JARO 14: 495–508 (2013)
DOI: 10.1007/s10162-013-0383-2

JARO
Journal of the Association for Research in Otolaryngology

Research Article

p27Kip1 Knockdown Induces Proliferation in the Organ of Corti in Culture after Efficient shRNA Lentiviral Transduction

Juan C. Maass[1,2,3,4], F. Andrés Berndt[1,2], José Cánovas[1,2], and Manuel Kukuljan[1,2]

[1]*Program in Physiology and Biophysics, Institute for Biomedical Sciences, Faculty of Medicine, Universidad de Chile, Av. Independencia 1027, 8380453, Independencia, Santiago, Chile*

[2]*Biomedical Neuroscience Institute, Faculty of Medicine, Universidad de Chile, Av. Independencia 1027, 8380453, Independencia, Santiago, Chile*

[3]*Department of Otolaryngology, Hospital Clínico Universidad de Chile, Santos Dumont 999, 8380456, Independencia, Santiago, Chile*

[4]*Department of Otolaryngology, Clínica Alemana de Santiago, Facultad de Medicina Clínica Alemana, Universidad del Desarrollo, Av. Vitacura 5951, 7650568, Vitacura, Santiago, Chile*

Received: 18 June 2012; Accepted: 25 February 2013; Online publication: 24 April 2013

ABSTRACT

The cells in the organ of Corti do not exhibit spontaneous cell regeneration; hair cells that die after damage are not replaced. Supporting cells can be induced to transdifferentiate into hair cells, but that would deplete their numbers, therefore impairing epithelium physiology. The loss of p27Kip1 function induces proliferation in the organ of Corti, which raises the possibility to integrate it to the strategies to achieve regeneration. Nevertheless, it is not known if the extent of this proliferative potential, as well as its maintenance in postnatal stages, is compatible with providing a basis for eventual therapeutic manipulation. This is due in part to the limited success of approaches to deliver tools to modify gene expression in the auditory epithelium. We tested the hypothesis that the organ of Corti can undergo significant proliferation when efficient manipulation of the expression of regulators of the cell cycle is achieved. Lentiviral vectors were used to transduce all cochlear cell types, with efficiencies around 4 % for hair cells, 43 % in the overall supporting cell population, and 74 % within lesser epithelial ridge (LER) cells. Expression of short hairpin RNA targeting p27Kip1 encoded by the lentiviral vectors led to measurable proliferation in the organ of Corti and increase in LER cells number but not hair cell regeneration. Our results revalidate the use of lentiviral vectors in the study and in the potential therapeutic approaches for inner ear diseases, as well as demonstrate that efficient manipulation of p27Kip1 is sufficient to induce significant proliferation in the postnatal cochlea.

Keywords: cochlear regeneration, supporting cell proliferation, p27Kip1 shRNA, Lentiviral vectors

INTRODUCTION

Hearing loss is a prevalent and important disease, affecting between one and three newborn infants per every 1,000 births, and increasing up to 89 % in the population over 80 years old (Erenberg et al. 1999; Cruickshanks et al. 1998). Commonly, hearing loss is a consequence of irreversible hair cells (HC) loss (Schuknecht 1993; McFadden et al. 2004). During intrauterine development of the organ of Corti (OC), cells permanently exit the cell cycle and differentiate (Ruben 1967). There is no cell replacement or regeneration during extrauterine life. The current therapies aimed to improve hearing do not address the HC loss problem.

Correspondence to: Juan C. Maass • Program in Physiology and Biophysics, Institute for Biomedical Sciences, Faculty of Medicine • Universidad de Chile • Av. Independencia 1027, 8380453, Independencia, Santiago, Chile. Telephone: +56-2-29786033; fax: +56-2-27776916; email: jcmaass@yahoo.com

Loss and gain of function studies of genes expressed in the inner ear during development have demonstrated that supporting cells (SC) retain some regenerative potential (Chen and Segil 1999; Löwenheim et al. 1999; Woods et al. 2004). Consequently, new HC have been generated in cochlear explants after Notch inhibition (Yamamoto et al. 2006) and AtoH1 overexpression (Zheng and Gao 2000). There is also evidence of functional HC generation after AtoH1 overexpression in vivo (Kawamoto et al. 2003; Izumikawa et al. 2005; Gubbels et al. 2008), but those strategies are not effective in severely damaged OC with absence of SC (Izumikawa et al. 2008). Therefore, it appears that increasing SC numbers is necessary for making transdifferentiation possible while preserving the epithelium physiology.

p27Kip1 controls the cell cycle progression and has been proposed as the main responsible for the permanent exit of SC from the proliferative cycle (Chen and Segil 1999; Löwenheim et al. 1999). White et al. (2006) showed that isolated SC in culture are competent to decrease p27Kip1 expression, and therefore proliferate and transdifferentiate into HC. There is also evidence that after decreasing p27Kip1 expression in vitro and in vivo it is possible to induce postnatal OC proliferation (Ono et al. 2009; Oesterle et al. 2011). These observations set the basis to explore the potential therapeutic use of p27Kip1 manipulation. Nevertheless, the ability of the OC to sustain significant proliferation after p27Kip1 knockdown through therapeutically feasible manners, such as viral vectors is not clearly established. Vectors aimed to modify gene expression and surgical approaches to deliver them into the OC in vivo have been developed (Lalwani et al. 1996; Staecker et al. 2001; Ishimoto et al. 2002; Bedrosian et al. 2006; Kilpatrick et al. 2011). Lentiviral vectors (LV) are widely used and efficient tools for gene delivery due to their wide tropism, ability to transduce dividing and non-dividing cells, potential for in vivo use, and long-term expression. Nevertheless, these vectors have not been successful in transducing the postnatal OC. Studies reported poor results, with lack of transduction of HC in vitro and lack of reaching the OC in vivo (Han et al. 1999; Pietola et al. 2008; Duan and Mi 2010).

Thus, we set to probe the hypothesis that the decrease of p27Kip1 expression in the postnatal cochlea achieved through viral delivered tools can induce significant proliferation of the epithelium. We efficiently transduced the OC with LV in rat and mouse explants and demonstrated their capacity to induce OC proliferation in rat cochlear cultures after delivery of short hairpin RNA (shRNA) targeting p27Kip1.

METHODS

shRNAs

Nineteen basepairs target sites were selected within the *Rattus norvergicus* p27Kip1 mRNA sequence (NM_031762.3) using a siRNA design software (http://i.cs.hku.hk/~sirna/software/sirna.php) (Yiu et al. 2005). Then those with significant sequence coincidences with other rat genes in "nr" database after "blastn" were removed (http://blast.ncbi.nlm.nih.gov). Sense and antisense oligonucleotides were synthetized (Sigma-Aldrich) to obtain chosen targets, as well as one scrambled sequence (control) containing adaptors for hairpin formation and cloning. After cloning, we tested their efficiency and selected the vectors containing the following oligonucleotides (target sequences shown italicized): p27Kip1-shRNA sense 5′-GATCCCC*GCCAGCGCAAGTGGAATTT*CCACACCAAATTCCACTTGCGCTGGCTTTTTGGAAA-3′ and p27Kip1-shRNA antisense 5′-AGCTTTTCCAAAAA*GCCAGCGCAAGTGGAATTT*GGTGTGGAAATTCCACTTGCGCTGGCGGG-3′; Control-shRNA sense 5′-GATCCCC*GCTCGGCAGCGATAATGCT*TTCAAGAGAAGCATTATCGCTGCCGAGCTTTTTGGAAA-3′ and Control-shRNA antisense 5′-AGCTTTTCCAAAAA*GCTCGGCAGCGATAATGCT*TCTCTTGAAAGCATTATCGCTGCCGAGCGGG-3′.

Lentiviral vectors

The oligonucleotides were sequentially cloned into two silencing plasmids: pZOFF and pFUGW-H1 following the Leal-Ortiz et al. (2008) procedure. The second plasmid expresses EGFP driven by the UbiC promoter and serves as a shuttle plasmid for a second generation LV also constituted by pCMVΔR8.91 and pVSV-G. Vectors were prepared in HEK 293T, harvested, and concentrated according to Sena-Esteves et al. (2004) protocol for high viral titers. Viral concentration was determined by flow cytometry with FACSCantoABD (Becton, Dickinson and Company) and modifying the protocol of Logan et al. (2004). Briefly, we transduced HEK 293T cells with serial viral dilutions: $1:10^3$, $1:10^4$, and $1:10^5$, and after 72 h we fixed the cells at 4 °C in PBS containing 2 % formaldehyde, 0.5 % bovine serum albumin, and 0.05 % sodium azide. Then, we determined the percentage of EGFP positive (+) cells and estimated the number of viral particles (VP) per microliter of viral stock present at transduction. The efficacy for p27Kip1 knockdown was initially evaluated by Western blot in E18.5 primary rat cortical neuron cultures (Goslin and Banker 1991) transduced or not transduced with LV with a multiplicity of infection (MOI) of 5 VP per cell. Subsequent work was performed only

with the vectors containing the most effective shRNA and the control shRNA.

Organotypic cochlear culture and transduction procedure

Newborn P0 Sprague Dawley rats and Carworth Farms 1 mice were euthanized and their cochleas were collected. The bony capsule, membranous lateral wall, and the spiral ganglia were removed. The hook was removed and the rest of the explants were divided into three portions and only the middle third was placed on Nuclepore Track Etch Membranes (Whatman) floating on DMEM-F12, supplemented with N2 (Invitrogen) and Penicillin G (Sigma) in a 5 % CO_2 incubator at 37 °C. Animal procedures were approved by the Ethics Committee in Animal Research, Faculty of Medicine, Universidad de Chile (protocol # 201).

To optimize the transduction procedure, we tested the addition of 10 µl of the control-shRNA LV to the cultures, with different concentrations (0.3×10^5 to 1.5×10^6VP/µl), in one or two doses, and with different routes of administration: alternatively, the concentrated viral stock was dissolved into the final culture medium (500 µl), used undiluted in 5 min incubation with the explants before culture or placed undiluted in a droplet on top of explants immediately after their attachment to the membranes. The latter was the most efficient and therefore the chosen method for subsequent procedures. The following experiments were performed by administering the vectors at 2×10^5VP/µl two times, separated by 8 h and followed by 12 h incubation. Culture media was replaced every second day. The EGFP fluorescence was checked (with no fixation) as reporter of transduction after 72 h and explants were cultured between 3 and 9 days.

Immunohistochemistry

After culture, explants were fixed with 4 % paraformaldehyde and stained. The primary antibodies used were anti-MyosinVI (MyoVI; Proteus Biosciences), anti-Prox1 (Chemicon), anti-p27Kip1 (Labvision Neomarkers), anti-BrdU (Abcam), anti-cleaved Caspase 3 (c-Casp3; Cell Signaling), anti-Phosphohistone3 (PH3; Cell Signaling), anti-Ki67 (Chemicon), and anti-GFP (Invitrogen). The secondary antibodies were Rhodamine Red-X, Cy5, and FITC (Jackson ImmunoResearch). The fluorescent dyes used were Hoechst 33258, DAPI, and Rhodamine-Phalloidin (Rhod-Phall; Invitrogen). For p27Kip1 staining, the samples were boiled in 10 mM citric acid pH 6 for 10 min to unmask epitopes. For BrdU staining, 2 N HCl incubation was performed for 10 min. Anti-GFP antibodies were used with BrdU staining to enhance the EGFP detection after acid treatment.

Image acquisition and analysis

Image acquisition was performed using a BX61WI DSU Olympus microscope, a LSM Pascal 5 Axiovert 200 Zeiss confocal microscope and an Axio Observer Z1 Zeiss microscope with Apotome.2. High magnification stacks of images were obtained across the epithelium. The pictures obtained with the DSU microscope were treated with a deconvolution algorithm using the program Huygens Professional 3.2.Op7 (Scientific Volume Imaging BV) and analyzed with ImageJ 139u (NIH) and CellR 2.7 (Olympus). Within these images, randomly selected 114× 114 µm fields aligned with HC rows and centered in the first row of outer HC were analyzed. Cell type markers detection, cell location, morphology, virtual slicing in the Z plane and 3D reconstruction of EGFP+cells were used in recognizing each cell type and determining the percentage of viral transduction per field in the tissue. Due to important differences in EGFP expression intensity found between the different cell types, images presented in the paper were optimized either for visualization of low or high EGFP expressing cells, according to the rationale of the figure. However, during analysis the EGFP+ and the EGFP negative (−) cells were quantitated using the information obtained from all of the brightness settings. Between the same cell type we found that the EGFP expression intensity did not varied significantly.

RNA extraction and quantitative PCR

The p27Kip1 mRNA level was determined in rat cochlear explants in duplicates per experimental condition using qPCR after transduction and 4 days in culture. We extracted total RNA from four cultures per replicate with the RNeasy mini kit (Qiagen) and synthesize cDNA using random primers with the SuperScript® III First-Strand Synthesis System (Invitrogen). The qPCR reaction was performed with SYBRGreen qPCR Master Mix in StepOnePlus Real Time PCR System (Applied Biosystems). The following primers were used: Gapdh forward 5′-CTCTCTGCTCCTCCCTGTTC-3′ and reverse 5′-GCCAAATCCGTTCACACC-3′; p27Kip1 forward 5′-GGAAGCGACCTGCGGCAGAA-3′ and reverse 5′-GCCAGCATTCGGGGAACCGT-3′. The analysis of relative gene expression level was performed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Proliferation assay

P0 cochlear cultures were studied after 6 days in vitro (DIV). The experiments were performed in triplicate for each of the following conditions: non transduced cultures (control), cultures transduced twice with 10 µl of control-shRNA LV (control shRNA), or p27Kip1-shRNA LV (p27Kip1 shRNA) at 2×10^5VP/µl. Ten fields per cochlear culture were analyzed (see sample size calculation in "Statistical analysis"). The proliferative status of the cochlear epithelia was assessed by BrdU, PH3, and Ki67 immunostaining and evaluated in the EGFP+ and in the EGFP– populations. The BrdU incorporation was evaluated after 2 h of incubation just before fixation using the BrdU labeling reagent kit (Invitrogen). The mitotic index was expressed as PH3+ cells/Ki67+ cells.

Immunostaining and morphological criteria were used to identify SC subtypes. The fields were divided into four regions to allocate the cells in proliferation: (a) OC region (OC), where the HC, phalangeal, pillar, and Deiters' cells were located; (b) lesser epithelial ridge (LER), containing Hensen's and Claudius' cells; (c) greater epithelial ridge region (GER), which contains inner sulcus cells and border cells; and (d) below the OC region (BeOC), constituted by tympanic border cells located under the OC basal membrane. The SC were distributed between OC and LER regions.

The rate of proliferation was calculated and expressed as the mean percentage of BrdU or PH3+ cells within total cell count per region (Hoechst+ cells). The efficacy of transduction to induce proliferation was expressed as the mean percentage of BrdU and EGFP+ cells within the total EGFP+ cell population per field; corrected by removing the number of BrdU and EGFP+ cells present in the control-shRNA LV and not considering the cells in GER and the HC population. A linear regression was performed between the percentage of EGFP+ cells and cells in proliferative cycle per field in cultures treated with p27Kip1-shRNA LV and control-shRNA LV.

Finally, for each condition ten fields per culture triplicate were analyzed to determine cell density per field for each OC cell type within EGFP positive and negative population.

Evaluation of hair cell transdifferentiation

The following features were analyzed to infer transdifferentiation: co-labelling of BrdU and MyoVI, increase in number or ectopic HC, increase in the numbers of contacts between HC or loss of spaces between HC in the epithelium and decrease in support cell markers. BrdU incorporation per HC was evaluated in transduced P0 rat cochlear cultures after 72 h BrdU incubation between 4th and 7th DIV. Incubation in 10 µM DAPT (Calbiochem) 5 DIV was used as positive control of transdifferentiation in our culture conditions.

Statistical analysis

All data are expressed as averages ± standard errors (SEM). The efficiency of transduction was described by the mean percentage of transduced cells per cell type and was analyzed by descriptive statistics. Statistical tests were performed with PAST2.01 (Hammer & Harper) and OpenEpi 2.3 (Emory University) softwares. The level of significance was defined as $p<0.05$. Sample size calculation was performed for proliferative study using Stat Calc (Epiinfo 3.4.3, CDC). The total number of cells and the basal rate of mitosis nearby the OC per explant (within the potential fields) was estimated using MyoVI and PH3 immunofluorescence pixels quantitation (Image J, NIH) in controls and considering OC cell types distribution per field. The number of fields to sample for significant differences detection was determined with a confidence of 95 %. The numbers of cells in proliferation, cells per region and field, and contacts between cells were compared using Mann–Whitney or Kruskal–Wallis nonparametric tests for single or multiple testing, respectively, since the samples did not accomplish normal distribution (Shapiro–Wilk test) or homocedasticity (F or Barlet test). The pairwise comparisons after Kruskal–Wallis test were performed by the Mann–Whitney test with the Bonferroni correction. For association analysis, linear correlation was used calculating coefficient of Pearson (r) and p values.

RESULTS

Lentiviral vectors transduce all cell types present in the postnatal rodent organ of Corti in culture

As a proof of the concept that decreasing p27Kip1 it is possible to induce proliferation in the OC, and in order to achieve it through a procedure with therapeutic potential, we selected the LV delivery as the method for stable expression of shRNA targeting p27Kip1. Despite a previous report showing low success of postnatal OC transduction in culture with LV (Han et al., 1999), we investigated the possibility of transduction with higher doses and different means of administration in order to improve the interaction between the vectors and the OC. First, we optimized the production of lentiviral stocks to regularly achieve viral titers from 2 to 3×10^5VP/µl. Then, the means of best viral administration was explored to select one that improved the transduction efficiency in the

cultured OC. We found that placing the concentrated preparation in a droplet on top of the cochlear explant already attached to a filter membrane allowed us targeting of the periphery of the explants and the OC (Fig. 1A, B). On the contrary, with the application of the vectors into the culture medium or by pre-incubation, the results revealed a more scattered distribution of transduced cells (EGFP+) through the epithelium with low transduction of OC cells, which was similar to the results reported by Han et al. (1999). Moreover, with this method, the highest efficiency of transduction was found in the cells actually facing the rim of the explant, which allowed the transduction of up to 63 % of HC and 81 % of SC when the dissection edge passed through the OC, thus exposing it to the vectors (not shown). In order to preserve the epithelium structure and include most OC cell types, explants were standardized to establish the dissection edge more laterally, approximately between Hensen's and Claudius' cells. After these procedures, all cell types were transduced (Fig. 1C–F and Table 1), and the obtained OC cultures showed normal morphology and EGFP expression for up to 9 DIV (not shown). The procedure of viral transduction was repeated to increase the transduction efficiency and avoid the use of higher viral titers (Table 1). Thus, we demonstrated that the cochlear explant transduction with LV is a feasible method to transduce all cell types that constitute the postnatal OC.

Then, we tested the efficiency of transduction in mouse and rat explants using different viral doses and concentrations and we evaluated the percentage of transduction for each particular cell type. We used EGFP, MyoVI, and Prox1 expression as references. Results showed different efficiencies between different cell types and species (Table 1) and higher transduction efficiency for SC (up to 43 %). The highest efficiency (74 %) and the highest EGFP fluorescence intensity was found in LER cells, the most lateral cells present in these explants. Transduction efficiency in the OC decreased from the lateral cells towards the medial region of the explants (Fig. 1E, F and Table 1), except in the HC, in which the percentage of EGFP+ cells was higher in inner HC than in outer HC (10.8±2.7 and 2.2±0.4 %, respectively, after double use of 2×10^6 VP in rat explants). The GER region accounted for the lowest percentage of transduction with a 0.05±0.0 %, and in the BeOC region the percentage of EGFP+ cells was 58.2±0.2 % and it did not vary from lateral to medial (double use of 2×10^6VP in rat explants). We found in mouse explants that the percentage of transduced cells increased with higher viral doses, and that the HC were transduced only after applying at least 2.0×10^6VP total per explant. When we compared rat and mouse explants transduced with similar viral amounts (2.0 and 2.2×10^6VP respectively) we found a higher transduction efficiency in rats than in mice for HC ($p=8.2\times10^{-3}$) and for SC ($p=3.0\times10^{-5}$; Table 1).

p27Kip1-shRNA lentiviral transduction induces a decrease in p27Kip1 expression and proliferation in the organ of Corti in culture

After demonstrating the ability to efficiently transduce SC in rat cochlear explants we designed and cloned rat oligonucleotides encoding shRNAs targeting p27Kip1 into the vectors to promote the SC re-entry into the cell cycle. We found one shRNA that markedly decreased p27Kip1 expression in primary rat neuronal cultures, dropping the protein level by 65 and 91 % after 4 and 6 DIV, respectively (Fig. 2A). Its capacity was confirmed in P0 rat mid portion cochlear cultures, which revealed a significant decrease in p27Kip1 immunostaining in transduced cells (Fig. 2C, D) and a mild reduction (17 %, $p=0.1479$) in total p27Kip1 mRNA level in the whole explant (Fig. 2B). This last modest response as measured in the entire cultures was probably due to p27Kip1 expression in other areas of the cochlear explants (not shown), while our transduction method targeted the most lateral part of the OC. Also immunostaining analysis showed that the level of reduction was related with the intensity of EGFP, with the lowest p27Kip1 labeling intensity found in the brightest EGFP+ cells (Fig. 2D).

After verifying that our vectors were effective to decrease p27Kip1 expression, we studied the appearance of proliferation markers in P0 rat cochlear cultures at 6th DIV. We compared non-transduced controls and cultures transduced twice either by control-shRNA LV or p27Kip1-shRNA LV (2.0×10^6 VP). Because other areas of the explants could constitute sources of proliferation, we evaluated the whole width of the explant near the OC by dividing the studied fields into four regions: OC, LER, GER, and BeOC (see "Proliferation assay" in "Methods"; Fig. 3A, B). In the SC holding regions (OC and LER) we found proliferating cells, a higher mitotic index and a significant increase in the overall number of positive cells for each proliferative marker per field after p27Kip1-shRNA LV transduction (Figs. 3C, D and 4 and Table 2). In the OC region we saw BrdU+ cells after 2 h of incubation only when the explants were transduced by p27Kip1-shRNA LV (Fig. 4A, B). In the LER and BeOC regions, although we found some BrdU+ cells in control explants we observed statistically significant increments of BrdU+ cells in the p27Kip1-shRNA LV-treated explants (Figs. 3D and 4C, D). Within the SC holding regions, the LER region contained most of the proliferation increase

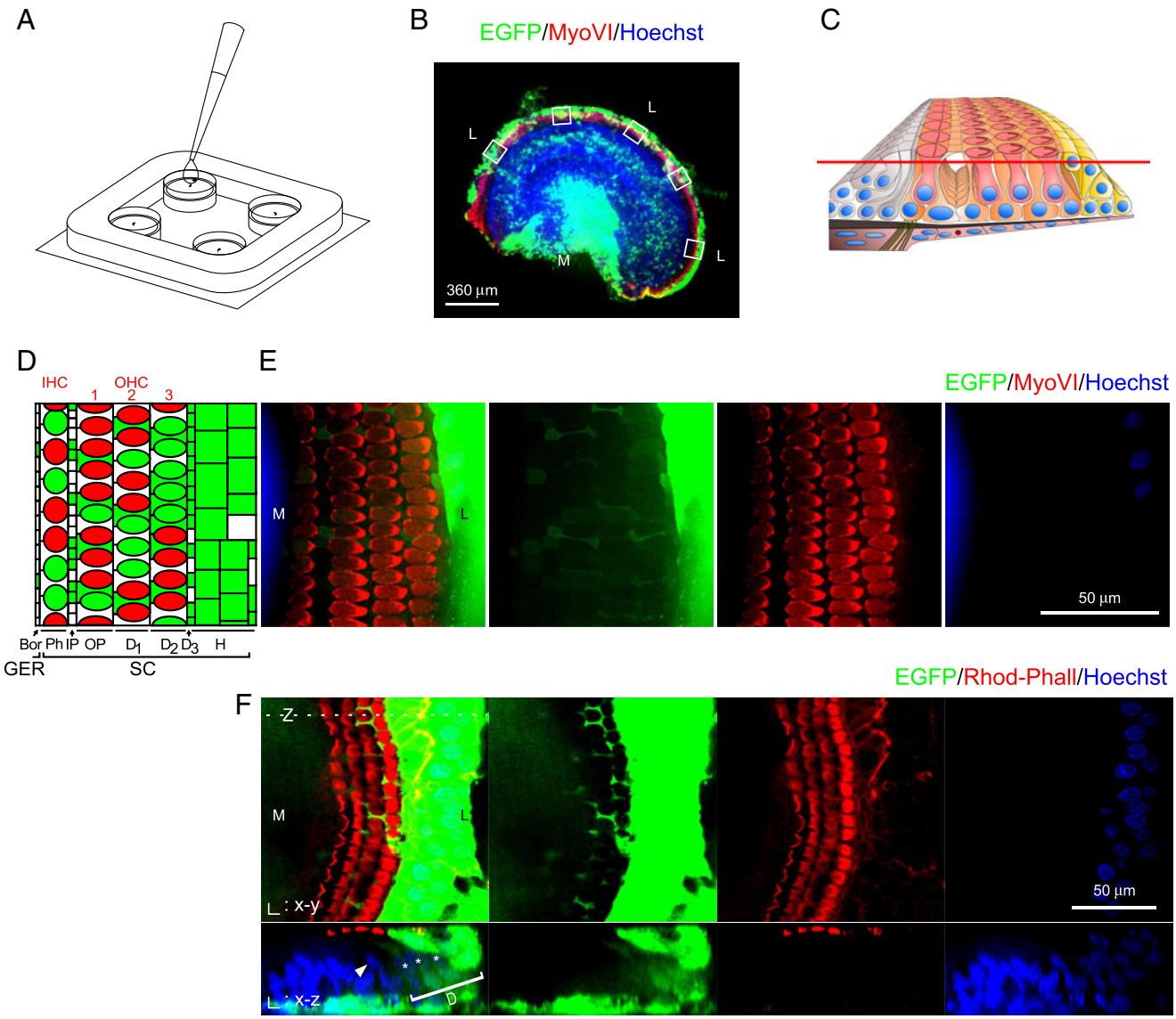

FIG. 1. Efficient lentiviral transduction of rodent postnatal cochlear explants. **A** Schematic view of the viral delivery procedure. Application of 10 µl of concentrated LV on top of cochlear explants floating in filter membranes. **B** Transduction of the middle third of cochlear explants of P0 mice cultured for 8 DIV with 10 µl of LV at 1.5×10^6VP/µl encoding a control shRNA. *White squares* represent the fields evaluated later in the study of proliferation. We show EGFP (*green*) as reporter of transduction, Myosin VI (*red*) and Hoechst (*blue*). **C** Representation of a cochlear explant indicating (*red line*) the level of the optical section pictured in (**D**) and (**E**). **D** Diagram of the apical domain of a transduced OC. Cells represented in the GER and OC: inner and outer HC (*IHC* and *OHC*), border (*Bor*), phalangeal (*Ph*), pillar (*IP* and *OP*), Deiters' (*D1*, *D2*, and *D3*), and Hensen's cells (*H*). **E** Cochlear mid portion explant obtained from a P0 rat and cultured for 5 DIV after control-shRNA LV transduction at 1.5×10^6VP/µl. **F** P0 rat cochlear mid portion explant cultured for 6 DIV after double control-shRNA LV transduction at 2×10^5VP/µl. *Arrow head* IHC. *Asterisks* OHC. *D* Deiters' cells. The explants exhibit moderate EGFP expression in most OC cell types and very intense EGFP expression in Hensen's cells. That difference impairs the detailed presentation of all transduced cells in this image. The pictures shown in (**E**) and (**F**) were optimized for EGFP visualization in the Deiters' cells. *M* medial, *L* lateral.

(Figs. 3D and 4D). In the whole field, the BeOC region had the greatest absolute increase and accounted for the majority of the rise of proliferating cells per field. However, when comparing the control-shRNA LV and p27Kip1-shRNA conditions, the relative increase in BrdU+ cells was 390 % in the LER and 210 % in the BeOC region. In confirming these findings in the cultures transduced by p27Kip1-shRNA LV, we also found PH3+ cells in the LER (Fig. 4C) and in the GER at 6th DIV, but with significant increases only in the BeOC region (p=0.04) and in the total count per field (p=0.02).

We evaluated cultures viability because we did not find any significant increase in PH3+ labeling between 7th to 9th DIV (not shown), we found a significant reduction in the number of BrdU+ cells at 6th DIV

TABLE 1

Transduced cells in mouse and rat cochlea

Explants from	*Concentration*	*Doses*	*Analyzed fields*	*Hair cells*		*Phalangeal cells*		*Pillar cells*		*Deiters' cells*		*LER cells*		*Supporting cells*[a]		*BeOC cells*	
Animal	*10^5 viral particles/µl*	*10 µl*	*n (F×S)*	*%*	*±*	*%*	*±*	*%*	*±*	*%*	*±*	*%*	*±*	*%*	*±*	*%*	*±*
Mouse	0.3	1	5 (5×1)	0.0	0.0	0.0	0.0	4.2	1.1	6.2	0.2	12.3	3.3	11.9	1.9	ne	
	1.2	1	5 (5×1)	0.0	0.0	1.5	1.7	0.6	0.6	7.2	3.1	14.3	3.7	8.4	1.9	ne	
	2.2	1	15 (5×3)	0.6	0.2	6.3	3.7	2.1	0.7	9.5	1.9	28.9	6.2	15.5	2.9	ne	
	5.0	1	15 (5×3)	0.8	0.5	3.4	1.6	4.3	1.0	10.9	2.0	38.6	7.5	19.5	2.8	ne	
	10.0	1	5 (5×1)	0.6	0.8	0.0	0.0	2.0	1.1	12.7	8.9	59.4	7.6	28.1	3.5	ne	
	15.0	1	5 (5×1)	0.9	0.6	10.0	9.0	11.5	5.0	31.0	13.9	75.1	12.9	48.0	15.2	ne	
Rat	2.0	1	15 (5×3)	4.1	0.6	1.6	1.0	9.3	2.8	13.1	3.3	66.0	5.9	36.6	3.0	ne	
	2.0	2	60 (10×6)	4.2	0.6	0.6	0.3	8.2	1.2	20.6	3.2	74.4	3.2	42.9	1.7	58.2	0.2

Data are presented for each cell type as mean percentage (%)±standard error (SEM). LER cells in the studied fields are predominantly Hensen´s cells including some Claudius' cells

[a]Supporting cells includes all cell types analyzed excluding hair cells. Beside the number of analyzed fields (*n*) we included the relation of fields per specimens (*F×S*). The explants were cultured between 3 to 6 DIV. *BeOC* region located below the OC. *ne* not explored

below the OC after the control-shRNA lentivirus transduction when comparing with the untransduced cultures (Fig. 3C, D), and there is evidence indicating that both these vectors and the knockdown of p27Kip1, acting independently, could be detrimental for the OC (Bedrosian et al. 2006; Kanzaki et al. 2006; Ono et al. 2009; Oesterle et al. 2011). However, we did not find morphological alterations or apoptosis in the fields by cleaved Caspase 3 (c-Casp3) immunostaining between 6th and 9th DIV at any condition (not shown).

To explain the origin of the increments in proliferation, we evaluated the number of BrdU+ cells in the EGFP+ and EGFP− population (Fig. 3D). We found that the significant increments in the proliferating cells per region and per field correspond to significant increases in the EGFP+ cells, and that the changes in the EGFP− cells were not significant, except in the BeOC region. Below the OC, the BrdU+ cells significantly increased within EGFP+ and EGFP− cells (p=1.0×10^{-7} and 3.0×10^{-3} respectively) even though the p27Kip1 immunostaining was negative (not shown).

In order to confirm that the increase in proliferation was due to our intervention we performed a linear regression analysis between the percentage of EGFP+ cells and cells in proliferative cycle on each region per field. We found positive and significant linear correlation between the percentage of transduction of Deiters' cells and the total number of proliferating cells per field (Pearson coefficient r=0.5, p=6.0×10^{-3}) in the explants treated with p27Kip1-shRNA LV and not with the control-shRNA LV. In the other cell types and regions the values did not have the linear behavior required to establish correlations between them (proliferating cells numbers close to "zero" and transduced cells per field near to 100 %). Thus, the Deiters' cells were a good indicator of the degree of transduction present per field and the total number of proliferating cells represented the proliferative response in the field.

Then, we studied the efficacy of our intervention to induce proliferation by defining it as the corrected percentage of BrdU and EGFP+ cells in the total EGFP population per field. And, we found a 4.3±0.2 % of proliferating cells per field after transduction.

Altogether, this evidence supports the assertion that it is possible to induce the SC to reenter the cell cycle and to induce an increase in proliferation in the whole explant after p27Kip1-shRNA LV transduction.

p27Kip1 knockdown after shRNA transduction induces an increase in support cell number in culture

In order to assess the impact of the proliferative response, we counted the number of cells per region

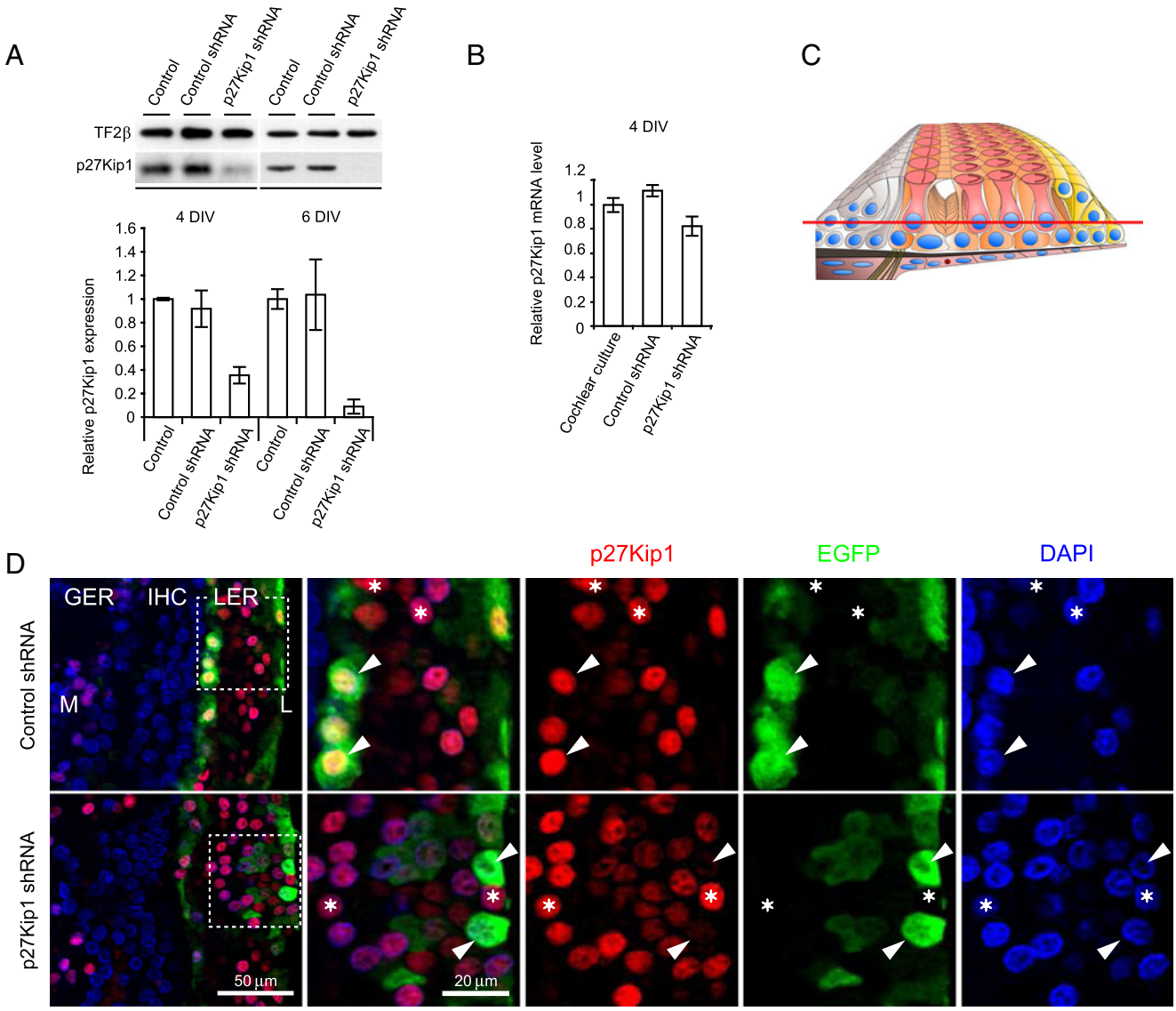

FIG. 2. Expression of shRNAs targeting p27Kip1 with LV effectively decreases p27Kip1 in rat neuronal cultures and cochlear explants. **A** p27Kip1 level in rat primary neuronal cultures. *Upper panel* representative immunoblot at 4 and 6 DIV. *Lower panel* quantification of p27Kip1 immunoblots normalized by TF2β expression. $n=2$ and 3 experiments for 4 and 6 DIV, respectively. Cultures transduced with MOI=5. **B** p27Kip1 mRNA levels in rat mid cochlear explants, 4 days after a double administration of 10 µl of LV at 2×10^5VP/µl. Relative quantification by $2^{-\Delta\Delta CT}$ method and Gapdh normalization. $n=3$ experiments. Data are expressed as means ± SEM. **C** Schematic representation of the cochlea showing the optical section level (*red line*) pictured in (**D**). **D** *Left panels* p27Kip1 immunostaining (*red*) 6 days after double application of 10 µl of 1×10^5VP/µl of either control-shRNA LV (*upper panel*) or p27Kip1-shRNA LV (*bottom panel*) to P0 mid cochlear explants. EGFP (*green*) is reporting transduction and DAPI (*blue*) is showing cell nuclei. The *dashed squares* show the regions magnified in the four right panels. Note that only in this last assay we reduced the viral amount enhancing the contrast between transduced and not transduced cells. *IHC* inner hair cells nuclei. *Arrow heads* examples of transduced cells. *Asterisks* examples of not transduced cells. *M* medial, *L* lateral.

and cell type. Although we did not find any association at the 6th DIV between the number of SC, the number of proliferating cells, and the percentage of transduction, we found a significant increment in the number of cells per field in the LER region in the p27Kip1-shRNA LV-treated explants, and an enlargement of that area in the cultures (Fig. 5B–D). Even though the total number of SC number significantly increased overall ($p=5.6\times10^{-5}$) we did not find significant changes in the "non LER" SC numbers.

To elucidate the source of the increase in the LER region, we evaluated the EGFP+ and EGFP– cells numbers. Results indicated significant increases in number of cells in both populations when comparing p27Kip1-shRNA LV and control-shRNA LV conditions ($p=1.5\times10^{-6}$ and 0.02 respectively). This result, and the fact that the increase in proliferation found in that region at 6th DIV was only significant in the transduced cells but was significant in both populations in the BeOC region (Fig. 4D),

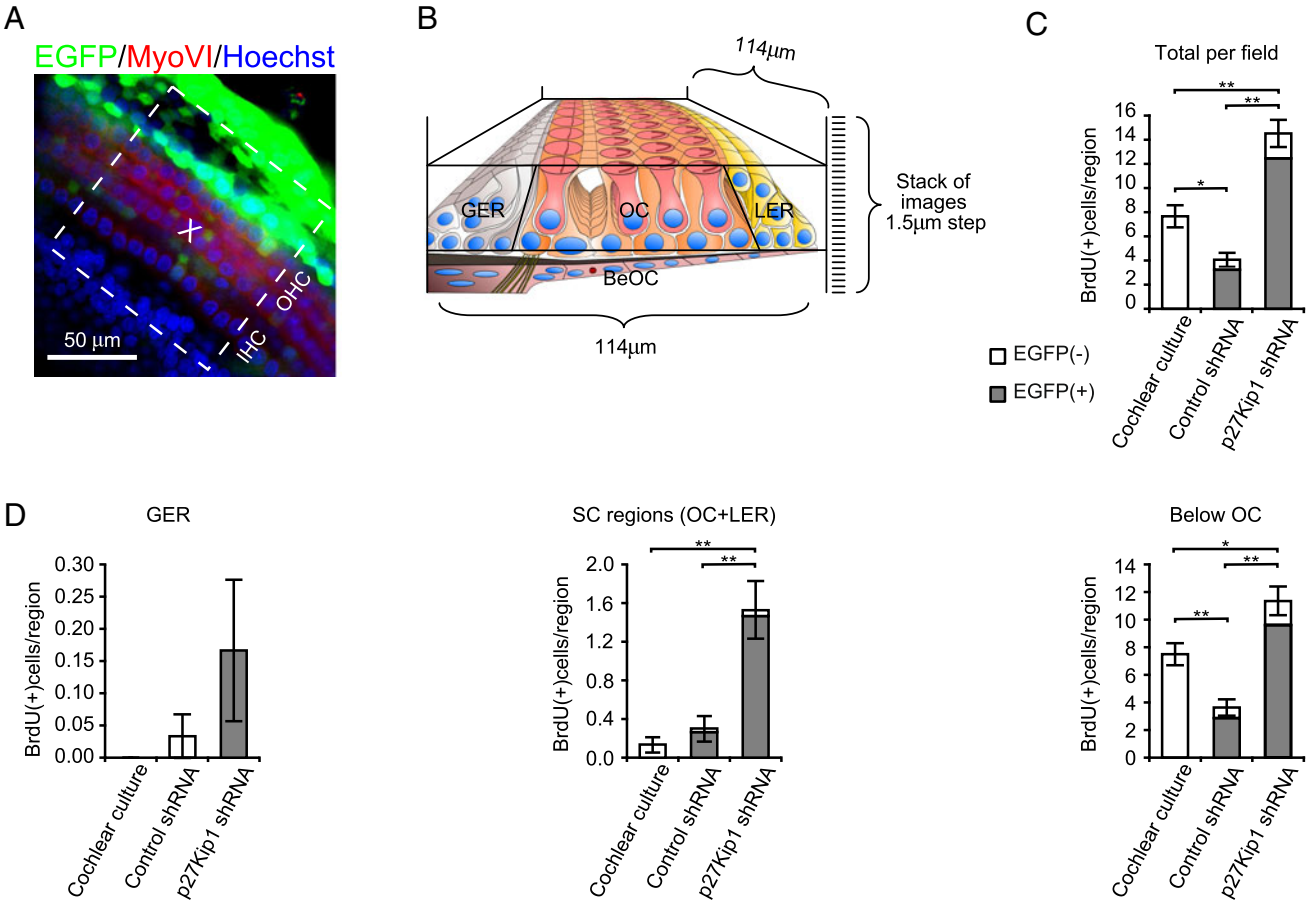

FIG. 3. Expression of shRNAs targeting p27Kip1 increases the proliferation in rat postnatal cochlear explants. **A** Example of an examined field in the proliferation study after transduction of LV encoding shRNAs targeting p27Kip1. The *dashed square* demarks a 114×114 µm field aligned and centered to the first row of outer HC (*OHC*) nuclei. EGFP (*green*) reporter of transduction, Myosin VI (*red*), and Hoechst (*blue*). **B** Schematic representation of the regions evaluated across the explants. Each field was divided in: GER, OC, LER, and BeOC regions. The SC are distributed into OC and LER regions. BeOC region contain cells located below the OC in the cultures (see "Proliferation assay" in "Methods"). **C** Number of BrdU+ cells per field at 6th DIV in cochlear explants transduced twice with 10 µl of 2×10^5 VP/µl. Multiple test overall significance: $p=1.4\times10^{-9}$ (Kruskal–Wallis test). Pairwise comparisons: $^*p<0.05$, $^{**}p<0.01$ (Mann–Whitney test with Bonferroni correction). **D** Number of BrdU+cells per region, merging OC and LER into SC regions. Multiple tests overall significance per region: GER $p=0.79$, SC regions $p=2.2\times10^{-4}$ and BeOC $p=6.3\times10^{-8}$ (Kruskal–Wallis test). Pairwise comparisons: $^*p<0.05$ and $^{**}p<0.01$ (Mann–Whitney test with Bonferroni correction). Data are means per region±SEM. *Gray and white bars* represent EGFP positive and negative cells, respectively.

could suggest that cell migration from below of the cultures could play a role in the increment of LER cells. Nevertheless, we did not find p27Kip1 negative cells in that region in the controls (Fig. 2D; unless decreasing their expression while proliferating) making that possibility unlikely, since the most plausible candidates for migration in the explants would be p27Kip1 negative cells from BeOC region.

p27Kip1-shRNA knock down does not associate to transdifferentiation of SC cells

Since there is evidence that cultured SC can proliferate and transdifferentiate into HC after decreasing p27Kip1 (White et al. 2006) we evaluated that possibility in our system. After 4 DIV, we incubated the transduced cultures with BrdU for 72 h and we looked for any MyoVI and BrdU double+ cells as an indication that proliferating cells (putative SC) later could express hair cell markers (HC differentiation). Although in our culture conditions SC were able to transdifferentiate into HC after 48 h of DAPT 10 µM incubation increasing the HC number (positive control, not shown), after knocking down p27Kip1 with this approach, we did not find any MyoVI and BrdU double+ cells or any other evidence of transdifferentiation, as inner, outer, or ectopic HC numbers did not change (Fig. 5A) and no morphologic changes suggesting transdifferentiation were apparent in the transduced explants (not shown).

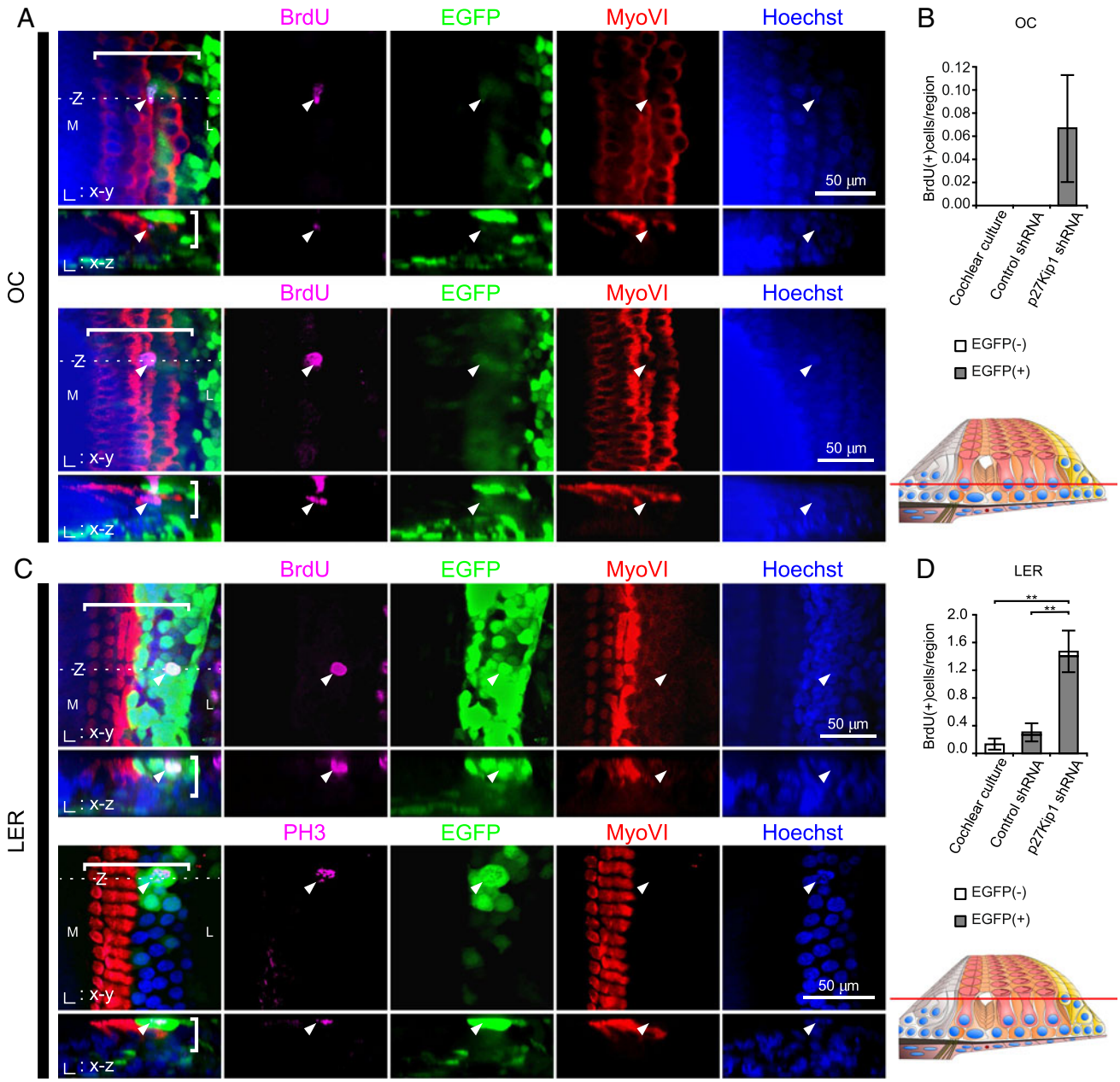

FIG. 4. p27Kip1 shRNA LV transduction increases proliferation rate in the regions containing SC in culture, the LER region (Hensen's and some Claudius' cells) accounts for the majority of the increase. **A**, **C** Proliferating cells and mitotic figures in OC and LER regions respectively in P0 rat mid cochlear portions transduced twice with 10 µl of 2×10^5VP/µl and cultured 6 DIV. EGFP (*green*) LV reporter, BrdU (2 h incubation) and PH3 (*both magenta*), MyoVI (*red*), and Hoechst (*blue*). *M* medial, *L* lateral. *Dashed line in upper panels* shows the location of the Z plane virtual slice presented in the *lower panels*. *Brackets* delimitates the area that holds SC. **B**, **D** Quantification of BrdU+cells in OC and LER regions, respectively. Overall significance for OC is $p=0.88$ and LER $p=6.4\times10^{-4}$ (Kruskal–Wallis test). Pairwise comparisons: $^*p<0.05$ and $^{**}p<0.01$ (Mann–Whitney test with Bonferroni correction). Data are expressed as means ± SEM. *Red lines* in the diagrams indicate the level of the optical section shown in (**A**) and (**C**). *Gray and white bars* represent EGFP positive and negative cells, respectively.

DISCUSSION

Our work confirms and extends previous observations about the role of p27Kip1 in the control of SC proliferation and the possibilities of manipulating it to induce cell division which may sustain therapeutic efforts towards functional regeneration. Secondarily, we established that the rodent postnatal hearing epithelium is amenable to being efficiently transduced by LV which allowed testing the significance of the proliferative capabilities of the epithelium.

This is the first report of successful transduction of postnatal HC and efficient SC transduction in rodents with LV. To the best of our knowledge, only four

TABLE 2

Proliferative status of transduced explants

		Cells in S[a] *(BrdU+/Hoechst+)*		*Cells in M (FH3+/Hoechst+)*		*Cells in proliferative cycle (Ki67+/Hoechst+)*		*Mitotic index (FH3+/Ki67+)*
shRNA	*Region*	*%*	*±*	*%*	*±*	*%*	*±*	*%*
p27Kip1	SC regions	0.82	0.17	0.02	0.02	0.56	0.17	3.57
	LER	1.38	0.34	0.04	0.04	1.29	0.38	3.10
	BeOC	8.16	0.70	0.78	0.12	6.27	0.87	12.44
	Field	3.94	0.35	0.41	0.06	3.61	0.43	12.92
Control	SC regions	0.18	0.07	0	0	0	0	-
	LER	0.44	0.18	0	0	0	0	-
	BeOC	1.49	0.25	0.53	0.11	5.08	1.01	10.04
	Field	0.98	0.16	0.24	0.04	1.58	0.47	10.04

Data are presented per region as mean percentage (%) ± standard error (SEM). S and M (mitosis phases)

[a]BrdU+cells after 2 h incubation. SC holding region (SC region) merges OC and LER regions. The cells in the GER region and the hair cells were excluded from this analysis. "Field" includes SC and BeOC regions

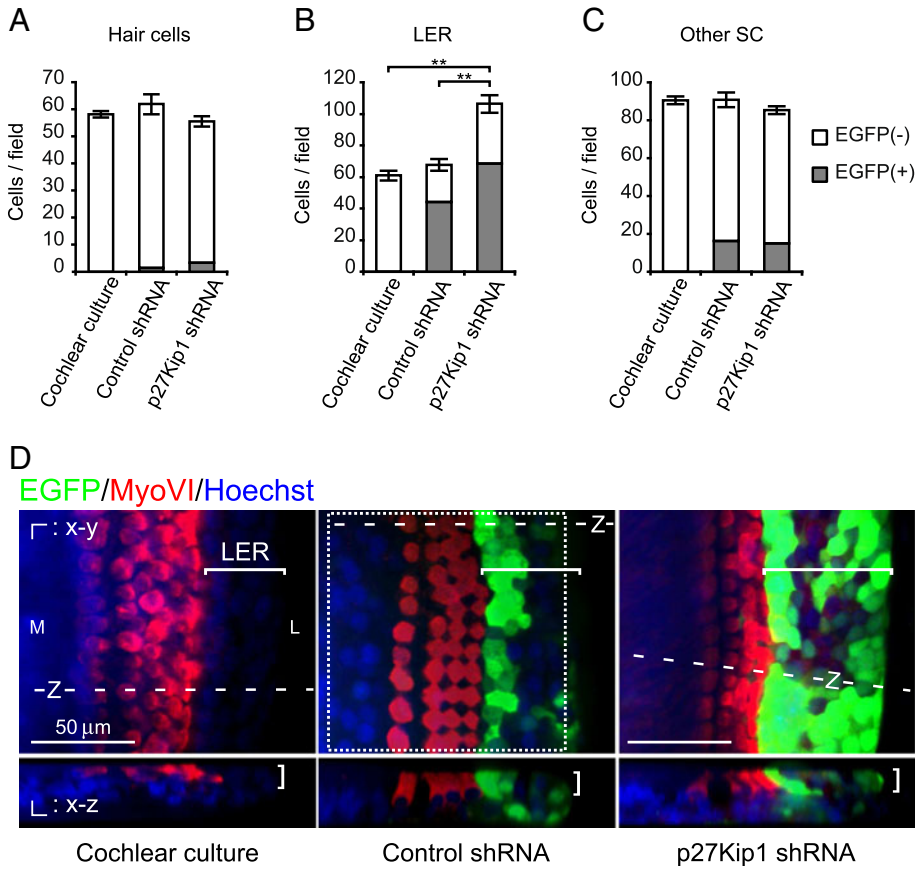

FIG. 5. Loss of p27Kip1 mediated by LV transduction increases the number of SC in the culture. Quantification of SC after transduction of p27Kip1-shRNA LV. **A–C** Number of cells in the sensory epithelium per field and per cell type after 6 DIV and double lentiviral administration of 10 µl of 2×10^5 VP/µl. "Other SC" includes supporting cells as Deiters', Pillar and Phalangeal cells excluding Hensen's and Claudius' cells. In the studied fields the cells in the LER are mostly Hensen's cells. *Gray and white bars* represent EGFP positive and negative cells, respectively. Overall significance per cell type: HC $p=0.16$, other SC $p=0.28$ and LER cells $p=2.5\times10^{-8}$ (Kruskal–Wallis tests). Pairwise comparisons: $*p<0.05$ and $**p<0.01$ (Mann–Whitney test with Bonferroni correction). Data are expressed as means ± SEM. **D** LER expansion in the p27Kip1-shRNA LV transduced explants. *Brackets* mark the width and depth of the region. EGFP (*green*) reporter of transduction, MyoVI (*red*), and Hoechst (*blue*). *Dashed line in upper panels* shows the location of the Z plane virtual slice presented in the *lower panels*. *Dotted square* delimitates the 114 x114 µm region quantitated in (**A–C**). The images have the same magnification. *M* medial, *L* lateral. Although EGFP was present in some hair cells and in other supporting cells, it is not apparent in this representation because these pictures are optimized for EGFP visualization in Hensen's cells due to differences in intensity of EGFP fluorescence between the different cell types (see "Image acquisition and analysis" in Methods).

previous reports addressed the LV administration to the rodent cochlea. One group transduced immature cochlear cells in utero, showing transgene expression and hearing impairment (Bedrosian et al. 2006), but did not explore the capacity to transduce postnatal OC cells. Three additional reports did not find evidence of OC transduction after perilymph injection (Han et al. 1999; Pietola et al. 2008; Duan and Mi 2010). Han et al. (1999) also used LV in postnatal cochlear explants but with a lower dose and different administration showing low rate of transduction in the OC and no HC transduction. Our method of vector delivery targeted the lateral region of the OC, thus obtaining a reproducible manner to transduce approximately 11 % of inner HC, 2 % of outer HC, 8 % of pillar cells, 21 % of Deiters' cells, and 74 % of LER cells (Hensen's and Claudius' cells). We explain our results and differences with previously published on the basis of our delivery technique and higher viral amount and concentration. We show that our approach associates to a higher transduction efficiency in SC; and also that it is more efficient transducing rat rather than mouse cochlear cells. Additionally, we did not detect any evidence of toxicity in the OC. This bunch of information will also serve to promote the use of LV in cochlear research taking advantage of their long-term expression and versatility of their design and tropism (reviewed by Mátrai et al. 2010).

There are still relatively few communications regarding the use of shRNA to manipulate gene expression in the inner ear and none published using LV in rodents to express shRNA in the OC. This could be due to the initial negative results with LV. However, we show that this strategy is useful and should be integrated into inner ear research, considering the availability of shRNA libraries already cloned into LV (Paddison et al. 2004; Moffat et al. 2006). Despite the negative results after perilymph injection by other groups (Han et al. 1999; Pietola et al. 2008), we propose to explore the potential role and efficiency of LV in vivo. For that purpose it is necessary to test higher doses and other delivery methods, such as injections into the *scala media*.

The effectiveness of transduction was associated to a significant effect on cell division, as expected for the stable loss of function of p27Kip1. Thus, despite a relative low rate of proliferation, we induced a significant increase in proliferative markers showing the feasibility to induce SC to reenter to the proliferative cycle with this methodology.

The proliferative response that we found (0.82 % of SC were BrdU+ after 2 h incubation at 6 DIV) was particularly significant taking into account the short period of action of our shRNAs, the short window of observation, and the low rate of basal and induced proliferation, which was also found in other studies. (Löwenheim et al. 1999) reported eight BrdU+ cells per 100 µm of the OC (approximately 4 % of the SC) in p27Kip1−/− mice after 4 days of BrdU administration. Another report with higher proliferation induction but with low gene delivery efficiency showed up to 13.8 BrdU+ cells per explant (approximately 0.35 % of SC) after cochlear explant electroporation with a different p27Kip1 shRNA and 3 days of BrdU incubation (Ono et al. 2009). Therefore, the mild change in the proliferative status of the epithelium that we observed could be of greater biological relevance because even a moderate response induced by our tools could have prolonged and more significant long-term effects.

The rate of proliferation found in the OC of p27Kip1 mutant mice decreased with age (Löwenheim et al. 1999). Nevertheless, Oesterle et al. (2011) showed lower but still measurable proliferative responses in the adult OC after conditionally deleting p27Kip1 in vivo in neonates and adult mice, raising the possibility to use approaches like ours to induce proliferation. Considering that cultured explants resemble the in vivo behavior of the epithelium and taking into account that at 6th DIV we still found a proliferative response to p27Kip1-shRNA LV, this method shows promise to be useful at least in early postnatal stages.

Although we did not identify the proliferating cells among all subtypes of SC using markers, this approach and the general morphology of the cultured explants allow us to propose that most, if not all of the induced proliferation affects native OC cells, in contrast with the migration of extrinsic cells.

In the LER region we found the highest relative increase in proliferation, the maximum percentage of transduction, and also the brightest fluorescence. The coincident observation of a higher proliferation rate in this region in mutant p27Kip1−/− mice (Chen and Segil 1999) and in p27Kip1-shRNA electroporated explants (Ono et al. 2009) could explain this not only based on a higher efficiency of transduction but also on a greater susceptibility to p27Kip1 downregulation.

We did not find a significant increase in proliferation in GER and OC regions. This could be due to the very low rate of proliferation found and insufficient power to detect differences in these regions. Nevertheless, it must be noted that we found evidences of proliferation in the OC region only in the p27Kip1-shRNA LV condition.

The BeOC region accounted for most of the absolute increment in proliferation observed in the explants transduced by p27Kip1-shRNA LV. We did not expect this increase since we could not detect p27Kip1 expression in this region. We do not have a plausible explanation of this, other than a 'tissue' response which is suggested by the occurrence of this increase in both EGFP+ and EGFP− populations and

the positive correlation found between the percentage of transduction in Deiters' cells and the number of BrdU+ cells per field. Conversely, in this region we also found a significant decrease in the proliferating cells in the explants treated with control-shRNA LV when compared with no treated explants. Thus, although we did not find c-Casp3+ cells, we cannot exclude a toxic effect of the lentiviral-shRNA system in this area. Should this be the case, this phenomenon could be masking a higher response to p27Kip1-shRNA LV. We report this information, because of the therapeutic potential of knocking down p27Kip1 with LV, and also because recently cells from this region, tympanic border cells, have been suggested as candidates for inner ear stem/progenitors cells (Chai et al. 2011; Jan et al. 2011), and their proliferative behavior was not previously described in the p27Kip1 loss of function reports (Chen and Segil 1999; Löwenheim et al. 1999; Kanzaki et al. 2006; Ono et al. 2009; Oesterle et al. 2011). All these changes in proliferation should be intensely evaluated in future research to corroborate the occurrence of a non-cell autonomous effect, as well as if they are also observed in vivo.

This is the first report suggesting an increase in SC number after inducing postnatal loss of function of p27Kip1. Previous studies did not show SC number counts (Ono et al. 2009; Oesterle et al. 2011). The increase in the number of cells in the LER region spatially coincides with the highest number of BrdU+ cells within the SC cells holding regions, the highest efficiency of transduction and the highest intensity of fluorescence per cell. However, we did not find association between the number of SC and the number of cells proliferating at 6th day or the percentage of EGFP+ cells per field. We believe that our mild but persistent proliferative response could explain the increments found in the SC number, although the possibility of migration from other areas of the explant cannot be completely refuted.

We did not find evidence of transdifferentiation from SC to HC. This is consistent with the observations in p27Kip1-shRNA electroporated explants (Ono et al. 2009) or in vivo after conditionally deleting p27Kip1 (Oesterle et al. 2011). And also with the idea that the higher number of HC present in mutant p27Kip1 cochleas (Chen and Segil 1999; Löwenheim et al. 1999; Kanzaki et al. 2006) was the result of an expanded population of progenitors before differentiation, and not persistent proliferation in the cochlea and further transdifferentiation.

These results sustain the concept that postnatal SC can be efficiently induced to proliferate by means of a viral-mediated decrease in p27Kip1 expression. The results also validate the role of LV as a useful tool to manipulate gene expression in the inner ear, by showing efficient transduction in postnatal cochlear explants. This sets the basis for designing in vivo experiments, seeking long-term effects and comparing differences in response based on age. Nevertheless, significant improvements in vector technology, including the control of delivered material, as well as the possibility of co-expressing molecules leading to transdifferentiation, should be reached before realistic therapeutic attempts are made.

ACKNOWLEDGMENTS

We thank Angelika Doeztlhofer (Johns Hopkins University), Neil Segil (House Ear Institute), Martin Basch, Alyssa Faught, and Andy Groves (Baylor College of Medicine) for advice and methodological support, and Pedro Zamorano (U. Antofagasta) for the silencing vectors. This work was supported by the grants from Sociedad Chilena de Otorrinolaringología, Medicina y Cirugía de Cabeza y Cuello (SOCHIORL) (Chile), Comisión Nacional de Ciencia y Tecnología (CONICYT) AT24071080 (Chile), Proyecto Anillo Ciencia y Tecnología ACT-47 (Chile), Iniciativa Científica Milenio ICM-PP07-048-F (Chile), and CONICYT fellowships (Chile) (to J.C.M. and J.C.).

REFERENCES

Bedrosian JC, Gratton MA, Brigande JV, Tang W, Landau J, Bennett J (2006) In vivo delivery of recombinant viruses to the fetal murine cochlea: transduction characteristics and long-term effects on auditory function. Mol Ther 14:328–335. doi:10.1016/j.ymthe.2006.04.003

Chai R, Xia A, Wang T, Jan TA, Hayashi T, Bermingham-McDonogh O, Cheng AG (2011) Dynamic expression of Lgr5, a Wnt target gene, in the developing and mature mouse cochlea. J Assoc Res Otolaryngol 12(4):455–469. doi:10.1007/s10162-011-0267-2

Chen P, Segil N (1999) p27Kip1 links cell proliferation to morphogenesis in the developing organ of Corti. Development 126:1581–1590

Cruickshanks KJ, Wiley TL, Tweed TS, Klein BE, Klein R, Mares-Perlman JA, Nondahl DM (1998) Prevalence of hearing loss in older adults in Beaver Dam, Wisconsin: the epidemiology of hearing loss study. Am J Epidemiol 148:879–886

Duan M, Mi Q (2010) Local delivery of reporter gene to the cochlea does not spread to brain tissue in an animal model. Acta Otolaryngol 130(1):25–30. doi:10.3109/00016480902963053

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. doi:10.1542/peds.103.2.527

Goslin K, Banker G (1991) Rat hippocampal neurons in low-density cultures. In: Banker G, Goslin K (eds) Culturing nerve cells, 2nd edn. MIT, Cambridge, pp 251–281

Gubbels SP, Woessner DW, Mitchell JC, Ricci AJ, Brigande JV (2008) Functional auditory hair cells produced in the mammalian cochlea by in utero gene transfer. Nature 445:537–541. doi:10.1038/nature07265

Han J, Mhatre AN, Wareing M, Pettis R, Gao WQ, Zufferey RN, Trono D, Lalwani AK (1999) Transgene expression in the

guinea pig cochlea mediated by a lentivirus-derived gene transfer vector. Human Gene Therapy 10:1867–1873. doi:10.1089/10430349950017545

ISHIMOTO S, KAWAMOTO K, KANZAKI S, RAPHAEL Y (2002) Gene transfer into supporting cells of the organ of Corti. Hear Res 173:187–197. doi:10.1016/S0378-5955(02)00579-8

IZUMIKAWA M, MINODA R, KAWAMOTO K, ABRASHKIN KA, SWIDERSKI DL, DOLAN DF, BROUGH DE, RAPHAEL Y (2005) Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. Nature Med 11:271–276. doi:10.1038/nm1193

IZUMIKAWA M, BATTS SA, MIYAZAWA T, SWIDERSKI DL, RAPHAEL Y (2008) Response of the flat cochlear epithelium to forced expression of Atoh1. Hear Res 240:52–56. doi:10.1016/j.heares.2008.02.007

JAN TA, CHAI R, SAYYID ZN, CHENG AG (2011) Isolating LacZ-expressing cells from mouse inner ear tissues using flow cytometry. J Vis Exp 58:e3432. doi:10.3791/3432

KANZAKI S, BEYER LA, SWIDERSKI DL, IZUMIKAWA M, STÖVER T, KAWAMOTO K, RAPHAEL Y (2006) p27Kip1 deficiency causes organ of Corti pathology and hearing loss. Hear Res 214:28–36. doi:10.1016/j.heares.2006.01.014

KAWAMOTO K, ISHIMOTO S, MINODA R, BROUGH DE, RAPHAEL Y (2003) Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. J Neurosci 23:4395–4400

KILPATRICK LA, LI Q, YANG J, GODDARD JC, FEKETE DM, LANG H (2011) Adeno-associated virus-mediated gene delivery into the scala media of the normal and deafened adult mouse ear. Gene Ther 18:569–578. doi:10.1038/gt.2010.175

LALWANI AK, WALSH BJ, REILLY PG, MUZYCZKA N, MHATRE AN (1996) Development of in vivo gene therapy for hearing disorders: introduction of adeno-associated virus into the cochlea of the guinea pig. Gene Ther 3:588–592

LEAL-ORTIZ S, WAITES CL, TERRY-LORENZO R, ZAMORANO P, GUNDELFINGER ED, GARNER CC (2008) Piccolo modulation of Synapsin1a dynamics regulates synaptic vesicle exocytosis. J Cell Biol 181(5):831–846. doi:10.1083/jcb.200711167

LIVAK KJ, SCHMITTGEN TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408. doi:10.1006/meth.2001.1262

LOGAN AC, NIGHTINGALE SJ, HAAS DL, CHO GJ, PEPPER KA, KOHN DB (2004) Factors influencing the titer and infectivity of lentiviral vectors. Human Gene Therapy 15:976–988. doi:10.1089/hum.2004.15.976

LÖWENHEIM H, FURNESS DN, KIL J, ZINN C, GÜLTIG K, FERO ML, FROST D, GUMMER AW, ROBERTS JM, RUBEL EW, HACKNEY CM, ZENNER HP (1999) Gene disruption of p27Kip1 allows cell proliferation in the postnatal and adult organ of Corti. Proc Nat Acad Sci U S A 96:4084–4088. doi:10.1073/pnas.96.7.4084

MÁTRAI J, CHUAH MK, VANDENDRIESSCHE T (2010) Recent advances in lentiviral vector development and applications. Mol Ther 18(3):477–490. doi:10.1038/mt.2009.319

MCFADDEN SL, DING D, JIANG H, SALVI RJ (2004) Time course of efferent fiber and spiral ganglion cell degeneration following complete hair cell loss in the chinchilla. Brain Research 997:40–51. doi:10.1016/j.brainres.2003.10.031

MOFFAT J, GRUENEBERG DA, YANG X, KIM SY, KLOEPFER AM, HINKLE G, PIQANI B, EISENHAURE TM, LUO B, GRENIER JK, CARPENTER AE, FOO SY, STEWART SA, STOCKWELL BR, HACOHEN N, HAHN WC, LANDER ES, SABATINI DM, ROOT DE (2006) A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell 124(6):1283–1298. doi:10.1016/j.cell.2006.01.040

OESTERLE EC, CHIEN WM, CAMPBELL S, NELLIMARLA P, FERO ML (2011) p27(Kip1) is required to maintain proliferative quiescence in the adult cochlea and pituitary. Cell Cycle 10(8):1237–1248. doi:10.4161/cc.10.8.15301

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. doi:10.1016/j.mcn.2009.08.011

PADDISON PJ, SILVA JM, CONKLIN DS, SCHLABACH M, LI M, ARULEBA S, BALIJA V, O'SHAUGHNESSY A, GNOJ L, SCOBIE K, CHANG K, WESTBROOK T, CLEARY M, SACHIDANANDAM R, MCCOMBIE WR, ELLEDGE SJ, HANNON GJ (2004) A resource for large-scale RNA-interference-based screens in mammals. Nature 428:427–431. doi:10.1038/nature02370

PIETOLA L, AARNISALO AA, JOENSUU J, PELLINEN R, WAHLFORS J, JERO J (2008) HOX-GFP and WOX-GFP lentivirus vectors for inner ear gene transfer. Acta Oto-Laryngologica 128:613–620. doi:10.1080/00016480701663409

RUBEN RJ (1967) Development of the inner ear of the mouse: a radioautographic study of terminal mitosis. Acta Otolaryngologica Supp 220:1–43

SENA-ESTEVES M, TEBBETS JC, STEFFENS S, CROMBLEHOLME T, FLAKE AW (2004) Optimized large-scale production of high titer lentivirus vector pseudotypes. Journal of Virological Methods 122:131–139. doi:10.1016/j.jviromet.2004.08.017

SCHUKNECHT HF (1993) Pathology of the ear, 2nd edn. Lea & Febiger, Philadelphia

STAECKER H, LI D, O'MALLEY BW JR, VAN DE WATER TR (2001) Gene expression in the mammalian cochlea: a study of multiple vector systems. Acta Otolaryngol 121:157–163. doi:10.1080/00016480130004330 7

WHITE P, DOETZLHOFER A, LEE YS, GROVES A, SEGIL N (2006) Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. Nature 441:984–987. doi:10.1038/nature04849

WOODS C, MONTCOUQUIOL M, KELLEY MW (2004) Math1 regulates development of the sensory epithelium in the mammalian cochlea. Nat Neurosci 7:1310–1318. doi:10.1038/nn1349

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 (Berl) 84(1):37–45. doi:10.1007/s00109-005-0706-9

YIU SM, WONG PW, LAM TW, MUI YC, KUNG HF, LIN M, CHEUNG YT (2005) Filtering of ineffective siRNAs and improved siRNA design tool. Bioinformatics 21:144–151. doi:10.1093/bioinformatics/bth498

ZHENG JL, GAO WQ (2000) Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. Nat Neurosci 3:580–586. doi:10.1038/75753