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Genetic and pharmacological intervention for treatment/prevention of hearing loss

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Abstract

Twenty years ago it was first demonstrated that birds could regenerate their cochlear hair cells following noise damage or aminoglycoside treatment. An understanding of how this structural and functional regeneration occurred might lead to the development of therapies for treatment of sensorineural hearing loss in humans. Recent experiments have demonstrated that noise exposure and aminoglycoside treatment lead to apoptosis of the hair cells. In birds, this programmed cell death induces the adjacent supporting cells to undergo regeneration to replace the lost hair cells. Although hair cells in the mammalian cochlea undergo apoptosis in response to noise damage and ototoxic drug treatment, the supporting cells do not possess the ability to undergo regeneration. However, current experiments on genetic manipulation, gene therapy, and stem cell transplantation suggest that regeneration in the mammalian cochlea may eventually be possible and may 1 day provide a therapeutic tool for hearing loss in humans.

Learning outcomes—The reader should be able to: (1) Describe the anatomy of the avian and mammalian cochlea, identify the individual cell types in the organ of Corti, and distinguish major features that participate in hearing function, (2) Demonstrate a knowledge of how sound damage and aminoglycoside poisoning induce apoptosis of hair cells in the cochlea, (3) Define how hair cell loss in the avian cochlea leads to regeneration of new hair cells and distinguish this from the mammalian cochlea where there is no regeneration following damage, and (4) Interpret the potential for new approaches, such as genetic manipulation, gene therapy and stem cell transplantation, could provide a therapeutic approach to hair cell loss in the mammalian cochlea.

1. Introduction

Nearly 35 million Americans suffer from measurable hearing impairment and related speech disorders. Hearing loss affects approximately 17 in every 1000 children under the age 18. The incidence increases with age: Approximately 314 in 1000 people over age 65 have a hearing loss and 40–50% of people 75 and older have a hearing loss (National Institute on Deafness & Other Communication Disorders, 2007 National Institute on Deafness and Other Communication Disorders [NIDCD], 2007). Hearing loss affects more people than epilepsy, multiple sclerosis, spinal injury, stroke, Huntington's and Parkinson's diseases combined (Hudspeth, 1997) and although it is rarely life-threatening, it has a huge financial impact on our economy and lifestyle. Two million Americans are completely deaf and two-to-three out

of every thousand children born are severely to profoundly deaf, half of those due to hereditary causes. The primary cause of these hearing impairments is thought to be damage to the sensory cells, supporting cells and neurons in the cochlea, and is referred to clinically as sensorineural or “nerve” deafness. The loss of these cells in the mammalian cochlea leads to permanent hearing loss because these cells are made only during embryonic development (Ruben, 1967) and must last throughout a person's lifetime.

However, 20 years ago my lab discovered that the bird cochlea could replace hair cells lost after sound damage (Cotanche, 1987). Since then, studies from our lab, the labs of Brenda Ryals and Ed Rubel, and many other groups have shown that birds can regenerate new hair cells after sound or aminoglycoside damage, leading to recovery of auditory function (Corwin & Cotanche, 1988; Cruz, Lambert, & Rubel, 1987; Lippe, Westbrook, & Ryals, 1991; Ryals & Rubel, 1988). This regeneration is thought to offer a potential treatment for sensorineural hearing loss in humans. Based on the recent clinical success of the electronic cochlear implant, the goal of the research in our laboratory is to develop a biological cochlear implant. We hope to be able to use the information we have gleaned from studies of regeneration in avians to produce functional hair cells, nerve cells and supporting cells in the mammalian cochlea through the induction of regeneration or by transplanting stem cells into the damaged inner ear.

2. The mammalian cochlea

The mammalian cochlea is a coiled, snail-shaped sensory organ encased in the dense temporal bone at the base of the skull. Within the cochlea, the organ of Corti is a long, thin strip of sensory epithelium that transduces sound waves into neural signals (Fig. 1). The cells that do the hearing are known as hair cells because they have a prominent tuft of stereocilia (“hairs”) on their apical surfaces that bend in response to a particular frequency. The bending of the cilia sends signals through the body of the hair cells to stimulate auditory nerves which form synapses at the bases of the hair cells. There are four rows of hair cells in the Organ of Corti, one row of inner hair cells and three rows of outer hair cells (Fig. 2). The inner hair cells are in contact with over 95% of the afferent auditory nerves (i.e. sending signals to the brain) and, thus, transmit most of the signals from the cochlea to the brain (Kiang et al., 1984; Spoendlin, 1972). The outer hair cells communicate with only 5% of the afferent fibers, but are heavily innervated by efferent nerve fibers, bringing signals from the brain to the cochlea. It is the efferent fibers and outer hair cells that are thought to fine tune the auditory signals that are sent to the brain, allowing us to focus on important speech signals or other critical sounds in a noisy background. The four rows of hair cells in the mammalian cochlea are organized in a tonotopic manner so that high frequency tones are heard at the base of the cochlea while low tones are heard at the more apical end of the cochlea. Tones in between are localized at progressive positions along the organ of Corti, so that it is organized like a piano keyboard with high notes at one end and low notes at the other.

The hair cells in the organ of Corti are surrounded by a group of supporting cells that physically hold the hair cells up and also provide growth factors and other nutrients to keep the hair cells alive and healthy. The supporting cells also function to remove excess potassium ions that build up in the hair cells during the transduction process. The supporting cells do this through a series of gap junctions that allow extensive intercellular communication between supporting cells and cells in the lateral wall of the cochlea, where the potassium ions are recycled into the scala media. It is these gap junctions that do not function properly in mutations of the Connexin 26 gene, the most common form of nonsyndromic genetic hearing loss (Denoyelle et al., 1999; Kelsell et al., 1997).

In the developing mammalian embryo, the hair cells and supporting cells arise from a common progenitor cell and then go on to develop specific patterns of gene expression specific to the various hair cell and supporting cell phenotypes (Kelley, 2002, 2007; Kelley, Xu, Wagner, Warchol, & Corwin, 1993). Neither hair cells nor supporting cells can be generated in the cochlea after embryonic development, so both types of cells are irreplaceable if lost due to damage or genetic mutations.

3. The avian cochlea

The avian cochlea functions similarly to the mammalian cochlea, but it looks much different. It is not coiled into a snail shell form, but is a short, curved tube that looks somewhat like a banana (Fig. 3, Smith, 1985). However, the bird cochlea does have a population of “inner” and “outer” hair cells that function similar to those in the mammal and are connected to afferent and efferent nerve fibers in a pattern quite similar to that of mammals (Fischer, 1992). The hair cells are arranged in a mosaic pattern, rather than four discrete rows, but the entire sensory epithelium exhibits a similar tonotopic organization to that seen in the mammalian organ of Corti. Birds have a much narrower range in frequency discrimination than most mammals, as they can hear up to only about 12 kHz. Humans can hear up to about 18 kHz (before we discover rock and roll music in our teen years and lose our higher frequencies), but this is still fairly low for most other mammals. Dogs, cats, mice and other rodents can hear up to around 60 kHz, while mammals such as bats, dolphins and whales can hear well beyond 100 kHz. So, humans are actually at the very lower end of frequency discrimination among mammals and have a hearing range fairly close to that of birds. Humans can hear at slightly higher frequencies than birds, but birds are very good at temporal discrimination, i.e. the speed of pitch changes in a signal. This is evidenced by the very complex patterns of songbird calls.

4. Damage and regeneration in the avian cochlea

Sound damage and aminoglycoside poisoning cause damage to the hair cells of the bird cochlea, just as they do in mammalian inner ears. Sound damage is represented in a tonotopic manner so that the region of damage is located along the avian cochlea in a position relative to the frequency of the sound stimulus (Cotanche, Saunders, & Tilney, 1987; Ryals & Rubel, 1982). Aminoglycoside damage usually occurs from the base to the apex in a dose dependent manner (Fig. 4). Low doses affect only the most basal, high frequency hair cells, while larger doses or several doses over a number of days will damage more hair cells further down the cochlea and involve progressively lower frequency ranges. The damage caused by sound or aminoglycosides leads to the death of the hair cells and their eventual ejection from the sensory epithelium. In the mammal, this leads to a permanent hearing loss, as these hair cells are not replaced. But we were surprised to find in our original studies 20 years ago that birds respond to the loss of these damaged hair cells by inducing the adjacent supporting cells to revert to a more plastic state and produce new hair cells to replace those that were lost (Corwin & Cotanche, 1988; Cotanche, 1987; Cruz et al., 1987; Ryals & Rubel, 1988). This replacement of lost hair cells leads to a reconnection with the nerves (Hennig & Cotanche, 1998; Ofsie & Cotanche, 1996; Ofsie, Hennig, Messana, & Cotanche, 1997) and a recovery of auditory function (McFadden & Saunders, 1989; Tucci & Rubel, 1990).

5. Damaged hair cells die by apoptosis

Recent studies in our lab and by others have shown that hair cells in the bird cochlea die in response to sound damage or aminoglycoside poisoning through a process known as apoptosis (Duncan et al., 2006; Forge & Li, 2000; Li, Nevill, & Forge, 1995; Mangiardi et al., 2004; Matsui, Ogilvie, & Warchol, 2002; Matsui et al., 2003; Torchinsky, Messana, Arsura, & Cotanche, 1999). Apoptosis is an active genetic program where a cell sensing that it is in danger begins to make a series of proteins that will lead to the death of that cell. It is, in fact, a

mechanism for cells to commit suicide when in an inhospitable environment or when the cells are no longer needed during development.

Apoptosis is a very complex series of events beginning when a cell first senses a dangerous environment. This initiates the onset of the apoptotic cascade. Once apoptosis is initiated, there is a period of assessment, as the cell determines whether conditions are so bad that they must continue the death process or if they can survive once conditions improve. The cell continuously monitors the situation and competing pro-apoptotic and anti-apoptotic signals released within the cell battle for dominance. This phase of the death process is known as “arbitration” (Adams & Cory, 1998; Crompton, 2000; Letai, 2005). If the anti-apoptotic signals dominate, the cell reverts to normal and death is averted. If the pro-apoptotic signals dominate, the cell continues into the final stages of the cell death cascade. Once this stage has been reached, a family of molecules known as caspases are activated within the cell (Cryns & Yuan, 1998; Earnshaw, Martins, & Kaufmann, 1999; Lavrik, Golks, & Krammer, 2005; Thornberry & Lazebnik, 1998). These molecules begin to break down vesicles, enzymes and the cytoskeleton in the cytoplasm and the DNA in the nucleus, leading to the eventual death of the cell (Fig. 5). Once a cell has died, its remaining components are broken up into small membrane-bound packets and taken up by neighboring cells, or in epithelial tissues the entire dying cell is ejected from the surviving layer of cells (Rosenblatt, Raff, & Cramer, 2001).

Research from my laboratory has been able to identify the timing of several of the steps in hair cell apoptosis and a number of the proteins involved. The first indication that apoptosis has been activated in hair cells of the avian cochlea appears in the base of the cochlea 12 h after a single gentamicin injection. At this point, a molecule known as TIAR moves out of the cell nucleus, where it normally resides, and forms dense spots, or stress granules in the hair cell cytoplasm (Mangiardi et al., 2004). TIAR is translocated only in the hair cells, as the adjacent supporting cells retain the TIAR within their nuclei (Fig. 6A and B). At this time, there is no physical evidence of damage to the hair cells. By 24 h, all the hair cells in the basal 30% of the chick cochlea exhibit a translocation of TIAR. By 30 h, hair cells in the very basal tip of the cochlea begin to show signs of physical damage to their stereocilia and apical cell surfaces. The region expressing damage to the hair cells extends throughout the basal 30% of the cochlea by 36 h. At this point another molecule, cytochrome *c*, alters its localization, indicating that the period of arbitration has been completed and the hair cells are beginning to self-destruct (Fig. 6C and D). In normal, healthy cells cytochrome *c* is restricted to the mitochondria, where it is involved in generating ATP, the fuel needed to run cells. As cytochrome *c* is released from the mitochondria into the cell cytoplasm, it interacts with two other molecules, APAF1 and caspase 9, to form an apoptosome. This protein complex acts as a catalyst to initiate the activation of caspase 3, known as the executioner caspase. Caspase 3 begins to devour up all the cellular proteins and nuclear DNA and also acts to catalyze the activation of more caspase 3, leading to the eventual degradation and death of the hair cell.

Caspase 3 activation appears at the same time that the dying hair cells begin to bleb up and be ejected from the sensory epithelium (Fig. 6E and F). Many dying hair cells with activated caspase 3 are seen up in the tectorial membrane overlying the sensory epithelium. More traditional models of apoptosis regard the activation of caspase 3 as the beginning of the terminal stage of cell death, but that it goes on for a while after the initial activation of caspase 3. Our results suggest that the activation of caspase 3 occurs just as the cells are being ejected and further experiments indicate that the dying hair cell are ejected while still alive and complete their death throes once they have been eliminated from the cochlear epithelium (Mangiardi et al., 2004). Moreover, recent studies have shown that the adjacent supporting cells play an active role in pushing the dying hair cells out of the sensory epithelium (Hordichok & Steyerger, 2007).

6. Therapeutic intervention in hair cell apoptosis

Damage to hair cells exposed to aminoglycosides appears to be caused by a release of large quantities of reactive oxygen species, or free radicals, into the hair cell cytoplasm (Chen et al., 2007; Schacht, 2007). Most cells release small quantities of free radicals as a byproduct of their normal metabolic processes and they have specific buffers that bind up these free radicals to keep them from damaging the cell. But in situations such as aminoglycoside overdose, the levels of free radicals are too high and they overwhelm the intrinsic protective systems. This, then, results in the initiation of apoptosis in the hair cells. It has been proposed that if we could supply the cochlea with additional free radical buffers during the drug exposure this abundance of buffers may be able to bind up all the excess free radicals and avert the initiation of apoptosis in the hair cells. Animal studies and pre-clinical trials in humans show support for this hypothesis by demonstrating a reduction in hair cell loss and functional impairment when subjects were treated systemically or directly into the middle ear with free radical scavengers (Campbell & Rybak, 2007; Chen et al., 2007; Rybak, Whitworth, Mukherjea, & Ramkumar, 2007). Thus, future therapies for preventing hearing loss may include systemic or localized application of free radical scavengers to prevent the buildup of reactive oxygen species during or shortly after a traumatic event. These scavengers may be able to effectively reduce the high levels of reactive oxygen species generated by aminoglycosides, chemotherapeutic agents or noise exposure and thus inhibit the initiation of hair cell apoptosis.

7. Two pathways for regeneration in the avian ear

Regeneration in the avian cochlea following sound damage or aminoglycoside treatment occurs because the supporting cells adjacent to the dying hair cells produce new hair cells to replace those that were lost (Corwin & Cotanche, 1988; Ryals & Rubel, 1988; Stone & Cotanche, 1994). The supporting cells utilize two different pathways for producing the new hair cells: direct transdifferentiation and mitotic regeneration. In direct transdifferentiation a supporting cell alters its gene expression pattern and starts expressing hair cell genes, eventually differentiating into a new hair cell. It does this without dividing, so this results in the loss of one supporting cell for each new hair cell gained through this pathway. Since there are only three to four supporting cells for each hair cell in the bird cochlea (Goodyear & Richardson, 1997), and these cells actually do physically support the hair cells in the sensory epithelium, only one or two supporting cells can pursue this pathway without causing a severe disruption of the cellular architecture of the cochlea. In mitotic regeneration, supporting cells adjacent to the dying hair cells leave quiescence, reenter the cell cycle and divide to produce two daughter cells, one that can become a hair cell and one that replaces the supporting cell that entered into mitosis. In some cases, where there is extensive hair cell loss, the daughters of the initial supporting cell that divided can also divide, leading to a pool of new cells that can then be distributed as needed into new hair cells and supporting cells (Stone & Cotanche, 1994).

Both of these pathways for regenerating new hair cells can occur within the same cochlea in response to hair cell loss (Fig. 7). Direct transdifferentiation seems to be the immediate, early response to hair cell loss, with some supporting cells entering this pathway as early as 12–15 h after a single gentamicin injection (Cafaro, Lee, & Stone, 2007; Roberson, Alosi, & Cotanche, 2004). The first new hair cells derived from direct transdifferentiation appear in the cochlear sensory epithelium 3 days after the gentamicin injection. Mitotic regeneration occurs later in the regeneration process, but eventually leads to a majority of the new hair cells made (Duncan et al., 2006; Roberson et al., 2004). Some supporting cells show evidence of entering the mitotic pathway as early as 24 h after the gentamicin injection (Bhave, Stone, Rubel, & Coltrera, 1995). The first of these proliferating cells enter into the S, or DNA Synthesis, phase of the cell cycle by 65 h and the peak of S phase is reached by 72 h. By 96 h the number of supporting cells entering S phase declines and it tapers back to control levels by 120 h (Bhave et al.,

1995;Duncan et al., 2006). The first new hair cells derived from mitotic regeneration can be identified 4.5 days after the gentamicin treatment (Duncan et al., 2006) but then go on to produce more than two-thirds of the regenerating hair cells (Roberson et al., 2004).

Thus, the regenerative response in the avian cochlea appears to occur in two waves. Direct transdifferentiation seems to be the quickest way to make a new hair cell and may be the “first-responder” pathway or may be used when only a few new hair cells are needed. However, if many new hair cells are required, direct transdifferentiation cannot be the answer because too many supporting cells would be lost. At this point, the ear must activate the mitotic regeneration pathway to produce not only new hair cells, but also new supporting cells. There is preliminary evidence that this pathway may result in the production of a large pool of cells that can then be parsed out into a group to make the number of new hair cells required to repair the epithelium and then the rest assume the role of supporting cells. It is even possible that there is an initial overproduction of both hair cells and supporting cells and that once the cochlea has been repaired the excess cells are removed by apoptosis (Mangiardi & Cotanche, unpublished observations).

Regardless of which regenerative pathway is chosen, it appears that both pathways are initiated and regulated by the apoptotic cascade in dying hair cells. The first steps in both direct transdifferentiation and mitotic regeneration are initiated as the hair cells first enter the apoptotic cascade. However, the supporting cells seem to be stalled at this first early step until the hair cells are actually ejected from the sensory epithelium. At this point the supporting cells resume their progression toward hair cell differentiation (for direct transdifferentiation) or into the S phase of the cell cycle (for mitotic proliferation). Thus, the dying hair cells must be continually signaling the neighboring supporting cells about their progression through the death cascade. If they start down the cascade, but then decide not to die, the supporting cells are sent back into quiescence (Matsui, Gale, & Warchol, 2004). But once they reach the point where they decide death is inevitable, they signal the supporting cells to continue on their task of regenerating and repairing the cochlea.

8. Pharmacological intervention of apoptosis or activation in regeneration

An accurate understanding of the signals in the apoptotic cascade that allow the cells to proceed through cell death or inhibit it may be instrumental in developing drugs that can block the early or middle stages of hair cell death and enable hair cells to survive a traumatic insult. Several studies in animal models have shown that specific inhibitors of apoptosis can prevent hair cells from dying and preserve their function (Cheng, Cunningham, & Rubel, 2003; Cunningham, Cheng, & Rubel, 2002; Matsui et al., 2002, 2003, 2004; Sugahara, Rubel, & Cunningham, 2006). Thus, it may be possible to develop specific blockers of apoptosis targeted to the cochlea for use in human patients that require systemic chemotherapy or aminoglycoside treatment for cancer or aggressive infections. This localized inhibition of hair cell death may preserve the hair cells and auditory function while enabling the otherwise ototoxic levels of these agents to perform their tasks in other tissues.

In terms of controlling regeneration, the working hypothesis that we have developed is that early and late apoptotic signals from the dying hair cells enable the supporting cells to initiate regeneration, wait until the hair cells are ejected, and then proceed through new cell proliferation and differentiation. If these signals from the dying hair cells could be identified and/or the receptors on the supporting cells that respond to the signals be determined, then we could experimentally manipulate the supporting cells to enter and complete regeneration without the need of initiating hair cell death. Once these signals can be controlled in the avian cochlea, then we can address whether manipulation of these same signals in the mammalian cochlea could lead to regeneration.

9. Potential therapies in the mammalian cochlea

Although hair cell regeneration has been found to occur in the cochleas of every species of bird examined and at all stages of their lifespan, repeated attempts by a number of research groups have found no evidence of inherent regeneration in the mammalian cochlea in response to trauma. There is a brief period during mouse embryonic development when new hair cells can be induced through direct transdifferentiation to supplement or replace the initial population (Kelley et al., 1993; Kelley, Talreja, & Corwin, 1995). Moreover, there is good evidence that limited hair cell regeneration occurs in the mammalian vestibular system, even in humans (Forge, Li, Corwin, & Nevill, 1993; Warchol, Lambert, Goldstein, Forge, & Corwin, 1993). But it appears that the mammalian cochlear sensory epithelium has lost its regenerative capacity. One possible reason for this is that the mammalian cochlea activates a number of anti-proliferation genes in both the hair cells and supporting cells of the organ of Corti as soon as the initial population of hair cells and supporting cells is established (Chen & Segil, 1999; Chen, Johnson, Zoghbi, & Segil, 2002; Chen et al., 2003; Kelley, 2002, 2007). These genes, called oncogenes, are used in all cells of the body to prevent unregulated cell division from going out of control and becoming cancerous. In the cochlea these oncogenes, once they are transcribed, inhibit cell division in the normal supporting cells and appear to be resistant to down-regulation in response to hair cell loss, thus prohibiting the re-activation of proliferation following trauma.

While the mammalian cochlea does not seem to be able to regenerate on its own after hair cell loss, recently developed tools in cellular and molecular biology have enabled scientists to manipulate cell function and change the normal fates or capabilities of mammalian cells. Preliminary attempts to extrinsically induce regeneration in the mammalian cochlea have utilized genetic manipulation, gene therapy, and stem cell transplantation and have achieved at least limited success. Each of these approaches is discussed below.

9.1. Genetic manipulation

As described above, the differentiating mammalian cochlea normally upregulates cell proliferation inhibitors once the structure of the cochlea is established during embryonic development. However, current techniques in molecular biology enable researchers to turn off or “knock out” specific genes in the fertilized mouse zygote and examine how the lack of a specific gene affects normal development. The value of this powerful technique was recently recognized by a 2007 Nobel Prize in Physiology or Medicine to Mario Capecchi, Martin Evans, and Oliver Smithies, the three scientists who developed it (Nobelprize.org, 2008).

Knockout mice were generated that lacked $p27^{kip1}$, $p19^{Ink4d}$, and $Rb1$, three of the most well-recognized proliferation inhibitors in cells (Chen & Segil, 1999; Chen et al., 2003; Lowenheim et al., 1999; Sage et al., 2005, 2006). The cochleas in mice lacking these inhibitors underwent normal embryonic development of the organ of Corti until the time when the oncogenes should have stopped proliferation. Instead, the cells in the knockout mice continued to proliferate and produced more hair cells and supporting cells than in normal ears. So, it appears that an ear lacking some of these oncogenes can continue to proliferate and produce new hair cells very much like a bird cochlea. Unfortunately, though, the lack of these oncogene proteins seems to also initiate programmed cell death in the proliferating cells, perhaps as an innate protective mechanism against cancer. As a result, all of the cells in the cochlea, and in some cases, the entire animal, die within a month of birth. This suggests that turning off these genes is an important first step in stimulating regeneration in the mammalian cochlea, but that we need to be able to turn them back on once new hair cells are made in order to preserve the integrity of the sensory epithelium. Future experiments in genetic regulation of the mammalian cochlea will be exploring ways to turn off and then turn back on these proliferation inhibitors and determine if that will lead to structural and functional hair cell regeneration.

9.2. Gene therapy

The differentiation of hair cells in the developing cochlea seems to be keyed by the expression of a gene known as *Atoh1* (formerly known as *Math1*, Bermingham et al., 1999). Once precursor cells begin to express this gene, they then go on to express a cascade of hair cell-specific genes. Furthermore, the lack of this gene in a knockout mouse results in the production of no hair cells during development (Bermingham et al., 1999; Chen et al., 2002). Experiments using cultures of mouse vestibular sensory epithelia and very immature cochlear epithelia demonstrated that some nonsensory cells in the inner ear could differentiate into hair cells when the *Atoh1* gene was extrinsically introduced into these cells (Woods, Montcouquiol, & Kelley, 2004; Zheng & Gao, 2000). Cells from other epithelia outside of the ear do not show an ability to differentiate into hair cells when *Atoh1* is overexpressed in them; rather they tend to form neurons or organ-specific cells. Thus, there seems to be some inherent pathway in the cochlear nonsensory supporting cells that can be readily diverted toward a hair cell fate if *Atoh1* is artificially introduced into these cells. Taking advantage of this, Yoash Raphael and his group at the University of Michigan have utilized a gene therapy approach to transfect *Atoh1* into the surviving supporting cells in a guinea pig cochlea following hair cell loss induced by aminoglycoside treatment. In the non-*Atoh1*-treated ear of the guinea pig there is an extensive loss of hair cells throughout a majority of the sensory epithelium. In the *Atoh1* treated ear, however, there is a significant regeneration of hair cells and a recovery in the complex organization of the organ of Corti that is also correlated with a modest recovery of auditory function (Izumikawa et al., 2005; Kawamoto, Ishimoto, Minoda, Brough, & Raphael, 2003). This is an exciting result in that the introduction of exogenous *Atoh1* into the supporting cells of the organ of Corti seemed to lead to functional hair cell regeneration in the mammal. However, the *Atoh1* gene was also taken up by other supporting cells outside of the organ of Corti and led to the development of numerous ectopic hair cells. Interestingly, though, these ectopic hair cells were able to release neurotrophic signals that enabled cochlear nerve processes to grow out and meet them, even in their abnormal locations. This suggests that a developing hair cell inherently generates signals that attract connections with nerve fibers, which greatly simplifies the job of reinnervating the regenerated hair cells. So the future challenges in this gene therapy approach to hair cell regeneration in the mammalian cochlea will be to restrict the transfection of *Atoh1* to only those supporting cells in the organ of Corti and induce hair cell regeneration in only the areas of the inner ear where it will lead to a structural recovery that can most effectively re-establish auditory function.

9.3. Stem cell transplantation

The experimental approaches described above for inducing regeneration in the mammalian cochlea have focused on altering the capacity and fate of intrinsic cochlear cells so that they can make new hair cells and supporting cells in response to damage. One other approach is to introduce a new population of cells into a damaged cochlea and enable them to integrate into and repopulate the damaged sensory epithelium. Stem cells are an obvious and popular choice to utilize in attempting this approach (Parker & Cotanche, 2004). By definition, stem cells are multipotent cells that have the ability to give rise to a number of different cell types, either within a single organ or throughout multiple organs. Many adult tissues have an intrinsic population of stem cells that can produce two or more different cell types within that tissue. These stem cells are seen as tissue-specific and their ability to produce cells for other tissues is thought to be limited. However, more recent theories suggest that these tissue-specific stem cells are more flexible in their fates and when moved to a new environment can recognize this change, alter their genetic programming, and give rise to new cells appropriate for the new environment. This emerging theory, which is known as the “microenvironment” theory, posits that all stem cells in a mature animal are derived from a similar population and that they become specialized to produce cells for the particular tissue in which they reside by getting specific cues from the microenvironment of that tissue. Thus, if these cells could be moved to a new

microenvironment, they would detect and respond to the new conditions and begin making cells characteristic of their new tissue. The implications of this “microenvironment” theory are that stem cells from tissues that are readily accessible, such as blood or skin, could be isolated and transplanted to a new tissue where they will then integrate and become a stem cell for that tissue.

Human embryonic stem cells have received quite a bit of popular press lately (both good and bad) and are probably the most well-known type of stem cell. They are derived from a small pool of cells in the very early embryo called the inner cell mass. It is this population of a few hundred undifferentiated cells that will eventually produce all the cells, tissues, and organs of the human body. Therefore, they have a tremendous potential for therapeutic repair and replacement in aging, diseased, and traumatically injured humans. Unfortunately, they also have some significant medical and ethical implications that currently limit their use in treating human disease. While many groups have been enmeshed in political debates over whether the humanitarian potential of embryonic stem cells outweighs their ethical concerns, other researchers have focused on using either “adult” stem cells (i.e. stem cells found in mature organs such as skin or blood) or stem cells derived from umbilical cord blood to pursue the clinical potential of stem cell transplantation.

In recent years many groups have been exploring the use of various stem cell types for repairing the auditory system. Stefan Heller and colleagues have pursued the isolation and identification of intrinsic stem cells from the mouse vestibular sensory epithelium and investigated whether they can be used to repair the damaged cochlear nerve or organ of Corti (Li, Liu, & Heller, 2003). This group has also shown that mouse embryonic stem cells and mesenchymal stem cells from bone marrow can be coaxed down a hair cell pathway and will express a number of hair cell-specific genes in culture (Jeon, Oshima, Heller, & Edge, 2007; Li, Roblin, Liu, & Heller, 2003; Oshima et al., 2007). They have also shown that neural progenitors derived from mouse embryonic stem cells can integrate into a damaged cochlear nerve and send out neural processes toward the cochlea (Corrales et al., 2006). Several other groups have explored the use of mouse embryonic stem cells, bone marrow cells, or neural stem cells for repopulation of the cochlear nerve, organ of Corti, and lateral cochlear wall (Hildebrand et al., 2005; Lang et al., 2006; Matsuoka, Kondo, Miyamoto, & Hashino, 2006; Matsuoka, Kondo, Miyamoto, & Hashino, 2007).

Research in my lab has focused on transplanting neural stem cells into the noise-damaged cochleas of mice and guinea pigs (Parker et al., 2007). We use an immortalized line of mouse neural stem cells developed by Evan Snyder (Snyder et al., 1992) that is advantageous because all of the cells express stem cell characteristics. In contrast, the neurosphere, the most commonly utilized form of neural stem cells, contains only a small percentage of true stem cells, with a majority of the cells being glial cells (Parker et al., 2005). We transplant the neural stem cells into the scala tympani of mice and guinea pigs 2 days after noise exposure, allowed the cells to integrate and survive for 2, 4, or 6 weeks. We then analyze whether the neural stem cells survive, whether they infiltrate into the cochlear tissues, and if they begin to express any characteristic of native cochlear cells. One difficult issue that we dealt with in these experiments is that the stem cells appear to turn off the genetic markers that are used to identify them after 4–6 weeks in the animal. Either that, or all the stem cells die after 4 weeks in their new environment. Data from other groups suggests that these cells tend to turn off their integrated markers as they begin to differentiate into mature cells. In order to overcome this problem, we utilize only male neural stem cells and transplant them into female guinea pigs. Subsequently, the presence of the stem cells in the transplanted ears can be detected using chromosome markers for the male Y chromosome. Female cells of the host guinea pig should only express XX chromosomes, so any cells in the transplanted ears that label with the Y chromosome marker should be derived from the stem cells.

At 6 weeks of survival, we find that a majority of the identifiable stem cells are located in the cochlear nerve, where they differentiate into satellite cells (a type of neural supporting cell) and neurons expressing markers characteristic of the normal cells found in the cochlear ganglion (Fig. 8). A quantitative analysis indicates that there is a significant increase in the number of neurons in the cochlear ganglion on the side where stem cells are transplanted when compared to the opposite side, which was noise exposed but no stem cells implanted. In the cochlea, we find fewer stem cells than in the nerve, but those that do infiltrate the organ of Corti or the lateral wall differentiate into cochlear-specific cell types (Fig. 9). This includes inner and outer hair cells, pillar cells, Deiter's cells, fibrocytes in the lateral wall and cells within the stria vascularis (Parker et al., 2007). These studies are still preliminary and we need to examine longer survival times or find ways to direct the stem cells to the cochlea, rather than the spiral ganglion. However, these early results do indicate that the stem cells are able to survive transplantation, integrate into the tissues of the cochlea, respond to the local signals in the cochlear microenvironment, and differentiate into specific cell types needed to repair the sensory epithelium. These proof-of-principal experiments with transplanting stem cells into a damaged mammalian cochlea along with experiments from other groups transplanting stem cells into the cochlear nerve and lateral wall of the cochlea suggest that this approach may offer a potential therapeutic approach to repairing the damaged or aging human cochlea. However, much more work in animal models needs to be done before attempting stem cell transplantations into human cochleas.

10. Conclusions

After 20 years of exploring hair cell regeneration in the cochlea of birds and mammals, we have a solid understanding of the process of hair cell death and the regenerative responses (or lack thereof) in the supporting cells. We still have not yet defined the specific signaling molecules that dying hair cells use to signal supporting cells to undertake regeneration, nor have we found the signals that stimulate direct transdifferentiation versus mitotic regeneration in supporting cells of the avian cochlea. However, the impending identification of these signals should allow us to experimentally regulate hair cell regeneration in the bird ear itself or in organ culture preparations of the cochlea. Although mammalian cochlear supporting cells do not normally regenerate like those of the bird, we are beginning to understand some of the signals and molecules that block mammalian supporting cells from responding to hair cell loss. We are at the earliest stages of experimentally manipulating mammalian supporting cells to artificially induce them to regenerate and in transplanting stem cells into the damaged ear to replace the lost hair cells and supporting cells. Continued advances over the next 20 years on why bird cochleas can regenerate while mammalian ones cannot may enable us to develop cellular and genetic therapies for treating hearing loss in the human cochlea.

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Appendix A. Continuing education (*correct answers)

1. Hair cell death, or apoptosis, following noise trauma or aminoglycoside treatment:
 - a. Causes a correlated death of the adjacent supporting cells.

- b. Is caused by biochemical and mechanical trauma completely outside of the control of the hair cells.
 - c. Is a genetically controlled program that enables a cell to regulate its own death through the dismantling of its cytoplasmic and nuclear contents.*
 - d. Is under direct neural control.
- 2. Hair cells and supporting cells in both the avian and mammalian cochlea:
 - a. Arise from two completely different embryonic sources.
 - b. Are derived from a common precursor cell.*
 - c. Function completely independent of one another.
 - d. Are sensory cells capable of turning sound waves into neural signals to the brain.
- 3. Supporting cells in the avian cochlea participate in regeneration:
 - a. Through two separate but closely related processes: direct transdifferentiation and mitotic regeneration.*
 - b. Through a process of cell division that is ongoing throughout the life of the bird.
 - c. By making new nerve fibers to connect to the hair cells.
 - d. By recruiting new hair cells from neighboring vestibular endorgans.
- 4. Current approaches to inducing regeneration in the mammalian cochlea include:
 - a. Genetic manipulation.
 - b. Gene therapy.
 - c. Stem cell transplantation.
 - d. All of the above.*
- 5. Potential therapies for prohibiting hair cell death in the mammalian cochlea include:
 - a. Blocking the onset of cell death by introducing excess free radical scavengers to the cochlea.
 - b. Throwing away your teenagers' iPod and banning them from ever listening to rock and roll music.
 - c. Blocking the progression of hair cell death, even once it has been initiated, using specific blockers of early and middle steps in the apoptotic cascade.
 - d. Both A and C are potential therapeutic options.*

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Fig. 1.

The human cochlea. A classic anatomical drawing from Frank Netter of a mid-modiolar cross-section through the human cochlea showing the centrally located cochlear spiral ganglion and the various coils of the cochlear duct. The higher magnification drawing on the left represents a cross-section through an individual turn and shows the location of the organ of Corti, the three scalae and the peripheral process of the cochlear nerve cells. Used with permission from the Netter Atlas of Human Anatomy, 4th Edition.

Fig. 2.

The organ of Corti. A confocal micrograph of the surface of the organ of Corti from a mouse cochlea showing the four rows of hair cells. This preparation was labeled with fluorescent phalloidin, a marker for actin filaments in cells. The intense labeling of the stereociliary bundle on each hair cell reveals the actin bundles that form the structural core of the stereocilia. IHC, inner hair cell; OHC1, first row of outer hair cells; OHC2, second row of outer hair cells; OHC3, third row of outer hair cells.

Fig. 3.

The avian cochlea. (A) This light micrograph shows an isolated preparation of the entire chick cochlea. The lateral stripes seen on the upper surface represent the folds of the tegmentum vasculosum, the ion-transporting epithelium in the chick cochlea. At the distal tip of the avian cochlea is the horseshoe-shaped lagena, a low-frequency sound and vibration detector. (B) This low power confocal micrograph shows a surface preparation of the basilar papilla, the sensory epithelium of the avian cochlea. The chick cochlea has a tonotopic organization, where high frequencies are heard in the narrow, proximal part to the left, and low frequencies are heard at the wide, distal end shown to the right. This preparation is labeled with fluorescent phalloidin, marking the actin filaments in the hair cells and supporting cells.

Fig. 4. Gentamicin damage in the chick cochlea. These two low power confocal micrographs show only the high frequency half of the bird cochlea. A normal high frequency region from a control bird is shown in A. The white dots represent hair cells. A gentamicin treated bird cochlea is shown in B, at 72 h after a single injection of gentamicin. There are no remaining hair cells in the high frequency end of this cochlea, as seen by the loss of the white dots (arrows). Regenerating hair cells in this region are not yet identifiable.

Fig. 5.

Apoptosis in hair cells. This low power confocal micrograph shows the proximal, high frequency region of the chick cochlea 72 h after gentamicin treatment. The dying hair cells contain activated caspase-3 (green), the executioner caspase in apoptosis. Surviving hair cells and other cell types in the cochlea are labeled with phalloidin (red) to detect actin filaments.

Fig. 6.

Apoptotic proteins in the death cascade. The earliest sign that apoptosis has been activated following gentamicin treatment is the translocation of TIAR out of the nucleus into the cytoplasm of hair cells. A normal cochlea is shown in A, where the TIAR is localized to all the cell nuclei of both hair cells and supporting cells (inset). In B, the TIAR in hair cells move out of the nucleus and forms brightly fluorescent granules in the hair cell cytoplasm (inset) only 24 h after gentamicin treatment. The next identifiable step in apoptosis is the release of cytochrome *c* from mitochondria into the hair cell cytoplasm. In C the distribution of cytochrome *c* is shown (green) in hair cells double-labeled with phalloidin (red). In D only the distribution of cytochrome *c* is shown for the same sample. The normal, punctate distribution

of cytochrome *c* in mitochondria is seen in the two cells at the very top of the micrograph. In the cells labeled with arrows cytochrome *c* has spilled out into the hair cell cytoplasm and fills it with a green fluorescence. Finally the executioner stage of apoptosis is reached with the activation of caspase-3. In E and F the distribution of the activated form of caspase 3 is shown in hair cells that are in the process of blebbing up and being ejected from the cochlear epithelium (at arrows, plus in other cells). The green label in E and F is the activated form of caspase 3. The red label in E represents phalloidin labeling of the hair cells.

Fig. 7.

Hair cell regeneration in the chick cochlea. This whole-mount confocal micrograph shows the development of new hair cells in the chick cochlea 7 days after a single gentamicin treatment. The new hair cells (green) are labeled with a marker for myosin VI, a protein found only in hair cells in the cochlear sensory epithelium. Some of the new hair cells are labeled with BrdU, a marker for DNA synthesis in dividing cells. The new hair cells that lack BrdU in their nuclei were generated by direct transdifferentiation (white arrows), while those that have BrdU-labeled nuclei were made through the mitosis of a supporting cell. Some labeled nuclei are in newly generated supporting cells, which don't label with myosin VI (red arrows).

Fig. 8.

Stem cell transplantation into the cochlear nerve. Male mouse neural stem cells were transplanted into female guinea pigs allowed to integrate into the cochlear nerve bundle for 6 weeks. In A, labeling with a marker for the male Y chromosome (green dot) showed that both satellite cells (white arrows) and neurons (green arrow) in the recovered cochlear nerve were derived from stem cells. Neurons are labeled with neurofilament 200 (red), a protein found only in neurons. All cell nuclei are labeled with DAPI (light blue), a marker for DNA. In B, a satellite cell derived from a stem cell (yellow arrow) is labeled with S100b (red), a protein found satellite cells and not neurons. In C, a neuron in the cochlear spiral ganglion derived

from a stem cell (green arrow) is labeled with neurofilament 200 (red). Cell nuclei in B and C are labeled with DAPI.

Fig. 9.

Stem cell transplantation into the organ of Corti. The two micrographs in A and B represent a brightfield/ fluorescence view (A) and a fluorescence-only view of the same section through a cochlea that was transplanted with stem cells. The third row outer hair cell in these images is labeled with a male Y chromosome marker (white arrow), while the first and second row outer hair cells (green arrow) and the inner hair cell (red arrow) are not. The hair cells are also labeled for Myosin VIIa, a marker found only in hair cells in the cochlea. All cell nuclei are labeled with DAPI (light blue).