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Cell autonomous functions of the receptor tyrosine kinase TIE in a late phase of angiogenic capillary growth and endothelial cell survival during murine development

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SUMMARY

TIE is a receptor tyrosine kinase expressed in both mature endothelial cells and their precursors, as well as in some hematopoietic cells. Mouse embryos homozygous for a disrupted *Tie* allele die at midgestation due to impaired endothelial cell integrity and resulting hemorrhage. Here we have performed chimeric analysis to study further the function of the murine TIE in the development of embryonic vasculature and in the hematopoietic system. Cells lacking a functional *Tie* gene (tie^{lcz}/tie^{lczn-} cells) contributed to the embryonic vasculature at E10.5 as efficiently as cells heterozygous for a targeted *Tie* allele (tie^{lcz}/+ cells). Thus, TIE does not play a significant role in vasculogenesis or in early angiogenic processes, such as formation of the intersomitic arteries and limb bud vascularization. At E15.5 tie^{lcz}/tie^{lczn-} cells still readily contributed to major blood vessels and to endothelial cells of organs such as lung and heart, which have been suggested to be vascularized by angioblast differentiation. In contrast, the tie^{lcz}/tie^{lczn-} cells were selected against in the capillary plexuses of several angiogenically vascularized tissues, such as brain and kidney. Our results thus support a role for TIE in late phases of angiogenesis but not vasculogenesis. Furthermore, the results suggest that different mechanisms regulate early and late angiogenesis and provide support for a model of differential organ vascularization by vasculogenic or angiogenic processes. Analysis of adult chimeras suggested that TIE is required to support the survival or proliferation of certain types of endothelial cells demonstrating heterogeneity in the growth/survival factor requirements in various endothelial cell populations.

Chimeric analysis of adult hematopoietic cell populations, including peripheral platelets and bone marrow progenitor cells, revealed that tie^{lcz}/tie^{lczn-} cells were able to contribute to these cell types in a way indistinguishable from tie^{lcz}/+ or wild-type cells. Thus, the primary function of TIE appears to be restricted to the endothelial cell lineage.

Key words: receptor tyrosine kinase, endothelial cell, microvasculature, angiogenesis, megakaryocyte, gene targeting, mouse

INTRODUCTION

The development of the vertebrate embryonic circulatory system begins soon after the mesodermal cells emerge from the primitive streak. Cell clusters, called blood islands, are formed extraembryonically in the yolk sac mesoderm possibly in response to an endoderm-derived signal (Wilt, 1965). Within these clusters, differentiation of vascular endothelial cells is closely linked to the appearance of primitive hematopoietic cells and thus these two lineages have been suggested to share a common ancestor, the hemangioblast (Sabin, 1920; Murray, 1932). Simultaneously, endothelial cell precursors called angioblasts differentiate in the mesoderm of the embryo proper. The angioblasts differentiate either in situ or after directed migration into endothelial cell cords (Coffin and Poole, 1988; Pardanaud et al., 1987; Coffin et al., 1991). The early vasculature, including the dorsal aortae and cardinal veins as well as heart endocardium, is formed by this de novo mechanism, which has been termed vasculogenesis. In contrast to yolk sac vasculogenesis, intraembryonic vasculogenesis is generally not accompanied by hematopoiesis. An exception is the para-aortic region, which is a rich source of hematopoietic stem cell activity and might harbor the precursors of the definitive hematopoietic system (Godin et al., 1993, 1995; Medvinsky et al., 1993).

The early vascular tree is expanded by endothelial cell migration and proliferation resulting in vessel sprouting, a process termed angiogenesis. The intersomitic arteries, the

limb bud vasculature as well as the vasculature of several organs such as brain and kidney are thought to be formed by this mechanism, as suggested by morphological studies and avian interspecies organ transplantation experiments (Coffin and Poole, 1988; Jotereau and LeDouarin, 1978; Stewart and Wiley, 1981; Pardanaud et al., 1989; Sariola et al., 1984; Ekblom et al., 1982). However, vasculogenesis has still been observed during avian organogenesis, especially in endoderm containing organ rudiments such as lung, liver, pancreas and intestine (Pardanaud et al., 1989). In the adult, most of the endothelium is in a quiescent state and the remaining vascularization in the female reproductive system and during wound healing or tumorigenesis occurs by angiogenic growth of capillaries.

Isolation and mutation of receptor tyrosine kinases expressed in endothelial cells has recently provided insight into intercellular molecular mechanisms regulating murine vasculogenesis and angiogenesis. The receptors for the vascular endothelial cell growth factor (VEGF) family, namely FLK-1, FLT-1 and FLT-4, as well as the orphan receptors TEK (TIE-2) and TIE (TIE-1; for a review see Mustonen and Alitalo, 1995) are expressed in succession in angioblasts and mature endothelial cells (Dumont et al., 1995). Disruption of signaling through FLK-1 in mouse embryos leads to a block in angioblast/hemangioblast differentiation and no mature endothelial cells or primitive hematopoietic cells are formed (Shalaby et al., 1995). In contrast, homozygosity for a mutation in *Flt*-1 results in strikingly abundant endothelial cell differentiation and failure in the correct assembly of the vessel endothelium (Fong et al., 1995). Thus an interplay between the two VEGF receptors appears to be required for controlled early vascular development. In addition to their role in embryonic vasculogenesis, VEGF and its receptors are involved in angiogenic growth both during embryonic development and tumor vascularization (Flamme et al., 1995; Millauer et al., 1994; Shweiki et al., 1992; Dumont et al., 1995). Embryos homozygous for a disrupted allele of the receptor tyrosine kinase *Tek* show abnormal development of the heart and vasculature and die around E9.5 (Dumont et al., 1994; Sato et al., 1995). In *Tek* mutant embryos endothelial cells initially form, but their survival and proliferation is affected, resulting in deficient early angiogenesis.

We and others have previously analyzed the phenotypic effect of a null mutation in the murine *Tek*-related gene *Tie* (Sato et al., 1995; Puri et al., 1995). In contrast to the early lethality of *Tek* mutant embryos, embryos homozygous for a *Tie* mutation appear to be normal until E13. Thereafter, mutant embryos display edema and localized hemorrhage, presumably due to impaired survival and integrity of capillary endothelial cells. As a result, the majority of the mutant embryos die before E15.5. A cell autonomous requirement for *Tie* in endothelial cells of the adult kidney was demonstrated by chimeric analysis using embryonic stem (ES) cell lines with both *Tie* alleles disrupted (Puri et al., 1995).We have here performed a detailed chimeric analysis of TIE function in the embryonic and adult vasculature. Our results show that in contrast to FLK-1, FLT-1 and TEK, TIE does not play a significant role in angioblast differentiation or early angiogenic vessel growth, but supports later angiogenesis and endothelial cell proliferation. The results also indicate that there is heterogeneity in the requirement for TIE in various endothelial cell populations and support the concept of differential organ vascularization, first suggested by the avian transplantation chimera experiments (Pardanaud et al., 1989).

TIE is also expressed in some hematopoietic cell lineages, including several human megakaryoblastic leukemia cell lines (Partanen et al., 1990; Armstrong et al., 1993; Hashiyama et al., 1996). Human and murine hematopoietic stem cells have also been suggested to express TIE in vivo (Hashiyama et al., 1996; Iwama et al., 1993; Batard et al., 1996). However, using chimeric mice we could detect no functional consequence of the loss of TIE in hematopoietic cell lineages.

MATERIALS AND METHODS

Generation of chimeras

The generation of the tie^{lcz} and tie^{lczn-} alleles has been described previously (Puri et al., 1995). Briefly, the tie^{lcz} allele was created by homologous recombination events placing a bacterial *lacZ* gene under the control of the *Tie* promoter. The tie^{lczn-} allele was derived from tie^{lcz} by removing a *neo* gene following *lacZ* by Cre mediated recom-

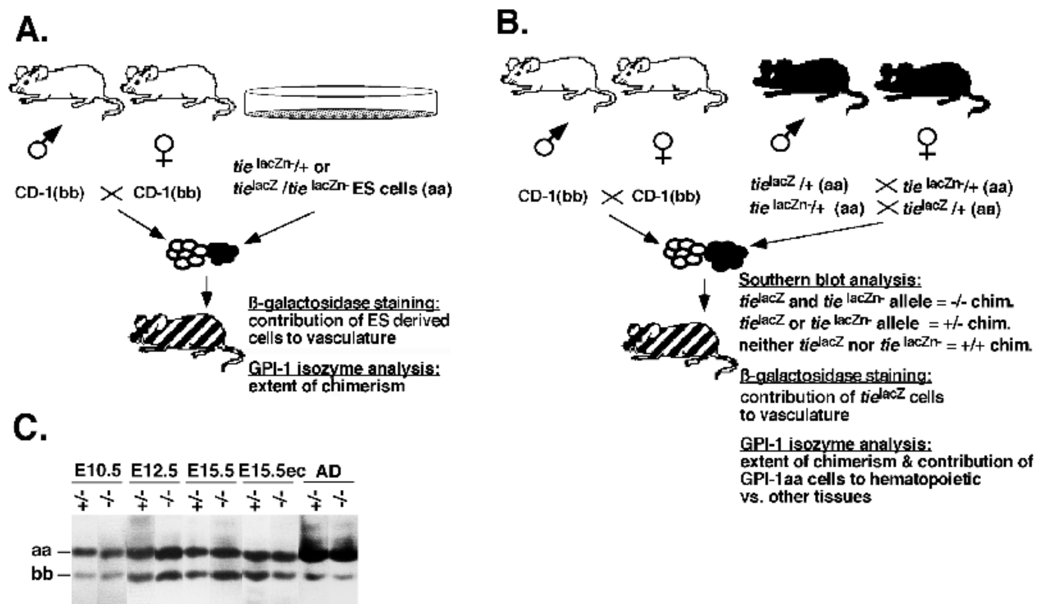

Fig. 1. Generation of chimeras. (A) ES cell↔morula aggregation strategy. (B) Morula↔morula aggregation strategy. (C) Determination of the extent of chimerism by GPI-1 isoenzyme analysis of tail or brain tissue (adult chimeras) samples of the chimeras shown in Figs 2-5. ec, morula↔morula chimeras; AD, adult chimeras; aa, GPI-1^{aa}; bb, GPI-1^{bb}.

bination. The tie^{lcz}/tie^{lczn-} and $tie^{lcz}/+$ ES cell lines (glucose phosphate isomerase-1 isotype aa, 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 (Fig. 1A).

In another set of experiments, both the tie^{lcz} and tie^{lczn-} alleles were transmitted through the germline by a tie^{lcz}/tie^{lczn-} ↔ CD-1 chimera. This allowed us to make chimeras using an embryo↔embryo aggregation strategy (Fig. 1B). Morula stage embryos from $tie^{lcz}/+$ (GPI-1^{aa}) × $tie^{lczn-}/+$ (GPI-1^{aa}) matings were aggregated with morula stage CD-1 (GPI-1^{bb}) embryos as described above. The yolk sac or tail DNA samples of the resulting chimeras were analyzed by Southern blotting for the presence of tie^{lcz} or tie^{lczn-} alleles (Puri et al., 1995). Samples from the tail or various other tissues of the chimeras were used for determination of the extent of chimerism by GPI-1 isoenzyme analysis.

β-galactosidase staining of chimeric embryos and tissues

Embryos or tissue samples to be stained were fixed in 0.2% glutaraldehyde, 1.5% formaldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 100 mM sodium phosphate pH 7.3 at room temperature for 30-90 minutes depending on the size. The E12.5 and E15.5 embryos were cut in half 30 minutes from the beginning of the fixation and fixation was continued for another 30-45 minutes. The embryos or tissue samples were then washed three times at room temperature in wash buffer (0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$, 100 mM sodium phosphate pH 7.3) for 20 minutes each. Samples were stained in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$, 100 mM sodium phosphate pH 7.3 at room temperature for 18-48 hours. Following β-galactosidase 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 counterstained with Nuclear Fast Red.

Isolation of platelets and bone marrow hematopoietic colony assays

After cervical dislocation of adult chimeras, 0.5-1 ml peripheral blood samples were taken from the heart and diluted with one volume of Rossi's buffer (140 mM NaCl, 5 mM glucose, 7 mM citric acid pH 6.5). The samples were repeatedly centrifuged at 160 ***g*** for 2 minutes until no red blood cells were pelleted and the platelets were collected by centrifugation at 2000 ***g***. The platelets were washed once in Rossi's buffer and their purity was checked under a microscope. Splenocytes and thymocytes were also collected and red blood cells were lysed from the preparations by incubation in 0.16 M ammonium chloride.

Hematopoietic colony assays were performed on single cell suspensions of bone marrow from chimeric mice. In brief, 2×10^5 cells were plated in 1.5 ml of 0.9% methyl cellulose (Terry Fox Laboratories) in IMDM (Gibco) supplemented with 25% FCS (Hyclone), 2% IL-3 conditioned medium (Karasuyama and Melchers, 1988) muEpo (2.5 U/ml, Boehringer) and monothioglycerol (4.5×10^{-4} M) and incubated in a humidified CO_2 atmosphere at 37°C. After 8-10 days colonies were pooled, washed with PBS and subjected to GPI-1 analysis.

Glucose phosphate isomerase-1 isoenzyme analysis

Samples from different hematopoietic cell populations and other tissues as well as tail samples of chimeric embryos were freeze-thawed and diluted in water for glucose phosphate isomerase-1 isoenzyme analysis. The samples were run on cellulose acetate plates (cat. no. 3024, Helena Laboratories, Beaumont, Texas) and the color reaction was performed as described by Nagy and Rossant (1993).

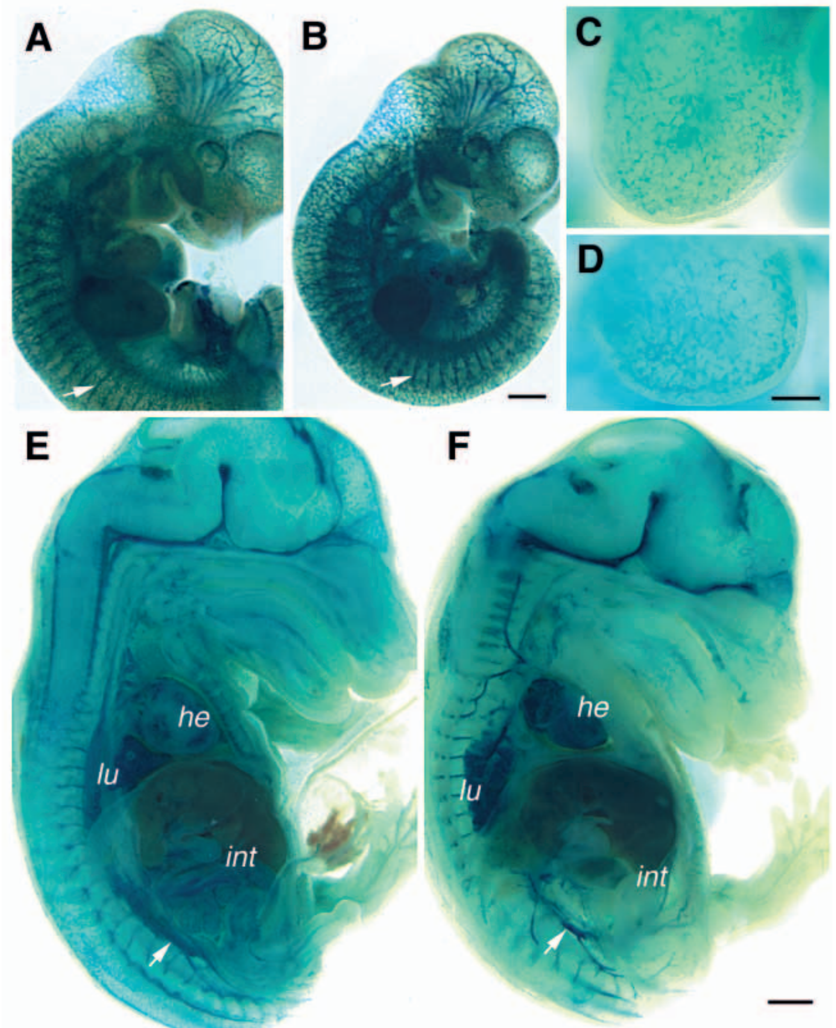

Fig. 2. Analysis of tie^{lcz}/tie^{lczn-} cell contribution in the endothelium of chimeras at E10.5 and E15.5. The $tie^{lcz}/+$ or tie^{lcz}/tie^{lczn-} cell derived capillaries were visualized by whole-mount β-galactosidase staining. An E10.5 $tie^{lcz}/+$ ↔ CD-1 chimera (A) and a slightly less advanced tie^{lcz}/tie^{lczn-} ↔ CD-1 chimera (B) show no observable differences in the abundance of the ES cell-derived endothelium. Arrows indicate intersomitic vessels. Close-ups of the front limb buds of the $tie^{lcz}/+$ ↔ CD-1 chimera (C) and the tie^{lcz}/tie^{lczn-} ↔ CD-1chimera (D) show no differences in the ES cell contribution to capillary endothelium. Both E10.5 chimeras shown were approximately 70% ES cell derived as judged by GPI-1 isoenzyme analysis. Comparison of a E15.5 $tie^{lcz}/+$ ↔ CD-1 chimera (E) and a corresponding tie^{lcz}/tie^{lczn-} ↔ CD-1chimera (F) shows unimpaired contribution of tie^{lcz}/tie^{lczn-} cells to the endothelium of major vessels such as aorta (arrow) as well as heart (he) and lung (lu). Compared with the $tie^{lcz}/+$ ↔ CD-1 chimera, fewer tie^{lcz}/tie^{lczn-} cell derived capillaries are observed, for example in the intestine (int). Both E15.5 chimeras were approximately 60% ES cell derived (see Fig. 1C). The embryos were cut in half before staining. Scale bars, (A,B) 500 µm; (C,D) 200 µm, (E,F) 1 mm.

RESULTS

tie^{lcz}/tie^{lczn-} cells contribute normally to the embryonic vasculature at E10.5

To analyze the function of TIE during early vascularization, tie^{lcz}/tie^{lczn-} ↔ CD-1 (n=9) and $tie^{lcz}/+$ ↔ CD-1 (n=5) chimeras were generated by ES-cell↔morula aggregation technique (Fig. 1A) and analyzed at E10.5. The presence of the *lacZ* gene in the *Tie* locus allowed us to identify tie^{lcz}/tie^{lczn-} or $tie^{lcz}/+$ cell-derived endothelial cells by β-galactosidase staining (Puri et al., 1995). At this stage of development there was no apparent difference in the ability of the tie^{lcz}/tie^{lczn-} or $tie^{lcz}/+$ cells to contribute to the embryonic endothelium (Fig. 2A,B) including intersomitic arteries as well as capillaries of the limb buds (Fig. 2C,D). As determined by GPI-1 isoenzyme analysis of tail samples of the embryos, both chimeras shown in Fig. 2A-D were approximately 70% ES-cell derived (Fig. 1C).

tie^{lcz}/tie^{lczn-} cells are underrepresented in capillaries undergoing angiogenic growth at E15.5

In more advanced ES-cell↔morula aggregation chimeras analyzed at E15.5 the tie^{lcz}/tie^{lczn-} cells still readily contributed to the endothelium of major vessels such as the aorta and intercostal arteries as well as to the endothelia of the heart and lung (Fig. 2E,F). In contrast, there was a marked reduction in β-galactosidase-expressing endothelial cells in several capillary plexuses including the capillaries of the midbrain, intercostal area, skin and the intestine of the tie^{lcz}/tie^{lczn-} ↔ CD-1 (n=4) chimeras compared to corresponding $tie^{lcz}/+$ ↔ CD-1 (n=4) chimeras (Figs 2E,F, 3C-H). The first signs of selection against tie^{lcz}/tie^{lczn-} cells in the midbrain capillaries were present already at E12.5 (Fig. 3A, B). All the E12.5 and E15.5 chimeras shown in Figs 2 and 3 were approximately 60% ES-cell derived as judged by GPI-1 isoenzyme analysis of tail samples of the embryos (Fig. 1C), indicating that the loss of tie^{lcz}/tie^{lczn-} cells in the capillary plexuses is specific.

Histological analysis of the E15.5 chimeras shown in Figs 2 and 3 revealed that the number of capillaries was normal in the tie^{lcz}/tie^{lczn-} ↔ CD-1 chimeras, but the capillaries were mostly made of wild-type endothelial cells not expressing the β-galactosidase marker. This was clearly seen in the midbrain, bladder and intercostal area (Fig. 4A,B,G,H,M). There was also a clear reduction in ES-cell-derived capillary endothelial cells in the kidney, adrenal gland and intestine of the tie^{lcz}/tie^{lczn-} ↔ CD-1 chimeras compared to corresponding $tie^{lcz}/+$ ↔ CD-1 chimeras (Fig. 4C-F and data not shown). Interestingly, tie^{lcz}/tie^{lczn-} cells still strongly contributed to the endothelium of the lung and aorta (Fig. 4I, J) as well as to the endocardium of the atria and the endothelium of the ventricles (Fig. 4K,L). These results were reproduced using

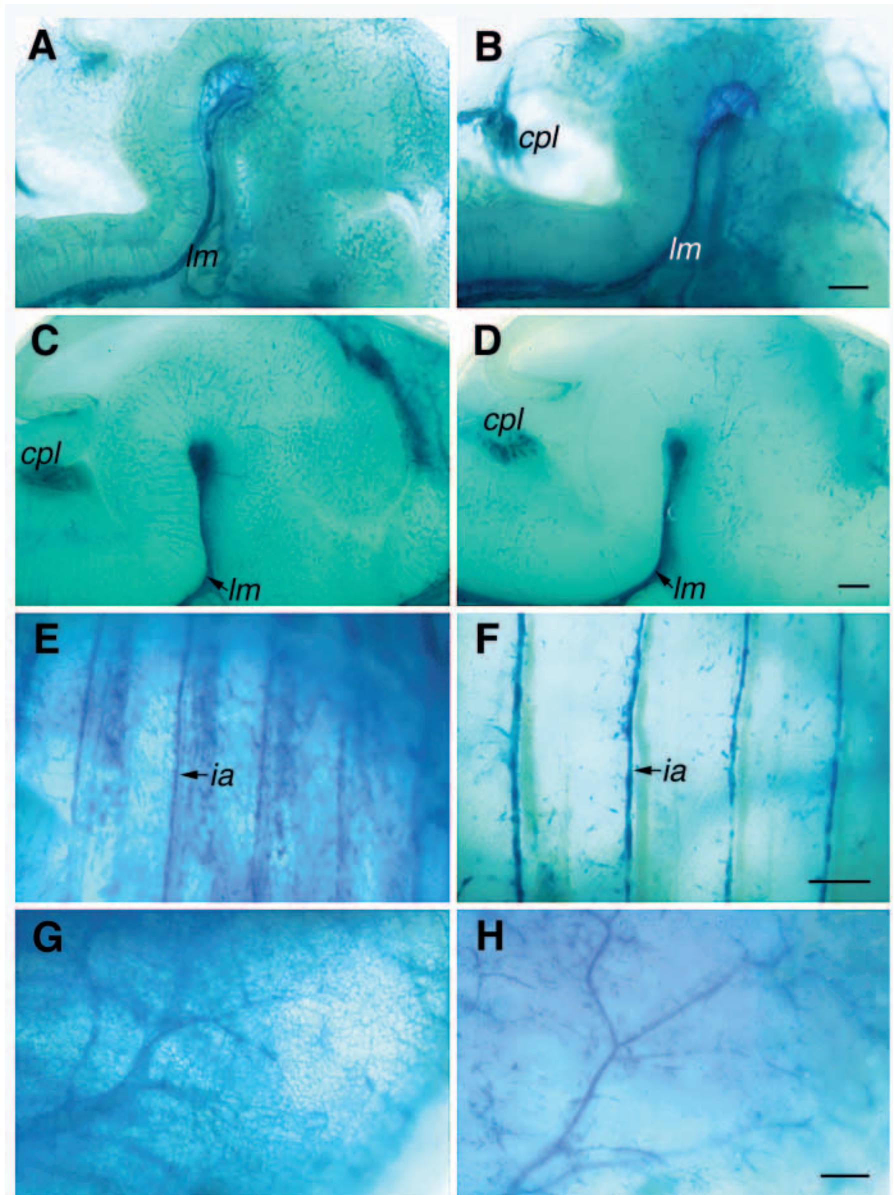

Fig. 3. Analysis of tie^{lcz}/tie^{lczn-} cell contribution into various capillary plexuses of chimeras at E12.5 and E15.5. Whole-mount β-galactosidase staining of the midbrain-hindbrain of an E12.5 $tie^{lcz}/+$ ↔ CD-1 chimera (A) and a corresponding tie^{lcz}/tie^{lczn-} ↔ CD-1 chimera (B) shows $tie^{lcz}/+$ or tie^{lcz}/tie^{lczn-} derived brain capillaries sprouting from the leptomeninges. Whole-mount β-galactosidase staining of the midbrain-hindbrain (C,D) intercostal area (E,F) and skin (G,H) of a E15.5 $tie^{lcz}/+$ ↔ CD-1 chimera (C,E,G) and a corresponding tie^{lcz}/tie^{lczn-} ↔ CD-1 chimera (D,F,H) demonstrates underrepresentation of tie^{lcz}/tie^{lczn-} cells in the capillary plexuses. A-D are from ES cell↔morula chimeras; E-H are from morula↔morula chimeras. All chimeras were approximately 60% GPI-1^{aa} cell derived (see Fig. 1C). Posterior choroid plexus, cpl; leptomeninges, lm; intercostal artery, ia. Scale bars, 200 µm.

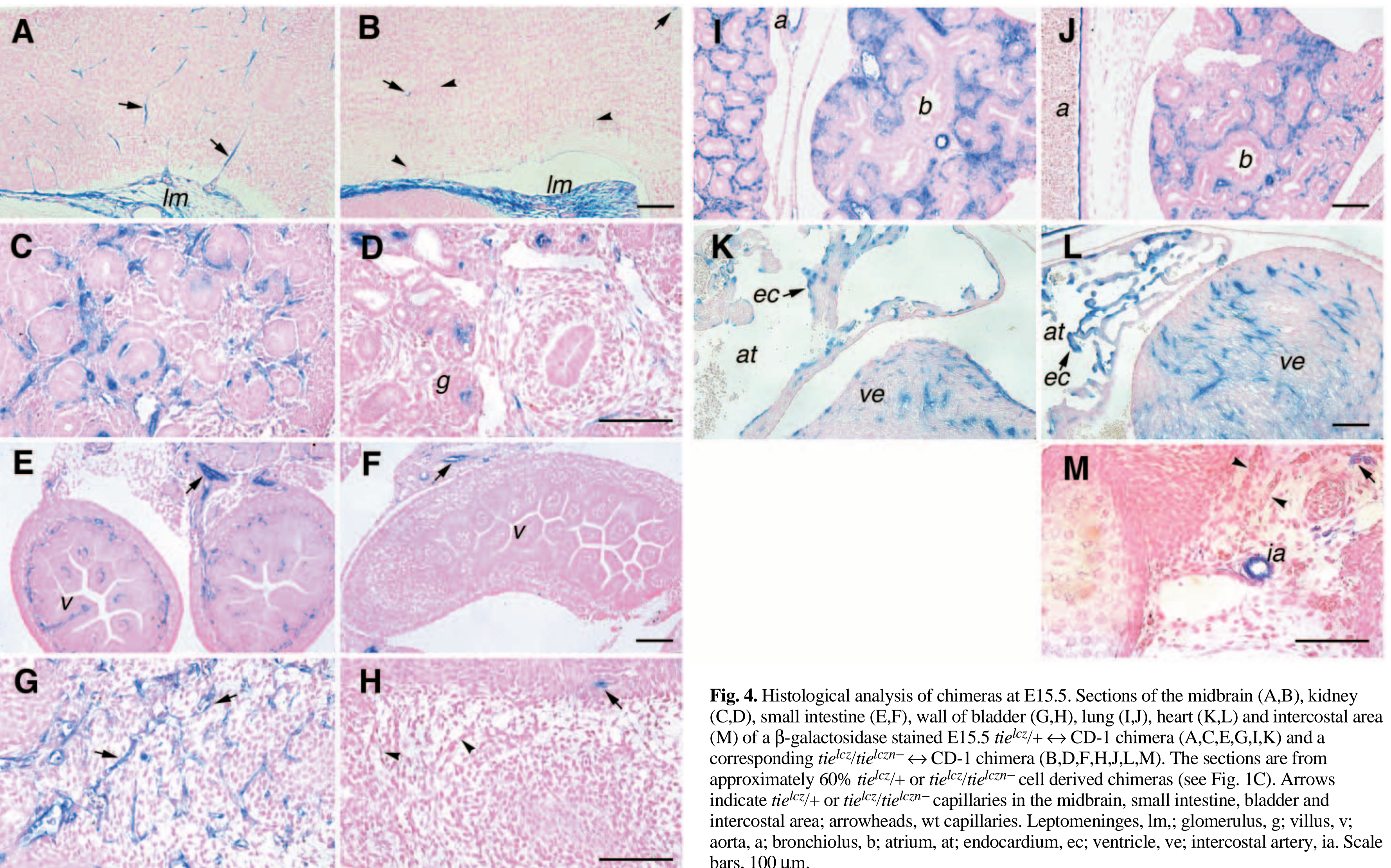

Fig. 4. Histological analysis of chimeras at E15.5. Sections of the midbrain (A,B), kidney (C,D), small intestine (E,F), wall of bladder (G,H), lung (I,J), heart (K,L) and intercostal area (M) of a β-galactosidase stained E15.5 $tie^{lcz}/+ \leftrightarrow$ CD-1 chimera (A,C,E,G,I,K) and a corresponding $tie^{lcz}/tie^{lczn-} \leftrightarrow$ CD-1 chimera (B,D,F,H,J,L,M). The sections are from approximately 60% $tie^{lcz}/+$ or tie^{lcz}/tie^{lczn-} cell derived chimeras (see Fig. 1C). Arrows indicate $tie^{lcz}/+$ or tie^{lcz}/tie^{lczn-} capillaries in the midbrain, small intestine, bladder and intercostal area; arrowheads, wt capillaries. Leptomeninges, lm,; glomerulus, g; villus, v; aorta, a; bronchiolus, b; atrium, at; endocardium, ec; ventricle, ve; intercostal artery, ia. Scale bars, 100 µm.

chimeras generated by the morula↔morula aggregation strategy (Fig. 1B). In approximately 90% mutant cell derived chimeras more tie^{lcz}/tie^{lczn-} cell derived capillary endothelial cells were detected but they were still at a clear disadvantage when compared with $tie^{lcz}/+$ cells (data not shown). Many of the strong tie^{lcz}/tie^{lczn-} ↔ CD-1 chimeras died before E15.5.

Analysis of adult chimeras suggests continued selection against specific tie^{lcz}/tie^{lczn-} cell derived capillary endothelial cells

Strong adult (12-week old) tie^{lcz}/tie^{lczn-} ↔ CD-1 (n=3) and $tie^{lcz}/+$ ↔ CD-1 (n=3) chimeras (approximately 90% mutant cell derived) were produced by both ES cell↔morula and morula↔morula aggregation techniques (Fig. 1A,B). No tie^{lcz}/tie^{lczn-} cell derived capillaries were detected in the kidneys and adrenals by β-galactosidase staining (Fig. 5A-D), despite some contribution in E15.5 and newborn chimeras (Fig. 4D and data not shown). In the lung the number of tie^{lcz}/tie^{lczn-} cell derived endothelial cells was also greatly reduced (Fig. 5E,F), although tie^{lcz}/tie^{lczn-} cells strongly contributed to various types of lung endothelium at E15.5 (Fig. 4J). Interestingly, patches of tie^{lcz}/tie^{lczn-} cell derived capillaries were still detected in this organ in 4-week old chimeras (n=3, data not shown). Thus there appears to be continuous selection against the tie^{lcz}/tie^{lczn-} endothelial cells after their initial differentiation in the lung. In contrast, the capillaries in the heart myocardium as well as the heart endocardium and some major vessels of the 12-week old chimeras still contained many tie^{lcz}/tie^{lczn-} endothelial cells (Fig. 5G-I). However, analysis of several sections suggested that the tie^{lcz}/tie^{lczn-} endothelial cells were still slightly selected against in the myocardial capillaries. The GPI-1 isoenzyme analysis of the chimeras in Fig. 5 is shown in Fig. 1C.

tie^{lcz}/tie^{lczn-} cells can contribute to several hematopoietic cell populations and bone marrow hematopoietic progenitors

To avoid chimeric drift resulting from differences in the genetic backgrounds of 129Sv-derived ES cells and CD-1 embryos as well as possible epigenetic changes occurring in the ES cells (Berger et al., 1995), the analysis of TIE function in various hematopoietic populations was performed using chimeras generated by the morula↔morula aggregation strategy. In this situation both mutant and host embryos have approximately the same genetic background (129Sv-CD-1 hybrid vs. CD-1, Fig. 1B). The tie^{lcz} and tie^{lczn-} alleles were bred into a CD-1 (GPI-1^{aa}) background, whereas the wild-type host embryos were CD-1 (GPI-1^{bb}), thus allowing the identification of the cells derived from the two embryos by GPI-1 isoenzyme analysis.

Ear punch (control tissue) and peripheral blood samples were taken from three week old +/+ ↔ CD-1 (n=7), $tie^{lcz}/+$ ↔ CD-1 (n=4) and tie^{lcz}/tie^{lczn-} ↔ CD-1 (n=9) chimeras and

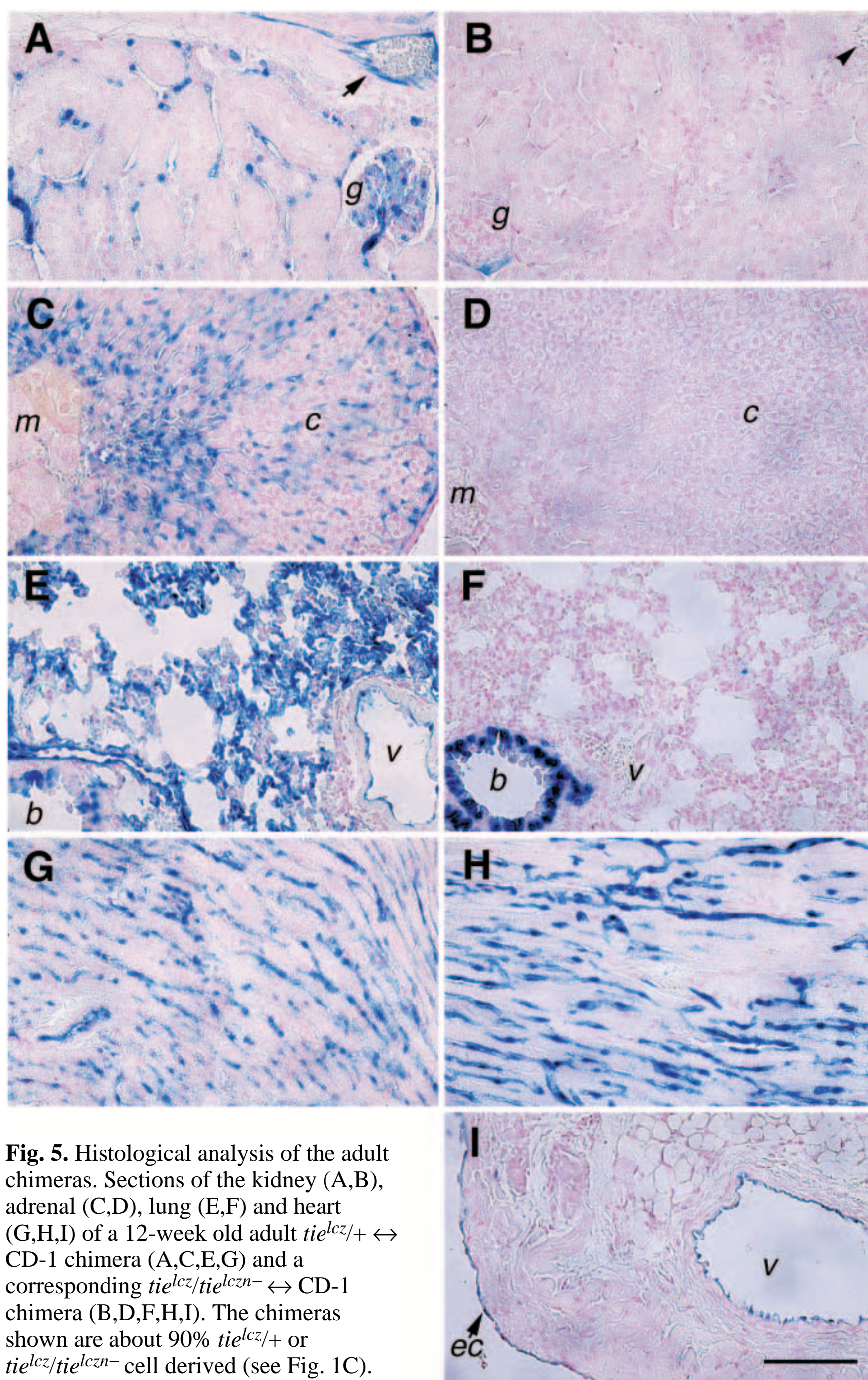

Fig. 5. Histological analysis of the adult chimeras. Sections of the kidney (A,B), adrenal (C,D), lung (E,F) and heart (G,H,I) of a 12-week old adult $tie^{lcz}/+$ ↔ CD-1 chimera (A,C,E,G) and a corresponding tie^{lcz}/tie^{lczn-} ↔ CD-1 chimera (B,D,F,H,I). The chimeras shown are about 90% $tie^{lcz}/+$ or tie^{lcz}/tie^{lczn-} cell derived (see Fig. 1C). Nonendothelial background staining was detected in the epithelium of bronchioli of the lung as well as Bowman's capsule of the glomeruli of the kidney. Arrow indicates $tie^{lcz}/+$ endothelium, arrowhead, wild-type endothelium. Artery, a; glomerulus, g; cortex, c; medulla, m; bronchiolus, b; vein, v; endocardium, ec. Scale bar, 100 µm.

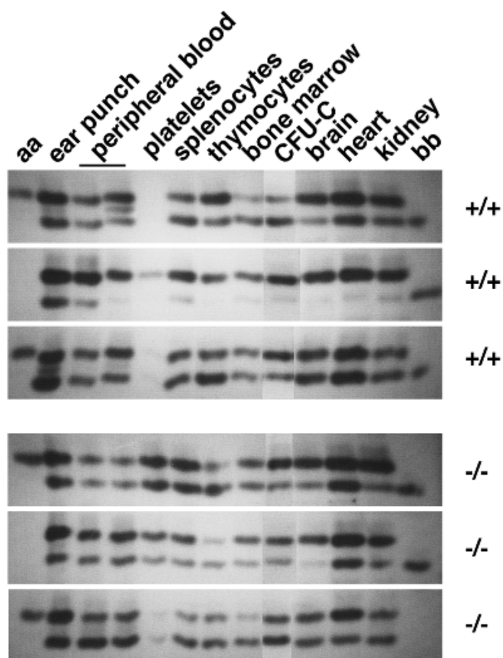

Fig. 6. Contribution of tie^{lcz}/tie^{lczn-} cells to various hematopoietic populations. GPI-1 isoenzyme analysis of various hematopoietic cell populations and bone marrow progenitor cells of three +/+ ↔ CD-1 chimeras (+/+), and three tie^{lcz}/tie^{lczn-} ↔ CD-1chimeras (–/–). The chimeras shown were 4 week old littermates. The first peripheral blood sample was taken at the age of 2.5 weeks.

analyzed for their GPI-1 isoenzyme composition. Generally, the ear punch and peripheral blood samples of +/+ ↔ CD-1, tie^{lcz}/+ ↔ CD-1 as well as tie^{lcz}/tie^{lczn-} ↔ CD-1 chimeras were more or less equivalent in their GPI-1^{aa} cell contribution, although some random variation was observed (data not shown). Analysis of the various hematopoietic cell populations of these chimeras revealed that the tie^{lcz}/tie^{lczn-} cells readily contributed to thymocyte, splenocyte and peripheral platelet populations as well as to in vitro cultured bone marrow progenitor cells (CFU-C; Fig. 6).

Histological sections of fetal liver of E15.5 tie^{lcz}/tie^{lczn-} ↔ CD-1 as well as tie^{lcz}/+ ↔ CD-1 chimeras revealed β-galactosidase expressing megakaryocytes in both cases (Fig. 7). Thus, TIE appears to be expressed in megakaryocytes in vivo, but this expression is not required for their differentiation.

DISCUSSION

Several factors have been observed to induce endothelial cell proliferation, vessel formation and capillary sprouting in both in vitro and in vivo assays (Folkman and Shing, 1992). How these findings relate to the formation of the vascular tree during embryogenesis is often less well understood. We have approached the question of developmental regulation of vascularization from a different angle using gene deletion experiments in the mouse. Our previous studies showed that homozygosity for a null mutation in the endothelial cell expressed receptor tyrosine kinase, *Tie*, results in embryonic lethality after E13.5, due to loss of vascular integrity (Puri et al., 1995). Using chimeras generated by aggregation of ES cells having no functional *Tie* gene with wild-type embryos we further demonstrated a cell autonomous requirement for TIE in the development of endothelial cells of the adult mouse kidney. However, the exact time and place of the requirement for TIE signaling remained unresolved.

Using chimeric analysis we have defined the function of TIE during different phases of vascular development more carefully. Analyses of both avian and murine embryos have suggested that de novo differentiation of angioblasts generates the major vessels, such as the dorsal aorta, cardinal and vitelline veins as well as heart endocardium (Coffin et al., 1991; Pardanaud et al., 1987; Coffin and Poole, 1988). Our results showed that up till E10.5 cells without a functional *Tie* gene were found in all of the embryonic vasculature suggesting no role for TIE alone in the early differentiation of endothelial cells despite the expression of TIE in the angioblasts (Korhonen et al., 1994). Soon after the de novo formation of the major vessels the vascular tree is expanded by vessel sprouting. Because *Tie* mutant cells readily contributed to the presumably angiogenically derived vessels, like the intersomitic arteries and limb bud vasculature (Coffin and Poole, 1988; Jotereau and LeDouarin, 1978), TIE does not appear to be necessary for an early phase of angiogenic vessel growth. Instead, early angiogenic growth as well as heart development seem to be dependent on expression of the closely related receptor tyrosine kinase TEK (Dumont et al., 1994; Sato et al., 1995).

The chimeric analysis described here has revealed a major function for TIE during organogenesis at midgestation. These results also further demonstrated differential contributions of vasculogenesis and angiogenesis to the formation of the vascular tree. Vascularization during organogenesis has been studied by quail-chicken interspecies organ rudiment transplantation chimeras. In these studies certain, preferentially endoderm containing rudiments, such as lung, liver and intestine, have been suggested to undergo vasculogenesis (Pardanaud et al., 1989). Based on morphological studies, differentiation of blood island-like structures in situ is also thought to give rise to the coronary capillary network of the heart (Hutchins et al., 1988; Hirakow, 1983) although their precursors have been suggested to have an extracardiac source

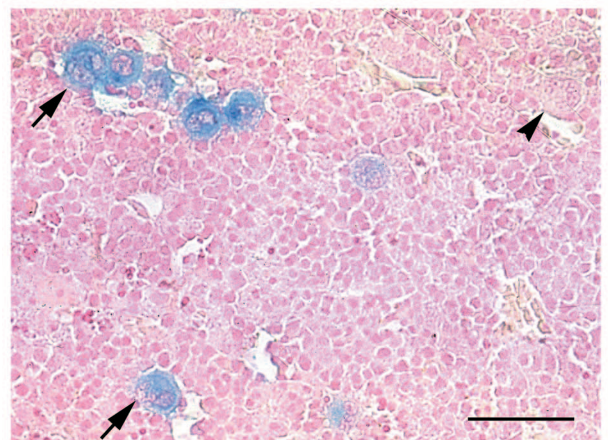

Fig. 7. Expression of *Tie* in megakaryocytes in vivo. Section of whole-mount β-galactosidase stained liver of an E15.5 tie^{lcz}/tie^{lczn-} ↔ CD-1 chimera, showing tie^{lcz}/tie^{lczn-} cell derived megakaryocytes (arrows) and wild-type megakaryocytes (arrowheads). Scale bar, 50 µm.

(Poelmann et al., 1993). Interestingly, abundant contribution of *Tie* mutant cells was detected at E15.5 in the endothelia of the heart and lung. Thus, consistent with our results from E10.5 embryos, TIE does not appear to be required in the presumably vasculogenic processes taking place during later development.

In contrast to the organs possessing vasculogenic activity, *Tie* mutant cells were underrepresented in capillaries of organs and tissues like kidney, brain and skin at E15.5. Analysis of quail-chicken transplantation chimeras has shown that these organs depend on an extrinsic source of endothelium, which grows into the organ rudiments as capillary sprouts (Ekblom et al., 1982; Stewart and Wiley, 1981). Thus, TIE appears to specifically support capillary endothelial cell proliferation and/or migration needed for angiogenic growth, although at this stage there is no absolute requirement for TIE in any endothelial cell type. The avian interspecies transplantation experiments have indicated that certain organ rudiments possess vasculogenic activity, but they do not suggest that it is the exclusive mechanism of vascularization, and presumably vessel sprouting plays a role after the initial angioblast differentiation in the rudiments. However, our results suggest that sprouting is either not very extensive or not TIE dependent in the embryonic lung and heart. It may play a more important role in the vascularization of the intestine, which also has vasculogenic potential (Pardanaud et al., 1989), as the *Tie* mutant cells were clearly selected against in the vasculature of this tissue. Alternatively, there might be a specific requirement for TIE in the vasculogenesis in the intestine. Our results demonstrate that there are obvious differences in the cellular or regulatory mechanisms of vessel sprouting at various stages of development. As suggested (Noden, 1991; Brand-Saberi et al., 1995), angioblast differentiation might contribute to the early vessel sprouting. The transition from the early TIE-independent phase of angiogenesis to the later TIE-dependent angiogenesis might also reflect down-regulation of the *Tek* gene expression during embryonic development.

In addition to its role in the development of the vasculature, TIE appears to be involved in supporting survival of endothelium in mature vessels. This is best illustrated in the endothelium of the lung, which initially can have a strong contribution from the *Tie* mutant cells (see above). Selection against these cells continues even after weaning age and by 12 weeks of age, most *Tie* mutant cells are eliminated from the lung endothelium of the chimeras. Also the remaining mutant cells contributing to kidney glomeruli and adrenals seem to be replaced by wild-type cells by the time the chimeras reach weaning age. In contrast, endothelial cells of some major vessels, the heart endocardium and several myocardial capillaries were still able to survive without TIE function. As an alternative to affecting endothelial cell survival directly, TIE could also support endothelial cell proliferation that replaces senescent endothelial cells. We feel this is somewhat unlikely, however, because of the very slow turnover of the mature endothelium (Hobson and Denekamp, 1984). Our results suggest a striking heterogeneity of endothelial cells in their requirements for environmental signals regulating growth and survival.

Since TIE is also expressed in some hematopoietic cell lineages in addition to the endothelium, we made use of the chimeras to ask whether there is a function for TIE in the developing hematopoietic system. The *Tie* mutant cells were able to contribute to several hematopoietic organs and populations, and were not at a disadvantage when compared with wild-type cells. Thus, we conclude that TIE expression in the hematopoietic system is either not functionally significant or there may be redundancy between TIE and another receptor, such as TEK, which has also been reported to be expressed in hematopoietic cells (Iwama et al., 1993; Batard et al., 1996; Hashiyama et al., 1996). The inability to detect a hematopoietic defect in the *Tie* mutant cells further supports our conclusion that TIE has no function in early vasculogenesis at the level of the hypothetical hemangioblast. In addition to hematopoietic stem cells, *Tie* is also expressed in megakaryoblastic cell lines in vitro. We have shown here that *Tie* is also expressed in megakaryocytes in vivo, but it is not required for their differentiation. We cannot, however, exclude the possibility that TIE plays a more subtle role in the maturation of the megakaryocytic and perhaps other hematopoietic lineages (Batard et al., 1996; Hashiyama et al., 1996). Our results are consistent with a recent report demonstrating that *Tie* mutant cells are still capable of hematopoietic colony formation in vitro and reconstitution of the hematopoietic system in vivo (Rodewald and Sato, 1996). The chimeric analysis presented here is even a more stringent test, as it can also detect a defect of the mutant cells that is only apparent when these cells are put in competition with the wild-type host cells.

Recent gene targeting studies have demonstrated a hierarchy of signals regulating the development of the vascular tree (Dickson et al., 1995; Fong et al., 1995; Puri et al., 1995; Sato et al., 1995; Shalaby et al., 1995; Dumont et al., 1994; Carmeliet et al., 1996; Ferrara et al., 1996). Our results indicate that in contrast to VEGF, which has functions both in vasculogenesis and angiogenesis, the signal mediated by the receptor tyrosine kinase TIE is specific for a late phase of angiogenesis and regulates endothelial cell survival and proliferation rather than their differentiation. In addition, there appear to be striking differences in the growth factor requirements of the endothelium in various parts of the vascular tree. Despite widespread expression in different types of endothelia, TIE is not required uniformly. Our results thus demonstrate fundamental differences in the mechanisms that regulate both the formation and maintenance of the various parts of the vasculature.

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