

## Chapter 2

# Development and Regeneration of Sensory Hair Cells

Matthew W. Kelley and Jennifer S. Stone

**Abstract** Hair cells are sensory receptors for hearing and balance, and for detection of water movement in aquatic animals. In mammals, the vast majority of hair cells are formed during embryogenesis and early postnatal development, whereas in other vertebrates hair cells are formed throughout life. Destruction of hair cells is caused by genetic, environmental, or aging factors and results in sensorineural deficits that are irreversible in humans. Research in the 1980s demonstrated that non-mammalian vertebrates fully replace hair cells after damage and recover function, suggesting hair cell regeneration may someday be coaxed in humans as a treatment for some forms of hearing and balance deficits. To facilitate this possibility, subsequent studies explored the molecular and cellular bases of hair cell formation during development and after damage in mature animals. This chapter reviews the findings in each of these areas, describing similarities and differences across species, sensory organs, and age. For instance, while mature mammals have a limited innate ability to regenerate hair cells in the vestibular inner ear, no hair cells are replaced in the cochlea. Further, although the transcription factor (*Atoh1*) drives cells toward a hair cell fate during development in all types of animals and in nonmammals after damage, it has limited ability to promote hair cell regeneration in mature mammals. Finally, we discuss some of the hurdles that remain, as well as new technologies that may be used to move the field forward.

**Keywords** Development • Hair cell • Inner ear • Lateral line • Molecular regulation • Regeneration • Supporting cell

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## Abbreviations

<i>Atoh1</i>	Atonal homolog 1
E	Embryonic day
HMG	High mobility group
<i>Lfn</i>	Lunatic fringe
mTOR	Mechanistic target of rapamycin
P	Postnatal day
PI3K	Phosphoinoside-3 kinase

## 2.1 Introduction

Hair cells are sensory receptors located in specialized sensory epithelia in the inner ear of all vertebrates and along the body surfaces of aquatic vertebrates. They transduce biologically relevant stimuli into signals that are used to avoid predators, locate prey, and regulate motor activities, including vocalizations and locomotion. Hair cells in auditory organs respond to sound waves and enable hearing, while those in vestibular organs encode head movements and help to maintain posture, coordinate active motions, and stabilize gaze. In aquatic vertebrates, hair cells are also located along the body surface in lateral line sensory organs called neuromasts. These organs sense water movements, enabling aquatic animals to determine current direction and sense predators.

All hair cell epithelia are composed of three cellular elements: hair cells, nonsensory supporting cells that resemble glia in many respects, and peripheral fibers of the VIIIth cranial (cochleovestibular) nerve. The number of hair cells in each type of organ varies substantially within and across species. For example, some fish sacculi contain hundreds of thousands of hair cells, while each zebrafish (*Danio rerio*) neuromast has around 10. In addition, the morphology and function of hair cells vary within and between organs. For instance, in mammals, cochlear hair cells are classified as “inner” or “outer” based on anatomical and physiological distinctions, while vestibular organs comprise type I and type II hair cells identified using similar criteria. In contrast, the criteria used to define supporting cells are limited, but they are typically elongated cells that extend from the lumen to the basal lamina. They provide structural and physiological support to hair cells and neurons and generate the overlying and underlying extracellular matrices of the sensory epithelium. Proportions of supporting cells to hair cells are approximately 1:1 in some organs (e.g., mouse utricle, Desai et al. 2005) but higher in other organs (e.g., chicken auditory epithelium, Goodyear and Richardson 1997). Peripheral fibers leading to and from the brain are distributed throughout most sensory epithelia and synapse on hair cells, although efferent fibers are limited with respect to the hair cell subtypes that they contact.

Section 2.2 of this chapter reviews hair cell formation during embryogenesis. Section 2.3 then discusses homeostasis and replacement of hair cells in mature animals. Although mechanisms of hair cell development are largely conserved across vertebrates, the ability to fully replace lost hair cells is confined to nonmammalian vertebrates. However, recent new discoveries indicate that there is partial replacement of hair cells in adult mammalian vestibular organs. Finally, factors that may inhibit hair cell regeneration in mammals, and potential methodologies to overcome those barriers, are discussed.

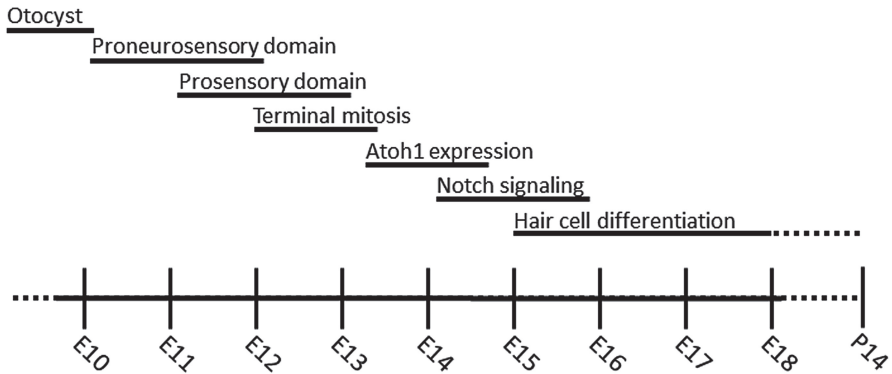
## 2.2 Hair Cell Development

### 2.2.1 *Sensorineural Development in the Inner Ear*

The inner ear is a remarkable structure. Initially derived from an ectodermal placode, individual cells proliferate to generate an appropriate number of cells, which then undergo complex lineage restriction and cell fate determination events to give rise to all of the cell types required to form the inner ear (Wu and Kelley 2012). Inner ear derivatives from the otocyst can be divided into three primary classes of cells: neuronal cells comprising the afferent neurons of cranial nerve VIII, sensory epithelial cells including mechanosensory hair cells and surrounding supporting cells, and nonsensory epithelial cells, which include all remaining otocyst-derived cells. It is important to note that many nonsensory structures such as the stria vascularis, endolymphatic duct, Reissner's membrane, and epithelial linings of the semi-circular canals are essential for inner ear function. However, these structures are not addressed in this chapter.

At the time of closure, the otic vesicle appears largely homogeneous in terms of both cellular and gross morphologies. Consistent with these observations, experiments performed in amphibians in the early 1900s demonstrated that early rotation of the otocyst along any one of the main body axes leads to development of an essentially normal otocyst, suggesting cells in the early otocyst are largely equipotential. However, fairly soon after closure cellular identities along the three body axes become restricted. It has been shown that the dorsal–ventral axis is established through opposing gradients of sonic hedgehog (ventral) and Wnt (dorsal) (Riccomagno et al. 2002, 2005), while the anterior–posterior axis is defined through a gradient of retinoic acid (Bok et al. 2011). For further details see Wu and Kelley (2012).

A key outcome of axis specification is the formation of a proneurosensory cell population in the anteroventral region of the otocyst (Raft et al. 2007) (Fig. 2.1). Understanding of the molecular basis for the formation of this region is still fairly limited. However, the transcription factor *Tbx1*, which inhibits proneurosensory formation, is expressed outside of this region (Raft et al. 2004). Disruption of *Tbx1*



**Fig. 2.1** Timeline of hair cell development in the mouse. The timing of specific events in the progression of cells from uncommitted neuroepithelial cells in the otocyst through several developmental restrictions en route to forming mature hair cells is illustrated

expression or function leads to increased neural and sensory structures at the expense of nonsensory regions of the inner ear.

Several markers of the proneurosensory region have been identified, including *Sox2* and *Lfng* (Morsli et al. 1998; Dabdoub et al. 2008). Otocyst-specific deletion of *Sox2* leads to loss of both neural and sensory structures, demonstrating the importance of this transcription factor in formation of the proneurosensory derivatives of the ear (Kiernan et al. 2005b; Dabdoub et al. 2008). In contrast, *Lfng* mutants are phenotypically normal, suggesting either a nonessential role in inner ear development or, possibly, functional compensation by one of two other *fringe* genes, *radical* and *manic* (Zhang et al. 2000).

Following specification of the proneurosensory domain, a subset of cells within this region becomes further determined toward a neural lineage (Ma et al. 2000; Appler and Goodrich 2011). These cells then undergo an epithelial-to-mesenchymal transition, delaminate through the basement membrane of the otocyst, and migrate a short distance medially toward the hindbrain. These statoacoustic neuroblasts divide extensively and then differentiate as auditory and vestibular neurons. Cells that remain within the proneurosensory region develop as prosensory cells (Brooker et al. 2006).

As development proceeds, the prosensory cells separate and redistribute to give rise to the sensory patches of the inner ear (Fig. 2.1). At present, three factors are known to be necessary and sufficient for formation of the prosensory domains. The first is *Sox2*, a high-mobility group (HMG) transcription factor known for its role in stem cell induction but that also plays a key role in the formation of neural progenitor cells (Zhang and Cui 2014; Takahashi and Yamanaka 2016). Two other factors that seem to play similar roles in specification of prosensory cells are the transcription factor *Eya1* and its binding cofactor *Six1* (Xu et al. 1999; Zheng et al. 2003). Mutations in either *Eya1* or *Six1* lead to branchial-oto-renal (BOR) syndrome, which cause defects in inner ear formation and hearing, and targeted deletion of

either gene results in complete absence of sensory epithelia. *Eya1* is strongly expressed within the prosensory domain beginning at a relatively early stage in otocyst development, while *Six1* shows broader expression within the otocyst. More recently, transfection experiments have shown that Sox2, Eya1, and Six1 can bind to and activate specific regions within a highly conserved *atonal homolog 1* (*Atoh1*) enhancer (Ahmed et al. 2012a, b; Kempfle et al. 2016). These results establish key roles for each factor in the onset of expression of *Atoh1*, the most potent known inducer of hair cell fate (Sect. 2.2.2.1). However, it is not clear whether the effects of any or all of these factors are limited to regulation of *Atoh1*.

One study examined this issue by forcing ectopic expression of *Atoh1* in embryonic and early postnatal cochlear cells from *Sox2* mutants (*Sox2<sup>Lcc/Lcc</sup>*, Puligilla and Kelley 2016). No *Atoh1* expression is observed in *Sox2* mutant ears. However, if the only role of Sox2 is to induce expression of *Atoh1*, then ectopic expression of *Atoh1* should be sufficient to induce limited hair cell formation. Interestingly, the results of these experiments indicated no hair cell formation following ectopic expression of *Atoh1* (Puligilla and Kelley 2016). This result suggests that Sox2 has critical roles in hair cell formation beyond its role in transcriptional activation of *Atoh1*. These roles might include activation of progenitor genes that change the state of the prosensory cells and/or epigenetic modifications that allow *Atoh1* to modulate key target genes.

Another intriguing aspect of the role of Sox2 in prosensory development is the observation that Sox2 is downregulated in all auditory hair cells and in type I vestibular hair cells as they differentiate. This pattern of expression is similar to that of the central nervous system, in which *Sox2* is transiently expressed as progenitors transition from highly proliferative blast cells to terminally differentiated neurons (Bylund et al. 2003) and has led to the suggestion that Sox2 can also act as an inhibitor of hair cell differentiation (Dabdoub et al. 2008). However, although the role of Sox2 in promoting *Atoh1* expression has been examined extensively, its inhibitory role remains unclear (Liu et al. 2012b).

The remainder of the discussion of hair cell development refers primarily to the mammalian cochlea. As development proceeds, the number of prosensory cells continues to increase through ongoing proliferation. As discussed in the text that follows, the total number of prosensory cells is tightly regulated, as programmed cell death within the epithelium is minimal and mutations that lead to prolonged proliferation also result in overproduction of hair cells and supporting cells. However, the factors that regulate ongoing proliferation within the prosensory domain are poorly understood. Recent work has demonstrated key roles for fibroblast growth factor 9 (Fgf9) and Fgf20 in the overall level of proliferation in the prosensory domain (Huh et al. 2015). However, this function may be indirect, as Fgf9 and Fgf20 arising from the epithelium appear to signal to adjacent mesenchyme, which then reciprocally influences epithelial proliferation through an undetermined mechanism.

Following prosensory specification, the next key step in formation of the sensory epithelia is terminal mitosis. Within the developing cochlear duct, terminal mitosis of prosensory cells occurs in a highly stereotyped and conserved pattern, beginning in the apex and extending in a gradient toward the base (Ruben 1967; Lee et al.

2006). In mice, apical cells become postmitotic beginning on embryonic day 12.5 (E12.5), with the last basal cells leaving the cell cycle by E14.5 (Fig. 2.1). Recent studies examined the roles of several cell cycle regulatory factors in controlling terminal mitosis in both auditory and vestibular prosensory cells (Schimmang and Pirvola 2013). The cyclin-dependent kinase inhibitor *Cdkn1b* (formerly *p27<sup>kip1</sup>*) plays a key role in the timing of cell cycle exit in the cochlear duct (Chen and Segil 1999). *Cdkn1b* protein and transcripts are expressed in a gradient that foreshadows the apical-to-basal gradient of terminal mitosis. Deletion of *Cdkn1b* leads to prolonged proliferation of prosensory cells, which in turn leads to formation of supernumerary hair cells and supporting cells and hearing loss. The formation of differentiated cell types in *Cdkn1b* mutants indicates that *Cdkn1b* is not essential for terminal mitosis. In fact, prosensory cells are thought to go through only one additional round of proliferation before cell cycle exit. Interestingly, in the absence of *Cdkn1b*, the gradient of terminal mitosis switches from apical-to-basal to basal-to-apical.

### 2.2.2 Initial Differentiation of Hair Cells

The onset of cellular differentiation within the cochlear duct appears to coincide with the arrival of the wave of terminal mitosis in the base (Chen and Segil 1999; Lee et al. 2006) (Fig. 2.1). As discussed in Sect. 2.2.1, differentiation begins near the base and extends both apically and, to a lesser extent, basally toward the future hook region (Rubel 1978). The earliest sign of hair cell development, expression of *Atoh1*, extends from the base of the cochlea toward the apex and hook (Chen et al. 2002; Woods et al. 2004). Expression onset is rapid, with apical progenitors becoming *Atoh1*-positive roughly 24 hours after progenitors in the base. However, the subsequent differentiation of hair cells proceeds more slowly. The first signs of differentiation are observed in basal hair cells around E13, but the extreme apex of the cochlea reaches a similar stage of maturity 2–3 days later. Two aspects of this developmental wave are remarkable. First, in most developing systems, the onset of cellular differentiation is coupled with completion of terminal mitosis. In contrast, in the cochlea, terminal mitosis and differentiation are largely uncoupled. As a result, whereas hair cell differentiation in the base begins soon after terminal mitosis, progenitor cells in the apex become terminally mitotic on E12 but may not differentiate until E16. This discrepancy highlights a second remarkable aspect of this developmental wave: progenitors at the apex of the cochlea remain in an undifferentiated, but postmitotic, state for approximately 4 days. The molecular factors that regulate this inhibition of differentiation are largely unknown, but it has been shown that a secreted molecule, sonic hedgehog (*Shh*), produced by the spiral ganglion, acts as an inhibitor of hair cell formation (Driver et al. 2008; Bok et al. 2013). Moreover, spiral ganglion expression of *Shh* is downregulated in a basal-to-apical gradient that precedes the gradient of differentiation. Deletion of *Shh* from the spiral ganglion or elimination of the ganglion disrupts the gradient of hair cell differentiation, leading

to synchronized hair cell formation along the length of the duct (Ma et al. 2000). The biological benefit of this gradient is unclear, but may play a role in region-specific patterning of hair cells in the cochlea.

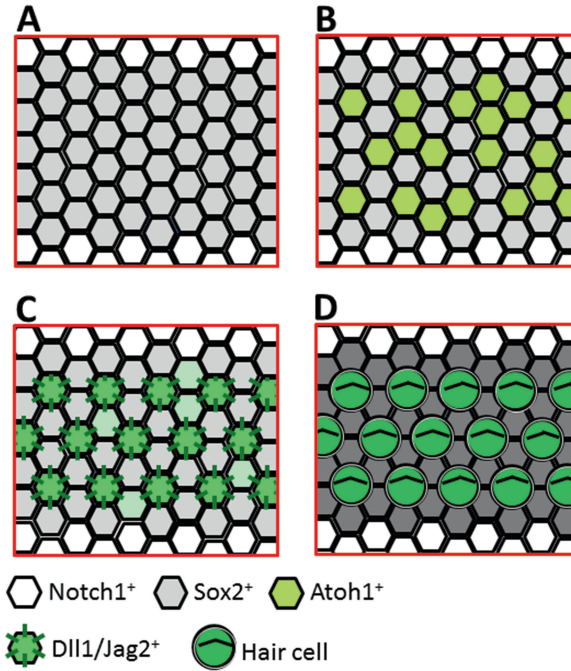
### **2.2.2.1 *Atoh1* Is Necessary and Sufficient for Hair Cell Formation**

The crucial role of *Atoh1* in hair cell formation was first demonstrated through the publication of the inner ear phenotype in *Atoh1* knockout mice (Bermingham et al. 1999). Although these animals die at birth, an analysis of cochleae at E18 indicated a complete absence of hair cells. Subsequent studies in which cells within the nascent cochlear duct were forced to express *Atoh1* demonstrated strong induction of a hair cell fate (Zheng and Gao 2000; Jones et al. 2006). Perhaps two of the best examples of the critical role for *Atoh1* are publications from Kelly et al. (2012) and Liu et al. (2012b) in which transgenic mouse lines were used to induce expression of *Atoh1* in the inner ears of embryos or postnatal animals in vivo. These mice showed extensive conversion of cells into hair cells throughout the organ of Corti, demonstrating that *Atoh1* acts as a powerful inducer of hair cell fate.

### **2.2.2.2 Regulation of Hair Cell and Supporting Cell Fates Through Notch Signaling**

The wave of *Atoh1* expression in the organ of Corti initiates a molecular program that ultimately leads to hair cell differentiation. However, not every cell that expresses *Atoh1* develops as a hair cell (Driver et al. 2013). These observations reveal a complex developmental regulatory mechanism that is still not fully understood (Fig. 2.2). The number of cells that maintain *Atoh1* expression and therefore go on to develop as hair cells is regulated through activation of the notch signaling pathway. This pathway includes notch receptor molecules and a family of ligands referred to as either deltas or jaggeds. Because both ligand and receptor are membrane bound, notch signaling can be activated only between cells in direct contact (reviewed in Palermo et al. 2014). Although the biological effects of notch activation are diverse, the primary outcome of notch receptor activation is inhibition of cellular differentiation. Within the cochlea, all prosensory cells express *notch1* before the onset of *Atoh1* expression (Lanford et al. 1999; Murata et al. 2006). As hair cells begin to develop, they upregulate expression of two notch ligands, *delta1* and *jagged2* (Lanford et al. 1999; Kiernan et al. 2005a). These ligands bind *notch1* and activate several known notch target genes (Lanford et al. 2000; Zine et al. 2001). Genetic deletion of any component of this signaling pathway results in an increase in the number of hair cells, demonstrating that at least one role of notch signaling is the inhibition of hair cell formation (Kiernan et al. 2005a). Moreover, lineage tracing studies have demonstrated that activated notch signaling downregulates *Atoh1* expression, explaining at a mechanistic level how this pathway can influence hair cell fate (Driver et al. 2013). Notch pathway genes are reactivated in hair cell





**Fig. 2.2** Regulation of hair cell development. Shown is a schematic view of the surface of a hypothetical hair cell sensory epithelium. **(A)** All cells express *notch1* (black border around cells), and a subset of these cells has been specified as prosensory through expression of *Sox2* (gray). **(B)** With the prosensory cell population, a limited number of cells initiates expression of *Atoh1* (light green). **(C)** Next, some of the *Atoh1*-positive cells begin to express two notch ligands, delta-like 1 (*Dll1*) and jagged2 (*Jag2*). Binding of these ligands to notch1 in neighboring cells leads to downregulation of *Atoh1* expression and induction of a supporting cell fate. **(D)** In the mature epithelium, cells that maintain expression of *Atoh1* become hair cells, while neighboring cells that have downregulated *Atoh1* develop as supporting cells

epithelia after damage in animals that regenerate hair cells, and inhibition of notch signaling causes overproduction of new hair cells (e.g., zebrafish neuromasts, Ma et al. 2008; chicken auditory epithelium, or basilar papilla, Daudet et al. 2009; and mouse utricle, Lin et al. 2011) (see Sect 2.3.6.2).

### 2.2.2.3 Onset of *Atoh1* Expression

The understanding of the factors that initiate *Atoh1* expression within the prosensory domain remains limited. However, several studies have implicated the Wnt signaling pathway, the transcription factor *Eya1*, and its coactivator *Six1* (Ahmed et al. 2012a; Jacques et al. 2012). Pharmacological activation of canonical Wnt signaling in vitro results in a significant increase in proliferation within the sensory epithelium and formation of supernumerary hair cells (Jacques et al. 2012).



Consistent with this result, genetic inactivation of the Wnt pathway in the developing cochlea beginning near E13 results in a complete absence of *Atoh1* expression and hair cell formation (Shi et al. 2013). Finally, Tcf/Lef, key Wnt targets, bind to the *Atoh1* enhancer in neural progenitor cells (Shi et al. 2010). Taken together, these results are consistent with a role for Wnt in upregulation of *Atoh1*.

BOR syndrome is a genetic disorder associated with mutations in *EYA1* and *SIX1* that includes defects in branchial arch derivatives, inner ear, and kidneys. Ahmed et al. (2012a) examined the roles of *Eya1* and *Six1* through forced expression in cells within Kolliker's organ. Coexpression of *Eya1/Six1* was sufficient to induce hair cell formation and expression of *Atoh1*. Moreover, *Six1* was shown to bind to specific regions within the *Atoh1* enhancer, demonstrating direct regulation.

### 2.2.3 Hair Cell Development Beyond *Atoh1*

Although much attention has been focused on *Atoh1* as the earliest inducer of hair cell formation, *Atoh1* expression marks only the beginning of hair cell development. Two additional transcription factors, *Pou4f3* and *Gfi1*, are both expressed specifically in hair cells with onsets of expression that closely follow that of *Atoh1* (Erkman et al. 1996; Hertzano et al. 2004). Although hair cells still form in mice with targeted deletions of either gene, they rapidly die, demonstrating roles in hair cell survival (Wallis et al. 2003). Forced expression of all three factors drives embryonic stem cells toward a more "hair cell-like" phenotype than *Atoh1* expression alone, suggesting that *Pou4f3* and *Gfi1* may play roles beyond cellular survival (Costa et al. 2015).

Given the crucial role of *Atoh1* in, at least, the early differentiation of hair cells, its downstream targets are of great interest. Studies in cerebellar granule neurons and dorsal spinal cord commissural neurons, two other cell types that depend on developmental expression of *Atoh1*, have used comprehensive profiling methods to define the genes that are specifically regulated by *Atoh1* in those systems (Klisch et al. 2011; Lai et al. 2011). Unfortunately, similar studies have not been completed in the inner ear. However, bioinformatics has been used to identify 233 putative *Atoh1* target genes in developing hair cells, identifying genes whose expression is reduced in mice lacking *Atoh1* and verifying *Atoh1* binding sites in the promoters of such genes (Cai et al. 2015). Although this study did not fully define the targets of *Atoh1*, it provided valuable insights regarding *Atoh1* regulation of hair cell formation.

The time between initial differentiation of a cell as an auditory hair cell (E14 for basal inner hair cells) and maturation of that cell (P14 or possibly later) is protracted. During that time, the cell must develop not only obvious apical and basal specializations, such as a stereociliary bundle and synaptic zones, but also the precise physiologic and anatomic structures required for mechanotransduction. The factors that mediate hair cell differentiation are poorly understood. As discussed in Sect. 2.2.3, *Pou4f3* and *Gfi1* may have a roles in differentiation, but

the rapid death of hair cells in the absence of either gene has made it difficult to pinpoint the role. Additional studies using temporal deletion of either factor may provide clues to the roles of these factors beyond survival. Two secreted molecules that regulate hair cell maturation are thyroid hormone and retinoic acid. Deletion of thyroid hormone receptors leads to marked delays in the overall differentiation of the cochlear duct, including hair cells (Forrest et al. 1996; Rusch et al. 1998). Further, several ion channels fail to develop at the appropriate time in inner hair cells (Rusch et al. 1998; Brandt et al. 2007), while in outer hair cells, expression of *Slc26a5* (prestin) and *Kcnq4* is delayed (Weber et al. 2002; Winter et al. 2006). Unfortunately, other specific targets of thyroid hormone in hair cells have not been identified.

### **2.2.4 Summary of Development**

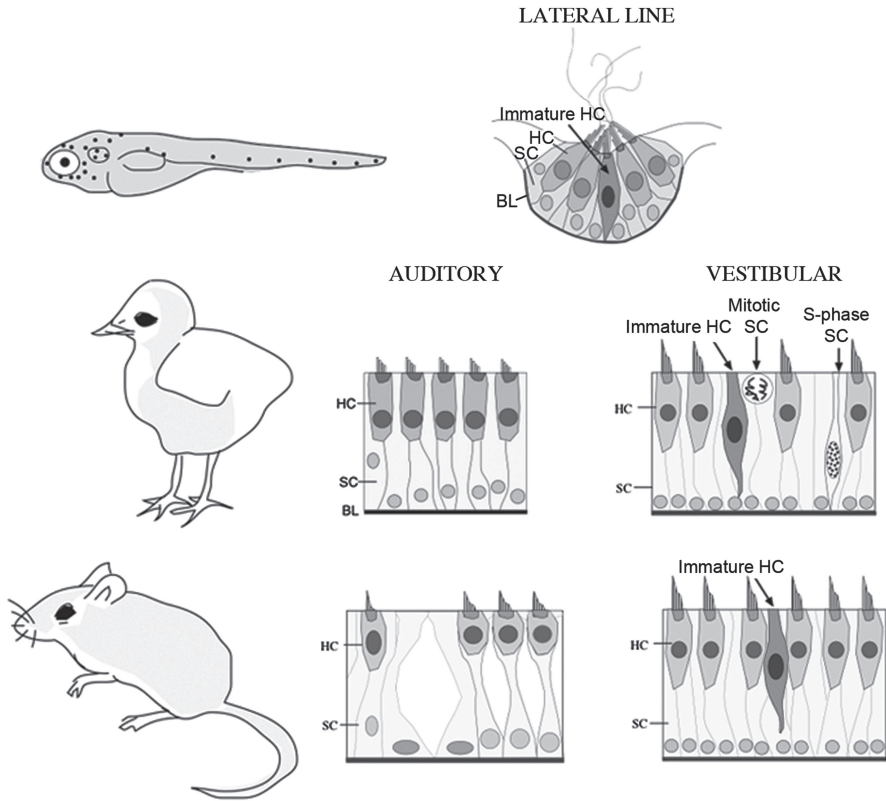
At the most basic levels, hair cell progenitors appear to pass through four stages of specification, first as otic cells, next as proneurosensory cells, then as prosensory cells, and finally as hair cells. At least some of the genetic factors that regulate each step have been identified, with the most attention focused on the final decision to become a hair cell. The identification of *Atoh1* as a key regulator of hair cell fate provided valuable insights regarding the developmental processes that lead to hair cell formation. However, subsequent work demonstrated that *Atoh1* is just part of the answer, and more research is needed before we can fully understand hair cell development.

## **2.3 Hair Cell Regeneration**

### **2.3.1 Overview**

As discussed, previous studies showed that in nonmammalian vertebrates of any age, lost hair cells are replaced through conversion of supporting cells, leading to maintenance or restoration of sensory function (Fig. 2.3). However, the situation is quite different in the cochleae of mature rodents, in which the supporting cells that remain after hair cell loss converge to form a permanent scar, and hair cells are not replaced. Some vestibular hair cells are regenerated in mature rodents after damage, but only a subpopulation is replaced, and the mechanisms for hair cell replacement are different from those used by nonmammals.

This section considers what is known about hair cell replacement in nonmammals and what is currently understood about hair cell regeneration in mammals.



**Fig. 2.3** The structure and regenerative capacity of hair cell epithelia in mammals and nonmammals. Sensory epithelia of vertebrates in the lateral line, auditory organs, and vestibular organs share a common composition of hair cells and supporting cells but vary with respect to their capacity for hair cell regeneration. Nonmammalian vertebrates—fish and birds are shown here—replace all hair cells after damage and also exhibit ongoing hair cell production, even in adulthood. By contrast, the auditory epithelium of mammals (the organ of Corti) cannot replace hair cells after damage, but vestibular epithelia regenerate a subpopulation of hair cells. HC = hair cell; SC = supporting cell; BL = basal lamina

Before proceeding, it is important to clarify a few terms. Hair cell *regeneration* refers to the replacement of hair cells in mature organs following trauma or toxic injury whereas the replacement of hair cells after injury in developing organs is not considered regeneration because organs at this time may retain the ability for repair from embryogenesis. Further, hair cell regeneration is distinct from hair cell *turn-over*, which involves the natural death and addition of hair cells. This section addresses hair cell regeneration, with only minor discussions of hair cell turnover.

### ***2.3.2 Hair Cell Injury, Death, and Repair***

Hair cell damage is a first step in all studies of hair cell regeneration. Acoustic overstimulation can be used to kill hair cells specifically in auditory organs while ototoxins, such as aminoglycoside antibiotics and heavy metals, are toxic to hair cells in all sensory organs. Ototoxins are injected, delivered directly to the labyrinth, or added to cultured organs to avoid system toxicity. Diuretics such as furosemide enhance aminoglycoside damage to the cochlea. Lasers can selectively ablate hair cells, and this method is easily scaled and site directed (Balak et al. 1990; Kelley et al. 1995). Further, genetic variants of mice allow destruction of different hair cell populations (e.g., Fujioka et al. 2011; Golub et al. 2012).

The numbers, types, and positions of hair cells that are destroyed depend on the method of damage, and all methods have off-target effects (e.g., Roberto and Zito 1988; Zheng and Gao 1996). Perhaps most relevant to regeneration, ototoxins can, in certain contexts, damage or kill supporting cells (Kevetter et al. 2000; Slattery and Warchol 2010), and high-level noise can induce migration of nonsensory cells into the sensory region, displacing supporting cells (Cotanche et al. 1995). Hair cell regeneration cannot proceed in these cases because supporting cells serve as progenitors to new hair cells in all species capable of regeneration (see Sect. 2.3.3.1, 2.3.4, and 2.3.5).

Hair cells can repair themselves when damage is mild. In the mammalian cochlea, moderate noise treatments result in degenerative changes in stereocilia (Mulroy and Whaley 1984), but bundles are subsequently repaired (Wang et al. 2002). Similar bundle repair occurs after direct mechanical injury in cultured organs of Corti (Sobkowicz et al. 1996) or after aminoglycoside treatments in vestibular organs (Zheng et al. 1999a; Gale et al. 2002). By contrast, when damage is severe, hair cell corpses are ejected or decompose within the epithelium, and cellular debris is cleared by macrophages and/or supporting cells (e.g., Jones and Corwin 1996; Abrashkin et al. 2006). The factors that tip a damaged hair cell from repair to cell death remain to be determined.

### ***2.3.3 Hair Cell Regeneration in Nonmammalian Vertebrates***

The first definitive demonstrations of hair cell regeneration after damage in homeothermic animals (birds) were published in the late 1980s. Proof of regeneration came in three forms: signs of embryonic-like hair cells in the region of damage (Cotanche 1987a); recovery of hair cell numbers over time (Cruz et al. 1987); and incorporation of a nucleotide analog, tritiated thymidine, into the DNA of hair cells and supporting cells, indicating that new cells arise through mitosis (Corwin and Cotanche 1988; Ryals and Rubel 1988). Subsequent studies showed hair cell regeneration in other nonmammalian vertebrates, including inner ears of reptiles, amphibians, and

fish (e.g., Baird et al. 1993; Lombarte et al. 1993; Avallone et al. 2003) and in lateral line neuromasts of amphibians and fish (Balak et al. 1990; Song et al. 1995).

Remarkably, all nonmammalian vertebrates studied to date can restore moderately damaged hair cell epithelia to original status, with only minor imperfections. Importantly, accessory structures that are also required for inner ear function, such as the tectorial membrane in the auditory organ or the otolithic membrane and cupulae in vestibular organs, are also repaired (e.g., Cotanche 1987b). As a result of this response, nonmammalian vertebrates recover function of hearing, balance, and lateral line systems throughout life (reviewed in Bermingham-McDonogh and Rubel 2003; Ryals et al. 2013).

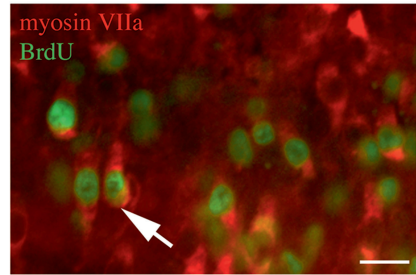
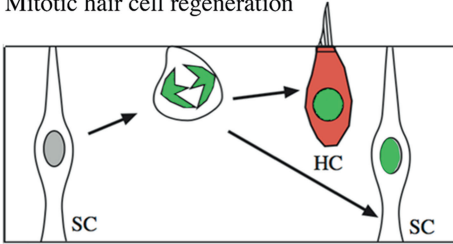
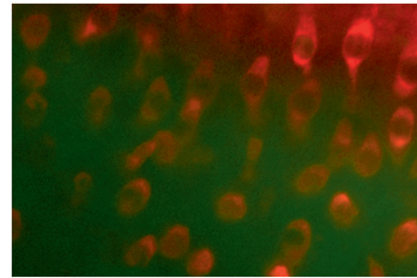
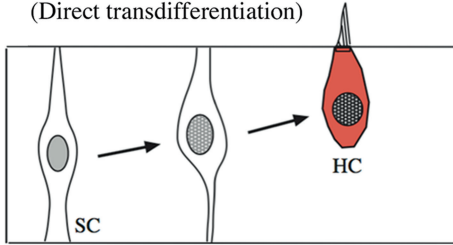
### **2.3.3.1 Hair Cell Progenitors in Nonmammalian Vertebrates: Identity and Behavior**

Supporting cells are the hair cell progenitors in avian inner ear epithelia (Fig. 2.4) (e.g., Hashino and Salvi 1993; Stone and Cotanche 1994). In the chicken basilar papilla, significant hair cell loss triggers supporting cell proliferation within 2 days after the onset of damage (e.g., Raphael 1992; Warchol and Corwin 1996). The extent and duration of supporting cell division are positively correlated with the amount of hair cell loss, and supporting cells stop dividing when new hair cells populate the damaged area. These findings suggest that the loss of hair cells enables supporting cells to exit growth arrest and divide, and replacement hair cells provide negative feedback to halt cell division.

In contrast to the basilar papilla, hair cells in vestibular organs and neuromasts of nonmammals undergo turnover throughout life (Jørgensen and Mathiesen 1988; Williams and Holder 2000). Therefore, there is always a small degree of supporting cell division in vestibular and neuromast organs. Upon hair cell loss, additional supporting cells are recruited to enter the cell cycle (Weisleder and Rubel 1993; Williams and Holder 2000) (Fig. 2.5). In birds, the time course of supporting cell proliferation following damage is similar in vestibular and auditory organs when lesions are comparable in scale (compare Stone et al. 1999 with Ku et al. 2014). Periods of increased supporting cell division following hair cell destruction are shorter in aquatic animals (e.g., Balak et al. 1990; Harris et al. 2003).

Although cell division is always observed in response to severe hair cell injuries, it is not required for hair cell regeneration in nonmammals. Approximately half of the hair cells regenerated in chicken basilar papillae are not labeled with a proliferation marker when it is continually infused after damage (Roberson et al. 1996, 2004), indicating new hair cells can arise without division. Moreover, hair cells are still regenerated in chickens, frogs, and salamanders when cell division is blocked (e.g., Adler et al. 1997; Baird et al. 2000; Taylor and Forge 2005). Finally, cells with phenotypes intermediate between those of supporting cells and hair cells have been observed in frogs and birds (Steyger et al. 1997; Cafaro et al. 2007). These studies indicate that some supporting cells convert into hair cells without dividing, a process called direct transdifferentiation (Fig. 2.4).

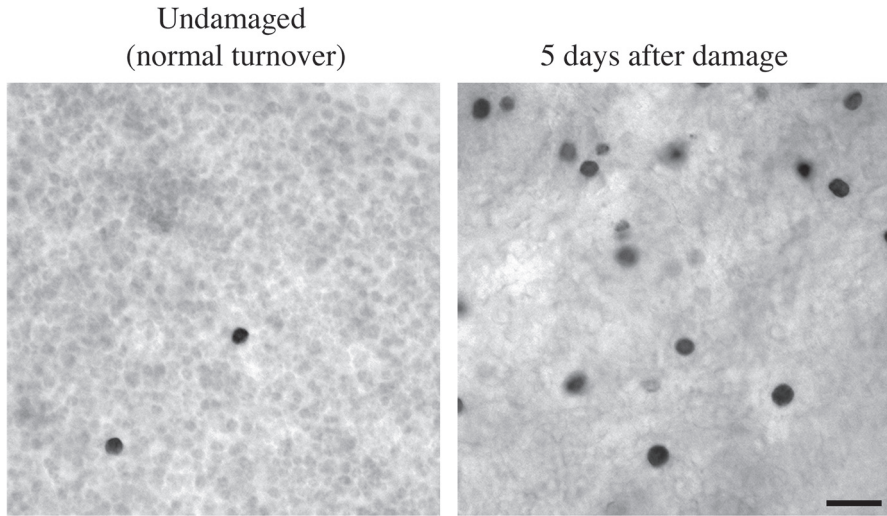
## Mitotic hair cell regeneration

Non-mitotic hair cell regeneration  
(Direct transdifferentiation)

**Fig. 2.4** Hair cells are regenerated using two distinct cellular processes. The two modes of hair cell regeneration in nonmammalian vertebrates are shown schematically in the *left panels* and in color digitized images from the regenerating chicken basilar papilla in the *right panels*. *Top panels*: Cells formed by mitosis are marked by BrdU (green), which is taken up by dividing supporting cells during DNA duplication and distributed to daughter cells during mitosis. Regenerated hair cells are labeled with antibodies to myosin VIIa (red). Hair cells formed by mitosis have red cytoplasm and green nuclei (arrow). *Bottom panels*: Regenerated hair cells formed by direct transdifferentiation lack BrdU because no cell division (DNA synthesis) occurs. Scale bar = 10  $\mu$ m

It is not clear why a given supporting cell would undergo mitotic versus nonmitotic regeneration. In addition, it remains to be determined if all supporting cells have the capacity to form new hair cells or if there are subsets of supporting cells with different intrinsic features, such as stem cells, committed progenitors, postmitotic hair cell precursors, or terminally differentiated cells (Morest and Cotanche 2004). Several studies suggest there is a renewable progenitor cell population in mature hair cell epithelia. For instance, hair cells are regenerated after multiple successive killings in the avian basilar papilla (Niemic et al. 1994) and in zebrafish neuromasts (Cruz et al. 2015). Further, when supporting cells are isolated and grown in culture, they can be passaged multiple times and retain the ability to differentiate into hair cells and supporting cells (Hu and Corwin 2007). However, outcomes of supporting cell divisions in birds and fishes *in vivo* are variable and appear to be spatially segregated in some cases, suggesting progenitors with different developmental potentials are present among supporting cells (Stone and Rubel 2000; Romero-Carvajal et al., 2015). In zebrafish neuromasts, supporting cells that divide and yield new supporting cells are enriched in the periphery of the organ, while divisions leading to two hair cells are enriched in the central zone (Romero-Carvajal





**Fig. 2.5** Supporting cell division is increased in the chicken utricle following hair cell loss. Dividing supporting cells, with their nuclei marked by the proliferation marker BrdU (black), are evident in the undamaged utricle from a post-hatch chicken (*left*) due to normal hair cell turnover. Numbers of dividing supporting cells increase significantly in utricles during the 5 days after streptomycin-induced hair cell destruction (*right*). Scale bar = 10  $\mu$ m

et al. 2015). Similarly, supporting cells in the neural half of the chicken basilar papilla are more likely to divide after hair cell damage, while those in the abneural half are biased toward direct transdifferentiation (Cafaro et al. 2007). Although these findings are consistent with specialized populations of supporting cells in hair cell epithelia, it is likely that spatially distributed signals also influence cell fate during regeneration.

### 2.3.3.2 Morphological and Functional Maturation of Regenerated Hair Cells in Nonmammalian Vertebrates

Maturation of regenerated hair cells in nonmammals has been most extensively studied in birds. Using electron microscopy and immunolabeling, investigators determined that new hair cells begin to populate the damaged auditory epithelia 3–5 days after damage (e.g., Girod et al. 1989; Ryals and Westbrook 1994). Stereociliary bundles of regenerated hair cells grow and establish proper sizes, shapes, and orientations (e.g., Cotanche and Corwin 1991; Duckert and Rubel 1993) before becoming mechanosensitive (Masetto and Correia 1997; Correia et al. 2001). Finally, synaptic connections are reestablished with peripheral nerves (Duckert and Rubel 1993; Ryals and Westbrook 1994).

In birds, several weeks are required to restore a full complement of hair cells, but it takes several months for sensory epithelia to reach full histological maturation



(e.g., Duckert and Rubel 1990; Dye et al. 1999). Once sensory epithelia and accessory tissues are repaired or regenerated, nonmammalian vertebrates recover auditory or vestibular function, even after extensive hair cell loss (reviewed in Bermingham-McDonogh and Rubel 2003; Ryals et al. 2013).

### ***2.3.4 Hair Cell Regeneration in Mammals: The Cochlea***

Following the discovery of hair cell regeneration in nonmammals, investigators began to examine whether any hair cells are naturally regenerated in the mammalian cochlea. In mice and other rodents, developmental addition of cochlear hair cells ceases before or very soon after birth (e.g., Ruben 1967; Chen and Segil 1999). During the late embryonic and early postnatal periods, there is some plasticity in the organ of Corti, with small numbers of hair cells replaced after damage. For example, Kelley et al. (1995) used laser ablation to remove nascent hair cells in cultured organs of Corti from mouse embryos. They found that surrounding cells (either supporting cells or uncommitted precursor cells) differentiated into hair cells without dividing, and that a similar, but much less robust, replacement could also occur in neonatal mice. Consistent with this, a few hair cell-like cells regrow in neonatal rats after aminoglycoside-induced hair cell damage (Romand et al. 1996). Fate mapping of supporting cells in the neonatal mouse cochlea demonstrated that the immature hair cells that arise after damage are derived from supporting cells (Cox et al. 2014). Replacement potential also varies regionally, with the cochlear apex having the greatest capacity, perhaps because it is the least developmentally mature region in the organ of Corti at the time of hair cell ablation.

Even this minimal degree of regeneration is transient because cochlear supporting cells lose their ability to divide and form new hair cells within 2 weeks after birth, both in vivo and in vitro (Cox et al. 2014; White et al. 2006). As a result, auditory hair cells are not regenerated in mature mammalian cochleae after damage (e.g., Raphael and Altschuler 1991; Roberson and Rubel 1994).

### ***2.3.5 Hair Cell Regeneration in Mammals: Vestibular Organs***

Contrary to the auditory system, a limited number of hair cells are regenerated in vestibular organs of mature rodents and, possibly, humans. Using tritiated thymidine as a tracer, Warchol et al. (1993) determined that small numbers of supporting cells divide in utricles from guinea pigs and humans after aminoglycoside treatment in culture. Similar observations were made in vestibular epithelia of other rodents after hair cell damage in vivo (Rubel et al. 1995; Li and Forge 1997). These results, although significant, contrasted markedly with observations nonmammalian utricles in which hundreds of supporting cells incorporate the mitotic tracer bromodeoxyuridine (BrdU) after streptomycin treatment (Ku et al. 2014). Despite limited

new cell production, cells with features typical of developing hair cells—small cell bodies or stereocilia bundles that were short and/or had a centrally located kinocilium—were detected in vestibular organs of adult rodents after damage, and numbers of these cells increased over time (Forge et al. 1993; Kawamoto et al. 2009; Golub et al. 2012). Most recently, Taylor et al. (2015) presented evidence for immature-appearing hair cells in utricles harvested from adult humans, suggesting some regeneration may also occur in primates.

One of the challenging aspects of studying vestibular hair cell regeneration in mammals is variable resistance of hair cells to damaging agents, either in vivo or in vitro. For example, in vivo injection of gentamicin to guinea pigs kills significant numbers of hair cells only in the striolar region of the utricle (Forge et al. 1993). Moreover, in some cases, hair cells may not be killed but instead may suffer apical damage that is subsequently repaired in a process that resembles the development of new hair cells (Zheng et al. 1999a), making it impossible to discriminate repaired hair cells from regenerated ones. To bypass this problem, several groups used genetic labeling to fluorescently mark supporting cells in mice and follow their fates after damage. The observation of fluorescent hair cells in utricles and cristae over time indicated some supporting cells had transdifferentiated into hair cells (Lin et al. 2011; Slowik and Bermingham-McDonogh 2013). As few postmitotic hair cells were observed in adult rodents after damage (see preceding paragraph), it is thought that mammalian vestibular hair cells are replaced predominantly by direct transdifferentiation of supporting cells (Fig. 2.4).

It is important to note that only a fraction of vestibular hair cells are regenerated after damage. In mature guinea pigs, about 70% of hair cells are replaced in the striolar region of the utricle, but this represents a small percentage of the total number of utricular hair cells (Forge et al. 1998). After nearly complete loss of hair cells was induced in utricles of transgenic *Pou4f3<sup>DTR</sup>* mice, only 17% of hair cells were replaced (Golub et al. 2012). Importantly, all regenerated hair cells appear to be type II. Although these new type II hair cells develop stereociliary bundles, mechanotransduction channels, and synapses (e.g., Golub et al. 2012; Forge et al. 1998), it is not clear whether they restore vestibular function.

### **2.3.6 Molecular Control of Hair Cell Regeneration in Nonmammals**

#### **2.3.6.1 Regulators of Supporting Cell Division in Nonmammals**

Because nonmammalian vertebrates naturally regenerate all types of hair cells in the inner ear and lateral line after injury, they provide excellent opportunities to study molecular signals controlling new hair cell production. Here, studies that defined the various signals regulating supporting cell division, differentiation of postmitotic cells into hair cells or supporting cells, and direct transdifferentiation of supporting cells into hair cells are discussed. However, it is important to consider that a

comprehensive understanding of the genetic programs that mediate functional recovery does not exist yet.

Investigators postulated that signals derived from healthy hair cells maintain supporting cells in a state of quiescence. For instance, mature avian hair cells express fibroblast growth factors (FGFs), and FGF receptors are downregulated in supporting cells in regions where cell division occurs following hair cell loss (Birmingham-McDonogh et al. 2001; Ku et al. 2014). These findings suggest FGF signaling could, under normal conditions, maintain supporting cell quiescence. Consistent with this idea, addition of FGFs to cultured utricles or basilar papillae reduces supporting cell division (Oesterle et al. 2000; Ku et al. 2014).

A similar line of thinking suggests that supporting cell transit through the cell cycle may be stimulated by mitogens released from hair cells or other injured cells. Consistent with this idea, receptors for activins, which are secreted members of the transforming growth factor (TGF)  $\beta$  family, are expressed in supporting cells (McCullar et al. 2010). Addition of activin A/B to cultured chick basilar papillae that were not treated with ototoxin increases supporting cell proliferation, suggesting release of activins from injured hair cells might trigger proliferation. Additional growth factors and growth factor-triggered enzymes have been implicated as positive regulators of supporting cell division in nonmammals, including protein kinase A (Navaratnam et al. 1996), mechanistic target of rapamycin (mTOR) and phosphoinoside-3 kinase (PI3K) (Witte et al. 2001), epidermal growth factor (White et al. 2012), insulin (Oesterle et al. 1997), TGF $\alpha$ , tumor necrosis factor  $\alpha$  (Warchol 1999), and canonical Wnts (Head et al. 2013; Jacques et al. 2014).

Structural changes in the epithelium during hair cell loss may also trigger supporting cells to reenter the cell cycle. After extrusion of hair cells, supporting cells expand, presumably to reestablish the integrity of the epithelial surface. Some investigators suggest rearrangement of actin at the supporting cell surface during expansion could trigger enzymatic reactions within supporting cells to promote division (Meyers and Corwin 2007; Collado et al. 2011). Another hypothesis is that conversion of supporting cells into hair cells, and the accompanying depletion of supporting cells, stimulate supporting cell division (Roberson et al. 2004).

### 2.3.6.2 Regulators of Hair Cell Differentiation in Nonmammals

A critical step in hair cell regeneration is the acquisition of a hair cell phenotype in newly born precursor cells or transdifferentiating supporting cells. Cell fate is influenced by intrinsic properties of progenitor cells (e.g., transcription factors that restrict cells to a particular lineage) and local signals from neighboring cells. An excellent example of the interplay of these two mechanisms during regeneration is provided by *Atoh1* and notch signaling. During development (Sects. 2.2.1 and 2.2.2), cells in prosensory regions of the otocyst become distinguished from non-sensory regions by expressing *Sox2*. Subsequently, some prosensory cells activate *Atoh1* expression, which is sufficient to induce the hair cell fate. In chicken basilar papillae following aminoglycoside-induced hair cell damage, the earliest steps of

regeneration mirror development: supporting cells reduce levels of Sox2 protein and accumulate Atoh1 protein (Cafaro et al. 2007). Many of these supporting cells progress to form hair cells, although some do not (Lewis et al. 2012). As in development, *Atoh1* transcription is modulated by notch signaling. In birds and fishes after ototoxic drug treatments, notch ligand (delta1) becomes expressed on the surface of early regenerating hair cells (Stone and Rubel 1999; Ma et al. 2008). Delta1 can bind to and activate notch receptors on neighboring supporting cells, increasing their expression of transcriptional repressors, such as Hes5. These repressors antagonize *Atoh1* transcription in some supporting cells and postmitotic precursor cells, preventing them from transdifferentiating into hair cells. Consequently, notch signaling directs cell patterning in regenerating epithelia to reestablish the precise mosaic of alternating hair cells and supporting cells. Indeed, when notch-mediated repression of *Atoh1* transcription is blocked pharmaceutically, nearly all supporting cells in the damaged area form new hair cells (Daudet et al. 2009).

### 2.3.6.3 Factors Limiting Hair Cell Regeneration in Mammals

There are several ideas to explain why hair cell regeneration does not occur (in the case of the cochlea) or is drastically attenuated (in the case of vestibular organs) in mature mammals. The failure of hair cell regeneration is likely due to progressive changes in supporting cells as well as signals that restrict supporting cells from responding to hair cell damage. These features of mammalian supporting cells may have evolved to ensure stability of inner ear epithelia in adulthood, which could be critical for mammalian auditory and vestibular function.

Loss of progenitor/stem cells may suppress regeneration. Oshima et al. (2007) noted a dramatic, age-related reduction in the number of stem cells that can be derived from mouse organ of Corti or utricle as defined by their ability to generate clonal colonies. This result is consistent with a loss of stem-like cells with development.

It is also possible that proregenerative signals are not activated in mammals after hair cell loss, or the signals that are turned on are not effective. To test this hypothesis, investigators treated tissues from mature mammalian inner ears with growth factors known to be mitogenic in other tissues and assessed effects on supporting cell division. In dissociated cell cultures derived from utricular epithelia of neonatal rats, Zheng et al. (1997) identified several mitogens that increased supporting cell division by two- to fourfold over untreated cultures, including fibroblast growth factor-2, TGF $\alpha$ , and insulin-like growth factor-1. In similar preparations, Montcouquiol and Corwin (2001) identified kinases downstream of mitogens that, when activated, trigger supporting cell division. However, the proproliferative effects of these growth factors and pathway activators were not replicated in other cellular contexts. For instance, investigators found only modest increases in supporting cell division after treatment with TGF $\alpha$  (with or without insulin) in organotypic cultures of mature mammalian utricles (Lambert 1994; Yamashita and Oesterle 1995) or after infusion of growth factor in vivo (Kuntz and Oesterle 1998).

Furthermore, even these modest responses are lost with age. For instance, epidermal growth factor and glial growth factor trigger supporting cell division in neonatal rat utricles (Zheng et al. 1999b; Gu et al. 2007) but have little or no effect in adult mouse utricles (Hume et al. 2003). Similarly, activation of  $\beta$ -catenin (the effector of canonical Wnt signaling) triggers a transient increase in supporting cell division in the cochleae of early postnatal mice (Chai et al. 2012; Shi et al. 2013) but not in juvenile mice (Kuo et al. 2015).

Investigators have proposed mechanisms that could explain the inability of mammalian supporting cells to respond to pro-regenerative signals as they mature. Corwin and colleagues demonstrated that mammalian supporting cells develop thick apical actin networks that could prevent cell spreading, a process that may be required for cell division (Collado et al. 2011; Burns and Corwin 2014). Other groups hypothesize that natural cell cycle blockers such as retinoblastoma protein (Rb) or cyclin-dependent kinase inhibitors (CKIs) prevent supporting cells from responding to mitogenic cues that exist under normal conditions or after hair cell damage. When genes encoding Rbs or CKIs are deleted in the organ of Corti or utricular maculae of adult mammals, both hair cells and supporting cells divide, but the number of cells is limited, and many cells die during or after mitosis (e.g., Löwenheim et al. 1999; Laine et al. 2007). Supporting cell division is also triggered when potent cell cycle activators, such as cyclins or their downstream effectors, Mycs, are misexpressed (Loponen et al. 2011; Burns et al. 2012). Furthermore, misexpression of *Sox4* and *Sox11* transcription factors in vestibular supporting cells of adult mice increases mitotic hair cell regeneration, but it is unclear how long new hair cells survive (Gnedeva and Hudspeth 2015). Expression of both transcription factors in supporting cells is high embryonically and low postnatally. These studies suggest activation of negative regulators such as CKIs and suppression of positive regulators such as *Sox4* and *Sox11* collectively limit supporting cell division in mature mammals.

Investigators have also examined signals that control differentiation of regenerated cells. Early studies found that diffusible factors that drive new hair cell differentiation in very young cochleae are not effective in more mature cochleae (e.g., Chardin and Romand 1995, 1997). Thereafter, many researchers shifted focus away from diffusible factors to new strategies for promoting new hair cell production, namely forced expression of *Atoh1* and inhibition of the notch pathway. Investigators reasoned that forced expression of *Atoh1* in mature cochlear or vestibular epithelia after damage could promote supporting cells to convert into hair cells. In the cochlea, virally delivered *Atoh1* stimulates formation of small numbers of new hair cells in mature guinea pigs (Kawamoto et al. 2003; Atkinson et al. 2014) and mice (Kraft et al. 2013), while *Atoh1* misexpression in damaged hair cells appears to trigger bundle repair (Yang et al. 2012). *Atoh1* overexpression using transgenic mice triggers considerable new hair cell production in neonates, but it yields fewer new hair cells in adults. Moreover, in adults, the new hair cells fail to mature, and many die (Kelly et al. 2012; Liu et al., 2012a). The discrepancy between these studies underscores the challenge of defining the capacity of *Atoh1* to act on its own to promote hair cell regeneration. A recent study points to the potential for activation

of canonical Wnt to enhance effects of *Atoh1* misexpression in neonatal mice (Kuo et al. 2015), but further studies are needed to ascertain effectiveness in adults.

The notch signaling pathway is a potent inhibitor of *Atoh1* transcription (Sect. 2.2.2.2). Inactivation of notch with  $\gamma$ -secretase inhibitors (GSIs) promotes considerable conversion of supporting cells into hair cells in the neonatal cochlea but very little or no conversion in juvenile or adult cochleae (e.g., Yamamoto et al. 2006; Korrapati et al. 2013). Why do GSIs become less effective in promoting supporting cell-to-hair cell conversion as rodents mature? One explanation was offered by Maass et al. (2015), who showed that, in contrast with the avian basilar papilla (Stone and Rubel 1999), notch pathway genes are not reactivated in the mature organ of Corti after damage. However, a similar study from a different research group did show reactivation of notch signaling and even some recovery of auditory function after GSI treatment (Mizutari et al. 2013). Thus, the ability of notch signaling to modulate hair cell formation and regeneration in the adult cochlea remains controversial.

Whereas auditory and vestibular systems of adult rodents seem to have comparable brakes on supporting cell division, adult vestibular epithelia are more responsive to signals promoting direct conversion of supporting cells into hair cells. This is most likely due to the fact that some hair cells are naturally regenerated in adult rodents (Sect. 2.3.5). Virally misexpressed *Atoh1* promotes significant hair cell regeneration in adult rodent utricles in vitro (Shou et al. 2003; Staecker et al. 2007) and in vivo, where type I hair cells may also be regenerated (Schlecker et al. 2011; Xu et al. 2012). In neonatal mice, inhibition of Wnt or notch leads to increased replacement of type I and II vestibular hair cells after damage, but only type II hair cells are regenerated in adult mice (Wang et al. 2015). Inhibition of notch activity in mice leads to higher numbers of replacement hair cells in vestibular epithelia (e.g., Collado et al. 2011; Lin et al. 2011), demonstrating that notch signaling actively antagonizes vestibular hair cell regeneration at all ages.

### **2.3.7 Future Considerations for Hair Cell Regeneration**

Hearing and balance dysfunctions are growing concerns in countries with increasing numbers of elderly individuals. To develop hair cell regeneration as a clinical therapy, researchers must determine how to more effectively drive hair cell replacement in adult mammals. Investigators are beginning to employ different scientific approaches to identify factors controlling mammalian hair cell regeneration. One of these is transcriptomics—the analysis of the set of transcripts (mRNAs) that are present in a cell at a particular time. Because the proteins that ultimately control a cell's behavior are translated from mRNAs, transcriptional profiling provides insights regarding a cell's state and potential. Several studies have published transcriptional profiles of hair cell epithelia from different species in normal conditions and after damage (e.g., Hawkins et al. 2003; Jiang et al. 2014). These studies enable comparisons of the signaling molecules, receptors, and transcription factors that are

being transcribed under different conditions and at a particular time. The results will, hopefully, reveal the factors that should be tested for regulatory roles in regeneration. These sorts of analyses should also provide greater insight into properties of supporting cells in each species. For instance, are some supporting cells genetically programmed to respond in a distinct manner to damage—e.g., by remaining quiescent, dividing, or converting?

Although transcriptional profiling offers a way to model protein expression within a cell, the behavior of that cell is also controlled by other factors. For instance, posttranscriptional events regulate which proteins are generated. MicroRNAs are endogenous noncoding RNAs that direct degradation of specific transcripts. Investigation of microRNAs in hair cells is at an early stage, but it is already clear they play key roles in hair cell production (reviewed in Ushakov et al. 2013). In addition, epigenetic modifications, including DNA methylation and histone acetylation, are potent regulators of transcription and therefore represent another level at which hair cell regeneration may be modulated in mammals. Consistent with this idea, *Atoh1* transcription becomes epigenetically blocked in mouse cochlear supporting cells during postnatal maturation (Stojanova et al. 2015). Further, inhibition of histone deacetylases reduces avian hair cell regeneration (Slattery et al. 2009), although the specific mechanisms have not been defined.

Investigators hope to use these tools to drive hair cell regeneration in mature mammals by delivering drugs or manipulating gene expression to alter the protein composition and the behavior of supporting cells. It is possible, however, that such treatments will be insufficient to drive hair cell regeneration and functional recovery in adult mammals. In this case, it may be necessary to introduce another cell population to achieve recovery. Several labs are studying the capacity of mammalian stem cells, derived from young embryos or induced from mature somatic cells, to form new hair cells in a dish or on delivery to the inner ear. This scientific approach, although at an early stage, has yielded promising results in vitro (reviewed in Hu and Ulfendahl 2013).

### 2.3.8 Summary

Several decades of research have revealed key facets of hair cell development and regeneration, including the cellular mechanisms and some of the molecules that control those processes. In nonmammals, which regenerate hair cells after damage, the signaling molecules that direct regrowth and differentiation of hair cells mirror those employed during development, suggesting genetic programs used in embryogenesis are reactivated in adult tissues. However, there is still much work to be done before the orchestration of complete genetic and molecular pathways necessary for hair cell regeneration can be described. Nonetheless, research has proven that a deepening understanding of hair cell development hastens progress toward determining how to stimulate hair cell replacement in adult mammals. Further, as more is learned about how hair cell replacement is controlled, new ideas are generated



about regulation of hair cell development. Thus, these two lines of investigation are complementary and critical for one another.

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