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Patterning and cell fate in ear development

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ABSTRACT The inner ear is a complex structure responsible for the senses of audition and balance in vertebrates. The ear is organised into different sense organs that are specialised to detect specific stimuli such as sound and linear or angular accelerations. The elementary sensory unit of the ear consists of hair cells, supporting cells, neurons and Schwann cells. Hair cells are the mechano-electrical transducing elements, and otic neurons convey information coded in electrical impulses to the brain. With the exception of the Schwann cells, all cellular elements of the inner ear derive from the otic placode. This is an ectodermal thickening that is specified in the head ectoderm adjacent to the caudal hindbrain. The complex organisation of the ear requires precise coupling of regional specification and cell fate decisions during development, i.e. specificity in defining particular spatial domains containing particular cell types. Those decisions are taken early in development and are the subject of this article. We review here recent work on: i) early patterning of the otic placode, ii) the role of neural tube signals in the patterning of the otic vesicle, and iii) the genes underlying cell fate determination of neurons and sensory hair cells.

KEY WORDS: *placode, otic vesicle, proneural, hindbrain, patterning*

State of the art

The inner ear is one major sensory organ of the head and it is responsible for the perception of sound and balance in vertebrates. In the adult, it is arranged in a highly complex three-dimensional structure, named the membranous labyrinth, composed of a closed epithelial layer that is diversified into specific regions that contain the sensory elements (Fig. 1A). The sensory epithelium consists of hair cells, and supporting cells disposed in a cellular mosaic (Fig. 1B) (Adam *et al*., 1998; Fritzsch *et al*., 2000). Mechanosensory information is transduced by the hair cells that release transmitters which activate afferent bipolar sensory neurons which, in turn, transmit the information to second order neurons in the brainstem. The membranous labyrinth is subdivided into vestibular and auditory regions. The vestibule forms the dorsal part of the labyrinth and is responsible for the senses of motion and position. It comprises the three cristae, the sensory organs located at the basis of three orthogonally arranged semi-circular canals, and the utricle and saccule, which contain two additional sensory organs, the maculae. The ventral auditory part is more diverse. In mammals it is composed of the cochlea, a coiled structure whose sensory epithelium is called the organ of Corti. In birds, the auditory region is composed of the basilar papilla, while in fish the saccule and lagena are both involved in hearing (Fig. 1A) (Popper and Fay, 1993; Riley and Phillips, 2003). In jawed vertebrates, the adult inner ear is highly regionalised along its three axes. In addition to the dorso-ventral (DV) subdivision into vestibular and auditory regions, an asymmetry along the medio-lateral (ML) axis is also obvious with, for instance, the endolymphatic sac and duct located in the medial part, close to the brain. The whole structure also shows pronounced antero-posterior (AP) asymmetry.

There has been a sustained interest in the development of the ear throughout the past century. Histological observation and the experimental manipulation on embryos contributed to an increase in our knowledge of several processes of ear development that were somehow defined by the end of the eighties (Cremers *et al*., 1988; Ciba Foundation Symposium, 1991). This view, however detailed, remained descriptive and phenomenological until the dissemination of molecular biology during the last two decades. It is common place to relate this enormous progress to the explosion of DNA recombination techniques, the access to genetic manipulation of organisms, the import of ideas from fly development into vertebrate studies and, more recently, the knowledge of whole genomes.

Albeit the structural complexity of the adult inner ear, it derives from a simple embryonic anlagen, the otic placode (for review see Torres and Giraldez, 1998). All cellular components of the inner ear, including the primary afferent neurons, derive from the otic placode, but with the only exception of the melanocyte cells of the

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secretory epithelium and Schwann cells of the ganglia, which are of neural crest origin (D'Amico-Martel and Noden, 1983, Rubel and Fritzsch, 2002). Cranial placodes are specialized areas of ectoderm outside the neural plate that contribute to all the cranial paired sensory organs and most of the sensory neurons from the peripheral nervous system of the head. The current view is that placodes share a common developmental origin, the preplacodal region (PPR), a horseshoe band encircling the neural plate from which individual placodes emerge (Jacobson, 1966; Torres and Giraldez, 1998; Streit, 2007). Olfactory, profundal and trigeminal, otic, lateral line, and epibranchial are all *neurogenic* placodes that give rise to sensory neurons among other specialized cell-types (Adam *et al*., 1998; Ma *et al*., 2000; Schlosser and Northcutt, 2000; Andermann *et al*., 2002; Begbie *et al*., 2002).

The complex organisation of the ear requires precise coupling between regional specification and cell fate decisions, that is, specificity in defining particular spatial domains with particular cell types. We shall review some of the recent work done on that issue, and discuss first, the early steps of regional specification of the otic placode, then patterning of the otic vesicle, and finally, the genetic networks underlying cell fate decisions and sensory organ development.

The regional specification of the otic vesicle: the patterning of the ear

The regional (or axial) polarity needed to develop the membranous labyrinth of the ear has long been recognised as providing the basis for ear function. Classical transplantation experiments showed that rotated otic placodes produce enantiomorphic twins (Harrison, 1945; Yntema, 1955), which somewhat resemble the symmetric ear of the hagfish. The early patterning of the ear is also set, at least in part, by interactions with surrounding tissues, particularly the neural tube. The first sign of otic regionalisation is that of the establishment of the otic neural and non-neural fields (Fig. 2). In the following, we review the molecular properties of these two domains and discuss the possible models by which this early specification is set in place. However, axial polarity extends to further complexity in the regionalisation of the otic vesicle, when it becomes necessary to establish the different domains of the inner ear. Inductive processes extend further into the development of the otic vesicle, and the neural tube seems to play an important role in establishing the final axial pattern of the ear (for late reviews see Choo, 2007; Schneider-Maunoury and Pujades, 2007; Whitfield and Hammond, 2007). We shall examine first, the establishment of the neural domain of the otic placode, including the role of Notch signalling in this process and, secondly, the function of the neural tube as a source of signals for the regionalisation of the otic vesicle.

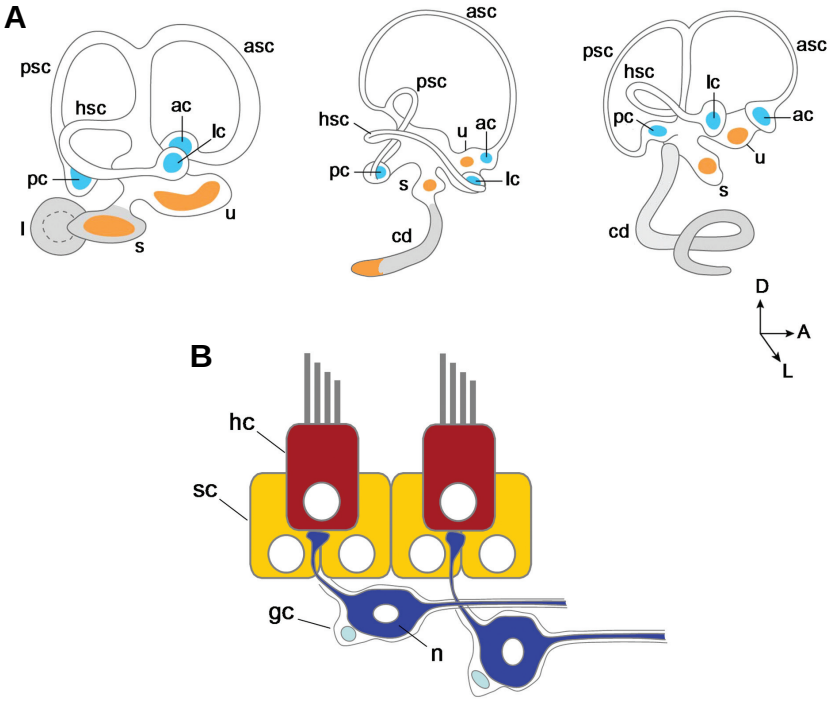

Fig. 1. The structure of the inner ear. (A) *The membranous labyrinth in three vertebrate species. From left to right: zebrafish,chicken and mouse. The vestibular (dorsal) part of the membranous labyrinth contains five sensory organs: the three cristae (blue) located at the basis of the three semicircular canals, and the utricular and saccular maculae (orange), surrounded by otoliths. The ventral, auditory part of the inner ear (grey) is highly variable in morphology and complexity in different vertebrates. In the mouse, the cochlear duct, acoiled structure, contains a finely patterned sensory organ, the organ of Corti. In chicken, the auditory organ, the basilar papilla, is also contained in the cochlear duct. In zebrafish, there is no ventral cochlear duct and the auditory function is carried by the saccular and lagenar maculae. ac: anterior crista; asc: anterior semicircular canal; cd:cochlear duct; hsc: horizontal semicircular canal; l: lagena; lc: lateral crista; pc:posterior crista; psc: posterior semicircular canal; s: saccule; u: utricle. Anterior is to theright ans dorsal to the top.* **(B)** *The functional unit of the ear. The four basic cellular elements of the functional unit are depicted: hair cells (red), supporting cells (orange), neurons (blue) and Schwann cells (white).*

The establishment of the otic neural competent domain

Current understanding of specification of the otic placode involves a two-stage mechanism by which first, an extended multipotent pre-placodal domain is specified at the head ectoderm, and then individual placode identities are specified (Jacobson, 1966; Streit, 2007). An initial set of genes (*Foxi*, *Msx* and *Dlx)* identify an ectodermal domain between the neural plate and the epidermis from which the preplacodal domain is segregated from the neural crest. Both the positioning of the preplacodal ectoderm and its capacity to express the specific *Six/Eya/Dach* cassette seem to require interactions between the presumptive ectoderm and the surrounding tissues (Streit 2007). How does the preplacodal ectoderm transit from a pluripotent ground state to one in which otic fate is specified? This apparently requires another round of interactions that position and specify the fate of individual placodes (Ohyama *et al*., 2007; Streit, 2007; Jayasena *et al.*, 2008). This notion of sequential rounds of interactions was anticipated by the classical studies of Yntema, 1955 and Jacobson, 1966.

Recent work has shown that the ear primordium is already patterned at the time of the development of the otic placode (Alsina *et al*., 2004; Vázquez-Echeverría

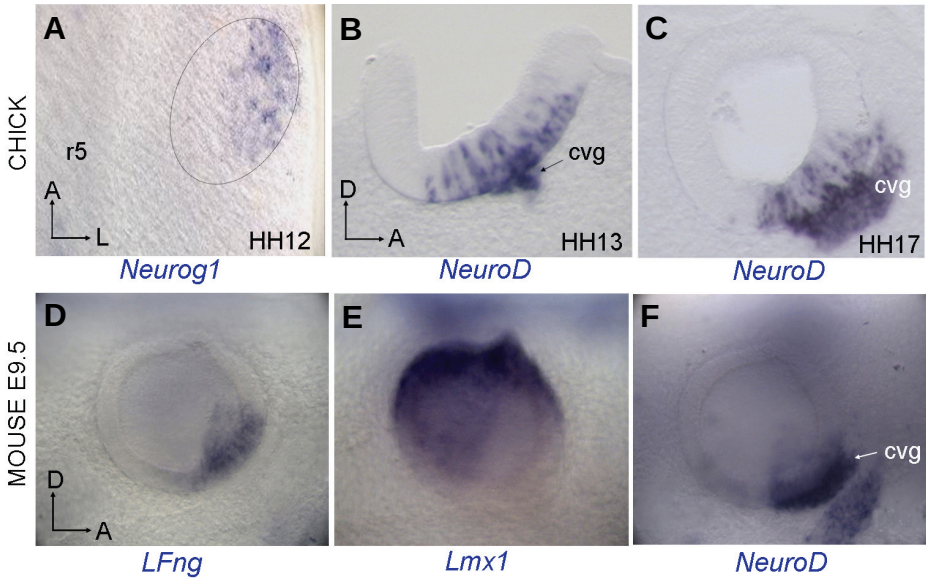

Fig. 2. Early genes of the inner ear. (A-C) Neurog1 *and* NeuroD *in the chick otic placode and otic vesicle.* **(A)** Neurog1 *expression in the early otic placode.* **(B,C)** *Para-sagittal sections showing* NeuroD *expression in epithelial and delaminating ganglionar neuroblasts of the cvg.* **(D-G)** *Spatially restricted genes in the mouse otic vesicle. Lateral views of whole mount* in situ *hybridisation with the indicated probes.* **(D)** LFng *expression in the otic vesicle is restricted to the anteroventral region prefiguring the neurogenic domain.* **(F)** Lmx1 *extends throughout the non-neural domain, which is complementary to* LFng *expression.* **(G)** NeuroD *expression in the neurogenic domain of the otic vesicle. cvg: cochleo-vestibular ganglion, r5: rhombomere 5. Hamburger & Hamilton stages are indicated in (A-C).*

et al., 2008; Bell *et al.,* 2008). This early regionalisation is related to the establishment of two complementary neural and non-neural territories in the otic placode and otic vesicle (Fig. 2). In the chick embryo, otic cell types emerge sequentially during development. First the neuroblasts are specified in the anterior otic cup, as revealed by *Neurog1*-positive cells (Fig. 2A). Mechanosensory hair cells do so later in development within the domains of the different sense organs (Fig. 1A). The activation of proneural genes is one of the firsts signs of neural determination, and in the case of the development of sensory neurons, *Neurog1* has been shown to be sufficient for the acquisition of neuronal fate. Over-expression of *Neurog1* drives formation of ectopic neurons (Perron *et al*., 1999; Kim *et al*., 2001), while targeted inactivation of *Neurog1* results in severe loss of proximal cranial sensory ganglia and hair cells (Ma *et al*., 2000). This suggests that *Neurog1* is related to the development of a common pro-neural and -sensory field that first gives rise to neurons and later on to hair cells, or alternatively to a common neural-competent field (Raft *et al*., 2007). In chick, a territory expressing *Neurog1*, *Delta1* and *LFng* emerges as a triangle in the anterior half of the flat otic placode. Initially the neural domain is the anterior-medial aspect of the otic placode to end up, after invagination, in an anterior-medial and ventral position of the otic vesicle (Alsina *et al*., 2004). In mammals, expression of *Neurog1* appears in a more lateral position, detected as an antero-ventro-lateral quadrant at otic cup stage, to extend also medially as development proceeds (Fig. 2B and Ma *et al*., 1998; Vázquez-Echeverría *et al.,* 2008). A recent analysis by Cre-loxP fate mapping in mouse shows that part of the vestibular sensory hair cells derive from a neurogenic region, and the study of mouse mutants provides evidences that a mutual antagonism between *Neurog1* and *Math1* regulates the transition to sensory cell production (Raft *et al*., 2007).

Experiments of otic cup rotation have suggested that AP patterning is not fixed until late otic cup stage (Bok *et al*., 2005), suggesting that the first signs of otic regionalisation detected by gene expression patterns require posterior signals to stabilize the neural/non-neural patterning.

But is the expression of *Neurog1* the first sign of neural commitment within the otic placode? Recent studies of indicate that *Sox3* is required early in otic neural development (Khatri and Alsina, unpublished results). *Sox3* is expressed before the otic placode is morphologically visible, within a broad band that contains the otic and epibranchial territories, to get restricted later on in development to the proneural region of the otic placode (Fig. 3). This suggests that neural fate specification takes place prior the formation of the otic placode, within a broad territory that contains the antero-medial otic region and the lateral epibranchial territory. Previous work has indicated that FGF signalling is

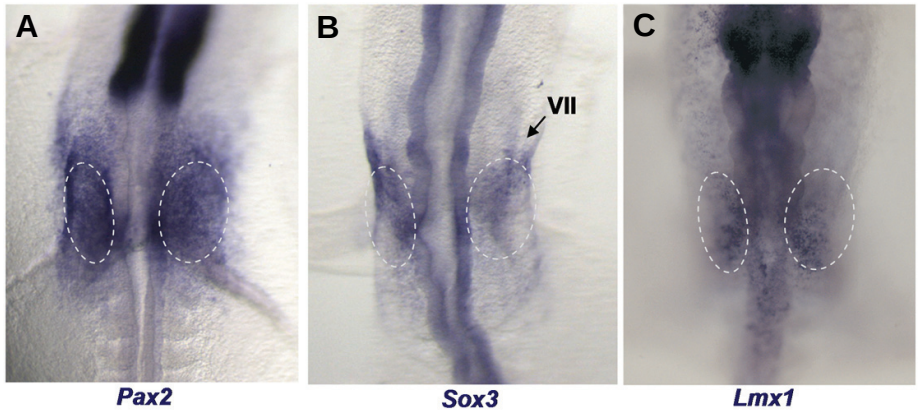

Fig. 3. Early patterning of the otic placode. *Expression of early otic genes in the chick HH10 otic placode.* **(A)** Pax2 *is expressed in a broad domain that includes otic and epibranchial placodes at the ectoderm adjacent to r3-r6.* **(B)** Sox3 *concentrates in a oblique band in the ectoderm at the level of adjacents r4-r5, which includes the otic and geniculate placodes.* **(C)** Lmx1 *starts to be expressed complementary to* Sox3*. Whole mount* in situ *hybridisation with the indicated genes. The dotted line indicates the location of the otic placode, and VII that of theVII/geniculate placode. Anterior is to the top.*

required for epibranchial induction in chick and zebrafish (Abu-Elmagd *et al*., 2001), and recent data reinforced the idea that otic and epibranchial placodes do not emerge as separate identities by different inducing signals but, instead, share the same developmental origin (Millimaki *et al*., 2007; Nikaido *et al*., 2007; Sun *et al*., 2007).

Two main questions arise here: first, does the process of placodal induction, by default, involve proneural induction or, alternatively, successive induction events lead first to placodal and subsequently to neural fate? And secondly, is the neural competence only acquired by a fraction of otic fated cells, or does it involve the repression of neural potential in the non-neural competent region? Graft experiments at different axial levels performed by Li *et al*., 1978 revealed that presumptive otic placode ectoderm could ectopically generate otic vesicles without the ability to form neurons. Groves and Bronner-Fraser (2000) observed that quail anterior epiblast grafted in the presumptive otic region of host of 3-10 somites could start to express *Pax2* and *Sox3*, while grafts performed at 11-21 somites, only expressed *Sox3* but not *Pax2*, suggesting that *Pax2*-inducing or maintaining signals are lost before *Sox3*-inducing signals. Thus, depending on the length of exposure of signals the appearance of some molecular markers, as *Pax2* and *Sox3* can be dissociated. In parallel, inhibition of FGF signalling in zebrafish indicates that induction of *Sox3* and *Pax2* requires FGF signalling in the otic-epibranchial region but not in an interdependent manner (Groves and Bronner-Fraser, 2000; Nikaido *et al*., 2007; Sun *et al*., 2007). Results of our laboratory indicate that suppression of FGF signalling at specific stages let the embryo allow the development of otic cups, but devoid of *Sox3* expression and neurons (Abello and Alsina, unpublished results). Altogether, this suggests a multistep model for otic development in which the induction of otic fate and the formation of the otic placode is followed by neural induction. During early development of the embryo, the Fibroblast Growth Factor (FGF), Wingless/wnt (Wnt) and Bone Morphogenetic Protein (BMP) signalling pathways repeatedly converge to induce neural fate in the neural plate and neural crest. The current view on this process is that FGF signals and/or Wnt signals are required to inhibit BMP signalling, the earliest pathway described to potentiate epidermal fate over neural fate (Stern, 2005). So far, FGF signalling has been shown to be required for otic *Neurog1* expression and sensory development (Pirvola *et al*., 2000; Leger and Brand, 2002; Alsina *et al*., 2004).

In summary, the data suggest that the early steps of regionalisation take place by the specification of the proneural field by *Sox* genes. The restricted neural function would result from the enhancement of proneural activity by FGFs.

Notch signalling during otic development

Notch signalling is involved in several developmental processes, such as cell fate specification, cell proliferation, patterning and boundary formation (reviewed in Bray, 2006; Louvi and Artavanis-Tsakonas, 2006). The transmembrane Notch receptor is activated upon binding to membrane-bound Delta (*Dl*) or Serrate (*Ser*) ligands present in adjacent cells. The most studied role of Notch pathway is its ability to influence the fate of neighbouring cells, by one cell adopting one state and the adjacent cell adopting the alternative state. This mechanism is called lateral inhibition and plays a role during neurogenesis, in which the *Delta*-positive cell adopts neuronal fate and the Notch-activated cell represses it (reviewed in Lewis, 1998). However, Notch pathway also has the ability to segregate specific cell lineages or territories from fields of developmentally equivalent cells.

Members of the Notch pathway are differentially expressed in the otic placode/cup. In chick, *Notch1* is expressed in the entire otic epithelium from 11 somites to late otocyst stage (Groves and Bronner-Fraser, 2000). *LFng* is expressed throughout the proneural domain, and *Dl1* is detected in a salt and pepper pattern (Adam *et al*., 1998; Cole *et al*., 2000; Alsina *et al*., 2004). *Hes5* is expressed in cells adjacent to *Dl1*-positive cells as a consequence of the N-Dl lateral inhibition process (Abello *et al*., 2007). As expected from the lateral inhibition process, disruption of Notch signalling leads to the production of excess neuronal precursors in the inner ear concomitantly to the suppression of *Hes5* activation (Haddon *et al*., 1998; Abello *et al*., 2007). Altogether, Notch signalling is required in otic neurogenesis to regulate the number of neural committed cells that enter into neuronal differentiation. Interestingly, *Ser1* (or *Jag1*) and *Hes1* are also expressed broadly in a domain complementary to the proneural domain at early otic cup stage, well before the appearance of prosensory patches. In summary, the non-neural region is initially characterized by the expression of *Jag1* and *Hes1* and devoid of *LFng* and *Dl1*. Studies on Notch function revealed that this complementary gene expression is required for early otic patterning. Blockade of Notch at 6-9 somites suppressed the restriction of *Lmx1* to the posterior domain of the otic placode, without affecting the extension of the neural domain (Abello *et al*., 2007; Daudet *et al*., 2007). The effects of Notch blockade were not due to intermingling between neural and non-neural cells, but to upregulation of posterior genes in the neural domain (Abello *et al*., 2007).

Hindbrain segmentation and otic patterning

Tissues surrounding the inner ear, such as the hindbrain, mesoderm and endoderm are potential sources of signals required for inner ear development (Giraldez, 1998; Fekete, 1999). Here we shall focus on the role of the hindbrain; however, although not discussed here, the contribution of the periotic mesenchyme and the cross-talk between the epithelium and the mesenchyme are also important (Montcouquiol and Kelley, 2003; Pirvola *et al*., 2004). The hindbrain and the otic placode keep an invariant spatial relation in all animal species (Fig. 4A), and the importance of the hindbrain for ear development has been demonstrated by the analysis of several mutants for regulatory genes that are expressed in the hindbrain, but not in the otic primordium (Fig. 4B). Those genes, such as *MafB*, *vHnf1* and *Hoxa1*, are involved in caudal hindbrain segmentation. Their ear phenotypes are attributed to defects in rhombomeres (r) 4 to 6, the region of the hindbrain juxtaposing the developing otocyst (Fig. 4A). While there are discrepancies among the results obtained in different species, available data point to an essential role of hindbrain signals, and particularly FGFs, in otic regionalisation.

The *kreisler* mutant mice are deaf, display a circling behaviour, and show many defects in otic development (Deol, 1964). This mutation is the consequence of suppression of *MafB* expression in r5 and r6, and since *MafB* is not expressed in the otocyst, it has been proposed that the deficit in FGF signalling is one major cause of the observed otic defects (McKay *et al*., 1996). In these

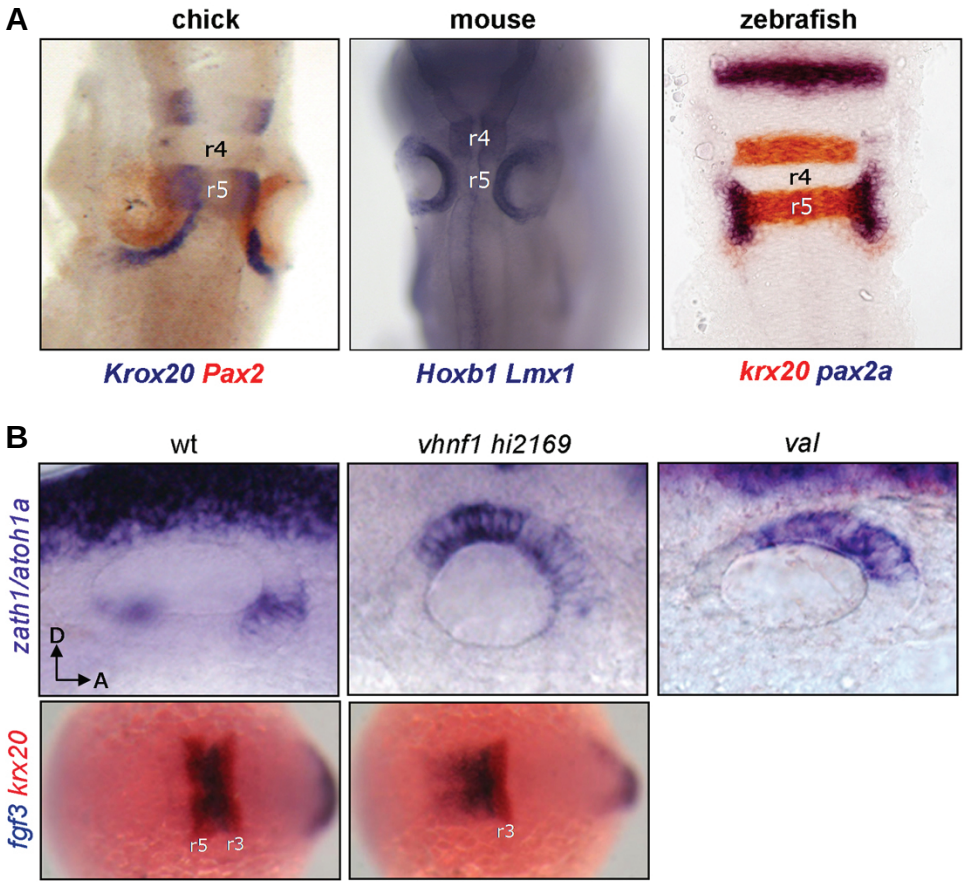

Fig. 4. The otic primordium develops in the ectoderm adjacent to the caudalhindbrain. (A) *Double* in situ *hybridisation in chick, mouse and zebrafish embryos for otic* (Pax2 orLmx1) *and hindbrain genes* (Krox20 or Hoxb1). *Note the intimate relationship betweenthe otic primordium and the r4-r6 region.* **(B)** *Zebrafish mutant embryos for genes that are expressed in the hindbrain and not in the otic primordium such as* vhnf1 *and* MafB/val, *display defects in otic patterning as shown by expansion of* zath1/atoh1a*marker.* vhnf1 *hypomorphic mutation leads to the caudal expansion of* fgf3 *expressionin the neural tube. In (A), anterior is to the top. In (B), anterior is to the right.*

mice, dorsomedial markers such as *Gbx2* and *Wnt2b* are lost, while the ventral *Otx2* domain is expanded, suggesting a role of the hindbrain in the specification of dorsomedial structures of the inner ear (Choo *et al*., 2006). The similarity of this phenotype with the observed in *Gbx2* null mutants (Lin *et al*., 2005) suggested that *Gbx2* was a target of hindbrain signalling in the otocyst. On the other hand, *kreisler* mutants display an expansion of the otic neurogenic region as revealed by the complementary changes in the early expression of patterning genes *LFng* and *Lmx1* (Vázquez-Echeverría *et al.,* 2008). Thus, in addition to the DV patterning defects, *kreisler* mutants display an early AP patterning defect, affecting mainly the neurogenic/non-neurogenic fate decision. As for *Hoxa1* mutants in which ear patterning defects are correlated to the loss of *Fgf3* expression in the hindbrain (Pasqualetti *et al*., 2001), *kreisler* mutants fail to upregulate *Fgf3* and *Fgf10* in r5 and r6 (Vázquez-Echeverría *et al.,* 2008).

The *MafB/val* mutation in zebrafish results mainly in AP patterning defects (Kwak *et al*., 2002). Anterior markers such as *hmx3* are expanded posteriorly, while the expression of caudal markers is reduced or absent. *val* mutants also present an excess of hair cells, ectopically produced between the anterior and posterior maculae (Kwak *et al*., 2002). The *val* mutation results in a posterior expansion of *fgf3* expression in the hindbrain. Reduction of *fgf3* RNA levels in *val* mutants using morpholinos rescues some of the otic defects, strongly suggesting that, in zebrafish as well as in mouse, FGF3 is a major signal involved in ear patterning, downstream of *MafB* (Kwak *et al*., 2002).

The analysis of the zebrafish *vhnf1* mutant has added more complexity to the picture. *vhnf1* positively controls *val* expression in the hindbrain (Wiellette and Sive, 2003; Hernandez *et al*., 2004). As expected, AP patterning phenotypes are observed in the inner ear of *vhnf1* mutants, which display an expansion or a duplication of the expression of anterior otic genes such as *hmx3, fgf8* and *pax5*. However, *vhnf1* mutants also show DV patterning defects, and a dorsal shift of intermediate markers such as *atoh1a,* which marks the future maculae (Lecaudey *et al*., 2007). *val* and *vhnf1* mutants display hair cells at ectopic positions all along the AP axis of the otic vesicle, suggesting that either an intact r5 identity or r5-signals are essential to restrict early hair cell specification to the otic region lateral to r4 and r6. There is a striking difference between mouse and zebrafish. Although for years it was thought that the main defects seen in mice are along the DV axis, while in zebrafish, defects have been found primarily along the AP axis, recent data have shown that this difference may be only apparent, since a closer examination of the mutants has allowed the detection of DV patterning defects in both *vhnf1* and *val* zebrafish mutants (Fig. 4B, Lecaudey *et al*., 2007; Schneider-Maunoury and Pujades, unpublished results), and AP defects in the otic vesicle of mice *kreisler* mutants (Vazquez-Echeverria *et al.,* 2008). It has to be kept in mind that the process of patterning is also concomitant to growth and large morphogenetic movements all of which will surely require of more elaborated models to be understood.

The hindbrain as a source of instructing molecules for otic regionalisation: a complex signalling network

Three main signalling pathways, the Hedgehog (Hh), Wnt and FGF pathways have been involved in otic patterning from the adjacent hindbrain. The function of FGFs in otic development has been extensively studied. Several *Fgfs* are expressed in the hindbrain with species-specific patterns, and loss of function of *Fgf* genes leads to smaller and malformed otic vesicles, demon-

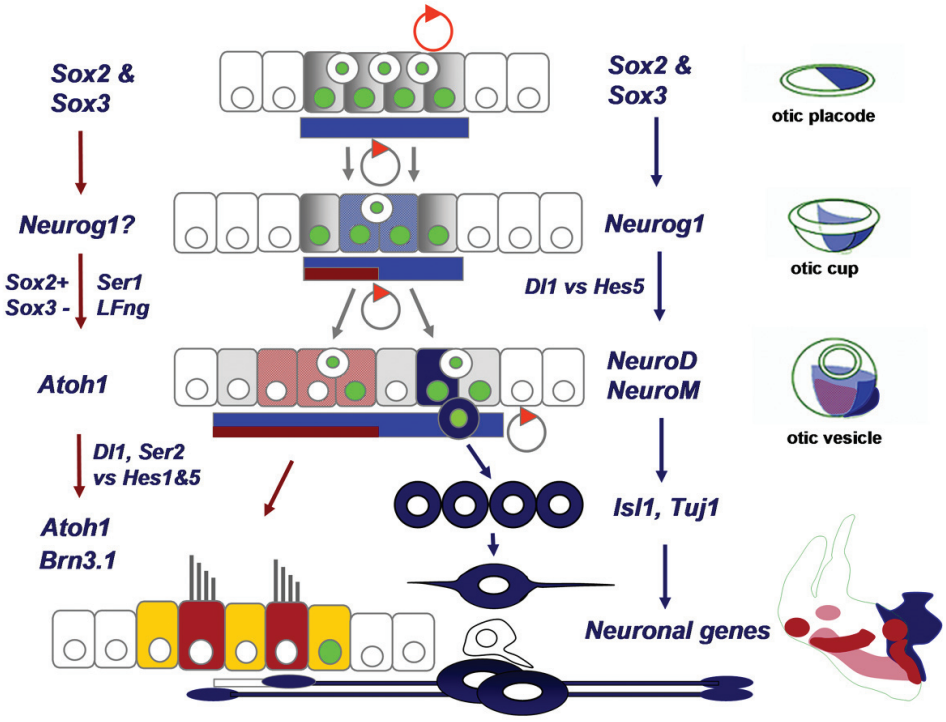

Fig. 5. Cell fate specification in the inner ear. *The diagram shows a model of hair cell and neuron specification during ear development in amniotes. The sequence of gene expression for sensory (left) and neuron development (right), is indicated. The neural competent domain is common for the two lineages and expresses genes of the* SoxB1 *group, which maintain the cell renewal state and commit progenitors to neural fate. Those are probably multipotent progenitors that generate all cell lineages (Satoh and Fekete 2005). This domain is specified either by temporal and/or spatial cues to give rise to the two main lineages: sensory and neuronal. The bars under the epithelia indicate neural competence (blue) and prosensory specification (brown).* Ser1 *is necessary for sensory specification as is* Sox2*, and probably the down-regulation of* Sox3. Neurog1 *expression is required for both lineages in* macula*, but not for crista or auditory epithelium. Neuronal specification then takes place by the enhanced expression of* Neurog1 *via the Delta-Notch pathway,and the subsequent expression of* NeuroD *and* NeuroM *proneural genes. The latter allow delamination and transient amplification of neuronal precursors within the ganglion. Hair cells are singled out within* Atoh1 *clusters, under the sustained expression of* Dl1/Ser2*, in a positive feed-back loop.*

strating a role for this signalling pathway in otic induction (for review see Schimmang, 2007). In zebrafish, this function is attributed mainly to FGF signals coming from the hindbrain, while in amniotes, other surrounding tissues such as the mesenchyme and endoderm are also sources of FGFs (Ladher *et al*., 2005; Freter *et al.,* 2008). FGF target genes are expressed in the otic epithelium, suggesting a direct effect of this signalling pathway (Chambers *et al*., 2000; Raible and Brand, 2001; Aragon and Pujades, unpublished results). The redundancy between different FGFs and their role in otic induction have hampered the analysis of their role in otocyst regionalisation (for review see Schimmang, 2007).

Shh signalling from the notochord and floor plate is essential for ear patterning in mice. The study of *Shh* mutants shows that this pathway is required for the formation of the cochlea, since ventral *Otx1/2* expression is reduced and dorsal *Dlx5* expression is expanded. While sensory specification is not affected, proneural gene expression is strongly reduced, and the SAG is absent. The reverse phenotype is seen after missexpression of *Shh* in the otocyst using transgenic mice: dorsal, vestibular structures are lost and ventral, auditory cell fates are expanded. Interestingly, neurogenesis appears increased and the SAG is larger (Driver *et al.,* 2008). These results led the authors to propose that Shh instructs ventral fates, but differently along the AP axis: anteriorly Shh activates *Ngn1* and *NeuroD* promoting neurogenesis, whereas posteriorly it activates *Pax2* and *Otx1/2* and promotes cochlear fate (Riccomagno *et al*., 2002). The Hh signalling targets, *Gli1* and *Ptc1,* are expressed broadly in the otic epithelium suggesting that Hh signalling may act directly. Different levels of Shh activity mediate the formation of inner ear structures, with Gli3 repressor required dorsally for vestibular formation and Gli activators functioning ventrally to form the cochlear duct (Bok *et al*., 2007).

Surprisingly, manipulating the Hh signalling pathway in zebrafish results, not in DV or ML, but in AP patterning defects (Hammond *et al*., 2003). Two strong Hh pathway mutants exhibit striking partial mirror image duplication of anterior sensory structures such as the utricular macula, concomitant with a loss of posterior otic domains. Hh signalling from both floor plate and notochord needs to be abolished to obtain this phenotype. The reverse phenotype, namely expansion of posterior structures at the expense of anterior ones, is obtained when the Hh pathway is constitutively activated by overexpression of Shh or by injection of a dominant negative form of PKA. Based on the expression patterns of Hh target genes in these experimental contexts, a direct effect of Hh signalling on posterior otic cells is proposed (Hammond *et al*., 2003). Given that the relevant receptors are expressed uniformly along the AP axis, how can we explain the different responses to Hh signalling of ventral otic cells along this axis? One possibility is that FGFs locally restrict the response to Hh signalling of ventral progenitors. Recent results in zebrafish *vhnf1* hypomorphs show that although these embryos present an expansion of *Fgf3* in the caudal hindbrain, they do not display any defects in the Hh pathway elements in the otic vesicle (Sapède and Pujades, unpublished data). Moreover, since *Fgf3* expression in the neural tube of the *Shh-/-* mice is not affected (Riccomagno *et al*., 2002), it will be interesting to explore the crosstalk between Shh and other pathways such retinoic acid.

The role of canonical Wnt signalling from the dorsal neural tube has been studied in mouse (Riccomagno *et al*., 2005). Surprisingly, while Wnt-responsive cells are distributed along the dorsomedial otic cup and later confined to the dorsal aspect of the

otic vesicle, both vestibular and cochlear structures are reduced in double *Wnt1/Wnt3a* mutants (Riccomagno *et al*., 2005). To explain these conflicting observations, lineage studies using an inducible genetic marker of Wnt-responsive cells were performed. These studies show that progenitors of the cochlea received Wnt signalling, suggesting that ventral cells of the otic placode originate from the dorsomedial part of the otic cup. This study underlines the contribution of cell migration and morphogenetic movements to otic patterning processes: otic cell groups originally located close to the dorsal neural tube end up at the ventral aspect of the otocyst after otic invagination and morphogenesis. Gain-of-function studies confirmed the role of canonical Wnt pathway in vestibular formation and showed a mutual repression between Wnt and Shh pathways in ear DV patterning. However, Wnt signals cannot be the only cues involved in auditory fate specification since ventral otic determinants are appropriately expressed in double mutants for *Wnt1* and *Wnt3a* (Riccomagno *et al*., 2005). Other dorsal secreted cues, such as BMPs, could play a role in this process.

Cell fate specification of the neural elements of the ear: the components of the mechanotransducing unit

The elementary sensory unit of the ear – the sensory patch- consists of: i) hair cells, which are the sensory receptor cells that contain the mechano-electrical transducing machinery, ii) supporting cells that hold and space the hair cells in a precise pattern, and collaborate to their maintenance, iii) otic neurons that innervate the sensory patches and connect the hair cells with the brain, and iv) glial-Schwann cells that enwrap neurons and their axons (see Fig. 1B). As mentioned above, with the exception of most Schwann cells that are of neural crest origin, all cell types derive form the otic placode (D'Amico-Martel and Noden, 1983).

The expression of proneural genes confers to cells the potential to become neural precursors, the ability to differentiate into neural elements, and in some instances they specify particular cellular identities (Bertrand *et al*., 2002). Proneural genes were related to the proneural achaete-scute complex (ASC) in flies (Garcia-Bellido, 1979), and the analysis of the complex lead to the identification of four genes (Ghysen and Dambly-Chaudiere, 2000). The vertebrate counterparts were unveiled by screening for homologous sequences in mouse (Bertrand *et al*., 2002). A further *Drosophila* proneural gene, *atonal,* was isolated later in a PCR-based screen to identify genes containing bHLH sequences. The orthologs of this gene subfamily have been shown by loss-of-function analysis to be critical for ear development (Jarman *et al*., 1993; Ma *et al*., 1998; Bermingham *et al*., 1999; Kim *et al*., 2001). It is now clear that *Neurog1*, *NeuroD1* and *Atoh1* are at the core of the proneural function in the ear. They are necessary and sufficient to promote neuronal and hair cell fates, respectively (reviewed by Kelley, 2007). The diagram in Fig. 5 summarises the sequence of cellular states that generate neurons and hair cells from neural competent epithelium. *NeuroD* acts after *Neurog1* and drives neuronal differentiation, and *Atoh1* is a proneural gene that confers competence to a prosensory cell cluster to develop into hair- or supporting cells and which persistence directs development towards hair cell fate. This decision requires lateral inhibition through the Delta-Notch mechanism and results in the characteristic cellular pattern of ear sensory epithelia (Whitfield *et al*., 1997).

Hair cell fate specification is concomitant with the morphogenetic process that foreshadows the appearance of the sensory organs (see diagram at the right in Fig. 5). They emerge as groups of sensory fated cells regionally restricted in what is called the sensory patches. It is still unclear how sensory patches emerge within the otocyst. Recent work suggests that sensory organs and their innervating neurons are spatially segregated in the otic placode (Bell *et al.,* 2008). The transition between the proneural domain, which is clearly defined at the otic vesicle stage and the prosensory patches, which are identifiable later on in development, has not yet been resolved unambiguously. In amniotes, this occurs after the otic vesicle is formed and it has been difficult to assess whether it is the result of the development of a common domain, or the result of the emergence of different, perhaps overlapping, independent prosensory patches. Some genes expressed in the neurogenic domain, like *LFng* and *Fgf10*, persist in the prospective sensory patches, during the stages of sensory organ development (Cole *et al*., 2000; Pauley *et al*., 2003; Pujades *et al*., 2006). Other genes, like *Bmp4* are absent from the initial proneural domain, but thereafter they foreshadow the sensory domains and precede *Atoh1* expression at the sensory patches (Pujades *et al*., 2006). *Ser1* is probably accompanying the prospective sensory domain since very initial steps of specification, and functional studies have shown that it is required for the development of sensory organs (Cole *et al*., 2000; Daudet and Lewis, 2005; Brooker *et al*., 2006; Kiernan *et al*., 2006). A recent study suggests that the *macula* emerge from a *Neurog1* positive domain that is common to the neurogenic domain, whereas *crista* and the auditory epithelium derive from other independent regions (Raft *et al*., 2007).

Sox genes in neurosensory fate

Sox genes contain an HMG-box closely related to that of the mammalian testes-determining gene *Sry*, and are highly conserved throughout evolution. The C-terminal region of the SOX protein carries a cryptic transactivating domain that uncovers only after specific interaction with partner factors. To date, twenty four vertebrate *Sox* genes have been identified and are classified into seven subgroups (A–G) based on sequence identity, and at least twelve members of the *Sox* gene family are expressed in the nervous system (Pevny and Placzek, 2005; Wegner and Stolt, 2005). Throughout evolution, the expression of the *SoxB1* genes (*Sox1, Sox2* and *Sox3*), directly correlates with: i) ectodermal cells that are competent to acquire neural fate; and ii) the commitment of cells to a neural fate (Rex *et al*., 1997; Pevny and Placzek, 2005). The *Drosophila SoxNeuro*, a putative ortholog of the vertebrate *Sox1-3* genes, is one of the earliest transcription factor to be expressed pan-neuroectodermally (Cremazy *et al*., 2000), and it acts upstream and in parallel with the *achaete-scute* genes. Interestingly, in *Drosophila*, *SoxNeuro* is only involved in central but not in peripheral nervous system development, suggesting that recruitment of SOX proteins into placode development is a novelty of craniates in order to rapidly expand the ectodermal anlage (Fritzsch *et al*., 2006).

SOX2 is expressed in multipotent neural stem cells at all stages during mouse ontogeny (Wegner and Stolt, 2005). *Sox2*

expression in the early embryonic Central Nervous System (CNS) is pieced together by separate enhancers with distinct spatio-temporal specificities, and the enhancers driving expression of *Sox2* to the lens and nasal/otic placodes have been identified (Uchikawa *et al*., 1999). *Sox2* belongs to the stem-cell cassette that maintains the self renewal state and pluripotency of progenitors (Takahashi and Yamanaka, 2006). *Sox1-3* interact with various partner transcription factors, and participate in defining distinct cell states that depend on the partner factors -*Pax6* for lens differentiation, *Oct3/4* for establishing the epiblast/ES cell state and, *Brn2* for the neural primordia. *Sox1-3* are co-expressed in proliferating neural progenitors of the embryonic and adult CNS. The *SoxB2* subgroup of *Sox* factors, including *Sox14* and *Sox21*, are very similar to *SoxB1* in their HMG-DNA binding domain, but act as transrepression domains. A key common feature of *SoxB1*, *SoxB2* and *SoxE*, however, is their ability to maintain neural progenitor or stem cell identity (for a review Wegner and Stolt, 2005). Studies in the chick embryo have provided evidence that neural inducing signals directly regulate SOX2 expression in the neural tube, and that SOX2 is responsible for commitment of actively proliferating cells to neural fate (Rex *et al*., 1997; Bylund *et al*., 2003; Graham *et al*., 2003). As mentioned before, recent studies also revealed that *Sox2*-regulatory region contains a domain that responds directly to neural inducing signals, which is conserved across diverse animal species (Takemoto *et al*., 2006).

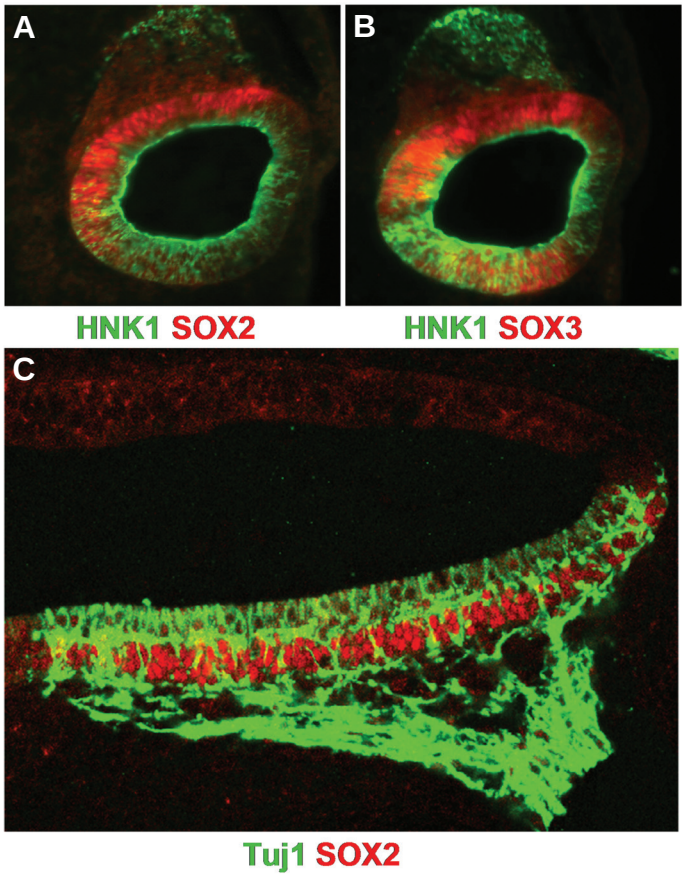

Fig. 6. SOX2 and SOX3 expression in the developing inner ear. (A,B) *SOX2 and SOX3 detected by immunofluorescence (red) and HNK1 surface epitope (green) in a HH18 chick otic vesicle. SOX2 and 3 overlap in the proneural domain of the otic vesicle and SOX3 seems to be more intense at the posterior pole of the otic vesicle.* **(C)** *SOX2 but not SOX3 maintains its expression in the supporting cells of the sensory patches after sensory organ formation. SOX2 immunofluorescence (red) and Tuj1 (green) are shown in a confocal section of the macula utriculi from an E6 chick embryo. In (A,B), anterior to the top.*

Sox2 and *Sox3* are expressed in the early proneural domain of the otic placode and otic vesicle (Fig. 5 and Fig. 6A and B, see also Uchikawa *et al*., 1999; Abello *et al*., 2007; Neves *et al*., 2007). Later in development, SOX2 expression foreshadows the prosensory patches and is expressed in all sensory organs (Kiernan *et al*., 2005; Neves *et al*., 2007). Two *Sox2*-deficient mice, *light coat and circling* (*Lcc*) and *yellow submarine* (*Ysb*), show hearing and balance impairment. *Lcc/Lcc* mutant mice fail to establish a prosensory domain and as a result of this, neither hair cells nor supporting cells differentiate (Kiernan *et al*., 2005). *Ysb/Ysb* mice show abnormal development with disorganized and fewer hair cells. These phenotypes are a direct consequence of the absence or reduced expression of the transcription factor SOX2 in the developing inner ear (Kiernan *et al*., 2005). Moreover, mutations of *Sox2* in humans cause anophthalmia, sensorineural hearing loss and global brain defects (Hagstrom *et al*., 2005) and regulates retinal neural progenitor competence (Taranova *et al*., 2006).

The role of *SoxB1* genes in cell fate specification in the ear is a subject of intense work. Otic neurons and hair cells are neural cell types in strict sense and both are born upon activation of proneural bHLH genes. The outcome of the terminal division of ear proneural progenitors is the withdrawal of the cell cycle and the expression of proneural differentiation genes, *NeuroD* for neurons (Alsina *et al*., 2004) and *Atoh1* for sensory cells (Pujades *et al*., 2006). This links SOX2 function with cell fate acquisition in the way it has been illustrated in the neural tube, where SOX2 maintains to repress the activity of proneural genes until cell cycle withdrawal, and the expression of the *Sox*B2 gene group counteracting this effect (Bylund *et al*., 2003). Hence, there seems to be some general principle for shifting the balance between two cell states: on one side a state where cells are committed, but maintain the capacity for self-renewal; on the other, a state of cell determination where cells make their terminal division and become determined to a particular fate. As discussed by Fritzsch *et al*. (2006), the vertebrate sensory organ requires a mechanism for rapidly expanding the basic sensory unit, so that placodal epithelial cells bear characteristics of stem cells. Therefore, it is expected that they express typical genes of the stem cell cassette (Takahashi and Yamanaka, 2006). The expression of SOX2 in the ear is reminiscent of this general stem-cell function, but restricted to neural committed progenitors. Early in development, during otic vesicle stages, SOX2 and SOX3 are found in proliferating cells, but only within the proneural domain of the early otocyst. SOX2 and SOX3 are expressed during the generation of neurons, but only SOX2 remains during sensory organ formation. The concomitant expression of SOX2 and SOX3 only during neuron generation suggests the possibility that at a given stage of development, SOX3 expression would be switched off and the persistent SOX2 expression would result in sensory cell generation. The possibility of a phenotypic switch of cycling neural progenitors from neuron to hair cell fate has been suggested to occur in the *Neurog1* null-mice (Matei *et al*., 2005).

Sox2 and *3* are expressed within ear domains that are also the domain of expression of Notch signalling pathway genes (Abello *et al*., 2007). A potential link between *Sox2* and Notch signalling is suggested by the observation that *Sox2* expression is missing in *Jagged1* conditional mutants and after Notch inhibition (Kiernan *et al*., 2006; Daudet *et al*., 2007), which would indicate that *Ser1* (*Jagged1* in mammals) is upstream of *Sox2* in the specification of the prosensory field. On the other hand, the loss of *Sox2* in the inner ear results in the loss of p27kip1, a regulator of terminal division in the cochlea (Kiernan *et al*., 2005). These results are consistent with a function of *Sox* genes in maintaining the self-renewal state along with a state of neural commitment, perhaps the latter being restricted by other patterning signals.

Concluding remarks

The question of coupling patterning and cell fate determination is central to development. Recent work has shed light into how those processes take place during ear development. The early specification of the neural competent domain, which ultimately gives rise to neurons and hair cells, seems to occur very early and concomitantly to the specification of the otic fate. It requires the activity of the Notch signalling pathway for maintenance, but not for its establishment, which involves FGF signals and the reinforced expression of *SoxB1* genes. Further work is required to understand these very initial steps of regionalisation and their genetic relation with the acquisition of the otic fate by the preplacodal ectoderm. Further regionalisation of the otic vesicle gives rise to the topological organisation of the ear and the role of the neural tube in this process has been studied extensively. At least three signalling systems, FGF, Wnt and Hh are known to contribute to pattern the otic vesicle, and two of them (FGF and Wnt) depend on hindbrain signals. The analysis of hindbrain gene mutants, such *MafB, Hoxa1* and *vHnf1*, is helping to dissect the genetic pathways that link hindbrain segmentation and otic patterning. Finally, the allocation of specific neural competent precursors to the neural domain of the otic placode and otic vesicle allows the development of neurons and hair cells. The genetic cassette involved in the expansion of these progenitor cells, and the one that further leads to determination of neurons and hair cells is starting to be unveiled. The *SoxB1* gene group and the proneural genes *Neurog1, NeuroD* and *Atoh1* appear as major cell fate determination factors. Precise genetic networks, cellular interactions and the interplay with signalling mechanisms are the subject of current studies in several laboratories, and a subject of great interest not only for the understanding of the development of the ear but for generating tools for ear repair.

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