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"Hair cell regeneration in the inner ear"

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BASIC SCIENCE REVIEW

EDWIN W RUBEL, PhD
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Hair cell regeneration in the inner ear

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Hearing and balance disorders caused by the loss of inner ear hair cells is a common problem encountered in otolaryngology–head and neck surgery. The postembryonic production of hair cells in cold-blooded vertebrates has been known for several decades, and recent studies in the avian inner ear after ototoxic drug and noise damage have demonstrated a remarkable capacity for both anatomic and functional recovery. The regeneration of sensory hair cells has been shown to be integral to this repair process. Current work is focusing on the cellular progenitor source of new hair cells and the trigger mechanism responsible for inducing hair cell regeneration. Preliminary studies suggest that reparative proliferation may also occur in the mammalian inner ear. Work in this field is moving at a rapid pace. The results thus far have yielded optimism that direct stimulation of hair cell production or transplantation of living hair cells may eventually become treatment modalities for the damaged human inner ear. These proposals would have been considered unrealistic less than 10 years ago, but they now have caught the full attention of both clinician and researcher. (OTOLARYNGOL HEAD NECK SURG 1994;111:281-301.)

Deafness is the second most common handicap in the United States.[1] Sensorineural hearing loss resulting from noise-, infection-, ototoxin-, or age-induced cochlear hair cell damage is the most common form of hearing loss seen today by the otolaryngologist–head and neck surgeon. Vestibular disorders are also common, with 30% of all Americans having experienced episodes of dizziness by the age of 65. As the population ages, hearing and balance disorders caused by inner ear hair cell loss are expected to increase. Consequently, developing methods to replace lost receptor cells through transplantation or regeneration is an active area of research.

Postembryonic proliferation and differentiation of vertebrate hair cells has been investigated in elasmobranchs, fish, and amphibians. Continual postembryonic production of hair cells occurs in the macula neglecta of sharks and rays and in the saccules of the "oscar" fish and toad.[3-8] Hair cell generation has also been demonstrated after experimentally induced hair cell loss. New hair cells are regenerated in the laser-ablated lateral line of the axolotl salamander and in the gentamicin-damaged vestibular end-organs of the bullfrog and "oscar" fish.[9-11]

In contrast to cold-blooded vertebrates, both birds and mammals were believed to have their full adult complement of hair cells at birth.[12,13] Thus any postnatal hair cell loss was thought to be irreversible and associated with a permanent functional deficit. Studies in the avian and mammalian inner ear, discussed below, have suggested that these assumptions must be reevaluated.

The Avian Inner Ear

The avian inner ear has become a popular model for studying peripheral sensory end-organ function. The avian cochlea, or "basilar papilla," is curvilinear and has a tonotopic organization.[14] It is much easier to examine histologically than the spiraled mamma-

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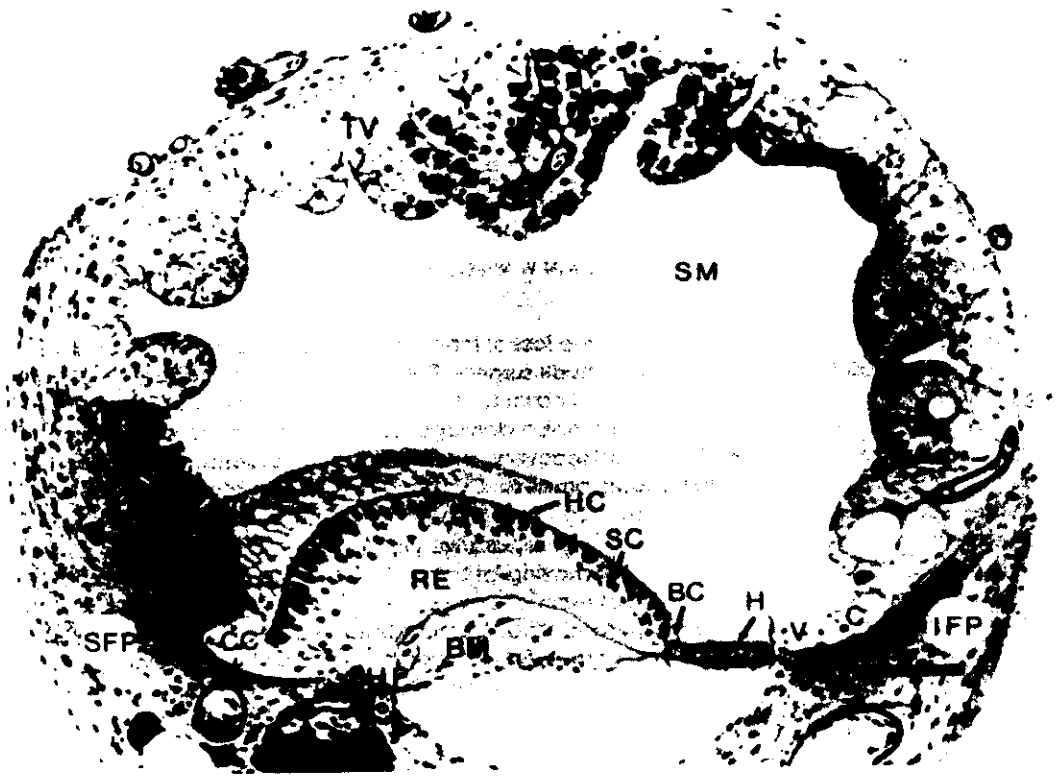

Fig. 1. Transverse light microscopic section through chick cochlea. The receptor epithelium *(RE)* lies on the basilar membrane *(BM)* and is composed of hair cells *(HC)* and supporting cells *(SC)*. Border cells *(BC)* and hyaline cells *(H)* lie inferior to the receptor epithelium. Superior fibrocartilaginous plate *(SFP)*, clear cells *(CC)*, tectorial membrane *(TM)*, habenula perforata *(HP)*, tegmentum vasculosum *(TV)*, scala media *(SM)*, vacuole cells *(V)*, cuboidal cells *(C)*, inferior fibrocartilaginous *(IFP)*. Scale bar = 100 μm.

lian cochlea. The auditory sensory epithelium consists of a continuous sheet of basal supporting cells and lumenal hair cells (Figure 1). One to four types of sensory hair cells have been described by several authors.[15-20] Tall hair cells (THC) and short hair cells (SHC) are in many ways analogous to the mammalian inner hair cells and outer hair cells, respectively.[21] The THC occupy the superior (neural) edge of the cochlea and have both afferent and efferent innervation. The SHC occupy the inferior (abneural) edge and receive predominately efferent innervation. Intermediate hair cells are frequently found between the THC and SHC. The supporting cells are located between the hair cells and basilar membrane, and have an apical process extending to the lumenal surface.

The avian vestibular system is also similar to the mammal's.[22] The vestibular labyrinth of birds contains two end-organ types: the orthogonal ampullary organs (lateral, superior, and posterior) and the otolithic organs (utricle, saccule, and lagena). Like the mammal, the avian vestibular sensory epithelium consists of supporting cells and two types of hair cells.[23] Type I hair cells are pear-shaped and enclosed by a nerve calyx, whereas type II hair cells are cylindrical and have multiple bouton-type nerve endings. Again, the supporting cells are predominately located between the sensory cells and the basal lamina.

Hair Cell Regeneration in the Avian Inner Ear

Two serendipitous studies led to the discovery of hair cell regeneration in the avian inner ear. Cruz et al.[24] investigated the temporal pattern of cochlear hair cell loss in the chick after a 10-day course of the ototoxic aminoglycoside gentamicin. Hair cell counts in treated cochlea were compared with counts from age-matched controls for survival times ranging from 1 day to 3 weeks after treatment. As time progressed, a basal to apical progression of hair cell loss was observed across the cochlea. Maximal hair cell loss was seen 1 week after the treatment,

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with over 60% of the cochlear length damaged. However, in gentamicin-treated chicks allowed to survive 2 weeks longer, partial restoration of hair cell number was observed throughout the basal region of the cochlea.

Independently, Cotanche[25] used scanning electron microscopy (SEM) to examine the chick cochlea after acoustic trauma. As early as 48 hours after severe acoustic overstimulation, immature hair cell stereociliary bundles were observed within the noise-damaged region. The new stereociliary bundles went through a sequence of maturational steps similar to that of developing stereocilia in the embryonic cochlea.

While other interpretations were possible, taken together, these two studies were suggestive of the production of newly created hair cells in the damaged avian auditory end-organ. Definitive evidence that the repopulation of hair cells after avian cochlear damage is due to the production and differentiation of a new generation or generations of cells was provided in two parallel studies using a cell-proliferation marker. Tritiated thymidine (^{3}H-thymidine), a radiolabeled analog of the nucleic acid thymidine, is readily incorporated into the replicating DNA of proliferating cells during "S-phase" of the cell cycle. After mitosis, the nucleus of each daughter cell contains the radioactive marker, which may then be detected using classic autoradiographic techniques.[26] Tissue sections are immersed in a radiosensitive emulsion, which, after an adequate exposure period, is developed, and this results in reduced silver grains overlying the nucleus of labeled cells.

Corwin and Cotanche[27] used intense sound (1.5 kHz at 120 dB SPL for 48 hours) to induce cochlear damage in neonatal chicks. After 10 days of ^{3}H-thymidine injections, the cochlea were removed and processed for autoradiography. Labeled hair cells and supporting cells were observed in the region of sensory epithelial damage. In an independent study, Ryals and Rubel[28] histologically examined cochlea from adult quail 10 days after acoustic trauma (1.5 kHz at 115 dB SPL for 12 hours). Birds were given twice-daily injections of ^{3}H-thymidine for 10 days after the noise exposure. As above, ^{3}H-thymidine-labeled hair cells and supporting cells were seen in the damaged area by 10 days after the damage (Figure 2). Hair cell counts revealed that the 70% hair cell loss observed 10 days after the damage was nearly fully recovered by 60 days after the damage (Figure 3).

More recently, evidence for a small amount of sensory epithelial proliferation has been reported in the normal, untreated avian cochlea. Ryals and Westbrook[29] observed a few ^{3}H-thymidine-labeled supporting cells and juxtaposed hair cells in normal adult quail. Oesterle and Rubel[30] reported a very low rate of production of cochlear supporting cells in normal neonatal chicks. Tritiated-thymidine labeled sensory epithelial cells had not been previously reported in control cochlea or in undamaged portions of acoustically overstimulated inner ears.[27,28,31,32] These more recent data suggest that the ability to restore lost receptor hair cells may be dynamically regulated; sensory epithelial mitosis may be regulated by tissue "needs."

Morphologic Recovery

These landmark studies have stimulated many laboratories to investigate the anatomic specifics of the avian cochlear repair process. Long duration studies demonstrated that the morphologic recovery of the sensory epithelium is near complete, with hair cell counts returning to normal and hair cell reinnervation occurring. In adult quail THC numbers were near normal by 60 days after acoustic insult, but SHC recovery had a slower time course.[33] Duckert and Rubel[34,35] examined the ultrastructure of the recovering basal chick cochlea after treatment with the ototoxic aminoglycoside gentamicin. Sensory cell numbers were equivalent to untreated control animals by 6 weeks, but full maturation of the SHC and their synaptic contacts was not complete until 20 weeks. During the maturational process, sensory cell apical surfaces expanded; numbers and complexity of cellular organelles increased; and SHC shape progressed from globular to the mature "squat pitcher" shape (Figure 4). In addition, stereociliary bundles grew in length, became more tightly packed and hexagonally arranged, and changed directional orientation (Figure 5). Similar morphologic changes have been described in the chick after acoustic trauma,[25,36-38] as well as in the adult budgerigar after treatment with the ototoxic aminoglycoside kanamycin.[39,40] Regarding synaptic patterns, initial afferent nerve terminals on regenerating SHC are replaced by efferent terminals, which steadily increase in number to 1 to 3 per cell. At 20 weeks after damage, the innervation pattern is qualitatively identical to that of normal animals.[35] Ryals et al.[41] saw a similar regeneration of normal hair cell synaptic patterns in adult quail recovering from acoustic overexposure.

Concomitant with the sensory epithelial recovery, the tectorial membrane also regenerates after

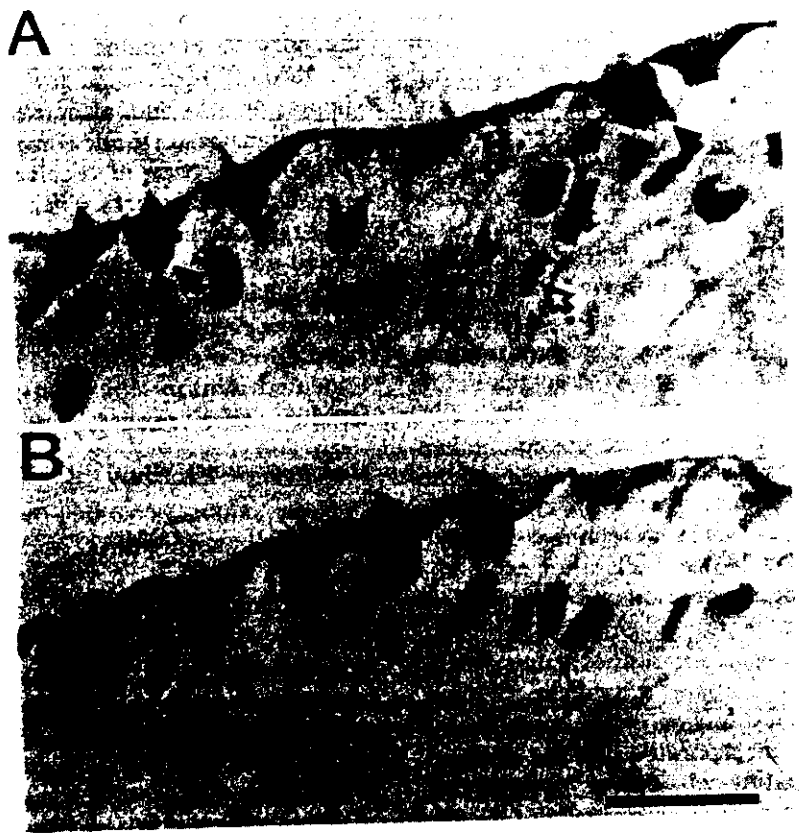

Fig. 2. Regeneration of quail hair cells. **A**, Photomicrograph of ^{3}H-thymidine-labeled hair cells *(solid arrows)* and supporting cells *(open arrows)* in the adult quail cochlea 10 days after acoustic trauma (1.5 kHz at 115 dB SPL for 12 hours). **B**, Photomicrograph of a cochlea from a normal quail injected with ^{3}H-thymidine. Note the absence of ^{3}H-thymidine-labeled epithelial cells. Scale bar = 20 μm. (From Ryals BM, Rubel EW. Science 1988;240:1774-6. By permission.)

acoustic trauma.[42-44] Cochlear supporting cells secrete new matrix materials to regenerate the honeycomb-like lower layer of the normally trilaminar tectorial membrane.

Physiologic Recovery

The surprisingly complete anatomic recovery of the avian cochlea after both acoustic and ototoxic insult has led to investigations of functional recovery. Are regenerated hair cells capable of restoring hearing? Both behavioral and electrophysiologic studies have suggested this may be true. Hearing recovers, but with some residual loss. Studies with postdamage recoveries longer than 22 weeks still remain to be undertaken to determine whether recovery is eventually complete. McFadden and Saunders[45] investigated cochlear nucleus evoked potentials in chicks after intense pure-tone exposure (0.9 kHz at 120 dB SPL for 48 hours). This stimulus resulted in an immediate 60 dB threshold shift, but near complete recovery was achieved within only 15 days, with the greatest recovery occurring within the first 3 days. Because of the fast functional recovery after noise damage, they postulated that repair of the tectorial membrane, not hair cell regeneration, played the major role in restoring hearing.

Studies utilizing a drug-damage paradigm, where the tectorial membrane appears unaffected by the drug, have enabled investigators to correlate hair cell regeneration and the functional restoration of hearing. Ototoxic aminoglycosides cause near complete hair cell loss in the basal cochlea without affecting the tectorial membrane.[39,46-48] The recovery of auditory perception after ototoxic drug damage has been studied in our laboratory by Marean et al.[48] Using European starlings operantly conditioned to respond to pure-tone stimuli, we compared behavioral detection thresholds before, during, and after

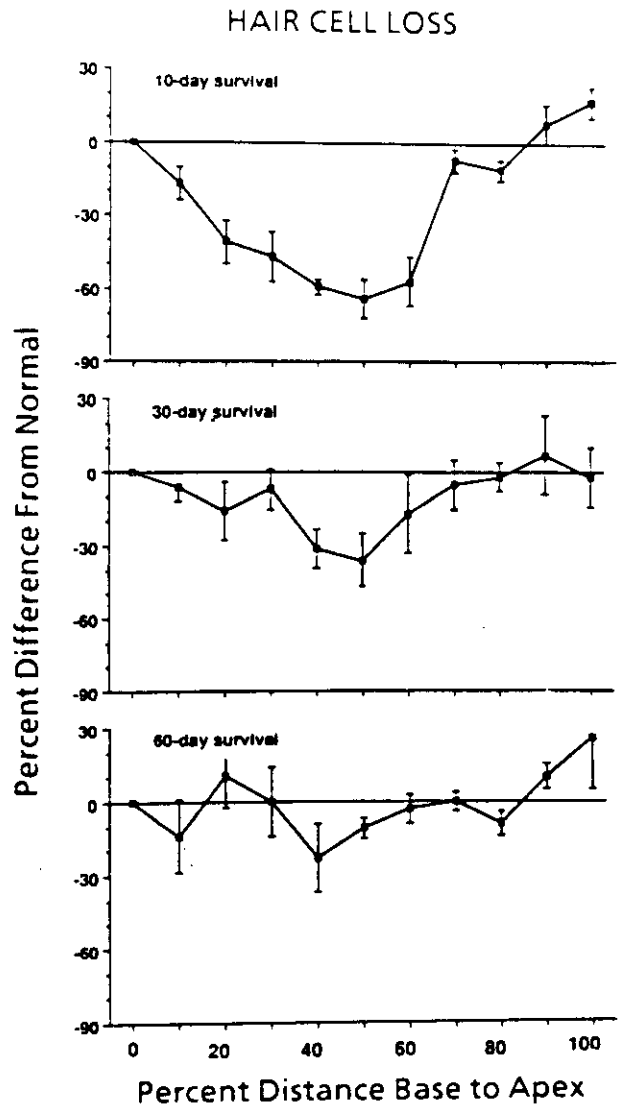

Fig. 3. Increase in hair cell number during regeneration in quail cochlea. The mean percentage difference in hair cell number, as compared with normal controls (n = 6), after acoustic trauma (1.5 kHz at 115 dB SPl for 12 hours) for *(top)* 10-day, *(middle)* 30-day, and *(bottom)* 60-day survival times. Normal hair cell number in controls is shown by a *straight line* at 0 along the cochlea from base to apex. Average percentage difference in hair cells from normal (± SEM) is shown in 10% intervals along the cochlea from base to apex at each survival time (n = 6, 5, and 3 for 10-day, 30-day, and 60-day survivals, respectively). (From Ryals BM, Rubel EW. Science 1988;240:1774-6. By permission.)

treatment with the aminoglycoside kanamycin (Figure 6). Using scanning electron microscopy, we investigated the extent and nature of cochlear hair cell loss and recovery. There was an immediate 60 dB high-frequency (4-7 kHz) hearing loss after treatment, which corresponded to a loss of hair cells across the basal 34% of the cochlea. Hearing progressively improved for the next 50 days, but some degree of hearing loss remained out to 141 days (25 dB threshold shift at 7 kHz). This threshold shift corresponded with a persistent stereociliary bundle disorientation in the basal cochlea. Destruction of the regenerated hair cells by retreating the animals with kanamycin reinstated the high-frequency hearing loss, strongly suggesting that the regenerated hair cells are responsible for the functional recovery. Hashino and Sokabe[49] studied behaviorally conditioned budgerigars treated with kanamycin. After

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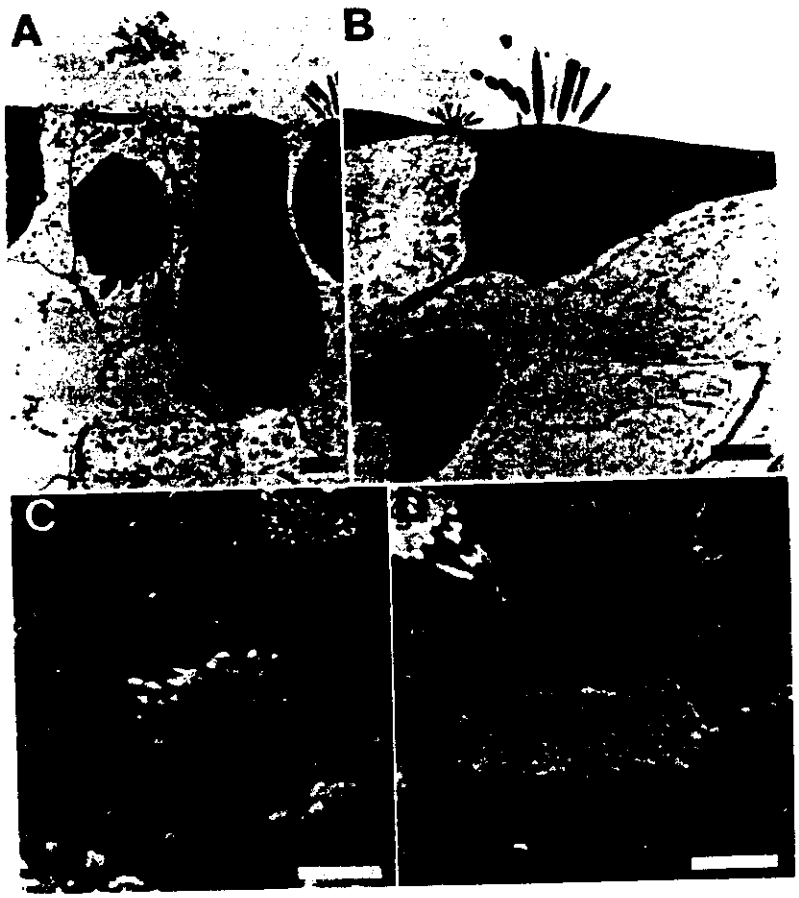

Fig. 4. Ultrastructural features of regenerated hair cell maturation process. Transmission electron photomicrographs of **(A)** an immature chick short hair cell (SHC) and **(B)** a mature appearing SHC at 6 and 10 weeks after gentamicin-induced cochlear damage, respectively. Note the pale cytoplasmic staining, fusiform shape, and small apical surface of the immature hair cell. The basally located nucleus, "squat pitcher" shape, and expanded apical surface are characteristic of a mature SHC. **C,** Scanning electron photomicrograph shows an immature hair cell stereociliary bundle 15 weeks after gentamicin treatment. Note the abnormal stereociliary alignment and presence of microvilli. **D,** Mature-appearing hexagonal array of stereocilia 20 weeks after gentamicin damage. The eccentric position of the stereociliary bundle is characteristic of a mature hair cell. Scale bar = 2 μm for all panels. (From Duckert LG, Rubel EW. J Comp Neurol 1993;331:75-96. Copyright © John Wiley & Sons, Inc.)

kanamycin, high-frequency thresholds returned to normal by 15 days, whereas some degree of low-frequency hearing loss persisted out to 42 days.

The recovery of hearing suggests that electrophysiologic function also recovers. Evoked potential thresholds recorded from the brain stem and concurrent morphologic changes in the cochlea were studied in our laboratory in chicks treated with gentamicin.[46,50] A significant hearing loss, especially in the high-frequency domain, was detected immediately after gentamicin treatment. Thresholds worsened, especially in the low- and mid-frequency ranges, up to 5 weeks after damage. This temporal pattern was matched by the immediate loss of hair cells in the basal cochlea, which progressed apically. At 16 to 20 weeks after the insult, total recovery of responses to low and middle frequencies and partial recovery of high frequencies thresholds was evident. Similarly, at 20 weeks, hair cell counts were normal, but a slight residual high-frequency loss was consistent with regenerated hair cell immaturity and stereociliary disarray. Interestingly, recovery of the response thresholds was slower than the anatomic restoration of the surface epithelium would have predicted.

Further studies suggest that the above delay may

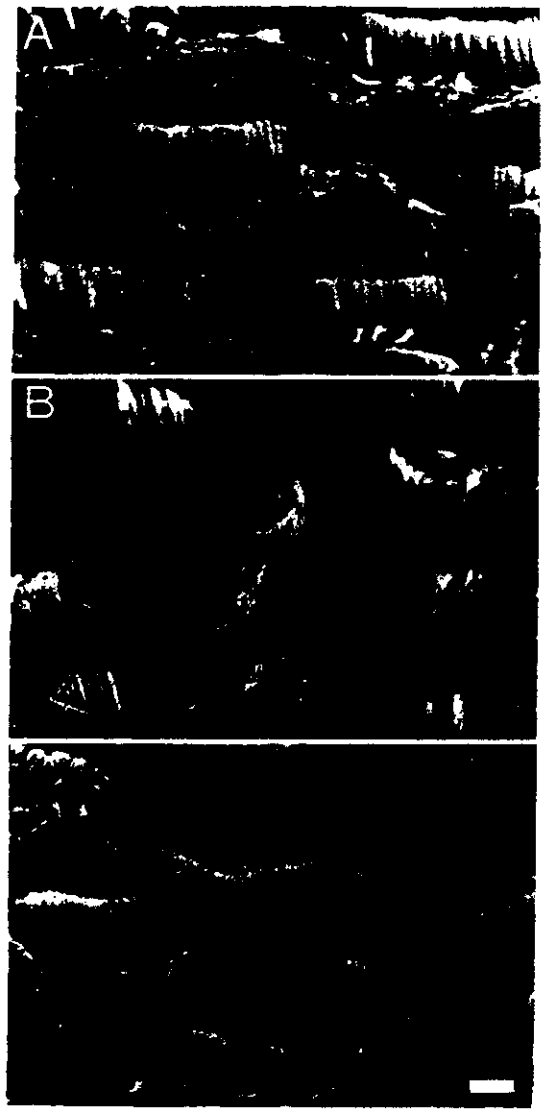

Fig. 5. Reorientation of regenerating hair cell stereociliary bundles. Scanning electron photomicrographs show chick stereociliary bundles from **(A)** a normal cochlea and from cochleas **(B)** 6 and **(C)** 20 weeks after gentamicin treatment. The stereociliary bundle long axes are initially disoriented compared with normals, but as the hair cells mature, the stereociliary bundle axes reorient to within 20 degrees of the normal axis. Scale bar = 2 μm for all panels. (From Duckert LG, Rubel EW. J Comp Neurol 1993;331:75-96. Copyright © John Wiley & Sons, Inc.)

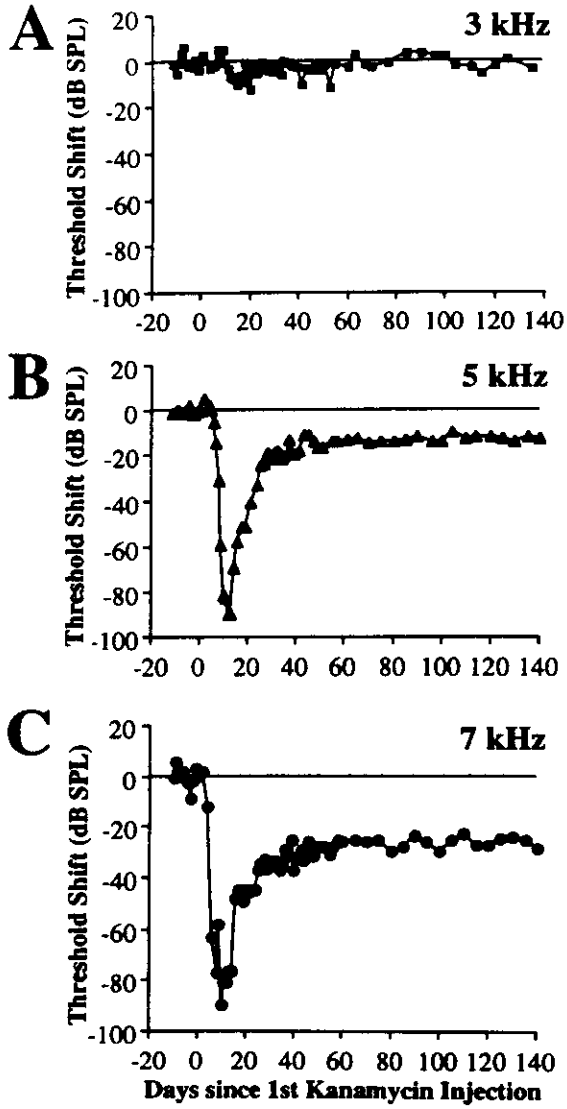

Fig. 6. Recovery of auditory perception in the European starling after kanamycin treatment. Mean daily pure-tone behavioral detection threshold shifts at three stimulus frequencies from 12 days before to 141 days after the first dose of kanamycin. **A,** No threshold shift was detected at 3 kHz stimulus frequency. **B,** A moderate threshold shift was observed at 5 kHz with eventual near normal recovery. **C,** After an immediate high-frequency threshold shift of greater than 80 dB, there is rapid recovery, but a residual threshold shift remained at long survival times. (From Marean GC, Burt JM, Beecher MD, Rubel EW. Hear Res 1994;71:125-36. By permission.)

be due to factors beyond the hair cell. Evoked otoacoustic emissions using acoustic distortion products (ADP) are a sensitive indicator of outer hair cell integrity in mammals and are independent of the integrity of the eighth cranial nerve.[51-54] Norton et al.[55] used ADP in an attempt to dissociate hair cell recovery from neural recovery. They studied the ADP and tone burst–evoked potentials from neonatal chicks after gentamicin ototoxicity (Figure 7). Recovery of evoked potential thresholds were similar to those observed by Tucci and Rubel.[50] Threshold elevations were first observed at high

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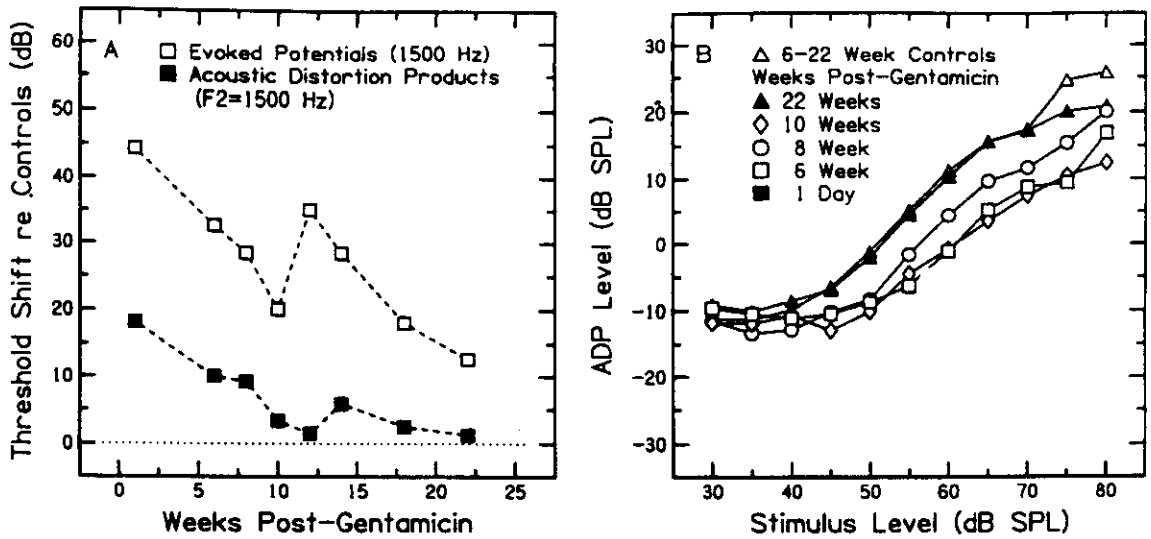

Fig. 7. Electrophysiologic recovery of hair cells and their neural connections. **A**, Comparison of brain stem evoked potential (EP) threshold and acoustic distortion product (ADP) threshold recovery after a 10-day gentamicin treatment of neonatal chicks. Mean threshold shift (compared with age-matched control animals) as a function of weeks after gentamicin treatment is illustrated. For EP, the stimulus was a 1.5 kHz tone burst. For ADP, F_2 = 1.5 kHz and F_1 = 1.15 kHz. Note that the ADP threshold recovered by 10 to 12 weeks, many weeks ahead of EP threshold recovery. **B**, ADP input-output functions during recovery. The mean ADP input-output function is plotted as a function of stimulus intensity at various times after gentamicin treatment (1 day to 22 weeks). Note that the ADP input-output function is fully recovered at most stimulus intensities by 22 weeks after gentamicin treatment. (From Dancer A, Henderson D, Salvi R, Hamernik RP, eds. Noise-induced hearing loss. St. Louis: Mosby–Year Book, 1992:204-27. By permission.)

stimulus frequencies, but rapidly progressed to the mid- and low-frequency domain. Between 1 and 22 weeks there was a gradual improvement in thresholds, first at low and mid frequencies, and later, at high frequencies. ADP thresholds recovered to within 5 dB of normal by 10 to 12 weeks after aminoglycoside damage, whereas evoked potential thresholds approached normal levels at 22 weeks. Thus, ADP emissions recovered before evoked auditory potentials. This result suggests that hair cell recovery precedes neural recovery by several weeks. The exact cause of this delay in the recovery of neural circuitry is unknown.

Overall, these studies demonstrated that regenerated hair cells are functional and can properly relay information centrally to ultimately influence behavior. Further, regenerated hair cells appear to develop the frequency-specific characteristics appropriate to their cochlear location.

Vestibular Hair Cell Regeneration

Because of developmental similarities between the auditory and vestibular systems, the investigation of postembryonic hair cell production has extended to the avian vestibular system. The germinal study on the avian vestibular epithelium was by Jørgensen and Mathiesen,[31] who gave twice-daily injections of ^{3}H-thymidine to normal adult budgerigars for 19 consecutive days and processed the vestibular end-organs for autoradiography. Tritiated-thymidine–labeled hair cells and supporting cells were observed throughout the vestibular sensory epithelium. This finding, recently replicated in the neonatal chick, suggests that there is a low level of ongoing hair cell production within the normal vestibular epithelium.[56] The level of ongoing hair cell production in the unmanipulated vestibular system is much greater than that described in the auditory end-organ.

The vestibular end-organs, like the cochlea, are sensitive to aminoglycosides. Streptomycin principally damages type I hair cells in the vestibular epithelium of neonatal chicks, but it also reduces the numbers of type II hair cells.[57,58] Weisleder and Rubel[57,58] treated chicks with streptomycin for 7 consecutive days. Beginning at 5 days of drug treatment, the birds were given concurrent twice-daily injections of ^{3}H-thymidine for 3 days. As in the

cochlea, the vestibular sensory epithelium was capable of self-repair (Figure 8). A fourfold increase in epithelial proliferative activity, as compared with control tissue, was observed early in the repair process (Figure 9). Essentially complete morphologic recovery was observed by 60 days after damage.

Like the cochlea, regenerated hair cells in the avian vestibular sensory epithelium appear to be functional and capable of relaying information to higher vestibular centers. Jones and Nelson[59] measured vestibular nerve compound action potentials in the aminoglycoside-treated chick. Immediately after streptomycin treatment, minimal vestibular nerve responses could be elicited by using pulsed linear accelerations. By 2 weeks, response thresholds had normalized, and by 6 to 8 weeks, activation latencies and input-output functions of the vestibular nerve compound action potential had fully recovered. In summary, these studies suggest that after hair cell loss, upregulated proliferative regeneration can provide both anatomic and functional recovery in the avian vestibular system.

Mechanisms of Hair Cell Regeneration

The studies described above on the avian auditory and vestibular systems have laid the foundation on which the mechanisms of postembryonic hair cell regeneration may be investigated. By understanding the specific mechanisms involved in the postembryonic production of hair cells, it is hoped that it may eventually be controlled. Two basic questions have been addressed by recent studies. First, what is the identity of precursor populations responsible for new hair cells? Second, what is the trigger mechanism for the regenerative production of new hair cells?

Hair Cell Precursors

Since ^{3}H-thymidine-labeled hair cells have been observed in the avian inner ear, mitosis must play a role in the production of new hair cells. Potential precursors for the new hair cells include hair cells that de-differentiate and reenter the mitotic cycle, stem cells located outside the sensory epithelium proper, and sensory epithelial supporting cells.

Girod et al.[32] first addressed this issue in the avian cochlea by determining the first cells to become mitotically active after noise-induced damage and attempting to follow the fate of their progeny. Neonatal chicks were exposed to an intense pure tone (1.5 kHz at 120 dB SPL) for 18 hours followed by injections of ^{3}H-thymidine. The animals were killed 6 hours to 30 days after noise damage, and their cochleas were processed for both autoradiography and SEM. These exposure conditions produced extensive loss of hair cells and supporting cells at the inferior (abneural) edge of the sensory epithelium in

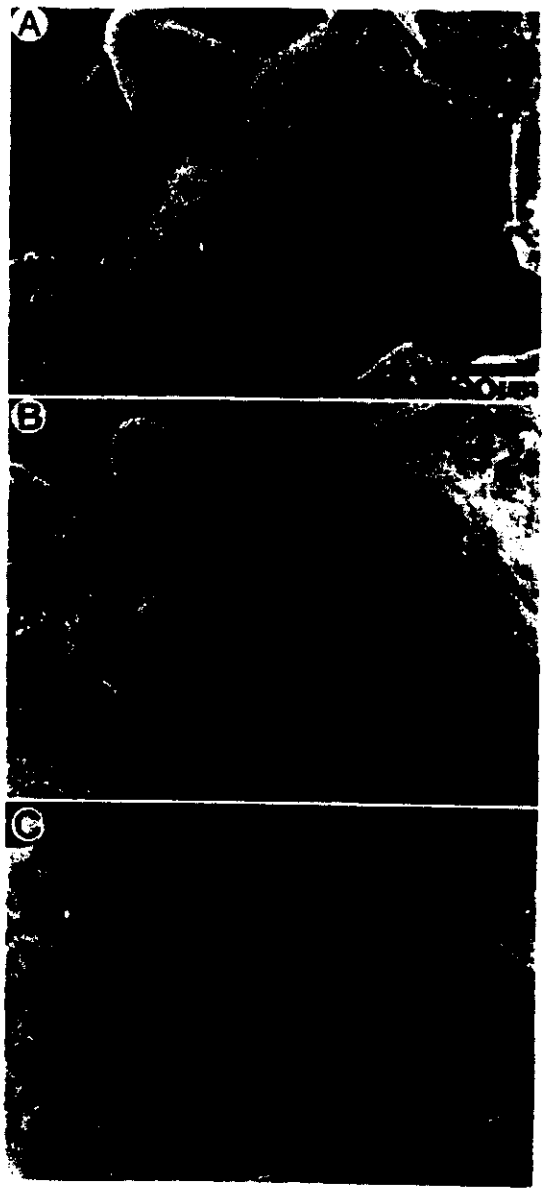

Fig. 8. Scanning electron photomicrographs of chick superior ampullae with cupulae removed. **A**, Crista ampullaris from a normal animal. **B**, Crista ampullaris after treatment with streptomycin for 7 consecutive days. Note the damage to the tissue characterized by the almost complete absence of stereocilia. **C**, Crista ampullaris 60 days after streptomycin treatment. The sensory epithelium has recovered most of its normal appearance. Scale bar applies to all panels. (From Weisleder P, Rubel EW. J Comp Neurol 1993;331:97-110. Copyright © John Wiley & Sons, Inc.)

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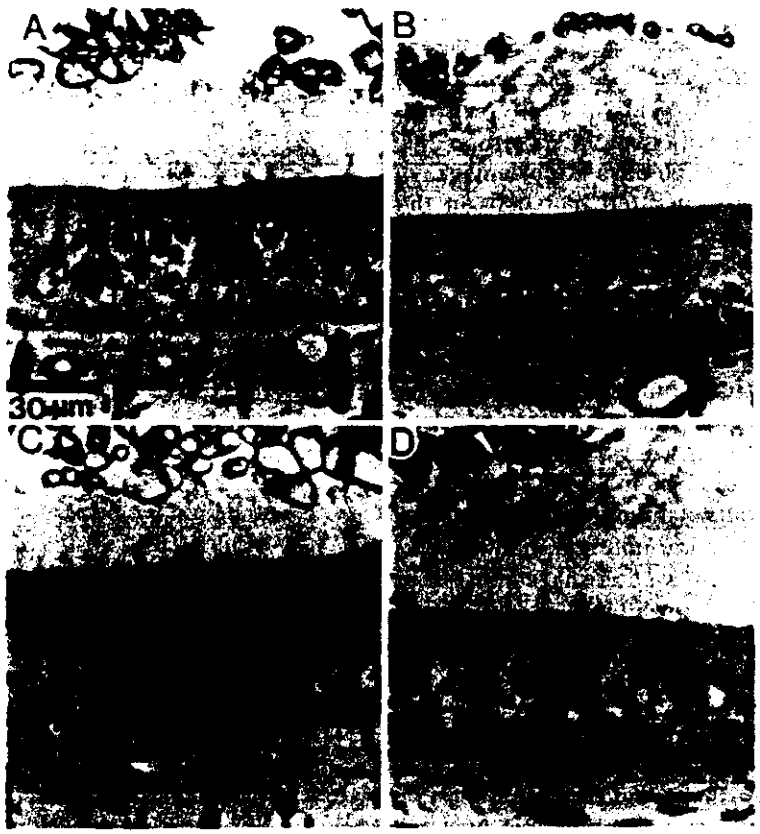

Fig. 9. Streptomycin toxicity and hair cell regeneration in the chick utricle. **A,** Utricle from a control animal allowed to survive 20 days after ^{3}H-thymidine injection. The sensory epithelium reveals both type I *(open arrows)* and type II *(solid arrows)* hair cells. Only two cells are labeled by ^{3}H-thymidine *(arrowheads).* **B,** One day after streptomycin treatment, no type I hair cells are seen in the sensory epithelium. Several supporting cells are ^{3}H-thymidine-labeled *(arrowheads).* **C,** Twenty days after streptomycin treatment, the number of type II hair cells *(solid arrows)* has increased slightly and a rare type I hair cell can be seen. Tritiated-thymidine-labeled type II hair cells and supporting cells *(arrowheads)* are observed. **D,** Sixty days after streptomycin treatment, the sensory epithelium has nearly recovered its normal appearance. Several labeled type I *(open arrows)* and type II *(solid arrows)* hair cells can be seen in the sensory epithelium. Scale bar applies to all panels. (From Weisleder P, Rubel EW. J Comp Neurol 1993;331:97-110. Copyright © John Wiley & Sons, Inc.)

most animals and variable damage more superiorly at the junction of the SHC and THC in some animals. By 15 hours after damage (33 hours after the beginning of noise damage), ^{3}H-thymidine-labeled undifferentiated epithelial cells were observed at the inferior edge of the damaged epithelium in animals with severe inferior hair cell loss. These cells apparently arose from the hyaline cells, nonsensory cells adjacent to the inferior edge of the cochlear epithelium (see Figure 1). In the more superior damage zone, labeled supporting cells were consistently seen immediately below newly generated hair cells. Labeled, regenerated hair cells were present 3 days after the noise exposure, and morphologic recovery from injury was nearly complete by 30 days (Figure 10). These data suggest two possible precursor cell types: hyaline cells and supporting cells. Each may be responsible for recovery in a different region of the sensory epithelium or after different severities of damage.

Direct evidence for the role of supporting cell mitosis in repopulating the sensory epithelium in noise-damaged chicks was provided by Raphael.[60] As early as 24 hours after noise damage (1.5 kHz at 120 dB SPL for 4 hours), supporting cells in the damaged zone incorporated the immunodetectable proliferation marker, bromodeoxyuridine (BrdU–thymidine analog). With use of a fluorescent DNA stain (Hoechst 33258), *en face* analysis of the cochlea demonstrated mitosis of supporting cells within the

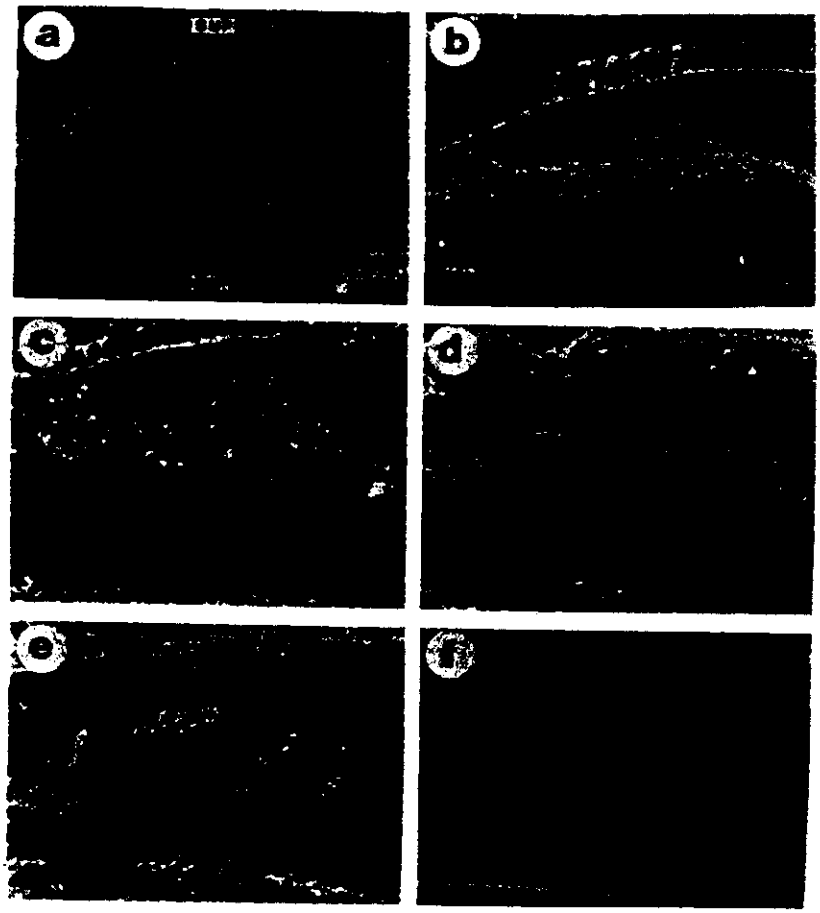

Fig. 10. Scanning electron photomicrographs of the 1.5 kHz region of the chick cochlear sensory epithelium. **a**, Normal control cochlea. Superior edge *(SUP)* and inferior edge *(INF)*. *Arrows* designate the hyaline cell region. **b**, Six hours after noise damage (1.5 kHz at 120 dB SPL for 18 hours). The surviving sensory epithelium has receded superiorly *(arrows)*. **c**, Twenty-four hours after sound damage. Note the apparent increase in cell density at the inferior edge of the surviving sensory epithelium. **d**, Three days after noise damage. Region of more severe loss of inferior hair cells. **e**, Three days after noise damage. Less severe superior band of hair cell loss *(arrows)*. **f**, Thirty days after noise damage. Note the significant morphologic recovery but persistent mild disorganization of the hair cell mosaic pattern. (Adapted from Girod DA, Duckert LG, Rubel EW. Hear Res 1989;42:175-94. By permission.)

damaged region. Fluorescent staining of F-actin (rhodamine phalloidin), a chief component in hair cell stereocilia, first revealed immature hair cells in the same cochlear region 96 hours after acoustic trauma. Stone and Cotanche[61] also examined noise-damaged chick cochleas after single injections of BrdU. The number of clustered BrdU-labeled daughter cells was correlated with the amount of postinjection survival time, suggesting that progenitor cells can undergo multiple divisions during the recovery process.

Supporting cells also appear to play a progenitor role in the avian vestibular system. Roberson et al.[56] described a statistically significant pairwise association between labeled supporting cells and hair cells in the normal utricular maculae and crista ampullae. Weisleder and Rubel[57,58] demonstrated that in the streptomycin-damaged avian vestibular system, supporting cells were the predominant ^{3}H-thymidine-labeled cell type initially after damage. The subsequent increase in labeled hair cells lagged temporally behind labeling in the supporting cell layer.

Tsue et al.[62] used a short pulse-fix autoradiographic protocol to isolate cells in "S-phase" (DNA synthesis) in the avian vestibular system. Both control and streptomycin-treated birds were killed 1 or

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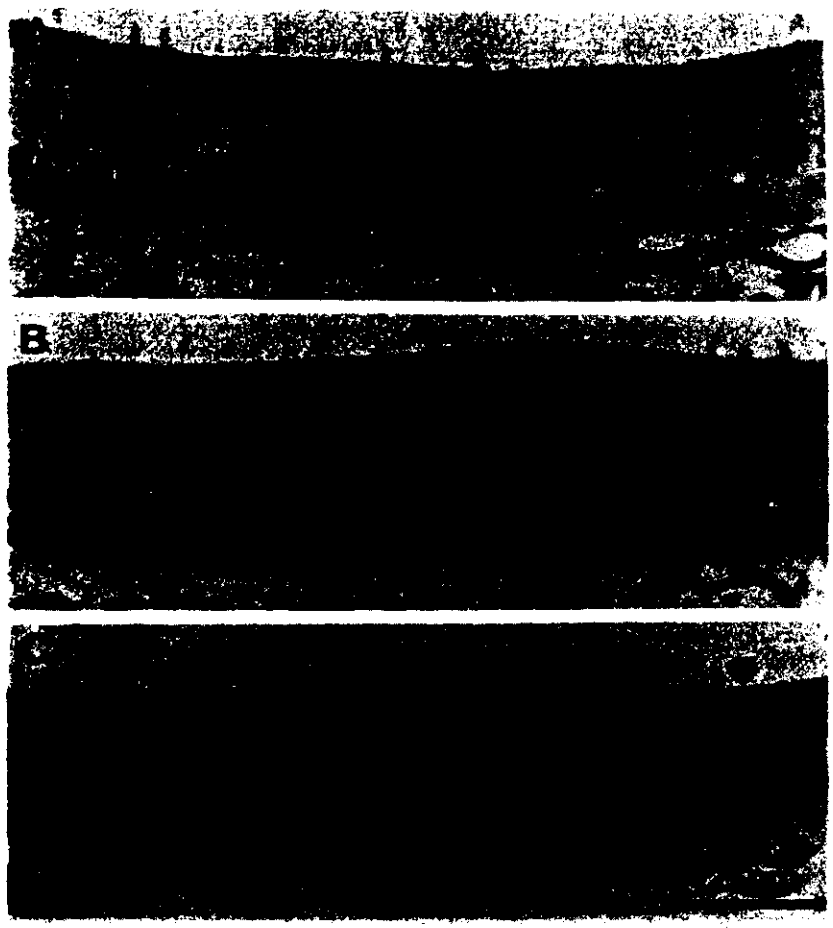

Fig. 11. Tritiated-thymidine labeling of proliferating cells in the chick saccular sensory epithelium. **A,** Normal saccule 1 hour after ^{3}H-thymidine injection. A single supporting cell near the basement membrane is labeled. Tritiated-thymidine-labeled supporting cells were seen in increased density in streptomycin-damaged saccules **(B)** 1 hour and **(C)** 3 hours after radioactive marker injection. No labeled hair cells were observed. Scale bar applies to all panels. (From Tsue TT, Watling DW, Weisleder P, Coltrera MD, Rubel EW. J Neurosci 1994;14:140-52. By permission.)

3 hours after a single ^{3}H-thymidine injection. These survival times were chosen to be short enough to prevent any cell from incorporating the proliferation marker during DNA replication (S-phase) and traversing the cell cycle to divide and produce two daughter cells (M-phase). Thus, only cells in S-, G2- (preparatory cell cycle phase for mitosis), or M-phases of the cell cycle could be labeled. In all experimental paradigms, labeling in the basal supporting cell layer was predominant, indicating that the majority of progenitor proliferative activity occurs within the supporting cell population (Figure 11).

No ^{3}H-thymidine-labeled hair cells were observed in the short survival times (1 and 3 hours), making hair cells themselves an unlikely progenitor source. A small amount of ^{3}H-thymidine-labeled nuclei were also located lumenally in the regenerating vestibular epithelium. These cells resembled supporting cells as well. If separate proliferating precursor populations existed in each epithelial nuclear layer (basal and lumenal), the percentage of total ^{3}H-thymidine label in each layer would remain the same for each end-organ type in both the 1- and 3-hour survival groups. However, after 3 hours' survival, there was a consistent increase in the percentage of labeled nuclei in the lumenal nuclear layer compared with analogous tissue from the 1-hour survival group. This can be explained only by migration of proliferating basally located supporting cells into the lumenal nuclear layer during the preparation for mitosis (Figure 12). Fluorescent

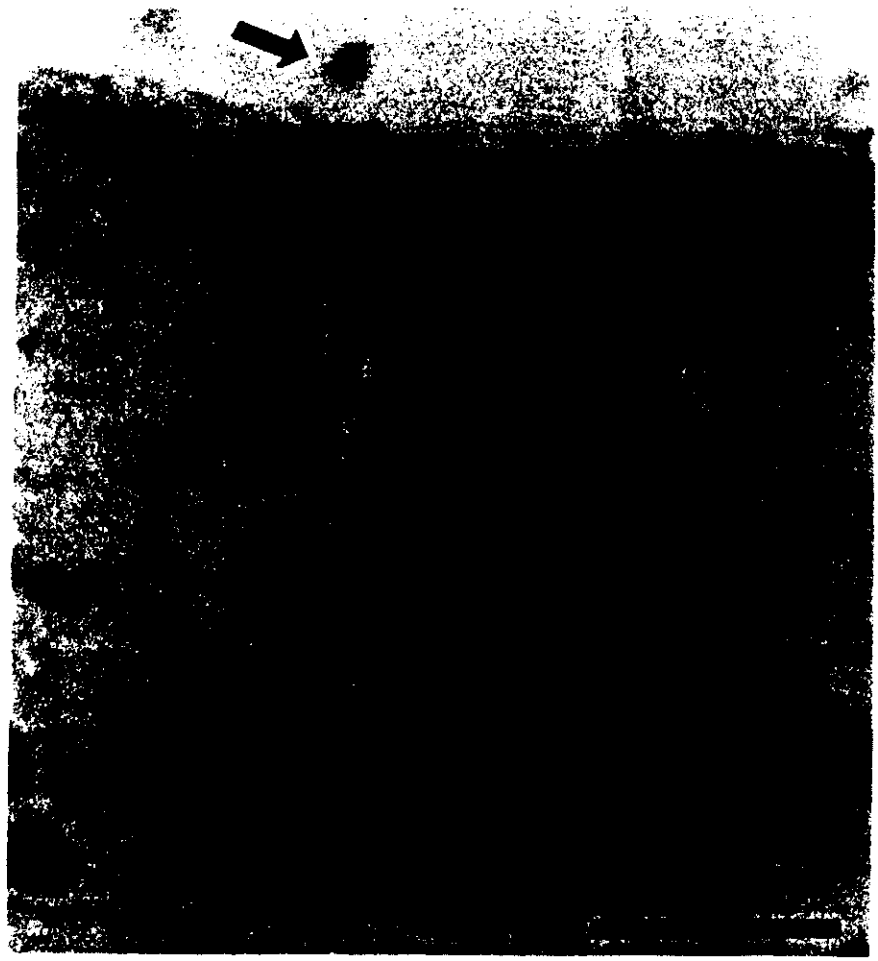

Fig. 12. Progenitor cell mitoses occur near lumenal surface in vestibular sensory epithelium. Photomicrograph shows condensed chromosomes of a mitotic figure near the lumenal sensory epithelium of a streptomycin-damaged chick saccule. *Arrow* points to a hair cell stereociliary bundle on the lumenal epithelial surface. (From Tsue TT, Watling DW, Weisleder P, Coltrera MD, Rubel EW. J Neurosci 1994;14:140-52. By permission.)

staining of sensory epithelial mitotic figures (Hoechst 33342), confirms that mitosis always occurs at or lumenal to the hair cell nuclear layer (Figure 13). Therefore progenitor cell nuclei in the normal and regenerating sensory epithelium migrate lumenally as the cell progresses from S-phase near the basement membrane to M-phase near the lumenal surface. This intraepithelial migration of progenitor cells, also seen in the regenerating chick cochlea and fish statoacoustic end-organ, is analogous to that observed in the developing neural tube and inner ear.[8,60,63,64]

The evidence thus far has been overwhelmingly in favor of the sensory epithelial supporting cell as a progenitor source for new differentiated hair cells. Whether all supporting cells or only a small subpopulation are potential precursors remains unanswered. Immunocytochemical heterogeneity between supporting cell populations has not been found, despite numerous attempts by our laboratory. Extensive ultrastructural investigation of these cells has not yet been performed. To determine whether all supporting cells are capable of proliferation, Roberson and Rubel[65] continuously infused the proliferation marker ^{3}H-thymidine into normal and gentamicin-treated chick inner ears. The gentamicin treatment resulted in a near complete hair cell loss in the basal cochlea. Twelve days after treatment, only about a third of the supporting cells in the regions of total hair cell loss were ^{3}H-thymi-

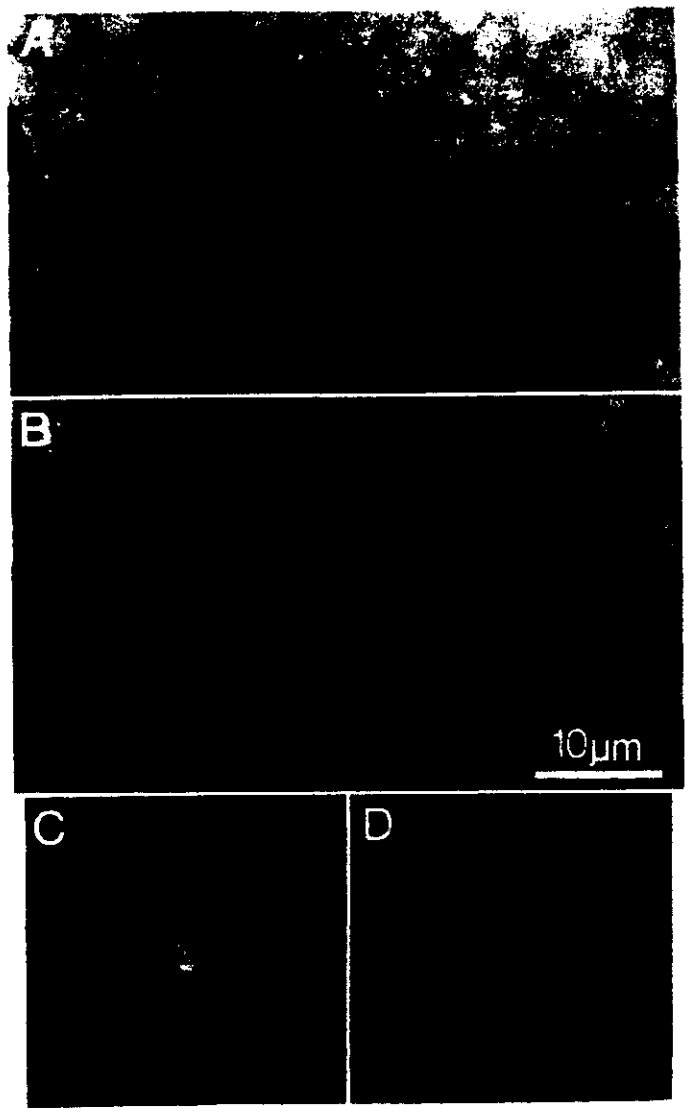

Fig. 13. *En face* views of a chick utricle stained with the fluorescent DNA stain Hoechst 33342, after 5 days of streptomycin treatment. Focal planes of the **(A)** tightly-packed supporting cell nuclear layer, and the **(B)** more lumenal, more sparsely packed hair cell nuclear layer are shown. Interphase nuclei show relatively homogenous staining throughout the nuclear area, whereas prophase, metaphase, anaphase, and telophase nuclei appear as condensed chromosomes characteristic of mitotic figures. A metaphase nuclei **(C)** is shown at or slightly lumenal to the hair cell nuclear layer, while an anaphase nuclei **(D)** is shown more near the lumenal surface. Scale bar applies to all panels. (From Tsue TT, Watling DW, Weisleder P, Coltrera MD, Rubel EW. J Neurosci 1994;14:140-52. By permission.)

dine labeled. Thus, despite a maximal regenerative stimulus, some supporting cells remained quiescent, suggesting that supporting cell subpopulations may exist.

Roberson and Rubel[65] also observed that, even after continuous ^{3}H-thymidine infusion, a minority of immature-appearing cochlear hair cells were not labeled with the proliferation marker (Figure 14). This finding suggests that new hair cells may also arise from transdifferentiation of supporting cells without mitotic activity.

Regardless of the progenitor cell source, an increase in proliferative activity, compared with control tissue, is observed in both the auditory and vestibular epithelium of birds after sensory epithelial damage.[27,28,57,58] Is this dynamic upregulation due

Fig. 14. Evidence for new hair cell generation by direct transdifferentiation without progenitor cell mitosis. Photomicrograph from the basal chick cochlear sensory epithelium 12 days after gentamicin treatment. The chick received a continuous infusion of ^{3}H-thymidine into the inner ear from 2 days before gentamicin treatment until 12 days after treatment. An unlabeled immature hair cell *(open arrow)* can be seen near ^{3}H-thymidine-labeled hair cells *(solid arrows)*. Both labeled and unlabeled supporting cells can be seen below the hair cells.[65]

to an increase in the number of progenitor cells dividing or an increase in the cell cycle rate? Tsue et al.[62] immunocytochemically labeled both control and streptomycin-treated vestibular organs with an antibody against the proliferating cell nuclear antigen (PCNA). PCNA is an auxiliary protein to DNA polymerase-δ, which has highest nuclear levels during S-phase of the cell cycle (DNA synthesis). Thus, PCNA is immunocytochemically detectable in cells actively proliferating. PCNA-labeled epithelial cells were greater in number in the damaged regenerating tissue, ranging from 3 × (lagenar macula) to 52 × (saccular macula) control tissue numbers (Figure 15). Thus, an increase in the number of dividing progenitor cells appears to play a major role in the proliferative upregulation observed during self-repair.

Trigger Mechanism

From the studies described above, certain characteristics about the triggering mechanism leading to a new differentiated hair cell may be elucidated. First, new hair cells are produced in both undamaged and damaged vestibular epithelia, and in damaged auditory epithelium. Thus, whatever triggers the production of a new hair cell is active in normal, as well as in acoustically overstimulated or aminoglycoside-treated, avian inner ears. Second, the trigger mechanism is functional in both neonatal and adult birds. Third, the trigger mechanism is activated during damage and persists until recovery is near complete. The initiation of recovery occurs near the beginning of noise overexposure and aminoglycoside damage. In contrast, new hair cells are still being produced up to 20 weeks after cochlear damage, when morphologic recovery is nearly complete in the chick.[35]

Finally, the trigger mechanism may be functional without neural activity. Weisleder and Rubel[66] treated streptomycin-damaged chick inner ears with tetrodotoxin, a neurotoxin that blocks any neural transmission. This silenced eighth cranial nerve activity. The regenerative response was slightly decreased in tetrodotoxin-treated end-organs, compared with controls, but still present. In addition, Warchol and Corwin[67] saw increased proliferation in cultured chicken cochleas after laser damage. Oesterle et al.[68,69] have developed an organotypic culture model using chick auditory and vestibular organs. Chick end-organs were grown in vitro in the presence of ^{3}H-thymidine, and both labeled hair cells and supporting cells were observed (Figure 16).

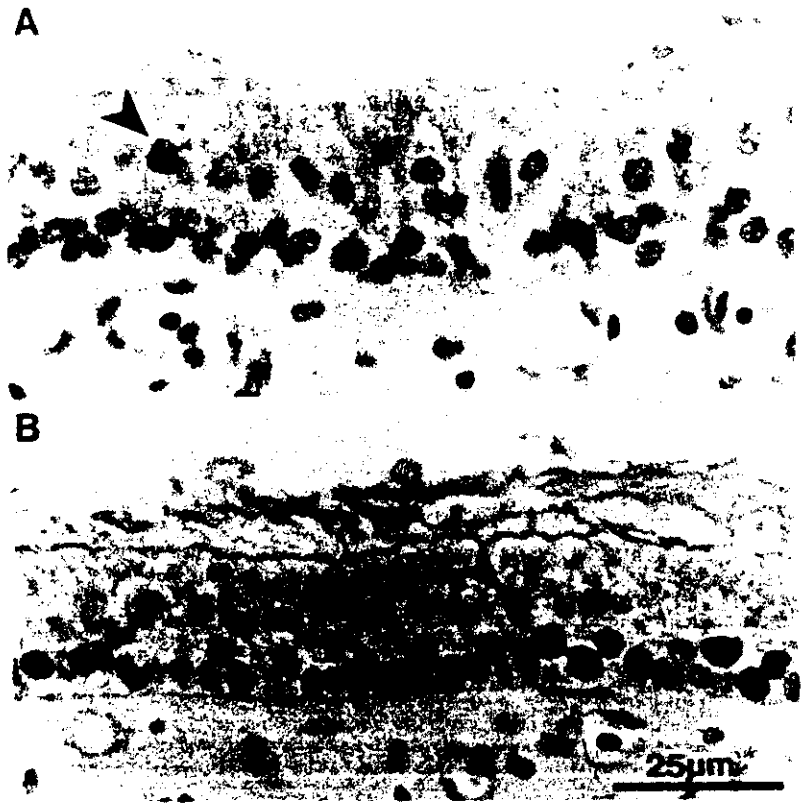

Fig. 15. Immunocytochemical labeling of the proliferating cell nuclear antigen (PCNA) in nuclei of proliferating vestibular sensory epithelial cells. PCNA immunolabel is observed in the **(A)** normal utricle and in increased numbers in the **(B)** streptomycin-damaged utricle. The *arrowheads* point to the characteristic nuclear stippling pattern of the immunolabel. Scale bar applies to both panels. (From Tsue TT, Watling DW, Weisleder P, Coltrera MD, Rubel EW. J Neurosci 1994;14:140-52. By permission.)

New labeled vestibular hair cells were also observed after explanation into serum-free culture media. Cultured streptomycin-damaged utricles demonstrated increased proliferation, compared with explanted normal utricles. Thus, whatever trigger mechanism is acting during hair cell regeneration, it appears to be local; it is independent of the exogenous neural and humoral environment of the in vivo state.

Two possible triggering mechanisms responsible for initiating postembryonic hair cell production in the avian inner ear have been proposed. First, the mechanical loss of hair cells may reverse an inhibitory influence on progenitor cells, allowing them to reenter the cell cycle and begin epithelial recovery.[70] In the auditory sensory epithelium, both noise- and aminoglycoside-induced epithelial damage appear to result in the extrusion of many dead or dying cells, although this may not be true for all dying cells.[47,71,72] Loss of the normal apposition of hair cells and supporting cells that occurs during hair cell damage could provide a signal to progenitor cells that the epithelium requires restoration. Conceivably, the inhibitory effect of these heterophilic cell-cell interactions could be mediated through membrane-spanning glycoproteins on both hair cell and supporting cell membranes.[73-75] In the auditory end-organ, regenerative proliferation is seen predominately in the region of epithelial injury, where a mechanical stimulus may be detected.[27,28,46,47] In addition, the number of ejected dead or dying hair cells appears to closely parallel the quantity of regenerated hair cells.[76]

The second hypothesis involves a paracrine mechanism, whereby damaged hair cells, scavenging phagocytic cells, or loss of receptor neural activity causes the release of a mitogenic substance. This mitogen can then diffuse to reach and stimulate

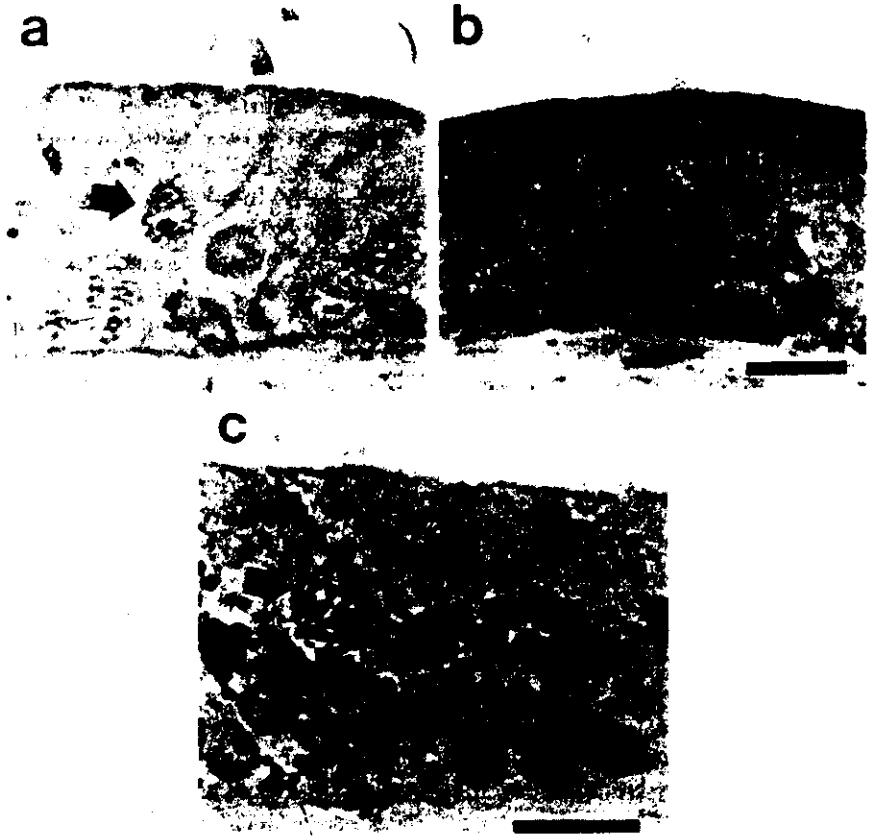

Fig. 16. *In vitro* hair cell regeneration. Tritiated-thymidine-labeled hair cells with immature (b,c) and more mature stereociliary bundles (a) from chick utricular explants grown *in vitro* for 2 days. The presence of labeled hair cells in cultures demonstrates the ability of the organotypic culture model to support the production and differentiation of progenitor cells into hair cells. Scale bars = 10 μm. The magnification is idential in a and b. (From Oesterle EC, Tsue TT, Reh TA, Rubel EW. Hear Res 1993;70:85-108. By permission.)

mitosis in progenitor cells. Similarly, epithelial damage could decrease production of a chalone or antimitotic factor. Previous observations of the isolation of proliferation to damaged regions of the auditory epithelium could be explained by diffusion limitations of the soluble factor. However, Raphael[72] recently observed new hair cells and expansion of supporting cells in undamaged cochlear regions of sound-damaged birds. Xu and Corwin[77] have recently described a small protein with some homology to epidermal growth factor that is expressed in the cochlear sensory epithelium after aminoglycoside damage and during the regenerative response.

Further evidence for the role of a soluble factor comes from recent studies in the vestibular system. Streptomycin is known to damage type I hair cells more than type II hair cells, causing a differential pattern of damage across the sensory epithelium.[57,58,62,78,79] Although damaged hair cells were seen throughout the epithelium, Oesterle et al.[69] observed a sickle-shaped area of greatest damage in the treated utricular sensory epithelium using scanning electron microscopy. In contrast, reparative proliferation appears relatively evenly distributed across the epithelial surface area.[57,58,62] In addition, the amount of upregulated mitotic activity of progenitor cells after aminoglycoside insult does not appear to be directly related to the extent of damage. The crista ampullae are more susceptible to the ototoxic effects of streptomycin than the maculae.[58,78,80] However, Tsue et al.[62] found that the maculae have a greater than two times reparative response to streptomycin treatment than the crista ampullae in terms of progenitor cells reentering the cell cycle. These mismatches between the extent of epithelial damage and the proliferative response

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suggest the role of a diffusible factor in regulating hair cell regeneration. Undamaged or less damaged epithelial zones would have less of a change in epithelial mechanical infrastructure, but may be easily penetrated by a soluble chemical factor.

Further studies are needed to help elucidate what role each of these two hypothetical mechanisms play in triggering hair cell regeneration. Both mechanisms could equally induce progenitor cell transdifferentiation, as well as cellular division. Studies involving an in vitro model of hair cell regeneration allow easy manipulation of the environment surrounding the regenerating end-organ.[67-69] Co-culture, conditioned media, and growth factor addition experiments are in progress in our laboratory.

Hair Cell Regeneration in the Mammal

In classic studies on development of the mouse inner ear, Ruben[12] reported that mammalian hair cell production ceased before birth in the cochlea and within 2 days of birth in the vestibular organs. Noise, ototoxic drugs, infection, and age have all been shown to cause damage and destruction of hair cells. Hair cell destruction was held to be irreversible and associated with a permanent sensorineural hearing loss or balance disorder. Nonetheless, the literature does contain reports of hearing recovery in human beings after aminoglycoside ototoxicity. Moffat and Ramsden[81] reported partial hearing recovery in one patient with a gentamicin-associated hearing deficit. In a prospective study of 20 patients receiving gentamicin therapy, Winkel et al.[82] demonstrated significant ototoxicity in half, which was eventually fully reversible in four patients. Fee[83] reported some auditory recovery in 55% of patients with aminoglycoside-associated hearing loss.

These reports and the rapid progress made in research of the postembryonic production of sensory hair cells in the avian model have given hope that this sensory regenerative process may be inducible in all warm-blooded vertebrates, including man. Consequently, a significant effort is being put forth to apply what has thus far been learned in the avian inner ear to the mammalian inner ear.

Recent findings in the mammalian auditory and vestibular systems are preliminary, but promising. Taken together, they suggest that the postembryonic production of mammalian hair cells is possible. In vestibular system studies, Forge et al.[84] investigated mature guinea pigs treated with gentamicin for 10 consecutive days. At 4 weeks after damage, immature-appearing stereociliary bundles were seen in the sensory epithelium by scanning electron microscopy. In addition, hair cell numbers in the areas of epithelial damage partially recovered in a period up to 4 weeks. New supporting cells were also seen in guinea pig utricles in vitro.[85] Explanted guinea pig utricles were treated with neomycin- or gentamicin-containing media to induce hair cell loss. The cultures were then grown for up to 4 weeks in the presence of the cell-proliferation markers ^{3}H-thymidine or BrdU. Some supporting cells were labeled, especially near the neural edge. In addition, a few cells with more lumenally labeled nuclei were detected. Similar experiments using three human utricles, obtained during surgical labyrinthectomy, also revealed evidence of supporting cell proliferation after aminoglycoside damage in vitro. Overall, the number of dividing cells was much lower and the proliferative time course much slower than that observed in the chick vestibular epithelium, but these results suggest that some spontaneous recovery of hair cells in the mammalian vestibular epithelium can occur. Thus, at least in the vestibular epithelium, a potential progenitor population seems to exist.

The immature mammalian organ of Corti has been studied in vitro as well.[86] Cochlear explants of 3-day postpartum rats were treated with neomycin for 48 hours. This treatment damaged the epithelium such that surface preparations failed to reveal actin-stained stereociliary bundles. When the damaged cultures were subsequently grown in the presence of fetal bovine serum and retinoic acid, stereociliary bundles reappeared as early as 7 days after the ototoxic injury. The concurrent presence of cytosine arabinoside, a poison that kills proliferating cells, prevented the recovery process, suggesting that cell division was crucial to these events. This study suggests that new hair cells may be produced in the immature mammalian cochlea, but final confirmation will require use of a proliferation marker, such as ^{3}H-thymidine or BrdU. Whether this is also true of the mature organ of Corti is under investigation.[87,88]

These studies, although preliminary, present encouraging evidence that the postembryonic production of the hair cell mechanoreceptor may also occur in mammals. Many questions remain to be answered: Are fully differentiated and functional hair cells produced? Does it occur in all mammals? Does it occur in human beings? If present in the mammal, the process is significantly reduced in robustness, compared with that in birds. Thus, the fine details of this extraordinary phenomenon and any hope of control lie in continued research in both avian and mammalian inner ears.

Clinical Implications

Further understanding of the process of hair cell regeneration may ultimately lead to direct stimulation of hair cell production in the damaged human inner ear. In addition, the in vitro models being developed to study the mechanism of hair cell regeneration may someday be useful in transplanting living hair cells into the damaged inner ear. Encouraging clinical results from cochlear implants have given hope that even a partial restoration of the normal mechanoreceptor sensory epithelium could result in remarkable benefits in auditory and vestibular perception. In situ repopulation of the inner ear sensory epithelium would have many advantages over surgical implantation of a multichannel electrode. These proposals would have been considered unrealistic less than 10 years ago; now they appear as possibilities.

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