Chapter 6

The role of FGFR2IIIb during otic induction *in vitro*

Introduction

In chapter 3 we observed that FGF3 and FGF10 induced the expression of most otic markers compared with cells grown in DFNB medium. This FGF-mediated otic differentiation is in agreement with the role of these ligands during inner ear development. However, we observed a large variation in the response to FGFs between cell lines. Later on, we found that the secretion of FGF10 in some cell lines could explain part of this variation, explaining their endogenous differentiation trend, and also validating the FGF-otic induction model in "disparate cell lines". Moreover, when time course expression of otic markers was considered, different lines presented a similar pattern of expression, which again was in agreement with developmental models. Nevertheless, some experiments with the FGFR inhibitor SU5402 produced some contradictory views with the findings just described.

Therefore, in this and the following chapter, we will try to dissect FGF-mediated otic induction in more detail. Targeting the role of FGFR2 isoform IIIb during otic differentiation in our *in vitro* system, this receptor isoform is hypothesised to be the mediator of otic placode induction by *Fgf3* and *Fgf10* in the mouse (Wright and Mansour, 2003).

In the general introduction (chapter 1) we extensively present the different aspect of FGF signalling. Here, we will focus our attention on the role of FGF receptors.

There are currently identified 23 members of the FGF family; most of them are short range secreted proteins, although many of them have intracellular isoforms. They are involved in tissue homeostasis (e.g. angiogenesis and wound healing) and in every morphogenic event during development. They signal through the binding to FGF receptors tyrosine kinases (Eswarakumar et al., 2005). This binding requires heparan sulphate proteoglycans (HSPG) to maintain stability. Upon FGF binding, receptor dimerization takes place, and autophosphorylation of their tyrosine residues triggers a signalling cascade that often involves ERK, PI3K/AKT and PLCγ signalling. The extracellular region of FGFRs 1-4 is composed by three immunoglobulin like domains, in the third of them (III Ig) an alternative splicing event leads to the formation of either of two isoforms, designated as IIIb or IIIc that present different binding affinities for specific member of the FGF family (Chellaiah et al., 1994; Johnson et al., 1991; Miki et al., 1992; Ornitz et al., 1996; Zhang et al., 2006). In

addition, the expression of either of these isoforms seems to be strictly regulated during development, being the IIIb isoform localized in epithelial structures and the IIIc is expressed mainly in the mesenchyme (Orr-Urtreger et al., 1993; Peters et al., 1992).

The effects of FGFR mutations are diverse and pleiotropic, hampering the analysis of their function in specific organs. For example FGFR1 mutation causes early embryonic lethality due to defects in mesoderm formation (Yamaguchi et al., 1994), and *FGFR2* mutant mice die at E10.5 due to a failure in placental development (Xu et al., 1998). Thus, developmental biologists have relied in the knowledge of tissue specific markers to conditionally inactivate FGFR in different organs. It is interesting that FGFR1-3 have all been linked to some aspect of inner ear development.

Specific inactivation of *Fgfr1* in the ear by using Foxg1-Cre mice has revealed an important function of this receptor in the development of hair cells in the cochlea (Pirvola et al., 2002). *Fgfr3* null mice are deaf due to a failure of pillar cells to develop (Colvin et al., 1996). *Fgfr4* has also been found in the mesenchyme surrounding the cochlea, but whether if it has a function or not in the ear has not been explored (Hayashi et al., 2010). Fgfr2 has also been observed in the mature cochlea and at early stages of otic induction. In this regard, although the mouse mutant generated by Xu et al died at E10.5, the otocyst at that stage was markedly reduced (Xu et al., 1998), reminiscent of the small otic vesicle sometimes formed in the double mutant *Fgf3-/-, Fgf10-/-*.

In vitro studies suggest that FGF3, FGF10 and FGF7 bind specifically the IIIb isoform of FGFR2. This is in agreement with the early expression of *Fgfr2IIIb* in the mouse during otic induction, and with the phenotype of the *Fgfr2IIIb* targeted mutation in a variety of epithelial structures (De Moerlooze et al., 2000), including the otic vesicle. The otocyst in *Fgfr2IIIb* mutants is smaller but always forms (Pirvola et al., 2000), contrary to what has been observed in the Fgf3/Fgf10 double mutant (Wright and Mansour, 2003). This milder phenotype could be the result of compensation by other FGF receptors expressed at early otic development, in particular *Fgfr1IIIb*, through which these FGF can also present some activity. Nonetheless, the data indicates that FGFs act through FGFR2IIIb isoform to induce the formation of the otic placode.

As discussed in the previous chapter, all inner ear development models indicate FGF signalling is necessary for otic induction. However expression of PAX genes and other early transcription factors has not been assessed in the Fgfr2IIIb mutant mouse. In addition, in the work of Maroon et al (2000) with the zebrafish, it was shown that FGFR inhibitor SU5402 abolished the expression of *pax2.1* but not *pax8*, suggesting that the latter one could be independent of FGF activity for its induction (Maroon et al., 2002). Therefore, our *in vitro* system represents a human model to evaluate the role FGFR2IIIb during the otic differentiation; it could also add extra evidence to the model presented in the mouse, through the analysis of *PAX8, PAX2*, and *FOXG1* otic markers, and ultimately validate our proposal of FGF-mediated otic induction in hES cells.

Thus, the aim of this and the following chapter (7) is to evaluate the possible role of FGFR2IIIb during otic differentiation of hESCs. We made use of two approaches; an overexpression system and an inducible knock down (chapter 7). Here we present *FGFR2IIIb* expression data in hES cells and differentiated otic progenitors, and also the technical hurdles related to the establishment of the overexpression system.

Results

FGFR2IIIb is upregulated by FGF3 and FGF10

Expression of all FGFRs is well documented in hES cells (Ding et al., 2010; Dvorak and Hampl, 2005; Sato et al., 2003; Sperger et al., 2003). Our PCR data confirms that undifferentiated cells express different types of FGFRs, including FGFR2IIIb (figure 6.1 A). In addition we observed a statistically significant difference between the ΔCts of cells treated with FGFs compared with those in DFNB control condition (paired t test, *p=0.026, n=11*), therefore, the data suggest that FGF3 and FGF10 treatment activate a positive feedback loop mechanism in the FGFR2IIIb.

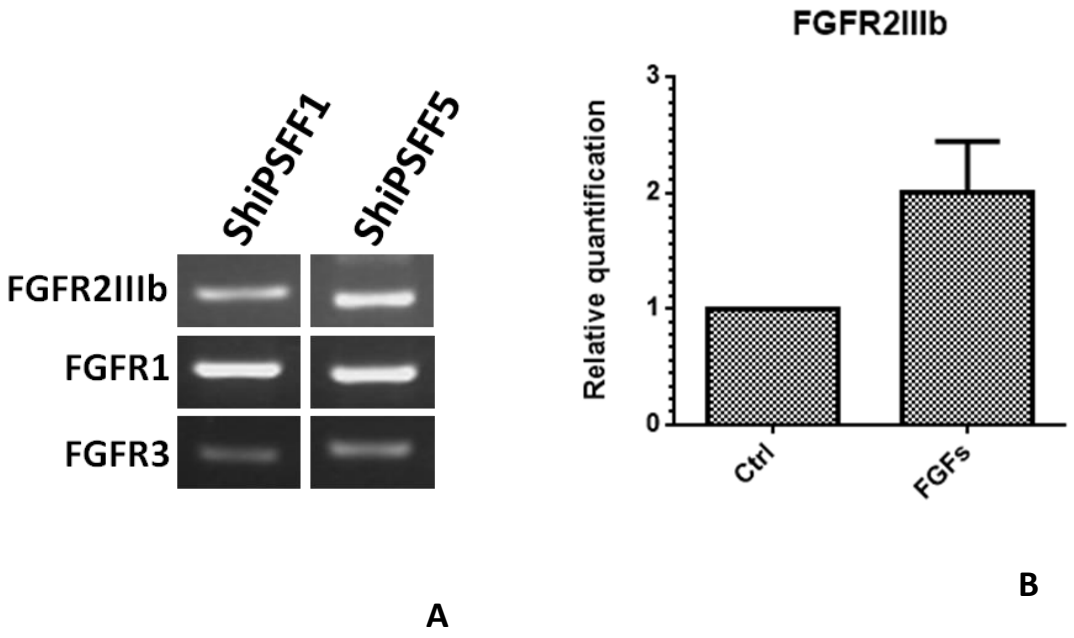


Figure 6.1 **FGFR2IIIb is expressed in undifferentiated hES cells and otic progenitors.** RT-PCR shows that undifferentiated hiPSCs express FGFR1, FGFR3 and more importantly FGFR2IIIb isoform (**A**). In **B**, QPCR data of hES and hiPS cells differentiated in FGF3 and FGF10 supplemented media or control (DFNB). Data is presented as relative expression using as calibrator cells in DFNB medium only (ctrl defined as "1"). Although we did not have any particular expectation regarding the behaviour of FGFR2IIIb when cells are treated with FGF3 and FGF10, we observed in general a higher expression of FGFR2IIIb in FGF condition compared with control. (paired t test, p=0.026, n=11).

Based on these data and in the literature, we were confident that hES cells express FGFR2IIIb. At this stage it was important to count with a reliable antibody for future knockdown studies and also for the optimization of the overexpression system.

The FGFR2 antibody from R and D system (MAB6843) was chosen based on its wide range of applications where it could be used; it does recognise all the FGFR2 isoforms and does not present cross-reactivity with other FGFRs.

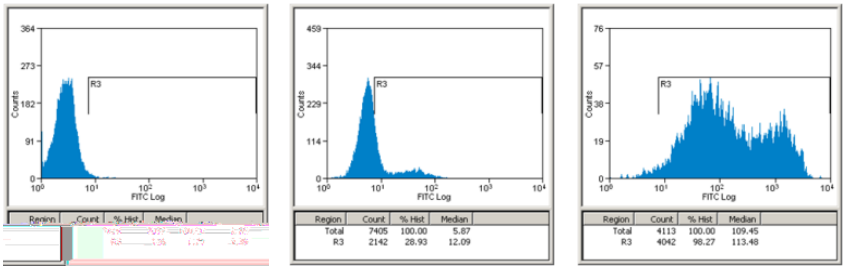
Undifferentiated hES cell lines were used to test if FGFR2 was detectable by flow cytometry. As negative control we used cells stained with 2ary antibody only. At the same time, markers of pristine hESCs, SSEA3 and TRA1-60, were used as positive controls to test that our hES cells were effectively undifferentiated and that there was no problem with the secondary antibody.

Flow cytometry charts of H7, H14 and Shef3 hES cells are presented in figure 6.2 (A, B and C respectively). In all the experiments we were unable to detect FGFR2, but not the antigens specific of undifferentiated hES cells TRA1-60 and SSEA3, ruling out the possibility that a differentiated progeny with no FGFR2 expression was the dominant population in the cultures. We corroborated this data by immunofluorescence (6.2 D).

Figure 6.2 **FGFR2 was not detected by flow cytometry or immunofluorescence of undifferentiated hESCs.** Undifferentiated cultures of hES cells were dissociated and stained with anti-FGFR2 (MAB6843; R and D systems). Secondary anti-mouse was used as negative control. As positive control for flow cytometry analysis, markers of undifferentiated hES cells TRA1-60 and SSEA3 were used. The cell line is indicated in the left part of the image and the antibody on the top. In addition, immunofluorescence of Shef1 with the same FGFR2 antibody was carried out (**D**' and **D''**), in **D** the bright field image of an undifferentiated colony. The data shows that FGFR2 was not detectable in undifferentiated hES cells.

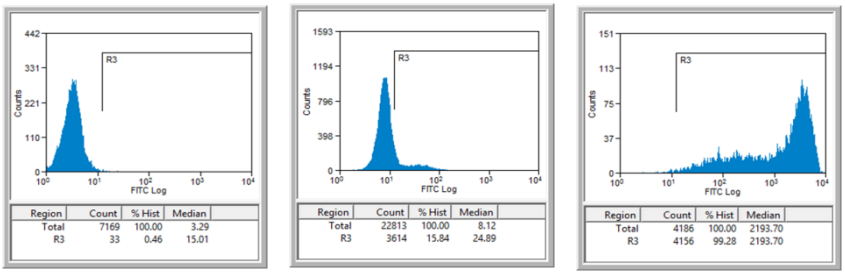
2ary only Anti-FGFR2 TRA1-60

H7



A

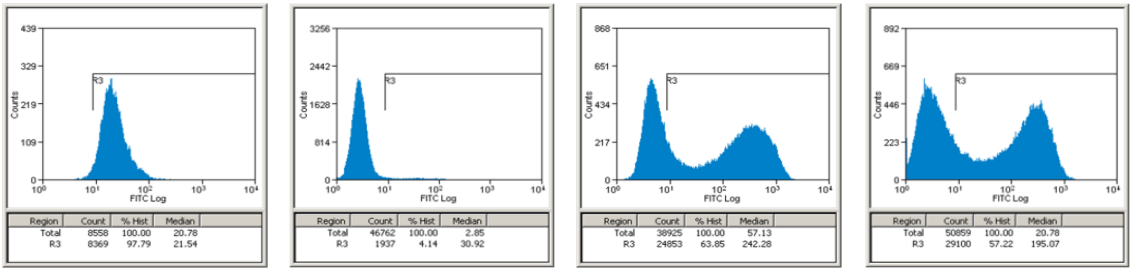
H14



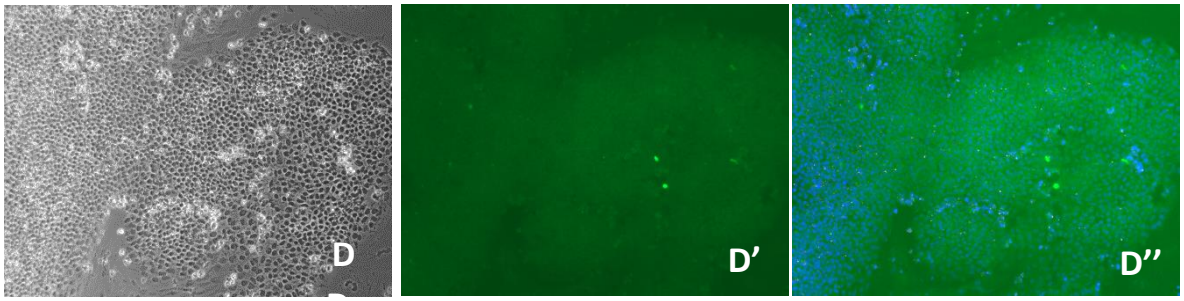
B

SSEA3

Shef3



C



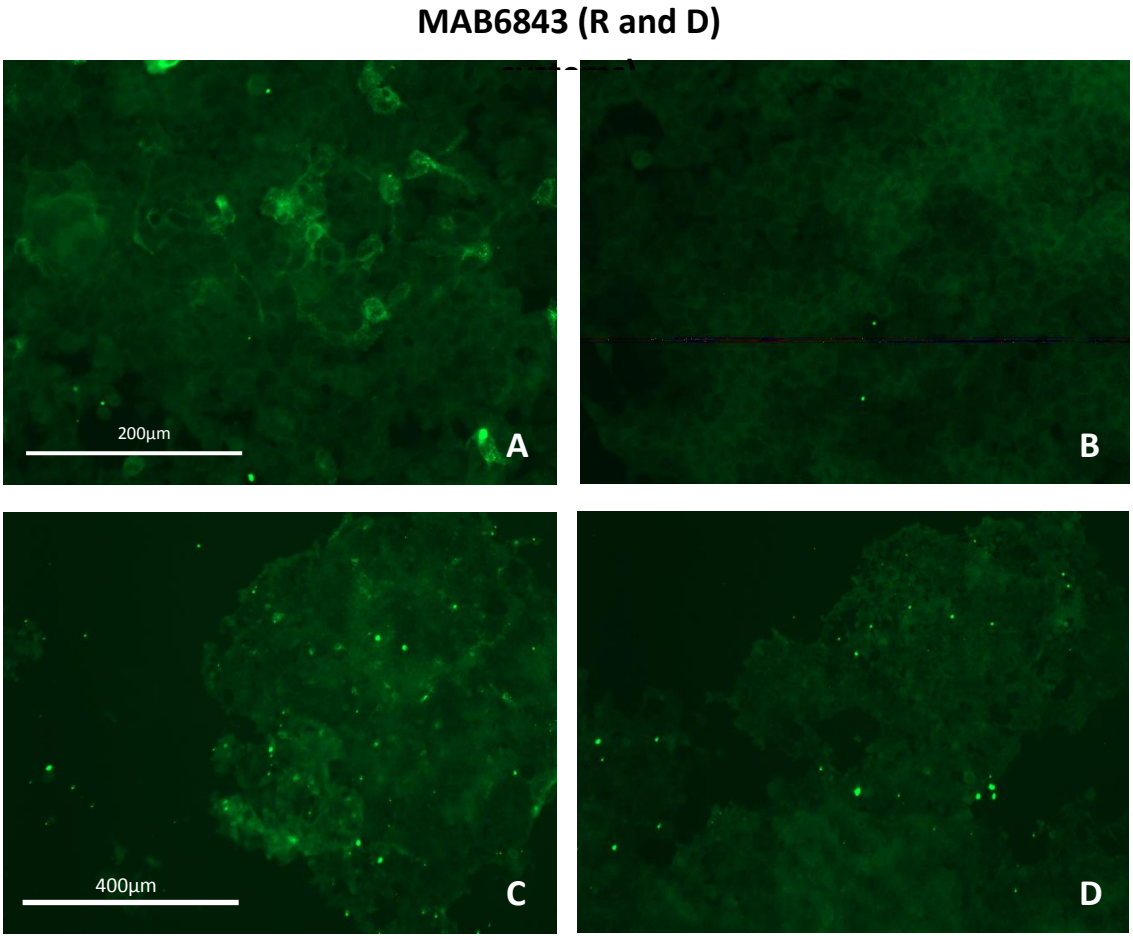
Next we considered that it would be better idea to look for a plasmid to overexpress FGFR2IIIb in hES cells, and at the same time, to be used as a positive control of our antibody. We obtained an LTR-FGFR2IIIb plasmid, kind gift from Dr. Ornitz.

There was a possibility that this vector may not be suitable for hES stem cells. In fact, it is known that hES cells possess strong silencing mechanisms that difficult the expression of exogenous proteins. When hES cells were transfected with LTR-FGFR2IIIb and selected with G418, most of the cells died during the first 48hrs (not shown). We suspect that the G418 resistance cassette from this vector was not strong enough to robustly protect the cells from G418 selection as other vectors in the lab are.

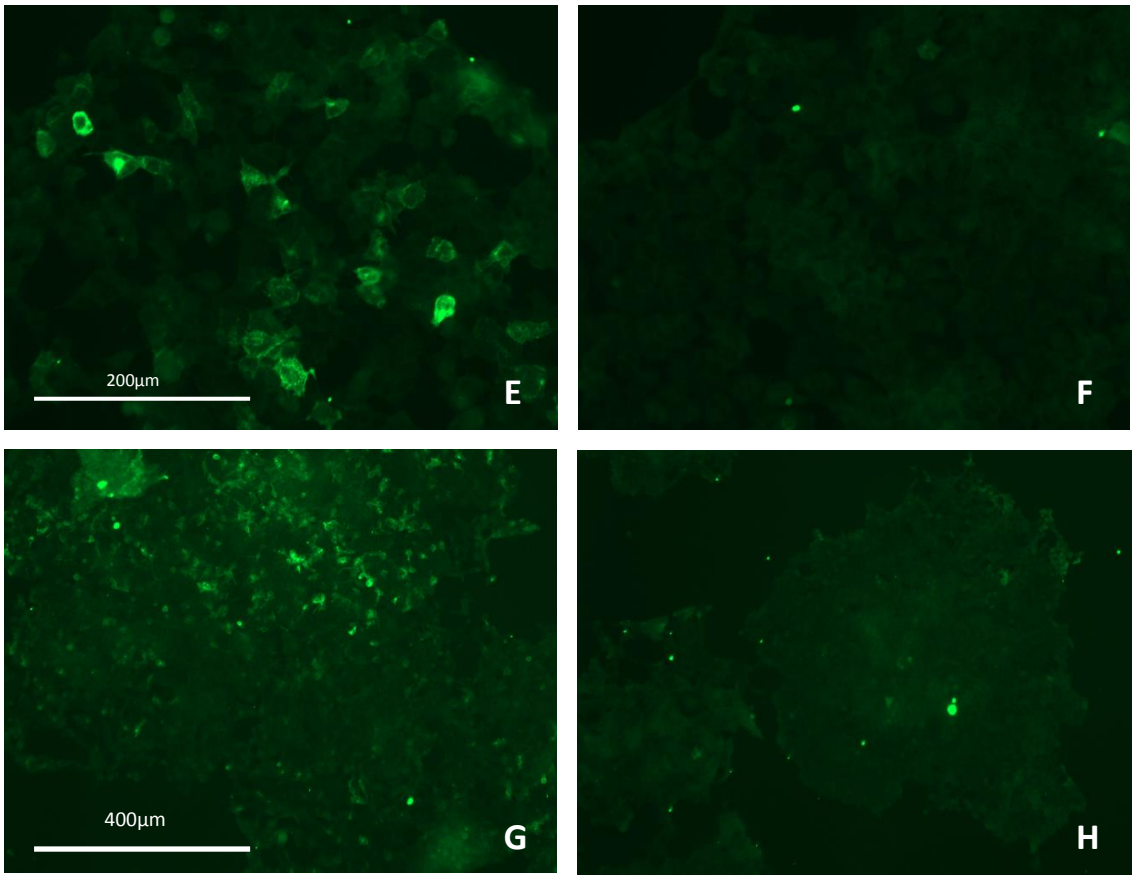
Therefore we decide to use an easy to transfect cell line, HEK-293. This was a good control for our FGFR2 antibody, since the cells do not express this receptor (Ahmed et al., 2008). At the same time, a second antibody raised against all FGFR2 isoforms (ab119237, Abcam) was used in parallel. HEK-293 cells were then transfected with LTR-FGFR2IIIb plasmid and immunofluorescence analysis with the mentioned antibodies was carried out 48 hrs post-transfection.

The results are shown in figure 6.3. We verified that the initial FGFR2 antibody (MAB6843) worked since transfected cells had some clear FGFR2IIIb positive cells compared with the minimal level of fluorescence observed in untransfected ones. However we confirmed that the affinity of this antibody was, as we originally thought, very weak. This conclusion comes from the immunofluorescence comparison between both antibodies in transfected cells (figure 6.3 A vs. E); the level of fluorescence signal in transfected cells was higher in cells stained with ab119237 compared with those stained with MAB6843. In addition, the background was much weaker in cells stained with ab119237 than the one observed with MAB6843 (B vs. F).

Figure 6.3 **Immunofluorescence analysis FGFR2 transfected cells demonstrate the low affinity of the FGFR2 antibody (MAB6843).** LTR-FGFR2IIIb plasmid from Dr Ornitz was used to transfect HEK-293 cells, followed by immunofluorescence with the previously used FGFR2 antibody (MAB6843) and another FGFR2 antibody (ab119237). Cells were stained 48hrs post-transfection. Immunofluorescence staining with MAB6843 in transfected (**A**) vs untransfected cells (**B**) is shown. In **E** and **F** transfected and untransfected cells respectively, stained with the new FGFR2 antibody (ab119237). **C** and **D** are low magnification pictures (10X) of **A** and **B** respectively, while **G** and **H** low magnification corresponding to **E** and **F.** Scale bars are indicated in the figure. The low affinity of MAB6843 was confirmed from the comparison **A** and **E**, the positive cells were more clearly identified in cells stained with ab119237 (**E**), compared to those stained with MAB6843 (**A**). In addition, the background fluorescence in untransfected cells was more in intense in cells stained with MAB6843 (**B**) compared with those stained with ab119237 (**F**). Scale bars are indicated in the figures: 400µm in low magnification images and 200µm in high magnification ones.



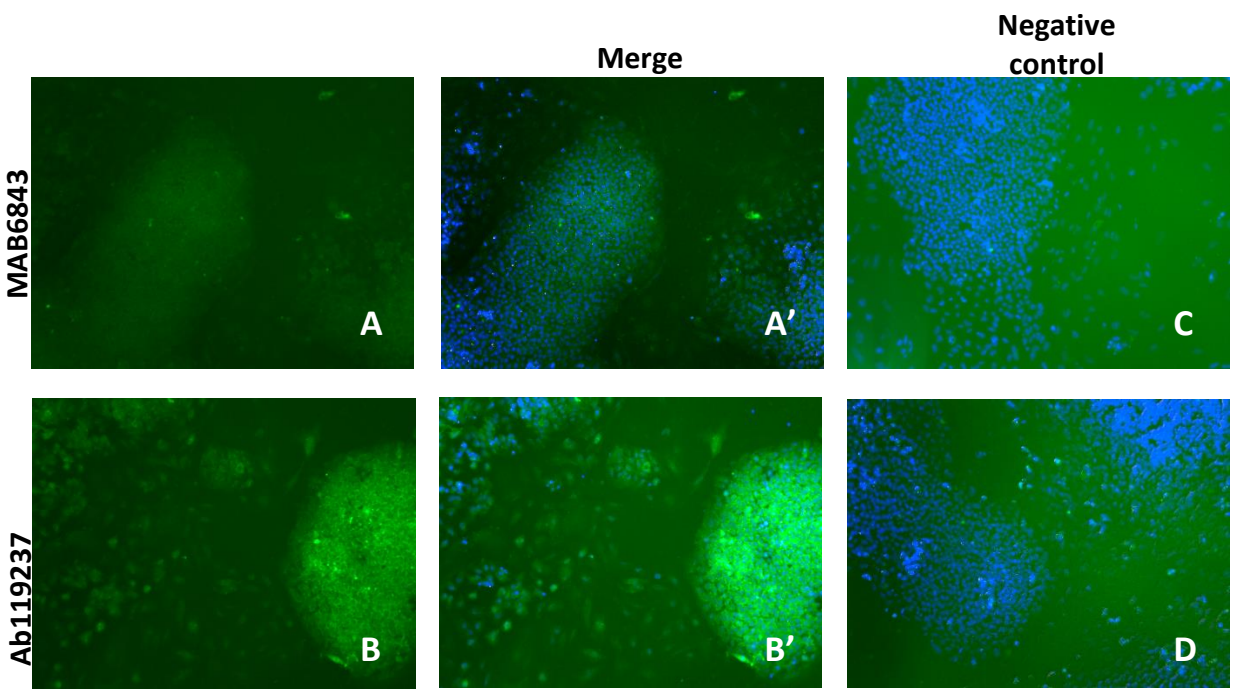
ab119237 (Abcam)

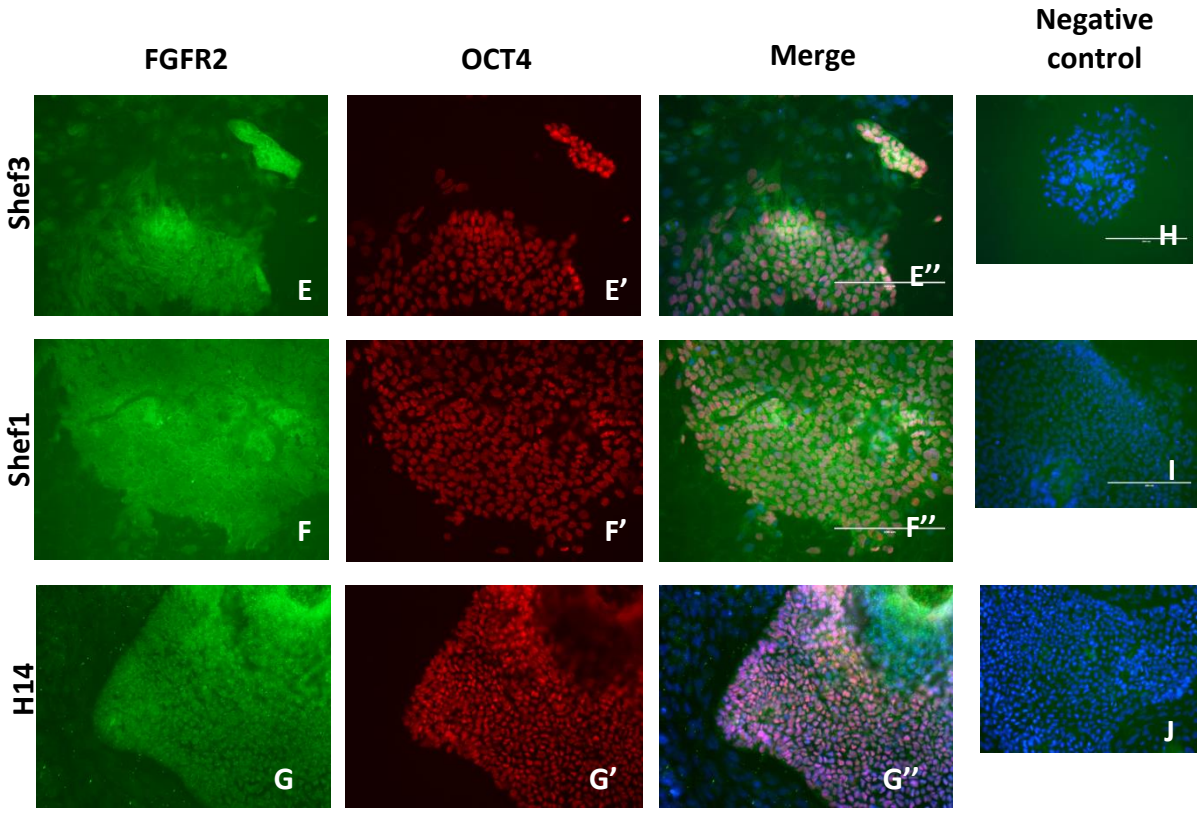


From this comparison, we were in a position of interrogate again if hES cells expressed FGFR2. First, we compared both antibodies in the hES cell line Shef1 (figure 6.4 A and B). The images show that the FGFR2 antibody (MAB6843) indeed had very low affinity for FGFR2 when compared with ab119237 FGFR2 antibody. Also with this experiment we corroborated the published information regarding FGFR2 expression in hES cells and our PCR results.

Although the morphology criteria to identify hES cell colonies is well accepted an robust, we confirmed the expression of FGFR2 in undifferentiated cells by double immunofluorescence with the hES cell marker OCT4 in the cells lines Shef3, Shef1 and H14 (figure 6.4 E, F and G respectively) .

Figure 6.4 **Undifferentiated hES cells express FGFR2 protein.** Shef1 cell line was stained with both FGFR2 antibodies: MAB6843 (**A , A'**) and ab119237 (**B, B'**). The signal was much stronger with the ab119237 antibody. This experiment and the one presented in figure 6.3 confirm that FGFR2 is expressed by hESCs and that it was not observed before due to the low affinity of the MAB6843 antibody. Negative controls for this immunofluorescence are shown in **C** and **D**. We confirmed this observation in the hES cell lines Shef3 (**E**), Shef1(**F**) and H14 (**G**) co-stained with the hES cell marker OCT4 (**E', F'** and **G'**). Merge images (**E'', F''** and **G''**) and negative controls (**H, I** and **J**) are shown for the corresponding cell lines stated before and indicated in the left part of the figure. Scale bars; 200µm


Shef1
Merge
Negative control
MAB6843
Ab119237
A
A'
C
B
B'
D

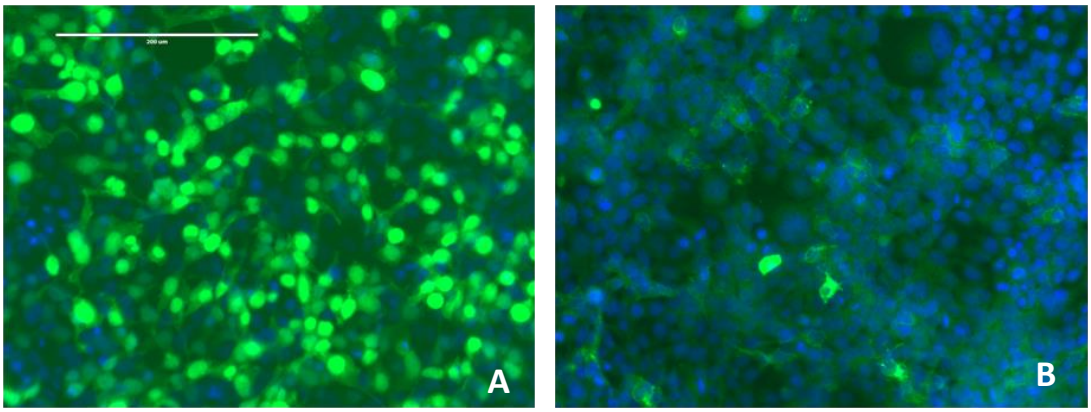

FGFR2
OCT4
Merge
Negative control
Shef3
Shef1
H14
E
E'
E''
H
F
F'
F''
I
G
G'
G''
J

In summary, we have established a control for the analysis of two antibodies to detect FGFR2 and concluded that MAB6843 has very low affinity. Later we have shown that hES cells express FGFR2 at the protein level and its isoform IIIb by QPCR (figure 6.1).

Robust expression of FGFR2IIIb can be achieved with the CAG promoter

A concerning aspect with the FGFR2IIIb transfection in 293 cells was the low expression of FGFR2IIIb compared with the GFP fluorescence normally observed with the pCAG vector (figure 6.5 A and B). Although some promoters may be more suitable than others for a particular cell type, CAG promoter has shown to drive strong expression in different mammalian cells (Miyazaki et al., 1989; Niwa et al., 1991). In addition, our current CAG vector has been shown to present robust and sustained expression in hESCs, with minimal silencing compared to other promoters like CMV (Liew et al., 2007). The pCAG vector is therefore, the one we commonly use in the centre to transfect hES cells.

Thus we aimed to subclone FGFR2IIIb gene from LTR vector into the pCAG vector to drive robust expression of this receptor during differentiation of hES cells.



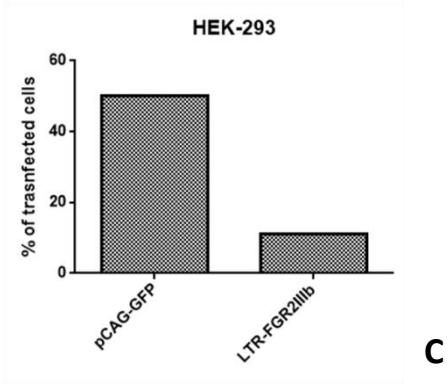


Figure 6.5 **pCAG-GFP vector drives more robust transgene expression than LTR-FGFR2IIIb vector.** HEK 293 cells were transfected with pCAG-GFP (**A**) and LTR-FGFR2IIIb vectors (**B**). 48hrs after transfection cells were stained with anti-FGFR2 (ab119237) and transfection efficiency determined (**C**). Although the expressed proteins are different and we cannot completely conclude that pCAG vector drives strongest transgene expression, the efficiency and the intensity of the signal was markedly different between both constructs. Also we did not observe an obvious cell death with the FGFR2IIIb transfection. Moreover, since the pCAG promoter vector has been shown to induce robust transgene expression without silencing in hES cells, we decided to subclone FGFR2IIIb downstream of the CAG promoter.

The LTR and CAG vectors with their main features are depicted in figure 6.6. Also a description of the strategy used for cloning plus the results is presented.

To make use of the CAG promoter is necessary to excise the EGFP cassette and substitute it with FGFR2IIIb. However, in the pCAG vector only two unique restriction sites can be found in the 5' end of the EGFP (XhoI is shown) limiting its plasticity for cloning. The LTR vector on the other hand, although contains multiple restriction sites at both extremes of the FGFR2IIIb, many of their sequences are also within the FGFR2IIIb insert. Fortunately, there was one compatible restriction site in both vectors in the 3' end, NotI. Therefore, the approach to follow was to digest the 5' end of both inserts, FGFR2IIIb and EGFP with KpnI and XhoI respectively. The cohesive ends generated in this way were blunted to make them compatible. Next, NotI digestion of the 3' end of both inserts generated cohesive compatible ends between FGFR2IIIb insert and CAG vector. When doing this procedure we found out there were extra KpnI sites in the LTR vector, which were not annotated in the vector map. As a result, after the double digestion, instead of having a single fragment corresponding to the FGFR2IIIb insert, we had 3 inserts of very similar size, making difficult to distinguish the one that contain our gene of interest (figure 6.6 B). Then, it was necessary to purify the different fragments and test them for cloning.

We tried different combination of ligation enzymes and bacterial strains, and at the end we were able of obtaining 3 colonies out of the ligation product between pCAG and smallest insert. Later on, we tested these new vectors by sequencing and transfection of 293 cells; none of them contained the FGFR2IIIb sequence (figure 6.6 C).

Although we kept trying to ligate the other 2 inserts into the CAG vector without any success, a PCR cloning strategy was considered (figure 6.6 D). In this case, amplification of the full length FGFR2IIIb is done with primers that contain restriction sites compatible with the recipient vector (XhoI and NotI in this case). Then, the PCR product and the vector are digested with the same pair of enzymes for further ligation. The advantage of the method is the high yield after PCR amplification and the certainty that the amplification product is the desired target. Our initial attempts to clone the vector in this way failed, but after many trials testing different reagents, strains and purification procedures, we obtained a large number of colonies with Ultragold strain (Stratagene)

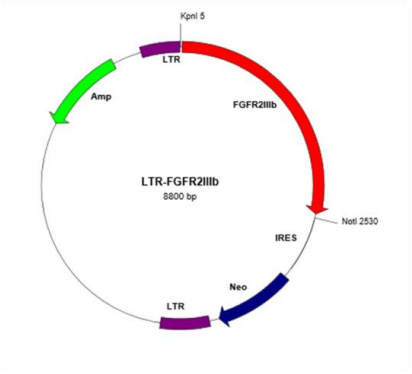
We verified that the cloned plasmids with different combination of restriction enzymes (figure 6.6 E). In addition, when HEK 293 cells were transfected with the new pCAG-FGFR2IIIb vector, a similar transfection efficiency to the one observed with the parental pCAG-GFP was observed, also the expression of the FGFR2IIIb was much stronger than the one driven by the LTR promoter in the original pLTR-FGFR2IIIb vector (figure 6.7).

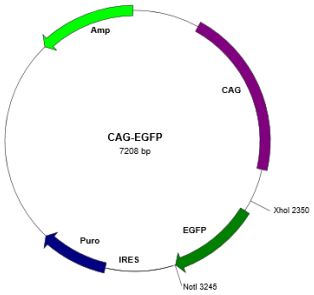
Finally, HEK-293 cells were transfected and phospho-ERK immunofluorescence was carried to test the functionality of the FGFR2IIIb protein, ruling out the possibility that during the cloning process a random mutation could have compromised the activity of the receptor (figure 6.8). ERK is often phosphorylated by FGFR activation.

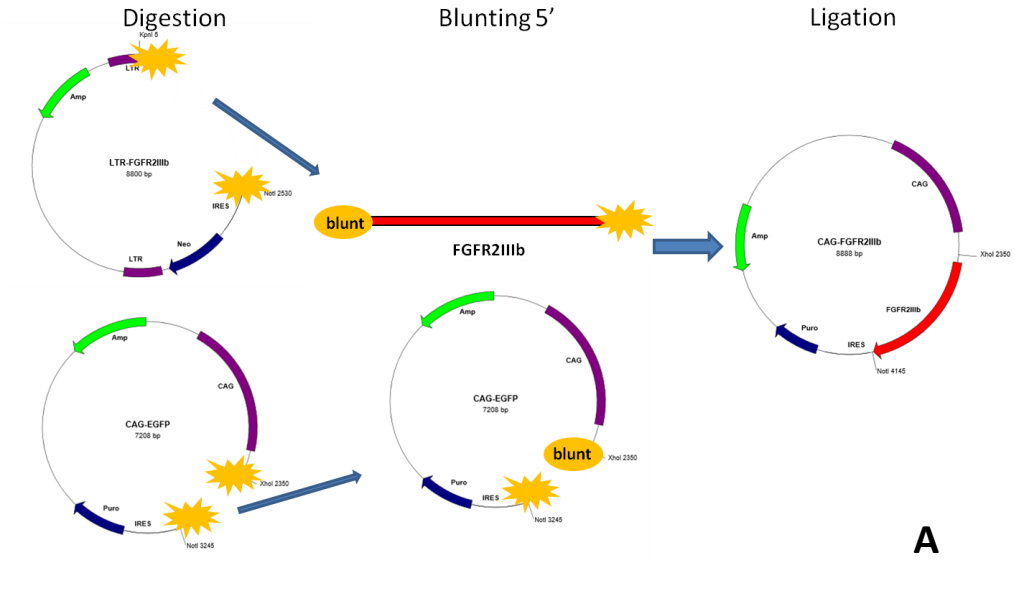
Figure 6.6 **FGFR2IIIb was subcloned into the pCAG-EGFP vector**. We aimed to remove the FGFR2IIIb cassette from the LTR plasmid and inserted downstream of the pCAG promoter to drive robust expression in hES cells. In A, top part of the figure shows the map of LTR-FGFR2IIIb and CAG-EGFP plasmids with their main features. They had a common restriction site in the 5' end of FGFR2IIIb and EGFP cassettes. In **A** below the vector maps, the strategy 1 for cloning is depicted. The 5' restriction site (NotI) was used to generate a compatible sticky end between FGFR2IIIb and CAG vector. In the 3' end no common restriction sites were found, therefore digestion followed by blunting was necessary to make the ends compatible. In **B**, inserts obtained after double digestion of LTR-FGFR2IIIb vector. We can observe that 3 inserts of similar size were purified, making difficult to determine which contained the FGFR2IIIb gene. In **C**, upon ligation, we could obtain 3 transformed colonies with the smallest digested fragment. These were later demonstrated to be negative for the FGFR2IIIb insert.

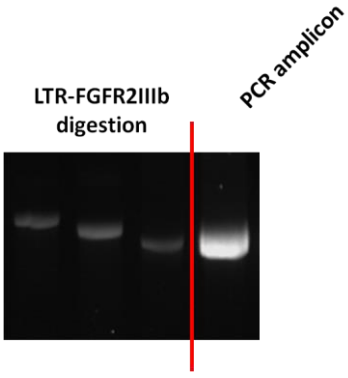
In **D**, second cloning strategy is shown; in this case FGFR2IIIb was amplified by PCR with primers containing restriction sites compatibles with the 3' and 5' ends of the CAG digested vector. Therefore, once the FGFR2IIIb is amplified, the product is digested and ligated with the vector. PCR product is shown in the forth lane of the gel shown in **B**. In **E**, XbaI-NotI digestion confirming that the FGFR2IIIb was inserted (bands indicated by the red arrow). CAG-GFP parental plasmid is presented in the gels to compare the pattern of bands of presumed positive plasmid. Our results were confirmed by sequencing.

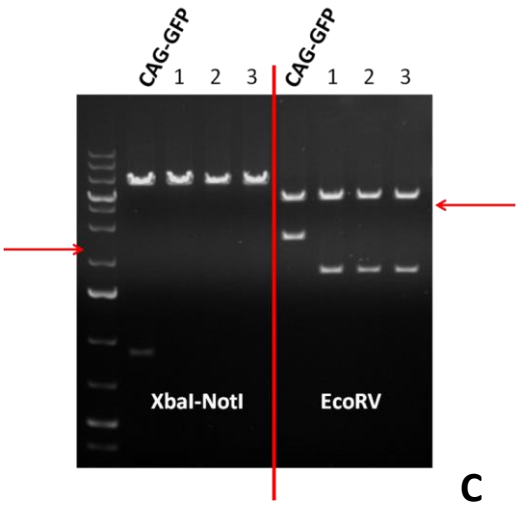
Cloning strategy 1



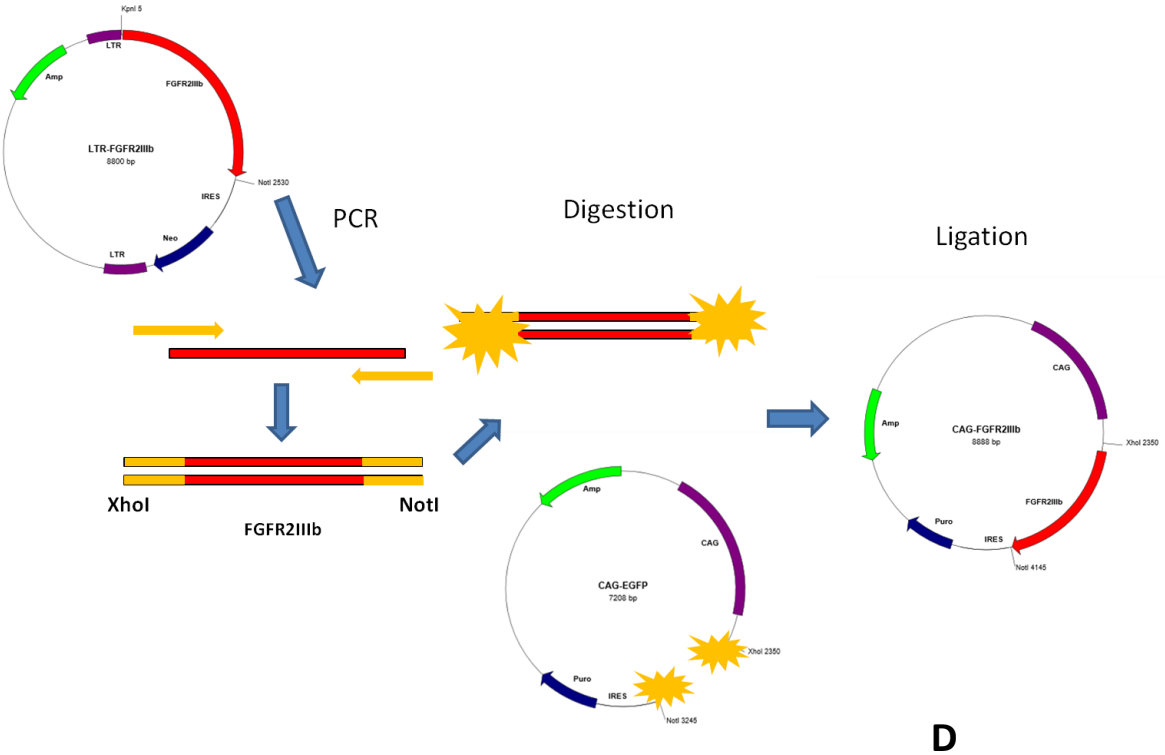




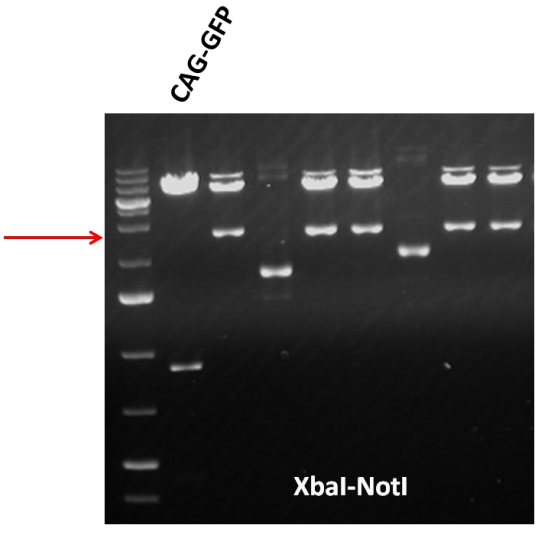




Cloning strategy 2



D



E

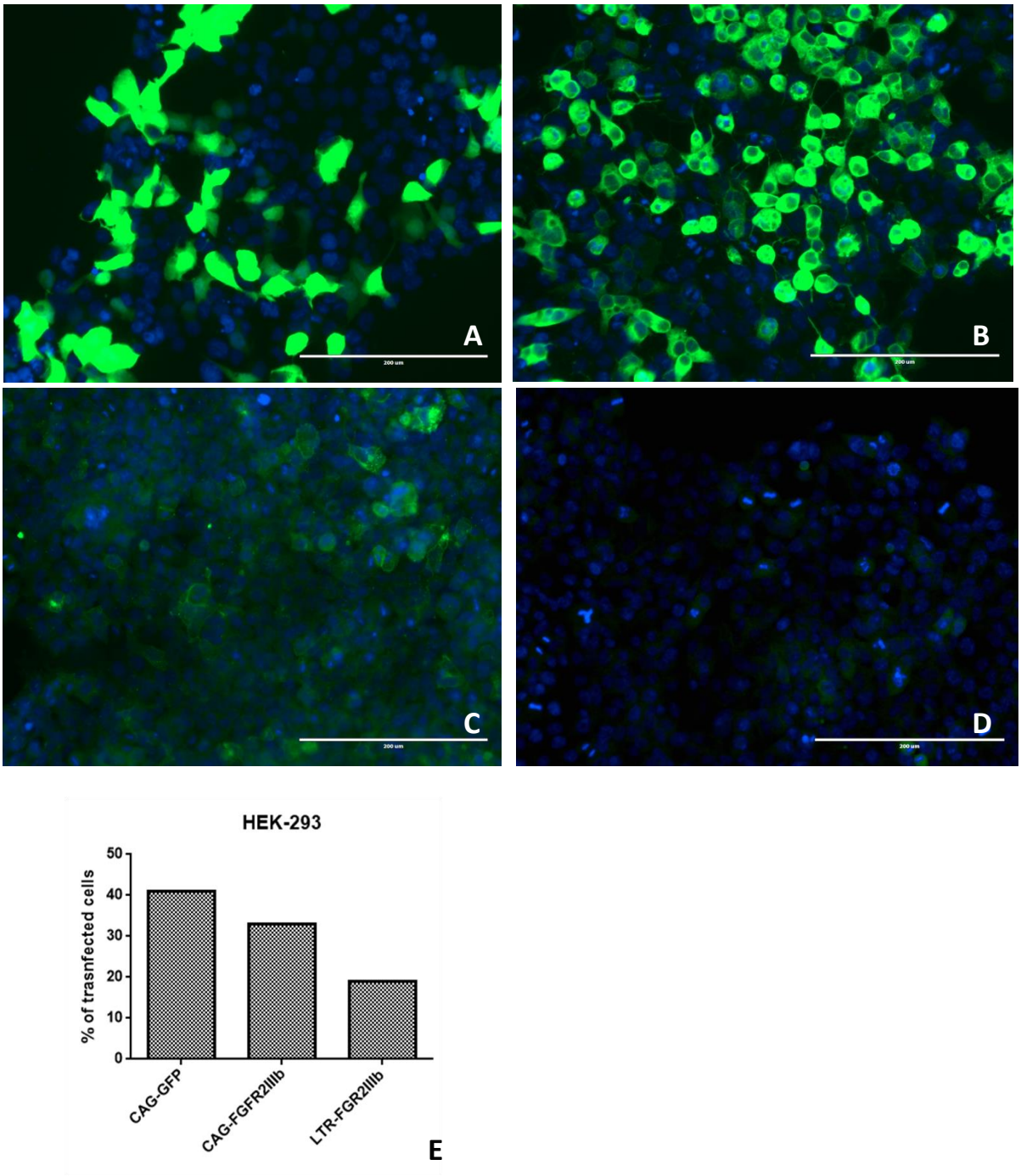


Figure 6.7 **The new pCAG vector induced more robust expression of FGFR2IIIb than the previous LTR vector.** HEK-293 cells were transfected with the new construct CAG-FGFR2IIIb (**B**), the parental plasmid CAG-GFP (**A**), and the LTR-FGFR2IIIb vector (**C**). Transfection efficiency was determined by immunofluorescence of FGFR2, 48hrs after transfection (**E**). Untransfected cells are shown in (**D**). The new CAG-FGR2IIIb plasmid induced robust expression of FGFR2IIIb protein, similar to the GFP induced by the CAG parental plasmid, and in striking contrast to the LTR vector. Scale bar; 200µm.

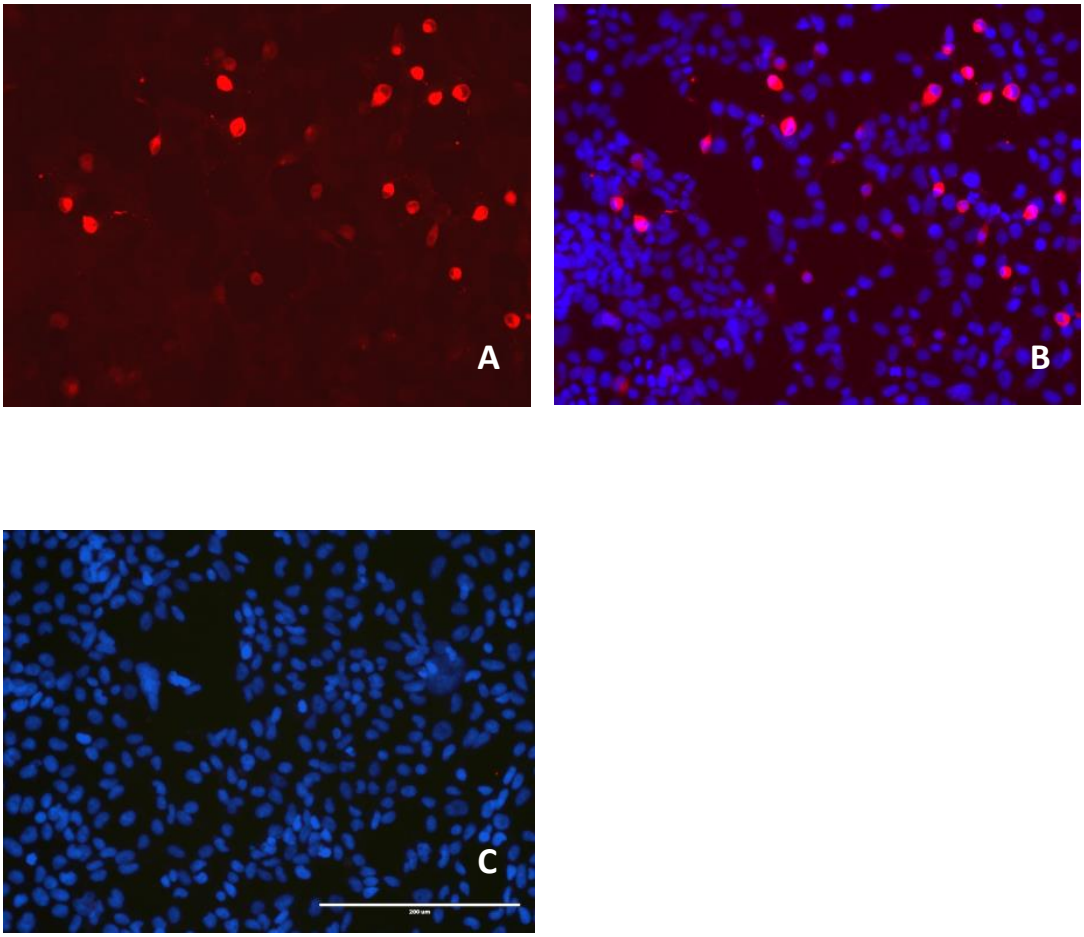


Figure 6.8 **FGFR2IIIb in transfected cells is functional.** In order to demonstrate that FGFR2IIIb is functional, immunofluorescence of phospho ERK (downstream target of FGFRs) was carried out. HEK-293 cells were transfected with CAG-FGFR2IIIb **(A** and **B**) and processed for immunofluorescence 48 hrs after transfection. Untransfected cells are shown in **C**. We can observe the expression of phosphor ERK in transfected cells but not in untransfected ones. Scale bar; 200µm

FGFR2IIIb overexpression in hESCs increases otic differentiation

Once this tool was ready, we aimed to test our hypothesis that FGFR2IIIb has a direct role during otic differentiation in hESCs. For this purpose however, there were many difficulties to overcome. The first challenge we encountered was the low transfection efficiency. In the past, different transfection methods have been tested and we have found that electroporation and lipofection yield similar efficiencies in hES cells (10-12%), contrasting with the common 40-50% observed in HEK-293 cells. Nevertheless, when doing stable cell lines, these levels of efficiency are not a problem since starting cell number is unlimited.

In our case, we cannot transfect hES cells with pCAG-FGFR2IIIb, since it is uncertain how the undifferentiated state will be affected. Therefore the transfection must be performed as soon as the cells start the induction process. In this regard, electroporation was the initial method of choice given the fast DNA delivery. In other words, cells can be transfected upon dissociation, just before the cells being transferred to the differentiating conditions.

We tried the electroporation method without any success, mainly because cell viability was seriously impaired by the method, and the additional problem of low seeding density (8000 cells/cm^2) increased the cell mortality. Therefore we decided to transfect with Lipofectamine LTX (Life technologies), a method less toxic for the cells. However, this and other reagents recommend to transfect cells at more than 60% confluence, something not attainable considering the low starting density needed for our differentiation experiments. An additional problem using lipofection is the speed of the process. Transfection is performed 1 day after seeding; we could miss a critical period in the differentiation process.

Having these potential problems in mind, we decided to carry out differentiation experiments with H14 and Shef3 cells. Cells were seeded at 8000 cells/ cm^2. The following day, transfection with CAG-FGFR2IIIb plasmid using lipofectamine LTX was carried out. Cells were differentiated during 6 days before being processed for QPCR analysis. As control for these experiments we used cells transfected with the CAG-GFP.

We monitored GFP cells during the 6 days course of the experiment, as an indirect way to confirm the presence of transfected cells. It is important to remember that both plasmids

are exactly the same with exception of the protein expressed (*GFP vs. FGFR2IIIb*). Figure 6.9 shows the results of these initial experiments in Shef3 cells. The results in H14 are not shown since only the DFNB control was completed, due to technical difficulties presented during the differentiation of FGF treated cells.

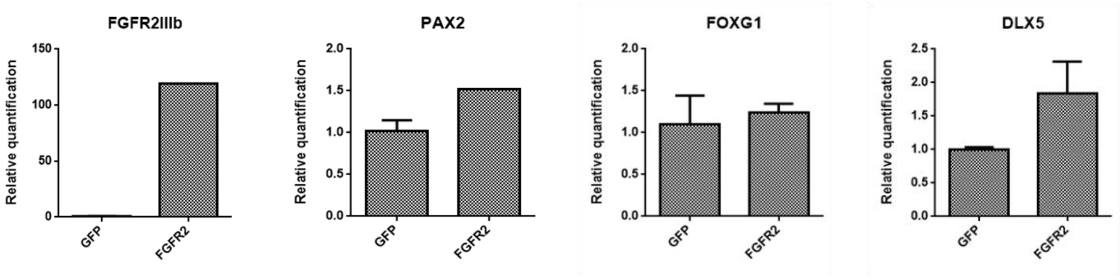
For the analysis, the DFNB and FGF parts of the experiment were separated and presented in different graphs. The purpose was to present more clearly the effect of *FGFR2IIIb* transfection compared with GFP one. We were not interested in the FGF vs. DFNB comparison at first place, and also, as will be presented in the discussion, a number of effects could be expected from the *FGFR2IIIb* transfection in FGF supplemented cells.

The results in figure 6.9 show that *FGFR2IIIb* expression was massively increased in FGFR2IIIb transfected cells compared with the GFP transfection, as expected. Regarding the otic markers, we observed a high increase in the expression of *PAX2*, followed by *FOXG1* in *FGFR2IIIb* transfected cells. DlX5 was in the other hand, downregulated. Importantly, this effect was only evident in the FGF treated cells, in agreement with the idea of Shef3 not secreting FGFs.

In the DFNB part of H14 (not shown), we observed that FGFR2IIIb transfection also induced *PAX2*, *FOXG1* and downregulated *DLX5.*

Although the results are interesting, there were some caveats with these two experiments. In both of them, the transfection efficiency was very low (5-10%) and the cell number just enough to extract some RNA, a situation that may produce some artifacts due to random representation of mRNAs during extraction.

DFNB



FGF3 and FGF10

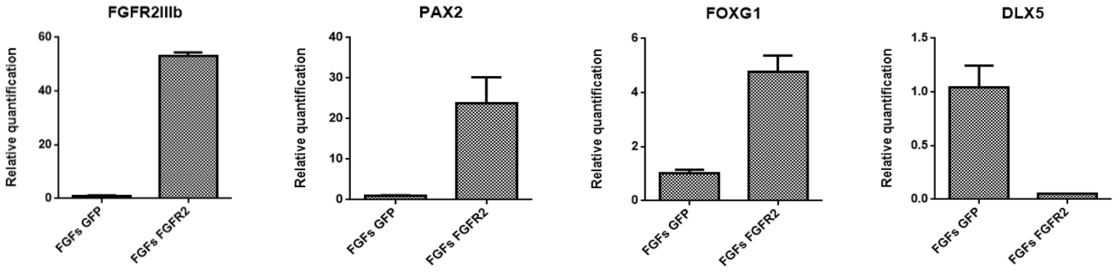


Figure 6.9 **FGFR2IIIb overexpression induces the upregulation of otic markers in Shef3 cell line**. Shef3 cells were plated at 8000 cells/cm². The next day cells were transfected with either CAG-FGFR2IIIb or CAG-GFP. They were maintained in differentiating condition during 6 days in DFNB medium (upper panel), or in FGF3 and FGF10 supplemented medium. Data was presented as relative expression taking as calibrator GFP transfected cells (defined as "1"). In this initial experiment *FGFR2IIIb* transfected cells upregulated the otic markers *PAX8*, *PAX2* and *FOXG1* in cells treated with FGFs compared with those transfected with GFP only. This is in agreement with the proposed role of this receptor in otic development. Error bars in this and the following experiments represent variation in PCR reactions within a single experiment.

In the next set of experiments, we decided to scale up the system so that we could obtain more material and potentially more representative data of the experiment.

The experiments were made in 3 cells lines; Shef3, Shef1 and H14. The cell density was deliberately scaled up to 24000 cells/cm² to make transfection more robust, with the downside that increasing cell density could affect the differentiation of cells but in any case reveal the role of FGFR2IIIb in our system.

In these set of experiments we obtained a large amount of material but transfection efficiency was as low as in previous ones (5-10% in all the cell lines). The results showed the same tendency than before, but the size the upregulation of otic markers in FGFR2IIIb transfected cells was lower (not shown).

We think that cell density could adversely affect otic differentiation and dilute the effect of *FGFR2IIIb* overexpression in the system. This problem could be compounded by the low transfection efficiency also observed at high density. Nonetheless, the fact that the same tendency was observed and that the absolute number of green cells was increased was encouraging.

Another aspect that has not been targeted in these experiments was the non-cell autonomous effect. It could be that increasing the expression of FGFR2IIIb does not lead to an upregulation of otic markers in those cells, but rather induce the expression of a second ligand.

We were in a position to perform the overexpression in a cleaner and more robust manner, and in the next set of experiments cells were seeded at 24000 cells/cm², transfected and immediately selected with Puromycin for 48hrs. In this manner, a high proportion of green cells at 6 days of differentiation were observed in the control.

The results of these experiments are presented in figure 10, 11 and 12 corresponding to Shef3, Shef1 and H14 respectively. The expression of the *FGFR2IIIb* is not presented in the figures, but it was robustly induced by the FGFR2IIIb transfection.

In Shef3 DFNB medium we can observe that *FGFR2IIIb* overexpression had little effect on the expression of otic markers. *FOXG1* was upregulated and *PAX2* was downregulated while

no change in *PAX8* or *DLX5* was observed. The reason for the downregulation of *PAX2* is not clear to us. On the other hand, when FGFR2IIIb was overexpressed in cells differentiated in FGF supplemented medium, strong upregulation of *PAX8* was observed, followed by *FOXG*1 and *PAX2*. These results agree with the tendencies observed in other experiments with Shef3 (figure 6.9). More important, the results add further support to the proposal that Shef3 cell line has a poor level of endogenous differentiation (in DFNB medium) due to low production of FGFs. So, even when FGFR2IIIb is overexpressed, this is not activated in cells in DFNB. In the contrary when FGF3 and FG10 are added, a more robust otic induction is observed in *FGFR2IIIb* transfected cells compared with *GFP* transfected ones.

Shef3

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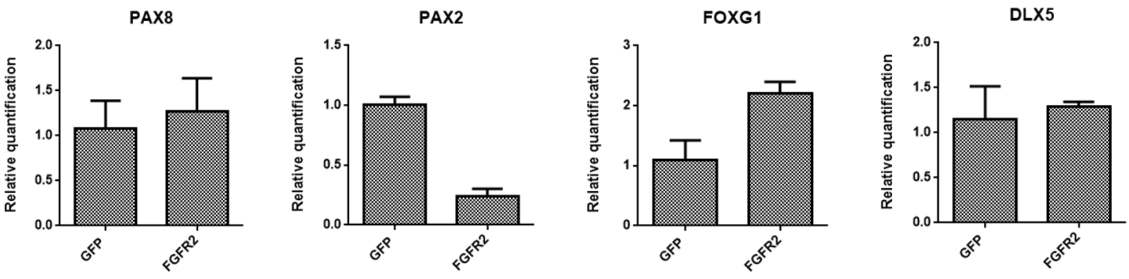

FGF3 and FGF10

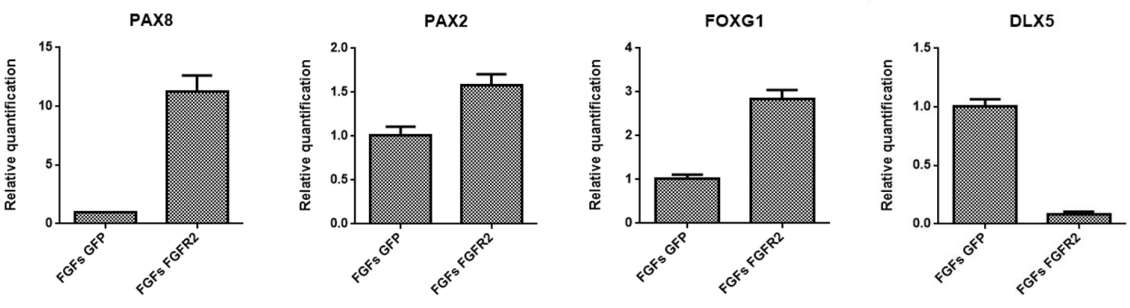

Figure 6.10 **FGFR2IIIb directly induces the upregulation of otic markers in Shef3 cell line**. Shef3 cells were plated at 24000 cells/cm². Transfection with either CAG-FGFR2IIIb or CAG-GFP took place the following day. Next day, Puromycin at 0.5µg/ml was added and removed two days later, and cells were kept for another two days (6 in total) in differentiating conditions either in DFNB medium (upper panel), or in FGF3 and FGF10 supplemented medium (lower panel). Data was presented as relative expression taking as calibrator GFP transfected cells (defined as "1"). As in the previous figure, we observed again that FGFR2IIIb overexpression induced the upregulation of otic markers (PAX8, PAX2 and FOXG1) in FGF medium. In this case the upregulation of otic markers most likely comes from transfected cells since the puromycin selection step kill almost all the untransfected cells.

In Shef1 upregulation of *PAX8, PAX2* and *FOXG1* was induced by *FGFR2IIIb* overexpression. However this trend was only observed in DFNB medium. Also in previous chapter it has been suggested that this cell line differentiates even in the absence of FGF supplementation.

In this experiment, in FGF treated cells, *FGFR2IIIb* overexpression only upregulates *PAX2*, there are no changes in *FOXG1* and *PAX8* is downregulated. Regarding *PAX8*, it is one of the genes that is already upregulated in FGF-GFP transfected compared with DFNB-GFP. Thus it could be that this level of expression is the maximum that can be induced by FGF signalling and further FGF activity may just result inhibitory. This idea has already been discussed in previous chapters.

Shef1

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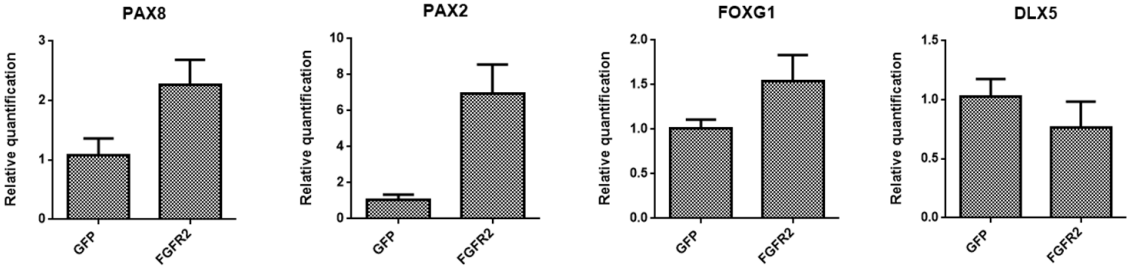


FGF3 and FGF10

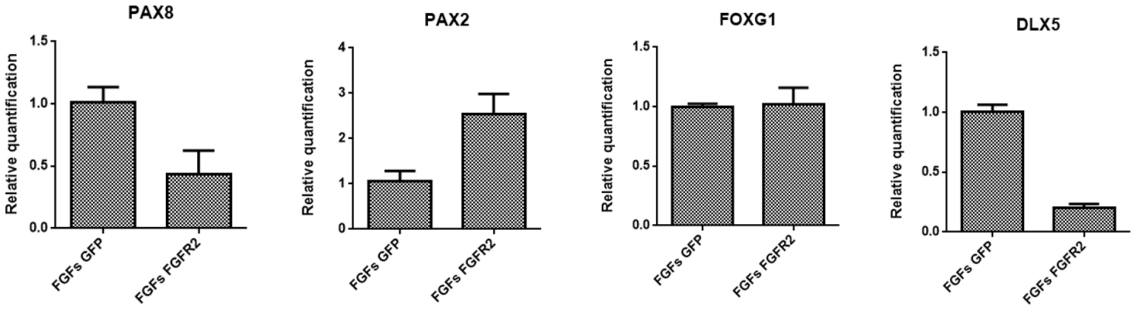


Figure 6.11 **FGFR2IIIb directly induces the upregulation of otic markers in Shef1 cell line**. Shef1 was differentiated in the same as She3 in figure 6.10. Data was presented as relative expression taking as calibrator GFP transfected cells (defined as "1"). As in the previous figure, we observed that FGFR2IIIb overexpression induced the upregulation of otic markers *PAX8, PAX2* and *FOXG1* in DFNB medium. While this time in FGF supplemented medium (lower panel) only *PAX2* was upregulated by FGFR2IIIb transfection. DLX5 has been seen downregulated by FGFR2IIib overexpression in all the cells lines. These results confirm our previous finding in Shef3 transfected cells.

In H14, we did also observed a positive effect of FGFR2IIIb overexpression in the upregulation of otic markers. *PAX8*, *PAX2* and *FOXG1* were all massively upregulated by in *FGFR2IIIb* transfection in cells differentiated in DFNB and FGF medium. In the case of *PAX8*, the apparent smaller upregulation induced by FGFR2IIIb in FGF medium, was probably due to the already upregulated level of this transcription factor by the FGF treatment. In other word, there are limits to the expression of these genes in response to FGF signalling activity.

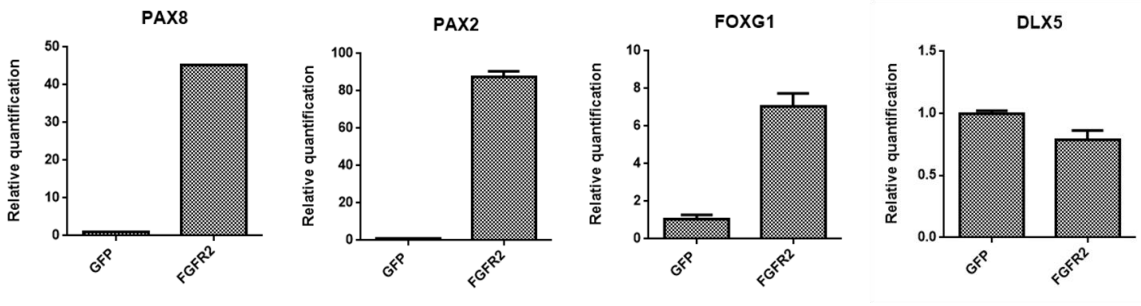
It was interesting to notice that in previous standard differentiation experiments with H14, we never observed the upregulation of any of the otic markers in FGF treated cells.

All together these data indicates that *FGFR2IIIb* is able to induce the expression of otic genes above the level induced by the simple FGF3 and FGF10 treatment. In those cell lines where spontaneous otic differentiation takes place in DFNB medium, FGFR2IIIb overexpression further increase the level of otic markers. In addition, these results help in the conciliation of the differences observed between cell lines, putting them together respect their response to an otic inducing factor, the *FGFR2IIIb*.

In all the cell lines we observed that *FGFR2IIIb* overexpression always caused the downregulation of the otic marker *DLX5* in FGF treated cells. This and other results will be covered in more detail in the discussion.

H14

DFNB



FGF3 and FGF10

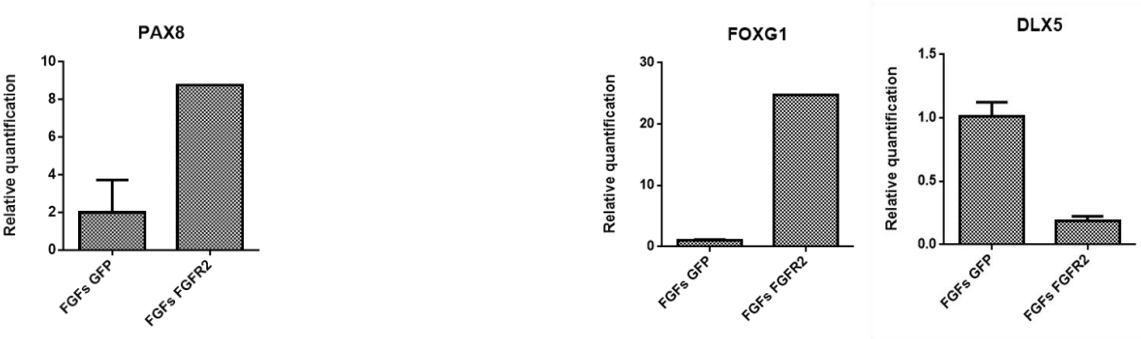


Figure 6.12 **FGFR2IIIb directly induces the upregulation of otic markers in H14 as well.** H14 was differentiated as the other cell lines. The data was presented in the same way. In the upper panel, cells in DFNB medium; in the lower, cells in FGF3 and FGF10 supplemented medium. *FGFR2IIIb* overexpression induced the expression of *PAX8*, *PAX2* and *FOXG1* as in the other cell lines. *PAX2* graph was removed from the FGF treated condition because the low RNA yield did not allow reliable amplification. DLX5 was downregulated in this experiment as well. Together, the results with the overexpression system add further support to the model of otic induction triggered by FGF signalling.

Discussion

FGFRs in human embryonic stem cells

This chapter covers mainly the technical challenges involved in the characterization of antibodies and in the standardization of an overexpression system for differentiating hESCs. Although this characterization is a time laborious process, it does not add any particular insight to the stem cell or inner ear research field. Nonetheless, it adds valuable technical information to the lab, and it should not be underestimated. In the future, the experience attained here should help in the solution of similar problems as the ones described in this chapter.

Apart from the technical challenges, we have also gained an important insight into the role of FGFR2IIIb during the differentiation of hESCs into otic progenitors.

We have observed that pluripotent stem cells expressed *FGFR1, FGFR3* and *FGFR2IIIb* in undifferentiated conditions (figure 6.1). Other studies have shown before the expression of these receptors in hESCs (Ding et al., 2010; Dvorak et al., 2005; Ginis et al., 2004; Sato et al., 2003) although little is known about the specific role that these receptors have in undifferentiated hESCs. In this regard, different FGFs have been shown to maintain ERK activation and NANOG expression in hES cells (Chen et al., 2012). Importantly, it is known that those FGFs act through different receptor isoforms; therefore it would seem that their activity is redundant in hESC maintenance.

The three FGFRs we have observed in undifferentiated conditions have been shown to be expressed at different stages of inner ear development in the mouse, and mutants for these receptors have inner ear defects. However the *FGFR2* and the isoform *IIIb* mutant mice are the ones with the most robust otic abnormalities, in agreement with their early expression pattern and with the FGF3 and FGF10 otic induction model. For these reasons, we focused our attention in FGFR2IIIb.

Based on the literature and our PCR results (figure 6.1) we were confident that hESCs presented the appropriate receptors to respond to the FGF3 and FGF10 treatment.

However, it was important for us to count with a reliable antibody to detect FGFR2IIIb, since we were planning to optimize an overexpression and downregulation systems. In addition, we have thought to use FGFR2IIIb as a tag for cell sorting in hESCs. In previous chapters (3 and 4) the heterogeneity in undifferentiated hESCs has been discussed, and we thought that FGFR2 expression could be one of those factors heterogeneously expressed. If that was the case, it would be possible to sort an FGFR2+ population more responsive to the FGF3 and FGF10 treatment, and potentially improve the otic differentiation. King et al (2009) recently found that there is a population CD133+ cells in the hESC niche, and this subset retained CD133 expression through passaging. More important, they found that the CD133+ cells differentiate towards ectoderm exclusively, while the CD133 negative gave produced cells of all germ layers (King et al., 2009).

In our analysis we had to rule out the flow cytometry results with FGFR2 antibody (MAB6843), since it was later demonstrated that those negative results came from the low affinity of this antibody. On the hand, after optimization with a second FGFR2 antibody, we proved that this receptor was expressed in hESCs as expected. Although the results in figure 6.4 showed a uniform expression FGFR2 in undifferentiated colonies, a more sensitive technique like flow cytometry could tell if FGFR2 marks different subsets. In this regard FGFR1 has been found to be heterogeneous in the hES cell niche (Carpenter et al 2003).

In our protein analysis we focused on the FGFR2 expression rather that the FGFR2IIIb isoform due to the antibodies available for this specific isoform showed less convincing set of data validation, but in the future, the specific isoform FGFR2IIIb expression can be analysed, especially considering robust positive control now available for its characterization.

Establishing an overexpression system

Overexpression and knockout are powerful approaches to study gene function. We choose to start with the overexpression system since we wanted to drive hESCs towards the otic fate. In other words, we don't know "how otic our differentiated cells are?", and for this

reason, trying to overexpressed a gene that is presumed to participate in inner ear development is more reasonable than knocking it out. Also, from the technical point of view, complete ablation of a gene by homologous recombination in hESCs is very difficult (Leavitt and Hamlett, 2011); they are hard to transfect (10-12% transfection efficiency in our hands), they do not survive single cell culture, and homologous recombination is extremely rare. In the original report from Zwaka and Thomson (2003), only 7 colonies out of 15 million cells were shown to be recombined specifically in the HPRT locus (Zwaka and Thomson, 2003).

An additional problem comes when the purpose is to study a gene presumed to be involved in a specific differentiation pathway; this applies for overexpression or knockout approaches. We must be sure that the targeted gene is only expressed in differentiated cells; otherwise long term characterization of undifferentiated hESCs is required. In these circumstances, inducible systems are very powerful molecular tools. The tet-on/off system originally developed by Gossen and Boujard (Gossen and Bujard, 1992) has been successfully used in mouse embryonic stem cells to change the level of Oct4 in tetracycline concentration dependant manner (Niwa et al., 2000). In the Centre, the dox inducible system has been used to knockdown OCT4 as well but through the expression of short hairpin (Zafarana et al., 2009). Another example from the utility of inducible transgenes comes from Jenish lab (Wernig et al., 2008), where they produced mouse iPS from different tissues through doxycycline exposure, in transgenic mice carrying a polycistronic dox inducible cassette to express the reprogramming factors.

We considered establishing an inducible system to overexpress FGFR2IIIb, but it is difficult to generate stable clones, and these could present a different behaviour from their parental hES line. In these circumstances, a transient expression approach could be more practical and easier to carry out.

For the overexpression, the vector LTR-FGFR2IIIb proved to be useful in 293 cells, but compared to the pCAG, the expression was at much lower level. In this regard the CAG has been show to drive robust expression in wide many mammalian cells in contrast to LTR (Miyazaki et al., 1989; Niwa et al., 1991). In addition the current version of the pCAG vector we use to express GFP, was developed in Centre for stem cells biology and carries polyoma

virus enhancer element that has been show to avoid silencing normally observed in hES cells transfected with vector containing different promoters. Although we wanted our vector for transient transfection, suppression of transgene promoters has been determined to be extremely high even in short term studies (5 days) in hES cells (Xia et al., 2007).

In light of these findings we thought it was important to subclone the FGFR2IIIb from the LTR promoter into the pCAG promoter, excising in this manner the EGFP cassette. Doing this, we confirmed in 293 cells that FGFR2IIIb expression was much stronger with the newly generated pCAG construct compared with the faint expression observed in the LTR-FGFR2IIIb construct (6.8 B and C), also transfection efficiency rise from 19% with LTR to 38% with the pCAG-FGFR2IIIb.

FGFR2IIIb induces the expression of otic markers in hESCs

We have already described the effect of FGFR2IIIb overexpression in hESCs in the results section. I observed a marked upregulation of *PAX8*, *PAX2* and *FOXG1* in *FGFR2IIIb* transfected cells as it was hypothesised. This effect was noticeable in all the cell lines in DFNB and FGF supplemented medium. Perhaps, the only exception was FGFR2IIIb transfected Shef3 cells in DFNB medium. This difference is in complete agreement with the result described in chapters 3 and 4 about the behaviour of this cell line in DFNB medium. It does not produce FGFs, and it does not have spontaneous otic differentiation if FGF3 and FGF10 are not added.

On the contrary in other cell lines (Shef1 and H14) that have a default high level of differentiation in DFNB medium (potentially through the secretion of FGFs), FGFR2IIIb overexpression induces the upregulation of otic markers in DFNB medium.

Another remarkable fact in all the experiments was the downregulation of DLX5 by FGFR2IIIb overexpression. This phenomenon only occurred in FGF treated cells in all the lines. In the otocyst different subdomains have been defined (Fekete and Wu, 2002). FGFR2IIIb is expressed in the most dorsal region while FGF10 is observed more ventrally (Pirvola et al., 2000). The same relation can be drawn for *Dlx5* and *Pax2* (Robledo and Lufkin,

2006; Streit, 2002), being *Dlx5* more dorsally localized. Now, in this scenario we would expect that *FGFR2IIIb* overexpression to enrich its own domain, the dorsal one. We are observing exactly the opposite; *PAX2* is upregulated while *DLX5* is downregulated. This could be a difference in the human than in the mouse. If this is the case, it will be necessary to confirm this by looking at the expression of markers for the different subdomains in the otocyst. An alternative hypothesis is that FGF signalling is arresting the cells at an early stage in the differentiation path. Freter et al (Freter et al., 2008) showed that over expression of FGF in the chick ectoderm increased the *PAX2* domain but it blocked the following stages of otic development that were assessed by the expression of otocyst markers like *NKX5.1*. In this work they showed that sustained FGF signalling impaired the progression of otic induction (Freter et al., 2008). This would make sense with one of the interpretations proposed in chapter 4 for the FGF signalling inhibition experiments, suggesting that if FGF signalling is inhibited the differentiation is delayed, while in these set of experiments, *FGFR2IIIb* overexpression increased the transcription of otic genes and it could also impede the progression of the cells to more advances stages of otic differentiation.

These insights add further support to the FGF-induced otic differentiation model, originated from *in vivo* studies. It also strengthens our proposal that otic differentiation *in vitro* in a human system shares many similarities with the different animal models. In addition, it conciliates the disparate behaviours observed in different cell lines in chapter 3, by putting them together here, in their response to an otic induction mediator as FGFR2IIIb.