Fibroblast Growth Factor Receptor-1 Expression Is Required for Hematopoietic but not Endothelial Cell Development

Peetra Ulrica Magnusson, Roberto Ronca, Patrizia Dell’Era, Pia Carlstedt, Lars Jakobsson, Juha Partanan, Anna Dimberg, Lena Claesson-Welsh

Objective — The purpose of this study was to clarify the role of fibroblast growth factors (FGFs) and FGF receptors (FGFRs) in hematopoietic/endothelial development.

Methods and Results — Using several different FGFR-1–specific antibodies and FGFR-1 promoter-driven LacZ activity, we show that FGFR-1 is expressed and active as a tyrosine kinase in a subpopulation of endothelial cells (≈20% of the endothelial pool) during development in embryoid bodies. In agreement, in stem cell–derived teratomas, expression of FGFR-1 was detected in some but not all vessels. The FGFR-1–expressing endothelial cells were mitogenically active in the absence and presence of vascular endothelial growth factor (VEGF). Expression of FGFR-1 in endothelial cell precursors was not required for vascular development, and vascularization was enhanced in FGFR-1–deficient embryoid bodies compared with wild-type stem cells. In contrast, hematopoietic development was severely disturbed, with reduced expression of markers for primitive and definitive hematopoiesis.

Conclusions — Our data show that FGFR-1 is expressed in early hematopoietic/endothelial precursor cells, as well as in a subpool of endothelial cells in tumor vessels, and that it is critical for hematopoietic but not for vascular development.

Key Words: angiogenesis ■ endothelial cells ■ FGF receptor-1 ■ hematopoiesis ■ vasculogenesis
continued for 8 days. To visualize the FGFR-1 promoter-driven gen, followed by instant covering with a second layer of collagen.

4 after LIF withdrawal) were distributed on the polymerized collagen gels. Embryoid bodies (day 1.25 mg/mL X-gal. Incorporation of bromodeoxyuridine (BrdUrd) was visualized using anti-BrdU+nuclease kit (Amersham Pharmacia Biotech) and secondary antibody Alexa 488 goat anti-mouse highly cross-absorbed IgG (Molecular Probes). Nuclei were visualized by incorporation of Hoechst 33342 (1 μg/mL).

Magnetic Cell Sorting to Enrich for Endothelial Cells

Embryoid bodies were dissociated by incubation in 0.25% collagenase and the single cell suspension was incubated with rat anti-mouse CD31 antibody (Becton Dickinson Biosciences), followed by mixing with magnetic cell sorting goat anti-IgG micro beads (Miltenyi Biotec). The CD31+ eluate was then incubated with mouse monoclonal FGFR-1 antibody (QED Bioscience) and goat anti-mouse IgG microbeads to isolate a CD31+FGFR-1+ fraction. Total RNA was extracted using the Qiagen RNeasy mini kit.

Fluorescence Activated Cell Sorting and Cell Cycle Assays

Embryoid bodies were dissociated by incubation in 0.25% collagenase. The single-cell suspension was incubated with mouse FGFR-1 monoclonal antibody (QED Bioscience) and secondary Alexa 488 goat anti-mouse highly cross-absorbed IgG (Molecular Probes). The cells were then stained with rat anti-mouse CD31-phycoerythrin antibody (Becton Dickinson). To identify the cell cycle profile, 5 μmol/L Hoechst 33342 was added to the single cell suspension in DMEM with 15% fetal bovine serum during 1 hour after antibody staining as described. Viable cells were identified by use of propidium iodide. The results were analyzed on a FACSVantage SE DiVa machine (Becton Dickinson) equipped with an Enterprise laser (Coherent Inc) for ultraviolet and 488 nm excitation using ModFit software (Verity Software House, Inc).

Embryoid Bodies in 3-Dimensional Collagen I Gels

Collagen gels were prepared by mixing 10x Ham’s F12 medium (Invitrogen) with 0.12% NaHCO3, 50 mmol/L HEPES, 5 mmol/L NaOH, and 1.5 mg/mL collagen I (Cohesion). Embryoid bodies (day 4 after LIF withdrawal) were distributed on the polymerized collagen, followed by instant covering with a second layer of collagen. Medium containing 30 ng/mL of VEGF-A was added and culture continued for 8 days. To visualize the FGFR-1 promoter-driven β-galactosidase activity, FGFR-1+/+Iy/xGyr-1 embryoid bodies in 3-dimensional collagen gel were fixed in 4% p-formaldehyde for 30 minutes, followed by overnight incubation at 37°C in 5 mmol/L K-ferricyanide, 5 mmol/L K-ferrocyanide, 4 mmol/L MgCl2, and 1.25 mg/mL X-gal. Incorporation of bromodeoxyuridine (BrdUrd) of 4 individual experiments is presented. G, Cell cycle profile by staining for DNA content using Hoechst 33342 of the dispersed CD31+/Hoechst 33342 of the dispersed CD31+/Hoechst 33342 of the dispersed CD31+/Hoechst 33342 of the dispersed CD31+ fraction. Total RNA was extracted using the Qiagen RNeasy mini kit.

Results

FGFR-1 Expression in Endothelial Cells During Development

We analyzed expression of FGFR-1 in endothelial cells during development in differentiating embryonic stem cell cultures, which display several hallmarks of vascular development in mouse embryos.14 Embryoid bodies (strain R1) were created by aggregation of stem cells and seeded individually in 8-well chambers in the presence of 20 ng/mL FGF-2 for 8 days, to induce vessel formation. Coimmunostaining showed that expression of FGFR-1 overlapped with that of CD31 on endothelial cells (Figure 1A). In embryoid bodies created from ES cells homozygous-null for FGFR-1, the anti-FGFR-1 antibody gave rise to an even, low background without vessel-specific staining (Figure 1B); in contrast, immunostaining for CD31 showed vessel-like structures. This result demonstrates the specificity of the anti-FGFR-1 antibody. To show that the FGFR-1 was functional in endothelial cells, an antibody was used that is specific for the activated receptor, phosphorylated at Y653 in the second kinase domain. Staining for Y653-phosphorylated FGFR-1 colocalized with CD31 expression in endothelial cells (Figure 2).
collagen gels. Some but not all endothelial cells contained pools (data not shown).

expression of Annexin V indicated that there was no significant
individually expressing either CD31 or FGFR-1. Staining for
are progressing through the cell cycle, compared with cells
behaved similar to wild-type R1 stem cells with regard to
response to VEGF-A. Embryoid bodies in
endodermal marker
expression of FGFR-1 in endothelial cells, compared with cells
To analyze expression of FGFR-1 in endothelial cells indepen-
study that the CD31 FGFR-1 also expressed
endothelial cell markers VEGFR-2 and VE-cadherin. The
endothelial marker α-fetoprotein served as a negative control
and was present in the flow through (CD31 FGFR-1) from
the magnetic bead separation, but absent in the CD31 FGFR-1
pool (Figure 1E).

To allow analysis of cell cycle parameters of the
CD31 FGFR-1 cells, fluorescence-activated cell sorting
analyses were performed on the collagenase-digested embry-
loid bodies. A relatively small pool of viable CD31 FGFR-1 cells was identified; 3% of all cells (Figure 1F), possibly
representing more than one population of cells. In total,
12% of all viable cells expressed CD31 and 23% expressed
FGFR-1. Staining with Hoechst 33342 allowed visualization
of the cell cycle profile in the different fractions. As shown in
Figure 1G, 70% of the CD31 FGFR-1 cells were in the
S/G2 phase, indicating that a larger proportion of these cells
are progressing through the cell cycle, compared with cells
individually expressing either CD31 or FGFR-1. Staining for
expression of Annexin V indicated that there was no significant
difference in the extent of apoptosis in the different cell pools (data not shown).

FGFR-1 Promoter-Driven LacZ Expression
Demonstrates FGFR-1 Expression in
Endothelial Cells

To analyze expression of FGFR-1 in endothelial cells indepen-
dently of FGFR-1 antibodies, we created an embryonic stem cell
line expressing β-galactosidase less than the control of the
FGFR-1 promoter (FGFR-1lacZfgfr-1). This strain of ES cells
behaved similar to wild-type R1 stem cells with regard to
proliferation rate and FGF-2-driven DNA synthesis (Figure 1, available online at http://atvb.ahajournals.org). To facilitate
analysis of individual endothelial cells, we used a sprouting angi-
genesis assay in which embryoid bodies are placed in
3-dimensional collagen gels. Inclusion of VEGF-A is required
to induce sprouting of vessel-like structure into the gel10 (Figure 2A). FGFR-1lacZfgfr-1 embryoid bodies were cultured between
days 4 and 12 in 3-dimensional collagen and β-galactosidase activity was identified by X-gal staining. Containing with anti-
CD31 and anti-α-smooth muscle cell actin antibodies was
performed to identify pericyte-coated vessel sprouts invading
the collagen gels. Some but not all endothelial cells contained
β-galactosidase activity as a result of FGFR-1 promoter activity (Figure 2B). By analyzing the distal ends of sprouts where cells
clearly could be classified as single endothelial cells, the number
of CD31 β-galactosidase-positive cells (ie, CD31 FGFR-1

cells) was estimated to 15% of all endothelial cells in the
sprouts. This indicates a lower fraction of coexpressing cells in
the angiogenic sprouts than in the total CD31-positive pool
(25%; see Figure 1F), however, the fluorescence-activated cell
sorting-derived value may be influenced by the fact that CD31 is
expressed also on hematopoietic cells. Moreover, in the sprout-
ing assay, only endothelial cells in the tip of the sprouts could be
clearly identified. Fluorescence-activated cell sorting analyses
combining the only commercial FGFR-1 antibody of several
tested that allowed specific visualization of the FGFR-1-expressing
cells with antibodies against other endothelial cell
markers were not technically feasible because of specificity of
the reagents.

Data in Figure 1G showed that the CD31 FGFR-1 cells
were actively going through the cell cycle, whereas cells
individually expressing CD31 or FGFR-1 were resting to a higher extent. We wished to test the ability of the cell pools to respond mitogenically to VEGF-A. Embryoid bodies in
3-dimensional collagen gels were treated with VEGF-A for
different periods of time; BrdUrd was added during the last
24 hours of culture. The embryoid bodies were then stained to
visualize CD31 β-galactosidase-positive cells (Figure 2C shows
10-day-old embryoid bodies). Quantification by counting the
number of X-gal-stained and BrdUrd-stained endothelial cells in
the angiogenic sprouts showed the fraction of mitogenically

Figure 2. Detection of FGFR-1 expression in endothelial cells using FGFR-1lacZfgfr-1 ES cells. A, 3-dimensional culture of embry-
oid body in collagen gel in the presence of VEGF-A induced angiogenic sprouting visualized by CD31 staining. Bars, 1 mm. B,
VEGF-A induced sprouting by FGFR-1lacZfgfr-1 embryoid body in
3-dimensional collagen gel shows FGFR-1 expressing (arrow) or nonexpressing (arrowhead) endothelial cells. Left panel, Fluores-
cence staining; red; CD31, green; α-smooth muscle cell actin, pur-
el; Hoechst staining. Right panel, Light microscopy in which X-gal
stain (blue) marks FGFR-1 promoter-positive cells. Bars, 200 μm.
C, Left panel shows fluorescence staining for BrdUrd (green) and
CD31 (red); right panel shows X-gal staining (blue); BrdUrd-positive
CD31 FGFR-1 (ring marked) and CD31 FGFR-1 (arrowhead)
donvastatin endothelial cells in FGFR-1lacZfgfr-1-derived sprouts. Bars, 50 μm.

Magnusson et al FGFR-1 Expression in Endothelial Cells 3

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The mitogenic activity of CD31/H11001-galactosidase–positive cells showed the same pattern with an initial activity in 50% of the CD31/H11001-FGFR-1 pool followed by a decrease to 25% to 30% (data not shown).

These data show that the total endothelial cell pool responds to exogenous VEGF-A with induction of a mitogenic response also engaging the CD31/H11001-FGFR-1− cells. However, in the absence of exogenous VEGF-A, the activity is markedly higher in the CD31/H11001-FGFR-1− pool than in the total CD31/H11001 pool (Figure 1G).

**FGFR-1 Is Expressed in Teratoma Vessels**

We next asked whether FGFR-1 is expressed on vessels in vivo. For this purpose, we analyzed vessels in teratomas, created by inoculation of immune-deficient mice with J1 or R1 embryonic stem cells expressing FGFR-1. As shown in Figure 3A (arrowheads), CD31/FGFR-1 coexpressing cells were readily detected in the J1 teratomas by use of an antibody reactive with the intracellular domain of FGFR-1. The result was further verified by use of a second commercial antibody against the FGFR-1 extracellular domain to stain sections of R1 teratomas. The FGFR-1 immunostaining colocalized with that for CD31 in vessel structures of the teratoma (Figure 3B). A third commercial FGFR-1 antibody specific for the unique NH2-terminal IgG loop of the receptor also costained CD31-positive vessel-like structures in R1 teratomas (Figure 3C). We estimated that ~25% of the endothelial cells in teratomas expressed FGFR-1, although the frequency varied between different regions of the tumors.

**Function of FGFR-1 in Endothelial Cells During Development**

Embryoid bodies derived from stem cells lacking one (1/2) or both (1/1) alleles of FGFR-1 still form blood vessels. In fact, even though VEGFR-2 expression is decreased in the FGFR-1−/− stem cells, blood vessels form to a considerably increased extent even in the absence of exogenous growth factors (Figure 4A). Thus, endothelial cell development still proceeds in the absence of FGFR-1; in contrast, hematopoietic development is attenuated. Development of the endothelial and hematopoietic lineages diverges downstream of a common mesodermal precursor cell, the hemangioblast.

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**Figure 3.** Immunostaining for FGFR-1 expression in mouse teratomas. A, FGFR-1 expression in J1 teratoma (green; antibody against FGFR-1 intracellular domain; Santa Cruz, sc-121). Arrowhead indicates coexpression with CD31. B, R1 teratoma (green; antibody against the extracellular domain of FGFR-1; Santa Cruz sc-4975). C, R1 teratoma (green; antibody against the unique NH2-terminal Ig loop of FGFR-1; QED Bioscience, mAb 30104). All panels show CD31 staining in red. Bars, 100 µm.

**Figure 4.** Endothelial and hematopoietic development in FGFR-1 lacking embryoid bodies. A, Increased vascularization in FGFR-1−/− embryoid bodies compared with FGFR-1+/− control embryoid bodies as visualized by staining for VEGFR-2. B, Real-time PCR analysis of expression in FGFR-1+/− or FGFR-1−/− embryoid bodies of hematopoietic or endothelial cell markers at day 8, or for brachyury at day 4, and CD45 at day 12. C, Localization of staining for CD41 (red) and VE-cadherin (green) in blood island-like structures in FGFR-1−/− embryoid bodies. D, Localization of CD41-positive (red) cells inside VE-cadherin-positive (green) vessels. E, Immunofluorescent staining for CD41 (red; arrow) and VE-cadherin (green) expression in FGFR-1−/− embryoid bodies. Bars, 100 µm.
pinpoint the level at which loss of FGFR-1 expression guides the common endothelial/hematopoietic development toward a preference for the endothelial lineage, expression of markers of primitive and definitive hematopoiesis, as well as endothelial cell markers, was investigated by real-time PCR in differentiating embryoid bodies (Figure 4B). Expression of brachyury, one of the earliest markers for mesoderm development,19 was slightly increased by day 4 in the absence of FGFR-1. Runx1, which is expressed at the earliest stage of blood island development,20 was not affected by elimination of FGFR-1 expression. Hemangioblasts are known to express brachyury, VEGF-R-2, and Tal-1/SCL.17,21,22 In the absence of FGFR-1, there was a 50% reduction in VEGF-R-2 transcripts, whereas the level of Tal-1 expression was <10% of that in control FGFR-1+/− stem cells. These data indicate that loss of FGFR-1 expression affects the endothelial/hematopoietic differentiation at the level of the hemangioblast. Further real-time PCR analyses showed that expression of the primitive hematopoietic marker CD4123 and the embryonic form of β-globin, β-H1, a marker for primitive erythropoiesis,24 was essentially lost. In accordance, there was little or no expression of GATA-1, a transcription factor expressed in hematopoietic but absent in endothelial cells,24 or of the pan hematopoietic marker CD4525 in FGFR-1−/+ stem cells at day 8 and day 12, respectively. Despite changes on the hemangioblast level as indicated by decreased expression of VEGF-R-2 and Tal-1/SCL, development of the endothelial cell lineage proceeded and expression of CD31 and VE-cadherin transcripts was higher or similar to the control in the FGFR-1−/+ stem cells at day 8. Transcript levels of the general smooth muscle cell marker α-smooth muscle actin was reduced (Figure 4B) in agreement with the impaired mesodermal development in the absence of FGFR-1 expression. Time course analyses at days 4, 8, and 12 (Figure II, available online at http://atvb.ahajournals.org) showed there was no rescue in expression of hematopoietic markers in the FGFR-1−/+ embryoid bodies excluding that the FGFR-1−/+ phenotype was caused by a delay in development.

Immunostaining for CD41 and VE-cadherin in FGFR-1−/+ embryoid bodies showed the presence of specifically stained cells in blood island-like structures, which form in the absence of exogenous angiogenic growth factors less than the conditions used here (Figure 4C). CD41-expressing cells were localized inside VE-cadherin–positive vessels (Figure 4D). In the FGFR-1−/+ embryoid bodies, a mature vascular plexus was visualized by immunostaining for VE-cadherin, but very few CD41-expressing cells were detected (Figure 4E, arrow), in agreement with the real-time PCR data in Figure 4B.

To verify that the vascular and hematopoietic phenotype of FGFR-1−/+ embryoid bodies was caused by loss of the receptor, FGFR-1 was reintroduced by lentivirus-mediated gene transfer26 into the gene-targeted ES cells to create Lv−hFGFR-1. Introduction of FGFR-1 restored CD41 expression in the Lv−hFGFR-1 embryoid bodies. Spontaneous vessel formation decreased and vessels assumed a more primitive morphology. Furthermore, CD45 expression in Lv−hFGFR-1 was similar to that in wild-type embryoid bodies at day 12 (Figure III, available online at http://atvb.ahajournals.org).

**Discussion**

FGFR-1 is expressed on endothelial cells in tissue culture and FGF-2 is a potent mitogen for such endothelial cells.27 It has not been generally accepted that this reflects the in vivo situation, because it is well known that explanted cells display an inflammatory response leading to induction of genes not normally expressed in the in vivo context.28 We report expression of FGFR-1 in a subpopulation of endothelial cells in embryoid bodies and in teratomas. Our data are in accordance with a report on expression of FGFR-1 in a subpopulation (4.5±2.1) of CD34+ endothelial progenitor cells.29

What distinguishes an FGFR-1–expressing endothelial cell from one that lacks FGFR-1 expression? It has been suggested that replicating but not quiescent endothelial cells express FGFR-1.30 Our data agree with this report in that fluorescence-activated cell sorting analyses of the CD31+FGFR-1− cells displayed an increased fraction of actively cycling cells compared with cells individually expressing CD31 or FGFR-1 (Figure 1). Exposure to VEGF-A induced a similar extent of mitogenic response in the CD31+ pool and the double-positive CD31+FGFR-1− pool (Figure 2C). Expression of FGFR-1 in endothelial cells in tumor vessels may promote vascularization of the tumor and facilitate tumor growth. This is indicated by the fact that expression of dominant-negative FGFR is accompanied by decreased vascularization and growth of different model tumors in mice.11,12 Giavazzi et al32 showed that overexpression of FGF-2 leads to increased vascularization and growth of endometrial carcinoma in mice. Suppression of tumor angiogenesis was also achieved by vaccination of mice with a xenogeneic FGFR-1.33 However, many endothelial cells in teratomas (Figure 3) as well as in human kidney cancer (data not shown) lack FGFR-1 expression; therefore, the different manipulations of FGF/FGFR function in tumor endothelial cells reported in these articles may be exerted via other types of FGFRs than FGFR-1. Alternatively, the treatment may target the tumor cells as well as the endothelial cells.

Our data show clearly that FGFR-1 expression is not required for endothelial cell development. This conclusion is further supported by the fact that endothelial cell–specific FGFR-1 gene inactivation is compatible with normal development and growth of the mouse (Partanen J and Rossant J, unpublished). In contrast, Lee et al34 showed that expression of dominant-negative FGFR delivered to the vasculature of E9 mouse embryos led to disrupted embryonic and extraembryonic vasculature. Possibly, the dominant-negative FGFR used in this study acted as a general FGF-binding decoy, blocking the function of all FGFRs. We have previously reported that although differentiating embryoid bodies express reduced levels of VEGF-2 transcript and protein, the VEGF-2 appears hyperactive and promotes elevated vascularization of embryoid bodies.14 In agreement with Faloons et al,17 we show that lack of FGFR-1 expression is accompanied by decreased expression of markers of primitive and definitive hematopoiesis and an apparent arrest in hematopoietic development. Furthermore, we pinpoint the FGFR-1–dependent switch toward endothelial cell development to the level of the hemangioblast. Expression of brachyury was slightly...
increased, whereas VEGFR-2 and Tal-1/SCL expression was reduced by 50% and 90%, respectively, in the FGFR-1–deficient embryoid bodies. This indicates that differentiation of the hemangioblast was disturbed in conjunction with segregation toward hematopoietic and endothelial cell development, and loss of FGFR-1 supports further development only of the endothelial cell lineage. FGFR-1 function in this context appears to be linked to that of Tal-1/SCL as inferred from the study by Robertson et al., who showed that elimination of Tal-1/SCL expression attenuates hematopoiesis but allows progression of endothelial development.

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Supplementary Materials and Methods

ES cell and embryoid body culture
Embryonic stem (ES) cells were cultured on growth-arrested murine embryonic fibroblast (MEF) feeder cells in ES medium composed of Dulbecco’s modified Eagle’s medium/glutamax (Invitrogen) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, 1.2 mM Na-pyruvate, 19 μM monothioglycerol and 1,000 U/ml recombinant leukemia inhibitory factor (LIF; Chemicon International). Before differentiation, the ES cells were cultured for 1-2 passages on gelatin-coated tissue culture plastic to remove feeder cells. Differentiation of embryoid bodies started day 0 when LIF was withdrawn from the medium. Growth factors were added as indicated from this point (20 ng/ml FGF-2 or 30 ng/ml VEGF-A165, Peprotech). Formation of embryoid bodies was induced in hanging droplets and on day 4, the bodies were flushed down and plated on 8-well glass culture slides (Becton Dickinson (BD) Bioculture/Falcon) or on tissue culture plastic dishes. All analyses were performed on four or more individual embryoid bodies at three or more individual occasions. To create lentivirus-human FGFR-1 (Lv-hFGFR-1), human FGFR-1 cDNA was cloned under control of the ubiquitous phosphoglycerate kinase (PGK) promoter in the pRRLsin.PPT .hPGK vector (1). Viral particles were produced by transfection of Lvαs in 293T cells and used to infect J1 FGFR-1-/- ES cells. Infected ES cells were screened for expression of FGFR-1 using a 125I-FGF-2 binding assay. All ES cell lines were cultured as previously described (2).

Staining and antibodies
Peroxidase and fluorescence staining of embryoid bodies was performed as described (2). For peroxidase staining, embryoid bodies were fixed in zinc fix (0.1 M Tris HCl, pH 7.5, 3 mM calcium acetate, 23 mM zinc acetate and 37 mM zinc chloride) over night at 4°C. After peroxidase treatment and blocking in 3% bovine serum albumin (BSA) diluted in Tris-buffered saline (TBS), samples were incubated with rat anti-mouse CD31 antibody (BD) diluted in 3% BSA blocking buffer and incubated over night at 4°C. This was followed by washing and incubation for one hour at room temperature with secondary biotinylated goat anti-rat antibody (Vector Laboratories Inc.) diluted in 3% BSA blocking buffer and finally, a 30-minutes incubation with streptavidin-HRP (Vector Laboratories Inc.). Immune reactivity was visualized by the use of the chromogen substance (AEC kit from Vector Laboratories Inc.). Slides were mounted with Ultramount aqueous mounting medium (DAKO) and photographed in a Nikon Eclipse E1000 microscope.

For fluorescence staining, embryoid bodies were fixed in ice cold acetone or methanol for 15 minutes followed by blocking of unspecific binding in 3% BSA for one hour at room temperature. Primary antibodies, i.e. rabbit anti-mouse FGFR-1 (sc-121, Santa Cruz Biotechnology), rabbit anti-mouse pY563/FGFR-1 (Cell Signaling Technologies), rat anti-mouse CD31 (BD) rat anti-mouse CD41 (BD), rat anti-mouse CD45 (BD) and goat anti-mouse VE-cadherin (R&D Systems) were diluted in 3% BSA and incubated on specimens over night at 4°C. The following day, the embryoid bodies were washed for 30 minutes with TBS and incubated for one hour at room temperature with appropriate secondary antibodies, Alexa 488 goat anti-rabbit IgG, Alexa 568 goat anti-rat IgG, Alexa 594 donkey anti-rat IgG or Alexa 488 donkey anti-goat IgG (all from Molecular Probes) diluted in 3% BSA. Slides were then washed in TBS, briefly dried and then mounted in Fluoromount-G (Southern Biotechnology), and photographed in a Nikon Eclipse E1000 microscope.
Proliferation assay
FGFR-1+/lacZfgfr-1 and R1 control ES cells were plated in the presence of LIF at various densities, 0.6 x 10^4/ml, 0.8 x 10^4/ml, 1.2 x 10^4/ml and 1.8 x 10^4/ml. Cells were cultured for 3 days, trypsinized in trypsin-EDTA (Gibco BRL) and counted in a particle coulter counter. The results were presented as fold induction.

DNA synthesis
FGFR-1+/lacZfgfr-1 and R1 control ES cells were cultured without LIF in ES medium for three days to induce expression of FGFR-1. On day 3, ES cells were seeded at either 2.5 x 10^4 or 1.0 x 10^4 cells/ml in a 24-well plate in starvation ES medium containing 0.2% FBS. After 5 hours, the medium was replaced with starvation medium containing 0.5 μCi/ml 3H-thymidine without (basal) or with 20 ng/ml FGF-2 (Boehringer/Mannheim). Cultures were incubated at 37°C for 18 hours. Medium was aspirated and cells were placed on ice and washed twice with ice-cold PBS, treated with 10% trichloro acetic acid to precipitate the 3H-thymidine and then incubated on ice for 20 minutes. Cells were dissolved in 0.2 M NaOH and incorporation of 3H-thymidine was estimated by scintillation counting. DNA synthesis was expressed as percentage of basal for both FGFR-1+/lacZfgfr-1 and R1 control cells.

Culturing of embryoid bodies in three-dimensional collagen I gels
At day four of embryoid development, collagen gels were prepared by mixing 10x Ham’s F12 medium (Invitrogen) with 0.12% NaHCO₃, 50 mM HEPES, 5 mM NaOH and 1.5 mg/ml collagen I (Cohesion). The collagen mix was deposited in the wells of a 12-well plate and let to polymerize at 37°C for three hours. Eight embryoid bodies were distributed in each well on the polymerized collagen, followed by an instant covering with a second layer of the collagen solution. Three hours after polymerization, medium containing 30 ng/ml of VEGF-A was added. Embryoid bodies were cultured for six or eight days in collagen gels.

X-gal and peroxidase staining of embryoid bodies in three-dimensional collagen I gels
To visualize the FGFR-1 promoter-driven β-galactosidase activity, FGFR-1+/lacZfgfr-1 embryoid bodies cultured in collagen gel I as described above, were exposed to the substrate X-gal. The gels were first washed for 20 minutes in PBS at room temperature and fixed in 4% p-formaldehyde in PBS for 30 minutes. The X-gal staining was achieved by incubation over night at 37°C in a buffer containing 5 mM K-ferricyanide, 5 mM K-ferrocyanide, 4 mM MgCl₂ and 1.25 mg/ml X-gal. The X-gal-stained embryoid bodies were processed for CD31 fluorescence staining the following day. For peroxidase staining, the embryoid bodies were fixed in 4% p-formaldehyde in PBS for 30 minutes and the staining was performed as described above.

Fluorescence staining of embryoid bodies in collagen I gels
After washes in TBS, fixation and exposure to X-gal as described above, the second layer of the collagen gel was removed and placed in a new plate for staining, followed by blocking and permeabilization in 3% BSA, 0.2% Triton X-100 in PBS for 2 hours at room temperature. Incubation with primary rat anti-mouse CD31 antibody (BD) in 3% BSA, 0.1% Tween in PBS, proceeded at 4°C over night. The X-gal staining was achieved by incubation over night at 37°C in a buffer containing 5 mM K-ferricyanide, 5 mM K-ferrocyanide, 4 mM MgCl₂ and 1.25 mg/ml X-gal. The X-gal-stained embryoid bodies were processed for CD31 fluorescence staining the following day. For peroxidase staining, the embryoid bodies were fixed in 4% p-formaldehyde in PBS for 30 minutes and the staining was performed as described above.

BrdU staining of embryoid bodies in collagen I gels
Culturing of FGFR-1+/lacZfgfr-1 embryoid bodies in collagen gels was performed as described above. At day 11 of differentiation, bromodeoxyuridine (BrdU) (Sigma) was added to the cultures at a final concentration of 10 μM, and cultures were incubated for 24 hours at 37°C and 5% CO₂ and then fixed in 4% p-formaldehyde in PBS for 30 minutes following X-gal staining as described above. After blocking and permeabilization in 3% BSA, 0.2% Triton X-100 in PBS for 2 hours at room temperature, the embryoid bodies were incubated with mouse monoclonal anti-BrdU antibodies and nuclease (Anti-BrdU+nuclease kit, Amersham Pharmacia Biotech) for another 2 hours at room temperature. After washing the embryoid bodies were analyzed and photographed using a Nikon Eclipse E1000 microscope.
in PBS 3x1 hours at room temperature, staining with primary rat anti-mouse CD31 antibody (BD) was performed as described above. Results were analyzed and photographed using a Nikon Eclipse E1000 microscope. Statistical analysis was done by unpaired Student t-test using the StatView® program.

**Semi-quantitative polymerase chain reaction (PCR) analysis**

Total RNA was extracted from eluate and flow through using the RNeasy mini kit (Qiagen), according to the manufacturer’s instructions. As control for FGFR-1 primer specificity, total RNA was extracted from 8 day old FGFR-1+/− and FGFR-1−/− embryoid bodies. Contaminating genomic DNA was digested with DNase I (Amersham Pharmacia Biotech) and 1 ug total RNA was used for first strand cDNA synthesis using oligo dT primers and the Advantage RT-for-PCR-Kit (Clontech). Primers used are listed in supplementary Table 1A. β-actin was used as an internal control. FGFR-1 primers yielded a 650 base pair product and the CD31, VEGFR-2, VE-cadherin, AFP and β-actin primers an 80 base pair product. PCR conditions for FGFR-1 detection was as follows: 10 min activation at 95°C, followed by 24 cycles at 95°C for 30 sec, 70°C for 30 sec and 72°C for 1 min. The cycles were followed by PCR for CD31, VEGFR-2, VE-cadherin and AFP: 10 min activation at 95°C, followed by 27 cycles at 95°C for 15 sec and 60°C for 1 min. PCR for β-actin: 10 min activation at 95°C, followed by 30 cycles at 95°C for 15 sec and 60°C for 1 min.

**Real-time PCR analysis**

Total RNA was extracted from eluate and flow through from the endothelial cell purification from day 8 embryoid bodies. cDNA was prepared as described above. Primers used are listed in supplementary Table 1B. β-actin was used as an endogenous reference and non-reverse transcribed RNA was used as a negative control. The PCR samples, containing cDNA, primers (0.25 μM final concentration) and 2x SYBR Green PCR master mix (Applied Biosystems), were run in triplicate on an ABI Prism 7700 Sequence Detection System instrument (Applied Biosystems) with an initial 10-minute activation at 95°C, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. Real-time PCR for Tal-1 was run with an initial 10-minute activation at 95°C, followed by 45 cycles at 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The threshold cycle (Ct) value was calculated for each sample by the ABI Prism 7700 instrument. Transcript levels were then normalized against β-actin levels and changes in transcript levels were expressed as relative values.

**Supplementary Tables**

Table 1A. Semi-quantitative PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer (5’-3’)</th>
<th>Anti sense primer (5’-3’)</th>
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</thead>
<tbody>
<tr>
<td>FGFR-1</td>
<td>GCTGACTCTGGCTCTCTACGCT</td>
<td>CAGGATCTGGACATAAGGCAA</td>
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<tr>
<td>CD31</td>
<td>TACTGCAAGCATCAGGCAA</td>
<td>GCATTTCGCACAAGGCTGAT</td>
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<td>VEGFR-2</td>
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<td>VE-cadherin</td>
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<tr>
<td>AFP</td>
<td>ACCCTTATTATCAGGCCC</td>
<td>GCAAGGAGCAAGGACCTTG</td>
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<td>β-actin</td>
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<td>TCCATACCCGAAAGGAGGC</td>
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Table 1B. Real-time PCR primers

<table>
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<th>Gene</th>
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<th>Anti sense primer (5’-3’)</th>
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<td>AAACGGATGGGGCAAGGTC</td>
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<td>VEGFR-2</td>
<td>TACAGACCCGCAACAAACA</td>
<td>TTTCCCCCTGGGAAATCCCT</td>
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<tr>
<td>Tal-1</td>
<td>GGCAGAGAGAGACTTCCCTG</td>
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<td>TCCCCGIGAACCATCGAA</td>
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<td>TGGGCCAGAGGGTTTG</td>
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<td>β-H1</td>
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<tr>
<td>VEGFR-2</td>
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<td>Tal-1</td>
<td>GGCAGAGAGAGACTTCCCTG</td>
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<td>β-actin</td>
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Supplementary references


Supplementary figures

Fig. I online supplement. Construct and characteristics of FGFR-1+/laczZgfr-1 ES cells. A. Creation of FGFR-1+/laczZgfr-1 ES cell line by homologous recombination (TMB, transmembrane domain). B. Proliferation of FGFR-1+/laczZgfr-1 and R1 control ES cells. C. FGF-2-induced $^3$H-thymidine incorporation in FGFR-1+/laczZgfr-1 and R1 ES cells.
**Fig. II online supplement.** Gene expression kinetics in FGFR-1+/− and FGFR-1−/− embryoid bodies day 4, day 8 and day 12. Real-time PCR analysis of mesodermal, hematopoietic, endothelial and mural gene transcripts in embryoid bodies day 4 (d4), day 8 (d8) and day 12 (d12). Dark grey bars represent FGFR-1+/− and light grey bars represent FGFR-1−/−. Values are relative to β-actin levels. 1k = 1000.

**Fig. III online supplement.** Vessel formation in Lv-hFGFR-1 embryoid bodies. A. Restored expression of CD41 (red) and VE-cadherin (green) in day 8 embryoid bodies after re-introduction of FGFR-1 (Lv-hFGFR-1). B. Comparison of CD45 (red) expression at day 12 in FGFR-1−/−, FGFR-1+/− and Lv-FGFR-1 embryoid bodies. Bars, 100 μm.