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 Partanen, 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

Fibroblast growth factors (FGF) constitute a family of at least 23 related heparin-binding growth factors with vital and broad functions in health and disease. There are 4 distinct FGF receptor (FGFR) tyrosine kinases, denoted FGFR-1, -2, -3, and -4.1 Binding of FGF to its receptor leads to receptor dimerization and activation of the kinase, followed by tyrosine phosphorylation of the receptor itself, as well as of downstream signal transduction molecules.2 FGFR-2 was the first angiogenic growth factor to be identified,1 and endothelial cells in culture respond mitogenically to FGF-2 mainly through FGFR-1.3 The question of whether endothelial cells in vivo express FGFR-1 and respond to various FGFs has remained a matter of debate. There is no clear vascular phenotype in FGF-2 or FGFR-1 knockout animals. Gene inactivation of FGFR-1 leads to embryonic death in conjunction with gastrulation between embryonic days 7.5 to 9.5.5,6 Embryos homozygous-null for FGFR-1 expression display severe reduction in paraxial mesoderm formation, whereas development of node, axial, and extraembryonic mesoderm is unaffected. Gene inactivation of FGFR-2, a ligand for FGFR-1, is compatible with normal development, although the gene–targeted animals display decreased vascular tone and low blood pressure.7 The possibility of compensation through any of the other FGFs or FGFRs cannot be excluded.

Vessels are established through vasculogenesis in embryonic development at approximately day 7.5 in the mouse. The vital role for vascular endothelial growth factor (VEGF)-A and its receptors VEGFR-1 and -2 for blood vascular development has been clearly demonstrated by gene inactivation.8 Cross-talk between the VEGF and FGF families of growth factors has been described; expression of VEGF-A and VEGFR-2 may be induced by FGF/FGFRs in vitro9 and in vivo.10–12 The purpose of this study was to determine whether FGFR-1 is expressed in endothelial cells using several different strategies and high-quality models.

Materials and Methods

Embryonic Stem Cell and Embryoid Body Culture

The following murine embryonic stem cell lines were used: R1,13 either wild-type or clone FGFR-1lacZfgfr-1; J1,6 homozygous or heterozygous for FGFR-1 gene inactivation; or clone Lv–hFGFR-1, in which FGFR-1 was reintroduced. For creation of FGFR-1lacZfgfr-1, the LacZ expression cassette was excised from NTR–LacZ and inserted into the pPNT–FGFR-1 vector encompassing FGFR-1.
exons 11 to 17, corresponding to part of the tyrosine kinase domain. The FGFR-1/LacZ cDNA was integrated into R1 Sv129 by homologous recombination. For generation of Lv–hFGFR-1 stem cells, see http://atvb.ahajournals.org. The ES cell lines were cultured as described.14

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-rat 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 in 0.25% collagenase. The single-cell suspension was incubated with mouse 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 followed by antibody staining as described. Viable cells were identified by use of propidium iodide. The results were analyzed on a FACS Vantage 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 gel. 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 Y653-phosphorylated FGFR-1 (antibodies directed against the N-terminal part or against FGFR-1 phosphorylated at Y653). Staining of FGFR-1+ embryoid bodies (B, D) was used as a negative control. Bars, 100 µm (A, C), 200 µm (B, D). E. Semi-quantitative PCR for CD31, FGFR-1, VEGFR-2, and VE-cadherin transcript expression in CD31+ FGFR-1+ purified endothelial cells. Analysis of α-fetoprotein and β-actin transcript expression in flow-through1 and CD31+FGFR-1+ cell pool.2 F. Fluorescence-activated cell sorting analysis of CD31 and FGFR-1 expression (blue) in embryoid bodies. The insert (red) shows background fluorescence of IgG-phycoerythrin and IgG-488. An average of 4 individual experiments is presented. G. Cell cycle profile by staining for DNA content using Hoechst 33342 of the dispersed CD31/FGFR-1 antibody-labeled cells. Figures indicate the fraction of cells in the different cell cycle phases, in percent.

Figure 1. Expression of FGFR-1 in embryoid bodies and in purified endothelial cells. Whole-mount immunostaining of day 8 wild-type R1 embryoid bodies (A, C) for CD31 or FGFR-1 (antibodies directed against the N-terminal part or against FGFR-1 phosphorylated at Y653). Staining of FGFR-1+ embryoid bodies (B, D) was used as a negative control. Bars, 100 µm (A, C), 200 µm (B, D). E. Semi-quantitative PCR for CD31, FGFR-1, VEGFR-2, and VE-cadherin transcript expression in CD31+ FGFR-1+ purified endothelial cells. Analysis of α-fetoprotein and β-actin transcript expression in flow-through and CD31+FGFR-1+ cell pool.2 F. Fluorescence-activated cell sorting analysis of CD31 and FGFR-1 expression (blue) in embryoid bodies. The insert (red) shows background fluorescence of IgG-phycoerythrin and IgG-488. An average of 4 individual experiments is presented. G. Cell cycle profile by staining for DNA content using Hoechst 33342 of the dispersed CD31/FGFR-1 antibody-labeled cells. Figures indicate the fraction of cells in the different cell cycle phases, in percent.

Preparing, Sectioning, and Staining of Teratomas
1×106 R1 (FGFR-1+/-) or J1 (FGFR-1+/-) ES cells were injected subcutaneously on the back flank of female NMRI-nude mice (n = 13; M&B Animal Models) and teratomas were grown for 30 days. Animal handling was performed with ethical permission approved by the Uppsala University ethics committee and according to the UKCCCR guidelines for the welfare of animals in experimental neoplasia. Acetone fixed frozen 6-µm sections immunostained with rabbit anti-mouse FGFR-1 (sc-121 and sc-4975; Santa Cruz Biotechnology) and mouse monoclonal FGFR-1 (30104; QED Bioscience Inc) were analyzed by confocal laser-scanning microscope (Zeiss LSM 510 META) or by use of a Nikon Eclipse E1000 microscope. Please see http://atvb.ahajournals.org.

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
Collagen gels. Some but not all endothelial cells contained pericyte-coated vessel sprouts invading the endothelial cell markers VEGFR-2 and VE-cadherin. The endodermal marker \(\alpha\)-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 embryoid 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, \(\approx 12\%\) 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, \(\approx 70\%\) 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 independently of FGFR-1 antibodies, we created an embryonic stem cell line expressing \(\beta\)-galactosidase less than the control of the FGFR-1 promoter (FGFR-1 \(^{\text{lacZfgfr-1}}\)). 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 angiogenesis 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 gel\(^{16}\) (Figure 2A). FGFR-1 \(^{\text{lacZfgfr-1}}\) embryoid bodies were cultured between days 4 and 12 in 3-dimensional collagen and \(\beta\)-galactosidase activity was identified by X-gal staining. Costaining with anti-CD31 and anti-\(\alpha\)-smooth muscle cell actin antibodies was performed to identify pericyte-coated vessel sprouts invading the collagen gels. Some but not all endothelial cells contained \(\beta\)-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/\(\beta\)-galactosidase-positive cells (ie, CD31 FGFR-1 cells) was estimated to \(\approx 15\%\) 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 also expressed in hematopoietic cells. Moreover, in the sprouting 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/\(\beta\)-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-1 \(^{\text{lacZfgfr-1}}\) ES cells. A, 3-dimensional culture of embryoid 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-1 \(^{\text{lacZfgfr-1}}\) embryoid body in 3-dimensional collagen gel shows FGFR-1 expressing (arrow) or nonexpressing (arrowhead) endothelial cells. Left panel, Fluorescence staining, red; CD31, green; \(\alpha\)-smooth muscle cell actin, purple. Hoechst staining. Right panel, Light microscopy in which X-gal stain (blue) marks FGFR-1 promoter-positive cells. Bars, 200 \(\mu\)m. 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) endothelial cells in FGFR-1 \(^{\text{lacZfgfr-1}}\)-derived sprouts. Bars, 50 \(\mu\)m.
active CD31⁺ endothelial cells was higher initially at day 6 (50%) and decreased with time in conjunction with stabilization of the endothelial sprouts (30%). The mitogenic activity of CD31/β-galactosidase-positive cells showed the same pattern with an initial activity in ~50% of the CD31⁺ 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⁺FGFR-1⁺ cells. However, in the absence of exogenous VEGF-A, the activity is markedly higher in the CD31⁺FGFR-1⁻ pool than in the total CD31⁺ 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 (−/−) or both (−/−) 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.
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, was slightly increased by day 4 in the absence of FGFR-1. Runx1, which is expressed at the earliest stage of blood island development, was not affected by elimination of FGFR-1 expression. Hemangioblasts are known to express brachyury, VEGFR-2, and Tal-1/SCL. In the absence of FGFR-1, there was a 50% reduction in VEGFR-2 transcripts, whereas the level of Tal-1 expression was <10% of that in control FGFR-11/– 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 CD41 was higher or similar to the control in the cell lineage proceeded and expression of CD31 and VE-VEGFR-2 and Tal-1/SCL, development of the endothelial gibbon level as indicated by decreased expression of blood island development, was not affected by elimination of FGFR-1. Runx1, which is expressed at the earliest stage of primitive and definitive hematopoiesis, as well as endothelial cell development and growth of the mouse (Partanen J and Rossant J, unpublished). In contrast, Lee et al. showed that overexpression of FGFR-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. 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 FGFR/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 al. showed that expression of dominant-negative FGFR delivered to the vasculature of E9 mouse embryos led to disrupted embryonic and extra-embryonic vasculature. Possibly, the dominant-negative FGFR used in this study acted as a general FGFR-binding decoy, blocking the function of all FGFRs. We have previously reported that although differentiating embryoid bodies express reduced levels of VEGFR-2 transcript and protein, the VEGFR-2 appears hyperactive and promotes elevated vascularization of embryoid bodies. In agreement with Faloon et al., 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 lower or 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. 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. 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.

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. 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. Giavazzi et al. showed that overexpression of FGFR-2 leads to increased vascularization and growth of endometrial carcinoma in mice. Suppression of tumor angiogenesis was achieved by vaccination of mice with a xenogeneic FGFR-1. 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 FGFR/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.
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.

Acknowledgments

This study was supported by grants from the Swedish Cancer Society (project no. 3820–B04–09XAC), the Swedish Science council (project no. K2005–32X–12552–08A), and the Novo foundation to Lena Claesson-Welsh. Expert assistance by Charlotte Wikner is deeply appreciated. The expert guidance of Dr Jan Grawe’s in the fluorescence-activated cell sorting analyses was crucial for our work. We are also grateful to Dr Andras Nagy, Samuel Lunenfeld Research Institute, Toronto, Canada, and Dr. Chuxia Deng, Mammalian Genetics Section GDDB, National Institute of Health Bethesda, MD, for R1 and FGFR-1 gene-inactivated ES cell lines, respectively. Vascular stromatosis virus-pseudotyped lentiviral vectors (LVs) were kind gifts of Drs Luigi Naldini and Michele De Palma, San Raffaele Telethon Institute for Gene Therapy, Milan, Italy.

References

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 Partanen, Anna Dimberg and Lena Claesson-Welsh

Arterioscler Thromb Vasc Biol. published online March 17, 2005;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2005/03/17/01.ATV.0000163182.73190.f9.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/03/17/01.ATV.0000163182.73190.f9.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
Fibroblast growth factor receptor-1 expression is required for hematopoietic but not endothelial cell development

Peetra Ulrica Magnusson1, Roberto Ronca2, Patrizia Dell’Era2, Pia Carlstedt1, Lars Jakobsson1, Juha Partanen3, Anna Dimberg1 and Lena Claesson-Welsh1*

1Uppsala University, Dept Genetics and Pathology, Rudbeck Laboratory, Dag Hammarskjöldsv. 20, 751 85 Uppsala Sweden
2Unit of General Pathology and Immunology, Department of Biomedical Sciences and Biotechnology, University of Brescia, Brescia, Italy
3Institute of Biotechnology, University of Helsinki, PO Box 56, 00014 Helsinki, Finland.
*To whom correspondence should be directed using the adress above, fax +46-18-558931 or email: Lena.Welsh@genpat.uu.se

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) Biosciences/Falcon) or on tissue culture plastic dishes. All analyses were performed on four or more individual embryoid bodies at three or more individual occasions.

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 zine 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 aceton 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 blocking buffer 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 10⁴/ml, 0.8 x 10⁴/ml, 1.2 x 10⁴/ml and 1.8 x 10⁴/ml. Cells were cultured for 3 days, trypsinized in trypsin-EDTA (Gibco BRL) and counted in a particle 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 10⁴ or 1.0 x 10⁴ 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% trichloroacetic 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 gels were then washed in 0.1% Tween in PBS 3x1 hours at room temperature, followed by incubation with secondary Alexa 568 goat anti-rat IgG (Molecular Probes) and fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal anti α-smooth muscle actin (ASMA) antibodies (Sigma) in 3% BSA, 0.1% Tween in PBS at 4°C over night. Extensive washing in PBS/Tween was followed by staining of nuclei with Hoechst 33342 in PBS (1μg/ml) for 10 minutes at room temperature. After further washing the embryoid bodies were analyzed and photographed using a Nikon Eclipse E1000 microscope.

**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 washes in 0.1% Tween in PBS 3x1 hours at room temperature, fluorescence staining of cells that had incorporated BrdU was done by use of Alexa 488 goat anti-mouse highly cross absorbed (HCA) IgG (Molecular Probes) antibodies which was added and incubated over night at 4°C. After new washes in 0.1% Tween...
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 μg 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 72°C at 7 min. 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>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR-1</td>
<td>GCTGACTCTGGCCTCTACGCT</td>
<td>CAGGATCTGGACATAAGGCACAA</td>
</tr>
<tr>
<td>CD31</td>
<td>TACTGCAGGCATCGGCACAA</td>
<td>GCATTTCAGACACCTGGA</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>TACAGACCCGGCCAAACAAA</td>
<td>TTTCCCCCTGGAAATCCT</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>AGGACAGCAACCTTACCTCCTCA</td>
<td>AACTGCCCCACACTTGACCTG</td>
</tr>
<tr>
<td>AFP</td>
<td>ACCCCTTCTATGTATGCCCT</td>
<td>GCATGGCCAGAAGCCTG</td>
</tr>
<tr>
<td>β-actin</td>
<td>CACTATTGGCAACGAGCGG</td>
<td>TCCATACCCAGAAGGAAGGC</td>
</tr>
</tbody>
</table>
Table 1B. Real-time PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer (5’-3’)</th>
<th>Anti sense primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachyury</td>
<td>TAAGGAACCACCGGTCATCG</td>
<td>TTGTCCGCATAAGTTGGAGAG</td>
</tr>
<tr>
<td>Runx1</td>
<td>CAACCTCCTCTGCTCGTGC</td>
<td>AAAAGCGATGGGCAAGTC</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>TACAGACCCGGCACAACAA</td>
<td>TTTCCCCCTGGAAATCTC</td>
</tr>
<tr>
<td>Tal-1</td>
<td>GGCAGCAGAGAGACTGATCTCTCG</td>
<td>AGAAGCAAAACAGACTTTGGA</td>
</tr>
<tr>
<td>CD41</td>
<td>TGGCATGTTCCTCAACCAGC</td>
<td>TCCCCGGTAACCATCAGA</td>
</tr>
<tr>
<td>GATA-1</td>
<td>TGGGCGAGGAGGTTCGG</td>
<td>GCGGCGTTGGCTGCA</td>
</tr>
<tr>
<td>β-H1</td>
<td>TGGGAAAACCCCGGATTA</td>
<td>AAACCCCAAGCCCAAGG</td>
</tr>
<tr>
<td>CD45</td>
<td>TGGGACTGCTGAGAGTGGCA</td>
<td>GGCAGGAGGGTTCATT</td>
</tr>
<tr>
<td>CD31</td>
<td>TACTGCAGGTCACCCCTCA</td>
<td>GCACTTCGACACCTGGAT</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>AGGACAGCACAACCCTCACCTCA</td>
<td>AAAGGATACCTTGACCAGT</td>
</tr>
<tr>
<td>ASMA</td>
<td>CTGCAGAGGCAACCACTGAA</td>
<td>AGAGGCATAGGGAGGACAGCA</td>
</tr>
<tr>
<td>β-actin</td>
<td>CACTATTGGCAACGAGCGG</td>
<td>TCCATACCAAGGAGGACGC</td>
</tr>
</tbody>
</table>

Supplementary references


Supplementary figures

**Fig. I online supplement.** Construct and characteristics of FGFR-1+/lacZfgfr-1 ES cells. A. Creation of FGFR-1+/lacZfgfr-1 ES cell line by homologous recombination (TMB, transmembrane domain). B. Proliferation of FGFR-1+/lacZfgfr-1 and R1 control ES cells. C. FGF-2-induced ³H-thymidine incorporation in FGFR-1+/lacZfgfr-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.