Sprouty2, a Mouse Deafness Gene, Regulates Cell Fate Decisions in the Auditory Sensory Epithelium by Antagonizing FGF Signaling

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Summary

The auditory sensory epithelium (organ of Corti), where sound waves are converted to electrical signals, comprises a highly ordered array of sensory receptor (hair) cells and nonsensory supporting cells. Here, we report that Sprouty2, which encodes a negative regulator of signaling via receptor tyrosine kinases, is required for normal hearing in mice, and that lack of SPRY2 results in dramatic perturbations in organ of Corti cytoarchitecture: instead of two pillar cells, there are three, resulting in the formation of an ectopic tunnel of Corti. We demonstrate that these effects are due to a postnatal cell fate transformation of a Deiters’ cell into a pillar cell. Both this cell fate change and hearing loss can be partially rescued by reducing Fgf8 gene dosage in Spry2 null mutant mice. Our results provide evidence that antagonism of FGF signaling by SPRY2 is essential for establishing the cytoarchitecture of the organ of Corti and for hearing.

Introduction

One of the most striking examples of cellular patterning in vertebrates is found in the auditory sensory epithelium or organ of Corti (oC). The oC is a specialized region of the inner ear (cochlear) epithelium composed of a highly ordered array of sensory (hair) and nonsensory (supporting) cell types (see Figure 3M; reviewed in Kiernan et al., 2002). The two types of mechanosensory hair cells in the oC, inner and outer hair cells (IHCs and OHCs), are arranged such that a single row of IHCs and three evenly spaced rows of OHCs run along the length of the snail-like cochlea from base to apex. The supporting cells of the oC are also organized in a precise pattern. For example, there are two rows of pillar cells (inner and outer) separating the IHC row from the OHC rows. In a mature oC, inner and outer pillar cells form a distinctive space, the tunnel of Corti, that appears as an open triangle in cross-section (see Figure 4I). Adjacent to the pillar cells, Deiters’ cells are also organized in an evenly spaced array such that a Deiters’ cell is invariably found underlying each OHC. Recent work has demonstrated roles for two signaling pathways in patterning the oC: Notch signaling plays a role in hair cell specification (reviewed in Kelley, 2003; Kiernan et al., 2002), and fibroblast growth factor (FGF) signaling is required to generate hair cell progenitor pools (via FGF receptor 1 [Fgfr1]) (Pirvola et al., 2002) and for pillar cell specification/differentiation (via FGF receptor 3 [Fgfr3]) (Colvin et al., 1996). However, little else is known about the molecular mechanisms whereby the exquisite cytoarchitecture of the oC is achieved during development.

The oC plays a pivotal role in hearing: it converts the mechanical energy resulting from complex sound pressure waves into electrical signals that are transmitted to the brain. Given the central role of the hair cell as the mechanosensory receptor, it is not surprising that there are many mutations that cause hearing deficits due to loss or malfunction of hair cells (reviewed in Steel and Kros, 2001). However, one might predict that hearing deficits would also be caused by perturbations in the cytoarchitecture of the oC due to effects on supporting cells. To date, the only mutant mice in which hearing loss has been attributed to abnormalities in the cellular pattern of the supporting cells are Fgfr3–/– mice (Colvin et al., 1996). Further genetic analysis is needed to determine how the cytoarchitecture of the supporting cells of the oC influences hearing function.

Here, we have explored the role in oC morphogenesis and hearing of Sprouty2 (Spry2), a member of the Sprouty family of negative feedback regulators of signaling via receptor tyrosine kinases (RTKs), including FGF receptors. The first member of this gene family, sprouty (spry), was identified in Drosophila, where it was shown to antagonize RTK signaling during tracheal branching (Hacohen et al., 1998), and other developmental processes (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999). In the mouse, four Sprouty genes (Spry1–Spry4) comprise a family of membrane-associated intracellular proteins that share a cysteine-rich carboxyl terminal region that is highly conserved across species (de Maximy et al., 1999; Minowada et al., 1999). Overexpression of Sprouty genes in vertebrates perturbs a number of developmental processes, including limb (Minowada et al., 1999), lung (Mailleux et al., 2001; Perl et al., 2003), and kidney development (Chi et al., 2004), as well as convergent extension movements during Xenopus gastrulation (Nutt et al., 2001). Like their Drosophila homologs, vertebrate Sprouty family members have been shown to act intracellularly to negatively regulate FGF and other RTK signaling through diverse biochemical mechanisms, primarily via effects on the MAPK pathway (reviewed in Dikic and Giordano, 2003; Guy et al., 2003; Kim and Bar-Sagi, 2004). Here, we describe the production of...
mouse lines carrying null and conditional null alleles of Spry2. We show that Spry2 plays a key role in the determination of supporting cell fate in the oC, and that Spry2 null mice are severely hearing impaired.

Results

Generation of Mice Carrying Spry2 Null and Conditional Null Alleles

To explore Spry2 function in mice, we used the same strategy we previously employed to generate an Fgf8 mutant allelic series (Meyers et al., 1998) to produce animals carrying the Spry2 mutant alleles illustrated in Figure 1. Animals homozygous for either the Spry2neo-flox or Spry2flox allele were viable and fertile and displayed no obvious defects. When crossed in reverse, animals heterozygous for Spry2LORF, a null allele in which the entire Spry2 open reading frame is deleted, produced Spry2LORF/LORF progeny at Mendelian frequency at birth (n = 11/42; 26%). By weaning (postnatal day [P] 21–28), almost half of the Spry2LORF/LORF mice had died, and many of the remaining homozygotes were significantly small than normal. Preliminary analysis of the vital organs pointed to abnormalities in gastrointestinal tract function as a possible cause of this lethality (P.-F. Lu, G.M., and G.R.M., unpublished data). Here, we report on a separate phenotype observed in Spry2LORF/LORF homozygotes, hereafter referred to as Spry2−/− mice or Spry2 null mutants.

Spry2 Null Mutants Are Severely Hearing Impaired

Spry2−/− mice that survived past weaning did not appear to respond to loud noises. Auditory brainstem response (ABR) tests revealed that at P21, Spry2−/− mice suffered from intermediate to severe hearing loss, with click-evoked ABR thresholds averaging 76 decibels sound pressure level (dB SPL), 60 dB higher than in their Spry2+/+ littermates (average threshold = 16 dB SPL) and 53 dB higher than in Spry2+/− littermate controls (average threshold = 23 dB SPL) (Figures 2A and 2B; see Figure S1A in the Supplemental Data available online). The initiation of hearing function was not simply delayed, because ABR tests showed that Spry2−/− mice at 7, 10, or 19 weeks were also hearing impaired (average threshold = 79 dB SPL). The absence of a wave 1 response in the ABR tests (Figures 2A and 2C)
The Middle Ear Ossicles, Inner Ear Labyrinth, and Spiral Ganglion Appear Normal in Spry2 Null Mutants

During hearing, sound pressure waves cause vibration of the tympanic membrane, leading to the transmission of vibrational energy through the ossicles of the middle ear to the oC of the inner ear. In the oC, this vibrational energy is converted into electrical impulses that are transmitted by the spiral ganglion to the brain. To explore the basis of the hearing defects in the Spry2 null mutants, we examined the middle ear, inner ear, and spiral ganglion. There were no gross defects in the shape of the ossicles (malleus, incus, stapes) or in the articulation of the malleus to incus, incus to stapes, or stapes to the oval window of the inner ear in Spry2−/− mice (Figures 3A and 3B, and data not shown). This suggests that their hearing defect is unlikely to be due to a failure in sound pressure conduction.

We next examined the inner ear labyrinth, which begins developing at ~E9.5 from an epithelial sac and undergoes a complex morphogenesis to achieve its nearly mature structure by ~E15.5 (Morsli et al., 1998). At that stage, it comprises the cochlea and the vestibule, which is required for balance. We found no abnormalities in the gross structure of the inner ear labyrinth of E15.5 Spry2 null mutants by using the paint-filling technique (Martin and Swanson, 1993) to visualize its lumen (Figures 3C and 3D). The cochlea appeared to have completed the appropriate number of turns, and the semicircular canals, utricle, and saccule of the vestibule were properly positioned. Consistent with the latter observation, vestibular function appeared unaffected in Spry2−/− adults as measured by multiple criteria (Bergstrom et al., 1998): (1) absence of hyperactivity, head-tossing, or circling behavior; (2) proper dorsoflexion and extension of forelimbs upon tail hanging; (3) ability to orient when inverted in a conical tube; and (4) ability to swim (data not shown).

In the cochlea, no gross abnormalities were detected by histological analysis at E18.5 and P21 in structures essential for normal hearing, including the stria vascularis, tectorial membrane, and basilar membrane (data not shown). Furthermore, although defects in the stereocilia of the hair cells are often the cause of severe hearing deficits, we found no obvious abnormalities in the hair bundles of the IHCs and OHCs of Spry2 null mutants by scanning electron microscopy (Figures 3E and 3F, and data not shown).

In addition, no obvious abnormalities were detected in the neurons that directly innervate the cochlea by either neurofilament staining at P0 or by histological analysis at P15 and P21 (Figures 3G, G’, H, and H’, and data not shown).

Extra Outer Hair Cells and Supporting Cells Are Present in the Spry2−/− Organ of Corti

We next looked more closely at the cytoarchitecture of the oC. As in normal mice (see Figure 3M), Spry2 null mutants contained a single longitudinal row of IHCs, as determined by examination of dissected cochlea stained in whole mount with rhodamine phalloidin, which labels the actin-rich hair cells intensely (Figures
Figure 3. Loss of Spry2 Function Causes an Increase in Outer Hair Cell and Supporting Cell Number

(A and B) Middle ear ossicles (stapes [St], incus [In], malleus [Ma]) of P8 control and Spry2−/− mice stained with Alcian blue and Alizarin red.

(C and D) Paint-filled inner ear labyrinths from E15.5 control and Spry2−/− embryos. Dorsal views of the cochlea (insets) show that it has completed one and a half turns in both control and Spry2−/− embryos. Location of the cochlea (Co), vestibule (Ve), and site of paint injection (arrowhead) are indicated.

(E and F) Scanning electron micrographs at P21 showing the stereocilia on the apical surface of individual outer hair cells (OHCs).

(G and H) Spiral ganglion from P0 control and Spry2−/− mice stained for neurofilament (NF) protein.

(G’ and H’) Higher magnification views in which the neuronal dendrites from the inner hair cell (nIHC) and OHC (nOHC) rows are indicated. The region shown in (H’) appears to contain four OHC rows (see below), rather than the normal three rows shown in (G’).

(I and J) Surface views of the oC at P0, stained for actin. The region of the Spry2−/− cochlea shown in (J) has four rows of OHCs instead of the three found in the control and elsewhere in the Spry2 null oC. The pillar cell (PC), inner hair cell (IHC), and OHC rows are indicated.

(K–L#) Cross-sections through the oC at P5 stained for p75, which labels the inner PC, Deiters’ cells (DC), Hensen’s cells (HC), and Claudius’ cells (CC), and counterstained with a green nuclear dye. (K’ and L’) The same photomicrographs as in (K) and (L), showing only the p75 staining. The Deiters’ cells are readily identified by a distinctive Y-shaped staining pattern: (L and L’) The Spry2 null mutant has four Deiters’ cells, supporting the four OHCs, and extra Claudius’ cells.

(M) Schematic diagram showing the normal morphology and cell types comprising the organ of Corti (oC) at P1.

In cross-sections through the oC at P0–P5, we found that in every transverse section in which a fourth OHC was observed in Spry2 null mutants, an extra supporting cell was also present underlying the extra OHC (Figures 3K and 3L). The extra supporting cell was identified as a Deiters’ cell based on the presence of a distinctive Y-shaped structure readily detected by staining for p75NGFR (von Bartheld et al., 1991) (Figures 3K’ and 3L’). We also observed multiple regions with more sup-

3I and 3J). However, instead of the normal three longitudinal rows of OHCs, we detected four. Over a 3 mm length of cochlea starting from the base (representing ~55% of its total length), we counted an average of 142 fourth row OHCs in Spry2−/− (n = 3), 6 in Spry2+/− (n = 3), and 0 (n = 2) or 1 (n = 1) in Spry2+/+ mice (see Table S1). The extra OHCs were found in patches that alternated with regions containing the normal three rows. These patches appeared more frequently and were longer toward the apex.
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porting Claudius’ cells, which are also marked by p75NGFR (Figures 3K, 3K’, 3L, and 3L’, and data not shown).

Spry2 Null Mutants Develop an Extra Pillar Cell between P5 and P10

At P0–P5, when we observed increased OHC, Deiters’, and Claudius’ cell numbers in Spry2−/− mice, pillar cell number appeared normal. At those stages, p75NGFR is detected in only one of the two normal pillar cells, the inner pillar cell. In Spry2 null mutants and littermate controls alike, we always saw a single p75NGFR-positive inner pillar cell, and between it and the nearby p75NGFR-positive Deiters’ cell, we always saw a single nucleus, presumably of the outer pillar cell (n = 6 Spry2−/− mice; n = 7 Spry2−/+ or Spry2+/+ controls; Figures 3K and 3L). Moreover, using an anti-tubulin antibody, we detected only the normal two pillar cells in both Spry2 null mutants and controls at P3 (Figures 4A’ and 4B’).

At P10, pillar cell differentiation is nearly complete: the nuclei of the inner and outer pillar cells have moved apart basally, and the cells have changed shape so that they touch one another only at their extreme apical and basal ends, forming the space that constitutes the tunnel of Corti (see Figure 4I). When stained with an antibody against tubulin, the apical process of the differentiated pillar cells has a distinctive pattern of staining, showing numerous microtubule bundles (Figure 4C). Strikingly, at this stage in Spry2 null mutants, we found that in addition to the two normal pillar cells there appeared to be an ectopic, third pillar cell, which like the normal inner and outer pillar cells, extended a heavily tubulin-labeled process from the basilar membrane to the lumenal surface (open arrowhead in Figure 4D). This third pillar cell was also clearly seen in sections stained for nuclei and S100, a protein that is enriched in pillar cell and Deiters’ cell bodies (Pack and Slepecky, 1995) (Figures 4E and 4F; Figure S2). The presence of this cell created an aberrant, second tunnel of Corti-like space between it and the normal outer pillar cell. This second tunnel always contained a single OHC that was stranded without a supporting Deiters’ cell.

Importantly, in contrast to the patchy increase in OHC/Deiters’ cell (OHC/DC) numbers, the third pillar cell was observed in virtually every transverse row of each Spry2−/− oC examined (n = 6 Spry2−/− mice; n = 5 Spry2−/+ controls); thus, in regions of the cochlea where four OHCs were present, we always observed a third pillar cell with three remaining Deiters’ cells (Figures 4D and 4F), and in regions of the cochlea where the normal stranded without a supporting Deiters’ cell (arrow).

Figure 4. An Extra, Third Pillar Cell Forms in Spry2 Null Mutants during Postnatal Differentiation of the Organ of Corti

(A–B’) Cross-sections through oC at P3 stained for tubulin (green) and a red nuclear dye. The Spry2 null mutant has two normal pillar cells and four Deiters’ cells.

(C and D) Similarly stained cross-sections through oC at P10. At this stage, the Spry2 null mutant has a third, extra pillar cell with its nucleus (filled arrowhead) near the basilar membrane, and a thick tubulin-stained process extending to the lumenal surface (open arrowhead). The adjacent Deiters’ cells extend thinner tubulin-stained processes to the lumenal surface. The third pillar cell appears at the expense of the Deiters’ cell nearest to the outer pillar cell, leaving three remaining Deiters’ cells and stranding an OHC without a supporting cell (arrow).

(E–H) Cross-sections through oC at the ages indicated, stained for S100 (red) and counterstained with a green nuclear dye. An extra pillar cell is present in the Spry2 null mutant (arrowheads), irrespective of whether it has (F) four or (H) three OHCs, leaving three or two remaining Deiters’ cells, respectively. In each case, an OHC is...
Very occasionally, two extra pillar cells were observed (data not shown).

Spry2 Expression in the Organ of Corti
Sprouty genes encode proteins thought to function intracellularly. Thus, to explore which cells might be directly affected by loss of Spry2 function, we performed an in situ hybridization analysis of Spry2 expression in whole mount and in cryosections taken along the length of the wild-type cochlea. At E11.5, in the cochlear portion of the otocyst, Spry2 RNA was detected in a broad but discrete medial domain of the inner ear epithelium (data not shown). In the cochlea at E14.5, before different cell types have become morphologically distinct, Spry2 RNA was detected in most or all of the presumptive oC region (Figure 5A). At E16.5 and E17.5, when the constituent cell types of the oC could be clearly identified, Spry2 RNA was detected in pillar cells, Deiters’ cells, Claudius’ cells, and Hensen’s cells, another supporting cell type. Reduced or no Spry2 expression was observed in IHCs and OHCs at this stage (Figure 5C and data not shown). By P5, just before the pillar cell phenotype becomes evident in Spry2 null mutants, Spry2 RNA in wild-type animals was clearly detected in Deiters’ cells, but appeared to be considerably less abundant in other cell types (Figures 5E and 5I, and data not shown). Thus, Spry2 is initially expressed in a broad domain in the oC prior to cytological differentiation of its constituent cell types, and by the end of oC maturation at P5–P7, it appears to be enriched in Deiters’ cells.

We found that at least one other member of the Sprouty gene family, Spry1, was coexpressed with Spry2 during oC development. At E14.5, Spry1 RNA was detected in a thin stripe of cells, ~2 cells wide, in the presumptive oC region, and in a small region of the cochlear epithelium outside of the oC (Figure 5B). At E16.5, after cell differentiation had commenced, and as Spry1 RNA was localized to just the two pillar cells (Figures 5D, 5F, and 5I). In situ hybridization analysis of the genes at the developmental stages indicated in wild-type cochlea sectioned to visualize the oC.

Spry2 Genetically Antagonizes FGF8 Signaling
Previous studies have shown that at E16.5, Fgf8 is expressed in the IHCs and its putative receptor, Fgfr3, is expressed in the adjacent pillar cells as well as in OHCs and Deiters’ cells (Pirvola et al., 2002). We detected a similar pattern in wild-type animals at P5, just before the pillar cell phenotype is observed in Spry2 mutants, except that Fgfr3 no longer appeared to be expressed in OHCs (Figures 5G–5I). Since both Fgf8 (U. Pirvola and A. Neubuser; B. Jacques, M. Lewandoski, and M. Kelley, personal communication) and Fgfr3 (Colvin et al., 1996) are required for pillar cell differentiation, this suggests a model in which FGF8, produced in the IHCs, stimulates the differentiation of pillar cells. One hypothesis that could explain our findings is that Spry2 function is required to limit the range of the FGF8 signal, so that only the two cells nearest the source of FGF8 differentiate as pillar cells. When SPRY2 is absent, the effective range of FGF8 is expanded so that now an Fgfr3-expressing Deiters’ cell takes on a pillar cell fate.
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Figure 6. Pillar Cell Development and Hearing Are Partially Rescued in Spry2−/− Animals by Reducing Fgf8 Gene Dosage

(A–E) Cross-sections through oC at P21 stained for S100 and counterstained with a green nuclear dye. The filled arrowhead in (B) and (E) points to the nucleus of the extra pillar cell, and the open arrowhead points to the apical process of the extra pillar cell. (A) Normal oC morphology in a Spry2+/+; Fgf8+/− animal. (B) Typical section of an Spry2−/−; Fgf8+/− oC showing an extra pillar cell. (C–E) Examples of the variation in phenotype observed within an individual Spry2−/−; Fgf8+/− oC. In (C) and (D), there are three or four rows of OHCs/Deiters’ cells, respectively, but no extra pillar cell (asterisk). In (E), an extra pillar cell is still observed. (F) ABR waveform patterns and thresholds for 3-week-old mice of the genotypes indicated are as described in the legend to Figure 2. Each ear was individually measured in n = 6 Spry2+/+; Fgf8+/−, n = 4 Spry2−/−; Fgf8+/+, and n = 9 Spry2−/−; Fgf8+/− mice.

(see Figure 7B). The consequent disruption of oC cytoarchitecture might be the cause of the observed hearing deficit.

One prediction of this hypothesis is that reducing the amount of FGF8 should prevent both the excess pillar cell phenotype (see Figure 7B) and the hearing impairment observed in Spry2 null mutants. To test this prediction, we produced Spry2 null mutants in which Fgf8 gene dosage was reduced, tested their hearing, and then examined the oC (Figures 6A–6E). In Spry2 null mutants with wild-type Fgf8 gene dosage, virtually every transverse section observed had an extra pillar cell by P21 (average of 39 third pillar cells over a 300 μm length of cochlea; n = 3; Figure 6B), whereas in their Spry2−/−; Fgf8+/− littermates, we saw extended regions in which there were the normal two pillar cells (average of 20 third pillar cells/300 μm; n = 5; Figures 6C–6E), representing an ~50% reduction in the incidence of an aberrant third pillar cell. In Spry2+/+; Fgf8+/− littermate controls, we never observed a third pillar cell (Figure 6A; see Table S2). The significant rescue of the pillar cell defect in Spry2−/− mice with reduced Fgf8 gene dosage suggests that the normal function of SPRY2 is to antagonize FGF8 signaling in Deiters’ cells. In contrast, there did not appear to be any rescue of the extra OHC/DC phenotype in Spry2−/−; Fgf8+/− mice (data not shown).

The ABR hearing tests conducted on these animals prior to examination of oC cytoarchitecture showed that, as expected, Spry2+/+; Fgf8+/− P21 controls had normal hearing (average ABR threshold = 11 dB SPL). Spry2−/−; Fgf8+/− littermates had the expected severe hearing loss, 55 dB higher than the Spry2+/+; Fgf8+/− controls (average ABR threshold = 66 dB SPL). However, Spry2−/−; Fgf8+/− littermates showed partial rescue of hearing function with average ABR thresholds 31 dB higher than the Spry2+/+; Fgf8+/− controls (average ABR threshold = 42 dB SPL; Figure 6F). These results reflected a range of rescue: some mice showed substantial improvement, with ABR thresholds as low as 24 dB SPL; others showed intermediate rescue of hearing function, with ABR thresholds as low as 24 dB SPL; and some mice showed little or no rescue of hearing function (see Figure S1B). However, the differences in mean ABR threshold values measured in Spry2−/−; Fgf8+/− versus Spry2−/−; Fgf8+/− mice were statistically significant by two independent tests (p = 0.001, Student’s two-tailed t test; p = 0.0021, Mann-Whitney test). These data demonstrate a positive correlation between rescue of the extra pillar cell phenotype and improvement in auditory function, consistent with the hypothesis that disruption of the cytoarchitecture of the organ of Corti caused by loss of Spry2 function is responsible for the hearing deficit.
Figure 7. Model of Spry2 Function during Organ of Corti Development

(A) Schematic diagram of the oC at the developmental stages indicated, showing stages at which Spry2 function is required.

(B) Diagrams of the oC at P5 (top row) and P10 (bottom row) for the genotypes indicated. At P5, the domains of Fgf8 expression in the IHCs and Spry2 expression in the Deiters’ cells are shaded orange and blue, respectively. Pillar cells are highlighted in purple at both P5 and P10. According to our model, in the wild-type oC the effective range of FGF8 signaling (triangle) is limited by intracellular Spry2 activity in the Deiters’ cells. In the Spry2−/− oC, SPRY2-negative regulation of FGF8 signaling is missing, resulting in a larger effective range of the FGF8 signal. This results in the formation of an extra pillar cell by P10. In the Spry2−/−; Fgf8+/− oC, although SPRY2-mediated negative regulation is absent, less FGF8 ligand is produced by the IHCs (indicated by stippling), and therefore the effective range of FGF8 signaling is decreased. Thus, only the normal two pillar cells are present at P10.

Discussion

The ability to hear depends upon normal form and function of the organ of Corti. We report here that Spry2−/− mice suffer from severe congenital hearing loss (Figure 2), and we have identified two aspects of oC development that are dependent on Spry2 function (Figure 7A). First, Spry2 is required to prevent the formation of excess OHCs, their underlying Deiters’ cells, and the nearby Claudius’ cells. Second, Spry2 is required to prevent a Deiters’ cell from undergoing a cell fate conversion into a pillar cell in the early postnatal period. The effect of loss of Spry2 function on pillar cell number and hearing can be at least partially rescued by reducing Fgf8 gene dosage, indicating that Spry2 normally functions to prevent this cell fate conversion by antagonizing FGF8 signaling.

SPRY2 Prevents the Formation of Excess Outer Hair Cells and Supporting Cells

In many cross-sections of Spry2 mutant cochlea, in addition to the normal three OHCs, each with an underlying Deiters’ cell, we observed a fourth OHC/DC pair as well as more Claudius’ cells than normal (Figures 3I–3L). No effects on these cells were detected other than this regional increase in number, as judged by actin, tubulin, and p75 staining and by morphology in histological sections. Furthermore, the extra OHCs develop morphologically normal stereocilia. Together, these data indicate that Spry2 is required to prevent the formation of excess OHCs, Deiters’ cells, and Claudius’ cells, but does not function to regulate their differentiation.

During normal development, cells in the prospective oC region undergo a round of terminal mitosis that progresses in a wave starting at the apex of the cochlea at E11.5 and finishing at the base by E15.5 (Ruben, 1967), after which cellular differentiation commences (Lim and Annilko, 1985; Sher, 1971). It seems likely that Spry2 function plays a role sometime between E11.5 and E15.5 in determining the numbers of OHC, Deiters’ cell, and Claudius’ cell progenitors that form (Figure 7A). One or a combination of mechanisms could explain how Spry2 functions in this context, including regulation of the time at which the progenitor cells exit mitosis. However, since there are normal numbers of IHCs and pillar cells in newborn Spry2 mutants, there must be some mechanism whereby loss of Spry2 function affects only OHC, Deiters’ cell, and Claudius’ cell progenitors. In addition, at birth, OHC and Deiters’ cell numbers always match in the Spry2 mutants. Thus, it seems that the mechanism(s) that ensure proper OHC/DC pairing is still intact in the absence of SPRY2.
SPRY2 Prevents Deiters’ Cells from Taking on a Pillar Cell Fate

Our data show that in the absence of Spry2 function, sometime between P5 and P10 a Deiters’ cell, which cups an OHC and sends a thin process to the luminal surface, transforms into a pillar cell, which extends a thick process to the luminal surface. Importantly, this cell fate transformation occurs in every row of the oC, irrespective of whether or not a fourth OHC/DC pair formed as an earlier consequence of loss of Spry2 function. Thus, the effect of loss of Spry2 function on pillar cell formation is independent of its effect on OHC/DC numbers. The finding that a Deiters’ cell can transform into a pillar cell after P5 indicates that once specified during embryogenesis, Deiters’ cells are not committed to a Deiters’ cell fate. Rather, cell fates remain plastic and cell fate information must be continuously provided over the course of oC maturation. Consistent with this idea, FGFR3 signaling is continuously required for pillar cell differentiation in cochlear explant cultures (Mueller et al., 2002). Our conclusion that Spry2 is required to prevent a cell fate transformation in the oC is consistent with observations in Drosophila that sprouty functions as a regulator of cell fate in multiple developmental settings (Casci et al., 1999; Hacohen et al., 1998; Kramer et al., 1999; Reich et al., 1999). Thus, it appears that this function of sprouty has been conserved through evolution.

We detected expression of another Sprouty family member, Spry1, in the developing oC. However, in contrast to Spry2, which is initially broadly expressed in oC progenitor cells and then becomes enriched in the Deiters’ cells by P5, Spry1 appears to mark only pillar cells from very early stages (Figure 5). Preliminary data suggest that Spry1 null mice (Basson et al., 2005) are not hearing impaired (not shown), but analysis of double mutants will be required to determine if Spry1 and Spry2 are functionally redundant in pillar cells, where they are coexpressed.

Spry2 Is a Negative Regulator of FGF Signaling in the Organ of Corti

Sprouty was first identified in Drosophila as an antagonist of FGFR signaling during tracheal branching (Hacohen et al., 1998), and most experiments assaying the effects of Sprouty gain-of-function in vertebrate embryos have produced phenotypes consistent with roles as FGF antagonists (Farhauer et al., 2002; Maillaux et al., 2001; Minowada et al., 1999; Nutt et al., 2001; Perl et al., 2003). However, genetic analysis in Drosophila revealed that sprouty also inhibits signaling via other RTKs (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999), and the only Sprouty gene knockout in mice studied thus far showed that SPRY1 inhibits the GDNF/RET signaling pathway during kidney development (Basson et al., 2005). In contrast, our data showing that the Spry2 loss-of-function phenotype can be rescued by decreasing Fgf8 gene dosage (Figure 6) indicate that Spry2 function antagonizes FGF signaling in the oC. Specifically, we propose that FGF8, which is produced in the IHCs, promotes pillar cell differentiation, and SPRY2, produced in the Deiters’ cells adjacent to the pillar cells, antagonizes FGF8 signaling and thus prevents a Deiters’ cell from taking on a pillar cell fate (Figure 7B).

There is substantial evidence that FGFR3 is the receptor that transduces the FGF8 signal for pillar cell differentiation. First, Fgfr3 is expressed in cells that normally develop into pillar cells (Pirvola et al., 2002), as well as in the Deiters’ cell that changes its fate in the Spry2 null mutants (Figure 5G). Second, pillar cells fail to develop in both Fgfr3−/− mice (Colvin et al., 1996) and mice in which Fgf8 has been inactivated in inner ear epithelium (U. Pirvola and A. Neubuser; B. Jacques, M. Lewandoski, and M. Kelley, personal communication). However, we found that reducing Fgfr3 gene dosage had no effect on the Spry2 null phenotype in the oC: Spry2−/−;Fgfr3−/− animals showed no rescue of either the hearing deficit or the excess pillar cell phenotype (data not shown), whereas both defects were partially rescued in Spry2−/−;Fgf8+/− mice. Perhaps animals are more sensitive to Fgf8 than Fgfr3 gene dosage because FGF8 ligand may be limiting, whereas the normal number of FGFR3 receptors may be so large that a reduction by roughly half still leaves sufficient numbers of receptors available in Fgfr3 heterozygotes to function at wild-type capacity. Alternatively, other FGFRs may function either redundantly or instead of FGFR3 to transduce the FGF8 signal in Spry2 null Deiters’ cells.

In both Drosophila and vertebrates, Sprouty gene expression is induced by the signaling pathway that it inhibits (reviewed by Dikic and Giordano, 2003; Guy et al., 2003; Kim and Bar-Sagi, 2004). In particular, Fgf8 expression has been found to be both necessary and sufficient to induce Spry2 expression in mouse embryos (Minowada et al., 1999), although it has yet to be determined whether this is the case in the oC. However, there is no simple relationship between FGF8 signaling and Spry2 expression since, at P5, when Fgf8 is expressed in only the IHCs, Spry2 expression is strongest in the Deiters’ cells, which are two and more cell diameters away. If Spry2 gene expression is entirely controlled by FGF8 signaling at this stage, some other factor must ensure that Spry2 expression is downregulated in the pillar cells closest to the FGF8 source. Spry1, expressed in pillar cells, is a candidate for such a factor.

Although SPRY2 antagonizes Fgf8 signaling, presumably via FGFR3, to prevent Deiters’ cells from undergoing a fate transformation to a pillar cell, its role in preventing the formation of excess OHCs, Deiters’ cells, and Claudius’ cells may be performed via an antagonistic effect on FGFR1 signaling activated by an as yet unknown FGF. Consistent with this hypothesis, loss of Spry2 function and loss of Fgfr1 function in the inner ear have opposite effects. Whereas Spry2 mutants have excess OHCs, Fgfr1 mutants show a dramatic reduction in hair cell number, with OHCs preferentially affected (Pirvola et al., 2002). The reduction in hair cells observed in Fgfr1 mutants correlates with reduced cell proliferation in the developing oC, suggesting that if SPRY2 does antagonize FGFR1 signaling, it may function to prevent excess cell proliferation. Although the data discussed here support the hypothesis that SPRY2 antagonizes FGF signaling, there is as yet no reason to think that this is the only signaling system it
Spry2 Is a Mouse Deafness Locus
What causes the hearing impairment observed in Spry2−/− mice? It is unlikely that the extra OHCs, Deiters’ cells, and Claudius’ cells contribute significantly to the observed hearing loss because extra OHC/DC pairs are observed at a low frequency in wild-type animals with presumably normal hearing (Mu et al., 1997). Furthermore, we found that decreasing Fgf8 gene dosage in Spry2−/− mice did not rescue the extra OHC/DC phenotype, even in mice in which hearing was rescued to a nearly normal level. This indicates that the increased numbers of OHC/DC pairs do not prevent normal hearing.

Although we cannot exclude the possibility that there are defects in the Spry2 mutants that were missed in our analysis, the presence of an extra pillar cell in virtually every transverse row in the Spry2 mutants provides a plausible explanation for their hearing deficit. During hearing, the mechanical vibrations of the middle ear ossicles ultimately cause displacement of the basilar membrane toward and away from the scala media. In the region(s) along the length of cochlea with the largest displacement, the stereocilia of the OHCs deflect in the excitatory direction. This results in contraction of the OHCs at just the right moment so as to amplify the displacement of the basilar membrane (Ashmore, 1987; Nilsen and Russell, 1999). This amplification by OHCs is essential for normal hearing, since Prestin knockout mice, in which OHC contraction cannot occur, suffer from a 40–60 dB hearing loss (Liber et al., 2002) comparable to that in Spry2 null mutants. It is known that very little relative displacement of the basilar membrane occurs underneath the outer pillar cell, which behaves as an inflexible strut, counteracting OHC contraction (Nilsen and Russell, 1999; Olson and Mountain, 1994; Tolomeo and Holley, 1997). In Spry2 null mutants, the presence of an extra pillar cell may further stiffen the cochlear partition, negating amplification by OHCs. In Fgfr3−/− mice, which also are severely hearing impaired, the lack of pillar cells may render the cochlear partition too flexible, preventing optimal shear of hair cell stereocilia against the tectorial membrane. Tests of this model will require direct biophysical measurements of displacement of the basilar membrane in Spry2 and Fgfr3 null mice.

In summary, our data show that Spry2 is required for normal hearing, and that Spry2 together with Fgfr3 comprises a class of mouse deafness genes in which the primary defect appears to involve effects on supporting rather than sensory cells in the OHC. Further studies of the dramatic and novel perturbations in the cytoarchitecture of OHC in Spry2 null mutant mice and their relationship to severe hearing loss should help to determine how supporting cells provide a cytoarchitectural scaffold whose physical properties orchestrate the ability of hair cells to function.

Experimental Procedures
Generation of Spry2 Mutant Mice
The targeting vector used to produce the Spry2neo−/− allele in ES cells was constructed by using ~5.5 kb of Spry2 genomic DNA isolated from a BAC strain 129/Ola ES cell library (Genome Systems, St. Louis), and was electroporated into E14tg2a.4 ES cells, derived from the 129/Ola strain (Hooper et al., 1987). Of ~70 ES cell clones assayed by Southern blotting, four were found to be correctly targeted. However, a PCR analysis revealed that three of these clones did not contain the loxP site downstream of the Spry2 open reading frame. Germline transmission of the Spry2neo−/− allele was obtained following injection into blastocysts (performed by the Stanford University Transgenic Research Facility) of the one homologous recombinant ES cell line that contained both loxP sites. Lines carrying the Spry2neo−/− conditional and Spry2LOXP null alleles were derived by crossing mice carrying Spry2neo−/− to Flip- and Cre-expressing mice, as described in the legend to Figure 1. We currently maintain the Spry2 null allele on both a mixed (FvB/ N; C57/B16; 129sv; Swiss black) and a C57BL/6J genetic background (n = 8 at present). The mutant animals described here were all of mixed genetic background, since most Spry2 null mutants on the inbred background die at birth. The sequences of primers illustrated in Figure 1 and conditions used for genotyping are given in Table S3.

Measurement of Hearing
ABR tests were conducted as described (Jero et al., 2001). Click stimuli were decreased in 5 dB steps, from 90-0 to 70-0 dB peak SPL. Threshold was identified as the midpoint between the stimulus intensity that produced a replicable waveform and at which the waveform could not be reproducibly detected. All procedures were performed in accordance with the regulations of the UCSF Animal Care Committee.

Bone and Cartilage Stain and Paint Filling
Middle ear bones were prepared and stained with Alcian blue and Safranin O, with cartilage stained with Alizarin red according to standard protocols. Paint-fill injections were performed as described (Martin and Swanson, 1993), except that a 1% solution of Correction Fluid in methyl salicylate was injected instead of paint.

Scanning Electron Microscopy
Cochlea from three Spry2−/− mice and two Spry2+/− littermate controls were dissected on P21 and fixed in 2.5% glutaraldehyde, 0.1 M phosphate buffer. The osmium tetroxide-thiocarbohydrazide (OTOTO) procedure was used to stain the cochlea prior to critical point drying and sputter coating with gold as described in Self et al., 1996. Specimens were examined on a Hitachi S-5000 field emission microscope.

Immunohistochimistry
Inner ears were dissected in phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde (PFA). After embedding in 4% agarose, 100 μm vibratome sections were cut through the modulus of the cochlea to expose the OHC. Sections were blocked in PBTr (PBS, 1% bovine serum albumin, 0.1% Triton X-100, 5% normal goat serum) and incubated in primary antibody overnight. After washing in PBT (PBS, 1% BSA, 0.1% Triton X-100), sections were incubated overnight with the appropriate fluorescently labeled secondary antibody (Jackson Immunoresearch) diluted in PBTr, then washed in PBT prior to mounting in Vectashield (Vector Laboratories). Samples were examined on either a Leica TCS NT or a BioRad 1024 MP confocal microscope. Primary antibodies at the dilutions indicated were against: p75 nerve growth factor receptor (Chemicon, 1:1000); α-tubulin (clone DM1A, Sigma, 1:500); S100 (DakoCytomation, 1:200); neurofilament (clone 2D3, Developmental Studies Hybriddoma Bank, 1:200); Rhodamine phalloidin (Molecular Probes) was used at 1:50 dilution. Nuclei were counterstained with TO-PRO-3 (far red) or YO-PRO-1 (green) (Molecular Probes).

In Situ Hybridization
Inner ears dissected from CD1 embryos or pups were fixed in 4% PFA, embedded in OCT, and cryo-sectioned through the modulus to cross-section the OHC. RNA in situ hybridization was performed on cryo-sectioned tissue as described on the Dr. Alexandra Joyner
lab website by using mouse probes for Spry2, Spry1, Fgf8, and Fgfr3.

Pillar Cell Quantification
Inner ears fixed in 4% PFA were decalcified in 10% EDTA, samples were embedded in 4% agarose, and 100 μm vibratome sections were cut in the transverse plane through the entire cochlea. Sections were processed for 100 staining and examined by confocal microscopy as described above. For each OC in which pillar cells could be clearly visualized, a z-stack was compiled with a total thickness of 50 μm and a step size of 2.5 μm or smaller. Pillar cell and hair cell numbers were determined from each 50 μm stack. Multiple 50 μm samplings, scattered along the length of the cochlea, were measured. For each cochlea, between 300 and 450 μm of total distance along its length was examined.

Supplemental Data
Supplemental Data showing hair and pillar cell quantification, ABR wave i and wave ii peak amplitudes, PCR primer information, and histological sections are available at http://www.developmentalcell.com/cgi/content/full/8/4/553/DC1/.

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