β1 Integrin Establishes Endothelial Cell Polarity and Arteriolar Lumen Formation via a Par3-Dependent Mechanism

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SUMMARY

Maintenance of single-layered endothelium, squamous endothelial cell shape, and formation of a patent vascular lumen all require defined endothelial cell polarity. Loss of β1 integrin (Itgb1) in nascent endothelium leads to disruption of arterial endothelial cell polarity and lumen formation. The loss of polarity is manifested as cuboidal-shaped endothelial cells with dysregulated levels and mislocalization of normally polarized cell-cell adhesion molecules, as well as decreased expression of the polarity gene Par3 (pard3). β1 integrin and Par3 are both localized to the endothelial layer, with preferential expression of Par3 in arterial endothelium. Luminal occlusion is also exclusively noted in arteries, and is partially rescued by replacement of Par3 protein in β1-deficient vessels. Combined, our findings demonstrate that β1 integrin functions upstream of Par3 as part of a molecular cascade required for endothelial cell polarity and lumen formation.

INTRODUCTION

Essential to vascular morphogenesis, the molecular events that regulate endothelial lumen formation have been difficult to dissect. The information currently available has been gathered from elegant in vitro studies in three-dimensional culture systems that recapitulate the initial steps in the angiogenic cascade. These in vitro studies have indicated that, at least in capillary vessels, lumens result from the coalescence of vacuoles (Davis et al., 2007; Downs, 2003; Folkman and Haudenschild, 1980). A similar process has also been observed in vivo, as zebrafish intersomitic vessel sprouts demonstrate a required alignment of vacuoles from preceding cells (Kamei et al., 2006). The events are thought to be initiated downstream of integrin-extracellular matrix signaling, and require the activation of Cdc42, Rac1, pak2/4, Raf kinases, and the Par3/6/atypical PKC complex (Koh et al., 2008, 2009). Further confirmation of these regulatory mechanisms in vivo has been more difficult to obtain, as these molecules are likely essential for multiple functions, and studies using loss or gain of function would require both temporal and cellular control. A more recent study has demonstrated that in the early murine aorta, a two-cell cord-like structure polarizes prior to downstream activation of aPKC and Rho-associated proteins to form a lumen, in lieu of vacuole accumulation (Strilic et al., 2009). Thus, the mechanisms of lumen formation may be as varied and specialized as the vascular beds in which they occur.

The integrin family of heterodimeric transmembrane proteins functions in cell-surface binding to extracellular matrix (ECM) and simultaneous association with internal actin cytoskeletal components (Hynes, 2002). These receptors are formed by pairs of α and β subunits, and have been associated with processes ranging from cell structure and adhesion to cell differentiation and survival (Giancotti and Ruoslahti, 1999; Hynes, 2002). Of the 22 currently recognized integrin heterodimers, at least seven (αvβ3, αvβ5, αvβ1, α1β1, α2β1, α3β1, and α5β1) are expressed by endothelial cells and have been implicated in vascular morphogenesis (Hynes and Bader, 1997; Rupp and Little, 2001; Stupack and Cheresh, 2002). However, it has been challenging to ascertain their specific contributions during blood vessel formation and homeostasis due to complex phenotypes, functional overlap, and in some cases early embryonic lethality. Because the β subunits associate with multiple α receptors, genetic inactivation of specific β subunits is fruitful, as they result in the lack of expression of multiple α-β pairs.
Three recent reports using a Tie-2 Cre to ablate β1 integrin in endothelial cells showed early lethality (embryonic day [E] 9.5–E10.5), with defects in vessel patterning typified by reduction in vascular branching from main vessels, and sac-like structures instead of uniform lumens (Carlson et al., 2008; Lei et al., 2008; Tanjore et al., 2008). As the severity of the phenotype precludes investigating the roles of β1 integrin in later angiogenic events, we studied the function of β1 integrin in endothelial cells by genetic inactivation using a VE-cadherin Cre (Alva et al., 2006). The VE-cadherin Cre line initiates deletion as early as the Tie-2 Cre (E6.5), but full penetration is noted only by E14.5 (Alva et al., 2006). The slower expression kinetics results in hypomorphic phenotypes and the possibility of investigating genetic contributions at later stages of vascular maturation. Indeed, deletion of β1 integrin using the VE-cadherin Cre results in later lethality, and has uncovered novel roles for this molecule in endothelial cell polarity and subsequent downstream events with far-reaching implications for lumen formation.

RESULTS

β1 Integrin Loss Results in Dose-Dependent Lethality

Cell-specific deletion of the Itgb1 gene (herein called β1 integrin) with homozygous floxed alleles by VE-cadherin Cre recombinase (β1f/f; Cre+) results in lethality that spans from E13.5 to E17.5 (Figure 1A, green). When a floxed allele is replaced by a “null” allele (β1f/n; Cre+), an earlier lethality is noted ranging from E10.5 to E14.5 (Figure 1A, blue). When inactivation of the β1 integrin gene is confined to exon 3 (β1e3/e3; Cre+), a rather sharp lethality at E13.5 is noted (Figure 1A, red). All deletions result in embryonic death, and exhibit vascular rupture and hemorrhage (Figure 1B). Whereas the impact of β1 integrin ablation is similar, the timing of VE-cadherin Cre expression combined with efficiency of recombination between floxed alleles of various sizes and numbers results in a gene-dosage-dependent phenotype (Figure 1C). Evaluation of β1 integrin protein expression within endothelial cell (EC) populations at E12.5 of each mouse line shows that
85% of β1^+/++; Cre+ ECs express β1 integrin, as compared to 89% of β1^+/−; Cre+ (Figure 1C; see Figure S1F available online). Cre-mediated inactivation can be traced when using the larger floxed allele (β1^+) by β-galactosidase (β-gal) staining, as a LacZ reporter becomes in-frame upon gene excision (Figure 1D; Figures S1A and S1D). Thus, β-gal indicates both the activity of the β1 integrin promoter and the efficiency of recombinase. It is clear that by E14.5 a large majority of endothelial cells have at least one allele recombined (Figure 1D; Figures S1A and S1D). However, evaluation of protein expression shows that β1-ablated endothelial populations comprise a small fraction of the total at E15.5. In fact, by E17.5, only 45% of the endothelium shows complete loss of β1 integrin protein (Figure 1E; Figure S1F). Therefore, genetic deletion may not result in protein loss for quite some time, which in this case provides a unique opportunity to evaluate β1 integrin ablation in the context of late vascular development. The multiple models of cell-specific deletion also allow for assessment of the β1 integrin protein requirement in ECs. Significant lethality by E12.5 does not occur until the β1 integrin protein expression within EC populations drops below 89% (Figure 1C), suggesting that the developing endothelium is exclusively sensitive to β1 integrin dosage. Evaluation of β3 integrin protein expression revealed a proportional correlation between absence of β1 integrin and increase of β3, indicating a potential compensatory mechanism (Figures S1C and S1F).

β1 Integrin Is Critical for Lumen Formation
Detailed histological examination of viable endothelial specific β1^+/−; Cre+ mutants revealed frequent occlusion of mid-sized vascular lumens (Figure 2; Figure S2A). By E14.5, the cells occluding the lumen have undergone Cre-mediated β1 integrin deletion, as evidenced by LacZ expression (Figure 2A), and strongly express the endothelial adhesion protein PECAM-1 by E15.5 (Figures 2A and 2B; Figure S2B). The observation of the phenotype in medium-sized vessels and not larger arteries may be attributed to the timing of complete deletion. The aorta, other large-caliber arteries, and the endocardium display multi-layered endothelium as well as cuboidal-shaped endothelial cells (Figures 2D and 2E). The abnormal endothelial cell shape is also noted in mouse lines with earlier lethality, β1^+/−; Cre+ (Figure 2D) and β1^+/−; Cre+ (Figure S1E), but at earlier time points (E12.5). The effect is exclusive to cells that have total loss of β1 integrin, as indicated by the β-gal staining in β1^+/−; Cre+ embryos (Figure 2D, arrows) and by β1 integrin immuno-staining in β1^+/−; Cre+ (Figure S3A, arrows). However, the full-occlusion phenotype is most prominent at later developmental stages (Figure S3C). Luminal occlusion and cuboidal endothelial cell shape also occur after postnatal β1 integrin deletion with an inducible system (Figure S2F). The data indicate that β1 integrin is required for maintenance of endothelial cell shape, single-layered morphology, and luminal patency of mid-sized arteries after formation of the major vessels. To examine whether the luminal occlusion was due to cell-autonomous defects in the endothelium, we evaluated smooth muscle cell morphology and function, ECM organization, and thrombus formation. Smooth muscle cells are recruited to β1-ablated vessels, but exhibit abnormal morphology and smooth muscle actin organization (SMA) (Figure 2B, arrow-heads). ECM deposition was noted to be disorganized (Figure S2C). These findings are likely due to aberrant patterning originating from the primary endothelial defect, as deletion of β1 integrin within the smooth muscle cell compartment does not result in luminal occlusion (Figure S2E). Luminal occlusion is also not a result of thrombus formation, as exhibited by the presence of nucleated cells (ECs as per PECAM-1 expression) within the lumen of mutant mice (Figure 3B; Figures S3A–S3C) and lack of fibrinogen staining (Figure S2D). To examine whether abnormal vascular smooth muscle tone could result in the observed endothelial phenotype, animals were perfused with muscle relaxants. The resultant effect was persistence of luminal occlusion and circumferential distribution of PECAM-1 (Figure 2C).

Loss of β1 Integrin Affects Arterial Endothelial Cell Polarity through Par3
To understand the molecular mechanisms underlying the observed phenotype, we performed microarray analysis and compared endothelial cells lacking β1 integrin (β1^+/−; Cre+) with ECs from the same mice that still expressed β1 integrin (Figure S1G) at E16.5. A clear pattern emerged in which cell-cell and cell-matrix adhesion proteins were increased and, in accordance with the FACS data, β1 integrin transcripts were decreased while β3 transcripts were increased (Figure 3A). These findings were reproduced by qRT-PCR (Figure S3D). β1 integrin protein loss was also confirmed in ablative vessels by immunohistochemistry (IHC) (Figure 3B; Figures S3A–S3C), and within the FACS-sorted population by western blotting (Figure 3C). An unexpected consequence of β1 integrin deletion was a reduction in transcripts coding for the polarity protein Par3 (Figure 3A). Par3 is a well-known polarity regulator that functions in a complex with aPKC and Par6 in epithelial cells (Suzuki and Ohno, 2006). Interestingly enough, other members of the complex were not transcriptionally affected by β1 integrin deletion as per our microarray and qRT-PCR analyses (Figure S3D, legend). However, Par6 and aPKC have been shown to interact independently of Par3 (Yamanaka et al., 2003). We also confirmed that the transcriptional decrease in Par3 translated to loss of protein in β1 integrin-ablated EC populations by western blot (Figure 3C).

Further investigation of specific cell-cell adhesion proteins in mutant mice demonstrated abnormalities in localization (Figure 3D). VE-cadherin, which is restricted to lateral cell-cell borders of luminized vessels (Figure 3D, arrows), was broadly redistributed in the absence of β1 integrin and present around the entire cell perimeter (Figure 3D, arrows). Another polarized protein, CD99, noted to be localized to cell junctions and have a reduction in transcripts coding for the polarity protein Par3 (Figure 3A). Par3 is a well-known polarity regulator that functions in a complex with aPKC and Par6 in epithelial cells (Suzuki and Ohno, 2006). Interestingly enough, other members of the complex were not transcriptionally affected by β1 integrin deletion as per our microarray and qRT-PCR analyses (Figure S3D, legend). However, Par6 and aPKC have been shown to interact independently of Par3 (Yamanaka et al., 2003). We also confirmed that the transcriptional decrease in Par3 translated to loss of protein in β1 integrin-ablated EC populations by western blot (Figure 3C).
Figure 2. β1 Integrin Endothelial Deletion Results in Luminal Occlusion, Cuboidal Cell Shape, and Stratification of the Endothelial Layer

(A) β-gal staining identifies β1-deleted endothelial cells (top panel) surrounding a nearly occluded lumen (arrow) in the E14.5 homozygous floxed (β1f/f; Cre+) animal. PECAM-1 (bottom, black) also demonstrates an occluded endothelial lumen (arrow) at E15.5. Note that vessels appear occluded by endothelial cells (PECAM-1+), as also demonstrated in (B) (arrows). Panels are histological sections of skin with either β-gal (blue) or PECAM-1 (black), and nuclear stain in red.

(B) Occlusion of the lumen was demonstrated in semithin sections of E15.5 β1f/f; Cre+ mice (arrows; top panel), and again with PECAM-1 expression (arrows; PECAM-1 in red). Endothelial deletion of β1 integrin also leads to abnormalities of smooth muscle cell (SMC) organization. Whereas SMC morphology appears near normal in the semithin sections (arrowheads), α smooth muscle actin staining (SMA; green) depicts disorganization within SMC layers (arrowheads).

(C) To ensure the occluded lumens were not a function of vascular constriction, muscle relaxants were administered to pregnant dams prior to sacrifice, and sections demonstrate no change in the occlusion phenotype (arrow). PECAM-1, black; nuclear stain, red.

(D) β1 integrin loss is associated with atypical cuboidal shape (black arrows) and stratification (brackets and white arrows) in contrast to the normally flattened appearance of other adjacent endothelial cells (arrowheads). Top panels: E12.5 β1f/f; Cre+ endocardial cells exhibit β1-LacZ expression (β-gal staining in blue) after Cre-mediated excision (counterstain in red or hematoxylin and eosin staining in purple). β1 integrin-ablated embryos also exhibit single-layered squamous-shaped endothelial cells (arrowheads) within the same section of cuboidal ECs or stratification, suggesting that full deletion does not occur within the entire endothelial population simultaneously. Lower panels: stratification is also seen in E16.5 β1f/f; Cre+ (arrows) large-vessel endothelium (PECAM-1, red; SMA, green).
**Figure 3E and 3F; Figure S4F).** When luminal patency was quantified according to vessel type, it became readily apparent that the occlusion phenotype was strictly arterial (Figure 3G). When Par3 localization was evaluated by IHC, it also exhibited a preferential arterial expression (Figure 3H).

Par3 was predominantly found in the basal aspect of arterial endothelial cells, and there it colocalizes with β1 integrin (Figures 3Ha and 3Hb, arrows). However, it is also expressed within the smooth muscle cell layer and to some extent in veins (Figures 3Ha and 3Hb, arrowheads; Figure 3Hc, arrows). To verify whether there was increased Par3 expression within the arterial compartment, aortas and vena cavae from wild-type mice were flushed with Laemmli buffer to enrich for endothelial protein and evaluated for Par3 protein by western blot (Figure 3I). Increased expression of all Par3 isoforms was noted within the arterial compartment, but especially the 150 kDa isoform. These differences were only found in young (4-week-old) animals (Figure 3I).

Par3 protein expression was also examined across cell lysates from primary human endothelial cell lines of arterial (HAEC), umbilical vein (HUVEC), and saphenous vein (HSVEC) origin (Figure 3I). There were no differences between endothelial cell types in culture, but the human EC lines did demonstrate a distinct Par3 isoform pattern as compared to mouse vessels. In β1 integrin-ablated vessels, Par3 was no longer basally localized but instead aggregated at cell borders (Figure 3J, arrows). In addition, recombined cells occluding the lumen no longer expressed either β1 integrin or Par3 (Figure 3J, arrowheads). This was noted to a larger extent in older embryos (Figure 3SC), suggesting that Par3 is mislocalized prior to its decrease in expression.

**Postnatal β1 Integrin Ablation Results in Reduction of Par3 in the Retinal Vasculature**

By using a tamoxifen-inducible VE-cadherin Cre line, we were able to impose Cre-mediated ablation of β1 integrin in the postnatal retinal vasculature. Recombination was assessed by either β-gal staining or EYFP expression from the ROSA26 locus (R26R) Cre reporter (Soriano, 1999). Western blots for Par3 of wild-type whole retinal lysates showed an early peak between postnatal day 3 (P3) and P6 (Figure S4A). However, as there exist multiple cell types in the retina that likely express Par3, endothelial cells were isolated and further analyzed (Figures S4B and S4E). Interestingly, the retinal endothelial cells demonstrate a different developmental expression pattern for Par3, with a peak at P9, which coincides with a period of intense retinal vascular remodeling (Figure 4A). In retinal sections, Par3 is clearly expressed in the endothelium (Figure 4B).

Postnatal β1 ablation resulted in discrete hemorrhagic sites in the retina (Figure S4C), endothelial cell clumping, and cyst-like outgrowths (Figures 4C and 4F; Figure S4D), phenotypes that resemble those found in the embryo (Figure S4G). Upon cross-sectioning, luminal occlusion was also observed in the retina as a result of β1 ablation (see below). However, the inducible system was notably less robust when compared to the constitutive VE-cadherin Cre, as evidenced by LacZ activity from the R26R locus (Figure 4D). Nonetheless, when Par3 protein was evaluated in β1 integrin-ablated retinal endothelial cells, a decrease in Par3 protein was detected in lysates that also demonstrated Cre expression and loss of β1 integrin (Figure 4E).

To ensure that the abnormalities were due to β1 integrin loss, we further evaluated β1 IHC in conjunction with an EYFP R26R line, thus labeling cells that underwent Cre recombination. As noted in Figure 4F, cells that undergo deletion (EYFP+; green) are abnormally situated in the vasculature (isolectinB4; red) and lack β1 integrin expression (blue). Therefore, the postnatal retinal model recapitulates the findings of embryonic β1 integrin deletion in the embryo.

**Pharmacological Blockade of β1 Integrin in the Retina**

To further confirm the role of β1 integrin in vascular morphogenesis, we induced global β1 integrin pharmacological blockade in postnatal (P4, P7, and adult) retinas and evaluated the vasculature 72 hr later. The blockade at P7 mimics the inducible β1 integrin ablation with abnormal cell aggregation and luminal occlusion (Figures 5A and 5B), but also demonstrates more severe effects with excess branching (Figure 5A). To evaluate whether pericyte coverage was affected, we investigated NG2 expression (Figure 5B, green) and noted that whereas there was a lack of coverage in abnormally located ECs (Figure 5B, arrows), the majority of vessels had NG2+ pericyte coverage equivalent to controls.

We also investigated whether arterial identity was affected, as the increased branching and loss of arterial-venous borders may suggest. Dil4, a Notch ligand known to be specific for arterial identity (Shutter et al., 2000), was evaluated. Even though the arteries exhibited increased branching and loss of the avascular zone surrounding the main arterial vessel, the expression of Dil4 was intact in arteries (Figure 5C). To understand the requirement of β1 integrin earlier in angiogenesis, and in quiescent vessels, pharmacological β1 integrin blockade was conducted at P4, a very early stage of retinal angiogenesis, and in the adult. Earlier blockade also appeared to demonstrate branching abnormalities and EC aggregates with luminal occlusion (Figure 5D), but there were also pericyte abnormalities in which NG2+ pericytes appeared to be increased in number (Figure 5D). As the P4 time point precedes the peak of retinal angiogenesis, it is likely the blockade also affects other populations that participate in vascular retinal patterning. In contrast, adult retinal inactivation of β1 integrin leads to very few consequential effects. Rarely,
Figure 3. Luminal Occlusion and Loss of Polarity Are Arterial Phenomena

(A) Microarray analysis of endothelial cells (ECs), sorted based on β1 integrin protein expression (from E16.5 β1f/f; Cre+ embryos), exhibited increased levels of adhesion-related genes and a decrease in the polarity gene Par3.

(B) Loss of β1 integrin protein (green) results in mislocalization of PECAM-1 (red) from laterally placed cell-cell contacts to global cell-surface expression (arrows). TOPRO-3 nuclear stain is in blue.

(C) To confirm the loss of β1 integrin and Par3 on a protein level, ECs sorted in the same manner were evaluated by western blot. Sorted β1+ and β1− endothelial cells from β1f/f; Cre+ mice and β1+ ECs from β1f/f; Cre− mice were loaded equally by cell number, and demonstrated loss of β1 and Par3 protein (α-enolase loading control).

(D) Normally polarized expression of VE-cadherin (top panel in red; SMA in green) at lateral cell-cell contacts (arrows) is dispersed and circumferentially expressed in cells occluding the lumen within β1f/f; Cre− vessels (arrows). CD99 (bottom panels; green) demonstrates a polarized apical expression (arrowheads) and lateral colocalization with PECAM-1 (yellow; arrows) that after β1 integrin ablation redistributes to surround the cell at E15.5 (arrows), much like PECAM-1 (in red).

(E) Luminal occlusion is distinctly noted in arteries (arrows), as delineated by PECAM-1 (top panels in black; bottom panels in red) and lack of EphB4 (bottom panels; green) expression. A, arteries; V, veins; L, lymphatics.

(F) The extent of occlusion can vary in mid-sized arteries (arrows) at E15.5 after endothelial β1 integrin deletion, but as compared to nearby veins is a distinctly arterial phenomenon. Lower panels are high magnification of vessels in upper panels. PECAM-1 is in black.

(G) Vessels were evaluated at E15.5, quantified for luminal patency, and confirmed that the phenotype is exclusively arterial (n = 5 each; *p < .001).

(H) Par3 protein expression is preferentially expressed in embryonic arteries, as evidenced in β1f/f; Cre− at E15.5 (Par3, red; β1 integrin, green; TOPRO-3 nuclear stain, blue). (a)–(c) Are higher magnifications of the arterial-venous pair above. (a and b) Par3 colocalizes with β1 integrin (yellow) in the basal aspect of the arterial endothelial layer (arrows), but is also prominent in the surrounding smooth muscle cell layer (red; arrowheads). (c) Veins also express Par3 in conjunction with β1 (yellow; arrows), but to a lesser extent than arterial vessels.

(I) To evaluate Par3 expression in vessel subtypes, dorsal aortas (A) and inferior vena cavae (V) of 4-week-old and adult animals were flushed with Laemmli buffer and evaluated by western blot. Primary human endothelial cells were evaluated for Par3 expression among vessel subtypes (human aortic endothelial cells, HAECs; human umbilical vein ECs, HUVECs; human saphenous vein ECs, HSVECs). GAPDH and α-enolase loading controls.

(J) Normal basal expression of Par3 (red) with coexpression of β1 integrin (green; colocalization in yellow) in β1f/f; Cre− vessels (arrows) is aggregated and mislocalized in β1f/f; Cre+ vessels (arrows), with complete absence in β1-deleted cells within the vessel lumen (arrowheads). TOPRO-3 nuclear stain is in blue. Scale bars are labeled for each row. See also Figure S3.
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an endothelial cell would be mislocalized (Figure 5D). But for the majority of adult vessels, both morphology and pericYTE coverage were undisturbed, suggesting that vascular β1 integrin may be most critical in the angiogenic stages following the primitive plexus, but prior to vessel quiescence.

As we observed an increase of β3 integrin in the absence of β1 integrin (Figures S1C and S1F), the retinal model allowed us to investigate whether this upregulation had a protective or detrimental effect on vascular development. When β3 integrin blockade was induced in the postnatal retina, the retinas exhibited defects in vessel patterning with an inability to progress past the primitive plexus stage and defects in pericyte coverage (Figure S5). If β3 and β1 integrins were both pharmacologically blocked, the phenotype resulted in a combination of the individual phenotypes observed: EC aggregates, loss of pericyte coverage (Figure S5).

**Viral-Mediated Par3 Delivery Partially Rescues Luminal Defects Imposed by β1 Integrin Deletion**

When retinal endothelial cells undergo β1 integrin deletion, the phenotype of luminal occlusion and cell aggregates was partially rescued by delivery of Par3 (Figure 6). Ocular lentiviral delivery of Par3 driven by a CMV promoter (Sfakianos et al., 2007) rescued the occlusion phenotype by almost 40% (p = .025) when retinal vessels were evaluated for patency in the context of the deletion background (Figures 6C and 6D). The extent of deletion was normalized after β-gal staining of endothelial cells within separate vascular beds, and was noted to be comparable between lentiviral empty vector control and Par3 rescue (Figures 6D and 6E). To ensure that the normalization of vessel morphology was specifically due to viral rescue, we developed a triple-flag-tagged Par3 lentivirus under the control of a VE-cadherin promoter (Figure S6), and rescued postnatal animals crossed to the EYFP R26R Cre reporter. Using this strategy, we were able to track virally rescued β1 integrin-ablated ECs within the retinal vasculature (Figure 6B). Deleted ECs that were not rescued retained their abnormal shape and morphology, whereas rescued ECs reverted back to a normal phenotype (Figure 6B).

**Lumen Formation Is Halted with Accumulation of Intracellular Vacuoles**

A final aspect of the vascular occlusion phenotype is that the ECs occluding the lumen exhibited an overaccumulation of vacuoles within their cytoplasm (Figure 7A). This accumulation is not likely due to cell death, as there were no observed changes in cleaved caspase-3 activity (data not shown) and the cells did not exhibit hallmarks of apoptosis by electron microscopy. However, the increase in vacuole number may be either the cause or consequence of increased Rab7 transcripts found upon β1 genetic ablation (Figure 3A). As Rab7 is noted in late-stage vacuole formation (Stenmark, 2009), the data may suggest that vacuole maturation is not arrested but vacuole fusion may be. When Rab7 protein is evaluated at late embryonic stages of β1 integrin deletion (E17.5), there is definitive increases of Rab7 in nonpolarized ECs that have lost β1 integrin (Figure 7B). As directed vacuole formation and fusion is one method of lumen formation (Iruela-Arispe and Davis, 2009), it may be that arrest of lumen formation due to β1 integrin deletion, and subsequent loss of polarity, results in abnormal vacuole accumulation. In addition, loss of β1 integrin also demonstrated endothelial cyst-like structures in larger vessels and the retina (Figure S4G; Figures 4C, 4F, and 5B). The cysts may also result from intracellular vacuole accumulation, and possibly associated with the observed increase of Rab7 (Figure 3A).

**DISCUSSION**

Our investigation defines an overarching sequence of events that begins initially with a loss of endothelial cell polarity as a result of β1 integrin ablation. Once cell polarity is lost, the mechanisms that maintain a single endothelial layer, as well as the formation of a patent lumen, are also lost. Arrest of lumen formation can also be seen with β1 integrin pharmacological blockade, both in the avian embryo (Drake et al., 1992) and the postnatal retina (Figure 5). Endothelial deletion of β1 integrin results in substantial increases and ubiquitous localization of cell-cell adhesion proteins Claudin-5, PECAM-1, VE-cadherin, and CD99 (Figure 3). This resembles patterns of abnormal lumen formation in Drosophila heart tubes, where gain-of-function E-cadherin mutants display increased cell adhesion and subsequent loss of lumenization (Santiago-Martinez et al., 2008). The data presented here suggest that at least within the endothelium, VE-cadherin and other cell-cell adhesion molecules operate downstream of cell-matrix cues and internal cell-polarity cues (Par3) to set up ordered polarized cell-cell contacts.

In models of in vitro endothelial lumen formation, events are initiated downstream of integrin-extracellular matrix signaling, but also require the activation of Cdc42, Rac1, Pak2/4, and the Par3/6/aPKC complex (Koh et al., 2008). Although we did not examine Cdc42 or other Rho-family GTPases, the genetic deletion of β1 integrin may affect its ability to activate Cdc42. Yet, our data also suggest a genetic link between β1 integrin and Par3, as ablation of β1 integrin results in loss of Par3. Another contrast to the in vitro data is that while lumen formation of mid-sized arteries was blocked upon β1 integrin endothelial ablation, lumens of single-cell capillaries were unaffected. An initial conclusion is that much like epithelial cells (Lubarsky and Krasnow, 2003), lumen formation in vessels of distinct type and caliber is likely to employ alternative processes or molecules.

Recent data in epithelial tube formation suggest that there are Cdc42-independent mechanisms in forming a patent lumen, and that separate and distinct polarity complexes can function as polarity regulators: the association of Cdc42 with polarity complex members Par6 and aPKC being one complex and separately, Par3 localized to tight junctions being another (Jaffe et al., 2008; Martin-Belmonte et al., 2007). Par6 and aPKC have been shown to interact independently of Par3 (Yamanaka et al., 2003). This divergent set of events fits nicely with our data, as we did not observe any transcriptional changes in other polarity complex members, Par6 and aPKC included (Figure S3D and data not shown). Specific to endothelium, Par3 can associate with VE-cadherin in the absence of aPKC and prior to the association of Par6 (Iden et al., 2006). Thus, it may be that cell-cell and cell-matrix contacts operate separately in maintaining
Figure 4. Postnatal β1 Integrin Deletion Results in Par3 Loss and Abnormal Endothelial Cell Polarity

(A) Wild-type (WT) retinal endothelial cell protein evaluated by western blot demonstrates that Par3 levels peak at P6–P9.
(B) Par3 (red) is expressed in WT retinal vessels (P10) with β1 integrin (green; arrows). TOPRO-3 nuclear stain is in blue.
(C) Postnatal β1 integrin ablation induced by tamoxifen injection (from P2 to P7) in the β1f/n; iCre+ retina results in large cyst-like outgrowths from the vasculature at P9 (arrows in right panel). Isolectin B4 (IsoB4) is in red.
(D) Postnatal tamoxifen induction when traced using a LacZ R26R Cre reporter line (R26R; iCre+) labels a small subset of retinal endothelia at P7, as compared to constitutive expression (R26R; Cre+). A, arteries; V, veins.
(E) After postnatal induction, retinal endothelial cells were isolated (at P9) and evaluated by western blot. β1 integrin protein is notably decreased in β1f/n; iCre+, as are Par3 protein levels (right column).
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cell polarity through convergent pathways, where both cadherins and integrins are capable of activating Cdc42 pathways (Desai et al., 2008; Koh et al., 2008) or alternatively regulating polarity proteins. In our study, cell-matrix cues (via β1 integrin) appear to exert initial and critical control of cell-cell adhesion protein distribution and the polarity protein Par3.

In epithelial cells, successful lumen formation requires directed centripetal vacuole movement toward the future lumen and eventual coalescence with the plasma membrane. When β1 integrin is deleted, endothelial cells are no longer polarized, and thus may become incapable of directed vacuole movement and fusion, leading to the retention of vacuoles. This is a likely cause or consequence of the increase in Rab7, a Rab GTPase that may prove critical to endothelial vacuole trafficking, analogous to other Rabs during epithelial lumen formation (Descluzeaux et al., 2008). Whereas the downstream mechanisms of lumen formation (vacuole formation and fusion) are employed in β1 integrin-ablated vessels, the phenotype is restricted to arterial endothelial cells. It is well known that subspecification within endothelial lineages includes identity markers for each endothelial subtype. Our study demonstrates that in addition to canonical arterial markers such as Dll4, Par3 (especially isoform 150 kDa) is preferentially expressed in nascent arterial vessels. However, the Par3 protein increase and isoform pattern in arteries is fleeting, and eventually becomes nonexistent in the adult. The significance of specific Par3 isoform expression is unclear, but differences in isoform binding abilities have been previously shown (Stakianos et al., 2007). It is likely that arteries activate a specific and exclusive set of events for lumen formation that are then silenced after successful patency.

The downstream consequence of vascular β1 integrin ablation, as dissected in this study, lends mechanistic insight into the process of polarity maintenance and lumen formation in arterial endothelium. The first and main cause of these subsequent events appears to be loss of Par3 and thus cell polarity, which includes abnormal distribution of cell-cell adhesion molecules. As a result, the endothelial cells adopt atypical cell shapes and increase their adhesive properties. Although intracellular vacuoles appear capable of forming in lieu of β1 integrin, they are no longer capable of directed migration and coalescence to form a lumen. However, lumen formation is restored with the addition of Par3 protein, suggesting that the adhesion changes and lumen defects are downstream of polarity cues, which are regulated by cell-matrix interactions via β1 integrin (Figure 7C).

**EXPERIMENTAL PROCEDURES**

**Animals**

Mouse lines (and their respective genotyping) VE-cadherin Cre (Alva et al., 2006), inducible VE-cadherin Cre (Monvoisin et al., 2006), floxed β1 integrin exons 2–7 (Potocnik et al., 2000), floxed β1 integrin exon 3 (Raghavan et al., 2000), β1 integrin null (Stephens et al., 1995), R26R LacZ (Soriano, 1999), and R26R EYFP (Srinivas et al., 2001), as described, were maintained in the Animal Research Facility at the University of California, Los Angeles. Care and experimental procedures were performed in accordance with university guidelines. Pregnancies were dated by the presence of a vaginal plug (day 0.5 of gestation). For survival data, 673 embryos were evaluated for the β1<sup>+/−</sup> deletion, 104 embryos for the β1<sup>ex3/ex3</sup> deletion, and 234 embryos for the β1<sup>−/−</sup> deletion.

**Immunohistochemistry**

Littermate controls were used throughout the study. Immunostaining and β-galactosidase protocols were as previously described (Alva et al., 2006; Monvoisin et al., 2006). Electron microscopy was as previously described (Lee et al., 2007).

Tissue sections underwent immunostaining with the following antibodies: PECAM-1 1:100 (BD Pharmingen), fibrinogen 1:100 (Abcam), incubation with biotinylated secondary antibodies, Vectastain ABC kit, DAB substrate, and nuclear fast red counterstain (all from Vector Laboratories). Separately, tissue sections also underwent the Tyramide signal amplification system with Alexa Fluor secondary antibodies (Invitrogen) per the manufacturer’s instructions with β1 integrin 1:500 (Chemicon), PECAM-1 1:500 (raised in rabbit; a generous gift from Josephine Enciso and Joseph Madri, Yale University), Par3 1:300 (Upstate Biotechnology), Rab7 1:500 (Cell Signaling), and TOPRO-3 (Invitrogen) nuclear stain. RetaWebakee-moat staining was done using Alexa Fluor-conjugated isoclin IB4 1:100 (Invitrogen) and NG2 1:100 (Chemicon) as described (Hellstrom et al., 2007), and anti-FLAG M2 1:100 (Sigma) using the M.O.M. kit (Vector), with Alexa Fluor-conjugated secondary antibodies (Invitrogen).

Alternatively, for vibratome sections (250 μm), the following primary antibodies were used: PECAM-1 1:500 (BD Pharmingen), paxillin 1:100 (BD Pharmingen), laminin 1:200 (Sigma), z-smooth muscle actin FITC-labeled 1:500 (Sigma Laboratories), CD99 1:100 (from Gabriele Bixel, Max Planck Institute), Texas red X phalloidin 3.5:100 (Molecular Probes), β1 integrin 1:100 (Chemicon), Claudin-5 1:100 (Zymed); EphB4 1:100 (R&D Sytems), and VE-cadherin 1:150 (BV 13/4; a generous gift of ImClone Systems). Primary antibodies were revealed with the appropriate secondary antibodies conjugated with either FITC or Cy3 (Jackson ImmunoResearch Laboratories). All fluorescent IHC was analyzed using a Bio-Rad confocal MRC1024 or a Zeiss LSM multichannel microscope.

**FACS Analysis and Antibodies**

Embryos (or retinas) were mechanically dissociated by collagenase digestion (Sigma), followed by red cell lysis, and filtered (70 μm and 40 μm). Cells were incubated with PE-conjugated PECAM-1, FITC-conjugated β1 integrin, biotin-conjugated β1 integrin with streptavidin-Tricolor, and APC-conjugated CD45 antibodies and analyzed on a FACSCaliber with the appropriate isotype controls. Antibodies and isotype controls were purchased from BD Pharmingen; streptavidin-Tricolor was from Caltag/Invitrogen.

**Cell Sorting and Microarrays**

FACSArise-sorted endothelial cells from β1<sup>+/−</sup>; Cre<sup>+</sup> whole embryos at E16.5 were gated for PECAM<sup>+/−</sup>CD45<sup>−</sup> and further sorted on β1 integrin protein expression. Approximately 2–4 μg of purified RNA was converted into double-stranded cDNA (Superscript; Invitrogen). Four hundred nanograms of biotin-labeled cRNA was synthesized from cDNA using an IVD labeling kit (Affymetrix). Affymetrix GeneChip Fluidics Station 400 hybridized fragmented biotinylated cRNA to the Affymetrix Mouse Expression Set 430 ChipA GeneChip array (over 14,000 full-length annotated mouse genes and nearly 4,000 expressed sequence tags). Arrays were stained and scanned using a Hewlett-Packard GeneArray scanner. Comparisons were performed between β1<sup>+/−</sup>; Cre<sup>+</sup> ECs that were β1<sup>+/−</sup> and β1<sup>+/−</sup> using a total of six Affymetrix GeneChips. Data generated following probe hybridization were analyzed with Microarray Suite 5.0 (Affymetrix). Array images were visually examined for array or probe hybridization abnormalities.

**Smooth Muscle Relaxation**

Pregnant dams were anesthetized and perfused at 120 mmHg with 10 mg/ml adenosine (Sigma), 4 mg/ml papaverine (Sigma), and 10 U heparin/ml in adenosine (Sigma), 4 mg/ml papaverine (Sigma), and 10 U heparin/ml in

(F) When β1<sup>+/−</sup>; iCre+ crossed to an EYFP R26R reporter undergoes β1 ablation, the β1-deleted retinal ECs (EYFP+ in green; arrows) become abnormally located in the vasculature in cyst-like structures (labeled by IsoB4 in red) and demonstrate loss of β1 protein (blue).

Scale bars are included for each row. See also Figure S4.
PBS to produce maximum smooth muscle relaxation. A perfusion of 2% PFA followed, and the embryos were removed and processed as described above.

Occlusion Counts
Littermates at E15.5 were genotyped and stained with PECAM-1. Skin sections 5 μm thick from embryos were evaluated 100 μm apart for the

Figure 5. β1 Integrin Antibody Blockade Recapitulates the Genetic Deletion Phenotype
(A) β1 integrin pharmacological blockade at P7 and evaluation at P10 demonstrate increased endothelial cell aggregation and cysts (arrows) with increased branching and decreased borders between arteries (A) and veins (V). Note the normally avascular area surrounding the retinal arteries is restricted after β1 antibody blockade (arrowheads). Isolectin B4 (IsoB4) is in red.
(B) Top panel: the cysts are composed of endothelial cells, as demonstrated by IsoB4 and TOPRO-3 (blue) nuclear staining (arrows). Bottom panels: when retinas are evaluated for pericyte coverage (NG2; green), there is no observed effect after β1 blockade; however, ECs that are abnormally positioned with respect to the vasculature, or in cysts, are not covered by pericytes (arrows). PECAM-1 staining (brown) of histological sections demonstrates abnormal vessel morphology (arrows) and luminal occlusion (inset).
(C) Dll4, an arterial marker (green), was examined to evaluate whether arterial identity was affected as a result of β1 antibody blockade. Whereas Dll4 expression was preserved (arrowheads), increased branching with closer proximity to the main arterial vessel is apparent (arrows, brackets).
(D) To evaluate the effects of β1 blockade at different ages, retinas were injected either at P4 or in the adult and evaluated 72 hr later. Left: the P4 blockade results in abnormal vascular patterning (IsoB4 in red) with excessive pericyte coverage (NG2 in green), and luminal occlusion (PECAM-1 in brown; arrows). Right: in the adult, very few abnormalities were encountered. Rarely, small cysts could be seen (IsoB4 in red; inset) as well as modest thickening of the endothelial layer (PECAM-1 in brown; arrow), whereas pericyte coverage was normal. TOPRO-3 nuclear stain is in blue.
Scale bars are shown for each row. See also Figure S5.
Figure 6. Par3 Partially Rescues Lumen Occlusion and Cyst Formation in Endothelial β1 Integrin Ablation

Postnatal animals (β1f/n; iCre+) were induced with tamoxifen (P2–P7) and retinal vasculature was evaluated from P9 to P12. A subset was then rescued with Par3 lentiviral ocular delivery 48 hr prior to evaluation.

(A) The large cysts observed with postnatal β1 integrin ablation were resolved significantly with lentiviral Par3 replacement, but a few endothelial cells still displayed smaller atypical aggregates (arrows). Isolectin B4 (IsoB4) is in red.

(B) When β1f/n; iCre+ crossed to EYFP R26R (green) retinas were rescued with FLAG-tagged Par3 lentivirus (blue), a rescued vessel demonstrates a patent lumen (arrowheads). In contrast, ECs that underwent β1 deletion (green) but were not rescued remain abnormally shaped with an occluded lumen (arrows). Asterisk denotes a EYFP+ red blood cell.

(C) On analysis of retinal semithin sections, β1 ablation resulted in vessel occlusion (arrowheads) that was partially rescued with Par3 protein (arrows). Boxed areas are magnified on the right.

(D) Retinal vessels were quantified for the percentage of deletion (left) by assessing β-gal-positive endothelial cells within the abdominal muscle; tamoxifen group (dark gray) and tamoxifen + Par3 rescue group (light gray). The number of occluded vessels was quantified and compared to percent deletion in a ratio (right). The β1f/n; iCre+ tamoxifen group (dark gray) demonstrates that the occlusion phenotype is in direct proportion to the amount of deletion. There is a significant (*) decrease in the ratio of occluded vessels (%) to percent deletion with Par3 rescue (light gray). Data are shown as mean ± SEM; n = 7 each group; p = 0.025.

(E) On a per-animal basis, the percentage of deletion varies (circles) but averages at approximately 30% for both groups, whereas the percentage of occlusion (triangles) is dramatically reduced from 27% in the nonrescue to 16% in the rescued group.

(F) Higher magnification depicting the vessel occlusion (or patency in the rescue) that was quantified in the retina (arrow). Scale bars are labeled for each row. See also Figure S6.
presence of lumen. EphB4 IHC was conducted on a subset of sequential slides to confirm venous versus arterial identity. A total of 250 arteries and veins and 620 capillaries were counted from five independent littermate pairs of $\beta_1^{fl/f};\text{Cre}^+$ and $\beta_1^{fl/f};\text{Cre}^-/\text{C}0$ embryos.

**Integrin Antibody Blockade**

Mice were anesthetized with isofluorane (Abbott Laboratories). For pups, eyelids were first pierced under sterile conditions with an insulin syringe (BD Pharmingen), followed by intravitreal injection of 1–2 μl of blocking antibody or isotype control with the aid of pulled glass micropipets and a micromanipulator; contralateral eye served as a noninjected control (3–4 μl used for adults). Low-endotoxin blocking antibodies for $\beta_1$ and $\beta_3$ integrins and isotypes (BD Pharmingen) were used at 1 mg/ml dilution.

**Par3 Lentivirus**

The entire 1333 nt open reading frame of mouse Par3 isoform 3 (NCBI reference sequence NP_296369) (see nomenclature in Supplemental Information) was fused to a triple-flag tag (MADYKDHDGDYKDHIDYKDDDDKGTSLYK KAG) to generate 3×Flag_mPar3 (plasmids were fully sequenced in both orientations) and then cloned into the multiple cloning site of the lentiviral entry plasmid pRRL-sin VECADivs NLS GFP-cPPT. Replication-deficient lentivirus directing expression of pRRL-sin VECADivs_3×Flag_mPar3-cPPT was then generated by the UCLA Vector Core Facility.

**Postnatal Induction of $\beta_1$ Integrin Deletion and Retina Manipulations**

Mice were fed or injected (intragastrically) with 0.1–0.2 mg (10–20 μl) of tamoxifen (MP Biomedicals) prepared as described (Monvoisin et al., 2006) every other day from P2 to P7. Controls included Cre– mice also fed with tamoxifen, and $\beta_1$-ablated mice fed or injected with vehicle (sunflower oil). The amount of deletion was quantified by counting $\beta$-gal-positive cells (nuclei of positive cells as compared to total nuclei) in the abdominal muscles of the respective mice. Purification of retinal endothelial cells was accomplished by immunobeads (see Supplemental Experimental Procedures for a full protocol).

Delivery of lentiviral proteins was accomplished by intravitreal injection of 0.5–1.0 μl of 10⁸ viral particles (see antibody blockade above) and evaluation 48 hr later. Injection of the Par3 or empty virus did not provoke any major infections or macroscopic alterations during that time period. The mice appeared to respond to light and visual cues 1 day after the procedure.

**Western Blotting**

Isolated mouse tissues or cells were placed in lysis buffer, loaded onto 8% acrylamide gels, and probed with Par3 (Upstate Biotechnology), tubulin (Sigma), GAPDH (Chemicon) Cre-recombinase (Abcam), or $\beta_1$ integrin (Chemicon). See Supplemental Experimental Procedures for full protocol and antibody dilutions.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.devcel.2009.12.006.

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