The Prosensory Function of Sox2 in the Chicken Inner Ear Relies on the Direct Regulation of Atoh1

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Abstract

The proneural gene Atoh1 is crucial for the development of inner ear hair cells and it requires the function of the transcription factor Sox2 through yet unknown mechanisms. In the present work, we used the chicken embryo and HEK293T cells to explore the regulation of Atoh1 by Sox2. The results show that hair cells derive from Sox2-positive otic progenitors and that Sox2 directly activates Atoh1 through a transcriptional activator function that requires the integrity of Sox2 DNA binding domain. Atoh1 activation depends on Sox transcription factor binding sites (SoxFBBS) present in the Atoh1 3’ enhancer where Sox2 directly binds, as shown by site directed mutagenesis and chromatin immunoprecipitation (ChIP). In the inner ear, Atoh1 enhancer activity is detected in the neurosensory domain and it depends on Sox2. Dominant negative competition (Sox2HMG-Engrailed) and mutation of the SoxFBBS abolish the reporter activity in vivo. Moreover, ChIP assay in isolated otic vesicles shows that Sox2 is bound to the Atoh1 enhancer in vivo. However, besides activating Atoh1, Sox2 also promotes the expression of Atoh1 negative regulators and the temporal profile of Atoh1 activation by Sox2 is transient suggesting that Sox2 triggers an incoherent feed-forward loop. These results provide a mechanism for the prosensory function of Sox2 in the inner ear. We suggest that sensory competence is established early in otic development through the activation of Atoh1 by Sox2, however, hair cell differentiation is prevented until later stages by the parallel activation of negative regulators of Atoh1 function.

Introduction

The inner ear provides the brain with accurate information on mechanical perturbations that result in the perception of sound and balance. Mechano-electrical transduction is initiated by the highly specialized hair cells, which transmit electrical signals to the primary afferent neurons that convey this information to the brain. There is good evidence that hair cell fate depends on the function of the proneural factor Atoh1, that behaves as a master gene for hair cell differentiation [1,2,3]. Atoh1 is an Helix-Loop-Helix (HLH) transcription factor regulated through a positive autoregulatory loop that maintains its expression in the sensory precursors [4,5], and through the negative regulation of other HLH proteins that prevent Atoh1 expression and function [3,6,7,8,9,10,11,12]. Yet, the molecular mechanisms underlying the onset of Atoh1 expression remain obscure.

Sox2 is a High Mobility Group (HMG) box domain transcription factor that belongs to the B1 subfamily of Sox proteins [13] and it behaves as a transcriptional activator [14]. Sox2 shows two seemingly contradictory functions in the developing inner ear. On one hand, it is expressed in neurogenic and sensory progenitors [15,16,17] and it is necessary for hair cell development [18]. Misexpression of Sox2 results in an increased number of neurons and ectopic hair cells [19,20]. On the other hand, Sox2 counteracts Atoh1 function and prevents hair cell formation when over-expressed in sensory precursors [21]. This is reminiscent of the function of SoxB1 genes in the Central Nervous System (CNS), where they promote neural competence but prevent neuronal differentiation [22,23,24]. Since neural commitment depends ultimately on the expression of proneural genes, the general question arises as to how Sox2 regulates proneural gene function.

In the present work, we show that Sox2 directly activates Atoh1 transcription in the early otic vesicle, providing a molecular mechanism for the prosensory function of Sox2 in the inner ear. Besides, we found that Sox2 regulates Atoh1 through an incoherent logic that promotes the expression of both Atoh1 and Atoh1 negative regulators. We suggest that as a result of this dual interaction, otic progenitors are committed to sensory fate early in development, but their differentiation deferred until later stages.

Methods

Plasmids and constructs

The NOP2-EGFP contains EGFP under the control of Sox2 nasal and otic enhancer [25]. Atoh1enh-BG-EGFP and Atoh1enh-BG-ZA (J. Johnson Lab, Dallas, USA) contain the 1.4 kb Atoh1 enhancer region 5’ to the β-globin basal promoter, the EGFP
or lacZ coding regions, respectively, and SV40 polyadenylation sequences [4]. The Atoh1enhmut-BG-EGFP and Atoh1enhmut-BG-ZA are similar to Atoh1enh-BG-EGFP and Atoh1enh-BG-ZA but each contains three point mutations in the SoxTFBS (see below, site-directed mutagenesis). Either pCMV/SV1-cSox2 or mSox2pCDNA3 (P. Scolding lab, Nottingham, UK) were used for Sox2 misexpression in vivo and in vitro with similar results. The pCMV/SV1-cSox2HMG-VP16/Engrailed has the C-terminal domain of Sox2 coding region (aa 184 till C-terminal) replaced by the VP16 trans-activator domain/Engrailed repressor domain. The pCMV/SV1-cSox2AHMG has the HMG domain (aa 3-202) removed. pDRed (Clontech), pCIG-EGFP (Elisa Marti, Barcelona, Spain) and pCMV-luciferase (R.Perona, Madrid, Spain) were used as controls for electroporation domains and cell transfection levels.

Site directed mutagenesis

The mutated reporter constructs Atoh1enhmut-BG-EGFP and Atoh1enhmut-BG-ZA were generated using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene). Briefly, mutually complementary primers (Invitrogen, sequence available upon request) aligning with the region of the Atoh1 enhancer containing the SoxTFBS were designed according to the manufacturer’s instructions to create three point mutations. The mutated reporter construct was replicated in a PCR reaction and the parental DNA digested with DpnI. Undigested mutated constructs were amplified in bacterial hosts and sequenced to detect the insertion of the desired mutation before using in subsequent functional assays.

Chicken (Gallus gallus) embryos and in ovo electroporation

Fertilized hens’ eggs (Granja Gibert, Tarragona, Spain) were incubated at 38°C for designated times and embryos were staged according to Hamburger and Hamilton [26]. HH11-14 chicken embryos were electroporated in ovo with the desired vector (1 µg/µl for Sox2 expression vectors, 1.5 µg/µl for Atoh1 reporter; 2 µg/µl for Sox2 reporter) mixed with fast green (0.4 mg/ml) that were injected onto theotic cup by gentle air pressure through a fine micropipette. Square pulses (8 pulses of 10 V, 50 Hz, 250 ms) were generated by an electroporator Square CUY-21 (BEX Co., Ltd, Tokiwasaianseu, Japan). Focal electroporation of HH12-21 otic vesicles was performed in ovo, using a method modified from Chang et al. [27].

HEK293T cell transfection

HEK293T cells were cultured in DMEM supplemented with glutamine, antibiotics and 10% fetal bovine serum. Before transfection, cells were cultured in serum and antibiotics-free medium. For transfection, the DNA was mixed with Polyethylene-imimine 1 mg/ml (PEI, Polysciences Inc, PA, USA) at the ratio of 4 µl of PEI/µg of DNA, incubated twenty minutes at room temperature and finally added to the cell culture. For Atoh1 enhancer activity assays, 1 µg of Sox2 expression vector (or Sox2HMG-VP16 or Sox2AHMG) was co-transfected with 0.5 µg of Atoh1 enh-BG-ZA and 0.2 µg of pCMV-Luciferase for βGal activity assays, or 0.5 µg of Atoh1enh-BG-EGFP and 0.2 µg of pDsRed for direct fluorescence assays. For Western blot and qRT-PCR analysis, 1 µg of Sox2 expression vector was co-transfected with 0.2 µg of pCIG-EGFP.

Immunohistochemistry

Embryos were sectioned and processed according to Neves et al. [15]. Primary antibodies were: β-Jag1 rabbit polyclonal (Santa Cruz Biotechnology, Inc, sc-8303, H-114,1:50); α-GFP mouse monoclonal (Invitrogen, 1:400); α-GFP rabbit polyclonal (Clontech, 1:400); α-Sox2 goat polyclonal (Santa Cruz Biotechnology, Inc, sc-17320, Y-17, 1:400); α-MyoVIImouse monoclonal (DSHB, 39.4D5, 1:400) and α-MyoVIImouse monoclonal (gift of Guy Richardson, D10, 1:500). Secondary antibodies were Alexa Fluor488, -594 and -568 conjugated and HRP-conjugated anti-goat or anti-rabbit (Dako, 1:500). HRP staining was developed with DAB substrate (Sigma). Sections were counterstained with DAPI (100 ng/ml, Molecular Probes) and mounted in Mowiol media (Calbiochem). Fluorescence was analyzed in whole embryos and in 20 µm cryostat sections by conventional fluorescence microscopy (Leica DMRB Fluorescence Microscope with Leica CCD camera DC300F). Images were processed with Adobe Photoshop.

Quantitative real time PCR (qRT-PCR)

Eight to twelve otic vesicles were dissected and total RNA isolated using RNeasy Mini kit (Qiagen). For HEK293T cells, total RNA from 6-well plates was isolated with a standard Trizol extraction (Invitrogen). Retrotranscription of 15 ng (chicken samples) or 1 µg (HEK293T samples) of purified mRNA was used to synthesize cDNA with Superscript III DNA polymerase (Invitrogen) and random primers (Invitrogen). Real time PCR was carried out using SybrGreen master mix (Roche), 1 µl of retrotranscribed cDNA and specific primers sets for each gene (Invitrogen, primer sequences are available upon request), in LightCycler480 (Roche). GAPDH and hPum1 were used as calibrator genes for chicken and HEK293T samples, respectively. Expression levels of each gene were normalized to the calibrator gene and then referred to the levels in control samples, which were arbitrarily set to 1. Transcription levels were further normalized to co-transfected GFP. Quantitative real-time PCR experiments were performed with cDNA from three independent biological replicates.

βGal and luciferase enzymatic assays

Protein extracts from cells were prepared using Reporter Lysis buffer (Promega) according to the manufacturer’s instructions. For βGal activity, triplicates of each protein extract (10 µl) was mixed with 90 µl βGal staining solution (100 mM PBS, 100 mM MgCl2, 4 mg/ml ONPG, 4,5 M βmercaptoethanol) in a 96-well ELISA plate and incubated for 2–20 h at 37°C. βGal activity was determined by the absorbance at 420 nm in a microplate reader (VERSAnax, Molecular Devices, Cape Cod). For luciferase activity, 10 µl of each protein extract was mixed with 20 µl of Luciferase Assay Reagent (Promega) and activity was determined with a Luminescence Microplate Reader (Clarity, BioTek). For each well, βGal activity was normalized for the level of transfection using luciferase activity and then the values in transfected samples were referred to the corresponding control, which was arbitrarily set to 1. Enzymatic activity was measured with protein extracts from three independent biological replicates.

Western Blot

Protein extracts were prepared using a mild protein extraction buffer (PBS-EDTA 1 mM, Na2VO4 100 µM, β Glycerophosphate 20 mM, PMSF 0.2 mM, 0.5% Triton). Proteins were separated in 12%polyacrylamide gels and transferred to a PVDF membrane (Immobilon-P, Millipore). Membrane was blocked with 5% milk in Tris buffered saline with 0.1% Tween (TBST) and incubated overnight at 4°C with primary antibodies diluted in 1% milk in TBST, with gentle shaking. Membranes were washed with TBST, incubated with secondary antibodies, washed first with
of either Goat IgG (Purified Immunoglobulin, Sigma, I9140) or α-Sox2 goat polyclonal antibody (Santa Cruz Biotechnology, Inc., sc-17320, Y-17, 1:500) or α-GFP rabbit polyclonal antibody (Clontech, 1:1000) and α-Tubulin monoclonal (Sigma, 1:2000). Secondary antibodies were HRP-conjugated donkey anti-goat or anti-rabbit (Jackson ImmunoResearch Laboratories, Inc, 1:5000) and HRP-conjugated rabbit anti-mouse (Dako, 1:2000).

Chromatin Immunoprecipitation (ChIP)

HEK293T cells or dissected otic vesicles were processed for ChIP as previously described [28]. Briefly, formaldehyde cross-linked cell or tissue extracts were sonicated in a Bioruptor (Diagenode), and the chromatin fraction incubated overnight with 5 μg of either Goat IgG (Purified Immunoglobulin, Sigma, 19140) or α-Sox2 goat polyclonal antibody (Santa Cruz Biotechnology, Inc., sc-17320, Y-17) in RIPA buffer, and precipitated with protein A/G-Sepharose (Amersham). Cross-linkage of the co-precipitated DNA-protein complexes was reversed, and DNA was analyzed by qRT-PCR as described above. Primers used to detect the different regions of chromatin are available upon request.

Results analysis and statistics

qRT-PCR analysis, reporter enzymatic activity and in vitro ChIP assays were performed with three independent biological replicates. In vivo ChIP assays were performed with two independent biological replicates. The results are shown as mean±SE for one typical experiment, and statistical significance was assessed using Student’s t test applied to the three independent experiments. p<0.001 is labeled with ***, p<0.005 is labeled with ** and p<0.05 is labeled with *. n.s., non significant.

Results

Hair cells and neurons derive from Sox2-positive progenitors

Previous work suggests that Sox2 promotes the competence to generate neurons and hair cells in the otic vesicle [19,20]. This predicts that in the embryo, both cell types derive from Sox2-positive progenitors. To analyze this possibility, we electroporated the NOP-2-EGFP in HH12 chicken embryos and followed the fate of the progeny with specific markers. The NOP-2-EGFP construct contains the EGFP reporter gene under the control of a Sox2 enhancer that drives expression specifically in otic and nasal placodes [25]. The stability of EGFP provides a cumulative labeling of cells that expressed Sox2 throughout the experiment and, hence, the lineage of Sox2-expressing progenitors (Fig. 1A–F).

In 11 samples, EGFP-positive cells were detected both in the prosensory domain (compare B and C) and in the cochleovestibular ganglion (dotted line, B). Neuronal fate of the Sox2 progeny was confirmed by co-labeling with Islet1 antibody (D, n = 4), and that of hair cells by co-labeling with MyoVIIa and Hair Cell Specific (HCA) antibodies (E and F, n = 4). The results indicate that both hair cells and neurons derive from Sox2-positive progenitors.

Sox2 induces the transcription of Atoh1

Hair cell formation depends on the function of the proneural gene Atoh1 [3], but it is not known which factors regulate the onset of Atoh1 expression in the ear. Since Sox2 function is required for Atoh1 expression and hair cell formation, we asked whether Sox2 was able to induce Atoh1 expression. HEK293T cells were used as a convenient model system for analysis of molecular interactions before testing their biological significance in vivo. HEK293T cells endogenously expressed Atoh1 and Sox2 mRNAs and proteins (Fig. 2A upper). Accordingly, Atoh1 transcriptional activity was detected after transfection with either EGFP or LacZ. Atoh1 reporter constructs (Fig. 2A, middle photograph and bar diagram, respectively). They contain the reporter genes under the control of Atoh1 enhancer elements that reside 3′ of the Atoh1 coding sequence and are sufficient to recapitulate the endogenous Atoh1 expression in several species, including the chicken [4,29,30]. Overexpression of Sox2 increased Atoh1 enhancer reporter activity as measured either by βGal activity on cell extracts (Fig. 2B, left bar diagram) or by EGFP fluorescence (Fig. 2B, photographs on the bottom left), confirming previous observations by Neves et al. [20]. Similarly, Sox2 transfection resulted in an increase in

Figure 1. Tracing Sox2-positive progenitors. A–C, Coronal section of an HH22 otic vesicle electroporated with pDsRed (A) and NOP-2–EGFP (B) at HH12 and immunostained for Jag1 (C). The dotted line labels the cochleovestibular ganglion (CVG). The arrow indicates an electroporated domain, outside the Jag1-positive region, where the reporter is not active. D–F, Detail of the electroporated epithelium showing the co-localization of EGFP driven from the NOP-2 reporter with Islet1 in neurons (D), and with MyoVIIa (E) and HCA (F) in hair cells. Arrows indicate double labeled cells. A, anterior; M, medial.

doi:10.1371/journal.pone.0030871.g001

The Regulation of Atoh1 by Sox2

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doi:10.1371/journal.pone.0030871.g001
endogenous Atoh1 mRNA levels (Fig. 2B, middle bar diagram) and in Atoh1 protein (Fig. 2B, low-right).

Since Sox2 is an activator transcription factor [14], the effects of Sox2 on Atoh1 transcription should be dependent on both DNA-binding and transcriptional activator function. HEK293T cells were co-transfected with the Atoh1 reporter and with either Sox2HMG-VP16 or Sox2ΔHMG (Fig. 2C, left diagram). The Sox2ΔHMG lacks the DNA binding domain and its co-transfection had no effect on Atoh1 reporter activity (Fig. 2C, grey bar). This shows that the regulation of Atoh1 requires the binding of Sox2 to DNA. The Sox2HMG-VP16 construct contains the Sox2 DNA binding domain fused to a potent trans-activator domain. The co-transfection with Sox2HMG-VP16 reproduced the effects of Sox2 on Atoh1 (Fig. 2C, blue bar).

Figure 2. Sox2 induces Atoh1 expression. A, Endogenous expression of Sox2 and Atoh1 in HEK293T cells. RT-PCR and Western blot showing the endogenous expression of Sox2 and Atoh1 mRNA and protein, respectively (top). Direct green fluorescence in HEK293T cells transfected with Atoh1enh-BG-EGFP (middle). βGal activity in protein extracts of HEK293T cells transfected with Atoh1enh-BG-ZA (bottom). B, Sox2 induces Atoh1 expression in HEK293T cells. Relative βGal activity in HEK293T cells co-transfected with Sox2 and Atoh1enh-BG-ZA one day after transfection (top, left bar diagram). Relative mRNA levels of Atoh1 and Sox2 in HEK293T cells transfected with Sox2 for one day (middle and right bar diagrams). Direct green and red fluorescence in HEK293T cells co-transfected with pDsRed (for transfection level control) and Atoh1enh-BG-EGFP (bottom left). Western blot analysis of HEK293T protein extracts one day after Sox2 transfection showing Atoh1 protein induction (bottom right). Endogenous Sox2 protein levels were too low to be detected in the same blot. All techniques show an induction of Atoh1 after Sox2 transfection. C, Atoh1 regulation depends on the function of Sox2 as a transcriptional activator. Structure of the Sox2 mutant constructs used in the experiment (left, see Methods). Analysis like in Fig. 2B, showing the relative βGal activity in HEK293T cells co-transfected with Atoh1enh-BG-ZA and Sox2ΔHMG (grey) or Sox2HMG-VP16 (blue) (right graph). Deletion of DNA binding domain eliminates the effects on Atoh1 enhancer activity while Sox2HMG-VP16 reproduces the effects of Sox2.

doi:10.1371/journal.pone.0030871.g002
These experiments show that Sox2 is able to induce Atoh1, that this depends on the function of Sox2 as an activator transcription factor, and that it requires Sox2 binding to DNA.

**Sox2 directly binds to the Atoh1 enhancer**

In order to test the possible binding of Sox2 to the Atoh1 regulatory regions, the enhancer sequence of Atoh1 was screened using Transfac database in rVista software and two overlapping Sox Transcription Factor Binding Sites (SoxTFBS) were found. They were conserved among human, mouse and chicken, mapping to the 3’ end of the Atoh1 enhancer A (Fig. 3A). In order to test the interaction between Sox2 and these binding sites, we performed a ChIP assay. Chromatin from HEK293T cells was immunoprecipitated with a Sox2 antibody and analyzed for the presence of the SoxTFBS with specific primers for the corresponding region of the Atoh1 enhancer. As controls, we used two regions located 5 kb upstream and downstream of the binding sites. Chromatin precipitated with Sox2 antibody was enriched in the SoxTFBS region of Atoh1 enhancer when compared to the chromatin precipitated with a goat IgG antibody (Fig.3B). Furthermore this enrichment was specific for this region of the chromatin and not detected in the control sites (n = 3).

Site-directed mutagenesis was used to evaluate whether the induction of Atoh1 by Sox2 was dependent on binding to these SoxTFBS. Briefly, we introduced three point mutations in the Atoh1 enhancer reporter construct, which destroys the ability of Sox2 to bind to the conserved SoxTFBS (Fig. 3C, left diagram). Co-transfection of Sox2 with the mutated Atoh1 enhancer reporter reduced βGal activity to half of the value obtained after co-transfection with the native Atoh1 enhancer reporter (Fig. 3C, right bar diagram, n = 3, native and mutated reporter activities compared in the same experiment). Interestingly, the mutation of SoxTFBS did not result in the complete reduction Atoh1 reporter activity to control values. This is likely due to the induction of endogenous Atoh1 protein after Sox2 transfection (See Fig.2B). Atoh1 is able to regulate its own expression through the ‘E-

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**Figure 3. A, In silico analysis of the 3’ Atoh1 enhancer.** Atoh1 locus and regulatory sequences as described in Helms et al. (2000). Arrow indicates the location of the consensus SoxTFBS in the 3’ end of the enhancer sequence A. Green represents the consensus TFBSs and black the immediate flanking sequence. Two lines are used to represent the same sequence in order to undisclosed the two overlapping sites. +5 and −5 label the regions used as controls in the Chip experiment. The table summarizes the location of Atoh1 gene and the 3’ enhancer in three different species and the conserved location of the SoxTFBS. B, ChIP assay in HEK293T cells. The bar diagram shows the relative amount of chromatin precipitated with Sox2 (blue) with respect to IgG (grey) containing the three different regions of the Atoh1 locus indicated in the cartoon. Chromatin precipitated with Sox2 was significantly enriched in the SoxTFBS of the Atoh1 enhancer. C, Site directed mutagenesis of the SoxTFBS. Three point mutations were introduced in the Atoh1 enhancer reporter construct and are indicated in red (left diagram). Bar diagram to the right shows the relative βGal activity in HEK293T cells co-transfected with Sox2 and Atoh1enhmut-BGZA compared to the native Atoh1 reporter. The mutated reporter activity was reduced to half.

doi:10.1371/journal.pone.0030871.g003
box' in the Atoh1 enhancer [4], and this site was intact in the construct.

In summary, these experiments show that Sox2 directly binds to the Atoh1 enhancer and that the regulation of Atoh1 by Sox2 is, at least in part, mediated by the SoxTFBS present in the Atoh1 regulatory regions.

Atoh1 transcriptional activity in the otic vesicle depends on Sox2

Sox2 is expressed throughout the neurosensory domain of the otic vesicle [15] and we sought to analyze whether this is able to activate Atoh1 transcription, by using the Atoh1enh-BG-EGFP reporter in vivo. Otic vesicles were electroporated with this reporter construct together with the tracer pDsRed (Fig. 4A–D). Atoh1 reporter was active in the otic vesicle but spatially restricted to the anterior-medial domain (Fig. 4A and C, n = 19 otic vesicles), corresponding to the Sox2-positive expression domain (Fig. 4D, n = 10 otic vesicles). Note that electroporated cells in the surface ectoderm and lateral aspect of the otic vesicle remained GFP-negative (asterisk in Figs. 4A–B and H–I). Reporter activity was also detected in the neuroblasts of the cochleo-vestibular ganglion, which is consistent with the previous observation these neurons derive from Sox2-positive progenitors (see Fig.1) and suggests that Atoh1 transcription is also activated by Sox2 in this type of progenitors (arrows in Fig. 4C). Later in development, the activity of the reporter was restricted to the nascent hair cells within the sensory patches (HH24, Fig. 4E–G, n = 11 otic vesicles).

We next tested whether the observed Atoh1 reporter activity in the otic vesicle indeed depended on Sox2 (Fig. 4H–O). For this purpose we co-electroporated Atoh1enh-BG-EGFP with Sox2HMG-Engrailed, which suppresses Sox2 function as a dominant negative [23]. This resulted in the suppression of the Atoh1 reporter activity (Fig. 4H–K) suggesting that the early activation of Atoh1 transcription is dependent on Sox2. Furthermore, the electroporation of the Atoh1enh-BG-EGFP reporter construct carrying the mutation in the SoxTFBS (Atoh1enhmut-BG-EGFP, see Fig. 3C)

Figure 4. A–D, Atoh1 reporter activity in the early otic vesicle. Direct green and red fluorescence in coronal sections of a HH17 otic vesicle co-electroporated at HH12 with pDsRed (B) and Atoh1enh-BG-EGFP (A). Coronal section of an HH17 otic vesicle electroporated with Atoh1enh-BG-EGFP (C) at HH12 and immunostained for Sox2 (D). Reporter activity specifically restricted to the anterior-medial aspect of the otic vesicle (compare A and B) and it overlapped with Sox2 expression (compare C and D). Arrow in C indicates reporter activity in the cochleo-vestibular ganglion (CVG), and asterisks indicate the lack of reporter activity in the ectoderm. E–G, Atoh1 reporter activity in early sensory organs. Coronal section of a crista from an HH24 embryo co-electroporated with Atoh1 enhancer (E) and pDsRed (F) in HH20. The reporter activity restricted to the prosensory domain as shown by Sox2 immunochemistry (G). Figs. 4D and G are HRP staining pseudocolored in blue. H–O, The Atoh1 enhancer activity in the otic vesicle is Sox2 dependent. H–K, Direct red and green fluorescence in coronal sections of a HH17 otic vesicle electroporated at HH12 with the Sox2HMG-Engrailed (J, K, HMG-En) or without (H, I, control). Embryos were co-electroporated with pDsRed and Atoh1enh-BG-EGFP. Green fluorescence derived from the reporter is lost in the presence of Sox2HMG-Engrailed. As above, asterisks indicate that the enhancer was silent in the ectoderm. L–O, Direct red and green fluorescence in coronal sections of a HH17 otic vesicle electroporated at HH12 with pDsRed and Atoh1enhmut-BG-EGFP (N–O). An equivalent electroporation of the same reporter construct is shown for comparison (L–M). The mutation of the SoxTFBS resulted in the complete loss of reporter activity. A, anterior; L, lateral.

doi:10.1371/journal.pone.0030871.g004
for mutation) resulted in none (Fig. 4N, n = 17/23 otic vesicles) or very low (n = 6/23 otic vesicles) reporter activity in the otic vesicle. This was evaluated by comparing EGFP expression after electro-poration of the native Atoh1 reporter (Fig. 4L, n = 9 otic vesicles) and the mutated Atoh1 reporter (Fig. 4N, n = 23 otic vesicles) for otherwise equivalent electroporations (pDsRed in Fig. 4M and O). Together, these experiments suggest that Sox2 switches on Atoh1 transcriptional activity in the early otic vesicle.

Since Atoh1 transcription is active in the neurosensory domain of the otic vesicle, one critical question is whether Sox2 binds to the endogenous Atoh1 enhancer during normal development. In order to test this possibility, we performed ChIP assay in vivo on dissected otic vesicles, as illustrated in Fig. 5 (left). Indeed, there was a significant enrichment in the SoxTFBS region of the Atoh1 enhancer in the chromatin fraction immunoprecipitated with Sox2 when compared to precipitation with IgG (Fig. 5, upper bar diagram). Furthermore, this enrichment was specific to this region of the genome as the fraction of SoxTFBS precipitated with Sox2 antibody was significantly higher than the fraction of control region precipitated under the same conditions (Fig. 5, lower bar diagram). This demonstrates that in the early otic vesicle, Sox2 is bound to the Atoh1 enhancer.

In summary, the regulation of Atoh1 by Sox2 in the otic vesicle relies on the direct binding of Sox2 to the SoxTFBS in the 3′ regulatory region of Atoh1 enhancer.

The transient activation of Atoh1 and the induction of inhibitors: an incoherent logic?

The above results suggest that Atoh1 is directly activated by Sox2 at early developmental stages. However, Atoh1 expression during pre-differentiation stages is very low or negligible [3,31]. Several HLH factors like Hes/Hey, Ids, Neurog1 and NeuroD have been involved in the inhibition of Atoh1 expression during otic development [5,6,7,8,9,10,11,12,32], and sequence analysis reveals the presence of bHLH binding sites in the Atoh1′ regulatory regions [4]. Therefore, these factors are potential candidates to counteract the induction of Atoh1 by Sox2. Besides, Sox2 has been also associated with the negative regulation of Atoh1 and hair cell formation during ear development [21], a function that is reminiscent of that of SoxB1 genes in CNS development [23,24]. However, the mechanism behind this seemingly paradoxical situation in which Sox2 is able to both induce and counteract Atoh1 is unknown. In order to gain insight into this problem we explored further the regulation of Atoh1 by Sox2. A time course analysis of Atoh1 expression following Sox2 transfection in HEK293 cells revealed that Sox2 counteracts its own activator effect on Atoh1. Sox2 transfection induced only a transient activation of Atoh1 as measured either by Atoh1 βGal reporter activity or by qRT-PCR analysis of Atoh1 mRNA (Fig. 6A). The loss of Atoh1 transcription occurred even though Sox2 levels increased monotonically throughout the time window of the

Figure 5. ChIP assay in vivo. Diagram of the experimental design for the ChIP assay in vivo. Otic vesicles (500/experiment) were dissected from HH18 chicken embryos and processed for ChIP as indicated (left). Semi-quantitative RT-PCR of the ChIP assay in vivo (bottom left). Bands represent the fragments amplified with primers for SoxTFBS and for a control region using the input chromatin or the fractions precipitated with Sox2 and IgG as templates. qRT-PCRs of the ChIP assay performed on otic vesicles (right bar diagrams). The bar diagram on the top shows that the chromatin precipitated with Sox2 was significantly enriched in the SoxTFBS of the Atoh1 enhancer. The bar diagram on the bottom shows the percentage of input chromatin precipitated with Sox2 that contained the three regions analyzed. The fraction of input containing the SoxTFBS of the Atoh1 enhancer was significantly higher than the ones containing the control regions.

doi:10.1371/journal.pone.0030871.g005
Several mechanisms may account for this behavior, but the following data suggest that both activation and inhibition require DNA binding and the transcriptional activator function of Sox2. The co-transfection of Sox2^D_HMG (Fig. 6B, graph, grey) had no effect on \( \text{Atoh1} \) reporter activity, while the co-transfection of Sox2^HMG-VP16 (Fig. 6B, graph, blue) reproduced the effects of Sox2, both the early up-regulation of \( \text{Atoh1} \) and the delayed return to baseline. This suggests that the inhibition of \( \text{Atoh1} \) by Sox2 is indirect and requires intermediate factors that change the sign of the activator function of Sox2. Hence, the concurrent activation of inhibitor factors is a plausible explanation. The transient behavior of the \( \text{Atoh1} \) response to Sox2 is well described by a genetic network where a gene triggers parallel opposing effects on its target (Fig. 6B, right diagram), the Incoherent Feed Forward Loop (I-FFL) as modeled by Allon [33].

The above observations lead us to think that since \( \text{Atoh1} \) expression and hair cell formation in vivo correlate with Sox2 down-regulation [15], it is possible that Sox2 cooperates with other signaling pathways that maintain \( \text{Atoh1} \) expression tuned down during pre-differentiation stages. If such a mechanism operates in vivo, one would expect the activation of \( \text{Atoh1} \) inhibitory factors after the overexpression of Sox2 in the otic vesicle. Therefore, we explored the ability of Sox2 to induce these factors in the otic vesicle. Variables: Sox2, Atoh1, Hes, Hey, Neurog1, NeuroD.

**Figure 6. The transient activation of Atoh1 and the induction of Atoh1 inhibitors.**

A. The time course of Atoh1 activation. Relative βGal activity at different time points in HEK293T cells co-transfected with Sox2 and Atoh1enh-BG-ZA. For each time point βGal activity is referred as the fold increase with respect to reporter alone, which was arbitrarily set to one (dashed line, left graph). Relative mRNA levels of Sox2 (red) and Atoh1 (blue) at different time points, after Sox2 transfection (right graph).

B. The time course of the HMG-VP16 activation. Structure of the Sox2 mutant constructs used in the experiment (left, see Methods). Time course like in Fig. 6A showing the relative βGal activity in HEK293T cells co-transfected with Atoh1enh-BG-ZA and Sox2^HMG-VP16 (blue, left graph) or Sox2^D_HMG (grey, left graph). Deletion of DNA binding domain eliminates the effects on Atoh1 enhancer activity while Sox2^HMG-VP16 reproduces the effects of Sox2. Right diagram: Type1 Incoherent Feed Forward loop (I-FFL, Alon, 2007). The regulator X regulates Y and Z, which is both regulated by X and Y. However, the two arms of the FFL act in opposition and the effect is a transient activation of the target Z. C. Sox2 induces the expression of Atoh1 negative regulators in the otic vesicle. A. Bar diagram showing the relative mRNA levels of Id1-3 (left), Hes-Hey (middle) and Neurog1 and NeuroD (right) in otic vesicles transfected with control plasmids (grey bars) or with Sox2 (blue bars) for one day (Id-3 and Hes-Hey) or two days (Neurog1 and NeuroD). Untransfected otic vesicles (white bar). doi:10.1371/journal.pone.0030871.g006
vesicle. Indeed, Sox2 induced the expression of *Id1-3* (Fig. 6C, left bar diagram), *Hes5* and *Hey1* (middle bar diagram) and *Neurog1* and *NeuroD* (right bar diagram) in the otic placode. This indicates that in parallel to *Atoh1* induction, Sox2 activates and/or modulates the expression of other genes that counteract *Atoh1*. *Neurogenin1* is a direct target of Sox2 in other model systems [34,35], but it remains to be explored whether this also the case in the otic placode. *Ids* are regulated by BMP signaling [9], and *Hes5* and *Hey1* are downstream targets of Notch [11], but it is unknown whether Sox2 directly regulates these genes, or if it rather cooperates at other steps in the signaling cascades (see Discussion). In summary, these data suggest that in parallel to the activation of *Atoh1*, Sox2 induces an incoherent response by promoting the expression of *Atoh1* negative regulators.

**Discussion**

The prosensory function of Sox2: sensory commitment and deferred hair cell differentiation

Throughout evolution, the expression and function of the Sox2 correlates with the commitment to neural fate [36]. However, Sox2 prevents proneural gene function and neuronal differentiation [23,24]. This is also the case during ear development: Sox2 is necessary for sensory fate specification [18], and the misexpression of Sox2 results in increased number of neurons and ectopic hair cells [19,20]. However, Sox2 shows also an antagonistic function with *Atoh1* that results in the prevention of hair cell differentiation [21]. The aim of this work was to shed light on the mechanism behind this dual function.

The results show that, both in vitro and in vivo, Sox2 is able to directly activate *Atoh1* transcription by binding to the SoxTFBS in the 3’ *Atoh1* enhancer region, as shown by the functional experiments with the mutated reporter and by ChiP analysis. In the early otic vesicle, ChiP assay reveals that Sox2 is bound to the 3’ *Atoh1* enhancer and, moreover, the mutation of the SoxTFBS in the 3’ regulatory region of *Atoh1* suppresses the activity of the enhancer in the otic vesicle. This suggests that *Atoh1* transcription is switched on early in otic development, well before hair cell differentiation, and that Sox2 may be one of the factors involved in the initiation of *Atoh1* expression. Interestingly, this inductive function seems not to be conserved in non amniotes where Sox2 expression in otic progenitors and their function is associated with the prevention of *Atoh1* function and premature differentiation [7,9,10,11,12,43]. In fact, the *Atoh1* enhancer contains a series of bHLH binding sites, which may account for the negative regulation exerted by these genes [4]. Taken together, these factors exert multiple and diverse functions in neural development, but they share a common inhibitory action on *Atoh1* that results in the maintenance of the undifferentiated state of neurosensory progenitors.

**Sox2 activation of Atoh1 inhibitors: an incoherent loop?**

Our results show that Sox2 induces the expression of several of the above mentioned inhibitory factors. Although most of them are under the control of specific signaling pathways, Sox2 is nevertheless able to promote their expression. This indicates that Sox2 operates with an incoherent logic with respect to *Atoh1*: it both activates *Atoh1* and promotes its inhibition. Several network motifs have been studied by Alon [33] as a set of recurrent gene regulation patterns that result in predictable functional behaviors. The activation of *Atoh1* by Sox2 fits well with the so-called Type1 Incoherent Feed Forward Loop (I1-FFL) in which the two arms of the FFL act in opposition. The result is a transient target gene activation, with amplitude and timing dependent on the thresholds and time constants of the individual interactions, while the final steady-state level depends on the strength of the inhibition [33]. This type of model predicts well the transient nature of the response of *Atoh1* in the presence of continuously increasing concentrations of Sox2 mRNA in vitro. Indeed, the fact that the same behavior is induced by the Sox2-HMG VP16 construct indicates that the decay must be induced by intermediate factors that change the sign of the original signal. In our case, Sox2 directly activates *Atoh1* transcription but, on the other hand, Sox2 also up-regulates several inhibitors of *Atoh1* that include *Neurog1, NeuroD, Hes/Hey* and *Id* genes. This probably causes a balance between activation and inhibition that results in the observed profile of transient activation and steady-state down-regulation of *Atoh1*. This molecular interaction offers a simple explanation for the intriguing dual effects of Sox2: the induction of neural competence and prevention of differentiation. Further experiments will be required to demonstrate the detailed kinetics and the modulation of this genetic network and to describe the detailed mechanisms by which Sox2 modulates the expression of *Atoh1* inhibitors.

*Neurog1* has been previously described as a direct target of Sox2 in neural crest cells [35] and recent work suggests that this may the case also in the inner ear [34]. This provides support to the operation of a I1-FFL in which Sox2 directly regulates *Atoh1* and also its negative regulator *Neurog1*. However, it remains to be proven that this direct interaction operates in vivo in the otic vesicle. But nevertheless, the suggestion that the direct regulation of *Atoh1* may extend to *Neurog1* provides an interesting model for the function of Sox2 in the specification of the neurosensory competence of the otic placode, and the sequential generation of neurons and hair cells (see below).
The other Atoh1 inhibitors regulated by Sox2 are Hes5, Hey1, and Id1-3. They have never been described as primary Sox2 targets, and their regulation during inner ear development is mainly dependent on non-autonomous signaling. Although we cannot exclude the possibility that Sox2 regulates them directly, it is likely that Sox2 cooperates with the signaling pathways that regulate their expression. The regulation of Hes5 and Hey1 in the ear is mostly Notch-dependent [11]. Sox2 misexpression does not affect the expression of Notch ligands in the ear [21]. But in the otic vesicle Sox2 does result in the induction of Notch1 (Neves et al., unpublished data), and Notch1 has been identified as a direct target of Sox2 in the retina [44]. On the other hand, Id genes are regulated by BMP signaling in the inner ear [9]. Apart from Ids, Sox2 electroporation up-regulates several elements of the BMP pathway that are upstream Id transcription. This includes the Smad Interacting Protein 1 (SIP1, Neves et al., unpublished data), which has been identified as a potential Sox2 target by in silico analysis [45]. Taken together, the data suggest that unlike Atoh1 or Neurog1, Sox2 may regulate these other inhibitors by interacting with the signaling pathways that regulate their expression.

Neurosensorv competence and the sequential generation of neurons and hair cells in the inner ear

The problem of cell fate specification is central to neural development. How do different cell types with defined phenotypic characteristics originate from multipotent progenitors? The functional unit of the ear consists of three elements of neural origin: the mechanotransducing hair cells, the supporting cells, and the primary afferent neurons. All three elements derive from the neurosensorv competent domain of the otic vesicle and their development follows a stereotyped spatial and temporal pattern, with neurons being specified prior to hair cells [46,47,48].

Neuronal fate is specified by the expression of the proneural genes Neurog1 and NeuroD [6,49,50]. Sensory fate specification occurs after neurogenesis, and commitment to the sensory fate is associated with the expression of Atoh1 [1,3].

The observation that both neurons and hair cells derive from Sox2-positive progenitors fits well with the idea of the common origin of both cell types, as suggested by viral and genetic tracing [5,51]. How does Sox2 specify this dual competence in the otic progenitors? Sox2 is able to induce the expression of proneural genes Neurog1, NeuroD and Atoh1 [34 and present work] which would be sufficient, in principle, to specify neuronal and hair cell fates. But the question then is how these fates are sorted out, and why hair cell fate is delayed with respect to neuronal fate. One possibility is that Sox2 establishes neurosensorv competence early in development, by the activation of the major proneural genes Neurog1 and Atoh1. However, the down-regulation of Atoh1 by Neurog1 and NeuroD would allow neurogenesis but not hair cell differentiation. Cell fate decisions would thus depend on selective repression of the initial neurosensorv potential, rather than the temporal acquisition of new properties. It is not until Sox2 is counteracted that Atoh1 expression would be permitted in hair cells, but not in supporting cells. Daudet and co-workers have recently shown that Sox21 is expressed during hair cell differentiation and that it is able to inhibit Sox2 expression (N. Daudet, personal communication). A similar interaction between Sox2 and Sox21 was described in the neural tube [32].

In summary, Sox2 promotes sensory fate in the otic vesicle by direct binding to Atoh1 regulatory sequences. However, Atoh1 activation is deferred and Atoh1 up-regulation and hair cell differentiation do not occur until later developmental stages. One possible explanation for this dual effect is that Sox2 triggers an incoherent response that results in a steady-state inhibition of Atoh1. This would provide a simple explanation for the dual function of Sox2 in neural development, i.e.: promotion of neural competence and suppression of differentiation.

Acknowledgments

We thank Donna Fekete and Thomas Schimmang for reading the manuscript; Pau Formosa and Marta Ibanes for inspiring comments; Marta Linares, Miquel Sas, Ivan Vachikov for excellent technical assistance; and Jordi Guin, Maria Mulero, Lluís Espinosa, and Pura’s Lab (Pura Muñoz, CEXS UPF) for technical advice. Hisato Kondoh (GFSB-Osaka University) helped this project from its very initial steps. Jane Johnson (CBN, University of Texas) kindly shared reporters constructs with us. Guy Richardson (University of Sussex, Brighton, UK) kindly provided us with the HCA antibody and Isel1 and MyoVIIa monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA).

Author Contributions

Conceived and designed the experiments: JN FG. Performed the experiments: JN. Analyzed the data: JN AB FG. Contributed reagents/materials/analysis tools: MU AB. Wrote the paper: JN FG.

References

1. Bermingham NA, Hassan BA, Price SD, Vollrath MA, Ben-Arie N, et al. (1999) Math1: an essential gene for the generation of inner ear hair cells. Science 284: 1837–1841.
2. Zheng JL, Gao WQ (2000) Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. Nat Neurosci 3: 500–506.
3. Woods C, Montcouquiol M, Kelley MW (2004) Math1 regulates development of the sensory epithilium in the mammalian cochlea. Nat Neurosci 7: 1310–1318.
4. Helms AW, Abney AL, Ben-Arie N, Zoghi HY, Johnson JE (2000) Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. Development 127: 1183–1196.
5. Raft S, Koundakjian EJ, Quinones H, Jayasena CS, Goodrich LV, et al. (2007) Cross-regulation of Ngn1 and Math1 coordinates the production of neurons and sensory hair cells during inner ear development. Development 134: 4405–4413.
6. Mates V, Pauley S, Kaing S, Rowitch D, Belis EW, et al. (2005) Smaller inner ear sensory epithilium in Neurog1 null mice are related to earlier hair cell cycle exit. Dev Dyn 234: 633–630.
7. Taneja JM, Montcouquiol M, Dabdoub A, Woods C, Kelley MW (2006) Inhibitors of differentiation and DNA binding (Ids) regulate Math1 and hair cell formation during the development of the organ of Corti. J Neurosci 26: 550–558.
8. Jahan I, Pan N, Kersigo J, Fritsch B (2010) Neurod1 suppresses hair cell differentiation in ear ganglia and regulates hair cell subtype development in the cochlea. PLoS One 5: e11661.
9. Kamai A, Neves J, Giraldes F (2010) Id gene regulation and function in the sensory domains of the chicken inner ear: a link between Bmp signaling and Atoh1. J Neurosci 30: 11426–11434.
10. Zine A, de Ribaupeire F (2002) Notch/Notch ligands and Math1 expression patterns in the organ of Corti of wild-type and Hes1 and Hes5 mutant mice. Hear Res 170: 22–31.
11. Dootzhofer A, Basch ML, Ohayama T, Geseler M, Groves AK, et al. (2009) Hey2 regulation by FGF provides a Notch-independent mechanism for maintaining pillar cell fate in the organ of Corti. Dev Cell 16: 58–69.
12. Tateya T, Imai T, Tateya I, Ito J, Kageyama R (2011) Cooperative functions of Hes/Hey genes in auditory hair cell and supporting cell development. Dev Biol 352: 329–340.
13. Uchikawa M, Kamachi Y, Kondoh H (1999) Two distinct subgroups of Group B Sox genes for transcriptional activators and effectors: their expression during embryonic organogenesis of the chicken. Mech Dev 84: 103–120.
14. Nolting TK, Johnson LR, Wiebe MS, Rizzino A (2000) Identification of the transcriptional domain of the transcription factor Sox2 and an associated co-activator. J Biol Chem 275: 3810–3818.
15. Neves J, Kamaid A, Alima B, Giráldez F (2007) Differential expression of Sox2 and Sox3 in neuronal and sensory progenitors of the developing inner ear of the chick. J Comp Neurol 503: 487–500.

16. Mak AC, Sztio IY, Fritsch B, Cheah KS (2009) Differential and overlapping expression pattern of SOX2 and SOX5 in inner ear development. Gene Expr Patterns 9: 444–453.

17. Hume CR, Bratt DL, Oesterle EC (2007) Expression of LHX3 and SOX2 during mouse inner ear development. Gene Expr Patterns 7: 798–807.

18. Kiernan AE, Pelling AL, Leung KK, Tang AS, Bell DM, et al. (2005) Sox2 is required for sensory organ development in the mammalian inner ear. Nature 434: 1034–1035.

19. Palgilla C, Dabdoub A, Brenowitz SD, Kelley MW (2010) Sox2 induces neuronal formation in the developing mammalian cochlea. J Neurosci 30: 714–722.

20. Neves J, Parada C, Chamizo M, Giráldez F (2011) Jagged 1 regulates the restriction of Sox2 expression in the developing chicken inner ear: a mechanism for sensory organ specification. Development 138: 735–744.

21. Dabdoub A, Palgilla C, Jones JM, Fritsch B, Cheah KS, et al. (2008) Sox2 signaling in progeny stem domain specification and subsequent hair cell differentiation in the developing cochlea. Proc Natl Acad Sci U S A 105: 18396–18401.

22. Pevny LH, Sockanathan S, Placzek M, Lovell-Badge R (1998) A role for SOX1 in neural determination. Development 125: 1967–1978.

23. Bylund M, Andersson E, Novitch BG, Mohr J (2003) Vertebrate neurogenesis is constricted by Sox1-3 activity. Nat Neurosci 6: 1162–1168.

24. Graham V, Khudyakov J, Ellis P, Pevny L (2003) SOX2 functions to maintain neural progenitor identity. Neuron 39: 749–763.

25. Urchikawa M, Ishida Y, Take moto T, Kamachi Y, Kondoh H (2003) Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals. Dev Cell 4: 509–519.

26. Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. 1951. Dev Dyn 195: 231–272.

27. Chang W, Lin Z, Kulesza H, Hebert J, Hogan BL, et al. (2008) Bmp1 is essential for the formation of the vestibular apparatus that detects angular head movements. PLoS Genet 4: e1000650.

28. Robert-Moreno A, Guin J, Ruiz-Herguido C, Lopez ME, Inglese-Esteve J, et al. (2008) Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1. EMBO J 27: 1886–1895.

29. Ebert PJ, Timmer JR, Nakada Y, Helms AW, Parab PB, et al. (2003) Zic1 represses Math1 expression via interactions with the Math1 enhancer and modulation of Math1 autoregulation. Development 130: 1949–1959.

30. Timmer J, Johnson J, Niswander L (2001) The use of in vivo electroretroporation for the rapid analysis of neural-specific murine enhancers. Genesis 29: 121–132.

31. Pujades C, Kamad A, Alima B, Giráldez F (2006) BMP-signaling regulates the generation of hair-cells. Dev Biol 292: 55–67.

32. Li S, Mark S, Raddke-Gallwitz K, Schlusner R, Chin MT, et al. (2008) Hey2 functions in parallel with Hes1 and Hes3 for mammalian auditory sensory organ development. BMC Dev Biol 8: 20.

33. Alon U (2007) Network motifs: theory and experimental approaches. Nat Rev Genet 8: 450–461.

34. Jeon SJ, Fujisaka M, Kim SC, Edge AS (2011) Notch signaling alters sensory or neuronal cell fate specification of inner ear stem cells. J Neurosci 31: 8351–8358.

35. Cimadamore F, Fishwick K, Giusto E, Gneveka K, Cattarossi G, et al. Human ESC-derived neural crest model reveals a key role for SOX2 in sensory neurogenesis. Cell Stem Cell 6: 338–351.

36. Pevny L, Placzek M (2003) SOX genes and neural progenitor identity. Curr Opin Neurobiol 13: 7–13.

37. Sweet EM, Vemaruju S, Riley BB, Sox2 and Fgf interact with Atoh1 to promote sensory competence throughout the zebrafish inner ear. Dev Biol 358: 113–121.

38. Amara V, Perry P, Sauer S, Spivakov M, Jorgensen HF, et al. (2006) Chromatin signatures of pluripotent cell lines. Nat Cell Biol 8: 532–538.

39. Fischer A, Gesler M (2007) Delta-Notch and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. Nucleic Acids Res 35: 4503–4506.

40. iso T, Kedès L, Hamamori Y (2003) HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 194: 237–255.

41. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H (1990) The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell 61: 49–59.

42. Norton JD (2000) ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J Cell Sci 113(Pt22): 3897–3905.

43. Hayashi T, Kobuko H, Har Iman BH, Ray CA, Reh TA, et al. (2008) Hes1 and Hes2 may act as early effectors of Notch signaling in the developing cochlea. Dev Biol 316: 87–99.

44. Tarasova OV, Magness ST, Fagan BM, Wu Y, Szerzenko N, et al. (2006) SOX2 is a dose-dependent regulator of retinal neural progenitor competence. Genes Dev 20: 1107–1202.

45. Chakravartthy H, Boer B, Desler M, Mallanna SK, McKeathan TW, et al. (2008) Identification of DPPA4 and other genes as putative Sox2-Oct-3/4 target genes using a combination of in silico analysis and transcription-based assays. J Cell Physiol 216: 631–662.

46. Adam J, Myat A, Le Roux I, Eddison M, Henrique D, et al. (1998) Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with Drosophila sense-organ development. Development 125: 4645–4654.

47. Bell D, Streit A, Gorporne I, Varela-Nieto I, Alima B, et al. (2008) Spatial and temporal segregation of auditory and vestibular neurons in the otic placode. Dev Biol 322: 109–120.

48. Abello G, Khatiri S, Radosievic M, Scouting PJ, Giráldez F, et al. (2010) Independent regulation of Sox3 and Lmx1b by Fgf and BMP signaling influences the neurogenic and non-neurogenic domains in the chick otic placode. Dev Biol 339: 166–178.

49. Ma Q, Anderson DJ, Fritsch B (2000) Neurogenin 1 null mutant ears develop fewer, morphologically normal hair cells in smaller sensory epithelia devoid of innervation. J Assoc Res Otolaryngol 1: 129–134.

50. Alima B, Abello G, Ulloa E, Henrique D, Pujades C, et al. (2004) FGF signaling is required for determination of otic neuroblasts in the chick embryo. Dev Biol 267: 119–134.

51. Satoh T, Kallstrom M, Muh J (2005) Sox21 promotes the progression of auditory cell fate specification from the ESC-derived neural crest model. J Cell Sci 118: 4717–4722.

52. Sandberg M, Kallstrom M, Muh J (2005) Sox21 promotes the progression of auditory cell fate specification from the ESC-derived neural crest model. J Cell Sci 118: 4717–4722.