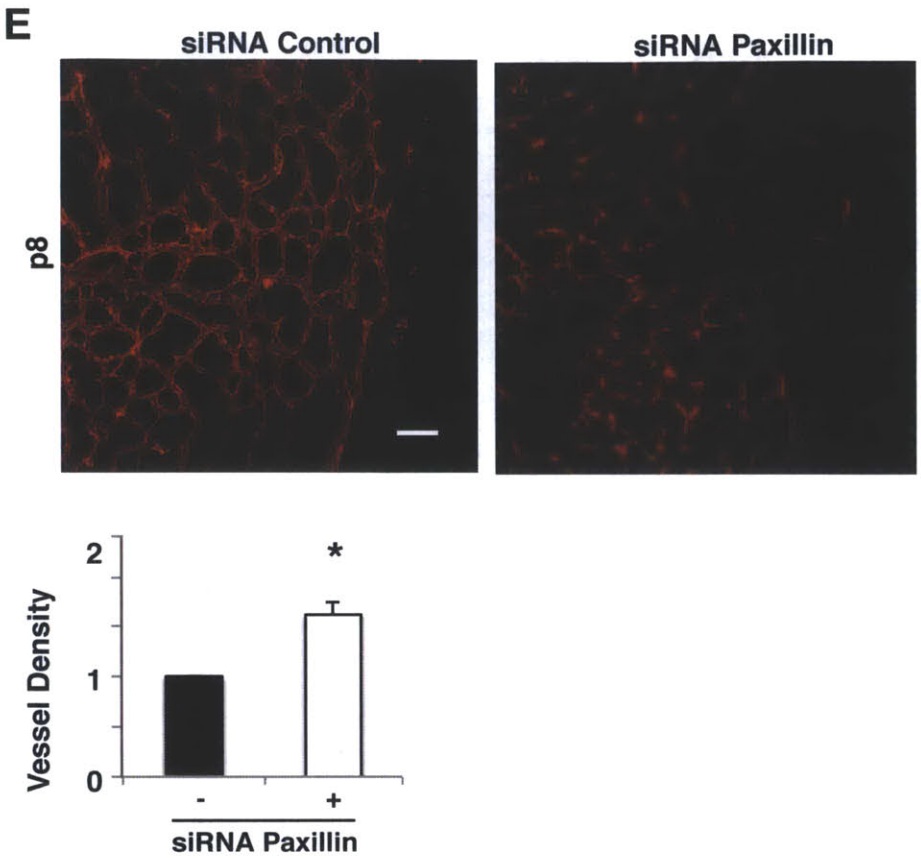

Figure 2.5E Confocal images of isolectin-stained retina from P10 neonatal mouse 2 days post intravitreous injection of control or paxillin siRNA. Scale bar; 0.1 mm. Graph showing the vessel density of peripheral region of retina treated with paxillin siRNA normalized with that from control siRNA treated retina (n=6, *, $p < 0.05$). Data are represented as mean +/- s.e.m.

2.3 DISCUSSION

Paxillin suppression enhances endothelial cell invasion *in vitro* and *in vivo* and subsequently increases microvessel density *in vivo* in Matrigel plugs and in developmental angiogenesis in the mouse neonatal retina. Similar to previous studies by our laboratory using fibroblasts (Sero et al., 2011), we showed that paxillin knockdown also decreases endothelial cell migration persistence, and this random migration phenotype results in a loss of sprout orientation *in vivo* in the developing retina microvessel network.

The mechanism by which paxillin knockdown results in enhanced microvascular network formation remains unclear. We previously showed that paxillin is not required for FA formation or overall cell migration in fibroblasts, but that it is required for tension sensing, proper localization of both FA and lamellipodia, and directional migration (Sero et al., 2011). Thus, suppression of paxillin, which is required for directional cell motility, appears to result in an inability of endothelial cells to properly localize FA remodeling and motile processes, resulting in haphazard and enhanced migration as observed in the tumor vasculature. Our wound healing studies of directional migration of endothelial cells *in vitro* involved collective cell monolayer migration where cells experience both anchorage forces to the ECM in addition to cell-cell adhesion forces (Friedl and Alexander, 2011; Friedl and Wolf, 2010; Trepat and Fredberg, 2011). However during single cell migration as in our invasion assay *in vitro* and Matrigel implantation model *in vivo*, cells may not experience reaction forces from adhesions to other cells (Friedl and Alexander, 2011; Friedl and Wolf, 2010; Trepat and Fredberg, 2011). Therefore, it is important to consider that since paxillin integrates mechanical inputs to localize FAs and lamelipodia and direct migration (Plotnikov et al., 2012; Polacheck et al., 2014; Sero et al., 2011), there may be differences in regulation depending on the presence or absence of adjacent cells. Furthermore, microenvironmental properties such as ECM density, ECM stiffness and proteases are also affected by adjacent cells and can therefore dictate paxillin-dependent independent or monolayer cell migration.

The molecular mechanism by which paxillin enhances cell migration remains unknown. It is known is that Tyr 31/118 phosphorylated-paxillin competes with p190RhoGAP for binding to p120RasGAP (Tsubouchi et al., 2002), and that unbound p190RhoGAP inhibits RhoA thereby effectively allowing cell polarization, membrane protrusion and cell elongation (Arthur and Burridge, 2001). p190RhoGAP was recently found to control angiogenesis and expression of VEGFR2 by controlling the balance between nuclear transport of two opposing transcription factors—GATA2 and TFII-I (Mammoto et al., 2009). Thus,

modulation of paxillin expression could indirectly alter interactions between p190RhoGAP and RhoA, and thereby modulate cell migration regulation, particularly in endothelial cells.

Mechanical forces also contribute to control of capillary cell growth, migration and fate determination (Mammoto and Ingber, 2009; Mammoto et al., 2012), and changes in mechanical forces elicited by altering ECM stiffness can regulate angiogenesis through p190RhoGAP (Mammoto et al., 2009). Since paxillin mediates force-dependent control of cell behaviors (Sawada and Sheetz, 2002; Zaidel-Bar et al., 2007), including directional cell motility (Sero et al., 2011), cell-derived mechanical forces may contribute to the control of angiogenesis through paxillin as well. In fact, a recently study in collaboration with our laboratory found that knockdown of paxillin in breast adenocarcinoma cells stimulated by 3D interstitial flow stresses resulted in enhanced formation of membrane protrusions that lacked polarization or oriented localization of vinculin and actin (Polacheck et al., 2014). Paxillin knockdown resulted in a loss of "rheotaxis" or flow-induced upstream migration with no observable change in migration speed. This, therefore, provides supporting evidence for a paxillin-dependent mechanism by which FA activation leads to oriented protrusion formation and migration in 3D porous scaffolds.

We found that paxillin knockdown enhances developmental angiogenesis by increasing the number of capillary sprouts in the forming retina. A similar increase in sprout formation has been seen with loss of DLL4/Notch signaling (Blanco and Gerhardt, 2013). Notch signaling is an attractive clinical target (Noguera-Troise et al., 2006) since inhibition of Notch signaling in tumor endothelial cells can restore sensitivity of tumors to anti-VEGF therapy. Tip-cell versus stalk-cell establishment in endothelial cells has been shown to be regulated by VEGF and DLL4/Notch signaling mediated by VEGFR2 and the neuropilins (Blanco and Gerhardt, 2013). As evidenced by our neonatal retinal angiogenesis studies, apparently paxillin also can control the tip- versus stalk-cell switch. Loss of NRP2 subsequent to paxillin knockdown (as we will show in

Chapter 3) could lead to inhibition of VEGF/VEGFR2-mediated DLL4 production and therefore increase sprouts and predominance of tip-cell status. Similarly, while total HUVE cell displacement into the wound in our scratch wound healing assay was not different in control versus paxillin knockdown cells, we did see decreased displacement of non-leading edge cells (persistence time and migration speed were both similar in leading edge and surrounding cell monolayer populations). This could indicate that paxillin knockdown changes the propensity for leading edge and trailing cells alike to turn on a tip-cell-like program which upregulates migration, and changes expression of proteins such as VEGFR2 (Blanco and Gerhardt, 2013) compared with the stalk-cell-like program that promotes adhesion and proliferation.

When we specifically studied the characteristics of endothelial cell migration, we found that while knocking down paxillin expression decreased persistence, it enhanced migration speed and ultimately there was no difference in the extent of wound closure between control and paxillin knockdown cells. One possible explanation is that paxillin is critical for controlling both directional persistence and migration speed. This appears to be endothelial cell-specific as paxillin knockdown was not found to control migration speed in mouse embryonic fibroblasts (Sero et al., 2011) or breast tumor epithelial cells (Polacheck et al., 2014). Alternatively, it is possible that paxillin only controls directional persistence, as has been shown (Plotnikov et al., 2012; Polacheck et al., 2014; Sero et al., 2011), and that endothelial cells upregulate migration speed as a compensatory mechanism during wound healing.

Our results probing the effects of paxillin knockdown on different cell types *in vivo* support past studies that showed paxillin can either promote or inhibit migration (Nayal et al., 2006; Nishiya et al., 2005). As described, in Matrigel implanted subcutaneously in mice, knocking down expression of paxillin enhanced endothelial cell migration but, paradoxically, it did not change total cell migration. When we analyzed the effects on different cell types *in vivo*, we found that paxillin knockdown did not change immune cell migration, and it suppressed

fibroblast migration. It is likely that the increased endothelial cell migration we observed was offset by this decrease in fibroblast migration, which is what led us to see no change in total cell migration in paxillin knockdown implants. Therefore, paxillin seems to have differential effects depending on the cell type.

Our lab previously showed that knocking down paxillin increased PDGF-stimulated fibroblast invasion in a 3D *in vitro* model, which conflicts with our *in vivo* findings here. This highlights how paxillin can have differential effects on cell migration depending on the experimental conditions. In fact, fibroblasts could display varying sensitivity to growth factors or ratios of growth factors, which ultimately determines the migration phenotype. In our *in vivo* system, fibroblasts integrate a number of chemical and mechanical signals, whereas an *in vitro* system with only one growth factor allowed us to probe specifically PDGF-driven, paxillin-dependent migration. Our studies suggest that paxillin-mediated control of migration is cell type specific and that it can either promote or inhibit migration depending on the cell analyzed.

Taken together, these studies show that paxillin controls both the degree of endothelial cell migration and specific migratory properties (e.g., persistence, speed), resulting in enhanced but deregulated microvessel networks *in vivo*. To determine the molecular mechanism through which paxillin elicits control of angiogenesis, I then carried out studies to explore whether paxillin controls expression of VEGF receptors, as these are important regulators of CE cell migration and guidance. The results of these studies are described in Chapter 3.

2.4 MATERIALS AND METHODS

Materials

Anti-paxillin monoclonal antibody was from Abcam (Cambridge, MA, USA), anti-CD31 monoclonal antibody was from BD Biosceinces (San Diego,

CA, USA), anti-SMA monoclonal antibody was from Epitomics (Burlingame, CA, USA), anti-GAPDH antibody was from Millipore (Billericka, MA, USA) and Alexa Fluor 488 phalliodin was from Invitrogen. rhVEGF was from R&D Systems (Minneapolis, MN, USA).

Cell Culture

HUVE cells (Lonza, Walkersville MD, USA) were cultured in EBM2 (Lonza, Walkersville MD, USA) medium with 5% FBS and growth factors (VEGF, b-FGF, PDGF) (Lonza, Walkersville MD, USA). Knockdown of paxillin (Ambion, Austin, TX, USA) was performed with siRNA (Table 4) and transfected into cells using siLentFect transfection reagent (Bio-Rad, Hercules, CA, USA). An siRNA duplex with irrelevant sequence served as a control (Ambion, Austin TX, USA). Effects of knockdown were confirmed via qRT-PCR and Western blotting at 48-72 hours after transfection.

Molecular Biology Assays

RNA was purified using RNeasy mini kit (Qiagen, Valencia, CA, USA). qRT-PCR was performed with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) and iTaq Sybr Green Supermix (Bio-Rad, Hercules, CA, USA) using CFX96 real-time PCR machine (Bio-Rad, Hercules, CA, USA). β2 microglobulin or cyclophilin controlled for complementary DNA content. Primers are described in Table 5.

Cell Biological methods

Transwell Migration Assays

$6x10^5$ HUVE cells/ml in 0.5% EBM2 were cultured on a 1% gelatin (Sigma, St. Louis, MI, USA)-coated membrane (Corning) and placed in a well with a chemoattractant (5% serum EGM2) (Mammoto et al., 2009). Cells were stained

with Giemsa solution (Sigma, St. Louis, MI, USA) 24hrs later and counted in 4 random fields with 3 or more independent experiments.

Transwell Invasion Assay

Matrigel was added to an 8μm-pore Transwell insert (Corning). $3x10^6$ cells/ml in 5% EGM2 were cultured on the underside of an inverted insert for 2 hours, the insert was then placed in a well with chemoattractant (described above) and incubated for 24 hours (Sero et al., 2011). Fixed inserts were incubated with Matrigel Immunofluorescence Wash Buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20) and stained with Alexa Fluor phalliodin (Invitrogen, Carlsbad, CA, USA) and DAPI. Images were taken at 25°C on a Leica TCS SP2 confocal laser scanning microscope at excitation 405 and 594 with a 63x/1.40 oil objective using Leica confocal software 2.61 at 3μm z-plane slices through the depth of the Matrigel and image analysis was performed with ImageJ software. Immunocytochemical image analysis was performed by measuring the depth of the center of the cell.

Wound Healing Assay

Confluent monolayers of siRNA-transfected HUVE cells were "scratched" with a 200 μl pipette tip and imaged immediately after in a live cell imaging chamber (37C, 5% C02) for >16 hours at 10 min intervals. Cells were tracked manually and persistence time, migration speed (Lauffenburger and Horwitz, 1996), wound closure was analyzed using Matlab software (Mathworks, Natick, MA, USA).

Tube Formation Assay

100 μl Matrigel was added to 24-well flat bottom plate wells. Previously transfected HUVE cells at $6x10^4$ were added to cured Matrigel and allowed to incubate at 37C for 6-8 hours. Bright field images at 10x, 20x were taken.

In vivo Matrigel Plug Assay

All animal studies were reviewed and approved by the Animal Care and Use Committee at Boston Children's Hospital. Matrigel plugs were cast in 7x7x2 mm PDMS molds and implanted subcutaneously on the back of a C57BL/6 mouse (Mammoto et al., 2009) (Fig. 2.3 A). 10 µg of siRNA paxillin or scramble control siRNA was injected locally at day 3 after implantation. Fluorescein-conA (Vector Laboratories, Burlingame, CA, USA) was injected into the retro-orbital vein prior to harvesting at day 7. Implants were fixed, frozen, sectioned and stained with hematoxylin and eosin (H&E), anti-CD31, paxillin, NRP2, VEGFR2 and DAPI antibodies. H&E stained images were taken on a Nikon Eclipse E600 microscope, fluorescent images were taken at 25°C on a Leica TCS SP2 confocal laser scanning microscope at excitation 405, 488 and/or 594 with a 63x/1.40 or 40x/1.25 oil objective and Leica confocal software version 2.61. Immunohistochemical vessel density analysis was performed by quantifying the expression of CD31 and fluorescent conA per three different 50x50 µm areas of Matrigel for more than three independent implants using ImageJ software. In brief, projected z-stack images of 30 µm thick gel sections stained with CD31 or perfused with fluorescent ConA were binarized and positively-staining areas were quantified using ImageJ software. Paxillin expression was measured when colocalized with CD31+ or ConA+ cells for three 50x50 µm areas of Matrigel for more than 3 implants. Invasion distance was measured by taking images through the depth of the gel, (beginning at the skin line and moving away from the skin) and measuring distance of CD31+ cells using ImageJ software. Migration of immune cells and fibroblasts was quantified by counting the number of CD45+ cells and smooth muscle actin (SMA+/CD31-) cells, respectively, in 50x50 µm areas of Matrigel for more than three independent implants using ImageJ software. Stacks of 5-20 immunohistochemical images (1-3 µm between images) were complied to form 3D images using Volocity 4.4 Software (Improvision).

In vivo Retinal Vessel Formation

0.5 µg of paxillin siRNA was injected intravitreally into one eye of a P4 or P8 neonatal C57BL/6 mouse and control siRNA was injected into the other eye. The eyeball was harvested 2 days later. Flat mounted, fixed tissues were stained with fluorescein-conjugated isolectin and imaged (Mammoto et al., 2009) at 25°C using a Leica TCS SP2 confocal laser scanning microscope at excitation 594 with a 5x air or 20x/0.70 oil objective, and Leica confocal software 2.61. Stacks of 5-20 images were complied with Volocity 4.4 Software as above. Vessel density was quantified using Adobe Photoshop as described above. Isolectin-staining sprouts within 100 µm of the retina edge were counted using ImageJ.

Statistical Analysis

All experiments were repeated independently three or more times and data are represented as mean +/- s.e.m. Statistical analyses to assess the difference between control and treatment groups were performed with Matlab R2012b software (Mathworks, Natick, MA, USA) using the unpaired Student's *t* test.

CHAPTER 3: PAXILLIN CONTROLS NEUROPILIN-2 EXPRESSION

INTRODUCTION

VEGF is one of the most ubiquitous angiogenic factors and a potent endothelial cell motility factor. In Chapter 2, I found that paxillin knockdown altered capillary development during retinal angiogenesis, which is driven by a VEGF gradient. Thus, to determine the mechanism through which paxillin exerts control over endothelial cell migration and angiogenesis, in this Aim I set out to explore whether paxillin modulates the expression of VEGF receptors.

VEGF controls CE cell migration by binding its receptors, VEGFR2, NRP1 and NRP2 (Chung and Ferrara, 2011; Koch et al., 2011; Lohela et al., 2009) (Favier et al., 2006; Takashima et al., 2002). VEGFR2 is the main receptor VEGF utilizes to stimulate angiogenesis, and it also plays a key role in controlling CE cell migration (Chung and Ferrara, 2011; Koch et al., 2011; Lamalice et al., 2007; Lohela et al., 2009) by acting directly on FAK, PI3K, p38, Cdc42, Rho and ROCK. Transcriptional control of VEGFR2 is complex and realized by multiple mechanisms (Mammoto et al., 2009) downstream of soluble and physical stimuli.

The cell surface VEGF receptors NRP1 and NRP2 control endothelial cell survival and migration in response to VEGF. NRP2 mediates binding of Sema3F— the chemorepulsive cytokine that normally suppresses motile process formation and inhibits endothelial cell movement (Bielenberg et al., 2004; Bielenberg et al., 2006; Carmeliet, 2005; Geretti et al., 2008). Interestingly, Sema3F-dependent chemorepulsion of CE cells appears to be disrupted during tumor progression (Bielenberg et al., 2004). NRP2 gene inactivation also suppresses SEMA3F-mediated responses to repulsive guidance events in neurons (Chen et al., 2000; Giger et al., 2000). Not much is known about the regulation of NRP2 expression beyond the recent report that NRP2 expression is controlled by the transcription factor GATA2 (Coma et al., 2013), which also

controls VEGFR2 expression in response to changes in ECM stiffness through modulation of p190RhoGAP activity (Mammoto et al., 2009). GATA2 belongs to a family of C_4 zinc finger transcription factors and it has been shown to control endothelial cell migration (Coma et al., 2013) and vascular integrity (Johnson et al., 2012; Mammoto et al., 2009). GATA2 is also important for lymphangiogenesis during development (Ishida et al., 2012; Kazenwadel et al., 2012; Lim et al., 2012; Orkin, 1992; Ostergaard et al., 2011), proliferation and differentiation of hematopoietic progenitors (Briegel et al., 1993; Kitajima et al., 2002; Tsai et al., 1994; Tsai and Orkin, 1997), and is expressed in endothelial cells where it regulates the promoter activity of PECAM-1 and endothelin (Gumina et al., 1997; Kawana et al., 1995; Lee et al., 1991).

RESULTS

3.1 Paxillin controls neuropilin 2 expression in vitro and in vivo

To understand regulation of directed CE cell motility by paxillin, I explored its effects on regulation of VEGF receptors that control activities of signaling molecules that are central to migratory control (Chung and Ferrara, 2011; Koch and Claesson-Welsh, 2012; Lohela et al., 2009; Rousseau et al., 1997). Specifically, I examined whether paxillin might control expression of the endothelial cell guidance molecule, NRP2 or the key VEGF receptor, VEGFR2. Using siRNA to suppress paxillin expression, I probed the expression of NRP2 in HUVE cells in culture. siRNA-mediated knockdown of paxillin resulted in a 50% decrease in NRP2 expression at both protein and mRNA levels in cultured HUVE cells (Fig. 3.1A, $p<0.05$).

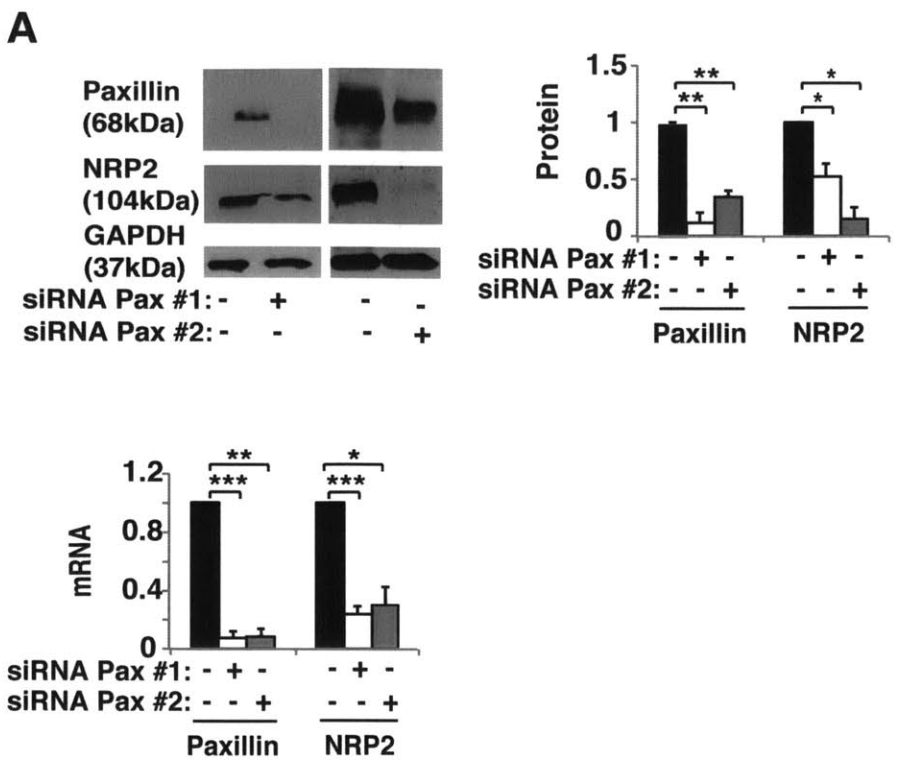

Figure 3.1A Immunoblots of paxillin, NRP2, and GAPDH in HUVE cells treated with control or paxillin siRNA. Graph showing quantification of protein levels of paxillin and NRP2 in HUVE cells treated with control or paxillin siRNA. Graph showing mRNA levels of paxillin and NRP2 in HUVE cells treated with control or paxillin siRNA (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$). Data are represented as mean +/- s.e.m.

To determine whether NRP2 expression changes are induced specifically by the FA protein paxillin, we explored whether other key focal adhesion proteins (vinculin and zyxin) also affect NRP2 expression. These key FA proteins also partake in similar signaling cascades that transduce chemical and mechanical signals into cellular responses. In fact, subsequent to VEGF binding, VEGFR2 initiates phosphorylation of FAK, which recruits and phosphorylates paxillin, which in turn recruits vinculin (Romer et al., 2006). Vinculin is a scaffold protein with no known catalytic activity that binds F-actin, talin, α-actinin, tensin, zyxin and others (Goldmann et al., 2013) and localizes to regions of applied force (Wang et al., 1993). It is critical for cell migration particularly in 3D and vinculin knockdown results in immobility (Mierke et al., 2010; Mitra et al., 2005; Plotnikov

and Waterman, 2013; Schaller, 2001; Schlaepfer and Mitra, 2004; Wang et al., 2001). Zyxin is a zinc-finger-containing FA protein that responds specifically to cyclic stretch by translocating from the FA to the nucleus thereby inducing changes in expression of genes, including endothelin-B receptor, tenascin-C and plasminogen activator inhibitor-1 (Cattaruzza et al., 2004). Zyxin is also required for actin polymerization at FAs after applied stretch (Hirata et al., 2008) and knockdown is associated with enhanced fibroblast migration and invasion (Hoffman et al., 2006). However, neither knockdown of vinculin nor zyxin altered NRP2 expression when measured by protein concentration or mRNA concentration (Figure 3.1B).

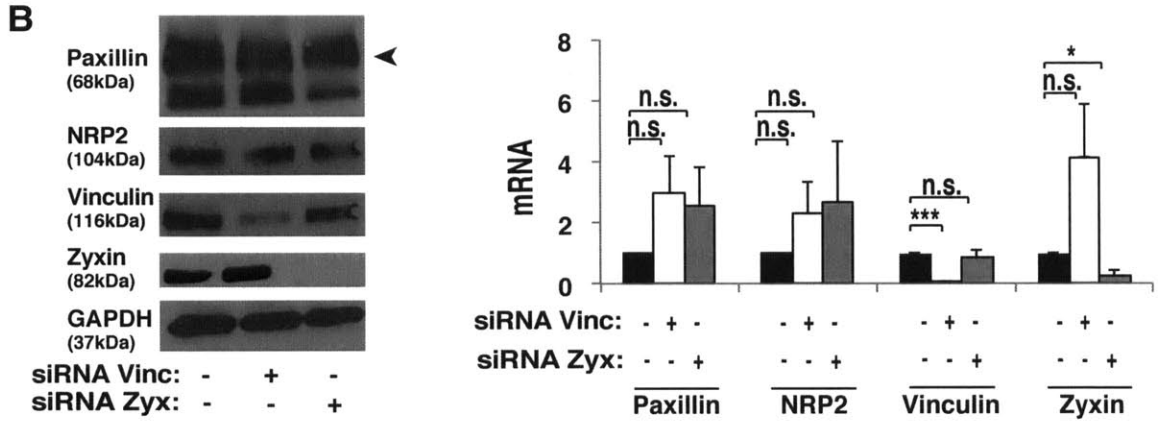

Figure 3.1B Immunoblots showing paxillin, NRP2, vinculin, zyxin and GAPDH expression in HUVE cells treated with vinculin or zyxin siRNA. Graph showing mRNA levels of paxillin, NRP2, vinculin and zyxin in HUVE cells treated with vinculin or zyxin siRNA (***, $p<0.001$, *, $p<0.05$). Data are represented as mean +/- s.e.m.

To determine the physiological relevance of these effects, we measured the expression of NRP2 in CE cells that infiltrated Matrigel plug implants in the Matrigel capillary invasion assay when we knocked down paxillin expression *in vivo*. NRP2 expression was decreased by 40% in CE cells, as measured by image analysis, when paxillin siRNA was injected subcutaneously (Fig. 3.1C, $p<0.05$), and a similar decrease in NRP2 mRNA level was measured by analyzing total mRNA in gel implants using qRT-PCR (Fig. 3.1C, $p<0.001$).

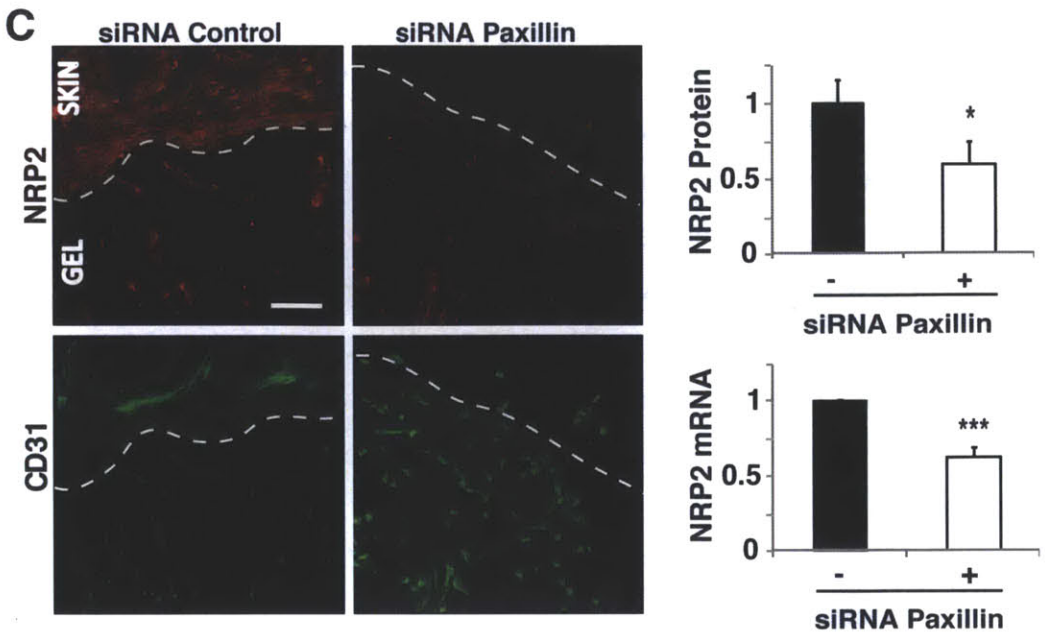

Figure 3.1C Confocal micrographs showing NRP2 expression (*top*) and CD31 stained endothelial cells (*bottom*) in Matrigel implanted subcutaneously on the back of a C57BL/6 mouse treated with control or paxillin siRNA. Scale bar; 50 μm. Graph showing NRP2 protein levels quantified via immunohistochemical image analysis where NRP2 colocalized to CD31+ cells as measured in 3 random 50x50 μm fields per gel (n=5, *, $p<0.05$) *top*. Graph showing mRNA level of NRP2 in the infiltrated cells in the implanted Matrigel (n=8, ***, $p<0.001$) *bottom*. Data are represented as mean +/- s.e.m.

3.2 Paxillin-neuropilin 2 signaling axis controls endothelial cell migration

To confirm that paxillin's control of NRP2 expression is the mechanism through which it controls endothelial cell migration and angiogenesis, we explored whether direct modulation of NRP2 gene expression also changed endothelial cell movement and invasion. When NRP2 was knocked down using siRNA (Fig. 3.2A), HUVE cell migration increased by 1.5-fold (Fig. 3.2B, $p<0.01$). Importantly, over-expression of NRP2, which increases NRP2 mRNA and protein levels in CE cells (Fig. 3.2A), was able to prevent the pax kd-induced increase of HUVE cell migration (Fig. 3.2B). Of note, neither NRP2 knockdown nor its over expression changed the expression of paxillin (Fig. 3.2A). Furthermore, when vinculin or zyxin was knocked down using siRNA, no change was seen in endothelial cell migration (Fig. 3.2C) in a Transwell migration assay. In contrast,

when NRP2 was knocked down, endothelial cell invasion into Matrigel doubled (Fig. 3.2D, $p<0.001$), much as it did when paxillin was knocked down in these cells (Fig. 2.1D). Over-expression of NRP2 plus siRNA mediated pax kd was able to prevent the pax kd-induced increase of HUVE cell invasion (Fig. 3.2D). Thus, NRP2 appears to mediate the effects of paxillin on endothelial cell motility and invasion *in vitro*.

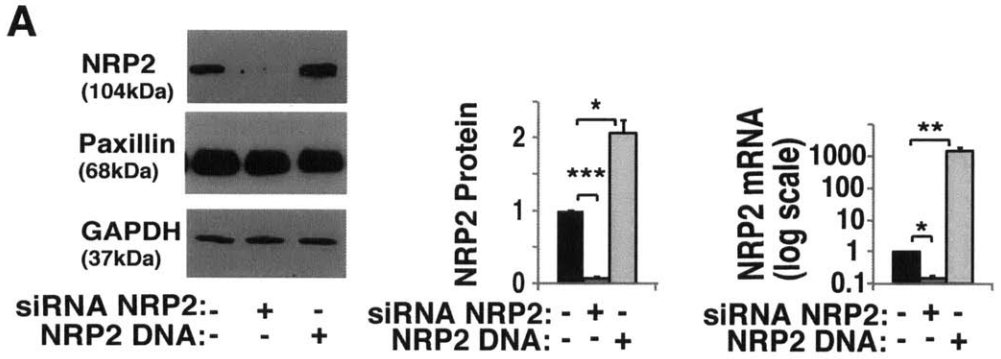

Figure 3.2A Immunoblots showing NRP2, paxillin and GAPDH expression in HUVE cells treated with NRP2 siRNA or NRP2 DNA. Graph showing quantification of immunoblots of NRP2 in HUVE cells treated with NRP2 siRNA or DNA (***, $p<0.001$, *, $p<0.05$) *left*. Graph showing mRNA level of NRP2 in HUVE cells treated with NRP2 siRNA or DNA (**, $p<0.01$, *, $p<0.05$) *right*. Data are represented as mean +/- s.e.m.

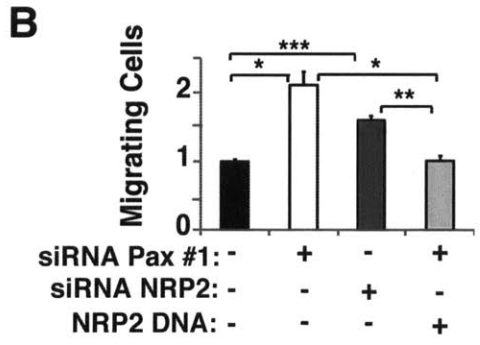

Figure 3.2B Graph showing number of migrating HUVE cells treated with paxillin siRNA, NRP2 siRNA, or paxillin siRNA plus NRP2 DNA normalized by control siRNA transfected cells in Transwell migration assay (*, $p < 0.05$, **, $p < 0.01$, ***, $p<0.001$). Data are represented as mean +/- s.e.m.

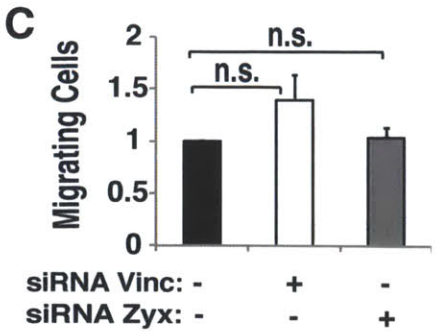

Figure 3.2C Graph showing the number of migrating HUVE cells transfected with control, vinculin or zyxin siRNA normalized to control siRNA-transfected cells in Transwell migration assay. The migratory stimulus is 5% serum EGM2. Data are represented as mean +/- s.e.m.

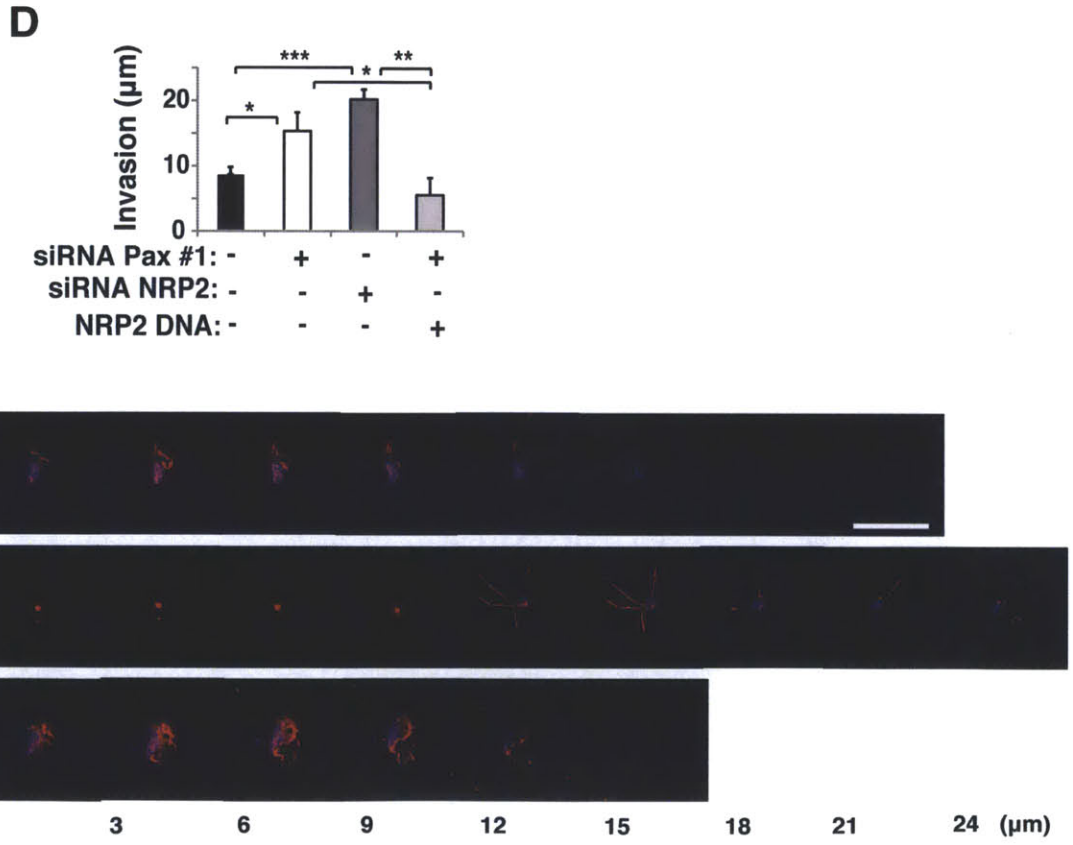

Figure 3.2D Graph showing invasion distance of HUVE cells treated with paxillin siRNA, NRP2 siRNA, or paxillin siRNA plus NRP2 DNA in Transwell invasion assay (*, $p < 0.05$, **, $p < 0.01$, ***, $p<0.001$). The migratory stimulus is 5% serum EGM2. Confocal micrographs showing HUVE cell invasion in Transwell invasion assay with 5% EGM2 as chemoattractant in combination with NRP2 siRNA or paxillin siRNA plus NRP2 DNA. Scale bar; 50 µm. Data are represented as mean +/- s.e.m.

3.3 Paxillin-neuropilin 2 signaling axis controls CE cell migration in vivo

To confirm that the paxillin-NRP2 signaling axis controls CE cell migration *in vivo,* I directly modulated NRP2 expression in Matrigel plugs and measured the subsequent effect on CE cell invasion and microvessel network formation. When I injected NRP2-specific siRNA subcutaneously into Matrigel implanted into mice as described previously, it produced ~ 60% knockdown of NRP2 protein levels in CD31+ CE cells (as measured by immunohistochemical image analysis). As expected, addition of siRNA against NRP2 also decreased NRP2 mRNA levels in all cells that migrated into the implanted Matrigel by 60% (Fig. 3.3A, $p<0.05$).

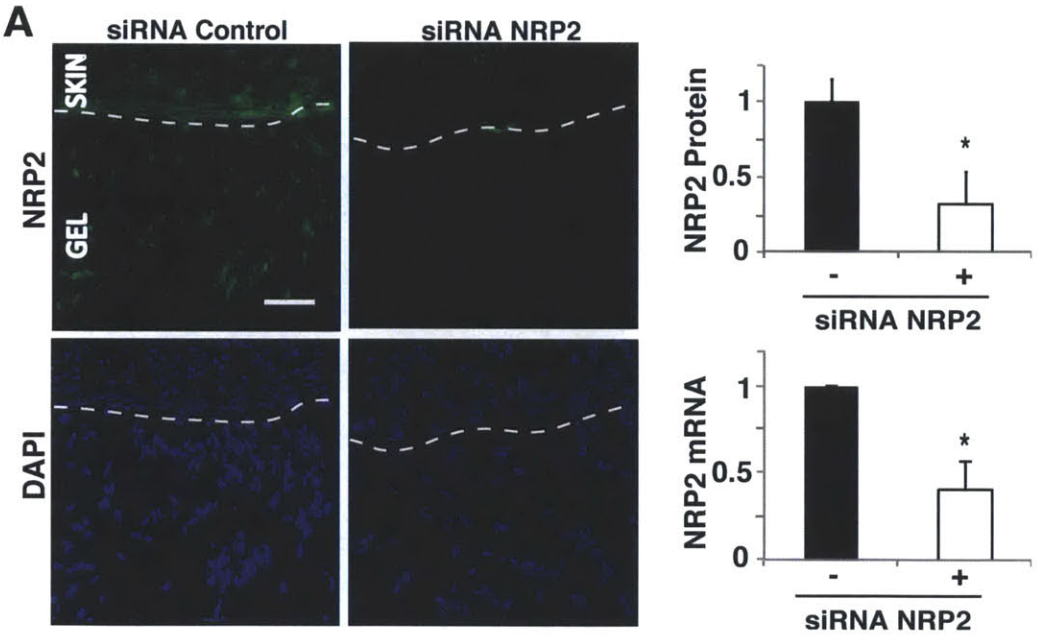

Figure 3.3A Confocal micrographs showing NRP2 expression (*top*) and DAPI (*bottom*) in control or NRP2 siRNA treated implants. Scale bar; 50 µm. Graph showing NRP2 protein levels quantified via immunohistochemical image analysis where NRP2 colocalized to CD31+ cells as measured in 3 random 50x50 µm fields per gel (n=5, *, $p<0.05$) *top*. Graph showing mRNA level of NRP2 in the infiltrated cells in the implanted Matrigel (n=8, *, $p<0.05$) *bottom*. Data are represented as mean +/- s.e.m.

Knockdown of NRP2 in the implanted Matrigel produced a specific increase in endothelial cell migration, which resulted in significantly ($p < 0.05$) enhanced capillary ingrowth into the gel (Fig. 3.3B), as determined by immunohistochemical image analysis of CD31 staining. NRP2 knockdown of NRP2 in the subcutaneous Matrigel implant also resulted in the appearance of a higher number of CE cells at increasing distances into the gel relative to control gels (Fig. 3.3C, $p<0.05$). This closely mimicked the increase in CE cell invasion that we observed by knocking down paxillin in this same assay (Fig. 2.4D). Furthermore, when I perfused the mouse vasculature with fluorescent conA prior to harvesting and visualized 3D reconstructions, I found that NRP2 knockdown increased the number of functional vessels that formed in the implanted Matrigel (Fig. 3.3D, $p<0.01$).

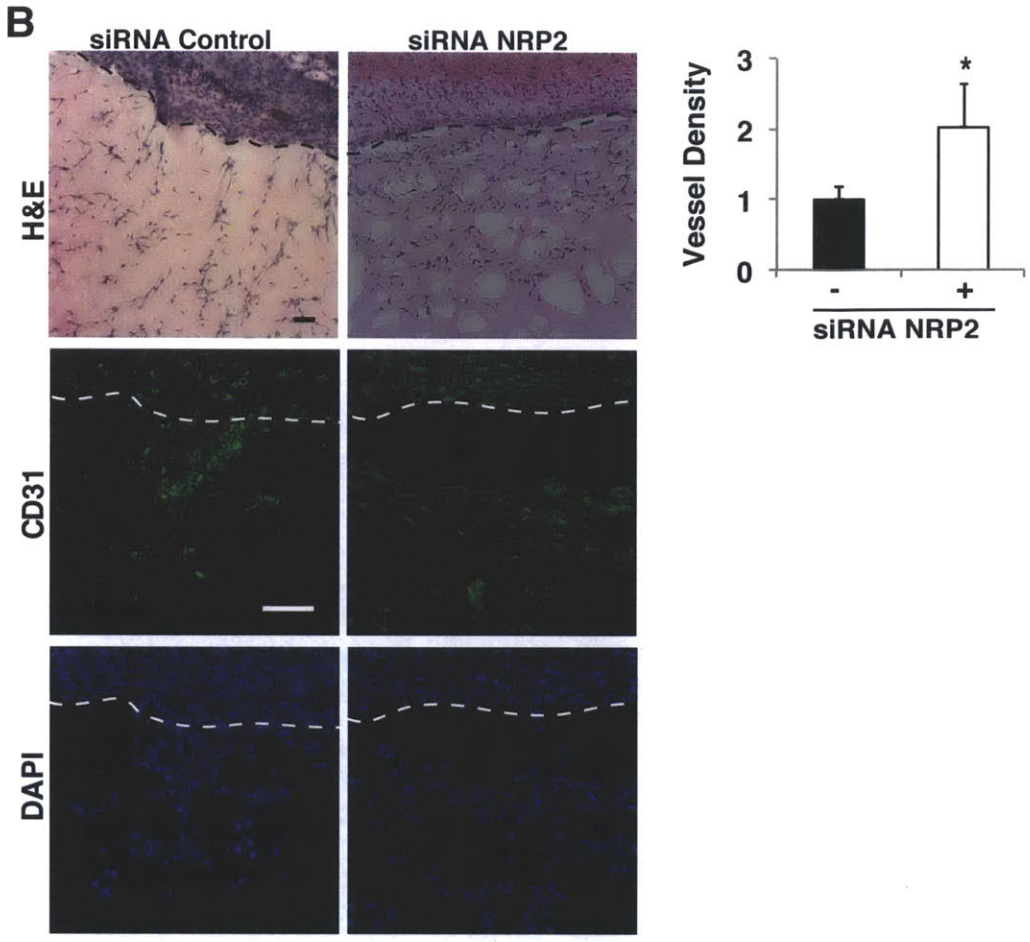

Figure 3.3B H&E stained micrographs showing host cell migration from C57BL/6 mouse skin into implanted Matrigel treated with control or NRP2 siRNA (*top*). Scale bar; 50 μm. Confocal micrographs showing CD31-stained endothelial cells migrated in the Matrigel implant with control or NRP2 siRNA (*middle*). Confocal micrographs showing DAPI-stained endothelial cells migrated in the Matrigel implant with control or NRP2 siRNA (*bottom*). Scale bar; 50 μm. Quantification of CD31+ endothelial cell migration into Matrigel implant treated with control or NRP2 siRNA as measured in 3 random 50x50 μm fields per gel (n=4, *, $p<0.05$). Data are represented as mean +/- s.e.m.

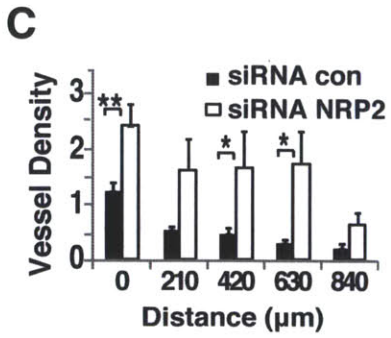

Figure 3.3C Graph showing migration distance of CD31+ endothelial cells into Matrigel implant treated with control or NRP2 siRNA (n=4, **, p<0.01, *, p<0.05). Data are represented as mean +/- s.e.m.

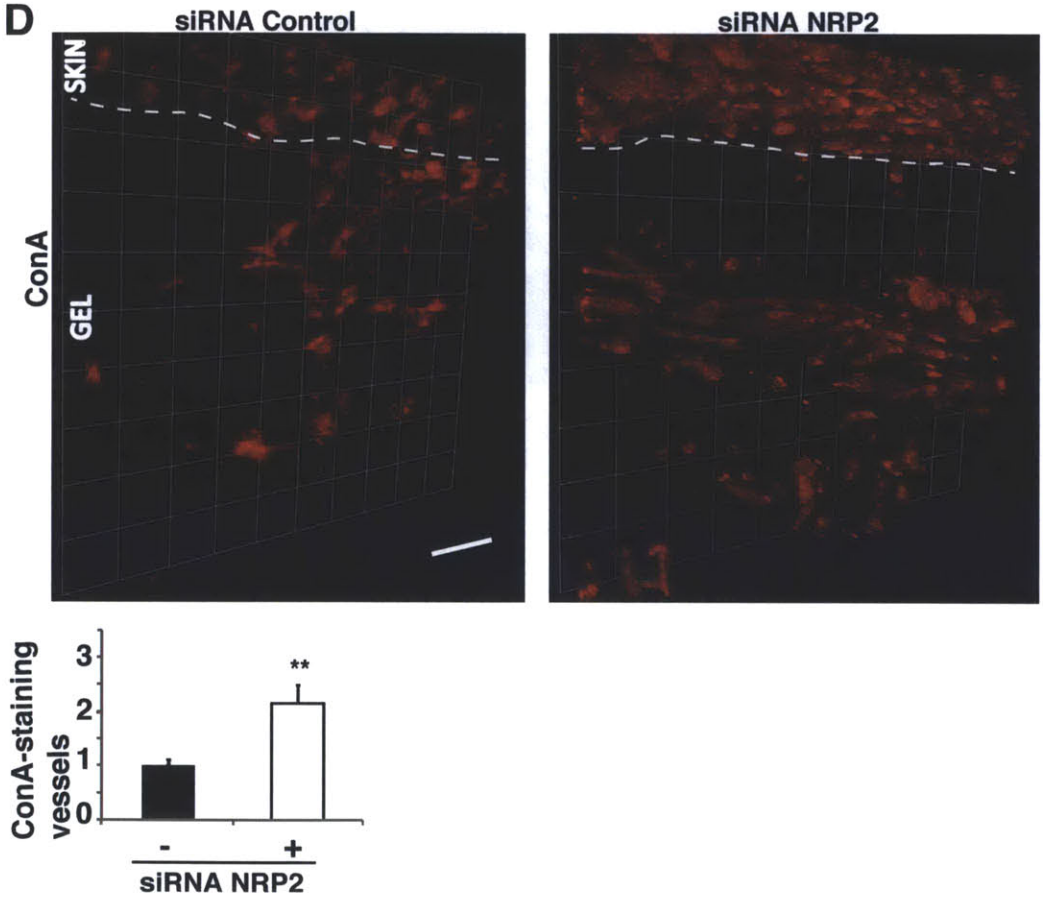

Figure 3.3D 3D reconstructed confocal micrographs of Matrigel implant treated with control or NRP2 siRNA perfused with fluorescein-conA. Scale bar; 50 μm. Quantification of endothelial cell migration into Matrigel implant treated with control or NRP2 siRNA via immunohistochemical fluorescein-conA image analysis as measured in 3 random 50x50 μm fields per gel (n=3, **, p<0.01). Data are represented as mean +/- s.e.m.

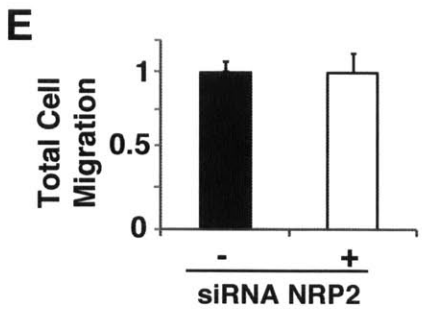

Figure 3.3E Quantification of total cell migration into Matrigel implant treated with control or NRP2 siRNA as measured in 3 random 50x50 μm fields per gel (n=4). Data are represented as mean +/- s.e.m.

These results indicate that paxillin controls NRP2 expression both *in vitro* and *in vivo*, and that siRNA-mediated knockdown of NRP2 enhances endothelial cell migration and microvascular invasion, resulting in a phenotype nearly identical to that produced by inhibiting paxillin expression. Direct knockdown of NRP2 also did not alter the total number of cells (e.g., immune cells, fibroblasts and CE cells) migrating into the Matrigel plug (Fig. 3.3E). This is similar to the result found by knocking down paxillin in the implanted Matrigel (Fig. 2.4D) and it indicates, similarly, that the particular effect of NRP2 expression on cell migration is endothelial cell-type specific.

3.4 Paxillin does not affect VEFGR2 expression

While NRP2 acts as a VEGF receptor, VEGFR2 is the key receptor VEGF utilizes to stimulate angiogenesis. More specifically, VEGFR2 has been known to control many cellular processes including endothelial cell migration (Chung and Ferrara, 2011; Koch and Claesson-Welsh, 2012; Lamalice et al., 2007; Lohela et al., 2009). I therefore explored whether paxillin controls CE cell migration by altering VEGFR2 expression. I found that pax kd via siRNA transfection decreased VEGFR2 expression *in vitro* when protein and mRNA levels were

analyzed (Fig. 3.4A, $p<0.05$). However, when I analyzed the affect of pax kd on VEGFR2 expression *in vivo,* I could not detect a significant change in VEGFR2 expression in CE cells that infiltrated into the implanted Matrigel plugs (Fig. 3.4B). Similarly, pax kd did not affect the total VEGFR2 mRNA level of all cells in the gel plugs (Fig. 3.4B). Thus, *in vivo*, paxillin appears to exert its angiogenesis-modulating effects by selectively altering NRP2 expression.

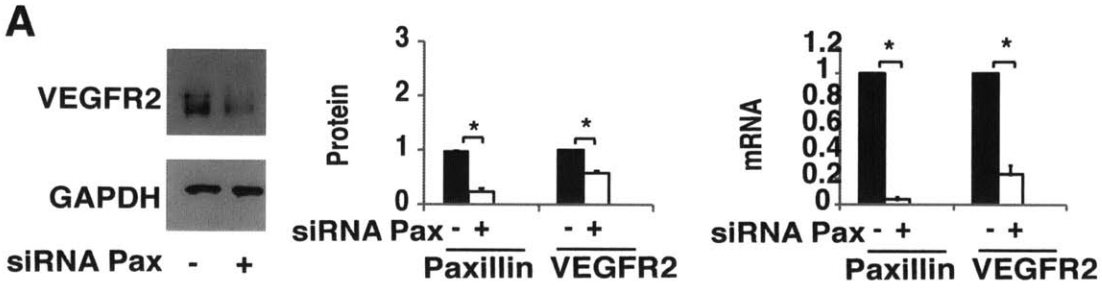

Figure 3.4A Immunoblots showing VEGFR2 and GAPDH expression in HUVE cells treated with paxillin siRNA. Graph showing quantification of immunoblots of paxillin and VEGFR2 in HUVE cells treated with paxillin siRNA (*, $p<0.05$) *left*. Graph showing mRNA level of paxillin and VEGFR2 in HUVE cells treated with paxillin siRNA (*, $p<0.05$) *right*. Data are represented as mean +/- s.e.m.

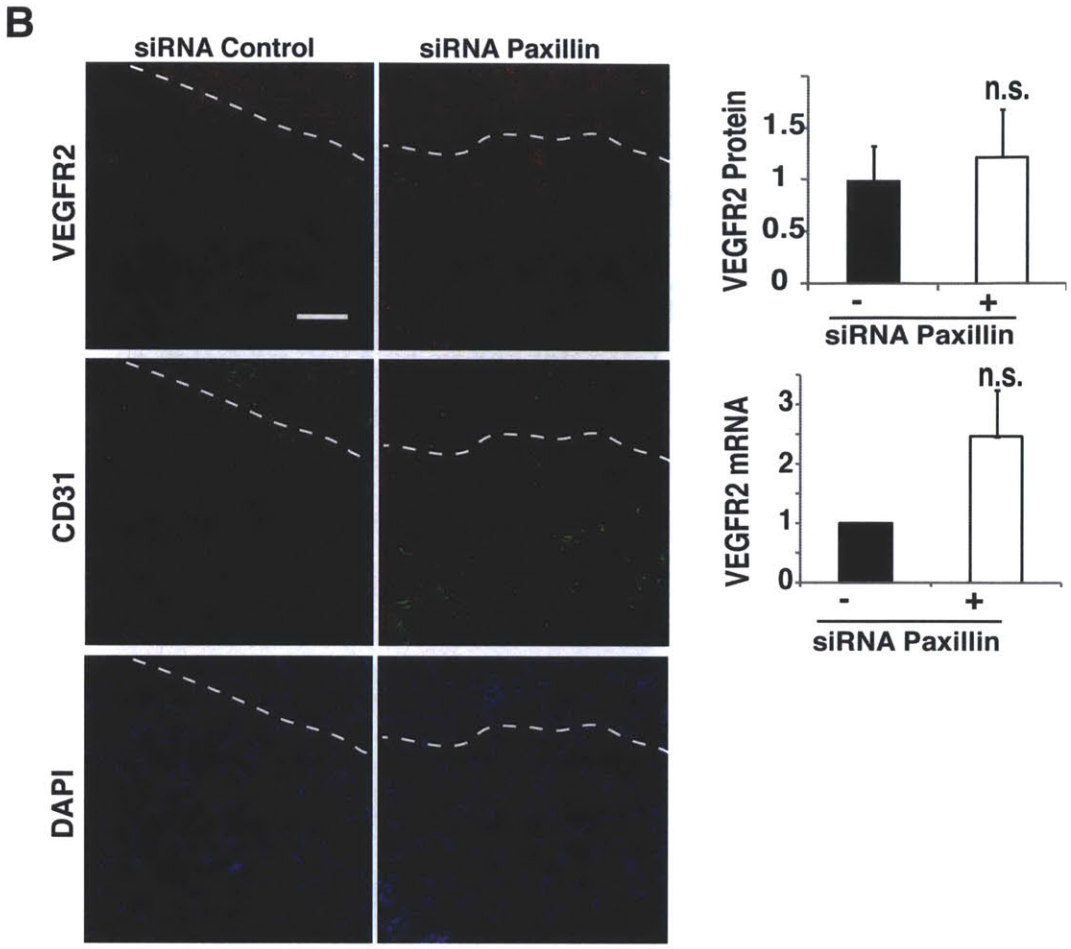

Figure 3.4B Confocal micrographs showing VEGFR2 expression (*top*), CD31-stained blood vessels (*middle*) and DAPI-stain (*bottom*) in control or paxillin siRNA treated implants. Scale bar; 50 µm. Quantification of VEGFR2 expression in Matrigel implant treated with control or paxillin siRNA via immunohistochemical analysis of VEGFR2 colocalized to CD31+ cells as measured in 3 random 50x50 µm fields per gel (n=4) *top*. Graph showing mRNA level of VEGFR2 in the infiltrated cells in the implanted Matrigel (n=8) *bottom*. Data are represented as mean +/- s.e.m.

3.5 GATA2 mediates the effects of paxillin on neuropilin 2

The mechanism through which paxillin controls NRP2 expression is unknown. In fact, control of NRP2 expression is largely unexplored beyond the recent finding that NRP2 expression is regulated by the transcription factor GATA2 in endothelial cells (Coma et al., 2013), which also controls VEGFR2 expression (Mammoto et al., 2009). GATA2 is a transcription factor expressed

by endothelial cells where it regulates the promoter activity of PECAM-1 and endothelin (Gumina et al., 1997; Kawana et al., 1995; Lee et al., 1991). GATA2 governs endothelial cell migration (Coma et al., 2013) and vascular integrity (Johnson et al., 2012; Mammoto et al., 2009). When I used siRNA to knockdown GATA2 in endothelial cells, I found that it decreases the expression of NRP2 (Fig. 3.5A) as analyzed by protein and mRNA levels, similar to what has been reported (Coma et al., 2013). To determine if GATA2 mediates paxillin-driven control of NRP2, I used immunocytochemistry to analyze the effects of knocking down paxillin on nuclear translation of GATA2. These studies revealed that ratio of nuclear GATA2 to total GATA2 was reduced by 50% in pax kd cells (Fig. 3.5B, $p<0.01$). Paxillin therefore appears to control expression of NRP2 by determining GATA2's ability to transit into the nucleus. When paxillin is knocked down, GATA2 becomes sequestered in the cytoplasm, which suppresses NRP2 transcription.

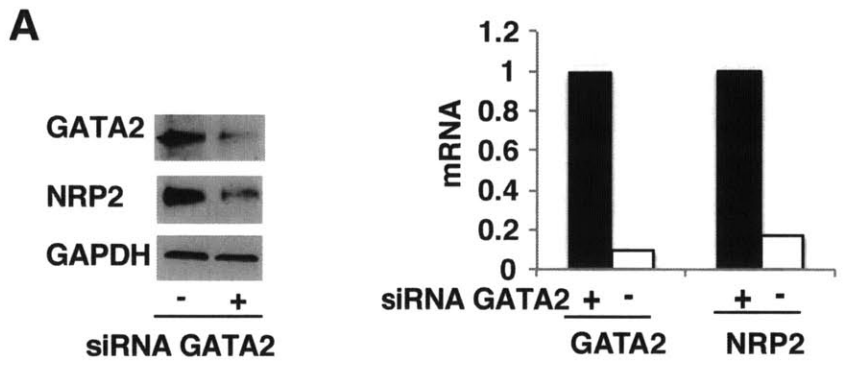

Figure 3.5A Immunoblots showing GATA2, NRP2 and GAPDH expression in HUVE cells treated with GATA2 siRNA. Graph showing mRNA level of GATA2 and NRP2 in HUVE cells treated with GATA2 siRNA.

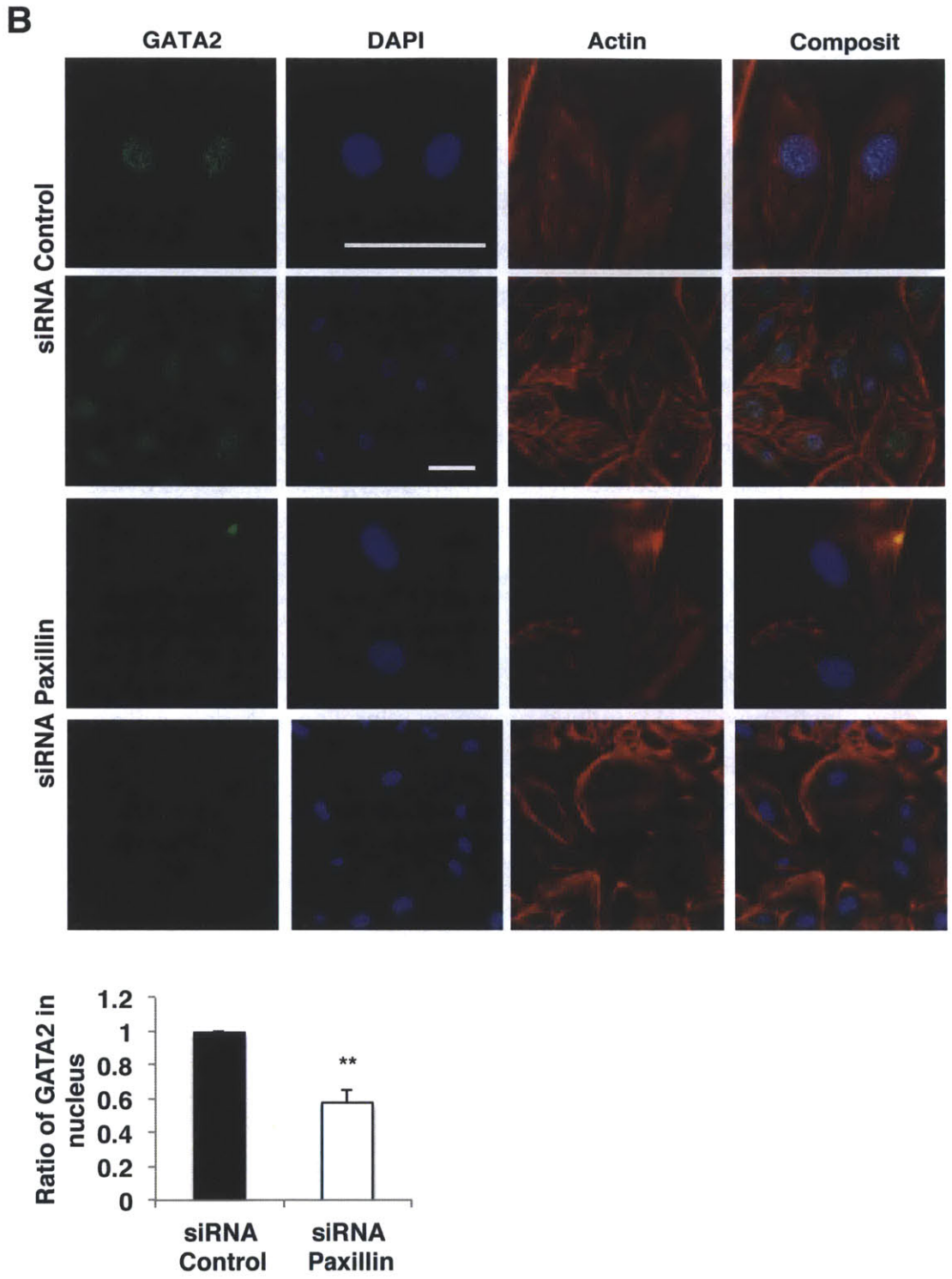

Figure 3.5B Confocal micrographs showing GATA2, DAPI and actin expression in HUVE cells treated with control and paxillin siRNA at high (*first and third rows*) and low (*second and fourth rows*) magnification. Scale bars; 50µm. Graph showing quantification of GATA2 protein in the nucleus relative to total GATA2 in HUVE cells treated with control and paxillin siRNA (n = 3, **, $p<0.01$). Data are represented as mean +/- s.e.m.

3.6 Paxillin N-terminus controls neuropilin 2 expression and endothelial cell migration

We previously showed that overexpression of a paxillin N-terminal (paxN) truncation mutant in paxillin knockout mouse embryonic fibroblasts resulted in a significant increase in their invasive behavior (Sero et al., 2011). To further analyze the molecular mechanism through which paxillin controls endothelial cell migration, we examined whether overexpression of paxillin truncation mutants could control endothelial cell migration. In fact, in paxillin-knockdown HUVE cells *in vitro*, overexpression of paxN (Fig. 3.6A) resulted in 20% increase in migration relative to paxillin knockdown cells (Fig. 3.6B), whereas paxC overexpression (Fig. 3.6A) displayed no change in migration relative to paxillin knockdown cells (Fig. 3.6B). Furthermore, paxN overexpression in paxillin knockdown HUVE cells also resulted in decreased NRP2 protein levels whereas paxC overexpression had no effect of NRP2 protein levels (Fig. 3.6A). Therefore, similar to studies with fibroblasts, paxillin N terminus promotes migration in endothelial cells by controlling NRP2 expression.

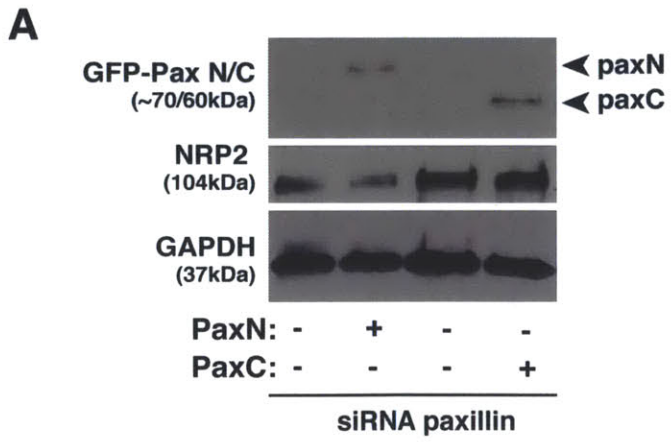

Figure 3.6A Immunoblots showing GFP, NRP2 and GAPDH expression in HUVE cells treated with paxillin siRNA with or without paxN transfection and paxC transfection.

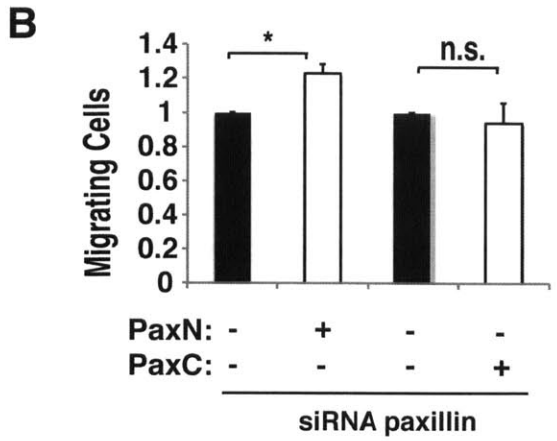

Figure 3.6B Graph showing the number of migrating HUVE cells transfected with paxillin siRNA with or without paxN transfection and paxC transfection normalized to paxillin siRNA cells. (*, $p<0.05$). Data are represented as mean +/- s.e.m.

DISCUSSION

These studies indicate that paxillin controls endothelial cell migration and angiogenesis by regulating the expression of NRP2 *in vitro* and *in vivo*. Direct modulation of NRP2 gene expression also changes endothelial cell migration and invasion *in vitro* and *in vivo* and this can be reversed by over expressing paxillin *in vitro*.

The suppression of NRP2 expression might enhance vascular network formation by decreasing binding of its ligand Semaphorin 3F (Sema3F), which is a chemorepulsive cytokine that normally suppresses motile process formation and endothelial cell movement (Bielenberg et al., 2004; Bielenberg et al., 2006; Carmeliet, 2005; Geretti et al., 2008). Although decreased expression of NRP2 also would decrease binding of its VEGF ligand, this angiogenic factor can still stimulate endothelial cells through its other receptor, VEGFR2. This hypothetical mechanism conflicts, however, with the finding that NRP2 knockdown can inhibit VEGF-induced migration of endothelial cells *in vitro* (Favier et al., 2006). But this response might be due to differences in experimental settings, for example, we used whole serum while the others used only VEGF. In addition to VEGF and

Sema3F, other molecules that can modulate cell migration, such as b-FGF, PDGF, HGF and TGF-β1 (Wild et al., 2012) have been shown to bind NRP2. In fact, semaphorins, including SEMA3F, are often down-regulated or mutated in tumors, allowing enhanced VEGF/NRP interactions (Grandclement and Borg, 2011). Thus, the balance of attractive and repulsive cues may control the degree and direction of cell migration as well as microvascular network formation through their common receptor NRP2.

It is as yet unknown whether paxillin controls the expression of receptors besides NRP2, or expression of other angiogenic factors. Although p190RhoGAP regulates VEGFR2 expression (Mammoto et al., 2009), paxillin did not significantly modulate VEGFR2 expression in our Matrigel plugs *in vivo*. Although in a pared down system such as HUVE cells stimulated with optimal growth factors *in vitro* I was able to see an effect of paxillin knockdown on VEGFR2 expression, this was not observed in my Matrigel plug implantation system *in vivo*. This may be because control of VEGFR2 expression is mediated by many cues, including various growth factors and ECM mechanics *in vivo* (Ferrara et al., 2003; Mammoto et al., 2009).

I found that knockdown of the other FA scaffold proteins vinculin and zyxin did not alter endothelial cell migration *in vitro,* nor did it alter NRP2 expression. Past studies have shown that vinculin knockdown has no effect on 2D chemotaxis (Mitra et al., 2005), which is consistent with our results. However, this contradicts other studies in which knockdown of vinculin and zyxin elicited changes in cell migration (Hoffman et al., 2006; Mierke et al., 2010; Mitra et al., 2005; Mori et al., 2009; Nguyen et al., 2010; Plotnikov and Waterman, 2013). Apparently, the effects of these FA proteins on cell migration can vary significantly depending on the cell type studied and the experimental conditions utilized.

My studies suggest that paxillin controls NRP2 expression through dictating intracellular localization of the transcription factor GATA2. Utilizing

immunocytochemistry, I showed that paxillin knockdown decreases GATA2 localization to the nucleus. To confirm this finding, further biochemical studies need to be done. For example, gel shift and CHIP studies would allow us to determine whether paxillin modulation changes GATA2 interaction with NRP2 mRNA. Our studies also reveal that the paxillin N-terminus may control NRP2 expression since overexpression of a paxillin N-terminal truncation mutant decreases NRP2 protein levels thereby leading to enhanced endothelial cell migration. In fact, the paxillin N terminus interacts with a number of signaling molecules including some known to regulate transcription and migration (Deakin and Turner, 2008). For example, p190RhoGAP has been shown to control the activity of Y31/118-phosphorylated paxillin (Tsubouchi et al., 2002), and p190RhoGAP controls VEGFR2 expression through a balance of the transcription factors GATA2 and TFII-I (Mammoto et al., 2009). Interestingly, NRP2 has a GATA2 binding site in its promoter region, and thus, paxillin N-terminus could control NRP2 expression through indirect interactions with p190RhoGAP and GATA2.

The mechanism by which paxillin controls NRP2 is not necessarily limited to action of the transcription factor GATA2. For example, the VEGF-phosphorylated transcription factor ELK1 (Murata et al., 2000) also can bind to the NRP2 promoter region. ELK1 has been reported to complex with nuclear paxillin to regulate cell functions (Sen et al., 2012), and so paxillin also could control NRP2 transcriptional activity through ELK1 in CE cells. In addition, it is possible that paxillin may control endothelial cell migration both upstream and downstream of NRP2 because even though paxillin interacts with p190RhoGAP (Tsubouchi et al., 2002) and controls NRP2 expression, p190RhoGAP can act downstream of NRP2 to inhibit motility in other cells (Shimizu et al., 2008).

In conclusion, I show that the control of endothelial cell invasion and angiogenesis by paxillin is mediated by the guidance molecule and VEGF receptor, NRP2, but not VEGFR2. Further studies suggest that paxillin controls

localization of the transcription factor GATA2 which controls NRP2 expression (Coma et al., 2013).

MATERIALS AND METHODS

Materials

Anti-NRP2 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-vinculin monoclonal antibody was from Sigma (St. Louis, MI, USA), anti-zyxin monoclonal antibody was from Abcam (Cambridge, MA, USA), anti-VEGFR2 antibody was from Cell Signaling, anti-GATA2 antibody was from Abcam (Cambridge, MA, USA), anti-GFP antibody was from BioVision (Milpitas, CA, USA).

Cell Culture

HUVE cells (Lonza, Walkersville MD, USA) were cultured as described. Knockdown of vinculin and zyxin (Ambion, Austin, TX, USA) and NRP2 (Sigma, St. Louis, MI, USA) was performed with siRNA (Table 4) and transfected as described. An siRNA duplex with irrelevant sequence served as a control (Ambion, Austin TX, USA). Over-expression of human NRP2 (gift from Michael Klagsbrun, Boston Children's Hospital, MA (Shimizu et al., 2008)) was performed using Superfect transfection reagent (Qiagen, Valencia, CA, USA). Transfection with vehicle alone served as control. Effects of knockdown and over-expression were confirmed via qRT-PCR and Western blotting at 48-72 hours after transfection. GFP-tagged human paxillin N-terminus (paxN) and human paxillin C-terminus (paxC) truncation mutants (Sero et al., 2011) were transfected into siRNA-based paxillin knockdown HUVE cells. We ensured that the siRNA did not also knockdown the transfected paxN and paxC mutants by using two sequences of siRNA against paxillin acting in the two different termini (e.g. paxillin siRNA #1 with target sequence in the paxillin C terminus was used in combination with paxN transfection, and paxillin siRNA #2 with target sequence in the N terminus was used with paxC transfection).

Molecular Biology Assays

RNA was purified as described. qRT-PCR was performed as described. Primers for zyxin, vinculin, NRP2 and VEGFR2 are described in Table 5.

Cell Biological methods

Transwell migration assay was performed as described. Transwell invasion assay was performed as described.

Nuclear translocation assay was performed first by serum starving HUVE cells plated on fibronectin for 24 hours in basal cell medium or 0.5% EGM2, then replenishing growth factors and serum to 1-2% EGM2 for 24 hours. Cells were fixed, permeabilized, stained and imaged with GATA2, DAPI and Actin. Nuclear expression of GATA2 was quantified by positive pixel count in the DAPI-stained nucleus. The cytosol and cell border was defined by actin+ staining and again GATA2 was quantified by positive pixel count in actin+/DAPI- cell body.

In vivo Matrigel Plug Assay

Matrigel plug assay was performed as described. 10 μg of siRNA against NRP2 or scramble control siRNA was injected locally at day 3 after implantation. Implants were harvested and processed as described. Additionally, implants were stained with NRP2 and VEGFR2 antibodies. NRP2 and VEGFR2 expression was measured when colocalized with CD31+ or ConA+ cells for three 50x50 μm areas of Matrigel for more than 3 implants.

Statistical Analysis

All experiments were repeated independently three or more times and data are represented as mean +/- s.e.m. Statistical analyses to assess the difference between control and treatment groups were performed with Matlab R2012b software (Mathworks, Natick, MA, USA) using the unpaired Student's *t* test.

CHAPTER 4: PAXILLIN CONTROLS TUMOR ANGIOGENESIS

INTRODUCTION

Formation of well-organized microvessels is necessary for normal organ development and deregulation of this process contributes to cancer progression, as well as development of tumor resistance to cancer therapies (Carmeliet and Jain, 2011; Chung and Ferrara, 2011). Specifically, deregulation of endothelial cell migration in the tumor microenvironment contributes to the formation of a disorganized tumor vasculature characterized by leakiness and fragility (Nagy et al., 2007) which reduces chemotherapy delivery to the solid tumor.

Excessive VEGF produced by tumor cells as well as surrounding stromal cells such as immune cells and fibroblasts is accompanied by abnormal expression and activity of VEGF receptors (Chung and Ferrara, 2011) and associated downstream signaling molecules important for migration (e.g. Cdc42, Rho and ROCK and adhesion proteins such as FAK) (Lamalice et al., 2007). NRP2 has been reported to be present on tumor microvessels and it is often the only VEGF receptor expressed by tumor cells (Grandclement and Borg, 2011). NRP2 mediates SEMA3F-driven chemorepulsion of endothelial cells induced by melanoma cells. SEMA3F is also often down-regulated or mutated in tumors, thereby reducing NRP2's chemorepulsion effects (Grandclement and Borg, 2011). However, little is known about how NRP2 expression is modulated in tumor endothelial cells.

My studies in Chapters 2 and 3 revealed that siRNA knockdown of paxillin and NRP2 result in enhanced formation of microvessel networks that lack oriented sprouts, which is similar to responses observed during tumor angiogenesis. I therefore hypothesized that tumor factors, such as VEGF, might modulate the paxillin-NRP2 signaling axis, thereby contributing to deregulation of CE cell migration and angiogenesis in cancer. In this Chapter, I explored this possibility experimentally.

RESULTS

4.1 Tumor factors control paxillin and neuropilin 2 expression in vitro

I first set out to explore whether paxillin is involved in control of tumor angiogenesis by examining whether tumor factors promote angiogenesis *in vitro* by altering paxillin expression and modulating NRP2 expression levels. I started by testing conditioned medium collected from cultured Lewis Lung Carcinoma (LLC) cells to stimulate HUVE cells *in vitro*. LLC cells are known to secrete VEGF as well as multiple other soluble growth factors (Satchi-Fainaro et al., 2005). Indeed, LLC conditioned medium decreased expression of both paxillin protein (Fig. 4.1A) and mRNA (Fig. 4.1B) levels by about 70% ($p<0.01$) and 50% ($p<0.001$), respectively. Moreover, NRP2 expression decreased by at least 50% ($p<0.001$) when the cultured HUVE cells were stimulated with LLC factors that also suppressed paxillin expression (Fig. 4.1A,B), and over-expression of paxillin restored normal NRP2 levels (Fig. 4.1A,B, $p<0.05$). Thus, again, NRP2 appears to be downstream of paxillin in this signaling pathway.

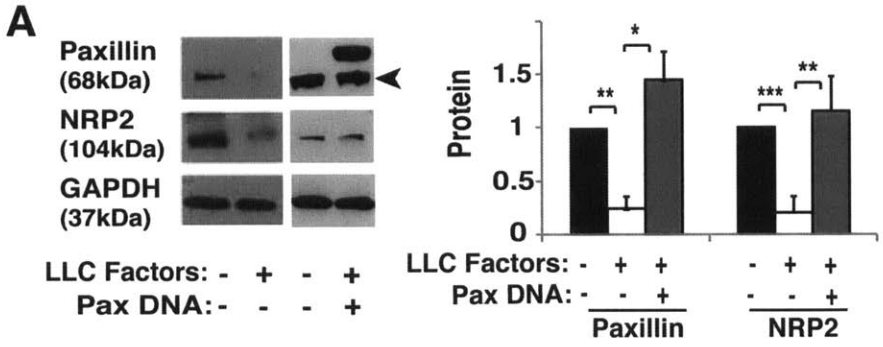

Figure 4.1A Immunoblots showing paxillin, NRP2, and GAPDH protein levels in HUVE cells treated with 0.5% serum EBM2 with or without LLC factors, or in combination with paxillin DNA. Arrowhead indicates paxillin. Quantification of immunoblots showing protein levels of paxillin and NRP2 (***, $p<0.001$, **, $p<0.01$, *, $p<0.05$). Data are represented as mean +/- s.e.m.

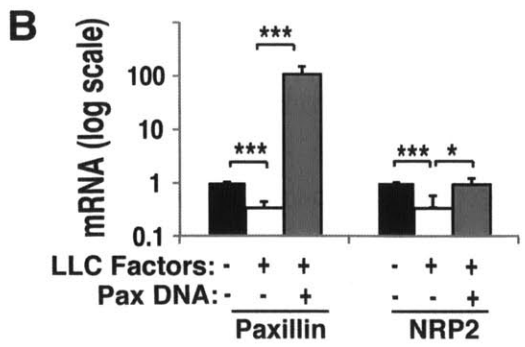

Figure 4.1B Graph showing mRNA level of paxillin and NRP2 in HUVE cells treated with 0.5% serum EBM2 with or without LLC factors, or in combination with paxillin DNA (***, $p<0.001$, *, $p<0.05$). Data are represented as mean +/- s.e.m.

4.2 Tumor factors control endothelial cell migration in vitro

When I examined the effects of LLC-derived factors on HUVE cell migration *in vitro*, the tumor factors increased the number of migrating cells in a Transwell migration assay by 1.5-fold (Fig. 4.2A, $p<0.05$). As expected, when I over-expressed either paxillin or NRP2 in LLC factor-treated cells, cell migration was restored to control levels (Fig. 4.2A, $p<0.05$). LLC-derived factors also stimulated a 2-fold increase in HUVE cell invasion into Matrigel plugs in a modified Transwell assay (Fig. 4.2B, $p<0.001$). Again, when I over-expressed either paxillin or NRP2 in LLC factor-treated cells, cell invasion was restored to control levels (Fig. 4.2B, $p<0.05$).

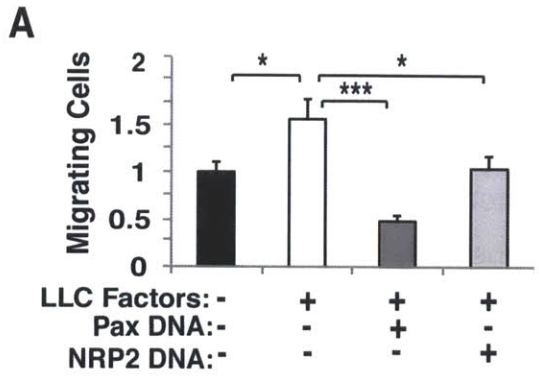

Figure 4.2A Graph showing number of migrating HUVE cells towards 0.5% serum EBM2 with or without LLC factors in combination with paxillin DNA or NRP2 DNA treatment in Transwell migration assay (*, $p<0.05$, ***, $p<0.001$). Data are represented as mean +/- s.e.m.

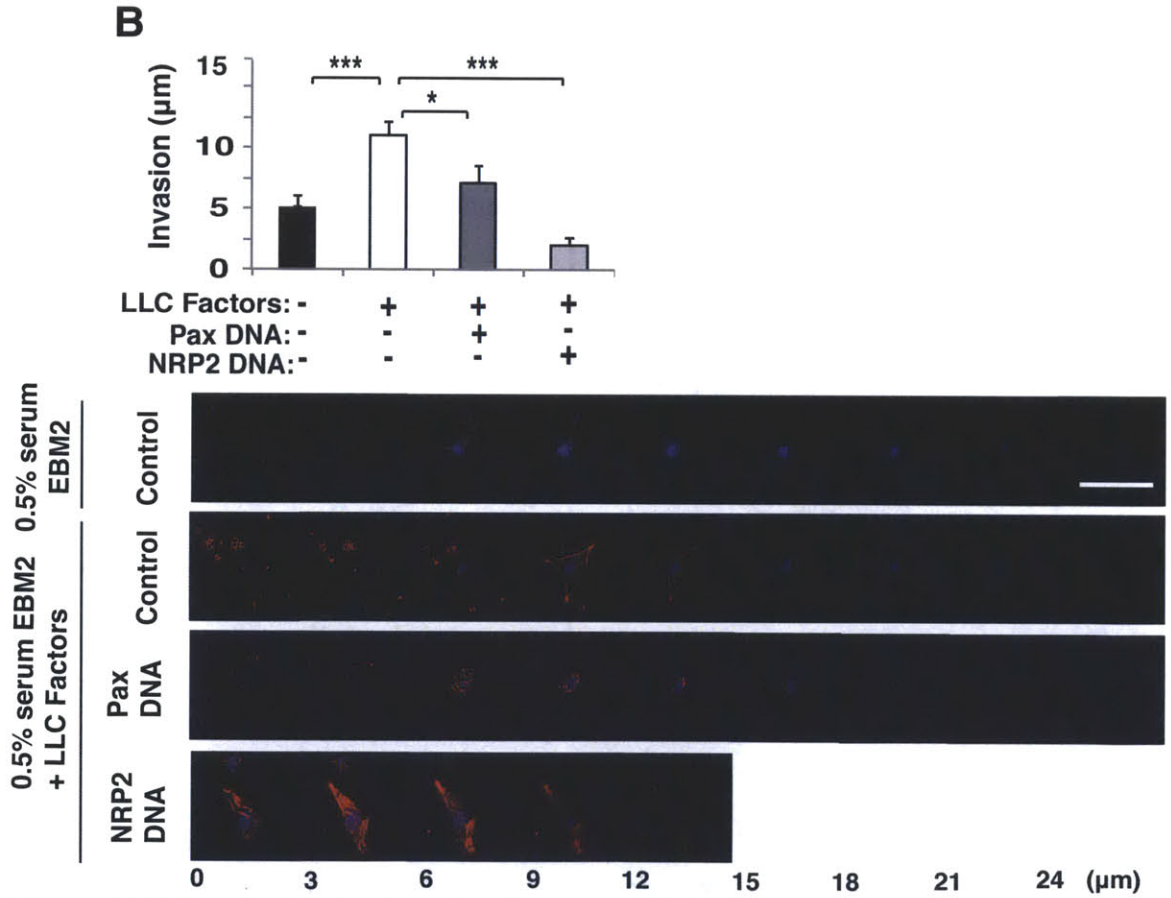

Figure 4.2B Graph showing invasion distance of HUVE cells to 0.5% serum EBM2 with or without LLC factors in combination with paxillin DNA or NRP2 DNA treatment in Transwell migration assay (*, $p<0.05$, ***, $p<0.001$). Confocal micrographs showing HUVE cell invasion in Transwell invasion assay with 0.5% serum EBM2 or paxillin DNA or NRP2 DNA with LLC-conditioned 0.5% serum EBM2. Scale bar; 50μm. Data are represented as mean +/- s.e.m.

4.3 Tumor factors control paxillin and neuropilin 2 expression in vivo

To determine whether tumor factors also control paxillin and NRP2 expression *in vivo*, I implanted subcutaneously a 2-chamber PDMS device that contained a Lewis lung carcinoma (LLC) cell-Matrigel suspension separated from a second chamber with Matrigel alone by a porous filter membrane (Fig. 4.3A). LLC cells are unable to escape, but the soluble factors they produce diffuse through pores of the membrane and act as chemoattractants to stimulate migration of host cells into the underlying Matrigel.

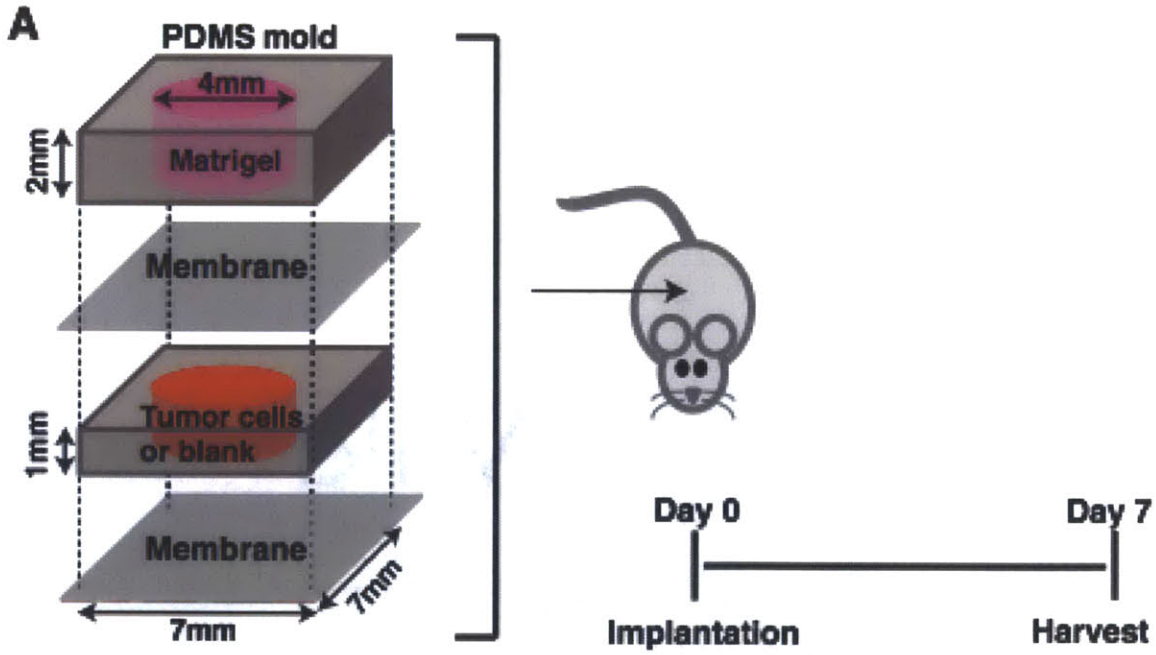

Figure 4.3A Schematic of dual chamber Matrigel plug implant and experimental timeline.

In these studies, I found that the presence of LLC-derived angiogenic factors decreased cellular expression of paxillin in endothelial cells by 40% ($p<0.05$) and 90% ($p<0.01$), as demonstrated by computerized image analysis of immunohistochemically stained sections (Fig. 4.3B) and qRT-PCR of extracted gels (Fig. 4.3C), respectively. The soluble factors secreted by LLC cells that decreased paxillin expression in CE cells also reduced CE cell expression of

NRP2 by 30%-50%, when analyzed by immunohistochemistry (Fig. 4.3D, $p<0.05$) or measuring total NRP2 mRNA levels in the Matrigel plug implants (Fig. 4.3E, $p<0.01$). Thus, it appears that the endogenous expression of paxillin is decreased by the presence of tumor factors and this, in turn, suppresses NRP2 expression *in vivo*, just as it does *in vitro*.

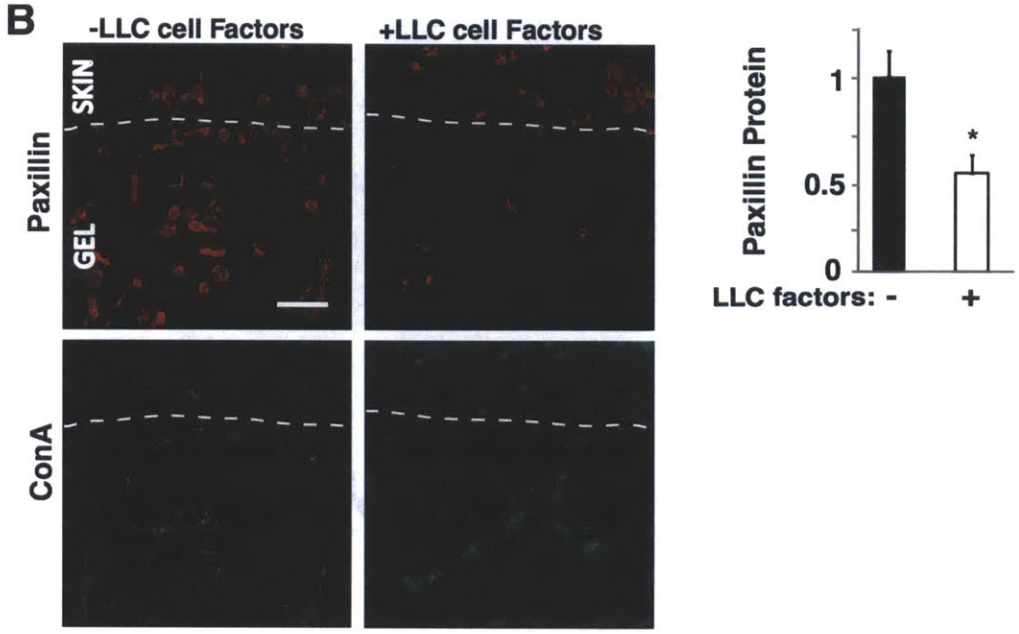

Figure 4.3B Confocal micrographs showing paxillin expression (*top*) and ConA stained vessels (*bottom*) in infiltrated cells in the Matrigel implant with or without LLC factors. Scale bar; 50μm. Graph showing paxillin protein levels quantified via immunohistochemical image analysis where paxillin colocalized to ConA+ cells as measured in 3 random 50x50 μm fields per gel (n=5, *, $p<0.05$). Data are represented as mean +/- s.e.m.

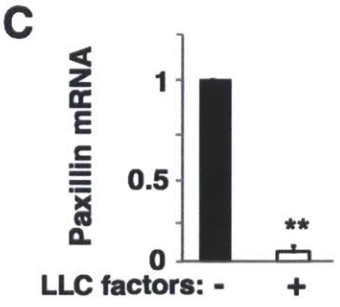

Figure 4.3C Graph showing mRNA level of paxillin in the infiltrated cells in the implanted Matrigel (n=6, **, $p<0.01$). Data are represented as mean +/- s.e.m.

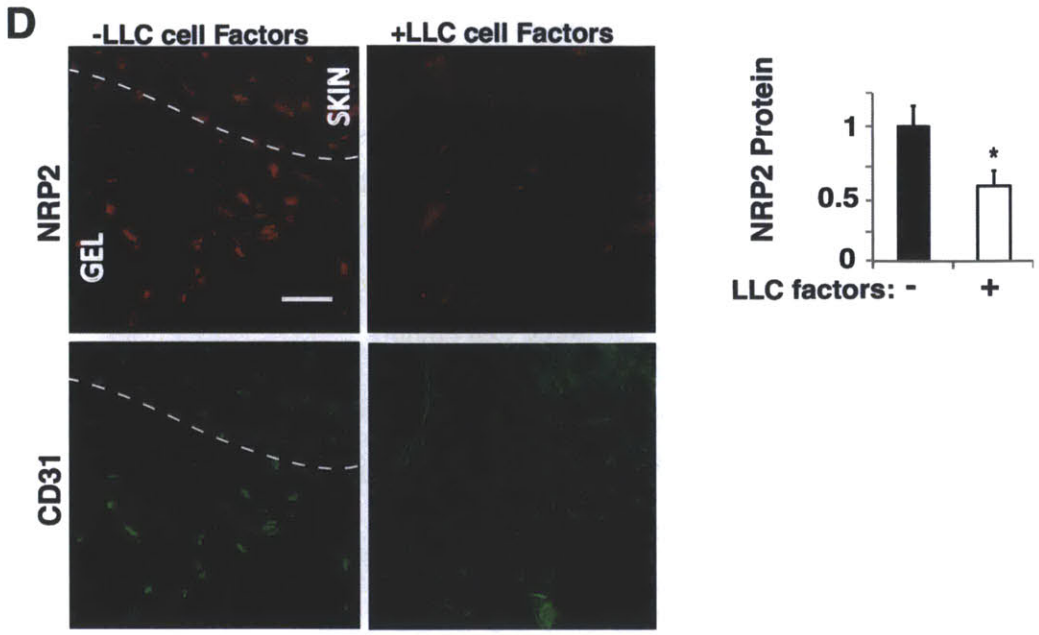

Figure 4.3D Confocal micrographs showing NRP2 expression (*top*) and CD31-stained endothelial cells (*bottom*) migrated into the implanted Matrigel with or without LLC factors. Scale bar; 50μm. Graph showing NRP2 protein levels quantified via immunohistochemical image analysis of NRP2 colocalized with CD31+ cells as measured in 3 random 50x50 μm fields per gel (n=4, *, $p<0.05$). Data are represented as mean +/- s.e.m.

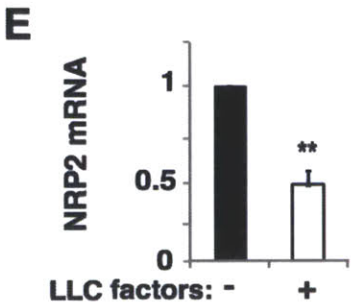

Figure 4.3E Graph showing mRNA level of NRP2 in the infiltrated cells in the implanted Matrigel (n=8, **, $p<0.01$). Data are represented as mean +/- s.e.m.

4.4 Tumor factors control CE cell migration through paxillin-NRP2 signaling in vivo

The presence of LLC-derived factors in a second chamber resulted in a significant ($p<0.05$) increase in total number of migrating cells (Fig. 4.4A). This is

consistent with the finding that LLC cells produce many potent chemoattractants (Satchi-Fainaro et al., 2005). LLC factors also specifically stimulated CE cell migration into the Matrigel (Fig. 4.4B, $p<0.001$), and significantly increased the number of CE cells that appeared at increasing depths into the gel compared to single-chamber implants (Fig. 4.4C, $p<0.001$). As expected, tumor-derived factors increased the total number of functional vessels as well, as indicated by perfusing with fluorescent conA and carrying out 3D reconstructions (Fig. 4.4D, $p<0.001$). Taken together, these results indicate that soluble angiogenic factors produced by the LLC tumor both suppress paxillin expression and greatly increase CE cell migration and microvascular invasion *in vivo*.

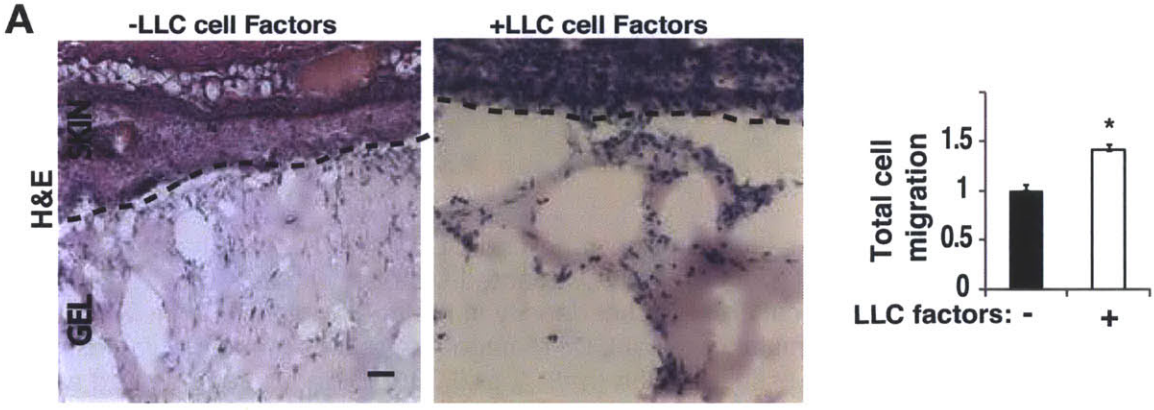

Figure 4.4A H&E stained micrographs showing total cell migration from C57BL/6 mouse skin into implanted Matrigel with or without LLC factors. Scale bar; 50μm. Quantification of total cell migration into implant as measured in 3 random 50x50um fields per tissue section (n=4, *, $p<0.001$). Data are represented as mean +/- s.e.m.

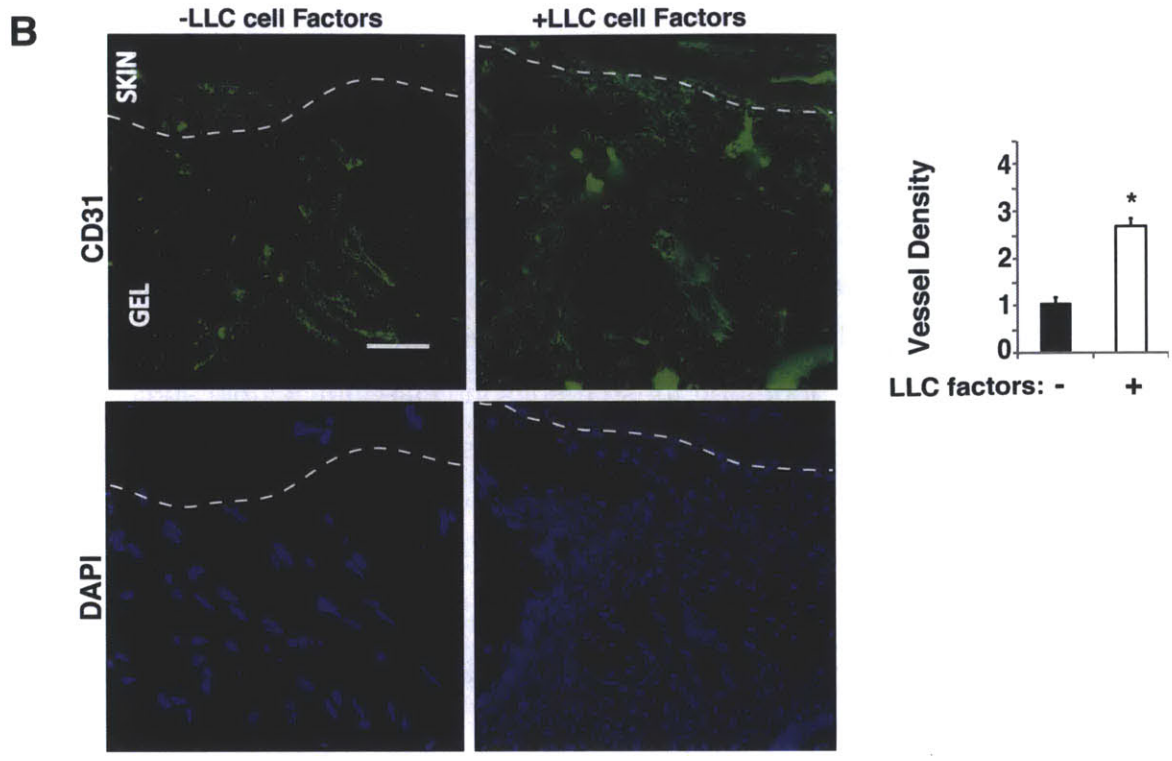

Figure 4.4B Confocal micrographs showing CD31-stained endothelial cells *(top)* and DAPI stained nuclei *(bottom)* in the implanted Matrigel with or without LLC factors. Scale bar; 50µm. Quantification of vessel density in Matrigel implant with or without LLC factors via immunohistochemical CD31 image analysis as measured in 3 random 50x50um fields per tissue section (n=9, *, p<0.001). Data are represented as mean +/- s.e.m.

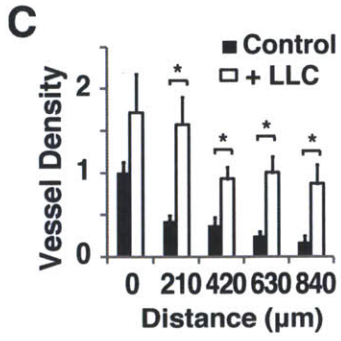

Figure 4.4C Graph showing migration distance of endothelial cells into Matrigel implant with or without LLC factors (n=7, *, p<0.001). Data are represented as mean +/- s.e.m.

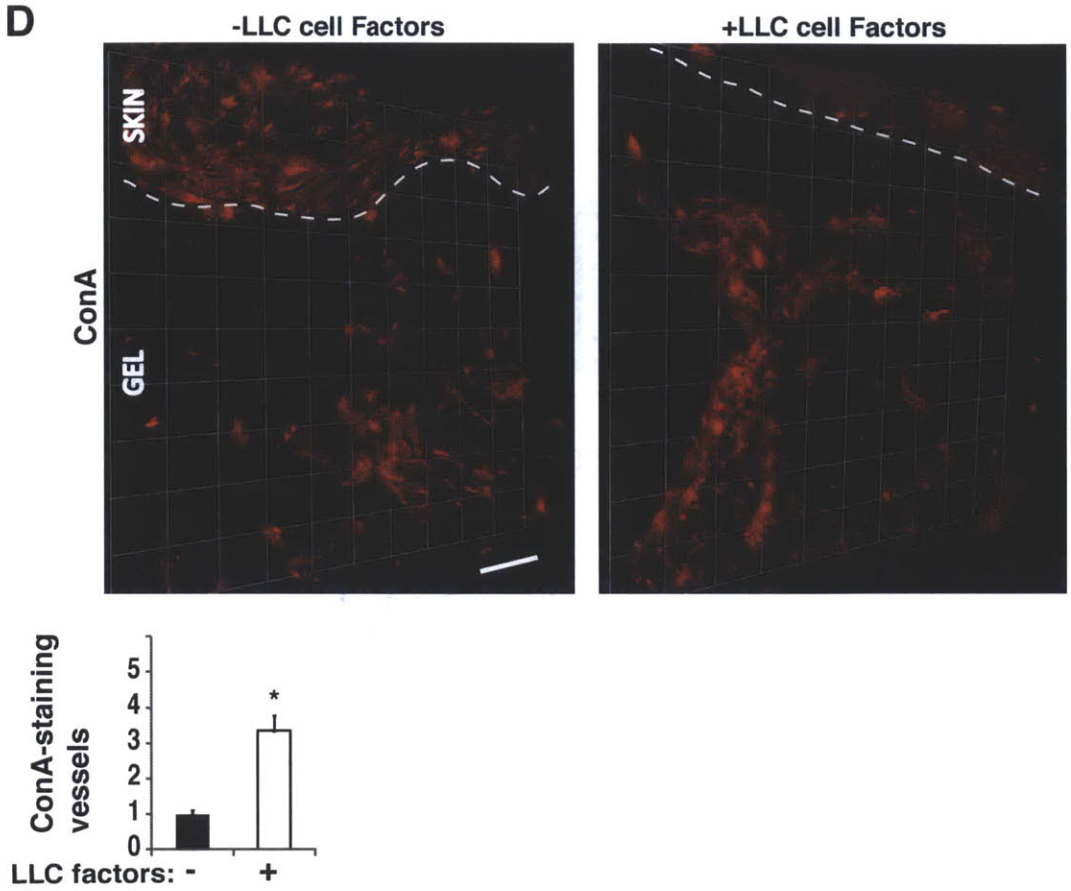

Figure 4.4D Corresponding 3D reconstructed confocal micrographs to Fig. 4.4B showing Matrigel implant with or without LLC factors perfused with fluorescein-conA. Scale bar; 50μm. Quantification of endothelial cell migration into Matrigel implant with or without LLC factors via immunohistochemical fluorescein-conA image analysis as measured in 3 random 50x50um fields per tissue section (n=4, *, $p<0.001$). Data are represented as mean +/- s.e.m.

4.5 VEGF controls paxillin and neuropilin 2 expression in vitro

As VEGF is a major angiogenic chemoattractant produced by LLC cells (Satchi-Fainaro et al., 2005) and NRP2 is a VEGF receptor, I explored whether VEGF could be responsible for the observed effects of tumor-derived factors on paxillin and NRP2 expression. VEGF alone decreased paxillin expression by about 80% when measured by qRT-PCR (Fig. 4.5A, $p<0.01$), in addition to suppressing expression of NRP2 mRNA (Fig. 4.5A, $p<0.05$), much as I observed in my studies with LLC-derived factors. Moreover, paxillin over-expression in

combination with VEGF was able to reverse the suppression of NRP2 mRNA levels (Fig. 4.5A, $p<0.05$).

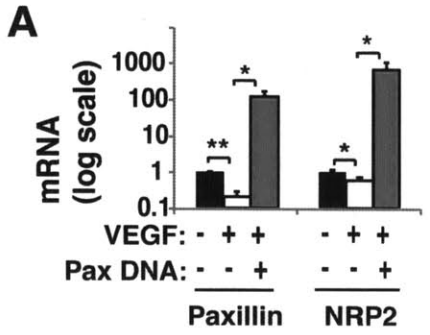

Figure 4.5A Graph showing paxillin and NRP2 mRNA levels from HUVE cells in 0.5% serum EBM2 with or without VEGF (30 ng/ml), or in combination with paxillin DNA (**, $p<0.01$, *, $p<0.05$). Data are represented as mean +/- s.e.m.

I also corroborated our finding of VEGF-induced paxillin expression decrease by RNA-seq via collaboration. HUVE cells were serum starved then stimulated with VEGF after which RNA was harvested at time points up to 12 hours. Interestingly, RNA-seq revealed that paxillin (PXN) and sister protein, leupaxin (LPXN) both displayed decreasing RNA levels over time, as measured by fragments per kilobase of exon per million fragments mapped (FPKM). In particular, paxillin RNA displayed a substantial total decrease over 12 hours, whereas vinculin (VCL), talin (TLN1, 2) and FAK (PTK2) did not display any significant changes in expression.

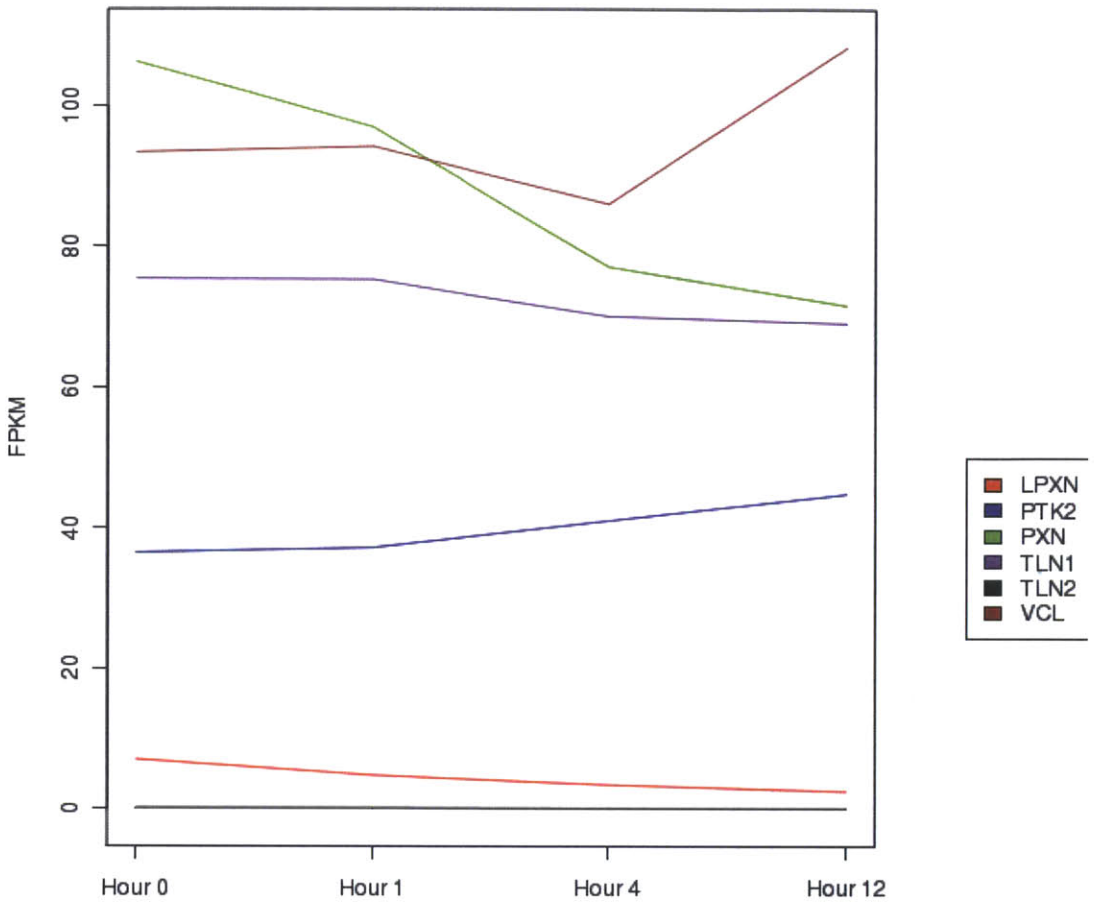

Figure 4.5B Graph showing leupaxin (LPXN), FAK (PTK2), paxillin (PXN), talin 1 (TLN1), talin 2 (TLN2) and vinculin (VCL) RNA levels from HUVE cells serum starved then stimulated with VEGF at t = 0, t = 1 hour, t = 4 hours, t = 12 hours. Fragments per kilobase of exon per million fragments mapped (FPKM) was determined via RNA-seq. Experiment and image courtesy of Daniel Day and Peter Park laboratory (Harvard Medical School).

4.6 VEGF controls endothelial cell migration though paxillin-NRP2 signaling

VEGF induced an increase in both cell migration (Fig. 4.6A, $p<0.05$) and invasion (Fig. 4.6B, $p<0.001$) of HUVE cells in in vitro assays, and these effects were suppressed by over-expressing NRP2 (Fig. 4.6A, B, $p<0.05$). Together, these experiments show that soluble tumor-derived factors, such as VEGF, stimulate microvessel ingrowth at least in part by decreasing endogenous

expression of paxillin, which in turn leads to suppression of NRP2, and an associated increase in migration and invasion of endothelial cells.

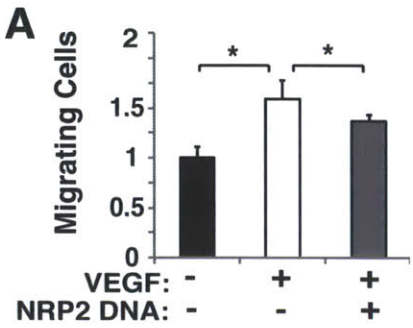

Figure 4.6A Graph showing number of migrating HUVE cells towards 0.5% serum EBM2 with or without VEGF in combination with NRP2 DNA in Transwell migration assay (*, $p<0.05$). Data are represented as mean +/- s.e.m.

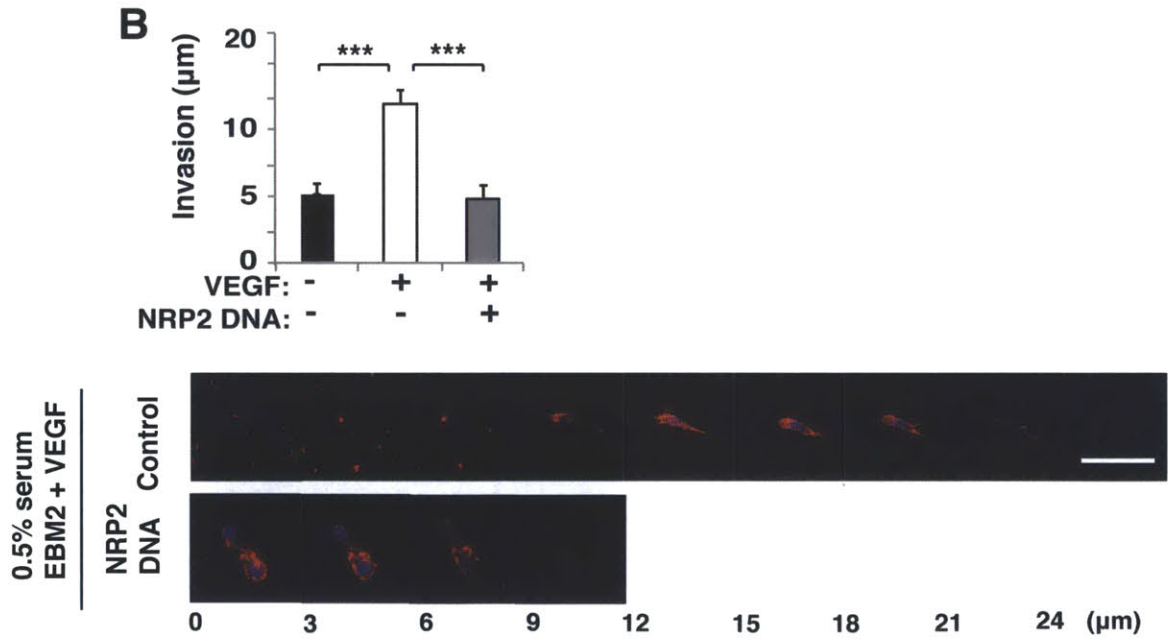

Figure 4.6B Graph showing invasion distance of HUVE cells to 0.5% serum EBM2 with or without VEGF in combination with NRP2 DNA in Transwell migration assay (***, $p<0.001$). Confocal micrographs showing HUVE cell invasion in Transwell invasion assay with or without NRP2 DNA with VEGF-conditioned 0.5% serum EBM2. Scale bar; 50µm. Data are represented as mean +/- s.e.m.

DISCUSSION

My studies revealed that soluble tumor factors decrease paxillin expression and thereby enhance the migration of CE cells that drives angiogenesis and microvascular invasion. Paxillin knockdown by tumor factors also resulted in a decrease in the expression of NRP2. Overexpression of paxillin subsequently reversed these changes seen in NRP2 expression, as well as associated effects on endothelial cell migration and invasion *in vitro*.

Because different tumors secrete different factors and different ratios of factors, I endeavored to determine the generality of my finding that factors secreted by LLC cells decrease paxillin and NRP2 expression. LLC cells secrete many growth factors including a modest amount of VEGF (Satchi-Fainaro et al., 2005). Because I observed changes in both endothelial cell migration and vascular network formation, which are regulated by VEGF and its downstream signals (Lamalice et al., 2007), I chose to explore whether VEGF is the factor responsible for controlling paxillin expression. Indeed, *in vitro* studies confirmed that VEGF alone is sufficient to decrease expression of paxillin and NRP2, albeit to a lesser degree than the LLC factors. These results were corroborated by RNA-seq studies that also showed that the effects of VEGF were specific in that they did not affect the expression of other FA proteins such as vinculin, talin or FAK. Others have shown that TGF-β controls the expression of paxillin in endothelial cells (Walsh JE and Young MR, 2010). Although I did not explore this possibility in our system, it is possible that other soluble factors that are often deregulated in tumors, including TGF-β, might also contribute to control of paxillin expression by LLC-conditioned medium.

Interestingly, my finding that soluble tumor-derived factors decreased paxillin and NRP2 expression *in vitro* and *in vivo* is consistent with past reports which showed that paxillin is decreased in lung, breast and colorectal tumors, among others (Deakin et al., 2012; Salgia et al., 1999; Yang et al., 2010). This seems to be controversial as paxillin expression also has been reported to be

elevated in lung, prostate, breast, cervical and other tumors (Deakin et al., 2012; Jagadeeswaran et al., 2008; Mackinnon et al., 2011; Salgia et al., 1999; Sen et al., 2012). These conflicting results may be because these studies did not address the specific expression of paxillin in tumor endothelial cells compared to other cellular components of cancers (e.g., parenchymal tumor cells, stromal fibroblasts, immune cells, endothelial cells) that can respond in different ways to the same tumor factors. Alternatively, there might be differences in growth factor secretion and/or requirements among these different tumor types.

The dual chamber Matrigel implantation model I used in which tumor cells are separated from the host tissue by a permeable membrane allowed me to probe tumor-factor driven chemotaxis. In tumors, endothelial cells exist in an environment where tumor cells can both secrete chemical factors and modulate ECM components and mechanics. Consequently, changes in ECM stiffness due to elaboration of degradative enzymes, exertion of contractile forces by stromal cells (e.g., fibroblasts, pericytes) and exposure to underlying interstitial collagen can all modulate endothelial cell behavior by altering the level of forces balanced across transmembrane integrin receptors when they interact with the ECM. Alterations in this force balance can modulate FA position and signaling that, in turn, controls cytoskeletal reorganization, directional migration (Xia et al., 2008) and capillary morphogenesis (Davis and Senger, 2005; Rhodes and Simons, 2007). Tumors have been shown to exhibit different mechanical properties relative to normal tissue, including increased ECM stiffness (Levental et al., 2009; Mammoto et al., 2013; Polacheck et al., 2013) and remodeling of the ECM in the tumor microenvironment (Murdoch et al., 2008), which can lead to cytoskeletal restructuring and activation of signaling cascades (Ingber, 2002; Mammoto et al., 2013). Past work, as reviewed here, suggests that paxillin plays a critical role in integrating soluble and physical cues to elicit directed cell motility (Plotnikov et al., 2012; Polacheck et al., 2014; Sero et al., 2011). So modulation of paxillin expression by soluble tumor factors could result in deregulated durotaxis (through altered cellular mechanotransduction) as well as chemotaxis.

MATERIALS AND METHODS

Materials

rhVEGF was from R&D Systems (Minneapolis, MN, USA).

Cell Culture

LLC cells were cultured in DMEM with 10% FBS (Gibco, Invitrogen, Carlsbad, CA, USA). LLC-conditioned media was generated from confluent LLC monolayers after 24 hours of culture in 0.5% serum EBM2. To stimulate cells with VEGF, I added rhVEGF to 0.5% serum EBM2 to obtain a concentration of 30 ng/ml.

Molecular Biological Assays

Over-expression of human paxillin (pSport-Paxillin, Open Biosystems) and human NRP2 (gift from Michael Klagsbrun, Boston Children's Hospital, MA (Shimizu et al., 2008)) were performed using Superfect transfection reagent (Qiagen, Valencia, CA, USA). Transfection with vehicle alone served as control. Effects of over-expression were confirmed via qRT-PCR and Western blotting at 48-72 hours after transfection.

RNA was purified as described. qRT-PCR was performed as described. Primers for paxillin and NRP2 are described in Table 5.

Cell Biology Assays

Transwell migration and invasion assays were performed as described. Chemoattractants were 0.5% serum EBM2 plus 30 ng ml^{-1} rhVEGF or 0.5% serum EBM2 + LLC-conditioned media at a 2:1 ratio.

RNA-seq Experiment

HUVE cells were serum starved and subsequently stimulated with VEGF.

RNA was harvested at t = 0, t = 1 hour, t = 4 hours, t = 12 hours. RNA-seq was performed to determine the RNA level (in fragments per kilobase of exon per million fragments mapped, FPKM) of paxillin, leupaxin, FAK, talin 1, talin 2 and vinculin. Experiment and RNA-seq analysis courtesy of Daniel Day, Peter Park laboratory, Harvard Medical School.

Matrigel Implantation Assay

Matrigel plug mouse implants described previously had an additional, attached, adjacent mold with $1x10^6$ LLC cells encased in 0.22 μm membranes (Whatman, Clifton, NJ, USA) (Fig. 4.3A). Implants were processed as described. Of note, RNA was harvested from Matrigel-containing chamber only, not from tumor-containing chamber. When implants were harvested for immunohistochemistry, tumor-containing chambers were separated from Matrigel chamber plus skin and the latter were fixed, frozen, sectioned and stained.

Statistical Anslysis

Statistical analysis was performed as described.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY

To gain better insight into the mechanism by which directional migration of endothelial cells is controlled during neovascularization and tumor angiogenesis, I explored whether the FA protein paxillin contributes to control of directed cell motility in CE cells because my mentor had previously shown that it is central to directed motility in fibroblasts. I found that paxillin knockdown enhances endothelial cell migration and invasion *in vitro* and *in vivo.* More specifically, utilizing a scratch wound healing assay, I showed that paxillin knockdown decreases directional persistence of endothelial cells while increasing their migration speed. I further determined that this enhanced endothelial cell migration speed and reduced migration persistence increased microvessel density while reducing angiogenic sprout orientation *in vivo*, resulting in microvessel morphology that more closely resembled the tumor phenotype.

To answer the question of *how* changes in expression of paxillin influence angiogenesis, I explored whether paxillin's control of CE cell migration is mediated by changes in expression of VEGF receptors, such as VEGFR2 and NRP2, which are both important for endothelial cell migration and guidance. I found that paxillin knockdown decreases the expression of NRP2 *in vitro* and *in vivo* in endothelial cells possibly by decreasing the nuclear localization of the transcription factor GATA2 (Fig. 5.1A). Importantly, direct NRP2 knockdown (which does not reciprocally control paxillin expression) also increased endothelial cell migration and invasion *in vitro* and *in vivo*. Finally, I endeavored to test whether the paxillin-NRP2 signaling axis is involved in tumor angiogenesis. I found that tumors inhibit expression of paxillin *in vitro* and *in vivo* in endothelial cells by secreting soluble factors, such as VEGF.

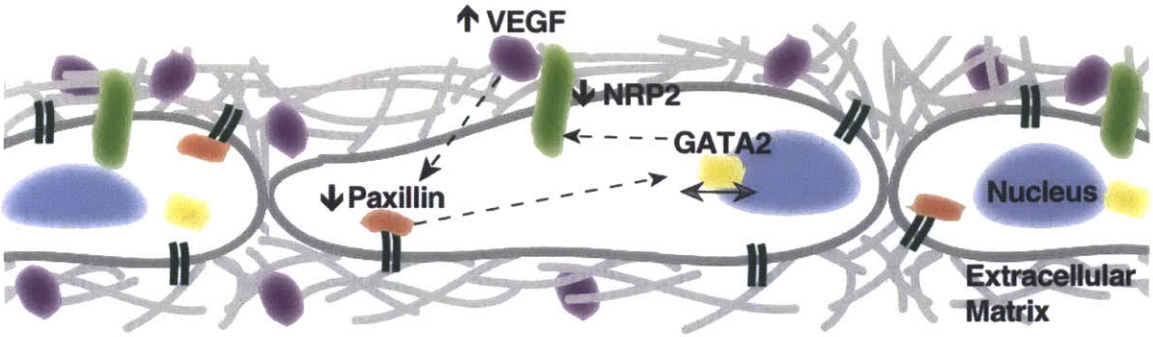

Figure 5.1A Diagram showing VEGF-paxillin-GATA2-NRP2 signaling axis in an endothelial cell. Abundant VEGF, as is secreted by tumor cells, decreases paxillin expression. This influences the intracellular localization of the transcription factor GATA2 toward the cytosol. NRP2 expression is subsequently decreased. The overall effect of this pathway is increased endothelial cell migration and invasion and eventually increased vascular density with loss of microvessel sprout orientation.

As evidenced by my work and other paxillin knockdown studies, I found that paxillin has a dual role in controlling migration: it negatively regulates chemotaxis, but positively regulates mechanotaxis—the directed movement of cells in response to mechanical cues such as substrate stiffness or fluid shear stress gradients. Although the stimuli, cell types and experimental settings are different in these studies, my studies are clearly consistent with the possibility that paxillin integrates chemical signals and mechanical signals to guide migration, but that it differentially regulates mechanotaxis versus chemotaxis. This conjecture is supported by various past studies, as described below. Differential regulation of mechanotaxis versus chemotaxis by paxillin gene suppression increases endothelial cell and fibroblast migration in response to chemical factor gradients (Sero et al., 2011) and promotes chemotaxis in breast cancer migration (Polacheck et al., 2014). Therefore, paxillin appears to negatively regulate chemotaxis (Table 1).

Paxillin is also required for durotaxis since paxillin knockdown in fibroblasts disturbs lamellipodia and FA localization in response to local increases in cytoskeletal stress (Sero et al., 2011). Similarly, blocking critical vinculin-binding paxillin phosphorylation sites prevents durotaxis (the movement of cells up an ECM stiffness gradient) (Plotnikov et al., 2012). Therefore, paxillin appears to positively regulate durotaxis (Table 1). Interestingly, this study did not

find an increase in chemotaxis by blocking paxillin phosphorylation (Plotnikov et al., 2012) suggesting that changes in paxillin expression compared with phosphorylation could have differential effects on migration.

Indeed, a recent study we carried in collaboration with the laboratory of Dr. Roger Kamm (MIT) revealed that paxillin is critical for localization of membrane processes and FAs in breast adenoma cells cultured in 3D ECM gels (Polacheck et al., 2014). Using a microfluidic platform to apply a fluid pressure gradient (at physiologically-relevant levels) through a 3D collagen scaffold, they showed that subsequent to β1-integrin polarization, FAK, F-actin, vinculin, paxillin and F-actin-based motile processes all localize upstream of flow (where flow-induced stress is highest) and cells migrate upstream (Polacheck et al., 2011). However, when paxillin is knocked down, cells reverse their directional bias and migrate downstream. This downstream migration could be driven by autologous chemotaxis where chemokines released by cells themselves accumulate downstream due to fluid flow thereby creating a chemokine gradient that drives downstream migration (Shields et al., 2007). Therefore, in breast cancer cells, paxillin controls the switch between mechanotaxis and chemotaxis (Table 1).

Migration mode	**Cell type**	**Stimulus**	**Effect of paxillin knockdown on migration**	**Source**
Chemo-taxis	Endothelial	Serum + GFs	+	(German et al., 2013)
	Tumor epithelial	Serum + GFs	+	(Polacheck et al., 2014)
	Fibroblast	PDGF	+	(Sero et al., 2011)
Mechano-taxis	Fibroblast	ECM stiffness	- *	(Plotnikov et al., 2012)
	Tumor epithelial	Interstitial fluid flow/pressure	-	(Polacheck et al., 2014)

+ = Pax kd increased migration relative to control
- = Pax kd decreased migration relative to control
* Paxillin phosphorylation sites were blocked in this study to prevent vinculin binding

Table 1 Effects of paxillin knockdown on cell migration

The ability of paxillin to differentially regulate chemotaxis and mechanotaxis is unique among FA proteins. In fact, most proteins important for regulation of migration function by turning on or off migration. For example, FAK knockdown prevents both chemotaxis and invasion (Mitra et al., 2005; Schlaepfer and Mitra, 2004; Wang et al., 2001) and vinculin knockdown prevents invasion (Mierke et al., 2010; Mitra et al., 2005; Plotnikov and Waterman, 2013). In no past study has it been shown that paxillin knockdown *prevents* any type of cell migration. The subtle but critical ability of paxillin to control directionality of migration and do so by integrating both chemotactic and mechanotactic signals is unique. This could be one mechanism by which paxillin knockdown disrupts sprout orientation and increases vessel density. Therefore, paxillin expression appears to govern directional CE cell motility and angiogenesis by modulating the response of endothelial cells to extracellular soluble and mechanical cues.

Paxillin-NRP2 signaling pathway

These studies also led to the identification of a previously unknown paxillin-NRP2 signaling pathway that regulates endothelial cell migration and vascular network development. Typically, approaches to the study of angiogenesis surround pathways downstream of VEGF/VEGF receptor interactions. In this case, I discovered that the FA protein, paxillin, regulates the expression of the VEGF receptor NRP2. The role of NRP2 in migration is complex in that NRP2 can be a pro-migration molecule or a pro-repulsion molecule depending whether its ligand is VEGF or SEMA3F. I observed decreased NRP2 expression when paxillin expression was inhibited. Therefore, this suppression of VEGF/NRP2 interaction could prevent downstream VEGF signaling pathways such as those that regulate cell migration. However, I observed increased CE cell migration upon suppression of the paxillin-NRP2 pathway. The loss of the SEMA3F/NRP2 chemorepulsion interaction also could promote migration, where VEGF signaling could proceed through its major

receptor VEGFR2.

VEGF specifically decreases the expression of paxillin and NRP2, but tumor conditioned media that includes VEGF and other soluble factors further reduced paxillin and NRP2 expression. These findings suggest that the paxillin-NRP2 pathway is regulated by other factors in addition to VEGF. In fact, TGF-β also controls the expression of paxillin in endothelial cells (Walsh JE and Young MR, 2010). A screen of major growth factor stimulators (TGF-β, PDGF, PGF, FGF) of endothelial cells analyzed with RNA-seq or RT-qPCR would allow us to determine which factors are responsible for control of paxillin expression in the future. In fact, datasets might already exist, for example in Gene Expression Omnibus, which could be mined for paxillin expression patterns. Furthermore, CHIP-seq also could be performed to determine which transcription factors are directly responsible for controlling the expression of paxillin.

Relevance beyond angiogenesis

Because directed motility is a critical component of many biological processes such as development, wound healing and immune responses, paxillin-NRP2 regulation of migration could be broadly important to a variety of biological processes. Paxillin is critical during development as paxillin knockout mice are embryonic lethal due to mesodermal development defects (Hagel et al., 2002). I show here that paxillin controls gene expression of NRP2, a neuronal guidance molecule (Chen et al., 2000; Giger et al., 2000) so it is possible that paxillin plays a role in neuronal development. In fact, during embryonic development, neuronal crest derivatives including migrating dorsal root ganglia and trigeminal ganglia express paxillin (Hagel et al., 2002) and it has been postulated that paxillin plays a role in regulating neural crest migration and function of neural crest-derived tissues. In addition, paxillin phosphorylation and up-regulation is initiated upon integrin ligand binding which then results in remodeling of the actin cytoskeleton, cell spreading and neurite formation *in vitro* (Leventhal and Feldman, 1996).

Paxillin is also important for peripheral versus central nervous system stiffness-dependent neuron outgrowth *in vitro* (Koch et al., 2012). Additional studies on the role of the paxillin-NRP2 signaling pathway in neuronal development would aid our understanding of paxillin's role in neuronal cell migration and neural network formation.

Also, it is likely that paxillin is critical for efficient fibroblast-based wound healing, based on our laboratory's *in vitro* fibroblast migration studies (Sero et al., 2011). Consequently, my data demonstrating the relationship between paxillin and NRP2 expression are implicated in a variety of biological processes motivate further investigation of paxillin in wound healing and development as well.

5.2 CLINICAL IMPLICATIONS

Antiangiogenic agents have been proposed to treat diseases characterized by enhanced angiogenesis. In addition to tumorigenesis, these include intraocular neovascular disorders, immunogenic rheumatoid arthritis and psoriasis. For example, retinal ischemia (Alon et al., 1995), proliferative retinopathy secondary to diabetes (Adamis et al., 1994; Aiello et al., 1994) and age-related macular degeneration (AMD) (Jager et al., 2008) are all characterized by increased neovascularization and increased VEGF-A. Importantly, VEGF-A inhibitors including pegatanib and ranibizumab have been approved for clinical use and proven to be beneficial in AMD (Ferrara, 2010). In the case of ranibizumab, vision loss was slowed and gains in vision were observed. However, many patients become resistant to therapy over time, and thus, there is a great need to identify new drugs that work through alternative pathways.

Much work in cancer has focused on developing therapeutic modulators to normalize the tumor vasculature transiently, thereby increasing efficiency of drug delivery (Jain, 2005). For example, VEGF-A targeted antibodies were shown to

suppress tumor angiogenesis and tumor growth in animal models (Kim et al., 1993) which led to the humanized VEGF-A monoclonal antibody, bevacizumab, that was approved by the FDA for cancer therapy (Ferrara and Kerbel, 2005). Other antiangiogenic drugs such as sorafenib, sunitinib and pazopanib inhibit tyrosine kinase inhibitors like VEGFRs and PDGFRs. Again, although these treatments increase progression-free or overall survival, they are not beneficial to all patients and are met with drug tolerance and side effects. Therefore, it is well recognized that it is important to identify new angiogenesis pathways as targets for drug intervention (Chung and Ferrara, 2011).

Recently, NRP2 has been recognized as a viable drug target and modulators of NRP2 have been developed. For example, a mutant NRP2 small molecule peptide that has 8-times the affinity for VEGF as wild type NRP2, decreased VEFG-induced endothelial cell sprouting *in vitro* and inhibited tumor growth when overexpressed in melanoma cells *in vivo* (Geretti et al., 2008). Similarly, an anti-NRP2 antibody developed by Genetech Inc. blocks VEGF-C binding to decrease lymphangiogenesis and tumor metastasis *in vivo* (Caunt et al., 2008). Importantly, these molecules both interfere with NRP2-VEGF signaling. However, my finding that tumor-induced decrease in NRP2 expression leads to enhanced endothelial cell migration, implicates the loss of NRP2-Sema3F repulsive signaling as critical for tumor angiogenesis. Therefore, an alternative or additional clinical target is the NRP2-semaphorin interaction. Finally, NRP2-specific siRNA and shRNA transfected into colorectal tumor cells decreased tumor mets *in vivo* (Gray et al., 2008). My studies again indicate that tumors decrease NRP2 in endothelial cells, so overexpression of NRP2 in endothelial cells may have a clinical benefit by modulating tumor angiogenesis. Previous studies have not addressed the role of NRP2 gene modulation specifically in endothelial cells.

As FA proteins are critical for cell migration and therefore metastasis, many have been investigated as therapeutic targets for cancer (Cox et al., 2010; Desgrosellier and Cheresh, 2010; Frame, 2002; Goldmann et al., 2013; Hao et

al., 2009; Lamalice et al., 2007; Rodríguez Fernández et al., 1993; Schlaepfer and Mitra, 2004). Although there is no drugs in clinical trial that act on paxillin, past findings have shown that modulation of paxillin has potential clinical implications for cancer (Table 2). For example, inhibition of paxillin phosphorylation by the sialytransferase inhibitor Lith-O-Asp or other small molecules suppresses lung tumor progression and metastasis (Chen et al., 2011; Huang et al., 2012) as well as breast cancer progression (Golubovskaya et al., 2008) *in vivo*. Interrupting α4-integrin interaction with paxillin alters migration of leukocytes, lymph endothelial cells and Jurkat T cells (Alon et al., 2005; Féral et al., 2006; Garmy-Susini et al., 2010) and it decreases lymphangiogenesis in tumors (Garmy-Susini et al., 2010).

Mode of Action	**Type**	**Effect**	**Reference**
Increases degradation of paxillin	Antibiotic produced by *Streptomyces luteoreticuli* Thiolutin	Decreases adhesion to vitronectin, decreases angiogenesis	(Minamiguchi et al., 2001)
Decreases phospho-paxillin, -FAK, RhoA-GTP in tumor cells	Novel indole compound SK228	Inhibits cell migration, induces apoptosis, inhibits tumor growth	(Huang et al., 2012)
Decreases amount of Y31 paxillin (and FAK y397)	Sialyltransferase inhibitor	Decreases capillary tube formation, decreases TW migration, decreases lung mets.	(Chen et al., 2011)
Blocks phosphorylation of paxillin by FAK	Small molecule inhibitor 1,2,4,5-Benzenetetraamine tetrahydrochloride	Increases cell detachment and inhibits cell adhesion, dose-dependent breast tumor regression *in vivo*	(Golubovskaya et al., 2008)
Microtubule-blocking agent that decreases phospho-pax exp. and localization to lamellipodia in HUVECs	9-bromonoscapine noscapinoid EM011	Decreases angiogenesis, decreased VEGF expression	(Karna et al., 2012)
(1) Point mutations in LIM domains (C-term) (2) N-pax transfection	Mutations in cortical neurons/ vitro	(1) Abrogates PTP-PEST & integrin interaction with paxillin (2) Decreases neuronal dystrophy - Decreases Aβ-induced neuronal dystrophy. - Dominant negative FAK had no effect on dystrophy	(Grace and Busciglio, 2003)
(1) Mimics binding of	(1) Small peptide	(1) Similar to normal	(Liu et al.,

paxillin to α9 integrin (2) Blocks binding of paxillin to α9 integrin	pentadecapeptide (2) Point mutations in small peptide	interaction, decreases cell spreading in CHO cells, dose dependent. (2) Point mutations block pax-a9 interaction Note that α9 activation can increase migration independent of pax (Young et al., 2001)	2001)
Blocks paxillin-a4-integrin interaction	Small molecule	Changes leukocyte migration to decrease inflammation	(Kummer et al., 2010)
	Small molecule inhibitor	Inhibits migration in Jurkat T cells	(Ambroise et al., 2002)
	A4 mutant Y991A	Decreases cell migration, impairs paxillin-mediated integrin anchorage, resistance to shear	(Alon et al., 2005)
	Knock-in mouse (mutation in α4β1)	Decreases LEC migration, adhesion, lymphangiogenesis Angiogenesis not affected in LLC tumors	(Garmy-Susini et al., 2010)

Table 2 Paxillin-targeted clinical molecules.

Interestingly, these molecules affect phosphorylation activity in the N-terminal region of paxillin (e.g. Y31, Y118, A127), and I found that expression of the N-terminal alone in paxillin knockdown endothelial cells increases their migration. Similarly, our laboratory previously showed that N-terminal expression substantially enhanced 3D invasion compared to intact paxillin when transfected into paxillin knockout fibroblasts, and the N-terminus and C-terminus of paxillin have complementary but opposite effects on cell migration and invasion (Sero et al., 2011) (Fig. 5.2A, Table 3). Thus, modulation of the N-terminus of paxillin could be responsible for mitigated migration and metastasis seen in past studies (Table 2). Together these observations suggest that modifying the expression and activity (i.e., phosphorylation) of paxillin may represent a potential future therapeutic strategy.

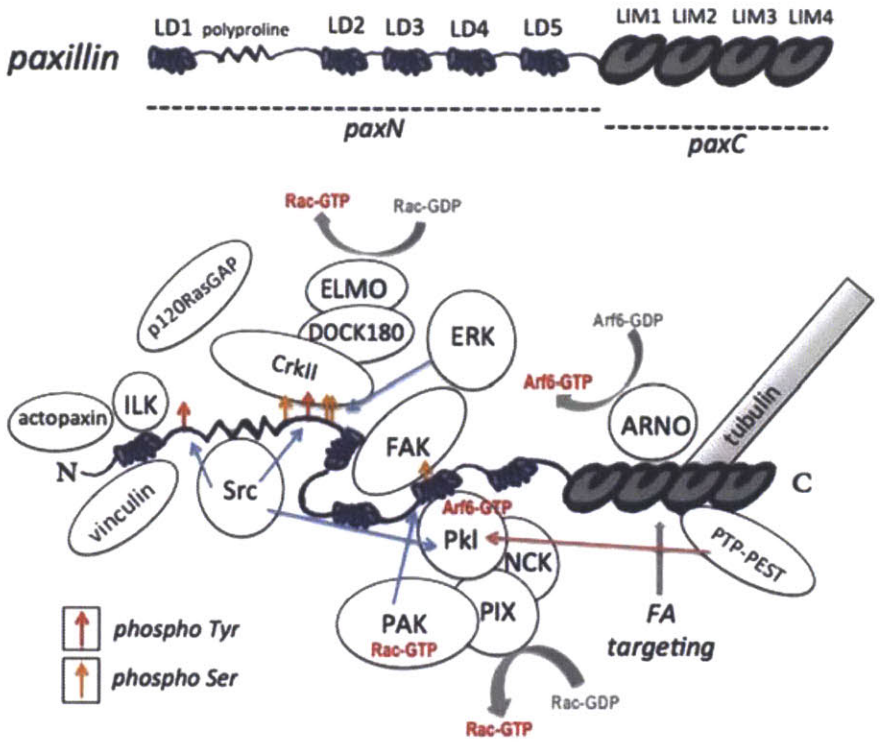

Figure 5.2A Paxillin structural domains and binding partners (Sero et al., 2012).

Terminal	Domain	Phosphorylation and/or interactions	Effect on migration
N	GENERAL		Suppresses membrane extension where appropriate, promotes directionality, increases migration, FA localization
	LD1	Binds actopaxin, ILK, vinculin	Promotes cell adhesion
	Between LD1 & LD2	Calpain cleavage site	Inhibits migration and FA turnover
		Tyr31 phosphorylation by Src/FAK, binds p120RasGAP	Promotes cell migration, promotes adhesion disassembly at leading edge
		Tyr118 phos. by Src/FAK, binds CrkII/DOCK180/ELMO complex (which interacts w/ Rac)	Promotes cell migration, promotes adhesion disassembly at leading edge
		Serine phosphorylation by ERK	
	LD2	Binds FAK (can be concurrent with LD4 and binding to both sites is optimal for signaling)	Promotes adhesion

	LD3	Phosphorylation sites at serine 188/190	Epithelial cells expressing the paxillin S188/190A mutant spread less, have reduced protrusive activity but migrate more actively. (Abou Zeid et al., 2006)
	LD4	Ser273 phosphorylation by PAK, decreased FAK affinity	Localized lamellipodium formation, Rac1 activity
		Rac1-, Src-, FAK-mediated binding to GIT/PAK/NCK/PIX complex (which interacts w/ Rac1)	Cell adhesion and integrin signaling, termination signal for Rac1
		PTP-PEST dephosphorylation of Ser273 = destabilizes PIX/PAK/GIT2	Decreases Rac1 activity
		Ser273 phos. involved in positive feedback regulation of Rac	
C	GENERAL		Promotes lamellipodia formation, suppresses FA formation where appropriate
	LIM2	Binds ARNO (which interacts with Arf6-GTP)	FA targeting
	LIM3	Binds tubulin & PTP-PEST	FA targeting
	LIM 1,2 LIM 3,4	Interacts with β-catenin in Rac-dependent manner	Monolayer integrity, adhesion junctions

Table 3 Paxillin domain interactions (Bellis et al., 1997; Bertolucci et al., 2005; Birge et al., 1993; Cai et al., 2006; Chen et al., 2004; Côté et al., 1999; Deakin and Turner, 2008; Gehmlich et al., 2007; Herreros et al., 2000; Huang et al., 2003; Ishibe et al., 2004; Jamieson et al., 2005; Manabe et al., 2002; Petit et al., 2000; Romanova et al., 2004; Sabe et al., 2006; Schaller and Schaefer, 2001; Tobe et al., 1996; Tsubouchi et al., 2002; Tumbarello et al., 2002; Turner et al., 1999; Turner and Miller, 1994; Webb et al., 2005).

To explore the clinical potential of paxillin as an antiangiogenic target, it would be necessary to carry out studies to elucidate its feasibility in the future. Because I have shown that tumor factors decrease the expression of paxillin in endothelial cells, it would likely be necessary to increase paxillin expression to decrease tumor angiogenesis. This potentially could be accomplished with gene therapy approaches; however, given that the effect of paxillin modulation appears to depend on the cell type, and that disparate changes in paxillin expression and mutations have been observed in many tumors (Deakin et al., 2012; Jagadeeswaran et al., 2008; Mackinnon et al., 2011; Salgia et al., 1999; Sen et

al., 2012), drug therapy inducing changes paxillin expression in tumor cells could have a detrimental effect.

I found that paxillin knockdown increased migration of endothelial cells in the *in vivo* Matrigel plug assay system but decreased migration of fibroblasts (and had no effect on immune cell migration). Fibroblasts, and particularly tumor-associated fibroblasts secrete proangiogenic factors such as VEGF and FGF and chemoattractants (Dong et al., 2004; Hlatky et al., 1994; Pietras et al., 2008). It is important to note that if antiangoigenic therapy via paxillin modulation is pursued, the effect on fibroblast migration would need to be carefully considered. Changes in the number of fibroblasts and their migratory potential could either enhance the effects of paxillin modulating therapy or undermine it. One solution to this potential pitfall would be to target paxillin-modulating molecules specifically to endothelium (e.g., using targeted nanoparticles).

In summary, I have demonstrated that paxillin controls directional migration of endothelial cells as well as microvessel invasion *in vitro* and *in vivo*, and that its expression is regulated by soluble tumor factors through VEGF-NRP2 signaling. Because paxillin expression is altered in various cancers that are characterized by enhanced angiogenesis, development of modifiers of paxillin expression could represent a new avenue for therapeutic development in cancer and other angiogenesis related diseases.

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APPENDICES

TABLE 4

Target sequences for human and mouse siRNA.

Human paxillin #1 (target sequence in the paxillin C-terminus)	5'-GUGUGGAGCCUUCUUUGGU-3'
Human paxillin #2 (target sequence in the paxillin N-terminus)	5'-CCACACAUACCAGGAGAUU-3'
Human NRP2	5'-CCAGAAGAUUGUCCUCAAC-3'
Human vinculin	5'-GGCAUAGAGGAAGCUUUAA-3'
Human zyxin	5'-CUGGACAUGGAGUUGGACCUGAGGC-3'
Mouse paxillin	5'-GAGCCCUCACCUACCGUCAU-3'
Mouse NRP2	5'-GAGCAGAGAGAAAGAAUAA-3'

TABLE 5

Sequences for primers for qRT-PCR.

	Forward	Reverse
Human paxillin	5'-CTGGCGGACTTGGAGTCTAC-3'	5'-CTCCTCCGACAAGAACACAGG-3'
Human NRP2	5'-CCAGAAGAUUGUCCUCAAC-3'	5'-GUUGAGGACAAUCUUCUGG-3'
Human vinculin	5'-CTCGTCCGGGTTGGAAAAGAG-3'	5'-AGTAAGGGTCTGACTGAAGCAT-3'
Human zyxin	5'-TCTCCCGCGATCTCCGTTT-3'	5'-CCGGAAGGGATTCACTTTGGG-3'
Human β2-micro-globulin	5'-GAATGGAGAGAGAATTGAAAAAGTGGAGCA-3'	5'-CAATCCAAATGCGGCATCTTCAAAC-3'
Mouse paxillin	5'-GGCATCCCAGAAAATAACACTCC-3'	5'-GCCCTGCATCTTGAAATCTGA-3'
Mouse NRP2	5'-GCTGGCTACATCACTTCCCC-3'	5'-CAATCCACTCACAGTTCTGGTG-3'
Mouse VEGFR2	5'-GCCCTGCCTGTGGTCTCACTAC-3'	5'-CAAAGCATTGCCCATTCGAT-3'
Mouse cyclophilin	5'-CAGACGCCACTGTCGCTTT-3'	5'-TGTCTTTGGAACTTTGTCTGCAA -3'

APPENDIX A: siRNA or DNA Transfection

Day 1 Seed HUVE cells using 5% EGM2
50,000 cells/well in 6-well plate

Day 2 siRNA or DNA transfection
siRNA transfection: Per well, combine (1 µl siRNA (at 20 µM) + 125 µl OptiMem) with (2 µl Silentfect + 125 µl OptiMem). Let incubate for 20 min at RT and add 250 µl solution/well.

DNA transfection: Per well, combine 1 µl DNA + 10 µl Superfect + 100 µl OptiMem. Let incubate for 10 min at RT and add 110 µl solution/well. Change media in 2 hours.

Day 3 *siRNA transfection:* Change media.

Day 4 Assay

APPENDIX B: Transwell migration assay

Day 1 Seed HUVE cells (see Appendix A)

Day 2 siRNA or DNA transfection (see Appendix A)

Day 3 Change media

Day 4

Day 5 Heat gelatin stock to 37C and dilute to 1% with PBS -/-

Add to 8 µm pore, 6.5mm diameter Transwell insert and incubate 30 min at 37C

Aspirate gelatin and allow residual to evaporate while prepping cells

Harvest cells and make 600,000 cells/ml suspension in 0.5% EGM2

Add 150 µl cell suspension to Transwell insert

Add 600 µl chemoattractant to 24-well plate well

5% EGM2

or 0.5% EGM2 + 30 ng ml^{-1} rhVEGF

or 0.5% EGM2 + LLC conditioned media at 1:2

Incubate overnight at 37C

Day 6 Rinse wells with PBS

Wipe inside of Transwell inserts with Q-tip

Add giemsa solution diluted 1:10 in ddH_20 to wells and inserts

Incubate RT overnight

Day 7 Place inserts on glass slide and count migrated cells using light microscope

APPENDIX C: Transwell invasion assay

Day 1 Seed HUVE cells (see Appendix A)

Day 2 siRNA or DNA transfection (see Appendix A)

Day 3 Change media

Day 4 Thaw Matrigel to 4C on ice overnight

Day 5 Add 75-100 µl Matrigel to 8 µm pore Transwell insert and incubate 30 min at 37C while prepping cells
Harvest cells and make 2,000,000 cells/ml suspension in 5% EGM2
Invert Transwell insert and add 50 µl cell suspension to membrane, incubate 2 hours at 37C to allow cells to settle
Add 600 µl 5% EGM2 to 24-well plate well
Place Transwell insert into wells (right side up), add 150 µl 5% EGM2 to insert and incubate overnight at 37C

Day 6 Aspirate media from well and insert
Add 0.5% EGM2 to 24-well plate wells
Add chemoattractant to inserts
5% EGM2
or 0.5% EGM2 + 30 ng ml^{-1} rhVEGF
or 0.5% EGM2 + LLC conditioned media at 1:2
Incubate overnight

Day 7 Rinse wells with PBS
Wipe bottom of Transwell insert membrane with Q-tip
Incubate at RT with 4% paraformaldehyde in PBS for 30 min
Wash 3x with PBS for 10min on shaker

Permeabilize Matrigel with IF buffer (below) for 30 min at RT

130 mM NaCl, 7 mM Na2HP04, 3.5 mM NaH2P04, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20

Actin and nuclei can be visualized by staining with 1:200 dilutions of Alexa594phalloidin (200 U/ml) and DAPI (5 mg/ml) in Matrigel IF buffer for 1hr at RT

Day 7+ Wash 2x with ddH_20

Place inserts on cover-glass bottom dish (MatTek) and image with scanning confocal microscope

APPENDIX D: Wound healing assay

Day 1 Seed HUVE cells in 24 well plate

Note: For some experiments, PDMS inserts were made from custom molds and placed firmly into wells. Essentially, cells were plated around a 1mm PDMS "barrier" which when removed served as the "scratch"

Day 2 siRNA transfection (see Appendix A)

Day 3 Change media

Day 4

Day 5 Just prior to imaging, use 200 µl pipette tip to "scratch" confluent monolayer

Note: making a scratch is not necessary if using a PDMS insert. Just remove insert

Image at 20x using live cell imaging chamber taking DIC or bright field images at 10 min intervals for > 16 hours.

Note: live cell nucleus stain (Hoechst) and/or UV exposure severely reduced HUVE cell viability and migration

Day 6+ Track cells manually or with software

Analyze persistence time, migration speed, wound closure using Matlab (Lauffenburger and Horwitz, 1996)

Matlab code adopted from Reinhart-King lab/Cornell resources (cellmechanics.org)

APPENDIX E: Tube formation assay

Day 1 Seed HUVE cells (see Appendix A)

Day 2 siRNA (see Appendix A)

Day 3 Change media

Day 4 Thaw Matrigel to 4C on ice overnight

Day 5 Add 100 µl Matrigel to 24 well flat bottom dish wells. Incubate 30 min at 37C while prepping cells
Harvest cells and make 60,000 cells/ml suspension in 5% EGM2 (Note: cell concentration is extremely important in this assay)
Add 150 µl to Matrigel and incubate for 8 hours
Image tubes using bright field microscope

Day 6+ Use ImageJ and Matlab to analyze tube length, number, angle

APPENDIX F: Single chamber Matrigel implantation assay (see Fig. 2.3A)

Day 0 Mix PDMS ingredients well, centrifuge at 1000 RPM for 5 min to remove bubbles

Note: A vacuum chamber can also be used to remove bubbles.

Pour onto 10 cm dish to reach 2 mm height and let cure at 60C overnight

Thaw Matrigel at 4C on ice overnight

Day 1 Remove PDMS sheet from dish and cut into 7 mm x 7 mm squares

Use 4 mm biopsy punch to cut hole in the middle of square

Place PDMS squares on parafilm in 10 cm dish and add ~30 µl Matrigel to hole avoiding bubbles. Cure at 37C for > 30 min

Note: Matrigel stiffness can be modulated by adding the crosslinker transglutaminase (Mammoto et al., 2009)

Implantation

Anesthetize mice and inject local anesthetic subcutaneously to mouse back

Make 1.5 cm incision in mouse skin on back. Cut open fascia if necessary to create pocket for implant

Place implant in pocket as far away from incision as possible

Close wound with > 3 resorbable suture stitches

Note: Implants can be placed in mammary fat pad using the same technique.

Day 2,3 Check wound and re-suture if necessary

Day 4 Inject 1-3 µl of 10-50 µg siRNA directly into Matrigel using microsyringe

Day 5-7 Check wound and re-suture if necessary

Day 8 Euthanize mice using CO_2

Note: To visualize functional vasculature, injection of Concanavalin A into retro orbital vein can be performed 30 min prior to harvesting

Cut skin around implant, keeping implant attached to skin. Follow A) or B)

Note: implants are stable and can be left in mouse for up to 14 days

A) Place Matrigel in 1.5 ml tube of RNA later (spin down and remove supernatant of RNA later prior to harvesting RNA)

B) Place skin + implant in 4% paraformaldehyde in PBS, 4C overnight

Day 9

B) Replace paraformaldehyde with sucrose to displace water, 4C overnight

Day 10

B) Carefully remove PDMS from sample while keeping Matrigel intact and attached to skin. Trim excess skin to make sample as small as possible
Embed in OCT-filled cryomold such that skin+gel plane of sample is facing the top of the mold (e.g. such that when sample block is sectioned, each section contains both skin and gel)
Allow freezing for 1 hr on dry ice
Place in -80C freezer for long-term storage

Day 11

B) Use microtome to cryosection samples into 20-35 μm sections
Store slides at -20C and perform immunohistochemistry

Note: A milk-based blocking solution versus serum-based blocking solution will help reduce the high background due to Matrigel non-specific binding. Overnight blocking at 4C is recommended.

APPENDIX G: Dual chamber Matrigel implantation assay (See 4.3A)

Day -3, -2 Make PDMS squares with holes as described (see Appendix F)

Culture Lewis lung carcinoma cells such that there is enough for 1 15 cm confluent dish/3 implants

Day -1 Make 1 ml PDMS

Cut 0.22 µm Watman membrane into 7 mm x 7 mm squares

Spread thin layer of wet PDMS on PDMS square and place membrane on top of chamber hole

Allow curing at 60C overnight

Day 0 For ½ the PDMS/membrane squares: spread thin layer of wet PDMS on membrane

Note: do not allow wet PDMS to get on membrane section over chamber hole opening or membrane will become impermeable

Stack dry PDMS/membrane square on top of wet PDMS layer such that the order of components is PDMS square, membrane, PDMS square, membrane (Fig. 4.3A)

Allow curing at 60C overnight

Thaw Matrigel at 4C on ice overnight

Day 1 Harvest LLC cells and spin down, keep on ice

Make cell solution such that 1 15 cm dish is resuspended in 1 ml PBS

Spin down again and resuspend in ~100 µl PBS, keep on ice

Inject ~30 µl cell suspension into membrane-encased chamber through the PDMS (do not inject through the membrane) avoiding bubbles/leaks

Add ~30 µl Matrigel to the open chamber, incubate 30 min 37C with small volume of medium in dish for LLC cells.

Implantation

See Appendix F

Note: Orient such that Matrigel chamber is adjacent to skin

Day 2-7 See Appendix F

Note: If injecting siRNA, take care not to pierce membrane (only inject into Matrigel)

Day 8 Euthanize mice using CO_2

Cut skin around implant, keeping implant attached to skin. Remove only LLC-containing chamber

Note: This can be accomplished by separating the chambers with a thin blade or syringe needle before attempting to pull off Matrigel chamber PDMS

See Appendix F

Day 9-11 See Appendix F