Conditional deletion of Atoh1 using Pax2-Cre results in viable mice without differentiated cochlear hair cells that have lost most of the organ of Corti

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1. Introduction

Proneural basic Helix–Loop–Helix (bHLH) genes have been known for thirty years as mediators of sensory cell differentiation (Ghysen and Richelle, 1979; Ghysen and Dambly-Chaudiere, 2000). It has been known for over ten years that loss of the bHLH genes Atoh1 (formerly Math1) and Neurog1 (formerly Ngn1) eliminates hair cell and neuron differentiation in the mouse ear, respectively (Bermingham et al., 1999; Ma et al., 1998). Further work showed that expression of Atoh1 in tissue culture (Zheng and Gao, 2000), embryonic ears (Gubbels et al., 2008), sensory ganglia (Jahan et al., 2010a) and even adult ears (Izumikawa et al., 2005; Kawamoto et al., 2003; Praetorius et al., 2010) can generate extra hair cells, leading to the perception that Atoh1 is both necessary and sufficient to drive hair cell differentiation in the ear (Kelley, 2006). While compelling based on this evidence, this conclusion nevertheless cannot be fully reconciled with some data.

For example, while early work showed that many hair cell precursors die in Atoh1 null mice (Chen et al., 2002), follow-up work revealed that at least some organ of Corti cells survive and continue to express Atoh1-lacZ at least until birth, the latest stage this null mutant could be analyzed (Fritzsch et al., 2005b). These cells have been identified already in the initial report (Bermingham et al., 1999) and have been variously referred to as ‘supporting cells’ or ‘hair cell precursors’. More perplexing is a report that indicates
that hair cells can form without Atoh1 if surrounded by Atoh1 expressing hair cells in chimaeric mice (Du et al., 2007). It was also shown that the prosensory domain that gives rise to hair cells is delineated much earlier by other markers such as certain neurotrophins (Farinas et al., 2001), transcription factors such as Sox2 (Kiernan et al., 2005), Gata3 (Karis et al., 2001; Lawoko-Kerali et al., 2004) and Eyal (Zou et al., 2008), supporting cell markers such as Prox1 (Bermingham-McDonogh et al., 2006; Fritzsch et al., 2010b) and several Fgf’s and their receptors (Hayashi et al., 2008; Pirvola et al., 2000), cyclin kinase inhibitors (Doetzlhofer et al., 2009) and members of the Delta/Notch signaling family (Doetzlhofer et al., 2009; Kiernan et al., 2006). In addition, Sox2 is known to be essential for this process and its absence leads to lack of hair cell formation (Kiernan et al., 2005). Furthermore, Prox1, Jag1 and Sox2 are at least partially retained in Atoh1 null mice (Dabdoub et al., 2008). This indicates that molecules associated with sensory precursor and supporting cell definition and differentiation can remain expressed without Atoh1 mediated regulation of the Delta/Notch lateral inhibition system (Doetzlhofer et al., 2009; Kageyama et al., 2009). Together these data suggest the possibility for a more sophisticated molecular interaction of Atoh1 during hair cell differentiation. Most importantly, if expressions of at least some of these genes are retained after hair cell loss, they could be of profound translational use for future therapies aiming to reconstitute the organ of Corti. Such genes could provide the molecular means to direct differentiation only in the organ of Corti precisely at the right position on the basilar membrane.

In order to understand how long such gene expression persists in the absence of hair cell differentiation, we bred a Tg(Pax2-cre) line (Ohyama and Groves, 2004) with a recently available floxed Atoh1 line (Maricich et al., 2009). In the Tg(Pax2-cre)::Atoh1fl/fl, we achieved near complete and early ear specific deletion of Atoh1 as evidenced by Atoh1 in situ hybridization. Only some cells in the posterior canal crista, which were positive for Atoh1 because of incomplete recombination, developed Myo7a expression and turned into histologically recognizable hair cells. There were no Atoh1 positive cells in the cochlea at any time and we demonstrated that most cells of the organ of Corti degenerate in late embryos. Nevertheless, some remaining organ of Corti cells become Myo7a positive in particular in older postnatal mice. A ‘flat’ epithelium, instead of an organ of Corti, forms that expresses Gata3, a transcription factor essential for normal ear and hearing development (Karis et al., 2001; Van Esch and Devriendt, 2001). The pattern of innervation of the Atoh1 conditional knockout (KO) was comparable to the control littermate at embryonic day 14.5 (E14.5) and to Atoh1 systemic null mice at E18.5/P0 (Fritzsch et al., 2005b), but showed interesting focal projections to spotty Sox2 expressing domains and Myo7a positive cells in neonates. These genetically engineered mice without differentiated hair cells can be used with Atoh1 containing viruses to test the window of opportunity during which Atoh1 expression can still induce the full differentiation and maintenance of hair cells out of these flat epithelia.

2. Material and methods

2.1. Mice and genotyping

All animal procedures were approved by the University of Iowa Animal Care and Use Committee (IACUC) guidelines for the use of laboratory animals in biological research (ACURF #0804066).

We bred mice carrying the Pax2-cre transgene (Ohyama and Groves, 2004) with mice carrying floxed Atoh1 (Maricich et al., 2009; Shroyer et al., 2007), resulting in conditional knockout mice with the genotype Tg(Pax2-cre)::Atoh1fl/fl at the expected Mendelian ratio. The littersmates with the genotype of Tg(Pax2-cre)::Atoh1fl/+ or Atoh1fl/ were used as controls. Offspring were genotyped by PCR analysis of the tail DNA using Cre-specific primers (forward: 5’-CCT GTT TGG CAC GTT CGC-3’ and reverse: 5’-ATG CTG TCT CCC GGC GTC CC-3’) which produced a ~280 bp product, and Atoh1 specific primers (forward: 5’-AGC GAT GAT GGC ACA GAA G-3’ and reverse: 5’-GAA GTC AGG TCG TTA CAT AC-3’) which produced a ~300 bp product from wild-type Atoh1 coding region and a ~500 bp product from the floxed allele.

All mice were perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) using a peristaltic pump following a lethal dose of Avertin anesthesia (1.25% of 2,2,2-tribromoethanol at a dose of 0.025 ml/g of body weight). Heads were isolated and fixed in 4% PFA at least for 24 h before the ears were dissected in 0.4% PFA for further processing.

2.2. In situ hybridization

In situ hybridization was performed using an RNA probe labeled with digoxigenin. The plasmids containing the cDNAs were used to generate the RNA probe by in vitro transcription. The dissected ears were dehydrated in 100% methanol and rehydrated in a graded methanol series and then digested briefly with 20 μg/ml of Proteinase K (Amersham, Austin, TX, USA) for 15–20 min. Then the samples were hybridized overnight at 60 °C to the riboprobe in hybridization solution containing 50% (v/v) formamide, 50% (v/v) 2× saline sodium citrate and 6% (w/v) dextran sulphate. After washing off the unbound probe, the samples were incubated overnight with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany). After a series of washes, the samples were reacted with nitroblue phosphate/5-bromo, 4-chloro, 3-indolil phosphate (BM purple substrate, Roche Diagnostics GmbH, Mannheim, Germany), which is enzymatically converted to a purple colored product. The ears were mounted flat in glycerol and viewed in a Nikon Eclipse 800 microscope using differential interference contrast microscopy and images were captured with Metamorph software.

For sections, the reacted ears were embedded in resin, sectioned (see below) and imaged with a Nikon E800 microscope.

2.3. Lipophilic dye tracing

The heads of the mice were cut sagittally at the midline and two different colored lipophilic dye-soaked filter strips (Fritzsch et al., 2005a; Tonniges et al., 2010) were inserted approximately into the alar plate of rhombomere 2 and the basal plate of rhombomere 4 to label the afferent and efferent fibers to the inner ear, respectively. Half heads were kept in 60 °C oven for about 24 h for proper diffusion. Then the ears were dissected out for analysis and images were taken with a Leica TCS SP5 confocal microscope.

2.4. Immunocytochemistry and apoptotic cell labeling

The ears were dehydrated in 100% ethanol and after rehydration with graded ethanol and PBS the samples were blocked with 2.5% normal goat serum in PBS containing 0.25% Triton-X-100 for 1 h. Then the primary antibodies for activated Caspase3 (1:100; Cell Signaling, 8661), Myo7a (1:200; Proteus Biosciences, 25-6790), Prox1 (1:500; Covance, PRB-238C), Sox2 (1:200; Millipore, AB5603), and Tubulin (1:800; Sigma, T7451) were added and incubated for 48 h at 4 °C. After several washes with PBS, corresponding secondary antibodies (1:500; Alexa fluor molecular probe 647, 532, or 488; Invitrogen) were added and incubated overnight at 4 °C. The PSVue480 (10 μM; Molecular Targeting Technologies, Inc., P-1003) and Hoechst (5 μg/ml; Polysciences, Inc., 90460) dyes were then added and incubated for 15 min at room temperature.
Fig. 1. Conditional deletion of Atoh1 is early, almost complete, and affects other bHLH genes but not early neuronal development. (A, B) The Pax2-Cre mediated recombination of floxed Atoh1 leads to reduction and loss of Atoh1 expression as shown by in situ hybridization. In E13.5 control mice, Atoh1 is expressed in all five vestibular endorgans with limited expression in the cochlea (A). In Atoh1 CKO mice, only some weak expression persists in the posterior crista and the anterior crista at this stage (B). (C–H) Absence of Atoh1 leads to expression changes in other bHLH genes. At E13.5 Neurog1 shows a wider expression in the utricle and saccule of Atoh1 CKO mice (D) compared to their control littermates (C). At E14.5, while Neurog1 is drastically downregulated in the control mice (G), it continues to be expressed in the CKO mice (H). An expanded expression is also seen for Neurod1 in the mutant ear at E14.5 (E, F). (I–J) Nhlh2, a neuronal marker shows near normal expression in developing ganglia in CKO mutants. However, the sizes of vestibular ganglia are obviously different: the superior vestibular ganglion is reduced but the inferior vestibular ganglion is enlarged in CKO mutants compared to control littermates (compare the bars in I and J). (K–L) Lipophilic dye tracing shows that both the afferent and the efferent innervation in the Atoh1 CKO mice (L, L’) is indistinguishable from the control littermate (K, K’). AC, anterior canal crista; Co, cochlea; HC, horizontal canal crista; IGSB, intraganglionic spiral bundle; IVG, inferior vestibular ganglion; PC, posterior canal crista; S, saccule; SG, spiral ganglion; SVG, superior vestibular ganglion; U, utricle. Bar indicates 100 μm.
The ears were washed with PBS and mounted in glycerol and images were taken with a Leica TCS SP5 confocal microscope.

2.5. Plastic embedding and Stevenel’s Blue staining

The ears were fixed in 2.5% glutaraldehyde overnight followed by several washes with 0.1 M phosphate buffer and then fixed with 1% osmium tetroxide for 1 h. Samples were then dehydrated in graded ethanol and propylene oxide, embedded with Epon 812 in beam capsules and polymerized at 60°C for at least 24 h. Two μm sections were cut using a Reichert Ultratome and stained at 60°C with Stevenel’s Blue (del Cerro et al., 1980) made of 2% potassium permanganate and 1.3% methylene blue.

2.6. TSLIM

2.6.1. Thin-Sheet Laser Imaging Microscopy (TSLIM)

Fixed three week old CKO and control littermate ears were dissected from the skull. Ears were decalcified with 10% EDTA, dehydrated and cleared with Spalteholz solution (5:3 methyl salicylate:benzyl benzoate). Specimens were stained in Rhodamine B isothiocyanate (1 mg/200 mL in Spalteholz for one day) and imaged with TSLIM at the University of Minnesota (Santi et al., 2009). Whole inner ears were nondestructively, serially sectioned in a mid-modiolar plane at 5 μm thickness. A complete z-stack of optical sections containing the full dimension of the inner ear resulted in about 300 well-registered images using a Retiga 2000 digital camera on TSLIM. Image voxel size was 1.5 × 1.5 × 5 μm. Images were adjusted for brightness and contrast.

2.6.2. 3D reconstruction

The z-stack for each inner ear was loaded into Amira ver. 5.2 (Visage Imaging, San Diego, CA) for 3D reconstruction of inner ear structures. To isolate different inner ear structures and compute their morphometric parameters, a process called segmentation was used. Using Amira’s semi-automated tools, the borders of each structure of interest were outlined in a different color in every section of the stack. By our definition the scala media was outlined to include the endolympathic space within the scala but it did not include the tectorial membrane or the inner sulcus. After segmentation, Amira provided isosurface volume reconstructions of individual inner ear structures as well as an estimate of their volume based on voxel size. Structure centroids were determined by the centerline tree module in Amira. To compute the spiral length of each structure, a smooth B-spline curve was fit computed from each structure’s centroid. A set (99 sections) of virtual orthogonal sections were generated by Amira along the length of the scala media using its centroid as a reference. From these orthogonal sections area of the scala and width of the basilar membrane were computed.

3. Results

3.1. Tg(Pax2-Cre) led to early and near complete loss of Atoh1 expression

We first wanted to establish that in the CKO mice the recombination of the floxed Atoh1 gene was complete and happened prior to Atoh1 expression, which starts in wild-type mice around E11 in the vestibular sensory epithelia and around E13.5 in the cochlea (Matei et al., 2005). We checked the expression of Atoh1 with in situ hybridization assuming that levels of Atoh1 mRNA that cannot be detected with this technique have little to no effect on hair cell differentiation. We could not detect even traces of Atoh1 mRNA in almost all sensory epithelia with this approach as early as E13.5 (Fig. 1B) except for limited expression in the posterior canal cristae.

Fig. 2. Loss of Atoh1 causes failure of hair cell differentiation and cell death of organ of Corti precursors and neurons. (A–B) Atoh1 in situ hybridization at P0 confirms the deletion of Atoh1 in almost all the hair cells of the mutant ear. The control ear has strong Atoh1 signal in almost all the hair cells of the mutant ear. The black patches in anterior canal cristae, posterior canal cristae, utricle and saccule are the pigments but not Atoh1 signal, which shows dark purple color. (C–F) Myo7a immunochemistry shows complete loss of hair cells in the mutant cochlea. Consistent with the remaining Atoh1 expression, a few Myo7a positive hair cells are present in the posterior canal cristae (F). (G–I) Absence of Atoh1 causes cell death of neurons and cells in the organ of Corti. In E18.5 mutant, activated Caspase3 positive cells are seen in spiral ganglion (G) and the region topographically equivalent to the organ of Corti (H). Degeneration of these cells is further evidenced by Hoechst staining of the pyknotic nuclei (H'). In E16.5 mutant, we find a streak of cells in the organ of Corti that are labeled by PSVue, which stains phosphatidylserine exposed on the cell membranes after the onset of apoptotic cell death (I).
and occasionally in the anterior canal crista (Fig. 1B), likely reflecting directional regulation of the Pax2-cre transgene on the heterogeneous genetic background generated with the crossing of these two lines. It was previously shown that genes expressed in the posterior canal may not be properly recombined with Pax2-cre (Soukup et al., 2009) and our data on Tg(Pax2-cre)::Atoh1CKO concur with this suggestion. Thus the posterior canal crista with some remaining Atoh1 expression can serve as an internal control for the effects of the successful elimination of Atoh1 in other epithelia. Previous work has shown massive hair cell reduction in Neurog1 null mice, in particular in the saccule, (Ma et al., 2000; Matei et al., 2005) possibly due to cross-regulation of Neurog1 and Atoh1 (Raft et al., 2007). Recent work suggested this cross-regulation may be in part mediated by another bHLH gene Neurod1 (Jahan et al., 2010a). Therefore we investigated the expression of Neurog1 and Neurod1 in Atoh1 CKO mice using in situ hybridization. Our data showed expanded expression of Neurog1 in the utricle and saccule at E13.5 and E14.5 (Fig. 1D, F, H) and also of Neurod1 at E14.5 (Fig. 1F), consistent with the cross-regulation of Neurog1 and Neurod1 by Atoh1. The expanded expression of Neurog1 and Neurod1 (Fig. 1D, F, H) resulted in reduction of the superior vestibular ganglion and expansion of the inferior vestibular ganglion, but did not result in any obvious change in reduction of the superior vestibular ganglion and expansion of the cochlea nerve (Fig. 2F). The cochlea in the CKO mutant went through near normal full extension (Table 1) without differentiation of any hair cells (Fig. 2D). It was previously shown in Atoh1 null mice that some undifferentiated cells survive and continue to express Atoh1-lacZ until E18.5, the latest stage these mice can be analyzed (Bermingham et al., 1999; Fritzsch et al., 2005b). Another study showed that many cells in the sensory epithelium, including supporting cells, undergo apoptosis in the Atoh1 null mutant ear, but this was not quantified (Chen et al., 2002). To further evaluate the magnitude of cell death, we performed whole mount immunocytochemistry of activated Caspase3 to directly examine the apoptotic cells in Atoh1 CKO ear. At E18.5, the control cochlea had very few apoptotic cells (Fig. S1A). In contrast, the Atoh1 CKO mutant cochlea had substantially more apoptotic cells in the spiral ganglion (Figs. 2G and S1B). This suggests extensive neuronal death in the absence of hair cell differentiation. We also observed some apoptotic cells in the area comparable to the organ of Corti (Figs. 2H, 2H′ and S1B). We further examined the cell death using a newly developed dye PSVue, which specifically binds to phosphatidylserine (PS) exposed on the membranes of dying cells (Krijnen et al., 2010). At E16.5, we observed a streak of PSVue positive cells in the upper middle part of the topographical organ of Corti in the mutant cochlea (Fig. 2I), but fewer in the base and none in the apex. Anti-activated Caspase3 antibody also detected dying cells with a relatively larger cell body that were rarely co-localized with PSVue (Fig. 2I). These results are in agreement with the suggested cell death progression in which the activation of Caspase3 is followed by the loss of PS flipase and the appearance of phosphatidylserine on the cell surface. These data suggest a prolonged and extensive apoptosis of cells near the lumen as well as near the basilar membrane (Fig. 2I′), possibly indicating cell death of both undifferentiated hair cells and supporting cells in the organ of Corti.

### Table 1

| Atoh1 CKO mice had near normal cochlear extension, but had no recognizable organ of Corti, narrowed basilar membrane and greatly reduced spiral ganglion. |
|-----------------|------------------|------------------|
| **Control** | **Atoh1 CKO** | **% Of reduction** |
| Length of cochlea (μm) | 5338 ± 341  | 5045 ± 374  | 5.5 |
| Number of efferent fibers | 475  | 44  | 91 |
| Number of nerve fibers in the cochlea nerve | 10000  | 547  | 95 |
| Length of basilar membrane (μm) | 6436  | 5861  | 8.9 |
| Average width of basilar membrane (μm) | 127.6  | 58.65  | 54 |
| Volume of basilar membrane (nL) | 9.980  | 4.488  | 55 |
| Volume of organ of Corti (nL) | 12.99  | 0th | 100 |
| Volume of Rosenthal's canal (nL) | 52.35  | 5.197  | 90 |
| Volume of tectorial membrane (nL) | 14.0  | 6.172  | 56 |
| Volume of scala media (nL) | 274.5  | 229.8  | 16 |
| Volume of scala vestibuli (nL) | 478.1  | 450.5  | 5.8 |
| Volume of scala tympani (nL) | 485.2  | 465.4  | 4.1 |

* Cochlea length was measured in whole mount preparation.

n = 3, shown here is average length ± S.D.

n = 3, shown here is average width ± S.D.

* Campbell and Hennon, 1988.


** Basilar membrane width was measured manually in orthogonal sections every 5% of scala media length (5–95%) and the average width is shown.

* No recognizable organ of Corti was found in Atoh1 CKO mutant ear.

3.2. Conditional deletion of Atoh1 resulted in near complete absence of differentiated hair cells in a near normal ear

Unlike the Atoh1 systemic null mice, the Atoh1 CKO neonates did not appear to have any breathing difficulty at birth. Although the mutant mice had lower body weight compared to their control littermates (data not shown), some can survive for about three weeks but died within a few days after weaning. The oldest Atoh1 CKO mice obtained thus far were kept with their mothers past littermates (data not shown), some can survive for about three weeks. In Atoh1 CKO neonates, we confirmed that Atoh1 was eliminated in all sensory epithelia except the posterior canal crista (Fig. 2B, B′), which had only a few hair cells as shown by Myo7a immunocytochemistry (Fig. 2F). The cochlea in the CKO mutant went through near normal full extension (Table 1) without differentiation of any hair cells (Fig. 2D). It was previously shown in Atoh1 null mice that some undifferentiated cells survive and continue to express Atoh1-lacZ until E18.5, the latest stage these mice can be analyzed (Bermingham et al., 1999; Fritzsch et al., 2005b). Another study showed that many cells in the sensory epithelium, including supporting cells, undergo apoptosis in the Atoh1 null mutant ear, but this was not quantified (Chen et al., 2002). To further evaluate the magnitude of cell death, we performed whole mount immunocytochemistry of activated Caspase3 to directly examine the apoptotic cells in Atoh1 CKO ear. At E18.5, the control cochlea had very few apoptotic cells (Fig. S1A). In contrast, the Atoh1 CKO mutant cochlea had substantially more apoptotic cells in the spiral ganglion (Figs. 2G and S1B). This suggests extensive neuronal death in the absence of hair cell differentiation. We also observed some apoptotic cells in the area comparable to the organ of Corti (Figs. 2H, 2H′ and S1B). We further examined the cell death using a newly developed dye PSVue, which specifically binds to phosphatidylserine (PS) exposed on the membranes of dying cells (Krijnen et al., 2010). At E16.5, we observed a streak of PSVue positive cells in the upper middle part of the topographical organ of Corti in the mutant cochlea (Fig. 2I), but fewer in the base and none in the apex. Anti-activated Caspase3 antibody also detected dying cells with a relatively larger cell body that were rarely co-localized with PSVue (Fig. 2I). These results are in agreement with the suggested cell death progression in which the activation of Caspase3 is followed by the loss of PS flipase and the appearance of phosphatidylserine on the cell surface. These data suggest a prolonged and extensive apoptosis of cells near the lumen as well as near the basilar membrane (Fig. 2I′), possibly indicating cell death of both undifferentiated hair cells and supporting cells in the organ of Corti.
a Hensen’s and Claudius’ cell-like appearance (Fig. 3G, G'). These ears had a well-developed spiral limbus with a tectorial membrane extending toward the undifferentiated area of the organ of Corti (Fig. 3C, E, E', G, G') comparable to the control littermate (Fig. 3A, A'). Sections through the vestibular sensory epithelia showed some hair cells only in the posterior canal crista (Fig. 3J'), consistent with our data on Atoh1 and Myo7a expression (Figs. 1B and 2B, B', F). However, other areas of the same crista and the other vestibular sensory organs were completely devoid of differentiated hair cells and an undifferentiated single cell layered epithelium was found (Fig. 3J). These data demonstrate the near normal development of the ear with topologically arranged sensory organs and acellular covering structures without formation of differentiated hair cells and supporting cells in most of the sensory organs, except for the posterior canal crista.

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**Fig. 3.** Conditional deletion of Atoh1 generates a flat epithelium and shows long-term retention of some spiral neurons in neonates. (A–H') Thin plastic sections through cochlea show histological changes of P14 CKO ear. The control mice form the spiral limbus and sulcus, the tectorial membrane and the organ of Corti on the basilar membrane (A, A'). While the basilar membrane is progressively shorter toward the base, there is no alteration in the flat epithelium that indicates the position of the organ of Corti through the approximation of the tectorial membrane (E, E', G, G'). Control mice have a large Rosenthal’s canal filled with sensory neurons (B, B'). In CKO mice the apex and the basal tip have clusters of sensory neurons (D, D', H, H'), whereas the middle turn has just a bony matrix or a few isolated neurons surrounded by bone (arrows in F and F'). Note that all bone spaces are filled with neurons in both control and Atoh1 CKO mutants, suggesting that the neuronal cell death phase is not continuing in neonates despite the transformation of the primordial organ of Corti into a flat epithelium in the absence of Atoh1. (I–J') Some hair cells are only found in the posterior canal crista in the mutant ear. Sections through the posterior canal crista of control mice show the large bundle of the posterior canal crista nerve approaching an organ filled with many hair cells that extend apical bundles into the cupula (I, I'). The nerve approaching the posterior canal crista of Atoh1 CKO mice are much smaller and some areas of the crista show no hair cells (J, J', arrow indicates a differentiated hair cell). (K–L') Sections through the cochlear nerve show obvious reduction of the number of nerve fibers in the CKO mutant in comparison to control (See Table 1). BM, basilar membrane; Cne, cochlear nerve; Eff, efferent nerve fibers; IHC, inner hair cell; IP, inner pillar cell; OHC, outer hair cell; OP, outer pillar cell; PC, posterior canal crista; PCN, posterior canal crista nerve; SG, spiral ganglion; SL, spiral limbus; TC, tunnel of Corti; TM, tectorial membrane; Vne, vestibular nerve. Bar indicates 100 μm in all except in A', B', C', D', E', F', G', H', K and L', where it indicates 10 μm.
Fig. 4. TSLIM reveals comparable cochlear development with near identical length of the cochlea in CKO mutant. (A–E) TSLIM 5 μm optical sections through the modiolus plane of the cochlea in P21 control and CKO mice show some reduction of the scalae and apparent radial reduction of basilar membrane. Labeled turns in A and B are orthogonal to scala media at 50% distance from base to apex. A color overlay of two sections at 30% of the length of scala media from control (red) and CKO (green) cochlea shows where structural differences occur (E). The sections are aligned at the lateral end of the basilar membrane (arrow in E) and the narrowing of the mutant basilar membrane is evident. (F–I) 3D reconstructions show that the overall structure of the CKO mutant cochlea is near normal despite the absence of an identifiable differentiated organ of Corti (bright red). The position of remaining Rosenthal’s canal (orange) in the mutant is consistent with the topology of neurons found in plastic sections shown in Fig. 3D, F, H, I. However, the volume reduction of scalae (Fig. 4A, E, H, I; Table 1) and a nearly identical length of the basilar membrane (Fig. 4F, G; Table 1) shows some reduction of about 50% suggesting that the shortening of the radial span of the basilar membrane and its likely effect on the biomechanics of the ear may affect postnatal growth of these essential features for non-cell based sound induced movement.

Spiral ganglia in Rosenthal’s canal were found in Atoh1 CKO mice only near the basal tip and in the apex (Fig. 3D, H). The middle turn had only a few neurons singly casted in bone (Fig. 3F, F’). In contrast to the neuronal cell death induced in postnatal mice, which leaves empty spaces in Rosenthal’s canal (Shibata et al., 2010), we found tightly fit bone space around the neurons, suggesting that little, if any cell death takes place after the initial phase of spiral neuron loss between E14.5 and P0. Close examination showed that the neurons were identical in size with no indication of Type II neurons (Fig. 3D’, F’, H’). With this exception, the overall appearance of the neurons was similar to their control littersmates. Cross sections through the cochlear nerves showed massive reduction of fibers, consistent with the reduction of the neurons (Fig. 3K, K’, L, L’). To estimate the total loss of spiral neurons we counted the number of cochlear fibers as they exited the ear. We observed an over 90% reduction of both the cochlea afferent and efferent fibers in the Atoh1 CKO mutant (Table 1).

We next used Thin-Sheet Laser Imaging Microscopy (TSLIM) to analyze the overall structures of the mutant ear. Our data showed a small reduction in overall volume of the scalae (Fig. 4A–E, H, I; Table 1) and a nearly identical length of the basilar membrane (Fig. 4F, G; Table 1). However, the width of the basilar membrane was reduced (Fig. 4C–G; Table 1) possibly due to the complete absence of a recognizable organ of Corti. Color overlay in Fig. 4E also indicated scalar displacement and minor structural changes in the mutant ear. The basilar and the tectorial membranes had comparable volume reductions of about 50% suggesting that the shortening of the radial span of the basilar membrane and its likely effect on the biomechanics of the ear may affect postnatal growth of these essential features for non-cell based sound induced movement.

3.3. Some innervation remained in the Atoh1 CKO neonates

We next investigated the defects on innervation caused by the complete absence of Atoh1 mediated hair cell differentiation. In agreement with previous work on E18.5 Atoh1 null mice (Fritzsch et al., 2005b), Tubulin immunochemistry showed some innervation to all sensory epithelia at birth (Fig. 5B, B’). However, the volume reduction of the neurons (Fig. 5B, B’). With this exception, the overall appearance of the neurons was similar to their control littersmates. Cross sections through the cochlear nerves showed massive reduction of fibers, consistent with the reduction of the neurons (Fig. 3K, K’, L, L’). To estimate the total loss of spiral neurons we counted the number of cochlear fibers (Fig. 4F, G; Table 1). The volume reduction in vestibular parts such as the canal cristae was also obvious with this approach (Fig. S2A–D).

Previous work has shown the continued presence of Prx1 and Sox2 proteins in the apex of Atoh1 null mice (Dabdoub et al., 2008; Fritzsch et al., 2010a). Using in situ hybridization we showed the
Sox2 mRNA in the CKO cochlea (Fig. 5I, J). Compared to the Sox2 protein expression in the apex of systemic Atoh1 null mice (Dabdoub et al., 2008), Sox2 expression in Atoh1 CKO mice was variable (Fig. 5I), whereas the apex (B') and lower middle turn show some innervation with higher spacing density of radial fibers toward the apex (B, B'). The fibers in the middle turn demonstrate abnormal loop formation near the greater epithelial ridge (B). The vestibular innervation is present in all sensory epithelia but greatly reduced (D, F) in comparison to a control littermate (C, E). (G–L') Expression of Sox2 persists but is reduced in mutant cochlea. Sox2 in situ hybridization reveals a discontinuous or patchy distribution throughout the mutant cochlea (I, J, J'). Combining Tubulin with Sox2 immunohistochemistry reveals Sox2 labeling in supporting cells and prosensory cells with dense innervation in control animals (K–K'). In some CKO mutants Sox2 protein is more restricted and patchy in the apex (L). Nerve fibers are attracted to those patches, branch, and end freely (L'). (M–N') Bdnf expression is greatly reduced in the Atoh1 CKO cochlea. In situ hybridization shows an apex to base gradient of Bdnf expression in the newborn control mice (M, M'), whereas in the mutant ear, Bdnf expression is patchy in the apical half and is lost in the base (N, N'). AC, anterior canal crista; GER, greater epithelial ridge; HC, horizontal canal crista; IGSB, intraganglionic spiral bundle; OC, organ of Corti; 'OC', putative organ of Corti in Atoh1 CKO; PC, posterior canal crista; RF, radial fibers; S, saccule; Sc, supporting cells; SG, spiral ganglion; U, utricle. Bar indicates 100 μm.

In areas of patchy expression of Sox2 protein we found that fibers branched and ended freely (Fig. 5L, L'). Since Sox2 protein is expressed in the supporting cells in wild-type mice at this stage (Dabdoub et al., 2008; Mak et al., 2009), we tested whether the residual expression of Sox2 correlates with the expression of Ntf3, a neurotrophin expressed primarily in the supporting cells of the developing cochlea (Farinas et al., 2001; Pirvola et al., 1992). While we could obtain a signal in control mouse cochlea, there was no detectable signal for Ntf3 in Atoh1 CKO mice (data not shown). We therefore investigated the expression of Bdnf, which was previously shown to be expressed in undifferentiated precursors of Atoh1 null mice (Fritsch et al., 2005b) and predominantly supported the apical spiral ganglia (Farinas et al., 2001). Our data showed a clear apex to base gradient of Bdnf expression in control mice (Fig. 5M). In the Atoh1 CKO mice, Bdnf intensity was greatly reduced in the apex, sometimes not detectable with in situ hybridization and showed a patchy expression pattern (Fig. 5N, N'). We could detect neither Ntf3 nor Bdnf in the basal tip where some innervation remained. These data suggest that some limited expression of sensory precursor/supporting cell specific genes, including Sox2 and Bdnf, is possible in the absence of Atoh1 mediated hair cell differentiation. Most importantly, this residual expression was well correlated with the remaining apical innervation of the cochlea.

We also investigated the efferent innervation using lipophilic dye tracing in P0 animals (Fig. S3A–F). Efferents grow along afferents and loss of afferents is closely followed by alterations in efferents.

**Fig. 5.** The pattern of residual innervation correlates with the distribution of Sox2 and Bdnf in Atoh1 CKO mice. (A–F) Immunolabeling with Tubulin shows the reduction of innervation in P0 Atoh1 CKO mice. Radial fibers are closely spaced in the cochlea of control mice (A). In contrast, the base of the Atoh1 CKO cochlea is almost free of any fibers (B) except for the basal tip (B'), whereas the apex (B') and lower middle turn show some innervation with higher spacing density of radial fibers toward the apex (B, B'). The vestibular innervation is present in all sensory epithelia but greatly reduced (D, F) in comparison to a control littermate (C, E). (G–L') Expression of Sox2 persists but is reduced in mutant cochlea. Sox2 in situ hybridization reveals a discontinuous or patchy distribution throughout the mutant cochlea (I, J, J'). Combining Tubulin with Sox2 immunohistochemistry reveals Sox2 labeling in supporting cells and prosensory cells with dense innervation in control animals (K–K'). In some CKO mutants Sox2 protein is more restricted and patchy in the apex (L). Nerve fibers are attracted to those patches, branch, and end freely (L'). (M–N') Bdnf expression is greatly reduced in the Atoh1 CKO cochlea. In situ hybridization shows an apex to base gradient of Bdnf expression in the newborn control mice (M, M'), whereas in the mutant ear, Bdnf expression is patchy in the apical half and is lost in the base (N, N'). AC, anterior canal crista; GER, greater epithelial ridge; HC, horizontal canal crista; IGSB, intraganglionic spiral bundle; OC, organ of Corti; 'OC', putative organ of Corti in Atoh1 CKO; PC, posterior canal crista; RF, radial fibers; S, saccule; Sc, supporting cells; SG, spiral ganglion; U, utricle. Bar indicates 100 μm.
Compared with the control ear (Fig. S3A, A', C, D), the Atoh1 CKO mutant ear had greatly reduced efferents at birth (Fig. S3B, B', E, F) as previously reported in late embryos in Atoh1 null mice (Fritzsch et al., 2005b). We also investigated the cochlear nucleus projection of spiral afferents. However, this analysis is compromised by a neonatal appearance of Pax2-Cre expression in the hindbrain that eliminates Atoh1 in cochlear nucleus neurons (data not shown). With this caveat in mind we found a reduction of afferents and the cochlear nucleus but also extensive branching of the basal turn afferents to the remaining cochlear nucleus (Fig. S3H, H') comparable to previous data on Ntf3 null mice that have a topographically comparable loss of spiral neurons (Fritzsch et al., 1997).

3.4. Loss of Atoh1 arrested hair cell and supporting cell differentiation

We next wanted to better understand the relationship of various expressions of genes as they correlate with the apparent reduction of the organ of Corti, the residual expression of Sox2, the remaining innervation, and the absence of differentiation of supporting cells. We first investigated the expression of Prox1, a marker for supporting cells, which is important in type II spiral neuron pathfinding (Bermingham-McDonogh et al., 2006; Fritzsch et al., 2010b). Like Sox2, in situ hybridization showed Prox1 expression in patches of two rows of cells in the P0 Atoh1 CKO cochlea instead of the five rows of supporting cells found in control (Fig. 6A, A', C, C'). The apical tip of the cochlea showed more continuous expression of Prox1 (Fig. 6C), consistent with Sox2 expression (Fig. 5I) implying that the reduction to two rows of Prox1 positive cells or their complete loss is secondary to the initial upregulation. Immunochemistry for Prox1 revealed the previously reported labeling of two pillar and three Deiter's cells in P7 control mice (Fig. 6B) but showed only very few labeled cells in the CKO mice (Fig. 6D). Pillar cells and Deiter's cells were positive for Tubulin at this stage in wild-type mice but showed only an irregular and low expression in the remaining organ of Corti in CKO mice (Fig. 6B, D).

We next analyzed the relationship of Sox2 expression to the organ of Corti in older mice. As in embryos, Sox2 expression was obvious in the greater epithelial ridge (GER) medial to the organ of Corti in both control and CKO mice (Fig. 6E–F'). In addition, the Sox2 expression overlapped with the Tubulin labeling of the supporting cells in the control organ of Corti (Fig. 6E–E'). In contrast, little Tubulin labeling and no Sox2 labeling beyond a few cells remained in CKO mice (Fig. 6F–F'). Nerve fibers ended as radial fibers near the middle of the Sox2 expression in the GER in the CKO mice (Fig. 6F', F'). These fibers could be followed in different focal planes to the organ of Corti in the control but formed loops in the CKO (Fig. 6F–G'). Occasionally a few fibers extended to the area of the organ of Corti in the CKO mice (Fig. 6G', G') where they formed a meshwork of fibers (Fig. 6H). The base showed projection of very limited fibers consistent with the newborn basal innervation (Fig. 6H).

We next wanted to analyze the continued decline of organ of Corti differentiation by following the supporting cells and innervation with Tubulin and the degree of differentiation using Myo7a, a hair cell marker. While nearly all of the perinatal and most of the early neonatal CKO mice showed no staining for Myo7a (Fig. 7B), we found some older mice with single cells or focal aggregates of Myo7a positive cells (Fig. 7C). These cells persisted at least until P31, the oldest stage we analyzed (Fig. 7E). These cells receive Tubulin positive radial fibers and were flanked laterally by Tubulin positive cells resembling pillar cells and phalangeal processes of Deiter's cells (Fig. 7E). This data suggest that a few cells can survive in the organ of Corti and can turn into Myo7a positive cells that receive most of the residual innervation (Fig. 7C, E). None of the mutant mice showed Atoh1 expression in the organ of Corti at any stage, suggesting...
incomplete recombination is an unlikely explanation for these Myo7a positive cells. We further confirmed the effective recombination of Atoh1 in these mice by checking the complete loss of cerebellar granule cells in these mice (Fig. S4B), which depend critically on Atoh1 for early development (Bermingham et al., 2001; Pan et al., 2009).

3.5. Bmp4, Fgf10 and Gata3 define the position of the organ of Corti in the absence of Atoh1 mediated hair cell differentiation

To evaluate the development of the structures associated with the organ of Corti in the near complete absence of hair cell development, we next examined the expression of Bmp4, a gene expressed lateral to the organ of Corti in developing Hensen’s and Claudius cells (Hwang et al., 2010; Morsli et al., 1998; Ohyma et al., 2010). A gradient of Bmp4 expression was observed in control mice with an apex to base progression and a gradual reduction of Bmp4 expression in most cells near the base (Fig. 8A, C). A more obvious apex to base gradient of Bmp4 expression was detected in Atoh1 CKO mice compared to control mice in P0 (Fig. 8B, B’), and in P7 Bmp4 expression was limited to the apical half of the cochlea with complete loss in the base (Fig. 8D–D’). This suggests that a differentiated organ of Corti is needed to maintain Bmp4 expression as much as Bmp4 is needed to induce differentiation of the organ of Corti (Ohyama et al., 2010).

We next investigated Fgf10, a gene expressed in the GER of the developing cochlea in wild-type mice (Pauley et al., 2003) partially overlapping with the expression of Sox2 in the GER (Fig. 8E, G). Fgf10 expression persisted in both P0 and P7 Atoh1 CKO cochleae mostly in the apex with gradual reduction near the base (Fig. 8F, H–H’). The Fgf10 expression was greatly reduced in P7 Atoh1 CKO mice (Fig. 8H–H’), consistent with the dissolution of the GER in neonates.

We next investigated the expression of Gata3, a gene that causes deafness in patients with a single allele mutation (Van Esch and Devriën, 2001) and likely acts as a proneural selector gene like its ortholog pannier in insects (Karik et al., 2001; Sato and Saigo, 2000). We observed expression of Gata3 in the approximate position of the organ of Corti in the Atoh1 CKO mice (Fig. 8J, J’, L, L’, N). Cells in the base that expressed Gata3 showed no indication of hair cell differentiation, but only a subset of these rather uniform epithelial cells displayed the Gata3 expression (Fig. 8N). There was both a progressive reduction in staining intensity and a narrowing of the stained area progressing from the apex to base in the CKO mice (Fig. 8J, J’).

These data suggest that Bmp4, Fgf10 and Gata3, which are expressed prior to Atoh1 in ear development, are not directly regulated by Atoh1 mediated hair cell differentiation. Apparently, absence of hair cell differentiation leads to the dedifferentiation of the organ of Corti which in turn affects the expression levels of these genes inside and outside the organ of Corti, in particular the basal half of the cochlea.

Given that Fgf10 and Bmp4 flank the organ of Corti medially and laterally, we wanted to determine how the apparent loss of marker genes affected the radial dimension

Fig. 7. Some Myo7a positive cells develop later in postnatal CKO mice. Consistent with the absence of Atoh1 in situ signal in the Atoh1 CKO mice, no Myo7a immunopositive cells are found in the mutant embryos, newborns, or in most postnatal mice up to P7 (B). However in some CKO cochleae, especially in late stages, there are few Myo7a positive cells detected (C, E). These few Myo7a positive cells are seen either individually or in small clusters. They receive the majority of the remaining nerve fibers and are more laterally flanked by Tubulin positive cells shaped either like pillar cells or Deiter’s cells with thin phalangeal processes (arrows in C and E). IHC, inner hair cell; Mid, the middle part of the cochlea; OHC, outer hair cell; P, pillar cells; RF, radial fibers. Bar indicates 100 μm in A–C and 10 μm in D and E.
Fig. 8. Expression of several genes defines the undifferentiated organ of Corti in Atoh1 CKO mutant. (A–D'). Bmp4 defines the lateral boundary of the organ of Corti and is retained in the apex of the mutant cochlea. Bmp4 in situ hybridization shows an apex to base gradient in Hensen’s and Claudius cells lateral to the hair cells of the organ of Corti in P0 and P7 control mice (A, A', C, C'). Likewise, Atoh1 CKO mice display a gradient of Bmp4 expression that is almost restricted to the apical half (B, B', D, D') and progressively lost in the base of the cochlea from P0 to P7 (B, D). Note the wider Bmp4 expression area in D' is closer to the apex than the area in B', both of which appear to be restricted to the Hensen’s cells and Claudius cells in the absence of differentiation of the organ of Corti (B', D'). (E–H') Fgf10 defines the medial boundary of the organ of Corti. Fgf10 is expressed in the greater epithelial ridge (GER) in both control animals (E, G, G') and also in CKO mice medial to the undifferentiated organ of Corti (F, H, H'). Like Bmp4, Fgf10 is also downregulated in the basal half of cochlea in the absence of Atoh1 compared to a control littermate. (I–N) Gata3 is expressed in the organ of Corti in P7 and P21 control mice and shows a similar expression pattern in the area of the putative organ of Corti in Atoh1 CKO mice. Higher magnifications and sections reveal the distribution of Gata3 in situ reaction product in the organ of Corti and adjacent cells in control mice (F, M). In Atoh1 CKO mice, Gata3 is expressed in undifferentiated cells that are topographically equal to the organ of Corti cells (J, N), CI, Claudius cells; GER, greater epithelial ridge; He, Hensen’s cells; IHC, inner hair cell; OC, organ of Corti; ‘OC’, putative organ of Corti in Atoh1 CKO; OHC, outer hair cell; SG, spiral ganglion. Bar indicates 100 μm in all except in A' and where it indicates 10 μm.
of the organ of Corti that is so obvious in the adult CKO ear (Fig. 4). We used a simultaneous in situ hybridization for both Fgf10 and Bmp4 to identify the organ of Corti both in whole mounts (Fig. 9A, A′, B, B′) and sections (Fig. 9A″, B″). The organ of Corti appears as an unlabeled strip of four rows of hair cells and five to six rows of supporting cells in the control cochlea (Fig. 9A–A″). In contrast, the two expression domains of Fgf10 and Bmp4 are much closer in the CKO mice and only one or two rows of cells intervene between them (Fig. 9B–B″). At this late developmental stage there is a change in Fgf10 expression not noted previously (Pauley et al., 2003) as Fgf10 appears to highlight inner hair cells (Fig. 9A′–A″). Consistent with our finding of occasional Myo7a positive cells that correspond topologically to inner hair cells (Fig. 7C, E) we found patchy aggregation of Fgf10 in single cells corresponding in radial and vertical position to inner hair cells in the CKO mice (Fig. 9B′, B″).

In summary, we show that the ear undergoes a near normal differentiation in the absence of Atoh1, including convergent extension of the cochlea (Hwang et al., 2010; Kelley et al., 2009). Associated structures such as spiral limbus, spiral sulcus and tectorial membrane form almost normally with the tectorial membrane touching the mostly single-layered epithelium that has replaced the developing organ of Corti by P14 (Fig. 3). The only area of near normal differentiation of hair cells is in the posterior canal, likely related to the inability of Tg(Pax2-Cre) to fully recombine the floxed Atoh1 as already shown for another floxed gene (Soukup et al., 2009). The pattern of innervation appears to be stable after P0, suggesting that there is limited further neuronal death after the end of the neurotrophins mediated cell death phase (Fritzsch et al., 2004) that is possibly related to the few Myo7a positive cells that remain in the organ of Corti. The profound cell death observed in late embryos seems to result in loss of the majority of cells of the organ of Corti, approximating flanking markers to the one to two rows of organ of Corti cells that remain in neonates (Fig. 9C, D). However, a few organ of Corti cells near the GER survive, express Fgf10 in neonates and later express Myo7a. Further studies are needed to understand the molecular basis for this survival and limited level of differentiation in the absence of Atoh1.

Fig. 9. Flanking markers define the cellular loss of the organ of Corti in the Atoh1 CKO mutant. (A–B″) Fgf10 and Bmp4 expression shown by double in situ hybridization defines the region between the two expression domains to be the organ of Corti. The P1 control mice have differentiated supporting and hair cells in this region (A, A′, with I, 1, 2, 3 in A′ indicating the inner and three rows of outer hair cells respectively), whereas only two rows of undifferentiated cells remain in the Atoh1 CKO mice (B, B′). Radial sections show the degree of differentiation of hair cells and supporting cells (stars in A″) in the control cochlea between the two areas of marker gene expression (Fgf10 medially and Bmp4 laterally). At this stage, Fgf10 also appears to be expressed in the inner hair cells (arrow in A″). However in the mutant cochlea, the two areas of marker gene expression are only separated by two rows of cells of the putative organ of Corti (B″), with patchy cells at the position of inner hair cells also expressing Fgf10 (arrows in B′ and B″). (C, D) Schematic diagrams summarize the cellular loss of organ of Corti and the marker gene expression pattern change in the Atoh1 CKO mutant. Cl, Claudius cells; GER, greater epithelial ridge; He, Hensen’s cells; IHC, inner hair cell; LER, lesser epithelial ridge; Mid, the middle part of the cochlea; OC, organ of Corti; OC′, putative organ of Corti in Atoh1 CKO; OHC, outer hair cells; SG, spiral ganglion; SL, spiral limbus. Bar indicates 100 μm in A, B and 10 μm in A′, A″, B′, B″.
4. Discussion

4.1. Absence of hair cell differentiation does not affect ear morphogenesis and histogenesis

Our data on Atoh1 CKO mice show that Atoh1 is not necessary for most of the normal differentiation of the ear, except for the differentiation of hair cells. Some transient expression of the Atoh1 enhancer occurs in pillar cells (Matei et al., 2005) and some expression of Atoh1-Cre in pillar cells has recently been reported (Yang et al., 2010), indicating that the initial expression of Atoh1 may not be specific only to future hair cells. However, mechanisms to suppress hair cell differentiation upon expression of Atoh1 seem to be in place specifically in developing pillar cells that can prevent Atoh1 mediated hair cell differentiation (Doetzlhofer et al., 2009). The near normal histological appearance of vestibular and cochlear areas including the appearance of the inner spiral sulcus and the spiral limbus indicates that the patterning processes defining these areas act independently of hair cell differentiation and may at best be dependent on the undifferentiated organ of Corti cells. This result is surprising given the ability of Atoh1 expressing hair cells to organize in vitro (Woods et al., 2004) and in vivo (Jahan et al., 2010a) the formation of supporting cells and even hairlike vesicles around them if misexpressed in the sensory ganglion. One explanation may be that Atoh1 expressing differentiating hair cells only reinforce in normal development the epithelial organization pattern generated by other genes. These data extend previous findings on expression of several organ of Corti specific genes in Atoh1 null mice (Dabdoub et al., 2008; Fritzsch et al., 2005b). Atoh1 seems to act in the ear mostly as a maturation factor for hair cells but does not act as a typical cell fate determining bHLH factor as in the cerebellum (Flora et al., 2009) and intestine epithelium (Ray and Leiter, 2007; Shroyer et al., 2007). This conclusion is consistent with our previous work on the expression of Atoh1-lacZ reporter in Atoh1 null mice (Fritzsch et al., 2005b) and the data suggesting expression of Atoh1 only after hair cells have exited the cell cycle (Lee et al., 2006; Matei et al., 2005). In contrast, Atoh1 is expressed in proliferating precursors in other developing systems (Flora et al., 2009; Pan et al., 2009).

Overall, our data show much less differentiation of the organ of Corti compared to previously reported Pou4f3 mutant mice, which show initial formation of hair cells followed by late embryonic hair cell loss (Hertziano et al., 2004; Xiang et al., 2003). Pou4f3 null mice show formation of some pillar cells, which may have had enough interaction with differentiating hair cells before hair cells die to initiate and partially complete their differentiation and maintain this differentiation for several months (Pauley et al., 2008). Interestingly, the few Myo7a positive cells that are found medial to the pillar cells in some older Atoh1 CKO mice suggest that at least some residual but incomplete differentiation is possible without Atoh1. This finding is comparable to the data recently obtained in Neurod1 null mice. In these mice most of the sensory neurons die but some appear to survive and are converted into hair cells inside the ganglia, apparently under the control of Atoh1 (Jahan et al., 2010a). It remains to be shown which other, possibly bHLH gene(s) (Fritzsch et al., 2010a) is (are) upregulated in postnatal mice to rescue and partially differentiate these cells in the organ of Corti to at least express Myo7a.

Our data do not directly challenge the results of clonal expression that suggest a regulation of hair cell differentiation of Atoh1 null cells by nearby Atoh1 positive hair cells (Du et al., 2007). However, if this were true we would have expected to see more histologically identifiable hair cells in the posterior canal cristata than what can be identified with Atoh1 in situ hybridization. This is clearly not the case, arguing that at least in the vestibular sensory epithelia our data do not support the notion of induction of hair cell differentiation of Atoh1 null cells by Atoh1 positive cells (compare Fig. 2B, 2F. However, the late appearance of Myo7a positive cells in the Atoh1 CKO cochlea could indicate that further differentiation of these cells might be possible if Atoh1 positive cells were near them as previously suggested (Du et al., 2007). Most importantly, we have generated a mouse model that will allow testing this idea by monitoring which cells most likely respond to Atoh1 using viral transfection (Izumikawa et al., 2005; Praetorius et al., 2010). Using GFP labeling with the vectors (Shibata et al., 2010) could also show if and how far such differentiation can spread from single transfected cells that differentiate as hair cells. Our mice can also help to reveal at which point in time the remaining cells of the flat epithelium will stop responding to Atoh1 transfection, as is the case with the chemically induced flat epithelium (Izumikawa et al., 2008).

4.2. Neurons develop and project normally, then undergo rapid loss in embryos but are stable in neonates

Our previous work has shown that neurons can develop and project to the sensory epithelia in the absence of any hair cell differentiation in Atoh1 null mice (Fritzsch et al., 2005b), indicating that hair cells per se do not provide an attraction beyond the expression of neurotrophins in supporting cells and hair cells. There is a rapid decline of the innervation density in Atoh1 CKO mice between E14.5 and P0 that is overlapping with the well known phase of extensive dependency on neurotrophins (Farinas et al., 2001; Fritzsch et al., 2004). We previously showed some residual expression of the neurotrophin Bdnf in the apex of newborn Atoh1 null mice correlating with the highest density of afferent innervation (Fritzsch et al., 2005b). Since we could not extend this observation into neonates due to neonatal lethality of Atoh1 null mice (Bermingham et al., 1999), we had to leave open whether this trend would continue leading to a rapid loss of all innervation in neonates.

Our data on Atoh1 CKO mice confirm the rapid loss of innervation in embryos but show a surprising maintenance of the pattern of residual innervation after this initial cell death. For at least two weeks there is no apparent change in the pattern of innervation. In addition, the empty spaces found in Rosenthal’s canal after neuronal death (Shibata et al., 2010) do not form. In Atoh1 CKO mice, areas in the basal tip and the apex that contain neurons at P0 (Fig. 5B—B’, L) remain until P14 (Fig. 3D, H). In contrast, solid bone instead of a canal is found in the middle turn where no neurons remain at P0 (Figs. 3F and 5B, L).

The data show that neuronal cell death comes to a near complete halt around birth in these mice. This is so despite the fact that the phase after P0 is characterized by the near complete demise of the embryonic organ of Corti with the formation of the spiral sulcus and a one cell layer thick ‘flat’ epithelium after P7 almost throughout the cochlea. These data on a genetically engineered mouse model with hair cell loss agree with data in humans where only a slow loss of sensory neurons after the demise of hair cells was reported (Linthicum and Fayad, 2009; Nadol and Eddington, 2006; Spoendlin and Schrott, 1990) and on data generated in another genetically engineered mouse model, the Pou4f3 null mice that also showed surprisingly long retention of some innervation (Pauley et al., 2008). Our CKO mice can provide a valuable model to test the molecular basis of this long-term sensory neuron retention in the absence of organ of Corti differentiation.

We report that there is some Bdnf in the undifferentiated cells that by their topology would represent ‘undifferentiated hair cells’ in the apex of the organ of Corti. There may also be expression of Ntf3 detectable by q-PCR (Morris et al., 2006) but we could not detect a clear signal using in situ hybridization. We are currently breeding mice with a Foxg1-Cre line which has less overlap with the Atoh1 expression in the brainstem (Hebert and McConnell, 2000; Pauley et al., 2006) to generate possibly even longer surviving
mice to test long-term effects of loss of hair cells on viability of sensory neurons.

4.3. Absence of hair cell differentiation does not affect expression of markers adjacent to and within the organ of Corti

Previous work has shown that genes such as Bdnf, Sox2, Jag1, and Prox1 that are expressed prior to Atoh1 in the undifferentiated sensory precursor cells remain expressed in Atoh1 null mice (Dabdoub et al., 2008; Fritzsch et al., 2010b, 2005b). We confirm and extend these previous findings and show that additional genes are expressed near the developing organ of Corti of Atoh1 CKO mice.

Previous work has shown that Bmp4 is expressed in Hensen’s and Claudius cells bordering the organ of Corti (Hwang et al., 2010; Morsli et al., 1998; Ohyama et al., 2010). Using Bmp4 in situ hybridization we show that this marker lateral to the organ of Corti is not affected by the absence of hair cell differentiation in its radial expression. However, there is progressive longitudinal loss of expression, paralleling the base to apex progression of differentiation. We also used an Fgf10 in situ hybridization, a marker for the greater epithelial ridge immediately medial to the inner hair cells (Pauley et al., 2008, 2003). Our data suggest that the apical expression of this gene is retained whereas the basal turn expression is nearly or completely gone prematurely compared to its loss in wild-type mice. We followed up on the fate of this expression during the transition period of greater epithelial ridge transformation to the inner spiral sulcus in neonates. Our data show that the Fgf10 expression disappears coincidentally with the loss of GER cells in the Atoh1 CKO and wild-type mice. These data suggest that flanking markers for the organ of Corti remain expressed to identify the shrinking organ of Corti.

This radial shrinkage comes about through the loss of precursor cells of the organ of Corti through late embryonic cell death (Fig. 21, J). Our data suggest that this is more extensive than previous data implied (Chen et al., 2002; Fritzsch et al., 2005b) and leads to the near complete loss of all cells of the organ of Corti between the flanking markers of Bmp4 and Fgf10 (Fig. 9C, D). Already at birth, only two rows of organ of Corti cells (instead of five rows of supporting cells found in wild-type mice) are positive for Prox1 (Fig. 6). Surprisingly, the few remaining cells of the organ of Corti can occasionally express the hair cell marker Myo7a and show pillar cell-like expression of Tubulin (Fig. 7). Why some of the cells of the organ of Corti survive and are even able to express Myo7a in the absence of Atoh1 requires further research.

Gata3 is highly expressed in the developing organ of Corti (Karais et al., 2001; Lawokoko-Kerali et al., 2004). Humans suffer from hearing loss when a single allele is mutated (Van Esch and Devriendt, 2001), indicating the importance of Gata3 for cochlear sensory development. Our data show that the expression of Gata3, which is clearly present several days prior to expression of Atoh1 in the ear (Karais et al., 2001; Matei et al., 2005), remains and defines the organ of Corti even in the absence of any overt cytological differentiation characterizing the organ of Corti as such. Gata3 is thus a good candidate gene to isolate promoter elements that may be useful to drive regeneration of the organ of Corti even in the flat epithelium that remains after hair cell loss. It remains to be shown if these genes are equally expressed in other mouse models with chemically induced hair cell loss or whether the antibiotic treatment causes additional thus far undetected effects in the gene expression profiles of the supporting cells.

5. Conclusion

Our data show that Atoh1 CKO mice undergo a rapid change of the embryonic prosensory epithelium through exaggerated cell death that results in near complete cellular loss of the organ of Corti. Some of the few surviving cells express the hair cell marker Myo7a. The expression of certain transcription factors and overall differentiation of the ear is not affected by this near complete loss of the organ of Corti. Identifying promoter regions of the remaining transcription factors might be useful to reconstitute the organ of Corti in these mice.

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Appendix. Supplementary data

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References


