

Research paper

# Strategies to regenerate hair cells: Identification of progenitors and critical genes

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## Abstract

Deafness commonly results from a lesion of the sensory cells and/or of the neurons of the auditory part of the inner ear. There are currently no treatments designed to halt or reverse the progression of hearing loss. A key goal in developing therapy for sensorineural deafness is the identification of strategies to replace lost hair cells. In amphibians and birds, a spontaneous post-injury regeneration of all inner ear sensory hair cells occurs. In contrast, in the mammalian cochlea, hair cells are only produced during embryogenesis.

Many studies have been carried out in order to demonstrate the persistence of endogenous progenitors. The present review is first focused on the occurrence of spontaneous supernumerary hair cells and on nestin positive precursors found in the organ of Corti. A second approach to regenerating hair cells would be to find genes essential for their differentiation. This review will also focus on critical genes for embryonic hair cell formation such as the cell cycle related proteins, the *Atoh1* gene and the Notch signaling pathway. Understanding mechanisms that underlie hair cell production is an essential prerequisite to defining therapeutic strategies to regenerate hair cells in the mature inner ear.

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## 1. Introduction

In mammals, permanent acquired hearing loss is commonly caused by the loss of sensory hair cells as a consequence of aging and environmental stresses, e.g. acoustic trauma or exposure to ototoxic drugs (cisplatin, aminogly-

cosides...) (Reviewed in (Hawkins et al., 1976; Saunders et al., 1985; Schacht, 1986)). In contrast, damaged vestibular organs retain some capacity for self-repair (Forge et al., 1993). After HC loss, limited numbers of supporting cells (SCs) divide in these sensory epithelia and several progenies appear to differentiate as HCs (Kuntz and Oesterle, 1998; Lambert et al., 1997; Warchol et al., 1993). The development of the organ of Corti involves the differentiation of placodal tissue into sensory HCs and non-sensory SCs. The mammalian organ of Corti is a complex structure containing a single row of inner HCs and three rows of outer HCs, supported by one row of phalangeal cells and three rows of Deiters cells (Fig. 1). Additional SC types can be distinguished including pillar cells, Hensen cells,

*Abbreviations:* HC, hair cell; SC, supporting cell; E, embryonic day; P, postnatal day; GER, greater epithelial ridge; LER, lesser epithelial ridge; DIV, day *in vitro*; EGF, epidermal growth factor; FGF2, fibroblast growth factor 2; GFP, green fluorescent protein; CDK, cyclin-dependent kinase; CDKI, CDK inhibitors; pRb, retinoblastoma protein; bHLH, basic helix-loop-helix

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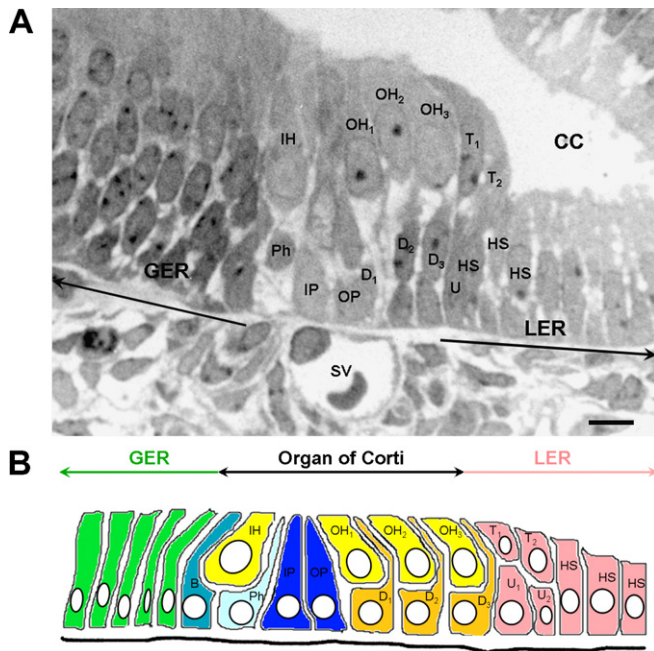


Fig. 1. The developing organ of Corti. (A) Cross-section through the organ of Corti in a E19 rat. (B) Schematic representation of the corresponding structure illustrating the organization of the organ of Corti and the adjacent structures, i.e. the GER and the LER. B = border cell; Ph = phalangeal cell; IP = inner pillar cell; OP = outer pillar cell; IH = inner hair cell; OH = outer hair cells; D = Deiters' cells; T = tectal cells; U = undertectal cells; HS = Hensen's cells. Scale bar in A represents 0.2  $\mu\text{m}$ .

tectal cells, undertectal cells and Claudius cells (Lim and Rueda, 1992; Lim, 1986; Malgrange et al., 2002b). In mammals, the HCs and SCs of the organ of Corti undergo their final round of division between embryonic day 12 (E12) and E16 (Ruben, 1967). After this critical period, mammalian HC generation no longer occurs. Potential strategies to replace lost sensory HCs include: (1) replacement of lost cochlear cells with transplanted stem cells in the damaged inner ear and (2) recruitment of inner ear endogenous stem cells or progenitor cells to generate new HCs. Embryonic stem cells (Li et al., 2003, 2004; Rivolta et al., 2006) or adult stem cells from various tissues (Doyle et al., 2006; Jeon et al., 2007) can be induced to differentiate into new HCs. However, these approaches are not yet possible in the adult mammalian organ of Corti *in vivo*. In addition, this strategy is technically challenging, since it requires (1) the design of surgical procedures to implant progenitor cells in the damaged cochlea without causing additional damage to the inner ear, (2) the isolation of precursor cells competent to integrate and ultimately to differentiate into functional HCs in the mature mammalian organ of Corti *in vivo* and (3) the recovery of functional HCs in the organ of Corti even if the cochlea cytoarchitecture is not restored. Recruiting cochlear precursor cells capable of dividing and further differentiating into specific cochlear end-phenotypes, such as HCs or SCs, represents the most elegant strategy for replacing HCs lost as a result of injury or dis-

ease. This review will focus on this approach for HC regeneration.

### 1.1. Identification of HC progenitors: focus on endogenous cells for replacing lost HCs

During rodent embryonic morphogenesis, the cochlear epithelial progenitor cells are formed in the otic vesicle at E11.5. The epithelial progenitor cells proliferate and expand to form two distinct regions in the dorsal epithelium of the cochlear canal, the greater epithelial ridge (GER) and the lesser epithelial ridge (LER), which contribute to the formation of HCs and SCs in the primitive organ of Corti from E15-P0 (postnatal day 0). Although disappearing gradually, the GER and LER, which can still be observed at birth, will give rise to inner spiral sulcus, outer spiral sulcus (primitive Hensen and Claudius cells) and other non-hair cell epithelial cells (Lim and Rueda, 1992). In the present review, we define the epithelium bordering on the organ of Corti at the most distant edge from the modiolus as LER and the epithelium bordering on the organ of Corti at the nearest edge from the modiolus as GER in the neonatal rat (Fig. 1). Alternative nomenclature for these regions includes outer spiral sulcus and inner spiral sulcus cells, respectively (Sobkowicz et al., 1975, 1990, 1993).

In order to generate new HCs, we have to develop strategies to change the fate of non-sensory cells (i.e. precursor cells) and to transdifferentiate them into new HCs with or without a mitotic cycle. Immature cells must be present and must retain the potential to undergo differentiation into HCs. These immature cells may arise from the GER and/or the LER. But SCs can also be a good candidate for HC progenitors. They can generate new HCs by transdifferentiation (conversion of the phenotype without cell division) (Baird et al., 2000; Minoda et al., 2004). Therefore, the SCs are attractive targets for interventions designed to produce new HCs.

#### 1.1.1. Mammalian vestibular progenitors

Stem cells are remarkable in their capacity to self-renew and to differentiate into a multiplicity of cell types (Raff, 2003). Such cells have been identified and isolated from several adult tissues. Taking advantage of some well-known characteristics of stem cells growing *ex-vivo*, Li et al. (2003) isolated cells from adult mouse utricular macula. After dissociation, these cells proliferated and expanded as free floating clonal spheres. After 8 days *in vitro* (DIV), these cells progressed to a progenitor cell state characterized by the expression of nestin. Nestin is an intermediate filament protein expressed by stem cells and progenitors early in development and throughout the early postnatal period in the central and peripheral nervous systems. Nestin is considered a neural stem cell marker (Lendahl et al., 1990). Li et al. (2003) found that some nestin positive cells amplified in culture from the utricular macula could be differentiated into HCs characterized by the expression of myosin VIIa, *Brn3c* and *espin* but not

p27<sup>Kip1</sup> and pan-cytokeratin, two specific markers of inner ear SCs. Moreover, Li et al. (2003) showed that these cells have filamentous actin-rich protrusions like normal HCs. Thus, adult stem cells were shown to reside in the sensory epithelium of the adult mouse utricle. They were seen to retain a high proliferative potential and to have the ability to generate cells possessing features of HCs.

### 1.1.2. Mammalian auditory progenitors

Nestin positive cells are present in the organ of Corti of both developing and mature cochleae, suggesting that immature HC and SC precursors may reside *in vivo* (Kojima et al., 2004; Lopez et al., 2004; Malgrange et al., 2002a). These nestin positive cells were found to be located at the same position throughout the maturation of the cochlea until P7. At P15, nestin positive cells were observed at the level of the spiral limbus. In adult animals, myosin VIIa (HCs) and p27<sup>Kip1</sup> (SCs) positive cells have never been shown to co-express nestin.

Cells can be mechanically dissociated from the organ of Corti of newborn rats and cultured in suspension in the presence of epidermal growth factor (EGF) and/or fibroblast growth factor 2 (FGF2). Under these conditions, they proliferate and form floating colonies that are mostly composed of nestin positive cells. Sixty percent of these cells incorporate BrdU, a thymidine analog incorporated into the DNA of dividing cells during the S-phase of the cell cycle. Some of these cells retain the ability to differentiate into SCs, i.e. p27<sup>Kip1</sup> positive cells or HCs, i.e. myosin VIIa positive cells. One third of the myosin VIIa positive cells that are produced are labeled with BrdU, indicating that these HCs have resulted from the differentiation of a mitotic precursor prior to the acquisition of an HC phenotype. Myosin VIIa positive cells, which do not incorporate BrdU, correspond to cells that may be either surviving pre-existing HCs or are newly formed HCs arising from the direct differentiation of postmitotic precursors (Malgrange et al., 2002a).

Another model for studying the biology of HC progenitors is based on the spontaneous occurrence of supernumerary HCs when the embryonic organ of Corti explants are cultured *in vitro* (Abdoh et al., 1993; Malgrange et al., 2002b). Abdoh et al. (1993) and Malgrange et al. (2002b) showed that, under unsupplemented culture conditions, production of supernumerary outer HCs occurs in the middle turn of the sensory epithelium of E19 rat organ of Corti. The supernumerary HCs were seen to arise as extra rows, respecting the architecture of the existing sensory epithelium, without any rearrangement of the existing HCs. Mitotic figures were never observed in the sensory epithelium in any sections of the explants. Moreover, in these experiments, the classically described mosaic including an invariant alternation of outer HCs and Deiters cells was still present and was conserved even in the area of supernumerary cells. In addition, the total number of cells per section was shown to remain constant in the organ of Corti even when the number of supernumerary outer

HCs or Deiters cells per section increased. Taken together, these results suggest that Hensen cells retain the capacity to transdifferentiate into either tectal cells, which themselves give rise to outer HCs, or into undertectal cells, which differentiate into Deiters cells (Malgrange et al., 2002b).

More recently, White et al. (2006) showed that SCs, purified from the cochlea of the postnatal mouse, are capable of division and transdifferentiation into new HCs in culture. They isolated p75<sup>NGFR+</sup> postmitotic SCs (i.e. pillar cells and Hensen cells) from a transgenic mouse line in which GFP (green fluorescent protein) is expressed in HCs under the control of the *Math1* enhancer. Due to the presence of this transgene, *Math1*-GFP positive HCs were eliminated by FACS purification, and *de novo* differentiation of HC from the p75<sup>NGFR+</sup> SC population was monitored by the expression of GFP. After 1 DIV, no *Math1*-GFP positive cells were observed in the cultures, but at 6 DIV, many new *Math1*-GFP positive cells were observed in the epithelial islands. This study demonstrates that postmitotic, postnatal SCs can transdifferentiate into HCs in culture.

In conclusion, HC progenitors are present in the mature mammalian cochlea, but their recruitment for transdifferentiation into HCs does not occur spontaneously after HC loss *in vivo*. Newly formed HCs can potentially arise from progenitor cells in the GER and/or the LER. However, HC progenitors are also found among the supporting cell population: i.e. in Hensen cells, pillar cells and tectal cells.

## 1.2. Identification of genes directly implicated in HC and/or SC differentiation

Understanding the molecular mechanisms that underlie the specification and differentiation of immature and/or proliferating epithelial cells into mature HCs and SCs is essential for elucidating the mechanisms that would be required to trigger cell repair and/or regeneration after injury. Identification and manipulation of candidate genes that control and regulate HC differentiation would help us to understand how to restore HCs in the damaged organ of Corti (Table 1).

### 1.2.1. Cell cycle-related proteins

Cell cycle-related proteins occupy a unique position among regulators of terminal differentiation, as they exhibit a dual role in regulating the cell cycle control and in maintaining the quiescence of postmitotic cells. During development, cell cycle progression is promoted by the activity of cyclins and CDKs. CDK inhibitors (CKI) promote cell cycle exit by binding and inactivating cyclin-CDK complexes (Sherr and Roberts, 1999). One of the key CDK substrates is the retinoblastoma protein (pRb), which is inactivated by phosphorylation (Zhu and Skoultschi, 2001). Inactivation of pRb relieves its repression of E2F transcription factors, which activates the expression of genes whose products promote cell proliferation (Wein-

Table 1  
Principal genes directly implicated in the patterning of the mammalian organ of Corti

Name	Nature of the gene or protein	Role	Expression pattern	Mouse mutant phenotype	References
<i>Atoh 1 (Math1)</i>	Basic helix-loop-helix transcription factor Homolog of the <i>Drosophila</i> proneural gene atonal ( <i>ato</i> )	Principal regulator of HC differentiation  Necessary and sufficient for HCs development in the mammalian cochlea	E13.5-E14.5 in progenitor cells committed to an HC fate	Null mutant: complete absence of HCs	Bermingham et al. (1999); Chen et al. (2002); Lanford et al. (2000); Woods et al. (2004)
<i>Foxg1</i>	Forkhead gene family	Action through participation of the protein binding domain in the Delta/Notch/Hes signaling pathway  Formation and size determination of sensory patches Organization of HCs	Developing otic placode (E9.5) Restricted to SCs and IHCs (E17.5)	Null mutant: shortened cochlea with multiple rows of HCs and SCs. Disorganization of cochlear HCs	Pauley et al. (2006)
<i>Notch signaling cascade proteins</i>					
<i>Notch1</i>	Transmembrane receptor	Differentiation of HCs in vertebrates  Lateral inhibition, restricting the proportion of cells that differentiate as HCs	Between E12-E14.5 in the developing cochlear duct Becomes restricted to SCs (E17.5)	<i>Foxg1-cre; Notch1<sup>fllox/-</sup></i> : increased number of HCs	Kiernan et al. (2005a); Lanford et al. (1999); Zine et al. (2000)
<i>Delta1 (Dll1)</i>	Transmembrane ligand for Notch	Lateral inhibition	Mouse nascent HCs at E14.5	<i>Foxg1-cre; Dll1<sup>fllox/fllox</sup></i> : auditory HCs developed earlier and in excess <i>Dll1<sup>hyp/-</sup></i> : some supernumerary HCs	Brooker et al. (2006); Kiernan et al. (2001); Kiernan et al. (2005a); Lanford et al. (2000); Morrison et al. (1999)
<i>Jagged1 (Jag1) (Serrate1)</i>	Transmembrane ligand for Notch	Lateral induction?	E12.5 and becomes restricted to SCs	<i>Foxg1-cre; Jag1<sup>fllox/fllox</sup></i> : reduced number of HCs Slalom ( <i>Slm</i> ) and Headturner ( <i>Htu</i> ): ( <i>Jag1</i> missense mutations) Reduction in the number of HCs	Brooker et al. (2006); Kiernan et al. (2001); Kiernan et al. (2006); Tsai et al. (2001); Zine et al. (2000)
<i>Jagged2 (Jag2) (Serrate2)</i>	Transmembrane ligand for Notch	Lateral inhibition	E14.5 in progenitor cells. Becomes restricted to HCs (E17.5)	Null mutant ( <i>Jag2<sup>ADSL</sup></i> ): extra rows of HCs	Jiang et al. (1998); Lanford et al. (1999); Lanford et al. (2000); Zine et al. (2000)
<i>Hes1</i> (mammalian hairy and enhancer-of-split homolog)	bHLH transcription factor	Negative regulator of HC differentiation	Mouse GER and LER at E16.5	Null mutant: increased number of IHCs	Zheng et al. (2000); Zine et al. (2001); Zine and de Ribaupierre (2002)

<i>Hes5</i>	bHLH transcription factor	Negative regulator of HC differentiation	Begins on E15 and becomes restricted to SCs (E16.5)	Null mutant: increased number of OHCs	Zheng et al. (2000); Zine et al. (2001); Zine and de Ribaupierre (2002)
<i>Hes6</i>	bHLH transcription factor	Not determined	E14.5 in progenitor cells and becomes restricted to HCs (E17.5)	Null mutant: no phenotypical abnormalities	Qian et al. (2006)
<i>Lunatic fringe (Lfng)</i>	Modulator of Notch signaling (Glycosyltransfer-ase)	Sensory HC and SC determination	Expressed at E9 in the ventral portion of the otic cup and is restricted at E16 to SCs	<i>Lfng</i> <sup>LacZ/LacZ</sup> : no phenotypical abnormalities	Morsli et al. (1998); Zhang et al. (2000)
<i>Pou4f3 (Brn3.1 or Brn3c)</i>	Class IV Pou Domain transcription factor	Development and survival of HCs in mouse	HCs as soon as they leave the cell cycle (E16)	Null mutant: complete loss of auditory HCs by P14	Hertzano et al. (2004); Huang et al. (2001); Xiang et al. (1997); Xiang et al. (1998); Xiang et al. (2003)
<i>p19<sup>Ink4d</sup></i>	Cyclin-dependent kinase inhibitor of the Ink4 family	Regulates cell cycle exit of HC progenitors Participates in HC apoptosis	Begins at E14.5 and persists in HCs at E16.5	Null mutant: HCs aberrantly re-enter the cell cycle and later undergo apoptosis	Chen et al. (2003)
<i>p27<sup>Kip1</sup></i>	Cyclin-dependent kinase inhibitor protein of the Cip/Kip family	Regulates cell cycle exit of HC progenitors Coordinating cell cycle exit with differentiation and patterning	Begins at E12.5 and becomes restricted to SCs (E16.5)	Null mutant: supernumerary HCs and SCs	Chen et al. (2003); Chen and Segil (1999); Lee et al. (2006); Lowenheim et al. (1999)
<i>Rb</i>	Nuclear phospho-protein encoded by the tumor suppressor retinoblastoma gene <i>Rb</i>	Regulates cell cycle exit of the inner ear sensory epithelial cells Maturation and survival of cochlear HCs	E12.5 in sensory precursor cells. Up-regulated at E15.5 in the developing organ of Corti and becomes restricted to HCs (E16.5)	<i>Coll1a1-pRb<sup>-/-</sup></i> or <i>mgRb:Rb<sup>-/-</sup></i> : overproduction of HCs during embryonic period  <i>Pou4f3-pRb<sup>-/-</sup></i> : ectopic proliferation in postnatal mice, increased number of HCs	Chen (2006); Mantela et al. (2005); Sage et al. (2005); Sage et al. (2006)
<i>Sox2</i>	High-Mobility-Group box transcription factor (groupB)	Establishment of the sensory progenitor cells	Ventral part of the otic placode (E9.5) and in HCs and SCs (E16.5)	<i>Ysb</i> ( <i>Sox2</i> expression reduced) and <i>Lcc</i> ( <i>Sox2</i> expression eliminated): disruption in the formation of the sensory epithelia and direct correlation between the number of HCs and the level of <i>Sox2</i> expression	Dong et al. (2002); Kiernan et al. (2005b); Wood and Episkopou (1999)

Abbreviations: HC = hair cell; SC = supporting cell; E = embryonic day; GER = greater epithelial ridge; LER = lesser epithelial ridge.

berg, 1995). Two families of CKI proteins have been described: the Ink4 family, which includes p16<sup>Ink4a</sup>, p15<sup>Ink4b</sup>, p18<sup>Ink4c</sup> and p19<sup>Ink4d</sup>, and the Cip/Kip family, which includes p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup> (Sherr and Roberts, 1995). In the mammalian auditory sensory epithelium, several cell cycle regulating proteins have been shown to regulate the transition from proliferation to differentiation (Chen et al., 2003; Chen and Segil, 1999; Lee et al., 2006; Lowenheim et al., 1999; Malgrange et al., 2003).

Targeted disruption of p19<sup>Ink4</sup>, a member of the Ink4 family of CKIs, leads to abnormal DNA synthesis in postnatal HCs (Chen et al., 2003). However, p19<sup>Ink4</sup> inactivation has not been shown to affect the quiescent state of HCs during late embryogenesis, even where p19<sup>Ink4</sup> has been reported to be expressed in the embryonic organ of Corti. This suggests that additional CKIs compensate for p19<sup>Ink4</sup> deficiency in developing cochlear HCs. Another CKI, p21<sup>Cip1</sup>, is expressed in developing HCs, however p21<sup>Cip1</sup>-null mice have been shown to exhibit an unaltered phenotype, suggesting a redundancy between p21<sup>Cip1</sup> and other CKIs (Mantela et al., 2005).

In the cochlea of mice lacking one or both alleles of p27<sup>Kip1</sup>, the proportion of mitotic cells has been shown to be prolonged for more than two weeks after the expected period of proliferation (Chen and Segil, 1999; Lowenheim et al., 1999). Proliferating cells appeared in clusters in the area of the Hensen cells, lateral to the outermost row of outer HCs, as well as in the pillar cell region. PCNA-positive cells were no longer seen in the Deiters cell region after E16. In addition, in the absence of p27<sup>Kip1</sup>, supernumerary HCs and SCs (including Deiters cells and pillar cells) arose spontaneously.

Interestingly, preliminary data presented in abstract form have shown an increase in BrdU-positive cells in the organ of Corti of mice that are heterozygous for p27<sup>Kip1</sup> after HC loss induced by systemic injection of amikacin (Kil et al., 2000). Other preliminary data suggest that inoculation of adenovirus encoding shRNA, which targets p27<sup>Kip1</sup> into the deafened guinea-pig cochlea, induces supporting cell proliferation and new stereociliary bundle hair cell production *in vivo* (Yamasoba et al., 2006). These findings support the idea that cochlear SCs are able to proliferate when p27<sup>Kip1</sup> is down-regulated and that HC loss triggers this proliferation.

In our laboratory, E19 rat organ of Corti explants treated with roscovitine, a pharmacological inhibitor of CDK1, 2, 5 and 7, have been shown to develop extra HCs and corresponding SCs (Malgrange et al., 2003). In the regions where supernumerary HCs were seen, the normal cytoarchitecture of the organ of Corti with alternating sensory and SCs was conserved. Roscovitine and other CDK1, 2, 5 or 7 inhibitors were shown to trigger the differentiation of precursor cells without affecting cell proliferation. This effect was far less intense at P0 than at E19. CDK1/2 expression and activity was high at E19 but barely detectable at P0, and roscovitine specifically inhibited CDK1/2 activity in E19 organ of Corti explants. These results sug-

gest that roscovitine induced the appearance of supernumerary HCs and corresponding SCs through the inhibition of CDK1/2 activity at E19 and that this effect disappears at the neonatal period owing to the absence of CDK1/2 activity. Hensen cells and cells located in the GER are the likely source of supernumerary HCs and corresponding SCs, as previously described (Kawamoto et al., 2003; Malgrange et al., 2002b; Zheng and Gao, 2000). Amazingly, p27<sup>Kip1</sup> is absent or slightly expressed in Hensen cells at the late embryonic stages but is present in neonatal mice (Chen and Segil, 1999) and rats (Malgrange et al., unpublished results). Therefore, it is tempting to speculate that the effect of roscovitine is possible on Hensen cells only during the embryonic period in the absence of p27<sup>Kip1</sup> and that the accumulation of p27<sup>Kip1</sup> in Hensen cells induces the decline of CDK1/2 activity and consequently the absence of the effect of roscovitine. Analysis of gene expression profiles of the developing mouse utricle has led to the identification of the retinoblastoma family members, *Rb*, *Rbl1* (p130) and *Rbl3* (p107) as candidate genes for cell cycle exit in the inner ear (Chen, 2006). Chen's preliminary studies on single and double p130 and p107 knockout mice did not reveal any major phenotype in the inner ear, in either the cochlea or vestibular systems. Unfortunately disruption of both *Rb* alleles (*Rb*<sup>-/-</sup> mice) resulted in embryonic lethality before HC differentiation. As a result of these studies, the role of pRb was evaluated in two transgenic mouse models with the *Rb* gene deleted in early progenitor cells of the inner ear. In the first model, partially rescued *Rb* mutants were examined (Mantela et al., 2005). These mutants were shown to have an *Rb* minigene allowing a low expression of pRb (*mgRb:Rb*<sup>-/-</sup>). In these animals, pRb was seen to be absent in the inner ear and the animals survived until birth. In the second transgenic model, *Rb*<sup>loxp/loxp</sup> mice were crossed with *Collagen1A1-cre* mice to produce *Col1A1-Rb*<sup>-/-</sup> embryos (Sage et al., 2005). Continuous cell division was observed in progenitor cells and in SCs in these transgenic mouse models. In addition, differentiated mammalian HCs were shown to keep dividing in the absence of pRb and electrically functional HCs were generated through the division of pre-existing HCs. Taken together, these results demonstrate that pRb is required for the cell cycle exit of developing inner ear sensory epithelial cells. The effect of pRb in postmitotic sensory epithelia has been addressed by studying the conditional deletion of *Rb* in *Brn3c* cells (*Brn3c-Cre* x *Rb*<sup>loxp</sup> i.e. *Brn3c-Rb*<sup>-/-</sup> transgenic mice). *Brn3c* (or *Pou4f3*) is a transcription factor restricted to postmitotic HCs and is absent in mitotic progenitors (Xiang et al., 1998). Conditional deletion of the *Rb* gene in the cochlear postmitotic progenitor cells has been shown to lead to the proliferation of both HCs and SCs even at postnatal stages (Sage et al., 2006). However, prominent cell death has been shown to occur in these postnatal cochleae in a pattern that is consistent with the maturation of cochlear HCs (Sage et al., 2006). As a result, *Brn3c-Rb*<sup>-/-</sup> mice were shown to exhibit total hearing loss. As mentioned previously, even

in the presence of p27<sup>Kip1</sup>, in *Brn3c-Rb*<sup>-/-</sup> mice, SCs re-enter the cell cycle. Therefore, *Rb* can override the p27<sup>Kip1</sup> function (Chen, 2006). Taken together, these results support *Rb* as a relevant target from the perspective of HC regeneration.

### 1.2.2. *Atoh1/Math1*

Basic helix-loop-helix (bHLH) transcription factors regulate the development of a variety of systems in vertebrates. Expression of the bHLH transcription factor *Atoh1* (also known as *Math1*), the mouse homolog of the *Drosophila* atonal gene, in the inner ear is required for the generation of HCs. Absence of *Atoh1* in mice leads to a complete loss of HCs (Bermingham et al., 1999). This gene has been shown to act as a “pro-hair cell gene” in the developing sensory epithelia and is required for the differentiation of sensory HCs from multipotent progenitors (Chen et al., 2002). Absence of *Atoh1* results in a complete loss of both HCs and SCs (Woods et al., 2004).

Experimental overexpression of *Atoh1* in non-sensory cells of the normal cochlea generates extra HCs. *In vitro* experiments in embryonic and postnatal rodent cochlear explants has shown that transfected cells with an *Atoh1* expressing plasmid lead to the production of ectopic new HCs (Jones et al., 2006; Woods et al., 2004; Zheng and Gao, 2000). These extra HCs come from the transdifferentiation of non-sensory epithelial precursors, the columnar epithelial cells located outside the sensory epithelium in the GER. In addition, overexpression of *Atoh1* in these regions of the cochlea is sufficient to induce the formation of sensory clusters that contain both HCs and SCs. It has been suggested that ectopic HCs can induce surrounding cells to develop as SCs, and that the loss of SCs in the cochlea of *Atoh1*-null mice is a consequence of the loss of HCs (Woods et al., 2004). The induction of these ectopic SCs would be due to inductive signals coming from the HCs and through the activation of the Notch signaling pathway. *In vivo* inoculation of adenovirus with the *Atoh1* gene insert into the endolymph of the mature guinea pig cochlea results in the production of immature HCs in the organ of Corti and of extra new HCs in the interdental cell, GER and Hensen cells regions (Kawamoto et al., 2003). In addition, these new ectopic HCs are able to attract auditory nerve fibers, suggesting that they might be functional. In 2005, Izumikawa et al. reported that transfection of *Atoh1* in young guinea pigs deafened by systemic administration of ototoxic drugs led to the generation of new HCs and improved auditory brain-stem response (ABR) thresholds (Izumikawa et al., 2005). At present, this experiment constitutes the first demonstration of a cellular and functional repair of the organ of Corti in a mature deaf animal. This represents a great potential strategy for cell replacement therapy in the mature mammalian cochlea.

### 1.2.3. Notch signaling

The Notch signaling pathway is widely used during both vertebrate and invertebrate development to regulate cell

fate by lateral signaling (Artavanis-Tsakonas et al., 1999; Lewis et al., 1998). The Notch family of cell-surface receptors are activated by the ligands Delta and Jagged. Upon ligand binding, the intracellular portion of the Notch receptor is cleaved and enters the nucleus where it interacts with a transcriptional complex including RBP-J (recombination signal binding protein for J-Kappa) (Greenwald, 1998; Mumm and Kopan, 2000). The Notch/RBP-J complex promotes the transcription of target genes such as *Hes1* and *Hes5* (Iso et al., 2003), which repress cell differentiation. Concomitantly, by preventing differentiation, *Hes1* and *Hes5* also repress Notch ligand expression or up-regulate the expression of *Notch* itself (Weinmaster, 1998). In the mammalian inner ear, the components of the Notch pathway are expressed in a pattern suggesting a role for *Notch* in lateral inhibition. The transmembrane receptor Notch is expressed ubiquitously (at least up to the time of HC differentiation), as are the transmembrane ligands *Delta1* and *Jagged2* in the nascent HCs (Lanford et al., 1999; Zheng et al., 2000; Zine et al., 2001). This is consistent with the idea that nascent HCs express Notch ligands and activate Notch in their neighbors, thereby preventing them from becoming HCs. Defective Notch signaling in the cochlea results in excess production of HCs. Indeed, a deletion of the *Jagged2* or *Delta1* gene has been shown to result in extra rows of HCs accompanied by a reduction in the number of p27<sup>Kip1</sup>-positive SCs (Brooker et al., 2006; Kiernan et al., 2005a; Lanford et al., 1999; Zine et al., 2000). Similarly, mice knockout for *Hes1* and *Hes5*, as well as mice lacking one copy of the *Notch* gene, have been shown to exhibit an increased number of HCs (Brooker et al., 2006; Kiernan et al., 2005a; Zhang et al., 2000; Zine et al., 2001). In addition, it has recently been shown that *Delta1* and *Jagged2* function synergistically in order to promote HC differentiation in the cochlea (Kiernan et al., 2005a). All these results match the prediction of lateral inhibition. However, it remains unclear (1) why homozygous *Jagged2* mutants were shown to have only a mild excess of HCs (Lanford et al., 1999), (2) why in *Delta1* mutant mice the loss of SCs was seen to be modest in comparison with the increase in HC numbers (Brooker et al., 2006; Kiernan et al., 2005a) and (3) why the loss of one copy of *Jagged1* in heterozygous Slalom (Tsai et al., 2001) and Headturner (Kiernan et al., 2001) mutant mice, instead of causing overproduction of HCs, were shown to result in a reduction in their number in the cochlea. The timing of gene expression in the wild type embryo also hints that the Notch signaling pathway does something more than cause lateral inhibition. Notch expression marks out the otic placode from the surrounding ectoderm before it has even begun to invaginate to form an otic vesicle (Daudet and Lewis, 2005). In addition, the expression of *Jagged1* becomes visible soon after this, before cell differentiation, suggesting an additional role of the Notch pathway at early stages of inner ear development. Indeed, in the inner ear of conditional knock-out mice, where *Jag1*<sup>loxpl/loxp</sup> mice were crossed with *Foxg1-cre* mice, *Sox2* and p27<sup>Kip1</sup>,

two early markers of the future organ of Corti, were shown to be down-regulated and a complete loss of outer HCs was observed (Brooker et al., 2006; Kiernan et al., 2006). *Jagged1* is also initially expressed in the prosensory region of the cochlea and becomes down-regulated in the nascent organ of Corti by E14.5 when the cells exit the cell cycle and differentiate (Kiernan et al., 2006). Taken together, these results demonstrate that *Jagged1*-mediated Notch signaling is essential during early development for establishing the prosensory region of the inner ear (Brooker et al., 2006; Kiernan et al., 2006). In addition, a detailed examination of the SC population in *Delta1* mutant and in *Foxg1-cre-Notch1<sup>flox/-</sup>* mice (Brooker et al., 2006), showed that there are discrepancies in SC loss. Deiters cells appeared to be the most drastically reduced SC population, suggesting a cell fate switch from Deiters cells to HCs. The other SC types (i.e. pillar cells and Hensen cells) underwent extra rounds of cell division, explaining the low drop in total SC numbers. Thus, in all these mutant mice, it is possible that an initial failure of lateral inhibition was followed by some compensatory proliferation of residual SCs.

## 2. Conclusions

- The modulation of gene expression levels by specific molecules or by using various viral gene transfer technologies in the inner ear might be used to stimulate the regeneration of inner ear HCs, and this could eventually benefit those suffering from hearing and balance disorders. The mammalian inner ear retains the ability to give rise to new HCs even after birth. Appropriate regulation of gene expression is essential for the generation of *de novo* HCs. Identification of the precursor cells of supernumerary HCs is an important step in elaborating a regeneration/repair strategy in the adult animal.
- Postnatal mice cochleae contain cells that under appropriate conditions are capable of proliferating and differentiating into sensory HCs, and these proliferating cells may be developed from outer spiral sulcus cells residing in the LER. We can also find progenitors of HCs in the subpopulation of SCs residing within the sensory epithelium and also in the GER. For many progenitors in the cochlea, one of the final cell fate choices is a binary choice between an HC and an SC. Finally, the non-sensory cochlear cells maintain the competence to become new HCs in mature animals (Kawamoto et al., 2003).
- Innervation of the new HCs would be a prerequisite for restoring hearing. The ability of new HCs to receive new nerve terminals has been demonstrated in the regenerating avian basilar papilla (Ofsje and Cotanche, 1996; Wang and Raphael, 1996). Raphael's lab showed that in *Atoh1* transfected cochleae, axons are extended from the bundle of auditory nerves toward some of the new HCs, suggesting that these new and ectopic HCs can provide signals to attract auditory neuron fibers. It remains to be established whether HCs generated by transdifferentiation survive in the cochlea and whether they would become physiologically functional in the organ of Corti to restore impaired hearing even if they are not located at their initial position.

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