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Cell-autonomous requirement for $\beta 1$ integrin in endothelial cell adhesion, migration, and survival during angiogenesis in mice

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Summary

$\beta 1$ integrin is established as a regulator of angiogenesis based upon the phenotypes of complete knockout of $\beta 1$ heterodimer partners or ligands and antibody inhibition studies in mice. Its direct function in endothelial cells (ECs) *in vivo* has not been determined because $\beta 1^{-/-}$ embryos die before vascular development. Excision of $\beta 1$ from ECs and a subset of hematopoietic cells, using *Tie2-Cre*, resulted in abnormal vascular development by embryonic day 8.5 (e8.5) and lethality by e10.5. *Tie1-Cre* mediated a more restricted excision of $\beta 1$ from ECs and hematopoietic cells and resulted in embryonic lethal vascular defects by e11.5. Capillaries of the yolk sacs were disorganized, and the endothelium of major blood vessels and of the heart was frequently discontinuous in mutant embryos. We also found similar vascular morphogenesis defects characterized by EC disorganization in embryonic explants and isolated ECs. $\beta 1$ -null ECs were deficient in adhesion and migration in a ligand-specific fashion, with impaired responses to laminin and collagens, but not fibronectin. Deletion of $\beta 1$ reduced EC survival, but did not affect proliferation. Our findings demonstrate that $\beta 1$ is essential for EC adhesion, migration, and survival during angiogenesis, and further validate that therapies targeting $\beta 1$ integrins may effectively impair neovascularization.

Keywords

angiogenesis; integrin; endothelial cell; vascular development; extracellular matrix

Introduction

Integrins are cell surface receptors that bind to the extracellular matrix (ECM) and propagate many crucial intracellular signals. Several integrins and their ECM protein ligands are expressed in endothelial cells (ECs) during the formation of new blood vessels, or angiogenesis (Hynes, 2002). This observation has led to intense investigation on integrin-ECM interactions as critical regulators of angiogenesis. Although it is established that integrin-ECM interactions are involved in angiogenesis, it is unclear which integrin-ligand pairs are required for the angiogenic process. For example, the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ are upregulated in ECs during wound healing and in certain tumor vasculatures, and antibody or small molecule inhibition of these integrins blocks angiogenesis *in vivo* (Eliceiri and Cheresch, 2001). However, the unexpected finding that $\beta 3^{-/-}/\beta 5^{-/-}$ mice are viable and fertile has challenged the notion that

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αv integrins are required for angiogenesis and calls for further investigation into the precise role of integrins in angiogenesis.

At least six $\beta 1$ integrins ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, and $\alpha v\beta 1$) are expressed in ECs, and several $\alpha\beta 1$ heterodimers are thought to be pro-angiogenic. The collagen receptors, $\alpha 1\beta 1$ and $\alpha 2\beta 1$, are upregulated by vascular endothelial growth factor, and antibody-based inhibition of these integrins blocks tumor angiogenesis (Senger et al., 1997). In addition, tumor angiogenesis in $\alpha 1^{-/-}$ mice is reduced compared to controls (Pozzi et al., 2000). Fibronectin and its primary integrin receptors, $\alpha 4\beta 1$ and $\alpha 5\beta 1$, are upregulated in blood vessels of several human tumors, and inhibition of $\alpha 4\beta 1$, $\alpha 5\beta 1$, or fibronectin with antibody- or peptide- based approaches blocks angiogenesis in vivo (Garmy-Susini et al., 2005; Kim et al., 2000a). In addition, $\beta 1$ integrins, and $\alpha 5\beta 1$ in particular, are critical for vascular network formation in embryoid bodies (Bloch et al., 1997; Francis et al., 2002). Finally, $\alpha 5^{-/-}$ or *fibronectin*^{-/-} mice are embryonic lethal due to cardiovascular and neuronal defects, although the precise role of these genes in vascular development remains unclear because of their widespread embryonic expression (Francis et al., 2002; George et al., 1997; George et al., 1993; Yang et al., 1999; Yang et al., 1993).

Direct in vivo genetic evidence for an angiogenic role for endothelial $\beta 1$ integrins is precluded because $\beta 1^{-/-}$ die at embryonic day (e) 5.5 due to implantation defects, prior to vascular development (Fassler and Meyer, 1995; Stephens et al., 1995). In this study, we deleted $\beta 1$ in a cell lineage-specific manner, using both *Tie2-Cre* and *Tie1-Cre*, to bypass the early implantation defects of $\beta 1$ complete null embryos, and gained significant insights into its role in EC adhesion, migration, proliferation, and survival during vascular development.

Results

EC expression of $\beta 1$ integrin is required for early embryonic vascular development

In order to delete $\beta 1$ integrins in ECs, we bred *Tie2-Cre*; $\beta 1^{lox/+}$ male mice with $\beta 1^{lox/flox}$ female mice (Graus-Porta et al., 2001). Cre was also active in a subset of hematopoietic cells in this line (Braren et al., 2006). No live *Tie2-Cre*; $\beta 1^{lox/flox}$ (from now on referred to as “*Tie2-Cre* mutant”) mice were born indicating that the mutation was embryonic lethal. Since Cre activity is observed as early as e7.5 in our *Tie2-Cre* line (Braren et al., 2006), we analyzed e8.5 embryos for $\beta 1$ deletion by several methods. First, we performed genomic PCR analysis of whole embryos using primers that flank the loxP sites and are capable of detecting wild type $\beta 1$, floxed $\beta 1$, and the recombined $\beta 1$ alleles. We found that only embryos carrying two floxed alleles and *Tie2-Cre* demonstrated recombination (Fig. 1A). Next, we performed immunofluorescence with antibodies against CD31, a pan-EC marker, and $\beta 1$ integrins to determine the cell type specificity of gene deletion. $\beta 1$ integrins were expressed in ECs of the primary head veins, dorsal aorta, and yolk sac blood islands as well as in other tissues in littermate controls, which consisted of embryos lacking *Tie2-Cre* or having only one floxed $\beta 1$ allele (Fig. 1B). Conversely, $\beta 1$ integrins were absent from ECs of *Tie2-Cre* mutants despite their persistent expression in non-EC tissues. Finally, we digested e8.5 embryos, plated the cells onto fibronectin-coated dishes, and stained the mixed population with anti-CD31, anti- $\beta 1$ integrin, and anti- $\beta 3$ integrin antibodies. While control ECs displayed prominent $\beta 1$ integrin focal contact staining, only about 10% of *Tie2-Cre* mutant ECs had detectable $\beta 1$ integrins in focal contacts (Fig. 1C,D). We also observed more prominent focal contact staining of $\beta 3$ integrins in *Tie2-Cre* mutant ECs compared with control ECs, suggesting compensation for the absence of $\beta 1$. These findings indicate that *Tie2-Cre* mediates efficient deletion of $\beta 1$ integrins from ECs in embryos by e8.5.

Tie2-Cre mutant embryos were indistinguishable from controls upon dissection at e8.5 (data not shown). At e9.0, *Tie2-Cre* mutants had normal sizes but appeared to have slightly enlarged pericardial sacs (see Fig. S1 in supplementary material). By e9.5, almost all *Tie2-Cre* mutants

displayed enlarged pericardial sacs indicative of edema, which is a common phenotype of mutations that affect the vasculature at this stage (Conway et al., 2003). Approximately half of e9.5 *Tie2-Cre* mutants were also growth-retarded, a phenotype that became more obvious as the embryos neared death around e10.5. No live *Tie2-Cre* mutants were observed at e11.5 (see Table S1 in supplementary material).

To verify that EC expression of $\beta 1$ integrins is required for vascular development, we also used the *Tie1-Cre* line, which mediates recombination of floxed genes in the majority of ECs and a small fraction of hematopoietic cells in embryos (Gustafsson et al., 2001). We bred *Tie1-Cre*; $\beta 1^{lox/+}$ males with $\beta 1^{lox/lox}$ females and found that among 8 litters of newborn pups, only one live *Tie1-Cre*; $\beta 1^{lox/lox}$ (from now on referred to as “*Tie1-Cre* mutant”) mouse was born, indicating that this genotype was also embryonic lethal. Timed embryo dissections yielded zero *Tie1-Cre* mutants at e14.5 or e12.5. At e11.5, 4 of 4 *Tie1-Cre* mutants were apparently dead in that they lacked a heartbeat and were growth retarded. e10.5 *Tie1-Cre* mutants were also slightly growth retarded, and the majority of embryos displayed hemorrhaging in the head veins (see Fig. S1 in supplementary material). Genomic PCR analysis of e10.5 embryos demonstrated recombination of $\beta 1$ in embryos carrying two floxed $\beta 1$ alleles and *Tie1-Cre* (Fig. 1E). Unlike *Tie2-Cre* mutants, the size and appearance of e9.5-e10 *Tie1-Cre* mutants were similar to those of controls, perhaps because $\beta 1$ deletion was incomplete in *Tie1-Cre* mutant ECs at e9.5 (Fig. 1F). Thus, most *Tie1-Cre* mutants die around e11.5, approximately one day later than *Tie2-Cre* mutants (see Table S1 in supplementary material).

EC disorganization occurs throughout the entire cardiovascular system in the absence of EC $\beta 1$ integrins

The embryonic yolk sac undergoes extensive angiogenic remodeling from e8.5–e10.5, and therefore we examined this tissue by anti-CD31 immunofluorescence. Although we observed rather homogenous capillary plexuses at e8.5 in both control and *Tie2-Cre* mutant yolk sacs, mutant capillaries were slightly thinner than controls in some regions (data not shown). More strikingly, subsequent arteriovenous remodeling of yolk sac blood vessels was severely affected in the *Tie2-Cre* mutants. Whereas large tubular vessels were evident in control yolk sacs at e9.0, mutant blood vessels failed to organize into tubular structures and instead assembled into sac-like structures (Fig. 2). At e9.5 and e10.5, a hierarchical network of arteries, veins, and capillaries was developed in control yolk sacs, whereas *Tie2-Cre* mutant yolk sacs contained a disorganized network of thin capillaries and sac-like structures containing CD31-positive ECs. Connections appeared to be missing among mutant blood vessels, and we frequently observed individual or small clusters of ECs between vessels at e9.5 and e10.5. We also examined the yolk sac vasculature of *Tie1-Cre* mutants and found that blood vessels were disorganized, irregularly shaped, and frequently contained prominent EC protrusions in between vessels (Fig. 2). Taken together, these findings suggest that $\beta 1$ integrins are required within ECs for proper vascular morphogenesis and that in their absence, ECs are disorganized and detached from one another.

Tie2- and *Tie1-Cre* were active in the endocardium, and therefore we also analyzed cardiac development. In controls, the primitive heart tube looped and developed into a four-chamber heart by e9.0–e9.5 (see Fig. S1 in supplementary material and Fig. 3A,C). During this period, the ventricular myocardium underwent trabeculation and the endocardium became more tightly associated with the myocardium (Fig. 3C,G). In *Tie2-Cre* mutants, the primitive heart remained tubular at e9.0, and the endocardium appeared less intricate than the control endocardium (Fig. 3B). Heart looping and four-chamber development eventually occurred in the mutants, but it was delayed by about a half day relative to controls (Fig. 3D,H and data not shown). The most striking phenotype in *Tie2-Cre* mutant hearts was the disorganization of endocardial cells, which appeared as speckles in whole mount anti-CD31 stains at e9.5 and later (Fig. 3D). Cross-

sectional analyses revealed that mutant endocardial cells formed clusters that failed to associate tightly with the ventricular or atrial myocardium (Fig. 3H). This contrasted the control endocardium, which formed a monolayer that closely apposed the entire trabecular surface of the myocardium (Fig. 3G,I). Like the *Tie2-Cre* mutants, *Tie1-Cre* mutants had cardiac defects that were characterized by poorly trabeculated ventricles containing rounded endocardial cells that had detached from the myocardium (Fig. 3J).

Next, we examined the patterning and structure of embryonic blood vessels. *Tie2-Cre* mutant dorsal aortae were formed and patent at e9.0 and the capillary beds appeared similar to controls (Fig. 3A,B). However, at e9.5 (Fig. 3D) and e10.5 (data not shown), *Tie2-Cre* mutant dorsal aortae had narrowed, capillaries were disorganized, and intersomitic vessels failed to form (Fig. 3D).

Several prominent vascular defects were observed in *Tie1-Cre* mutants at e10.5. First, the endothelium of *Tie1-Cre* mutant (Fig. 4B,B') blood vessels was frequently discontinuous, unlike the continuous monolayer observed in controls (Fig. 4A,A'). This phenotype was most clearly observed when the basement membrane to which ECs adhere was labeled with anti-fibronectin antibodies (Fig. 4C,D), and was detected in approximately 75% of all dorsal aortae and cardinal vein sections analyzed (Fig. 4E). Second, the fibronectin staining was more diffuse and less prominent in mutant basement membranes as compared with controls, where it stained brightly beneath the continuous EC monolayer (Fig. 4C). A similar reduction in the intensity of fibronectin staining was observed in the vascular walls of *Tie2-Cre* mutants as well (data not shown). Third, cranial blood vessels in mutants were frequently dilated (Fig. 4B), unlike controls. Finally, we detected blood vessel patterning defects in mutant neural tubes. Whereas numerous capillaries that co-stained prominently with laminin were present within control neural tubes (Fig. 4A,F,H), mutant neural tubes were completely devoid of capillaries (Fig. 4B,G,H), even though the laminin boundary between the mesenchyme and neural tube was present (Fig. 4G). In summary, deletion of $\beta 1$ integrins via *Tie2-* or *Tie1-Cre* leads to widespread EC disorganization resulting in embryonic lethality at mid-gestation.

Abnormal vascular morphogenesis is due to EC-intrinsic defects upon $\beta 1$ integrin deletion

Fluid shear stress resulting from blood flow is known to contribute to vascular remodeling in the chick and mouse yolk sacs (le Noble et al., 2004). In order to eliminate the influence of shear stress and examine the EC-intrinsic effects of $\beta 1$ integrin deletion on capillary morphogenesis, we performed two in vitro angiogenesis assays. In the first, we monitored vascular development in para-aortic splanchnopleural (P-Sp) explants (Takakura et al., 1998). To do so, we dissected the P-Sp region of e8.5 control and *Tie2-Cre* mutant embryos and cultured it on top of a feeder layer of OP9 mouse stromal cells. We then monitored vascular development in cultures expressing the green fluorescent protein (GFP) in ECs via a *Tie1-GFP* transgene (Iljin et al., 2002) in real-time with timelapse fluorescence videomicroscopy. In cultures lacking *Tie1-GFP*, we used endpoint whole mount anti-CD31 immunostaining. Vascular network formation began in control cultures when spindle-shaped tip ECs sprouted outwards from the P-Sp. The tip ECs retained contact with ECs at their rears, and the sprouts grew more or less as single-file lines of ECs. The complexity of control networks was increased when two or more sprouts came into contact with each other, and multiple ECs within each sprout made stable connections with neighboring ECs (Fig. 5A,C and Movie 1,2). In contrast to the ordered development of EC networks in control P-Sp cultures, *Tie2-Cre* mutant ECs, while highly motile, were rounded and did not maintain their migration paths (Fig. 5B,D and Movie 1,2). Mutant ECs migrated in clusters and either failed to make EC-EC connections, or made transient ones. We also observed that mutant ECs died more frequently than control ECs (Movie 1,2). The inability of mutant ECs to make and maintain stable EC-EC connections led

to the appearance of EC clusters at the endpoint of the cultures (Fig. 5D), which contrasted the vascular networks that formed in control cultures (Fig. 5C).

We next tested whether $\beta 1$ -null embryonic ECs were capable of forming capillary networks on Matrigel, a gelatinous basement membrane mixture consisting of mostly laminin and collagen IV that promotes EC network formation. These experiments required large numbers of purified ECs, so we generated immortalized embryonic ECs from e9.5 $\beta 1^{flox/flox}$ embryos (called ECs $^{\beta 1flox/flox}$), and infected them with control adenovirus containing GFP (Ad-GFP or control) or Ad-Cre (or mutant). Within 4 hr of seeding single cell suspensions onto Matrigel, control ECs $^{\beta 1flox/flox}$ formed elaborate networks reminiscent of capillary beds (Fig. 5E). In contrast, mutant ECs $^{\beta 1flox/flox}$ remained as single cells or isolated groups at 4 hr (Fig. 5F). The mutant cells did not form networks at later time points, indicating that the morphogenic process is blocked in the absence of $\beta 1$ integrins and not simply delayed (data not shown). To confirm the role of $\beta 1$ integrins in this process, we seeded control ECs $^{\beta 1flox/flox}$ in the presence of function-blocking anti-integrin antibodies, and found that anti- $\beta 1$ antibodies (Fig. 5H), but not the combination of anti- αv plus anti- $\beta 3$ antibodies, phenocopied the response of mutant ECs $^{\beta 1flox/flox}$ (Fig. 5G). To verify that the anti- αv and anti- $\beta 3$ antibodies were function-blocking, we included the combination in a cell adhesion assay and found that they significantly inhibited the adhesion and spreading of control ECs $^{\beta 1flox/flox}$ on vitronectin (data not shown). The EC disorganization observed in the P-Sp and Matrigel assays support our *in vivo* findings, and indicate that $\beta 1$ integrins are required in an EC-intrinsic manner for angiogenesis.

$\beta 1$ -null ECs are defective on collagens and laminin, but not fibronectin *in vitro*

The embryonic vascular ECM consists of fibronectin, laminin, and collagen, and $\beta 1$ integrins are thought to be the primary EC receptors for each of these proteins (George et al., 1997; Kalluri, 2003). We therefore examined the requirement for $\beta 1$ integrins for interactions with these ECM proteins *in vitro*. To determine whether $\beta 1$ integrins are required for EC adhesion, we plated control and mutant ECs $^{\beta 1flox/flox}$ onto ECM-coated plates in serum-free medium. We found that while control ECs $^{\beta 1flox/flox}$ adhered well to all tested ECM, mutant ECs $^{\beta 1flox/flox}$ failed to adhere to laminin, collagen I, collagen IV, or Matrigel, even though they adhered as well as controls to fibronectin or vitronectin (Fig. 6A). Next, we tested whether $\beta 1$ integrins are required for EC haptotaxis, or migration in response to an immobilized substrate, in the absence of serum. Control and mutant ECs $^{\beta 1flox/flox}$ were plated onto ECM-coated filters and allowed to migrate for 4 hr. Fibronectin, laminin, Matrigel, or vitronectin each supported the migration of control ECs $^{\beta 1flox/flox}$ (Fig. 6B). Conversely, mutant ECs $^{\beta 1flox/flox}$ were incapable of migrating on laminin or Matrigel, even though they migrated as well as controls on fibronectin and significantly more so than controls on vitronectin.

Next, we examined the phenotype of primary ECs by dissociating e9.0 embryos, plating them onto ECM-coated plates in serum-containing medium, and labeling ECs with DiI-Ac-LDL. We then monitored EC behaviors with timelapse fluorescence videomicroscopy. We found that mutant ECs spread on fibronectin or laminin as well as control ECs (Fig. 6C). The ability of primary mutant ECs, but not mutant ECs $^{\beta 1flox/flox}$ (Fig. 6A), to adhere to laminin may be due to the presence of serum or to the longer duration of attachment required for the culture of primary cells. We also measured the motility of primary ECs, and found that the average speed of mutant ECs was similar to controls on fibronectin (Fig. 6D–F). In contrast, mutant ECs were significantly less motile than control ECs on laminin (Fig. 6D,G,H). Finally, we examined focal contact formation on fibronectin, but did not detect any obvious differences in the staining patterns of activated focal adhesion kinase (FAK, Fig. 6I,J) or paxillin (data not shown) among control and mutant ECs.

β 1 integrins regulate EC growth by affecting cell survival, but not proliferation

Integrins regulate cell proliferation and survival in adherent cell types (Giancotti and Ruoslahti, 1999). To test whether decreased proliferation or survival contributed to the vascular defects of mutants, we performed BrdU incorporation and TUNEL assays on control and *Tie2-Cre* mutant embryos at e9.0. Approximately 25.1 \pm 1.8% of control and 23.9 \pm 1.4% of *Tie2-Cre* mutant EC incorporated BrdU within 2 hr (Fig. 7A). We observed a slight increase in mutant EC apoptosis, as 0.85 \pm 0.4% of control and 1.35 \pm 0.3% of *Tie2-Cre* mutant ECs were TUNEL positive (Fig. 7B). Mutant ECs were substantially more apoptotic than controls at e9.5, but mutant non-ECs were highly apoptotic, which may indicate that systemic defects also contributed to cell death at this stage (data not shown).

We also measured the growth rate of ECs ^{β 1^{flox/flox}} cultured in two different conditions: either on gelatin, a direct ligand for β 3 integrins (Davis, 1992) and an indirect ligand for β 1, β 3, and β 5 integrins due to its high affinity for fibronectin present in serum (Engvall and Ruoslahti, 1977), or on vitronectin, a ligand for β 3 and β 5, but not β 1, integrins. We found that while mutant ECs grew significantly slower than control ECs on gelatin, their growth was unaffected on vitronectin (Fig. 7C). Mutant ECs incorporated BrdU into DNA as efficiently as controls on both ECM (Fig. 7D). Mutant ECs were significantly more permeable than control ECs to the apoptotic cell dye YO-PRO-1 on gelatin, but not on vitronectin (Fig. 7E). These findings, considered together with the P-Sp results, suggest that β 1 integrin deletion impairs EC growth primarily through effects on cell survival pathways.

Discussion

We set out to determine what role β 1 integrins play within ECs during embryonic vascular development using a cell lineage-specific gene deletion approach. Both *Tie2-* and *Tie1-Cre-* mediated gene excisions show that β 1 integrins in ECs are essential for embryonic angiogenesis by regulating cell adhesion, migration, and survival. β 1-null ECs fail to interact properly with β 1 ligands such as collagens and laminin, but behave normally on a non- β 1 ligand, vitronectin. β 3 integrins compensate for the absence of β 1 during EC interactions with fibronectin in vitro, but may be insufficient to completely mediate the essential roles of fibronectin in vivo.

Integrins are multi-functional proteins, and our results suggest that β 1 integrins primarily regulate EC adhesion, migration, and survival during embryonic angiogenesis. For example, we demonstrate a discontinuous endothelium in the mutant blood vessels, isolated ECs in between blood vessels in mutant yolk sacs, and clusters of rounded endocardial cells in mutant hearts. These phenotypes are consistent with the adhesion defects that we observe in β 1-null ECs in vitro. Furthermore, ECs do not invade the neuroepithelium in *Tie1-Cre* mutants. Since capillary growth into the neural tube occurs exclusively via sprouting angiogenesis (Kurz et al., 1996), it is likely that β 1-null ECs are defective in cell migration. Cell migration is a dynamic process not readily tractable in mouse embryos, and therefore we examined this behavior in vitro. β 1-null ECs do not migrate on laminin or Matrigel and fail to maintain their migration paths in P-Sp explants. A rounded cellular phenotype in vivo and similar in vitro cell adhesion and migration defects are observed when β 1 integrins are deleted in vascular smooth muscle cells, indicating that they also control these processes in the other major cell type of the vascular wall in vivo (Abraham et al., 2008). In addition, we observe a slight increase in EC apoptosis in e9.0 *Tie2-Cre* mutant embryos and more substantial EC apoptosis in mutant P-Sp explants and cultured ECs. While others have reported no effect of β 1 deletion on vascular smooth muscle cell survival (Abraham et al., 2008), our results in ECs are consistent with findings in mammary epithelial cells, which are 1.5-fold more apoptotic than controls when β 1 is deleted in vivo (Li et al., 2005), and are substantially more apoptotic when β 1 is blocked with antibodies in vitro (Boudreau et al., 1995). Finally, we find that proliferation is unaffected by β 1 deficiency, which is consistent with results from β 1 integrin-deficient enteric neural crest

cells (Breau et al., 2006). Conversely, keratinocyte (Raghavan et al., 2000), mammary epithelial cell (Li et al., 2005), and cerebral granule cell (Blaess et al., 2004) proliferation is reduced, and vascular smooth muscle cell proliferation is increased (Abraham et al., 2008) in the absence of $\beta 1$ integrins. It is possible that $\beta 3$ integrins, which we find to be upregulated in the absence of $\beta 1$, can compensate for $\beta 1$ function in EC proliferation, but not survival. All together, our results offer a mechanistic explanation for the multitude of studies indicating that inhibition of $\beta 1$ integrins with antibody-or peptide- based approaches blocks angiogenesis in vivo (Garmy-Susini et al., 2005; Kim et al., 2000b; Pozzi et al., 2000; Senger et al., 1997).

The ECM ligands for $\beta 1$ integrins that are likely to be most relevant to vascular development are collagens, laminin, and fibronectin, since all of these molecules are expressed in the EC basement membranes of e8.5 mouse embryos (Francis et al., 2002). The main EC collagen receptors are $\alpha 1\beta 1$ and $\alpha 2\beta 1$, and therefore it is not surprising that $\beta 1$ -null ECs fail to adhere to collagens. Similarly, the main EC receptors for laminin, $\alpha 3\beta 1$ and $\alpha 6\beta 1$, are deleted in our system. However, $\alpha \nu\beta 3$ and $\alpha 6\beta 4$ are also expressed in EC and have demonstrated functions as laminin receptors. For example, the G domain of the $\alpha 4$ laminin chain, which is pro-angiogenic, binds $\alpha \nu\beta 3$ and $\alpha 3\beta 1$, and EC adhesion to this domain is blocked by anti- $\alpha \nu\beta 3$ or $-\beta 1$ (Gonzales et al., 2001; Gonzalez et al., 2002). In addition, $\beta 4$ integrins colocalize with laminin in tumor blood vessels, and blood vessels in mice expressing a truncated $\beta 4$ integrin lacking the intracellular domain do not develop in Matrigel implants to the extent that they do in control mice (Nikolopoulos et al., 2004). These results suggest that $\alpha \nu\beta 3$ and $\alpha 6\beta 4$ interactions with laminin are pro-angiogenic. However, we find that $\beta 1$ -null EC express $\beta 3$ integrins and adhere and migrate on vitronectin, the main ligand for $\alpha \nu\beta 3$ and $\alpha \nu\beta 5$, yet fail to mount appropriate angiogenic responses. Therefore, $\alpha \nu\beta 3$ and $\alpha 6\beta 4$ interactions with laminin, if they indeed exist in embryonic ECs, are not sufficient to support embryonic angiogenesis.

Although $\alpha 5^{-/-}$ or *fibronectin*^{-/-} mice are embryonic lethal (Francis et al., 2002; George et al., 1997; George et al., 1993; Yang et al., 1999; Yang et al., 1993), it was unclear from these studies whether the cardiovascular phenotypes were due to gene deletion within ECs or supporting tissues. Since the phenotypes of our mutants are similar to these mutants in many regards, it is likely that EC-intrinsic defects play a major role in the lethality of $\alpha 5^{-/-}$ and *fibronectin*^{-/-} embryos. For example, cranial blood vessels in $\alpha 5^{-/-}$ are dilated, branch less frequently, and contain less fibronectin than wild type vessels (Francis et al., 2002), which is similar to what we observe in our mutants. In addition, ECs within $\alpha 5^{-/-}$ or *fibronectin*^{-/-} embryoid bodies are disorganized and fail to form networks (Francis et al., 2002), which reflect our observations of $\beta 1$ -null ECs in P-Sp cultures or grown on Matrigel. Somewhat unexpectedly, we find that EC adhesion, migration, and focal contact formation on fibronectin appear to be independent of $\beta 1$ integrin expression. These data are consistent with studies of $\alpha 5^{-/-}$ embryonic cells (Yang and Hynes, 1996), but differ from data obtained with $\beta 1^{-/-}$ embryonic cells (Fassler et al., 1995; Stephens et al., 1993), which do not adhere or migrate on fibronectin. Despite the apparent lack of a requirement for $\beta 1$ integrins in EC interactions with fibronectin in vitro, it is a prominent component of embryonic vascular basement membranes. Since we show EC adhesion and migration defects in our mutant embryos, we hypothesize that compensatory pathways that support fibronectin interactions with $\beta 1$ -null ECs in vitro fail to compensate fully for $\beta 1$ interactions with fibronectin in vivo. This may be due to additional in vivo factors, such as hemodynamics, a three dimensional environment, and the complex ECM composition of vascular basement membranes, which are difficult to model in vitro, but are essential to consider when assessing the function of EC $\beta 1$. Our results, considered together with the phenotypes of $\alpha 5^{-/-}$ and *fibronectin*^{-/-} embryos and tumor models in which these molecules are inhibited (Garmy-Susini et al., 2005; Kim et al., 2000b), strongly support the notion that EC $\beta 1$ integrin interactions with fibronectin are required for angiogenesis.

Our conclusion that $\beta 1$ integrins within ECs are required for angiogenesis is supported by two additional reports that were published after we submitted our manuscript (Lei et al., 2008; Tanjore et al., 2008). Lei et al., using different *Tie2-Cre* and $\beta 1^{flox/flox}$ lines, report that deletion of $\beta 1$ integrin is lethal around e9.5, with growth retardation and gross vascular defects that include dilation and reduced branching in the head region as well as abnormal patterning in the yolk sac (Lei et al., 2008). Using the same *Tie2-Cre* line as Lei et al. and a different $\beta 1^{flox/flox}$ line from both Lei et al. and us, Tanjore et al. report that mutants die by e10.5 and are growth retarded. Large vessels in mutants are developed equally well as controls, but mutants display less sprouting and branching of smaller vessels (Tanjore et al., 2008). While the general conclusion regarding the requirement for $\beta 1$ integrins within ECs is similar among the published reports and ours, our work provides several unique morphological, cellular, and molecular findings. First, a major phenotype in both of our *Tie2-* and *Tie1-Cre* mutants is the histological evidence of EC adhesion defects, seen most prominently in the hearts of *Tie2-Cre* mutants and in the dorsal aortae and cardinal veins of *Tie1-Cre* mutants. By contrast, Tanjore et al. report a lack of detectable histological abnormalities in mutant embryos at e9.5, and instead conclude that general hypoxia is the major contributor to lethality (Tanjore et al., 2008). Second, we provide evidence from P-Sp explant and in vitro analyses that $\beta 1$ deficient ECs are defective in cell adhesion and migration. Third, we identify that cell survival defects contribute to the slowed growth of $\beta 1$ -null EC in vitro and are likely to contribute to the demise of *Tie2-Cre* mutants in vivo. Finally, we demonstrate reduced fibronectin in mutant blood vessels, and suggest a critical role of EC $\beta 1$ for interactions with this molecule, despite the apparent compensation by $\beta 3$ integrins, which also bind to fibronectin. Thus, our results not only demonstrate that $\beta 1$ integrins within ECs are required for vascular development, but also extend the published reports by providing mechanistic insights into the functions of EC $\beta 1$ integrins.

Our findings have implications that extend beyond embryonic vascular development and may be of therapeutic importance. For example, anti-angiogenic compounds targeting $\alpha 5\beta 1$ are currently in development for cancer therapy (Ramakrishnan et al., 2006). The data presented herein provide genetic evidence of an EC-intrinsic requirement for $\beta 1$ integrins during angiogenesis, and contribute a mechanistic explanation for the anti-angiogenic actions of $\beta 1$ -targeted therapies. Moreover, they support the further development of targeted therapies to block EC $\beta 1$ function during pathological angiogenesis or to promote its activity in ischemia or other diseases in which the growth of new blood vessels is desirable.

Materials and Methods

Mice

Tie2-Cre (Braren et al., 2006), $\beta 1^{flox/flox}$ (Graus-Porta et al., 2001), *Tie1-Cre* (Gustafsson et al., 2001), and *Tie1-GFP* (Iljin et al., 2002) have been described. Genotyping was performed with PCR for 40 cycles under the following conditions: denature: 95°C, 1 min; anneal: 56°C, 1 min; extend 72°C, 1 min. The Cre primers were 5'-GCCTGCATTACCGGTCGATGCAACGAGTG and 5'-CTGGCAATTTCCGGCTATACGTAACAGGGTG. For routine genotyping, the $\beta 1^{flox/flox}$ primers were 5'-GGAAAGTAGGCTGGACATGG and 5'-TGCCACTCCAAACATAGAGC. For detection of the wild type, floxed, and recombined $\beta 1$ alleles in the same reaction, 5'-TTCTGCAAGTGTGGTGTGAAG and 5'-TGCCACTCCAAACATAGAGC were used. The GFP primers were 5'-GCCGACCACTACCAGCAGAACACCCCATC and 5'-ATTTTATGTTTCAGGTTTCAGGGGGAGGTGT. All animals were treated in accordance with the guidelines of the University of California, San Francisco Institutional Animal Care and Use Committee.

Immunological staining of embryo whole mounts and sections

Embryo harvest, processing, and immunostaining were performed as described (Braren et al., 2006). The following antibodies were used at a dilution of 1:100: anti-CD31 (MEC13.3, BD Biosciences), anti-VE-cadherin (11D4.1, BD Biosciences, San Jose, CA), anti- β 1 integrin (Ha2/5, biotinylated, BD Biosciences, San Jose, CA), and anti-BrdU (Zymed/Invitrogen, Carlsbad, CA). Polyclonal anti-laminin (1:1000) and anti-fibronectin (1:2500) antibodies were obtained from Dr. Alex Morla. Secondary reagents were obtained from Invitrogen (Carlsbad, CA) and 4',6-diamidino-2-phenylindole (DAPI)-containing Vectashield mounting medium was from Vector Laboratories (Burlingame, CA).

Primary EC isolation and culture

Embryonic cells were prepared as described (Braren et al., 2006), and cultured in F12 + 10% FBS + penicillin/streptomycin + 150 μ g/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA) + 25 mM HEPES + 0.1 mM 2-Mercaptoethanol + non-essential amino acids, sodium pyruvate, and glutamine. For immortalization, cells were infected with a retrovirus containing polyoma middle T antigen (pMIG-PyMT-IRES-GFP, obtained from Dr. Alana Welm at UCSF), which selectively immortalizes embryonic EC (Balconi et al., 2000). Further purification was performed with anti-CD31 antibodies and anti-rat IgG conjugated magnetic Dynabeads (Invitrogen, Carlsbad, CA), and purity was confirmed by anti-CD31 and anti-VE-cadherin immunofluorescence and DiI-Ac-LDL (Biomedical Technologies, Stoughton, MA) uptake (data not shown). Immortalized EC were cultured on 0.1% gelatin (Sigma, St. Louis, MO) in DMEM:F12 + 10% FBS + penicillin/streptomycin + 50 μ g/ml heparin (Sigma, St. Louis, MO) + 25 μ g/ml endothelial mitogen. Adenoviral-mediated deletion of β 1 integrins was performed with Ad-GFP or Ad-Cre-GFP (obtained from Dr. Hillary Beggs at UCSF) at a multiplicity of infection of 100. Infections were performed in serum-free medium for 2 hr. Up to 4 rounds of infection were performed to achieve >95% β 1 deletion by FACS (data not shown).

P-Sp explants

P-Sp explants obtained from e8.5 embryos were cultured and monitored as described (Braren et al., 2006; Takakura et al., 1998). EC fluorescence was derived from a *Tie1-GFP* transgene bred into the *Tie2-Cre; β 1^{lox/+}* line. Anti-CD31 immunostaining was performed on cultures lacking GFP.

In vitro capillary morphogenesis

ECs were seeded onto Matrigel in 24-well plates and cultured in a timelapse chamber at 37°C in normal growth medium in a humidified mixture of 5% CO₂/95% air. Phase contrast images were captured at 15 min intervals, 1–24 hr after plating. Function-blocking anti- β 1 (Ha2/5), - β 3 (2C9.G2), and - α v (H9.2B8) integrin antibodies, used at 10 μ g/ml, were from BD Biosciences (San Jose, CA).

Cell adhesion, spreading, migration, and focal contact formation

Timelapse analyses of primary EC were performed as described (Braren et al., 2006). Imaging and measurements were performed using Slidebook software (Intelligent Imaging Innovations, Denver, CO). Cell adhesion and modified Boyden migration (ChemoTx 101-8, Neuro Probe, Gaithersburg, MD) studies were performed as described (Carlson et al., 2001), except that DMEM:F12 + 0.5% bovine serum albumin (Sigma, St. Louis, MO) was the medium. Immunofluorescence for focal contacts was performed as described (Carlson et al., 2001) after overnight culture of primary ECs on 10 μ g/ml fibronectin-coated glass coverslips. The following antibodies, diluted at 1:100 were used: anti- β 1 integrin (HM β 1-1-biotinylated, BioLegend, San Diego, CA), anti- β 3 (2C9.G2-Alexa Fluor 647, BioLegend, San Diego, CA),

anti-FAK^{pY397} (BD Biosciences, San Jose, CA), or anti-paxillin (Zymed/Invitrogen, Carlsbad, CA). ECs were identified with anti-CD31 (MEC13.3, BD Biosciences, San Jose, CA) at 1:100. The sources of ECM were: Human fibronectin, Roche (Indianapolis, IN); Natural mouse laminin, Invitrogen (Carlsbad, CA); Rat collagen I, Upstate Biotechnology/Millipore (Billerica, MA); Mouse collagen IV, Sigma (St. Louis, MO); Growth factor reduced Matrigel, BD Biosciences (San Jose, CA); Human vitronectin, Chemicon/Millipore (Billerica, MA).

Cell proliferation and survival

In vivo analyses were performed as described (Braren et al., 2006), except that BrdU was injected at 100 µg/g body weight 2 hr prior to dissection at e9.0. Apoptosis was detected with a Fluorescein In Situ Apoptosis Detection Kit (Chemicon/Millipore, Billerica, MA) in combination with anti-CD31 immunofluorescence. In vitro EC growth was measured by counting cells with a hemacytometer every day for one week after seeding 30,000 cells/well in 0.1% gelatin- or 1µg/ml vitronectin-coated 12-well tissue culture plates. 0.5% bovine serum albumin (heat-inactivated for 10 min at 85°C) was added to vitronectin-coated wells to block the additional adsorption of serum components prior to cell seeding. In vitro proliferation was assessed by culturing ECs on ECM coated glass coverslips with 125µM BrdU (Sigma, St. Louis, MO) for 6 hr prior to staining with anti-BrdU antibodies (Zymed/Invitrogen, Carlsbad, CA). In vitro survival was assessed by culturing ECs on ECM coated glass coverslips overnight prior to staining with YO-PRO-1 (Vybrant Apoptosis Assay kit #4, Invitrogen, Carlsbad, CA). In both assays, the coverslips were mounted with Vectashield containing DAPI, and the percentages of BrdU or YO-PRO-1 positive ECs were calculated after manual counting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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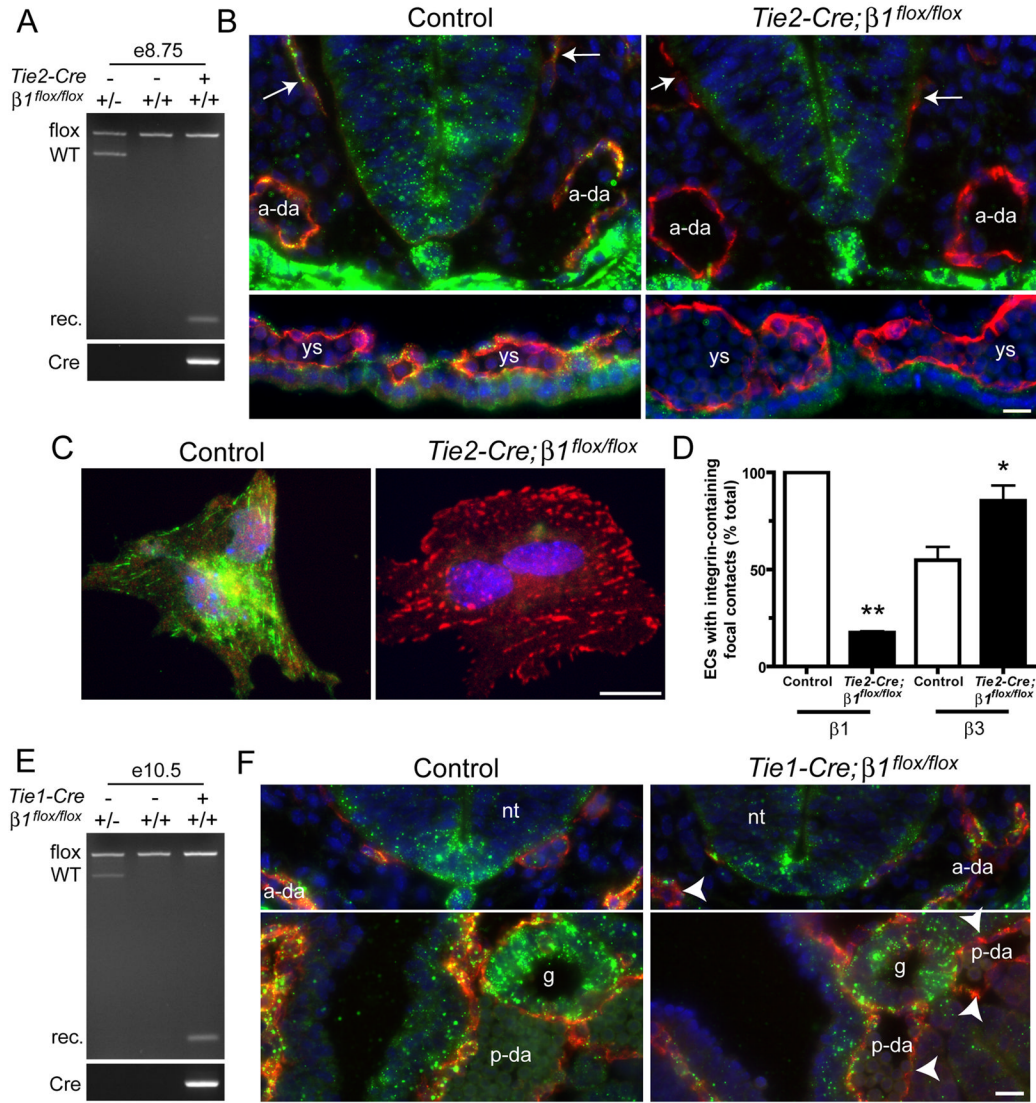


Figure 1. *Tie2-* and *Tie1-Cre* mediate efficient deletion of $\beta 1$ integrins in embryos
 Gene deletion analyses of *Tie2-* (A–D) and *Tie1-* (E,F) mutants. (A) Genomic PCR analysis of e8.5 embryos demonstrates recombination (rec.) of $\beta 1$ in embryos carrying *Tie2-Cre* and two floxed alleles of $\beta 1$. (B) e8.5 cryosections were stained with anti-CD31 (red), anti- $\beta 1$ integrin (Ha2/5, green), and DAPI (blue). (C) Collagenase-dissociated e8.5 embryonic cells were plated onto FN and stained with anti- $\beta 1$ (HM $\beta 1$ -1, green), anti- $\beta 3$ (red), and DAPI (blue). EC identity in C was determined by co-staining with anti-CD31 (not shown). (D) Focal contacts in isolated ECs. Bars are means + SEM of 2 ($\beta 1$) or 3 ($\beta 3$) experiments. ** $p < 0.01$ by one sample T-Test and * $p < 0.05$ by Student’s T-test. (E) Genomic PCR analysis of e10.5 embryos from *Tie1-Cre* matings. (F) e9.5 cryosections were stained with anti-CD31 (red), anti- $\beta 1$ integrin (green), and DAPI (blue). Arrows, primary head veins; arrowheads, $\beta 1$ -negative endothelium; ys, yolk sac blood islands; a-da, anterior dorsal aortae; p-da, posterior dorsal aortae; nt, neural tube; g, gut; ve, visceral endoderm. Also see Fig. 3F for diagram of embryonic structures. Bars, 20 μ m.

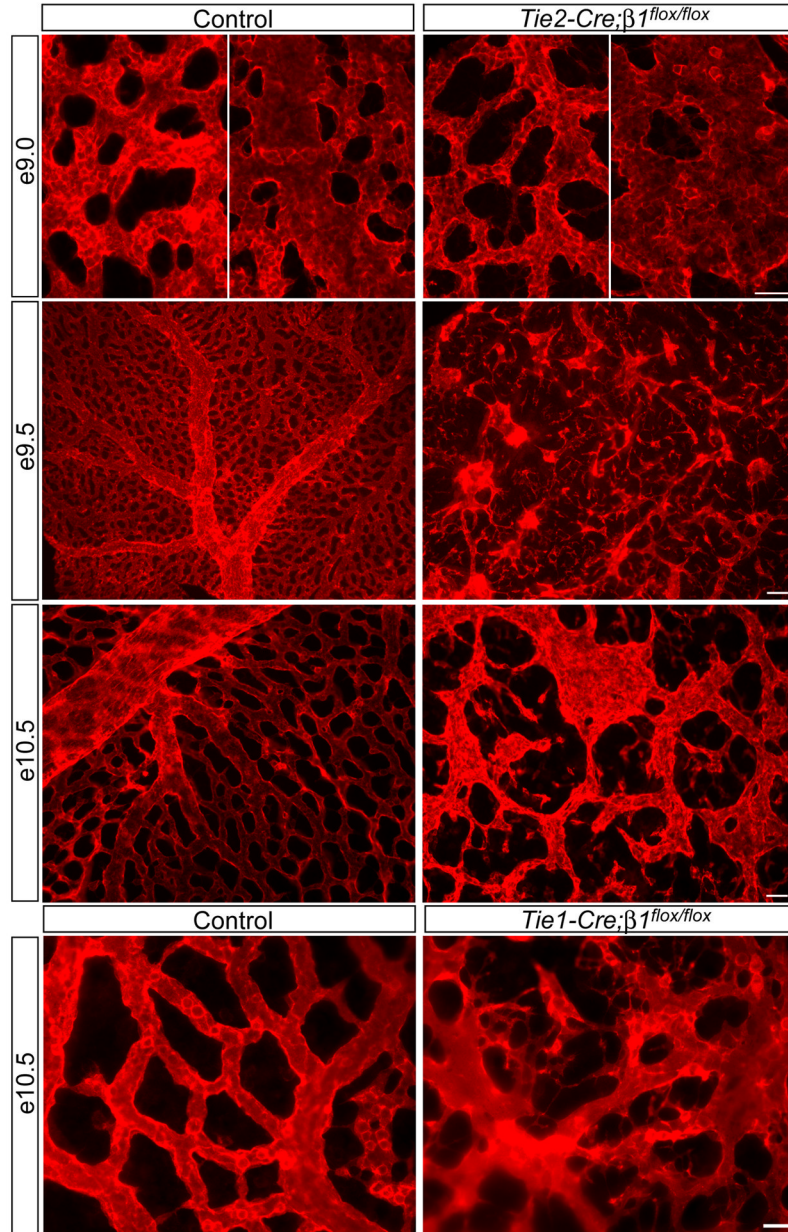


Figure 2. Endothelial deletion of $\beta 1$ integrins causes EC disorganization leading to cardiovascular defects and lethality at midgestation

Whole mount anti-CD31 immunostained yolk sacs at the indicated embryonic stages. The left panels of e9.0 are capillary regions and the right panels are regions apparently undergoing arteriovenous remodeling. Note the disconnected capillaries and overall vascular disorganization in all mutants. Images are representative of at least 5 control/mutant embryo pairs at each stage. Bars, 100 μm (e9.5), 50 μm (e9.0 and e10.5, *Tie2-Cre*), and 25 μm (*Tie1-Cre*).

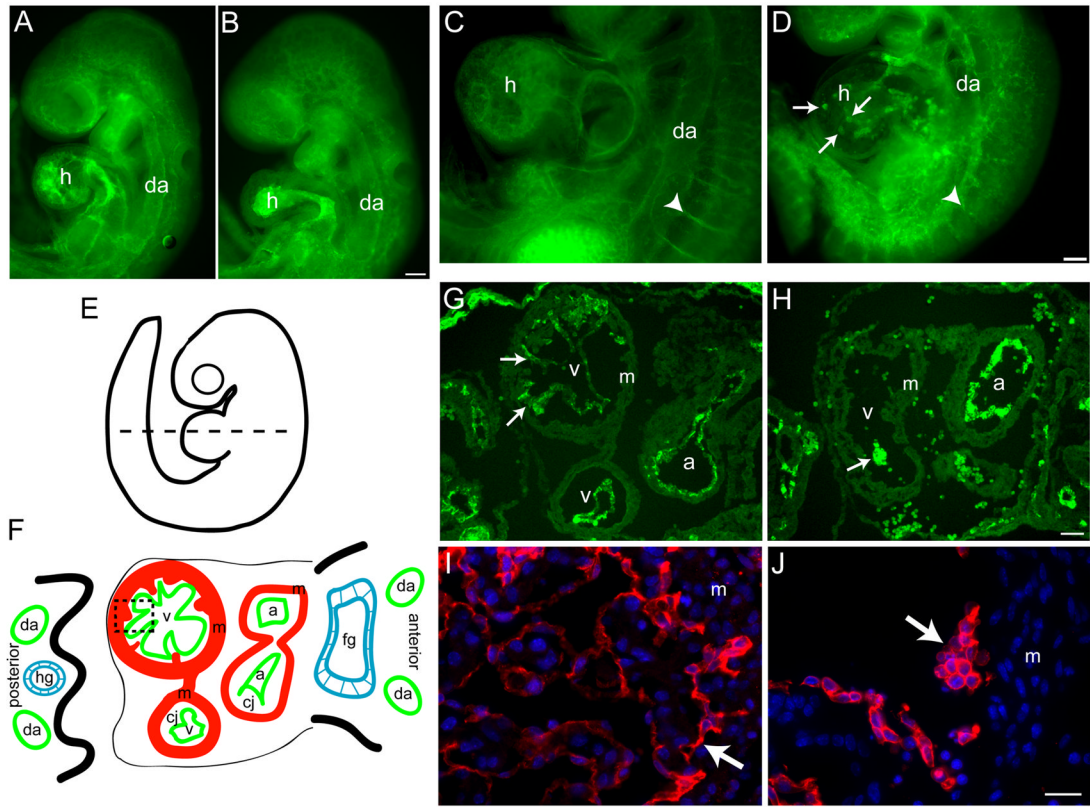


Figure 3. Endocardial cell patterning defects and abnormal cardiac morphogenesis in the absence of $\beta 1$ integrins

Endothelial staining of *Tie2-Cre* (A–F) and *Tie1-Cre* (G,H) mutants. Whole mount anti-CD31 immunostained control (A,C) and *Tie2-Cre* mutant (B,D) embryos at e9.0 (A,B) and e9.5 (C,D). (E,F) Diagram of an e9.5 embryo. Dashed line indicates the approximate plane of the cross-section diagrammed in F, and of the e9.5 control (G) and *Tie2-Cre* mutant (H) cryosections stained with anti-CD31 antibodies. The dashed box in F indicates the approximate location of the e10.5 control (I) and *Tie1-Cre* mutant (J) paraffin sections stained with anti-VE-cadherin antibodies and DAPI. Arrows, endocardium; arrowheads, intersomitic vessels; da, dorsal aorta; h, heart; a, atrium; v, ventricle; m, myocardium; fg, foregut; hg, hindgut; In F, the endocardium (green) is separated from the myocardium (red) by the cardiac jelly (cj). Bars, 100 μ m (A–D, G,H), 20 μ m (I,J). All images are representative of at least 4 control/mutant embryo pairs.

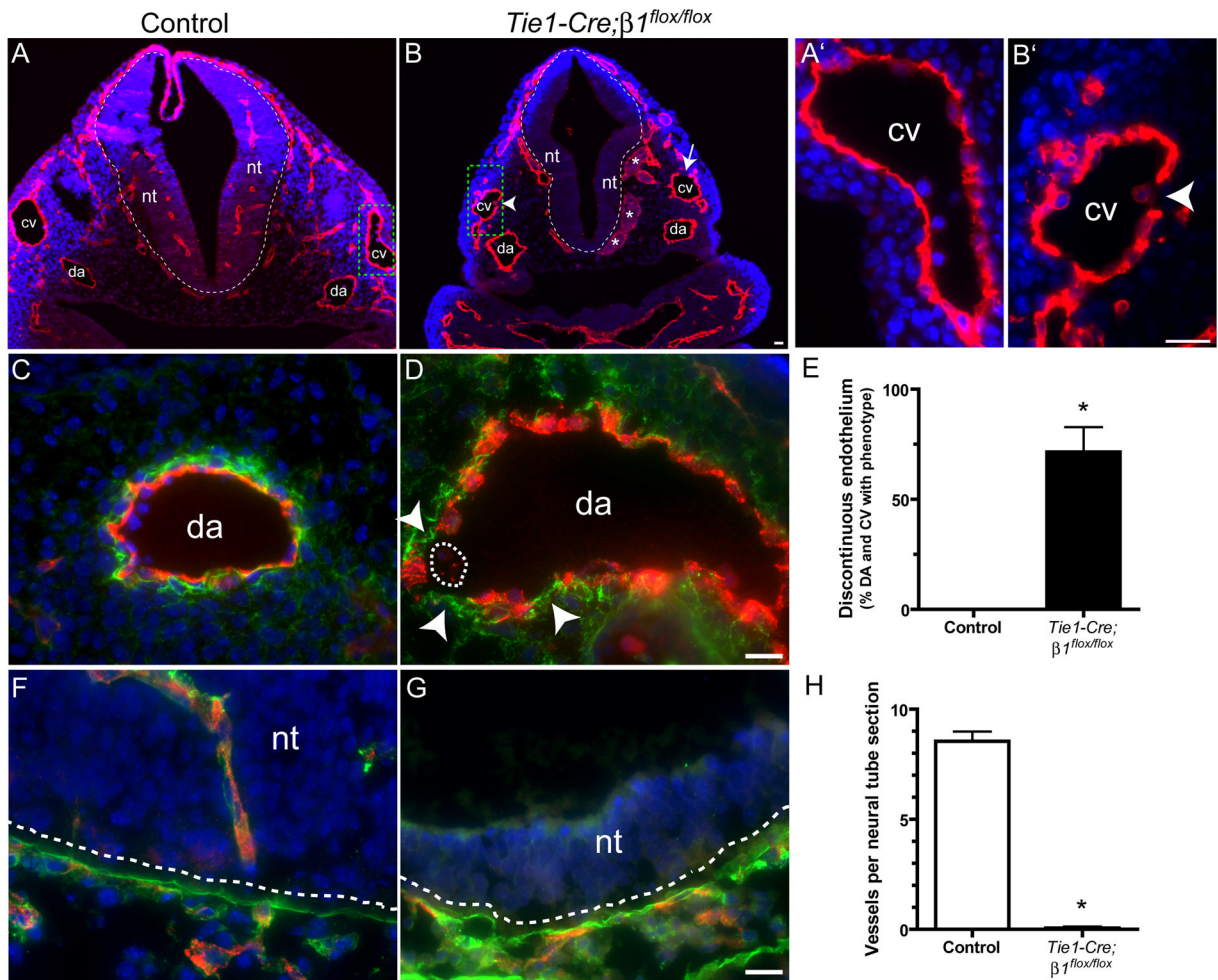


Figure 4. EC detachment and absence of neural tube invasion upon *Tie1-Cre*-mediated deletion of $\beta 1$ integrins at e10.5

(A,B) Embryos were whole mount stained with anti-VE-cadherin (red), embedded in paraffin, sectioned, and counterstained with DAPI (blue). The dashed line indicates the boundary between the neural tube (nt) and the mesenchyme. The arrow indicates an area of discontinuous endothelium in the cardinal vein (cv) and the arrowhead indicates an endothelial cell apparently undergoing detachment. Asterisks indicate dilated blood vessels. Dorsal aortae (da) are labeled. The cardinal veins in the dashed boxes are shown at high magnification in A' and B'. (C,D,F,G) Embryo cryosections stained with anti-CD31 (red), DAPI (blue), and anti-fibronectin (green, C,D) or anti-laminin (green, F,G). Arrowheads in D indicate discontinuous endothelium along the vascular basement membrane, and the dashed line encircles an endothelial cell within the lumen that has apparently detached. In F and G, the laminin-rich basement membrane (green) indicated by the dashed lines separates the nt from the mesenchyme. Note the absence of ECs in the neural tubes in mutants, and that significant laminin surrounds ECs within the control neural tubes. (E,H) Bars are the mean values + SEM of 4 control/mutant pairs at e10.5. * $p < 0.001$ by Student's T-test. Bars, 20 μm .

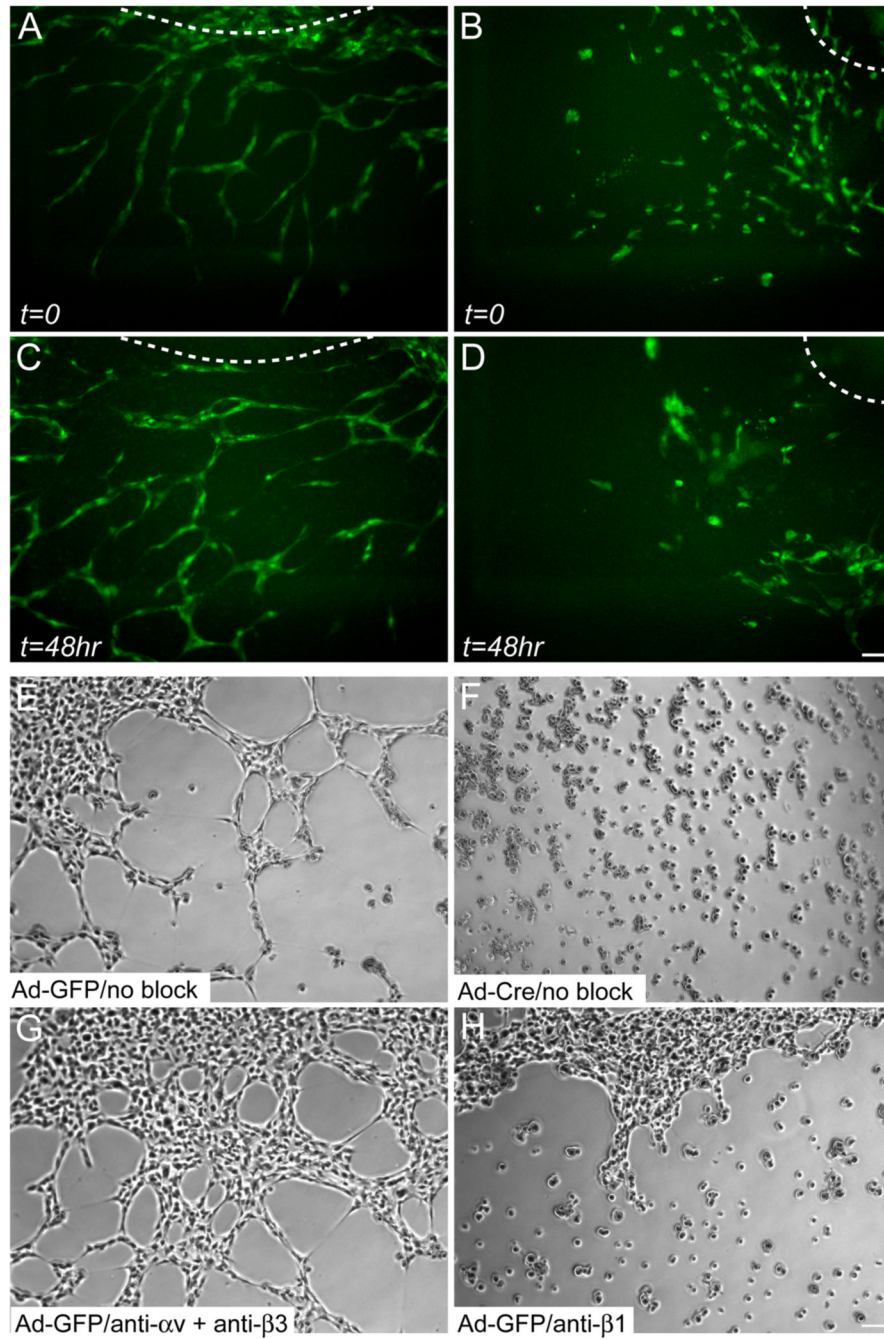


Figure 5. EC-autonomous role for β 1 integrins in angiogenic remodeling

Still images of control (A,C) and *Tie2-Cre* mutant (B,D) P-Sp explant cultures at the indicated timepoints. GFP-positive ECs were visualized by a *Tie1-GFP* transgene. Aberrant EC clusters are evident in *Tie2-Cre* mutant explants, but not in control explants. The OP9 feeder cells, not visible in the fluorescence images, were a confluent monolayer on top of which the ECs grew out from the P-Sp. The dashed lines indicate the approximate location of the P-Sp explants. See also supplemental online movies. (E–H) Capillary morphogenesis of embryonic β 1^{fl/fl} ECs immortalized with PyMT and subsequently infected with adenovirus (Ad), in the absence of any antibodies (E,F) or in the presence of anti- α plus anti- β 3 (G) or anti- β 1

(H) function-blocking integrin antibodies. Phase contrast images were captured after 4 hr in culture. Bars, 100 μm .

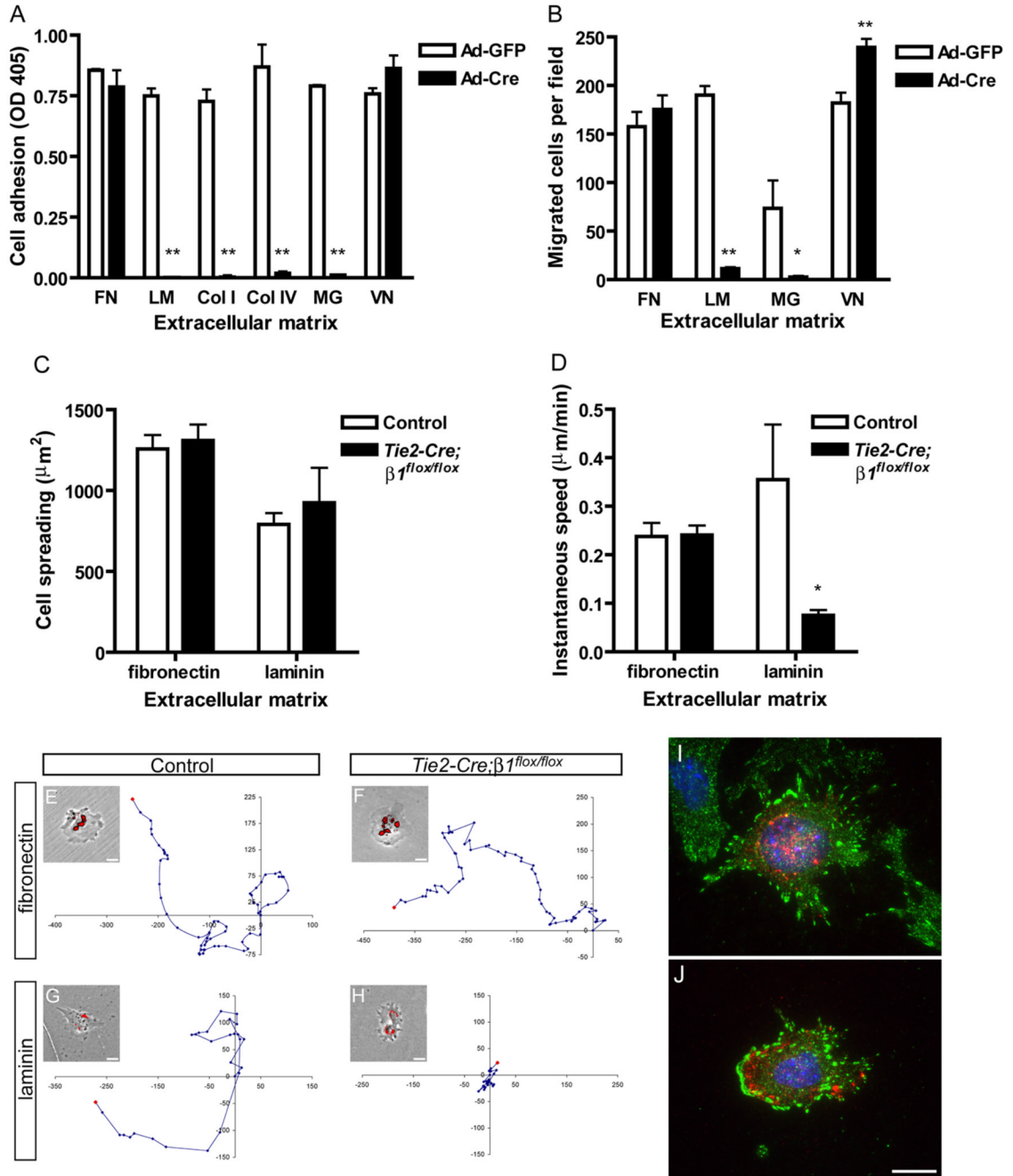


Figure 6. $\beta 1$ integrins are required for EC adhesion and migration in a matrix-specific manner
 Analysis of immortalized embryonic $\beta 1^{flx/flx}$ ECs (A, B) or primary embryonic ECs (C–J). (A) Adhesion of adenovirus (Ad) infected embryonic $\beta 1^{flx/flx}$ ECs in serum-free medium after 30 min. Microplate coating concentrations were: fibronectin (FN), 20 nM; laminin (LM), 25 nM; collagen I (Col I), 55 nM; collagen IV (Col IV), 40 nM; Matrigel (MG), 125 $\mu\text{g}/\text{ml}$; vitronectin (VN), 20 nM. (B) Haptotactic migration of embryonic $\beta 1^{flx/flx}$ ECs in serum-free medium after 4 hr. The ECM that served as the stimulus for migration was coated to the underside of a filter at the following concentrations: FN, 40 nM; LM, 50 nM; MG, 500 $\mu\text{g}/\text{ml}$; VN, 20 nM. (C) Cell spreading after 20 hr culture. (D) Migration speed measured over 24 hr (fibronectin) or 14 hr (laminin) by timelapse videomicroscopy. (E–H) Representative

migration tracks along with a phase contrast image overlaid with DiI-Ac-LDL uptake fluorescence. Units on migration tracks are pixels. Coating concentrations were: fibronectin, 40 nM; laminin, 15 nM. Focal adhesion formation in control (**I**) and mutant (**J**) embryonic ECs on fibronectin as assessed by anti-FAK^{pY397} (green), anti-CD31 (red), and DAPI (blue) staining. Values in panels A and B are means + SD, and in panels C and D are means + SEM. **p < 0.01 and *p < 0.05 by Student's T-test. All experiments were performed at least twice and representative results are shown. Bars, 20 μ m.

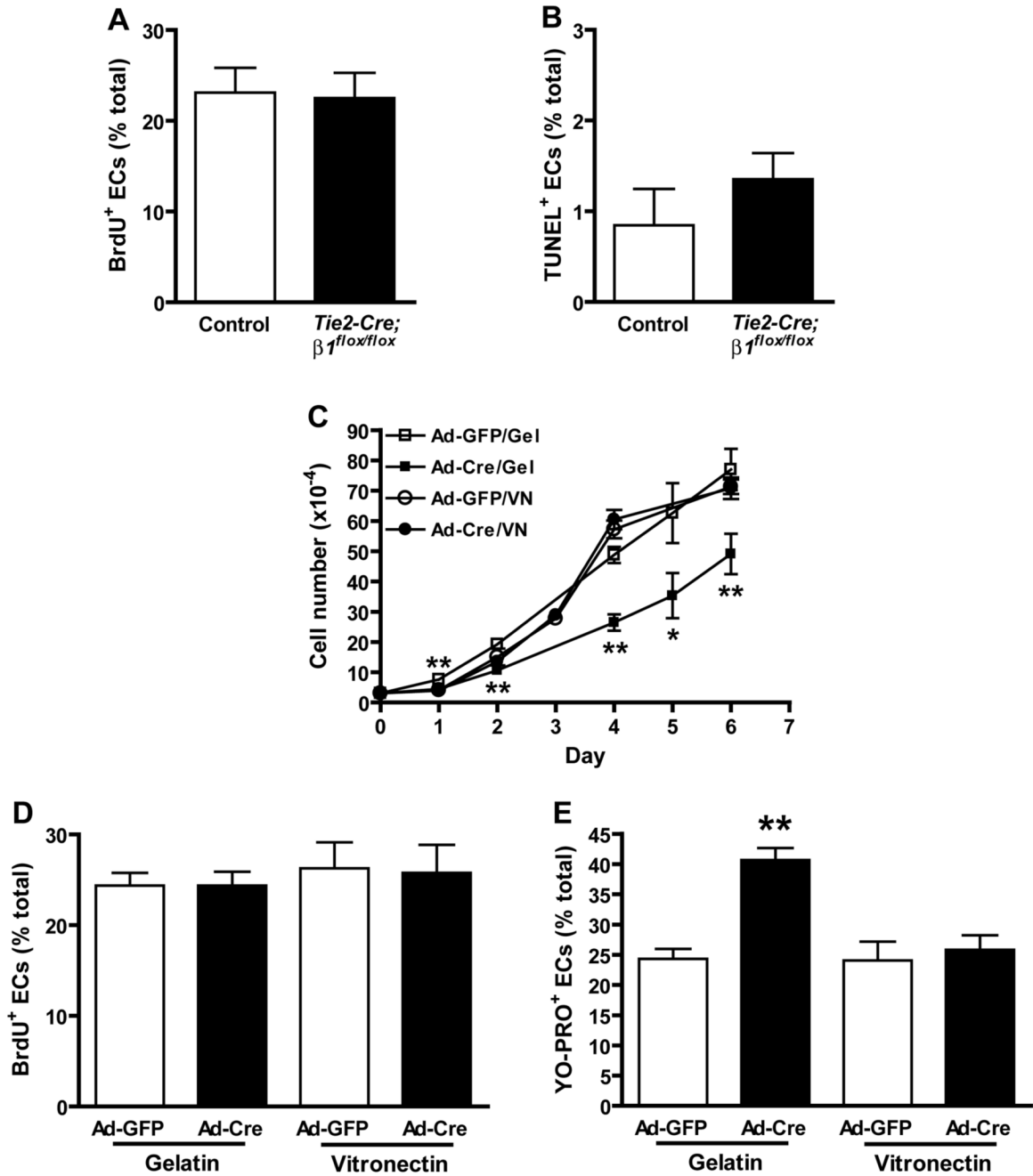


Figure 7. $\beta 1$ integrins regulate EC growth through effects on survival rather than proliferation In vivo (**A**, **B**) and in vitro (**C**–**E**) effects of $\beta 1$ deletion on EC growth. The ratios of BrdU/CD31 (**A**) and TUNEL/CD31 (**B**) double positive ECs relative to total ECs were calculated from multiple immunostained cryosections prepared from 3 (**A**) or 4 (**B**) pairs of control and *Tie2-Cre* mutant embryos at e9.0 prior to the onset of overt morbidity. Bars in panels **A** and **B** are means + SEM, and differences are not statistically significant by Student’s T-test (**A**, n = 1200 control and 965 *Tie2-Cre* mutant EC, p = 0.625; **B**, n = 1299 control and 1116 mutant EC, p = 0.36). (**C**) Growth rate of embryonic $\beta 1^{flox/flox}$ ECs cultured on gelatin (Gel) or vitronectin (VN). (**D**) Incorporation of BrdU into DNA after 6 hr culture. (**E**) Entry of the apoptotic cell permeant dye, YO-PRO-1, into proliferating EC cultures. Panel C-E data are

means + SD of replicate measurements, and each experiment was repeated with similar results.
* $p < 0.01$ and * $p < 0.05$ by Student's T-test.