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A Requirement for Flk1 in Primitive and Definitive Hematopoiesis and Vasculogenesis

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Summary

Mouse embryos lacking the receptor tyrosine kinase, Flk1, die without mature endothelial and hematopoietic cells. To investigate the role of Flk1 during vasculogenesis and hematopoiesis, we examined the developmental potential of *Flk1*$^{-/-}$ embryonic stem cells in chimeras. We show that Flk1 is required cell autonomously for endothelial development. Furthermore, *Flk1*$^{-/-}$ cells do not contribute to primitive hematopoiesis in chimeric yolk sacs or definitive hematopoiesis in adult chimeras and chimeric fetal livers. We also demonstrate that cells lacking *Flk1* are unable to reach the correct location to form blood islands, suggesting that Flk1 is involved in the movement of cells from the posterior primitive streak to the yolk sac and, possibly, to the intraembryonic sites of early hematopoiesis.

Introduction

During embryogenesis, development of the hematopoietic system occurs at various anatomical sites, including the extraembryonic yolk sac, fetal liver, spleen, and finally, adult bone marrow. Earlier evidence suggested that both fetal and adult pluripotent hematopoietic stem cells (HSCs) originate in the yolk sac and subsequently colonize the fetal liver and then the bone marrow. More recently, studies in avian, amphibian, and now mouse embryos have supported the view that there are two discrete anatomic origins of hematopoietic activity, one extraembryonic and one intraembryonic (Dzierzak and Medvinsky, 1995). During the primitive streak stage, groups of mesodermal cells aggregate in the developing yolk sac to form the extraembryonic blood islands. Cells at the periphery of these aggregates differentiate into endothelial cell precursors (angioblasts), while cells in the interior become primitive blood cells (Risau, 1991). The close spatial and temporal association between the development of these two cell lineages has led to the hypothesis that they have a common precursor, the ''hemangioblast'' (Sabin, 1920; Murray, 1932; Wagner, 1980). Although the yolk sac contains hematopoietic activity as early as 7–8.5 days postcoitum (dpc), these progenitors have limited potential (Moore and Metcalf, 1970; Wong et al., 1986; Liu and Auerbach, 1991; Cumano et al., 1993; Huang and Auerbach, 1993). Pluripotent HSCs with long-term repopulating potential (LTR-HSC) and cells capable of forming macroscopic spleen colonies in irradiated recipients (CFU-S) appear later in the yolk sac, between 9 and 11 dpc (Moore and Metcalf, 1970; Perah and Feldman, 1977; Symann et al., 1978; Sonoda et al., 1983; Medvinsky et al., 1993; Muller et al., 1994). However, recent evidence suggests that these cells populate the yolk sac secondarily from an intraembryonic source. Lymphoid and multipotent myeloid precursors can develop from the intraembryonic posterior splanchnopleure as early as 7.5 dpc in the absence of any yolk sac circulation (Cumano et al., 1996). By 10.5 dpc, LTR-HSCs originate in the embryo proper in the aorta, genital ridge, and mesonephros region (AGM), which is lineally related to the earlier posterior splanchnopleure region (Medvinsky and Dzierzak, 1996). HSCs from this region are then presumed to colonize both the yolk sac and fetal liver, where they give rise to definitive hematopoietic precursors after 12.5 dpc (Dzierzak and Medvinsky, 1995).

An association between vasculogenesis and hematopoiesis is clearly seen in the development of the yolk sac blood islands, but is also present during intraembryonic hematopoiesis. Explants of embryonic tissue that give rise to hematopoietic progenitors always include the developing dorsal aorta (Godin et al., 1993, 1995; Medvinsky et al., 1993; Cumano et al., 1996; Medvinsky and Dzierzak, 1996), and clusters of hematopoietic cells have been reported intravascularly, contiguous to the inner surface of posterior arterial endothelia in the chick (Dieterlen-Lievre and Martin, 1981) and the mouse (Garcia-Porrero et al., 1995). This intimate proximity between endothelial and hematopoietic precursors in the embryo proper is again consistent with the possibility that these two cell lineages are derived from a common precursor.

The relationship between vasculogenesis and hematopoiesis has also been established from the analysis of the expression and function of receptor tyrosine kinases that are largely expressed in endothelial cells. The Tek/Tie-2 (Dumont et al., 1992; Sato et al., 1993) and Tie/Tie-1 (Partanen et al., 1992; Sato et al., 1993) receptors have similar extracellular domains and define one class of endothelial cell-specific tyrosine kinases. These receptors and their ligands appear to have important roles in endothelial cell survival, integrity, and function (Dumont et al., 1994; Puri et al., 1995; Sato et al., 1995; Davis et al., 1996; Partanen et al., 1996; Suri et al., 1996)

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but do not affect the initial generation of endothelial precursors. Chimeric analysis has also shown that the development and function of the hematopoietic system proceeds normally in the absence of the Tie/Tie-1 receptor, although expression of *tie* and *tek* has been reported in hematopoietic cells (Iwama et al., 1993; Batard et al., 1996; Hashiyama et al., 1996; Partanen et al., 1996).

The expression of *tek/tie-2* and *tie/tie-1* is preceded by that of *Flk1* (Matthews et al., 1991; Yamaguchi et al., 1993) and *Flt1* (Shibuya et al., 1990). These two receptors, along with Flt4, define a second class of endothelial cell-specific tyrosine kinases based on their structural similarities. Both the Flk1 and Flt1 receptors bind vascular endothelial growth factor (VEGF) with high affinity (de Vries et al., 1992; Millauer et al., 1993; Quinn et al., 1993). The early expression of *Flk1* and *Flt1* in the developing blood islands of primitive streak stage embryos (Yamaguchi et al., 1993; Fong et al., 1995; Shalaby et al., 1995) is consistent with a dual role for this signaling pathway in the onset of vasculogenesis and hematopoiesis in the yolk sac. In addition, *Flk1*, but not *Flt1*, is also expressed in the posterior splanchnopleure (Yamaguchi et al., 1993; Fong et al., 1995; Shalaby et al., 1995) and in fetal liver hematopoietic precursors (Matthews et al., 1991), suggesting that it could play a role in establishing definitive hematopoiesis.

Embryos homozygous for the *Flk1*-null mutation die at midsomite stages, lacking both mature endothelial and hematopoietic cells, as a result of a failure to form blood islands during the primitive streak stage (Shalaby et al., 1995). These results clearly demonstrate a role for Flk1 in yolk sac hematopoiesis, but these data do not distinguish whether the hematopoietic defect in these embryos is primary or is secondary to the lack of a suitable endothelial-lined microenvironment. In addition, because the mutation is lethal early in development, any requirement for Flk1 in later adult hematopoiesis could not be assessed.

In this study, we examined the developmental potential of embryonic stem (ES) cells homozygous for the *Flk1* mutation in chimeras generated by aggregation with wild-type embryos. The endothelia of chimeric embryos and mice were always derived from wild-type cells, demonstrating a cell-autonomous requirement for Flk1 in endothelial cell differentiation. In these chimeras, *Flk1* mutant cells were also absent in the hematopoietic lineages derived from the hematopoietic progenitors of both the yolk sac and the fetal liver, indicating an intrinsic requirement for Flk1 during embryonic and definitive hematopoiesis. For at least the development of yolk sac hematopoiesis, this requirement does not appear to be absolute, since erythroid colonies were observed in embryoid bodies developed from homozygous mutant *Flk1* ES cells in vitro. Finally, we present evidence that the role of Flk1 is to ensure the correct location in the yolk sac of the mesodermal precursors of the blood islands, and we suggest that Flk1 may play a similar role during the development of the intraembryonic blood-forming region.

Results

Flk1-Deficient Cells Do Not Contribute to the Endothelial Lineages in Chimeras

Homozygous *Flk1*$^{-/-}$ ES cells were generated by selecting *Flk1*$^{+/-}$ ES cells (Shalaby et al., 1995) in high G418 concentrations (Mortensen et al., 1992). Two homozygous mutant clones (5547 and 5548) and two heterozygous clones (5539 and 5540) were isolated after the selection and were used to generate chimeras. The clones were genotyped by PCR amplification of genomic DNA with primers specific for the wild type and the mutant alleles (Shalaby et al., 1995).

The targeted *Flk1*-null allele contains a bacterial *lacZ* gene inserted under the transcriptional control of the endogeneous *Flk1* promoter (Shalaby et al., 1995), facilitating analysis of the contribution of *Flk1* mutant cells to endothelial lineages in chimeras. Thus, β-galactosidase staining marks endothelial cells derived from the *Flk1*$^{+/-}$ or *Flk1*$^{-/-}$ ES cells in a chimera. Ten *Flk1*$^{+/-}$ <–> CD1 chimeric embryos and 25 *Flk1*$^{-/-}$ <–> CD1 chimeric embryos were stained for β-galactosidase activity. The contribution of ES cells was estimated for each chimeric embryo by GPI analysis on a portion of the embryo and varied from 10% to 80% (data not shown). β-galactosidase staining of chimeric embryos at 9.5 dpc demonstrated that *Flk1*$^{+/-}$ cells readily contributed to embryonic endothelia, such as the endothelium of the dorsal aorta, the endocardium, and the developing vasculature of the yolk sac (Figures 1A–1F). Expression of *Flk1* was also observed in nonendothelial cells in the base of the allantois and the posterior splanchnopleure region of the embryo (Figure 1D). In contrast, no *Flk1*$^{-/-}$ cells were observed in the embryonic or extraembryonic endothelia of *Flk1*$^{-/-}$ <–> CD1 embryos (Figures 1G–1L). Thus, the vasculature in *Flk1*$^{-/-}$ <–> CD1 chimeric embryos was entirely derived from wild-type cells. In these embryos, β-galactosidase-expressing cells were observed near locations that normally give rise to embryonic blood vessels, including the head mesenchyme, but failed to form endothelium (Figure 1H). In addition, some *lacZ*-positive mutant cells were seen in ectopic sites, such as the neural tube (Figure 1I).

The chimeric analysis demonstrates that there is an absolute cell-autonomous requirement for Flk1 in the development of the embryonic vasculature, consistent with the phenotype of the *Flk1* mutant embryos. The inability of *Flk1*$^{-/-}$ cells to participate in vasculogenesis in the embryo implies that the endothelial lineages in adult chimeras will also be entirely wild type in origin. We could not assess this directly for all endothelia because *Flk1* expression, and hence the expression of the *lacZ* reporter, is largely downregulated in adult endothelial cells. However, considerable expression persists in the glomerular endothelia of the kidney and the growing follicles of the ovary. No β-galactosidase staining was detected in these regions in *Flk1*$^{-/-}$ <–> CD1 chimeras (data not shown). Thus, these results are consistent with a complete inability to generate mature endothelial cells in the absence of Flk1 signaling.

Flk1 Mutant Cells Are Deficient in Yolk Sac Hematopoiesis

Because the chimeric embryos had a wild-type yolk sac vasculature, it was possible to ask whether Flk1-deficient cells have the potential to develop into yolk sac hematopoietic cells in the presence of a normal endothelial-lined microenvironment. Hematopoietic colonies obtained from the yolk sacs of chimeras at 9.5

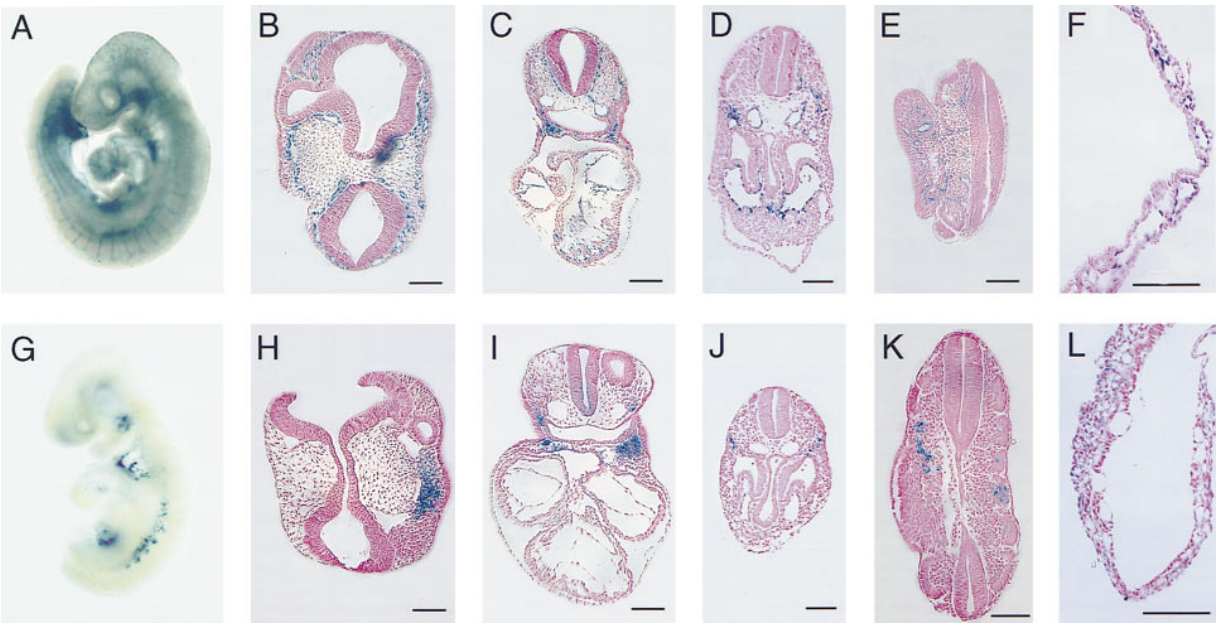

Figure 1. Analysis of the Contribution of Flk1-Deficient Cells to the Endothelium of 9.5 dpc Chimeras

β-galactosidase whole-mount staining of *Flk1*$^{+/-}$ <–> CD1 (A) and *Flk1*$^{-/-}$ <–> CD1 (G) 9.5 dpc chimeras. Sections of β-galactosidase-stained embryos showed *lacZ* expression in the vasculature of chimeric embryos derived from *Flk1*$^{+/-}$ ES cells, including blood vessels of the head (B), the endocardium (C), dorsal aorta (D), intersomitic vessels (E), and yolk sac blood vessels (F). In contrast, chimeric embryos generated from *Flk1*$^{-/-}$ ES cells lacked *lacZ* expression in their endothelia (H–L), although *lacZ* expression was detected in nonendothelial cells. Scale bar = 100 μm.

dpc were examined by GPI analysis (Figure 2). While the *Flk1*$^{+/-}$ cells contributed efficiently to yolk sac hematopoietic colonies, including macrophage, granulocyte-macrophage, and mixed granulocyte-macrophage-erythroid colonies, *Flk1*$^{-/-}$ cells from chimeras failed to do so (Figure 2). Four of eleven *Flk1*$^{-/-}$ chimeric yolk sacs produced colonies that were entirely derived from wild-type cells (GPI-BB). In the other seven yolk sacs, a faint GPI-AA band was detected, which may represent contamination from nonhematopoietic cells or a very small proportion of *Flk1*$^{-/-}$ hematopoietic colonies (Figure 2B). Thus, we could detect few, if any, *Flk1*$^{-/-}$ hematopoietic progenitors in chimeric yolk sacs, suggesting that there is an intrinsic defect in the ability of *Flk1*$^{-/-}$ cells to colonize extraembryonic hematopoietic lineages.

Flk1-Deficient Cells Accumulate in the Amnion of 7.5 dpc Chimeras

This analysis could not distinguish between whether *Flk1* mutant cells were initially present in the blood islands but failed to differentiate into hematopoietic cells, or whether they were never in the correct location to contribute to yolk sac hematopoiesis. Because *Flk1* is expressed in the common progenitor of the angioblasts and hematopoietic cells of the blood islands, we could examine this question further by following expression of β-galactosidase in chimeras. In situ analysis of both *Flk1*$^{+/-}$ and *Flk1*$^{-/-}$ cells in chimeras at 7.5 dpc showed that *Flk1*$^{+/-}$ cells readily contributed to the extraembryonic mesoderm fated to become blood islands (Figure 3A). In contrast, β-galactosidase staining did not extend over the inner surface of the yolk sac in chimeras made with *Flk1*$^{-/-}$ ES cells. Instead, blue cells accumulated on the surface of the amnion, where *Flk1* is not normally expressed, suggesting that mutant cells were diverted from their normal yolk sac fate (Figure 3B).

Endothelial and Hematopoietic Development in Flk1-Deficient Embryoid Bodies

Heterozygous and homozygous mutant *Flk1* ES cells were analyzed for their ability to generate endothelial and hematopoietic cells in vitro. Undifferentiated ES colonies were treated with dispase and grown in suspension in the absence of leukemic inhibitory factor (LIF). Under these conditions, ES colonies spontaneously differentiate into cystic embryoid bodies, which resemble the yolk sac in overall morphology and pattern of gene expression (Doetschman et al., 1985; Schmitt et al., 1991; Wiles and Keller, 1991). Cystic embryoid bodies from *Flk1*$^{+/-}$ and *Flk1*$^{-/-}$ cells were grown and stained for β-galactosidase activity. Although *Flk1*$^{+/-}$ cystic embryoid bodies formed an extensive vascular endothelial network (Figure 4A), *Flk1*$^{-/-}$ cystic embryoid bodies did not (Figure 4B).

To determine if the *Flk1*$^{-/-}$ embryoid bodies contained hematopoietic cells, precystic embryoid bodies were transferred from suspension cultures onto tissue culture plates (Bautch et al., 1996). The attached embryoid bodies were stained for β-galactosidase activity. The *Flk1*$^{+/-}$-attached embryoid bodies developed hemoglobinized blood islands adjacent to vascular endothelium (Figure 4C). The *Flk1*$^{-/-}$-attached embryoid bodies also generated hemoglobinized blood islands next to the β-galactosidase-expressing mesodermal endothelial cell precursors (Figure 4D). These observations demonstrate that Flk1-deficient cells do not have a complete

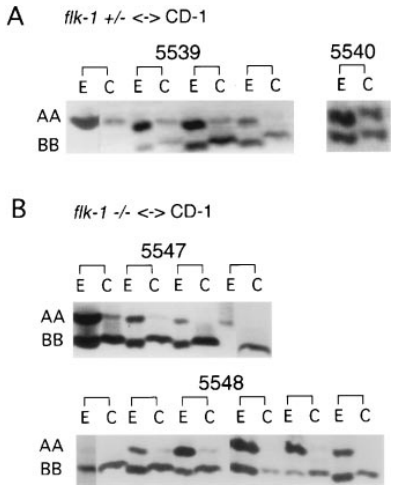

Figure 2. Analysis of the Contribution of Flk1-Deficient Cells in 9.5 dpc Chimeric Yolk Sacs

Hematopoietic colony assays were performed on the yolk sacs of 9.5 dpc chimeras. Colonies (C) from each yolk sac were pooled, lysed, and analyzed for the presence of the GPI-AA and -BB isoforms. The embryos (E) corresponding to each yolk sac were identified as chimeras by GPI as well. *Flk1*$^{+/-}$ ES cells were able to contribute to hematopoietic progenitors (A), but *Flk1*$^{-/-}$ ES cells (B) only did so rarely.

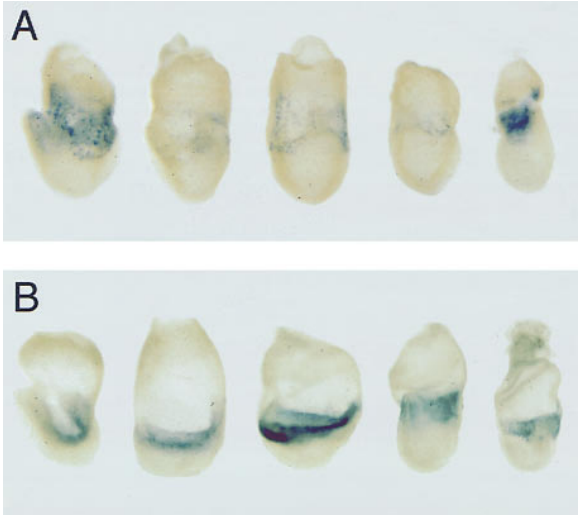

Figure 3. Distribution of Flk1-Deficient Cells in 7.5 dpc Chimeric Embryos

β-galactosidase staining of 7.5 dpc *Flk1*$^{+/-}$ <–> CD1 (A) and *Flk1*$^{-/-}$ <–> CD1 (B) chimeras showed a lack of *Flk1*$^{-/-}$ cells in the extraembryonic mesoderm believed to give rise to the blood islands. The *Flk1*$^{+/-}$ cells readily contributed to the presumptive precursors of the blood islands, but the *Flk1*$^{-/-}$ cells appeared to be diverted to other mesodermal populations such as the amnion of these embryos.

block in the generation of hematopoietic cells, but rather can generate at least erythroid cells under these in vitro culture conditions.

Definitive Hematopoiesis Is Deficient in Flk1 Mutant Chimeras

The development of viable *Flk1*$^{-/-}$ <–> CD1 chimeras allowed us to determine whether Flk1 was also required for the development of adult hematopoiesis. GPI analysis showed that both *Flk1*$^{+/-}$ and *Flk1*$^{-/-}$ ES cells could contribute to many different adult organs (Figures 5A and 5B). Samples of peripheral blood, bone marrow, spleen, and thymus tissues of adult chimeras were analyzed for their GPI isoenzymes. These tissues were essentially derived from the wild-type embryo cells (GPI-BB) in chimeras generated with the *Flk1*$^{-/-}$ ES cells (Figures 5D and 6). The GPI-AA band observed in some spleen and thymus preparations was not present in splenocytes and thymocytes derived from these tissues and therefore likely represents nonhematopoietic stromal cells derived from the *Flk1*$^{-/-}$ ES cells in these organs. In chimeras generated with the *Flk1*$^{+/-}$ ES cells, the heterozygous cells contributed efficiently to all hematopoietic tissues (Figures 5C and 6).

There are two possible models to explain the absence of *Flk1*$^{-/-}$ cells in the hematopoietic compartment of adult chimeras. First, *Flk1* mutant cells may be unable to contribute to the generation of definitive hematopoietic progenitors. Alternatively, HSCs may differentiate from *Flk1*$^{-/-}$ cells at earlier developmental stages, but these clones may then be subsequently selected against in the presence of wild-type cells in chimeric animals. There is considerable evidence from chimeric mouse studies that selective effects can cause marked skewing in the contribution of cells of different genotypes to the hematopoietic system when assayed in mature animals (Berger et al., 1995). To address this question, we examined the contribution of *Flk1*$^{-/-}$ cells to definitive hematopoiesis during fetal development in 14.5 dpc chimeras. By this stage, progenitors with full adult hematopoietic potential are normally present in the fetal liver (Medvinsky et al., 1993; Muller et al., 1994). Individual tissues from 14.5 dpc chimeras made with *Flk1*$^{-/-}$ or *Flk1*$^{+/-}$ ES cells were analyzed by GPI isozyme analysis to confirm the chimerism of the embryos (Figures 5E and 5F). The hematopoietic colonies obtained from five 14.5 dpc fetal livers were entirely wild type (GPI-BB) in chimeras generated with *Flk1*$^{-/-}$ ES cells. In contrast, *Flk1*$^{+/-}$ cells contributed efficiently to myeloid (macrophage and granulocyte-macrophage) and mixed myeloid-erythroid colonies from four 14.5 dpc chimeric fetal livers (Figures 5E and 5F). Taken together, the chimeric analysis demonstrates that Flk1 is necessary for both primitive and definitive hematopoiesis.

Flk1 Is Expressed in Regions of Intraembryonic Hematopoiesis

The absence of *Flk1*$^{-/-}$ cells contributing to definitive hematopoiesis led us to reexamine whether *Flk1* expression marks the putative early sites of intraembryonic hematopoiesis. Examination of β-galactosidase expression in *Flk1*$^{+/-}$ embryos between 8.5 and 10.5 days of gestation revealed broad expression of *Flk1* in the posterior splanchnopleure, as previously reported (Figure 7) (Yamaguchi et al., 1993; Shalaby et al., 1995). Within this region, there were small clumps of isolated *lacZ*-positive cells, which might represent specific hematopoietic precursors (Figure 7A). In slightly older embryos,

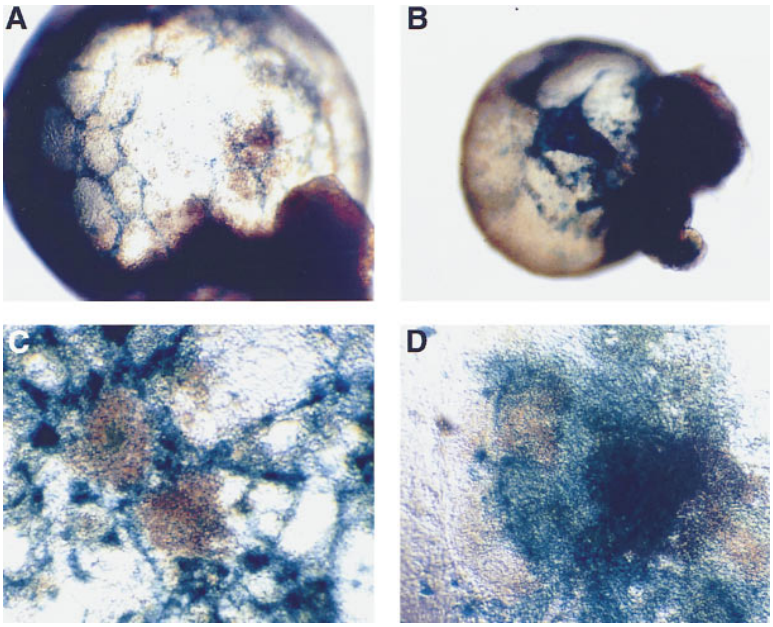

Figure 4. Hematopoietic Development in the Absence of Mesodermal Migration in Flk1-Deficient Embryoid Bodies

In vitro differentiation and β-galactosidase staining of *Flk1*$^{+/-}$ ES cells (A and C) and *Flk1*$^{-/-}$ ES cells (B and D). The *Flk1*$^{+/-}$ cells form an extensive vascular endothelial network in cystic embryoid bodies (A), whereas *Flk1*$^{-/-}$ cells form endothelial cell precursors but do not form mature endothelial cells in cystic embryoid bodies (B). Day 8 attached embryoid bodies demonstrate that both *Flk1*$^{+/-}$ (C) and *Flk1*$^{-/-}$ (D) ES cells are capable of primitive hematopoiesis as defined by the formation of hemoglobinized blood islands.

where hematopoietic potential has been associated with the AGM region, small clumps of strongly *lacZ*-positive cells were observed intravascularly in the omphalomesenteric artery, apparently in close association with the endothelial lining (Figures 7B–7D). Histological studies have suggested that these cells may be the intraembryonic hematopoietic progenitors (Garcia-Porrero et al., 1995).

Discussion

Both endothelial and hematopoietic cells fail to develop in mouse embryos lacking the Flk1 receptor tyrosine kinase, and embryos die by 9.5 days of gestation, precluding a detailed analysis of the functions of this signaling pathway during development (Shalaby et al., 1995). By generating chimeras between *Flk1* mutant ES cells and wild-type embryos, we have been able to rescue the mutant phenotype and provide insights into the roles of Flk1 signaling in both endothelial development and hematopoiesis. Flk1-deficient ES cells were incapable of developing into endothelial cells at any stage of development analyzed, as judged by the expression of the β-galactosidase reporter that was introduced during the disruption of the *Flk1* gene. *Flk1*$^{-/-}$ cells were absent in the dorsal aorta, cardinal veins, intersomitic arteries, and yolk sac blood vessels of chimeric embryos as early as 9.5 dpc, consistent with the previously observed lack of mature endothelium in *Flk1* mutant embryos (Shalaby et al., 1995). They could, however, effectively contribute to various other tissues and cell types examined, producing viable adult chimeric mice. Based on these results, we conclude that Flk1 is required cell autonomously for endothelial development, both within the embryo and in extraembryonic locations. Interestingly, β-galactosidase expression indicative of activation of *Flk1* expression was still observed in chimeras containing *Flk1*-null mutant cells. However, such expression was confined to areas where *Flk1* is expressed in nonendothelial cells, such as the base of the allantois and cells in the branchial arches. Some blue cells were also seen in ectopic sites in mutant but not heterozygous chimeras, suggesting that endothelial precursors lacking the ability to signal through Flk1 may not die but rather may be redirected into other fates.

The generation of chimeras with wild type endothelia allowed us to ask whether Flk1 was directly required for the early development of the hematopoietic system. As judged by GPI isozyme contribution, *Flk1*$^{-/-}$ cells were strongly underrepresented in the in vitro colonies representing the primitive hematopoietic progenitors derived

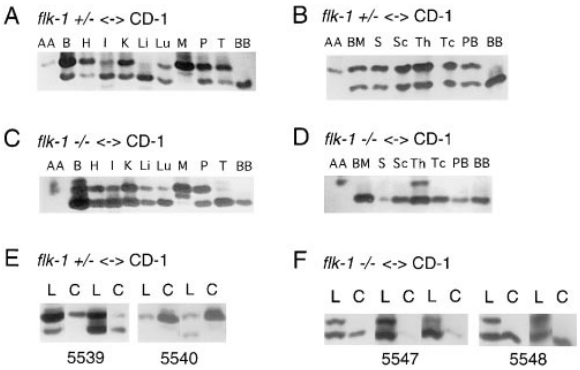

Figure 5. GPI Analysis of the Contribution of *Flk1*$^{+/-}$ and *Flk1*$^{-/-}$ Cells to Hematopoietic Lineages in Chimeric Mice

GPI isoenzyme analysis of various tissues taken from a *Flk1*$^{+/-}$ <–> CD1 chimera (A) and from a *Flk1*$^{-/-}$ <–> CD1 chimera (C) demonstrates that the *Flk1* ES cells are pluripotent. GPI analysis of various hematopoietic tissues from a chimera generated with *Flk1*$^{+/-}$ ES cells (B), and one generated with *Flk1*$^{-/-}$ ES cells (D), shows that *Flk1*$^{-/-}$ cells are incapable of contributing to hematopoietic tissues. Colonies from the fetal livers of four *Flk1*$^{+/-}$ <–> CD1 chimeras (E) and five *Flk1*$^{-/-}$ <–> CD1 chimeras were pooled and analyzed for the presence of GPI isoenzymes (F). L, limb; C, colonies.

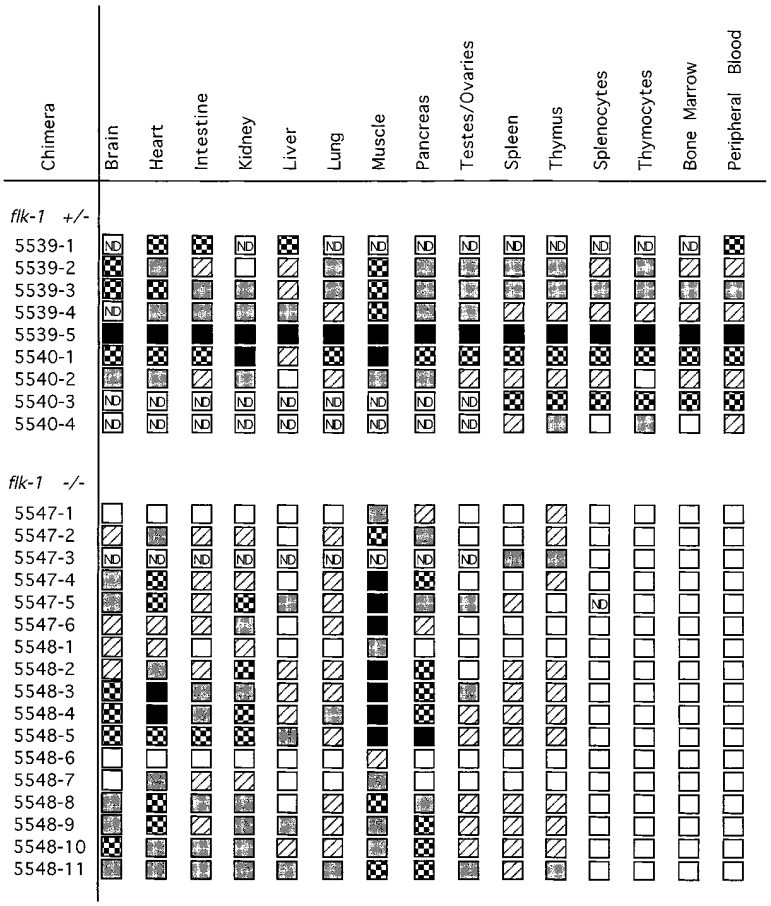

Figure 6. Distribution of the *Flk1*$^{+/-}$ and *Flk1*$^{-/-}$ Cells to Various Tissues from Chimeric Mice

Tissues of chimeras obtained by aggregation of wild-type CD1 embryos and the *Flk1*$^{+/-}$ and *Flk1*$^{-/-}$ ES cells were electrophoresed on cellulose acetate membranes. The contribution of ES cells to each tissue was estimated visually and categorized as follows: 0%, open boxes; 1%–25%, hatched boxes; 26%–50%, shaded boxes; 51%–75%, checkered boxes; or 76%–100%, closed boxes. ND, no data. Flk1-deficient cells failed to contribute to hematopoietic lineages.

from chimeric yolk sacs. The low contribution from the mutant cells may reflect true contribution by *Flk1*$^{-/-}$ cells to yolk sac hematopoiesis, or may simply be due to the presence of some nonhematopoietic cells in the yolk sac that survived the in vitro culture conditions. In either case, there was clearly a major deficiency in the primitive hematopoietic potential of *Flk1*$^{-/-}$ cells. These data argue for either a dual role for Flk1 signaling in the progenitor cells of both the endothelial and hematopoietic lineages, or for a common Flk1-dependent precursor of both lineages.

Primitive hematopoietic and endothelial progenitors arise within the yolk sac blood islands from precursor cells that all appear to express *Flk1* (Shalaby et al., 1995). *lacZ*-expressing *Flk1*$^{-/-}$ cells were not seen in the developing blood islands of the yolk sac at 7.5 dpc, suggesting that *Flk1*$^{-/-}$ cells are excluded from the blood islands and hence fail to contribute to both yolk sac endothelial and hematopoietic lineages. As within the embryo itself at later stages, *lacZ*-expressing cells were observed in the chimeras, but they appear to be diverted to other mesoderm populations such as the amnion. We propose that activation of Flk1 signaling is required as cells exit the posterior primitive streak if these cells are to move to the correct yolk sac location, where they are then able to undergo further development into endothelial and hematopoietic progenitors. The ligand for Flk1, VEGF, is expressed in the endoderm of the yolk sac and may attract cells to this location (Dumont et al., 1995). In the absence of such signaling, it is possible that mesoderm cells respond to other signals and thereby become committed to alternative fates. Thus, Flk1 may indeed be required in a common precursor of both hematopoietic and endothelial lineages, but this precursor may be a pluripotent, not bipotent, mesoderm cell that has multiple developmental options.

The failure of *Flk1* mutant mesoderm cells to colonize the blood islands raises the question of whether *Flk1*$^{-/-}$ cells could differentiate into primitive hematopoietic progenitors if they were located in the right environment. Examination of embryoid body differentiation of *Flk1*$^{-/-}$ ES cells confirmed the inability of endothelial cell precursors to develop into mature blood vessels. However, the *Flk1*$^{-/-}$ ES cells were able to form hemoglobinized blood islands adjacent to the *lacZ*-expressing endothelial cell precursors, suggesting that *Flk1*$^{-/-}$ mesodermal cells have the capability to differentiate into primitive erythroid progenitors. Indeed, more detailed analysis suggests that under such in vitro conditions, ES cell-derived *Flk1*$^{-/-}$ and *Flk1*$^{+/-}$ primitive hematopoiesis is indistinguishable (A. C. S. et al., unpublished data). Thus, a comparison of the in vitro embryoid body data and the in vivo chimeric data suggests that Flk1 signaling is not necessarily required in a cell-autonomous manner for primitive hematopoiesis per se, but for ensuring that mesodermal progenitors are placed in the right environment to respond to the appropriate signals for hematopoiesis. This contrasts with the autonomous role for Flk1 in the endothelial lineages. In mutant and in chimeric embryos, *Flk1*$^{-/-}$ precursors are observed in the correct

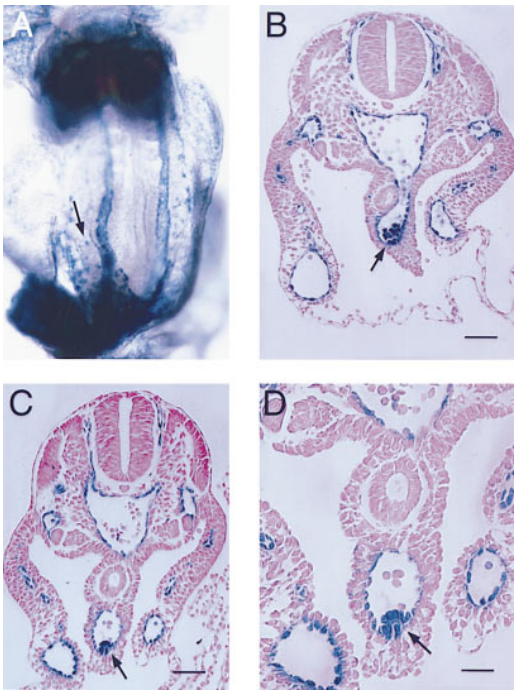

Figure 7. Flk1 Expression in 8.5 and 9.5 dpc Embryos

β-galactosidase staining of 8.5 and 9.5 dpc *Flk1*$^{+/-}$ embryos. Isolated *Flk1*-expressing cells (arrow) were detected in the posterior splanchnopleure of 8.5 dpc embryos (A). Histological transverse sections through 9.5 dpc *Flk1*$^{+/-}$ embryos showed a clump of *lacZ*-positive cells (arrows) in the omphalomesenteric artery (B), as well as near the connection of this artery with the posterior dorsal aorta (C). Scale bar = 100 μm. These clumps appear in close association with the endothelial lining of the artery (D). Scale bar = 50 μm.

embryonic location in the head mesenchyme, where they would develop into embryonic blood vessels but fail to do so. Similarly, in embryoid bodies, *Flk1*$^{-/-}$ cells fail to form mature blood vessels, even though they do not have to undergo the morphogenetic events of streak formation. Thus, there appears to be an absolute requirement for Flk1 in the specification of the endothelial lineages, and an additional earlier role in the allocation of the progenitors of the extraembryonic blood islands.

The derivation of viable chimeras allowed us to examine whether Flk1 was also required for definitive hematopoiesis in vivo. Flk1-deficient ES cells were not detected among bone marrow cells, peripheral blood, splenocytes, or thymocytes in adult chimeras, nor could *Flk1*$^{-/-}$ hematopoietic progenitors be detected in the fetal livers of *Flk1*$^{-/-}$ <–> CD1 chimeras. Thus, we conclude that there is an intrinsic requirement for Flk1 signaling during the development of the progenitors of the adult hematopoietic system. The absence of *Flk1*$^{-/-}$ cells from all compartments of the hematopoietic system, along with the inability to detect *Flk1*$^{-/-}$ hematopoietic progenitors in in vitro colony assays, suggests there is an early requirement for Flk1 in the generation of the pluripotent adult HSC.

Until recently, it was widely accepted that pluripotent HSCs first arise in the yolk sac, then migrate to the fetal liver, and finally colonize the bone marrow (Moore and Metcalf, 1970; for review, see Dzierzak and Medvinsky, 1995). According to this view, the absence of adult hematopoietic cells in *Flk1*$^{-/-}$ <–> CD1 chimeras would be a direct consequence of the earlier absence of yolk sac progenitors. However, recent work has convincingly supported the idea of a separate intraembryonic origin for definitive hematopoiesis in mice, as in other vertebrates (Godin et al., 1993, 1995; Medvinsky et al., 1993; Medvinsky and Dzierzak, 1996). The presence of LTR-HSCs in the isolated AGM in organ culture has demonstrated that this region is capable of initiating definitive hematopoiesis without cellular exchange with the yolk sac (Medvinsky and Dzierzak, 1996). Furthermore, when the paraaortic splanchnopleure region is isolated and cultured before circulation begins, the presence of lymphoid and myeloid progenitors can be detected (Cumano et al., 1996). In the light of this evidence supporting a separate origin for definitive hematopoietic progenitors, the absence of *Flk1*$^{-/-}$ cells in the adult hematopoietic system suggests that Flk1 plays a separate role in definitive hematopoiesis.

The nature of the requirement for Flk1 signaling in the development of the adult hematopoietic system is unclear. Flk1 is expressed in fetal liver hematopoietic progenitors (Matthews et al., 1991) and may therefore play an intrinsic role in definitive hematopoiesis. However, given the association between blood vessels and hematopoietic progenitors in the AGM, and evidence presented here that *Flk1* is expressed in putative hematopoietic precursors in the AGM, as well as the developing vessels, it is also possible that Flk1 may be acting within the embryo in a similar manner to its role in extraembryonic hematopoiesis, namely to regulate the developmental behavior of the common precursor of the hematopoietic and angioblast lineages. A role for VEGF signaling in promoting development of the earliest hematopoietic progenitors has also recently been proposed (Kennedy et al., 1997) based on the effects of VEGF on the differentiation of ES cells in vitro.

A role for Flk1 signaling in both yolk sac and definitive hematopoieisis contrasts with the role for signaling through the Kit receptor tyrosine kinase, which appears to be restricted to definitive hematopoiesis. Mutations in the c-*kit* gene (mouse *W* locus) lead to multiple developmental abnormalities, including defects in hematopoiesis (for review, see Russell, 1979; Reith and Bernstein, 1991). Mice injected with anti-Kit antibody do not display overt YS hematopoietic defects, suggesting that the Kit receptor is not required for YS hematopoiesis (Ogawa et al., 1993). Recently, Sanchez et al. (1996) have demonstrated that the Kit receptor tyrosine kinase is expressed on hematopoietic stem cells in the AGM region. It is interesting to note that *Flk1* and c-*kit* map within 2 Mb in both the mouse and human genomes (Spritz et al., 1994; Brunkow et al., 1995). Thus, just as the genes within the β-globin gene cluster undergo switching during the transition from primitive to definitive hematopoiesis, there may also be a transition from an early requirement for Flk1 through a dual requirement for Flk1 and the closely linked Kit receptor in fetal hematopoiesis, followed by a reliance on Kit signaling in adult life.

Although many different regulators of hematopoietic development have been identified, mutational analysis has shown that most have roles confined to the development of definitive hematopoiesis. However, mutations in the *SCL/tal-1* and *rbtn2* transcription factor genes

both lead to defects in early yolk sac hematopoiesis (Warren et al., 1994; Shivdasani et al., 1995). Chimeric analysis has shown that SCL/tal-1 is also required for definitive hematopoiesis (Porcher et al., 1996), showing that some aspects of the transcriptional programs in both primitive and definitive hematopoietic progenitors are shared. Our evidence suggests that signaling through Flk1 is also a common property of early progenitors of both types of hematopoietic cells. A further understanding of the Flk1 signaling pathway and how it affects the behavior of mesodermal precursors of the endothelial and hematopoietic lineages may thus shed light on the fundamental mechanisms of acquisition of hematopoietic potential.

Experimental Procedures

Production of *Flk1*$^{-/-}$ ES Cells and Chimeras

ES cells heterozygous for the *Flk1*-null allele (Shalaby et al., 1995) were seeded at 5×10^4 cells/100 mm plate in ES cell medium (Wurst and Joyner, 1993). One day later, 640 μg/ml of G418 was added to the medium. Colonies were picked after one week and screened for homozygosity at the *Flk1* mutant allele by Southern blot and PCR analysis. Three primers were used for PCR amplification as previously described (Shalaby et al., 1995).

ES cell lines that were either homozygous or heterozygous for the *Flk1*-null allele (which are also glucose phosphate isomerase isotype *Gpi-1*^{aa}) were aggregated with CD1 (*Gpi-1*^{bb}) embryos, and the aggregates transferred into pseudopregnant females (Nagy and Rossant, 1993).

Glucose Phosphate Isomerase Isoenzyme Analysis and β-galactosidase Staining of Cells and Tissues

Cells and tissue lysates were freeze thawed in water as described by Nagy and Rossant (1993). Bone marrow cells were obtained by flushing the femur with 1 ml of phosphate-buffered saline. Splenocytes and thymocytes were obtained by teasing the spleen and the thymus with forceps and collecting the released cells in 10 ml PBS. Erythrocytes were removed from these cell preparations by specific lysis in 0.16 M ammonium chloride on ice until the cell pellet obtained by centrifugation was visually free of erythrocytes.

The samples were subjected to electrophoresis on cellulose acetate plates (cat. no. 3024, Helena Laboratories), and the GPI isoenzyme bands were visualized as previously described (Nagy and Rossant, 1993). The contribution of ES cells to the samples was visually estimated by comparison to a set of standard mixtures of GPI-AA and GPI-BB cells.

Embryos and adult tissues were fixed, stained for *lacZ* expression, and counterstained as previously described (Shalaby et al., 1995).

Hematopoietic Colony Assays

Individual yolk sacs from 9.5 dpc chimeric embryos were treated with 0.5 ml of 0.1% collagenase/20% fetal calf serum for 3.5 hr at 37°C as described (Wong et al., 1986) and disaggregated with a 26-gauge needle. The cells were then plated in 1.5 ml of 0.9% methylcellulose (Methocult, Stem Cell Technologies) in IMDM (GIBCO) supplemented with 25% FCS (Hyclone), 4.5×10^{-4} M monothioglycerol, 2 U/ml murine erythropoietin (Boehringer), and 2% IL-3-conditioned medium (Karasuyama and Melchers, 1988). Corresponding embryos were analyzed by GPI to determine the degree of chimerism. Colonies were incubated in a humidified CO_2 atmosphere at 37°C, scored visually at day 10, and all colonies from one yolk sac were pooled for GPI analysis.

Fetal livers from 14.5 dpc chimeric embryos were disaggregated with a 26-gauge needle in 1 ml IMDM and plated on semisolid medium as described above at $1–5 \times 10^5$ cells/ml. Viable cells were counted with Trypan blue (GIBCO). The growth factors added were 2 U/ml erythropoietin, 0.2 ng/ml GM-CSF (Pharmingen), and IL-3 (Karasuyama and Melchers, 1988). The colonies that were produced from one fetal liver were scored visually and pooled for GPI analysis on day 10 of incubation.

In Vitro Differentiation of ES Cells

In vitro differentiation of ES cells was performed as previously described (Bautch et al., 1996). In brief, ES cells were passaged twice on gelatinized plates. On day 6, after the second passage, ES colonies were treated with dispase, washed, and grown in suspension in bacterial dishes in ES media without LIF. For attached embryoid body cultures, embryoid bodies were transferred to tissue culture plates on day 3 postdispase treatment. On day 8 postdispase treatment, β-galactosidase activity was detected as before.

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