p120-Catenin Is Required for Mouse Vascular Development

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Rationale: p120-catenin (p120) is an armadillo family protein that binds to the cytoplasmic domain of classical cadherins and prevents cadherin endocytosis. The role of p120 in vascular development is unknown.

Objective: The purpose of this study is to examine the role of p120 in mammalian vascular development by generating a conditionally mutant mouse lacking endothelial p120 and determining the effects of the knockout on vasculogenesis, angiogenic remodeling, and the regulation of endothelial cadherin levels.

Methods and Results: A conditional Cre/loxP gene deletion strategy was used to ablate p120 expression, using the Tie2 promoter to drive endothelial Cre recombinase expression. Mice lacking endothelial p120 died embryonically beginning at embryonic day 11.5. Major blood vessels appeared normal at embryonic day 9.5. However, both embryonic and extraembryonic vasculature of mutant animals were disorganized and displayed decreased microvascular density by embryonic day 11.5. Importantly, both vascular endothelial cadherin and N-cadherin levels were significantly reduced in vessels lacking p120. This decrease in cadherin expression was accompanied by reduced pericyte recruitment and hemorrhaging. Furthermore, p120-null cultured endothelial cells exhibited proliferation defects that could be rescued by exogenous expression of vascular endothelial cadherin.

Conclusions: These findings reveal a fundamental role for p120 in regulating endothelial cadherin levels during vascular development, as well as microvascular patterning, vessel integrity, and endothelial cell proliferation. Loss of endothelial p120 results in lethality attributable to decreased microvascular density and hemorrhages. (Circ Res. 2010; 106:941-951.)

Key Words: cadherin ■ endothelial ■ adhesion

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 for their role in vascular biology and pathophysiology. In particular, vascular endothelial (VE)-cadherin has been implicated in the regulation of vascular barrier function,1–3 inflammatory cell transmigration,4 and endothelial cell proliferation and morphogenesis during neovascularization.5

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.1,6 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.1,7 β-Catenin and plakoglobin have been shown to mediate associations between cadherins and the cytoskeleton,8,9 although the precise molecular interactions that lead to this linkage remain unclear. p120-catenin binds to the juxtamembrane domain of classical cadherins.10,11 First discovered as a Src phosphorylation substrate,12 p120 was later shown to be an armadillo family protein.13 A central function of p120 is to regulate cadherin stability.14 Previous studies have shown that p120 prevents clathrin-dependent endocytosis of VE-cadherin and thus stabilizes VE-cadherin at the plasma membrane.15–17 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.18–20 Additionally, p120 is an important regulator of members of the Rho family of small GTPases,21 and functions in the nucleus to regulate transcription through interactions with Kaiso.22

Although many of these activities of p120 have been elucidated using in vitro studies, the functions of p120 in vivo are less clear. In Drosophila23,24 and C. elegans,25 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,26,27 and embryonic lethality in mice28 and zebrafish.29 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.30 Conditional p120 knockout in forebrain neuroepithelia resulted in reduced density of neuronal spines and synapses, an effect owing more to the dysregulation of Rho GTPases than changes in N-cadherin levels.31 Furthermore, an epidermal conditional p120 knockout mouse displayed a chronic inflammatory response caused by nuclear factor κB activation, also likely downstream of altered regulation of Rho family GTPases.32 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. To explore the functions of this junctional protein in vivo, a conditional mouse knockout approach was used 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.

Methods
Mice with LoxP sites in introns 2 and 8 of the p120 gene were generated as described previously.30 Tie2-Cre–expressing C57BL/6 mice were obtained from The Jackson Laboratory (no. 004128).

Detailed descriptions of tissue processing, antibodies used for immunofluorescence, microscopy and image quantitation, primary cell isolation, and cultured cell experiments are provided in the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

Results
Loss of Endothelial p120-Catenin Is Embryonic Lethal
A conditional gene ablation strategy was used to define the function of p120 in mouse vascular development. Mice harboring a LoxP-flanked allele of the p120 gene30 were crossed with transgenic mice expressing Cre recombinase driven by the Tie2 promoter (Online Figure I, A), resulting in endothelial Cre expression beginning at embryonic day (E)7.5.31 Fewer than expected pups harboring the p120^flox/flox, Cre^+ (conditional mutant) genotype were observed in litters (Online Figure I, 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 (Online Figure I, 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 platelet endothelial cell adhesion molecule (PECAM)-1.34 p120 colocalized with PECAM-1 in the vasculature of control mice (Figure 1A through 1C) but not in vessels of conditional mutant animals (Figure 1D through 1F). Similar results were observed in embryonic tissue (see Online Figure II). 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 control mice (Figure 1G through 1I) 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 1J through 1L). In contrast, p120 expression appeared normal in genotypically mutant animals that survived into adulthood with no obvious phenotype (Figure 1M through 1O). 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 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.35,36 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 (Online Figure II, D through 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. Whole-mounted E9.5 embryos from six litters showed no defects in intersomitic vessel organization or vertebral arteries in mutant animals (Online Figure II, G and 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 (Online Figure I). 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, a reduction in vascular density was observed among conditional mutants (Online Figure III, C and D) compared to wild type animals (Online Figure III, A and 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 (Online Figure III, E through H). PECAM-1 staining of the brain of whole-mounted E13.5 conditional mutant embryos (Online Figure III, J and L) revealed disorganized and less dense vascular networks compared to control littermates (Online Figure III, I and K). Defects were particularly striking in the hyaloid vascular system of the developing eye (Online Figure III, M and 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 2A and 2B), whereas subsequent steps in vessel remod-
eling and expansion were compromised. In control animals, yolk sac vessels remodeled into a well-organized hierarchy of large and small vessels (Figure 2A, 2C, 2E, and 2G). In contrast, conditional p120 mutants exhibited reduced vascular branching and increased numbers of blind-ending vessels (Figure 2I and 2J). Furthermore, morphometric analysis of vascular and avascular space revealed that mutant vessels failed to form homogeneous networks. As shown in Figure 2K, a linear relationship was apparent between vessel diameter and avascular space diameter in control animals ($r^2=0.82$). In contrast, mutant vessels exhibited highly variable avascular space diameter ($r^2=0.27$). 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 3D and 3G, compared to 3A) and other organs (data not shown) of conditional mutant animals, and histological staining of brain sections revealed both large hemorrhages (Figure 3E) and leaky microvessels (Figure 3F and 3H). 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 endocy-

**Figure 2. 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 (A through H). 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 versus 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). Scale bar is 100 μm.

**Figure 3. Conditional mutant mice exhibit hemorrhages.** E13.5 control (A) and mutant (D and G) embryos photographed using light microscopy. Hemorrhages in mutant mice are indicated by arrowheads. Hematoxylin/eosin staining of paraffin-embedded sections reveal normal microvessels in the brains of control embryos (B and C) and hemorrhaging (E) and leaky vessels (indicated by arrowheads) (F and H) in the brains of mutant embryos. Asterisks in B and C mark red blood cells in microvessels. Scale bars are 20 μm.
tosis and degradation. To determine whether 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 4). In control tissues, p120 (A) and VE-cadherin (B) colocalize in blood vessels (C). In conditionally mutant tissue, VE-cadherin expression is retained (E), but vascular p120 is absent (D, F). Tissue from genotypically mutant mice which exhibit mosaic p120 expression (G, I) have reduced levels of VE-cadherin in areas lacking p120 (blue arrowheads) (H), 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 through 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, whereas 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 for PECAM (O). (t test, P<0.001.) Scale bar is 20 μm.

Figure 4. 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 (B) colocalize in blood vessels (C). In conditionally mutant tissue, VE-cadherin expression is retained (E), but vascular p120 is absent (D, F). Tissue from genotypically mutant mice which exhibit mosaic p120 expression (G, I) have reduced levels of VE-cadherin in areas lacking p120 (blue arrowheads) (H), 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 through 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, whereas 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 for PECAM (O). (t test, P<0.001.) Scale bar is 20 μm.

N-cadherin is also expressed in endothelial cells and plays important roles in vascular development. Previous work has shown that p120 also regulates N-cadherin levels. 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 5A through 5D). However, in mutant littermates, N-cadherin was not detected in corresponding p120-negative vessels (Figure 5E through 5H, quantified in 5I and 5J). N-cadherin has been implicated in the recruitment of pericytes to developing vessels. These cells associate with endothelial cells and are important regulators of vessel remodeling and stabilization. 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. Importantly, pericyte coverage was signifi-
cantly reduced in mutant embryos (Figure 6, compare 6B and 6E; quantified in 6G). Thus, loss of p120 leads to a substantial decrease in endothelial cadherin expression levels and is associated with reduced pericycle 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\textsuperscript{flox/flox}; Cre\textsuperscript{H11002} mice.

To cause p120 deletion, Cre recombinase was introduced using an adenoviral vector.\textsuperscript{44} Following a 72-hour period to allow for turnover of previously transcribed p120, the cells were fixed and examined by immunofluorescent microscopy (Figure 7). Control cells infected with empty adenoviral vector retained p120 expression (Figure 7A and 7G). Additionally, these cells also expressed both VE-cadherin (Figure 7B) and PECAM-1 (Figure 7H), confirming the endothelial identity of the cells. Expression of Cre resulted in near complete ablation of p120 (Figure 7D and 7J). Furthermore, loss of p120 resulted in a striking reduction in VE-cadherin (Figure 7E) but not PECAM-1 levels (Figure 7K). This result was confirmed by western blot analysis (Figure 7M). 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\textsuperscript{flox/flox}; Cre\textsuperscript{H11002} 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 7N). 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 7O). 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,\textsuperscript{45–47} 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. To determine whether the endothelial growth defect in p120-null cells is RhoA- or VE-cadherin–dependent, exogenous p120 and VE-cadherin were reexpressed in p120-null primary endothelial cells. In addition to wild-type p120 1A, a p120 mutant unable to inhibit RhoA (p120 4A K622,628A) was used to determine whether inhibition of RhoA is involved in regulating VE-cadherin expression levels and/or endothelial proliferation. Similar to the results shown in Figure 7, loss of endogenous p120 resulted in significantly decreased VE-cadherin levels (Figure 8C and 8D) and a corresponding decrease in endothelial proliferation as measured by bromodeoxyuridine (BrdUrd) uptake (Figure 8K). Expression of exogenous wild-type p120 1A rescued both VE-cadherin levels (Figure 8E and 8F) and BrdUrd 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 BrdUrd uptake. These studies indicate that p120 regulates endothelial proliferation through a cadherin-dependent mechanism.

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 labyrin-
thine was markedly reduced (Online Figure III, A through 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 2I through 2K). 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 (Figures 7 and 8). 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, and recent studies have implicated VE-cadherin in the transcriptional regulation of claudin-5, 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 (Online Figure IV). In addition to tight junctions, pericytes associate with small vessels and have been shown to regulate capillary diameter and vascular permeability in the brain. Loss of pericytes in the developing mouse results in lethality attributable to microvascular hemorrhaging and edema. Importantly, deletion of p120 resulted in reduced pericyte coverage in mutant vessels in the brain (Figure 6). 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. Previous studies have demonstrated that a central function of p120 is to posttranslationally stabilize cadherins. In cultured endothelial cells, knockdown of p120 using siRNA leads to VE-cadherin endocytosis and degradation. In the present study, we observed that VE-cadherin levels were significantly reduced in vessels lacking p120 (Figure 4), demonstrating that p120 also regulates VE-cadherin levels in vivo. Similarly, in endothelial cells isolated from p120flox/flox; Cre mice, deletion of the p120 gene on expression of

Figure 8 (Continued). 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). Reexpression of wild-type p120 (E and F) or the Rho-uncoupled p120 mutant (p120 4A K622,628A) (G and H) restored VE-cadherin expression in p120-null cells. Exogenously expressed VE-cadherin assembled at cell–cell junctions in the absence of p120 (I and J). Deletion of p120 reduced endothelial proliferation rates. BrdUrd 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 1-way ANOVA on ranks, P<0.001.) Furthermore, reexpression 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 vs control group by Dunnett’s method, P<0.05). Scale bar is 20 μm.
Cre recombinase leads to a significant reduction in both p120 and VE-cadherin (Figure 7). These cells also exhibit dramatically reduced proliferation (Figure 8). Loss of endothelial proliferation during embryonic development provides a reasonable explanation for the loss of microvascular density observed in vivo (Figure 2; Online Figure III). A number of studies have implicated VE-cadherin in regulating endothelial proliferation.54,55 Consistent with these previous studies, the endothelial proliferation defect of p120-null endothelial cells could be rescued by expression of exogenous VE-cadherin (Figure 8), 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 5). N-cadherin is required for vascular development and has been implicated in pericyte recruitment to developing vessels.39,40 Together, these results suggest that the reduction in VE-cadherin and N-cadherin on 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.48 To determine whether RhoA inhibition is important for regulating VE-cadherin levels and/or endothelial cell proliferation, we used 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 8). These observations are consistent with our recent report that p120 inhibits cadherin endocytosis in a RhoA-independent manner.17 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 because of severe vascular remodeling defects.57,58 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 4M). 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.39,47,56 In addition, a recent study using a zebrafish model demonstrated that even a modest reduction in VE-cadherin resulted in brain hemorrhaging.29 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 dysregulation of Rho-family GTPases.57 Consistent with this notion, VE-cadherin was recently shown to reduce vessel sprouting by suppressing Rac1 activity and enhancing actomyosin contractility.48 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.

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Disclosures

None.

References

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Novelty and Significance

What Is Known?

- p120-catenin is a widely expressed adherens junction protein that is essential for vertebrate development.
- p120-catenin stabilizes cell surface VE-cadherin in cultured endothelial cells by regulating cadherin endocytosis.
- The role of p120-catenin in vertebrate vascular development is not yet known.

What New Information Does This Article Contribute?

- Loss of endothelial p120-catenin results in midgestational embryonic lethality in the developing mouse.
- The vascular p120-null phenotype includes hemorrhaging, angiogenic remodeling defects, endothelial cadherin reduction, and decreased pericyte recruitment.
- p120-catenin ablation results in a reduction in endothelial proliferation which is VE-cadherin-dependent.

p120-catenin is an armadillo family protein that localizes to intercellular adherens junctions in many cell types and is required for vertebrate development. Previous studies from our laboratory and others have shown that by binding to members of the cadherin family of adhesion receptors, p120-catenin stabilizes their expression at the cell surface. However, the role of p120-catenin during mammalian vascular development is not understood. We reveal here an essential role for endothelial p120-catenin in the developing mouse embryo and demonstrate that ablating endothelial p120-catenin causes hemorrhages and microvascular patterning defects which result in midgestational lethality. Furthermore, we show that the loss of p120-catenin leads to a reduction of endothelial cadherins and a cellular proliferation defect which is VE-cadherin-dependent. This study demonstrates for the first time that p120-catenin is required for mammalian vascular development and that its ablation compromises vascular integrity and microvascular morphogenesis. This work provides novel insights into the regulation of intercellular adhesion during the process of vascular development.
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SUPPLEMENT MATERIAL

Online Figure I. 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 p120<sup>lox/lox</sup>;Cre<sup>+</sup> (x) p120<sup>lox/wt</sup>;Cre<sup>-</sup> 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 littersmates.

Online Figure II. 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.

Online Figure III. 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 littersmates (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 littersmates (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.

Online Figure IV. 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).

EXPANDED METHODS

Animals

Mice (Mus musculus) with LoxP sites inserted in introns 2 and 8 of the p120 gene were generated as described previously<sup>1</sup>. Tie2-Cre expressing C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) #004128<sup>2</sup>. 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 littersmates.

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 (Dv) 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 (Dα) ³. The paired measurements were plotted (Dv vs. Dα) and a linear regression was performed in SigmaPlot. The average ratio, Dv / Dα, was also compared between mutant and control groups.
Cell culture

Endothelial cells were obtained using methods previously described 4, 5. Briefly, hearts and lungs were removed from mice of the p120flox/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 6. 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 confluency 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 7. 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% confluency 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°C. 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 p120flox/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 Laemmlin 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\textsuperscript{flx/flx} mice and cultured in complete growth medium. An equal number of cells were seeded into gelatin-coated 1.9cm\textsuperscript{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.

SUPPLEMENTAL REFERENCES

References
Viability of Neonatal Mice

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Online Figure II