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Expression of LHX3 and SOX2 during Mouse Inner Ear Development

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Abstract

A cascade of transcription factors is believed to regulate the coordinate differentiation of primordial inner ear cells into the subtypes of hair cells and supporting cells. While candidate genes involved in this process have been identified, the temporal and spatial patterns of expression of many of these have not been carefully described during the extended period of inner ear development and functional maturation. We systematically examined the expression of two such transcription factors, LHX3 and SOX2, from the time of hair cell terminal mitoses into adulthood. We show that LHX3 is expressed specifically in auditory and vestibular hair cells soon after terminal mitoses and persists into the adult in vestibular hair cells. While SOX2 expression is widespread in the inner ear sensory epithelia prior to hair cell differentiation, it has a unique pattern of expression in the mature auditory and vestibular organs.

Keywords

LHX; LIM-homeodomain; MATH-1; inner ear; cochlea; ATOH1; SOX; hmg box; hair cell; deafness; HLH; bHLH; genetics

1. Results and Discussion

The mature mammalian inner ear is a complex membranous labyrinth that contains six distinct sense organs that respond to auditory (cochlea) and vestibular (utricle, saccule, three cristae) stimuli. During development, the inner ear forms as an invagination from a region of thickened ectoderm adjacent to the hindbrain, called the otic placode (reviewed in: Fritzsch et al., 1998; Kikuchi and Hilding, 1965; reviewed in: Lim and Anniko, 1985). As development proceeds, the otic epithelium differentiates into numerous distinct post-mitotic cell types with specialized structural and functional characteristics, including sensory patches with sensory receptors, called hair cells and non-sensory support cells. In the auditory and vestibular organs, there are two types of hair cells, inner and outer, and Type I and Type II, respectively. In the cochlea, there are also multiple morphologically identifiable subtypes of support cells. The absence of auditory hair cells and their associated sensory neurons account directly or indirectly for most acquired and congenital hearing loss. Because hair cells are not regenerated in mature

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mammals, this damage is permanent (reviewed in: Bermingham-McDonogh and Rubel, 2003). Similar damage in the vestibular compartment of the inner ear is the basis for many balance disorders. Over the past few years, there has been rapid progress in identifying the genetic basis of inherited hearing loss. More than 100 loci have been mapped that can lead to non-syndromic hearing loss (Friedman and Griffith, 2003). In addition to components of the auditory transduction pathway, some of these mutations lie in critical transcription factors that regulate inner ear embryonic development including BRN3C, ATOH1, SOX2, NGN1 and GFI1 (reviewed in: Barald and Kelley, 2004; Housley et al., 2006).

We have compared the sequential patterns of expression of three transcription factors, SOX2, ATOH1 and LHX3, present during critical periods of inner ear development and maturation using immunohistochemistry. These transcription factors fall into three general families: SOX (a subset of HMG box), basic helix-loop-helix (bHLH) and LHX (LIM homeodomain), respectively. Limited information has been reported regarding the expression of these genes during inner ear development (Bermingham et al., 1999; Bermingham-McDonogh et al., 2006; Chen et al., 2002; Kiernan et al., 2005; Lumpkin et al., 2003; Sage et al., 2005; Woods et al., 2004). We focused on the time interval between initial hair cell differentiation and the adult. During this time period, there is a dramatic change in the finely detailed structure of the inner ear as it matures into a functioning organ. In some developmental contexts, including the pituitary and spinal cord, the basic helix-loop-helix (bHLH) and LHX families of transcription factors regulate gene expression in a combinatorial fashion, but little is known about their interactions during inner ear development (Allan and Thor, 2003; Lee and Pfaff, 2003). SOX2, an HMG box transcription factor, appears to be required for the development of inner ear sensory domains, however, its role in later cell differentiation events in the ear is unknown (Kiernan et al., 2005). Prior to hair cell differentiation, SOX2 is expressed in the ventral and lateral otocyst corresponding to the developing sensory epithelium domain, and mutants for SOX2 have absent or disordered formation of sensory domains and hair cells (Kiernan et al., 2005). The bHLH transcription factor ATOH1 (Math-1) appears to act at a later point in this pathway, but is also essential for hair cell differentiation and potentially for determination of support cells (Bermingham et al., 1999; Woods et al., 2004). Ectopic expression of ATOH1 has been shown to induce the differentiation of hair cells *de novo* in the embryonic and adult inner ear (Izumikawa et al., 2005; Kawamoto et al., 2003; Woods et al., 2004; Zheng and Gao, 2000). Other members of the bHLH family, including NGN1 and NEUROD2, play a central role in sensoriepithelial and cochleovestibular ganglion development (Kim et al., 2001; Liu et al., 2000; Ma et al., 2000). LHX3 is involved in the differentiation of multiple cell types, including several neuronal subpopulations in the spinal cord and in the pituitary (Hunter and Rhodes, 2005; Shirasaki and Pfaff, 2002). The expression of LHX3 in the inner ear has not been extensively described (Sage et al., 2005). To begin to understand the role of these genes in inner ear development and the potential for their interaction, we have compared the onset of their expression to hair cell specific myosins using immunohistochemistry (MYO6 and MYO7A, reviewed in: Hasson et al., 1997).

1.1. Early labyrinth- E13

The time period of terminal mitoses of hair cells and support cells in the vestibular sensory epithelium begins at approximately embryonic day (E)12 in mouse, peaks around E14, and declines rapidly before birth (Chen and Segil, 1999; Lee et al., 2006; Ruben, 1967). Terminal mitoses in the auditory sensory epithelium are delayed by approximately one day compared to the vestibular organs (Ruben and Sidman, 1967). As noted above, of the markers we studied, only SOX2 is expressed in the inner ear before this period (Kiernan et al., 2005). Immunoreactivity for ATOH1, LHX3 and MYO6 is first detected in the vestibular epithelia at E12 in a small subset of cells within the SOX2 domain (not shown). LHX3 and ATOH1 are not detectable in the inner ear prior to this age, although immunoreactivity is found in spinal

motor neurons and dorsal spinal cord at E10.5 (not shown) (Bermingham et al., 2001; Sharma et al., 1998; Tsuchida et al., 1994). Comparison of SOX2, ATOH1, LHX3 and MYO6 expression in the vestibular region of the inner ear at E13 is shown in Figure 1 (A-D). While SOX2 expressing nuclei span the full thickness of the sensory epithelium, ATOH1 and LHX3 labeled nuclei are found predominantly in the more luminal layer corresponding to differentiating hair cells. Cytoplasmic labeling for the hair cell-specific myosin, MYO6 is detectable in cells with structural features of hair cells in the same region of the luminal layer of the vestibular sensory epithelium as ATOH1 and LHX3 (Figure 1, D).

We treated embryos with bromodeoxyuridine (BrdU) beginning at E13.5 to label nuclei of cells undergoing division between E13.5 and E14 in a pulse-fix paradigm. This time period corresponds to the peak of terminal mitosis of hair cells, but did not identify any ATOH1+/BrdU+ or LHX3+/BrdU+ double-labeled cells (Figure 1, E-H). The pattern of onset of LHX3 expression parallels that found in the embryonic spinal cord, where LHX3 is first detected in motor neurons after terminal mitoses (Sharma et al., 1998; Tsuchida et al., 1994). No expression of ATOH1 or LHX3 is detected at this age in the auditory sensory epithelium, consistent with the delay in terminal mitoses within the auditory relative to the vestibular regions (data not shown).

1.2. Late Labyrinth- E15-16

Between E12.5 and E15, the individual vestibular organs expressing ATOH1, LHX3, MYO6 and MYO7A become identifiable as more hair cells begin to differentiate (Compare Figure 1, A-D with Figure 1, I-L). In the E15 auditory sensory epithelium (Figure 1, M), SOX2 expression is limited to a domain that encompasses parts of adjacent areas of both the lesser epithelial ridge (LER) and greater epithelial ridge (GER) (Kiernan et al., 2005; Kiernan et al., 2006). Immunoreactivity for SOX2 was consistently more intense in the LER than the GER. These regions are separated by a morphological furrow corresponding to the future site of the tunnel of Nuel (Corti) and will give rise to multiple cell types including outer hair cells (LER) and inner hair cells (GER) (Lim and Rueda, 1992; Sher, 1971). No immunoreactivity for ATOH1, LHX3 or MYO6 is detected at this age in the auditory epithelium (Figure 1, N-P). This contrasts with previous descriptions of ATOH1 expression detected by *in situ* hybridization at E13 within the auditory region, or as revealed in transgenic mice expressing beta-galactosidase or green fluorescent protein under control of the ATOH1 promoter in the basal turn of the cochlea at E14.5 (Bermingham et al., 1999; Bermingham-McDonogh et al., 2006; Lumpkin et al., 2003; Woods et al., 2004). There is also one description of immunologic detection of ATOH1 in the basal cochlea at E14.5 (Chen et al., 2002). Strain differences, inconsistency of staging criteria and variations in the sensitivity of these techniques may explain this variability.

Analysis of a number of differentiation markers suggests that maturation of the auditory epithelium (organ of Corti) occurs along the longitudinal axis of the cochlea, progressing from the mid-base towards the apex along a temporal gradient (Chen et al., 2002; Chen and Segil, 1999). Beginning at E15.5, ATOH1, LHX3, MYO6 and MYO7A immunoreactivity are detectable in the organ of Corti at the basal end of the cochlea in a single row of cells adjacent to the lumen of the cochlear duct, corresponding by position to inner hair cells (E16, Figure 1, Q-S). Although outer hair cells can be recognized earlier by nuclear position relative to the furrow between the GER and LER and nuclear/cytoplasmic ratio, expression of ATOH1, LHX3 and MYO6 is consistently delayed in outer hair cells as compared to inner hair cells and in the apical versus basal cochlea. Weak immunoreactivity for ATOH1 in outer hair cell nuclei is also seen in Figure 1Q. By comparison of adjacent sections, expression of ATOH1 slightly precedes temporally LHX3, MYO6 and MYO7A. As differentiation continues, the domain of

expression of each of these proteins expands radially to include outer hair cells, and apically along the longitudinal axis of the cochlea.

1.3. Late embryonic- E17

In vestibular epithelia, SOX2 immunoreactivity is detected in both hair cell and support cell nuclei (Figure 2A). In contrast, ATOH1, LHX3 and MYO6 are detected exclusively in the hair cell layer of the epithelium (Figures 2B-D).

SOX2 immunoreactivity in the E17 cochlea is detected in a longitudinal axial band of nuclei spanning the full depth of the epithelium above the basement membrane from the base to apex (Figure 2E, I, M). In the basal and middle regions of the E17 cochlea, two distinct layers of nuclei can be identified, corresponding to the location of hair cells (luminal) and underlying support cells. ATOH1, LHX3 and MYO6 expression is detectable in the basal and middle cochlea in both inner and outer hair cells (Figure 2E-L), but no staining is observed for these proteins in the mouse apical cochlea (Figure 2M-P), consistent with the base to apex wave of differentiation.

Along the medial to lateral axis of the mid-basal cochlea, SOX2-expressing nuclei are detected in both hair cell (MYO6+) and support cell layers spanning from inner hair cells and inner phalangeal cells to outer hair cells, Deiters' cells and lateral tunnel cells (Figure 2Q, R) (Spicer et al., 2003). PROX1, a homeobox transcription factor, is expressed some support cells in the cochlea at this stage of development, therefore, we tested whether these populations coincided (Bermingham-McDonogh et al., 2006). We find that PROX1 is expressed in a subset of SOX2-positive cells that does not include the inner phalangeal and more medial support cells (Figure 2R). In contrast to SOX2, expression of PROX1 is gradually lost from the cochlea during the early postnatal period (see below and: Bermingham-McDonogh et al., 2006).

1.4. Early postnatal maturation

Expression of ATOH1, LHX3 and MYO7A persist in vestibular hair cells at this age (Figure 3A-E). Nuclear SOX2 protein expression is declining in the majority of vestibular hair cells, but it is maintained in support cells and a subset of hair cells (Figure 3A).

During the early postnatal period in the cochlea, the domain of SOX2 expression expands medially into the GER and is maintained in support cells, including inner phalangeal cells, pillar cells, Deiters' cells and Hensen's cells (Figure 3A, E). In contrast, SOX2 immunoreactivity is gradually lost from the nuclei of both inner and outer hair cells, although some expression does persist transiently in the hair cell cytoplasm (Figure 3E). The sub-cellular distribution of other SOX family members (e.g., Group E) is important for regulation of their activity (Rehberg et al., 2002), and this property has also been described for SOX2 (Avilion et al., 2003; Rehberg et al., 2002). At this stage of inner ear development, cells in the SOX2-expressing region of the GER are undergoing cellular rearrangements that eventually give rise to the inner sulcus. GER cells are believed to be relatively undifferentiated, as demonstrated by their ability to transdifferentiate into hair cells with ectopic expression of ATOH1 (Woods et al., 2004; Zheng and Gao, 2000). At postnatal day 2, ATOH1, LHX3 and MYO6 are detectable along the entire axis of the cochlea in inner and outer hair cells (Figure 3E-P).

1.5. Mature adult

In the mature vestibular organs, SOX2 immunoreactivity persists at a low level in support cells (not seen at this level of exposure in Figures 4A,D, but seen in Figure 5B) and at a higher level in a subset of hair cell nuclei distributed across the sensory epithelia (arrowheads in Figure 4A, D). This pattern of expression is similar in both macular organs (utricle, saccule) and the cristae of the semicircular canals. We also noted that immunodetection of SOX2 in support cells at

this age was sensitive to extended periods of decalcification. To help identify the subset of SOX2-positive vestibular hair cells, some macular organs were double labeled with an anti-calbindin antibody that outlines the afferent calyces surrounding Type I hair cells in the striolar region (green in Figure 5A) (Dechesne and Thomasset, 1988; Dechesne et al., 1988). In a confocal z-series spanning the width of the hair cell nuclear layer of the saccule, no SOX2+/calbindin+ cells were identified. This suggests that in the striola, SOX2 expression is limited to Type II vestibular hair cells (Figure 5A). In extrastriolar regions, approximately one-half of the hair cell nuclei were labeled for SOX2, but markers to distinguish Type I and Type II hair cells outside the striola are not available (see discussion in: Desai et al., 2005). A detailed quantitation of SOX2 expression in vestibular hair cell subtypes is in preparation (Oesterle et al., 2007). A confocal z-series spanning the layer of support cell nuclei shows that SOX2 is uniformly expressed in support cells (Figure 5B). Some gaps are seen where nerve fibers penetrate the support cell layer (arrowhead in Figure 5B). LHX3 expression is maintained in nuclei of Type I and Type II vestibular hair cells (Figure 4B, E). ATOH1 expression in the inner ear declines soon after birth in both auditory and vestibular hair cells and is undetectable in the adult (not shown). MYO6 is a component of the sensory transduction apparatus, and its expression persists in both vestibular and auditory hair cells into adulthood (Figure 4C, F, I) (Hasson, 1997; Hasson and Mooseker, 1994; Hasson and Mooseker, 1996).

The tunnel of the organ of Corti forms during the first two postnatal weeks in mouse and the inner ear acquires its adult structure coincident with the onset of hearing. While SOX2 and MYO6 expression persist in the adult (Figure 4G, I), LHX3 and ATOH1 expression are lost from the auditory sensory epithelium (Figure 4H and data not shown). (Hasson, 1997) SOX2 expression is detected in a subset of differentiated support cells, including Deiters', pillar, inner phalangeal, tectal and lateral tunnel cells (Figures 4G, 5C, D) (Spicer et al., 2003). Because of the three-dimensional organization of the adult organ of Corti, the full extent of SOX2 expression is best appreciated in the brightest point projection Z-series of an immunolabeled whole-mount preparation (Figure 5C, D). This compressed confocal stack spans the full thickness of the sensory epithelium from the basilar membrane to the apices of outer hair cells. Parvalbumin, a calcium binding protein, is used to identify hair cells (Xiang et al., 1998).

Figure 5D shows the same field as 5C, with the red (SOX2, support cell) and green (parvalbumin, hair cell) channels separated along the midline to emphasize the breadth of the SOX2-positive domain and the absence of expression in hair cell nuclei (arrowheads). In contrast to the early postnatal period, cytoplasmic expression of SOX2 is no longer detected in adult cochlear hair cells (Figures 4G, 5C,D).

1.6. Conclusion

We show that members of the SOX, LHX and bHLH families have unique, but overlapping patterns of expression during critical periods of inner ear development. SOX2 is expressed in the developing inner ear and mutations lead to malformations and deafness in mice (Kiernan et al., 2005). The expression of SOX2 in the mature inner ear has not been described. In the early auditory and vestibular sensory epithelia, SOX2 is expressed in a broad domain encompassing prospective sensory and non-sensory cell types. As the inner ear matures, the expression of SOX2 becomes restricted to highly differentiated cell types in both the vestibular and auditory organs. We find that in the adult vestibular epithelia, SOX2 is expressed in support cells and a subset of Type II hair cells. In the organ of Corti, SOX2 is progressively restricted to support cells during the early neonatal period. The SOX2-positive population of support cells is distinct from, but includes that delineated by expression of PROX1 (Bermingham-McDonogh et al., 2006). The expression of SOX2 in support cells persists into adulthood. Although the SOX2 transcription factor has been implicated in maintenance of stem cell

phenotype (reviewed in: Ellis et al., 2004; reviewed in: Wegner and Stolt, 2005), its role in the post-mitotic inner ear is unclear.

LHX3 is involved in the regulation of differentiation of multiple cell types (Hunter and Rhodes, 2005; Shirasaki and Pfaff, 2002), but its expression in the inner ear has not been extensively described (Sage et al., 2005). LHX3 is expressed in vestibular and auditory hair cells shortly after they are generated by a wave of terminal mitoses. In our study, this time course closely follows that of the bHLH transcription factor, ATOH1 and is similar to that of MYO6 and MYO7A. LHX3 expression is detected first in the vestibular organs around E12, followed by inner hair cells in the base of the cochlea at E15.5, and then extending apically and peripherally to include all inner and outer hair cells. Unlike ATOH1, LHX3 expression is maintained into adulthood in vestibular hair cells. In the cochlea, LHX3 expression is downregulated in postnatal hair cells. Individuals with LHX3 mutations have no gross hearing deficit, indicating that its expression is not essential for some degree of hearing (Gruters et al., 2004; Netchine et al., 2000). The effect of the loss of LHX3 on vestibular function is unknown.

2 Experimental Procedures

2.1. Animal husbandry

Mice (Swiss-Webster) were time-mated by the breeder (Charles River, ATL, Taconic) with the date of vaginal plug considered as embryonic day 0.5 (E0.5). Embryos were individually staged according to standard tables of mouse development (<http://genex.hgu.mrc.ac.uk/>). For BrdU experiments, E13.5 pregnant mice were injected intraperitoneally (50 mg/kg) at two-hour intervals three times. Two hours after the final dose (total of eight hours), the animals were sacrificed. Animal care and sacrifice were conducted according to methods approved by the University of Washington Institutional Animal Care and Use Committee.

2.2. Tissue preparation

Heads of staged embryonic mice were fixed in ice-cold, fresh 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for 2 hours at 4°C and washed extensively in PBS. The temporal bones of neonatal mice (postnatal day 1-7) were dissected free from the skull and processed in a similar fashion. The temporal bones from adult mice were directly perfused with fixative through the oval window after removal of the stapes and making a small apical cochleostomy. The temporal bones were then immersed in fixative for two hours at 4°C. After several washes in PBS, adult temporal bones were decalcified at room temperature in 0.4M EDTA (pH 8.0) for up to 5 days with daily solution changes. To obtain cryostat sections, heads or decalcified temporal bones were sequentially infiltrated with 30% sucrose and O.C.T. compound (Tissue Tek, Miles Laboratories), frozen rapidly on dry ice, and cut into 10 µm sections with a cryostat. Sections were mounted onto Platinum Superfrost (+) slides (Mercedes Medical). For wholemount immunostaining, individual turns of the cochlea or vestibular organs were dissected from the temporal bone after fixation. Further processing was as described for tissue sections below.

2.3. Immunohistochemistry

Polyclonal antibodies to LHX3 and ATOH1 were generously provided by S. L. Pfaff (Salk Institute, La Jolla, CA) and Thomas M. Jessell (Columbia University, NYC, NY) (Helms and Johnson, 1998; Sharma et al., 1998; Tsuchida et al., 1994). Similar results were obtained using anti-LHX3 antibodies from Chemicon (AB3202, 1:2000). A polyclonal antibody (goat) to SOX2 (sc-17320) was obtained from Santa Cruz Biochemicals. Specificity of this antibody was verified by blocking with a SOX2 peptide see (supplementary data). Comparable results were also obtained using a rabbit-anti-SOX2 polyclonal antibody (Chemicon AB5603, 1:1000). Anti-MYO6 and MYO7A rabbit polyclonal antibodies were obtained from T. Hasson

(UC San Diego) or from Proteus Biochemicals (25-6791, 25-6790; 1:1000) (Hasson et al., 1997; Hasson and Mooseker, 1994). Antibodies to parvalbumin and calbindin were obtained from SIGMA (parv19, 1:1000) and Chemicon (AB1778, 1:2000) respectively (Cunningham et al., 2002; Dechesne et al., 1994; Dechesne and Thomasset, 1988). Rabbit anti-PROX1 antibody was obtained from Chemicon (AB5475, 1:2000) (Bermingham-McDonogh et al., 2006). A rat anti-BrdU monoclonal antibody (Accurate) was used at 1:20 to label mitotic cells. Secondary antibodies with fluorescent labels (Alexa 488, 555, 594) were purchased from Invitrogen/Molecular Probes (Eugene, OR) and used at dilutions recommended by manufacturer (1:500-1:2000).

Primary antibody incubations were performed for 12 hours at 4°C in PBS, 0.03% saponin, 3% goat serum, 2 mg/ml bovine serum albumin, and 0.1% Triton X-100. For BrdU experiments, sections were pretreated with 1 N HCl (1 hour, 37°C), prior to incubation with the primary antibody. Fluorescent-labeled secondary antibodies (Alexa 488, 555, 594, Invitrogen/Molecular Probes) were used at a dilution of 1:1000 in the same buffer for 2-4 hours at room temperature. In some cases, the TSA system (Invitrogen/Molecular Probes) or ABC system (Vector Laboratories) was used for signal amplification as recommended by the manufacturer. Comparable results were obtained with both techniques. For mouse antibodies against parvalbumin, the M.O.M. kit was used as specified by the manufacturer (Vector Labs). In some experiments, nuclei were counterstained with bisbenzimidazole (bis) or DAPI. Sections were mounted in Vectashield (Vector Laboratories) or Prolong Gold (Molecular Probes) and examined with epifluorescence using a Zeiss AxioPhot microscope. At least three individual animals representative of each age were analyzed for each antibody. For laser scanning confocal microscopy of whole-mount preparations, we used an Olympus FluoView-1000, equipped with 405 nm blue diode, 457 nm, 488 nm and 514 nm multi-line argon, 543 nm helium neon green and 637 nm helium neon red lasers. FluoView version 1.4a acquisition software was used. Sequential image acquisition was performed when bleed-through between channels was an issue. Files were imported into ImageJ and/or Adobe Photoshop for processing and analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Embryonic day 13-16. Immunofluorescence was used to localize the expression of SOX2 (green, A, E, I, M), ATOH1 (green, B, F, J, N, Q), LHX3 (green, C, G, K, O, R) and MYO6 (green, D, H, L, P, S) in the vestibular and auditory sensory epithelia of the embryonic mouse. E13 (A-D), E14 (E-H), E15 (I-P) E16 (Q-S). In each image, nuclei are counterstained with bisbenzimidazole (pseudocolored red A-B and I-S; blue E-H). Expression of each protein was detected in all five vestibular epithelial patches at these ages. In panels E-H, timed-mated pregnant animals were injected with BrdU at E13.5 and sacrificed 8 hours later. Adjacent sections were double-labeled with antibodies for BrdU (red) and the indicated marker (green). For E-H, each image shows the brightest point projection of a z-series confocal stack. Double-

labeled cells are seen only in the SOX2 panel (E). A mid-modiolar section of the E15 basal cochlea is shown (M-P), however, expression of each gene is comparable in the later maturing, more apical regions (i.e., only SOX2 is detected, not shown). One day later, ATOH1 (Q), LHX3 (R) and MYO6 (S) are expressed in inner hair cells of the basal cochlea. ATOH1 expression is also faintly detectable in OHC at this age (closed arrowheads Q). HC indicates the depth of the hair cell layer; SC indicates the depth of the support cell layer. GER = greater epithelial ridge, LER = lesser epithelial ridge, OHC = outer hair cell, IHC = inner hair cell. The dashed lines in E-H indicate the borders of the sensory epithelium. The asterisks indicate non-specific label (Q, S). Scale bars shown in D, H, P and S = 20 μ m in A-D, E-H, I-P and Q-S, respectively.

Figure 2.

Embryonic day 17. Immunofluorescence was used to localize the expression of SOX2 (green, A, E, I, M; red, Q, R), ATOH1 (green, B, F, J, N), LHX3 (green, C, G, K, O), MYO6 (green, D, H, L, P, Q) and PROX1 (green, R) in the vestibular and auditory sensory epithelia of the embryonic mouse at E17. In each image, nuclei are counterstained with bisbenzimidazole (pseudocolored red A-P; blue Q,R). Expression of each protein was detected in all five vestibular epithelial patches although only a representative series of sequential sections of the saccule is shown (A-D). Mid-modiolar sections through the base, middle and apex of the cochlea are shown (E-R). Note that the inner phalangeal cells and more medial support cells express SOX2, but not PROX1 (Q, R). Downward and upward arrows indicate hair cells (IHC=

Inner hair cell, OHC= Outer hair cell) and support cell (SC, IPhC = Inner phalangeal cell, PC = Pillar cells) nuclei, respectively. HC indicates the depth of the hair cell layer. GER = greater epithelial ridge, LER = lesser epithelial ridge. BM indicates the level of the basement membrane, below the support cell nuclei. Scale bars in P and R = 20 μm and 10 μm respectively for panels A-P and Q, R. Asterisks in O indicate non-specific overlying debris.

Figure 3.

Postnatal day 2. Immunofluorescence was used to localize the expression of SOX2 (green, A, E, I, M), ATOH1 (green, B, F, J, N), LHX3 (green, C, G, K, O) and MYO6 (green, D, H, L, P) in the vestibular and auditory sensory epithelia of the embryonic mouse at postnatal day 2. For each image, nuclei were counterstained with bisbenzimidazole and pseudo-colored red.

Expression of each protein was detected in all five vestibular epithelial patches although only a representative section of the saccule is shown (A-D). Representative mid-modiolar sections through the base, middle and apex of the cochlea are shown. HC indicates the depth of the hair cell layer; SC indicates the depth of the support cell layer (A-D). Arrowheads in A indicate SOX2 expressing nuclei in the HC layer. Downward and upward arrows in G and I indicate hair cell (IHC= Inner hair cell, OHC= Outer hair cell) and support cell (SC) nuclei, respectively. Scale bar (P) = 20 μ m in all panels.

Figure 4.

Adult. Immunofluorescence and immunohistochemistry were used to localize the expression of SOX2, MYO7A (A, D, G), LHX3 (B, E, H) and MYO6 (C, F, I) in the vestibular and auditory sensory epithelia of adult mice. A representative mid-modiolar section through the middle turn of cochlea is shown for each antibody (G, H, I). LHX3 and MYO6 while were analyzed by immunofluorescence (A, D, G; red = SOX2, green = MYO7A, blue = bisbenzimidide).

Downward arrowheads in A, D and G indicate SOX2-expressing nuclei in the HC layer.

Upward pointing arrows in G and I indicate subtypes of support cells (SC). PC= pillar cells; DC= Deiters' cells; IPhC= Inner phalangeal cells. IHC= Inner hair cells; OHC= Outer hair cells;. Bars in A-C indicate the depth of the hair cell (HC) and support cell (SC) layers. Scale bar (I) = 20 μ m in all panels.

Adult

Figure 5.

Confocal images of SOX2 expression in the adult inner ear. Whole-mount preparations of normal adult mouse saccule (A, B) or cochlea (C, D) were immunolabeled for SOX2 (red, A-D) and the calcium-binding protein calbindin (green, A) which labels afferent calyces and delineates the striolar region, or the HC-specific marker calbindin (green, C, D). Nuclei were counterstained with bisbenzimidazole (blue). Each image shows the brightest point projection of a z-series stack taken parallel to the luminal surface of the epithelium (step size = 1 μ m). For images of the saccule, adjacent segments of a single stack spanning through either the luminal hair cell (A) or more basal support cell nuclear layers (B) are shown. In panel A, Type I HCs in the striolar region are surrounded by calbindin-positive nerve fibers and are SOX2-negative. Hair cells in the extrastricular region are either SOX2-positive or SOX2-negative. In B, all support cell nuclei in the basal layer are SOX2 positive. Calbindin labeling of afferent fibers is not shown in B for clarity. In the organ of Corti images (C, D), the projection spans the full

thickness of the epithelium to emphasize the medial and lateral extent of SOX2 expression relative to the position of hair cells. Arrowheads indicate the 3 rows of outer hair cells (OHC) and the single row of inner hair cells (IHC). The red (SOX2) and green (Parvalbumin) channels of panel 5C are separated in the left and right halves of panel 5D. TC/LTC, Tectal cell/Lateral tunnel cell. Scale bar = 20 μm for A; 10 μm for B-C.