Chapter 7

Knockdown of FGFR2IIIb

Introduction

RNA-mediated silencing

In addition to the FGFR2IIIb overexpression experiments, we decided to test the function of this gene by two complementary, loss-of-function approaches. In the first one, we established a knockdown inducible system in the hES cell line Shef3. The second approach consisted of a competitive assay where a soluble form of FGFR2IIIb is supplemented in the media during differentiation. This recombinant protein should be able to sequester any FGF able to activate the FGFR2IIIb during differentiation.

From the initial discovery that double stranded RNA (dsRNA) was able to induce robust and specific silencing of gene expression (Fire et al., 1998), a great progress has been made in the field. This phenomenon has been correlated with previous silencing mechanisms observed in plants, and now RNA interference, as it became known, is considered a well established mechanism of gene silencing in plants and animals. In this regard, components of the molecular machinery in charge of processing dsRNA have been identified in *Caenorhabditis, Arabidopsis* and the fruit fly *Drosophila* (Hammond et al., 2001; Sharp, 2001). Although the discovery of gene-specific silencing through dsRNA was an exciting breakthrough to study gene function, specific gene knockdown was still a challenge in mammalian cells, which were known to switch on RNA-activated protein kinase (PKR) pathway in response to dsRNA. This pathway switches off non-specific translation and protects against viral infection and stress, often culminating in apoptosis (Garcia et al., 2006). An important step in the study of RNA interference came from the works of Tuschl and colleagues, who discovered that small double stranded RNA entities (22nt aprox) originated from the dsRNA were able to induce strong silencing in *Drosophila* embryo extracts (Elbashir et al., 2001b; Tuschl et al., 1999). This opened the possibility of silencing gene expression in mammals, which was later demonstrated by Tuschl group (Elbashir et al., 2001a) and Caplen (Caplen et al., 2001).

These short sequences of small interfering RNA (siRNA) have been widely used to target specific mRNA degradation. However in most cases, this approach is only transient, and sequential transfections are needed to maintain the protein of interest downregulated. In an attempt to circumvent these hurdles, RNA polymerase III based vectors have been shown to be effective tools either to produce siRNA *in vitro* or stem loop structures called short hairpin RNA (shRNA) that have the same structure as siRNA with the exception of a loop that connects the sense and antisense strands. These shRNA have resulted in some instances more effective that the same siRNA version (Brummelkamp et al., 2002; Paddison et al., 2002; Yu et al., 2002), perhaps because the loop structure is more similar to the micro RNA (miRNA), a large class of RNA endogenously produced by the cells to control gene function by silencing.

In summary, shRNAs have allowed to target specific gene function in a variety of cells. Here, we sought to exploit this technology. As briefly mentioned in the previous chapter, some of the problems typically encountered when studying hESCs are the low transfection efficiency and the silencing of exogenous expression vectors. In addition, when knocking down a gene that may carry an important function, cell survival may be compromised, hampering the study of the gene in question. For this reason, we decided to use an inducible system based on the Tet operon (Gossen and Bujard, 1992) that has been previously employed to knockdown OCT4 and SMAD4 in hESCs (Avery et al., 2010; Zafarana et al., 2009). Briefly, for the system to work it is necessary to generate a stable cell line constitutively expressing the Tet repressor protein (TetR). In the CSCB (Centre for Stem Cell Biology) this has been made by cloning the Tet repressor protein from pcDNA6/Tr (life technologies) downstream the pCAG promoter, a suitable vector for hESCs. The second component is the transfection of TetR hESCs with a vector named pSUPERIOR. This vector drives the expression of specific hairpin RNAs from a modified polymerase III H1 RNA promoter sequence. The modification in the H1 promoter creates a binding site for the TetR, making the site inactive for transcription. When tetracycline or doxycycline is added, there is conformational change in the Tet repressor protein that renders it unable to block the H1 promoter, permitting the shRNA transcription and the consequent gene silencing (figure 7.1). The selection of the short hairpin sequences is important to avoid off-target effects, as well as to maximise the chances of detecting a knock down.

In our project, the selection of an inducible system instead of siRNA transient transfection or constitutive expression of shRNA was very important. Firstly; because we did not want to disturb the hESC undifferentiated state; we wanted to knockdown FGFR2IIIb at specific time points during differentiation uniformly in all the cells (a transient transfection would imply a selection step); finally, the system has already been used with efficacy in the CSCB.

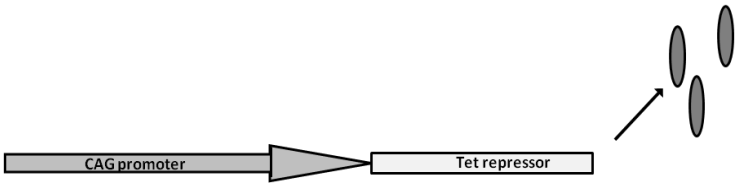
The importance of FGFs during inner ear development was discussed in previous chapters, as well as the role of FGFR2 and its isoform IIIb *in vivo*. FGFR2 has been linked to ovarian, prostate and bladder cancers among others (Carstens et al., 1997; Kwabi-Addo et al., 2004; Steele et al., 2001; Yan et al., 1993). In an attempt to investigate the role of FGFR2 in gastric cancer cell lines characterized by gene amplification and overexpression of *FGFR2*, Kunni et al used small molecule inhibitors and shRNAs, targeting *FGFR2* (Kunii et al., 2008). Since a robust decrease in the expression of FGFR2 was observed in two out of three cell lines with the panel of shRNA used, we decided to use the same sequences in our study. While we are primarily interested in knocking down the IIIb isoform of this receptor, the region specific to this variant is relatively small limiting the possible repertoire of target sites. Moreover, published shRNAs sequences targeting this isoform didn't show a robust downregulation. We have therefore decided to target the entire FGFR2 subfamily, which would include the FGFR2IIIb.

In mESCs, shRNA-mediated silencing of FGFR2 has been carried out only once (Coumoul et al., 2005). However, the purpose of that study was to validate a different inducible knockdown system based on the Cre lox, rather that gaining an insight into the function of FGFR2. In fact, most of the work with this receptor has been done *in vivo* through knockouts (De Moerlooze et al., 2000; Xu et al., 1998) and the ear has been studied in detail only in the work of Pirvola (Pirvola et al., 2000). In this regard, it is important to stress that this is the first study aiming to address the role of FGFR2 during otic differentiation in hESCs. So far, our data is in agreement with the animal models of inner ear development.

A competitive specific inhibitor of FGFR2IIIb

Apart from mRNA inhibition, we can make use of soluble forms of FGFRs able to bind FGFs that would otherwise activate endogenously produced FGFRs. The use of these soluble forms was the method originally chosen by Ornitz and Chellaiah to propose the FGFs-FGFRs binding pairs as well as the requirement of heparan sulphate proteoglycans upon binding (Chellaiah et al., 1999; Chellaiah et al., 1994; Ornitz et al., 1992). In those studies, the extracellular fraction of FGFR1 and FGFR3 was fused to secreted placental alkaline phosphatase, generating soluble FGFRs with an unaffected extracellular domain necessary for the binding-specificity studies. The alkaline phosphatase domain was necessary for secretion, and also for purification. Their results demonstrated the extracellular domain can be uncoupled from the rest of the FGFR without affecting the binding specificity. This characteristic has been exploited to engineer an extracellular secreted form of FGFR2IIIb that was hypothesised to work as dominant negative isoform (DNR) and test its functionality *in vivo* (Celli et al., 1998). The results were in agreement with the expression pattern of FGFR2IIIb isoform in epithelial tissues (Orr-Urtreger et al., 1993), and the mice expressing the DNR presented gross abnormalities of the limbs, skeleton, skin and inner ear, similar to the observed phenotype in the KO mice developed later (De Moerlooze et al., 2000). A soluble FGFR2IIIb has also been used in a model system for pancreas development (Miralles et al., 1999), and the results were similar to the ones observed with antisense RNA to inhibit FGFR2IIIb. However, the effects with the soluble form of FGFR2IIIb were milder.

For our work, we decided to use a commercially available recombinant form of human FGFR2IIIb isoform (663-FR-050; R and D systems), hypothesising it would compete against FGFR2 for any FGF ligand activating this receptor, in particular FGF3 and FGF10 normally added during differentiation experiments. For these experiments it was necessary to titrate FGF3 and FGF10, so that the minimal amount of each and soluble recombinant receptor would be used.



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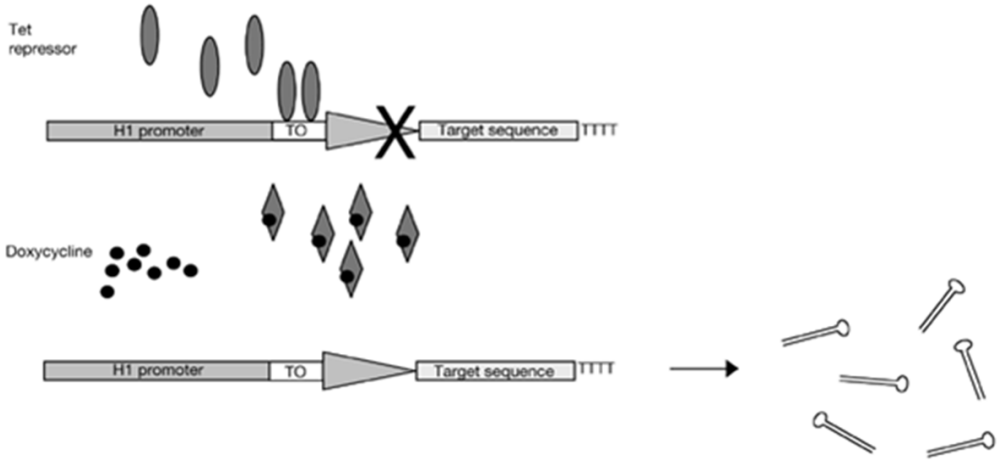


A'

Figure 7.1 **Tetracycline (dox) inducible knockdown system**. **A** and **A**', schematic representation of knockdown system. In **A** the 1st element required is shown; the constitutive expression of the TetR protein by the cells. Stable hESC lines have been previously made in the stem cell centre (e.g. Shef3). In **A'** is depicted the second element, Short hairpin oligos are cloned in the pSUPERIOR plasmid downstream the H1 polymerase III promoter region. This promoter has Tet operon responsive element as well. When TetR cells are transfected with this vector, the TetR proteins bind to the promoter region stopping transcription by polymerase III. In the contrary, when doxycycline is added, the TetR binds to it and renders the H1 promoter available for transcription, short hairpin production and gene silencing.

Results

The Dox inducible system

Schematic representation of the Dox inducible system is presented in figure 7.1. A description of the process is explained in the introduction.

Short hairpin sequences were obtained from Kunii et al (Kunii et al., 2008) because they caused a decrease in the expression of FGFR2 at the protein and mRNA levels in different cancer cell lines. All the sequences were blast in the NCBI nucleotide database, and they showed to be specific for human FGFR2.

The sequences used to construct the vectors are depicted in figure 7.2 A 5' BglII overhang in the sense oligo and an extra XhoI overhang in the 5' of the antisense oligo were added for further ligation into the pSUPERIOR vector. In addition a terminator signal for RNA polymerase III is added upstream of the XhoI restriction site. The oligos were then aligned as the pSUPERIOR manual recommends. Meanwhile, the pSUPERIO.neo vector was digested with BglII and XhoI restriction enzymes (figure 7.2 B and C), creating compatible overhangs. We made all the cloning optimization with 1 short hairpin first. In our first attempt, we could not get any bacterial colony after transformation.

The problem encountered was the high molecular ratio between insert and vector (30:1), which should be normally 1:1 or 5:1 insert: vector. We should not forget that in this case the insert is 100 times smaller than the vector (note: this is common mistake when ligating small inserts). Then we readjusted the molar ratios 6:1. At the same time, we tested dephosphorylated vector only against gel-purified vector. Phosphatase treatment of the vector helps to reduce the number of background colonies product of re-ligation between the vectors without having insert. Gel purified vector should in theory yield a higher amount of transformed colonies due to vector to vector ligation plus the additional positive ligations (vector and insert). Nevertheless, we obtained hundreds of colonies upon transformation with the phosphatase treated vector but not with the gel purified one, despite that the same molar ratios were used in the ligations, indicating that some kind of contaminant was

present in the gel purified plasmids, inhibiting the ligation. All the transformed colonies from the ligation with dephosphorylated vector contained the insert (figure 7.2 D). This was confirmed by BglII digestion and sequencing. In this system, although the vector was digested with BglII, and the overhang generated was compatible with the short hairpins, the sequence in 5' sense strand of the hairpins corresponded to BamHI restriction site, destroying the BglII restriction site upon ligation. Therefore, we would not expect any BglII digestion, as can be seen from the comparison between uncut and "digested" in figure 7.2D. Moreover, and as highlighted before, the clones used in this study were confirmed by sequencing.

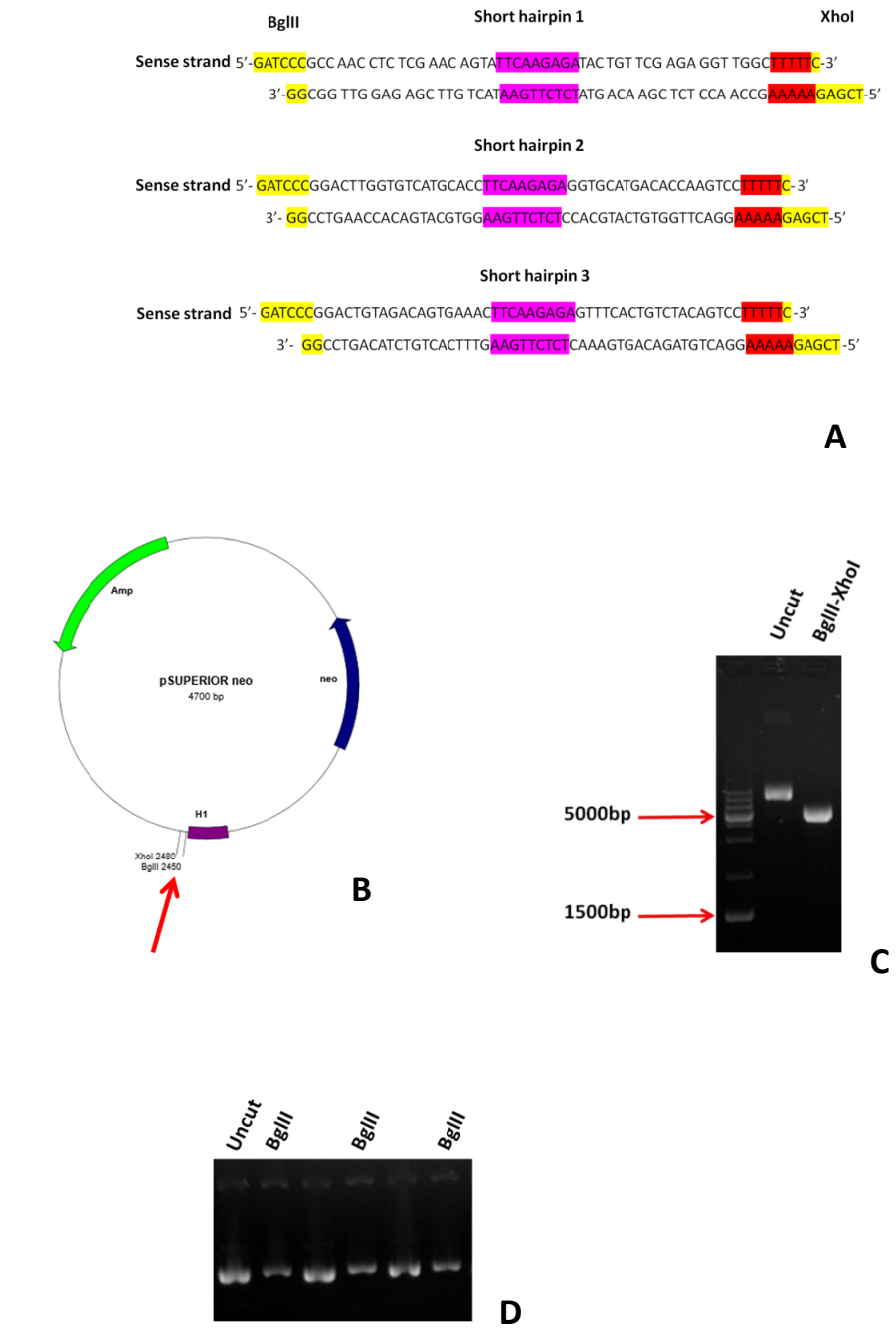


Figure 7.2 **Short hairpin sequences and cloning process**. (**A**) Short hairpin sequences were obtained from Kunni et al 2008. The oligos were then synthesised with BglII and XhoI overhangs (yellow) for cloning, in addition a terminator sequence (red) and middle loop (purple) were included as Clontech recommends. (**B**) pSUPERIOR map highlighting the restriction points (red arrow) for BglII and XhoI. The digested migration pattern of double digested plasmid is shown in the gel in **C**, compare to uncut, red arrow show the sizes of 1500 and 5000bp bands. The digested plasmid was ligated with the annealed short hairpin sequences, destroying the BglII restriction site. Therefore, no difference between BglII digested and uncut plasmid were seen (just prep of positive bacs are shown).

I have described here the cloning process, because although it is a very well established technique, it can be problematic and often involves a long troubleshooting procedure, as it was in this case.

Once our vectors were ready, the next step was to create stable cell lines. For this purpose we used the parental cell line Shef3, from which we knew that differentiation was efficient in response to FGF3 and FGF10 treatment (chapter 3 and 4). In addition, a Shef3 TetR stable line had been previously generated in the CSCB. This cell line constitutively expressed Tet repressor protein driven by the CAG promoter. As already explained, the presence of TetR will keep the short hairpin silenced upon transfection of pSUPERIOR-Sh.

To further check the efficiency of our plasmids in knocking down FGFR2 expression, NTERA cells (teratocarcinoma cell line) were transfected and selected, or just transiently transfected with the pSUPERIOR-Sh vectors. In theory, since no Tet repressor is expressed in these cells, expression of the short hairpin should take place immediately after transfection. Relative expression data showed that although not very efficient, there was a clear downregulation of FGFR2 in cells transfected with the three short hairpin plasmids (figure 7.3). The low efficiency of the knockdown (50% downregulation) could be consequence of not all cells being transfected. We did not control for this variable in this experiment, but our previous experience suggests that transfection efficiency with this cell line is around 30% with the pCAG vector.

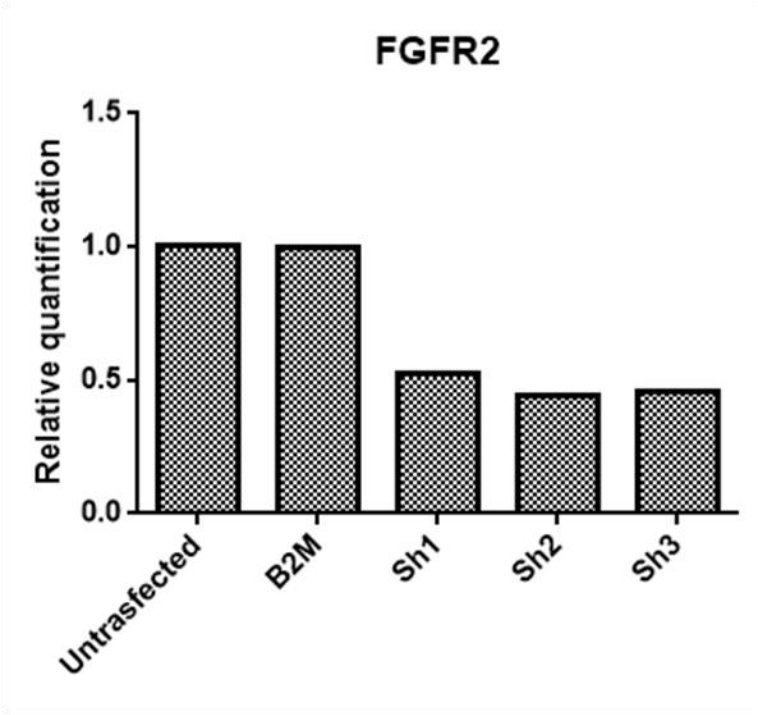


Figure 7.3 **Sh vectors downregulate FGFR2 NTERA cells**. To further check the efficiency of the short hairpin sequences, NTERA cells were transiently transfected with the pSUPERIOR-Sh (1, 2 and 3), and the pSUPERIOR-Shβ2M (β-Microglobulin) control. The cells were collected 72 hrs post transfection and analysed by Q-PCR. Data is presented as relative expression, taking as calibrator the untransfected cells.

Establishing a dox-inducible FGFR2 knockdown

We made a kill curve with G418 antibiotic in Shef3 TetR cells to determine the selection pressure necessary to obtain stable clones with high expression levels of short hairpins (figure 7.4). Most of the cells died after 48hrs of exposure to G418 at all concentrations tested. Therefore the lowest concentration, 50μg/ml, was the one we used to make stable lines. Although the protocols recommend selecting for 10-15 days to obtain stable lines, we were more stringent and applied G418 selection for 21 days at the forementioned concentration.

Following selection, individual colonies were picked, expanded and frozen. Approximately, 40 clones in total, transfected with the different short hairpin vectors were collected.

The next stage consisted in the screening of the individual clones to choose those ones with a more robust dox- induced downregulation of FGFR2. Because the large amount of work that represents to culture many hES cell lines, groups of 4 individual clones were grown, screened and frozen again. The cells were maintained for 72hrs in mTESR medium (undifferentiated conditions) with and without Doxycycline (1μg/ml).

We screened for downregulation of the FGFR2IIIb isoform by QPCR. In figure 7.5, relative quantification of most of the clones is presented. In red squares are the ones that showed the strongest silencing of FGFR2IIIb when cells were treated with Doxycycline. These clones were chosen for further analysis and differentiation experiments. Despite that we obtained enough clones to work with, it was unexpected to observe that most of the clones did not show any downregulation and that some of them showed the opposite effect. This phenomenon remains unexplained.

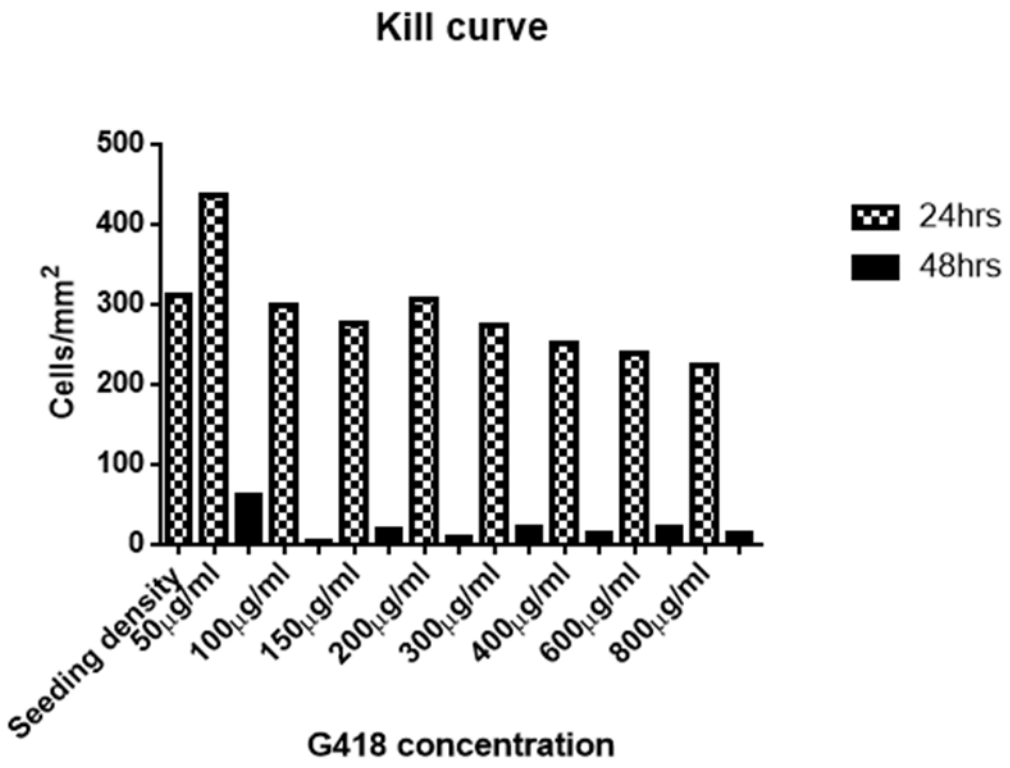
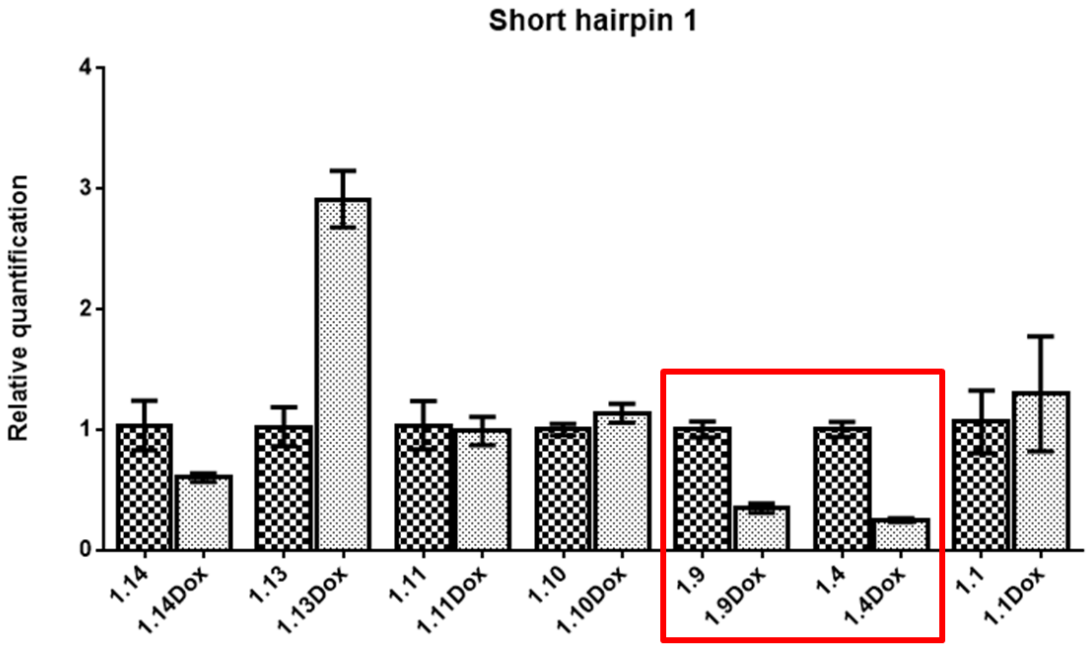
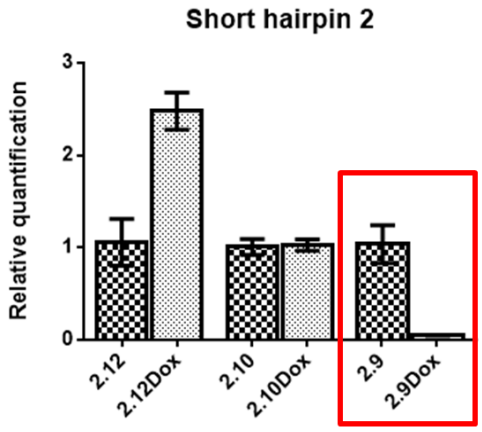


Figure 7.4 **Kill curve of Shef3 TetR cells**. A kill curve was made to determine the concentration of antibiotics necessary to obtain stable lines with from pSUPERIOR-Sh transfection. Cells were seeded at 30 000 cells/cm², antibiotic was added the following day and cells quantified at 24hrs and 48hrs. Even the lowest concentration of G418 (50µg/ml) killed most of the cells by 48hrs. Therefore, this was the one used to create stable lines.

FGFR2IIIb relative quantification





FGFR2IIIb relative quantification

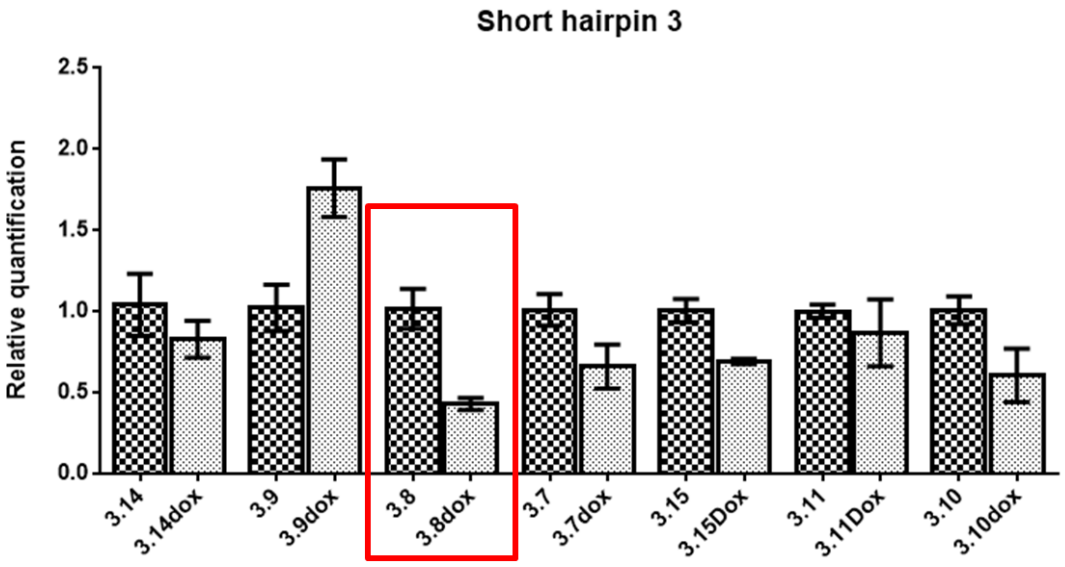
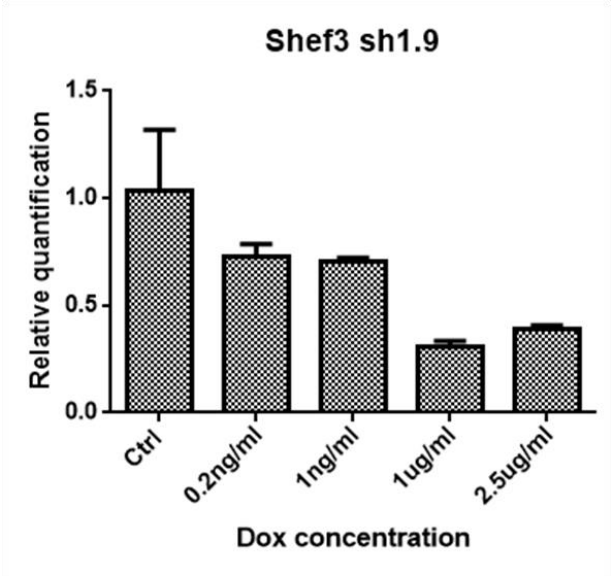


Figure 7.5 **Screening of Shef3 TetR clones shows dox-induced downregulation of FGFR2IIIb**. Undifferentiated clones for the different short hairpins were cultured in mTESR1 medium in groups of 4 clones at a time, each line was treated with 1µg/ml of Doxycycline for 72 to induce short hairpin expression, pair of treated vs untreated cells were analysed by Q-PCR and normalised against the untreated condition ("1"). The short hairpin type of clone (1, 2 or 3) is indicated in every graph. Clones generated with the three short hairpin vectors were shown to downregulate FGFR2IIIb upon doxycycline addition. Te ones in red squares are those that presented the strongest knockdown.

In the previous experiment we used 1µg/ml of doxycycline to induce knockdown. This concentration is considerably higher compared to what has been previously reported in the CSCB publications (Zafarana et al 2009; Avery et al 2010). However, we decided to go for this high concentration since we did not know much about the efficiency of the system, and this concentration was still low enough not to have any deleterious effect on the cells. We made a dox-response study in one of our selected clones (sh1.9) to know if we could downregulate FGFR2IIIb even more, or if that level could be regulated by dox concentration in future experiments.

The dose response indicated that the concentration used before (1µg/ml) was the correct one, and that more dox did not have any additional effect. Moreover, we observed that the system was slightly inefficient since at lower doses that should induce silencing of FGFR2IIIb, the downregulation was minimal (figure 7.6 A).

As part of the initial characterization, we wanted to know how fast the downregulation took place, for this purpose one of the positive clones was grown with and without doxycycline for 24, 48, and 72hrs. We observed that as soon as 24hrs, dox treatment was effective inducing the silencing of FGFR2. Although downregulation was consistently observed at every time point, the decrease in FGFR2 expression was not large, reaching a maximum of 50% reduction at the transcript level at 72hrs of dox treatment (figure 7.6 B).



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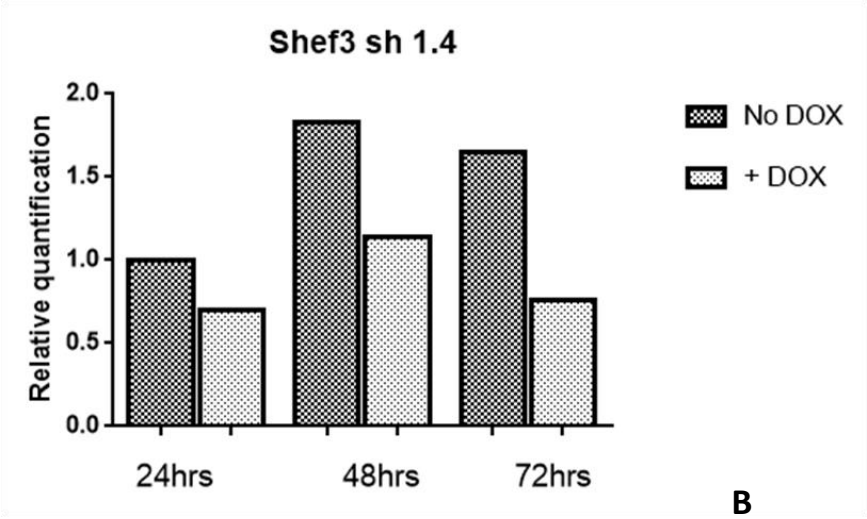


B

Figure 7.6 **Dox-inducible system knockdowns FGFR2III in a dox-dependant manner, as soon as 24hrs after dox addition**. Two of the clones chosen in the previous screening were used to characterize the dox response in the system. In **A**, Sh1.9 clone was grown in mTESR1 and treated with the indicated concentrations of doxycycline for 72hrs. The little knockdown observed at lower doses indicates the system is not very efficient compared to Zafarana et al 2009 . The graph showed in **B**, indicates that knockdown of FGFR2IIIb can take place as early as 24hrs after dox addition, suggesting that it could be used in narrow windows of differentiation. Data represent relative expression calibrated with untreated cells with each experiment. Error bars in **A** are s.e.m of reaction triplicate of one experiment

Differentiation of clones in the presence of Dox

The clones chosen from the screening were then differentiated in FGF3 and FGF10 and control DFNB medium (DMEM:F12 plus N2, B27 supplements). In the initial set of experiments, we did not observed any downregulation of FGFR2 when dox was present during differentiation (not shown). All together the results suggested that the system was not working very efficiently. One of the reasons could be the low expression level of Tet repressor protein due the strong silencing mechanisms present in hESCs. If Tet repressor expression is low, there is always a higher baseline level of short hairpins silencing FGFR2. In this situation, the lower the expression of TetR, the smaller the downregulation will be when doxycycline is added.

Therefore, cells were maintained with the selection antibiotics puromycin and G418 from here onwards to maintain the levels of TetR and short hairpins stable. We checked the presence of TetR in two of the clones, grown in both undifferentiated and differentiating conditions to confirm that the level of expression was maintained even when cells were differentiated. There was a small possibility that TetR expression could be affected by the differentiation since all the experiments where a downregulation had been observed were carried out in undifferentiated cells.

A low level of expression of TetR was maintained in all conditions in the two clones analysed (Figure 7.7 A). In this manner we were sure that TetR expression was maintained when cell were differentiated. Nonetheless, it must be said that the expression level of TetR is low compared to what was published by Zafarana et al (Zafarana et al., 2009).

These clones (sh1.4 and sh2.9) were differentiated in DFNB and FGFs media. A downregulation of FGFR2 in DFNB control medium was observed when cells were exposed to doxycycline (figure 7.7 B and C). However, in the FGF-treated condition we did not observe any difference between dox and non-dox control. In fact, in clone sh2.9 FGFR2 was upregulated in response to dox. There are some aspects that deserve some attention in these experiments: in both clones FGFR2 expression was lower in FGF treated cells than in those in control medium, contrary to what has been previously observed in hESCs, where

FGFR2 expression is induced by FGF3 and FGF10 treatment (chapter 6 figure 6.1). Shef3 TetR is subclone of Shef3, and the short hairpin clones are subclones of Shef3 TetR, and giving the heterogeneity in the hES cell niche, there is a possibility we could have selected clones that are markedly different to parental Shef3. A final aspect to consider is that in control conditions (DFNB) were the absolute level of FGFR2 expression was higher, the addition of doxycycline indeed downregulated FGFR2, suggesting that the system is also working in differentiated cells as it does in hESCs in mTESR1 medium, we also observed that the downregulation of FGFR2 was more efficient in DFNB of sh1.4 than in sh2.9, and this correlated with a higher FGFR2 expression level in sh1.4. In other words, it seems that the system works better if FGFR2 is expressed above certain threshold, and if this is not reached, no further dowregulation can be observed. These results have been confirmed in short term differentiation experiments (not shown), and the same pattern is observed.

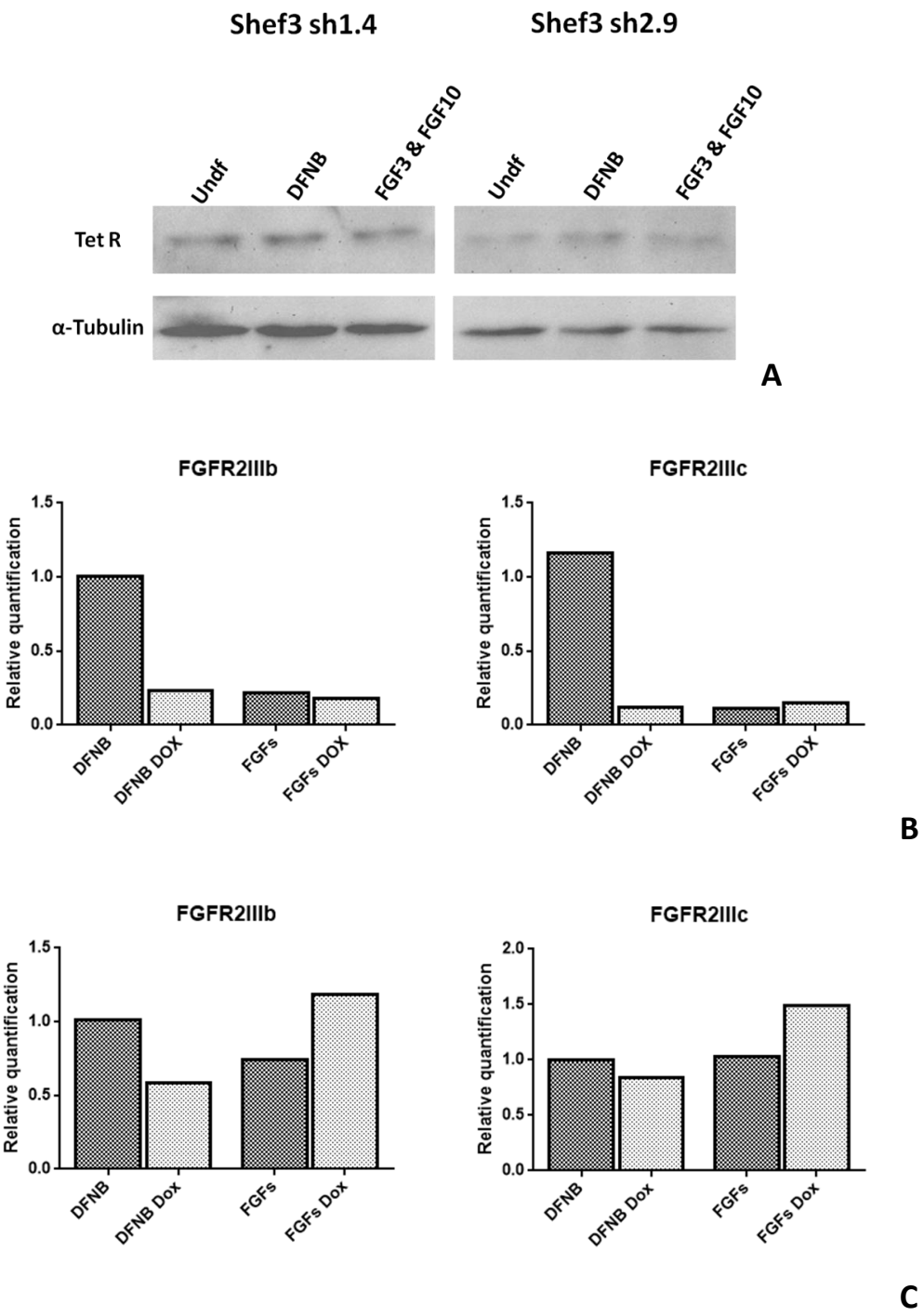
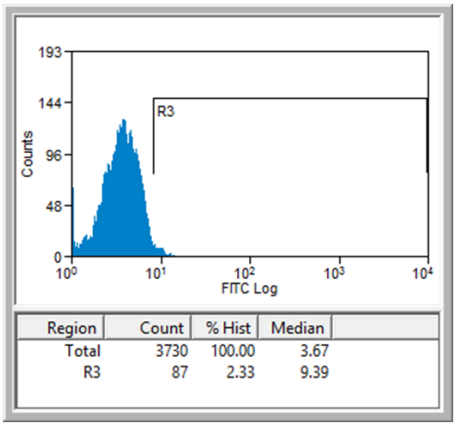


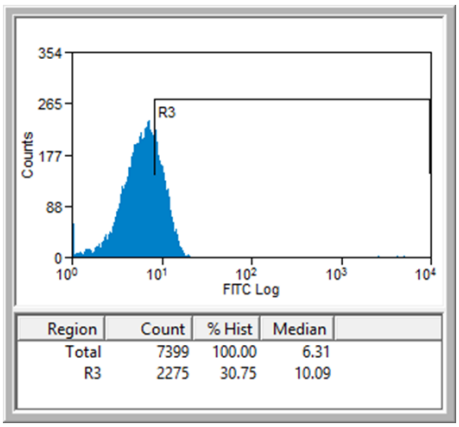
Figure 7.7 **Dox-inducible system knockdowns FGFR2 during hESCs otic differentiation**. The clones selected in the first screening were differentiated in standard conditions w and w/o dox. Since no downregulation of FGFR2 was observed in these conditions, selection antibiotics were added again to maintain level of TetR and short hairpins. After selection clones were differentiated again. In **A**, western blot showing that the levels of TetR were unaffected by the differentiating condition in clones Sh1.4 and Sh2.9. (**B)** Sh1.4 was differentiated 12 days in DFNB control medium or FGFs and *FGFR2* expression analysed with primers for FGFR2IIIc and FGFR2IIIb isoforms. (**C**) Sh2.9 clone was differentiated in the same way. In both cases, dowregulation of *FGFR2* by dox treatment only occurred in DFNB medium and more clearly in Sh1.4, correlating with the higher expression of FGFR2 in this clone in this condition (DFNB).

In parallel to the relative expression analysis, we have tried to measure any possible downregulation of FGFR2 at the protein level. In chapter 6 we showed that undifferentiated hESCs express FGFR2 (chapter 6). Moreover, we have found by flow cytometry analysis of differentiated Shef3, a higher percentage of FGFR2 positive cells in FGF treatment compared with DFNB ones (figure 7.8), in agreement with the QPCR analysis showing an upregulation of *FGFR2IIIb* in response to FGFs (chapter 6 figure 6.1). However, the results obtained by flow cytometry have not been confirmed, and since the shift in the peak of the histogram produced by the FGF treatment was minimal compared with the negative control (figure 7.8), it will require more optimization before we can use such analysis in the dox-inducible knockdown system (figure 7.8). In other words, we do not observe two populations of FGFR2+ and FGFR2– in the FGF treated cells, instead the whole population would seem to express FGFR2 (evidenced by a single peak in the histogram) but in a level just above of the fluorescence detected in the control (secondary antibody only), making difficult to conclude that cells express FGFR2 b flow cytometry analysis.



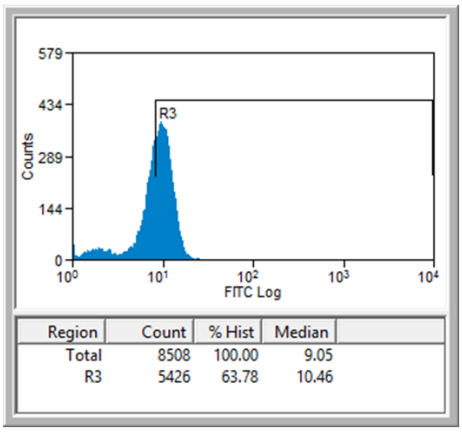
Region	Count	% Hist	Median
Total	3730	100.00	3.67
R3	87	2.33	9.39

A



Region	Count	% Hist	Median
Total	7399	100.00	6.31
R3	2275	30.75	10.09

B



Region	Count	% Hist	Median
Total	8508	100.00	9.05
R3	5426	63.78	10.46

C

Figure 7.8 **FGFs increase the percentage of FGFR2 positive cells**. Shef3 cells were differentiated for 12 days in FGF and DFNB control medium. Then, cells were harvested and stained with anti-FGFR2. (**A**) FGF treated cells stained with secondary antibody only used for gating (negative control). (**B**) Cells in DFNB medium. (**C**) Cells in FGF supplemented medium. There was twice the amount of FGFR2 positive cells induced by FGF treatment. Nonetheless, the shift in the signal intensity was minimal, and we haven't confirmed these results. Therefore, the use of flow cytometry for the analysis of the knockdown is still uncertain.

Altogether, it seems that the system has not worked efficiently. We were able to select some clones from the original screening although in most of them no silencing of *FGFR2* was observed when cells were treated with doxycycline. Time point analysis in clone sh1.4 indicated that downregulation occurred quickly after doxycycline addition, but the level of downregulation was 50% maximum. Equally in the dose response, the level of downregulation was minimal when compared with the previously published results. These results correlated with low expression level of TetR (figure 7.7). Future experiments should be aimed to test if there is any change in the downregulation of otic markers correlated to the low expression level of FGFR2 in response to doxycycline, and to verify if we can induce downregulation of β-2microglobulin in the control clones.

An alternative approach: a competitive inhibitor of FGFR2IIIb

In addition, we have also tried to test the effect of FGFR2IIIb during differentiation of otic progenitors by blocking specifically the activity of this isoform through a competitive assay. For this purpose we made use of the soluble recombinant FGFR2IIIb (663-FR-050; R and D systems).

In order to use this inhibitor, it was necessary to titrate FGF3 and FGF10, most of the systems use lower concentrations of FGFs than the one we currently use (50ng/µl). It has been suggested that in order to saturate the FGFRs in cells where these are highly expressed, lower FGFs concentration are needed (Presta et al., 1998; Roghani and Moscatelli, 1992). In addition, there are indications that FGFs may play a dual role depending of the level of activation of the pathway (Nelson and Svendsen, 2006). For example in previous chapters it was mentioned that FGFs induced a strong upregulation of otic markers in Shef3 but not in some other cell lines (e.g. Shef1). Furthermore, when cells were treated with the FGFR inhibitor SU5402, otic genes were dramatically downregulated in Shef3 as expected, while the effect was less prominent in H14 and, in the case of Shef1 an opposite effect was observed for some markers. Therefore, titrating the ligands could help us to understand how much FGFs are required to induce otic differentiation in these cells.

Figure 7.9 shows titration results of Shef3. Cells were differentiated for 6 days in with 50ng/ml (std concentration), 25ng/ml and 0.5ng/ml of FGF3 and FGF10. The graphs indicate that low FGF concentrations (0.5 ng/ml, 100 times lower than the standard used), *PAX8* is induced more strongly compared to the expression in standard FGF concentrations (50ng/ml). PAX2 and *DLX5* do not change, and there is minimal drop in the expression of *FOXG1*. Therefore, even at this low concentration, a similar level of otic induction to the one observed at concentration 100 times higher can be obtained. It would be important to explore lower FGF concentrations in the future, especially considering the possible inhibitory effect that high concentration of FGFs could have evidenced here in the expression of PAX8.

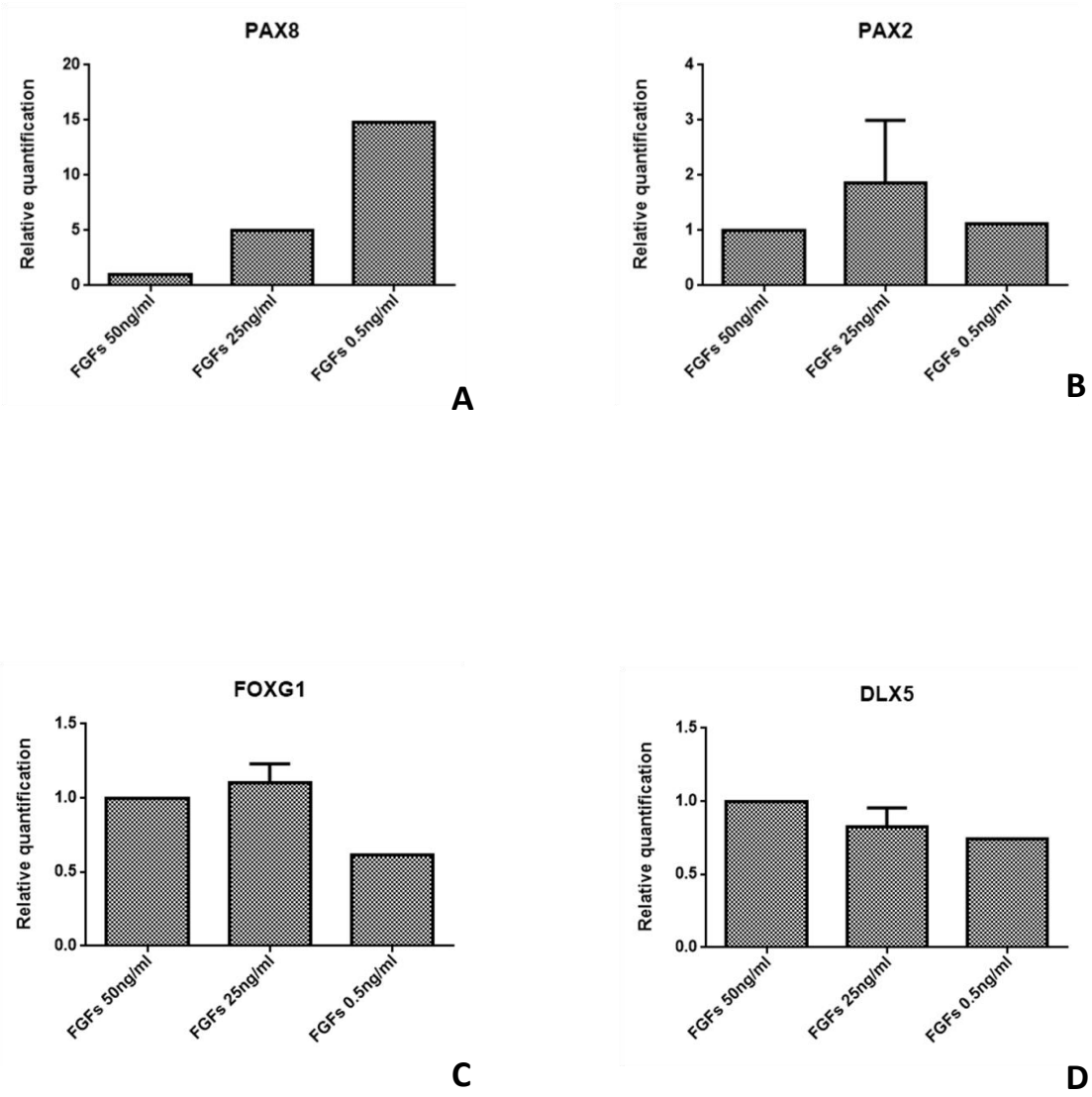


Figure 7.9 **Low concentration of FGFs trigger strong PAX8 expression.** In order to test the soluble FGFR2IIIb competitor, titration of FGFs was necessary. Shef3 cells were differentiated for 6 days at different concentration of FGF3 and FGF10. Only three concentrations are shown; 50, 25 and 0.5ng/ml. Relative expression QPCR data is presented. Cells treated with standard concentrations of FGFs (50ng/ml) were used as a calibrator. PAX8, PAX2, FOXG1 and DLX5 are shown in **A**, **B**, **C** and **D** respectively. The level of expression of the otic markers at 0.5ng/ml was similar to the one observed at 50ng/ml. interestingly, in the case of PAX8, lowering the FGF concentration increased the level of expression of this otic marker. Error bars are s.e.m, n=2

When we tried to block specifically FGFR2IIIb-mediated activity through the competitive soluble isoform, a downregulation of the otic markers was expected, similarly to what has been previously shown in these cells when treated with the FGFR inhibitor SU5402 (chapter 4) and in agreement with the otic markers upregulation by FGFR2IIIb overexpression. Nonetheless, the results were not as robust as those observed with the SU5402 (figure 7.10). In this regard, there was a chance that a different result was observed since the soluble FGFR2IIIb is a more specific inhibitor. Only PAX2 and DLX5 were downregulated, while PAX8 and FOXG1 were slightly upregulated. These results indicate an incomplete penetrance of the soluble FGFR2IIIb inhibitor, milder to the one seen using the SU5402 inhibitor.

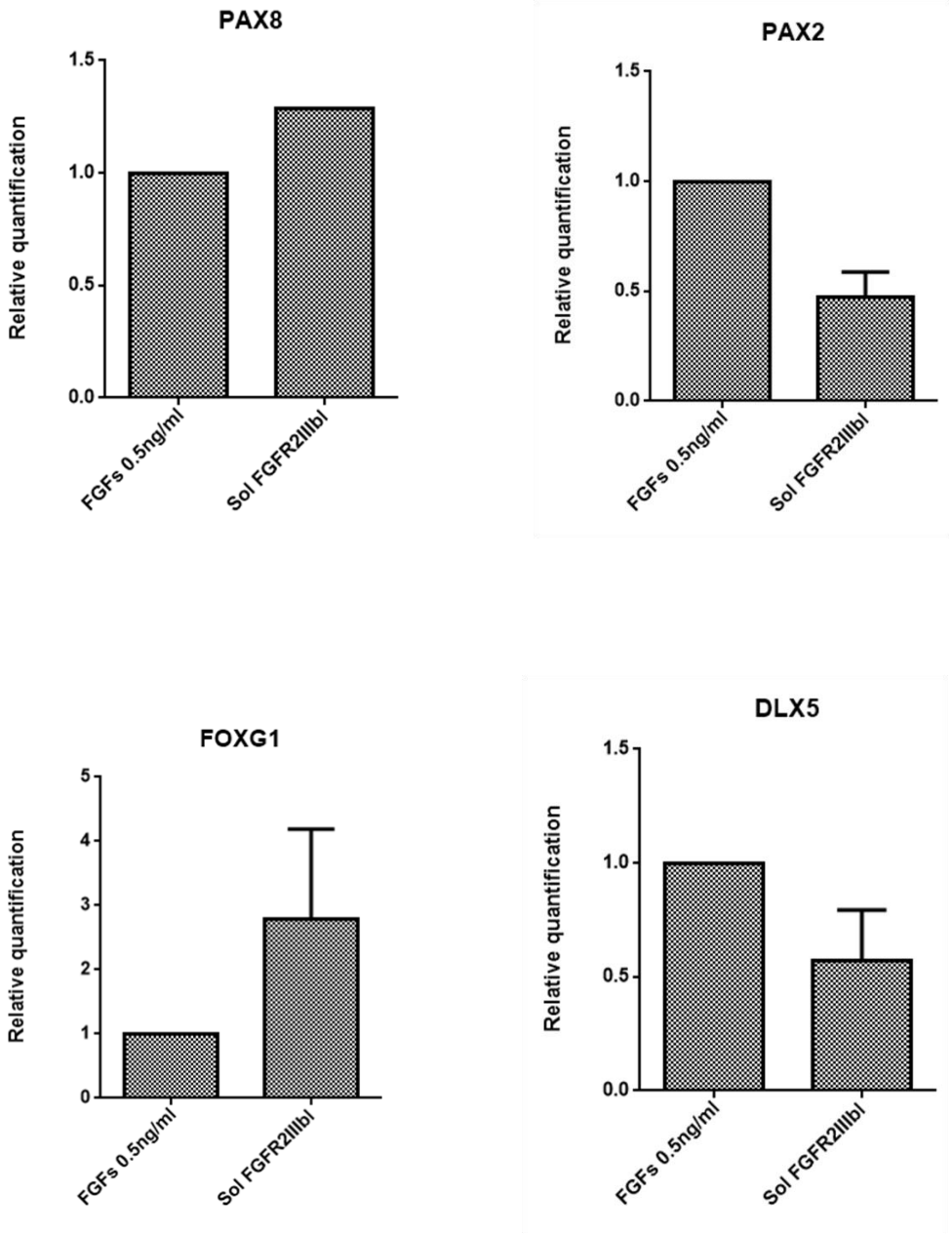


Figure 7.10 **Soluble FGFR2IIIb has a milder effect than SU5402.** Shef3 cells were differentiated for 6 days with low concentration of FGFs (0.5ng/ml), or FGFs 0.5ng/ml plus 10ng/ml sol hFGFR2IIIb (R and D). Samples were analysed by RT-PCR of the main otic markers (indicated in each graph). PAX8 and FOXG1 were upregulated with the FGFR2IIIb inhibitor. In the contrary, PAX2 and DlX5 were both downregulated with this type of inhibitor as observed with SU5402 (chapter 4). The effect of FGFR2IIIb inhibitor in PAX8 expression is agreement with the more inductive role of low FGFs in PAX8 induction (figure 7.9). Error bars are s.e.m. n=2

In summary, although most of the results presented in this chapter need further confirmation and are more difficult to interpret than previous ones, they still support a positive role for FGFR2IIIb during otic differentiation of hESC. It must be said however, that the FGFR2IIIb overexpression produced a more reliable result than our attempt to block this receptor with a competitive, soluble fragment. In our *in vitro* approach we are trying to find out the right cues to push the cells towards a fate resembling the progenitors found in the inner ear *in vivo*, but we are not there yet and in this context it would be more reasonable to overexpress the FGFR2IIIb than to knock it down.

Discussion

Inducible knockdown is inefficient

We screened a large set of clones that were transfected with the different pSUPERIOR-short hairpin versions (1, 2 and 3). Most of them showed either none or a minimal downregulation of FGFR2IIIb. Nonetheless, we had a group of 4-5 belonging to the three classes of short hairpins that showed more than 50% downregulation at the transcript level. In Kunni's work (2008), where these short hairpins were taken from (Kunii et al., 2008), they reported that two of their cell line showed a robust decrease in FGFR2 protein when transduced with the different shRNA. In another cell line of that study, shRNA transduction never downregulated FGFR2. The authors explained this phenomenon by the poor transduction efficiency in that cell line. When they tried to circumvent the problem by creating stable cell lines, none of the clones showed downregulation of FGFR2 anymore. This raises many questions, for example; is that a common general phenomenon in knockdown studies?, is it related to the function of FGFR2 (do cells have strong mechanisms to keep it "on")?. There are examples where dsRNA can activate gene expression, but in all these cases, the RNA sequence is targeted to the promoter region of a gene (Li et al., 2006; Portnoy et al., 2011). If it is a common problem in knock down studies is also difficult to determine, since being a negative unexpected result, it is often ignored. In addition, most of the studies have looked at the knock down for short periods of time. We don't know if there

is particular mechanism of surveillance regarding FGFR2 expression that may hamper the ability of short hairpin sequences to knockdown the receptor in some cell types or for longer periods of time. In this regard, conditional knockdown of *Fgfr2* in the mouse by means of a Cre-Lox system produces offspring with variable degrees of developmental defects despite being derived from the same transgenic strain (Coumoul et al., 2005). This phenotypic variation could be also consequence of the variable degree of Cre activity in the germ cells, or compensation by other fibroblast growth factors in a genotype dependent manner (offspring variation).

If knocking down FGFR2 has deleterious effect on the cells, this should be overcome by the inducible system that would not allow the downregulation of FGFR2, unless doxycycline is added. In this regard, since we later showed that expression of TetR was very low in our clones (figure 7.7) compared to what was previously published by Zafarana et al (Zafarana et al., 2009), our system may have not worked entirely as an inducible one. In other words, a continuous downregulation of FGFR2 by pSUPERIOR.sh transfection could have blocked the short hairpin-mediated silencing observed in most of the clones, similar to what Kunni et al. observed when trying to generate stable sh-expressing cell lines that at the end of the selection process did not present any knockdown.

Differentiation of dox-inducible sh clones

In any case, some clones downregulated FGFR2 when exposed to doxycycline. Thus they were used for differentiation experiments (figure 7.7). In those experiments we noticed that the inducible downregulation worked only in DFNB treatment, and this correlated with a higher level of expression of FGFR2IIIb in this condition. This means that if there is little or no FGFR2 expression, an inefficient production of short hairpins will have a minimal effect. On the contrary, if there is high expression of FGFR2, it will be easier to observe the effect of short hairpins. This is what was hypothesised, but what it seems more contradictory in our differentiation experiments is the higher expression of FGFR2 in DFNB than in FGF treated cells (figure 7.7 B). This different behaviour in the Sh-clones could be explained by the

selection of clonal cells from the undifferentiated Shef3 population that present a markedly different behaviour to the parental cell line. Another possibility is that the media formulation used to maintained undifferentiated hESCs may impact in how the cells differentiate, for example the sh-clones were maintained in mTESR1, while the rest of the hESCs were maintained in hES medium (methods, chapter 2) on inactivated mouse embryonic fibroblasts.

The different subsets of cells present in a culture has been an idea already introduced in previous chapters. Gene arrays of single cells have found differences in the expression profile between single cells derived from SSEA4+ fraction of pluripotent cells (Narsinh et al 2011). What it seems particularly interesting of this heterogeneity is the fact that is not random. Using a retroviral tagging approach, Stewart et al showed that within the hES culture different types of cells can be identified, and those are part of a self-renewing population and therefore remains present in the culture through passaging. Interestingly they observed that the developmental potential of these single cells was different, some of them being enriched during differentiation and some others having clonogenic capacity, and importantly this potential of particular clones was the same if assessed latter at different passage (Stewart et al 2010). Therefore, cells with different functional properties coexist in the hES cells culture. The clonal origin of Shef3-TetR-Sh cells could have selected those with a radically different behaviour to parental Shef3. It is interesting to mention than in Stewart work the clonogenic subset oh hESCs was positive for insulin-like growth factor 1 receptor (IGF1R). In the previous chapter it was also discussed that FGFR1 can be found in small subset of hES cells (Carpenter et al 2004). Therefore, differences in behaviour between cells lines could be explained by their sensitivity to important signalling molecules.

As mentioned before, the difference between the sh-clones and Shef3 parental cell line could have also arisen from differences in the culture medium used for the maintenance of the undifferentiated cells. For example mTESR1 was used in the sh-clones and MEFs in the rest of hESCs stocks. In this regard, in mouse ESCs it has been found that the cell culture medium used has a strong impact in the epigenetic status and expression profile of several genes (Marks et al., 2012). Particularly interesting was the finding that serum-based medium enriched the expression of genes related to ectoderm specification compared to 2i-medium, a medium supposed to maintain a more stable “ground” state in mESCs. The

media choice has been shown to be relevant in the maintenance of the undifferentiated state (International Stem Cell Initiative et al., 2010) but careful examination of the effect that hES-medium choice has on the differentiation potential has not been made yet.

Concentration-dependent role of FGFs

The results obtained in the titration of FGFs in Shef3 cells line are in agreement with a concentration-dependant, dual mode of action of FGFs. Low concentrations of FGFs are inductive while high concentrations are inhibitory for otic differentiation (figure 7.9). Nevertheless, the results indicate the different otic markers are induced at different threshold of FGFs. In the case of Shef3 for example, as the FGF concentration is decreased, *PAX8* is augmented even more when compared with cells differentiated in standard FGFs concentration (50ng/ml). At some point the concentration of FGF will be too low (e.g. SU5402), and in that case no *PAX8* will be observed (chapter 4; figures 4.1, 4.2). The soluble FGFR2IIIb decreases the FGF activity even more but not completely, causing in this manner a slight increase in *PAX8* expression (figure 7.10). In other words, *PAX8* is a very sensitive marker to FGF concentration and high concentrations can have an inhibitory effect on its expression. Regarding the other markers, *PAX2* followed by *DLX5* are less sensitive to FGF concentration. There is no inhibitory effect of high concentration of FGFs (50ng/ml) in *PAX2* expression, and *DLX5* expression is slightly higher at high FGF concentration. In accordance, when FGFs are reduce *PAX2* is unchanged and *DlX5* is decreased, moreover, if the soluble inhibitor is added and therefore FGF are reduced, we start to see a drop in the expression of both markers, but not at the level of the one observed when SU5402 is added. Thus, the FGFR2IIIb lowers down the FGFs available for the cells, but it does not completely blocks their activity. The incomplete penetrance of soluble FGFRs has been observed in the mouse as well (Celli et al., 1998) and the phenotypes are more variable than FGFR2IIIb knockout mouse (Xu et al., 1998).

The concentration-dependent mode of action of FGFs is well supported by other systems. In the previous chapter, the work of Nelson and Svendsen was discussed, they showed that low concentrations of FGFs induced neurogenesis and high concentration induced proliferation of human neural progenitors cells (Nelson and Svendsen, 2006; Qian et al., 1997). Similarly in the lens progenitors, cell differentiation is affected by the amount of FGFs, low FGFs induce proliferation but not differentiation, while high concentration induce both. (Iyengar et al., 2007; Lovicu and McAvoy, 2001). In the future single cell analysis differentiation in FGF titration will help in the understanding of the important effects that FGFs could have in our system.