Targeted \textit{Vezf1}-Null Mutation Impairs Vascular Structure Formation During Embryonic Stem Cell Differentiation

Zhongmin Zou, Pauline A. Ocaya, Huiqin Sun, Frank Kuhnert, Heidi Stuhlmann

\textbf{Objective}—\textit{Vezf1} encodes an early zinc finger transcription factor that is essential for normal vascular development and functions in a dose-dependent manner. Here, we investigated the role of \textit{Vezf1} during processes of endothelial cell differentiation and maturation by studying mutant \textit{Vezf1} embryonic stem (ES) cells using the in vitro embryoid body differentiation model and the in vivo teratocarcinoma model.

\textbf{Methods and Results}—\textit{Vezf1}−/− ES cell–derived embryoid bodies failed to form a well-organized vascular network and showed dramatic vascular sprouting defects. Our results indicate that the retinol pathway is an important mediator of \textit{Vezf1} function and that loss of \textit{Vezf1} results in reduced retinol/vitamin A signaling and aberrant extracellular matrix (ECM) formation. Unexpectedly, we also uncovered defects during in vitro differentiation of \textit{Vezf1}−/− ES cells along hematopoietic cell lineages. \textit{Vezf1}−/− ES cell–derived teratocarcinomas were able to spontaneously differentiate into cell types of all 3 germ layers. However, histological and immunohistochemical examination of these tumors showed decreased cell proliferation, delayed differentiation, and large foci of cells with extensive deposition of ECM. Embryoid bodies and teratocarcinomas derived from heterozygous ES cells displayed an intermediate phenotype.

\textbf{Conclusion}—Together, these results suggest that \textit{Vezf1} is involved in early differentiation processes of the vasculature by regulating cell differentiation, proliferation, and ECM distribution and deposition. (\textit{Arterioscler Thromb Vasc Biol.} 2010;30:1378-1388.)

\textbf{Key Words:} angiogenesis ■ biology, developmental ■ endothelium ■ gene mutations ■ vascular biology ■ embryonic stem cells ■ \textit{Vezf1} ■ embryoid body differentiation ■ gene knockout ■ teratocarcinoma

Development of the vascular system involves 2 distinct processes: coalescence of mesoderm-derived angioblasts to form a primitive vascular plexus (vasculogenensis) and subsequently sprouting, branching, remodeling, and maturation of the primary plexus to give rise to the mature blood vasculature (angiogenesis).1–3 These complex developmental processes require the precise temporal and spatial expression of a large number of genes. Much progress has been made over the past 15 years to identify relevant genes and to understand their participation in signaling pathways and their role in vascular development and disease.1,4,5 In contrast, much less is understood about how the precise expression of vascular-specific genes is mediated by the coordinated action of transcription factors. Promoter analysis of endothelial-specific genes in vitro identified crucial transcriptional regulatory factors. Their roles in vascular development and in the adult endothelium have been elucidated through transgenic and gene targeting studies (see reviews 6–8). The emerging picture from these studies suggests that most if not all transcription factors that are important for blood vessel formation are not endothelial specific either in their expression or in their function. In fact, several of these factors are involved in both hematopoiesis and vascular development. Furthermore, expression of endothelial genes during blood vessel formation and homeostasis often requires unique combinations of multiple transcription factors, possibly in combination with tissue-specific cofactors or bridging proteins.5,7

In a gene trap screen for early cardiovascular genes, we have identified a novel zinc finger transcription factor, \textit{Vezf1}.9–11 The C2H2 (Kruppel-like) zinc finger protein VEZF1 is highly conserved among vertebrates, and its 518 amino acid sequence shows only 6 amino acid differences between mouse and human,12 suggesting that \textit{Vezf1} plays a conserved role in mammals. \textit{Vezf1} is unique among endothelial transcription factors; although it is expressed in the endothelium as well as in neuronal and mesodermal tissues,10 (D. Lemons, H. Stuhlmann, unpublished data, 2009) its function appears to be restricted to the vascular system. In vitro studies indicate that \textit{Vezf1} regulates the proliferation, migration, and network formation of endothelial cells.13

Received on: January 13, 2009; final version accepted on: April 12, 2010.
From Department of Cell Biology, Division of Vascular Biology, Scripps Research Institute, La Jolla, Calif (Z.Z., H.S., F.K., H.S.); Department of Cell and Developmental Biology, Weill Cornell Medical College, New York, NY (P.A.O., H.S.). Present addresses: Department of Toxicology, State Key Laboratory of Burn, Trauma and Combined Injury, Third Military Medical University, Chongqing, People’s Republic of China 400038 (Z.Z., H.S.); Department of Medicine, Division of Hematology, Stanford University Medical Center, Stanford, Calif (F.K.).
Dr Zou and Dr Ocaya contributed equally to this work.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.109.200428

1378
addition, its human ortholog DB1/ZNF161 functionally interacts with Rho GTPases and associated regulatory proteins and regulates expression of the human endothelin-1 gene.\textsuperscript{14,16} Targeted inactivation of Vezf1 in mice results in embryonic lethality at midgestation, and Vezf1 was found to control development of the blood vascular and lymphatic system in a dose-dependent fashion.\textsuperscript{17} Homozygous mutant embryos showed defects in vascular remodeling and loss of vascular integrity resulting in localized hemorrhaging, concomitant with defects in endothelial cell adhesion and junction formation in the mutant vessels. Haploinsufficiency was observed in 20% of heterozygous embryos, and those displayed lymphatic hypervascularization associated with hemorrhaging and edema.

Here, we address the specific role(s) of Vezf1 in endothelial cell proliferation, sprouting, and remodeling by using an in vitro differentiation assay of mutant embryonic stem (ES) cells into embryoid bodies (EBs). Vasculogenesis and angiogenesis occur in successive steps during EB formation, and these can be easily followed by using immunohistochemical methods,\textsuperscript{18–21} making this in vitro model suitable for studies of targeted mutations in ES cells.\textsuperscript{19,22,23} In addition, pluripotent ES cells, when transplanted into an ectopic location in syngeneic or athymic host mice, can develop tumors of different tissue types called teratocarcinoma.\textsuperscript{24} We have used this in vivo differentiation model to examine Vezf1 gene function during tissue differentiation and tumor development. Our results show that Vezf1 is involved in multiple processes of vascular formation by regulating cell differentiation and proliferation, as well as extracellular matrix (ECM) distribution and deposition.

### Methods

#### ES Cell Culture, EB Differentiation, and Angiogenic Sprouting Assay

Wild-type (wt) R1 (Vezf1/+/+), Vezf1+/− (clone V2), and Vezf1−/− ES cells (clone F3)\textsuperscript{17} were maintained on γ-irradiated primary mouse embryo fibroblast feeder cells in high-glucose DMEM (Gibco-BRL) supplemented with 20% fetal bovine serum (Gemini), 0.1 mmol/L β-mercaptoethanol, 1 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 20 mmol/L HEPES and 1000 U/mL of leukemia inhibitory factor (LIF) (ESGRO; Chemicon). In vitro differentiation of ES cells into EBs was initiated using the hanging drop culture protocol.\textsuperscript{19} Briefly, drops containing ES cells (2500 cells in 30 μL) were spotted onto the lid of 150-mm dishes and incubated as hanging drops for 1 day in suspension culture on gelatin-coated tissue culture plates for spontaneous differentiation. Lymphatic endothelial cell differentiation was induced in EBs at day 5 or day 14 in suspension culture by adding 20 ng/mL recombinant human vascular endothelial growth factor (VEGF)-A165 (R&D Systems) and 200 ng/mL recombinant human VEGF-C (R&D Systems)\textsuperscript{20,21} and culturing for up to 21 days postinduction. At the indicated time points, EBs were collected and grown in suspension culture for 1 to 3 days after transduction. Alternatively, EBs grown for 2 days in hanging drops were cultured for 1 day in suspension culture and then transferred to 2D attachment cultures on gelatin-coated tissue culture plates for spontaneous differentiation. Lympathic endothelial cell differentiation was induced in EBs at day 5 or day 14 in suspension culture by adding 20 ng/mL recombinant human vascular endothelial growth factor (VEGF)-A165 (R&D Systems) and 200 ng/mL recombinant human VEGF-C (R&D Systems)\textsuperscript{20,21} and culturing for up to 21 days postinduction. At the indicated time points, EBs were collected, fixed in 4% parafomaldehyde, and embedded in OCT. Angiogenic sprouting assays were performed in semisolid 3D collagen gels.\textsuperscript{21} Briefly, EBs from day 12 suspension culture were harvested, embedded in type I collagen gel containing 20 ng/mL VEGF, and cultured in small droplets for 2 to 5 days. EBs were fixed in situ, transferred to glass slides, air-dried, and stored at −20°C for immunostaining.

#### Immunofluorescence and Immunohistochemistry

Protocols for immunofluorescence and immunohistochemistry were essentially as described.\textsuperscript{17} Primary antibodies used were rat anti-mouse PECAM-1/CD31 (1:200; Pharmingen), rat anti-mouse MECA-32 (1:15; Pharmingen), intercellular adhesion molecule-1 (ICAM-1) (1:100; Seikagaku Corp.), goat anti-mouse VE-cadherin (1:20; R&D Systems), rabbit anti-mouse collagen IV (1:200; Chemicon), rabbit anti-human caspase-3 (1:200; Cell Signaling Technology, Inc), rabbit Annexin V (1:200; Abcam), and rabbit anti-mouse LYVE1 (1:200; AngioBio). For each antibody, an IgG isotype antibody was used as a control. Secondary antibodies included tetramethylrhodamine B isothiocyanate–conjugated (TRITC) goat anti-rabbit IgG, F(ab’)_2, fluorescein isothiocyanate isothiocyanate–conjugated goat anti-rat IgG (1:100), Cy3-conjugated donkey anti-rabbit IgG (1:300), Cy3-conjugated goat anti-rabbit IgG (1:300), Cy5-conjugated donkey antigeno IgG (1:500), horseradish peroxidase–conjugated donkey anti-rabbit IgG (1:200), and horseradish peroxidase–conjugated donkey anti-rabbit IgG(H+L) (1:200). Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) using Vectashield mounting medium (Vector Laboratories). Images were taken under a Zeiss Axioscan 2 IE MOT microscope using an ORCA-ER-1394 digital camera.

#### Quantitative RT-PCR

Total RNA was extracted from ES cells following mouse embryonic fibroblast depletion and from day 5 EBs using TRIzol reagent (Invitrogen). cDNA was prepared from 1 μg of RNA using SuperScript III reverse transcriptase and random hexamers (Invitrogen) in a 40-μL reaction. A 1:20 aliquot of the cDNA reaction was used for real-time polymerase chain reaction in a 25-μL reaction using SYBR Green technology (Applied Biosystems). Gene expression was measured on a BioRad IQ5 Multicolor PCR Cycler (BioRad) using the primers listed below and was normalized using primers against mouse β-actin. To confirm specificity of the reaction, melting point analysis was incorporated at the end of the polymerase chain reaction (Table).

#### Protein Isolation and Western Blots

Conditioned medium was collected, filtered, and snap frozen from wt and Vezf1−/− ES cells cultured for 24 hours, and from EBs maintained in medium supplemented with 50 ng/mL VEGF-A and 100 ng/mL basic fibroblast growth factor (R&D Systems). Protein concentration was determined by using the BCA protein assay (Pierce). Fifty micrograms of protein from conditioned media was separated on 4% to 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman GmbH, Dassel) using the NUPAGE Electrophoresis and Transfer System (Invitrogen). Prestained low-range molecular weight standard (Invitrogen) and purified RBP4 (a kind gift from Dr William Blaner, Columbia University) were also included. Membranes were blocked in 2% skim milk in Tris-buffered saline (0.05% Tween 20; 1 hour at room temperature) and incubated overnight with rabbit polyclonal anti-RBP4 antibody (1:500; provided by Dr William Blaner, Columbia University). Secondary antibody used was horseradish peroxidase-

### Table. Primers for Quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP4</td>
<td>gacaaggctgtttcttg</td>
<td>aagaggctaccccgct</td>
<td>243</td>
</tr>
<tr>
<td>TTR</td>
<td>gcagaggtctccttgg</td>
<td>cccagcatgttgcagcttg</td>
<td>117</td>
</tr>
<tr>
<td>RAR-β</td>
<td>gctctgagatggcttgaagt</td>
<td>gtccttggatcttcgta</td>
<td>150</td>
</tr>
<tr>
<td>Col-Iv(a1)</td>
<td>ttcatatgtcagctgcttcttc</td>
<td>ttctcatgcacatgttgccagc</td>
<td>310</td>
</tr>
<tr>
<td>Col-Iv(a2)</td>
<td>ttggtgtagggaatagcagcag</td>
<td>aatgtgtggctcagaggt</td>
<td>232</td>
</tr>
<tr>
<td>b-Actin</td>
<td>gcggcccgctctgcaccaaa</td>
<td>cttcgtgatcgcacgcatttc</td>
<td>539</td>
</tr>
</tbody>
</table>
conjugated donkey anti-rabbit (1:5000, 1 hour at room temperature). The ECL Plus Western blotting reagent (Amersham Biosciences) was used to visualize RBP4 protein.

**Flow Cytometry**

At indicated time points, EBs and undifferentiated ES cells were dissociated to single-cell suspension using 0.2% collagenase A (Sigma). Dissociated ES cells were mouse embryoblast depleted by incubation on gelatin-coated dishes (0.7%; Sigma). ES cells and EB-derived cells were fixed in 2% paraformaldehyde and washed in staining buffer (0.01M Na3; in PBS). For 0.3% expression, cells were incubated with 1% saponin (Sigma) for 15 minutes before washing. Cells were incubated for Fc receptor blockage with rat anti-mouse CD16/CD32 (1 g/106 cells) and stained either alone with Alexa 647–conjugated rat anti-mouse Oct3/4 (1 g/106 cells; eBiosciences), Alexa 488–conjugated rat anti-mouse VE-cadherin (1 g/106 cells; eBiosciences), or in combination with phycoerythrin-Cy7 (PE-CY7)-conjugated rat anti-mouse PECAM-1/H11546 (0.5 g/106 cells; eBiosciences), Alexa 488–conjugated rat anti-mouse VE-cadherin (1 g/106 cells; eBiosciences), and PE-conjugated rat anti-mouse CD41 (1 g/106 cells; Becton Dickinson). Cells were washed, resuspended in PBS, and filtrated using cell-strainer caps. Unstained cells were used to set the cutoff for background fluorescence. Data acquisition was done using FACS-Canto (Becton Dickinson), and analysis was performed using FlowJo software.

**Generation of ES Cell Clones With LacZ Knock-In Into the Vezf1 Locus**

The original Vezf1 targeting construct contained a deletion of the transcription start site, resulting in a nonfunctional LacZ gene. A new construct, termed Vezf1lacZ, was generated, in which the transcriptional start site was restored and the IRES-łacZgt1.2neo cassette was replaced by an SDK-łacZ-PGKneo cassette. Xhol-linearized DNA was introduced into R1 ES cells by electroporation, and 6 correctly targeted clones were identified by Southern blot analysis as described. Expression of LacZ in ES cells derived from targeted ES cell clones was detected by using a β-galactosidase staining kit (ActiveMotif).

**Teratocarcinoma Formation**

A total of 1x10⁷ cells of clone F3 (Vezf1+/−) or clone V2 (Vezf1+/+) suspended in 150 μL of PBS was injected subcutaneously into the left flank of 6- to 8-week-old male nude mice. The right flank was injected with wt ES cells as a control. Two weeks after injection, animals were euthanized, and the teratocarcinomas were disseected from the subcutaneous fascial planes, weighed, and stained either alone with Alexa 647–conjugated rat anti-mouse CD41 (1 g/106 cells; Becton Dickinson), or wt for a targeted Vezf1-null allele. We have shown previously that Vezf1 mRNA is absent in Vezf1−/− ES cells and EBs and is reduced to about half in Vezf1+/− EBs. The in vitro differentiation of ES cells was initiated in hanging drop cultures, followed by growth either in suspension culture or in 2D attachment culture. We first assessed the ability of EB to attach to substrate and for outgrowth of differentiating cells. After overnight culture on gelatin-coated dishes, almost all EBs derived from Vezf1+/− and wt ES cells were attached and had formed a rim of spreading, differentiating cells, whereas about one third of Vezf1−/− EBs did not firmly attach overnight, or even within 6 days of culture (not shown). Furthermore, cellular outgrowth from those Vezf1−/− EBs that attached to the culture dishes was significantly delayed and slower, with diameters of attached Vezf1−/− EBs 50% reduced compared with Vezf1+/− and wt EBs (Figure 1A). Interestingly, when grown in suspension culture, mutant and wt EBs formed cystic structures; however, both Vezf1+/− and Vezf1−/− EBs grew significantly slower than wt EBs, and by day 6 they were about one third smaller compared with wt controls (Figure 1B).

**Vascular Structure Defects in Mutant Vezf1 EBs**

We next examined the differentiation of endothelial cells and formation of vascular structures in EBs. For this, EBs were grown in 2D attachment culture and double-stained at days 3, 6, 9, and 12 with antibodies specific for the mouse pan-endothelial marker PECAM-1/CD31 and the ECM protein collagen type IV. Differences between mutant and wt EBs became apparent at day 6 when endothelial cell cords were beginning to develop, and were most obvious at day 9 (shown in Figure 2A). Thus, wt (Vezf1+/+) EBs displayed cords with PECAM-1/collagen IV–positive endothelial cells that were organized into extensive vascular networks (Figure 2A, left panel). In contrast, Vezf1−/− EBs formed only short PECAM-1–positive cell cords that did not develop into a proper vascular network. Overall, vascular coverage, as measured by the area covered by PECAM-1–positive structures, was reduced from 14.5% in wt to 3.6% in Vezf1−/− EBs (Figure 2B). In addition, most of the PECAM-1 staining in Vezfl-null EBs was localized in patches of cells with undifferentiated morphology (Figure 2A, top right panel). High levels of collagen IV expression were visible on large sheets of undifferentiated cells (Figure 2A, bottom right panel) and generally did not colocalize with PECAM-1 expression (35% colocalization of PECAM-1 and collagen IV in Vezf1+/− EBs versus 98% in wt EBs; Figure 2C). No changes in vascular structure formation were detected when 2D cultures were extended to 12 days, indicating that the observed defects were not due to delayed vascular differentiation. Interestingly, Vezf1+/− EBs displayed an intermediate phenotype with shortened and thickened cell sheets that contained PECAM-1/collagen IV–positive cells; however, these had failed to form an extensive vascular network (Figure 2A, middle panel).

We also examined vascular differentiation in EBs grown for 3, 6, and 9 days in suspension culture (representative sections of day 9 cystic EBs are shown in Figure 2D). PECAM-1 and collagen IV in wt and Vezf1+/− EBs colocalized to endothelial cell cords that were mostly restricted to a region underneath an outer layer of tightly packed visceral endodermal cells (Figure 2D, left and middle panel). These vascular structures were well developed. In contrast, Vezf1−/− EBs contained a broad, presumably mesodermal layer that was filled with collagen IV–positive cells but devoid of PECAM-1–positive cells. PECAM-1–positive cells...
were restricted to the center of the EBs, but these failed to coalesce into cords (Figure 2D, right panel).

The expression patterns in wt and mutant EBs of endothelial ICAM-1 in 2D attachment culture (supplemental Figure I, available online at http://atvb.ahajournals.org) and of endothelial VE-cadherin (supplemental Figure II) and MECA-32 (not shown) in suspension culture, respectively, were similar to that observed for PECAM-1. Thus, we observed patches of endothelial cells that fail to properly form vascular cords. VE-cadherin staining partially overlapped with PECAM-1 in Vezf1-null EBs (supplemental Figure II, bottom row). Together, these results confirm that endothelial lineage determination and differentiation are unaffected but that vascular structure formation is defective in EBs derived from Vezf1−/− ES cells. To test for the possibility that apoptosis could be the cause of the observed vascular defects, EBs were grown for 9 days in 2D attachment culture and stained for PECAM-1 and caspase-3. However, no increase in the numbers of caspase-3-positive cells was detected within the PECAM-1-positive cell population or overall in the EBs (supplemental Figure III). Similarly, when sections of day 9 suspension culture EBs were stained for Annexin V, we observed no increase in apoptosis in PECAM-1-positive or -negative cells in mutant Vezf1 EBs (supplemental Figure IV).

Finally, we investigated the formation of lymphatic endothelial structures in EBs derived from wt and mutant Vezf1 ES cells. LYVE-1+/MECA-32− or LYVE-1+/PECAM-1+ cells became apparent in ∼80% of wt EBs at day 7 in suspension culture and were found up to day 26 in cord-like structures. Fewer LYVE-1-positive EBs were obtained from Vezf1+/− and Vezf1−/− ES cells and at later time points of suspension culture. No obvious differences in the morphology of the putative lymphatic structures were found (data not shown).

Suppression of Retinol Metabolism in Vezf1−/− EBs

A recent microarray screen for Vezf1 target genes in embryonic day 9.5 embryos identified several candidate genes involved in retinol metabolism (S. Chitnis, H. Stuhlmann, unpublished data, 2010). Specifically, expression of retinol binding protein 4 (Rbp4), a retinol/vitamin A carrier in the plasma, and transthyretin (Ttr), which binds to RBP4 and prevents it from renal clearance,27,28 was significantly decreased in Vezf1−/− embryos. Intriguingly, several studies reported that all-transretinoic acid inhibits collagen type IV expression in vitro in human and rat cells.29–31 To investigate whether expression of key retinol pathways genes and collagen IV are similarly affected during in vitro differentiation in mutant EBs, we performed quantitative RT-PCR on ES cells and day 5 EB. Expression of Rbp4, Ttr and the retinoic acid target gene RAR-β32,33 was significantly reduced in Vezf1−/− day 5 EBs but not in undifferentiated ES cells (Figure 2E, left panel). The levels of secreted RBP4 protein were also decreased in supernatant medium harvested from Vezf1−/− EBs, and to a lesser extent in ES cells, compared with wt controls (Figure 2F). Consistent with the observed strong and aberrant immunostaining (Figure 2A and D), increased expression of α1 and α2 chains of collagen type IV was detected in Vezf1−/− EBs (Figure 2E, right panel). Together, these results support our model (Figure 2G) that Vezf1 activates the retinol/vitamin A pathway, either directly by transactivation of Rbp4 and Ttr or indirectly. Deregulation of retinol/vitamin A signaling in Vezf1-null EBs results in elevated collagen IV expression and accumulation (Figure 2G).

Sprouting Angiogenesis Is Impaired in Vezf1−/− EBs

When ES-derived EBs are embedded in a 3-dimensional matrix of type I collagen in the presence of angiogenic factors, sprout-
Figure 2. Aberrant vascular structure formation and collagen IV deposition in mutant EBs. A, EBs in 2D culture. Shown are representative images of day 9 EBs derived from wt (Vezf1+/+), Vezf1+/−, and Vezf1−/− ES after fixation and immunostaining for PECAM-1 and type IV collagen. Cell nuclei were counterstained with DAPI. The bottom row depicts merged images from PECAM-1, collagen IV, and DAPI staining. Note the extensive vascular network in wt EBs. In contrast, vascular structures did not form properly in Vezf1−/− EBs. Vezf1+/− EBs displayed an intermediate phenotype. Images were taken under a Zeiss Axioplan 2 IE MOT using a 20 objective. B, Quantitative analysis of vascular coverage. The vasculature shown by PECAM-1 staining was analyzed for area density and expressed as percentage of the PECAM-1 area. At least 3 independent measurements were performed in each group. Comparison of Vezf1−/− versus Vezf1+/− and Vezf1+/+ groups were performed by the Student t test; **P<0.01. C, Quantitative analysis of coexpression of PECAM-1 and collagen IV. The percentage of collagen IV areas that was adjacent to or overlapped with PECAM-1+ areas. Comparisons between 2 groups were done using the Student t test. **P<0.01, Vezf1−/− versus Vezf1+/− and Vezf1+/+; #P<0.01, Vezf1−/− versus Vezf1+/−. D, Suspension culture EBs. Sections of EBs grown for 9 days in suspension culture were immunostained for PECAM-1, collagen IV and DAPI staining. Well-developed PECAM-1+ positive vascular structures were visible in wt and, to a lesser degree, in Vezf1+/− EBs. In Vezf1−/− EBs, PECAM-1+ positive sheets of cells were visible in the center, but these failed to form cell cords and they did not colocalize with collagen IV. E, Expression of retinol pathway genes Rbp4, Ttr, and RAR-β, and collagen 4α1 and 4α2 in ES cells and EBs. Gene expression levels were determined by quantitative RT-PCR and normalized to β-actin. Shown are changes in expression in Vezf1−/− versus wt ES cells and day 5 EBs. Comparisons between wt and Vezf1−/− ES cells and EBs were performed by the Student t test (n=3 for each genotype). F, Detection of secreted RBP4 protein by Western blot analysis. Shown is a representative Western blot using conditioned media (50 μg of protein) from wt and Vezf1−/− ES cells and day 5 EBs. Fifty nanograms of purified 23-kDa RBP4 protein was loaded as a control. RBP4 levels are reduced in Vezf1−/− EBs and, to a lesser extent, in Vezf1+/− ES cells. G, Model proposing how Vezf1 regulates the retinol/vitamin A pathway. Left: Vezf1 activates expression of the all-trans retinol carrier Rbp4 and transthyretin, directly or indirectly. TTR binds to RBP4:all-trans retinol, preventing its renal clearance and resulting in increased cellular uptake. Retinol is converted to retinal and retinoic acid, which directly transactivates expression of its receptor RAR-β and indirectly represses that of collagen IV. Right: Loss of Vezf1 function in EBs results in deregulation of the retinol/vitamin A pathways, with reduced expression of Rbp4, Ttr, and RAR-β and accumulation of collagen IV.
ing outgrowth of endothelial cells from the initial cord-like vascular structures in EBs can be observed that closely mimics sprouting angiogenesis in the developing embryo.\textsuperscript{34,35} The 3-dimensional system is a powerful tool to study this angiogenesis process on a cellular and molecular level. To examine the ability of endothelial cells derived from Vezf1-null ES cells to undergo sprouting angiogenesis, EBs were grown in suspension culture for 12 days and subsequently embedded in collagen type I gels in the presence of the proangiogenic growth factor VEGF. Generally, initial spouting was detected after 24 hours in wt and Vezf1+/− EBs in the collagen matrix. The sprouts continued to grow in number and length, and after 3 to 4 days the primary sprouts began to interconnect and form secondary sprouts. Coimmunostaining for PECAM-1 and collagen IV of day 5 cultures indicated that the sprouts contained endothelial cells (Figure 3A, left and middle columns, and Figure 3B). In contrast, no or few sprouts were visible after 24 hours in collagen-embedded Vezf1−/− EBs. At day 5 in collagen matrix, Vezf1−/− EBs had significantly fewer primary sprouts and rarely formed secondary sprouts (Figure 3A, right column, and Figure 3B). In conclusion, sprouting angiogenesis of mutant ES cell–derived endothelial cells was severely impaired.

**Quantitative Analysis of Undifferentiated, Endothelial, Hematopoietic Cell Populations**

We further examined the differentiation potential of wt and Vezf1 mutant ES cells along the endothelial and hematopoietic lineages by using flow cytometry. Cell suspensions of wt, Vezf1+/−, and Vezf1−/− ES cells and of day 9 EBs were analyzed for the presence of undifferentiated cells (Oct3/4-positive fraction), endothelial cell lineage (PECAM-1-positive and VE-cadherin-positive fractions), and hematopoietic cell lineage (CD41 fraction) (Figure 4). wt and Vezf1 mutant ES cells were able to differentiate at comparable levels, as judged by a similar decrease in Oct-3/4-positive cells (6- to 8-fold decrease during 9 days of in vitro differentiation; Figure 4, upper left panels). Importantly, we did not detect a significant difference in endothelial marker gene expression between wt and mutant cells, as shown by the percentage of PECAM-1-positive and VE-cadherin-positive cells before and after differentiation (Figure 4, bottom panels). Thus, loss of function of Vezf1 does not appear to block endothelial lineage determination and differentiation. We noted a 1.6-fold increase in VE-cadherin-positive cells with all 3 genotypes. We also observed an overall 3- to 4-fold decrease in PECAM-1-positive cells on differentiation; the large fraction of PECAM-1-positive ES cells is consistent with its previously reported expression in the inner cell mass of blastocysts.\textsuperscript{36} Unexpectedly, significantly lower numbers of CD41-positive hematopoietic cells were detected on differentiation in Vezf1 mutant EBs as compared with wt EBs. Thus, whereas the fraction of CD41-positive cells increased during wt EB differentiation by 19-fold, and during Vezf1+/− EB differentiation by 4-fold, no significant change was found during differentiation of Vezf1−/− EBs (0.75% versus 0.85% CD41+ cells; Figure 4, upper right panel). To determine which specific hematopoietic lineages are affected, hematopoietic progenitor assays\textsuperscript{37,38} were performed (supplemental Figure V). Day 6 EBs derived from wt and Vezf1+/− ES cells gave rise to all types of hematopoietic progenitors with similar efficiencies, including primitive erythroid, definitive erythroid, macrophage, bipotential definitive erythroid/macrophage, and multipotential precursor colonies. In contrast, greatly reduced numbers of primitive erythroid and bipotential colonies, and no definitive erythroid, macrophage, and multipotential progenitor colonies were obtained from Vezf1−/− day 6 EBs. Together, these results indicate that Vezf1-null ES cells differentiate efficiently in vitro into PECAM-1-positive and VE-cadherin-positive endothelial cells but that differentiation into cells of the hematopoietic lineages is compromised.

**Aberrant Cell Differentiation in Teratocarcinomas Derived From Vezf1-Null ES Cells**

To study the differentiation of mutant Vezf1 ES cells in vivo, Vezf1−/− or Vezf1+/− ES cells were transplanted subcutaneously into one flank of athymic nude mice, and wt ES cells were injected as control into the opposite flank. Two weeks later, teratocarcinomas were dissected, weighed, and fixed. The growth of Vezf1−/− tumors and, to a lesser extent, of Vezf1+/− tumors was significantly reduced compared with wt controls.
H&E staining of tumor sections revealed that both mutant and wt-derived teratocarcinomas contained a spectrum of somatic tissues derived from ecto-, endo-, and mesoderm; these included epithelium, ciliatum, cartilage, bone, muscle cells, adipose tissue, neural tissue, and connective tissue (Figure 5B). Overall, neural tissue was prominent in the tumors and consisted of neural rosettes and tubes, and central nervous system–like tissue. In addition, an abundance of columnar epithelial cells lining cystic spaces formed glands and solid nests (Figure 5B). Although Vezf1−/− ES cells were able to differentiate into every cell type found in the control teratocarcinomas, we detected notable differences. First, neural tube structures in Vezf1−/− teratocarcinomas were typically less differentiated (Figure 5C), whereas wt tumors contained neural tissue of the more differentiated central nervous system–like phenotype. A second pathological feature was the large number of ECM-rich foci in connective tissue present in Vezf1−/− tumors, some of which formed bone-like structures (Figure 5C). In contrast, wt tumors contained overall more gland-like structures with well-defined spaces that were surrounded by columnar epithelium, similar to those shown in the far right panel of Figure 5B.

Figure 4. Analysis of Oct3/4, CD41, PECAM-1, and VE-cadherin gene expression in undifferentiated ES cells and differentiated cells from day 9 EBs. wt, Vezf1+/−, and Vezf1−/− ES cells (10⁶ cells each) and dissociated cells from day 9 suspension culture EBs (550, 880, and 3300 EBs from wt, Vezf1+/−, and Vezf1−/− EBs, respectively) were analyzed by flow cytometry for the expression of Oct3/4, CD41, PECAM-1, and VE-cadherin. The gates correspond to Oct3/4−, CD41−, PECAM-1−, or VE-cadherin− populations. The histograms depict the number of day 9 EB-derived cell population (black), ES cell population (gray), positive cells from day 9 EB cell population (green), and positive cells from the ES cell population (red). SSC indicates side scatter.
Reduced Blood Vessel Formation in Teratocarcinomas

We generated ES cell clones with a LacZ knock-in into the endogenous Vezf1 locus (Vezf1lacZ+/+) (supplemental Figure VI). This approach allowed us to visualize Vezf1 gene expression during in vivo and in vitro differentiation. β-Galactosidase staining of Vezf1lacZ/+ EBs grown in 2D attachment culture revealed that Vezf1 expression was widespread but not ubiquitous. Before day 7, LacZ expression was highest in the center of attached EBs in presumably mesodermal cells. Beginning at day 8 in culture, localized and elevated expression became apparent in cell clusters and tubes similar in appearance and shape to PECAM-1-positive structures, suggesting that these correspond to areas of early endothelial differentiation (supplemental Figure VI, bottom).

Teratocarcinomas derived from Vezf1−/− ES cells were smaller, and they contained fewer and less organized vascular structures that contained with PECAM-1 and collagen IV compared with wt or Vezf1+/− tumors (Figure 6 and not shown). In addition, vascular structures in mutant teratocarcinomas rarely displayed ZO-1-positive cells (not shown). This prompted us to investigate whether blood vessels in teratocarcinomas are derived from the host, from differentiating ES cells, or both. For this, we injected Vezf1lacZ/+ ES cells into nude mice, allowing us to visualize ES cell–derived cells in the blood vessels. Sections from the resulting tumors were examined for colocalization of PECAM-1 and collagen IV immunostaining with β-galactosidase staining. The majority of PECAM-1-positive small vessels and the surrounding tissue were negative for LacZ (Figure 5D, upper panel) and thus were presumably host-derived. However, a small portion of PECAM-1-positive cells within the vascular structures showed β-galactosidase activity, and these cells were interspersed with apparently host-derived endothelial cells within the same vessel (Figure 5D, arrows in lower panel). Thus, our results suggest that Vezf1-null ES cells are capable to differentiate into endothelial cells and to contribute to the formation of vessels within the teratocarcinomas.

Strikingly, distinct areas in Vezf1−/− ES cell–derived teratocarcinomas displayed low cellularity and high ECM content, as evidenced by H&E staining (Figure 5C, Figure 6, far right Vezf1−/− panel). In consecutive sections, these regions showed high levels of collagen IV deposition but few PECAM-1-positive cells, indicating rudiment and disorganized vascular structures (Figure 6). In contrast, wt teratocarcinomas contained well-organized vascular structures with PECAM-1 and collagen IV colocalization in ECM-rich regions (Figure 6, Vezf1+/+) panels). These areas were similar in their morphology to meso-
Figure 6. Vascular structure defects in Vezf1−/− teratocarcinomas. Representative sections from 2 wt and 2 contralateral Vezf1−/− teratocarcinomas are shown. Sections were immunostained for PECAM-1 and collagen IV, and cell nuclei were counterstained with DAPI. Merged images are from single PECAM-1, collagen IV and DAPI images. Bottom row: close-by H&E-stained section. Note the absence of well-formed vascular structures around glandular epithelial structures in Vezf1−/− but not wt teratocarcinomas (left 2 columns). Mesenchyme-rich areas in wt teratocarcinomas contained PECAM-1, collagen IV–positive vessels. In contrast, Vezf1−/− teratocarcinomas contained large mesenchymal foci with high collagen IV deposition and much lower PECAM-1 expression. These areas did not contain recognizable vascular structures (right 2 columns).

**Discussion**

Blood vessel development during mammalian embryogenesis is a finely tuned process that is controlled by a wide variety of genes. We have recently reported on the crucial and dose-dependent role of the zinc finger transcription factor gene Vezf1 during vascular system development. Although Vezf1 is expressed in the endothelium of the developing vasculature and in mesodermal and neuronal cells, the primary defect in Vezf1-null embryos appears to have its origin in the endothelium. Thus, ultrastructural and immunohistochemical studies demonstrated loss of vascular wall integrity concomitant with defects in endothelial cell junctions and the ECM as the underlying cause of hemorrhaging and embryonic death. In this study, we investigated in greater detail the involvement of Vezf1 in various processes of angiogenesis. We used ES cells that are heterozygous or homozygous for a targeted Vezf1 allele and induced their differentiation in vitro into EBs and in vivo during teratocarcinoma formation. EB formation recapitulates the key developmental processes during perimplantation and early postimplantation development; these include vascular lineage differentiation and the formation of a network of endothelial cell cords and tubes. The EB system is particularly useful to study loss-of-function mutations when the phenotype is embryonic lethal, as is the case with Vezf1-null embryos.

Our results add new insights into the role of Vezf1 during vascular development. We observed 3 major phenotypes in EBs derived from mutant ES cells: delay in growth and attachment, defects in the formation of vascular structures, and extensive and abnormal collagen IV deposition. Importantly, these phenotypes were recapitulated when mutant ES cells were induced to form teratocarcinomas. In contrast to wt EBs that formed a well-organized vascular network surrounded by a defined collagen IV–positive ECM, Vezf1−/− EBs contained mostly dispersed endothelial cells that failed to form a recognizable network. We also observed reduced angiogenic sprouting and branching in mutant EBs. It is unlikely that these defects are solely a consequence of the slower growth rate of the mutant EBs because overall differentiation was not affected, whereas vascular cord formation did not catch up on extended periods of culture. Rather, it is likely that the defects are the result of differences in endothelial cell polarization, migration, or alignment. Of interest, intermediate phenotypes were observed in mutant and wt EBs. Because Vezf1 mRNA levels are reduced to half in Vezf1-null embryos. Because Vezf1 mRNA levels are reduced to half in heterozygous EBs, these results provide further support for the notion that Vezf1 acts in a strictly dose-dependent fashion.

Loss of Vezf1 gene function resulted in the abnormal distribution and deposition of collagen IV. Loss of collagen IV colocalization with PECAM-1-positive endothelial cells is consistent with the reduced levels of collagen deposition found around capillaries of Vezf1-null embryos. Collagen IV is a main component of the vascular basement membrane, a specialized ECM on the basal side of endothelial cells, and connective tissue, where the majority of blood vessels are normally localized. The area density of blood vessels in wt and Vezf1−/− ES-derived teratocarcinomas was 21.59±2.54% and 6.67±4.50%, respectively (P<0.01). Because the majority of the vessels are host-derived, indirect, nonendothelial effects may contribute to the reduced vascularity. The results suggest that the cellular microenvironment inside the teratocarcinomas may play an additional role in the formation of the tumor vasculature.
which is important for endothelial differentiation and proliferation and for providing mechanical support.\textsuperscript{42,43} Mice with a targeted inactivation of the Col4α1/2 locus die between embryonic days 10.5 and 11.5 because of structural failure of the basement membrane.\textsuperscript{44} We propose that loss of \textit{Vezf1} causes aberrant accumulation on nonendothelial cells and incomplete deposition of collagen IV on endothelial cells, resulting in the destabilization of the endothelial basement membrane and the emerging vascular structures, vascular remodeling defects, and the loss of vascular integrity. Importantly, our studies have uncovered retinol/vitamin A metabolism as a potential molecular pathway linking \textit{Vezf1} function to collagen IV expression and its distribution in the ECM (model in Figure 2G). Consistent with this model, vitamin A deficiency in rats has been shown to increase the expression of collagen type IV.\textsuperscript{45} We propose that \textit{Vezf1} regulates the retinol/vitamin A pathway by activating expression of Rbp4 and Ttr. Regulation of Rbp4 and Ttr by \textit{Vezf1} could be achieved either through direct transcriptional transactivation or indirectly via the transcription factor HNF4α.\textsuperscript{46}

Previous studies using \textit{Vezf1} antisense RNA resulted in reduced numbers of endothelial cells in G2/M and an increase in apoptosis.\textsuperscript{14} In contrast, we did not observe increased apoptosis in \textit{Vezf1}/-- EBs, and therefore apoptosis can be excluded as a cause of the observed angiogenesis defects. Certain metabolic products of collagen IV, including tumstatin\textsuperscript{47,48} and canstatin,\textsuperscript{49} inhibit angiogenesis by inducing apoptosis. The accumulation of collagen IV in \textit{Vezf1}/-- EBs and teratocarcinomas possibly may be explained by increased local expression or low metabolic turnover, which in turn may explain the low number of apoptotic cells.

Unexpectedly, we uncovered striking defects during in vitro differentiation of \textit{Vezf1}-null ES cells along the definitive hematopoietic lineages; in addition, differentiation into primitive erythrocytes was significantly reduced. It should be noted that in \textit{Vezf1}-null embryos formation of primitive erythrocytes and megakaryocytes appeared to be normal.\textsuperscript{17} However, lethality of homozygous mutant embryos before the onset of definitive hematopoiesis precluded the analysis of potential differentiation defects in vivo.

Histological analyses of teratocarcinomas revealed that loss of \textit{Vezf1} does not result in a general block of cell lineage differentiation in these tumors; however, it does affect the tumorigenic ability of ES cells in vivo. Thus, \textit{Vezf1}/---derived tumors were significantly smaller and displayed an overall less differentiated phenotype, including an increase in neural tube-like structures, and reduced formation of epithelia with well-defined lumen. It is important to note that significant fewer PECAM-1-positive vascular structures were detected in \textit{Vezf1}/-- teratocarcinomas around and within differentiated tissue structures compared with wt controls. Lower vascularity may lead to reduced supply of the tumors with nutrients and oxygen, likely resulting in reduced tumor growth. Interestingly, we found that the majority of vascular structures within the tumors were host-derived, whereas ES cell-derived endothelial cells contributed in a mosaic fashion to the vascular cords. Thus, the lower vascular content cannot be attributed solely to reduced differentiation or proliferation of mutant ES cell-derived endothelial structures; rather, differences in angiogenic factors that are present in the tumor microenvironments may play a role as well. It is possible that loss of function of \textit{Vezf1} in nonendothelial cells contributes to the observed phenotypes observed in EBs and teratocarcinomas. However, experimental evidence from the \textit{Vezf1}-null mice suggested that endothelial cells are the origin of the primary defects. Results presented here substantiate this notion further: our findings indicate that overall differentiation of ES cells, as judged by cavitation and appearance of cells of the endothelial lineage, is very similar in wt and mutant EBs.

In conclusion, our studies using the EB and teratocarcinoma model provide new insights into the role of \textit{Vezf1} in the vascular system. Our data further support our hypothesis that \textit{Vezf1} is a transcription factor that controls the formation of a functional 3-dimensional vascular network in a strictly dosage-dependent manner. Importantly, they suggest a direct involvement of \textit{Vezf1} in regulating endothelial differentiation, proliferation, and ECM distribution and deposition, with collagen IV being an important mediator. A complete understanding of how \textit{Vezf1} exerts its role on a molecular level is still lacking. Several potential downstream target genes have been identified in vitro; these include endothelin-\textit{1,15,16} and stathmin/OP18.\textsuperscript{13} We have recently performed a microarray screen using RNA from \textit{Vezf1}/-- embryos and identified a small number of potential \textit{Vezf1} target genes; these include members of the retinoic acid metabolism pathway, members of the Apoa/b family of lipid transport proteins, fibrinogens, and members of the serpin family of proteases (S. Chitnis, H. Stuhlmann, unpublished data, 2010). In addition, increasing evidence suggests that \textit{Vezf1} function is modulated by protein-protein interactions with components of the Rho GTPase machinery, including RhoB and p68RacGAP.\textsuperscript{14,16} Finally, an intriguing new role for \textit{Vezf1} in epigenetic regulation of gene expression has recently been uncovered.\textsuperscript{50} \textit{Vezf1} loss of function in ES cells results in downregulation of the de novo methyltransferase Dnmt3b and affects widespread loss of DNA methylation of genomic DNA. Results from the present study suggest that the EB system will be a powerful in vitro model that will allow studying genetic and epigenetic regulation of vascular system development in a quantitative fashion.

Acknowledgments
We thank Derek Lemons (University of California, San Diego) for generating the \textit{Vezf1}\textsuperscript{lacZ+} targeting vector, W. Blaner (Columbia University) for providing RBP4 antibody and purified RBP4 protein, G. Keller and M. Kennedy (McEwen Centre for Regenerative Medicine) for advice on the hematopoietic progenitor assay, Wendy LeVine for expert technical assistance with histology, and James Mtui for assistance with differentiating ES cells into lymphatic endothelial cells.

Sources of Funding
This work was supported by National Institutes of Health grants R01-HL65738 and R01-HL68648 (to H.S.) and a postdoctoral fellowship from the Swedish Research Council (to P.A.O.).

Disclosures
None.

References
Targeted Vezf1-Null Mutation Impairs Vascular Structure Formation During Embryonic Stem Cell Differentiation
Zhongmin Zou, Pauline A. Ocaya, Huiqin Sun, Frank Kuhnert and Heidi Stuhlmann

Arterioscler Thromb Vasc Biol. 2010;30:1378-1388; originally published online April 29, 2010; doi: 10.1161/ATVBAHA.109.200428
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/30/7/1378

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2010/04/29/ATVBAHA.109.200428.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Methods.

**Hematopoietic precursor colony assay.** EBs and blast colonies were prepared essentially as described \(^{40}\). To generate EBs, ES cells were trypsinized into a single-cell suspension and plated at 1,000 cells/ml into differentiation medium containing IMDM supplemented with 15% FCS, 2 mM L-glutamine (Life Technologies), 0.5 mM ascorbic acid (Sigma), and \(4.5 \times 10^{-4}\) M MTG in 60 mm Petri-grade dishes. After 6 days in suspension culture, dissociated cells from EBs were plated in 1% methylcellulose containing 10% plasma-derived serum (PDS; Antech), 5% protein-free hybridoma medium (PFHM-II; Life Technologies) plus the following cytokines: c-kit ligand (KL; 1% conditioned medium), IL-11 (25 ng/ml), IL-3 (1% conditioned medium), GM-CSF (3 ng/ml), G-CSF (30 ng/ml), M-CSF (5 ng/ml) and IL-6 (5 ng/ml). Primitive erythroid colonies were scored at days-5 to -6 of culture, whereas definitive erythroid (BFU-E), macrophage, mast cell, granulocyte/macrophage and mixed colonies were counted at days-7 to -10 of culture.

**Supplemental Figure Legends.**

**Supplemental Figure I.** ICAM-1 expression in EBs grown in 2D culture. Attachment cultures of representative day-9 EBs were fixed and stained for ICAM-1. Nuclei were counterstained with DAPI. In *Vezf1*\(^{+/+}\) EBs, ICAM-1 positive cells formed a well-organized network. In contrast, *Vezf1*\(^{-/-}\) EBs showed ICAM-1 staining in cell clumps that failed to form vascular-like structures. *Vezf1*\(^{+/}\) EBs showed an intermediate phenotype. Images were taken using a 20X objective.

**Supplemental Figure II.** VE-Cadherin expression in EBs grown in suspension culture. Embryoid bodies derived from *Vezf1* null ES cells show aberrant expression of VE-Cadherin. EBs maintained in suspension culture for 9 days were sectioned and stained for PECAM-1 and
VE-Cadherin. Expression of VE-Cadherin was observed in EBs derived from wt, Vezf1+/− and Vezf1−/− ES cells. Co-localization of VE-Cadherin with PECAM-1 was observed in vascular structures of wt EBs and the majority of Vezf1+/− EBs. In contrast, in Vezf1−/− EBs VE-Cadherin and PECAM-1 co-localization was observed only at very low frequency (bottom panels). Nuclei were counterstained with DAPI. Images were taken using a 20X objective.

Supplemental Figure III. Attached embryoid bodies derived from Vezf1 null ES cells do not show increased apoptosis. Shown are representative images of day-9 EBs derived from wt (+/+), Vezf1+/-, and Vezf1−/− ES after fixation and immunostaining for PECAM-1 and Caspase-3. Cell nuclei were counter-stained with DAPI. Bottom row depicts merged images from PECAM-1, Caspase-3 and DAPI staining. Note the extensive vascular network in wt EBs, and the loosely aggregated vascular structures in Vezf1−/− EBs. No increase in apoptotic nuclei (red) is visible. Images were taken using a 20X objective.

Supplemental Figure IV. Suspension culture EBs derived from Vezf1 null ES cells do not show increased apoptosis. EBs maintained in suspension culture for 9 days were sectioned and stained for Annexin V and PECAM-1. Bottom row shows merged images of Annexin V, PECAM-1 and DAPI counter-staining. Annexin V-positive cells were less frequent in Vezf1−/− derived EBs compared to wt and Vezf1+/- EBs. Images were taken using a 20X objective.

Supplemental Figure V. Hematopoietic progenitor assay. Day-6 EBs were analyzed for the potential to give rise to hematopoietic precursor colonies. Error bars indicate standard deviations from three independent experiments, performed in triplicates. Ery/p, primitive erythroid; Ery/d, definitive erythroid; Mac, macrophage; M/E, bipotential E/d/Mac; Mix, mixed colonies.
Supplemental Figure VI. Generation of Vezf1lacZ/+ ES cells and β-galactosidase staining in EBs.

Top: Schematic map of the endogenous Vezf1 locus and the targeting vector. Sequences in exon 1 immediately downstream of the Vezf1 transcription start and part of intron 1 were replaced by a SDK-lacZ–PGKneo expression cassette with a Kozak-ATG sequence, placing the LacZ gene under transcriptional control of the Vezf1 promoter. The position of 5’ and 3’ flanking probes for Southern blot analysis are shown.

Bottom: Southern blot analysis of BamHI digested genomic DNA. Wt ES cells showed a specific 16-kb band, whereas heterozygous Vezf1 clone showed an additional 9-kb band (left panel). Representative EBs from day-9 attachment cultures were stained for β-galactosidase (middle panel), and immunostained using PECAM-1 antibodies (right panel). *Apparent vascular cord structures.
Zou et al.
Suppl. Figure II

PECAM

VE-Cad

DAPI

Merged

Vezf1+/+

Vezf1+/-

Vezf1-/-
<table>
<thead>
<tr>
<th>Gene</th>
<th>+/+</th>
<th>+/-</th>
<th>-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>PECAM</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Caspase3</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>DAPI</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>Merged</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>Vezf1+/+</td>
<td>Vezf1+-</td>
<td>Vezf1-/-</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>---------</td>
<td>----------</td>
</tr>
<tr>
<td><strong>AnnexinV</strong></td>
<td><img src="image" alt="AnnexinV" /></td>
<td><img src="image" alt="AnnexinV" /></td>
<td><img src="image" alt="AnnexinV" /></td>
</tr>
<tr>
<td><strong>PECAM</strong></td>
<td><img src="image" alt="PECAM" /></td>
<td><img src="image" alt="PECAM" /></td>
<td><img src="image" alt="PECAM" /></td>
</tr>
<tr>
<td><strong>DAPI</strong></td>
<td><img src="image" alt="DAPI" /></td>
<td><img src="image" alt="DAPI" /></td>
<td><img src="image" alt="DAPI" /></td>
</tr>
<tr>
<td><strong>Merged</strong></td>
<td><img src="image" alt="Merged" /></td>
<td><img src="image" alt="Merged" /></td>
<td><img src="image" alt="Merged" /></td>
</tr>
</tbody>
</table>
Suppl. Figure V

The figure shows a bar chart with data on colonies per 100,000 cells for different conditions labeled M/E, Mac, Ery/p, Ery/d, and Mix. The chart includes three categories representing wt, het, and KO.