

## Interaction of the TEK and TIE receptor tyrosine kinases during cardiovascular development

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### SUMMARY

**TEK (TIE2) and TIE (TIE1) are structurally related receptor tyrosine kinases expressed in endothelial cells and their precursors. Genetic studies in the mouse have revealed essential functions of both receptors in angiogenic expansion of the vasculature during development. As previously shown, mouse embryos homozygous for a disrupted *Tek* allele die by day 10.5 of embryogenesis due to endocardial defects, hemorrhaging, and impaired vascular network formation. Furthermore, TIE is required cell autonomously for endothelial cell survival and extension of the vascular network during late embryogenesis. Here we have investigated possible redundancy in the TEK and TIE signalling pathways during vascular development. Vasculogenesis proceeds**

**normally in embryos lacking both TEK and TIE, although such embryos die early in gestation of multiple cardiovascular defects. Mosaic analysis revealed an absolute requirement for TEK in the endocardium at E10.5, whereas TEK and TIE are dispensable for the initial assembly of the rest of the vasculature. In contrast, both receptors are required in the microvasculature during late organogenesis and in essentially all blood vessels of the adult. This analysis demonstrates essential functions for TEK and TIE in maintaining the integrity of the mature vasculature.**

Key words: Endothelial cell, Embryogenesis, Chimera, Receptor tyrosine kinase, Angiogenesis

### INTRODUCTION

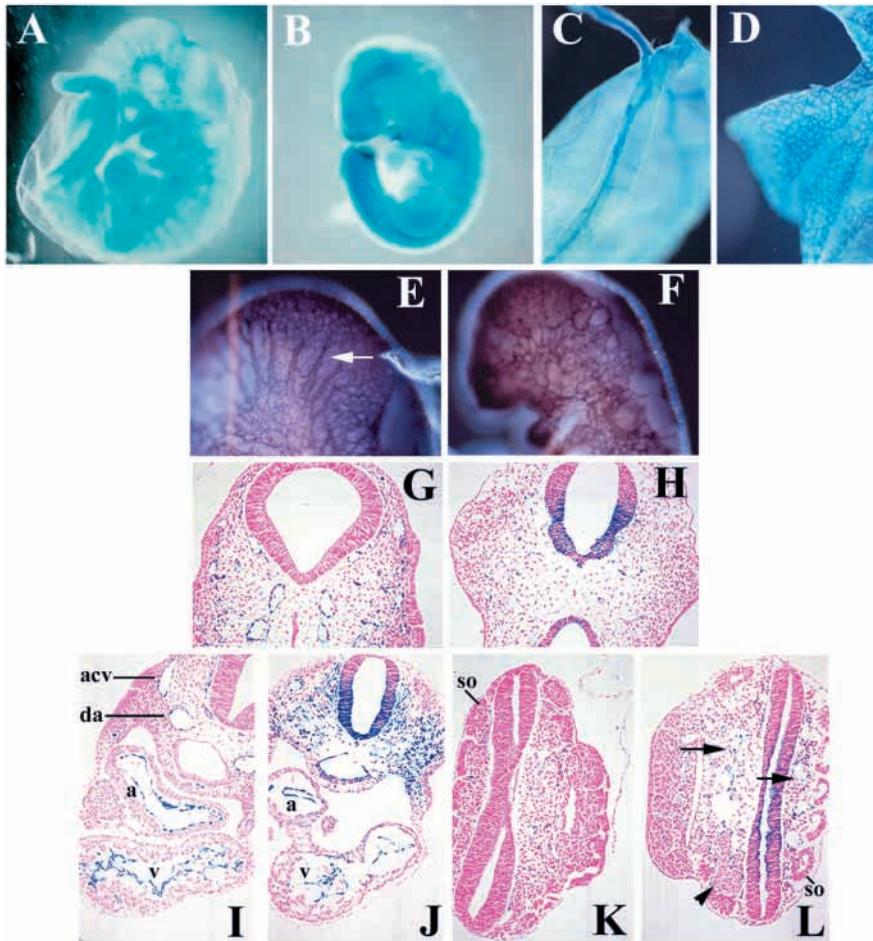
Mammalian cardiovascular development is a complex process involving the coordinated differentiation and interaction of distinct cell lineages to form the heart and the diverse array of arteries, veins and capillaries required to supply oxygen and nutrients to all tissues of the adult organism. Perturbations in this process can result in a wide variety of disorders ranging from fetal mortality to vascular malformations (Schaffer and Avery, 1971).

Embryonic endothelial cells (ECs) differentiate early in development and provide a framework around which the vascular system is subsequently organized (Risau, 1997; Wilting and Christ, 1996). Blood vessel formation is initiated by the process of vasculogenesis: the differentiation of mesodermally derived EC precursors, the angioblasts, and either their immediate aggregation to form endothelium, or their migration through the embryo and organization into vascular channels. The major vascular structures of the early embryo, including the dorsal aorta, the heart endocardium and yolk sac vasculature are thought to arise by this process (Pardanaud et al., 1987; Coffin et al., 1991). Subsequently, a branching network of blood vessels is assembled in the embryo by proliferation, sprouting and organization of preformed ECs

in a process termed angiogenesis. The vascularization of several organs, including the brain and kidney, as well as formation of the smaller vessels and the microvasculature occur by angiogenesis (Pardanaud et al., 1989; Stewart and Wiley, 1981; Noden, 1991b; Kurz et al., 1996; Sariola et al., 1983). Finally, as organogenesis proceeds, pericytes, vascular smooth muscle cells and fibroblasts are recruited to the ECs of primitive vessels to promote the assembly and stabilization of the mature blood vessel wall (Beck and D'Amore, 1997; Risau, 1997).

Insight into the molecular mechanisms that mediate these distinct stages of blood vessel assembly has been provided by studies of two subfamilies of signalling molecules and their receptors that are critical for these processes. One subfamily includes the vascular endothelial growth factor (VEGF) family of polypeptides and their receptors: VEGF receptor-1 (VEGFR-1 or Flt-1), VEGFR-2 (Flk-1) and VEGFR-3 (Flt4) (Hanahan, 1997; Ferrara and Davis-Smith, 1997). The second subfamily consists of TEK (TIE2) the receptor for Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2), and TIE (TIE1), an orphan receptor (Partanen and Dumont, 1999).

VEGF-deficient mice die between embryonic day 8.5 (E8.5) and E9.0 and display severe defects in the formation of the earliest vascular structures (Carmeliet et al., 1996; Ferrara et



**Fig. 1.** Phenotypic comparisons of *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>* and *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//+* embryos at E9.0 to E9.5. (A) E9.5 *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//+* embryo showing *Tie*-specific *lacZ* expression in embryonic blood vessels and endocardium compared to *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>* littermate (B) exhibiting overall size reduction, developmental arrest, poor heart development, edema and uniform *lacZ* expression. (C) Whole-mount view of E9.5 *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//+* X-gal stained yolk sac vascular network composed of large vessels and smaller branches. (D) In yolk sac of *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>* littermate of the embryo in C, vessels are of uniform size. (E) Cephalic vasculature of PECAM1 stained E9.0 wild-type embryo showing delineated branches of the anterior cardinal vein and capillary plexi (arrow). (F) PECAM1-stained littermate of the embryo in E. Note absence of large branches of anterior cardinal vein and arrest of angiogenesis to dorsal aspect of embryo. (G) Transverse section of anterior portion of X-gal stained E9.0 *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//+* embryo. (H) Corresponding region of *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>* embryo exhibits weak X-gal staining, loss of inter-endothelial cell contact, absence of branches of anterior cardinal vein, decreased number and poor organization of remaining vessels, and non-EC staining in neural tube. (I) Transverse section through heart region of X-gal stained E9.0 *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//+* embryo compared with (J) *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>* littermate showing sparse endocardium, reduced ventricular trabeculation, poor vascular organization dorsally including perineural vascular plexus and collapsed dorsal aortae, and extensive non-EC  $\beta$ -galactosidase activity in the mesenchyme and neuroectoderm. (K) Section through dorsal posterior of X-gal stained E9.0 *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//+* embryo and (L) *Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>//Tie1<sup>lacZ</sup>; tek<sup>Δsp</sup>* littermate showing vessel dilation (arrows) blood pooling (arrowhead), edema and somite deterioration and necrosis. Acv, anterior cardinal vein; da, dorsal aorta; a, atrium; v, ventricle; so, somite.

al., 1996). Similarly, embryos homozygous for a targeted null mutation in the gene for VEGFR-2 die early in gestation due to the lack of formation of both ECs and hematopoietic cells (Shalaby et al., 1995), as a result of the blocked migration of angioblasts to the initial embryonic sites of vasculogenesis

(Shalaby et al., 1997). ECs develop in embryos lacking VEGFR-1, but these embryos still die before E9.5 as a result of severe defects in the organization of the primitive vascular system (Fong et al., 1995). Also, inactivation of the gene for VEGFR-3 affects lumen formation in large vessels during early development (Dumont et al., 1998).

In contrast to the defects in vasculogenesis revealed in embryos lacking VEGF, VEGFR-2 or VEGFR-1, targeted mutations in *Tek*, *Tie* or *Ang-1* all result in embryonic lethality during embryogenesis as a consequence of the failure of the embryonic vasculature to expand and survive (Dumont et al., 1994; Sato et al., 1995; Puri et al., 1995; Suri et al., 1996). Notably, the initial differentiation and assembly of ECs is unperturbed in these mutants. For example, *Tek*<sup>-/-</sup> embryos undergo normal vasculogenesis, but die by E10.5 as a result of hemorrhaging and cardiac failure. Endocardial development and myocardial trabeculation are impaired in these mutant embryos and they fail to form a branched network of small and large vessels (Dumont et al., 1994; Sato et al., 1995). Similarly, targeted disruption of *Ang-1*, the ligand for TEK, leads to embryonic lethality by E12.5, with heart and blood vessel defects similar to those observed in *Tek*<sup>-/-</sup> embryos (Suri et al., 1996). The absence of Ang-1 or TEK is also associated with a lack of peri-vascular cells at the sites of deficient vessel branching and reorganization (Suri et al., 1996; Patan, 1998). These observations suggest that TEK signalling is required for angiogenic sprout formation, vessel remodelling and vessel maturation, but not for EC differentiation. Consistent with this model, Ang-1 induces both sprout formation and migration in vitro (Koblizek et al., 1998; Witzenbichler et al., 1998) and an increase in vessel branching in vivo (Suri et al., 1998).

Genetic analysis has also revealed a role for the TIE receptor after the initial formation of the embryonic vasculature. Embryos homozygous for a mutant *Tie* allele survive with a normal cardiovascular system until at least E13.5, but subsequently manifest edema and/or aneurysms of the microvasculature, eventually succumbing to widespread hemorrhage (Puri et al., 1995; Sato et al., 1995). In addition, in chimeric embryos derived from normal embryos and *Tie*<sup>-/-</sup> embryonic stem (ES) cells, *Tie*-deficient cells participate in vasculogenesis, early angiogenesis, and contribute fully to the formation of major blood vessels, but are unable to give rise to the capillaries that form in the brain and kidney by

angiogenesis during late embryogenesis (Partanen et al. 1996). In contrast to the *Ang-1* and *Tek* mutants, disruption of the *Tie* gene does not lead to obvious cardiac defects, and ECs lacking TIE can contribute the heart endocardium even in adult mice (Puri et al., 1995; Partanen et al., 1996).

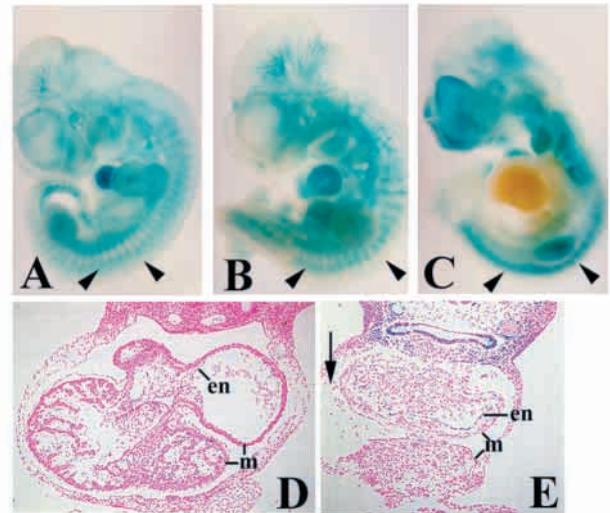
These genetic analyses suggest that TEK and TIE are both required for vascular development but play somewhat different roles. However, several observations suggest that they may also have overlapping functions in vascular development. First, the genes for both receptors are co-expressed in virtually all ECs throughout embryogenesis (Dumont et al., 1995; Sato et al., 1993). Second, TEK and TIE share a number of unique structural features including fibronectin type III repeats, immunoglobulin loops and epidermal growth factor like domains in their extracellular domains (Sato et al., 1993). Furthermore, the presence of several conserved tyrosine residues in the intracellular domain suggests that these receptors may share a common signalling transduction pathway (Lyons et al., 1998). Importantly, neither *Tek* nor *Tie* mutant embryos exhibit any defects in angioblast differentiation or early vessel assembly although both receptors are expressed in angioblasts. Thus, it is possible that a role for TEK and TIE signalling at an early stage of vascular development is obscured by their overlapping functions and expression.

In this report we have investigated possible redundant functions of TEK and TIE by analysing embryos doubly homozygous mutant for both genes. Interaction between the TEK and TIE signalling pathways was also investigated by manipulating the gene dosage of both receptors. Our results show that EC differentiation from mesodermally derived angioblast precursors occurs normally even in embryos lacking both TEK and TIE. In addition, to define more precisely the role of TEK and TIE in the developing and mature vasculatures, we studied the ability of cells lacking both TEK and TIE to contribute to the endothelium of chimeric animals in early development, midgestation and in the adult. This analysis revealed an absolute requirement for TEK in the differentiation of the heart endocardium by E10.5 of gestation; however, these receptors were dispensable for the initial assembly of the rest of the vasculature in chimeras. An essential requirement for both receptors became evident in the microvasculature during late organogenesis and in virtually all blood vessels in the adult mouse.

## MATERIALS AND METHODS

### Mutant alleles and generation of *Tek*, *Tie* compound mutants

The generation of *tie<sup>lcz</sup>*, *tie<sup>lczn</sup>*- and *tek<sup>Δsp</sup>* mice has been described previously. (Puri et al., 1995; Dumont et al., 1994). Because the murine *Tek* and *Tie* genes map within 12.2 cM on mouse chromosome 4 (Sato et al., 1993), doubly heterozygous recombinant parents were required in order to produce and analyse embryos homozygous mutant



**Fig. 2.** Phenotypic comparison of *tie<sup>lcz</sup>tek<sup>Δsp</sup>/+/+* and *Tek* gene dosage compromised *Tie* mutant embryos. (A) Whole-mount X-gal stained E11.5 *tie<sup>lcz</sup>tek<sup>Δsp</sup>/+/+* embryo compared to (B,C) *tie<sup>lcz</sup>tek<sup>Δsp</sup>/+/+tie<sup>lcz</sup>+*  littermates. Embryo in B shows slight pericardial edema and vessel disorganization, compared to that in C which exhibits serious enlargement of the heart, diminished endocardial staining, and extensive deterioration of vessels in the intersomitic region and head. Arrowheads indicate intersomitic vessels. (D) Transverse section through heart of *tie<sup>lcz</sup>tek<sup>Δsp</sup>/+/+* embryo and (E) *tie<sup>lcz</sup>tek<sup>Δsp</sup>/+/+tie<sup>lcz</sup>+*  embryo showing intra-cardial blood pooling (arrow) and reduced myocardial trabeculation. En, endocardium; m, myocardium.

for both alleles (Table 1). After intercrossing *tie<sup>lcz</sup>/+* and *tek<sup>Δsp</sup>/+* single heterozygotes, non-recombinant double heterozygotes (*tie<sup>lcz</sup>/+/+tek<sup>Δsp</sup>*) were produced and subsequently bred to CD-1 wild-type animals to generate recombinant double heterozygous founders (*tie<sup>lcz</sup>tek<sup>Δsp</sup>/+/+*). Two doubly heterozygous recombinant males (TR37 and TR84) as well as two single heterozygous littermates: (TR91 (*tie<sup>lcz</sup>/+*) and TR4 (*tek<sup>Δsp</sup>/+*), were bred to wild-type CD-1 females to generate large numbers of animals of all three genotypes to perform the phenotypic analyses on a uniform genetic background. The analyses described here (Table 2) were performed in F<sub>3</sub> to F<sub>5</sub> generations of mice on the CD-1 background.

### Genotyping of mice carrying *tek<sup>Δsp</sup>* and *tie<sup>lcz</sup>* alleles

Screening for *tek<sup>Δsp</sup>* and *tie<sup>lcz</sup>* alleles by restriction digestion and Southern analysis was performed as described previously (Dumont et al., 1994; Puri et al., 1995). Polymerase chain reaction (PCR) based screening was also used to genotype embryonic tissues. Yolk sac fragments were dissected and incubated overnight at 55°C in lysis buffer containing Proteinase K (Boehringer).

PCR screening to distinguish wild-type *Tie* and *tie<sup>lcz</sup>* alleles was performed using the *Tie* exon 1-specific sense primer 1: (5'CCTTCCAGAGACTTCCAGGCCACA3') and an antisense primer 2 specific to the deleted portion of exon 1 (Puri et al., 1995) (5'CATACTCTGGAAGCTGCCCTG3'). The mutant *tie<sup>lcz</sup>* allele was amplified using primer 1 and an antisense *lacZ*-specific primer 3 (5'GATGTGCTGCAAGGCGATTAAG3'). Wild-type *Tek* and *tek<sup>Δsp</sup>*

**Table 1. Genetic nomenclature and derivation of *Tek* and *Tie* compound mutant and control mouse strains**

Generation	Male	×	Female	Offspring of interest
F <sub>0</sub>	( <i>tie<sup>lcz</sup>/+/+</i> )		(+ <i>tek<sup>Δsp</sup>/+/+</i> )	( <i>tie<sup>lcz</sup>/+/+tek<sup>Δsp</sup></i> ) Non-recombinant double het.
F <sub>1</sub>	( <i>tie<sup>lcz</sup>/+/+tek<sup>Δsp</sup></i> )		(+/+/+)	( <i>tie<sup>lcz</sup>tek<sup>Δsp</sup>/+/+</i> ) Recombinant double het. ( <i>tie<sup>lcz</sup>/+/+</i> ) Control (+ <i>tek<sup>Δsp</sup>/+/+</i> ) Control

**Table 2. Compound mutational analysis**

Male	×	Female	Offspring of interest
<i>(tie<sup>lcz</sup>tek<sup>Δsp</sup>/++)</i>		<i>(+//+tek<sup>Δsp</sup>)</i>	<i>(tie<sup>lcz</sup>tek<sup>Δsp</sup>/+tek<sup>Δsp</sup>)</i> <i>(tie<sup>lcz</sup>tek<sup>Δsp</sup>/++)</i> (control) <i>(+//+tek<sup>Δsp</sup>)</i> (control)
<i>(tie<sup>lcz</sup>tek<sup>Δsp</sup>/++)</i>		<i>(+//tie<sup>lcz</sup>+)</i>	<i>(tie<sup>lcz</sup>tek<sup>Δsp</sup>/tie<sup>lcz</sup>+)</i> <i>(tie<sup>lcz</sup>tek<sup>Δsp</sup>/++)</i> (control) <i>(+//tie<sup>lcz</sup>+)</i> (control)
<i>(tie<sup>lcz</sup>tek<sup>Δsp</sup>/++)</i>		<i>(tie<sup>lcz</sup>tek<sup>Δsp</sup>/++)</i>	<i>(tie<sup>lcz</sup>tek<sup>Δsp</sup>/tie<sup>lcz</sup>tek<sup>Δsp</sup>)</i> <i>(tie<sup>lcz</sup>tek<sup>Δsp</sup>/++)</i> (control)

alleles were amplified in a reaction containing *Tek* exon 1-specific primer 4 (5'AGCGAGTGGACCATGCGAGC3') and the antisense primer 5 (5'AGGAGCAAGCTGACTCCACAG3') specific to the deleted portion of exon 1 (Dumont et al., 1994). The *tek<sup>Δsp</sup>* allele was amplified using antisense *neo* gene-specific primer 6 (5'CCATTGCTCAGCGGTGCTGTCCAT3') and primer 5 as above.

### Chimeric analysis

Chimeras were generated by the morula-morula aggregation technique as described previously (Wood et al., 1993) and as shown in Fig. 3. Morulae from a cross between *tie<sup>lcz</sup>tek<sup>Δsp</sup>/++* and *tie<sup>lczn</sup>-tek<sup>Δsp</sup>/++* (GPI<sup>aa</sup>) parents were aggregated with CD-1 wild-type (GPI<sup>bb</sup>) and transferred to the uteri of CD-1 foster mothers. Screening of yolk sac and tail DNA for *tie<sup>lcz</sup>* and *tie<sup>lczn</sup>-* alleles by restriction enzyme digestion and Southern blotting has been described previously (Puri et al., 1995).

### β-galactosidase staining of embryos and adult tissues

Embryos or tissue samples were dissected, fixed, washed and stained as described previously (Puri et al., 1995; Partanen et al., 1996).

### Whole-mount immunohistochemistry

Embryos for whole-mount PECAM (CD31) antibody staining were dissected in ice-cold PBS, fixed on ice in 4% paraformaldehyde for 2-4 hours and washed overnight in PBS at 4°C. Embryos were then dehydrated through a graded methanol series and stored at -20°C. Embryos were rehydrated at room temperature through graded methanols and incubated twice on a rotating platform for 1 hour in PBSMT (3% skim milk powder, 0.1% Triton X-100 in PBS), followed by overnight incubation at 4°C with a 1:1000 dilution of preabsorbed rat anti-mouse PECAM-1 antibody (Pharmingen) in PBSMT. Embryos were washed five times for 1 hour each at 4°C in PBSMT, and staining was completed using the Vectastain ABC Elite kit (Vector Labs) according to the recommendations of the manufacturer. Colour resolution was achieved using diaminobenzidine (DAB; Vector Labs).

### Glucose phosphate isomerase-1 (GPI) isozyme analysis

Tissue samples from organs of adult animals, as well as tail and/or limb samples of E10.5 and E15.5 chimeric embryos were collected in Eppendorf tubes, crushed using an Eppendorf sized pestle, stored in distilled water and freeze-thawed at least three times before GPI analysis. After spinning briefly in a microfuge, 10 μl of sample was

run on cellulose acetate plates (Helena Laboratories) at 150 Volts for 45 minutes as described by Nagy and Rossant (1993). The colour reaction was performed as described previously (Nagy and Rossant, 1993) and stopped in 5% acetic acid.

## RESULTS

### Generation of embryos lacking both TEK and TIE

We have previously described the construction of mutant null alleles of both the *Tek* and *Tie* genes by gene targeting in embryonic stem (ES) cells (Dumont et al., 1994; Puri et al., 1995). *Tek* and *Tie* genes are localized to within 12.2 cM on mouse chromosome 4 (Sato et al., 1993). Thus, in order to initiate a phenotypic analysis of doubly homozygous mutant embryos, recombinant doubly heterozygous mice were produced according to the scheme shown in Table 1 and described in Materials and Methods. After interbreeding *tie<sup>lcz</sup>* and *tek<sup>Δsp</sup>* heterozygotes, double heterozygotes were bred to wild-type CD-1 mice and the offspring genotyped for their *Tek* and *Tie* alleles. Doubly heterozygous recombinants (*tie<sup>lcz</sup>tek<sup>Δsp</sup>/++*) were present at a frequency of approximately 10%, indicating either slightly closer linkage of the two genes than previously described, or reduced survival to adulthood of a subset of doubly heterozygous animals. The nomenclature used for all combinations of *Tek* and *Tie* alleles is shown in Table 1. In the studies described below, we focused on the doubly homozygous mutant (*tie<sup>lcz</sup>tek<sup>Δsp</sup>/tie<sup>lcz</sup>tek<sup>Δsp</sup>*) embryos and homozygous *Tie* mutant mice with half *Tek* gene dosage (*tie<sup>lcz</sup>tek<sup>Δsp</sup>/tie<sup>lcz</sup>+)*. The *tie<sup>lcz</sup> tek<sup>Δsp</sup>/+tek<sup>Δsp</sup>* mice displayed phenotypic characteristics that were indistinguishable from *Tek* nullizygotes (Table 3 and data not shown).

### Vascular development in the absence of both TEK and TIE

To determine whether the TEK and TIE signalling pathways are functionally redundant in early EC differentiation, recombinant mice doubly heterozygous for null alleles of both genes (*tie<sup>lcz</sup>tek<sup>Δsp</sup>/++*) were inter-crossed (Table 2) and the offspring were analysed at embryonic day (E) 10.5 and earlier (Table 3). The *lacZ* gene targeted into the *Tie* locus provided an endogenous marker for ECs in *tie<sup>lcz</sup>tek<sup>Δsp</sup>/tie<sup>lcz</sup>tek<sup>Δsp</sup>* mutant and *tie<sup>lcz</sup>tek<sup>Δsp</sup>/++* control embryos. E9.5 double mutant embryos were considerably smaller than their doubly heterozygous littermates, and most had an enlarged pericardial sac and a poorly developed heart, under-developed forebrain structures as well as edema (Fig. 1A,B). In addition, most double mutant embryos were extremely pale compared to their littermates. Despite these overt defects, many developmental

**Table 3. Summary of phenotypic analysis of double homozygous and gene dosage compromised mutant embryos**

Embryonic day	No. of offspring of each genotype (% abnormal)											
	<i>tie<sup>lcz</sup> tek<sup>Δsp</sup>/++ × tie<sup>lcz</sup> tek<sup>Δsp</sup>/++</i>			<i>tie<sup>lcz</sup> tek<sup>Δsp</sup>/++ × tie<sup>lcz</sup> +/+</i>				<i>tie<sup>lcz</sup> tek<sup>Δsp</sup>/++ × tek<sup>Δsp</sup>/++</i>				
	<i>Tek:</i>	+/+	+/-	-/-	+/+	+/+	+/-	-/-	+/+	+/-	+/-	-/-
E8.0-8.5		17 (0)	27 (0)	17 (82)	ND				ND			
E9.0-9.5		22 (0)	43 (18)	24 (100)	ND				6 (0)	14 (0)	10 (0)	4 (100)
E9.5-10.5		31 (0)	56 (25)	24 (100)	10 (0)	4 (0)	5 (0)	6 (25)	8 (0)	13 (0)	10 (0)	6 (100)
E10.5-11.5		ND			28 (0)	17 (0)	12 (8)	12 (66)	ND			
E11.5-12.5		ND			13 (0)	9 (0)	12 (0)	7 (86)	ND			
E12.5-13.5		ND			15 (0)	12 (0)	13 (8)	7 (100)	ND			

processes were completed, including neural tube closure and somitogenesis.

Double mutant embryos could also be distinguished from their doubly heterozygous littermates by their underdeveloped yolk sac vasculature lacking major blood vessels, likely caused by a failure of remodeling of the primary yolk sac capillary plexus into a more complex network of blood vessels and their branches (Fig. 1C,D). Despite this striking defect, also observed in *Tek*<sup>-/-</sup> embryos (Dumont et al., 1994; Sato et al., 1995), there appeared to be a normal connection between the embryo and the yolk sac vasculature as well as chorio-allantoic fusion (data not shown).

The failure of blood vessels to become organized into large and small vessels was also apparent in the cephalic region as determined by whole-mount analysis of embryos stained with PECAM-1, an EC-specific antigen (Fig. 1E,F). Blood vessel development in *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lcz</sup>tek<sup>Δsp</sup>* embryos failed to proceed to the dorsal portion of the body, and the vessels that were present were not properly organized into small and large channels (Fig. 1F) when compared to a wild-type littermate (Fig. 1E). Mutant embryos also exhibited poor branching structures of the blood vessels in the head (Fig. 1H). The presence of some cells weakly expressing *lacZ* outlining the blood vessels suggests that some vascular channels had arisen at an earlier stage, but clearly formation of a vessel network was greatly reduced compared to *tie<sup>lcz</sup>tek<sup>Δsp</sup>//++* control littermates (Fig. 1G). Moreover, vessel integrity in *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lcz</sup>tek<sup>Δsp</sup>* embryos was noticeably compromised, a feature noted in *Tie*<sup>-/-</sup> mutant embryos (Puri et al., 1995; Sato et al., 1995).

Another prominent feature of *Tek*- and *Tie*-deficient embryos was the striking reduction of endocardial tissue in the common atrial and ventricular chambers and the accompanying lack of myocardial trabeculation in the ventricle, although both endocardial and myocardial cell layers were clearly present in these embryos at E9.0 (Fig. 1J). A thin myocardial layer, poor maintenance of endocardial contact and absence of trabeculations in *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lcz</sup>tek<sup>Δsp</sup>* mice likely contributed to cardiac failure as a result of poor contractility.

We frequently observed reporter gene activity in non-EC types such as the neural ectoderm and surrounding mesenchyme in doubly homozygous mutant but not control embryos (Fig. 2I,J and A,B). Such ectopic β-galactosidase expression did not represent the site of new blood vessel formation because PECAM staining of double mutant embryos did not follow the same pattern (Fig. 1B vs F and data not shown). Instead, β-gal expression in non-endothelial sites may reflect ectopic expression of the *Tie* gene whose expression is responsive to hypoxia (McCarthy et al., 1998). Compromised circulation leading to hypoxia in mutant embryos is the likely cause of this response.

Doubly homozygous mutant embryos also contained enlarged and highly distorted blood vessels instead of the small vessels seen in the dorsal, posterior portion of doubly heterozygous littermate embryos (Fig. 1K,L). Moreover, mutant somites were disorganized and necrotic. Additional sites of tissue necrosis were occasionally observed in E9.5 mutants, and by E10.5 it was consistently severe throughout the embryo.

At E8.5 and earlier, mutant embryos were the same size as their *tie<sup>lcz</sup>tek<sup>Δsp</sup>//++* and wild-type littermates (Table 3 and

data not shown). However, X-gal staining showed that although the major blood vessels such as the dorsal aortae and cardinal veins had formed normally, the endocardium of *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lcz</sup>tek<sup>Δsp</sup>* embryos was less extensive compared to *tie<sup>lcz</sup>tek<sup>Δsp</sup>//++* littermates.

The above observations and the data in Table 3 suggest that the developmental failure in *Tek*- and *Tie*-deficient embryos begins by E8.5 and is fully penetrant by E9.5, the period in which yolk sac vasculogenesis is normally completed, the embryonic and extraembryonic vasculatures are fused and embryonic blood circulation begins. The *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lcz</sup>tek<sup>Δsp</sup>* phenotype qualitatively resembles that of *Tek* nullizygous embryos (Dumont et al., 1994; Sato et al., 1995). However, double mutants consistently showed an increased severity and earlier onset of the mutant characteristics, particularly poor cardiac development, a likely cause of the pronounced growth delay.

### Genetic interaction between *Tek* and *Tie* in cardiovascular development

Embryos lacking TEK die at E10.5, three full days before the lethality associated with a null mutation in *Tie* (Dumont et al., 1994; Sato et al., 1995; Puri et al., 1995), suggesting that the presence of the TIE receptor is not sufficient to rescue embryos lacking TEK. To test directly whether TEK and TIE have partly redundant functions later in development, we generated embryos with one or two functional *Tek* genes in a genetic background in which the *Tie* gene was absent (Table 2). Embryos were scored at E13.5 and earlier for developmental abnormalities since *tie<sup>lcz</sup>* homozygous mutant mice develop normally to this gestational stage (Sato et al., 1995; Puri et al., 1995). Beginning at E9.5, development of the cardiovascular system was sensitive to *Tek* gene dosage in embryos lacking the TIE receptor (Table 3). The most prominent phenotype observed in *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lcz</sup>+* embryos was heart failure as evidenced by swelling of the pericardium (Fig. 2B,C) and poor maintenance of the atrial and ventricular endocardium leading to hemorrhage within the heart (Fig. 2E). Many embryos were also reduced in size (data not shown) and showed poor maintenance of the blood vessels of the head and extremities (Fig. 2C), as well as edema and localized hemorrhage (data not shown).

Together, these data suggest that the survival of embryos lacking the TIE receptor up to E13.5 and later depends on TEK, and therefore that these receptors share some overlapping functions during cardiovascular development. The severity of the *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lcz</sup>+* mutant phenotype varied in the outbred genetic background (Table 3; Fig. 2A-C), suggesting that additional genetic modifier loci are affecting TEK and TIE function. It is important to note, however that the *Tek* and *Tie* single mutant phenotypes in this genetic background closely followed the pattern demonstrated in previous studies (data not shown).

### Generation of double mutant chimeras

The rapid deterioration of *Tek* and *Tie* double mutant embryos by E9.5 hindered an examination of the ability of ECs lacking TEK and TIE to undergo angiogenic sprouting and vessel maturation, processes that have previously been suggested to be mediated by Ang1/TEK and TIE (Sato et al., 1995; Suri et al., 1996; Folkman and D'Amore, 1996). We therefore produced

chimeras by aggregating morula stage CD-1 wild-type embryos and embryos generated in a cross between two strains of doubly heterozygous parents which carried the distinguishable mutant *tie<sup>lacZ</sup>* and *tie<sup>lacZn-</sup>* alleles (Fig. 3 and Materials and Methods). The presence of the *lacZ* gene targeted into the *Tie* locus made it possible to identify ECs derived from doubly homozygous (*tie<sup>lacZ</sup>tek<sup>Δsp</sup>//tie<sup>lacZn-</sup>tek<sup>Δsp</sup>*) or doubly heterozygous (*tie<sup>lacZ</sup>tek<sup>Δsp</sup>//++* or *tie<sup>lacZn-</sup>tek<sup>Δsp</sup>//++*) embryos by staining for β-galactosidase and to compare their behaviour within the context of a wild-type environment. Chimeric animals of all genotypes were analysed at E10.5, E15.5 and as adults (Table 4).

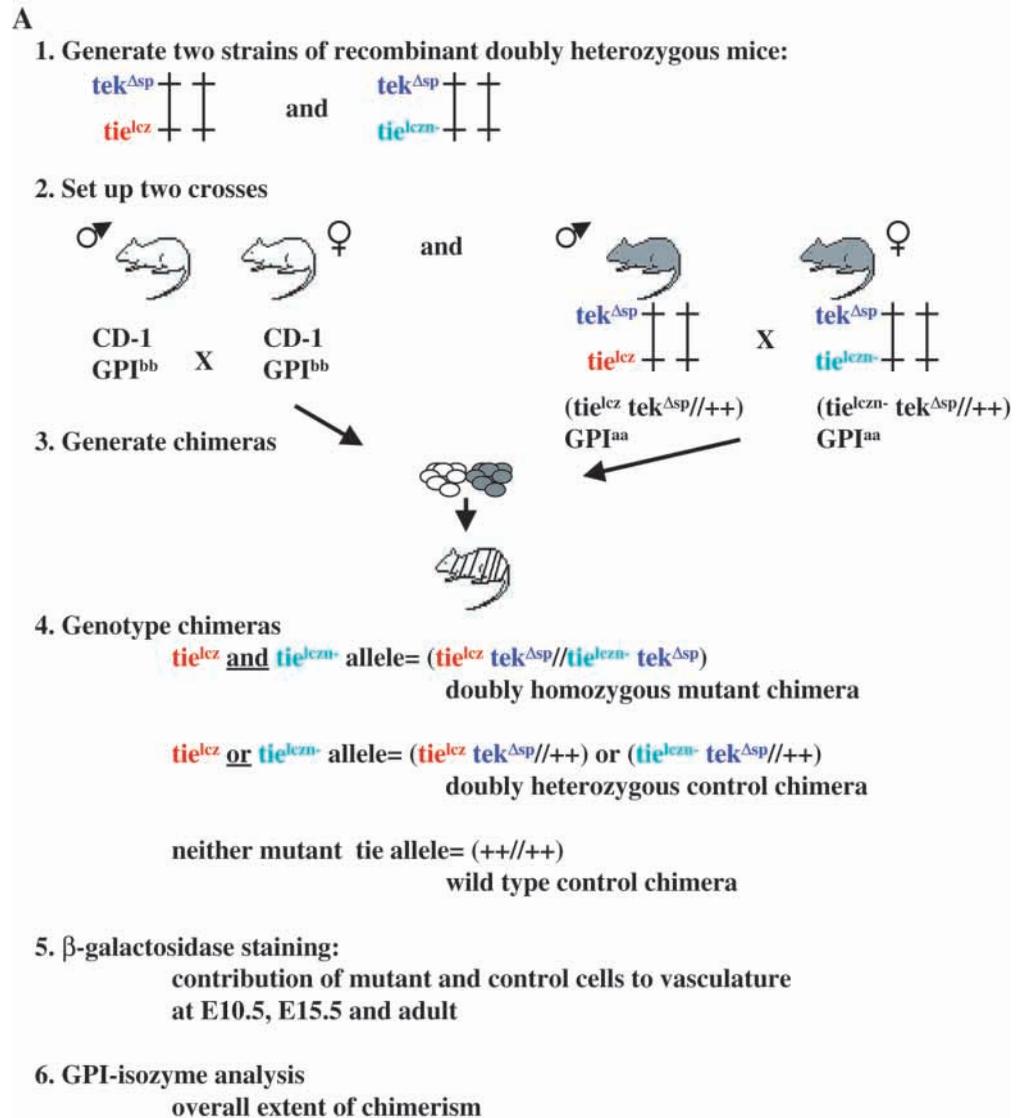
### ECs lacking both TEK and TIE contribute normally to all vascular structures except the atrial and ventricular endocardium at E10.5

There was a clear difference in the ability of cells lacking both TEK and TIE to contribute to the atrium and ventricle of the heart (Fig. 4C,D) compared to doubly heterozygous cells (Fig. 4B). Histological analysis confirmed the presence of a large proportion of β-galactosidase-positive endocardial cells in the atrium and ventricle of *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//++* ↔ CD-1 chimeras (Fig. 4M,O) but mostly wild-type, (unstained) endocardial cells in these structures in corresponding *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//tie<sup>lacZn-</sup>tek<sup>Δsp</sup>* chimeras (Fig. 4N,P). However, the endocardium of the outflow tract showed a similar contribution of both mutant and heterozygous cells (Fig. 4B-D and M,N).

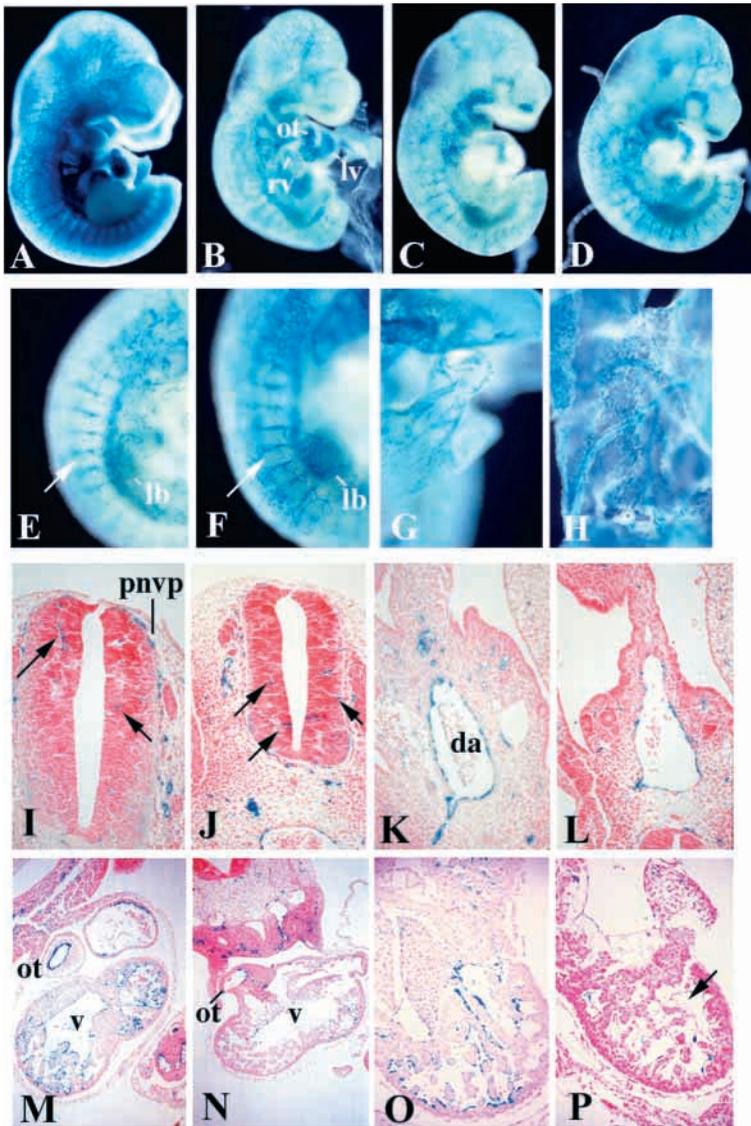
In contrast to the exclusion of *Tek*- and *Tie*-deficient cells from the endocardium, there were no obvious differences in the ability of such cells to contribute to the blood vessels permeating all other anatomical sites in the embryo, including the head, branchial arches and limb buds (Fig. 4B-F). Moreover, double mutant cells contributed readily to blood vessels of various sizes, showing an obvious presence in large vessels such as the dorsal aorta (Fig. 4L) as well as in smaller vessels and capillaries throughout the body (Fig. 4J,L). In these chimeras, double mutant ECs were also capable of giving rise to angiogenic sprouts in the

**Table 4. Distribution of genotypes of embryonic and adult chimeras**

Stage	+/+/+	<i>tie<sup>lacZ</sup>tek<sup>Δsp</sup>//++</i> or <i>tie<sup>lacZn-</sup>tek<sup>Δsp</sup>//++</i>	<i>tie<sup>lacZ</sup>tek<sup>Δsp</sup>//tie<sup>lacZn-</sup>tek<sup>Δsp</sup></i>	Total no. animals
E10.5	47 (26.5%)	92 (52%)	38 (21.5%)	177
E15.5	20 (25.3%)	48 (60.7%)	11 (14%)	79
Adult	14 (58.3%)	6 (25%)	4 (16.7%)	24



**Fig. 3.** (A) Production of chimeras by the morula-morula aggregation strategy. (B) Determination of the extent of chimerism by GPI-1 isoenzyme analysis of tail, limb or liver tissue samples of the chimeras shown in Figs 4-7. Lanes 1 and 2: 100% and 50% *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//++* cell derived E10.5 chimeras shown in Fig. 4A and B, respectively. Lane 3: 50% *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//tie<sup>lacZn-</sup>tek<sup>Δsp</sup>* cell derived E10.5 chimera shown in Fig. 4C. Lanes 4 and 5: 100% and 50% *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//++* cell derived E15.5 chimeras shown in Fig. 5A,B and 5C,D respectively. Lane 6: 50% *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//tie<sup>lacZn-</sup>tek<sup>Δsp</sup>* cell derived E15.5 chimera shown in Fig. 5E,F. Lane 7: 70% *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//tie<sup>lacZn-</sup>tek<sup>Δsp</sup>* cell derived adult chimera shown in Fig. 7C,F,I,L. Lane 8: 70% *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//++* cell derived adult chimera shown in Fig. 7B,E,H,K.

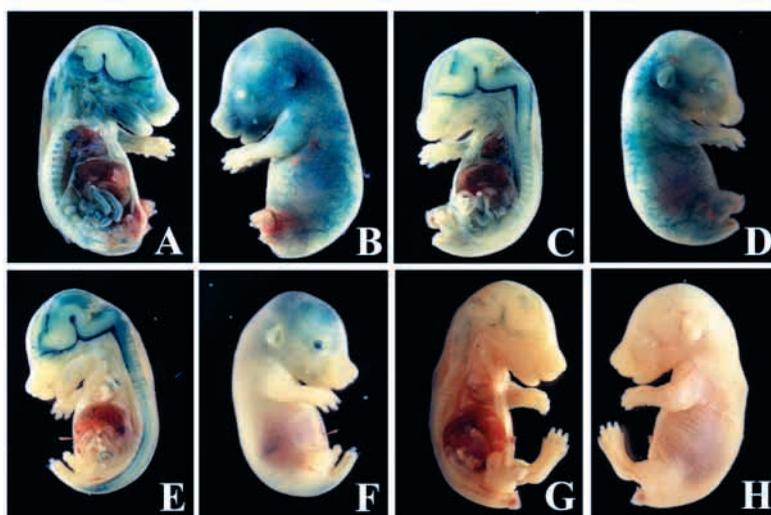


**Fig. 4.** Analysis of *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* cell contribution to the heart and vasculature of chimeras at E10.5. (A-D) Whole-mount X-gal stained chimeras at E10.5. (A) 100% *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* embryo derived chimera as control for X-gal staining in all blood vessels and the endocardium at this stage. (B) Control chimera derived from 50% *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* cells shows uniform contribution to all blood vessels and the endocardium. (C,D) Chimeras composed of 50% *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* cells exhibit normal growth and development, and mutant cell contribution to all vascular structures except the atrium and ventricle. (E) Trunk region of X-gal-stained chimeric embryo derived from 50% *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* cells and (F) equivalent *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* chimera which shows *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* EC contribution to limb bud vasculature, intersomitic vessels (arrows) and their branches, and numerous dorsal capillaries. (G) Yolk sac of X-gal stained chimeric embryo derived from 50% *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* cells and (F) equivalent *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* chimera which shows normal vasculature and contribution of mutant cells to all vessels. (I-P) Histological analysis of 50% *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* cell derived (I,K,M,O) or *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* derived (J,L,N,P) chimeras. (I,J) Transverse section through neural tube. *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* chimera (J) shows normal sprout formation into the neuroectoderm from the perineural vascular plexus (arrows) and contribution of mutant cells to capillaries permeating the surrounding mesenchyme. (K,L) Descending dorsal aorta. (L) *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* cell contribution to endothelium of this vessel is as extensive as by *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* cells (K). (M,N) Transverse section through the heart. *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* cells readily contribute to endocardium of atrium, ventricles, and outflow tract (M) but endocardial cells are primarily wild-type derived in atrium and ventricles of *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* chimera (N). Note contribution of *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* cells to endocardium of outflow tract. (O,P) High power view of ventricles. *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* cells are reduced in the endocardium (arrow) compared to *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* chimera, but trabeculation is normal. Ot, outflow tract; rv, right ventricle; lv, left ventricle; lb, limb bud; pnvp, perineural vascular plexus; da: dorsal aorta; v: ventricle

intersomitic area (Fig. 4E,F) which arise in part from the dorsal aorta (Coffin and Poole, 1988) and in the neural tube (Fig. 4I,J) which is vascularized by angiogenic sprout formation from the

surrounding perineural vascular plexus and by invasion of migratory angioblasts (Stewart and Wiley, 1981; Noden, 1991b; Kurz et al., 1996). Finally, we observed that *Tek*- and *Tie*-deficient cells were also present in the blood vessels of the extra-embryonic circulation including the vessels of the yolk sac (Fig. 4G,H) and those of the fetal component of the placenta (data not shown).

Remarkably, all *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup> ↔ CD-1* chimeric embryos of the type shown in Fig. 4 were



**Fig. 5.** Whole-mount analysis of *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* cell contribution to the vasculature of E15.5 chimeras. X-gal stained chimeras derived from *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* embryos (A-D) and *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* embryos (E-H). (A,B) Strong (90-100% by GPI isoenzyme analysis) *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* chimera showing contribution of *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* cells to all ECs of the embryo. (C,D) Moderate *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* chimera (50% overall contribution by *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* cells) showing contribution of *tie<sup>lc</sup>tek<sup>Δsp</sup>/+* cells to endothelium of the internal organs, meninges, facial structures and skin. (E-H) Moderate *tie<sup>lc</sup>tek<sup>Δsp</sup>/tie<sup>lc</sup>zn-tek<sup>Δsp</sup>* chimeras (50% mutant cell contribution) demonstrating lack of mutant cell contribution to most vascular structures.

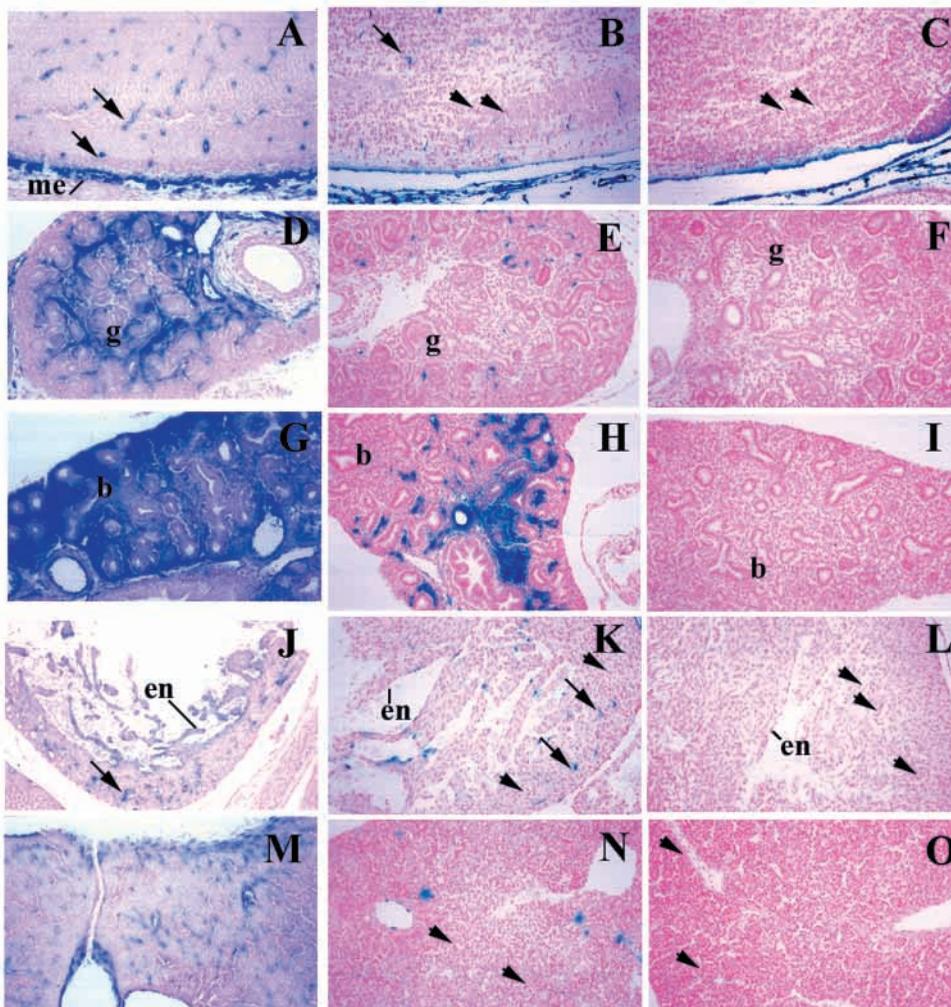
rescued of all of the deleterious characteristics associated with the doubly homozygous mutant phenotype. For instance, normal trabeculae were present in the ventricles of such chimeras and the major blood vessels of the head were correctly remodelled into large and small sized vessels, as were the vessels of the yolk sac vasculature (Fig. 4B,D,G,H). Additionally, the size of rescued chimeric embryos was comparable to their equivalent littermate chimeras derived from doubly heterozygous or wild-type cells (Fig. 4A-D and data not shown).

The doubly homozygous mutant chimeras described above represented approximately 40% of all *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lczn</sup>-tek<sup>Δsp</sup>↔CD-1* chimeras, and all were composed of 60% or less mutant cells as judged by GPI analysis (Fig. 3B). In contrast, an equal number of *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lczn</sup>-tek<sup>Δsp</sup>↔CD-1* chimeras displayed phenotypic features identical to those of the doubly homozygous mutant embryos shown in Fig. 1. These chimeras (13/15) were essentially derived from mutant cells as judged by GPI analysis. Other strong chimeric embryos containing endocardium derived from mutant cells, but which were healthy, were found at approximately the predicted recombination frequency between *Tek* and *Tie* loci. Phenotypically, these embryos resembled *Tie*<sup>-/-</sup> chimeras as described previously (Partanen et al., 1996), and we therefore assume they are chimeras derived from recombinant morulae.

### Doubly homozygous mutant cells were significantly underrepresented in most blood vessels in E15.5 chimeras

We next evaluated chimeras at E15.5, an advanced stage of fetal development when many events of organogenesis and vascularization of organs have been completed. The contribution by doubly homozygous mutant cells to the vasculature in chimeras ( $n=8$ ) in which 40-60% of the cells were derived from the mutant embryo, was significantly reduced (Figs 5E-H, 3B). In contrast, moderate chimeras generated from doubly heterozygous (*tie<sup>lcz</sup>tek<sup>Δsp</sup>//++* or *tie<sup>lczn</sup>-tek<sup>Δsp</sup>//++*) embryos, composed of 40-60% doubly heterozygous cells ( $n=16$ ), as well as stronger chimeras with doubly heterozygous cells ( $n=26$ ) contained significant numbers of  $\beta$ -galactosidase-positive cells in most organs (Fig. 5C,D and A,B respectively; Fig. 3B).

The blood vessels of chimeric embryos derived from double mutant morulae were essentially only of wild-type origin in the capillaries of the midbrain, kidney, lung, heart and liver (Fig. 6C,F,I,L,O), as well as stomach intestine, and skeletal muscle (data not shown). In most *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lczn</sup>-tek<sup>Δsp</sup>↔CD-1* chimeras, the aorta was also devoid of ECs of double mutant cell origin (data not shown). In the heart, although the endocardium was derived from wild-type cells in all chimeras examined, mutant ECs occasionally populated the blood vessels that permeate the myocardial tissue (data not shown). Weak



**Fig. 6.** Histological comparison of *tie<sup>lcz</sup>tek<sup>Δsp</sup>//++* and *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lczn</sup>-tek<sup>Δsp</sup>* cell contribution to E15.5 embryonic vasculature. Brain (A-C), kidney (D,E,F), lung (G,H,I), heart (J,K,L) and liver (M,N,O) sections from X-gal-stained E15.5 chimeras. A strong (90%) *tie<sup>lcz</sup>tek<sup>Δsp</sup>//++↔CD-1* chimera (A,D,G,J,M) shows strong contribution of *tie<sup>lcz</sup>tek<sup>Δsp</sup>//++* cells to ECs of all organs. A moderate (40-50%) *tie<sup>lcz</sup>tek<sup>Δsp</sup>//++↔CD-1* chimera (B,E,H,K,N) shows *tie<sup>lcz</sup>tek<sup>Δsp</sup>//++* cell contribution to ECs, but contribution in kidney, brain and liver is reduced. A *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lczn</sup>-tek<sup>Δsp</sup>↔CD-1* chimera (C,F,I,L,O) shows no contribution of mutant cells to vasculature of any tissues except the meningeal layer of the brain. Arrows indicate vessels showing EC of double mutant or doubly heterozygous cell origins; arrowheads denote vessels with ECs of wild-type origin. All sections photographed under 20 $\times$  objective. Me, meninges; g, glomerulus; b, bronchiolus; en, endocardium.

contribution by double mutant cells to larger vessels of the skin and to blood vessels of the meninges surrounding the brain and spinal chord was also observed in these chimeras (Figs 5E,F, 6C).

In contrast to the behaviour of doubly homozygous mutant cells in the E15.5 embryo, doubly heterozygous cells were capable of contributing to virtually all blood vessels and capillaries of the E15.5 embryos in strong chimeras (>80% doubly heterozygous cells as judged by GPI analysis), (Fig. 6A,D,G,J,M). Similar observations were made for other organs including the intestine, stomach, skeletal muscle and limbs as well as for the aorta and blood vessels of the intercostal region (data not shown).

In all moderate chimeras composed of 40-60% *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//++* or *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//++* cells ( $n=16$ ), doubly heterozygous cells contributed to the blood vessels in the brain, meninges, kidney, the microvasculature of the heart, the endothelium of the lung, (Fig. 6B,E,H,G,K) skin (Fig. 5D), intestine, stomach, skeletal muscle and aorta and intercostal vessels (data not shown). Contribution to the endocardium of the heart, capillaries in the brain and kidney glomeruli was markedly reduced in these moderate chimeras (Fig. 6B,E,K) and doubly heterozygous cells in all such chimeras were almost entirely excluded from the vessels of the liver (Fig. 6N).

The results presented above indicate that there is an absolute requirement for both TEK and TIE in virtually all vascular structures of the embryo by E15.5 of gestation. Also, even doubly heterozygous cells are at a competitive disadvantage with wild-type cells during development of endothelium of certain tissues such as kidney, brain and liver.

### Both doubly homozygous and doubly heterozygous cells were absent from blood vessels in adult chimeras

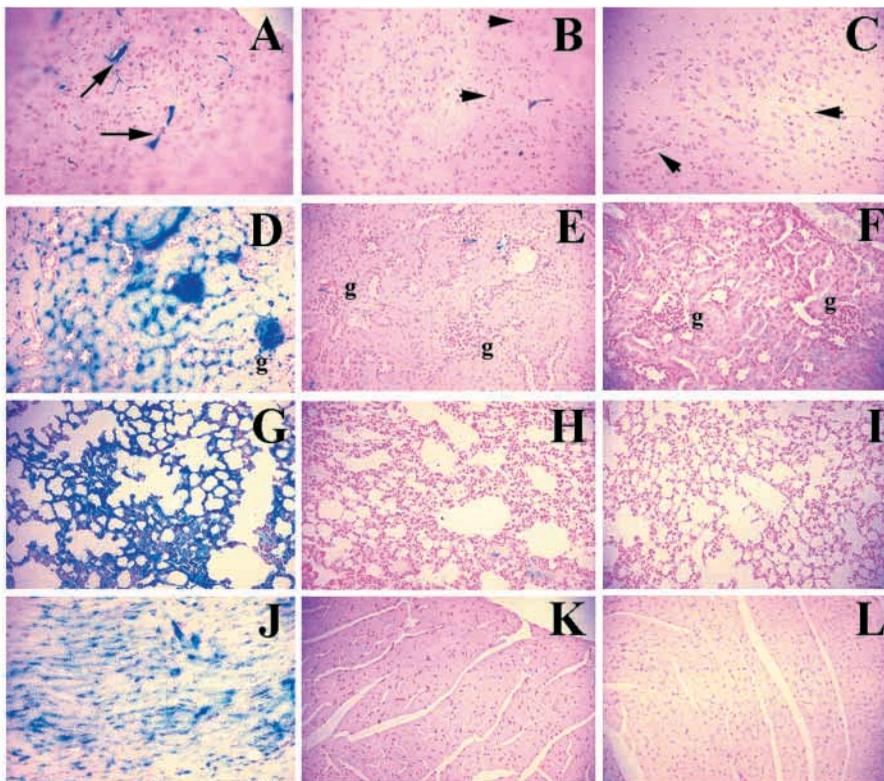
To address the role of TEK and TIE in the fully mature

vasculature, we allowed some aggregation chimeras to reach adulthood. The organs were then dissected and assessed for their overall levels of contribution by GPI analysis, as well as for X-gal staining. As shown in Table 4, there was a severe reduction in the number of adult chimeras with measurable contribution from either mutant or even doubly heterozygous cells. The majority of surviving adult animals were genotypically wild type with a depletion of chimeras derived from double mutant or doubly heterozygous morulae.

Of four adult chimeras derived from doubly homozygous mutant morulae, one showed a contribution level of over 50% in all organs (Fig. 3B), whereas the remaining three chimeras were composed of only 15-30% mutant cells as assessed by GPI isozyme analysis. Histological analysis of the  $\beta$ -galactosidase staining profiles indicated that mutant ECs were completely absent in blood vessels of virtually all organs tested including the brain, kidney, lung, and heart microvasculature (Fig. 7C,F,I,L), as well as skeletal muscle (not shown).

Interestingly, the blood vessels of equivalent chimeras generated from doubly heterozygous embryos were also lined almost entirely with wild-type endothelium (Fig. 7B,E,H,K). The lung occasionally showed some contribution by these cells, although their contribution was generally not extensive (data not shown).

All organ staining patterns were compared to the pattern for an age matched control *tie<sup>lacZ</sup>/+* animal (Fig. 7) to rule out the possibility that the lack of EC-specific staining in the chimeras was not the result of downregulation of the *lacZ* reporter gene. Moreover, both doubly mutant and doubly heterozygous cells still gave rise to non-vascular cells in all of the tissues tested, indicating the specificity of the lack of contribution to the endothelium. One exception to this result was in the peripheral blood: although we invariably detected mutant and heterozygous circulating blood cells by GPI isozyme analysis,



**Fig. 7.** Histological comparison of *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//++* and *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//tie<sup>lacZ</sup>tek<sup>Δsp</sup>* cell contribution to adult vasculature. Brain (A-C), kidney (D-F), lung (G-I) and heart (J-L) sections from X-gal stained organs of adult *tie<sup>lacZ</sup>/+* control mouse (A,D,G,J); *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//++*  $\leftrightarrow$  CD-1 chimera (B,E,H,K) and *tie<sup>lacZ</sup>tek<sup>Δsp</sup>//tie<sup>lacZ</sup>tek<sup>Δsp</sup>*  $\leftrightarrow$  CD-1 chimera (C,F,I,L). Arrows indicate vessels showing EC contribution by double mutant or doubly heterozygous cells; arrowheads denote vessels with ECs of wild-type origin. All sections photographed under 20 $\times$  objective. g, glomerulus.

the contribution to the peripheral hematopoietic system was always at a lower level than for other organs from the same animal.

## DISCUSSION

The studies reported here have focused on the elucidation of the function of TEK and TIE RTKs in the cardiovascular system using mutational and mosaic analyses in the mouse embryo. The results presented show that these receptors play critical roles in ECs at different stages of development: first in the heart endocardium in early gestation, subsequently during maturation and angiogenic expansion of the vasculature in late embryogenesis, and finally throughout the quiescent endothelium of the adult.

### Function of TEK and TIE during embryogenesis

Previous studies clearly demonstrated that *Tek* and *Tie* are both expressed in angioblasts, that is, in EC precursors prior to tube formation and vessel maturation (Dumont et al., 1995; Sato et al., 1993; Schnürch and Risau, 1993; Korhonen et al., 1994). Despite this overlapping expression pattern, embryos lacking both receptors have ECs and survive up to at least E9.0 with a basic, albeit poorly functioning circulatory system. Furthermore, *Tek*- and *Tie*-deficient embryonic cells contribute to virtually all blood vessels of the E10.5 chimeric embryo. Thus we conclude that TEK and TIE do not share redundant essential functions in vasculogenesis, the initial differentiation of ECs.

However, we have also demonstrated genetic interaction and overlapping biological function for TEK and TIE during development. Embryos lacking both copies of *Tie* are particularly sensitive to *Tek* gene dosage in cardiovascular development, indicating that these receptors mediate a common subset of functions during cardiogenesis and development of the vasculature.

A major objective of the chimeric analysis of embryos at E10.5 was to elucidate the primary defects leading to the multiple cardiovascular abnormalities exhibited by embryos lacking both TEK and TIE. The presence of doubly homozygous ECs in essentially all vascular structures except the endocardium implicates compromised circulation due to poor endocardial function as a fundamental cause of death in doubly homozygous mutant embryos. The initial specification of the endocardium does not require TEK and TIE since cells with endocardial morphology are clearly present even in double mutant embryos. Since heart development is normal in *Tie* mutant embryos (Puri et al., 1995) and the endocardial defect is completely penetrant in *Tek* homozygous mutants (Dumont et al., 1994; Sato et al., 1995), signalling by TEK in this tissue is more critical than that of TIE. This view is supported by our previous finding that *Tie* homozygous mutant ECs were unimpaired in their ability to contribute to and remain part of the mature endocardium in adult chimeras (Partanen et al., 1996). The stringent requirement for TEK in the endocardium is underscored by the exclusion of *Tek*- and *Tie*-deficient cells from this structure in all E10.5 chimeras that were visibly rescued of the deleterious features of the double mutant phenotype.

Recently, Takakura et al. (1998) reported a defect in early

definitive hematopoiesis in *Tek* nullizygotes which are pale and anemic at dissection, as are *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lcz</sup>tek<sup>Δsp</sup>* embryos. This observation suggests that there is either an intrinsic cell autonomous requirement for TEK in hematopoiesis, or that TEK is required indirectly to establish an appropriate microenvironment for hematopoiesis. The appearance of *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lczn</sup>-tek<sup>Δsp</sup>* cell derived hematopoietic cells in the peripheral blood of chimeric adults suggests that the apparent block in hematopoietic cell differentiation in *Tek* nullizygotes has been overcome by restoration of the appropriate microenvironment. Thus we conclude that TEK is involved in establishing the correct vascular microenvironment for hematopoiesis to proceed.

### TEK and TIE are not essential for angiogenic sprout formation and vessel remodelling during early embryogenesis

Previous studies have suggested that TEK is required for angiogenic sprout formation during embryogenesis (Sato et al., 1993; Suri et al., 1996). *Tek*-deficient ECs are also incapable of sprout formation in vitro in response to Ang-1 (Koblizek et al., 1998). In addition, *Tek*<sup>-/-</sup> and *Ang-1*<sup>-/-</sup> embryos exhibit a prominent defect in blood vessel remodelling. A paucity of pericytes and smooth muscle cells at sites of defective vessel branching was noted in *Ang-1*- and *Tek*-defective embryos, as well as in human patients with vascular dysmorphogenesis disorder due an activating mutation in TEK (Suri et al., 1996; Patan, 1998; Vikkula et al., 1996). These findings have led to the suggestion that Ang-1/TEK signal transduction mediates perivascular cell recruitment to the vessel wall during development perhaps by stimulating the release of chemoattractant growth factors (Vikkula et al., 1996; Folkman and D'Amore, 1996).

These phenotypes are seen either in vitro or in dying embryos, making it difficult to distinguish between primary and secondary phenomena. To circumvent this dilemma, we analysed the developmental potential of embryos lacking both TEK and TIE in chimeras in which normal heart function is restored due to the presence of wild-type cells. In this situation, double mutant cells contributed to virtually all blood vessels, including those participating in angiogenic sprouting. Furthermore, the presence of even a large number of *Tek*- and *Tie*-deficient cells in the developing vasculature in this chimeric environment did not lead to abnormal vessel morphology, nor did it prevent the early steps of vessel remodelling such as the reorganization of uniform capillary plexi to networks of variously sized vessels. This outcome was also not the result of wild-type rescue of the extra-embryonic circulation since double mutant *Tek*- and *Tie*-deficient cells readily contributed to both the yolk sac and placental vasculatures. These results strongly suggest that TEK and TIE are not directly necessary for the processes of early angiogenic sprout formation and peri-vascular cell recruitment in extra-embryonic and embryonic tissues and that these deficiencies are secondary to the endocardial and hematopoietic defects in *Tek*<sup>-/-</sup> and *tie<sup>lcz</sup>tek<sup>Δsp</sup>//tie<sup>lcz</sup>tek<sup>Δsp</sup>* mice. Due to the nature of the chimeric assay, we cannot exclude the possibility that growth factors necessary for perivascular cell recruitment are provided by wild-type cells inherently present in these animals. However, given the strong selection against doubly homozygous mutant ECs in later stage chimeric embryos and

in the adult, we favour the view that TEK and TIE play a cell autonomous role in the endothelium at all stages of life.

### Requirement for TEK and TIE in the mature vasculature

Our studies have also addressed the roles of TEK and TIE in the mature vasculature using chimeric rescue to reveal functions obscured by earlier embryonic lethality of the double knockout embryo. ECs lacking functional TEK and TIE were clearly absent from virtually all vascular endothelia of adult tissues. We conclude that there is an essential, ongoing role for TEK and TIE in quiescent ECs, perhaps in EC maintenance or survival. The continuous expression of *Tek*, *Tie* and *Ang-1* in a variety of adult tissues (Partanen et al., 1996; Wong et al., 1997; Maisonpierre et al., 1997) as well as the phosphorylation of TEK in the quiescent adult endothelium (Wong et al., 1997) are consistent with this conclusion. The physiological importance of TEK/TIE mediated signalling in mature endothelia is underscored by the competitive disadvantage displayed by doubly heterozygous ECs in adult chimeras, and also by the strikingly diminished survival to adulthood of chimeras derived from doubly homozygous mutant embryos.

Our previous studies of *Tie* chimeras also demonstrated a requirement for TIE in the adult endothelium (Partanen et al., 1996). However, in these chimeras, selection against *Tie*<sup>-/-</sup> ECs from several tissues including lung, kidney and adrenal gland occurred gradually during the late embryonic and postnatal period (Partanen et al., 1996). In contrast, *tie*<sup>lcz</sup>*tek*<sup>Δsp</sup>/*tie*<sup>lczn</sup>-*tek*<sup>Δsp</sup> cells were excluded from virtually all endothelia by E15.5 of embryogenesis. This difference implies that TEK is required earlier than TIE by the maturing vascular endothelium in particular vascular beds.

A specific requirement for TEK in maturing endothelia is illustrated in the heart, which when fully developed contains discrete populations of ECs that arise at distinct gestational stages from diverse angioblast populations. We have previously shown that TIE is not strictly required in any heart ECs (Partanen et al., 1996), provided wild-type levels of TEK are present (this work). Selection against *tie*<sup>lcz</sup>*tek*<sup>Δsp</sup>/*tie*<sup>lczn</sup>-*tek*<sup>Δsp</sup> cells was first evident by E10.5 in endocardial cells of the atria and ventricles. These cells differentiate during the late primitive streak stage and play an active role in subsequent myocardial differentiation. At E10.5, when these endocardial cells were at a clear competitive disadvantage relative to wild-type cells, *tie*<sup>lcz</sup>*tek*<sup>Δsp</sup>/*tie*<sup>lczn</sup>-*tek*<sup>Δsp</sup> cells contributed normally to the outflow tract (OT) endothelium. Fate mapping studies in the chick have demonstrated that OT endothelium arises independently of and after endocardial differentiation (Noden, 1991a). Thus, at E10.5, the OT endothelium represents a younger EC population whose requirement for TEK is not manifested until its further differentiation into inner lining of the vessel derivatives of the OT. Accordingly, *tie*<sup>lcz</sup>*tek*<sup>Δsp</sup>/*tie*<sup>lczn</sup>-*tek*<sup>Δsp</sup> cells were absent from the endothelium of OT-derived vessels. Finally, the myocardial microvasculature which forms by invasion of angioblasts from an extra-cardiac source at an even later stage of embryogenesis (Gittenberger-de Groot et al., 1998; Poelmann et al., 1993), showed some contribution by *Tek*- and *Tie*-deficient cells at E15.5, but was composed primarily of wild-type cells when analysed in the adult doubly homozygous mutant chimeras. These examples strongly support a primary and cell

autonomous role for TEK in maturing endothelia during embryonic development.

In conclusion, our studies have demonstrated that TEK and TIE do not mediate a redundant function in angioblast differentiation during early vascular development. Contrary to previous inferences, TEK does not appear to be required for early angiogenic sprout formation, angiogenic growth, or perivascular cell recruitment. Rather, we conclude that TEK and TIE support maturation and maintenance or survival of ECs from late gestation throughout the life of the animal. This ongoing requirement for TEK and TIE to maintain the integrity of the adult vasculature makes these receptors and their intracellular signalling pathways likely targets in diseases involving angiogenesis and neovascularization such as cancer and cardiovascular disease.

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### REFERENCES

- Beck, L. J. and D'Amore, P. A. (1997). Vascular development: cellular and molecular regulation. *FASEB J.* **11**, 365-373.
- Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kiekens, L., Gertsenstein, M., Fahrig, M., Vandenhoek, A., Harpal, K., Eberhardt, C., et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-439.
- Coffin, D. J., Harrison, J., Schwartz, S. and Heimark, R. (1991). Angioblast differentiation and morphogenesis of the vascular endothelium in the mouse embryo. *Dev. Biol.* **148**, 51-62.
- Coffin, J. D. and Poole, T. J. (1988). Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development* **102**, 735-748.
- Dumont, D. J., Fong, G.-H., Puri, M. C., Gradwohl, G., Alitalo, K. and Breitman, M. L. (1995). Vascularization of the mouse embryo: a study of *flk-1*, *tek*, *tie* and vascular endothelial growth factor expression during development. *Dev. Dynam.* **203**, 80-92.
- Dumont, D. J., Gradwohl, G., Fong, G.-H., Puri, M. C., Gertsenstein, M., Auerbach, A. and Breitman, M. L. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, *tek*, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8**, 1897-1909.
- Dumont, D. J., Jussila, L., Taipale, J., Mustonen, T., Pajusola, K., Breitman, M. and Alitalo, K. (1998). Failure of cardiovascular development in embryos deficient of the lymphatic vascular endothelial growth factor-C receptor, VEGFR-3. *Science* **282**, 946-949.
- Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-442.
- Ferrara, N. and Davis-Smith, T. (1997). The biology of vascular endothelial growth factor. *Endocrine Rev.* **18**, 4-25.
- Folkman, J. and D'Amore, P. A. (1996). Blood vessel formation: what is its molecular basis? *Cell* **87**, 1153-1155.
- Fong, G.-H., Rossant, J., Gertsenstein, M. and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376**, 66-70.
- Gittenberger-de Groot, A. C., Vranken-Peters, M.-P. F. M., Mentink, M.

- M. T., Gourdie, R. G. and Poelmann, R. E.** (1998). Epicardium-derived cells contribute a novel population to the myocardial wall and the atrioventricular cushions. *Circ. Res.* **82**, 1043-1052.
- Hanahan, D.** (1997). Signaling vascular morphogenesis and maintenance. *Science* **277**, 48-50.
- Koblizek, T. I., Weiss, C., Yancopoulos, G. D., Deutsch, U. and Risau, W.** (1998). Angiopoietin-1 induces sprouting angiogenesis in vitro. *Curr. Biol.* **8**, 529-532.
- Korhonen, J., Polvi, A., Partanen, J. and Alitalo, K.** (1994). The mouse tie receptor tyrosine kinase gene: expression during embryonic angiogenesis. *Oncogene* **9**, 395-403.
- Kurz, H., Gartner, T., Eggl, P. S. and Christ, B.** (1996). First blood vessels in the avian neural tube are formed by a combination of dorsal angioblast immigration and ventral sprouting of endothelial cells. *Dev. Biol.* **173**, 133-147.
- Lyons, M. S., Bell, B., Stainier, D. and Peters, K. G.** (1998). Isolation of the zebrafish homologues for the *tie-1* and *tie-2* endothelium specific receptor tyrosine kinases. *Dev. Dyn.* **212**, 133-140.
- Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T., et al.** (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277**, 55-60.
- McCarthy, M. J., Crowther, M., Bell, P. R. F. and Brindle, N. P. J.** (1998). The endothelial receptor tyrosine kinase *tie-1* is upregulated by hypoxia and vascular endothelial growth factor. *FEBS Lett.* **423**, 334-338.
- Nagy, A. and Rossant, J.** (1993). Production of completely ES cell derived fetuses. In *Gene Targeting, a Practical Approach*. (ed. A. Joyner), IRL press, New York.
- Noden, D.** (1991a). Origins and patterning of avian outflow tract endocardium. *Development* **111**, 867-876.
- Noden, D. M.** (1991b). Origins and assembly of avian embryonic blood vessels. *Annals. New York Acad. Sci.* **1**, 236-249.
- Pardanaud, L., Altmann, C., Kitos, P., Dieterlen-Lievre, F. and Buck, C. A.** (1987). Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* **100**, 339-349.
- Pardanaud, L., Yassine, F. and Dieterlen-Lievre, F.** (1989). Relationship between vasculogenesis, angiogenesis, and haemopoiesis during avian ontogeny. *Development* **105**, 473-485.
- Partanen, J. and Dumont, D. J.** (1999). Functions of Tie1 and Tie2 receptor tyrosine kinases in vascular development. *Curr. Top. Microbiol. Immunol.* **237**, 159-272.
- Partanen, J., Puri, M. C., Schwartz, L., Fischer, K.-D., Bernstein, A. and Rossant, J.** (1996). Cell autonomous functions of the receptor tyrosine kinase TIE in a late phase of angiogenic capillary growth and endothelial cell survival during murine development. *Development* **122**, 3013-3021.
- Patan, S.** (1998). TIE1 and TIE2 receptor tyrosine kinases inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth. *Microvasc. Res.* **56**, 1-21.
- Poelmann, R. E., Gittenberger-de Groot, A. C., Mentink, M. M. T., Bokenkamp, R. and Hogers, B.** (1993). Development of the cardiac coronary vascular endothelium, studied with antiendothelial antibodies, in chicken-quail chimeras. *Circ. Res.* **73**, 559-568.
- Puri, M. C., Rossant, J., Alitalo, K., Bernstein, A. and Partanen, J.** (1995). The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J.* **14**, 5884-5891.
- Risau, W.** (1997). Mechanisms of angiogenesis. *Nature* **386**, 671-674.
- Sariola, H., Ekblom, P., Lehtonen, E. and Saxen, L.** (1983). Differentiation and vascularization of the metanephric kidney grafted on the chorioallantoic membrane. *Dev. Biol.* **96**, 427-435.
- Sato, T. N., Qin, Y., Kozak, C. and Audus, K. L.** (1993). *tie-1* and *tie-2* define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc. Natl. Acad. Sci. USA* **90**, 9355-9358.
- Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maquire, M., Gridley, T., Wolburg, H., Risau, W. and Qin, Y.** (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376**, 70-74.
- Schaffer, A.J. and Avery, M.E.** (1971). *Diseases of the Newborn*. W.B. Saunders Co. Philadelphia.
- Schnürch, H. and Risau, W.** (1993). Expression of *tie-2*, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage. *Development* **119**, 957-968.
- Shalaby, F., Ho, J., Fischer, K.-D., Schuh, A. C., Schwartz, L., Bernstein, A. and Rossant, J.** (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89**, 981-990.
- Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X.-F., Breitman, M. L. and Schuh, A. C.** (1995). Failure of blood island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376**, 62-66.
- Stewart, P. A. and Wiley, M. J.** (1981). Developing nervous tissue induces formation of blood-brain characteristics in invading endothelial cells: a study using quail-chick chimeras. *Dev. Biol.* **84**, 183-192.
- Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N. and Yancopoulos, G. D.** (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87**, 1171-1180.
- Suri, C., McClain, J., Thurston, G., McDonald, D. M., Zhou, H., Oldmixon, E. H., Sato, T. N. and Yancopoulos, G. D.** (1998). Increased vascularization in mice overexpressing angiopoietin-1. *Science* **282**, 468-471.
- Takakura, N., Huang, X.-L., Naruse, T., Hamaguchi, I., Dumont, D. J., Yancopoulos, G. D. and Suda, T.** (1998). Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* **9**, 677-686.
- Vikkula, M., Boon, L. M., Carraway III, K. L., Calvert, J. T., Diamonti, A. J., Goumnerov, B., Pasyk, K. A., Marchuk, D. A., Warman, M. L., Cantley, L. C., et al.** (1996). Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. *Cell* **87**, 1181-1190.
- Wiltling, J. and Christ, B.** (1996). Embryonic angiogenesis: a review. *Naturwissenschaften* **83**, 153-164.
- Witzenbichler, B., Maisonpierre, P. C., Jones, P., Yancopoulos, G. D. and Isner, J.** (1998). Chemotactic properties of Angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2. *J. Biol. Chem.* **273**, 18514-18521.
- Wong, A. L., Haroon, Z. A., Werner, S., Dewhirst, M. W., Greenberg, C. S. and Peters, K. G.** (1997). Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. *Circ. Res.* **81**, 567-574.
- Wood, A. S., Allen, N. D., Rossant, J., Auerbach, A. and Nagy, A.** (1993). Non-injection methods for the production of embryonic stem cell-embryo chimeras. *Nature* **365**, 87-89.