Chapter 8

Conclusions

Through this thesis we have learned a number of things regarding the differentiation of hESCs towards otic progenitors *in vitro*. The general message we should take is that the process takes place with many resemblances observed during inner ear development *in vivo*. However, the second message should be that in our hands the system is still highly variable, and this leaves room for improvement in the future.

Importantly, through a series of analysis we have gained an insight into the sources of variation in the system, and in this final chapter we propose future directions that should give us valuable and interesting information for the delineation of a better differentiation protocol. Finally we think that through the improvement of the differentiation conditions, the transplantation of otic progenitors will produce better hearing restoration. In addition, a clear understanding of the types of cells transplanted in the inner ear will be necessary for the translational application of hES cell based therapies.

Variation between pluripotent stem cell lines

We started this work studying the otic differentiation induced by FGF3 and FGF10 in the hES cell line Shef3. We observed that these FGFs induced the expression of *PAX8, PAX2* and *FOXG1*, important transcription factors with conserved function during inner ear development. The robust effect observed by FGF3 and FGF10 was in agreement with all the animal models of inner ear development, stressing the crucial role of FGFs during the induction of the otic placode. However, the finding also raises some questions regarding the identity of the cells induced by FGF3 and FGF10 in our protocol. In the chicken, it has been observed that FGFs can induce the expression of *PAX2* only in a region of the ectoderm that surrounds the head neural plate (Litsiou et al., 2005; Martin and Groves, 2006). This region has been characterized in other models organisms as well. We don't know if such requirement exists in our system, but we know that expression of the neuroectodermal marker nestin can be observed as early as 4 hrs after differentiation of mouse embryonic stem cells (Tropepe et al., 2001). Therefore, there the progenitors competent to respond to FGF3 and FGF10 are likely to be produced very quickly during differentiation. The otic inductive role of FGFs in our protocol received further support from the observation that

blocking FGF signalling with the FGF receptor inhibitor SU5402, wiped out the expression of the otic markers in Shef3 (figure 4.1 and 4.2).

Endogenous production of FGFs

In the hES cell field it is very important that once a model of differentiation is established, to look at other cell lines, since these are known to be heterogeneous (Mehta et al., 2010; Osafune et al., 2008). In our study, we observed large variation in the results across differentiation experiments with different cells lines. In light of this variation, we carried out different types of analysis in the QPCR data set of all the differentiation experiments. Through this analysis, we tried to figure out which were the possible sources of the variation in our system. In the first place, as hypothesised, we found that cell lines could be clustered in different groups depending in their response to FGF3 and FGF10, when compared with cells in control differentiation medium (DFNB). We observed that Shef3 and hiPS cell line FF1 were similar to each other, but different to FF5, Shef1, or H14. While Shef3 and FF1 responded to FGFs upregulating *PAX8*, *PAX2* and *FOXG1*, the other cell lines did not. What seems to be happening in this group of cells, is that there is a high level of expression of otic transcription factors in the DFNB control media, similar to the one observed in FGF-treated Shef3 and FF1. In addition, when we looked at the growth rate in different cell lines, we observed that in Shef3, there was robust growth induced by the FGF treatment but not in cells maintained in DFNB medium, in agreement with the role of FGFs in proliferation (although proliferation or apoptosis were not strictly measured). In the contrary, in FF5 and H14, we observed that the growth rate was equally robust in DFNB and FGF supplemented medium (figure 4.6). Together, this was an indication that FGFs are endogenously produced in some cells and not in others. In fact we observed that FGF10 was produced by the hiPSC line FF5 in DFNB medium, while this was not expressed in the cell line Shef3. This raised another question, are FGF3 and FGF10 equivalent to each other? In the past we never considered this possibility because FGF3 and FGF10 are redundant during the formation of the inner ear (Wright and Mansour). However, when we made the comparison between FGF3 and FGF10, FGF10 induced a higher expression of otic markers when compared to

both FGFs together or FGF3 alone. This highlights that *in vitro* and *in vivo* approaches have their own strengths and can be complementary.

From these experiments we concluded that cell lines are not different in their response to FGFs, but rather in the endogenous level of FGF production (FGF10 in particular). In this regard, hES cells have been found to secrete enough FGF2 to sustain their undifferentiated growth. Consequently at higher cell densities more FGFs are found in the media (Dvorak et al., 2005). This argument leads me to explain another of our findings; the effect of cell density. When analysing the whole data set of differentiation experiments, we observed negative correlation between cell density and the absolute level of otic genes expression (figure 3.6). At higher cell density, a decrease in the expression of otic genes is observed. If endogenous secretion of FGFs is responsible for the differentiation observed in DFNB medium, then, increasing cell seeding density should have a positive effect in differentiation. The explanation that we found for this paradoxical observation was that FGFs play a dual role depending on concentration. In other system, like lens progenitors or neural progenitors, it has been observed that FGF triggers divergent outcomes depending on the concentration, low FGF induce proliferation, high induce differentiation (Lovicu and McAvoy, 2001) or the opposite (Nelson and Svendsen, 2006). In support we observed that the slope of the correlation curve is increased in FGF treated cells, meaning that the cell density inhibition is potentiated by the extra addition of FGFs in the medium. These findings suggest that FGFs may be secreted in high amount in the medium. In the future, it will be interesting to quantify FGF10 in conditioned medium of differentiating cells. The effect of cell density has been observed in other experiments as well. In Shef3, differentiated cells seeded at 4000 and 8000 cells/cm² showed that the expression of otic markers decreased when cells were seeded at higher density. It is important to mention that we still use 8000 cells/cm² as a seeding density since lower ones compromise the survival of the cells. ROCK inhibitor has been found to increase the survival of hESCs after dissociation into single cells (Watanabe et al., 2007), this could be a possible candidate to add in the differentiation medium.

The cell density and the endogenous secretion of FGF10, could explain the divergent response to SU5402 between FF5and Shef3 as well. In Shef3 we observed a complete inhibition of otic markers, but in FF5 there was an upregulation of otic genes when the

inhibitor was added. The explanation in this case would be that the inhibitor is reducing FGF activity in Shef3 so that otic induction is blocked, but since FF5 (and potentially Shef1 as well) produce FGFs in high amount, the inhibitor in this case is reducing FGFs activity to a level where they are otic inductive. In fact, in the titration experiments in chapter 7, we observed a higher expression of PAX8 when the level of FGFs is reduced.

We have observed that part of the variation between cells lines is due to endogenous production of FGFs. Still our data is in agreement with the development model that stresses FGFs as key molecules in the formation of the inner ear. In this regard, despite the variation in our system, there was significant difference in the expression level of otic markers between cells treated with FGFs and those in DFNB medium only.

Time course of otic gene expression

We thought that part of this variation could be due to length of the differentiation. When working in cell culture system is easier to observe the most direct response to an experimental variable within a short period of time. In addition, we have observed in our PCR data a lower expression of the otic marker PAX8 compared to PAX2 and FOXG1. Given the early and transient expression of this marker during inner ear development (Heller and Brandli, 1999; Pfeffer et al., 1998) we thought to analyse the expression of these genes before day 12 of differentiation (the standard length of the experiments). Through this analysis we observed that all the otic markers are expressed at a higher level during the first half of the differentiation protocol. Immunofluorescence analysis also indicated that the percentage of double positive cells for PAX8/PAX2 also dropped from day 6 to day 12. In addition, the same tendency was observed in Shef3 and in Shef1 cell lines, indicating that they follow the same developmental route with the same pattern of expression when the temporal analysis is considered. More important, this pattern of expression is similar to the observed *in vivo*, *Pax8* having an early transient expression in the prospective otic placode, followed by *Pax2* that initially present a wide expression domain and then becomes restricted to a subdomain in the otic vesicle (Bouchard et al., 2010; Ohyama and Groves,

2004). The same occur in the case of *Foxg1* that appears later in the ear (Pauley et al., 2006).

The role of FGFR2IIIb

Finally, another of the aims in this project was to define to possible role of FGFR2IIIb, the receptor for FGF3 and FGF10. We attempted to see its role during differentiation by a loss or gain of function approaches. We established an inducible knockdown system that resulted being very inefficient and therefore did not provide the experimental insights we wanted. In contrast, the overexpression of FGFR2IIIb robustly induced the expression of *PAX8, PAX2* and *FOXG1* in both, FGF treated cells and those in DFNB. These results were observed in all the cells lines analysed H14, Shef1 and Shef3. Therefore, the experimental system conciliates differences observed across cell lines by showing that otic differentiation is enhanced if one of the putative components of the pathway is increased. In addition, these results are in agreement with the early expression pattern of this receptor in the mouse before the formation of the otic placode (Wright and Mansour, 2003), and also with the knock out mouse of this receptor isoform that shows gross morphological abnormalities in the formation of the inner ear (Pirvola et al., 2000). Nonetheless, it is must be mention that early expression of otic genes has not been analyzed in the knock out mouse, and this work provides direct evidence of the involvement of this receptor in the expression of early otic genes in human system.

All together the results of this study are in agreement with many aspects of otic induction in animal models of inner ear development. In addition, our results provided insights to investigate in more detail key aspects that could improve otic differentiation in the future, for example: the use of FGF10, low concentration of FGFs, perhaps the use of specific heparan sulfate proteoglycans that could enhance the activity through FGFR2IIIb, and the sorting of FGFR2IIIb positive cells from the hESC niche to drive more homogeneous differentiation.