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# A network of growth and transcription factors controls neuronal differentiation and survival in the developing ear

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**ABSTRACT** Inner ear neurons develop from the otic placode and connect hair cells with central neurons in auditory brain stem nuclei. Otic neurogenesis is a developmental process which can be separated into different cellular states that are characterized by a distinct combination of molecular markers. Neurogenesis is highly regulated by a network of extrinsic and intrinsic factors, whose participation in auditory neurogenesis is discussed. Trophic factors include the fibroblast growth factor, neurotrophins and insulin-like peptide families. The expression domains of transcription factor families and their roles in the regulation of intracellular signaling pathways associated with neurogenesis are also discussed. Understanding and defining the key factors and gene networks in the development and function of the inner ear represents an important step towards defeating deafness.

**KEY WORDS:** *otic neurogenesis, IGF-I, FGF, inner ear, auditory ganglion, cochlear microarray*

## Otic neurogenesis

The inner ear develops from the otic placode, an ectodermal patch located close to the neural tube that invaginates to form a transitory structure, the otic cup. This cup subsequently closes and pinches off from the ectoderm to generate the otic vesicle (Figure 1). The otic vesicle is an autonomous structure that contains the information required to generate most of the cell types and structures of the adult inner ear (Bissonnette and Fekete, 1996; Bever *et al.*, 2003; reviewed in Varela-Nieto *et al.*, 2004). The mammalian inner ear is composed of six distinct sensory organs: the three cristae of the semicircular canal, the two maculae of the saccule and utricle and the organ of Corti in the cochlea. The cristae and the maculae are vestibular organs, whereas the organ of Corti is the organ of hearing (reviewed in Moller, 2006). The inner ear cochlear ganglion is formed by bipolar neurons that connect the peripheral sensory receptors or hair cells with central neurons in auditory brain stem nuclei. The correct organogenesis of the inner ear involves a dynamic balance of cell proliferation, differentiation, survival and death, processes that are tightly regulated by a network of extrinsic and intrinsic factors (Frago *et al.*, 2000; Varela-Nieto *et al.*, 2003 and 2004; Leon *et al.*, 2004).

The process of otic neurogenesis can be separated into different cellular states, each characterized by a distinct combination of molecular markers (summarized in Figure 1A). The first

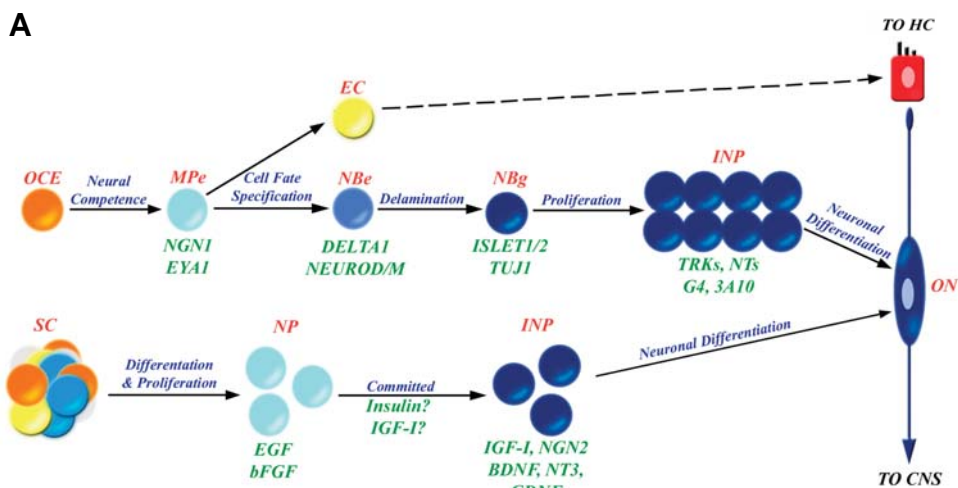
visible output of otic neurogenesis is the delamination of neural cells (otic neuroblasts) from the otic cup (Fig. 1B). These neuroblasts are committed to generate otic neurons and they populate the cochleo-vestibular ganglion (CVG). This ganglion is generated from a region of the otic vesicle epithelium denominated the prospective neural-sensory domain and that is defined by the domain in which *Ngn1* and *Delta1* are co-expressed at early stages (Adam *et al.*, 1998; Abu-Elmagd *et al.*, 2001). It is believed that both neurons and sensory cells derive from a common multipotent progenitor cell (MPe, in Figure 1A) (Brigande *et al.*, 2000; Lang and Fekete, 2001). Indeed, there is a loss of ganglion neurons and a decrease in the number of hair cells in the mouse *Ngn1* null-mutant (Ma *et al.*, 2000). Interestingly, *Notch* and its ligands *Delta1*, *Serrate1* and *Serrate2* (*Ser1* in the chicken, *Jag1* in the mouse) are expressed in the otic placode (Adam *et al.*, 1998; Eddison *et al.*, 2000; Cole *et al.*, 2000). While *Ser1* is expressed at the anterior and posterior poles of the otic cup, *Ser2* overlaps with the neural sensory domain defined by *Delta1* (Lang and Fekete, 2001). The Delta-Notch pathway is again essential at later steps when the cell fate decision of becoming a hair cell or a supporting cell is taken (Lanford *et al.*, 1999; Lang and Fekete,

*Abbreviations used in this paper:* BDNF, brain-derived neurotrophic factor; bHLH, basic helix-loop-helix; CVG, cochleovestibular ganglion; FGF, fibroblast growth factor; IGF, insulin-like growth factor; NGF, nerve growth factor; NT, neurotrophin.

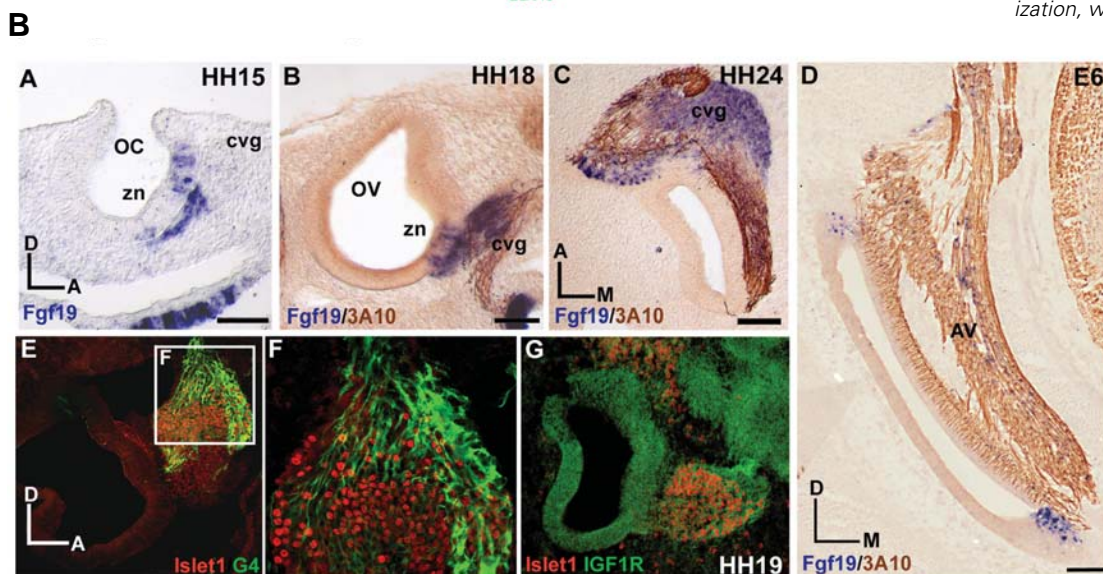
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**Fig. 1. Early inner ear neurogenesis. (A)** Scheme representing the different steps and factors implicated in otic neuron development. **(B)** Early inner ear neurogenesis. The inner ear arises from the otic placode, which invaginates to form the otic cup (oc) and then the otic vesicle (ov), from which the adult inner ear ultimately derives **(C,D)**. **(A-D)** *Fgf19* transcripts were detected in delaminating neuroblasts by in situ hybridization, while 3A10 immunoreactivity identifies otic axons (Modified from Sánchez-Calderón et al., 2007). *Islet-1* and *G4* expression in ganglionic neuroblasts **(E,F)** and *Islet-1* and *IGF1R* expression in the otic vesicle **(G)**. Abbreviations: CVG, cochlear-vestibular ganglion; HC, hair cell; INP, immature neural precursor; MPE, multipotent progenitor; NBE, epithelial neuroblast; NBg, ganglionic neuroblast; NP, neuronal precursor; OC, otic cup; OCE, otic cell epithelial; ON, otic neuron; OV, otic vesicle; SC, stem cell; ZN, neurogenic zone. Orientation: A, anterior; D, dorsal; M, medial. Scale bars: A, 50µm; B-D, 100µm.



2001; Brooker *et al.*, 2006).

Another set of proneural genes, *NeuroD* and *NeuroM*, are expressed both in the otic cup epithelium and in the delaminating neuroblasts (Liu *et al.*, 2000; Abu-Elmagd *et al.*, 2001), suggesting that they act after the selection and specification of neural precursors. The null mouse for *NeuroD* exhibits deficient neuroblast delamination but it does not display alterations in the generation of neuronal precursors (Liu *et al.*, 2000; Kim *et al.*, 2001; reviewed in Chae *et al.*, 2004). Therefore, the expression of *NeuroD* defines a second cell state in the neural lineage after neural fate specification, the epithelial neuroblast (NBE, Figure 1A).

Delamination of otic neuronal precursors has been studied extensively in the chicken embryo (D'Amico-Martel and Noden, 1983; Alvarez *et al.*, 1989; Hemond and Morest, 1991). This delamination starts at the otic cup stage and their migration peaks when the otic cup closes to form the early otic vesicle. The delaminating cells are a population committed to a neural fate able to proliferate that constitutes a transit-amplifying cell population (Adam *et al.*, 1998; Begbie *et al.*, 2002). We can identify this third cellular state as the ganglionic neuroblast (NBg, Figure 1A).

The LIM-homeodomain gene family of transcription factors is crucial to specify the identity of neurons derived from the neural tube (Hobert and Westphal, 2000). *Islet-1* is one member of this family that is expressed in a few cells within the epithelium, but it is intensely expressed in cells located at the sites of delamination and in those that have delaminated (Figure 1B, and Adam *et al.*, 1998; Camarero *et al.*, 2003; Li *et al.*, 2004b). In addition to *NeuroD* and *Islet-1*, the mouse epithelial neuroblasts also express the  $\alpha 6$  integrin subunit, which interestingly is not expressed in delaminated neuroblasts (Davies, 2007). The ganglionic neuroblasts make up a population characterised by the expression of *NeuroD*, *Islet-1*, neuron-specific  $\beta$ III-tubulin, *Tuj1* (Memberg and Hall, 1995; Camarero *et al.*, 2003) and proliferation markers such as the proliferating cell nuclear antigen (PCNA) and phospho-histone 3 (Camarero *et al.*, 2003). Although there are other fundamental elements involved, as discussed later, ganglionic neuroblasts depend on insulin-like growth factor I (IGF-I) for their survival, expansion and differentiation (Camarero *et al.*, 2003). The mouse transcription factor *Eya1* modulates the expression of the transcription factors *Tbx1* and of the proneural genes *Ngn 1* and *NeuroD*, as well as that of the neurotrophins,

brain derived neurotrophic growth factor (BDNF) and neurotrophin 3 (NT-3) (Friedman *et al.*, 2005). Since *Tbx1* is expressed in the otocyst epithelium but is excluded from the prospective endolymphatic duct and proneural regions, it has been postulated that this gene could inhibit neural fate by suppressing *Ngn1* and *Delta1* expression (Raft *et al.*, 2004; Xu *et al.*, 2007).

After the last cycle of cell division, neuroblasts are post-mitotic and they become immature neurones, which then differentiate and extend projections towards their peripheral and central targets (Whitehead and Morest, 1985). These cells are immature neuronal precursors (INP, Figure 1A), a fourth cell state characterised by being post-mitotic but not yet differentiated. Accordingly, these precursors down-regulate the expression of most of the early neural genes and start to express another set of markers related to neurite extension and survival. These markers include fasciculin G4 (Fig. 1B; Camarero *et al.*, 2003), the neurofilament associated antigen 3A10 (Adam *et al.*, 1998; Camarero *et al.*, 2003) and the TrkB and TrkC neurotrophin receptors (Brumwell *et al.*, 2000; Kim *et al.*, 2001, Sanchez-Calderon, unpublished observations). The final cellular state is that of a mature otic neuron (ON, Figure 1A), which generates action potentials and expresses synaptic receptors and neurotransmitters (Raphael and Altschuler, 2003).

Adult inner ear cells retain certain capacity to proliferate. Natural regeneration of the sensory hearing epithelia is displayed by teleosts, amphibians and birds. In contrast, there is no evidence of regeneration in the cochlear sensory epithelium or the cochlear ganglion neurons in mammals and only vestibular hair cells retain some capacity for regeneration. This lack of regeneration in the adult mammalian inner ear has made cell replacement therapy by transplanting extrinsic stem cells into the inner ear, or by activating intrinsic stem cells residing in the inner ear, an interesting proposition to counteract the degeneration and loss of sensory and neuronal cells (Hu and Ulfendahl, 2006). Several growth factors, including epidermal growth factor (EGF), IGF-I and basic fibroblast growth factor (bFGF) promote the proliferation of embryonic stem cells to produce inner ear precursors (Li *et al.*, 2003a and 2003b; Hu *et al.*, 2005), in accordance with observations implicating these factors in the proliferation, differentiation and survival of developing inner ear cells (Leon *et al.*, 1995; Zheng *et al.*, 1997; Hossain and Morest, 2000; Ladher *et al.*, 2000; Varela-Nieto *et al.*, 2004). In particular, IGF-I has been shown to promote neural stem cell proliferation and survival in

different contexts and to induce differentiation in combination with neurotrophins (Arsenijevic and Weiss, 1998; Arsenijevic *et al.*, 2001) (Figure 1A).

It is clear that hair cells can be generated from embryonic and adult mammalian stem cells and that in some cases they can integrate into the developing chicken sensory field (Malgrange *et al.*, 2002; Tateya *et al.*, 2003; Li *et al.*, 2003a, 2003b and 2004a). In contrast, there is only limited evidence for the presence of neuronal stem cells in the mammalian ear (Rask-Andersen *et al.*, 2005). Therefore, an attractive alternative for the replacement of neurons lost from the cochlear ganglion is the transplantation of neural stem cells (Okano *et al.*, 2005). These stem cells play a major role in the development of the embryonic central nervous system and their expansion depends on signals from the microenvironment in which they are found, including a number of growth factors such as IGF-I and bFGF (reviewed in Merkle and Alvarez-Buylla, 2006; Doetsch, 2003). Adult mouse neural stem cells differentiate into a neuronal fate but only few survive in the inner ear. Nevertheless, it is promising that the survivors migrate to the spiral ganglion, the auditory nerve tract and the organ of Corti, where they differentiate and extend neurites into the auditory system of adult mammals (Hu *et al.*, 2005; Hu and Ulfendahl, 2006).

## Otic neurogenesis is regulated by trophic factors

### The Fibroblast Growth Factor family

The FGF family is comprised of 25 members that have been implicated in different cell functions, including cell differentiation, proliferation, motility and survival (Wright and Mansour, 2003). Two of them, FGF24 and FGF25, have been identified in zebrafish (Fischer *et al.*, 2003; Katoh and Katoh, 2005). FGF's act through the specific FGF receptors produced from four genes in mammals, which generate many different protein isoforms through alternative splicing (Wright and Mansour, 2003).

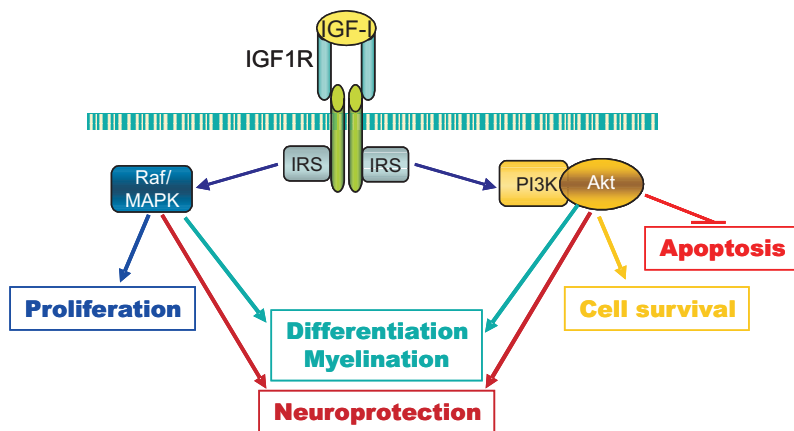
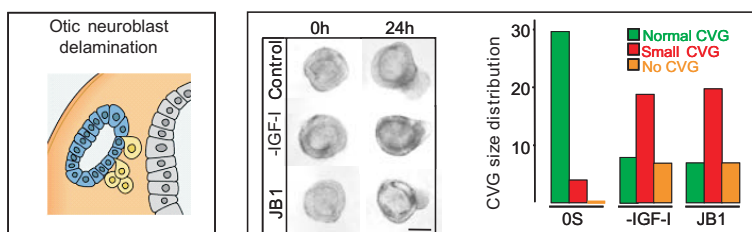
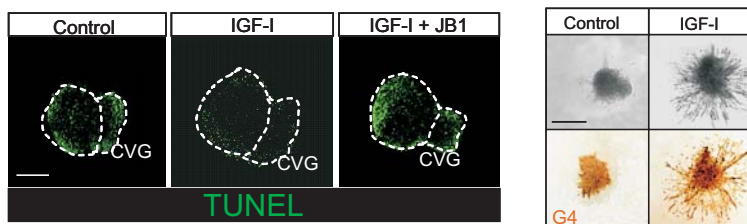
FGF2, FGF3, FGF8, FGF10 and FGF19 participate in otic neurogenesis (Table 1), and FGF16 is present in the otic vesicle epithelium in areas not associated with neurogenesis but rather with sensory organ specification (Chapman *et al.*, 2006). *Fgf2* (*bFGF*) is expressed in mouse and chicken otic placode and otic vesicle (Vendrell *et al.*, 2000). This FGF augments the migration and differentiation of CVG neurons (Hossain *et al.*, 1996; Zheng *et al.*, 1997; Adamska *et al.*, 2001) and it has been implicated in

TABLE 1

### DIFFUSIBLE FACTORS INVOLVED IN INNER EAR NEUROGENESIS

Factor	Migration	Proliferation	Survival	Apoptosis	Early Differentiation	References
Insulin	nd	+	nd	nd	-	Leon <i>et al.</i> , 1995 and 1998.
IGF-I	-	+	+	-	+	Leon <i>et al.</i> , 1995; Frago <i>et al.</i> , 2003
IGF-II	nd	+	nd	nd	nd	León <i>et al.</i> , 1995
BDNF/TrkB	+	+	+	-	+	Farinas <i>et al.</i> , 2001; Fritsch <i>et al.</i> , 2004
NT-3/TrkC	+	+	+	-	+	Fritsch <i>et al.</i> , 2004
NGF/p75 <sup>NTR</sup>	nd	-	-	+	nd	Frago <i>et al.</i> , 1998; Sanz <i>et al.</i> , 1999a.
FGF2	+	+	+	-	+	Adamska <i>et al.</i> , 2001; Nicholl <i>et al.</i> , 2005
FGF3	+	nd	+	nd	nd	Wright and Mansour, 2003
FGF8	nd	-	nd	nd	nd	Adamska <i>et al.</i> , 2001
FGF10	-	-	-	-	+	Alsina <i>et al.</i> , 2004
FGF19	+	nd	nd	nd	+	Sanchez-Calderon <i>et al.</i> , 2007

Data compiled from transgenic mouse and from *in vitro* experiments performed in mouse and chicken otic cultures. The list is not intended to be comprehensive. +, positive modulation; - negative modulation; nd, not determined.

**A** Intracellular pathways for IGF-I**B** Endogenous IGF-I promotes CVG generation**C** Exogenous IGF-I induces neuronal differentiation and survival

the differentiation of sensory cells in the chicken embryo. However, the inner ear forms normally in mice lacking *Fgf2* and no abnormalities are observed in adults (Carnicero *et al.*, 2004; Sliwinska-Kowalska *et al.*, 2005). Interestingly, FGF2 promotes BDNF activity by up-regulating its high affinity TrkB receptor in cultured mouse auditory neurons (Brumwell *et al.*, 2000). Significantly, the sequential interaction between FGF2, BDNF, NT-3 and their receptors define critical periods of the development of auditory neurons (Hossain *et al.*, 2002). Moreover, co-administration of FGF2 and glial cell line-derived neurotrophic factor (GDNF) supports the survival of adult mouse cochlear neurons *in vitro*, whilst FGF2 alone promoted neurite generation (Wei *et al.*, 2006). Indeed, either FGF2 or FGF1 induced bipolar neurite formation in cultures of US/VOT-N33 (N33) cells, which are derived from mouse cochlear neuroblasts, a characteristic of mature cochlear neurons (Nicholl *et al.*, 2005).

In mice and *Xenopus*, *Fgf3* (*Int2*) mRNA is confined to the otic vesicle sensory epithelium and to the CVG (Wilkinson *et al.*, 1989; Tannahill *et al.*, 1992), but not in the chicken embryo (Mahmood

**Fig. 2. IGF-I and neurogenesis: intracellular pathways and their implications in CVG generation, proliferation, neuronal differentiation and survival of otic neuroblasts.**

**(A)** Proposed signalling pathways involved in IGF-I-mediated neurogenesis and related process. Binding of IGF-I to the extracellular  $\alpha$ -subunits of its high affinity receptor (IGF1R) provokes its auto-phosphorylation on tyrosine residues in the  $\beta$ -subunits and the subsequent docking and phosphorylation of intracellular signalling proteins. The figure shows the two main pathways specifically activated by IGF-binding to IGF1R, the PI-3K/Akt pathway and the Raf/MAPK pathway and their general role in neurogenesis (summarized from Feldman *et al.*, 1997; Varela-Nieto *et al.*, 2003; Russo *et al.*, 2005; Ye and D'Ercole, 2006). Abbreviations: IRS, insulin-receptor substrate; PI3K, phosphoinositide-3 kinase; MAPK, mitogen-activated protein kinase.

**(B)** Endogenous IGF-I is involved in chicken CVG generation. The function of IGF-I was explored in explants of otic vesicles by assessing the formation of the CVG in medium without additives (control) or in the presence of anti-IGF-I antibodies ( $\alpha$ -IGF-I) or the competitive receptor antagonist JB1. The scheme to the left represents the process of neuroblast delamination from the ventromedial part of the otic vesicle. The middle part illustrates representative photomicrographs of otic vesicles at the start of culture (0 h) and after 24 hours in culture with serum-free medium (Control), with  $\alpha$ -IGF-I antibodies and with JB1. The histogram to the right, presents the size distribution of ganglia generated from otic vesicle explants cultured under different conditions. Scale bar, 150  $\mu$ m. Modified from Camarero *et al.*, 2003. **(C)** Exogenous IGF-I promotes chicken neuroblast differentiation and survival. Photomicrographs show the effect of IGF-I on survival (TUNEL, left) and on differentiation in cultured CVG explants (G4, right). Scale bar: C, Control, 150  $\mu$ m. Modified from Camarero *et al.*, 2003.

*et al.*, 1995). The CVG ganglion is diminished in mice carrying a null-mutation for *Fgf3*, on a background of morphogenetic defects (Mansour *et al.*, 1993). Indeed, mouse embryos lacking both *Fgf3* and *Fgf10* fail to form otic vesicles and therefore, these two genes are clearly implicated in otic induction (Wright and Mansour, 2003; Alvarez *et al.*, 2003). It was

recently shown that a human syndrome characterized by type I microtia, microdontia and profound congenital deafness due to the complete absence of inner ear structures is caused by any of three different homozygous mutation in *FGF3* (Tekin *et al.*, 2007).

*Fgf8* (*AIGF*) is expressed in the chicken otic vesicle and later in the CVG (Colvin *et al.*, 1999) and it is implicated in zebrafish, chicken and mouse otic induction (Leger and Brand, 2002; Ladher *et al.*, 2005; Hans *et al.*, 2007). Both loss of function of *Fgf8* in zebrafish and the knock down of *Fgf3* with antisense morpholinos cause profound alterations in the induction and formation of the otic vesicle (Phillips *et al.*, 2001; Hans *et al.*, 2007). However, little is known about the alterations of sensory neurons or sensory receptors under these circumstances. The formation of the otic placode in the chicken and mouse requires at least two signals, one from the mesoderm and one from the ectoderm. In the chicken, the mesodermal signal is FGF19 while in the mouse it is FGF10. Moreover, a signal from the endoderm is necessary for *Fgf8* to induce mouse and chicken otic development (Ladher *et al.*, 2005). Previous studies in the chicken showed that the ectopic



application of FGF2 and FGF8 enhances the transcription of several otic patterning genes and enlarges the CVG (Adamska *et al.*, 2001), supporting a role for FGF8 in the generation of the CVG (Hossain *et al.*, 1996). Moreover, delaminating neuroblasts are observed at the boundary between the domains of chicken *Fgf8* and *Otx2* expression (Hidalgo-Sanchez *et al.*, 2000; Sanchez-Calderon *et al.*, 2004).

*Fgf10* mRNA is expressed in the chicken and mouse otic placode and otic vesicle, within the proneural-sensory epithelium. Indeed, *Fgf10* in the mouse is also expressed in CVG neurons (Pirvola *et al.*, 2000; Alsina *et al.*, 2004; Lilleväli *et al.*, 2006) and overexpression of chicken *Fgf10* increases the number of cells expressing *NeuroD* and *NeuroM*, but not *Delta1* (Alsina *et al.*, 2004). The number of otic neurons is reduced in mouse null mutant for *Fgf10* and defects are detected in the sensory epithelium (Pauley *et al.*, 2003). Based on binding studies, FGFR-2IIIb is thought to be the target of FGF10, although this factor also binds to FGFR-1 (Powers *et al.*, 2000). *Fgfr-2* is expressed dorsally in the prospective non-sensory epithelium of the otocyst, whereas *Fgf10* and *Fgf3* are co-expressed with neurotrophins in the ventral domain, suggesting that FGFs and FGFRs may act in a paracrine manner (Pirvola *et al.*, 2000). FGFR-2IIIb null mutants fail to form the CVG at early stages and although the detailed mechanism remains unknown neural precursors are probably affected. Disruption of *Fgfr1*, the other potential receptor for FGF10, causes defects in the organ of Corti associated with the reduced proliferation of the precursor pool that generates the auditory sensory epithelium (Pirvola *et al.*, 2002). Moreover, FGF10 signalling was proposed to be regulated by GATA3, a zinc finger transcription factor (Ohuchi *et al.*, 2005; Lillevali *et al.*, 2006).

Finally, mouse FGF15 is the homologue of chicken and human FGF19 (Wright *et al.*, 2004). Mesodermal chicken *Fgf19* has been implicated in otic induction (Ladher *et al.*, 2000; Wright *et al.*, 2004) and it is expressed both in the ganglionic neuroblasts delaminating from the otic epithelium and in the CVG (Figure 1B). FGF19 transcripts remain in the CVG until E8, suggesting a relationship between this factor and neural differentiation (Figure 1B) (Sanchez-Calderon *et al.*, 2007). *Fgf19* and *Fgf8* mRNA expression determine two subdomains within the *Fgf10*-positive proneural-sensory territory (Sanchez-Calderon *et al.*, 2007). Human FGF19 appears to bind only to FGFR4 (Xie *et al.*, 1999), which is expressed in the chicken and mouse neuroectoderm and in the chicken mesoderm (Wright *et al.*, 2004). In the mouse, *Fgf15* is not expressed in the CVG and accordingly, *Fgf15* null mutants do not display clear otic abnormalities (Wright *et al.*, 2004). Thus, FGF signalling clearly plays an important role in every aspect of the development and innervation of the ear. Hence, it will be interesting to explore of FGF-cooperation or looping and the interactions of these factors with other growth factors during otic neurogenesis.

### **Nerve growth factor and neurotrophins**

The neurotrophin (NT) family is made up of the small, basic, secreted proteins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). These proteins are essential for neuronal survival and differentiation (reviewed by Bibel and Barde, 2000; Huang and Reichardt, 2001; Pirvola and Ylikoski, 2003) and they mediate their effects through two types of receptors: the low affinity p75

neurotrophin receptor (p75<sup>NTR</sup>), a modulator of cell survival and cell death; and the Trk tyrosine kinase receptors that interact specifically with NGF (TrkA), BDNF (TrkB) and NT3 (TrkC) (Casaccia-Bonnel *et al.*, 1999; Barrett, 2000; Lee *et al.*, 2001; Chao and Bothwell, 2002). Both NT-3 and BDNF, but not NGF or NT-4/5, are expressed in the inner ear sensory epithelia and in delaminating neuroblasts suggesting an early source of neurotrophic support for newborn CVG cells (Farinas *et al.*, 2001; Friedman *et al.*, 2005). The high affinity receptors TrkB (BDNF) and TrkC (NT3) receptors are expressed in otic sensory neurons and, while cochlear neurons depend on NT-3, vestibular neurons depend on BDNF for survival and differentiation in chicken, mouse and humans (Table 1) (reviewed in Fritzsche *et al.*, 2004). Indeed, the majority of cochlear neurons are lost during inner ear development between E13.5 and E15.5 in *NT3*<sup>-/-</sup> mutant mice. The double *BDNF* and *TrkB* mutant mainly lose vestibular neurons and in *BDNF/NT-3* or *TrkB/TrkC* double homozygous mutants all cochlear and vestibular neurons are lost (Bianchi *et al.*, 1996; Fritzsche *et al.*, 1998 and 2004; and reviewed in Pirvola and Ylikoski, 2003). Specific binding of NGF to the TrkA receptor is involved in growth, survival and differentiation of neurons in the central nervous system (Williams *et al.*, 2006). NGF is not expressed in the inner ear, but *TrkA* is localised in the early-developing CVG (Pirvola *et al.*, 1994; Schecterson and Bothwell, 1994; Farinas *et al.*, 2001) and in the adult mouse and rat organ of Corti. Hence, NGF may exert a specific effect on the peripheral auditory system (Dai *et al.*, 2004). The development and survival of spiral ganglion neurons depends on neurotrophic factors such as NT-3 and GDNF (reviewed in Roehm and Hansen, 2005). In stem cells obtained from adult human and guinea pig spiral ganglions, neural differentiation and neurite outgrowth is induced by BDNF, GDNF and NT-3 (Rask-Andersen *et al.*, 2005). Neurons differentiated from human progenitors co-express receptors for neurotrophic factors such as TrkB and TrkC (Rask-Andersen *et al.*, 2005), consistent with the co-expression of these receptors in auditory neurons (Pirvola *et al.*, 1992; Farinas *et al.*, 2001). On this basis, neurotrophic factors have been proposed as candidates for pharmacological treatment to prevent secondary auditory nerve degeneration and to promote neurite re-growth following hearing loss (reviewed in Atar and Avraham, 2005; Holley, 2005).

All neurotrophins bind to p75<sup>NTR</sup>, which also interacts with Trk receptors to modulate ligand binding specificity, affinity, and function in certain cell types (Benedetti *et al.*, 1993; Bibel *et al.*, 1999). Mature neurotrophins preferentially bind to Trk receptors whereas the immature "proforms" of these proteins have higher affinity for p75<sup>NTR</sup>, which fulfils a critical role in controlling the balance between cell survival/death. Sortilin acts as a co-receptor for p75<sup>NTR</sup> essential for pro-neurotrophin-induced cell death (Nykjaer *et al.*, 2004). The p75<sup>NTR</sup> receptor is encoded by two proteins, the full-length (FL-p75<sup>NTR</sup>) and short (S-p75<sup>NTR</sup>) isoforms of p75 (Von Schack *et al.*, 2001). Indeed, NGF induces apoptotic cell death in organotypic cultures of otic vesicles and in the CVG through binding to p75<sup>NTR</sup> in the chicken (Table 1) (Frago *et al.*, 1998; Sanz *et al.*, 1999a). Moreover, NGF-induced cell death only occurs in specific areas of the otic vesicle and CVG. The presence of p75<sup>NTR</sup> has been reported at different stages of inner ear development in several animal species, and p75<sup>NTR</sup> expression has been associated with the early specification of vestibular

sensory patches in the chicken embryo (von Bartheld *et al.*, 1991; Schecterson and Bothwell, 1994; Wu and Oh, 1996; Frago *et al.*, 2003 and 2006). The precise signalling pathway(s) used by p75<sup>NTR</sup> to activate cell death remain unclear. They may involve the generation of ceramide (Frago *et al.*, 1998 and 2006; Hirata *et al.*, 2001) and the activation of Jun-N-terminal kinase (Sanz *et al.*, 1999a; Harrington *et al.*, 2002), the caspase cascade (Gu *et al.*, 1999; Troy *et al.*, 2002; Frago *et al.*, 2003) and cyclin-dependent kinases (Frade, 2000). The pro-apoptotic activity of NGF/p75<sup>NTR</sup> during early chicken neurogenesis is strictly controlled by survival factors such as IGF-I (Frago *et al.*, 2003). In mice carrying a null mutation for the FL-p75<sup>NTR</sup> but that still expressing S-p75<sup>NTR</sup>, there is progressive hearing loss four months after birth, which is associated with the degeneration of cochlear neurons and hair cell loss at the basal turn of the cochlea. It was proposed that p75<sup>NTR</sup> may play a significant role in the maintenance of cochlear function and that mice carrying a mutation in the p75 gene could represent a new animal model of early onset progressive hearing loss (Sato *et al.*, 2006). These data contrast with that suggesting that the activation of p75<sup>NTR</sup> is associated with cell death in the adult cochlea. In a normal cochlea, the hair and supporting cells of the organ of Corti produce mature BDNF that activates TrkB receptors on cochlear neurons, triggering their survival. After aminoglycoside administration, sensory cells of the organ of Corti progressively degenerate, reducing the availability of mature BDNF and producing a decrease in TrkB expression. In parallel, p75<sup>NTR</sup> expression and c-Jun phosphorylation augments in cochlear neurons, suggesting an activation of apoptotic pathways when pro-survival neurotrophic support is diminished (Tan and Shepherd, 2006).

New insights into the activity of profactors and the transcriptional regulation of the genes encoding neurotrophins and their receptors have re-kindled interest in these elements. Thus, by controlling cell death and survival decisions the NGF family of neurotrophins contributes to inner ear development and the maintenance of its activity. Therefore, these elements may be of interest for hearing protection and regeneration.

### **The insulin-like growth factor system in inner ear neurogenesis**

The insulin gene family is made up of insulin, the insulin-like growth factors (IGF) I, II, relaxin and several insulin like peptides in different species (reviewed in Russo *et al.*, 2005). Indeed, the gene encoding IGF-I is highly conserved in mammals, birds and amphibians (reviewed in Russo *et al.*, 2005). Insulin-like peptides bind to specific receptors in the plasma membrane of target cells which can be grouped into two categories, the tyrosine kinase receptors (insulin receptor and IGF1R) and the mannose-6-phosphate IGF2R. In addition, the activity of IGF is modulated by a family of plasma transporters referred to as the IGF binding proteins (IGFBP).

The actions of IGF-I are mediated by IGF1R, whose gene has also been highly conserved during evolution (Fig. 2A). IGF1R is a heterotetrameric transmembrane protein containing two  $\alpha$  (extracellular ligand-binding) and two  $\beta$  (intracellular) subunits with protein tyrosine kinase activity (LeRoith *et al.*, 1995). Proinsulin and insulin are also able to bind to this receptor with high affinity and both are abundant in serum, typically masking the activity of IGF-I. The IGF-I protein displays an amino acid identity above 91% in the tyrosine kinase encoding domain in animals as divergent as *Xenopus*, fish, chicken, rat and human (Russo *et al.*, 2005). In the zebrafish embryo, *Igf1r* is expressed abundantly, particularly in the

tail, eye, ear primordia and brain (Ayaso *et al.*, 2002). The inhibition of IGF1R signalling during zebrafish embryogenesis causes significant defects in the inner ear that are accompanied by reduced embryonic growth, arrested development, increased lethality and induced neuronal apoptosis (Schlueter *et al.*, 2007).

During embryogenesis, *Igf-1* and *Igf1r* mRNA are expressed in brain regions where there is active nerve sprouting in spinal and sensory ganglia and in cranial and spinal nerves (reviewed in Varela-Nieto *et al.*, 2003). However, the transcription of the IGF-I and IGF1R genes declines significantly postnatally and reaches very low levels in the adult, a decrease that is correlated with the degree of cell maturation (reviewed in Varela-Nieto *et al.*, 2003). Nonetheless, both proteins are still present in neurons and Schwann cells of the autonomic and peripheral nervous systems, as well as in structures of the central nervous system that undergo cell renewal in adulthood, such as the olfactory bulb, choroid plexus and the dentate gyrus of the hippocampus (reviewed in Varela-Nieto *et al.*, 2003). Both IGF-I and IGF-II appear to be able to increase the number of neural brain cells and prevent neuronal apoptosis (reviewed in Varela-Nieto *et al.*, 2003; Russo *et al.*, 2005; Ye and D'Ercole, 2006). IGF induces growth and DNA synthesis in a variety of neuronal cell types *in vitro*, including the neurons of the CVG (Leon *et al.*, 1995). IGF-I shortens the length of the cell cycle in neuronal progenitors during embryonic life and influences the growth of all neural cell types (reviewed in Varela-Nieto *et al.*, 2003; Ye and D'Ercole, 2006). IGF-I may also act as a competence factor, a factor required for other growth factors to act. For example, in the absence of IGF-I neither EGF nor FGF-2 were able to stimulate proliferation of cultured neural stem cells from E14 mouse embryos (Arsenijevic *et al.*, 2001). However, the role of IGF-I in cell lineage specification remains controversial, possibly due to species specific activity.

IGF-I is also required for the early differentiation and survival of neuroblasts in the chicken otic vesicle (Table 1; Fig. 2B) (Camarero *et al.*, 2003). IGF-I and IGF1R are expressed in the developing chicken otic epithelium and CVG (Camarero *et al.*, 2003) and in the postnatal cochlear and vestibular ganglia (Camarero *et al.*, 2001 and 2002; reviewed in Varela-Nieto *et al.*, 2003 and 2004). In organotypic cultures of chicken otic vesicles, the addition of exogenous IGF-I causes an increase in cell number in the otic vesicle and its associated CVG, mimicking the normal pattern of *in vivo* morphogenesis (Leon *et al.*, 1995). Addition of exogenous IGF-I to the isolated chicken CVG increases cell proliferation, causes neurite outgrowth and elevates the expression of the neuronal differentiation marker G4 (Camarero *et al.*, 2003) (Fig. 2C). Blockage of endogenous IGF-I activity inhibits CVG formation in growth factor-free medium and increases cell death, revealing that endogenous IGF-I activity is essential for ganglion generation and survival (Camarero *et al.*, 2003). These studies suggest that proliferation, differentiation and survival of neural progenitors and neurons of the inner ear are dependent on IGF-I.

Even though much progress has been made in recent years, the intracellular signaling pathways that mediate organ-specific IGF-I activity remain to be clarified. The response of target cells to IGF-I is mediated by its high affinity receptor, IGF1R, a classic transmembrane tyrosine kinase receptor. IGF1R is expressed in multiple neural tissues in the embryo and its activation plays an important role in both proliferation and neuronal differentiation during embryonic development and also in regeneration (reviewed

in Varela-Nieto *et al.*, 2003). Binding of IGF-I to IGF1R activates distinct signalling cascades, which in turn mediate the trophic effects of IGF. Tyrosine kinase activity results in the autophosphorylation of IGF1R and triggers the activation of two main intracellular signalling pathways: the phosphatidylinositol-3 kinase/Akt (PI-3K/Akt) survival pathway; and the Raf/mitogen-activated protein kinase (Raf/MAPK) that activates the proliferation of neuronal cells during development (reviewed in Varela-Nieto *et al.*, 2003; Russo *et al.*, 2005). Recently, IGF-I treatment has been shown to promote a neuronal phenotype in mouse olfactory bulb stem cells by regulating the PI-3K/Akt pathway (Otaegi *et al.*, 2006; Kalluri *et al.*, 2007) and significantly, IGF-I can activate these pathways during early development of the otic vesicle (Sanz *et al.*, 1999b; Frago *et al.*, 2003). The activation of the Raf/MAPK cascade is involved in the proliferation of the epithelial inner ear cells, whereas the activation of PI3-kinase/Akt pathway controls otic cell survival (Sanz *et al.*, 1999b; Frago *et al.*, 2003; Leon *et al.*, 2004). In addition to canonic intracellular pathways, ceramide kinase has also been proposed as a key target in IGF-I protective pathways (Sugiura *et al.*, 2002; Frago *et al.*, 2003 and 2006).

Insight into the role of IGFs in inner ear cochlear ganglion neurogenesis has come from the study of genetically modified mice. While there is an increase in cell size and less apoptosis in a transgenic mouse over-expressing IGF-I (reviewed in Varela-Nieto *et al.*, 2003 and 2004), conversely there is growth retardation, reduced brain size, loss of selective neuronal populations, hypomyelination and reduced peripheral conduction velocities in mice lacking IGF-I (*Igf1<sup>-/-</sup>*) (Figure 3) (reviewed in Varela-Nieto *et al.*, 2003 and 2004). In addition, postnatal cochlear development is severely impaired in mice *Igf1<sup>-/-</sup>*, which develop smaller cochlea and cochlear ganglia, an immature tectorial membrane and they display a significant decrease in the number and size of auditory neurons (Camarero *et al.*, 2001 and 2002). The marked reduction in neural cell number seems to be due to multiple processes,

including increased cell death (Camarero *et al.*, 2001). These results demonstrated that IGF-I contributes to the maturation and maintenance of cochlear neurons. In addition, the myelin sheath that envelops the bipolar neurons of the postnatal vertebrate cochlear nerve is severely affected (Camarero *et al.*, 2002). In summary, lack of IGF-I in mice affects the postnatal survival, differentiation and maturation of cochlear ganglion cells and causes abnormal innervation of the sensory cells in the organ of Corti. Interestingly, mutations in the gene encoding human IGF-I cause syndromic neurosensorial hearing loss (Woods *et al.*, 1996 and 1997; Walenkamp *et al.*, 2005; Walenkamp and Wit, 2006). Furthermore, IGF-I deficit in the mouse causes all-frequency bilateral sensorineural hearing loss and a delayed response to acoustic stimuli (Cediel *et al.*, 2006) (Figure 3N). In the diseased or injured nervous system, IGF-I infusion enhances nerve regeneration and *in vitro*, stimulates regeneration of adult sensory neurons (reviewed in Varela-Nieto *et al.*, 2003). Hence, IGF-I may be used as a treatment cofactor to combat hearing loss.

### Transcription factors and gene networks

During the development of the inner ear there are several families of transcription factors that are implicated in defining boundaries and polarity and in the regulation of intracellular signalling (Table 2). It follows a brief overview of the transcription factors that are expressed in the inner ear during the stages of early neurogenesis.

#### Zinc finger transcription factors

Two of the six members of the GATA family of zinc finger transcription factors are expressed in the inner ear, GATA3 and GATA2 (Lillevali *et al.*, 2004). Their expression overlaps in the mouse otic vesicle at E9.5-10.5 but during subsequent development, it diverges considerably. GATA3 is confined to the sensory

TABLE 2

#### TRANSCRIPTION FACTORS IMPLICATED IN OTIC NEUROGENESIS

Gene Name	Description	Family	Proces	References
Gata3	Gata3 zinc finger	Zinc fingers	Expressed only in auditory neurons. Selective loss of cochlear ganglion neurons in the null mouse.	Lillevali <i>et al.</i> , 2004; Lawoko-Kerali <i>et al.</i> , 2002 and 2004; Karis <i>et al.</i> , 2004
Gata2	Gata2 zinc finger	Zinc fingers	nd	Lillevali <i>et al.</i> , 2004; Holley <i>et al.</i> , 2007
Gfi1	Growth Factor Independence 1	Zinc fingers	Hair cells differentiation and survival	Wallis <i>et al.</i> , 2003
Brn3a	POU domain, class 4, transcription factor 1	POU domain	Growth and migration of sensory neurons; innervation and axon guidance	Huang <i>et al.</i> , 2001a
Brn3c	POU domain, class 4, transcription factor 3	POU domain	Hair cell differentiation	Xiang <i>et al.</i> , 1997; Zheng <i>et al.</i> , 2000
Ngn1	Neurogenin 1	Basic helix-loop-helix (bHLH)	Differentiation of all inner ear sensory neurons	Ma <i>et al.</i> , 1998
NeuroD	Neurogenic differentiation 1	bHLH	Survival of inner ear sensory neurons. The NeuroD null mouse presents a general loss of otic neurons, being more affected the cochlear ones.	Liu <i>et al.</i> , 2000†; Kim <i>et al.</i> , 2001
Hes1	Hairy and enhancer of split 1	bHLH	Ear histogenesis; effectors for the Notch pathway	Zine <i>et al.</i> , 2001; Kageyama <i>et al.</i> , 2005
Hes3	Hairy and enhancer of split 3	bHLH	Effectors for the Notch pathway	Kageyama <i>et al.</i> , 2005
Hes5	Hairy and enhancer of split 5	bHLH	Ear histogenesis; effectors for the Notch pathway	Zine <i>et al.</i> , 2001; Kageyama <i>et al.</i> , 2005
Atoh1	Atonal homolog 1	bHLH	Hair cell differentiation	Birmingham <i>et al.</i> , 1999
Isl1	Isl1	LIM homeodomain (LIM-HD)	Development of both sensory and neuronal lineages of inner ear	Radde-Gallwitz <i>et al.</i> , 2004; Li <i>et al.</i> , 2004b

Data compiled from null mouse studies. The list does not pretend to be comprehensive. nd, not determined.

domains, epithelial cells, auditory sensory neurons (spiral ganglion neurons) and periotic mesenchyme (Lawoko-Kerali *et al.*, 2002) and by E18.5 it becomes restricted to the cochlea. Without GATA3 a small otic vesicle forms while the cochlear duct, the semicircular canals and auditory ganglion fail to develop (Karis *et al.*, 2001). Its expression is particularly high in spiral ganglion neurons during migration and differentiation (Lawoko-Kerali *et al.*, 2004) and it is specifically down-regulated in hair cells when they start to differentiate within the sensory fields (Rivolta and Holley, 1998). There is evidence that GATA3 regulates the expression of the transcription factor *NeuroD* (Lawoko-Kerali, 2004), as well as that of *Fgf10* in otic epithelium (Lillevali *et al.*, 2006).

The role of GATA2 in the inner ear is not yet clear and no abnormalities in early otic development have been observed in *Gata2* deficient embryos (Lillevali *et al.*, 2004). However, a recent microarray study of GATA2 functionally related genes based on the similarity of expression patterns in inner ear cell lines have identified interesting co-regulated genes such as the signal transducer and activator of transcription, *Stat3* (Holley *et al.*, 2007).

Another zinc finger transcription factor that is expressed in the inner ear is the growth factor independence 1 (*Gfi1*), which is essential for hair cell differentiation and survival (Wallis *et al.*, 2003; Hertzano *et al.*, 2004).

#### **POU domain transcription factors**

There are two members expressed in the inner ear: *Brn3a* and *Brn3c*. *Brn3a* is required for proper growth and migration in the inner ear and gustatory sensory neurons and it is critically involved in target innervation and axon guidance by spiral and vestibular ganglion neurons. *Brn3a* controls survival and differentiation of sensory neurons by regulating different downstream genes. Loss of *Brn3a* results in severe retardation in the development of axon projections to the cochlea and the posterior vertical canal as early as E13.5. In addition, efferent axons that use the afferent fibers as a scaffold during pathfinding also show severe misrouting (Huang *et al.*, 2001).

*Brn3c* is specifically expressed by hair cells within the adult mice inner ear; moreover, *Brn3c* null mutant mice contain immature hair cells but have a normal development of cochlear innervation (Xiang *et al.*, 1997 and 2003). Ectopic overexpression of *Brn3c* does not lead to the production of hair cells, indicating that it is only required for the later aspects of hair cells differentiation (Zheng and Gao, 2000).

#### **bHLH transcription factors**

During development of the otocyst the auditory and vestibular neuroblasts are amongst the first cell types to be specified (Hemond and Morest, 1991; Hossain and Morest, 2000; Fekete and Wu, 2002). They migrate from the prospective neural-sensory domain epithelium at E9.5–10.5, simultaneously down-regulating epithelial cytokeratin. Their differentiation depends on the two basic helix-loop-helix (bHLH) transcription factors, neurogenin 1 (*Ngn1*) and *NeuroD*. In null mutants for *Ngn1*, both auditory and vestibular neurons are absent, showing that this gene is essential for the differentiation of all inner ear sensory neurons (Ma *et al.*, 1998). Indeed, it was suggested that *NeuroD* and  $\beta$ III-tubulin are part of the same regulatory cascade downstream of *Ngn1* (Ma *et al.*, 1998). In null mutants for *NeuroD*, both auditory and vestibular

neuroblast migration is compromised, although there is greater depletion of auditory ganglion neurons (Liu *et al.*, 2000; Kim *et al.*, 2001). Other bHLH genes are necessary to realize the full proliferative capacity of progenitor neurons: *Hes1*, *Hes3*, *Hes5* (Kageyama *et al.*, 2005). *Hes5* and *Hes1* are relevant for ear histogenesis (Zine *et al.*, 2001) since they appear to interact with Notch signalling in supporting cells and with *Atoh1* in hair cells (Zheng *et al.*, 2000; Lanford *et al.*, 2000). *Atoh1* is a bHLH transcription factor and it is 'necessary and sufficient' for hair cell differentiation (Bermingham *et al.*, 1999).

#### **LIM-HD transcription factors**

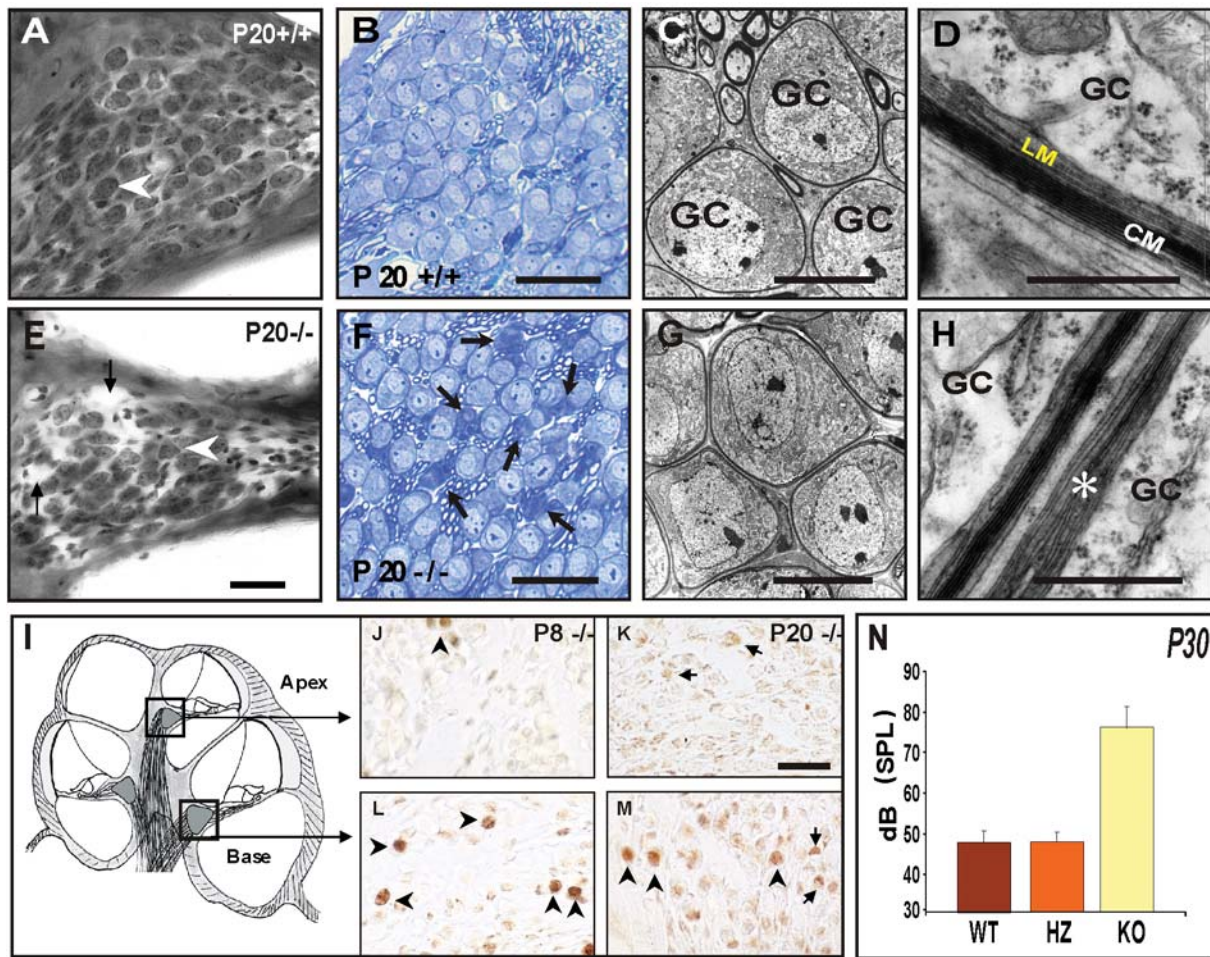
*Islet-1* (*Isl-1*) is a transcription factor belonging to the LIM homeodomain (LIM-HD) family. In the nervous system, the cooperation between the bHLH and LIM-HD transcription factors is responsible for generating cell diversity. As described earlier, *Islet-1* is expressed in the otic neurogenic zone (Li *et al.*, 2004b). Its expression is later maintained in otic neurons but is lost in the sensory lineages (Radde-Gallwitz *et al.*, 2004).

#### **Microarrays and gene networks**

One very unique aspect of the ear is the complex combination of forms and functions that enable the reception and the transduction of specific physical sensations. As an additional level of complexity, the precise geometry of the inner ear permits the interpretation of only certain mechanical stimuli. Very little is known about the molecular interactions and the genetic hierarchies that guide the morphogenesis of such a specialized epithelium. In the effort to unravel the regulatory gene networks that characterize the development and the maturation of the inner ear organs, new experimental techniques such as microarray analyses have been employed.

Microarrays provide a practical means to measure the expression of thousand of genes simultaneously (Schena *et al.*, 1995; Lockhart *et al.*, 1996). They have been successfully applied to the study of gene expression patterns in inner ear cell differentiation *in vitro* (Rivolta *et al.*, 2002) and more recently, in the studies of gene expression changes in the cochlea following exposure to noise and ageing (Kirkegaard *et al.*, 2006; Gong *et al.*, 2006). Very early attempts have also been made to analyse changes in gene expression during embryonic stem cell differentiation along the inner ear cells pathways (De Silva *et al.*, 2006). However, this technology is associated with many significant sources of experimental uncertainty that makes the data analysis very challenging. In studies of complex biological systems like the inner ear, the gene expression signal of fundamental processes may often be in the same order of magnitude as the background noise and therefore, very difficult to detect. For this reason the uncertainty generated by the experimental and biological noise must be carefully analyzed and quantified in order to obtain meaningful information from the data. An estimate of uncertainty is not fully achieved using repeat experiments because outliers are often due to flaws in the microarray technique, or to problems in the hybridization of the biological material. The most commonly used microarrays are high-density oligonucleotide arrays, such as Affymetrix GeneChip®. They are characterized by multiple probes associated with each target paired as perfect match (PM) probes and mismatch (MM) probes. The probe-set is used to measure levels of target gene expression and this measurement is then





**Fig. 3. Cochlear ganglion abnormalities in the *Igf1*<sup>-/-</sup> mutant mouse.** Normal *Igf-1*<sup>+/+</sup> ganglia are shown in (A-D) and mutant *Igf-1*<sup>-/-</sup> ganglia in (E-H). (A) Aspect of a normal P20 cochlear ganglion. (B) Toluidine blue staining of the cochlear ganglion. (C) Electron micrographs of the normal aspect of ganglion cells (GC), with their intact myelin sheaths. (D) Detail of the myelin sheath of a normal neuron with the external compact myelin (CM) and the internal loose myelin (LM). (E) Aspect of a mutant P20 cochlear ganglion. (F) Toluidine blue staining of the cochlear ganglion in mutant mice. Note the pyknotic cells (arrows) that are probably degenerating neurons. (G) Electron micrograph of an apparently normal group of ganglion cells. (H) Detail of the myelin sheath of a mutant mouse neuron. Note the thinner compact myelin and the non-compacted sheath (asterisk). (I) Schematic drawing of the cochlea in which the squares indicate the apical and basal turn cochlear ganglia. (J-M) TUNEL labelling from normal (*Igf-1*<sup>+/+</sup>) and mutant (*Igf-1*<sup>-/-</sup>) mice at postnatal days 8 and 20. The number of apoptotic neurons increases in the cochlear ganglion of *Igf-1*<sup>-/-</sup>. (N) Average ABR thresholds for click stimulus of *Igf-1*<sup>+/+</sup> (n = 21), *Igf-1*<sup>+/-</sup> (n = 29) and *Igf-1*<sup>-/-</sup> (n = 11) mice. Data are presented as mean ± SEM. The difference was statistically significant (\*\*P < 0.01) between the null group and the wild-type and heterozygous groups. Scale bars: (A,E) 30 μm; (B,F) 30 μm; (C,G) 10 μm; (D,H) 0.5 μm. Modified from: Camarero et al., 2001 and 2002; Cediel et al., 2006.

utilized to detect genes differentially expressed between different conditions, to visualize clustering or to define gene networks. To estimate gene expression levels a summary of these probe-level values is required. The level of uncertainty associated to low gene expression signals is very high and a summary that is less sensitive to the noise is needed. The most popular methods to analyse probe-levels at present only provide a single point estimate of the gene expression levels but most of them are unable to provide credibility intervals for each measurement. (Affymetrix, Microarray Suite User Guide version 5.0., 2001, Affymetrix Inc.) (Irizarry *et al.*, 2003). Probabilistic models have been introduced more recently that describe the probe data set in terms of probabilistic functions. The Bayesian Gene Expression (BGX, <http://www.bgx.org.uk>; Hein *et al.*, 2005) has developed a Hier-

archical Bayesian model for probe-level analysis but it is computationally very expensive and therefore not feasible to run on very large datasets. An affordable alternative are the models developed by PUMA group (Propagating Uncertainty in Microarray Analysis, <http://www.bioinf.man.ac.uk/resources/puma/>) and the gMOS models (gamma model of oligonucleotide signal) show improvements both in accuracy and computational efficiency over the BGX model. They estimate gene expression levels with credibility intervals that quantify the measurement variance associated with the estimates of target concentration within a sample. This within sample variance is a significant source of uncertainty in oligonucleotide arrays, especially for weakly expressed genes. These models also run efficiently and perform accurately on different types of data including inner ear data (Milo *et al.*, 2003;

Liu *et al.*, 2005; Holley *et al.*, 2007). A still ongoing microarray study on the *Igf1<sup>-/-</sup>* null mutant carried out by Sanchez-Calderon *et al.*, has shown that using a sensitive probe-level analysis, such as gMOS and related models, very low expression of signals can be detected in highly variable biological samples. Such types of gene expression analysis were not successful when performed with models that do not quantify variance at the probe-level. The high number of false positives made the detection of true positives almost impossible. However, the results obtained from gene expression analysis are now being validated *in vivo*.

Although microarray technology is becoming more affordable, there is still an increasing demand for robust bioinformatics tools to analyse large scale gene expression data. The high cost of arrays and the limitations in performing biological experiments, due to the lack of biological material and/or the complexity of the system makes it even more important to have robust predictions *in silico* before undertaking *in vitro* or *in vivo* experiments. Developing mathematical models that integrate the experimental data with a statistical inference is advantageous to identify and describe the dynamic changes in a network of genes of interest and ultimately to infer changes in key signalling pathways. In an organ like the mammalian inner ear where there are several different biological processes regulated in a very precise manner, it is more informative to look at these processes as dynamic networks of genes of which key components are transcription factors and their gene expression patterns.

Neurosensory hearing loss is the most common disabling disease worldwide, a large proportion of which involves the loss of hair cells and their associated neurons. Understanding and defining the key gene networks in the development and function of the mammalian inner ear would represent an important step towards defeating deafness. Such advances will potentially lead to new signalling pathways to assist hypothesis generation in regenerative studies and discovery of drug targets.

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