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Cell cycle regulation in the inner ear sensory epithelia: Role of cyclin D1 and cyclin-dependent kinase inhibitors

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ABSTRACT

Sensory hair cells and supporting cells of the mammalian cochlea and vestibular (balance) organs exit the cell cycle during embryogenesis and do not proliferate thereafter. Here, we have studied the mechanisms underlying the maintenance of the postmitotic state and the proliferative capacity of these cells. We provide the first evidence of the role of cyclin D1 in cell cycle regulation in these cells. Cyclin D1 expression disappeared from embryonic hair cells as differentiation started. The expression was transiently upregulated in cochlear hair cells early postnatally, paralleling the spatiotemporal pattern of unscheduled cell cycle re-entry of cochlear hair cells from the $p19^{Ink4d}/p21^{Cip1}$ compound mutant mice. Cyclin D1 misexpression *in vitro* in neonatal vestibular HCs from these mutant mice triggered S-phase re-entry. Thus, cyclin D1 suppression is important for hair cell's quiescence, together with the maintained expression of cyclin-dependent kinase inhibitors. In contrast to hair cells, cyclin D1 expression was maintained in supporting cells when differentiation started. The expression continued during the neonatal period when supporting cells have been shown to re-enter the cell cycle upon stimulation with exogenous mitogens. Thereafter, the steep decline in supporting cell's proliferative activity paralleled with cyclin D1 downregulation. Thus, cyclin D1 critically contributes to the proliferative plasticity of supporting cells. These data suggest that targeted cyclin D1 induction in supporting cells might be an avenue for proliferative regeneration in the inner ear.

Introduction

The inner ear sensory epithelia, the organ of Corti of the cochlea and the sensory epithelium of vestibular organs, comprise sensory hair cells (HCs) and various types of supporting cells. Normal hearing and balance functions depend on the generation and maintenance of correct numbers of HCs. Also supporting cells are required for proper inner ear function. Mammalian HCs and supporting cells are postmitotic cells. Similar to many other types of terminally differentiated cells, such as neurons, HCs do not divide even after trauma-induced loss of neighboring cells. Mammalian supporting cells do not normally proliferate, but mitogens can trigger their cell cycle re-entry *in vitro*. This response is prominent during neonatal period and declines significantly thereafter (Montcouquiol and Corwin, 2001a,b; White et al., 2006; Gu et al., 2007; Lu and Corwin, 2008). In contrast to the inner ear sensory epithelial cells, some other types of differentiated cells show a well-developed ability to produce daughter cells, one example being hepatocytes whose robust proliferation following injury underlies the distinct regenerative capacity of the liver (reviewed in Fausto, 2000). Interestingly, in contrast to mammals, inner ear supporting cells of non-mammalian species can divide and generate progeny that transdifferentiates into functional HCs. These events underlie the remarkable regenerative capacity of the inner ear sensory cells of birds, fishes and amphibians (Corwin and Cotanche, 1988; Ryals and Rubel, 1988).

Cyclin-dependent kinase inhibitors (Ckis), the negative cell cycle regulators, are key regulators of the timing of cell cycle exit in developing tissues. There are two Cki subfamilies, the Ink4 subfamily consisting of $p15^{Ink4a}$, $p16^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$, and the Cip/Kip subfamily consisting of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ polypeptides (reviewed in Besson et al., 2008). HCs and supporting cells have common precursors that exit the cell cycle during embryogenesis (Ruben, 1967). Earlier studies have highlighted the importance of $p27^{Kip1}$ in this process (Chen and Segil, 1999; Lee et al., 2006). In addition, $p27^{Kip1}$ controls the nonproliferative status of differentiated supporting cells (Löwenheim et al., 1999; White et al., 2006). The maintenance of the postmitotic state of HCs is controlled by the Ckis 19^{Ink4d} and $p21^{Cip1}$ (Chen et al., 2003; Mantela et al., 2005; Laine et al., 2007) and the prototypical member of the pocket protein family, the retinoblastoma protein (pRb) (Mantela et al., 2005; Sage et al., 2005).

Despite the data on Ckis and pRb, the core cell cycle machinery in the inner ear is to a large extent unexplored. Progression through the cell cycle is governed by cyclin-dependent kinases (Cdks) that are activated by binding to the regulatory subunits, the cyclins. Cyclin–Cdk complexes phosphorylate (inactivate) pRb and other pocket proteins (p107, p130), leading to E2F transcription factor-mediated

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activation of genes that promote cell cycle progression. Initial pRb inactivation is catalyzed by cyclin D–Cdk4 and cyclin D–Cdk6 complexes that trigger transition through the restriction point in G1-phase and, thus, commitment to a new round of cell division. Thereafter, full pRb inactivation is accomplished by cyclin E/A–Cdk2 complexes. The positive actions of cyclin–Cdk complexes are countered by Ckis. Members of the Ink4 subfamily inhibit cyclin D–Cdk4/6 complexes, while the Cip/Kip proteins can also inhibit cyclin D–, E–, A– and B–Cdk holoenzymes. In addition, there is another level of interaction between D-type cyclins and Cip/Kip inhibitors, in that these cyclins can titrate Cip/Kip proteins from cyclin E/A–Cdk2 complexes and, thus, also by this function promote cell cycle progression (reviewed in Besson et al., 2008).

The family of D-type cyclins is composed of cyclin D1 to D3 (cD1 to D3) in mice. They are often expressed in mutually exclusive cell types and developmental stages, but can also be expressed in overlapping patterns. D-type cyclins have at least partly non-redundant functions, as evidenced by developmental defects, albeit rather subtle, in mice in which a single family member is inactivated. However, these defects are exaggerated when 2 or 3 of these genes are simultaneously disrupted. This fact together with the data that inactivation of a single family member can cause compensatory upregulation of its relative suggest for collaborative functions as well (Fantl et al., 1995; Sicinski et al., 1995; Ciemerych et al., 2002). cD1 has broadest expression. In addition that cD1 regulates cell proliferation under normal conditions, its overexpression has been linked with the loss of cell cycle control in tumors (Sicinski et al., 1995; Landis et al., 2006). D-type cyclins are induced by mitogens and their levels decrease when mitogens are removed or when anti-mitogenic factors are added (Matsushime et al., 1991). Thus, D-cyclins serve as a link between the extracellular environment and the core cell cycle machinery.

It has been shown in the $p19^{Ink4d}$ single knock-out and more prominently in the $p19^{Ink4d}/p21^{Cip1}$ double knock-out (dko) mice that postnatal cochlear HCs abnormally re-enter the cell cycle (Chen et al., 2003; Laine et al., 2007). Analysis of these dko mice showed that cell cycle reactivation is initiated in the upper basal part of the cochlea at P3 and that it peaks at P7, when high numbers of proliferating HCs are found in the upper basal and middle parts of the cochlea. Thereafter, cell cycle re-entry abruptly decreases, so that of the 2 cochlear HC subtypes—the inner hair cells (IHCs) and outer hair cells (OHCs)—only a small number of IHCs and very few OHCs are proliferating at P10 and thereafter (Laine et al., 2007). Importantly, unscheduled proliferation of cochlear HCs from the $p19^{Ink4d}$ single mutant and $p19^{Ink4d}/p21^{Cip1}$ dko mice resulted in the death of these cells (Chen et al., 2003; Laine et al., 2007). In contrast to cochlear HCs, $p19^{Ink4d}/p21^{Cip1}$ inactivation did not trigger cell cycle reactivation of vestibular HCs (Laine et al., 2007). Inspired by this spatiotemporally very distinct pattern of cell cycle re-entry, we aimed to identify additional factors that regulate the maintenance of the postmitotic state of HCs and their proliferative capacity. We provide here evidence of the role of cD1. These results are likely to be important for the field of therapeutic HC regeneration and for understanding the regulation of the postmitotic state of differentiated cells.

Materials and methods

Mice

$p19^{Ink4d}/p21^{Cip1}$ dko mice were generated and genotyped by PCR using tail DNA extracts, as previously described (Laine et al., 2007). Control NMRI mice were used for mRNA and protein expression analyses. The *BAT-gal* reporter mice (Maretto et al., 2003) were used to study the activity of canonical Wnt/β-catenin signaling. Genotyping was done as described by Maretto et al. (2003). For timed pregnancies, the morning of vaginal plug identification was taken as embryonic day 0.5 (E0.5) and the day of birth as postnatal day 0 (P0). Animal care was in accordance with institutional guidelines.

Tissue preparation and immunohistochemistry on sections

Inner ears at E13.5, E15.5, E16.5, P0, P2, P3, P4, P7, P10, P15 and P60 were analyzed. Dissected inner ears were fixed overnight in 4% paraformaldehyde (PFA) in PBS. Inner ears from P3 and older mice were decalcified in 0.5 M EDTA, pH 8.0, followed by embedding in paraffin and cutting to 5-μm-thick sections. Epitopes were unmasked by microwave heating (800 W) in 10 mM citrate buffer, pH 6.0, for 10 min. An overnight incubation was performed with the following primary antibodies: rabbit polyclonal myosin 7a (Hasson et al., 1997); rabbit monoclonal cD1, mouse monoclonal cD2, rabbit monoclonal Ki-67, mouse monoclonal $p27^{Kip1}$ (LabVision/Thermo Scientific); and goat polyclonal $p57^{Kip2}$ (Santa Cruz Biotechnology). Detection was done with Vectastain Elite ABC kit or Vectastain Mouse-On-Mouse kit, and diaminobenzidine substrate (Vector Laboratories). Sections were counterstained with 2% methyl green and mounted in Permount (Fisher Scientific). In double-labeling experiments, myosin 7a staining was detected by immunofluorescence using Alexa 568-coupled secondary antibodies (Molecular Probes/Invitrogen) and thereafter $p27^{Kip1}$ was reacted as described above. Vectashield (Vector Laboratories) was used for mounting. In Ki-67/$p27^{Kip1}$ double-immunofluorescence, Alexa 568- and Alexa 488-coupled secondary antibodies were used. Analysis was done with a BX61 microscope (Olympus) using bright- and dark-field and Nomarski optics and epifluorescence. Images were acquired through CCD camera (DP70) and analySIS software (Olympus), and processed using Adobe Photoshop CS2 (Adobe Systems).

In situ hybridization

In situ hybridization was performed with riboprobes labeled with ^{35}S-UTP and with PFA-fixed, paraffin-embedded sections, as described (Wilkinson and Green, 1991). Inner ears at P0, P2, P3, P7, P10, P15 and P60 were analyzed for $p15^{Ink4a}$, $p16^{Ink4d}$, $p18^{Ink4c}$, $p21^{Cip1}$ and $p57^{Kip2}$ expressions. $p19^{Ink4d}$ and *cD1* expressions were studied at these stages as well as at E13.5, E15.5, E16.5 and P4. Sections were counterstained with hematoxylin. Images were processed using Adobe Photoshop CS2. Autoradiographic silver grains in the dark field image were selected, colored red and superimposed onto the brightfield image.

Cochlear wholemounts

Cochlear ducts were dissected from $p19^{Ink4d}/p21^{Cip1}$ dko and control mice at P7. Lateral wall and tectorial membrane were removed and specimens were fixed with PFA overnight. Wholemounts were incubated overnight with antibodies against cD1, myosin 7a or Ki-67, followed by incubation with Alexa 488- and Alexa 568-coupled secondary antibodies, and mounting in Vectashield. Images were acquired with a TSC SP5 confocal microscope using HCX PL APO 20×/0.7 (glycerol) and HC PL Fluotar 5×/0.15 (air) objectives (Leica). Three-dimensional image stacks were processed and analyzed using the Imaris 5.7.1 software (Bitplane).

Utricular explants infected with adenoviruses

Organotypic cultures of the utricular sensory epithelium were prepared from the $p19^{Ink4d}/p21^{Cip1}$ dko mice and wildtype littermates at P5. Explants were maintained on pieces of Nuclepore filter membrane (Whatman) in DMEM/F-12 medium supplied with 2 mM L-glutamine, penicillin (100 U/ml) (Gibco/Invitrogen) and 10% fetal bovine serum (HyClone/Thermo Scientific). Incubations were done in a humidified 5% CO_2 atmosphere at 37 °C. The adenoviral