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Manipulating cell cycle regulation in the mature cochlea

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Abstract

Sensorineural hearing loss, which is often caused by degeneration of hair cells in the auditory epithelium, is permanent because lost hair cells are not replaced. Several conceptual approaches can be used to place new hair cells in the auditory epithelium. One possibility is to enhance proliferation of non-sensory cells that remain in the deaf ear and induce transdifferentiation of some of these cells into the hair cell phenotype. Several genes, including *p27^{Kip1}*, have been shown to regulate proliferation and differentiation in the developing auditory epithelium. The role of p27^{Kip1} in the mature ear is not well characterized. We now show that p27^{Kip1} is present in the nuclei of non-sensory cells of the mature auditory epithelium. We determined that forced expression of *Skp2* using a recombinant adenovirus vector, resulted in presence of BrdU-positive cells in the auditory epithelium. When *SKP2* over-expression was combined with forced expression of *Atoh1*, ectopic hair cells were found in the auditory epithelium in greater numbers than were seen with *Atoh1* alone. *Skp2* over-expression alone did not result in ectopic hair cells. These findings suggest that the p27^{Kip1} protein remains in the mature auditory epithelium and therefore *p27^{Kip1}* can serve as a target for gene manipulation. The data also suggest that induced proliferation, by itself, does not generate new hair cells in the cochlea.

Keywords

Adenovirus; gene transfer; p27^{Kip1}; Atoh1; Skp2; guinea pig; hair cell; supporting cell; BrdU

1. Introduction

The sensory epithelium of hearing in mammals consists of terminally differentiated epithelial cells: sensory hair cells and non-sensory supporting cells. These cells are quiescent in

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mammals. Therefore, hair cell degeneration is irreversible and leads to sensorineural hearing loss. One potential therapy for hearing loss is induction of hair cell regeneration in the organ of Corti, the sensory region of the auditory epithelium.

Recent data demonstrate that forced expression of genes encoding hair cell development can induce transdifferentiation of non-sensory cells into new hair cells in the developing (Woods et al., 2004; Zheng and Gao, 2000) and mature organ of Corti (Izumikawa et al., 2005; Kawamoto et al., 2003; Shou et al., 2003). As therapy, this procedure would be suboptimal because transdifferentiation of supporting cells into new hair cells does not involve mitosis in the tissue. Thus, formation of new hair cells would reduce the number of supporting cells and compromise the ability to restore normal cochlear structure and function. In birds, where hair cell regeneration leads to functional recovery (Dooling et al., 1997; Marean et al., 1995; Niemiec et al., 1994; Saunders et al., 1992), non-sensory cells divide after a lesion to the epithelium (Hashino and Salvi, 1993; Raphael, 1992; Stone and Cotanche, 1994). To induce proliferation in the mature organ of Corti as part of the reparative process, it may be necessary to manipulate expression of genes that regulate cell cycle.

Among the genes that regulate cell-cycle proteins in the developing inner ear are $p27^{kip1}$ (Chen and Segil, 1999; Lowenheim et al., 1999), Ink4d (Chen et al., 2003) and Rb1 (Sage et al., 2005). Cell proliferation past the normal developmental cessation of mitosis has been shown in these transgenic mice. The ability to remove the inhibition of cell cycle in the mature inner ear, in a cell or organ specific manner, may potentially be used for developing clinical therapy for hair cell regeneration. One important step for inducing proliferation in the mature auditory epithelium is to identify and localize the cell cycle regulating molecules that are present in the tissue. This set of experiments was designed to determine whether $p27^{Kip1}$ is present in the mature guinea pig auditory epithelium and to test outcome of blocking this protein with Skp2.

$p27^{Kip1}$ is a cyclin-dependent kinase-2 (cdk-2) inhibitor (Sherr and Roberts, 1999). $p27^{kip1}$ acts as a negative regulator of the G1–S transition of the cell cycle (Harper, 2001). Skp2 is an F-box protein and substrate of recognition component of Cullin 1 (CUL1) for SCF ubiquitin ligase (Nakayama et al., 2000). Skp2 induces the G1 to G0 transition of the cell cycle through ubiquitination of $p27^{kip1}$ and cyclin E (Nakayama KI, 2001). As such, it may be used to antagonize the inhibition exerted on cell cycle by $p27^{Kip1}$. During inner ear development in the mouse embryo, a down-regulation of *Skp2* expression was noted to coincide with onset of $p27^{Kip1}$ expression in the non-sensory cells of the auditory epithelium (Dong et al., 2003).

Removal of inhibition on cell cycle in the auditory epithelium may not necessarily lead to formation of new hair cells. In birds and other non-mammalian vertebrates, the process of hair cell regeneration occurs spontaneously, with or without mitosis (Cotanche, 1997; Stone and Rubel, 2000). In mice with dysfunctional $p27^{Kip1}$ supernumerary hair cells are formed (Chen and Segil, 1999; Kanzaki et al., 2006; Lowenheim et al., 1999). The outcome of inducing cell proliferation in the mature auditory epithelium is unknown. If new cells do not take up the hair cell phenotype, it may be necessary to induce transdifferentiation with forced expression of genes such as *Atoh1*. *Atoh1* (formerly *Math1*) is a basic helix-loop-helix (bHLH) transcription factor that is essential for generating hair cells in developing inner ear (Bermingham et al., 1999; Chen et al., 2002; Zine et al., 2001).

After maturation of hair cells in developing mammals, the expression of *Atoh1* is down-regulated (Zheng et al., 2000). However, over-expression of *Atoh1* (or its homologs) in cultures of developing or mature rat cochleae results in the production of ectopic hair cells derived from non-sensory epithelial cells (Shou et al., 2003; Zheng and Gao, 2000). Over-expression of *Atoh1* has also been shown to generate new hair cells in mature guinea pig cochleae *in vivo* (Izumikawa et al., 2005; Kawamoto et al., 2003). The goal of our experiments was to localize

p27^{Kip1} in the mature auditory epithelium, to determine if forced expression of *SKP2* can induce proliferation in the tissue and to assess the potential for generation of new ectopic hair cells by *SKP2* alone versus *SKP2* in combination with *Atoh1* over-expression. We demonstrate that p27^{Kip1} is present in numerous types of non-sensory cells in the mature auditory epithelium and that over-expressing *Skp2* can induce proliferation but no ectopic new hair cells are formed. Forced expression of *SKP2* in combination with *Atoh1* increases the number and alters the pattern of ectopic hair cell generation as compared with *Atoh1* alone.

2. Materials and methods

Animal care and use were approved by institutional UCUCA committee and conformed to National Institutes of Health guidelines.

Adenovirus vectors

The vectors Ad.*Atoh1* (5.2×10^{11} pfu/ml) and Ad.empty (5.1×10^{11} pfu/ml) were based on human adenovirus serotype 5 with E1, E3 and E4 regions deleted, as described previously (Brough et al., 1996). Ad.*SKP2* (1.0×10^{12} pfu/ml) was constructed using the AdEasy system (He et al., 1998). Expression of the transgene insert in each of these vectors was driven by the human cytomegalovirus promoter. The recombinant adenoviruses were amplified and propagated as described previously (Gervais et al., 1998).

Animals and inoculation surgery

We used adult guinea pigs weighing 300–500 g at the beginning of the experiment. We inoculated 5 μl of the adenovirus vector or control solution into the 2nd turn scala media of the left ear, as previously described (Ishimoto et al., 2002). Briefly, animals were anesthetized with Rompun (i.m., xylazine, 10 mg/kg, Bayer, Shawnee Mission, KS) and Ketalar (i.m., ketamine HCl, 40 mg/kg, Parke Davis, Morris Plains, NJ). Chloramphenicol sodium succinate (i.m., 30 mg/kg) was administered as prophylaxis and 0.3 ml of 1% lidocaine HCl was injected subcutaneously in the post-auricular and neck areas, for local anesthesia. The animals were placed in a supine position on a thermo-regulated heated pad. Ventral skin was incised paramedially and the tympanic bulla was exposed. After opening the bony bulla, the lateral cochlea was revealed. A small perforation was made in the bone above the pigmented area of the stria vascularis using a fine surgical needle. A microcanula was inserted into the scala media through the perforation. The circumference of the inserted microcanula was sealed and covered with carboxylate cement (Durelon, 3M, St. Paul, MN).

To inoculate the fluid into the endolymph, we used a microcanula driven by an electromechanical infusion pump (Harvard Apparatus, Holliston, MA) operated at a rate of 1 μl/ml over 5 min. To inoculate Ad.*SKP2* and Ad.*Atoh1* combined, the two vector solutions were combined at a volume ration of 1:1 resulting in 50% reduction in the concentration of each vector. Once the inoculation was complete, a layer of carboxylate cement was placed over the inoculation site to minimize the leak from the fenestration after removing the canula. The incision was closed in two layers.

Scanning electron microscopy

SEM was performed in order to determine the distribution and the number of ectopic hair cells. We used 8 animals for the group receiving both Ad.*SKP2* Ad.*Atoh1*, 5 animals for the *Atoh1* alone group, 5 animals for the *SKP2* alone group, 5 animals for the artificial endolymph group and 5 animals for the Ad.empty group. Animals were deeply anesthetized, exsanguinated and systemically perfused with glutaraldehyde (2% in phosphate buffer). Immediately following the perfusion, animals were decapitated, the temporal bones removed from the skull and the cochleae opened at the apical tip and the round and oval windows and immersed in fixative.

Two hours later, the bony wall at was removed along with the lateral wall tissues (stria vascularis and spiral ligament) to reveal the surface of the sensory epithelium. In all samples, SEM evaluation was performed in areas including (from medial to lateral) the interdental cell region, the inner sulcus and the organ of Corti. Images were collected from all cochlear turns.

SEM analysis was performed for localizing and counting ectopic hair cells. To be counted, hair cells had to be localized to an ectopic site in the interdental cell area or the inner sulcus, and exhibit two or more stereocilia on the apical surface. The area immediately adjacent to the site of inoculation was excluded from the statistical analysis, because tissue in this area may have responded to the mechanical trauma (of the inoculation) as well as the presence of the transgenes.

Immunohistochemistry

Whole mounts of the auditory sensory epithelium and surrounding tissues were used to localize $p27^{Kip1}$, Atoh1 and SKP2. To localize $p27^{Kip1}$ in the auditory epithelium, we stained normal guinea pig cochleae from 4 animals with a monoclonal anti-mouse antibody specific to $p27^{Kip1}$ (Neomarkers, Fremont, CA), diluted 1:200. To localize Atoh1 and SKP2 after combined Ad.*Atoh1* and Ad.*SKP2* inoculation, we obtained cochleae from 4 animals, 4 days after the inoculation. Contralateral ears served as controls. We fixed cochleae in 4% paraformaldehyde in phosphate buffer, pH 7.4, removed the spiral ligament, stria vascularis and tectorial membrane and then permeabilized the tissue with 0.3% Triton X-100 in PBS with 1% goat serum for 10 min. Nonspecific binding of secondary antibodies was blocked with 5% BSA in PBS for 20 min. Tissues were reacted with primary antibody, rinsed and incubated with the secondary antibody. Specimens were mounted on glass slides using Crystal Mount (Biomeda, Foster City, CA). To perform double staining of Atoh1 and SKP2, we used a primary anti-Atoh1 monoclonal antibody (1:4 dilution, University of Iowa Hybridoma Core) and a rabbit polyclonal anti-SKP2 antibody (1:300 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were TRITC-conjugated goat anti-mouse (diluted 1:200, Jackson ImmunoResearch, West Grove, PA) and FITC-conjugated goat anti-rabbit (diluted 1:300, Jackson ImmunoResearch). Samples were evaluated under a Leica DMRB epifluorescence microscope (Leica, Eaton, PA) using 40x and 100x oil objectives and a CCD-Cooled SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

BrdU study

This experiment was done to determine the uptake of BrdU by cells undergoing DNA synthesis. BrdU was given to 6 animals from the Ad.*SKP2* group and 4 animals that received Ad.empty. Contralateral ears of these 10 animals served as additional controls. BrdU was administered in the drinking water from day 1 to day 14 after the inoculation of the viral vector(s). Two weeks after the surgery, all animals were euthanized and their ears and gut (positive control) were prepared for immuno-staining with anti-BrdU antibody. The cochleae were fixed with 4% paraformaldehyde in phosphate buffer, pH 7.4, permeabilized in 0.3% Triton X-100, incubated with 3% hydrogen peroxide for 30 min, immersed in 2N HCl for 30 min, and then incubated with mouse monoclonal antibody against BrdU (Sigma, Saint Louis, MO) for 30 min. A peroxidase-conjugated secondary anti-mouse antibody (ABC kit, Vector Laboratories, Burlingame, CA) was used, followed by DAB. After completion of immuno-staining, specimens were decalcified in 3% EDTA for 7 days, embedded in JB-4 resin (Electron Microscopy Sciences, Hatfield, PA) and sectioned (5 μm thickness). Every third section was collected, so that a given nucleus could only be counted once. Sections were mounted on glass slides and observed using light microscopy. The presence of BrdU-positive cells was assessed in 50 sections taken from serial sections of inoculated ears.

Data analysis

All analyses were performed using SPSS 13. Because of the small sample sizes, Fisher's exact test for the 2×2 contingency table comparing BrdU uptake between Ad.*SKP2* treated animals and controls. A one-sided t-test was used to test the hypothesis that treatment with both Atoh1 and SKP2 produced more ectopic hair cells than treatment with Atoh1, alone.

3. RESULTS

$p27^{Kip1}$ is expressed in the mature auditory epithelium in and around the organ of Corti

We used antibodies to localize to $p27^{>Kip1}$ in whole-mounts of the normal mature auditory epithelium. The whole-mounts included tissues from the interdental cell layer (medially) to the outer sulcus (laterally). At a focal plane just above the basilar membrane, staining was detected in nuclei of Deiters and pillar cells within the sensory epithelium, and in Hensen cells (Fig. 1A). The staining intensity in Hensen cells was invariably higher compared to supporting cells in the sensory epithelium. At a slightly higher focal plane Hensen cell nuclei were strongly positive whereas hair cells were all negative (Fig. 1B). Other non-sensory cells that were $p27^{Kip1}$ positive were inner sulcus cells and the interdental cells on the limbus (data not shown).

Over-expression of Skp2 induces proliferation in the auditory epithelium

In animals that received BrdU for 2 weeks following the Ad.*SKP2* inoculation, and sacrificed 2 weeks later, several BrdU positive cells were found in the interdental cell area (Fig. 2) and the inner sulcus (data not shown). Some of the BrdU positive cells appeared in pairs. In the plastic sections we examined, cells within the organ of Corti did not exhibit BrdU staining. All 6 Ad.*SKP2* inoculated animals that received BrdU had BrdU-positive cells, whereas none of the 4 control animals that received BrdU, but not Ad.*SKP2*, had positive cells. Fisher's exact test of the corresponding 2×2 contingency table indicates this difference in BrdU uptake is significant ($p=0.005$). The number of BrdU positive cells in each section was between 5 and 10, suggesting that with extrapolation the total number per ear could have reached many hundreds.

Gene Expression of Atoh1 and Skp2

The dual inoculation with Ad.*Atoh1* and Ad.*SKP2* was done by mixing equal amounts of each vector prior to inoculation. This leads to a dilution of each vector to half its original concentration and therefore to an overall reduction in the efficiency of gene expression. As such, the number of cells that are transduced by both vectors is limited. Cells expressing the *Atoh1* and *SKP2* transgenes are seen in the interdental cell region (Fig. 3A), in the organ of Corti, and in Hensen cell area (Fig. 3B). Staining for these transgenes was confined to the nucleus. The images were obtained at a focal plane immediately beneath the luminal surface. As expected, most transduced cells expressed only one transgene but dually transduced cells were found in all samples of the experimental group. Animals that received control inoculations did not show any positive staining with antibodies to these two proteins.

Ectopic hair cells

SEM analysis revealed numerous ectopic hair cells in ears inoculated simultaneously with Ad.*Atoh1* and Ad.*Skp2* (Fig. 4 A-C). Inoculation of Ad.*Atoh1* alone also induced generation of ectopic hair cells (Fig. 4B), but their number was smaller than that seen in the combined *Atoh1* and *SKP2* group. The mean number of ectopic hair cells ± standard deviation in the 2^{nd} turn of the 8 cochleae that received the combined (*Atoh1* and *SKP2*) inoculation was 17.8 ± 17.4. In the group receiving the Ad.*Atoh1* alone (5 animals) the mean number of ectopic hair cells was 1.40 ± 1.67. Thus the combined treatment produced more ectopic hair cells than did treatment with only Atoh1 ($p=0.017$).

In the combined group, the highest number of ectopic hair cells was found near the site of inoculation. In this area, some of the ectopic hair cells existed in pairs (Fig. 4D). Some of the new hair cells exhibited a long and/or thick kinocilium-like projection in addition to the stereocilia (Fig. 4C and E). There were no ectopic hair cells in the cochleae of guinea pigs inoculated with the Ad.*SKP2* vector alone. Ad.empty or artificial endolymph inoculations did not lead to formation of ectopic hair cells either (data not shown).

4. DISCUSSION

Our data show that *p27*^{Kip1} is expressed in non-sensory cells flanking the organ of Corti of the mature guinea pig. We demonstrate that forced expression of *SKP2* leads to proliferation in non-sensory cells around the organ of Corti and that combined inoculation of Ad.*SKP2* and Ad.*Atoh1* enhances the number of newly generated ectopic hair cells as compared to over-expressing *Atoh1* alone.

The presence of p27^{Kip1} in non-sensory cells of the membranous labyrinth has previously been documented in the developing mouse cochlea (Chen and Segil, 1999; Dong et al., 2003; Lowenheim et al., 1999). The present study extends the finding to the guinea pig model and provides whole-mount analysis that allows for the detection of the protein in large experimental fields. The data reveal that *p27*^{Kip1} expression is maintained into adulthood in supporting cells of the organ of Corti as well as in areas outside the organ of Corti. Interestingly, the staining intensity appeared weaker in the supporting cells of the sensory epithelium (Deiters and pillar cells) as compared to Hensen cells which flank the sensory epithelium. The reason for this difference is unclear.

In *p27*^{Kip1} null mice, proliferation of non-sensory cells continues in the organ of Corti, leading to generation of supplementary hair cells (Chen and Segil, 1999; Lowenheim et al., 1999). In the mature guinea pig ears examined in this study, forced expression of *SKP2* did not cause a notable presence of BrdU-positive cells in the organ of Corti proper. BrdU-positive cells were localized in areas flanking the sensory epithelium. It is presently unclear why the localization of proliferation site differs between the two models.

The number of ectopic cells generated by *Atoh1* over-expression alone is limited (Izumikawa et al., 2005; Kawamoto et al., 2003). The ability of *SKP2* to increase the number of ectopic cells generated by *Atoh1* suggests that therapies for cell cycle enhancement and transdifferentiation can be combined. It also indicates that mature non-sensory cells that undergo cell division can respond to the developmental signals provided by *Atoh1*. The data suggest that in areas adjacent to the organ of Corti the expression of *SKP2* leads to proliferation of the non-sensory cells, which by itself is not sufficient for generating new hair cells. These findings are in agreement with the outcome of disrupted *p27*^{Kip1} in mice, where the number of hair cells is increased only in the organ of Corti and not in areas flanking the sensory epithelium (Chen and Segil, 1999; Kanzaki et al., 2006; Lowenheim et al., 1999), despite the fact that cell division continues in flanking areas (Chen and Segil, 1999). Our overall interpretation of these data is that p27^{Kip1} blocks proliferation in non-sensory cells in and around the organ of Corti, and that in the absence of this block, new cells that are generated in the organ of Corti proper can become new hair cells without further intervention. In ectopic areas, however, it is necessary to force expression of hair cells genes such as *Atoh1* to generate new hair cells.

The area immediately adjacent to the site of inoculation was excluded from the statistical analysis, because tissue in this area responded to the mechanical trauma (related to the inoculation) as well as the presence of the transgenes. Therefore the numbers presented here may represent an underestimate of the total number of newly generated hair cells. The area of

the organ of Corti was also excluded from the counting, because the procedure was performed on non-deafened guinea pigs. Many hair cells degenerate in response to the procedure of endolymphatic inoculation, and regenerated hair cells could have taken their place. However, we were unable to distinguish between original hair cells and possibly regenerated hair cells within the organ of Corti and therefore did not count hair cells in this area.

The increase in ectopic hair cell number and the presence of paired hair cells following the combined treatment with *SKP2* and *Atoh1* indicate that new hair cells may be generated via mitotic division followed by transdifferentiation. This finding has important implications for future clinical use of hair cell regeneration therapy. The fact that post-mitotic cells can attain the hair cell phenotype is encouraging in that these cells are less likely to continue dividing and form a tumor. It is also important to observe that non-sensory cells retain their responsiveness to *Atoh1* after division. This is not trivial, because these cells de-differentiate morphologically in order to divide. However, the data corroborate finding in vertebrates other than mammals, including avian species, where mitotic transdifferentiation is the main spontaneous route of hair cell regeneration.

Ectopic hair cells were not found following forced expression of *SKP2* alone, despite the presence of BrdU positive cells. This suggests that proliferation by itself is insufficient for inducing generation of new hair cells in the mature ear. This finding appears to contrast with the situation in the developing ear of mice with loss of function of $p27^{Kip1}$ or *Rb1*, where the cell cycle regulation is disrupted and the phenotypic outcome is excessive number of hair cells (Chen and Segil, 1999; Lowenheim et al., 1999; Sage et al., 2005). It is possible that once an animal is mature, the addition of supernumerary hair cells due to the defect in cell cycle regulation is reduced, as seen in mature mice deficient for $p27^{Kip1}$ (Kanzaki et al., 2006).

In this study, the forced expression of *SKP2* and *Atoh1* was accomplished by two different viral vectors, one for each gene. For several reasons, this approach leads to a small number of cells that are transduced by both vectors. First, mixing Ad.*Atoh1* and Ad.*SKP2* prior to inoculation dilutes the concentration of each vector by 50%, thereby lowering the overall efficiency of transgene expression. Second, the chance for a cell to be transduced by both vectors is further reduced. The timing of vector delivery may also matter for maximizing the effect on proliferation and transdifferentiation. In these experiments, the vectors were applied simultaneously, which may have further compromised production of a large number of new hair cells. The efficiency of generating new hair cells would likely be enhanced by utilization of a virus vector encoding both *Skp2* and *Atoh1*, which would increase the rate of cells simultaneously expressing both transgenes, and/or by optimized timing for sequential use of the vectors.

The presence of newly generated cochlear hair cells does not necessarily imply that hearing improves toward normal hearing. It is possible that tinnitus may also result from the new hair cells, and the presence of ectopic hair cells further complicates the physiological outcome. Interestingly, mice with deficient $p27^{Kip1}$ expression have very poor hearing (Chen and Segil, 1999; Kanzaki et al., 2006; Lowenheim et al., 1999). It is currently unclear whether the functional quality of hair cells the arise due to $p27^{Kip1}$ deficiencies is lacking in some way, or if other problems in these mice, in the ear and elsewhere, contribute to the deficiency. The next step in assessing the feasibility of the combined *SKP2* / *Atoh1* treatment would be histological and physiological assessment of such therapy in deafened mature mammals.

Manipulation of cell cycle regulation for therapeutic purposes is usually aimed at treating cancer, by stabilizing and enhancing $p27^{Kip1}$ expression (Sumimoto et al., 2005; Supriatno et al., 2005). In contrast, our study looked at the effects of antagonizing $p27^{Kip1}$ by over-expressing *SKP2*. This strategy is relevant to cases where adding new cells may contribute to

the therapeutic goals. Promoting cell cycle with SKP2 has also been attempted in cultured primary hepatocytes and in hepatocytes in vivo and resulted in mitosis in hepatocytes that were otherwise quiescent (Nelsen et al., 2001). Although this therapy is attractive, it would be important to improve the control of gene expression to ascertain that proliferation remains limited in place and time so as not to promote tumor formation.

In addition to $p27^{Kip1}$, enhanced proliferation in the post-mitotic organ of Corti has been shown with disruption of cell cycle regulatory gene *Rb1* (Sage et al., 2005). It is presently unclear if the two genes act on the same signaling cascade in regulating cell cycle arrest in non-sensory cells of the auditory epithelium. Based on studies in other tissue models, it is possible that Rb1 represses Skp2 resulting in stabilizing $p27^{Kip1}$, leading to arrest of the cell cycle (Ji and Zhu, 2005). Better understanding of the specific role of each gene in regulating proliferation in the auditory epithelium will help design robust yet well regulated means for increasing the number of cells in the tissue. This is important, because therapy for hair cell regeneration based on transdifferentiation of non-sensory cells requires that a large enough number of supporting cells remain in the deaf auditory epithelium.

The results we present do not provide evidence for a direct causative relationship between *SKP2* and $p27^{Kip1}$. It is possible that proliferation due to *SKP2* forced expression is accomplished by another signaling cascade, not directly involving $p27^{Kip1}$. Further work is necessary for elucidating the molecular signaling initiated by *SKP2* in non-sensory cochlear cells resulting in a proliferative response in these cells.

In conclusion, we determined that $p27^{Kip1}$ is expressed in the mature auditory epithelium in the organ of Corti and in adjacent regions. Co-expression of Ad.*Atoh1* and Ad.*SKP2* enhances the number of ectopic hair cells compared to *Atoh1* over-expression alone. Forced expression of *SKP2* alone induces proliferation but does not enhance generation of new hair cells. These findings demonstrate that targeted enhancement of proliferation is by itself insufficient for inducing hair cell regeneration, but when combined with forced expression of *Atoh1*, regeneration in the mature auditory epithelium can be enhanced. The data suggest that therapies can be designed to induce proliferation in the auditory epithelium by removing the inhibition on cell cycle.

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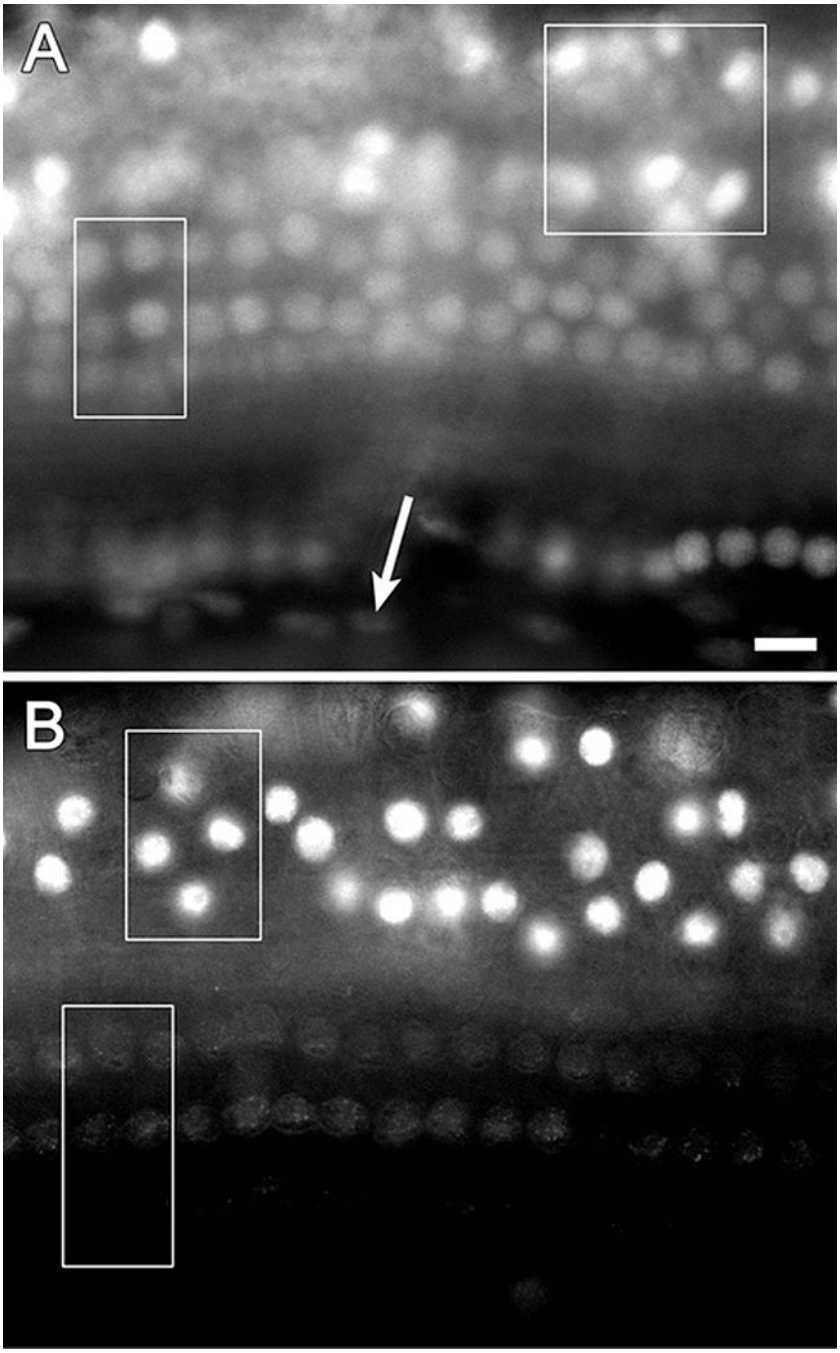


Figure 1.

The normal distribution of $p27^{Kip1}$ in mature guinea pig ears shown by immunofluorescence on whole-mounts of the auditory epithelium. A. At a focal plane immediately above the basilar membrane positive nuclei are found in Deiters cells (rectangle), Hensen cells (square) and inner pillar cells (round nuclei at bottom of image). Spindle shaped mesothelial cells located beneath the basilar membrane display background level staining (arrow). B. At a higher focal plane, $p27^{Kip1}$ positive nuclei are found in Hensen cells (top rectangle) whereas nuclei of hair cells are at background staining level (bottom rectangle). Bar, 10 μm for A and B.

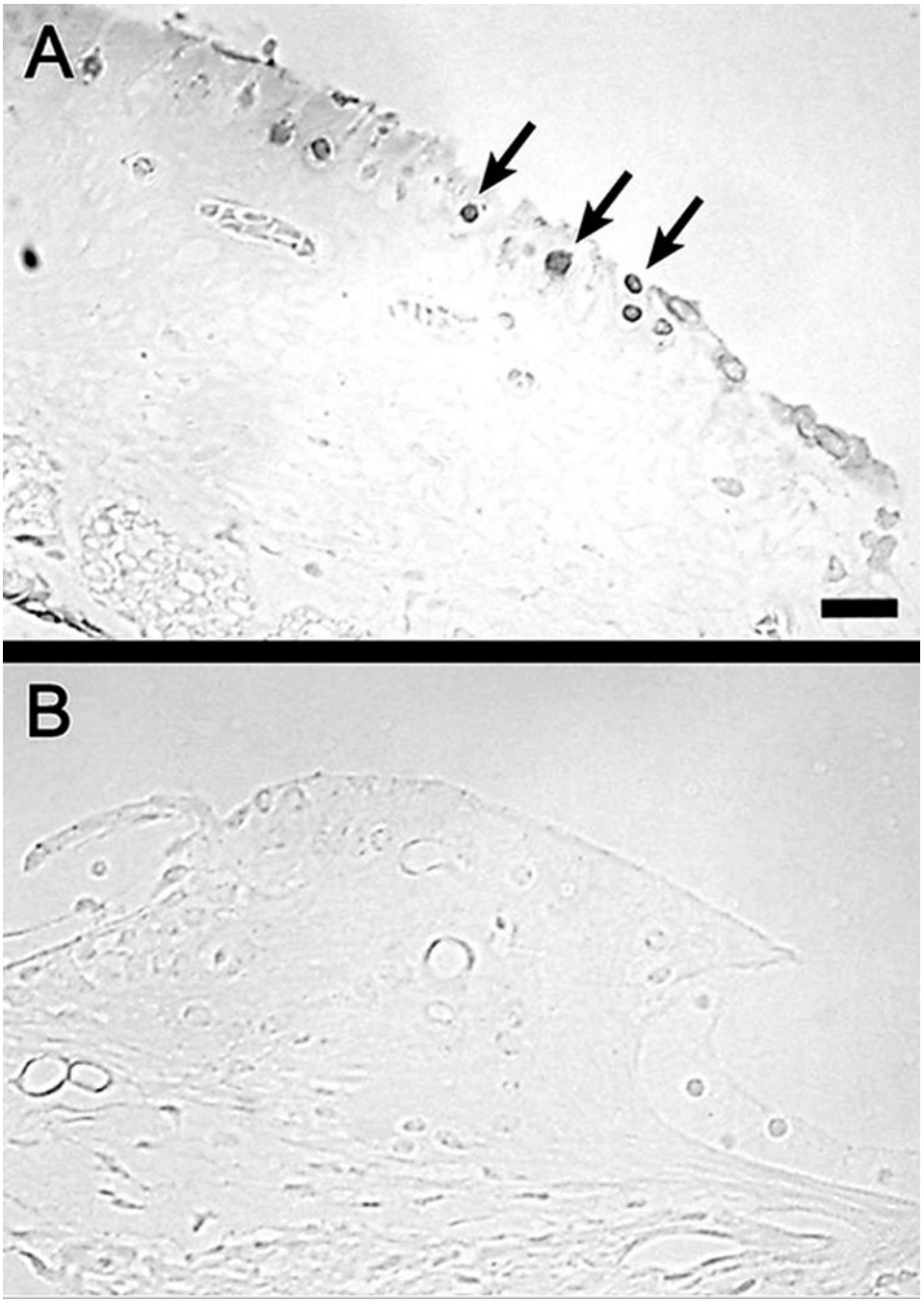


Figure 2.
Plastic sections of the auditory epithelium of an Ad.*SKP2* treated ear (A) and an Ad.empty control ear (B) stained with antibody to BrdU. A. Numerous BrdU-positive cells are found in the interdental cell region (arrows). B. No BrdU-positive cells are seen in a control ear. Bar, 25 μm for A and B.

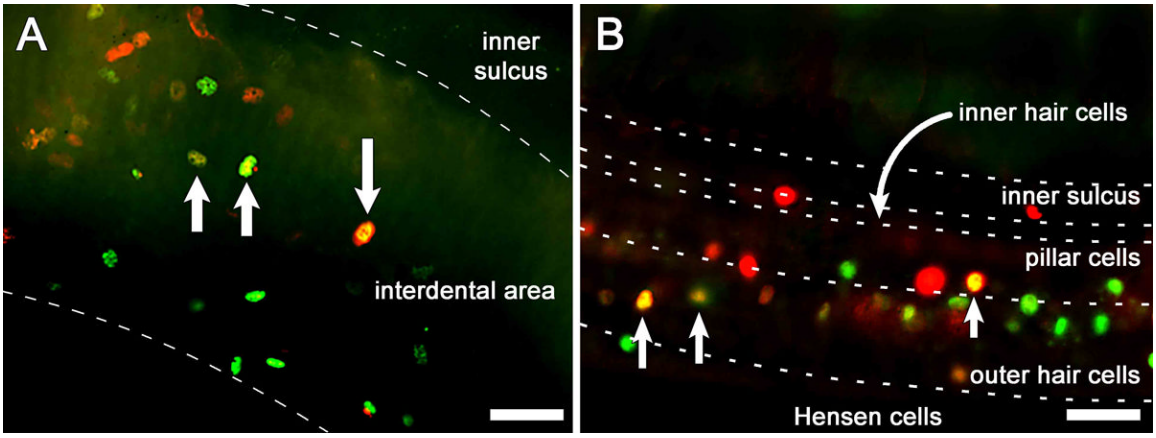


Figure 3.

Epi-fluorescence images of whole-mounts of the organ of Corti double immuno-labeled for Atoh1 (green) and SKP2 (red) 4 days after Ad.*Atoh1* and Ad.*SKP2* inoculation. Images were obtained immediately beneath the luminal surface. A. In the interdental cell region, numerous cells are stained for *Atoh1* or *SKP2*, and a small number of cells (yellow) express both proteins (arrows). B. In the organ of Corti and Hensen cell area, several cells (arrow) are yellow indicating dual expression of SKP2 and Atoh1 while others express either Atoh1 (green) or SKP2 (red). Dashed lines delimit regions within the tissue. Bar, 20 μm.

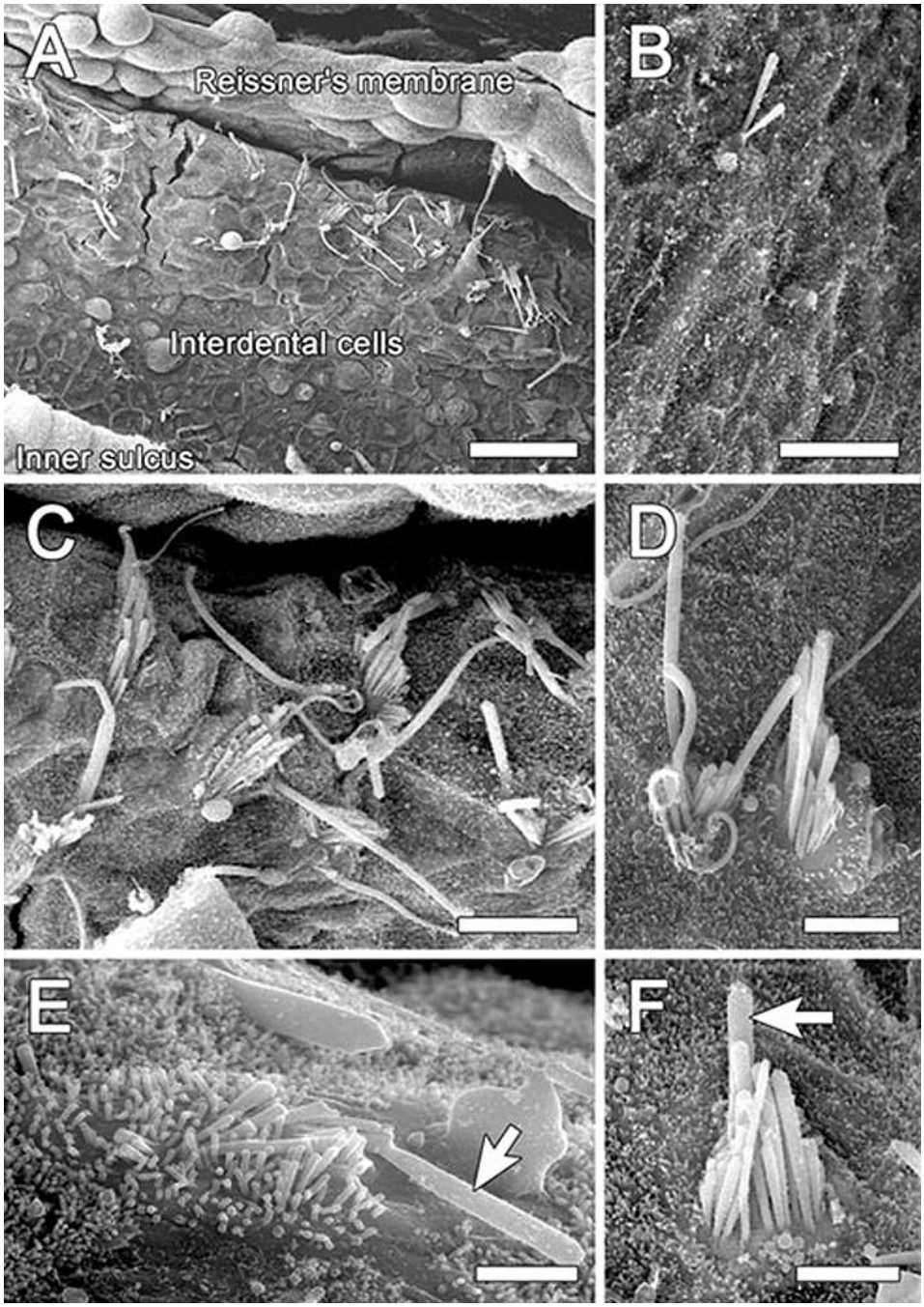
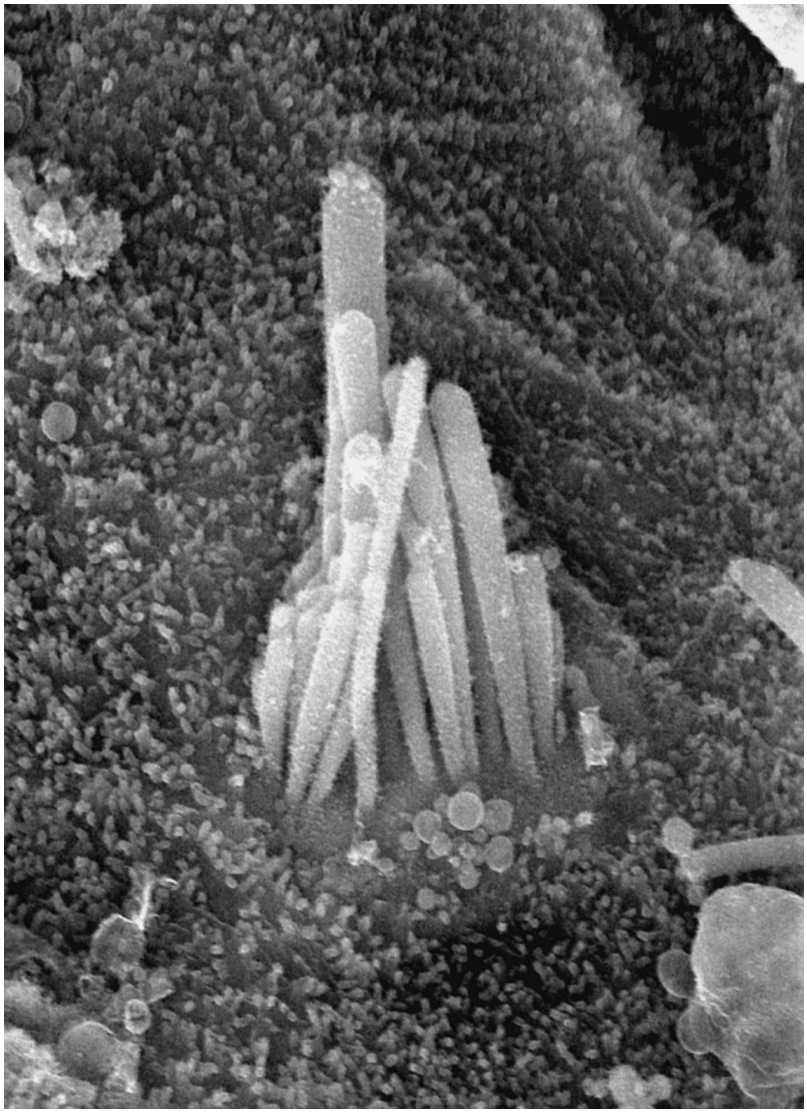


Figure 4.

SEM images of interdental cell area (2nd turn) 2 months after inoculation of Ad.*Atoh1* and Ad.*SKP2* (A and C-F) or Ad.*Atoh1* alone (B). A. Numerous ectopic stereocilia bundles on the limbus, reaching to the area where Reissner's membrane is inserted. B. A single ectopic bundle among interdental cells on the limbus. C. An enlarged area in (A) showing stereocilia bundles in an ectopic location on the limbus. Some bundles contain a graded array of stereocilia and others are rather disorganized. A kinocilium-like projection is seen on some of the bundles. D. Two ectopic hair cells appearing as a pair . E. Some ectopic hair cells appear like immature cells and exhibit a long and/or thick kinocilium-like protrusion (arrow). F. An ectopic hair cell

with a staircase organization of stereocilia and a projection that appears like a kinocilium (arrow). Bars, 30 μm in A, 10μm in B, C, 5μm in D, 2 μm in E, and 3 μm in F.



Suggested Cover Image.
SEM micrograph of an ectopic hair cell residing on the spiral limbus following inoculation of adenoviral vectors expressing Atoh1 and SKP2 into the cochlear endolymph of guinea pigs.