

Receptor FGFR1 does not promote cell proliferation but induces cell adhesion

XIAOCHEN YANG¹, FLORIAN STEINBERG^{1,2}, LEI ZHUANG¹, RALPH BESSEY¹ and BEAT TRUEB^{1,3}

¹Department of Clinical Research, University of Bern, CH-3008 Bern, Switzerland;

²Center for Biological Systems Analysis, University of Freiburg, D-79104 Freiburg, Germany;

³Department of Rheumatology, University Hospital, CH-3010 Bern, Switzerland

Received January 28, 2016; Accepted April 19, 2016

DOI: 10.3892/ijmm.2016.2601

Abstract. Fibroblast growth factor receptor (FGFR)-like protein 1 (FGFR1) is the most recently discovered member of the FGFR family. Owing to the fact that it interacts with FGF ligands, but lacks the intracellular tyrosine kinase domain, several researchers have speculated that it may function as a decoy receptor and exert a negative effect on cell proliferation. In this study, we performed overexpression experiments with TetOn-inducible cell clones and downregulation experiments with siRNA oligonucleotides, and found that FGFR1 had absolutely no effect on cell growth and proliferation. Likewise, we did not observe any influence of FGFR1 on ERK1/2 activation and on the phosphorylation of 250 other signaling proteins analyzed by the Kinexus antibody microarray. On the other hand, with bacterial petri dishes, we observed a clear effect of FGFR1 on cell adhesion during the initial hours after cell seeding. Our results suggest that FGFR1 is a cell adhesion protein similar to the nectins rather than a signaling receptor similar to FGFR1-FGFR4.

Introduction

The family of the fibroblast growth factor receptors (FGFRs) comprises five transmembrane receptors that control the proliferation, differentiation, migration and apoptosis of most cell types (1,2). The classical four receptors, FGFR1 to FGFR4, function by binding to FGFs and heparin. This interaction triggers the phosphorylation of selected residues in the intracellular part of the polypeptides, followed by the activation of various

signaling cascades, such as the RAS-MAP kinase pathway, the phospholipase C γ pathway and the PI3-kinase pathway.

FGFR-like protein 1 (FGFR1) is the fifth member of the FGFR family (3). Similar to the classical receptors, FGFR1 contains three extracellular Ig-like domains and a single transmembrane domain. It also interacts with FGF ligands and heparin. However, in contrast to the classical receptors, it does not possess any tyrosine kinase activity in the intracellular domain and consequently, it cannot signal by transphosphorylation. It has therefore been speculated that FGFR1 may function as a negatively acting receptor (decoy receptor) that binds and neutralizes FGFs (4,5). The intracellular domain of FGFR1 harbors a tandem tyrosine motif (YXX Φ YXX Φ) and a peculiar histidine-rich sequence. Both motifs act as sorting signals, which target the receptor to endosomes and lysosomes, and control its retention time at the cell membrane (6). It has also been suggested that the tyrosine motif may interact with SHP-1 phosphatase and induce the activation of ERK1/2 protein in β cells (7).

FGFR1 must fulfill a crucial function during embryonic development, as shown by the fact that Fgfr1 knockout mice die during birth with a penetrance of 100% (8,9). When compared to their wild-type littermates, the Fgfr1-deficient mice reveal a striking phenotype: they lack metanephric kidneys (10), they show a malformed diaphragm (8) and they have a dome-shaped skull (9). In a recent study, we showed that the alterations of the diaphragm are caused by the absence of slow muscle fibers (11). Without slow muscle fibers, the diaphragm is too weak to inflate the lungs and the mice will die by suffocation within minutes after birth.

We have recently compared the expression profiles of tissues from wild-type and Fgfr1 knockout mice by gene arrays and RT-PCR (12). Unexpectedly, we found that neither of the typical target genes of FGF signaling was expressed at higher levels in Fgfr1-deficient mice as would be expected after deletion of a negatively acting regulator. It is therefore unlikely that Fgfr1 acts as a decoy receptor, which attenuates FGF signaling. We have also produced mice with a targeted disruption of the intracellular domain of Fgfr1 (13). To our surprise, such mice are fully viable and do not display any obvious alterations when compared to their wild-type littermates. Thus, the intracellular domain is dispensable for normal life and most, if not all, of the vital functions of Fgfr1 must be conducted by the extracellular domain.

Correspondence to: Dr Beat Trueb, Department of Clinical Research, University of Bern, Murtenstrasse 35, CH-3008 Bern, Switzerland
E-mail: beat.trueb@dkf.unibe.ch

Abbreviations: FGF, fibroblast growth factor (human); Fgf, fibroblast growth factor (mouse); FGFR, fibroblast growth factor receptor (human); Fgfr, fibroblast growth factor receptor (mouse); FGFR1, fibroblast growth factor receptor-like protein 1

Key words: fibroblast growth factor, fibroblast growth factor receptor, fibroblast growth factor receptor-like protein 1, cell proliferation, cell adhesion

In this study, we examined the effects of FgfrL1 on cell proliferation and FGF signaling. We found that FgfrL1 did not affect cell proliferation, but that it promoted cell adhesion. It is therefore likely that FGFR1 is a cell adhesion protein, rather than a signaling receptor.

Materials and methods

Cell culture. HEK-TetOn cells were purchased along with the advanced TetOn expression system from Clontech Laboratories (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). All other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA): 293 cells (CRL-1573), A204 rhabdomyosarcoma cells (HTB82), MG63 osteosarcoma cells (CRL-1427), FaDu squamous carcinoma cells (HTB-43) and Detroit 562 pharyngeal carcinoma cells (CCL-138). The cells were grown under standard conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). For cell adhesion assays, the HEK-TetOn cells were cultivated on bacterial (non-tissue culture) petri dishes obtained from Sarstedt AG (Nümbrecht, Germany) (35 mm, 82.1135) or Falcon (60 mm, 1007; Thermo Fisher Scientific AG, Reinach, Switzerland). In some experiments, FGF signaling was stimulated with 15-100 ng/ml of human recombinant FGF2 (PeproTech, London, UK and Sino Biological, Inc., Beijing, China). The growth and shape of the cells were examined under a Nikon E800 eclipse microscope (Nikon AG, Elgg, Switzerland).

Generation of tetracycline-inducible cell clones. The HEK-TetOn cells were co-transfected with two cDNA plasmids. One plasmid contained human full-length FGFR1 or human FGFR1ΔC (encoding amino acids 1-417) in the pTRE expression vector, which harbors a tetracycline responsive promoter element. The other was a linearized hygromycin selection marker (Clontech). Following transfection, the cells were selected with 100 µg/ml hygromycin until resistant colonies could be observed (2-4 weeks). Individual colonies were isolated with the help of cloning cylinders and expanded. Approximately 40 individual cell clones were isolated from each transfection experiment. The clones were pre-screened for inducible FGFR1 expression by immunostaining with a monoclonal antibody against human FGFR1 as described elsewhere (6). For further cultivation of the stable cell clones, hygromycin was used at 60 µg/ml to maintain the selection pressure.

Northern blot analysis. Total RNA was isolated from the confluent cell cultures with the GenElute miniprep kit from Sigma-Aldrich (St. Louis, MO, USA). The RNA was resolved on 1% agarose gels in the presence of formaldehyde. The gels were stained with ethidium bromide to visualize the 18S and 28S ribosomal RNAs. The RNAs were quantitatively transferred to a nylon membrane by vacuum blotting. The blot was hybridized under standard conditions with probes that contained full-length cDNAs for FGFR1 and GAPDH, respectively. Beforehand, these probes had been radiolabeled by random primed oligolabeling with ³²P-dCTP (New England Nuclear/PerkinElmer, Waltham, MA, USA). The blot was extensively

washed with 1X SSC and exposed to X-ray film (Carestream Kodak BioMax MS; Sigma). For quantification of the bands, the blot was scanned with a phosphorimager (Storm 840; Molecular Dynamics, Sunnyvale, CA, USA).

Proliferation assay. Cells grown in tissue culture flasks were collected by trypsinization and seeded into the wells of 24-well plates at a density of approximately 2x10⁴ cells/well. One day after seeding, half of the wells was stimulated with 1 µg/ml doxycycline, while the other half remained untreated. During the following 5 days, the cell number was determined with a colorimetric assay that measures the amount of the endogenous enzyme hexosaminidase (14). To this end, the media were removed from the cell layers by aspiration and 100 µl of a solution containing 3.75 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide, 50 mM sodium citrate, 0.25% Triton X-100, pH 5.0 was added to each well. After 45 min, the reaction was terminated by the addition of 200 µl of 50 mM glycine, 5 mM EDTA, pH 10.4 and the absorbance of the yellow product was determined at 405 nm using an Infinite M200 microplate reader (Tecan AG, Männedorf, Switzerland).

Western blot analysis. Cell cultures were rinsed with cold phosphate-buffered saline (PBS) and directly dissolved in hot SDS sample buffer containing 2% mercaptoethanol. Proteins were separated under standard conditions on 10% SDS polyacrylamide gels containing 5% stacking gels. Resolved polypeptides were transferred from the gels to nitrocellulose membranes by semi-dry blotting (Trans-Blot SD; Bio-Rad Laboratories, Hercules, CA, USA). Unspecific sites of the membranes were blocked with 3% milk powder in PBS. The membranes were incubated with affinity-purified antibodies against phosphorylated human ERK1/2 (E7028, 1:1,000; Sigma). After 12 h, the membranes were rinsed with PBS and incubated for 2 h with phosphatase-conjugated secondary antibodies (711-055-152; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Bound antibodies were detected by reaction with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium substrate. After stripping, the blots were stained in a similar way with a monoclonal antibody against total p42/44 MAP kinase (L34F12; 1:2,000; Cell Signaling Technology Inc., Danvers, MA, USA).

Kinexus antibody microarrays. Protein extracts were prepared from HEK-TetOn cells grown on 10 cm tissue culture plates in the presence or absence of the inducer, doxycycline. Special care was taken to ensure that the two samples were always treated in parallel in the same way. The cell layers were rinsed with ice-cold PBS and lysed with 0.2 ml lysis buffer containing 20 mM MOPS, 1% Triton X-100, pH 7.0 and proteinase and phosphatase inhibitors as suggested by Kinexus Bioinformatics Corp. (Vancouver, BC, Canada). To rupture the cell bodies, the suspensions were sonicated twice for 15 min with an ultrasonic processor (Sonics Vibra-Cell; Sonics & Materials, Inc., Newtown, CT, USA). The homogenates were cleared in a Centrikon T-2170 ultracentrifuge at 100,000 x g for 30 min. Subsequently, the clear homogenates were analyzed on Kinex Antibody Microarrays (KAM-1.1; Kinexus Bioinformatics Corp.) that contained >650 different antibodies against signaling proteins. Selected proteins were

further verified by the Kinetworks multi-immunoblotting service.

Downregulation by siRNA. The expression of FGFR1 was downregulated with siRNA oligonucleotides from Dharmacon (ON-TARGETplus SMARTpool siRNA; Thermo Fisher Scientific, Waltham, MA, USA). To target the human FGFR1 mRNA, a pool of the following four oligonucleotides was utilized: GGCUGAAGCGCGUGGAGUA, GGACACUGAGCCUGAAGAA, GCUCCUACCUCAUAAGCU and GAA CACGACGGUGGACUUC. As a control, a pool of four non-targeting oligonucleotides was used (D-001810-10-05). To form lipid complexes, the oligonucleotides were mixed with Lipofectamine 2000 reagent in Opti-MEM medium (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) and incubated for 5 min at room temperature. The complexes were added dropwise to the cells grown either in 35 mm dishes (for northern blot analysis) or 24-well plates (for proliferation assays) at a final concentration of 25-50 nM.

Results

Effect on cell proliferation. To measure the effects of FGFR1 on cell growth and proliferation, and to avoid problems of reproducibility that are frequently observed by transient transfection experiments, we used a stable, tetracycline-inducible expression system. Commercially available HEK-TetOn cells, which constitutively express the Tet transactivator protein, were stably transfected with two different tetracycline-inducible FGFR1 constructs, namely the human wild-type construct FGFR1 and the human construct FGFR1ΔC, which lacks the intracellular domain (Fig. 1A). Following selection with antibiotics, colonies were isolated and pre-screened for FGFR1 expression by immunostaining with an antibody against FGFR1. Positive clones were further checked for strong inducibility of FGFR1 expression by northern blot analysis (Fig. 1B and C). Clones with a relatively high induction of FGFR1 expression and relatively low background expression before the addition of doxycycline were selected for further experiments (full-length clones: K3F, K5F, K9F, K17F, K19F, K24F; truncated clones FGFR1ΔC: K13ΔC, K14ΔC, K33ΔC, K35ΔC, K41ΔC). In the following, we will describe several experiments performed with these clones and present data for some selected clones. However, it should be emphasized that most experiments were performed with all of the clones and that we never observed any qualitative differences among the clones.

On a northern blot analysis, our clones showed a relatively tight repression before, but a relatively strong expression of FGFR1 after the addition of the inducer, doxycycline (Fig. 1B and C). Nevertheless, when the blot was overexposed, bands corresponding to the FGFR1 mRNA could also be detected in the lanes without doxycycline, suggesting that our expression system showed some leakiness. The extent of inducibility was determined by quantification of the northern blotting with the help of a phosphorimager. Clone K17F could be induced >180-fold, and clone K24F >30-fold.

A slight heterogeneity was noted between the individual clones with respect to the length of the mRNAs. This heterogeneity may originate from different sizes of the 3'UTR and the poly(A) tract due to integration of the cDNA constructs at different sites in

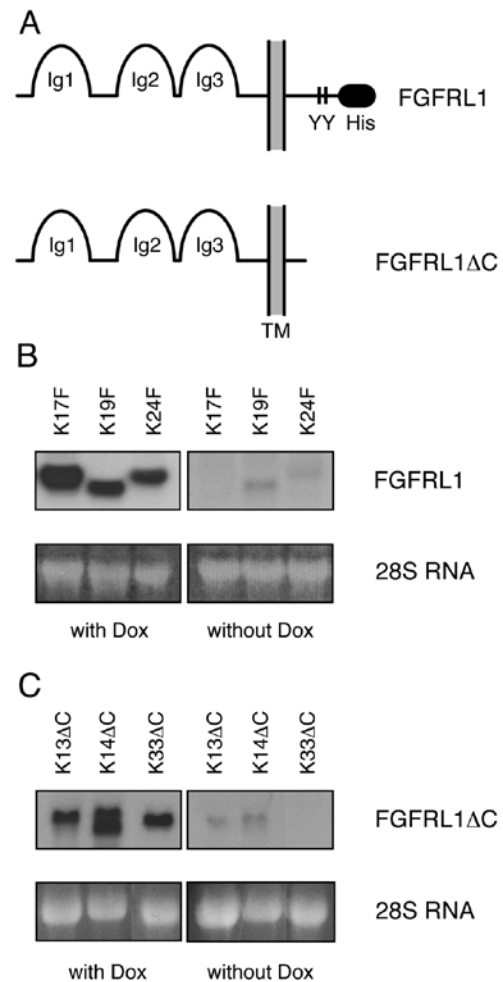


Figure 1. Generation of HEK-TetOn cell lines with tetracycline-inducible fibroblast growth factor receptor-like protein 1 (FGFR1) expression. (A) Schematic representation of the two FGFR1 constructs that were stably transfected into HEK-TetOn cells. FGFR1 represents the full-length, wild-type receptor, while FGFR1ΔC contains a truncated C-terminal domain lacking the histidine-rich sequence and the tandem tyrosine motif. The two motifs are known to control the retention time of the receptor at the cell membrane. Hygromycin resistant cell clones were analyzed by northern blotting for inducible FGFR1 expression. (B) Three representative full-length clones and (C) three representative C-terminally truncated clones are shown before and after induction with 1 μg/ml doxycycline. As a loading control, the 28S ribosomal RNA stained with ethidium bromide is included.

the genome. In two selected cases (K17F, K13ΔC) we subcloned the expressed cDNA by RT-PCR and fully confirmed the open reading frame of the FGFR1 sequence.

Fig. 2 shows two examples of FGFR1 expression in cell culture by immunofluorescence with a monoclonal antibody against human FGFR1. In the absence of the inducer doxycycline, no expression was observed. In the presence of doxycycline, a strong fluorescent signal was observed, indicating strong inducibility of FGFR1 expression. As expected, the fluorescent signal was predominantly found at the cell membrane in the case of the FGFR1ΔC construct (K13ΔC), but mainly in intracellular compartments in the case of the full-length construct (K3F). As previously reported, this difference in the distribution of the expressed protein is explained by the presence or absence of two sorting signals in the transfected FGFR1 constructs (6). Thus, we have successfully generated cell lines, in which we can overexpress FGFR1 by 30 to 180-fold.

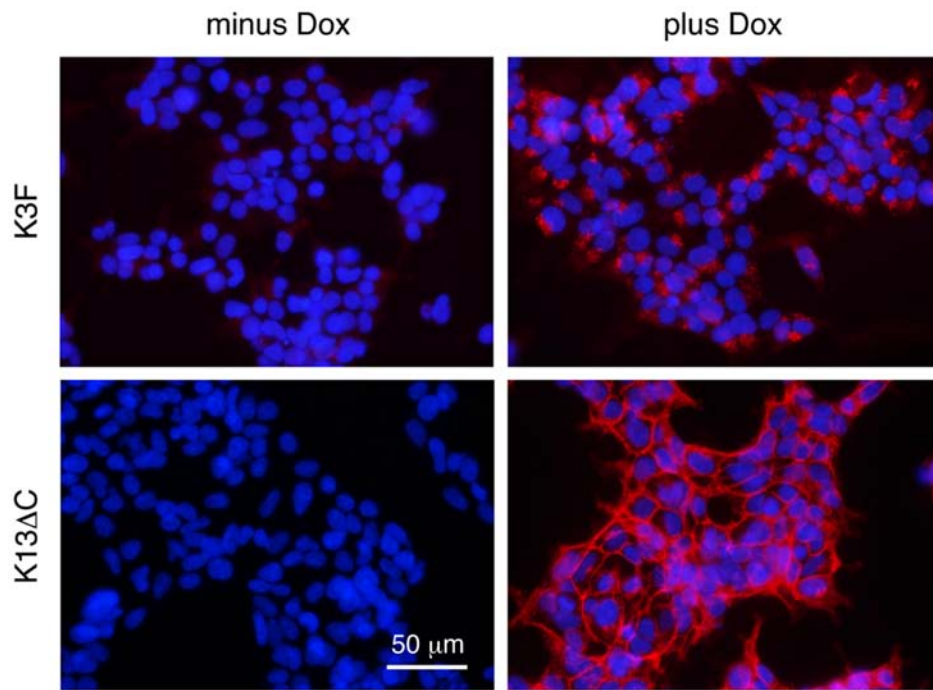


Figure 2. Inducible expression of fibroblast growth factor receptor-like protein 1 (FGFR1) demonstrated by immunofluorescence. A representative full-length clone (K3F) and a representative truncated clone (K13ΔC) were cultivated in the presence (plus Dox) or absence (minus Dox) of the inducer doxycycline. Expression was visualized with a monoclonal antibody against human FGFR1. Note that the wild-type receptor resided primarily in intracellular compartments, while the truncated form accumulated at the plasma membrane.

The inducible TetOn clones were utilized to examine the effects of FGFR1 on cell proliferation. Individual clones were seeded on cell culture plates and grown for up to 5 days in the presence or absence of the inducer, doxycycline. As shown in Fig. 3, the addition of doxycycline at 1 μ g/ml had absolutely no effect on cell growth. Albeit we noted a slight difference in the growth curves between individual clones, a given clone always showed the same growth characteristics in the absence or presence of doxycycline. This observation was true for full-length clones (e.g., clones K3F, K19F) as well as for C-terminally truncated clones (e.g., clones K13ΔC, K33ΔC). Thus, the presence of FGFR1 does not appear to have any effect on cell growth, at least not on the proliferation of our HEK-TetOn cells.

We also tried to prepare other stable cell lines (HT1080, A204) that would express FGFR1 in an inducible manner. However, we failed to obtain any other stable cell clones. This finding is in keeping with the remarks of the distributor of the TetOn expression system that it is extremely difficult to select cell lines, which stably express the Tet transactivator protein.

ERK1/2 signaling. Since we had expected that FGFR1 would be involved in FGF signaling, we examined the effects of FGFR1 on the activation of the MAP/ERK signaling pathway. These experiments were conducted only with the full-length clones, K3F, K5F, K17F, K19F and K24F, which possess an intact intracellular domain. The clones were grown on cell culture plates for 1-2 days in the presence of fetal bovine serum, starved overnight and then stimulated with FGF2 at 15 ng/ml or 30 ng/ml (a concentration of 15 ng/ml has a maximal effect on ERK phosphorylation according to a previous publication) (15). At the same time, half of the cultures were treated with doxycycline to induce the expression of FGFR1, while

the other half were left untreated. After different time intervals, the cell layers were dissolved in SDS sample buffer and analyzed by western blotting with specific antibodies against phosphorylated ERK (Fig. 4A). Before the addition of FGF2, the level of phosphorylated ERK of our HEK-TetOn cells was below the detection level. After the addition of FGF2, we observed two faint bands that should correspond to phosphorylated ERK1 and ERK2. Most importantly, we did not observe any differences in the levels of phosphorylated ERK between cultures grown in the absence or the presence of the inducer, doxycycline. To verify that our samples contained equal amounts of unphosphorylated ERK, we treated the same western blot after stripping with an antibody to total ERK. Two bands corresponding to ERK1 and ERK2 were observed and these bands had similar intensities in the samples before and after doxycycline treatment. Thus, the expression of FGFR1 did not appear to have any effect on ERK1/2 signaling.

Since the ERK signal detected in Fig. 4A was rather faint, we transfected a full-length clone for FGFR1 into our cell lines in order to enhance FGF signaling. In fact, we were able to increase the ERK signal significantly by this treatment (Fig. 4B). At the beginning of the experiment (0 min), no ERK phosphorylation was detected, but 5 and 15 min after FGF stimulation, the bands of phosphorylated ERK1/2 became much stronger than in the experiment without FGFR1 transfection. Nevertheless, we could not detect any differences in ERK phosphorylation between cultures grown in the presence or absence of doxycycline.

Kinexus antibody microarray. Since FGFR1 may also affect a pathway other than ERK signaling, we subjected two selected clones (K3F, K17F) to a global analysis of signaling proteins. As

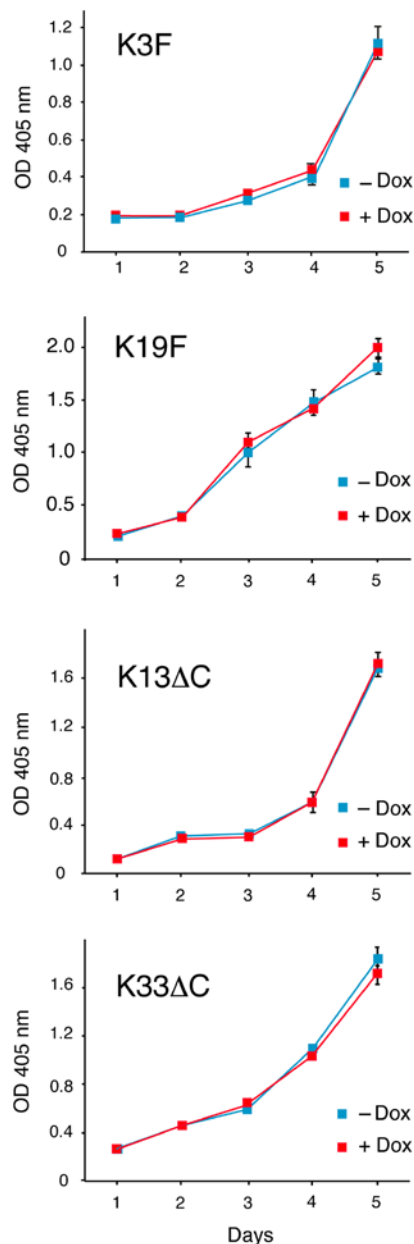


Figure 3. Effect of fibroblast growth factor receptor-like protein 1 (FGFRL1) expression on cell proliferation. Stable HEK-TetOn clones were cultivated in the presence or absence of doxycycline in 24-well plates. Cell proliferation was measured over the course of five days with the hexosaminidase assay. No effect on cell proliferation was observed by the presence of FGFRL1 or FGFRL1ΔC. Standard errors are given at some time-points where meaningful (n=3).

above, the cells were grown for 32 h in the presence or absence of the inducer doxycycline and protein extracts were prepared according to an established protocol. The extracts were analyzed by the Kinexus antibody microarray service, which utilizes >650 verified antibodies against most well-studied signaling proteins, including phospholipase C γ , PI3-kinase, STAT and Akt. Differences in the absolute amount of the signaling proteins (using 380 pan-specific antibodies), as well as differences in their phosphorylation status (using 270 phospho-site specific antibodies) were investigated. To our surprise, we did not find any significant differences between the doxycycline-treated and untreated cell cultures. Nevertheless, 18 proteins that had shown slight (but not significant) changes between the two

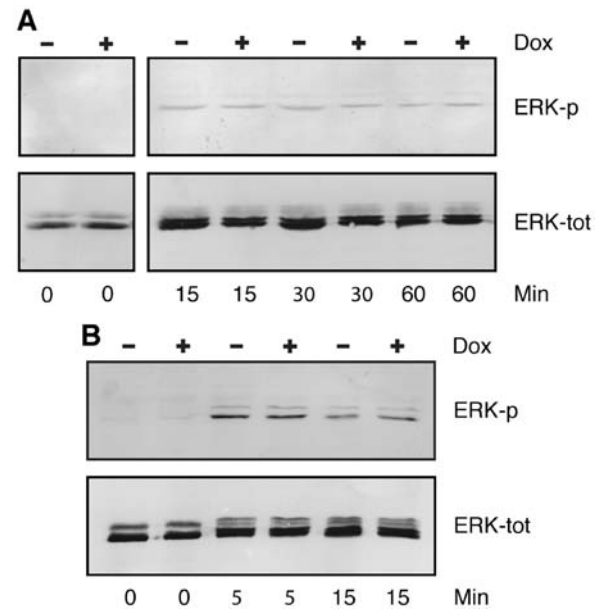


Figure 4. Effect of fibroblast growth factor receptor-like protein 1 (FGFRL1) on ERK phosphorylation. FGFRL1 inducible HEK-TetOn cells were cultivated in multi-well plates, starved overnight in medium lacking fetal bovine serum and then stimulated for 0-60 min as indicated with human FGF2 (15 ng/ml). Cells were lysed with hot SDS sample buffer. Cellular proteins were resolved on polyacrylamide gels and processed for western blotting with antibodies against phosphorylated ERK1/2 (ERK-p). The blots were stripped and reprobed with antibodies against total ERK (ERK-tot). An experiment conducted with clone K24F is depicted in panel A. Panel B shows an experiment with clone K17F. However in this case, the cells had been transfected - prior to starvation and FGF2 stimulation - with a full-length clone for human FGFR1 to increase signaling. ERK1/2 phosphorylation was extremely low before stimulation, but clearly visible after stimulation with FGF2. No difference in ERK phosphorylation was observed between doxycycline-induced and uninduced cells.

culture conditions were selected for further investigation. To this end, the two protein extracts were subjected to western blotting with a set of specific, verified antibodies against the 18 signaling proteins. However, as demonstrated by the immunoblot of Fig. 5, we could not detect any significant differences in the absolute amount or in the phosphorylation status of the selected signaling proteins. Thus, the expression of FGFRL1 in our cell clones did not appear to have any major effect on the signaling pathways that are covered by the Kinexus antibody microarray.

Downregulation of FGFRL1 expression by siRNA. As mentioned before, our doxycycline-inducible FGFRL1 TetOn clones show, in addition to their strong inducibility, a low level of leakiness. If a low level of FGFRL1 expression would already elicit a full response, we would not see any additional effect upon the overexpression of FGFRL1. To tackle this problem, we performed a set of converse experiments, in which we tried to downregulate FGFRL1 expression. For this purpose, we selected three different cell lines that express either low levels of FGFRL1 (293 kidney cells), moderate levels of FGFRL1 (A204 rhabdomyosarcoma cells) or relatively high levels of FGFRL1 (MG63 osteosarcoma cells), as demonstrated by northern blotting with a probe for human FGFRL1 (Fig. 6A). A pool of siRNA oligonucleotides was obtained from Dharmacon, which should effectively downregulate FGFRL1 mRNA levels following transfection into cells. In fact, when used at 25 nM, these oligonucleotides decreased

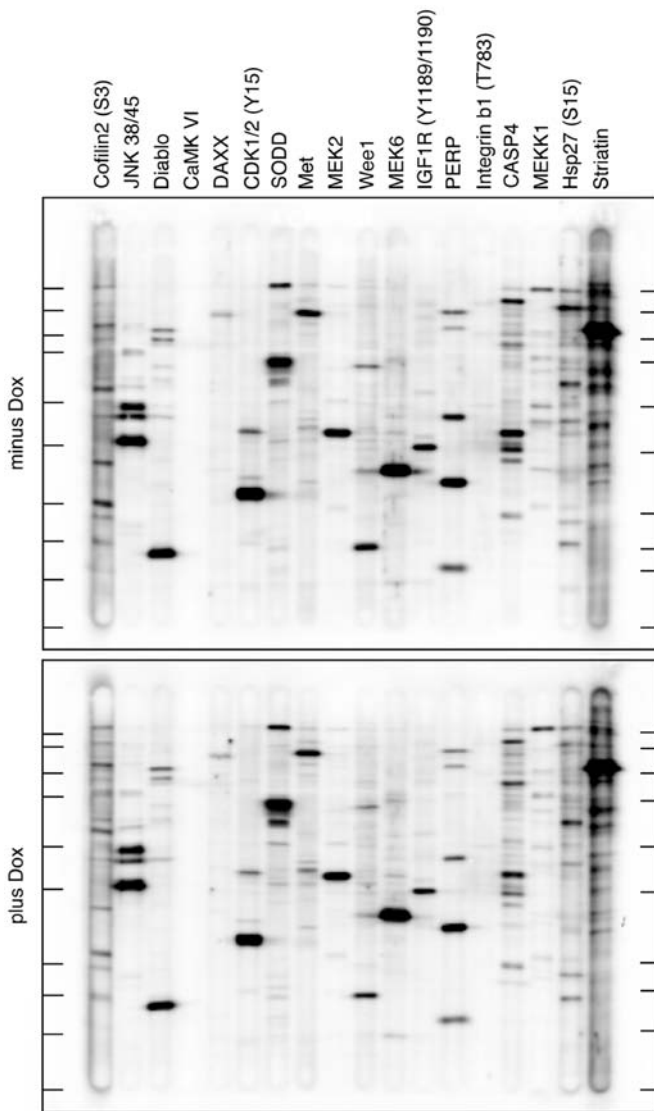


Figure 5. Kinexus antibody microarray. Fibroblast growth factor receptor-like protein 1 (FGFR1) inducible cells (clone K3F) were cultivated for 32 h in the presence or absence of the inducer doxycycline. Protein extracts were prepared and analyzed on Kinexus antibody microarrays containing more than 650 different antibodies against various signaling proteins. No significant differences were observed between doxycycline-treated and untreated cells (not shown). To verify some of the results, the two protein extracts were separated on SDS polyacrylamide gels, transferred to an immunoblot membrane and probed with antibodies against 18 selected proteins as indicated using a multiscreen apparatus from BioRad. The immunoblot was analyzed with a chemiluminescence imager. No significant differences were observed between doxycycline-induced (lower panel) and uninduced (top panel) cells. The migration positions of molecular weight markers are shown in the left and right margins by short horizontal lines. These lines correspond (from top to bottom) to 250 K, 150 K, 100 K, 75 K, 50 K, 37 K, 25 K, 20 K and 15 K.

FGFR1 levels in the 293, A204 and MG63 cells by 75-84%, as demonstrated by northern blotting and quantification with a phosphorimager (Fig. 6B). However, the same siRNA oligonucleotides had no effect on the levels of the GAPDH mRNA or 28S RNA. Likewise, a pool of control RNA oligonucleotides had no effect on the level of the FGFR1 mRNA, demonstrating the specificity of the FGFR1 siRNA pool.

Cell proliferation was determined by the hexosaminidase assay two days following transfection of the siRNAs or control RNAs into the three cell types. Although the siRNA pool had

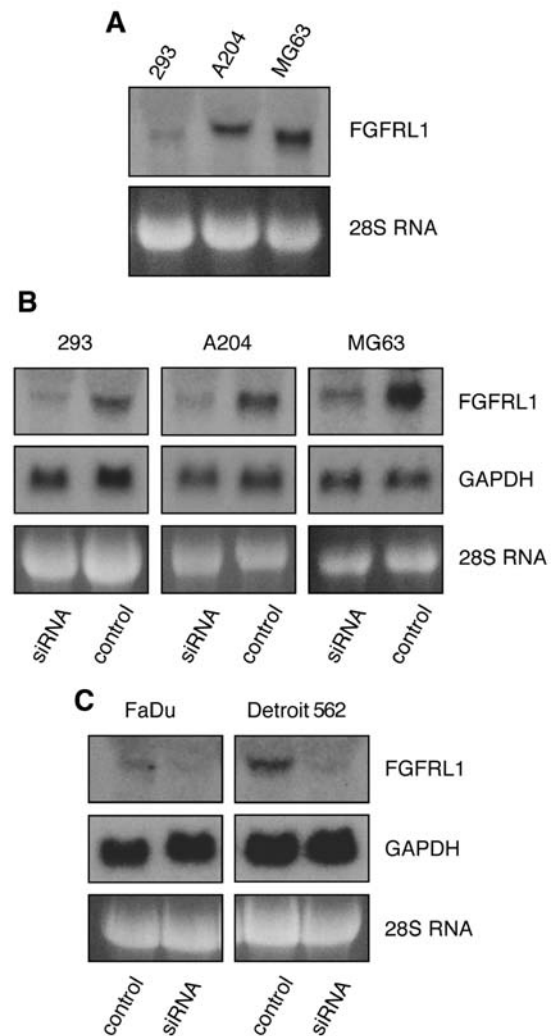


Figure 6. Downregulation of fibroblast growth factor receptor-like protein 1 (FGFR1) by siRNA oligonucleotides. (A) A northern blot demonstrates the levels of FGFR1 mRNA in three different cell lines. Osteosarcoma cells (MG63) express relatively high levels of FGFR1, A204 rhabdomyosarcoma cells moderate levels and 293 cells very low levels. (B) The cells were transfected with a pool of siRNA oligos against FGFR1 or with control RNA oligos. In each of the three cell lines, the FGFR1 mRNA levels were downregulated to near zero, while the levels of GAPDH and 28S RNA remained unaffected. (C) An analogous experiment was performed with two cell lines from esophageal cell carcinomas. Detroit 562 cells express moderate levels of FGFR1, FaDu cells extremely low levels. In both cases, the FGFR1 levels could be downregulated to near zero with siRNA oligos.

downregulated FGFR1 mRNA levels to near zero, we did not detect any changes in the proliferation rate of the 293, A204 and MG63 cells (Fig. 7A). Obviously, the downregulation of the FGFR1 mRNA had no significant effect on cell proliferation.

Recently, another research group reported that FGFR1 would have a stimulatory effect on the proliferation of certain tumor cells (16,17). These authors studied primary and established cell lines derived from human esophageal squamous cell carcinoma (ESCC). We therefore extended our siRNA experiments on esophageal squamous carcinoma cells. As before, we used a cell line that expressed low levels of FGFR1 (FaDu cells) and another cell line that expressed relatively high levels of FGFR1 (Detroit 562 cells) as assessed by northern blotting (Fig. 6C). When our pool of siRNA

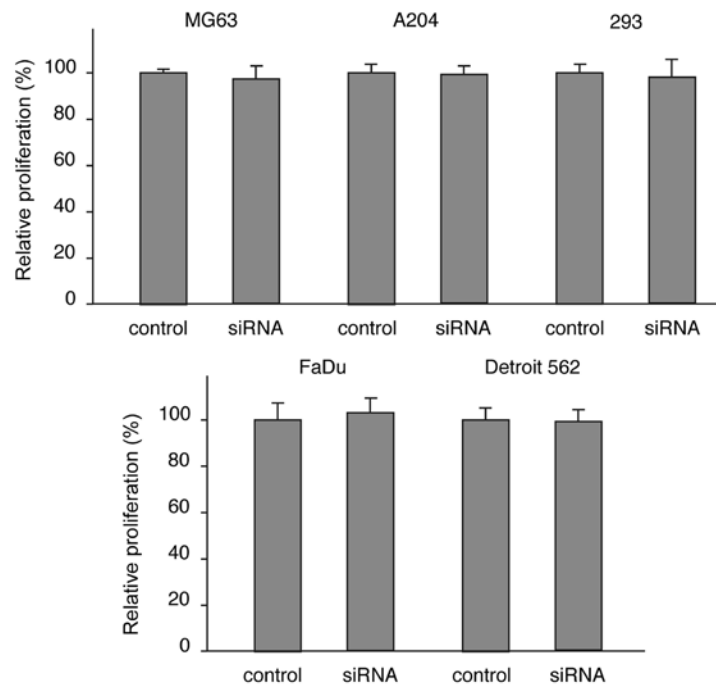


Figure 7. Effect of fibroblast growth factor receptor-like protein 1 (FGFRL1) downregulation on cell proliferation. Expression of FGFRL1 was downregulated with siRNA oligos in different cells lines as indicated and cell proliferation was measured with the hexosaminidase assay after two days. The results are expressed relative to a control treated with unrelated RNA oligos (100%). The bars indicate standard errors from four independent samples. No differences in cell proliferation were noted between control and FGFRL1 downregulated cells.

oligonucleotides was transfected into these cells, we observed a downregulation of FGFRL1 mRNA levels to nearly zero. However, cell proliferation, measured by the hexosaminidase assay, did not significantly differ between doxycycline-treated and untreated cells after two days in culture (Fig. 7B). Thus, it is very unlikely that FGFRL1 has any stimulatory effect on the proliferation of our esophageal tumor cells.

Cell adhesion. If FGFRL1 does not have any effect on cell growth and proliferation, it would be of interest to determine what function it has, if any. We have previously observed that recombinant FGFRL1 induced cell adhesion when coated on bacterial plastic dishes (18).

We therefore investigated whether the inducible expression of FGFRL1 in our HEK-TetOn cells would have any effect on cell adhesion. To this end, our cell clones were seeded into untreated (bacterial) petri dishes and grown in the absence or presence of the inducer, doxycycline. Cell adhesion was inspected under a microscope 10-15 h after seeding. As demonstrated in Fig. 8, neither of the cell lines was able to attach to the bacterial plastic plates in the absence of doxycycline within this short period of time. However in its presence, the cells began to attach to the plastic surface and to spread. Moreover, they aggregated and began to form large clusters, probably by cell-cell adhesion. This adhesion and clustering phenomenon was observed with the full-length clones as well as with the C-terminally truncated clones. A slight difference was noted between individual clones in the extent of clustering. This difference may reflect different amounts of FGFRL1 protein synthesized by individual cell clones within a given period of time. After 24 h, the differences between doxycycline-treated and untreated cells were no longer dramatic and the cells

began to attach and cluster also in the absence of doxycycline. This may be explained by the leakiness of inducible FGFRL1 expression and/or by the synthesis of adhesive proteins other than FGFRL1 (fibronectin, collagen, NCAM). Nevertheless, our experiments show that FGFRL1 has a clear effect on cell adhesion during the initial hours after seeding.

Discussion

In the present study, we presented compelling evidence that the novel receptor, FGFRL1, does not control cell growth and proliferation, but that it rather exerts a positive effect on cell adhesion. This conclusion was drawn from various experimental approaches, including: i) the inducible overexpression of FGFRL1 in HEK-TetOn cells; ii) the downregulation of FGFRL1 by siRNA oligonucleotides in established cell lines; iii) the determination of ERK phosphorylation in the presence and absence of FGFRL1; iv) the analysis of 650 signaling molecules by Kinexus antibody microarrays; and v) cell adhesion on bacterial petri dishes upon induction of FGFRL1 synthesis. Originally, we as well as other researchers had expected that FGFRL1 would have a negative effect on cell growth as it interacts with growth factors of the FGF family but cannot signal by phosphorylation as it lacks the intracellular tyrosine kinase domain (5,19). The interaction with FGF ligands has been confirmed in different laboratories using various approaches, such as coprecipitation, dot blot assays, cell-based ligand binding assays and surface plasmon resonance, and it was found that FGFRL1 mainly interacted with FGF3 and FGF8 (3). Obviously, we must face the fact that this interaction does not trigger cell proliferation. Nevertheless, the new findings are compatible with the domain structure of FGFRL1 as

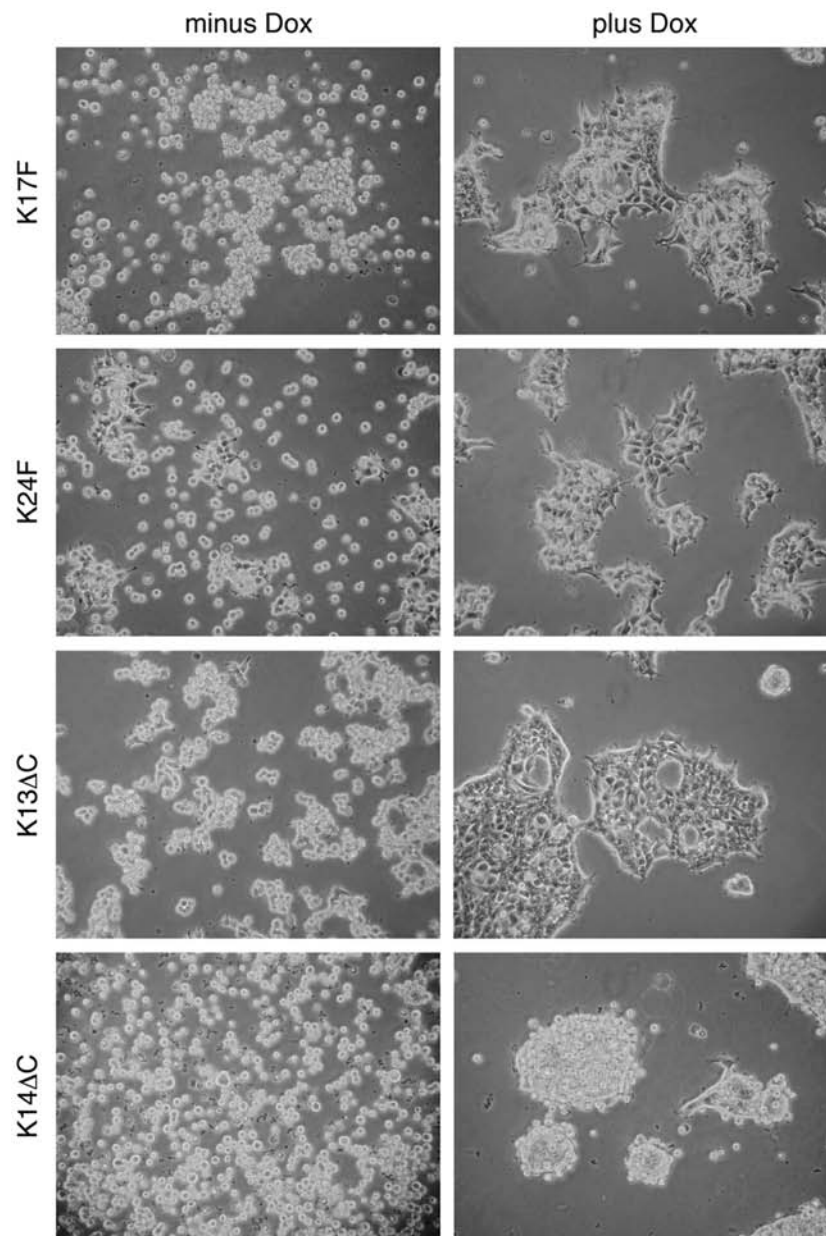


Figure 8. Effect of fibroblast growth factor receptor-like protein 1 (FGFR1) on cell adhesion. FGFR1 inducible HEK-TetOn cells were seeded into bacterial (non-tissue culture) petri dishes and cultivated for 10-15 h in the absence (minus) or presence (plus) of the inducer doxycycline. Note that expression of FGFR1, in its full-length form as well as its truncated form, promoted cell-cell adhesion and cell clustering. In sharp contrast, most cells remained in their single-cell state in the absence of FGFR1 within this short period of time. A control with original HEK-TetOn cells (prior to transfection with FGFR1 constructs) did not show any differences between the two conditions.

this receptor is lacking the intracellular tyrosin kinase domain that would be required for phosphorylation. The new findings are also in accordance with the observation that genetically manipulated mice with a deletion of the intracellular domain of FgfrL1 are viable and do not exhibit any abnormal phenotype when compared with wild-type littermates (13). Obviously, the intracellular domain of FGFR1 is dispensable for survival.

The only definite effect of FGFR1 that we could document in our study was a positive influence on cell adhesion. Upon expression of FGFR1 in our inducible HEK-TetOn clones, the cells started to aggregate and to form large clusters. Without FGFR1 induction, no similar clustering was observed. However, the effect was detected only during the initial hours after cell seeding and after two days, we did not notice any

differences between FGFR1 induced and uninduced cell cultures. It is possible that after two days, other cell adhesion molecules, such as fibronectin, collagen and NCAM, have taken over the adhesive function of FGFR1. Furthermore, the effect could not be demonstrated with surface-treated culture dishes that are normally utilized for cell culture experiments. A critical function of FGFR1 in cell adhesion is compatible with our previous observations that FGFR1 can bind to heparin and to heparan sulfate proteoglycans on cell surfaces (18). At that time, we showed that the second Ig domain was responsible for the heparin-binding activity and that it could be blocked with soluble heparin as well as with heparin-binding peptides.

An important function of FGFR1 in cell-cell adhesion had already been suggested by our studies with knockout

mice. FgfrL1-null mice lack metanephric kidneys because the metanephric mesenchyme is not able to undergo the essential conversion into the nephrogenic epithelium (10). Obviously, FgfrL1 must be expressed at the cell surface of mesenchymal cells in order to induce proper alignment and tight cell clustering as required for differentiation into renal epithelium. A similar effect was also noticed in the diaphragm. FgfrL1 knockout mice die because their diaphragm is too weak to inflate the lungs after birth (8,11). During development of the diaphragm muscle, individual muscle precursor cells must align with each other in order to fuse into myotubes and myofibers. Obviously, FGFR1 is required at the cell surface of muscle precursor cells to initiate such an alignment.

A critical function in cell adhesion is also in keeping with our observations that FGFR1 can induce cell-cell fusion in culture. With CHO cells and similar cell types, the mere over-expression of FGFR1 leads to fusion of the cells into large syncytia containing several dozens of nuclei (20). It is unlikely that this fusogenic activity is a normal, physiological function of FGFR1, as it is not observed with other cell types. However, it demonstrates that FGFR1 can pull together two cell surfaces with high tensile strength. In a recent publication, we have found evidence that FGFR1 binds to a target molecule on neighboring cells to induce cell-cell fusion (21). A hydrophobic pocket in the third Ig domain of FGFR1 appeared to interact with this target protein. However, the identity of the target protein remained elusive.

With its domain structure and its function during cell adhesion, FGFR1 resembles another family of proteins, the nectins (22,23). Four different nectins (nectin-1 to nectin-4) have been identified that possess a domain structure similar to FGFR1 with three extracellular Ig domains, a single trans-membrane domain and a relatively short intracellular domain. The nectins are involved in cell-cell adhesion and immune modulation and play an important role during host-pathogen interactions. The adhesive function is accomplished by homophilic as well as heterophilic interactions of the nectins. During the adhesive process, the C-terminal cytoplasmic domain is required to remodel the actin cytoskeleton. It interacts with the PDZ domain of the adapter molecule afadin, which in turn recruits filamentous actin. So far, we do not have any evidence that FGFR1 would interact with afadin or with any of the four nectins. Furthermore, the C-terminal domain of FGFR1 appears to be dispensable for its function (13). Thus, the molecular mechanism of inducing cell adhesion must be quite different between FGFR1 and the nectins, although the proteins share structural and functional homology.

Acknowledgements

This study was supported by grants from the Swiss National Science Foundation (no. 31003A-143350) and the Novartis Foundation for Medical-Biological Research (15A006).

References

- Goetz R and Mohammadi M: Exploring mechanisms of FGF signalling through the lens of structural biology. *Nat Rev Mol Cell Biol* 14: 166-180, 2013.
- Ornitz DM and Itoh N: The fibroblast growth factor signaling pathway. *Wiley Interdiscip Rev Dev Biol* 4: 215-266, 2015.
- Trueb B: Biology of FGFR1, the fifth fibroblast growth factor receptor. *Cell Mol Life Sci* 68: 951-964, 2011.
- Wiedemann M and Trueb B: Characterization of a novel protein (FGFR1) from human cartilage related to FGF receptors. *Genomics* 69: 275-279, 2000.
- Sleeman M, Fraser J, McDonald M, Yuan S, White D, Grandison P, Kumble K, Watson JD and Murison JG: Identification of a new fibroblast growth factor receptor, FGFR5. *Gene* 271: 171-182, 2001.
- Rieckmann T, Zhuang L, Flück CE and Trueb B: Characterization of the first FGFR1 mutation identified in a craniosynostosis patient. *Biochim Biophys Acta* 1792: 112-121, 2009.
- Silva PN, Altamentova SM, Kilkenny DM and Rocheleau JV: Fibroblast growth factor receptor like-1 (FGFR1) interacts with SHP-1 phosphatase at insulin secretory granules and induces beta-cell ERK1/2 protein activation. *J Biol Chem* 288: 17859-17870, 2013.
- Baertschi S, Zhuang L and Trueb B: Mice with a targeted disruption of the Fgfr1 gene die at birth due to alterations in the diaphragm. *FEBS J* 274: 6241-6253, 2007.
- Catela C, Bilbao-Cortes D, Slonimsky E, Kratsios P, Rosenthal N and Te Welscher P: Multiple congenital malformations of Wolf-Hirschhorn syndrome are recapitulated in Fgfr1 null mice. *Dis Model Mech* 2: 283-294, 2009.
- Gerber SD, Steinberg F, Beyeler M, Villiger PM and Trueb B: The murine Fgfr1 receptor is essential for the development of the metanephric kidney. *Dev Biol* 335: 106-119, 2009.
- Amann R, Wyder S, Slavotinek AM and Trueb B: The Fgfr1 receptor is required for development of slow muscle fibers. *Dev Biol* 394: 228-241, 2014.
- Gerber SD, Amann R, Wyder S and Trueb B: Comparison of the gene expression profiles from normal and Fgfr1 deficient mouse kidneys reveals downstream targets of Fgfr1 signaling. *PLoS One* 7: e33457, 2012.
- Bluteau G, Zhuang L, Amann R and Trueb B: Targeted disruption of the intracellular domain of receptor Fgfr1 in mice. *PLoS One* 9: e105210, 2014.
- Shibata H and Yagi T: Rate assay of N-acetyl-beta-D-hexosaminidase with 4-nitrophenyl N-acetyl-beta-D-glucosaminide as an artificial substrate. *Clin Chim Acta* 251: 53-64, 1996.
- Zhu H, Duchesne L, Rudland PS and Fernig DG: The heparan sulfate co-receptor and the concentration of fibroblast growth factor-2 independently elicit different signalling patterns from the fibroblast growth factor receptor. *Cell Commun Signal* 8: 14, 2010.
- Tsuchiya S, Fujiwara T, Sato F, Shimada Y, Tanaka E, Sakai Y, Shimizu K and Tsujimoto G: MicroRNA-210 regulates cancer cell proliferation through targeting fibroblast growth factor receptor-like 1 (FGFR1). *J Biol Chem* 286: 420-428, 2011.
- Shimada Y, Okumura T, Nagata T, Hashimoto I, Sawada S, Yoshida T, Fukuoka J, Shimizu K and Tsukada K: Expression analysis of fibroblast growth factor receptor-like 1 (FGFR1) in esophageal squamous cell carcinoma. *Esophagus* 11: 48-53, 2014.
- Rieckmann T, Kotevic I and Trueb B: The cell surface receptor FGFR1 forms constitutive dimers that promote cell adhesion. *Exp Cell Res* 314: 1071-1081, 2008.
- Trueb B, Zhuang L, Taeschler S and Wiedemann M: Characterization of FGFR1, a novel fibroblast growth factor (FGF) receptor preferentially expressed in skeletal tissues. *J Biol Chem* 278: 33857-33865, 2003.
- Steinberg F, Gerber SD, Rieckmann T and Trueb B: Rapid fusion and syncytium formation of heterologous cells upon expression of the FGFR1 receptor. *J Biol Chem* 285: 37704-37715, 2010.
- Zhuang L, Pandey AV, Villiger PM and Trueb B: Cell-cell fusion induced by the Ig3 domain of receptor FGFR1 in CHO cells. *Biochim Biophys Acta* 1853 (10 Pt A): 2273-2285, 2015.
- Mandai K, Rikitake Y, Mori M and Takai Y: Nectins and nectin-like molecules in development and disease. *Curr Top Dev Biol* 112: 197-231, 2015.
- Samanta D and Almo SC: Nectin family of cell-adhesion molecules: structural and molecular aspects of function and specificity. *Cell Mol Life Sci* 72: 645-658, 2015.