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The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells

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Vascular endothelial cells are critical for the development and function of the mammalian circulatory system. We have analyzed the role of the endothelial cell-specific receptor tyrosine kinase TIE in the mouse vasculature. Mouse embryos homozygous for a disrupted *Tie* allele developed severe edema, their microvasculature was ruptured and they died between days 13.5 and 14.5 of gestation. The major blood vessels of the homozygous embryos appeared normal. Cells lacking a functional *Tie* gene were unable to contribute to the adult kidney endothelium in chimeric animals, further demonstrating the intrinsic requirement for TIE in endothelial cells. We conclude that TIE is required during embryonic development for the integrity and survival of vascular endothelial cells, particularly in the regions undergoing angiogenic growth of capillaries. TIE is not essential, however, for vasculogenesis, the early differentiation of endothelial cells.

Keywords: angiogenesis/embryology/endothelial cell/gene targeting/receptor tyrosine kinase

Introduction

The embryonic cardiovascular system is the first organ system to form during development in order to accommodate the metabolic needs of growing tissue. Perhaps due to this fundamental importance for the vertebrate embryo, no naturally occurring mutations affecting development of the vasculature are known. Endothelial cells are the first cells of the cardiovascular system to differentiate and play an essential role in mediating the characteristic pattern of formation of the vasculature (Noden, 1991; Poole and Coffin, 1991; Risau, 1991). However, the intercellular signals regulating the differentiation and growth of embryonic endothelial cells have remained elusive.

Two distinct cellular processes have been observed to mediate blood vessel formation during avian and mammalian embryogenesis. The *in situ* differentiation of mesodermally derived endothelial cell precursors, the angioblasts and their assembly into vascular channels are referred to as vasculogenesis. Interaction of mesoderm with adjacent endoderm has been suggested to be important for angioblast differentiation (Pardanaud *et al.*, 1989). The larger vascular structures of the embryo, including the heart endocardium and the major blood vessels such as the dorsal aorta, arise by this process (Pardanaud *et al.*, 1987; Coffin and Poole, 1988; Coffin *et al.*, 1991). In contrast, the vascularization of many organs, especially ones lacking an endodermal component such as the brain and kidney, as well as formation of the smaller vessels and the microvasculature, occurs by the process of angiogenesis, the proliferation of pre-existing endothelial cells to expand the vascular network (Stewart and Wiley, 1981; Sariola *et al.*, 1984; Pardanaud *et al.*, 1989).

The biochemical mechanisms that regulate the processes of vasculogenesis and angiogenesis during development are not well understood and the molecules responsible for formation of the vasculature by these two processes are only beginning to be defined. The identification of two subfamilies of mammalian receptor tyrosine kinases whose expression is virtually restricted to endothelial cells and their precursors has provided a novel entry point to the genetic investigation of mammalian vasculogenesis and angiogenesis (Mustonen and Alitalo, 1995). These receptor tyrosine kinases consist of the members of the vascular endothelial cell growth factor (VEGF) receptor family, namely FLT-1, FLK-1 and FLT-4, as well as the TEK (TIE-2) and TIE (TIE-1) orphan receptors. Mutant analyses of TEK, FLT-1 and FLK-1 have indicated that these receptors play critical roles in vascular development (Dumont *et al.*, 1994; Fong *et al.*, 1995; Shalaby *et al.*, 1995).

We have here focused on the *in vivo* function of the receptor tyrosine kinase TIE (also known as TIE-1; Partanen *et al.*, 1992; Iwama *et al.*, 1993; Maisonpierre *et al.*, 1993; Sato *et al.*, 1993), the ligand of which remains to be identified. The *Tie* gene is expressed specifically in endothelial cells and their precursors during embryonic development (Korhonen *et al.*, 1994), as well as in some hematopoietic cell lineages (Partanen *et al.*, 1992; Armstrong *et al.*, 1993; Iwama *et al.*, 1993). Suggestive of a role in endothelial cell proliferation, up-regulation of *Tie* expression has been observed during wound healing and ovarian follicle maturation (Korhonen *et al.*, 1992), as well as tumor angiogenesis (Hatva *et al.*, 1994; Kaipainen *et al.*, 1994), processes which involve growth of new capillaries. However, *Tie* is also expressed in most of the non-proliferating adult endothelium.

To investigate the role of TIE in the development and function of endothelial cells we generated mice carrying a germline mutation in the *Tie* locus by gene targeting in embryonic stem (ES) cells. The analysis of these mutants indicated that TIE is not required for differentiation of

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the endothelial cell lineage, but is essential later for maintenance of the microvasculature.

Results

Generation of embryonic stem cells and mice lacking a functional Tie gene

To analyze the biological role of murine TIE a mutation in the *Tie* gene was generated by gene targeting in ES cells using the positive–negative selection strategy (Mansour *et al.*, 1988). The targeting vector was designed so that a homologous recombination event places the bacterial gene encoding β-galactosidase (*lacZ*) under the control of the transcriptional regulatory sequences of *Tie* and deletes the beginning of the TIE protein coding region (Figure 1A). Furthermore, because the splice donor site of the signal sequence encoding exon of the *Tie* gene is retained in the targeted allele, any putative transcripts derived from this allele would be processed into a form where the protein coding region of *Tie* would not be in an open reading frame, thus generating a predicted null allele, tie^{lcz}. We included the *lacZ* gene in our targeting construct in order to follow easily the *in vivo* pattern of *Tie* gene expression and the fate of endothelial cells either hetero- or homozygous for the mutation. R1 ES cells (Nagy *et al.*, 1993) were electroporated with the vector described above and several correctly targeted ES cell lines were obtained after screening of G418/GANC double resistant colonies by Southern blotting using external probes (data not shown). Two independent ES cell lines (1C4 and 1A9) were used to make CD-1 aggregation chimeras (Wood *et al.*, 1993), which passed the tie^{lcz} allele into the germline. Most of the analyses described below were performed with 1C4-derived mouse line, but identical results were obtained with the 1A9-derived line. tie^{lcz} heterozygous mice appeared phenotypically normal. Genotyping of a litter of an F1 intercross at E13.5 is shown in Figure 1B.

Tie gene regulatory sequences drive endothelial cell-specific reporter gene expression throughout embryonic development

Using β-galactosidase activity as a marker for expression of the endogenous *Tie* gene in tie^{lcz} heterozygous mice we confirmed that *Tie* is first expressed in vascular structures at E8.0 of gestation, after formation of yolk sac blood islands (data not shown; Korhonen *et al.*, 1994; Dumont *et al.*, 1995) and by E8.5 (Figure 2A) β-galactosidase staining was clearly observed in the heart, paired dorsal aortae and allantois, but only weakly in the extra-embryonic yolk sac membrane. Similarly, by mid-gestation at E9.5–10.0 (Figure 2B) β-galactosidase stained tissues included the heart, major blood vessels such as the dorsal aorta and intersomitic vessels, as well as the smaller vessels that penetrate the head region. Later during the development of the tie^{lcz} heterozygous mice β-galactosidase continued to be expressed in virtually all endothelial cells of the embryo proper, as demonstrated in a section of an E13.0 tie^{lcz} heterozygous embryo (Figure 2C). Thus the pattern of β-galactosidase activity in tie^{lcz} heterozygotes accurately reflected expression of the endogenous *Tie* gene as previously detected by RNA *in situ* hybridization (Korhonen *et al.*, 1994).

Mice lacking TIE lose endothelial cell integrity and die at mid-gestation

In order to determine the consequence of homozygosity for the tie^{lcz} allele, F1 tie^{lcz} heterozygous mice were intercrossed and analyzed for their *Tie* genotype and for any phenotypic abnormalities. All neonates were healthy and survived to weaning (Table I). No tie^{lcz} homozygous animals were found in the F2 generation, indicating that homozygosity for the targeted mutation at the *Tie* locus was lethal during embryogenesis.

Analysis of litters of F1 intercrosses at mid-gestation demonstrated that until E13.0 there were no visible phenotypic differences between tie^{lcz} homozygotes and their heterozygous littermates (Table I). All genotypically homozygous embryos had formed a functioning vascular system by this stage, as demonstrated by comparing the β-galactosidase staining profiles of tie^{lcz} homozygous and heterozygous embryos (Figure 2C and D). In contrast, by E13.5 of gestation all tie^{lcz} homozygous embryos manifested small hemorrhages distributed throughout the body surface (Figure 2E and F). Histological analysis of β-galactosidase stained embryos at E13.5 revealed that, except at the sites of vascular hemorrhage, there were no gross morphological differences between tie^{lcz} homozygotes and their heterozygous littermates. Most large vessels of the tie^{lcz} homozygotes showed β-galactosidase staining and appeared normal (data not shown). In contrast, diminished staining was observed in the microvasculature of tie^{lcz} homozygous embryos when compared with heterozygous littermates, suggesting that the condition of the mutant endothelium was somehow compromised by the absence of TIE at this stage. The lack of staining was often associated with breakdown of the vessels and hemorrhage, as shown in a view of the meningeal layer of the midbrain (Figure 2G and H). By day 14.5 of gestation all homozygous mutant embryos had died, exhibiting extensive hemorrhage and abdominal edema (Figure 2I and Table I).

Cells lacking TIE are compromised in their ability to contribute to the endothelium of adult kidney

To further analyze the fate of endothelial cells lacking TIE function we analyzed the ability of ES cells with both *Tie* alleles mutated (tie^{lcz}/tie^{lczn-}) to contribute to various cell lineages, including the endothelium, in chimeras with wild-type embryos. The tie^{lcz}/tie^{lczn-} ES cells were isolated by the double targeting strategy described in Figure 1. Utilizing Cre-mediated recombination of loxP sites the *neo* gene was removed from a $tie^{lcz}/+$ ES cell line to create G418-sensitive $tie^{lczn-}/+$ cell lines, which could be re-targeted with the same targeting vector to obtain tie^{lcz}/tie^{lczn-} ES cell lines. Completely tie^{lcz}/tie^{lczn-} ES cell-derived embryos produced by aggregation with tetraploid embryos (Nagy *et al.*, 1993) displayed the identical phenotype to tie^{lcz} homozygotes described above, demonstrating the unimpaired developmental potential of the cells (data not shown). In chimeras with tetraploid embryos the embryo proper is largely derived from the diploid donor ES cells, while the extra-embryonic structures are mostly derived from the tetraploid host. Thus this result demonstrates that extra-embryonic tissues containing a wild-type *Tie* gene cannot rescue the phenotype of tie^{lcz}/tie^{lczn-} embryos.

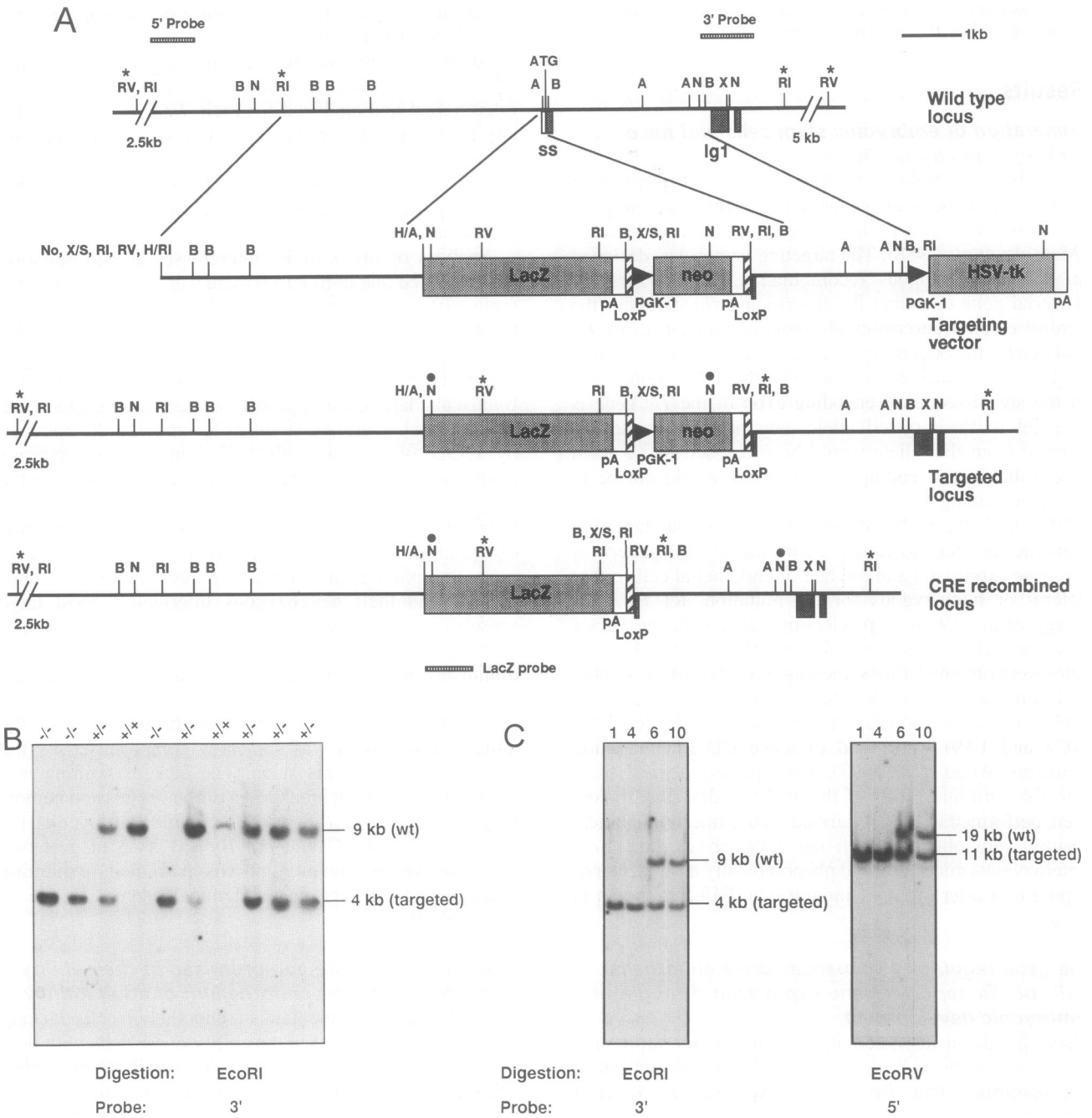

Fig. 1. Gene targeting of the murine *Tie* locus. (**A**) Targeting strategy. The homologous recombination event deletes the start of the protein coding region of the *Tie* gene, placing the *lacZ* gene under control of the *Tie* promoter. The loxP sites (shaded boxes) around the *neo* gene were included for subsequent Cre recombinase-mediated excision of this selectable gene, allowing re-targeting of the cells. A, *Apa*I; B, *Bam*HI; H, *Hin*dIII; N, *Nco*I; No, *Not*I; RI, *Eco*RI; RV, *Eco*RV; S, *Sal*I and X, *Xho*I. (**B**) Genotyping of a litter of a F1 intercross at E13.5. Expected lengths of *Eco*RI fragments hybridizing with the 3′ probe: 9 kb (wild-type) and 4 kb (targeted). +/+, wild-type; +/–, tie^{lcz} heterozygote; –/–, tie^{lcz} homozygote. (**C**) Generation of the tie^{lcz}/tie^{lczn-} ES cells. The *neo* gene was excised from the tie^{lcz}/+ ES cell line 1C4 by transient Cre recombinase expression (Sauer, 1993). Cre-mediated excision events were screened by Southern blotting using *Nco*I digestion and a *lacZ* probe (data not shown). The resulting G418-sensitive cell lines (tie^{lczn-}/+) were re-electroporated with the *Tie* targeting vector and clones were screened with 3′ and 5′ external probes using *Eco*RI and *Eco*RV digests respectively. Expected lengths of *Eco*RI fragments hybridizing with the 3′ probe: 9 kb (wild-type) and 4 kb (targeted). Expected lengths of *Eco*RV fragments hybridizing with the 5′ probe: 19 kb (wild-type) and 11 kb (targeted). Clones 1 and 4 are tie^{lcz}/tie^{lczn-}; clone 6, tie^{lcz}/+; clone 10, tie^{lczn-}/+.

In order to analyze the ability of the tie^{lcz}/tie^{lczn-} ES cells to contribute to the adult endothelium, diploid chimeras were made between these cells and CD-1 morula stage embryos (Wood *et al.*, 1993). As a control chimeras were also made with a tie^{lczn-}/+ heterozygous ES cell line. Adult kidney tissues were chosen for analysis of the chimeras because of their strong endothelial-specific staining and low background. The amount of ES cell-derived tissue in the analyzed chimeras was ~50%, as judged by coat color and Southern blot analysis (Figure 3). Samples of tie^{lcz}/tie^{lczn-}–CD-1 (n = 4) and tie^{lczn-}/+–CD-1 (n = 3) chimeras, as well as tie^{lcz} heterozygous

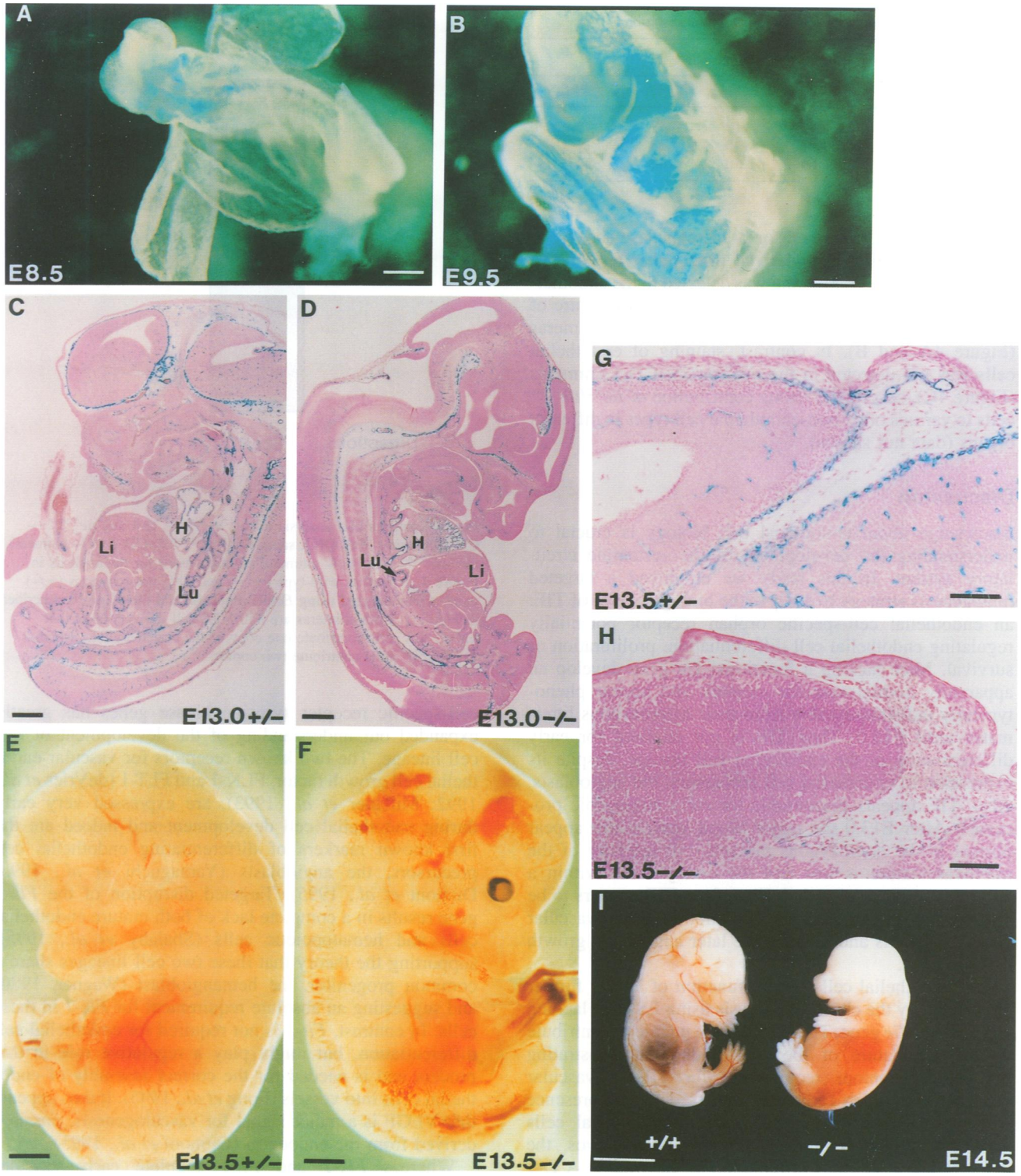

Fig. 2. The *Tie* promoter-driven β-galactosidase expression pattern and analysis of the phenotype of tie^{lcz} homozygote animals. (**A**) *Tie* expression, as assayed by staining for β-galactosidase activity, was detected at the early somite stage (E8.5) of embryonic gestation in the developing heart, paired dorsal aortae, allantois. Bar 100 μm. (**B**) At E9.5 most vascular structures of the embryo proper stain positively for β-galactosidase. Bar 100 μm. (**C**) Parasagittal section of E13.0 tie^{lcz} heterozygous (+/–) embryo. β-Galactosidase activity corresponds to cells of the endothelial lineage lining blood vessels and the heart (H). Lu, lung; Li, liver. Bar 500 μm. (**D**) tie^{lcz} homozygous (–/–) littermate to embryo in (C) shows no visible morphological differences and an identical β-galactosidase staining pattern. H, heart; Lu, lung; Li, liver. Bar 500 μm. (**E**) Unstained tie^{lcz} heterozygous (+/–) embryo at E13.5 compared with (**F**) tie^{lcz} homozygous (–/–) littermate which shows localized hemorrhaging distributed over the body surface. Bar 1 mm. (**G**) Section of the midbrain region showing the leptomeninges of a E13.5 tie^{lcz} heterozygous (+/–) embryo stained for β-galactosidase. Bar 100 μm. (**H**) A corresponding region of a tie^{lcz} homozygous (–/–) embryo at E13.5 showing loss of endothelial cell integrity and a local hemorrhage. A marked reduction in staining in the microvasculature proximal to the hemorrhage is observed. Bar 100 μm. (**I**) Unstained wild-type (+/+) and tie^{lcz} homozygous (–/–) embryos at E.14.5, demonstrating the severe hemorrhage and abdominal edema of tie^{lcz} homozygote embryos at this stage. Bar 100 μm.

Table I. *Tie*^{lcz} homozygotes die before E15.5 of gestation

Developmental stage	No. of live animals			
	Total	–/–	+/–	+/+
E12.5	59	10	37	12
E15.5	47	0	28	19
F2 weanlings	64	0	40	24

mice (n = 3), were stained for β-galactosidase activity to identify endothelial cells derived from either *tie*^{lcz}/*tie*$^{lczn-}$ or *tie*$^{lczn-}$/+ ES cells. Staining was detected in the endothelial cells of the glomeruli and other kidney vasculature of the *tie*^{lcz} heterozygous mice and *tie*$^{lczn-}$/+–CD-1 chimeras (Figure 4A and B). In contrast, staining of endothelial cells was not detected in the *tie*^{lcz}/*tie*$^{lczn-}$–CD-1 chimeras (Figure 4C), despite the strong contribution of *tie*^{lcz}/*tie*$^{lczn-}$ cells to various other non-endothelial cell types in different organs (data not shown).

Discussion

Knowledge about endothelial cell biology is crucial to understanding the development of the vertebrate circulatory system. In this study we employed a targeted mutagenesis strategy to analyze the biological role of TIE, an endothelial cell-specific orphan receptor potentially regulating endothelial cell differentiation, proliferation or survival. Mice lacking a functional *Tie* gene develop an apparently normal vascular network and appear phenotypically indistinquishable from their heterozygous littermates until E13.0. Thus TIE is not required for early differentiation of the endothelial cell lineage, despite its expression in endothelial cell precursors, the angioblasts. Also, TIE appears dispensable for early angiogenic processes. After E13.0 the major blood vessels still appear normal in the homozygous mutants, but integrity of the microvasculature is lost and the embryos die within a strikingly short time period. Therefore, TIE appears to be required for survival or proliferation of microvascular endothelial cells and thus for the later angiogenic growth of capillaries.

The endothelial cell-specific function of TIE is further underlined by the chimeric analysis using ES cells lacking a functional *Tie* gene. These cells were unable to contribute to the vasculature of the adult kidney, demonstrating the cell-autonomous requirement for TIE in vascular endothelium, which cannot be bypassed by the presence of a wild-type environment. Because endothelial cells survive until E13.0 in *tie*^{lcz} homozygous embryos, the defect leading to their elimination in *tie*^{lcz}/*tie*$^{lczn-}$–CD-1 chimeras likely takes place later in gestation, either by cell death or competitive proliferation of wild-type cells. The kidney is vascularized by the angiogenic process fairly late in embryonic development (Sariola *et al.*, 1984), which is consistent with TIE having a role in endothelial cell survival or growth during capillary sprouting. However, in a competitive situation the *tie*^{lcz}/*tie*$^{lczn-}$ cells might already be at a disadvantage at earlier stages of angioblast differentiation. Resolution of this issue will require a time course study of chimeric embryos.

Recent isolation and mutational analyses of endothelial cell-specific receptor tyrosine kinase genes has greatly expanded our understanding of the development of this cell lineage. The two known receptors for vascular endothelial cell growth factor, FLK-1 and FLT-1 (deVries *et al.*, 1992; Millauer *et al.*, 1993), are expressed very early during endothelial cell development and indeed are the first known markers of differentiating endothelial cell precursors, the angioblasts (Yamaguchi *et al.*, 1993; Dumont *et al.*, 1995). Targeted disruption of the *Flk-1* gene results in a complete lack of both mature endothelial cells and hematopoietic cells (Shalaby *et al.*, 1995), supporting the theory that these two cell lineages share a common progenitor, the hemangioblast (Wagner, 1980) and suggesting an absolute requirement for *Flk-1* in these cells. In contrast, *Flt-1* is not required for endothelial cell differentiation, but might play a regulative role in this process and is needed for the correct assembly of endothelial cells into vessels (Fong *et al.*, 1995). It thus appears that VEGF is a major signal for vasculogenesis, possibly derived from the endoderm (Dumont *et al.*, 1995).

Whereas *Flk-1* and *Flt-1* regulate early vascular development, *Tie* and its closely related family member *Tek* (also known as *Tie-2*; Dumont *et al.*, 1992; Iwama *et al.*, 1993; Sato *et al.*, 1993; Schnurch and Risau, 1993) are expressed somewhat later during endothelial cell differentiation (Korhonen *et al.*, 1994; Dumont *et al.*, 1995). The phenotype of *tie*^{lcz} homozygous mice, together with chimeric analysis, suggests that *Tie* is required for the later maintenance and/or proliferation of vascular endothelial cells, but not for their early differentiation. Interestingly, the *Tie* mutant embryos resemble the phenotype of *Tek* mutants, which die around E9.5, showing

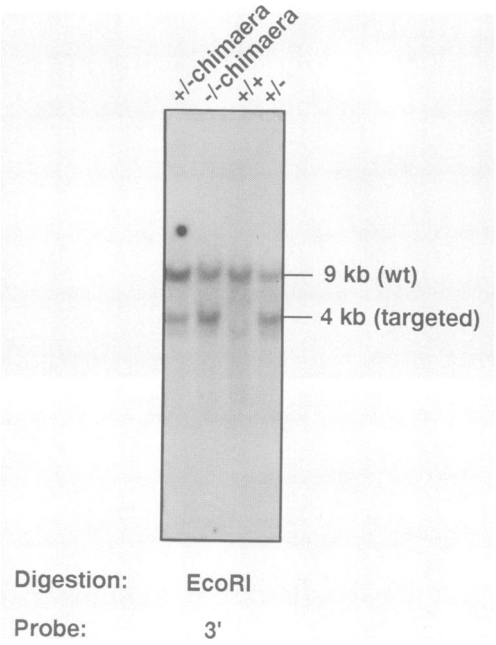

Fig. 3. Southern blot analysis of *tie*^{lcz}/*tie*$^{lczn-}$–CD-1 and *tie*$^{lczn-}$/+–CD-1 chimeras. Tail DNA samples of *tie*$^{lczn-}$/+–CD-1 (+/– chimera), *tie*^{lcz}/*tie*$^{lczn-}$–CD-1 (–/– chimera) chimeras used for the analysis in Figure 4, as well as wild-type (+/+) and *tie*^{lcz} heterozygous (+/–) mice, were analyzed using *Eco*RI digestion and the 3′ probe described in Figure 1. Both chimeras are ~50% ES cell derived. Note that *tie*^{lcz}/+ ES cells contribute one copy of the targeted allele, whereas *tie*^{lcz}/*tie*$^{lczn-}$ cells contribute two copies.

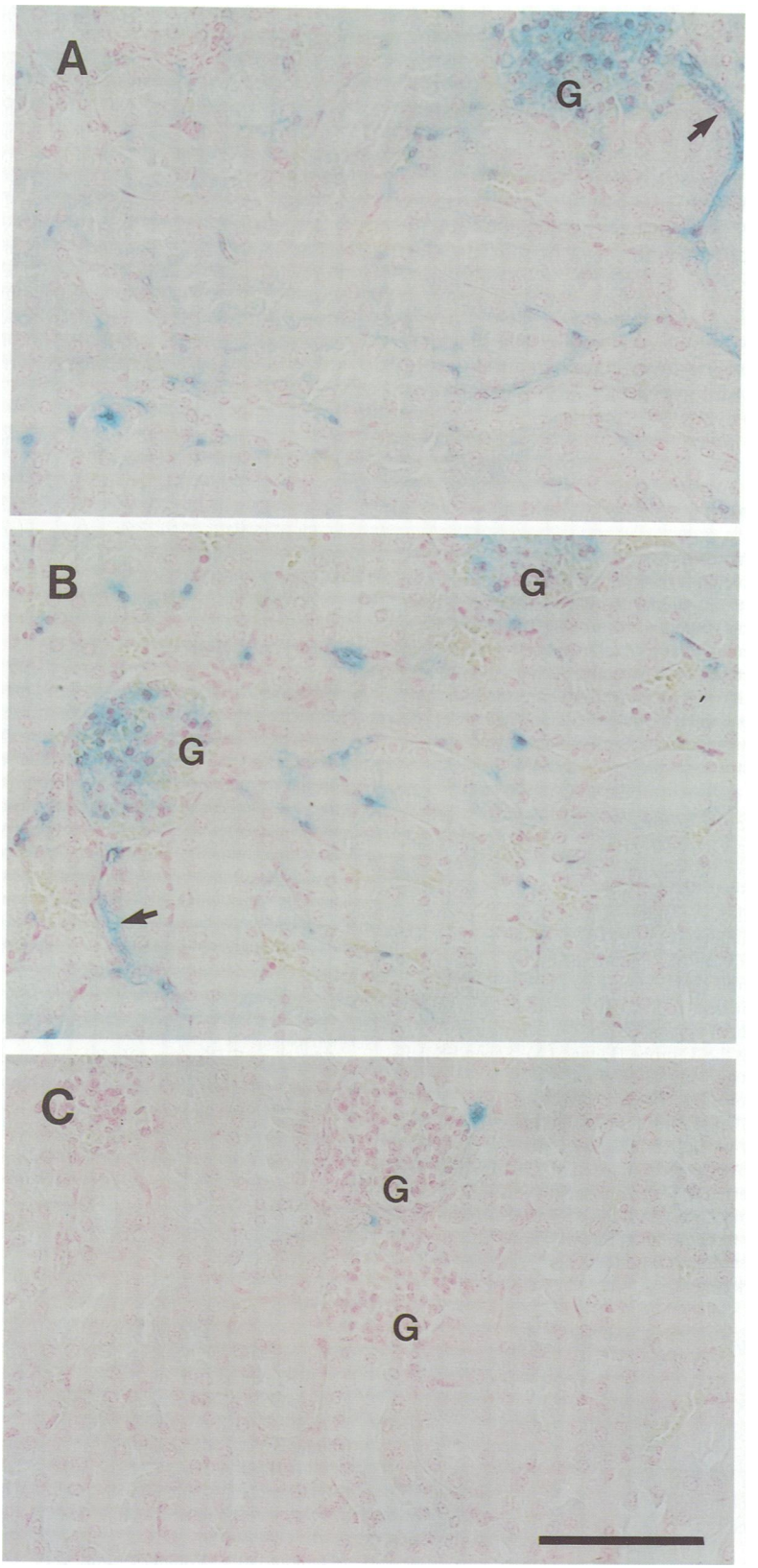

Fig. 4. Analysis of $tie^{lczn-}/+$ and tie^{lcz}/tie^{lczn-} ES cell contribution to the endothelial cells of chimeric adult kidney. β-Galactosidase staining of the kidney of (**A**) a tie^{lcz} heterozygous animal, (**B**) a $tie^{lczn-}/+$–CD-1 chimera and (**C**) a tie^{lcz}/tie^{lczn-}–CD-1 chimera. $Tie^{lczn-}/+$ (clone 10) or tie^{lcz}/tie^{lczn-} (clone 1) ES cells were aggregated with CD-1 embryos and adult chimeras were analyzed for ES cell contribution to the endothelium by β-galactosidase staining. The extent of chimerism in $tie^{lcz}/+$–CD-1 and tie^{lcz}/tie^{lczn-}–CD-1 chimeras was approximately the same (50%), as judged by coat color and Southern blot analysis (Figure 3). Interestingly, a few intensely staining non-endothelial cells were detected in the capsule of some glomeruli in all of the samples. The identity of these cells was not analyzed further. Glomeruli (G) and arterioles (arrows) are indicated. Bar 100 μm.

endothelial cell deficiency and severe hemorrhage (Dumont *et al.*, 1994). The later onset of the *Tie* phenotype is consistent with its slightly later onset of expression relative to *Tek*. Moreover, whereas *Tie* expression persists in most of the adult endothelium (Korhonen *et al.*, 1995; unpublished data), *Tek* expression is down-regulated during late embryonic development (Schnurch and Risau, 1993; Puri *et al.*, in preparation). Whether the down-regulation of *Tek* is coincident with onset of the vascular defect in *Tie* mutant embryos remains to be elucidated. The continued expression of *Tie* and its possible role in survival/proliferation of endothelial cells suggest that TIE and its ligand(s) may also be key factors in modulating human endothelial cell biology in several pathological situations (Folkman, 1995).

Materials and methods

Construction of the targeting vector

Murine (strain 129Sv) genomic clones flanking the signal sequence (ss) and the first Ig domain (Ig1) exons of the *Tie* gene (Korhonen *et al.*, 1994) were used to generate the targeting vector pPNT-TIE-LACZ. A 4.5 kb *Eco*RI–*Apa*I fragment containing 5′ flanking and 5′ untranslated sequences was fused to the bacterial *lacZ* gene and cloned as the 5′ arm into a modified pPNT vector having loxP sites flanking the *neo* gene (Shalaby *et al.*, 1995). A 3 kb *Bam*HI fragment containing half of the signal sequence exon and sequences from the first intron was used as the 3′ arm of the targeting vector.

Generation of tie^{lcz}/+ ES cells and tie^{lcz} mice

The pPNT-TIE-LACZ targeting vector (400 μg) was electroporated into 5×10^7 R1 ES cells derived from 129Sv strain (Nagy *et al.*, 1993) using a Bio-Rad gene pulser set at 250 V, 500 mF and plated on culture dishes coated with 0.1% gelatin. Cells were cultured for 7 days in positive–negative selection media containing G418 (150 μg/ml) and gancyclovir (2.2 μM). Addition of gancyclovir to the culture medium resulted in an 8-fold enrichment factor for homologous recombination events. Surviving colonies were isolated, trypsinized and replated onto 96-well plates coated with 0.1% gelatin. Southern blot analysis was performed on DNA purified from 55 colonies using the 3′ probe (Figure 1A). Seven ES cell clones were found to contain targeted events at the *Tie* locus (tie^{lcz}/+), giving a targeting frequency of ~1/8. These clones were subjected to Southern analysis using the 5′ probe to confirm the targeting events. Rearrangements in the vicinity of the *Tie* locus were not detected. Two independent clones (1C4 and 1A9) were aggregated to CD-1 blastomeres (Wood *et al.*, 1993) and transferred to foster mothers to generate several strong chimeras, which were mated to CD-1 mice. Chimeras from both lines transmitted the mutation through the germline, giving rise to two independent mouse lines. All analyses described here were performed in the CD-1/129Sv hybrid background.

Generation of tie^{lczn-}/+ and tie^{lcz}/tie^{lczn-} ES cells

One of the tie^{lcz}/+ ES cell lines (1C4; 5×10^6 cells) was electroporated with 40 μg pBS185 expression vector encoding the Cre recombinase of bacteriophage P1 (Sauer, 1993). The electroporated cells were plated on mouse embryonic fibroblast feeder cells at low density and random ES cell colonies were picked after 7 days in culture. The Cre-mediated recombination events between the two loxP sites around the *neo* gene were screened by Southern blotting using a *lacZ* probe and *Nco*I digest (Figure 1). Correctly recombined tie^{lczn-}/+ ES cell lines were obtained with a frequency of 5/44.

The tie^{lczn-}/+ ES cell lines were re-targeted with the pPNT-TIE-LACZ vector as described above to obtain tie^{lcz}/tie^{lczn-} ES cell lines (2). Untargeted tie^{lczn-}/+ ES cell lines which had undergone the same selection procedure were saved as control lines.

Genotyping of progeny

To identify mice containing the tie^{lcz} allele Southern blot analysis was performed on genomic DNA purified from tail biopsies of 3- to 4-week-old mice and from the yolk sacs of mouse embryos.

Whole mount β-galactosidase staining of embryos and tissues

Embryos or kidney samples to be stained were dissected in phosphate-buffered saline, fixed in 0.2% glutaraldehyde, 1.5% formaldehyde, 2 mM $MgCl_2$, 5 mM EGTA in 100 mM phosphate buffer at room temperature for 60–90 min, depending on the size, and then washed three times at room temperature in wash buffer (100 mM phosphate buffer plus 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$) for 20 min each. Samples were then stained in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% deoxycholate in 100 mM phosphate buffer at room temperature for 12–18 h (kidney samples up to 48 h). Following LacZ staining, samples were washed at 4°C in the above wash buffer overnight, followed by fixation in 3.7% formaldehyde overnight. Fixed embryos were dehydrated through graded ethanols and embedded in paraffin. Sections were cut at 5 μm, mounted onto glass slides, dewaxed and stained with nuclear fast red.

Chimeric analysis

The tie^{lcz}/tie^{lczn-} and tie^{lczn-}/+ ES cell lines (GPI-1 AA) were aggregated with CD-1 (GPI-1 AA) or CD-1 (GPI-1 BB) embryos using the morula aggregation technique (Wood *et al.*, 1993) and the aggregates were transferred into uteri of CD-1 foster mothers. Samples of the tails of resulting adult tie^{lcz}/tie^{lczn-}–CD-1 and tie^{lczn-}/+–CD-1 chimeras were taken for Southern blot analysis and the kidneys were used for staining for β-galactosidase activity.

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Note added in proof

Embryos homozygous for the tie^{lacz-} allele show a phenotype identical to the tie^{lacz} homozygotes described here. Sato *et al.* (*Nature*, **376**, 70–74) have also recently reported the phenotype of mice with a targeted mutation in *Tie*. In their study most of the homozygous *Tie* mutant mice died as neonates and displayed edema and localized hemorrhage. The different phenotypes of *Tie* mutant embryos between the two studies may be attributed to a difference in the genetic backgrounds of the two mutations. This possibility is further supported by our recent observation that viability of homozygous tie^{lacz} embryos is influenced by further breeding of the tie^{lacz} allele into CD-1 mouse background.