Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice

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SUMMARY

We previously demonstrated the essential role of the flt-1 gene in regulating the development of the cardiovascular system. While the inactivation of the flt-1 gene leads to a very severe disorganization of the vascular system, the primary defect at the cellular level was unknown. Here we report a surprising finding that it is an increase in the number of endothelial progenitors that leads to the vascular disorganization in flt-1⁻/⁻ mice. At the early primitive streak stage (prior to the formation of blood islands), hemangioblasts are formed much more abundantly in flt-1⁻/⁻ embryos. This increase is primarily due to an alteration in cell fate determination among mesenchymal cells, rather than to increased proliferation, migration or reduced apoptosis of flt-1⁻/⁻ hemangioblasts. We further show that the increased population density of hemangioblasts is responsible for the observed vascular disorganization, based on the following observations: (1) both flt-1⁻/⁻ and flt-1⁺/+ endothelial cells formed normal vascular channels in chimaeric embryos; (2) wild-type endothelial cells formed abnormal vascular channels when their population density was significantly increased; and (3) in the absence of wild-type endothelial cells, flt-1⁻/⁻ endothelial cells alone could form normal vascular channels when sufficiently diluted in a developing embryo. These results define the primary defect in flt-1⁻/⁻ embryos at the cellular level and demonstrate the importance of population density of progenitor cells in pattern formation.

Key words: Flt-1, Vasculogenesis, VEGF, Hemangioblast, Gene targeting

INTRODUCTION

The development of the extraembryonic vasculature begins as early as the onset of gastrulation. At this stage, hemangioblasts arise from the nascent extraembryonic mesenchyme and are recognized by their expression of specific molecular markers, flk-1 and flt-1, both of which encode receptor-type protein tyrosine kinases (Shibuya et al., 1990; Matthews et al., 1991; Yamaguchi et al., 1993; Fong et al., 1996). By late streak/early headfold stages (7.5-8.0 d.p.c.), blood islands are formed from hemangioblasts. Blood islands are solid or luminal structures with elongated angioblasts at the periphery and round hematopoietic progenitors in the core or cavity (Risau, 1991). Thus, hemangioblasts serve as common progenitors to both endothelial and hematopoietic lineages. Concomitant with these processes, adjacent groups of early as well as mature blood islands anastomose to form primary cords and vascular channels. This developmental process is characterized by the formation of endothelial cells and blood vessels from mesenchymal progenitors, and has been referred to as vasculogenesis (Pardanaud et al., 1989; Risau, 1991). At the cellular level, vasculogenesis is distinct from angiogenesis, the latter of which describes vascular growth and maturation by sprouting and remodeling from existing vessels. Despite the apparent differences at the cellular level, vasculogenesis and angiogenesis may overlap in their molecular controlling mechanisms, since several genes that are important for angiogenesis are also important for vasculogenesis (for reviews, see Folkman and D’Amore, 1996; Risau, 1997).

The vascularization events in the embryo proper lag behind those of the yolk sac. However, early molecular markers that are expressed in the endothelial progenitors of the extraembryonic mesenchyme are also expressed in the embryo proper (Yamaguchi et al., 1993; Dumont et al., 1995; Thomas et al., 1998), indicating that the development of the embryonic vasculature is governed by a closely related molecular program. Indeed, mutant phenotypes of several genes are identical or similar between the yolk sac and embryonic vascular systems (Dumont et al., 1994; Fong et al., 1995; Puri et al., 1995; Sato et al., 1995; Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996).

The early developmental stages of both vascular systems are critically regulated by vascular endothelial growth factor (VEGF) (Keck et al., 1989; Leung et al., 1989; Breier et al., 1992; Carmeliet et al., 1996; Ferrara et al., 1996). VEGF is the prototype of a rapidly growing family of growth factors, including, but not limited to, VEGF itself (now also called VEGF-A) (Albini et al., 1996), VEGF-B (Olofsson et al., 1996), VEGF-C and VEGF-D (Carmeliet et al., 1996; Dvorak et al., 1996).
MATERIALS AND METHODS

Mice

flt-1+/− and flk-1+/− mice were in 129 Sv, CD-1 and ICR backgrounds. flk-1+/− mice were initially generated by Shalaby et al. (1995) and a breeding pair was purchased from Jackson Laboratory. The mutant phenotypes of these mice were not affected by the differences in genetic background. In all mating procedures, the noon of the day when a vaginal plug was observed was considered as 0.5 d.p.c. CD-genetic background. In all mating procedures, the noon of the day breeding pair was purchased from Jackson Laboratory. The mutant flt-1 results in a severely disorganized vasculature, and an increase in the number of endothelial cells. However, it has been unclear whether the expansion of the endothelial lineage is a secondary consequence, or a primary defect.

We now report that increased mesenchymal-hemangioblast transition is the primary defect in flt-1 knockout mice, whereas the formation of disorganized vascular channels is a secondary phenotype due to the overcrowding of the endothelial population. When the overcrowding problem is alleviated by different means, flt-1−/− endothelial cells are capable of forming normal vascular structures. These results demonstrate a primary role of Flt-1 in regulating hemangioblast development, and provide an explanation at the cellular level for the formation of the disorganized vasculature in flt-1−/− embryos.

Analysis of ES cell contributions in chimaeric embryos

To determine the overall ES cell contribution in a chimaeric embryo, a piece of ectoplacental cone (EPC) was saved from each embryo, and cultured in ES cell medium for 2 weeks. DNA samples were isolated from the resulting cultures and analyzed by PCR, using the following primer pairs: CTGTGTCCCGCAGCCGGA TA (flk-1 F5B) (Shalaby et al., 1995) and GCACAGAGCCAGTTTCTCA (flk WT3R) for the wild-type allele, yielding a 190 bp band, and flk-1 F5B plus LacZ1 (AAGCGGATTCGCCATTC) for the targeted allele, generating a band of 250 bp. Since only moruli-derived cells may contribute to the developing EPC, the genotypes of the cultured trophoblasts represent those of the original moruli used for aggregation.

Histological and immunohistochemical analysis

Embryos or cystic embryoid bodies were fixed in glutaraldehyde for...
β-gal staining, or in 4% paraformaldehyde (or methanol) for immunohistochemical staining with antibodies against murine PECAM-1 (Mec13.3, Pharmingen) or *E. coli* β-galactosidase (polyclonal, Sigma). For sections, stained specimens were postfixed overnight with 3.7% formaldehyde, dehydrated and embedded in paraffin. Sections were cut to 5 μm and counterstained with nuclear Fast Red (Sigma). For cell counting, intact transverse sections of the yolk sac or the prospective yolk sac (such as those shown in Fig. 1C,D) were chosen, and endothelial cells (or progenitors) were counted in the entire circumference of each section.

**RESULTS**

**Increased hemangioblast development is a primary defect**

We reported that *flt-1*−/− embryos developed highly disorganized blood islands and vascular channels, and contained more angioblasts and endothelial cells (Fong et al., 1995). (Note that at the early gastrulation stage, the term ‘embryo’ is often used to refer to both the extraembryonic region and the prospective embryo proper). The coexistence of two distinct defects at an early stage rendered it difficult to appreciate which might be the earlier and hence possibly primary defect: excessive formation of endothelial progenitors or loss of normal structural organization?

To segregate these two variables, it was important to find a stage when *flt-1* was already expressed at an appreciable level but the structural organization of blood islands was not yet apparent. This corresponded to a very narrow time window (several hours) between the early and mid-streak stages (E7.0), based on the staining of *flt-1*+/− embryos for the expression of β-galactosidase (β-gal). (In our original *flt-1* gene targeting experiment, we inserted the *E. coli lacZ* gene into the *flt-1* locus and confirmed its expression in developing endothelial cells and their progenitors).

We observed a significant increase in β-gal staining at E7.0 in the absence of Flt-1 (Fig. 1A-D), suggesting that there was an increase in the number of hemangioblasts in *flt-1*−/− embryos. To unequivocally demonstrate this possibility, we analyzed the expression of PECAM-1 (CD31) (Baldwin et al., 1994) by immunohistochemical staining of E7.25 embryos. Indeed, *flt-1*−/− embryos stained much more strongly than *flt-1*+/− (Fig. 1E) or wild-type embryos (not shown). Double staining of the same embryos with anti-β-galactosidase antibody also revealed much stronger staining in *flt-1*−/− embryos, as expected (Fig. 1F). Thus, we concluded that there were more hemangioblasts in *flt-1*−/− embryos. Consistent with this conclusion, *flt-1*−/− embryos contained a significantly higher number of primary erythrocytes at E8.5, in addition to the previously found increase in endothelial cells (Table 1).

**Abnormal differentiation program leads to the excessive formation of *flt-1*−/− hemangioblasts**

To investigate which specific aspect of hemangioblast development was affected by the lack of Flt-1, we exploited the unique properties of in vitro differentiation of embryonic stem (ES) cells (Risau et al., 1988; Wang et al., 1992). ES cells, when grown in suspension in the absence of leukemia inhibitory factor (LIF), form aggregates that develop into cystic embryoid bodies (CEBs). The

![Fig. 1. An increased number of hemangioblasts are present prior to the formation of blood island structures. (A-D), β-gal staining of E7.0 embryos. (A,B) Whole mount; (C,D) cross sections at the level of stained extraembryonic regions. (E,F) Double immunohistochemical staining by anti-PECAM-1 (E) and anti-β-galactosidase (F) antibodies. The large arrow in E indicates an area of strong signals, the smaller arrows indicate weaker PECAM-1-positive signals. Overall weaker PECAM-1 staining than β-gal or anti-β-galactosidase staining is most likely due to the fact that onset of PECAM-1 expression is later than flt-1. En, extraembryonic endoderm; m, extraembryonic mesenchyme; Ec, extraembryonic ectoderm. Bars, 200 μm (A,B,E,F); 50 μm (C,D).]

<table>
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<th>Stage</th>
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<td>E7.0</td>
<td>Hemangioblast</td>
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<tr>
<td>E8.5</td>
<td>Erythrocyte</td>
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<tr>
<td>E8.5</td>
<td>Endothelial</td>
<td>154±14</td>
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Means for 5 sections of each embryo are at the left of each column. For E7.0 embryos, sections in the extraembryonic region were analyzed.

For E8.5 embryos, yolk sac sections at the level of headfold were chosen for cell counting.
membrane of a CEB is extremely similar to the yolk sac in its cellular organization, and contains vascular channels that closely resemble microvessels both in their structure and expression of molecular markers. In fact, it has been reported that the developmental mechanism of vascular channels in CEBs is rather similar to that in the yolk sac (Bautch et al., 1996; Vittet et al., 1996; Choi et al., 1998; Nishikawa et al., 1998). To take advantage of this system, we first verified that \textit{flt-1} was expressed in differentiating ES cells (data not shown). We subsequently isolated \textit{flt-1}\textsuperscript{-/-} ES cell lines and confirmed that, upon differentiation in vitro, a mutant phenotype was observed in \textit{flt-1}\textsuperscript{-/-} CEBs that was nearly identical to that in \textit{flt-1}\textsuperscript{-/-} embryos (Fig. 2A-D). As in \textit{flt-1}\textsuperscript{-/-} embryos, the significantly more widespread \textit{b}-gal staining corresponded to a dramatic increase in the number of endothelial cells, as confirmed by immunohistochemical staining for the expression of PECAM-1 (Fig. 2E-H). Therefore, this in vitro system was suitable for the study of the functional mechanisms of Flt-1 and, as in embryos, lacZ expression was a faithful marker for the developing endothelial lineage.

To study the origin of increased formation of hemangioblasts, we focused our attention to the early stages of CEB development. After 3 days of in vitro differentiation, most of the initially undifferentiated single ES cells had aggregated to form clusters of different sizes. At this stage, less than 30\% of aggregates in a typical \textit{flt-1}\textsuperscript{+/+} ES cell culture contained \textit{b}-gal-positive cells (Fig. 2I). In contrast, the vast majority (>80\%) of \textit{flt-1}\textsuperscript{-/-} ES cell aggregates

**Fig. 2.** In vitro differentiation of \textit{flt-1}\textsuperscript{-/-} ES cells replicates \textit{flt-1}\textsuperscript{-/-} embryonic phenotype. (A-D) \textit{b}-gal staining of mature cystic embryoid bodies (CEBs) (we define those CEBs with a typical cystic structure and an estimated diameter of 1.5-2.5 mm by day 12 as mature CEBs). Arrow in C, \textit{b}-gal-positive cells. (E-H) Immunohistochemical staining of CEBs with anti-CD31 (PECAM-1) antibody to visualize endothelial cells. (I,J) \textit{b}-gal staining of differentiating ES cell aggregates after 3 days in suspension culture. Bars, 500 μm (A,B,E,F,I,J); 50 μm (C,D,G,H).
Increased hemangioblasts in flt-1<sup>−/−</sup> embryos were already β-gal-positive by this time (Fig. 2J). This result was in agreement with the increased hemangioblast development in flt-1<sup>−/−</sup> embryos. However, one subtle but critical difference did exist between the two systems. In flt-1<sup>−/−</sup> embryos, we could not rule out uncontrolled migration of flt-1<sup>−/−</sup> hemangioblasts as a possible mechanism for the expansion of the lineage. In differentiating ES cell cultures, however, each aggregate was a physically isolated entity, and so hemangioblasts within an individual aggregate must have an internal origin. By comparing Figs 1A and 2I, we noticed that the latter may be viewed as if it were a very high magnification of the extraembryonic region in Fig. 1A. A similar comparison can also be made between Figs 1B and 2J. If extra aggregates in Fig. 2J became β-gal-positive by a migration-independent event, then it is reasonable to propose the same for the more widespread staining in Fig. 1B.

In flt-1<sup>−/−</sup> embryos, the number of hemangioblasts increased sharply at the beginning of gastrulation, over a very short period of time (a few hours). This was difficult to explain by increased proliferation, which would require a period of time sufficient for at least a few cell cycles to allow cell accumulation. Indeed, comparison of cell proliferation by BrdU labeling failed to reveal noticeable differences between wild type, flt-1<sup>+/−</sup> and flt-1<sup>−/−</sup> embryos at the time of hemangioblast differentiation (Fig. 3G-I). One could also hypothesize that there might have been massive cell death in normal embryos and that the lack of Flt-1 might have slowed down this process, leading to the accumulation of hemangioblasts. However, this is also very unlikely, since Hakem et al. (1996) reported that there was virtually no apoptosis in normal embryos at this stage.

We next directly tested hemangioblast development during ES cell differentiation in the absence of proliferation. We expected and verified that when ES cell cultures were treated with mitomycin C at a high dosage, cell proliferation was effectively blocked. However, since mitomycin C works by inhibiting DNA replication, various cellular activities not immediately requiring DNA synthesis would remain active for sometime. Thus, if a cell was already poised to undergo hemangioblast differentiation prior to mitomycin C treatment, it might still be able to carry on its differentiation program until it eventually dies. Based on these arguments, we speculated that the optimal time point for mitomycin treatment would be when the first few hemangioblasts were just formed, but prior to any noticeable difference occurring between flt-1<sup>+/−</sup> and flt-1<sup>−/−</sup> cultures.

After 2 days in culture, the vast majority of ES cell aggregates contained few or essentially no hemangioblasts (Fig. 3A,B). At 3.5 days, the controls without mitomycin C treatment contained a significant number of β-gal-positive

**Fig. 3.** Generation of extra flt-1<sup>−/−</sup> hemangioblasts is not due to increased cell proliferation. (A-D) Controls without mitomycin C treatment. ES cell aggregates were subjected to β-gal staining after 2 (A,B) or 3.5 (C,D) days of in vitro differentiation. (E,F) Cultures were treated with mitomycin C at 2 days and cultured for a further 1.5 days prior to β-gal staining. (G,H) Incorporation of BrdU into proliferating cells in E7.0 embryos. Most cells in both +/- and -/- embryos are labeled by BrdU. Arrows indicate examples of BrdU-positive cells in the extraembryonic mesenchyme. (I) Maternal decidua as a control. Only a small fraction of cells have BrdU-positive nucleus (arrows), demonstrating the specificity of nuclear staining. Note that although the intercellular junctions in the maternal decidua stained non-specifically, the corresponding areas in the embryos are clear, indicating that staining in G and H is specific to the nucleus. Bars, 200 μm (A-F); 50 μm (G-I).
aggregates, especially in the flt-1−/− culture (Fig. 3C,D). In parallel samples treated with mitomycin C at 2 days of differentiation and cultured for a further 1.5 days, hemangioblast development was also significant. Most importantly, the flt-1−/− culture (Fig. 3F) developed a much higher percentage (over 80%) of β-gal-positive aggregates than its flt-1+/− counterpart (Fig. 3E). Based on these results, we concluded that altered cell differentiation was the primary cause for the increased number of hemangioblasts in flt-1−/− embryos.

Development of flt-1−/− hemangioblasts is normal in a wild-type environment

A negative role of Flt-1 in the development of hemangioblasts presented an apparent paradox, because it was well known that flt-1-expressing cells were able to become endothelial cells later during development. To accommodate both of these facts, we hypothesized that the expression of flt-1 in hemangioblasts did not hinder their own development, but rather prevented yet undetermined mesenchymal cells from adopting a hemangioblastic fate. This hypothesis predicted a cell non-autonomous property of the flt-1−/− phenotype, which could be tested by chimaerism analysis. In chimaeric embryos containing both wild-type and flt-1−/− cells, wild-type hemangioblasts might inhibit the excessive formation of flt-1−/− hemangioblasts from their undetermined mesenchymal progenitors and therefore rescue the mutant phenotype.

We constructed chimaeric embryos by in vitro aggregation between flt-1−/− ES cells and wild-type morula embryos derived from ICR mice (referred to as wt:−/− chimaeras). Control chimaeras were also constructed between flt-1+/− ES cells and wild-type morula, and were found to be normal, as expected (data not shown). From two independently isolated flt-1−/− ES cell lines, over 70 chimaeric embryos were constructed and analyzed at various stages. Since the extent of ES cell contribution varied from embryo to embryo (as an intrinsic property of the technique), the overall percentage of ES cell-derived cells in a chimaera was determined by evaluating relative band intensities of targeted and wild-type flt-1 alleles in Southern blots. For embryos at early stages where material was limited, a comparative PCR procedure was developed (see Materials and methods). Among 17 viable E11.5 chimaeras analyzed, all embryos contained normal blood vessels that were composed of flt-1−/− and wild-type endothelial cells (e.g. Fig. 4A). Interestingly, some short stretches of normal blood vessels contained essentially only flt-1−/− endothelial cells (Fig. 4C). In histological sections, normal lumens that were lined only by flt-1−/− endothelial cells were frequently found (Fig. 4E). In addition, vascular lumens with mixed wild-type and flt-1−/− endothelial cells were also abundant (Fig. 4F), indicating that a distinction was not made between these two populations of endothelial cells. Analysis of chimaeras at earlier stages demonstrated similar rescue effects of flt-1+/− cells (data not shown). These results indicated that Flt-1 indeed acted in a cell non-autonomous manner and suggested that the role of Flt-1 was to negatively control the development of hemangioblast from undetermined mesenchymal cells. In addition, these experiments raised the possibility that flt-1−/− endothelial cells were not intrinsically defective in forming tubular structures. Instead, formation of disorganized vasculature in flt-1−/− embryos was likely a result of overpopulation by endothelial progenitors.

The efficiency of rescue, however, appeared to be dependent...
Increased hemangioblasts in \( fli-1^{-/-} \) embryos

**Fig. 5.** Overcrowding of endothelial progenitors leads to vascular disorganization. (A) A yolk sac section from an E8.5 chimaera whose endothelial cells were mostly \( fli-1^{-/-} \). Only the junction between a wild-type and a mutant area is shown, which together form a contiguous large endothelial sheet lining a greatly expanded cavity. (B) An example showing that wild-type endothelial cells (arrowheads) can also find themselves in the abnormal intraluminal locations in these chimaeras. (C) Non-chimaeric \( fli-1^{+/+} \) yolk sac, to show that \( \beta \)-gal staining condition was sufficient for uniform staining of all \( fli-1^{-/-} \) endothelial cells. (D) \( fli-1^{+/+} \) yolk sac (E8.5). Bars, 50 \( \mu \)m.

on the presence of a sufficient amount of wild-type cells. In chimaeras with over 50% of \( fli-1^{-/-} \) ES cell contribution, disorganized vessels were also present in addition to normal ones (Fig. 5), and were typically growth-arrested by E10.5 or earlier (regular \( fli-1^{-/-} \) embryos all die at E8.5). This is because fewer wild-type cells would mean reduced rescue capacity.

**Overcrowding of endothelial population leads to disorganized vasculature.**

We took advantage of the fact that an extremely high proportion of \( fli-1^{+/+} \) ES cell contribution led to disorganized vasculature in chimaeras, and asked what might happen to wild-type endothelial cells in such chimaeras. We focused our attention to the yolk sac, due to the ease of identifying endothelial cells in this tissue. In histological sections, the structure of E8.5 yolk sac membrane is very simple, consisting of a linear series of vascular lumens which are lined with the endothelial cells, and sandwiched between the endoderm-derived cuboidal cell layer and epithelium monolayer (Fig. 5D). Even in the highly disorganized vasculature of the \( fli-1^{-/-} \) yolk sac, the endothelial cells can still be easily recognized based on their characteristic elongated morphology, since the only other cell types inside the disorganized vascular lumen are the round hematopoietic cells. Therefore, in wt:\( -/- \) chimaeras, wild-type endothelial cells in \( \beta \)-gal-stained yolk sac membranes were identified as \( \beta \)-gal-negative, elongated cells lining or within vascular lumens. In chimaeras mostly contributed by \( fli-1^{-/-} \) ES cells, it was not surprising to find that \( fli-1^{-/-} \) endothelial cells dominated most of the defective lumens. Nevertheless, some wild-type endothelial cells did exist and, as shown in Fig. 5, they also participated in the formation of abnormal vascular lumens, rather than sorting themselves out to form normal tubular structures. These results demonstrate that, even for wild-type endothelial cells, disorganized vasculature can arise from an overcrowded endodermal environment, and further raises the possibility that Flt-1 might not directly regulate tube formation per se.

**\( fli-1^{-/-} \)-expressing hemangioblasts suppress mesenchymal-hemangioblast transition.**

Normal vascular development in wt:\( -/- \) chimaeras implied that \( fli-1^{+/+} \) hemangioblasts were responsible for the suppression of the excessive formation of \( fli-1^{-/-} \) hemangioblasts. Other subtle possibilities, however, also existed. For instance, although the cells of the endoderm were considered not to express \( fli-1 \), what if they did express this gene at a level below detection and that such expression was responsible for the rescue effect? To confirm that the presence of \( fli-1^{-/-} \)-expressing hemangioblasts was indeed essential, we needed chimaeric embryos that were deficient in \( fli-1^{-/-} \)-positive hemangioblasts. A wt:\( -/- \) chimaera predominantly contributed by \( fli-1^{-/-} \) ES cells would satisfy the requirement of the near-absence of \( fli-1^{-/-} \)-positive hemangioblasts, but at the same time it would also have few other wild-type cells. As a result, one would not be able to conclude if the development of defective vessels in such a chimaera was due to the diminished number of \( fli-1^{-/-} \)-positive hemangioblasts or other wild-type cells. This technical difficulty was overcome by constructing chimaeras between \( flk-1^{-/-} \) (\( fli-1^{+/+} \)) moruli and \( fli-1^{-/-} \) (\( flk-1^{+/+} \)) ES cells (referred to as KT chimaera). Since \( flk-1^{-/-} \) (\( fli-1^{+/+} \)) hemangioblasts fail to survive in a cell-autonomous manner (Shalaby et al., 1997), KT chimaeras would be deficient in \( fli-1^{+/+} \)-hemangioblasts, but not other \( fli-1^{+/+} \) cells. We examined 12 KT chimaeras that had moderate to significant levels of \( fli-1^{-/-} \) ES cell contribution (1/4 or over) and found that all of them developed extremely disorganized vasculatures and died at about E8.5 (Fig. 6A). These results argue strongly that the presence of a critical mass of \( fli-1^{-/-} \)-positive hemangioblasts is essential for the suppression of excessive mesenchymal-hemangioblast transition.

**\( fli-1^{-/-} \) endothelial cells alone can form normal vascular channels.**

Our finding that vascular disorganization could arise from overcrowding of the endothelial population suggested that \( fli-1^{-/-} \) endothelial cells might have normal properties in vascular tube formation. To thoroughly test this possibility, we decided to investigate if, in the absence of \( fli-1^{+/+} \) endothelial cells, sparsely populated \( fli-1^{-/-} \) endothelial cells could form normal vascular channels. For this experiment, we again took advantage of the properties of the KT chimaeras mentioned above. Despite the lack of a mechanism for the prevention of uncontrolled development of \( fli-1^{-/-} \) hemangioblasts in KT chimaeras, we expected that if the contribution of \( fli-1^{-/-} \) ES cells was extremely low, we might be able to obtain KT
chimaeras that contained relatively few flt-1−/− hemangioblasts. Although the exact level of contribution could not be controlled precisely, we maximized our chance of obtaining such KT chimaeras by reducing the number of ES cells used. In this way we were able to obtain three KT chimaeras that had very low levels of flt-1−/− ES cell contribution (significantly less than 10%), and all of them formed normal vascular channels both in the yolk sac and in the embryo proper (Fig. 6B,C,E). This result convincingly demonstrates that the existence of flt-1-positive hemangioblasts is only required for the negative control of hemangioblast development but not for tubular structure formation per se. When other means are available to achieve population control, flt-1−/− endothelial cells have the full capacity of forming normal vascular channels in the absence of wild-type endothelial cells.

Note that in the same KT chimaeras that had normal vascular channels, large patches of intense blue staining were also present. These patches of endothelial cells probably came from relatively large blocks of flt-1−/− mesenchymal cells, which generated locally overcrowded populations of hemangioblasts and later endothelial cells. On the other hand, smaller blocks or individual mesenchymal cells, despite their fundamental property of being prone to undergoing hemangioblast commitment due to the absence of wild-type hemangioblasts, could only form a limited number of endothelial progenitors, simply because there are only a few cells to begin with. This interpretation appears to be reasonable since cell movement during development may mostly occur as clusters of various sizes, although some single cells may also be involved. For instance, coat colours of chimaeric mice derived from ES cells and CD1 or ICR combinations mostly appear as patches of different sizes, with only a minor fraction of the coat where hair mixing is nearly complete.

The presence of large patches of disorganized endothelial population in these KT chimaeras also indicates that any remnant amount of flk-1−/− (flt-1+/+) hemangioblasts in such chimaeras were far from sufficient to provide a rescue effect. Therefore, it is extremely unlikely that the formation of the normal vessels was due to the presence of a trivial amount of flt-1+/− endothelial progenitors rather than due to the reduced population density of flt-1−/− hemangioblasts.

While it would be interesting to see if a completely normal vascular system could be formed when the relative contributions between flk-1−/− and flt-1−/− cells were optimized, we were never able to do so. This was because normal vessels could only be obtained when the contribution by flt-1−/− ES cell was very low, and in such cases there were not enough vessels to support embryonic development. These KT chimaeras behaved essentially like flt-1−/− mutants and died at E8.5-9.0. With even a moderate increase in ES cell contribution, flt-1−/− cells begin to form too many large patches of hemangioblasts, leading to the flt-1−/− phenotype.

DISCUSSION

Early in gastrulation, both hemangioblasts and undifferentiated mesenchymal cells are abundant in normal embryos. In flt-1−/− embryos, however, the vast majority of the extraembryonic mesenchymal cells become hemangioblasts. Notably, this increase in the number of endothelial progenitors

Fig. 6. Wild-type hemangioblasts are required for negative regulation of mesenchymal-hemangioblast transition, but not for subsequent vascular organization. (A) A yolk sac section of a typical KT chimaera. Since flk-1−/− cells fail to contribute to the endothelial lineage in a cell-autonomous manner, all of the endothelial cells in KT chimaeras are flt-1−/−. The arrow indicates entrapped endothelial cells in a large cavity. (B,C,E) In KT chimaeras with very little flt-1−/− ES cell contribution, normal vascular channels are formed, both in the embryo proper (C, arrowhead) and in the yolk sac (E, arrowhead). (B) A histological section to show normal vascular lumens in a KT chimaera. (D,F) Non-chimaeric flk-1−/− embryo and yolk sac for comparison. Blue staining in D reflects flk-1 expression in non-endothelial tissues. Bars, 50 μm (A,B); 250 μm (C-E).
occurs prior to the development of blood islands, the first structurally organized units formed during normal vasculogenesis. Therefore, the increased development of endothelial progenitors is the primary defect.

Several possible mechanisms were initially considered to explain the origin of extra hemangioblasts in the absence of Flt-1, including altered cell fate determination, migration, increased proliferation and reduced cell death. We excluded increased motility as a possible mechanism based on the following reasoning. ES cell aggregates are physically unlinked to one another, and so obviously hemangioblast development depends on internal events within an aggregate rather than migration from another one. Therefore, the dramatic increase in the number of hemangioblast-containing aggregates in flt-1−/− cultures was taken as evidence for the presence of an in situ, rather than migratory, process that led to the increased hemangioblast development.

Increased proliferation or reduced cell death would need sufficient time to allow the accumulation of extra hemangioblasts. In flt-1−/− embryos, however, the dramatic increase of hemangioblasts is obvious at the very beginning of the mesenchymal-hemangioblast transition. In addition, we were unable to detect any difference in proliferation between flt-1+/+, flt-1+/− and flt-1−/− embryos (E7.0), indicating that increased proliferation was not a likely mechanism. More convincingly, treatment of differentiating ES cell cultures with a strong inhibitor of DNA replication, mitomycin C, did not prevent the excessive generation of hemangioblasts in flt-1−/− cultures. Reduced apoptosis was also an unlikely explanation, as Hakem et al. (1996) reported that there was virtually no apoptosis in early gastrulating embryos. Therefore, we concluded that an alteration of the mesenchymal differentiation program was the primary cause for the increased development of hemangioblasts in flt-1−/− embryos.

We further demonstrated that the loss of normal vascular organization was a consequence of the presence of an overcrowded population of endothelial cells, rather than a separate, independent defect. In chimaeric embryos, the behaviour of flt-1−/− and wild-type endothelial cells was indistinguishable: both were able to form normal vascular channels if not overcrowded and both could be involved in disorganized vessels if overcrowded. Moreover, flt-1−/− endothelial cells were able to form normal vascular structures when their population density was reduced experimentally, indicating that these cells were not intrinsically defective in forming blood vessels.

The reason why disorganized vessels are formed if endothelial cells are too crowded can be explained as follows. Under normal circumstances, each blood island is kept at some distance from its neighboring groups of endothelial progenitors, and the vascular network pattern is established by the formation of cellular interactions among these groups. When each group of endothelial progenitors is surrounded by many others, interactions occur in many different directions, resulting in highly disorganized vascular cavities. Orange spots are non-hemangioblastic mesenchymal cells.

Increased hemangioblasts in flt-1−/− embryos.
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