

Silencing p27 reverses post-mitotic state of supporting cells in neonatal mouse cochleae

Kazuya Ono¹, Takayuki Nakagawa¹, Ken Kojima¹, Masahiro Matsumoto¹, Takeshi Kawauchi², Mikio Hoshino³, Juichi Ito¹

¹Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, 606-8507 Kyoto, Japan, ²Department of Anatomy, Keio University School of Medicine, 160-8582 Tokyo, Japan and ³Department of Biochemistry and Cellular Biology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 187-8502 Tokyo, Japan

Corresponding author: Takayuki Nakagawa, MD, PhD
Department of Otolaryngology, Head and Neck Surgery,
Graduate School of Medicine, Kyoto University,
Kawaharacho 54, Shogoin, Sakyo-ku, 606-8507 Kyoto, Japan
Tel: +81-75-751-3346 Fax: +81-75-751-7225
E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

Abstract

The post-natal cochlear mammalian epithelium have no capacity to proliferate in tissue, however, dissociated supporting cells exhibit the ability to divide and trans-differentiate into new hair cells *in vitro*, with this process found to be correlated with the downregulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$. Here we show that knockdown of $p27^{kip1}$ with short hairpin RNA-expressing vectors results in the cell-cycle reentry of post-mitotic supporting cells in the post-natal mouse cochleae *ex vivo*. The $p27^{kip1}$ -knockdown cells incorporated BrdU, and then divided into two daughter cells. However, there was also activation of the apoptotic pathway in some supporting cells. These results indicate that the use of RNA interference to target $p27^{kip1}$ is an effective strategy for inducing cell-cycle reentry in post-mitotic supporting cells in the post-natal mammalian cochleae, although additional manipulations of the supporting cells are required to achieve hair cell regeneration.

Key words: RNA interference; cyclin-dependent kinase inhibitor; cochlea; mitosis, apoptosis; regeneration

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities. Auditory hair cells in the cochlea are mechanoreceptors that play a crucial role in hearing. In mammals, if the hair cells are damaged or lost, the resulting SNHL is permanent. Therefore, one of the best approaches for improving hearing in mammals would be to find a way to induce hair cell regeneration.

The cochlear epithelium is composed of hair cells and supporting cells (SCs). In the avian auditory epithelium, SCs are able to reenter the cell cycle and proliferate in response to hair cell loss, which gives rise to both new hair cells and SCs (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). In mice, progenitors of the cochlear epithelium stop dividing by embryonic day 14.5 (Ruben, 1967), with differentiation of the hair cells and SCs occurring after this terminal mitosis. Both hair cells and SCs maintain a post-mitotic state throughout life in adult mammals, and do not exhibit any spontaneous capacity to divide under normal conditions or in response to damage *in vivo* (Roberson and Rubel, 1994).

It has been determined that the cyclin-dependent kinase inhibitor (CKI), p27^{kip1}, plays a crucial role in the entry of mammalian SCs into the G0 phase (Chen and Segil, 1999; Lowenheim et al., 1999). CKIs function by binding to and inhibiting the activity of the cyclin-dependent kinases that promote cell cycle progression and fulfill the cell cycle checkpoint functions (Sherr and Roberts, 1999). The p27^{kip1} acts as a negative regulator of the G1-S transition in the cell cycle (Harppper, 2001). Studies on p27^{kip1}-deficient mice have shown that when deleted, it results in the continued proliferation of SCs in postnatal mouse cochlear epithelia (Chen and Segil, 1999; Lowenheim et al., 1999). Recently, White *et al.* have reported that after dissociation,

some of the mammalian SCs are able to recover their proliferative abilities (White et al. 2006). In addition, these dissociated SCs can also trans-differentiate into hair cells *in vitro*. These findings suggest that while these mammalian SCs might have a regenerative potential similar to avian auditory epithelia, this potential is suppressed within the tissue. This previous study also found that there was a correlation between the reduced expression levels of p27^{kip1} and the ability of the mammalian supporting cells to reenter the cell cycle *in vitro*. Overall, these previous results suggest that manipulation of the p27^{kip1} levels could be used therapeutically to stimulate the proliferation of mammalian SCs in tissue.

Discovery of gene inactivation by RNA interference (RNAi) has led to the development of a new targeted therapy for inner ear disease at the molecular level (Fire et al., 1998). RNAi is a two-stage intracellular process that converts the double-stranded RNA molecule precursors into functional small interfering RNAs. These small interfering RNAs are then incorporated into RNA-inducing silencing complexes. Subsequently, these duplexes unwind, with one strand used to target sequence-specific cleavage of the messenger RNAs that ultimately cause the knockdown of the expression of the targeted proteins (Elbashir et al., 2001).

Although studies on p27^{kip1}-deficient mice have shown that its deletion results in continued SC proliferation in postnatal mouse cochlear epithelia (Chen and Segil, 1999; Lowenheim et al., 1999), the consequences of acute removal of p27^{kip1} from differentiated SCs have yet to be examined. Therefore, the aim of the current study was to examine the efficacy of RNAi in silencing p27^{kip1} in cochlear explant cultures from post-natal mice, and its potential for inducing mitosis in post-mitotic SCs in the cochlear epithelia after birth.

Results and Discussion

Co-transfection efficiency

In this study, the two different plasmids were simultaneously introduced into the cochlear explants in order to label cells in which short hairpin RNAs (shRNAs) were transfected. To examine the efficiency of co-transfection, we introduced two different plasmids, EGFP- and DsRed-expressing vectors, into five cochlear explants via electroporation. One day after the electroporation, EGFP⁺ cells were found in all explants, with the majority co-expressing DsRed (**Figs. 1a, b**). Immunostaining for p27^{kip1} identified SCs within the cochlear epithelia (**Fig. 1c**). DsRed was expressed in $32.5 \pm 11.0 / 33.0 \pm 10.9$ EGFP⁺, p27^{kip1}⁺ cells (98.5%), and there were $32.5 \pm 11.0 / 33.5 \pm 12.4$ DsRed⁺, p27^{kip1}⁺ cells (97.0%) that expressed EGFP. These results demonstrated that electroporation was able to simultaneously introduce two different plasmids into the SCs.

Silencing p27^{kip1} expression in SCs by RNAi

ShRNA for p27^{kip1}-expressing vectors, sh-p27a, or the control scrambled shRNA vector, sh-scr, were co-electroporated with pEGFP-N1 into cochlear explants (n = 5 in each condition). Two days after the introduction of a mixture of sh-scr and pEGFPN-1, 48.4 ± 13.7 EGFP-expressing cells were found in the whole single cochlear explant, with all the EGFP-expressing cells positive for p27^{kip1} (**Figs. 2g-h'**). These results indicate that transfection of plasmids into SCs did occur and that there was no silencing of the expression of p27^{kip1}. In sh-p27a-transfected explants, $28.4 \pm 7.8 / 29.7 \pm 7.7$ EGFP-expressing cells (95.4%) exhibited no expression of p27^{kip1} (**Figs. 2i-l**), indicating that silencing of the expression of p27^{kip1} occurred in these SCs.

To confirm that EGFP⁺ and p27^{kip1}-cells were SCs after the knockdown of p27^{kip1}, we performed immunostaining for myosin VIIa and Sox2 in explants in which sh-p27a has been introduced. Cochlear specimens of post-natal day 3 (P3) mice 1 day *in vitro* were used as the immunohistochemistry controls. In controls, immunostaining for myosin VIIa and p27^{kip1} clearly demonstrated the location of the hair cells (**Figs. 2a, d**), and the p27^{kip1}-labeled SC layer (**Figs. 2b, e**), which was located underneath the hair cell layer. Sox2 was strongly expressed in the SCs in the organ of Corti and in the inner sulcus region (which corresponds to the interior of the organ of Corti), but in the outer sulcus region (which corresponds to the exterior of the organ of Corti), it was either faintly seen or not found at all within the SCs (**Figs. 2c, f**), which is identical to previous findings (Oesterle et al., 2008; Hume et al., 2007). In sh-p27a-transfected specimens (n = 5), double labeling for myosin VIIa and p27^{kip1} demonstrated that the EGFP⁺ and p27^{kip1}- cells were located underneath the myosin VIIa+ hair cell layer (**Fig. 2i'**), which was coincident with the location of the SCs. Double labeling for Sox2 and p27^{kip1} demonstrated that all EGFP⁺ and p27^{kip1}- cells that were located in the organ of Corti (41 cells in four explants) were positive for Sox2 (**Figs. 2k-m**). Overall, these results demonstrated that the introduction of sh-p27 efficiently silenced p27^{kip1} expression in the SCs.

In addition, to examine the efficacy of transfection in pillar cells and Deiters' cells, immunostaining for Prox1 was performed (Kirjavainen et al. 2008). In P3 mouse cochleae, the expression of Prox1 was found in pillar cells and Deiters' cells (Fig. 3a). In sh-p27a-transfected specimens (n = 7), a few EGFP⁺ Prox1⁺ cells were found (Figs. 3b-d). The mean was 2.1 ± 1.9 cells in the whole single cochlea, which is $4.4 \pm 3.5\%$ of total EGFP⁺ cells in the whole single cochlea. Other EGFP-expressing cells were

located in the outer sulcus region. In the inner sulcus region, no EGFP-expressing cells were found.

S-phase reentry of SCs by silencing $p27^{kip1}$

To examine whether $p27^{kip1}$ silencing had an effect on the reentry of SCs into the cell cycle, a 5-bromo-2-deoxyuridine (BrdU)-labeling assay was employed. To confirm that the S-phase reentry of the SCs is specific to the knockdown of $p27^{kip1}$, we used two different shRNAs to target $p27^{kip1}$ in this experiment. Explants after introduction of the plasmid mixtures, pEGFP-N1 and, sh-p27a (n = 9), sh-p27b (n = 5) or sh-scr (n = 5) were used. Three days after the introduction of a mixture of sh-scr and pEGFPN-1, there were no double-positive BrdU+ EGFP+ cells seen in the cochlear epithelia (**Figs. 4a-d**). While we found that a few mesenchymal cells located under the basement membrane of the cochlear epithelium were positive for BrdU, all of these were found to be negative for EGFP (**Figs. 4a'-d'**). In an explant transfected with sh-p27a, $13.8 \pm 6.8 / 48.2 \pm 15.2$ EGFP+ cells (28.6%) were labeled by BrdU with no expression of $p27^{kip1}$ (**Figs. 4e-h'**). In cochlear explants transfected with sh-p27b, BrdU+, EGFP+ cells were also found in the cochlear epithelia (**Figs. 4i-l'**). BrdU incorporation was identified in $7.6 \pm 4.7 / 24.6 \pm 15.0$ EGFP+ cells (30.9%). These results demonstrated that silencing $p27^{kip1}$ expression occurred after transfection of shRNA that was directed against $p27^{kip1}$ and led to the initiation of S-phase reentry of the post-mitotic SCs. However, the knockdown of $p27^{kip1}$ did not induce S-phase reentry in approximately 70% of transfected cells. There are two possible explanations for this. One is that insufficient suppression of $p27^{kip1}$, which was not enough for S-phase reentry, occurred in 70% of transfected cells. Another is that other cell-cycle inhibitors compensate the function of $p27^{kip1}$.

Mitosis in SCs induced by RNAi with p27^{kip1}

We performed time-lapse observations and propidium iodide (PI) staining in order to document the characteristic morphology for mitosis in SCs in which the p27^{kip1} had been silenced. Two days after the introduction of a mixture of sh-p27a and pEGFPN-1, time-lapse observations of EGFP⁺ cells demonstrated mitosis in SCs within the explants (**Figs. 5a, b, S1**). We observed 10 EGFP⁺ cells in the outer sulcus region. In five out of the 10 EGFP⁺ cells that were recorded, the cells became rounded in shape and then divided into two daughter cells, which stained for BrdU but did not show any labeling for p27^{kip1} (**Figs. 5c, d**). In addition, cross sections clearly showed nuclear migration to the luminal portion of the SCs (**Figs. 5c', d'**). During this process, PI staining of the nuclear chromatin demonstrated that there was segregation of the chromosomes in the EGFP⁺ cells (**Figs. 5e, f**). Thus, these results showed that silencing of the p27^{kip1} expression in post-mitotic SCs induced mitosis of the SCs in postnatal cochlear epithelia. This provides direct evidence that p27^{kip1} plays a central role in controlling the proliferation of the SCs in neonatal cochleae. White *et al.* have reported that the capacity of SCs for proliferation is diminished depending on ages (White et al. 2006). Therefore, further experiments are required to date the efficacy of RNAi for p27^{kip1} for induction of SC proliferation in adult mouse cochleae.

Fate of SCs after p27^{kip1} silencing

To examine the effect of silencing p27^{kip1} expression on the survival of post-mitotic SCs, we counted the number of EGFP⁺ cells in the cochlear epithelia that had been introduced a mixture of pEGFPN-1 and sh-p27a or sh-scr (**Fig. 6**). Quantitative analysis

of the EGFP⁺ cell survival demonstrated no significant differences in the number of surviving cells among culture periods in explants transfected with sh-scr, while there were significant reduction in the number of transfected cells was found in explants transfected with sh-p27a (**Fig. 6**). The number of EGFP⁺ cells on day 5 or 7 was significantly lower than that on day 1 or 2. These findings indicate that introduction of sh-p27a induced the loss of transfected SCs. We also examined the numbers of EGFP⁺, BrdU⁺ cells, cell-cycle reentering cells, in explants transfected with a mixture of sh-p27a and pEGFPN-1. BrdU incorporation was not found in EGFP⁺ cells on day 1, while on days 2-7, EGFP⁺, BrdU⁺ cells were observed in explants that had been introduced sh-p27a. There were significant differences in the number of EGFP⁺, BrdU⁺ cells between day 2 and day 3, 5, or 7 (**Fig. 6**). **Fig. 6** indicates that the reduction of EGFP⁺ cells between day 2 and 3 mainly caused by the reduction of EGFP⁺, BrdU⁺ cells, and that the reduction of EGFP⁺ cells between day 3 and 5 was due to the loss of EGFP⁺, BrdU⁻ cells. Therefore, both reentry of cell cycle by silencing p27^{kip1} expression and silencing p27^{kip1} expression itself may induce degeneration of transfected SCs.

We then investigated the cell death mode that occurred after silencing p27^{kip1} expression. Immunohistochemical labeling for cleaved caspase 3 along with PI staining was performed in the explants 3 days after introduction of a mixture of sh-p27a and pEGFPN-1. The activation of caspase 3 was identified in 9/327 EGFP⁺ cells in six explants (**Fig. 7a**). PI staining showed the fragmentation of nuclear chromatin in EGFP⁺ and cleaved caspase 3⁺ cells (**Fig. 7b**). In addition, we examined the effects of the addition of a caspase 3 inhibitor to the culture medium. Addition of the caspase 3 inhibitor significantly increased the number of EGFP⁺ cells in explants transfected with

sh-p27a (two-way factorial ANOVA, $p < 0.0001$, **Fig. 8**). However, the caspase 3 inhibitor did not completely prevent the loss of EGFP+ cells, as over time, the explants treated with the caspase 3 inhibitor exhibited a decrease in their numbers (**Fig. 8**). All these results indicated that silencing the p27^{kip1} expression in post-mitotic SCs initiated apoptosis in at least some, but not all of the cells. These results are consistent with previous data reported for apoptosis in mouse hair cells after the deletion of pRb (Mantela et al., 2005; Sage et al., 2005; Weber et al., 2008), p19^{ink4d} (Chen et al., 2003) or both p19^{ink4d} and p21^{cip1} (Lane et al., 2007). On the other hand, the results indicate that other mechanisms rather than degradation via apoptosis are involved in the loss of transfected SCs. Hence, further investigations are necessary to reveal mechanisms for the loss of transfected SCs.

On the other hand, even at 7 days after the introduction of a mixture of sh-p27a and pEGFPN-1, EGFP+, BrdU+ cells were still observed in cochlear epithelia that had been transfected with sh-p27a. To examine the potential of silencing p27^{kip1} expression for trans-differentiation of SCs into hair cells, specimens that had been transfected with sh-p27a were immunohistochemically labeled for myosin VIIa. The results showed that there was no myosin VIIa labeling noted in the EGFP+ cells on days 5 and 7 (data not shown). These findings indicate that no SCs in which the p27^{kip1} expression had been silenced underwent the trans-differentiation into hair cells. On the other hand, a separate study that used dissociated SCs showed the ability of post-mitotic SCs to trans-differentiate into hair cells in combination with the downregulation of p27^{kip1} *in vitro* (White et al., 2006). Therefore, it appears that further manipulations are required in order to achieve hair cell regeneration in tissue.

When there is manipulation of Notch signaling by gene transfer (Kawamoto et al.,

2003) or by pharmacological inhibitors (Yamamoto et al., 2006), the formation of ectopic hair cells in the outer sulcus region was observed, suggesting that SCs in this region are able to retain their ability to trans-differentiate into hair cells. In the avian auditory epithelium, the loss of hair cells is a key trigger that leads to the conversion of SCs to hair cells, a process for which Notch signaling is also involved (Stone and Rubel., 1999; Cafaro et al., 2007). In the mammalian cochlear epithelium, transient activation of Notch signaling has been observed in SCs following hair cell loss (Hori et al., 2007; Batts et al., 2009), suggesting that additional manipulation of Notch signaling in SCs could possibly contribute to the regeneration of hair cells once there is induction of SC proliferation by RNAi with p27^{kip1}.

Sage *et al.* (2005) demonstrated that there was proliferation of SCs in the cochlear epithelia of pRb^{-/-} mice, indicating that pRb can be a target to induce cell-cycle reentry of post-mitotic SCs in tissue. However, pRb expression is undetectable in SCs in post-natal cochlear epithelia in normal mice (Mantela et al., 2005). Therefore, pRb expression does not appear to be an attractive candidate for induction of mitosis in post-natal SCs. In contrast, post-natally in normal mice, the p27^{kip1} expression is strong and stable in the SCs (Chen and Segil, 1999; Lowenheim et al., 1999; Endo et al., 2002; White et al., 2006). Based on these reports, we considered p27^{kip1} to be an appropriate target for induction of SC proliferation in the mammalian cochlear epithelium.

In conclusion, this is the first report showing induction of mitosis in post-mitotic mammalian SCs in tissue by RNAi targeting of p27^{kip1}. The present findings indicate that p27^{kip1} expression is sufficient for maintaining the post-mitotic state of the SCs in tissue, and that RNAi is an efficient strategy for knockdown of the expression of p27^{kip1} in the SCs of post-natal mammalian cochlear epithelia. On the other hand, our data have

also identified obstacles that need to be overcome in order to achieve hair cell regeneration via the stimulation of SC proliferation. The most critical of these factors is the activation of the apoptotic pathways in SCs after silencing the expression of p27^{kip1}. Induction of trans-differentiation of the SCs into hair cells is also indicated obstacles to be overcome. Therefore, further manipulations are required for preventing apoptosis and induction of trans-differentiation into hair cells to achieve hair cell regeneration by downregulating p27^{kip1}.

Experimental Methods

Animals

ICR mice (Japan SLC Inc., Hamamatsu, Japan) were maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Japan. Experimental protocols were approved by the Animal Research Committee of Kyoto University Graduate School of Medicine (MedKyo07062), and complied with the US National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Explant culture

P3 ICR mice (were deeply anesthetized with carbon dioxide and decapitated. The temporal bones were dissected out and the cochleae removed from the surrounding tissue in 0.01 M phosphate-buffered saline (PBS), pH 7.4, which was supplemented with 0.2% glucose. The lateral walls were removed from the cochleae and the cochlear epithelia were dissected from the cochlear modiolus and placed intact on sterile

membranes in culture inserts (12 mm Millicell CM, Millipore, Billerica, MA). These explants were maintained in 24-well culture plates (Iwaki, Tokyo, Japan) in minimum essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 3 mg/ml glucose and 0.3 mg/ml penicillin G potassium salt (Nacalai Tesque, Kyoto, Japan), at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 24 h. The cultured explants were then used for the electroporation experiments.

Plasmids

ShRNAs, are RNA sequences that make tight hairpin turns and can silence gene expression via RNAi (Paddison et al., 2002). These sequences are expressed in vectors and can be used to target the *p27^{kip1}* coding sequence (sh-p27) or to act as a control scrambled sequence (sh-scr). The two different sh-p27s prepared for this experiment were sh-p27a and sh-p27b. The sequences were 5'-AGACAATCAGGCTGGGTTA-3' for sh-p27a, 5'-GAAGCGACCTGCTGCAGAA-3' for sh-p27b and 5'-TACGCGCATAAGATTAGGG-3' for the sh-scr control sequence. These complementary sequences were inserted into the mU6 pro vector. The efficacy of sh-p27s in silencing the *p27^{kip1}* expression in neuronal cells has been previously demonstrated (Kawauchi et al., 2006). pEGFP-N1 and pDsRed-Express-N1 vectors were purchased from Clontech (Palo Alto, CA). Each plasmid DNA was propagated in DH5α *Escherichia coli* and purified using an Endo Free Plasmid Maxi Kit (Qiagen, Valencia, CA). The yield and purity of the plasmid DNA was evaluated using an Ultrospec 3300pro spectrophotometer (GE Healthcare, Tokyo, Japan).

Electroporation

For the electroporation experiments, we prepared four kinds of plasmid mixtures that included, pDsRed-Express-N1 and pEGFP-N1, sh-p27a and pEGFP-N1, sh-p27b and pEGFP-N1 and sh-scr and pEGFP-N1. All vectors were diluted in electroporation buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 10 mM glucose, 20 mM Hepes, pH 7.4) to a concentration of 1.5 mg/ml. A CUY21 electroporator (Nepa Gene, Chiba, Japan) was used to transfer the plasmid mixtures into the auditory epithelial cells from the explant cultures. Culture inserts were placed on the lower platinum electrode, with 10 µl of the plasmid DNA solution then applied between them. Eight rectangular pulses (14 V, 50 ms duration at 100-ms intervals) were passed from the upper to the lower electrodes, followed by an additional eight pulses that were applied from the lower to the upper electrodes. Afterwards, explant cultures were maintained in culture medium supplemented with 3 µg/ml BrdU (Sigma-Aldrich, St. Louis, MO) for 1-7 days. The culture medium was changed daily. Ten cochlear epithelia were used for a single culture, with ten independent cultures performed.

Immunohistochemistry

Explants were fixed in 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature, washed with 0.2% Triton-X 100 in PBS (Triton-PBS) and stained as whole mounts. After blocking with 10% normal goat serum in Triton-PBS at 4°C for 60 min, the explants were incubated overnight with the following primary antibodies: anti-mouse p27^{kip1} rabbit polyclonal antibody (1:500; Lab Vision, Fremont, CA), anti-myosin VIIa rabbit polyclonal antibody (1:500; Proteus Bioscience, Ramona, CA), anti-Sox2 goat polyclonal antibody (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Prox1 rabbit polyclonal antibody (1:1000; Chemicon, Temecula CA),

anti-BrdU mouse monoclonal antibody (1:100; Becton Dickinson, Franklin Lakes, NJ), and anti-cleaved caspase-3 mouse monoclonal antibody (1:100; #9661S, Cell Signaling Technology, Danvers, MA). Alexa Fluor 594-conjugated anti-rabbit antibody, Alexa Fluor 633-conjugated anti-mouse, or anti-rabbit antibody (1:500; Invitrogen) was used as the secondary antibody and Vectashield (Vector Laboratories, Burlingame, CA) was used for mounting the samples. For BrdU staining, the specimens were pre-treated in 2N HCl for 20 min at 37°C, and neutralized with 0.01M PBS (pH 8.5) for 10 min at room temperature. To label for both myosin VIIa and p27^{Kip1} simultaneously, a Zenon™ Alexa Fluor 647 Rabbit IgG Labeling Kit (Invitrogen) was used. PI (Invitrogen) was used to stain nuclear chromatin. Images were acquired using a confocal laser scanning system (TCS SP2, Leica Microsystems, Wetzlar, Germany).

Co-transfection efficiency

To examine the efficiency of co-transfecting two different plasmids, we used five explants that were transfected with a mixture of pEGFPN-1 and pDsRed-Express-N1 vectors. To identify the location of the SCs in the cochlear epithelia, the explants were labeled immunohistochemically for p27^{Kip1} 1 day after the electroporation. We then quantified the number of p27^{Kip1}⁺ cells that were also EGFP⁺ and/or DsRed⁺.

Silencing p27^{Kip1} expression in SCs by RNAi

To examine the efficiency of silencing p27^{Kip1} in sh-p27-transfected SCs, explants were transfected with a mixture of sh-scr and pEGFPN-1, sh-p27a and pEGFPN-1 or sh-p27b and pEGFPN-1 2 days prior to being immunolabeled for p27^{Kip1} (n = 5 in each condition). We then quantified the number of p27^{Kip1}-EGFP⁺ and EGFP⁺ cells in the

cochlear epithelia. To examine the location of EGFP⁺ cells in the cochlear epithelium, we counted the number of EGFP⁺ cells in the outer sulcus region, in the organ of Corti and in the inner sulcus region in sh-p27a-transfected specimens.

To confirm that EGFP⁺ and p27^{kip1}-cells were SCs after the knockdown of p27^{kip1}, immunohistochemistry for p27^{kip1} and myosin VIIa or Sox2 was performed in explants 2 days after sh-p27a introduction (n = 5 for myosin VIIa, n = 4 for Sox2). To examine the efficacy of transfection in pillar cells and Deiters' cells, immunostaining for Prox1 was performed in explants 2 days after sh-p27a introduction (n = 7). In order to demonstrate normal distributions of these molecules, we used frozen sections or whole mounts of P3 ICR mouse cochleae after 1 day *in vitro* (n = 10) for the immunohistochemistry controls. At the end of the staining procedures, we used FITC-phalloidin (Invitrogen) to counterstain the specimens.

S-phase reentry of SCs by silencing p27^{kip1}

To examine the effect of silencing p27^{kip1} expression on the S-phase reentry of SCs, we used cochlear explants that were introduced a mixture of sh-p27a (n = 9), sh-p27b (n = 5) or sh-scr (n = 5) and pEGFPN-1 2 days prior to labeling the explants for p27^{kip1} and BrdU. We then counted the number of BrdU⁺ EGFP⁺ cells and EGFP⁺ cells for each group.

Mitosis in SCs induced by RNAi with p27^{kip1}

To examine the effect of p27^{kip1} silencing on SC division, we used cochlear explants that were introduced a mixture of sh-p27a and pEGFPN-1 (n = 10) 2 days prior to making time-lapse recordings of the explants using the BZ-9000 system (Keyence, Osaka,

Japan). In the different explants, still images were captured for 10 EGFP-expressing cells. Immediately after the time-lapse observations were recorded, the specimens were fixed with 4% PFA for 15 min at room temperature, and then stained immunohistochemically for p27^{kip1} and BrdU or with PI.

Fate of SCs after p27^{kip1} silencing

To determine the effect of p27^{kip1} silencing on the survival and differentiation of post-mitotic SCs, mixtures of sh-p27 and pEGFPN-1 or sh-scr and pEGFPN-1 were introduced into cochlear explants at 1, 2, 3, 5 and 7 days (n = 5-10) prior to the counting of the number of EGFP+ or EGFP+, BrdU+ cells.

Six explants into which a mixture of sh-p27a and pEGFPN-1 had been introduced 3 days earlier were labeled immunohistochemically for cleaved caspase 3 and stained with PI in order to investigate the activation of the apoptotic pathways. In addition, we also clarified the caspase 3-dependency of SC death following the silencing of p27^{kip1} expression by examining explants that were cultured in medium supplemented with 100 μ M Caspase-3 inhibitor III (Calbiochem, San Diego, CA) after a mixture of sh-p27a and pEGFPN-1 had been introduced. The numbers of EGFP+ cells after 2-7 days of culturing were counted (n = 5 for each time point).

To examine the trans-differentiation of SCs into hair cells after silencing the p27^{kip1} expression, myosin VIIa was labeled immunohistochemically in explants into which a mixture of sh-p27a and pEGFPN-1 had been introduced 5 or 7 days earlier (n = 5 for each condition). Results were used.

Statistical analysis

The effects of silencing p27^{kip1} expression and inhibiting caspase 3 on the survival of EGFP+ cells in auditory epithelia were examined using a two-way factorial analysis of variance (ANOVA). When an interaction was determined to be significant, pair-wise comparisons were analyzed using the Tukey-Kramer test for multiple comparisons. A one-way factorial ANOVA with the Tukey-Kramer test was used for analysis of differences for the number of EGFP+, BrdU+ cells among cultured periods in cochlear explants that were introduced a mixture of pEGFPN-1 and sh-p27a. The data was presented as the means \pm standard deviations.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a Grant-in-Aid for Researches on Sensory and Communicative Disorders from the Ministry of Health, Labour and Welfare of Japan. We thank Tatsunori Sakamoto and Norio Yamamoto for critical review of this manuscript.

References

- Batts, S.A., Shoemaker, C.R., Raphael, Y., 2009. Notch signaling and Hes labeling in the normal and drug-damaged organ of Corti. *Hear. Res.* 249, 15-22.
- Cafaro, J., Lee, G.S., Stone, J.S. 2007. Atoh1 expression defines activated progenitors and differentiating hair cells during avian hair cell regeneration. *Dev. Dyn.* 236, 156-170.
- Chen, P., Segil, N. 1999. p27 (Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. *Development* 126, 1581-1590.
- Chen, P., Zindy, F., Abdala, C., Liu, F., Li, X., Roussel, M.F., Segil, N., 2003, Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d. *Nat. Cell. Biol.* 5, 422-426.
- Corwin, J.T., Cotanche, D.A., 1988, Regeneration of sensory hair cells after acoustic trauma. *Science* 240, 1772-1774.
- Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T. 2001, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494-498.
- Endo, T., Nakagawa, T., Lee, J.E., Dong, Y., Kim, T.S., Iguchi, F., Taniguchi, Z., Naito, Y., Ito, J., 2002, Alteration in expression of p27 in auditory epithelia and neurons of mice during degeneration. *Neurosci. Lett.* 334, 173-176.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806-811.
- Harper, J.W., 2001, Protein destruction: adapting roles for Cks proteins. *Curr. Biol.* 11, R431-435.

- Hori, R., Nakagawa, T., Sakamoto, T., Matsuoka, Y., Takebayashi, S., Ito, J., 2007, Pharmacological inhibition of Notch signaling in the mature guinea pig cochlea. *Neuroreport* 18, 1911-1914.
- Hume, C.R., Bratt, D.L., Oesterle, E.C., 2007, Expression of LHX3 and SOX2 during mouse inner ear development. *Gene. Expr. Patterns.* 7, 798-807.
- Kawamoto, K., Ishimoto, S., Minoda, R., Brough, D.E., Raphael, Y., 2003, Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. *J. Neurosci.* 23, 4395-4400.
- Kawauchi, T., Chihama, K., Nabeshima, Y., Hoshino, M., 2006, Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration. *Nat. Cell Biol.* 8, 17-26.
- Kirjavainen, A., Sulg, M., Heyd, F., Alitalo, K., Ylä-Herttuala, S., Möröy, T., Petrova, T.V., Pirvola, U., 2008, Prox1 interacts with Atoh1 and Gfi1, and regulates cellular differentiation in the inner ear sensory epithelia. *Dev. Biol.* 322, 33-45.
- Laine, H., Doetzlhofer, A., Mantela, J., Ylikoski, J., Laiho, M., Roussel, M.F., Segil, N., Pirvola, U., 2007, p19(Ink4d) and p21(Cip1) collaborate to maintain the postmitotic state of auditory hair cells, their codeletion leading to DNA damage and p53-mediated apoptosis. *J. Neurosci.* 27, 1434-1444.
- Löwenheim, H., Furness, D.N., Kil, J., Zinn, C., Gültig, K., Fero, M.L., Frost, D., Gummer, A.W., Roberts, J.M., Rubel, E.W., Hackney, C.M., Zenner, H.P., 1999, Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of Corti. *Proc. Natl. Acad. Sci. U S A.* 96, 4084-4088.
- Mantela, J., Jiang, Z., Ylikoski, J., Fritzsche, B., Zacksenhaus, E., Pirvola, U., 2005, The retinoblastoma gene pathway regulates the postmitotic state of hair cells of the

- mouse inner ear. *Development* 132, 2377-2388.
- Oesterle, E.C., Campbell, S., Taylor, R.R., Forge, A., Hume, C.R., 2008, Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. *J. Assoc. Res. Otolaryngol.* 9, 65-89.
- Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J., Conklin, D.S., 2002, Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes. Dev.* 16, 948-958.
- Roberson, D.W., Rubel, E.W. 1994, Cell division in the gerbil cochlea after acoustic trauma. *Am. J. Otol.* 15, 28-34.
- Ruben, R.J., 1967, Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. *Acta Otolaryngol. Suppl.* 220, 221-244.
- Ryals, B.M., Rubel, E.W., 1988, Hair cell regeneration after acoustic trauma in adult *Coturnix* quail. *Science* 240, 1774-1776.
- Sage, C., Huang, M., Karimi, K., Gutierrez, G., Vollrath M.A., Zhang, D.S., García-Añoveros, J., Hinds, P.W., Corwin, J.T., Corey, D.P., Chen, Z.Y. 2005, Proliferation of functional hair cells in vivo in the absence of the retinoblastoma protein. *Science* 307, 1114-1118.
- Stone, J.S., Rubel, E.W. 1999, Delta1 expression during avian hair cell regeneration. *Development* 126, 961-973.
- Sherr, C.J., Roberts, J.M. 1999, CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes. Dev.* 13, 1501-1512.
- Weber, T., Corbett, M.K., Chow, L.M., Valentine, M.B., Baker, S.J., Zuo, J., 2008, Rapid cell-cycle reentry and cell death after acute inactivation of the retinoblastoma gene product in postnatal cochlear hair cells. *Proc. Natl. Acad. Sci. U. S. A.* 105,

781-785.

White, P.M., Doetzlhofer, A., Lee, Y.S., Groves, A.K., Segil, N., 2006, Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. *Nature* 441, 984-987.

Yamamoto, N., Tanigaki, K., Tsuji, M., Yabe, D., Ito, J., Honjo, T., 2006, Inhibition of Notch/RBP-J signaling induces hair cell formation in neonate mouse cochleas. *J. Mol. Med.* 84, 37-45.

Figure legends

Fig 1. Electroporation-mediated co-transfection of two different plasmids into cochlear supporting cells.

Co-expression of EGFP (**a**) and DsRed (**b**) are found in supporting cells, which is identified by p27^{kip1} immunostaining (**c**) 1 day after electroporation (arrows). IS, inner sulcus; OC, organ of Corti; OS, outer sulcus. Scale bar = 30 μ m.

Fig 2. Knockdown of p27^{kip1} expression by RNAi occurred in supporting cells. Expression of myosin VIIa, p27^{kip1} and Sox2 in sections (**a-c**) or in whole-mounts (**d-f**) are shown. Green fluorescence in **a-c** shows phalloidin staining. Asterisks in **b,c** indicate the location of hair cells. In cochlear epithelia transfected with sh-scr (**g,h**), EGFP+ cells (arrowheads) express p27^{kip1}. Cross-section images along with white dotted lines in **g-j** show in **g'-j'**. In cochlear epithelia transfected with sh-p27a (**i-m**), EGFP+ cells (arrows) are negative for p27^{kip1}. Double-labeling for myosin VIIa and p27^{kip1} demonstrates the location of a transfected cell (arrow in **i'**) corresponding to the supporting cell. Double-labeling for Sox2 and p27^{kip1} (**k-m**) demonstrates the expression of Sox2 in EGFP+, p27^{kip1}- cells (arrows), indicating that p27^{kip1} silencing occurs in supporting cells. A part of EGFP+, p27^{kip1}- cells lacks Sox2 expression (arrowhead in **k-m**). Myo7a, Myosin VIIa; IS, inner sulcus; OC, organ of Corti; OS, outer sulcus. Scale bars = 30 μ m.

Fig. 3. Transfection in Prox1-positive supporting cells.

In normal P3 cochlear epithelia, Prox1 expression (red) is seen in pillar cells and

Deiters' cells (**a**). Green fluorescence in **a** shows phalloidin staining (Pha), and blue shows nuclear staining with DAPI. Asterisks in **a** indicate the location of hair cells. Some EGFP+ cells exhibit Prox1 expression (arrows in **b-d**), while other EGFP+ cells in the outer sulcus are negative for Prox1 (arrowheads in **b-d**). IS, inner sulcus; OC, organ of Corti; OS, outer sulcus. Scale bars = 20 μ m.

Fig. 4. Supporting cells silenced $p27^{kip1}$ by RNAi reenter S-phase.

In cochlear epithelia following introduction of sh-scr, EGFP+ cells exhibit expression of $p27^{kip1}$ and are negative for BrdU (arrowheads in **a-d**). In cross-section images (**a'-d'**) along with white dotted lines in **a-d**, a cell located underneath the cochlear epithelium shows BrdU incorporation (asterisk). In cochlear epithelia following introduction of sh-p27a (**e-h**) or sh-p27b (**i-l**), some of EGFP+, $p27^{kip1}$ - cells exhibit BrdU incorporation (arrows). Other EGFP-positive cells (arrowheads in **e-h**) show silencing of $p27^{kip1}$, but no incorporation of BrdU. IS, inner sulcus; OC, organ of Corti; OS, outer sulcus. Scale bar = 30 μ m.

Fig. 5. Mitosis in supporting cells silenced $p27^{kip1}$ by RNAi.

Time-lapse observation demonstrates division of sh-p27a-transfected supporting cells (**a,b**). A dividing cell expressing EGFP is positive for BrdU, but not for $p27^{kip1}$ (arrows in **c,d**). Cross-section images along with white dotted lines in **c,d** show migration of a BrdU+ nucleus to the luminal surface of the supporting cell (arrowheads in **c',d'**). An EGFP+ supporting cell in the outer sulcus region exhibits the segregation of chromosomes (arrowheads in **e,f**). PI, Propidium iodide, IS, inner sulcus; OC, organ of Corti; OS, outer sulcus. Scale bar = 30 μ m.

Fig. 6. Effects of silencing p27^{kip1} and cell-cycle reentry on transfected supporting cell survival.

The surviving EGFP⁺ cell numbers in sh-scr-transfected explants (black column) show no significant alteration among culture periods, while those in sh-p27a-transfected explants (white column) significantly decreased over time. Significant decreases in numbers EGFP⁺, BrdU⁺ cells that have reentered cell-cycle, in sh-p27a-transfected explants (grey column) are observed on days 3-7 in comparison with day 2. Asterisks indicate statistical significance with the Tukey-Kramer test. Bars represent standard deviation.

Fig. 7. Activation of caspase 3 in supporting cells that had been silenced p27^{kip1} in the outer sulcus region.

The expression of cleaved caspase 3 is found in sh-p27a-transfected supporting cells (a). PI staining exhibits the fragmentation of nuclear chromatin in sh-p27a-transfected supporting cells expressing EGFP (b). Scale bar = 10 μ m.

Fig. 8. Effect of a caspase 3 inhibitor on the number of surviving supporting cells following p27^{kip1} silencing.

Closed squares show the numbers of EGFP⁺ cells in sh-p27a-transfected explants cultured without a caspase 3 inhibitor, and open circles show those with a caspase 3 inhibitor. An overall effect of application of a caspase 3 inhibitor is significant at $p < 0.001$ with two-way factorial ANOVA. No significant differences in the numbers of EGFP⁺ cells were identified with pair-wise comparisons with Tukey-Kramer test. Bars

represent standard deviation.

SUPPLEMENTARY MATERIAL

S1. Time-lapse observation showing division of supporting cells that had been transfected with sh-p27a and pEGFPN-1 two days earlier. Moving images were recorded every 30-second for 15 min. Scale bar = 30 μm .















