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Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL

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The *TEL* gene, which is frequently rearranged in human leukemias of both myeloid and lymphoid origin, encodes a member of the Ets family of transcription factors. The *TEL* gene is widely expressed throughout embryonic development and in the adult. To determine the requirement for the *TEL* gene product in development we generated *TEL* knockout mice (*TEL*$^{-/-}$) by gene targeting in embryonic stem cells. *TEL*$^{-/-}$ mice are embryonic lethal and die between E10.5–11.5 with defective yolk sac angiogenesis and intra-embryonic apoptosis of mesenchymal and neural cells. Two-thirds of TEL-deficient yolk sacs at E9.5 lack vitelline vessels, yet possess capillaries, indicative of normal vasculogenesis. Vitelline vessels regress by E10.5 in the remaining *TEL*$^{-/-}$ yolk sacs. Hematopoiesis at the yolk sac stage, however, appears unaffected in *TEL*$^{-/-}$ embryos. Our findings demonstrate that TEL is required for maintenance of the developing vascular network in the yolk sac and for survival of selected cell types within the embryo proper.

Keywords: angiogenesis/apoptosis/Ets transcription factor/gene targeting/leukemia

Introduction

The *TEL* (ETV6) gene, which encodes a member of the Ets family of transcription factors, was first identified by virtue of its rearrangement in human chronic myelomonocytic leukemia associated with a t(5;12) chromosomal translocation (Golub *et al*., 1994). Subsequently, the *TEL* gene has been shown to be rearranged in a variety of human leukemias, each subtype being associated with the formation of a chimeric protein product. Fusion partners for *TEL* include the platelet-derived growth factor receptor-β (PDGFRβ) and Abelson (ABL) tyrosine kinases (Golub *et al*., 1994, 1996; Papadopoulos *et al*., 1995), the transcription factors AML-1 (Golub *et al*., 1995; Romana *et al*., 1995a), EVI1 (Raynaud *et al*., 1996a) and proteins of unknown function (Buijs *et al*., 1995). The *TEL*/AML-1 fusion is common in childhood acute lymphoblastic leukemia, where this gene rearrangement accounts for 25–30% of cases (Romana *et al*., 1995b; Shurtleff *et al*., 1995; McLean *et al*., 1996; Raynaud *et al*., 1996b). However, the contribution of TEL to the properties of chimeric proteins is not fully understood. In the *TEL*/PDGFRβ, *TEL*/ABL and *TEL*/AML1 fusions, an amino-terminal conserved region referred to as the helix-loop-helix (HLH) or pointed domain, is contributed to the fusion protein. This domain, which is able to serve as a self-association motif, is critical to the biological activity of the fusion protein (Carrol *et al*., 1996; Golub *et al*., 1996; Hiebert *et al*., 1996; Jousset *et al*., 1997). Conversely, the MN1/*TEL* fusion, the consequence of a t(12;22) translocation in myeloid leukemias, results in the aberrant expression of the *TEL* DNA-binding Ets domain, suggesting that this fusion protein may initiate leukemic transformation through the dysregulation of genes normally regulated by TEL (Buijs *et al*., 1995). Finally, it has been observed that loss of heterozygosity at the *TEL* gene locus is associated with the *TEL*/AML-1 gene rearrangement in childhood leukemia (Cave *et al*., 1995; Golub *et al*., 1995; Romana *et al*., 1995b; Stegmaier *et al*., 1995; Raynaud *et al*., 1996b; Takeuchi *et al*., 1996;). These findings have been interpreted to suggest that TEL loss of function may also contribute to the process of leukemogenesis.

TEL is a widely expressed nuclear protein which recognizes DNA sequences containing a GGAA core motif through a highly conserved 85 amino acid ETS domain (T.R.Golub *et al*., unpublished data; Poirel *et al*., 1997). The amino-terminal HLH or pointed domain is also highly conserved among a subset of Ets proteins, including Ets-1, Ets-2, Erg, Fli-1, GABPα and the *Drosophila* proteins *Yan* and *Pointed* (Wasylyk *et al*., 1993; O'Neill *et al*., 1994). While this domain facilitates self-association of TEL, such oligomerization properties have not been observed for other members of the family, where the function of this domain remains obscure. Nonetheless, it has been established recently that the corresponding region of Ets-1 and Ets-2 is required for full transactivation and particularly for synergy with the Ras pathway (Galang *et al*., 1994; Yang *et al*., 1996). The transcriptional properties of TEL are not fully characterized. Studies suggest that the fusion of *TEL* to AML-1 results in conversion of AML-1, a transcriptional activator, to a transcriptional repressor (Hiebert *et al*., 1996). Preliminary studies of the normal TEL protein similarly suggest that TEL may function as a transcriptional repressor (T.R.Golub *et al*., unpublished data). *In vivo* targets for TEL, however, are not known.

Members of the ETS family of transcription factors are important in diverse developmental processes. For example, the *Drosophila Yan* protein acts as a negative regulator of photoreceptor cell development in the eye (Lai and Rubin, 1992) and is itself negatively regulated by the Ras/MAPK pathway (O'Neill *et al*., 1994). In mice, the expression patterns of Ets-1 and Ets-2 during mouse development suggest potential roles in cell proliferation and differentiation (Maroulakou *et al*., 1994). Indeed,

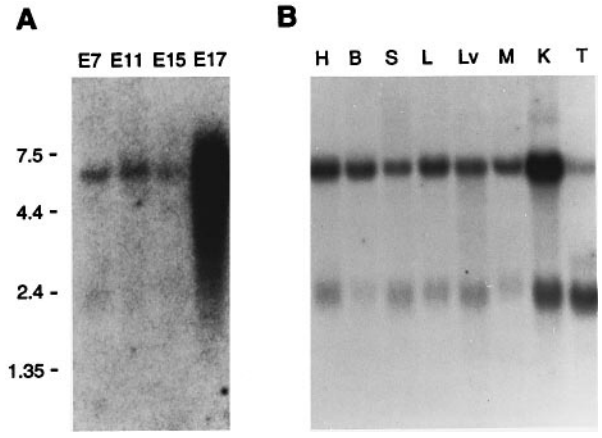

Fig. 1. Northern blot analysis of *TEL* mRNA on staged mouse embryos and adult tissues. (**A**) Poly-A mRNA from embryonic (E) day 7, 11, 15, 17 and (**B**) adult mouse tissues (MTN blots, Clontech) were hybridized with *TEL* cDNA probes as described in Materials and methods. Two principal transcripts were detected in these tissues. The large transcript represents full-length *TEL* mRNA; the nature of the small transcript, however, has not been analyzed. H, heart; B, brain; S, spleen; L, lung; Lv, liver; M, skeletal muscle; K, kidney; T, testis.

studies in chimeric mice have shown that Ets-1 is essential for the survival of T lymphoid cells and for the maintenance of a normal pool of B lymphoid cells (Bories *et al*., 1995; Muthusamy *et al*., 1995). Ets-2 is required for early embryonic development (R.Oshima *et al*., personal communication). Other Ets-related proteins, including PU.1 and Fli-1 are essential for the commitment or differentiation of hematopoietic lineages (Scott *et al*., 1994; McKercher *et al*., 1996; Melet *et al*., 1996).

To pursue the normal role of the TEL protein we have examined the expression of the *TEL* gene in mouse development and generated knockout mice by gene targeting in embryonic stem (ES) cells. We report here that *TEL* is widely expressed with increased expression in neural tissues, developing kidney, lung and liver in the embryo. Analysis of loss of TEL function demonstrates that TEL is essential for normal development. While not strictly required for yolk sac hematopoiesis, the TEL protein is essential for maintaining integrity of the developing vascular network in the yolk sac and for survival of neural and mesenchymal cells within the embryo. Thus, these studies establish TEL as a critical regulator in the survival of multiple cell types during early embryonic development.

Results

Widespread expression of TEL in the embryo and adult mouse

First we examined the pattern of *TEL* expression in the developing embryo and in adult tissues. By Northern blot analysis and *in situ* hybridization, *TEL* mRNA is detected in the embryo as early as E7.0; expression is markedly increased at E17 (Figure 1A). At E8.5 and E9.5, *TEL* is expressed throughout tissues of the embryo proper and the yolk sac (Figure 2A–D and data not shown). At E12.5, higher transcript levels are seen in multiple tissues and organs, including developing lung, kidney and liver. Notably, higher *TEL* expression is also detected in the cranial nerve ganglia, the dorsal root ganglia, and the ventral region of the caudal neural tube (Figure 2E–G). Finally, *TEL* mRNA is expressed in several adult tissues (Figure 1B).

Targeted disruption of TEL results in early embryonic lethality

To inactivate the *TEL* gene, two exons comprising the *TEL* DNA-binding domain and an additional 26 bp of the immediate 3′ adjacent exon were replaced by a PGK-neo cassette (Figure 3A). Of 287 G418- and gancyclovir-resistant ES clones analyzed, five contained an appropriately targeted *TEL* locus (Figure 3B). Two clones were injected into C57BL/6 blastocysts and led to germline transmission of the mutation. In addition, three independently derived double knockout *TEL* ES clones ($TEL^{-/-}$) were obtained by targeting the second allele with a replacement vector containing a PGK-hygromycin resistance cassette (Figure 3B)

To establish that the targeting event resulted in a null mutation, Northern analysis was performed on three $TEL^{-/-}$ ES cell clones. As shown in Figure 3C, no *TEL* mRNA transcripts were detected in these clones, whereas *TEL* mRNA was readily detected in wild-type ES cell samples. In addition, when sets of primers specific for the *TEL* HLH domain, DNA-binding domain, and exons downstream of these domains were used in an RT–PCR assay to amplify *TEL* mRNA, no *TEL* transcripts were detected in $TEL^{-/-}$ embryos; products of the predicted size were seen in control or heterozygous littermates (Figure 3D, and data not shown). Thus, the engineered mutation produces a null allele of the *TEL* locus.

F_1 mice heterozygous for disruption of the *TEL* gene appeared normal in size, fertility, and overall development (data not shown). Heterozygous mice were intercrossed to generate homozygous mutants. Of 168 neonates genotyped, no homozygotes ($TEL^{-/-}$) were identified (Table I). Likewise, no $TEL^{-/-}$ embryos were found after E13.5. Although $TEL^{-/-}$ embryos could be retrieved at E11.5, they were under-represented and grossly retarded in their development (Table I and data not shown). These findings indicate that loss of TEL function results in early embryonic lethality.

Failure to maintain yolk sac blood vessel formation in $TEL^{-/-}$ mice

Analysis of E8.5 embryos from $TEL^{+/-}$ F_1 intercrosses revealed no discernible differences between $TEL^{-/-}$ and control embryos (Table I and data not shown). However, at E9.5, 65% of $TEL^{-/-}$ embryos (Table I, type I mutant) exhibited yolk sacs that lack the branching vitelline vessels normally present in controls (Figure 4A and B). Instead, as highlighted by anti-PECAM antibody staining, a marker for endothelial cells (Baldwin *et al*., 1994), a honeycomb-like network of interconnecting sinusoids was observed (Figure 4C and D). Histologically, branching vitelline vessels of wild-type yolk sacs appear in cross-section as large luminal spaces attached to the undersurface of the mesodermal layer (Figure 4E). Although $TEL^{-/-}$ mutant yolk sacs develop ample sinusoidal spaces or blood islands with lumens, similar in diameter to wild-type and containing pooled primitive red cells, larger lumens are absent (Figure 4F). Endodermal and endothelial cells lining the sinusoids in mutant yolk sacs appear normal

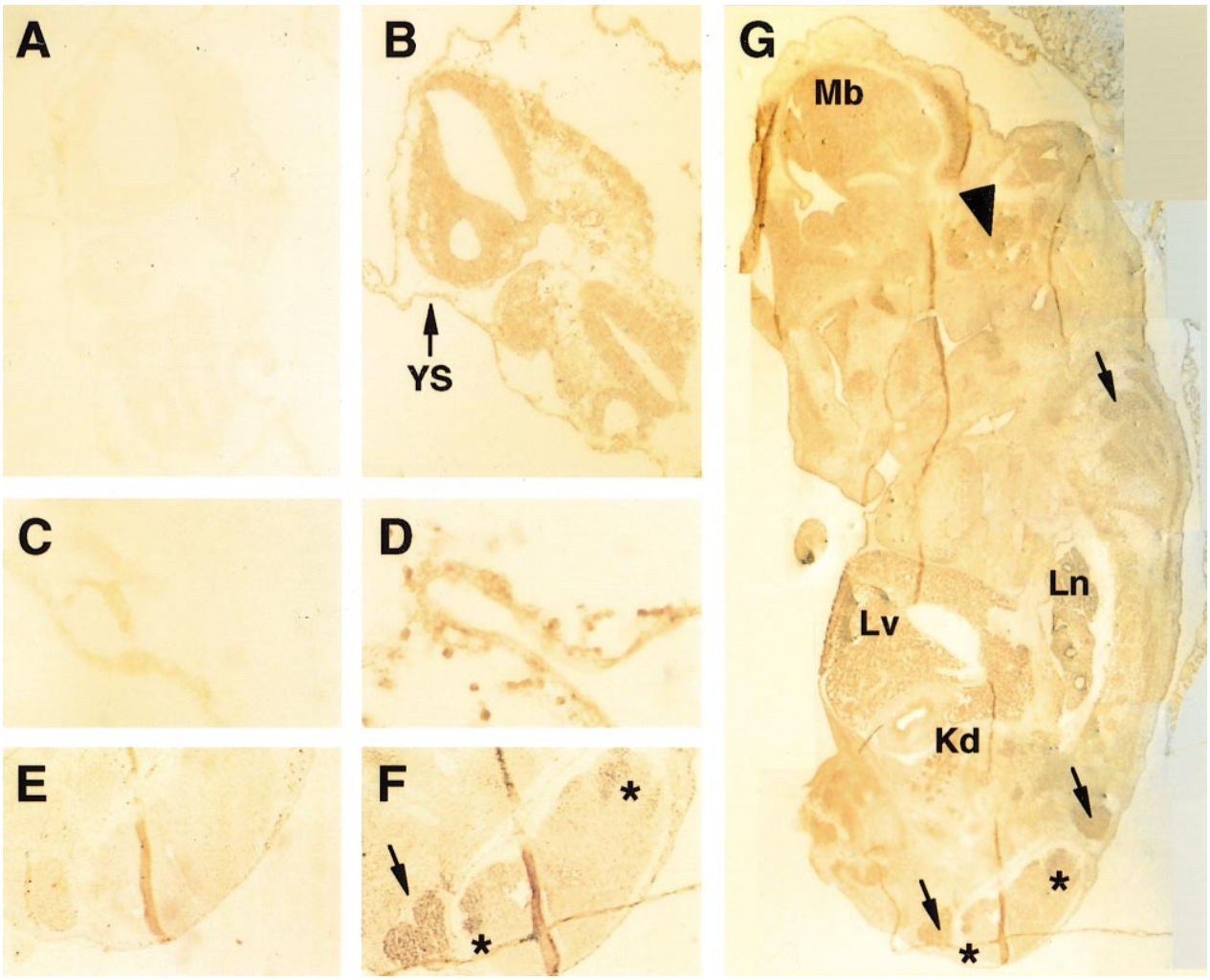

Fig. 2. *In situ* hybridization of *TEL* mRNAs on mouse embryos. Frozen embryo sections from E 8.5 (**A**–**D**) and E 12.5 (**E**–**G**) were hybridized with digoxigenin-11-UTP labeled sense (A, C and E) and antisense (B, D, F and G) TEL probes as described in Materials and methods.
(E) and (F) represent higher magnification of the caudal neural tube region depicted in (G). YS, yolk sac; Mb, midbrain; Lv, liver; Kd, kidney; Ln, lung. Arrowhead indicates trigeminal ganglia; arrows indicate dorsal root ganglia; asterisks indicate ventral region of the caudal neural tube.

(Figure 4E and F). This yolk sac blood vessel defect is seen as early as E9.0 (data not shown). At E9.0–9.5, *TEL*$^{-/-}$ embryos are grossly appropriate in appearance (Figure 4G and H) with regularly beating hearts and visible blood in the circulation. In addition, the number of somites and appearance of the cranial prominence are similar in controls and *TEL*$^{-/-}$ embryos (Figure 4G and H). Placento-allantoid fusion also occurs normally (data not shown). Interestingly, in contrast to the abnormal appearance of yolk sac blood vessels, the dorsal aorta, intersomitic vessels and branching head veins of *TEL*$^{-/-}$ embryos proper are evident by whole mount staining with anti-PECAM antibody, and appear histologically normal (data not shown). Yolk sac-derived embryonic red blood cells are present in the blood vessels of *TEL* $^{-/-}$ embryos, suggesting that yolk sac sinusoidal spaces communicate with the embryonic circulation through small anastomosing channels rather than the vitelline vessels.

Some 35% of *TEL*$^{-/-}$ embryos (Table I, type II mutant) displayed normal-appearing yolk sac vitelline vessels at E9.5 (Figure 4I). However, by E10, all mutants (type I and type II) lack normal vitelline vessels. Occasionally, some residual remnants of vessels formed earlier in type II embryos can be seen as streak-like structures (Figure 4J). At this stage (E10), type II embryos are also developmentally less severely retarded than type I embryos (Figure 4K) correlating with the later onset of the yolk sac blood vessel defect.

By E10.5, all *TEL*$^{-/-}$ embryos are grossly abnormal. The majority of embryos (about two-thirds) are markedly growth-retarded and exhibit an enlarged pericardial sac (Figure 4L). This most likely reflects the fraction of type I mutants that failed to develop normal yolk sac vasculature at E9.5. Histologically, these embryos are largely necrotic (data not shown). The remaining one-third of embryos, presumably type II mutants—those that had developed normal yolk sac vasculature earlier—lose the vitelline vessels completely and exhibit the same honeycomb-like vasculature as type I mutants (data not shown). Therefore, the phenotype of type II mutant embryo indicates that vascular cells of the yolk sac develop, but that they can not be maintained after initial remodeling of primary

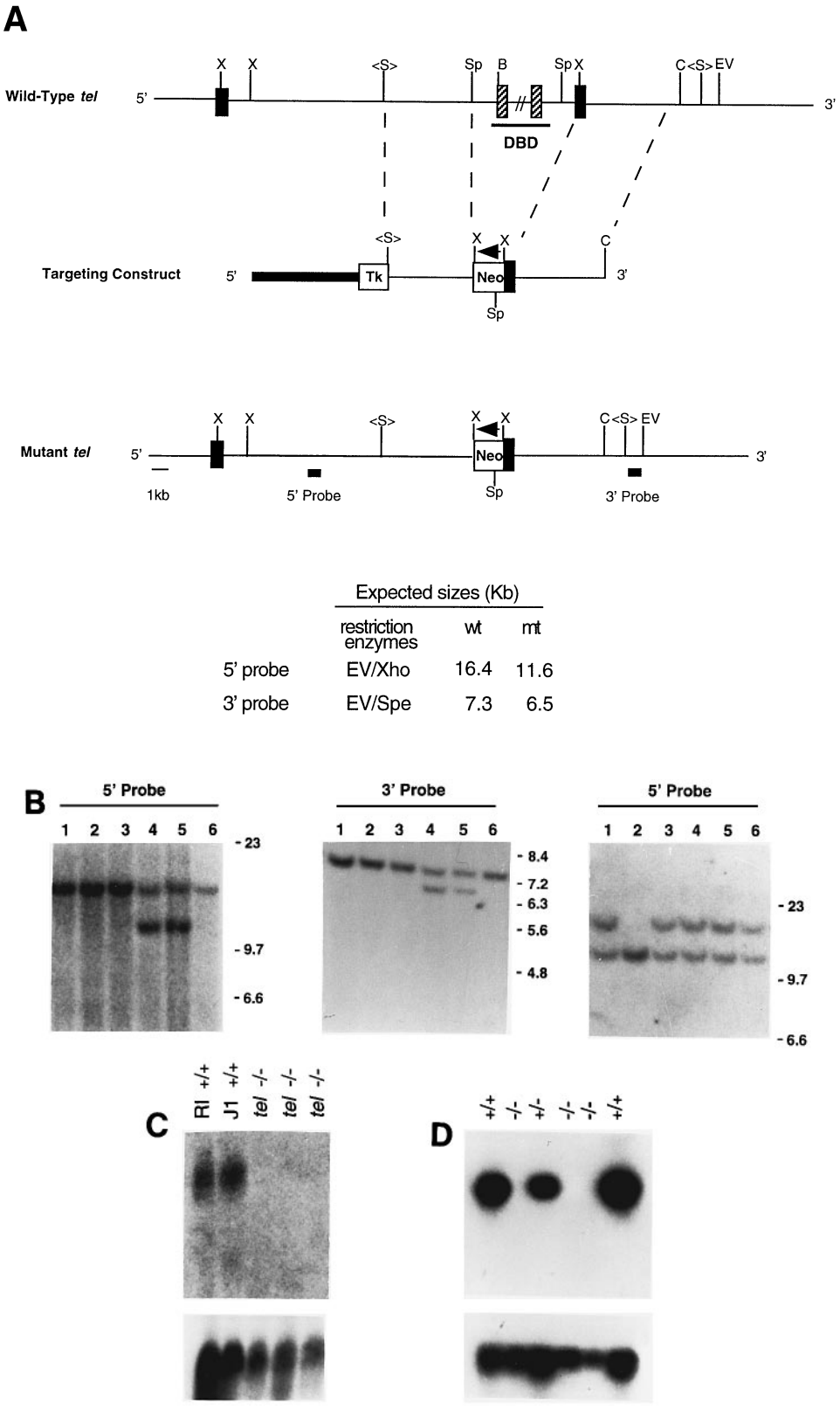

Fig. 3. Gene targeting of the murine TEL locus. (**A**) Targeting strategy. The homologous recombination event replaces the *TEL* DNA-binding domain (DBD) exon 5, 6 and portion of exon 7 with a PGK-neo cassette. X, *Xho*I; S, *Sal*I; Sp, *Spe*I; B, *Bam*HI; EV, *Eco*RV; C, *Cla*I. (**B**) Generation of targeted ES cells. G418- and gancyclovir-resistant ES cell clones were screened by Southern blot analysis with both 5′ (left panel) and 3′ (middle panel) external probes using EV/*Xho* and EV/*Spe* digests respectively. Expected fragments hybridize with both probes as depicted in (A). $TEL^{-/-}$ ES cell clones were obtained by targeting the second wild-type *TEL* allele with a PGK-hygromycin cassette. Hygromycin-resistant clones were screened as described above. Three $TEL^{-/-}$ ES cell clones were obtained and one representative clone is shown (right panel) (**C** and **D**) Absence of *TEL* expression in $TEL^{-/-}$ ES cell clones and $TEL^{-/-}$ embryos. Northern blot (C) and RT–PCR analyses (D) were performed on $TEL^{-/-}$ ES cell clones (C) and E 9.5 embryos (D) respectively. Upper panel in (C) shows *TEL* mRNA, lower panel, β-actin: upper panel in (D) shows amplified 304 bp *TEL* PCR product, lower panel, HPRT. Note the absence of *TEL* mRNA or PCR products in $TEL^{-/-}$ ES and embryos.

Table I. *TEL* homozygotes die before E 11.5 of gestation

Embryonic stage	No. of litters	No. of embryos	+/+	+/–	–/–
E 8.5	4	29	9	14	6
E 9.0	2	17	2	10	5
E 9.5	24	197	51	101	45
YS abnormal, embryo abnormal (type I)					30
YS normal, embryo normal (type II)					15
E 10.5					
YS abnormal, embryo abnormal	10	85	26	45	24
E 11.5	7	56	17	28	11
E 13.5	1	9	3	6	0
Neonates	25	168[a]	52	116	0

[a]Liveborn

vessels into a more complicated vascular network. Thus, absence of TEL leads to a defect in angiogenesis in the developing yolk sac.

Apoptotic cell death in TEL$^{-/-}$ embryos

The embryo proper of both type I and type II *TEL*$^{-/-}$ mutants appears grossly normal with respect to overall development and vascular structure. However, histological examination reveals cell death restricted to particular regions. This cell death can be attributed to apoptosis as revealed by labeling 3′-OH DNA ends (ApoTag; Gavrieli *et al*., 1992). Regions of apoptosis include the developing neural tube (Figure 5A–C), the mesenchymal tissues immediately adjacent to the primitive gut (Figure 5D–F) and along the entire body length (Figure 5G and data not shown). In addition, neural crest-derived cranial nerve ganglia also contain numerous apoptotic cells (Figure 5H–L). In general, those areas with evident apoptosis correlate with regions in which *TEL* is most highly expressed. Specifically, apoptosis is prominent in the cranial nerve ganglia V trigeminal and VII–VIII facial–acoustic regions (Figure 2E). Thus, in addition to the requirement of TEL in maintaining the integrity of yolk sac vascular network, TEL is also essential for the survival of mesenchymal cells and neural tissues.

Normal myeloerythroid hematopoiesis in TEL$^{-/-}$ embryos at the yolk sac stage

As the *TEL* gene was first identified through a chromosomal translocation in human leukemia (Golub *et al*., 1994), we have assessed the requirement for TEL in hematopoiesis in the embryo. Several other transcription factors identified in a similar fashion have been shown to be essential for normal hematopoiesis. For example, AML-1 (CBFαA2) protein is required for fetal liver, but not yolk sac, hematopoiesis (Okuda *et al*., 1996; Wang *et al*., 1996), whereas SCL/tal-1 is essential for development of all hematopoietic lineages (Porcher *et al*., 1996; Robb *et al*., 1996). Hematopoietic colony-forming assays were performed from E9.5 yolk sacs by plating cells onto methylcellulose-containing media supplemented with appropriate cytokines (KL/Epo for erythroid colonies and IL-1/IL-3/GM-CSF/G-CSF for macrophage colonies). In addition, mixed erythroid–myeloid colonies were obtained by growth in a combination of these growth factors. As shown in Figure 6A, the total number and content of the erythroid, macrophage and mixed colonies derived from precursors present in *TEL*$^{-/-}$ yolk sacs do not differ from control littermates. Moreover, similar numbers of primitive and definitive erythroid colonies are observed upon *in vitro* differentiation of wild-type and *TEL*$^{-/-}$ ES cells (Figure 6B). Thus, TEL is not strictly required for the differentiation and maturation of erythroid and macrophage cell lineages at the yolk sac stage.

Discussion

The *TEL* gene is frequently rearranged by chromosomal translocation in human leukemias of both myeloid and lymphoid origins. In chronic myelomonocytic leukemia (CMML), one *TEL* allele is disrupted and fused in-frame to the tyrosine kinase domain of the platelet-derived growth factor beta (PDGFβ) receptor to generate a dominantly-acting oncogene (Golub *et al*., 1994; Carroll *et al*., 1996; Jousset *et al*., 1997). Loss of the normal *TEL* allele in association with translocation of the *TEL* gene to the AML-1 (CBFαA2) locus in childhood acute lymphoblastic leukemia suggests that *TEL* loss of function may also contribute to pathogenesis of leukemia (Cave *et al*., 1995; Golub *et al*., 1995; Romana *et al*., 1995b; Stegmaier *et al*., 1995; Raynaud *et al*., 1996; Takeuchi *et al*., 1996). To initiate study of its *in vivo* role, we have used gene targeting in ES cells to inactivate the *TEL* gene in mice. Our results demonstrate that TEL is essential for normal development and is specifically required for maintaining blood vessel integrity within the developing yolk sac and for survival of different cell types in the developing embryo.

The formation of blood vessels involves two distinct cellular processes: (i) vasculogenesis, the *in situ* differentiation of angioblast and the subsequent assembly into primary vascular channels (Risau, 1995; Risau and Flamme, 1995); and (ii) angiogenesis, the proliferation of pre-existing endothelial cells to expand and remodel the vascular network (Pardanaud *et al*., 1989). The latter process is believed to include the formation of the vascular wall by recruitment of pericytes/smooth muscle cells from mesenchymal progenitor cells and neural crest cells (Nakamura, 1988; Kirby and Waldo, 1995). In the absence of TEL, vasculogenesis in the yolk sac and embryo appears to occur normally, suggesting that TEL is not required for the proliferation or differentiation of endothelial cells *per se*. In contrast, a defect in maintenance of the vascular network in the yolk sac is seen. In two-thirds of the *TEL*$^{-/-}$ yolk sacs, branching vitelline vessels are not observed at E9.5. However, one-third of the yolk sacs exhibit normal

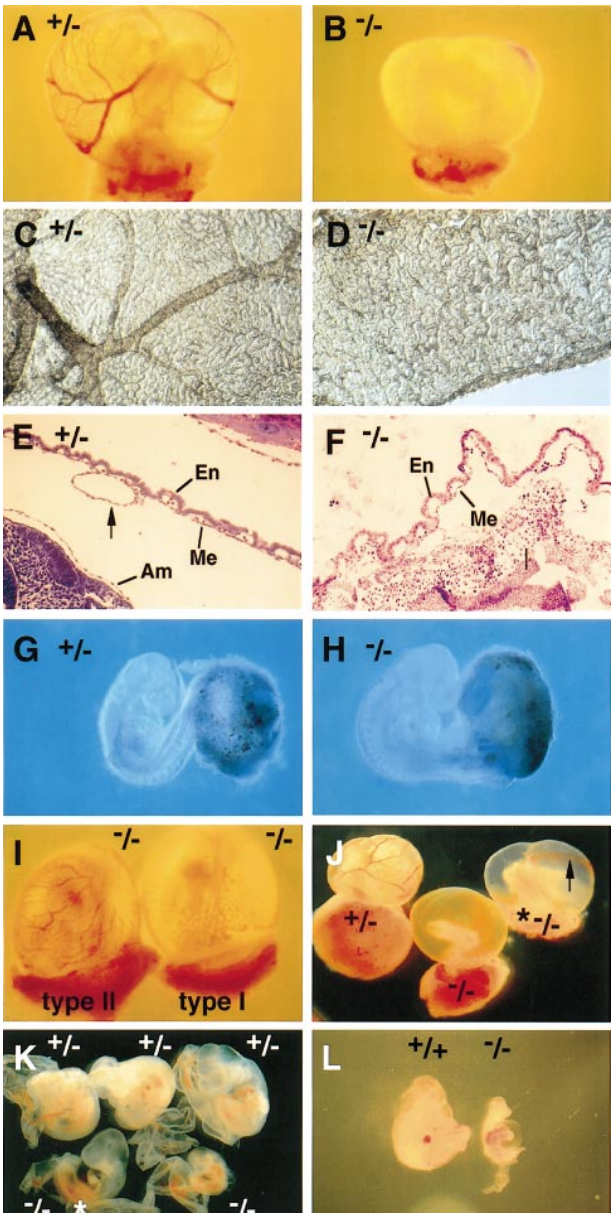

Fig. 4. Yolk sac angiogenic defects in *TEL*$^{-/-}$ embryos. (**A** and **B**) E9.5 *TEL*$^{+/-}$ and *TEL*$^{-/-}$ yolk sacs. Note the lack of branching vitelline vessels in *TEL*$^{-/-}$ yolk sac. (**C** and **D**) Whole mount anti-PECAM antibody staining of E9.5 *TEL*$^{+/-}$ and *TEL*$^{-/-}$ yolk sacs shows presence of honeycomb-like vasculature in both *TEL*$^{+/-}$ and *TEL*$^{-/-}$ yolk sacs, but lack of branching vitelline vessels in *TEL*$^{-/-}$ yolk sac. (**E** and **F**) Hematoxylin and eosin staining on paraffin-embedded *TEL*$^{+/-}$ and *TEL*$^{-/-}$ yolk sacs. Arrow in (E) identifies a large lumen indicative of large vitelline vessel in (A) and (C) that is observed in *TEL*$^{+/+}$ and *TEL*$^{+/-}$, but not *TEL*$^{-/-}$ yolk sacs. En, extra-embryonic endoderm; Me, extra-embryonic mesoderm; Am, Amnion. (**G** and **H**) E 9.5 *TEL*$^{+/-}$ and *TEL*$^{-/-}$ embryos. Note the stage appropriate development of the *TEL*$^{-/-}$ embryo. (**I**) Two types of *TEL*$^{-/-}$ phenotypes at E 9.5. Two-thirds of *TEL*$^{-/-}$ yolk sacs (type I) exhibit yolk sac phenotype as described in (B), (D) and (F), one-third of *TEL*$^{-/-}$ yolk sacs (type II) exhibit branching vitelline vessels indistinguishable from that of the control littermates as in (A). (**J**) Type I and Type II *TEL*$^{-/-}$ yolk sacs at E 10. Note the disintegration of the vitelline vessels of the type II mutant as indicated by the arrow pointing to a residual vessel. (**K**) Embryos from the same litter of E 10 shown in (J). Asterisk indicates a type II embryo; note that it is developmentally less retarded as compared with the other *TEL*$^{-/-}$ embryo (type I). (**L**) *TEL*$^{-/-}$ embryos at E 10.5. Both type I and type II mutant embryos are grossly retarded; approximately two-thirds of these mutants exhibit enlarged pericardial sac.

branching vessels at E9.5 which are not present at E10.5. These observations indicate that, while initiation of yolk sac angiogenesis occurs in the absence of TEL, the integrity of a more complex vascular network cannot be maintained. This phenotype is remarkably similar to that of embryos lacking tissue factor (TF) (Carmeliet *et al*., 1996b). In contrast to *TF*$^{-/-}$ yolk sacs, we observe an apparently normal, rather than reduced, number of mesenchymal cells expressing α-actin in the *TEL*$^{-/-}$ yolk sacs (data not shown). Such differences suggest diverse mechanisms by which yolk sac angiogenesis is regulated and the integrity of the vascular network is maintained.

It has been hypothesized that organs of ectodermal or mesenchymal origin, such as brain and kidney, are vascularized by angiogenic mechanisms (Bar, 1980; Sariola *et al*., 1983). Similar to *TF*$^{-/-}$ embryos, *TEL*$^{-/-}$ embryos display normal vasculature within the embryo proper E9.5, at which time the yolk sac vascular defect is first apparent. It is likely that a complex vascular network within the embryo has yet to develop at this stage and the early death of these mutant embryos precludes the appreciation of an intra-embryonic angiogenic defect, if present. A particularly striking and specific feature of the *TEL*$^{-/-}$ embryos is prominent mesenchymal cell apoptosis. Whether some of these apoptotic mesenchymal cells are progenitors of pericyte/smooth muscle cells is unknown. It is attractive to speculate that the failure to maintain a complex yolk sac vascular network is related to a function for TEL in preventing apoptosis in a critical cell population, either of endothelial or mesenchymal origin. In addition to mesenchymal cell apoptosis, *TEL*$^{-/-}$ embryos also exhibit apoptosis in regions of the neural tube and neural crest-derived ganglia which normally display the highest levels of *TEL* mRNA transcripts. We speculate that apoptosis in these cell populations may lead to neural defects at later stages of the mouse development.

Several genes encoding receptors, their ligands, or a transcription factor involved in hypoxia response have recently been shown to be essential for proper yolk sac vascular development. These include receptor tyrosine kinases, such as Flk-1 (Shalaby *et al*., 1995), Flt-1 (Fong *et al*., 1995), Tie-1 (Puri *et al*., 1995; Sato *et al*., 1995), and Tie-2 (Dumont *et al*., 1994; Sato *et al*., 1995); the vascular endothelial growth factor VEGF (Carmeliet *et al*., 1996a; Ferrara *et al*., 1996), the Tie-2 ligand, angiopoietin-1 (Suri *et al*., 1996), TF (Carmeliet *et al*., 1996b); and arylhydrocarbon receptor nuclear translocator, ARNT (Maltepe *et al*., 1997). Recent study of the role of angiopoietin-1 during embryonic angiogenesis (Suri *et al*., 1996) suggests that interactions between Tie-2-expressing endothelial cells and angiopoietin-1-producing mesenchymal cells underlies blood vessel remodeling (Folkman and D'Amore, 1996; Vikkula *et al*., 1996). We envision that loss of TEL function in an as yet unknown manner disrupts maintenance of these critical cellular interactions.

It is worth noting that Ets family of transcription factors, including Ets-1, Ets-2, *yan* and *pointed*, have been shown to be downstream targets of the Ras signaling pathway (O'Neill *et al*., 1994; Yang *et al*., 1996). Biochemical studies suggest that molecules involved in Ras signaling pathway, including rasGAP complex, GRB2 and SH-PTP may be substrates for Flk-1, Flt-1 and Tie-2 tyrosine kinase activity (Guo *et al*., 1995; Huang *et al*., 1995;

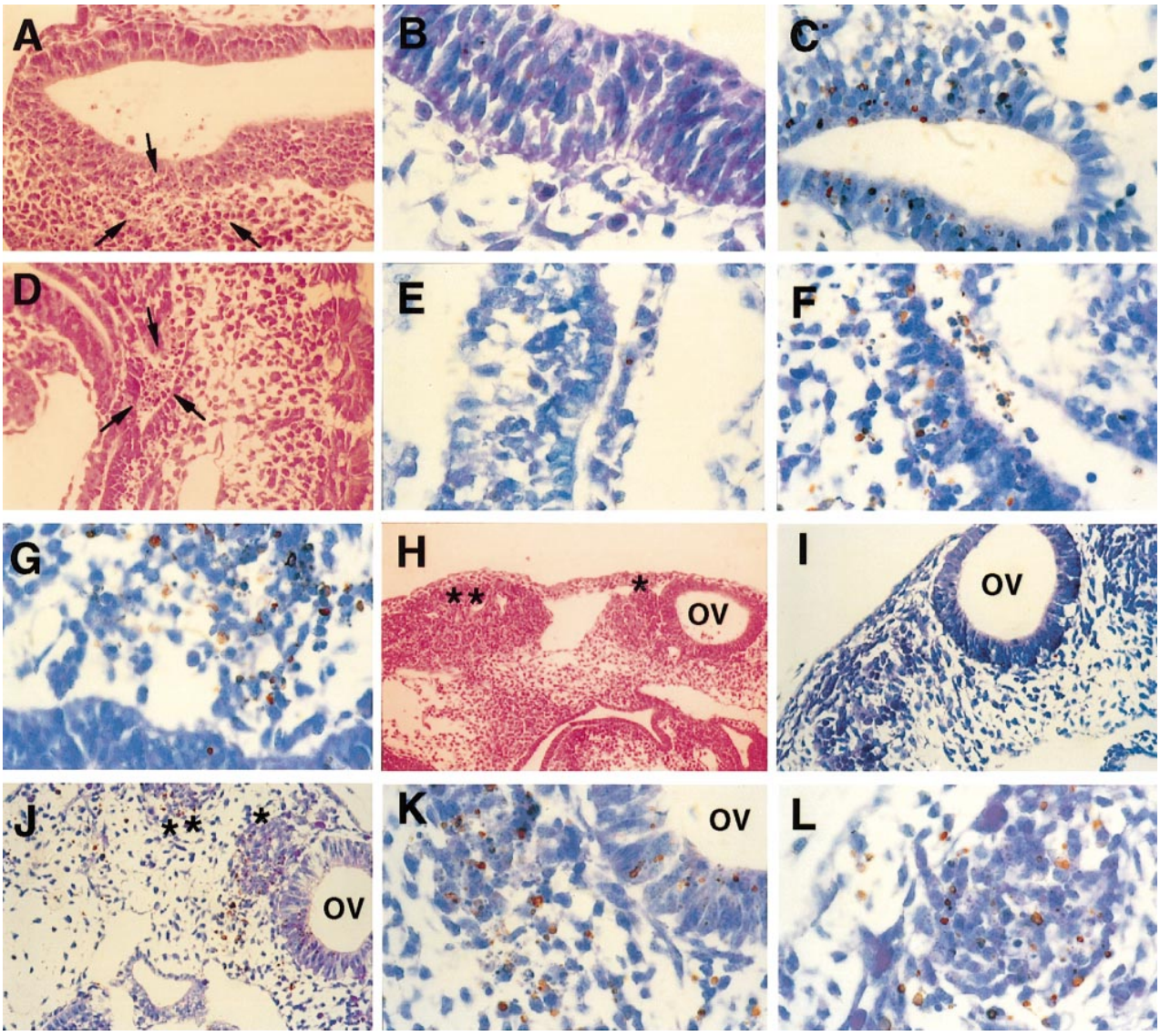

Fig. 5. Apoptosis in E10 *TEL*$^{-/-}$ embryos. (**A**, **D** and **H**) Hematoxylin and eosin staining of the *TEL*$^{-/-}$ embryos. (A) Transverse section of the neural tube; (D) sagittal sections of the primitive gut; and (H) cranial nerve ganglia regions. Arrows in (A) and (D) point to pyknotic nuclei indicative of cell death; magnification ×400. The single asterisk in (H) and (**J**) indicates V trigeminal region and double asterisk indicates VII–VIII facial–acoustic nerve regions; ×200. OV, otic vesicle. (**B** and **C**) TUNEL assay was performed on the *TEL*$^{+/+}$ (B) and **TEL**$^{-/-}$ (C) neural tube tissue sections; ×1000. (**E** and **F**) TUNEL assay on the *TEL*$^{+/+}$ (E) and *TEL*$^{-/-}$ (F) embryo sections of the primitive gut; ×1000. (**G**) Representative TUNEL assays show mesenchymal cell death observed along the entire body length of the *TEL* $^{-/-}$ embryos; magnification, ×1000. (**I** and **J**) TUNEL assay on the *TEL*$^{+/+}$ (I) and *TEL* $^{-/-}$ (J) tissue sections of the cranial nerve ganglia regions; ×400. (**K**) TUNEL assay on the V trigeminal region; ×1000. (**L**) TUNEL assay on the VII–VIII facial–acoustic nerve region; ×1000. Note the high degree of apoptosis, as indicated by brownish nuclei staining, only observed in *TEL*$^{-/-}$ embryos.

Seetharam *et al*., 1995). Whether Ras signaling events result from interaction between these endothelial cell-specific receptor tyrosine kinases and ligands lead to activation of an Ets-like transcription factors, such as TEL, remains to be determined. Interestingly, recent knockout studies on mutants lacking the Ras signaling molecules GTPase-activating protein (GAP) and GTP-binding protein α_{13} subunit demonstrate yolk sac angiogenic defects (Henkemeyer *et al*., 1995; Offermanns *et al*., 1997) and neuronal apoptosis (Henkemeyer *et al*., 1995) similar to those in *TEL*$^{-/-}$ embryos. In addition, expression of Tie-1, Tie-2, Flk-1, Flt-1 and GAP mRNAs is not appreciably affected in the *TEL*$^{-/-}$ embryos (data not shown), suggesting that *TEL* might lie downstream of a receptor tyrosine kinase signal transduction pathway in yolk sac angiogenesis.

Members of the Ets-family of transcription factors, especially Ets-1, have been indirectly implicated previously in blood vessel formation based on the pattern of expression (Pardanaud and Dieterlen-Lièvre, 1993) and the presence of putative ets-binding sites in relevant genes (Risau and Flamme, 1995). Although our results establish TEL as essential for normal yolk sac vascular development, Ets-1 appears to be dispensable at this stage (J.M.Leiden, personal communication), though it is required for the survival of T lymphoid cells (Bories *et al*., 1995; Muthusamy *et al*., 1995). Whether Ets-family members other than TEL are critical for blood vessel formation

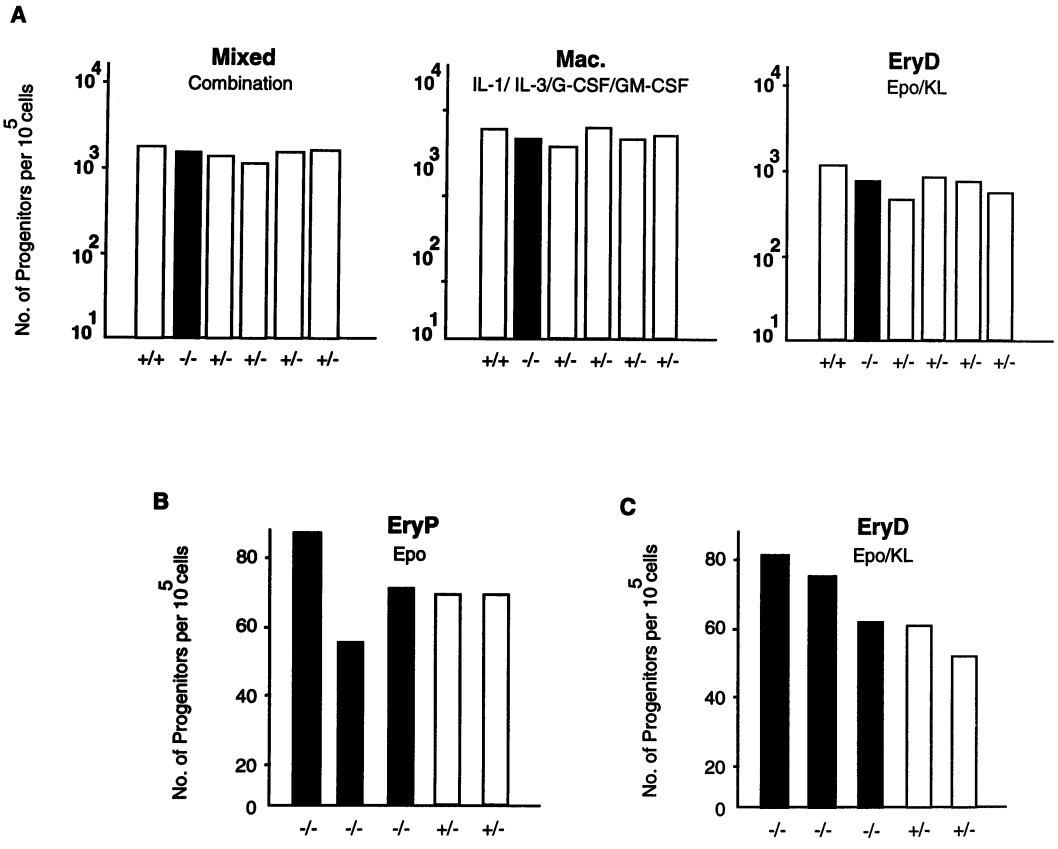

Fig. 6. Normal myeloerythroid hematopoiesis in *TEL* $^{-/-}$ mice at the yolk sac stage. (**A**) Yolk sac progenitor assays were performed as described in Materials and methods. The data shown were obtained from one litter at E9.5. Three independent litters were assayed at E9.5 with similar results. (**B**) *In vitro* differentiation of ES cells was performed on three independently targeted *TEL*$^{-/-}$ ES cell clones and two *TEL*$^{+/-}$ ES cell clones. Solid bars indicate the numbers of progenitors from *TEL*$^{-/-}$ yolk sacs or ES cell clones. Colonies were counted and harvested for cytospin and May–Grunwald–Giemsa staining after 4–7 days of *in vitro* culture with the indicated growth factors.

remains to be determined. Our data add to the increasing evidence that Ets-proteins serve to control apoptosis in diverse cell types.

Chromosomal translocations in human leukemia often activate expression of transcription factors or generate chimeric proteins containing a portion of a transcription factor. In several instances, notably SCL/tal-1(Porcher *et al*., 1996), Rbtn-2/LMO2 (Warren *et al*., 1994), and AML-1/CBFαA2 (Okuda *et al*., 1996; Wang *et al*., 1996), the relevant factors are also essential for normal hematopoietic development. Our analysis, however, demonstrates that TEL is not strictly required for the proliferation or differentiation of erythroid and myeloid lineages at the yolk sac stage. However, these findings do not preclude roles for TEL in later fetal liver or adult hematopoiesis, or in lymphopoiesis. Analysis of chimeric mice made with *TEL*$^{-/-}$ ES cells injected into wild-type or *RAG-2*$^{-/-}$ blastocysts may permit more direct study of blood cell development in the absence of TEL.

In summary, by gene targeting in ES cells, we have demonstrated that the widely expressed Ets-family member TEL is essential for the integrity of remodeled blood vessels in the developing yolk sac and is also required to prevent apoptosis in a variety of cell types within the embryo. Identification of target genes for TEL and the creation of developmentally regulated or tissue-restricted knockouts should allow for further characterization of its function in blood vessel formation, neural development and hematopoiesis.

Materials and methods

Construction of the targeting vector

Murine *TEL* genomic sequences were isolated from a 129/sv strain λFIXII library (Strategene) with a 3.5 kb *TEL* cDNA probe which encompassed the *TEL* DNA-binding domain. Inserts from positive phage were isolated as *Sal*I fragments, subcloned into pUC18 plasmid, and subjected to restriction enzyme mapping. A 4.8 kb *Xho*I–*Spe*I fragment spanning two exons of the *TEL* DNA-binding domain were replaced with a 1.8 kb PGK-neo cassette. The resulting *Sal*I fragment was then fused with *Xho*I-digested BlueScript-SK vector (Strategene). A 2 kb HSV-TK cassette was then inserted into *Sal*I site of this vector to generate the final targeting construct.

To obtain *TEL*$^{-/-}$ ES clones, a similar construct was assembled in which a PGK-hygromycin-resistant cassette replaced the PGK-neo cassette.

Gene targeting in ES cells and generation of mutant mice

The *TEL* targeting construct was linearized with *Not*I and electroporated into J1 ES cells as described previously (Shivdasani *et al*., 1995). 5′ flanking 0.7 kb *Pst*I fragment and 3′ flanking 0.7 kb *Xba*I–*Cla*I fragments were used to identify appropriately targeted ES cell clones by Southern blot analysis. *TEL*$^{+/-}$ ES cell clones were subjected to karyotyping to check for chromosomal integrity. Two ES clones with a normal karyotype were injected into C57BL/6 blastocysts to generate chimeras which contributed to the germline. Chimeras exhibited >90% contribution from ES cells on the basis of agouti coat color were used to mate with C57BL/6 mice. Genotyping of pups was performed by Southern blot analysis. Breeding of the *TEL*$^{+/-}$ mice and subsequent analysis of the homozygous mutant mice generated from two independently targeted ES cell clones were performed and yielded the same phenotype.

To confirm the null mutation in *TEL*$^{-/-}$ ES clones and embryos, total RNAs from the ES cells and embryos were prepared by extraction with RNAzol B (Tel-Test Inc.) and subjected to either Northern analysis or RT–PCR. A combination of two probes: 0.63 kb and 0.56 kb of *Xho*I fragments from the mouse *TEL-7* cDNA plasmid were used for Northern

analysis. For RT–PCRs, primers amplifying 304 bp of the *TEL* DNA-binding domain (*TEL*-1184:ACAAACATG-ACCTATGAGAAA; *TEL*-1487R:AGAAGTGTCCCTGCTATTCCC and 249 bp of constitutively expressed HPRT (Keller *et al*., 1993) were used under the following conditions: 94°C, 1 min; 56°C, 2 min; 72°C, 1 min for 30 cycles in a reaction mix containing 10% DMSO. The samples were then subjected to Southern analysis using a fragment spanning the DNA-binding domain as probe. Control experiments without reverse transcriptase in the cDNA synthesis reactions did not show specific PCR products (data not shown).

Whole mount and in situ hybridization

Whole mount immunohistochemistry using anti-PECAM antibody (Pharmigen) was performed as described previously (Schlaeger *et al*., 1995). *In situ* hybridization was performed according to the protocol of Wilkinson (1992). Frozen sections (12 μm) of staged embryos and yolk sac were prepared and hybridized at 65°C with digoxigenin-11-UTP (Boehringer-Mannheim) labeled probes. A 350 bp *Bam*HI–*Sma*I fragment representing the 3′ end of the *TEL* coding region and a 550 bp *Eco*RI–*Eco*47III fragment spanning the 5′ untranslated region were subcloned into BlueScript vectors. Both sense and antisense RNA were transcribed using T3 or T7 primers. Both antisense probes yielded similar results.

Histological analysis and TUNEL assay

Mouse embryos were fixed overnight in 10% buffered formalin and embedded in paraffin. Some sections (12 μm) were used for the TUNEL assay, while others were stained with hematoxylin and eosin for histological examination. The TUNEL assay was performed according to the manufacturer's protocol (Oncor) with some modification. Briefly, paraffin-embedded tissue sections were de-waxed and rehydrated through series of decreased concentration of ethanol. Following proteinase K reaction (20 μg/ml, 15 min) and quenching (2% H_2O_2, 10 min) treatments, sections were incubated with reaction mix as suggested by the manufacturer, except that 1 μl of TdT was used to reduce non-specific labeling. After washing, the sections were incubated with peroxidase conjugated anti-digoxigenin antibody for 30 min at RT and developed with DAB substrate (Vector). A DNase I-treated section was included in each experiment as a positive control. Sections were then counterstained with methyl green according the manufacturer's suggestion (Oncor). Controls without added TdT enzyme did not show specific staining (data not shown).

Yolk sac progenitor and in vitro ES cell differentiation assay

E9.5 embryos were dissected under sterile conditions and were used for genotyping. Yolk sacs from embryos were incubated in Ca^{2+}/Mg^{2+}-free PBS with 20% fetal calf serum (FCS) and 0.1% collagenase (Sigma) at 37°C for 1 h. Cells were then disaggregated by passage through a syringe and 22-gauge needle (Wong *et al*., 1986). The yield from each yolk sac was $1–5\times10^4$ cells. Subsequently, 5×10^3 cells of each yolk sac were plated in α-minimal essential medium supplemented with 0.9% α-methylcellulose, 30% FCS, 1% BSA and growth factors. For erythroid colonies: 2 units/ml erythropoietin (Epo), 50 ng/ml recombinant c-kit ligand (KL) were added; for macrophage colonies: IL-1 (10^3 U/ml), IL-3 (10 ng/ml), granulocyte colony-stimulating factor (G-CSF, 1 ng/ml), granulocyte-macrophage CSF (GM-CSF, 5 ng/ml) were added; for mixed erythroid–myeloid colonies: IL-1, IL-3, IL-11 (5 ng/ml), KL, Epo, GM-CSF, G-CSF were added (combination). *In vitro* ES cell differentiation was performed as described (Keller *et al*., 1993; Porcher *et al*., 1996). Briefly, ES cell clones ($TEL^{+/-}$, $TEL^{-/-}$ and wild-type) were permitted to form embryo bodies. At day 9 of culture, embryo bodies were disaggregated and 2×10^4/ml of cells were replated onto methycellulose medium supplemented with Epo (2 U/ml) for differentiation of primitive erythroid colonies and KL (50 ng/ml) + Epo for differentiation of definitive erythroid colonies.

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