Chapter 2

Methods

Cell culture

hES cells

Pluripotent cells were maintained in mytomycin-inactivated mouse embryonic fibroblast, in knock out DMEM (Gibco), 20% knock out serum replacement (Gibco), 1mM Glutamax (Gibco), 0.1mM β-Mercaptoethanol, and 4ng/ml human recombinant bFGF (Life Technologies), cells were incubated at 5%CO_2 37^oC.

Alternatively, mTESR1 (Stem cell Technologies) ,a defined medium was used to generate stable cells lines..

Mouse embryonic fibroblasts for hES cell culture were thawed and seeded every week at 24000 cells/cm² in DMEM, 10% FBS (GIBCO), incubated in 10%CO_2 37^oC.

Otic differentiation

The day before the experiment, cell culture dishes were coated with mouse laminin (Cultrex) at 2.5µg/cm². Mouse laminin was thawed for 30 to 40 min at 4^oC, and then diluted in cold cell culture PBS (Sigma), 90µl PBS per 1µg of Laminin.

On the day of the experiments, undifferentiated cells hES medium or mTESR1 were rinsed twice with Hanks buffer (Sigma), trypsinised for 5 min with Trypsin/EDTA (Sigma) at 0.5X concentration, 1.5ml of trypsin solution for a T12.5 flask was added. Next, trypsin activity was stopped with Soybean trypsin inhibitor at 0.5mg/ml (Sigma) at 1:1 volume ratio, cells were spin at 200g 5 min and resuspended in 2ml of pre-warmed defined DFNB medium (DMEM (Gibco) and F12 (Gibco) 1:1, plus N2 (Gibco) and B27 (Gibco) supplements). Next, the cell suspension was passed through a sieve 100µm pore size (BD biosciences). Then cells were counted with automated TC20 cell counter (Biorad) and seeded in 4000 or 8000cells/cm² on laminin coated dishes, with either DFNB, or in otic induction medium, DFNB plus recombinant human FGF3 (R and D systems) and FGF10 (R and D systems).

Medium was changed every other day. Cells were collected for further analysis at day 12 of differentiation, considering as day 0 the day that cells were seeded.

Otic differentiation in FGFR inhibitor experiments

The same process protocol as the one described before was followed, with the exception that seeding was always made on DFNB medium only. The following day, medium was changed to either DFNB or DFNB-FGFs (FGF3 and FGF10). The FGFR inhibitor SU5402 (Merck) was added at the same time when the medium was changed at a concentration of 10µM. Stocks of this inhibitor were at 20mM in DMSO following manufacturer instructions. The same volume of DMSO as the one added with the inhibitor was used in the controls. The day(s) that the SU5402 was added during the time window inhibition is indicated in results of these experiments.

Neural differentiation of otic progenitors

The day before the end of the differentiation experiment (11^{th} day in FGF3 and FGF10 medium), 35 mm dishes (Nunc) were coated with gelatine (Millipore) for neural induction, 1ml/35mm dish, and incubated overnight in 5%CO_2 37^{o}C.

On day 12^{th} of differentiation cells, otic progenitors were rinsed twice with HANKS, trypsin (Sigma) at 0.5X was added for 5min, stopped with trypsin inhibitor 0.5mg/ml 1:1 volume ratio, cells were spin at 167g 5min and resuspended in DFNB medium for counting. Cells were seeded in neuralising conditions and maintained there for period of 15 days. On day 1 to 3 the neutralising media was made of DFNB plus 20ng/ml bFGF and 500ng/ml human recombinant SHH (R and D systems). On day 4 and day 5 of neuralising conditions 10ng/ml of BDNF (Peprotech) and 10ng/ml of NT3 (Peprotech) were added. From day 6 onwards the SHH was withdrawn from the neuralising conditions.

RNA extraction and cDNA synthesis

RNA was extracted with RNeasy Micro kit (Qiagen) following the manufacturer's instructions. 0.1 to 1.5 µg of RNA (depending on the yield) were retrotranscribed using Superscript III retrotranscriptase (Life technologies). Briefly, samples were lysed by RLT buffer (kit), DNA digestion was carried in the purification column through DNase supplied with the kit. RNA was measured with the Nanodrop ND-1000 (Thermo Fisher), purity was estimated by the 260/280nm and the 230/260nm ratios to measure protein and guanidine isothiocyanate carryovers. The same amount of RNA in each treatment was hybridised with 10µg/ml of 15 nucleotides length oligo-dT primers (Promega), volume was adjusted to 11µl with Rnase Dnase-free H_20. Samples were incubated 5 min 65^oC and leaved to cool down at room temperature 10 min.

Oiligo-dT primed RNA was mixed with first strand buffer 1X, DTT (5mM), dNTPs (100µM), 40 units of RNase inhibitor plus (Promega) and 200 units of Superscript III retrotranscriptase (Life technologies). The cDNA synthesis was made at 50^oC 90 min followed by70^oC 15 min protein denaturation step.

RT-PCR, Q-PCR and gel electrophoresis

RT-PCR.

Table 2.1 shows the PCR and QPCR primers used in this project as well as the annealing temperature for each pair. All primer pairs span at least one intron and were Blasted against the nucleotide collection in the NCBI, to select the more specific ones. The RT-PCR products were analysed by gel electrophoresis to corroborate specificity. For RT-PCR, Go-Taq polymerase (Promega) and the accompanying reagents were used at a final concentration of 1X PCR green buffer, 2mM $MgCl_2$, 1µM of each primer, 10µM dNTPs, 2.5units of Go-Taq

were used in a 10µl reaction containing 1µl of cDNA synthesis reaction (1/20 of the final cDNA mix)

The amplification was carried out in Master cycler (Eppendorf), an initial denaturation step of 95^{o}C 5 min, followed by 24 to 36 cycles, 95^{o}C 30 seconds, the specified annealing temperature (table 2.1) 30 seconds, and extension 72^{o}C 30 seconds, followed by a final extension step of 10 min at 72^{o}C . The PCR products were analysed by gel electrophoresis in 2% agarose, TBE buffer, run at 90v 30 min.

Quantitative RT-PCR

For QPCR, Jump start reaction mix (Sigma) was prepared following the manufacturer's instructions. Previously, primers were diluted 1:20 each in Rnase/Dnase-free water (Fisher Scientific) and 1:10 of this dilution was added in the final PCR mix to have final concentration of 0.5µM each primer. 1.5 or 3µl cDNA were added to 60µl PCR mix and divided in 3 wells of a QPCR plate (Cell projects). Reaction plate was sealed with transparent PCR lids (Biorad), and run at according to the protocol. The specificity of the primers was evaluated by looking single peaks in melting curves and by gel electrophoresis. Once primers with high specificity were selected, observation of melting curves was routinely checked as a quality control.

Each protocol contained two extension temperatures: 72^{o}C and 84^{o}C for PAX8, PAX2; 72^{o}C and 78^{o}C for FOXG1; 72^{o}C and 86^{o}C for DLX5; 72^{o}C and 82^{o}C for FGFR2 primers. The purpose of the different extension temperature was to quantify exclusively fluorescence of the specific target and exclude any possible "noise" fluorescence, derived from primer-dimer that would melt at the higher extension temperature. After 50 cycle amplification, a melting curve step was added, starting at 55^{o}C 30 seconds, and increasing 0.5^{o}C in very step until 95^{o}C temperature was reached. It is important to mention that primers were chosen base on the observation of a single peak in the melting curves, although sometimes a primer-dimer peak could be observed due to the low amount of cDNA target.

Table 2.1 **Primers used for RT-PCR or Q-PCR analysis.** Primers are in 5'-3' orientation. The forward primer is in the first row of every primer pair. Ta is the recommended annealing temperature. All primers have been blasted to confirm specificity.

	Sequence	Size	*Ta*	Reference
EYA1	TCAAGCCAGTTCAGATGTTGC	628, 741, 756	57	(Morisada et al., 2010)
	ATGTGCTGGATACGGTGAGC			
SIX1	AAGGAGAAGTCGAGGGGTGT-3	205	57	(Ng et al., 2006)
	TGCTTGTTGGAGGAGGAGTT			
PAX6	AACAGACACAGCCCTCACAAAC	274	63	(Nat et al., 2007)
	CGGGAACTTGAACTGGAACTGAC			
LFNG	CGCGCCACAAGGAGATGACGTTC	551	66	(Besseyrias et al., 2007)
	TGGGCACCTGCTGCAGGTTCT			
DLX5	GCCACAACAGCAAGGACAG	439	57	(Schule et al., 2007)
	TTTGCCATTCACCATTCTCA			
DLX6	TCGCTTTCAGCAGACACAGT-3	456	57	
	CGGCTTCTTGCCACACTTAT			
FGF4	GCGTGGTGAGCATCTTCG	229	53	(Li et al., 2010)
	GGTGACCTTCATGGTGGG			
HPRT	AATTATGGACAGGACTGAACGTC	387	52	
	CGTGGGGTCCTTTTCACCAGCAAG			
FOXI3	CGCCCGAGCGCAAACTCAC	257	61	
	ATTGCTGGCCTCAGAGCGGC			
FGFR2IIIc q-PCR pp2	TGAGGACGCTGGGGAATATACG		60	(Naimi et al., 2002)
	TAGTCTGGGGAAGCTGTAATCTCCT			
FGFR2IIIb q-PCR pp1	GATAAATAGTTCCAATGCAGAAGTGCT	95	60	(Naimi et al., 2002)
	TGCCCTATATAATTGGAGACCTTACA			

FGFR2IIIb q-PCR pp2	CGTGGAAAAGAACGGCAGTAAATA	80	60	(Naimi et al., 2002)
	GAACTATTTATCCCCGAGTGCTTG			
FGFR2IIIb	CACTCGGGGATAAATAGTT	461	60	(Steele et al., 2001)
	ACTCGGAGACCCCTGCCA			
PAX8	ACCCCCAAGGTGGTGGAGAAGA	448		(Minagawa et al., 2002)
	CTCGAGGTGGTGCTGGCTGAAG			
PAX8 q-PCR	CTTGGCAGGTACTACGAGAC	128	60	
	GCAAACATGGTAGGGTTCTG			
PAX2	GAGCGAGTTCTCCGGCAAC			(Tavassoli et al., 1997)
	GTCAGACGGGGACGATGTG			
PAX2 q-PCR	CTTTAAGAGATGTGTCTGAGGG	185	60	
	CCTGTTCTGATTTGATGTGCT			
FOXG1 q-PCR	CTAAATAGTGACTGCTTTGCCA	135	61.5	
	TTTAGGTTGTTCTCAAGGTCTG			
DLX5 q-PCR	CCCTACCAGTATCAGTATCAC		62	
	GTCACTTCTTTCTCTGGCTG			
GAPDH	GTCCACTGGCGTCTTCACCA	260	60	(Harper et al., 2003)
	GTGGCAGTGATGGCATGGAC			
RPLP0	GAAGGCTGTGGTGCTGAT GG	103	58	
	CCGGATATGAGGCAGCAG TT			

Immunofluorescence

For immunolabeling, cells were rinsed twice with cold PBS, fixed with 4% Paraformaldehyde solution for 15min, the edge of the well where they were grown was marked with wax pen (Vectorlabs). Cells were then washed 3 times, 5 min/each with PBS 0.1% triton (Fisher Scientific), PBS/T for permeabilization. Next, 1 hr room temperature incubation in blocking buffer was carried. As blocking buffer, 10% donkey serum (Sigma), 1% BSA in PBS/T was used.

Primary antibodies (Table 2.2) were incubated overnight at 4^{o}C in blocking buffer at the concentrations stated in the table. Next day, cells were washed 4 times with PBS/T, 5 min each time, followed by incubation with secondary antibody mix, at 1hr 30 min room temperature in the dark. Cells were washed 3 times with PBS/T as described before, but in the dark. Then, samples were incubated 20 min incubation in Hoechst (Sigma) at 1:1000 dilution. Finally, 3 washes with PBS were performed and wells were covered with PBS for microscope visualization. Alternatively, PBS was completely removed, and a drop of Vectashield (Vectorlabs) added to 35mm dishes, and samples were sealed with a coverslip and nail varnish.

Microscopy visualization was made mainly with EVOS microscope (AMG), or with Leica immunofluorescence microscope. For automated immunofluorescence analysis, samples were processed in the same way for In cell analyser automated microscope (General Electric), when using this system, 40-60 pictures per well were taken (in 12 well plate format).

Table 2.2 **Antibodies used for immunofluorescence.** The antibody, product key, manufacturer, host species where it was produced and the working concentration are indicated. When used for flow cytometry, the same concentration indicated in this table was used, with the exception of secondary antibodies that were used at 1:100 for Flow.

Antibody/product code	Host Species	Concentration	Manufacturer
PAX8 (60145-4)	Mouse monoclonal	1:100	Proteintech
PAX8 (66073-1)	Mouse monoclonal	1:100	Proteintech
PAX8 (13611)	Goat polyclonal	1:100	Abcam
PAX2 (38738)	Rabbit polyclonal	1:100	Abcam
FOXG1 (18259)	Rabbit polyclonal	1:50	Abcam
DLX5 (64827)	Rabbit polyclonal	1:200	Abcam
FGFR2 (MAB6843)	Mouse monoclonal	1:50	R and D systems
FGFR2 (119237)	Mouse monoclonal	1:100	Abcam
PhosphoERK (4377)	Rabbit monoclonal	1:100	Cell signalling
OCT4 (2890)	Rabbit monoclonal	1:100	Cell signalling
β-TUBULIN (	Mouse monoclonal	1:250	SIGMA
BRN3A (AB5945)	Rabbit polyclonal	1:100	Chemicon
Alexa 568 anti-goat (A11055)	Donkey polyclonal	1:250	Life Technologies
Alexa 488 anti-rabbit (A21206)	Donkey polyclonal	1:250	Life Technologies
Alexa 488 anti-mouse (A21202)	Donkey polyclonal	1:250	Life Technologies
Alexa 568 anti-rabbit (A10042)	Donkey polyclonal	1:250	Life Technologies

Flow cytometry

Undifferentiated hESCs were rinsed twice with HANKS buffer and trypsinised using trypsin/EDTA 0.5X for 5 min, in the same way it was described during otic differentiation experiments. Trypsin activity was stopped with washing buffer (WB: PBS, 5%FBS), and cells collected in Falcon tube. The cell suspension was spin down at 167g 5 min. Next, cells were counted and separated into as many tubes as conditions required keeping the cell number from 100 000 to 250 000 cells per tube.

Cells were spin again (167g, 5 min), the supernatant was removed leaving 200µl at the bottom of the tube (with the cells). Then primary antibody mix was incubated for 2 hrs at 4^{o}C in rocking table, primary antibodies were used at the concentration indicated in table 2.3 for the cell surface markers, or the same concentration used during immunofluorescence withFGFR2 (MAB6843) in table 2.2 in a total volume of 500µl/tube. After incubation, cells were washed 3 times with 1ml WB, 5 min each time at 4^{o}C in rocking table. Cells were spin down (167g 5 min) after each wash and supernatant was removed, leaving again 200µl with the cells at the bottom of the tube.

After last wash, secondary antibody mix was added and incubated for 30 min at 4^{o}C in rocking table in the same total volume as primary mix (500ul), in the concentrations stated in table 2.2. Finally, cells were washed 3 times as described before, and samples processed with CyAn cell analyser (Beckman Coulter).

Table 2.3 **Antibodies used for flow cytometry.** The antibody, product key, manufacturer, host species where it was produced and the working concentration are indicated. CSCB are produced in house by Peter Andrews lab. They are hybridoma culture supernatants concentrated.

Antibody/product code	Host Species	Concentration	Manufacturer
SSEA3	Mouse	1:10	CSCB
SSEA4	Mouse	1:100	CSCB
TRA1-60	Mouse	1:10	CSCB
Anti-mouse FITC conjugated (M30801)	Goat	1:100	Caltag

Transfection

Transfection of hES cells

The day before transfection 60 mm dishes were coated with growth factor reduced Matrigel (356230, BD biosciences), 2.5ml per 60mm dish . Matrigel should be thawed for 30 min at 4^{o}C, and diluted 1 in 15 in cold Knockout DMEM before use.

On the day of transfection, undifferentiated hESC cultures were rinsed twice with Hanks buffer and dissociated with 3ml trypsin/EDTA 0.5X for 5min. Trypsin was stopped with trypsin inhibitor 0.5mg/ml at 1:1 volume ratio. Cell suspension was spin at 167g 5min,supernatant removed and cells were re-suspended in 3.5ml mTESR1 (05850, Stem cell Technologies). A 70µm sieve was used to pass the cell suspension and eliminate large cell clumps. hESCs were then counted and seeded into the previously coated 60mm dish at a seeding density of 1 million cells per dish, adding 10µM of ROCK inhibitor Y-27632 (MercK) for the first 24 hrs to increase single cell survival.

The following day, Lipofectamin transfection was carried out, 10µg of plasmid DNA per million of cells was used. Lipofectamin LTX (Life technologies) was used following manufacturer instructions and maintaining a ratio DNA to LipoLTX, 1:3. Therefore in this case, 30µl of Lipofectamin were used. Plus reagent was also added at 1:1 ratio as recommended. Thus, for a 60mm dish, an aliquot of 700µl of OptiMEM (Gibco) was prepared,10µg of DNA plus 10µl of plus reagent were mixed and incubated for 15 min. Following, 30µl of Lipofectamine LTX were added to the mix and incubated 40 min room temperature, with mild rocking every 10 min. Finally, transfection mix was added to the cells and removed the next day.

To generate stable cell lines, the appropriate selection antibiotic was added starting from 48 hrs post transfection and maintaining the selection pressure for 3 weeks. Medium was changed every two days. Colonies were carefully picked with the help of pipette and microscope, and expanded independently as any other hESC line.

Alternatively, hESC were electroporated to produce stable cell lines. Cell culture dishes were coated with Matrigel the day before electroporation, as described in the previous method. The following day cells were trypsinised for 5 min, stopped with trypsin inhibitor added, then cell suspension was spin, and supernatant removed as described before. Then cells were resuspended again in cell culture PBS, counted and spin down again. Next, the pellet was resuspended in Ingenio solution (Mirus) at 2.5 million cells per ml, plasmid DNA was then added at the same concentration used in lipofection (1µg/100 000 cells) and cells suspension split into 4mm cuvettes (Cell projects), using 250µl as minimum for that size cuvette. The electroporation conditions were 2 pulses 1ms/each, 100ms interval at 240V in square wave porator (ECM830). Colonies were picked after three weeks selection with the appropriate antibiotic.

Transfection of HEK 293 cells and NTERAs

HEK-293 and NTERA-2 cells were always transfected with Lipofectamine LTX. Cells were trypsinised in the same way as hESCs with the difference that trypsin activity was stopped with DMEM 10%FBS. Cells sere seeded in 12 well plates at 100 000 cells/well. The following day, 100µl OptiMEM, 1µg DNA, 1µl Plus reagent and 3µl LTX were mix per each experimental condition. The incubation times of DNA, Plus and LTX were the same as those used in hESCs. Transfected cells were analysed at 24 to 48 hrs after transfection (indicated in the experiment).

Stable transfection was also carried out in NTERAs, the transfection process was the same as the one just described, with the only difference that cell culture surface was augmented so that 100 000 cells were seeded in well of 6 well plate instead of 12 well plate. Selection of stable cells was made with 800µg/ml of G418 during 3 weeks.

Protein extraction and western blot

60-80% confluent T12.5 cell culture flasks (Nunc) were used for western blot analysis. Samples were rinsed 3 times with cold PBS, and lysed with 150µl of Radio Immunoprecipitation assay (RIPA) buffer (Sigma), containing "complete" protease inhibitor cocktail (Roche). Cells were collected with the help of cell scraper and sonicated 3 times, 8 seconds each (10 amplitude microns), with 20 seconds ice-rest intervals. 1/15 protein dilution was used for quantification with the BCA standard calibration method (Thermo Fisher) as described by the manufacturer. A linear regression curve was obtained and protein concentration determined. 120µg of protein were mixed with 5X sample buffer (1.5M Tris-HCl, 25% β-Mercapto-OH, 50% glycerol, 10% SDS, 0.01% bromophenol blue) and heated for 2 min 95^{o}C.

Once having sample ready, 12% separating gel was prepared as follows: For 10ml gel, 4ml 30% bis-acrylamide solution (Biorad), 2.5ml separating gel buffer (375mM Tris HCl, 0.1% SDS, pH 8.8),100µl of 10% SDS, 5ul of TEMED, and 75µl ammonium persulfate were mixed and added to running chamber.

4% stacking gel was prepared as follows: 4ml of 30% bis-acrylamide solution (Biorad), 2.5% separating gel buffer (125mM Tris HCl, 0.1% SDS, pH 6.8), 100µl of 10% SDS, 10ul of TEMED, 100µl ammonium persulfate were mixed and added on top of separating gel.

20-30µg of protein were loaded into each lane of the gel, and run at 40mA constant current from 1hr to 1hr 30 min in electrophoresis buffer (25mM Tris, 250mM glycine, 0.1% SDS).

Following electrophoresis, proteins were transferred at 15V 1hr in transfer buffer (48 mM Tris, 39 mM glycine, 0.04% SDS, 20% methanol) in semidry transfer box (Biorad).

Membrane with the proteins was carefully transferred to 1X TBST buffer, and incubated in blocking buffer (2.5% skimmed milk, 2.5% BSA in 1X TBST) 1hr 30 min. Then 3 washes of 10 min each with 1X TBST were made. Primary antibody was then incubated for 1hr 30 min. Mouse monoclonal anti α-tubulin (Sigma) was used as loading control at 1:10000 and mouse monoclonal anti-Tet repressor protein (Clonetech) was used at 1:1000. Then, the membrane was washed 3 times with 1X TBST and secondary goat anti-mouse peroxidase

1:5000. 3 additional washes with TBST were made. Proteins were then visualized with Pierce ECL western blot substrate kit according to manufacturer instructions. Detection was made in the Biorad Gel-doc system.

Cloning

Since cloning involved a considerable amount of time, it was important to include the overall process in the result chapters (6 and 7). Here we described some details of reagents used without further explanation or the troubleshooting process.

For the construction of short hairpin vectors, pSUPERIOR.neo vector was used following the manufacturer's instructions. Short hairpin sequences shown in table 2.4 that were published and showed to down regulate FGFR2 (Kunii et al., 2008) were blasted, confirming to be FGFR2 specific. Then 0.12µg/µl of every oligo were mixed annealed as follows 94^{o}C 4 min, 85^{o}C 4 min, 82^{o}C 4 min, 78^{o}C 4 min, 75^{o}C 4 min, 70^{o}C 4 min and then decreasing at rate of 1^{o}C per min until 35^{o}C then temperature was decreased at 10^{o}C. Next, annealed oligos were phosphorylated with PNK (NEB) following the NEB protocol. In the meantime, 10µg pSUPERIOR vector were digested in 100µl reaction with XhoI and BglII following NEB conditions. Vector was treated with Antarctic phosphatase (NEB) following the manufacturer's conditions. 6 to 1 molar ratio between insert and vector respectively with 140ng of digested vector were used for ligation in a final 20µl ligation reaction with T4 ligase (NEB). Reaction was incubated at 15^{o}C overnight. The following day, 1 or 2 µl of reaction mix were used with 50µl XL-1 blue electrocompetent bacteria (Stratagene). Bacterial colonies were grown overnight, DNA extracted with plasmid quick spin prep (QIAGEN) and screened by sequencing.

For the cloning of FGFR2IIIb into the pCAG-GFP vector, the full FGFR2IIIb was amplified with Q5 polymerase (NEB). Degenerated forward primer carrying XhoI restriction and reverse carrying NotI restriction site in the 5'end plus 4 random base pairs (table 2.4) were used during amplification of FGFR2IIIb. FGFR2IIIb amplicon and pCAG-GFP were digested with XhoI, NotI overnight. Then vector was phosphatase treated with Antartic phosphatase. T4

ligation with 3 to 1 molar ratio insert vector. 100ng vector, 107ng insert, 1µl T4 ligase in final 20 µl reaction were the conditions that worked. Ultragold chemical competent bacteria (Stratagene) were used according to manufacturer instructions. Colonies were screened as before and sequencing confirmed the cloning was successful.

Table 2.4 **Sequences of Short hairpin oligos and cloning primers.** Short hairpin oligos wee designed as recommended by the manufacturer (Clonetech). The forward is on top of each pair and has a BglII restriction site, while the reverse has a XhoI site for cloning into pSUOERIOR.neo. The FGFR2IIIb primers were designed with restriction sites compatible with pCAG-GFP vector (XhoI-Not) for FGFR2IIIb amplification, digestion and ligation.

	Sequence	Notes
FGFR2 short hairpin 1	GATCCCGCCAACCTCTCGAACAGTATTCAAGAGATACTGTTCGAGAGGT TGGCTTTTTC	(Kunii et al., 2008)
	TCGAGAAAAAGCCAACCTCTCGAACAGTATCTCTTGAATACTGTTCGAG AGGTT GGCGG	
FGFR2 short hairpin 2	GATCCCGGACTTGGTGTCATGCACCTTCAAGAGAGGTGCATGACACCA AGTCCTTTTTC	
	TCGAGAAAAAGGACTTGGTGTCATGCACCTCTCTTGAAGGTGCATGAC ACCAAGTCCGG	
FGFR2 short hairpin 3	GATCCCGGACTGTAGACAGTGAAACTTCAAGAGAGTTTCACTGTCTACA GTCCTTTTTC	
	TCGAGAAAAAGGACTGTAGACAGTGAAACTCTCTTGAAGTTTCACTGTC TACAGTCCGG	
FGFR2IIIb	**Fwd** GACA CTC GAG ATG GTC AGC TGG GGT CGT TTC ATC TG	These primers were used for FGFR2IIIb subcloning
	Rev GGA ATT GC GGCC GC TCT AGA ACT AGT	

Statistical analysis

Q-PCR results were analysed the ΔΔCt method (Livak and Schmittgen, 2001). Essentially, in every sample, the Ct (cycle threshold) of a housekeeping gene not affected by the experimental condition is subtracted from the Ct of the gene of interest. This is the ΔCt, and gives us an idea of the absolute expression of the gene of interest. Then we want to know how that level of expression changes in relation to the experimental variable to be tested (e.g. FGFs), therefore, an experimental condition is defined as calibrator for the experiment, and ΔCt-ΔCt for that calibrator equals "0". Then the level of expression in the other treatment is subtracted from the one in the calibrator; ΔCt FGF-ΔCt calibrator. This number is the ΔΔCt and because we are talking of an exponential reaction, this is better expressed as logarithm base 2. The power of this conversion is what is represented graphically.

For statistical analysis, the ΔCTs of every experimental variable were compared with a paired T test, one tailed when our hypothesis was that the treatment was changing the ΔCt in one specific direction. Data was analysed in Prism Graphpad 6.0