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Expression of the Pax2 Transcription Factor is Associated with Vestibular Phenotype in the Avian Inner Ear

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Abstract

The paired-domain transcription factor Pax2 is involved in many facets of inner ear development, but relatively little is known about the expression or function of Pax2 in the mature ear. In this study, we have used immunohistochemical methods to characterize the expression patterns of Pax2 in the sensory organs of inner ears from post-hatch chicks. Immunoreactivity for Pax2 was observed in the nuclei of most hair cells and supporting cells in the vestibular organs. In contrast, Pax2 expression in the chick cochlea was limited to hair cells located in the very distal (low frequency) region. We then used organotypic cultures of the chick utricle to examine changes in Pax2 expression in response to ototoxic injury and during hair cell regeneration. Treatment with streptomycin resulted in the loss of most Pax2 immunoreactivity from the luminal (hair cell) stratum of the utricle. During the early phases of regeneration, moderate Pax2 expression was maintained in the nuclei of proliferating supporting cells. Expression of Pax2 in the hair cell stratum recovered in parallel with hair cell regeneration. The results indicate that Pax2 continues to be expressed in the mature avian ear, and that its expression pattern is correlated with a vestibular phenotype.

Keywords

Hair Cell; Supporting Cell; Utricle; Saccule; Lagena; Cochlea; Regeneration

Introduction

The sensory organs of the inner ear are responsible for the detection of sound and head movement. Mechanotransduction within the ear is accomplished by hair cells, whose sensitivity is strongly influenced by their position and orientation within individual sensory epithelia. As a result, the inner ear is one of the most precisely-patterned structures in the vertebrate body. Studies of ear development in zebrafish, *Xenopus*, chick and mouse embryos have identified a number of transcription factors that are essential for the establishment of otic morphology. In particular, the paired-domain transcription factor Pax2 has been shown to play a crucial role in several facets of ear development. Expression of

Pax2 is one of the first indicators of otic placode induction and Pax2 continues to be expressed in various regions of the ear throughout subsequent development (e.g., Groves and Bronner-Fraser, 2000; Burton et al., 2004; Sanchez-Calderon et al., 2005). In the developing chick ear, Pax2 is expressed in regions of high cell proliferation (Li et al., 2004). In mice, genetic deletion of Pax2 results in variable defects in the formation of the cochlea and saccule, altered structure of the semicircular canals, and a decrease in the numbers of auditory and vestibular neurons (Torres et al., 1996; Burton et al., 2004).

The expression of particular transcription factors in the mature ear is likely to be a key influence on the capacity for repair after injury (e.g., Hawkins et al., 2007). Hair cells in the mature ear can be injured or lost as a consequence of noise exposure, ototoxic drug treatment or aging. The regenerative ability of the post-natal mammalian ear is very limited, and the loss of hair cells typically leads to permanent deficits in hearing and equilibrium. In contrast, the ears of nonmammalian vertebrates are capable of producing new sensory hair cells throughout life, and can quickly regenerate hair cells after injury (e.g., Corwin and Oberholtzer, 1997). Previous studies of Pax2 expression in the ear have focused exclusively on embryonic development, and the expression of Pax2 in the mature ear has not been closely examined. In the present study, we show that hair cells in the mature vestibular organs of birds maintain strong Pax2 immunoreactivity and that weaker Pax2 immunoreactivity is also present in vestibular supporting cells. Closer examination of the maculae revealed that relatively few cells within the striolar reversal zones of the utricle and lagena expressed Pax2. Cells in these regions have previously been shown to uniquely express the GATA3 transcription factor (Warchol and Speck, 2007). Treatment with the ototoxic antibiotic streptomycin resulted in the loss of most Pax2 expression in the hair cell stratum of the utricle, but expression of Pax2 recovered in parallel with the reappearance of hair cell stereocilia bundles. Finally, Pax2 expression in the mature cochlea was limited to hair cells at the very distal tip of the sensory epithelium. The results suggest that Pax2 expression is correlated with a vestibular (as opposed to auditory) hair cell phenotype.

Methods

Animals

Experiments were conducted on chicks at 7-21 days post-hatch (P7-21). Animals were hatched from fertile eggs or were obtained as hatchlings from local suppliers. All animals were housed in heated brooders prior to use. Experimental protocols that utilized animals were approved by the Institutional Animal Care and Use Committees of both Washington University and the Marine Biological Laboratory and conformed to the National Institutes of Health animal use guidelines.

Organ Culture Techniques

Chickens were euthanized with CO₂ and decapitated. The lower jaw and the skin covering the head were removed and the heads were immersed in 70% ethanol for about 5-10 min., in order to kill surface pathogens. The temporal bones were opened with forceps and utricles were quickly removed and transferred to chilled Medium-199 (with Hank's salts and HEPES; Invitrogen). Otoconia and otolithic membranes were removed and individual utricles were transferred to 1 cm diameter culture wells (Mat Tek, Ashland, MA) that contained 100 µl of medium (Medium-199 supplemented with Earle's salts, 2,200 mg/L sodium bicarbonate, 0.69 mM L-glutamine, 25 mM HEPES, and 2.5% Fetal Bovine Serum (FBS; Invitrogen). Cultures were maintained at 37°C in a humidified 5% CO₂/95% air environment for 2-8 days. For the first 24 hours of culture, the medium also contained 1 mM streptomycin sulfate (Sigma). Specimens were then rinsed 3× in fresh aminoglycoside-free culture medium and maintained in aminoglycoside-free medium for an additional 1-7 days.

Proliferating cells were labeled by the addition of 5-bromo-2-deoxyuridine (BrdU; 3 µg/ml; Sigma) to the culture medium for the final four hours *in vitro*.

Tissue Processing and Immunohistochemistry

Explanted cochleae, saccules, utricles, lagenae and cristae were fixed for 15-20 min. with 4% paraformaldehyde (in 0.1 M phosphate buffer, pH=7.4). Specimens were then rinsed 5× with 0.01M phosphate buffered saline (PBS; Sigma), and nonspecific antibody binding was blocked by incubating specimens in 5% normal horse serum (NHS) and 0.2% Triton X-100 (in PBS) for 2 hours at room temperature. Specimens were incubated overnight in primary antibodies at 4° C. They were then rinsed 5× in PBS and incubated for 2 hours in secondary antibodies (at room temperature). Secondaries were conjugated to either Cy3 (Jackson Research) or Alexa-488 (Invitrogen/Molecular Probes). Some specimens were also labeled with Alexa-488-labeled phalloidin (Invitrogen/Molecular Probes, 30-60 min, in PBS/0.2% Triton X-100). Nuclear DNA in all specimens was labeled with DAPI. After labeling, whole sensory organs were placed upright on glass microscope slides in a drop of glycerol:PBS (9:1). Coverglasses were then placed directly over the specimens and the edges were sealed with nail polish.

Processing of sectioned specimens was conducted according to a similar protocol. Utricles, saccules and cochleae were fixed (as above) and cryoprotected by overnight incubation in 30% sucrose (in PBS, at 4° C). Specimens were then embedded and frozen in Tissue-Tek OCT compound (Miles, Elkhart IN) and sectioned at 10-12 µm thickness. Sections were mounted on subbed microscope slides and processed for immunohistochemistry as described above.

Background on Primary Antibodies

The Pax2 transcription factor was detected using a rabbit polyclonal antibody raised against a fusion protein from the C-terminal domain (amino acids 188-385) of murine Pax2 (Dressler et al., 1992; Zymed 71-6000, diluted 1:200). Identical protocols have used this antibody to label Pax2 in the inner ears of chicks (Hutson et al., 1999; Groves and Bronner-Fraser, 2000, Li et al., 2004) and mammals (Lawoko-Kerali et al., 2002) and in lateral line neuromasts of zebrafish (Riley et al., 1999). Specificity has been demonstrated by the fact that the patterns of Pax2 expression in chick ear that are observed with this antibody (e.g., Li et al., 2004) are in agreement with those determined using *in situ* hybridization methods (e.g., Sanchez-Calderon et al., 2005). The zinc finger transcription factor GATA3 was labeled with a mouse monoclonal antibody raised against human recombinant GATA3 (clone HG3-31, 1:200, Santa Cruz Biotechnology). Full characterization of this antibody in the chick ear has been reported previously (Hawkins et al., 2003; Warchol and Speck, 2007). Hair cells in the auditory and vestibular organs were labeled with the HCS-1 antibody, a mouse monoclonal that recognizes otoferlin (Cyr et al., 2006; diluted 1:100; a gift from Jeffrey Corwin, University of Virginia, Charlottesville VA). Finally, immunolabeling of BrdU incorporation was carried-out following a previously-published protocol (Warchol and Corwin, 1996).

Imaging and Analysis of Data

Specimens were imaged on inverted microscopes (either Nikon Eclipse 2000 or Zeiss Axiovert 135), equipped with epifluorescence illumination and cooled CCD cameras (either Q-Imaging, Burnaby BC, Canada or Photonics Cool Snap ES, Roper Scientific, Tucson AZ). Standard laboratory software (Q-Capture or IP-Lab) was used to capture images, which were then colorized and sharpened with Adobe Photoshop and/or ImagePro (Media Cybernetics, Silver Springs MD). Confocal images were obtained using a Bio-Rad Radiance 2000 MP system and were constructed with Volocity software. Quantification was carried-out directly

from stored images and final cell densities were normalized to 10,000 μm^2 fields. Statistical tests were performed with Microsoft Excel. Finalized figures were assembled with Adobe Illustrator software.

Results

Expression of Pax2 in the Avian Ear is Correlated with Vestibular Phenotype

Prior studies have characterized the expression of Pax2 during the embryonic development of the inner ear (e.g., Groves and Bronner-Fraser, 2000; Li et al., 2004; Burton et al, 2004). In order to determine whether Pax2 continues to be expressed in the mature ear, we examined Pax2 immunoreactivity in the auditory and vestibular organs of the chick at post-hatch days 7-21 (P7-21). As noted in an earlier preliminary report (Warchol, 2007), strong immunoreactivity for Pax2 was observed in hair cell nuclei in the utricle (Fig. 1A), saccule (Fig. 1C), lagena (Fig. 4B) and in the anterior, posterior and horizontal cristae (data not shown). Quantification of Pax2-labeled nuclei in the hair cell stratum of the utricle revealed a density of 133.1 ± 7.8 Pax2-labeled cells/10,000 μm^2 ($n=10$ samples from the extrastriolar regions of 3 specimens). Weaker labeling for Pax2 was also observed in supporting cell nuclei in all of these sensory organs (e.g., Fig. 1). Pax2 expression in hair cells could be easily distinguished from expression in supporting cells, since hair cell nuclei were larger than supporting cell nuclei and were located in the luminal (upper) stratum of the sensory epithelium.

Immunoreactivity for Pax2 was also observed in a very limited region of the cochlea. Specifically, expression of Pax2 was confined to hair cells in the inferior portion of the cochlear sensory epithelium, within approximately 150 μm of the distal tip (Figs. 2A, 3A). No immunoreactivity for Pax2 was observed in the more proximal regions of the sensory epithelium (Figs. 2C, 3B, 3C). Notably, some Pax2-labeled hair cells appeared to be slightly outside of the distal border of the cochlear epithelium (Fig. 3A).

Regional Variation in Pax2 Expression

Expression of Pax2 in the saccule and in the three cristae appeared to be uniform throughout those sensory organs. In contrast, the sensory epithelia of the utricle and lagena displayed a more complex pattern of Pax2 immunoreactivity. Hair cells in the extrastriolar region of both sensory organs were uniformly labeled for Pax2 (Fig. 4A, B), while the striolar regions contained a strip of cells that contained distinctly fewer Pax2-expressing cells (Fig. 4C-F). In both sensory organs, the locus of diminished Pax2 expression corresponded to the striolar reversal zone, a region that has previously been shown to uniquely express the GATA3 transcription factor (Warchol and Speck, 2007). Consistent with that outcome, we found that Pax2-labeled nuclei appeared to completely surround the GATA3-expressing region (Fig. 5). Notably, a few Pax2-expressing cells were also observed within the GATA3 region, but cells that were clearly immunoreactive for *both* Pax2 and GATA3 were not observed.

Pax2 Expression During Ototoxic Injury and Regeneration

The previous results indicate that Pax2 is expressed in the majority of vestibular hair cells in the chick ear. We then used organ culture methods to characterize Pax2 expression after ototoxic injury and during regeneration. Utricles were initially cultured for 24 hr in 1 mM streptomycin, and were then rinsed 3 $\times$ in fresh (streptomycin-free) medium and maintained for an additional 1-7 days. After fixation, specimens were processed for Pax2 immunoreactivity, and Pax2-expressing cells in the upper (hair cell) stratum of the sensory epithelium were imaged and quantified. Streptomycin treatment resulted in a sharp reduction in the numbers of Pax2-labeled cells in the luminal stratum (Figs. 6, 7). In spite of this loss, however, the general patterns of Pax2 and GATA3 expression appeared unchanged. The few

remaining Pax2-labeled cells were located in the extrastriolar regions and on the borders of the GATA3-expressing reversal zone (Fig. 6). Also, as reported previously (Warchol and Speck, 2007), we observed no injury-evoked changes in the expression of GATA3.

Additional specimens were maintained in culture for 2-7 days after streptomycin treatment, in order to characterize the regeneration of Pax2 expression and recovery of hair cell stereocilia. At all recovery times the numbers of Pax2-labeled nuclei were significantly higher than the numbers of stereocilia ($p < 0.0001$; Fig. 8). Specimens incubated for two days after streptomycin treatment contained 9.2 ± 3.1 cells/ $10,000 \mu\text{m}^2$ in the upper stratum of the sensory epithelium ($n=14$ samples from 3 specimens). After five days recovery, the density of Pax2 labeling in this region increased to 25.1 ± 8.1 cells/ $10,000 \mu\text{m}^2$ ($n=16$ samples from six specimens). Expression of Pax2 further increased (to 46.3 ± 8.1 cells/ $10,000 \mu\text{m}^2$, $n=18$ samples from 6 specimens) after 7 days recovery. We also examined Pax2 expression and the presence of stereocilia in utricles ($n=5$) that were maintained in culture for eight days, but did not receive streptomycin treatment. Although the overall hair cell density in these specimens appeared to be slightly less than in utricles that were fixed *in vivo* (e.g., Fig. 4A), these control utricles still contained numerous phalloidin-labeled stereocilia and associated Pax2-labeled nuclei (Fig. 7A).

Maintenance of Pax2 Expression in Proliferating Supporting Cells

Previous studies have shown that hair cell regeneration in the avian vestibular organs occurs mainly through the renewed proliferation of epithelial supporting cells (e.g., Weisleder and Rubel, 1993; Stone et al., 1999; Matsui et al., 2000). In the present study, we observed moderate immunoreactivity for Pax2 in the supporting cells of unlesioned sensory organs (e.g., Fig 1). In order to determine whether Pax2 expression was maintained during the onset of regenerative proliferation, we double-labeled supporting cells for Pax2 and for BrdU (a mitotic tracer that was added to the cultured medium and is incorporated into newly-synthesized DNA). Cultured utricles received BrdU for the final four hours of a 48 hour recovery period following streptomycin treatment (the time window of maximal cell proliferation – Matsui et al., 2000). Specimens were then fixed and processed for immunohistochemical labeling of BrdU and Pax2. Doubled-labeled nuclei were observed throughout the regenerating sensory epithelia (Fig. 9).

Discussion

Transcription factors of the paired-domain (Pax) family are involved in the development of many somatic tissues (e.g., Lang et al., 2007). Depending on the context and cell type, Pax factor expression can promote either cellular proliferation or differentiation, or regulate apoptosis. Expression of Pax2 has been characterized in the developing ear, retina, central nervous system and urogenital system (e.g., Dressler et al., 1990), and mice that possess mutations in Pax2 show defects in the development of the inner ear, visual system and kidney (e.g., Torres et al., 1995; 1996; Burton et al., 2004). The expression patterns of Pax2 during the embryonic development of the inner ear have been extensively profiled, but no previous studies have examined Pax2 in the mature ear or during sensory regeneration. The results presented here demonstrate that Pax2 expression in post-hatch chickens is maintained within most regions of the vestibular sensory organs. In contrast, Pax2 expression in the cochlea is limited to a small region at the distal tip. Immunoreactivity for Pax2 is particularly strong in hair cell nuclei, but weaker immunoreactivity for Pax2 is also observed in the nuclei of vestibular supporting cells. In addition, Pax2 continues to be expressed in many proliferating supporting cells during sensory regeneration. Taken together, these results suggest that one function of Pax2 may be to direct sensory differentiation in the ear toward a vestibular - as opposed to auditory - phenotype.

Role of Pax2 Expression in Inner Ear Development

Prior studies have shown that regionalized Pax2 expression is one of the earliest markers of otic placode induction (Hutson et al., 1999; Groves and Bronner-Fraser, 2000). During the subsequent stages of ear development, Pax2 expression is observed in several distinct regions of the labyrinth (Burton et al., 2004). Analysis of the ears of Pax2 mutant mice yields some insight into the diverse roles of Pax2 in otic development. Such mice typically contain fewer neurons in the spiral and vestibular ganglia, a small (or absent) saccule, and defects in the outgrowth of the cochlear duct (Torres et al., 1996; Burton et al., 2004). Pax2-null mice also show reduced levels of cell proliferation and increased levels of apoptosis in the developing cochlear duct (Burton et al., 2004). Although the sensory organs of Pax2-deficient mice are often smaller than their counterparts in normal mice, those sensory organs usually contain some normal-appearing hair cells. This observation indicates that expression of Pax2 is not necessary for hair cell differentiation (Burton et al., 2004).

Factors Associated with Pax Signaling in the Inner Ear

Pax transcription factors are upstream members of the Pax/Eya/Six transcriptional pathway and both Eya and Six family members have also been shown to be involved in inner ear development. Four Eya1 genes have been identified in mammals and all four are expressed in the developing mouse ear (Xu et al., 1997). Similarly, Six1 is expressed in all sensory organs of the developing mouse ear and appears to interact with Eya1 and/or Pax2 (Zheng et al., 2003; Zou et al. 2006). Genetic knockout of Eya1 or Six1 leads to significant defects in mouse inner ear development (Zheng et al., 2003; Zou et al. 2006). In humans, mutations in either Eya1 or Six1 result in brachial-oto-renal (BOR) syndrome, which often includes severe hearing loss (e.g., Kalatzis et al., 1998). Although the expression of Eya and Six factors in the mature ear has not been carefully examined, changes in the expression levels of Eya1, Six1, Six2, and Six3 have been observed during the early phases of regeneration in the avian auditory and vestibular organs (Hawkins et al., 2007). These findings further are consistent with a suggested role for Pax2 during regeneration.

Pax2 Expression and Hair Cell Phenotype

The differing patterns of Pax2 expression in the vestibular vs. the auditory organs suggest that Pax2 expression might be associated with the induction of vestibular specializations in hair cells. Although both auditory and vestibular hair cells belong to the same generic phenotype, close comparison of these two classes of hair cell reveals numerous differences in structure, ion channel expression, and innervation (e.g., Eatock et al., 2006). Development of the hair cell phenotype initially depends on expression of the Atoh1 transcription factor (e.g., Kelley, 2006), but it is not known what signals cause a newly-differentiated hair cell to acquire either auditory or vestibular characteristics. The data presented here suggest that Pax2 expression might be involved in this fate choice. Strong immunoreactivity for Pax2 was observed in most vestibular hair cells. Significantly, hair cells in the reversal zones of the utricle and lagena that show reduced Pax2 expression also have a distinctly different morphology from hair cells found elsewhere in the avian vestibular organs (Jørgensen and Andersen, 1973; Jørgensen, 1989). There are presently no published physiological data from these unique hair cells or from afferents that innervate them, so it is impossible to know whether the lack of Pax2 expression is correlated with differences in membrane properties or frequency sensitivity. It is notable, however, that Pax2 was also observed in hair cells located at the very distal tip of the cochlea, which have previously been described as possessing a surface morphology and bundle configuration that resembles a vestibular (as opposed to auditory) phenotype (Lavinge-Rebillard et al., 1985). Such distal hair cells respond to sound frequencies of 5-20 Hz (Warchol and Dallos, 1989), a stimulus range that is normally associated with vestibular hair cells.

Pax2 Expression During Regeneration

During the development of the avian ear, regionalized expression of Pax2 is correlated with zones of high cell proliferation (Li et al., 2004). Notably, the avian vestibular organs can regenerate hair cells via renewed supporting cell proliferation (Weisleder and Rubel, 1993; Stone et al., 1999; Matsui et al., 2000), but it was not clear whether Pax2 expression is permissive for cell division in the mature ear. In the present study, we labeled proliferating supporting cells with BrdU and observed numerous cells that were immunoreactive for both Pax2 and BrdU. This observation suggests that Pax2 expression does not prevent cells from re-entering the cell cycle. Since specimens received BrdU for only four hours, most of the double-labeled cells were probably in the S-phase of the cell cycle, and we did not attempt to determine how Pax2 expression changed during the subsequent phases of the cell cycle. We did, however, quantify luminal (hair cell) Pax2 expression at later recovery times. Our observation that the numbers of Pax2-immunoreactive cells increased throughout the time-course of regeneration suggests that the onset of Pax2 expression might correspond to the differentiation of new hair cells. Also, at all recovery times, the number of Pax2⁺ cells was significantly greater than the numbers of stereocilia bundles. The Pax2⁺ nuclei that were observed at 2 days after streptomycin treatment (Fig. 7B) probably represented hair cells that had lost their stereocilia bundles, but had otherwise survived the ototoxic injury (e.g., Gale et al., 2002). The disparity between Pax2 labeling and stereocilia bundles at later recovery times probably indicates that newly-regenerated hair cell begin to express Pax2 prior to growing a new stereocilia bundle.

Possible Mechanisms of Pax2 Signaling

Comparative studies suggest an association between Pax2 and mechanosensory organs. An evolutionary precursor of Pax2/5/8 is expressed in the statocyst sensory organs of jellyfish and mollusks (O'Brien and Degan, 2003; Piatigorsky and Kozmik, 2004). In *Drosophila*, Pax2 is involved in the differentiation of the bristle sensory organs that cover most of the body surface. Notably, Pax2 is expressed in all bristle precursor cells prior to differentiation, and characterization of loss-of-function mutants suggests that Pax2 is necessary for differentiation of two classes of 'supporting' cells (shaft and sheath cells) within the mature bristle organ (Kavaler et al., 1998). Hair cells in zebrafish also express of Pax2.1, a teleost orthologue of Pax2. Fish that contain a mutated form of the Pax2.1 gene contain supernumerary hair cells, suggesting the involvement of Pax2.1 in notch-delta-mediated lateral inhibition during hair cell differentiation (Riley et al., 1999).

Such findings support a linkage between Pax2 and the development of mechanoreceptors, but it is not clear how these results directly apply to the development or maintenance of the avian ear. At present, we possess little mechanistic information pertaining to how Pax2 influences ear development. More detailed knowledge of the function of Pax2 in otic development and regeneration must await the identification of specific genes that are regulated by Pax2. Gene array studies of the developing nervous system have revealed a few Pax2-regulated genes, including *En2*, *Brn3.1*, *Sef* and *FGF8* (Bouchard et al., 2005). Notably, both *Sef* and *FGF8* are involved in mammalian ear development (Abraira et al., 2007; Jacques et al., 2007). It is also intriguing to note that, in late-stage chick embryos, *FGF8* is expressed in all vestibular organs, but not in the sensory epithelium of the cochlea (Sanchez-Calderon et al., 2004). This observation suggests that *FGF8* expression in the chick ear may be associated with Pax2, a prediction that can be tested using gene-knockdown methods.

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Figure 1.

Pax2 expression in the utricle and saccule of the post-hatch chick. Strong nuclear immunoreactivity for Pax2 (red) was observed in frozen sections of the utricle (A) and saccule (C). Hair cells in both images are labeled with the HCS-1 antibody (green). Supporting cell nuclei (lower stratum of each epithelium) showed weaker labeling for Pax2. Images B and D show all cell nuclei, as labeled with DAPI (blue). Scale bar = 20 μ m.

Figure 2.

Expression of Pax2 in frozen sections of the chick cochlea. Pax2-labeled nuclei (red) were observed only in the inferior portion of the distal tip of the cochlea (A, arrows), and were not detected in the more proximal regions of the sensory epithelium (C). Hair cells in images A and C are labeled with HCS-1 (green). Cell nuclei are shown in images B and D, as labeled with DAPI (blue). Scale bar = 20 μm .

Figure 3.

Expression of Pax2 in whole-mounts of the chick cochlea. Pax2-labeled hair cells are present at the distal tip of the cochlea (A, arrows). In contrast, no Pax2 immunoreactivity was observed in the middle (B) or proximal (C) regions of the sensory epithelium. Hair cell stereocilia are labeled with phalloidin (green). Scale bar = 20 μm .

Figure 4.

Reduced Pax2 expression in the reversal zones of the utricle and lagena. Images on the left (A, C, E) show Pax2 (red) and phalloidin (green) double-labeling, while the images on the right (B, D, F) show Pax2 alone. Hair cells in the extrastriolar regions of the utricle and lagena were uniformly immunoreactive for Pax2 (A, B). The striolar regions of both the utricle (C, D) and lagena (E, F) possessed a 6-10 cell-wide region in which most hair cells did not express Pax2. Notably, however, those regions did contain a few Pax2-expressing cells (arrows). Scale bar = 40 μm .

Fig. 5. The region of diminished Pax2 expression corresponds to the region of GATA3 expression. Images show immunoreactivity for Pax2 (A, red) and GATA3 (B, green) in the striola of the utricle; these images are merged in (C). Note that the narrow strip of reduced Pax2 immunoreactivity corresponds to the region of GATA3 expression. Scale bar = 30 μ m.

Figure 6.

Loss of Pax2 expression after ototoxic injury. Treatment for 24 hours in 1 mM streptomycin resulted in many fewer nuclei that possessed strong Pax2 immunoreactivity (red). The overall pattern of Pax2 expression, however, remained unchanged. Some Pax2-labeled nuclei were present near the borders of the GATA3-expressing reversal zone (A, green). Labeling for HCS-1 (green) in the extrastricular region (B) shows a few surviving hair cells (arrows) as well as fragments of degenerating hair cells (green spots). Scale bar = 20 μ m.

Figure 7.

Recovery of Pax2 expression following ototoxic injury parallels the time-course of hair cell regeneration. In all images, hair cell stereocilia and cell-cell junctions are labeled with phalloidin (green). (A) Numerous Pax2-labeled nuclei (red) were present in utricles that were cultured for 8 days, but did not receive streptomycin. (B) Treatment for 24 hr with 1 mM streptomycin strongly reduced the number of stereocilia bundles and Pax2-expressing nuclei in the hair cell stratum. (C) Pax2-expressing cells (red) began to recover after 5 days of culture. (D) After 7 days recovery, numerous Pax2-labeled cells and recovered stereocilia bundles were observed. Higher magnification images show that, after 7 days recovery, Pax2-labeled nuclei were usually associated with hair cell stereocilia (E, F, G). Scale bar = 40 μ m (A, B, C, D) or 10 μ m (E, F, G).

Figure 8.

Regeneration of hair cell stereocilia bundles and Pax2-labeled nuclei in cultured utricles. Quantitative data were obtained at 2, 5 and 7 days recovery from streptomycin treatment. At all time points, the density of Pax2-labeled nuclei in the upper stratum of the sensory epithelium was greater than the density of stereocilia bundles (mean $\pm$ SD; $p < 0.0001$; 2-tailed Students t-test).

Figure 9. Pax2 expression in proliferating supporting cells. Utricles were cultured for 24 hr in 1 mM streptomycin and then maintained *in vitro* for an additional 48 hr. The mitotic tracer BrdU was added to the specimens for the final 4 hr of culture, and the utricles were processed for visualization of both Pax2 and BrdU. (A) Pax2 immunoreactivity, (B) BrdU immunoreactivity, (C) merged. Note that several nuclei are double-labeled for both Pax2 and BrdU (arrow). Scale bar = 20 μ m.