Chapter 4

Otic differentiation propensity in human pluripotent stem cells

Introduction

hESCs are typically maintained on inactivated mouse embryonic fibroblast (MEF) supplemented with bFGF. In fact, most of the hES cell media formulations are supplemented with bFGF. Moreover, it was proposed that hES cells could be maintained in feeder-free culture if high amounts of bFGF are added (100ng/ml). Therefore, FGF signalling activation by FGF2 has been seen as an essential component for the maintenance of undifferentiated hES cells. In line with this idea, Dvorak et al observed ERK activation in hES cells grown in MEF-conditioned media, evidence of active FGF signalling (Dvorak et al., 2005). More important, they also found that if FGF signalling is blocked by a specific inhibitor of FGF receptors, SU5402, cells started to differentiate within 2 days. In a separate study, Ding and colleagues, using a panel of inhibitors for specific pathways downstream of FGF activation, concluded that PI3K/AKT pathway is essential for hES cell maintenance (Ding et al., 2010). In both studies however, it was observed the endogenous production of FGFs was enough to keep hES cells undifferentiated for up to 6 days. Contrasting with these insights, Dr Na (Na et al., 2010) showed that hESCs can be kept in undifferentiated state in the presence of SU5402 if high concentration of Activin is supplied. Although the precise role of FGF in hES cells is still debated, it is clear that FGF function depends heavily on the balance with other signalling molecules present in the different media formulations.

In any case, it is interesting to notice that hES cells are very responsive to FGF activation. They express all the FGFRs and a number of FGF ligands (Ding et al., 2010; Dvorak et al., 2005; Ginis et al., 2004; Sato et al., 2004; Sperger et al., 2003). Therefore, they are an ideal starting population for FGF-based differentiation protocols.

As mentioned in the first chapter, FGFs are very important for otic development and mutants for FGFs and FGF receptors have different defects in inner ear morphogenesis. In agreement with this, in our system we have also showed that FGF3 and FGF10 induce the expression of the main otic markers: *PAX8, PAX2, FOXG1* and *DLX5*. However, the results were highly variable and cell line dependent. In other words; while in some experiments there was a clear induction of otic markers in the presence of FGF3 and FGF10 compared to the DFNB medium ctrl, in others, there was no difference between these two conditions. In

this section, we will explore and discuss the possibility that endogenous production of FGFs may mask the effect of exogenous FGF3 and FGF10 supplementation. In this regard, undifferentiated and differentiated hESCs express high endogenous level of FGF2 and differentiate towards the neuronal lineage if FGF signalling is blocked. However, there are contradictory views regarding the role of FGF signalling in differentiating hESCs and it seem to depend heavily in the cell on other variables in the protocol used (chapter 1). For example, it has been shown that inhibition of FGF signalling with FGFR inhibitors like SU5402 can impair neuronal differentiation (Chen et al., 2010; Cohen et al., 2010). In this work, Cohen et al also showed that although FGF activity is necessary for neuronal differentiation, endogenous secretion of FGFs by the cells is sufficient to trigger endogenous differentiation.

The take home message from these studies should be that hESCs are able to produce FGFs and switch on an endogenous differentiation route without the addition of growth factors to the medium. It is also important to stress the potential of small molecules inhibitors to dissect the FGF-mediated differentiated or activation. Therefore, in this chapter we will make use of the FGFR inhibitor SU5402 to dissect the role of FGF signalling in differentiating cells.

Results

FGF inhibition impedes otic differentiation in hESCs

As a starting point we wanted to know if the level of otic induction measured by gene expression of the main otic markers *PAX8, PAX2* and *FOXG1* was affected by the FGFR inhibitor SU5402. We decided to do these experiments in the cell line Shef3, which has been previously shown to be the one with the most robust otic differentiation when FGF3 and FGF10 are added. Relative expression data of Shef3 cell line differentiated for 12 days in FGFs (FGF3 and FGF10) or control medium (DFNB) with and without SU5402 is presented in figure 4.1. We can observe first, the robust induction of otic progenitors in cells treated with FGF3 and FGF10 compared to DFNB (ctrl) and undifferentiated hESCs. More important, the FGFR inhibitor SU5402 completely suppresses the expression of the otic markers in cells grown with FGF3 and FGF10, confirming that the FGF-mediated otic induction takes place through FGFR activation of the canonical FGF signalling pathway.

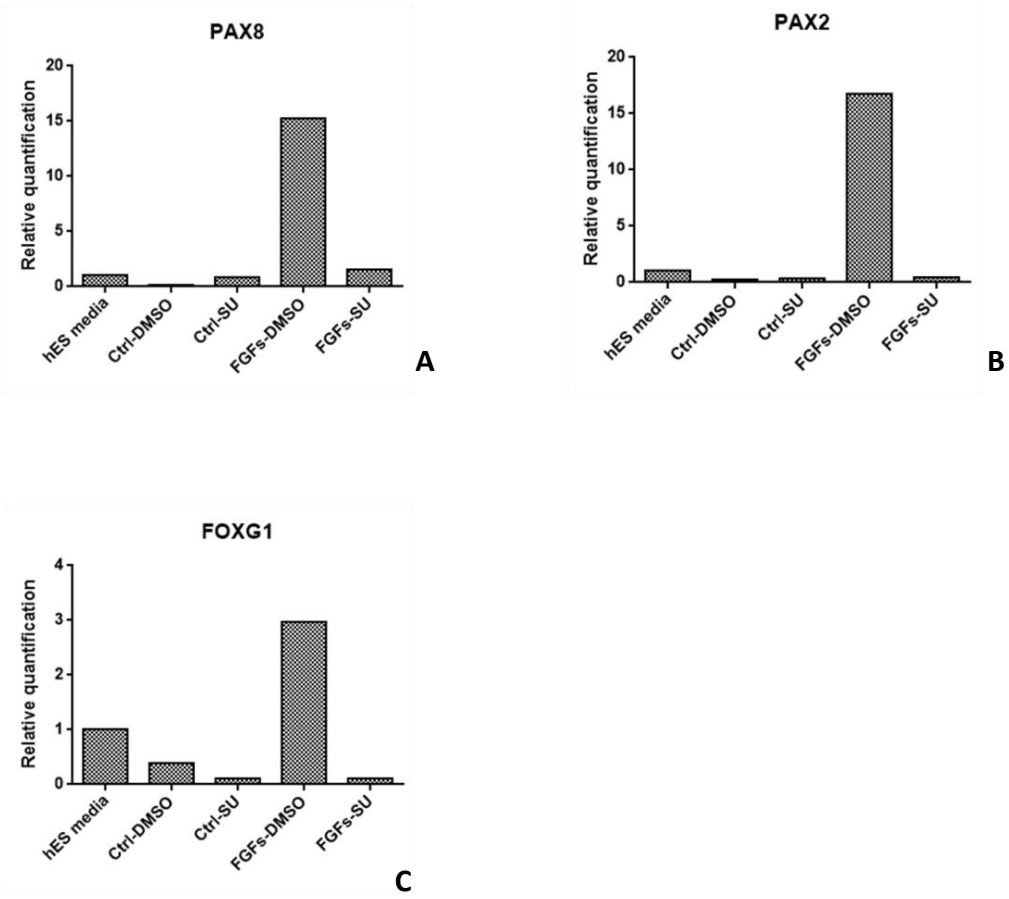
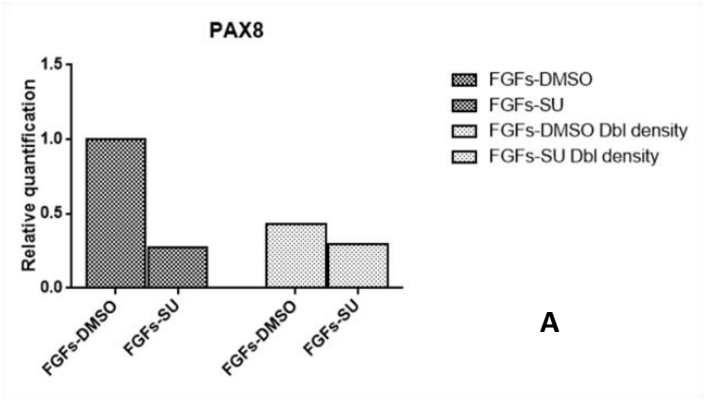


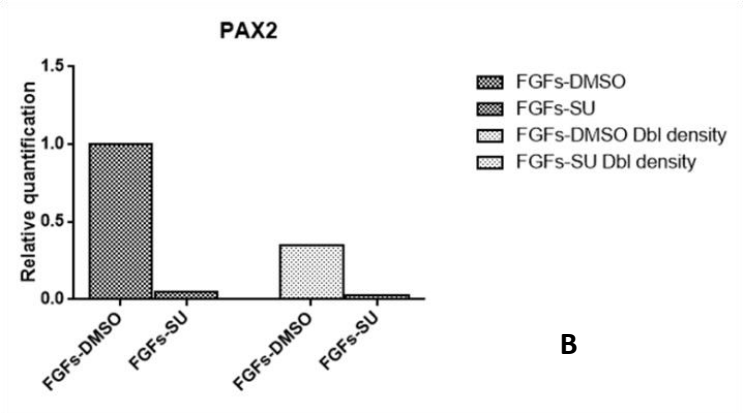
Figure 4.1 **FGF signalling is necessary for otic differentiation.** Cell were differentiated in standard conditions in FGF supplemented medium (FGFs) or DFNB only (control). FGFR inhibitor SU5402 or vehicle (DMSO) was added for the length of the experiment in both types of media. QPCR data was then presented as relative expression using undifferentiated cells as a calibrator (defined as "1"). Relative expression data of PAX8 (**A**), PAX2 (**B**) and FOXG1 (**C)** is shown. FGF signalling inhibition decreased the level of expression of all otic markers induced by FGF3 and FGF10, indicating that the FGF pathway is necessary for otic induction.

The strong inhibition produced by SU5402 was reproducible in this cell line. However, as introduced in chapter 3, the cell density could have an effect, manifested as a lower expression level of otic markers in cells seeded at higher density (double 8000 cells/cm²).

Consequently, we decided to test if FGF inhibition was less robust in cells seeded at higher densities. We differentiated cells at 4000 and 8000 cells/cm² in similar conditions as in the previous experiment. When we analysed the relative expression of markers using as calibrator FGF-DMSO treated cells seeded at low density (4000 cells/cm²), we observed a drop in the expression of *PAX8, PAX2* and *FOXG1* when seeding density was increased, confirming our previous observation that high density was inhibitory for otic induction. Even in this situation, SU5402 was able to suppress the expression of the otic markers, although the drop was less pronounced in cells seeded at double density (8000 cells/cm²) than those seeded at 4000 cells/cm² (figure 4.2), perhaps as consequence of the lower expression levels of otic genes in cells seeded at higher densities.

In any case, despite the cell density effect, a strong reduction in the expression of otic genes was observed.





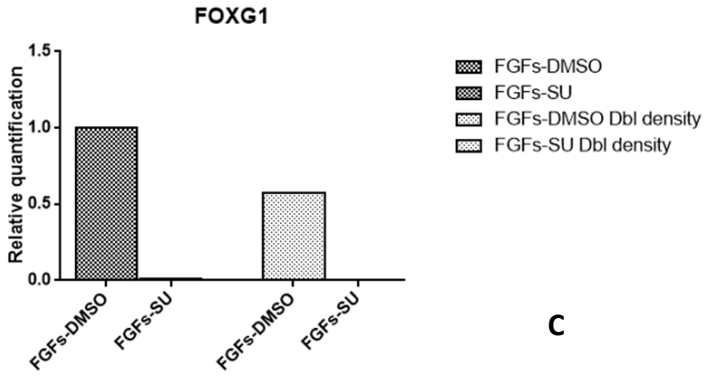


Figure 4.2 **FGF inhibition also blocks otic differentiation in cells seeded at high density.** Shef3 cells were differentiated in the same way as explained in the text and in the previous figure. Cells were seeded at two densities, 4000 and 8000 cells/cm². In this figure, QPCR data was represented as relative expression using as calibrator the level of expression in FGF-DMSO treated cells seeded at 4000 cells/cm² ("1"). Cells seeded at 8000 cells/cm² (dbl density) expressed lower levels of otic markers compared with the levels in cells seeded at low density. SU5402 reduced the expression of PAX8 (**A**), PAX2 (**B**) and FOXG1 (**C**), independently of the starting cells density.

Endogenous FGF secretion induces otic differentiation in control medium

Shef1, H14 and shiPSFF5 lines have been shown previously to present a small difference between FGF treated and DFNB control cells. For example Shef1 consistently upregulated *PAX8* but at a minimal level above the DFNB control, a similar phenomenon is observed in ShiPSFF5 with *FOXG1* and in the case of H14 a mild downregulation of all otic marker is observed in FGF treated cells compared with the DFNB control. We hypothesised that perhaps the endogenous level of FGF secretion in DFNB control media was big enough to hide the effect of FGF3 and FGF10 supplementation in those cells lines. In an attempt to test this idea, we compared the endogenous production of FGF3 and FGF10 in the cell lines Shef3 and ShiPSFF5 that readily differentiate into otic progenitors, but Shef3 only does it when FGFs are added to the medium while the ShiPSFF5 differentiates even in the absence of FGFs (figure 4.3). We observed that in DFNB medium (control) FGF10 was expressed in all the ShiPSFF5 experiments but not in Shef3, suggesting that FGF10 accounts for the strong differentiation observed in ShiPSFF5 in DFNB media (figure 4.3 G). Moreover, a third cell line ShiPFF1 was also included. This cell line presented behaviour between Shef3 and FF5, upregulating *FOXG1* and *PAX2* slightly, but downregulating *PAX8* in FGF treated cells. FF1 cell line expressed FGF10 as FF5 did, but it also expressed FGF3 as Shef3 did in DFNB medium. These results suggest that FGF3 and FGF10 may not be equivalent in our system and that FGF10 is more important for otic induction than FGF3.

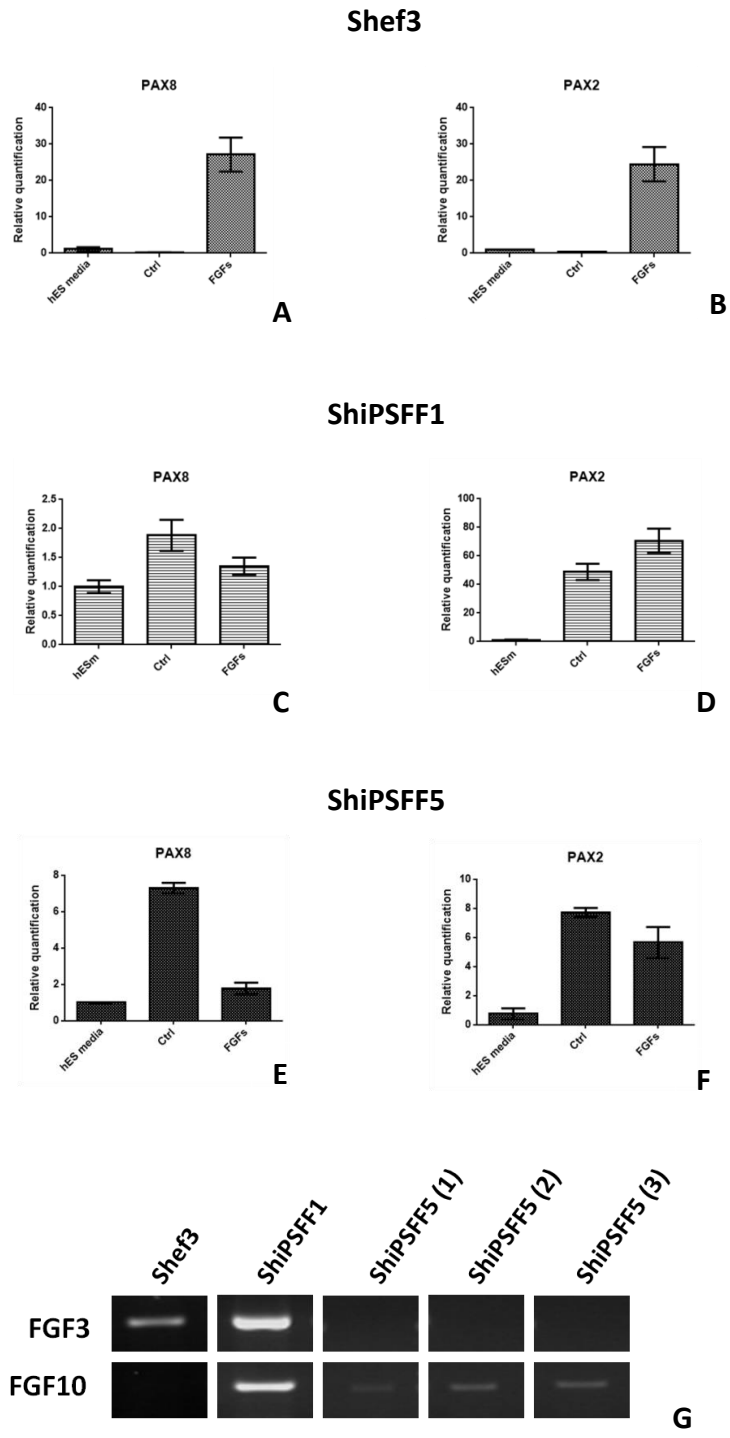


Figure 4.3 **Endogenous production of FGFs accounts for a default otic differentiation without the addition of FGF3 and FGF10.** Relative expression data of the hES cell line Shef3 (A and B) and the hiPS cell lines FF1 (**C** and **D**) and FF5 (**E** and **F**) is presented. Undifferentiated cells were used as calibrator ("1"). Shef3 differentiated when treated with FGF3 and FGF10, while ShiPSFF5 differentiate even when cells were grown in the DFNB medium only. In fact FGFs resulted inhibitory for the expression of *PAX8* and *PAX2* in ShiPSFF5. ShiPSFF1 behaved between Shef3 and ShiPSFF5. They differentiated in DFNB medium, while FGF treatment increased PAX2 even more and inhibit slightly PAX8 expression. In **G**, RT-PCR of FGF3 and FGF10 in Shef3, ShiPSFF1 and ShiPSFF5 cells grown in DFNB medium. Shef3 produced FGF3 only while the hiPSCs produced FGF10, indicating that FGF10 could be more important for otic differentiation than FGF3.

FGF10 induces higher expression of otic genes than FGF3

In light of these results we decide to differentiate Shef3 cell line for 12 days in either FGF3 or FGF10 supplemented medium. If there was a difference between FGF3 and FGF10 in otic induction, we would expect FGF10 alone to be a better otic inducing signal than FGF3, since the latter was produced by the cells in DFNB control in the previous experiment (figure 4.3 A), and otic markers were not induced despite of that.

The QPCR relative expression data of a single factor experiment are shown in figure 4.4. In this experiment we could not detect *PAX8* reliably, and therefore it is not presented. However, we did notice a tendency of FGF10 to upregulate the otic genes PAX2, FOXG1, and DLX5, to a level above both FGFs and FGF3. We have observed this similar tendency in another single factor experiment in Shef3 cell line. However, at the moment such experiments has not been performed with the hiPSCs FF5 and FF1, so we cannot yet categorically support these conclusions, but it would seems that FGF10 could be better for otic induction than FGF3, and that FGF3 is perhaps inhibitory for otic induction at higher concentrations.

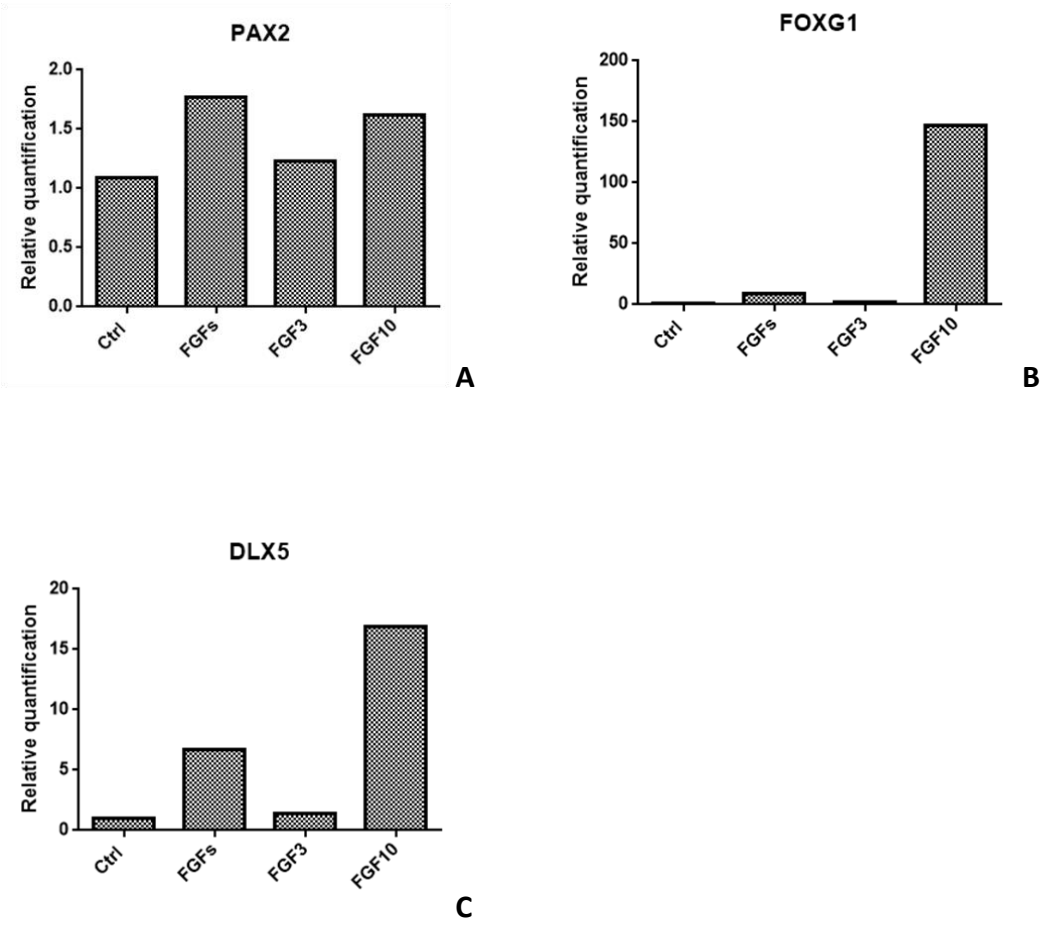


Figure 4.4 **FGF10 alone is more potent otic inducing signal than FGF3 in Shef3 hESCs.** Shef3 cells were for 12 days in DFNB (ctrl), FGF3 and FGF10 (FGFs), FGF3 only (FGF3) or FGF10 only FGF10 supplemented medium. QPCR relative expression data of PAX2 (A), FOXG1 (B) and DlX5 (C) is shown. Cells grown in DFNB control were used as calibrator ("1"). FGF10 alone induced the upregulation of FOXG1 and DLX5 at a level higher than cells treated with both FGFs, and FGF3 alone, in agreement with the data presented in figure 4.3. This tendency has been observed in other experiments with Shef3 cell line. Single factor experiments have not been made in the hiPSCs FF1 and FF5 to correlate more accurately the endogenous production of FGF10 with the level of expression of otic markers.

A logical conclusion of the previous experiments was that any cell line, including those with a spontaneous level of otic differentiation (due to the endogenous production of FGFs) if differentiated in the presence of the FGFR inhibitor SU5402, would downregulate otic genes, in a similar fashion to that observed in Shef3 cells. We carried out the standard differentiation protocol in the cell lines H14, Shef1 and ShiPSFF5 with and without SU5402. Unexpectedly we did not observe a clear downregulation of otic genes in cells treated with SU5402.

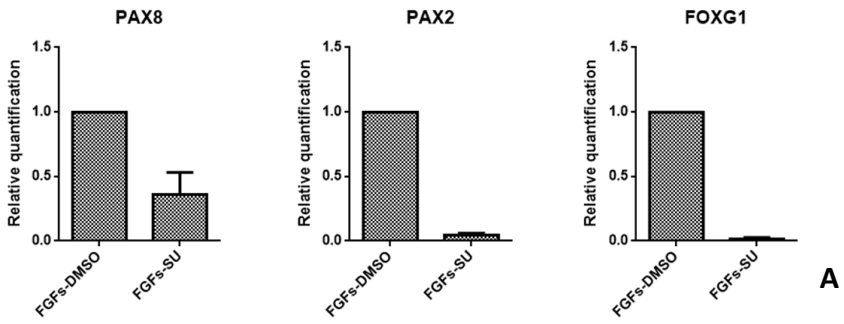
In figure 4.5, cells grown in FGFs-DMSO and FGFs-SU5402 are shown. As we can observe, SU5402 strongly reduced the expression of all otic genes in Shef3 cell line (previous figure and in 4.5 A) and inhibited the expression of *FOXG1* in the hES cell lines H14 and Shef1 as expected. However *PAX2* was only minimally downregulated by SU5402 in H14, and upregulated in Shef1, while *PAX8* was upregulated in both cell lines when the inhibitor was present. Therefore, it seems that these two hES cell lines are intrinsically different to Shef3, and that some otic genes are more responsive than other to FGF activity. In summary, while FGF supplementation is required to trigger the expression of inner ear transcription factors in Shef3, their inclusion in the media seems to be less necessary for H14 and Shef1.

When the hiPS cell line ShiPSFF5 was taken into consideration for the analysis, its response to the inhibitor SU5402 was even more different to Shef3. ShiPSFF5 upregulated *FOXG1* when it was differentiated in the presence of SU5402, a transcription factor that had been shown to be inhibited in all the hES cell lines presented so far. ShiPSFF5 also upregulated *PAX2* when the inhibitor was added, in the same way that Shef1 did. The response to SU compared to DMSO vehicle was the same either in cells in DFNB medium or in FGF treated cells.

Therefore, through this set of experiments we observed a cell-dependent gradual requirement for FGF-mediated otic induction in all the cells, being Shef3 the one presenting the strictest requirement for FGFs during otic differentiation, while ShiPSFF5 presenting an opposite response to FGFs during otic induction.

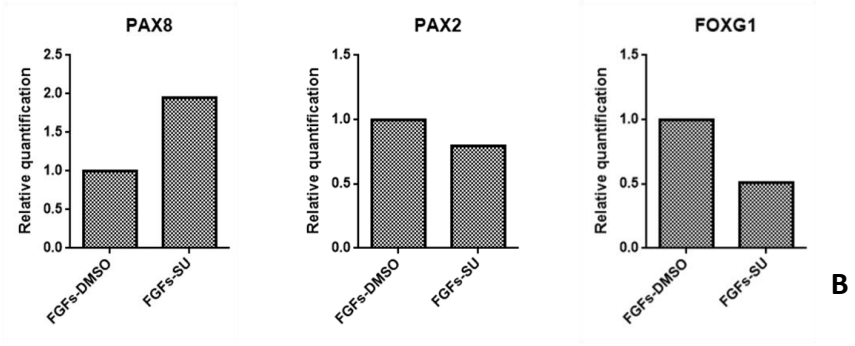
Figure 4.5 **Cell line-dependant requirements of FGF signalling during otic differentiation** Shef3, H14, Shef1, and ShiPSFF5 were differentiated in standard conditions in the presence of FGFs with and without the FGFR inhibitor SU5402. Relative expression is presented in the graphs. Cells treated with FGF3 and FGF10 plus DMSO were used calibrator (defined as "1"). We hypothesise that FGF signalling inhibition with SU5402 would suppress the expression of otic genes. However, there was a different requirement for FGFs during otic differentiation between cell lines. In Shef3 (**A**) all the otic markers were downregulated when cells were exposed to SU5402, in H14 (**B**) the same tendency was observed but the drop in the expression of otic genes was minimal with the exception of *FOXG1* that was strongly downregulated by SU5402. Similarly, *FOXG1* and *DLX5* were downregulated in Shef1 (*C*), but PAX8 and PAX2 were upregulated by SU5402. In ShiPSFF5 (**D**) only DLX5 was downregulated by SU5402. (Error bars are s.e.m. *n*=2 in Shef3 and Shef1).

Shef3



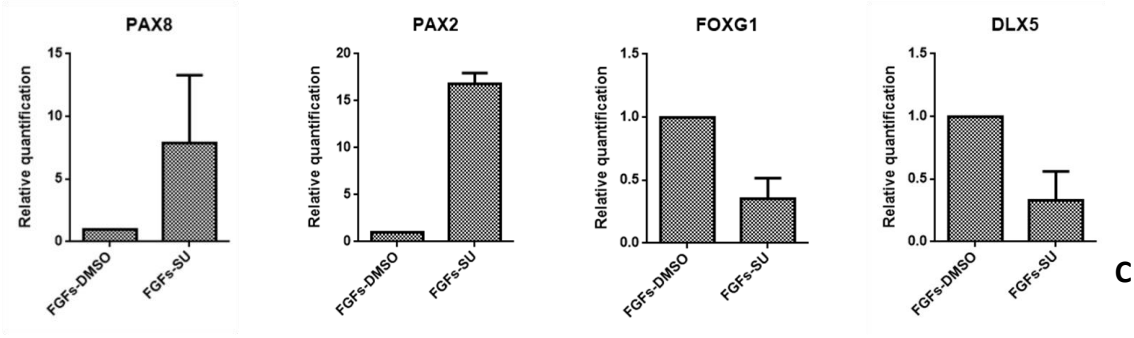
A

H14



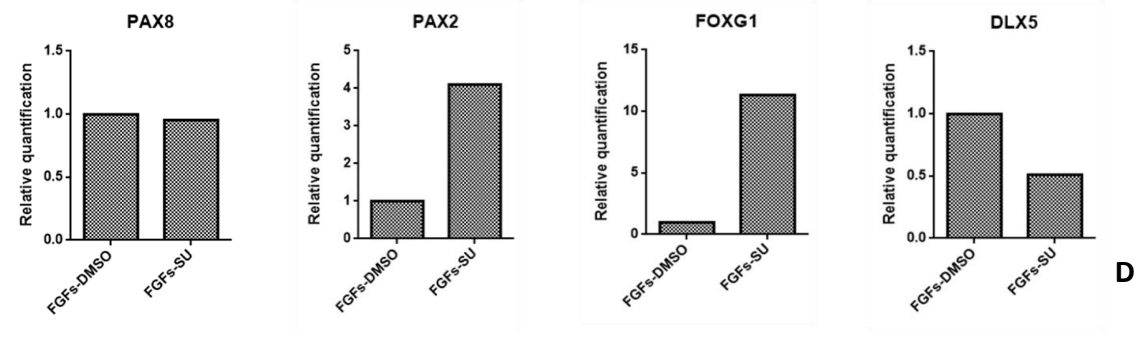
B

Shef1



C

ShiPSFF5



D

The apparent opposite response to SU5402 in the different cells lines could indicate that cells are markedly different to the point that they differentiate towards the same fate with a variable requirement for FGF signalling, indicating perhaps that other pathways may be more relevant.

An alternative explanation to the variable response to FGFs could be due to the strict requirement of a specific level of FGF activity in each cell line. In other words, while some cells would need a high amount of FGFs to differentiate (e.g. Shef3), others lines like ShiPSFF5 readily differentiate with minimal amounts of FGFs, and the extra addition could result inhibitory for otic differentiation. In this case, the SU5402 inhibitor would decrease the level of FGF signalling without completely blocking it, therefore allowing for a lower level of FGF signalling, sufficient to be inductive.

Finally, it would seem that the role of FGFs in proliferation is separate from the otic inducting activity. We observed in chapter 1 that the addition of FGFs to Shef3 cell line increased the growth of these cells compared to those maintained in DFNB medium only. In contrast, the growth rate of ShiPSFF5 was the same in cells maintained in DFNB or FGF medium, in agreement with the endogenous production of FGF10 in DFNB medium.

Thus when we compared the growth of cells differentiated with and without the FGFR inhibitor SU5402, we observed that FGFs are essential for proliferation, independently of the cell line (figure 4.6).The Shef3 graph shows that without the extra addition of FGF3 and FGF10, cell proliferation is reduced (Ctrl-DMSO in figure 4.6 A), and therefore, no further reduction is observed with SU5402 inhibitor. Equally if the inhibitor is added together with the FGFS, the growth is similar to the one observed in Ctrl-DMSO, but reduced compared with in FGFs-DMSO control cells. On the contrary, in the cell lines ShiPSFF5 and H14 that endogenously produce FGFs (H14 not checked), and otic differentiation of cells takes place in DFNB medium, we observed robust growth in DFNB condition, that was strongly reduced by the application of the FGFR inhibitor. In an experiment with H14 (4.6 C), only the DFNB control survived due to a technical problem with the FGF treated cells. Nonetheless, a robust growth was observed in this condition, which was reduced upon the addition of SU5402.

These experiments indicate that FGFs are necessary for the growth of differentiating cells. However taking into consideration the somehow variable response to FGF signalling inhibition in the expression of otic markers between different cell lines (figure 4.5), the growth rates would suggest that FGFs induce the proliferation in a non-selective manner in the different subpopulations of cells, and that the role of FGFs in proliferation can be separated from their otic induction activity.

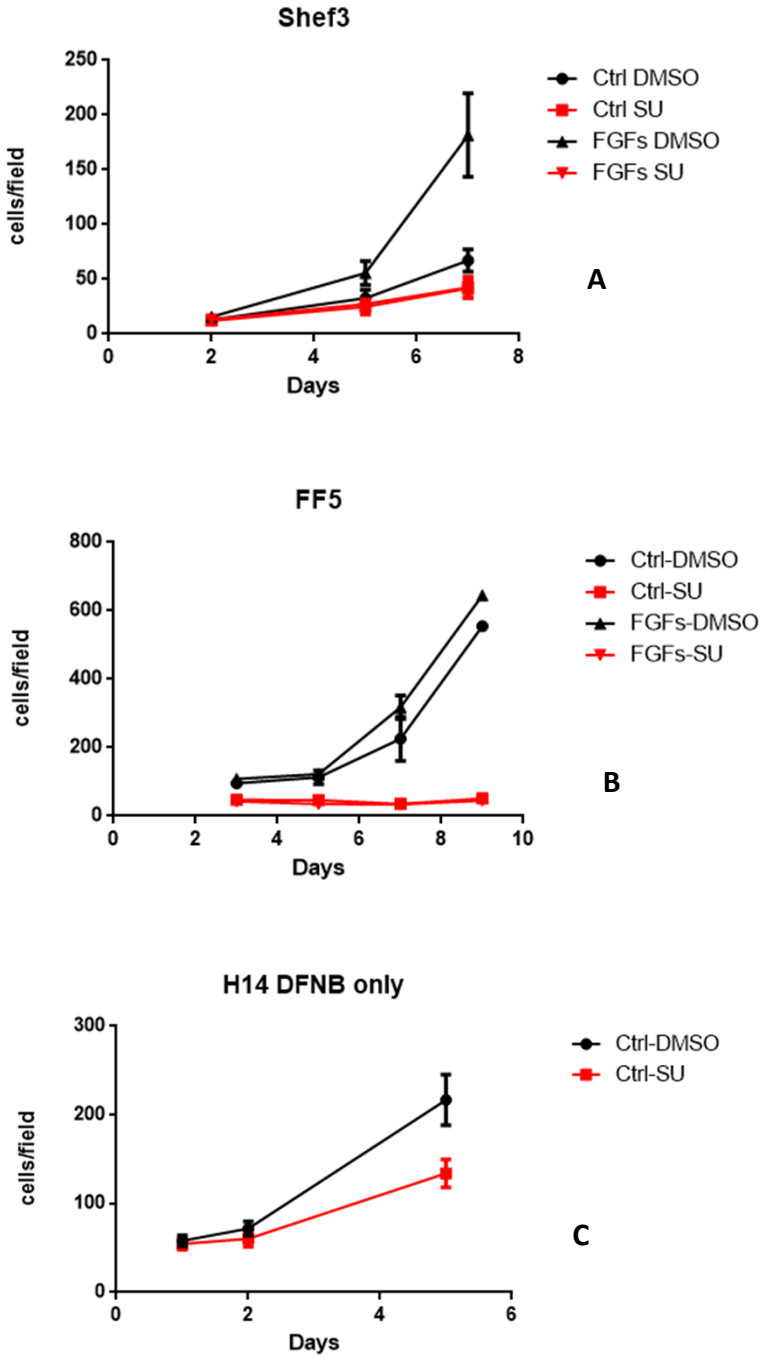


Figure 4.6 **FGF signalling is necessary for the cell proliferation in differentiating cells.** Cells were differentiated in standard conditions in control or FGF (FGF3 and FGF10) supplement medium plus the addition of either FGFR inhibitor (SU5402) or DMSO control. Cell counts were taken at different intervals. In Shef3 the growth rate of FGF-DMSO was more robust that cells without FGFs or cell treated with the SU5402 (**A**). In ShiPSFF5 control and FGF treated cells proliferated at similar rate (**B** black curves), in agreement with endogenous expression of FGF10. In H14, there is an endogenous level of differentiation in DFNB medium as there is ShiPSFF5, and similarly the robust growth in this condition is impaired by the addition of SU5402 (**C**). Therefore these data shows that FGF activity is required for the proliferation of differentiating cells. FGF condition is missing in H14 due to technical circumstances. Black curves are DMSO controls; red ones, SU5402 treatments. Error bars are s.e.m of counts in different fields.

Discussion

FGF signalling is required for otic differentiation

The previous chapter indicated that although highly variable, there was a small but significant difference in the expression levels of cells treated with FGF3 and FGF10 compared with their controls in DFNB medium only. This difference is more robust in Shef3 cell line, where the effects of FGF3 and FGF10 are more reminiscent of mouse inner ear development. Therefore, Shef3 constitutes a good starting point to study otic differentiation *in vitro.* An alternative approach to test the involvement FGFs during the differentiation of otic progenitors, is through the use of a specific FGFR inhibitor SU5402 (Mohammadi, 1997). When Shef 3 cell line was differentiated with FGF3 and FGF10 in the presence of the SU5402, we observed strong suppression in the expression of otic genes when compared to FGF3 and FGF10 treated cells (figure 4.1), in agreement with the role of FGFs in inner ear development in all animal models (Introduction chapter 3). It is remarkable that in some of these studies, the inhibitor SU5402 has resulted to be very useful to dissect the precise role of FGF signalling during otic development. In this regard, it has been shown that prospective placode chick explants of 0-4ss fail to express the otic marker *PAX2* when exposed to SU5402, indicating a critical timing for induction in this model (Martin and Groves, 2006). Maroon and colleagues have also used the SU5402 inhibitor to dissect the specific timing of otic induction in the Zebrafish (Maroon et al., 2002). Their conclusion supports the strict requirement of FGF signalling for the induction of the otic marker *pax2.1* at 60% epiboly.

Otic differentiation is affected by cell density

In chapter 3, we presented a correlation curve showing the negative effect cell density has in the expression of otic genes, particularly *PAX8*. However, cell line differences and other variables at the time of performing the experiments accounted for the large dispersion in ΔCTs. Using the robustness of FGF-dependant otic differentiation observed in Shef3, we tested if cell density could have an inhibitory effect in the expression of otic markers, and if this effect could diminish the efficiency of SU5402 to suppress otic differentiation. The data confirmed that cell density cell had an inhibitory effect in otic gene expression (figure 4.2). Nonetheless, the effect of SU5402 at the two seeding densities tested was very robust.

Cell to cell contact or media conditioning could be the responsible for the inhibitory effect in cells. In this regard, in the previous chapter we suggested that the inhibitory effect could be due to FGFs since the cell density inhibition was less pronounced in DFNB medium than in FGF treated cells. Thus we decided to compare the expression of FGF3 and FGF10 in cells that present a different response when differentiated. In figure 4.3 we observed that ShiPSFF5 cell line differentiated in DFNB medium while Shef3 did not, this correlated with the endogenous production of FGF10 in ShiPSFF5, but not in Shef3, suggesting first that FGF10 is more important for otic differentiation and second, that this endogenous level of FGF10 production is sufficient to induce a high level of expression of otic markers ShiPSFF5 in DFNB, and more FGFs are unnecessary or could even be inhibitory (4.3 C and E). In this regard, Nelson and Svendsen showed that human neural progenitor cells can present dual response to FGF2 stimulation depending on the concentration of the ligand (Nelson and Svendsen, 2006). They showed that maximal neurogenesis is observed at 2pg/ml of FGF2 compared to the control. However a substantial neurogenesis (statistically significant) is observed already at 0.2pg/ml of FGF2 stimulation. This range of concentration is 250,000 times lower to the one we currently use in our differentiation experiments (50ng/ml). If our system presents such sensitivity, this phenomenon could easily account for variation we observe. In Nelson and Svendsen work they also show that neurogenesis is inhibited if the concentration of FGF2 is raised up to 20ng/ml, but not cell proliferation. Therefore, in their system, FGF2 plays a dual role depending on concentration. As it will be discussed shortly,

we suspect that FGFs are also playing at least two separate activities in our differentiation protocol.

FGF3 and FGF10 are not equivalent during otic induction

In conclusion we found that cells produce their own FGF3 and FGF10, the latter one correlating with the spontaneous differentiation observed in ShiPSFF5. We tested the possibility that FGF10 could be more important to induce the expression of the otic markers in Shef3 cells, by differentiating them in FGFs (3 &10), FGF3 or FGF10 only. We found that FGF10 was a more potent otic inducing factor than FGF3 or both FGFs together. In addition, we observed an inhibitory effect in FGF3 treated cells when compared with cells exposed to both FGFs. Although more optimization is needed to corroborate this finding, the data is in agreement with work of Alvarez who found that ectopic otic vesicles can only be induced by missexpression of FGF10 but not FGF3 in the mouse. In this study and also, in Wright and Mansour, the conclusion was that FGF3 and FGF10 act redundantly during inner ear induction since no phenotype was observed in single mutants. Nonetheless, careful examination of single mutant indicates that FGF10 may have a more important role in the formation of the dorsal domain of the otic vesicle, characterized by the expression of *Dxl5*, while Fgf3 plays its role in the maintenance of the ventromedial domain (*Pax2*). In our experiments we also observed that FGF10 induced a stronger upregulation of *DLX5* than *PAX2* when compared with cells treated with both FGFs. In addition *FOXG1* was also robustly upregulated by FGF10. In the ear, it has been found that both genes are involved in the formation of the vestibular canals, and *Foxg1* mutant present a stronger phenotype when combined with FGF10 heterozygous mutants, suggesting *Foxg1* as component of the same developmental circuitry than Fgf10 (Pauley et al., 2006). Thus our data supports the not equivalent role of FGF3 and FGF10 during otic differentiation. In this regard, FGF2 but not other FGFs, was found to induce the expression of *PAX2* in chick explants of anterior ectoderm (Martin and Groves, 2006).

Cell line heterogeneity can be caused by the endogenous production of FGFs

The data presented before, suggested that the endogenous production of FGFs was responsible for the minimal difference between DFNB and FGF treated cells observed in the relative expression of otic markers. We decided to test is FGF signalling was indeed necessary for otic differentiation in the rest of the cell lines (figure 4.5), as it was the case in Shef3.

Cells were differentiated in FGFs alone or FGFs plus SU5402 (figure 4.5). The results of this set of experiments were somehow paradoxical.

In this regard, differentiation propensity across cell lines is accepted and well documented (Hu et al., 2010; Mehta et al., 2010; Osafune et al., 2008). The common view is that pluripotent stem cells share common transcriptional networks, but there are differences in their epigenetic status that become evident during differentiation, accounting for the variation in the differentiation efficiency among different cell lines. Even though, it was completely unexpected to find lines with almost opposite response to FGF inhibition (e.g. Shef3 vs. ShiPSFF5, Shef1). This variability is unlikely to have been generated by changes in the pluripotency state of the original population, since the cells are assessed at periodic intervals at the Centre for Stem Cell Biology, and expression of pristine pluripotent markers has been routinely observed by colleagues and me through flow cytometry analysis. It is noteworthy that some of the cell lines used in Hu's work (Hu et al., 2010) responded differentially to growth factors. In that study they used either bFGF or SMAD inhibitors to differentiate hES and hiPS cells towards neuroepithelial progenitors. Some of the lines increased the number of PAX6+ cells when treated with bFGF and others almost didn't respond, and surprisingly, one line responded negatively to it, showing a decrease in the percentage of PAX6+ cells. Perhaps there are variable responses to the level of FGF in pluripotent stem cells. It has been shown before that the level of Oct4 in mouse ES cells dictates if cells become endoderm (Oct4 upregulation), trophoectoderm (Oct4 downregulation) or if they stay undifferentiated with a steady level of Oct4 expression (Niwa et al., 2000). A similar phenomenon could be happening with FGF signalling during

differentiation, where otic differentiation is accomplished within certain levels of FGF activity.

This level of FGF activity required for otic differentiation could be determined intrinsically by the endogenous production of some components of the pathway like FGFs, FGFRs, ERk1/2, docking proteins etc, and the balance with other pathways. In this regard, it has been observed that there is heterogeneity in the level of endogenous WNT signalling activation within the stem cell niche, and cells with low WNT activity differentiate better into neuroectodermal lineage (Blauwkamp et al., 2012).

As already discussed earlier in this chapter, there are other systems known to be highly sensitive to FGF concentration (Nelson and Svendsen, 2006), where no FGF triggers neurogenesis that is further enhanced by increasing FGF2 concentrations up to a threshold, above which an opposite response is observed. If this is true here, it could explain the paradoxical results obtained with the inhibitor. If a cell line with high endogenous FGF secretion like ShiPSFF5 is supplemented with exogenous FGFs, it could trigger an inhibition of otic markers when compared to the DFNB control (Fig 4.3 E and F). On the other hand, when cells under this treatment are exposed to the inhibitor, the FGF activity is dampened to a level in the range to be inductive. Therefore the explanation would be that SU5402 is working below saturation, without completely blocking the pathway. In the future, it would be interesting to carry out titration experiments with the SU5402 to test this idea. In this regard titration experiments with FGFs are discussed in chapter 7.

Another possibility contemplated briefly in this chapter is the role FGFs during the proliferation of cells as a different activity separate from their otic inductive role. Cells that presented a variable repose to FGF signalling inhibition evidenced at QPCR level were compared in their growth patterns. In all of them, we observed that SU5402 suppressed the growth of the cells. In mice double mutant for *Fgf3/Fgf10*, sometimes smaller otic vesicles were seen, but these were later shown to be caused by lack of induction rather than defects in proliferation or apoptosis (Wright and Mansour, 2003). The same conclusion was observed in the fish double mutant for *fgf3/fgf8* (Maroon et al., 2002). On the contrary, the mouse Fgfr2 mutant dies at E11.5 due to defects in placental development, associated with impaired proliferation in the trophoblast (Xu et al., 1998). The isoform IIIb of Fgfr2 is

hypothesised to be the responsible the Fgf3 and Fgf10 otic induction, consequently, mouse mutants of Fgfr2IIIb phenocopy Fgf3 and Fgf10, although in a milder way. However in the Fgfr2IIIb mutant, increased apoptosis is observed in the in vestibulocochlear ganglia at E13 (Pirvola et al., 2000). Therefore, as observed in mouse development, our data indicates that FGFs play dual role in otic induction and in cells proliferation. In chapter 6 and 7, we will discuss the role of FGFR2IIIb in our system. At the moment, it is important to stress that FGF signalling induce the growth of all the cell lines, but this activity is not specific to the otic progenitors, otherwise we would expect a downregulation of otic markers FGFs-SU compared with the DMSO control. Therefore it could be that cells produce other FGFs apart from FGF3 and FGF10, that could stimulate the proliferation of other progenitors as well, or that FGF3 and FGF10 are supporting the growth of cells originated independently of the FGF pathway.