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FGFR3 Expression during Development and Regeneration of the Chick Inner Ear Sensory Epithelia

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Several studies suggest fibroblast growth factor receptor 3 (FGFR3) plays a role in the development of the auditory epithelium in mammals. We undertook a study of FGFR3 in the developing and mature chicken inner ear and during regeneration of this epithelium to determine whether FGFR3 shows a similar pattern of expression in birds. FGFR3 mRNA is highly expressed in most support cells in the mature chick basilar papilla but not in vestibular organs of the chick. The gene is expressed early in the development of the basilar papilla. Gentamicin treatment sufficient to destroy hair cells in the basilar papilla causes a rapid, transient downregulation of FGFR3 mRNA in the region of damage. In the initial stages of hair cell regeneration, the support cells that reenter the mitotic cycle in the basilar papilla do not express detectable levels of FGFR3 mRNA. However, once the hair cells have regenerated in this region, the levels of FGFR3 mRNA and protein expression rapidly return to approximate those in the undamaged epithelium. These results indicate that FGFR3 expression changes after drug-induced hair cell damage to the basilar papilla in an opposite way to that found in the mammalian cochlea and may be involved in regulating the proliferation of support cells.

Key Words: **Cek2; basilar papilla; chicken; hair cell; growth factors; receptors; cochlea.**

INTRODUCTION

Hair cells are the sensory receptors of the vestibular and auditory epithelia. In birds, hair cell production occurs throughout life in the vestibular epithelia (Jorgensen and Mathiesen, 1988; Roberson *et al.*, 1992), but not the auditory epithelium (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Oesterle *et al.*, 1993). Excessive noise, certain antibiotics, and normal aging cause hair cell loss in the auditory epithelium of both birds and mammals, but in birds, new hair cells replace those that degenerate (Cotanche, 1987; Cruz *et al.*, 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Lippe *et al.*, 1991). Since the discovery of hair cell regeneration in the avian inner ear, a substantial effort has been made to more fully understand the mechanisms of hair cell development and regeneration in these animals. The regenerated hair cells are thought to arise from supporting cells within the sensory epithelium. Within 24 h after the damage, the first support cells have entered the mitotic cycle (Girod *et al.*, 1989; Raphael, 1992; Hashino and Salvi, 1993; Stone and Cotanche, 1994). The first identifiable new hair cells differentiate as early as 5 days after the damage (Duckert and Rubel, 1990; Stone *et al.*, 1996). While most of the new hair cells appear to arise from mitotic divisions of support cells, there is also some evidence that support cells can transdifferentiate into hair cells independently of mitosis (Roberson *et al.*, 1996; Adler *et al.*, 1997). The regeneration of hair cells results in the functional recovery of the auditory and vestibular systems (McFadden and Saunders, 1989; Tucci and Rubel, 1990; Marean *et al.*, 1993; Carey *et al.*, 1996; Goode *et al.*, 1999; Smolders, 1999). Although birds show a robust regenerative response to auditory hair cell damage, an analogous response has not been observed in the mammalian cochlea

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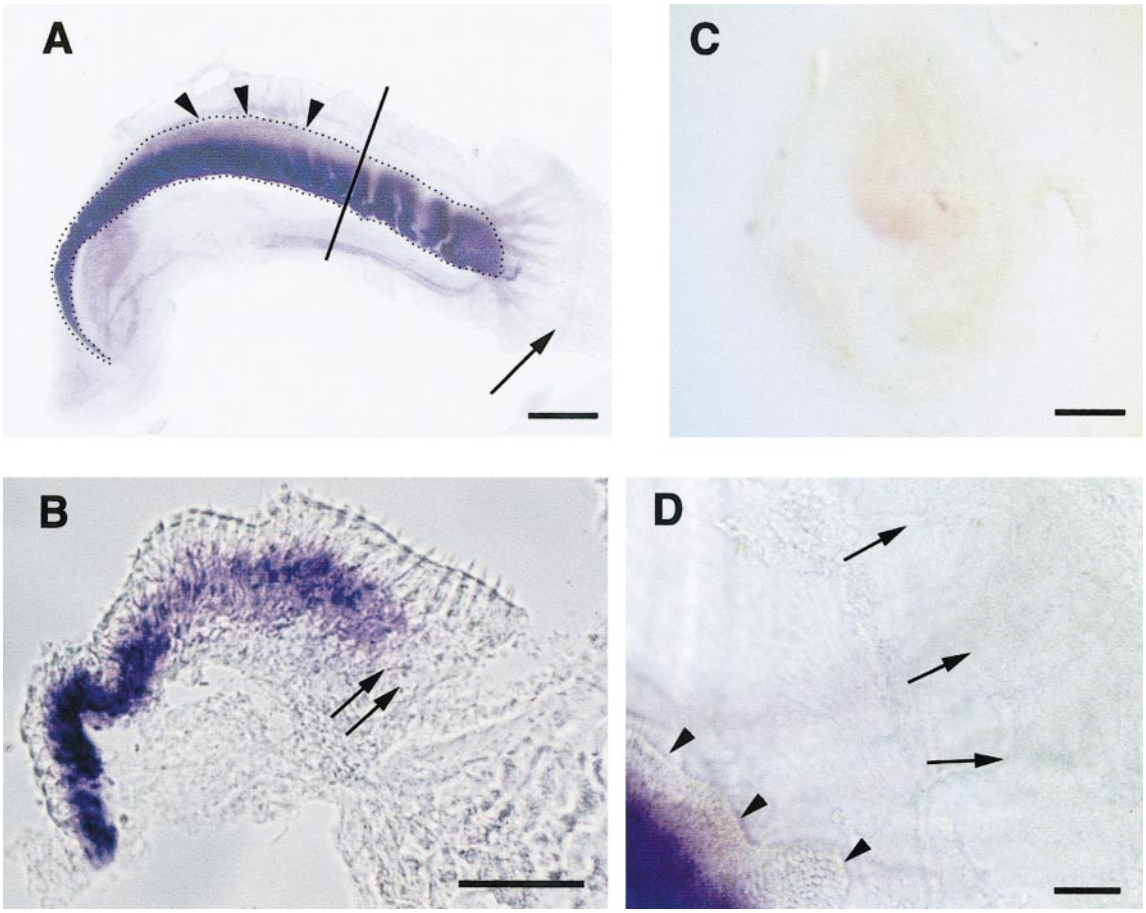

FIG. 1. FGFR3 mRNA is expressed in the support cells of the adult chicken basilar papilla but not in the vestibular system. *In situ* hybridization for FGFR3 mRNA expression in mature chicken auditory and vestibular sensory epithelia. (A) In the whole mount shown the organ is positioned so that the wider distal end of the basilar papilla is toward the right of the micrograph and the narrower proximal end (or base) is near the bottom left corner. The neural side is toward the top of the figure. The dotted line indicates the area covered by the sensory epithelium. A long stripe of labeled cells can be seen extending the full length of the epithelium. Arrowheads point to the area of the sensory epithelia (SE) where support cells do not express FGFR3 mRNA. Arrow points to the lagena macula. (B) A cross section through the epithelium at approximately the position of the line in A. In this section the hair cells are at the top of the section and the stereocilia can be seen as small hairs on the top of the hair cells. The FGFR3 mRNA expression is confined to the support cell layer, and the gene is not expressed in the hair cells. Arrows point to the area of the SE where support cells do not express FGFR3 mRNA. (C) *In situ* hybridization of a whole-mount chicken utricle processed together with the basilar papilla shown in A; there is no expression of FGFR3 mRNA in this vestibular organ. (D) *In situ* hybridization of a whole-mount chicken lagena; arrows point to the SE of the lagena to show the lack of FGFR3 mRNA expression in this vestibular organ; arrowheads point out the adjacent basilar papilla. Scale bar = 200 μm in A and C and = 50 μm in B and D.

(Roberson *et al.*, 1992; Lowenheim *et al.*, 1999; Romand and Chardin, 1999). No conditions have yet been reported that enable hair cell replacement in the mammalian auditory or vestibular epithelium, although there is some evidence of limited support cell proliferation (Forge *et al.*, 1993; Rubel *et al.*, 1995; Lopez *et al.*, 1997; Zheng *et al.*, 1997; Kuntz and Oesterle, 1998).

Several investigators have attempted to stimulate the process of hair cell regeneration in the chick and mammalian inner ear epithelia by the addition of peptide growth factors or through the manipulation of second messenger pathways. In the chick vestibular epithelia, IGF-1 and insulin stimulate the proliferation of the progenitor cells (Oesterle *et al.*, 1997), whereas in the mammalian vestibular epithelia, TGF-α stimulates new mitotic activity (Lambert, 1994; Yamashita and Oesterle, 1995; Kuntz and Oesterle, 1998). In the chick basilar papilla or the mammalian cochlea, it has been more difficult to identify factors that can stimulate new hair cell production or even proliferation of the support cells. Nevertheless, the results of experiments in the vestibular system suggest that the receptor tyrosine kinase signal transduction cascade is critical for

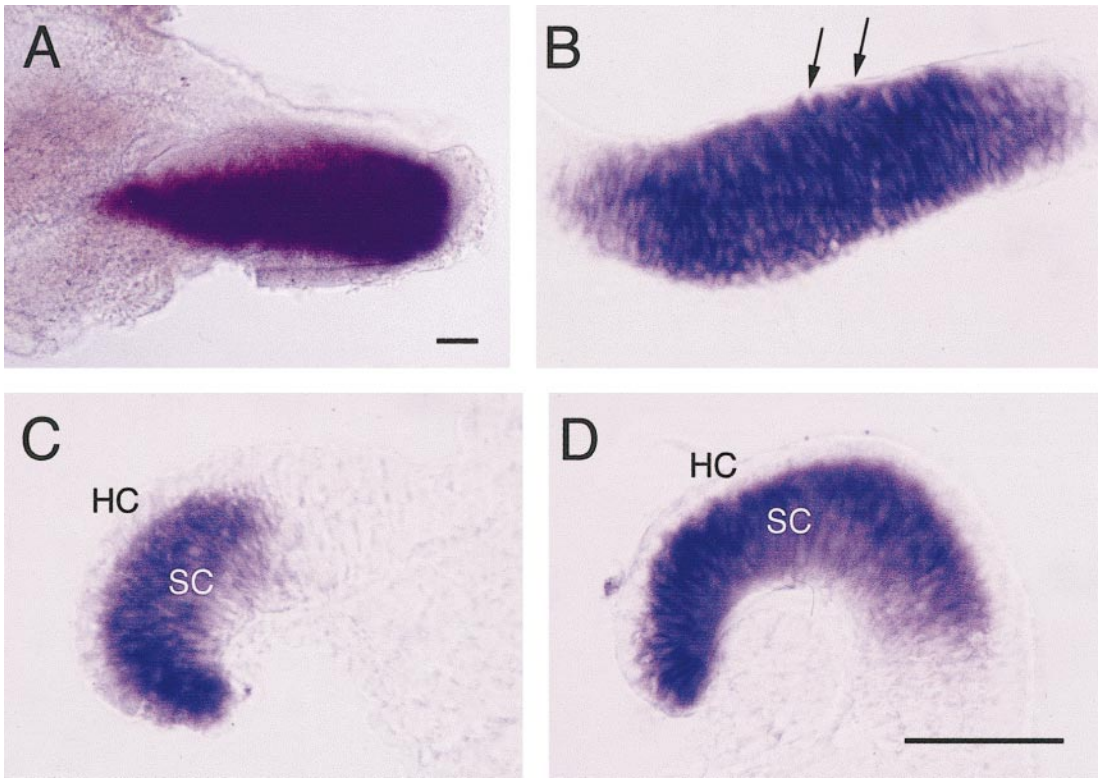

FIG. 2. FGFR3 mRNA is initially expressed in the progenitors of both hair cells and support cells. The expression of FGFR3 mRNA by *in situ* hybridization in the chick basilar papilla at various stages of embryonic development is shown. (A) At E5.5 FGFR3 mRNA marks the extent of the developing basilar papilla. (B) In cross section, the FGFR3 mRNA is expressed throughout the width of the epithelium in the region where hair cells and support cells will develop. The arrows point to mitotic on the lumenal side of the epithelium where the hair cells will develop. In cross sections of the developing epithelium at E11 (C) and E14 (D), when hair cells (HC) have differentiated, the FGFR3 mRNA expression appears to be expressed only in the support cells (SC). Scale bar = 50 μm in both A and D; scale bar in D applies also to B and C.

the control of proliferation and differentiation in the inner ear epithelia.

Several recent studies point to a specific receptor tyrosine kinase, fibroblast growth factor receptor 3 (FGFR3), as playing a key role in the development and maintenance of the auditory epithelium in mammals. FGFR3 is highly expressed in the developing and mature mammalian organ of Corti (Peters *et al.*, 1993; Pirvola *et al.*, 1995; Colvin *et al.*, 1996; Pickles *et al.*, 1998). Following noise damage to the rat cochlea, FGFR3 is rapidly upregulated in the support cells, suggesting that this receptor plays a role in the response to hair cell loss (Pirvola *et al.*, 1995). In addition, deletion of this gene through homologous recombination in mice results in abnormal cochlear development; a particular type of support cell, the pillar cell, fails to develop in knockout mice (Colvin *et al.*, 1996). We undertook a study of the FGFR3 mRNA in the developing and mature chicken basilar papilla to determine whether FGFR3 shows a similar pattern of expression in birds. We were particularly interested in determining whether FGFR3 in the chicken showed the same change in expression following damage to hair cells as has been reported in mammals, given that in birds damage is followed by regeneration, while in mammals it is not. We have found that FGFR3 changes after drug-induced damage to the basilar papilla, but in an opposite way to that found in the mammalian cochlea, and we discuss this finding in the context of a potential role for this molecule in the regeneration process.

MATERIALS AND METHODS

Animals

White leghorn chickens or fertilized eggs were obtained from H&N International (Redmond, WA). Hatchlings were housed in heated brooders with free access to food and water, according to the guidelines of the Department of Comparative Medicine at the University of Washington. The University of Washington Animal Care Committee approved all procedures. Eggs were incubated at 37°C and embryos staged according to Hamburger and Hamilton.

RNA Isolation and Cloning of Receptor Tyrosine Kinases

Seven-day-old chickens were euthanized by overdose of pentobarbital (100 mg/kg; intraperitoneal injection) and decapitated. Cochlear ducts were dissected from the temporal bone and placed in cold Hank's buffered saline solution (HBSS; Gibco/BRL, Grand Island, NY). The tegmentum vasculosum was removed with fine microforceps, and the remaining tissue was placed in 0.01% Type I collagenase (Sigma Chemical, St. Louis, MO) in HBSS for 3 min. The tectorial membrane was removed by grasping it with microforceps at the distal (apical) end of the organ and pulling it off the entire length of the sensory epithelium (SE). Incubation in collagenase was continued for a further 5 min and the sheet of sensory epithelium was lifted from the basilar membrane using an insulin syringe needle to separate the layers. The dissected tissue was homogenized immediately in Trizol (Gibco-BRL/Life Technologies, Rockville, MD) and total RNA isolated according to the manufacturer's instructions. A 3.6 μg sample of total RNA was obtained from the basilar papillas of 24 chicks; 1.5 μg of total RNA was reverse transcribed using random primers and MMLV reverse transcriptase (Stratagene, La Jolla, CA). This cDNA was then used as a template for PCR with degenerate primers directed to the kinase domain, XTK-1 and XTK-2, from Brandi and Kirschner (1995). The products from the PCR reaction products were separated on a 2% agarose gel (Nusieve; FMC BioProducts, Rockland, ME) and the 220-bp reverse transcriptase-dependent fragment was isolated and reamplified by PCR using the original primers. The products of the reamplification were cloned into pBluescript using the TA cloning system (Invitrogen, San Diego, CA) according to the manufacturer's instructions.

In Situ Hybridization

The FGFR3 coding sequence was cloned by RT-PCR using as a template RNA derived from the sensory epithelium of the chick basilar papilla (primer sequences: forward: GGAGCGAGACCGCCTTTCTG; reverse: GGGTCAGGCGAGAACGTGCC). These primers generated an FGFR3 fragment, containing nucleotides 269–1553 of the Cek2 gene (Pasquale, 1990), which was subsequently cloned into pBluescript. To obtain sense and antisense probes, respectively, the plasmid was linearized with *Xho*I or *Not*I and 1 μg DNA incubated with T7 or T3 RNA polymerase and digoxigenin–UTP according to the protocol provided by the manufacturers (Life Technologies). *In situ* hybridization was carried out essentially as described in Henrique *et al.* (1995). Embryos were removed from eggs, staged, decapitated, and the temporal bones dissected. The cochlear duct was further dissected and fixed in 4% formaldehyde/2 mM EGTA in phosphate-buffered saline (PBS) for 2 h at 4°C. Hatchlings were terminated with an overdose of pentobarbital and decapitated. The cochlear ducts were removed and fixed in 4% formaldehyde/2 mM EGTA in PBS. The basilar papilla was then isolated from the surrounding tissue, and the tectorial membrane was removed. For both the embryos and the hatchlings, after fixation, the tissue was rinsed in PBS/0.1% Tween 20 (PTW), then incubated in 50% methanol/PTW and then stored at −20°C in 100% methanol. Tissue was rehydrated, treated with proteinaseK (10 μg/ml) in PTW, and postfixed in 4% formaldehyde/0.1% glutaraldehyde. Following a rinse in PTW, the tissue was incubated with hybridization mix, lacking the probe, for 1 h at 65°C. Tissue was then hybridized in approximately 1 μg/ml digoxigenin-labeled RNA probe for FGFR3/cek2 overnight at 65°C. After hybridization, the tissue was washed in hybridization solution, and then bound probe was revealed with an antibody to digoxigenin conjugated to alkaline phosphatase (Boehringer-Mannheim, Germany).

In some cases, the chicks were also given bromodeoxyuridine (BrdU) injections to detect mitotically active cells (see below); the BrdU-labeled cells were detected by immunohistochemistry, as follows. After the *in situ* hybridization and color reaction, the tissue was incubated in 4% paraformaldehyde for 20 min, incubated in 2 M HCl for 1 h, washed twice with 0.1 M sodium borate (pH 8.5), and then rinsed with PBS prior to blocking in 10% normal goat serum and subsequent overnight incubation with an antibody against BrdU (Becton Dickinson, San Jose, CA) (Biffo *et al.*, 1992). The primary antibody was detected with the appropriate secondary antibody conjugated to Bodipy/FL; Molecular Probes, Eugene, OR) and visualized with fluorescent optics. The tissue was then examined and photographed as whole-mount preparations, and in some cases, embedded in OCT compound for cryostat sectioning or in Historesin for plastic sectioning.

Experimental Damage Protocol

The induction of hair cell damage and regeneration in hatchling chicks was carried out as follows. A single injection (400 mg/kg) of the ototoxic aminoglycoside antibiotic gentamicin (Fugisawa) was given to 7-day-old chicks. This dose of the drug kills all hair cells in the proximal (basal) one-third to one-half of the basilar papilla (Stone *et al.*, 1996). A single injection of 100 mg/kg of BrdU (Sigma) in PBS was given intraperitoneally to some of the gentamicin-treated animals, 2 h prior to euthanasia. The animals were euthanized 1–7 days after gentamicin injection as described above, and the cochlear ducts were removed and fixed in 4% formaldehyde/2 mM EGTA in PBS.

After *in situ* hybridization for FGFR3 mRNA, the treated basilar papillas and age-matched controls were analyzed as follows. Video images of the whole-mount preparations were captured with an MTI CCD camera coupled to a Quadra 950, using NIH Image software. The length of FGFR3 mRNA expression was measured along the sensory epithelium from the distal end (apex) to the most proximal point where consistent expression was observed. In treated basilar papillas the FGFR3 mRNA expression was substantially reduced in the proximal regions, with little or no reduction in the distal region. Thus, the length of FGFR3 mRNA expression serves as a valid measure of both the region and the amount of change in expression after treatment. A total of 33 basilar papillas were analyzed (see Fig. 4 legend below for details). As described above, some of the animals received a 2-h pulse of BrdU to label dividing support cells in the basilar papilla. To quantify the relationship between the S-phase cells and the changes in FGFR3 mRNA expression, we counted every BrdU-labeled cell from 8 basilar papillas at 3 days postgentamicin treatment and determined whether each had detectable levels of the *in situ* hybridization reaction product.

FGFR3 Antibody Production

A peptide corresponding to amino acid residues 795–811 at the carboxy-terminus of chick FGFR3 was synthesized. This peptide was used to immunize two rabbits. One rabbit mounted robust immune response and sera were harvested. The serum was tested at various concentrations and found to be effective at a 1:10,000 dilution. The serum was also affinity purified on a peptide affinity column. The affinity-purified material was not significantly better

than the crude serum, so crude serum was used for the Western blots.

Western Blot Analysis

Whole cochlear ducts were harvested at various time points after chicks were treated with gentamicin (see above). The tissue was immediately homogenized in SDS gel-loading buffer (50 mM Tris, pH 6.8, 100 mM DTT, 4% SDS, 0.2% bromphenol blue, and 20% glycerol), heated to boiling for 10 min and centrifuged at 10,000*g* for 5 min. The supernatant was loaded on a 7.5% polyacrylamide gel and electrophoresed in running buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS). After electrophoresis was complete, the gel was removed from the glass plates and the proteins were electrophoretically transferred to PVDF membrane (Bio-Rad, Richmond, CA). The blot was then washed in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min, twice. The blot was then washed in blocking solution (0.2% nonfat dry milk in TBS) for 1 h. The blot was then incubated overnight at room temperature in FGFR3 antiserum at a dilution of 1:10,000 in blocking solution containing 0.1% Tween 20. Antibody binding was detected using an Immuno-Star Chemiluminescent protein detection system (Bio-Rad). The blot was exposed to hyperfilm-ECL (Amersham, Arlington Heights, IL) for between 10 s and 1 min.

RESULTS

FGFR3 mRNA Is Highly Expressed in Most Support Cells of the Basilar Papilla

The chick basilar papilla is an elongated, sickle-shaped sensory epithelium (SE), with a tonotopic organization in which high frequencies are encoded toward the narrow proximal end and progressively lower frequencies at the much wider distal region. The basilar papilla is a stratified epithelium, composed primarily of two cell types: the hair cells that act as the sensory receptors and the intercalated support cells. The support cell nuclei reside in a layer under the hair cells, although the support cell bodies extend from the basal lamina to the lumenal surface. Using a degenerate PCR strategy (see Materials and Methods) to identify which receptor tyrosine kinase receptors are expressed in the SE, we identified FGFR3/Cek2 (Pasquale, 1990). In fact more than 80% of the clones that we isolated, out of a total of 150 clones analyzed, corresponded to FGFR3.

Figure 1A shows the expression of FGFR3 mRNA in the mature chicken cochlear duct prepared as a whole mount for *in situ* hybridization. FGFR3 mRNA is expressed from the most proximal region of the SE to the distal end (Fig. 1A). This gene does not appear to be expressed anywhere else in the cochlear duct. The expression does not cover the entire width of the SE and so appears narrower than the SE itself, particularly in the middle regions at the neural (superior) edge. In Fig. 1A the area of the sensory epithelium is indicated by the dotted line and the arrowheads point to the region not expressing FGFR3. Sections through whole-mount preparations processed for *in situ* hybridization revealed that the FGFR3 mRNA expression is confined to the support cells (Fig. 1B). Figure 1B is taken from a section about one-third the distance from the distal tip. The labeled support cells can be clearly seen, whereas the overlying hair cells are unlabeled. The expression of FGFR3 mRNA in support cells is not uniform, however; support cells at the abneural (inferior) edge are consistently more intensely labeled, while several rows of support cells at the neural (superior) edge are unlabeled (Fig. 1B, arrows). This pattern is observed in the midregions of the SE, but in the most distal region and the most proximal regions, all support cells express FGFR3 mRNA.

We also examined expression of FGFR3 mRNA in chick vestibular organs. The chick inner ear has six vestibular SE including three maculae (the utricle, the sacculus, the lagena) and the crista of each of the three semicircular canals. We examined all of the vestibular organs in post-hatch chicks and found no detectable expression of FGFR3. Figure 1C shows an example of a utricle that was processed for *in situ* hybridization along with the basilar papilla shown in Fig. 1A. Even after long reaction times, we saw no expression in the utricle. The lagena is a smaller vestibular receptor organ located at the distal end of the basilar papilla (see arrow in Fig. 1A) and it also lacks detectable expression of FGFR3 mRNA (Fig. 1D).

FGFR3 mRNA Is Expressed Early in the Development of the Basilar Papilla

Because FGFR3 mRNA is confined to the support cells in the mature basilar papilla, we were interested in determining the pattern of expression during development of hair cells and support cells. Birthdating studies of hair cell production in the chick basilar papilla have shown that cells in the SE first withdraw from the cell cycle on embryonic day 5 (E5) (Katayama and Corwin, 1989). A wave of hair cell differentiation sweeps across the epithelium from superior–proximal to inferior–distal, and hair cell generation is complete by E9 (Cotanche and Sulik, 1983, 1984). Support cells and hair cells are generated concurrently, and lineage analysis has shown that these cells arise from a common progenitor (Fekete *et al.*, 1998).

We analyzed the expression of FGFR3 mRNA in embryonic chick basilar papilla from E3 to E14 by *in situ* hybridization. We did not find any specific expression in the otic vesicle prior to E3.5. However by E5.5, FGFR3 mRNA is expressed throughout the growing basilar papilla (Fig. 2A). The expression of the gene at this stage extends through the thickness of the epithelium, in what appear to be the progenitors of both hair cells and support cells (Figs. 2A and 2B). The arrows in Fig. 2B point to two cells with mitotic figures that show expression of FGFR3 mRNA. As the hair cells differentiate in the epithelium, they lose the expression of the gene, while the differentiating support cells retain the FGFR3 mRNA expression (Figs. 2C and 2D). This pattern can be seen in the photomicrographs through the developing SE at both E11 (Fig. 2C) and E14 (Fig. 2D). Note that at both of these ages the lumenally located hair cells

are completely devoid of reaction product, while the underlying support cells are strongly labeled.

FGFR3 mRNA Is Downregulated after Gentamicin Treatment

Previous reports of FGFR3 expression in the mammalian organ of Corti demonstrated that FGFR3 mRNA and protein are upregulated after acoustic overstimulation and suggested that this receptor upregulation may represent an adaptive response to the injury (Pirvola *et al.*, 1995). We therefore asked whether the FGFR3 expression in chicken basilar papilla is under a similar form of regulation. The chicken auditory epithelium regenerates new hair cells after they are lost as a result of antibiotic treatment or acoustic overstimulation (see Introduction). Therefore, we were able to follow the changes in FGFR3 mRNA expression during both the loss of hair cells that results from aminoglycoside damage and the subsequent regeneration of new hair cells.

When we examined the expression of FGFR3 mRNA after gentamicin treatment in posthatch chickens, we found a striking change in the pattern of expression. As early as 2 days after the gentamicin treatment, there is a clear decline in the level of FGFR3 mRNA expression in the proximal region of the basilar papilla, where the hair cells are degenerating. Figure 3 shows the expression pattern of FGFR3 mRNA in the basilar papilla at 2–5 days after gentamicin treatment in whole-mount preparations of the basilar papilla. The area between the two arrows indicates the region that has lost hair cells as a result of the gentamicin treatment. By 2 days after treatment there is a reduction in expression of FGFR3 mRNA in the region of damage. The reduction in expression has reached a maximum by Day 3. We can see weak expression returning by 4 days postgentamicin treatment and by 5 days after the treatment expression in the support cells has returned. The downregulation of FGFR3 mRNA expression in the region of damage occurs in every case, but in some it is more pronounced than in others. The decline in FGFR3 mRNA expression is at times somewhat patchy (see Fig. 6 below).

The decline in FGFR3 mRNA expression reverses as regeneration progresses. As shown in Fig. 3, by 4 days there is some recovery in expression levels and by 5 days the expression level has returned to near normal. To further analyze this change in FGFR3 mRNA expression, we took measurements of the length of the basilar papilla that express detectable levels of FGFR3 mRNA in the gentamicin treated animals, and compared the values with those of the control animals. Figure 4 shows that FGFR3 mRNA expression is rapidly lost from the proximal one-third of the basilar papilla, but it approaches control levels within a few days. The length of FGFR3 mRNA expression does not fully recover; even after 14 days following the gentamicin treatment it remains at approximately 75% of the untreated value. It is possible that the failure of FGFR3 mRNA expression to fully recover in the proximal end of the basilar papilla is attributable to differential shrinking of treated versus untreated basilar papillas.

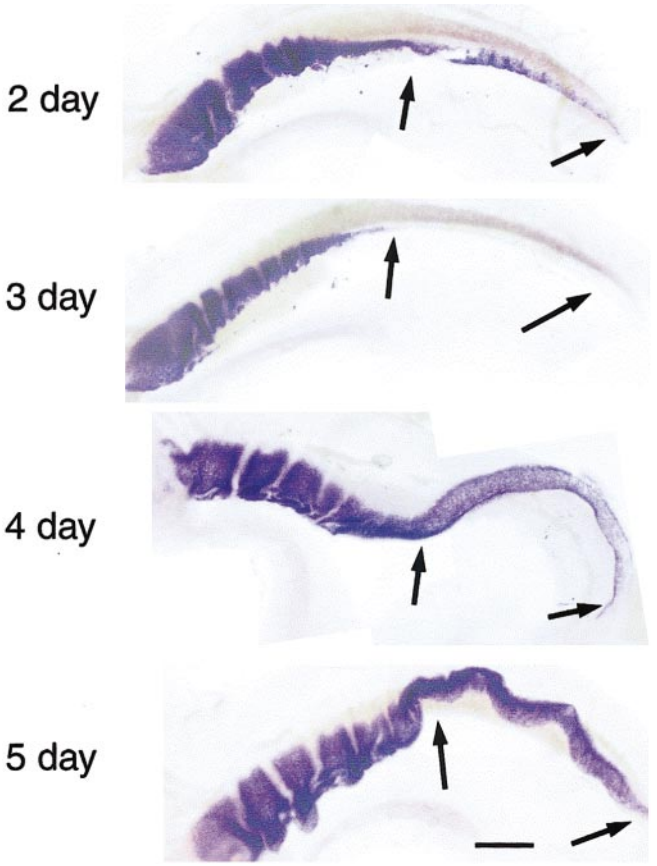

FIG. 3. FGFR3 mRNA expression declines in the proximal end but not the distal end of the basilar papilla after gentamicin treatment. Photomicrographs of whole-mount *in situ* hybridization for FGFR3 mRNA in basilar papillas at 2, 3, 4, and 5 days after gentamicin treatment. The decline in FGFR3 mRNA expression is primarily in the proximal region of the basilar papilla (demarcated by arrows), where the hair cells have been completely lost. The distal region in which hair cells remain shows little if any reduction in FGFR3 mRNA expression levels (compare to Fig. 1A). The FGFR3 mRNA in the support cells in the proximal end of the basilar papilla begins to decline within 2 days after the treatment and gradually recovers over the next few days. Scale bar = 200 μm.

The downregulation and recovery of FGFR3 mRNA expression after gentamicin treatment is reminiscent of the regenerative response of this tissue. We directly related the changes in FGFR3 mRNA expression to the proliferative response of the support cells using the same damage protocol. We superimposed on the plot of FGFR3 mRNA expression the number of BrdU-positive cells per square mm of tissue in the damaged area at the corresponding dates after damage, as assessed by a 2-h pulse of BrdU (see Fig. 5 in Stone *et al.*, 1999). These data show that FGFR3 mRNA is downregulated prior to the upregulation of BrdU incorpora-

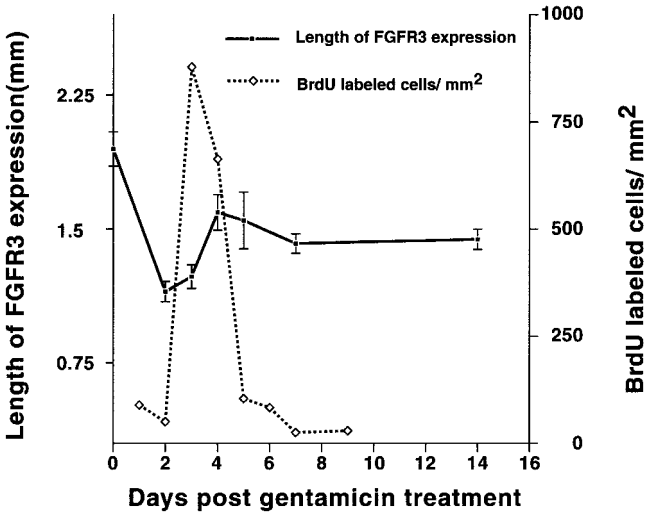

FIG. 4. Quantitation of the extent of FGFR3 mRNA expression after gentamicin treatment. The extent of FGFR3 mRNA expression along the length of the basilar papilla was measured as described in the text and is shown as a function of days after gentamicin treatment. The rapid downregulation in expression at 2 days after treatment is followed by a slower recovery that does not reach pretreatment levels even after 14 days postgentamicin. Values shown are means ± SD (at 0, 2, 3, 4, 5, 7, and 14 days posttreatment; n = 7, 5, 5, 5, 2, 6, and 3 of basilar papillas, respectively). The dotted line depicts quantification of BrdU-labeled cells in chick basilar papillas 3 days after treatment with gentamicin and 2 h after administration of BrdU. The data to generate this graph came from the study of Stone *et al.* (see Fig. 5 in Stone *et al.*, 1999).

tion and that the recovery of FGFR3 mRNA expression roughly follows the same time course as the cessation of mitotic activity by support cells in the basilar papilla.

To look directly at the FGFR3 protein, we made an antibody directed to the C-terminus of the chick FGFR3. This polyclonal serum was used to probe Western blots of extracts of chick cochlear duct. The antiserum detected a prominent band at approximately 118 kDa (Fig. 5A), which is similar to the size of the mammalian FGFR3 protein (Keegan *et al.*, 1991a,b; Monsonego-Ornan *et al.*, 2000). When we used the antiserum to examine the FGFR3 protein levels in the auditory organ after gentamicin damage, we found that the protein expression was rapidly and dramatically decreased as early as 1 day postgentamicin treatment (see band at ~118 kDa in Fig. 5B). The protein appears to return to normal levels between Day 3 and Day 4; thus the protein expression closely correlates with the mRNA expression pattern. We see what appear to be breakdown products running between 20 and 40 kDa (Fig. 5B, arrowheads). These bands increase in intensity as the intensity of the 118-kDa band decreases. We used the preimmune serum as a control and detected no bands on the Western blot (Fig. 5A).

FGFR3 mRNA Expression during Hair Cell Regeneration

We next directly compared the proliferative response in the gentamicin-treated animals with the spatial pattern of expression of FGFR3 mRNA in the support cells by double labeling for BrdU incorporation and for FGFR3 mRNA. We found that nearly all of the mitotically active cells cluster in regions where FGFR3 mRNA expression has been lost or dramatically diminished. Figure 6 shows these results in animals that were studied 3 days after gentamicin treatment, the time at which the mitotic activity peaks (see Stone *et al.*, 1999). To study cells in S phase, all animals received BrdU injections 2 h prior to termination. Figure 6B shows BrdU-labeled cells in the region of the SE where hair cells are lost. Figure 6A shows that the same areas have undergone a marked decline in the expression of FGFR3 mRNA, as detected by *in situ* hybridization. Figures 6C–6E show the same correlation at a higher magnification and, although a few cells are labeled with both the FGFR3 probe and the BrdU label, the majority of BrdU-labeled cells do not express detectable levels of FGFR3 message. In some cases, we observed patches of expression of the FGFR3 gene that completely exclude the proliferating support cells, while in other cases, there are a few BrdU-labeled cells in the region of FGFR3 mRNA expressing support cells. Table 1 summarizes counts of labeled cells from 8 basilar papillas from chicks given a 2-h pulse of BrdU at 3 days following drug damage. The counts show that in all basilar papillas over 85% of the BrdU-labeled cells are not labeled with the FGFR3 probe. Thus, there is a marked downregulation in the expression of this gene as the support cells enter the mitotic cycle.

DISCUSSION

We found that FGFR3 mRNA is highly expressed in most support cells in the mature chick basilar papilla. The gene is expressed early in the development of the basilar papilla, with detectable levels of transcripts observed as early as embryonic day 5 (E5). This gene is not expressed, however, in the mature vestibular organs of the chick, suggesting that it has a function specific for the auditory SE. We also found that FGFR3 mRNA and protein are transiently downregulated in the support cells in the region of the basilar papilla in which hair cells degenerate as a result of gentamicin treatment. At the peak of the proliferative response in the initial stages of the regeneration process after drug damage (i.e., 3 days postgentamicin treatment), over 85% of the support cells that reenter the mitotic cycle in the basilar papilla do not express detectable levels of FGFR3 mRNA. However, once the hair cells have regenerated in this region, the levels of FGFR3 expression rapidly return to approximate those in the undamaged epithelium.

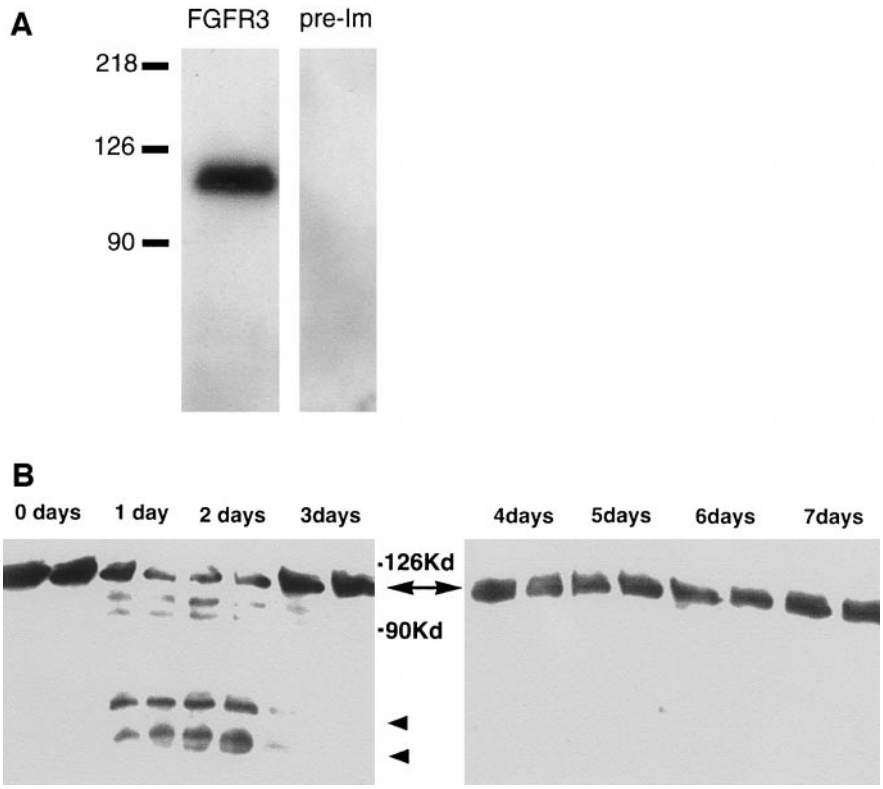

FIG. 5. Western analysis of FGFR3 expression after gentamicin treatment in the chick basilar papilla. Extracts of cochlear ducts were run on a 7.5% gel and electrophoretically transferred to PVDF membrane. Each lane corresponds to a single cochlear duct equivalent. Each time point is run in duplicate. (A) Comparison of preimmune and immune sera; (B) the protein expression at various times after gentamicin treatment. A band can be seen at approximately 118 kDa. Bands can also be detected at lower molecular weights between 20 and 40 kDa (see arrowheads). The lower-molecular-weight bands occur in lanes in which a dramatic decrease in the amount of full-length FGFR3 protein is seen, so it seems reasonable to assume these are proteolytic fragments of the receptor.

FGFR3 Expression in Mature and Developing Inner Ear Sensory Epithelium

FGFR3 is a highly expressed receptor tyrosine kinases in the chick auditory epithelium. FGFR3 mRNA is expressed in the support cells of the basilar papilla but not in the hair cells. This result is similar to what has been observed in the mature mammalian organ of Corti. FGFR3 has been shown to be expressed by two sets of support cells: the pillar cells and the Deiter cells (Pirvola *et al.*, 1995). The vestibular epithelia do not appear to express detectable levels of this receptor in the chick, consistent with the observation that no vestibular phenotype is seen in mice homozygous null mutants of FGFR3 (Colvin *et al.*, 1996).

The analysis of FGFR3 mRNA expression in the developing basilar papilla shows that this gene is initially expressed throughout the basilar papilla at E5.5. The expression pattern suggests that it is probably present in the precursors of both the hair cells and the support cells (Fekete *et al.*, 1998). The expression, which is not present in the hair cells because they differentiate at the lumenal surface of the epithelium, is retained in support cells to maturity. This pattern of expression, in which a gene is initially expressed ubiquitously in the sensory precursor cells, and then later becomes restricted to support cells, is reminiscent of the pattern of BMP7 described by Oh *et al.* (1996). However, in the case of BMP7, the expression does not become restricted to the support cells until much later in embryonic development at E16. Another difference is that BMP7 is also expressed in the vestibular epithelia where we did not detect any FGFR3 mRNA expression. BMPs and FGFs are thought to interact in a complex feedback loop in the developing limbs and bones (Buckland *et al.*, 1998), and it is possible that some similar type of interaction may occur in the basilar papilla. In addition it has been reported that BMP2 can upregulate the expression of FGFR1 in PC12 cells (Hayashi *et al.*, 2001). Other genes that show a somewhat similar pattern and time course of expression include those involved in Notch signaling. Serrate and lunatic fringe are initially expressed in all auditory precursor cells in chick and mouse, and then appear to become restricted to the support cells (Adam *et al.*, 1998; Morsli *et al.*, 1998).

As noted above, previous studies of the developing chick

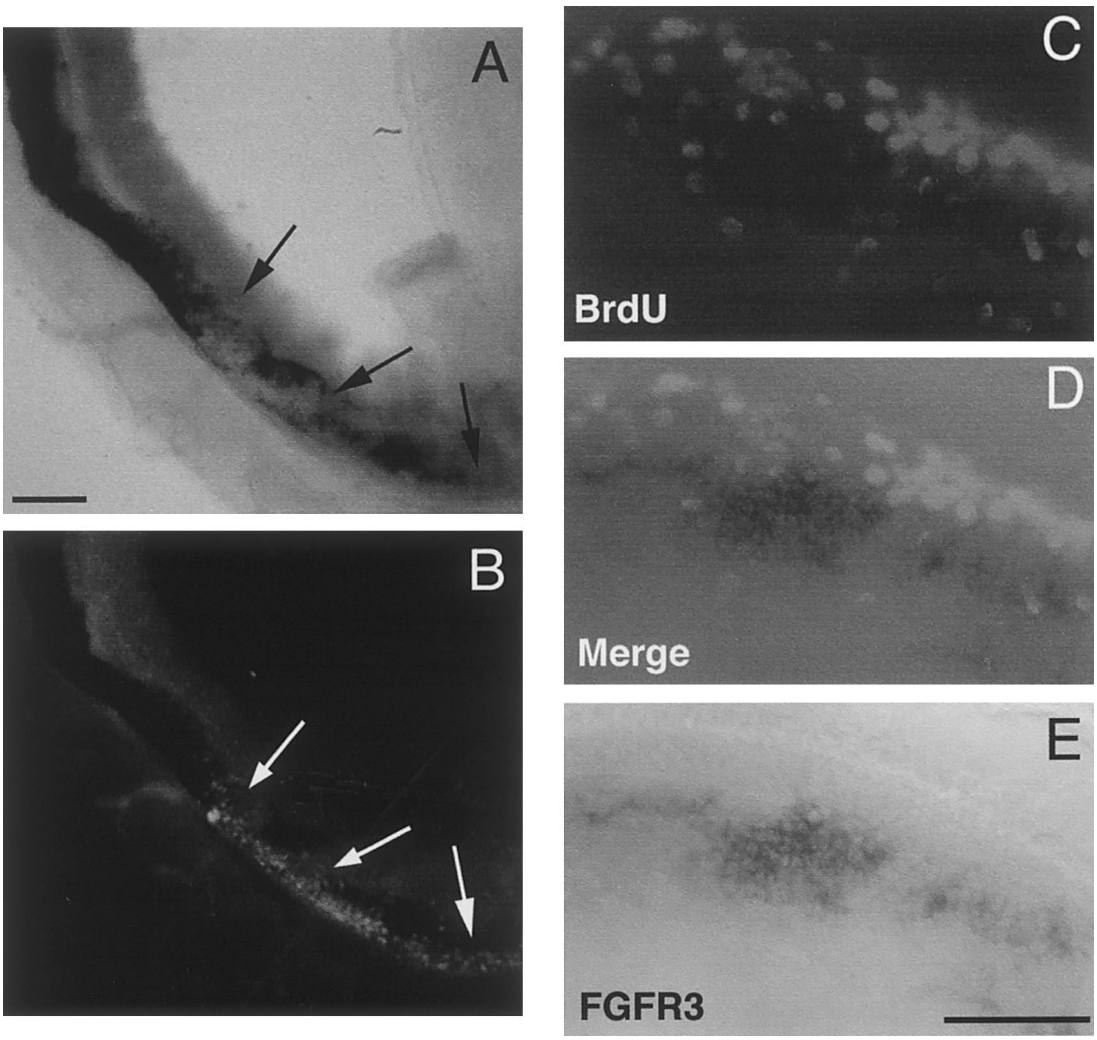

FIG. 6. FGFR3 mRNA expression declines during regeneration of the hair cells in the chick basilar papilla. Photomicrographs showing bright-field and fluorescent views of a cochlear duct that has been doubled labeled for FGFR3 mRNA by *in situ* hybridization (A) and an antibody to BrdU (B), 3 days after gentamicin damage. At the particular concentration of gentamicin used in this experiment, the hair cell loss is primarily in the proximal end. As a result, the regeneration is occurring only in the proximal half of the papilla, as evidenced by the large number of BrdU-labeled cells in this area (arrows in B). Coincident with the increase in mitotic activity in this area is a decline in FGFR3 mRNA expression (arrows in A). (C–E) Fluorescent, merged, and bright-field views of a basilar papilla that was doubled labeled for FGFR3 mRNA by *in situ* hybridization (E) and BrdU (C). The receptor expression has declined in the areas where the proliferating support cells are found, but is still highly expressed in a patch that lacks proliferating cells. Scale bar = 100 μm in A and 50 μm in E.

basilar papilla have shown that a common precursor to hair cells and support cells generates both types of cells from E5 to E8 (Katayama and Corwin, 1989; Fekete *et al.*, 1998). The newly generated hair cells begin to express cell-specific antigens within a day of their withdrawal from the cell cycle (e.g., HCA) (Goodyear and Richardson, 1997; Molea *et al.*, 1999). Thus, the restriction of FGFR3 to the support cells appears to roughly coincide with the withdrawal of these cells from the cell cycle. However, our analysis was not at sufficiently fine grain to determine precisely when a differentiating hair cell ceases FGFR3 expression. In addition, it is clear that during development proliferating cells in the SE are expressing FGFR3 mRNA.

FGFR3 Is Downregulated after Gentamicin Treatment

We found that FGFR3 mRNA and protein are downregulated in the proximal end of the basilar papilla after gentamicin treatment. The region of FGFR3 mRNA downregulation corresponds to the region of the basilar papilla that sustains the loss of hair cells after the antibiotic treatment.

TABLE 1
The Majority of BrdU Incorporating Support Cells No Longer Express FGFR3 mRNA

$BrdU^+/FGFR3^-$	$BrdU^+/FGFR3^+$		$\%BrdU^+/FGFR3^-$
171	11		93.9
114	21		84.4
29	3		90.6
79	6		92.9
105	12		89.7
54	2		96.4
136	10		93.1
123	12		91.1
		Average	91.5

Notes. Three days after gentamicin treatment, posthatch chicks received a pulse of BrdU to label dividing support cells in the basilar papilla. After 2 h, the basilar papillas were dissected and processed for detection of both FGFR3 (by *in situ* hybridization) and BrdU (by immunohistochemistry). The number of labeled cells was quantified from the entire basilar papilla, and although there was considerable variation in the number of BrdU-labeled cells in the different animals (usually due to the fact that the preparation of the tissue sometimes leads to loss of the very proximal end of the epithelium), in all cases the majority of dividing cells no longer express detectable levels of FGFR3.

The downregulation of FGFR3 mRNA is rapid and confined to the region of damage, suggesting that the presence of healthy hair cells is required to maintain normal levels of FGFR3 expression by support cells. Moreover, the FGFR3 levels are restored to near normal levels when hair cells have regenerated. Thus, we propose that hair cells are necessary to maintain the expression levels of FGFR3 in the support cells.

The local nature of the effect suggests that the signal may also be a short-range one. One such signaling system that was previously studied during regeneration is the interactions between the Notch receptor and its ligands delta and serrate. Stone and Rubel (1999) have shown that delta is expressed during hair cell regeneration in the damaged region in the regenerating hair cells. Therefore, it is possible that delta in some way regulates FGFR3 expression during development, although delta is not present in the mature basilar papilla under normal conditions. Even though interactions between these signaling systems were not previously reported, we observed a pattern of FGFR3 expression in the chick embryo hindbrain that is similar to the pattern of delta expression (T. Reh and O. Bermingham-McDonogh, unpublished observations).

It is also possible that the signal from the hair cells that regulates FGFR3 expression is one of the many FGFs. FGF8 has been reported to be expressed in the hair cells of the mammalian cochlea (Pirvola *et al.*, 1998, 2000), and FGF2 is expressed in rat utricular hair cells (Zheng *et al.*, 1997). FGF2 has been shown to regulate the expression of FGFR2 in bone development (Iseki *et al.*, 1997). Might the loss of FGF with hair cell loss cause the downregulation of the FGFR3 mRNA? We examined FGF8 expression and have not found it to be expressed in the chick hair cells of the basilar papilla at any stage of development (unpublished observations). However, there are now at least 22 members of the FGF family of peptides (for recent review, see Ornitz and Itoh, 2001). Therefore, it is possible that one or more other members of the family are expressed in the hair cells. FGF9 is known to be a relatively specific, high-affinity ligand for FGFR3, but it is not known whether it is expressed in the inner ear of any species. FGF17 and 18 are in the same subfamily of FGFs as FGF8 and are also potential ligands but to date these genes encoding chick forms have not been cloned. Alternatively, the innervation of the basilar papilla is also thought to undergo some changes following gentamicin treatment (Ryals *et al.*, 1992; Ofsie and Cotanche, 1996; Hennig and Cotanche, 1998). The spiral ganglion peripheral processes are known to contain FGF (Luo *et al.*, 1993). It is therefore possible that these terminals change in their release of these factors resulting in the downregulation of FGFR3. At this time, however, we have no direct evidence that FGFs regulate FGFR3 expression in this system and further experiments will be necessary to determine whether this is indeed the case.

Is the Decline in FGFR3 Expression Related to the Ability of Support Cells to Reenter the Mitotic Cycle in Chickens?

The results and discussion presented above are consistent with a role for FGFR3 in the control of proliferation and

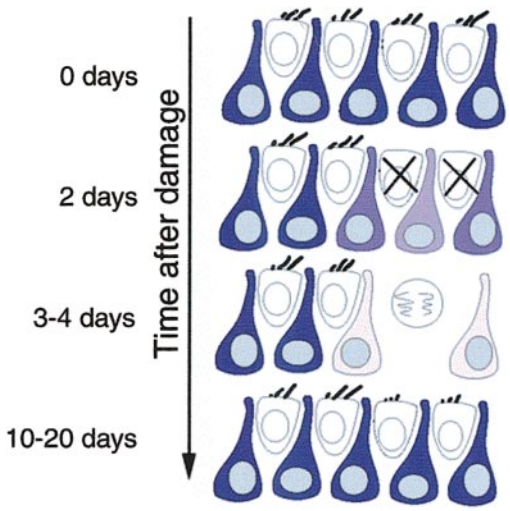

FIG. 7. A working model of FGFR3 regulation of the support cells during hair cell regeneration. Top row depicts undamaged adult basilar papilla, where the support cells express FGFR3 (dark purple). Second row shows dying hair cells after gentamicin treatment, and the FGFR3 expression in the adjacent support cells has begun to decline (lighter purple). Third row shows a support cell devoid of FGFR3 expression that has reentered the mitotic cycle. Last row shows the regenerated SE with normal expression of FGFR3 in support cells. Arrow indicates the progression of time.

differentiation of the hair cells and support cells in the developing and regenerating basilar papilla of the chick. A working model of FGFR3 regulation of the support cells is presented in Fig. 7. In the adult basilar papilla (top), the support cells express FGFR3 and the adjacent hair cells secrete a ligand that activates the receptor and inhibits their proliferation by maintaining their differentiated state. When hair cells are lost either by drug or noise damage (Xs), the FGF they release is no longer available to suppress the support cells from proliferation. As a consequence, the support cells downregulate their FGFR3 expression and are no longer inhibited from entering the mitotic state. The support cells then go on to divide and replace the lost hair cells. After a sufficient number of hair cells begin to regenerate, the support cells begin to express FGFR3 and their proliferation is inhibited (bottom).

There are three additional lines of evidence that support the hypothesis that FGFR3 is important in maintaining the support cells in a quiescent state. First, this gene is not expressed in the support cells of the vestibular SE. The hair cells and support cells of the vestibular epithelium are very similar to those of the auditory epithelium of the basilar papilla, and the two epithelia share the same fluid space. However, vestibular hair cells are constantly turning over and, consequently, some vestibular support cells are continually in a state of mitotic activity (Jorgensen and Mathiesen, 1988; Roberson *et al.*, 1992). Second, as the proliferation ceases after the hair cells have regenerated in the basilar papilla, the levels of FGFR3 are restored to those observed in the undamaged epithelium. Third, as noted above, FGFR3 is upregulated in its expression in the mammalian cochlea, after noise damage that destroys hair cells, and the support cells do not reenter the mitotic cycle in mammals. Thus the changes in FGFR3 expression are consistent with this receptor's playing an inhibitory role on the proliferation in both birds and mammals. However, it remains a formal possibility that the difference in the changes in FGFR3 expression following hair cell loss in the chick basilar papilla and the mammalian cochlea are ascribed not to a species difference but, rather, to the type of insult that caused the damage in our study versus that of Pirvola *et al.* (1995).

This gene may be critical for support cell differentiation in the chick and may regulate the transition from proliferation to differentiation in these cells. This hypothesis is consistent with other observations concerning the function of FGFR3 in other tissues of the body. Mutations in the FGFR3 gene that cause a constitutive activation are the most common cause of dwarfism in humans (achondroplasia) (Horton, 1997; Webster and Donoghue, 1997); this phenotype typically includes severe shortening of the long bones. Conversely, FGFR3 null mutant mice have the opposite phenotype, skeletal overgrowth (Colvin *et al.*, 1996). In developing bone tissue, FGFR3 is expressed in the proliferating and differentiating chondrocytes. The mutations in the FGFR3 gene that result in achondroplasia, thanatophoric dysplasia, and hypochondroplasia all cause a ligand-independent activation of the receptor (Webster and Donoghue, 1997). Thus, this kinase appears to act predominantly either to inhibit growth or to promote differentiation.

One potential mechanism by which the FGFR3 may be regulating proliferation and differentiation in the auditory epithelium is through the enhancement of expression of cell cycle inhibitor proteins. The p27 cell cycle inhibitor has recently been shown to be critical in controlling the exit from the cell cycle of the precursor cells in the mouse auditory epithelia (Chen and Segil, 1999; Lowenheim *et al.*, 1999). Coincidentally, in a recent study of the molecular mechanisms that result in skeletal dysplasias, mice carrying the lys644glu mutation in FGFR3 were examined (Li *et al.*, 1999). This mutation causes a decrease in the number of [^{3}H]thymidine-labeled cells and an upregulation of the cell cycle inhibitor proteins p16, p18, and p19. The authors suggest that activating mutations of FGFR3 inhibit proliferation and/or terminal differentiation of chondrocytes. We find that FGFR3 is expressed by dividing cells during the development of the auditory epithelia, suggesting that the ligand to activate FGFR3 is not yet present. Thus, if the ligand is produced by the hair cells, it may be required both for the initial differentiation of the support cells and for the maintenance of their phenotype in the mature basilar papilla.

There is a region in the middle of the basilar papilla at the superior edge of the sensory epithelium that does not appear to express FGFR3. This result may indicate that these support cells are a distinct class that may not have the capacity to reenter the cell cycle. In the damage paradigm used in the preceding experiments hair cells are lost from the basal one-third of the papilla; however, this does not include the region that contains the nonexpressing support cells. Thus it is difficult from our experiments to determine whether these support cells would fail to reenter the cell cycle after damage.

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