Deregulation of Flk-1/vascular endothelial growth factor receptor-2 in fibroblast growth factor receptor-1-deficient vascular stem cell development

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Summary

We have employed embryoid bodies derived from murine embryonal stem cells to study effects on vascular development induced by fibroblast growth factor (FGF)-2 and FGF receptor-1, in comparison to the established angiogenic factor vascular endothelial growth factor (VEGF)-A and its receptor VEGF receptor-2. Exogenous FGF-2 promoted formation of morphologically distinct, long slender vessels in the embryoid bodies, whereas VEGF-A-treated bodies displayed a compact plexus of capillaries. FGF-2 stimulation of embryonal stem cells under conditions where VEGF-A/VEGFR-2 function was blocked, led to formation of endothelial cell clusters, which failed to develop into vessels. FGFR-1$^{-/-}$ embryoid bodies responded to VEGF-A by establishment of the characteristic vascular plexus, but FGF-2 had no effect on vascular development in the absence of FGFR-1. The FGFR-1$^{-/-}$ embryoid bodies displayed considerably increased basal level of vessel formation, detected by immunohistochemical staining for platelet-endothelial cell adhesion molecule (PECAM)/CD31. This basal vascularization was blocked by neutralizing antibodies against VEGFR-2 or VEGF-A and biochemical analyses indicated changes in regulation of VEGFR-2 in the absence of FGFR-1 expression. We conclude that VEGF-A/VEGFR-2-dependent vessel formation occurs in the absence of FGF-2/FGFR-1, which, however, serve to modulate vascular development.

Key words: Vasculogenesis, Angiogenesis, Embryoid body, Hemostasis, Thrombosis, Development, FGFR-1, VEGFR-2

Introduction

During vasculogenesis, blood vessels are formed through in situ differentiation of endothelial cells from precursor cells, termed angioblasts (Flamme et al., 1997). Aggregates of mesodermal angioblasts form blood islands, which are organized as a central core of hematopoietic precursor cells with an outer lining of endothelial cell precursors. The existence of a common stem cell for these two lineages has been demonstrated, which is called the hemangioblast (Risau et al., 1995; Choi et al., 1998). Maturation of the vasculature involves pruning of vessels and eventually angiogenesis: formation of capillaries from pre-existing vessels. Morphologically, angiogenesis involves the formation of cytoskeletal filopodia, which extend from the sprouting tip cell in the direction of a stimulus, i.e. a growth factor. Formation of new vessels in the adult, either because of physiological or pathological demands, occurs through angiogenesis (Folkman et al., 1995). Recent work has demonstrated the existence of circulating endothelial precursor cells, which contribute to pathological angiogenesis by integration at sites of vascular injury, and subsequent differentiation to mature endothelial cells (Isner et al., 2001).

Vascular development is guided by growth factors such as vascular endothelial growth factor-A (VEGF-A) and fibroblast growth factor-2 (FGF-2). Growth factors exert their effects by binding to cell surface-expressed receptors, which possess intrinsic tyrosine kinase activity. Kinase activation initiates intracellular signaling pathways, which lead to the establishment of biological responses. VEGF-A, which is also known as vascular permeability factor (VPF) (Dvorak et al., 1995), exerts its effect via two tyrosine kinase receptors, VEGFR-1 and VEGF receptor-2 (VEGFR-2) expressed on vascular endothelial cells. VEGFR-2 (Flk1/KDR for murine or human species, respectively) is the earliest specific marker for vascular endothelial cells (Kabrun et al., 1997). Inactivation of the genes for VEGF-A and VEGFR-2 severely impairs vasculogenesis and hematopoiesis in the embryo (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1997). In contrast, VEGFR-1 plays a negative regulatory role during vascular development. Thus, mice lacking VEGFR-1 expression die at day 8.5-9 because of an increase in the number of endothelial cell progenitors, leading to obstruction of the vessel lumen (Fong et al., 1995).

The ever-expanding family of FGF peptides and their four receptors (FGF receptor-1, -2, -3 and -4) play important roles in many cellular functions (for a review, see Powers et al., 2000). FGF-2 appears to be crucial in inducing angioblast formation from uncommitted mesoderm in the quail (Poole et al., 2001). Endothelial cells as well as endothelial progenitor cells express FGF receptor-1 (FGFR-1) in vitro and in vivo, at least in certain

tissues, and respond to FGF-2 (Cross and Claesson-Welsh, 2001; Burger et al., 2002). However, the role of FGF-2/FGFR-1 in vascular development remains controversial and it has not been clarified through genetic analyses. Gene inactivation of FGF receptor-1 (FGFR-1) leads to embryonal death during gastrulation between embryonal day 7.5-9.5. Lack of FGFR-1 expression is compatible with mesoderm formation, but the embryos display patterning defects (Deng et al., 1994). Gene inactivation of FGF-2 leads to a decreased vascular tone and low blood pressure in otherwise phenotypically normal mice (Dono et al., 1998). The role of FGF/FGF receptors in physiological and pathological vessel formation is complex and this subject merits further studies.

We have exploited embryoid bodies derived from pluripotent murine embryonal stem cells established on day 3.5 post conception (dpc) from the inner cell mass of blastocysts (Nagy et al., 1993), as a model for analysis of vascular development and the role of angiogenic growth factors in this process. Removal of leukemia inhibitory factor (LIF) from the culture medium in combination with culture conditions that favor self-aggregation, promotes spontaneous differentiation of the pluripotent embryonal stem cells. After 24-48 hours, a primitive endoderm layer surrounds the aggregated stem cells, the embryoid body. Thereafter visceral and parietal endoderm develops around a core of primitive ectoderm cells. Subsequently, the core will develop into the three germ layers; endoderm, mesoderm and ectoderm (Doetschman et al., 1985; Keller, 1995; Rathjen and Rathjen, 2001). The earliest stages of hematopoietic development within embryoid bodies follows an ordered sequence of events mimicking those observed in the developing embryo (Keller, 1995; Choi et al., 1998). At later stages of differentiation (approximately day 8) in the EBs, sprouting angiogenesis occurs, which is further increased and modified by growth factor treatment. Embryoid bodies have also been reported to faithfully reproduce cardiomyogenesis (Metzger et al., 1995; Müller et al., 2000), neurogenesis (Strubing et al., 1995; Kawasaki et al., 2000) and muscle cell development (Yamashita et al., 2000).

In this study, we have employed the embryoid body model to study the effect of FGF-2/FGFR-1 on vascular development. We show that FGF-2 stimulates formation of endothelial cells in the absence of VEGFR-2 expression, based on morphology and endothelial cell marker expression, but these cells fail to form vessels. However, there is no strict requirement for FGFR-1 function during vascular development, as VEGF-A was found to stimulate formation of a characteristic capillary plexus in FGFR-1$^{-/-}$ embryoid bodies. Embryoid bodies lacking expression of FGFR-1 displayed a marked increase in basal vessel formation, which was dependent on VEGF-A/VEGFR-2. Therefore, inactivation of FGFR-1 appears to result in deregulation of VEGFR-2 function.

Materials and Methods

Tissue culture

R1 murine embryonal stem (ES) cells derived from mouse strain SvJ129 (Nagy et al., 1993), were a kind gift from Andras Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada. Murine ES cells lacking expression of FGFR-1 (FGFR-1$^{-/-}$) (Deng et al., 1994) were a kind gift from Chuxia Deng, Mammalian Genetics Section GDDB, NIDDK, National Institute of Health Bethesda, MD 20892, USA, and VEGFR-2$^{-/-}$ (Shalaby et al., 1995), a kind gift from Janet Rossant, Samuel Lunenfeld Research Institute Mount Sinai Hospital, Toronto, Ontario, Canada, were also employed. ES cells were cultured in the presence or absence of a murine embryonal fibroblast (MEF) feeder layer, as indicated, in ES medium composed of Dulbecco's modified Eagle's medium/glutamax (Invitrogen, Rockville, MD) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 25 mM Hepes, 1.2 mM sodium pyruvate (Invitrogen), 19 μM monothioglycerol (Sigma, St. Louis, MO) and 1,000 U/ml recombinant leukemia inhibitory factor (LIF; Chemicon International, Harrow, UK) (Hilton and Gough, 1991). Cells were grown at 37°C with 5% CO_2. Medium was changed every day and splitting was done every second or third day. MEFs were growth arrested by treatment with 10 μg/ml of mitomycin C (Sigma) dissolved in ES medium without LIF (EB medium) at 37°C for 2 hours and then washed twice in phosphate-buffered saline (PBS). MEF cells were cultured in ES medium for 1 hour before plating of ES cells. To induce differentiation, LIF was withdrawn from the medium. Aggregation was induced by placing 20 μl drops of 1200 cells/drop on the lid of a non-adherent tissue culture dish placed over sterile PBS. The medium was supplemented with FGF-2 (a kind gift from Andrew Baird, Ciblex Corporation, San Diego, CA) and VEGF-A165 (Peprotech, Rocky Hill, NJ) at different concentrations as indicated. The day when LIF was removed from the medium and droplets were formed was denoted day 0. The drops were left hanging on the lid for 3-5 days, in 37°C and 5% CO_2, and then plated one by one in 8-well glass culture slides (Falcon, Frankling Lakes, NJ). All analyses were performed on four or more embryoid bodies at three or more individual occasions.

Peroxidase staining

Embryoid bodies (EBs) cultured on glass culture slides were washed with Tris-buffered saline (TBS) and 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) overnight at 4°C. The EBs were permeabilized for 15 minutes with 0.2% Triton X-100 in TBS, followed by blocking of endogenous peroxidase activity by 3% H_2O_2 in methanol, for 30 minutes. The EBs were washed in TBS and blocked in 0.1 M Tris-HCl/0.15 M NaCl/blocking (TNB) buffer (kit NEL700; PerkinElmer Life Sciences, Boston, MA) for 1 hour, and incubated with one of the following primary antibodies: rat anti-mouse CD31 (Becton Dickinson (BD) Biosciences, Erembodegem, Belgium), rat anti-mouse VEGFR-2 (BD), rat anti-mouse vascular endothelial (VE)-cadherin (BD) or rabbit anti-mouse VE-cadherin (kind gift from Rupert Timpl, Max-Planck Institute, Martinsried, Germany) diluted in TNB buffer overnight at 4°C. The following day, EBs were washed 3× 5 minutes in TBS and stained with appropriate biotinylated secondary antibodies: goat anti-rat IgG (Vector Laboratories Inc., Burlingame, CA) or goat anti-rabbit IgG (Vector), diluted in TNB buffer for 1 hour at room temperature. After a 30-minute incubation with streptavidin-HRP (Vector) and washing for 30 minutes with TBS, the chromogen substance (AEC kit from Vector) was added for 10-15 minutes at room temperature. After counterstaining with 0.1% Hematoxylin solution (Sigma) and mounting with Ultramount aqueous mounting medium (Dako, Glostrup, Denmark) the result was studied and photographed using an inverted Nikon Eclipse TE 300 microscope or a transmitted light Nikon Eclipse E1000 microscope (Nikon, Kanagawa, Japan). Quantification of the area (without holes) and length of CD31 staining was performed with Easy Image Analysis software (Tekno Optik AB, Stockholm, Sweden). Compensation for background was performed to avoid quantification of unspecific staining. Statistical analysis was done by analysis of variance (ANOVA) followed by multiple comparison by Fisher's method using the StatView® computer program.

Neutralizing antibodies

Rat anti-mouse VEGFR-2 antibody (DC101, ImClone Systems, Inc,

New York, NY) or goat anti-mouse VEGF antibody (AF-493-NA, R&D Systems, Minneapolis, MN) was added to FGFR-$1^{-/-}$ or FGR-$1^{+/-}$ EBs from day 6 to 8 at a final concentration of 30 μg/ml and 5 μg/ml respectively, followed by fixation and peroxidase staining with CD31 as described above. As control, EBs treated with anti-rat IgG (30 μg/ml; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or anti-goat IgG (5μg/ml; Jackson) were analyzed in parallel.

Fluorescence staining

EBs were grown and fixed as described above, and then permeabilized for 15 minutes with 0.2% Triton X-100 in TBS, followed by blocking with TNB buffer at room temperature for 1 hour. The primary antibodies rat anti-mouse CD31 (BD), goat anti-mouse CD31 (Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-mouse VEGFR-2 (BD), rabbit anti-VE-cadherin (BD), rabbit anti-human von Willebrand factor antigen (vWF) (DAKO), goat anti-mouse T-cell acute leukemia/stem cell leukemia (TAL-1/SCL, Santa Cruz) and fluorescein isothiocyanate (FITC)-labeled mouse monoclonal anti-α smooth-muscle actin (ASMA) (Sigma) were incubated in single or mixed solution overnight at 4°C as indicated. The following day, the EBs were washed for 30 minutes with TBS and incubated for 1 hour at room temperature with appropriate secondary antibodies; Alexa 568 goat anti-rat IgG, Alexa 488 goat anti-rabbit IgG, Alexa 568 donkey anti-goat IgG, or Alexa 488 donkey anti-rat IgG (all from Molecular Probes, Eugene, OR) diluted in TNB buffer. For TAL-1/SCL staining, biotinylated secondary rabbit anti-goat IgG (Vector) incubation for 1 hour was followed by Alexa 488 Streptavidin for another hour. The EBs were then washed in TBS for 30 minutes, dried and mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL).

Benzidine and benzidine/CD31 staining

Erythroblasts in EBs were stained for hemoglobin using benzidine. EBs plated on 8-well glass culture slides were washed with phosphate-buffered saline (PBS), then incubated in a freshly made staining solution containing 0.87 mg/ml benzidine (Sigma), 23 mM sodium acetate buffer (pH 4.7), 0.9% H_2O_2, 61.22% ethanol at room temperature for 15 minutes. EBs were then fixed in 4% *p*-formaldehyde (*p*-FA) for 10 minutes at room temperature. When the rat anti-mouse CD31 antibody (BD) was combined with benzidine staining, the protocol for peroxidase staining was followed except that after the incubation with secondary biotinylated goat anti-rat IgG antibody (Vector), the TSA kit (NEL700 kit, PerkinElmer Life Sciences, Inc., Boston, MA) was used to amplify the staining after *p*-FA fixation. After a 30-minute incubation with streptavidin-HRP from the NEL700 kit, the EBs were washed 3× 5 minutes with 0.1 M Tris-HCl and 0.15 M NaCl (TN) wash buffer. Thereafter, the samples were incubated for 8 minutes with biotinyl tyramide (amplification reagent) diluted 1:50 in 1× Amplification Diluent from the NEL700 kit. After washes with TN wash buffer, the streptavidin-HRP incubation was repeated for 30 minutes. After further washes, the chromogen substance (AEC kit from Vector) was added for 10-15 minutes at room temperature.

Immunoblotting

EBs were plated out on day 4 in 3.5 or 10.0 cm tissue culture dishes to allow them to attach. After indicated times of culture in basal medium the EBs were lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 500 μM Na_3VO_4, 1% aprotinin, 10 μg/ml leupeptin and 1 mM fenylmethyl sulfonylfluoride. Growth factor-treated EBs were starved for 16 hours in 0.2% BSA in ES medium without LIF and then stimulated with growth factors as indicated for 20-30 minutes prior to cell lysis. Cell lysates were centrifuged for 15 minutes at 4°C and the protein concentration of the supernatant was measured using the BCA protein detection kit (Pierce, Rockford, IL). Total cell lysates with approximately 10 μg protein/lane was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 7% or 10% gels and transferred to Hybond C-Extra nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). The membrane was blocked in TBS-T (10 mM Tris HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20) containing 5% bovine serum albumin (BSA, Boehringer Mannheim, Mannheim, Germany) for 3 hours and then incubated with rat anti-mouse VEGFR-2 antibody (BD), goat anti-mouse CD31 antibody (Santa Cruz), or the rabbit anti-mouse VE-cadherin antibody (from Rupert Timpl) or rabbit anti-mouse β-catenin antibody (Cell Signalling Technologies, Beverly, MA) overnight at 4°C. After vigorous washing with TBS/0.1% Tween 20 (TBS-T), the membrane was incubated with peroxidase-conjugated anti-rat antibody, anti-rabbit antibody (Amersham Biosciences) or anti-goat antibody (Santa Cruz). Immunoreactive bands were then visualized using the ECL western blotting detection reagents (Amersham Biosciences). The intensity of protein bands was quantified, after scanning of the blot, by software Image Gauge, Fujifilm.

Semi-quantitative PCR analysis

Total RNA was extracted from 8-day-old basal treated R1 and FGFR-$1^{-/-}$ EBs using the RNeasy mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Contaminating genomic DNA was digested with DNase I (Amersham Pharmacia Biotech, Uppsala, Sweden) and 1 μg total RNA was used for first strand cDNA synthesis using oligo(dT) primers and the Advantage RT-for-PCR-Kit (Clontech, Palo Alto, CA). Primers were made (Invitrogen) for:

FGFR-1 sense: (5′-3′) GCTGACTCTGGCCTCTACGCT
FGFR-1 antisense: (5′-3′) CAGGATCTGGACATACGGCAA
β-actin sense: (5′-3′) CACTATTGGCAACGAGCGG
β-actin antisense: (5′-3′) TCCATACCCAAGAAGGAAGGC

β-actin was used as an internal control.

FGFR-1 primers yielded a 650 base pair product and the β-actin primers an 80 base pair product. PCR for FGFR-1: 10 minutes activation at 95°C, followed by 30 cycles at 95°C for 30 seconds, 70°C for 30 seconds and 72°C for 1 minute. PCR for β-actin: 10 minutes activation at 95°C, followed by 30 cycles at 95°C for 15 seconds and 60°C for 1 minute.

Real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from ES cells and 8-day-old FGFR-$1^{+/-}$ and FGFR-$1^{-/-}$ EBs using the RNeasy mini kit (Qiagen). Contaminating genomic DNA was digested with DNase I (Amersham Biosciences) 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 were made (Invitrogen) for:

CD31 sense: (5′-3′) TACTGCAGGCATCGGCAAA
CD31 antisense: (5′-3′) GCATTTCGCACACCTGGAT
VEGF-C sense: (5′-3′) AAGACCGTGTGCGAATCGA
VEGF-C antisense: (5′-3′) ACACAGCGGCATACTTCTTCAC
Ephrin B2 sense: (5′-3′) AAGTACCGCAGGAGACACCG
Ephrin B2 antisense: (5′-3′) GGCCAGTGTGCTGAGAGACA
VEGFR-2 sense: (5′-3′) TACAGACCCGGCCAAACAA
VEGFR-2 antisense: (5′-3′) TTTCCCCCCTGGAAATCCT
VEGFR-1 sense: (5′-3′) GGGCAGACTCTTGTCCTCAACT
VEGFR-1 antisense: (5′-3′) CAGCTCATTTGCACCCTCGT
VEGF-A sense: (5′-3′) AAGGAGAGCAGAAGTCCCATGA
VEGF-A antisense: (5′-3′) CTCAATTGGACGGCAGTAGCT
β-actin sense: (5′-3′) CACTATTGGCAACGAGCGG
β-actin antisense: (5′-3′) TCCATACCCAAGAAGGAAGGC

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 2×

SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), 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. The threshold cycle (C_T) value, was calculated for each sample using the ABI Prism 7700 instrument. Transcript levels were then normalized against β-actin levels and changes in transcript levels were expressed as relative values.

In vitro kinase assay

EBs cultured in basal conditions were starved in EB medium, 0.2% BSA for 16 hours and stimulated as indicated with 100 ng/ml of VEGF-A for 10 minutes. After stimulation, the EBs were washed twice with TBS containing Na_3VO_4 and lysed in NP40 lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP40, 100 μM Na_3VO_4, 1 mM DTT, 1% aprotinin and 1 mM PMSF). After mechanical dissociation of the EBs with a 21G syringe, followed by centrifugation, the supernatant was immunoprecipitated with anti-VEGFR-2 antibody (RS-2) on ice for 1 hour. The immunocomplex was collected using protein A-Sepharose, washed with lysis buffer and resuspended in kinase buffer (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.05% Triton X-100 and 1 mM DTT). In vitro phosphorylation was carried out in the presence of 1.0 μCi [γ-^{32}P]ATP/sample for 10 minutes at room temperature. The reactions were stopped by addition of sample buffer (100 mM Tris-HCl, pH 6.8, 10% SDS, 10% glycerol, 0.2% Bromophenol Blue and 4% β-mercaptoethanol). The boiled samples were separated by SDS-containing 7% polyacrylamide gels. The gels were then incubated in destain for 30 minutes, 2.5% glutaraldehyde for 30 minutes, 1 M KOH at 55°C for 30 minutes and destain for 30 minutes. After drying, the gels were analyzed using a Phospho Imager, Fujifilm BAS-1800 II.

Results

Creation of embryoid bodies

Embryoid bodies (EBs) are spheres of aggregated, differentiating embryonal stem (ES) cells. We wished to evaluate the accuracy and versatility of EBs as a model for signal transduction in vasculogenesis and angiogenesis. There are different options for the formation and culture of EBs; as suspension culture (Risau et al., 1988), as spinning flask culture (Sauer et al., 2000) or by use of the hanging drop technique (Mummery et al., 1990). To induce embryoid body formation in hanging drops, a defined cell suspension was placed in 20 μl droplets on the lid of a culture dish, left for 3-5 days, and thereafter flushed down, analyzed or cultured further. This strategy makes it possible to control the size of the EB and circumvents paracrine stimulation between EBs, and therefore allows a very high degree of reproducibility.

Expression of endothelial cell markers

We studied vascular development in the EBs by analyzing protein expression of known endothelial cell markers over time. Expression of VEGFR-2, CD31 and VE-

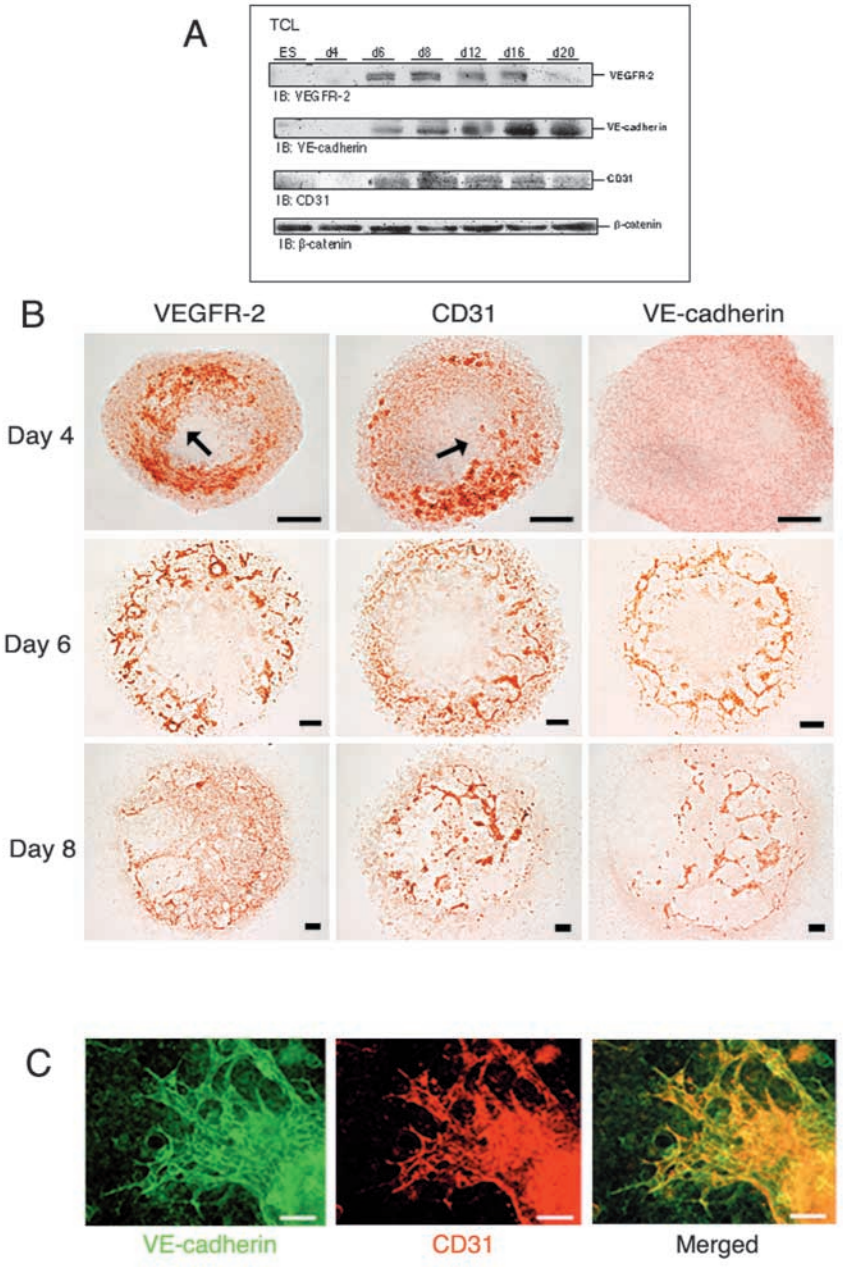


Fig. 1. Expression of endothelial-specific markers during vascular development in EBs. (A) Immunoblotting was performed on total cell lysates of ES cells and EBs cultured for the indicated number of days, using antibodies against VEGFR-2, VE-cadherin and CD31. β-catenin expression was analyzed as a loading control. (B) Immunohistochemical staining for VEGFR-2, VE-cadherin and CD31 in EBs cultured for the indicated number of days. One representative EB of eight for each condition is shown. Each condition was examined on two or more separate occasions. Arrows indicate blood islands visualized by VEGFR-2 and CD31 staining in day 4 EBs. Scale bars: 100 μm day 4 and 6; 200 μm day 8. (C) VE-cadherin was expressed in a 10-day old EB at the cell-cell contacts of the endothelial cells and the expression overlapped with CD31 expression. Scale bars: 100 μm.

cadherin was followed from day 0 to day 20, by immunoblotting (Fig. 1A). VEGFR-2 expression appeared at day 4, and reached maximal levels by day 8. Thereafter, the expression level declined and was essentially gone by day 20. A similar pattern was seen for CD31 expression. CD31 expression was barely detectable in the stem cells in the presence of LIF, in contrast to previous reports (Vittet et al., 1996). These results confirm and extend previous studies (Redick and Bautch, 1999) and agree with data reported for vascular development in vivo (Kabrun et al., 1997). VE-cadherin expression appeared at day 6 and reached a maximum at day 16.

To follow changes in morphological patterns, immunohistochemical staining was performed (Fig. 1B). At day 4, expression of CD31 and VEGFR-2 appeared in immature clusters of cells possibly representing blood islands (Fig. 1B arrows). Expression of VE-cadherin was not detected at this point. On day 6, all three markers were expressed in morphologically immature vessels, which developed from the blood-island-like clusters. VE-cadherin staining was localized to endothelial cell-cell borders and showed an overlapping staining pattern with CD31 from day 6 onwards (Fig. 1C).

Around day 8, fine capillary-like structures were evident from staining for all three markers (Fig. 1B). Vessel remodeling continued further up until day 20, whereafter the vessels disintegrated and the quality of the EB cultures declined (data not shown). Together, these results indicate that expression of endothelial cell markers was faithfully reproduced in the EB model.

Vasculogenesis and angiogenesis in the EBs

In order to show the presence of endothelial precursor stages in the EB, we examined the expression of the hematopoietic lineage marker TAL-1/SCL (Porcher et al., 1996). Fig. 2A shows that 6- and 8-day-old EBs contained clusters of endothelial cells and immature vessels that co-expressed TAL-1/SCL and VEGFR-2, which is compatible with the identification of these cells as hemangioblasts (Faloon et al., 2000; Drake and Fleming, 2000). At day 12 onward, the EBs displayed clear signs of angiogenesis as detected by anti-CD31 staining, with characteristic sprouting vessels whose tip cells extended multiple filopodia (Fig. 2B). These sprouting vessels were negative for TAL-1/SCL, indicating maturation of the hemangioblasts to endothelial cells (data not shown). In EBs cultured for 16 days, clusters of erythroblasts were visualized inside the vessel lumen, as shown by in situ benzidine staining for hemoglobin (Fig. 2C).

Von Willebrand Factor (vWF) marks endothelial cells relatively late in development, around day 8 in EBs, and has been reported to be expressed preferentially in embryonal veins (Thurston et al., 1998). In the EBs, expression of vWF was confined to a relatively small population of larger, non-sprouting CD31-positive vessels (Fig. 2D, arrowhead). In contrast, sprouting CD31-positive vessels lacked vWF expression (Fig. 2D, arrow).

Thus, in a highly reproducible manner, the EBs underwent a temporally correct development with clear indications of vasculogenesis in an early phase and sprouting angiogenesis, yielding lumen-containing vessels, in a later phase.

Effects of exogenous growth factors on vascular development in the EBs

Treatment of EBs with VEGF-A and FGF-2 promoted formation of characteristic vascular networks unique for each growth factor, as shown by immunohistochemical staining for CD31. The growth factors were added on day 0 to the hanging drops and again at regular intervals until day 8, at

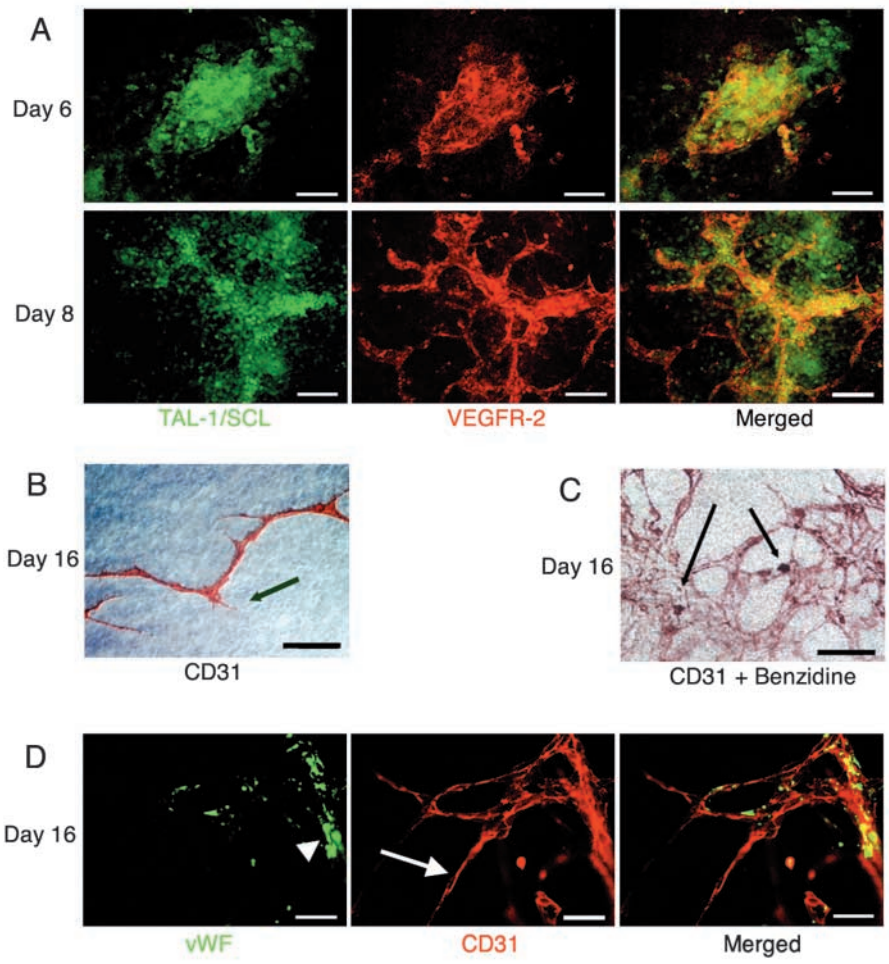


Fig. 2. Hallmarks of vascular development in the EBs. (A) Co-localization of TAL-1/SCL and VEGFR-2 at day 6 and 8. Note that more mature vessels did not stain positive for TAL-1. (B) At day 16, sprouting angiogenesis could be readily identified morphologically in the EBs, as fine vessels ending in a brush of lamellopodia from the endothelial cell in the distal tip of the vessel (arrow). (C) Benzidine-stained erythroblasts visualized as dark clusters (arrows) inside the CD31-positive vessels. (D) While CD31 expression was seen in sprouting vessels (arrow), vWF staining appeared in larger vessels (arrowhead). Scale bars: 100 μm.

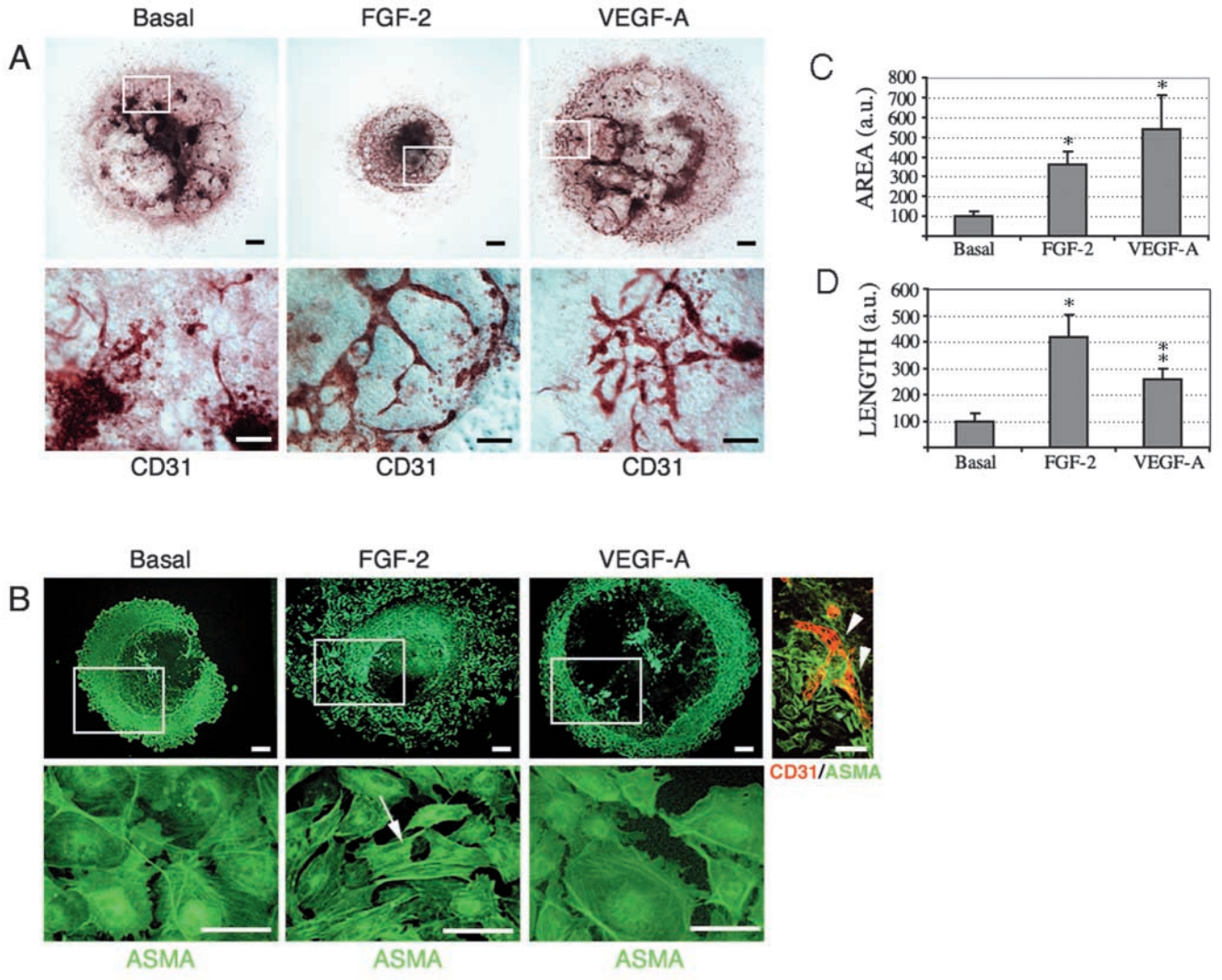
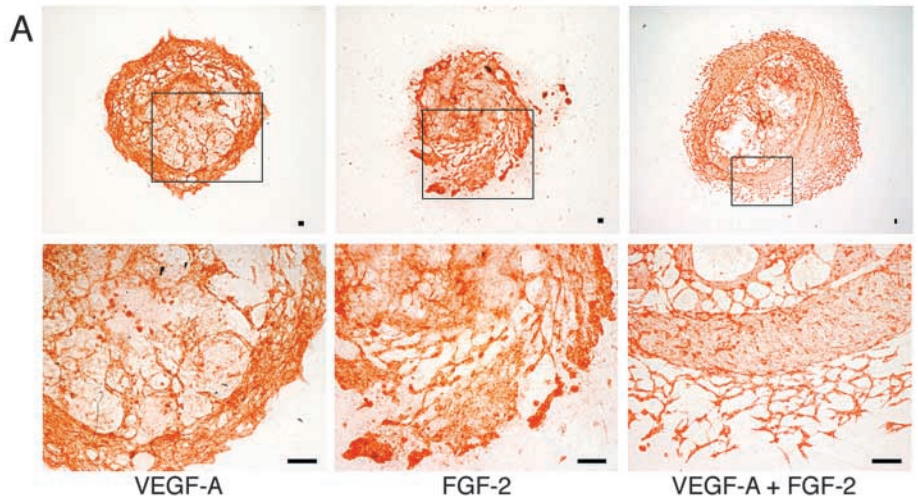


Fig. 3. Distinct effect of exogenous FGF-2 and VEGF-A on vascular development. EBs were cultured in the absence (basal) or presence of 20 ng/ml VEGF-A or FGF-2 from day 0 to day 8 and stained immunohistochemically or by fluorescence using antibodies against CD31 (A) or α-smooth muscle actin (ASMA) (B) as indicated. Each growth factor produced distinct morphologies of the endothelial and smooth muscle cell pools. The bottom panels in A and B are higher magnification images of part of the boxed regions in the upper panels. Scale bars: A, 300 μm; B, upper panel 200 μm, lower panel 100 μm. (B) Co-localization (arrowheads) of CD31-positive cells (red) with ASMA cells (green) is shown in the far right image. (C) Quantification of CD31 staining as area (i.e. area without holes) in 8-day EBs (mean±s.d., n=6). *P<0.0001 FGF-2 compared to basal and FGF-2 compared to VEGF-A. (D) Quantification of CD31 staining as vessel length in 8-day EBs (mean±s.d., n=6). *P<0.0001 FGF-2 compared to basal, **P=0.0002 VEGF-A compared to basal.

which point the EBs were processed for immunostaining (Fig. 3A). VEGF-A treatment promoted migration of the endothelial cells to the EB margin, where short discontinuous vessels formed a dense plexus at day 8. In contrast, FGF-2 stimulated formation of long and slender vessels, which extended from the center of the EB to the periphery, slightly twisted around a central point. Quantification of vessel area (Fig. 3C) and vessel length (Fig. 3D) shows that vessels in VEGF-treated bodies covered a larger area, whereas vessels in the FGF-2-treated bodies were longer.

The treatment also affected the smooth muscle cell compartment, detected by staining for α-smooth muscle actin (ASMA). As shown in Fig. 3B, VEGF-A treatment led to a peripheral displacement of smooth muscle cells, whereas FGF-2 promoted scattering of these cells, which were arranged in parallel bundles, and displayed increased formation of actin stress fibers (arrow in Fig. 3B). Occasionally, the endothelial cells were in close contact with the smooth muscle cells (Fig. 3B arrowheads indicate CD31 in red and ASMA in green) and co-localization was increased in EBs treated with platelet-derived growth factor (C.R., unpublished). Thus, both endothelial and smooth muscle cell compartments appeared to be distinctly affected, both temporally and morphologically, by FGF-2 and VEGF-A.

Dose response analyses of the effects of VEGF-A and FGF-2 showed that increasing concentrations up to 80 ng/ml enhanced the tendency of VEGF-A to promote a compact peripheral plexus, and of FGF-2 to induce formation of long, twisted vessels (Fig. 4A). EBs treated with a combination of both growth factors (20 ng/ml FGF-2 and 30 ng/ml VEGF-A),



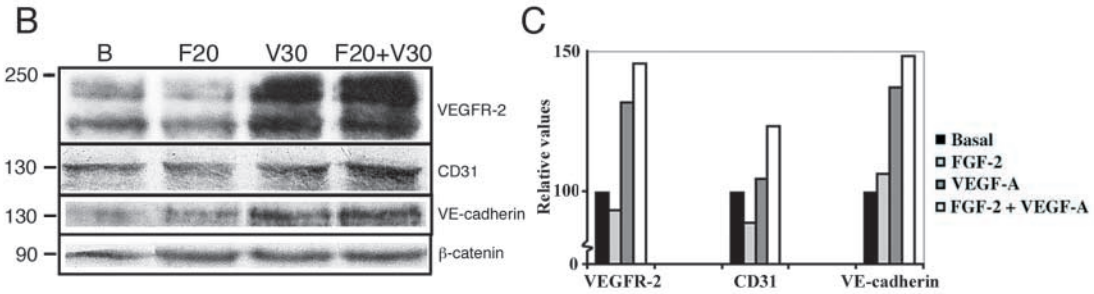


Fig. 4. Combined effects of FGF-2 and VEGF-A. (A) EBs were cultured in the presence of individual factors VEGF-A (80 ng/ml) or FGF-2 (80 ng/ml) or a combination of the two (20 ng/ml FGF-2 and 30 ng/ml VEGF-A) as indicated from day 0 to day 8, followed by immunohistochemical anti-CD31 staining. Scale bars: 100 μm (upper) and 200 μm (lower). Boxed regions are shown at a higher magnification in the lower panels. (B) Western blotting of total lysates of 8-day EBs showed growth factor-regulated expression of VEGFR-2, CD31 and VE-cadherin. β-catenin was used as loading control. B, basal; F20, FGF-2 at 20 ng/ml; V30, VEGF-A at 30 ng/ml; F20+V30, FGF-2 at 20 ng/ml and VEGF-A at 30 ng/ml). (C) Quantification of protein bands in western blot shown in Fig. 4B.

displayed the characteristic signs of each factor; the peripheral truncated capillaries typical for VEGF-A and the rotation typical for FGF-2 (Fig. 4A). The combined treatment also gave rise to sheets of endothelial cells, possibly implicating hyperstimulation of the endothelial cells. Growth factor-treated EBs were lysed and analyzed for expression of VEGFR-2, CD31 and VE-cadherin (Fig. 4B). Treatment with FGF-2 alone (20 ng/ml) did not affect expression of these endothelial cell markers. Exposure to VEGF-A (30 ng/ml) substantially elevated VEGFR-2 and VE-cadherin levels, whereas the expression level of CD31 remained unaffected. Combined treatment (FGF-2, 20 ng/ml and VEGF-A, 30 ng/ml, respectively) further increased expression of all three markers. Fig. 4C shows quantification of the intensities of VEGFR-2, CD31 and VE-cadherin expression relative to basal levels. Thus gene regulation in response to VEGF-A and FGF-2 appeared quite distinct.

Vascular development in FGFR-1$^{-/-}$ and VEGFR-2$^{-/-}$ EBs

To determine the direct contribution of FGF-2/FGFR-1 to vascular development, EBs derived from ES cells with targeted inactivation of the VEGFR-2 gene (Shalaby et al., 1995) or the FGFR-1 gene (Deng et al., 1994) were examined. The VEGFR-2$^{-/-}$ EBs lacked vessels in the basal condition. VEGF-A treatment failed to promote vessel formation at any of the doses tested (from 30 to 80 ng/ml; Fig. 5A). FGF-2-treatment led to formation of CD31-positive clusters of cells located in the periphery in a slightly rotated pattern. Further culturing of these EBs in the presence of FGF-2 did not result in connection of the clusters and formation of vessels (data not shown).

To determine whether VEGF-A-induced vascular development was critically dependent on FGF-2/FGFR-1, we examined FGFR-1$^{-/-}$ EBs, which were treated with FGF-2 or VEGF-A for 8 days and stained for CD31 (Fig. 5A). In the FGFR-1$^{-/-}$ EBs, the basal level of vessel formation was considerably increased and vessel formation was not further enhanced by treatment with FGF-2 at a wide range of concentrations. In contrast, VEGF-A at 80 ng/ml, gave rise to the peripheral capillary plexus characteristic for this growth factor. Quantification of CD31 staining showed that both the area (Fig. 5B) and length (Fig. 5C) of vessels were increased 3-fold and close to 2-fold, respectively, in the FGFR-1$^{-/-}$ EBs compared to FGFR-1$^{+/-}$ EBs. In heterozygous FGFR-1$^{+/-}$ EBs (Fig. 5A), FGF-2- and VEGF-A-stimulated vessel formation was indistinguishable from that observed in the wild-type R1 EBs, demonstrating that the features of vascular development in the FGFR-1$^{-/-}$ EBs was not due to a random clonal variation.

ASMA-positive cells in the basal and VEGF-A treated cultures of FGFR-1$^{-/-}$ EBs were morphologically indistinguishable from those in the wild-type EBs (Fig. 3B). In response to FGF-2, the FGFR-1$^{-/-}$ ASMA-positive cells arranged in parallel streaks and individual cells showed actin stress fiber formation (arrow in Fig. 5D). This indicates that FGFR-1 is not essential for transduction of FGF-2 effects on smooth muscle cells and agrees with recent data from Dell'Era and coworkers (Dell'Era et al., 2003) who showed intact expression of FGFR-2 and -3 but decreased expression of FGFR-4 in FGFR-1$^{-/-}$ bodies. Furthermore, in agreement with

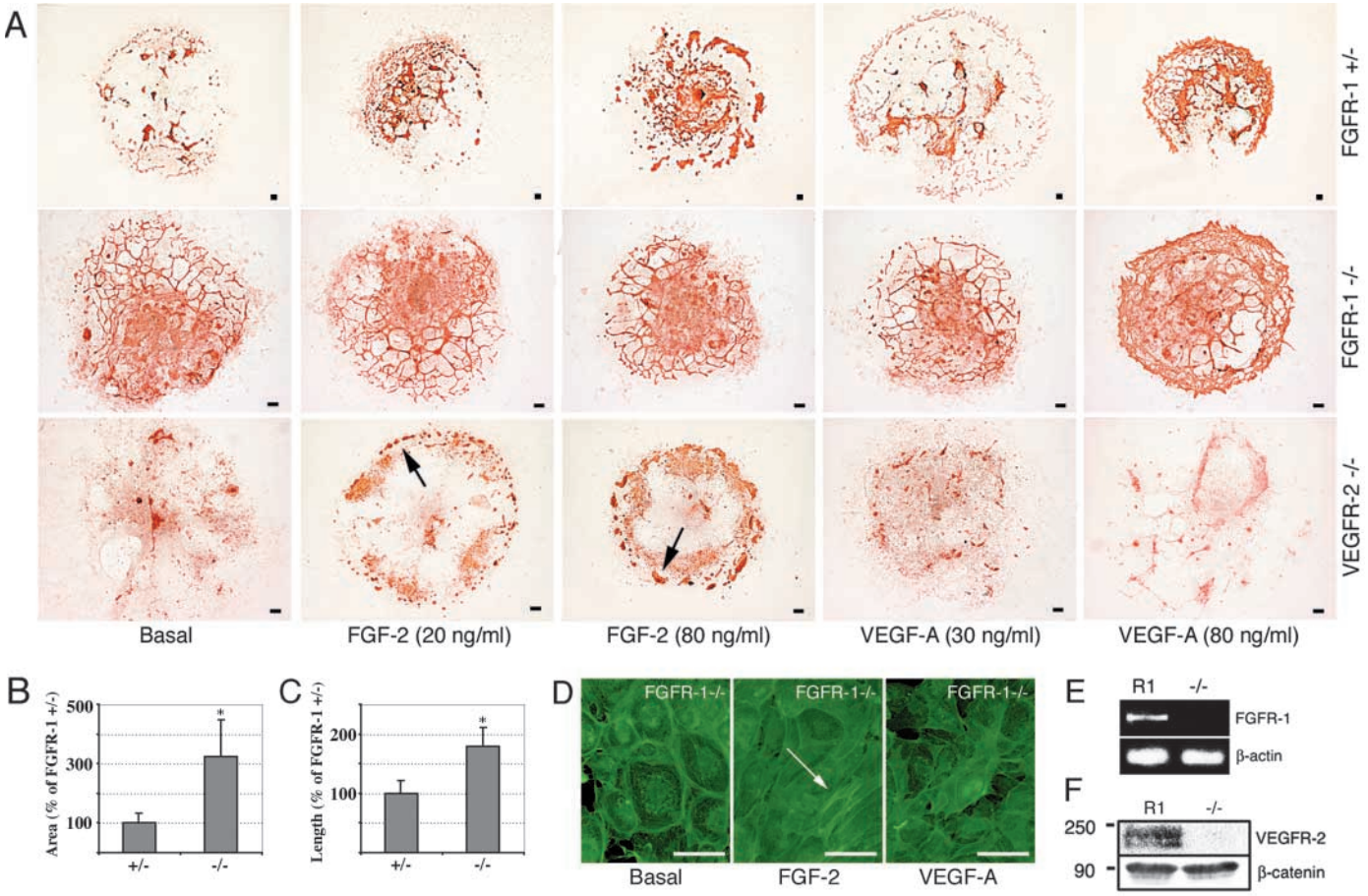


Fig. 5. Effects of angiogenic growth factors on vascular development in FGFR-1$^{-/-}$ and VEGFR-2$^{-/-}$ EBs. (A) EBs lacking expression of FGFR-1 or VEGFR-2 (indicated by $^{-/-}$) and control FGFR-1$^{+/-}$ EBs were treated with VEGF-A or FGF-2 at indicated concentrations from day 0 to day 8 and stained for CD31. FGFR-1$^{-/-}$ showed a marked increase in basal vascularization compared to FGFR-1$^{+/-}$ control EBs. Treatment with FGF-2 had no additional effect on vascularization in the FGFR-1$^{-/-}$ EBs, whereas VEGF-A at 80 ng/ml stimulated formation of a typical peripheral capillary plexus. In the VEGFR-2$^{-/-}$ EBs, FGF-2 at different concentrations stimulated CD31 positive cells to migrate and form clusters in the periphery (arrows). VEGF-A was essentially without effect. Scale bars: 200 μm. (B) Quantification of area of CD31 staining (i.e. area without holes) in 8-day FGFR-1$^{+/-}$ (+/–) and FGFR-1$^{-/-}$ (–/–) EBs. Results are expressed as percentage of control FGFR-1$^{+/-}$ EBs (mean±s.d., n=5). *P=0.0438 relative to control. (C) Quantification of CD31 staining as vessel length in 8 day FGFR-1$^{+/-}$ (+/–) and FGFR-1$^{-/-}$ (–/–) EBs. Results are expressed as percentage of control FGFR-1$^{+/-}$ EBs (mean±s.d., n=5). *P=0.0019 FGFR-1$^{-/-}$ compared with FGFR-1$^{+/-}$. (D) Immunofluorescence staining for α-smooth muscle actin in FGFR-1$^{-/-}$ EBs. Treatment with FGF-2 resulted in stress fiber formation and lining up of cells (arrow) in agreement with the effect of FGF-2 in the R1 EBs (cf. Fig. 3B). Scale bars: 100 μm. (E) Semi-quantitative PCR on cDNA derived from 8-day-old R1 and FGFR-1$^{-/-}$ EBs using FGFR-1 specific primers; as a control, β-actin transcript levels were analyzed. (F) Immunoblotting for VEGFR-2 on total cell lysates from R1 or VEGFR-2$^{-/-}$ EBs cultured for 8 days. β-catenin was used as a loading control.

Dell'Era's findings, the FGFR-1$^{-/-}$ EBs lacked contracting cardiomyocytes (data not shown).

To confirm that the targeted FGFR-1$^{-/-}$ ES cells were of correct identity, we performed PCR reactions using FGFR-1-specific primers and β-actin control primers, on RNA isolated from wild-type R1 or from FGFR-1$^{-/-}$ EBs, cultured for 8 days (Fig. 5E). There was no detectable expression of FGFR-1 in the targeted EBs. VEGFR-2$^{-/-}$ EBs were shown to lack expression of VEGFR-2 by immunoblotting, in comparison to wild-type R1 EBs (Fig. 5F). Expression of β-catenin was employed as a loading control.

Expression of endothelial cell markers in FGFR-1$^{-/-}$ EBs

Expression levels of angiogenic factors were quantified by real time PCR in 8-day-old FGFR-1$^{+/-}$ and FGFR-1$^{-/-}$ EBs (Fig. 6A). Expression of CD31, VEGF-C, EphB2, VEGF-A and VEGFR-1 was similar in FGFR-1$^{-/-}$ and FGFR-1$^{+/-}$ EBs. In contrast, the level of VEGFR-2 transcripts was decreased by 50% in the FGFR-1$^{-/-}$ EBs. We examined the level of VEGFR-2 protein in homozygous and heterozygous FGFR-1 inactivated EBs. As shown in Fig. 6B, there was a reduced expression of VEGFR-2 protein in the FGFR-1$^{-/-}$ EBs (60% of the level in FGFR-1$^{+/-}$ EBs), but still the protein was clearly detectable both in western analysis and immunohistochemical staining for VEGFR-2. The capacity of the VEGFR-2 in the FGFR-1$^{-/-}$ and FGFR-1$^{+/-}$ EBs to respond to VEGF-A by increased kinase activity was examined by an immunocomplex kinase assay (Fig. 6C). VEGFR-2 was immunoprecipitated from EBs, which were either

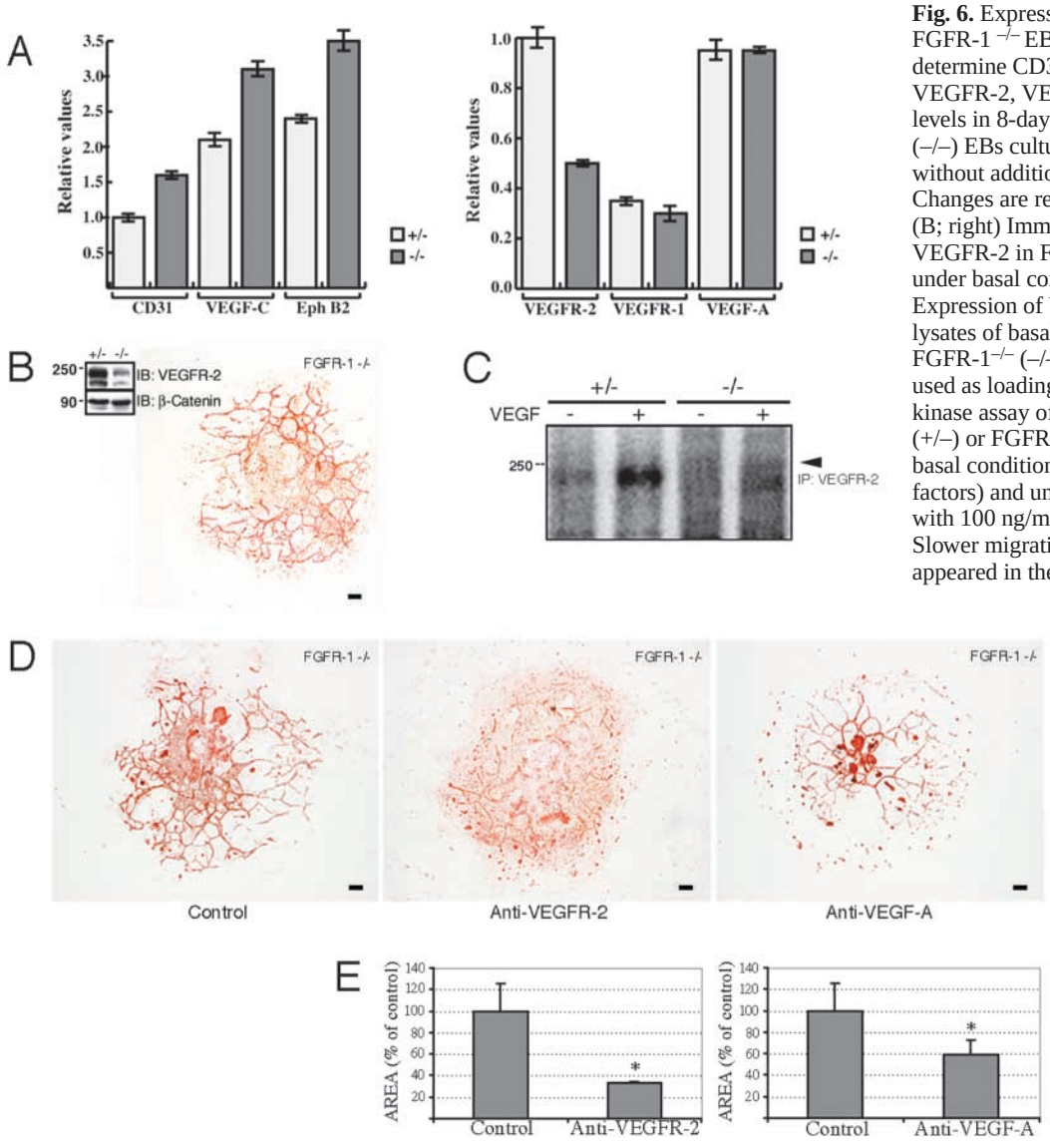


Fig. 6. Expression of endothelial cell markers in FGFR-1 $^{-/-}$ EBs. (A) Real-time PCR to determine CD31, VEGF-C, Ephrin B2 and VEGFR-2, VEGFR-1, VEGF-A transcription levels in 8-day-old FGFR$^{+/-}$ (+/–) or FGFR$^{-/-}$ (–/–) EBs cultured in basal conditions (i.e. without addition of exogenous growth factors). Changes are relative to β-actin transcript levels. (B; right) Immunohistochemical staining for VEGFR-2 in FGFR-1$^{-/-}$ EBs cultured for 8 days under basal condition. Scale bars: 200 μm. (Left) Expression of VEGFR-2 protein in total cell lysates of basal treated FGFR-1$^{+/-}$ (+/–) and FGFR-1$^{-/-}$ (–/–) 8-day-old EBs. β-catenin was used as loading control. (C) Immunocomplex kinase assay of VEGFR-2 on day-8 FGFR-1$^{+/-}$ (+/–) or FGFR-1$^{-/-}$ (–/–) EBs cultured under basal conditions (without exogenous growth factors) and unstimulated (–) or stimulated (+) with 100 ng/ml of VEGF-A for 10 minutes. Slower migrating, kinase active VEGFR-2 appeared in the FGFR-1$^{-/-}$ EBs (arrowhead). (D) CD31 staining of FGFR-1$^{-/-}$ EBs in basal conditions treated with either control anti-rat IgG, neutralizing anti-VEGFR-2 (30 μg/ml), or anti-VEGF-A (5 μg/ml) antibodies from day 6-8. Scale bars: 200 μm. (E) Quantification of CD31 staining as area (i.e. area without holes) in 8-day FGFR-1$^{-/-}$ EBs treated with anti-VEGFR-2 or anti-VEGF-A antibodies from day 6-8. Results are expressed as percentage of FGFR-1$^{-/-}$ EBs treated with control IgG (mean±s.d., n=4). *P=0.002 for anti-VEGFR-2 treatment compared to control. *P=0.0306 for anti-VEGF-A treatment compared to control.

unstimulated or stimulated for 10 minutes on day 8 with VEGF-A. In the FGFR-1$^{+/-}$ EBs, there was basal kinase activity of VEGFR-2, which increased with stimulation of the cells with exogenous VEGF-A. In the FGFR-1$^{-/-}$ EBs, basal and VEGF-induced VEGFR-2 kinase activity was identified and, interestingly, slower migrating, kinase active VEGFR-2 species were detected in response to VEGF-A treatment (arrowhead in Fig. 6C).

To examine the role of VEGF-A/VEGFR-2 in the increased basal vessel formation in the homozygous knockout, the FGFR-1$^{-/-}$ EBs were treated with neutralizing antibodies against VEGFR-2 or VEGF-A between days 6-8 and stained for CD31 (Fig. 6D). In both cases, vessel formation was decreased considerably and only a few vessel fragments remained. Quantification of the results showed a threefold reduction in vessel formation upon VEGFR-2 neutralization and blocking VEGF-A decreased vessel formation by 50% (Fig. 6E). We interpret these biochemical and functional data to indicate a changed regulation of VEGFR-2 in the FGFR-1$^{-/-}$ embryoid bodies, leading to increased signaling through VEGFR-2 and thus increased basal vessel formation.

Discussion

In this report, we show that vascular development in embryoid bodies resembles that in embryos, in kinetics of expression of marker proteins such as VEGFR-2, CD31 and VE-cadherin and in kinetics of morphological development, including identification of hemangioblasts/blood islands, and eventually, sprouting angiogenesis. In agreement with earlier literature, we detected erythroblasts within the vessels; contraction of cardiomyocytes in the bodies causes these erythroblasts to move inside the vessels, indicating a continuous lumen (Risau and Flamme, 1995). Thus, despite the fact that the embryonal layers may be relatively disorganized in the embryoid body, which consequently may lack the characteristic in vivo axial patterning (Rathjen and Rathjen, 2001), our data indicate that embryoid bodies represent a useful model for analysis of

vascular development and for analysis of the molecular mechanisms of angiogenic growth factors.

The advantages of using the embryoid bodies include easy access, no need for ethical consideration, the relatively low cost and the possibility to follow the effect of manipulation from day 0 to day 20. Balconi et al. (Balconi et al., 2000), showed that endothelial cells isolated from embryoid bodies displayed morphological and functional similarities to endothelial cells isolated from the yolk sac and embryo proper at day 9.5. Furthermore, it should also be possible to isolate large quantities of endothelial cells, e.g. for co-administration in conjunction with transplantation of insulin-producing β-cells, in order to facilitate grafting. In accordance, Marchetti et al. (Marchetti et al., 2002) have reported that endothelial cells derived from embryonal stem cells formed functional vessels facilitating vascularization in a mouse tumor model.

The VEGF/VEGFR family is required for migration and differentiation of stem cells to generate appropriately positioned, differentiated endothelial cells (Risau and Flamme, 1995). VEGFR-2 is one of the earliest markers of endothelial and hematopoietic cells (Kabrun et al.,1997). We failed to identify VEGFR-2 expression on undifferentiated stem cells, and detected weak expression of VEGFR-2 at day 4 and considerably induced expression on day 6, in agreement with previous reports (Vittet et al., 1996; Nishikawa et al., 1998). VEGF-induced vascular development is independent of FGFR-1 as demonstrated by the induction of capillaries by VEGF-A in the FGFR-1$^{-/-}$ EBs. In contrast, in the VEGFR-2$^{-/-}$ embryoid bodies, vascular development was completely arrested as judged from immunostaining for CD31. However, these embryoid bodies appeared not to be impaired in their development; they expanded to a normal size and contained contracting cardiomyocytes. Even though the VEGFR-2$^{-/-}$ embryoid bodies failed to respond to VEGF-A treatment, FGF-2 stimulated expansion and clustering of a CD31-positive pool of cells. Future plans include isolation and characterization of these cells in more detail with regard to expression of endothelial cell markers.

What is the role of FGF-2/FGFR-1 in vascular development and angiogenesis? FGF-2 has been implicated in regulation of very early stages in vascular stem cell development (Risau and Flamme, 1995). Furthermore, FGF-2 is regarded as an angiogenic factor (Javerzat et al., 2002), based on its potent induction of angiogenesis in various in vitro and in vivo models, such as the chicken chorioallantois membrane assay and the rabbit and mouse cornea model, and its ability to drive tumor vascularization (Giavazzi et al., 2001). It has been shown that FGF-2 may exert its effects on endothelial cells indirectly, by inducing expression of VEGF-A (Seghezzi et al., 1998; Auguste et al., 2001; Tille et al., 2001). However, expression of dominant-negative (DN) FGFR-1 has been shown to adversely affect vessel development in a manner that indicates VEGF-independent effects on the vasculature. In an elegant approach using adenovirus-mediated expression of soluble receptors in a Rip1Tag2 transgenic mouse model for β-cell carcinogenesis, Compagni et al. (Compagni et al., 2000) showed that DN VEGFR predominantly affected initiation of tumor angiogenesis whereas DN FGFR impaired maintenance of tumor angiogenesis. Moreover, Matsumoto et al. (Matsumoto et al., 2002) showed that FGF-2 but not VEGF-A could induce activation of p38 MAPK in endothelial cells in the chicken chorioallantois membrane. The responding FGFR subtype was not identified in these studies. Clearly, the pleiotrophic effects of the large family of FGFs and FGFRs have hampered genetic dissection of their roles in the vasculature. Our data support the view that FGFR-1 is an indirectly acting component in vascular development. This is based on the fact that VEGF-driven vascular development occurred independently of FGFR-1. Moreover, the remarkable increase in basal vessel formation in the FGFR-1$^{-/-}$ embryoid bodies is most probably not a direct effect of loss of this receptor, but can be explained on the basis of changes in regulation of the VEGF/VEGF receptor family. Also, biochemical analyses showed a twofold decrease in expression of VEGFR-2 protein, which responded to VEGF-A with increased kinase activity. The immunoprecipitated kinase active VEGFR-2 in FGFR-1$^{-/-}$ EBs, moreover, migrated more slowly than in the heterozygous condition, indicating changes in regulation of phosphorylation of VEGFR-2 in the absence of FGFR-1. How would lack of FGFR-1 expression affect VEGFR-2 phosphorylation? One possibility would be that loss of FGFR-1 could be accompanied by changes in expression levels of phosphatases. In accordance with this Takahashi et al. (Takahashi et al., 2003) showed that loss of function of the tyrosine phosphatase CD148 causes defects in vascular development and reduced branching of vessels (Takahashi et al., 2003). Alternatively, loss of FGFR-1 could affect clearance of VEGFR-2 by changes in internalization or ubiquitination of the receptor (Duval et al., 2003). The reduced expression levels of VEGFR-2 in the absence of FGFR-1 may thus be a consequence rather than a cause of this deregulation. In conclusion, our data provide novel information on the complexity of the interplay between FGF and VEGF growth factors and receptors during vascular development.

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