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RESEARCH ARTICLE

Cellular Growth and Rearrangement During the Development of the Mammalian Organ of Corti

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The sensory epithelium of the mammalian cochlea, the organ of Corti, is comprised of ordered rows of cells, including inner and outer hair cells. Recent results suggest that physical changes in the overall size and shape of the cochlear duct, including possible convergence and extension, could play a role in the development of this pattern. To examine this hypothesis, changes in cell size and distribution were determined for different regions of the cochlea duct during embryonic development. In addition, changes in the spatial distribution of sensory precursor cells were determined at different developmental time points based on expression of $p27^{kip1}$. Unique changes in luminal surface area, cell density, and number of cell contacts were observed for each region of the duct. Moreover, the spatial distribution of $p27^{kip1}$-positive cells changed from short and broad early in development, to long and narrow. These results are consistent with the hypothesis that convergence and extension plays a role in cellular patterning within the organ of Corti. *Developmental Dynamics 229:802–812, 2004.* Published 2004 Wiley-Liss, Inc.†

Key words: hair cell; convergent extension; cochlea; $p27^{kip1}$

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INTRODUCTION

In mammals, sounds are perceived through mechanosensory hair cells located within the sensory epithelium of the cochlea (referred to as the organ of Corti). Within the organ of Corti, hair cells and several types of specialized supporting cells are arranged in a regular mosaic pattern that extends along the basal-to-apical axis of the cochlear duct (reviewed in Kelley and Bianchi, 2001). One of the most striking aspects of this mosaic is that specific cell types are arranged in discreet rows. The edge of the organ of Corti located closest to the modiolus (modiolar edge) is composed of a single row of alternating inner hair cells and inner phalangeal cells (Fig. 1). Directly adjacent to the lateral edge of the inner hair cell row is a single row of inner pillar cells and a single row of outer pillar cells. Finally, the edge of the organ of Corti located closest to the stria vascularis (strial edge) is composed of three or four rows of outer hair cells and Deiter's cells that are also arranged into a regular alternating mosaic. This cellular pattern represents one of the most highly ordered structures in any vertebrate system; however, the factors that are required for the formation of this pattern are poorly understood.

The cochlear duct develops as an outpocketing that extends from the ventromedial region of the otocyst beginning around embryonic day 11 (E11) in mice (reviewed in Bryant et al., 2002). As development proceeds, the duct extends and coils such that, by E14, it is approximately 1 turn, and by postnatal day 0 (P0), it encompasses 1 and 3/4 turns (Sher, 1971). Birthdating studies have demonstrated that virtually all of the cells that will contribute to the organ of Corti are already present and postmitotic at E14 (Ruben, 1967). In contrast, cells located within the cochlear duct that will contribute to the inner or outer sulci continue to actively proliferate throughout the embryonic period (Ruben, 1967). Because the cochlear duct continues to extend in length along its basal-to-apical axis well beyond the period of terminal mitoses for cells within the developing sensory epithelium, these observations suggest that cellular rearrangements could play a role in the development of cellular patterning within the organ of Corti. Moreover, it has been suggested recently that some aspects

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of the development of the organ of Corti are consistent with convergent extension (Chen et al., 2002; Montcouquiol et al., 2003). Convergent extension refers to a morphogenetic process in which the general shape of a structure elongates along a central axis as it narrows along a perpendicular axis (reviewed in Keller, 2002). As these changes occur, cells within the structure converge toward the midline of the central axis and subsequently extend along the perpendicular axis. Convergent extension has been shown to occur during the formation of several different structures, including the vertebrate neural tube and notochord, the ascidian notochord, and the *Drosophila* germ band. To begin to examine the potential roles of cellular rearrangements and convergent extension in cellular patterning of the organ of Corti, the growth and spatial arrangements of cells in each region of the cochlear duct were examined at different time points during embryogenesis.

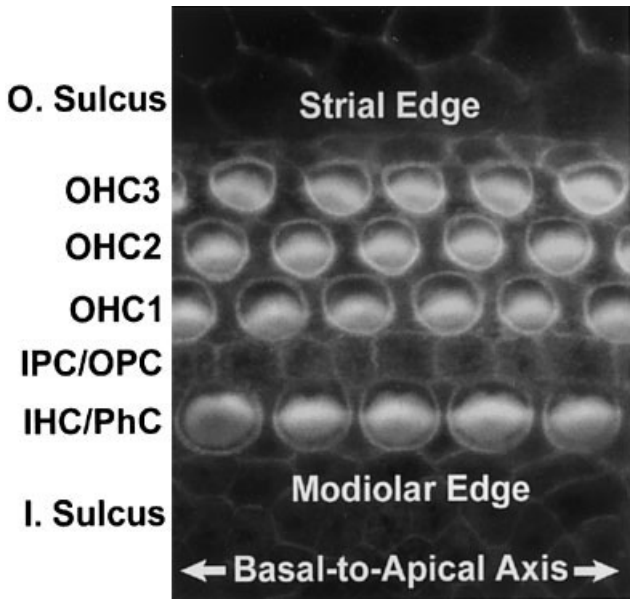

Fig. 1. The cellular mosaic in the organ of Corti. Luminal surface of the organ of Corti at postnatal day 0 illustrating the ordered cellular mosaic. I. Sulcus, inner sulcus; IHC/PhC, inner hair cells/phalangeal cells; IPC/OPC, inner pillar cells/outer pillar cells; OHC1– 3, outer hair cell rows; O. Sulcus, outer sulcus.

RESULTS

Changes in Cell Density and Cell Size

At E13, images of the luminal surface of the cochlear epithelium indicated that the entire epithelium is composed of an apparently homogenous population of epithelial cells (Fig. 2A,B). No significant differences in cell density, luminal surface area, or cellular organization were observed along either the basal-to-apical or modiolar-to-strial axes of the cochlea. Similarly, analysis of cross-sections of the cochlear duct were also consistent with a homogeneous population of cells at this time point (Fig. 2C).

By E14.5, heterogeneities in density and apical surface area could be identified across the modiolar-to-strial axis of the cochlea in the basal region of the duct (Fig. 2D). In particular, the density of cells appeared to be increased in the developing inner sulcus relative to the developing sensory epithelium and outer sulcus. In addition, developing inner hair cells could be identified based on the accumulation of actin at their lateral borders and by their arrangement into a somewhat disorganized single row. Putative hair cells could be identified occasionally in the middle or apical regions of the duct and slight differences in cell density and luminal surface area were also present (Fig. 2E). Analysis of cross-sections indicated similar changes in the development of the cochlea. In the basal region of the duct, developing hair cells could be identified in the region adjacent to the spiral vessel based on the presence of slightly enlarged nuclei and a more luminal position within the epithelium (Fig. 2F). In the same sections, the inner sulcus contained large numbers of mitotic figures while the outer sulcus was comprised of a relatively smaller number of larger cells (Fig. 2F). In contrast, no obvious differences in cellular morphology were evident in the more apical regions (data not shown).

At E16, developing inner hair cells in the basal region of the duct were arranged into a well-ordered row (Fig. 2G). In addition, developing outer hair cells could be identified in a band located adjacent to the single row of inner hair cells; however, these cells were not arranged in regular rows (Fig. 2G). Distinct changes in cellular morphology were now evident in all three regions along the modiolar-to-strial axis. In the inner sulcus, cells were present at high density and had relatively small luminal surface areas, while in the outer sulcus, cell density was low and the cells were large in the size. Cell density in the developing sensory epithelium was intermediate between the inner and outer sulci. The apical region of the duct appeared similar to the condition in the basal region on E14.5. A single irregular row of inner hair cells was present at the boundary between the inner sulcus and sensory epithelium, but there was no indication of outer hair cells (Fig. 2H). The different cell morphologies along the modiolar-to-strial axis of the epithelium were also evident in cross-sections through the basal region of the cochlea (Fig. 2I). The inner sulcus contained a thickened epithelium containing five to six layers of cells, some of which were still mitotically active. In contrast, in the sensory epithelium and outer sulcus, the epithelium was considerably thinner and developing inner and outer hair cells could clearly be identified.

By E17, cells located in the sensory epithelium in the basal regions of the cochlea were clearly arranged in the cellular pattern that is characteristic for the organ of Corti. A single row of inner hair cells, two rows of pillar cells, and three rows of outer

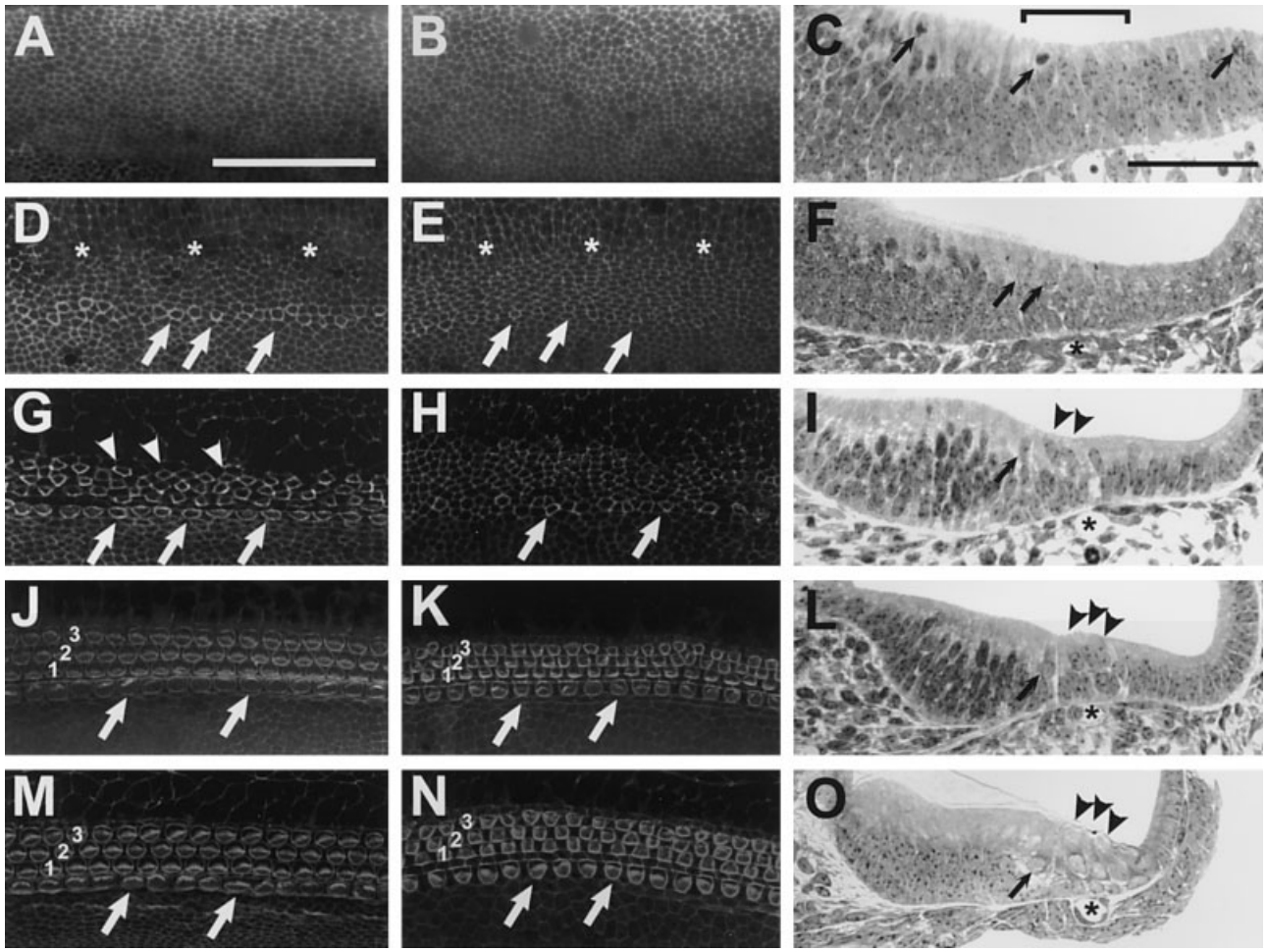

Fig. 2. Cellular patterning in the developing cochlear duct. For all luminal images, basal is to the left and the strial edge is located up. All cross-sections are through the basal region of the duct and are oriented with the modiolar edge to the left. In addition, all cross-sections have been aligned based on the presence of the spiral vessel (asterisks in F,I,L, and O). **A:** Basal region (25% position) of the cochlear duct at embryonic day 13 (E13). Cells located in all regions of the duct are hexagonally packed. No differences in cell density or packing are evident between regions of the duct that will develop as the inner sulcus, sensory epithelium, or outer sulcus. **B:** Apical region (75% position) of the cochlear duct at E13. The pattern appears very similar to the base. Cells are hexagonally packed with no obvious differences in cell density or arrangement. **C:** Cross-section of the duct at E13 illustrating the lack of differentiated cell types. Mitotic figures are present in the inner and outer sulci (arrows). Similar mitotic figures were observed in the region that will develop as the sensory epithelium (bracket; data not shown). **D:** Basal region of the cochlear duct at E14.5. A small number of cells with increased levels of filamentous actin at their borders (arrows) are located in a region of the duct that corresponds with the modiolar edge of the sensory epithelium. In addition, a distinct change in cell size and density is evident in a region that corresponds with the transition between the sensory epithelium and the outer sulcus (asterisks). **E:** Apical region (75% position) of the duct at E14.5. This particular image illustrates the leading edge of the row of developing inner hair cells (arrows). The strial boundary is marked as in D. **F:** In a cross-section from an E14.5 cochlea, putative developing hair cells can be identified within the region of the developing sensory epithelium (arrows). These cells are characterized by a slightly larger nucleus, as compared with surrounding cells, and by a more luminal position within the epithelium. **G:** Basal region of the duct at E16. Developing inner hair cells are now arranged in an ordered row (arrows) and developing outer hair cells can now be identified based on accumulation of filamentous actin at the lateral cell membranes (arrowheads). Also note the continued increase in luminal surface area in the developing outer sulcus. **H:** Apical region of the duct at E16. Developing inner hair cells (arrows) appear similar to the basal region of the duct at E14.5. **I:** Cross-section through the basal region of the duct at E16 illustrating the presence of developing inner (arrow) and outer hair cells (arrowheads) and a thinning in the overall thickness of the epithelium in the regions of the developing sensory epithelium and outer sulcus. **J:** Basal region of the duct at E17. A relatively mature cellular pattern including inner hair cells (arrows) and three rows of outer hair cells (numbered) are present. **K:** Apical region of the duct at E17. The overall cellular pattern is comparable to the basal region; however, the luminal surface area of the hair cells is markedly smaller. **L:** The characteristic pattern of inner (arrow) and outer hair cells (arrowheads) is present in the base of the duct by E17. **M:** Basal region of the duct at P0. Cellular pattern and luminal surface area appear largely unchanged from E17. **N:** Apical region of the duct at P0. Overall cellular pattern appears similar to E17; however, luminal surface areas for hair cells have increased. **O:** By P0, the epithelium has thinned to a single layer of hair cells and a single layer of supporting cells. Scale bars = 50 μm in A (applies to A,B,D,E,G,H,J,K,M,N), 50 μm in C (applies to C,F,I,L,O).

hair cells were evident both at the luminal surface and in cross-section (Fig. 2J,L). Cellular density remained high in the inner sulcus, while luminal surface area continued to increase in the outer sulcus. In the apex, cellular arrangement, cell density, and luminal surface area appeared similar to the basal region at E16 (Fig.

2K). Finally, by P0, a characteristic pattern of inner and outer hair cells was observed along the entire basal-to-apical axis of the cochlea (Fig. 2M–O).

To better understand the changes that occur in different regions of the cochlear duct during development, changes in cell density and luminal surface area were quantified for cells located in the developing inner sulcus, sensory epithelium, or outer sulcus at specific time points during development. As would be expected, results indicated a progressive increase in luminal surface area in all three regions of the cochlea throughout development (Fig. 3). Moreover, consistent with the observations reported above, the magnitude of the increase in luminal surface was markedly greater in the developing outer sulcus (Fig. 3C). Analysis of changes in luminal surface area at specific positions indicated a marked increase in the rate of change of luminal surface area in both the inner and outer sulci in the more basal regions of the cochlea between E16 and E17 (Fig. 3A,C).

This observation suggested that there might be a corresponding expansion of the basal portion of the cochlear duct during this time period. To examine this possibility, the length and overall shape of the cochlear duct was determined at specific time points between E14 and P0. Results indicated that the overall length of the cochlear duct increases by 25% between E16 and E17 compared with only 5% between E14.5 and E16 and only 7% between E17 and P0 (Fig. 4). Moreover, examination of changes in the shape of the cochlear duct during the same time period indicated that the majority of the growth between E16 and E17 occurs in the basal region of the duct (Fig. 4).

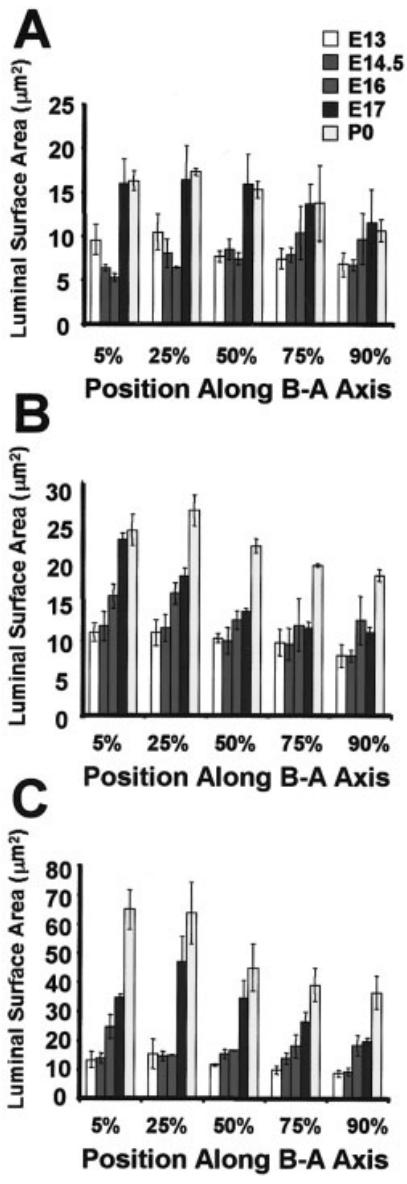

Fig. 3. Changes in luminal surface area during development. The average luminal surface area was determined for cells within a 50 μm × 25 μm rectangle in the inner sulcus (A), sensory epithelium (B), or outer sulcus (C) at the indicated positions along the basal-to-apical axis (B-A Axis) of the duct. **A:** Luminal surface area in the inner sulcus is stable or decreases slightly between embryonic day 13 (E13) and E16 in the basal half of the duct. Between E16 and E17, there is a significant increase in luminal surface area in the basal region. Luminal surface area also increases in the apical half of the duct; however, the increase is more gradual and begins between E14.5 and E16. **B:** In the developing sensory epithelium, average luminal surface area begins to increase between E14.5 and E16 and continues progressively through P0. Regional variations are minimal. **C:** In the outer sulcus, luminal surface area is largely stable along the length of the duct between E13 and E14.5, but surface areas undergo a striking increase beginning on E16. Between E14.5 and postnatal day 0 (P0), outer sulcus cells in the basal region of the duct increase in size by nearly 400%. Values are mean ± standard error of the mean. (Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.)

Changes in Number of Contacts per Cell

One of the most striking characteristics of the cellular mosaic of the organ of Corti is that each cell contacts only four other cells. To examine how this spatial pattern develops, the average number of contacts per cell was determined for the developing inner sulcus, sensory epithelium, and outer sulcus at the 25% and 75% positions along the basal-to-apical axis of the cochlear duct at different developmental time points. Results indicated a progressive decrease in the average number of cell contacts per cell within the developing sensory epithelium such that, by P0, the average number of contacts per cell had dropped from 6 to 4 (Fig. 5). This change began to occur by E14.5 at the 25% position along the cochlea and by E16 at the 75% position. In contrast, no changes in the average number of cell contacts were observed for cells located in either the inner or outer sulci.

Changes in the Spatial Arrangement of Cells Expressing $p27^{kip1}$

The results discussed above indicated that, as the length of the cochlear duct increases, the number of cell contacts within the developing sensory epithelium decreases. Because this population of cells is largely postmitotic by E14.5 (Ruben, 1967) and there is also apparently limited cell death within this population (Nishizaki et al., 1998; Knipper et al., 1999; Nishikori et al., 1999), these results are consistent with a progressive extension of the population of sensory precursor cells over time. Moreover, the results from two recent studies have similarly suggested that extension-like movements may occur during the development of the organ of Corti (Chen et al., 2002; Montcouquiol et al., 2003). Therefore, to examine this hypothesis directly, the population of sensory precursor cells within the developing cochlear duct was visualized by detection of expression of the cell cycle-inhibitor $p27^{kip1}$. The results of previous studies have dem-

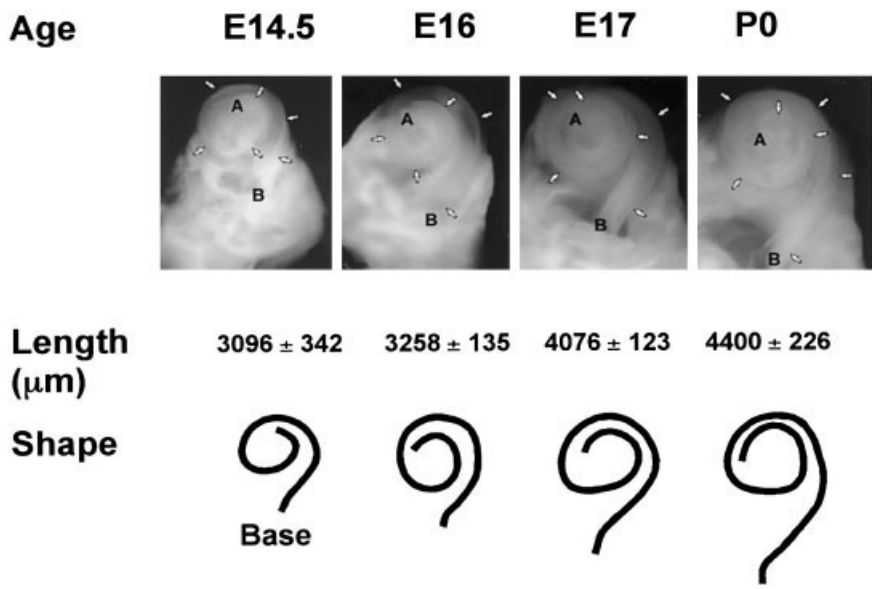

Fig. 4. Overall changes in length and shape of the cochlear duct. Images of the developing cochlear duct at the time points indicated demonstrate the extension and coiling of the epithelium (white arrows). The average length of the cochlear duct and its overall shape change with time as it develops. Representative outlines of cochlear spirals are all oriented similarly, with the basal end of the duct indicated for the sample from embryonic day 14.5 (E14.5; Base). Note the increased rate of growth between E16 and E17 (25% change) versus the change between E14.5 and E16 (5% change) and between E17 and postnatal day 0 (P0; 8% change). Based on the change in the shape of the duct, most of the growth of the cochlea between E16 and E17 appears to be associated with an elongation in the basal region. A, apex; B, base.

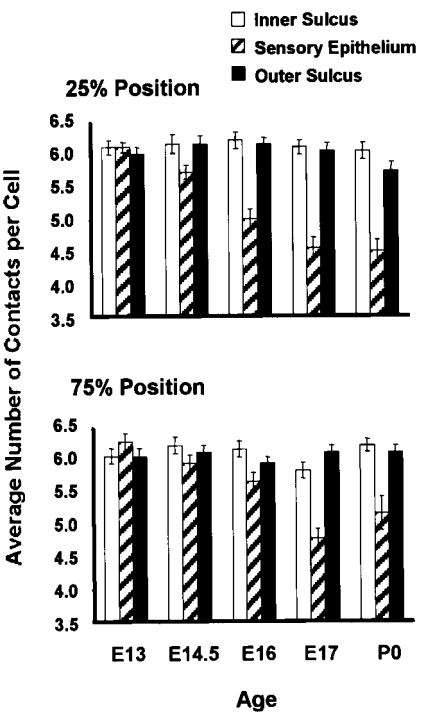

Fig. 5. Changes in the average number of cell contacts during development. The average number of cells contacted by individual cells in each region of the cochlear duct was determined for cells located at the 25% and 75% positions along the basal-to-apical axis of the cochlea. While the number of cell contacts in the inner and outer sulcus remained at six, indicating hexagonal packing, cell contacts in the sensory epithelium decreased to just above four at the 25% position and to five at the 75% position. Values are average ± SEM. E, embryonic day; P, postnatal day.

onstrated that $p27^{kip1}$ is expressed in a restricted region of the developing cochlea that appears to correlate with the population of cells that will give rise to the organ of Corti (the prosensory domain; Chen and Segil, 1999; Chen et al., 2002). Moreover, deletion of $p27^{kip1}$ results in ectopic cellular proliferation within the sensory cell population (Chen and Segil, 1999; Lowenheim et al., 2000), consistent with the hypothesis that expression of $p27^{kip1}$ is an early marker for the sensory cell precursor population. To examine changes in the spatial distribution of developing sensory epithelial cells over time, intact $p27^{kip1}$-labeled cochleae were double-labeled with phalloidin and then flat-mounted. The number and distribution of cells expressing $p27^{kip1}$ was determined at specific positions along the length of the cochlea (Fig. 6). Based on these results, it was possible to estimate the total number of $p27^{kip1}$-positive cells per cochlea and to determine the spatial distribution of these cells along the length of the duct at E14.5 and P0. The average number of $p27^{kip1}$-positive cells per cochlea at E14.5 was 13,081 ± 254 (439.7; mean ± SEM (SD); Fig. 7A). By comparison, the average number at P0 was 10,280 ± 1,250 (2,165.9; Fig. 7A). This result indicates that there is a decrease in the number of $p27^{kip1}$-positive cells per cochlea between E14.5 and P0; however, the decrease is not significant ($P = 0.05$). In contrast, the spatial distribution of these cells is strikingly different between the two stages (Fig. 7B,C). At E14.5, the average length of the duct for these samples was 2,735.0 ± 314.4 (506.2) μm and the average number of $p27^{kip1}$-positive cells within a 50 μm length along the basal-to-apical axis of the duct varied from a minimum of 185.0 ± 18.5 (29.7) in the basal region to a maximum of 300.7 ± 39.7 (63.9) at the extreme apex. In contrast, at P0, the average length of the duct for the assayed samples was 5,214.7 ± 178.0 (286.5) μm and the average number of $p27^{kip1}$-positive cells within a 50 μm length along the basal-to-apical axis varied from a low of 79.0 ± 11.0 (17.8) in the base to a high of 125.7 ± 21.3 (34.3) in the apex. The change of the length of the $p27^{kip1}$-positive domain along the basal-to-apical axis between E14.5 and P0 represents a significant increase ($P = 0.01$), whereas the change in the average number of cells within a 50 μm length of the basal-to-apical axis represents a significant decrease in both the basal and apical regions of the duct ($P = 0.01$; Fig. 7B,C).

Changes in Cellular Alignment and Position

The redistribution of $p27^{kip1}$-positive cells as the cochlea extends is consistent with convergent extension. If this is the case, then cells located near the boundaries of the developing sensory epithelium would be expected to move toward the midline as development proceeds. To exam-

ine this possibility, we analyzed the positions of developing inner hair cells at E14.5 and P0. At E14.5, several examples were observed in which some hair cells were located on the modiolar side of the row of developing inner hair cells (Fig. 8A). However, a similar analysis at P0 indicated that virtually all inner hair cells were aligned in a single row at this stage (Fig. 2M). Moreover, line tracings of the arrangement of developing inner hair cells in the basal region of the duct at E14.5 indicated unordered rows with numerous examples of cells located on the neural side of the developing inner hair cell row (Fig. 8B). In contrast, at P0, inner hair cells were aligned in a highly ordered and essentially perfect single row (Fig. 8B). These results suggest that a subset of inner hair cells initially develop on the modiolar side of the row of inner hair cells and subsequently move in a strial direction to assume their final position within the sensory epithelium.

Similarly, if cells within the sensory epithelium extend along the basal-to-apical axis of the cochlear duct, then an overall reduction in the number of cells at any specific position within the epithelium should occur over time. To examine this possibility, the number of cells was determined in the basal region (25% position) of the cochlear duct at E15 and P0. E15 was selected as the initial time point, because this was the earliest time point at which the third row of outer hair cells could be identified (see section on Changes in Cell Density and Cell Size). Because the overall size of the cells within the sensory epithelium increases with development, the number of cells was determined for a region bounded by the strial edge of the inner hair cells and the modiolar edge of the third row outer hair cells. The length of the region along the basal-to-apical axis was normalized to a distance equivalent to three inner hair cells at either time point (Fig. 8C). The average number of cells within this region decreased from 27.8 ± 1.0 at E15 to E18.8 ±0.3 at P0 (Fig. 8C). These results demonstrate that changes within the sensory epithelium are consistent with both convergence toward the midline and extension along the basal-to-apical axis. While these results do not definitively demonstrate convergent extension, they strongly support the existence of this type of morphogenetic movement.

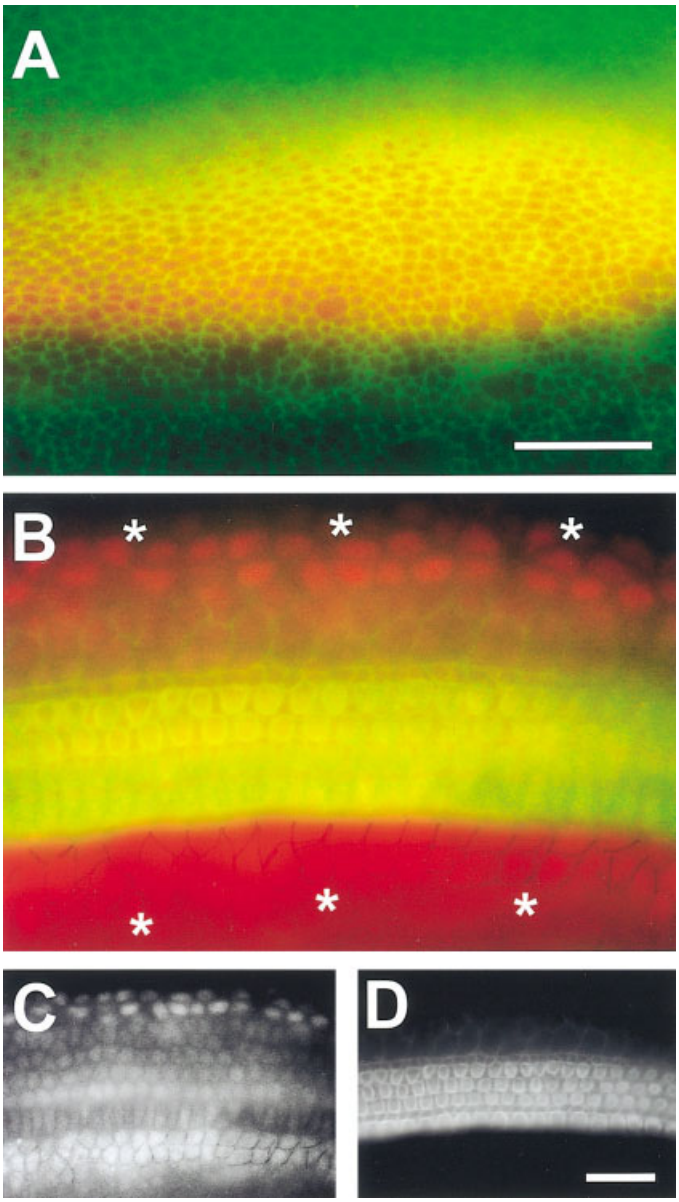

Fig. 6. Expression of $p27^{kip1}$ in the cochlear duct. Basal is to the left, and the strial edge is up. **A:** Expression of $p27^{kip1}$ in the basal region of the cochlea at embryonic day (E14). In this particular sample, developing inner hair cells could not be identified. $p27^{kip1}$ is labeled in red and cell boundaries are labeled in green. Note that $p27^{kip1}$ is expressed in a band of undifferentiated cells. **B:** Expression of $p27^{kip1}$ in the basal region of the cochlea at postnatal day 0 (P0). At this stage, $p27^{kip1}$ has been down-regulated in hair cells but is still expressed in supporting cells and also in cells located adjacent to the sensory epithelium. The boundaries of $p27^{kip1}$ expression are illustrated by asterisks. **C:** $p27^{kip1}$-labeling from the image in B. Expression of $p27^{kip1}$ in supporting cells is clearly illustrated. **D:** Phalloidin labeling from B, illustrating the position of the hair cells relative to the expression of $p27^{kip1}$. Scale bars = 25 μm in A (applies to A,B), 25 μm in D (applies to C,D).

DISCUSSION

Growth of the Cochlea

Several previous studies have analyzed the growth and density of specific cell types, in particular inner and outer hair cells, within the late-embryonic and postnatal cochleae of various animals including mice, rats, and hamsters (Burda et al., 1988; Pirvola et al., 1991; Roth and Bruns, 1992; Romand et al., 1993; Kaltenbach and Falzarano, 1994; Mu et al., 1997). In this study, the

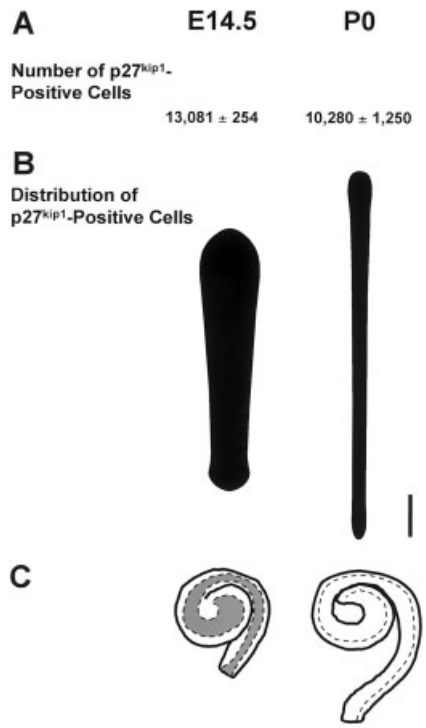

Fig. 7. Number and distribution of $p27^{kip1}$-positive cells in the cochlea at embryonic day 14.5 (E14.5) and postnatal day (P0). **A:** The average number of $p27^{kip1}$-positive cells per cochlea at E14.5 and P0 was determined as described. Values are average ± SEM. There is a decrease in the average number of cells per cochlea between E14.5 and P0, but the overall decrease is not significant ($P < 0.01$). **B:** Spatial distribution of $p27^{kip1}$-positive cells along the basal-to-apical axis of the cochlea at E14.5 and P0, basal is down. Spatial distributions were determined using the average length of the sensory epithelium at each time point and the average number of $p27^{kip1}$-positive cells at specific positions along the length of the cochlea. In contrast with cell number, there is a significant change in the spatial distribution of $p27^{kip1}$-positive cells. Note that the population of cells has converged along the modiolar-to-strial axis at the same time that it has extended along the basal-to-apical axis. **C:** Outlines of the overall shape of the cochlear duct at E14.5 and P0. The approximate spatial distribution of $p27^{kip1}$-positive cells is illustrated by the gray box with dashed lines at E14.5 and by the dashed line at P0. See text for further details. Scale bar = 500 μm in B.

period of analysis was extended to include the mid-embryonic period beginning at E13. In addition, changes in cell size and density were also examined outside the developing sensory epithelium in the inner and outer sulci. The results presented here are consistent with previous studies in terms of the

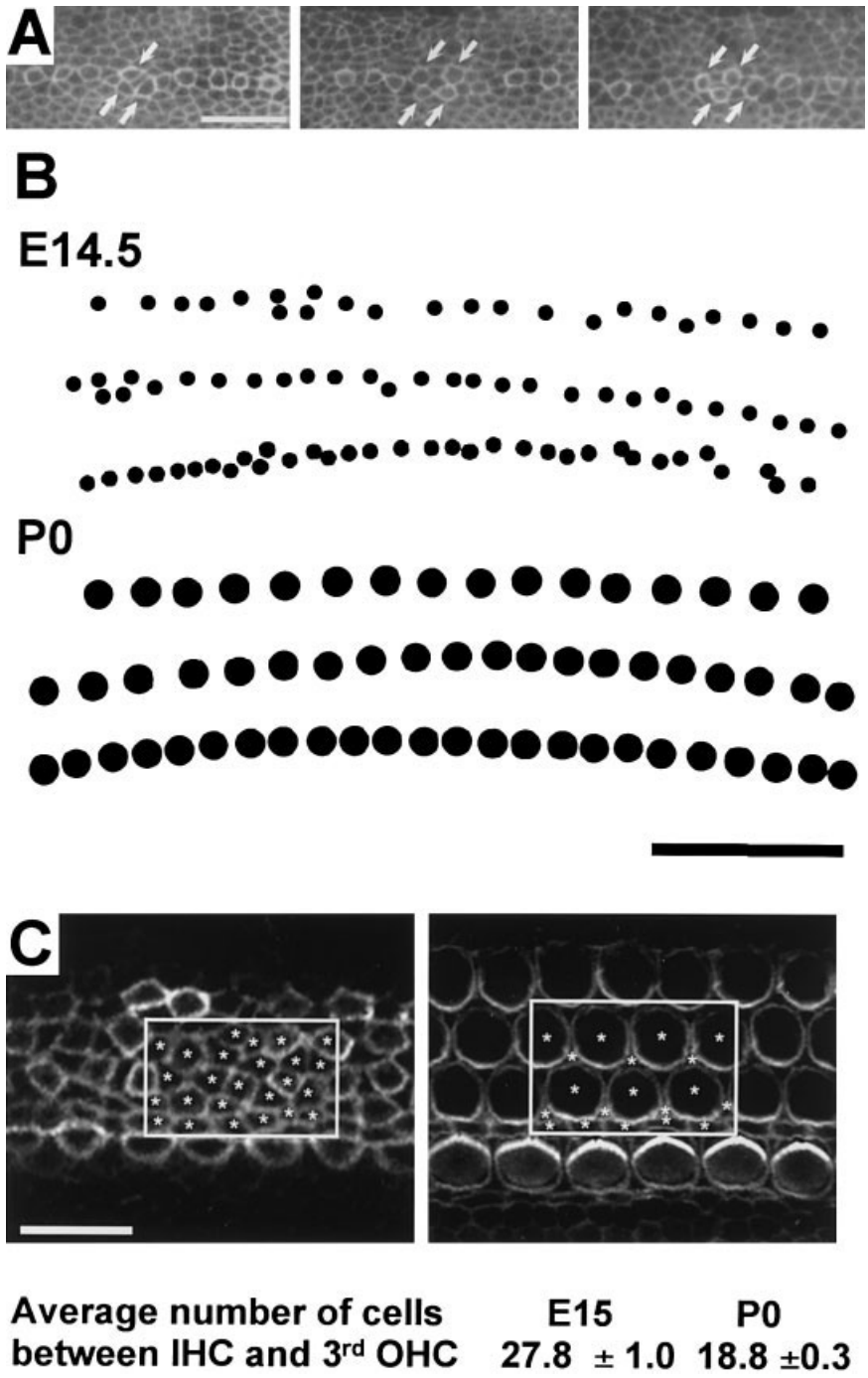

Fig. 8. Changes in cell position and number are consistent with cellular intercalation. **A:** Representative examples of inner hair cell positions in the basal turn of the cochlear duct at embryonic day 14.5 (E14.5). Basal is to the left and strial is up. Note that, in each case, some developing inner hair cells (arrows) are located on the modiolar side of the single ordered row. **B:** Diagrammatic illustrations for the positions of developing inner hair cells in the basal region of the cochlea from three samples at E14.5 and three samples at postnatal day 0 (P0). Note that, at E14.5, the developing row of inner hair cells is poorly organized and each sample contains multiple examples of hair cells that are located on the modiolar side of the developing row. In contrast, by P0, all inner hair cells are perfectly aligned within a single ordered row. **C:** Changes in the number of cells between the developing row of inner hair cells and third row of outer hair cells. At E15, the boxed region (equivalent to the distance covered by three inner hair cells along the basal-to-apical axis) includes numerous small, undifferentiated cells (asterisks). In contrast, at P0, the number of cells within the same three inner hair cell region is reduced (asterisks), even though the overall size of the boxed region has increased. See text for details. Scale bars = 50 μm in B, 10 μm in C.

growth of developing hair cells within the sensory epithelium. However, changes in luminal surface areas were also observed in both the inner and outer sulci. The change in surface area in the inner sulcus was comparable with the sensory epithelium (56% vs. 122%, respectively) between E13 and P0. In contrast, surface area changes in the outer sulcus increased by greater than 450% during the same time period. The greater degree of change in the outer sulcus is consistent with the location of these cells at the outer edge of the coiled cochlear duct. However, it is not clear whether the growth of these cells acts as a driving force for cochlear coiling or occurs merely in response to physical forces exerted on these cells as a result of growth in other regions of the developing labyrinth.

There was a marked change in rate of surface area growth between E16 and E17, in particular in cells located in the developing inner and outer sulci in the basal regions of the cochlea. These changes were reflected in the overall as well as regional growth of the cochlear duct between these time periods. As is the case for cochlear coiling, it remains to be determined whether these changes in cell size play an active role in the overall growth of the cochlea.

Cellular Rearrangements

The organ of Corti is characterized by ordered rows of both hair cells and supporting cells that extend along the basal-to-apical axis of the cochlea. As a first step toward understanding how this pattern is generated, we wanted to characterize its morphologic development. At E13, cells throughout the duct are arranged in a hexagonal packing pattern that is consistent with undifferentiated epithelial cells. A limited number of mitotic figures are observed in the region of the developing sensory epithelium, and no signs of differentiating hair cells are present either at the luminal surface or in cross-sections. However, by E14.5, boundaries between the inner sulcus, developing sensory epithelium, and outer sulcus can be clearly identified in the basal and, to a lesser extent, apical regions of the duct. As had been reported previously in rats (Pirvola et al., 1991; Romand et al., 1993), the modiolar boundary of the sensory epithelium can be identified by the presence of an imperfect row of developing hair cells, characterized by an accumulation of filamentous actin at their lateral membranes. At the same time, the strial boundary of the epithelium can be identified as a result of increased growth in the luminal surface areas of cells in the developing outer sulcus. These results suggest that cellular differentiation and patterning begins between E13 and E14.5. This conclusion is consistent with the timing of terminal mitoses within the sensory epithelium (Ruben, 1967) and with previous analyses of cytologic development (Anniko, 1983; Lim and Anniko, 1985) as well as with the reported onset of expression of the cell-cycle inhibitor $p27^{kip1}$ and the transcription factor Math1 between E12 and E14 (Bermingham et al., 1999; Chen and Segil, 1999; Lanford et al., 2000). However, it is important to consider that a limited number of cells have been shown to express the hair cell marker myosin 6 in the basal regions of cochleae as early at E13.5 (Montcouquiol and Kelley, 2003), suggesting that the onset of differentiation may precede any obvious morphologic changes within the epithelium.

The molecular factors that specify the boundaries of the prosensory domain are largely unknown. Chen and Segil (1999) demonstrated that the expression of $p27^{kip1}$ correlates with the population of cells that will develop as the prosensory domain. In addition, it has been suggested that all cells within the prosensory domain also express the transcription factor Math1 (Lanford et al., 2000). However, the factors that regulate the expression of either $p27^{kip1}$ or Math1 within the cochlear duct have not been determined. The appropriate size and location of the prosensory domain is clearly a crucial step in the normal development of the organ of Corti, because overproduction of cells within this domain results in patterning defects and deafness (Chen and Segil, 1999), suggesting that the epithelium has no way to correct for inappropriate numbers of cells within this domain. This observation also suggests that immigration or emigration of cells into or out of the prosensory domain is limited.

Between E14.5 and E16, a subset of the cells located between the boundaries of the developing sensory epithelium begin to develop as outer hair cells. The initial patterning of these cells is nonuniform, but as development proceeds, these cells become arranged into three ordered rows. The specification of cells within this region of the prosensory domain as either hair cells or supporting cells is presumed to be regulated through lateral inhibitory interactions that are mediated through the Notch signaling pathway (Lanford et al., 1999, 2000; Zheng et al., 2000; Zine et al., 2001); however, the factors that regulate the subsequent arrangement of these cells into ordered rows have not been identified. Outer hair cell patterning defects, independent of changes in cell fate, have been reported in mice containing targeted deletions of several different Notch pathway genes, including *Notch1*, *Jagged2*, and *HES5* (Lanford et al., 1999; Zhang et al, 2000; Zine et al., 2001). Similar defects may also occur in mice containing mutations in *Jagged1* (Kiernan et al., 2001; Tsai et al., 2001), another member of the Notch pathway; however, further study is required to determine the relative contributions of defects in cell fate versus patterning.

Convergent Extension

Convergent extension refers to a morphogenetic process in which the general shape of a structure elongates along a central axis as it narrows along a perpendicular axis (reviewed in Keller, 2002). Convergent extension has been shown to occur during the formation of several different structures, including vertebrate neural tube and notochord, the ascidian notochord, and the *Drosophila* germ band. Moreover, Chen et al. (2002) recently suggested that convergent extension could play a role in the develop-

ment of the organ of Corti as well. One of the fundamental processes of convergent extension is intercalation of cells in the direction of the midline that results in extension along the midline axis. Many of the results presented here are consistent cellular intercalation and convergent extension. The population of $p27^{kip1}$-positive cells thins along the modiolar-to-strial axis of the cochlear duct at the same time that it extends along the basal-to-apical axis. In addition, the observed changes in the position of developing inner hair cells are consistent with intercalation in a strial direction followed by, or concomitant with, extension along the basal-to-apical axis.

The driving force for the intercalative movements required for convergent extension are not well understood. However, a recent series of studies have demonstrated that an evolutionarily conserved signaling pathway referred to as the planar cell polarity (PCP) pathway because of its role in the development of PCP in *Drosophila* is also required for convergent extension in vertebrates (Mlodzik, 2002). Intercalation apparently requires cellular polarization leading to the production of active protrusions that act to generate that forces required for cellular movement (Davidson and Keller, 1999; Goto and Keller, 2002). Although active protrusions have not been observed in cells within the developing organ of Corti, recent results suggest that, during epithelial convergent extension, protrusions may be restricted to the basal surface of the cells and may be less than a micron in size (Munro and Odell, 2002). Based on these observations, it seems possible that similar events could occur in the developing cochlear duct.

Members of the PCP pathway have been shown to be necessary for convergent extension in vertebrates. In particular, *vangl2*, a novel membrane protein, was demonstrated recently to be required for convergent extension during gastrulation in *Xenopus* (Goto and Keller, 2002). This result is particularly intriguing because mutations in mouse *vangl2* lead to a shortened cochlear duct and a disruption in cellular patterning in the organ of Corti (Montcouquiol et al., 2003). In particular, in the apical region of *vangl2* cochleae, multiple additional rows of both inner and outer hair cells are present and the number of cell–cell contacts is greater than four per cell (Montcouquiol et al., 2003). These results are consistent with a defect in convergent extension, suggesting that this event plays a role in the development of the organ of Corti and that these movements are dependent on vangl2 (Montcouquiol et al., 2003).

In addition to the members of the PCP pathway, another key factor in the movement of epithelial cells is the formation of adherins junctions by cellular adhesion molecules (CADs). Epithelial morphogenesis is perturbed if the function of CADs is disrupted (reviewed in Schock and Perrimon, 2002). Several different CADs are expressed in the developing cochlear duct during a time period that would be consistent with a role in convergent extension (Whitlon, 1993; Whitlon et al., 1999). In particular, N-Cad and E-cad recently have been demonstrated to be expressed in the greater and lesser epithelial ridges, respectively, in the rat cochlea at E16 (equivalent to approximately E14.5 in the mouse; Simonneau et al., 2003). While it is not possible to draw any conclusions regarding function based on the pattern of expression for these molecules, their presence in the epithelium during this time period suggests that they could play a role in convergent extension.

Another possible source of the driving forces for the observed cellular movements would be that mesenchymal cells underlying the developing cochlear duct provide the actual driving force for convergent extension and that the overlying epithelial cells are passively carried along with the mesenchymal cells (Keller and Danilchik, 1988; Shih and Keller, 1992; Elul and Keller, 2000). Although this possibility cannot be excluded, it is important to note that expression of *vangl2* was not detected in developing cochlear mesenchyme, suggesting that the effects of a deletion of *vangl2* could not have a direct effect on mesenchymal cell movements (Montcouquiol et al., 2003).

Convergent extension can explain the driving force that leads to the progressive decrease in the number of cell contacts in the developing sensory epithelium. As cells extend along the basal-to-apical axis, the density of cells per unit area would decrease. However, the factors that regulate the formation of ordered rows of cells are still unknown. It is possible that signals from underlying mesenchyme, acting directly or through the basement membrane, could act as guides for establishment of cellular pattern (Rhee et al., 2003). However, no obvious cellular pattern is present in the underlying mesenchymal cells (unpublished observations), and a relatively normal cellular pattern can develop in cochlear explants in which all mesenchymal cells have been removed as early as E12 (Montcouquiol and Kelley, in press). Therefore, it seems likely that the signals that regulate the fine aspects of pattern formation and cellular arrangement are intrinsic to the epithelium. As discussed, the factors that regulate this patterning are unknown but could include differential adhesion and/or local cell signaling (reviewed in Tepass et al., 2002) and may be regulated, at least in part, by the Notch signaling pathway (Zhang et al., 2000).

In summary, the results presented here describe the cellular rearrangements that occur during the development of the cochlear duct in mice. Changes in the patterns of cellular growth and number of cell contacts are evident in different regions of the duct by as early as E14.5, suggesting that each region has become distinct by this time. Finally, changes in the spatial distribution of the population of $p27^{kip1}$-positive cells and of BrdU-labeled cells are consistent with a role for convergent extension in the development and patterning of the organ of Corti.

EXPERIMENTAL PROCEDURES

Cochlear Dissections and Actin-Labeling

Timed-pregnant CD-1 mice were killed in accordance with the *NIH*

Guide for the Care and Use of Animals. Embryos were removed and staged according to Kaufman (1992). Cochleae were dissected and fixed in 4% paraformaldehyde. To visualize cell–cell interactions, cochlear ducts were dissected to expose the apical surface of the developing cochlea and filamentous actin was labeled by incubation in Alexa 488-conjugated phalloidin (Molecular Probes). Briefly, fixed cochleae were incubated in 0.1% Triton X-100 in 1× phosphate buffered saline (PBS) with gentle agitation for 30 min followed by incubation in 3 units/ml of Alexa 488-conjugated phalloidin in 1× PBS for 1 hr.

Determination of Cell Density, Cell Size, and Cochlear Shape

Individual cochleae were flat-mounted with the sensory epithelium facing up. The entire length of the cochlear duct was measured and used to identify the 5%, 25%, 50%, 75%, and 90% positions along the duct. At each position, the number of cells within a 25 × 50-μm rectangle oriented with its long edge parallel to the basal-to-apical axis of the cochlea was determined (Fig. 1). In addition, to examine differences in cell density in different regions of the cochlea, similar measurements were taken for cells located in the inner sulcus, developing sensory epithelium, and outer sulcus. A minimum of three samples were analyzed for each developmental time point, and statistically significant changes were determined by using a *t*-test. To determine the overall shape of the cochlear duct at different developmental time points, intact cochlear ducts were imaged through the cartilage of the temporal bone.

Cochlear Histology

To examine the histologic development of the cochlear duct, individual cochleae were staged and dissected as described and then fixed in 3% glutaraldehyde/2% paraformaldehyde for 8 hr to overnight at 4°C. After fixation, samples were dehydrated, embedded in methacrylate (Immuno-Bed, Polysciences, Inc.), sectioned at a thickness of 3 μm and stained with thionin.

Expression of $p27^{kip1}$

Changes in the distribution of $p27^{kip1}$-positive cells were determined by labeling cochleae with an antibody against $p27^{kip1}$ (Cell Signaling), followed by labeling with phalloidin as described. For detection of $p27^{kip1}$, cochleae were incubated in 0.1% Triton X-100 as described, followed by blocking with 1.0% goat serum in 1× PBS for 1 hr. After blocking, cochleae were incubated overnight in anti-$p27^{kip1}$ at a dilution of 1:100 in 1× PBS with 0.1% Triton X-100. Antibody labeling was detected by using a goat anti-rabbit secondary antibody conjugated to Alexa 563 (Molecular Probes) at a dilution of 1:1,000 in 1× PBS. Cochleae were flat-mounted, the overall length was determined, and the total number of $p27^{kip1}$-positive cells was determined at specific positions along the length of the cochlea. These data we used to estimate the total number of $p27^{kip1}$-positive cells within the cochlear duct. These data are presented at mean ± standard error of the mean (SEM). For reference the standard deviations (SD) are also provided.

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