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Hair cell regeneration in the avian auditory epithelium

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ABSTRACT Regeneration of sensory hair cells in the mature avian inner ear was first described just over 20 years ago. Since then, it has been shown that many other non-mammalian species either continually produce new hair cells or regenerate them in response to trauma. However, mammals exhibit limited hair cell regeneration, particularly in the auditory epithelium. In birds and other non-mammals, regenerated hair cells arise from adjacent non-sensory (supporting) cells. Hair cell regeneration was initially described as a proliferative response whereby supporting cells re-enter the mitotic cycle, forming daughter cells that differentiate into either hair cells or supporting cells and thereby restore cytoarchitecture and function in the sensory epithelium. However, further analyses of the avian auditory epithelium (and amphibian vestibular epithelium) revealed a second regenerative mechanism, direct transdifferentiation, during which supporting cells change their gene expression and convert into hair cells without dividing. In the chicken auditory epithelium, these two distinct mechanisms show unique spatial and temporal patterns, suggesting they are differentially regulated. Current efforts are aimed at identifying signals that maintain supporting cells in a quiescent state or direct them to undergo direct transdifferentiation or cell division. Here, we review current knowledge about supporting cell properties and discuss candidate signaling molecules for regulating supporting cell behavior, in quiescence and after damage. While significant advances have been made in understanding regeneration in non-mammals over the last 20 years, we have yet to determine why the mammalian auditory epithelium lacks the ability to regenerate hair cells spontaneously and whether it is even capable of significant regeneration under additional circumstances. The continued study of mechanisms controlling regeneration in the avian auditory epithelium may lead to strategies for inducing significant and functional regeneration in mammals.

KEY WORDS: *proliferation, transdifferentiation, cell cycle, cell fate, Notch, apoptosis, BrdU, Atoh1*

Introduction

Sensory hair cells (HCs) are mechanoreceptors that are located in specialized epithelia in the inner ear and in lateral line neuromasts. A full complement of HCs in the auditory and vestibular epithelia of the inner ear is required for normal hearing and balance function. In humans, once auditory or vestibular HCs are damaged or lost, the resulting sensory deficits are permanent. Although deleterious changes occur in the auditory nerve and at higher levels following auditory HC loss, it is generally believed that restoration of healthy HCs could lead to substantial hearing improvements.

In mature mammals, regeneration of sensorineural structures and function after damage is uncommon. Exceptions include receptors in the olfactory receptor epithelium and in taste buds, which undergo continual turnover throughout life (e.g., for recent reviews, see Beites *et al.,* 2005; Miura *et al.,* 2006). Similar to the sensory epithelia of the inner ear, these cells are developmentally derived from surface ectoderm. In contrast, regeneration of sensory receptors and neurons derived from the neural tube (e.g., retinal ganglion neurons) or neural crest (e.g., dorsal root ganglion neurons) in mature mammals is atypical (but see Gould, 2007).

Several studies in experimental mammals have examined the inner ear's response to HC loss with the goals of finding evidence for initiation of HC regeneration or identifying ways to trigger the process. During development of the mouse auditory epithelium,

Abbreviations used in this paper: BP, basilar papilla; HC, hair cell; SC, supporting cell.

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known as the organ of Corti, progenitor cell division has ceased by embryonic day 14.5 (Ruben, 1967; Lee *et al.,* 2006; Matei *et al.,* 2005). In adult mammals, the organ of Corti shows no spontaneous ability to form new HCs after noise or drug damage *in vivo* (e.g., Roberson and Rubel, 1994; Forge *et al.,* 1998), although *in vitro* repair of injured HCs has been documented (Sobkowicz *et al.,* 1997; Zheng *et al.,* 1999). In contrast, the vestibular epithelium in adult rodents shows a small but significant increase in cell proliferation in response to HC damage *in vivo* (Rubel *et al.,* 1995; Kuntz and Oesterle, 1998). The cells that divide (progenitor cells) are believed to be supporting cells (SCs), which are non-sensory cells that surround HCs in the sensory epithelia. Unfortunately, several studies show that *in vivo* differentiation of newly produced cells into HCs is rare or non-existent (Rubel *et al.,* 1995; Kuntz and Oesterle, 1998; Ogata *et al.,* 1999; Oesterle *et al.,* 2003, but see Forge *et al.,* 1993).

In contrast to mammals, the post-embryonic replacement of damaged auditory and vestibular HCs is robust in many non-mammalian vertebrates. Early studies demonstrated that cold-blooded animals, cartilaginous fish and toads, form new vestibular HCs as part of their normal body growth (Corwin, 1981; Corwin, 1985; Popper and Hoxter, 1984). In addition, HCs in the lateral line neuromasts are regenerated after tail amputation (Stone, 1933; Stone, 1937; Balak *et al.,* 1990) or after laser-ablation of individual HCs (Jones and Corwin, 1993; 1996). Remarkably, avian species also form new HCs in vestibular epithelia on an ongoing basis in reaction to normal HC apoptosis (Jørgensen and Mathiesen, 1988; Roberson *et al.,* 1992; Kil *et al.,* 1997) and rates of regeneration are increased upon HC damage (Weisleder and Rubel, 1993). Regeneration of HCs in the avian auditory epithelium (also called the basilar papilla) occurs only in response to externally triggered HC trauma and death (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Oesterle and Rubel, 1993). Importantly, HC regeneration in birds occurs after HC epithelia and hearing function have matured. Morphological and functional recovery, including re-innervation, is rapid and thorough (reviewed in Cotanche, 1999; Smolders, 1999; Stone and Rubel, 2000a; Bermingham-McDonogh and Rubel, 2003).

In the late 1980s, two studies in post-hatch chickens initially demonstrated that birds have the capacity to regenerate HCs after they are damaged in the basilar papilla (BP; Cotanche, 1987a; Cruz *et al.,* 1987). In these studies, two methods were used to induce HC damage; Cotanche exposed chickens to intense pure-tone noise, while Cruz *et al.* gave birds systemic injections with the ototoxic aminoglycoside antibiotic, Gentamicin. Pure-tone noise exposure creates relatively focal areas of HC damage and extrusion in corresponding tonotopic regions of the BP. In contrast, Gentamicin treatment induces complete HC loss in the high-frequency (proximal) end of the BP, starting at the proximal tip and moving toward the low-frequency (distal) end over time to encompass different total areas of the BP depending on the number of drug exposures (e.g., Hashino *et al.,* 1991; Janas *et al.,* 1995). In both studies, morphological analysis of the epithelium was performed at different times after HC damage. Cotanche (1987a) discovered that around 2 days (d) after the noise exposure had ended, cells with the appearance of embryonic HCs had emerged in the area of HC loss. These cells matured over the next 2 weeks and the normal cellular patterning of the BP was restored. Cruz *et al.* (1987) counted HCs at different times after Gentamicin treatment and discovered that HC numbers fell immediately but showed partial restoration by 3-4 weeks. Both studies interpreted their results to reflect the regeneration of sensory HCs, by an unknown mechanism that up to that point had not been considered feasible.

The discovery of HC regeneration in post-hatch birds raised considerable excitement about the potential for stimulating HC regeneration in adult mammals. Accordingly, some subsequent research has focused on identifying HC progenitors and on characterizing cellular and molecular mechanisms that direct HC regeneration in avian species, with the hopes that comparative studies in mammals might unveil critical differences between the two animal classes with respect to their response to HC loss. Here, we review the salient features of avian HC regeneration, with special emphasis on characteristics and regulation of HC progenitors in the BP.

Avian supporting cells give rise to new hair cells through two distinct mechanisms

Since initial reports by Cotanche and Cruz *et al.* in 1987, investigators explored cellular and molecular mechanisms employed by birds to regenerate new HCs in maturity. Seminal studies showed that new HCs arise through cell division of non-sensory supporting cells (SCs; Fig. 1), which are interspersed among HCs. In the BPs of chicken embryos, mitotic activity ceases by embryonic day 9 (Katayama and Corwin, 1989) and little or no cell division normally occurs after hatching (Oesterle and Rubel, 1993). However, exposure of post-hatch chickens or quail to intense noise stimulates SCs in the area of HC loss to leave growth-arrest and re-enter the cell cycle and the newly formed precursor cells then differentiate into HCs and SCs (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). This was first demonstrated by delivery of the traceable nucleoside analog tritiated thymidine to birds following noise exposure in order to record proliferative activity. After 10d of recovery, the nuclei of HCs and nearby SCs contained the analog, demonstrating they arose through renewed cell division. Subsequent studies went on to show that it is the SCs themselves, rather than an extrinsic cell type, that divide after HC damage in the BP, whether due to noise exposure, drug treatment, or laser ablation of individual HCs (Raphael, 1992; Hashino and Salvi, 1993; Stone and Cotanche, 1994; Stone *et al.,* 1999; Warchol and Corwin, 1996). SCs also serve as HC progenitors during regeneration of the avian vestibular epithelium (Jørgensen and Mathiesen, 1988; Roberson *et al.,* 1992) and during regeneration of all HC-epithelia and neuromasts in other non-mammalian vertebrates (e.g., Balak *et al.,* 1990; Yan *et al.,* 1991; Baird *et al.,* 1993). Analysis of avian sensory epithelia after HC damage revealed that, as SCs traverse from growth arrest to mitosis, their nuclei migrate from their normal position near the basal lamina to the lumenal surface (Raphael, 1992; Tsue *et al.,* 1994; Fig. 1). Precursors born at the lumen then undergo specific nuclear translocation and differentiation of cytoplasmic and apical features depending on the cell fates they acquire (Stone *et al.,* 1996; Stone and Rubel, 2000b; Roberson *et al.,* 2004; Duncan *et al.,* 2006).

SC division is not the sole means for generating new HCs in non-mammalian vertebrates; SCs also have the ability to spontaneously convert into HCs (Fig. 1) via a process called *direct*

transdifferentiation. While there are many examples of differentiated cell types that alter their phenotypes to stably acquire another differentiated state (transdifferentiation), this process usually involves transit through the cell cycle (e.g., Nathanson, 1986). In contrast, direct transdifferentiation occurs without cell cycle re-entry (Beresford, 1990). For example, neural retina can directly convert into lens cells in embryonic chickens (Opas and Dziak, 1998). There is significant evidence for HC regeneration via direct transdifferentiation from SCs in mature amphibian saccules and avian BPs. After ototoxic drug treatment, HC numbers are restored in cultured saccules from frogs and newts despite continuous inhibition of SC division by the S-phase blocker, Aphidicolin (Baird *et al.,* 1996; Baird *et al.,* 2000; Taylor and Forge, 2005). A similar finding was reported in the chicken BP after HC damage (Adler *et al.,* 1996), although inhibition of SC division was only transient. Roberson *et al.* (1996) implanted a pump to continuously deliver a traceable nucleoside analog (bromodeoxyuridine or tritiated thymidine) into the chicken perilymph and subsequently administered Gentamicin to trigger HC loss. At 12d post-Gentamicin, only 50% of regenerated HCs had incorporated the nucleoside, while the other half had not. In a similar study, at 10d post-Gentamicin, 70% of regenerated HCs showed incorporated nucleoside while 30% did not (Roberson *et al.,* 2004). Since the HC lesion was complete in the area of analysis, HC repair was ruled out as an explanation of the finding. Rather, since the mitotic tracer was available to SCs before, during and after HC death, the result suggested that a subpopulation of SCs phenotypically converted directly into HCs without dividing. Further evidence for SC transdifferentiation into HCs was provided by a recent study showing that Atoh1, a proneural transcription factor whose translation during organ of Corti development is thought to be limited to early differentiating HCs (Chen *et al.,* 2002; Woods *et al.,* 2004; Matei *et al.,* 2005), becomes highly expressed in the nuclei of SCs shortly after HC damage and is later highly expressed in regenerated HCs (Cafaro *et al.,* 2007). These studies provide strong evidence that direct transdifferentiation is a significant source of new HCs during avian HC regeneration.

The first new HCs to emerge after HC damage are produced by direct transdifferentiation, while new HCs generated by mitosis appear later and eventually comprise a substantial proportion of the new sensory cells (Roberson *et al.,* 1996; Roberson *et al.,* 2004; Cafaro *et al.,* 2007). Transdifferentiating SCs are detected as early as 15 hours (h) after Gentamicin treatment, prior to the extrusion of HCs in the region (Cafaro *et al.,* 2007). Thus, SCs are activated to transdifferentiate in response to cues that occur shortly after HC damage is initiated. Significant numbers of SCs in the damaged region enter S phase around 72h (Bhave *et al.,* 1995; Stone *et al.,* 1999; Roberson *et al.,* 2004; Duncan *et al.,* 2006). At this time, there are already regenerated HCs present in the region where SC division occurs, as illustrated by labeling with early HC antigens (e.g., MyosinVI and TuJ1; Stone and Rubel, 2000b; Roberson *et al.,* 2004). However, none of these first-appearing regenerated HCs is derived from newly post-mitotic

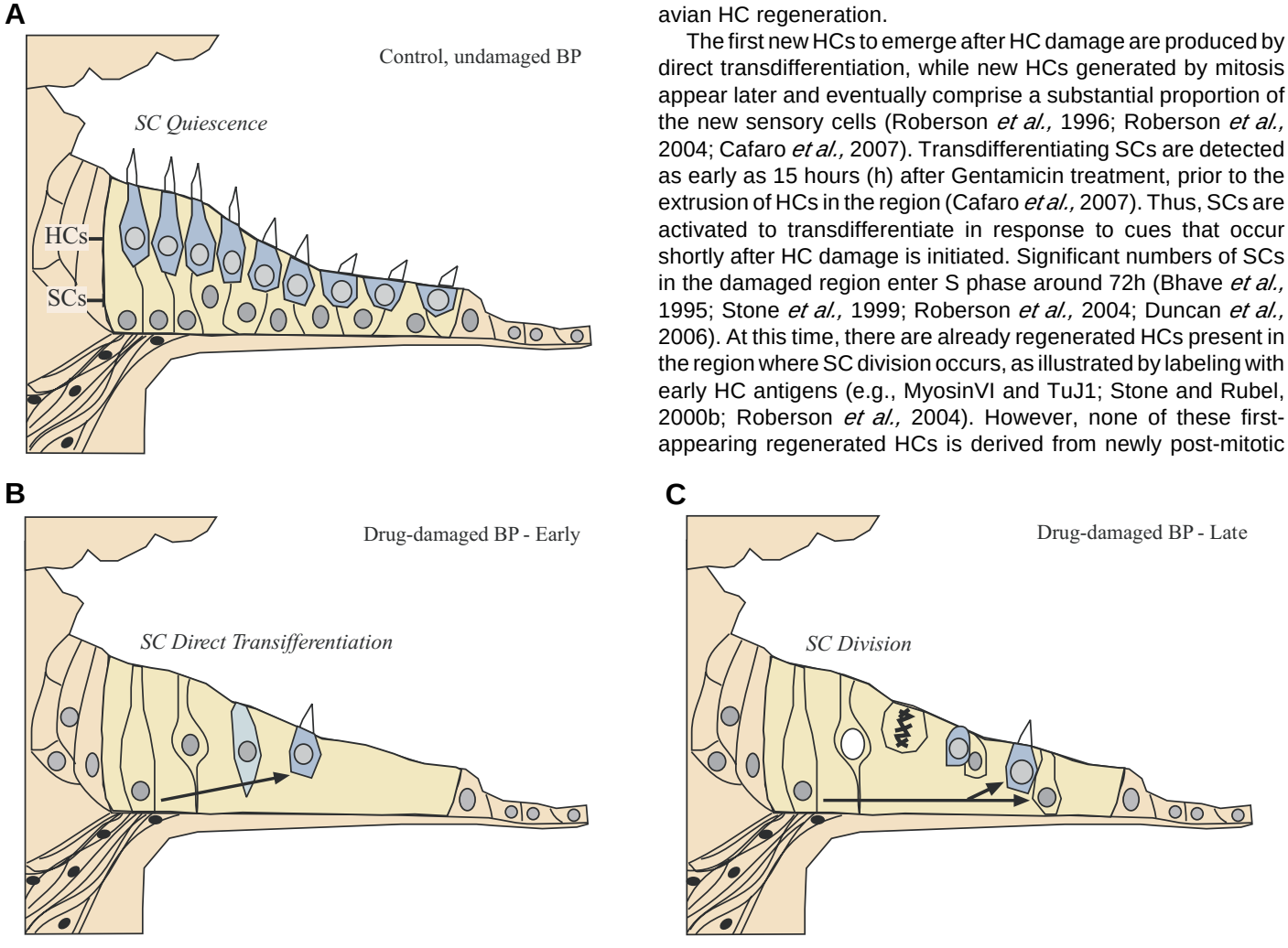

Fig. 1. Supporting cell behaviors in the chicken basilar papilla. *These schematics depict supporting cells (SCs) in the mature chicken basilar papilla (BP) in different states.* **(A)** *SCs in quiescence in an undamaged BP. Hair cells (HCs).* **(B,C)** *SCs in the drug-damaged BP undergoing either direct transdifferentiation* **(B)** *or cell division* **(C)**. *In (B,C), arrows indicate the temporal progression of each SC response, starting with a single SC on the left and ending in a single new HC on the right (B, direct transdifferentiation) or starting with a single SC on the left and ending in two new cells (a HC and a SC here) on the right (C, cell division). Abbreviations: BP, basilar papilla; HC, hair cell; SC, supporting cell.*

cells (Roberson *et al.,* 2004). Rather, HCs regenerated via mitosis are not detected by antibodies to early HC-specific antigens until around 96h (4d; Fig. 2) and by later antigens, such as MyosinVIIa, until 120h (5d). The number of HCs formed by direct transdifferentiation peaks at 144h (6d) and remains unchanged at 240h (10d), whereas the number of mitotically regenerated HCs increases significantly after 5d, so that by 10d, they comprise 50-70% of the new HCs (Roberson *et al.,* 1996; Roberson *et al.,* 2004). These data indicate that the first new HCs created during the early stages of regeneration are produced by direct transdifferentiation and that later, when more HCs are lost, mitosis takes over to produce additional new HCs. Once mitosis is initiated, direct transdifferentiation is down-regulated, so that by 10d, most HCs present in the epithelium have been derived through cell division. However, signals that regulate the initiation of direct transdifferentiation and stimulate the switch to mitosis are not yet identified (discussed below).

In addition to this temporal separation of non-mitotic and mitotic regeneration, SCs in different regions along the width-wise axis of the BP are more likely to give rise to new HCs using one mechanism rather than another. Cafaro *et al.* (2007) showed that, after Gentamicin treatment, the density of dividing SCs is much higher in the neural half of the epithelium than in abneural half. Further, the majority (81%) of regenerated HCs in the neural half of the BP are formed by SC division while in the abneural half, the majority (66%) of new HCs are formed by direct transdifferentiation. Since the timing, mechanism and degree of HC loss are essentially the same in these two regions of the BP, this observation likely reflects intrinsic differences in SCs in each region or divergence in local signals.

Properties of avian supporting cells

Unlike HCs, whose cell bodies are confined to the lumenal region of the sensory epithelium, most SCs have elongated cell bodies that appear to contact both the lumenal and basal surfaces of the epithelium. When quiescent, SC nuclei are typically located basal to HC nuclei. Ultrastructural studies show that SCs of the avian BP are fairly undifferentiated, with poorly developed cytoskeletons and a low density of organelles (e.g., Takasaka and Smith, 1971; Hirokawa, 1978; Hirose *et al.,* 2004). In fact, a study of the fish saccule demonstrated that actively dividing SCs bear high resemblance to quiescent SCs with respect to their gross and fine morphological features (Presson *et al.,* 1996). Nonetheless, in the avian BP, SCs serve many functions, including production of extracellular matrix for the overlying tectorial membrane (Cotanche, 1987b; Epstein and Cotanche, 1995; Killick *et al.,* 1995; Goodyear *et al.,* 1996; Coutinho *et al.,* 1999), establishment of a pathway for potassium clearance via gap junctions (Wangemann, 2002; Forge *et al.,* 2003; Nickel *et al.,* 2006), neurotrophic support of HCs and neurons (Stankovic *et al.,* 2004) and anchoring of the sensory epithelium to the basilar membrane (Cotanche *et al.,* 1992; Cotanche *et al.,* 1995). As discussed above, after HC damage, some SCs display behaviors characteristic of progenitors, undergoing cell division, while others resemble precursor cells, forming new HCs through staged transdifferentiation. Quantitative analyses after ototoxic HC damage demonstrate that approximately 1/4 of SCs divide, 1/4 of SCs transdifferentiate and 1/2 of SCs exhibit neither response (Roberson *et al.,* 1996). It is logical that these SCs responses must be balanced in order to establish the proper number and type of new cells in the BP while maintaining important SC functions. Thus, a critical question is how individual SCs are instructed to select each response. One possibility is that subsets of SCs are restricted to behave only as stem/progenitor cells or precursor cells poised for transdifferentiation and the remaining SCs are terminally differentiated and incapable of either response. Alternatively, all SCs may have equivalent potential, but subpopulations respond in a given manner due to locally regulated signals. If the former is true – that SCs are specialized – then subsets of SCs with distinct properties should be identifiable, but to date, few studies have directly addressed this issue. A cell lineage study revealed two distinct SC shapes in the late embryonic BP, which is nearly mature (Fekete *et al.,* 1998). A small subpopulation of SCs (4%) had a basally located nucleus and a thin cytoplasmic process leading to the lumenal surface. The remaining SCs that were observed had a thicker appearance, with a nucleus located more lumenally. This is an intriguing observation, particularly because approximately 4% of SCs show stem cell-like behavior during regeneration, dividing more than once (Stone *et al.,* 1999). However, it remains to be demonstrated that SCs with the delicate appearance are in fact stem cells. In the quiescent BP, several molecular markers appear to label all SCs (e.g., Kruger *et al.,* 1999). However, only a few markers define subsets of SCs. For example, Bhave *et al.* (1995) found that 3% of quiescent SCs express detectable levels of proliferating cell nuclear antigen (PCNA). In addition, SCs across the neural-abneural axis of the BP express different levels of the transcription factor Prox1 (Stone *et al.,* 2004) or transcripts for fibroblast growth factor receptor 3 (Bermingham-McDonogh *et al.,* 2001). The functional significance of these different expression profiles has not been established. However, given the different degrees of SC division seen in the neural and abneural regions of the BP after HC loss (Cafaro *et al.,* 2007), it is tempting to hypothesize that these molecules play a role in regulating cell cycle progression or stem cell properties in SCs.

Which properties might render SCs likely to respond in one manner or another? Molecular analyses demonstrate that mature SCs are not simply progenitor cells retained from development. For example, the transcription factor Prox1 is detectable in all progenitor cells in the sensory epithelia of the chick otocyst as they are dividing (Stone *et al.,* 2003). But, in the BP, Prox1 protein is downregulated in SCs as post-mitotic cells differentiate, such that by hatching, only a subpopulation of SCs in the neural half express detectable levels of the protein. While the function of Prox1 in the BP remains to be determined, homologues of Prox1 are required for proliferative activity and cell lineage decisions in fruit flies (e.g., Reddy and Rodrigues, 1999) and mice (e.g., Wigle and Oliver, 1999; Dyer *et al.,* 2003). Thus, one possibility is that SCs that retain Prox1 expression in maturity are embryonic-like and more apt to respond by dividing. This may also be true of SCs that express high levels of PCNA (Bhave *et al.,* 1995). At this point, however, the relative homogeneity of SCs favors the interpretation that a SC responds in a given way not due to its degree of specialization but rather in response to distinct cues encountered in its microenvironment (Morest and Cotanche, 2004). Analyses in other tissues have stimulated investigators to theorize that direct transdifferentiation occurs because of shifts in

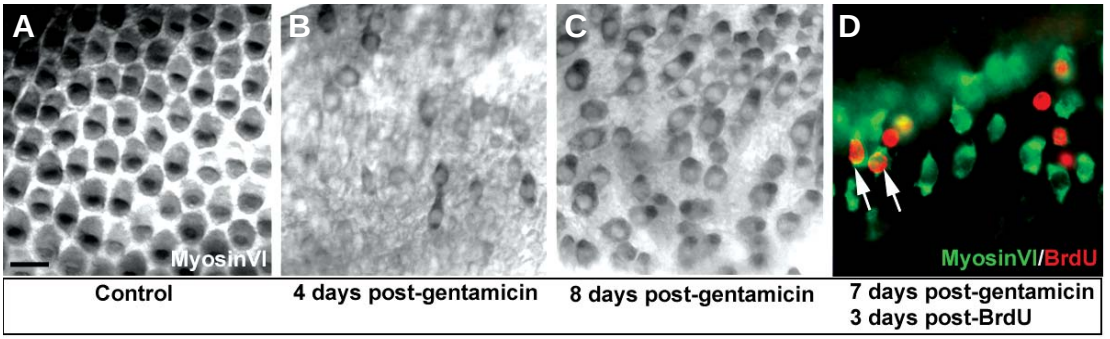

Fig. 2. Hair cell regeneration after drug damage in the chicken basilar papilla. *The proximal end of the basilar papilla (BP) is shown in whole-mount preparations in all panels. Panels* **(A-C)** *show immunolabeling for the hair cell (HC)-specific protein, MyosinVI (gray), in the control, undamaged BP* **(A)***, in the BP at 4 days post-Gentamicin* **(B)** *and in the BP at 8 days post-Gentamicin* **(C)***. The apical surfaces of normal mature HCs (A) appear round. At 4 days post-Gentamicin (B), note the loss of mature HCs in the proximal region and the reemergence of new, immature HCs with fusiform cell shapes. By 8 days post-Gentamicin, more new HCs have emerged and they appear more mature.* **(D)** *Double-labeling for MyosinVI (green) and BrdU (red). BrdU was applied as a single pulse at 4 days post-Gentamicin and animals were euthanized 3 days post-BrdU, at 7 days post-Gentamicin. Hair cells formed by mitosis are double-labeled (arrows). Scale bar, 10 μm.*

intrinsic properties of progenitors in response to microenvironmental changes (discussed in Opas and Dziak, 1998). Future experiments in the chicken BP should aim to characterize molecular profiles associated with each SC behavior after HC death.

Avian supporting cells respond to the progression of hair cell death

In the normal post-hatch BP, SCs are in a state of quiescence; there is no mitotic activity and no evidence for ongoing transdifferentiation. Shortly after HC damage is initiated, SCs are activated to alter this quiescence. Somewhat surprisingly, this response is not limited to the area of ultimate HC loss. Rather, SCs throughout the entire sensory epithelium, including in areas that are a millimeters from where HC loss will occur, progress from growth arrest to G1, an early phase of the cell cycle (Bhave *et al.,* 1995). Therefore, an early signal triggering a change in SC properties is globally distributed throughout the BP. However, SCs that show clear signs of transdifferentiating as HCs and SCs that undergo DNA synthesis and divide, are only seen near where HC loss will occur or is occurring (Corwin and Cotanche, 1988; Raphael, 1992; Stone and Cotanche, 1994; Cafaro *et al.,* 2007). Therefore, critical local signals regulate the ultimate behavior of SCs after HC damage in the avian BP.

What might these global and local signals be? One strategy for identifying potential regulators of SC behavior in the chicken BP is to identify specific steps in cellular injury and death during sound-damage or Gentamicin-damage in the avian cochlea and to correlate each step with specific regenerative responses in nearby or distant SCs (e.g., Torchinsky *et al.,* 1999; Hirose *et al.,* 2004; Mangiardi *et al.,* 2004; Dai *et al.,* 2006; Duncan *et al.,* 2006; Warchol and Speck, 2007). It appears that damaged HCs undergo apoptosis, which is a carefully orchestrated genetic program that results in a cell deliberately killing itself (Kerr *et al.,* 1972; Wyllie, 1980). Apoptosis is thought to involve three phases: initiation, arbitration and execution. Initiation is defined as the internal damage or external signal that starts the cell death program. Once apoptosis is initiated, there is a period of arbitration where cell survival signals compete with the cell death signals in an attempt to rescue the cells. If the death signals dominate at this stage, the cell dies; but if the survival signals dominate, the cell lives. It is not clear at this point which specific changes in HCs occur early enough to result in the initiation of transdifferentiation in nearby SCs. For many cell types, the execution stage of the pathway involves the release of Cytochrome C from mitochondria and the activation of Caspase-3 (McDonald and Windebank, 2000; Bouchier-Hayes *et al.,* 2005). This subsequently mobilizes a regulated enzyme cascade that results in the breakdown of multiple cellular components and eventual cell death. The most prominent and well-studied components of the execution phase are Caspases (Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998; Earnshaw *et al.,* 1999 Lavrik *et al.,* 2005). Specific Caspases are thought to be involved in the early, upstream phase, such as Caspases 8, 9 and 10, while others are restricted to the later, downstream stages, such as Caspases 3, 6 and 7.

Gentamicin enters HCs shortly after a single systemic injection (by 6h; Dai *et al.,* 2006). Initially, Gentamicin is detected in the most proximal HCs, but by 9h, it has accumulated in HCs throughout the BP. SCs show no evidence of Gentamicin uptake, suggesting that changes in SC behaviors are not caused by cell-autonomous effects of the drug. Following a single Gentamicin injection, HCs first begin to be ejected from the proximal tip of the BP at 30h, or 24h after the Gentamicin first reaches these HCs. Hair cell loss progresses down the cochlea to encompass the proximal 30% of the BP by 42h and by 54h, all HCs have been ejected from this region (Mangiardi *et al.,* 2004). The lack of Myosin-labeled cells within the proximal 30% of the BP by 54h demonstrates that no surviving HCs remain in the Gentamicin-damaged region of the BP.

The first indication that damaged HCs are undergoing apoptosis appears at 12h post-Gentamicin, with the translocation of T-cell restricted intracellular antigen-related (TIAR) protein from the nucleus into the cytoplasm of HCs in the proximal tip of the BP. Cellular translocation of TIAR is an early indicator of apoptosis in many tissues (Taupin *et al.,* 1995). This step occurs 6h after Gentamicin first enters the HCs and 18h before HCs will be ejected from the sensory epithelium. No changes in TIAR location have been detected in areas where HC loss and regeneration will

not occur (e.g., in the distal half after Gentamicin treatment). TIAR translocation is the only known molecular sign that the HCs have entered the apoptotic cascade at this early time-point. Since TIAR translocation occurs around the time that SCs begin to show overt signs of transdifferentiating (Cafaro *et al.,* 2007), it is intriguing to hypothesize that TIAR translocation may in some way be connected with early signals that trigger SC transdifferentiation. While TIAR appears to be involved in the formation of stress granules in damaged cells (Kedersha *et al.,* 2000), the specific downstream molecules that it regulates are not known.

Two additional apoptosis-associated events occur in HCs around 30h post-Gentamicin, as HCs are just beginning to be ejected from the BP: the release of Cytochrome C from mitochondria into the cytoplasm and the activation or cleavage of Caspase-3 (Mangiardi *et al.,* 2004; Cheng *et al.,* 2003). Release of Cytochrome C and Caspase-3 activation are both first seen in the proximal end and spread distally over time, as the HC lesion increases in size. Neither response occurs in areas where HC loss and regeneration will not occur. These changes occur prior to the initiation of SC re-entry into cell cycle and therefore may be part of the cascade of events leading to SC division.

Recent studies have used inhibitors of the apoptotic pathway to block ongoing or drug-induced HC cell death in the avian vestibular epithelium *in vivo* (Matsui *et al.,* 2002; Matsui *et al.,* 2003; Matsui *et al.,* 2004) and in the mammalian utricle and chick BP *in vitro* following aminoglycoside treatment (Cunningham *et al.,* 2002; Cheng *et al.,* 2003; Sugahara *et al.,* 2006). These studies suggest that inhibiting steps in the cell death pathway can block apoptosis in HCs. It would be interesting to determine how SC responses progress in these cases, in which HC damage is initiated but survival signals predominate, preventing HC death. Another important area of study is the distal end of the BP, where HCs undoubtedly are affected by short Gentamicin treatments but ultimately survive. While it has been demonstrated that distal SCs initiate transition from quiescence into the cell cycle (Bhave *et al.,* 1995), it is not clear if distal SCs begin steps toward transdifferentiation. It is of particular interest to determine how HCs and SCs are differentially altered in the proximal and distal ends of the BP after Gentamicin treatment, because such information could help to identify the extent of HC damage required to initiate SC withdrawal from quiescence and progression toward transdifferentiation or cell division.

Signals directing a SC's pathway for regeneration

Which signals regulate SC quiescence and activation after HC loss? One working hypothesis is that direct transdifferentiation is the principal route for regenerating HCs and that SC division occurs only to repopulate depleted SCs after they have converted to HCs (Roberson *et al.,* 2004). In this model, the loss of SCs that accompanies the initial transdifferentiation phase of regeneration may trigger adjacent SCs to divide. This hypothesis represents an interesting divergence from the classical idea that normal HCs laterally inhibit SC division (e.g., Corwin and Cotanche, 1988). However, this hypothesis is currently difficult to test because there is no known tool for experimentally blocking direct transdifferentiation.

Regardless of the signals' source, the following signals must be critical for regulating SC behavior: those that maintain SCs in a differentiated quiescent state (preventing transdifferentiation and division), those that trigger SC transit from quiescence toward transdifferentiation or division and those that assure only a limited number of SCs respond to active signals. Clues toward identifying these signals may be derived from our knowledge of pathways that direct the initial development of inner ear epithelia. Several molecular pathways are known to regulate embryonic HC progenitors and some of these pathways are reactivated in mature HC epithelia after HC loss and therefore may be important in directing SC behavior. Sensory epithelia, including the BP, form from the otic placode, a specialized region of ectoderm. During embryogenesis, the placode undergoes growth, morphogenesis and differentiation, establishing specific auditory or vestibular epithelia as well as non-sensory structures. Induction of the otic placode is controlled by several molecules, including Wnts and Fibroblast Growth Factors (reviewed in Fekete and Wu, 2002; Brown *et al.,* 2003). After induction, placodal cells express distinct sets of transcription factors and later, as the otic epithelium develops, distinct regional patterns of gene expression emerge. Specification of regions as sensory patches is associated with expression of bone morphogenetic proteins (*BMPs 4* and *7*; Wu and Oh, 1996; Cole *et al.,* 2000), Notch-associated genes (*Serrate1, Lunatic Fringe [Lnfg], Notch1 and Delta1*; Adam *et al.,* 1998; Cole *et al.,* 2000) and some transcription factors (e.g., Prox1, Atoh1 [Stone *et al.,* 2003] and Sox2 [Kiernan *et al.,* 2005; Neves *et al.,* 2007]. Since mature SCs in the avian BP are distinct from embryonic progenitors, it is of great interest to determine if SCs must transit to an immature state (dedifferentiate) before they can divide or directly transdifferentiate into HCs. If this were the case, SCs would show regressive changes in their genetic profiles after HC damage. Analysis and perturbation of genes expressed in embryonic progenitors (e.g., *Prox1, BMP4, Serrate1,* or *Lnfg*) could provide insight into this question. It would also be important to identify anchored or diffusible signaling molecules that regulate such regressive genetic changes in SCs, as they are likely required for regeneration. In mature mammals, regeneration of HCs may fail because SCs are inhibited from dedifferentiating, they are unable to respond to signals promoting dedifferentiation, or signals promoting regression are lacking.

In the mature chicken BP after HC loss, the basic helix loop helix (bHLH) proneural transcription factor Atoh1 (atonal, Ath1; Jarman *et al.,* 1993; Ben-Arie *et al.,* 2000) becomes reactivated in transdifferentiating and mitotically active SCs (Cafaro *et al.,* 2007). Atoh1 has a critical function in HC development, which has been best studied in the mouse inner ear. There, loss of *Atoh1* (*Math1*) leads to failed specification and/or differentiation of HCs (Bermingham *et al.,* 1999; Woods *et al.,* 2004; Matei *et al.,* 2005). SCs also fail to differentiate, but this may occur as a result of failed HC development rather than in direct response to *Atoh1* deletion (Woods *et al.,* 2004). Similarly, knockdown of *Atoh1a* and/or *Atoh1b* in zebrafish results in the production of fewer inner ear HCs (Millimaki *et al.,* 2007). During development, *Atoh1* expression emerges in sensory patches (Bermingham *et al.,* 1999; Stone *et al.,* 2003; Woods *et al.,* 2004; Matei *et al.,* 2005), becomes highly elevated in HCs after terminal mitosis (Chen *et al.,* 2002; Lumpkin *et al.,* 2003) and is down-regulated after HC differentiation (Lanford *et al.,* 2000; Zheng *et al.,* 2000). Misexpression of *Atoh1* in the inner ear of developing or mature rodents leads to ectopic HC differentiation (Zheng and Gao, 2000;

Kawamoto *et al.,* 2003; Shou *et al.,* 2003; Izumikawa *et al.,* 2005). In the mature undamaged chicken BP, Atoh1 protein is not detected in HCs or SCs (Cafaro *et al.,* 2007). However, within hours of HC damage, while HCs are still intact, Atoh1 protein becomes detected in the nuclei of SCs in the proximal, damaged end of the BP. Later, Atoh1 protein is seen in regenerated HCs. During development of the chicken otocyst, *Atoh1* transcripts are not detected in progenitor cells but rather, they seem to first emerge in differentiating HCs (Pujades *et al.,* 2006). Thus, it seems unlikely that upregulation of Atoh1 in activated SCs represents a return to an immature progenitor-like state. Nonetheless, given Atoh1's critical role in regulating HC specification and/or differentiation in rodents, Atoh1 probably directs the SC to HC fate switch during regeneration in the chicken BP. Therefore, it is of great interest to determine how *Atoh1* expression is regulated in quiescent and activated SCs and whether changes in Atoh1 activity are sufficient to direct one SC behavior or another. One study – Cafaro *et al.,* 2007 – shows that elevated expression of Atoh1 in SCs or in post-mitotic precursor cells is not sufficient to irreversibly specify that cell as a HC. Rather, Atoh1 expression is highly dynamic in some post-mitotic cells during the first 24-48h after it is upregulated and some of the cells that initially show high levels of Atoh1 protein go on to differentiate as SCs. Therefore, cell fate specification is a complex process that likely depends on several inputs from cells in the epithelia microenvironment.

Regulation of *atonal/Atoh1* transcription is combinatorial; atonal/Atoh1 (e.g., Helms *et al.,* 2000), Ngn1 (Gowan *et al.,* 2001), Wingless/Wnt (e.g., Niwa *et al.,* 2004), Decapentaplegic/BMPs (e.g., Niwa *et al.,* 2004), epidermal growth factor (zur Lage *et al.,* 2004), fibroblast growth factor (e.g., Millimaki *et al.,* 2007) and

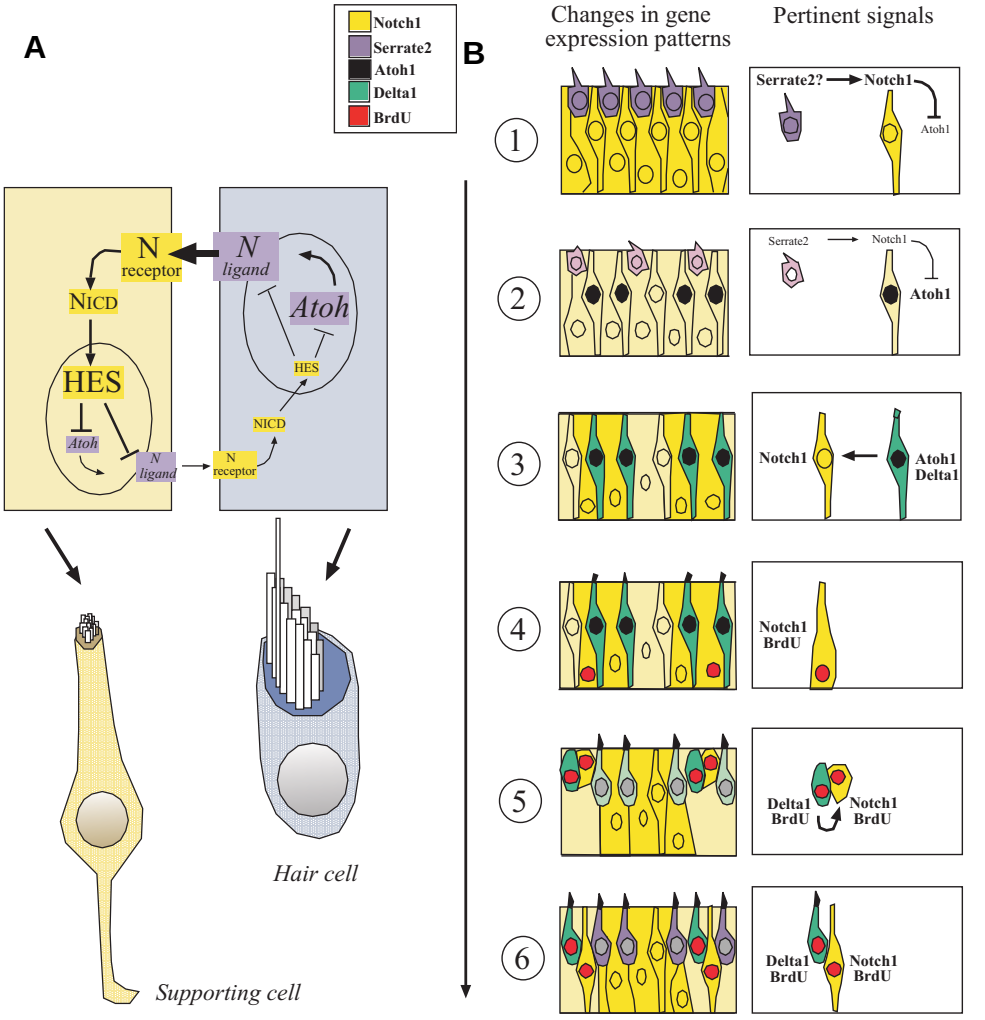

Fig. 3. Notch signaling in hair cell epithelia. (A) *Major molecules involved in the Notch signaling pathway and how they cooperate in two adjacent cells to lead to the production of different cell types, a supporting cell and a hair cell in this case. The cell on the right (purple) is the "signaling cell". Its relatively high levels of Notch (N) ligand bind and activate Notch (N) receptors on the cell on the left (yellow), the "receiving cell. As a result, Notch activation is high in the receiving cell and high levels of the Notch intracellular domain (NICD) are released from the membrane and travel to the nucleus. As a result, HES repressor activity is increased and transcription of* Atoh1 *and* N ligand *is inhibited. In response, the receiving cell's ability to activate N receptor on the signaling cell is diminished and transcription of* Atoh1 *and* N ligand *becomes increased in the signaling cell due to low HES repressor activity. In this manner, signaling cells acquire the sensorineural fate (HC) and receiving cells acquire a non-sensory fate (SC).* **(B)** *A working model for how Notch signaling progressively alters SC behaviors in the chicken basilar papilla after HC damage, with stages numbered and later stages shown at lower levels in the figure. On the left, progressive changes in gene expression patterns are shown. On the right, the pertinent signals regulating/reflecting SC behavior at a given time are shown.* **1)** *In the mature, undamaged BP, Notch ligand (Serrate2?) expressed in HCs activates Notch1 in SCs, maintaining them in a quiescent state.* **2)** *After ototoxin treatment, HCs are damaged and lose Notch ligand function; in nearby SCs, Notch1 activity is decreased and Atoh1 levels are increased.* **3)** *Atoh1-positive SCs begin to transdifferentiate and upregulate* Delta1*; increased Notch1 activity in some SCs prevents them from expressing Atoh1 and initiating direct transdifferentiation.* **4)** *SCs with increased Notch activity either undergo cell division (incorporate BrdU) or remain mitotically quiescent; Delta1/Atoh1-positive cells continue to differentiate as HCs.* **5)** *Some post-mitotic cells upregulate Atoh1 and Delta1 (shown) and activate Notch1 in other post-mitotic cells (siblings?); transdifferentiated HCs down-regulate Delta1 and Atoh1.* **6)** *Delta1/Atoh1-positive post-mitotic cells differentiate as HCs; neighboring cells with high Notch1 activity differentiate as SCs; transdifferentiated HCs approach maturity and reexpress Serrate2.*

Notch (e.g., Baker *et al.,* 1996) are all examples of molecules thought to exert direct or indirect influence on *atonal/Atoh1* transcription. Of these, Notch has been most thoroughly studied in the vertebrate inner ear. Notch's role in limiting expression of *atonal/Atoh1* is effectively illustrated by its role in fruit fly sensory organ development. Initially, all progenitor cells in sensory primordia express *atonal*. Over time, *atonal* becomes limited to specific cells that will acquire sensory or neural fates (e.g., Baker *et al.,* 1996; Baker and Yu, 1997). Initially, Notch activity is required for *atonal* transcription in cells across the sensory primordium. Later, Notch activity restricts *atonal* transcription to limited cells. Repression of proneural genes such as *atonal* occurs through another bHLH transcription factor called Hairy/Enhancer of Split (HES; Heitzler *et al.,* 1996). HES's repressor activity becomes activated when the Notch receptor is bound by one of its ligands, Delta or Serrate (Artavanis-Tsakonas and Simpson, 1991; Muskavitch, 1994), which are located on adjacent cells. When bound, a portion of Notch is released from the membrane and moves into the nucleus, where it alters HES function (Fig. 3A). A similar transduction pathway occurs in mammals (reviewed in Selkoe and Kopan, 2003; Kageyama *et al.,* 2005). Since HES and atonal/Atoh1 have opposing effects on transcription of Notch ligands, (Kageyama *et al.,* 1995; Heitzler *et al.,* 1996), adjacent cells ultimately require different levels of atonal/Atoh1 and therefore, distinct fates.

During HC development in the vertebrate inner ear, Notch plays several important roles (Lewis, 1996; Kelley, 2006). In the early otocyst, activation of a Notch receptor by Jagged1 (analogous to Serrate1 in chick and fruit flies) is required for specifying regions of the otic epithelium as "sensory". Either loss of *Jagged1* in mice (Kiernan *et al.,* 2001; Kiernan *et al.,* 2006) or complete inhibition of Notch activity with the pharmaceutical inhibitor DAPT in chickens (Daudet *et al.,* 2007) causes decreased size or loss of sensory patches. In contrast, misexpression of the Notch intracellular domain in chickens leads to the formation of ectopic sensory patches (Daudet and Lewis, 2005). Later, as sensory epithelia differentiate, signaling through Notch regulates cell fate specification, primarily by inhibiting the HC fate. Disruption of Notch1 receptor, Jagged2/Delta-1-like, or downstream effector function leads to premature differentiation and overproduction of HCs (Haddon *et al.,* 1998; Lanford *et al.,* 1999; Riley *et al.,* 1999; Lanford *et al.,* 2000; Zhang *et al.,* 2000; Zheng *et al.,* 2000; Zine *et al.,* 2000; Kiernan *et al.,* 2001; Tsai *et al.,* 2001; Zine *et al.,* 2001; Kiernan *et al.,* 2005; Brooker *et al.,* 2006), as well as limited transdifferentiation of SCs into HCs (Yamamoto *et al.,* 2006). Thus, as expected given Notch's antagonism of *Atoh1*, loss of Notch1 activity generates the opposite phenotype as seen with *Atoh1* deletion (Bermingham *et al.,* 1999). Notch1's negative regulation of *Atoh1*, mediated by HES1/5, is a likely mechanism for its inhibition of HC specification in the inner ear (Lanford *et al.,* 2000; Zine and deRibaupierre, 2002). By suppressing *Atoh1* during development, Notch-mediated lateral inhibition appears to inhibit progenitor/precursor cells from acquiring the HC fate. By preventing progenitor/precursor cells from differentiating as HCs, Notch activation essentially conserves these cells in the sensory epithelium, perhaps allowing mitotic or non-mitotic production of HCs (or SCs) later in development, perhaps even postnatally. This role for Notch is also seen in several other tissues (reviewed in Kageyama *et al.,* 2005). For example, in the developing cerebral cortex, loss of *HES1/5* leads to significant decreases in clonal growth of neural stem cells in culture (Ohtsuka *et al.,* 2001).

Since Notch has several functions in the development of inner ear sensory epithelia, it is tempting to hypothesize that these functions are reiterated in the chicken BP during HC regeneration. Expression analyses in the mature chicken BP support this hypothesis (Stone and Rubel, 1999). In the quiescent (undamaged) chicken BP, SCs actively transcribe *Notch1* and *Serrate1*, but no transcription of *Delta1* is seen in either HCs or SCs. A third Notch ligand, Serrate2, is expressed in developing HCs (Eddison *et al.,* 2000) and may also be expressed in mature HCs. This general pattern of Notch-related gene expression is comparable to what is seen in the mature organ of Corti. By 3d post-Gentamicin, *Delta1* transcripts become increased in dividing SCs and are later highly upregulated in daughter cells as they differentiate into HCs. *Delta1* levels are low in post-mitotic, regenerated SCs and expression of *Delta1* during transdifferentiation has not been explored. By 10d post-Gentamicin, *Delta1* is highly downregulated. Changes in *Notch1*, *Serrate1* and *Serrate2* have not been extensively described after HC loss.

The precise roles for Notch in the quiescent and regenerating BP have not been functionally tested, although some hypotheses can be drawn based on these expression data (Fig. 3B). In the quiescent (undamaged) state, the Notch1 receptor in SCs may be activated by neighboring HCs via Serrate2 (or by SCs via Serrate1). This activation may maintain SC identity and/or SC quiescence, preventing SCs from converting into HCs. Shortly after Gentamicin treatment, Notch receptor or ligand function may be diminished, leading (in SCs) to decreased Notch activity, increased *Atoh1* transcription and transdifferentiation toward the HC phenotype. Retention of Notch activation in some SCs could prevent them from transdifferentiating, conserving them for critical functions in the BP or for mitotic regeneration at a later time. In post-mitotic cells, Notch activation, presumably via Delta1, would also specify cell fate determination (Stone and Rubel, 1999). Alternatively, Notch activity may be low or absent in the quiescent BP and activation of Notch may occur only after HC damage. In this case, increased Notch activity may be required to respecify progenitor cells (SCs) toward a sensory fate, as occurs during early development. In any event, once the function of Notch signaling is elucidated in the quiescent and damaged chicken inner ear, it will be important to investigate the degree to which Notch signaling is active in the mature mammalian inner ear after damage and which roles Notch plays in regulating SC responses after HC damage. Notch activity may limit the regenerative response in mammals, by preventing spontaneous SC transdifferentiation after HCs are lost or damaged.

Signals regulating SC division

A few studies have examined signals that trigger SCs to leave mitotic quiescence and enter the cell cycle. Although most evidence has been collected for vestibular SCs (reviewed in Oesterle and Hume, 1999; see below), some well known intracellular signaling pathways have been implicated in triggering SC division in the chicken BP. Using cochlear duct organ cultures, Navaratnam *et al.* (1996) showed that activation of adenylate cyclase increases the number of SCs that divide after Gentamicin exposure and modulation of this signaling leads to decreased SC division.

Using cultures of pure utricular epithelium, a study by Witte *et al.* (2000) implicates additional second messengers, including PI-3 kinase, TOR-kinase and MAP-kinase, in the signaling cascade leading to SC division. Which extracellular factors might alter these signaling pathways? Addition of basic FGF (bFGF) to cultured cochlear ducts leads to decreased SC division (Oesterle *et al.,* 2000). Consistent with this, expression of FGF receptor3 is abundant in quiescent SCs of the BP and becomes highly decreased in areas where numerous SCs are dividing, suggesting that attenuated signaling through this receptor must occur before SC re-entry into the cell cycle (Bermingham-McDonogh *et al.,* 2001). In the avian vestibular epithelium, *in vitro* experiments demonstrate SC proliferation is inhibited by bFGF (Oesterle *et al.,* 2000), Retinoic Acid (Warchol, 2002), N cadherin (Warchol, 2006) and Dexamethasone (Warchol, 1999). In contrast, Insulin, Insulin-like Growth Factor 1, Transforming Growth Factor alpha and Tumor Necrosis Factor alpha have mitogenic effects on vestibular SCs, as does the extracellular matrix molecule, Fibronectin (Oesterle *et al.,* 1997; Warchol, 2002). At this point, effects of the various signaling molecules just described remain to be tested *in vivo*. Further, localization of critical ligands and receptors is required. These observations suggest that multiple signaling pathways interact in a SC to regulate its cell cycle progression. Important future challenges are to link extracellular signals with specific intracellular pathways and to determine which pathways are dispensable versus critical to mitogenic regeneration in chickens. This information will guide additional experiments in mammals toward identifying whether SC division is stalled due to the absence of mitogenic signals or due to the activity of inhibitory signals in the mammalian sensory epithelium after HC loss.

Differentiation in post-mitotic cells

Very little information exists about how post-mitotic cells acquire features of SCs during regeneration of the chicken BP. However, differentiation of cells along the HC pathway has been studied to some extent (e.g., Stone and Rubel, 2000b; Roberson *et al.,* 2004; Stone *et al.,* 2004; Duncan *et al.,* 2006; Cafaro *et al.,* 2007). For example, some of the earliest known markers of HC differentiation are Myosins VI and VIIa. In cochlear and vestibular end-organs of vertebrates, including chickens, immunolabeling has shown that Myosin VI and Myosin VIIa protein is specifically localized to HCs (Hasson *et al.,* 1997; Torchinsky *et al.,* 1999; Mangiardi *et al.,* 2004; Roberson *et al.,* 2004; Duncan *et al.,* 2006). MyosinVI protein has been documented as one of the earliest protein markers of HC differentiation in mouse embryos after *Atoh1* is upregulated (Montcouquiol and Kelley, 2003; Kelley, 2006). In the regenerating chick BP, HCs formed through either direct transdifferentiation or cell division show elevated levels of MyosinVI early during their differentiation. However, SCs undergoing direct transdifferentiation appear to take a relatively longer time to express MyosinVI compared to precursor cells formed by cell division. As discussed earlier, Atoh1 protein is detected in the nuclei of transdifferentiating SCs by 15h post-Gentamicin (Cafaro *et al.,* 2007). MyosinVI is detected in transdifferentiating SCs by 78h post-Gentamicin, or 63h later (Roberson *et al.,* 2004). In contrast, newly post-mitotic cells exhibit detectable levels of MyosinVI by 108h post-Gentamicin (Roberson *et al.,* 2004), which is only 36h after the first proliferating cells complete mitosis (at 72h). There are at least two possible explanations for this delay in Myosin expression in HCs arising directly from SCs. First, it may take a considerable amount of time for quiescent SCs to down-regulate SC-specific genes and to upregulate HC-specific genes. In contrast, newly formed post-mitotic precursor cells are probably in a very immature, labile state in terms of how readily new genes can be activated and accordingly, they are able to rapidly proceed down the HC differentiation pathway. A second explanation is that completion of cell fate specification and differentiation is delayed in early transdifferentiating cells because injured HCs remain in the epithelium, while in contrast, most injured HCs have been completely ejected by the time SC division is triggered. (The peak period of HC ejection occurs between 30h and 42h [Mangiardi *et al.,* 2004], which overlaps with initial SC transdifferentiation but precedes the initiation of SC division.) This form of regulation would suggest that dying HCs control the progression of SCs through the two regeneration pathways.

Controlling regeneration: balancing modes of regeneration and re-establishing SC quiescence

Sensory epithelia of the inner ear and lateral line neuromasts are highly specialized and their function depends upon the precise arrangement of HCs and SCs. Therefore, it is of utmost importance that the correct number and type of cells be re-established during regeneration. Direct transdifferentiation poses a specific problem, because each time a new HC is formed using this mechanism, a SC is lost from the epithelium. Tight regulation of direct transdifferentiation is essential, because, at a ratio of only 2-4 SCs per HC (Goodyear and Richardson, 1997), excessive direct transdifferentiation would lead to SC depletion. Since direct transdifferentiation is initiated early, it is tempting to hypothesize that SC conversion into HCs has evolved as an early, rapid way to make new HCs if only a few are lost. However, if a large number of HCs die, then the cochlea must activate mitosis to maintain the integrity of the sensory epithelium. The selective differentiation of post-mitotic precursors into SCs would counteract SC depletion to direct transdifferentiation. SC divisions can generate either HCs or SCs (Raphael, 1992; Stone and Cotanche, 1994) and during early stages of SC proliferation, each mitotic event is equally likely to give rise to symmetrically differentiating pairs of daughter cells (2 SCs or 2 HCs) or an asymmetric pair (1 HC and 1 SC; Stone and Rubel, 2000b). However, specific temporospatial patterns of mitotic regeneration have not been characterized at different periods of regeneration, so it is not clear at this time whether mitotic regeneration is sufficient to compensate for direct transdifferentiation or if other mechanisms (e.g., cell death, cell rearrangement, or immigration of cells from outside the epithelium) are also involved.

Inhibition of SC activity in the BP is also highly important for establishing the correct number and type of new cells. Mechanisms for halting regenerative behavior in SCs have received considerably less attention than those initiating it and are therefore poorly understood. One attractive control that has been documented in other sensory epithelia is a negative feedback mechanism, whereby regenerated HCs and/or SCs inhibit nearby SCs from further division or transdifferentiation (e.g., see Bermingham-McDonogh *et al.,* 2001). Negative feedback may

indeed be active in the regenerating inner ear epithelium. It is clear, however, that if negative feedback for SC division were derived from regenerating HCs, then the signaling HCs would be quite immature, because SC division becomes highly attenuated before well differentiated new HCs emerge in the epithelium. The peak of BrdU uptake during regeneration occurs at 72h after a single Gentamicin injection and this begins to taper off by 96h and is greatly reduced by 120h (Bhave *et al.,* 1995; Duncan *et al.,* 2006). The first identifiable markers of HC differentiation in newly divided cells (Myosin VI and TUJ1) appear at 96-108h, just about the same time that BrdU labeling begins to decrease.

Comparisons with mammals

Mammalian inner ear SCs are similar to those in birds in that they share a common progenitor cell with HCs during development and they remain in close contact with HCs in maturity. Yet, SCs of the mammalian organ of Corti fail to show any regenerative response to HC loss, via either direct transdifferentiation or mitosis (e.g., see Forge *et al.,* 1998). In contrast, there is evidence that considerable spontaneous regeneration of HCs does occur in the mammalian vestibular epithelium, most likely through direct transdifferentiation (Forge *et al.,* 1998).

Several studies suggest that the capacity to regenerate HCs via either mitotic or non-mitotic avenues is lost from the mammalian organ of Corti over the course of post-embryonic maturation. For example, cultured SCs from the early post-natal organ of Corti show robust proliferation and a fraction of post-mitotic cells can differentiate into new HCs (Malgrange *et al.,* 2002; Doetzlhofer *et al.,* 2004; White *et al.,* 2006), but this capacity is lost with time (White *et al.,* 2006; Oshima *et al.,* 2007). It is not clear if this developmental change reflects an intrinsic shift in SC properties or an absence of appropriate mitogenic signals in maturity. However, recent studies provide support for the former hypothesis. During embryogenesis, cells in the embryonic cochlear epithelium upregulate expression of the cyclin-dependent kinase inhibitor, $p27^{kip1}$, around the time that terminal mitosis is beginning (Chen and Segil, 1999; Löwenheim *et al.,* 1999). Levels of $p27^{kip1}$ protein remain robust in differentiated SCs in the mature organ of Corti, suggesting that $p27^{kip1}$ imposes strong inhibition on cell cycle progression in mature SCs and may prevent them from dividing in response to HC loss. Deletion of the *$p27^{kip1}$* gene supports this hypothesis, since knockout mice show extended periods of developmental progenitor cell division and supernumerary production of both HCs and SCs *in vivo* (Löwenheim *et al.,* 1999; Chen and Segil, 1999) and increased rates of proliferation *in vitro* (White *et al.,* 2006). Similar results are seen when the negative cell cycle regulator, Rb, is deleted (Sage *et al.,* 2005; Sage *et al.,* 2006). Although loss of cell cycle inhibitors also results in increased apoptosis, these knockout experiments indicate that the inhibition of SC proliferation seems to be a major step in blocking HC regeneration in the mammalian cochlea. Similarly, SCs may lose the ability to directly convert into HCs over development. Kelley *et al.* (1995) showed that new HCs are generated in the mouse organ of Corti prior to E16 when existing HCs are ablated using a laser, but this capability is lost after E16. In post-natal rats, treatment with the otoxin, Amikacin, causes a large HC lesion and leads to the emergence of cells that resemble HC-SC hybrids (Daudet *et al.,* 1998). While these cells have been hypothesized to be SCs that have initiated direct transdifferentiation, they fail to differentiate many HC features. Together, these findings suggest that the ability of auditory SCs to undergo direct transdifferentiation is lost over development. Mature SCs may be too differentiated to undergo direct transdifferentiation, or there may be signaling mechanisms that prevent it. As we begin to understand more about how the SC to HC conversion is regulated in the avian BP, we will be able to better dissect the degree to which SCs initiate direct transdifferentiation in the mammalian organ of Corti.

A few studies suggest that HC regeneration may occur in the vestibular epithelium of mature mammals. For example, Forge *et al.* (1998) performed careful morphologic analyses of vestibular epithelia in guinea pigs following ototoxic drug treatment at different time-points. Hair cells appeared to be completely lost from the striolar region one-to-two weeks after treatment. However, HC numbers were increased in this region by 2-4 weeks after treatment. Since little SC division occurs spontaneously after HC damage in the utricle of adult guinea pigs (Rubel *et al.,* 1995), new HCs likely arose through direct transdifferentiation of SCs. This interpretation is supported by the finding that SC numbers decreased after recovery, which is predicted to occur if SCs had converted into HCs without mitotic replacement. In addition, studies of cultured vestibular end organs show that addition of serum or growth factors significantly upregulates SC division in the mature vestibular epithelium, *in vitro* (Warchol *et al.,* 1993; Lambert, 1994; Yamashita and Oesterle, 1995; Hume *et al.,* 2003) and *in vivo* (Kuntz and Oesterle, 1998). However, it remains to be clearly demonstrated if a significant number of post-mitotic cells is able to differentiate into HCs.

Summary and future directions

Studies of non-mammalian vertebrates, birds in particular, have revealed several important features of the cellular and molecular processes associated with development and regeneration of sensory hair cells (HCs). Investigations of progressive morphological and molecular changes that occur in the damaged sensory epithelium have begun to pinpoint intracellular signaling pathways that may regulate HC apoptosis and trigger surrounding non-sensory, supporting cells (SCs) to become activated to leave quiescence and to de-differentiate toward a more primitive progenitor-like state. Additional candidate regulatory molecules, including diffusible factors and transcription factors, then direct SCs to undergo phenotypic conversion into HCs or to divide, forming progeny that differentiate into new HCs and SCs. Despite this progress in our understanding, considerable work remains to be done in defining critical signaling pathways involved in these stages of regeneration. In particular, signals that maintain SCs in quiescence in the undamaged state and/or retain stem-like progenitors during regeneration must be identified. Further, we must determine the degree to which SC behavior after HC damage is determined by intrinsic cellular changes versus locally released signals. These aspects of cellular regulation are highly relevant to determining why mammalian SCs generally remain in a quiescent state after HC loss.

Recent advances in techniques used to study non-mammalian HC regeneration promise to broaden our understanding of molecular signaling required to trigger SCs to generate new HCs. For

example, two recent studies (Hawkins *et al.,* 2003; Hawkins *et al.,* 2007) used genomic profiling to identify a large number of genes that are altered in the avian auditory and/or vestibular epithelium after HC damage. This characterization was accomplished with gene microchips, which define relative levels of tens of thousands of transcripts in a given tissue and allow one to compare levels of expression across tissue samples. Microchip analyses enable the identification of molecular markers and signaling pathways heretofore unknown to be relevant for HC regeneration. These studies have confirmed differential expression levels of some candidate genes using quantitative polymerase chain reaction and *in situ* hybridization, which provides additional assurance of the validity of the approach. Follow-up technologies must now be developed to enable investigators to accept or reject hypotheses regarding the role of specific candidate genes in HC regeneration. Two examples of these are gene knock-down and gene misexpression. Inhibition of gene function through knockdown can be accomplished with RNA interference or RNA antisense approaches. Gene misexpression can be accomplished through delivery of conditionally or constitutively active genes into cells of interest. To our knowledge, no studies involving genetic perturbation in the post-embryonic amphibian or avian inner ear have been performed. However, delivery of modified nucleic acids into the developing chicken otocyst has been accomplished using retrivirus (e.g., Fekete *et al.,* 1998), electroporation (e.g., Daudet and Lewis, 2005) and antisense morpholinos (Gerlach-Bank *et al.,* 2004). The challenge is now to bring these methods for gene transfection and transduction into tissues of the mature inner ear.

Another new area of study in the genetics of HC loss and regeneration has recently been developed in zebrafish, a classic model for genetics experiments. Hair cells of the lateral line neuromast are positioned along the external body wall of these fish. Neuromasts are therefore highly accessible to aminoglycosides and allow visual monitoring during HC damage and regeneration. Recent studies have investigated lateral line HCs and SCs after treatment with the aminoglycoside antibiotic, Neomycin (e.g., Williams and Holder, 2000; Harris *et al.,* 2003). At this point, published studies have characterized only the basic morphologic events that occur. Nonetheless, these studies provide proof of concept that the behavior of lateral line HCs and SCs after aminoglycoside exposure is highly analogous to the inner ear of birds and amphibians. One group (E.W. Rubel, D. Raible *et al.*) are now conducting mutagenesis studies in zebrafish aimed at identifying genes that confer either protection or increased susceptibility of lateral line HCs to Neomycin, as well as genes that alter the regenerative process after Neomycin exposure. In addition, parallel studies are screening small molecule libraries to identify drugs that alter HC damage after Neomycin. It would be interesting to determine the degree to which blockade of HC loss at various stages of the process alters SC responses. This information could help to pinpoint which HC changes must occur in order for SCs to become activated and initiate HC regeneration.

In conclusion, since the discovery of HC regeneration in birds over 20 years ago, significant progress has been made in characterizing HC progenitors and their activities following HC loss in the inner ear of mature non-mammalian vertebrates. It is anticipated that future studies of molecular interactions governing HC development will continue to point the way for investigators interested in unraveling regulation of post-embryonic HC production. In addition, pioneering experimental approaches just described, as well as others, should help to identify new genes of interest and to reveal their function during HC regeneration.

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