Chapter 1

Introduction

Abstract

Deafness is a chronic condition affecting more than 250 million people worldwide. Most of the cases are associated with the loss of hair cells and/or auditory neurons, neither of which regenerate in humans. In this context, cell transplantation represents a potential therapy. Our lab has recently demonstrated that human embryonic stem cell (hESCs)-derived otic progenitors are able to engraft and produce a functional recovery in an animal model of deafness. We have established a protocol to induce the differentiation of hESCs into otic progenitors through exposure to FGF3 and FGF10, ligands known to be important for the induction of inner ear development in the mouse.

We are now exploring in greater detail the role of FGF signalling during otic induction in hESCs. We have found that in the absence of FGF signalling, expression of otic genes is impaired, but high concentration of FGFs can be inhibitory for otic differentiation as well. Moreover, although our lab previously established a 12 days differentiation protocol, our time point analysis suggests that otic genes expression is higher during the first half of the protocol, a profile reminiscent of inner ear development *in vivo*.

Mouse development studies and biochemical approaches indicate that that FGF3 and FGF10 primarily bind to the isoform IIIb of FGFR2. In this work, we subcloned FGFR2IIIb into a potent vector to overexpress it during hES cells otic differentiation. Our results indicate that FGFR2IIIb overexpression strongly upregulates the otic markers *PAX8, PAX2* and *FOXG1*. As complementary approach, an inducible knockdown system for FGFR2 in hES cells has been set up. However, the results with the knockdown approach are incomplete, and further optimization will be required.

Also, although it has been suggested that FGFs are interchangeable and redundant, in our system we have shown that FGF3 and FGF10 are not equivalent, FGF10 with a more potent otic induction signal than FGF3. Moreover, endogenous expression of FGF10 correlates with high level of otic differentiation in cells maintained in control medium (no FGFs).

Understanding how FGF signalling controls otic differentiation of human pluripotent stem cells *in vitro* should help to improve the yield and purity of the progenitors, facilitating their translational application.

Inner ear development

The inner ear originates from the otic placode, an epithelial thickening of the ectoderm in the embryonic head region characterized by the expression of DLX, PAX and SIX protein-family members. This domain is induced by the concerted action of signals originating from neighbouring tissues: the hindbrain, the underlying mesenchyme and the endoderm. The main molecules involved at this induction stage are components of the FGF family (Ohyama et al., 2007; Schimmang, 2007). The role of FGFs in different species as inductive ligands is conserved, however the use of the individual members, the timing and localization varies in different species (Alvarez et al., 2003; Ladher et al., 2005; Lombardo et al., 1998; Maroon et al., 2002; Phillips et al., 2001; Wright and Mansour, 2003; Zelarayan et al., 2007). For example, mesodermal FGF19 is essential for inner ear induction in the chicken (Ladher, 2000; Ladher et al., 2005) while its orthologue in the mouse, Fgf15, has no known effect during otic induction (Wright et al., 2004). In figure 1.1 a diagram of otic induction is presented based on mouse inner ear development (Alvarez et al., 2003; Ladher et al., 2005; Wright and Mansour, 2003).

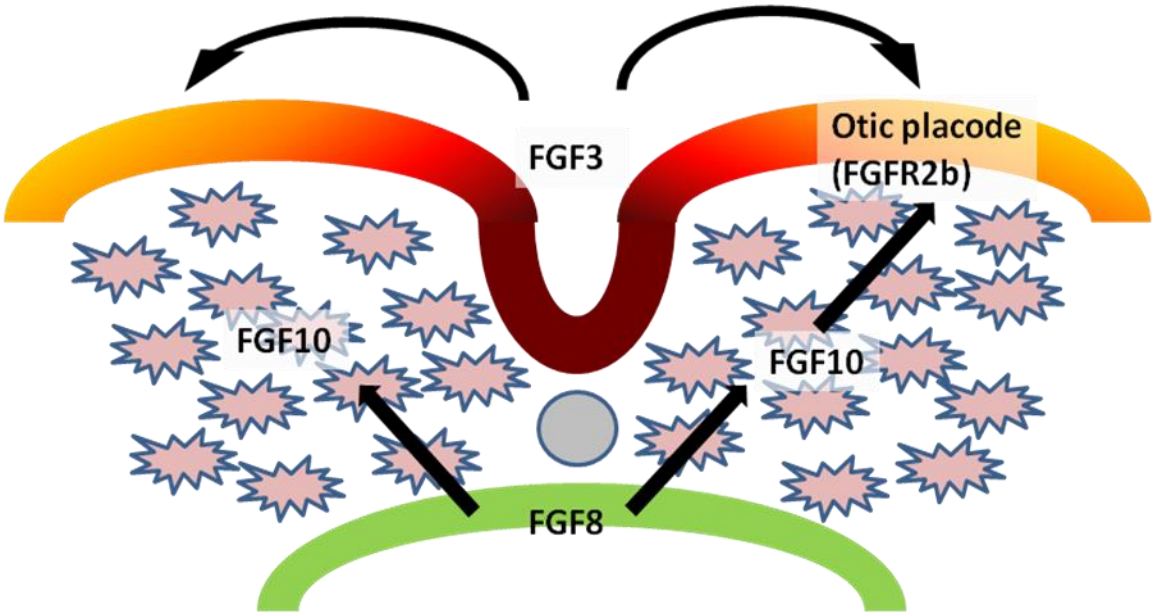

Fig 1.1. **Schematic representation of the early stages of otic induction**. The yellow portion corresponds to the ectoderm were the otic placode arise by means of induction signals from the hindbrain e. g. Fgf3 and Wnt (dark red); the periotic mesenchyme (pink stars) and the pharyngeal mesoderm (green) that express Fgf10 and Fgf8 respectively. At the same time we can observe the expression of early otic genes

Once the otic placode has been formed, it starts to invaginate in the ectoderm until it gives rise to the otic vesicle or otocyst. Within the otocyst, different subdomains are identified by the differential expression of molecular markers, for example *Dlx5* in the dorsal part and *Pax2* in the ventral region (Fekete and Wu, 2002; Wright and Mansour, 2003). Soon after the otocysts have formed, a group of cells delaminates from its ventral region to form the stato acoustic ganglion (SAG) and the rest of the otic vesicle experiences a complex morphogenic process to give rise to the endolymphatic duct, the vestibular system and the cochlea where the Organ of Corti resides (Barald and Kelley, 2004). Alongside the early role of FGFs during otic induction, FGFs play other key activities in otic development, like otic vesicle proliferation, SAG formation, pillar cell development and hair cell maturation in the Organ of Corti.

FGF receptors and FGF ligands have been observed at early stages of otic development (Pirvola et al., 2000; Wright et al., 2003) and analysis of mouse mutants for Fgfr3, Fgfr2IIIb and Fgfr1 has revealed abnormalities in the inner ear. However, a detailed analysis of the role of these receptors at earlier developmental windows is missing (Mueller et al., 2002; Pirvola et al., 2000; Pirvola et al., 2002). From the description above, it is clear that the FGF pathway is essential to trigger the formation of the otic placode. However, before that, the ectoderm in the border of the neural plate must acquire competence to respond to the otic induction signals (Hans et al., 2007; Martin and Groves, 2006). This requirement was originally described by Jacobson, where he transplanted a strip of non-neuronal ectoderm surrounding the neural plate and rotated in its anterior-posterior axes leaving the presumptive anterior placodes (nasal and lens) located in a more posterior position and vice versa. When such a manipulation took place at open neurula stage, normal development of cranial placodes was observed in the right location, but if performed later, the fate of placodes was already fixed leading to incorrect location of placodes. (Jacobson, 1963, 1966; Ohyama et al., 2007; Streit, 2004, 2007). More recently, thanks to the molecular markers available, more support for the existence and functional significance of a common preplacodal domain has emerged. For example in the chick, ectoderm explants from any region in the border of the neural plate express the otic marker PAX2 when cultured in the presence of FGF2, on the contrary, explants outside the “border region” do not respond to FGF2 but they do so if previously transplanted into the border region (Martin and Groves,

2006). Another piece of evidence comes from lineage tracing studies where precursors for lens and olfactory placodes originally overlap in a large area at the edge of the neural plate but they become restricted to their definitive position once placode formation starts (Bhattacharyya et al., 2004). It is noteworthy that preplacodal precursors at the border region express PAX6 and develop into lens placode if explanted and cultured in the absence of any morphogenetic cue, suggesting a default model that is abolished *in vivo* by the presence of locals signals that induce the different placodes (e.g. trigeminal, lens , nasal, otic and epibranchial) (Bailey et al., 2006).

Molecular markers also support the idea of common origin of all cranial placodes from a preplacodal region originally characterized by the expression of DLX and FOXI protein family members (Hans et al., 2007; McLarren et al., 2003; Ohyama and Groves, 2004; Woda et al., 2003; Yang et al., 1998) and there is functional evidence in the fish (*Danio rerio*) that expression of *dlx3* and *foxi1* is a prerequisite for otic induction (Hans et al., 2007). After this border region has been specified, there is a further segregation between the neural crest next to the neural ectoderm and the preplacodal region, which can be clearly identified through expression of a number of transcription factors e.g. Pax6, Six and Eya genes (Brugmann et al., 2004; Li et al., 1994). This set of genes form a transcriptional network that has been better studied in Drosophila eye development. Although mutations for Eya1, Six1 and Dlx genes produce alterations in inner ear development and in some cases other placodes are affected as well, none of them affects absolutely all the cranial placodes. This could be explained perhaps, by a compensation effect produced by the expression of orthologues with similar function (e.g. Eya2, Six5 etc.). In any case, in humans, Eya1 mutations have been associated with deafness and some eye defects.

The preplacodal region described above is established by the cooperation of different signals emanating from the epidermis, the neural plate and the head mesoderm, which secrete members of the WNT, BMP and FGF pathways. Streit and Stern (Streit and Stern, 1999) showed the neural plate border expressing *BMP4* and its target *MSX1* in response to FGF from the organiser and suggested that once this border region is established, its maintenance depends upon autocrine bmp activity. Similarly, in *Xenopus*, fgf8 has been shown to be able to induce the preplacodal marker *six1*, with bmp again interacting with Fgf pathway to set up the preplacodal domain (Ahrens and Schlosser, 2005). However, in this

study they also show that BMP inhibition is necessary for maintaining and expanding the *six1* domain. Therefore it seems there is a discrepancy between both works regarding BMP activity; in one of them BMP helps to maintain border region, while in the other, it diminish the size of it. The explanation could be that there are species differences in the requirements to set up the preplacodal domain, or it could be a result of the type of markers used in their analysis, for example, although *MSX1* and *SIX1* are first expressed in all the border region, this region is then divided into the neural crest domain and the proper preplacodal domain, and MSX1 is maintained only in the neural crest domain, while in the preplacodal region *six1* remain expressed. In this situation although both markers are widely expressed in the neural plate border, then they become restricted to different domains, and once this segregation of fates occurs BMP activity may increase the size of the neural crest domain at the expense of the preplacodal. In this regard, *Dlx5* has been found expressed in the border region (Yang et al., 1998), and it has been suggested that MSX1 and DLX5 counteract each other to create the preplacodal (DLX5) and neural crest (MSX1) domains (Luo et al., 2001; McLarren et al., 2003; Woda et al., 2003). In these experiments, neither FGF activation (McLarren et al., 2003) nor BMP inhibition alone were able to induce ectopic preplacodal markers. In this regard, Litsiou and colleagues elegantly showed that a combination of ectopic FGF expression and concerted inhibition of BMP and WNT was enough to induce preplacodal markers in naïve ectoderm, and they presented a model where WNT produced in the trunk ectoderm and epidermis restricts the preplacodal region to the head where first FGF activity and BMP gradient established the border (Litsiou et al., 2005) (figure 1.2). A detail explanation of preplacodal region can be found in Streit 2007 (Streit, 2007).

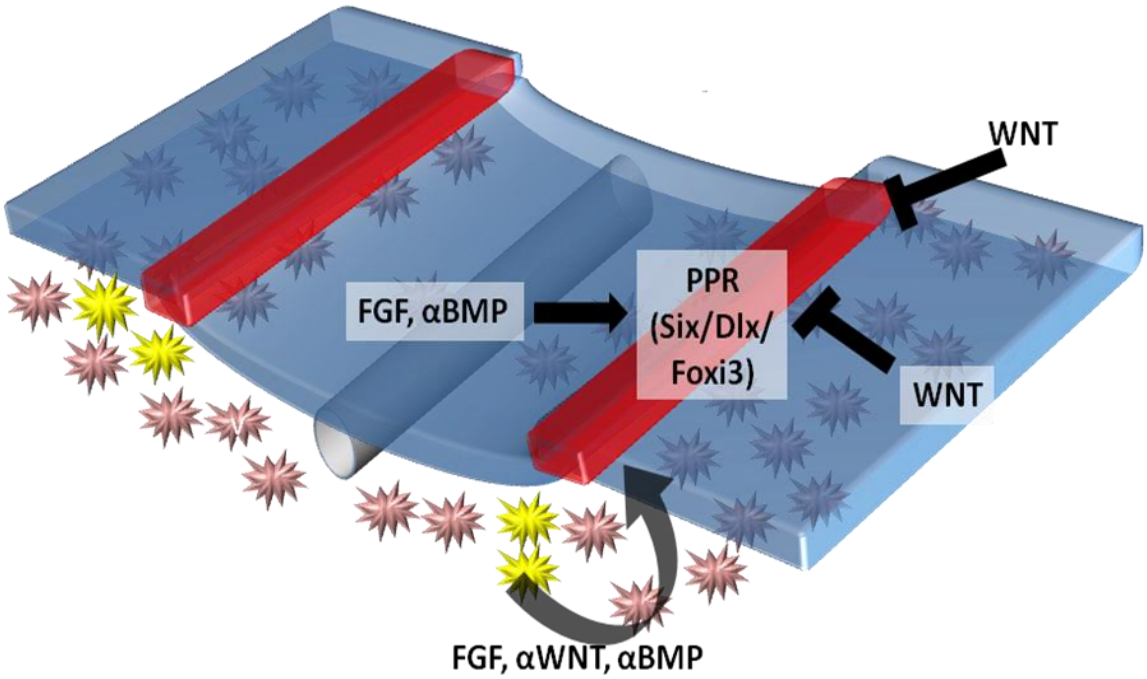

Fig 1.2. **Establishment of the preplacodal region (PPR).** The PPR (red) is characterized by the expression of transcription factors like Foxi3, Six1, DLX and EYA family members. The induction of this region depends on the concerted action WNT and BMP inhibitors plus FGFs derived from the mesoderm (yellow stars). In addition the trunk and lateral ectoderm (blue) express Wnt that limits the PPR to the head ectoderm. This region is the common ground where all the cranial placodes arise.

In summary, it is clear that inner ear development is a complex process and that FGFs are involved first in inducing the preplacodal domain and then during specification of the otic placode. Roles for FGF signalling have also been highlighted in the development of other cranial placodes (Bhattacharyya et al., 2004; Garcia et al., 2011; Nechiporuk et al., 2005), and critically, for further auditory development, the pathway is also essential for otocyst patterning and sensory cells formation within the cochlea. This evidence showing how FGFs are able to induce disparate cell fate decisions depending on the cell context also indicates the intricate nature of the FGF pathway.

Our *in vitro* system offers the opportunity to explore some aspects of FGF pathway relevant for otic induction without the confounding factors of a complex animal model, but with the disadvantage of the unpredictability of the cell culture.

FGF signalling

Since the characterization of a mitogen present in bovine pituitary extracts, able to induce the growth of fibroblasts, and therefore the name 'fibroblast growth factor' (Gospodarowicz, 1974) more than 23 members of the FGF family have been identified. These proteins have diverse functions largely dependent on the cell context. They are important in many developmental processes e.g. mesoderm formation, bone elongation, branching morphogenesis of the lungs and kidney, limb growth, and of course inner ear development. They are also important in the adult, for tissue homeostasis, wound healing and angiogenesis. Most FGFs are short range secreted molecules, although FGF11-FGF14 are intracellular, while others like FGF2 have different isoforms with some cytoplasmic while others are secreted. They exert their actions through 5 FGF receptors (FGFRs 1-5) that belong to the receptor tyrosine kinase superfamily. In their external part they have either 2 or 3 immunoglobulin-like domains followed by a juxtamembrane domain, a single pass membrane region and two tyrosine kinase intracellular domains. Expression data of some FGFRs and FGFs during limb and bone formation, together with *in vitro* studies of receptor activation and proliferative response in *FGFRs* transfected cells have provided the basis to define the specificity of FGFRs to different members of the FGF family (Chellaiah et al., 1994;

Johnson et al., 1991; Miki et al., 1992; Ornitz et al., 1996; Zhang et al., 2006). The conclusion from these studies has been that multiple FGFs bind to each single receptor isoform. Interestingly, in the study of Ornitz group (Ornitz et al., 1996; Zhang et al., 2006), chimeric FGFRs that share the same intracytoplasmic domain but differ in the extracellular portion, are able to elicit the same proliferative response in transfected cells despite being stimulated with different FGFs; therefore, it is thought that any specific outcome of FGF activation depends more on the FGF receptor involved and that FGFs are only redundant triggers of the receptor. FGFs bind to the second and third immunoglobulin like domain of FGFRs. It is noteworthy that an alternative splicing event in the third immunoglobulin-like domain (Ig-III) of FGFR1-FGFR3 produces two isoforms (IIIb and IIIc) with completely different binding properties and mutually exclusive expression patterns *in vivo* (Orr-Urtreger et al., 1993; Peters et al., 1992), with the IIIb isoform expressed mainly in epithelial structures and IIIc confined to the mesenchyme. For the binding of any specific FGF to its respective FGFR isoform to occur, a second molecule belonging to the heparan sulphate proteoglycans (HSPG) must also bind to both receptor and ligand to stabilise the joint. Once this has occurred, receptor dimerization and autophosphorylation of tyrosine residues in the receptor intracytoplasmic domain takes place. I have summarized how the initiation of FGF signalling takes place. However, this is a simplification of a process far from being completely dissected despite the pathway has been studied for more than 30 years. For instance, as mentioned earlier, it has been observed that some FGFs are cytoplasmic and their role it is not clearly defined. It is thought that intracellular FGFs may trigger gene expression as there are reports where Fgfs and their receptors are observed in the nucleus (Johnston et al., 1995; Kiefer and Dickson, 1995; Maher, 1996; Schmahl et al., 2004). Further research is also necessary on the activities of the FGFRs as, although specificity of FGFRs is accepted, formation of heterodimers is a possibility and their consequences on cell behaviour poorly understood (Shi et al., 1993). Furthermore, it is unknown if different HPSGs bind preferentially to different FGFRs and if that has any consequence in the pathway activation.

Downstream of FGFR activation, there are a number of molecules involved in the signalling process. PI3k-AKt, RAS-ERK1/2, and PLCγ pathways are known to be activated by FGFR phosphorylation. However, the relative contribution of each of these pathways seems

specific to the cell type. Therefore, while ERK activation may be observed in most of the cases, its blockage could have no consequences in actual particular biological phenomenon studied (differentiation, apoptosis or proliferation) as in any single situation other pathways could be more relevant. In addition, contrary to what has been observed in other signalling pathways where common transcription factors are recognised (e.g. WNT activation cause LCF/TCF transcription factor expression and BMP/TGFβ activates SMAD members), the FGFR family does not have a common transcription factor. Another complication added to the understanding of FGF signalling, is the crosslink with components of other pathways, for instance as mentioned in the previous section the cooperation with WNTs and BMPs during preplacodal domain formation or with WNTs for the early cell fate decision between epidermal or neuronal in the epiblast (Wilson et al., 2001). Another example comes from osteoblast differentiation where FGF activity blocks the WNT pathway through Sox2 expression (Mansukhani et al., 2005). Also, activating mutations in FGFR3 that lead to achondroplasia involve nuclear translocation and activation of STAT1 (Hart et al., 2000; Legeai-Mallet et al., 2004) and downregulation of components of SHH and BMP pathways (Naski et al., 1998).

When the link between mutations in FGFR3 and achondroplasia was observed in 1994 (Rousseau et al., 1994; Shiang et al., 1994) an increased interest for understanding the FGF pathway emerged. Since then, more mutations in FGFR1-3 have been found to be related with skeletal development, such as FGFR2 mutations causing Appert and Crouzon syndromes (Jabs et al., 1994; Ornitz, 2005; Reardon et al., 1994; Wilkie et al., 1995), characterized by a premature suture of craniofacial bones. In these cases the increased activity of FGFR signalling, lead to an arrest in proliferation of chondrocytes and a quick progression to hypertrophic chondrocytes. In other words, an early transition from the cartilaginous structure made by the chondrocytes into a mature osificated bone is due to the fast exit from cell cycle in chondrocytes as a consequence of FGFR activity (Aikawa et al., 2001; Sahni et al., 1999). Many forms of cancer have also been found to be related to incorrect FGF signalling activation (Grose and Dickson, 2005), for example mutations that affect splicing from IIIb into IIIc isoform of FGFR2 cause a loss of communication between epithelia and the mesenchyme, and are involved in prostate and ovarian cancer progression and increased invasiveness (Carstens et al., 1997; Cunha et al., 2003; Kwabi-Addo et al.,

2004; Steele et al., 2001; Yan et al., 1993). Indeed, FGFR2IIIb overexpression has been shown to be a tumour suppressor in prostate and bladder epithelial cell lines, reverting them into a state where their growth depends of the presence of stromal cells (Cunha et al., 2003; Feng et al., 1997; Matsubara et al., 1998; Ricol et al., 1999). FGFR3 has also being implicated in bladder cancer (Cappellen et al., 1999) and in myelomas (Chesi et al., 2001; Li et al., 2001). As mentioned earlier, FGFR3 activity can inhibit proliferation of chondrocytes while in other circumstances behaves as an oncogene, stressing again the importance of cell context in the FGF pathway outcome. Finally, FGFR1 mutations where the intracytoplasmic domain is fused with ZNF198 or BCR genes can trigger myoproliferative disease (Reiter et al., 1998; Roumiantsev et al., 2004). These fusions dimerise and stabilize FGFR1 in way that is constitutively activated.

In the same way that FGFR activation is a common component in many types of cancer, inappropriate FGF expression is also an important element of cancer progression. In the mouse model of mammary tumours caused by insertional mutagenesis of mouse mammary tumour virus (MMTV), activation of either *Fgf3*, *Fgf10 Fgf4* or *Fgf8* can induce tumorigenesis (MacArthur et al., 1995; Shackleford et al., 1993; Theodorou et al., 2004). In addition, in a mouse model of fibrosarcoma, vascularization of tumours is associated with the production of FGF2 (Kandel et al., 1991). Supporting this insight but in a different model of bladder carcinoma, induced expression of FGF2 and FGF1 accelerates tumour progression and induces angiogenesis (Jouanneau et al., 1994; Jouanneau et al., 1997).

Most of the cases described so far are consequences of activating mutations in FGF signalling. These works have been supported by *in vitro* studies and mouse genetics, which have also been used to dissect the role of individual FGFs and their receptors. However, isolating the specific roles of FGFs in the development of different organs has been hampered by their pleiotropic effects and the redundancy of some of their components. For example, the *Fgfr1* mouse mutant is arrested in early development because of a defect in mesoderm formation (Yamaguchi et al., 1994). In this case conditional KO will be necessary to study *Fgfr1* defects in other organs. *Fgfr3* mutants have inner ear malformation and overgrown skeletons (Colvin et al., 1996), which makes sense considering the activating mutation causing achondroplasia in humans. However in the case of the inner ear, detailed analysis of early stages has not been made. *Fgfr2* mutants also die early at E10.5, with

already evident defects in limb buds, salivary glands and lungs. Of particular interest for us is the reduced size of the otic vesicle in these mutants (Xu et al., 1998), a phenotype recapitulated in the *Fgfr2IIIb* mutant (De Moerlooze et al., 2000) and the double KO *Fgf3* and *Fgf10* discussed in the previous section (Wright and Mansour, 2003). No obvious phenotype has been described for an Fgfr4 mutant (Weinstein et al., 1998).

The effects of *Fgfs* KO are less severe in some instances, for example the ear where *Fgf3* and *Fgf10* work redundantly. Another example is *Fgf1* and *Fgf2* for which deletion does not cause a phenotype despite its important function in wound healing and angiogenesis (Miller et al., 2000). The rest of the mutations in the FGF family cover a wide range of phenotypes. However, whilst it is important to understand the broad range of roles that FGFs play in living organisms, it is those involved in inner ear development the ones that are most critical for our work (Fgf3, Fgf10, Fgf8, Fgfr2, Fgfr3 and Fgfr1).

It is evident that FGF signalling plays a role in almost every morphogenetic event and that the outcome of FGFR activation depends heavily on the cell type. This fact stresses the need to assess the FGF pathway in any particular system used. Our interest is in the differentiation of human embryonic stem cells into otic progenitors; therefore it will be necessary to revisit the regulation of the hES undifferentiated state and the action that FGFs play on it. This will give us an insight of what is occurring during our differentiation conditions and provide a framework for future modifications to our protocol.

Human embryonic stem cell state maintenance

In addition to the described activity of FGFs during early inner ear formation *in vivo*, and the myriad of effects they trigger in other systems, the FGF pathway also plays a role in hES cell maintenance and differentiation, and importantly that role differs from the one in mouse embryonic stem cells (mESCs).

hESCs and mESCs are considered to be developmentally different from each other, and therefore differ in the signalling activation requirements for their undifferentiated growth. For example, mESCs are typically maintained with LIF protein, which negates the need for feeders, and BMP in place of serum (Ying et al., 2003a). However, BMPs induce trophoblast

differentiation in hESCs (Xu et al., 2002) while the LIF-Stat pathway does not have any role in the self-renewal of hESCs (Thomson et al., 1998)Thomson 1998). In contrast, hES cells are typically maintained in the presence of mouse embryonic fibroblasts (MEFs) and FGF2, conditions which trigger differentiation towards neuroectoderm in mESCs (Kunath et al., 2007; Stavridis et al., 2007; Ying et al., 2003b). Indeed, most media formulations developed to keep hESCs undifferentiated contain FGF2 as one of the main components, highlighting the importance of FGF signalling in maintaining pluripotency in these cells (Greber et al., 2010; Levenstein et al., 2006; Ludwig et al., 2006a; Ludwig et al., 2006b; Yao et al., 2006).

Apart from the different responses to extracellular signals seen between mESCs and hESCs, there are differences in morphology and in the expression of cell surface markers, for instance SSEA3 and SSEA4, markers of pristine hESCs whilst SSEA1 expression marks this state in mESCs (Draper et al., 2004). The transcriptional profile of mESCs and hESCs has also been found to be different (Sato et al., 2003). Altogether, these differences stress the need to study any particular pathway involved in differentiation in both systems; mESCs and hESCs, since the outcome is not always the same. More recently a new type of pluripotent stem cell from postimplantation mouse embryos has been derived called mouse epiblast stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007). These cells share a number of characteristics with hESCs, including their differentiation potential and extracellular signal requirements; therefore it is thought that hESCs fall developmentally between mESCs and EpiSCs. The understanding of the hESCs status will help in the interpretation of the available differentiation protocols and in the design of new ones.

Regarding the FGF pathway, hESCs express all 4 FGFRs (Ding et al., 2010; Dvorak and Hampl, 2005; Sperger et al., 2003) and produce a number of FGFs, in particular FGF2 (Dvorak et al., 2005; Ginis et al., 2004; Sato et al., 2004). FGF2 is typically used in conjunction with the MEF or MEF-conditioned medium; however the endogenous production of FGF2 is enough to keep hESCs undifferentiated. ERK1/2 is phosphorylated even in the absence of exogenous FGF2, indicative of active FGF signalling. Nonetheless, when FGF signalling is completely blocked by the FGFR inhibitor SU5402, differentiation occurs within two days (Dvorak et al., 2005). In a separate study, Ding et al found that is in fact the PI3K-AKT branch rather than the ERK1/2 downstream of FGFR activation the one responsible for hESCs maintenance (Ding et al., 2010). In this study they propose that AKT inhibits GSK3β and potentiates

β-Catenin nuclear translocation. In support of this hypothesis, an inhibitor of GSK3β has been used to maintain hESCs undifferentiated without feeders (Sato et al., 2004). In an attempt to remove the need for feeders from hESCs culture, a higher dose of FGF2 (100ng/ml) was used (Levenstein et al., 2006); contrasting with the typical 4ng/ml in the standard culture conditions. That concentration is above the saturation point of FGFR by FGFs in other systems with high FGFR expression (Presta et al., 1998). The explanation proposed in Levenstein's work is that in the absence of feeders or conditioned media, FGF2 is quickly degraded below the threshold required to keep hES cells undifferentiated. In this regard, it is important to mention that the amount of FGF2 remaining after 24hrs on cell cultures is still higher than the one observed in MEF cultures, indicating that perhaps low affinity binding of FGF2 may be responsible for the observed effect. In another study from the same group they showed that MEF-conditioned media was suppressing BMP activity in hESCs and that noggin (BMP inhibitor) in conjuction with FGF2 was able release hESCs from MEF dependency (Xu et al., 2005). Activin, another member of the TGFβ pathway, antagonistic to BMP, was shown to cooperate with FGF2 to maintain hESCs in chemically defined media without feeders (Vallier et al., 2005), in agreement with the work described before (Xu et al., 2005). Moreover, Greber and collaborators have shown that FGF2 is not only working on hESCs, it is also directly acting on the feeder cells by inducing the expression of Activin (Greber et al., 2007). Therefore, the interplay between TGFβ and FGF pathways has been demonstrated in different studies.

In an attempt to dissect the role of FGF, Hans Scholler's group, using inhibitors for FGF, Activin and BMP in undifferentiated hESCs, found that FGF helps in the maintenance of the hESC self-renewal by inhibiting the expression of the neuroectodermal markers *OTX2* and *PAX6* in the absence of BMP. By contrary, when BMP was present, extra embryonic ectoderm or mesoderm markers were observed during FGF inhibition (Greber et al., 2011), confirming results obtained before by Vallier et al regarding the function of BMP using a similar approach (Vallier et al., 2009). However, in Vallier's work they concluded that FGF was necessary for neuroectoderm differentiation. This claim was backed-up by Na et al, who showed that ERK inhibition (downstream target of FGFR activation) blocks neuroectoderm differentiation in predifferentiated cells (Na et al., 2010). The discrepancy between the findings of Scholler and those of Vallier and Na could be explained by their use of different

markers, Scholler focused on *OTX2* and *PAX6*, not seen by Vallier. Also in the Scholler publication, when SB431542 (Activin inhibitor), NOGGIN and FGF2 were present, *SOX2* was indeed maintained in hESCs though *OCT4* and *NANOG* were already downregulated, indicative perhaps of a "neuroectoderm bias". It is interesting to mention that in the work of Dr. Na, a predifferentiation step towards neuroectoderm without FGF2 or activin took place before inhibiting ERK. There is still the possibility that inhibition of another pathway/ pathways (e.g. AKT, PLCγ) triggers neuroectoderm differentiation in Scholler's work, while ERK inhibition helps in hESCs self renewal.

Another example of the different roles played by FGF on hESCs and its interplay with BMP signalling comes from a study by the Thomson lab (Yu et al., 2011). They suggested that FGF2 plays a role downstream of BMP, which normally induces extraembryonic differentiation (Xu et al., 2002) but if FGF2 is present, mesoderm differentiation is enhanced instead through the maintained level of NANOG expression. Of note in Vallier's experiments mentioned before, mesoderm differentiation was also enhanced when FGF2 and BMP were present and Activin was blocked, however in that study a predifferentiation stage existed (Vallier et al., 2009). The idea of FGF2 sustaining *NANOG* expression has been postulated before (Greber et al., 2007; Greber et al., 2010; Wang et al., 2009). In addition, *NANOG* overexpression has been shown to prompt hESCs toward a primitive ectoderm fate, suggesting that *NANOG* not only marks the undifferentiated state, but also, an early exit towards an ectodermal fate (Darr et al., 2006).

In summary, evidence suggests that FGF has multiple roles in hESCs depending on the balance with other signalling pathways. FGF2 inhibits neural differentiation in the absence of BMPs, but if the latter is present, mesoderm differentiation is induced. If we block FGF2 completely, extraembryonic differentiation takes place. FGF2 and Activin cooperate to maintain NANOG expression in the undifferentiated state, whereas if Activin is completely blocked, hESC self-renewal is lost and early neuroectoderm is observed despite the presence of FGF2. Furthermore, if FGF2 is blocked in this background, neuroectoderm differentiation is enhanced.

Therapeutic approaches for hearing impairment

An understanding of the hES cell state will help us both to develop our differentiation conditions, and in the interpretation of our results. It is also important to realise that hESCs express the receptors and the intracellular machinery to respond to all developmental signalling molecules, giving them their pluripotent character. This is one of the main reasons that hESCs are attractive agents as potential cell therapeutics.

There has been a myriad of efforts to treat hearing impairment in the experimental field; however, an ideal treatment is still a long term goal despite the encouraging results. Currently, at the clinical level the only available option is the cochlear implant. Although this device has proven to be useful in many cases, there are a number of patients for whom, such a strategy is not an option, or recovery observed is very limited (Wilson and Dorman, 2008). The loss of the spiral ganglion neurons is one of the situations where a cochlear implant would not offer any improvement. For these patients, a different electronic device has been developed, the auditory brainstem implant. However the medical care, and associated risks like meningitis, cerebrospinal fluid leak, plus the most limited auditory aid compared with the cochlear implant, limits a wide application of this device (Schwartz et al., 2008).

Alternative therapies to replace the hair cells and the associated neurons have been proposed, for example, gene and drug delivery approaches, *in vitro* differentiation protocols for a wide variety of stem cells, and transplantation of different cell types are currently under investigation.

Gene therapy in the inner ear

During the last stages of hair cell differentiation *in vivo*, the prosensory domain characterized by *Sox2* expression gives rise to hair and supporting cells. Within the prosensory domain, *Atoh1* has been proposed as the key factor to trigger the terminal differentiation towards hair cell fate, and simultaneously induce the expression of NOTCH

ligands capable of inhibiting neighbouring cells from becoming hair cells (Kelley, 2006; Lanford et al., 1999; Murata et al., 2012). The mouse mutant for *Atoh1* fails to develop hair cells in the cochlea or vestibular system (Bermingham et al., 1999). Moreover, cochlear rat explants produce extra hair cells in ectopic locations when transfected with *Atho1* (Zheng and Gao, 2000). These findings make *Atoh1* a promising candidate for gene therapy. In adult guinea pigs exposed to ototoxic insult, *Atho1* transduction in the cochlea has been shown to be enough to replace hair cells through mitosis of supporting cells or direct transdifferentiation (Izumikawa et al., 2005). It is remarkable that the regenerated hair cells preserved the typical architecture within the cochlea and were capable of restoring hearing function in deafened animals, suggesting that the cues that instruct the right localization and orientation to the hair cells still exist after the acoustic trauma. A different mechanism of hearing recovery by *Atho1* transduction has been suggested recently by Yang et al (Yang et al., 2012). They claim that disruption of the hair bundle by loud noise preludes hair cell death, and it is in this period that *Atho1* is able to bring restoration of hair cells. Their idea is that *Atho1* in supporting cells indirectly helps the repair of the hair cells already present, however the evidence supporting this point is not strong since a time point analysis looking for cell division and apoptosis in the damaged cochlea was not undertaken. Also, in a previous study by Kawamoto, hair cells originated directly from *Atho1* transduced cells (Kawamoto et al., 2003), implying that supporting cells have the competence to differentiate into hair cells and that *Atho1* is directly responsible for that. However, what is clear from Yang's work is that hair cell restoration by *Atho1* can only take place within a short period of time after otoacoustic insult. The fact that supporting cells from the mature cochlea can be converted into new hair cells by *Atho1* expression and the observed spontaneous recovery of hair cell observed in birds has driven an effort to find a stem cell population in the mature mammalian cochlea. So far, only neonatal mammalian cochlea has been shown to contain supporting cells able to re-enter the cell cycle and differentiate *in vitro* into hair cells, and this capacity remains in the mouse until the third postnatal week, after that no proliferative capacity is observed (White et al., 2006). In contrast, in the mouse vestibular system, a stem cell population in the adult has been described (Li et al., 2003a). Those cells were characterized by their *in vitro* self-renewal capacity and were shown to be pluripotent in some *in vivo* assays that were not as robust as those developed in embryonic stem cells, and the number of cells expressing neuronal and hair cell markers was relatively

low (6-8%). These data, plus the limited capacity of hair cell regeneration in the adult vestibular epithelia (Warchol et al., 1993), makes unlikely the possibility to induce endogenous recovery of the cochlear sensory epithelia at this stage. Thus, it urges us to study if an inhibitory signal that stops regeneration could exist in the adult cochlea or if there is a way to revert the non-sensory cells into a state where they could regenerate new hair cells again (Ronaghi et al., 2012).

Another strategy currently under investigation is the delivery of growth factors into the inner ear. As mentioned earlier, the efficacy of the cochlear implant depends on the proper connectivity and stimulation of the spiral ganglion neurons, which often degenerates following hair cell death (Bichler et al., 1983; Dodson and Mohuiddin, 2000). It is known that hair cells and supporting cells secrete neurotrophins to maintain the survival of the spiral ganglion neurons. Moreover, the spiral ganglion neurons express the neurotrophin receptors Trkb and Trkc, specific for neurotrophins Bdnf and Nt3 respectively (Ylikoski et al., 1993), and mouse double mutants for these neurotrophins completely lack vestibulocochlear innervation (Ernfors et al., 1995; Fritzsch et al., 1997; Fritzsch et al., 2004). *In vitro* studies support the idea that neurotrophins not only help in the survival of neurons but also in their sprouting and as tropic factors (Avila et al., 1993; Malgrange et al., 1996). Animal models of deafness have provided an insight into the degenerative process undergone in the cochlea. After hair cell death, spiral ganglion neuron terminals retract and eventually the cell bodies also degenerate within weeks or months (Bichler et al., 1983; Spoendlin, 1975). This is in striking contrast to what has been observed in humans, where neural degeneration can take place many years after hair cell damage (Nadol, 1997; Nadol and Eddington, 2006). Despite this difference, animal models represent the most reliable source of information to test neuroprotective approaches and their functional significance in a controlled system. The data is promising, pointing to neurotrophins as protective agents to avoid neural degeneration *in vivo* (Ernfors et al., 1996; Glueckert et al., 2008; Staecker et al., 1996; Wise et al., 2005). For example, adult guinea pigs have been shown to maintain a higher density of spiral ganglion neurons after hair cell death when their cochleas are transduced with *Bdnf* or *Nt3* (Shibata et al., 2010; Wise et al., 2010). In addition, there is evidence that cochlear implant function and hearing threshold reductions occur when animals are transduced with *Bdnf*, and this could be correlated with the spiral ganglion cell

density (Chikar et al., 2008). Therefore there is considerable effort to preserve the spiral ganglion through neurotrophic factor application and cochlear implant stimulation.

Adult stem cells for hearing impairment

A different approach to potentially increase the efficacy of the cochlear implant would be cell replacement of auditory neurons and hair cells. There is evidence that the performance of cochlear implants can be augmented if some remaining hair cells are still present (Turner et al., 2010), therefore even replacing a small proportion of them could represent a step forward in the treatment of deafness. Eventually, we would like to replace the entire sensory epithelium and auditory neurons, a situation that would restore hearing to normal levels without the aid of prosthetics. Although this goal could be far away based on current knowledge, great progress has been made in the field of cell replacement strategies.

There are many aspects to be considered when developing a cell transplantation therapy towards use in the clinical setting, for example the cell source, the *in vitro* manipulation and differentiation, the plasticity of the cell type at the time of transplantation, the method of delivery, the length of the effects *in vivo* and the functional recovery that can be achieved. Regarding the cell type of origin, our lab and many others have explored the possibility of using a variety of cell types. Adult stem cells are interesting since they could potentially be used in an autologous manner, there are no ethical concerns regarding their use and some of them can be easily harvested. For example, bone marrow stem cells have been pushed to differentiate *in vitro* towards inner ear hair cells and neurons (Boddy et al., 2012; Duran Alonso et al., 2012; Jeon et al., 2007; Kondo et al., 2005). In the study by Jeon et al, the induction of hair cells was accomplished by forced expression of *Atoh1* and injection into developing chick otocyst, leaving the question if this induction can take place in a mature deafened cochlea. In another type of work, Kondo (Kondo et al., 2005) focused on the differentiation potential of mouse mesenchymal stem cells (mMSCs) *in vitro*, and showed that culture with embryonic otocyst conditioned media was necessary to induce the full repertoire of sensory neuron markers (*Brn3a*, *Ngn1*, *NeuroD* and *Gata3*). Such an undefined system cannot be used for clinical applications but the proof of concept drives further

investigation to elucidate which components of the conditioned media are responsible for the induction process. In a similar manner, our lab studied the possibility to differentiate bone marrow stem cells of human origin (hMSCs) towards the inner ear sensory cells. To achieve that, we used conditioned media from human cochlea foetal auditory stem cells (hFASCs). We found that culture of hMSCs in hFASC-conditioned media was able to induce some early otic markers (*PAX8, PAX2*) followed by the expression of hair cells and neuronal markers. Moreover, we found that within the conditioned media, WNT activity was necessary to induce *PAX8* expression (Boddy et al., 2012). Although these results are promising, it must be said that the percentage of neuron and hair cells produced in these experiments is generally low, and together with the heterogeneity and the limited lifespan of MSCs *in vitro,* more research is necessary before we can use them in cell replacement therapies of the inner ear. Initial results obtained so far from transplanting mMSCs into the cochlea of deafened chinchillas are still incomplete since engrafting has been the only aspect evaluated and functional recovery has not been demonstrated (Naito et al., 2004).

Another source of adult stem cells that has been recently explored is the olfactory epithelia of mouse and human. This epithelium is easily harvested and is responsible for replacing the olfactory neurons throughout life in mammals. Olfactory epithelium cells are presumed to have a neural crest origin (Delorme et al., 2010). These cells have been shown to express some of the hair cell markers when differentiated in vitro (Doyle et al., 2007). In addition, when human olfactory epithelium cells are transplanted in a mouse model of deafness (A/J mice), a decline in the rate of natural hearing loss is observed 4 weeks after transplantation (Pandit et al., 2011). Therefore, although an increase of ABR threshold still occurs in transplanted animals, the pace is reduced, suggesting a protective function conferred by the olfactory epithelium cells. In this regard, no hair cells or auditory neurons of human origin were found in the mouse cochlea (Pandit et al., 2011). This raises the possibility that secreted growth factors produced by the cells could be responsible for the protective effect. Such an effect has already been proposed in other transplantation studies in the inner ear. Revoltella et al showed that human hematopoietic stem cells (HSCs) transplanted in the mouse cochlea after ototoxic insult help in the preservation of the organ of Corti (including hair cells) as assessed morphologically (Revoltella et al., 2008). No hair cells or neurons of human origin were observed in this case either. In line with a protective role of HSCs in the

inner ear, ABR threshold increases caused by cochlear ischemia in adult gerbils were reduced when animals were previously transplanted with HSCs (Yoshida et al., 2007).

Embryonic stem cells: progress and challenges

There have been a number of studies exploring the use of embryonic stem cells as a potential target to treat hearing impairment. ES cells are being investigated at the core of regenerative medicine for two main reasons: First, their pluripotent status is clearly established through robust assays like teratoma formation, embryo complementation for mouse ESCs, and directed differentiation protocols. Secondly, the endless self-renewal of ESCs in vitro provides the opportunity to produce specific cell types in high numbers from clonal origin, and to study any particular differentiation path more in depth.

Regarding inner ear therapies, some protocols for the differentiation of ESCs into hair cells and auditory neurons have been described (Chen et al., 2012; Corrales et al., 2006; Li et al., 2003b; Oshima et al., 2010; Ouji et al., 2012; Shi et al., 2007), and in some of these cases, cells have been transplanted into the cochlea (Chen et al., 2012; Corrales et al., 2006; Hildebrand et al., 2005; Sakamoto et al., 2004). However, despite encouraging results, there is so far no ideal protocol to produce auditory neurons and hair cells because the culture conditions have not been thoroughly defined, and many protocols use undefined conditions like; cell coculture, conditioned media, embryoid bodies, serum etc. For example the Heller group developed a protocol to differentiate mouse ES cells into hair cell-like cells (Li et al., 2003b). In their report, expression patterns of early inner ear development were followed by the expression of hair cells markers as it happens *in vivo*, however, only a small percentage of cells coexpressed hair cell markers *in vitro* and no morphological characteristics of hair cells were ever observed. For full differentiation to take place, it was necessary to transplant progenitors in developing otic vesicles. This protocol started with embryoid body formation, which primes mES cells towards different lineages in a random manner, perhaps explaining the low efficiency of hair cell differentiation observed in the end. In addition, no physiological measurements were carried out and questions regarding the nature of the signals in the developing chick otocyst that could trigger differentiation

remain. More recently, Ouji and colleagues have developed an alternative protocol for the differentiation of mESCs into hair cell-like cells (Ouji et al., 2012). However, the use of undefined components like embryoid body formation and conditioned media from ST2 stromal cells makes the protocol unsuitable from a clinical perspective. Moreover, Ouji et al did not carry out any electrophysiological examinations on their hair cells-like cells. Nonetheless, the protocol is simple and the percentages of immunopositive cells were higher than in Li's work. SEM analysis also showed cells presenting bundle-like structures reminiscent of hair cells.

Recently, in a second protocol proposed by Stephan Heller's group, mESCs and mouse induced pluripotent stem cells (miPSCs) were aggregated in embryoid bodies which were treated with a cocktail of WNT and TGF-β inhibitors to block mesoderm formation plus IGF-1 to produce head ectoderm. The purpose of this treatment was to enrich a population of neuroectodermal progenitors within the embryoid body. The progenitors obtained in this manner were exposed to FGF2 to induce otic differentiation (Oshima et al., 2010). The rationale behind this protocol was that if neuroectoderm is generated first, then cells will be competent to otic induction signals (e.g. FGF2) and therefore a more directed developmentally-reminiscent differentiation would be achieved. Through this approach, Oshima et al obtained 20-25% of PAX2+ otic progenitors from mESCs and miPSCs. It is important to mention that FGF treatment only worked when inhibitors for both WNT and TGF-β pathways and IGF-1 were present, any other combination dramatically reduced the percentage of PAX2+ cells, stressing the importance of the early differentiation stage. Moreover, when cells were differentiated further, co-expression of hair cell markers was observed. Perhaps the most remarkable aspect of this study was the generation of hair cell bundles observed when PAX2+ progenitor cells were cultured on inactivated chicken utricle stromal cells. Although the factors produced by the stromal cells are unknown and the possibility of cell fusion cannot be ruled out, the bundles had all the morphological and physiological characteristics of immature hair cells. Nevertheless, a functional assessment of hearing function of these progenitors is lacking.

So far I have described some of the efforts to recreate and regenerate hair cells for future medical applications. The other "ultimate" promise of ESCs in the treatment of hearing impairment would be through the replacement of spiral ganglion neurons. In this respect,

our lab and others have made significant progress. Albert Edge's group have used a chemically defined medium to create mESCs-derived sensory neurons and transplanted them into deafened gerbils (Corrales et al., 2006), the transplanted neuroblasts extended processes that reach the hair cells and lasted for up to 2 months, the longest time point analysed. It is noteworthy that the protocol used in this case differs significantly from the one used by Oshima et al (2010). In the Corrales study, they used a chemically defined culture protocol developed by Austin Smith lab to produce neuroectodermal progenitors from mESCs (Ying et al 2003). In that protocol cells are grown in adherent monolayer in DFNB medium (DMEM:F12 plus N2 and B27 supplements), which yields a high percentage of neuroectodermal cells in only 4 days, contrasting with the protocol employed by Oshima that required the blocking of alternative cells fates produced during embryoid body formation. The idea that mESCs convert into neuroectodermal derivatives when grown in adherent monocultures in the absence of externally added growth factors has been claimed as default trend (Munoz-Sanjuan and Brivanlou, 2002; Tropepe et al., 2001). It is interesting that the otic progenitors we produce from hESCs in our lab are generated in a similar way to the ones produced by Corrales et al, supporting the idea that neuroectodermal derivatives are also spontaneously produced in hESCs, and that additional suppression of endoderm and mesoderm may not be necessary in our monolayer approach (Chen et al., 2012).

Following Corrales report described above, Edge's group produced a second study, this time with hESCs. Embryoid bodies were first generated to produce neural progenitors that were later co-cultured with dissected cochlear epithelia to prouce sensory neurons (Shi et al 2007). Although the percentage of neurons obtained in this manner was high (22%), a few PAX2+ cells were seen at the neural progenitor stage, suggesting that even a partial otic placode induction may be enough for further differentiation. In this study Shi used cells aggregates in suspension instead of a monolayer during the first differentiation stage, a situation that may hamper the purification of different subsets of neural progenitors and impede its future clinical application.

Our hESC differentiation protocol

The approaches covered so far involving the transplantation of ESC-sensory neural progenitors into the deafened cochlea of animals have successfully demonstrated *de novo* innervations of hair cells, but examination of hearing function after transplantation has not yet been made (Corrales et al., 2006; Shi et al., 2007). In fact, only the work of Hildebrand et al (2005) assessed the ABR thresholds of animals after transplanting mESCs and differentiated mESC into the deafened inner ears of guinea pigs and the results in that study showed no improvement.

Recently published work from our lab represents a step forward in the treatment of deafness, since it overcomes many of caveats encountered in the protocols discussed before (Chen et al., 2012). Some of the advantages of our system are: the use of hES cells instead of mouse ones, differentiation in a chemically defined medium, low density monolayer differentiation allowing purification of specific cell types, and the use of FGF3 and FGF10 instead of FGF2, a situation more reminiscent of *in vivo* otic development. As a result, we have obtained otic progenitors characterized by the expression PAX2, PAX8, FOXG1, and SOX2 at a high percentage. Currently, we are dissecting the different roles of FGF signalling in the published protocol, so that in the future the differentiation conditions can be improved further.

The critical outcome of the Chen et al (2012) study is that for the first time, a functional hearing recovery in a gerbil model of auditory neuropathy has been observed after otic progenitor transplantation.

Aims

Although the results obtained in our lab are encouraging, the outcome of the differentiation protocol is variable, and the effect of FGF3 and FGF10 seems to be peripheral in many experiments. Therefore it is important to dissect the role of these ligands in our system.

As discussed, it is presumed that FGF3 and FGF10 act specifically through the FGFR2IIIb, and I have designed a number of tools to test if this receptor is also relevant in our differentiation protocol. Also in the future, it would be interesting to know if there is a subset of FGFR2b positive cells in the hES cell niche, so that population more competent to differentiation could be enriched. The insights from this work will be translated in experiments with better outcome in terms of differentiation efficiency, homogeneity and financial expense.

Particular aims

- Investigate if FGF3 and FGF10 can induce the expression of otic markers in different hES and hiPS cell lines.
- Measure the relevance of FGF signalling in our system by Q-PCR, using the FGFR inhibitor SU5402.
- Validate our *in vitro* model of otic differentiation by defining the window of FGF activity and examining the temporal expression of otic markers.
- Since it is accepted FGF activity depends on the specific FGFR activated rather than the ligand, investigate the presumed equivalency of FGF3 and FGF10.
- Define the expression of FGFR2 and FGFR2IIIb, the specific isoform presumed to bind FGF3 and FGF10, in hESCs
- Establish an overexpression construct to determine the role of FGFR2IIIb during otic differentiation. This receptor isoform is presumed to bind FGF3 and FGF10.
- As a complementary approach, I will set up an inducible knockdown system in hES cells to downregulate FGFR2.
- Alternatively, a soluble form FGFR2IIIb will be used to sequester any FGF acting through this receptor, working as a specific competitive inhibitor of this receptor isoform.