

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

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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 hall-

mark 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)

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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 al³). 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-Smyth⁹ and Neufeld et al¹⁰). 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% NaN₃ 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 MgCl₂ 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 MgCl₂, 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 ³²P-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).

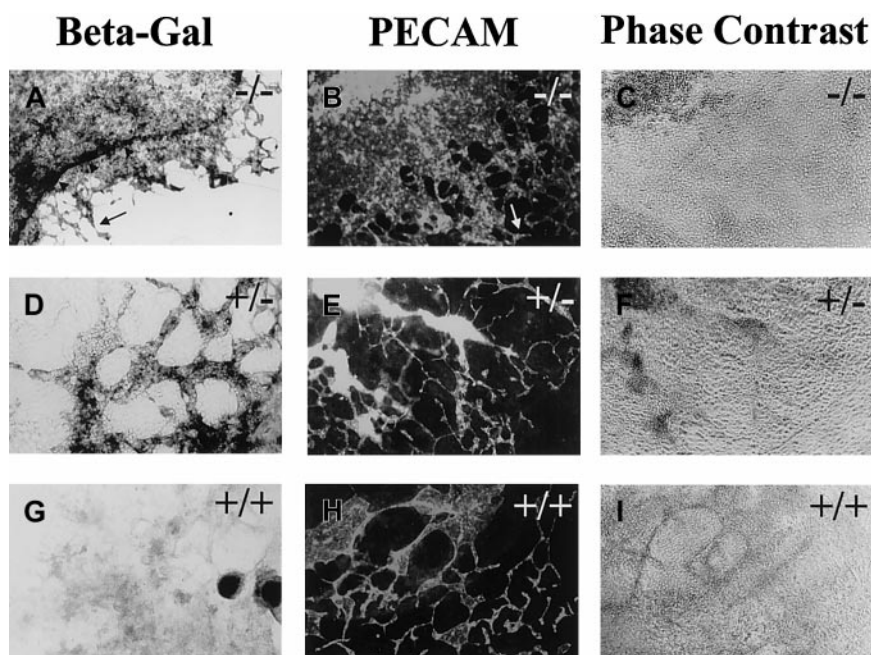
Fluorescent-activated cell sorter analysis

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 fixative⁴⁷ 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% H₂O₂ 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-horse radish 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 NiSO₄ 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 H₂O 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} Hemoendothelial development begins when a mesodermally derived hemaangioblast 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

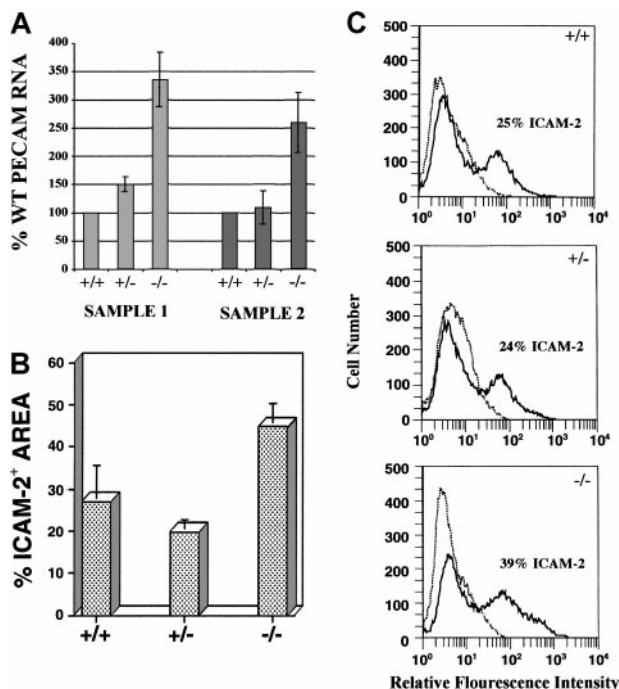


Figure 2. *Flt-1*^{-/-} ES cell cultures have increased numbers of endothelial cells. (A) RNase protection assay using an antisense PECAM RNA probe on day 8 WT, *flt-1*^{+/-}, and *flt-1*^{-/-} attached cultures. Protected fragments were separated on a polyacrylamide-urea gel and quantified by using 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.

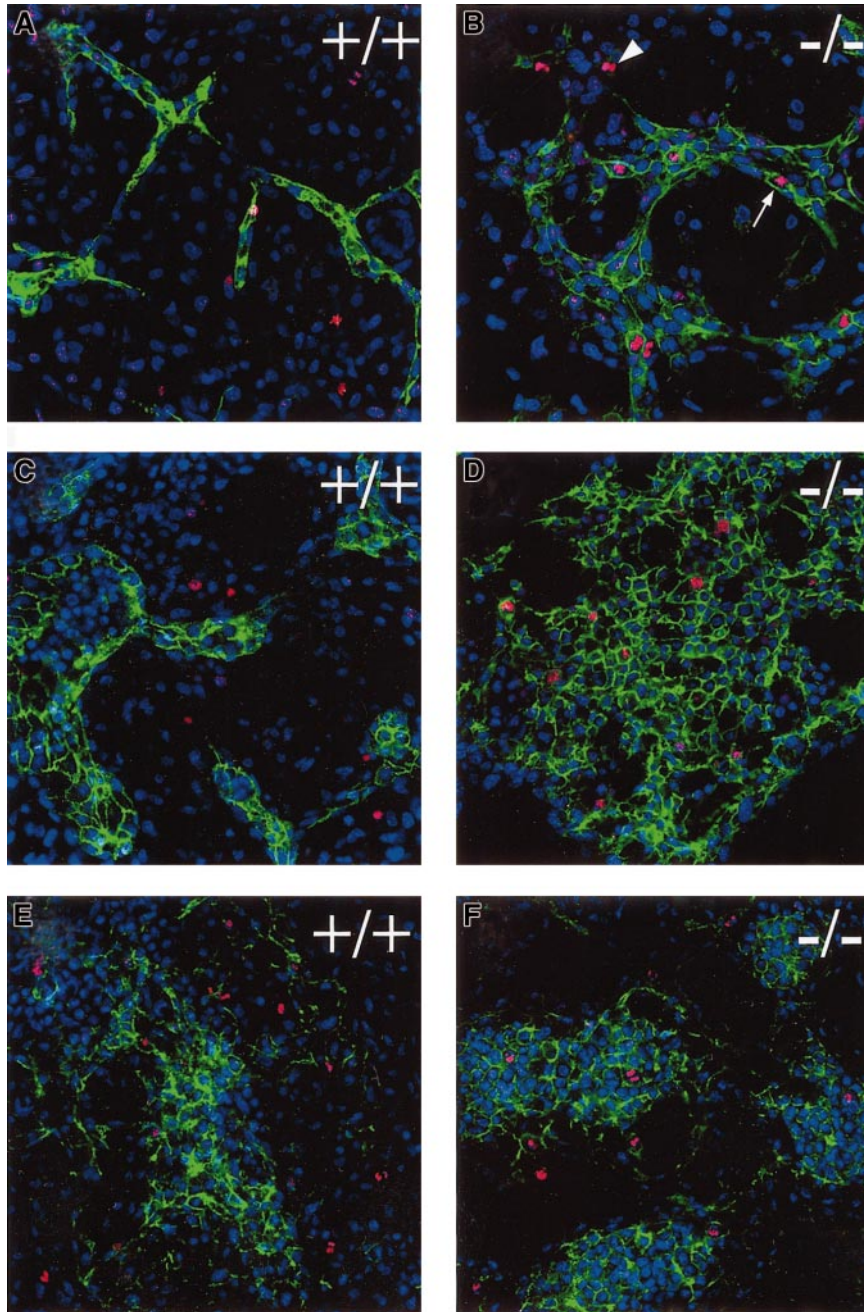


Figure 3. *Flt-1*^{-/-} ES cell cultures have mitotic endothelial cells. Day 7 (A-D) or day 6 (E,F) WT (A,C,E), and *flt-1*^{-/-} (B,D,F) attached cultures were labeled with antibodies to PECAM (green) and phosphohistone H3 (red), then stained with the nuclear marker topro-3 (blue). The arrowhead (B) 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 $\times 40$ 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 -ga-

lactosidase- 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†		EI/NEI × 100
	Endothelial‡	Nonendothelial§	Endothelial	Nonendothelial	
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).

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

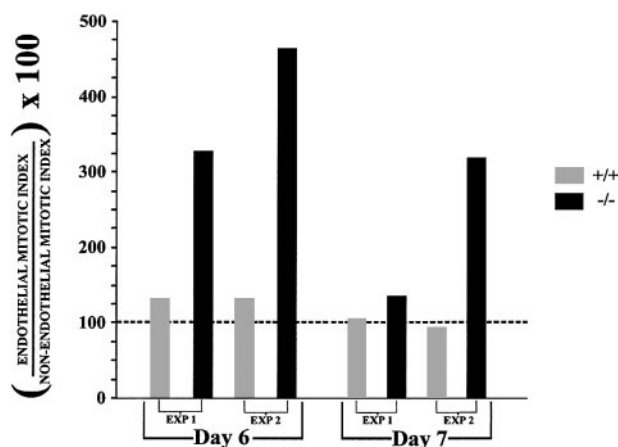


Figure 4. *Flt-1*^{-/-} ES cell cultures have an elevated endothelial cell mitotic index. Days 6 and 7 WT (+/+) and *flt-1*^{-/-} triple-labeled images were used to calculate nonendothelial cell and endothelial cell mitotic indices for 2 separate differentiation experiments (Table 1). Endothelial cell 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.

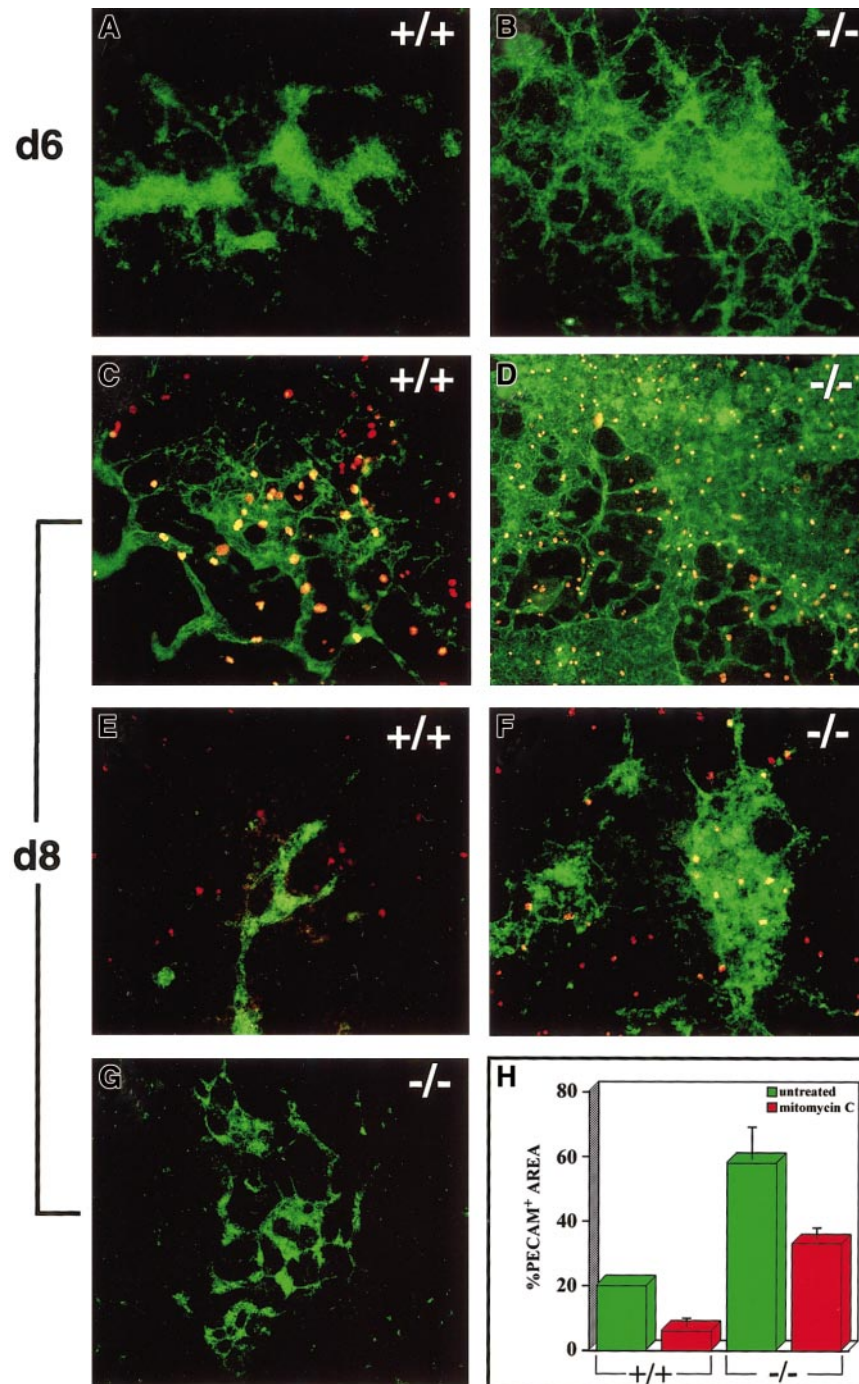


Figure 5. Mitomycin C treatment partially rescues the *flt-1*^{-/-} vascular phenotype. Day 6 ES cell cultures were fixed (A,B), left untreated (C,D), or treated with mitomycin C (E-G). Some cultures (C-G) were differentiated for an additional 48 hours. Cultures were labeled with an antibody to PECAM (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 $\times 10$ except C ($\times 20$) and G ($\times 4$).

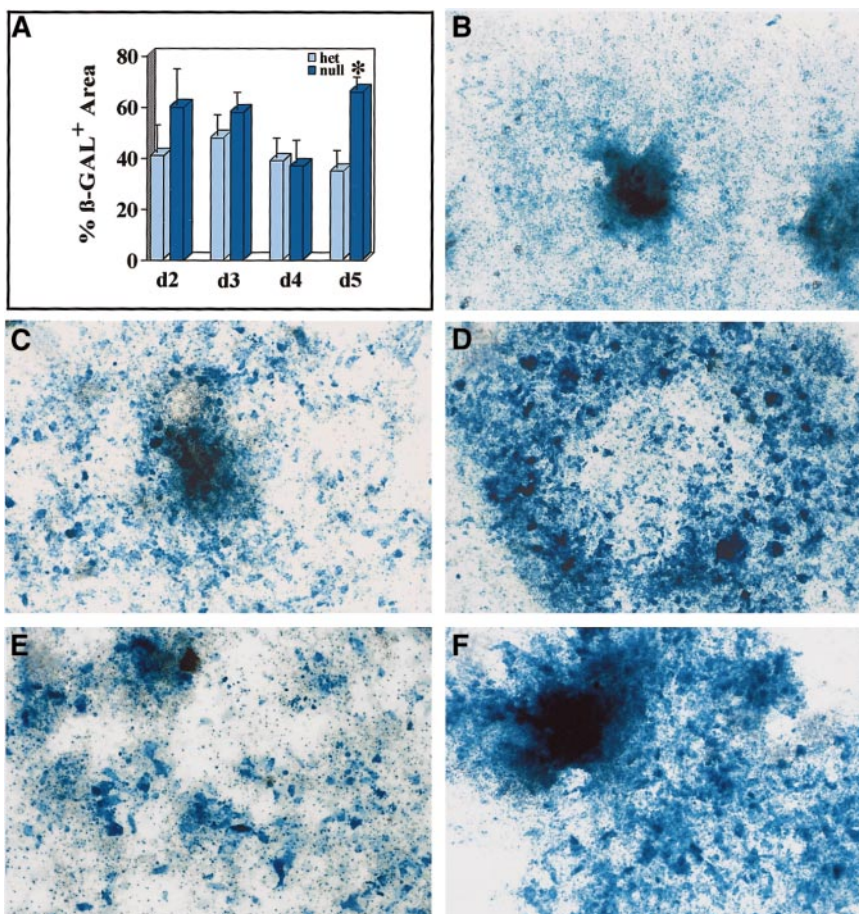
because blood vessel formation requires endothelial cell division. The treated WT cultures had 2- to 3-fold less vasculature and less branching than untreated controls (compare Figure 5C with E,H). Thus, treatment with mitomycin C, an inhibitor of replication, partially rescues the *flt-1* mutant vascular 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, $\times 20$.



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 differentia-

tion, 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

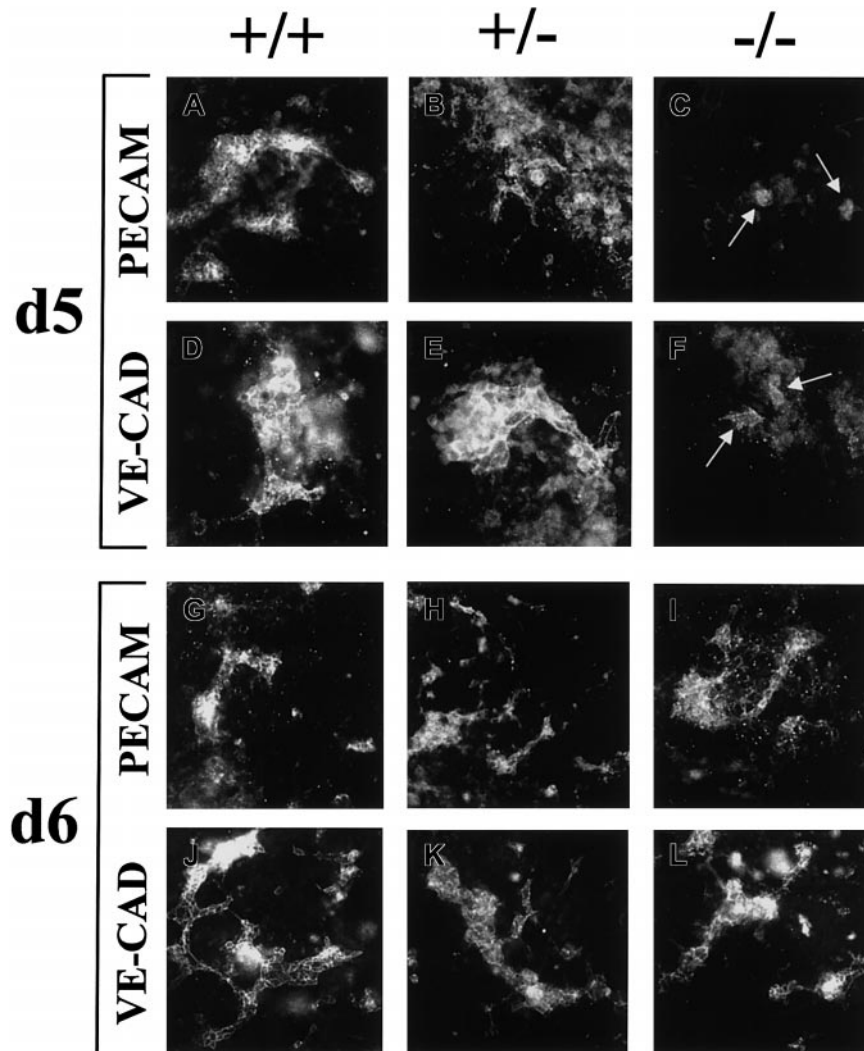


Figure 7. Expression of vascular markers in differentiating ES cell cultures. Wt (+/+) (A,D,G,J), *flt-1*^{+/-} (B,E,H,K), and *flt-1*^{-/-} (C,F,I,L) ES cell cultures were fixed on day 5 (A-F) or day 6 (G-L) and labeled with antibodies to PECAM (A-C,G-I) or VE-cadherin (D-F,J-L), and the appropriate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

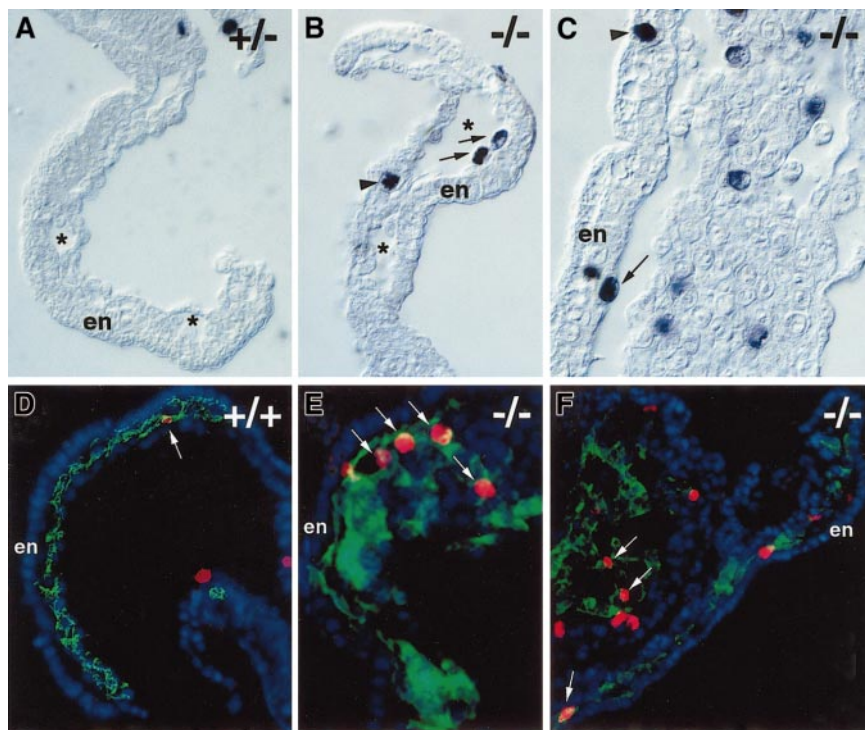
vascular lineage. The identification of this cellular mechanism of *flt-1* action suggests that *flt-1* is critical for the fine tuning of VEGF-mediated vessel growth that is required to form proper blood vessels. It also strongly suggests that *flt-1* may affect blood vessel formation in similar ways 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 antiphosphohistone 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 development⁶¹ 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.

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