FGF18 in colorectal tumour cells: autocrine and paracrine effects

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Fibroblast growth factors (FGFs) and their high-affinity receptors contribute to the autocrine growth stimulation in several human malignancies. Here, we describe that FGF18 expression is up-regulated in 34/38 colorectal tumours and is progressively enhanced during colon carcinogenesis reaching very high levels in carcinoma. Moreover, our data suggest that FGF18 affects both tumour cells and tumour microenvironment in a pro-tumorigenic and pro-metastatic way. Addition of recombinant FGF18 to the culture media of slowly growing colorectal tumour cell lines LT97 and Caco-2 stimulated proliferation. Phosphorylation of externally regulated kinase 1/2 and S6 was increased already 5 min after growth factor addition. SW480 cells, endogenously producing large amounts of FGF18, were not affected in this setting, but recombinant FGF18 supported tumour cell survival under conditions of serum starvation. Down-modulation of endogenous FGF18 production by small interference RNA (siRNA) significantly reduced clonogenicity of SW480 cells and restored sensitivity to exogenous FGF18. With respect to the tumour microenvironment, both recombinant and tumour-derived FGF18 stimulated growth of colon-associated fibroblasts at 0.1 ng/ml and migration at 10 ng/ml. In addition, recombinant FGF18 (10 ng/ml) induced tube formation in human umbilical vein endothelial cells. siRNA knock down demonstrated that tube-forming activity of colon cancer cell supernatants depended to a large part on tumour cell-derived FGF18. In summary, this study demonstrates that FGF18 is almost generally over-expressed in colon cancer and exerts pro-tumorigenic effects both in the epithelial and the stromal compartments by stimulating growth and survival of tumour cells, migration of fibroblasts and neovascularization. Together, these data strongly support an oncogenic role of FGF18 in colorectal cancer.

Introduction

Colorectal cancer develops through a series of pre-malignant (adenomatous) lesions. Molecular analysis has shown the accumulation of tumour-specific mutations during this process along with, and responsible for, progressive de-regulation of growth (1,2). A prominent mechanism of de-regulation is the establishment of autocrine growth stimulation by transforming growth factor alpha (TGFB) that is induced at the adenoma stage and increases with tumour progression (3–5). Recent studies suggest that also members of the fibroblast growth factor (FGF) family are de-regulated in colon carcinogenesis (6–8).

FGFs are a family of 22 peptide growth factors with essential functions in normal cell growth, morphogenesis, tissue repair and angiogenesis (9). In many tumour types, however, FGFs and their tyrosine kinase receptors are inappropriately expressed (10–12). Like other FGFs, FGF18 is an essential mitogen in embryonic limb development and its loss causes severe skeletal defects (13,14). It has been shown that FGF18 plays a crucial role in the development of bone and cartilage, but over-expression in tumours has also been demonstrated (6,15,16). FGF18 expression is up-regulated through the constitutive activation of the Wnt pathway observed in most colorectal carcinomas (6) and, together with FGF16, FGF20 and the FGF signal regulator sprouty 4, it represents a β-catenin target gene (17). FGF18 stimulates growth in NIH3T3 cells, osteoblasts, chondrocytes and glial cells (6,18–20). As a secreted protein, FGF18 can thus affect both the tumour and the connective tissue cells of the tumour microenvironment. To assess the pathophysiological impact of FGF18 in colorectal cancer, we have performed a comprehensive study of gene expression in tissue specimens and cell cultures of normal, pre-malignant and malignant colons. In addition, we have characterized autocrine and paracrine effects of FGF18 on colorectal cancer cells, fibroblast and endothelial cells and suggest an oncogenic function of this FGF family member in human colon cancer.

Materials and methods

Tissue specimen

Tissue specimens of colorectal carcinoma and normal mucosa were obtained from patients undergoing surgery for colorectal cancer. Informed consent was obtained from all the patients. Immediately after surgery, tissue specimens were frozen in liquid N2 until the extraction of RNA.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue specimens of adenomas, carcinomas and adjacent normal tissue from the resection margin were used to prepare serial sections from the same tissue block (1–3 μm). They were deparaffinized, rehydrated and stained according to the standard methods with a polyclonal goat anti-human FGF18 antibody diluted 1:50 (FGF18 C-16, sc-16380, Santa Cruz, Biotechnology, Santa Cruz, CA) and a biotinylated polyclonal rabbit anti-goat second antibody diluted 1:400 (E0466, Dakocytomation, Glostrup, Denmark).

Cell lines

SW480, SW620, T84 and Caco-2 colon carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The cell lines were kept under standard tissue culture conditions using minimum essential medium containing 10% fetal calf serum (FCS). The LT97 colon adenoma cell line has been established in our laboratory (21). This cell line was kept under standard tissue culture conditions using Ham F-12 medium containing 20% L-15 medium, 2% FCS, 10 μg/ml insulin, 2 × 10 −10 M triiodothyronine, 2 μg/ml transferrin, 1 μg/ml hydrocortisone, 5 × 10 −9 M Na-selenite and 30 ng/ml epidermal growth factor. WI38, a normal embryonic lung fibroblast cell line, and F331, a colon-associated fibroblast cell line, were kept in Dulbecco’s minimal essential medium containing 10% fetal calf serum (FCS). The LT97 colon adenoma cell line has been established in our laboratory (21). This cell line was kept under standard tissue culture conditions using Ham F-12 medium containing 20% L-15 medium, 2% FCS, 10 μg/ml insulin, 2 × 10 −10 M triiodothyronine, 2 μg/ml transferrin, 1 μg/ml hydrocortisone, 5 × 10 −9 M Na-selenite and 30 ng/ml epidermal growth factor. WI38, a normal embryonic lung fibroblast cell line, and F331, a colon-associated fibroblast cell line, were kept in Dulbecco’s minimal essential medium containing 10% FCS. Human umbilical vein endothelial cells (HUVECs) were provided by DiT.Mohr (Institute of Cancer Research, Vienna) and cultured in EBM-2 MV medium (Cambrex, East Rutherford, NJ) containing 10% FCS, EGM-2MV SingleQuots (Cambrex) and 60 μg/ml endothelial cell growth supplement. Serum starvation experiments in these cells were done in M199 medium (PromoCell, Heidelberg, Germany) with 1% FCS and 50 U heparin/ml.

Abbreviations: ERK, externally regulated kinase; FCS, fetal calf serum; FGF, fibroblast growth factor; GAPDH, glyceraldehyde phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; IGFBP, insulin-like growth factor; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; RT–PCR, reverse transcription–polymerase chain reaction; siRNA, small interference RNA; TGFs, transforming growth factor alpha.
Isolation of RNA and cDNA synthesis
Total RNA was isolated from sub-confluent cultures or frozen colon tissue specimens using Trizaf reagent according to the instructions of the manufacturer (PeqLab, Erlangen, Germany). First, strand cDNA was synthesized using mouse Moloney leukemia virus reverse transcriptase (Fermentas, Burlington, Ontario, Canada) and random hexamer primers (GE Healthcare, Piscataway, NJ).

Quantitative reverse transcription–polymerase chain reaction analysis of FGF18 messenger RNA
The expression of FGF18 and glyceraldehyde phosphate dehydrogenase (GAPDH) messenger RNAs (mRNAs) was determined by quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR) with Taqman assays from Applied Biosystems (Foster City, CA). Reactions were carried out on an ABI Prism 7000 system (Applied Biosystems) using the standard Taqman assay conditions. FGF18 gene expression in the tumours was calculated as x-fold change compared with the corresponding normal mucosa using GAPDH as a control gene for normalization. Selected RT–PCR experiments for the evaluation of FGF18 gene expression were done using cycles of 40 s denaturation at 94°C, 40 s annealing at 53°C and 40 s extension at 72°C. FGF18 primer sequences were sense 5′-ACTTGGCTGTGTACATTCTCC-3′ and antisense 5′-GCGAGACCTCTCGAATGAC-3′. For GAPDH, primers were sense 5′-GGGAGAATGTGATCAATGG-3′ and antisense 5′-GGGGTGTATGCTGAGTCG-3′. Amplifications were done for 35 and 23 cycles in the case of FGF18 and GAPDH, respectively. PCR products were separated on 6% acrylamide gels. Bands were stained with ethidium bromide and quantified using a GelDoc system (Bio-Rad, Hercules, CA) and ImageQuant 5.0 (GE Healthcare) software.

Luciferase assay
To analyse β-catenin-dependent gene expression, we used reporter constructs that contained wild-type (TOP) or mutant (FOP) Tcf4-binding sites to determine the transactivational activity of endogenous β-catenin/Tcf4 (22). Higher ratios of these two reporter activities (TOP:FOP) indicated a higher β-catenin/Tcf4 activity. A total of 2 × 10^4 cells per well were seeded in 24-well plates, and after 48 h they were co-transfected with 1 μg of TOP-plasmid DNA, respectively, FOP-plasmid DNA and 1 ng Renilla-plasmid DNA using Lipofectamine 2000 (Gibco, Grand Island, NY). After 24 h, cell lysates were obtained by using passive lysis buffer provided with the Dual Luciferase Assay (Promega, Madison, WI) and luciferase activity determined according to the manufacturer’s instructions. Quantification was calculated using the activity of Renilla as an internal control.

Modulation of FGF18 expression
To achieve over-expression of FGF18, a FGF18 cDNA German Research Center for Genome Research (RZPD, Berlin, Germany; IRAW p69B0732D6; NM 003862) was sub-cloned into an adenoviral construct expressing the gene under the control of a cytomegalovirus (CMV) promoter. SW480 and Caco-2 cells were infected with the virus at 1 and 20 MOI (multiplicity of infection), respectively, 24 h after plating. Growth assays of the infected cultures were initiated 48 h after infection and cell number determined another 48 h later.

For knock down of FGF18 expression, cells were seeded at a density of 2 × 10^4 cells per well in six-well plates in a medium containing 10% serum. After 24 h, the cells were transfected with 5 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 h, cell lysates were obtained by using passive lysis buffer provided with the Dual Luciferase Assay (Promega, Madison, WI) and luciferase activity determined according to the manufacturer’s instructions. Quantification was calculated using the activity of Renilla as an internal control.
and further to strongly positive in malignant tumour including both primary and metastatic lesions. Typical examples are shown in Figure 1B.

To clarify whether this increase is also reflected by *in vitro* models for colorectal carcinogenesis, RNA was isolated from five cell lines with increasing malignant potential—LT97, Caco-2, T84, SW480 and SW620. FGF18 expression analysis by RT–PCR (Figure 1C) and by quantitative real-time RT–PCR (data not shown) yielded comparable results. FGF18 expression was low in the adenoma cell line LT97 and in the well-differentiated, slowly growing carcinoma cell lines Caco-2 and T84, whereas it was highest in SW620 cells that have been isolated from a metastatic lesion.

As FGF18 is a known β-catenin target gene, activity of a luciferase-coupled Tcf-promotor was analysed in these cell lines (Figure 1D). Activity of this promoter was found to correlate with the level of FGF18 mRNA expression in a highly significant manner, with *P* = 0.0001 and *r*² = 0.6959, calculated for linear regression (Figure 1E).

**Growth stimulation by exogenous FGF18**

To investigate the cellular effects of FGF18, cells were kept in serum-reduced culture medium (1% FCS, LT97 and Caco-2) or serum-free medium (SW480) for 2 days. Then recombinant FGF18 (1–10 ng/ml) was added and cell number was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 6 days later. FGF18 stimulated growth in the slowly growing, well-differentiated, tumour cell lines LT97 and Caco-2 in a dose-dependent manner (Figure 2A). Growth stimulation ranged from 40 (LT97, 1 ng/ml FGF18) to 100% (Caco-2, 10 ng/ml FGF18) in these cell lines. The poorly differentiated cell line SW480, which expressed large amounts of endogenous FGF18, was not growth stimulated by exogenous FGF18 in this setting (Figure 2A). To study the effects of endogenous FGF18 production, Caco-2 and SW480 cells were infected with an adenovirus expressing FGF18 (FGF18 virus). Cell number was increased in Caco-2 cells infected with FGF18 virus compared with uninfected Caco-2 cells or Caco-2 cells infected with an adenovirus-expressing green fluorescence protein virus.
Exogenous addition of FGF18 did not further increase the cell number of FGF18v-infected Caco-2 cells but increased the cell number of uninfected or green fluorescence protein virus infected Caco-2 cells to the level of FGF18v-infected cells. SW480 cultures was not affected by either over-expression or exogenous addition of FGF18 (Figure 2B). Growth stimulation with time was analysed in LT97 and Caco-2 cell lines, which produced low endogenous FGF18 and found to be progressively increased with prolonged exposure time (Figure 2C and D). The cell line SW480 showed no growth factor-dependent increase of cell number even after 6 days (data not shown).

Measuring DNA synthesis by [3H]-thymidine incorporation checked the impact of FGF18 on SW480 cells after prolonged serum starvation. Under these settings and also in the case of SW480 cells, DNA synthesis was increased by FGF18 up to ~50% (data not shown). Accordingly, cell cycle analysis of SW480 cells by fluorescence-activated cell sorter demonstrated that after prolonged starvation (5 days) and growth factor exposure (4 days), FGF18 significantly increased the fraction of cells in S phase (Figure 2E).

The consequences of FGF18 on apoptosis induction by complete serum starvation were measured by determination of the mitochondrial membrane potential using JC-1. After 5 days of serum starvation,
apoptosis was induced in 13% of the cells, while 10 ng/ml FGF18 reduced this value to 8% (Figure 2F; \( P < 0.05 \)).

The data indicate that under low serum conditions colonic tumour cells require FGF18 for growth and survival and that it may come from exogenous or endogenous sources. Consequently, the FGF18 insensitivity in SW480 cells at early time points might be caused by the high production of endogenous growth factor. Thus, we have used siRNA to knock down FGF18 expression and checked for re-sensitization to FGF18. The two different siRNAs reduced FGF18 mRNA expression to 22 and 35%, respectively, whereas a scrambled siRNA had no effect on FGF18 expression (Figure 3A). These results were also reflected at the level of FGF18 secreted into the culture supernatant as determined by western blot (Figure 3B). Consequently, for all further experiments the most potent siRNA (si-FGF18-1) was employed. Overall, growth of SW480 under standard conditions was not affected in cultures transfected with si-FGF18, but sensitivity to growth stimulation by recombinant FGF18 under total FCS reduction was restored (Figure 3C). Scrambled siRNA had no effect on stimulation of growth by FGF18.

The effect of endogenous FGF18 on colony formation was investigated in SW480 cells plated at low density (100 cells per cm²; Figure 3D, upper panel). Compared with scrambled siRNA, FGF18 siRNA resulted in a 30% decrease in the number of colonies formed (Figure 3D, lower panel), demonstrating paracrine growth stimulation by FGF18 under conditions of low-density plating.

**Activation of FGF18 downstream pathways**

To analyse the signalling pathways that mediate FGF18-induced cell proliferation, Caco-2 carcinoma cells were serum starved for 24 h and then stimulated with FGF18 10 ng/ml for 5–30 min before total protein was harvested. Phosphorylation of ERK and S6 was analysed by western blotting as a measure of the MAPK and the phosphoinositide-3 kinase pathways, respectively. ERK was strongly phosphorylated 5 min after growth factor addition (a 3-fold increase was obtained, \( P = 0.0068 \)), but phosphorylation was lost within 30 min (Figure 4A). Weak phosphorylation of S6 was observed at 5 min of FGF18 exposure (Figure 4B). Similar results were obtained using LT97 adenoma cells (data not shown). Reflecting the lack of growth response in SW480 cells, no phosphorylation of signalling kinases could be observed (data not shown). However, FGF18 response in terms of downstream pathway activation was again restored when endogenous FGF18 was knocked down by si-FGF18. The resulting ERK stimulation was

**Fig. 3.** Consequences of FGF18 knock down in SW480 cells. (A) siRNA oligonucleotides complementary to FGF18 mRNA (si-FGF18) or scrambled siRNA (si-scrambled) were introduced into SW480 cells by lipofection and expression of FGF18 mRNA after 48 h was determined by real-time RT–PCR. Data are normalized to the scrambled siRNA control set as 1. (B) Effects of the siRNA molecules described under (A) on FGF18 secretion by SW480 cells were detected by western analysis (upper panel) and semi-quantified using ImageQuant software (lower panel). Lane 1, 100 ng recombinant FGF18 and lane 2, 100 ng recombinant FGF18 recollected from growth medium by heparin–sepharose. FGF18 secreted by SW480 cells within 48 h treated with scrambled siRNA (lane 3), si-FGF18-2 (lane 4) and si-FGF18-1 (lane 5) were prepared by heparin–sepharose affinity chromatography. (C) The impact of FGF18 knock down by the indicated siRNAs on growth stimulation by recombinant FGF18 was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 48 h. Data are given normalized to the respective control groups without FGF18 set as 100%. (D) Impact of FGF18 knock down on colony-forming ability was determined by plating 100 transfectants/cm² into six-well plates in a medium containing 1% serum. After 7 days, cells were fixed and stained with crystal violet (upper panel) and colonies counted (lower panel). Results are means ± SDs of three experiments in duplicates. * indicates a significant increase and # a decrease as compared with control at \( P < 0.05 \) by Student’s \( t \)-test.
pronounced (55%, \( P = 0.003 \); Figure 4C), while phosphorylation of S6 was increased only by \( \sim 15\% \) (\( P = 0.039 \); Figure 4D).

**Paracrine effects of FGF18 on mesenchymal cells**

To analyse possible paracrine effects of FGF18 on mesenchymal cells, fibroblasts obtained from the colon (F331) or the lung (WI38) were exposed to the growth factor. These cell lines express very little (WI38) or no (F331) FGF18 on mRNA level (data not shown). Both cell lines grew slowly with doubling times of 5 and 2 days, respectively. Addition of FGF18 at concentrations 0.05–1 ng/ml significantly stimulated cell growth in both cell lines, with WI38 being significantly more responsive as compared with F331 (Figure 5A).

In addition, cell migration induced by FGF18 in fibroblast cultures was analysed by scratch tests (Figure 5B–D). F331 cells were characterized by a high-intrinsic migration potential so that scratches healed in \(< 24\text{ h} \) (20\% of the population doubling time). Serum starvation reduced the scratch closure capability from 100 to 50\%. A total of 10 ng/ml of recombinant FGF18 restored migration, leading to \( 80\% \) scratch closure (Figure 5C). In WI38 cells, serum starvation completely abolished migration capability and treatment with FGF18 did not increase migration within 24 h. However, induction of migration by 10\% FCS was observed (Figure 5D). Conditioned media obtained from Caco-2 cells induced a 1.5-fold increase in tube formation; SW480 and SW620 culture supernatants caused a doubling (Figure 5B). Addition of FGF18 to the conditioned media further increased induction of tubes by Caco-2 but not by SW480 supernatants.

Media obtained from SW480 cultures with knocked down FGF18 had significantly reduced tube induction, which could also be restored by the addition of recombinant FGF18. This demonstrates angiogenic activity of colon cancer cell-derived FGF18 (Figure 6C).

**Discussion**

FGF18 is a secreted heparin-binding polypeptide growth factor essential for normal embryogenesis and development. Although its function in normal skeletal development has been extensively studied (26), its role in malignant growth and progression is largely unexplored.
FGF18 has been found up-regulated as a β-catenin target gene in colorectal tumours (6). Our study confirms these results using a larger number of tissue specimens and demonstrates progressive FGF18 over-expression during colon carcinogenesis from adenoma to carcinoma. In vitro cell models perfectly mirrored the clinical situation with highest FGF18 expression in carcinoma-derived cell lines. In the in vitro models, FGF18 expression correlated with the activity of a β-catenin-dependent promoter measured with Top/Fop reporter constructs (22). Levels of free β-catenin, as the major effectors of the Wnt pathway, are limited in normal cells by both ubiquitination and degradation via the proteasome and sequestration at the cell periphery due to interaction with E-cadherin (27). The hyperactivation of the Wnt pathway early in colon carcinogenesis is mainly based on the characteristic adenomatous polyposis coli (APC) mutation (28). Binding of β-catenin in a complex with APC, axin and glycogen synthase kinase 3β is essential for degradation of β-catenin. Accordingly, our adenoma cell model established from a patient suffering from familial adenomatous polyposis harbours a mutated APC gene (21). In addition, loss of E-cadherin (27,29) in poorly differentiated colorectal cancer cells may further contribute to pathway activation explaining the gradual increase of Wnt-pathway activity and hence also target gene products like FGF18 from adenoma to carcinoma.

In addition, our results demonstrate growth stimulatory effects of both recombinant FGF18 and endogenous over-expression of FGF18 in colorectal tumour cell lines with low endogenous production of the growth factor. This corresponds well to the proliferative effect of

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**Fig. 5.** Impact of FGF18 on growth and migration of fibroblasts. (A) Cultures of colonic F331 and lung WI38 fibroblasts were exposed to increasing concentrations of FGF18 diluted in a medium containing 0.2% FCS. Cell viability was determined by 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Results represent means ± SDs of three experiments using triplicates. (B–D) Impact of FGF18 on migration of colon F331 fibroblast (C) as well as lung WI38 fibroblast (D) cultures was determined by scratch assays. Control cultures received medium without FCS (control) or 10% FCS as indicated. Four positions in each scratch as representatively shown in (B) were measured at the indicated time points and migration distance was calculated from the differences of scratch width. The results are given in micrometre migrated distance and represent the means ± SDs of two respective experiments. (E) F331 cells were exposed to conditioned media obtained from Caco-2 and SW480 cells with and without substitution by recombinant FGF18. (F) FGF18 production in SW480 cells was knocked down by transfection with si-FGF18. Control cells received si-scrambled as indicated and migration determined by scratch assays. Conditioned media with or without recombinant FGF18 were used. * and #, significantly increased or decreased above control at *P* ≤ 0.05, respectively.
recombinant FGF18 on hepatic and intestinal epithelia of the mouse (18). In contrast, carcinoma-derived cell lines with high endogenous FGF18 production were insensitive to exogenous growth factor under standard cell culture conditions. Specific survival effects were, however, observed under prolonged serum starvation, where recombinant FGF18 normalized cell cycle distribution and prevented apoptosis induction. These observations suggest that carcinoma cells like SW480 support their own growth and survival by autocrine FGF18 production. Comparable findings have been reported with regard to other FGFs, for example in lung cancer (30). Insensitivity due to endogenous growth factor production is believed to be based on saturation of available receptor growth factor-binding sites on the cell surface and subsequent receptor internalization (31,32). To prove whether this concept also holds true in our models, we knocked down endogenous FGF18 production in SW480. Indeed, sensitivity to recombinant FGF18 was restored not only in terms of growth stimulation but also of downstream signal pathway activation.

This implies that colon carcinoma cell lines like SW480 utilize endogenous FGF18 in an autocrine manner. Accordingly, knock down of FGF18 by siRNA significantly reduced colony-forming capability of SW480 cells confirming a growth- and survival-promoting effect of endogenous FGF18 under more stringent culture conditions. Analysis of the intracellular signalling pathways demonstrated significant phosphorylation of ERK and S6 as indicators of the growth-promoting MAPK and the survival-promoting phosphoinositide-3 kinase/Akt pathways (33,34), respectively. Phosphorylation of S6 was relatively weak as compared with ERK, suggesting that FGF18 signalling is mainly mediated by the MAPK pathway. Together, these results indicate that FGF18 is capable of exerting autocrine stimulation in colorectal tumour cells enhancing both cell proliferation and survival. Until now, autocrine TGFβ mainly mediated by the MAPK pathway. Together, these results in-weak as compared with ERK, suggesting that FGF18 knock down by siRNA on tube formation capacity of SW480 supernatants with or without FGF18 was determined in comparison to scrambled siRNA. The extent of tube formation in all experiments was quantified by measuring tube lengths from digitized photomicrographs using Imaged software. The results (B) and (C) represent means ± SDs of two experiments. * and #, significantly increased or decreased above control at P ≤ 0.05, respectively.

**Fig. 6.** Effect of FGF18 on tube formation by HUVECs. (A) HUVECs were suspended in 50 μl matrigel and tube formation was induced with 10 ng/ml FGF18. Respective photomicrographs are shown. (B) Tube formation of HUVECs (control) was stimulated by the indicated growth factors (solid bars) or conditioned media of the indicated colon carcinoma cell lines with or without FGF18. (C) Impact of FGF18 knock down by siRNA on tube formation capacity of SW480 supernatants with or without FGF18 was determined in comparison to scrambled siRNA. The extent of tube formation in all experiments was quantified by measuring tube lengths from digitized photomicrographs using Imaged software. The results (B) and (C) represent means ± SDs of two experiments. * and #, significantly increased or decreased above control at P ≤ 0.05, respectively.

During embryogenesis and wound healing, FGFs play an important role in tissue interactions, stimulating connective tissue fibroblasts and endothelial cells (9,26). FGF18 mainly functions as a mesenchymal growth factor by stimulating fibroblasts and chondrocytes via the FGF receptor 3 variant IICs (15,41,42). It is increasingly recognized that the stroma has distinct functions in tumour growth and spread (43). In colorectal tumours, myofibroblasts have been shown to accumulate at the invasion front where they provide pro-invasive signals (44) and produce an altered extracellular matrix (45) and the matrix degrading protease urokinase (46). Our work used colon-associated fibroblasts that have previously been shown to interact with normal colonic epithelial cells and provide them with survival signals (47,48). FGF18 produced by the tumour cells exerts paracrine effects on these fibroblasts stimulating both proliferation and cell migration, which is in agreement with a previous study in the mouse (18). Fibroblasts of lung origin (W138), in contrast, were not induced to migrate, even though their growth response to FGF18 was much stronger. These data suggest organ-specific responsiveness of mesenchymal cells to tumour-derived growth factors (49,50). Tumour cell supernatants also stimulated migration of F331 fibroblasts and the differences
between Caco-2 and SW480-derived conditioned media as well as knock-down experiments demonstrate that FGF18 contributes a considerable part of this activity and consequently participates in the reorganization of the tumour-specific tissue (49,51,52).

In addition to fibroblasts, HUVECs were used as a model for the endothelial compartment of the microenvironment. Recombinant FGF18 did not induce growth of HUVECs; however, it stimulated vessel tube formation comparably to vascular endothelial growth factor, a well-known vascularization factor in several tumour types including colon cancer (53). Accordingly, Antoine et al. (50) found enhanced migration but not growth of HUVECs in response to FGF18. The contribution of tumour-derived FGF18 in the conditioned medium from metastasizing colorectal tumour cells (SW620 and SW480) was shown by comparison with the media from Caco-2 cells, producing low FGF18 or cells transfected with FGF18 siRNA. The obtained data demonstrate the capability of secreted FGF18 to stimulate vascularization as a prerequisite of tumour progression (54). Together, these data indicate a role of FGF18 in neoangiogenesis.

Thus, the paracrine effects of FGF18 can produce a condition reminiscent of wound healing and tissue repair—a process that has been shown to exert pro-tumour effects (43,55).

In summary, our results show that FGF18 is increasingly up-regulated during malignant progression in the colon. It affects both the epithelial cell compartment of colorectal tumours by stimulating tumour cell growth and the connective tissue compartment by promoting growth and migration of fibroblasts as well as vessel formation in vascular endothelial cells. This suggests that FGF18 exerts autocrine and paracrine oncogenic functions in colorectal cancers.

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References


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