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Robust generation of new hair cells in the mature mammalian inner ear by adenoviral expression of *Hath1*

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Abstract

Although hair cells regenerate spontaneously in birds and lower vertebrates following injury, there is yet no effective way to stimulate hair cell regeneration in mature mammalian inner ears. Here we report that a large number of hair cells are produced in the sensory epithelium of cultured adult rat utricular maculae, via adenovirus-mediated overexpression of *Hath1*, a human *atonal* homolog. The generation of new hair cells via *Hath1* expression does not involve cell proliferation based on bromodeoxyuridine immunocytochemistry. Furthermore, using a similar approach, hair cells are regenerated following aminoglycoside injury in these cultures. These data show conclusively that mature mammalian inner ears have the competence to produce a large number of new hair cells. Local adenoviral gene therapy in the inner ear may be a potential approach to treatment of hearing and balance disorders.

Introduction

Hair cells of the inner ear are mechanosensory receptors capable of transducing sound and body motion signals. Hair cell loss due to loud sound, aminoglycoside toxicity, and aging is one of the major causes of hearing and balance impairments. Although it is well demonstrated in birds and lower vertebrates that hair cells regenerate spontaneously following injury (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Jones and Corwin, 1996), there is yet no effective way to stimulate hair cell regeneration in the mature mammalian inner ear. Identification of effective methods to stimulate hair cell regeneration in the inner ear would be of therapeutic value for treatment of hearing and balance disorders.

Recent studies on hair cell development have demonstrated that the basic helix–loop–helix transcription factor *Math1*, a mouse *atonal* homolog, is not only required (Bermingham et al., 1999), but also sufficient (Zheng and Gao, 2000), for production of hair cells in certain types of cells in immature inner ears. However, it is unclear whether overexpression of *Math1* in the mature mammalian inner ear can induce production of new hair cells. In rodents, the inner ear tissue undergoes dramatic structural, immunocytochemical, and physiological changes within the first 3 weeks after birth (Lim and Rueda, 1992; Walsh and Romand, 1992). These changes include apoptosis (Zheng and Gao, 1997), maturation of stereociliary bundles (Lim and Rueda, 1992), functional synaptogenesis (Walsh and Romand, 1992), and acquisition of specific ionic channels (Rusch et al., 1998) in hair cells. The cells in the sensory epithelia of mature mammalian inner ears become highly differentiated and show reduced response to growth factors (Gu et al., 1997). Consequently, it is unknown whether cells in the mature ear have the same potential as the immature ear to differentiate into hair cells. Demonstration of a robust production of new hair cells in mature inner ears is an important proof-of-concept experiment for potential hair cell regeneration in humans and is eagerly awaited (Cho, 2000; Fekete, 2000; Larkin, 2000; Seppa, 2000).

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In the present experiments, we set forth to determine whether overexpression of *Hath1*, a human *atonal* homolog, would induce production of new hair cells in mature inner ears. As viral gene transfer is more practical than electroporation (Zheng and Gao, 2000) for potential therapeutic applications, we designed an adenoviral vector to misexpress *Hath1*. We performed adenoviral infection in postnatal rat cochlear explant and mature rat utricular whole mount cultures. We found that *Hath1* has the same ability as *Math1* to induce production of hair cells. The mature mammalian inner ear can be induced to generate a large number of new hair cells. The robust production of new hair cells does not involve cell proliferation. Moreover, production of new hair cells can occur via *Hath1* expression in mature inner ears damaged by gentamicin, an ototoxic aminoglycoside antibiotic.

Results

Construction of ad-Hath1-EGFP vector

As shown in Fig. 1A, the adenoviral construct we designed also contains a reporter gene *EGFP* (enhanced green fluorescent protein), allowing us to identify infected cells. We also constructed a vector containing *EGFP* alone. These vectors were named ad-Hath1-EGFP and ad-EGFP, respectively. After collection of the active adenovirus, the faithfulness of the ad-Hath1-EGFP vector was confirmed by Western blot analysis of the cell lysate from the virus-infected human LnCaP cells, using a polyclonal antibody against Math1 (Helms and Johnson, 1998). As shown in Fig. 1B, infected cells expressed Hath1 protein of the correct size (approximately 40 kDa). Additionally, immunocytochemistry of infected postnatal rat cochlear explant cultures (Zheng and Gao, 2000) revealed that all ad-Hath1-EGFP-infected cells in the lesser epithelial ridge (LER) area were labeled by the anti-Math1 antibody (Helms and Johnson, 1998) (arrows in Fig. 1C), indicating expression of Hath1 protein. In contrast, uninfected cells were negative for Math1 immunostaining (arrowheads in Fig. 1C).

Production of cochlear hair cells by Hath1 expression

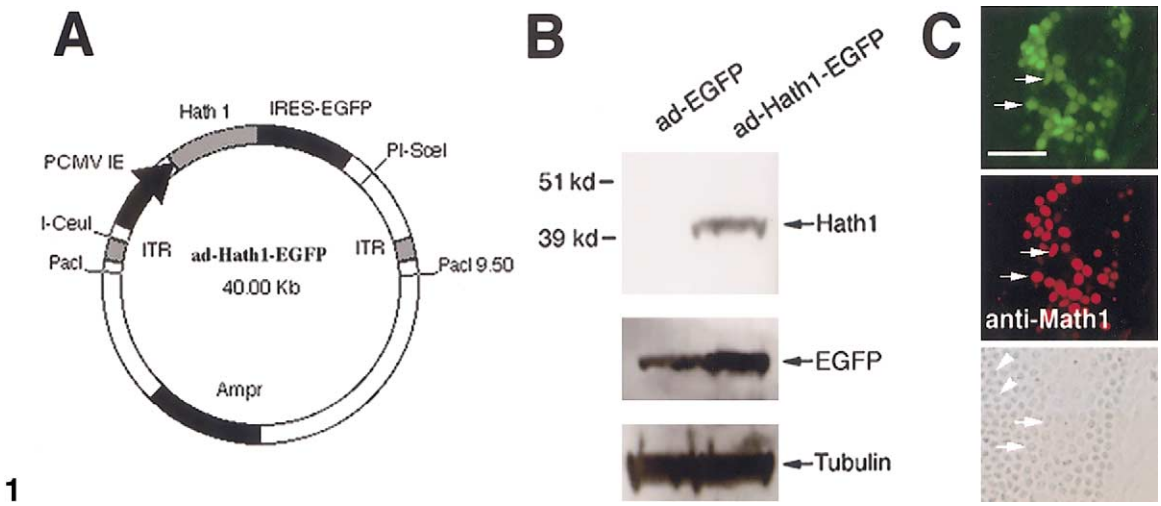
To test whether *Hath1* has the same ability as *Math1* (Zheng and Gao, 2000) to induce hair cell differentiation, we examined postnatal day 0 rat cochlear explant cultures infected with the ad-Hath1-EGFP virus. Consistent with previous electroporation experiments (Zheng and Gao, 2000), many of the infected greater epithelial ridge (GER) cells were double labeled by anti-myosin VIIa antibody, a hair-cell-specific marker (Hasson et al., 1995; Zheng and Gao, 2000) (Figs. 2A–C), when the cultures were fixed at 3–6 days following viral infection. Interestingly, in addition to GER cells, infected cells in the LER region, which is lateral to the organ of Corti (OC) and spans about 10 to 14 cell widths in the superficial layer, were also able to convert into hair cells (Figs. 2D–F). Normally, these LER cells in postnatal cochleae would give rise to Hensen's and Claudius' cells (Lim and Rueda, 1992) as development proceeds. In agreement with previous electroporation experiments (Zheng and Gao, 2000), none of the infected cells in the spiral ganglion or in the connective tissue regions differentiated into hair cells (data not shown). None of the GER or LER cells in any of the 12 control cultures infected with the ad-EGFP virus stained with anti-myosin VIIa antibody, indicating failure to become hair cells (Figs. 2G–I). Cell counts from 11 randomly selected cultures revealed that approximately 35.5 ± 9.8% of LER cells infected with the ad-Hath1-EGFP virus became myosin VIIa-positive. In all of the 36 cultures examined, neither the ad-Hath1-EGFP nor the ad-EGFP virus infected any hair cells in the OC region (Figs. 2A–I). Adenoviral infection of selective cell types in the inner ear tissue appears to depend upon how adenovirus is produced, as some show preferential infection of hair cells (Holt et al., 1999; Luebke et al., 2001), while others report no infection of hair cells (Yagi et al., 1999; Dazert et al., 2001; Jero et al., 2001).

Production of new hair cells in mature inner ears

To address whether overexpression of *Hath1* can induce production of new hair cells in the mature inner ear sensory epithelium, we attempted to infect the adult rat cochlea with adenovirus in culture. Unfortunately, because of the tortu-

Fig. 1. Construction of ad-Hath1-EGFP vector. (A) The Ad-Hath1-EGFP construct map. (B) Western blot of the ad-Hath1-EGFP- or ad-EGFP-infected cells. The anti-Math1 antibody used recognizes Hath1 protein. Note that ad-Hath1-EGFP-infected, but not ad-EGFP-infected, cells show a band at approximately 40 kDa size, representing Hath1. Expression of EGFP and α-tubulin is observed for both samples. (C) EGFP (top), anti-Math1 immunostaining (middle), and phase constrast (bottom) images of ad-Hath1-EGFP-infected LER cells in postnatal rat cochlear explant cultures. The nuclear staining by the anti-Math1 antibody indicates expression of the Hath1 protein in ad-Hath1-EGFP-infected cells (marked by arrows), but not uninfected cells (marked by arrowheads). Bar in C, 40 μm.

Fig. 2. Induction of hair cell differentiation in GER and LER cells in postnatal cochlear explant cultures via adenoviral expression of *Hath1*. (A–C and D–F) Overexpression of *Hath1* in GER (C) and LER cells (F) in the postnatal rat cochlear explant cultures induces hair cell differentiation. Many of the ad-Hath1-EGFP-infected cells (green) are myosin VIIa positive (red). The double-labeled cells are in yellow (examples are indicated by arrows in C and F). The insets in C and F are high-magnification images of the area containing the example double-labeled cells (indicated by arrows). (G–I) Shown is a control culture infected with ad-EGFP virus. All ad-EGFP-infected LER cells (green) are myosin VII (red) negative (marked by arrowheads). The anti-myosin VIIa antibody also labels all normal hair cells in the organ of Corti (OC) in all cultures. Bar, 40 μm, 20 μm for the insets in C and F.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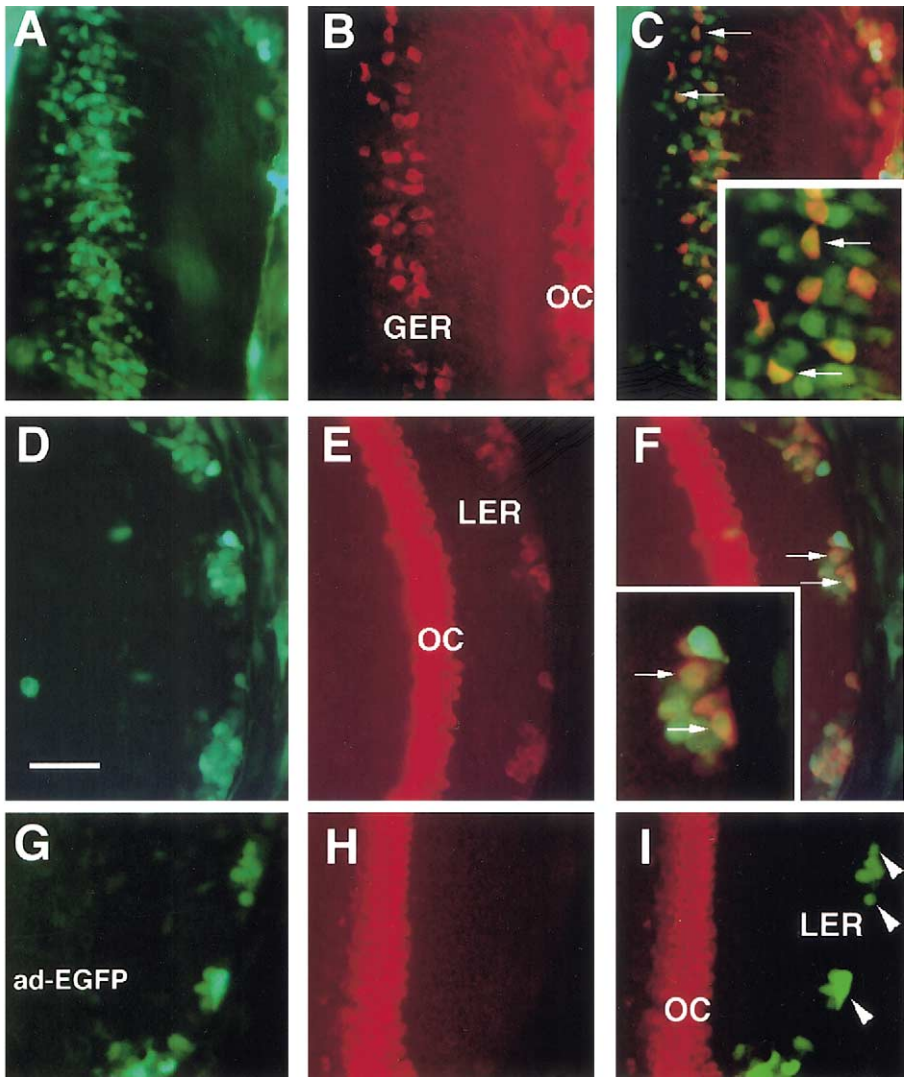
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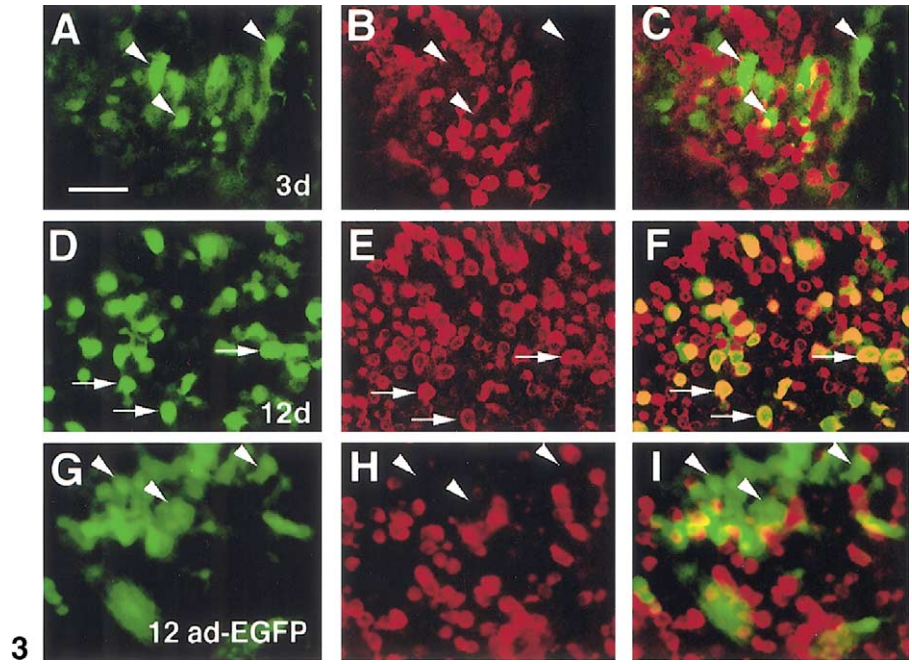
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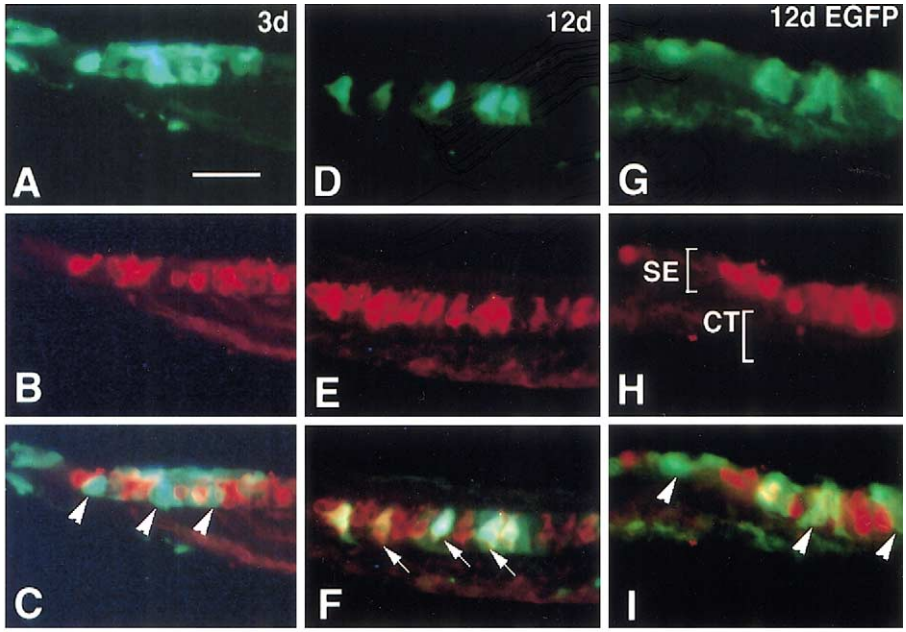
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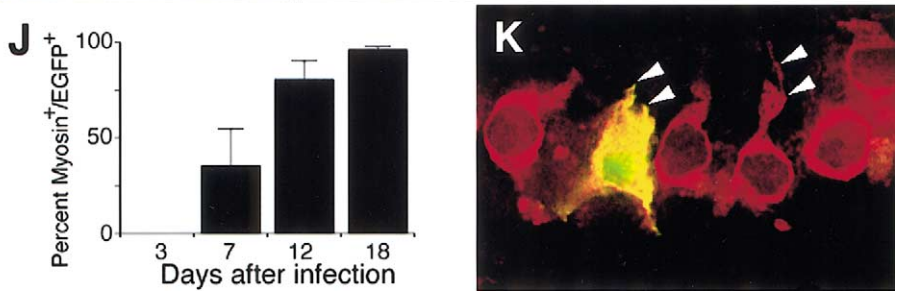
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ous bony structure, it was not feasible to dissect out the mature cochlea tissue without damaging it to an extent that precludes successful tissue culture. We therefore focused our attention on the mature utricular macula, a vestibular end organ in the inner ear, responsible for body motion and position. Unlike the cochlea, this tissue could be easily isolated and maintained in culture as previously reported (Warchol et al., 1993). The vestibular end organs share many similarities with the cochlea: both are derived from the otic placode during embryogenesis; hair cells in both organs are sensitive to aminoglycoside toxicity; and the transduction of auditory and body motion signals to the brain by hair cells in the cochlea or vestibular end organs is achieved through the same eighth cranial nerve. Furthermore, the genes that act as either positive or negative regulators of hair cell differentiation, such as *Math1* (Bermingham et al., 1999; Zheng et al., 2000; Chen et al., 2002), or *Hes1* and *Hes5* (Shailam et al., 1999; Lanford et al., 2000; Zheng et al., 2000; Zine et al., 2001), are not only expressed in comparable patterns, but also act similarly in both the developing cochlea and vestibular end organs.

When adult rat utricular whole mount cultures were infected with the ad-Hath1-EGFP virus, many cells fluoresced green when live cultures were observed under UV light 1 day following viral infection. When the infected cultures were fixed and double labeled with anti-myosin VIIa antibody at 3 days following viral infection, careful examination of the whole mount preparations under a fluorescence microscope by focusing up and down revealed that no infected cells in the cultures were myosin VIIa positive (arrowheads in Figs. 3A–C), even though a large number of cells were infected. Cell counts from 5 of 17 randomly selected cultures revealed that there were 584 adenovirus-infected cells in total (approximately 117 infected cells per utricle) within the sensory epithelium, indicating that infection was not a rare event. Infected cells included supporting cells within the sensory epithelium, epithelial cells in the marginal epithelial zone, and cells in the connective tissue. The failure to observe EGFP/myosin VIIa double-labeled cells in any of the cultures at 3 days after viral infection indicates that no hair cell was infected by the virus. The lack of virally infected hair cells was consistent with the experiments in the postnatal cochlear explant cultures described above. A large number of EGFP-positive but myosin VIIa-negative cells observed within the sensory epithelium at 3 days following viral infection strongly suggests that these infected cells are supporting cells, because the sensory epithelium essentially contains hair cells and supporting cells. At present we are limited by the lack of reliable, mammalian-supporting cell-specific markers. We examined the immunostaining in the tissue very carefully by focusing up and down, and we did not observe any EGFP/myosin VIIa double labeled cells in either ad-Hath1-EGFP- or ad-EGF-infected cells at 3 days following viral infection, a time point at which the maximal expression of transgenes by the adenovirus is achieved (Holt et al., 1999). It should be pointed out that although the nuclei of supporting cells are located at the bottom layer of the sensory epithelium, the cytoplasmic region of supporting cells can extend to the surface of the sensory epithelium, passing the hair cell layer. Therefore, the EGFP staining was frequently observed either below or above the focusing plane of hair cells (Figs. 3C and I; also see Figs. 4C and I).

Excitingly, when the cultures were examined at 7–18 days following infection with the ad-Hath1-EGFP virus, the majority of infected cells in the sensory epithelium became myosin VIIa positive (arrows in Figs. 3D–F). On average, 76 of 107 (approximately 71%) infected supporting cells per utricle became myosin VIIa positive, based on cell counts from 16 of 52 randomly selected cultures fixed at 7–18 days after infection. In sharp contrast, all of the infected cells in each of the 16 control cultures infected with the ad-EGFP

Fig. 3. Overexpression of Hath1 induces production of hair cells in adult rat utricular whole mount cultures. (A–C and D–F) Double immunocytochemistry of EGFP (green) and myosin VIIa (red) of cultures fixed at 3 and 12 days after ad-Hath1-EGFP infection, respectively. While no infected cells are labeled by anti-myosin VIIa antibody at 3 days after infection (arrowheads in C), the majority of the infected cells become myosin VIIa positive at 12 days after infection, (examples are indicated with arrows in F). (G–I) Double immunocytochemistry of EGFP (green) and myosin VIIa (red) of a culture fixed at 12 days after ad-EGFP infection. In contrast to the ad-Hath1-EGFP-infected cells in (F), all ad-EGFP-infected cells remained myosin VIIa negative (arrowheads in I). Images were acquired from a given focusing plane of the whole mount cultures. The myosin VIIa-positive/EGFP-negative cells are preexisting, normal hair cells. Bar, 50 μm.

Fig. 4. Gradual conversion of supporting cells into hair cells in adult rat utricular whole mount cultures. (A–C and D–F) Myosin VIIa immunocytochemistry of sections from the ad-Hath1-EGFP-infected cultures at 3 and 12 days after infection, respectively. (G–I) Myosin VIIa immunocytochemistry of a section of an ad-EGFP-infected culture at 12 days after infection. Note that while the ad-Hath1-EGFP-infected cells (green) are intermingled with hair cells (red) within the sensory epithelium at 3 days after infection (arrowheads in C), the majority of them become myosin VIIa positive (examples are indicated by arrows in F) at 12 days after infection (F). In contrast, the ad-EGFP-infected cells remain myosin VIIa negative within the sensory epithelium (arrowheads in I). Because of the fact the cytoplasmic region of supporting cells extends from the bottom to the surface of the sensory epithelium, the superimposed green/red images (C, I) appeared to have some overlaps of EGFP (green) and myosin VIIa (red) staining, even though they are located at different focusing planes. Careful examination of all the cultures on a microscope by focusing up and down revealed no double-labeled cells at 3 days after ad-Hath1-EGFP infection or 12 days after ad-EGFP infection. (J) Cell counts of myosin VIIa-positive cells versus total ad-Hath1-EGFP-infected cells in the SE indicate a gradual increase of myosin VIIa/EGFP double-labeled cells as a function of time. Data collected from five to six cultures for each group are expressed as means ± SD. (K) A high-magnification confocal image from EGFP/myosin VIIa double-labeled tissue infected with ad-Hath1-EGFP virus. Note that myosin VIIa immunostaining (red) that labels both the cytoplasm and the stereociliary bundles of normal, preexisting hair cells (arrowheads over the red cells) shows formation of short stereocilairy bundles from the cell that just converted into a hair cell due to Hath1 expression (arrowheads over the yellow cell). Bar, 30 μm for A–I; 5 μm for K. Abbreviations: SE, sensory epithelium; CT, connective tissue.

virus did not stain with the myosin VIIa antibody and failed to convert into hair cells at 12 days after infection (arrowheads in Figs. 3G–I).

Examination of cross-sections of the utricular whole mount cultures provided further evidence for the conversion of supporting cells into hair cells. At 3 days after infection, many of the ad-Hath1-EGFP-infected supporting cells were intermingled with hair cells within the sensory epithelium and showed elongated morphology, but remained myosin VIIa negative (Figs. 4A–C). By 12 days after infection, however, the vast majority of the ad-Hath1-EGFP-infected cells within the sensory epithelium were myosin VIIa positive and had assumed pear-shaped morphology (Figs. 4D–F). In contrast, the ad-EGFP-infected cells remained myosin VIIa negative 12 days after infection and displayed irregular morphology with large cell bodies and variable numbers of processes (Figs. 4G–I).

Cell counts of the EGFP/myosin VIIa double-labeled cells versus total ad-Hath1-EGFP-infected cells within the sensory epithelium revealed a gradual conversion of supporting cells into hair cells as a function of time (Fig. 4J). As previously noted, no infected cells were myosin VIIa positive at 3 days following infection. Starting at 7 days after infection, approximately 36% of infected cells became hair cells. At 12 days, approximately 81% of infected cells had converted into hair cells, and by 18 days the percentage of hair cell conversion had risen to 96%.

Hair cells induced by Hath1 misexpression display stereociliary bundles

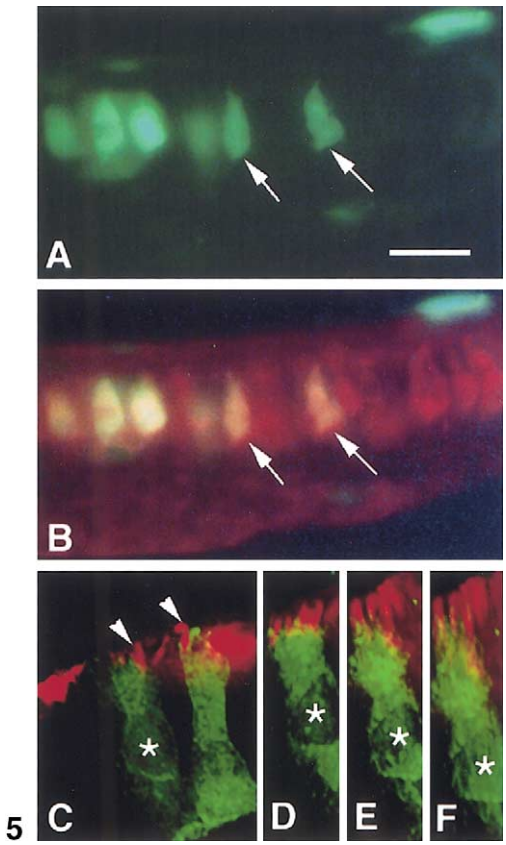
To provide further evidence that expression of *Hath1* induces production of new hair cells in the mature utricular sensory epithelium, we examined EGFP/myosin VIIa double-labeled cells in the tissue infected with the ad-Hath1-EGFP virus using confocal microscopy at high magnification. Previously it has been shown that anti-myosin VIIa antibody not only labels the cytoplasmic region of hair cells, but also the stereociliary bundles (Hasson et al., 1995), one of the important hair cell features. As shown in Fig. 4K, anti-myosin VIIa antibody labeled the stereociliary bundles of not only pre-existing normal hair cells (arrowheads on a myosin VIIa-positive cell), but also those of newly converted hair cells (marked by arrowheads on a EGFP/myosin VIIa double-labeled cell). In addition, we double labeled some of these cultures with another selective vestibular hair cell marker, anti-calretinin antibody (Zheng and Gao, 1997). Many of the ad-Hath1-EGFP-infected cells also became calretinin positive at 11 days following infection (arrows in Figs. 5A and B). Furthermore, because phalloidin is another more commonly used marker for hair cell stereociliary bundles, we double labeled a few of these cultures with rhodamine-conjugated phalloidin at 11 days following ad-Hath1-EGFP infection. As shown in Fig. 5C, two ad-Hath1-EGFP-infected cells showed EGFP-labeled columnar cell bodies and phalloidin-stained stereociliary bundles on the apical surface (marked by arrowheads). Displaying *Z*-series of confocal images from different angles shows more clearly that the ad-Hath1-EGFP-infected cells in the sensory epithelium grew stereociliary bundles (Figs. 5D–F).

Production of new hair cells does not involve cell proliferation

Based on earlier work on chicken inner ears, previous studies emphasized proliferation of supporting cells as a necessary event for the production of new hair cells in the inner ear sensory epithelia (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Warchol et al., 1993). Therefore, in our study, we determined the number of proliferating cells in 15 of the ad-Hath1-EGFP-infected cultures by inclusion of bromodeoxyuridine (BrdU) in the culture medium for 11–13 days. BrdU immunocytochemistry of these cultures showed that the total number of dividing supporting cells in the sensory epithelium was extremely small (2.6 ± 0.9, SEM). This result indicates that production of new hair cells via expression of *Hath1* does not have to involve cell proliferation.

Regeneration of new hair cells in gentamicin-damaged mature inner ears

Clinically, many patients suffer from hearing and balance impairments resulting from hair cell loss. Hence, we determined whether the mature inner ear epithelium has the capacity to generate new hair cells following injury. We treated the adult rat utricular macula with gentamicin, which is an ototoxin that kills a majority of the hair cells in the mature ear (Warchol et al., 1993), and subsequently infected the tissue using the ad-Hath1-EGFP virus. Consistent with experiments with normal utricular macula tissue, we found that many of the infected cells in the sensory epithelium were myosin VIIa positive 12 days after infection. An example of the cultures is shown in Figs. 6A–C (marked by arrows). Cell counts of 5 of 15 representative cultures revealed a total of 167 EGFP/myosin VIIa double-labeled cells. Although a small number of hair cells (see Zheng et al., 1999) that were myosin VIIa positive but EGFP negative survived gentamicin treatment, the majority of the EGFP-positive cells (approximately 70% of the total 205 infected cells) were myosin VIIa positive. In contrast, none of the ad-EGFP-infected cells were stained by the anti-myosin VIIa antibody in the gentamicin treated control cultures (Figs. 6D–F). This observation not only indicates that no ad-EGFP-infected cells became hair cells, but also suggests that adenovirus did not infect those hair cells that survived genetamicin treatment. Like the cultures of normal utricular whole mounts, the ad-EGFP virus-infected cells in the sensory epithelium displayed large cell bodies and irregular morphology with numerous processes (arrowheads in Fig. 6F). Consistent with our previous findings with GER cells in the postnatal cochlear explant cultures (Zheng and



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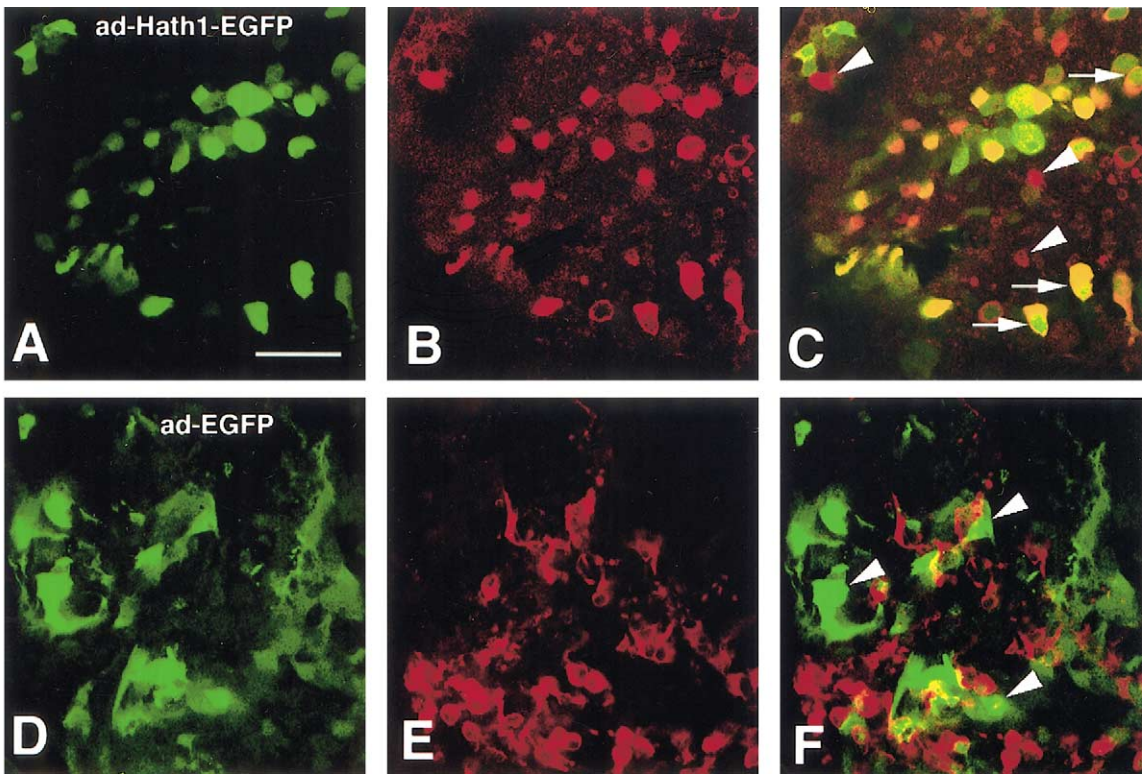

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Fig. 5. Newly induced hair cells express other hair cell markers. (A and B) Calretinin immunocytochemical labeling (red) of a section from the ad-Hath1-EGFP-infected cultures at 11 days after infection. Note that the ad-Hath1-EGFP-infected cells (green in A) become calretinin positive (orange in B, as pointed by arrows). (C) Texas red-conjugated phalloidin staining of the ad-Hath1-EGFP-infected cells (green) displays hair cell phenotypes including barrel-shaped morphology and formation of stereociliary bundles (arrowheads) at 11 days after infection. (D–F) Shown are *Z*-series of confocal images of

Gao, 2000), the observations in the present experiments suggest that while these ad-EGFP-infected cells maintained their supporting cell phenotype, those infected with ad-Hath1-EGFP underwent a morphological conversion into a smaller, pear-shaped cell body, in addition to showing hair cell markers including myosin VIIa, calretinin, and stereociliary bundles.

Discussion

A robust production of new hair cells in normal and gentamicin-injured utricular macula after adenoviral gene transfer of *Hath1* is significant. Although previous studies suggested that hair cell regeneration occurs in mature mammalian vestibular end organs (Forge et al., 1993; Warchol et al., 1993; Zheng and Gao, 1997; Forge et al., 1998), the regeneration capacity appears to be very limited (Rubel et al., 1995; Warchol et al., 1995), because of the very small number of putative new hair cells (Warchol et al., 1993; Zheng and Gao, 1997) and the possibility that partially damaged hair cells might repair their stereociliary bundles (Zheng et al., 1999; Gale et al., 2002). Even though tritiated thymidine (Warchol et al., 1993) or bromodeoxyuridine (Zheng and Gao, 1997) is shown to be incorporated into normally postmitotic hair cells as evidence for formation of new hair cells derived from dividing supporting cells, there is a concern of DNA repair in the damaged hair cells (Sandler and Swenne, 1985). The present experiments were carried out with well-characterized and commonly used hair cell markers, myosin VIIa (Hasson et al., 1995; Xiang et al., 1998; Zheng and Gao, 2000; Zheng et al., 2000), calretinin (Zheng and Gao, 1997), and phalloidin. The newly generated hair cells grow stereociliary bundles as indicated by myosin VIIa (Fig. 4K) and phalloidin labeling (Figs. 5C–F). In addition, the experiments were performed using *EGFP* as an internal indicator for infected cells and the control ad-EGFP virus. The data were analyzed at different time points following viral infection. Together, these observations unambiguously confirm the production of a large number of new hair cells in the mature inner ear.

It is noteworthy that although our previous study showed that the GER cells in developing cochleae have the capacity to differentiate into hair cells, it was not known whether other cells in the cochlea have the competence to become hair cells, because of the limited ability of electroporation to transfect cells in the cochlea (Zheng and Gao, 2000). To date, a cell lineage study using lineage tracers was performed only in the avian inner ear (Fekete et al., 1998), but not in the mammalian cochlea. Therefore, whether other epithelial cells in the cochlea have the capacity to differentiate into hair cells is still an important, but unanswered question. In the present experiments, the adenovirus was able to infect GER cells, LER cells, cells in the spiral ganglion, and cells in the connective tissue. We found that LER cells also have the competence to give rise to hair cells. This finding provides the first line of direct evidence that LER and GER may act as hair cell progenitors. Therefore, the present experiments provide important insights for potential hair cell regeneration in the mature cochlea. While some GER cells are believed to undergo apoptosis during maturation of the cochlear tissue (Lim and Rueda, 1992), the majority of LER cells become Hensen's and Claudius' cells that remain in the mature cochlea. These data suggest that Hensen's and Claudius' cells might also be good target cells that can be induced to *trans*-differentiate into new hair cells in the mature cochlea.

Our finding that expression of *Hath1* induces hair cell production in the rodent inner ear suggests that the functions of the *Drosophila atonal* homologs are conserved during revolution. Initially isolated as a proneuronal gene that preferentially promotes the formation of chordotonal organs (Jarman et al., 1993), the *Drosophila atonal* gene regulates neurogenesis in the vertebrate nervous system and differentiation of the secretory epithelial cells in the mammalian intestine. In *Xenopus* (Kanekar et al., 1997) and zebrafish (Kay et al., 2001), *atonal* homologs direct the production of retinal ganglion cells. In mammals, *Math1* is an essential transcription factor regulating differentiation of cerebellar granule neurons (Ben-Arie et al., 1997), spinal cord dorsal commissural interneurons (Helms and Johnson, 1998), and intestinal secretory epithelial cells (Yang et al., 2001). Acting together with an *achaete-scute complex* homolog, mammalian *atonal* homologs are also found to regulate neuronal versus glial cell fate determination (Tomita et al., 2000; Inoue et al., 2001). More interestingly, misexpression of *Math1* in the *Drosophila* induces ectopic chordotonal organs and partially rescues chordotonal organ loss in the *atonal* mutant embryos (Ben-Arie et al., 2000). In the present study we showed that the human *atonal* homolog,

the cell marked by an asterisk in C, which are displayed at different angles, demonstrating that the ad-Hath1-EGFP-infected cells in the sensory epithelium grew stereociliary bundles on the apical surface. Bar, 15 μm for A and B; 4 μm for C–F.

Fig. 6. Regeneration of hair cells in adult rat utricular whole mount cultures following gentamicin treatment. (A–C) EGFP (A), myosin VIIa (B), and double labeling (C) of a gentamicin-treated culture fixed at 12 days following ad-Hath1-EGFP infection. The cultures were treated with 0.5 mM gentamicin for 2 days prior to ad-Hath1-EGFP infection. Although a small number of hair cells survived gentamicin treatment (red cells marked by arrowheads in C), many new hair cells (yellow) are generated owing to *Hath1* overexpression (examples are indicated by arrows in C). (D–F) EGFP (D), myosin VIIa (E), and double labeling (F) of a gentamicin-treated culture fixed at 12 days following ad-EGFP infection. No ad-EGFP-infected cells become myosin VIIa positive (marked by arrowheads in F). The myosin VIIa-positive cells (red) are those hair cells that survived genetamicin treatment. Note that unlike those ad-Hath1-EGFP-infected cells that acquire pear-shaped morphology, the ad-EGFP-infected cells (green cells marked by arrowheads in F) maintain their supporting cell phenotype: large cell body and irregular morphology with numerous processes. Bar, 40 μm.

Hath1, is capable of substituting for its rodent counterparts in generating new hair cells from supporting cells in the inner ear. Therefore, the functions of the *Drosophila atonal* gene and its homologs are evolutionarily conserved and production of hair cells in humans is likely regulated by *Hath1*. Such conservation is not only biologically interesting, but also raises the possibility of using *Hath1* as a gene therapy target for hearing and balance impairments in humans.

In conclusion, this study is the first to report a robust conversion of supporting cells into hair cells by overexpression of *Hath1* in mature mammalian vestibular end organs; as many as 185 of 192 infected supporting cells converted into hair cells in a single utricular sensory epithelium. Although supporting cell proliferation might be one early step of hair cell regeneration as proposed in previous studies (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Warchol et al., 1993), our work suggests that potent hair cell production can occur without involving cell proliferation. We believe that animal studies are warranted in the near future to determine whether new hair cells can be generated in vivo. Given that adenoviral gene therapy in the inner ear can be performed locally (Yagi et al., 1999; Dazert et al., 2001; Luebke et al., 2001) or through middle ear round window membrane (Jero et al., 2001), the adverse effects, if any, can be minimized. As hearing and balance disorders affect millions of people, we believe that our study is very encouraging and represents another important step toward potential hair cell regeneration. This could eventually be helpful for the recovery from hearing and balance impairments caused by hair cell loss.

Experimental methods

Adenoviral constructs and generation of active virus

The adenoviral vector was constructed using the Adeno-X expression system (K1650-1, Clontech) according to the instruction manual. *Hath1* coding region was isolated by PCR amplification of the human genomic DNA (Applied Biosystems) using primers flanking the *Hath1* open reading frame. The 1.1-kb PCR product was sequenced and cloned into pIRES2-GFP (6029-1, Clontech). The resulting 2.4-kb fragment containing Hath1-IRES-EGFP was then popped out and inserted into the pShuttle vector (Clontech). The pCMV-Hath1-IRES-EGFP expression cassette was then cut from the pShuttle and subsequently ligated into pAdeno-X vector, which was derived from wild-type Ad5 genome with deletion in E1 and E3 regions, to generate the replication-incompetent recombinant adeno-Hath-IRES-EGFP construct. The viral DNA was linearized with *Pac* I digestion and transfected into early-passage HEK 293 cells. Transfected HEK 293 cells were maintained in DMEM/F12 (1/1) plus 10% fetal bovine serum (Gibco-BRL) in a 5% CO_2 humidified tissue culture incubator. Virus collection and propagation were performed according to the instruction manual (Clontech). The control adeno-EGFP virus was generated in parallel without inclusion of the *Hath1* open reading frame. The PCR primer sequences used are listed as follows: *Xho*I–Hath1 (forward), TGCTCGAGCAATGTCCCGCCTGCTGCAT; Hath1–*Eco*RI (reverse), TCGAATTCCACCTTCCTAACTTGCCTCATCCG.

Western blot

Human LnCaP cells were infected with either ad-Hath1-EGFP or ad-EGFP virus for a total of 3 days. Cells were then lysed and approximately 75 μg of each sample was loaded in the gel and processed for Western blot as described (Shou et al., 1999). Primary antibodies used are rabbit anti-Math1 (gift from J. Johnson), chicken anti-GFP (Chemicon), and mouse anti α-tubulin (Sigma) antibodies. Briefly, proteins were separated on Nupage 4%–12% BT gels (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). After incubation with primary and HRP-conjugated secondary antibodies and extensive rinses, the membrane was dipped in ECL chemiluminescence substrate and exposed to Hyperfilm (Amersham) to visualize the bands.

Cell cultures and immunocytochemistry

Postnatal rat cochlear explant cultures were prepared as previously described (Zheng and Gao, 2000). The freshly dissected postnatal cochlear explants were incubated in ad-Hath1-EGFP or ad-EGFP viral supernatant for 3 h before they were plated on collagen-coated eight-well Lab-Tek slides in serum-free medium as described (Zheng and Gao, 2000). The cultures were then fixed at 3–6 days following viral infection and processed with EGFP/myosin VIIa double immunocytochemistry as described (Zheng and Gao, 2000). The mature utricular whole mount tissue was dissected from 10- to 12-week-old rats. The freshly dissected tissue was incubated with ad-Hath1-EGFP or ad-EGFP viral supernatant for 3 h before transfer to transwell culture inserts (Becton Dickinson) and maintained in 300 ml serum-free medium (Zheng and Gao, 2000) in 24-well plates. The cultures were fixed at 3, 7, 12, and 18 days following viral infection and then processed with myosin VIIa (gift from T. Hasson) or calretinin (Chemicon) immunocytochemistry as described (Zheng and Gao, 2000). For some mature utricular whole mount cultures, BrdU (1:1000, Amersham) was added to the culture medium following viral infection for 11–13 days before the cultures were fixed for immunocytochemistry as described (Zheng and Gao, 1997). Some mature utricular whole mount cultures were treated with 0.5 mM gentamicin for 2 days prior to ad-Hath1-EGFP infection. The myosin VIIa doubled-labeled whole mount cultures were carefully examined under a fluorescence microscope by focusing up and down to locate and count double-labeled cells. The sensory epithelium was defined as

described (Zheng et al., 1999). Some of the cultures were sectioned with a cryostat machine as described (Zheng et al., 1999). Images and cell counts were obtained as described (Zheng et al., 1999; Zheng and Gao, 2000).

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