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Research paper

Manipulating cell cycle regulation in the mature cochlea ☆

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Abstract

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

Keywords: Adenovirus; Gene transfer; *p27^{Kip1}*; *Atoh1*; *Skp2*; Guinea pig; Hair cell; Supporting cell; BrdU

1. Introduction

The sensory epithelium of hearing in mammals consists of terminally differentiated epithelial cells: sensory hair cells and non-sensory supporting cells. These cells are quiescent in mammals. Therefore, hair cell degeneration is irreversible and leads to sensorineural hearing loss. One potential therapy for hearing loss is induction of hair cell regeneration in the organ of Corti, the sensory region of the auditory epithelium.

Recent data demonstrate that forced expression of genes encoding hair cell development can induce transdifferentiation of non-sensory cells into new hair cells in the developing (Woods et al., 2004; Zheng and Gao, 2000) and mature organ of Corti (Izumikawa et al., 2005; Kawamoto et al., 2003; Shou et al., 2003). As therapy, this procedure would be suboptimal because transdifferentiation of supporting cells into new hair cells does not involve mitosis in the tissue. Thus, formation of new hair cells would reduce the number of supporting cells and compromise the ability to restore normal cochlear structure and function. In birds, where hair cell regeneration leads to functional recovery (Dooling et al., 1997; Marean et al., 1995; Niemiec et al.,

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1994; Saunders et al., 1992), non-sensory cells divide after a lesion to the epithelium (Hashino and Salvi, 1993; Raphael, 1992; Stone and Cotanche, 1994). To induce proliferation in the mature organ of Corti as part of the reparative process, it may be necessary to manipulate expression of genes that regulate cell cycle.

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

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

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

After maturation of hair cells in developing mammals, the expression of *Atoh1* is down-regulated (Zheng et al., 2000). However, over-expression of *Atoh1* (or its homologs) in cultures of developing or mature rat cochleae results in the production of ectopic hair cells derived from non-sensory epithelial cells (Shou et al., 2003; Zheng and Gao, 2000). Over-expression of *Atoh1* has also been shown to generate new hair cells in mature guinea pig cochleae in vivo (Izumikawa et al., 2005; Kawamoto et al., 2003). The goal of our experiments was to localize p27^{Kip1} in the mature auditory epithelium, to determine if forced expression of *SKP2* can induce proliferation in the tissue and to assess the potential for generation of new ectopic hair cells by *SKP2* alone versus *SKP2* in combination with *Atoh1* over-expression. We demonstrate that p27^{Kip1} is present in numerous types of non-sensory cells in the mature auditory epithelium and that over-expressing *Skp2* can induce proliferation but no ectopic new hair cells are formed. Forced expression of *SKP2* in combination with *Atoh1* increases the number and alters the pattern of ectopic hair cell generation as compared with *Atoh1* alone.

2. Materials and methods

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

2.1. Adenovirus vectors

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

2.2. Animals and inoculation surgery

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