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Central Roles of $\alpha_5\beta_1$ Integrin and Fibronectin in Vascular Development in Mouse Embryos and Embryoid Bodies

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Abstract—Vascular development and maturation are dependent on the interactions of endothelial cell integrins with surrounding extracellular matrix. Previous investigations of the primacy of certain integrins in vascular development have not addressed whether this could also be a secondary effect due to poor embryonic nutrition. Here, we show that the α_5 integrin subunit and fibronectin have critical roles in blood vessel development in mouse embryos and in embryoid bodies (EBs) differentiated from embryonic stem cells (a situation in which there is no nutritional deficit caused by the mutations). In contrast, vascular development in vivo and in vitro is not strongly dependent on α_v or β_3 integrin subunits. In mouse embryos lacking α_5 integrin, greatly distended blood vessels are seen in the vitelline yolk sac and in the embryo itself. Additionally, overall blood vessel pattern complexity is reduced in α_5-null tissues. This defective vascular phenotype is correlated with a decrease in the ligand for α_5 integrin, fibronectin (FN), in the endothelial basement membranes. A striking and significant reduction in early capillary plexus formation and maturation was apparent in EBs formed from embryonic stem cells lacking α_5 integrin or FN compared with wild-type EBs or EBs lacking α_v or β_3 integrin subunits. Vessel phenotype could be partially restored to FN-null EBs by the addition of whole FN to the culture system. These findings confirm a clear role for α_5 and FN in early blood vessel development not dependent on embryo nutrition or α_v or β_3 integrin subunits. Thus, successful early vasculogenesis and angiogenesis require α_5-FN interactions. **(*Arterioscler Thromb Vasc Biol.* 2002;22:927-933.)**

Key Words: $\alpha_5\beta_1$ integrins ■ fibronectin ■ vascular development ■ angiogenesis

Blood vessels in vertebrate embryos can develop through either of 2 processes, vasculogenesis or angiogenesis.[1,2] In vasculogenesis, blood vessels are generated from mesodermally derived angioblasts, whereas in angiogenesis, vessels arise as sprouts from preexisting vessels by bridging or by intussusception.[2] In the mouse, vessels are formed by the migration of angioblasts in the embryo and in the blood islands in the extraembryonic tissues. During vessel development, the endothelial precursors and differentiated cells are regulated by a number of environmental cues, including growth factors (such as fibroblast growth factors and vascular endothelial growth factors), cytokines, proteoglycans, extracellular adhesive glycoproteins, and interactions with the extracellular matrix.[3–6]

See cover

Endothelial cell interactions with the extracellular matrix are mediated in large part by the integrin family of adhesion receptors, heterodimeric transmembrane glycoproteins, consisting of α and β subunits. At the cell surface, integrins can play adhesive as well as signaling functions.[7,8] Ligand specificity and signaling ability of specific integrins are determined by their heterodimeric composition. Endothelial cells have been shown to express a variety of integrins, including the following: $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$, which are laminin and collagen receptors; $\alpha_5\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_5$, which are receptors for fibronectin (FN); $\alpha_6\beta_1$, a laminin receptor; and $\alpha_v\beta_3$, a receptor for FN, vitronectin, osteopontin, von Willebrand factor, laminin, and collagen.[4,7,9]

A number of inhibition experiments in vivo and in vitro have indicated a role for endothelial-FN interactions in vascular development.[10–12] Moreover, knockouts of FN have shown that it is essential for the organization of heart and blood vessels.[13,14] In the absence of FN, no blood vessels form in the vitelline yolk sac, whereas aortic endothelial cells in the embryo proper are scattered and disorganized. Furthermore, ablation of the α_5 integrin in mice results in extensive vascular as well as mesodermal defects and early embryonic lethality,[15,16] and Kim et al[17] have reported that antibody or peptide blockade of the $\alpha_5\beta_1$-FN interaction interferes with angiogenesis. The α_v integrins, in particular, $\alpha_v\beta_3$, have previously been implicated in a number of angiogenic func-

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tions through peptide- or antibody-blocking experiments.[18–21] Surprisingly, however, mouse embryos lacking all α_v integrins display extensive vasculogenesis, angiogenesis, and organ development, leading to questions about the primacy of the α_v integrins in vascular development.[22] Mouse knockouts of the β_3 integrin are also viable and fertile, with normal developmental angiogenesis and postnatal neovascularization of the retina.[23]

In the present study, we have addressed the role of the α_5 integrin and its major ligand, FN, in mouse vascular development in greater detail with the use of whole embryos and quantifiable embryoid bodies (EBs) in in vitro assays. We report that α_5-null embryos display marked decreases in the complexity of the vasculature that can be correlated with decreased FN matrix assembly and organization in α_5-null endothelial basement membranes. In addition, in embryonic stem (ES) cells preferentially differentiated toward an endothelial lineage, primitive vessel formation is significantly reduced in α_5-null and FN-null EBs compared with wild-type, β_3-null, or α_v-null EBs. Notably, vascular phenotype could be partially restored in FN-null EBs by the addition of whole FN to the culture system. These results strongly support a critical role for α_5 integrin–FN interactions in the normal cellular processes involved in generating the embryonic vasculature.

Methods

The Methods section can be accessed online (please see http://www.ahajournals.org).

Results

Swollen Vessels and Reduced Vessel Complexity in α_5-Null Embryos

The heads of α_5-null and wild-type embryos were of similar sizes at the stages observed. To obtain a more detailed examination of blood vessels in the α_5-null embryos, whole-mount immunohistochemistry using an antibody recognizing platelet and endothelial cell adhesion molecule (PECAM)-1, a marker for endothelial cells, was performed at E8.5 and E9.5 stages (where E indicates embryonic day) in α_5-null and wild-type littermates (Figure 1). PECAM-1 staining highlighted the abnormally swollen blood vessels in the α_5-null vitelline membranes at E8.5 (Figure 1A and 1B) and E9.5 stages. Cross sections through the yolk sacs stained for PECAM-1 showed that the enlarged vessels in α_5-null embryos were due to separation of the endodermal and mesodermal layers of the yolk sacs (Figure 1C and 1D). However, PECAM-1 staining also revealed a lining of endothelial cells around the walls of the dilated vessels. The staining also revealed a decrease in the complexity of the vascular network of the primary perineural plexus in α_5-null embryos (Figure 1E through 1H). The cranial plexus mainly consisted of large vessels that branched less frequently in the null embryos compared with age-matched wild-type littermates.

Decreased FN Expression In Vivo in the Absence of α_5

Because development and maintenance of the endothelium involves attachment to adhesive glycoproteins and the basement membrane,[4,9] the abnormal vessel patterning in the α_5 knockouts led us to a closer examination of the endothelial basement membrane. It has been shown previously that abundant levels of FN are present in blood islands and the capillary plexus,[13] whereas laminin, collagen, and other extracellular matrix molecules are produced by endothelial cells later in vasculogenesis.[3]

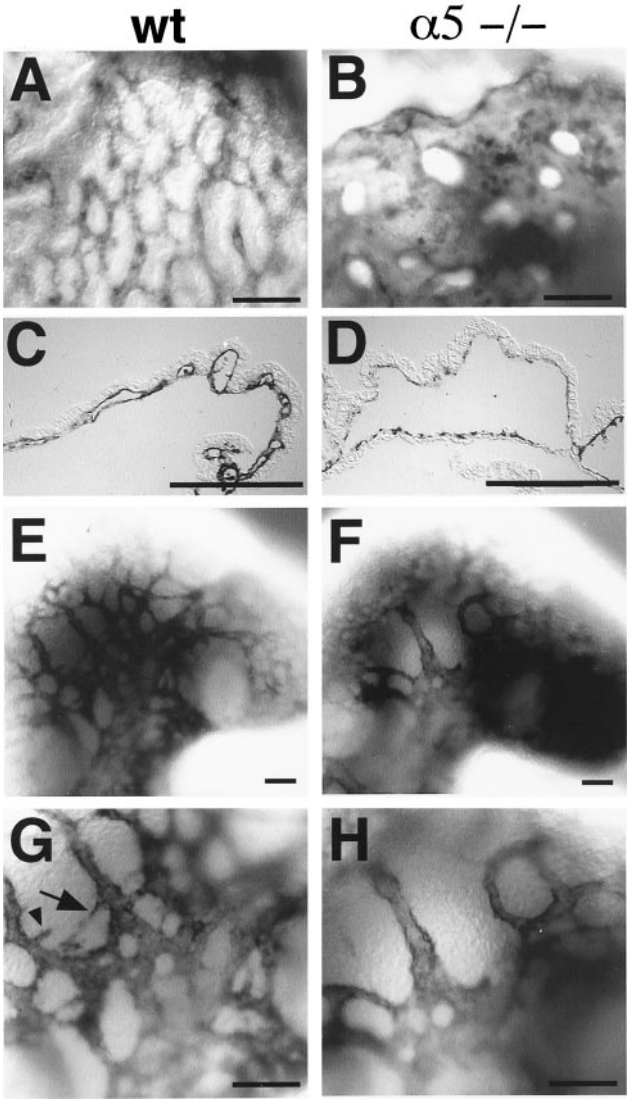

Figure 1. PECAM-stained vasculature in E8.5 wild-type (A, C, E, and G,) and α_5-null ($\alpha_5-/-$; B, D, F, and H) yolk sacs and embryos. A and B, PECAM-stained yolk sacs showing the absence of the large- and small-vessel patterning and more swollen vessels in $\alpha_5-/-$ relative to wild-type yolks sacs. C and D, Sections through PECAM-stained whole-mount yolk sacs. Dark-colored cells lining vessels are PECAM-positive endothelial cells. E and F, PECAM-stained head vessels showing decrease in vessel complexity in $\alpha_5-/-$ embryos. G, Higher magnification of panel E. H, Higher magnification of panel F. Arrow points to small branching vessels, and arrowhead indicates vessel sprouts, both of which are less prevalent in the null embryos. Bar=200 μm.

There was no reduction in the amount of mesoderm in the α_5 compared with the wild-type yolk sacs.[16] Staining of E8.5 yolk sac blood vessels and dorsal aortas for FN showed that less FN is deposited/retained in the matrix (Figure 2). In yolk sacs, FN was decreased in particular at the endoderm–endo-

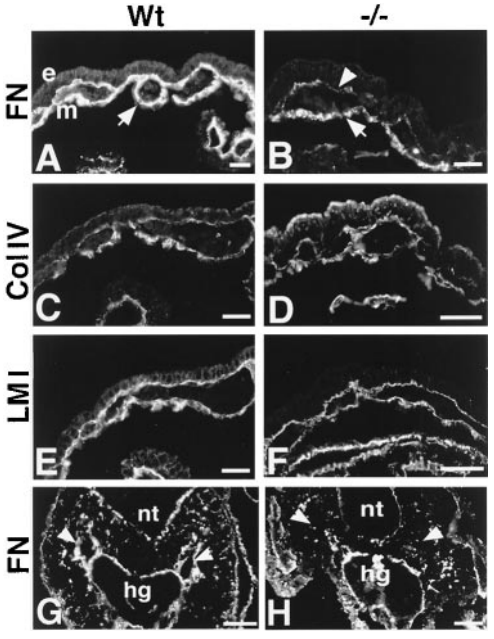

Figure 2. In vivo FN expression in E8.5 $\alpha_5-/-$ and wild-type yolk sacs and dorsal aortas. Reduced FN expression is seen in the null yolk sacs (A and B). Arrows point to vitelline blood vessels. e indicates endodermal layer of cells overlying the endothelium; m, mesodermal layer of cells underlying the endothelium. Wild-type and null vessels express similar levels of collagen IV (Col IV, C and D) and laminin I (LM I, E and F). Reduced FN is also seen in dorsal aortas (G and H). Arrowheads indicate positions of dorsal aortas in sections through the embryos' posteriors. nt indicates neural tube; hg, hindgut. Bar=100 μm.

thelial basement membrane interface (Figure 2A and 2B), whereas laminin and collagen IV expression were similar in the 2 strains (Figure 2C through 2F). The expression of entactin was also unchanged (data not shown). Similarly, in the embryo, dorsal aortic expression of FN was decreased in null relative to wild-type embryos (Figure 2G and 2H, arrowheads), whereas FN expression in epithelial basement membranes, such as those surrounding the neural tube and the hindgut, remained equally strong in the null embryos compared with wild-type embryos.

These results extend earlier descriptions of the defects in α_5-null embryos and show reduced FN deposition. FN-null embryos show similar or more severe defects,[13,14] confirming a key role for α_5-FN interactions in vessel development in vivo. However, these results could be, in part, a secondary consequence of other defects in these embryos, such as nutritional deficiencies arising from vascular or other defects. To analyze the roles of α_5 and FN in more detail without these attendant complications, we turned to an in vitro system.

Primitive Vessel Formation in Wild-Type and Integrin-Deficient EBs

To determine more clearly the role of the different integrins, we used a well-established model of early vascular plexus formation, the formation of EBs from ES cells.[24,25] EB development was monitored at 3, 4, 5, 7, 11, and 15 days after seeding by using planimetry. In view of the severe phenotype observed in the α_5-null embryos, we anticipated difficulties in performing the EB assays. As predicted from the in vivo data, α_5-deficient EBs were difficult to grow because the ES cells lacked cohesion and because EBs tended to break down as they grew larger (from day 11 onward). Despite this, cells that formed EBs did not differ significantly in size from wild-type cells (Table).

Characteristics of Wild-Type and Integrin-Null EBs

EB	EB Diameter at 11 Days, μm±SEM (n=10)	% Area of EB Occupied by PECAM-1$^+$ Staining±SEM
Wild-type (no EC gfs)	175.8±17	7±0.6
Wild-type+EC gfs	167±14	54.3±2.8
αv-Null	174.8±11	47±3.9
β3-Null	168±14	40±4.8
α5-Null	188±13	22±2.8
FN-null	213±18	6±0.7
FN-null (add-back expt)	196±16	26±3.5*

*$P<0.05$ compared with FN-null.

EB diameters were not significantly different at any time point for the ES cells lacking any of the various integrins or when endothelial growth-promoting factors were omitted from the methylcellulose-containing medium (Table). All EBs began to pulsate at day 8 to day 9 of culture, indicating the development of cardiomyocytes.

By use of confocal laser scanning microscopy of EBs stained for the expression of the endothelial marker PECAM-1, no vascular network was visible at 11 days in EBs cultured in the absence of endothelial growth-promoting factors (please see online Figure IA, which can be accessed at http://www.ahajournals.org). However, a complex lattice of PECAM-1–positive cells was visible in wild-type EBs from day 7 of culture under endothelial growth-promoting conditions, with large lacuna-like structures visible from day 11 (please see online Figure IB and Figure 3A). The diameters of these early vessel-like structures varied somewhat among EBs (wild type, 50±7 μm; α_v null, 30±4 μm [$P<0.05$ compared with wild type]; and β_3 null, 40±7 μm [all n=10]).

For wild-type, α_v-null, α_5-null, β_3-null, and FN-null EBs, individual z series were combined and projected in 2D (Figure 3), and the percent area occupied by PECAM-1–positive cells was measured by drawing around the area bounded by PECAM-1–positive staining with the use of a hand-held mouse (Table). These data confirmed that there were no differences in EB diameter regardless of whether specific integrins were present or not. However, confocal sectioning of the EBs revealed marked differences in the occupation of the body by PECAM-1–positive cells. In wild-type EBs, >50% of the total area of the EB was occupied by PECAM-1–positive cells in contrast to 22% for α_5-null cells (Table). Montages of confocal slices through these EBs (Figures 4 [wild type] and 5 [α_5-null]) and 3D reconstruction of volume-rendered 3D images (data not shown) indicated that specification of cells occurred in both cases but that in α_5-null EBs, the ability of the cells to form tubes and, therefore, lacuna-like structures appeared to be inhibited, with "islands" of PECAM-1–positive cells making

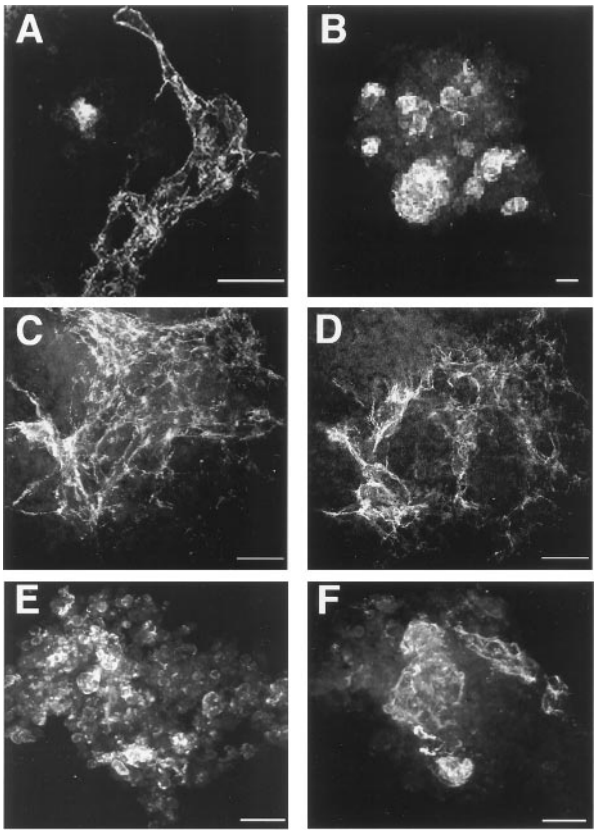

Figure 3. Projected confocal image stacks of PECAM-1–positive cell structures in wild-type (wt) and integrin-null EBs on day 11 of differentiation. A, EB (wt) with endothelial growth factors. B, EB (α_5 null) with endothelial growth factors. C, EB (α_v null) with endothelial growth factors. D, EB (β_3 null) with endothelial growth factors. E, EB (FN null) with endothelial growth factors. F, Same as panel E but cultured in the presence of 100 μg/mL whole mouse FN. All images use data collected from at least 40 consecutive optical sections with the use of 1-μm z step. Bar=50 μm.

only occasional contact (Figures 3B and 5). Compared with wild-type cells, cells within the islands tended to be densely packed in layers, and the percent area occupied by PECAM-1–positive staining was reduced significantly (Table). In contrast to this, wild-type EBs exhibited a complex weblike pattern of PECAM-1–positive cells (Figures 3A and 4). Similarly, EBs lacking α_v or β_3 also developed extensive PECAM-1–positive structures (Figure 3C and 3D and Table).

FN-Null EBs Contain Endothelial Cells, but These Do Not Organize Into Islands or Form Vascular Structures

EB assays were also performed by using FN-null ES cells. FN-null, like α_5-null, EBs exhibited PECAM-1 staining but with no distinct pattern or organization within the EB (Figure 3E). In rescue experiments, in which FN-null EBs were cultured in the presence of 100 μg/mL whole FN, partial rescue of the null phenotype (an increase in PECAM-1–positive cells) occurred in all EBs examined (26±3.5% [rescued] versus 6±0.7% [(FN null], $P<0.05$; Table and Figure 3F.

Discussion

The establishment and regulation of blood vessel growth is critical for normal mammalian embryonic development and for pathological processes in the adult, such as wound repair and tumorigenesis. Numerous studies have illustrated the importance of cell–extracellular matrix interactions (in particular, via the integrin family of adhesion/signaling receptors) in the mechanisms behind vascular development.[4,26–33] A major focus of these investigations has been on the roles of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Indeed, much evidence has suggested the importance of the α_v integrins in angiogenesis[19] (see reviews[21,34]).

Recent studies have pointed to a more central role for β_1 integrins in vascular development. In the absence of β_1 integrins, fewer blood vessels form in a teratoma assay, and formation of a complex vasculature is delayed.[35] The basement membrane also lacks laminin 1 in EBs.[36] Senger et al[37] have shown that angiogenesis induced by vascular endothelial growth factor can be inhibited by antibodies against $\alpha_1\beta_1$ and $\alpha_2\beta_1$. Knockouts of the α_v[22] and β_3[23] integrins have shown much milder vessel defects than anticipated: α_v-deficient mice display a complex embryonic vasculature, whereas β_3-null mice are viable and fertile and show no vessel defects in perinatal retinal neovascularization. In the present study, we have shown that α_5-null embryos exhibit a lower complexity of blood vessel formation correlating with reduced FN matrix assembly in vivo. Because there may be concerns that these integrin-deficient embryos could be nutritionally limited, we have reinforced these data by performing ES cell differentiation, a technique in which these limitations do not apply. In EB assays in vitro, α_5-null and FN-null EBs are both unable to form any significant primitive vasculature. It is of interest that some vascular phenotype can be restored in FN-null EBs by the addition of whole FN to the culture system. Taken together, these data suggest that $\alpha_5\beta_1$-FN interactions are necessary for basic cellular processes involved in normal vessel development and that the endothelial functions of α_5 can be separated from those of α_v and β_3.

Using whole-mount PECAM staining of embryos and EBs, we have shown that the initial generation of endothelial cells occurs normally in the absence of α_5 integrin, similar to the situation seen in FN-null embryos.[38] Contrary to the extreme picture in the FN-deficient embryos, the α_5-null endothelial cells do appear to organize themselves into vessels in the yolk sac and the embryo proper and into islands of endothelial cells in EBs. Because FN has been shown to be necessary for normal tube formation in the yolk sac, the initial vessel formation seen in the α_5 knockout is probably due to the function of other FN receptors, such as the α_v integrins, which, as we have shown (K.L. Goh, unpublished data, 2001), are expressed in α_5-null primary endothelial cells. However, the α_5-null blood vessels seen in the embryo are not completely normal, inasmuch as they are enlarged and, as illustrated by the head vessels, lack the complexity of pattern seen in the wild-type control vessels. Less capillary branching, ie, angiogenesis, seems to occur in the α_5-null cranial plexus. This effect is also seen in α_5-null EBs, in which (although islands of endothelial cells form) their ability to

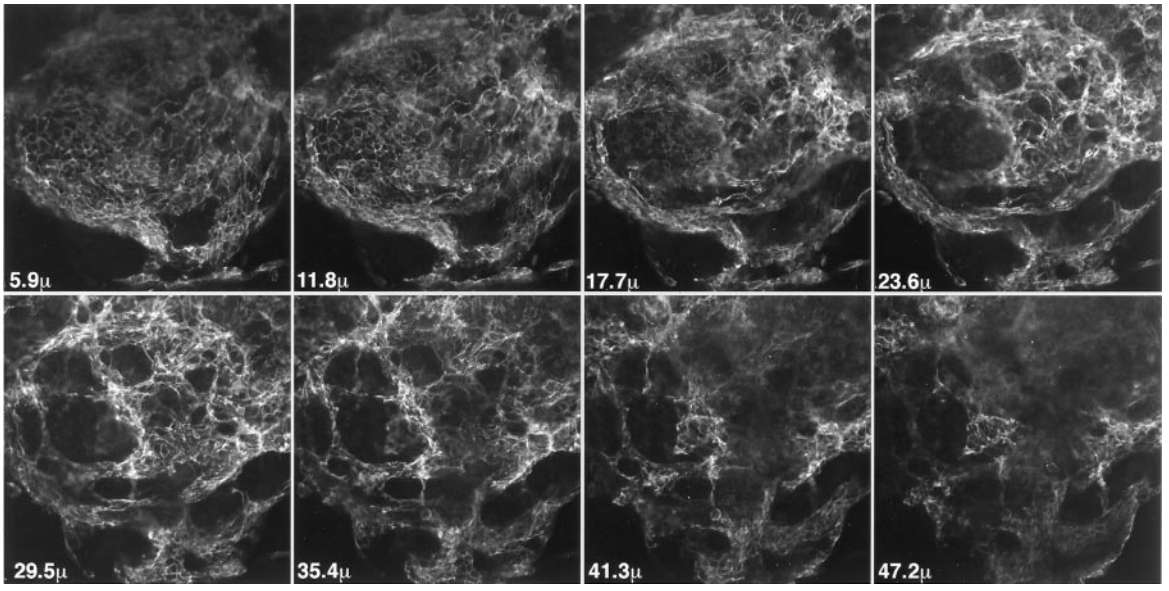

Figure 4. Selected confocal slices of PECAM-1–positive structures in a wt EB at day 11 in culture. Note complex lacunae of PECAM-1–positive cells within the EB and the emerging pattern of tubelike networks. Figures at bottom left of each panel indicate position of each confocal slice within the EB. Original magnification ×200.

contact one another to form a network of tubes appears to be limited.

Preliminary analyses of α_5-null cells in vivo and in EBs and of α_5-null endothelial cells cultured from embryos suggest that cell proliferation and survival are somewhat reduced in culture (authors' unpublished data, 2001). Similar results have been observed in α_5-null teratocarcinomas,[39] although they were not evident at early stages in the embryos.[16] As for cell adhesion, α_5-null endothelial cells show reduced adhesion to FN, as expected, but are normally adherent to other matrix proteins (laminin, vitronectin, and collagen IV; authors' unpublished data, 2002).

In the α_5-null yolk sacs, the distended blood vessels are accompanied by a separation between the endodermal and mesodermal layers, which is also seen in a more severe form in the FN-null yolk sacs.[38] This separation is interesting because in situ differentiation of endothelial cells occurs primarily from mesodermal cells in contact with the endoderm,[5,40,41] and separation of the endoderm from the mesoderm has been shown previously to result in the absence of a vascular network.[42] The endoderm is of importance because it is thought to be the main source of basic fibroblast growth factor (bFGF), a factor required for normal vasculogenesis.[2] Interestingly, treatment with bFGF has been shown to cause a significant increase in the surface expression of the $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_5$ integrins in microvascular endothelial cells.[43] In contrast, the levels of expression of $\alpha_1\beta_1$ and $\alpha_v\beta_3$ were decreased in bFGF-treated cells. The addition of transforming growth factor-β1 and bFGF results in a synergistic induction of α_5, with no significant changes in the expression of β_1.[44] Thus, it is possible that the failure of normal signaling from the endoderm could contribute to the vessel defects seen in the α_5-null embryos.

Another possibility suggested by our data is that endothelial cells play an active role in organizing and assembling the FN matrix and that the failure to organize the matrix appropriately in the endothelial basement membrane could lead to defective endothelial cell adhesion and migration and, hence, to defects in vessel remodeling and angiogenesis. It has been previously shown that the profile of the subendothelial basement matrix changes as vascular development proceeds in the embryo, with FN being the earliest and most abundantly expressed matrix molecule (Risau and Lemmon[3] and the present study). Moreover, the assembly of an FN matrix has been shown to influence a number of cellular functions, including the organization of intracellular cytoskeletal structures and changes in signaling pathways; eg, assembly of a native FN matrix has been shown to induce rapid formation of actin stress fibers and colocalization of $\alpha_5\beta_1$ integrin, focal adhesion kinase, vinculin, and paxillin to regions of cell-matrix contact[45] and is required for Rho GTPase activation and cell-cycle progression.[46] In addition, and reinforcing the importance of FN as a primary matrix molecule in vessel formation, FN-null EBs exhibit a more severe defect in endothelial cell organization than that seen in α_5-null EBs: PECAM-1–positive cell content is markedly reduced compared with other EBs, and no islands of endothelial cells are observed. It is indeed noteworthy that a partial vascular phenotype could be restored by the addition of whole mouse FN to the culture system. A possible reason that only a partial rescue was observed may be the difficulty of access and/or inadequate concentration of FN available to the growing EB.

All the experiments performed have suggested that α_5 may be a critical player in organizing the FN matrix underlying the endothelial cells during periods of blood vessel development in the embryo or in angiogenesis in teratomas, contributing to the normal assembly of the endothelial basement membrane. This matrix-organization function of $\alpha_5\beta_1$ may explain in part the observations in β_1-null teratomas, in which diffuse pat-

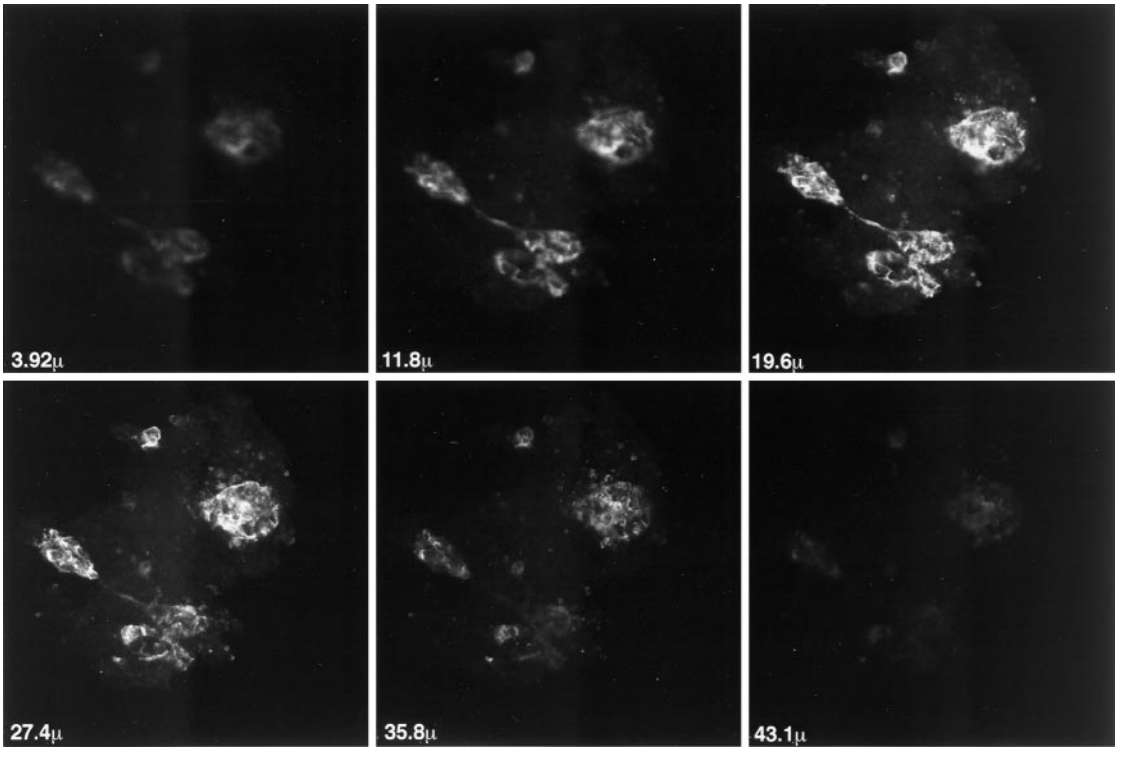

Figure 5. Selected confocal slices of PECAM-1–positive structures in an α_5 EB at day 11 in culture. Note that there are very few PECAM-1–positive cells/islands compared with wt and limited connections between islands of PECAM-1–positive cells. Figures at bottom left of each panel indicate position of each confocal slice within the EB. Original magnification ×200.

terns of FN matrix, irregular basement membranes, and a poor vasculature have been detected.[35,36]

Our results may serve as an explanation for discrepancies in the literature concerning the role of integrins in angiogenesis. As mentioned previously, an important role for angiogenesis has been suggested for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins.[47] However, examination of β_1-null teratomas, which display abnormally developed vasculature, by Bloch et al[35] revealed that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins were unchanged. The recently described mild vascular phenotypes of α_v knockouts,[22] in which 20% survive to birth, and β_3 knockouts,[23] which are viable and fertile, also raise the question of the necessity for α_v integrins in embryonic vasculogenesis and angiogenesis. Our results suggest that it is probable that $\alpha_5\beta_1$, perhaps along with $\alpha_1\beta_1$ and $\alpha_2\beta_1$, has critical functions in regulating early vessel formation, independent of α_v and β_3 integrins.

In conclusion, we present a detailed look at the critical involvement of α_5 integrin in the cellular processes involved in vascular development in vivo and in ES cell cultures. Using α_5-deficient mice and EBs, we have revealed the importance of α_5 integrin and FN interaction in vascular development and have shown that EB vasculogenesis is not strongly dependent on either α_v or β_3.

Acknowledgments

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On-line materials and methods

1. Materials

Rabbit antiserum against rat FN (28STU) was made in-house, mouse collagen IV antibody was purchased from Collaborative Biomedical Products, (Becton Dickinson Labware, Bedford, MA), rat anti-mouse PECAM/CD 31 antibody, MEC13.3 was purchased from Pharmingen (San Diego, CA), and mouse laminin-1 antibody from Sigma (St. Louis, MO). Anti-mouse entactin/nidogen was a kind gift from A. Chung (University of Pittsburgh, PA).

2. Mouse ES cell lines

ES cells containing homozygous null mutations in the α5 (clones 154 and 305) [1] FN [2] β3 [3] and αv [4] integrin genes have been described. All except the FN-null ES cells were derived from heterozygous ES cells by selection in high concentration of G418 [5]. FN-null ES cells were established from FN-null E3.5 embryos arising from FN +/- intercrosses (Robinson S, Hynes RO. unpublished). All cell lines were originally derived from the 129Sv D3 ES cell line.

All ES cells were maintained on γ-irradiated mouse embryonic feeder fibroblasts in the presence of leukaemia inhibitory factor (LIF) as described previously [6].

3. *In vitro* culture system for ES cells.

Subconfluent undifferentiated D3/129Sv or null ES cell lines were allowed to differentiate in DMEM High Glucose supplemented with 1.4 % methylcellulose (Stem Cell Technologies, Vancouver, Canada), 6% fetal calf serum (Intergen, CA), 450μM monothioglycerol, 10μg/ml human insulin (both Sigma), 50U/ml penicillin and 50μg/ml streptomycin. To optimise vascular differentiation,

growth factors were added to medium containing methylcellulose [7-8]: Recombinant human VEGF (Peprotech, Rocky Hill, NJ), 50ng/ml; mouse erythropoetin (Boehringer Mannheim, Germany), 2U/ml; human basic fibroblast growth factor, 100ng/ml and recombinant murine IL-6, 10ng/ml, both from Genzyme, Cambridge, Massachusetts. Mouse fibronectin was from Gibco BRL, Grand Island, NY).

ES cells were seeded at 1.25 X 10^3 cells/ml in a volume of 2ml of complete methylcellulose-containing medium in 35 mm bacterial-grade petri dishes (Becton Dickinson, Plymouth, UK). Cultures were fed at day 11 after seeding with 0.5 ml of complete medium containing methylcellulose and endothelial growth-promoting factors. For all experiments with FN-null ES cells, including 'add-back' experiments, FN-depleted serum was used [9]. FN depletion by gelatin-Sepharose is widely used to purify FN and to yield a depleted serum that is a good growth supplement for numerous cells. EB diameter *in vitro* was measured using planimetry of the *in situ* EBs using an inverted microscope.

EBs were collected at 3,4,5,6,7,11 and 15 days by dilution of the methylcellulose medium with PBS and EBs were prepared for confocal microscopy analysis.

4. Whole-mount PECAM staining

PECAM staining was performed essentially as described in Bader *et al.* [4]. In brief, embryonic day (E) 8.0-9.5 litters from C57BL/6;129Sv/Jae α5 heterozygous crosses were dissected into ice-cold PBS (vaginal plug designates E0.5 stage). Yolk sacs and embryos were fixed overnight in 4% paraformaldehyde (PFA) or Dent's fixative (80% methanol: 20% DMSO). Samples were either stored in methanol at -20°C at this stage or processed further by treatment with 6% hydrogen peroxide in methanol for 1 hour at room temperature. After rehydration into PBST (PBS + 0.1% tween 20), samples were blocked in 4% BSA,

and primary antibody solution (PECAM-1 diluted 1:200 into 10% goat serum, 4% BSA in PBST) was added. After overnight incubation at 4°C, samples were washed for 2 x 5 min, followed by 5 x 1 h washes in PBST, and then incubated with secondary antibody overnight (1:200 alkaline phosphatase (AP)-conjugated goat anti rat IgG (H+L), Pierce) at 4°C. Washes were followed by color development using NBT/BCIP substrates (Boehringer Mannheim, Indianapolis, IN). Samples were then fixed in 4% PFA, 0.1% glutaraldehyde overnight at 4°C. Photographs of whole-mounts were taken using an Axiophot microscope (Carl Zeiss, Thornwood, NY). For whole-mount sections, samples were paraffin-embedded and 6-8 μm sections were taken.

5. Immunofluorescence microscopy

Sections were prepared as described in Bader et al [4]. In brief, after trypsinization and blocking steps (using FN-depleted goat serum in the case of FN antibody preparations), primary antibodies were applied 2 h to overnight at 37°C. Sections were washed and secondary antibody applied for 2 h at 37°C. Sections were mounted in gelvatol with Dabco (Sigma) and photographed using an Axiophot microscope. Final figures were scanned and processed using Adobe Photoshop 5.02 on Power Macintosh 7100/80.

6. Confocal Laser Scanning Microscopy

EBs were harvested at 7, 11 and 15 days for whole-mount confocal microscopy. EBs were permeabilised and fixed in methanol-dimethyl sulphoxide (4:1) overnight at 4°C while rotating. EBs were then rehydrated in sterile ice-cold PBS and stored at 4°C. For confocal analysis, EBs were pre-incubated in 2% BSA/PBS containing 0.1% Tween-20 for 1 hour at room temperature. All incubations were performed while keeping the EBs gently rotating. Incubation with a rat

monoclonal anti-mouse platelet cell adhesion molecule (1:200, PECAM) was carried out overnight at 4°C. After 4 successive washes in PBS/BSA/Tween, a secondary FITC-conjugated antibody (1:500, Biosource International, Camarillo, CA) was applied for 2 hours in the dark at room temperature. After 4 successive washes as above, EBs were mounted on glass slides in Gelvatol (Monsanto, St Louis, MO) containing the anti-fade agent DABCO (Sigma, St Louis, MO) and stored at 4°C until analysis.

Confocal analysis was performed using an MRC 1024 system (Biorad, Hercules, CA) connected to an inverted microscope (Axioplan, Zeiss, Germany). The system was equipped with a Krypton/Argon laser which was used at 3% power. Excitation was at 488nm for FITC and 568nm for Texas Red. A Plan Neofluar 25X, a Plan Apochromat 63X and an Aeroplan 40X water immersion objectives were used. Full frame images (512 x 512 pixels) were acquired. In brief, for the purposes of reconstruction, at least 30 full frame images separated by a distance of 1μm in the z direction were recorded. Additional data, at least 10 full frame images, were also taken at distances of 5μm to give full information on capillary area and spatial organization in an EB of 50-60μm diameter. From the acquired images, an overlay image giving a projection of the vascular structures in the EB was generated. Pixel intensity in optical sections of EBs treated only with secondary antibody was subtracted from the overlay image. The % area of PECAM staining and lumen diameters was calculated using image analysis software (LaserSharp V3.1 Beta 7, Biorad, Hemel Hempstead, UK).

7. 3D Reconstruction of confocal optical sections of EBs.

Complete z series of wild-type and α5 -/- EBs were surface-rendered using a Woolz algorithm to generate a solid surface image of the cells which could be rotated to arbitrary viewing angles and viewed under different lighting and

shading conditions. Images were displayed by turning through 360 degrees with a snapshot taken every 36 degrees.

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On-line results: Table I

Table I. Characteristics of wild-type and integrin-null EBs

EB	**EB Diameter at 11 days μm ± SEM (n=10)**	**% area of EB occupied by PECAM-1+ staining ± SEM**
Wild type (no EC gfs)	175.8 ± 17	7 ± 0.6
Wild type + EC gfs	167 ± 14	54.3± 2.8
αv null	174.8 ± 11	47 ± 3.9
β3 null	168 ± 14	40 ± 4.8
α5 null	188 ± 13	22 ± 2.8
FN null	213 ± 18	6 ± 0.7
FN null (add-back expt)	196 ± 16	26± 3.5*

***** P<0.05 compared to FN null