Published in final edited form as:
Curr Opin Otolaryngol Head Neck Surg. 2008 October ; 16(5): 465–471. doi:10.1097/MOO.0b013e32830f4ab5.

Recent advances in hair cell regeneration research

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Abstract

Purpose of the review—This review discusses recent progress in research that seeks to understand the regeneration of hair cells and it highlights findings that may hold importance for the eventual development of regenerative therapies for hearing and balance impairments.

Recent findings—Signaling via the Notch receptor and the bHLH transcription factors has important roles in the development and regeneration of hair cells. The cytoskeletal properties and cell-matrix interactions of supporting cells in mice of different ages may hold part of the explanation for the age-related differences in their proliferative responses to damage and the differences between mammals and non-mammals in hair cell regeneration. Progress also has been made in deriving stem cells from inner ear tissues and other sources and in the evaluation of their potential uses as source of new hair cells and tools for biomedical research.

Summary—Much has been accomplish since the discovery of postembryonic hair cell production and hair cell regeneration in non-mammals decades ago. No therapies for hair cell regeneration are under clinical trials, but research is yielding potentially important discoveries that are likely to lead to the development of therapeutic methods for inducing hair cell regeneration in the mammalian inner ear.

Keywords

ear; repair; regeneration; proliferation; hair cell

INTRODUCTION

Hair cell loss in humans and other mammals appears to be permanent and cumulative, but thousands of hair cells are added throughout life in the ears of many non-mammalian vertebrates (reviewed in [1]). When hair cells in non-mammals are killed by trauma, toxicity, or other causes they are replaced and begin to restore hearing and balance sensitivity within weeks. This article will address the possibility for therapeutic regeneration while highlighting recent important findings related to the molecules that may regulate inner ear regeneration, postnatal changes in mammalian ears that may limit regeneration, and potential roles for stem cells in the treatment of ear disorders.

Signals that control proliferation

Embryonic signals that control when, where, and which cells divide and when they will stop dividing and develop into the specialized cells of the ear may have importance for the eventual development of regenerative therapies. Nearly all the cells stop dividing in the auditory

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epithelia of birds and mammals before birth [2,3]. But damage to the auditory epithelium in birds evokes an innate capacity for renewed supporting cell proliferation that leads to the regeneration of hair cells [4,5]. Molecules such as retinoblastoma protein, pRb, an important tumor suppressor that prevents cell proliferation have recently been explored. Several years ago it was discovered that Rb deletion can lead to overproduction of mammalian hair cells via renewed cycling [6,7,8]. More recent investigations in postnatal ears have confirmed that hair cells that lack pRb will replicate their DNA as they reenter the cell cycle, but that is quickly followed by hair cell death [9••]. Thus, deletion of Rb by itself does not lead to lasting hair cell replacement in postnatal ears.

Cell production in the inner ear is also regulated by Cip/Kip and Ink4 cyclin-dependent kinase inhibitors, CKIs. Mice that carry a mutant form of the widely expressed CKI, $p27^{Kip1}$, develop supernumerary hair cells and supporting cells in the organ of Corti [10,11]. Another Cip/Kip family member, $p21^{Cip1}$, and the Ink4 family member, $p19^{Ink4d}$, also help to maintain the post-mitotic state of hair cells. In mice where $p21^{Cip1}$ and $p19^{Ink4d}$ have been deleted auditory hair cells re-enter the cell cycle, but quickly die by apoptosis while the vestibular hair cells appeared to remain intact [12].

Forced expression of Skp2, which triggers the degradation of $p27^{Kip1}$, has been found to increase proliferation in cells adjacent to the organ of Corti. Ectopic hair cells were also observed, but only when Skp2 was overexpressed together with Atoh1 [13•]. Taken together, the recent results suggest that the development of therapeutic regeneration is likely to require something more than the absence of cell cycle inhibitors. The importance of tumor suppression mechanisms has led to the evolution of multiple redundant controls, such as p53-mediated apoptosis, that act to limit cell proliferation in mammals.

Signals that influence cell fate

Considerable gains have been made in understanding the molecules that regulate cell fate during development and regeneration in the ear. Atoh1 (formerly known as Math1) is a basic helix-loop-helix (bHLH) transcription factor protein necessary for hair cell differentiation. In its absence, mice do not develop hair cells and their cochleae do not label for supporting cell markers, consistent with a vital role for Atoh1 in the establishment of the prosensory domain and cell fate determination [14,15]. Similar roles recently have been attributed to two Atoh1 genes in Zebrafish [16••].

Atoh1 mRNA is expressed in embryonic cochlear progenitor cells in mice, but the Atoh1 protein is only found in differentiating hair cells [14,17–20]. In chickens, Atoh1 protein is expressed in the cells of developing sensory patches [21,22]. Atoh1 expression is found in the avian utricular sensory epithelium, where the hair cell population turns over continually, but is not found in the undamaged and quiescent auditory sensory epithelium. However, ototoxic damage leads to the expression of Atoh1 in supporting cells and regenerated hair cells in both types of sensory epithelia [23•].

The bHLH transcription factors of the Inhibitor of Differentiation (Id) family are expressed within the developing cochlea, and appear to strongly inhibit the expression of *Atoh1* and the differentiation of hair cells [24]. Another bHLH transcription factor, Neurogenin1, and Atoh1 have recently been found to cross-inhibit each in the early otocyst as it transitions from neurogenesis to sensory cell formation, and Atoh1 was found to positively regulate its own expression [25••]. Fgf3 and Fgf8 act as upstream activators of Atoh1 in the Zebrafish otic vesicle and fox1, pax8, and dlx genes regulate Atoh1 in the preplacode [16••]. A recent study has provided evidence that Atoh1 activates Hes6 transcription through binding to three E-boxes in its promoter [26•].

Several years ago it was discovered that ectopic hair cells could be induced to develop in Kollicker's organ through the forced overexpression of Atoh1, and forced expression in vitro also resulted in new hair cells in damaged postnatal utricles [27,28]. Nearly complete in vivo recovery of hair cells and supporting cells and partial recovery of auditory function are reported to follow adenoviral delivery of the Atoh1 gene into the cochlea of guinea pigs after toxic injury [29]. More recently, adenoviral delivery of Atoh1 in vivo has been reported to lead to vestibular function recovery in mice after aminoglycoside damage [30]. Caution may be warranted, however, in interpreting these remarkable early reports. A scientific axiom is that extraordinary claims require extraordinary evidence. In the absence of evidence for intermediate stages in the recovery process the data reported thus far have not reached that level and they await replication in other laboratories.

The Notch signaling pathway

Notch signaling influences the development and specialization of cells in diverse tissues, and Notch mutations have been identified in certain forms of cancer and other conditions. When the cell-attached ligands of the DSL family bind to Notch receptors that leads to cleavage and release of the Notch intracellular domain (NICD), which translocates to the nucleus where it converts the CBF1 repressor complex (formerly known as Rbpsuh) into an activator complex. The NICD/CBF1 activator complex upregulates the basic helix-loop-helix (bHLH) transcriptional regulators *Hes* and *Hes*-related protein genes, which antagonize proneural genes like neurogenin and the "prosensory" gene Atoh1. This antagonism is central to the inhibition of hair cell differentiation that followed Notch activation (Figure 1).

Notch signaling activated by binding to the ligands Jagged1, Jagged 2, and Delta1 has been implicated in early the establishment of prosensory domains [31–34], and the lateral inhibitory interactions that determine hair cell and supporting cell fate during later development of the mammalian ear [31,32,35–40]. In the developing inner ear, genetic deletions of Notch ligands [31,36,37] or the CBF-1/Rbpsuh gene [41] result in the development of supernumerary hair cells. This effect has also been produced pharmacologically using γ-secretase inhibitors [41•, 42•] as well by oligonucleotide knockdowns directed at Jagged1 and Notch1 [40].

Notch signaling is reactivated during regeneration in the avian ear [43] and the zebrafish lateral line where it appears to limit the hair cell production by acting as a brake on supporting cell proliferation [44••]. Recently, in epithelia of adults guinea pigs that had been damaged by ototoxic treatments, Notch signaling components were found to be upregulated, and when γ-secretase inhibitors were administered that led to the formation of ectopic auditory hair [45•]. An increasing number of receptors have been discovered to undergoγ-secretase mediated cleavage so specific disruption of Notch signaling will be required to establish the mechanism of the γ-secretase inhibitors in the inner ear. γ-secretase inhibitors and other pharmacological agents that influence intermediates in the Notch-bHLH transcription factor pathway may provide important potential candidates for regenerative therapies, but much remain to be discovered in these future studies.

Other signaling pathways

The canonical Wnt, sonic hedgehog (Shh), bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) signaling pathways have important roles in regionalization and early embryonic specification of the developing ear (reviewed in [46–48]). Those pathways are likely to have important roles in hair cell regeneration, but much remains to be investigated.

Gene expression analysis

The development of microarray technology for assessing gene expression can provide comprehensive and relatively unbiased information about molecular regulation at the

transcriptional level. These approaches are being used to investigate inner ear development, hair cell death and regeneration. Investigations of avian sensory epithelia determined that the expression of 605 genes in the cochlea and 188 genes in the utricle changed hours after they were damaged by laser ablation or ototoxicity [49••]. Another study of the developing mouse inner ear identified genes whose expression patterns corresponded with the organogenesis of the cochlea, utricle, and saccule and provided novel evidence of roles for β-adrenergic, amyloid, estrogen receptor, circadian rhythm, and immune pathways in inner ear development [50••].

MicroRNAs

Short noncoding, RNAs (20-22 nucleotides) termed microRNAs (miRNAs) have recently emerged as important regulators of protein expression [51,52]. miRNAs play a role in fin regeneration in zebrafish [53,54] and other regenerative processes (review in [55]). Recently, Tsonis et al. reported that members of the let-7 family of miRNAs are downregulated during hair cell regeneration in newts, and suggested that they may have a role in phenotypic conversion of supporting cells into hair cells [56]. These findings may presage an important, but still largely unexplored role for miRNAs in hair cell regeneration.

Gene therapy

The inner ear may provide a privileged site for the therapeutic delivery of genes in an isolated compartment. As a first approach to that potential goal, an in vitro system for study gene therapy in the human inner ear has been developed and was successfully used to demonstrate expression of exogenous genes delivered into hair cells and supporting cells via second-generation adenovirus transfection [57••]. Other approaches have been recently reviewed [58].

Potential limits to regeneration in mammals

When balance epithelia from newborn rodents are isolated and cultured with appropriate mitogens the majority of the supporting cells replicate their DNA and begin to proliferate (Figure 2) [59–65]. But a sharp, decline in cell production capacity progresses during the first postnatal weeks and appears to leave mammalian ears uniquely vulnerable to permanent hair cell loss (Figure 2) [60,61••]. That decline was recently correlated with a progressive loss of the capacity for supporting cells to change from their normal, columnar shapes to thin and widely spread, squamous cells that have a high incidence of proliferation (Figure 2) [66••]. Wounding assays in cultures of whole utricles, assessed spreading of supporting cells on their natural substrate and demonstrated that the number of cells that begin to proliferate is correlated with number of cells spreading to cover a wound [67••].

Cell shape may also influence differentiation in the inner ear. As will be discussed below, when widely spread proliferating cells derived from passaged cultures of avian hair cell progenitors were lifted off a 2D culture substrate and aggregated in suspension the cells took on columnar shapes and differentiated into hair cells and supporting cells [68••]. Also, after the cochlea has been damaged by high concentrations of ototoxic drugs, a flattened epithelium consisting of thinly spread and highly proliferative nonsensory cells can replace the organ of Corti [69]. When transfected with Atoh1 those cells do not differentiate into hair cells [70], which may indicate that a flattened shape prevents them from differentiating, as it does for human epidermal keratinocytes [71].

The cytoskeleton and matrix interactions

Recent preliminary investigations in utricles from mice revealed substantial differences in the amount of filamentous actin that bracket the apical junctions between supporting cells in the utricles of young and old mice, suggesting that increases in cytoskeletal stiffness may

contribute to the loss in their capacity to spread and proliferate [67]. The cytoskeleton also appears to have a role in determining how hair cells are lost. A recent study revealed that cultured bullfrog saccules which were treated with myosin-contraction inhibitors lost nearly twice as many hair cells as controls [72•]. The substantial loss of hair cells after these treatments points to myosin-mediated tension as a potential mechanism for retaining hair cells within the epithelium. Disruption of microtubule polymerization also increased the loss of hair cell, and treatments with actin polymerization inhibitors appeared to slow the closure of voids left by extruded hair cells [72•].

Age-related changes in integrin expression and activation also may contribute to restrictions in shape change and proliferation in mammalian hair cell epithelia. In sensory epithelia isolated from the utricles of embryonic and postnatal day 6 mice cellular spreading and proliferation were dependent onα6 integrin, which disappeared from lateral cell membranes by P6 and co-localized with β4 integrin near the basement membrane at both ages [66]. α6β1 promotes migration in multiple cell types while α6β4 promotes firm adhesion and structural stability, these developmental changes could contribute to stronger substrate adhesions and the decreased spreading and proliferation that develops postnatally in mammalian ears. Given the intimate relations between shape change, the cytoskeleton, and the cell's connections to its substrate and other cells, further work to characterize potential differences between species that differ in their capacity for innate regeneration will be of interest.

Potential roles of inner ear stem cells

Stem cells are defined by their capacity for self-renewal and differentiation into more than one cell type. Supporting cells are important stem cells in the inner ear, but there is the potential for the existence of other tissue resident stem cells that might be held in reserve and could contribute to the regenerative replacement of hair cells. If such cells exist, they remain to be identified, but recent studies have shown that the mammalian ear contains cells that have the ability to self-renew and differentiate into a variety of cell types both in vitro and after xenograft transplantation into the ears of chick embryos developing in ovo [73]. Cells that have been isolated from the greater epithelial ridge of the neonatal rat cochlea [74] and from the organ of Corti of the newborn mouse cochlea [75,76] also appear to be able to proliferate and generate cells that exhibit some properties of hair cells.

FACS sorting has been used to produce samples enriched for viable supporting cells from the cochlea of p27(kip1)-GFP expressing mice. These supporting cells retain the ability to divide and appear to be able to adopt the phenotype of hair cells in culture [77••]. Embryonic stem cells (ES cells) [78••], bone marrow mesenchymal stem cells [79], and adult mouse olfactory precursor cells [80] also can differentiate into cells that express proteins and other characteristics normally found in hair cells. Transplantation of ES-cell-derived cells into the early otocysts of chicken embryos resulted in the differentiation of more normal appearing hair cells, indicating that the inner ear microenvironment may facilitate hair cell differentiation [78]. Cells isolated from the inner ears of the mouse embryos were shown to aggregate in vitro and develop into the normal alternating patterns of hair cells surrounded by supporting cells, indicating that the cells are likely to express signals that normally direct pattern formation in vivo [77••,81]. Elements of the Notch signaling pathway and the bHLH transcription factors are likely to contribute to the development of these patterns, and these in vitro systems hold the potential to reveal other signals as yet unidentified that may be important for regeneration.

Supporting cells derived from dissociated sheets of utricular sensory epithelium from embryonic chickens were found to undergo an epithelia-to-mesenchymal transition and proliferate for more than 20 population doublings when cultured on 2-D substrates. Those cultures can be frozen, shipped to other laboratories, thawed and greatly expanded as a source of hair cells. When dissociated and cultured in suspension, these cells aggregate and pass

through a mesenchymal-to-epithelial transition that gives rise spherical epithelia which develop hair cells that are crowned by hair bundles, comprised of a single kinocilium and an asymmetric array of stereocilia [68]. Vials of these frozen cells provide the capacity to produce bona fide hair cells completely in vitro and may be useful for increasing the pace of various research approaches to treatments for hearing loss and other inner ear disorders.

CONCLUSION

Recent research has identified molecules that regulate the production and differentiation of hair cells during development. Such knowledge is likely to be important for the development of methods for stimulating hair cell regeneration in mammalian ears. Questions remain as to what limits hair cell regeneration in mammals, but recent cellular studies have revealed candidate mechanisms. Stem cells have been isolated from the inner ear and derived from other sources. These cells are likely to become important tools for inner ear research and they hold the potential for therapeutic uses if major challenges can be overcome.

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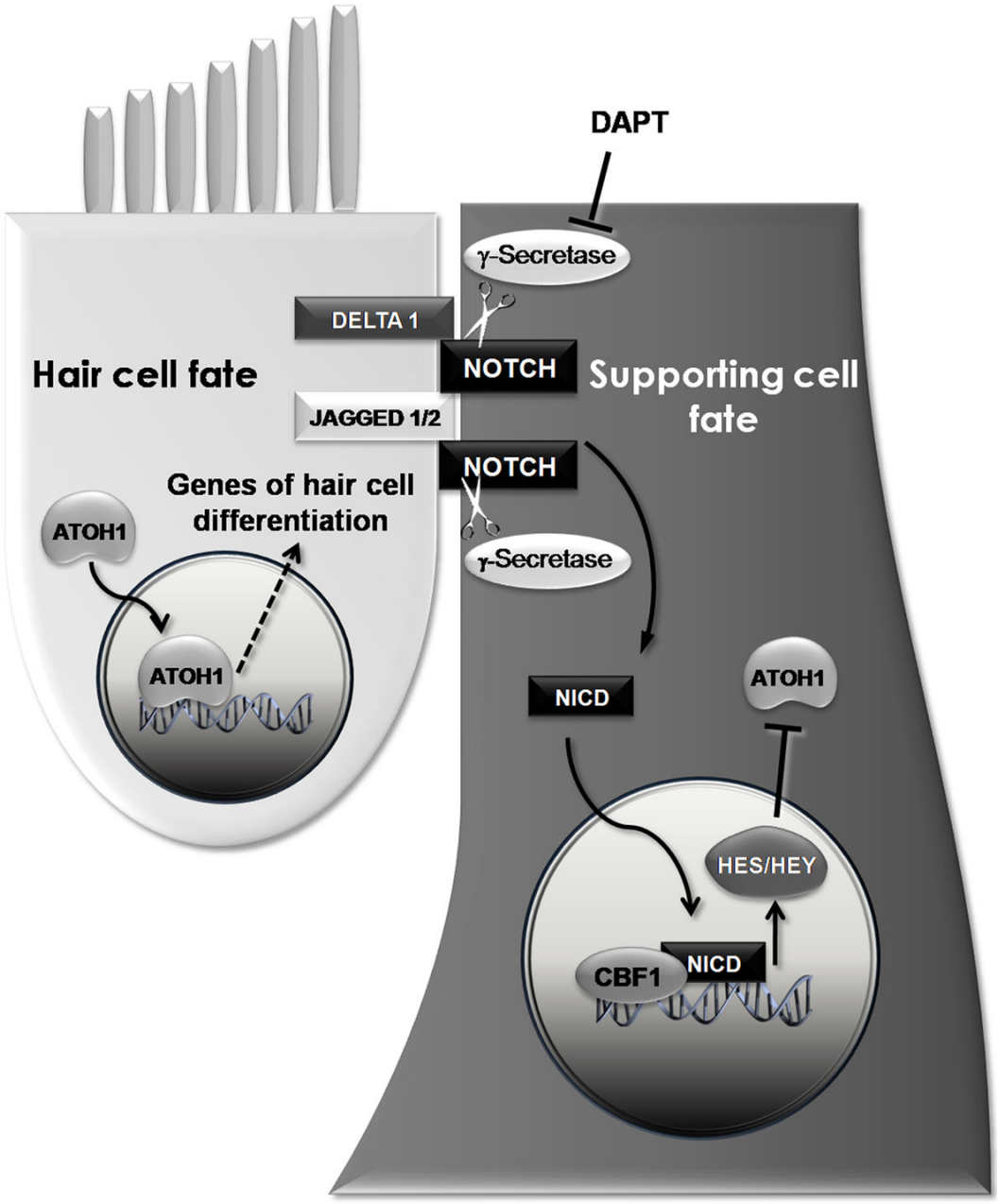

Figure 1. Notch signaling in cell fate determination

Developing hair cells express the ligands Jagged and Delta. Those ligands bind to the Notch receptors of adjacent cells, and that leads to γ-secretase mediated cleavage and generation of a Notch intracellular domain (NICD). After tranlocation to the nucleus, NICD forms a complex with CBF1 and other proteins, and upregulates the expression of Hairy and enhancer of split (HES) and related HEY genes, which block the effects of Atoh1, leading to inhibition of hair cell fate and the development of the cell as a supporting cell. Developing hair cells express Atoh1 which is necessary for hair cell differentiation. Inhibitors of γ-secretases such as DAPT appear to interrupt Notch signaling by blocking the generation of the NICD.

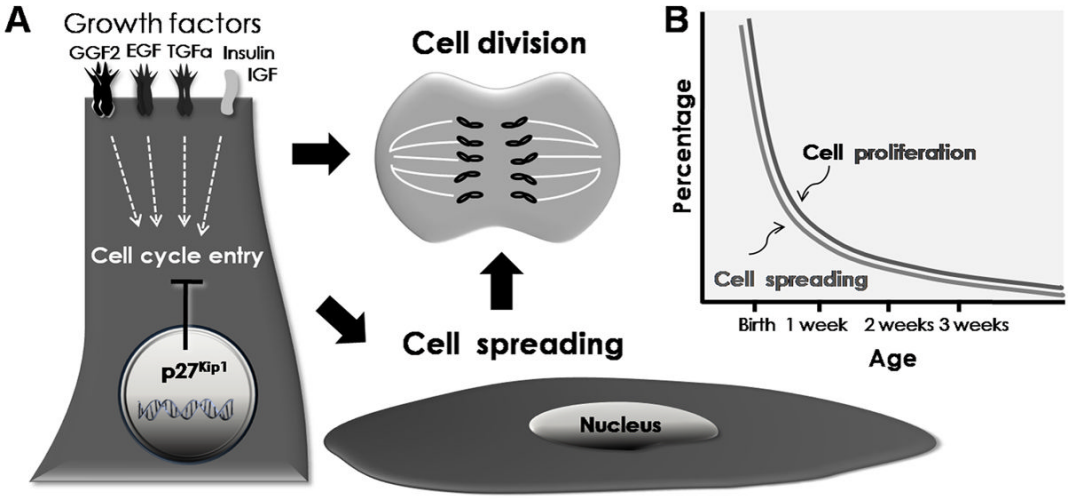

Figure 2. Cell proliferation in mammalian supporting cells (SC) correlates with SC spreading and is influenced by growth factors and cyclin-dependent kinase inhibitors

A. Glial Growth factor (GGF2), Epidermal growth factor (EGF), Transforming growth factor (TGFα), Insulin, Insulin-like Growth Factor and other extracellular signals can promote robust cell production in sensory epithelia from neonatal mammals. On the other hand, the cyclin-dependent kinase inhibitor $p27^{Kip1}$ helps to maintain supporting cell in a mitotically quiescent state. When supporting cells change from their normal elongated columnar shape to spread and more flattened shapes their capacity for proliferation is enhanced. **B.** In mammalian vestibular epithelia, the capacity for cellular spreading and cell proliferation decline during the first week after birth and that appears to contribute to the limited regeneration that is characteristic of mammals.