p27Kip1 links cell proliferation to morphogenesis in the developing organ of Corti

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SUMMARY

Strict control of cellular proliferation is required to shape the complex structures of the developing embryo. The organ of Corti, the auditory neuroepithelium of the inner ear in mammals, consists of two types of terminally differentiated mechanosensory hair cells and at least four types of supporting cells arrayed precisely along the length of the spiral cochlea. In mice, the progenitors of greater than 80% of both hair cells and supporting cells undergo their terminal division between embryonic day 13 (E13) and E14. As in humans, these cells persist in a non-proliferative state throughout the adult life of the animal. Here we report that the correct timing of cell cycle withdrawal in the developing organ of Corti requires p27Kip1, a cyclin-dependent kinase inhibitor that functions as an inhibitor of cell cycle progression. p27Kip1 expression is induced in the primordial organ of Corti between E12 and E14, correlating with the cessation of cell division of the progenitors of the hair cells and supporting cells. In wild-type animals, p27Kip1 expression is downregulated during subsequent hair cell differentiation, but it persists at high levels in differentiated supporting cells of the mature organ of Corti. In mice with a targeted deletion of the p27Kip1 gene, proliferation of the sensory cell progenitors continues after E14, leading to the appearance of supernumerary hair cells and supporting cells. In the absence of p27Kip1, mitotically active cells are still observed in the organ of Corti of postnatal day 6 animals, suggesting that the persistence of p27Kip1 expression in mature supporting cells may contribute to the maintenance of quiescence in this tissue and, possibly, to its inability to regenerate. Homozygous mutant mice are severely hearing impaired. Thus, p27Kip1 provides a link between developmental control of cell proliferation and the morphological development of the inner ear.

Key words: p27Kip1, Organ of Corti, Inner ear, Cell cycle, Cyclin-dependent kinase inhibitor

INTRODUCTION

Developmental control of morphogenesis requires that cell proliferation be coordinated with growth and differentiation. Recent studies in Drosophila have begun to identify some of the developmental signals that are able to influence cell cycle progression (Go et al., 1998; Johnston and Edgar, 1998; Weigmann et al., 1997), as well as some of their likely cell cycle targets (Edgar and Datar, 1996). In vertebrates, the existence of analogous pathways linking development to control of the cell cycle machinery that controls cell proliferation remains poorly understood.

Development of the organ of Corti, the auditory sense organ of mammals, involves the differentiation of two types of mechanosensory hair cells (inner and outer) and four types of supporting cells (Deiters’, Hensen’s, Claudius’ and pillar) (Fig. 1). Each of these cell types has a distinct morphology that contributes to the complex structural and functional properties of the organ of Corti. Both the auditory and vestibular sense organs of the inner ear develop collectively from a thickening of the epithelial sheet in the dorsolateral region of the head known as the otic placode (Fritzsch et al., 1998). In the mouse, after its formation on embryonic day 8.5 (E8.5), the placode invaginates to form the otocyst on E11. By this time, specification of the regions that will give rise to sensory neuroepithelia containing the mechanosensory hair cells and supporting cells, as well as non-sensory epithelia, which will give rise to tissues such as Reisner’s membrane and the endolympathic tube and sac, have already been specified (Morsli et al., 1998). By E12, the otocyst has begun to develop morphologically into the cochlea and vestibule. The organ of Corti is recognizable at this time as a thickened ridge in the cochlear portion of the otocyst (Fig. 2B, arrow). At E12, the progenitors of the hair cells and supporting cells are still dividing (Ruben, 1967).

Between E12 and E16, the cells of the primordial organ of Corti exit the cell cycle to form a postmitotic neuroepithelial
sheet suspended in the spiral duct of the cochlea (Ruben, 1967). Greater than 80% of these cells exit the cell cycle between E13 and E14, with a slightly increased probability of cells located in the apex of the mature cochlea having exited the cell cycle earlier than those in the base (Ruben, 1967). Starting on E15, a gradient of differentiation, which radiates in both directions from the mid-basal region of the cochlea (Sher, 1971; Lim and Anniko, 1985) turns these newly postmitotic cells into the various morphologically and functionally differentiated cell types illustrated in Fig. 1.

These cells remain postmitotic for the life of the animal. In mammals, the loss of auditory hair cells does not appear to lead to proliferative regeneration (Chardin and Romand, 1995, but see Lefebvre et al., 1995) and represents the major cause of deafness in humans. In the mammalian vestibular system, damage to hair cells appears to lead to a very limited degree of repair or regeneration, but whether supporting cell proliferation leads to the differentiation of new hair cells is still controversial (Forge et al., 1993, 1995; Warchol et al., 1993, 1995; Rubel et al., 1995). In lower vertebrates, hair cell death leads to renewed proliferation of supporting cells, which subsequently leads to the differentiation of both hair cells and supporting cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988).

In higher eukaryotes, the decisions concerning whether to proceed through another round of cell division, exit the cell cycle and become quiescent, or re-enter the cell cycle from quiescence are all regulated by a family of proteins known as cyclin-dependent kinases (CDK). Exit from the cell cycle usually occurs during G1 phase and distinct CDKs, CDK4/6 and CDK2, regulate transit through G1 and entry into the following S phase, respectively (see Elledge, 1996 for review). CDK activity, in turn, is regulated by multiple mechanisms, including covalent modification of the CDK catalytic subunit; the relative abundance of required positive cofactors, one of the several members of the cyclin family of proteins, and by the activity of negative regulators, the cyclin-dependent kinase inhibitors (CKI) (see Sherr and Roberts, 1995; Elledge et al., 1996; Harper and Elledge, 1996 for reviews). In vertebrates, CKIs are represented by two unrelated families of proteins, the CIP/KIP family and the INK family. These families interact with CDKs in different ways, but both work by binding to, and inhibiting the activity of cyclin-dependent kinases (CDKs) which can lead to withdrawal from the cell cycle. In spite of the advances in our knowledge of the regulation of CDK activity, little is known about how regulation of CKIs is integrated into specific developmental programs to coordinate cell proliferation with morphogenesis.

Here we report that between E12 and E14, the cyclin-dependent kinase inhibitor (CKI) p27Kip1 is induced in the progenitors of the primordial organ of Corti, correlating with the time when these cells are withdrawing from the cell cycle. We also report that, in mice with a targeted deletion of the p27Kip1 gene (p27<sup>−/−</sup>) (Fero et al., 1996), the cells of the developing neuroepithelium undergo a period of prolonged cell division relative to wild-type littermates, leading to the appearance of supernumerary hair cells and supporting cells. This observation suggests that p27Kip1 is responsible for determining the size of the progenitor population and thus the morphology of the array of sensory hair cells. These animals are severely hearing impaired, indicating the importance of precise developmental control of the cell cycle to the normal development of function. Finally, we have observed that p27<sup>Kip1</sup> is rapidly downregulated in differentiating hair cells, although its expression persists in postmitotic supporting cells of the mature organ of Corti, suggesting a role in maintaining the normally quiescent state of these cells. Consistent with this hypothesis, we have observed continued proliferation of cells in the postnatal organ of Corti of p27<sup>−/−</sup> mice.

**MATERIALS AND METHODS**

**Immunohistochemistry**

Antibody raised against p27Kip1 was purchased from NeoMarker and assayed for specificity by immunoblotting (Fig. 2A). Extracts from mouse N1E-115 neuroblastoma cells (ATCC) were prepared according to Kranenburg et al. (1995) before (Fig. 2A, lane 2) and after (Fig. 2A, lane 3) differentiation and used as controls. Results indicated that the antibody is monospecific and recognizes a band in E14 (data not shown) and neonatal (Fig. 2A, lane 1) cochlear extracts, that comigrates with p27<sup>Kip1</sup> from the neuroblastoma cells. Differences in abundance of p27<sup>Kip1</sup> before and after differentiation of N1E-115 cells are consistent with previously published results (Kranenburg et al., 1995). The more slowly migrating band visible in lanes 2 and 3 most likely represents the previously reported phosphorylated form of p27<sup>Kip1</sup>. Paraffin sections of temporal bones were prepared according to Yoho et al. (1997) and immunohistochemistry was done according to standard procedures (Harlow and Lane, 1988). Immunohistochemistry of p27<sup>Kip1</sup> (NeoMarkers, 1/100 dilution) included an antigen retrieval step carried out by boiling deparaffinized sections in 10mM Citric acid buffer, pH 6.0 for 10 minutes. Other antibodies used in this study included: polyclonal anti-myosin VIIa (courtesy of Christine Petit, Pasteur Institute) (1/2000 dilution), monoclonal anti-PCNA (NeoMarker, 1/200 dilution) and monoclonal anti-neurofilament 68 (Sigma, 1/100 dilution). Antibody incubations were done overnight at 4°C and binding was visualized with fluorescein- or rhodamine-conjugated secondary antibodies (1/200, Jackson ImmunoResearch). Organs of Corti from at least three wild-type and three p27<sup>Kip1</sup> mutant animals were examined with these antibodies at each developmental stage described, except in the PCNA experiment shown in Fig. 6, where two mutant animals were examined.
Confocal imaging

Surface preparations of organ of Corti were prepared from animals aged P1 to 5 months old. Following fixation in paraformaldehyde, cochleae were dissected in PBS buffer, stained with rhodamine-phalloidin (Molecular Probes) and mounted on slides. Z series scans were made on a Zeiss LSM 410 inverted laser scanning microscope. Hair cell counts were made from photographs of these surface preparations and analyzed with a non-parametric statistic (the Mann-Whitney U test, StatView).

Animals

For production of mutant mice, male (129/Sv) mice, heterozygous for an induced mutation at the p27Kip1 locus (Fero et al., 1996), were mated with female (C57BL/6NHSd) mice to produce an F1 generation. p27+/− offspring were identified by polymerase chain reaction (PCR) as described (Fero et al., 1996). Heterozygotes were mated and animals homozygous for the induced mutation were identified by PCR. Wild-type, p27−/− and p27+/− animals used in this study were from this F2 generation. Genotyping was verified by western blotting of brain extracts and also by immunohistochemistry of sections taken through the cochlea, using anti-p27Kip1 antibody (NeoMarkers) (data not shown). Animal care was in accordance with institutional guidelines.

Auditory brainstem response (ABR)

ABRs were recorded from two subcutaneous needle electrodes placed behind the pinna and on the vertex. A third electrode was placed on the hindleg as a ground. The Tucker Davis ABR Workstation with TDT SigGen and BioGen software were used for stimulus generation and data acquisition. Broadband clicks were delivered through an
Intelligent Hearing System Insert transducer. The broadband click was calibrated in peak equivalent sound pressure level (peSPL), in situ for two wild-type animals (ER-7 probe tube microphone system). Click-evoked ABR threshold was defined as the last level (dB peSPL) at which an ABR waveform could be visually detected.

RESULTS

The onset of expression of p27Kip1 in the developing organ of Corti

In mice, greater than 80% of the cells of the developing organ of Corti stop dividing between E13 and E14 of embryonic development (Ruben, 1967). At this time, the vestibular system has acquired its gross morphology and the cochlea has made one and one quarter of its ultimate one and three quarter turns. Based on morphological criteria, the primordial organ of Corti develops along the greater epithelial ridge of the otocyst (Fig. 2B, arrow). Just prior to this time, on E12, no p27Kip1 protein expression is observed in any region of the otocyst (Fig. 2B). However, 2 days later, correlating with the time of cessation of cell division, p27Kip1 is strongly expressed at this site (Fig. 2C, arrow). Confirmation that this is the site of organ of Corti development was obtained by staining alternate sections with a neurofilament antibody (Fig. 2D) to visualize the nerve fibers from the spiral ganglion cells that are known to reach the primordial organ of Corti before the morphological differentiation of hair cells (Pujol et al., 1998). At this time, supporting cells and hair cells are not distinguishable morphologically or by antibodies to one of the earliest markers of hair cell differentiation, myosin VIIa (Sahly et al., 1997).
Fig. 5. A comparison of the structure of organ of Corti from p27<sup>+/+</sup> wild-type (A-C), p27<sup>+/−</sup> heterozygous (D-F) and p27<sup>−/−</sup> homozygous mutant (G-I) mice, age P6. Loss of p27<sup>Kip1</sup> expression due to a targeted gene deletion causes the development of supernumerary hair cells and supporting cells in the organ of Corti. (A,D,G) Confocal images of surface preparations used to compare the overall arrangement of sensory cells in the organ of Corti. They were stained with rhodamine-conjugated phalloidin to visualize the actin-rich stereocilia of the sensory hair cells. (B,E,H) Cross sections through the organ of Corti in the mid-cochlear region stained with antibody to the hair-cell-specific antigen, myosin VIIa. (C,F,I) Alternate sections stained with hematoxylin to reveal the cellular architecture of the organ of Corti. Brackets mark the multiple rows of outer hair cells, three in the p27<sup>+/+</sup> and p27<sup>+/−</sup> animals, four in the p27<sup>−/−</sup> animals. Arrowheads point to the single row of inner hair cells present in the wild-type organ of Corti, as well as to the rows of inner hair cells containing supernumerary hair cells in the p27<sup>+/−</sup> and p27<sup>−/−</sup> mutant animals. Sections from wild-type animals contain the normal number of inner and outer hair cells (B,C). The occasional presence of supernumerary inner hair cells in heterozygotes is illustrated in E but not in F. Note supernumerary cells present in the pillar cell region of the organ of Corti from p27<sup>−/−</sup> animals where normally one inner pillar and one outer pillar cells are present (compare C with I, asterisks).

Fig. 6. Anti-PCNA staining of E16 organ of Corti from p27<sup>−/−</sup> (A,B) and p27<sup>+/+</sup> embryos (C,D) reveals actively cycling cells in the mutant organ of Corti. Sections through the mid-basal region of the developing cochlea of E16 p27<sup>−/-</sup> and p27<sup>+/+</sup> animals were double-labeled with antibody against PCNA (A,C, red) to stain cycling cells, and myosin VIIa (B,D, green) to stain differentiating hair cells. The hair cell region is indicated by brackets, while the region of Deiters’ cells is indicated by clustered arrows. Note PCNA-stained nuclei (red) in a position above the arrows in the p27<sup>−/−</sup> organ of Corti (A) indicating the continued proliferation at this site, as well as the absence of PCNA staining in the comparable region in p27<sup>+/+</sup> animal (C). Size bar, 50 μm.
between p27Kip1 downregulation and the differentiation of hair outer hair cells and show the close temporal correlation These results suggest that the inner hair cells mature before the to stain all of the cells in the primordial sensory epithelium. time, ranging from E14 to P3 (Ruben, 1967). Developmentally, organs withdraw from the cell cycle over a longer period of origin of these cells is unknown. In contrast to their hair cells (Fig. 3B), as it is throughout the cochlea at slightly myosin VIIa is observed in the apical turn present in the same a gradient of expression of the hair cell marker myosin VIIa. In a turn of this cochlea from the basal region (Fig. 3A), we show both apical and basal turns and indicate the presence of a gradient of expression of the hair cell marker myosin VIIa. In a turn of this cochlea from the basal region (Fig. 3A), we found that inner hair cells (arrowhead) are labeled relatively strongly with myosin VIIa (green) while outer hair cells (bracket) are more weakly labeled. In contrast, no staining of myosin VIIa is observed in the apical turn present in the same cochlea at this stage. p27Kip1 staining is present in the supporting cell layer of the basal turn, but absent from all the hair cells (Fig. 3B), as it is throughout the cochlea at slightly later embryological times. However, in the apical turn, where no myosin VIIa staining is observed (Fig. 3A), p27Kip1 appears to stain all of the cells in the primordial sensory epithelium. These results suggest that the inner hair cells mature before the outer hair cells and show the close temporal correlation between p27Kip1 downregulation and the differentiation of hair cells as seen by the onset of myosin VIIa expression. They also demonstrate a gradient of differentiation within the cochlea, consistent with that previously reported in morphological studies (cited in Rubel, 1978).

p27Kip1 is also expressed in the vestibular system

In the vestibular sensory organs, p27Kip1 is also localized in the non-sensory supporting cells of neonatal (P6) animals (Fig. 4A-D). As shown in Fig. 4B, occasional p27Kip1-positive cells are seen in the sensory cell layer as well, although the type and origin of these cells is unknown. In contrast to their counterparts in the cochlea, the cells of the vestibular sensory organs withdraw from the cell cycle over a longer period of time, ranging from E14 to P3 (Ruben, 1967). Developmentally, we observed p27Kip1 expression in subregions of the sensory component of the differentiating vestibular epithelia as early as E14 (data not shown). This suggests that a similar sequence of developmental events involving p27Kip1 in exit from the cell cycle takes place in the vestibular system. However, the levels of p27Kip1 expressed in the developing and mature vestibular system appear to be lower than in comparable sections of organ of Corti making their analysis more difficult. Consequently, we have not precisely correlated the expression of p27Kip1 with the cessation of cell division in the developing vestibular epithelia.

Hyperplasia of the organ of Corti in p27Kip1 knockout mice

The restricted pattern of p27Kip1 expression in the developing sensory epithelium led us to examine the developmental consequences of an induced mutation in the p27Kip1 gene (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). In the absence of p27Kip1, animals are larger than wild-type littermates as a result of multiorgan hyperplasia (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Wild-type mice normally contain a single row of inner hair cells (arrowhead) and three rows of outer hair cells (brackets, Fig. 5A-C). In mice homozygous for a p27Kip1 mutation (p27+/−), supernumerary hair cells developed in both the inner and outer rows of hair cells (Fig. 5G-I). The inner row contains a partly disorganized line of at most two hair cells (Fig. 5G-I, brackets). Representative samples of outer and inner hair cells in the mid-basal region of the cochlea were counted and revealed significantly more cells in mutant (n=4) versus wild-type (n=4) mice (P<0.05). Outer hair cell numbers in the mutant animals were increased by a mean of 36% and inner hair cell numbers were increased by a mean of 23%. In addition, an excess number of supporting cells, including pillar cells separating inner from outer hair cells and occupying the area of the tunnel of Corti, are present in p27+/− animals (Fig. 5I, asterisks). Normally, only one inner pillar cell and one outer pillar cell are present in this region (Figs 2B, 5C, asterisks). These results indicate that there is an increase in both hair cells and supporting cells in the absence of p27Kip1.

In mice heterozygous for a p27Kip1 mutation (p27+/−), the outer hair cell population of the heterozygous animals appears normal (Fig. 5D-F). However, we observed occasional supernumerary inner hair cells throughout the organ of Corti in 6-day-old heterozygous animals (Fig. 5D,E). This phenomenon was never observed in wild-type littermates (Fig. 5A-C). Thus, the severity of this abnormality appears to be dose dependent in that homozygotes are more severely affected than heterozygotes (Fig. 5), although the degree of dose dependence has not been quantified.
hearing deficit in homozygous animals could lie in the peripheral abnormalities that we have described or it could lie in central abnormalities that result either from the \( p27^{kip1} \) mutation or as a consequence of the peripheral anatomical abnormalities. These possibilities are currently under investigation.

**Cell proliferation in the organ of Corti of \( p27^{kip1} \) knockout mice**

The abnormalities observed in the organ of Corti of \( p27^{-/-} \) mice suggest that \( p27^{kip1} \) functions as a growth inhibitor during development, and its ablation leads to prolonged or unscheduled proliferation of hair and supporting cell progenitors, ultimately leading to the differentiation of supernumerary cells. We tested this hypothesis by comparing the expression of Proliferating Cell Nuclear Antigen (PCNA), a marker for cells that are in, or about to enter, the DNA replication phase of the cell cycle and thus are scheduled to undergo another round of cell division (Galand, 1989), in \( p27^{+/+} \) and \( p27^{-/-} \) animals. Since the onset of \( p27^{kip1} \) expression (Fig. 2C) correlates with the cessation of cell division of organ of Corti progenitor cells on E14 (Ruben, 1967), we studied the incorporation of PCNA into replicating DNA in \( p27^{-/-} \) and \( p27^{+/+} \) embryos between E15 and E16 when cell division should have ceased. In \( p27^{-/-} \) embryos, PCNA-positive cells are observed in the Deiters’ cell region (Fig. 6A, arrows) beneath the newly differentiated hair cells stained with anti-myosin VIIa (Fig. 6B, bracket), indicating that proliferation continues in these animals past the normal time of cell cycle withdrawal. In contrast, in wild-type embryos PCNA-positive cells are absent from the Deiters’ cell region (Fig. 6C, arrows) of the organ of Corti, beneath the myosin VIIa-stained hair cells (Fig. 6D, brackets).

To test the duration of this aberrant proliferative activity, we stained P6 cochleas from \( p27^{-/-} \) and \( p27^{+/+} \) animals for expression of the PCNA marker (Fig. 7). In \( p27^{-/-} \) animals, PCNA-positive cells are no longer seen in Deiters’ cell region as they are at E16, but appear in clusters in the region of Hensen’s cells, lateral to the outermost row of outer hair cells (Fig. 7A, arrow), as well as in the pillar cell regions separating inner and outer hair cells (Fig. 7B, arrows). As expected, no PCNA staining is apparent in the organ of Corti (Fig. 7C, bracket and arrow) from \( p27^{+/+} \) animals, and no PCNA-positive cells were observed in comparable sections through the organ of Corti of heterozygous animals (data not shown). These results suggest that \( p27^{kip1} \) is required for maintaining the supporting cells of the mature organ of Corti in their normally quiescent state.

**DISCUSSION**

While the control of cell proliferation, growth and differentiation are separable phenomena, coordination between these phenomena is required if correct morphogenetic patterning is to occur (see Skaer, 1998; Johnston, 1998 for reviews). By studying the effects of an induced mutation in the cell cycle regulator \( p27^{kip1} \), we have identified one element required for coordinating the cessation of cell division with differentiation and morphogenetic patterning in the developing organ of Corti. Our results show that \( p27^{kip1} \) activity is required for the timely withdrawal from the cell cycle of the cells of the developing sensory epithelium and its absence leads to the appearance of supernumerary cells throughout the mature organ of Corti. Additional studies of the pattern of expression of \( p27^{kip1} \) during normal development of the inner ear, suggest that \( p27^{kip1} \) may play a subsequent role in cellular differentiation and homeostasis of the various cell types in the sensory epithelium.

**Coordinating cell proliferation with morphogenetic events**

The ability of \( p27^{kip1} \) to coordinate cell proliferation with morphogenetic events has been suggested by its well-known capacity to block cell cycle progression by inhibiting the activity of CDK4/6 and CDK2 (Elldegge et al., 1996), by its ability to respond in vitro to a variety of known cellular growth regulators (Sher and Roberts, 1995), and finally by the developmental abnormalities that are caused by its targeted deletion in mice (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Regulation of \( p27^{kip1} \) protein levels
in vitro is governed at the transcriptional (Kawasaki et al., 1998), translational (Hengst and Reed, 1996; Millard et al., 1997) and, perhaps most importantly, the post-translational (Pagano et al., 1995; Vlach et al., 1997) level. However, to our knowledge, the specific mechanisms used to regulate p27\(^{Kip1}\) levels in vivo have not been studied, and the molecular machinery underlying the temporally and spatially restricted induction of p27\(^{Kip1}\) protein that we have described (Fig. 2) is currently unknown.

Morsli et al. (1998) have shown that the gene lunatic fringe, a modulator of the Notch signaling pathway, is expressed in the sensory regions of the developing cochlea in a restricted region that appears to presage p27\(^{Kip1}\) expression. While it is not clear whether the expression of this gene in the cochlea has the exact same boundaries as p27\(^{Kip1}\), it is possible that the Notch signaling pathway affects morphogenesis of the organ of Corti through the regulation of cell proliferation in a manner similar to that shown for wing development in Drosophila (Johnston and Edgar, 1998). Although the specific stimulus for p27\(^{Kip1}\) induction is not known, the sharp boundaries of p27\(^{Kip1}\) expression that arise in the otocyst between E12 and E14 (Fig. 2C) are likely to reflect boundaries that have been set up by morphogenetic gradients analogous to those seen during the patterning of other embryonic anlage and, as such, may serve as a downstream marker for the specification of the boundaries of the sensory epithelia.

### Control of cell proliferation in the developing organ of Corti

During development of the organ of Corti, p27\(^{Kip1}\) levels rise abruptly between E12 and E14 (Fig. 2) and the majority of the cells that will make up the mature sensory epithelium exit the cell cycle over the period of 24–48 hours that follow (E13 and E15, Ruben, 1967). In animals that lack p27\(^{Kip1}\), proliferation within the E16 sensory epithelium continues (as seen by the presence of PCNA-positive cells in the Deiters’ cell region, Fig. 6A), even as some of the newly generated cells are beginning to differentiate into hair cells (Fig. 6B). The supernumerary cells that we observe (Fig. 5) are the consequence of this abnormal proliferation. At later times (P6, Fig. 7) PCNA-positive cells are restricted to the region of Hensen’s, Claudius’ and pillar cells and are absent from the Deiters’ cell region. The reason for this change is not known, but is likely to be related to the mechanism governing hair cell and supporting cell differentiation discussed below.

The fact that both hair cells and supporting cells are able to stop dividing and differentiate in the organ of Corti of p27\(^{–/–}\) animals indicates that there are multiple levels of cell cycle control at work. The nature of these other controls on cell proliferation in the organ of Corti is not clear. In studies of developing oligodendrocytes in vitro, it was found that the absence of p27\(^{Kip1}\) leads to a prolonged period of cell division (Casaccia-Bonnefil et al., 1997; Durand et al., 1998), correlating with an increased number of cells in vivo in p27\(^{–/–}\) animals (Casaccia-Bonnefil et al., 1997). While p27\(^{–/–}\) oligodendrocytes are able to undergo cell cycle arrest and differentiation in vivo, under specific in vitro growth conditions, differentiation of these cells is partially impaired (Casaccia-Bonnefil et al., 1997; Tikoo et al., 1998). These observations, like our own, are consistent with the overall phenotype displayed by p27\(^{–/–}\) animals, namely the generalized hyperplasia observed in many, if not all tissues, along with a range of abnormalities in tissue organization (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). In spite of the increase in cell number, the function of most tissues is not grossly perturbed (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996) and homozygous mutant animals are viable. The presence in some tissues of more than one CKI (Franklin et al., 1998; Zhang et al., 1998), as well as the existence of potential CKI-independent pathways able to downregulate CDK activity (Elledge et al., 1996), suggests that overlapping pathways regulating the cell cycle during development may be the norm. However, as our results indicate, p27\(^{–/–}\) animals are severely hearing impaired, probably reflecting a strict requirement for precise cellular organization in the organ of Corti.

### Specification of cell type in the organ of Corti

The primary mechanism specifying hair cell versus supporting cell differentiation remains an important question for the field. While alternative models exist, several investigators have suggested that at the time of their terminal mitosis, the cells of the sensory epithelium are not determined to be either hair cells or supporting cells, and only sometime after cell cycle exit do they differentiate into one or the other cell type, possibly through a process of lateral inhibition (see Fekete, 1996 for review). The specification of hair cells versus supporting cells following terminal mitosis is consistent with results from recent studies of terminal differentiation in the basilar papilla of the chicken using retroviral tracing methods (Fekete et al., 1998), as well as studies on the differentiation of sensory hair cells during in vitro organ culture of the mouse organ of Corti (Kelley et al., 1993, 1995). These workers show that for a limited period of time following terminal cell division, cells of the sensory epithelium can be induced to become supernumerary hair cells, either through retinoic acid treatment (Kelley et al., 1993) or by the death of a neighboring, already differentiated hair cell (Kelley et al., 1995). This is accomplished without additional cell division, suggesting that the new cells are likely to have been derived from cells that would otherwise have differentiated into supporting cells or from a population of cells that would normally have undergone apoptosis.

If these arguments are correct, the initial induction of p27\(^{Kip1}\) in the otocyst, which occurs prior to hair cell and supporting cell differentiation, is likely to be responsible for regulating the correct number of postmitotic precursor cells on which these later specification processes can act. By this argument, the supernumerary cells produced because of the absence of p27\(^{Kip1}\) are the result of the normal patterning within an increased population of progenitors. In contrast, the supernumerary cells that are produced in response to retinoic acid, in the absence of new cell division (Kelley et al., 1993), likely result from a more direct influence on the process of postmitotic differentiation.

While the very existence of both hair cells and supporting cells in p27\(^{–/–}\) animals argues strongly against a required, positive role for p27\(^{Kip1}\) in the terminal differentiation of either cell type, it leaves open the possibility of a negative role in regulating the differentiation of one or the other. In this case, the rapid downregulation of p27\(^{Kip1}\) that occurs in differentiating hair cells (Fig. 2) could be a necessary consequence of the
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initiation of differentiation in these cells. We have observed that the induction of myosin VIIa, a marker of hair cell differentiation, occurs after the last cell division and is temporally correlated with the downregulation of p27Kip1. Just how tightly correlated is perhaps best seen by our demonstration of the gradient of differentiation within the E15 cochlea, where basal sections reveal hair cells expressing myosin VIIa and lacking p27Kip1 expression, at the same time as postmitotic progenitor cells in more apical regions of the cochlea have yet to begin expression of myosin VIIa and still express p27Kip1 (Fig. 3). At no time do we observe myosin VIIa and p27Kip1 double-labeled cells in our preparations. Nor do we observe cells in the postmitotic organ of Corti that lack both markers. This suggests that the expression of these two markers may be mutually exclusive. Although no causative relationship has been demonstrated, a mutually exclusive pattern of expression exists between one of the other CIP/Kip family members (p21Cip1), and markers of cell differentiation in postmitotic keratinocytes (Di Cunto et al., 1998) suggesting that p21Cip1 has a direct role in the differentiation of these cells, beyond its role in regulating cell proliferation. The downregulation of p27Kip1, which appears to be part of the program of hair cell differentiation, could serve an analogous function.

Quiescence and regeneration

The presence of proliferating cells in the postnatal organ of Corti of p27Kip1-/- animals (Fig. 7), as well as the persistence of p27Kip1 expression in mature supporting cells (Fig. 2G,H, and data not shown), reveals a second role for p27Kip1, that of maintaining these cells in a quiescent state. While its persistence may be required in mature supporting cells to maintain this state, it may also offer a possible explanation for the lack of regeneration in mammals. In lower vertebrates such as chickens, the loss of hair cells due to acoustic trauma or ototoxic shock leads to re-entry of supporting cells into the cell cycle and the subsequent differentiation of their progeny into hair cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). We hypothesize that, if supporting cells in chickens also contain high levels of a p27Kip1 homologue, it is rapidly degraded upon loss of hair cells, in order to allow them to re-enter the cell cycle. The difference between mammals and birds could lie in the level of p27Kip1 in mature supporting cells or the efficiency with which it is downregulated following injury. Alternatively, other cell cycle regulators besides p27Kip1 may be involved. Regardless, the implication is that the high levels of p27Kip1 that persist in supporting cells of mammals could be an impediment to re-entry into the cell cycle following loss of hair cells.

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