Chapter 3

FGF3 and FGF10 induce the expression of otic markers in human pluripotent stem cells

Introduction

FGFs during inner ear induction

The developmental events and the molecular cues involved in the formation of the inner ear have started to be revealed. It is clear that FGFs are between the main otic-inductive molecules in different animal models. In the case of the Zebrafish, Maroon et al (2002) used antisense morpholinos to prove that fgf3 and fgf8 from the hindbrain are necessary for the induction of the otic placode (Maroon et al., 2002). They showed that co-injection of both morpholinos or inhibition of FGF signalling with the small molecule SU5402 were able to block the expression of the early otic markers *dlx3, pax8* and *pax2.1*. They further demonstrated that the observed effect arose from an impaired induction process since proliferation and cell death were not affected in the presumptive otic region. These results were in agreement with the study of Phillips et al (Phillips et al., 2001), who used a *fgf3*-morpholino injection in an *fgf8* mutant background to show that both fgfs play a redundant role during otic placode induction, and in their absence, the expression of *pax8* and *pax2.1* was strongly decreased. Moreover, conditions that expand the endogenous *fgf3* and *fgf8* expression domain also expanded the otic placode domain characterized by *pax8*, and later generated supernumerary otic vesicles within that domain, suggesting these ligands were sufficient to trigger the first stages of otic development.

In the chick and the mouse FGF signalling has been demonstrated to be essential for the formation of the otic vesicle, FGFs from the mesoderm and ectoderm cooperate to induce the formation of the otic placode in a region of the ectoderm adjacent to the hindbrain. In the chicken for example, mesodermal *FGF19* has been identified as the responsible molecule to act directly in the ectoderm to induce additional signalling molecules with which it cooperates to induce the formation of the otic placode (Ladher, 2000). However, the cascade of signalling events that lead to the formation of the otic placode starts even before *FGF19* appears. *FGF8* expression in the endoderm marks the start of otic induction signals in both the chicken and the mouse (Ladher et al., 2005). In this work they show that siRNA targeted to *FGF8* is able to block mesoderm *FGF19* expression and the future otic

placode formation in the chick, indicating the requirements for this particular FGF upstream of FGF19. Moreover, in this work it was also shown that the activity *Fgf8* during otic induction was conserved in the mouse, since *Fgf8* hypomorphic/*Fgf3* mutant mice did not form otic vesicles and the expression of otic markers in the presumptive otic placode was absent. However *Fgf8* expression in the mouse is more complex than in the chicken, and its specific role has been difficult to dissect since *Fgf8* null mouse present early embryonic lethality and transgenic lines missexpressing *Fgf8* are also lethal (Alvarez et al., 2003; Meyers et al., 1998). The mesoderm FGF signal involved in inner ear induction is another difference between the mouse and the chicken development. For example, as already mentioned FGF19 is the direct inducer of otic development in the chick, but its homologue in the mouse FGF15 does not have any role during otic induction (Wright et al., 2004). Instead, *Fgf10* expressed in the mouse mesoderm is the one that cooperates with *Fgf3* to trigger inner ear development. This has been clearly demonstrated in the studies of Wright and Mansour (Wright and Mansour, 2003) and those from Shimmang's group (Alvarez et al., 2003; Zelarayan et al., 2007). They found that double mutant mice for *Fgf3* and *Fgf10* don't form otic vesicles and if they do, those are pretty small and lack the expression of the early otic markers *Pax2, Gbx2* and *Dlx5* (Wright and Mansour, 2003). It seems that both FGF signals are redundantly required since no phenotype is observed in the single mutant of either of those Fgfs. Alvarez et al (Alvarez et al., 2003) also concluded FGF3 and FGF10 were playing a redundant role in otic induction based on that their initial *Fgf3* mutant didn't have an otic phenotype. To assess the possible contribution of other FGF ligands, transgenic lines missexpressing *Fgf3*, *Fgf10* and *Fgf2* were later developed. Only *Fgf10* missexpression caused the production of ectopic otic vesicles, expressing the characteristics otic marker with the exception of *Pax2*, which agrees with Wright and Mansour work (2003). FGF10 would therefore appear to have a behaviour equivalent to FGF19 in the chick, while FGF3 is necessary for otic induction in all the animals models described so far, although redundantly with FGF10 in the mouse.

Our differentiation protocol: a developmental biology approach

These important insights from developmental biology constitute the basis of our current protocol to differentiate hES cells into otic progenitors characterized be the expression of *PAX8, PAX2, FOXG1* and *DLX5.* Our protocol has several advantages over other protocols discussed in the introduction (chapter 1). For example we use a completely defined media (DMEM:F12 plus N2 and B27 supplements) to differentiate cells. Also, our cells are seeded at low density as a monolayer, so that cell to cell contact and media conditioning, two confounding factors, are minimized. This protocol contrast to the one used by Oshima (Oshima et al., 2010) who also produced otic progenitors from mouse ESCs, characterized by expression of *Pax2*. In their approach, progenitors were produced through two stages: in the first one they enriched the proportion of neuroectoderm during embryoid body differentiation, and in the second one, bFGF was applied to instruct neuroectoderm to differentiate towards the otic lineage. *In vivo*, the FGF signals that trigger otic development can only act at a very specific developmental window in a region of the ectoderm that resides between the neural and epithelial ectoderm (Martin and Groves, 2006), called the preplacodal domain. Therefore, in Oshima's work, they tried to mimic the normal developmental path before otic induction by producing ectoderm using embryoid bodies. This is a procedure commonly used with mouse ESCs and when they are left to aggregate, they form spheroid structures that resemble 5 day mouse embryos which gives rise to all germ layers in a couple of days (Doetschman et al., 1985; Martin, 1981; Martin and Evans, 1975). In these circumstances, it is necessary to block undesired germ layers within the embryoid body to enrich for neuroectoderm formation. Oshima et al (2010) did that by blocking TGFβ and WNT pathways involved in mesendoderm formation (Gadue et al., 2006; Schuldiner et al., 2000) plus adding IGF to produce head ectoderm at the expense of trunk (Pera et al., 2001). Our protocol does not appear to require an ectoderm enrichment stage, and FGFs are applied as soon as the cells are dissociated from the hES cell niche. It has been proposed that cells of the early embryo have a default tendency to differentiate into neuroectoderm (Munoz-Sanjuan and Brivanlou, 2002), and that this tendency is abolished by activation of TGFβ signalling due to BMP in the developing embryo; in this context the organizer secretes BMP inhibitors that set up the start of neuroectoderm development.

Therefore, in order to see the default tendency of cells towards the neuroectodermal lineage it would be necessary to abolish BMP activity occurring within the embryo. When human ES cells are dissociated and plated at low density instead of being aggregated in embryoid bodies, BMP activity is reduced and cells differentiate into neuroectoderm in the absence of external growth factors (Tropepe et al., 2001; Ying et al., 2003), confirming the default model. It is interesting to mention that in this manner more than 80% nestin+ neuroectodermal stem cells are observed as early as 4hrs after seeding under low density conditions (Tropepe et al., 2001). This population of "primitive ectodermal progenitors" originated as part of a default trend, is most likely the one that is competent to respond to FGF3 and FGF10 in our protocol, alleviating the need of ectoderm enrichment seen in Oshima's protocol.

Thus, the purpose of this chapter was to characterize the response of human induced embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) when differentiated in the presence of FGF3 and FGF10. This initial characterization is important for the further interpretation of the results when studying the different aspects of FGF-mediated otic induction. Although we took as a model Shef3 cell line for most of this thesis, we did not ignore the behaviour in other cell lines. A very important aspect when working with hESCs as it is known that there is variation across cell lines (Osafune et al., 2008).

Results

Otic induction by FGF3 and FGF10

Continuing with previous work in the lab, we first asked if FGF3 and FGF10 treatment could induce the expression of early otic markers genes (e.g *PAX8, PAX2, FOXG1*) in the hESCs. We first looked at Shef3 cell line since there was previous evidence indicating these cells differentiated into otic progenitors, and some Shef3 transgenic lines that we anticipated were to be useful for our project, had been already generated in the CSCB. The cells were trypsin-dissociated and differentiated for 12 days in a chemically defined medium composed of DMEM, F12 plus N2 and B27 supplements (DFNB), to which FGF3 and FGF10 were added. The growth of the cells was monitored for the length of the experiment (figure 3.1 A). It was hypothesised that cells would grow faster when exposed to FGF3 and FGF10 when compared with those seeded in DFNB medium only. Cells were collected on day 12 of differentiation and the expression of the main otic markers (*PAX8, PAX2* and *FOXG1*) was quantified by Q-PCR.

The results are presented as relative expression taking as calibrator undifferentiated cells (hES media in figure 3.1) or cells differentiated in DFNB without FGFs (rest of the figures).

FGF3 and FGF10 treatment strongly induced the upregulation of the main otic markers *PAX8*, *PAX2* and *FOXG1* in Shef3 cell line (figure 3.1 B, C and D). In addition, the cells presented robust proliferation under these conditions compared with cells grown in DFNB control (figure 3.1 A). These results confirm our hypothesis that FGF3 and FGF10 are important inducer of an otic fate in our *in vitro* system, in agreement with the animal models.

Interestingly, despite the clear difference observed between cells in DFNB and those treated with FGFs, a variety of cell morphologies was found in both conditions (figure 3.1 E), indicative the heterogeneity of the system even when cells are differentiated under these stringent conditions.

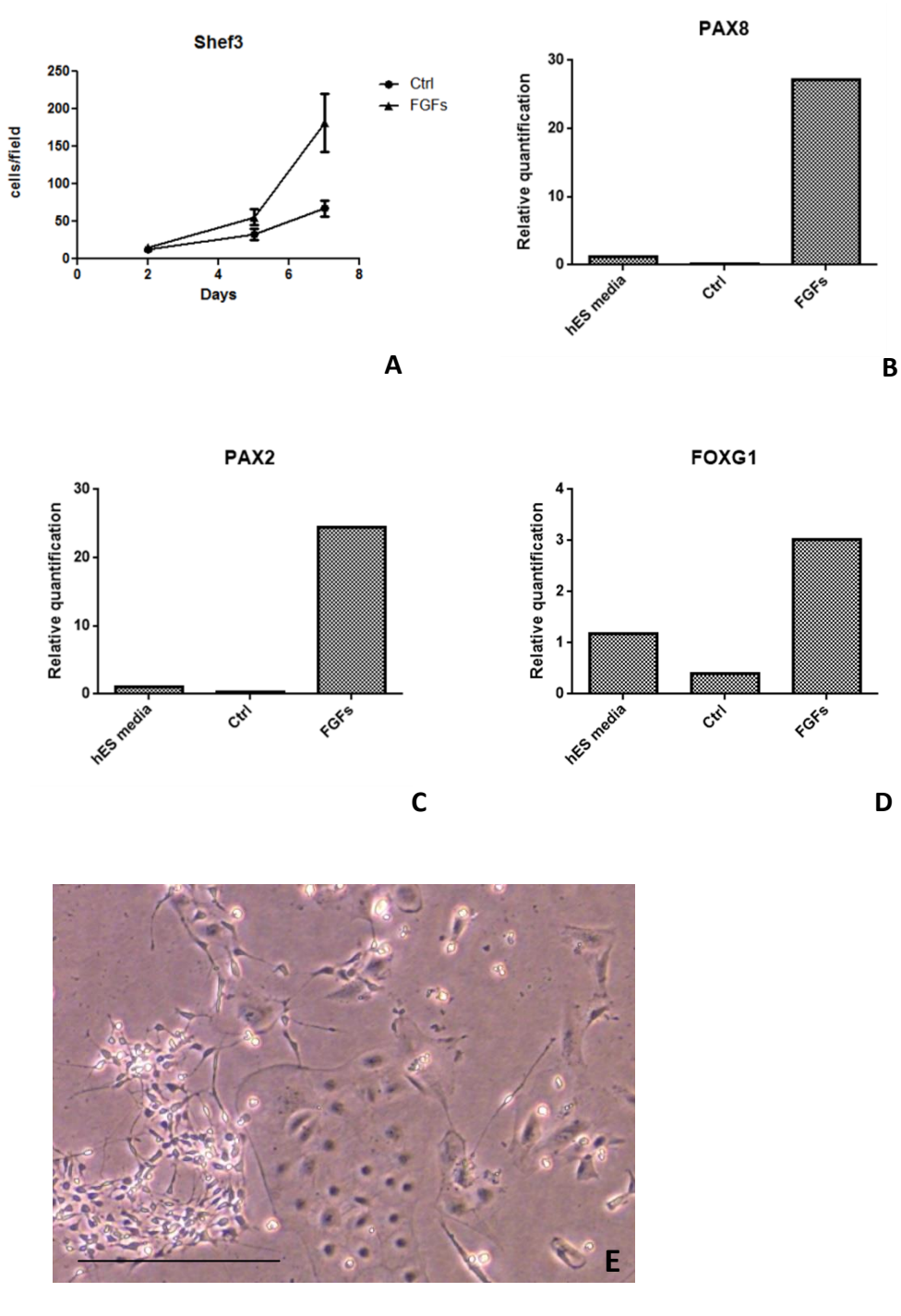


Figure 3.1 **Otic differentiation is induced by FGF3 and FGF10**. Cells were differentiated for 12 days in chemically defined media with and without FGF3 and FGF10. In **A,** a comparison of the growth of cells treated with FGF3 and FGF10 (FGFs) and those grown without them (Ctrl). In **B**, **C**, and **D**, QPCR relative expression data of *PAX8*, PAX2 and FOXG1 respectively, in cells collected at day 12 of differentiation. Undifferentiated cells (hES media) were used as a calibrator. The types of morphologies observed when cells were differentiated are presented in **E**. All together these data indicate the robust otic differentiation of Shef3 cell line when cells are exposed to FGF3 and FGF10, evidenced by the upregulation of otic markers and an increased growth rate. Error bars represent s.e.m. Scale bar is 50µm in **E**

Although the FGF-induced otic differentiation in Shef3 cell line in is robust and reproducible, we have observed that such tendency is more variable in the rest of the cells lines. In order to troubleshoot the source of this variation, it has been necessary to perform multiple comparisons in the Q-PCR data. We have collected a substantial amount of information from different cells lines, and in this chapter we will be exploring this variability.

In this manner, since the first thing we wanted to know was the effect of FGF3 and FGF10 in the otic differentiation of the whole set of pluripotent cell lines, we pooled together the Q-PCR results of each differentiation experiment of the hES cell lines Shef1, Shef3, H14 and hiPS cell lines ShiPSFF1 and ShiPSFF5, totalling 22 experiments . As calibrator for each experiment we used cells differentiated in DFNB medium without FGFs (Ctrl), and presented the data as relative expression (figure 3.2).

When other cell lines were brought into the analysis, although all otic markers were upregulated by the FGF3 and FGF10 treatment (calibrated against DFNB) the magnitude of the upregulation was less robust than in Shef3. In addition, there was a large variation among experiments (figure 3.2). Nevertheless, the large sample size makes these results very solid. It is noteworthy that even under these circumstances; there was a robust differentiation when both FGF and DFNB samples were compared to the undifferentiated cells in hES media (not shown in here).

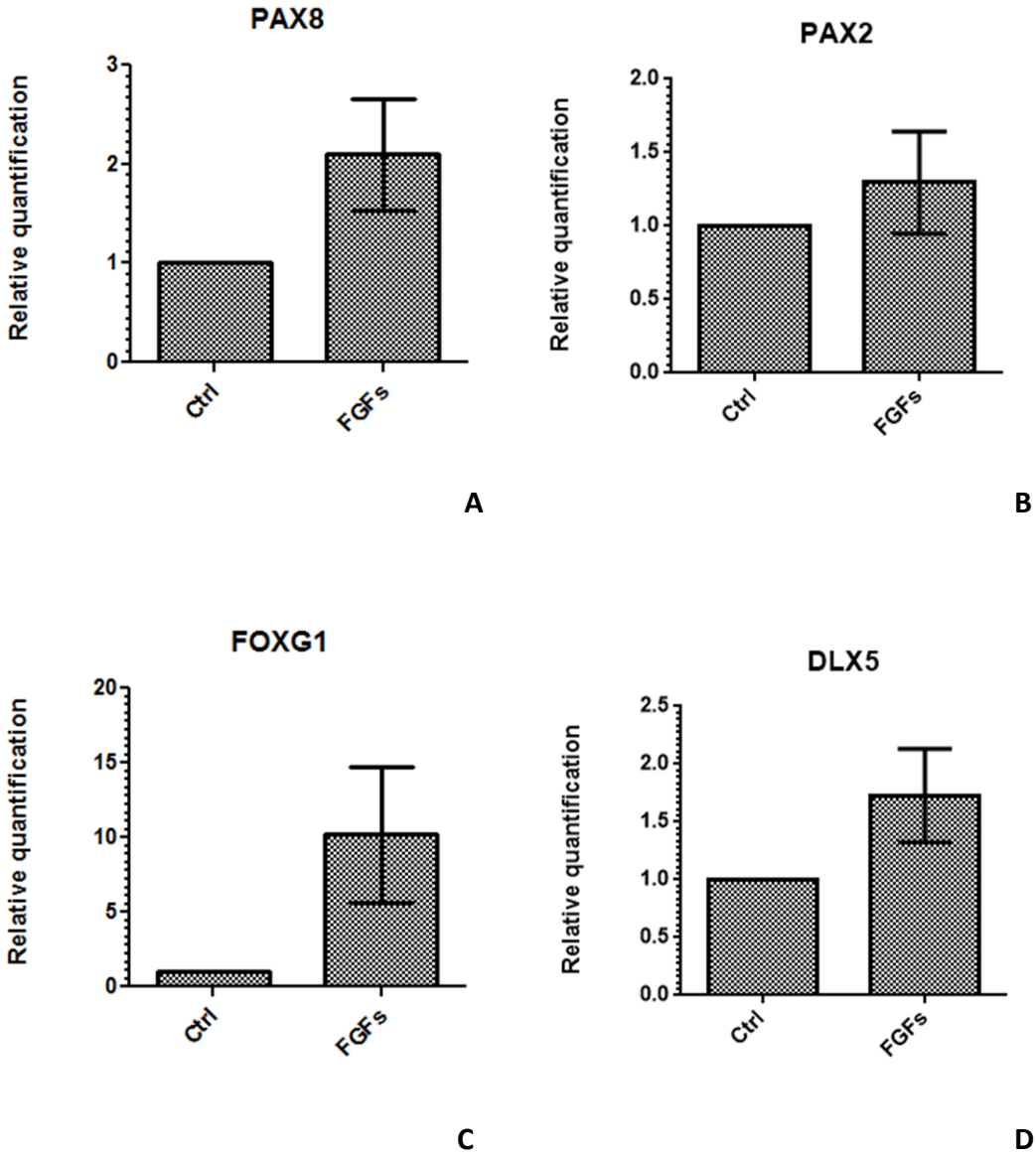


Figure 3.2 **Induction of otic genes by FGF3 and FGF10 treatment**. Comparison between DFNB vs. FGFs (FGF3 and FGF10) considering the whole set of differentiation experiments with all the cell lines. Experiments were analysed by Q-PCR and the data presented as relative expression, using as calibrator within each experiment cells grown in DFNB medium (control). PAX8 (**A**) and FOXG1 (**C**) were the markers most upregulated in FGF treated cells. Error bars are s.e.m. (n= 22).

We carried out a statistical analysis to determine the effect of FGFs on individual genes. We were interested in the magnitude of the upregulation when cells were FGF-treated and in the reproducibility of that upregulation. In other words, we could have an otic marker that is upregulated in every experiment by the FGF treatment, but the magnitude of that upregulation could be just above its DFNB control. Alternatively, another marker could display a strong upregulation when compared with the control, but that tendency could occur only in fraction of the experiments, indicative perhaps of a cell line-dependant response, or another variable having an important role more independent of the FGF treatment. We normalised all experiments using the median of the reference gene (*RPLP0*) and the linear shift of the target gene (e.g. *PAX8*) (methods, chapter 2). Then we obtained the ΔCt for DFNB controls and FGFs conditions and compared them by paired T-test.

The results of such analysis are presented in table 3.1. This table also contains frequency data of the number of experiments where upregulation of individual markers was observed as a consequence of FGF3 and FGF10 treatment. If the fold change in FGF treated cells was >1 in that particular experiment, then it was counted as an upregulation event. On the contrary, if the fold change in FGF treated cells ≤1, it was considered to be "not upregulated". In this way the data of table 1 was used to make contingency tables and carry out Fisher statistic of distributions.

From this analysis we can conclude that although there was a tendency of FGFs to induce the expression of the otic markers *PAX8, PAX2, FOXG1* and *DLX5,* the effect of FGFs on individual factors differed considerably among the experiments. Nonetheless, there was clear tendency of FGFs to upregulate all otic markers, and such tendency was statistically significant in the case of *PAX8, FOXG1* and *DLX5*. Moreover, despite the variation observed between different experiments in the magnitude of the upregulation, *PAX8* was the most consistently induced gene by the FGF treatment, where upregulation induced by FGFs was observed in 15 out of 22 experiments (P=0.0168). However, the magnitude of *PAX8* induction was lower when compared to *FOXG1*, being *FOXG1* the one with a more sizable level of induction (P=0.0277). In other words, while *PAX8* was consistently upregulated by FGF independently of the cell line or other variables present in the experiments, the magnitude of the fold change was modest, only inducing PAX8 at a level slightly above the control as shown in figure 1. On the other hand, *FOXG1*, is a transcription factor that was

strongly induced by the FGF treatment and its level of expression was well above the baseline (Ctrl) in those experiments where it was induced. However, its upregulation was less consistent, failing to be detected in 10 out of 24 experiments.

Table 3.1. **Statistical analysis of Q-PCR data comparing control vs. FGF treated cells**. Q-PCR ΔCt values of individual genes in cells differentiated in FGF or DFNB control medium were compared using one tailed paired t test. In addition, the frequency of experiments where upregulation was observed for each of otic marker is presented in the first raw. We based this comparison on relative expression of Q-PCR data. An upregulation event was defined as a value >1 in the FGF treated cells, since the cells in control DFNB were defined as 1 in each experiment. The results of this table indicate that even with the large variation observed there was a statistically significant increase in the expression of PAX8, FOXG1 and DLX5 when cells were treated with FGF3 and FGF10.

	PAX8	**PAX2**	**FOXG1**	**DLX5**
No of experiments upregulated	15	8	13	8
Association FGF and upregulation (Fisher exact test)	*P=0.0168* YES	NO	*P=0.500* NO	*P=0.1102* NO
Difference Ctrl vs. FGFs (paired T-test) significant?	*P=0.0463* *YES*	*P=0.4335* NO	*P=0.0277* YES	*P=0.0410* *YES*
No of experiments	22	22	25	12

Are hES and hiPS cells the same?

As mentioned earlier, different hES and hiPS cell lines were used in the analysis. Therefore, we decided to split the data into hES cells and hiPS cells and carry out a similar analysis as the one presented before. It must be said however, that the number of hiPS cell lines used was small and do not cover all the possible variables that may affect the behaviour of hiPSCs (e.g. cell type of origin, set of factors used, transduction method, etc). Thus, if any conclusion is presented at this stage, it should be seen as an initial insight for future exploration.

Relative expression was calculated as before, taking as calibrator within each experiment cells grown in DFNB condition (ctrl). As observed in figure 3.3, FGF treatment induced the expression of all the otic markers in hES and hiPS cells, however in hiPSCs the magnitude of the upregulation (normalised against control) was smaller for the 3 genes. In addition there was a large variation in the level of upregulation of *FOXG1* hiPSCs. Data was also sorted out in table 3.2, showing the proportion of experiments in hES and hiPS cells where each transcription factor was unregulated by the FGF treatment. The criteria to consider an upregulation was as before, if FGF-treated cells had a relative value of expression larger than the calibrator of that experiment, in other words >1.

There was a marked tendency in hES cells to upregulate *PAX8* (12 out of 14 experiments) in response to FGFs, despite the size of that upregulation was relatively small when compared with *FOXG1* for example (figure 3.3 A and table 3.2). The other two markers (*PAX2* and *FOXG1*) showed upregulation in 50% of the cases approximately when cells were treated with FGFs, indicating therefore two classes of results. This could indicate that the effect of FGFs may depend from other variables present in the experiment (e. g. cell density, cell line, differentiation period, etc). Nonetheless, the level of upregulation of *FOXG1* was very high when compared to other markers.

Regarding hiPSCs, *FOXG1* was upregulated in most experiments, although the level of that upregulation was very variable among them (figure 3.3 F). Also the other two markers were upregulated like in hESCs, in 50% of the experiments, and the size of that upregulation was

smaller than the one observed in hESCs. My conclusion from this comparison is that hiPSCs are not different to hESCs. It seems that there are two classes of response in both cell types, and therefore splitting the data into cell lines could bring some explanation of that. However, it must be stressed that despite other variable affecting the results, *PAX8* is most of the time upregulated by FGF treatment.

The fact that some hiPSCs experiments do not show the expected tendency to upregulate otic markers in response to FGFs, or that this upregulation is smaller than the one observed in hESCs, should not be interpreted as hiPSCs being refractory to otic differentiation. On the contrary, data presented later in this thesis (chapter 4) and also gathered by other members of the lab demonstrate that the apparent lack of induction by FGF is due that hiPSCs grown in DFNB control medium present an enhanced spontaneous otic differentiation without the extra addition of FGF3 and FGF10.

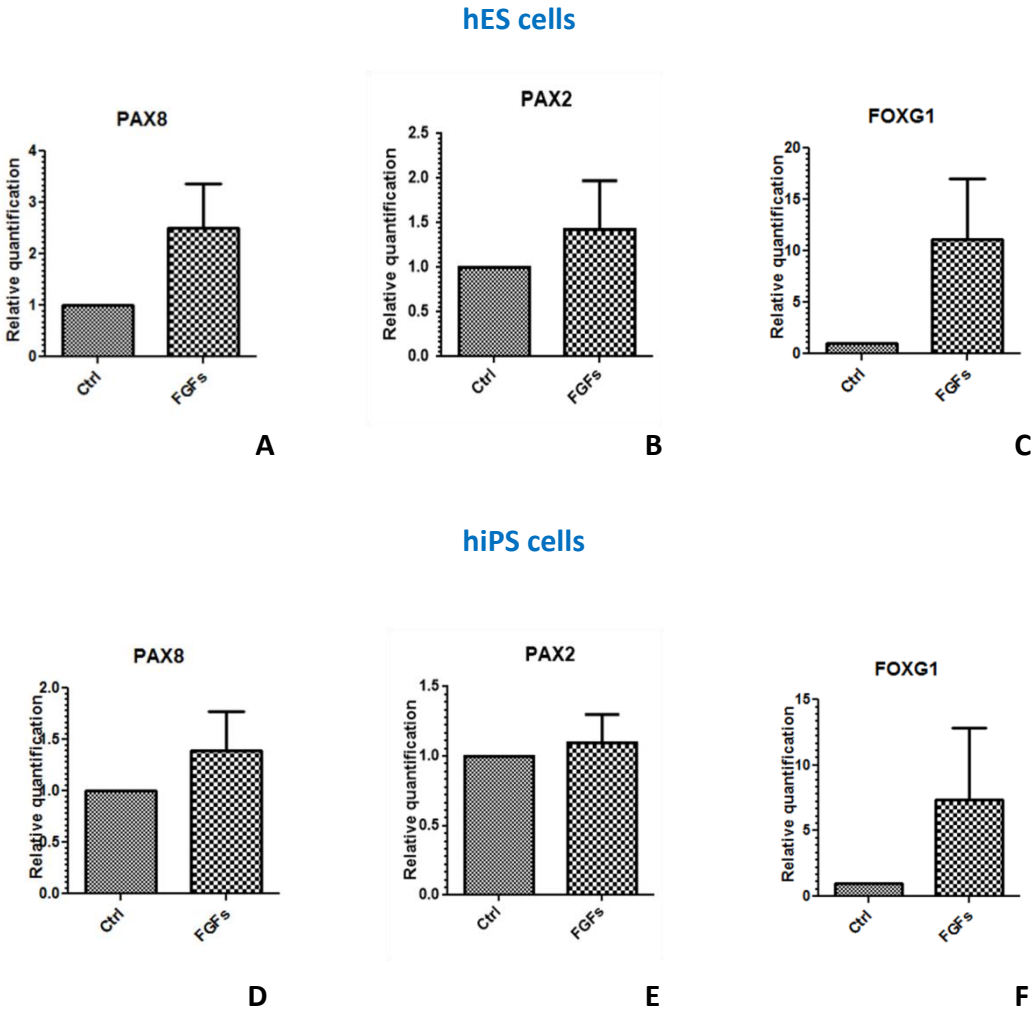


Figure 3.3 **Comparison between hES cells and hiPS cells**. Q-PCR data from differentiation experiments was split in hESCs and hiPSCs, and represented as relative expression, using as calibrator cells differentiated without FGFs (ctrl). In **A**, **B** and **C** relative expression data of *PAX8, PAX2* and *FOXG1* respectively in hESCs experiments. hiPSCs relative expression is presented below in **D**, **E** and **F,** for the indicated otic markers. Both cell types presented similar patterns although the level of upregulation was smaller in hiPSCs. Also the variation in the expression level of *FOXG1*, was larger in hiPSCs than in hESCs.

Table 3.2. **Reproducibility of upregulation of otic markers in hESCs and hiPSCs**. The data represents the proportion of experiments where the different otic markers were upregulated by the FGF treatment compared with the DFNB medium (ctrl). An upregulation event was defined as an expression value in FGF treated cells larger that the value in DFNB control medium (> 1). Although FOXG1 was upregulated in most of the experiments with hiPSCs, the level of expression was highly variable. In addition the fact that the rest of the marker were not consistently upregulated, suggest that cells lines may have a different response.

	Proportion of experiments where otic markers were upregulated	
	hES cells	**hiPS cells**
PAX8	12/14	5/8
PAX2	5/14	4/8
FOXG1	8/19	5/6

Taking into consideration the differences between these two cell types and the inconsistency in the upregulation of some otic markers both in hES cells and hiPS cells, we decided to analyse individual cell lines and to study the effect that other variables like the starting cell density could have had in the experiment results.

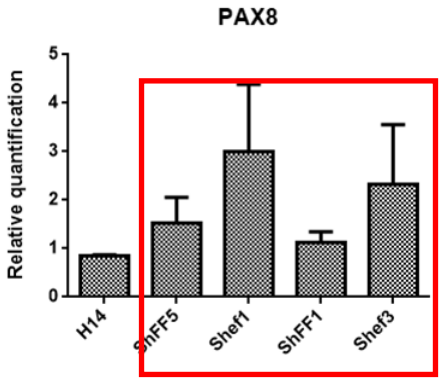
In figure 3.4, a comparison between the levels of expression of otic markers in all cell lines is depicted. Every bar represents relative expression values in FGF condition normalised against the DFNB medium control ("1"). Also, in the right part of the figure, there are stacked graphs of the proportion of experiments where upregulation of otic markers was observed in FGF condition. Looking at the data of all the cells lines plus the current knowledge regarding the FGF induced otic differentiation in these lines, we decided to arrange the cell lines that show a more robust differentiation in FGF condition when compared with the DFNB controls in the right part of the graph and keep the same order in all the graphs. The cell lines that showed the most prominent upregulation of otic genes or that upregulated those genes with more consistency across experiments were highlighted in the figure 3.4.

Through this analysis we observed Shef3 and the hiPS cell line ShFF1 stand out as the cell lines that respond most efficiently to the FGF treatment, they upregulated all the otic markers at a higher level more consistently than other cell lines. In the other side of the spectrum, H14 was a cell line that didn't respond to the FGF treatment, none of the markers was upregulated in response to FGFs. The hESC line Shef1 and the hiPSC line ShFF5 presented a response in the middle between the H14 and Shef3 classes. Each of these lines upregulated one of the otic marker more consistently and robustly than the other markers. For example, when exposed to FGFs, a consistent *PAX8* upregulation was observed in Shef1 (figure 3.4 C and D), while in ShFF5 a similar upregulation occurred in the case of *FOXG1* (figure 3.4 E and F).

The plots from figure 3.4 supports the idea of a degree of interdependency between the different otic markers, suggesting that their expression is integrated into a coherent

differentiation program (inner ear) and that they could be regulatory linked. For example, the pattern observed in the *PAX2* and *FOXG1* plots is very similar indicating that the behaviour of one maker mimics the other, and the application of FGFs, induced the expression of both genes in a similar trend, although in a cell line-dependant manner. It is important to notice again that the otic marker *PAX8* was induced by the FGF treatment independently of the cell line.

Figure 3.4 **Otic differentiation varies between cell lines**. In the left part, relative expression data of *PAX8* (A), *PAX2* (C) and *FOXG1* (E) in FGFs treated cells is shown. Data was normalised against DFNB (control). In the right the proportion of experiments that showed upregulation for the different otic markers. Upregulation was defined as in table 3.1 and 3.2. We can observe that the hiPSC line ShFF1 and the hESC line Shef3 presented the most robust and consistent upreguation of all the otic makers in (red squares in C to F) indicative of their bigger competence to differentiate in FGF3 and FGF10. *PAX8* was upregulated by FGFs independently of the cell line, with the exception of H14, that did not upregulated any of the otic markers.
The similarity in the patterns of the graphs indicates that the genes are part of the same regulatory program but some cell lines are more competent to FGF induced differentiation than others. Error bars represent s.e.m.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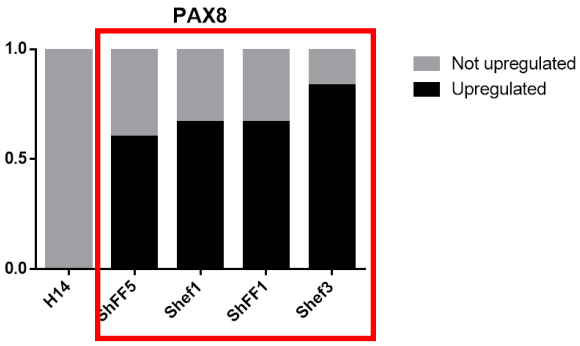
3
2
1
0
H14
ShFF5
Shef1
ShFF1
Shef3

A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Shef3
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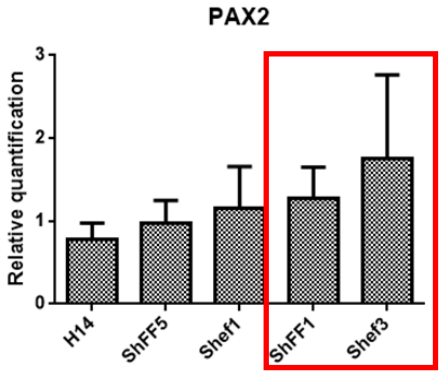
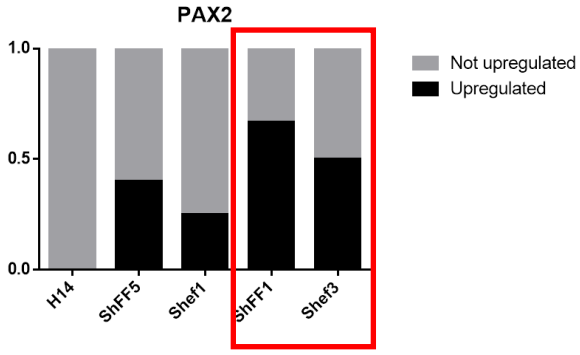
Upregulated

B


PAX2
Relative quantification
3
2
1
0
H14
ShFF5
Shef1
ShFF1
Shef3

C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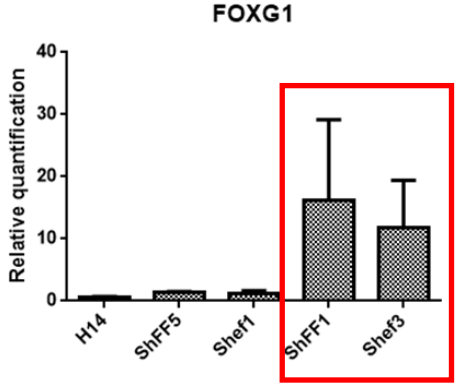
Upregulated

D


FOXG1
Relative quantification
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30
20
10
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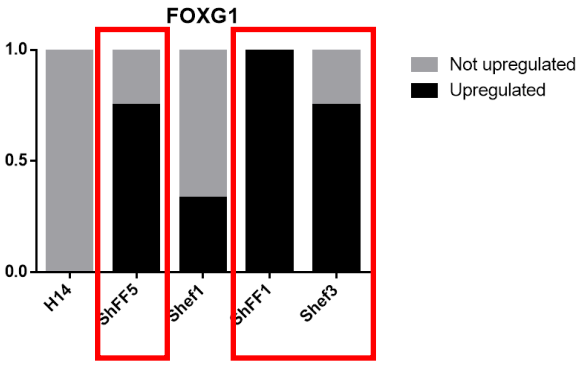
H14
ShFF5
Shef1
ShFF1
Shef3

E


FOXG1
1.0
0.5
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H14
ShFF5
Shef1
ShFF1
Shef3
Not upregulated
Upregulated

F

The effect of cell density

During the course of the differentiation experiments, we often observed cells not attaching properly or not surviving more than a couple of days after induction. In fact, most cells would die early during differentiation while a small fraction will remain and continue proliferating for the length of the experiment (12 days). In figure 3.5 A and B, there are example of the appearance of Shef3 and Shef1 cells, 48 hrs after otic induction. We have observed that cells keep a steady growth during the first 4 days of differentiation, and then the rate increases exponentially beginning to reach confluency at day 6 (figure 3.5 C and D). It is worth highlighting that the final density of the FGF-treated cells was larger in the hESC line analysed (Shef3), while the FF5 iPSC line showed no difference between the FGF sample and the control. The FF5 profile represents well the higher endogenous differentiation detected in hiPSCs, perhaps contributing to the variability described before. In order words, it would seem that the differentiating hiPS cell line FF5 is more likely to behave like FGF-treated, even without supplementation with the ligands. This paradoxical result is potentially explained by findings presented in chapter 4 that explore more in detail the behaviour of hiPSC cell line compared to Shef3 when differentiated. In this scenario, increasing the starting cell density could increase the endogenous levels of FGFs in the medium, and potentially induce the differentiation without FGF supplementation.

In order to know if cell density played a role in differentiation efficiency, we plotted the ΔCT values of otic genes of FGF-treated cells of every experiment against their initial, seeding cell densities (4000, 8000 and 12000 cells/cm²). By using the ΔCTs of the otic markers (Gene of interest Ct- Reference gene Ct) we are expressing an estimate of the absolute level of expression of these genes. Most of the experiments were run at a density of 8000 cells/cm², since cells seeded at this density were most likely to survive for the length of the experiment. The fact that only three densities were analysed and that one of them is underrepresented in terms of number of experiments makes the analysis complicated. Nevertheless, this correlation analysis shows that there is a tendency of cells to express higher levels of *PAX8 and PAX2* when cells are seeded at lower cell densities in the FGF condition. *FOXG1* in the other hand seems unaffected by cell density (figure 3.6).

If FGFs are being secreted by the cells and in this manner creating a self-induction loop, we would expect the ΔCt to be smaller (higher expression) in cells seeded at higher densities. However, the opposite tendency is observed in figure 3.6, almost implying that FGFs are inhibitory for otic differentiation. Moreover when we look at the tendency of expression of *PAX8* in cells grown in DFNB medium (figure 3.6 A'), the slope of the line is less prominent that the one in cells grown in FGF3 and FGF10 medium. These results, together with the ones presented in figure 3.2-3.4 indicate that FGFs could have a dual role depending on their concentration, they could trigger otic differentiation at some level of activity, but if this is surpassed, an opposite effect could be observed.

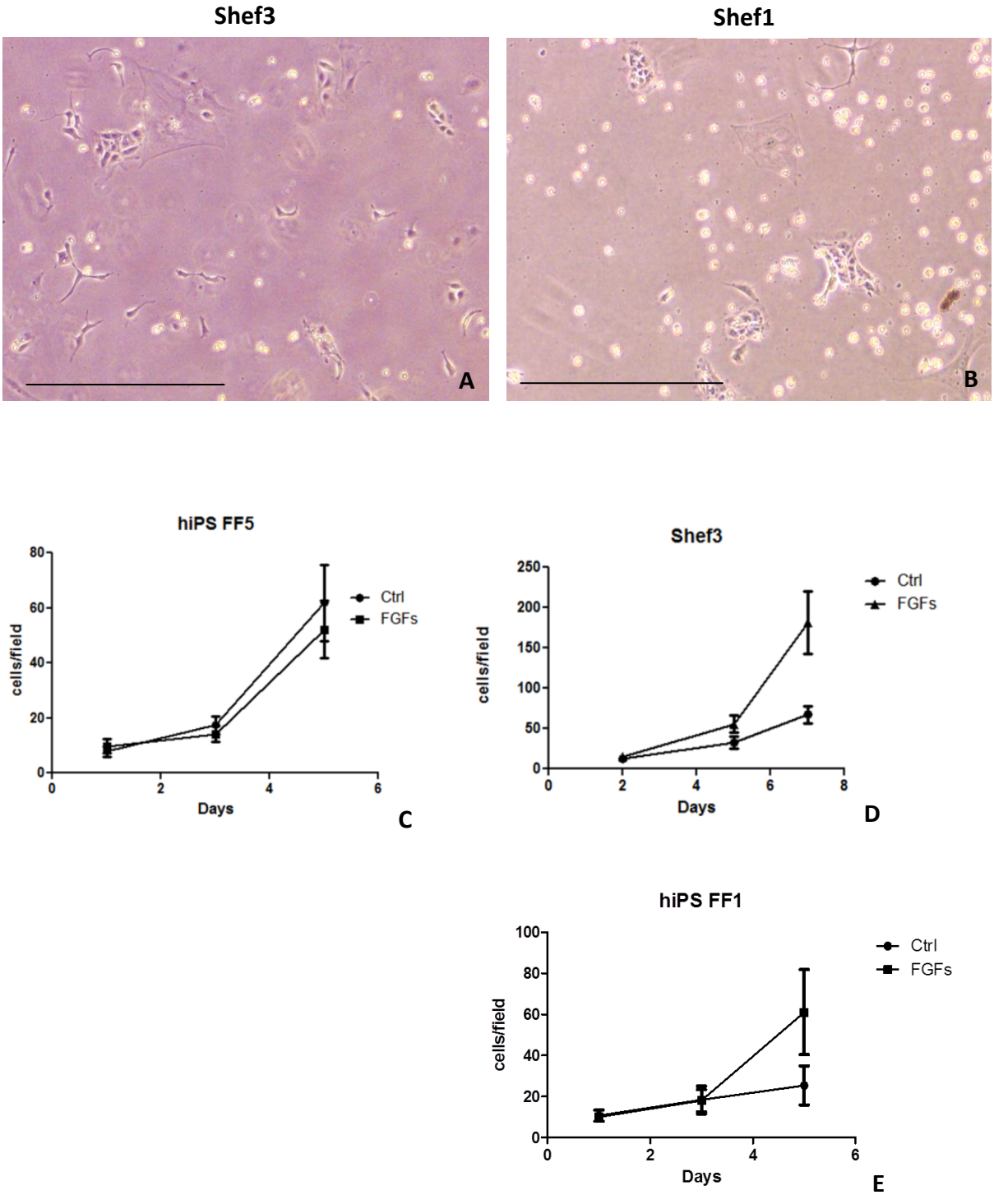

Figure 3.5 **Dynamic of differentiating cells.** In **A**, and **B** appearance of Shef3 and Shef1 cells respectively 48hrs after being seeded in induction medium (DFNB plus FGF3 and FGF10), most of the seeded cells die within a couple of days after seeding. The surviving clusters are nevertheless, able to proliferate exponentially (**C**, **D** and **E**) and become confluent at the end of the differentiation period. The difference between the hiPS cell line FF5 and other cell lines like ShiPSFF1 and Shef3 that was evidenced by QPCR is also clear when we compare the growth rate of cells. FGFs are mitogenic in Shef1 and FF1 but in FF5 the DFNB control behaves as cells supplemented with FGFs. It could be that endogenous behaviour of cells hides the effect of FGF supplementation. Scale bar 200µm.

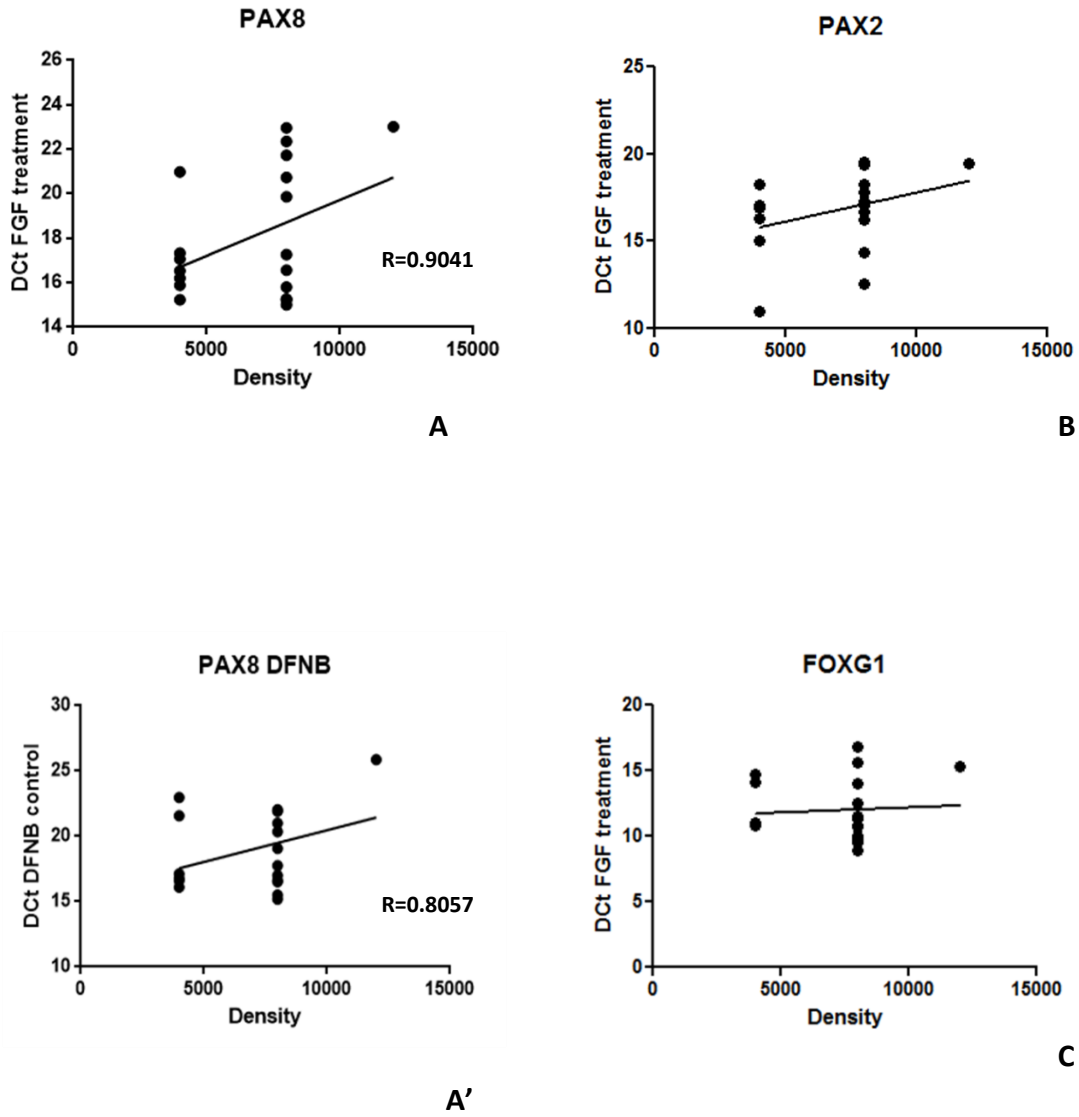

Figure 3.6. **Effect of cell density on otic differentiation**. Correlation plots of Q-PCR ΔCts of cells grown in FGF media (**A**, **B** and **C**) seeded at three different densities 4000, 8000 and 12000cell/cm². In **A'** the correlation plot of the PAX8 ΔCts of cells grown in DFNB control medium, compare with **A**. The ΔCts were calculated based on the expression level of the housekeeping gene *RPLP0.* There is negative correlation between the level of expression of otic genes and the starting cells density in the culture. The comparison in the ΔCts pattern of PAX8 in cells differentiated in FGF vs. DFNB medium suggests that FGFs could play an inhibitory role in otic differentiation.

Discussion

When studying any particular pathway *in vitro*, is important to establish a reliable model to investigate that phenomenon at the molecular level with the minimal "noise". Cancer cell lines are used for this purpose, because in general they are easy to maintain, present a robust growth and are suitable for different *in vitro* manipulations, e.g. transfection, silencing, clonal assays, etc. On the contrary, hESCs are costly to maintain, most media formulation must be supplemented with bFGF, serum or serum-related mixtures plus the co-culture with mouse embryonic fibroblast. These undefined conditions potentially create variations when studying a pathway. Cell culture-defined media to keep hESCs in undifferentiated state have been developed, but the cost of these systems is considerably high, hampering the routine use of it in most of the labs. Differentiation is another source of variation in the system, having the pluripotency of human embryonic stem cells been usually established by teratoma formation and *in vitro* differentiation into derivatives of all germ layers. This potential for differentiation is one of the main motivations behind hESC research. The need to generate functional cell types that could have a translational application either as models or for cell-replacement therapy requires a reproducible system with a robust understanding of the sources of variability.

It is important in any case, to work in one cell line first to establish a model of otic induction. For our initial work, we made use of the cell line Shef3, since we had some evidence that this cell line differentiated in the presence of FGF3 and FGF10, and because we knew of the existence of some Shef3 transgenic lines that could be used in our project (e.g. TetR Shef3 line).

We observed a robust differentiation of Shef3 when cells were treated with FGF3 and FGF10, evidenced by strong upregulation of the otic markers *PAX8, PAX2* and FOXG1 (figure 3.1). Together with that, proliferation was increased by the FGF treatment, in agreement with a role of FGFs in proliferation. For example, the mouse mammary tumor virus can increase the expression of Fgf3, Fgf10, Fgf4 and Fgf8 consequence of viral integration in their regulatory sequences leading to uncontrolled growth in the mammary epithelium (MacArthur et al., 1995; Shackleford et al., 1993; Theodorou et al., 2004). It has been

hypothesised that this tumour arises from the autocrine increased activity of FGF signalling by Fgfs.

The upregulation of *PAX8, PAX2* and *FOXG1* by the FGF3 and FGF10 treatment is in agreement with the role of FGFs in the induction of inner ear development, extensively presented in the introduction of this chapter and in the general introduction (chapter 1). Nonetheless, *in vivo,* the predominant approach to study the role of FGFs has been through the use of knockouts models in the fish and mouse or by the use of FGFR inhibitors in the chicken. However, two publications in particular deserve our attention since they make use of a gain of function approach that has more resemblance to our system at this stage. In the first one, when Alvarez et al (Alvarez et al., 2003) missexpressed Fgf3 or Fgf10 in other regions of the hindbrain not involved in otic induction, they observed the formation of ectopic otic vesicles, characterized by some but not the entire set of otic markers. In line with this, in the chicken, overexpression of *FGF3* and *FGF19* expanded the otic domain characterized by *PAX2* (Freter et al., 2008). Therefore these findings support a direct involvement of FGFs in the induction of an otic fate outside the normal domain of otic development, and are in agreement with the results observed in the differentiation of Shef3 cell line.

Variation between cell lines in their response to FGFs

When studying hESCs it is very important to analyse a range of cell lines with the purpose of developing a coherent differentiation protocol of general use with hESCs, rather than the fine dissection of a molecular event of a particular signalling pathway. The first thing we noticed when other hESCs and hiPSCs were brought into the analysis is the large variation among cell lines and experiments. It must be stressed, however, that despite this variation we observed a significant difference in the expression level of cells treated with FGF vs those grown in DFNB control medium (Figure 3.2), in agreement with the results observed in the Shef3 cell line.

The QPCR data was sorted in different cell lines, and through this analysis we were able to distinguish three groups of pluripotent cells depending of their response to the addition FGF3 and FGF10; the most responsive ones (FF1 and Shef3), the ones that only upregulated two otic markers at a lower level than the previous group (Shef1 and FF5), and H14, that presented downregulation of otic genes when exposed to FGF3 and FGF10. However, it must be stressed that for instance in the case of the hiPSFF5, despite a small upregulation of otic genes when compared to the DFNB control, the expression level was considerably higher when compared with the undifferentiated cells (chapter 9). The same has been true for H14 (Chen et al., 2012).

The type of variation observed is common when studying hESCs differentiation and it appears to be related to an endogenous propensity in the cells to produce specific cell types. In this regard Osafune et al (Osafune et al., 2008) showed that the expression levels of markers for different cells lineages differed between hESCs when differentiated through embryoid bodies. These differences were later shown to correlate with the capability of cells to produce specific cell types in targeted differentiation protocols. Even hESC obtained in the same laboratory and differentiated in stringent conditions have been shown to produce neurons with a different identity related to the cell line from which they were derived (Wu et al., 2007). In addition to the inter-cell line variation, the hESC niche is known be heterogeneous (Blauwkamp et al., 2012), and this could affect the outcome of any particular experiment.

PAX2, PAX8 and FOXG1 are part of the same otic differentiation route

Through the cell line comparison, it was interesting to notice that the pattern of expression of an otic marker (e.g. PAX2) in relation to the cell line was similar for the pattern observed in the rest of the otic markers, indicative of the an integrated regulatory pathway (inner ear). In other words, although the response of one gene was dependant on the cell line, the response was similar for the other genes. These genes are all coexpressed in the otic placode and vesicle, and mutations in *PAX2* and *FOXG1* present similar inner ear morphological defects (Burton et al., 2004; Pauley et al., 2006). In addition to the link of

these genes during inner ear development, PAX2 binding sites in the FOXG1 promoter have been predicted based in the SABiosciences database (figure 3.7 from QIAGEN).

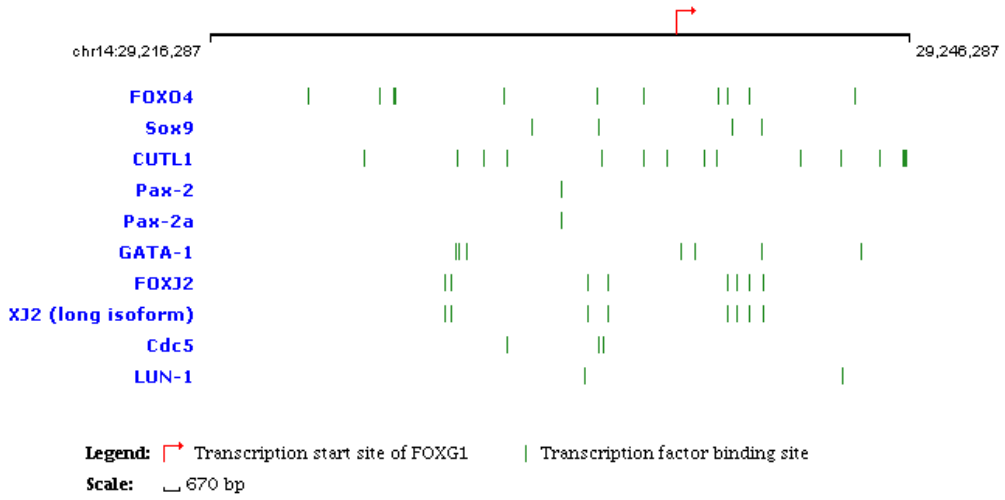

Figure 3.7 **PAX2 binding sites in FOXG1 regulatory region.** This image taken from QIAGEN, shows the predicted biding sites for different transcription factors in the promoter region of FOXG1. Putative biding sites are predicted using the SABiosciences text mining application from QIAGEN. It supports the develolopmental view and our data that this transcription factors are part of the same regulatory pathway.

In an effort to troubleshoot sources of variation in our system, we thought of cell density as a possible source of variation. Most of the experiments were made at 8000 cells/cm, a density that is double than the previously established in our protocol. However, increasing cell density could have a downside; it could hide the effect of FGFs and enrich media conditioning and cell to cell interactions, a consequence of overconfluent cultures at the end of the differentiation process. Thus we performed a correlation analysis between the level of expression of individual otic markers (ΔCts) and the starting cell density.

An inverse correlation between cell density and otic marker gene expression level was observed, suggesting that an inhibitory signal (either soluble or contact-mediated) is produced by the cells and counteracts the FGF effect. The nature of this inhibitory activity

could be the endogenous secretion of FGFs. In other words, if FGFs are secreted by cells, the extra addition of FGF3 and FGF10 could be inhibitory. Although FGFs are known to play different role depending on the cell context, there is a report where NIH 3T3 cells were transfected with either full length FGFR3 or a shorter version of it without the extracellular FGF binding domain. The cells were also cotransfected with *c-Fos,* a reporter of FGF-induced transcription (Webster and Donoghue, 1997). Interestingly in this work, it was observed that the level of c-fos activity was higher in cells transfected with the deleted form of the FGFR3 than with the full length, an unexpected finding considering that the activation of the receptor depends on ligand-binding activity. This suggests that FGFs may activate the pathway through the receptors, but they could also limit their activity. More interesting in Webster and Donoghe's work (1997) is the finding that, compared cells transfected with the active FGFR3 without the extracellular binding domain showed higher c-Fos activity than cells transfected with the full length constitutively active version of the FGFR3. In our experiments, if too much FGF activity is being inhibitory, we would expect the slope of the curve to be opposite when cells are grown in DFNB medium. This is not exactly the case, but a decrease in the slope of the correlation curve of PAX8 is observed in DFNB grown cells (figure 3.6 A').

Although the idea FGFs having a dual role depending on their concentration is covered in other chapters (4 and 7); it is one of the findings that we have made through this analysis and we are still investigating this aspect.

In the future, if it is important to decrease cell density, a possible way to overcome the low survival associated with the low density plating could be by the use of ROCK inhibitor, a caspase inhibitor proved to increase the survival of hES cells upon dissociation (Watanabe et al., 2007). The effect of cell density in hESC differentiation protocols has been documented. In an in vitro system of neural differentiation, Chambers and colleagues (Chambers et al., 2009) blocked SMAD signalling to produce a large proportion of neuroectodermal cells characterized by the expression of PAX6, and the formation of neural rosettes. If the starting cell density was decreased, the cells then differentiated into neural crest-like cells that did not formed rosettes and expressed the neural crest marker HNK1 and p75.