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A historical to present-day account of efforts to answer the question, “What puts the brakes on mammalian hair cell regeneration?”

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Abstract

Hearing and balance deficits often affect humans and other mammals permanently, because their ears stop producing hair cells within a few days after birth. But production occurs throughout life in the ears of sharks, bony fish, amphibians, reptiles, and birds allowing them to replace lost hair cells and quickly recover after temporarily experiencing the kinds of sensory deficits that are irreversible for mammals. Since the mid 1970's, researchers have been asking what puts the brakes on hair cell regeneration in mammals? Here we evaluate the headway that has been made and assess current evidence for various alternative mechanistic hypotheses that have been proposed to account for the limits to hair cell regeneration in mammals.

The title question arose in 1974 when the senior author was a first-year graduate student and fortuitously discovered that the ears of sharks add hundreds of thousands of new hair cells throughout life. That finding naturally led to the prediction that regenerative repair of hair cell deficits might be possible in such animals, and it raised the possibility that research on hair cell regeneration, which has been the primary focus of our lab since its start in 1981, might eventually reveal how to overcome whatever was responsible for the permanence of clinically prevalent hearing and balance deficits caused by hair cell loss (Corwin, 1977; Corwin, 1978; Corwin, 1981; Corwin, 1983; Corwin, 1985a; Corwin, 1985b; Corwin, 1986). When Douglas Cotanche presented striking scanning electron microscope images at the meeting of the Association for Research in Otolaryngology in February of 1986 and 1987, many others began to join in the effort and some expanded the interpretation of older data (Cruz et al., 1985; Cruz et al., 1987). Cotanche's SEM images brought greater credibility to the idea that hair cell regeneration was worthy of study, because they provided vividly clear and incontrovertible evidence that rapid and remarkably complete self-repair had occurred in chicken auditory epithelia within days after they had been damaged by loud sound

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(Cotanche et al., 1986; Cotanche, 1987b; Cotanche, 1987a). Evidence from subsequent research has shown that the self-repair in chickens is the result of cell replacement and that numerous non-mammalian species replace lost hair cells spontaneously. Yet, the title question still remains to be answered. This article reviews evidence that appears to have brought us closer to an answer, and it outlines some pieces of the puzzle that have not been addressed.

Prior to the discoveries in shark ears and chickens, it had long been known that salamanders regenerate lateral line organs when they regrow amputated tails (Stone, 1933; Stone, 1937; Speidel, 1947; Wright, 1947). Results from histology and scanning electron microscopy also had suggested that amphibian ears could add limited numbers of hair cells during postembryonic life (Alfs and Schneider, 1973; Lewis and Li, 1973; Lewis and Li, 1975). Nevertheless, convincing evidence showed that mammalian ears were different. In rodents, hair cell production peaks during the second half of gestation and sharply declines by birth (Fig. 1; Ruben, 1967; Sans and Chat, 1982; Mbiene et al., 1984). In addition, the permanence of many clinical forms of hearing impairment is consistent with the belief that inner ear hair cells could be produced in substantial numbers only before birth. In fact, recent investigations have confirmed that hair cell production occurs rarely at best in the ears of mature mammals (Lambert, 1994; Lambert et al., 1997; Kirkegaard and Nyengaard, 2005; Lee et al., 2006; Collado et al., 2011b; Lin et al., 2011; Burns et al., 2012c).

In contrast with mammals, the ears of sharks, rays, bony fish, amphibians, reptiles, and birds all can produce hair cells throughout life (Fig. 2; Corwin, 1981; Corwin, 1983; Popper and Hoxter, 1984; Corwin, 1985b; Jorgensen and Mathiesen, 1988; Lombarte and Popper, 1994; Kil et al., 1997; Goodyear et al., 1999; Severinsen et al., 2003). In some non-mammalian vertebrates, new hair cells arise and accumulate during lifelong growth of the body, while in others they are produced continually as hair cell populations turnover, or when the ear heals by spontaneously replacing hair cells that died as the result of acoustic trauma, poisoning, or other causes.

What prevents hair cell production and regeneration from occurring in mammals? Do mammals lack elements that are essential to the regenerative replacement of hair cells that occurs in non-mammals? Recent results suggest that might not be the case. Instead, it has been found that the supporting cells in mammalian ears develop unique characteristics as they mature, and it appears that those characteristics may block regenerative responses to hair cell loss. Such a block to regeneration may be loosely analogous to the way a parking brake blocks the movement of a car. This article will review evidence relating to the “parking brake hypothesis” and evidence for alternative hypotheses that hold the potential to account for differences between the regeneration responses that mammalian and non-mammalian ears exhibit after hair cell losses.

1.1 How do non-mammalian ears produce hair cells after birth?

Supporting cells appear to be the source of the hair cells that are produced in non-mammalian vertebrates during postembryonic life (Fig. 3). When 3H-thymidine was given to postembryonic sharks, bony fish, and amphibians whose ears were later preserved and examined, the label was localized to the nuclei of hair cells and supporting cells, which often occurred in isolated cell pairs that contained one small labeled hair cell and a labeled supporting cell (Corwin, 1981; Popper and Hoxter, 1984; Corwin, 1985b). Lineage tracing via time-lapse recordings after laser ablations of hair cells and after antibiotic treatments also has shown that supporting cells give rise to regenerated hair cells in lateral line organs (Corwin et al., 1989; Balak et al., 1990; Jones and Corwin, 1996; Ma et al., 2008).

Do supporting cells in non-mammalian ears need to dedifferentiate before they divide? If so, what differentiated characteristics do they shed, and are there identifiable cell-specific characteristics that they retain which could be used to identify those cells as they divide and for lineage tracing that does not require time-lapse imaging? Such questions may be answered definitively with the development of better molecular markers for supporting cells. In particular there is a need for stage-specific markers – some expressed in nascent supporting cells as they are differentiating and others expressed only after they have become fully differentiated.

Do sensory epithelia in the ears of non-mammalian vertebrates contain a special cell subpopulation comprised of undifferentiated stem cells residing in reserve amongst the fully differentiated supporting cells? If so, what characteristics identify those cells? Could the mature mammalian ear's failure to regenerate hair cells be due to irreversible differentiation or loss of such yet-to-be-identified stem cells, which are able to give rise to hair cells early during embryonic development in mammals? Or are the supporting cells in non-mammals able to dedifferentiate and function as stem cells more readily than their counterparts in mammals? Progress toward answers to questions like these has been constrained by the small size of otic sensory epithelia as well as the paucity of distinctive cytological characteristics and molecular markers that are known to identify differentiated supporting cells. For supporting cells in vestibular epithelia just a few have been described and better ones are needed. Fortunately, the situation is different for the organ of Corti, which is unique to mammals and where the epithelium contains five readily distinguishable types of supporting cells, with excellent markers available for some of the most highly differentiated types (reviewed in Cox et al., 2012).

In fact, for most auditory epithelia in non-mammalian vertebrates and the vestibular epithelia in all vertebrates it remains to be shown that the supporting cell populations contain reliably identifiable subtypes of supporting cells that differ in form or function. It is possible and likely that the population of cells, which are lumped together under the name “supporting cell” may harbor distinct subtypes that have yet to be identified.

The seminal 1987 article by Cotanche included the suggestion that mature supporting cells in chicken ears could respond to damage and give rise to replacement hair cells. That view was subsequently supported in an expanded interpretation of limited data from an earlier study of ototoxic damage in chickens (Cruz et al., 1985; Cruz et al., 1987), with the latter accepted for publication on 28 July 1987. It was also supported by conclusive evidence from microautoradiographic labeling studies in chickens and quail that used ^3H -thymidine, which served as a radioactive tracer that cells can pass to their progeny after they incorporate it in replicated DNA as they prepare to divide (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Stone and Cotanche, 1994), with the definitive radioactive thymidine results from Corwin and Cotanche first presented in June of 1987 (at an international meeting reported in Cremers et al., 1988). Subsequent laser microbeam ablation experiments in cultured chicken cochleas showed that the supporting cells that were within $\sim 200\ \mu\text{m}$ of small groups of lesioned hair cells were the first to label with 5-bromo-2'-deoxyuridine (BrdU), a non-radioactive thymidine analogue that can be used to label replicating DNA (Warchol and Corwin, 1996). Other experiments have shown that after aminoglycoside antibiotic poisoning of hair cells in the avian cochlea the nuclei of supporting cells downregulate G0 markers and pass through the same interkinetic migrations that progenitor cell nuclei pass through when hair cells are first produced in chicken embryos (Katayama and Corwin, 1993; Bhavé et al., 1995). Such evidence suggests that regeneration in the avian cochlea does not depend on a special population of reserve stem cells. Instead, it seems that many mature supporting cells in avian ears retain a latent capacity to dedifferentiate. Hair cell loss appears to reactivate that capacity, so that supporting cells reenter the cell cycle and produce

replacement cells that are able to differentiate as either hair cells or supporting cells. This occurs even in the avian cochlear epithelium where the supporting cells are mitotically quiescent in the absence of damage (Corwin and Cotanche, 1988). Some supporting cells in those epithelia are able to convert directly into hair cells, with cell fate choices controlled through Notch-Delta signaling interactions during both processes (Lewis, 1998; Daudet et al., 2009; Slattery and Warchol, 2010; Lewis et al., 2012).

Although the evidence covered thus far suggests that most, if not all, supporting cells in non-mammalian ears may be capable of giving rise to new hair cells, there are regional differences. Proliferating supporting cells and newly produced hair cells are more common near the outer edges of the continuously growing hair cell epithelia in elasmobranchs and amphibians, while proliferation occurs equally across growing hair cell epithelia of teleost fish and in the vestibular organs of birds where hair cell populations turn over (Lewis and Li, 1973; Li and Lewis, 1979; Corwin, 1981; Corwin, 1983; Popper and Hoxter, 1984; Corwin, 1985b; Jorgensen and Mathiesen, 1988; Popper and Hoxter, 1990; Roberson et al., 1992; Lombarte and Popper, 1994; Lanford et al., 1996; Kil et al., 1997; Goodyear et al., 1999). Similar to the embryonic growth pattern in the avian cochlea and the postembryonic growth patterns in elasmobranchs and amphibians, the first cells that exit the cell cycle in developing vestibular epithelia in rodents are located centrally, near or in the region that becomes the postnatal striola. Cells produced later occupy zones that expand concentrically towards the outside medial edge of the utricle (Sans and Chat, 1982; Katayama and Corwin, 1989; Denman-Johnson and Forge, 1999). In the utricles of newborn mice, there is a significant amount of cell proliferation inside the macula's lateral edge, and some proliferation in the medial striola (Burns et al., 2012c). Proliferation normally ends by the third day after birth, but the number of hair cells continues to increase for 12 days after birth as progeny from the earlier cell divisions differentiate (Figs. 1-2, 4). It remains for future research to determine whether the mechanisms and signals that sustain the temporary growth zone and proliferation of cells at lateral edge of the macula in newborn mice are related to those that operate in the growth zones and proliferation that persist in the maculae of fish and amphibians throughout life.

1.2 Do the supporting cell populations harbor a special subpopulation of reserve stem cells?

Experiments specifically designed to determine whether postembryonic production of hair cells in fish ears depends on a distinct subset of stem cells provided evidence consistent with the conclusion that most, if not all, supporting cells are able to give rise to hair cells (Presson et al., 1995). In that study the chemotherapy agent cytosine arabinoside (Ara-C) was used to kill proliferating progenitor cells. Although the Ara-C killed the preexisting progenitor cells throughout the hair cell epithelium, a new wave of proliferating cells arose within days in the treated epithelia, and those new progenitors, which are presumed to be supporting cells and not a special resident stem cell, replaced those progenitors that had been killed by the Ara-C and gave rise to new hair cells (Presson et al., 1995). In addition, a subsequent investigation localized replicating cells using methods that required light microscopy, then reembedded those specimens for a tour de force electron microscopic examination of the same replicating cells, showing that the progenitors of hair cells are ultrastructurally indistinguishable from mature supporting cells, in fish ears at least (Presson et al., 1996). Still, *in vivo* lineage tracing is needed to definitively determine whether subsets of supporting cells more readily act as hair cell progenitors than others.

Although the evidence reviewed above is consistent with the view that special stem cells are not required for the regeneration of hair cells that occurs in non-mammalian ears, experiments by Heller and colleagues have shown that the vestibular and cochlear sensory epithelia in rodents contain cells that are able to proliferate like neural stem cells when the

cells of those epithelia are dissociated and cultured in suspension. Such cultures give rise to clonal spheres that resemble neurospheres, and can be dissociated and serially passaged to generate more spheres, which give rise to hair-cell-like cells after adherence and growth factor removal (Li et al., 2003; Oshima et al., 2007).

To look for the source of the cells that exhibit stem cell properties after dissociation and culturing in suspension, cells from the mouse organ of Corti were isolated and sorted by FACS (fluorescence activated cell sorting; White et al., 2006; Sinkkonen et al., 2011; Chai et al., 2012; Shi et al., 2012). One study used labeling with different cell surface markers and FACS to separate four populations of cells from the organ of Corti in neonatal mice, pinpointing the cells of the Lesser Epithelial Ridge as those which are capable of giving rise to hair-cell-like cells when cultured with mitotically-inactive mesenchymal cells from the chicken ear (Sinkkonen et al., 2011). Others have examined cells in mouse ears that express Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5) and shown that they have the potential to give rise to hair cells and hair-cell-like cells *in vivo* and *in vitro* (Chai et al., 2011; Chai et al., 2012; Shi et al., 2012). Lgr5 is a Wnt target gene that has been used to identify one type of intestinal stem cell (Barker et al., 2007; Sato et al., 2009). Wnt signaling is important for the proliferative expansion of cells in the skin, the gut, and in early development of inner ear sensory epithelia in rodents and in non-mammals (Dabdoub et al., 2003; Koebernick et al., 2003; Stevens et al., 2003; Dabdoub and Kelley, 2005; Riccomagno et al., 2005; Ohyama et al., 2006; Jayasena et al., 2008; Lu and Corwin, 2008; Park and Saint-Jeannet, 2008; Sienknecht and Fekete, 2008; Sato et al., 2009; Jacques et al., 2012; Vendrell et al., 2012). Treatments with Wnt pathway agonists promote the proliferation of mammalian otic Lgr5+ cells in culture. Overexpression of β -catenin, a downstream component in the Wnt pathway, leads to proliferation in the neonatal organ of Corti itself (Chai et al., 2012). In the vestibular epithelia changes in Wnt signaling appear to influence the decline in proliferative potential that develops later as mammalian ears mature, but the results suggest that this is not the primary cause of the decline (Lu and Corwin, 2008).

2.1 The relative likelihood of regeneration in mammalian cochlear and vestibular organs

The organ of Corti presents a number of challenges for regeneration research as well as potential promise, since its third row of Deiter's cells and the inner pillar cells maintain expression of the Wnt pathway stem cell marker, Lgr5, even at adult ages (Chai et al., 2011; Shi et al., 2012). Amongst the challenges is the fact that the organ of Corti is a highly specialized sensory epithelium that is only found in the cochlea of mammals, where it provides frequency sensitivity ranges that are unmatched in the animal world (Fay, 1988). Also, in contrast to the vestibular epithelia, where dozens of hair cells and supporting cells appear functionally equivalent to their neighbors, individual cells in the organ of Corti have precisely tuned frequency-specific sensory functions that differ from cell to cell even amongst their close neighbors. With its inner hair cells arranged in a long single-file row, that is separated by pillar cells and a fluid-filled extracellular tunnel from three precisely arranged, close-packed rows of outer hair cells, the organ of Corti is one of the most highly ordered tissues known. Rows of specialized supporting cells also maintain intricate interrelationships with individual outer hair cells, and each cell's position along the longitudinal and radial axes influences its frequency sensitivity or that of its neighbors. The uniquely mammalian evolutionary origin of the organ of Corti, and the tight linkage between its precise cellular architecture and the tuned sensory functions of its individual cells suggest that this tissue may hold unusual challenges for studies of regeneration.

Much of what is known about signals that influence cell fate, proliferation, planar cell polarity, and cellular differentiation in developing hair cell epithelia has come from studies

of the embryonic organ of Corti. In part, that is because mutations that cause even minor disruptions to its highly ordered cellular architecture are readily recognizable and frequently cause hearing impairment (Cantos et al., 2000). Thus, from an evolutionary standpoint it seems likely that selective pressures would have favored redundancy in regulatory mechanisms so as to limit perturbations to the precise cellular organization in the organ of Corti, since even minor changes could lead to loss of sound sensitivity that has an extremely high adaptive value.

In addition, regeneration is often a messy process, where tissue organization is disrupted by the engulfment or jettisoning of cells and remodeling of intercellular junctions or other subcellular elements as cells dedifferentiate, proliferate, and become motile (Gale et al., 2002; Kalluri, 2009; Bird et al., 2010). Like embryonic development, regeneration can involve overproduction of cells in some cases, with a subset of the newly produced cells selected for survival and other cells dying or removed after the regenerative events have made considerable progress (Jones and Corwin, 1993; Jones and Corwin, 1996; Nikolaev et al., 2009; Eisenhoffer et al., 2012; Marinari et al., 2012). Disruptions such as these appear incompatible with the precision and high order of the organ of Corti.

In contrast to the organ of Corti, vestibular epithelia originated early in evolution of the vertebrates, with the three semicircular canals, the saccule, and the utricle all present in species derived from the first jawed vertebrates. Mammalian and non-mammalian vestibular epithelia also share many structural similarities, which has led to the suggestion that hair cell regeneration may be less difficult to unleash or stimulate in mammalian vestibular epithelia than in the mammalian organ of Corti. If the hypothetical braking mechanism that limits regeneration can be suspended in a mouse vestibular epithelium, that may be sufficient to allow proliferation and differentiation and the addition of hair cells that could be sufficient for restoring some sensory function, without requiring that regeneration reestablish the precise architecture required for sensory function in the organ of Corti. The best way to find out will be to identify and target the limiting factor or factors that may contribute to the braking function, so we will now review some of the most likely candidates in the following section.

3.1 What are the signals that drive proliferation and differentiation within the developing sensory epithelium that could be repressed by the brake?

The signals that guide hair cell production during development are likely to have important roles in regeneration responses, and it is conceivable that the repression of such signals in mammalian ears could contribute to putting the brakes on regeneration. Fibroblast growth factors (FGFs), insulin, insulin-like growth factors (IGFs), Wnts, bone morphogenetic proteins (BMPs), sonic hedgehog (Shh), thyroid hormone, and retinoids all appear to have roles in guiding the induction, specification, and patterning of cells that occurs as inner ear sensory epithelia develop during embryogenesis (reviewed in Kelley, 2006; Schimmang, 2007; Groves and Fekete, 2012). Those signals and signals in the Notch pathway control inner ear and hair cell development by regulating the expression and activity of transcription factors such as Pax2/8, Sox2, Dlx5, Otx2, Foxg1, Pou4f3, Gata3, and basic helix-loop-helix (bHLH) transcription factors (reviewed in Kelley, 2006; Bermingham-McDonogh and Reh, 2011; Murata et al., 2012; Pan et al., 2012). Atoh1, Neurog1, and Neurod1, are highly conserved bHLH transcription factors that are important for guiding otic epithelial cells to become hair cells and neurons (reviewed in Mulvaney and Dabdoub, 2012; Pan et al., 2012). Whether these signals have a role in the braking mechanism or might be used to overcome or bypass its effects remains to be determined at present, but recent investigations have provided insights into binding partners and signaling pathways that influence the actions of these transcription factors, which will be reviewed in the next section.

3.2 Could Notch signaling and the transcription factors Atoh1 and Sox2 be part of the brake?

The upregulation of Atoh1 precedes the generation of new hair cells in zebrafish lateral line organs and the auditory and vestibular organs of chickens, where experiments have shown that Atoh1 expression is antagonized by activation of the Notch pathway (Cafaro et al., 2007; Ma et al., 2008; Daudet et al., 2009; Slattery and Warchol, 2010; Lewis et al., 2012). When notch activity is blocked by treatment with a gamma-secretase inhibitor (GSI) or when Atoh1 is overexpressed in the neonatal mouse cochlea and utricle, substantial numbers of greater epithelial ridge cells and vestibular supporting cells differentiate as hair cells (Lanford et al., 1999; Kiernan et al., 2005; Brooker et al., 2006; Yamamoto et al., 2006; Doetzlhofer et al., 2009; Collado et al., 2011b; Zhao et al., 2011; Kelly et al., 2012; Liu et al., 2012a). But the effects of most of those treatments have not appeared to persist in undamaged inner ear sensory epithelia of mice that are two weeks old or older. However, when hair cells in adult mouse utricles have been killed a number of supporting cells upregulate Atoh1, and when hair cells are killed in organ cultures of adult mouse utricles and that is followed by an 18-day GSI treatment, significant numbers of supporting cells convert directly into hair cells (Collado et al., 2011b; Lin et al., 2011). Yet, there are limits to the number of supporting cells that respond in that way, and as in the cochlea, those limits appear to become pronounced as the ears of rodents mature during the first two weeks after birth (Collado et al., 2011b). These results, combined with the observation that Atoh1 is induced after damage in relatively few cells in the adult mouse utricle (Lin et al., 2011; Golub et al., 2012), suggest that the cell fate regulation that appears to be mediated by Notch signaling and Atoh1 expression is likely to be downstream of the brake that limits the plasticity of mature mammalian supporting cells.

Sox2 is an HMG (high mobility group) box transcription factor that has an important role in maintaining pluripotency and self-renewal capacity in cells. It also is one of the four transcription factors that can induce pluripotency in differentiated cell types (Takahashi and Yamanaka, 2006). Sox2 is expressed in supporting cells (Hume et al., 2007), and it has been found to cooperate with the transcription coactivator Eya1 and its partner Six1 to control the differentiation of neurons and hair cells in the inner ear through the regulation of the SWI/SNF chromatin remodeling complex (Ahmed et al., 2012b; Ahmed et al., 2012a). In the brain, Sox2 is expressed in neural stem cells that can self-renew and give rise to differentiated, post-mitotic neurons. That finding suggests that the continued expression of Sox2 that occurs in auditory and vestibular supporting cells of mammals may be linked to a latent, but persistent capacity for self-renewal and the production of postmitotic hair cells that is blocked by some other factor and may be more persistently blocked in mammals after the brake is set as the ear matures.

When hair cells in the utricle die in newborn mice, mammalian supporting cells divide and produce replacement hair cells *in vivo*, like supporting cells do in non-mammals, but in mice such repair responses disappear during the first postnatal week (Burns et al., 2012d). Current evidence provides an incomplete picture of the changes that may cause the rodent vestibular epithelium to lose plasticity early in postnatal life, but it appears that the changes persist in the cells of the epithelium itself, since age-related decreases in plasticity are observed even after hair cell epithelia have been delaminated from the underlying stroma and cultured as sheets (Gu et al., 1996; Gu et al., 1997; Zheng and Gao, 1997; Zheng et al., 1999; Montcouquiol and Corwin, 2001a; Montcouquiol and Corwin, 2001b; Gu et al., 2007).

3.3 Are cell cycle control proteins likely to be or contribute to the brake?

Cyclin dependent kinase inhibitors (CDKIs) and the pocket proteins, Rb1, Rb1/p107, and Rb12/p130 have critical roles in turning off the proliferation machinery as the cells in

eukaryotes stop DNA replication and cell division. One hypothesis is that these proteins restrict proliferation in a way that blocks regeneration in mammalian ears. Mutations and deletions of Rb1 and p19^{Ink4d} lead to aberrant cell cycle reentry in hair cells at embryonic and neonatal ages (Chen et al., 2003; Mantela et al., 2005; Sage et al., 2005; Sage et al., 2006; Weber et al., 2008). While p21^{Cip1}-null mice showed normal cell cycle exit, co-deletion of p19^{Ink4d} and p21^{Cip1} enhanced the low levels of proliferation observed in p19^{Ink4d} mutants (Mantela et al., 2005; Laine et al., 2007). When p27^{Kip1}, Rb1, or Rb12/p130 in supporting cells were individually mutated, deleted, or silenced by shRNA (short hairpin RNA), that caused aberrant cell cycle reentry at embryonic and early postnatal ages (Chen and Segil, 1999; Lowenheim et al., 1999; Lee et al., 2006; Ono et al., 2009; Rocha-Sanchez et al., 2011; Liu et al., 2012b). However, the limited increases in proliferation that have thus far resulted from eliminating the expression of these cell cycle genes declined with age or led to apoptosis or disorganization of the sensory epithelium, and impaired sensory function (Chen et al., 2003; Mantela et al., 2005; Sage et al., 2006; Laine et al., 2007; Weber et al., 2008; Ono et al., 2009; Huang et al., 2011; Oesterle et al., 2011; Rocha-Sanchez et al., 2011; Liu et al., 2012b).

Positive regulators of the cell cycle also could have influences on the brake since cyclin D1 expression declines as hair cells and supporting cells exit the cell cycle. Consistent with that notion, forced overexpression of cyclin D1 has been shown to trigger S-phase entry in hair cells and supporting cells in neonatal and adult mouse utricles (Laine et al., 2010; Lopenen et al., 2011). However, when a type 5 adenovirus was used to express cyclin D1 in a large fraction of the supporting cells in adult mouse utricles, only a few of the many cells that entered S-phase appeared to progress to mitosis and divide, which led the authors to conclude that there is some other intrinsic block preventing adult utricular supporting cells from progressing through proliferation (Lopenen et al., 2011).

It is likely that whatever is responsible for causing the postnatal decline in the proliferation capacity of mature mammalian supporting cells involves changes in cell cycle proteins, with the signals that coordinate transcriptional changes arriving from upstream through the actions of transcription factors like those from the *Myc* gene family. *c-Myc*, *N-Myc*, and *L-Myc* encode bHLH transcription factors that influence cell proliferation by regulating the expression of cyclin D1 and a constellation of other cell cycle proteins. Conditional deletion of *N-Myc* in the embryonic ear drastically reduces growth by inhibiting proliferation (Dominguez-Frutos et al., 2011; Kopecky et al., 2011). Conditional deletion of *c-Myc* has no phenotype, but forced expression of c-Myc in whole organ cultures of adult mouse utricles causes supporting cells to reenter the cell cycle (Dominguez-Frutos et al., 2011; Burns et al., 2012b). The results suggest that *Myc* genes and the cell cycle proteins they regulate may be downstream targets of a braking mechanism. When supporting cells in adult mouse utricles are forced to overexpress c-Myc that appears to be sufficient to bypass or overcome the “brake” that normally appears to prevent proliferation (Burns et al., 2012a). Other important transcription factors include Sox2, which directly activates p27^{Kip1} to maintain quiescence in inner pillar cells (Liu et al., 2012b), and Atoh1, which can stimulate proliferation after forced expression in cochlear supporting cells from neonatal mice (Kelly et al., 2012).

3.4 Other potential components of the brake

Other potential candidate “braking mechanisms” could include chromatin remodeling, endocrine signaling, and microRNA (miRNA) expression. Chromatin remodeling is likely to play a role in inner ear development and appears to be a suitable mechanism for installing a persistent change like the “setting of the brake.” Although there has not been adequate exploration of that possibility, chromatin remodeling has been shown to influence supporting cell proliferation and expression of E-cadherin in utricles from neonatal mice as

well as the proliferation of supporting cells in avian utricles (Lu and Corwin, 2008; Friedman et al., 2009; Slattery et al., 2009). Growth hormone treatments also have been shown to promote proliferation in the zebrafish inner ear (Schuck et al., 2011; Sun et al., 2011), and estrogen signaling has been implicated in inner ear regeneration and reviewed elsewhere. Members of the let-7 family of miRNAs are downregulated during hair cell regeneration in newts and may play a role in the direct conversion of supporting cells into hair cells (Tsonis et al., 2007). miRNA-183 family members are expressed in inner ear hair cells and neurons during development, and they appear to be necessary for hair cell differentiation (Lewis et al., 2009; Mencia et al., 2009; Sacheli et al., 2009; Soukup et al., 2009; Kuhn et al., 2011; Weston et al., 2011). miRNA microarrays and other high throughput profiling strategies are being employed together with knockdown approaches to identify other miRNAs expressed in the inner ear and to determine their function (Friedman et al., 2009; Wang et al., 2010; Elkan-Miller et al., 2011; Patel and Hu, 2012). Results to date suggest that miRNAs are likely to have important roles in hair cell production and regeneration, but it remains to be determined whether they may have roles in limiting the regenerative potential of mammalian hair cell epithelia.

3.5 A decreased capacity for shape change in supporting cells may restrict the regeneration of hair cells in mammals

One of the earliest responses to loss of hair cells that occurs *in vivo* is the expansion of the apical surfaces of supporting cells into and near the sites where hair cells have been lost (Forge, 1985; Cotanche et al., 1987; Cotanche and Dopyera, 1990; Marsh et al., 1990; Li et al., 1995). In the avian cochlea, the expansion of the supporting cell surfaces appears to precede cell cycle entry in those supporting cells, and their return to normal surface dimensions occurs as replacement hair cells and supporting cells differentiate (Corwin and Cotanche, 1988; Marsh et al., 1990). These data are all consistent with the hypothesis that shape change in supporting cells may be an important step in triggering the cell cycle reentry that occurs in non-mammalian hair cell regeneration.

The strong connection between cellular spreading and proliferation has been appreciated since early experiments by Folkman and Moscona (1978) reported compelling results obtained in their investigations of isolated epithelial cells in culture. Many subsequent studies have born that connection (Kulesh and Greene, 1986; Watt et al., 1988; Chen et al., 1994; Chen et al., 1997; Huang et al., 1998; Huang and Ingber, 2005; Liu et al., 2006; Liu et al., 2007). Studies of isolate sheets of hair cell epithelia from the ears of chickens, rats, and mice that were enzymatically delaminated from the underlying stroma through incubation in a thermolysin solution also show strong connections between supporting cell shape change and proliferation.

Thermolysin-isolated sheets from rodent vestibular maculae respond to insulin and neuregulin growth factor containing culture media with over 40% of the cells entering S-phase in 72 hrs, while utricular epithelia from 3-week-old rodents show little or no proliferation in such 72-hr experiments (Fig. 5; Gu et al., 1996; Gu et al., 1997; Zheng and Gao, 1997; Zheng et al., 1999; Montcouquiol and Corwin, 2001a; Montcouquiol and Corwin, 2001b; Gu et al., 2007). Also, when utricular epithelia from adult rats were cultured, their incidence of proliferation remained low in comparison to neonatal tissue, but when culture time for the adult epithelia was extended to a week or longer, some of the supporting cells began to change from columnar to spread cell shapes. After that spreading, the adult epithelia exposed to the neuregulin growth factor rhGGF2 showed 20-fold proliferation increases over controls, although those levels were still modest when compared with the levels in early neonatal epithelia, which rapidly spread when cultured (Gu et al., 1997; Gu et al., 2007).

Neuregulin growth factors ligate ErbB receptors, which appear to be expressed continuously in inner ear sensory epithelia, and insulin mediated enhancement of proliferation, which acts through a different intercellular signalling pathway exhibits a parallel age-related decline in proliferative response, so it is unlikely that the declines are caused by receptor downregulation (Zheng et al., 1999; Hume et al., 2003; Gu et al., 2007). Also, the reduced responsiveness observed in a range of extracellular contexts suggests that the changes that limit supporting cell plasticity become intrinsic to the cells of the epithelium itself, even though they may be influenced to develop by the extracellular environmental context in which the epithelium matures (Davies et al., 2007). Independent of what cues initiate the changes, it is understanding the subcellular mechanisms that control the postnatal decline and maintain the reduced plasticity and proliferative responsiveness of mature mammalian supporting cells that is likely to be fundamental to unleashing the mature mammalian ear's potential for hair cell regeneration.

Sheets of utricular sensory epithelia delaminated from the utricles of young rodents and sheets of sensory epithelia that were cultured after delamination from the utricles of chickens have shown many instances where proliferation is more common near the outer edges of the sheets where supporting cells changed most quickly from their normal columnar shapes to spread shapes as the sheets grew in culture (Montcouquiol and Corwin, 2001a; Montcouquiol and Corwin, 2001b; Witte et al., 2001). In following up on these observations, the age-related reduction in the propensity for murine utricular supporting cells to spread was documented in time-lapse microscopy (Davies et al., 2007). During 72 hrs of culture, supporting cells in sheets of sensory epithelia from E18.5 and P1 mice spread appreciably, but sheets from the ears of mice that were six days old or older exhibited much less cellular spreading and a much lower incidence of cell proliferation (Fig. 6). Age-related declines in spreading and proliferation were observed regardless of whether the epithelia were grown on fibronectin, collagen IV, or a mixture of entactin, collagen, and laminin. Yet, both spreading and proliferation were significantly reduced when P6 epithelia were cultured on laminin instead of fibronectin. The restriction of spreading also appeared to correlate with an age-related increase in the expression of integrin alpha 6, which normally heterodimerizes with integrin beta 4 in epithelia to function as the laminin receptor. Subsequent culture experiments demonstrated that delaminated sheets of utricle epithelium from chicken ears continue to spread and proliferate at high rates that are independent of whether they originated from the ears of hatchlings or fully mature adults (Fig. 6; Burns et al., 2008).

The healing of excision lesions in hair cell epithelia from whole utricles showed that the association between the spreading of supporting cells and their proliferation was not an artifact of culture on artificial substrates, since the age-related changes occurred in sensory epithelia that remained *in situ* on their native extracellular matrix (Meyers and Corwin, 2007). Time-lapse microscopic recordings of the changes in the epithelium that occurred shortly after the creation of excision lesions and BrdU labeling of proliferating cells showed that utricle sensory epithelia from embryonic day 18.5 (E18.5) mice closed excision wounds within 24 hrs with many supporting cells proliferating after wound closure. In contrast, when comparable excision wounds were made in utricles from mice that were 14 days old or older, the wounds remained open for the 48-hr duration of the experiments, eventually closing later. The closure of wounds that occurred in the younger utricles was driven by the formation and contraction of an actin purse string with the supporting cells that covered the lesion changing from columnar to spread shapes.

When P14 and older utricles were treated with lysophosphatidic acid (LPA) in order to stimulate myosin-II-mediated contraction of the actin purse string, wound closure and cellular shape change occurred more rapidly than in controls. Furthermore, the wound

closure that was stimulated in those older LPA-treated utricles resulted in increased BrdU labeling of supporting cells that changed from columnar to spread shapes. In fact, quantitative analysis showed that after the LPA treatments the mature supporting cells that had spread to planar area that was greater than $300\ \mu\text{m}^2$ had an 85% of probability for entering S-phase, whereas only 10% of those supporting cells that had apical planar areas less than $100\ \mu\text{m}^2$ entered S-phase (Meyers and Corwin, 2007).

Additional experiments examining wound healing *in situ* showed that avian utricles exhibit rapid, age-independent wound closure that is followed by robust proliferation, and adult mouse utricles can eventually close wounds after 72 hrs, even without LPA stimulation. Similar to the supporting cells in LPA-treated utricles from juvenile mice, supporting cells in utricles from adult mice had an 86% probability of entering S-phase when they spread to an apical planar area greater than $300\ \mu\text{m}^2$. However, supporting cells in neonatal utricles only required two-fold changes in apical diameter to elicit similar proliferative responses. This shows that the signals that are associated with large degrees of cellular shape change are powerful enough to override whatever CDKIs and other cell cycle controls are expressed in supporting cells in the utricles of adult mice (Collado et al., 2011a). It also shows that the amount that supporting cells expand their apical area to reseal the epithelium when hair cells die may be insufficient to elicit a proliferative response in an adult mammal. Thus, the mechanisms that limit regeneration may partially suppress the mechanoreceptors and signaling networks that sense or respond to cellular shape change.

3.6 Is the brake connected to specializations of the intercellular junctions of mammalian supporting cells?

The tight linkage between cellular shape change and proliferation pointed to the need to understand what was restricting shape change as mammalian supporting cells were becoming mature during postnatal life. A preliminary clue was that phalloidin labeling of F-actin in the sensory epithelia in utricles from older mice was much more intense than the labeling in the sensory epithelia in neonates, and much more intense than that in the non-sensory epithelium that surrounds the macula (Meyers and Corwin, 2007). Subsequent measurements showed that the circumferential assemblies or “belts” of F-actin that bracket the junctions between utricular supporting cells increase in thickness 1,300% as mice age postnatally (Figs. 7-9). The belts grow so large that they fill 89% of the adult supporting cell at the level of its intercellular junction (Burns et al., 2008). Similar-sized belts were found when adult human vestibular epithelia were examined, but no cells in the non-sensory epithelia of the ear and no cells outside of the ear appear to contain actin belts that come close to showing the massive thickening of the circumferential F-actin belts that develop in supporting cells as mammalian ears mature. The thickening of those F-actin belts in murine supporting cells is nearly perfectly correlated with the age-related reductions in the cellular spreading and proliferation that the supporting cells in murine utricular epithelia show in culture ($r = -0.985$ and $r = -0.975$, respectively Burns et al., 2008).

In contrast with mammals, actin belts in the utricular supporting cells of chickens remain thin from hatching through adulthood, consistent with their lifelong capacity for spreading and proliferation (Fig. 6-7). Further analysis has shown that vestibular supporting cells in sharks, bony fish, frogs, turtles, and birds, all contain F-actin belts that remain thin even in adults (Fig. 9; Burns et al., 2012a).

In other epithelial cells, adherens junction molecules associated with circumferential belts like E-cadherin, beta-catenin, and p120 catenin can translocate to the nucleus where they alter transcription and promote or inhibit proliferation and differentiation (Matter and Balda, 2003; McCrea et al., 2009). It is therefore conceivable that belt reinforcement may restrict junctional molecules from shuttling to the nucleus, thereby limiting their ability to bind

transcriptional elements that induce cell cycle entry and cell fate changes. Actin itself may be a critical signal, since it has recently been established that depletion of globular β actin is a key mediator of quiescence in epithelial cells (Spencer et al., 2011). Alternatively, if belt reinforcement does not restrict the function of junctional molecules that positively regulate cell fate choices, or through the effects of actin itself, the signals that lead to F-actin accumulation could stimulate overexpression of junctional molecules that negatively inhibit proliferation and differentiation. Consistent with that notion, at supporting-cell-supporting-cell junctions in mice, E-cadherin, the prototypical membrane adhesion protein and an important tumor suppressor, increases by six-fold from P1 to adulthood (Collado et al., 2011b). Similar reinforcement of junctional E-cadherin is also observed in humans, while E-cadherin expression remains low or absent throughout life at supporting cell junctions in fish, frogs, turtles, and chickens (Warchol, 2007; Collado et al., 2011b; Burns et al., 2012a). It remains to be seen whether experimental results will support the hypothesis that the exceptionally thick and stable junctional specializations in mammalian supporting cells cause them to be less responsive or able to participate in regeneration, but a possible model for such a mechanism is outlined below.

3.7 The potential braking effect of increasing the mechanical stiffness of the supporting cells

In the excision-wounded utricles from neonatal mice, many supporting cells that were distal to the initial wound edge changed shape and participated in closure (Collado et al., 2011a). In adults, however, only supporting cells that were at or near the wound edge participated in the closure of the epithelial hole by spreading into the lesioned area. Since only a small number of cells participated in closure in such adult specimens, those that did had to spread to large areas before the excision area was reepithelialized. The lack of shape change in cells that are two or three rows back from the edges of such wounds suggests that supporting cells in adults are probably more resistant to deformation than those in utricles of juvenile mice. It is likely, but not established, that an increase in resistance to deformation would result from the unique and massive thickening of the F-actin assemblies that are associated with the intercellular junctions in adult supporting cells of mammals. It is also interesting that the adult supporting cells that change from columnar to spread shapes as they participate in closing wounds have much thinner actin belts when compared to the neighboring supporting cells that do not spread. And it is those spread supporting cells that enter S-phase, label with BrdU and proliferate (Meyers and Corwin, 2007; Collado et al., 2011a). Combined with the postnatal decline in the capacity for isolated murine utricular epithelia to spread on a variety of different substrates (Davies et al., 2007; Collado et al., 2011a), the results strongly suggest that supporting cells become increasingly stiffer with age, with clear effects on cellular functions that are necessary for repair.

Models predict that the majority of stress generated by a spreading epithelial sheet is transferred to the intercellular junctions, and just the increase in the diameter of the circumferential belts is estimated to significantly enhance their bending rigidity, priming them to resist deformation (Burns et al., 2008; Treppe et al., 2009; Vedula et al., 2012). Given the highly differentiated state of F-actin, microtubules, and intermediate filaments that has been documented in immunofluorescence and EM studies of supporting cells in the organ of Corti and vestibular organs, it is likely that other components of the supporting cell cytoskeleton become reinforced and contribute to enhanced stiffness as well (Slepecky and Chamberlain, 1983; Slepecky and Chamberlain, 1986; Raphael et al., 1993; Ogata and Slepecky, 1995; Kuhn and Vater, 1996; Leonova and Raphael, 1997). In fact, a recent study used atomic force microscopy (AFM) to investigate the Young's modulus of inner pillar cells in E18, P0, P3, and P5 mice and found that on average the stiffness of these cells increases with age, particularly at the borders between neighboring cells (Szarama et al.,

2012). Pharmacological manipulations indicated that the enhanced Young's modulus was almost entirely due to microtubules, and not actin filaments. Microtubules have a significantly greater persistence length and flexural rigidity compared to actin filaments (Gittes et al., 1993), which makes them the predominant load-bearing component in compressive tests like AFM. Therefore, stretch tests and rheometry may be more appropriate for revealing the important roles actin filaments must have in supporting cell mechanics. In addition, it is important to note that actin filaments in supporting cells from adult mouse utricles are largely unperturbed at the light microscopy scale after treatments with cytochalasin D and latrunculin A (Burns and Corwin, unpublished observation), so other non-pharmacological methods of perturbing the actin cytoskeleton may have to be utilized to assess its contribution to supporting cell stiffness.

Increased supporting cell stiffness might be responsible for increased resistance to shape change, and could also account for the decreased sensitivity of adult supporting cells to the small shape changes that appear to be sufficient to promote proliferation in supporting cells from mice at younger ages. External forces can be transmitted to distant locations within a cell, which affects signaling and alters transcription (Ingber, 2000; Ingber, 2008). The Linker of Nucleoskeleton and Cytoskeleton (LINC) complex is one mechanism by which cells transmit this so-called “action at a distance” mechanotransduction signal (Lombardi and Lammerding, 2011; Martins et al., 2012). LINC complex proteins connect and transmit forces between the actin cytoskeleton and the nuclear membrane, and they are essential for interkinetic nuclear migration that occurs during cell division in retinal photoreceptor cells and neurons (Zhang et al., 2009; Yu et al., 2011). Increased cellular stiffness could dampen the input to sensors like the LINC complex and this could drastically inhibit the ability to detect mechanical stresses created during hair cell death and removal. Also, the formation of the massively thick F-actin assemblies in the supporting cells of maturing mammalian ears may serve to reduce the levels of globular actin resulting in its depletion from the nucleus, which appears to be sufficient to cause epithelial cell quiescence (Spencer et al., 2011).

In birds, time-lapse recording have shown that the process of hair cell loss is strikingly dynamic, with rapid deformations and active participation of the supporting cells that surround the dying hair cell (Bird et al., 2010). Our preliminary results suggest that this process is also dynamic in newborn mice, but is significantly less so in utricle from adults. Consistent with our hypotheses derived from observations of sensory epithelial spreading, the compliance of avian supporting cells also appears to translate to a larger scale since sensory epithelia from hatchling and adult chickens show an undiminished capacity to rapidly spread as explants, and they close excision wounds rapidly when on their native substrates (Burns et al., 2008; Collado et al., 2011a). Perhaps most compelling though are our recent investigations showing a lack of junctional reinforcement in five classes of non-mammals, which suggest that supporting cells in most regenerating non-mammals are more compliant and capable of mechanosensing a hair cell death event since only mammalian supporting cells develop extremely reinforced and nearly solid F-actin assemblies at the level of the intercellular junctions (Burns et al., 2012a). Yet, vestibular supporting cells in at least one species of lizard, *Anolis carolinensis*, possess a reticulated network of F-actin that extends far from the intercellular junction in adult supporting cells (Fig. 9). While wide in extent, the structure of the belts in *Anolis* supporting cells is reticular and porous and therefore distinctly different from the structures in mammalian supporting cells (Burns et al., 2012a). Evidence for hair cell regeneration in one other species of lizard has been presented, but the extent of functional sensory recovery remains unclear, and it is possible that the extent of regeneration could vary among non-mammals (Avallone et al., 2003; Avallone et al., 2008).

4. Conclusion

Since the 1970's it has been recognized and the evidence has grown clearer that whatever provides the brake to regeneration in mammalian ears must differ in the ears of non-mammalian species that are able to make hair cells and replace dead ones throughout life. Fossils of the earliest jawed fishes from more than 450 ma have shown that they possessed inner ears with three semicircular canals and the utricular and saccular organs in the form that has been retained in their descendants, including all sharks, bony fish, amphibians, reptiles, mammals, and birds. Hair cells are highly specialized and the mechanisms that form them are unlikely to have evolved independently more than once in the vertebrates. Therefore, it is likely that the major cellular processes, protein expression patterns, and interacting regulatory signaling events in the various signal pathways required to build a hair cell will have been largely conserved throughout vertebrate evolution. Such conserved mechanisms of formation are likely to be observed, independent of whether the hair cells are produced in the embryonic ear of a mammal or the ear of an adult non-mammalian species that naturally adds hair cells during continuous growth or in replacing lost cells. It therefore seems reasonable to expect that the principal elements of the intercellular mechanisms and molecular interactions that control the activation and inhibition of the cycling in hair cell progenitors, the establishment of cell fate choice in their progeny, and their differentiation will be shared amongst mammals and non-mammals. Thus, the key to identifying and understanding the brake that prevents hair cell regeneration in mammals is likely to be a difference in a secondary, or lesser element or combination of elements that operate in those processes in mammalian and non-mammalian ears, rather than a difference in a major element of such a process.

Substantial progress has been made in tests that have definitively established that the inhibition of Notch signaling and the forced expression of a major hair cell transcription factor Atoh1 can cause numerous supporting cells to convert or differentiate as hair cells in young rodent ears, and in adult ears in more limited numbers. It also has been shown that knockout of the a major cell cycle inhibitor protein p27kip1 can cause embryonic mammalian ears to make more hair cells during their development and that overexpression of the major cell cycle protein cyclin D1 in adult utricular supporting cells can cause them to reenter the cell cycle and replicate DNA, but neither of those manipulations appears to allow adult supporting cells to progress completely through the processes to produce substantial numbers of new hair cells in adult ears.

Although none of those major elements are known to differ between the regeneration capable ears of non-mammals and the mammalian ear, and few have been assessed in that way, non-mammalian and mammalian inner ears have been found to differ in two secondary characteristics of the cytological differentiation of the intercellular junctions in supporting cells. Mammalian vestibular supporting cells continue to thicken the assemblies of F-actin that form circumferential belts associated with the intercellular junctions in all epithelia, but in these cells thickening fills nearly 90% of the cell at the level of its intercellular junction. There also is a near perfect correlation between the thickening of those actin assemblies and mammalian supporting cells' capacity to proliferate. Supporting cells in mammals and non-mammals differ as well in their expression of E-cadherin at those supporting-cell-supporting-cell junctions, another secondary differentiation characteristic. Still, correlation is not equivalent to causation, and it remains to be determined whether either of these secondary differences might be components of the brake.

Is there a quiescent stem cell population residing as supporting cells or amongst the supporting cells in the inner ears of adult mammals? Could such cells be poised to become responsive if the brakes are identified and targeted for temporary suspension? Or do stem/

progenitor cells that produce hair cells during the development of mammalian ears lose that capacity as they proceed through differentiation as a result of a brake mechanism that comes about through chromatin modifications? Experiments on supporting cells in lesioned utricles of adult mice suggest that may not be the case, since they readily reenter the cell cycle if they change shape and expand in the diameter of their apical domains by four-fold. At present the identity of the brake remains to be determined, but it seems highly likely that an understanding of the brake will be important for efforts to learn how to unleash the mammalian ear's potential to regenerate hair cells.

List of Abbreviations

AFM	atomic force microscopy
Ara-C	cytosine arabinoside
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
CDKI	cyclin dependent kinase inhibitor
E#	embryonic day #
FACS	fluorescence activated cell sorting
F-actin	filamentous actin
FGF	fibroblast growth factor
GSI	gamma-secretase inhibitor
HMG	high mobility group
IGF	insulin-like growth factor
Lgr5	leucine-rich-repeat-containing G-protein-coupled receptor 5
LINC	Linker of Nucleoskeleton and Cytoskeleton
LPA	lysophosphatidic acid
P#	postnatal day #
miRNA	microRNA
rhGGF2	recombinant human glial growth factor 2
SEM	scanning electron microscope
Shh	sonic hedgehog
shRNA	short hairpin RNA
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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Highlights

- A past to present-day review of studies on hair cell regeneration is presented.
- Early work showed that supporting cells give rise to new hair cells in non-mammals.
- Recent evidence suggests mammalian ears may harbor reserve stem cell populations.
- Hypotheses for mechanisms that limit hair cell regeneration in mammals are proposed.

Figure 1.

Cell cycle exit proceeds in precise spatiotemporal patterns in the murine cochlea and utricle. Top: Confocal images of cochlear whole mounts from embryonic day 12.5 (E12.5), E13.5, and E14.5 CD-1 mice that were killed 2 hrs after a single BrdU injection. Antibody labeling for BrdU is shown in red. White dots indicate the regions within the cochlear duct that did not label with BrdU, where cells have presumably exited the cell cycle. Cell cycle exit progresses along an apex-to-base gradient. Images modified from Lee and Segil (2006). Middle: Confocal images of whole mount utricles from E17.5, postnatal day 0 (P0), and P4 Swiss Webster mice that were labeled with antibodies to Ki-67 (red), a protein expressed at high levels in actively cycling cells. The white dashed lines outline the sensory epithelium. During embryogenesis, cells first appear to become quiescent in the lateral-striola. By birth, most cells near the medial edge of the sensory epithelium have also exited the cell cycle. The lateral edge is the predominant site of postnatal proliferation, and to a lesser extent, the

medial-striola. At P4, significant levels of proliferation are still detected on the nonsensory-side of the lateral sensory-nonsensory border. Image of E17.5 utricle modified from Burns et al. (2012b). Image of P0 utricle modified from Burns et al. (2012c). A region in the upper-left corner of this image that showed Ki-67 labeling in the nonsensory regions of the adjoining anterior and lateral cristae has been selectively deleted to facilitate comparison with the other images. Bottom: Graphs of the mean number of labeled cells in the sensory regions of cochleas and utricles from CBA-J mice that were given a single injection with 3H-thymidine at the indicated ages, and then were killed and processed for autoradiography at adulthood. Data in graphs obtained from Ruben (1967). Ruben reported labeling for individual cell types, so each data point for the cochlea is a sum of the number of labeled inner hair cells, outer hair cells, inner supporting cells, Deiter's cells, inner pillar cells, and outer pillar cells, and each data point for the utricle is a sum of the number of labeled supporting cells and hair cells.

Figure 2.

The maculae of non-mammals continue to expand and add hair cells into adulthood, whereas macular expansion and hair cell addition is limited after the first two postnatal weeks in mouse utricles. Left: representative outlines of sensory epithelia within the macula neglecta auditory detector of the ray (*Raja clavata*), the saccule of tadpole larvae and postmetamorphic adult bullfrogs (*Rana catesbeiana*), the utricle of E7-18 embryonic and posthatch 2- to 112-day-old chickens (*Gallus gallus*), and utricles of E18.5 and postnatal, P0 to P80 mice (*Mus musculus*). Note the different scales for the outlines from each species. Macular outlines were re-drawn from Corwin (1983) for rays, Lewis and Li (1973) for frogs, Goodyear et al. (1999) for chickens, and Burns et al. (2012c) for mice. Right: graphs of the

number of hair cells versus age or body length in each of these species. The graph of the data from chickens shows the hair cell production rate and death rate estimated from counts of total hair cell numbers (Goodyear et al., 1999) and the number of apoptotic cells observed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) at different ages (Kil et al., 1997). Hair cells continue to be added to the vestibular epithelia in adult chickens, but ongoing hair cell death is balanced with that production, so hair cells continuously turn over. Graphs for each species were obtained from the corresponding studies listed above, except the graph for anuran amphibians, which was obtained from determinations made in *Bufo marinus* (Corwin, 1985). T

Figure 3.

Hair cell loss can be repaired when supporting cells divide and produce new cells that can differentiate as replacement hair cells and in some cases when supporting cells convert directly into hair cells without an intervening passage through S-phase of the cell cycle. (A) is a schematic depiction of stages that occur in proliferative hair cell regeneration. When hair cells die (1-2), nearby supporting cells change shape as they spread into the spaces the dead cells had occupied (3). Supporting cell nuclei replicate DNA during interkinetic migration as they rise toward the apical surface where mitoses occur (3-5). It appears that supporting cells maintain contacts with the basal lamina throughout various stages in this process (Katayama and Corwin, 1993; Raphael et al., 1994; Loonen et al., 2011; Lewis et al., 2012). The progeny of the supporting cell divisions become hair cells or supporting cells and establish synaptic contacts with afferent neurons (5-6). (B) is a schematic depiction of stages in the conversion of a supporting cell directly into a hair cell without an intervening passage through S-phase. These diagrams from Meyers et al. (2009) have been modified to reflect current understanding.

Figure 4.

Hair cell production in the murine utricle begins and ends a few days after the onset and completion of terminal mitoses. The graph of cell cycle exit in the murine utricle from Fig. 1 was re-plotted as a smoothed line graph. Embryonic data points were taken from Ruben (1967) and postnatal data points were extrapolated from Burns et al. (2012c). The graph of hair cell numbers in the murine utricle in Fig. 2 was extrapolated to an x-intercept of E13, which is when hair cells are first observed in the murine utricle (Denman-Johnson and Forge, 1999), and was re-plotted as a smoothed line graph and overlaid on the graph of cell cycle exit.

Figure 5.

Supporting cells in the sensory epithelia from young rodents can respond to exogenous factors and exhibit robust proliferation. Compared to control sensory epithelia cultured for 72 hrs with 2.5% FBS (left), a 15 minute stimulation with 1 μ M forskolin followed by 72 hrs in 50 ng/ml rhGGF2 strongly increases the incidence of S-phase entry in sensory epithelia cultured from the utricles of 2-day-old rats (right). Nuclei that have replicated DNA and labeled with the thymidine analog, BrdU, are black. Images from Montcouquiol and Corwin (2001a).

Figure 6.

Sensory epithelia cultured after delamination from mouse utricles exhibit an age-dependent decline in outward spreading and proliferative capacity, while those delaminated from chicken utricles spread and proliferate extensively with no appreciable decrease as chickens age. Left: outlines of representative utricular sensory epithelia derived from embryonic day 18, postnatal day 1 (P1) and P15 mice, and post-hatch day 0 (P0), P60, and P180 chickens after 1, 24, 48 and 72 hrs in culture. The explants from juvenile mice spread little if at all during 72 hrs in culture. Images of mouse explants are from Davies et al. (2007). Images of chicken explants are from Burns et al. (2008). Right: graphs of the percentages of proliferating cells within the explants at various culture times, assessed by continuous BrdU labeling. The chickens used for these experiments were obtained from two different suppliers, which could contribute to the variability in labeling between age groups. The dashed line in the chicken graph indicates the average of all the age groups. The mouse graph is from Lu and Corwin (2008). The chicken graph is re-plotted from data in Burns et al. (2008).

Figure 7.

The supporting cells in mouse utricles develop unique junctional specializations that contain reinforced circumferential F-actin belts and high levels of E-cadherin, whereas supporting cells in chicken supporting cells retain thin F-actin belts and appreciably lower levels of E-cadherin expression throughout life. (A) Confocal images of utricles from hatchling and adult White Leghorn chickens (*Gallus gallus*) labeled with fluorescent phalloidin. Yellow brackets indicate the apical junction region comprised of the circumferential belts and intercellular junction between neighboring supporting cells. (B) Confocal images of anti-E-cadherin labeling (green) in a positive control tissue (stomach epithelium, left) and the utricular sensory epithelium (right) from an adult White Leghorn chicken. Control tissue and vestibular sensory epithelia were stained with the same antibody solution and imaged at identical and fixed gain and offset settings in the confocal microscope. (C) Confocal images of phalloidin labeling in newborn and adult Swiss Webster mice (*Mus musculus*). (D) Confocal images of anti-E-cadherin labeling in control tissue and the utricular sensory epithelium from an adult Swiss Webster mouse. This figure is modified from images in Burns et al. (2012a).

Figure 8.

A 3D schematic diagram of the utricular sensory epithelium in an adult mammal. Reinforced circumferential belts, which contain a dense network of F-actin that fills almost 90% of the average adult supporting cell's area at the level of the junction, are depicted in green.

Figure 9.

Schematic diagram of the actin belts in supporting cells from ears of non-mammals and mammals. The upper and lower transverse and longitudinal views depict the relative thickness and density of circumferential F-actin belts in the utricular sensory epithelia in adult non-mammals and mammals. The belts in adult sharks, bony fish, amphibians, birds, and turtles are relatively thin compared to those in lizards, mice, and humans. The thickness of the belts in frogs and turtles is greater than in sharks, bony fish, and birds, but the belts in those species do not appear to grow appreciably throughout life like the belts in mammals (Burns et al. 2012a). Also, the belts in lizards are considerably more porous compared to the

denser belts in mammals. F-actin is colored green, supporting cells are colored light blue, and hair cells are colored purple.