Neurod1 Suppresses Hair Cell Differentiation in Ear Ganglia and Regulates Hair Cell Subtype Development in the Cochlea

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Abstract

Background: At least five bHLH genes regulate cell fate determination and differentiation of sensory neurons, hair cells and supporting cells in the mammalian inner ear. Cross-regulation of Atoh1 and Neurog1 results in hair cell changes in Neurog1 null mice although the nature and mechanism of the cross-regulation has not yet been determined. Neurod1, regulated by both Neurog1 and Atoh1, could be the mediator of this cross-regulation.

Methodology/Principal Findings: We used Tg(Pax2-Cre) to conditionally delete Neurod1 in the inner ear. Our data demonstrate for the first time that the absence of Neurod1 results in formation of hair cells within the inner ear sensory ganglia. Three cell types, neural crest derived Schwann cells and mesenchyme derived fibroblasts (neither expresses Neurod1) and inner ear derived neurons (which express Neurod1) constitute inner ear ganglia. The most parsimonious explanation is that Neurod1 suppresses the alternative fate of sensory neurons to develop as hair cells. In the absence of Neurod1, Atoh1 is expressed and differentiates cells within the ganglion into hair cells. We followed up on this effect in ganglia by demonstrating that Neurod1 also regulates differentiation of subtypes of hair cells in the organ of Corti. We show that in Neurod1 conditional null mice there is a premature expression of several genes in the apex of the developing cochlea and outer hair cells are transformed into inner hair cells.

Conclusions/Significance: Our data suggest that the long noted cross-regulation of Atoh1 expression by Neurog1 might actually be mediated in large part by Neurod1. We suggest that Neurod1 is regulated by both Neurog1 and Atoh1 and provides a negative feedback for either gene. Through this and other feedback, Neurod1 suppresses alternate fates of neurons to differentiate as hair cells and regulates hair cell subtypes.

Introduction

Neuronal and hair cell development of the inner ear critically depends on the basic Helix-Loop-Helix (bHLH) genes Neurog1 and Atoh1, respectively [1,2]. However, while several bHLH genes are known in the otocyst, their interplay in prosensory cells to determine neuronal and hair cell differentiation in interaction with other factors remains unclear [3,4,5]. Previous work has identified several genes that are co-expressed in both neurosensory primordia in the ear as well as delaminating and differentiating sensory neurons outside the ear, indicating that common upstream regulatory elements may exist for these topologically distinct cells that apparently differentiate into unique adult cells [6,7,8,9]. For example, neurotrophins delineate future sensory areas and are transiently expressed in delaminating neurons that exit the ear adjacent to or overlapping with prosensory regions [10]. Based on this circumstantial evidence it was suggested that these delaminating neurons may have some lineal relationship with the prosensory areas in the ear [6]. If true, ES or iPS cells could be made to develop into both neurons and hair cells, and could regenerate all neurosensory cells lost in deaf patients [11,12,13].

This idea of some relationship of neurosensory precursors was further substantiated by studies of two inner ear bHLH genes (Neurog1, Neurod1). Loss of either gene affects both sensory neuron and hair cells to a variable degree across all epithelia [14,15,16]. Neurod1 is the earlier expressed of the two genes and its absence substantially reduces hair cells in all sensory epithelia [2,14]. In addition, non-sensory cells such as cells in the cruciate eminence [14], greater epithelial ridge and ductus reuniens [17] are converted into hair cells. In contrast Neurod1, which is regulated by Neurog1 expression in neurons, shows a less profound effect on sensory epithelia [15,16]. Neurog1 or Neurod1 may have distinct cell-autonomous effect and thus not all cells that are positive for Neurog1 will be positive for Neurod1 which results in some disparity between these two mutations. The simplest explanation for this combined effect on sensory neurons and hair cells by these two bHLH genes is a possible lineage or even clonal relationships of some sensory neuron and hair cell precursors [18,19]. The observed reduction in hair cells in the respective null mutants
could be a consequence of loss of neurosensory precursors [14] or their conversion into hair cells [17]. This idea of lineage relationship is supported by lineage tracing for some neurons and hair cells in mice [20,21] and the clonal relationship of a small set of neurons and hair cells has been established in chicken [22]. However, technical limitations have thus far precluded establishing unequivocally the degree of this lineage/clonal relationship between all sensory neurons and hair cells.

While these data establish some molecular and in certain cases, lineage and clonal relationship of neurons and hair cells, the molecular basis for the distinct differentiation of either cell type has not been investigated beyond the transcriptional regulation of Neurog1 and Atoh1 [20] or short range interactions mediated by delta-notch [23]. This could either happen through de novo differentiation of distinct, unspecified otic cells or through successive refinement of cell fate within a given lineage of potentially ambivalent precursor cells. Using a conditional deletion approach we provide here evidence that Neurod1, a gene regulated by Neurog1 in neurons [2] and by Atoh1 in hair cells [17], suppresses an alternate Atoh1-mediated hair cell fate in cells within the ganglia and aids in differentiation of specific hair cell types in the cochlea.

Neurod1 is regulating multiple transcription factors in the neurosensory precursors, which are prematurely expressed in a different pattern in the absence of Neurod1. These data provide for the first time a detailed molecular mechanism for cell fate switching in neurosensory precursors of the mammalian inner ear and show that it hinges on suppression of alternate fates in neurosensory precursors by Neurod1.

Materials and Methods

Ethics Statement

All animal procedures were approved by the University of Iowa Animal Care and Use Committee (ACURF) and conducted according to their guidelines (ACURF #0804066).

Mice and genotyping for generation of conditional Neurod1 knockout mice (CKO)

Previously, lethality of newborn Neurod1 systemic null mice due to severe diabetes arrested the analysis in postnatal mice. To overcome this problem, we extended our analysis in the inner ear using Neurod1 conditional knockout mice [Neurod1f/f, Tg(Pax2-cre)]. By generating the Neurod1 conditional knockout (CKO) mice we could successfully circumvent the effect of Neurod1 in pancreatic β-cell development and could rescue mice to adulthood in Mendelian ratio.

To generate the Neurod1 conditional knockout mice we crossed the Pax2-cre line [24] with the floxed Neurod1 line [25]. For this study we used crosses between homozygotic floxed Neurod1 mice (Neurod1f/f) with heterozygous Neurod1f/+; Tg(Pax2-cre) mice. The resulting Neurod1f/-; Tg(Pax2-cre) mice are conditional knockout (CKO) mutant and the Neurod1f/+; Tg(Pax2-cre) heterozygous siblings serve as controls, here referred to as wild-type. To show the endogenous Neurod1 expression by the lacZ reporter, we have used Neurod1f/f; Tg(Pax2-cre) mice as control and Neurod1f/+; Tg(Pax2-cre) mice as control.

We also analyzed the Neurod1 CKO using Tg(Atoh1-cre). We generated the mice by breeding the homozygous floxed Neurod1 (Neurod1f/f) [25] and Tg(Atoh1-cre) with a ROSA26 reporter [17] as previously described [26].

Offspring were genotyped by PCR analysis of tail DNA using Cre-specific primers which produce a 280 bp product, and Neurod1-specific primers which produce a 400 bp product from Neurod1 coding region and a 600 bp product from the floxed allele. Embryos were collected from timed pregnant females at embryonic day 10.5 (E10.5), E11.5, E12.5, E14.5, E16.5 and E18.5 counting noon of the day the vaginal plug was found as E0.5. We have also analyzed post-natal day 0 (P0), P7, P14, P16 and P90 mice. Pregnant mothers or juvenile mice were anesthetized with a lethal dose of (1.25% of 2.2,2-tribromoethanol at a dose of 0.925 ml/g of body weight). Embryos were dissected from the uterus and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) using a peristaltic pump. Heads were isolated and fixed in 4% PFA for further analysis.

X-gal staining

After perfusion with 4% PFA, mice were hemisected and ears were dissected in 0.4% PFA. After brief washes with phosphate buffer, the samples were stained in a solution containing 0.1 M phosphate buffer, 0.01% deoxycholic acid, 0.02% NP40, 2 mM magnesium chloride, 5 mM potassium ferriyanide, 5 mM potassium ferrocyanide and 0.1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) for up to 24 hours at room temperature [27].

In situ hybridization

In situ hybridization was performed using the RNA probe labeled with digoxigenin. The plasmids containing the cDNAs were used to generate the RNA probe by in vitro transcription. The following probes were graciously provided: Atoh1; Dr. Zoghbi; Neurog1; Dr. Ma; Fgf8; Dr. Pirvola; Pou4f3; Dr. Xiang; Nkhi1 and Nkhi2; Dr. Braun; Sox2; Dr. Cheah. The dissected ears were fixed in 0.4% paraformaldehyde, dehydrated in 100% methanol and rehydrated and then digested briefly with 20 μg/ml of Proteinase K (Ambion, Austin, TX, USA) for 15–20 minutes. Then the samples were hybridized overnight at 60°C to the riboprobe in hybridization solution containing 50% (v/v) formamide, 50% (v/v) 2X saline sodium citrate (Roche) and 6% (w/v) dextran sulphate. After washing off the unbound probe, the samples were incubated overnight with an anti-digoxigenin antibody (Roche Diagnostics GmbH, Mannheim, Germany) conjugated with alkaline phosphatase. After a series of washes, the samples were reacted with nitroblue phosphate/5-bromo, 4-chloro, 3-indolyl phosphate (BMPII; Roche Diagnostics, Germany) conjugated with alkaline phosphatase. After washing off the unbound probe, the samples were incubated overnight with an anti-digoxigenin antibody (Roche Diagnostics GmbH, Mannheim, Germany) conjugated with alkaline phosphatase. After a series of washes, the samples were reacted with nitroblue phosphate/3-bromo, 4-chloro, 3-indolyl phosphate (BM purple substrate, Roche Diagnostics, 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 Image-Pro software.

Immunofluorescence

For immunofluorescence staining, the ears were dehydrated in graded ethanol overnight and rehydrated in graded ethanol and PBS. Samples were then blocked with 0.25% normal goat serum in PBS containing 0.01% Triton-X-100 for 1 hour. Then the primary antibodies for Myo VIIa (Myosin VIIa, Proteus Biosciences), Tubulin (Sigma), Caspase 3 (Cell Signaling Technology) and espin (a gift from Dr. J. Bartles JR) were used in dilutions of 1:200, 1:800, 1:100 and 1:5 respectively and incubated for 24–48 hours at 4°C. After several washes with PBS, corresponding secondary antibodies (1:500) (Alexa fluor molecular probe 647 or 532 or 488; Invitrogen) were added and incubated overnight at 4°C. The ears were washed with PBS and mounted in glycerol and images were taken with a Leica TCS SP5 confocal microscope.

Plastic embedding and Stevenel’s Blue staining

The end organs of ears were fixed in 2.5% glutaraldehyde overnight followed by several washes with 0.1 M phosphate buffer.
we performed counterstained with Stevenel’s blue for more detailed histology. For higher resolution and co-localization of probes and proteins, we performed in situ hybridization for Figβ, followed by Myo VIIa immunocytochemistry on same ears. Some of these ears were embedded in resin, sectioned and imaged with epifluorescent and transmitted light on a Nikon E800. Some of these sections were counterstained with Stevenel’s blue for more detailed histology.

SEM
P30 mice were lethally anesthetized and perfused with 4% PFA. Ears were dissected, decalcified in EDTA and osmicated [1% OsO4 in 0.1 M phosphate buffer (pH 7.4)]. Osmicated ears were washed several times in distilled water to remove all ions, dehydrated in a graded ethanol, critically point dried, mounted on stubs and coated with gold/palladium. Stubs were viewed with a Hitachi S-3400N Scanning Electron Microscope with 2MeV acceleration.

Results
Ectopic hair cells form in sensory ganglia of Neurod1 conditional null mice
Previous work has shown that most cochlear and many vestibular sensory neurons are lost in Neurod1 null mice [15] with the surviving neurons projecting aberrantly to the sensory epithelia of the ear and into the brain [29]. When we studied the detailed distribution of sensory epithelia in whole mounted ears using Myo VIIa as a marker for hair cells to quantify the effects of loss of Neurod1 on hair cell development, we found numerous Myo VIIa positive cells scattered in the two remaining neuronal aggregations of vestibular and cochlear sensory neurons near the saccule and utricle (Fig. 1A, B’–K’). We also observed the appearance of these positive cells scattered in the two remaining neuronal aggregations of vestibular and cochlear sensory neurons near the saccule and utricle (Fig. 1A, B’–K’). Many of these cells were grouped around multiple vesicles that were present within these ganglia (Fig. 1H,H’). Immunofluorescence labeling with anti-β-tubulin antibody revealed that each of the Neurod1 positive cells was also immunopositive for Myo VIIa antibody revealed that each of the Neurod1 positive cells was also immunopositive for Myo VIIa (data not shown) identical to Atoh1 (Fig. 2A, A’). However, in the Neurod1 CKO mice, Atoh1 expression continued in these ganglia past the transient expression found in control animals (Fig. 2D, D’). Atoh1 expression was more profound in later stages and was found in a cluster of cells in the remaining ganglia next to the utricle and saccule, mostly around the ectopic vesicles (Fig. 2E–G’). Immunofluorescence labeling with Myo VIIa antibody revealed that each of the Atoh1 positive cells was also immunopositive for Myo VIIa (Fig. 2H, H’).

While these data would normally be considered as proof of the hair cell nature of these cells [12], we worked with additional specific hair cells markers to establish that these cells were indeed hair cells and not transformed neurons with abnormal properties. Two POU domain factors are uniquely expressed in hair cells and neurons [33,34], Pou4f3 and Pou4f1 (formerly Brn3c and Brn3a, respectively). We demonstrated that Pou4f3, an exclusive marker for hair cells in the ear with limited expression outside the ear [33,35], was expressed in hair cell-like cells in the remaining ganglia of the Neurod1 CKO ear (Fig. 3H) and colocalized with Neurod1 (data not shown) identical to Atoh1 (Fig. 2H, H’). The expression of these three markers (Atoh1, Pou4f3 and Myo VIIa) is uniquely associated with hair cells in wild-type inner ears (Fig. 2C, 3). Therefore, their expression in cells within the remaining ganglia in Neurod1 CKO mice provides evidence that these cells are hair cells. Differentiation of these hair cells started around the same time those markers were upregulated in the nearby vestibular sensory epithelia and, transiently, in the delaminating sensory neurons.

To further expand the notion that these cells were genuine hair cells, we next studied another set of bHLH genes Nhlh1 and Nhlh2 which are associated with hair cells and neurons, respectively [36]. Nhlh1 and Nhlh2 are primarily expressed in sensory neurons in early embryos with an additional later expression of Nhlh1 in inner ear sensory epithelia [36]. We observed Nhlh1 mRNA expression in the vestibular ganglia and in delaminating cells in early embryos with progressive upregulation in the sensory epithelia in later stages (Fig. 3B,C). In the absence of Neurod1 the expression of Nhlh1 was massively reduced in neurons with some residual expression in cells below the saccule and utricle (Fig. 3B’,C’). In contrast to Nhlh1, Nhlh2 expression was exclusively in sensory neurons (Fig. 3D). In Neurod1 CKO mice, Nhlh2 expression was retained only in a small set of cells near the utricle (Fig. 3D’). Loss of expression of both genes in neurons was likely associated with early onset of apoptosis in Neurod1 mutant [29].

We also investigated Sox2, a protein required for hair cell differentiation [37] and later is highly expressed in supporting cells in the ear [38]. Expression of this gene was also found within the ganglia next to the utricle and saccule (Fig. 3F’). This could indicate that some cells in these ganglia were possibly supporting cells.

Gene expression suggests that lack of Neurod1 transforms some surviving neurons into hair cells
Atoh1 is a hair cell differentiation marker with a well established role in the ear only in the differentiation of hair cells [1,31,32]. Only limited expression of Atoh1 has been reported with sophisticated techniques in sensory neurons [17] and none in the neural crest derived Schwann cells or mesenchyme derived perineural fibrocytes. Using in situ hybridization we observed only a transient Atoh1 expression in the vestibular ganglia of wild-type mice at E11.5 (Fig. 2A, A’). However, in the Neurod1 CKO mice, Atoh1 expression continued in these ganglia past the transient expression found in control animals (Fig. 2D, D’). Atoh1 expression was more profound in later stages and was found in a cluster of cells in the remaining ganglia next to the utricle and saccule, mostly around the ectopic vesicles (Fig. 2E–G’). Immunofluorescence labeling with Myo VIIa antibody revealed that each of the Atoh1 positive cells was also immunopositive for Myo VIIa (Fig. 2H, H’).
Another factor exclusively expressed in some hair cells and only transiently in sensory neurons is **Fgf8** [39]. Consistent with data on several transcription factors, we found transient expression of **Fgf8** in delaminating sensory neurons of both wild-type and **Neurod1** CKO mice (Fig. 4H–J'). At later stages, when other markers for hair cells are expressed, we found **Fgf8** expression in the ganglia in a pattern reminiscent of the hair cells identified by other markers (Fig. 3 E').

In summary, the presence of **MyoVIIa**, **Atoh1**, **Pou4f3**, **Sox2**, **Nhlh1**, **Nhlh2** and **Fgf8** expression in cells near intraganglionic vesicles inside the remaining ganglia implied a substantial modification of the cellular identity of these cells in **Neurod1** CKO mice. We suggest that at least some surviving neurons are converted into hair cells which organize the surrounding tissue into vesicles inside ganglia and possibly regulate supporting cell differentiation of nearby neurons, fibroblasts or Schwann cells to form epithelia-like structures. Such organizing capacity of **Atoh1** expressing cells has already been demonstrated in vitro [40] and is known for **Fgf8** in vivo [41]. Since none of these markers ever appear in neural crest derived Schwann cells or mesenchyme derived fibroblasts, it seems unlikely that they are de novo expressed in these cells in the absence of **Neurod1** expression in differentiating neurons. In contrast, several of these factors are known to be expressed in sensory neurons [17]. We therefore suggested that

![Image of Neuronal and Cellular Structures](https://www.plosone.org/doi/10.1371/journal.pone.0011661)
among the three cell types found in wild-type ear ganglia, it is the sensory neurons that are converted into hair cells. While highly suggestive of a neuronal origin, these data cannot fully exclude the alternative but more complex scenario of a Schwann cell or fibroblast transformation into hair cells in the absence of Neurod1.

Neurod1 affects hair cell type differentiation through regulation of multiple genes

A role of Neurog1 in inner ear neurosensory lineage acquisition is well established along with Neurod1 as a downstream activator of Neurog1 [2,15,16]. We now provide evidence for a novel function of Neurod1 consistent with its early expression in the prosensory domain to suppress an alternate hair cell fate. To further understand the possible interactions we analyzed in a Neurod1 lacZ reporter the level of β-galactosidase expression in delaminating neuroblasts (Fig. 4A). We previously showed by in situ hybridization that Pax2-Cre results in complete and early deletion of Neurod1 in mutant mice [29]. We therefore needed to use the reporter as both wild-type neurons and neurons lacking Neurod1 will show the reporter. In contrast to wild-type mice, the expression of lacZ reporter was increased in the delaminating neurons of Neurod1 CKO mice (Fig. 4A) while the expression in the otocyst was reduced. This early knock out of Neurod1 resulted in considerable changes in expression pattern of several genes analyzed in different embryonic stages with in situ hybridization (Fig. 4).

For example, Neurog1 persisted longer in its expression in the delaminating neurons of Neurod1 CKO mice (Fig. 4A') while the expression in the otocyst was reduced. This early knock out of Neurod1 resulted in considerable changes in expression pattern of several genes analyzed in different embryonic stages with in situ hybridization (Fig. 4).
Among twenty-three different Fgfs and four FGF receptors, several are known to function in early otic induction and sensory specification [8,44,45,46,47]. We investigated Fgf8 expression due to its known early expression in delaminating neurons and later function in organ of Corti in supporting cell development [41,48,49]. We found Fgf8 expression in the early stage of development, which is substantially modified in the absence of Neurod1 (Fig. 4H–J'). Fgf8 was expressed in the prosensory domain and transiently in the delaminating neuroblasts (Fig. 4H,I,J) of wild-type mice. In Neurod1 CKO mice, Fgf8 was expressed in delaminating cells with moderate loss of expression in ganglia (Fig. 4H',I',J') consistent with the neuronal loss in the absence of Neurod1 [29].

In summary, we suggested a compelling role of Neurod1 in specification of neurosensory precursors beyond its role in neuronal differentiation, possibly through an interaction with important genes like Neurog1, Sox2 and Fgf8. This suggestion is based on the fact that early deletion of Neurod1 results in substantial changes of expression of several genes that may directly or indirectly affect cell fate determination of the precursor population.

Inactivation of Neurod1 results in a shortened and disorganized organ of Corti

Having now established that Neurod1 affects cell fate acquisition in the ear ganglia we investigated the effect of loss of Neurod1 on hair cells. Previous work has shown a surprising overlap of apparent cell specific bHLH genes in inner ear development. For example, Neurog1 null mice not only lose all sensory neurons but also have truncated development of hair cells in several sensory epithelia [14,17]. Recently, a lineage relationship between neurons and some hair cells was demonstrated [20]. Interestingly, despite absence of Neurog1, hair cells strongly express Neurod1 [17], suggesting that hair cell specific factors such as Atoh1 are also
able to upregulate Neurod1 expression and can do so more effectively in the absence of Neurog1. Since Neurod1 is immediately downstream and directly regulated by Neurog1 [2], we investigated the effects of Neurod1 on hair cell development as they are affected by the absence of either Neurog1 or Neurod1 [14,15,16,20]. Consistent with an expression of Neurod1 in hair cells [29] and previous suggestions about a shortened cochlea with disorganized hair cells in Neurod1 systemic null mice [13,16,50], we also found a truncation of the cochlea. When compared to wild-type, Neurod1 mutant cochlea was not as much shortened as Neurog1 null cochlea (Table 1). Size reduction in Neurod1 CKO mice was also apparent in other epithelia and canal cristae which were approximately 30% shorter than the control (Table 1; Fig. S1). These data suggest that Neurod1 exerts a comparable effect on hair cell formation as Neurog1 albeit at a reduced scale. The somewhat less severe effect could relate to the fact that Neurod1 is downstream to Neurog1 and some common sensory neuron/hair cell precursors may already have separated.

We next wanted to investigate whether the truncated cochlea has multiple rows of disorganized hair cells as previously reported in other mutants with shortened cochlear growth [9,14]. There was a gradient of malformation of the hair cells in the Neurod1 CKO mice (H–J). In the wild-type, Fgf8 disappears in the otic vesicle after E11.5 but remains transiently restricted to the ganglia (I, J). In contrast, this Fgf8 expression remains in the absence of Neurod1 inside the otic vesicle in areas identified to be composed of sensory precursor cells (Fig. H', I', J'). G, vestibular ganglion; AC, anterior canal cristae; HC, horizontal canal cristae; PC, posterior canal cristae; S, saccule; U, utricle; Co, cochlea; Ed, endolymphatic duct. Boundary of otic vesicle is marked with dotted lines. Bar indicates 100 μm.

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**Figure 4. Absence of Neurod1 results in aberrant expression of genes responsible for prosensory specification.** At E11.5 embryo, β-galactosidase histochemistry show Neurod1-lacZ expression in the delaminating neuroblast (A, A') and in the vestibular ganglion (G) which is moderately larger in the Neurod1 CKO mice (A'). In contrast, expression in the otic vesicle is reduced in Neurod1 CKO mice (arrow in A'). Neurog1, an upstream regulator of Neurod1, show a larger expression in the absence of Neurod1 (B', C', D'). In particular the prosensory domain is remarkably enlarged inside the otic vesicle with aberrant migration of the prosensory precursors (arrow in B') in Neurod1 CKO mice. After the specification of the sensory epithelia, Neurog1 expression is progressively downregulated in wild-type mice (C, D) but some expression remains in the Neurod1 CKO mutant (C', D'). In the mutant, Sox2 expression is expanded in the otocyst (compare E, E') and shows more profound expression in the neurosensory precursor domain (F', G'). We also investigate Fgf8 expression which is strongly positive in the delaminating neuroblasts both in wild-type and in Neurod1 CKO mice (H–J). In the wild-type, Fgf8 disappears in the otic vesicle after E11.5 but remains transiently restricted to the ganglia (I, J). In contrast, this Fgf8 expression remains in the absence of Neurod1 inside the otic vesicle in areas identified to be composed of sensory precursor cells (Fig. H', I', J'). G, vestibular ganglion; AC, anterior canal cristae; HC, horizontal canal cristae; PC, posterior canal cristae; S, saccule; U, utricle; Co, cochlea; Ed, endolymphatic duct. Boundary of otic vesicle is marked with dotted lines. Bar indicates 100 μm.

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The basal half of mutant cochlea was more obvious in later stages (P7 shown here) and demonstrated two rows of IHCs and four to five rows of OHCs (Fig. 5F,F'). In addition, Myo VIIa was more prominently expressed in IHCs in the apex, as compared to the uniform expression in the base, and similar high levels of expression of Myo VIIa was found in scattered OHCs in the apex (Fig. 5F,F'). We therefore interpreted these cells as 'ectopic IHC's'. The apical tip consisted exclusively of IHCs in two disorganized rows without any OHCs (Fig. 5F').

To evaluate the apical specialization of the organ of Corti of Neurod1 null mice, we performed the immunofluorescence labeling combined with the Myo VIIa. We found similar abnormality of stereocilia with espin as with Myo VIIa in hair cells in the apical half. The respective immunolabeling confirmed the disorganization of organ of Corti in the apex of Neurod1 CKO mice where multiple rows of IHCs and OHCs were found with two distinct types of stereocilia (Fig. 5H–H'). In the newborn mice, density of the stereocilia of both IHCs and OHCs were almost equal, but at later stages the density of stereocilia of OHCs was remarkably reduced with few displaced highly dense inner hair stereocilia in place of weakly labeled outer hair stereocilia (Fig. 5I). Near the apical tip only the inner type of stereocilia were found (data not shown).

We next wanted to understand how the irregularity of hair cells affected the supporting cells. In situ hybridization with Pdx1, a marker for Deiter’s and Pillar cells [51,52] revealed disorganized supporting cells (Fig. 6). We also used β-tubulin immunofluorescence labeling in P7 and P16 Neurod1 CKO mutant mice (Fig. 6E–F',G). We found the organization of supporting cells in the basal half of the cochlea was regular with thick processes of two (Fig. 6E–F',G). We therefore interpreted these cells as 'ectopic IHC’s'. The apical tip consisted exclusively of IHCs in two disorganized rows without any OHCs (Fig. 5F').

Table 1. Length of the Cochlea in wild-type, Neurod1 CKO and Neurog1 null mutations.

| Length of Cochlea [μm] | WT (n = 4) | Neurod1 CKO (n = 4) | Neurog1 null (n = 3) |
|------------------------|-----------|---------------------|---------------------|
| Mean                   | 5907      | 2695                | 2449                |
| SD                     | 408       | 138                 | 320                 |

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In summary, Myo VIIa, Atoh1, Pouf4b3 and Nhlh1 showed an altered pattern of expression in Neurod1 CKO cochlea with premature expression in an apex-to-base instead of a base-to-apex progression as in wild-type littermates (Fig. 5A–B', 7). This spatiotemporal aberration of the bHLH gene expression might alter the onset of differentiation of apical hair cells. The premature expression of these transcription factors in hair cells that show a delayed differentiation compared to their early cell cycle exit [17] may relate to the disorganization of apical hair cells in Neurod1 CKO mice.

Atoh1 expression was not only altered spatiotemporally but also persisted longer in the absence of Neurod1. For example, Atoh1 expression progressively reduced in the wild-type cochlea from base-to-apex started from P0 to onward with restricted expression only in the inner two rows of OHCs (Fig. S1A–A'; shown in P0). In contrast, Atoh1 expression persisted longer in all the hair cells throughout the cochlea of Neurod1 CKO mice (Fig. S1B–B'). Atoh1 was also aberrantly expressed in the non-sensory compartment which was most obvious in the cruciate eminence of the anterior crista (AC) and also in the crista region of the sacculus and utricle (Fig. S1D–D'). Consistent with Atoh1 expression, we also found persistence of Pouf4b3 expression in the base and apex of the Neurod1 CKO cochlea in contrast to wild-type littermates (data not shown). We previously reported that loss of Neurod1 resulted in continued expression of Atoh1 in cerebellar granule cells [26]. Apparently, Neurod1 exerts a similar inhibitory influence on the expression of Atoh1 and its downstream genes Nhlh1 and Pouf4b3.

Altered Fgf8 expression may relate to the cochlear histological changes

In mice, FGF3, FGF8 and FGF10 play a role in the early inductive events of the otic vesicle formation [8,39,46,55,56,57,58].
Figure 5. *Neurod1* is necessary for development of an orderly patterned organ of Corti. Myo VIIa immunocytochemistry shows upregulation of Myo VIIa in the wild-type starts around E16.5 from the mid-base and later progresses toward both base and apex with a regular organization of one row of inner and three rows of outer hair cells throughout the cochlea (A, A’, C, C’, E). In contrast, Myo VIIa is already expressed throughout the cochlea with disorganization of hair cells in the apical half (B, B’, D) in E16.5 *Neurod1* CKO mutant littermates. In later stages, the basal half of the CKO mutant shows normal orientation of the hair cells (D, D’) whereas the apex of *Neurod1* CKO mice shows multiple rows of both IHCs and OHCs with reduction of Myo VIIa intensity in most outer hair cells (F, F’). In addition, clusters of higher intensity of Myo VIIa positive cells are found in between outer hair cells with equivalent staining intensity to inner hair cells (arrows in F). The apical tip of the mutant cochlea shows a partially duplicated row of inner hair cells with complete absence of outer hair cells (F”). Using espin immunocytochemistry we confirm the disorganization of the apical half of the mutant cochlea where two rows of inner hair stereocilia and four to five rows of outer hair stereocilia are observed (H–H’) along with some unusually displaced strongly stained inner hair stereocilia (arrow in I) in between faintly labeled outer hair stereocilia (I). IHC, inner hair cells; OHC, outer hair cells. Bar indicates 100 μm except F”; 10 μm in F”. doi:10.1371/journal.pone.0011661.g005
We observed Fgf8 expression in the delaminating sensory neuron in both wild-type and Neurod1 CKO mice as early as E10.5 (Fig. 4). Consistent with expression changes in Atoh1 and other downstream hair cell specific genes, Fgf8 was also expressed prematurely in the apex of the Neurod1 mutant mice (Fig. 8B'). Fgf8 was transiently expressed in the apex of wild-type mice but disappeared after E11.5 (compare Fig. 8A and B). However, absence of Neurod1 resulted in continued Fgf8 expression in the apex from E11.5 onward and thus resulted in premature and reversed expression pattern (Fig. 8A', B', C'). In contrast, in wild-type mice, Fgf8 expression started at E14.5 from the base of the cochlea progressing over time to the apex (Fig. 8C).

In the cochlea, Fgf8 is expressed in IHCs from where it diffuses to bind to its receptor, Fgfr3, which leads to the development of Pillar cells instead of Deiter’s cells [41,59,60]. Our data on Myo VIIa expression in the Neurod1 CKO mutant suggested that some OHCs may achieve an inner hair cell-like phenotype (‘ectopic IHCs’; Fig. 5) and this may be due to an altered Fgf8 expression. We therefore examined Fgf8 expression to verify that these cells are ‘ectopic IHCs’ as Fgf8 is a marker of IHCs of the organ of Corti [44]. In wild-type mice, Fgf8 was expressed exclusively in the single row of IHCs (Fig. 8D,D'). In the Neurod1 CKO mutant, we found single rows of Fgf8 positive IHCs only in the basal half of the cochlea (Fig. 8E,G) whereas in the apical half, multiple rows of
Fgf8 positive IHCs was observed as well as ectopic expression in some IHCs replacing OHCs (Fig. 8E,F,H,I) consistent with the observation of Myo VIIa expression (Fig. 5F,F'). Closer to the apical tip we found only Fgf8 expression in multiple rows of IHCs without OHCs (Fig. 8J). In conclusion, absence of Neurod1 altered Fgf8 expression in the apex of the mutant cochlea which directly or indirectly related to the change of stereotyped pattern and differentiation of apical hair cells in the organ of Corti.

To analyze more closely the effect of Fgf8 expression in the organ of Corti, we performed plastic sections of Fgf8 in situ reacted P0 ears sequentially immunolabeled with anti Myo VIIa antibody. Sections through the base showed the expected distribution of a single row of Fgf8 and Myo VIIa positive IHCs and three rows of OHCs without OHCs (Fig. 8F,F'). In conclusion, absence of Neurod1 altered Fgf8 expression in the apex of the mutant cochlea which directly or indirectly related to the change of stereotyped pattern and differentiation of apical hair cells in the organ of Corti.

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Figure 7. Neurod1 controls hair cell specific gene expression in the apex. Normally hair cell cycle exit and differentiation is delayed in the cochlea compared to vestibular epithelia and shows a pattern of apex-to-base progression of cell cycle exit. In contrast, there is a base-to-apex progression of differentiation, including upregulation of Atoh1 (A), Pou4f3 (C) and Nhlh1 (E) in wild-type mice. In Neurod1 CKO mice, the expression of these genes not only happens earlier but progresses from the apex to the base (compare A and B; C and D, D', E, E' and F, F', F''). Atoh1 and Pou4f3 are essential for hair cell differentiation and maintenance, respectively. Their expression suggests a premature initiation of hair cell differentiation that normally is delayed in the apex compared to the base. Note that the apex in B is shown with 90° anti-clockwise rotation in B'. AC, anterior canal crista; HC, horizontal canal crista; PC, posterior canal crista; S, saccule; U, utricle. Bar indicates 100 μm.

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Fgf8 positive IHCs was observed as well as ectopic expression in some IHCs replacing OHCs (Fig. 8E,F,H,I) consistent with the observation of Myo VIIa expression (Fig. 5F,F'). Closer to the apical tip we found only Fgf8 expression in multiple rows of IHCs without OHCs (Fig. 8J). In conclusion, absence of Neurod1 altered Fgf8 expression in the apex of the mutant cochlea which directly or indirectly related to the change of stereotyped pattern and differentiation of apical hair cells in the organ of Corti.

To analyze more closely the effect of Fgf8 expression in the organ of Corti, we performed plastic sections of Fgf8 in situ reacted P0 ears sequentially immunolabeled with anti Myo VIIa antibody. Sections through the base showed the expected distribution of a single row of Fgf8 and Myo VIIa positive IHCs and three rows of OHCs without OHCs (data not shown). While, radial sections through the apex confirmed both clusters of inner hair cells as well as single or multiple Fgf8 positive cells among the OHCs in Neurod1 CKO mice (Fig. 8K–K'') consistent with the whole mounted data.

We further investigated the consequences of Fgf8 misexpression and analyzed the differentiation of the stereocilia in P30 mice using SEM. Consistent with the patchy expression of Fgf8 in only some topographic OHCs we found a patchy aberration of stereocilia where some ‘OHCs’ had stereocilia twice as thick as others, resembling the diameter of inner hair cell stereocilia (Fig. 8M,N). The changes in stereocilia supported the evidence of ‘ectopic IHCs’, dispersed among OHCs.

In summary, absence of Neurod1 leads to premature upregulation of hair cell differentiation genes in the apex, severe disorganization of the apical hair cells and supporting cells, misexpression of Fgf8 in some ‘OHCs’, and development of inner hair cell-like stereocilia among OHCs. Consistent with its early expression in the inner ear prosensory region, Neurod1 plays a significant role in hair cell maturation through the suppression of several genes in the apex, most prominently Atoh1 and Fgf8. We tested whether a delayed knockout of Neurod1 using Tg(Atoh1-cre) could achieve these altered differentiation of hair cells. Despite a massive cerebellar phenotype of this CKO mouse [26], our data showed no effect in inner ear development or any alteration of phenotype of neurosensory cells (Fig. S2). Once the prosensory domain is specified in early embryonic stage, later deletion of Neurod1 has no effect in refinement of hair cell fate.
Neurod1 is essential for neuronal differentiation in the cerebellum [26] and the ear [29] and can convert non-neuronal cells into neurons [61] through the regulation of over 500 downstream genes [62]. We analyzed the role of Neurod1 in inner ear neurosensory cell development using a newly generated Neurod1 conditional knockout mouse. We previously reported [15,29] substantial loss of inner ear sensory neurons and disorganization of remaining afferent projections in Neurod1 systemic and conditional null mice. The expression of other bHLH genes such as Nhlh1 and Nhlh2 [36] may be responsible for partial rescue of those few

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**Figure 8.** Fgf8 misexpression correlates with formation of ‘ectopic inner hair cells’. In situ hybridization shows a persistent expression of Fgf8 in neurosensory precursors in Neurod1 mutant cochlea as early as E12.5 (arrow in B’) with premature expression in particular in the apex of the cochlea (C) in comparison to wild-type (C). Fgf8 is expressed transiently in the prosensory domain in wild-type mice (arrow in A) and later is shown to be upregulated in the cochlea with a base-to-apex gradient (A,B,C). In newborn mice, Fgf8 is uniformly expressed in all inner hair cells almost along the entire length of the cochlea in wild-type mice (D, D’). In contrast, Neurod1 CKO mice display an increased expression level in the apex (E, F, H, I, J) and deviate from the single row labeling of only inner hair cells seen in the base (G). Two or more rows of inner hair cells are positive for Fgf8 and scattered single and multiple cells are interspersed among the multiple rows of outer hair cells which are also positive for Fgf8 (arrows in I). The apical tip shows up to three rows of Fgf8 positive cells (J). Radial sections through the apex of Fgf8 ISH reacted and Myo VIIa immunostained cochlea reveals co-localization of Fgf8 and Myo VIIa in inner as well as ‘ectopic inner hair cells’ scattered among outer hair cells (arrows in K–K’). SEM of P30 Neurod1 CKO mice reveals a normal organization of inner hair cells in a single row and three rows of outer hair cells in the base of Neurod1 CKO mice (L). In contrast, the apex shows inner hair cell sized stereocilia (arrows) interspersed among normal sized stereocilia bearing outer hair cells (arrowhead; M,N). Dotted line in H indicates border between normal and disorganized organ of Corti. IHC, inner hair cells; OHC, outer hair cells. Bar indicates 100 μm in A–F except D’; 10 μm in D’,G–J, K–K’ and 1 μm in L–N.

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**Discussion**

Neurod1 is essential for neuronal differentiation in the cerebellum [26] and the ear [29] and can convert non-neuronal cells into neurons [61] through the regulation of over 500 downstream genes [62]. We analyzed the role of Neurod1 in inner ear...
Neurod1 suppresses differentiation of ganglion cells into hair cells

A cascade of pro-neuronal bHLH genes transforms ectodermal cells into neurons and can do so by simply being misexpressed in the developing ectoderm [61] or the ear [43]. These bHLH proteins also determine cell fate in other tissues such as pancreas [16], gut [63] and Merkel cells [64]. bHLH genes are important for cell fate switch. Without expression of Atoh1, cells can change from a secretory to an absorptive phenotype [65].

In this study, we observed the formation of vesicles lined by hair cells in place of remaining ganglia in the Neurod1 CKO mice (Fig. 1). To further understand the molecular basis of formation of these ‘intraganglionic hair cells’, we analyzed expression of multiple hair cell specific genes. We found positive expression of Atoh1 and Pou4f3 and the hair cell marker Myo VIIa in two ‘intraganglionic hair cells’. Nhlh1 and Neurod1 are limited early on to neuronal expression but are found later in hair cells, including the ‘intraganglionic hair cells’ (shown with Nhlh1 in situ hybridization and Neurod1 lacZ expression). The overlapping function of Neurod1 and Nhlh1 may not only lead to the survival of some neurons but the absence of Neurod1 may allow premature and persistent expression of Atoh1 in these remaining ganglion neurons (Fig. 2D, D'). Consistent with the previous report of Atoh1 expression in some neurons [17], our in situ data also show a transient Atoh1 expression in the delaminating sensory neurons in the wild-type embryo. This limited expression of Atoh1 may normally be restrained by the expression of Neurod1 and absence of Neurod1 seems to allow continued expression. We suggest that this continued expression of Atoh1, combined with the absence of Neurod1 expression can result in the differentiation of ‘intraganglionic hair cells’ as well as the expression of other hair cell markers such as Myo VIIa, Pou4f3 and Nhlh1. In addition to hair cell markers, these or other cells within the ganglia show neurosensory markers such as Sox2, Nhlh2 and Fig8.

Only three cell types with different embryonic origins are found in vestibular and cochlear ganglia: inner ear derived sensory neurons [66], mesoderm derived fibroblasts and neural crest derived Schwann cells [67]. A transformation of Schwann cells or fibroblasts, which never express Neurod1, is theoretically possible. However, the presence of multiple genes known to be expressed in neurosensory cells but not in Schwann cells or fibroblasts makes this a very improbable scenario. We therefore interpret our data to suggest that some inner ear derived ‘sensory neuron precursors’ adopt a hair cell fate in the absence of Neurod1. Such ‘hair cell’ formation from delaminated neurosensory precursors suggests a degree of flexibility of cell fate acquisition and is consistent with the emerging concept of lineage and possibly clonal neurosensory relationships in the ear [18,19,20]. In addition, these Myo VIIa positive ‘hair cells’ express marker genes otherwise only associated with hair cells in the ear, and reside around vesicles inside vestibular ganglion aggregations near the utricle and saccule. Since none of these markers ever appear in neural crest derived Schwann cells or in fibroblasts but at least Atoh1 is known to be expressed in sensory neurons [17], we suggest that all three cell types found in wild-type ear ganglia is the sensory neurons that are converted to hair cells. We name these hair cells as ‘intraganglionic hair cells’. Further work is needed to analyze in details the transformation of sensory neuron precursors into ‘intraganglionic hair cells’ and demonstrate the suppression of Atoh1 by Neurod1 at the molecular level either within a given cell or between cells via the delta/notch system.

Neurod1 helps to organize the organ of Corti by controlling spatiotemporal gene expression

Previous work showed that Neurod1 is expressed in hair cells [16,17] in a shorter and disorganized cochlea [15,50] of Neurod1 null mice. It has also been noted that some of the first row of OHCs obtain inner hair cell like appearance [16] and the IHCs may form multiple disorganized rows. We demonstrate for the first time the degree of disorganization of the cochlear apex with the formation of ectopic IHCs in place of OHCs in Neurod1 CKO mice and show the complete absence of OHC formation in the most apical part of the cochlea. We show expression of IHC marker genes in cells that topologically should be outer hair cells and the histological alteration of these cells such as diameter of stereocilia. Neurod1 mimics Neurog1 with respect to shortening, disorganization and gene expression alteration in the cochlea [14]. Like in Neurog1 null mice, Atoh1 is prematurely upregulated in the apex of Neurod1 CKO mice, suggesting a fate change of common precursors toward hair cells [17]. The most reduced sensory epithelia in Neurog1 null mice is the saccule [14,31], now known to be affected because of lineage relationship of saccular neurons and hair cells [20].

In contrast, Neurod1 CKO mice show the most profound size reduction in canal cristae and cochlea (Table 1, Fig. S1B,D,D'). Effects of Neurod1 on overall growth are thus not simply a milder extension of Neurog1 effects. It is possible that simple premature expression of Atoh1 and its downstream genes disrupts convergent extension [68] and thus leads to the observed histological alteration of the cochlear apex. This is in agreement with the complete extension of the cochlea in the absence of Atoh1 expression and any differentiation of hair cells [31]. Other mutants with reduction in growth and multiple rows of hair cells show no mixing of inner and outer hair cells [9,14]. Combined with the enhanced cell death as early as E9.5 in Neurod1 null mice [29], the early appearance of truncated growth in the cochlea suggests that common neuronal/hair cell precursors may die in the absence of Neurod1, reducing the growth of the organ of Corti and canal cristae. The expression of other bHLH genes such as Nhlh1 and Nhlh2 may rescue some common neurosensory precursors in the utricle and saccule resulting in near normal size growth. What additional gene(s) may mediate these differential sensory epithelia effects is unknown.

How can Neurod1 affect neuronal and hair cell differentiation?

Neurod1 regulates several genes involved in hair cell differentiation. Atoh1, Pou4f3, Fig8 and Nhlh1 are prematurely expressed in the apical half of the cochlea in Neurod1 CKO mutants (Fig. 9A,A') and appear in hair cells within the sensory ganglia of the ear. There is also a transient change in Sox2 expression and in Neurog1 expression. Our results are best compatible with a suggestion that Neurod1 expression in neurosensory precursors suppresses specific downstream genes (Atoh1, Pou4f3, Nhlh1, Fig8, Sox2) necessary for general neurosensory and specific hair cell differentiation (Fig. 9B,B'). For example, the upregulation of Fig8 in some ‘outer hair cells’, which may change their fate to ‘inner hair cells’, suggest a more specific function of Neurod1 in regulation of Fig8. The effect of Neurod1 on Neurog1 is likely due to a direct, intracellular feedback loop (Fig. 9B,B') and is in line with previous reports of such a
feedback loop in olfactory receptor cell development [69]. In contrast, the effect of Neurod1 on Atoh1 expression could be either directly in the same cell as in the cerebellum [26] or could be mediated through an intermediary such as Fgf8, Sox2 or an as yet to be determined factor within or between cells. Further analysis of other developing systems in which Neurod1 and Atoh1 are sequentially expressed or co-expressed, such as the dorsal cochlear nucleus [70] or the enteroendocrine intestine cells [71], are needed to establish generality of this feedback loop beyond the ear and the cerebellum.

Cross-regulation of Neurog1 and Atoh1 have been proposed for the spinal cord [72] and the mammalian ear [20] in which hair cells are massively reduced in Neurog1 null mice [14]. However, in none of these cases has the interaction been directly demonstrated at the cellular or molecular level. Our data on the effect of Neurod1 CKO mutants suggests that Neurod1 is at least in the ear an intermediate factor that mediates such cross-inhibitory interactions between Neurog1 and Atoh1 (Fig. 9B, B'). The differences in effects of either loss of Neurog1 or Neurod1 on overall hair cell formation and specific hair cell developmental changes suggests that other downstream factors specific to Neurog1 or Neurod1 must exist that also mediate such cross-inhibitory interactions. Fgf8 and possibly Sox2 seem to be appropriate candidates to play this role. Further analysis of expression of these and other genes now identified as being changed in Neurod1 CKO mice are needed for Neurog1 null mice to fully understand the complexity of interaction of the genes in this developing system.

In summary, we propose that inner ear development resembles other developing systems insofar as sophisticated interactions of bHLH genes determine neuronal fate [3,73]. In cooperation with other genes known to be expressed in the developing sensory neurons and hair cells [3,4,5,74,75], Neurod1 may achieve neuronal differentiation not only through upregulation of appropriate downstream target genes [62] but also through suppression of other bHLH genes that mediate other states of cellular differentiation such as Neurog1 [69] or Atoh1 [26]. In the absence of Neurod1, several cell fate determining genes that would normally be suppressed are prematurely or continuously expressed (Figs 4, 9). These expression changes result most likely in a cell fate change of neurosensory precursors into topologically inappropriate cells such as ‘intraganglionic hair cells’ and ‘ectopic inner hair cells’ (Fig. 9B'). Through some of the 500 genes directly regulated by Neurod1 [62], Neurod1 may actually mediate the cross-regulation of Neurog1 and Atoh1 as recently suggested [20]. Our findings show a more refined action of Neurod1 in the developing ear than the previously suggested simple effects on neuronal survival and differentiation [15,16,29]. Neurod1 may interact with other genes expressed during neurosensory development [36], likely mimicking better described systems in the complexity of their interaction [76] particularly at the promoter level [77]. Fully understanding this interplay is

**Figure 9. Neurod1 regulates neuronal differentiation by suppression of premature hair cell differentiation of neurons possibly interacting with several target genes.** In the absence of Neurod1 several hair cell specific genes such as Myo VIIa, Atoh1, Pou4f3 and Nhlh1 are prematurely expressed with an inverse gradient of apex-to-base progression of hair cell differentiation instead of usual base-to-apex progression (cochlear expression shown with bars in A, A'). In addition, these genes are also expressed ectopically in the differentiating vestibular ganglia near the utricle and saccule. This substantial alteration of gene expression changes the organization of the apical part of the cochlea and results in the formation of ‘intraganglionic hair cells’. Our data and those of others suggest the following interaction of Neurod1 with Neurog1, Atoh1, Sox2 and Fgf8 to regulate inner ear cellular identity (B, B'). We propose that after early and transient activation of Neurod1 by Neurog1 and Atoh1 to differentiate neuron, Neurod1 suppresses Neurog1 to inhibit precursor proliferation and Atoh1 to inhibit hair cell differentiation in neurons. These three way interactions result in formation of neurons with delayed hair cell differentiation. Cross inhibition of Neurod1 and Atoh1 was previously suggested [17, 20] and we suggest that Neurod1 is a key intermediary player. Neurod1 also regulates other cell fate determining genes like Sox2 and Fgf8 which may more directly related to the observed cell fate switch. We suggest that Neurod1 deletion in early embryos disrupts this gene network and, as a consequence, the coordinated sequential neurosensory development of inner ear resulting in the transformation of some surviving neurons into ‘intraganglionic hair cells’ and alteration of the cell type specific differentiation of outer hair cells in the cochlea.

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necessary to allow, through regulation of the levels of expression of Neurod1, the generation of intraganglionic hair cells in deaf patients. Such ‘intraganglionic hair cells’ could imitate regular hair cells and sustain long-term cochlear implant function by maintaining viable neurons.

Supporting Information

**Figure S1** Atoh1 in situ hybridization shows a progressive base-to-apex reduction in wild-type cochlea (A, A’, A’’) with faded expression in IHC and outermost OHCs. In contrast, Atoh1 remains uniformly expressed throughout the cochlea in Neurod1 CKO mice (B, B’, B’’). Direct comparison of vestibular sensory epithelia shows that canal cristae are more reduced than utricle and saccule (C–F) which show mostly alterations in shape. Such qualitative changes are also apparent in anterior canal cristae where hair cells form in the non-sensory region of cruciate eminence in absence of Neurod1 (arrow in G,H). AC, anterior canal cristae; HC, horizontal canal cristae; S, saccule; U, utricle. Bar indicates 100 μm.

**Figure S2** Our data suggest that Neurod1 specifies neurosensory precursors by refining cellular identity during early embryonic stage. We confirm this assumption studying the delayed knock out of Neurod1 using Tg (Atoh1cre), a CKO mutation that results in massive cerebellar defects (26). The in situ hybridizations of Atoh1 and Fgf8 show normal organization of inner ear with four rows of hair cells throughout the cochlea (A–B). We conclude that once Atoh1 has regulated its downstream target genes to specify the hair cell precursor’s fate, later loss of Neurod1 cannot alter hair cell differentiation. IHC, inner hair cell; OHC, outer hair cell. Bar indicates 100 μm.

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