REVIEW

Sensory hair cell development and regeneration: similarities and differences

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ABSTRACT

Sensory hair cells are mechanoreceptors of the auditory and vestibular systems and are crucial for hearing and balance. In adult mammals, auditory hair cells are unable to regenerate, and damage to these cells results in permanent hearing loss. By contrast, hair cells in the chick cochlea and the zebrafish lateral line are able to regenerate, prompting studies into the signaling pathways, morphogen gradients and transcription factors that regulate hair cell development and regeneration in various species. Here, we review these findings and discuss how various signaling pathways and factors function to modulate sensory hair cell development and regeneration. By comparing and contrasting development and regeneration, we also highlight the utility and limitations of using defined developmental cues to drive mammalian hair cell regeneration.

KEY WORDS: Wnt, FGF, Shh, Notch, Atoh1, p27^{Kip1}, Cdkn1b

Introduction

The inner ear sensory organs, which comprise the cochlea, utricle, saccule and the crista ampullaris of the three semicircular canals, serve to detect sound and motion. The hair cells that reside in these six sensory organs are mechanoreceptors, and their development and survival are crucial for these sensory functions. In the cochlea, hair cells are derived from prosensory cells in the floor of the cochlear duct (Fig. 1), which, in response to a variety of signals, enter terminal mitosis and begin to express Atoh1, a transcription factor that is necessary and sufficient for hair cell differentiation (Bermingham et al., 1999; Chen et al., 2002). Multiple events then occur within a relatively short time window as sensory hair cells appear in the prosensory region of the cochlear duct, eventually giving rise to one row of inner hair cells, three rows of outer hair cells, and supporting cell subtypes (Deiters' cells, pillar cells, inner phalangeal/border cells and Hensen's cells; Fig. 1). This orderly matrix of hair cells interdigitated by supporting cells forms the organ of Corti, which extends along the length of the cochlear duct and is the receptor organ for hearing.

In the mature mammalian cochlea, hair cells are susceptible to damage from a variety of insults, ranging from aminoglycoside antibiotics to noise exposure. Once lost, hair cells are unable to spontaneously regenerate and the resulting hearing loss is thus permanent (Fig. 2). In stark contrast, the avian cochlea (the basilar papilla) is able to regenerate lost hair cells and auditory function can fully recover (Corwin and Cotanche, 1988; Cruz et al., 1987; Ryals and Rubel, 1988). During regeneration in the avian cochlea, the supporting cells that are interdigitated with hair cells serve as the source of

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regenerated hair cells through two mechanisms (Fig. 2): (1) via mitotic regeneration, whereby a supporting cell divides and then one or both of the daughter cells differentiates into a hair cell; and (2) via direct phenotypic conversion of a supporting cell into a hair cell without undergoing mitosis, a phenomenon termed direct transdifferentiation (Stone and Cotanche, 2007). Since the discovery of spontaneous hair cell regeneration in the avian cochlea, significant advances have been made towards elucidating the molecular mechanisms that underpin hair cell regeneration in this organ. In addition, studies of sensory organs with ongoing turnover and production of hair cells, including the chicken utricle, the zebrafish lateral line system and other non-mammalian vertebrate models (e.g. frogs, newts, lizards), have also shed light on potential key regulators of hair cell development and regeneration.

Here, we review the key factors (*Atoh1* and *p27^{Kip1}*) and cell signaling pathways [the Notch, fibroblast growth factor (FGF), Wnt and sonic hedgehog (Shh) pathways] that are involved in the development of hair cells, with a focus on the auditory organ, the cochlea. By highlighting similarities and differences in how these pathways and factors modulate hair cell development and regeneration, we then discuss the potential applicability and limitations of manipulating these signals individually or in combination to induce hair cell regeneration.

The transcription factor *Atoh1* plays a key role in hair cell development

Atoh1 is the mammalian homolog of the *Drosophila* gene *atonal*, which was first described as a proneural gene involved in regulating the formation of mechanoreceptors and photoreceptors in *Drosophila* (Jarman et al., 1993, 1994). In the developing mouse cochlea, *Atoh1* expression marks the prosensory region, and the *Atoh1*-positive lineage gives rise to sensory hair cells (Driver et al., 2013). In the absence of *Atoh1* expression, prosensory cells fail to form hair cells and supporting cells and instead undergo cell death (Bermingham et al., 1999; Pan et al., 2011; Woods et al., 2004). Although *Atoh1* is transiently expressed in developing hair cells until the early postnatal period, the deletion of *Atoh1* at various developmental time points revealed that it is necessary for many of the processes needed to generate fully functional hair cells. Specifically, *Atoh1* is crucial for hair cell survival soon after differentiation, which occurs from E15.5 to E17.5 (Cai et al., 2013; Chonko et al., 2013). Later on, *Atoh1* expression is also required for hair cell maturation, including the development of stereociliary bundles (Cai et al., 2013). Collectively, these findings indicate that *Atoh1* is necessary for hair cell differentiation, survival and maturation in the developing cochlea.

Conversely, the ectopic expression of *Atoh1* has generated phenotypes that vary depending on the developmental stage of the cochlea. In the embryonic cochlea, *Atoh1* overexpression results in the formation of supernumerary hair cells (Gubbels et al., 2008;

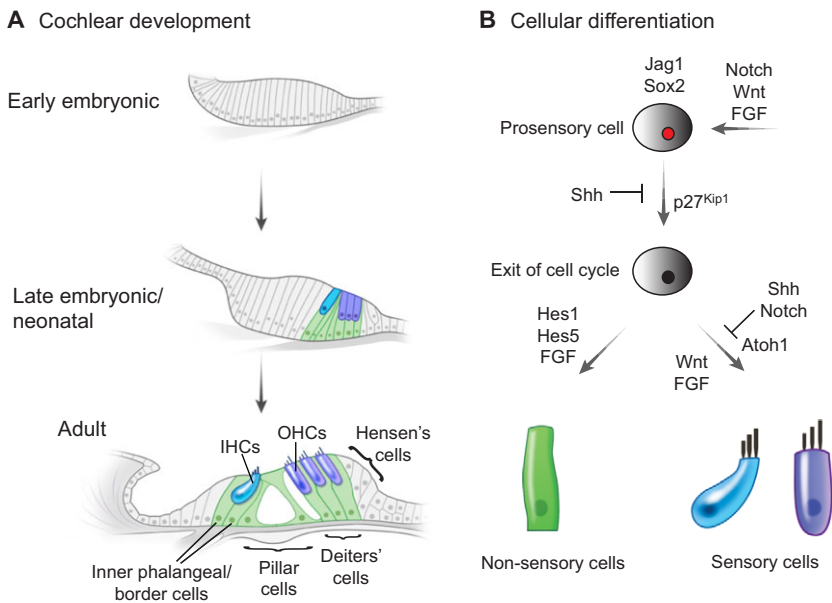**A** Cochlear development   **B** Cellular differentiation

Fig. 1. Cell fate decisions and the development of the mammalian organ of Corti. (A) An overview of cochlear development, highlighting the location of inner hair cells (IHCs, blue), outer hair cells (OHCs, purple) and various supporting cell subtypes (green). (B) A simplified schematic highlighting some of the signaling pathways and factors responsible for the specification of prosensory, sensory and non-sensory cells. The FGF, Notch and Wnt signaling pathways, together with the transcription factor Sox2, are required for prosensory cell specification and/or maintenance. The timing of terminal mitosis and the upregulation of the cell cycle inhibitor $p27^{Kip1}$ in Sox2-positive prosensory cells are governed by Shh signaling. Wnt and FGF signaling are required for *Atoh1* expression and the induction of sensory cell fate, which is restricted by the Notch and Shh pathways. By contrast, the activation of Notch target genes (*Hes1* and *Hes5*) promotes a supporting cell fate, with FGF signals regulating differentiation into supporting cell subtypes.

Woods et al., 2004). In neonatal mice, supporting cells remain competent to form hair cells in response to *Atoh1* overexpression, especially in the Kölliker's organ, which is directly medial to the organ of Corti (Kelly et al., 2012; Liu et al., 2012a, 2014; Shou et al., 2003; Yang et al., 2013; Zheng and Gao, 2000). However, this competence is significantly diminished in the undamaged, mature cochlea when assessed via either a transgenic approach or direct viral inoculation (Kawamoto et al., 2003; Liu et al., 2012a).

Building upon its crucial role in hair cell development, numerous studies have focused on *Atoh1* in an attempt to regenerate hair cells in the damaged, mature cochlea. For example, Izumikawa and colleagues reported that ectopic expression of *Atoh1* immediately after ototoxic injury results in the formation of immature hair cells and the rescue of hearing function in guinea pigs (Izumikawa et al., 2005). However, two subsequent studies showed that the efficacy of this approach might be variable and dependent on timing of expression after damage (Atkinson et al., 2014; Izumikawa et al., 2008). Although these studies illustrate the limited value of using *Atoh1* as the sole agent to coerce a hair cell fate in the mature cochlea, they have served as foundations for other studies examining the signaling pathways that cooperatively govern *Atoh1* expression and hair cell differentiation, several of which are discussed below.

Cell signaling pathways involved in hair cell development and regeneration
Notch signaling
The Notch pathway mediates short-range cell-cell communication and controls diverse cellular processes, including proliferation, differentiation and cell death in a context-dependent manner. Upon ligand activation, the Notch receptor is enzymatically cleaved, resulting in the release of the Notch intracellular domain (NICD). NICD then translocates into the nucleus, where it interacts with the DNA-binding protein and core effector of the canonical Notch pathway, RBPjk. Readers are referred to the following reviews for a more in-depth discussion of Notch signaling (Kiernan, 2013; Kopan, 2012; Louvi and Artavanis-Tsakonas, 2012).

The Notch pathway plays multiple roles during cochlear development. Several independent lines of research show that

Notch signaling, acting via the process of lateral induction, is sufficient to specify prosensory cells. First, the Notch ligand jagged 1 (*Jag1*) is expressed prior to and during specification of the prosensory region, which is identified by *Sox2* expression (Brooker et al., 2006; Kiernan et al., 2006). Second, conditional deletion of *Jag1* leads to the downregulation of this prosensory marker, resulting in a malformed cochlear duct that contains few hair cells and supporting cells (Brooker et al., 2006; Kiernan et al., 2006). Third, cultured explants exposed to Notch inhibitors fail to form a prosensory domain (Munnamalai et al., 2012). Conversely, gain-of-function studies using virus-mediated overexpression of NICD in the chick cochlear duct (Daudet and Lewis, 2005) or transgenic approaches in the mouse inner ear (Hartman et al., 2010; Liu et al., 2012b; Pan et al., 2013) result in ectopic sensory patches composed of hair cells and supporting cells. Together, these studies suggest that the Notch pathway is both necessary and sufficient for specifying prosensory cells.

However, several other studies have challenged this interpretation. For example, an early *in vitro* study of the developing chick cochlea showed that Notch inhibition disrupts the maintenance but not the initial specification of prosensory cells (Daudet et al., 2007). Following up on this finding, two groups examined the *in vivo* effects of ablating *RBPjk* (*Rbpj*) prior to prosensory specification in mice (Basch et al., 2011; Yamamoto et al., 2011). They both observed the appearance of prosensory cells that subsequently underwent extensive cell death, further supporting the notion that the canonical Notch pathway is important for the maintenance rather than the specification of the prosensory region. Although canonical Notch signaling is classically mediated by RBPjk, one untested hypothesis for these conflicting results is that Notch signaling exerts its effects on prosensory specification in an RBPjk-independent manner. However, our understanding of the molecular machinery executing non-canonical Notch signaling is limited, and future studies to identify additional Notch effectors are required to clarify the role(s) of the Notch pathway during prosensory development.

After the initiation of hair cell differentiation, Notch-mediated lateral inhibition is involved in determining whether a prosensory cell becomes a hair cell or a supporting cell; differentiating hair cells express Notch ligands, and these activate Notch signaling in neighboring supporting cells, which, in turn, are prevented from
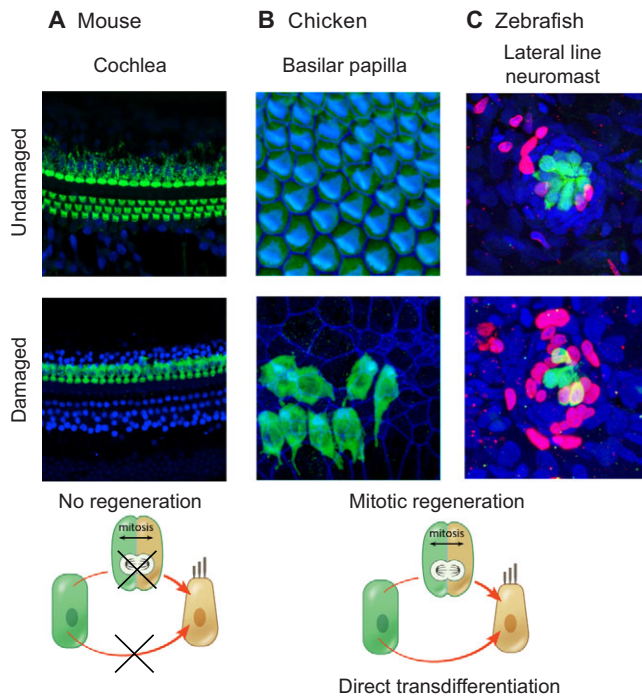
A Mouse

Cochlea

B Chicken

Basilar papilla

C Zebrafish

Lateral line neuromast

Fig. 2. Regenerative capacities of the adult mouse cochlea, the chicken basilar papilla and the zebrafish lateral line system. (A) No sensory hair cell regeneration occurs in the adult mouse cochlea. Shown are the basal turns of postnatal day (P) 28-30 cochleae from control (undamaged) mice and from mice that were treated with sisomicin and furosemide at P21. Immunostaining was performed to detect myosin VIIa (green) and Sox2 (blue); the reduction in myosin VIIa-labeled hair cells indicates that the damaged hair cells are not able to regenerate. (B) Hair cell regeneration in the chicken basilar papilla. Organs were harvested from control chicks and from those that had received daily injections of streptomycin in the previous week. Myosin VIIa-labeled hair cells are in green and phalloidin staining (F-actin) is in blue. Image courtesy of M. Warchol. (C) Hair cell regeneration in the zebrafish lateral line system. Images show posterior lateral line neuromasts from control zebrafish and from those that were damaged with neomycin. Hair cells are labeled by GFP in green, while BrdU (red) marks proliferating cells and DAPI staining (blue) marks nuclei. Regeneration in the neuromast populations, as indicated by the number of BrdU-positive cells, occurs primarily through mitotic regeneration. Image courtesy of T. Piotrowski and A. Romero-Carvajal.

adopting a hair cell fate. As a result, a mosaic pattern of organ of Corti cells is generated. Accordingly, mice deficient for the Notch ligand *Jag2* exhibit an increase in hair cell number at the expense of surrounding non-sensory cells. This phenotype is more severe when another Notch ligand, delta-like 1 (*Dll1*), is also deficient, suggesting a graded regulation of this pathway by these ligands (Kiernan et al., 2005; Lanford et al., 1999). Unlike *Jag1*-deficient mice, *Jag2* null and *Jag2;Dll1* double mutants still form hair cells (Kiernan et al., 2005; Lanford et al., 1999). Similarly, conditional deletion of the Notch receptor *Notch1* does not prevent formation of the prosensory domain, but instead leads to supernumerary hair cell formation and a concomitant reduction in the number of supporting cells (Kiernan et al., 2005). These studies suggest that different combinations of Notch ligands/receptors mediate prosensory specification and hair cell differentiation. This notion is supported by a recent study of the developing chick cochlea, which showed that different Notch ligands (Jag1 and Delta1) can activate the pathway at various levels (Petrovic et al., 2014).

Given the integral roles of the Notch signaling pathway during hair cell development, extensive work has been undertaken to characterize its involvement during hair cell regeneration. These

studies have shown, for example, that hair cell damage in the avian cochlea and utricle, as well as in the zebrafish lateral line, results in the upregulation of Notch target genes (Daudet et al., 2009; Ku et al., 2014; Ma et al., 2008; Stone and Rubel, 1999). Furthermore, although the regenerative capacities of the mammalian cochlea and utricle are limited, the expression of Notch target genes in both of these organs also increases after damage (Batts et al., 2009; Korrapati et al., 2013; Lin et al., 2011; Mizutari et al., 2013; Wang et al., 2010).

Many studies have also characterized the effects of Notch inhibition on hair cell regeneration. Ma and colleagues, for example, demonstrated that mitotic hair cell regeneration after damage increases when γ-secretase inhibitors are used to block Notch signaling in the zebrafish lateral line system, whereas hair cell turnover during homeostasis is unaffected (Ma et al., 2008). Likewise, a study of the chick cochlea showed that treatment with γ-secretase inhibitors increases the number of regenerated hair cells, in this context via both mitotic and non-mitotic mechanisms (Daudet et al., 2009). Studies on the undamaged neonatal (immature) mammalian cochlea have also shown that supporting cells can acquire a hair cell fate when treated with Notch inhibitors (Doetzlhofer et al., 2009; Li et al., 2015; Yamamoto et al., 2006). Building on these findings, two recent studies examined the damaged neonatal mouse cochlea and demonstrated a similar ability of supporting cells to regenerate nascent hair cells after Notch inhibition (Bramhall et al., 2014; Korrapati et al., 2013). This pharmacological approach has also been applied to the damaged, mature mammalian cochlea, but yielded a significantly less robust regenerative response (Mizutari et al., 2013; Tona et al., 2014); unlike the effects observed in the zebrafish lateral line system and chick cochlea, the regenerated hair cells were attributed to direct transdifferentiation of supporting cells, as no cell proliferation was found. Although it is commonly postulated that the regeneration of a functional organ of Corti requires the coordination of supporting cell proliferation and hair cell differentiation such that a balanced ratio of sensory and non-sensory cell types is maintained, a modest functional improvement was observed using this pharmacological approach (Mizutari et al., 2013).

In contrast to the mature mammalian cochlea, the mammalian utricle, saccule and crista ampullaris of the three semicircular canals do exhibit spontaneous hair cell regeneration, albeit at a lower level than that observed in other organisms (Forge et al., 1993, 1998; Golub et al., 2012; Tanyeri et al., 1995; Warchol et al., 1993). In the absence of damage, pharmacological inhibition of Notch signaling in the adult utricle failed to promote a hair cell fate among supporting cells (Collado et al., 2011). However, after hair cell loss, the inhibition of Notch signaling via either inhibition of the target gene *Hes5* or pharmacological inhibition of γ-secretase was able to significantly increase *Atoh1* expression, with a subset of supporting cells maturing to myosin VIIa-expressing hair cells (Burns et al., 2012; Jung et al., 2013; Lin et al., 2011). Similarly, more hair cells were detected following Notch inhibition in the damaged crista ampullaris (Slowik and Bermingham-McDonogh, 2013).

Taken together, these studies draw similarities between the roles of Notch signaling in specifying hair cell fate during development and in regulating hair cell regeneration. Importantly, these findings also underscore the value of Notch inhibition as a promising approach for enhancing hair cell regeneration. In addition, they suggest that mitotic divisions among supporting cells are likely to be guided by Notch-independent signals. These advances also highlight the diminishing competence of mammalian cochlear supporting cells to respond to cues and regenerate as the organ

matures. This notion is further supported by work on the postnatal cochlea, which, unlike the embryonic organ, does not generate ectopic hair cells following NICD overexpression (Liu et al., 2012b). If the process of hair cell regeneration were to simply recapitulate the process of hair cell development, one may schematize a strategy using sequential Notch activation and inhibition. However, the utility of this approach has not been tested and will require a better understanding of the factors that confer competence to respond to Notch manipulation in the developing cochlea – a topic to which we return later.

FGF signaling

The FGF pathway regulates the development of multiple sensory organs, including the inner ear where it plays an essential role in the induction of the otic placode, development of the otic vesicle and regulation of ensuing inner ear morphogenesis (Pirvola et al., 2000; Schimmang, 2007; Wright and Mansour, 2003). FGF also regulates the later stages of inner ear development and hair cell formation: tissue-specific deletion of FGF receptor 1 (*Fgfr1*) reduces prosensory cell proliferation, resulting in fewer prosensory cells and thus decreased numbers of hair cells and accompanying supporting cells (Ono et al., 2014; Pirvola et al., 2002). The treatment of cultured embryonic cochlear explants with chemical inhibitors of FGF signaling at a stage after terminal mitosis (E14) causes a decrease in *Atoh1* expression, suggesting that FGF signaling is also required for hair cell differentiation (Hayashi et al., 2008). Two independent studies pinpointed FGF20 as the candidate ligand for FGFR1 in the cochlea, with both FGF20 and FGFR1 being required for the development of at least a subset of hair cells and supporting cells (Hayashi et al., 2008; Huh et al., 2012). The prosensory effect of FGF20 was further shown to act downstream of Notch signaling; FGF20 treatment rescues prosensory specification that is impeded by Notch inhibition (Munnamalai et al., 2012). Remarkably, deletion of *Fgf20* did not lead to vestibular dysfunction, suggesting that the role of FGF20 in hair cell specification might be specific to the cochlea.

Following the initiation of hair cell differentiation, interactions between FGF8, which is secreted by nascent inner hair cells, and FGFR3, which is expressed by supporting cells, are required for the proper development of pillar cells (Colvin et al., 1996; Jacques et al., 2007). At this stage, deficient FGF signaling (as apparent in *Fgfr3* mutant mice) increases the number of outer hair cells at the expense of pillar cells and also distorts the relative proportion of supporting cell subtypes (Hayashi et al., 2007; Mansour et al., 2013; Puligilla et al., 2007). Unlike other supporting cell subtypes (e.g. Deiters' cells), pillar cells do not normally convert to hair cells after inhibition of Notch signaling. However, FGF receptor blockage or *Hey2* deficiency renders pillar cells responsive to Notch inhibition (Doetzlhofer et al., 2009), indicating that the two pathways – Notch and FGF – coordinate to maintain the fate of cochlear supporting cells.

The role of FGF signaling during hair cell regeneration has been more thoroughly investigated in the chicken utricle. When analyzing the transcriptome during regeneration of the chicken utricle *in vitro*, Ku and colleagues found a decrease in *Fgf20* and *Fgfr3* expression at the time when supporting cells robustly proliferate (Ku et al., 2014). Furthermore, although FGF20 treatment decreases the number of proliferative supporting cells, which is an effect previously observed with broad (basic) FGF treatment (Oesterle et al., 2000), no change was observed when a general FGF receptor inhibitor was applied (Ku et al., 2014). This suggests that FGF signaling inhibits proliferation during regeneration but that blocking FGF signaling alone is not

sufficient to enhance proliferation. A decrease in *Fgfr3* expression has also been observed after damage in the chicken cochlea and in the zebrafish lateral line (Bermingham-McDonogh et al., 2001; Jiang et al., 2014), whereas *Fgfr3* upregulation occurs in the damaged, mammalian cochlea (Pirvola et al., 1995). Whether this difference in FGF receptor expression between mammalian and non-mammalian cochleae accounts for their contrasting proliferative capacities is currently unclear.

In summary, these studies indicate that FGF signaling helps to govern the specification of prosensory cells and their subsequent differentiation into hair cells and supporting cells during cochlear development. By contrast, FGF signaling appears to play a role in limiting proliferation during regeneration in the chick utricle. These studies also elucidated interactions between the Notch and FGF signaling pathways during multiple stages of cochlear development, raising the question of whether and how these pathways might also intersect during regeneration.

Wnt signaling

The Wnt signaling pathway is involved in a wide spectrum of processes during development, including cell proliferation, cell fate determination, maintenance of stem/progenitor cells, and several aspects of cellular and subcellular polarization. The binding of Wnt protein to a receptor complex on Wnt-responsive cells activates the pathway, which is generally separated into the canonical/β-catenin and non-canonical signaling pathways (Logan and Nusse, 2004). A large body of literature describes the key roles of Wnt signaling during inner ear development (reviewed by Groves and Fekete, 2012; Munnamalai and Fekete, 2013). Here, we focus on the roles of canonical Wnt signaling during prosensory cell specification and hair cell differentiation. It is important to point out that non-canonical Wnt signaling also plays a key role during inner ear development by mediating planar cell polarity of hair cells and convergent extension (Dabdoub et al., 2003; Qian et al., 2007). However, it is not clear how Wnt-secreting and Wnt-responsive cells transition from utilizing canonical Wnt signals for guiding cell fate decisions to using non-canonical signals for establishing cell polarity a few days later. This is an important issue to address if we are to understand whether Wnt activation can be applied to stimulate hair cell regeneration.

The examination of two reporter mouse strains (Lgr5-EGFP-IRES-CreERT2 and TCF/LEF:H2B-GFP) (Barker et al., 2007; Ferrer-Vaquer et al., 2010) indicates that Wnt signaling is active in the cochlear duct prior to hair cell differentiation (Chai et al., 2011; Jacques et al., 2012; Shi et al., 2012). Using cochlear explants, it was shown that the application of Wnt inhibitors prevents the proliferation of prosensory cells and their subsequent differentiation into hair cells *in vitro* (Jacques et al., 2012). The opposite effects were observed when chemical activators of Wnt signaling were applied: an increase in the proliferation of prosensory cells and in the number of hair cells (Jacques et al., 2012). Wnt activation stimulates hair cell proliferation via the upregulation of cyclin D1 in Sox2-positive prosensory cells, both before and after their terminal mitosis, giving rise to an expanded Sox2-expressing domain. The subsequent determination of cell fate in the expanded Sox2 domain occurs as in the normal Sox2-expressing domain, indicating that Notch-mediated lateral inhibition takes place in these tissues (Jacques et al., 2012). By contrast, when cochlear explants from late embryonic or neonatal mice were treated with Wnt activators, a mitogenic response was only seen among a subset of supporting cells, and no ectopic hair cell formation was observed (Jacques et al., 2012; Jan et al., 2013). These observations indicate that the

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canonical Wnt pathway can regulate both the proliferative state and the fate of prosensory cells during cochlear development. In agreement with this interpretation, a follow-up study used a transgenic approach to ablate β-catenin (*Ctnnb1*) during cochlear development *in vivo*, and this resulted in diminished proliferation of prosensory cells and reduced hair cell formation (Shi et al., 2014). Moreover, overexpression of β-catenin in the developing chicken otocyst results in ectopic sensory patches (Stevens et al., 2003), suggesting that the competence to respond to the prosensory effects of Wnt signaling, like those of Notch activation (Hartman et al., 2010; Liu et al., 2012b; Pan et al., 2013), is broader than the prosensory domain at this earlier stage of development.

The roles of canonical Wnt signaling during hair cell regeneration have also been closely examined in recent years, given its mitogenic and prosensory effects during development. In the neonatal cochlea, a subset of supporting cells expresses the Wnt target gene *Lgr5* (Chai et al., 2011), which labels Wnt-responsive stem/progenitor cells in a variety of tissues (Barker and Clevers, 2006; Jaks et al., 2008). Although these Lgr5-positive supporting cells in the cochlea are normally quiescent both *in vivo* and *in vitro*, they become proliferative when isolated as single cells and can convert into hair cell-like cells in culture (Chai et al., 2012; Shi et al., 2012). Experiments using Wnt activators and inhibitors have shown that Wnt activity controls the proliferation of isolated Lgr5-positive supporting cells *in vitro* (Chai et al., 2012; Shi et al., 2012). In addition, the overexpression of β-catenin in colonies derived from isolated supporting cells modestly increases *Atoh1* expression and hair cell formation *in vitro* (Shi et al., 2012). This finding is concordant with previous work demonstrating that the enhancer region of *Atoh1* is a direct target of β-catenin in neural progenitors (Shi et al., 2010). In a similar vein, neonatal transgenic mice that conditionally express a stabilized form of β-catenin exhibit increased proliferation of supporting cells in the cochlea (Chai et al., 2012; Shi et al., 2013). Remarkably, among these Wnt-responsive supporting cells, the inner pillar cells also upregulate *Atoh1*, indicating that Wnt/β-catenin signaling can still exert a prosensory response in a limited fashion at this developmental stage. However, no mitogenic or prosensory effects were observed when this pathway was activated in supporting cells of the mature adult cochlea (Shi et al., 2013).

The role of Wnt signaling during hair cell regeneration has also been studied in the zebrafish lateral line. In this context, Wnt activators have been shown to increase the degree of regeneration after damage (Head et al., 2013; Jacques et al., 2014). Similarly, recent studies of the damaged neonatal mouse utricle revealed that Wnt/β-catenin activation increases both supporting cell proliferation and, subsequently, hair cell regeneration *in vivo* (Wang et al., 2015). It remains to be tested whether supporting cells in the damaged, adult mammalian cochlea can proliferate and regenerate in response to Wnt/β-catenin activation.

Collectively, these findings demonstrate that activating the canonical Wnt pathway can force neonatal cochlear supporting cells, which are otherwise mitotically quiescent, to proliferate and form new hair cells. It is thus feasible to formulate a regenerative strategy by first employing Wnt activation to stimulate progenitor/ supporting cell proliferation before introducing a prosensory signal, such as Notch inhibition (via lateral inhibition) or *Atoh1* overexpression, to direct hair cell differentiation. Although the interaction between the Notch and Wnt pathways is well characterized in other tissues, studies examining this interplay in the inner ear are rather limited (Jayasena et al., 2008; Li et al., 2015). Thus, more work is needed to further characterize this interaction

and the mechanisms involved in reducing Wnt responsiveness as the cochlea matures, particularly in the context of damage paradigms.

Shh signaling

The Shh signaling pathway is essential for various aspects of organogenesis, including growth and patterning (Varjosalo and Taipale, 2008). In a simplified model, the secreted ligand Shh binds its receptor patched 1, resulting in the derepression of another membrane protein, smoothened (Smo), in target cells. This in turn activates the Gli transcription factors, which are the effectors of Shh signaling, leading to the expression or repression of Shh target genes.

Prior to hair cell differentiation in the developing mouse cochlea, *Shh* is expressed by spiral ganglia neurons (Liu et al., 2010). The subsequent expression of *Shh* is dynamic, and Shh levels decrease from basal to apical neurons, with the overall level of expression further declining to undetectable levels around birth. This spatiotemporal pattern closely matches that of hair cell differentiation in the cochlear duct, raising the hypothesis that Shh regulates the timing of hair cell differentiation. This is indirectly supported by the finding that loss of the spiral ganglia in *Neurod1*$^{-/-}$ and *Neurog1*$^{-/-}$ mice leads to premature cell cycle exit and hair cell differentiation in the cochlear duct (Jahan et al., 2010; Matei et al., 2005).

Several elegant studies have been undertaken to characterize the role of Shh signaling in governing prosensory cell formation. Both *Gli3*-deficient cochlea and cochlear explants treated with chemical inhibitors of Shh signaling display an expanded prosensory domain, whereas Shh treatment prevents prosensory domain formation (Driver et al., 2008). Using a transgenic approach, it was shown that the ablation of *Shh* from the spiral ganglia at several developmental time points causes premature cell cycle exit and an abnormal wave of hair cell differentiation extending from the apex towards the base, in the opposite direction to normal development (Bok et al., 2013). In another study, Tateya and colleagues demonstrated that the conditional ablation of *Smo* in the developing cochlear epithelium results in accelerated hair cell differentiation in the apical region (Tateya et al., 2013). Conversely, the expression of a constitutively active allele of *Smo* in the cochlear epithelium prevents outer hair cell differentiation, with prosensory cells remaining largely undifferentiated (Tateya et al., 2013). This excessive Shh signaling inhibits hair cell formation by upregulating *Fgf20*; anti-FGF20 antibodies or FGF receptor blockage partially restored hair cell differentiation (Tateya et al., 2013). More recently, it was shown that the ablation of both *Hey1* and *Hey2*, which are both well-characterized Notch target genes, results in premature hair cell differentiation without affecting the proliferation of prosensory cells (Benito-Gonzalez and Doetzlhofer, 2014). This study also demonstrated that Shh signals regulate *Hey1* and *Hey2* expression, suggesting that Shh signaling governs the timing of hair cell differentiation via *Hey1* and *Hey2*, possibly by regulating FGF signaling as an intermediate step.

Although much work has been done to elucidate the roles of Shh signaling during cochlear development, its roles during hair cell regeneration remain largely unknown. One study has demonstrated that Shh treatment of cultured cochlea from neonatal rats inhibits retinoblastoma expression and induces the proliferation of supporting cells after hair cell damage (Lu et al., 2013). The crucial roles of Shh signaling in restricting both proliferation and hair cell differentiation during development, together with cumulative evidence of its interaction with other signaling pathways, should serve as an impetus for additional investigations to delineate the extent of these interactions during development and regeneration.

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Regulation of the cell cycle during hair cell development

As highlighted above, many signaling molecules appear to affect the proliferation and differentiation of cells during cochlear development. Given this intimate relationship between the cell cycle and cell differentiation, the potential roles of cell cycle inhibitors during the development and regeneration of hair cells have been extensively explored (Schimmang and Pirvola, 2013). In the developing mammalian cochlea, prosensory cells enter terminal mitosis and begin expressing the cell cycle inhibitor $p27^{Kip1}$ (Cdkn1b) in a wave extending from the apex towards the base (Chen and Segil, 1999; Ruben, 1967). Germline deletion of $p27^{Kip1}$ leads to ongoing proliferation of organ of Corti cells extending into the postnatal period, when sporadic mitotic events are observed (Chen and Segil, 1999; Lowenheim et al., 1999). However, this overproduction of cells results in a dysfunctional organ, as $p27^{Kip1}$ null mice exhibit hearing loss (Chen and Segil, 1999). In another mouse model, deletion of the cycle inhibitor $p19^{Ink4d}$ (Cdkn2d) similarly leads to prolonged proliferation of organ of Corti cells and to the eventual degeneration of hair cells, again causing hearing loss (Chen et al., 2003). These studies indicate that the expression of cell cycle inhibitors is required for the proper control of hair cell and supporting cell numbers, but not for the differentiation of hair cells.

A major difference between the regenerating avian cochlea and the non-regenerating mammalian counterpart is the ability of supporting cells in the former to restart proliferation upon damage (Warchol and Corwin, 1996) (Fig. 2). Furthermore, in the chicken utricle and the zebrafish lateral line system, hair cells are continuously replenished and new hair cells are generated via supporting cell division and differentiation. In these contexts, mitotic regeneration is increased when hair cells are damaged by treatment with ototoxins such as aminoglycosides (Harris et al., 2003; Hernández et al., 2007; Matsui et al., 2000). Conversely, in the mature mammalian organ of Corti, no spontaneous cell cycle re-entry of the supporting cells is observed (Fig. 3). This differential response to damage is postulated to be one of the reasons why the mature mammalian cochlea is unable to regenerate hair cells after damage.

Supporting cells in both the neonatal and mature mammalian cochlea are mitotically quiescent. When $p27^{Kip1}$ is acutely ablated *in vivo* or suppressed *in vitro*, supporting cells in the neonatal cochlea proliferate but do not acquire a hair cell fate (Liu et al., 2012c; Maass et al., 2013; Oesterle et al., 2011). In the mature cochlea, the degree of mitotic response was significantly lower than in the neonatal organ (Liu et al., 2012c; Oesterle et al., 2011). These findings suggest that $p27^{Kip1}$ also contributes to the mitotic quiescence of supporting cells in the postnatal period.

Like supporting cells, hair cells are mitotically quiescent. Deletion of $p27^{Kip1}$ from hair cells in the neonatal mouse cochlea allowed cell cycle re-entry and the survival of newly added hair cells into the mature organ without compromising hearing function (Walters et al., 2014). This phenotype contrasts with that observed following deletion of retinoblastoma (*Rb1*) (a tumor suppressor gene downstream of $p27^{Kip1}$) or co-deletion of $p19^{Ink4d}$ and $p21^{Cip1}$ (Cdkn1a), in which proliferating hair cells apoptosed with ensuing hearing loss (Laine et al., 2007; Sage et al., 2005, 2006; Weber et al., 2008). Although the effect of blocking cell cycle inhibitors in the damaged cochlea is unclear, these studies posit $p27^{Kip1}$ as another potential target to induce supporting cell divisions prior to the differentiation of regenerating hair cells. Alternatively, one tantalizing route to induce regeneration is to directly renew the proliferative capacity of surviving hair cells, a regenerative
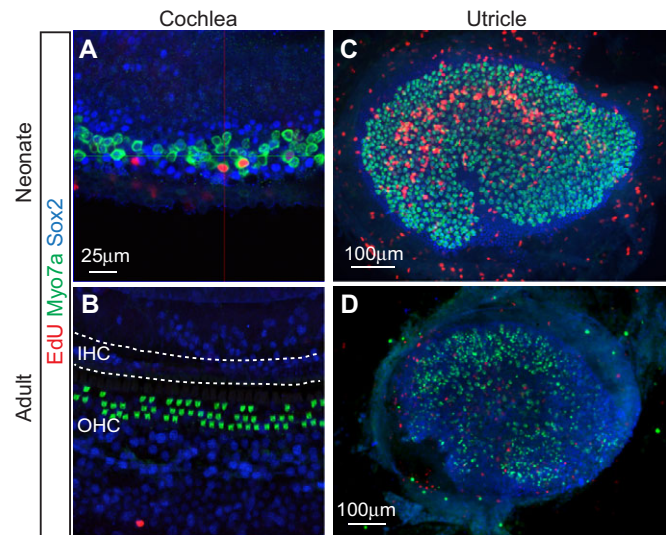

Cochlea   Utricle

Neonate   Adult

EdU Myo7a Sox2

A   C
25μm   100μm

B   D
IHC   100μm
OHC

Fig. 3. Comparison of the modes and capacity of hair cell regeneration in the neonatal and adult mouse cochlea and utricle. (A) Limited mitotic hair cell regeneration occurs in the neonatal cochlea following diphtheria toxin-induced damage. Shown is the apical turn of the cochlea from P7 $Pou4f3^{DTR/+}$ mice, which received diphtheria toxin at P1 to ablate hair cells. Immunostaining for myosin VIIa (green) highlights hair cells, while Sox2-positive supporting cells are in blue. The presence of hair cells labeled with EdU (red) indicates mitotic hair cell regeneration. Image courtesy of R. Chai. (B) Conversely, no mitotic response (EdU labeling) or hair cell regeneration occurs in the damaged adult cochlea. The image shows a cochlea from $Pou4f3^{DTR/+}$ mice that received diphtheria toxin one week previously. There is a complete loss of inner hair cells (IHC) and a partial loss of outer hair cells (OHC; green). (C) By contrast, robust mitotic hair cell regeneration (as indicated by the abundance of EdU-labeled hair cells) is observed in the neonatal utricle. The image is of a utricle from P30 $Pou4f3^{DTR/+}$ mice that received diphtheria toxin at P1 to ablate hair cells. Image courtesy of R. Chai and T. Wang. (D) Scant proliferation and regeneration are observed in the damaged mature utricle. Shown is a utricle from an adult mouse previously treated with a vestibulotoxin.

mechanism that is exhibited by differentiated cell types in other end-organs (Dor et al., 2004; Georgia and Bhushan, 2004; Porrello et al., 2011).

Age-dependent changes to regenerative capacity: implications for hair cell regeneration

A number of organs and tissues exhibit age-related changes to their regenerative capacity. For example, in the immature heart and lungs of neonatal mice, injury is met with a robust degree of regeneration/repair, but this phenomenon decreases with age (Jesty et al., 2012; Paxson et al., 2011; Porrello et al., 2011). Neural stem cells also decrease in number as the animal ages (Maslov et al., 2004), and this decrease in the stem cell reserve is implicated in the decline in tissue repair seen with aging (Jin et al., 2004). In the inner ear, a loss of competence to respond to multiple signaling pathways occurs as the cochlea matures (Walters and Zuo, 2013). As discussed above, this difference in responsiveness between the neonatal and adult cochlea is observed when overexpressing *Atoh1*, activating Wnt signaling, or manipulating Notch signaling and $p27^{Kip1}$. When hair cells are damaged in the embryonic or neonatal immature cochlea, spontaneous hair cell replacement has been observed (Bramhall et al., 2014; Cox et al., 2014; Kelley et al., 1995). Remarkably, damage in the neonatal cochlea results in a limited degree of proliferation among supporting cells and in mitotic hair cell regeneration (Bramhall et al., 2014; Cox et al., 2014). This body of work illustrates that the mature cochlea is less capable than the neonatal organ of proliferating and forming new hair cells. In parallel,

the mitotic response and degree of hair cell regeneration observed in the immature neonatal utricle are also more robust than in the mature adult organ (Burns et al., 2012; Golub et al., 2012) (Fig. 3).

Although our insights into the mechanisms responsible for such differences in responsiveness to gene manipulations in the neonatal and mature inner ear organs are limited, organs of these ages do display rather different gene expression profiles (reviewed by Walters and Zuo, 2013) and epigenetic differences, such as those at Sox2 enhancers (Waldhaus et al., 2012). In light of the important roles of epigenetic regulation in cellular senescence in other organs, one may postulate that epigenetic changes play a role in suppressing the expression of hair cell genes in supporting cells, possibly limiting the efficacy of reintroducing *Atoh1*. Thus, more studies are urgently needed to further elucidate epigenetic changes in neonatal and adult sensory organs, especially after damage.

Numerous studies have also closely probed for the existence of hair cell progenitors in the neonatal cochlea. Although cells from the neonatal mouse organ of Corti are mitotically quiescent, cochlear cells isolated from this stage can behave as progenitor cells by proliferating and forming new hair cells *in vitro* (Oshima et al., 2007; Savary et al., 2007; White et al., 2006). However, the quantity of colony-forming cells rapidly decreases from the neonatal to adult period (Oshima et al., 2007; White et al., 2006), implying a loss of stem cells and/or their accompanying niche. Several groups have used various defined markers to segregate distinct populations of cells in the neonatal cochlea (Chai et al., 2012; Jan et al., 2013; Oshima et al., 2007; Savary et al., 2007; Shi et al., 2012; Sinkkonen et al., 2011; White et al., 2006). Together, these studies suggest that *Lgr5*, which marks somatic stem cells in self-renewing organs (Barker et al., 2007; Jaks et al., 2008), is a promising enrichment marker for supporting cells that behave as hair cell progenitors *in vitro*. Specifically, isolated Lgr5-positive cells proliferate and form more hair cell-like cells than Lgr5-negative cells (Chai et al., 2012; Shi et al., 2012). Moreover, after damage *in vitro* and *in vivo*, Lgr5-marked supporting cells in the neonatal cochlea are capable of regenerating hair cells via a mitotic mechanism (Bramhall et al., 2014; Cox et al., 2014). Despite these promising findings that hair cells can indeed be regenerated via a mitotic mechanism, these cells undergo delayed cell death (Cox et al., 2014). Although informative as proof-of-principle experiments that putative hair cell progenitors exist in the neonatal cochlea, these studies have left several questions unanswered: which factors confer the competence to neonatal Lgr5-positive cells to mitotically regenerate hair cells; which survival factors are missing in these short-lived regenerated hair cells; and what is the function of Lgr5-positive cells in the adult, mature cochlea where regeneration does not occur? Below, we propose some possible explanations and approaches that could be used to tackle these open questions.

Future perspectives
Exploring the intersections between signaling pathways
Our understanding of the signaling mechanisms regulating cochlear development has leapt forward in recent years, aided by numerous studies characterizing the context-dependent roles of each signaling pathway. The availability of genetic tools has enabled us to further dissect components of these pathways. Moreover, these studies have begun to reveal the complex interplay that exists among different pathways at different developmental stages, ranging from otic placode specification (Jayasena et al., 2008) and sensory cell specification (Benito-Gonzalez and Doetzlhofer, 2014; Bok et al., 2013; Munnamalai et al., 2012; Tateya et al., 2013) to the maintenance of cell fate (Doetzlhofer et al., 2009; Li et al., 2015). As insights into

the roles of additional pathways in these diverse contexts culminate (e.g. TGFβ, Hippo, microRNAs, retinoic acid, insulin growth factor), the extent to which they interact with one another is likely to prove extensive. Understanding these interactions is a challenging but important next step, as the concurrent or sequential manipulation of several pathways is more likely to be fruitful as we advance towards the goal of regenerating hair cells in the adult mammalian cochlea. The manipulation of individual pathways has already been shown to exert multiple effects (e.g. proliferative and prosensory roles of the canonical Wnt pathway), so titrating the activities of several signaling pathways will undoubtedly bring additional layers of complexity to future investigations.

Akin to the approaches used to guide the differentiation of embryonic stem cells and induced pluripotent stem cells, directing adult mammalian cochlear supporting cells to proliferate and regenerate hair cells is likely to involve the rekindling and orchestration of multiple developmental cues. The findings of studies that have probed the dynamics of gene expression during regeneration, such as those in the chicken inner ear organs and the zebrafish lateral line, have already illustrated such multiplex changes during the different phases of regeneration (Alvarado et al., 2011; Hawkins et al., 2007; Jiang et al., 2014; Ku et al., 2014; Steiner et al., 2014). As we gain knowledge about the transcriptome of the non-regenerating mammalian cochlea, we can begin to dissect its gene expression patterns and contrast them with those seen in regenerating sensory organs. We might also be able to decipher whether differences exist between the mammalian cochlea immediately and long-term after damage.

Cellular reprogramming
Another novel approach used in regenerative medicine is *de novo* cellular reprogramming, or 'direct reprogramming', using defined factors. The introduction of such putative reprogramming factor(s) has met with success in regenerating cardiomyocytes (Efe et al., 2011; Ieda et al., 2010; Jayawardena et al., 2012; Qian et al., 2012) and neurons (Guo et al., 2014; Kim et al., 2011; Niu et al., 2013; Vierbuchen et al., 2010). An analogous method to regenerate hair cells has also been developed using Atoh1 as the sole reprogramming factor, which, as mentioned above, yielded limited success in preclinical models of hearing loss. Nonetheless, an active clinical trial, in which an adenoviral vector carrying *Atoh1* is directly inoculated into the inner ear, is still under way (ClinicalTrials.gov identifier: NCT02132130). Its results should reveal, first and foremost, whether such an approach is safe and, second, whether it is effective at promoting hair cell regeneration.

Other transcription factors that are crucial for hair cell development have also proven to be useful for generating new sensory cells (Ahmed et al., 2012; Masuda et al., 2012). For example, Ahmed and colleagues showed that the combined use of *Eya1* and *Six1* induces a hair cell fate in Sox2-expressing non-sensory cells in embryonic cochlear explants (Ahmed et al., 2012; Masuda et al., 2012). Interestingly, myosin VIIa-positive hair cells can be generated through Atoh1-dependent and -independent pathways, providing support for the existence of putative hair cell reprogramming factors aside from Atoh1. Additionally, transcription factors that are upregulated or downregulated during regeneration in non-mammalian sensory organs might prove to be valuable candidate reprogramming factors.

Possible differences between development and regeneration
In addition to the known and aforementioned similarities and differences in the functions of individual signaling pathways during
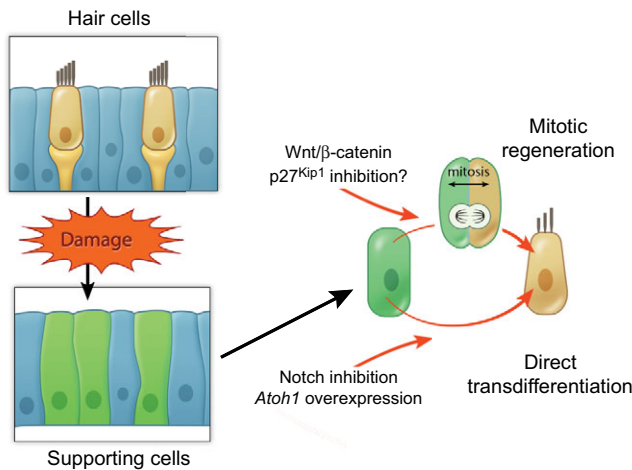
Fig. 4. Proposed mechanisms of hair cell regeneration. Supporting cells can classically regenerate hair cells via two mechanisms: mitotic hair cell regeneration and direct transdifferentiation. Enhancing the Wnt/β-catenin signaling pathway or inhibiting cell cycle inhibitors such as p27^{Kip1} may constitute approaches to enhance mitotic regeneration. By contrast, Notch inhibition and *Atoh1* overexpression may promote the direct conversion of supporting cells into hair cells.

prosensory signals (e.g. Notch and Atoh1) can drive the formation of sensory cells in regions outside the prosensory domain, possibly facilitating the characterization of novel factors that confer competence to generate sensory cells. Lastly, knowledge gained from analyses of hair cell progenitors in other species and the identification of mammalian progenitors in the immature cochlea and vestibular organs should accelerate additional discoveries on the path towards regenerating the mammalian cochlea.

development and regeneration, it is important to emphasize two major distinctions between the two processes.

The first concerns the timing of mitosis and hair cell formation. During development, prosensory cells enter terminal mitosis prior to differentiating into hair cells, yet the timing of these two events differs dramatically along the length of the cochlea, with the prosensory cells in the apical turn being the first to stop dividing and the last to change fate. By contrast, mitotic regeneration from supporting cells in avian inner ear tissues and the zebrafish lateral line system is much more synchronized. If a two-step strategy were employed (i.e. by first stimulating proliferation via Wnt activation or by inhibiting a cell cycle inhibitor, and then promoting hair cell induction through Notch inhibition or Atoh1 overexpression; Fig. 4), it will probably be necessary to tailor the timing of the two steps (and of other factors) to regeneration.

The second distinction relates to hair cell differentiation, maturation and survival. After overcoming the challenge of inducing proliferation and a cell fate change among supporting cells in the damaged, mature mammalian cochlea, another obstacle may arise from the lack of hair cell maturation and survival cues that are native to the embryonic and neonatal cochlea. This difference might be attributed to a deficient progenitor cell response to damage. For example, regenerating hair cells in the neonatal cochlea lack the survival factor Pou4f3 and undergo delayed cell death (Cox et al., 2014). This difference might also stem from several other structural and/or physiological changes to the developing cochlea (e.g. the loss of the greater epithelial ridge, establishment of the tectorial membrane and endocochlear potential, a decrease in the number of tympanic border cells), which may also influence the maturation and survival of hair cells.

Conclusions

In recent years, tremendous progress has been made in revealing the complex mechanisms involved in the development of the mammalian cochlea. These studies have helped to define the functions of individual signaling pathways at different stages of cochlear development, and also the complex interplay among them. In addition, gain-of-function studies have identified that

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