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13. ABSTRACT (Maximum 200 Words)

Breast cancer progression is dependent on the ability of breast tumors to recruit new blood vessels by angiogenesis. At the level of cell biology, angiogenesis is the migration and proliferation of endothelial cells to form vascular sprouts. These hallmarks of angiogenic endothelial cell biology are conserved between cancer angiogenesis and embryonic angiogenesis. Thus, understanding the molecular cues that regulate the migration and proliferation of endothelial cells during embryonic vascular development has the potential to elucidate novel breast cancer therapies. This proposal involves the analysis of the *flt-1* gene, a presumptive negative regulator of vascular outgrowth during embryonic development. To date, we have shown that *flt-1* functions to limit endothelial cell numbers during embryonic vascular development by negatively regulating endothelial cell proliferation both *in vivo* and *in vitro*. Furthermore, we have demonstrated that the vascular overgrowth phenotype of *flt-1* null mutant embryonic stem cell cultures can be partially rescued by co-culturing *flt-1* null mutant ES cells with wild type ES cells and by treatment of *flt-1* null mutant ES cultures with a soluble version of the flt-1 receptor. These data indicate that *flt-1* regulates embryonic angiogenesis and suggest a potential therapeutic role for *flt-1* in regulating the pathological angiogenesis that is essential for breast cancer progression.

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Table of Contents

Introduction

Angiogenesis, the migration and proliferation of endothelial cells to form new blood vessels, is a critical component of tumor growth and metastasis [1-3]. Thus, angiogenesis is a plausible target for the development of breast cancer therapies. Vascular endothelial growth factor-A (VEGF-A) is a potent endothelial cell mitogen and has been a target in previous anti-angiogenic strategies [4-6]. Our work focuses on elucidating the role of the VEGF-A receptor *flt-1* in vascular formation and patterning during angiogenesis. *flt-1* is essential for normal vascular development in mice and, presumably, in humans as well [7]. The *flt-1* gene produces both a membrane bound receptor tyrosine kinase (memFlt-1) and a soluble receptor (sFlt-1) [8, 9]. Each of these isoforms binds VEGF-A with high affinity. To date it is unclear whether these isoforms have distinct functions in blood vessel formation, and since *flt-1* null mutants do not produce either isoform, it is unclear what the relative contribution of each is to establishing *flt-1* function.

To better understand how *flt-1* affects endothelial cell biology, we have used *in vitro* differentiation of *flt-1* mutant embryonic stem (ES) cells as a model for vascular development in the absence of *flt-1* gene product. This cell culture system allows a level of quantification and analysis that is difficult to achieve *in vivo*, especially when dealing with embryonic lethal genetic backgrounds [10, 11]. We have shown that the absence of *flt-1* leads to vascular overgrowth, indicating that *flt-1* functions as a negative regulator of vascular development. We have demonstrated that this vascular overgrowth occurs both *in vitro* and *in vivo* and is due to increased endothelial cell division, indicating that *flt-1* normally functions as a negative regulator of endothelial cell proliferation. Further

experiments demonstrate that this negative regulatory function is partially cell non-autonomous, suggesting that sFlt-1 protein plays a major role in regulating vascular growth. Since endothelial cell division is a key component in tumor angiogenesis, we feel that the ability of *flt-1* to inhibit this process can be harnessed as an anti-angiogenic breast cancer therapy.

Results

Specific Aim 1: the effect of *flt-1* on endothelial cell division

To test the hypothesis that vascular overgrowth in *flt-1* null mutant differentiated ES cell lines was due to increased cell division, endothelial cell mitotic indices were established for day 6 and day 7 *flt-1* null mutant ES cultures as well as wild type control cultures. A triple-labeling technique, using the mitotic marker anti-phosphohistone H3, the endothelial marker PECAM, and the nuclear marker DAPI, was employed to determine mitotic endothelial indices. The absence of *flt-1* caused a 2.5-fold increase in the endothelial cell mitotic index, indicating that excess cell proliferation is a major contributor to vascular overgrowth in the absence of *flt-1* (for details see appended manuscript, Figs. 3 and 4; Table 1). This increase in cell proliferation was also detected in *flt-1* null mutant embryos, which exhibited a 2-fold increase in the percentage of replicating endothelial cells (appended manuscript, Fig. 8 and text). These experiments confirm our central hypothesis that *flt-1* normally functions to negatively regulate blood vessel growth by inhibiting endothelial cell division.

The original statement of work included experiments that would complement *the in situ* mitotic index approach outlined above using BrdU-labeling and fluorescent activated cell sorting. These approaches proved difficult to apply to our system. BrdU-labeling

experiments were intended as an alternative approach to determining *in situ* endothelial cell mitotic indices with the mitotic marker anti-phosphohistone H3. BrdU-labeling of ES cultures was attempted, however, the results were difficult to interpret due to low signal-to-noise ratio of fluorescent labeling. This made determining *in situ* BrdU-labeling indices impossible. Since anti-phosphohistone H3 proved to be a stronger and more appropriate marker for visualizing endothelial cell proliferation, we decided to use this marker to perform *in vivo* labeling experiments. Florescent-activated cell sorting was proposed as an independent means of determining endothelial mitotic indices of ES cultures. Preliminary FACS experiments suggested that the sensitivity of this approach was below the level necessary to detect the endothelial cell mitotic index differences seen in the *in situ* mitotic index experiments.

Specific Aim 2: analysis of memFlt-1 and sFlt-1 expression and function

1. memFlt-1/sFlt-1 expression in differentiated ES cell cultures. To detect expression of memFlt-1 and sFlt-1 during vascular development, reverse transcription PCR (polymerase chain reaction) using memFlt-1 and sFlt-1 specific primer sets was performed on day 8 RNA lysates from wild type, *flt-1* heterozygous and *flt-1* null mutant differentiated ES cell cultures (Fig. 1A and B, p.15). Both memFlt-1 (Fig. 1A) and sFlt-1 (Fig. 1B) were detected in WT and *flt-1*$^{+/-}$ lysates, indicating that both memFlt-1 and sFlt-1 RNA are present during day 8 of ES cell differentiation. Faint bands were detected in *flt-1*$^{-/-}$ lysates, however this represents small amounts of non-coding truncated transcript that are generated by the targeted *flt-1* locus [7].

RNase protection assays were proposed as a means of determining the relative levels of full-length flt-1 mRNA and sflt-1 (soluble flt-1) mRNA (an alternative transcript made

from the *flt-1* locus) in wild type, *flt-1* heterozygous, and *flt-1* mutant ES cultures. Due to the unavailability of RNase protection probes that can distinguish between the full-length and soluble transcripts, the RT-PCR approach was employed instead.

Expression of memFlt-1 and sFlt-1 protein was also analyzed in day 8 protein lysates from WT, *flt-1* heterozygous, and *flt-1* null mutant differentiated ES cell cultures. Western analysis of day 8 lysates with an antibody (clone flt-11, Sigma) that detects both memFlt-1 and sFlt-1 revealed expression of sFlt-1 in WT and *flt-1* heterozygous lysates (Fig 1C, arrow, p.15). As expected, no detectable sFlt-1 protein was observed in *flt-1* null mutant lysates. Furthermore, *flt-1* heterozygous cultures exhibit an approximate 50% decrease in sFlt-1 protein product compared with wild type controls, confirming that *flt-1* heterozygosity confers lower *flt-1* expression levels. Surprisingly, we were unable to detect full-length memFlt-1 protein in any of the lysates analyzed. This result suggests that sFlt-1 protein is expressed more abundantly than memFlt-1 protein. In an attempt to enrich for memFlt-1 protein in these lysates, immunoprecipitations (IPs) with the anti-Flt-1 antibody were performed. The antibody was unable to IP either *flt-1* isoform. Regardless, these data demonstrate expression of both full-length Flt-1 and sFlt-1 in ES cell cultures, and suggest that sFlt-1 is the more abundant of the *flt-1* isoforms.

2. Co-culture of WT and *flt-1* null mutant differentiated ES cell cultures partially rescues the *flt-1* mutant vascular phenotype. Since the *flt-1* gene encodes a membrane-bound receptor tyrosine kinase and a secreted receptor (sFlt-1), it could potentially have both cell autonomous and cell non-autonomous effects on blood vessel formation. To begin to assess how *flt-1* affects vascular development, we co-cultured day 3 differentiated WT embryoid bodies (EBs) and *flt-1* null mutant EBs during the

remainder of the differentiation period and assayed for rescue of the *flt-1* null mutant vascular phenotype. The wild type cells were ES cells engineered to express green fluorescent protein (GFP) ubiquitously [12]. β-galactosidase detection distinguished *flt-1* null mutant derived vasculature in co-cultures (Fig. 2, A and B, p.17). Co-cultures plated at a 3:1 or higher ratio of WT:*flt-1* null mutant EBs contained areas of partially rescued *flt-1* null mutant vasculature (Fig. 2B), while lower plating ratios had no detectable effect on the *flt-1* null mutant vasculature. These partial rescues were characterized by reduced vascular area of *flt-1* null mutant blood vessels (Fig 2B, compare to Fig 2A). Similar results were obtained when *Rosa26.1* WT EBs were used in place of GFP EBs (data not shown), strongly suggesting that this rescue effect is not an artifact induced by the GFP ES cells. These results indicate that the *flt-1* null mutant vascular phenotype can be partially rescued by WT EBs during differentiation and that this effect is non-cell autonomous, suggesting that it is mediated by sFlt-1.

We previously proposed to use the wild type GFP ES cell line in co-culture experiments with the *flt-1* null mutant ES cell line to quantify the levels of reduced *flt-1* null mutant vascular growth observed in co-culture experiments. These experiments proved technically difficult due to the inability to detect GFP signal (to determine total wild type culture area) and β-galactosidase protein expression (to determine *flt-1* mutant derived vascular area) simultaneously. Although we were unable to quantify the co-culture effect, these experiments do indicate that *flt-1* has a cell non-autonomous component that may influence vascular growth during pathological neovascularization.

3. Soluble Flt-1 treatment partially rescues the *flt-1* null mutant phenotype.

One of the major goals of this project was to understand the functions of the two *flt-1*

isoforms during vascular development by assessing the effects of isoform-specific expression on the *flt-1* null mutant vascular phenotype. To begin to assess the effects of sFlt-1 protein expression on *flt-1* null mutant vascular phenotype, I treated *flt-1* null mutant differentiated ES cell cultures with a commercially available fusion protein, flt-1/Fc (R&D Systems), that mimics sFlt-1 protein in its ability to bind VEGF-A with high affinity. Treatment of *flt-1* null mutant ES cultures with flt-1/Fc from days 3-8 of *in vitro* differentiation caused a dose-dependent decrease in vascular growth as assessed by anti-PECAM, an endothelial cell marker (Fig. 3, p.19). This result was quantified using image analysis of PECAM-labeled cultures (for details see Methods of appended manuscript). *flt-1* null mutant cultures treated with flt-1/Fc exhibited a significant dose-dependent reduction in $PECAM^+$ vascular area, with the 1000ng/ml treatment reducing vascular growth to wild type control level (Fig. 3B graph). This result shows that a soluble version of the Flt-1 receptor is capable of rescuing vascular overgrowth in the absence of endogenous Flt-1 protein, and indicates that vascular overgrowth in *flt-1* null mutant cultures is VEGF-dependent.

4. Isoform-specific transgenic ES cell lines. To better understand how memFlt-1 and sFlt-1 isoforms contribute to *flt-1* function, I proposed to generate transgenic *flt-1* null mutant ES cell lines expressing either the mouse memFlt-1 or sFlt-1 cDNAs in an endothelial-specific manner and analyze their vascular phenotypes. After testing several endothelial-specific promoters such as Tie-2, ICAM-2, and Flt-1 in the ES culture system, our lab determined that the PECAM promoter (gift from S. Baldwin) confers robust endothelial-specific transgene expression during ES cell differentiation (C. Ellerstrom, unpublished result). Thus, I generated DNA constructs containing the murine

memFlt-1 or murine sFlt-1 cDNAs linked to the PECAM promoter. To follow memFlt-1/sFlt-1 expression, PECAM-sFlt-1 or PECAM-memFlt-1 was co-electroporated with PECAM-GFP DNA into ES cells. ES cell clones were selected for stable transfection of these constructs by hygromicin resistance (*hygro*^{R} expressed PECAM-memFlt and PECAM-sFlt DNAs) and GFP expression.

After selection and analysis of a small set of putative memFlt-1 and sFlt-1 transgenics, we obtained only lines that expressed Flt-1, sFlt, and GFP transgenes in embryonic stem cells (Fig. 4B, p.21) and not in *in vitro* differentiated blood vessels. A few ES lines did have very minimal expression of GFP in a subset of vascular structures (Fig 4A, p.21); however, this was not enough to do a careful phenotypic and functional analysis of *flt-1* isoform-specific expression. Thus, I was unable to determine the relative roles of memFlt-1 and sFlt-1 in negatively regulating vascular growth and endothelial cell division. Ongoing efforts in our lab are designed to increase the scale of this transgenic approach in the hopes of obtaining *flt-1* isoform-specific ES cell lines for analysis.

Specific Aim 3: the effect of *flt-1* heterozygosity on mammary tumorigenesis. Our previous data indicates that *flt-1* is a negative regulator of vascular growth during embryogenesis. To test the effects of *flt-1* on pathological neovascularization during mammary tumorigenesis, I proposed to analyze the effects of *flt-1* gene dosage on the growth and metastasis of mammary tumors. More specifically, I proposed to characterize the growth and metastasis of tumors in MMTVPyMT female transgenic mice, which form spontaneous mammary tumors around 8 weeks of age [13]. Analysis of these mice in both wild type and *flt-1* heterozygous genetic backgrounds was designed to test the

effects of reduced *flt-1* expression on mammary tumor progression. Our previous data indicated that *flt-1* expression is reduced by approximately 50% in *flt-1* heterozygotes (Fig. 1C, p.14), indicating that *flt-1* heterozygosity results in decreased flt-1 protein levels. I hypothesized that reduced levels of memFlt-1/sFlt-1 protein would lead to increased mammary tumor vascularization and subsequently increased tumor size and metastasis in the MMTVPyMT transgenic mouse model.

Unfortunately, we were unable to obtain the MMTVPyMT transgenic mice from the lab of Dr. William Muller in a timely manner to complete the proposed experiments, however we were able to obtain MMTVPyMT transgenic mice from the lab of Dr. Terry VanDyke. Thus far, I have performed 3 separate MMTVPyMT hemizygous x *flt-1* heterozygous crosses and obtained 4 *flt-1* heterozygous females and 2 littermate control wild type females carrying the MMTVPyMT transgene as confirmed by PCR genotyping. Thus far, mammary tumors have not been detected in 3-week old *flt-1* heterozygous females carrying the MMTVPyMT transgene or littermate controls. Thus, in this small group of animals, *flt-1* heterozygosity does not appear to drastically increase the speed of mammary tumor incidence; however, these experiments will have to be repeated multiple times to verify these results. Continued analysis of these mice throughout their lifespan will determine any effects of *flt-1* gene dosage on mammary tumor incidence and metastasis. Since I have yet to obtain tumors in these mice, I have not been able to assay for tumor microvessel density or *flt-1* expression in tumor associated blood vessels. Thus, our preliminary results indicate that *flt-1* heterozygosity does not drastically increase tumor formation, however further analysis must be conducted to confirm these results.

Key Research Accomplishments

The following are results generated in this study (see appended manuscript and **Results** section for research details):

- *flt-1* null mutant embryonic stem (ES) cell cultures have a 2.5-fold increased endothelial cell mitotic index on days 6 and 7 of differentiation compared with day-matched wild-type ES cell cultures (Fig. 3, Fig. 4, and Table 1 of appended manuscript Kearney et al., 2002).
- E 8.5 *flt-1* null mutant embryos exhibit a 2-fold increased endothelial cell mitotic index compared to wild-type littermate controls (Fig. 8 and text, appended manuscript).
- Day 8 in vitro differentiated wild type ES cell cultures express both soluble Flt-1 (sFlt-1) and full-length Flt-1 (memFlt-1) RNA (Fig 1A and 1B, p.15), however sFlt-1 protein is expressed at much higher levels than memFlt-1 protein (Fig. 1C, p.15).
- Co-culture of *flt-1* null mutant ES cells with GFP wild type ES cells partially rescues the *flt-1* null mutant vascular phenotype (Fig. 2, p.17).
- Treatment of flt-1 null mutant ES cell cultures with a soluble Flt-1 protein (flt-1/Fc) partially rescues the flt-1 null mutant vascular overgrowth in a dose-dependent manner (Fig. 3, p.19).

Reportable Outcomes

- Publication: Kearney et al. Vascular endothelial growth factor receptor Flt-1 negatively regulates blood vessel formation by modulating endothelial cell division. Blood. 2002. **99**: 2397-407.
- Book chapter: Kearney JB and Bautch VL. In vitro differentiation of mouse ES cells: hematopoietic and vascular development. Methods in Enzymology. In press.
- Submitted paper: Roberts DM, Kearney JB, Johnson JH, Rosenberg MP, Kumar R, and Bautch VL. The VEGF receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. 2003. The American Journal of Pathology. Submitted.
- Poster presentation: "Flt-1 functions to limit endothelial cell numbers *in vitro* via a mitotic inhibition mechanism." July 2000 Gordon Conference on Vascular Cell Biology.
- Poster presentation: "Flt-1 functions to limit endothelial cell numbers *in vitro* via a mitotic inhibition mechanism." July 2001 National Society for Developmental Biology Conference, Seattle, Washington.

- Poster presentation: "Flt-1 negatively regulates blood vessel growth by modulating endothelial cell division." October 2002 DOD-BCRP Era of Hope Meeting, Orlando, Florida
- Degree: Doctor of Philosophy (PhD) in Genetics and Molecular Biology from the University of North Carolina, June 19th, 2003

Conclusions

Our finding that *flt-1* mutant differentiated ES cell cultures have an increased endothelial cell mitotic index indicates that *flt-1* normally functions to repress endothelial cell division. This *flt-1* activity is conserved *in vivo*, as indicated by an increased endothelial cell mitotic index in *flt-1* mutant embryos. From these results we conclude that *flt-1* is an endogenous negative regulator of endothelial cell division, which is a key component of vascular expansion during tumor angiogenesis [1, 14]. The ability of wild type tissue to rescue the *flt-1* mutant vascular phenotype indicates *flt-1* has a cell non-autonomous component that is likely sFlt-1, and western analysis suggests that sFlt-1 is the more abundant of the *flt-1* isoforms indicating that sFlt-1 activity is important for negatively regulating vascular growth.

Our experiments with the VEGF-A antagonist flt-1/Fc indicate that VEGF-A is required for endothelial cell overgrowth in *flt-1* null mutants and suggest that *flt-1* normally suppresses vascular growth by negatively regulating VEGF-A activity. Previous studies have shown that blocking VEGF-A activity with specific antibodies can inhibit tumor growth and metastasis [4, 5]. Thus, manipulating *flt-1* activity during mammary tumor-induced angiogenesis may provide an alternative and more potent means of preventing VEGF-A-dependent tumor recruitment of host blood vessels. This could be achieved by *flt-1* gene delivery to sites of tumor-induced neovascularization or by acute administration of sFlt-1 to sites of tumor growth. In sum, we have identified the

flt-1 gene as a negative regulator of blood vessel growth, and we suggest that this inhibitory activity may be harnessed during mammary tumorigenesis to limit tumor growth and metastasis by negatively regulating blood vessel formation.

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Figure 1. memFlt-1 and sFlt-1 are expressed in day 8 differentiated ES cell cultures. memFlt-1 **(A)** and sFlt-1 **(B)** cDNA were detected by RT-PCR in day 8 differentiated ES cell culture-derived WT and *flt-1* heterozygous RNA lysates using isoform-specific primers. *flt-1* null mutant differentiated ES cell culture RNA exhibits faint PCR products for each isoform. These correspond to non-coding RNA sequences. β-actin product is included as an RNA quality control. **(C)** Western analysis of day 8 protein lysates using an antibody that is specific for the Flt-1 extracellular domain shows expression of the 110 kD sFlt-1 protein in WT and *flt-1* heterozygous differentiated ES cell culture lysates. The 210 kD band corresponding to full-length Flt-1 was not detected in these lysates.

Figure 1

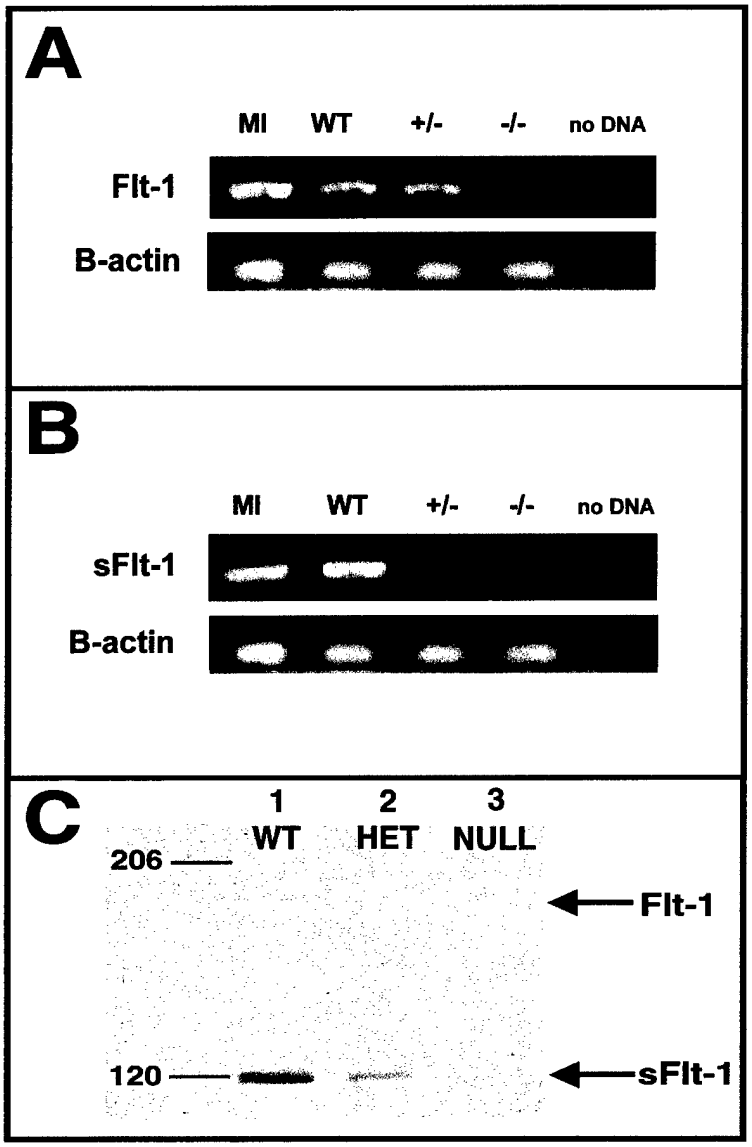
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Figure 2. The *flt-1* null mutant vascular phenotype can be partially rescued by co-culture with WT embryoid bodies. **(A)** β-galactosidase staining (blue) of control day 8 *flt-1* null mutant differentiated ES cell culture at 4X magnification. **(B)** β-galactosidase staining (blue) of day 8 differentiated co-culture plated 75% WT GFP /25% *flt-1* null mutant embryoid bodies at 4X magnification. The *flt-1* null mutant-derived vasculature appears reduced in total stained area and more organized into a vascular plexus compared to control cultures **(A)**. Embryoid bodies were co-cultured from day 3-8, fixed in glutaraldehyde and X-gal stained to detect β-galactosidase expression, which identifies *flt-1* null mutant-derived vasculature.

Figure 2

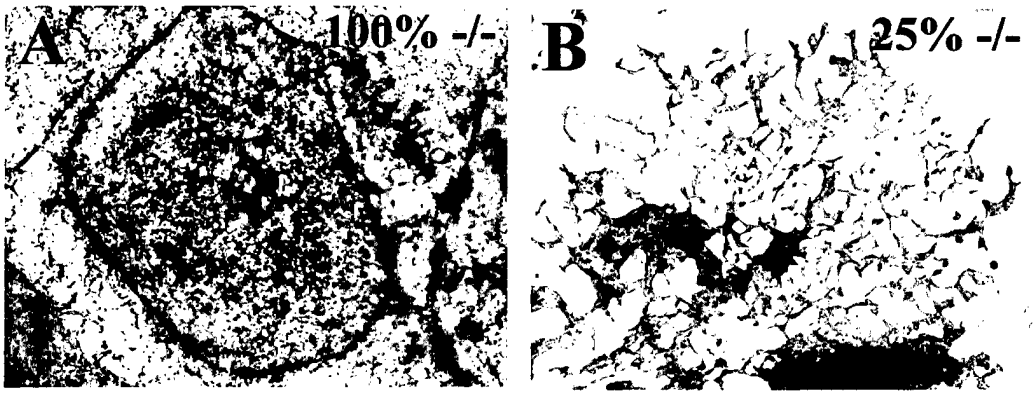

Figure 3. The flt-1 null mutant vascular phenotype can be partially rescued in a dose-dependent manner by treatment with a soluble version of the Flt-1 receptor. PECAM antibody staining of cultures that were untreated **(A)**, or treated with the indicated concentrations of flt-1/Fc protein (R&D Systems) on days 3, 5, and 7 of *in vitro* differentiation **(C-F)**. **(A)** Control *flt-1* null mutant culture displaying the typical vascular overgrowth phenotype. **(C-D)** Treatment with 300 ng/ml generated some areas of reduced vascular growth **(C)**, but areas displaying vascular overgrowth were also observed at this treatment concentration **(D)**. **(E-F)** 500 and 1000 ng/ml treatments significantly reduce vascular area. The graph in **(B)** shows PECAM quantitative image analysis at different flt-1/Fc treatment levels. The 1000 ng/ml treatment (1μg) reduces $PECAM^+$ vascular area to wild type (WT) levels.

Figure 3

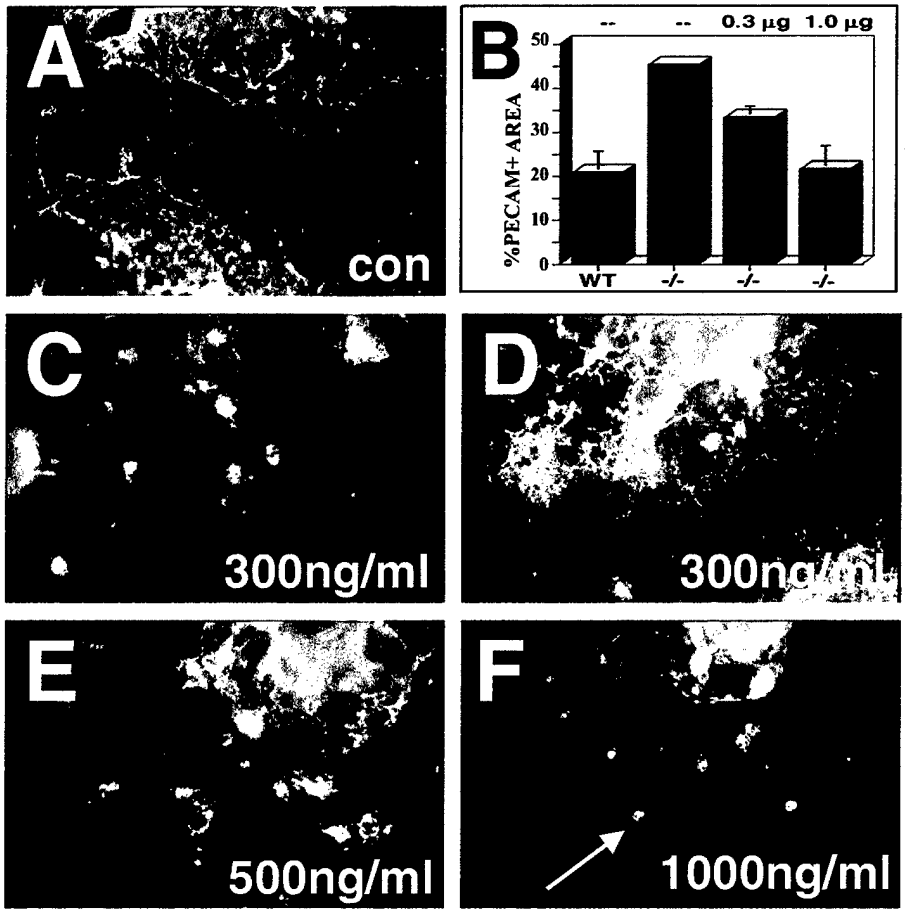
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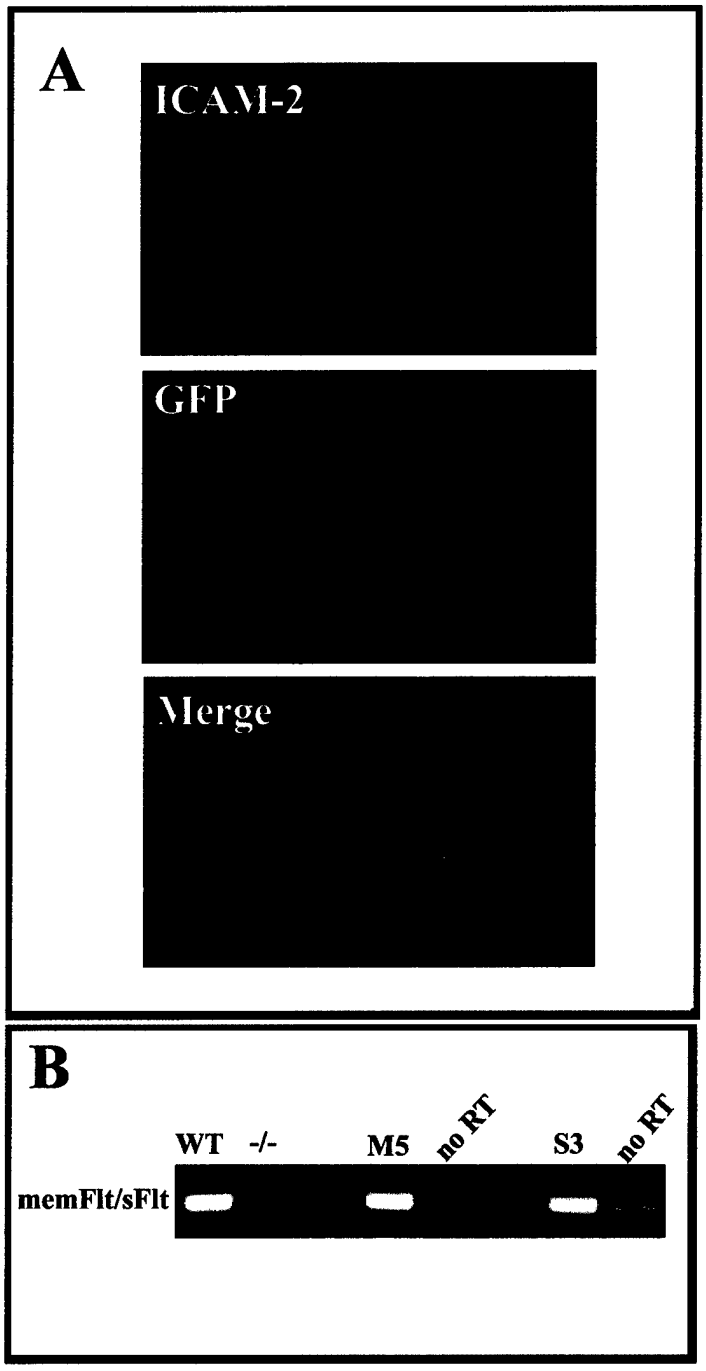
Figure 4. memFlt-1/sFlt-1 transgenic ES cell lines express memFlt/sFlt in ES cells but only in a small subset of vascular endothelial cells upon in vitro differentiation. **(A)** 40X epifluorescent images of a differentiated memFlt-1 transgenic line illustrating a field of ICAM-2^+ blood vessels (red) that express GFP (green) in a subset of their endothelial cells. In this transgenic line GFP expression is a marker for memFlt-1 expression. **(B)** Reverse transcription PCR with primers that recognize both memFlt-1 and sFlt-1 RNA was performed on ES cell RNA from the memFlt transgenic line **(M5)** and the sFlt-1 transgenic line **(S3)**. No RT controls show transgenic DNA contamination. Notice expression of memFlt-1/sFlt-1 in transgenic ES cells compared with control day 8 differentiated wild type RNA **(WT)**. **-/-** indicates day 8 *flt-1* null mutant RNA that serves as a negative control for memFlt-1/sFlt-1 detection. ES cells of both M5 and S3 expressed GFP (data not shown).

Figure 4



Vascular endothelial growth factor receptor Flt-1 negatively regulates developmental blood vessel formation by modulating endothelial cell division

Joseph B. Kearney, Carrie A. Ambler, Kelli-Ann Monaco, Natalie Johnson, Rebecca G. Rapoport, and Victoria L. Bautch

Mice lacking the vascular endothelial growth factor (VEGF) receptor flt-1 die of vascular overgrowth, and we are interested in how flt-1 normally prevents this outcome. Our results support a model whereby aberrant endothelial cell division is the cellular mechanism resulting in vascular overgrowth, and they suggest that VEGF-dependent endothelial cell division is normally finely modulated by flt-1 to produce blood vessels. *Flt-1*$^{-/-}$ embryonic stem cell cultures had a 2-fold increase in endothelial cells by day 8, and the endothelial cell mitotic index was significantly elevated before day 8. *Flt-1* mutant embryos also had an increased endothelial cell mitotic index, indicating that aberrant endothelial cell division occurs in vivo in the absence of flt-1. The *flt-1* mutant vasculature of the cultures was partially rescued by mitomycin C treatment, consistent with a cell division defect in the mutant background. Analysis of cultures at earlier time points showed no significant differences until day 5, when *flt-1* mutant cultures had increased β-galactosidase^{+} cells, indicating that the expansion of flt-1 responsive cells occurs after day 4. Mitomycin C treatment blocked this early expansion, suggesting that aberrant division of angioblasts and/or endothelial cells is a hallmark of the *flt-1* mutant phenotype throughout vascular development. Consistent with this model is the finding that expansion of platelet and endothelial cell adhesion molecule^{+} and VE-cadherin^{+} vascular cells in the *flt-1* mutant background first occurs between day 5 and day 6. Taken together, these data show that flt-1 normally modulates vascular growth by controlling the rate of endothelial cell division both in vitro and in vivo. (Blood. 2002;99:2397-2407)

Introduction

Blood vessels form by coordinating several cellular processes, including cell division and morphogenesis (reviewed in Folkman & D'Amore,[1] Weinstein,[2] and Conway et al[3]). Some of the mitogenic signals that promote division of endothelial cells and their precursors are known, but how these signals are modulated to initiate cell divisions only when and where they are needed is not known in detail. After blood vessels initially form, maturation and remodeling steps involve the recruitment of ancillary cells, such as smooth muscle and pericytes. These cells and the extracellular matrix that is also produced can negatively modulate endothelial cell division.[4-8] However, modulators of endothelial cell mitogenesis at the earliest stages of blood vessel formation have not been identified.

The vascular endothelial growth factor (VEGF) signaling pathway is clearly critical to both early endothelial cell division and morphogenesis, and its regulation is complex (reviewed in Ferrara & Davis-Smyth[9] and Neufeld et al[10]). Mouse embryos lacking even one copy of the VEGF gene die in utero with severe vascular defects, and vascular development in differentiating embryonic stem (ES) cells is compromised in *VEGF-A*$^{+/-}$ and *VEGF-A*$^{-/-}$ ES cells in a dose-dependent manner.[11-13] Moreover, even modestly elevated levels of VEGF lead to vascular abnormalities,[14] and large doses of VEGF invariably severely compromise both vascular development and neovascularization in adult organisms.[15-17] These findings suggest that VEGF signaling must be precisely controlled during vascularization to result in proper vessels. The location and duration of VEGF expression provide the first level of control,[18-21] but other components of the pathway are likely to be involved in fine-tuning the signal.

Two high-affinity receptors, flk-1 and flt-1, participate in VEGF signal transduction and are candidates to be involved in fine-tuning mechanisms. Both receptors are membrane-spanning receptor tyrosine kinases that bind VEGF with high affinity,[22-26] but their effects on VEGF signaling are very different. Mice or ES cells lacking flk-1 have little or no blood vessel formation, suggesting that many downstream effects of VEGF on endothelial cells are mediated through flk-1.[27,28] Specifically, numerous studies show that VEGF signaling through flk-1 produces a strong mitogenic signal for endothelial cells.[29-32]

In contrast, VEGF binding to flt-1 does not produce a strong mitogenic signal, and *flt-1*$^{-/-}$ mice die at mid-gestation with vascular overgrowth and disorganization.[23,29,33] This phenotype was reported to result from increased numbers of cells called hemangioblasts that can give rise to both hematopoietic and endothelial cells.[34] However, invoking control of an early cell fate switch as the exclusive cellular mechanism of flt-1 action is inconsistent with evidence that flt-1 is expressed in mature endothelial cells, including tumor vasculature.[35-37] It is also inconsistent with a molecular model of flt-1 action, suggesting that flt-1 can sequester VEGF ligand and, thus, modulate signaling through

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flk-1, because flk-1 signaling affects multiple endothelial processes, including cell division.[38,39] Moreover, VEGF addition to flt-1–expressing trophoblast cells inhibits cell division, and 2 recent studies using chimeric receptors suggested that flt-1 signaling may counteract the positive mitogenic signal from flk.[40-42]

Thus, we asked if flt-1 could negatively modulate endothelial mitogenesis developmentally, and to address this question we analyzed the cellular mechanism responsible for the *flt-1*$^{-/-}$ phenotype in both ES cell cultures and embryos. The *flt-1* mutant ES cell cultures and embryos had vascular overgrowth that was caused primarily by aberrant endothelial cell division, and this deregulated mitogenesis in the vascular lineage was seen throughout the stages of vascular development. Thus, flt-1 acts early in vascular development to modulate vessel formation by affecting the rate of cell division in embryonic endothelial cells and their precursors.

Materials and methods

Cell culture and in vitro differentiation

Wild type (WT, $^{+/+}$), hemizygous mutant (*flt-1*$^{+/-}$), and homozygous mutant for the targeted *flt-1* mutation (*flt-1*$^{-/-}$)[33] ES cells were maintained and differentiated in vitro as attached cultures as described previously.[43]

For mitomycin C treatment, ES cell cultures were differentiated to day 6, then incubated with mitomycin C (Sigma) at 30 μg/mL diluted in differentiation media for 2 hours at 37°C. After incubation in fresh differentiation medium for 48 hours (to day 8), cultures were fixed and stained with the appropriate antibodies. For earlier times, cultures were incubated with mitomycin C as described earlier on day 4 or day 5, then incubated in fresh medium for 24 hours (to day 5 or day 6) before fixation and staining.

Antibody staining and image analysis

ES cell cultures were rinsed in phosphate-buffered saline (PBS) and fixed for 5 minutes in ice-cold methanol:acetone (50:50) or fresh 4% paraformaldehyde (for VE-cadherin staining). Fixed cultures were reacted with antibodies as described previously.[13,43] In double-labeling experiments, cultures were first incubated with rabbit anti–β-galactosidase or rabbit antiphosphohistone H3 antibodies and the appropriate secondary, then blocked in staining media (3% fetal bovine serum [FBS], 0.1% NaN_3 in PBS) with 5% donkey serum before the addition of rat antimouse platelet and endothelial cell adhesion molecule (PECAM). In triple-labeling experiments, rabbit polyclonal antiphosphohistone H3 incubation was followed by incubation with rat antimouse PECAM and, subsequently, staining with the DNA dye topro-3 (Molecular Probes) at 1:1000 for 5 minutes at room temperature. All cultures were rinsed in PBS and viewed with an Olympus IX-50 inverted microscope by using epifluorescence or a Zeiss LSM 410 confocal microscope.

Primary antibodies and dilutions used were rat antimouse PECAM at 1:1000 (MEC 13.3; Pharmingen); rat antimouse intercellular adhesion molecule 2 (ICAM-2) at 1:500 (3C4; Pharmingen), rabbit polyclonal anti–β-galactosidase at 1:300 (Cappel Labs), rabbit polyclonal antiphosphohistone H3 at 1:500 (Upstate Biotechnology), and rat antimouse VE-cadherin at 1:100 (11D4.1; Pharmingen). Secondary antibodies and dilutions used were donkey antirabbit immunoglobulin G (IgG; H + L) TRITC cross-absorbed at 1:100 (Jackson Immunoresearch) for antiphosphohistone H3 and β-galactosidase, donkey antirat IgG (H + L) B-phycoerythrin cross-absorbed at 1:300 (Jackson Immunoresearch) for PECAM and ICAM-2, donkey antirat IgG (H + L) fluorescein isothiocyanate (FITC) cross-absorbed at 1:100 (Jackson Immunoresearch) for PECAM, and goat antirat IgG (H + L) Alexa 488 cross-absorbed at 1:100 (Molecular Probes) for PECAM and VE-cadherin.

Quantitative image analysis of day 8 ES cell cultures reacted with the appropriate antibodies was performed as previously described.[13] Sequential nonoverlapping areas completely covered with cells were photographed at ×10 magnification, so that the total area photographed per well was more than 60% of the well area. For earlier time points, β-galactosidase–stained wells were photographed, and only areas covered with cells were used for analysis. Digital images were generated and analyzed by using Adobe Photoshop (version 5.0, Adobe Systems). Quantitation of the stained area for each image was performed by using an Image Processing Tool Kit (Rev. 2.1; Reindeer Games, Asheville, NC). Stained area averages for each well were calculated, and the average of 3 to 4 wells for each condition was used to determine SD values.

β-Galactosidase detection

β-Galactosidase detection was performed by using a modified protocol.[44] Cultures were rinsed twice in 0.1 M phosphate buffer (pH 7.3) and fixed with glutaraldehyde fix solution (0.2% glutaraldehyde, 5 mM EGTA [pH 7.3], 2 mM $MgCl_2$ in 0.1 M phosphate buffer [pH 7.3]) for 5 minutes. After washing 3 times for 5 minutes with phosphate buffer, cultures were incubated for 3 hours (day 8 ES cultures) or 5 hours (early time course experiments) at 37°C in X-gal staining solution (0.625 mg/mL X-gal; Sigma), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, in wash buffer (2 mM $MgCl_2$, 0.02% Nonidet-P40 in 0.1 M sodium phosphate buffer [pH 7.3]), then rinsed and stored in wash buffer at 4°C.

RNA analysis

Total RNA was isolated from day 7 ES cell cultures by centrifugation through a CsCl gradient.[45] RNase protection assays for PECAM were performed by using a modified protocol.[13,46] In vitro transcription of PECAM-dCPa (nt 1425-1904) was used to generate a ^{32}P-labeled antisense RNA probe. Overnight hybridization at 45°C with the PECAM probe and a β-actin internal control probe was followed by digestion with RNase A and RNase T1. Protected fragments were then electrophoresed through a 5% acrylamide urea (8 mM gel) and quantified by using a PhosphorImager (Molecular Dynamics).

Fluorescent-activated cell sorter analysis

Day 8 ES cell cultures were rinsed twice with PBS and dissociated with 0.2% collagenase (Sigma; 0.15% type II, 0.05% type XI in PBS) for approximately 2 hours with repeated passage through a 20-gauge needle. The cells were rinsed in FBS/PBS (1:1), resuspended in cold staining media (3% FBS + 0.01% sodium azide in PBS), and incubated on ice for 20 minutes. Cells were then incubated with 100 μg/mL biotin-coupled ICAM-2 antibody in staining medium for 45 minutes at 4°C. After 3 washes with cold staining medium, cells were resuspended in staining medium with 25 μg/mL streptavidin-phycoerythrin (Southern Biologicals) and incubated for 45 minutes at 4°C. After 3 washes with cold PBS, the cells were fixed and stored at 4°C in 1% paraformaldehyde. Flow cytometry data were collected with a Becton Dickinson FACSCAN.

Mitotic index calculations

WT and *flt-1*$^{-/-}$ ES cell cultures were differentiated in chamber slide wells (Nunc) to day 6 or 7, fixed, and triple-labeled with rabbit antiphosphohistone H3, rat antimouse PECAM, and the DNA binding dye topro-3. Slides were mounted in AquaPolymount (LifeSciences). Confocal images were analyzed by using Adobe Photoshop (version 5.0, Adobe Systems) software. Triple-labeled images were counted in the following 4 ways: (1) the total number of cells per field, (2) the total number of phosphohistone H3$^+$ cells per field, (3) the number of PECAM$^+$ cells with endothelial morphology per field, and (4) the number of PECAM$^+$/phosphohistone H3$^+$ cells with endothelial morphology per field. Endothelial mitotic indices were calculated on a per field basis by dividing the number of PECAM$^+$, phosphohistone H3$^+$ cells by the total number of PECAM$^+$ cells. Nonendothelial mitotic indices were also calculated on a per field basis by dividing the number of PECAM$^-$, phosphohistone H3$^+$ cells by the total number of PECAM$^-$ cells. Data were collected from multiple fields of multiple wells and averaged for each day.

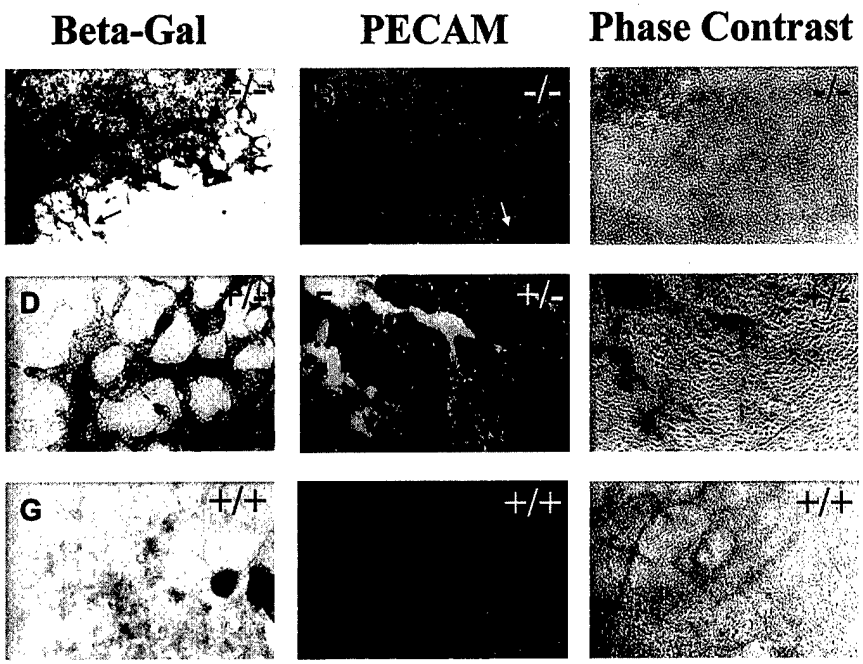

Figure 1. *Flt-1*$^{-/-}$ ES cell cultures have increased vascularization. Day 8 differentiated *flt-1*$^{-/-}$ (A-C), *flt-1*$^{+/-}$ (D-F), and WT (G-I) cultures were processed for β-galactosidase detection (A,D,G) or reacted with an antibody to PECAM (B,E,H). A and B show one quadrant of the relatively large β-galactosidase^{+} (A) or PECAM^{+} (B) sheet of cells that characterizes the *flt-1*$^{-/-}$ phenotype. In contrast, an extensive vascular plexus is found in both *flt-1*$^{+/-}$ (E) and WT (H) ES cell cultures. Arrowheads (A) outline an intensely stained β-galactosidase^{+} ring of cells that surrounds most of the β-galactosidase^{+} cells. Arrows (A,B) point to *flt-1*$^{-/-}$ vasculature that looks WT. (C,F,I) Phase contrast images of PECAM-labeled fields in B,E,H. Magnification is ×10.

Embryo immunohistochemistry

Flt-1$^{+/-}$ mice maintained on the CD-1 background were intercrossed to obtain embryos. Embryos were dissected from the maternal decidua at day 8.5 (the morning of the plug is day 0.5), heads were removed and saved at −20°C for genotyping by using a modification of a published protocol,[33] and the rest of the embryo was fixed in Serra fixative[47] or cold 4% paraformaldehyde at 4°C overnight. The embryos were dehydrated through a methanol series and stored at −20°C in 100% methanol. Embryos were embedded in paraffin, sectioned at 10 μm on a Zeiss Microm, dewaxed in Histoclear, and rehydrated. Sections fixed in paraformaldehyde were incubated in 0.02% Protease XXIV (Sigma) in PBS for 4 minutes, then washed 3 times in PBS. After blocking in 0.25% H_2O_2 in PBS for 15 minutes, primary antibody (1:250 dilution in 5% goat serum/PBS) was added, and sections were incubated overnight at 4°C in humidified chambers. After 3 washes in PBS, secondary antibody (1:300 dilution of goat antirabbit or antirat IgG–horseradish peroxidase [Accurate] in 5% goat serum/PBS) was added, and incubation was overnight as before. After 3 washes in PBS, sections were incubated in 3′-diaminobenzidine tetrahydrochloride substrate to which 3 mg/mL $NiSO_4$ was sometimes added (for blue color) for 15 minutes. Slides were rinsed in PBS, incubated in a 1:10 000 dilution of DAPI (1 mg/mL stock) in H_2O for 10 minutes, mounted using Glycergel (Dako), and visualized with a Nikon Eclipse E800 microscope outfitted with DIC optics and epifluorescence. To count mitotic endothelial nuclei, alternate sections were stained with PECAM and phosphohistone H3. The DAPI-stained nuclei were used to overlay digital images.

Results

Flt-1$^{-/-}$ ES cell cultures have increased vascularization

ES cells undergo a differentiation program in vitro that mimics early murine yolk sac development, including primitive hematopoietic development and blood vessel formation.[43,48-51] Hematoendothelial development begins when a mesodermally derived hemangioblast population arises at days 2 to 3 of differentiation,[52] and angioblasts can also differentiate directly from mesoderm. The endothelial cells of primitive blood vessels are differentiated from angioblasts by coexpression of PECAM and ICAM-2, both adhesion receptors of the immunoglobulin superfamily.[13,53,54] We

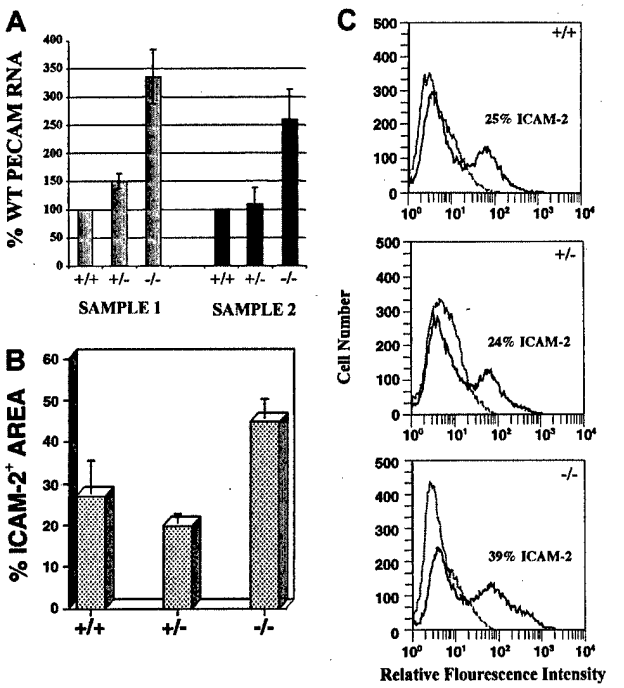

Figure 2. *Flt-1*$^{-/-}$ ES cell cultures have increased numbers of endothelial cells. (A) RNase protection assay using an antisense PECAM RNA probe on day 8 WT, *flt-1*$^{+/-}$, and *flt-1*$^{-/-}$ attached cultures. Protected fragments were separated on a polyacrylamide-urea gel and quantified by using a PhosphorImager. Protected PECAM signal was normalized to a β-actin signal, and the normalized PECAM band densities for *flt-1*$^{+/-}$ and *flt-1*$^{-/-}$ samples were compared with WT ($^{+/+}$) samples. Sample 1 and sample 2 are RNAs from separate differentiations. Each bar is the average of 3 experiments performed on a particular sample. (B) Quantitative image analysis of the ICAM-2^{+} area on day 8 WT ($^{+/+}$), *flt-1*$^{+/-}$, and *flt-1*$^{-/-}$ attached cultures. Each bar represents the average area stained with ICAM-2 antibody from 3 wells. This experiment was repeated (data not shown), and similar quantitative trends were obtained. (C) Fluorescent cell sorting of ICAM-2–labeled day 8 WT ($^{+/+}$), *flt-1*$^{+/-}$, and *flt-1*$^{-/-}$ ES cell cultures. The plots in dotted lines are controls without primary antibody.

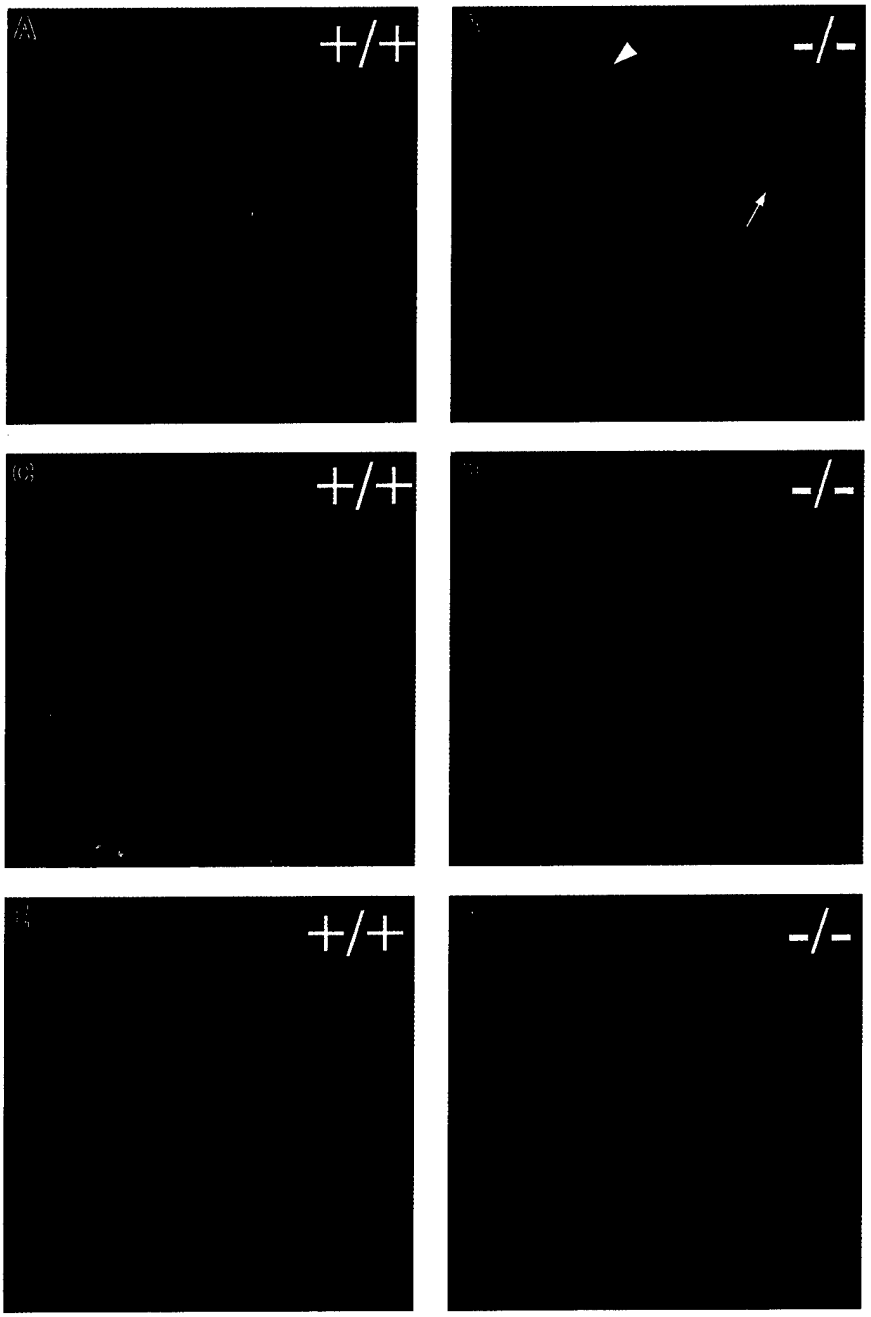

Figure 3. *Flt-1*$^{-/-}$ ES cell cultures have mitotic endothelial cells. Day 7 (A-D) or day 6 (E,F) WT (A,C,E), and *flt-1*$^{-/-}$ (B,D,F) attached cultures were labeled with antibodies to PECAM (green) and phosphohistone H3 (red), then stained with the nuclear marker topro-3 (blue). The arrowhead (B) shows a phosphohistone H3$^+$ nonendothelial cell (PECAM$^-$), whereas the arrow (B) points to a phosphohistone H3$^+$ endothelial cell (PECAM$^+$). Notice the increase in phosphohistone H3$^+$/PECAM$^+$ cells in *flt-1*$^{-/-}$ cultures relative to WT cultures. All panels are confocal images at ×40 magnification.

initially investigated the cellular mechanism of flt-1 in day 8 cultures, when the PECAM$^+$/ICAM-2$^+$ vasculature is well established.

Flt-1$^{+/-}$ and *flt-1*$^{-/-}$ ES cells were engineered so that *Escherichia coli lacZ* is expressed under *flt-1* regulatory control in the targeted gene.[33] These ES cells and WT ($^{+/+}$) controls were stained for β-galactosidase activity or for PECAM expression at day 8 (Figure 1). The *flt-1*$^{-/-}$ cultures had a dramatically expanded β-galactosidase expression domain compared with the *flt-1*$^{+/-}$ cultures (Figure 1A). The β-galactosidase$^+$ cells in the *flt-1*$^{-/-}$ cultures were found in large circular sheets, with areas of normal-looking vasculature at the edge of the sheets (Figure 1A,B, arrow). Immunofluorescent antibody staining for PECAM, ICAM-2, or VE-cadherin showed a similar pattern in the *flt-1*$^{-/-}$ cultures (Figure 1B and data not shown), suggesting that most of the β-galactosidase–expressing cells were endothelial cells. The β-galactosidase– and antibody-stained cells were elongated and interconnected, indicating that they were endothelial cells. This criterion is important, because subsets of hematopoietic cells can also react with the antibodies to PECAM or ICAM-2. Only a ring of intensely β-galactosidase$^+$ cells (Figure 1A, arrowheads) did not appear to stain for PECAM or ICAM-2 by double-label immunofluorescent antibody staining (data not shown). These cells were found in both WT and mutant cultures, and they reacted with a flt-1 antisense RNA probe in the WT background (data not shown), indicating that they are nonvascular flt-1–expressing cells.

The increase in endothelial cells observed in mutant cultures was quantitated in several ways (Figure 2). RNase protection analysis of day 8 cultures with a PECAM antisense RNA probe revealed that PECAM RNA levels were 2.5- to 3.3-fold higher in *flt-1*$^{-/-}$ cultures compared with WT cultures (Figure 2A). Quantitative image analysis on day 8 ICAM-2–labeled cultures used digital

Table 1. Comparison of endothelial and nonendothelial mitotic indices in wild type and *flt-1*$^{-/-}$ embryonic stem cell cultures

	Cell no.*		Mitotic index†		
	Endothelial‡	Nonendothelial§	Endothelial	Nonendothelial	EI/NEI × 100‖
Day 6					
WT Exp 1	1 543	4 006	2.31	1.61	143.5
−/− Exp 1	2 719	2 052	4.02	1.22	329.5
WT Exp 2	1 050	3 060	2.66	1.99	133.7
−/− Exp 2	1 072	2 374	6.14	1.32	465.2
Day 7					
WT Exp 1	3 554	11 303	1.88	1.78	105.6
−/− Exp 1	4 797	7 820	2.83	2.10	134.8
WT Exp 2	1 777	6 498	1.18	1.24	95.2
−/− Exp 2	3 216	4 703	3.25	1.02	318.6

EI, endothelial index; NEI, nonendothelial index; WT, wild type; Exp, experiment.
*Total number of nuclei counted.
†Percentage of replicating cells as determined by labeling with the mitotic marker phosphohistone H3.
‡Number of nuclei with PECAM-labeling along the cell border.
§Nuclei of cells that did not exhibit PECAM-labeling.
‖Formula used to express each endothelial index as a percentage of its companion nonendothelial index.

images of vascular immunofluorescence to determine the percentage area stained, which approximates the amount of vasculature (see "Materials and methods" section for detailed protocols). *Flt-1*$^{-/-}$ cultures exhibited nearly a 2-fold increase in ICAM-2 staining area over WT, whereas *flt-1*$^{+/-}$ cultures had essentially WT levels (Figure 2B). ICAM-2 antibody-stained cultures were also processed for fluorescent-activated cell sorting (FACS; Figure 2C). *Flt-1*$^{-/-}$–attached cultures contained a population of ICAM-2^{+} cells that was significantly increased over WT levels (compare 39% with 25%, respectively), whereas *flt-1*$^{+/-}$ cultures had WT numbers of ICAM-2^{+} cells. Similar FACS results were obtained with antibodies to PECAM (data not shown). Taken together, these data show that the lack of flt-1 results in increased numbers of vascular endothelial cells.

Lack of flt-1 leads to increased endothelial cell division

To investigate the cellular mechanism(s) responsible for the increased vascularization seen in the absence of flt-1, the hypothesis that *flt-1*$^{-/-}$ endothelial cells have a higher rate of cell division than WT endothelial cells was tested. Day 6 and day 7 ES cell cultures were labeled with antibodies to the vascular marker PECAM and to the mitotic marker phosphohistone H3,[55] then stained with a DNA-binding dye (topro-3; Figure 3). Visual observation suggested that day 6 and day 7 *flt-1*$^{-/-}$ ES cell cultures had more PECAM^{+} cells that colabeled with the antiphosphohistone H3 antibody than WT controls (compare Figure 3A,C with B,D and E with F).

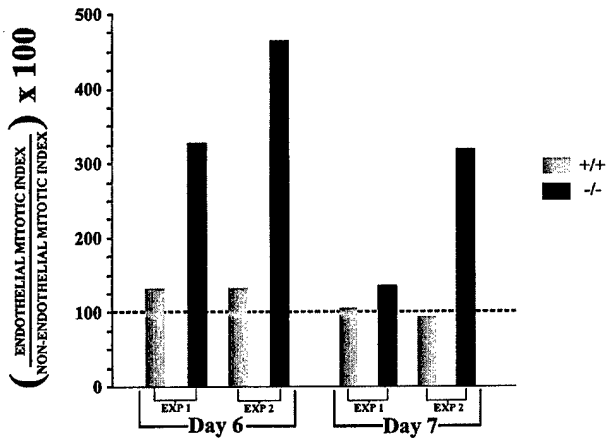

Figure 4. *Flt-1*$^{-/-}$ ES cell cultures have an elevated endothelial cell mitotic index. Days 6 and 7 WT ($^{+/+}$) and *flt-1*$^{-/-}$ triple-labeled images were used to calculate nonendothelial cell and endothelial cell mitotic indices for 2 separate differentiation experiments (Table 1). Endothelial cell mitotic indices were expressed as a percentage of the nonendothelial cell mitotic index calculated for each experimental condition. The dotted black line represents the nonendothelial mitotic index for each experiment converted to 100%, and it was used as a baseline for comparison of endothelial mitotic indices.

To quantitate the apparent increase in mitotic PECAM^{+} cells in *flt-1*$^{-/-}$ cultures, confocal images from day 6 or day 7 fixed cultures processed as in Figure 3 were used to calculate cell counts and mitotic indices for both endothelial and nonendothelial cell populations (Table 1 and Figure 4; see "Materials and methods" section for details). In all cases endothelial cells of the *flt-1*$^{-/-}$ cultures had a higher mitotic index than WT endothelial cells. To control for differential growth rates, a nonendothelial cell mitotic index was obtained for each experiment (Table 1). There was little difference between WT and *flt-1*$^{-/-}$ nonendothelial cell mitotic indices within a given experiment, in contrast to increases in the *flt-1*$^{-/-}$ endothelial cell mitotic index. Each endothelial cell mitotic index was normalized to its companion nonendothelial cell mitotic index (Figure 4; Table 1, far right column). Day 6 *flt-1*$^{-/-}$ cultures had normalized endothelial cell mitotic indices that were 3- to 4-fold higher than normal, and similar but less dramatic trends were observed in day 7 cultures (Figure 4, compare black bars with gray bars). These results indicate that the increased vascularization seen in day 8 *flt-1*$^{-/-}$ ES cell cultures is caused, at least in part, by an increased endothelial cell division rate in the absence of flt-1.

If aberrant endothelial cell division contributes to the *flt-1* mutant phenotype, then blocking cell division during ES cell differentiation may affect the phenotype. Thus, day 6 ES cell cultures were treated with the replication inhibitor mitomycin C before incubation for an additional 2 days (Figure 5). Untreated *flt-1*$^{-/-}$ cultures fixed on day 6 had slightly increased numbers of PECAM^{+} cells compared with day 6 WT cultures (compare Figure 5A with B). Treated day 8 *flt-1*$^{-/-}$ had half as much vasculature as untreated genotype-matched controls, accompanied by a dramatic decrease in the labeling of nuclei with antiphosphohistone H3 (compare Figure 5D with F,H). In some cases, mitomycin C-treated *flt-1*$^{-/-}$ vasculature at day 8 was branched and appeared WT in morphology (Figure 5G), suggesting that blocking cell division during days 6 to 8 of differentiation can partially compensate for the lack of flt-1 in vascular development. Mitomycin C treatment also affected vascular growth in WT cultures, which is predicted

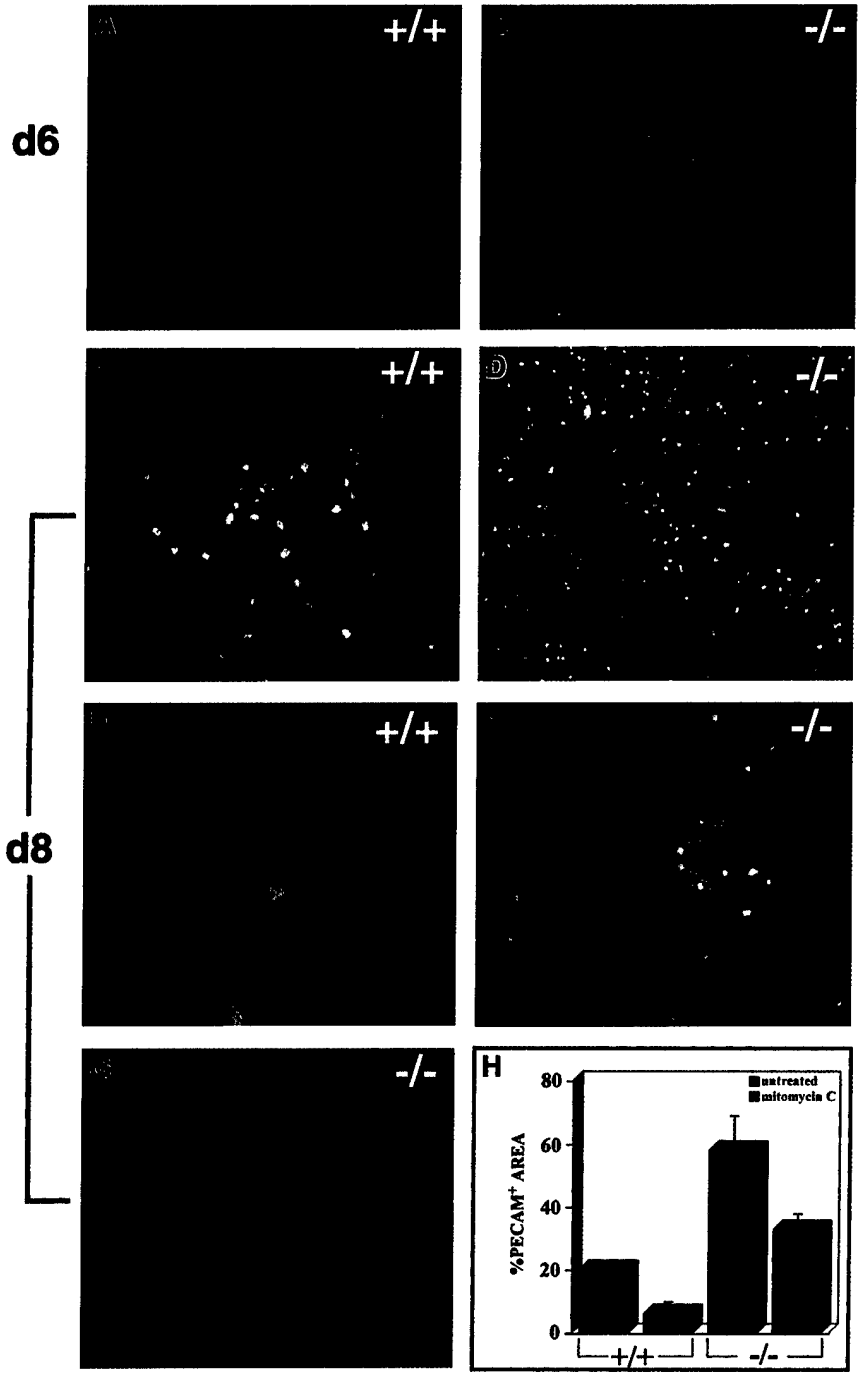

Figure 5. Mitomycin C treatment partially rescues the *flt-1*$^{-/-}$ vascular phenotype. Day 6 ES cell cultures were fixed (A,B), left untreated (C,D), or treated with mitomycin C (E-G). Some cultures (C-G) were differentiated for an additional 48 hours. Cultures were labeled with an antibody to PECAM (green), and some cultures (C-F) were also labeled with the mitotic marker antiphosphohistone H3 (red). Notice the abundance of phosphohistone H3–labeled figures in untreated (C-D) cultures compared with treated (E-F) cultures. (G) Example of a treated *flt-1*$^{-/-}$ culture that morphologically resembled WT vasculature. (H) Quantitative image analysis of the PECAM$^+$ area of day 8 WT ($^{+/+}$) and *flt-1*$^{-/-}$ ($^{-/-}$) cultures treated with mitomycin C (red) or left untreated (green). Each bar represents the average stained area from at least 3 wells stained with PECAM antibody. Magnification was ×10 except C (×20) and G (×4).

because blood vessel formation requires endothelial cell division. The treated WT cultures had 2- to 3-fold less vasculature and less branching than untreated controls (compare Figure 5C with E,H). Thus, treatment with mitomycin C, an inhibitor of replication, partially rescues the *flt-1* mutant vascular phenotype.

Flt-1 mutation affects division of vascular precursor cells

To determine when the flt-1 mutation first affects vascular development, we investigated earlier time points of ES cell differentiation. To establish when cells expressing lacZ under control of the flt-1 promoter were first affected by the lack of flt-1 protein, we analyzed an early time course of ES cell differentiation. We plated cells directly after dispase treatment, then processed wells of each genotype for lacZ expression on days 2 to 6 of differentiation (Figure 6). The percentage of lacZ-expressing cells was equivalent between *flt-1*$^{+/-}$ and *flt-1*$^{-/-}$ cultures on days 2 to 4, and only on day 5 was there a significant increase in the percentage of lacZ-expressing cells in the flt-1 mutant background (Figure 6A). To determine if this expansion was the result of aberrant cell division, wells were treated with mitomycin C on day 4 or day 5, then compared with control untreated wells 24 hours later. Day 5 *flt-1*$^{-/-}$ mutant cultures treated with mitomycin C 24 hours earlier had fewer lacZ-expressing cells than paired untreated controls (compare Figure 6C with D). The day 5 mitomycin C-treated wells were, in fact, similar to untreated wells fixed at day 4 (compare Figure 6B with C). Day 6 *flt-1*$^{-/-}$ mutant cultures treated with

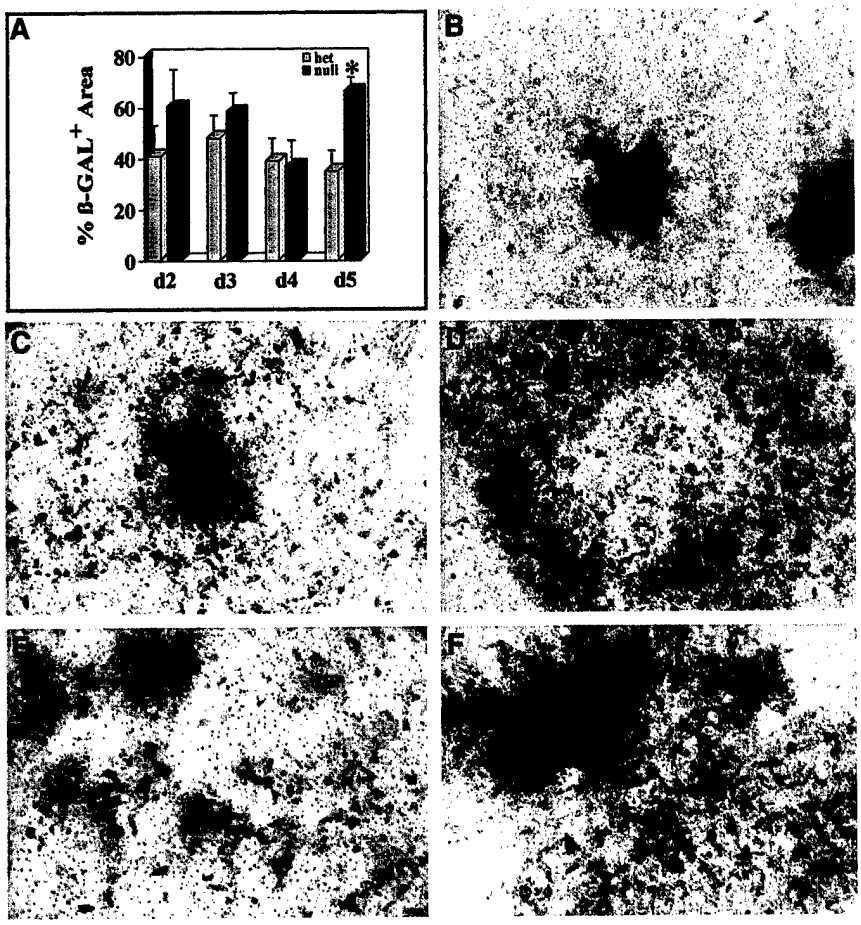

Figure 6. Mitomycin C–sensitive expansion of β-galactosidase–expressing cells in *flt-1*$^{-/-}$ ES cell cultures at earlier times. (A) Quantitative image analysis of the β-galactosidase$^+$ areas of *flt-1*$^{+/-}$ (light blue bars) and *flt-1*$^{-/-}$ (dark blue bars) ES cell cultures on days 2 to 5 of in vitro differentiation. For days 2 and 3, the bars represent the average β-galactosidase$^+$ area for 9 individual attached ES cell clumps. For days 4 and 5, the bars represent the average β-galactosidase$^+$ area for 2 culture wells. The asterisk (*) indicates significance at $P < .001$. (B-F) Days 4 to 6 *flt-1*$^{-/-}$ ES cell cultures untreated (B,D,F) or treated with mitomycin C (C,E) and stained for β-galactosidase activity. (B) Day 4 *flt-1*$^{+/-}$ culture. (C) Day 5 *flt-1*$^{-/-}$ culture treated on day 4 with mitomycin C. Note decrease in stained area relative to (D) untreated day 5 *flt-1*$^{-/-}$ culture. (E) Day 6 *flt-1*$^{-/-}$ culture treated on day 5 with mitomycin C. Note decrease in stained area relative to (F) untreated day 6 *flt-1*$^{-/-}$ culture. Original magnification, ×20.

mitomycin C 24 hours earlier also had fewer lacZ-expressing cells than paired untreated controls (compare Figure 6E with F). These results show that the earliest expansion of lacZ-expressing cells in the *flt-1*$^{-/-}$ mutant cultures can be inhibited by mitomycin C, suggesting that the expansion results from aberrant cell division.

Because both endothelial cells and a nonendothelial cell population express flt-1 promoter-driven β-galactosidase, we investigated the expression of several vascular markers in the ES cell cultures. Cultures were stained with PECAM or VE-cadherin from days 2 to 6 of differentiation (Figure 7), because both markers are expressed early in vascular development. PECAM was expressed throughout the time course, but before day 5 only clumps of PECAM$^+$ cells were seen, and no significant differences were seen among the different genotypes (data not shown). By day 5 both WT and *flt-1*$^{+/-}$ cultures had some areas of PECAM$^+$ vasculature, but surprisingly the *flt-1*$^{-/-}$ mutant day 5 cultures had few PECAM$^+$ cells and most were still in clumps (Figure 7A-C). By day 6 all cultures had PECAM$^+$ vasculature, and the *flt-1*$^{-/-}$ mutant cultures had as much or more PECAM$^+$ vessels compared with WT or *flt-1*$^{+/-}$ cultures (Figure 7G-I). Treatment of *flt-1*$^{-/-}$ cultures from days 5 to 6 with mitomycin C reduced the number of PECAM$^+$ cells (data not shown). VE-cadherin$^+$ cells were not seen in any cultures until day 5 (data not shown). Similar to the PECAM pattern, on day 5 WT and *flt-1*$^{+/-}$ cultures had VE-cadherin$^+$ vasculature, whereas the *flt-1*$^{-/-}$ mutant cultures had only a few VE-cadherin$^+$ cells that were not organized into vessels (Figure 7D-F). By day 6 cultures of all genotypes had VE-cadherin$^+$ vessels (Figure 7J-L). These results show that *flt-1*$^{-/-}$ mutant cultures did not have expansion of either PECAM$^+$ or VE-cadherin$^+$ vascular cells until between days 5 and 6 of differentiation, when expansion of both β-galactosidase–expressing cells and PECAM-expressing cells was sensitive to mitomycin C.

Flt-1$^{-/-}$ embryos have increased mitoses

To determine if the aberrant endothelial cell division seen in the absence of flt-1 during ES cell differentiation also occurred in vivo, day 8.5 embryos were stained with the antiphosphohistone H3 antibody (Figure 8). The *flt-1*$^{-/-}$ mutant embryos had numerous mitotic nuclei in several vascular areas, including the lining of yolk sac blood islands (Figure 8B,C,E,F) and the allantois (Figure 8F). In contrast, nonmutant embryos had far fewer mitotic nuclei in those areas (Figure 8A,D). The increase in mitotic nuclei was specific to vascular areas in vivo, because embryonic structures such as the neural tube and somites had roughly equivalent numbers of mitotic nuclei regardless of the genetic background (data not shown). Digital overlays of alternate sections stained with PECAM and phosphohistone H3 (Figure 8D-F) were used to calculate the endothelial mitotic indices in vivo. The endothelial mitotic index of flt-1$^{-/-}$ embryos was 2.8% (n = 1270), double that of WT$^{+/+}$ embryos whose endothelial mitotic index was 1.4% (n = 425). Thus, the aberrant endothelial cell division documented during ES cell differentiation in the absence of flt-1 is also a hallmark of the mutant phenotype in vivo.

Discussion

Our data support a model whereby flt-1 normally affects early vascular development by negatively modulating cell division in the

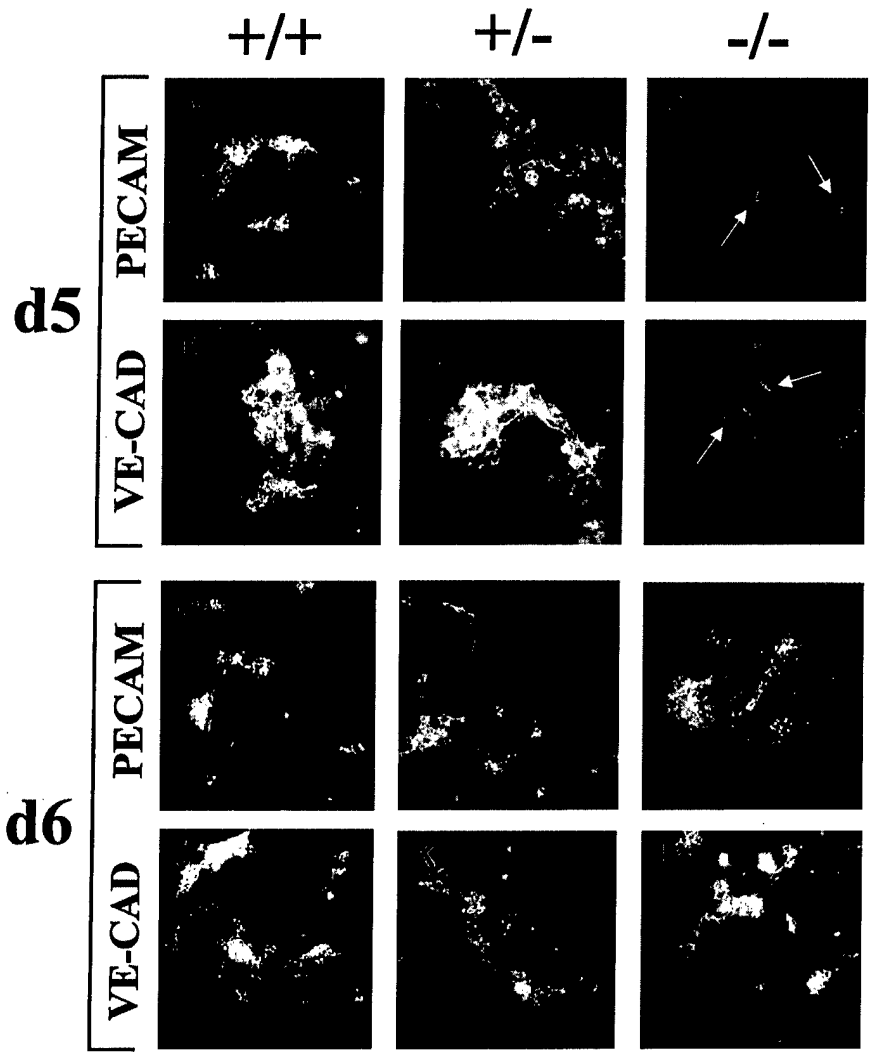

Figure 7. Expression of vascular markers in differentiating ES cell cultures. Wt ($^{+/+}$) (A,D,G,J), *flt-1*$^{+/-}$ (B,E,H,K), and *flt-1*$^{-/-}$ (C,F,I,L) ES cell cultures were fixed on day 5 (A-F) or day 6 (G-L) and labeled with antibodies to PECAM (A-C,G-I) or VE-cadherin (D-F,J-L), and the appropriate fluorescent-labeled secondary antibody. Arrows (C,F) point to sparse PECAM$^+$ and VE-cadherin$^+$ cells in day 5 *flt-1*$^{-/-}$ cultures. Original magnifications ×20, except D-F at ×40.

vascular lineage. The identification of this cellular mechanism of flt-1 action suggests that flt-1 is critical for the fine tuning of VEGF-mediated vessel growth that is required to form proper blood vessels. It also strongly suggests that flt-1 may affect blood vessel formation in similar ways in both the embryo and the adult. Embryos and differentiated ES cells lacking flt-1 have increased vascularization and numbers of endothelial cells accompanied by an increased endothelial cell mitotic index. In contrast, the nonendothelial cell mitotic index is similar in both genetic backgrounds, indicating that the increased mitotic rate in the *flt-1*$^{-/-}$ background is endothelial cell specific.

The ability of mitomycin C to partially rescue the *flt-1*$^{-/-}$ vascular phenotype further supports the conclusion that deregulated endothelial cell division is responsible for the *flt-1* mutant phenotype. The WT cultures were also affected, which was expected because endothelial cell division is a critical component of normal blood vessel formation.[56] A caveat is that mitomycin C inhibits division in all cells, so lack of division in nonendothelial cells could indirectly affect the endothelial cell phenotype. This scenario cannot be ruled out, but the increased endothelial mitotic index in the *flt-1* mutant background and its diminution with mitomycin C suggest that a substantial part of the rescue is likely to result from direct effects on endothelial cell division. This model can be more precisely tested by expressing genes that modulate cell division under the control of endothelial-specific regulatory sequences in the mutant ES cells.

Flt-1 modulates cell division in the vascular lineage at the earliest stages of vascular development. The first documented difference in ES cell cultures was at day 5, when *flt-1*$^{-/-}$ mutant cultures had more cells expressing β-galactosidase under control of the flt-1 promoter than *flt-1*$^{+/-}$ cultures. The exact identity of these cells is unclear because we have identified a nonvascular, flt-1–expressing cell population in ES cell cultures, and several cell types such as trophoblasts and monocyte/macrophages express flt-1 in vivo.[40,57-59] However, because endothelial cells also express flt-1, it is likely that at least a subpopulation of these cells are vascular precursor cells. In any case, the expansion of β-galactosidase–expressing cells in the *flt-1*$^{-/-}$ mutant background could be blocked by mitomycin C from days 4 to 5 onward, indicating that the expansion of this cell population resulted from aberrant cell division. Interestingly, vascular cells expressing PECAM and/or VE-cadherin were much less prevalent in the *flt-1*$^{-/-}$ mutant cultures on day 5, suggesting that different subpopulations of vascular precursor cells may be affected by the flt-1 mutation at different times. The expansion of PECAM$^+$ and/or VE-cadherin$^+$ vascular cells was not evident until day 6 in the *flt-1*$^{-/-}$ mutant background, and this expansion was also blocked by mitomycin C. Thus, flt-1 has a major role in modulating cell division in the

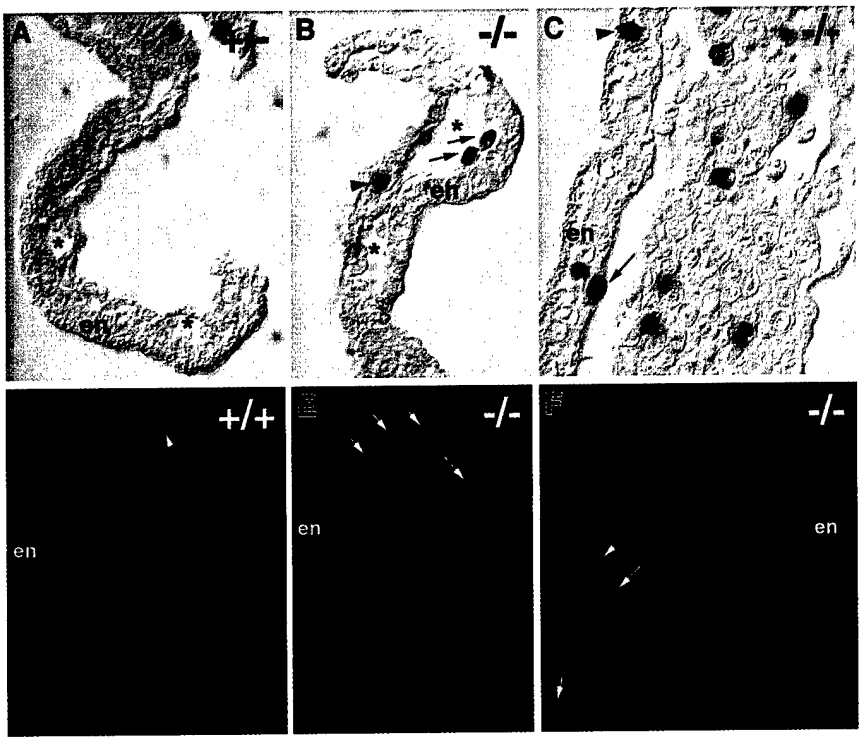

Figure 8. Flt-1 $^{-/-}$ embryos have increased mitoses in endothelial cells. Transverse sections of day 8.5 embryos were processed for immunohistochemistry by using antiphosphohistone H3 to detect mitotic nuclei (A-C), and overlays of adjacent sections were processed individually (see "Materials and methods" section) for immunohistochemistry with antiphospohistone H3 (red), anti-PECAM (green), and DAPI (blue) (D-F). Visualization of yolk sacs of *flt-1*$^{+/-}$ (A) or WT ($^{+/+}$) (D) embryos that were phenotypically normal showed few mitotic nuclei in vascular areas (arrow in D). In contrast, *flt-1*$^{-/-}$ embryos (B,C,E,F) exhibited vascular overgrowth and numerous mitotic nuclei (red; E,F) in PECAM^{+} regions (green; E,F) of the yolk sac and allantois (F, left part of panel). (A-C) Asterisks denote the lumina of blood islands in the yolk sac, and arrows point to mitotic nuclei abutting the endoderm with the long axis perpendicular to the long axis of the endoderm cells, a characteristic of dividing endothelial cells. In contrast, the arrowhead in C points to a mitotic nucleus in the endoderm with the long axis parallel to the long axis of the endoderm cells, a characteristic of dividing endoderm. The arrowhead in B points to a mitotic nucleus of unknown cell type. (D-F) Arrows point to mitotic nuclei of PECAM^{+} cells. En, visceral endoderm of the yolk sac.

vascular lineage starting at days 4 to 5 of ES cell differentiation, just before formation of the first primitive blood vessels.

Other processes can also affect the number of endothelial cells, including cell fate decisions and programmed cell death. Appreciable endothelial cell death is not observed during days 5 to 8 of normal ES cell differentiation (V.L.B., unpublished observation), so inhibition of apoptosis is unlikely to make a major contribution to the flt-1 mutant phenotype. Our results do not formally exclude that, in addition to an effect on vascular cell division, flt-1 may alter cell fate by affecting hemangioblast formation,[34] but our results are not consistent with this model. We see no significant differences between normal and mutant cultures until day 5, well beyond the peak of hemangioblast formation at days 2.5 to 3.0.[52] In the hemangioblast study, increased PECAM and β-galactosidase staining during differentiation of *flt-1*$^{-/-}$ EBs was interpreted as increased hemangioblast numbers, but the lack of a definitive hemangioblast marker makes it impossible to distinguish between hemangioblasts, angioblasts, and differentiated endothelial cells using these criteria. Moreover, in our hands the expansion of the vascular lineage was blocked by mitomycin C at its earliest detection on days 4 to 5, suggesting that the major effect of the flt-1 mutation on vascular growth results from aberrant cell division.

The identification of flt-1 as an early modulator of cell division in vascular development is consistent with several elegant studies showing that flt-1 affects endothelial cell mitogenesis in cultured endothelial cells.[31,41,42] Extending this model of flt-1 action to the earliest stages of development has several implications. First, it suggests that deregulation of proliferation can be sufficient to disrupt developmental processes. Other recent investigations of the role of the cell cycle in development support this hypothesis.[60] Second, the data suggest that flt-1 can modulate the endothelial cell cycle developmentally by affecting one or more molecular signaling pathways, although which pathways are affected is not entirely clear. Deletion of the flt-1 tyrosine kinase domain does not disrupt vascular development,[58] suggesting that signaling through this domain is not necessary for flt-1 to affect the endothelial cell cycle developmentally. Signaling through flk-1 does produce a strong endothelial mitogenic signal, and flk-1 selective inhibitors partially rescue the *flt-1*$^{-/-}$ phenotype in ES cell cultures (D. Roberts and V.L.B., unpublished results). This finding suggests that flt-1 affects vascular development at least in part by modulating VEGF-mediated flk-1 signaling, and this modulation could occur in several ways.

A soluble form of flt-1, sflt-1, is expressed during development[61] and ES cell differentiation (J.B.K. and V.L.B., unpublished results), and it can inhibit VEGF-dependent endothelial cell division.[38,39] Thus, sflt-1 can bind VEGF and prevent ligand-induced dimerization of the flk-1 receptor. The full-length receptor can also theoretically form an inactive heterodimer with flk-1, as suggested by a recent study using chimeric receptors.[41] In addition, ligand engagement of flt-1 may modulate flk-1 signaling at points downstream in the signal transduction pathway. This model is supported by the inhibitor sensitivity of chimeric receptors and a study implicating nitric oxide as a mediator of flt-1 effects on the flk-1 mitogenic pathway.[42,62] Importantly, these models of flt-1 action are not mutually exclusive, and it is likely that flt-1 uses some combination of these actions to modulate endothelial cell division developmentally. The identification of the cellular mechanism of flt-1 action suggests ways to test these molecular models.

Flt-1$^{-/-}$ mutant embryos had increased mitoses in areas rich in endothelial cells and an increased mitotic index as well, indicating that aberrant endothelial cell division contributes to the mutant phenotype in vivo. To our knowledge this is the first demonstration that flt-1 affects endothelial cell division in vivo. The ability of flt-1 to negatively modulate endothelial cell division in vivo indicates that it is an endogenous negative regulator of blood vessel formation. Our data show that flt-1 regulates vascular growth from the earliest stages of vascular development, and it is likely to modulate angiogenesis in the adult organism by a similar cellular mechanism. Several molecules, such as angiostatin and endostatin, negatively regulate pathologic blood vessel formation when administered exogenously, and some of these regulators are likely to

control pathologic vascularization by endogenous production.[63-65] However, these angiogenesis inhibitors have surprisingly little effect on normal blood vessel formation. Clearly, our knowledge of how blood vessel formation is negatively regulated is sparse compared with what is known of positive regulation.

VEGF expression is up-regulated in many pathologies with vascular components, such as cancer and chronic inflammation.[66-70] Thus, flt-1 could potentially negatively modulate pathologic vascularization, as described here for vascular development, and therapeutics that specifically block flt-1 action may help rather than hinder pathologic vascularization. Conversely, VEGF treatment can in some cases promote vascularization of ischemic limbs,[71,72] but our lack of understanding about how VEGF signaling is normally exquisitely fine-tuned has hampered our ability to produce functional vessels therapeutically. Flt-1 clearly participates in the modulation of VEGF-mediated vascular growth, and understanding the role of flt-1 in controlling this process should help in designing better therapies. In any case, defining the cellular mechanism of flt-1 action in endothelial cells developmentally suggests alternative ways to modulate blood vessel formation in vivo.

Acknowledgments

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