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Fibroblast Growth Factor Signaling Regulates Pillar Cell Development in the Organ of Corti

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One of the most striking aspects of the cellular pattern within the sensory epithelium of the mammalian cochlea is the presence of two rows of pillar cells in the region between the single row of inner hair cells and the first row of outer hair cells. The factors that regulate pillar cell development have not been determined; however, previous results suggested a key role for fibroblast growth factor receptor 3 (FGFR3).

To examine the specific effects of FGFR3 on pillar cell development, we inhibited receptor activation in embryonic cochlear explant cultures. Results indicated that differentiation of pillar cells is dependent on continuous activation of FGFR3. Moreover, transient inhibition of FGFR3 did not inhibit the pillar cell fate permanently, because reactivation of FGFR3 resulted in the resumption of pillar cell differentiation. The effects of increased FGFR3 activation were determined by exposing cochlear explants to FGF2, a strong ligand for several FGF receptors. Treatment with FGF2 led to a significant increase in the number of pillar cells and to a small increase in the number of inner hair cells. These effects were not dependent on cellular proliferation, suggesting that additional pillar cells and inner hair cells were a result of increased recruitment into the prosensory domain. These results indicate that FGF signaling plays a critical role in the commitment and differentiation of pillar cells. Moreover, the position of the pillar cells appears to be determined by the activation of FGFR3 in a subset of the progenitor cells that initially express this receptor.

Key words: cochlea; auditory system; hair cell; p75^{ntr}; ear; FGFR3

The sensory epithelium of the mammalian cochlea (the organ of Corti) is composed of at least six distinct cell types that are arranged in a rigorous cellular pattern (see Fig. 1). One of the most intriguing aspects of this pattern is the presence of the tunnel of Corti, an extracellular space that extends along the basal-to-apical axis of the organ of Corti between the single row of inner hair cells and the first row of outer hair cells (for review, see Lim, 1986). The walls of the tunnel of Corti are formed by single rows of inner and outer pillar cells that also extend along the length of the cochlea (see Fig. 1). Pillar cells and the existence of a tunnel within the hair cell sensory epithelium appear to be unique to mammalian cochleae (for review, see Slepecky, 1996; Manley and Koppl, 1998), suggesting that pillar cells represent a derived cell type. The results of previous studies have demonstrated that the development of pillar cells and the formation of a normal tunnel of Corti are required for normal hearing (Colvin et al., 1996; Chen and Segil, 1999); however, the factors that play a role in pillar cell development are, in large part, unknown.

One signaling pathway that has been implicated in pillar cell development is the fibroblast growth factor (FGF) signaling pathway (for review, see Ornitz, 2000; Ornitz and Itoh, 2001). Mice containing a targeted disruption of the fibroblast growth factor receptor 3 (*fgfr3*) gene are profoundly deaf; however, the only obvious defect in the auditory system of these mice is the incomplete development of the pillar cells and the tunnel of Corti (Colvin et al., 1996). In addition, expression of messenger RNA for *fgfr3* in the developing organ of Corti has been localized to a region of the cochlea that corresponds to the developing sensory epithelium (Peters et al., 1993; Pirvola et al., 1995). These results suggest that FGFR3 is required for the development of pillar cells; however, the specific effects of FGFR3 and the FGF signaling pathway have not been determined. The results presented here demonstrate that activation of FGFR3 is required throughout the embryonic period for the ongoing differentiation of the pillar cells. Moreover, increased activation of FGFR3 by treatment with fibroblast growth factor 2 (FGF2) leads to an increase in the number of cells that develop as pillar cells. These results demonstrate roles for the FGF signaling pathway in both the commitment and differentiation of cells as pillar cells.

MATERIALS AND METHODS

Immunohistochemistry. Cochleae were dissected from mouse embryos of specific ages between gestational day 13 (E13) and postnatal day 1 (P1) and were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Then some cochleae were cryoprotected in sucrose, embedded in TissueTek OCT (Ted Pella, Redding, CA), and sectioned in a cryostat at a thickness of 12 μm. Expression of p75^{ntr} (Chemicon, Temecula, CA) or FGFR3 (Santa Cruz Biotechnology, Santa Cruz, CA) at different developmental time points was determined in whole mounts and, for p75^{ntr}, cryosections. Briefly, developing scala vestibuli, scala tympani, and Reissner's membrane were dissected to expose the developing cochlear sensory epithelium. Samples then were permeabilized with either 0.1% Triton X-100 in PBS (p75^{ntr}) or 100% acetone (FGFR3), followed by overnight incubation in the primary antibody at 4°C with rocking. Antibody binding was detected by using either a biotin-linked secondary antibody and the Vector Elite ABC (peroxidase) kit (Vector Laboratories, Burlingame,

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CA) or an Alexa-568-conjugated secondary antibody (Molecular Probes, Eugene, OR).

Cochlear explant cultures. Cochlear explant cultures were prepared as described previously (Kelley et al., 1993; Raz and Kelley, 1999). Briefly, timed pregnant ICR strain mice were anesthetized deeply by the inhalation of CO_2 gas and then killed by creating a double pneumothorax on E13 or E14. Embryos were removed from the uterus and staged according to Kaufman (1992). All procedures involving animals met the guidelines described in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and had been approved previously by National Institutes of Health Institutional Animal Care and Use Committee.

After removal of the embryos, cochleae were dissected and oriented with the lumenal surface of the sensory epithelium facing upward onto MatTek dishes (MatTek, Ashland, MA) that had been coated with a 0.01% layer of poly-L-lysine (Sigma, St. Louis, MO), followed by a layer of Matrigel (1:70 dilution; BD Biosciences, San Jose, CA). Cultures were maintained in media composed of MEM, glucose, HEPES, sodium bicarbonate, N1 supplements, and 10% fetal bovine serum.

Inhibition of FGFR3. Activation of FGFR3 was inhibited by SU5402 (Calbiochem, San Diego, CA), a member of a new class of FGFR antagonists that block the tyrosine kinase activity of the receptor by interacting with the catalytic domain (Mohammadi et al., 1997). A stock solution of SU5402 was dissolved in DMSO and then diluted to specific concentrations between 1 and 50 μM in culture medium. Medium containing either SU5402 (experimental) or an equivalent amount of DMSO (control) was added to explant cultures at specific time points that corresponded with embryonic ages between E14 and E18. Cultures were maintained until hair cells could be identified along the entire length of the developing sensory epithelium, typically 6 d *in vitro* (6 DIV) for cultures established on E13. At the end of each experiment the cultures were fixed in either 4% PFA or 3% glutaraldehyde/2% PFA for 20 min at room temperature.

After fixation the pillar cells were labeled with an antibody against $p75^{ntr}$ (Chemicon), and the hair cells were labeled with either an antibody against myosin VI (a gift from Tama Hasson, University of California San Diego; Hasson et al., 1997) or VIIa (antibodies kindly provided by both Tama Hasson and Christine Petit, Institut Pasteur, Paris, France; Hasson et al., 1997; Sahly et al., 1997) or with *Griffonia simplicifolia* lectin (Vector Laboratories) (Lanford et al., 1999; Warchol, 2001). Primary antibody labeling was detected by appropriate secondary antibodies conjugated to Alexa-488 (Molecular Probes), Alexa-568 (Molecular Probes), or biotin (Vector Laboratories). Binding of secondary antibodies conjugated to biotin was detected via the Vector Elite ABC peroxidase staining kit (Vector Laboratories). *G. simplicifolia* labeling was detected by direct fluorescence or with the Elite ABC alkaline phosphatase staining kit (Vector Laboratories). To visualize cellular borders, we stained filamentous actin with Alexa-488-conjugated phalloidin (Molecular Probes). To examine cellular histology, we imbedded some cultures in Immuno-Bed (Polysciences, Warrington, PA) and sectioned them at a thickness of 3 μm.

Activation of FGFR3. FGF2 (R & D Systems, Minneapolis, MN) was dissolved in culture medium containing 2 μg/ml heparin and 1% DMSO (to improve penetration into the epithelium) and then was added to cochlear cultures at specific time points. Controls received medium containing 2 μg/ml heparin and 1% DMSO. Cultures were maintained until hair cells could be identified along the complete length of the developing organ of Corti, a total of 6 DIV for cultures established on E13. At the end of the experiment the cultures were fixed in 4% PFA. Pillar cells and hair cells were labeled as described above.

Detection of proliferating cells. To identify proliferating cells within cochlear explants, we added the thymidine analog 5-bromodeoxyuridine (BrdU; Sigma) to culture medium at a concentration of 3 μg/ml (Montcouquiol and Corwin, 2001a,b). Uptake of BrdU was determined by labeling with an anti-BrdU antibody (BD Biosciences), followed by a biotinylated secondary antibody and the Vector Elite ABC staining kit (Vector Laboratories). Because the BrdU antibody used for these studies was generated in a mouse, nonspecific labeling was inhibited by using the Mouse-On-Mouse kit (Vector Laboratories) before the addition of the primary antibody.

Determination of numbers of pillar heads, hair cells, and ITO distances. Changes in the number of pillar heads (the combined apical extensions of both the inner and outer pillar cells that give rise to the roof of the organ of Corti), inner and outer hair cells, and distances between inner and outer hair cells [inner to outer (ITO) distances] were determined as follows. First, the total length of the sensory epithelium was determined on the basis of the extent of inner and outer hair cells. Based on this length, positions that were equivalent to distances of 10, 20, 30, and 40% from the extreme basal end of the epithelium were identified. For each position the number of inner hair cells, outer hair cells, pillar heads, and ITO distances was determined. The length of the region to be counted always included a minimum of 10 inner hair cells, as determined by counting the number of myosin VI- or myosin VIIa-positive cells along the inner curve of the sensory epithelium. Outer hair cells within the same region also were counted on the basis of the number of cells that expressed myosin VI or myosin VIIa. The number of pillar heads was determined by one of the two following methods. First, cultures were labeled with anti-$p75^{ntr}$, and individual labeled pillar heads were counted at the apical surface of the sensory epithelium. Alternatively, filamentous actin was labeled with phalloidin. As a result, individual pillar heads were outlined in the region between the inner and outer hair cells. For all experiments a minimum of three independent samples from at least two separate experiments was analyzed.

RESULTS

Expression of FGFR3 in the organ of Corti

Results of immunolocalization studies indicated no expression of FGFR3 in the cochlea before E16 (data not shown). However, by E16 the FGFR3 is expressed in a band of cells that extends along the length of the cochlear duct (Fig. 2*A*). As was reported in Peters et al. (1993), cells that express FGFR3 are located in the region of the cochlear duct that will develop as the pillar cells, outer hair cells, and Deiter's cells (Fig. 2*A*). By P0, expression of FGFR3 in the sensory epithelium is restricted to pillar cells (Fig. 2*B*).

Developing pillar cells express $p75^{ntr}$

The results of previous studies have demonstrated that $p75^{ntr}$ is a specific marker for pillar cells in the organ of Corti of neonatal (P0–P3) mice (von Bartheld et al., 1991; Gestwa et al., 1999; Sano et al., 2001). At later developmental time points the expression of $p75^{ntr}$ apparently is downregulated in pillar cells; however, two published results disagree regarding the timing of this downregulation (Gestwa et al., 1999; Sano et al., 2001). To examine the embryonic expression of $p75^{ntr}$, we dissected cochleae from embryos at E15; the expression of $p75^{ntr}$ was determined by immunolabeling. Results indicated that, at E15, $p75^{ntr}$ is expressed in a relatively broad band of cells (Fig. 3*A*) extending along the length of the basal-to-apical axis of the cochlea (data not shown). In cross section the band of expression of $p75^{ntr}$ correlates with the region of the epithelium that will develop as pillar cells; however, the number of cells expressing $p75^{ntr}$ appears greater than the number of cells that ultimately will develop as pillar cells (Fig. 3*C*). In addition, a second, less intense, region of $p75^{ntr}$ expression is present in a position within the epithelium that correlates with developing Hensen's cells (Fig. 3*C*, *arrowhead*). By P0, as has been reported previously, $p75^{ntr}$ is expressed intensely in a narrow band (Fig. 3*C*) that extends along the length of the cochlea (data not shown). Analysis of cross sections indicates that this intense band of expression of $p75^{ntr}$ correlates with expression in both the inner and outer pillar cells (Fig. 3*D*); however, a second, less intense, band of staining is also apparent in the region of the Hensen's cells (Fig. 3*B*, *arrowheads*, *HeC*; 3*D*, *arrowhead*). Finally, double labeling of cochlear explant cultures with antibodies against myosin VI and $p75^{ntr}$ demonstrates that the band of $p75^{ntr}$ expression is located between the single row of inner hair cells and the first row of outer hair cells (Fig. 3*E*). Based on these results, expression of $p75^{ntr}$ in the region between the row of inner hair cells and the first row of outer hair cells was used as a marker for pillar cell development at P0.

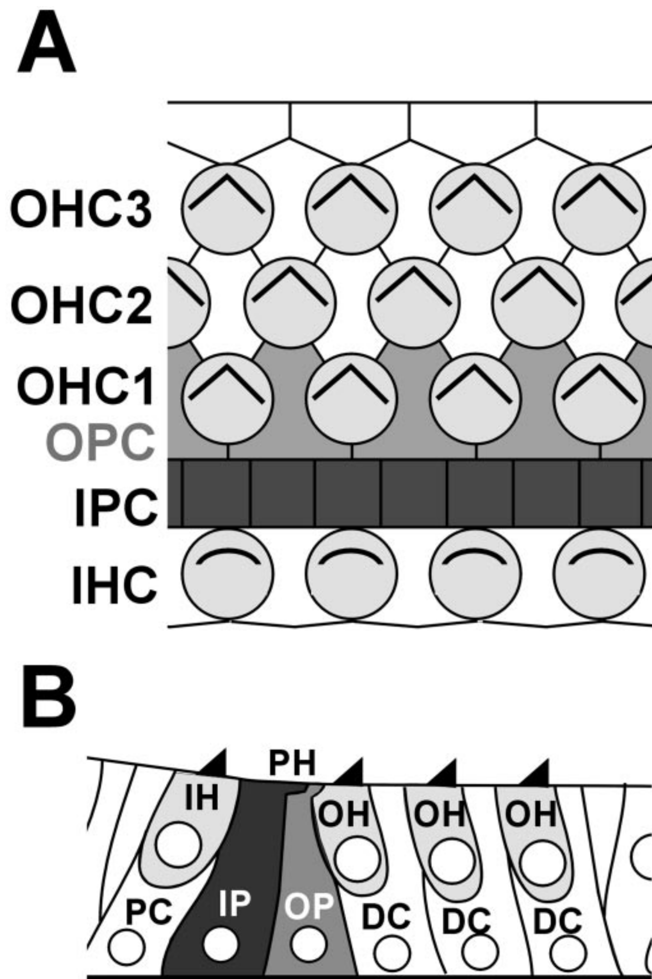

Figure 1. Cellular pattern in the organ of Corti. Schematic drawings of tangential (*A*) and cross-sectional (*B*) views of the organ of Corti in a mouse at P0. *A*, Mechanosensory hair cells are arranged in a single row of inner hair cells (*IHC*) and three or four rows of outer hair cells (*OHC1, OHC2, OHC3*). A single row of inner pillar cells (*IPC*, *dark gray*) and a single row of outer pillar cells (*OPC*, *lighter gray*) are located in the region between the inner hair cells and the first row of outer hair cells (*OHC1*). Outer pillar cells also extend cytoplasmic processes that interdigitate between the first row outer hair cells. *B*, The pillar head is composed of apical projections from both the inner (*IP*) and outer (*OP*) pillar cells. Note that the lumenal surface of the pillar head (*PH*) is derived primarily from the inner pillar cell. However, as illustrated in *A*, the lumenal surfaces of outer pillar cells can be visualized in the interdigitations between first row outer hair cells. As development continues, the tunnel of Corti will form in the region between the inner and outer pillar cells. *DC*, Deiter's cells; *IH*, inner hair cell; *OH*, outer hair cells; *PC*, phalangeal cell.

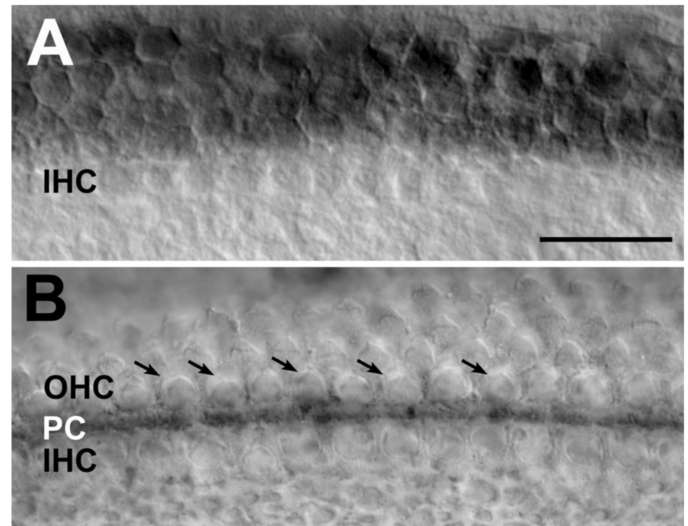

Figure 2. Expression of FGFR3 in the embryonic cochlea. *A*, Lumenal surface of the developing organ of Corti in the middle turn of the cochlea at E16. The band of expression of FGFR3 (*dark region*) is located adjacent to the row of developing inner hair cells (*IHC*) and appears to correspond with the region of the epithelium that will develop as pillar cells, outer hair cells, and Deiter's cells. *B*, Lumenal surface of the developing organ of Corti in the middle turn of the cochlea at P0. FGFR3 expression is restricted to the developing pillar cells (*PC*) located between the single row of inner hair cells and the first row of outer hair cells (*OHC*). Stereociliary bundles are evident in the outer hair cell region (*arrows*) but are not in the plane of focus for inner hair cells. Scale bar: *A* (for *A*, *B*), 50 μm.

Inhibition of FGFR3 disrupts pillar cell development

To determine the effects of FGFR3 during the development of the embryonic organ of Corti, we established cochlear explant cultures on E13 or E14. After 18 hr *in vitro* either 10 μM SU5402 or a vehicle control was added to the culture medium. SU5402 was maintained in the culture medium for the duration of the experiment. SU5402 has been shown to inhibit the tyrosine kinase activity of all four FGFRs by interacting with the catalytic domain (Mohammadi et al., 1997). The results of previous studies have suggested that FGFRs 1, 2, and 4 are not expressed in the cochlear sensory epithelium (Pirvola et al., 1995); however, recent unpublished findings have suggested that FGFR1 may be present in the developing cochlea (Pirvola et al., 2002).

Explants maintained in control medium developed a single row of $p75^{ntr}$-positive heads that appeared similar to the pattern of $p75^{ntr}$ expression at P0 *in vivo* (Fig. 4*A*). Analysis of double-labeled samples indicated that the row of $p75^{ntr}$-positive cells was located between the single row of inner hair cells and the first row of outer hair cells (Fig. 4*C*). Although expression of $p75^{ntr}$ at the apical surface appeared as a single line, analysis that used confocal microscopy indicated that both inner and outer pillar cells expressed $p75^{ntr}$ (data not shown). In contrast with controls, $p75^{ntr}$-positive cells were absent in explants exposed to SU5402 (Fig. 4*B*). A single row of inner hair cells and three to four rows of outer hair cells were still present in these cultures; however, the distance between the row of inner hair cells and the first row of outer hair cells was reduced (Fig. 4*D*).

To determine whether exposure to SU5402 led to the elimination of developing pillar cells, we analyzed cross sections from the basal turn of control and SU5402-treated explants. In sections from controls a pair of inner and outer pillar cells was present in the region between the inner hair cell and first row outer hair cell in most sections (Fig. 4*E*). In addition, projections from the two pillar cells extended to the apical surface to form a developing pillar head process. Two cells were also present in the region between the inner hair cells and first row of outer hair cells in explants that had been exposed to SU5402; however, these cells did not give rise to a developing head process (Fig. 4*F*). To determine whether these cells extended an apical process, we analyzed cell–cell junctions at the surface of the organ of Corti in control explants and explants that had been exposed to SU5402. In control explants, a row of approximately cuboidal pillar heads was present in the region between the row of inner hair cells and

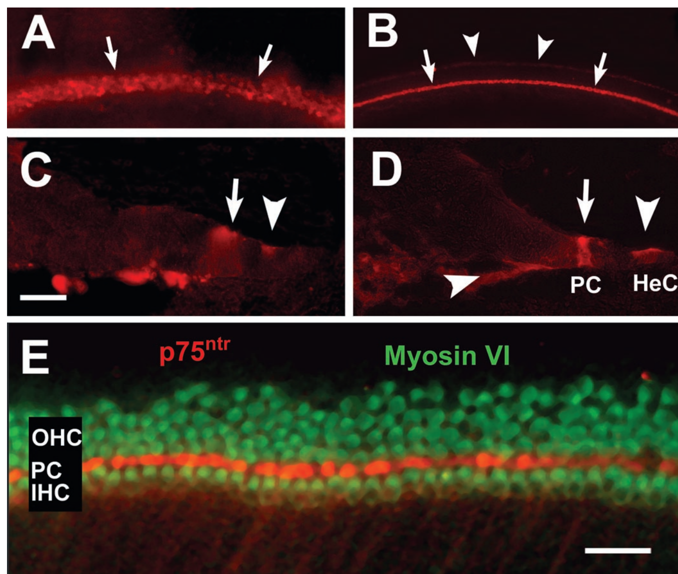

Figure 3. Developing pillar cells express p75^{ntr}. Shown is expression of p75^{ntr} at E15 (*A*, *C*) and P0 (*B*, *D*). *A*, Low magnification image of the lumenal surface in a whole mount of the cochlear at E15. p75^{ntr} is expressed broadly in a band of cells that correlates with the position of developing pillar cells, outer hair cells, and Deiter's cell (*arrows*). *B*, Low magnification image of the lumenal surface of the organ of Corti in the basal turn at P0. At this stage, p75^{ntr} is expressed intensely in the row of pillar heads (*arrows*). A second band of p75^{ntr} expression is present at the lateral edge of the sensory epithelium (*arrowheads*). *C*, Cross section of the developing sensory epithelium from the middle turn of the cochlea at E15. p75^{ntr} is expressed diffusely within a group of cells that correlates with the position of developing pillar cells (*arrow*). p75^{ntr} also is expressed in a more lateral region of the epithelium that appears to correlate with the development of Hensen's cells (*arrowhead*). *D*, Cross section of the organ of Corti from the middle turn of the cochlea at P0. p75^{ntr} is expressed strongly in the inner and outer pillar cells (*arrow*, *PC*) and more diffusely in the Hensen's cells (*arrowhead*, *HeC*). There is also expression of p75^{ntr} in neurites extending from the spiral ganglion to the sensory epithelium (*arrowhead*). *E*, Lumenal surface of the sensory epithelium in a cochlear explant culture established on E14 and fixed after 5 DIV. A single line of P75^{ntr}-positive pillar cells (*red*) is located between the single row of myosin VI-positive inner hair cells (*green*; *IHC*) and the first row of myosin VI-positive outer hair cells (*green*; *OHC*). Spiral ganglion neurites that innervate inner hair cells are labeled with p75^{ntr} also. Scale bars: *A*, *B*, 500 μm; *C–E*, 50 μm.

first row of outer hair cells (Fig. 4*G*). In contrast, in explants exposed to SU5402 a clear row of pillar heads was not evident and, and as discussed, the distance between the inner and outer hair cells was decreased. However, a small number of apical projections were observed in the region between the inner and outer hair cells (Fig. 4*H*), suggesting that some cells within this region did extend limited apical projections. These results are consistent with previous observations from *fgfr3* mutant mice and support the hypothesis that FGFR3 is necessary for pillar cell commitment and/or differentiation. In addition, because the overall phenotype in explants exposed to SU5402 appeared to match the phenotype in *fgfr3* mutants, it seems likely that the effects of treatment with SU5402 in the developing organ of Corti are restricted to inhibition of FGFR3.

Effects of SU5402 on pillar cell development are dependent on dosage

If FGFR3 activation is required for the development of cells as pillar cells, then changes in the level of activity of FGFR3 should lead to proportional changes in the number of cells that develop

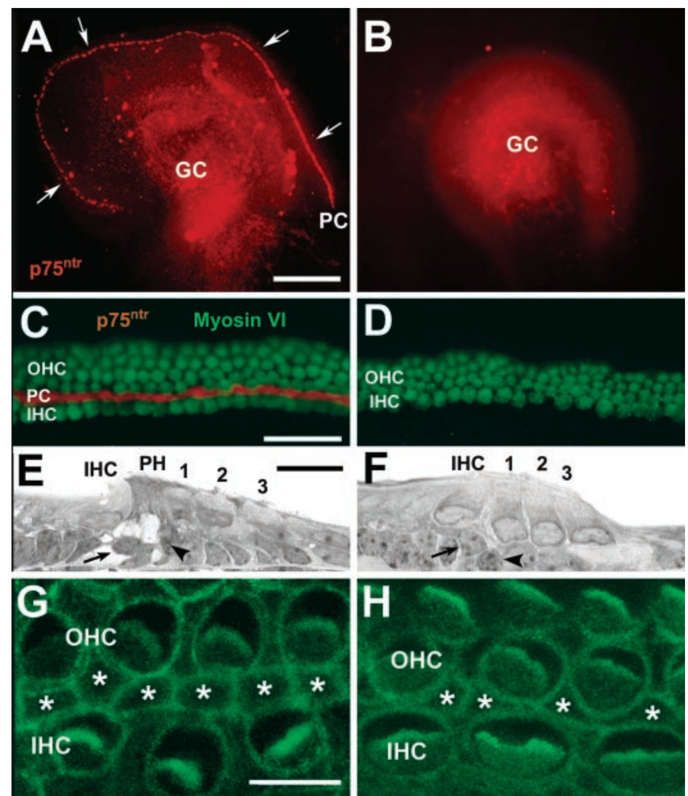

Figure 4. Inhibition of FGFR3 disrupts development of pillar cells. *A*, Low magnification image of an E14.5 control culture after 5 DIV. Expression of p75^{ntr} is present in the pillar cells (*PC*, *arrows*) and spiral ganglion neurons (*GC*). *B*, Low magnification image of an E14.5 cochlear culture treated with 10 μM SU5402 beginning after 18 hr *in vitro*. p75^{ntr} expression is still present in the spiral ganglion neurons (*GC*), but no expression is detected in the region of the pillar cells. *C*, High magnification image of the apical surface of the sensory epithelium from an E13 control explant after 6 DIV. The row of pillar cells (*red*) is located between the single row of inner hair cells (*green*; *IHC*) and the first row of outer hair cells (*green*; *OHC*). *D*, High magnification image of an E13 explant treated with 10 μM SU5402 beginning after 18 hr *in vitro* and maintained for a total of 6 DIV. Cells are labeled as in *C*. Note the absence of pillar cell labeling and the close apposition of the row of inner hair cells and the first row of outer hair cells. *E*, Cross section of the sensory epithelium from an E13 control explant after 6 DIV. A single inner hair cell (*IHC*), three outer hair cells (*1*, *2*, *3*), and single inner and outer pillar cells (*arrow* and *arrowhead*, respectively) are present. Note that a pillar head (*PH*) is present in the space between the inner and first row outer hair cells. *F*, Cross section through the sensory epithelium from an E13 explant treated with 10 μM SU5402 beginning after 18 hr *in vitro* and maintained for a total of 6 DIV. A single inner hair cell (*IHC*) and three outer hair cells (*1*, *2*, *3*) are present. Two cell nuclei (*arrow* and *arrowhead*) are present in the region between the inner hair cell and first outer hair cell; however, no pillar head is present, and neither of these cells appears to contact the lumenal surface. *G*, Apical surface of the sensory epithelium from an E13 control explant after 6 DIV. Cell–cell junctions and stereociliary bundles have been stained with phalloidin. A row of pillar heads (*asterisks*) is present in the region between the row of inner hair cells (*IHC*) and first row of outer hair cells (*OHC*). *H*, Apical surface of the sensory epithelium from an E13 explant treated with 10 μM SU5402 beginning after 18 hr *in vitro* and maintained for a total of 6 DIV. Cell–cell junctions are labeled as in *G*. The separation between the single row of inner hair cells (*IHC*) and first row of outer hair cells (*OHC*) is decreased noticeably compared with the control; however, a limited number of apical projections (*asterisks*) are present in the region between the *IHC* and *OHC*. Scale bars: (in *A*) *A*, *B*, 200 μm; (in *C*) *C*, *D*, 50 μm; (in *E*) *E*, *F*, 10 μm; (in *G*) *G*, *H*, 10 μm.

as pillar cells. To examine this hypothesis, we exposed cochlear explants to specific concentrations of SU5402 between 1 and 50 μM. As discussed, the addition of 10 μM SU5402 led to the loss of expression of p75^{ntr} along the entire length of the sensory epithelium. In explants exposed to 5 μM SU5402, a 63% reduction ($\pm$17%) in the length of the sensory epithelium with expression of p75^{ntr} was observed, whereas exposure to 1 μM SU5402 did not affect the number of cells that developed as pillar cells (Fig. 5*A*). Exposure to SU5402 at concentrations $>$10 μM resulted in general toxicity as determined by the observation of large-scale death of multiple cell types including epithelial, neuronal, and mesenchymal cells. Most cells appeared rounded and were no longer in contact with substrate. In addition, extensive cell debris was observed in many samples.

Exposure to SU5402 does not change the number of cells that develop as hair cells

Although two cells consistently were observed in the space between the inner and first row outer hair cells in explants treated with 10 μM SU5402, it seemed possible that progenitor cells that were inhibited from developing as pillar cells might develop as other cell types within the organ of Corti. To examine this possibility, we determined the number of inner and outer hair cells in both control explants and explants exposed to 10 μM SU5402. Results indicated no significant change in the number of inner or outer hair cells in cultures treated with SU5402 (Fig. 6). This result suggests that the inhibition of FGFR3 does not lead to changes in cell fate.

FGFR3 regulates pillar cell differentiation

The results of the initial experiments in this study confirmed a requirement for FGFR3 during pillar cell development. To determine whether there is a critical period for FGFR3 activation during pillar cell development, we established cochlear explants from embryos at E13 as described. SU5402 then was added after 18 hr (equivalent to E14), 36 hr (equivalent to E15), or 60 hr (equivalent to E16) *in vitro*. All explants were maintained for a total of 6 DIV. Results indicated that expression of p75^{ntr} was disrupted along the length of the sensory epithelium regardless of the timing of the addition of SU5402 (data not shown). These results suggest that the activation of FGFR3 is required for pillar cell development throughout most of the embryonic period.

To learn whether the effects of inhibition of SU5402 changed over developmental time, we determined the distance between the row of inner hair cells and the first row of outer hair cells (ITO distance) for explants from each of the time points listed above. In addition, a final time point, 108 hr (equivalent to E18), was added also. Previous morphological studies have demonstrated that the ITO distance progressively increases as pillar cells develop (Ito et al., 1995; Kaltenbach and Falzarano, 1997); therefore, changes in the ITO distance should be reflective of changes in pillar cell development. In addition, because hair cell differentiation along the cochlear duct progresses in a gradient that begins in the midbasal turn and extends both toward the apex and base, ITO distances for each experimental condition were determined at specific positions along the length of the sensory epithelium. Results for control explants indicated that after 6 DIV (P0 equivalent) the overall average ITO distance from positions located 10, 20, 30, and 40% from the basal end of the sensory epithelium was 5.77 μm (Fig. 5*B*). The addition of SU5402 beginning after 18 hr *in vitro* resulted in a mean ITO distance of 0.81 μm (Fig. 5*B*), a significant decrease from control. The addition of SU5402 after 36, 60, or 108 hr *in vitro* also resulted in significant decreases in the mean ITO distance (Fig. 5*B*). However, the average change in ITO distance decreased progressively, depending on the timing of the addition of SU5402. These results strongly suggest that continuous activation of FGFR3 is required for ongoing pillar cell differentiation.

To determine whether the inhibition of FGFR3 results in a permanent disruption of pillar cell differentiation, we maintained cochlear explants established at E13 in SU5402 for 48 hr beginning after 18 hr *in vitro*. At the end of the 48 hr time period SU5402 was washed out of the culture medium, and the cultures were maintained for an additional 4 DIV (P0 equivalent) before fixation. Results indicated normal expression of p75^{ntr} along the length of the sensory epithelium (data not shown). In addition, analysis of the mean ITO distance indicated a significant increase by comparison with explants that had been exposed to SU5402 continuously beginning after 18 hr *in vitro* (Fig. 5*C*). These results demonstrate that transient inhibition of FGFR3 results in a transient disruption of pillar cell differentiation. Although the mean ITO distance in explants exposed to SU5402 for 48 hr was significantly greater than in continuously exposed cultures, the distances were approximately one-half of those in control explants, suggesting that removal of the FGFR3 antagonist resulted in a resumption of pillar cell differentiation. However, the degree of decrease in ITO distance ($\sim$50% by comparison with control) is consistent with a 48 hr inhibition, suggesting that pillar cell differentiation may proceed at a relatively constant rate.

Exogenous FGF induces an increase in the number of cells that develop as pillar cells

To determine whether increased activation of FGFR3 also would influence pillar cell development, we treated cochlear explants with exogenous FGF2. FGF2 was used because it has been shown to be a strong activator of FGFR3c (Ornitz et al., 1996), the FGFR3 splice variant that is thought to be expressed in the developing cochlea (Pickles, 2001). Analysis of explants established on E13 and exposed to 300 ng/ml FGF2 for 6 DIV beginning after 18 hr *in vitro* indicated a marked increase in the number of pillar heads in the region between the row of inner hair cells and the first row of outer hair cells (Fig. 7*A,B*). A similar increase in the expression of p75^{ntr} also was observed in the pillar cell region (Fig. 7*C,D*). The effects of FGF2 were quantified by determining the average number of pillar heads and hair cells at specific positions along the basal half of the sensory epithelium. Results indicated that exposure to FGF2 induced a significant increase in the number of pillar heads (Fig. 8*A*). A significant increase in the number of inner hair cells was observed also (Fig. 7*B*); however, the magnitude of this increase was considerably less by comparison with the change in the number of pillar heads (Fig. 8*A*). Finally, the number of outer hair cells was unaffected in explants treated with FGF2, and outer hair cells were separated from one another by supporting cells, suggesting that there was no change in the number of Deiter's cells.

To determine whether changes in the number of pillar heads and inner hair cells also were dependent on the level and timing of activation of FGFR3, we varied the concentration and time of the addition of FGF2 (Fig. 8*B,C*). A concentration of 150 ng/ml induced an increase of $\sim$50% in the density of pillar heads, whereas explants exposed to 500 ng/ml developed almost six times as many pillar heads as controls (Fig. 8*B*). In contrast, inner hair cells were not affected in explants exposed to 150 ng/ml FGF2 but were increased significantly in explants treated with a

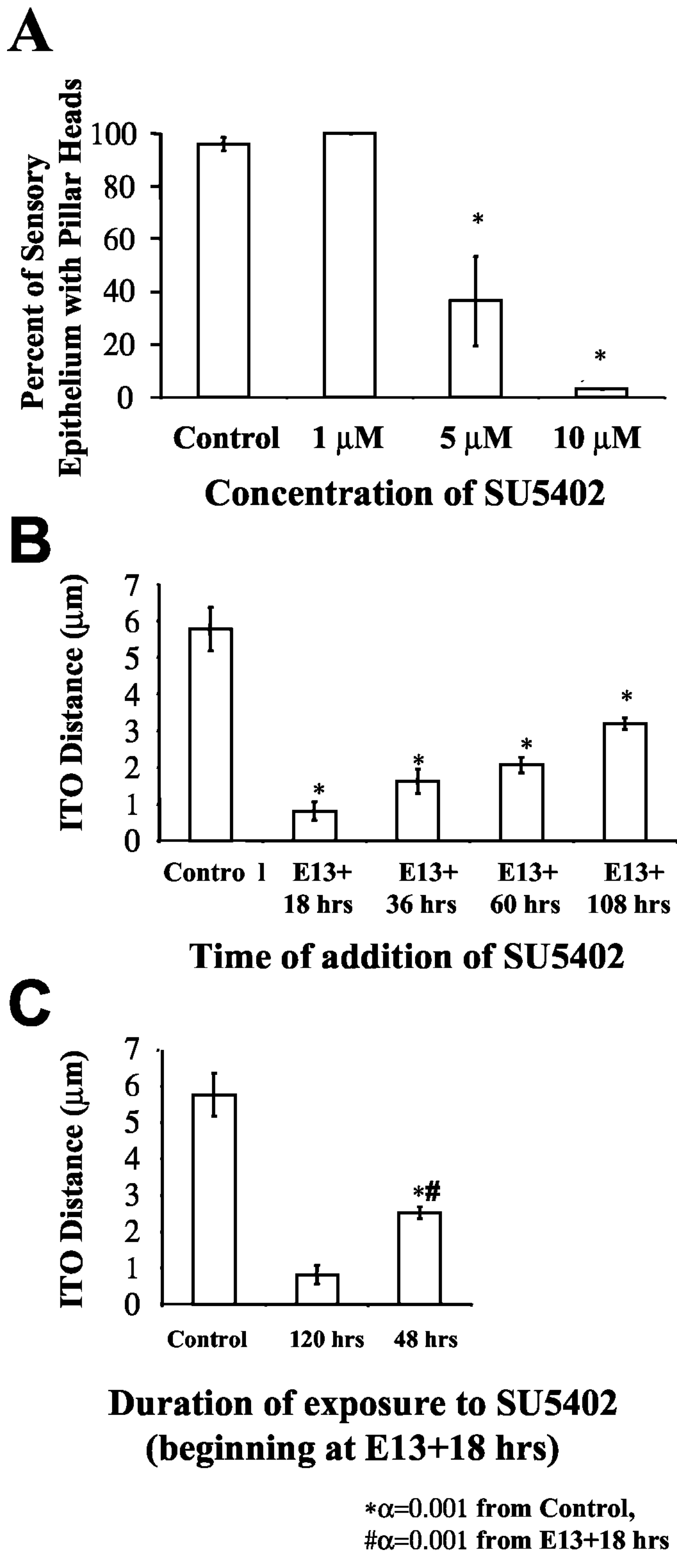

Figure 5. Effects of SU5402 on pillar cell development are dependent on dose, duration, and time of addition. *A*, E13 cochlear explants were exposed to different doses of SU5402 for 6 d beginning after 18 hr *in vitro*. Results indicate a dose-dependent decrease in the percentage of the sensory epithelium in which p75^{ntr}-positive pillar heads were present. *B*, E13 cochlear explants were exposed to 10 μM SU5402 beginning at the time points that are indicated. All explants were maintained for a total of 6 DIV. Exposure to SU5402 induced a significant change in the distance between the row of inner hair cells and the first row of outer hair cells (*ITO Distance*) regardless of the time of addition. However, the effects of SU5402 were clearly dependent on the timing of addition, suggesting an ongoing requirement for activation of FGFR3. *C*, E13 cochlear explants were exposed to 10 μM SU5402 beginning after 18 hr *in vitro*. Removal of SU5402 after 48 hr resulted in a significant increase in the ITO distance compared with explants maintained in SU5402 for the entire culture period (*120 hr*). All explants were maintained for a total of 6 DIV. Values for control and SU5402 exposure for 120 hr are from the same experiment as in *B*. Error bars indicate SEM.

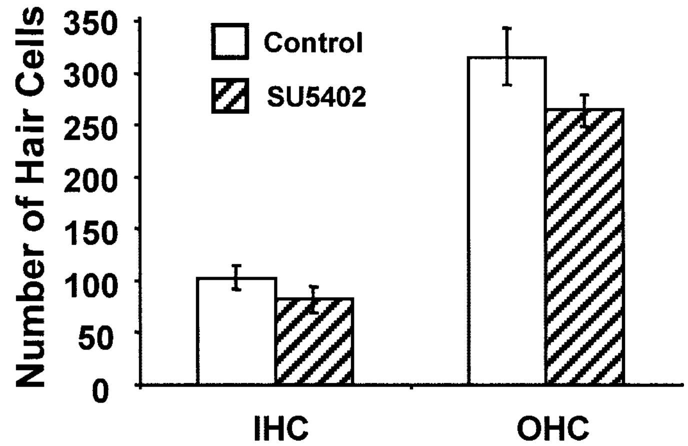

Figure 6. Exposure to SU5402 does not affect the development of hair cells. The total number of hair cells in the basal 50% of the sensory epithelium was determined for control and SU5402-treated cochlear explants established on E13 and maintained for 6 DIV. Error bars indicate SEM.

concentration of 300 ng/ml or greater (Fig. 8*B*). A graded decrease in the density of inner hair cells and pillar heads was observed when the addition of 300 ng/ml FGF2 was delayed by either 48 or 96 hr (Fig. 8*C*). To learn whether the decreased effects of FGF2 at later time points were a result of the maturation of the organ of Corti, we determined the changes in the number of pillar cells and inner hair cells for specific positions along the basal half of the sensory epithelium. Results indicated that the effects of the addition of FGF2 after either 48 or 96 hr *in vitro* were restricted to more apical positions along the sensory epithelium (data not shown). These results are consistent with the hypothesis that there is a critical period for the effects of the FGF signaling pathway and that this period is related to the developmental progression of the organ of Corti.

The observation that treatment with FGF2 induced an increase in both inner hair cells and pillar cells suggested that the effects of FGF2 on pillar cell development could be mediated indirectly via the increased number of inner hair cells. To determine whether an increase in inner hair cells was required to induce an increase in the number of pillar cells, we analyzed the effects of FGF2 on inner hair cells and pillar heads for different positions along the length of the sensory epithelium (Fig. 9). Results indicated that FGF2 induced a significant increase in the number of pillar heads at all four positions along the length of the sensory epithelium (Fig. 9). In contrast, the number of inner hair cells was increased significantly only at the 30 and 40% positions (Fig. 9). Therefore, at the 10 and 20% positions FGF2 induced a significant increase in the number of pillar heads independent of an increase in the number of inner hair cells. These results suggest that the FGF signaling pathway may mediate multiple events

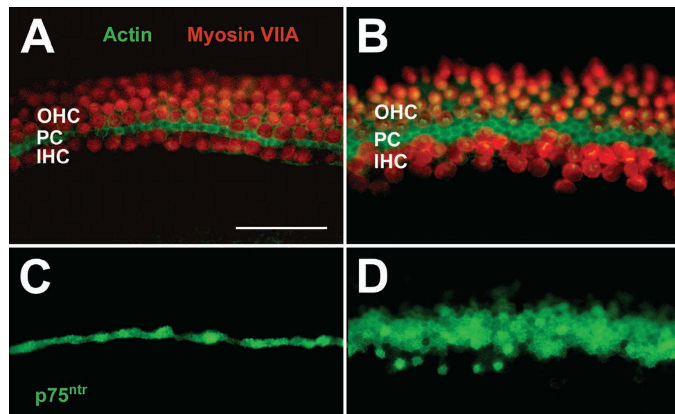

Figure 7. Exogenous treatment with FGF induces an increase in the number of cells that develop as pillar cells. *A*, Lumenal surface of the organ of Corti from an E13 control explant after 6 DIV. Cell boundaries are labeled with phalloidin in *green*, and hair cells are labeled with antimyosin VIIA in *red*. A single row of pillar cell heads (*PC*) is present in the region between the single row of inner hair cells (*IHC*) and the first row of outer hair cells (*OHC*). *B*, Lumenal surface of the organ of Corti from an E13 explant exposed to 300 ng/ml FGF2 beginning after 18 hr *in vitro* and maintained for a total of 6 DIV. Labeling is as in *A*. There is a marked increase in the number of pillar heads in the region between the inner and outer hair cells. An increased number of inner hair cells is also present. *C*, Lumenal surface of the organ of Corti from an E13 control explant after 6 DIV. Pillar cells have been labeled with anti-p75^{ntr}. A single band of pillar heads is present. *D*, Lumenal surface of the organ of Corti from an E13 explant exposed to 300 ng/ml FGF2 beginning after 18 hr *in vitro* and maintained for a total of 6 DIV. Labeling is as in *C*. There is a marked increase in the number of individual pillar heads. Scale bar: *A* (for *A–D*), 50 μm.

during the development of the organ of Corti, including the development of both inner hair cells and pillar cells.

FGF2 does not induce proliferation within the developing organ of Corti

To determine whether the effects of FGF2 on the production of supernumerary inner hair cells and pillar cells were dependent on mitotic proliferation, we treated explant cultures with 300 ng/ml FGF2 and maintained them in culture medium containing 3 μg/ml BrdU. There was extensive incorporation of BrdU in the nuclei of fibroblasts and other cell types in both control and FGF2-treated explants. However, no BrdU labeling was observed in the nuclei of either inner hair cells or pillar cells in either control or FGF2-treated cultures (data not shown).

DISCUSSION

FGFR3 is expressed in the embryonic cochlea

The results of previous studies have demonstrated that *fgfr3* is expressed in the organ of Corti (Peters et al., 1993; Pirvola et al., 1995); however, the time course for the expression of FGFR3 protein during embryonic development had not been determined. Consistent with previous findings, the results presented here demonstrate that FGFR3 initially is expressed broadly in progenitor cells that will develop as pillar cells, outer hair cells, and Deiter's cells. In contrast, by P0 FGFR3 was detected only in inner and outer pillar cells, suggesting that the activation of FGFR3 may be required for maintenance of expression.

FGFR3 activation is required for pillar cell differentiation

Deletion of *fgfr3* was known to lead to defects in pillar cell development, but the specific role of FGF signaling had not been determined (Colvin et al., 1996). Two rows of cells were observed in the region between the row of inner hair cells and the first row of outer hair cells in *fgfr3* mutants (Colvin et al., 1996); however, whether these cells represented uncommitted progenitor cells or pillar cells that had become arrested in development was not clear. The results presented here support the hypothesis that the activation of FGFR3 is required for pillar cell differentiation. As in *fgfr3* mutant mice, pillar cell development was disrupted in cochlear explants exposed to the FGFR inhibitor SU5402; however, the cells that would have developed as pillar cells did not undergo a change in fate and instead remained in the epithelium in an apparently undifferentiated state. After the removal of SU5402 these cells were able to resume their development as pillar cells, but ongoing activation of FGFR3 was required for their continued differentiation. FGFR3 also has been shown to control the rate of differentiation in developing lens fibers (Govindarajan and Overbeek, 2001) and osteoblasts (Chen et al., 1999; Funato et al., 2001), suggesting that this may be a conserved function of this receptor.

It is important to consider that FGFR3 also may play a role in the commitment of progenitor cells to the pillar cell fate. Although inhibition of FGFR3 did not lead to a change in the fates of the cells that would have developed as pillar cells, these cells may have remained in an uncommitted state until FGFR3 activity was restored. As will be discussed, the effects of treatment with FGF2 are consistent with a role for FGF signaling in cell fate.

The FGF signaling pathway regulates the number of cells that will develop as pillar cells

Treatment with FGF2 led to a dose-dependent increase in the number of cells that developed as pillar cells, suggesting that FGF signaling plays a key role in the regulation of pillar cell number. Moreover, the overproduction of pillar cells in response to exogenous FGF2 demonstrates that the number of cells with the potential to develop as pillar cells is significantly greater than the number of cells that normally assume this fate. These results suggest that the abundance of ligands for FGFR3 within the developing organ of Corti may be a limiting factor for the determination of cells as pillar cells.

The source of supernumerary pillar cells is not clear. Because BrdU labeling did not indicate an increase in cellular proliferation, the most likely source of additional pillar cells appears to be the population of FGFR3-positive cells that is present in the cochlear duct at E16. As discussed, during normal development of the organ of Corti the number of cells that express FGFR3 at E16 is considerably larger than the number of cells either that maintain expression of FGFR3 or that develop as pillar cells. Increased activation of FGFR3 in these cells by the addition of FGF2 is apparently sufficient to increase the number of these cells that will become committed to develop as pillar cells.

The population of progenitor cells that expresses FGFR3 at E16 appears to include cells that will develop as both pillar cells and hair cells (Peters et al., 1993; this study) (Fig. 2). Based on this observation, a second expected outcome of exposure to FGF2 might be a decrease in the number of cells that develop as outer hair cells. Surprisingly, the number of outer hair cells in explants exposed to FGF2 did not differ from control. This result suggests that the increased number of pillar cells, treatment with FGF2, or a combination of these factors resulted in the recruitment of additional cells into the prosensory domain. In fact, there have been several reports suggesting that the position of the boundary of the prosensory domain at its outer hair cell edge may be

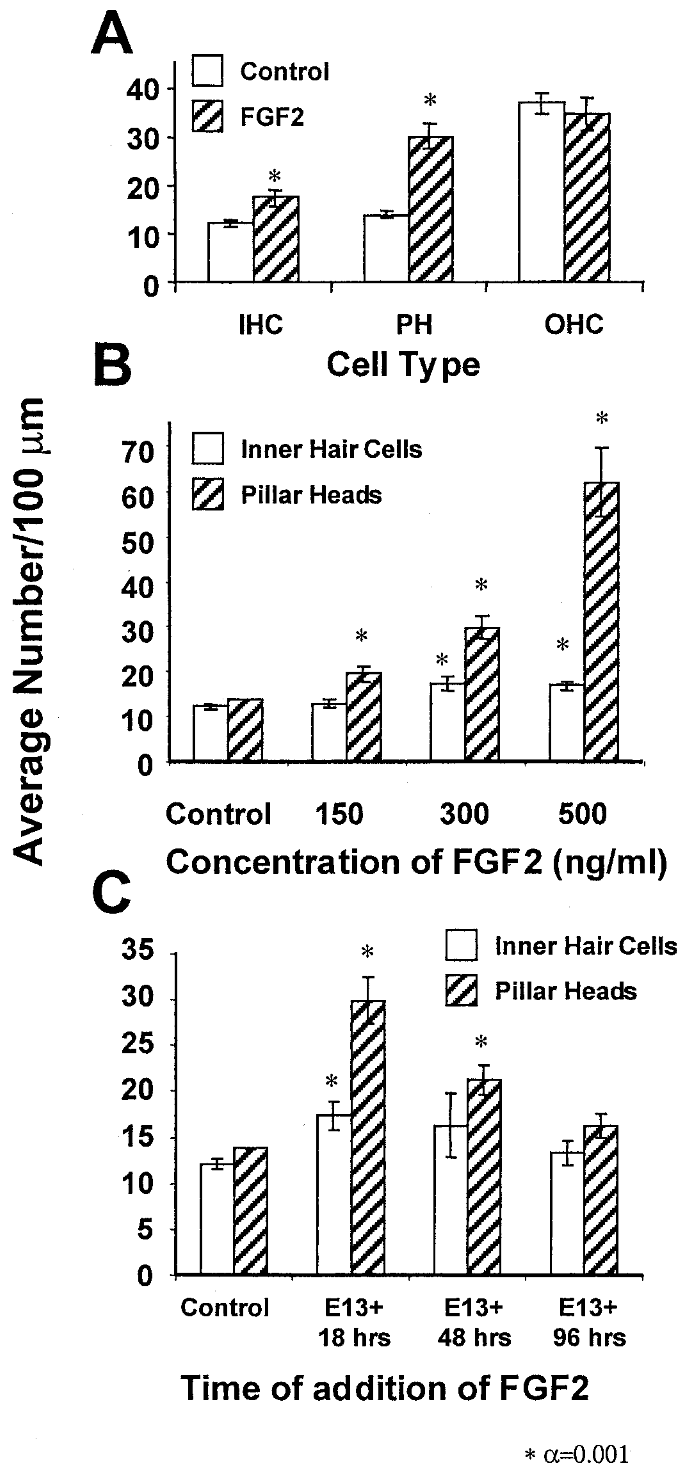

Figure 8. Treatment with FGF2 induces an increase in the number of pillar cells and inner hair cells. *A*, The average number of pillar heads and inner and outer hair cells was determined at specific locations along the basal half of the sensory epithelium in E13 explants maintained in either control medium or medium containing 300 ng/ml FGF2 for 6 DIV. Exposure to FGF2 led to an approximate doubling in the number of pillar heads. A significant increase in the number of inner hair cells also was observed; however, the magnitude of this increase was considerably lower by comparison with the change in the number of pillar heads. Outer hair cell number was unchanged. *IHC*, Inner hair cells; *OHC*, outer hair cells; *PH*, pillar heads. *B*, Average number of inner hair cells and pillar heads in E13 explants exposed to different concentrations of FGF2 for 6 DIV. Increased concentrations of FGF2 led to a graded increase in the average number of inner hair cells and pillar heads. *C*, Average number of inner hair cells and pillar heads in E13 explants exposed to 300 ng/ml FGF2 beginning on E14 (*E13 + 18 hr*), E15 (*E13 + 48 hr*), or E17 (*E13 + 96 hr*) and maintained for a total of 6 DIV. The effects of FGF2 on both the number of inner hair cells and the number of pillar heads were dependent on the time of administration. Significant increases in inner hair cells were observed only when FGF2 was added on E14. Pillar heads were increased significantly when FGF2 was added on E14 or E15. By E17, treatment with FGF2 did not induce a significant change in either the number of inner hair cells or the number of pillar heads. Error bars indicate SEM.

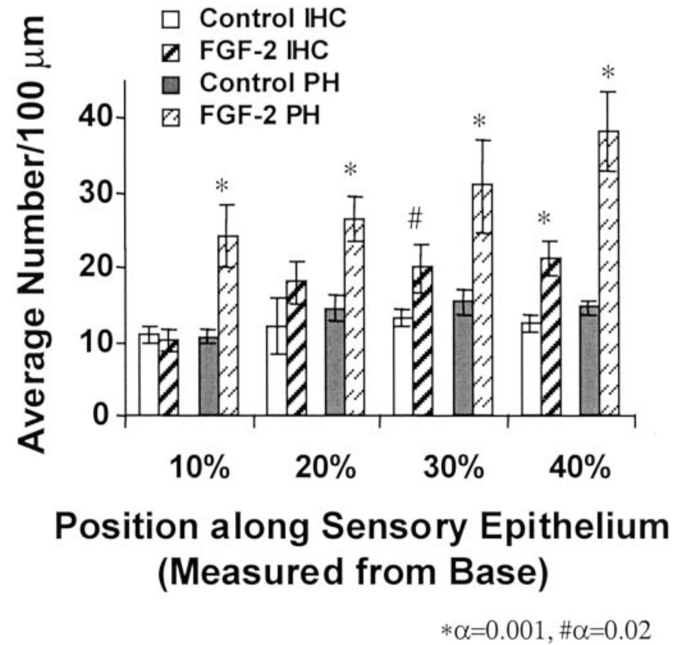

Figure 9. Effects of FGF2 on pillar cell development are independent of changes in inner hair cells. Effects of exposure to 300 ng/ml FGF2 on E13 explants after 6 DIV were determined at different positions along the length of the cochlea. Significant increases in the number of pillar heads were observed at all four locations. In contrast, inner hair cells were increased significantly only at the 30 and 40% positions. These results indicate that the increase in pillar heads is not dependent on an increase in inner hair cells. *IHC*, Inner hair cells; *PH*, pillar cells. Error bars indicate SEM.

variable. Supernumerary outer hair cells have been observed both *in vivo* and *in vitro* (Lavigne-Rebillard and Pujol, 1986, 1987; Abdouh et al., 1993, 1994), and treatment with retinoic acid induces a significant increase in the number of cells that develop as outer hair cells presumably via increased recruitment (Kelley et al., 1993).

An alternative hypothesis for the source of supernumerary pillar cells could be related to the expression of $p75^{ntr}$ in this cell type. The results of recent experiments have demonstrated that $p75^{ntr}$ can act as a mediator of apoptotic cell death (Chao and Bothwell, 2002); therefore, it seems possible that cell death could play a role in the number of cells that develop as pillar cells. However preliminary results indicate that neither activation of $p75^{ntr}$ by NGF or NT-3 nor loss of $p75^{ntr}$ activity by genetic manipulation results in a change in pillar cell number (B. E. Jacques and M. W. Kelley, unpublished results).

FGF2 also induces an increase in inner hair cells

In addition to an increase in the number of pillar cells, a more limited increase in the number of inner hair cells also was ob-

served in explants exposed to FGF2. The basis for this effect is not clear. FGFR3 is not expressed in the region of the cochlear duct that will develop as inner hair cells, suggesting that the effects of FGF2 on inner hair cell number probably are regulated via a different FGF receptor. One possibility would be FGFR1, which has been reported to be expressed in the embryonic cochlear duct (U. Pirvola, personal communication) and would be activated by treatment with FGF2 (Ornitz et al., 1996). However, treatment with SU5402, which antagonizes all FGFRs (Mohammadi et al., 1997), did not lead to a change in inner hair cell number, suggesting that the FGF signaling pathway is not required for inner hair cell development. This result raises the possibility that the effects of FGF2 on inner hair cell development could be indirect. A potential source of this interaction could be the increased number of pillar cells. If reciprocal signaling interactions between inner hair cells and pillar cells play a role in regulating the ratio of inner hair cells to pillar cells, then an increase in the number of pillar cells potentially could lead to a subsequent increase in the number of inner hair cells. In fact, decreases in the number of pillar cells have been reported in response to the loss of inner hair cells in the *Bronx waltzer* mutant mouse (Tucker et al., 1999). Although this does not demonstrate a link in the ratio of the two cell types, it does support the potential for cell–cell interactions.

It is important to consider that the addition of a relatively high concentration of FGF2, compared with the physiological concentration, was required to elicit an effect on pillar cell development. The basis for this disparity is not clear. However, previous results have suggested that the presence of the developing tectorial membrane (Rau et al., 1999) along with robust expression of different cadherins (for review, see Whitlon, 1993) may inhibit the penetration of some molecules into the developing sensory epithelium (Zheng and Gao, 2000). In addition, a number of endogenous inhibitors of FGFR activation have been identified recently (Tefft et al., 1999; Wakioka et al., 2001; Furthauer et al., 2002; Tsang et al., 2002), and the presence or some of these molecules within the developing cochlea also could play a role in modulating the effective concentration of FGF2 within individual explants.

FGF signaling and development of pillar cells

It is not clear which FGFs might act as endogenous ligands for FGFR3 in the developing cochlea. Previous reports (U. Pirvola, personal communication) and preliminary data from our laboratory indicate that FGF8 is expressed specifically in developing inner hair cells, suggesting that it could act as an endogenous ligand for FGFR3. As discussed, on E16, FGFR3 is expressed in a band of cells that appears to correspond with the region of developing pillar cells and outer hair cells. Therefore, inner hair cells expressing FGF8 would be located directly adjacent to the domain of FGFR3 expression. Presumably, the effective concentration of FGF8 would be limited to one or two cell diameters from each inner hair cell, leading to activation of FGFR3 and placement of the pillar cells adjacent to inner hair cells. It is not clear why the number of cells that initially express FGFR3 is considerably larger than the number of cells that ultimately develop as pillar cells; however, the group of FGFR3-positive cells could serve as a pool of potential pillar cells. This pool could represent a fail-safe mechanism to ensure pillar cell development.

The spatial expression of FGF8 suggests that it is a candidate for the endogenous ligand for FGFR3; however, existing data also suggest that other FGFs are present in the epithelium as well. In particular, FGF1 and FGF3 have been reported to be present in the developing cochlea (Luo et al., 1993; Pickles, 2001) although the specific cellular pattern of expression has not been determined. In addition, preliminary results from our laboratory indicate that FGF17 is also present in the sensory epithelium and that treatment of cochlear explants with a mixture of FGF8/17/18 leads to an increase in the number of pillar cells (our unpublished results). In contrast, FGF2 is not expressed in the embryonic cochlea (Luo et al., 1993), suggesting that it is unlikely to be an endogenous ligand for FGFR3.

In summary, the results presented here indicate that the number of cells that develop as pillar cells is regulated via the FGF signaling pathway and specifically via the activation of FGFR3. It seems likely that developing inner hair cells express one or more FGFs, leading to local activation of FGFR3 in adjacent cells. Because the number of cells with the potential to develop as pillar cells is considerably greater than the normal complement, we hypothesize that the activation of FGFR3 is limited to the cells located nearest to the developing inner hair cells.

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