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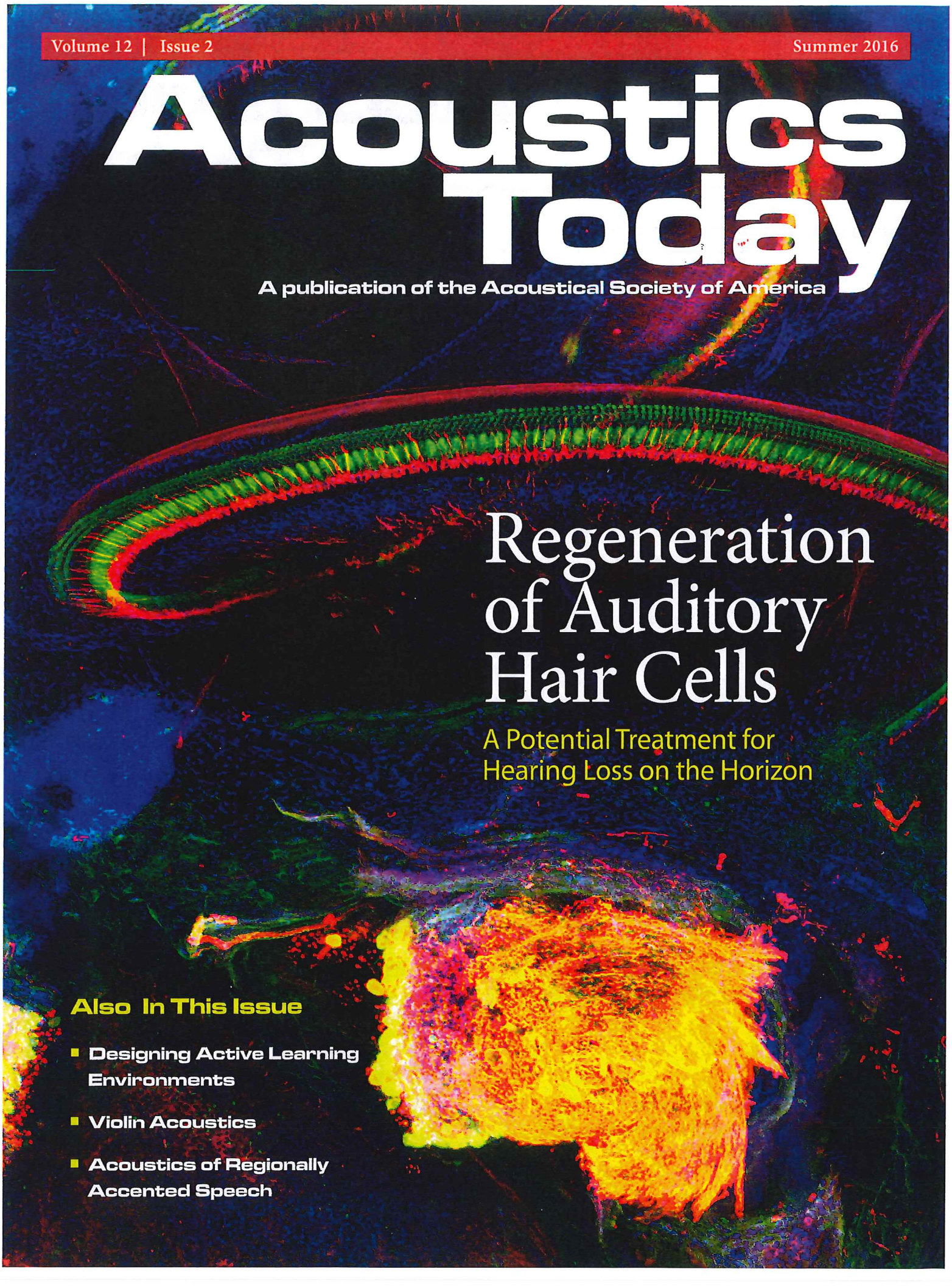

Regeneration of Auditory Hair Cells

A Potential Treatment for Hearing Loss on the Horizon

Also In This Issue

- Designing Active Learning Environments
- Violin Acoustics
- Acoustics of Regionally Accented Speech

Volume 12 | Issue 2 | Summer 2016

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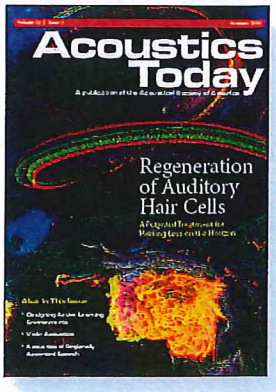
TABLE OF CONTENTS

About The Cover



The cover image is from the article, Regeneration of Auditory Hair Cells: A Potential Treatment for Hearing Loss on the Horizon, by Rebecca Lewis, Edwin Rubel, and Jennifer Stone, located on pages 40-48 of this issue (see Figure 2a). It is a photomicrograph of a side view the mammalian cochlea stained by fluorescent antibodies. The apex, encoding low frequencies, is toward the top and it spirals toward the base (high frequencies). The rows of hair cells (green) and adjacent nerve fibers (red) are seen along with the spiral ganglion neurons (yellow mass at the bottom). Image provided by Glen MacDonald and Edwin Rubel.

Regeneration of Auditory Hair Cells: A Potential Treatment for Hearing Loss on the Horizon

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Regeneration of cochlear hair cells is being investigated as a potential therapy for hearing impairments.

Introduction

The process of hearing involves a complex chain of events, and each one is important to ensure the proper detection and processing of sounds. In the first step, sound waves traveling through the environment enter the ear canal and vibrate the eardrum. This energy is transmitted through the three bones of the middle ear to the inner ear. Within the inner ear, the energy derived from the sound waves is transmitted to the basilar membrane of the cochlea, on which lies the sensory organ for hearing, the organ of Corti (**Figures 1** and **2A**).

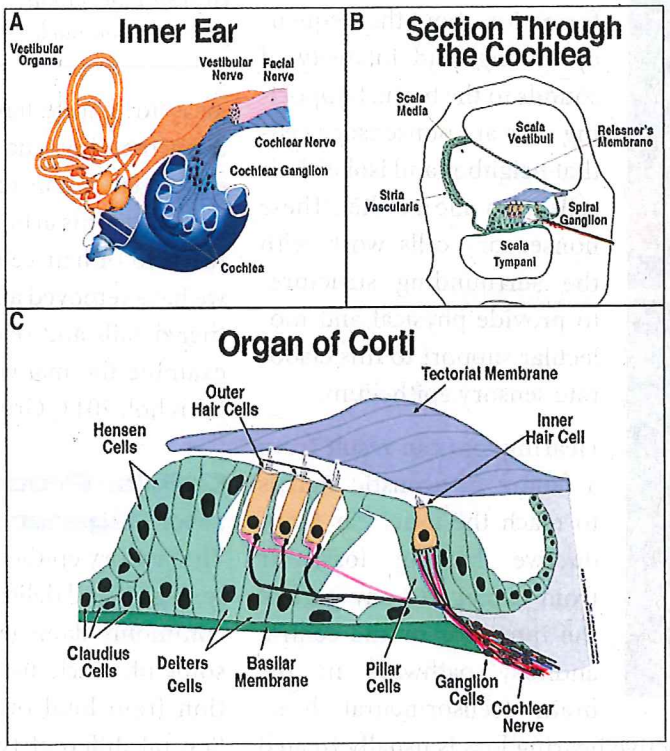

Figure 1. *Schematic diagrams principal structures of the inner ear tissues (A), a slice through one turn of the cochlea (B) and the organ of Corti (C). Note that in the organ of Corti a single inner hair cell and three outer hair cells are shown along with the supporting cells. This pattern is repeated about 3,000 times along the spiraled cochlea in humans.*

The organ of Corti is composed of sensory hair cells as well as a group of specialized cell types, collectively called supporting cells, and the peripheral processes of auditory neurons. Hair cells are sensory receptors. Responding to the mechanical signals derived from sound waves, hair cells transduce this energy into electrical signals that are transmitted via the auditory nerve to the brain. In the normal human ear, there are about 3,000 inner hair cells and 12,000 outer hair cells (Bredberg, 1967). Inner hair cells (**Figure 2B**) are the true sensory receptors. On stimulation, the inner hair cells activate auditory nerve fibers that in turn activate auditory brainstem nuclei. The major function of the outer hair cells (**Figure 2C**) is to modulate the function of the organ of Corti by enhancing signal processing of low-ntensity auditory signals. These two types of hair cells work together such that the auditory nerve

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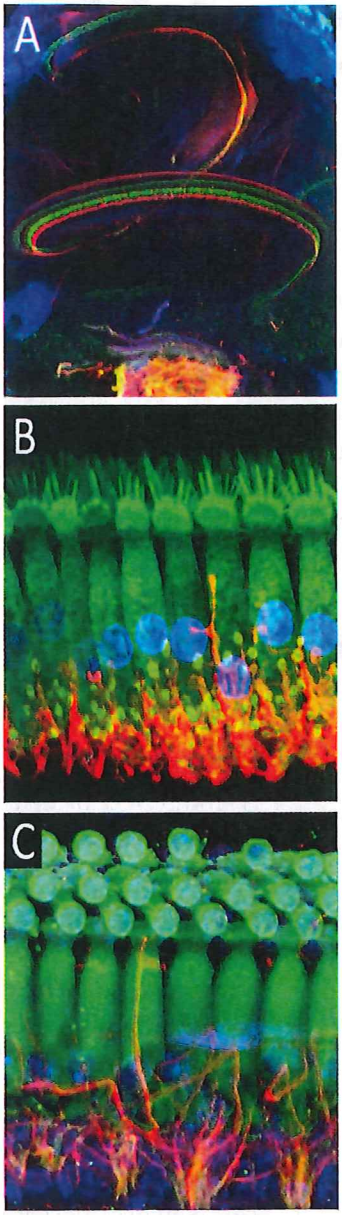

Figure 2. A: Mammalian cochlea with hair cells (green), nerves (red) and spiral ganglion neurons (yellow/orange). B: Inner hair cells and stereocilia (green), with nuclei (blue) and nerve fibers of neurons that transmit information to the brain (red; McLean et al., 2009). C: Top of the three rows of outer hair cells (green dots at top) and the tubular single row of inner hair cells innervated by neural process (red/orange).

transmits highly selective information about the frequency, timing, and intensity of sounds to the brain. Supporting cells are nonsensory cells that neighbor and isolate hair cells from one another. These nonsensory cells work with the surrounding structures to provide physical and molecular support to this elaborate sensory epithelium.

Hearing loss can result from a failure of acoustic signals to reach the inner ear (conductive hearing loss) or from damage to any part of the inner ear or the central auditory pathways in the brain (sensorineural hearing loss[SNHL]). Conductive hearing loss is usually treated by medical or surgical means. The most common form of SNHL results from damage or dysfunction of hair cells in the organ of Corti. When hair cells in the mammalian cochlea die, they do not regenerate; this form of SNHL is permanent (**Figure 3**). If hearing loss is moderate, patients can be fit with hearing aids, which amplify sounds to enhance hearing. If it is severe, patients can receive cochlear implants to bypass the injured hair cells and directly stimulate the auditory nerve. Neither form of treatment restores normal hearing or addresses the cause of hearing loss, the missing hair cells.

Around 30 years ago, the discovery that hair cells regenerate in birds raised the possibility that we could someday find

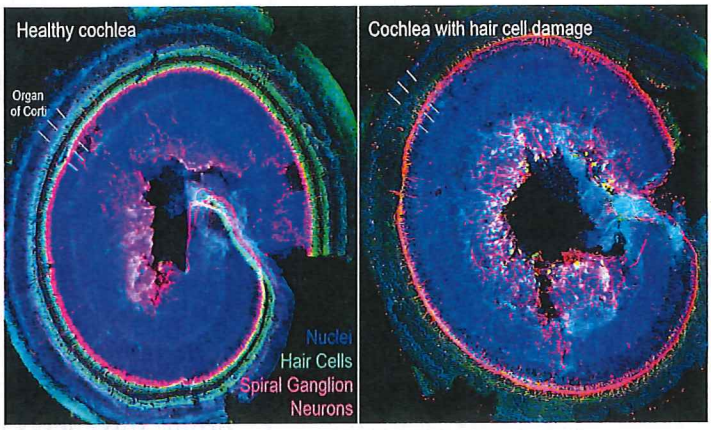

Figure 3. Left: Surface view of a healthy cochlea with hair cells (green), neural processes (red), and nuclei (blue).Right:A damaged cochlea that no longer contains hair cells but has preserved neural processes and nuclei. White arrows: Organ of Corti boundaries.

away to replace hair cells in mammals, including humans. Since that time, many advances in our understanding of hair cell regeneration in birds, fishes, and mammals have been achieved. This article reviews the current state of research in the field of hair cell regeneration. Due to space limitations, we have removed all but the most essential citations. For further details and relevant citations, we encourage readers to examine the many review papers related to this field (e.g., Warchol, 2011; Groves et al., 2013; Rubel et al., 2013).

Cellular Processes of Hair Cell Damage and Regeneration

The sensory epithelium of the cochlea is a cytoarchitecturally elegant and delicate structure (**Figure 1**). The hair cells are commonly damaged by a variety of environmental events, some of which are known, including acoustic overstimulation from loud or prolonged noise or concussive stimuli. Several different types of medications kill hair cells when administered at high doses or for prolonged periods. These include, but are not limited to, aminoglycoside antibiotics such as gentamicin and heavy metal anticancer drugs such as cisplatin. Hair cells also die as we age; in most cases, this is due to unknown causes. Finally, genetic mutations exist that cause hair cells to die during embryonic development or at later stages of life.

Until 1985, it was believed that regeneration of inner ear hair cells was not possible in vertebrates. While studying processes of hair cell damage in the chicken auditory epithelium, however, investigators noted a reappearance of hair cells in the area of damage. The immature morphology of these cells appeared similar to that of embryonic hair cells in

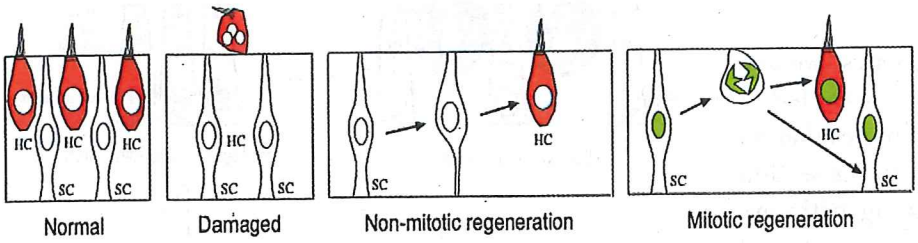

Figure 4. The undamaged auditory epithelium of the bird contains hair cells (HC; red) interdigitated with supporting cells (SC; white). On damage, hair cells are removed from the epithelium and supporting cells are triggered to regenerate hair cells. Nonmitotic regeneration allows a supporting cell to change its shape and genetic profile to that of a hair cell. Mitotic regeneration requires a supporting cell to divide and differentiate into two daughter cells, a hair cell and a supporting cell.

the cochlea of chickens (Cotanche, 1987; Cruz et al., 1987). During this same period, it was also discovered that regeneration of hair cells occurs readily in the vestibular portions of the avian inner ear (Jørgensen and Mathiesen, 1988). Soon, researchers learned that the hair cells of the inner ear and lateral line system of fish, frogs, and salamanders also readily regenerate after damage, which led to the conclusion that regeneration occurs in hair cell epithelia of all vertebrates except mammals. Further analysis revealed that the supporting cells that normally surround the hair cells are the source of these newly differentiating hair cells. Supporting cells may either mitotically divide to achieve hair cell differentiation or phenotypically convert to a hair cell in a process called direct transdifferentiation (**Figure 4**) (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Roberson et al., 1996). With these two methods of replacing hair cells, nonmammalian vertebrates provide valuable models to study these processes and their ability to restore hearing after sustained SNHL.

In mammals, the situation is quite different. When hair cells die in the mature mammalian organ of Corti, supporting cells fill in the gaps where hair cells were located to form permanent scars, and no new hair cells are formed. Moreover, supporting cells neither divide nor convert into hair cells after hair cell damage (e.g., Roberson and Rubel, 1994; Chardin and Romand, 1995).

In contrast to the organ of Corti, adult mammals can spontaneously replace a small number of hair cells in the vestibular organs of the inner ear. New hair cells are largely formed by nonmitotic regeneration (Forge et al., 1998; Kawamoto et al., 2009; Golub et al., 2012). There appears to be a small degree of supporting cell division triggered in response to hair cell loss (Li and Forge, 1997; Kuntz and Oesterle, 1998), but no newly formed cells become replacement hair cells (Oesterle et al., 2003).

The big challenge facing researchers today is to determine why hair cells are not readily regenerated in mammals. Regeneration could fail in the adult cochlea because the hearing organ loses the population of progenitor cells capable of forming new hair cells during development. Alternatively, cells with the potential to replace hair cells may exist in the cochlea but are unable to respond to damage due to active inhibition or lack of stimulatory signals.

Stimulating Native Progenitors to Form New Hair Cells in the Adult Cochlea

Researchers have examined whether the cells capable of forming new hair cells still exist in the cochlea of mature mammals. Many tissues in our body undergo continual renewal. One common feature of these tissues is that they contain stem cells that divide and form new specialized cells throughout life. Several lines of evidence show that the cochlea and vestibular organs possess stemlike progenitors to hair cells during early development but lose them as the organs mature (Oshima et al., 2007). Consistent with this, new hair cells can be formed by supporting cells from the organ of Corti of neonatal mammals (White et al., 2006; Cox et al., 2014), but not in adult mammals (e.g., Roberson and Rubel, 1994; Forge et al., 1998).

Investigators are using three general strategies to identify ways to trick supporting cells in the mature mammalian inner ear to regenerate hair cells. First, we are finding clues in cochlear development. Hair cells in the organ of Corti form during the embryonic period through a complex series of cellular steps controlled by a cascade of molecular interactions. Some researchers have postulated that, before any cell in the mature cochlea can form a new hair cell, it will need to relive these same stages of development.

Second, we look to other regenerative tissues. Many tissues in the body are continuously replaced under normal conditions and/or after damage, including cells in the skin, intestine, and some regions of the brain. We reason that many of the molecular cascades leading to regeneration in these other tissues could be co-opted to trigger regeneration in the cochlea.

Third, using the new tools of molecular genetics, we can directly query the molecular cascades that are activated in the sensory epithelia of nonmammalian vertebrates that do regenerate hair cells, such as birds and fishes. In the section below, we describe several genes and signaling pathways that met one or more of these criteria and were evaluated for their capacity to stimulate hair cell regeneration in mammals. These analyses revealed signaling molecules that are important for facilitating regeneration.

Forced Atoh1 Expression: Pushing Mature Supporting Cells to Transdifferentiate Into Hair Cells

A proneural transcription factor named atonal homolog 1 (Atoh1) is a potential therapeutic agent for promoting hair cell regeneration. Atoh1 helps to direct the generation of hair cell-specific proteins that give the hair cell its morphological and physiological identity (Cai et al., 2015). When the gene encoding Atoh1 is deleted, hair cells in the organ of Corti do not form (Bermingham et al., 1999). Thus, Atoh1 is a very powerful activator of hair cell features and could trigger cells to transdifferentiate into hair cells.

In tissues that regenerate hair cells, Atoh1 expression is activated in supporting cells shortly after hair cell damage (Cafaro et al., 2007; Wang et al., 2010; Lin et al., 2011). In cultured auditory organs from chickens, forced expression of Atoh1 influences supporting cells to form new hair cells by promoting division and direct transdifferentiation (Lewis et al., 2012). In rodents, forced expression of Atoh1 by viral injection into the organ of Corti or nearby regions of developing mice forces more cells to differentiate as hair cells (Zheng and Gao, 2000; Gubbels et al., 2008). These findings suggested Atoh1 misexpression might be sufficient to trigger supporting cells to transdifferentiate into hair cells after damage in the cochlea of adult mammals. Indeed, some studies suggest that Atoh1 may drive production of new hair cells in auditory (Izumikawa et al., 2005) and vestibular (Schlecker et al., 2011) organs, which might result in small improvements in hearing and balance function.

However, recent studies are less encouraging. Misexpression of Atoh1 in pillar and Deiters' cells, two supporting cell subtypes (**Figure 1**), in the mature mouse cochlea stimulates early stages of transdifferentiation into hair cells, but this process is not completed and many "forced" cells die (Liu et al., 2012). Indeed, Atkinson et al. (2015) noted no significant improvement in hearing after virally induced Atoh1 misexpression in the organs of Corti of guinea pigs. Hence, an important current challenge is to determine what factors

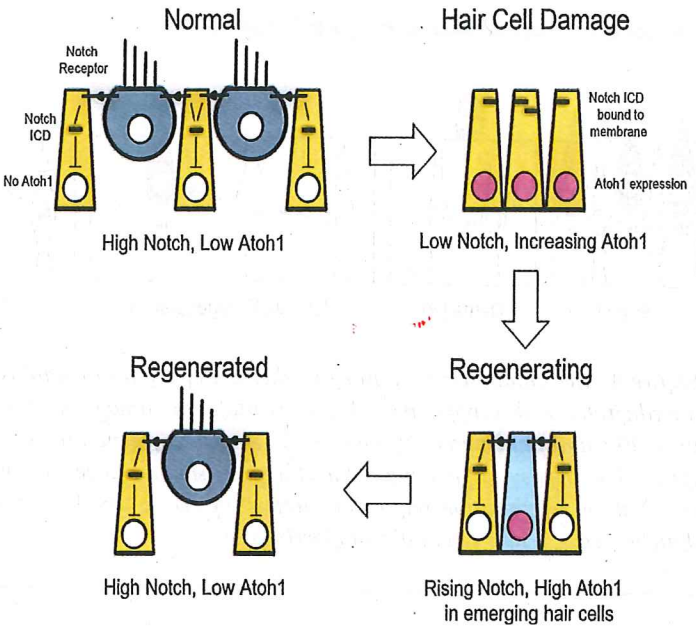

Figure 5. *Expression patterns of the Notch receptor and Atoh1 transcription factor in supporting cells (yellow) and hair cells (blue) under normal, damaged, and regenerating conditions. In supporting cells in undamaged epithelia, there is high Notch receptor activity and the Notch intracellular domain (Notch ICD) travels to the nucleus, inhibiting Atoh1 expression. In supporting cells after hair cell damage, Notch receptor activity is reduced, Notch ICD remains at the membrane, and Atoh1 levels increase, driving the supporting cell to transdifferentiate into a hair cell. Once the new hair cell matures, Notch activity is increased again and Atoh1 transcription is reduced to normal levels.*

limit the ability of Atoh to drive hair cell regeneration in the mature cochlea. Currently, a human clinical trial testing the ability of viral infection of Atoh1 to improve hearing is underway. Results are not available at this time.

Suppression of Notch Signaling: Can This Enhance Proregenerative Effects of Atoh1?

As discussed above, it is evident that, although Atoh1 misexpression reliably promotes supporting cells and other cells around the organ of Corti to become hair cells in neonatal mammals, unidentified factors appear to hinder the effects of Atoh1 in the mature organ of Corti. One likely suspect is the factor is signaling through the Notch receptor (Lewis, 1998).

Notch is a receptor on the surface of cells that is activated by molecules on adjacent cells (**Figure 5**). Notch has many functions in a variety of cells, but its most pertinent role with respect to hair cell regeneration is the inhibition of hair cell formation. During development, Notch ligands are expressed in young hair cells and influence surrounding supporting cells to maintain their identity rather than differentiate into hair cells (reviewed in Kelley, 2006). Notch signaling executes this function, at least in part, by blocking Atoh1 synthesis (Lanford et al., 2000). In the developing

cochlea, inhibition of Notch signaling results in a significant increase in the number of hair cells (e.g., Hayashi et al., 2008; Doetzlhofer et al., 2009). Similar effects of Notch inhibition have been documented during hair cell regeneration in fishes (Ma et al., 2008), birds (Daudet et al., 2009), and mouse vestibular organs (Lin et al., 2011). One study suggests that infusion of Notch inhibitors into live mice can promote supporting cells to convert into hair cells in the organ of Corti of adult mice after hair cell damage (Mizutari et al., 2013). However, another study clearly describes a precipitous loss of efficacy of Notch inhibitors to stimulate hair cell regeneration (Maass et al., 2015). Hopefully, these apparently conflicting interpretations of Notch inhibition will be resolved in future studies.

Lifting the Blockade on Supporting Cell Division in Native Progenitors

As discussed above, supporting cells in the mature organ of Corti are strongly inhibited from dividing even after hair cells have been killed. Although Atoh1 misexpression and/or Notch inhibition appears to encourage supporting cells to form hair cell-like cells in mature animals, neither treatment has a significant effect on supporting cell division. Therefore, as a therapy alone, either manipulation would likely deplete supporting cells, which would almost certainly reduce the function of the organ of Corti. Investigators are attempting to determine how to promote supporting cells to divide mitotically and either replace themselves or form new hair cells. At this point, there are no known manipulations that have these effects in the mature organ of Corti. However, we know some ways in which supporting cell division can be promoted in the young cochlea.

For cochlear supporting cells to divide, they must exit their normal state of mitotic inactivity and enter the cell cycle. $p27^{Kip1}$ is a molecule that blocks progenitor cells (or supporting cells) in the organ of Corti of mice from dividing during embryonic and postnatal development. Embryonic deletion of the gene encoding $p27^{Kip1}$ causes an excess of cells to be formed in the organ of Corti, including hair cells (Chen and Segil, 1999; Löwenheim et al., 1999). In mature mice, blocking the synthesis of $p27^{Kip1}$ causes a small but significant increase in cell division in some types of supporting cells in the organ of Corti (Oesterle et al., 2011). Inhibition of $p27^{Kip1}$ and similar molecules is under investigation as a way to promote mammalian hair cell regeneration. It is particularly important at this stage that investigators determine if $p27^{Kip1}$ deletion in adult rodents leads to the production of functional, stable hair cells.

Activity of $p27^{Kip1}$ and other regulators of cell division is controlled by extracellular signaling molecules. One set of molecules that drives cell division in many tissues is Wnts, which binds receptors on the surface of cells and activates a transcriptional coactivator called ß-catenin (reviewed in Jansson et al., 2015). Wnt/ß-catenin signaling is required for progenitor cell division during cochlear development; when inhibited, significantly fewer hair cells form (Shi et al., 2014). Forced overexpression of Wnt promotes supporting cells in the organ of Corti to divide in very young mice but not in mature mice (Chai et al., 2012; Shi et al., 2013). Therefore, activation of Wnt alone cannot overcome other inhibitory signals present in the mature mammalian organ of Corti. In contrast, pharmacological activation of Wnt promotes hair cell regeneration in lateral line functional neuromasts of larval zebrafish (Head et al., 2013; Jacques et al., 2014).

Epidermal growth factor (EGF) is another molecule that drives supporting cell division in the supporting cells in the organ of Corti of neonatal mice as well as in supporting cells in the regenerating auditory epithelium of mature chickens (White et al., 2012). Treatment of cultured organs of Corti with EGF in newborn rats increases the formation of supernumerary hair cells (Lefebvre et al., 2000). Once again, this effect rapidly declines with age (Hume et al., 2003).

Could Transient or Combinatorial Treatments Improve Hair Cell Regeneration?

As discussed above, we now know several powerful genes or signaling pathways that, when manipulated in very young rodents, cause supporting cells to divide and form new hair cells. But these same manipulations have very little effect or even deleterious effects in mature rodents. These findings tell us that promotion of hair cell regeneration in mature humans will be more challenging than originally thought. One strategy that scientists are testing is whether transient activation or suppression of gene activity has a better outcome than sustained alterations. During development, signals turn on and off in cells, whereas many of the manipulations discussed above are permanent and therefore unnatural. Modern techniques for transient gene silencing, such as siRNA, might enhance the effects of treatment by better recapitulating nature. Another hypothesis being tested is whether combinatorial manipulations of genes and pathways can more effectively promote regeneration than single

manipulations. This has proven to be fruitful in the cochlea of neonatal rodents in experiments that activate Atoh1 and inhibit Notch simultaneously (Zhao et al., 2011) or activate Atoh1 and Wnt simultaneously (Kuo et al., 2015). These dual approaches acknowledge the complexity of growth regulation in mature tissues as well as the critical interactions that occur between pathways.

Transplantation of Cells to Replace Hair Cells

In the prior section, we discussed strategies for promoting native cells in the damaged organ of Corti to divide or directly transdifferentiate to replace lost hair cells. It is possible, however, that a responsive population may not persist in the adult cochlea. On the other hand, we may fail to find appropriate treatments to stimulate resident cells to regenerate hair cells. In either case, it will be necessary to adopt an alternative approach and to transplant cells to the inner ear that can replace hair cells. The obvious choice is to transplant stem cells, which have the potential to divide and differentiate into a range of mature cell types. Stem cells can be grown in a dish and guided toward a desired cell fate (in this case, hair cell) by certain chemical agents or culture conditions. Stem cells hold great promise for treating several types of pathology, including heart disease, blindness, and leukemia.

Some of the first studies to test the usefulness of different types of stem cells to replace damaged hair cells were performed with pluripotent stem cells or neural stem cells derived from mouse embryos. Li et al. (2003) conditioned mouse embryonic stem cells with various compounds in culture to drive them to differentiate hair cell-like features. On transplantation into the embryonic chicken ear, conditioned cells incorporated into hair cell epithelia and acquired hair cell-like properties. Fujino et al. (2004) found that neural stem cells introduced into cultured inner ear organs from rats integrated into the sensory epithelia of vestibular organs but not the cochlea. Subsequently, Oshima et al. (2010) identified treatments that drive induced pluripotent stem cells (derived from fibroblasts) to differentiate advanced features of hair cells in culture, including hair bundles and mechanotransduction currents. More recently, stem cells from human embryos were found to be capable of forming hair cell-like cells in culture (Ronaghi et al., 2014).

The true test of the therapeutic usefulness of a stem cell is whether it can become integrated into the organ of Corti, become innervated by the auditory nerve, differentiate mature features, and survive. Introduction of stem cells into the organ of Corti is a challenge because the organ is surrounded by a fluid-filled cavity that is embedded within the temporal bone and is easily disrupted by surgical intervention. It would seem very difficult to place transplanted cells into the organ of Corti given the tiny nature and delicacy of the tissue and the fact that fluid barriers would need to be disrupted. Nonetheless, several approaches for cell delivery are under investigation. Scientists have introduced embryonic stem cells into the fluids of the organ of Corti (scala media) and into the perilymphatic spaces surrounding the scala media (Coleman et al., 2006; Hildebrand et al., 2005). Although some stem cells seem to persist in these spaces and integrate into some tissues around them, there is little evidence that stem cells integrate into the organ of Corti. However, Parker et al. (2007) reported that neural stem cells injected into the noise-damaged cochlea became incorporated into the sensory epithelium. Clearly, more studies are needed to identify ways to coax stem cells to integrate into damaged hair cell epithelia, acquire mature features, and restore function.

Clinical Considerations

Although progress toward hair cell regeneration has been significant given the limited time elapsed since its discovery, several challenges remain to determine how effective hair cell replacement could be for improving hearing in humans. For instance, we do not know how many hair cells of each type must be regenerated to adequately restore hearing in impaired individuals. Although we know that inner hair cells are critical, we can only guess how well they will restore hearing in the absence of outer hair cells. Many forms of hearing loss are caused by selective destruction of outer hair cells; regeneration of outer hair cells alone could be helpful in such patients. Furthermore, we lack the capability to accurately test which type of cells need repair in patients. This assessment requires development of more cell-specific and noninvasive diagnostic procedures. In addition, high-resolution imaging of the inner ear, enabling quantitative assessment of each cell type, would be very helpful and is currently under investigation. Although there are challenges to restoring hair cells after damage in mammals, many hurdles have already been conquered, with promising research on the horizon to introduce a potential treatment for hearing loss.

Acknowledgments

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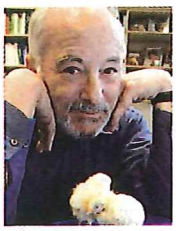
Biosketches

Rebecca M. Lewis studied speech and hearing sciences during her undergraduate training at the University of Washington, Seattle. She is currently enrolled in the dual AuD/PhD program at the University of Washington in speech and hearing sciences and is being mentored by Jennifer Stone in otolaryngology. She is enrolled in her clinical externship at the Veterans Affairs Puget Sound Health Care System and completed her AuD/PhD graduate training in May, 2016. She plans to continue practicing clinical audiology while remaining engaged in research in further treatments for audiology patients with sensorineural hearing loss or vestibular balance disorders.



Edwin W Rubel received PhD in physiological psychology from Michigan State University, Lansing. Since 1986, he has been a Professor in the Departments of Otolaryngology and Physiology and Biophysics and Adjunct Professor in the Department of Psychology at the University of Washington, Seattle. He is the founding Director of the Virginia Merrill Bloedel Hearing Research Center and currently holds an endowed Chair. His research interests include development and plasticity of the central and peripheral auditory system, inner ear hair cell regeneration, and modulation of inner ear hair cell death. His laboratory, along with Dr. Douglas Cotanche, discovered hair cell regeneration in birds.

Jennifer S. Stone studied biology and studio art at Skidmore College, Saratoga Springs, NY, and then completed PhD graduate training in anatomy and neurobiology at Boston University. She performed a postdoctoral fellowship in otolaryngology at the University of Washington School of Medicine, Seattle. Now, she is a Research Professor in Otolaryngology at the University of Washington School of Medicine.

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NEWS *from the* Acoustical Society Foundation Fund

Leo and Gabriella Beranek

Leo Beranek's manifest contributions, as discussed in Acoustics Today, Volume 10, Issue 4, are legend. In resonance with Leo, Gabriella Beranek, Leo's wife, shared these observations recently: "Of course we love to hear great performances in fine concert halls, but many other aspects of our acoustics environment deserve study and attention."

She and Leo together are referring to noise control in public spaces, reducing outdoor noise pollution, building better harmony in multifamily dwellings, understanding human perception to sound in spaces, and much more—all related to the fields of architectural acoustics and noise control.

They recognize that achieving these hopes depends on the training and education of future generations of acousticians. So they have chosen to support these goals through a significant donation to the Acoustical Society Foundation Fund (ASFF). The Leo and Gabriella Beranek Scholarship in Architectural Acoustics and Noise Control will be initiated by the first $30,000 stipend in 2016.

Your donation to the ASFF can be in tune with the Beraneks to provide similar support for the many educational opportunities funded through ASA.

Carl Rosenberg
Chair, Acoustical Society Foundation Board
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