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Fibroblast growth factor 16 and 18 are expressed in human cardiovascular tissues and induce on endothelial cells migration but not proliferation

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Abstract

Endothelial cells line the blood vessel and precursor endothelial cells appear to have a pivotal effect on the organ formation of the heart, the embryonic development of the kidney, and the liver. Several growth factors including the fibroblast growth factors (FGF) seem to be involved in these processes. Ligands such as basic FGF produced and secreted by endothelial cells may also coordinate cellular migration, differentiation, and proliferation under pathological conditions including wound healing, tumorgenesis, and fibrogenesis in the adult. Recently we demonstrated the expression of two secreted FGFs, FGF16, and FGF18, in HUVEC and in rat aortic tissue. In the present report, we confirmed by RT-PCR analysis that FGF18 is wildly expressed in the cardiovascular tissue, while FGF16 showed a more restricted expression pattern. HUVEC clearly demonstrated chemotaxis towards FGF16 and FGF18. Both FGFs also enhanced cell migration in response to mechanical damage. However, recombinant FGF16 and FGF18 failed to induce endothelial cell proliferation or sprouting in a three-dimensional in vitro angiogenesis assay. Fgf18 expression was earlier reported in the liver, and we detected FGF18 expression in liver vascular and liver sinusoidal endothelial cells (LSECs), but not in hepatic parenchymal cells. Recombinant FGF18 stimulated DNA synthesis in primary hepatocytes, suggesting, that endothelial FGF18 might have a paracrine function in promoting growth of the parenchymal tissue. Interestingly, FGF2, which is mitogenic on endothelial cells and hepatocytes stimulates a sustained MAPK activation in both cell types, while FGF18 causes a short transient activation of the MAPK pathway in endothelial cells but a sustained activation in hepatocytes. Therefore, the difference in the time course of MAPK activation by the different FGFs appears to be the cause for the different cellular responses.

Keywords: Fibroblast growth factor-18; Cardiovascular tissue; Fibroblast growth factor-16; Ras-mitogen activated protein kinase (MAPK)

The FGF ligand family comprises at present at least 23 structurally related polypeptides. FGFs are important modulators of cellular proliferation, migration, and differentiation depending on cell type and tissue context. Recently, we reported a systematic survey of the mRNA expression of the FGF ligands and FGF receptors in primary endothelial cells (HUVEC) and vascular smooth muscle cells (HUASMC). Most interestingly, HUVEC and HUASMC express the identical FGF ligand and receptor panels, suggesting that regarding the FGF family both types of cells act more as an entity than as two separate cell systems. In addition, we tested rat and human aortic tissue for the presence of those FGF ligands and found both FGFs expressed by the vascular cells. Next to FGF1, FGF2, and FGF7 expression, we also detected in human aortic tissue protein and mRNA expression of two secreted FGFs, FGF16, and FGF18. FGF18 and FGF16 immunoreactivity was found in the aortic endothelial cells and the smooth muscle cells. Interestingly, a substantial part of the FGF18 staining appears to be localized in the cell nucleus, in particular in the nucleus of the smooth muscle cells [1]. FGF stimulation leads to tyrosine auto-phosphorylation of four high-affinity

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tyrosine kinase FGF receptors (FGFR1–FGFR4). The extreme FGF signal is transduced by recruitment of signalling proteins to the tyrosine auto-phosphorylation sites on the activated FGF receptor and to linked docking proteins creating additional binding sites to further signalling molecules. The docking protein SNT-1/FRS2 (fibroblast growth factor receptor substrate2) binds constitutively to the juxtamembrane domain of FGFR1 (aa 407–433) and links FGFR activation to the RAS/MAPK signalling pathway [2–4]. FRS2 appears to play the leading role in signalling via FGF receptors and is crucial for mitogen-activated protein kinase (MAPK) activation, PI-3 kinase stimulation, chemotactic response, and proliferation. The extracellular signal-regulated kinase (ERK) 1 (p44 MAPK) and ERK2 (p42 MAPK) have been shown to be critical for induction of cell proliferation as well as for differentiation and migration [5].

FGF2 induces a wide range of effects on endothelial cells including cell proliferation, production of ECM modifying proteases, and cell migration. In many in vivo and in vitro systems FGF2 has proved its effectiveness to induce neovascularization and endothelial cell sprouting [6–12]. The effects of others than the prototypical FGFs FGF1 and FGF2 are not well documented in the vascular system. Biological activities promoted by different FGFs may account for the pleiotrophic responses of endothelial cells after stimulation of the FGF signal pathway.

In the present work, we reported the expression in the cardiovascular system and activities of two secreted FGFs, FGF16, and FGF18, in endothelial cells. Significant differences were observed in the biological responses of HUVE cells when stimulated with FGF2 or FGF16 and FGF18. We employed a three-dimensional angiogenesis system to analyse the sprouting capacity of the FGFs and a wound assay to investigate migration and cell proliferation. In contrast to FGF2, FGF16, and FGF18 stimulated endothelial cell migration without cell proliferation and failed to induce a sustained activation of ERK1/2 as seen with FGF2. Our data demonstrate that different FGF molecules may exert different cell responses by the longevity of MAP kinase activation.

Materials and methods

Antibodies and growth factors. Recombinant human VEGF, FGF1, FGF2, FGF16, and FGF18 were obtained from Peprotech (London, GB). FGF antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphorylated Akt polyclonal antibodies, anti-phosphorylated p44/42 MAPK polyclonal antibodies and anti-p44/42 MAPK polyclonal antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-phosphorylated p38 monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture. Human umbilical vein endothelial cells (HUVEC), Human umbilical artery smooth muscle cells (HUASMC), endothelial cell growth medium (ECGM), endothelial cell basal medium, and smooth muscle cell growth medium 2 (SMCGM2) were purchased from Promocell (Heidelberg, Germany). Fetal Calf Serum (FCS) was obtained from Biochrom (Berlin, Germany). Cells were cultured at 37 °C in 75-cm^2 tissue culture dishes in the corresponding media, routinely split in a 1:4 ratio, and frozen in liquid nitrogen at passage 4. HUVE cells cultured from passage 5 to 8 were used for experiments. Hepatocytes were essentially isolated from male Sprague–Dawley rats following the collagenase method of Seglen as described [13]. Hepatocytes were seeded at a density of 5×10^4 cm^2 on collagen-coated dishes in Hepatozyme–SFM (Gibco). Isolation and purification of rat liver sinusoidal endothelial cells (LSEC) was performed as described [14]. The endothelial cells were either directly used for RNA preparation or seeded on collagen-coated dishes for further experiments. Purity was assessed by staining with Dil-Ac-LDL (Paesel & Lorei, Frankfurt) and FITC coupled latex beads (Fluoresbrite plain YG 1.0 μm microspheres, Polysciences, Eppelheim). Purity of viable LSE cells was at least 95%.

Cell proliferation assay. A cell proliferation kit supplied by Roche (Roche Diagnostics) was used. Briefly, HUVE cells were grown on collagen-coated 8-chamber slides to about 50% confluence and serum-starved (1% ECGM in basal medium, 5 μg/ml heparin) for 48 h. Quiescent cells were then stimulated with the different growth factors mentioned above. After 16 h, cells were pulse-labelled with 5-bromo-2′-deoxyuridine (BrdU) for 4 h. After being labelled, the cells were fixed and the incorporated BrdU was detected with a monoclonal antibody and visualized with a second, peroxidase-conjugated anti-mouse immunoglobulin G antibody.

To determine proliferation of primary rat hepatocytes, 100,000 cells per well were seeded in 24-well plates encoated with collagen (rat tail collagen type I, BD Bioscience) in 1 mL hepatozyme–SFM (HSFM, Gibco). One day after seeding, the medium was changed to basal medium (Williams modified Eagle's medium, Sigma, St Louis, MO) supplemented with glutamine (2 mM), insulin (1.67 μM), dexamethasone (3 μM), and heparin (0.5 μg/mL). Twenty-four hours later, the medium was changed to serum-free conditions with and without various concentrations of growth factors as indicated and 16 h later exposed to [^{3}H]thymidine (673 GBq/mmol; 2 μCi/mL medium; Hartmann Analytic GmbH, Braunschweig). [^{3}H]thymidine incorporation was measured after a labelling period of 3 h, by trichloroacetic acid precipitation of cells as described [15]. As positive control served Hepatozyme–SFM. LSEC were stimulated in endothelial basal medium containing heparin and growth factors, 16 h later exposed to [^{3}H]thymidine for a labelling period of 6 h. As negative control served basal medium (BM) with heparin and as positive control served endothelial growth medium. Endothelial cell growth medium (ECGM) and endothelial cell basal medium (BM) were purchased from Promocell (Heidelberg, Germany). The mean (±SD) activity was determined from at least three independent experiments. Recombinant FGFs were obtained from PeproTech (London, UK).

Wound assay. Confluent HUVE cells grown in collagen-coated six-well plates were cultured in serum-reduced medium (1% ECGM in basal medium, 5 μg/ml heparin) for 2 days. The monolayers were mechanically wounded to obtain a denuded area of 2×10 mm. One side was scraped in a way to result in a cell barrier therefore, allowing cells only to migrate into the wounded area from one side. Wounded cells were incubated with or without FGFs in control medium (1% ECM in basal medium) for at least 24 h, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stained with crystal-violet.

Generation of endothelial cell spheroids. Endothelial cell spheroids were generated as previously described [16]. Briefly, HUVE cells were suspended in culture medium containing 0.25% carboxymethylcellulose and seeded in non-adherent round-bottomed 96-well plates (Greiner, Frickenhausen, Germany). The suspended cells integrate into a single spheroid of defined size and cell number. After 24 h of cultivation at 37 °C (5% CO_2, 100% humidity), the spheroids were used for the experiments or for RNA extraction.

In vitro angiogenesis assay. In vitro angiogenesis assay was performed as described previously [17]. Spheroids generated of 750 HUVE cells were embedded in collagen gels. For that purpose collected spheroids were suspended in ECGM basal medium (PromoCell, Heidelberg, Germany) containing 40% FCS and 0.5% (w/v) carboxymethylcellulose, and mixed with an equal portion of cold (at 4 °C) rat tail collagen solution (2 mg/ml, BD Biosciences, Bedford, MA) containing 10% medium 199 (10-fold, Sigma, Deisenhofen, Germany) and 0.1 N NaOH (to adjust the pH to 7.4). The 50 spheroids containing gel was transferred in a prewarmed 24-well

plate and allowed to polymerize at 37 °C. One hundred microliters basal medium containing test substances were pipetted on the polymerized gel which was further incubated at 37 °C in 5% CO_2 and at 100% humidity. Quantification was performed by digital imaging and calculating cumulative length of the three longest sprouts of each spheroid, analysing at least 10 spheroids per group and experiment.

Immunoblot analysis. The procedures used for preparing cell lysates have been described in detail elsewhere [18]. Samples from equivalent numbers of cells were fractionated by SDS–PAGE in 12.5% gels, transferred to nitrocellulose membranes (Schleicher and Schuell), and then probed with the indicated antibodies followed by horseradish peroxidase-linked secondary antibodies (Amersham–Pharmacia Biotech). Immunocomplexes were detected by enhanced chemiluminescence (Amersham–Pharmacia Biotech).

RNA isolation and RT-PCR. Total RNA from HUASMC, hepatocytes, and HUVE and liver sinusoidal cells were extracted by using the RNeasy Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions. Purified RNA samples were diluted at 1 mg/ml for RT-PCR or stored at −80 °C until further use. Total RNA was reverse transcribed with Omniscript Reverse Transcriptase Kit (QIAgen, Hilden, Germany) using oligo(dT) as a primer. cDNAs [(reverse transcribed from primary cells and cDNAs from human cardiovascular tissues (MTC panel) purchased from Clontech (Mountain View, CA)] were amplified using the *Taq* DNA Polymerase Kit from QIAgen. PCR primers were obtained from Invitrogen (Heidelberg, Germany), primer sequences are available upon request. PCR products were DNA sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany). PCRs were separated on agarose gels and scanned on a Kodak Image Station 440 CF (Perkin-Elmer).

Northern hybridization. Total RNA isolated from monolayer HUVEC and HUASMC cultures was separated using 1% formaldehyde agarose gels and blotted onto nylon membrane (Hybond N+, Amersham–Pharmacia Biotech, Buckinghamshire, England). cDNA probes for hybridization were prepared by labelling a 472 bp DNA sequence confirmed PCR product of human fgf18 cDNA with [^{32}P]dCTP using a Random Primers Labeling System (Invitrogen, Germany).

Immunocytochemical analysis. Rat liver sections and sections from rat liver slice cultures from 350 g male Wistar rats were fixed and processed for paraffin embedding [19]. Paraffin sections were stained for FGF18 using a polyclonal goat antibody and the avidin–biotin complex method. Secondary antibodies were biotinylated anti-goat IgG (Vector Laboratories Burlingame, CA), exposed to Streptavidin peroxidase, developed with diaminobenzidine, and counterstained with hematoxylin.

Endothelial cell transmigration activity. Endothelial cell transmigration was performed using Transwell membrane filters (Corning Costar) containing a polycarbonate filter with 8 μm pores. The filter was coated with 10 mg/L fibronectin at 4 °C overnight. Endothelial basal medium (EBM) containing 0.2% FCS with or without the indicated concentration of FGFs was added to the wells of the lower chamber. Then 100 μl of HUVEC (3×10^5 cells/mL) resuspended in EBM containing 0.2% FCS was added to each well of the upper chamber. After a 4 h incubation in 5% CO_2 at 37 °C, non-migrating cells were scraped from the upper surface of the filter. Migrating cells on the lower surface were washed, fixed with methanol and stained with Giemsa solution. The number of HUVEC on the lower, surface of the filter was determined by counting three microscopic fields per well. The number of cells counted on the untreated control filters was set to 100%.

Results

FGF16 and FGF18 and FGF receptors expressed in human cardiovascular system

Recently, we reported a full panel RT-PCR expression profiling of all known FGFs in primary endothelial cells (HUVEC) and smooth muscle cells (HUASMC). The expression of Fgf2, Fgf7, Fgf16, and Fgf18 genes was detectable in the endothelial and smooth muscle cells as well as in aortic tissue. We extended the expression analysis of Fgf16 and Fgf18 onto a panel of human cardiovascular tissues. While Fgf18 specific transcripts were revealed in all analysed cDNAs of the cardiovascular multi-tissue panel, Fgf16 mRNA expression showed an asymmetric distribution with a pronounced restriction to the left heart including left atrium, auricle sinistra, left ventricle and aorta and the adjacent interventricular septum and atrioventricular node (Fig. 1A). FGF16 and FGF18 mediate their effects on cells by binding to three FGF receptors FGFR2c, FGFR3c, and FGFR4. As before reported for HUVEC and HUASMC FGFR4 specific transcript could not be detected in any of the analysed cDNAs. Weak expression was found for the FGFR3c in most cDNAs prepared from the cardiovascular tissue except in aortic tissue and in the cDNA derived from the total adult heart expression was under detection limit. FGFR2c demonstrated a more differential expression pattern while FGFR1c was strongly expressed in all cDNAs with exception of adult heart (Table 1). In addition, the novel transmembrane FGF receptor (FGFR5/FGFRL1) without a kinase domain is found highly expressed in all cardiovascular tissues derived cDNAs (Fig. 1A). FGF18 appears to be constantly expressed in most if not all cardiovascular cells. To exclude that HUVEC and HUASMC may express different variant RNAs, the transcripts encoding the FGF18 were further examined by Northern blotting. Total RNA prepared from HUVE and HUASM cells was blotted and hybridized against a ^{32}P-labelled human FGF18 471 bp PCR fragment prior confirmed by DNA sequencing. Northern blotting revealed a single transcript of approximately 2.7 kb in both RNA probes (Fig. 1B).

Lack of wound-induced proliferation in EC treated with FGF16 or FGF18

Exogenous FGF2 induces a variety of endothelial cell responses including cell migration, proliferation, migration in mechanical wounding of monolayer cultures of EC, and sprouting and tube formation in angiogenesis assays. To study the biological activity of FGF16 and FGF18, we performed different biological assays to compare their activity with the effect of FGF2. Monolayers of confluent EC were mechanically wounded in three areas sized approximately 2×10 mm. rFGF1, rFGF2, conditioned medium from FGF4 transfected COS-1 cells, rFGF16, rFGF18 or control medium without growth factors were added immediately after the wounding. Cell migration was measured by staining the cultures with crystal violet. In serum-reduced medium few cells migrated into the denuded area, while in FGF2 treated cultures the total area was filled and wound healing was nearly completed (Fig. 2). This appeared to be a combined effect of migration and proliferation. Gradually fewer cells were present in the wound area when EC were treated with FGF1 or FGF4. Although

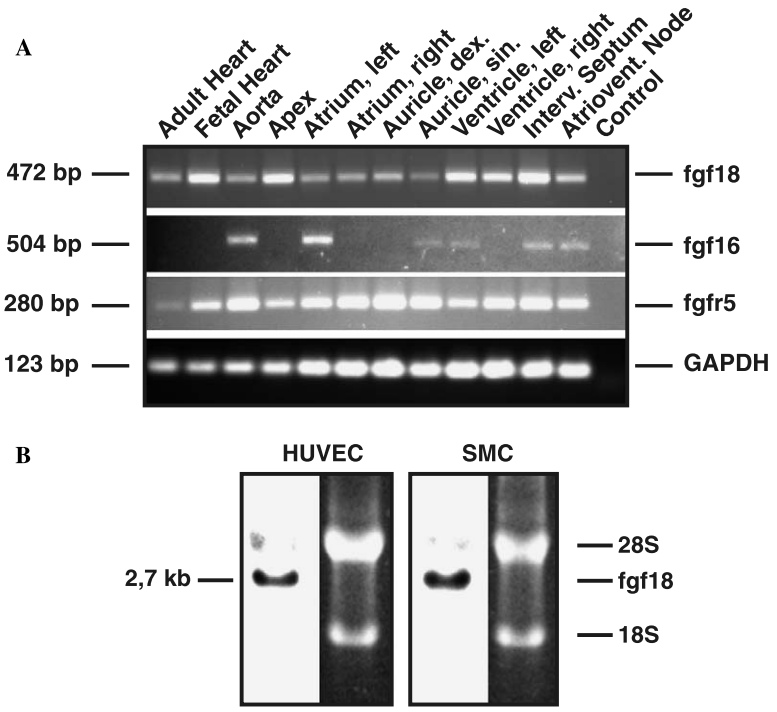

Fig. 1. (A) RT-PCR analysis of the expression of Fgf16, Fgf18, and fgfr5 in a human cardiovascular cDNA panel of 12 different cardiovascular tissues. The specific PCR amplification products and their expected sizes are indicated. Amplification of GAPDH transcripts in the cDNA templates was used as internal control. (B) Expression of Fgf18 transcripts in HUVEC and HUASMC. Northern blot containing 10 μg of total RNA was hybridized with a ^{32}p-labelled human cDNA probe. The size of the single transcript is indicated and ethidium bromide stained total RNA was used as loading control.

Table 1
Summary of the RNA expression of FGF16, FGF18, and FGF receptors in cardiovascular tissues

	FGF16	FGF18	FGFR1c	FGFR2c	FGFR3c
Adult heart	0	+	0	0	0
Fetal heart	0	+++	++	+	(+)
Aorta	++	+	++	++	0
Apex	0	+++	++	0	(+)
Atrium, left	+++	+	+++	+	+
Atrium, right	0	+	++	+	(+)
Autricle, dextra	0	+	++	++	+
Autricle, sinistra	+	+	++	+	(+)
Ventricle, left	+	+++	+++	++	+
Ventricle, right	0	+++	++	0	(+)
Interventr. Septum	+	+++	+++	0	(+)
Atrioventr. Node	+	+	++	++	(+)

FGF16 and FGF18 appear to have a significantly lower effect on the wound healing as compared with FGF2, in presence of both FGF16 and FGF18 more than twice as many cells migrated into the denuded area compared to the control without FGFs. These data suggest that FGF16 and FGF18 might have an effect on cell migration but not on cell proliferation.

FGF2 but neither FGF16 nor FGF18 stimulates outgrowth of capillary sprouts in an endothelial cell spheroid based angiogenesis assay

During angiogenesis, EC are induced to migrate, proliferate and to invade the surrounding tissue to form three-dimensional tube structures. When EC spheroids of a defined cell number were seeded into collagen, they formed capillary-like three-dimensional structures (Fig. 3). Even in presence of 20% FCS HUVEC had a low baseline sprouting activity without exogenous added FGFs. This finding may reflect the effects of antagonists of FGF signalling counteracting the endogenous FGFs activities [16]. Quantitative assessment of the in vitro angiogenesis assay was achieved by estimating the size of the capillary sprouts from the digital microscope images. FGF2 clearly stimulated the outgrowth of capillary-like sprouts in the assay. Although, FGF1 and conditioned medium from FGF4-expressing COS-1 cells stimulated sprouting, it was still to a much lower extent as compared

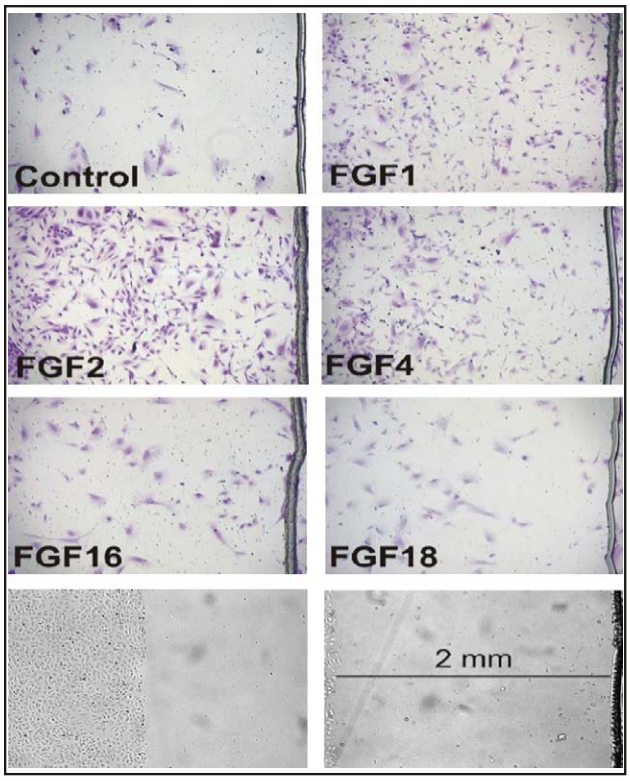

Fig. 2. Wound-induced HUVEC migration and proliferation in presence or absence of FGFs. ECs were wounded, incubated with FGFs (25 ng/ml), fixed, and stained as described in Materials and methods. A representative experiment after 6 days is shown. This experiment was repeated with similar results.

with FGF2 and VEGF as positive control. FGF16 and FGF18 did not show a significant stimulation in this in vitro angiogenesis assay (Fig. 3).

FGF16 and FGF18 failed to induce cell proliferation in HUVEC, but induce a strong chemotaxic response

To evaluate their ability to induce cell proliferation, serum-starved HUVEC were stimulated with FGFs and pulse-labelled with BrdU, BrdU incorporation was then detected by using a specific monoclonal antibody (Fig. 4A). The results show that BrdU incorporation in cells treated with FGF2 was three times higher compared to the control cells without FGFs and compared to FGF16 and FGF18 stimulated cells. Repeating the assay with higher concentrations of FGF16 or FGF18 did not reveal a stimulation effect on cell proliferation. Thus, it appears that neither FGF16 nor FGF18 is able to induce the entry of HUVEC into S phase of the cell division cycle. The results from both complex biological assays suggest that FGF16 and FGF18 might be capable to induce migration but not the whole process of sprouting in an in vitro angiogenesis assay. Therefore, we next assessed their ability to induce a chemotaxic response of EC. HUVEC were plated on the upper fibronectin-coated Transwell membrane filters and the inserts were placed into wells pre-filled with EBM containing 0.2% FCS with or without FGFs. As shown in Fig. 4B, FGF2 markedly stimulated chemotaxis of HUVEC. Importantly, FGF16 and FGF18 too demonstrated in this migration assay a pronounced effect that was

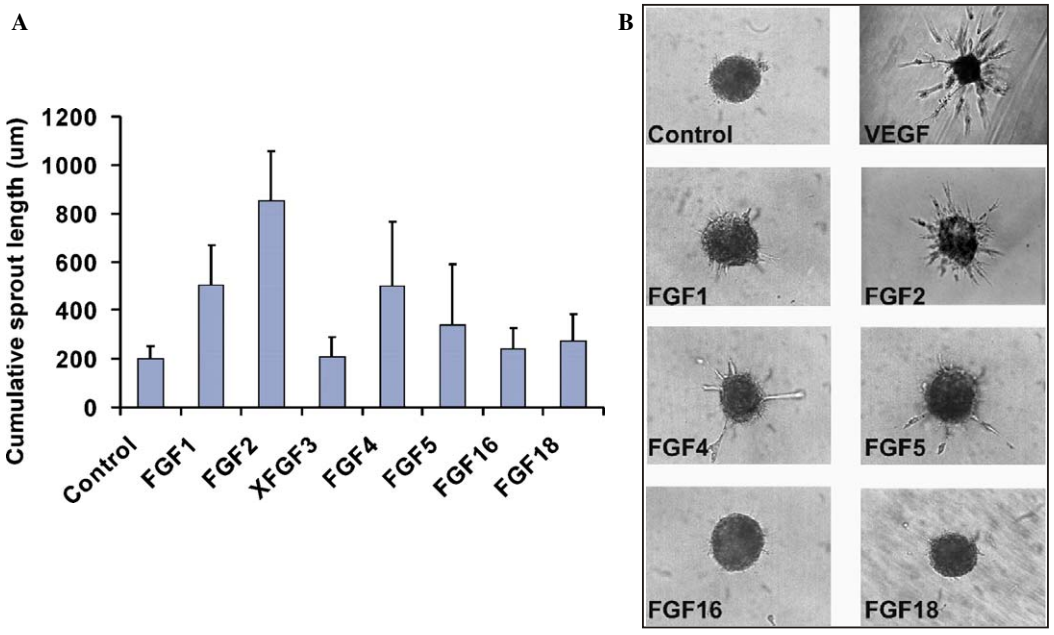

Fig. 3. Quantitative in vitro angiogenesis assay based on collagen embedded EC spheroids. (A) Spheroids of HUVEC were embedded into collagen and the capillary sprouting was quantified by digital imaging. (B) Representatives demonstrate the sprouting activity of different FGFs (rFGF1, rFGF2, rFGF16, and rFGF18, 25 ng/ml) and VEGF (50 ng/ml). FGF16 and FGF18 exert no sprouting activity in the spheroid angiogenesis assay. Conditioned medium from XFGF3, FGF4, and FGF5 expressing transfected COS-1 cells was used for these experiments.

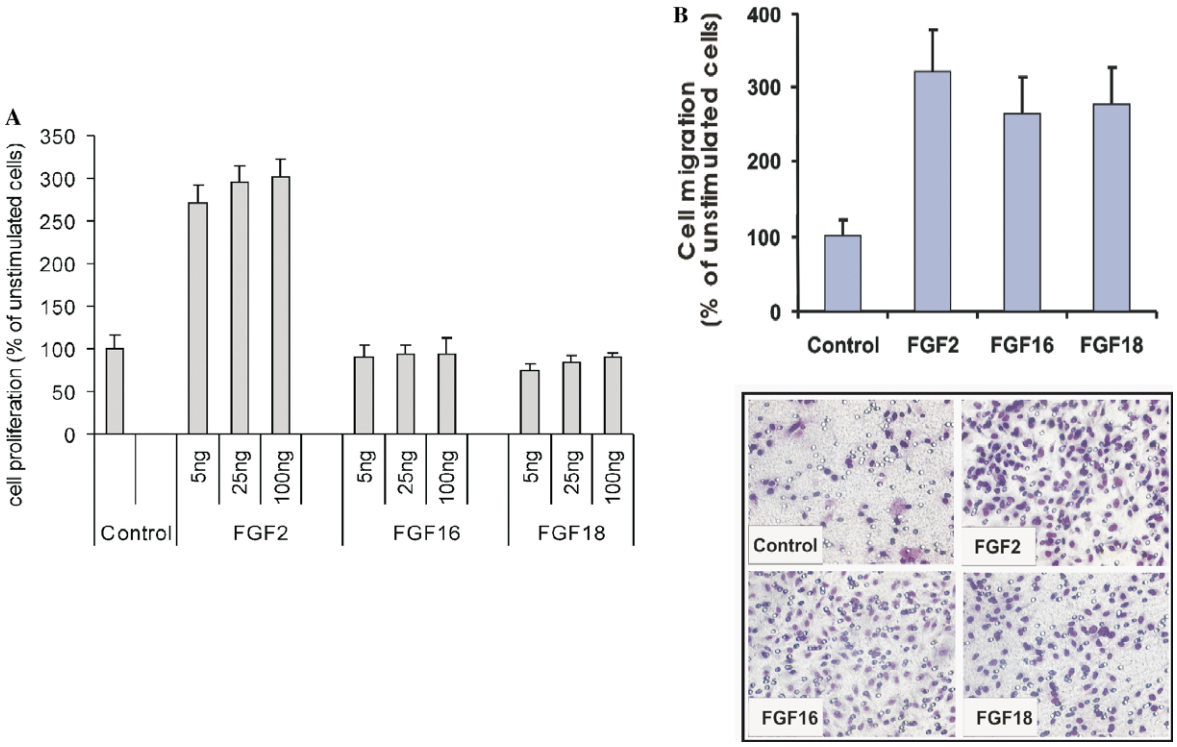

Fig. 4. FGF16 and FGF18 induce chemotaxis but not proliferation of HUVE cells. (A) Comparison of the mitogenic activity of different FGFs on HUVEC. To monitor DNA synthesis, serum-starved HUVEC were stimulated with the indicated FGFs and assessed for their ability to incorporate BrdU. BrdU incorporation was measured immunocytochemically. Values are means ± SD of three independent experiments. (B) HUVEC were plated on the fibronectin-coated upper well membranes of Transwell plate inserts in EBM containing 0.2% FCS. The inserts were placed in wells prefilled with EBM supplemented with 0.2% FCS and with (25 ng/ml) or without FGFs. After incubation at 37 °C for 4 h, cells were fixed and stained as described in Materials and methods. The cells present on the upper well membranes were removed and the cells migrated onto the lower surface membranes counted. Data are shown as means ±SD for triplicate wells. Similar results were obtained in three independent experiments.

as strong as with FGF2. Taken together, the results demonstrate that both FGF16 and FGF18 are able to stimulate cell migration of HUVEC but without cell proliferation, suggesting that endogenous expressed FGFs induce distinct cellular responses.

FGF18 is expressed in rat liver sinusoidal endothelial cells and induces cell proliferation in primary rat hepatocytes

Since in an earlier publication the liver specific expression of FGF18 was reported and since we could detect FGF18 expression in all analysed endothelial tissues, we isolated sinusoidal endothelial cells from rat livers and tested them for FGF18 transcripts by RT-PCR analysis. RT-PCR analysed with rat fgf18 specific primers revealed an amplification product of expected size. The same RT-PCR analysis did not obtain a detectable product when cDNAs derived from rat hepatocytes were used (Fig. 5A). However, Fgf18 transcripts were detectable in all analysed liver cancer cell lines (Fig. 5A). Immunostaining for FGF18 in rat liver tissue exhibited an expected strong expression of FGF18 in the large vascular endothelial cells and the adjacent smooth muscle cells. In addition, FGF18 immunoreactivity was detected in cells of the sinusoidal compartment. The staining was partly localized into the cytoplasm partly into the nucleus. In liver slices cultured for two days the sinusoidal compartment is more pronounced and immunohistochemistry revealed a clear FGF18 staining in cells lining the liver sinus, very likely representing sinusoidal endothelial cells (Fig. 5B). Since FGF18 stimulation failed to induce cell proliferation in vascular endothelial cells, we tested if FGF18 is able to induce cell proliferation of liver sinusoidal endothelial cells. As shown in Fig. 5C FGF18 is not able to stimulate DNA synthesis of LSEC, but FGF18 induced [^{3}H]thymidine incorporation of rat primary hepatocytes with a similar high activity as hepatocyte complete medium (HSFM) or FGF1 which is able to activate all known FGF receptors (Fig. 5C).

FGF2 induces a sustained activation of MAPK, while FGF18 stimulates a transient and short MAPK activation in HUVEC

It is well known that extracellular-regulated kinases (ERKs, p44/p42 MAPKs) are key effectors in the transduction of FGF-mediated effects including cell migration and cell proliferation. HUVEC were grown to confluence and incubated in the presence or absence of 25 ng/ml FGF2, FGF1, FGF16 or FGF18 for 30 min. The state of

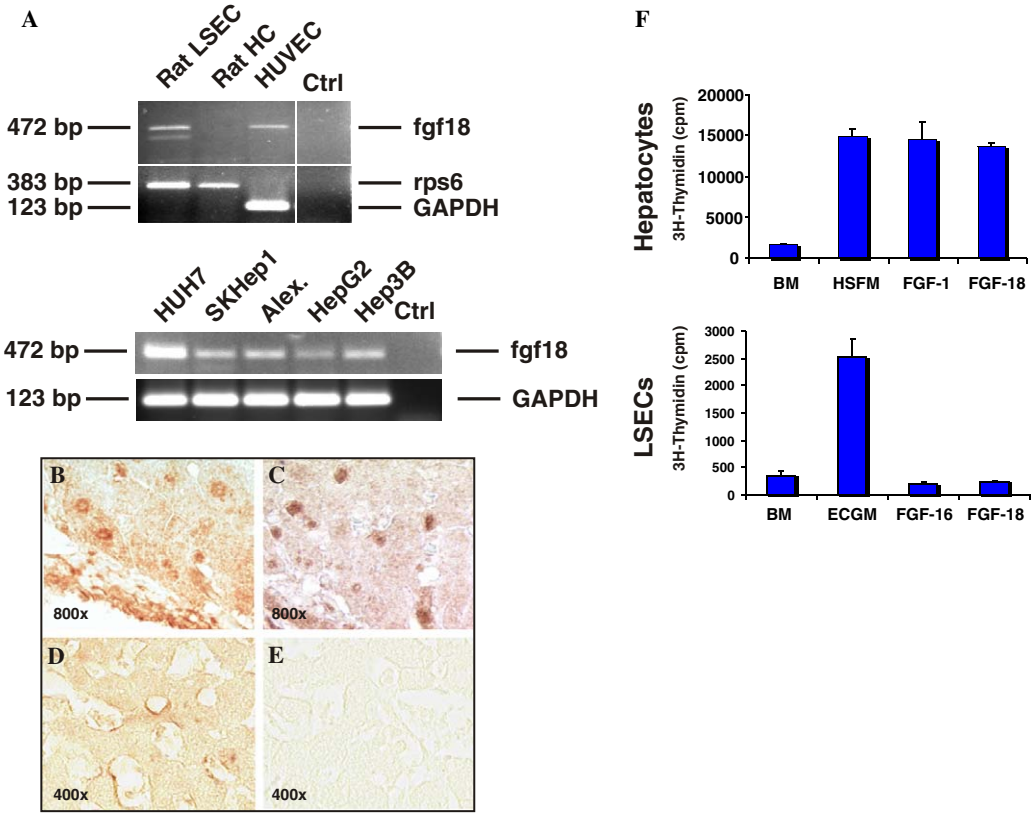

Fig. 5. FGF18 is expressed in liver vascular and sinusoidal endothelial cells and is highly mitogenic for hepatocytes. (A) RT-PCR analysis of Fgf18 RNA expression in rat primary liver sinusoidal endothelial cells (LSEC) and in rat primary hepatocytes. Amplification of the expected size was only detected in rat LSEC, but not in hepatocytes. cDNA prepared from HUVEC was used as positive control (upper panel). RT-PCR analysis of Fgf18 RNA expression in human liver cancer cell lines (lower panel). (B–E) Immunohistochemistry of FGF18 in normal rat liver tissue. Liver section from freshly embedded livers (B,C) and from two-day-old liver slice cultures (D) was processed for immunohistochemistry with a goat polyclonal antibody against FGF18. Negative control (E). (F) To examine DNA synthesis, serum-starved primary liver cell cultures were stimulated with the indicated recombinant FGFs (50 ng/ml). BM, basal medium; HSFM, hepatocyte-serum-free medium; and ECGM, endothelial cell growth medium. Values are means ± SD of three independent experiments.

phosphorylation of ERK1/2 was investigated by Western blotting using an antibody that specifically recognizes phosphorylated ERK1 and ERK2. In cell extracts prepared from cells without FGF2 treatment the amount of active ERK1/2 was low, a marked activation was detected in FGF2, FGF1, and serum stimulated cells. FGF18 activated the MAP kinase just above the base-line state, while FGF16 stimulation did not result in a detectable change of the phosphorylation state of p44/p42 MAP kinases (Fig. 6A). Since there is convincing evidence that sustained or transient activity of MAPK depending on stimulation with EGF or FGF directs the cellular response of neuronal cells either to proliferation or differentiation, we determined the kinetic of ERK1/2 MAPK activation after stimulation with FGF2 and FGF18, respectively [20,21]. In FGF2-stimulated HUVEC, the activation kinetic of ERK1/2 was sustained for 2 h after stimulation with a maximal activation already after 5 min. On the contrary, FGF18 stimulated a transient activation of ERK1/2 at 5 min, which is already declined to base-line levels after 15 min. In addition, we determined the phosphorylation status of the c-Jun N-terminal kinases (JNKs) in FGF18 and FGF2 stimulated HUVEC extracts, respectively. FGF2 but not FGF18 led to a strong but transient activation of a p54 kDa JNK2/3 corresponding band and a smaller amount of activated p46 kDa JNK1. JNK1 and JNK2 have regulatory effects on cell proliferation.

Akt, also known as protein kinase B is considered as a major downstream signalling substrate of the phosphatidylinositol-3-kinase (PI3K) and might play a role in FGF2-stimulated angiogenic response, since expression of kinase inactive c-Akt in EC inhibits FGF2-promoted capillary morphogenesis of IBE [22]. In our experiments, none of the FGFs was able to change Akt phosphorylation above the control levels in HUVEC, suggesting that FGF-mediated effects on HUVEC are not accompanied by an over-phosphorylation of Akt (Fig. 6B). Since FGF18 is mitogenic on hepatocytes, we analysed the activation kinetic of MAP kinase p44/p42 in FGF2 and FG18 stimulated hepatocytes. Both FGFs induced a sustained (over two hours) phosphorylation of ERK1/2 in hepatocytes, whereas FGF2 demonstrated slightly higher earlier phosphorylation levels of the MAP kinases than FGF18 (Fig. 6C). These data suggest that sustained

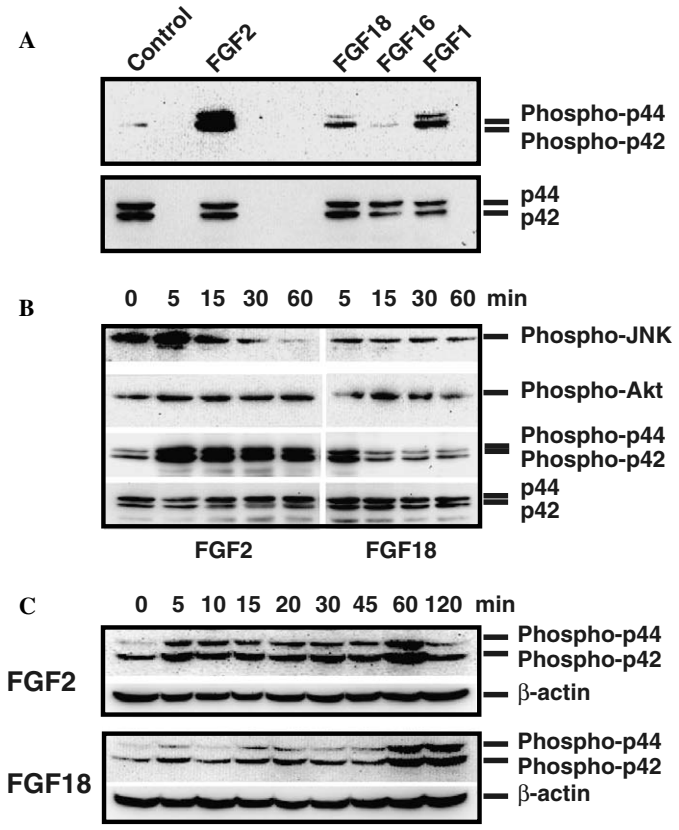

Fig. 6. Kinetic analysis of ERK1/2 activation by FGFs in HUVEC. Cell lysates were generated from FGF-stimulated HUVEC (A,B) or from FGF-stimulated primary rat hepatocytes treated for the indicated time periods (C). (A) Western blot analysis of extracts from untreated (control) HUVE cells or cells stimulated with the indicated rFGFs (25 ng/ml). The cells were harvested 30 min after addition of FGFs, subjected to SDS–PAGE, and examined for active ERK by immunoblotting using anti phospho-p44/42 MAP kinase antibodies. The membranes were stripped and reprobed with control p44/42 MAP kinase antibodies to control equal loading and protein transfer. (B) JNK, AKT, and MAPK ERK1/2 activation kinetics stimulated by FGF2 and FGF18 in HUVEC were evaluated by Western blot analysis using anti-phospho-JNK, -Akt, and -p44/p42 specific antibodies. Protein loading was examined using anti- p44/42 MAP kinase antibodies. Representative data from three independent experiments are shown. (C) FGF18 induces a sustained MAPK activation in hepatocytes. Corresponding MAPK activation in hepatocytes induced by FGF2 and FGF18 was evaluated by probing Western blot of hepatocyte lysates with anti phospho-p44/42 MAP kinase antibodies and reprobed the blots with anti-β-actin antibodies to control equal loading and protein transfer.

phosphorylation of ERK1/2 in HUVEC and hepatocytes induced by FGFs is associated with cell proliferation while a short transient activation of MAPK p44/42 by FGF18 is sufficient to induce on HUVEC migration but not proliferation.

Discussion

FGFs and their receptors have been implicated in blood vessel growth and tissue plasticity in different physiological and pathological conditions. FGFs may exert their effects on endothelial cells via an autocrine mode when secreted endothelial FGFs bind directly to cell surface receptors or when FGFs released by EC are again mobilized from the extracellular matrix. However, FGFs secreted by endothelial cells may also play an important role in a paracrine mode on adjacent cells, e.g., tumor cells. In addition to tumor growth, FGFs produced and released by endothelial cells might have an impact on tissue repair or on organogenesis during development. Therefore, EC which stay in a closed association with a developing organ may be an important source of growth factors to drive its growth and differentiation [23].

In a recent study, we found that HUVEC expressed a whole panel of FGFs capable to interact with all known FGF receptors and reported the expression of FGF16 and FGF18 in endothelial cells [1]. It had been shown that the ability of endothelial cells to respond to mechanical damage is correlated with the level of endogenous FGF2 as well as with that of exogenous FGF2 [24]. Although our results showed that addition of FGF16 and FGF18 in a wound assay appeared to affect the number of cells which have been mobilized and migrated into the wounded area, FGF16 and FGF18 do not seem to influence the proliferation of these cells. Consistent with this finding is that FGF16 and FGF18 effectively induced chemotaxis of HUVEC, while they failed to stimulate cell proliferation in a BrdU incorporation experiment. We used an EC spheroid-based three-dimensional in vitro angiogenesis assay which has some advantages over other assays using EC aggregates: EC spheroids contain a defined number of cells which allows standardized assay conditions and since a large number of endothelial cells can be applied, the assay is less dependent on the proliferation rate of the tested cells. In addition, the cell aggregates are focalized and embedded into a three-dimensional matrix, which allows testing EC invasion into the matrix under quasi in vivo conditions [17]. Sprouting in these assays relies on EC functions very much similar to those important in the in vivo situation; first mobilization of EC out of the quiescent cell layer, followed by cell migration and cell proliferation. FGF2 very efficiently induced capillary sprouting, while FGF1 and FGF4 were clearly less effective. When we tested conditioned medium from Xenopus FGF3 (XFGF3) transfected COS-1 cells in the angiogenesis assays, XFGF3 failed to induce the sprouting process (Fig. 3A). We employed XFGF3, since XFGF3 binds with the same high affinity to both mammalian FGFR2 isoforms IIIb and IIIc, but interacts with low affinity with FGFR1IIIc. In addition, a very weak affinity could be demonstrated for the FGFR3IIIc isoform [25]. Since mouse FGF4 is apparently less efficient than FGF2 in the sprouting assay and binds to FGFR1IIIc with 10-fold lower affinity than to FGFR2IIIc but with high affinity to FGFR3IIIc, our results with the two FGFs suggest that activation of FGFR1 is the key event to induce sprouting and proliferation in HUVEC [26]. FGF16 shares the highest sequence homology with FGF9 and might have a similar binding affinity to the FGF receptors as shown for

FGF9 which binds and activates FGFR4 as well as the IIIc isoforms of FGFR2 and of FGFR3. A similar receptor binding specificity was recently reported for FGF18 [27].

FGF receptor activation leads to recruitment of different intracellular binding partners to activate several important signal pathways such as the MAP kinases and the PI3 kinase (PI3K) signal cascades. Recently, it has been shown that the p38 MAP kinase is also an essential regulator of FGF2-mediated angiogenesis. In a three-dimensional collagen culture system, FGF2 induced activation of p38 kinase. Inhibiting phosphorylation of p38 enhanced the effects of FGF2 on tubular morphogenesis, cell proliferation, and cell differentiation, whereas p38 may serve as a negative feedback mechanism to limit the effect of FGF2 on vessel formation [28]. In agreement with reported results from different groups, we found that FGF1 and FGF2 activate the p38 MAP kinase. However, both FGF16 and FGF18 did not significantly change the phosphorylation of p38 (data not shown). Both secreted FGFs also failed to enhance the phosphorylation of JNKs in endothelial cells, while FGF2 does. Activation of Akt by PI3K may be another signal pathway involved in FGF2-mediated stimulation of proliferation of endothelial cells. At least for proliferation of choriocapillary endothelial cells, it had been shown that activation of Akt appears not to be necessary [29]. Interesting enough, none of the FGFs tested were able to stimulate phosphorylation of Akt above the control levels.

Several studies documented that FGF2 induced a rapid and robust phosphorylation of ERK1/2 MAP kinases and, that activation of the Raf/MEK/ERK signal cascade mediates cell proliferation and migration in endothelial cells. Endothelial cells transfected with the FGFR2IIIb isoform demonstrated FGF7-mediated chemotaxis but not proliferation. The cells stimulated with FGF7 clearly showed a weaker MAP kinase activation than cells stimulated with FGF2, suggesting that signalling via FGFR2 induces cell migration in EC, but leads to an activation of the ERK1/2 pathway that is not sufficient to promote cell proliferation [30]. Consistent with this conclusion are results obtained with chimeric receptor constructs composed of the cytoplasmic domains of FGFR1, FGFR3, and FGFR4 linked to the extracellular ligand binding domains of the PDGF-receptor demonstrating that all FGFRs essentially activate same signal pathways but with different intensity which leads in turn to different activation levels of the downstream localized protein kinases.

FGF16 and FGF18 which failed to stimulate cell proliferation and sprouting of EC in the in vitro angiogenesis assay, in contrast to the mitogenic FGF2 both secreted FGFs do not induce a sustained activation of ERK1/2 MAP kinase suggesting that the longevity of phosphorylation of ERK1/2 may be a prerequisite for cell proliferation of HUVEC. However, the short and transient phosphorylation of ERK1/2 observed with FGF16 and FGF18 seems not sufficient to induce cell proliferation but cell migration on HUVEC. In a recently reported model analysing the difference between EGF and FGF signal pathways, the sustained MAPK activation is correlated to the number of Grb2-Sos complexes recruited to the plasma membrane by FRS2 and EGF stimulation induced a sustained MAPK phosphorylation in cells overexpressing the EGF receptor [21]. The main FGF receptor expressed by hepatocytes is the FGFR4, which is lacking HUVEC and binds to FGF18 and FGF16 [31,32]. Therefore, it seems reasonable to suggest that the mitogenic response of hepatocytes is at least partly due to the FGF18 activation of FGFR4. Taken together, our data support the conclusion that certain members of the FGF family appear to induce a different time course of MAPK activation by signalling via different FGF receptors. The different time frame in which MAPK activation occurred has been considered to be the main cause of diverse cellular responses stimulated by the different FGFs very similar as reported for different growth factors including FGFs and EGF [21,32].

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References

[1] M. Antoine, W. Wirz, C.G. Tag, M. Mavituna, N. Emans, T. Korff, V. Stoldt, A.M. Gressner, P. Kiefer, Expression pattern of fibroblast growth factors (FGFs), their receptors and antagonists in primary endothelial cells and vascular smooth muscle cells, Growth Factors 23 (2005) 87–95.

[2] J.K. Wang, H. Xu, H.C. Li, M. Goldfarb, Broadly expressed SNT-like proteins link FGF receptor stimulation to activators of Ras, Oncogene 13 (1996) 721–729.

[3] H. Xu, K.W. Lee, M. Goldfarb, Novel recognition motif on fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins, J. Biol. Chem. 273 (1998) 17987–17990.

[4] H. Xu, M. Goldfarb, Multiple effector domains within SNT1 coordinate ERK activation and neuronal differentiation of PC12 cells, J. Biol. Chem. 276 (2001) 13049–13056.

[5] Z. Chen, T.B. Gibson, F. Robinson, L. Silvestro, G. Pearson, B. Xu, A. Wright, C. Vanderbilt, M.H. Cobb, MAP kinases, Chem. Rev. 101 (2001) 2449–2476.

[6] R. Giuliani, M. Bastaki, D. Coltrini, M. Presta, Role of endothelial cell extracellular signal-regulated kinase1/2 in urokinase-type plasminogen activator upregulation and in vitro angiogenesis by fibroblast growth factor-2, J. Cell Sci. 112 (Pt 15) (1999) 2597–2606.

[7] A. Gualandris, M. Rusnati, M. Belleri, E.E. Nelli, M. Bastaki, M.P. Molinari-Tosatti, F. Bonardi, S. Parolini, A. Albini, L. Morbidelli, M. Ziche, A. Corallini, L. Possati, A. Vacca, D. Ribatti, M. Presta, Basic fibroblast growth factor overexpression in endothelial cells: an autocrine mechanism for angiogenesis and angioproliferative diseases, Cell Growth Differ. 7 (1996) 147–160.

[8] P. Dell'Era, M. Belleri, H. Stabile, M.L. Massardi, D. Ribatti, M. Presta, Paracrine and autocrine effects of fibroblast growth factor-4 in endothelial cells, Oncogene 20 (2001) 2655–2663.

[9] P.W. Rieck, R.M. von Stockhausen, S. Metzner, C. Hartmann, Y. Courtois, Fibroblast growth factor-2 protects endothelial cells from

damage after corneal storage at 4 degrC, Graefes Arch. Clin. Exp. Ophthalmol. 241 (2003) 757–764.
[10] G. Seghezzi, S. Patel, C.J. Ren, A. Gualandris, G. Pintucci, E.S. Robbins, R.L. Shapiro, A.C. Galloway, D.B. Rifkin, P. Mignatti, Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis, J. Cell Biol. 141 (1998) 1659–1673.
[11] G. Pintucci, B.M. Steinberg, G. Seghezzi, J. Yun, A. Apazidis, F.G. Baumann, E.A. Grossi, S.B. Colvin, P. Mignatti, A.C. Galloway, Mechanical endothelial damage results in basic fibroblast growth factor-mediated activation of extracellular signal-regulated kinases, Surgery 126 (1999) 422–427.
[12] G. Pintucci, P.J. Yu, R. Sharony, F.G. Baumann, F. Saponara, A. Frasca, A.C. Galloway, D. Moscatelli, P. Mignatti, Induction of stromelysin-1 (MMP-3) by fibroblast growth factor-2 (FGF-2) in FGF-2−/− microvascular endothelial cells requires prolonged activation of extracellular signal-regulated kinases-1 and -2 (ERK-1/2), J. Cell Biochem. 90 (2003) 1015–1025.
[13] H. Fehrenbach, R. Weiskirchen, M. Kasper, A.M. Gressner, Up-regulated expression of the receptor for advanced glycation end products in cultured rat hepatic stellate cells during transdifferentiation to myofibroblasts, Hepatology 34 (2001) 943–952.
[14] F. Braet, R. De Zanger, T. Sasaoki, M. Baekeland, P. Janssens, B. Smedsrod, E. Wisse, Assessment of a method of isolation, purification, and cultivation of rat liver sinusoidal endothelial cells, Lab. Invest. 70 (1994) 944–952.
[15] P. Kiefer, M. Mathieu, I. Mason, C. Dickson, Secretion and mitogenic activity of zebrafish FGF3 reveal intermediate properties relative to mouse and *Xenopus* homologues, Oncogene 12 (1996) 1503–1511.
[16] T. Korff, S. Kimmina, G. Martiny-Baron, H.G. Augustin, Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness, FASEB J. 15 (2001) 447–457.
[17] T. Korff, H.G. Augustin, Tensional forces in fibrillar extracellular matrices control directional capillary sprouting, J. Cell Sci. 112 (Pt 19) (1999) 3249–3258.
[18] P. Kiefer, G. Peters, C. Dickson, The Int-2/Fgf-3 oncogene product is secreted and associates with extracellular matrix: implications for cell transformation, Mol. Cell. Biol. 11 (1991) 5929–5936.
[19] M. Antoine, C.G. Tag, W. Wirz, E. Borkham-Kamphorst, I. Sawitza, A.M. Gressner, P. Kiefer, Upregulation of pleiotrophin expression in rat hepatic stellate cells by PDGF and hypoxia: implications for its role in experimental biliary liver fibrogenesis, Biochem. Biophys. Res. Commun. 337 (2005) 1153–1164, Epub 2005 Oct 1156.
[20] J. Schlessinger, Common and distinct elements in cellular signaling via EGF and FGF receptors, Science 306 (2004) 1506–1507.
[21] S. Yamada, T. Taketomi, A. Yoshimura, Model analysis of difference between EGF pathway and FGF pathway, Biochem. Biophys. Res. Commun. 314 (2004) 1113–1120.
[22] S. Kanda, Y. Miyata, H. Kanetake, Fibroblast growth factor-2-mediated capillary morphogenesis of endothelial cells requires signals via Flt-1/vascular endothelial growth factor receptor-1: possible involvement of c-Akt, J. Biol. Chem. 279 (2004) 4007–4016.
[23] E. Lammert, O. Cleaver, D. Melton, Role of endothelial cells in early pancreas and liver development, Mech. Dev. 120 (2003) 59–64.
[24] R. Tsuboi, Y. Sato, D.B. Rifkin, Correlation of cell migration, cell invasion, receptor number, proteinase production, and basic fibroblast growth factor levels in endothelial cells, J. Cell Biol. 110 (1990) 511–517.
[25] M. Mathieu, P. Kiefer, I. Mason, C. Dickson, Fibroblast growth factor (FGF) 3 from *Xenopus laevis* (XFGF3) binds with high affinity to FGF receptor 2, J. Biol. Chem. 270 (1995) 6779–6787.
[26] Y. Li, C. Basilico, A. Mansukhani, Cell transformation by fibroblast growth factors can be suppressed by truncated fibroblast growth factor receptors, Mol. Cell. Biol. 14 (1994) 7660–7669.
[27] J.L. Ellsworth, J. Berry, T. Bukowski, J. Claus, A. Feldhaus, S. Holderman, M.S. Holdren, K.D. Lum, E.E. Moore, F. Raymond, H. Ren, P. Shea, C. Sprecher, H. Storey, D.L. Thompson, K. Waggie, L. Yao, R.J. Fernandes, D.R. Eyre, S.D. Hughes, Fibroblast growth factor-18 is a trophic factor for mature chondrocytes and their progenitors, Osteoarthritis Cartilage 10 (2002) 308–320.
[28] T. Matsumoto, I. Turesson, M. Book, P. Gerwins, L. Claesson-Welsh, p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis, J. Cell Biol. 156 (2002) 149–160.
[29] A. Zubilewicz, C. Hecquet, J.C. Jeanny, G. Soubrane, Y. Courtois, F. Mascarelli, Two distinct signalling pathways are involved in FGF2-stimulated proliferation of choriocapillary endothelial cells: a comparative study with VEGF, Oncogene 20 (2001) 1403–1413.
[30] T. Nakamura, Y. Mochizuki, H. Kanetake, S. Kanda, Signals via FGF receptor 2 regulate migration of endothelial cells, Biochem. Biophys. Res. Commun. 289 (2001) 801–806.
[31] C. Yu, F. Wang, C. Jin, X. Huang, W.L. McKeehan, Independent repression of bile acid synthesis and activation of c-Jun N-terminal kinase (JNK) by activated hepatocyte fibroblast growth factor receptor 4 (FGFR4) and bile acids, J. Biol. Chem. 280 (2005) 17707–17714, Epub 12005 Mar 17704.
[32] V.P. Eswarakumar, I. Lax, J. Schlessinger, Cellular signaling by fibroblast growth factor receptors, Cytokine Growth Factor Rev. 16 (2005) 139–149, Epub 2005 Feb 2001.