Hearing Research 373 (2019) 10–22

Contents lists available at ScienceDirect

Hearing Research

journal homepage: www.elsevier.com/locate/heares

Research Paper

p27^{Kip1} down-regulation as achieved by two clinically feasible means did not induce proliferation of supporting cells in the rat neonatal cochlea *in vivo*

Sebastián A. Silva [a], Juan C. Maass [a, b, *]

[a] Department of Otolaryngology, Hospital Clínico Universidad de Chile and Interdisciplinary Program of Physiology and Biophysics, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, Universidad de Chile, Av. Independencia 1027, 8380453, Independencia, Santiago, Chile
[b] Department of Otolaryngology, Clínica Alemana de Santiago, Facultad de Medicina Clínica Alemana-Universidad del Desarrollo, Av. Vitacura 5951, 7650568, Vitacura, Santiago, Chile

ARTICLE INFO

Article history:
Received 13 October 2017
Received in revised form
27 November 2018
Accepted 4 December 2018
Available online 6 December 2018

Keywords:
Inner ear
Labyrinth supporting cells
Cell proliferation
cdkn1b protein
shRNA
Alsterpaullone

ABSTRACT

In mammals, the cochlear sensory epithelium becomes quiescent early during development. After the first postnatal week, there is no cell replacement or proliferation, and severe damage leads to permanent deafness. Supporting cells' trans-differentiation has been suggested as a way to regenerate cochlear hair cells after damage. However, they are also needed for proper functionality. Cdkn1b (p27^{Kip1}) participates in the cochlear terminal mitosis state achieved during development. Its expression is maintained in adult supporting cells and its postnatal deletion has induced cochlear proliferation *in vitro* and *in vivo*. Therefore, its manipulation has been proposed as a feasible way to induce proliferation of supporting cells after birth. Nevertheless, the literature is scarce regarding feasible methods to directly decrease p27^{Kip1} in the clinical domain. The effects of p27^{Kip1} knockdown using viral vectors are not completely elucidated and no pharmacological approaches to decrease p27^{Kip1} in the cochlea have been tested *in vivo* before. This study explores the ability of p27^{Kip1} messenger knockdown and pharmacological transcriptional inhibition to induce proliferation of supporting cells in the P0 neonatal rat cochlea *in vivo*. Respectively, lentiviral vectors transducing shRNA against p27^{Kip1} were administered into the *scala media* or Alsterpaullone 2-Cyanoethyl into the round window niche. Cell markers and gene expression were assessed through immunostaining and qRT-PCR. Despite both methods significantly decreasing p27^{Kip1} expression *in vivo*, signs of toxicity in the organ of Corti were not found; however, relevant proliferation was not found either. Finally, cochlear damage was added to increase the response *in vitro*, achieving only a mild to moderate proliferation induction. We conclude that our approaches were not able to stimulate the recall of supporting cell proliferation despite significantly decreased p27^{Kip1} levels *in vivo*. Considering the evaluation of the cochlea at a very responsive stage, we propose that the level of isolated modification of p27^{Kip1} expression in living mammals achievable through these approaches is insufficient to induce proliferation of supporting cells. Future proliferation induction experiments in the cochlea should study other methods and genes.

1. Introduction

Hearing loss is a huge global health problem. It affects most of the population over the age of 75, as well as a significant proportion of young people (Cruickshanks et al., 1998; Erenberg et al., 1999). Considering the overall cost of this disability, it is a heavy economic burden for most countries in the world. Globally, the annual cost of unaddressed hearing loss has been estimated between 750 and 790 billion dollars (Olusanya et al., 2014; World Health Organization, 2017). The currently available treatments are expensive, and they do not directly assess the cochlear damage (Bishop and Eby, 2010; Manchaiah et al., 2017; Wilson, 2017; World Health Organization, 2017).

Like all other mammals, we are born with a limited number of

* Corresponding author. Department of Otolaryngology, Hospital Clínico Universidad de Chile and Interdisciplinary Program of Physiology and Biophysics, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, Universidad de Chile, Av. Independencia 1027, 8380453, Independencia, Santiago, Chile.
E-mail addresses: juanmaass@uchile.cl, jmaass@alemana.cl (J.C. Maass).

https://doi.org/10.1016/j.heares.2018.12.002
0378-5955/

cells within the organ of Corti. During early intrauterine development, the progenitors stop proliferating and differentiate (Ruben, 1967). Subsequently, functional cells involved in auditory mechanisms can decrease in number after damage, resulting in hearing loss (Merchant et al., 2010). However, other vertebrates like birds and fishes continuously experience cell proliferation and replacement of their hearing epithelium and can spontaneously regenerate their sensory cells (Corwin and Cotanche, 1988; Harris et al., 2003; Ryals and Rubel, 1988).

During the last 20 years significant breakthroughs have been achieved in the field of cochlear regeneration. The genetic manipulation of developmental genes has shown that there is regenerative potential in the mammalian organ of Corti. Trans-differentiation and proliferation of cochlear cells have been successfully induced in young mammals *in vitro* and *in vivo* (Izumikawa et al., 2005; Löwenheim et al., 1999; Mizutari et al., 2013; Oesterle et al., 2011; Sage et al., 2005; Walters et al., 2014a; White et al., 2006). Nevertheless, the degree of the response has been significantly reduced in older animals (Maass et al., 2015; Oesterle et al., 2011; White et al., 2006). Maturation apparently makes the hearing epithelium progressively unresponsive to regeneration induction, and unfortunately that is precisely when hearing loss becomes more frequent with age (Maass et al., 2016).

In the organ of Corti, the sensory cells—the hair cells—are the cell type that is more sensitive to damage. Trans-differentiation of supporting cells into hair cells has traditionally been the main goal of cochlear regeneration. However, it should not be considered the only purpose of the field, since both hair cells and supporting cells are necessary for normal cochlear physiology, and trans-differentiation necessarily decreases the number of supporting cells (Cai et al., 2013; Gubbels et al., 2008; Hori et al., 2007; Izumikawa et al., 2005; Kawamoto et al., 2003; Woods et al., 2004). Moreover, during regeneration experiments persistence of supporting cells is important to preserve the regenerative potential. Although the regeneration obtained is higher when some degree of hair cell damage is induced, no regeneration is achieved in severely damaged cochlear epithelia that are devoid of supporting cells (Izumikawa et al., 2005, 2008; Kawamoto et al., 2003; Mizutari et al., 2013; Raphael et al., 2007). Therefore, it is as important to have a means for increasing the number of supporting cells as it is having tools for producing more hair cells.

$p27^{Kip1}$ protein, or CDKN1B, is a cyclin-dependent kinase inhibitor that blocks the cell cycle progression from G1 to S. During development, a wave of $p27^{Kip1}$ expression precedes the cell cycle exit in the inner ear pro-sensory domain (Chen and Segil, 1999; Löwenheim et al., 1999; White et al., 2006). From there, its expression is preserved in the cochlear supporting cells until adulthood. Thus, this gene is believed to be one of the main factors responsible for preserving the quiescent state in the mature organ of Corti. We and others have successfully manipulated the $p27^{Kip1}$ messenger levels to induce proliferation of supporting cells in postnatal rodents (Maass et al., 2013; Oesterle et al., 2011; Ono et al., 2009). However, the responses obtained have been variable, and the direct modification of the $p27^{Kip1}$ messenger levels has not been tested *in vivo* in animal models through practicable methods in humans, which raises questions about the actual importance of decreasing $p27^{Kip1}$ for regenerative therapies.

In theory, to induce proliferation and end up with a normal ear, the effect should be transitory. The knockout mice of $p27^{Kip1}$ shows persistent proliferation in the organ of Corti, along with morpho-functional abnormalities (Chen and Segil, 1999; Kanzaki et al., 2006; Löwenheim et al., 1999; Oesterle et al., 2011). There are many ways to induce a decrease in gene expression, but fewer that could be theoretically delivered to patients and that are capable of inducing a transitory effect. In our previous study, we used $p27^{Kip1}$-shRNA (short hairpin RNA) lentiviral vectors to significantly decrease $p27^{Kip1}$ expression, induce cell proliferation and increase the number of supporting cells in newborn cochlear cultures (Maass et al., 2013). However, we did not test the effects *in vivo*. Recently, Alsterpaullone 2-Cyanoethyl (A2CE) was described as a potent $p27^{Kip1}$ transcriptional inhibitor; nonetheless, its capacity to induce cochlear supporting cell proliferation has not been studied (Walters et al., 2014b). Conceptually, both strategies can be performed *in vivo* in animals and patients (Izumikawa et al., 2005; Raphael et al., 2007; Goycoolea et al., 1988; Mizutari et al., 2013). We believe that studying the effects of these methods in living rodents could give a pre-clinical hint into the usefulness of targeting the $p27^{Kip1}$ messenger in the inner ear of humans. Therefore, we conducted an *in vivo* study to evaluate whether any of these clinically feasible approaches could induce proliferation and increase the number of supporting cells in healthy or damaged rat neonatal cochleas at the moment when the regenerative potential is supposed to be highest.

2. Material and methods

2.1. Experimental animals

Postnatal day zero (P0) Sprague Dawley rats were obtained from the Central Animal Facility of the Faculty of Medicine of Universidad de Chile. After the experimental procedures, the animals were euthanized or kept with their mothers in the animal station of the Interdisciplinary Program of Physiology and Biophysics of the Institute of Biomedical Sciences (ICBM) of the Faculty of Medicine of Universidad de Chile. All animal procedures were approved by the Faculty of Medicine of Universidad de Chile Bioethics Committee concerning the protocols number 585 and 992 and were conducted following the NIH guide for the care and use of laboratory animals.

2.2. Lentiviral vectors

Second generation lentiviral vectors, including silencing plasmids $p27^{Kip1}$-shRNA LV and control-shRNA LV were used, both of which have been previously described in the study conducted by Maass et al. (2013). The vectors were prepared in HEK293T cells, collected and concentrated by ultracentrifugation according to the protocol by Sena-Esteves et al. (2004). The lentiviral concentration used fluctuated between 2 and 5×10^6 Vp/μl, which was determined by flow cytometry, as described in the study conducted by Logan et al. (2004). All viral vectors procedures were performed under the institutional bio-safety guidelines of the Faculty of Medicine of Universidad de Chile.

2.3. Cochlear cultures

Cochlear explants were collected from P0 rats as described earlier (Maass et al., 2013). Briefly, the heads were bisected, the temporal bones were extracted and the otic capsules were removed to obtain the membranous cochlea, from which the Reissner's membrane, *Modiolus* and lateral wall were collected. The remnant cochlear epithelia were cultured on Nuclepore Track Etch Membranes (Whatman, Maidstone, UK) floating on DMEM/F-12 medium (buffered with Hepes; Thermo Fisher Scientific, Waltham, MA) supplemented with B27 (Thermo Fisher Scientific), 1 mM N-acetylcysteine (Sigma-Aldrich, St.Louis, MO), 5 ng/ml EGF (Thermo Fisher Scientific), 2.5 ng/ml FGF2 (Thermo Fisher Scientific) and 67 μg/ml penicillin (Laboratorio Chile, Santiago, Chile) in a 37 °C humidified incubator with 5% CO_2. For $p27^{Kip1}$ transcriptional inhibition experiments, the cultures were treated with 5 and 10 μM Alsterpaullone 2-Cyanoethyl

(A2CE; Calbiochem EMD, Burlington, MA) or 0.04% v/v DMSO (vehicle control; Thermo Fisher Scientific) for 24 h for mRNA level determination or for 72 h for immunostaining. For damage experiments, the explants were cultured for three to four days *in vitro*, completing two initial days in 320 μM of Gentamicin (Laboratorio Chile) and one to three final days in A2CE 10 μM or vehicle.

2.4. In vivo experiments

P0 pups were anesthetized using hypothermia. The skin on the neck posterior and inferior to the left ear was disinfected with 70% EtOH and incised. The retro-auricular fat and the muscles were dissected toward the *bulla*. The *bulla* was opened from its inferior aspect, the *annulus* was displaced laterally, and the middle ear mucosa was removed from the promontory. The stapedial artery, round window and basal turn of the cochlea were identified. This was followed by treating the rat pups with a *scala media* injection or a round window drug administration, according to the specific phase of the study.

2.4.1. Scala media injection

The *scala media* at the basal turn of the cochlea was punctured by transparency with a Bonn micro-probe, and 0.6 μl of lentiviral vectors were injected through a 0.0045″ ID and 0.0005″ wall polyimide micro-cannula (A-M Systems, Sequim, WA) at 0.4 μl/min. After the injection, a plug of muscle was placed in the middle ear.

2.4.2. Round window treatment

1 mm^3 of Surgispon (Aegis Lifesciences, Gujarat, India) gelfoam embedded in 20 μM or 1 mM Alsterpaullone 2-Cyanoethyl (A2CE; Calbiochem EMD) was placed over the round window niche.

After both of these procedures were completed, the *annulus* and the muscles were re-positioned, and the skin was closed with Histoacryl (B. Braun, Melsungen, Germany). The pups recovered in a heating pad and were kept with their respective mother for four days after the lentiviral vector injections and for three to eight days after the A2CE administrations.

For the BrdU incorporation experiments, the pups received an intra-peritoneal injection one day before euthanasia, according to the instructions of the manufacturer (Thermo Fisher Scientific).

After the completion of the *in vivo* experiments, the pups were sacrificed, and their ears were dissected for surface preparations of the hearing epithelium (see dissection in Cochlear cultures section). These explants were cultured in supplemented DMEM/F-12 medium for 1 h to allow adherence to the Nuclepore Track Etch Membranes (Whatman).

2.5. Immunostaining

The cochleas obtained from *in vivo* or *in vitro* experiments were washed in the membranes with PBS and fixed in 4% PFA for 1 h at 20 °C. Subsequently, they were washed in PBS, permeabilized and blocked in PBS containing 0.2% TritonX-100 and 10% donkey serum. Then, they were incubated with primary antibodies overnight at 4 °C, washed in PBST (0.1% TritonX-100 in PBS) and incubated with secondary antibodies for 2 h at room temperature. Finally, the explants were treated with Hoechst 33258 (Thermo Fisher Scientific) for nuclear staining or Rhodamine-Phalloidin (Phalloidin; Thermo Fisher Scientific) for actin filaments staining. The primary antibodies were anti-Myosin VI (MYO6; 25–6791, Proteus Biosciences), anti-$p27^{Kip1}$ (DCS-72.F6, Thermo Fisher Scientific), anti-SOX2 (SOX2; AB5603, Millipore), anti-cleaved Caspase 3 (c-CASP3; D175, Cell Signaling), anti-BrdU (ab6326, AbCam), anti-KI67 marker of proliferation (MKI67; AB9260, Chemicon), anti-proliferating cell antigen (PCNA; M0879, Agilent Dako) and anti-GFP (Thermo Fisher Scientific). The secondary antibodies were Rhodamine Red-X, Cy5 and FITC (Jackson ImmunoResearch). All the antibodies were used at 1:200. For $p27^{Kip1}$ and PCNA immunostainings, the samples were boiled for 10 min in 10 mM citric acid (pH 6) for epitope retrieval. For BrdU staining, the samples were treated with 2N HCl for 30 min. The images were obtained using a BX61WI DSU Olympus microscope and analyzed with ImageJ 139u (NIH) and CellR 2.7 (Olympus). The experiments were performed and analyzed in three to eight biologic replicates. Whole cochlear explants were evaluated unless specified. The stained cells were manually counted, and the counts were normalized to 100 μm. The percentages of positive cells were calculated using the number of positive cells for each evaluated stain within the total number of cells in the evaluated region. The quantification of the relative mean fluorescence intensity obtained after $p27^{Kip1}$ protein immunostaining was performed using ImageJ 139u (NIH) for at least six randomly selected cells in three different experiments. The pictures used for this purpose were taken under the same conditions, and the brightness and contrast settings were adjusted to the closest possible values. Supporting cells were identified by SOX2 expression, $p27^{Kip1}$ expression and position in Hoechst staining. The supporting cell counts were limited to phalangeal, pillar, Deiters', Hensen's and the closest two rows of the Claudius cells.

2.6. qRT-PCR

Per replicate, total RNA from four whole cochlear explants was extracted using the PureLink RNA Micro kit (Thermo Fisher Scientific), and cDNA was synthesized using random primers with the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). The number of biologic replicates used were three to four. SYBRGreen qPCR Master Mix was used for qRT-PCR reactions in the StepOne Real Time PCR System (Applied Biosystems). *Gapdh* forward 5′-CTCTCTGCTCCTCCCTGTTC-3′ and reverse 5′-GCCAAATCCGTTCACACC-3′, and $p27^{Kip1}$ forward 5′-GGAAGCGACCTGCGGCAGAA-3′ and reverse 5′-GCCAGCATTCGGGGAACCGT-3′ primers were used. The analysis of the relative gene expression levels was performed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

Statistical tests were performed using the Past 3.15 software (Hammer and Harper). Parametric or non-parametric tests were performed according to the distribution, variance, sample size and the characteristics of the variables included in the data. Therefore, Mann-Whitney and Kruskall-Wallis tests for independent samples were used for assessing the differences between the groups when one independent variable was used. When the effect of two factors or independent variables were evaluated, the two-way analysis of variance (ANOVA) test was used for comparison. For pairwise comparisons within the samples, Mann Whitney, and Dunn's post-hoc analysis with Benjamini-Hochberg procedure for 0.1 false discovery rate control were used. Assumptions of normality and homoscedasticity required for each statistical test were evaluated using visualization of the data, Shapiro-Wilk, F and Levene's tests. A p-value < 0.05 was significant. For non-normally distributed data, the box plots including the median and interquartile range or the whole data set were presented as descriptors. Medians, interquartile ranges (IQR) and ±1.5 IQR whiskers in the box plots were calculated using the interpolation method. Arbitrarily, when the sample size was 3, the dot box was used for data representation.

2.8. Research data repository

Raw data obtained and used in this research was included in

Mendeley's repository as suggested by Elsevier.

3. Results

3.1. Lentiviral vectors were capable of transducing supporting cells in the neonatal rat cochlea in vivo

As mentioned earlier, we previously found that a $p27^{Kip1}$-shRNA knockdown mediated by lentiviral vectors can induce a mild proliferation of the cochlear supporting cells *in vitro* (Maass et al., 2013). To confirm and extend these findings *in vivo*, we tested the abilities of our vectors to transduce the cochlear epithelium in pups after injecting them through the *scala media*. Concentrated lentiviral vectors were administered to P0 rats, which were then analyzed at P4 by EGFP immunostaining as a reporter of transduction (Fig. 1A–E). To assess the efficiency of our gene transfer, the percentage of transduction was calculated as the number of EGFP positive cells divided by the total number of cells in the evaluated fields per region and per cell type (Fig. 1D). The basal portion of the cochlea located near the puncture site was found to be the segment that achieved the highest transduction efficiency, and from that point, the number of transduced cells decreased toward the apex. In the basal segment of the organ of Corti, 6–8% of supporting cells and 12% of hair cells were transduced per given field, but no cells were transduced in the apex. All cell types in the organ of Corti were transduced except the phalangeal cells. We also evaluated the transduction efficiencies beyond the organ of Corti. Interestingly, the cells located in the Reissner's membrane and the *Stria vascularis* showed higher transduction efficiencies than in the sensory epithelium (Fig. 1C and D). In the region of the cochlear duct closer to the spiral ganglion, a high percentage of EGFP + cells were also found. However, in the spiral ganglion neurons, only 20% transduction was found after co-labeling with Beta 3 tubulin (TUBB3). We did not find significant transduction efficiency differences between control and $p27^{Kip1}$-shRNA lentiviral vectors (Fig. 1A–D). Thus, our vectors transduced the organ of Corti through *scala media* injection, which made them suitable for exploring the effects of $p27^{Kip1}$-shRNA transduction *in vivo*.

3.2. $p27^{Kip1}$-shRNA transduced by lentiviral vectors decreased $p27^{Kip1}$ protein but did not induce supporting cell proliferation in the neonatal rat cochlea in vivo

After evaluating the transduction feasibility, we evaluated whether our vectors were capable of significantly decreasing the $p27^{Kip1}$ protein *in vivo* in the organ of Corti. Therefore, four days after the injection, $p27^{Kip1}$ expression was explored by immunostaining (Fig. 2A). There was a moderate, but significant, decrease in the relative fluorescence corresponding to the $p27^{Kip1}$ protein expression in the $p27^{Kip1}$-shRNA treated EGFP positive cells (Fig. 2B). This indicated that our vectors could induce a decrease in the $p27^{Kip1}$ protein *in vivo*. We also noticed that the difference was greater and more significant when comparing these EGFP positive cells to the control-shRNA treated cells, rather than to the $p27^{Kip1}$-shRNA treated EGFP negative cells.

Since there are some concerns about the safety of decreasing the $p27^{Kip1}$ messenger (Kanzaki et al., 2006; Oesterle et al., 2011), and because it might be possible that a part of the protein decrease could be explained by cellular damage, we evaluated the cochlear structure and the presence of cleaved Caspase 3 (c-CASP3) by immunostaining. Within 4 days of the exploration period of our study, no gross morphology defect or c-CASP3 positive cell was found in the organ of Corti (Fig. 2C). Only some c-CASP3 positive cells were found under and nearby the organ of Corti of treated ears and controls. These findings led to the conclusion that there was no evidence of toxicity with our approach.

Finally, we explored whether the decrease in the $p27^{Kip1}$ messenger levels obtained with our lentiviral vectors was enough to induce proliferation and consecutively to increase the number of supporting cells *in vivo*. Initially, BrdU incorporation was assessed by immunostaining after a single intra-peritoneal injection in the treated and control animals at P4. There were no BrdU positive cell within or nearby the organ of Corti, despite ten replicates and the finding of BrdU positive cells in distant areas of the same explants (Fig. 2D). A single pulse was used, rather than multiple pulses or continuous BrdU administration, to strictly assess the proliferating cells at the time when the knockdown should already be effective and to skip prior developmental proliferation. Then, we explored the cell cycle progression in the treated organs by assessing the proliferation marker MKI67. Theoretically, this marker should be expressed in most of the proliferating stages of the cell cycle, omitting quiescent G0 cells. However, there were not any MKI67 positive cells, nor a mitotic figure in the organ of Corti, across the entire explants obtained from eight cochleas treated with the $p27^{Kip1}$-shRNA lentiviral vectors or their controls *in vivo*, although we obtained MKI67 positive cells immediately below the organ of Corti and in other areas of the explants (positive control of proliferation in our *in vivo* model; Fig. 2E). Moreover, the relative number of supporting cells and hair cells did not increase in the $p27^{Kip1}$-shRNA treatment relative to its control (Fig. 2F and G). Despite the fact that some proliferation could have been missed with these assessments, the results suggest that this method to decrease $p27^{Kip1}$ was not strong enough for inducing a significant proliferative response *in vivo* under our conditions.

3.3. A transcriptional inhibitor of $p27^{Kip1}$ significantly decreased the $p27^{Kip1}$ levels in the neonatal rat cochlea in vitro and in vivo

Considering the more diffuse and efficient *in vitro* transduction experiments shown in our previous study concerning these vectors (Maass et al., 2013), and the finding that the transduction obtained in this *in vivo* study was more limited to some cells and areas of the cochlea, we sought for a more extensive way to decrease the cochlear $p27^{Kip1}$ levels *in vivo*. A few years ago, Walters et al. (2014) performed a drug screen and reported that Alsterpaullone 2-Cyanoethyl (A2CE) was a potent $p27^{Kip1}$ transcriptional inhibitor. Thus, to see whether decreasing the $p27^{Kip1}$ messenger levels *in vivo* in a wider portion of the organ of Corti and in a higher proportion of cells would lead to a more significant proliferation response, we tested A2CE *in vitro* and *in vivo*. To check that A2CE could decrease the $p27^{Kip1}$ messenger levels, cochlear cultures were incubated in 5 and 10 μM A2CE for 24 h. Immunostaining and qRT-PCR results showed a significant decrease in the $p27^{Kip1}$ protein expression and $p27^{Kip1}$ messenger levels (Fig. 3A–C). Next, to reproduce these results *in vivo*, 1 m^3 gelfoams soaked in 20 μM or 1 mM A2CE were placed in the round windows of P0 neonatal rats. This caused a significant decrease in the $p27^{Kip1}$ levels at the highest concentration used, along with no evidence of damage in the immunostainings (Fig. 3D–G). Therefore, treatment of the middle ear with A2CE was successful in decreasing the $p27^{Kip1}$ levels in the organ of Corti *in vivo*.

3.4. Alsterpaullone did not induce relevant supporting cell proliferation in the neonatal rat cochlea, even in the presence of cochlear damage

Next, we tested the ability of A2CE to induce proliferation in the cochlea *in vitro* and *in vivo*. Cochlear explants were incubated in 10 μM A2CE for three days, while P0 neonate ears were treated with 1 mM A2CE gelfoams to analyze them on the third day of life. In

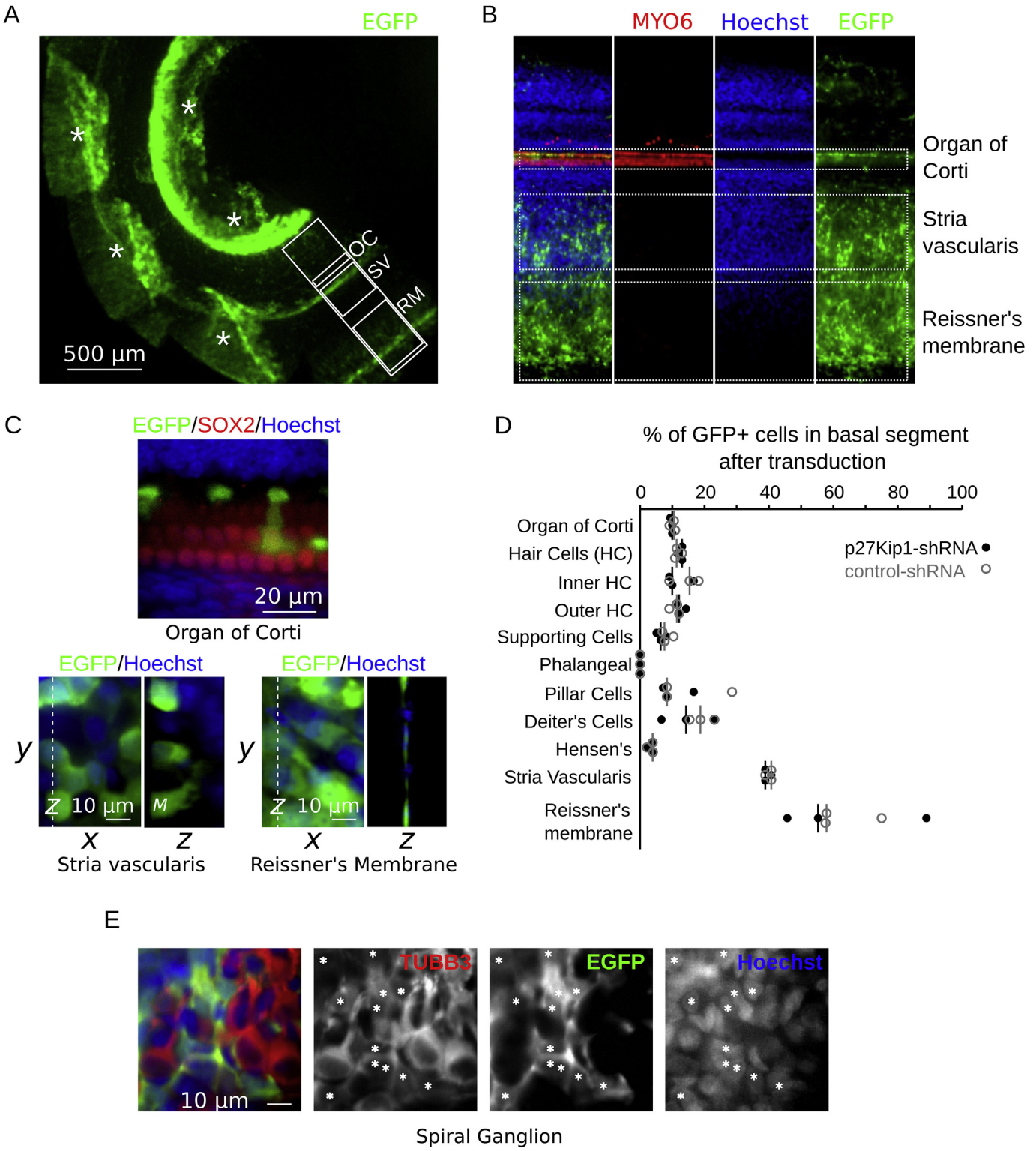

Fig. 1. *In vivo* transduction of neonatal cochlear epithelium with lentiviral vectors. P4 neonatal cochlear explants four days after the *in vivo scala media* injection of control-shRNA and $p27^{Kip1}$-shRNA lentiviral vectors at P0. A. The whole basal segment of a cochlear duct transduced with control-shRNA. In rectangles, the approximated regions of a part of another explant, shown in B. OC: organ of Corti. SV: *stria vascularis*. RM: Reissner's membrane. *: RM bent over the cochlear explant. EGFP: green and reporter of transduction. B. Portion of a basal segment transduced with control-shRNA lentiviral vectors. Most of the spiral ganglia was removed during dissection. EGFP: green. Hoechst: blue. MYO6: red. The dotted rectangles show the approximate areas corresponding to the organ of Corti, the *stria vascularis* and the Reissner's membrane. C. Some cell types transduced by control-shRNA lentiviral vectors. Top: Supporting cells transduced in the organ of Corti. Bottom left: *Stria vascularis* marginal Cells (*M*). Bottom right: Reissner's membrane. EGFP: green. Hoechst: blue. SOX2: red. The dashed lines in left panels show the location of the Z plane's virtual slice that is presented in right panels. D. The percentage of EGFP + cells per cell type and region after transduction with lentiviral vectors. Gray circles: % after control-shRNA transduction. Black dots: % after $p27^{Kip1}$-shRNA transduction. Vertical lines indicate the median for each case, n = 3. There were no significant differences between control and $p27^{Kip1}$-shRNA conditions ($p = 0.8524$ for both; two-way ANOVA). The percentages of transduction were significantly higher in the *stria vascularis* and in Reissner's Membrane cells ($p = 0.00000$ for both; two-way ANOVA). E. Transduced cells in the spiral ganglion. Left panel: merged pseudo-colored images shown in the right panels. TUBB3: red. EGFP: green. Hoechst: blue. Right panels from left to right: TUBB3, EGFP and Hoechst in gray. Stars mark a few transduced cells.

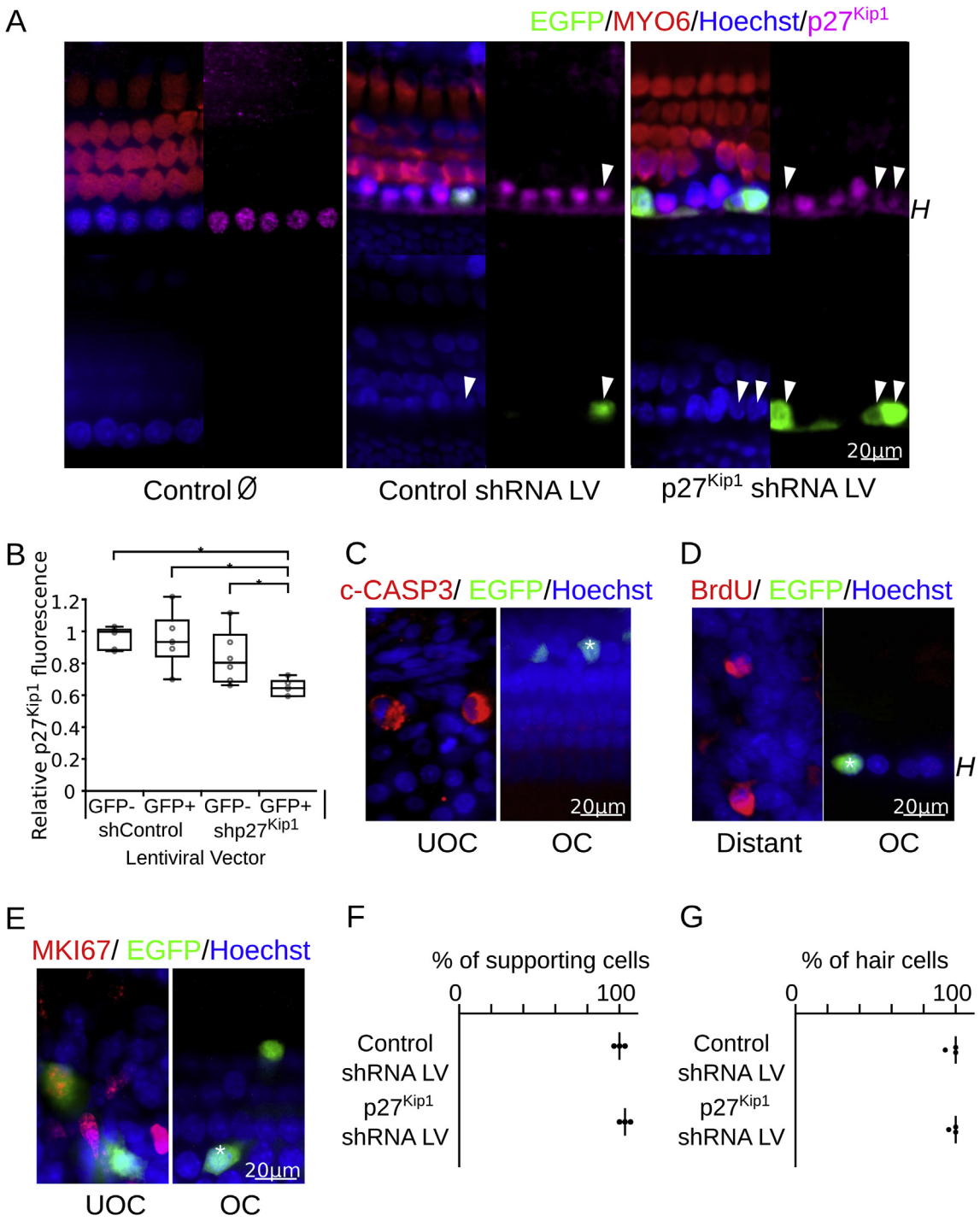

Fig. 2. The effects of p27^{Kip1} knockdown in neonatal rat cochleas using p27^{Kip1}-shRNA lentiviral vectors *in vivo*. A. p27^{Kip1} expression in the basal segments of P4 cochlear explants transduced by p27^{Kip1} or control-shRNA lentiviral vectors *scala media* injection at P0. Left: non-transduced explant. Center: explant transduced by p27^{Kip1}-shRNA lentiviral vectors. Right: explant transduced by control-shRNA. EGFP: green and reporter of transduction. p27^{Kip1}: magenta. Hoechst: blue. MYO6: red. The panels are divided to show the green, blue and magenta channels separately, along with the merged channel in the upper right corner of each panel. Arrow heads show transduced cells. Hensen's Cells (*H*). B. Relative p27^{Kip1} fluorescence in transduced or non-transduced cells by p27^{Kip1} or control-shRNA, n = 6 per condition. Line: median calculated by interpolation. Empty gray dots: the data set. Boxes: inter quartile range (IQR). Error bars: ± 1.5 IQR. $p = 0.00426$ using the Kruskal-Wallis test. *: $p = 0.00499$, 0.00812 and 0.02002, respectively, from left to right, using Mann-Whitney pairwise comparison. Benjamini-Hochberg procedure was used to assign significance. C, D and E. Respectively, c-CASP3, BrdU and MKI67 labeling in cochlear explants transduced *in vivo* by p27^{Kip1}-shRNA lentiviral vectors in the organ of Corti (OC), under it (UOC) or distant from it. c-CASP3, BrdU or MKI67: red. EGFP: green. Hoechst: blue. Stars show transduced supporting cells. F and G. Relative number of supporting and hair cells after p27^{Kip1}-shRNA or control-shRNA, respectively. The percentages relative to the median in the control condition are shown. The plot shows the actual data set in dots and the medians in vertical lines, n = 3 per group. $p = 0.36869$ and 1, respectively (Mann-Whitney test).

both experiments, we did not find any MKI67 positive cells in the organ of Corti, which would have been an indication of proliferation in the treated or control groups (Fig. 3H). Moreover, after using A2CE, the number of hair cells or supporting cells did not increase relative to the control group (Fig. 3I and J). Additionally, to see whether other proliferation markers and further time points would add a more manifest effect, we performed MKI67-p27^{Kip1} immunostaining on the fourth and eight days of life and PCNA-SOX2 immunostaining on the fourth day of life after a single *in vivo* application of A2CE at P0. Despite the significant decrease that was detected in the p27^{Kip1} expression in the basal segment of the cochlea at P4 (Fig. 3K), there were not any proliferating cells in the whole cochlea, nor was there a significant increase in the number of supporting cells either (Fig. 3L–M).

Finally, considering these cumulative negative results, we explored the effects of adding cochlear damage as a factor to induce proliferation. Damage triggers inner ear regeneration in other vertebrates (Corwin and Cotanche, 1988), and most of the relatively successful attempts to induce trans-differentiation *in vivo* have included cochlear damage (Izumikawa et al., 2008, 2005; Kawamoto et al., 2003; Mizutari et al., 2013). Thus, mild to moderate damage to the cochlear epithelium *in vitro* was induced prior to the reduction of p27^{Kip1} expression. For that purpose, cochlear explants were cultured in 320 μM Gentamicin for the initial 48 h and in 10 μM A2CE for the final 24 h to find evidence concerning apoptosis and proliferation by immunostaining (Fig. 4A–C). Interestingly, few proliferating cells were found only in the cochlear cultures that were treated with A2CE and Gentamicin; however, no significant increase in the number of SOX2 positive cells after the treatment was found (Figs. 4B and 5). To see if a longer incubation with A2CE plus damage would induce a stronger proliferative response, two additional sets of experiments were prepared *in vitro* by modifying the A2CE incubation length and by keeping the initial 48 h Gentamicin treatment. In the first set, one additional day in A2CE was included by completing a total of 48 h of p27^{Kip1} inhibition after Gentamicin incubation. In the second set, the cochleas were cultured in A2CE for 72 h or the entire experiment (Fig. 4C). With the first approach, no signs of proliferation were obtained after assessing with SOX2-PCNA double immunostaining. However, after three days of incubation in A2CE, SOX2-PCNA and MKi67-p27^{Kip1} double positive cells were identified, which suggests a moderate proliferation induction in the damaged organ of Corti. The number of supporting cells did not significantly increase after using A2CE (Fig. 4B and C and Fig. 5). Although no damage was previously detected *in vivo* or *in vitro* after A2CE incubation, signs of cochlear damage were detected in these final experiments (Fig. 4B and C and Fig. 5), possibly reaching the limit of tolerance concerning our *in vitro* set up. To further evaluate the therapeutic ability of this approach, we tried to reproduce these results *in vivo* in P0 neonates but were not capable of inducing any significant cochlear damage by means of direct *scala media* inoculation or topical round window administration of 80 mM Gentamicin as a preceding step to A2CE administration for proliferation induction. Although it is possible to induce damage in older animals *in vivo,* P0 rats were used because it is the extra-uterine stage that is temporally closer to the cell cycle exit and theoretically more amenable to the induction of proliferate. These mild to moderate effects combined suggests that the decrease of p27^{Kip1} expression achieved by these therapeutically feasible means was insufficient to induce relevant proliferation in rats' supporting cells.

4. Discussion

A significant amount of knowledge, along with potential therapeutic implications, is generated in animal models. Before translation into clinical practice, however, it is essential to comprehensively explore their realistic applicability. We have tested two clinically feasible methods to decrease p27^{Kip1} *in vivo*. After achieving moderate reductions in the protein level with both approaches, no relevant amounts of proliferation were detected, and thus, there was no increase in the number of supporting cells in the neonatal rat organ of Corti. Only *in vitro* and after adding cochlear damage to the p27^{Kip1} knockdown was there a mild proliferation response in the supporting cells, although a significant increase in their numbers was not detected. Still, we cannot rule out some proliferation induction in adults or long-term effects using these methods. The data presented here strongly suggest that the sole and moderate reduction of p27^{Kip1} obtained by either of these two clinically feasible approaches will not yield robust supporting cell proliferation *in vivo* in the rat cochlea.

This study is one of the few that has reported a successful transduction of the organ of Corti *in vitro* or *in vivo* with lentiviral vectors (Han et al., 1999; Maass et al., 2013; Wang et al., 2013; Wei et al., 2013). These vectors are frequently used in research, but their use concerning the inner ear has been scarce. In this study, we transduced them into the basal portion organ of Corti with mild to moderate success but achieved very high rates of transduction in the *stria vascularis* and in the Reissner's membrane. This might be useful for future studies involving these regions; however, it is also important in the context of this research. The cells in the cochlear duct are functionally connected through gap junctions. It is possible that some amount of p27^{Kip1}-shRNA could circulate (Zhu et al., 2015), causing RNA interference in the EGFP negative cells. Although this possibility was not evaluated directly, it supports the idea that the p27^{Kip1}-shRNA transduction might be distributed enough to decrease p27^{Kip1} in transduced and non-transduced cells of the cochlear duct. Supporting this idea, there is evidence that it is possible to induce gene knockdown in the organ of Corti after shRNA middle ear injections (Oishi et al., 2013; Yu et al., 2012). In case a more diffuse effect might be achieved, it could also determine the milder reduction in p27^{Kip1} relative fluorescence that we detected after p27^{Kip1}-shRNA transduction in the EGFP positive cells, compared to EGFP negative ones (Fig. 2B).

In the literature there are still questions regarding the pertinence of p27^{Kip1} expression manipulation for proliferation induction of supporting cells in deafness treatment. As we can all likely agree, in order for any regenerative approach to be truly beneficial in a clinical setting, it should induce robust supporting cell proliferation. This is one of the few reports that evaluates the ability *in vivo* to induce proliferation through the manipulation of the p27^{Kip1} levels, using approaches that could be used in patients. Previously, there was only one report that used clinically feasible methods to modify a component of the p27^{Kip1} pathway. Minoda et al. (2007) explored the effects of *Skp2* over-expression in the organ of Corti to stimulate the p27^{Kip1} protein degradation but did not explore the p27^{Kip1} expression in the transduced cells; thus, they could not directly link the results to p27^{Kip1} down-regulation. Although they found some proliferation of the interdental cells, correlating with our results, they did not find any evidence of proliferation in the organ of Corti after *Skp2* over-expression (Minoda et al., 2007). Regarding the amount of proliferation obtained from direct p27^{Kip1} level-reduction experiments, previous results are diverse. The constitutive mutants of p27^{Kip1} underwent persistent cell division in postnatal supporting cells (Chen and Segil, 1999; Kanzaki et al., 2006; Löwenheim et al., 1999), and the conditional mutants in the postnatal cochlea induced strong supporting cell growth (Oesterle et al., 2011). However, fewer amounts of proliferation were reported in the literature when the p27^{Kip1} messenger levels were only decreased in the postnatal cochlea. Interestingly, Löwenheim et al. (1999) also explored the amount of proliferation

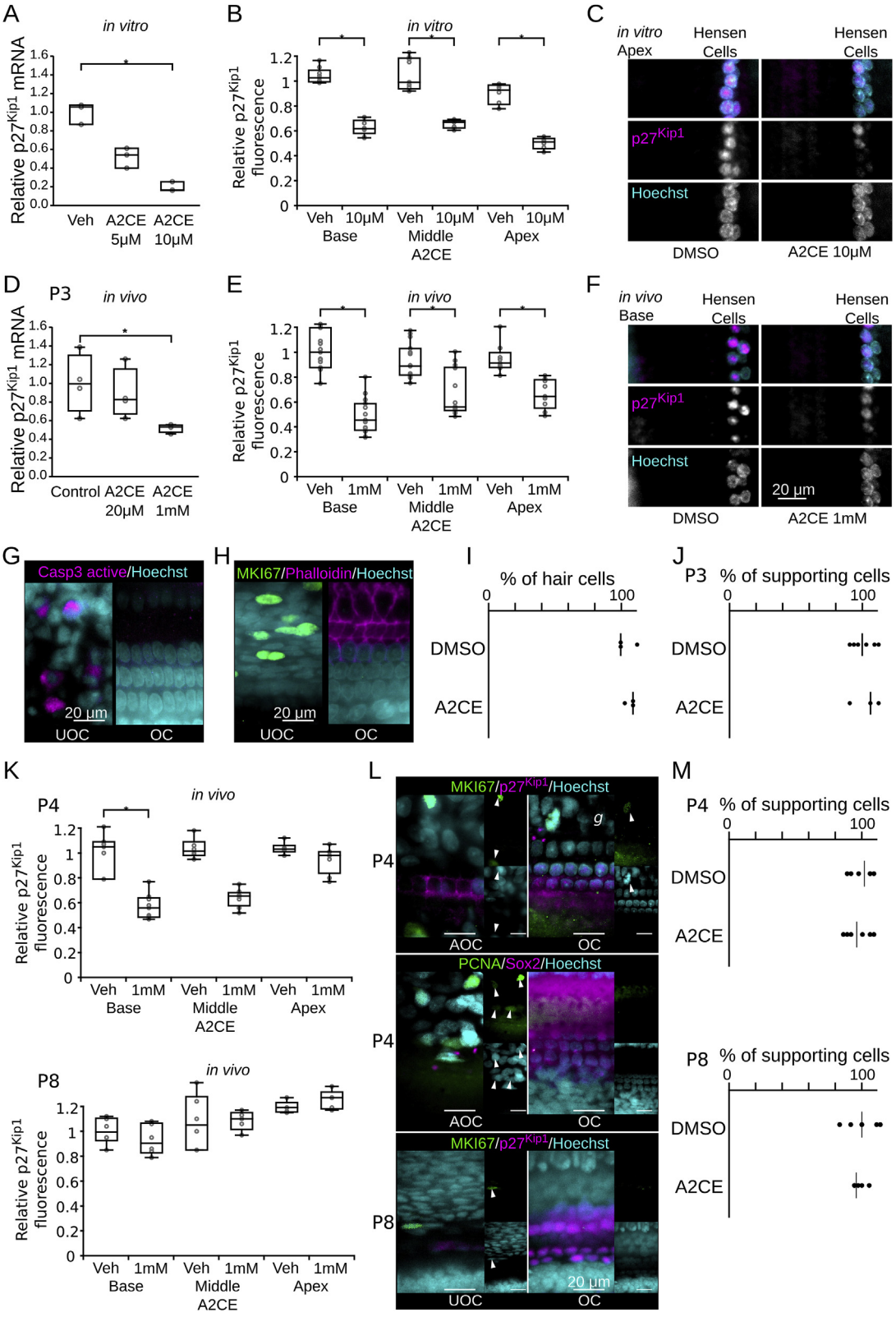

Fig. 3. The effects of pharmacological inhibition of p27^{Kip1} transcription on neonatal rat cochleas *in vitro* and *in vivo*. A. qRT-PCR results showing the p27^{Kip1} mRNA levels in P0 whole cochlear explants cultured for 1 day *in vitro* (DIV) in 10 μM Alsterpaullone 2-Cyanoethyl (A2CE) or vehicle, n = 3 per condition. $p = 0.02732$ using Kruskal-Wallis test. *: $p = 0.00729$ using Dunn's post-hoc analysis. B. p27^{Kip1} relative fluorescence in cochlear cultures treated with 10 μM A2CE or vehicle for 3 DIV. $p = 0.00000$ using Kruskal-Wallis test. *: $p = 0.00040$, 0.00039 and 0.005075, respectively, from left to right using Mann Whitney pairwise comparison, n = 6 to 9 per condition. C. p27^{Kip1} expression in the apex of P0 cochlear explants cultured in 10 μM A2CE or vehicle for 3 DIV. p27^{Kip1}: magenta. Hoechst: cyan. D. qRT-PCR results showing the p27^{Kip1} levels in whole P1 neonatal rat cochlear explants treated *in vivo* at P0 with the topical administration of 20 or 1 mM A2CE or vehicle to the round window, n = 4 for each condition. *: $p = 0.03038$ using Mann-Whitney pairwise comparison. $p = 0.02307$ using Kruskal-Wallis test. E. Relative p27^{Kip1} fluorescence in P3 neonatal rat cochlear explants treated *in vivo* at P0 with the topical administration of 1 mM A2CE or vehicle. $p = 0.00000$ using Kruskal-Wallis test. *: $p = 0.00000$, 0.00299 and 0.000048, respectively, from left to right using Mann-Whitney test pairwise

in heterozygous mutant $p27^{Kip1}$ mice during the first week of life, reporting postnatal proliferation in the organ of Corti, but it was 28 times weaker than in the homozygous mutant. Hence, when only a reduction in $p27^{Kip1}$ gene expression is achieved, the magnitude of the responses weaker and closer to this study findings, which were mild to negative (Maass et al., 2013; Ono et al., 2009). We believe that when comparing the data presented in this paper to all the cumulative $p27^{Kip1}$ reduction data there is a clear pattern suggesting that $p27^{Kip1}$ levels should be severely reduced, if not completely abolished, to induce a robust supporting cell proliferation.

Despite the fact that we were able to prove that it is possible to significantly reduce the $p27^{Kip1}$ expression by 40–50% *in vivo* with our methods, we could not bring its levels to zero. Therefore, we think that for a truly beneficial regenerative method other stronger approaches should be pursued. Furthermore, we believe that CRISPR-Cas9 technologies should be considered, since it might offer advantages over the methods evaluated in this study. Targeted gene disruption in humans might be possible with these technologies in the future (Hsu et al., 2014), and they were recently tested *in vivo* in the mammalian inner ear (Gao et al., 2017). However, *in vivo* gene editing has not yet been tested for this regenerative purpose. In theory, a permanent deletion or disruption of the $p27^{Kip1}$ gene could abolish the protein levels, but it will not induce a transient effect, compromising the safety of the approach. Although theoretically possible, inducing transitory proliferation of supporting cells by conditional genome modifications in non-mutant mammals seems to be less feasible than just modifying the gene expression. Alternatively, a nuclease null form of Cas9 fused to KRAB transcription repressor has also been used for highly specific and efficient genetic knockdown *in vitro* and *in vivo* in mice neurons and blood cells (Braun et al., 2016; Zheng et al., 2018). Using this CRISPR-based interference approach, it is theoretically possible to reduce nearly 90% of the gene expression of the targeted genes. These are very promising tools, but they have some limitations in targeting and still have not been used in cochlear cells (Gilbert et al., 2013; Qi et al., 2013).

Literature suggests that regenerative potential is increased by damage (Izumikawa et al., 2005; Kil et al., 2011; Mizutari et al., 2013), and we obtained *in vitro* an incremental change in the proliferation in the organ of Corti after adding cochlear damage to A2CE incubation. Nevertheless, the proliferating cells found were only assessed using SOX2 and $p27^{Kip1}$ immunostainings instead of more stable cell fate tracers. The expression of SOX2 is decreased between P1 and P6 (Maass et al., 2016), making its detection harder in later postnatal stages. Additionally, it is possible that the damage induced by aminoglycosides could disrupt the basal membrane of the epithelium. Also, SOX2 is expressed by the cells of the greater epithelial ridge. Thus, we cannot rule out the fact that the little incremental gains in proliferation found in the organ of Corti was caused by infiltration of cells coming from other areas of the explants. Furthermore, we believe that even if those proliferating cells were genuinely coming from supporting cells, the small amount of proliferation found would hardly mean a clinically relevant response. However, we could not confirm these ideas *in vivo*.

In addition to not finding relevant proliferation in the organ of Corti after $p27^{Kip1}$ decrease, we did not see any significant signs of collateral cell death *in vivo*. Although we recognize we did not evaluate other cell death types than apoptosis, we believe that this result is noteworthy. First, because it discourages the possibility that cell death might explain the decrease in the $p27^{Kip1}$ levels and the absence of proliferation obtained in this study *in vivo*. Second, it is interesting since it is possible that the lack of signs of cell death found in this study *in vivo* might be related to the lack of proliferation itself. Most $p27^{Kip1}$ reduction experiments that have achieved proliferation have also shown evidence of ototoxicity (Chen and Segil, 1999; Kanzaki et al., 2006; Löwenheim et al., 1999; Oesterle et al., 2011; Ono et al., 2009). Similarly, in the *in vitro* part of this study, we did find some cochlear cells positive for apoptosis markers together with other cells positive for proliferation markers (Fig. 4A–C and Fig. 5). The idea of co-occurrence is supported further by other reports of cochlear cell death after forced proliferation induction in $p27^{Kip1}$ independent fashion (Chen et al., 2003; Laine et al., 2007; Yu et al., 2010). However, since this study was not designed for detecting that association, we could not directly link forced proliferation to cell death. Finally, the lack of apoptosis markers is relevant because the moderately reduced levels of $p27^{Kip1}$ achieved in this research do not appear to cause cell death like what has been observed in the $p27^{Kip1}$ knockout papers. Aggregating the results of this research with the findings of our previous study *in vitro* (Maass et al., 2013) we do not have evidence of cell death after $p27^{Kip1}$ knockdown with shRNA transduced in lentiviral vectors. Nevertheless, we found apoptosis markers *in vitro* after A2CE. Despite the fact that the increment in cell death markers was not significant, the finding of some positive cells suggests that the drug might be toxic for the inner ear under certain conditions.

Unlike this research, our previous *in vitro* study detected some proliferation in the organ of Corti after $p27^{Kip1}$ knockdown (Maass et al., 2013). Even though we used the same viral strategy in the first part of this report, there are many differences between both studies. One is that in this study, the *scala media* injection was placed at the base of the cochlea, getting the best transduction rate in the basal segment. However, in our previous paper, we focused the analysis in the more immature, less differentiated and more responsive, mid-portion of the cochlear duct. Another difference is that our *in vivo* transduction was less efficient than our previous *in vitro* approach, which limited the possible amount of $p27^{Kip1}$ reduction. Finally, *in vitro* experiments are essentially distant from real conditions. In cultures, growth factors are added and the

comparison, n = 9 to 15 per condition. F. $p27^{Kip1}$ expression in the basal segment of P3 neonatal cochlea treated *in vivo* at P0 with the topical administration of 1 mM A2CE to the round window. $p27^{Kip1}$: magenta. Hoechst: cyan. G. Apoptosis in basal portion of P3 cochlear explants treated *in vivo* at P0 with 1 mM A2CE or vehicle. OC: in the organ of Corti. UOC: under the organ of Corti. c-CASP3: magenta. Hoechst: cyan. H. Proliferation markers in the basal portion of P3 cochlear explants treated *in vivo* with 1 mM A2CE or vehicle evaluated by MKI67 expression. Phalloidin: magenta. MKi67: green. Hoechst: cyan. OC: in the organ of Corti. UOC: under the organ of Corti, n = 3 to 6 per condition. I and J: Relative number of hair cells and supporting cells, respectively. The percentages relative to the median in the control condition. Medians calculated by interpolation. The plots show the actual data set in dots and the medians in vertical lines, n = 3 to 6 per group. $p = 0.6531$ and 0.89642, respectively (Mann-Whitney test). K. $p27^{Kip1}$ relative fluorescence in P4 and P8 neonatal rat cochlear explants treated *in vivo* at P0 with the topical administration of 1 mM A2CE or vehicle. Top: P4. $p = 0.004815$ for P4 using Kruskal-Wallis test. *: *p-value* = 0.001024, using Mann-Whitney test pairwise comparison. In the mid portion the $p = 0.002388$, but not considered significant after correction, n = 6 to 9 per condition. Bottom: P8. $p = 0.002806$ using Kruskal-Wallis test. After comparing treatment and control conditions, the lowest p-value obtained was 0.1988 using Mann-Whitney test pairwise comparison, n = 5 to 6 per condition. L. Proliferation markers in the basal portion of P4 and P8 cochlear explants treated *in vivo* with 1 mM A2CE or vehicle evaluated by MKI67 and PCNA expression. Upper most panels: P4. Bottom: P8. Right panels show cells in the organ of Corti (OC) or at the same level in the greater epithelial ridge (*g*). Left panels show cells located above the organ of Corti (AOC) or under the organ of Corti (UOC). $p27^{Kip1}$ and SOX2: magenta. MKi67 and PCNA: green. Hoechst: cyan. Right and left panels are divided to show the merged and the green and cyan channels separately, n = 3 to 6 per condition. M. Relative number of supporting cells at P4 and P8, top and bottom graphs respectively. Percentages relative to the median in the control condition. Medians calculated by interpolation. Plots show the actual data set in dots and the medians in vertical lines, n = 3 to 6 per group. $p = 0.6267$ and 0.68471, respectively (Mann-Whitney test). Benjamini-Hochberg procedure was used for significance determination after post-hoc analyses.

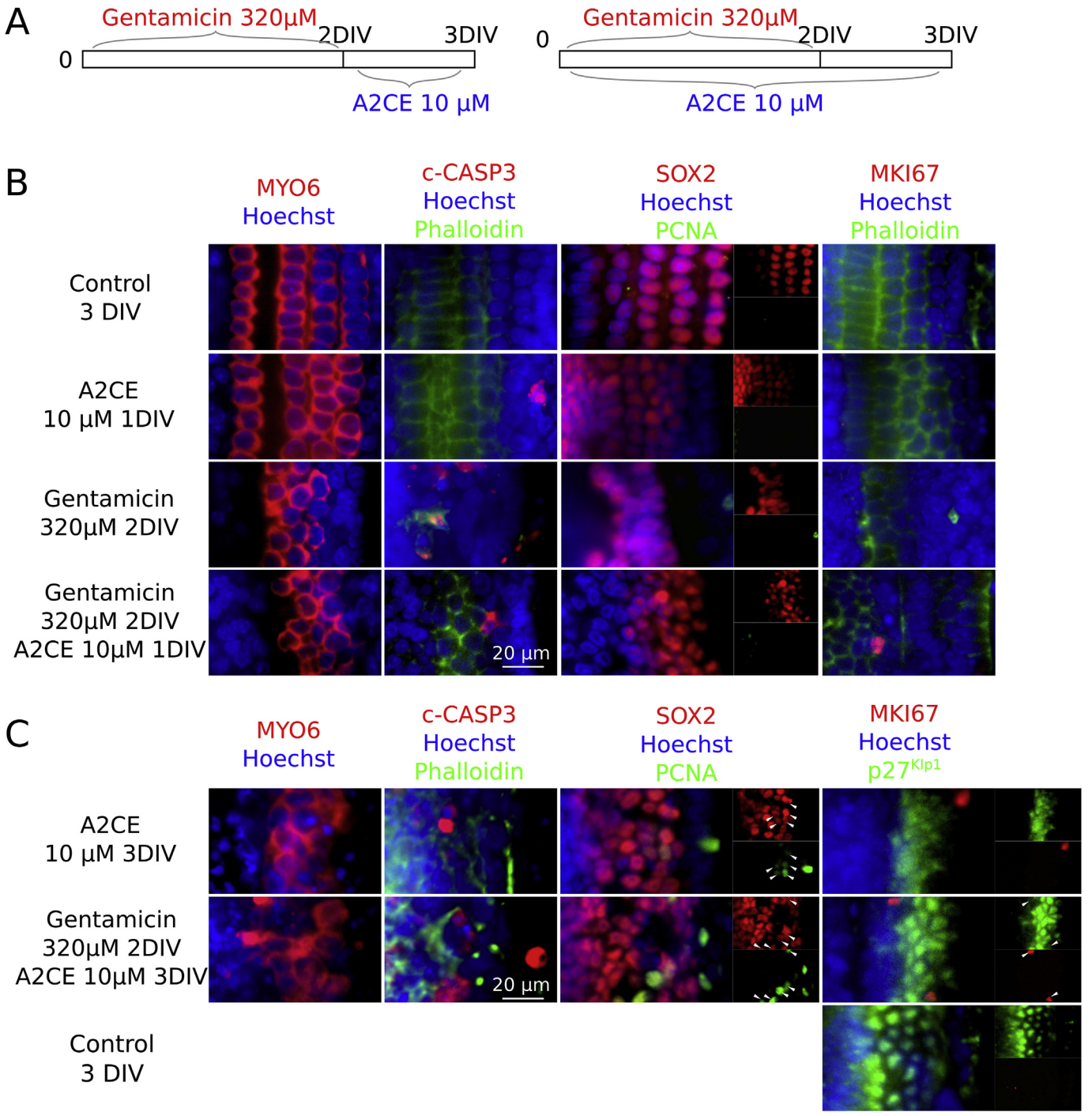

Fig. 4. The effects of Gentamicin and Alsterpaullone 2-Cyanoethyl (A2CE) incubation on the cochlear cultures. A. Schematic view of the experiments shown in B and C. B. Portion of the apex of P0 cochlear explants cultured for 3 days *in vitro* (3 DIV). During the initial 48 h, the explants were cultured in Gentamicin 320 μM or vehicle, and during the last day *in vitro* cultured in the presence of 10 μM A2CE or vehicle. The panels from left to right show MYO6, c-CASP3, SOX2 or MKI67: red. Hoechst: blue. PCNA and Phalloidin: green. C. Explants cultured for 3 days as in B but were exposed to A2CE during the 3 days of incubation. Same immunostaining as well but using $p27^{Kip1}$ instead of Phalloidin in the third column of panels: green. Arrowheads: double positive cells. For SOX2-PCNA and MKI67-$p27^{Kip1}$ immunostaining, red and green channels are shown separately.

proximity to cell cycle reentry or to cell death is not necessarily the same as that of *in vivo* experiments. Moreover, the structure of the cultured epithelium might be untidier, having more chances of migration of proliferating cells from other areas of the explants. Despite these differences, after comparing them we believe that the lack of proliferation found in this study still makes the point that we did not obtain more proliferation than the already mild proliferation found in our previous *in vitro* study (Maass et al., 2013). Thus, after evaluating the clinical implications of this approach, we believe that not having evidence of significant proliferation is more conclusive and relevant in this study, compared to our previous results (Maass et al., 2013).

Within the limitations of this study, we did not evaluate the effect of our approaches in adult animals, or after longer periods of

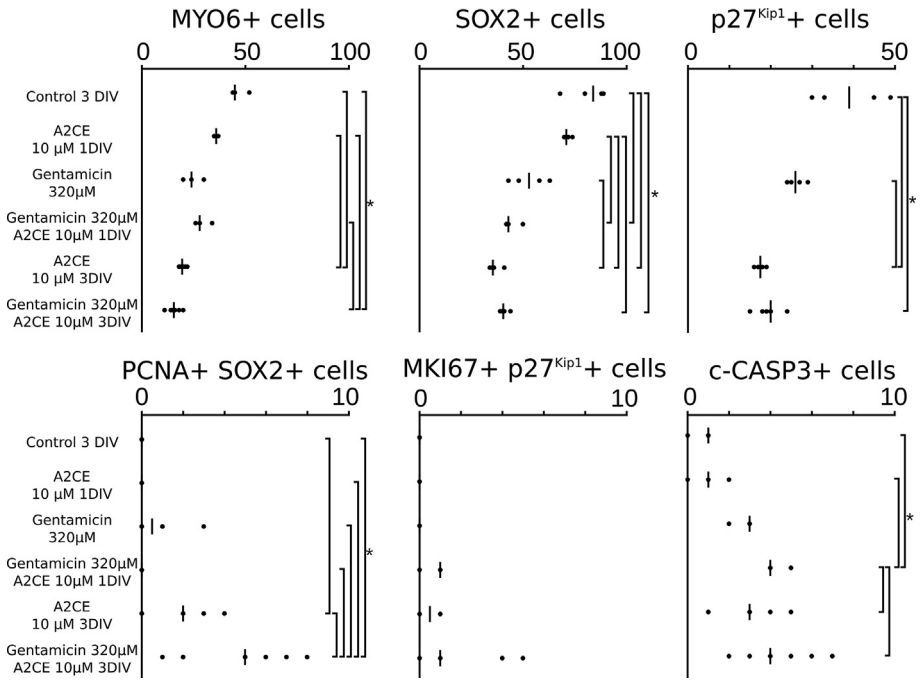

Fig. 5. Cell counts in the organ of Corti *in vitro* after Gentamicin and Alsterpaullone 2-Cyanoethyl (A2CE) incubation on the cochlear cultures. The number of positive cells for markers per 100 μm of the apex of the cochlea in the evaluated conditions in Fig. 4B and C: 3 days' control cultures, 10 μM A2CE for one day, 320 μM Gentamicin for two days, 320 μM Gentamicin for two days plus 10 μM A2CE for one day, 10 μM A2CE for three days and 320 μM Gentamicin for two days plus 10 μM A2CE for the whole length of the incubation. The cell counts are shown from left to right and from top to bottom as follows: MYO6+ cells, SOX2+ cells, $p27^{Kip1}$+ cells, PCNA + SOX2+ cells, MKI67 + $p27^{Kip1}$+ cells, and c-CASP3+ cells. The graphs show the actual data set in dots and the medians in vertical lines. Medians calculated by interpolation. n = 3 to 9 per group. $p = 0.00107$ for MYO6+ data, $p = 0.00104$ for SOX2+ data, $p = 0.00191$ for $p27^{Kip1}$+ data, $p = 0.00041$ for PCNA + SOX2+ data, $p = 0.1805$ for MKI67 + $p27^{Kip1}$+ data, and $p = 0.03415$ in c-CASP3+ data using Kruskal-Wallis test. *: the p-values obtained during Dunn's post-hoc analyses were significant after Benjamini-Hochberg procedure, $p < 0.05$.

observation. We also did not test the effect of damage *in vivo*. Although lentiviral vectors and osmotic pumps can provide long-term effects (Mátrai et al., 2010), after our mild results in the neonatal cochlea we decided not to test older animals. We did it considering that in the conditional mutant of $p27^{Kip1}$ the best proliferation rates were observed when harvesting the mutant cochleas just after birth. Later in life, the number of proliferating cells decreases, suggesting the involvement of other regulatory genes (Oesterle et al., 2011). Moreover, there was evidence supporting the argument that the lentiviral vectors' genetic information could be progressively silenced by epigenetic modifications in the host genome (Hofmann et al., 2006; Wang et al., 2010). We believe that during the first week of life or *in vitro* we had more chances to induce and detect stronger responses, and we supposed that later *in vivo* we would not get more relevant responses. However, this was only a supposition based on previous evidence, and we cannot precisely predict the results of a more prolonged intervention in more mature individuals or in damaged cochleas *in vivo*. It is also important to remark that this study was performed on newborn rats, and most of the previous related information was obtained from neonatal mice. Although the rat and mouse development of cochlear epithelia are very similar and responsive to trans-differentiation methods, we cannot fully extrapolate our results to mice. However, we believe that our approach is still accurate for testing feasibility in our preclinical context. Mice are not the ultimate target species that we are aiming for, and our methods are conceptually practicable regarding other species as well. Another limitation is that this study relies only on morphologic evidence of proliferation and damage. Even though we recognize that only using immunostaining proliferation markers could have decreased our detection rate, the fact that we got MKI67-positive supporting cells after $p27^{Kip1}$ down-regulation in our previous *in vitro* study supports the notion that the response obtained in this *in vivo* study was weaker than our previous study, instead of simply being negative. Although we tried to increase our detection rate and reproducibility by detecting different proliferation antigens, the fact that PCNA staining detected more cells could induce some error, since it is also expressed during DNA repair (Essers et al., 2005) and we induced damage *in vitro* in this study. Likewise, our exploration of toxicity was also limited to the evaluation of sensory epithelium morphology and Caspase 3 active immunostaining. A more extensive evaluation of cell death and hearing would be needed to definitively rule out the secondary damage induced by $p27^{Kip1}$ knockdown *in vivo*. Finally, our approaches mostly acted in the basal turn of the cochlea—precisely the portion of the cochlea that is less responsive to regeneration (Cox et al., 2014; Maass et al., 2016, 2015). Therefore, it is possible that our negative results were influenced by the aforementioned fact as well. We recognize that other strategies aiming at the middle or apical turns might be more effective inducing proliferation.

If we conceive the supporting cell trans-differentiation into hair cells as a future tool to treat deafness, we will need to have efficient means to increase the number of supporting cells to preserve the appropriate physiology. In agreement with our results, the previous literature and the available possibilities for the clinical context, we believe that it will be more beneficial to attempt to manipulate factors other than $p27^{Kip1}$, or perhaps to target these factors in combination with $p27^{Kip1}$ knockdown approaches. However, we cannot rule out the possibility that other pharmacological agents that target $p27^{Kip1}$, other dosing regimens or durations of treatment with A2CE or other drugs, or other potential gene therapeutic approaches that target more cells and induce greater knockdown of $p27^{Kip1}$ could induce a robust supporting cell proliferation.

To perform future experiments within this topic, we believe that

the cell cycle control in the supporting cells should be revisited. In a recent transcriptome analysis of supporting cells at P1 and P6 (Maass et al., 2016) and within a list of known genes canonically involved in the cell cycle control (Schimmang and Pirvola, 2013), we only detected significant expression of $p27^{Kip1}$ in supporting cells, whereas we found expression of many genes in the Notch and Wnt pathways. Furthermore, modifications in both pathways have been recently proposed as targets for proliferation induction in the neonatal cochlea (Hu et al., 2016; McLean et al., 2017; Ni et al., 2016b, 2016a). We believe that future experiments on this topic will need to add other gene expression modifications to briefly promote proliferation before trans-differentiation induction. It would be reasonable to test modifications of multiple genes alone or in combination with $p27^{Kip1}$ knockdown. If more than one gene is involved, then more complex approaches will be needed. For instance, current inducible viral vectors technologies could be used, since they can orchestrate intricate patterns of gene expression through multi-step modifications (Mátrai et al., 2010), as well as CRISPR interference for simultaneously targeting different genes (Braun et al., 2016; Zheng et al., 2018). Finally, we have shown that neonatal cochleas are useful for testing initial methodological feasibility, but we believe that the ultimate clinical relevance of the forthcoming approximations should be evaluated *in vivo* in older deaf animals and assessed for longer periods of time.

Many studies have proposed the manipulation of $p27^{Kip1}$ to induce proliferation, but none had evaluated its feasibility. We have presented cumulative evidence that it is possible to decrease the $p27^{Kip1}$ levels in cochlear supporting cells of the non-mutant animals *in vivo*. However, the presented reduction was not enough to induce relevant proliferation *in vivo*. Our results were obtained during early postnatal development and after damage, when the supporting cells would theoretically be more prone to proliferation. Therefore, we believe that it is unlikely to have better results later, when deafness is more common. Additionally, the studied period of rat inner ear development roughly corresponds to 14–15 weeks of human gestation (Locher et al., 2013), which is very far from the current reach concerning fetal surgery. Thus, our results suggest that it would be a major achievement to induce proliferation using the approaches tested in this study, even in fetal supporting cells. In this way, our results add additional preclinical context to the previous literature that suggested $p27^{Kip1}$ as a target to induce proliferation of supporting cells. We believe that the major impact of this research is in providing evidence that an isolated and moderate reduction of $p27^{Kip1}$ levels, such as that achieved *in vivo* by these clinically feasible means, might not be sufficient to induce proliferation after birth in the organ of Corti. Thus, future research should seek other methods, perhaps other genes and combined approaches to effectively stimulate inner ear proliferation in animals, and hopefully in patients.

Funding

This research was supported by CONICYT (Chile) grants FONDECYT 11130247 and ACT1403.

Acknowledgments

We would like to thank Lorena I. Tapia and Andrew K. Groves for their critical review of the manuscript along with Silvia Cantellano for her methodological advice.

References

Braun, C.J., Bruno, P.M., Horlbeck, M.A., Gilbert, L.A., Weissman, J.S., Hemann, M.T., 2016. Versatile in vivo regulation of tumor phenotypes by dCas9-mediated transcriptional perturbation. Proc. Natl. Acad. Sci. 113, E3892–E3900. https://doi.org/10.1073/pnas.1600582113.

Bishop, C.E., Eby, T.L., 2010. The current status of audiologic rehabilitation for profound unilateral sensorineural hearing loss. Laryngoscope 120, 552–556. https://doi.org/10.1002/lary.20735.

Cai, T., Seymour, M.L., Zhang, H., Pereira, F.A., Groves, A.K., 2013. Conditional deletion of Atoh1 reveals distinct critical periods for survival and function of hair cells in the organ of Corti. J. Neurosci. 33, 10110–10122. https://doi.org/10.1523/JNEUROSCI.5606-12.2013.

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. https://doi.org/10.1038/ncb976.

Corwin, J.T., Cotanche, D.A., 1988. Regeneration of sensory hair cells after acoustic trauma. Science 240, 1772–1774.

Cox, B.C., Chai, R., Lenoir, A., Liu, Z., Zhang, L., Nguyen, D.-H., Chalasani, K., Steigelman, K.A., Fang, J., Rubel, E.W., Cheng, A.G., Zuo, J., 2014. Spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo. Development 141, 1599–1599. https://doi.org/10.1242/dev.109421.

Cruickshanks, K.J., Wiley, T.L., Tweed, T.S., Klein, B.E., Klein, R., Mares-Perlman, J.A., Nondahl, D.M., 1998. Prevalence of hearing loss in older adults in beaver dam, Wisconsin. The epidemiology of hearing loss study. Am. J. Epidemiol. 148, 879–886.

Erenberg, A., Lemons, J., Sia, C., Trunkel, D., Ziring, P., 1999. Newborn and infant hearing loss: detection and intervention. American academy of pediatrics. Task force on newborn and infant hearing, 1998–1999. Pediatrics 103, 527–530.

Essers, J., Theil, A.F., Baldeyron, C., Cappellen, W.A. van, Houtsmuller, A.B., Kanaar, R., Vermeulen, W., 2005. Nuclear dynamics of PCNA in DNA replication and repair. Mol. Cell Biol. 25, 9350–9359. https://doi.org/10.1128/MCB.25.21.9350-9359.2005.

Gao, X., Tao, Y., Lamas, V., Huang, M., Yeh, W.-H., Pan, B., Hu, Y.-J., Hu, J.H., Thompson, D.B., Shu, Y., Li, Y., Wang, H., Yang, S., Xu, Q., Polley, D.B., Liberman, M.C., Kong, W.-J., Holt, J.R., Chen, Z.-Y., Liu, D.R., 2017. Treatment of autosomal dominant hearing loss by in vivo delivery of genome editing agents. Nature 553, 217–221. https://doi.org/10.1038/nature25164.

Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., Stern-Ginossar, N., Brandman, O., Whitehead, E.H., Doudna, J.A., Lim, W.A., Weissman, J.S., Qi, L.S., 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154, 442–451. https://doi.org/10.1016/j.cell.2013.06.044.

Goycoolea, M.V., Muchow, D., Schachern, P., 1988. Experimental studies on round window structure: function and permeability. Laryngoscope 98, 1–20. https://doi.org/10.1288/00005537-198806001-00002.

Gubbels, S.P., Woessner, D.W., Mitchell, J.C., Ricci, A.J., Brigande, J.V., 2008. Functional auditory hair cells produced in the mammalian cochlea by in utero gene transfer. Nature 455, 537–541. https://doi.org/10.1038/nature07265.

Han, J.J., Mhatre, A.N., Wareing, M., Pettis, R., Gao, W.Q., Zufferey, R.N., Trono, D., Lalwani, A.K., 1999. Transgene expression in the Guinea pig cochlea mediated by a lentivirus-derived gene transfer vector. Hum. Gene Ther. 10, 1867–1873. https://doi.org/10.1089/10430349950017545.

Harris, J.A., Cheng, A.G., Cunningham, L.L., MacDonald, G., Raible, D.W., Rubel, E.W., 2003. Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (*Danio rerio*). J. Assoc. Res. Otolaryngol. 4, 219–234. https://doi.org/10.1007/s10162-002-3022-x.

Hofmann, A., Kessler, B., Ewerling, S., Kabermann, A., Brem, G., Wolf, E., Pfeifer, A., 2006. Epigenetic regulation of lentiviral transgene vectors in a large animal model. Mol. Ther. 13, 59–66. https://doi.org/10.1016/j.ymthe.2005.07.685.

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. https://doi.org/10.1097/WNR.0b013e3282f213e0.

Hsu, P.D., Lander, E.S., Zhang, F., 2014. Development and applications of CRISPR-Cas9 for genome engineering. Cell 157, 1262–1278. https://doi.org/10.1016/j.cell.2014.05.010.

Hu, L., Lu, J., Chiang, H., Wu, H., Edge, A.S.B., Shi, F., 2016. Diphtheria toxin-induced cell death triggers Wnt-dependent hair cell regeneration in neonatal mice. J. Neurosci. 36, 9479–9489. https://doi.org/10.1523/JNEUROSCI.2447-15.2016.

Izumikawa, M., Minoda, R., Kawamoto, K., Abrashkin, K.A., Swiderski, D.L., Dolan, D.F., Brough, D.E., Raphael, Y., 2005. Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. Nat. Med. 11, 271–276. https://doi.org/10.1038/nm1193.

Izumikawa, M., Batts, S.A., Miyazawa, T., Swiderski, D.L., Raphael, Y., 2008. Response of the flat cochlear epithelium to forced expression of Atoh1. Hear. Res. 240, 52–56. https://doi.org/10.1016/j.heares.2008.02.007.

Kanzaki, S., Beyer, L.A., Swiderski, D.L., Izumikawa, M., Stöver, T., Kawamoto, K., Raphael, Y., 2006. p27Kip1 deficiency causes organ of Corti pathology and hearing loss. Hear. Res. 214, 28–36. https://doi.org/10.1016/j.heares.2006.01.014.

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.

Kil, J., Gu, R., Fero, M., Lynch, E.D., 2011. The cyclin dependent kinase inhibitor p27Kip1 maintains terminal differentiation in the mouse organ of Corti. Open Otorhinolaryngol. J. 5, 25–34. https://doi.org/10.2174/1874428101105010025.

Laine, H., Doetzlhofer, A., Mantela, J., Ylikoski, J., Laiho, M., Roussel, M.F., Segil, N.,

Pirvola, U., 2007. p19Ink4d and p21Cip1 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. https://doi.org/10.1523/JNEUROSCI.4956-06.2007.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Locher, H., Frijns, J.H.M., van Iperen, L., de Groot, J.C.M.J., Huisman, M.A., Chuva de Sousa Lopes, S.M., 2013. Neurosensory development and cell fate determination in the human cochlea. Neural Dev. 8, 20. https://doi.org/10.1186/1749-8104-8-20.

Logan, A.C., Nightingale, S.J., Haas, D.L., Cho, G.J., Pepper, K.A., Kohn, D.B., 2004. Factors influencing the titer and infectivity of lentiviral vectors. Hum. Gene Ther. 15, 976–988. https://doi.org/10.1089/hum.2004.15.976.

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., 1999. Gene disruption of p27Kip1 allows cell proliferation in the postnatal and adult organ of Corti. Proc. Natl. Acad. Sci. 96, 4084–4088.

Maass, J.C., Berndt, F.A., Cánovas, J., Kukuljan, M., 2013. p27Kip1 knockdown induces proliferation in the organ of Corti in culture after efficient shRNA lentiviral transduction. J. Assoc. Res. Otolaryngol. 14, 495–508. https://doi.org/10.1007/s10162-013-0383-2.

Maass, J.C., Gu, R., Basch, M.L., Waldhaus, J., Lopez, E.M., Xia, A., Oghalai, J.S., Heller, S., Groves, A.K., 2015. Changes in the regulation of the Notch signaling pathway are temporally correlated with regenerative failure in the mouse cochlea. Front. Cell. Neurosci. 9, 110. https://doi.org/10.3389/fncel.2015.00110.

Maass, J.C., Gu, R., Cai, T., Wan, Y.W., Cantellano, S.C., Asprer, J.S.T., Zhang, H., Jen, H.I., Edlund, R.K., Liu, Z., Groves, A.K., 2016. Transcriptomic analysis of mouse cochlear supporting cell maturation reveals large-scale changes in Notch responsiveness prior to the onset of hearing. PloS One 11, e0167286. https://doi.org/10.1371/journal.pone.0167286.

Manchaiah, V., Taylor, B., Dockens, A., Tran, N., Lane, K., Castle, M., Grover, V., 2017. Applications of direct-to-consumer hearing devices for adults with hearing loss: a review. Clin. Interv. Aging 12, 859–871. https://doi.org/10.2147/CIA.S135390.

Mátrai, J., Chuah, M.K., VandenDriessche, T., 2010. Recent advances in lentiviral vector development and applications. Mol. Ther. 18, 477–490. https://doi.org/10.1038/mt.2009.319.

McLean, W.J., Yin, X., Lu, L., Lenz, D.R., McLean, D., Langer, R., Karp, J.M., Edge, A.S.B., 2017. Clonal expansion of Lgr5-positive cells from mammalian cochlea and high-purity generation of sensory hair cells. Cell Rep. 18, 1917–1929. https://doi.org/10.1016/j.celrep.2017.01.066.

Merchant, S.N., Nadol, J.B., Schuknecht, H.F., 2010. Schuknecht's Pathology of the Ear. McGraw-Hill Medical; McGraw-Hill [distributor, New York; London.

Minoda, R., Izumikawa, M., Kawamoto, K., Zhang, H., Raphael, Y., 2007. Manipulating cell cycle regulation in the mature cochlea. Hear. Res. 232, 44–51. https://doi.org/10.1016/j.heares.2007.06.005.

Mizutari, K., Fujioka, M., Hosoya, M., Bramhall, N., Okano, H.J., Okano, H., Edge, A.S.B., 2013. Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma. Neuron 77, 58–69. https://doi.org/10.1016/j.neuron.2012.10.032.

Ni, W., Lin, C., Guo, L., Wu, J., Chen, Y., Chai, R., Li, W., Li, H., 2016a. Extensive supporting cell proliferation and mitotic hair cell generation by in vivo genetic reprogramming in the neonatal mouse cochlea. J. Neurosci. 36, 8734–8745. https://doi.org/10.1523/JNEUROSCI.0060-16.2016.

Ni, W., Zeng, S., Li, W., Chen, Y., Zhang, S., Tang, M., Sun, S., Chai, R., Li, H., 2016b. Wnt activation followed by Notch inhibition promotes mitotic hair cell regeneration in the postnatal mouse cochlea. Oncotarget 7, 66754–66768. https://doi.org/10.18632/oncotarget.11479.

Oesterle, E.C., Chien, W.-M., Campbell, S., Nellimarla, P., Fero, M.L., 2011. p27Kip1 is required to maintain proliferative quiescence in the adult cochlea and pituitary. Cell Cycle 10, 1237–1248. https://doi.org/10.4161/cc.10.8.15301.

Oishi, N., Chen, F.Q., Zheng, H.W., Sha, S.H., 2013. Intra-tympanic delivery of short interfering RNA into the adult mouse cochlea. Hear. Res. 296, 36–41. https://doi.org/10.1016/j.heares.2012.10.011.

Olusanya, B.O., Neumann, K.J., Saunders, J.E., 2014. The global burden of disabling hearing impairment: a call to action. Bull. World Health Organ. 92, 367–373. https://doi.org/10.2471/BLT.13.128728.

Ono, K., Nakagawa, T., Kojima, K., Matsumoto, M., Kawauchi, T., Hoshino, M., Ito, J., 2009. Silencing p27 reverses post-mitotic state of supporting cells in neonatal mouse cochleae. Mol. Cell. Neurosci. 42, 391–398. https://doi.org/10.1016/j.mcn.2009.08.011.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., Lim, W.A., 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific Control of gene expression. Cell 152, 1173–1183. https://doi.org/10.1016/j.cell.2013.02.022.

Raphael, Y., Kim, Y.-H., Osumi, Y., Izumikawa, M., 2007. Non-sensory cells in the deafened organ of Corti: approaches for repair. Int. J. Dev. Biol. 51, 649–654. https://doi.org/10.1387/ijdb.072370yr.

Ruben, R.J., 1967. Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. Acta Otolaryngol. (Stockh.) 220, 1–44.

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. https://doi.org/10.1126/science.1106642.

Schimmang, T., Pirvola, U., 2013. Coupling the cell cycle to development and regeneration of the inner ear. Semin. Cell Dev. Biol. 24, 507–513. https://doi.org/10.1016/j.semcdb.2013.04.004.

Sena-Esteves, M., Tebbets, J.C., Steffens, S., Crombleholme, T., Flake, A.W., 2004. Optimized large-scale production of high titer lentivirus vector pseudotypes. J. Virol. Methods 122, 131–139. https://doi.org/10.1016/j.jviromet.2004.08.017.

Walters, B.J., Lin, W., Diao, S., Brimble, M., Iconaru, L.I., Dearman, J., Goktug, A., Chen, T., Zuo, J., 2014. High-throughput screening reveals Alsterpaullone, 2-Cyanoethyl as a potent p27Kip1 transcriptional inhibitor. PLoS One 9, e91173. https://doi.org/10.1371/journal.pone.0091173.

Walters, B.J., Liu, Z., Crabtree, M., Coak, E., Cox, B.C., Zuo, J., 2014. Auditory hair cell-specific deletion of p27Kip1 in postnatal mice promotes cell-autonomous generation of new hair cells and normal hearing. J. Neurosci. 34, 15751–15763. https://doi.org/10.1523/JNEUROSCI.3200-14.2014.

Wang, Y., Song, Y., Liu, Q., Liu, C., Wang, L., Liu, Y., Zhou, X., Wu, J., Wei, H., 2010. Quantitative analysis of lentiviral transgene expression in mice over seven generations. Transgenic Res. 19, 775–784. https://doi.org/10.1007/s11248-009-9355-z.

Wang, Y., Sun, Y., Chang, Q., Ahmad, S., Zhou, B., Kim, Y., Li, H., Lin, X., 2013. Early postnatal virus inoculation into the scala media achieved extensive expression of exogenous green fluorescent protein in the inner ear and preserved auditory brainstem response thresholds. J. Gene Med. 15, 123–133. https://doi.org/10.1002/jgm.2701.

Wei, Y., Fu, Y., Liu, S., Xia, G., Pan, S., 2013. Effect of lentiviruses carrying enhanced green fluorescent protein injected into the scala media through a cochleostomy in rats. Am. J. Otolaryngol. 34, 301–307. https://doi.org/10.1016/j.amjoto.2012.12.011.

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. https://doi.org/10.1038/nature04849.

Wilson, B.S., 2017. The modern cochlear implant: a triumph of biomedical engineering and the first substantial restoration of human sense using a medical intervention. IEEE Pulse 8, 29–32. https://doi.org/10.1109/MPUL.2017.2649039.

Woods, C., Montcouquiol, M., Kelley, M.W., 2004. Math1 regulates development of the sensory epithelium in the mammalian cochlea. Nat. Neurosci. 7, 1310–1318. https://doi.org/10.1038/nn1349.

World Health Organization, 2017. Global Costs of Unaddressed Hearing Loss and Cost-effectiveness of Interventions.

Yu, J., Xiang, M., Wu, H., Shen, C., 2012. Intratympanic injection of shRNA-expressing lentivirus causes gene silencing in the inner ear in chicken. Neurosci. Lett. 510, 132–137. https://doi.org/10.1016/j.neulet.2012.01.021.

Yu, Y., Weber, T., Yamashita, T., Liu, Z., Valentine, M.B., Cox, B.C., Zuo, J., 2010. In vivo proliferation of postmitotic cochlear supporting cells by acute ablation of the retinoblastoma protein in neonatal mice. J. Neurosci. 30, 5927–5936. https://doi.org/10.1523/JNEUROSCI.5989-09.2010.

Zheng, Y., Shen, W., Zhang, J., Yang, B., Liu, Y.N., Qi, H., Yu, X., Lu, S.Y., Chen, Y., Xu, Y.Z., Li, Y., Gage, F.H., Mi, S., Yao, J., 2018. CRISPR interference-based specific and efficient gene inactivation in the brain. Nat. Neurosci. 21, 447–454. https://doi.org/10.1038/s41593-018-0077-5.

Zhu, Y., Zong, L., Mei, L., Zhao, H.-B., 2015. Connexin26 gap junction mediates miRNA intercellular genetic communication in the cochlea and is required for inner ear development. Sci. Rep. 5. https://doi.org/10.1038/srep15647.