Chapter 5

Expression time-course of otic transcription factors during differentiation

Introduction

During development, there are a number of key transcription factors orchestrating a cascade of morphogenetic events whose function has been shown to be conserved from flies to humans. Within this category we can find the PAX gene family members. For example, the pioneering work of Halder et al (Halder et al., 1995) showed that missexpression of *ey* or its homologue *Pax6* was able to trigger the development of ectopic eyes in *Drosophila*, demonstrating its conserved role as a master regulator of the eye development. However, *Pax6* is not an exception, in fact the rest of the PAX family share similar functions in neurogenesis, myogenesis and the development of sensory placodes in different animals (Chi and Epstein, 2002). In the case of inner ear development, *Pax8* and *Pax2* are observed from the earliest stages of otic development, and their function is essential for the development of this structure. *pax8* has been shown to be one of the earliest markers being expressed in the prospective otic placode of Zebrafish (Pfeffer et al., 1998) and *Xenopus* (Heller and Brandli, 1999). The expression of *pax8* is early and transient, and it is followed by *pax2* expression, which overlaps at the very beginning with *pax8* but then it remains switched on for the rest of the otic developmental period (Heller and Brandli, 1997, 1999). In the mouse, a similar expression pattern has been described for both genes (Bouchard et al., 2004; Nornes et al., 1990; Puschel et al., 1992), *Pax8* being detected slightly before an otic placode can be morphologically distinguished from the rest of the ectoderm, and then is followed by *Pax2* (Bouchard et al., 2010; Ohyama and Groves, 2004), which is strongly expressed in the ventromedial part of the otocyst and later on, in the developing cochlear duct, vestibular epithelia and inner hair cells (Burton et al., 2004; Lawoko-Kerali et al., 2004). In agreement with its expression pattern, *Pax2* mutant mice present a reduction in the size of the cochlea or its complete absence (Burton et al., 2004; Torres et al., 1996). It is interesting to mention that these mice also lack spiral ganglion, perhaps an indirect consequence of malformation of the cochlea, since *Pax2* mutation does not affect the neurogenic domain (Burton et al., 2004) and neither is expressed in the vestibulocochlear ganglion (Lawoko-Kerali et al., 2004). In summary, both markers are master regulators of otic development, *Pax2* with a more conserved and relevant function

than *Pax8*, but Pax8 being the earliest one and *Pax2* the one expressed for a longer developmental window. These elements justify the focus of our analysis in these genes, but also raise the issue of the importance of temporal analysis during differentiation, the core of this section.

Apart from the *Pax* genes, other transcription factors commonly used to define otic progenitors in our assays are *FOXG1* and *DLX5*. Both genes are observed from 1 somite stage (ss) in the mouse in a wide expression domain that includes all the head ectoderm surrounding the neural plate (Hatini et al., 1999; Quint et al., 2000; Yang et al., 1998). This horseshoe shaped region that has been identified as the preplacodal domain is the common ground where all the cranial placodes arise (Chapter 1). After this early pattern of expression, *Foxg1* remains switched on in all cranial placodes, and in the otic vesicle in particular, is strongly expressed in the cochlea and in the delaminating neuroblasts that give origin to the vestibulocochlear ganglia (Hatini et al., 1999). Opposite to *Foxg1* expression, *Dlx5* is expressed exclusively in the dorsolateral region of the otocyst that will develop into the vestibular system of the ear. This expression pattern is in agreement with the absence of vestibular structures in the *Dlx5/Dlx6* null mouse (Robledo and Lufkin, 2006). In the case of the *Foxg1* mutant, the otocyst and vestibulocochlear ganglia are formed normally, but at a later stage the cochlea and the spiral ganglion are massively reduced in size, with a few remaining fibres failing to project to the brain stem (Pauley et al., 2006). In is interesting to notice that some of the inner ear defects in the *Foxg1* mutant are mimicked by the *Pax2* mutant. In this regard, in the analysis presented in chapter 3, we observed that the expression pattern of *PAX2* generally follows the one of *FOXG1* in a number of cell lines, indicating that our protocol may trigger differentiation towards all subdomains found in the otocyst.

Until now we have described the importance of these transcription factors in inner ear development and their temporal expression pattern. In our differentiation experiments, we have noticed that the expression level of *PAX* genes is generally low compared with the expression levels of *FOXG1*, and taking into account the early and transient expression of PAX genes during development, we considered the possibility that it may be easy to detect them if we carry out our gene expression analysis at an earlier stage in the protocol. Also, In line with this hypothesis, *FOXG1* was the marker with the highest level of expression in

every experiment consistent with its prolonged expression during inner ear development in the mouse (Pauley et al., 2006). Therefore we decided to look at the expression of *PAX8, PAX2* and *FOXG1* at earlier time points.

Results

The time course of otic differentiation

We focus our analysis in two human hES cells lines: Shef1, a cell line with a poor response to the FGF3 and FGF10 treatment, that upregulates *PAX8* marginally in most of the experiments while PAX2 or FOXG1 are almost never induced by FGFs; and Shef3, the cell line that differentiates most efficiently in FGFs, upregulating the three markers in most of the experiments (chapter 2). We then differentiated these cells for 12 days, collecting FGF treated samples at 2, 4, 6, 8, 10 and 12 days from the start of the experiment. Also, as a calibrator for the relative expression analysis in these experiments, we used cDNA from undifferentiated hES cells. In this regard, undifferentiated hES is a control that should be interpreted with care when used as reference, since the cells are maintained in a metastable state and fluctuations in gene expression change from batch to batch. To minimize this, we used the average expression value of different batches of hESCs stocks, and use the same sample to normalise each experiment, the purpose was to put the relative expression values of both cell lines at the same level.

Interestingly, when we compared the course of expression of otic markers we found that all of them were present at a much higher level during the first half of the protocol (figure 5.1). Although the data was still with some outliers and variation, the tendency and overall curve shape was similar in both cell lines and for the three otic markers. Therefore, despite that one of the ideas developed in the previous chapter was that Shef1 and other cell lines were intrinsically different to Shef3, when including this temporal dimension on the analysis, the cell lines ended up being more similar to each other than previously thought. These profiles are in agreement with the temporal expression pattern of those markers during inner ear development mentioned earlier; supporting the view that otic induction takes places very early on during our differentiation protocol. Noticeable, was the quick and robust drop in the expression of *PAX8* in both cell lines (figure 5.1), in agreement with in transient requirement in inner ear development, and consistent with the low expression levels of this transcript when looking at the raw data in other experiments (not shown).

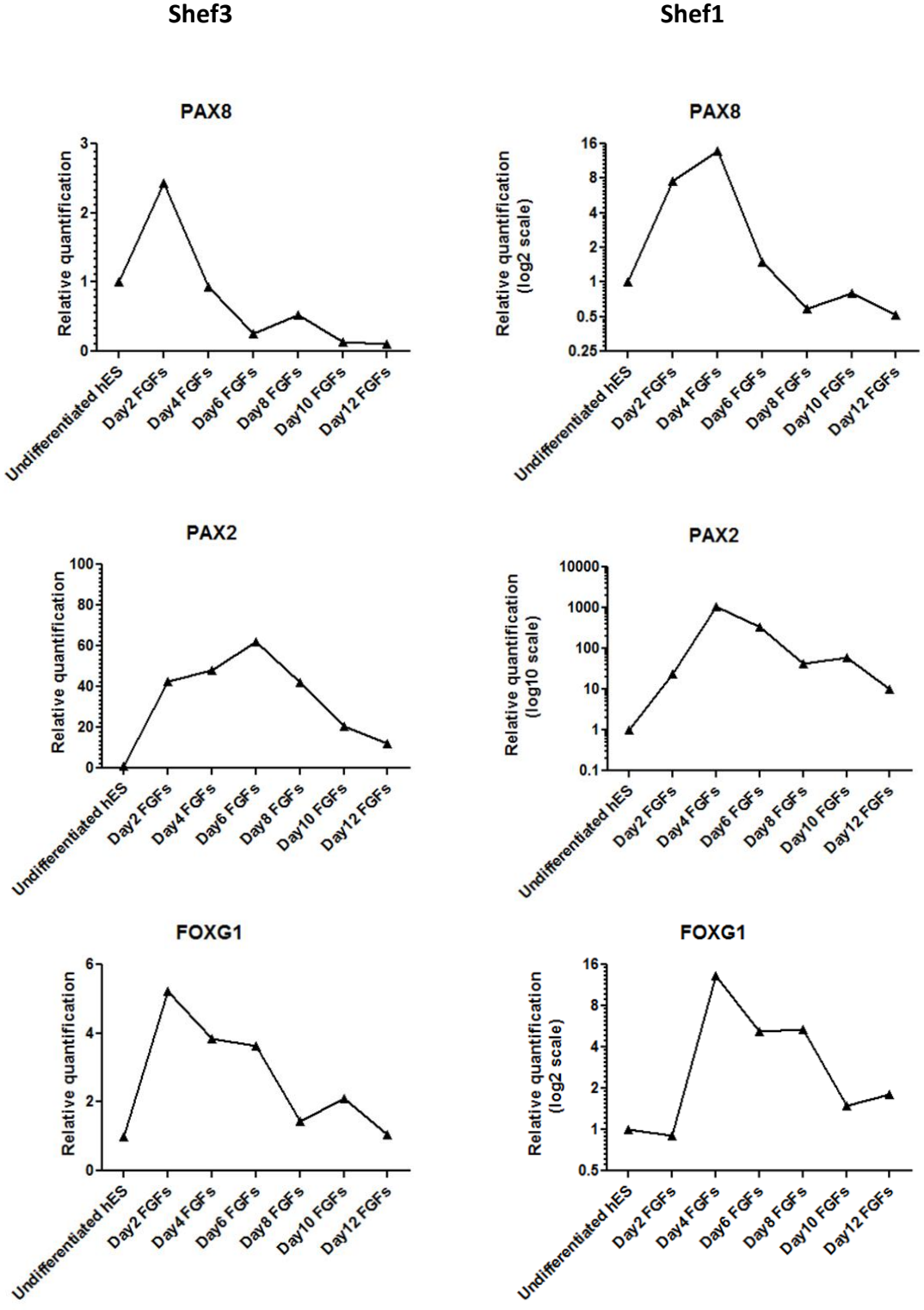


Figure 5.1. E**xpression time course of otic markers during differentiation**. Shef3 and Shef 1, left and right column respectively, were differentiated for 12 days in FGF3 and FGF10 medium with samples being collected every 2 days starting at day 2. Relative expression of Q-PCR data is presented. The same pull of undifferentiated hESCs were used as a calibrator (defined as "1"), in both lines. We can observe that the pattern of expression is very similar in both Shef3 and Shef1 for all the otic markers analysed. *PAX8, PAX2* and *FOXG1* were highly expressed during the first half of the differentiation protocol when compared with the day 12 (standard length of the experiment). These data indicate that Shef1 and Shef3, cell line considered different in their response to FGFs, are indeed similar when the expression time-course of otic genes is analysed. n=2 in Shef3; n=1 in Shef1

PAX8 and PAX2 proteins confirm early otic differentiation

We wanted to confirm these findings by immunofluorescence. Nevertheless, in the past it has been difficult to detect PAX8 protein expression for a number of reasons; either because we were looking at a very advanced stage of otic induction were this gene was already being downregulated (especially if some cell lines were differentiating faster than others), or because we were using conditions that were suboptimal (i.e. wrong cell densities, etc). To compound the problem, the antibodies available have shown a very low affinity, making it even more difficult to detect a low-abundance protein.

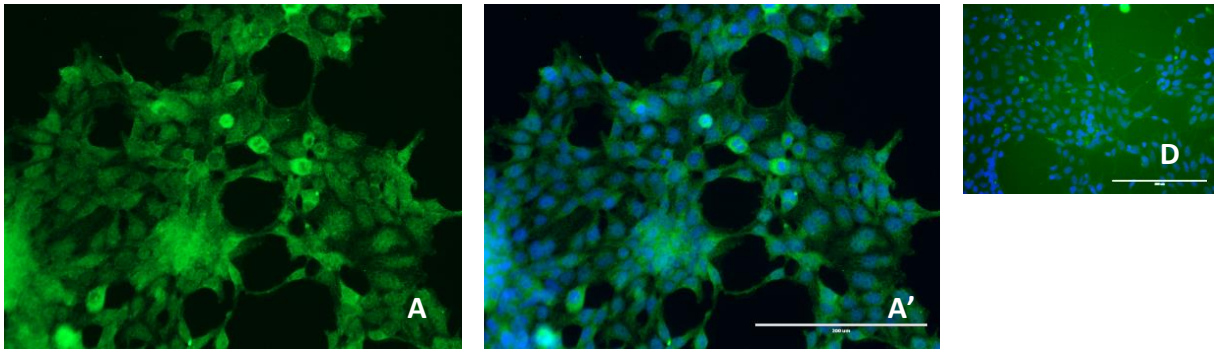
Therefore, we decided to test a panel of antibodies in HEKs293 cells. We confirmed by using the human protein atlas (http://www.proteinatlas.org/) that PAX8 protein should be expressed in 293 cells. In addition, the fact that this cell line comes from embryonic kidney (Graham et al 1977), although its precise cell of origin is unknown, makes them an ideal control to test for PAX proteins, since they are also involved in kidney development. Moreover, this cell line is easy to grow and can save considerable amount of time when testing transfections, antibodies, etc. Pictures of immunofluorescence carried out in these cells are shown in figure 5.2.

We tested three different antibodies: a goat polyclonal (ab13611 ABCAM) and two mouse monoclonal from Proteintech (60145-4 and 66073-1). Immunofluorescence protocol can be found in methods (Chapter 2).

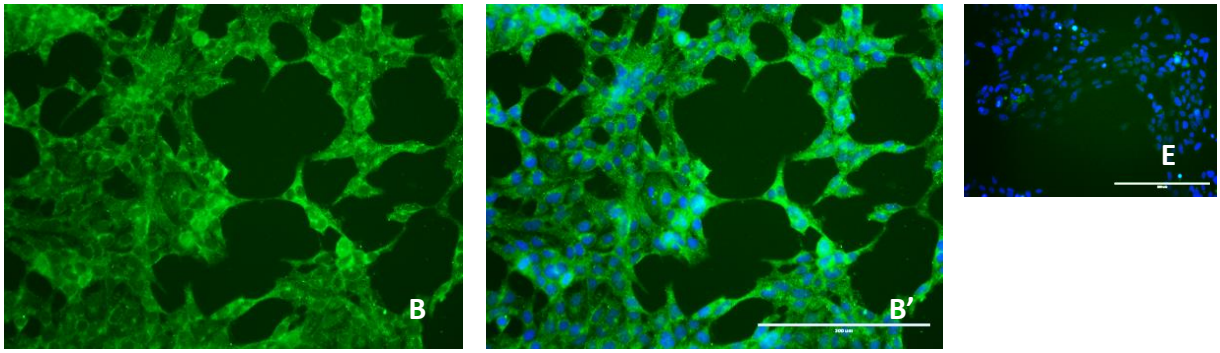
From the three antibodies, only the anti-PAX8 66073-1 (figure 5.2 C, C') showed the expected pattern of expression, with a clear nuclear localization, and was therefore selected for our differentiation experiments. The other two antibodies stained very strongly the cytoplasm and the cell membrane, the reason for that is not completely clear, but we have observed that pattern of expression in other cell lines when using these antibodies.

HEK-293 cells

Goat anti-PAX8



Mouse anti-PAX8



Mouse anti-PAX8 (66173-1)

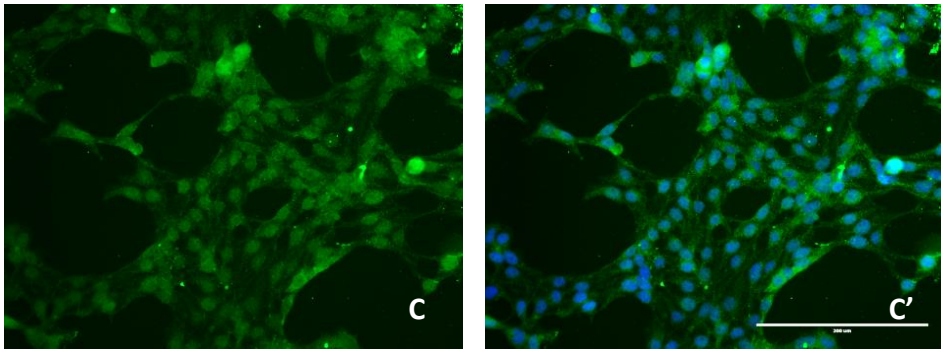


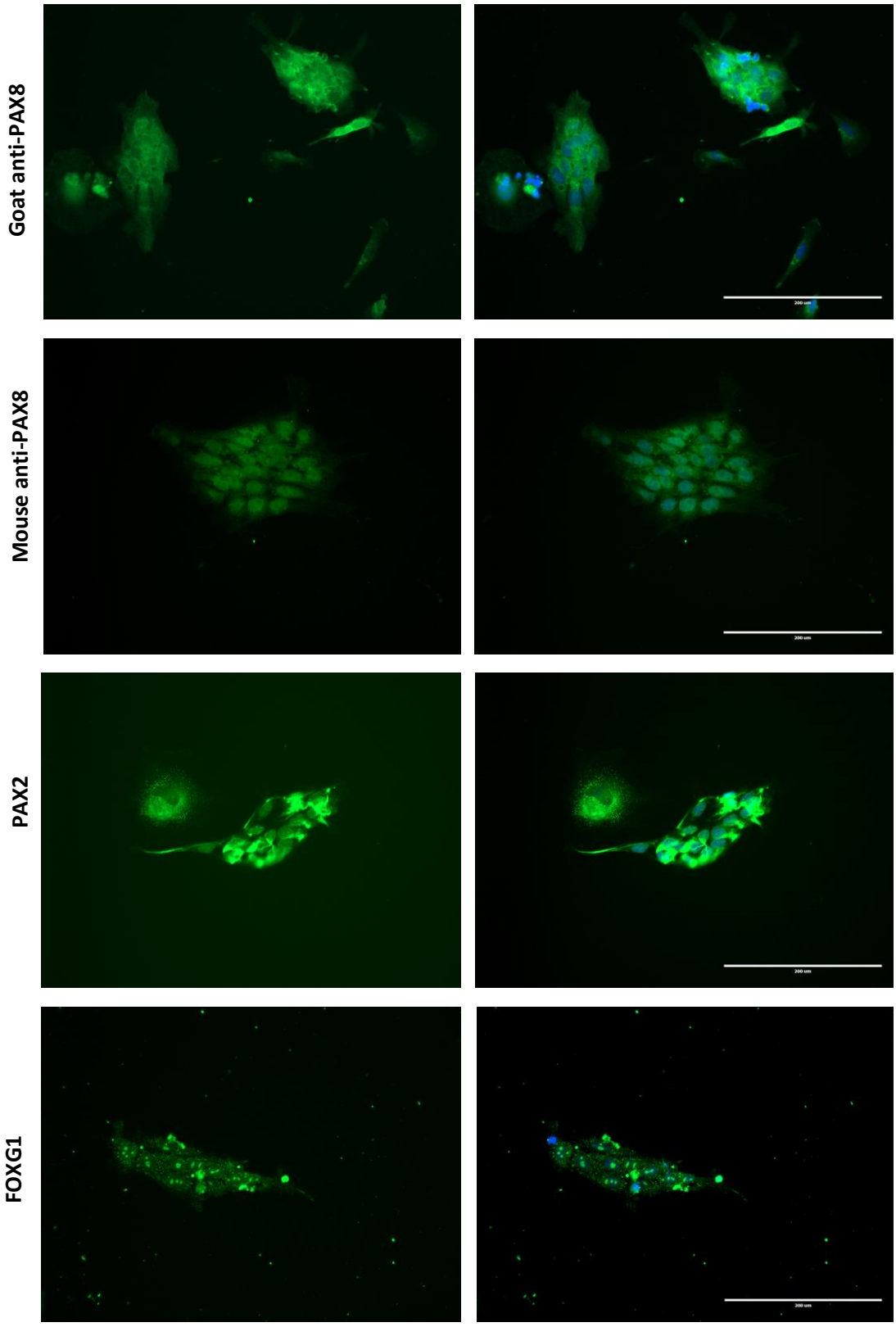
Figure 5.2 **PAX8 antibody test in 293 cells**. HEKs 293 cells were used as a positive control to test three different antibodies. In **A** and **A'** cells labelled with Goat polyclonal anti-PAX8 (13611, Abcam). **B** and **B'** show staining with a mouse monoclonal (60145-4, Proteintech). Although the signal was strong, the cells showed a lot of cytoplasmic background and the nuclear staining was diffuse with goat anti-PAX8 (C), while the mouse monoclonal did not showed any nuclear expression (**B**). In **C** and **C'** cells immunostained with the mouse monoclonal 66173-1 (Proteintech), showing the clear localization expected for a transcription factor. Negative control for goat and mouse antibodies is shown in **D** and **E** respectively. In merge images, nuclei were stained with Hoechst. Scale bar, 200µm

We next interrogated, if hESCs Shef1 and Shef3 differentiated for 48 hrs in FGFs (FGF3 and FGF10), expressed otic markers at this early time point. In this experiment we also verified if the goat anti-PAX8 had the same cytoplasmic background as the one observed in 293 cells (figure 3). There was a possibility that the cytoplasmic background observed in 293 and in other experiments using this antibody corresponded to proteolytic degradation of the PAX protein, given the late time point in the analysis (12 days).

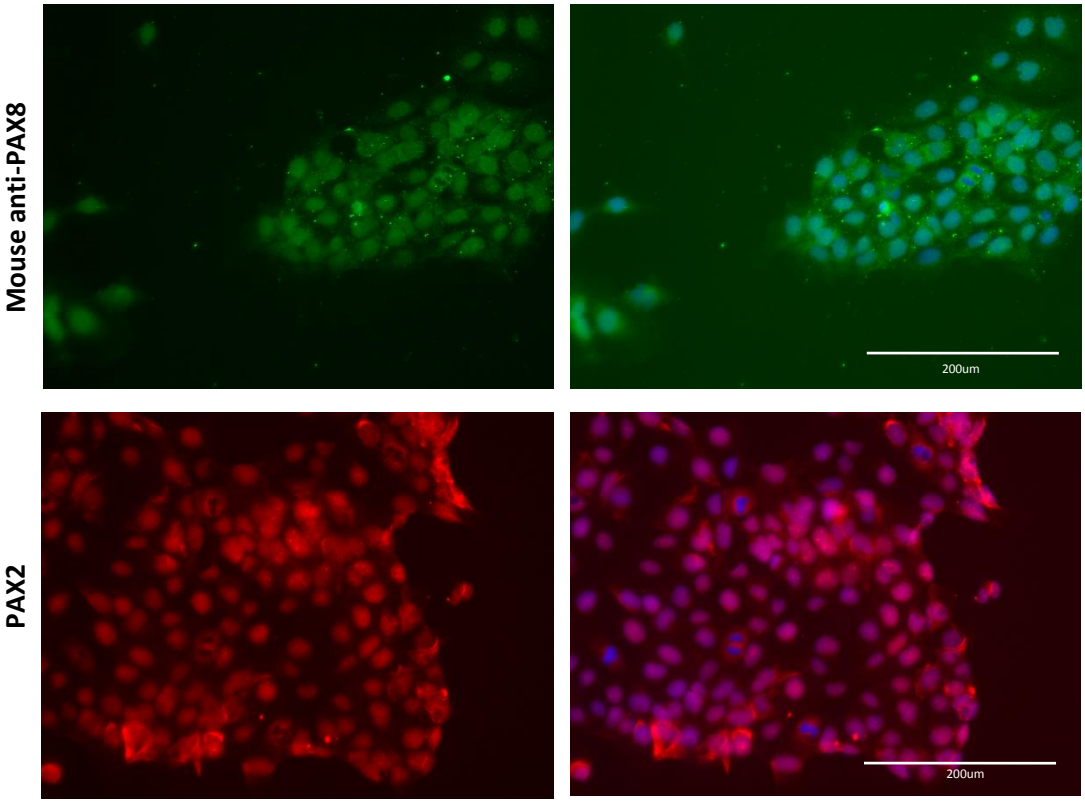
We observed that all the otic markers analysed were already expressed at 48hrs, confirming our Q-PCR results and suggesting that otic induction is taking place very early during the differentiation process. In line with these results, Tropepe et al found expression of the neuroectodermal marker Nestin in differentiating mES cells as early as 4hrs after induction (Tropepe et al., 2001). Although they use a different species, and their report refers to neural stem cell differentiation, it was remarkable how fast the induction took place. In addition, the fact that they use a similar protocol to the one we are currently using supports our idea of a quick differentiation process.

Figure 5.3 **Otic markers can be found by immunofluorescence at 48hrs of differentiation**. The hES cell lines Shef1 and Shef3 were differentiated in FGF medium (FGF3 and FGF10) and otic markers expression was assessed at 48hrs after induction by immunofluorescence. Goat anti-PAX8 (**A**) staining is shown first. We observed the same cytoplasm background found in 293 cells (figure 5.2). The monoclonal anti-PAX8 66173-1 on the other hand presented a defined nuclear localization in Shef1 (**B, B'**) and Shef3 (**E, E'**). **C** and **F** show immunofluorescence of PAX2 in Shef1 and Shef3 respectively. In **D** immunofluorescence of FOXG1 in Shef1 is shown. Merge image with nuclear Hoeschst staining is presented on the right side of every marker. Scale bar 200µm

Shef1 48hrs differentiation

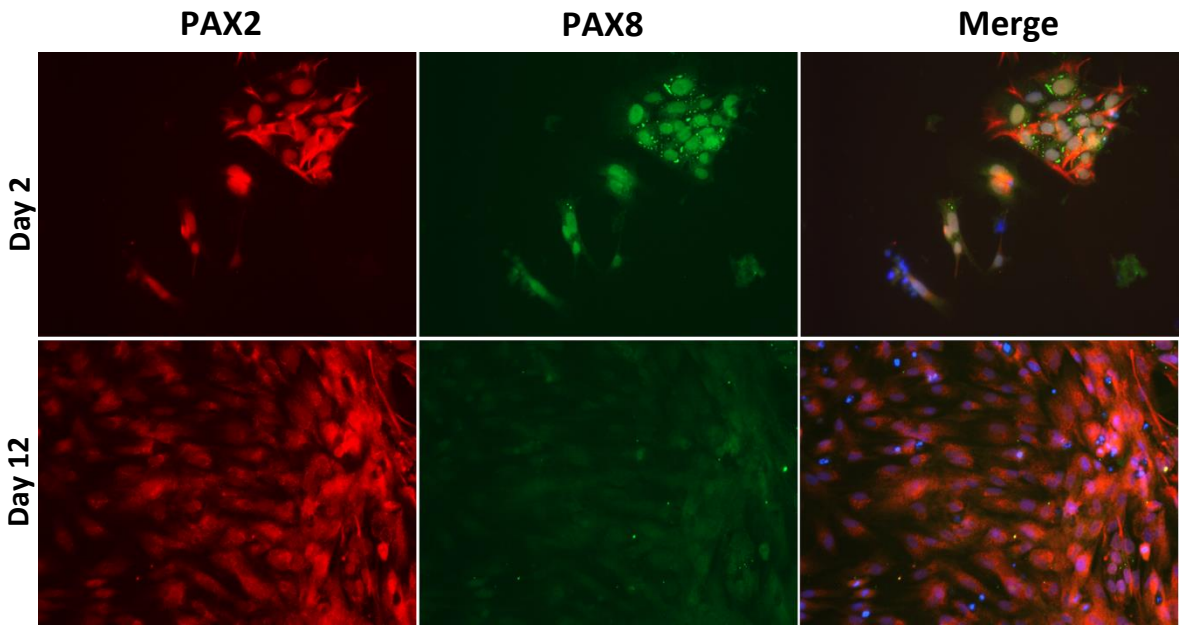


Shef3 48hrs differentiation

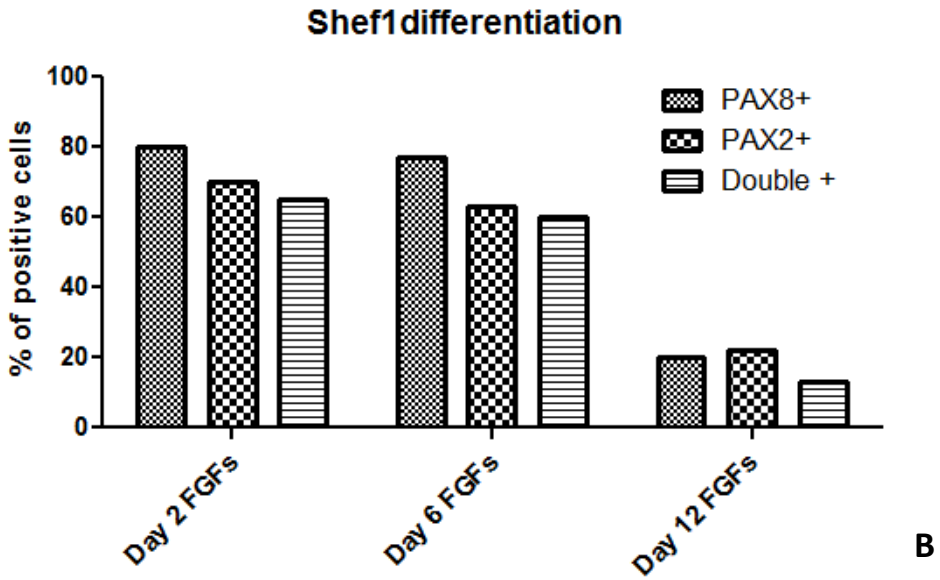


Next, immunopositive cells for PAX8 and PAX2 were quantified at different time points of the differentiation protocol (day 2, 6 and 12). The results shown in figure 5.4 clearly illustrate a dramatic drop in the percentage of double positive for PAX8 and PAX2 by day 12, in agreement with the Q-PCR analysis and the transient requirement of PAX genes during inner ear development. Cells presenting a nuclear signal for PAX8 and PAX2 strong enough to observe a defined nucleus were counted as positive. Nevertheless, in my opinion, all cells quantified as positive at day 12 for PAX8 (20%) fall in a different category even when a nucleus was clearly defined. The cells at this time point presented a much weaker signal compared with those observed at day 2 and 6 (figure 5.4 C and D). On the contrary, the PAX2 nuclear signal was equally strong during all the time points considered. However, there was a clear drop in the percentage of PAX2 positive cells at the end of the differentiation period. Concomitant with this drop, we also observed a number of cells with a fibroblastic-like morphology presenting a cytoplasmic red signal (PAX2) excluding the nucleus. These cells counted here as negatives, were almost non-existent at day 2 and 6 of differentiation. We don't know if those negative cells at day 12 were initially positive and subsequently downregulated the expression of PAX2 (implying that the cytoplasm signal may represent protein in route to be degraded) or, if on the other hand, they belonged to a marginal negative population that was present from the beginning of the induction and then proliferate robustly during the second half of the differentiation.

In summary, these results indicate that a 6 days induction period is sufficient to observe the expression of otic markers. Moreover, it is a window that is even more appropriate to detect these transcripts, since the expression level of all them resulted higher at day 2-6 when compared with the expression at day 12. In addition, the similar pattern of expression of otic genes between Shef1 and Shef3 supports the idea that these cells lines are not as different as previously thought. Furthermore, the fact that other cells from unknown identity proliferate while otic markers start to be downregulated from day 6 onwards, explain part of the variation observed in the analysis in the whole set of differentiation experiments. In other words, other subsets of cells more abundant at day 12 add "noise" to the system. Finally, this finding can save considerable amount of time and constitute a basis for future optimization of the differentiation protocol.



A

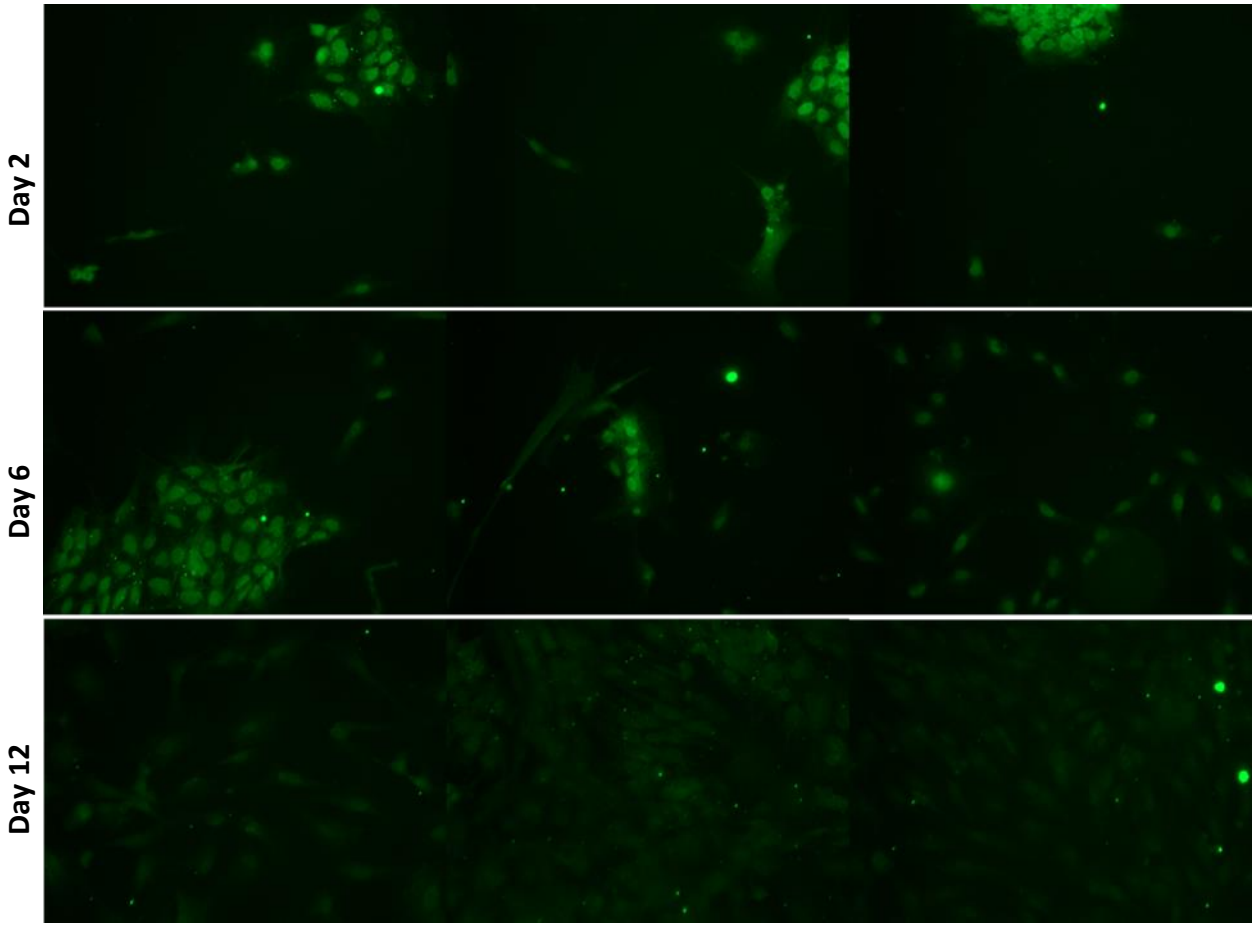


B

Figure 5.4 **Drop in the percentage of the otic progenitors through the course of differentiation**. Shef1 cell line was differentiated for 12 days in FGF3 and FGF10 medium. Cells were collected for immunofluorescence analysis at day 2, 6 and 12 of differentiation. Cells presenting a distinctive stained nucleus were counted as positive. In **A**, an example of double immunofluorescenece for PAX8 (green) / PAX2 (red) at day 2 and day 12 of differentiation is shown. In **B**, the graph shows the percentage of double positive cells through the course of differentiation protocol. In **C** and **D** (next pages), random pictures were taken to illustrate the intensity of the signal of PAX8 and PAX2 respectively during the length of the experiment. The percentage of positive cells was reduced by day 12 of the differentiation period. In addition, the intensity of the PAX8 signal also decreased at 12 days of differentiation, in agreement with its early and transient role during mouse, and Zebrafish inner ear development. Scale bar, 200µm.

Shef1 time course differentiation

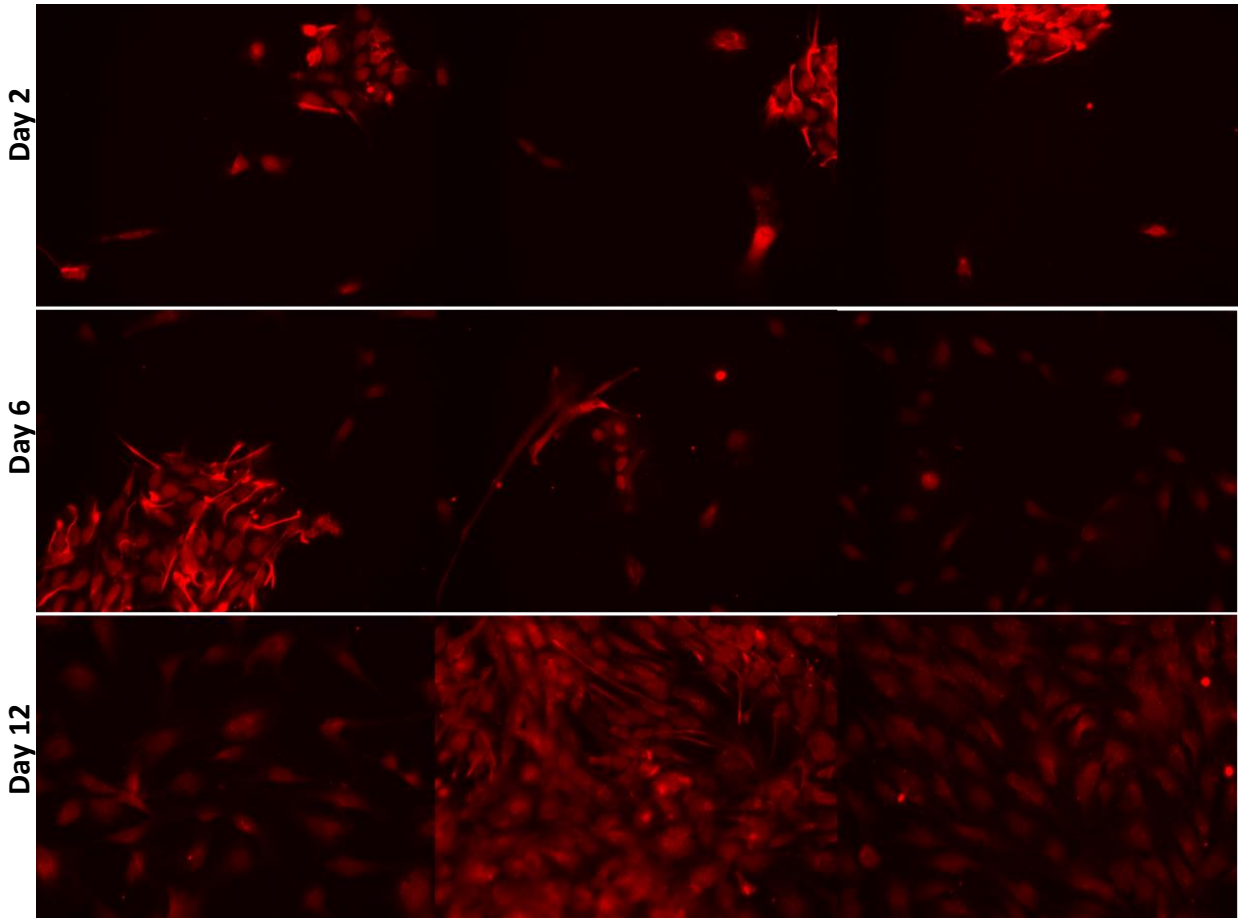
PAX8



C

Shef1 time course differentiation

PAX2



D

The fact that otic markers were robustly induced during the first days of the protocol compared with day 12 suggested a critical timing for otic induction at the very beginning of the differentiation process. Moreover, despite that cells remained in FGF supplemented media, otic genes were not sustained. In animal models, PAX8 is transiently switched on while PAX2 and FOXG1 remain expressed in different subsets of the otocyst for a longer period of time.

In the previous chapter, we observed that *FOXG1* was downregulated in most of the cell lines when these were treated with the FGFR inhibitor SU5402. Now, considering its early peak in expression, it was a good candidate to evaluate the timing of otic induction and otic commitment in our system. Thus, for this purpose, cells were differentiated in FGF supplemented medium plus the SU5402 inhibitor added in day 1-4 or 9-12, then, cells were collected and compared with cells in DFMB medium or treated with FGF3 and FGF10 for the full length of the experiment.

The results in figure 5.5 show that SU5402 is able to block the expression of FOXG1 when added at day 1-4 of differentiation in Shef3 and Shef1 as expected (figure 5.5 A and B respectively). However, when the inhibitor is added at the end of the differentiation period, FOXG1 is also downregulated, implying that FGFs are still required by otic progenitors. We noticed that inhibition of FGFs at an early time point was not as effective downregulating FOXG1 as inhibition in day 9-12. The fact is that in Shef3 cell line where the effect of SU during the course of the experiment has been demonstrated to be robust, it had little impact on the expression of FOXG1 when applied at an early window. Moreover when a third hES cell lines H14 was included in the analysis, a similar tendency was observed (figure 5.5 C). Therefore, these results suggest that the induction process is still taking place in our system, that otic induction is independent of FGFs, or that the first part of otic induction is independent of FGFs and second stage requires of them, however it is difficult to conciliate this possibility with the course of expression of this marker during the 12 days of differentiation. These alternatives will be discussed in brief in light of previous results and further chapters.

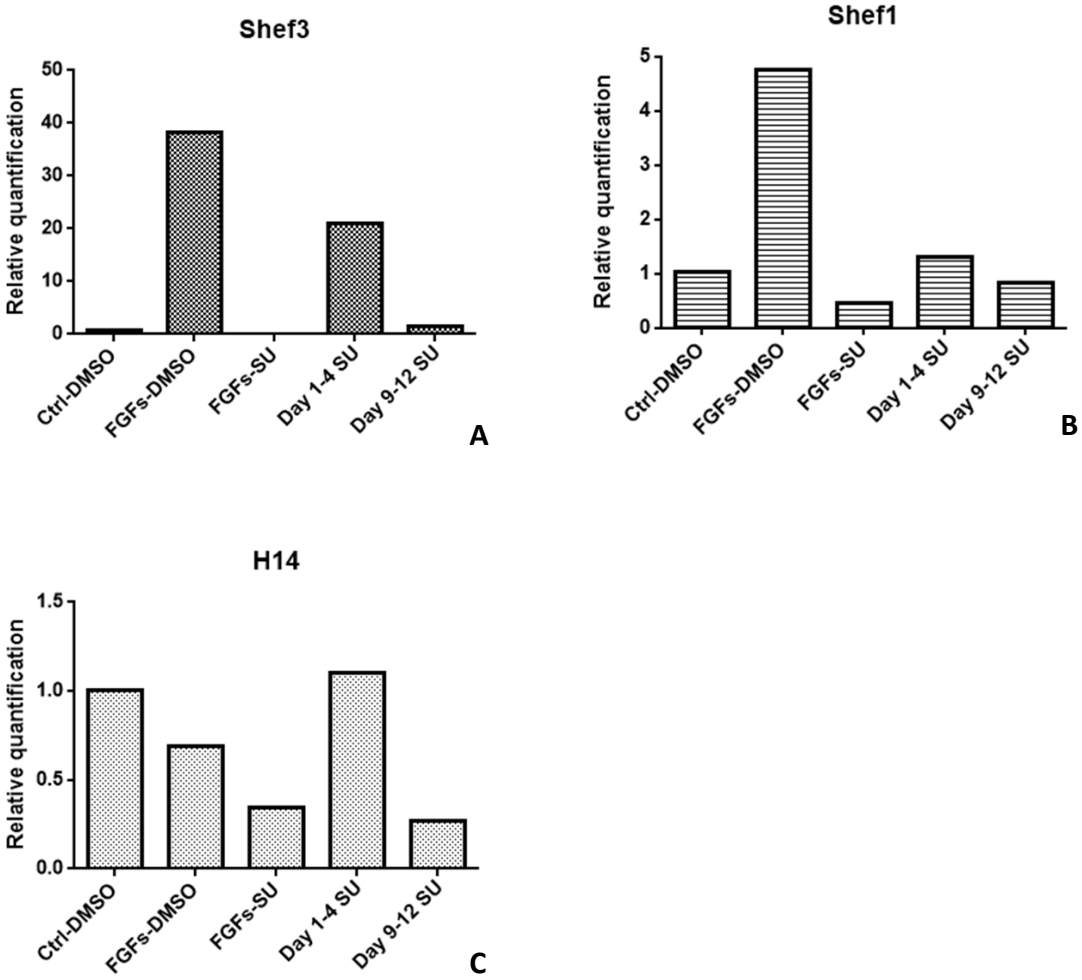


Figure 5.5 **Otic induction and commitment is different to animal models.** Shef3, Shef1 and H14 were differentiated for 12 days in control DFNB medium, or FGF supplemented medium. In the FGF supplemented treatment, another two conditions were compared; in the first one FGFR inhibitor SU5402 was added during day 1-4, and in the second SU5402 was added at day 9-12. Cells in DFNB (Ctrl-DMSO) were used as a calibrator of the relative expression data. The results show that despite FOXG1 is being downregulated from day 8 onwards (figure 5.1), the addition of SU5402 decrease its expression even more. In the other hand, when the inhibitor is added at the beginning (SU1-4), the expression of this marker is less affected in all the cell lines, particularly in Shef3 and H14 (**A** and **C** respectively). These results seem to be particular to our *in vitro* system and suggest a more complex model of FGF requirement than animal models.

Discussion

Otic progenitors express otic markers in a similar pattern to inner ear development in vivo

The temporal dimension is an important aspect during inner ear development and also for hES cell differentiation studies. The course of expression of *Pax8, Pax2* and *Foxg1* in animal models of inner ear development is in complete agreement with the data presented here. *PAX8* is the one most quickly downregulated after its early peak of expression, in agreement with its transient requirement in the Zebrafish and Xenopus models (Heller and Brandli, 1999; Pfeffer et al., 1998). On the other hand, *FOXG1* and *PAX2* are expressed very early and then become restricted to specific subdomains of the otic vesicle. In our experiments, we don't know if the otic progenitors acquire a particular regional identity, but if a mix of progenitors is observed (as evidenced by the different cell morphologies), the downregulation of these markers would also be expected.

Chick studies have shown that the competence of the ectoderm to respond to otic induction signals is time restricted. For example Groves and Bronner-Fraser elegantly showed that quail ectoderm explants that do not normally form otic vesicles are able to do so if transplanted into the presumptive otic placode region. However, such explants lose their competence to respond to otic inductive signals as developmental time progress, and by 10ss, they do not express any otic marker upon transplantation into the presumptive otic placode (Groves and Bronner-Fraser, 2000). In this work, they also demonstrated that the otic inducing signals (later shown to be members of the FGF family) are secreted at an early developmental window, since otic-competent explants transplanted into the ectoderm adjacent to the hindbrain readily formed otic vesicles and expressed a number of otic markers (e.g. *Pax2*) only if transplanted into a host ectoderm of 3 to 10ss. Older hosts were unable to induce an otic fate in competent explants as otic signals were not present anymore.

In a separate study, Martin and Groves (2006) cultured explants of presumptive otic placode from 0 to 8ss in the presence of the FGFR inhibitor SU5402 (Martin and Groves, 2006). They

observe that before the 5ss, SU5402 abolished the expression of the otic markers *PAX2* and *EPHA4,* but not in explants of older age, implying that FGFs are required at an early stage to induce an otic character, but once otic commitment is achieved, FGF inhibition does not have a major impact. In this regard our data is different. Based on the early expression of otic genes, it was hypothesised that FGFs are more important at that stage. However, when the SU5402 is applied at day 9-12, a robust reduction in the expression of *FOXG1* is observed in all the cell lines, suggesting that FGFs are still required by the progenitors to mantain the expression of *FOXG1*. What was more surprising was the fact that FGFR inhibitor applied at an early window had less impact on the downregulation of *FOXG1* when compared with cells treated with SU during the whole length of the experiment. This finding contrast with the results presented by Martin and Grooves and the model of FGF-mediated otic induction described by Freter et al (2008), who showed that FGF signalling is required at the beginning, but then it is necessary to be attenuated to proceed with otic development in chicken embryos, (Freter et al., 2008).

One possibility is that FGF inhibition impairs the differentiation process by blocking the expression of otic transcription factors but also by delaying the differentiation process. Therefore, in this set of experiments the cells treated with SU at an early window would have a delayed induction of FOXG1, but also a delayed downregulation of this gene, accounting for the higher expression level of this marker in cells treated with SU from day 1-4 compared with cells treated with SU during the whole experiment. In order asses if FGF signalling inhibition is delaying the differentiation process, it would be necessary to carry out time point expression analysis in experiments where the inhibitor is added for short periods of time (e.g 24 hrs) at the begging of the differentiation protocol. It is important to stress that in these experiments, the inhibitor at day 1-4 also dowregulated FOXG1 when compared with cells treated with FGFs only, stressing the importance of the pathway for this marker in any case.

Our results show a dynamic process of otic induction. We observed that PAX8, PAX2 and FOXG1 expressed as soon as 48hrs after induction (the earliest point analysed), and a dramatic drop in the expression of all these markers during the second half of the differentiation protocol.

From a practical perspective, a clear understanding of the course of expression of otic genes during differentiation could be the starting point to test new hypothesis aimed to improve differentiation conditions. For example, hypothetically, we could be interested in studying the application of a new ligand known to have a role during inner ear development, and find out that it is effectively increasing the expression of panel of transcription factors at the end of the differentiation period. However, in this example, our compound could be actually delaying the expression of the otic transcription factors. This situation would be revealed only if a time course expression analysis is carried out. Therefore, the conclusions derived from a differentiation experiment can be quite different if the temporal dimension is ignored, and the long term cost could be decreased if this type of analysis is performed.

Here we found that FGFs play an inductive role very early, but they are unable to maintain the expression of otic markers for the length of the protocol. A phenomenon that could partially explain the modest difference between Ctrl and FGFs presented in chapter 3.

Moreover, our findings also give us confidence that we can test experimental variables after 6 days of differentiation if these otic markers are to be used as a readout.