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Rebecca G. Oas Date

p120-catenin in vascular development and endothelial adhesion strengthening

by

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p120-catenin in vascular development and endothelial adhesion strengthening

by

Rebecca G. Oas
B.S., Michigan State University, 2002

Advisor: Andrew P. Kowalczyk, Ph.D.

An abstract of
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Abstract

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By: Rebecca Oas

Adhesion between endothelial cells is subject to dynamic regulation to enable both the protection of vascular barrier function and the controlled passage of solutes and white blood cells between the bloodstream and surrounding tissues. Endothelial adherens junctions contribute to vascular morphogenesis and the maintenance of tissue integrity through the homophilic, calcium-dependent binding of vascular endothelial (VE)-cadherin molecules on adjacent cells. p120-catenin (p120) binds to the cadherin cytoplasmic tail and has been shown to regulate the stability of cadherins at the cell surface by preventing their endocytosis and subsequent degradation. The interaction between p120 and cadherins has also been shown to support strong adhesion in cell-based assays. However, the specific role of p120 in vascular development and the nature of its contribution to adhesion strengthening in an endothelial context are not fully understood.

The purpose of this research was to explore the role of p120 in vascular morphogenesis by using a mouse developmental model, and to further explore the contribution of the p120-VE-cadherin interaction in strengthening of cell adhesion. These experiments reveal that p120 is essential to vascular development in the mouse, and that its conditional endothelial knockout results in embryonic lethality in midgestation due to hemorrhaging and defects in the remodeling and organization of vascular networks in the yolk sac, placenta and embryo proper. The loss of endothelial p120 results in a specific reduction in VE-cadherin and N-cadherin levels *in vivo* and is accompanied by a decrease in pericyte recruitment to microvessels in mutant tissues. Furthermore, we identify a proliferation defect in endothelial cells lacking p120, and we show that this effect occurs independently of RhoA regulation but is dependent on the expression of VE-cadherin.

Using biophysical approaches, we also present evidence that p120 binding to the VE-cadherin tail supports increased adhesion strength by promoting cell spreading, and we propose that this occurs by localized Rac1 activation and actin reorganization at the cell membrane. These studies reveal that endothelial p120 plays an indispensible role in mammalian development by promoting the angiogenic remodeling, expansion, and stabilization of blood vessels in a cadherin-dependent manner.

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Chapter 1

Adherens junctions in the vascular endothelium

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Part 1: Cadherins and intercellular adherens junctions

1.1 Adherens junctions and the cadherin family

Endothelial and epithelial cells are connected by different types of junctions which enable adhesion and communication of signals between adjacent cells, aid in the establishment of cellular polarity, and confer selective permeability to a cellular monolayer. Gap junctions create channels by which small molecules can pass between neighboring cells, tight junctions regulate permeability across an epithelial monolayer, and adherens junctions initiate contact between cells and regulate the formation of mature junctions, which is essential to the process of tissue morphogenesis. Both tight and adherens junctions provide structural adhesion between cells and associate with the actin cytoskeleton. In epithelial cell layers, tight junctions localize to the apical side of the intercellular contact region and serve as a regional boundary separating the apical surface of the cell from the basolateral surface, with adherens junctions localized basally to tight junctions. Endothelial adherens junctions occur throughout regions of intercellular interaction, and their location relative to tight junctions is less clearly demarcated (Bazzoni and Dejana, 2004; Wallez and Huber, 2008). Both adherens and tight junctions are formed the by interactions between transmembrane receptors on adjacent cells. Adherens junctions are characterized by the presence of members of the cadherin family of transmembrane adhesion receptors. In addition to promoting the structural integrity of tissues by mediating cell adhesion, adherens junctions play a key developmental role by enabling cell sorting by type. Since cadherins interact in a homophilic manner, cells expressing the same type of cadherin tend to aggregate with one another, thus enabling tissue organization as cells differentiate.

The cadherin family contains over 80 known members, which include classical cadherins, desmosomal cadherins, protocadherins and others. They are found in vertebrates and invertebrates, and all family members are single-pass transmembrane proteins which are characterized by extracellular cadherin repeat domains. Cadherins were named for their properties as calcium-dependent cell adhesion proteins. In the context of adherens junctions, cadherins engage in homophilic, calcium-dependent trans-interactions with cadherins on neighboring cells and mediate linkage to the actin cytoskeleton through intercellular proteins which form a complex with the cytoplasmic tail. The N-terminal domain of classical cadherins consists of five extracellular cadherin (EC) repeats, each of which must bind to calcium in order to mediate adhesion (Hartsock and Nelson, 2008). The cadherin family is subdivided into type I cadherins, which feature a conserved histidine-alanine-valine (HAV) sequence in their EC1 domain and exhibit a high level of sequence similarity to E-cadherin in that domain, and type II or atypical/divergent cadherins which lack the HAV sequence and display more variance in their amino acid sequences (Nollet et al., 2000). Other cadherins include the desmosomal cadherins desmoglein and desmocollin, protocadherins, and some isolated members not assigned to a larger group. The C-terminal cytoplasmic domain of classical cadherins consists of the juxtamembrane domain (JMD) and the catenin-binding domain (CBD). Both domains interact with a class of proteins called catenins, which are named for their properties as "linkers" – specifically, to the actin cytoskeleton (Figure 1.1). Further study has revealed that the roles of catenins in adherens junctions are far more complex, and this will be explored further below.

Vascular endothelial cadherin (VE-cadherin) is the principal cadherin family member found in endothelial cells, although they are also known to express neuronal (N)-cadherin and T-(or H)-cadherin. Unlike epithelial cells, endothelial cells do not express the desmosomal cadherins desmoglein and desmocollin. VE-cadherin was first identified and designated as cadherin 5 through a cDNA study designed to explore the diversity of the cadherin family (Suzuki et al., 1991), and it was subsequently shown to localize to the intercellular junctions of endothelial cells (Lampugnani et al., 1992) and regulate permeability across an endothelial monolayer (Breviario et al., 1995). It is designated as a classical type II cadherin, although sequence analysis reveals it to be a distant relative of the other members of that group (Nollet et al., 2000; Vincent et al., 2004).

In the presence of calcium, which rigidifies the structure of the extracellular portion of the cadherin, the EC1 domains of cadherins on adjacent cell membranes mediate adhesion through a process of "strand swapping" in which the β-strands of interacting EC1 domains are exchanged (Chen et al., 2005). Conserved tryptophan residues are crucial to this process, and their deletion results in a loss of adhesion. Trp-2 and Trp-4 are both necessary in type II cadherins (such as VE-cadherin), while in Type I cadherins only Trp-2 is required (Shapiro and Weis, 2009). Interestingly, VE-cadherin is unusual in that the strand-swapped dimer more closely resembles the interaction seen in Type I cadherins, despite having the requirement for Trp-4 common to Type II cadherins (Brasch et al., 2011).

Much of the information that has been gained by exploring the functions of individual cadherin family members can provide insights into those of other cadherins in

different cell types; however, the regulation of intercellular adhesion can vary both contextually and developmentally within an organism. Therefore, empirical analyses of individual cadherins in cultured cells and animal models are essential to our understanding of cadherin-mediated adhesion and its dynamic regulation.

1.2 Catenins

The catenin family of proteins were first identified and defined as linker proteins which associated with the cytoplasmic domains of cadherins at cell junctions. Most catenins are related by sequence and structure, and are characterized by having Armadillo domains, although α-catenin is a notable exception (McCrea and Gu, 2010). The Armadillo domain is named after the Drosophila protein of the same name, which is a homolog of mammalian β-catenin and plakoglobin (Peifer et al., 1992), and the domain's structure consists of 9 to 12 repeats of about 40 amino acids which fold into a superhelix with a groove that forms a binding interface (Choi and Weis, 2005; Shapiro and Weis, 2009). Within the armadillo family of proteins are three subfamilies defined by their best-known members: β-catenin, p120, and plakophilin. While β-catenin and p120-catenin are commonly associated with adherens junctions and plakoglobins and plakophilins are considered to be desmosomal armadillo proteins, all of them except β-catenin have been shown to localize to both adherens junctions and desmosomes, although with differing affinities (Delva et al., 2009). Catenins perform diverse cellular functions including mediating linkage between cellular junctions and the actin cytoskeleton (as well as the intermediate filament cytoskeleton in the case of desmosomes), influencing cellular morphology and motility through the regulation of

small GTPases, regulating junction complex stability, and translocating to the nucleus to act in conjunction with transcription factors. Since they are of central importance to this research, I will provide a further introduction to the major catenins found at endothelial adherens junctions: β-catenin, and p120.

1.2.1 β-catenin

β-catenin binds to the catenin-binding domain of cadherin family members at adherens junctions (fellow armadillo protein plakoglobin performs an analogous role at desmosomes), and interacts with α-catenin, which interacts directly with actin. In addition to its role as a linker between adherens junctions and the actin cytoskeleton, β-catenin is also a key regulator of Wnt signaling. Although β-catenin binds preferentially to cadherins, the interaction between them is dynamic and modulated by phosphorylation of the cadherin tail (Choi et al., 2006). Cytosolic β-catenin is rapidly degraded via its association with the adematous polyposis coli (APC) protein complex. However, in the presence of Wnt signaling, the cytoplasmic pool of β-catenin is stabilized and able to translocate to the nucleus, where it interacts with the lymphoid enhancer factor and T-cell factor (LEF/TCF), which are DNA-binding proteins, to regulate transcription of Wnt target genes. This process is important in epithelial-to-mesenchymal transition (EMT) in which cells abandon their epithelial layer morphology and become motile as individual cells. Consequently, mutations that result in heightened Wnt signaling are correlated with poor prognoses in cancer (Brembeck et al., 2006; Heuberger and Birchmeier, 2010).

1.2.2 p120-catenin

p120 was first discovered as a Src phosphorylation substrate in conjunction with the process of transformation (Reynolds et al., 1989) and later identified as an armadillo family protein (Reynolds et al., 1992). p120 belongs to a subfamily of armadillo proteins which also includes (ARVCF), δ-catenin, and the more divergent p0071-catenin (Anastasiadis and Reynolds, 2000; Hatzfeld, 2005). p120 was shown to interact with E-cadherin (Reynolds et al., 1994) and other members of the cadherin family, and the disruption of this interaction by deletion of the p120-binding portion of the cadherin tail resulted in compromised cell adhesion strength and blocked the lateral clustering of cadherins on the cell surface (Thoreson et al., 2000; Yap et al., 1998). In a cancer cell line which was p120-null, cell-cell adhesion was disrupted (though not completely eliminated), and a reduction in E-cadherin levels was observed. Both aspects of the phenotype could be rescued by re-expression of p120 (Ireton et al., 2002). This work established p120 as an upstream regulator of cadherin levels, although the mechanism remained unknown. In 2003, work from two groups revealed a specific role for p120 in regulating the localization of members of the cadherin family at the cell surface. The knockdown of p120 by siRNA showed that, once at the plasma membrane, cadherins require association with p120 to remain stable and maintain intercellular adhesion (although p120 family members δ-catenin and ARVCF can perform a compensatory function) (Davis et al., 2003). However, since these other related proteins are expressed at markedly lower levels, they are unable to functionally compensate for the loss of p120 (Mariner et al., 2000). A study from our lab using endothelial cells demonstrated that p120 acts as a rheostat, or set point, for the membrane expression of cadherins in a

specific and dose-dependent manner (Xiao et al., 2003a). Furthermore, the direct binding of p120 to the cadherin juxtamembrane domain was required to block endocytosis (Xiao et al., 2005).

p120 is essential to vertebrate life and its classical knockout is nonviable in mice (Reynolds, 2007) and *Xenopus laevis* (Fang et al., 2004). In the frog, directed p120 loss in the anterior neural region resulted in malformations in the face and eyes due to defects in embryonic morphogenic movement (Ciesiolka et al., 2004). Uniquely, the loss of ARVCF in *Xenopus* resulted in a similar phenotype to the loss of p120, and both proteins were able to rescue the knockout phenotype of the other, indicating important distinct yet overlapping roles for both in ranine development (Fang et al., 2004). In contrast, the Kucherlapati group found that the knockout of ARVCF in the mouse yielded no obvious phenotype (Reynolds, 2007). Studies in invertebrate development revealed a supporting but nonessential role for p120. In *Caenorhabditis elegans*, the one p120 family member is JAC-1, and while the knockdown of 90% of its expression using RNAi did not result in an apparent phenotype, the effects of a mutation in α-catenin were exacerbated when JAC-1 was concurrently knocked down. This resulted in a failure in the elongation process in the developing worm due to defects in maintaining cadherin at cell junctions and linking junctions to actin fibers (Pettitt et al., 2003). Like *C. elegans*, *Drosophila* have only one p120 family member and p120-null mutants are viable and fertile, although the loss of p120 enhances the phenotype of other mutations of adherens junction proteins (Myster et al., 2003). Based on these studies, p120 performs similar functions across a wide variety of species, but its function is only indispensible among vertebrates. For a

more detailed overview of the p120 family of armadillo proteins and their evolutionary roles, this is an excellent review (McCrea and Park, 2007).

Like β-catenin, p120 and other p120 family members are responsive to Wnt signaling (Hong et al., 2010; Park et al., 2005). A yeast-2-hybrid study showed that p120-catenin associates with the transcriptional repressor Kaiso, leading to speculation that it might also translocate the nucleus and regulate gene expression like β-catenin (Daniel and Reynolds, 1999). Subsequently, p120 was discovered to harbor a nuclear localization signal (NLS), and interaction with p120 in the nucleus was shown to relieve Kaiso's repression of the transcription of its targeted genes (Daniel, 2007; Kelly et al., 2004). In contrast to β-catenin, p120 is stable in the cytoplasm (Thoreson et al., 2000), and depending on its subcellular localization, exerts an important regulatory role on the Rho family of small GTPases, which are discussed further below.

1.3 Rho family GTPases

When p120 was overexpressed in fibroblasts lacking cadherins, the resulting phenotype was a dramatic increase in the extension of cellular processes called "branching" (Reynolds et al., 1996). Further work revealed that this dendritic phenotype was suppressed by cadherins (with a specific requirement for the p120-binding juxtamembrane domain) and could be enhanced or suppressed by manipulating the activity of members of the Rho family of small G proteins.

The Rho family of GTPases is also known to play an important role in the assembly of mature adherens junctions. The major subfamilies within this group are

Rho, Rac, and Cdc42, and they are collectively known to regulate actin cytoskeletal dynamics and thereby influence cell shape and movement. Rho family members specifically regulate the formation of actin stress fibers and the formation of focal adhesions and the promotion of cellular motility. Rac proteins regulate actin dynamics at the cell membrane by inducing ruffling and the extension of lamellipodia. Cdc42 regulates filopodia (or microspike) formation. Two studies confirmed the importance of Rho GTPase family members in the formation and maintenance of cadherin-based junctions. Expression of constitutively active Rac in MDCK cells was shown to promote adherens junction formation by increasing E-cadherin levels at the cell surface. In contrast, functionally blocking the Rho subfamily resulted in their inability to form and maintain both tight and adherens junctions. Manipulations of Rho and Rac were notably found to specifically affect actin-linked adherens junctions, while desmosomes (which connect to intermediate filaments) were not disturbed (Braga et al., 1997; Takaishi et al., 1997).

The discovery of p120's ability to influence cell morphology through Rho family GTPases led to the discovery that p120 inhibits RhoA and activates Rac1 and Cdc42. Since p120 can localize to the cell membrane when bound to cadherins and also remains stable in the cytoplasm, its regulation of Rho GTPase activity is in turn dependent on the expression and stability of cadherins. Overexpression of cadherins sequesters p120 at cell junctions, whereas if cadherin is absent, limiting, or uncoupled from p120 by mutation, cytosolic p120 accumulates and leads to the "branching" morphology observed in the E-cadherin-null cells. (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). Additionally, these studies showed that the overexpression of p120 resulted

in an increase in cell motility, which was later shown to be dependent on the inhibition of RhoA (Yanagisawa et al., 2008). The exact mechanisms by which p120 regulates these GTPases is not fully understood. Studies have provided evidence for p120 locally inhibiting RhoA at the cell membrane through p190RhoGap (Wildenberg et al., 2006) and other work supports a direct inhibition of Rho by p120 (Anastasiadis et al., 2000). p120 has also been shown to directly interact with Vav2, a Rho-GEF which in turn activates Rac1 and Cdc42 (Noren et al., 2003; Noren et al., 2000). These experiments indicate some level of redundancy in these regulatory mechanisms. Additionally, the importance of Rho GTPase activity in the process of adherens junction assembly led to the hypothesis that the mechanism by which p120 stabilizes cadherin expression at the cell surface might require localized RhoA inhibition. This hypothesis was supported by a study that demonstrated the necessity for p120 and p190RhoGAP at the plasma membrane during the formation of adherens junctions (Wildenberg et al., 2006). However, a recent study from our group found that the prevention of VE-cadherin internalization is RhoA-independent and instead occurs by preventing interaction of the cadherin tail with the clathrin endocytic complex machinery (Chiasson et al., 2009). The interactions between cadherins, Rho family GTPases, and p120 are very complex, and have been reviewed in greater depth elsewhere (Anastasiadis, 2007; Xiao et al., 2007).

1.4 Regulation of cadherin function by catenins

As summarized above, adherens junctional cadherins mediate intracellular adhesion, link indirectly to to the actin cytoskeleton, and provide a scaffold for the regulation of signaling through bound catenins via the Wnt pathway and Rho family

GTPases. These functions of cadherins are all dependent on their ability to interact with catenins through their intracellular domains. The interaction of cadherins on adjacent cells requires both their stable expression at the cell surface and the presence of calcium to facilitate homophilic binding. However, the discovery that p120 acts as a regulator of cadherin stability at the membrane led to the observation that adhesive defects observed in cells expressing dominant-negative cadherins (lacking functional ectodomains) could be phenocopied by the knockdown of p120 or by abolishing its interaction with the cadherin tail (Xiao et al., 2003a). Furthermore, the clustering of cadherins into adhesive domains within the plasma membrane, and subsequently into mature adherens junctions, occurs in response to the formation of initial contact, but further strengthening of adhesion over time requires the p120-binding juxtamembrane domain, which is sufficient to cause lateral clustering even in the absence of the cadherin ectodomain (Yap et al., 1998). Directly disrupting E-cadherin-p120 interaction was demonstrated to disrupt strong adhesion and the compaction of cells (Thoreson et al., 2000), although a separate study found conflicting results suggesting p120's role in promoting cell aggregation may be less important or dependent on cel type (Ozawa, 2003). Taken together, these findings indicate that interaction with catenins is essential to all the major functions of cadherins, including adhesion via the extracellular domain.

In the absence of trans-interactions, cadherins are endocytosed constitutively in a clathrin-dependent manner and recycled back to the plasma membrane by way of early endosomes or send to lysosomes for degradation (Izumi et al., 2004; Le et al., 1999; Paterson et al., 2003; Xiao et al., 2003b). Association with p120 at the plasma membrane was shown to decrease the rate of internalization. Furthermore, endocytosed cadherins in

endosomal compartments did not colocalize with p120, suggesting that the loss of p120 binding precedes internalization (Xiao et al., 2005). A recent study from our group found that VE-cadherin endocytosis required association with the clathrin adapter protein AP-2, and since p120 is known to inhibit VE-cadherin endocytosis, it is possible that binding of p120 to the cadherin tail physically blocks interaction with AP-2, thus preventing internalization (Chiasson et al., 2009) (Figure 1.2). Although the proposed AP-2 binding site is VE-cadherin-specific, a recent study analyzing the crystal structure of the interaction between p120 and E-cadherin provided further insight into the cadherin-p120 interaction, and did not rule out the possibility that binding by p120 to the VE-cadherin tail could be sufficient to mask the motif which interacts with endocytic machinery (Ishiyama et al., 2010). These findings have underscored the importance of p120 as a regulator of cadherins, and, by extension, of the adherens junctional complex.

Some cell types, such as endothelial cells, express more than one member of the cadherin family. While endothelial cells express both VE-cadherin and N-cadherin, their localization is distinct: VE-cadherin is found at intercellular junctions, whereas N-cadherin is nonjunctional (a phenomenon which only occurs in endothelial cells; in other cell types it serves as a junctional cadherin) (Salomon et al., 1992). Furthermore, VE-cadherin is implicated in the exclusion of N-cadherin from endothelial junctions (Navarro et al., 1998). More recently, a study using p120-uncoupled VE-cadherin demonstrated a necessary function for p120 in regulating the relative distribution of endothelial cadherins, and, specifically, the recruitment of VE-cadherin into lipid raft domains at the membrane (Gentil-dit-Maurin et al., 2010). In addition to the formation of stable

junctions, this membrane-level organization of cadherins may have important signaling implications.

Strong cadherin-mediated adhesion requires connection to the actin cytoskeleton. Blocking the interaction between the cadherin tail and β-catenin has been demonstrated to decrease endothelial barrier function (independently of cadherin levels at the cell surface) (Navarro et al., 1995), and block temporal adhesion strengthening measured in a laminar flow assay (Yap et al., 1998). The specific mechanism by which β-catenin and α-catenin link adherens junctions to the actin cytoskeleton has been a matter of some dispute arising from a study showing α-catenin is unable to bind simultaneously to β-catenin and actin, and proposing a more active role for α-catenin in the regulation of actin at the cell membrane than was previously posited (Drees et al., 2005; Gates and Peifer, 2005; Yamada et al., 2005). A recent study demonstrated the requirement for both α- and β-catenin in the Rap1-dependent enhancement of VE-cadherin-mediated adhesion. In cells stimulated with cAMP, both catenins were required for the actin reorganization which supported VE-cadherin clustering at cell-cell contacts. This work both illustrated the importance of actin regulation in the formation of adherens junctions and provided support for the model proposing direct linkage between cadherins and the actin cytoskeleton through α- and β-catenin (Noda et al., 2010). Additionally, while β-catenin binding to the cadherin tail does not regulate the stability of cadherins (Xiao et al., 2003a), it is an important component of the Wnt signaling pathway, which promotes epithelial-to-mesenchymal transition through transcriptional regulation, including the repression of cadherin expression (Heuberger and Birchmeier, 2010). In summary, all of these findings reveal key roles for catenins in the dynamic regulation of cadherin

function. In particular, the importance of both p120 and β-catenin in strengthening of cadherin-mediated adhesion has been demonstrated in many of these studies. In a later chapter, I will present research exploring the contributions of catenins and the cadherin cytoplasmic tail to adhesion strengthening and cell spreading using a hydrodynamic assay. This work will focus on the mechanism by which the interactions of adherens junction components promote strong adhesion over time in endothelial cells.

Within the context of cultured cells, adherens junctional proteins have been shown to regulate aspects of adhesion, morphology, motility, and transcription. In the following section, I will discuss the role of p120 and the adherens junction complex in the multicellular processes of mammalian growth and development, as well as misregulation in cancers and other disease states, with a focus on the vascular endothelium.

Part 2: Adherens junctions in the vascular endothelium: development, maturation, and pathology

1.5 The vascular endothelium

The development, growth, and maintenance of mammalian tissues are dependent on a steady and locally available supply of blood which in turn carries oxygen and nutrients throughout the body. The vascular system, ranging from the heart to major veins and arteries to networks of small capillaries, is supported by the vascular endothelium, which forms a thin layer lining the interior of blood vessels. Endothelial cells form a single layer over the basal lamina, interact laterally with other endothelial cells, and form a monolayer which is directly exposed to the lumen of the blood vessel. The junctions between endothelial cells must be selectively permeable to allow for the passage of solutes between the vessel and the surrounding tissues. Endothelial junctions are also important to the immune system, as the adhesive interfaces between cells must be dynamically regulated to allow for the passage of leukocytes into and out of the blood vessel (Vestweber, 2008). During the process of vascular growth and remodeling known as angiogenesis, endothelial cells must be responsive to mechanical and chemical signaling cues to proliferate and move as necessary to maintain an uninterrupted lining throughout the entire vascular system. Vascular morphogenesis proceeds from vasculogenesis to angiogenesis in the developing embryo, and angiogenic processes continue throughout adulthood in the form of wound healing, menstrual cycles, and adipose tissue growth (Figure 1.3). The following is an overview of the morphogenic

processes involved in vascular development, maintenance, and pathology, with emphasis on adherens junctions.

1.6 Vasculogenesis and early vascular development

In the early stages of embryonic development, the process of gastrulation gives rise to the endoderm and the ectoderm, with the mesoderm between them. In addition to other supportive and connective tissues, the mesoderm also gives rise to the vascular system. The first stages of vascular development are the formation of blood islands in the yolk sac and shortly thereafter the assembly of early vessels *in situ* within the embryo proper. These blood islands consist of endothelial precursors known as hemangioblasts, which give rise to erythrocytes (blood cells) and, in the case of those making up the outer layer of the blood island, endothelial cells. As these endothelial cells proliferate and migrate, they form connections between adjacent blood islands, giving rise to the primitive vascular plexus (Carmeliet, 2004; Risau and Flamme, 1995). In the developing mouse, the first appearance of blood islands in the yolk sac occurs at E8.25 (Ferkowicz and Yoder, 2005). The vascular plexus, which is established by E9.5, is characterized by a network of vessels of roughly equal size, distinct from the organized hierarchy of large to small vessels which arise as the maturation process proceeds (Figure 1.3 A).

This preliminary stage of vascular development is known as vasculogenesis, which specifically refers to the formation of blood vessels *in situ* as a product of differentiation, as opposed to those which arise from existing vessels and networks, which is known as angiogenesis. Certain major vessels, including the cardinal vein and dorsal aortae, are formed by the vasculogenesis. This process utilizes distinct signaling

molecules, most importantly vascular endothelial growth factor (VEGF). VEGF, also known as vascular permeability factor (VPF) is a member of the platelet-derived growth factor (PDGF) family and each of the five VEGF subtypes is essential in mouse embryonic development, as are the three VEGF receptors and the coreceptors Neuropilin 1 and 2 (Jin and Patterson, 2009). Mice lacking the most common VEGF receptor (VEGFR2, also known as Flk-1) exhibit embryonic lethality at developmental day E8.5-9.0 due to a failure to form blood vessels by vasculogenesis and a lack of blood islands (Shalaby et al., 1995). Soluble VEGF is secreted by a variety of cell types in response to hypoxic conditions, and upon binding to endothelial VEGF tyrosine kinase receptors, induces a variety of cellular responses including motility, proliferation, and increased vascular permeability (Dvorak et al., 1995; Larcher et al., 1998). VEGF is unique among vascular growth factors in that it both acts to induce the formation of new blood vessels and destabilizes existing blood vessels, which can lead to pathological conditions such as vascular leakage and edema associated with cancer or ischemic injury (Weis and Cheresh, 2005). However, this effect can be remediated by the coexpression of other vascular growth factors such as angiopoietin-1 (Ang-1) (Yamakawa et al., 2003). VE-cadherin is known to interact with VEGFR2 and, through resulting signaling, exert a limit on cell proliferation in a contact-dependent manner (Lampugnani et al., 2003). VE-cadherin was subsequently demonstrated to slow the internalization of VEGFR2 which is induced by the binding of VEGF (Lampugnani et al., 2006).

The first expression of VE-cadherin occurs at E7.5 in hemangioblasts, and its constitutive expression in endothelial cells occurs from then onward (Breier et al., 1996). When VE-cadherin expression is ablated in a mouse model, embryonic lethality occurs

by E9.5. VE-cadherin-null mice exhibit high levels of endothelial apoptosis which results in a failure of some major vessels to develop and others to regress or fail to form connections. Because VE-cadherin is absent and unable to interact with VEGF receptors, cells are unable to receive pro-survival signaling from VEGF (Carmeliet et al., 1999; Gory-Faure et al., 1999). These two studies presented the key question of whether VE-cadherin was necessary for the initial formation of blood vessels by vasculogenesis or whether it functioned by relaying the signals necessary to prevent their disassembly. Previous work in cultured VE-cadherin null endothelial cells revealed a defect in their ability to form vascular structures compared to wild-type controls (Vittet et al., 1997), but in the mouse models initial assembly of endothelial cells into structures occurred prior to lethality. Two monoclonal antibodies against VE-cadherin which blocked its angiogenic function with or without an accompanying increase in vascular permeability (Corada et al., 2002; Liao et al., 2000) were applied to mouse allantois explants and demonstrated that the failure of vasculogenesis was linked to increased permeability. This evidence, combined with the observation that certain major vessels in the VE-cadherin-null mouse formed normally before exhibiting defects, led to the conclusion that VE-cadherin is not necessary for initial vascular formation, but is required for maintenance (Crosby et al., 2005).

While it is not specific to vascular tissues, N-cadherin is also found in endothelial cells, and when it is knocked out in the mouse, early vascular defects result. Ablating N-cadherin, which affects neural tissues in addition to the endothelium, resulted in defective yolk sac vasculature and major cardiac morphogenic defects by E9.5, with lethality occurring shortly thereafter (Radice et al., 1997). Unlike the VE-cadherin knockout,

however, the vasculogenic defects outside of the heart appear to be confined to the yolk sac. When the N-cadherin knockout was targeted to the endothelium, mortality was delayed by roughly one day, but cardiac defects were again observed, along with defects in the vascular plexus. Interestingly, VE-cadherin levels were also reduced in the absence of N-cadherin, leading to the conclusion that N-cadherin has an upstream regulatory effect (Luo and Radice, 2005). Subsequent studies, however, have challenged this conclusion – among other discrepancies, the loss of N-cadherin in endothelial cells was not found to affect levels of VE-cadherin, in contrast to the findings of the Radice group (Gentil-dit-Maurin et al., 2010; Tillet et al., 2005).

VEGF has been shown to increase endothelial permeability and induce tyrosine phosphorylation of junction proteins including VE-cadherin, β-catenin, and PECAM-1 (Esser et al., 1998). Further research revealed a specific serine residue found in VE-cadherin (but not E- or N-cadherin) that is phosphorylated in response to VEGF signaling and induces rapid internalization of VE-cadherin (Gavard and Gutkind, 2006). These results reveal further functions for VE-cadherin during early vascular development, as well as providing insight into the mechanism by which VEGF signaling induces endothelial permeability. While such permeability is associated with vascular pathologies later in development, the highly dynamic nature of vascular remodeling during these earlier stages may demand a certain tolerance of porous vasculature, or render increased barrier function impossible, particularly before the onset of blood flow at E8.5.

A detailed overview of developmental knockout models exhibiting defects in vasculogenesis highlights three major areas of importance that are perturbed by genetic manipulation: cell-cell interactions (as evidenced by the VE-cadherin knockout and others), signaling through the VEGF pathway and other receptors for soluble ligands, and cell-matrix interactions (Argraves and Drake, 2005). However, the classical or conditional knockout of other genes involved in endothelial adhesion result in vascular defects during later developmental stages, typically during midgestation, and these will be explored in the following section.

1.7 Angiogenesis and blood vessel maturation

Once the primitive vasculature has been established, it is subject to a process of vascular remodeling known as angiogenesis. In the developing embryo, this is the process which transforms the primitive vascular plexus into an ordered hierarchy from major vessels to capillaries, supports the recruitment of mural cells which stabilize mature vessels, and enables the vascular system to keep pace with tissue growth into adulthood (Figure 3.1 B). The physical processes of angiogenesis include sprouting, intussusception, and pruning. While larger vessels mature by increasing their luminal width, capillaries will enhance their networks by sprouting. Specific endothelial cells lining capillaries become designated as tip cells expressing the Delta-like-4 ligand which interacts with Notch receptors on nearby cells, preventing them from behaving as tip cells. The tip cell's migration is regulated by a VEGF gradient and the growing vessel is comprised of migrating endothelial cells forming a phalanx which follow the tip cell, or supported by increased proliferation (Adams and Alitalo, 2007; Gerhardt et al., 2003).

The tip cells migrate chemotactically toward the wall of an existing vessel or toward another tip cell to join two sprouts into one new vessel. An example of this process is intersomitic vessels, which sprout from the dorsal aortae, extend dorsally, and fuse with the wall of the vertebral artery. Intussusception occurs when the lumen of a vessel is split by the formation of an intussusceptive pillar, and the process of pruning involves the regression of vessels in response to the loss of signaling cues involved in vascular maintenance. This can involve the expression of factors such as Angiopoietin-2 which promote regression of vessels such as the hyaloid vasculature in the eye, which performs a transient function during the growth of the lens (Thomas and Augustin, 2009).

The transition from vasculogenesis to angiogenesis represents a shift from the initial formation of vessels to the maturation and remodeling of vascular networks, and this is accompanied by a change in the dominant signaling molecules directing the process. While VEGF signaling remains important during angiogenesis, angiopoietins 1 and 2 (Ang-1 and Ang-2) and their associated Tie receptors become key players in promoting the stability of vessels in addition to their growth and the proliferation of endothelial cells. The genetic knockouts of Tie1 and Tie2 in the mouse result in embryonic lethality at E13.5 and E10.5, respectively, due to deficiencies in vascular patterning, mural cell recruitment defects, and loss of endothelial barrier function (Rodewald and Sato, 1996; Sato et al., 1995). While the differences in the timing and presentation of their phenotypes show the roles of the receptors are complementary but not redundant, it is notable that in both cases, as well as the double knockout (Puri et al., 1999), the process of vasculogenesis is not perturbed.

While the classical knockout of VE-cadherin induces lethal defects during vasculogenesis during the early stages of embryonic development, its continued expression and function are necessary for the maintenance of blood vessels in later development and throughout adulthood. Even in early development, VE-cadherin performs a necessary role in maintaining vessels and preventing their disassembly (Crosby et al., 2005). The injection of function-blocking antibodies against VE-cadherin into adult mice caused an increase in vascular permeability and edema (Corada et al., 1999). Additionally, some recent work in zebrafish demonstrated some key developmental angiogenic functions for VE-cadherin that were likely masked by early lethality in the mouse. In one study, the partial knockdown of VE-cadherin levels resulted in a dose-dependent phenotype which featured hemorrhaging from leaky vessels and excessive angiogenic sprouting due to a failure of vessels to form completed contacts (Montero-Balaguer et al., 2009). It is notable that although the mouse phenotype was more severe, there was no haploinsufficiency, indicating that a 50% reduction in VE-cadherin levels could be tolerated, in contrast to the requirement for full expression in the zebrafish. Another study examined the mechanism by which VE-cadherin reduced vessel sprouting and found that it was Rac1-dependent and resulted from VEGF signaling which was, in turn, suppressed by VE-cadherin (Abraham et al., 2009). These findings suggest a balance within the antagonistic relationship between VE-cadherin and VEGF signaling from permeability during vasculogenesis to heightened sprouting during angiogenesis, and ultimately to the quiescent state of mature vessels.

As described in previous sections, adherens junction catenins perform a variety of key functions which are dependent on their interactions with cadherin family members in

different cell types, as well as functions independent of cadherin association. Because of the ubiquitous nature of their expression in vivo, analysis of the distinctly endothelial aspects of their function in developing and adult organisms must be carried out using conditional and/or inducible approaches. When β-catenin is conditionally knocked out in the mouse using the Cre-LoxP system and using the endothelial-specific Tie2 promoter to drive Cre expression, the mice die during midgestation between E11.5 and E13.5. These mice exhibited disorganized vascular networks with increased blind-ending vessels compared to control littermates, and some major vessels such as the vitelline vessels had smaller diameters. Furthermore, the mice were frequently hemorrhagic, particularly in areas of high blood flow, evidencing a failure of vascular barrier function (Cattelino et al., 2003).

Conditional mouse knockout studies involving p120 have been performed in several tissues, including neurons and multiple epithelial tissues. The knockout of p120 in the salivary gland resulted in a loss of E-cadherin (by about 50%), which was also seen in other tissues surveyed in the same study, including skin, lacrimal glands, small intestine, and colon epithelia (Davis and Reynolds, 2006). The salivary glands of these mutant mice went on to develop a neoplastic phenotype, which is consistent with previous observations that the loss of E-cadherin is frequently seen in cancers (Reynolds and Carnahan, 2004). Other p120 knockouts in the intestine (Smalley-Freed et al., 2010) and the tooth enamel organ (Bartlett et al., 2010) yielded phenotypes that were classified as cadherin-dependent. In a study in which p120 knockout was targeted to the epidermis, mice exhibited epidermal hyperplasia and a strong inflammatory response. Although E-cadherin loss was observed, the phenotype was instead ascribed to an increase in NFκB

signaling, which was in turn attributed to RhoA activation in the absence of p120's inhibitory effects (Perez-Moreno et al., 2006). One notable aspect of this inflammatory phenotype was cellular hyperproliferation, although a further study from the same group demonstrated that the loss of p120 in cultured keratinocytes (in the absence of heightened NFκB signaling) led to a reduction in proliferation instead (Perez-Moreno et al., 2008). A neuronal p120 knockout caused defects in dendrite formation and a reduction in synapses along those dendrites. In this study, both reductions in N-cadherin levels and aberrant Rho signaling were implicated in the phenotype (Elia et al., 2006). Based on the results of its tissue-specific ablation, it is clear that p120 is required for a wide range of processes during development and beyond. One recurring question in these conditional knockout studies is whether the ensuing defects are dependent on cadherins, GTPases, or both. Due to its key role in regulating VE-cadherin expression in endothelial cells, as well as the potential consequences of Rho GTPase misregulation, the loss of vascular p120 was predicted to cause severe developmental defects in a mouse model system. We tested this possibility by generating a conditional endothelial knockout of p120, and our findings from that study form a major part of this dissertation (Oas et al., 2010).

A key component of the stabilization of mature blood vessels is the recruitment of mural cells, which includes the pericytes that sparsely cover the exterior of capillaries and the smooth muscle cells which coat the exterior of larger vessels (Figure 1.3 C). Mural cells are associated with the maintenance of vascular integrity, and the failure to recruit or maintain associations with them can result in hemorrhage (Braun et al., 2007). Angiopoietins are required for the recruitment of pericytes and smooth muscle cells, and the recruitment of pericytes is further promoted by platelet-derived growth factor (PDGF)

(Cleaver and Melton, 2003) and N-cadherin. In a study using N-cadherin-null murine embryonic stem cells, endothelial differentiation and angiogenic sprouting were not adversely affected, but pericyte recruitment was impaired (Tillet et al., 2005). It is likely that defects in pericyte recruitment would have been observed in the constitutive or endothelial-specific knockouts of N-cadherin, but for the fact that lethality in both cases occurred prior to mural cell investment (Luo and Radice, 2005). In addition to assisting in the maintenance of vascular barrier function, associations between vessels and pericytes have been associated with reduced sprouting (Adams and Alitalo, 2007; Bergers and Song, 2005), which further associates their presence with mature vessels – as does the fact that their recruitment is suppressed by VEGF (Greenberg et al., 2008).

In summary, the process of vascular development can be loosely divided into two stages: vasculogenesis, which is characterized by the differentiation of endothelial precursor cells, VEGF signaling, and a greater tolerance of vascular permeability, and angiogenesis, whose features include a rise in angiopoietin signaling and sprouting and splitting of existing vessels. Following angiogenesis, which continues beyond development, there is an increased requirement for vascular stability and barrier function, which is supported by and mural cell recruitment. However, in some situations this stability can be disrupted, or new vessels can arise abnormally. These pathologies are explored in the following section.

1.8 Endothelial junctions in vascular disorders and diseases

Misregulation of vascular morphogenesis is associated with a variety of disease states, and the hallmarks of these include vascular leakage, inappropriate

neovascularization, and inflammation. Many vascular disorders involve alterations to cell junctions and their maintenance. Some vascular malformations are genetic in nature (see review (Brouillard and Vikkula, 2007)), others arise secondarily from other pathologies. This section will primarily focus on the latter group, although two notable hereditary conditions in which vascular integrity is perturbed are cerebral cavernous malformation (CCM) and hereditary hemorrhagic telangiectasia (HHT), which are both autosomal dominant. In CCM, which is characterized by seizures and headaches, there are defects in the blood-brain barrier and tight junctions are disrupted (Clatterbuck et al., 2001). Based on the signaling pathways affected by perturbations of the genes implicated in this disorder, it is likely that other junction proteins may be involved (Dejana et al., 2009). It has recently been reported that VE-cadherin and other adhesive receptors are upregulated in CCM patient samples (Burkhardt et al., 2010). In the case of HHT, patients have small vascular malformations on the skin, mucous membranes, and other organs, and nosebleeds and intestinal bleeding can also result. The genes identified as causes of this disorder are members of the transforming growth factor beta (TGFβ) signaling pathway, of which VE-cadherin is a positive regulator (Rudini et al., 2008).

Defective vascular barrier function is a common feature of many diseases, and hyperpermeability with resulting edema typically results from VEGF signaling or inflammation, which occur by different mechanisms. In the case of inflammation, endothelial adhesion is disrupted to allow for diapedesis of monocytes from the vascular lumen into the surrounding tissue (Wallez and Huber, 2008). Leukocyte transmigration across the endothelial layer induces a temporary loss of VE-cadherin at the sites of adhesion (Allport et al., 2000), after which the junction is reestablished through a

mechanism that requires the small GTPase Cdc42 (Kouklis et al., 2004). Endothelial permeability resulting from VEGF signaling is dependent on Src family kinases (Dejana et al., 2008; Eliceiri et al., 1999) and occurs in response to hypoxia. In addition to vascular development, hyperpermeability caused by VEGF is found in localized hypoxic regions following ischemic injury (stroke or myocardial infarction) and in some tumors (Weis and Cheresh, 2005).

Aberrant angiogenesis, resulting in disorganized and leaky neovessels, has been associated with VEGF both in ocular disease and in cancers, making the VEGF signaling pathway a well-established target for treatments. Leaky or hemorrhagic neovasculature of the retina is a feature of several ocular disorders including diabetic retinopathy, retinopathy of prematurity, "wet" age-related macular degeneration, and macular telangiectasias (Bradley et al., 2007). The vasculature of tumors is often characterized by hyperpermeability and unusually high levels of angiogenic sprouting, both of which are associated developmentally with immature vascular networks. When tumor cells express PDGF-B, they are able to recruit pericytes, but the inability of the pericytes to attach normally to blood vessels abrogates their ability to stabilize the vessels and inhibit sprouting (Abramsson et al., 2003). The influence of pericytes on tumor vessel stability, levels of proliferation, and dependence on VEGF signaling, as well as possible implications for metastatic potential, suggest that both antipericyte and propericyte treatments may have context-dependent uses in cancer treatment (Raza et al., 2010).

1.9 Cadherins, catenins, and cancer

In addition to the issue of neovascularization in tumors, adherens junction proteins have long been studied in tumor biology, particularly since the loss of cadherins

is associated with a mesenchymal phenotype and is often seen in cancer cells. E-cadherin has been shown to be absent in many studies of epithelial-derived cancer (reviewed in (Wheelock et al., 2008) and (Birchmeier and Behrens, 1994)), and has been classified as a tumor suppressor as well as a suppressor of metastasis. Cadherin switching, in which the transcription of cadherin isoforms is altered leading to a change in the cadherin family members present at the cell surface, is a feature of non-pathological EMT, but an aberrant shift in the expression of E-cadherin to N-cadherin has also been identified in tumors (Wheelock et al., 2008). While this switch has been shown to occur at the transcriptional level, inducing the overexpression of N-cadherin in epithelial cells led to a reduction in E-cadherin (Islam et al., 1996), and coupled with the finding that cadherins compete for limiting p120 binding (Maeda et al., 2006), it is likely that the loss of E-cadherin could result in an increase in the stability of N-cadherin, which has also been associated with increased motility in a study surveying breast cancers (Nieman et al., 1999). While misregulation of cadherins in tumors has been observed in the presence of p120 expression, it was also noted that p120 expression was also reduced or absent in many tumors (Thoreson and Reynolds, 2002), and the discovery of p120's role in regulating cadherin stability led to increased interest in the possible implications for cancer (Kowalczyk and Reynolds, 2004; Reynolds and Carnahan, 2004; Reynolds and Roczniak-Ferguson, 2004).

In addition to regulating cadherin stability, p120 has been shown to play numerous other roles in cancer biology. In tumors where E-cadherin is lost and p120 is retained, p120 can promote the stability and surface expression of mesenchymal

cadherins such as N-cadherin and also activate Rac at the cell surface. Additionally, p120 uncoupled from cadherins can translocate to the cytoplasm and inhibit RhoA. Both of these functions for p120 have been shown to promote cell migration (Yanagisawa and Anastasiadis, 2006). Further work from the same group revealed that of the several isoforms of p120 that are expressed (all of which can activate Rac1), only the full-length isoform 1 can potently inhibit RhoA, and this is the isoform is associated with increased invasiveness and the potential for metastasis in tumor cells (Yanagisawa et al., 2008). In vascular tumors such as angiosarcomas, VE-cadherin levels are decreased compared to normal vascular tissues or benign hemangiomas (Martin-Padura et al., 1995). In a further study in which vascular tumors were induced in mice, the loss of VE-cadherin was correlated with invasiveness as well as the incidence of hemorrhaging (Zanetta et al., 2005). Kaposi's sarcoma is a highly angiogenic tumor caused by human herpesvirus 8 (HHV8) and most commonly seen in immunocompromised patients such as those with AIDS. Recent work has shown that VE-cadherin junction localization was disorganized and internalization was increased in response to expression of the HHV8 G protein . Furthermore, the authors found that Rac activation, independent of VEGF signaling, was required to induce the vascular permeability that is associated with these tumors (Dwyer et al., 2011). Taken together, these studies provide evidence that cadherins, p120, and Rho GTPases and the interactions between them have profound implications for tumor biology, particularly in the area of invasiveness.

The Wnt signaling pathway, and in particular β-catenin, are also key players in oncogenesis. Since a key aspect of Wnt transcription is the regulation of proliferation,

mutations that lead to increased stability of cytoplasmic β-catenin (such as mutations to APC) have been associated with tumors (Moon et al., 2004). However, cadherin loss alone is not sufficient to induce Wnt signaling, as β-catenin in the cytoplasm is rapidly degraded under normal circumstances. Confirming this, a study showed that the loss of E-cadherin contributes to tumor progression in a Wnt-independent manner (Herzig et al., 2007). Since β-catenin and p120 are both involved in Wnt signaling (with some overlap in the regulatory processes), it is possible that transcriptional misregulation through p120 and Kaiso may also have effects in cancers (Hong et al., 2010).

In summary, the vascular adherens junction complex performs a variety of essential functions throughout development, including maintenance of barrier function, responses to growth factor signaling, and transitioning from primitive to mature blood vessels. In addition to forming interendothelial junctions, vascular cadherins also mediate key interactions with other cell types, such as mural cells. Perturbations to endothelial junctions are seen in many vascular pathologies including tumor neovasculature. Furthermore, the interactions between cadherins and catenins at the cellular level introduced in earlier sections have profound implications for the invasiveness and progression of cancers. In the following chapters I will present two studies in which the functions of p120 in murine vascular development and the strengthening of endothelial adhesion are examined.

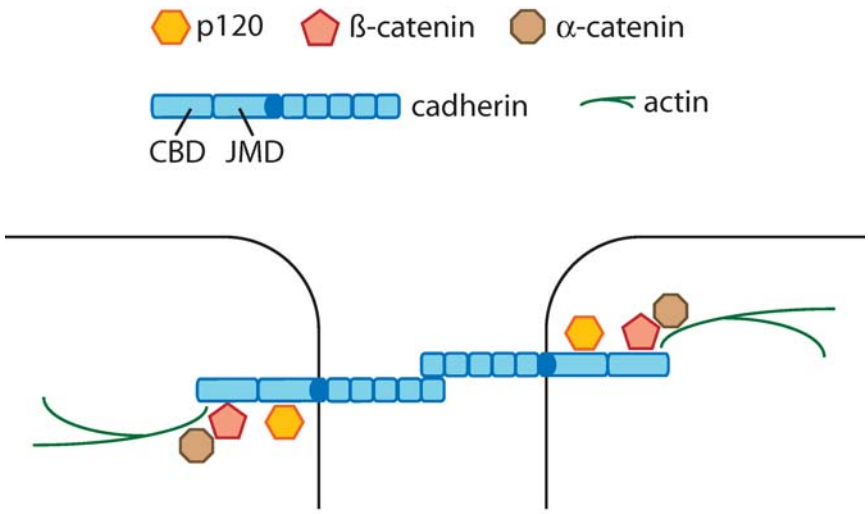

Figure 1.1

Figure 1.1

The adherens junction complex. Adherens junctions are characterized by the presence of members of the cadherin family, which mediate calcium-dependent, homophilic adhesion between cells. The extracellular (EC) part of the cadherin consists of five repeats, the most distal of which (EC1) is involved in contacting cadherins on adjacent cells. Inside the plasma membrane, the cytoplasmic tail consists of two domains: the juxtamembrane domain (JMD) and the catenin-binding domain (CBD). The JMD interacts with p120-catenin and the CBD is bound by β-catenin, which in turn interacts with α-catenin and provides linkage to the actin cytoskeleton. p120 has been shown to prevent the internalization of cadherins, thus stabilizing them at the cell surface.

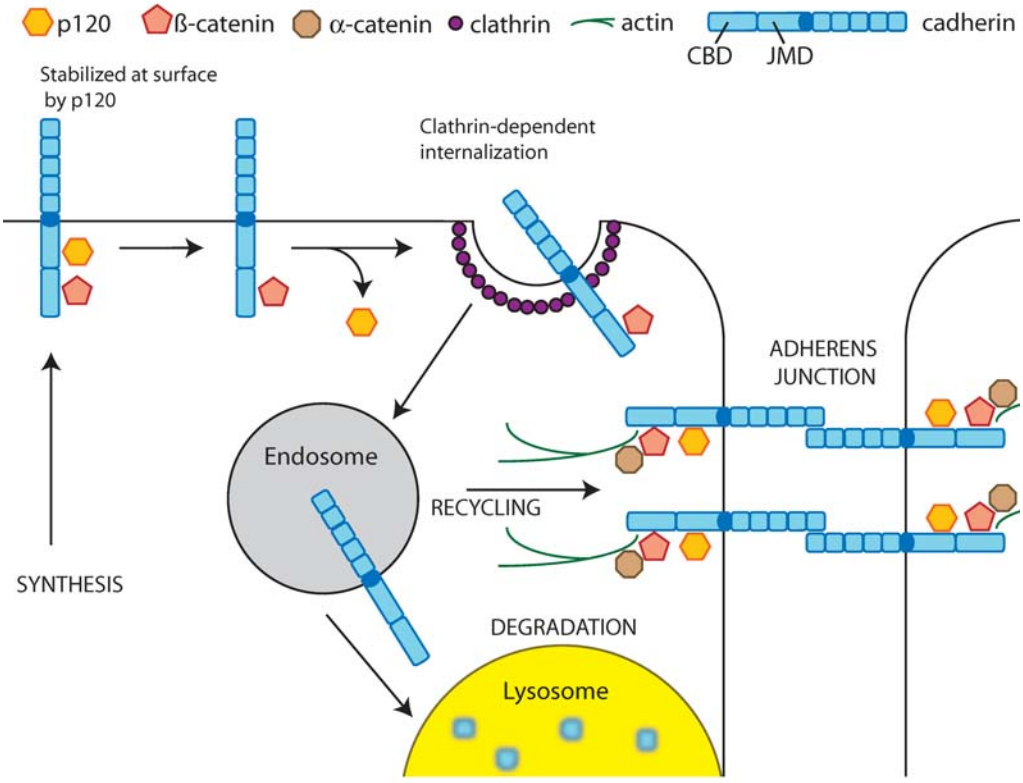
p120
ß-catenin
α-catenin
clathrin
actin
cadherin
CBD
JMD
Stabilized at surface by p120
Clathrin-dependent internalization
ADHERENS JUNCTION
Endosome
RECYCLING
SYNTHESIS
DEGRADATION
Lysosome

Figure 1.2

Figure 1.2

Model of cadherin recycling and stabilization at the membrane by p120. Cadherin dynamics are regulated by several cellular trafficking processes. Following its synthesis, cadherins are sorted through the Golgi apparatus and delivered to the cell surface, where they form junctional complexes. If the stabilizing interaction between the cadherin juxtamembrane domain and p120 is disrupted, the cadherin is endocytosed through a clathrin-dependent mechanism and delivered to an endosomal compartment. From there, it is either sent to the lysosome and degraded or recycled back to the cell membrane to form junctions. This process of turnover, which occurs throughout development and is required to maintain intercellular interactions, is subject to regulation and allows for the alteration of adhesion in response to morphogenic, inflammatory, or pathologic cues.

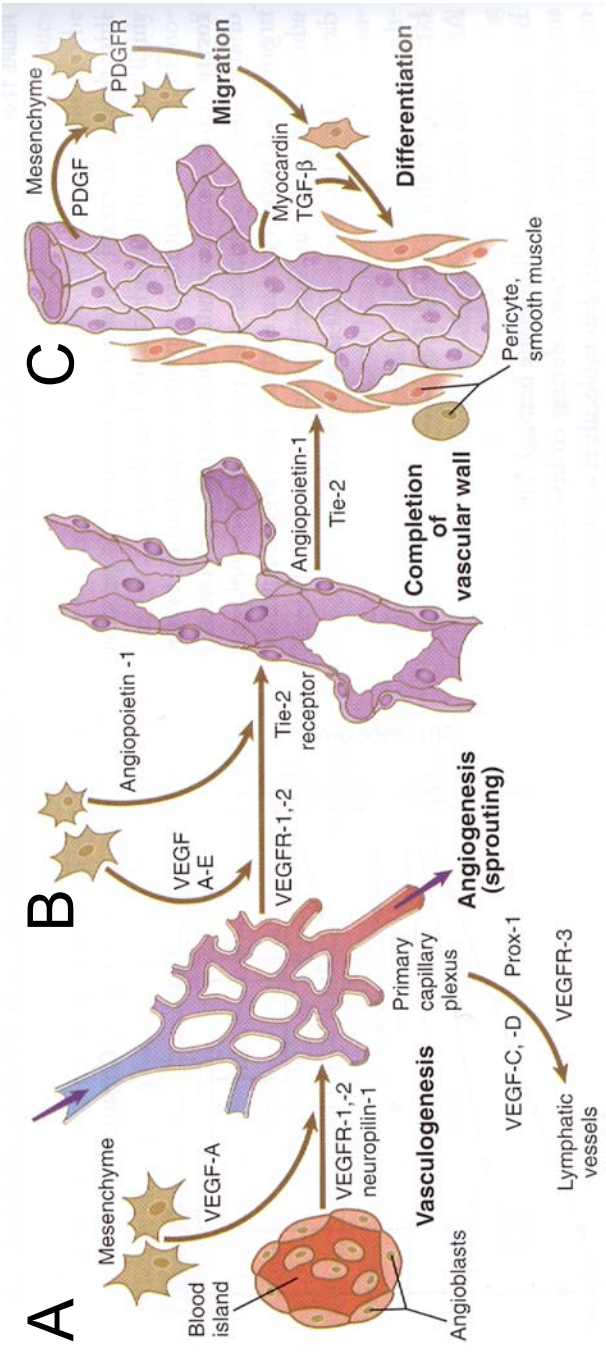

Figure 1.3

(From Carlson, B.M. Human Embryology and Developmental Biology (4th ed.), p. 442, Elsevier, 2009)

Figure 1.3

Model illustrating the processes of vasculogenesis, angiogenesis, and vascular wall stabilization. (A) During vasculogenesis, angioblasts expressing VEGF receptors respond to VEGF secreted from the mesenchyme to form into the primitive vascular plexus. Early endothelial cells respond to additional signaling and begin to form sprouts. (B) During angiogenesis existing primitive vessels are remodeled into a mature vasculature through sprouting, pruning, and intussusception. In this stage, the signaling shifts from VEGF to angiopoietins and the Tie-2 receptor, which promote growth without increasing vascular permeability. (C) Once mature blood vessels are established, mural cells such as pericytes are recruited to stabilize the vessel and enhance its ability to withstand increased blood flow. These mural cells are induced to differentiate from the surrounding mesenchyme by signaling through myocardin and transforming growth factor (TGF)-β. Adapted from (Carlson, 2009).

Chapter 2

p120-catenin is required for mouse vascular development

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2.1 Introduction

Vascular endothelial cells line blood vessels and regulate the movement of solutes, fluids, and cells between the plasma and tissue extracellular space. In addition, endothelial cells are active participants in inflammatory responses and wound healing and undergo dynamic alterations in cell surface adhesive potential, migratory activity, and proliferative capacity. Endothelial cell adhesion molecules have long been appreciated to play key roles in vascular biology and pathophysiology. In particular, vascular endothelial cadherin (VE-cadherin) has been implicated in the regulation of vascular barrier function (Bazzoni and Dejana, 2004; Dejana et al., 2008; Vincent et al., 2004), inflammatory cell transmigration (Wallez and Huber, 2008), and endothelial cell proliferation and morphogenesis during neovascularization (Czirok et al., 2008).

VE-cadherin mediates adhesion through homophilic, calcium-dependent interactions between neighboring endothelial cells and couples this adhesive activity to the actin cytoskeleton at the adherens junction (Bazzoni and Dejana, 2004; Vestweber, 2008). Cytoplasmic interactions between the cadherin tail and armadillo family proteins such as β-catenin, plakoglobin, and p120-catenin (p120) are thought to regulate cadherin adhesive function (Pokutta and Weis, 2007; Vincent et al., 2004). β-catenin and plakoglobin have been shown to mediate associations between cadherins and the cytoskeleton (Hartsock and Nelson, 2008; Miyoshi and Takai, 2008), although the precise molecular interactions that lead to this linkage remain unclear. p120-catenin binds to the juxtamembrane domain of classical cadherins (Thoreson et al., 2000; Yap et al., 1998). First discovered as a Src phosphorylation substrate (Reynolds et al., 1989), p120 was

later shown to be an armadillo family protein (García et al., 1998). A central function of p120 is to regulate cadherin stability (Reynolds and Carnahan, 2004). Previous studies have shown that p120 prevents clathrin-dependent endocytosis of VE-cadherin and thus stabilizes VE-cadherin at the plasma membrane (Chiasson et al., 2009; Xiao et al., 2005; Xiao et al., 2007). Through this activity, cellular levels of p120 act as a set point, or rheostat, for control of cell surface and steady state cadherin expression levels (Davis et al., 2003; Iyer et al., 2004; Xiao et al., 2003a). Additionally, p120 is an important regulator of members of the Rho family of small GTPases (Anastasiadis, 2007), and functions in the nucleus to regulate transcription through interactions with Kaiso (Daniel, 2007).

While many of these activities of p120 have been elucidated using in vitro studies, the functions of p120 in vivo are less clear. In Drosophila (Myster et al., 2003; Pacquelet et al., 2003) and C. elegans (Pettitt et al., 2003), p120 plays a supporting but nonessential role in cadherin stability and cell adhesion. However, global loss of p120 is lethal in vertebrates, resulting in severe morphogenetic defects in Xenopus (Ciesiolka et al., 2004; Fang et al., 2004), and embryonic lethality in mice (Reynolds, 2007) and zebrafish (Montero-Balaguer et al., 2009). Tissue-specific p120 ablation in the mouse likewise results in a variety of defects. The conditional knockout of p120 in the salivary gland using the Cre/LoxP system resulted in disorganized ducts, reductions in E-cadherin levels, and the formation of epithelial masses that followed a cancer-like growth progression (Davis and Reynolds, 2006). Conditional p120 knockout in forebrain neuroepithelia resulted in reduced density of neuronal spines and synapses, an effect owing more to the misregulation of Rho GTPases than changes in N-cadherin levels (Elia

et al., 2006). Furthermore, an epidermal conditional p120 knockout mouse displayed a chronic inflammatory response caused by NFκB activation, also likely downstream of altered regulation of Rho family GTPases (Perez-Moreno et al., 2006). These and other unpublished results demonstrate that tissue-specific ablation of p120 produces a wide range of phenotypes with differing degrees of severity.

The role of p120 in mammalian vascular development has not been addressed. In order to explore the functions of this junction protein in vivo, a conditional mouse knockout approach was utilized to ablate endothelial p120 expression. The results presented here demonstrate that p120 is essential for vascular development and remodeling and that its conditional endothelial ablation results in embryonic lethality. Mice lacking endothelial p120 exhibit a reduction in VE-cadherin and N-cadherin levels, as well as hemorrhages, decreased microvascular density, reduced pericyte coverage, and disorganized vascular networks in both embryonic and extraembryonic tissues. These findings reveal a fundamental role for p120 in vascular development and endothelial function in vivo.

2.2 Materials and Methods

Animals: Mice (*Mus musculus*) with LoxP sites inserted in introns 2 and 8 of the p120 gene were generated as described previously (Davis and Reynolds, 2006). Tie2-Cre expressing C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) #004128 (Peifer and Yap, 2003). All animal care and experimentation was performed in accordance with local and national regulations. For viability assessments, embryos that were discolored and partially or completely reabsorbed were scored as nonviable. All comparisons were made between mutant and wild-type littermates.

Tissue Processing and Staining: Tissue samples from adult and embryonic mice were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A., Torrance, CA) and cut in 5μm sections using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). Sections were then mounted on glass Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA), and fixed using methanol (Acros Organics, Geel, Belgium) or 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline with calcium and magnesium (PBS+) containing 2% bovine serum albumin (BSA) (Fisher Scientific), followed by permeabilization with 0.1% Triton (Roche Diagnostics Corporation, Indianapolis, IN) in PBS+, then subsequently stained. E9.5 embryos used for dorsal aorta analysis were incubated in 20% sucrose in PBS+ overnight following paraformaldehyde fixation, then embedded and cryosectioned. Whole-mounted embryos and yolk sacs were also fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton, and stained before mounting on slides. Paraffin-embedded sections from mouse embryos were also stained using hematoxylin and eosin.

Mouse anti-pp120 monoclonal antibody (mAb), rat anti-mouse VE-cadherin (CD144) mAb, and rat anti-mouse PECAM-1 (CD31) mAb were purchased from BD Biosciences (San Jose, CA). Rabbit polyclonal anti-NG2 chondroitin sulfate proteoglycan antibody was purchased from Millipore (Temecula, CA). Mouse anti-claudin 5 antibodies were obtained from Zymed (San Francisco, CA). Rat anti-mouse VE-cadherin mAb BV13 was a gift from Dr. E. Dejana (FIRC Institute of Molecular Oncology, Milan, Italy).

Microscopy: Whole unfixed embryos were photographed with a QIMAGING Retiga EXi-Fast camera and a Leica M2FLIII microscope (Leica Microsystems). Image manipulation was carried out using Adobe Photoshop CS software (Adobe Systems Incorporated, San Jose, CA). Stained tissue sections were analyzed using either a wide-field fluorescence microscope (model DMR-E; Leica, Wetzlar, Germany) equipped with narrow bandpass filters and a digital camera (model OrcaER; Hamamatsu Corporation, Sewickley, PA) or an inverted Leica DMI-6000B microscope equipped with an Infinity II confocal scanning module, 561- and 491-nm lasers, and a Hamamatsu CCD camera (C9100-12). Images were captured and processed with Simple PCI software (Hamamatsu).

For analysis of relative cadherin levels, line scan pixel intensity plots were generated from linear regions of interest (ROI) drawn perpendicular to vessels in Simple PCI. The peak fluorescence of the marker of interest (VE-cadherin, PECAM, or N-cadherin) was recorded along with the corresponding peak fluorescence of p120 from the image intensity 2D profile. Linear ROIs were repeated at 5 or 10 pixel increments along

the entire vessel. A minimum of 150 paired data points were collected per field for 3 mosaic E10.5 embryos for VE-cadherin and PECAM comparison. Paired data points were sorted into p120 positive and negative groups based on a background threshold peak fluorescence measured from a non-vascular area. VE-cadherin or PECAM-1 fluorescence levels were plotted relative to p120 fluorescence. A minimum of 75 paired data points were collected from 2 control and 2 mutant E11.5 embryos and average N-cadherin fluorescence of p120-positive vessels and p120-negative vessels was compared.

Yolk sac vascular network analysis: Analysis of vessel branch point number and blind-ending vessels per field was performed by a blinded observer on paired mutant and control whole-mounted yolk sacs from five separate litters of E12.5 embryos. Branches and blind-ending vessels per field were averaged and a t-test was performed in SigmaPlot. For morphometric analysis of vascular networks, a magnification bar image was superimposed on images of PECAM-1 stained whole mount yolk sac samples in Adobe Photoshop CS to measure the diameter of a minimum of five vessels and adjacent avascular space per field for two mutant and two control E12.5 embryos. Vascular diameter (D_V) measurements were made from a straight line drawn perpendicularly across the vessel at a point equidistant from adjacent vessel branches. A contiguous line drawn to bisect the neighboring avascular space into approximately equal halves was measured as the paired avascular diameter (D_A) (DeFouw and DeFouw, 2000). The paired measurements were plotted (D_V vs. D_A) and a linear regression was performed in SigmaPlot. The average ratio, D_V/ D_A, was also compared between mutant and control groups.

Cell culture: Endothelial cells were obtained using methods previously described (Lim and Luscinskas, 2006; Springhorn et al., 1995). Briefly, hearts and lungs were removed from mice of the $p120^{flox/flox}$; cre^{-} genotype between 8 and 10 days of age, or skins were removed from mice at 3 days of age. Tissues were finely minced and incubated with 2mg/mL collagenase type I (Worthington, Lakewood, NJ) at 37°C on a shaker for 30 minutes before trituration with a cannula. To purify endothelial cells, magnetic Dynal® Dynabeads (Invitrogen, Carlsbad, CA) were coated with PECAM-1 mAb (BD Biosciences) and added to the cell suspension. The beads and attached cells were washed to remove non-endothelial cells. Purified cells were plated on dishes coated with 0.1% gelatin or fibronectin (Sigma-Aldrich, St. Louis, MO) and re-purified during a later passage using magnetic beads coated with ICAM-2 mAb (BD Biosciences). The endothelial identity of the cells was verified by staining with antibodies to PECAM-1 and VE-cadherin (see above). Primary mouse endothelial cells were cultured in high-glucose DMEM (Mediatech, Herndon, VA) with 20% antibiotic/antimycotic solution (Mediatech), 100µg/mL heparin (Sigma-Aldrich), 100µg/mL endothelial cell growth supplement (ECGS) (Biomedical Technologies, Stoughton, MA), 1mM non-essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 2mM L-glutamine (Mediatech), and 25mM HEPES (Mediatech). To induce p120 knockout in cell culture, the cells were infected with an adenovirus expressing Cre (gift from Dr. L. Yang, Winship Cancer Institute, Emory University School of Medicine). Wild-type and mutant p120 adenoviruses were generated as previously described (Chiasson et al., 2009). To ensure turnover of previously transcribed p120, a period of 72 hours was allowed before experiments. Control cells were infected with an empty adenoviral vector.

Endothelial barrier function and proliferation assays: Primary mouse endothelial cells were grown to confluence on Costar 3460 Transwell cell culture chambers (Corning Costar, Cambridge, MA). Following p120 ablation by adenoviral Cre (or infection with an empty adenoviral vector), Texas Red-labeled dextran (0.1 mg/mL) in growth media was added to the upper chamber and fluorescence readings were taken from the lower chamber at regular intervals (every 30 minutes) for two hours using a HTS 7000 Plus BioAssay Reader (Perkin Elmer, Waltham, MA) to measure the rate of dextran diffusion across the monolayer of cells, as described previously (Wang et al., 2003).

The proliferation potential of both p120-null cells and the control cells was evaluated using the in Situ Cell Proliferation Kit, FLUOS (Roche). Briefly, primary mouse endothelial cells were grown to 60-70% confluence on fibronectin-coated coverslips and infected with adenoviral Cre or empty virus. The cells were then incubated with BrdU labeling reagent for 1 hour at 37°. The incorporated BrdU was detected with anti-BrdU FLUOS and DAPI (Sigma-Aldrich) staining was performed to visualize nuclei. The results were analyzed by fluorescence microscopy and the percentages of BrdU positive cells were compared between the p120-null cells and control cells. Cells were also examined for p120 expression by immunofluorescence to verify successful Cre-mediated gene excision.

Western blot: Primary mouse microvascular endothelial cells were isolated from $p120^{flox/flox}$ mice and cultured in complete growth medium. Cells were infected with an adenoviral empty vector or adenoviral Cre recombinase and cultured for 4 days to allow for p120 deletion and turnover. Cells were harvested in Laemmli sample buffer (Bio Rad

Laboratories, Hercules, CA) and samples were boiled for 5 minutes before loading on 7.5% SDS-PAGE gel for protein separation. Proteins were transferred to nitrocellulose membrane for immunoblotting and probed with antibodies against VE-cadherin: (eBioscience, San Diego, CA #16-1441-82 and BD Pharmingen #550548, diluted to 1:100 each), PECAM-1 (Santa Cruz, Santa Cruz, CA #SC-1506), p120 (Santa Cruz #SC-1101), or β-actin (Sigma-Aldrich #A5441). HRP-conjugated secondary antibodies (Bio-Rad Laboratories) were used at 1:3000 dilution and blots were developed with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ #RPN2106) or Amersham ECL Plus (GE Healthcare #RPN2132).

Growth curve: Primary mouse microvascular endothelial cells were isolated from $p120^{flox/flox}$ mice and cultured in complete growth medium. An equal number of cells were seeded into gelatin-coated 1.9cm^2 wells on day 0. Cells were allowed to attach for 4 hours and then infected with an empty adenoviral vector or adenoviral Cre recombinase. On day 1, cells were harvested by trypsinization and counted on a hemocytometer, with at least 3 wells per condition and 4 samples per well counted by two individual observers. Loss of p120 was confirmed in Cre-infected cells by immunofluorescence microscopy. Data are representative of four separate growth curve experiments.

2.3 Results

Loss of endothelial p120-catenin is embryonic lethal

A conditional gene ablation strategy was utilized to define the function of p120 in mouse vascular development. Mice harboring a LoxP-flanked allele of the p120 gene (Davis and Reynolds, 2006) were crossed with transgenic mice expressing Cre recombinase driven by the Tie2 promoter (Figure 2.1 A), resulting in endothelial Cre expression beginning at developmental day E7.5 (Peifer and Yap, 2003). Fewer than expected pups harboring the $p120^{flox/flox}$; Cre^{+} (conditional mutant) genotype were observed in litters (Figure 2.1 B), suggesting that deletion of the p120 gene in endothelial cells resulted in an embryonic lethal phenotype. A series of timed mating experiments revealed that the conditional mutant animals began to exhibit lethality at developmental day E12.5, with approximately 40% lethality observed by E14.5 (Figure 2.1 C). Additionally, a fraction of the conditional mutant pups died shortly after birth. The remaining genotypic mutants survived into adulthood, with some animals exhibiting small size and failure to thrive and others exhibiting no obvious abnormalities.

To verify loss of p120 protein expression, yolk sacs were cryosectioned and examined by immunofluorescence microscopy for p120 and the endothelial adhesion molecule PECAM-1 (platelet/endothelial cell adhesion molecule 1) (Muller et al., 2002). p120 colocalized with PECAM-1 in the vasculature of control mice (Figure 2.2 A-C) but not in vessels of conditional mutant animals (Figure 2.2 D-F). Similar results were observed in embryonic tissue (see Figure 2.3 and Figure 2.7). p120 expression in endothelial cells was also examined in surviving adult animals harboring the conditional

mutant genotype ($p120^{flox/flox}$; Cre^{+}). Analysis of p120 expression in lung tissue of control mice (Figure 2.2 G-I) revealed extensive colocalization between p120 and PECAM-1. p120 expression could also be detected in endothelial cells of surviving mutants. Interestingly, in a mutant animal that was significantly smaller than control littermates, endothelial p120 expression was mosaic and microvascular density was dramatically reduced (Figure 2.2 J-L). In contrast, p120 expression appeared normal in genotypically mutant animals that survived into adulthood with no obvious phenotype (Figure 2.2 M-O). The inefficient deletion of this p120 allele in some animals was also observed in the mammary gland and prostate of mice expressing Cre driven by the MMTV (mouse mammary tumor virus) promoter and in intestinal epithelium of mice expressing Cre using the villin promoter (Reynolds et al., unpublished). Several other genes have been reported to exhibit mosaic deletion using Cre-loxP systems in the mouse (Casper and McCarthy, 2006; Ryding et al., 2001). In the model system reported here, this mosaic deletion of p120 results in reduced rates of lethality. However, we examined many tens of embryos that exhibited efficient p120 ablation as assessed by immunostaining. In the phenotypic analysis described below, we summarize the phenotype representative of animals with complete endothelial p120 deletion. Importantly, genotypically mutant animals survived because of inefficient deletion of endothelial p120 rather than overcoming loss of p120 expression. From these findings, we conclude that the expression of p120 in mouse vascular endothelial cells is required for survival, and loss of p120 in endothelial cells results in embryonic lethality beginning around E12.5.

Deletion of endothelial p120 causes defects in microvascular patterning and hemorrhages

To define the vascular defects resulting from the ablation of the p120 gene in endothelial cells, a series of timed mating experiments was conducted and vessel formation analyzed beginning at E9.5. PECAM-1 staining of transverse sections revealed no apparent defect in dorsal aortae lacking p120 (Figure 2.3 D-F). These findings suggest that formation of large vessels by vasculogenesis proceeds normally in the absence of endothelial p120. In contrast to the dorsal aortae, intersomitic vessels form by sprouting angiogenesis (Coffin and Poole, 1988). Whole-mounted E9.5 embryos from six litters showed no defects in intersomitic vessel organization or vertebral arteries in mutant animals (Figure 2.3 G, H). Thus, major vessels formed by both vasculogenesis and sprouting angiogenesis were indistinguishable in conditional mutant and control embryos at E9.5.

Conditional mutant embryos began to exhibit lethality around E12.5 (Figure 2.1). Therefore, embryonic and extraembryonic tissue was examined at midgestational stages. At E11.5, mutant embryos displayed significant defects in placenta vasculature. In the labyrinthine layer, which contains capillaries of fetal origin(Takata et al., 1997), a reduction in vascular density was observed among conditional mutants (Figure 2.4 C, D) compared to wild type animals (Figure 2.4 A, B). Severe defects in microvascular density were also observed in the embryo proper. By E14.5, conditional mutant embryos were often visibly pale or exhibited a dramatic reduction in vessel density when examined under low power light microscopy (Figure 2.4 E-H). PECAM-1 staining of the

brain of whole-mounted E13.5 conditional mutant embryos (Figure 2.4 J, L) revealed disorganized and less dense vascular networks compared to control littermates (Figure 2.4 I, K). Defects were particularly striking in the hyaloid vascular system of the developing eye (Figure 2.4 M-N). To further investigate the defects in microvascular patterning resulting from p120 loss, time course experiments were performed by examining vascular plexus formation and microvessel remodeling in the yolk sac. Yolk sacs were isolated at developmental time points from E9.5 to E13.5 and stained for PECAM-1 expression. The formation of the initial vascular plexus appeared normal in p120 conditional mutant yolk sacs (Figure 2.5 A, B), whereas subsequent steps in vessel remodeling and expansion were compromised. In control animals, yolk sac vessels remodeled into a well-organized hierarchy of large and small vessels (Figure 2.5 A, C, E, and G). In contrast, conditional p120 mutants exhibited reduced vascular branching and increased numbers of blind-ending vessels (Figure 2.5 I, J). Furthermore, morphometric analysis of vascular and avascular space revealed that mutant vessels failed to form homogeneous networks. As shown in Figure 2.5 K, a linear relationship was apparent between vessel diameter and avascular space diameter in control animals ($r = 0.82$). In contrast, mutant vessels exhibited highly variable avascular space diameter ($r = 0.27$). Together, these data indicate that loss of p120 results in severe defects in vascular patterning and morphogenesis. In addition to defects in vascular organization, the absence of endothelial p120 also resulted in hemorrhages. In E12.5 embryos, hemorrhages were commonly observed in the brain (Figure 2.6 D and G, compared to A) and other organs (not shown) of conditional mutant animals, and histologic staining of brain sections revealed both large hemorrhages (Figure 2.6 E) and leaky microvessels

(Figure 2.6 F, H). These results demonstrate that deletion of endothelial p120 leads to striking defects in mouse microvascular density and patterning as well as compromised vessel integrity in both embryonic and extraembryonic tissue.

Cadherin expression and pericyte recruitment are decreased in p120-null endothelial tissues

Previously, p120 was found to stabilize VE-cadherin expression at the plasma membrane by inhibiting cadherin endocytosis and degradation (Xiao et al., 2005). To determine if deletion of the p120 gene caused a corresponding loss of VE-cadherin in mouse vessels, p120 and VE-cadherin colocalization were examined in embryonic vessels of E10.5 animals (Figure 2.7). In control vessels, VE-cadherin colocalized extensively with p120 (Figure 2.7 A-C). VE-cadherin was also detected in p120-null vessels but appeared to be reduced compared to control vessels (Figure 2.7 D-F). To determine if the relative levels of cadherin were reduced in the absence of p120, we took advantage of the mosaic nature of p120 expression in a subset of embryos, thereby allowing comparison of VE-cadherin levels within the same tissue section. Line scan pixel intensity plots revealed that the levels of VE-cadherin were significantly reduced in vessels that were p120-null (Figure 2.7, G-I, quantified in M). In contrast to VE-cadherin, no alteration in PECAM-1 levels was observed in vessels lacking p120 (Figure 2.7 J-L, quantified in N; see also Figure 2.2). Furthermore, we were unable to detect any differences in expression or localization of the tight junction protein claudin-5 (Figure 2.8).

N-cadherin is also expressed in endothelial cells and plays important roles in vascular development (Luo and Radice, 2005; Tillet et al., 2005). Previous work has shown that p120 also regulates N-cadherin levels (Davis et al., 2003; Elia et al., 2006; Ferreri et al., 2008). Therefore we examined N-cadherin expression levels in E11.5 mouse embryos. In control animals, vessels expressing N-cadherin were readily observed adjacent to the neural tube (Figure 2.9 A-D). However, in mutant littermates, N-cadherin was not detected in corresponding p120-null vessels (Figure 2.9 E-H, quantified in I and J). N-cadherin has been implicated in the recruitment of pericytes to developing vessels (Tillet et al., 2005). These cells associate with endothelial cells and are important regulators of vessel remodeling and stabilization (Hellstrom et al., 2001). Therefore, we surveyed the degree of pericyte coverage of blood vessels in the brains of E11.5 mutant and control embryos using the pericyte antigen NG2 (Tigges et al., 2008; Tillet et al., 2005). Importantly, pericyte coverage was significantly reduced in mutant embryos (Figure 2.10, compare B and E; quantified in G). Thus, loss of p120 leads to a substantial decrease in endothelial cadherin expression levels and is associated with reduced pericyte coverage of developing vessels.

Primary endothelial cells lacking p120 exhibit proliferation defects

To explore the cellular mechanisms underlying the vascular defects in the conditional p120-null mouse, primary endothelial cells were isolated from newborn $p120^{flox/flox}$; Cre^{-} mice. To cause p120 deletion, Cre recombinase was introduced using an adenoviral vector (Stec et al., 1999). Following a 72-hour period to allow for turnover of previously transcribed p120, the cells were fixed and examined by immunofluorescent

microscopy (Figure 2.11). Control cells infected with empty adenoviral vector retained p120 expression (Figure 2.11 A, G). Additionally, these cells also expressed both VE-cadherin (Figure 2.11 B) and PECAM-1 (Figure 2.11 H), confirming the endothelial identity of the cells. Expression of Cre resulted in near complete ablation of p120 (Figure 2.11 D, J). Furthermore, loss of p120 resulted in a striking reduction in VE-cadherin (Figure 2.11 E) but not PECAM-1 levels (Figure 2.11 K) This result was confirmed by Western blot analysis (Figure 2.11 M). In addition to expression of adhesion molecules, cultured mouse endothelial cells were also examined for barrier function and proliferation potential. For barrier studies, monolayers of endothelial cells from $p120^{flox/flox}$; Cre^{-} mice were cultured on filter membranes and infected with adenovirus to express either GFP or Cre. Diffusion of Texas red-labeled dextran across the cell layers was monitored as an assay of barrier function (Figure 2.11 N). Although a slight increase in dextran diffusion was observed in some experiments, we were unable to demonstrate a statistically significant change in dextran flux in p120-null cells compared to controls. However, endothelial cells lacking p120 exhibited significantly reduced growth rates (Figure 2.11 O). These results suggest a role for p120 in endothelial proliferation.

Previous studies have shown that VE-cadherin plays a key role in endothelial growth control (Carmeliet et al., 1999; Caveda et al., 1996; Venkiteswaran et al., 2002), and more recent studies have shown that p120-null keratinocytes exhibit a growth-arrested phenotype. This latter study also implicated p120 inhibition of RhoA as the mechanism underlying this mitotic defect (Perez-Moreno et al., 2008). To determine if the endothelial growth defect in p120-null cells is RhoA- or VE-cadherin-dependent, exogenous p120 and VE-cadherin were re-expressed in p120-null primary endothelial

cells. In addition to wild type p120 1A, a p120 mutant unable to inhibit RhoA (p120 4A K622,628A) (Yanagisawa et al., 2008) was utilized to determine if inhibition of RhoA is involved in regulating VE-cadherin expression levels and/or endothelial proliferation. Similar to the results shown in Figure 2.11, loss of endogenous p120 resulted in significantly decreased VE-cadherin levels (Figure 2.12 C, D) and a corresponding decrease in endothelial proliferation as measured by BrdU uptake (Figure 2.12 K). Expression of exogenous wild type p120 1A rescued both VE-cadherin levels (Figure 2.12 E, F) and BrdU uptake. Furthermore, VE-cadherin levels were also restored by the Rho-uncoupled p120 mutant (p120 4A K622,628A). Interestingly, the Rho-uncoupled mutant also rescued the proliferation defect observed in p120-null cells, suggesting that decreased VE-cadherin expression underlies the reduction in proliferation. Consistent with this interpretation, exogenous expression of VE-cadherin in p120-null cells also restored BrdU uptake. These studies indicate that p120 regulates endothelial proliferation through a cadherin-dependent mechanism.

3.4 Discussion

The findings reported here demonstrate for the first time a central and indispensable role for p120-catenin in mammalian vascular development. Deletion of endothelial p120 results in downregulation of both VE-cadherin and N-cadherin, as well as decreased endothelial proliferation and reduced pericyte coverage of developing microvessels. These alterations are associated with microvascular patterning defects, hemorrhaging and midgestational embryonic lethality.

One of the most striking phenotypes resulting from the selective inactivation of the p120 gene in endothelial cells is the failure of microvessels to properly pattern. The earliest defects in vessel patterning were observed in the placenta at E11.5, where vascular density in the labyrinthine was markedly reduced (Figure 2.4 A-D). Analysis of vascular patterning in the yolk sac at different developmental stages suggests that microvessels form normally, but then either regress or are unable to expand with tissue growth (Figure 2). Morphological analysis of yolk sac microvessels revealed decreased vessel branching and increased avascular space (Figure 2.5 I-K). These observations suggest that microvessel growth into avascular areas is insufficient to keep pace with the rapid midgestational growth of the embryo. Consistent with this notion, deletion of p120 in cultured endothelial cells resulted in significantly reduced endothelial cell growth and proliferation (Figure 2.11 and 2.12). It is likely that the inability of endothelial cells to proliferate efficiently in the absence of p120 explains, at least partially, the defect in vascular density observed in vivo.

In addition to microvascular patterning defects, mutants lacking endothelial p120 also exhibited hemorrhaging, particularly in the brain. Tight junction proteins are essential to maintaining the blood-brain barrier (Sandoval and Witt, 2008), and recent studies have implicated VE-cadherin in the transcriptional regulation of claudin-5 (Taddei et al., 2008), suggesting that alterations in tight junctions may also underlie some of the p120-null defects. However, we were unable to demonstrate any significant alterations in the expression or localization of claudin-5 in the absence of endothelial p120 (Figure 2.8). In addition to tight junctions, pericytes associate with small vessels and have been shown to regulate capillary diameter and vascular permeability in the brain (Casper and McCarthy, 2006). Loss of pericytes in the developing mouse results in lethality due to microvascular hemorrhaging and edema (Hellstrom et al., 2001). Importantly, deletion of p120 resulted in reduced pericyte coverage in mutant vessels in the brain (Figure 2.10). These findings suggest that reduced pericyte recruitment, rather than alteration of tight junctions, is the underlying cause of brain hemorrhaging in the p120 mutant embryos.

Pericyte recruitment and endothelial growth are both regulated by endothelial cadherins (Luo and Radice, 2005; Tillet et al., 2005; Vestweber, 2008). Previous studies have demonstrated that a central function of p120 is to post-translationally stabilize cadherins (Delva and Kowalczyk, 2009). In cultured endothelial cells, knockdown of p120 using siRNA leads to VE-cadherin endocytosis and degradation (Xiao et al., 2003a). In the present study, we observed that VE-cadherin levels were significantly reduced in vessels lacking p120 (Figure 2.7), demonstrating that p120 also regulates VE-cadherin levels in vivo. Similarly, in endothelial cells isolated from $p120^{flox/flox}$; Cre^{-}

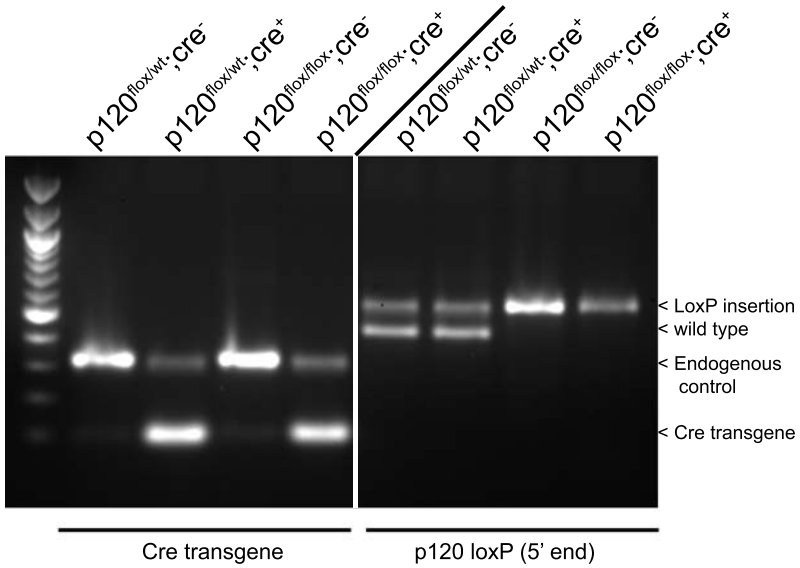
mice, deletion of the p120 gene upon expression of Cre recombinase leads to a significant reduction in both p120 and VE-cadherin (Figure 2.11). These cells also exhibit dramatically reduced proliferation (Figure 2.12). Loss of endothelial proliferation during embryonic development provides a reasonable explanation for the loss of microvascular density observed in vivo (Fig 2, Figure 2.4). A number of studies have implicated VE-cadherin in regulating endothelial proliferation (Niessen and Yap, 2006; Ryding et al., 2001). Consistent with these previous studies, the endothelial proliferation defect of p120-null endothelial cells could be rescued by expression of exogenous VE-cadherin (Figure 2.12), suggesting that reduced VE-cadherin levels play an important role in both endothelial proliferation and in the loss of vessel density in the mutant embryos. Similar to VE-cadherin, levels of N-cadherin are also reduced in p120-null vessels (Figure 2.9). N-cadherin is required for vascular development and has been implicated in pericyte recruitment to developing vessels (Luo and Radice, 2005; Tillet et al., 2005). Together, these results suggest that the reduction in VE-cadherin and N-cadherin upon deletion of endothelial p120 contributes to the reduced microvascular density and hemorrhaging that characterizes these mutants.

In addition to regulating cadherin endocytosis, p120 inhibits RhoA activity. Furthermore, recent studies indicate that RhoA inhibition can rescue proliferation defects observed in p120-null keratinocytes (Perez-Moreno et al., 2008). To determine if RhoA inhibition is important for regulating VE-cadherin levels and/or endothelial cell proliferation, we utilized a p120 mutant that associates with cadherin but is unable to inhibit RhoA. This approach revealed that decreased endothelial VE-cadherin levels and reduced proliferation could both be rescued by re-expression of wild type p120 1A or a

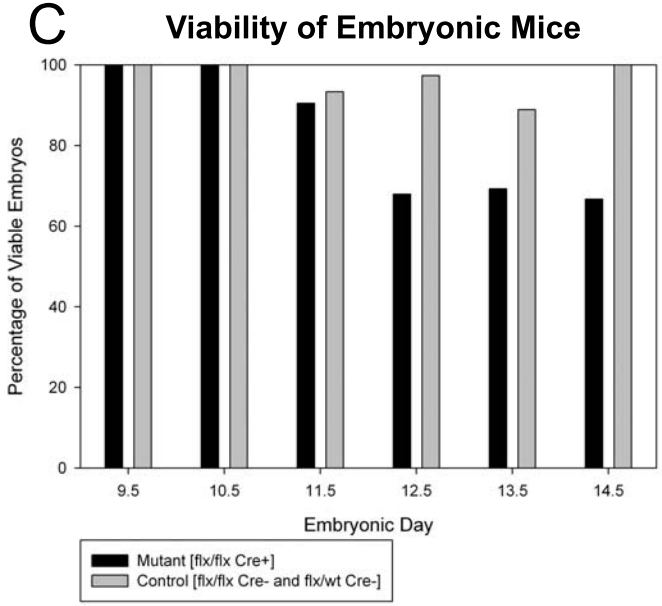
mutant p120 defective in RhoA inhibition (Figure 2.12). These observations are consistent with our recent report that p120 inhibits cadherin endocytosis in a RhoA-independent manner (Chiasson et al., 2009). Furthermore, the ability of the Rho-uncoupled mutant to rescue both VE-cadherin levels and endothelial proliferation further couples loss of VE-cadherin to the proliferation defect in the p120-null endothelial cells.

Previous studies demonstrated that VE-cadherin null animals die embryonically due to severe vascular remodeling defects (Carmeliet et al., 1999; Gory-Faure et al., 1999). However, heterozygous mice in which VE-cadherin protein levels were reduced by approximately 50% were phenotypically normal. In the absence of p120, VE-cadherin levels in vivo were reduced by approximately 50% as assessed by fluorescence intensity measurements of control and p120-null vessels (Figure 4 M). However, it is important to note that deletion of p120 results in reduced levels of both N-cadherin and VE-cadherin, both of which are required for vascular development (Carmeliet et al., 1999; Gory-Faure et al., 1999; Luo and Radice, 2005). In addition, a recent study using a zebrafish model demonstrated that even a modest reduction in VE-cadherin resulted in brain hemorrhaging (Montero-Balaguer et al., 2009). The severity of the phenotype correlated with the degree to which VE-cadherin levels were reduced, suggesting that loss of VE-cadherin may also contribute directly to the hemorrhaging observed in our conditional p120 mutant animals. Lastly, it is also likely that loss of p120 compromises VE-cadherin adhesive functions independently from control of cadherin expression levels. Likely possibilities include misregulation of Rho-family GTPases(Nelson, 2008). Consistent with this notion, VE-cadherin was recently shown to reduce vessel sprouting by suppressing Rac1 activity and enhancing actomyosin contractility (Abraham et al., 2009).

Given the role of p120 in regulating Rho-family GTPases, this activity of p120 may also contribute to some aspects of endothelial cell function in developing vessels, including tubule formation and barrier function. Additional mouse genetic models and other approaches will be required to distinguish between these possibilities. Lastly, we should note that the Tie-2 promoter has been shown to be active in hematopoietic cells. Although it is formally possible that functions of p120 are important in this lineage, the data presented here provide clear evidence for a crucial role of p120-catenin in endothelial cells, both in vivo and in vitro. Together, these findings demonstrate a central role for p120 catenin in vascular integrity, microvascular patterning, and endothelial proliferation.



C



B

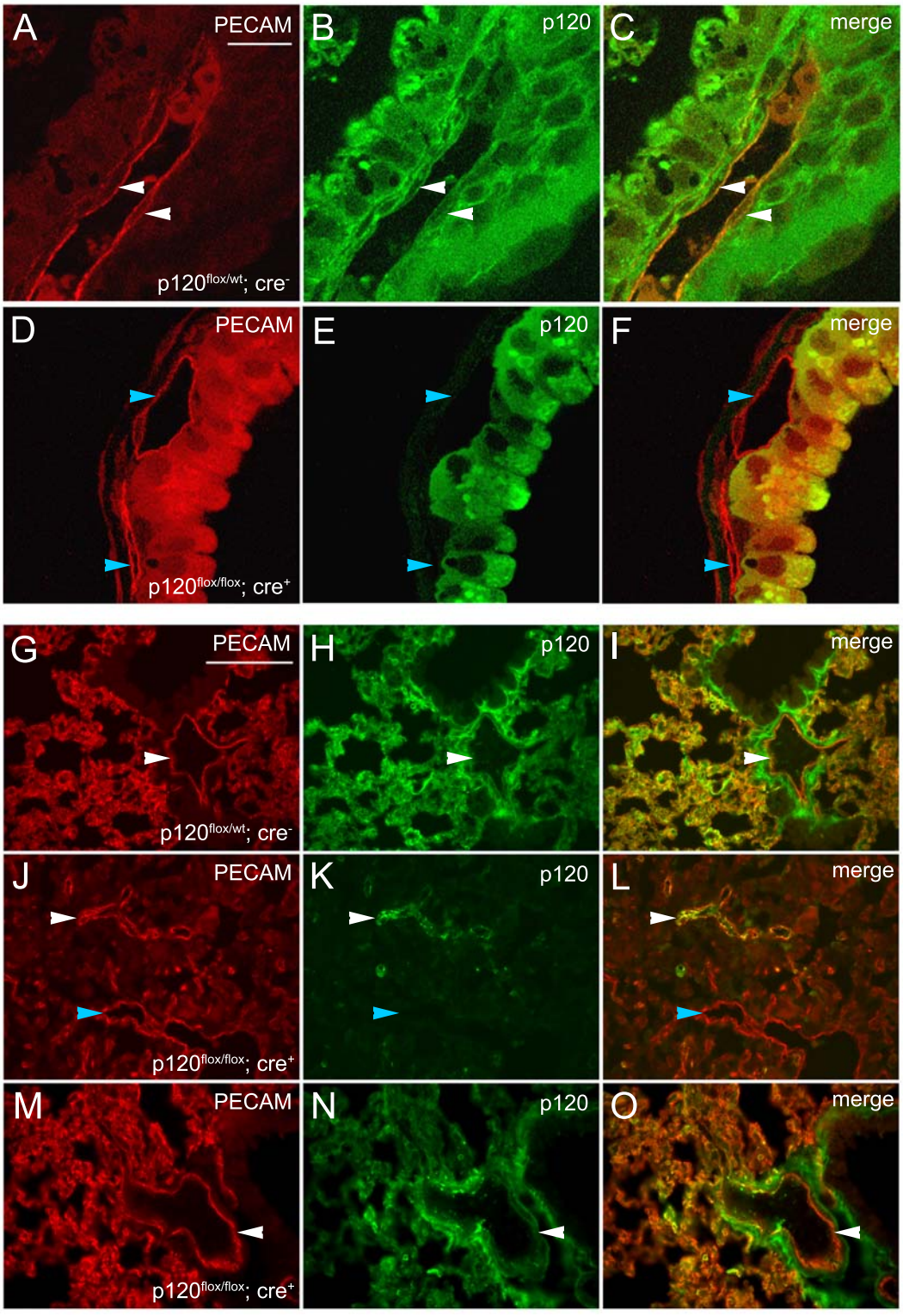
Viability of Neonatal Mice

Genotype	Number	% Actual	% Expected
$p120^{flox/wt};cre^{-}$	95	35.2	25.0
$p120^{flox/wt};cre^{+}$	55	20.4	25.0
$p120^{flox/flox};cre^{-}$	76	28.1	25.0
$p120^{flox/flox};cre^{+}$	44	16.3	25.0

Figure 2.1

Figure 2.1

Endothelial p120 conditional knockout is embryonic lethal. A p120-null allele was obtained by crossing mice harboring LoxP sites in the p120 gene with mice expressing Cre recombinase driven by the Tie2 promoter. Genotypes were confirmed using PCR (A). Breeding pairs of p120flox/flox; Cre- (x) p120flox/wt; Cre+ were established to obtain mutant mice. Genotypes of neonatal mice revealed lower numbers of conditional mutant mice than the predicted 25% (B). Timed matings were conducted to determine the stage of development at which lethality occurred (C). Embryos were isolated at different time points and graded as viable or nonviable and then genotyped. Embryonic lethality among mutants first began to occur at E11.5, and continued to rise over three consecutive days compared to control littermates.


A
PECAM
p120flox/wt; cre-
B
p120
C
merge
D
PECAM
p120flox/flox; cre+
E
p120
F
merge
G
PECAM
p120flox/wt; cre-
H
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I
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J
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p120flox/flox; cre+
K
p120
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M
PECAM
p120flox/flox; cre+
N
p120
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Figure 2.2

Figure 2.2

p120 is successfully ablated in conditional mutant mice but deletion is mosaic in some animals. E13.5 yolk sacs were cryosectioned and stained for p120 and PECAM-1 to identify blood vessels. The markers colocalized in control tissues (C), but mutant blood vessels lacked p120 (F), demonstrating that the Cre/LoxP-mediated deletion of p120 was effective. Representative blood vessels expressing p120 are marked by white arrowheads and vessels lacking p120 are indicated by blue arrowheads. Scalebar in A is 20μm. Lung tissues from young adult mice were cryosectioned and stained for p120 and PECAM-1. Representative large vessels expressing p120 are labeled with white arrowheads; blue arrowheads indicate large vessels lacking p120. Control lung tissue exhibited robust p120 expression (H) and dense microvasculature (G). p120 colocalized with the endothelial marker PECAM-1 (I). Genotypically mutant lung tissue from a physically small littermate revealed extensive p120 loss (K) with small patches of p120 expression which colocalized with PECAM-1 (L). Note reduced microvascular density and intact large vessels (J). Another genotypically mutant, surviving littermate displayed normal body size and exhibited normal p120 levels (N) and microvascular density (M) comparable to control animals. Scalebar in G is 100μm

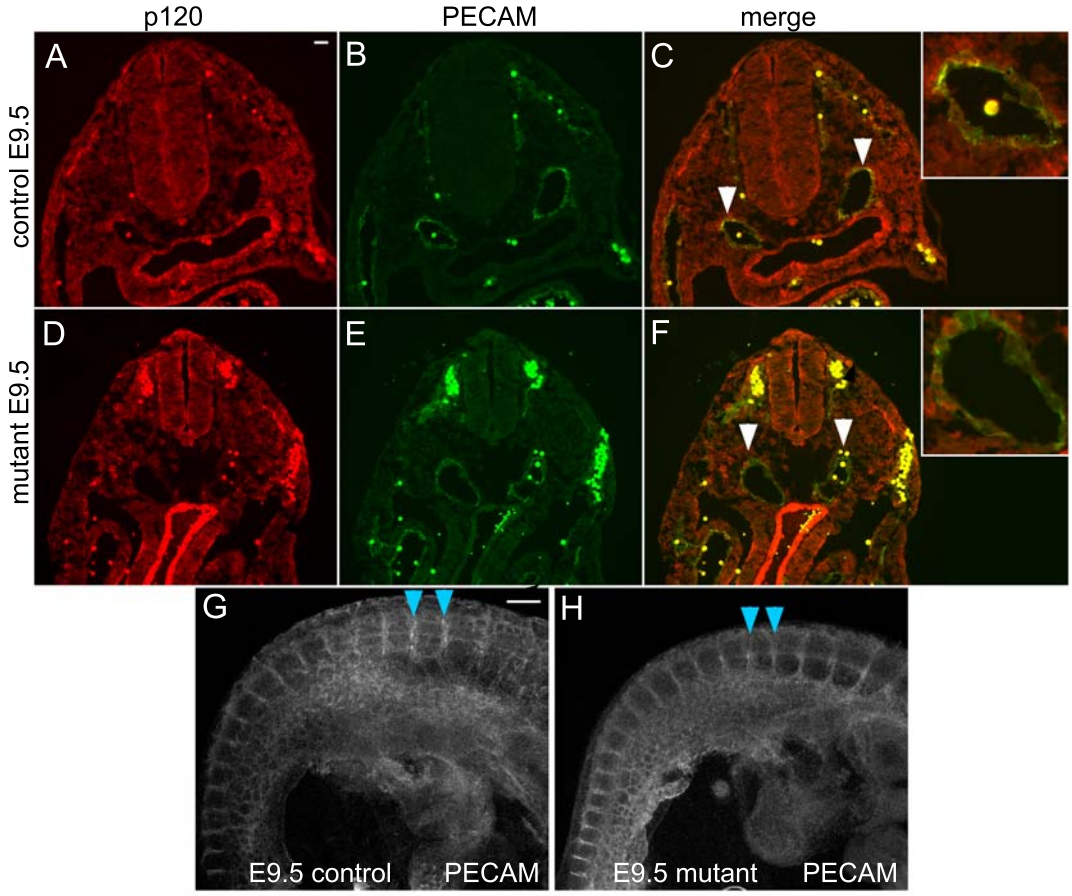
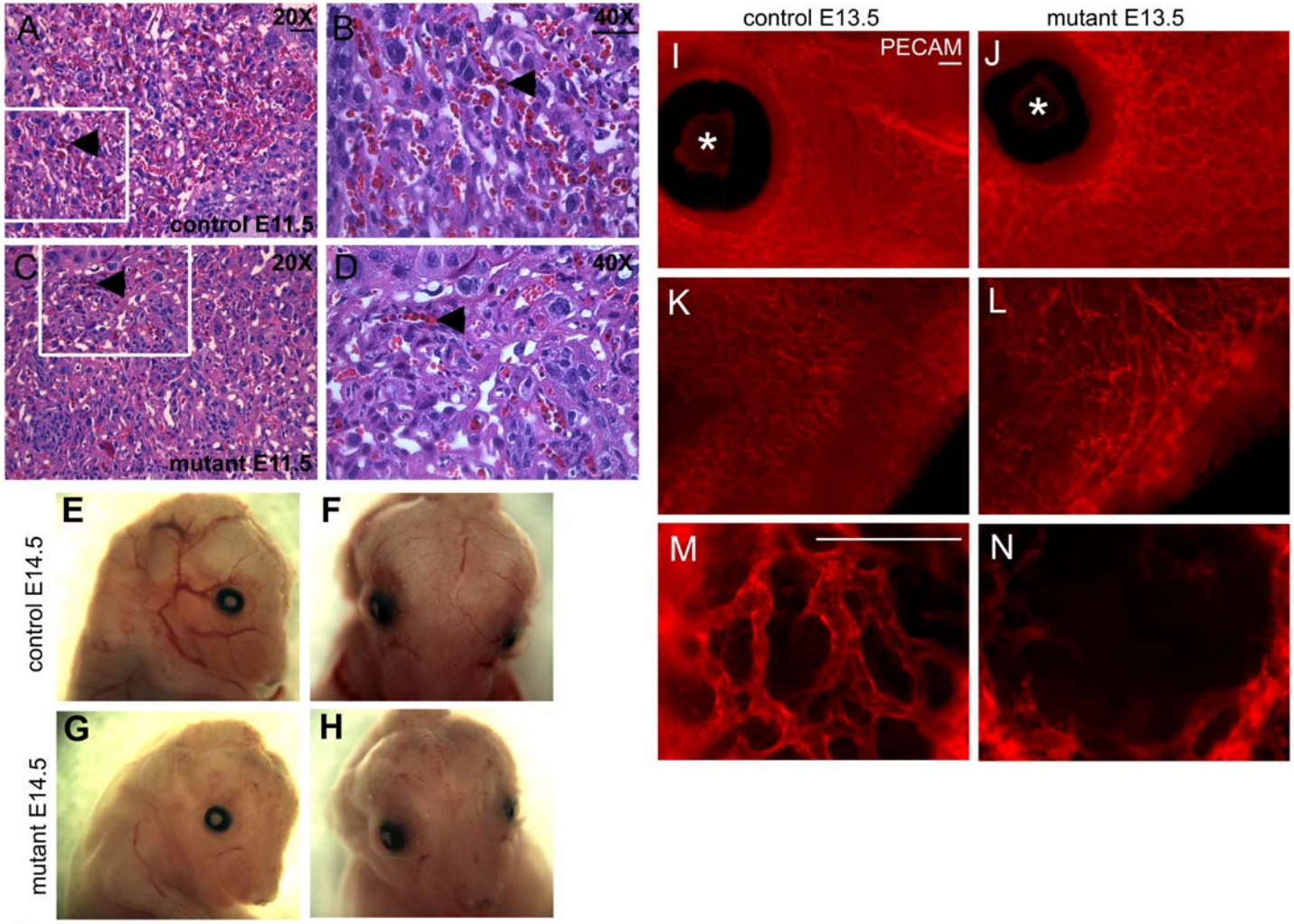

p120
PECAM
merge
A
B
C
D
E
F
control E9.5
mutant E9.5
G
H
E9.5 control
PECAM
E9.5 mutant
PECAM

Figure 2.3

Figure 2.3

Major vessels form normally in p120 endothelial conditional mutants. E9.5 embryos were cryosectioned transversely at heart level and stained for PECAM-1 and p120. p120 loss in the dorsal aortae (indicated by arrowheads) was verified by lack of colocalization between the two markers (C and F, inserts). Sections are shown with dorsal side upward. In all mutants analyzed, dorsal aortae were intact and showed no obvious defects. Scalebar in A is 20μm. To monitor intersomitic vessel formation, E9.5 embryos were whole-mounted and stained for PECAM-1. No defects were seen in mutant embryos (G) compared to control (H). Representative intersomitic vessels are indicated by blue arrowheads. Scalebar in G is 200μm.


A
20X
control E11.5
B
40X
C
20X
mutant E11.5
D
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control E14.5
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H
mutant E14.5
control E13.5
mutant E13.5
I
PECAM
J
K
L
M
N

Figure 2.4

Figure 2.4

p120-null mutants exhibit decreased microvascular density and disorganized vascular networks. Placentas from E11.5 control and mutant embryos were stained with hematoxylin and eosin. Microvascular density in the labyrinthine layer was reduced in mutant tissue (C and D) compared to control (A and B). Boxes indicate areas enlarged in B and D, and arrowheads indicate representative microvessels. Scalebar in A is 100μm. Surviving E14.5 conditional mutant embryos exhibit reduced blood vessels in the head (G and H) compared to control littermates (E and F). PECAM-1-stained whole mount analysis revealed that microvascular networks in E13.5 brains are disorganized and exhibit decreased density in conditional mutant mice (J and L) compared to control littermates (I and K). Enlarged views of the eye show that the hyaloid vascular network (indicated by asterisks in I and J) is reduced in the mutant embryo (N, compared to M). Scalebar in I is 100μm.

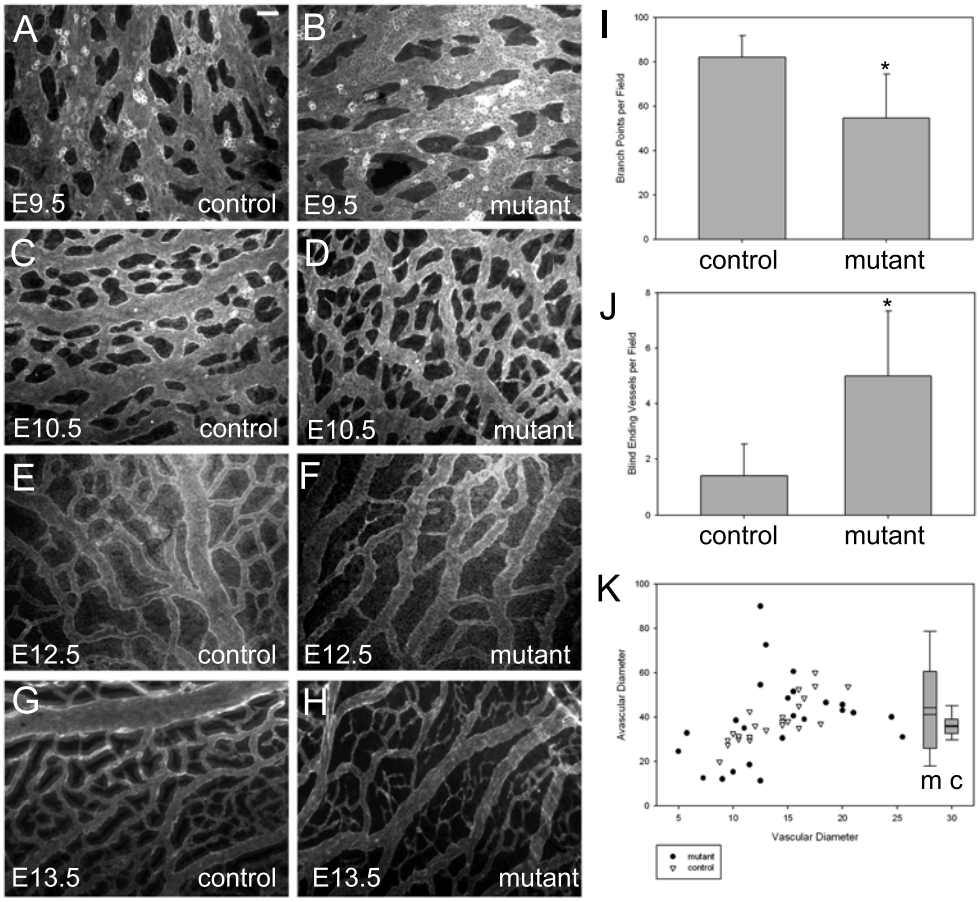
A
E9.5
control
B
E9.5
mutant
C
E10.5
control
D
E10.5
mutant
E
E12.5
control
F
E12.5
mutant
G
E13.5
control
H
E13.5
mutant
I
Branch Points per Field
control
mutant
*
J
Blind Ending Vessels per Field
control
mutant
*
K
Avascular Diameter
Vascular Diameter
m c
mutant
control

Figure 2.5

Figure 2.5

Yolk sac vessels of p120 conditional mutant mice reveal angiogenic remodeling defects. Whole mount yolk sacs from control and mutant embryos at various developmental time points were processed for immunofluorescence microscopy using PECAM-1 antibodies to highlight vessels. Analysis of E12.5 yolk sacs revealed decreased branch points (intersections) in mutant tissues compared to control ($p = 0.024$) (I), accompanied by an increase in blind-ending vessels ($p = 0.015$) (J). A plot of vascular vs. avascular diameters in E12.5 yolk sacs revealed a lack of uniformity in blood vessel networks in mutant tissues compared to control (K, scatter plot). The ratio of vascular/avascular diameters showed greater variability in mutant tissues (K, box plot). Scalebar is 100μm.

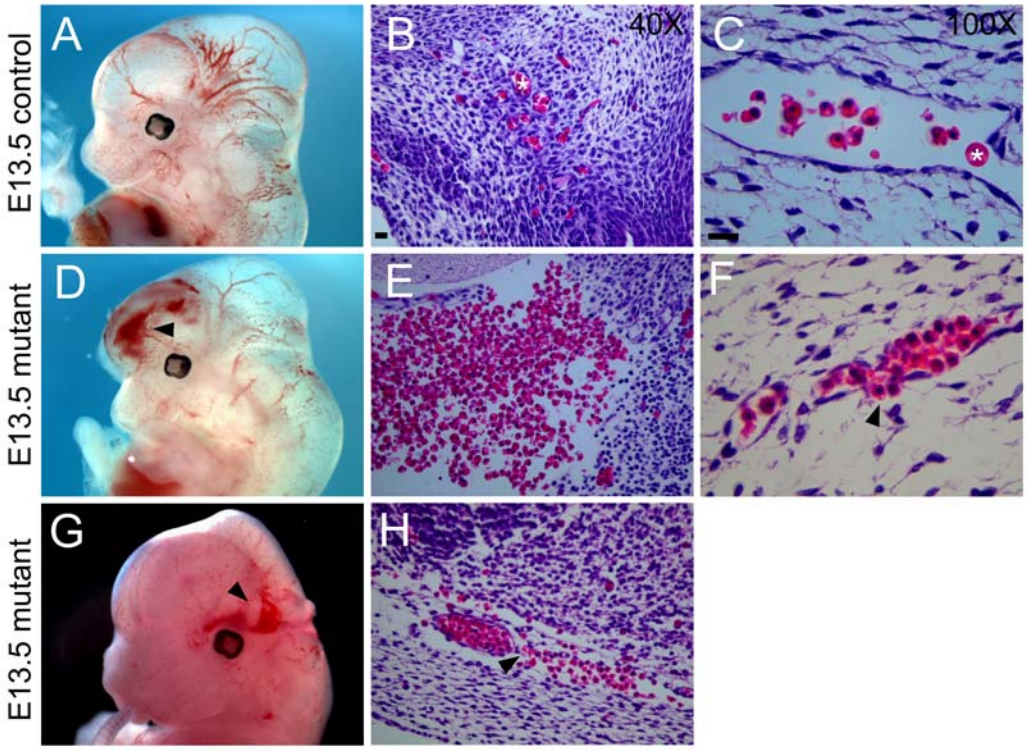

Figure 2.6

Figure 2.6

Conditional mutant mice exhibit hemorrhages. E13.5 control (A) and mutant (D,G) embryos photographed using light microscopy. Hemorrhages in mutant mice are indicated by arrowheads. Hematoxylin and eosin staining of paraffin-embedded sections reveal normal microvessels in the brains of control embryos (B,C) and hemorrhaging (E) and leaky vessels (F,H – indicated by arrowheads) in the brains of mutant embryos. Asterisks in B and C mark red blood cells in microvessels. Scalebars are 20μm.

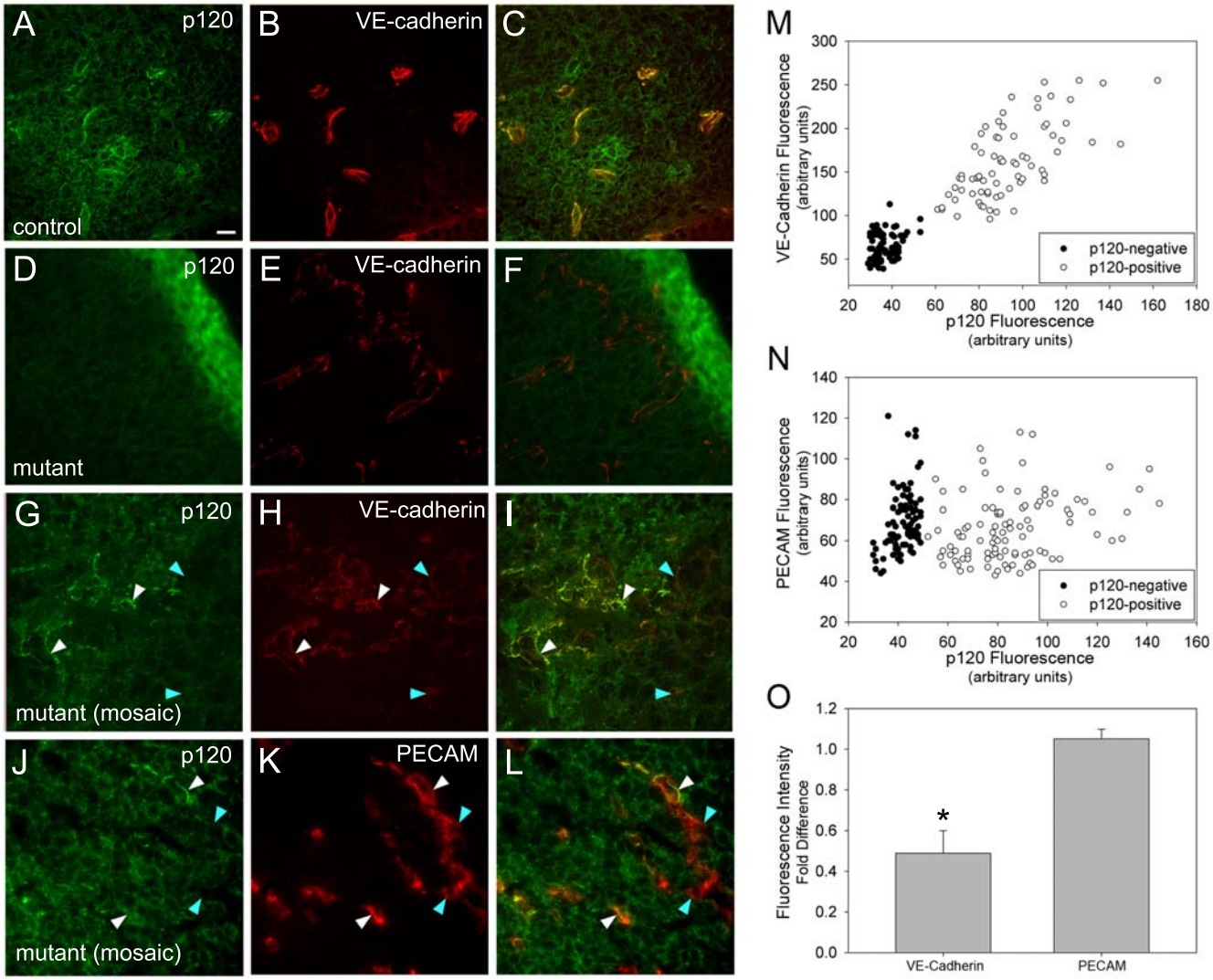
A
p120
B
VE-cadherin
C
control
D
p120
E
VE-cadherin
F
mutant
G
p120
H
VE-cadherin
I
mutant (mosaic)
J
p120
K
PECAM
L
mutant (mosaic)
M
VE-Cadherin Fluorescence (arbitrary units)
p120 Fluorescence (arbitrary units)
p120-negative
p120-positive
N
PECAM Fluorescence (arbitrary units)
p120 Fluorescence (arbitrary units)
p120-negative
p120-positive
O
Fluorescence Intensity Fold Difference
VE-Cadherin
PECAM

Figure 2.7

Figure 2.7

VE-cadherin levels are decreased in embryonic tissues lacking endothelial p120. E10.5 embryos were fixed, cryosectioned, and stained for p120, VE-cadherin, and PECAM-1. In control tissues, p120 (A) and VE-cadherin colocalize in blood vessels (C). In conditionally mutant tissue, VE-cadherin expression is retained (E), but vascular p120 is absent (D). Tissues from genotypically mutant mice which exhibit mosaic p120 expression (G) have reduced levels of VE-cadherin in areas lacking p120 (H - blue arrowheads), compared to areas which have positive staining for vascular p120 (white arrowheads). To verify the specificity of this effect, mosaic mutant tissues were stained for PECAM-1 (J,K,L). Note that PECAM-1 expression is similar in endothelial cells that express p120 (white arrowheads) and those lacking p120 (blue arrowheads). To quantify the reduction in VE-cadherin in p120-negative vessels, peak fluorescence of VE-cadherin (M) or PECAM (N) was plotted against peak fluorescence of p120. VE-cadherin levels were significantly reduced in a manner dependent on p120, while PECAM levels were independent of p120 levels. The fold difference of the average fluorescence intensity of VE-cadherin in p120-negative over p120-positive vessels was 0.489, compared to 1.052

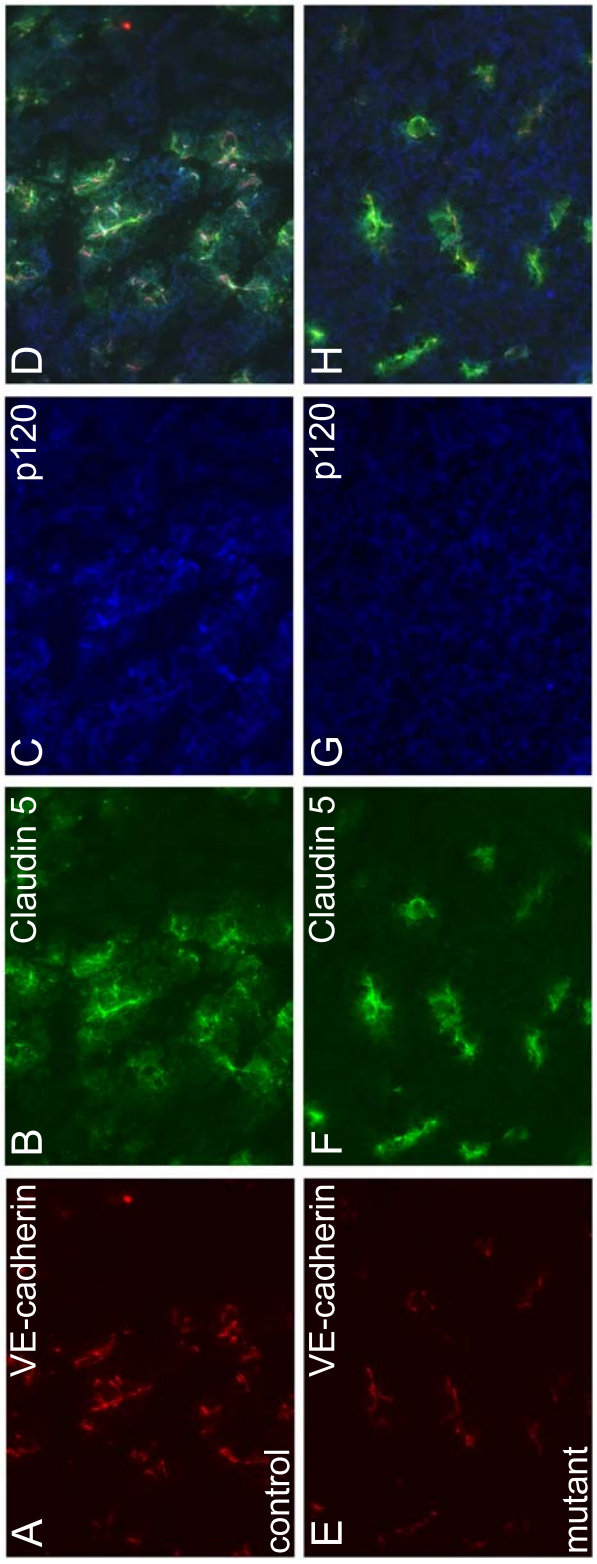
A
VE-cadherin
control
B
Claudin 5
C
p120
D
E
VE-cadherin
mutant
F
Claudin 5
G
p120
H

Figure 2.8

Figure 2.8

Claudin 5 expression is not altered by endothelial p120 deletion. Cryosectioned E11.5 embryos were labeled for claudin 5, VE-cadherin, and p120 to identify blood vessels and verify the vascular ablation of p120 in mutant tissues. Representative fields from control (A-D) and mutant (E-H) mice are shown. No apparent changes in Claudin 5 expression or localization were observed in mutant animals (compare B to F). Scalebar is 20μm.

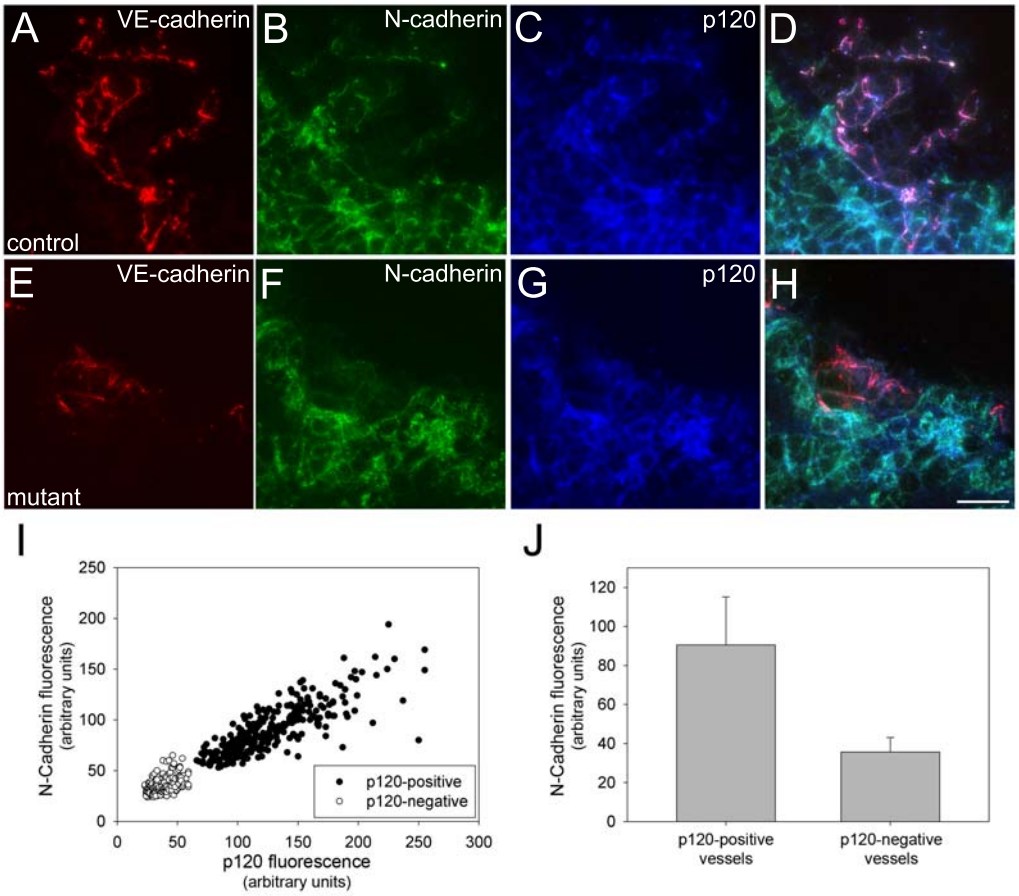
A
VE-cadherin
B
N-cadherin
C
p120
D
control
E
VE-cadherin
F
N-cadherin
G
p120
H
mutant
I
N-Cadherin fluorescence (arbitrary units)
p120 fluorescence (arbitrary units)
p120-positive
p120-negative
J
N-Cadherin fluorescence (arbitrary units)
p120-positive vessels
p120-negative vessels

Figure 2.9

Figure 2.9

N-cadherin is absent in endothelial cells lacking p120 in vivo. Cryosectioned E11.5 embryos were fixed and stained for N-cadherin, VE-cadherin (to identify blood vessels) and p120 (to verify its absence in mutant endothelial tissue). In the area surrounding the neural tube, N-cadherin-positive blood vessels were frequently seen in control mice (A-D). Corresponding vessels of mutants lacking vascular p120 also lacked N-cadherin (E-H). N-cadherin expression was quantified in mutant and control blood vessels relative to p120 as described for VE-cadherin (see Figure 2.7). The average N-cadherin fluorescence intensity of p120-positive vessels was more than 2.5 times that of p120-negative vessels (J). (Mann-Whitney Rank Sum, $p \leq 0.001$). Scalebar is 20μm.

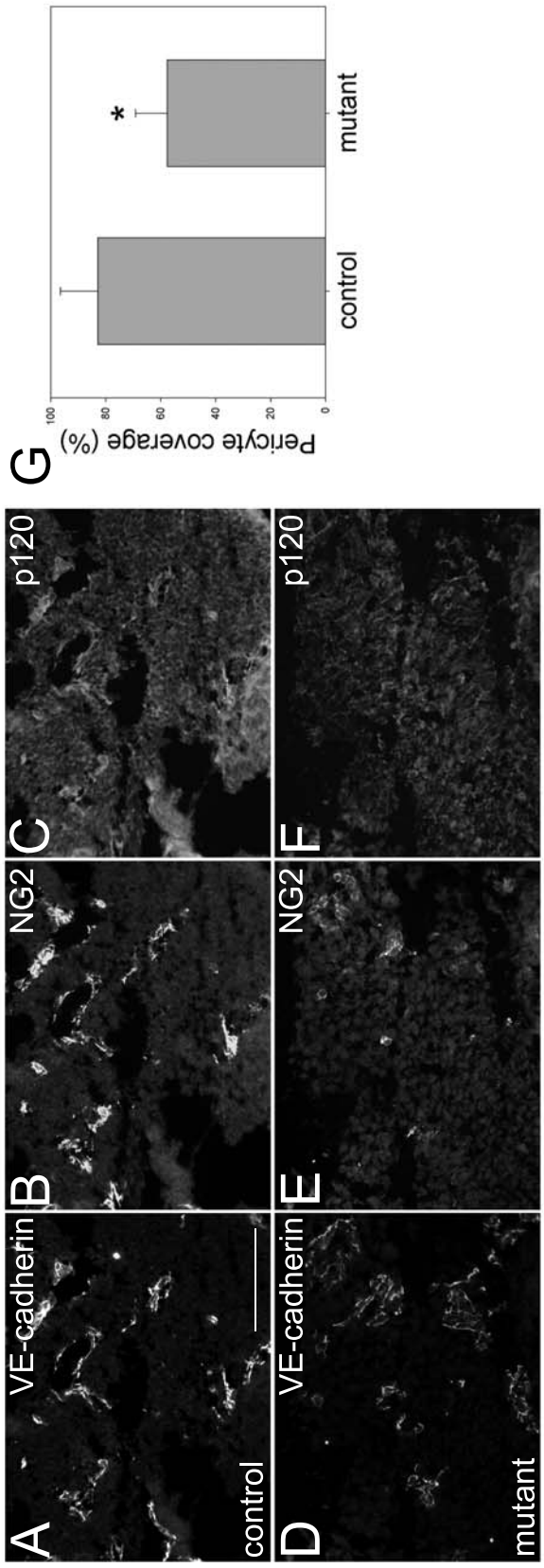
A
VE-cadherin
control
B
NG2
C
p120
D
VE-cadherin
mutant
E
NG2
F
p120
G
Pericyte coverage (%)
100
80
60
40
20
0
control
mutant
*

Figure 2.10

Figure 2.10

Reduced pericyte recruitment in brains of mutant mice. 12-μm cryosections were cut from the brains of E11.5 mice and stained for NG2 to identify pericytes, VE-cadherin to identify blood vessels, and p120. Representative fields are shown for control (A-C) and mutant (D-F) mice. To quantify pericyte coverage of blood vessels, multiple images were collected from a series of two mutant and three control mice from the same litter. 500 individual vessels from each population were scored based on their contact with pericytes. The percent pericyte coverage per field was compared between mutant and control (G). Control vessels had pericyte coverage of nearly 83% compared to only 58% pericyte coverage in mutant vessels (t-test, $p \leq 0.001$). Scalebar is 100μm.

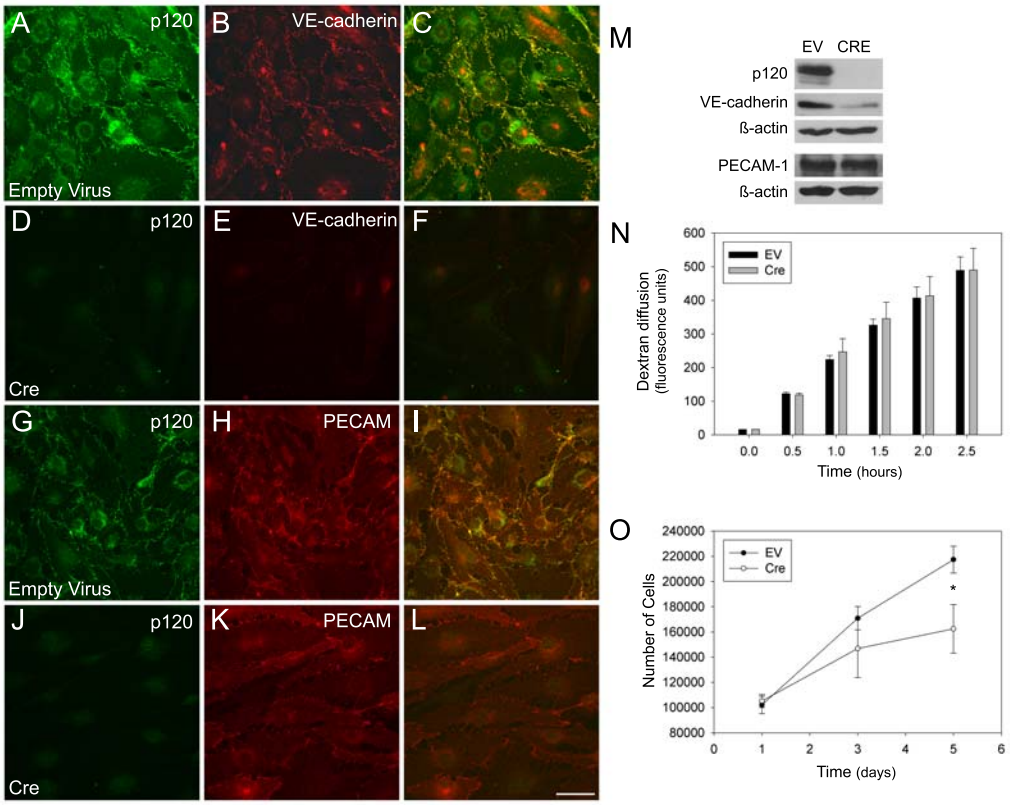
A
p120
B
VE-cadherin
C
Empty Virus
D
p120
E
VE-cadherin
F
Cre
G
p120
H
PECAM
I
Empty Virus
J
p120
K
PECAM
L
Cre
M
EV CRE
p120
VE-cadherin
ß-actin
PECAM-1
ß-actin
N
Dextran diffusion (fluorescence units)
EV
Cre
Time (hours)
O
Number of Cells
EV
Cre
Time (days)

Figure 2.11

Figure 2.11

Ablation of p120 in cultured mouse endothelial cells leads to decreased VE-cadherin levels and reduced proliferation. Immunofluorescence microscopy of primary mouse endothelial cells infected with adenoviral Cre or an empty vector show successful ablation of p120 (compare A and D, G and J). In the absence of p120, VE-cadherin levels are reduced (compare B and E), but PECAM-1 levels are not affected by p120 loss (compare H and K). Western blot analysis confirms the reduction of VE-cadherin, but not PECAM-1, in p120-null cells (M). Measurement of the diffusion of Texas Red-labeled dextran across a confluent monolayer of p120-null cells shows no statistically significant change in barrier function compared to control cells (N) Growth curve analysis demonstrates that p120 null endothelial cells exhibit slower growth rates compared to control cells (Mann-Whitney Rank Sum, $p=0.029$ at day 5). Scalebar is 50μm.

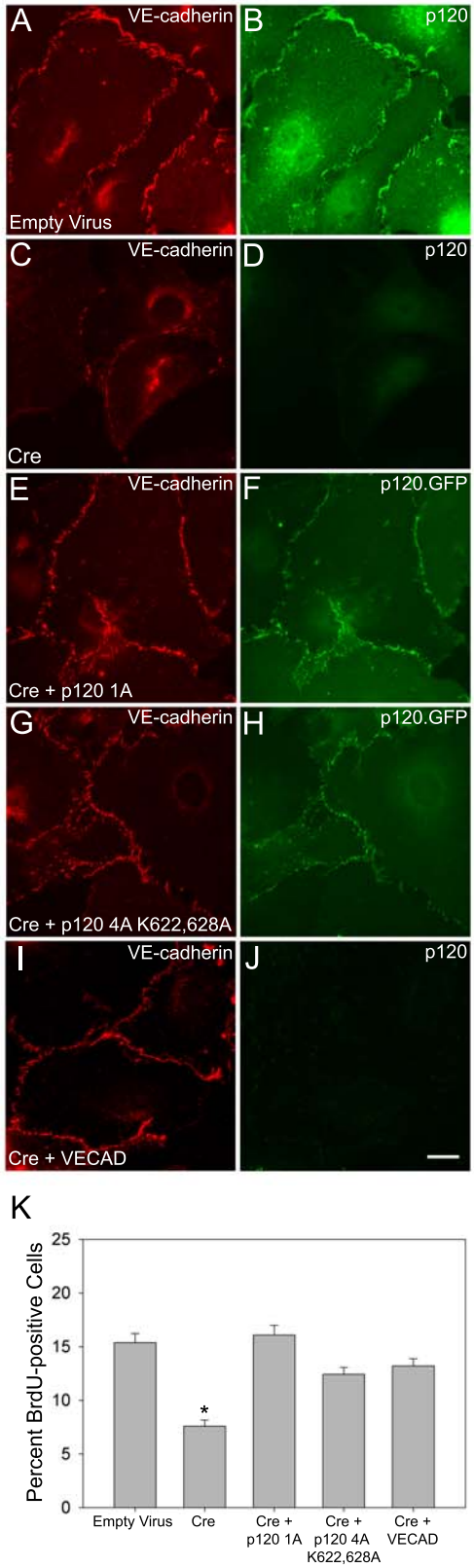
A
VE-cadherin
B
p120
Empty Virus
C
VE-cadherin
D
p120
Cre
E
VE-cadherin
F
p120.GFP
Cre + p120 1A
G
VE-cadherin
H
p120.GFP
Cre + p120 4A K622,628A
I
VE-cadherin
J
p120
Cre + VECAD
K
Percent BrdU-positive Cells
25
20
15
10
5
0
*
Empty Virus
Cre
Cre + p120 1A
Cre + p120 4A K622,628A
Cre + VECAD

Figure 2.12

Figure 2.12

Re-expression of p120 rescues VE-cadherin expression and restores proliferation rates in p120-null endothelial cells. Immunofluorescence microscopy of primary mouse endothelial cells infected with adenoviral Cre demonstrates ablation of p120 (compare B and D). In the absence of p120, VE-cadherin levels are reduced (compare A and C). Re-expression of wild type p120 (E, F) or the Rho-uncoupled p120 mutant (p120 4A K622,628A) (G, H) restored VE-cadherin expression in p120-null cells. Exogenously expressed VE-cadherin assembled at cell-cell junctions in the absence of p120 (I, J). Deletion of p120 reduced endothelial proliferation rates. Bromodeoxyuridine (BrdU) uptake by p120-null and control cells was measured and compared to total nuclei (stained by DAPI). Cells lacking p120 showed a decrease in proliferation compared to control (N). (Kruskal-Wallis One Way Analysis of Variance on Ranks, $p \leq 0.001$). Furthermore, re-expression of wild type p120 restored endothelial proliferation (K). Similarly, both the Rho-uncoupled p120 mutant and exogenously expressed VE-cadherin also rescued endothelial proliferation in p120-null cells (Multiple Comparisons versus Control Group by Dunnett's Method, $p < 0.05$). Scalebar is 20μm.

Chapter 3

p120-catenin and β-catenin differently regulate endothelial cell spreading and adhesion strengthening

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3.1 Introduction

Cell adhesion enables tissues to maintain their structural integrity and withstand mechanical stress. Cadherins are a family of transmembrane adhesion receptors which enable cells to form initial contacts which mature into adherens junctions as other cadherins and cytoplasmic binding partners are recruited to facilitate cytoskeletal linkages. The vascular endothelium, which forms a thin layer lining the interior of blood vessels, must sustain strong intercellular adhesion in order to maintain vascular barrier function and prevent hemorrhage and edema. However, the adhesion between endothelial cells must be dynamically regulated to enable angiogenesis during growth and development and to allow the passage of leukocytes between the vascular lumen and the surrounding tissue at sites of inflammation. The major cadherin family member found in endothelial cells is vascular endothelial (VE)-cadherin, which mediates homophilic, calcium-dependent adhesion through its extracellular domain and binds to Armadillo family proteins p120-catenin (p120) and β-catenin inside the cytoplasm through its juxtamembrane and catenin-binding domains, respectively. β-catenin provides linkage between adherens junctions and the actin cytoskeleton, while p120 regulates cadherin stability at the plasma membrane by preventing its internalization (Davis et al., 2003; Xiao et al., 2003a). Studies using mouse models have demonstrated a requirement for both p120 and β-catenin to maintain vascular barrier function, and the conditional endothelial knockout of either catenin results in hemorrhages, particularly in areas subject to increased vascular flow during development (Cattelino et al., 2003; Oas et al., 2010).

In previous studies involving C-cadherin and E-cadherin, the juxtamembrane domain and its specific interaction with p120 were implicated in the strengthening of cell adhesion (Goodwin et al., 2003; Yap et al., 1998). However, the mechanisms by which strong cadherin-based adhesion is achieved in endothelial cells, and the contribution of catenins to this process, are not fully understood. The loss of endothelial p120 *in vivo* results in a reduction in VE-cadherin levels (Oas et al., 2010), consistent with a role for p120 in regulating cadherin turnover. A study using cultured endothelial cells demonstrated that the interaction between p120 and VE-cadherin at the plasma membrane is required for the maintenance of endothelial barrier function (Iyer et al., 2004). However, the knockout of VE-cadherin is recessive embryonic lethal (Carmeliet et al., 1999; Gory-Faure et al., 1999), and the reduction of VE-cadherin levels by 50% in the heterozygotes did not lead to hemorrhaging or other vascular defects. This raises the question of whether p120 could be acting to strengthen VE-cadherin-dependent adhesion independently of cadherin levels alone. Additionally, p120 is a potent regulator of the Rho family of small GTPases, which regulate actin cytoskeletal dynamics and play important roles in the establishment of cell-cell contacts and vascular barrier function (Anastasiadis, 2007; Beckers et al., 2010). Specifically, p120 activates Rac1 and inhibits RhoA (Anastasiadis et al., 2000; Noren et al., 2000). Work by Goodwin and colleagues showed that the adhesion defects introduced by blocking p120 binding to the E-cadherin tail could be rescued by expression of constitutively active Rac1 (Goodwin et al., 2003). Therefore, it is likely that the contribution of p120 to strong adhesion through VE-cadherin involves not only the stabilization of the cadherin at the cell surface, but also the localization of p120 near the membrane to locally activate Rac.

To examine the role of p120 in strengthening endothelial cell adhesion, we expressed chimeric proteins in which the interleukin-2 receptor (IL-2R) extracellular domain is fused to the cytoplasmic domain of VE-cadherin. This approach enables us to use mutations to separate the functions of the specific domains and interactions of the VE-cadherin tail in the process of strengthening adhesion. Chimeric receptors have been used in studies of cell adhesion mediated by cadherins as well as integrins (O'Toole et al., 1994; Yap et al., 1997). Additionally, by using antibodies directed against the IL-2R extracellular domain as adhesive ligands, we are able to limit the contacts between the cells and substrates to those mediated by the chimeric receptors and exclude other confounding interactions. Using a hydrodynamic spinning disk assay to measure the strength of cell adhesion, we determined that the interaction between p120 and the cadherin tail did not detectably alter adhesion strength. In contrast, linkage to the actin cytoskeleton through the β-catenin-binding domain was crucial to strengthening adhesion. However, we found that the interaction between p120 and VE-cadherin was necessary to promote cell spreading. We therefore propose that the contribution of p120 to the strengthening of cell adhesion is dependent on its role in advancing membrane extension, which we propose to be Rac1-dependent. Furthermore, we offer a model in which both p120-dependent cell spreading and mechanical stiffening mediated by actin cytoskeletal interactions through the catenin-binding domain provide necessary but experimentally distinguishable contributions to strong endothelial adhesion.

3.2 Materials and Methods

Cell culture: Primary cultures of dermal microvascular endothelial cells (MECs) from human neonatal foreskin were isolated and cultured in Microvascular Endothelial Cell Growth Media-2 (EGM-2MV) (Lonza, Basel, Switzerland) supplemented with cAMP (Sigma-Aldrich, St. Louis, MO). Mouse heart endothelial cells were isolated from mice harboring a floxed allele of p120 as previously described (Oas et al., 2010), immortalized by transfection with SV40 DNA according to a previously published method (Ades et al., 1992). Clonal cell populations were expanded and a cell line was selected on the basis of morphology and the expression of endothelial markers VE-cadherin and PECAM-1. To induce p120 knockout, the cells were infected with an adenovirus expressing Cre (gift from Dr. L. Yang, Winship Cancer Institute, Emory University School of Medicine) so that parallel wild-type and p120-null lines were generated. These cells were cultured in high-glucose DMEM (Mediatech, Herndon, VA) with 20% FBS (Sigma-Aldrich), antibiotic/antimycotic solution (Mediatech), 100µg/mL heparin (Sigma-Aldrich), 100µg/mL endothelial cell growth supplement (ECGS) (Biomedical Technologies, Stoughton, MA), 1mM non-essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 2mM L-glutamine (Mediatech), and 25mM HEPES (Mediatech). HeLa cells were cultured in DMEM supplemented with FBS and antibiotic/antimycotic solution. Cells were grown on 0.1% gelatin-coated plates to ~80% confluence for experiments.

Adenoviruses: The IL-2R-VE-cad$_{cyto}$, IL-2R-VE-cad$_{\Delta CBD}$, and IL-2R-VE-cad$_{JMD\text{-}AAA}$ constructs were generated as described previously (Xiao et al., 2003a). Having been

subcloned into the pAd-Track vector which coexpresses green fluorescent protein (GFP), these and the IL-2R and wild-type p120 constructs were added to cells 16-20 hours prior to seeding for experiments, and infection rates of ~80% were used as monitored by GFP expression.

Micropatterned surfaces: Micropatterned coverslips with adhesive islands surrounded by a non-adhesive background were prepared as previously described (Gallant et al., 2002). Briefly, to generate a regular array of adhesive islands 10μm in diameter and 75μm from center to center, a PDMS stamp was generated from a template (Dumbauld et al., 2010) and used for microcontact printing of self-assembled monolayers of alkanethiols on gold-coated coverslips. Glass coverslips 25mm in diameter were cleaned, then coated with titanium (100Å) and then gold (150Å) using an electron beam evaporator (Thermonics, San Leandro, CA). Prior to microcontact printing, the stamp was sonicated in 70% ethanol for 15 minutes and allowed to dry. Using a cotton swab, the patterned surface of the PDMS stamp was coated with 1.0mM hexadecanethiol in 200-proof ethanol, dried using a N_2 stream, and laid on the gold-coated coverslip for 30 seconds under 50-100g of weight to ensure uniform contact. This process generates a surface with regularly spaced adhesive islands which adsorb ligands such as extracellular matrix proteins or cell-membrane-associated adhesive receptors. To prevent adhesion to the areas between islands, patterned coverslips were incubated for 2 hours in tri(ethylene glycol)-terminated alkanethiol to create a nonadhesive and nonfouling background. The coverslips were then washed three times with absolute ethanol, once with sterile ddH_2O, and once with PBS+ before coating with IgG2a directed against the IL-2 receptor (purified from ATCC hybridoma #HB8784) at a concentration of 20μg/mL for 1 hour. Following ligand

adsorption, the patterned coverslips were blocked in heat-inactivated bovine serum albumin (1% w/v) for 30 minutes and incubated in PBS until seeding. Cells expressing IL-2R-containing constructs were removed from culture plates using trypsin/EDTA and seeded onto the micropatterned coverslips at a density of 225 cells/mm^2. The coverslips were returned to the 37° incubator for 16 hours.

Hydrodynamic spinning disk assay: Cell adhesion strength was measured as previously described (Gallant et al., 2002; García et al., 1998). Using a spinning disk, a micropatterned coverslip with adherent cells was mounted on the spinning platform (Figure 3.4 A), stabilized by vacuum pressure, submerged in a solution of 2mM dextrose in PBS+ (Figure 3.4 B), and spun for 5 minutes (Figure 3.4 C). The hydrodynamic forces present on the surface of the coverslip are well-defined by the following equation (García et al., 1998):

$$\tau = 0.8\mathrm{r}\sqrt{\rho\mu\omega^3} \qquad \text{(Equation 1)}$$

where τ is the applied shear stress (force/area), r is the radial position relative to the center of the coverslip, ρ is the density of the solution, μ is the viscosity of the solution, and ω is the angular speed. After being spun, the samples were fixed in 3.7% formaldehyde, permeabilized in 0.1% Triton, and stained with ethidium homodimer. The number of remaining adherent cells were counted on a fluorescence microscope with a motorized stage, ImagePro image analysis software (Media Cybernetics, Silver Spring, MD), and an algorithm that analyzed 61 fields per sample ranging from the center of the coverslip to the outer edges. The fraction of adherent cells (f) was calculated by comparing the number of cells present at each field with the number present at the center,

where the shear forces are close to zero. Detachment profiles (f vs. τ) were fit to a sigmoidal curve using SigmaPlot statistical software: $f = 1.0/1.0 + \exp [b(\tau-\tau_{50})]$, where τ_{50} is the value of shear stress at which 50% of the cells detach. This value was used as a measure of mean adhesion strength. For comparisons between groups, ANOVA was used, and if significant differences were detected, the Tukey test was used to perform pairwise comparisons.

Cell spreading assay: Adhesive substrates were generated using the same method as for the micropatterned samples described above, except that instead of stamping, the entire coverslip was coated with 1mM hexadecanethiol in ethanol prior to incubation in the IL-2R IgG2a ligand. Cells expressing the constructs containing IL-2R were seeded sparsely on these surfaces and allowed to attach at 37°C for 30 minutes. The samples were then gently washed in PBS, fixed with paraformaldehyde, and mounted on microscope slides. Using light microscopy, fields were photographed at random and for each condition, the spread areas of a total of 100 individual cells (not bordering any other cell) were measured. To determine whether the difference in median values between groups was statistically significant, we performed the Kruskal-Wallis test (with p values less than 0.001 indicating significance), followed by pairwise comparisons between groups using the Tukey test (with p values less than 0.05 indicating significance).

Immunofluorescence: Cells were fixed using methanol (Acros Organics, Geel, Belgium) or 3.7% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline with calcium and magnesium (PBS+) containing 2% bovine serum albumin (BSA) (Fisher Scientific), followed by permeabilization with 0.1% Triton

(Roche Diagnostics Corporation, Indianapolis, IN) in PBS+, then subsequently stained using antibodies against IL-2R (R&D #MAB223 clone 22722), and DAPI. Cells attached to micropatterned coverslips were washed with PBS+ and washed once in cytoskeleton buffer (CSK) containing 10mM PIPES buffer, 50mM NaCl, and 3mM $MgCl_2$. Protease inhibitors (1mM PMSF, 1μg/mL aprotinin, and 1μg/mL pepstatin) were added immediately before use. The cells were then washed twice in CSK containing 0.5% (v/v) Triton X-100 and fixed in 4% paraformaldehyde. Cells were subsequently blocked in 5% goat serum with 0.01% NaN_3 and stained using antibodies against IL-2R (R&D), p120 (BD Biosciences #610135), and β-catenin (Sigma-Aldrich #A5441).

Immunoprecipitation: Immunoprecipitations were carried out as described previously (Chiasson et al., 2009), without crosslinking. Briefly, HeLa cells were grown to confluence and infected with IL-2R-containing chimeric constructs. On the day of the experiment, cells were placed on ice, rinsed with PBS, and lysed with Buffer A (150mM NaCl, 10mM HEPES, 1mM EGTA, and 0.1mM $MgCl_2$, pH 7.4) + 0.5% Triton-X-100, scraped from the dish, and incubated on ice for 30 min. Cell lysates were centrifuged at 16,100 x g for 10 min, and supernatants were diluted to 1 mg/ml in 0.5ml of buffer A + 0.5% TX-100. The supernatants were incubated overnight at 4°C with sheep anti-mouse Dynal magnetic beads (Invitrogen) conjugated to mAbs against IL-2R (Santa Cruz #N-19). The beads were then washed with buffer A + 0.1% TX-100 and eluted with SDS-PAGE sample buffer at 75°C for 5 min before performing Western blots, which were probed with antibodies against p120 (Santa Cruz #SC-1101) and β-catenin (Sigma-Aldrich, #A5441).

Western blotting: Cells were cultured in complete growth medium and infected with adenoviruses for 16-24 hours before being harvested in Laemmli sample buffer (Bio Rad Laboratories, Hercules, CA) and samples were boiled for 5 minutes before loading on 7.5% SDS-PAGE gel for protein separation. Proteins were transferred to nitrocellulose membrane for immunoblotting and probed with antibodies against IL-2R (Santa Cruz #N19), and p120 (Santa Cruz #SC-1101). HRP-conjugated secondary antibodies (Bio-Rad Laboratories) were used at 1:3000 dilution and blots were developed with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ #RPN2106) or Amersham ECL Plus (GE Healthcare #RPN2132).

3.3 Results

Chimeric adhesion receptors are expressed at comparable levels at the cell surface

To examine the functional significance of the cytoplasmic domains and interactions of the VE-cadherin cytoplasmic tail in endothelial adhesion strengthening, we used adenoviral vectors to express a series of chimeric receptor proteins in primary human microvascular endothelial cells (MECs) (Figure 3.1). The extracellular adhesive domain of VE-cadherin was replaced with the interleukin-2 receptor (IL-2R) to generate the IL-2R-VE-cad$_{cyto}$ construct. Two additional variants were generated by further mutating the VE-cadherin cytoplasmic domain: a triple-alanine mutation in the juxtamembrane domain which blocks binding by p120 (IL-2R-VEcad$_{JMD\text{-}AAA}$) and a complete deletion of the catenin-binding domain which interacts with β-catenin (IL-2R-VEcad$_{\Delta CBD}$) (Xiao et al., 2003a). IL-2R alone was used as a control. Expression of the receptors in MECs was verified by immunofluorescence (Figure 3.2 A) and Western blot (Figure 3.2 B), which additionally confirmed that the constructs were expressed at comparable levels and at the correct molecular weight. Because the number of receptors at the cell surface could have implications for adhesion strength, flow cytometry assays were performed to assess surface IL-2R levels between the four constructs and compared with an uninfected control. The results showed that the surface expression levels were comparable between groups (Figure 3.2 C).

Chimeric receptors directly interact with catenins and colocalize with them at the adhesive interface

To ensure that the chimeric receptors were able to interact with p120 and β-catenin despite the absence of the cadherin extracellular domain, immunoprecipitations were performed in which antibodies against IL-2R were used to pull down the receptors which were subsequently probed by Western blot for p120 and β-catenin (Figure 3.3 A). Catenins did not associate with IL-2R, whereas p120 formed complexes with VE-cad$_{cyto}$ IL-2R-VE-cad$_{\Delta CBD}$ and β-catenin with IL-2R-VE-cad$_{cyto}$ and IL-2R-VE-cad$_{JMD\text{-}AAA}$. This result demonstrates that the chimeric receptors are able to interact with catenins except where those interactions have been specifically abrogated by mutation or truncation of the VE-cadherin cytoplasmic tail.

We next sought to determine whether the chimeric cadherins were able to recruit β-catenin and p120 to a site of adhesion mediated by the IL-2R extracellular domain. Cells expressing the chimeric constructs or IL-2R were seeded on micropatterned coverslips which presented a regular array of 20-μm adhesive islands prepared as previously described (Dumbauld et al., 2010). The adhesive areas were coated with antibodies directed against the IL-2 receptor and cells expressing the chimeric constructs were seeded on the micropatterned surfaces. Since the area of the islands was smaller than the full spread area of the cells, they were maintained in a uniform, rounded state, spaced regularly across the surface of the coverslip. To verify the requirement for IL-2R expression for adhesion and rule out the possibility of nonspecific binding, uninfected cells and cells transfected with an empty adenoviral vector were seeded on both patterned and unpatterned coverslips coated with IL-2R antibodies. These cells lacking expression of the IL-2R extracellular domain were unable to adhere (Figure 3.6 B). Using immunofluorescence microscopy, we verified that samples expressing IL-2R-VE-cad$_{cyto}$

and IL-2R-VE-cad$_{\Delta CBD}$ revealed strong colocalization between IL-2R and p120 (Figure 3.3 B), whereas samples expressing IL-2R-VE-cad$_{cyto}$ and IL-2R-VE-cad$_{JMD\text{-}AAA}$ displayed colocalization between IL-2R and β-catenin (Figure 3.3 C). Cells expressing IL-2R alone did not yield demonstrable colocalization with either catenin. These results show that the chimeric receptors expressing wild-type cytoplasmic VE-cadherin domains are able to recruit catenins to the site of adhesion and that mutations selectively abrogate either p120 or β-catenin recruitment.

The catenin-binding domain of VE-cadherin is necessary for strong adhesion

To define the contributions of the VE-cadherin cytoplasmic domains to cell adhesion strengthening, cells expressing the chimeric receptors were tested for their ability to mediate strong adhesion. Measurements of steady state adhesion strength were made using a spinning disk apparatus which applies hydrodynamic shear force to cells attached to patterned coverslips by rotating them in a fluid-filled chamber, generating a well-characterized range of shear values across the radius of the coverslip (Figure 3.4). Cells expressing the IL-2R constructs or IL-2R alone were seeded on patterned coverslips and spun to measure adhesion strength. The cells expressing IL-2R-VEcad$_{cyto}$ showed significantly higher adhesion values than those expressing the IL-2R alone – a difference of about 50% – demonstrating the importance of the cadherin cytoplasmic tail in mediating strong attachment (Figure 3.5 A, C). Unexpectedly, the cells expressing the IL-2R-VEcad$_{JMD\text{-}AAA}$ construct produced adhesion values statistically indistinguishable from those obtained with the wild-type cadherin tail. In contrast, cells expressing the IL-2R-VEcad$_{\Delta CBD}$ construct yielded adhesion comparable to the IL-2R, as defined by a

comparison of their τ_{50} values: the level of shear force at which 50% of cells remain attached to the coverslip. To verify that p120 levels in the cell were not limiting, exogenous p120 was co-expressed with IL-2R-VEcad$_{cyto}$ and IL-2R-VEcad$_{JMD\text{-}AAA}$. Since this did not result in higher adhesion strength values in cells expressing IL-2R-VE-cad$_{cyto}$ compared to IL-2R-VE-cad$_{JMD\text{-}AAA}$ (Figure 3.5 B), we concluded that p120 levels were not limiting in comparison to the chimeric constructs. This result suggests a requirement for linkage to the actin cytoskeleton through the catenin-binding domain and its association to β-catenin in order to produce strong steady-state adhesion, but surprisingly p120 appears to be dispensable.

As there was no detectable reduction in steady state adhesion strength when the VE-cadherin juxtamembrane domain was uncoupled from p120, we considered the possibility that p120 might play an important role in the earlier stages of adhesion strengthening, the effects of which might be undetectable after 16 hours post-seeding. To address this hypothesis, samples were prepared as before and spun at a range of time points ranging from 15 minutes to 4 hours post-seeding. The resulting adhesion strengthening profiles revealed no reproducible difference between cells expressing IL-2R-VEcad$_{cyto}$ and IL-2R-VEcad$_{JMD\text{-}AAA}$, although cells expressing IL2R were consistently less adhesive than those expressing chimeric constructs which contained the catenin-binding domain, even from the earliest time points (data not shown). These results indicate that the loss of p120 binding to the VE-cadherin juxtamembrane domain does not significantly reduce cell adhesion strength.

p120 binding to the VE-cadherin tail is necessary to promote cell spreading

Due to the limited size of the adhesive islands on micropatterned coverslips, the attached cells are maintained in a uniform shape, thus removing the ability of cells to alter their shape by spreading from analyses of adhesion strength. Since we were unable to show a measurable contribution from the interaction of p120 and VE-cadherin to adhesion strengthening using micropatterned surfaces subjected to shear force, we next tested the ability of cells expressing the chimeric constructs to spread on unpatterned substrates (Figure 3.6). Cells expressing IL-2R and the three chimeric receptors were able to adhere to the surface, while cells that did not present the IL-2R at the membrane were unable to form attachments (Figure 3.6 B). Cells expressing IL-2R-VE-cad_{cyto} flattened and spread substantially within 30 minutes, whereas those expressing IL-2R without the cadherin tail remained rounded and did not extend lamellipodia or spread over a larger surface area (Figure 3.6 A). Interestingly, while IL-2R-VE$cad_{\Delta CBD}$-expressing cells did not exhibit a significant spreading defect compared to IL-2R-VE-cad_{cyto}, the p120-uncoupled chimera was statistically indistinguishable from IL-2R, suggesting a p120-dependent spreading defect (Figure 3.6 A).

To verify that this effect was specifically the result of disrupting the VE-cadherin-p120 interaction, we next expressed IL-2R, IL-2R-VE-cad_{cyto}, and IL-2R-VE-$cad_{JMD\text{-}AAA}$ in a p120-null endothelial cell line and in a control cell line which retained p120 expression (Figure 3.7). In the p120-expressing cells, we again observed a significant increase in the spread surface area of cells expressing IL-2R-VE-cad_{cyto} compared to IL-2R or IL-2R-VE-$cad_{JMD\text{-}AAA}$ (Figure 3.7 A). However, in a p120-null background, there

was no statistically significant difference between the three groups, and the increased spreading seen previously in the IL-2R-VE-cad$_{cyto}$-expressing cells was eliminated (Figure 3.7 B). This result indicates cell spreading requires both the expression of p120 and its binding to the VE-cadherin juxtamembrane domain.

3.4 Discussion

Using chimeric receptors which fused the IL-2R extracellular domain to the cytoplasmic tail of VE-cadherin, we were able to separate the functions of the cadherin catenin-binding domain and juxtamembrane, and the requirements for binding to p120 and β-catenin, in the processes of cell adhesion and spreading. While the deletion of the catenin-binding domain substantially weakened adhesion strength, it did not significantly affect the ability of the cell to spread. In contrast, the knockout of p120 or the uncoupling of p120 from the cadherin tail was associated with a dramatic decrease in spreading, but blocking p120 from interacting with the juxtamembrane domain of the chimeric receptors did not affect adhesion strength. This latter observation was surprising in light of previous work from the Yap group, which showed that the cadherin juxtamembrane domain, and, specifically, its ability to bind to p120, were important in strengthening cell adhesion(Goodwin et al., 2003; Yap et al., 1998). In one study, a laminar flow assay was performed in which cells expressing C-cadherin constructs were allowed to adhere to a tube coated with the C-cadherin extracellular domain and subjected to fluid shear force. Cells expressing wild-type C-cadherin showed an increase in adhesion strength between 10 and 40 minutes post-seeding (Yap et al., 1998). However, this adhesion strengthening effect was lost in those cells in which the juxtamembrane domain was deleted. A further study examining E-cadherin in which the cadherin tail mutation was confined to the p120 binding site alone reported a similar loss of adhesion (Goodwin et al., 2003), indicating that the effect on adhesion from deleting the entire juxtamembrane domain was likely due to the loss of p120 binding.

The difference between our results and those reported previously can most likely be attributed to differences in the assays used to measure adhesion strength. Two clear differences between the spinning disk and laminar flow methods are the time frame in which cellular adhesion is assayed and the presence or absence of a restriction on the ability of the cell to spread after achieving initial adhesion. To address the former issue, we performed time course experiments in which the samples were spun at intervals ranging from 15 minutes to 4 hours post-seeding. Previous work in the García lab studying focal adhesion assembly demonstrated that steady-state adhesion levels were established by 4 hours and maintained up to 16 hours (Gallant et al., 2005). However, we were not able to detect a p120-dependent delay in adhesion strengthening in our system (data not shown). With regard to cell spreading, Yap and colleagues had reported a spreading reduction in cells expressing C-cadherin constructs lacking the juxtamembrane domain (Yap et al., 1998). Using unpatterned surfaces, we showed a significant and reproducible defect in spreading within 30 minutes of adhesion in cells in which the binding of p120 to the cadherin tail was disrupted either by mutation to the juxtamembrane domain or the loss of endogenous p120 (Figure 3.6 and 3.5.) Based on this observation, it is likely that in a shear-based adhesion assay, cells in which p120 is able to bind to the cadherin tail will flatten and spread in comparison to cells in which this interaction is disrupted. Cells that are less spread and more rounded will be exposed to greater levels of shear, which will increase their likelihood of detachment. If shape is controlled, as in the context of micropatterned coverslips, the ability of p120 to increase adhesion by binding to the cadherin tail is lost. Therefore, p120 contributes to adhesion strengthening by promoting cell spreading in a cadherin-dependent manner.

As mentioned previously, p120 binding to cadherins stabilizes them at the cell surface. However, the localization of p120 near the plasma membrane also influences actin dynamics through the Rho family GTPase Rac1. p120 has been well-characterized as a potent regulator of Rho family GTPases Rho, Rac, and Cdc42 (Anastasiadis, 2007). In particular, the initiation of adhesion by cadherins was demonstrated to stimulate Rac1 activity (Kovacs et al., 2002; Noren et al., 2001), and this activation is dependent on binding of p120 to the cadherin tail (Goodwin et al., 2003). Rac1 activity at the plasma membrane causes actin reorganization and membrane ruffling (Ridley et al., 1992) and is known to be important in the formation of lamellipodia (Ridley, 2001; Tan et al., 2008), particularly at newly formed adhesive contacts (Ehrlich et al., 2002). In the vascular endothelium, Rac1 has been shown to regulate cellular adhesion in both a positive and negative manner, depending on the maturity of the cell junction (Beckers et al., 2010). Rac1-null endothelial cells also exhibit an inability to form initial adhesive attachments when seeded onto fibronectin (Tan et al., 2008). However, Rac1 is also associated with a reduction in vascular barrier function in response to VEGF signaling. In the presence of VEGF, Rac1 is activated by the guanosine exchange factor (GEF) Vav2 in a Src-dependent manner and induces membrane ruffling and increased motility, accompanied by increased VE-cadherin endocytosis (Garrett et al., 2007; Gavard and Gutkind, 2006). Consistent with this, the knockout of Rac1 in cultured endothelial cells abolished their ability to respond to VEGF with increased permeability (Tan et al., 2008). The adhesive defects reported by Goodwin and colleagues when p120 binding to the E-cadherin tail was blocked were rescued when constitutively active Rac1 was expressed (Goodwin et al., 2003). To determine whether

the p120-dependent cell spreading defect we observed might be Rac1-dependent, we performed a spreading assay in which constitutively active Rac1 was coexpressed with IL-2R-VE-cad$_{JMD\text{-}AAA}$ and was able to rescue the spreading impairment (data not shown). Based on these previous findings, in combination with our recent observations, we hypothesize that the mechanism by which p120 promotes cell spreading is by attaching to the cadherin juxtamembrane domain and locally activating Rac1 signaling at the site of nascent adherens junctions, which in turn leads to actin alterations and membrane extension. We therefore propose a model in which the interaction between p120 and VE-cadherin strengthens endothelial cell adhesion by promoting spreading (Figure 3.8). The cell initially forms contact with its substrate through surface receptors, such as the chimeric receptors used in this work (Figure 3.8 A). Cadherin-bound p120 locally activates Rac1 at the periphery of the cell, which increases the available membrane for spreading by altering actin dynamics (Figure 3.8 B).

While the deletion of the VE-cadherin catenin-binding domain did not exert a significant influence on cell spreading (Figure 3.6 A), we found decreased levels of adhesion in cells expressing IL-2R and IL-2R-VE-cad$_{\Delta CBD}$, both of which are unable to link to the actin cytoskeleton through β-catenin (Figure 3.5 A). In the previously-reported laminar flow assay, cells expressing C-cadherin with a truncated catenin-binding domain did not exhibit a defect in adhesion strengthening over time (Yap et al., 1998), although as p120-cadherin binding was not disrupted in those cells, it is likely that they were able to spread and, thus, decrease their exposure to shear forces. One of the major properties of cytoskeletal networks is their ability to stiffen cells, particularly in situations of physiological fluid shear, such as in the vascular endothelium. A variety of

mechanosensory pathways in endothelial cells enable them to stiffen in direct response to shear force, including alterations to actin regulation (Stroka and Aranda-Espinoza, 2010; Walpola et al., 1993). Cells in which β-catenin is excluded from the adhesive interface by deletion of the cadherin catenin-binding domain (Figure 3.3 C), and in which spreading is physically blocked, are thus unable to withstand fluid shear forces and are detached at much lower shear values. We therefore propose that linkage between adherens junctions and the actin cytoskeleton provide a local stiffening effect which can be likened to adding a stiff backing to an otherwise pliable adhesive surface; while such a surface might ordinarily be removed by peeling from the edges, the addition of the backing greatly increases the amount of force required to remove it (Figure 3.8 C). In the case of cell adhesion, the stiffening effects are mediated by actin, and the ability of the cell to withstand shear force is likely to be limited by the spread area of the cell (Figure 3.8 D).

An important additional test of our hypothesis will be addressed by subjecting cells expressing the chimeric constructs to spinning assays on unpatterned coverslips within 30 minutes of seeding; we predict that a p120-dependent decrease in adhesion will be observed under those conditions in which cell spreading will not be limited. Additionally, we are currently developing reagents to perform similar experiments in the context of full-length VE-cadherin, using the VE-cadherin extracellular domain fused to the Fc domain of IgG as the adhesive ligand (VE-cad-Fc). Preliminary results indicate that the range of adhesion values we observed using chimeric receptors are similar to those obtained by spinning MECs expressing endogenous VE-cadherin adherent to VE-cad-Fc, (C. Esimai (Georgia Institute of Technology, personal communication). Further

experimentation will be necessary to explore the role of Rac1, as well as potential roles for RhoA, in p120-dependent cell spreading.

Physiologically, endothelial barrier function requires cells to form strong adhesive interactions, a process which requires the ability to alter their shape, form mature adherens junctions following initial contact, and retain the ability to remodel these junctions in response to inflammatory or angiogenic signals. The vascular endothelium must additionally maintain its integrity in the presence of luminal shear forces. Studies *in vivo* have demonstrated the necessity of endothelial cadherins (Carmeliet et al., 1999; Gory-Faure et al., 1999), catenins (Cattelino et al., 2003; Oas et al., 2010), and GTPases (Tan et al., 2008) for the maintenance of vascular integrity during development. By limiting the adhesion of cells to the chimeric proteins which in turn interact with other adherens junction components (Figure 3.3), we are able to model intercellular cadherin-based adhesive junctions as opposed to focal adhesions. In the present study, we utilized mechanical approaches to separate the effects of time, spreading area, and the different intracellular domains of the VE-cadherin tail on the strengthening of cell adhesion. Through these findings, we present a mechanism by which the cytoplasmic domains of VE-cadherin and the catenins that associate with them contribute to strong cellular adhesion in distinct and complementary ways.

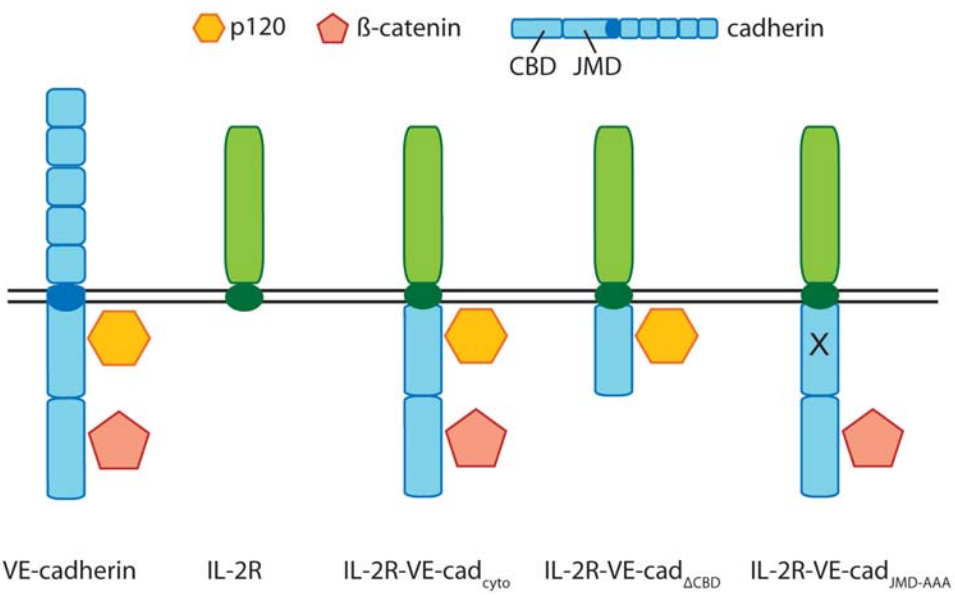
p120
ß-catenin
cadherin
CBD
JMD
X
VE-cadherin
IL-2R
IL-2R-VE-cad$_{cyto}$
IL-2R-VE-cad$_{\Delta CBD}$
IL-2R-VE-cad$_{JMD-AAA}$

Figure 3.1

Figure 3.1

Diagram of chimeric receptors combining the interleukin-2 receptor extracellular domain and the VE-cadherin cytoplasmic tail. The four chimeric constructs used in this work as adhesive receptors are compared to wild-type VE-cadherin. The extracellular domain of VE-cadherin is replaced with IL-2R to generate IL-2R-VE-cad_{cyto}. The catenin-binding domain is deleted to generate IL-2R-VE-$cad_{\Delta CBD}$. A triple alanine mutation in the p120 binding site which uncouples the cadherin tail from p120 is introduced to generate IL-2R-VE-$cad_{JMD\text{-}AAA}$. The four constructs are introduced into cells by way of adenoviral vectors.

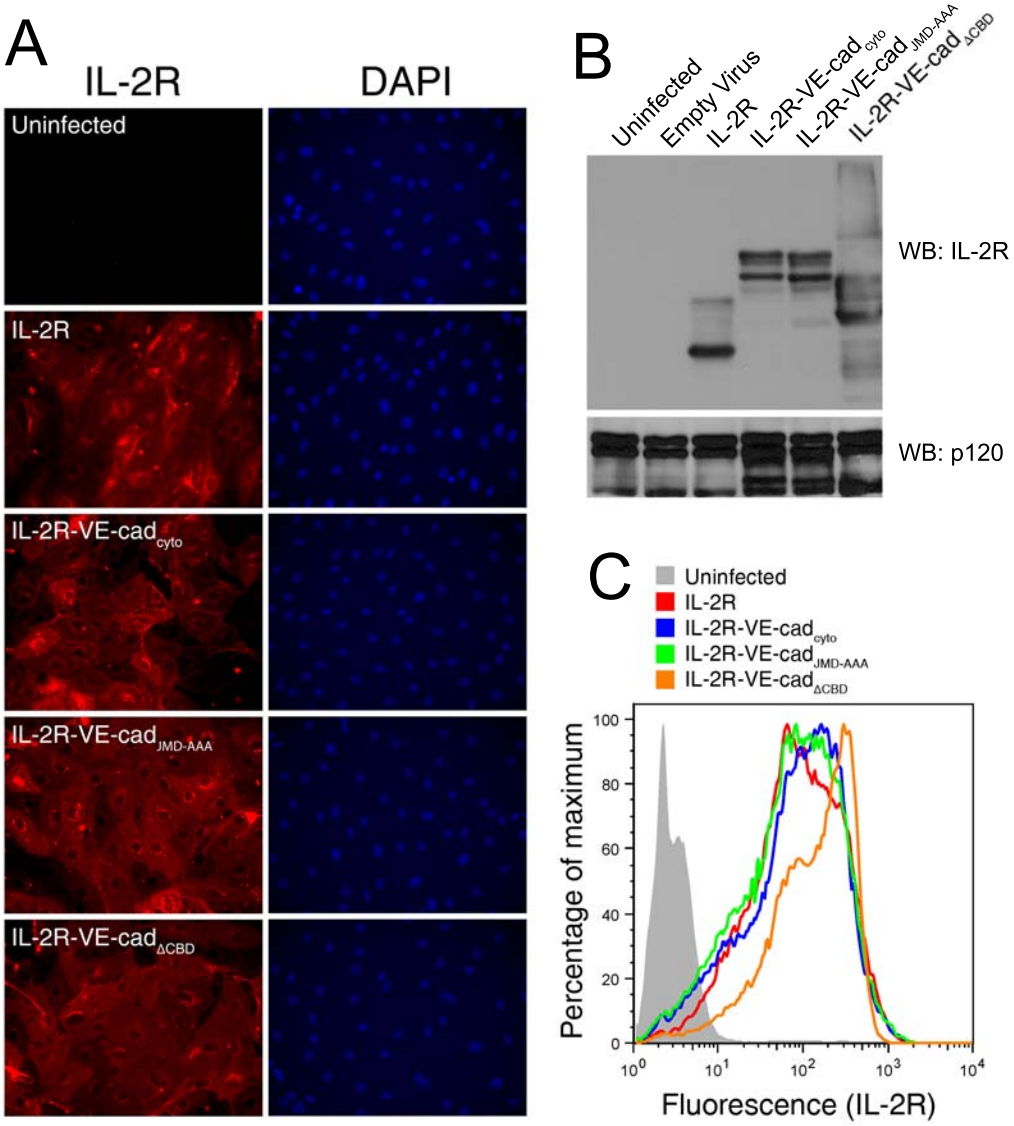
A
IL-2R
DAPI
Uninfected
IL-2R
IL-2R-VE-cad$_{cyto}$
IL-2R-VE-cad$_{JMD-AAA}$
IL-2R-VE-cad$_{\Delta CBD}$
B
Uninfected
Empty Virus
IL-2R
IL-2R-VE-cad$_{cyto}$
IL-2R-VE-cad$_{JMD-AAA}$
IL-2R-VE-cad$_{\Delta CBD}$
WB: IL-2R
WB: p120
C
Uninfected
IL-2R
IL-2R-VE-cad$_{cyto}$
IL-2R-VE-cad$_{JMD-AAA}$
IL-2R-VE-cad$_{\Delta CBD}$
Percentage of maximum
Fluorescence (IL-2R)

Figure 3.2

Figure 3.2

Chimeric receptors are expressed at comparable levels both in total protein and at the plasma membrane. Human microvascular endothelial cells were transfected with chimeric constructs containing the IL-2R extracellular domain fused to the cytoplasmic domain of VE-cadherin. Expression of the constructs was verified by immunofluorescence microscopy (A). Cells were stained for IL-2R to detect the receptors and DAPI to show the total nuclei and enable evaluation of infection rates. IL-2R staining was absent in uninfected cells, and for all four constructs we observed infection rates of 80% or higher. Expression levels were also assessed by Western blot (B) in which whole cell lysates were probed for IL-2R and with p120 as a loading control. The constructs were expressed at comparable levels and at their predicted molecular weights. The levels of expression of the chimeric receptors at the cell surface were assessed using flow cytometry. Unpermeabilized cells were fixed and stained for IL-2R and tested for their peak fluorescence values as compared to those of uninfected control cells. Peak values for each construct occurred within a similar range, indicating that their surface expression was comparable and the total number of receptors at the membrane was not widely variable between constructs.

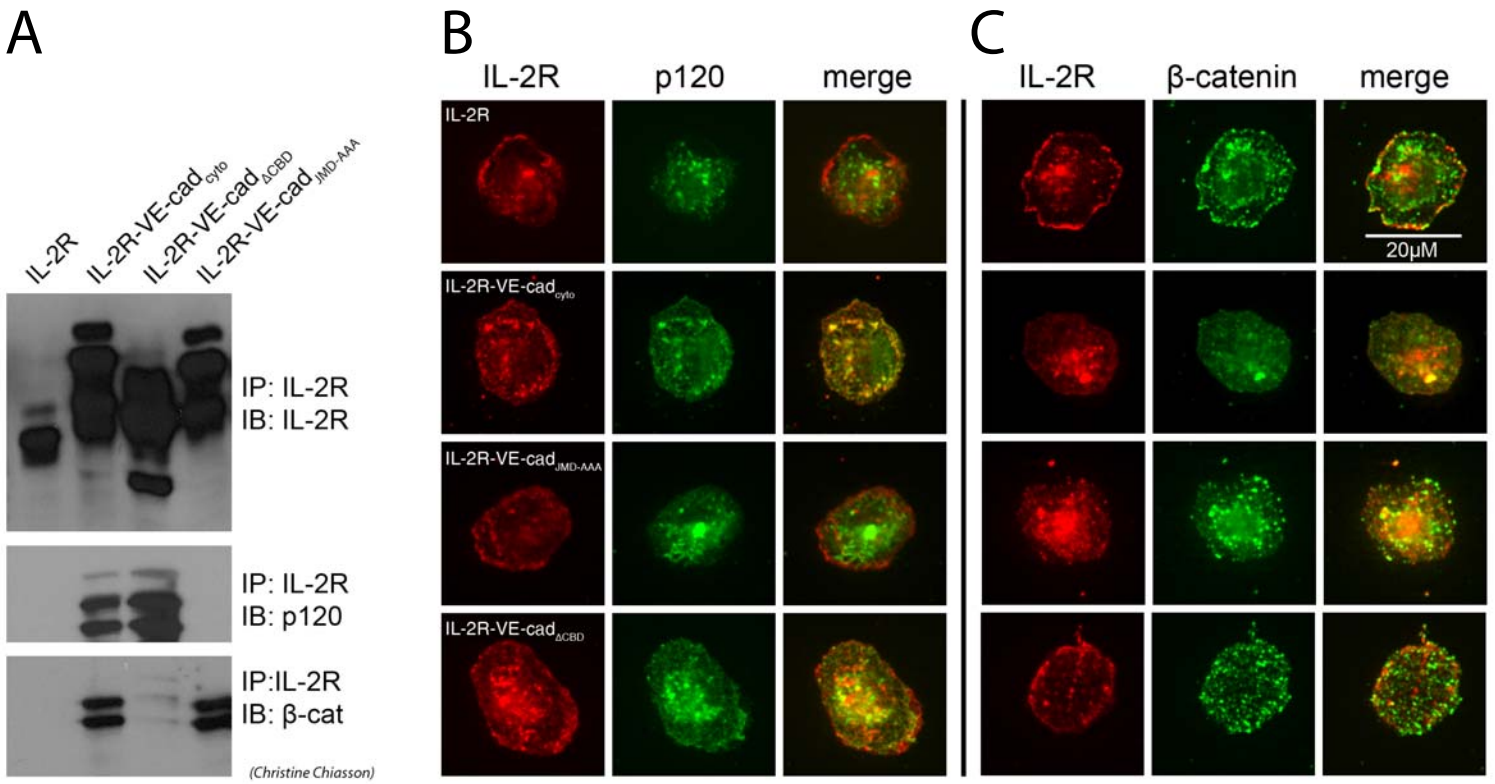
A
IL-2R
IL-2R-VE-cad cyto
IL-2R-VE-cad ΔCBD
IL-2R-VE-cad JMD-AAA
IP: IL-2R
IB: IL-2R
IP: IL-2R
IB: p120
IP:IL-2R
IB: β-cat
(Christine Chiasson)
B
IL-2R
p120
merge
IL-2R
IL-2R-VE-cad cyto
IL-2R-VE-cad JMD-AAA
IL-2R-VE-cad ΔCBD
C
IL-2R
β-catenin
merge
20μM

Figure 3.3

Figure 3.3

Cytoplasmic domains of chimeric receptors interact directly with p120 and β-catenin at the site of adhesion. Immunoprecipitations were performed to verify that the chimeric receptors were able to complex with the appropriate catenins (A). Magnetic beads conjugated to antibodies against IL-2R were used to pull down the chimeric constructs, which were subsequently probed for p120 and β-catenin. p120 was pulled down by IL-2R-VE-cad$_{cyto}$ and IL- 2R-VE-cad$_{\Delta CBD}$ and β-catenin was pulled down by IL-2R-VE-cad$_{cyto}$ and IL-2R-VE-cad$_{JMD\text{-}AAA}$. To ensure that catenins were recruited to the sites of adhesion mediated by the chimeric constructs, cells expressing the constructs were seeded on micropatterned coverslips using IL-2R antibodies as an adhesive ligand (B and C). The cells were then extracted using Triton X-100 in a cytoskeleton stabilization buffer and stained for p120, β-catenin, and IL-2R. p120 colocalized with IL-2R-VE-cad$_{cyto}$ and IL- 2R-VE-cad$_{\Delta CBD}$ (B), while β-catenin colocalized with IL- 2R-VE-cad$_{cyto}$, and IL- 2R-VE-cad$_{JMD\text{-}AAA}$ (C). Scalebar is 20μm, which is the diameter of the adhesive islands.

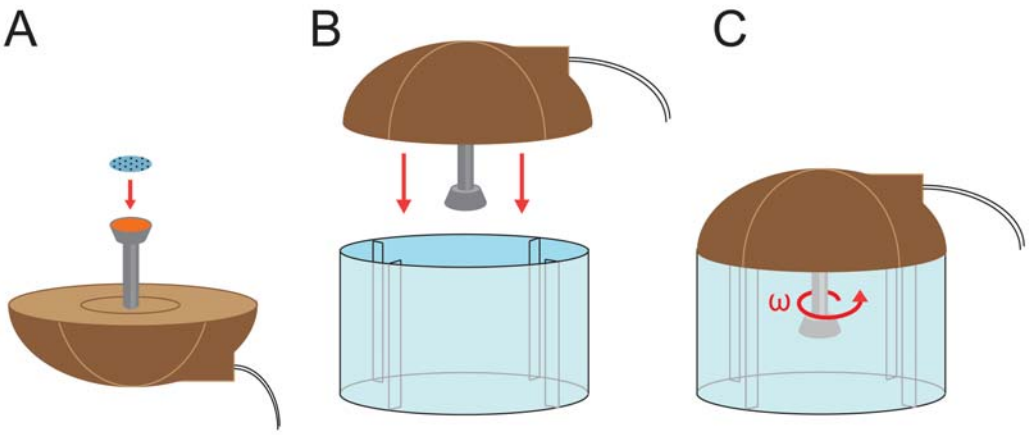

Figure 3.4

Figure 3.4

Diagram of the hydrodynamic spinning disk apparatus for measuring cell adhesion strength. Cells were seeded onto micropatterned coverslips and allowed to adhere for 16 hours. A coverslip with adherent cells were mounted onto the spinning disk apparatus and a vacuum pump was used to hold the sample in place (A). The sample was then submerged into the spin chamber filled with PBS+ with 2mM dextrose (B). The chamber was equipped with baffles at the edges which prevented the spinning motion of the sample from creating a vortex. The sample was then spun for 5 minutes at a controlled speed (ω) (C). Samples were then fixed, permeabilized, and stained for microscopy and quantitation of adherent cells remaining on the coverslip.

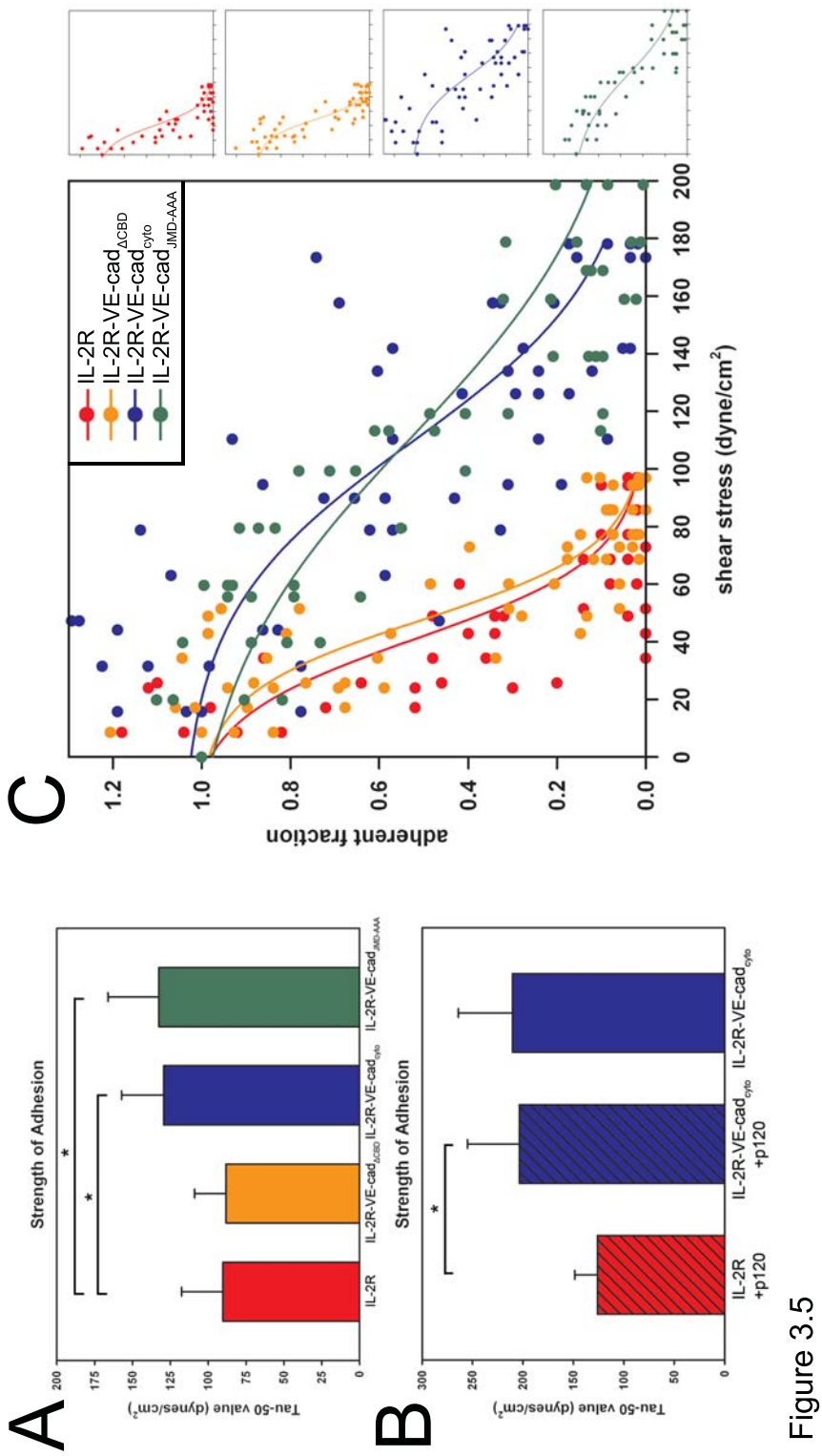
A
Strength of Adhesion
Tau-50 value (dynes/cm²)
IL-2R
IL-2R-VE-cad ΔCBD
IL-2R-VE-cad cyto
IL-2R-VE-cad JMD-AAA
B
Strength of Adhesion
Tau-50 value (dynes/cm²)
IL-2R +p120
IL-2R-VE-cad cyto +p120
IL-2R-VE-cad cyto
C
adherent fraction
shear stress (dyne/cm²)
IL-2R
IL-2R-VE-cad ΔCBD
IL-2R-VE-cad cyto
IL-2R-VE-cad JMD-AAA

Figure 3.5

Figure 3.5

Linkage between cadherins and the actin cytoskeleton is necessary to strengthen steady-state adhesion. (A) Comparisons of adhesions strength values (τ_{50}) between the chimeric constructs. IL-2R was significantly less adhesive than IL-2R-VE-cad$_{cyto}$ and IL-2R-VE-cad$_{JMD\text{-}AAA}$ (Tukey test; $p < 0.050$) but not significantly different from IL- 2R-VE-cad$_{\Delta CBD}$. (B) p120 is not limiting in cells expressing IL-2R-VE-cad$_{cyto}$. No difference in adhesion strength was observed when p120 and IL-2R-VE-cad$_{cyto}$ was co-expressed with exogenous p120, as compared to cells expressing IL-2R-VE-cad$_{cyto}$ alone. (C) Representative detachment profiles for the four receptors, with the individual profiles in smaller graphs to the right. For adhesion analysis, the number of samples per group = 8 and error bars are standard error of the mean (SEM).

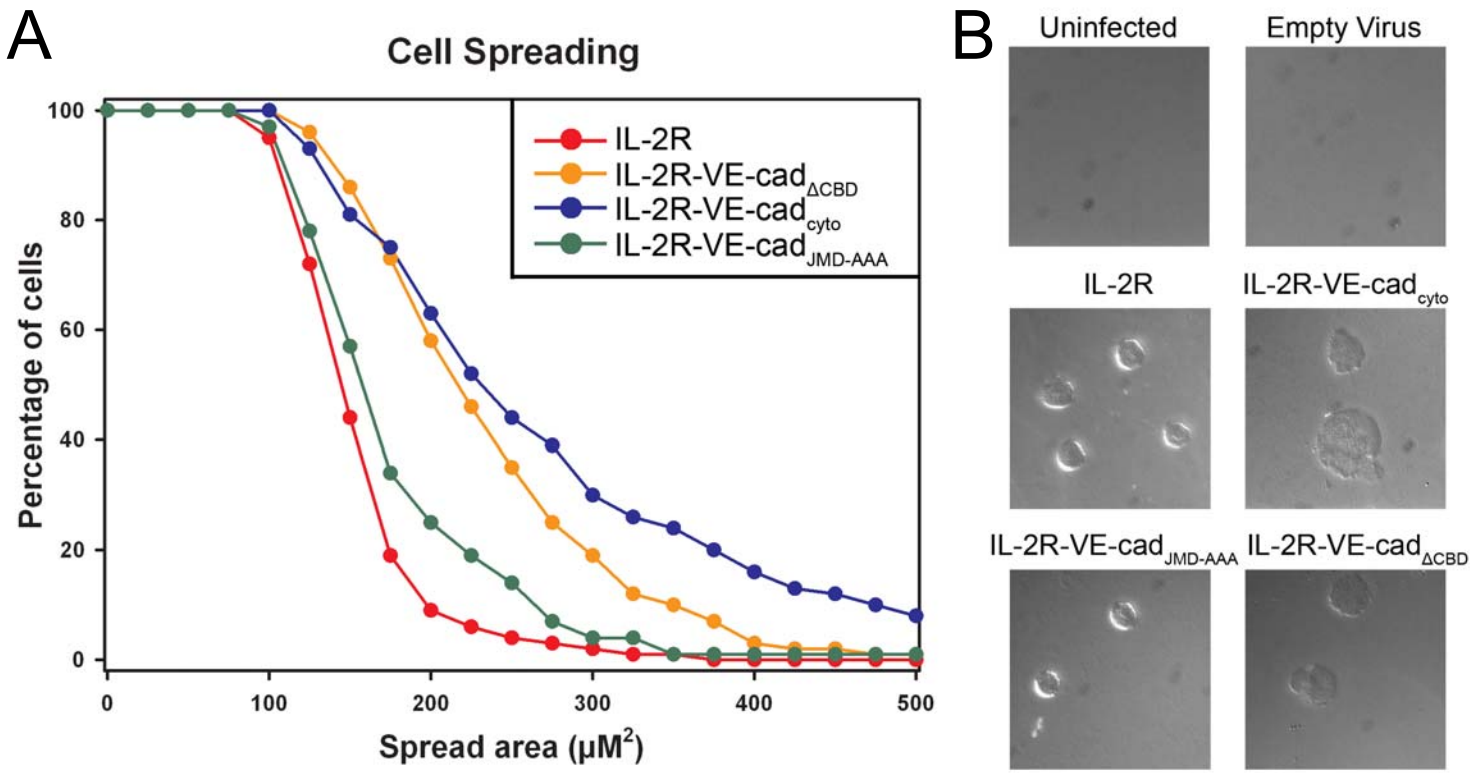
A
Cell Spreading
Percentage of cells
Spread area (μM²)
IL-2R
IL-2R-VE-cad ΔCBD
IL-2R-VE-cad cyto
IL-2R-VE-cad JMD-AAA
B
Uninfected
Empty Virus
IL-2R
IL-2R-VE-cad cyto
IL-2R-VE-cad JMD-AAA
IL-2R-VE-cad ΔCBD

Figure 3.6

Figure 3.6

The interaction between p120 and the cadherin juxtamembrane domain is required to promote cell spreading. Cells expressing the chimeric constructs were seeded sparsely on unpatterned coverslips coated with antibodies against IL-2R. The cells were allowed to adhere for 30 minutes before fixation. The spread areas of 100 cells per condition, chosen at random, were measured and plotted by quantifying the number of cells per condition whose spread areas were larger than a given area in microns (A). The mean values of the different groups were found to be statistically different (Kruskal-Wallis test; $p < 0.001$). IL-2R and IL- 2R-VE-cad$_{JMD\text{-}AAA}$ were not statistically different from each other, and IL- 2R-VE-cad$_{cyto}$ and IL- 2R-VE-cad$_{\Delta CBD}$ were not statistically different from each other. However, both members of the former pair were statistically different from both members of the latter pair (Tukey test; $p < 0.05$). (B) Representative images of adherent cells expressing the IL-2R constructs. Uninfected cells and those infected with an empty adenoviral vector were not able to attach to the IL-2R-antibody-coated coverslips.

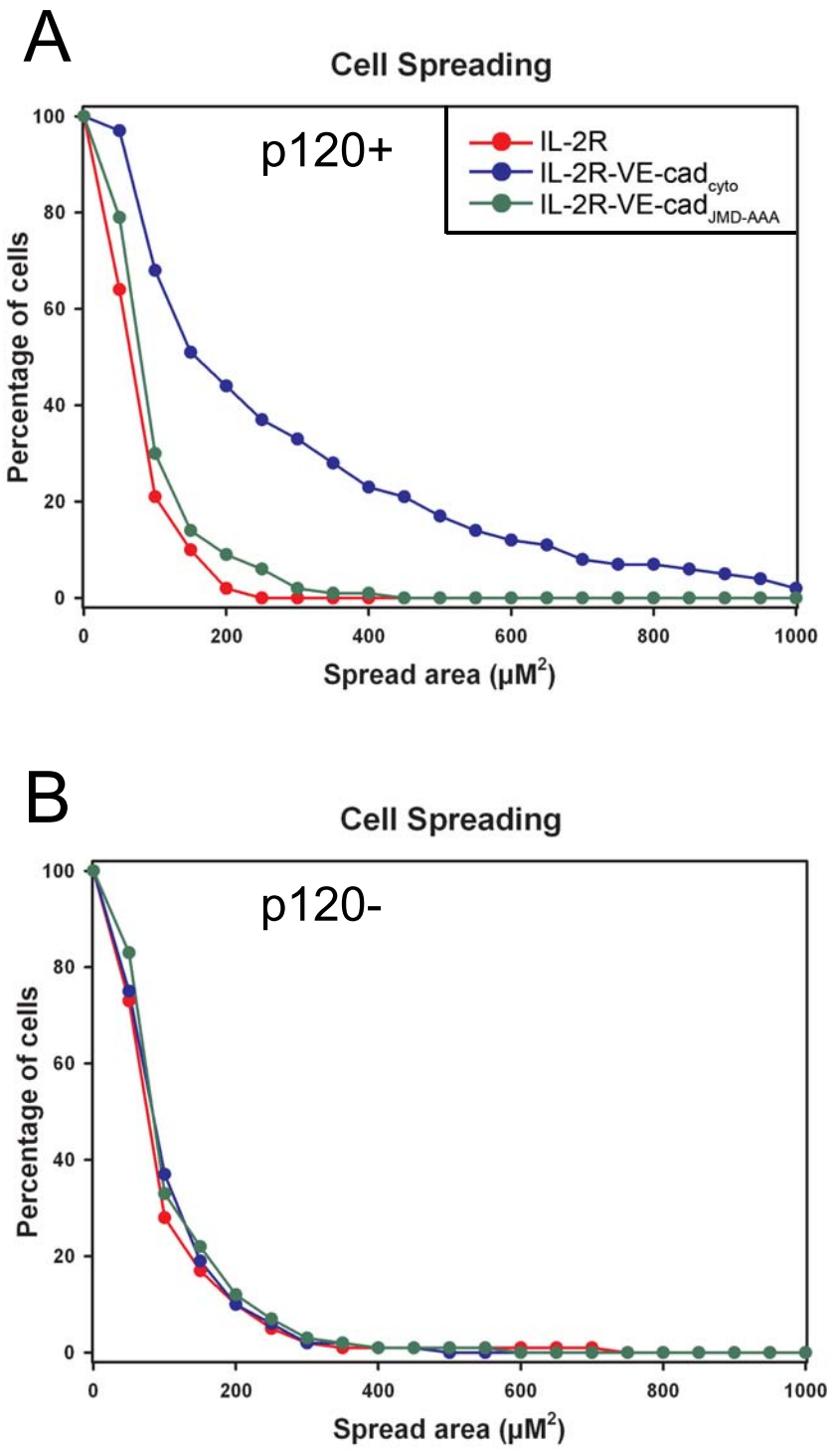
A
Cell Spreading
p120+
IL-2R
IL-2R-VE-cad cyto
IL-2R-VE-cad JMD-AAA
Percentage of cells
Spread area (μM2)
0
20
40
60
80
100
0
200
400
600
800
1000
B
Cell Spreading
p120-
Percentage of cells
Spread area (μM2)

Figure 3.7

Figure 3.7

Cell spreading is impaired in p120-null endothelial cells. IL-2R, IL- 2R-VE-cad$_{cyto}$, and IL- 2R-VE-cad$_{JMD\text{-}AAA}$ were expressed in mouse endothelial cells that were either control or p120-null and their spreading ability was measured as in the previous experiment. (A) In control cells, IL-2R-VE-cad$_{cyto}$ exhibited significantly increased spreading over IL-2R and IL- 2R-VE-cad$_{JMD\text{-}AAA}$. (Kruskal-Wallis test; $p < 0.001$. Tukey test; $p < 0.05$). (B) In p120-null cells, the increased spreading previously observed in IL-2R-VE-cad$_{cyto}$ was lost; the three conditions were not statistically different from one another (Kruskal-Wallis test; $p = 0.230$). A comparison between IL-2R-VE-cad$_{cyto}$ in control cells vs. p120-null cells revealed a significant difference (Tukey test; $p < 0.05$).

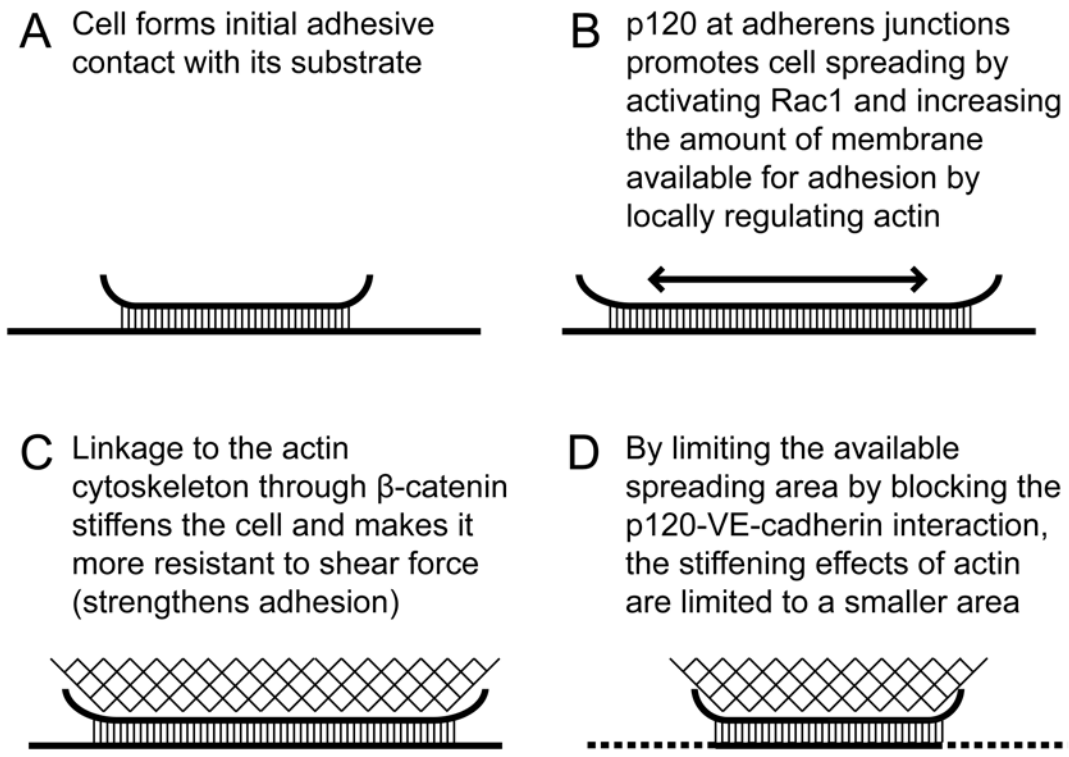
A Cell forms initial adhesive contact with its substrate
B p120 at adherens junctions promotes cell spreading by activating Rac1 and increasing the amount of membrane available for adhesion by locally regulating actin
C Linkage to the actin cytoskeleton through β-catenin stiffens the cell and makes it more resistant to shear force (strengthens adhesion)
D By limiting the available spreading area by blocking the p120-VE-cadherin interaction, the stiffening effects of actin are limited to a smaller area

Figure 3.8

Figure 3.8

Interaction between p120 and cadherins strengthens cell adhesion by promoting cell spreading. A proposed model demonstrating the distinct contributions of p120 and β-catenin to strengthening adhesion.

Chapter 4

Summary and Future Directions

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Overview of the Dissertation

The first goal of the work presented in this dissertation was to examine the role of p120 in mouse vascular development. Previous studies had explored the functions of p120 in cultured endothelial cells, but the effects of its conditional endothelial deletion in the mammalian context had not yet been determined. We sought to test whether the loss of endothelial p120 would produce a phenotype, whether that phenotype would be dominant or recessive, and at what developmental stage it would be manifest. We further explored which aspects of vascular development would be affected: initial formation of early vessels by vasculogenesis, sprouting and remodeling of networks during angiogenesis, or the maintenance of vascular integrity through the formation and stabilization of adhesive junctions and the recruitment of mural cells. We determined that mice lacking endothelial p120 were recessive embryonic lethal in midgestation, and that while the formation of major vessels produced by vasculogenesis and sprouting angiogenesis were not impaired, the remodeling of vascular networks was defective, resulting in sparse and disorganized microvessels in the embryos, as well as in mutant yolk sacs and placentas. Furthermore, we observed hemorrhages in the mutant mice, particularly in the brain, which indicated a loss of barrier function.

To understand the mechanisms underlying the phenotype, we examined the effects of endothelial p120 loss on VE-cadherin and N-cadherin, both of which are expressed in endothelial cells and are stabilized at the cell surface by p120. Using a quantitative immunofluorescence approach, we found that the expression of both cadherins was reduced in the absence of p120, although low levels of VE-cadherin were

retained. While VE-cadherin mediates adhesion between endothelial cells at adherens junctions, N-cadherin is required for the recruitment of pericytes, which stabilize capillaries and enable them to withstand the stresses exerted by blood flow. Accordingly, we found a significant reduction in pericyte coverage of capillaries in the brains of mutant embryos, which likely contributed to the occurrence of hemorrhages we had observed in that region. To examine the mechanisms underlying the angiogenic remodeling defects in p120-null microvessels, we isolated cells from mice which were homozygous for the floxed allele of p120 and used adenoviral Cre to knock out p120. While assays of barrier function and migration did not reveal significant defects in the p120-null cells, we observed a reduction in their proliferation compared to control cells. We determined that the proliferation defect was independent of p120's inhibition of the small GTPase RhoA, but could be rescued by the re-expression of VE-cadherin. We propose that a reduction in endothelial proliferation could underlie the observed defects in microvascular density and organization in the mouse, resulting in an inability of the vasculature to keep pace with the growth of the embryo. Both the brain hemorrhages and the likely insufficiency of vascular networks to support tissue growth are predicted to contribute to the embryonic lethality of the mutant mice, and both aspects of the phenotype are attributable to cadherin-dependent causes.

However, the exact role of VE-cadherin in the maintenance of strong intercellular adhesion was not fully understood. The knockout of VE-cadherin in the mouse resulted in a recessive embryonic lethal phenotype, but there was no evidence of haploinsufficiency among the heterozygous mice with a 50% reduction in VE-cadherin expression (Carmeliet et al., 1999; Gory-Faure et al., 1999). The endothelial knockout of

p120 likewise resulted in a ~50% loss of VE-cadherin, but this was accompanied by defects in vascular barrier function. While the loss of N-cadherin and pericyte coverage provided some explanation, previous studies had demonstrated the importance of the VE-cadherin cytoplasmic tail and, specifically, binding by p120 in the strengthening of adhesion (Goodwin et al., 2003; Yap et al., 1998). We therefore utilized a biophysical approach to examine the contributions of the VE-cadherin cytoplasmic domains to strong adhesion. To accomplish this, we used a series of chimeric receptors in which the extracellular domain of the interleukin-2 receptor was fused to the VE-cadherin cytoplasmic tail, as well as variants in which the p120 binding site or the β-catenin-binding domain were mutated. Using a combination of hydrodynamic adhesion strength assays and measurements of cell spreading, we found that both β-catenin and p120 contribute to adhesion strength, although through different mechanisms: β-catenin mediates linkage to the actin cytoskeleton and connects the stiffening properties of actin to the site of adhesion, and p120 at the cell surface is required to promote spreading, likely through Rac1. These results provide novel insights into the ways in which p120 and β-catenin regulate cadherin-mediated adhesion, in addition to raising some important new questions which will be explored in the following section.

Future Directions

How does p120 regulate endothelial cell spreading?

We have provided experimental evidence that the interaction between p120 and the VE-cadherin juxtamembrane domain is required to promote cell spreading, and that if p120 is lost or uncoupled from the cadherin tail, spreading is impaired. Preliminary results indicate that the spreading defect resulting from a mutation to the p120-binding site of the cadherin juxtamembrane domain can be rescued by the expression of constitutively active Rac1. This is consistent with previous data showing that a cell adhesion defect caused by a similar mutation to the E-cadherin tail could be rescued with dominant active Rac1 (Goodwin et al., 2003). We therefore predict that the expression of constitutively active Rac1 in cells seeded on micropatterned coverslips would not exhibit increased adhesion strength in a spinning disk assay. Notably, the cadherin tail sequesters p120 at the cell surface, enabling localized Rac1 activation. When the p120 binding site is mutated, spreading is decreased although p120 is still present in the cytoplasm. In order to explore this issue, we intend to continue to examine the effects of constitutively active and dominant negative Rac1 alongside the chimeric IL-2R-VE-cadherin constructs in addition to using pharmacological inhibitors of Rac1 or its downstream effectors. Our preliminary experiments rescuing the p120-dependent spreading defect by expressing dominant active Rac1 were performed in cells expressing IL-2R-VE-cad$_{JMD\text{-}AAA}$. We intend to repeat this experiment in the context of cells expressing IL-2R, which exhibit a similar decrease in spreading to IL-2R-VE-cad$_{JMD\text{-}AAA}$, but which lack linkage to the actin cytoskeleton through the catenin-binding domain. If

constitutively active Rac1 is able to rescue spreading in those cells as well, it is likely that its activation in a localized manner is overwhelmed by the level of overexpression. However, if there are differences between cells expressing IL-2R and IL-2R-VE-cad$_{JMD\text{-}AAA}$ in the presence of dominant active Rac1, it would suggest a greater contribution by the catenin-binding domain than our spreading results with IL-2R-VE-cad$_{\Delta CBD}$ have indicated thus far, as the deletion of the catenin-binding domain did not significantly affect spreading. This would suggest that the presence of p120 binding to the cadherin tail and locally activating Rac1 was sufficient to regulate actin at the cell surface even if the chimeric cadherins mediating adhesion had no physical linkage to the actin cytoskeleton. Additionally, based on the result showing constitutively active Rac1 was able to restore spreading when VE-cadherin was uncoupled from p120, we predict that the overexpression of dominant negative Rac1 or the use of pharmacological agents that inhibit Rac1 would reduce spreading in cells expressing IL-2R-VE-cad$_{cyto}$. If so, this would support our hypothesis that p120 promotes cell-spreading in a Rac1-dependent manner.

What are the developmental effects of uncoupling p120 and VE-cadherin?

One of the major questions to answer when characterizing any tissue-specific conditional knockout of p120 is to what extent the resulting phenotype is dependent on cadherins and to what extent it involves the regulation of Rho family GTPases. Clearly, these are difficult to separate since changes in the localization of p120 in the cell affects RhoA and Rac1 regulation, and the GTPases in turn have important roles in the formation of mature adherens junctions. However, further insights into the role of endothelial p120

in vivo may be obtained by the generation of a mouse in which a targeted mutation to the VE-cadherin gene disrupts p120 binding. Among the benefits of such an approach are the fact that it would be restricted to endothelial cells and the elimination of complications relating to mosaicism, which can be a confounding issue in conditional knockout animals. The mutation would also be present from the earliest stages of development, unlike a conditional knockout in which the expression of Cre recombinase requires the temporal activation of the promoter driving its expression as well as sufficient time for the turnover of the targeted protein that has already been synthesized. Furthermore, comparisons between these animals and the conditional p120 mutant animals previously described would provide insights into the extent to which the endothelial null phenotype was dependent on binding by p120 to the VE-cadherin tail. In a mouse expressing p120-uncoupled VE-cadherin, endogenous p120 would still be able to bind to N-cadherin and thereby promote pericyte recruitment. As a result, if N-cadherin loss was the underlying cause of the brain hemorrhages in the conditional knockout mice, we would expect that his particular phenotype would be absent in mice expressing p120-uncoupled VE-cadherin. The possibility remains that the mice expressing mutant VE-cadherin might exhibit a greatly reduced phenotype, or none at all. If this occurs, it would suggest that the lethality and vascular defects observed in the conditional p120-null mice occurred independently of VE-cadherin and may have involved other players such as N-cadherin, RhoA, or Rac1. If the phenotype in the mice harboring the mutant allele of VE-cadherin is more severe than that of the conditional p120 knockout, it could be due to a delay in the knockout and turnover of p120 in the conditional mutant embryos, or the lack of mosaicism which enabled some p120 mutant

mice to escape lethality. Another factor may be the preferential binding of p120 to N-cadherin in the absence of competition by VE-cadherin or the increased cytoplasmic localization of p120, where it is known to exert a potent inhibitory effect on RhoA. Both the increased presence of p120 in the cytoplasm and cadherin switching have been associated with increased motility and EMT in epithelial cells, and are associated with poor prognoses in tumors (Yanagisawa and Anastasiadis, 2006). The increased presence of p120 in the cytoplasm and the loss of p120 at adherens junctions would likely induce dramatic decreases in the regulation of Rho GTPases, which could impact motility and proliferation, both of which are key aspects of vascular growth and remodeling by angiogenesis. Given the intricate and finely regulated mechanisms by which p120, cadherins, and Rho GTPases interact, comparisons between these two mouse models would be of great interest, both *in vivo* and in cultured cells where further manipulations such as rescue experiments would be possible. One example would be to revisit the proliferation assay using cells from both mice. If the cells expressing p120-uncoupled VE-cadherin exhibited normal levels of proliferation, this would be consistent with our observation that re-expressing wild-type VE-cadherin in p120-null cells was able to rescue their proliferative defect. However, if the cells expressing mutant VE-cadherin also proliferated at reduced rates, a possible explanation could involve localized Rac1 signaling. The proliferation defect in p120-null cells was found to be RhoA-independent, but Rac1, which is known to be involved in proliferation (Fryer and Field, 2005), was not tested.

The work presented in this dissertation represents a significant addition to the understanding of how p120 influences endothelial cell biology by regulating adhesion,

proliferation, and cadherin stability. We demonstrate an essential role for p120 in mammalian vascular development and propose a mechanism by which it increases the adhesion strength of endothelial cells by promoting their ability to alter their shape and spreading. Ultimately, additional studies will be necessary to elucidate the full pathways by which p120 accomplishes these functions, particularly with regard to the regulation of Rho family GTPases, and to further examine the implications of p120 alterations in the context of vascular development and maintenance in human diseases.

Chapter 5

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