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Pocket proteins and cell cycle regulation in inner ear development

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ABSTRACT Loss of neurosensory cells of the ear, caused by genetic and non-genetic factors, is becoming an increasing problem as people age, resulting in deafness and vestibular disorders. Unveiling useful mechanisms of cell cycle regulation may offer the possibility to generate new cells out of remaining ones, thus providing the cellular basis to induce new hair cell differentiation in the mammalian ear. Here, we provide an overview of cell cycle regulating genes in general and of those studied in the ear in particular. We categorize those genes into regulators that act upstream of the pocket proteins and into those that act downstream of the pocket proteins. The three members of the pocket protein family essentially determine, through interaction with the eight members of the E2F family, whether or not the cell cycle will progress to the S-phase and thus cell division. The abundant presence of one or more members of these families in adult hair cells supports the notion that inhibition of cell cycle progression through these proteins is a lifelong process. Indeed, manipulating some of those proteins, unfortunately, leads to abortive entry into the cell cycle. Combined with recent success to induce hair cell differentiation through molecular therapy, these approaches may provide a viable strategy to restore lost hair cells in the inner ear.

KEY WORDS: *hair cell regeneration, transdifferentiation, cell cycle, E2F transcription factor, pocket protein*

Introduction

The mammalian inner ear is shaped through a series of highly synchronized molecular steps, proceeding from proliferation of epidermal cells (the otic placode), mitotic exit and differentiation of specific cell types to generate the elaborate three-dimensional structures and neurosensory components, which make up the fully formed, functional inner ear. With the recent advances in the understanding of the development and maturation of the peripheral auditory system, restoration of hearing using biological approaches are being actively pursued, with the goal of regeneration and replacement of cochlear hair cells (HCs). Hearing loss has three major etiological components: genetics, environmental insults and aging. In general the cochlear degeneration occurs in a basal to apical progression with an initial loss of outer hair cells (OHCs), followed by the loss of inner hair cells (IHCs), a subsequent protracted deterioration or "dedifferentiation" of the supporting cells (SCs) and finally a retraction of nerve fibers away from the organ of Corti (OC). At end-stage disease, a single layer of cells remains on the basilar membrane, the flat epithelium (Kim and Raphael, 2007). Severity of the OC deterioration ranges in frequency of occurrence from partial to complete loss of HCs in the base of the cochlea, to extensive loss of HCs throughout the cochlea with retention of the SCs and least frequently of all, the eventual formation of the flat epithelium. Therapeutic strategies must be targeted to the severity and extent of pathology and will require proliferation of HCs and/or supporting cells with or without SC proliferation and transdifferentiation into HC. The ultimate success of these biological approaches may also be based on restoration of the normal cytoarchitecture of the organ of Corti. In this review, we will discuss the biology of HC replacement, provide an overview of the molecular regulation of cellular proliferation, detail the biological roles of pocket proteins and present the current state of hair cell regeneration.

Hair cell regeneration in vertebrates and mammals

As the otic placode invaginates to form the otic vesicle, sufficient molecular changes accumulate in the responding tissue making the process irreversible (Fritzsch and Beisel, 2001, Fritzsch

Abbreviations used in this paper: CDK, cyclin-dependent kinase; CDKN, cyclin-dependent kinase inhibitor; HC, hair cell; IHC, inner hair cell; OC, organ of Corti; Rb, retinoblastoma; SC, supporting cell.

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et al., 2006b, Groves, 2005). Morphogenesis of the roughly spherical otic vesicle gives rise to the cochlea with its sensory and non-sensory regions. The mature sensory endorgans are composed of post-mitotic mechanosensory HCs and their associated SCs, which are unable to spontaneously proliferate after birth (White *et al.*, 2006). Overall, HCs and SCs are arranged within a given epithelium in an organized and invariant mosaic, such that each HC is surrounded by SCs. In the mammalian cochlea this pattern is even more ordered, in that the cells are further arranged into tightly organized rows, three rows of OHCs and one row of IHCs, making up the OC (Kawamoto *et al.*, 2003, Mansour and Schoenwolf, 2005). Similar to the loss of the post-mitotic sensory cells, disturbance of the well designed cytoarchitecture of the organ of Corti can adversely affect hearing and lead to irreversible deafness. Regeneration of the mammalian organ of Corti is not a naturally occurring process. In contrast, non-mammalian vertebrates can regenerate lost sensory HCs (Stone and Cotanche, 1992).

Regeneration capabilities have been documented in both the auditory and vestibular endorgans of fish and amphibians, where regeneration results as part of the continuous replacement process or is triggered by damage of the sensory cells. In addition to cellular replacement, morphological restoration also occurs as reflected by the recovery of normal auditory and balance functional capabilities (Adler and Raphael, 1996, Baird *et al.*, 1993, Bhave *et al.*, 1995, Fritzsch *et al.*, 2006a, Lanford *et al.*, 1996b, Smith *et al.*, 2006, Taylor and Forge, 2005). Robust spontaneous and induced HC proliferation is observed in the inner ear and lateral line neuromasts of fish and amphibians over the life of the animal (Hernandez *et al.*, 2007, Jones and Corwin, 1993, Jones and Corwin, 1996, Lanford *et al.*, 1996a, Lanford *et al.*, 1996b, Lopez-Schier and Hudspeth, 2006, Popper and Hoxter, 1990). HC regeneration capacity, however, vary among the different sensory endorgans, as well as sensitivity to the types of injuries that triggers the regeneration phenomena. Yet, the molecular mechanism(s) for HC and SC increase and/or turnover in these species is still poorly understood.

New HCs are also continuously produced in the avian vestibular epithelium. In the basilar papilla, HC and SC turnover does not occur and regeneration is only induced after HC loss (Cotanche *et al.*, 1991, Girod and Rubel, 1991, Jorgensen and Mathiesen, 1988, Oesterle and Rubel, 1993, Oesterle *et al.*, 1993, Roberson *et al.*, 1992, Weisleder and Rubel, 1992, Weisleder *et al.*, 1995). Regeneration of the basilar papilla HCs can transpire through two different molecular paths, both of which are dependent on the associated SCs (Adler and Raphael, 1996, Cruz *et al.*, 1987, Duckert and Rubel, 1990). One mechanism is through phenotypic conversion of the SCs, without cell division, to directly form new HCs by transdifferentiation (TD) (Adler and Raphael, 1996, Duncan *et al.*, 2006, Roberson *et al.*, 2004). The second process requires that SCs re-enter the cell cycle, giving rise to new supporting cells and additional newly formed HCs (Cafaro *et al.*, 2007, Duncan *et al.*, 2006, Morest and Cotanche, 2004, Oesterle and Rubel, 1993, Oesterle *et al.*, 1993, Roberson *et al.*, 2004).

Mammalians have little to no capacity for HC proliferation, except in vestibular endorgans, where HCs were shown to exhibit some regeneration capacity after injury (Forge *et al.*, 1998, Li and Forge, 1997). However, the mechanism(s) and many of the factors underlying vestibular SC plasticity to go back into proliferation or directly transdifferentiate into HCs are still unknown. Currently, there is no reliable *in vivo* production of new HC in the postnatal OC either spontaneously or after trauma, leaving mammals vulnerable to permanent hearing deficits caused by hair cell loss. Interestingly, mature rodent cochlear SCs still seem to keep some of their original capacity to proliferate after HC injury (Yamasoba and Kondo, 2006, Yamasoba *et al.*, 2003), making those cells the best candidates for HC replacement in mammals as do their counterparts in non-mammalian vertebrates. More recently, *in vivo* TD of mammalian cochlear HCs was proven to be possible through adenovirus-mediated overexpression of *Atoh1* (Izumikawa *et al.*, 2005, Kawamoto *et al.*, 2003). However, TD depletes the organ of Corti of SCs and thus leads to functional restoration of a questionable duration because of the severe disruption of the normal OC cytoarchitecture. A better approach would be to initiate SCs proliferation first, followed by differentiation of those additional cells into HCs, which essentially mimics the non-mammalian vertebrate regeneration process.

Cell cycle regulation

Replicating the perfectly synchronized formation of the OC may be one of the biggest barriers to overcome when working on HC regeneration. The fast succession of events that promotes the development of a microscopic sheet of cells into a macroscopic structure with many different types of cells imply that the proper development of the inner ear depends on a well-regulated pattern of cell division and histogenic patterning (Groves, 2005, Mansour and Schoenwolf, 2005, Pauley *et al.*, 2005). Nevertheless, the molecular signaling pathways underlying and regulating controlled cell progression and cellular differentiation during mammalian otogenesis are largely unknown; hence, to better understand the regulation of otogenesis paradigms from other tissues can be utilized.

Cell cycle components

The pocket protein (pRBs) family, composed of *Rb1* (retinoblastoma 1), *Rbl1* (p107) and *Rbl2* (p130), has well-established roles in a wide variety of tissues for the regulation of cellular proliferation, differentiation and apoptosis through interactions with different molecules (Figure 1). Besides the E2F transcription factors, there are currently over 100 proteins that are reported to interact with the pRBs (Morris and Dyson, 2001). The pRBs can interact with both the E2F transcription factors and a complex comprising of cyclin (CCN) and cyclin-dependent kinase (CDK). It is the differential interactions among these proteins/complexes that distinguish the properties of these three pocket proteins in the regulation of the cell cycle (Cobrinik *et al.*, 1996, Lee *et al.*, 1996b). pRBs are hypo-phosphorylated in cells at G0 phase and becomes increasingly phosphorylated during the transition to G1 which is maintained throughout the remaining phases of the cell cycle in proliferating cells. Dynamic variation in the levels of all three hyper-phosphorylated pocket proteins are directly correlated with cell cycle progression and duration (Figure 2A) (Chatterjee *et al.*, 2004, Karantza *et al.*, 1993, Toppari *et al.*, 2003). pRBs phosphorylation is known to be directed during G1 by the complex of cyclin D (CCND) and cyclin dependent kinases 4 and 6 (CDK4/6) in association with CCNE-CDK2 complex (Cobrinik, 2005, Cobrinik *et al.*, 1996, Wang and Timchenko, 2005). The activity of CCND-CDK4/6 is in turn regu-

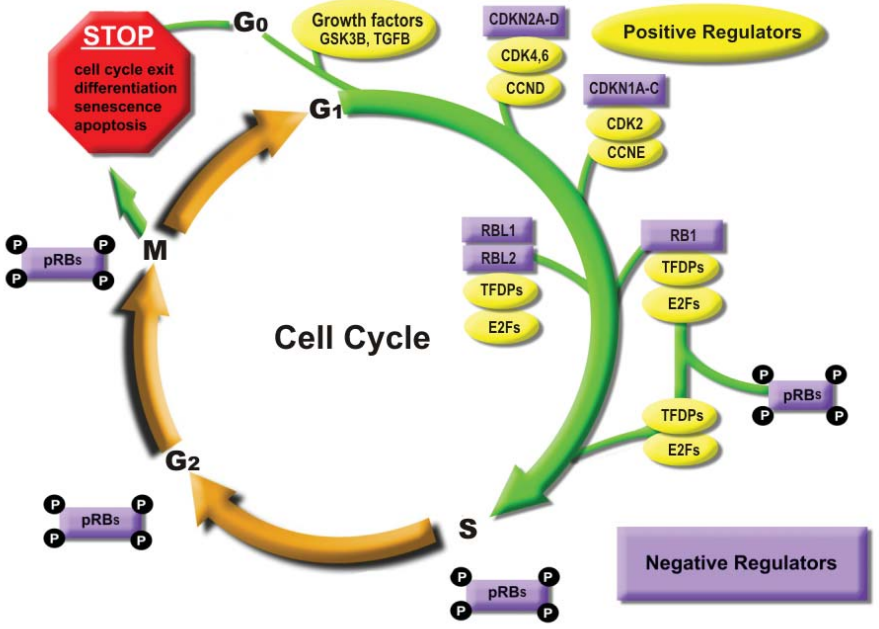

Fig. 1. Simplified schematic representation of the Rb1 pathway in the cell cycle regulation. *Regulation of cell proliferation is dependent on integration of positive regulatory systems that are activated by external signal molecules such as the growth factors. The complex formed by the pocket proteins and their associated E2Fs block cells in G_1 phase of the cell cycle. Dissociation of these complexes is regulated by the action of cyclins, CDKs and CDKNs; phosphorylated pRBs are unable to bind and inhibit E2F positive continuation of the cell cycle and cells progresses into S phase. Dependent on the correct interaction between the different components of this signaling cascade, the fate of the cells is determined in G0 phase either by going back in the G_1 phase of the cycle to continue proliferating or alternatively being arrested out of the cell cycle and induced to differentiate, become senescent, or to undergo apoptosis. Note that under certain circumstances cell cycle arrested cells may re-enter the cell cycle. Modified after Cobrinik (2005).*

lated by the cyclin-dependent kinase inhibitor 2a (CDKN2A, alias: p16) (Jin *et al.*, 2001, Omura-Minamisawa *et al.*, 2001), constituting one of the major pathways for the control of pRBs activity in virtually all cell types. The significance of this growth control pathway is emphasized by the frequent mutation of genes encoding CDKN2A and pRB in human cancers (Sherr, 1996, Sherr, 2000). RBL1 and RBL2 bind and inhibit the kinase activities of the CCNE/CDK2 and the CCNA/CDK2 complexes through a protein domain that is absent in RB1. This protein motif is similar to the CDK2 binding domain of the CDKN family members (Dagnino *et al.*, 1995, Schneider *et al.*, 1994, Zhu *et al.*, 1994, Zhu *et al.*, 1995a, Zhu *et al.*, 1993, Zhu *et al.*, 1995b).

Roles played by the pRB family members in development have been studied through numerous cellular and biochemical analyses as well as studies of genetically engineered null mutant mice. These studies have revealed distinct aspects of the *in vitro* and *in vivo* functions of *Rb1*, *Rbl1* and *Rbl2*. Investigation of double null mutants of the pocket proteins has demonstrated functional overlap within this gene family. Most notably, the lethality associated with *Rb1* ablation can be, at least partially, compensated by *Rbl1* and *Rbl2* expression during development (Table 1). Combined deletion of *Rb1* and either one of the remaining pocket protein genes results in much earlier mortality than in *Rb1* null embryos (Table 1) (Clarke *et al.*, 1992, Dannenberg *et al.*, 2004, Lipinski and Jacks, 1999, Robanus-Maandag *et al.*, 1998). Moreover, *Rbl1* and *Rbl2* also have overlapping roles as double homozygous null mice for these two pocket proteins die at birth, while single knockout mice survive and develop normally (Table 1) (Cobrinik *et al.*, 1996). *Rbl1* can fully compensate for *Rbl2* deficiency. However, the absence of both can be partially compensated by *Rb1*, since these animals survive to birth"(Mulligan *et al.*, 1998). Additional examples of the dynamics of pRBs compensation can be observed in the developing heart (MacLellan *et al.*, 2005), skin (Ruiz *et al.*, 2004) and primary mouse embryonic fibroblasts (Dannenberg *et al.*, 2004, Dannenberg and te Riele, 2006, Dannenberg *et al.*, 2000). Unlike the role of mutated RB1 in human retinoblastomas, mouse retinoblastomas are observed only when both *Rb1* and either *Rbl1* or *Rbl2* functions are lost (Spencer *et al.*, 2005). Taken together, these studies indicate that despite their diversification and specialization, the pRB family of proteins act in a coordinated fashion to regulate at least some cellular functions, such as E2F transcriptional activity, during cell cycle progression.

Compared to other tissues, the biochemical and molecular pathways of the cell cycle occurring during ear genesis is relatively unexplored. Immunohistochemical investigation of cell cycle proteins are primarily restricted to inner ear expression of RB1 and the CDKN1 proteins (Chen and Segil, 1999, Chen *et al.*, 2003, Laine *et al.*, 2007, Mantela *et al.*, 2005, Sage *et al.*, 2005, Sage *et al.*, 2006, White *et al.*, 2006). Likewise, analysis of genetically manipulated mouse models for *RBL1* and *RBL2* and their associated inner ear phenotype is also incomplete (Table 1). Furthermore, *Rbl1 and Rbl2* expression patterns are yet to be described in any detail in the developing and adult inner ear. Nevertheless, recent microarray analysis of E18.5 mouse (Table 2) and immunohistochemical staining of adult wild type and $Rb1^{-/-}$ mice (Figures 2 B,C) suggest that all three pRBs are present in the inner ear, although differentially expressed at different stages, cellular compartments and different cell types during and after OC development. Our preliminary data so far resemble those described in other systems showing a dynamic pattern of pRBs expression throughout the cell cycle (Cobrinik, 2005, Jiang *et al.*, 1997, Lee *et al.*, 1996a, Mulligan and Jacks, 1998, Schneider *et al.*, 1994, Sherr, 1996, Sherr, 2000, Sherr and McCormick, 2002, Zhu *et al.*, 1994, Zhu *et al.*, 1993).

Regulatory roles of cell cycle components

Coordination between cell proliferation and differentiation is important for normal development. Like in many, if not all, mammalian tissue, this process seems to follow an antagonistic dynamic process, in that differentiation is blocked in proliferative

cells and differentiated cells become postmitotic and no longer divide. In some instances, cyclins, cyclin-dependent kinases (CDKs), their inhibitors (CDKNs) (Chen *et al.*, 2002, Chen and Segil, 1999, Chen *et al.*, 2003, Harper and Elledge, 1996, Takebayashi *et al.*, 2005), as well as pRB (Mantela *et al.*, 2005, Sage *et al.*, 2005, Sage *et al.*, 2006) and members of the E2F family of proteins play important roles in this antagonistic regulation; however nothing is really known about this last component in the inner ear. Insights gained from cancer research shows that D-type cyclins associated with CDK4 or CDK6 are critical during G1 phase, as is CCNE-CDK2. Once activated by cyclin binding, these CDKs phosphorylate the pRBs. The activated pocket proteins directly regulate a

TABLE 1

SELECTION OF PHENOTYPES OBSERVED IN MICE DEFICIENT FOR SOME CELL CYCLE CONTROL GENES

Disrupted Gene	Ear Phenotype	Other Phenotypes	References
Rb1$^{-/-}$	Increased HC proliferation and apoptosis; SCs were also affected but to less extent.	Embryonic lethality (E13.5- E15.5); abnormal proliferation and apoptosis in CNS/PNS; defective hematopoiesis; Increased lens proliferation/ apoptosis.	(Clarke *et al.*, 1992, Mantela *et al.*, 2005, Sage *et al.*, 2006, Sage *et al.*, 2000)
Rbl1$^{-/-}$	Not analyzed	Embryonic lethality is not common; strain-dependent phenotype varying from complete normality to augmented proliferation in CNS neural progenitor cells and apoptosis; craniofacial, skeleton and limbs abnormality, growth deficiency, accelerated cell cycle and myeloid hyperplasia, etc.	(Cobrinik *et al.*, 1996, LeCouter *et al.*, 1998a, Lee *et al.*, 1996a, Vanderluit *et al.*, 2004)
Rbl2$^{-/-}$	Not analyzed	Lethality at mid-embryogenesis otherwise normal; strain-dependent phenotypes varying from normal to many adverse phenotypes involving abnormal neural, muscle and heart development; lethality age varies among strains; craniofacial, skeleton and growth abnormalities also observed in some backgrounds.	(Cobrinik *et al.*, 1996, LeCouter *et al.*, 1998b)
Rb1$^{-/-}$/ *Rbl1*$^{-/-}$	Not analyzed	Embryonic lethality 2 days earlier than embryos homozygous for *Rb1* alone; focal murine retinoblastoma, increased apoptosis in liver and CNS, deregulation of epidermal homeostasis.	(Lee *et al.*, 1996a, Ruiz *et al.*, 2004, Spencer *et al.*, 2005)
Rb1$^{-/-}$/ *Rbl2*$^{-/-}$	Not analyzed	Embryonic lethality 2 days earlier than embryos homozygous for *Rb1* alone; retinoblastoma, increased proliferation in myocytes and apoptosis in CNS and liver.	(Dannenberg and te Riele, 2006, Lipinski and Jacks, 1999, MacLellan *et al.*, 2005, Spencer *et al.*, 2005)
Rbl1$^{-/-}$/ *Rbl2*$^{-/-}$	Not analyzed	Neonatal lethality; decreased number of hair follicles and a clear developmental delay in hair, whiskers and tooth germs. Abnormal chondrocyte growth, defective endochondral bone development, shortened limbs.	(Cobrinik *et al.*, 1996, Ruiz *et al.*, 2004)
E2F1$^{-/-}$	Not analyzed	Animals are viable but prone to various tumors as they age; testicular atrophy and exocrine gland dysplasia; defect in T-cell differentiation due to fail in thymocytes apoptosis.	(Garcia *et al.*, 2000, Pan *et al.*, 1998, Wu *et al.*, 2001)
E2F2$^{-/-}$	Not analyzed	Animals are fully viable and do not show obvious developmental phenotypes; late-onset autoimmune disease with enhanced T-lymphocytes proliferation.	(Murga *et al.*, 2001, Wu *et al.*, 2001)
E2F3$^{-/-}$	Not analyzed	Reduced viability attributed to defects in proliferation of different cell types; Growth delay in the surviving animals followed by premature death probably due to congestive heart failure.	(Cloud *et al.*, 2002, Humbert *et al.*, 2000)
Rb1$^{-/-}$/ *E2F1*$^{-/-}$	Not analyzed	Late gestation lethality; significant reduction in formation of tumors otherwise commons in *Rb*$^{-/-}$ background; rescue of proliferation/unscheduled apoptosis in CNS, PNS and lens; partial rescue of hematopoiesis; defective skeletal muscle and lung development.	(Hyde and Griep, 2002, Pan *et al.*, 1998, Saavedra *et al.*, 2002, Tsai *et al.*, 1998)
Rb1$^{-/-}$/ *E2F2*$^{-/-}$	Not analyzed	Animals are viable; partial suppression of abnormal proliferation in CNS, retina and lens without unscheduled apoptosis.	(Saavedra *et al.*, 2002)
Rb1$^{-/-}$/ *E2F3*$^{-/-}$	Not analyzed	Late gestation lethality; rescue of proliferation in CNS, PNS, retina and lens without unscheduled apoptosis in CNS and PNS but not lens; no effects on apoptosis in retina; defective skeletal muscle and lung development; limited rescue of hematopoiesis.	(Ziebold *et al.*, 2001)
Cdkn2a$^{-/-}$	Not analyzed	Animal develop normally in spite of being highly cancer prone.	(Serrano *et al.*, 1996)
Cdkn2d$^{-/-}$	Sporadic reactivation of mature HC cell cycle followed by apoptosis.	Animals are viable and have a normal life span; no spontaneous development of tumors; testicular atrophy associated with increased apoptosis of germ cells without effecting fertility.	(Chen *et al.*, 2003)
Cdkn1a$^{-/-}$	Normal	Animals are viable and have a normal life span; increased T cell proliferation; increased autoantibody level; increased incidence of sarcomas and liver tumors and a decreased incidence of lymphomas.	(Arias *et al.*, 2007, Lebel *et al.*, 2001, Mantela *et al.*, 2005, Philipp-Staheli *et al.*, 2004, Salvador *et al.*, 2002)
Cdkn2d$^{-/-}$/ *Cdkn1a*$^{-/-}$	Profuse S-phase re-entry of postnatal HC, proliferation and apoptosis	Animals are viable (Observed phenotypes in double mutant mice: not described).	(Laine *et al.*, 2007)
Cdkn2d$^{-/-}$/ *Cdkn1b*$^{-/-}$	Not analyzed	Animals are viable but die at P18 days; bradykinesia, proprioceptive abnormalities and seizures, probably due to ectopic neuronal cell divisions and apoptosis in CNS system neurons that are normally quiescent.	(Chen *et al.*, 2003)
Rb1$^{-/-}$/Id2$^{-/-}$	Not analyzed	Animals survive to birth; erythropoiesis and neurogenesis are restored, but are followed by a slight increase in apoptosis in the PNS; abnormal muscle cells proliferation, differentiation and apoptosis.	(Lasorella *et al.*, 2000)

PNS, Periphral nervous sytem; CNS, Central nervous system.

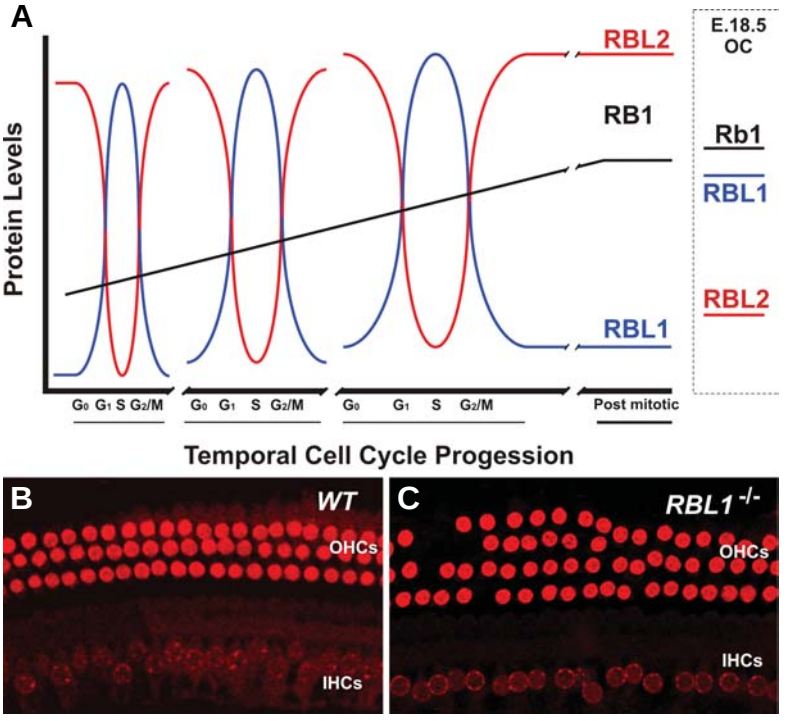

Fig. 2. Hypothetical relative expression of pRB family of proteins throughout the cell cycle. (A) *Cell cycle progression is regulated by a series of temporally coordinated phosphorylation events that target members of the pRB family. Progressive increasing in phosphorylated RB1 levels stimulate cell proliferation, whereas coordinated variation in RBL1 and RBL2 expression provide extra mechanisms for the control of cell cycle progression and duration. Moreover, pRB phosphorylation is often reverted by dephosphorylation, which normally occurs in late mitoses. Conversely, as the cell cycle progresses over time, dephosphorylated form of pocket proteins, particularly RB1, accumulate over time what seems to precede G1/G0 transition (Dailey* et al.*, 2003, Ludlow* et al.*, 1993, Moreno* et al.*, 2004). In post mitotic cells, including the HCs, RB1 levels tend to stabilize and become relatively steady throughout the cell life (Mantela* et al.*, 2005, Spencer* et al.*, 2005); nevertheless, the remaining pocket proteins levels seems to be variable and dependent on the cell type (MacLellan* et al.*, 2005, Spencer* et al.*, 2005). Microarray analysis of the pocket protein genes expression in the inner ear suggest RB1 and RBL1 as the predominant pocket proteins in the post-mitotic cells of the OC; whereas RBL2 levels seems to be lower (Detail in figure A). Immunofluorescence analyses of RBL1 expression in the OC of a P21 wild type* **(B)** *and* Pax2-Cre/Rb1$^{-/-}$ **(C)** *mice. Note that RBL1 is predominantly expressed in the multiple rows of out hair cells. Scale bar, 10 μm.*

controlled entry into and passage through the G1 phase of the cell cycle and then the progression into S phase by interacting with and regulating the activity of the E2F family of transcription factors (Figure 1) (Dulic *et al.*, 1994, Elledge and Harper, 1994, Harper *et al.*, 1993, Harper and Elledge, 1996, Matsuoka *et al.*, 1995, Sanchez *et al.*, 1997, Skowyra *et al.*, 1997, Takebayashi *et al.*, 2005, Zhang *et al.*, 1997, Zhang *et al.*, 1998, Zhang *et al.*, 1999). An additional level of CDK regulation is provided by CDKNs, which are divided into two families, the CDKN1 (previously Cip/Kip) family currently composed of CDKN1A (p21^{Cip1}), CDKN1B (p27^{Kip1}) and CDKN1C (p57^{Kip2}) and the CDKN2 (previously Ink4) family that includes CDKN2A (p16^{Ink4a}), CDKN2B (p15Ink4b), CDKN2C (p18Ink4c) and CDKN2D (p19^{Ink4d}). Both families of CDKNs seems to be involved in inner ear cell cycle arrest, as the loss of some members from both families results in cell cycle deregulation in HCs and SCs (Chen and Segil, 1999, Chen *et al.*, 2003, Laine *et al.*, 2007). These data suggest a role of both *CDKN1* and *CDKN2* genes in coordinately ensuring cell cycle exit and reaching a postmitotic state in both HCs and SCs (Laine *et al.*, 2007).

The expanding influence of the CDKs and CDKNs network in the inner ear has been the subject of several recent publications (Chen and Segil, 1999, Chen *et al.*, 2003, Laine *et al.*, 2007, White *et al.*, 2006). We will focus here on mechanisms through which pocket proteins are involved and control both proliferation and differentiation process through interaction with different E2F family members and provide additional insights into the inner ear cell cycle machinery.

Pocket protein and E2F protein interactions constitute one of the main nodes in cell cycle regulation

Ever since their first identification as a cellular factors that bound to and activated the adenovirus E2 promoter (Kovesdi *et al.*, 1986a, Kovesdi *et al.*, 1986b), the E2Fs are now considered key mediators of the pocket proteins activity, performing functions that go beyond the control of G1-S transition and include cellular proliferation, differentiation and apoptosis. Individual E2Fs have distinct mechanisms of action and regulation and are associated with particular types of biological activities (Cobrinik, 2005, Dimova and Dyson, 2005). These transcription factors can act by forming heterodimeric protein complexes with members of the DP family of transcription factors (TFDP1 and TFDP2) and work both as transcriptional activators and repressors of gene expression when bound to different members of the pocket protein family (Cobrinik, 2005, DeGregori *et al.*, 1997). 'Activating' E2F complexes are maximally expressed late in G1 and regulate cell cycle progression in proliferating cells. The 'repressing' E2F complexes are expressed throughout the cell cycle and are believed to be required for cell cycle exit and differentiation. Eight family members of *E2F* genes have been identified so far and are categorized as activator (E2F1, E2F2 and E2F3a), repressor (E2F3b, E2F4 and E2F5) and RB-independent repressor (E2F6, E2F7 and E2F8) (Adams *et al.*, 2000, Leone *et al.*, 2000). RB1 can interact and regulate the activity of both groups, but predominantly it interacts with 'activating' E2F1, E2F2, E2F3a; whereas RBL1 and RBL2 typically associate and control the 'repressing' E2Fs, e.g. E2F4 and E2F5 (Dimova and Dyson, 2005, Dyson, 1998, Leone *et al.*, 2000, Li *et al.*, 1997). The other family members, E2F6, E2F7 and E2F8 do not bind pocket proteins and repress E2F-responsive genes through other mechanisms (Cobrinik, 2005, Cobrinik *et al.*, 1992, Dannenberg *et al.*, 2004, Dimova and Dyson, 2005, Dyson, 1998, Li *et al.*, 1997, Logan *et al.*, 2005, Maiti *et al.*, 2005). In post-mitotic cells, inactivation of E2F function is mediated by binding to unphosphorylated pRBs. Moreover, pocket proteins acts as a transcriptional repressor complex by recruiting histone deacetylase (HDAC) and remodeling chromatin. When

cells move in a transcriptionally active status, phosphorylation of the pocket proteins by G1 cyclins and CDKs leads to an inability to bind E2F, which is released and becomes transcriptionally active (Figure 1) (Cobrinik, 2005, Dannenberg *et al.*, 2004, Dyson, 1998, Li *et al.*, 1997).

Among the group of E2F family members that interact with the pocket proteins (Figure 3), E2F1, E2F2 and E2F3a are considered potent transcriptional activators, binding according to our current understanding, exclusively to RB1 and peaking in expression during the G1-S transition (Dyson, 1998). Consequently, as expected, combined ablation of all three activator *E2Fs* results in severe deregulation of E2F-target gene expression, impairing the capacity of cell proliferation and highlighting the importance of the activator complexes in cell cycle progression (Wu *et al.*, 2001). In contrast, repressor E2Fs are expressed in quiescent cells and appear to be involved with cell cycle exit and differentiation (Dimova and Dyson, 2005). Given the crucial role of the activator E2Fs in the cell cycle regulation and direct interaction with RB1, one would predict that, similar to the loss of *Rb1* or any of the remaining pocket proteins (Table 1), specific loss of any of the activators should have devastating effects on development and organogenesis. However, the conditional deletion of the activator E2Fs have failed to demonstrate a unique requirement for any single E2F, suggesting that loss of individual E2Fs or specific combinations can be functionally compensated by other related family members (Wu *et al.*, 2001). The only exception was observed in embryos lacking E2F3, which have reduced viability presumably because of a defect in proliferation in certain cell types, such as fibroblasts (Humbert *et al.*, 2000, Saavedra *et al.*, 2002). Another contradiction is that the majority of double null mice for both *Rb1* and any of the activator E2Fs survive to birth (Table 1), especially the combination of $Rb^{-/-}E2F2^{-/-}$ seems to rescue in part the deleterious effects of *Rb1* loss in CNS, retina and lens without the ensuing apoptotic response. These latter data suggest the existence of a 'backup system' in these tissues, capable of rescuing and compensating for the combined loss of these genes (Saavedra *et al.*, 2002).

Although it seems paradoxical, taken together, all evidence in the inner ear and other systems suggest that the best way to manipulate *Rb1* expression is to avoid direct elimination of *Rb1*. Instead, other components of the *Rb1* regulatory network should be used to impact on *Rb1* controlled cell cycle progression in the inner ear either upstream (CDKN's) or downsteam (E2F's) or through the other pocket proteins. This could be accomplished by using DNA tumor virus proteins, SV40 large tumor antigen (LT), that bind to pRBs and displace E2Fs (Moreno *et al.*, 2004), by inappropriate RB1 phosphorylation due to overexpression of CCNDs, due to loss of CDKNs, loss of the CDKN2A, or by mutation or amplification of the CDK4 or CDK6 genes (Chen and Segil, 1999, Chen *et al.*, 2003, Cheng *et al.*, 2000, Jin *et al.*, 2001, Laine *et al.*, 2007, Sherr, 1996, Sherr and McCormick, 2002, White *et al.*, 2006). Another alternative would be to temporarily manipulate some of the pRB-specific binding partners, the E2F1, E2F2 and E2F3a. All three activating E2Fs are capable of interacting specifically with *Rb1* and not with the other pocket proteins; like the pRBs they are tightly regulated during cell proliferation and accumulate as cells progress into the cell cycle (Cobrinik, 2005, Saavedra *et al.*, 2002). Experimental evidences are already available showing that ectopic expression of any of these E2Fs can efficiently induce S phase entry in postmitotic cells. Overexpression of *E2F1* is sufficient to reactivate DNA synthesis in cells that would otherwise be in cell cycle arrest. Moreover, microinjections of *E2F1* cDNA into post mitotic cells can induce S-phase entry (Johnson *et al.*, 1994, Johnson *et al.*, 1993). However, both E2F1 and E2F3, but not E2F2, are known to play important roles in the control of apoptosis and deregulation of their interaction with RB1 leads to greatly exaggerated cell death (DeGregori and Johnson, 2006, DeGregori *et al.*, 1997, Hou *et al.*, 2002, Saavedra *et al.*, 2002). Of great importance, E2F2 has been described to efficiently activate DNA synthesis in quiescent fibroblasts to an extent equal to E2F1, but without induction of apoptosis, suggesting that $E2F1^{-/-}/E2F3^{-/-}$induced apoptosis is not simply a default response triggered by the induction of an irregular S phase entry but rather may represent an intrinsic property of those specific transcription factors (DeGregori and Johnson, 2006, DeGregori *et al.*, 1997, Dimova and Dyson, 2005, Hou *et al.*, 2002, Pan *et al.*, 1998, Saavedra *et al.*, 2002). Indeed most recent evidence suggests that E2F2 can be used to induce new heart muscle cell formation without inducing apoptosis (Ebelt *et al.*, 2006).

Cell cycle mechanisms in the organ of Corti

A general notion among many ear researchers is that lack of proliferative ability is part of the adult mammalian HC and SC identity, a characteristic shared with most neurons in the CNS and heart muscle cells. As such, only a few studies examined the process of cell cycle regulation in the adult HCs/SCs of the OC, as they are believed to have undergone terminal mitosis and thus these studies were deemed an exercise in futility. Currently, a growing body of evidence attests to the ability of both HCs and SCs to be released from their 'permanent' post mitotic arrest, re-enter cell cycle and proliferate (Laine *et al.*, 2007, Mantela *et al.*, 2005, Sage *et al.*, 2005, Sage *et al.*, 2006, White *et al.*, 2006). Key regulators of the cell cycle were identified in the inner ear cells, notably *Cdkn1b* (Endo *et al.*, 2002, Kanzaki *et al.*, 2006, Kim and Raphael, 2007, Lee *et al.*, 2006, White *et al.*, 2006), *Cdkn2d* (Chen *et al.*, 2003, Laine *et al.*, 2007) and *Rb1* (Mantela *et al.*, 2005, Sage *et al.*, 2005, Sage *et al.*, 2006). It is thought that these regulators play crucial roles in the maintenance of the post-mitotic state of both HCs and SCs. CDKN1B expression is induced in the embryonic organ of Corti between E12 and E14, correlating with the end of cell division in HC and SC progenitors (Chen and Segil, 1999). During development of the postmitotic progenitor cells, CDKN1B expression is down-regulated in HCs, but persists at high levels in the SCs and spiral ganglion of the mature organ of Corti (Chen and Segil, 1999, Endo *et al.*, 2002). Mice bearing a targeted deletion of the *Cdkn1b* gene show proliferation of the sensory cell progenitors beyond E14, leading to the appearance of supernumerary HCs and SCs and severe hearing loss (Chen and Segil, 1999, Kanzaki *et al.*, 2006). Similarly, targeted disruption of the *Cdkn2d* gene (Chen *et al.*, 2003) have been shown to lead to abnormal DNA synthesis in postnatal cochlear HCs, resulting in a aberrant proliferation pattern, followed by apoptosis and progressive hearing loss (Chen *et al.*, 2003, Laine *et al.*, 2007). Mutations in *Cdkn1a* does not cause alterations in the inner ear structure, although co-deletion of both *Cdkn2d* and *Cdkn1a* triggered profuse S-phase entry of postnatal HCs, followed by p53-mediated apoptosis (Laine *et al.*, 2007). Altogether,

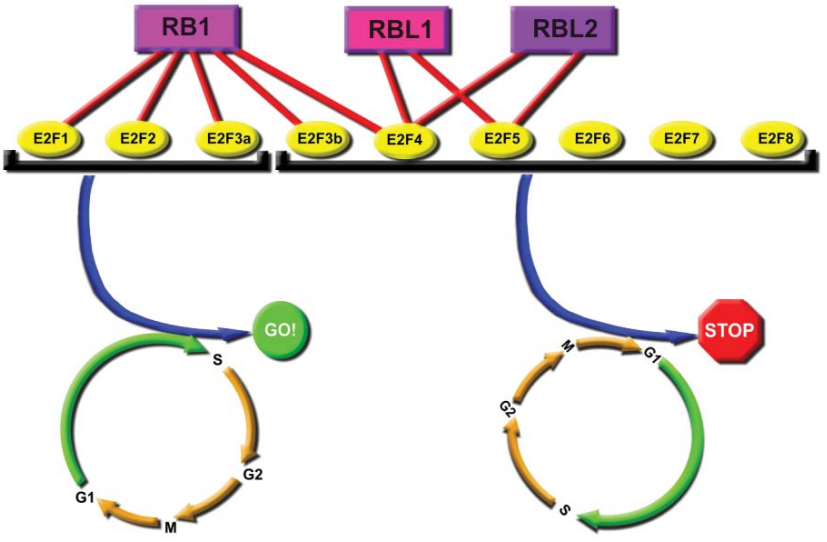

Fig. 3. Interaction of the pRBs with the E2F family of transcription factors make up one of the major features distinguishing the three pocket proteins from each other. *Eight E2F family members have been identified to date, which are divided in two subgroups: E2F1-E2F3a are considered transcriptional "activators", while E2F3b-E2F8 act by "repressing" the cell cycle continuity. RB1 can interact with both activating and repressor E2Fs, but generally bind activating E2Fs to promote cell cycle progression. RBL1 and RBL2 associate with but may not be limited to the repressing E2F4s and E2F5s and are believed to be required for cell cycle exit and differentiation. The remaining E2Fs form transcriptional repressor complexes in a pocket protein independent manner. Modified after Cobrinik (2005).*

these studies highlight the critical role of the CDKNs in the maintenance of the postmitotic state of the OC cells and suggest that loss of specific cell cycle inhibitors in the OC leads to pathology and dysfunction (Kanzaki *et al.*, 2006).

In addition to the CDKNs, *Rb1* plays an essential role in cell-cycle exit, maturation and survival of sensory HCs and SCs. RB1 expression seems to be up-regulated in the HCs of the developing OC, but it is confined to the IHCs and, at lower levels, the SCs of the mature OC (Mantela *et al.*, 2005). Loss of *Rb1* in postnatal HCs leads to aberrant HCs and, to some extent, SCs proliferation (Mantela *et al.*, 2005, Sage *et al.*, 2005, Sage *et al.*, 2006). Nevertheless, apoptotic loss of supernumerary HCs in the *Rb1* null mice seems to be spatially and temporally controlled, ranging from 'mild', as in both cochlea and vestibular system of a early postnatal *Rb1* null mice (Mantela *et al.*, 2005) and in the vestibular end-organs of adult conditional *Rb1* knockout (Sage *et al.*, 2006), to massive as detected in the OC of both the conditional *Tg(Pou4f3-Cre)/Rb1*$^{tm(loxP)/tm(loxP)}$and the tamoxifen-inducible conditional *Tg(Atoh1-CreER)/ Rb1*$^{tm(loxP)/tm(loxP)}$ adult mice (Sage *et al.*, 2006, T. Weber and J. Zuo, Personal communication). Overall, these studies reveal crucial, yet distinct roles of RB1 in the inner ear cells, which can also be dependent on the presence and level of expression of the remaining pocket proteins, suggesting that there is more to be learned about the inner ear sensory epithelia cells' cell cycle regulation than just cyclins, CDKs, CDKNs and RB1 alone. If indeed both HCs and SCs have permanently exited the cell cycle, how can we explain even the limited capacity of adult mammalian vestibular supporting cells to proliferate and replace lost hair cells (Roberson and Rubel, 1994, Warchol *et al.*, 1993)? Likewise, how can we explain the ability of the non-sensory cells of the OC flat epithelium to re-enter the cell cycle and proliferate (Kim and Raphael, 2007)? To be able to answer these questions we need a better understanding of the mechanics behind inner ear cells cell cycle regulation. Restricting our focus to just a handful of molecules and what we already know about their roles in inner ear development may blind us to the full repertoire of elements which may also be required for the proper control of HCs and SCs formation, maturation and rescue. As outlined above, alternative approaches have already been implemented successfully in other, equally post mitotic systems (Ebelt *et al.*, 2006) and should, in principle, be transferable to the ear, with proper adjustments.

Future perspectives

Cancer cells and developmental abnormalities that occur in pocket protein depleted mice models have provided key lessons to be learned on their function that should be extrapolated to their role in the inner ear (Table 1) (Gallie *et al.*, 1992, Gallie *et al.*, 1990). During the development of the inner ear *Rb1* has been shown to be important in both maturation and survival of HCs and SCs (Mantela *et al.*, 2005, Sage *et al.*, 2005, Sage *et al.*, 2006), manipulating RB1 or another pRB signal molecule(s) (either upstream through CDKN's or downstream through E2Fs) may provide the answer for the development of appropriate alternatives for the new proliferation of lost sensory HCs. Available data suggests that complete elimination of *Rb1* in the inner ear results in massive proliferation of HCs and to some extent SCs, followed by massive apoptotic death of all sensory HCs (Mantela *et al.*, 2005, Sage *et al.*, 2005, Sage *et al.*, 2006, T. Weber and J. Zuo, personal communication). A more transitory and possibly incomplete, inactivation of *Rb1* should be a more promising approach for HC regeneration. Such approaches may be possible using siRNA to transiently knockdown RB1 mRNA and should be safe if driven by SC-specific promoters such as *Gfap* or *Plp1* (Morris and Dyson, 2001, Rio *et al.*, 2002). Based on these insights and those derived from other systems, we predict that the inhibition of mammalian hair cell division is a life-long process in these cells. Reinforcement for this assumption comes from the fact that abnormal proliferation of hair cells precedes the activation of DNA damage response and *TP53*-dependent apoptosis (Laine *et al.*, 2007). Although safe manipulation and re-initiation of the cell cycle of the post mitotic inner ear HCs may be difficult if not impossible to achieve, these results suggest that more specific strategies need to be developed which target other cells and different cell cycle components than the ones that were targeted so far. Indeed, the combination of experimental evidence of the role of RB1 role in the inner ear and the fact that all three activating E2Fs are expressed in the developing organ of Corti (Table 2) suggest that, comparable to RB1, the cell cycle control and progression in the inner ear's sensory epithelia is

E2F-dependent and offers new opportunities to manipulate RB1 activity in the HCs and SCs.

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