The transcriptional programs that regulate blood vessel formation are largely unknown. In this paper, we examine the role of the zinc finger transcription factor LKLF in murine blood vessel morphogenesis and homeostasis. By in situ hybridization and immunohistochemistry, we show that LKLF is expressed as early as embryonic day 9.5 (E9.5) in vascular endothelial cells throughout the developing mouse embryo. To better understand the function of LKLF, we used homologous recombination in embryonic stem (ES) cells to generate LKLF-deficient (LKLF−/−) mice. Both angiogenesis and vasculogenesis were normal in the LKLF−/− mice. However, LKLF−/− embryos died between E12.5 and E14.5 from severe intra-embryonic and intra-amniotic hemorrhaging. This bleeding disorder was associated with specific defects in blood vessel morphology. Umbilical veins and arteries in the LKLF−/− embryos displayed an abnormally thin tunica media and aneurysmal dilatation before rupturing into the amniotic cavity. Similarly, vascular smooth muscle cells in the aortae from the LKLF−/− animals displayed a cuboidal morphology and failed to organize into a compact tunica media. Consistent with these findings, electron microscopic analyses demonstrated endothelial cell necrosis, significant reductions in the number of vessel-wall pericytes and differentiating smooth muscle cells, and decreased deposition of extracellular matrix in the LKLF−/− vessels. Despite these defects, in situ hybridization demonstrated normal expression of platelet-derived growth factor B, Tie1, Tie2, transforming growth factor β, and heparin-binding epidermal growth factor in the vasculature of the LKLF−/− embryos. Therefore, LKLF defines a novel transcriptional pathway in which endothelial cells regulate the assembly of the vascular tunica media and concomitant vessel wall stabilization during mammalian embryogenesis.

[Key Words: LKLF; gene targeting; vascular endothelial cells; hemorrhage]
giopoietin-1 and its receptor tyrosine kinase, Tie2, have been shown to be required for the processes of embryonic angiogenesis and vascular remodeling (Dumont et al. 1994; Sato et al. 1995; Davis et al. 1996; Suri et al. 1996). Both angiopoietin-1- and Tie2-deficient mice display normal vasculogenesis but die between E10.5 and E12.5 and fail to form a branched capillary network. They also display severe defects in endocardial development and myocardial trabeculation. The final stage of vascular development involves the assembly and stabilization of the vessel wall. This process is mediated by growth factors such as platelet-derived growth factor B (PDGF-B) and heparin-binding epidermal growth factor (HB-EGF) produced by endothelial cells as well as by transforming growth factor-β (TGF-β), which is activated by interactions between endothelial and mesenchymal cells (Leveen et al. 1994; Kume and Gimbrone 1994; Dickson et al. 1995). Despite this recent progress in identifying the soluble growth factors and cell-surface receptors that regulate blood vessel formation, much less is known currently about the nuclear transcriptional pathways that are regulated by these extracellular signals, and which, in turn, control endothelial cell differentiation and the migration and subsequent assembly and stabilization of the vessel wall.

The erythroid Krüppel-like factor (EKLF) was first identified as an erythroid lineage-specific zinc finger transcription factor that binds to a CACCC sequence motif in the promoter of the β-globin gene (Miller and Bieker 1993; Bieker and Southwood 1995). Gene targeting experiments demonstrated that EKLF is required for the switching from γ- to β-globin during the terminal stages of erythroid maturation (Nuez et al. 1995; Perkins et al. 1995). More recently, three related Krüppel-like zinc finger proteins, BKLF (Crossley et al. 1996), GKLF (Shields et al. 1996), and LKLF (Anderson et al. 1995), were identified simultaneously by several groups. All of these Krüppel-like factors share extensive sequence homologies within their carboxy-terminal zinc finger DNA-binding domains. The non-DNA binding domains of each of the proteins, however, are highly divergent. Unlike EKLF, BKLF is expressed widely in both the developing embryo and the adult mouse (Crossley et al. 1996). In contrast, GKLF and LKLF each display a more restricted pattern of expression, with the highest levels of adult expression seen in gut and lung, respectively (Anderson et al. 1995; Shields et al. 1996).

In the studies described in this paper, we examine the role of LKLF in murine blood vessel formation. We demonstrate that LKLF is expressed in vascular endothelial cells during early mouse embryogenesis. To better understand the role of LKLF in blood vessel formation and function in vivo, we used homologous recombination in murine embryonic stem cells to generate LKLF-deficient (LKLF−/−) mice. These mice demonstrate normal vasculogenesis, angiogenesis, and cardiac development. They do, however, display a specific defect in a later stage of blood vessel formation—the recruitment of pericytes and VSMCs to form the normal vascular tunica media. As a result, they have compromised blood vessel integrity and die from intra-embryonic and intra-amniotic hemorrhaging between E12.5 and E14.5. Therefore, LKLF defines a novel transcriptional pathway that regulates blood vessel assembly and homeostasis during mammalian embryogenesis.

Results

LKLF is expressed in embryonic vascular endothelial cells

We used in situ hybridization to examine LKLF expression during mouse embryogenesis. Beginning at E9.5, LKLF is expressed in the developing vasculature throughout the embryo (Fig. 1A,B). At E12.5, LKLF continued to be expressed at high levels in the blood vessels, especially the umbilical arteries and veins (Fig. 1C,D) and was also expressed in the primitive vertebrae. The

![Image of Figure 1](image-url)

**Figure 1.** Embryonic expression of LKLF. In situ hybridization analyses of E9.5 (A,B) and E12.5 (C,D) wild-type mouse embryos using radio-labeled sense and antisense LKLF cRNA probes. Note LKLF expression in the developing vasculature including the dorsal aorta (da) of the E9.5 embryo. The areas of highest LKLF expression in the E12.5 embryo are in the umbilical vessels (uv), the intervertebral arteries (iva), the vertebrae (vert), and the smaller vessel structures in the head and tail regions. Bar, 1 mm.
positive hybridization signals seen in the livers of the E12.5 embryos with both the sense and anti-sense probes reflect autofluorescence of hematopoietic precursors in the fetal liver and not LKLF gene expression. At E14.5, LKLF continued to be expressed in the vasculature throughout the embryo and was also expressed in the lung buds, the vertebral column, and the developing bony structures of the head and the rib cage (data not shown). By E18.5, the LKLF gene was expressed abundantly in the lungs as well as in blood vessels (data not shown).

To identify the vascular cell lineage(s) that expressed LKLF in the mouse embryo more precisely, we performed immunohistochemical assays in conjunction with in situ hybridization on embryonic tissue sections. Hematoxylin and eosin (H+E) staining of sections of developing brain from E9.5 and E12.5 embryos revealed the presence of both capillaries and large vessels (Fig. 2A,B). In situ hybridization using an LKLF antisense cRNA probe demonstrated that LKLF was expressed at low levels on E9.5, and at high levels on E12.5 in both types of blood vessels (Fig. 2D,E,G,H). Immunohistochemical staining of adjacent sections with a monoclonal antibody against CD34, which is expressed on the surface of vascular endothelial cells, but not on other vascular cells (Fina et al. 1990) demonstrated that LKLF expression was restricted to CD34+ endothelial cells (Fig. 2J,K) in the brain. Similarly, LKLF was expressed in the CD34+ endothelial cells of the umbilical arteries and veins and the aorta but not in VSMCs in the tunica media of these same vessels (Fig. 2C,F,I,L; data not shown).

Generation of LKLF−/− animals
LKLF genomic clones were isolated from a 129sv mouse genomic library by hybridization with a murine LKLF cDNA probe. Restriction endonuclease and DNA sequence analyses demonstrated that the LKLF gene is 2.5 kb in length and consists of three exons corresponding to nucleotides 1–117, 118–935, and 936–1557 of the murine cDNA (Fig. 3A) (Anderson et al. 1995). To produce a null mutation of the LKLF gene, we constructed a targeting vector in which the entire LKLF gene was replaced with a PGK-neomycin (neo) cassette (Fig. 3A). This targeting construct has been described previously (Kuo et al. 1997). Following electroporation of the linearized targeting vector into RW embryonic stem (ES) cells, G418- and gancyclovir-resistant ES cell colonies were screened for homologous recombination by Southern blot analysis. Eleven of 96 neo- and gancyclovir-resistant ES cell colonies were shown to be homologous recombinants (data not shown). Each of these LKLF−/− ES cell clones contained a single integration of the targeting vector as determined by Southern blot analysis with a neo probe (data not shown).

Three independently-derived LKLF−/− ES cell lines were injected into C57BL/6 blastocysts to generate male chimeric mice. Chimeric males derived from one of these ES cell lines when crossed to C57BL/6 females transmitted the targeted allele through the germ line as determined by Southern blot analysis. LKLF−/− heterozygous mice were viable and phenotypically normal. They were bred to wild-type C57BL/6 and CD1 mice, generating heterozygotes that were subsequently crossed to generate the LKLF−/− homozygous mice. The phenotypes of the LKLF−/− mice were identical on all genetic backgrounds studied. As shown in Table 1, no LKLF−/− animals were observed among more than 100 live-born offspring from LKLF−/−×LKLF−/− matings. Genotyping of E11.5 embryos from LKLF−/−×LKLF−/− matings showed Mendelian segregation of the LKLF−/− genotype (Fig. 3B and Table 1). The E11.5 LKLF−/− embryos appeared phenotypically normal and were alive with clearly visible beating hearts (Fig. 4A,B). LKLF−/− embryos died between E12.5 and E14.5 (Fig. 4C–H, Table 1).

To confirm that we had produced a null mutation of the LKLF gene, we performed in situ hybridization on E11.5 wild-type and LKLF−/− embryos using LKLF cRNA probes derived from both the coding and 3′ untranslated regions (UTRs) of the LKLF cDNA (Fig. 3C,D; data not shown). High-level LKLF expression was observed in vascular endothelial cells of the wild-type embryos. In contrast, no LKLF hybridization signal was detected in the LKLF−/− embryos.

Figure 2. LKLF expression in embryonic vascular endothelial cells. Histological (A–C), in situ hybridization (D–I), and immunohistochemical (J–L) analyses of developing vessels in E9.5 wild-type mouse embryos (A,D,G,J), E12.5 embryonic brain (B,E,H,K), and E12.5 umbilical vessels (C,F,I,L). (A–C) H + E staining. (D–I) In situ hybridizations using radio-labeled sense and antisense LKLF cRNA probes. Note the intracerebral vessels (v) and capillaries (c), the umbilical arteries (ua), and veins (uv). The fluorescent signal in the lumens of the umbilical vessels seen with both sense and antisense probes reflects RBC autofluorescence, not hybridization. (J–L) Immunohistochemical analyses using an anti-CD34 monoclonal antibody. Note CD34+ endothelial cells lining the endoluminal surface of the intracerebral vessels as well as the umbilical veins and arteries.
Intra-amniotic and intra-embryonic hemorrhage in the LKLF−/− embryos

As described above, LKLF−/− embryos appeared morphologically normal at E11.5 (Fig. 4A, B). Consistent with this observation, histological sections of E11.5 LKLF−/− embryos were indistinguishable from those of wild-type littermates (data not shown). By E12.5, ∼30% of the LKLF−/− embryos demonstrated internal hemorrhages in areas around the cardiac outflow tract and within the abdomen (Fig. 4D). Although these LKLF−/− embryos contained normal numbers of yolk-sac blood vessels, these vessels were strikingly devoid of blood (Fig. 4C). The observed hemorrhages were not the result of the dissection of the embryos, as they were also reproducibly seen in LKLF−/− embryos in which the amnion, yolk sac, and placenta remained undissected (Fig. 4C). In addition to the obvious intra-embryonic hemorrhages, the head size of the LKLF−/− embryos was significantly smaller than that of their wild-type littermates. This difference, which was particularly noticeable in the hindbrain areas, might have been a primary abnormality or instead may have reflected decreased head development from anemia and tissue necrosis in these animals.

The LKLF−/− embryos that survived to E13.5 began to

| Age   | ++ | +/- | -- (Hemorrhage) |
|-------|----|-----|-----------------|
| 11.5 dpc | 10 | 23  | 11 (0)          |
| % of total | 23 | 52  | 25              |
| 12.5 dpc | 17 | 37  | 18 (6)          |
| % of total | 24 | 51  | 25              |
| 13.5 dpc | 15 | 29  | 17 (16)         |
| % of total | 25 | 48  | 27              |
| 14.5 dpc | 6  | 15  | 7 (7)           |
| % of total | 21 | 54  | 25              |
| Newborn | 56 | 101 | none            |
| % of total | 36 | 64  |                  |
bleed profusely into the amniotic cavity (Fig. 4E). In addition, like the E12.5 embryos, they demonstrated internal hemorrhages in regions surrounding the cardiac outflow tract and within the abdomen. As shown in Table 1, nearly all of the E13.5 LKLF−/− embryos had intra-amniotic and/or intra-embryonic hemorrhages. Following dissection, the E13.5 LKLF−/− embryos appeared bloated and their heads and limbs were pale in comparison to wild-type littermates (Fig. 4F). Histological analyses of these embryos showed widespread tissue necrosis that was probably secondary to the severe anemia caused by intra-embryonic and intra-amniotic bleeding (data not shown). By E14.5, the LKLF−/− embryos had exsanguinated into the amniotic cavity. Following dissection, these embryos were significantly smaller than their wild-type counterparts and were strikingly pale, due both to blood loss and severe tissue necrosis (Fig. 4G,H). No LKLF−/− embryos survived beyond E14.5 (Table 1).

Normal vasculogenesis and angiogenesis in the LKLF−/− embryos

Because LKLF is expressed at high levels in the developing vasculature, we reasoned that the hemorrhagic defect observed in the LKLF-deficient embryos might have reflected defects in blood vessel morphogenesis or function. Whole-mount immunohistochemical staining of E12.5 LKLF−/− embryos using a monoclonal antibody against PECAM-1 revealed apparently normal vascular patterning that was indistinguishable from that seen in age-matched wild-type littermates (Fig. 5A). High-power photomicrographs of these embryos demonstrated a normal capillary plexus with both small and large vessels as well as normal capillary sprouting (Fig. 5B,C). Histological sections of H + E-stained E12.5 LKLF−/− embryos demonstrated normal cardiac development as well as normal-sized dorsal aortae and normal numbers of yolk sac, umbilical, and intervertebral vessels (Figs. 4A, 5D,E; Figure 5. Normal vasculogenesis and angiogenesis in the LKLF−/− embryos. (A–C) Whole-mount immunohistochemical staining with an anti-PECAM-1 monoclonal antibody. Wild-type (wt) and LKLF−/− (−/−) embryos were stained with an anti-PECAM-1 antibody and visualized by low power (A) or high power (B,C) photomicroscopy. Note the normal vascular patterning, capillary plexes, and capillary sprouting in the LKLF−/− embryo. (D,E) H + E-stained embryo sections from E12.5 wild-type (wt) and LKLF−/− (−/−) embryos. Note the normal cardiac morphology and the normal size of the dorsal aorta (da) in the LKLF−/− embryo. (i) Right atrium; (iv) right ventricle; (ii) left atrium; (iv) left ventricle.
Taken together, these data demonstrated that LKLF is not required for the formation of the primary capillary plexus (vasculogenesis) nor for capillary sprouting (angiogenesis) or remodeling of the capillary plexus into small and large vessels.

**Umbilical vessel defects in the LKLF**<sup>−/−</sup> embryos

To assess the later stages of blood vessel wall morphogenesis and stabilization, we examined histological sections of the umbilical vessels from E12.5 LKLF<sup>−/−</sup> embryos without grossly detectable hemorrhages. Wild-type umbilical arteries and veins contained an endoluminal layer of CD34<sup>+</sup> endothelial cells, surrounded by well-organized layers of differentiating pericytes and smooth muscle α-actin (SMαA)-expressing VSMCs (Fig. 6A,C,E,G,I,K), which formed tunica media of uniform thickness. In marked contrast, the LKLF<sup>−/−</sup> umbilical vessels displayed a number of severe histological abnormalities. These vessels clearly contained both CD34<sup>+</sup> endothelial cells and SMαA-expressing VSMCs (Fig. 6F,H,J,L). Therefore, LKLF is not required for the specification of either of these cell lineages. The LKLF<sup>−/−</sup> umbilical veins and arteries, however, displayed a dramatic reduction in the thickness of the tunica media (Fig. 6B,D). This thinning, which was patchy in the umbilical arteries and more uniform in the umbilical veins, resulted in multiple aneurysmal dilations of both umbilical vessels. In many cases, we observed areas of frank dissection of the umbilical veins and arteries and associated hemorrhage at the sites of aneurysmal dilation of these vessels (Fig. 6B,D). These abnormalities were never observed in wild-type or LKLF<sup>+/−</sup> embryos (Fig. 6A,C; data not shown).

The blood vessel defects in the LKLF<sup>−/−</sup> embryos were analyzed further by electron microscopy. Consistent with the light microscopic analyses, there was a marked reduction in the number of pericytes and VSMCs in the tunica media of the LKLF<sup>−/−</sup> umbilical vessels. In addition, the tunica media of these vessels contained significantly reduced amounts of extracellular matrix as compared with wild-type control vessels (Fig. 7). Some endothelial cells in the LKLF<sup>−/−</sup> umbilical veins appeared both enlarged and necrotic, as evidenced by a highly vacuolated cytoplasm and large nuclei (Fig. 7). To determine if the LKLF<sup>−/−</sup> endothelial cells were undergoing apoptotic cell death, we performed TUNEL assays on tissue sections from E12.5 LKFL<sup>−/−</sup> animals. There was no evidence of increased endothelial cell apoptosis in these animals (data not shown).

**Aortic defects in LKLF**<sup>−/−</sup> embryos

We also analyzed the morphology of the dorsal aortae in E12.5 LKLF<sup>−/−</sup> mice that did not show evidence of intra-embryonic bleeding. Aortae from E12.5 wild-type embryos displayed an intact endothelial cell layer surrounded by a compact tunica media composed of circumferentially elongated VSMCs (Fig. 8A,C,E). In contrast, LKFL<sup>−/−</sup> aorti contained an endoluminal layer of CD34<sup>+</sup> endothelial cells surrounded by a poorly organized tunica media composed of SMαA-expressing VSMCs that...
displayed a cuboidal morphology (Fig. 8B,D,F). The LKLF−/− VSMCs had prominent nuclei and stained less intensely for SMαA than their wild-type counterparts (Fig. 8F).

Expression of angiogenic growth factors and growth factor receptors in the LKLF−/− mice

Previous studies have demonstrated the importance of several growth factors and growth factor receptors in blood vessel formation during mouse embryogenesis. These include the Tie1 and Tie2 receptor tyrosine kinases, which are expressed on endothelial cells; PDGF-B and HB-EGF, which are expressed by endothelial cells and required for VSMC recruitment to the blood vessel wall; and TGF-β, which has an important role in the differentiation of pericytes and smooth muscle cells. Vascular expression of Tie1, Tie2, PDGF-B, and HB-EGF, and TGF-β were qualitatively normal in the LKLF−/− animals as assessed by in situ hybridization (data not shown). Therefore, although we cannot exclude quantitative defects in the expression of these growth factors or receptors, LKLF does not appear to be required for the vascular expression of these molecules.

Normal erythroid, myelomonocytic, and megakaryocyte development in the LKLF−/− embryos

The LKLF-related zinc finger transcription factor, EKLF, is required for normal erythroid development. Therefore, it was important to investigate the possibility that LKLF might have a critical role in the development of the erythroid and/or megakaryocyte lineages and that the hemorrhagic disorder and subsequent anemia observed in the LKLF-deficient mice might have reflected, at least in part, a defect in red blood cell (RBC) and/or platelet production in these animals. Three sets of studies were carried out to assess these possibilities. First, LKLF−/− ES cells were differentiated in vitro under conditions that promote hematopoiesis. Colonies derived from the LKLF−/− ES cells contained both erythroid and myelomonocytic cells, as well as megakaryocytes (data not shown). Therefore, at least in vitro, LKLF is not required for erythropoiesis or myelopoiesis. In a second series of experiments, fetal liver cells from E11.5 wild-type and LKLF−/− embryos were cultured in vitro for seven days and the resultant erythroid and mixed (erythroid + myelomonocytic) colonies were quantitated (Fig. 9A). Fetal liver cells from the LKLF−/− animals produced normal numbers of both erythroid and mixed colonies. Therefore, there was no evidence for decreased fetal liver hematopoiesis in the LKLF−/− mice. Finally, we examined Wright-Giemsa-stained blood smears from E12.5 wild-type and LKLF−/− embryos. Both nucleated and enucleated RBCs, as well as platelets, were present in the peripheral blood of the LKLF−/− embryos (Fig. 9B). Taken together, these experiments demonstrated that LKLF is not required for the differentiation of mature RBCs, platelets, or myelomonocytic cells in vitro or in vivo and thereby supported the hypothesis that the hemorrhagic
during later stages of murine embryogenesis including this transcription factor in mammalian development. As deficient mice do not exclude other nonvascular roles for vessel wall.

cell-mediated assembly, and stabilization of the blood stage in blood vessel morphogenesis, the endothelial

define LKLF as a novel transcriptional regulator of a late the LKLF-deficient mice. Taken together, these studies

throid, myelomonocytic, or megakaryocytic lineages in not detect abnormalities in the formation of the ery-

roles in blood vessel stabilization. Importantly, we did detect abnormalities in the expression of growth factors ing to aneurysmal dilatation and both arterial and ve-

nitrous rupture. Despite these defects, we were unable to detect abnormalities in the expression of growth factors and growth factor receptors, such as Tie1, Tie2, PDGF-B, HB-EGF, and TGF-β, that are known to have important roles in blood vessel wall stabilization. Importantly, we did not detect abnormalities in the formation of the erythroid, myelomonocytic, or megakaryocytic lineages in the LKLF-deficient mice. Taken together, these studies define LKLF as a novel transcriptional regulator of a late stage in blood vessel morphogenesis, the endothelial cell-mediated assembly, and stabilization of the blood vessel wall.

It should be emphasized that our studies of the LKLF-deficient mice do not exclude other nonvascular roles for this transcription factor in mammalian development. As described above, LKLF is expressed in multiple tissues during later stages of murine embryogenesis including bone, lung, and T and B lymphocytes. Although LKLF does not appear to be required for the normal development of the erythroid, myelomonocytic, or megakaryocytic lineages, our recent studies using LKLF−/−RAG2−/− chimeras have demonstrated a critical role for LKLF in programming the quiescent phenotype of single-positive (CD4+ and CD8+) thymocytes and T cells (Kuo et al. 1997). LKLF−/−T cells display a spontaneously activated phenotype and die in the peripheral lymphoid organs of activation-induced cell death. Similar chimera experiments may demonstrate important roles for LKLF in regulating the development of bone and pulmonary cell lineages. It will be of interest to determine if the diverse abnormalities seen in different LKLF-deficient cell lineages reflect a common molecular defect or, instead, define multiple distinct roles for LKLF in these different cell types. In this regard, it is of interest that EKLF regulates the terminal differentiation of erythroid cells, whereas LKLF appears to regulate a similar stage of T-cell development. Therefore, it is tempting to speculate that LKLF may also regulate the late stages of endothelial cell differentiation, which in turn, are required for vessel wall assembly and stabilization.

**Vessel defects in LKLF-deficient mice**

Mechanism of LKLF-mediated blood vessel wall stabilization

The molecular mechanism(s) by which LKLF regulates blood vessel wall stabilization remain unknown. The fact that LKLF is expressed in endothelial cells, but not in VSMCs or vascular adventitial cells, suggests that it regulates a signaling pathway by which endothelial cells recruit and/or promote the differentiation or survival of mesenchymal cells to form the mature blood vessel wall. Such a pathway could involve the secretion of a soluble morphogen or growth factor, or expression of a cell-surface or extracellular matrix molecule that is involved in endothelial cell-mesenchymal cell interactions. Alternatively, LKLF may be required for the survival of vascular endothelial cells, thereby enabling them to promote blood vessel wall assembly indirectly.

Our studies appear to exclude a number of mechanisms of LKLF action. For example, LKLF does not appear to be an important regulator of either vasculogenesis, the initial patterning of the circulatory system, or angiogenesis, the sprouting of capillaries from the pre-existing network of intra-embryonic vessels. Unlike the VEGF-, flk-1-, and flt-1-deficient mice, which die between E8 and E9.5 with similar but distinct severe defects in endothelial cell differentiation, abnormal vasculogenesis, angiogenesis, and yolk sac hematopoiesis, the LKLF-deficient mice survived until E12.5-14.5 and displayed apparently normal endothelial cell specification (as measured by CD34 and PECAM-1 staining), normal hematopoiesis, and normal vascular patterning and capillary branching. Therefore, it seems quite unlikely that the vascular defects seen in the LKLF-deficient mice reflect defects in the expression of VEGF, flk-1, or flt-1.

Similarly, angiotensin-1 and its receptor tyrosine kinases, Tie1 and Tie2, have been implicated as important
regulators of both angiogenesis and vascular remodeling. Tie2-deficient mice die by E10.5 with severe defects in capillary sprouting and branching and an inability to remodel the capillary network into large and small vessels. In contrast, Tie1 appears to have an important role in maintaining the structural integrity of blood vessels. Tie1-deficient mice survive until birth and display a grossly normal vascular system. Vessels from these animals, however, are leaky, resulting in diffuse edema and microhemorrhages. Both of these phenotypes are quite distinct from that of the LKLF-deficient animals. Moreover, we have shown by in situ hybridization that the expression of both Tie1 and Tie2 are qualitatively normal in the LKLF−/− embryos. Therefore, it appears unlikely that the vascular defects seen in the LKLF−/− animals are caused by abnormal expression of Tie1 and/or Tie2 on endothelial cells.

Finally, recent studies have also identified several growth factors that appear to have important roles in modulating the reciprocal interactions between vascular endothelial cells and peri-endothelial mesenchymal cells that are important for blood vessel morphogenesis and stabilization. PDGF-B and HB-EGF synthesized by endothelial cells are required for normal pericyte and VSMC recruitment to form the vascular tunica media. Similarly, angiopoietin-1 synthesized by peri-endothelial mesenchymal cells binds to the Tie2 receptor on endothelial cells and this interaction is required for the maturation and stabilization of the blood vessel wall. Activation of TGF-β has been implicated as an important regulator of VSMC differentiation, endothelial cell quiescence, and extracellular matrix production during vessel morphogenesis. Given the phenotype of the LKLF-deficient animals, each of these factors is a putative target for LKLF. At the present time, however, there is no direct evidence to implicate dysregulated expression of any of these molecules in the LKLF−/− phenotype. By in situ hybridization, the expression of PDGF-B, HB-EGF, and TGF-β are qualitatively normal in the LKLF−/− animals. Moreover, the phenotypes of the PDGF-B- and angiopoietin-1-deficient animals differ significantly from that of the LKLF−/− mice.

Regulation of LKLF in endothelial cells

Given the importance of LKLF in regulating blood vessel wall formation, it is of interest to determine both the endothelial cell targets of LKLF and the pathways that regulate its expression in endothelial cells. In T cells, LKLF is regulated at both the transcriptional and post-translational levels by signals from the T-cell antigen receptor (Kuo et al. 1997). Transcription of the LKLF gene is induced during the differentiation of immature double-positive (CD4+/CD8+) to mature single-positive (CD4+ or CD8+) thymocytes and LKLF expression remains high in quiescent circulating T cells. Following activation through the T-cell antigen receptor, the transcription of the LKLF gene is extinguished and pre-existing LKLF protein is degraded. It is important to determine if transcription of the LKLF gene is induced during the process of endothelial cell maturation by known vascular growth factors and their receptors, including angiopoietin-1/Tie2, and VEGF/flk1-flt1, and whether the expression of this gene changes in endothelial cells following vascular injury, or during the processes of angiogenesis and atherogenesis.

Materials and methods

Low-stringency hybridization

The murine LKLF cDNA was isolated by low stringency hybridization to a radio-labeled degenerate oligonucleotide probe corresponding to the zinc finger region of EKLF as described (Kuo et al. 1997). LKLF genomic clones were isolated from an 129 genomic library (Stratagene) by hybridization to the LKLF cDNA and characterized by restriction enzyme and DNA sequence analyses.

In situ hybridization

In situ hybridizations were as described previously (Kuratani et al. 1987). The 35S-cRNA probe used to detect embryonic expression of LKLF was derived from a SalI-Pac fragment from the 3' UTR of the LKLF cDNA. Dark-field photomicrographs were obtained using a Zeiss Axioskop.

Generation of LKLF−/− mice

The neo+ targeting vector was generated by inserting a 7.1-kb PacI-NotI genomic fragment of the murine LKLF locus into the XhoI-NotI site of pPNT (Tybulewicz et al. 1991), followed by ligation of a 1.4-kb HindIII–SalI genomic fragment located 5' of the LKLF exons into the EcoRI site of pPNT as described previously (Kuo et al. 1997). The resulting targeting construct was linearized with NotI before electroporation into RW ES cells. Neo+ transfectants were selected by growth in G418 (200 μg/ml) and gancyclovir (1 μM). DNA from ES cell clones was characterized by Southern blot analysis using EcoRI digestion and a radio-labeled 0.5-kb HindIII–Mscl genomic fragment. ES cells from three independently derived LKLF−/− clones were micro-injected into C57BL/6 donor blastocysts, which were subsequently implanted into CD1 pseudopregnant females (Bradley 1987). The resulting male chimeras were mated with C57BL/6 females and agouti offspring were genotyped by Southern blot analysis. One of the LKLF−/− ES cell lines generated LKLF−/− heterozygous mice. These LKLF−/− heterozygous mice were bred with C57BL/6 and CD1 animals to generate heterozygous LKLF−/− offspring that were interbred for phenotypic analysis. All animal experimentation was performed according to National Institutes of Health (NIH) guidelines in the Franklin McLean Memorial Research Institute of the University of Chicago.

Histological and electron microscopic analyses

Embryos were dissected into PBS and fixed overnight at 4°C in 4% paraformaldehyde in PBS. After dehydration in increasing concentrations of ethanol (50%, 70%, 95%, and 100%), the embryos were transferred into xylene and embedded in paraffin before sectioning at 5 μm. Histological sections were stained with H&E. For electron microscopic analyses, embryos were washed in PBS, then fixed in 2.5% glutaraldehyde in PBS at 4°C for 30 min. After subsequent washes in PBS, the embryos were post-fixed with 1% osmium tetroxide for 1 hr. Following post-fixation, the embryos were washed and stained with 1% uranyl
acetate for 1 hr, washed with 0.2 M maleate buffer (pH 5.1), then dehydrated in increasing concentrations of ethanol. The dehydrated embryos were infiltrated directly and embedded with LK-112 medium. Ultrathin sections of the embedded embryos were cut with a diamond knife, mounted onto uncoated 200-mesh grids, and stained with uranyl acetate and lead citrate. Electron micrographs were made with a JEOL-CXII electron microscope operating at 2.7 kV. Whole embryos were photographed using a Leica M3Z. Photomicrographs were made with a Zeiss Axioskop.

Immunohistochemical analysis

For CD34 and smooth muscle α-actin staining, paraffin-embedded sections were baked at 60°C for 1 hr, cleared in xylene, and hydrated in descending alcohol concentrations ending with distilled water. The hydrated tissue sections were placed in 0.01 M citrate buffer at pH 7.0. Endogenous peroxidase activity was blocked by treating the sections with 3% hydrogen peroxide in methanol for 20 min. Tissue sections were then incubated overnight at 4°C with monoclonal antibodies generated against CD34 (PharMingen) and smooth muscle α-actin (Sigma). Immunohistochemical staining was performed on a Ventana Gen System (Ventana Medical Systems), and immunostained sections were counterstained with hematoxylin. Whole-mount immunohistochemical staining of E12.5 embryos was performed as described previously (Schlaeger et al. 1995).

In vitro differentiation of fetal liver and embryonic stem cells

In vitro differentiation of ES cells, E11.5 fetal liver cells, and collection of E12.5 embryonic peripheral blood were performed according to procedures previously described (Olson et al. 1995; Okuda et al. 1996). Cytospin preparations of hematopoietic colonies and peripheral blood were stained with the Diff-Quik Stain Set (Baxter) according to manufacturer’s protocol. Photomicrographs of blood cells were made with a Zeiss Axioskop at a magnification of 400×.

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Note added in proof

Expression of both angioptotin-1 and angiopoietin-2 are quantitatively normal in the LKLF−/− embryos as assessed by in situ hybridization (data not shown).

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