Differential Expression of Sox2 and Sox3 in Neuronal and Sensory Progenitors of the Developing Inner Ear of the Chick

JOANA NEVES, ANDRÉS KAMAI, BERTA ALSINA, and FERNANDO GIRALDEZ

ABSTRACT

The generation of the mechanosensory elements of the inner ear during development proceeds in a precise temporal and spatial pattern. First, neurosensory precursors form sensory neurons. Then, prosensory patches emerge and give rise to hair and supporting cells. Hair cells are innervated by cochleovestibular neurons that convey sound and balance information to the brain. SOX2 is an HMG transcription factor characteristic of the stem-cell genetic network responsible for progenitor self-renewal and commitment, and its loss of function generates defects in ear sensory epithelia. The present study shows that SOX2 protein is expressed in a spatially and temporally restricted manner throughout development of the chick inner ear. SOX2 is first expressed in the neurogenic region that gives rise to sensory neurons. SOX2 is then restricted to the prosensory patches in E4 and E5 embryos, as revealed by double and parallel labelling with SOX2 and Tuj1, MyoVIIa, or Islet1. Proliferating cell nuclear antigen labelling showed that SOX2 is expressed in proliferating cells during those stages. By E5, SOX2 is also expressed in the Schwann cells of the cochleovestibular ganglion, but not in the otic neurons. At E8 and E17, beyond stages of sensory cell specification, SOX2 is transiently expressed in hair cells, but its level remains high in supporting cells. SOX3 is concomitantly expressed with SOX2 in the neurogenic domain of the otic cup, but not in prosensory patches. Our data are consistent with a role for SOX2 in specifying a population of otic progenitors committed to a neural fate, giving rise to neurons and hair cells. J. Comp. Neurol. 503:487–500, 2007.

Indexing terms: Sox; otic; ear progenitors; proneural; sensory organ

The basic functional unit of the inner ear consists of four elements of neural origin: the mechanotransducing hair cells (HCs), the supporting cells (SC), the primary afferent neurons, and the ganglion Schwann cells. Those elements develop in a stereotyped manner, with small variations among species (Adam et al., 1998; Torres and Giraldez, 1998; Fritzsch et al., 2006). The specification of the cell types of the chick inner ear proceeds sequentially and in a precise spatial pattern. First, neuroblasts are specified in the ventral epithelium of the otic cup, and they delaminate to form the cochleovestibular ganglion (CVG, VIII cranial nerve). Ganglionar neuroblasts still proliferate, and they expand generating the bipolar otic neurons that connect the hair cells to the brainstem (D’Amico-Martel, 1982; Whitehead and Morest, 1985; Alsina et al., 2003). Delayed by one day in the chick, sensory patches emerge in the otocyst at precise positions and develop into the sensory organs that contain the mechanotransducing hair cells (HCs) and the supporting cells (SCs). During the past...
years, we have expanded the identification and knowledge of the genes involved in the specification and differentiation of neurons and hair cells, particularly the expression and function of proneural genes (Lewis, 1998; Binghamham et al., 1999) and the role of cell communication signals (Alsin et al., 2004; Barald and Kelley, 2004; Pujades et al., 2006). However, we are still far from understanding the mechanisms by which the different neural cell types of sensory patches are generated from cell progenitors. Evidence has been obtained supporting the idea that neurons and hair cells can originate from a common progenitor cell (Lage and Puelles, 2001; MatSOX2 rd., 2005; Seidensticker and Fekete, 2005), and common genetic networks may underlie the development of sensory cells of the eye and the ear (Fritzsche et al., 2006).

The SOX2 transcription factor is expressed early in embryonic and neural stem cells. SOX genes contain an HMG-box closely related to that of the mammalian testes-determining gene SRY and are highly conserved throughout evolution. The C-terminal region of the SOX protein carries a cryptic transactivating domain that is uncovered only after specific interaction with partner factors. To date, 24 vertebrate SOX genes have been identified and are classified into seven subgroups (A–G) based on sequence identity, and at least 12 members of the SOX gene family are expressed in the nervous system (Wegner and Stolt, 2005; Pevny and Placzek, 2005). Studies in the chick embryo have provided evidence that neural inducing signals directly regulate SOX2 expression in the neural tube and that SOX2 is responsible for commitment of actively proliferating cells to a neural fate (Rex et al., 1997; Graham et al., 2003). Recent studies have also revealed that the SOX2 regulatory domain contains a domain that responds directly to neural-inducing signals, which is conserved across diverse animal species. Sox2-deficient mice such as light coat and circling (Lcc) and yellow submarine (Ysb) show hearing and balance impairment. Lcc/Lcc mutant mice fail to establish a prosensory domain, and neither hair cells nor supporting cells differentiate (Kiernan et al., 2005). Moreover, Ysb/Ysb mice show abnormal development, with disorganized and fewer hair cells. These phenotypes are a direct consequence of the absence or reduced expression of the transcription factor SOX2 in the developing inner ear (Kiernan et al., 2005). Moreover, mutations of SOX2 in humans cause anophthalmia, sensorineural hearing loss, and global brain defects (Hagstrom et al., 2005) and also regulate retinal neural progenitor competence (Tarunova et al., 2006).

This background suggests that SOX2 plays an important role in the development of neural elements of the ear, particularly in the regulation of the function of neural progenitors. Here, we report the first detailed analysis of SOX2 expression in the developing inner ear, from the early otic vesicle until postdifferentiation stages. The results show that SOX2 is expressed in proliferating cells of neurogenic and prosensory domains from the otic cup to early otocyst. As development proceeds, SOX2 is not expressed in differentiated neurons and hair cells but remains intensely expressed in supporting cells. We propose that SOX2 may play critical roles in ear development. First, it is associated with the self-renewal state and the proneural character of ear neurosensory progenitors, becoming an early marker for prosensory progenitor cells. Second, SOX2 is absent from hair cells and neurons after terminal division but it is retained by supporting cells along with their capacity to divide, suggesting that SOX2 may be a key element in hair cell regeneration after damage.

MATERIALS AND METHODS

Embryos

Fertilized hens' eggs (Granja Gibert, Tarragona, Spain) were incubated at 38°C for the designated times, and embryos were staged according to Hamburger and Hamilton (1992). Embryos were dissected from the yolk and fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (pH 7.5; 4% PFA/PBS) overnight at 4°C, dehydrated with consecutive washings in 25%, 50%, 75%, and 100% methanol in PBST (PBS 0.1% Tween) and stored at –20°C.

Cryostat sectioning

For cryostat sectioning, embryos fixed in 4% paraformaldehyde were immersed in 15% sucrose and embedded in 7.5% gelatin/15% sucrose. Blocks were frozen in isopentane to improve tissue preservation and then sectioned at 20 µm thickness onto Superfrost Plus Slides (Fisher, Pittsburgh, PA) and stored at –20°C. Sections were used either for immunohistochemistry or in situ hybridization.

Immunostaining

For immunodetection, the sections were thawed and permeabilized for 20 minutes with PBST. For antigen retrieval, sections were treated with citrate buffer (pH 6.1) at 95°C for 30 minutes. Nonspecific binding was blocked for 1 hour at room temperature in 0.1% Tween, 10% heat-inactivated goat serum in PBS (blocking solution). Slides were incubated overnight at 4°C with diluted antibodies and covered with Parafilm.

The antibody against SOX2 protein (Abcam ab15830, lot 103889) was a rabbit polyclonal antibody raised against the synthetic peptide of residues 9–20 of human SOX2 (GNQKNSPDRVKR) conjugated to KLH. The immunogen has 100% identity with the chicken protein. The antibody stained the nucleus of human ES cells (H9 line) and detected bands of apparent mw 34–42 kDa (predicted size 34 kDa) in Western blots of different human and mouse stem cells, where it did not cross-react with SOX1 (Abcam http://www.abcam.com/index.html?datasheet=15830). Antigen amino acid identity with other SOX proteins was 42% with SOX3 and SOX1 and less than 25% for other SOX proteins (SOX4, -8, -9, -10, -11, -12, -14, -21). We performed four controls for immunostaining with SOX2 antibody. First, specificity of antibody binding was tested by preabsorption of the antiserum against the synthetic peptide (Suppl. Fig. 1). All staining was abolished from neural tube and otocyst when 1 ml of rabbit polyclonal anti-SOX2 antibody was incubated with 2.5 ml of SOX2 antigen peptide used to raise the antibody (Abcam ab15831, synthetic peptide derived from residues 9–20 of human SOX2). Positive controls in the absence of peptide were run in alternate parallel sections. Second, we carried out a Western blot analysis of chick neuroectoderm extracts, which were compared with mouse ES-CGR8 cells and extraembryonic membrane extracts as positive and negative controls, respectively (Suppl. Fig. 2). A band of an apparent mw 38 kDa was detected in nuclear extracts of chick neuroectoderm and in mouse stem cells. This band was absent from extraembryonic tissues and from cytoplasmatic extracts of neural ectoderm. Chick embryonic neuroectoderm also showed another band of 35 kDa. This is
the expected mw of the SOX2 protein. SOXB proteins are known to undergo posttranslational modifications, including SUMOylation (Savare et al., 2005), and the chicken SOX2 protein has five possible SUMOylation sites (SUMOPLOT; Abgent). The two bands observed in chick extracts may therefore correspond to posttranslational modifications of the SOX2 protein and are within the range of molecular weights observed in mouse and human extracts (see above). Third, to ensure that the SOX2 antibody did not react with SOX3, which was also expressed at early developmental stages, we checked for cross-reactivity of the SOX2 antibody with the chSOX3 protein. For this purpose, the chicken Sox3-GFP fusion gene was transfected in HeLa CCL-2 cells, which were then probed by immunofluorescence with SOX2 or SOX3 antibodies (Suppl. Fig. 3). These experiments showed that anti-SOX2 antibody was unable to recognize the chicken SOX3 protein when expressed in HeLa cells. The expression of SOX3 was assessed by the concomitant detection of green fluorescent protein (GFP) in the same cells and also by immunofluorescence with the SOX3 antibody in parallel plates. Finally, the SOX2 immunostaining showed a close overlapping and colocalized with Sox3 in situ hybridization for the Sox2 mRNA (see Fig. 7). The anti-SOX2 antibody was used at a dilution of 1:400.

The anti-SOX3 antibody was a rabbit polyclonal antisem, kindly provided by Thomas Edlund and Jonas Muhr. The immunogen was a fusion protein expressed in E. coli that contained the 76 C-terminal amino acids of chicken SOX3 protein (Thomas Edlund, unpublished data). This antibody has been used to detect chicken SOX3 protein (Wilson et al., 2001; Bylund et al., 2003). The authors used this antibody to compare the expression of SOX1, -2, and -3 (Fig. 1 in Bylund et al., 2003), and they showed that transcriptional activity of the Sox3 gene is correlated by corresponding levels of SOX3 protein as detected by the SOX3 antibody (Figs. 3, 4 in Bylund et al., 2003) and that immunoreactivity is down-regulated by proline-rich proteins and neural-inducing signals, both in vitro and in vivo (Wilson et al., 2001; Bylund et al., 2003). In our hands, the SOX3 antibody corresponded well with Sox3 mRNA expression in the otic cup and neural tube (see Fig. 7). Whereas SOX3 expression in the neural tube persisted along with that of SOX2 throughout development, SOX3 staining was negative in the otocyst beyond E3 (see Fig. 7). Because SOX2 was distinctly expressed in sensory patches at these stages, we concluded that the SOX3 antibody did not cross-react with SOX2 in the developing ear. The SOX3 antibody was used at a dilution of 1:300.

Other antibodies used and dilutions were as follows. 1) Mouse monoclonal anti-HNK1 (CD57, clone HNK1, supernatant; Becton Dickinson; gift from López-Botet, Universitat Pompeu Fabra, Barcelona, Spain). This antibody was derived from hybridization of mouse P3-X63-Ag8.653 myeloma cells with lymph node cells from BALB/c mice immunized with membrane extracts of the HSB-2 T-lymphoblastoid cell line. It recognizes a carbohydrate-structure antigen from myelin-associated glycoproteins expressed in a subset of natural killer (NK) and T lymphocytes (Abo and Balch, 1981). Dilution was 1:400. 2) Mouse monoclonal antineuronal class III β-tubulin (Tuj1, MMS-435P; Berkeley Antibody Company, Berkeley, CA). This antibody was raised against microtubules derived from rat brain and recognizes the epitope CEAGGPK, corresponding to the N-terminal, isotype-defining domain of class III β-tubulin. This antibody is well characterized and highly reactive to neuron-specific class III β-tubulin (βIII). Tuj1 does not identify β-tubulin in glial cells (Lee et al., 1990), and it has been extensively characterized in the developing ear by Molea et al. (1999). Dilution was 1:400. 3) Mouse monoclonal anti-Islet1 (clone 39.4D5, cell culture supernantant, Developmental Studies Hybridoma Bank, University of Iowa). This antibody was developed by Thomas M. Jessel (Columbia University) and was raised against a bacterial expression generated peptide corresponding to the fragment extending from the beginning of the homeodomain to the C-terminal end of the protein. This antibody has been characterized in the developing ear by Li et al. (2004). Dilution was 1:400. 4) Rabbit polyclonal antimosin VIIα (MiyosinVIIα; Tama Hasson, University of California, San Diego). This antibody was raised against human myosinVIIα tail, and it is specific to the portion comprising amino acids 880–1,070. The antibody was developed and characterized by Tama Hasson et al. (1995). Dilution was 1:5,000. 5) Mouse monoclonal antiproliferating cell nuclear antigen (PCNA; ab29, clone pc-10; Abcam, Cambridge, United Kingdom). This antibody was raised against the protein A-rat PCNA fusion obtained from pC2T. It is specific for PCNA p36 protein expressed at high levels in proliferating cells (Waseem and Lane, 1990). It reacts with human, mouse, rat, chicken, and fruit fly (Drosophila melanogaster) and detects a 30-kDa band in WB of chicken DT40 cell total extract (see abcam Abreviews at http://www.abcam.com/index.html?pageconfig=reviews&intAbID=29). Dilution was 1:200.

All antibodies were diluted in blocking solution. Unbound antibody was removed by successive washes with PBST for 6 hours and incubated for 2 hours with Alexa-488- and -594-conjugated goat anti-mouse and anti-rabbit, respectively, secondary antibodies (Molecular Probes, Eugene, OR), diluted 1:400 in blocking solution. Slides were washed overnight in PBST and mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA), which provided DNA counterstaining. Slides were analyzed by conventional fluorescence microscopy (Leica DMRB Fluorescence Microscope with Leica CCD camera DC300F). Images were acquired with Leica IM50 image manager, and Adobe Photoshop 7.0.1 was used to obtain the merged images and to adjust contrast and brightness. Figures 2b,c,h,i and 7k–n were corrected for uneven illumination with the Photoshop dodging toolbar.

In situ hybridization

In situ hybridization in sections was performed essentially as described by Nieto et al. (1996), using digoxigenin (DIG)-labelled riboprobes. DIG was detected with anti-DIG-AP and NBT/BCIP (Roche), which gives a purple staining. The riboprobes were chicken Sox2 and Sox3 (Rex et al., 1997). After the in situ hybridization protocol, sections were immunostained with Tuj1 antibody, following the method described above.

RESULTS

SOX2 is expressed in the neurogenic domain of the the otic vesicle (E2 and E3)

The experiments that follow show the expression of the SOX2 protein throughout the development of the inner
ear, between early otic vesicle and prehatching stages. The proneural domain of the inner ear is specified early in development, during initial stages of the formation of the otic primordium. It is located in the anterior aspect of the otic cup and otic vesicle, and it is complementary to the posterior nonneural region of the otic cup. The latter is characterized by the expression of patterning genes and the carbohydrate epitope HNK1 (Alsina et al., 2004). The expression of SOX2 in an E2.5 (HH16) otic vesicle is shown in Figure 1. Parasagittal sections of HH16 otic vesicles for SOX2 protein (red), and the HNK1 epitope (green; a–d). SOX2 expression was detected in the nuclei of the cells located in the anterior domain (b) and HNK1 expression in a complementary fashion in the posterior domain (c). Double immunostaining of parasagittal sections of HH18 otic vesicles against SOX2 protein (red) and Islet1 (green; e–h). Islet1 expression in the cochlear-vestibular ganglion (CVG, arrow in e). The scarce yellow nuclei corresponded to cells positive for Islet1 and SOX2 (h, arrowheads). Expression of SOX2 and Tuj1 (i–l). Tuj1 expression was restricted to the cochlear-vestibular ganglion (CVG, arrow in e). No overlapping between Tuj1 and SOX2 positive cells was detected (l). DAPI is shown in a, e, and i. Dashed frames indicate the proneural domain enlarged at the right. D, dorsal; A, anterior; CVG, cochleovestibular ganglion; OV, otic vesicle. Scale bars = 50 μm in a (applies to a–d); 100 μm in e (applies to e,i); 25 μm in f (applies to f–h, j–l).

SOX2 is expressed in the prosensory patches of the otocyst (E4 and E5)

By E3.5–E4 (HH22–24), ear prosensory domains became specified, first the vestibular and then the auditory sensory organs, and, by E5 (HH26), the different sensory organs can be clearly identified (Wu et al., 1998; Cole et al., 2000; Sanchez-Calderon et al., 2005). Figure 2 shows serial coronal sections of an E4 and E5 embryo that were
stained for SOX2 and Tuj1. Tuj1 recognizes a neuron-specific βIII tubulin epitope that is also expressed in nascent hair cells (Stone et al., 1996; Stone and Rubel, 2000). At E4, the prosensory patches can be detected by gene expression domains that anticipate future sensory organs (see diagram in Fig. 2a; see also Cole et al., 2000; Sanchez-Calderon et al., 2005). The experiment shown in Figure 2b–f, illustrates the expression of SOX2 in the prospective cristae (Fig. 2b), macula utriculi (Fig. 2c,d), macula sacculi (Fig. 2e), and basilar papilla (Fig. 2f). The correspondence

The Journal of Comparative Neurology. DOI 10.1002/cne

SOX2 IN THE CHICK INNER EAR

Fig. 2. SOX2 expression is restricted to the prosensory patches of otocysts in the E4 and E5 embryo. Diagrams represent otocysts from E4 (a) and E5 (g) stage embryos. Sensory patches are represented in red, as defined by gene expression, and were modified from Sánchez-Calderon et al. (2005). Planes represent the extreme levels of the sections shown for each stage. All sections were immunostained for SOX2 (red), Tuj1 (green), and DAPI (blue). Serial coronal sections of an E4 otocyst, in a dorsal-ventral sequence (b–f). SOX2 expression in vestibular (b–e) and auditory (f) prosensory patches. Serial coronal sections of an E5 otocyst in a dorsal to ventral sequence (h–i). SOX2 expression in vestibular (h–k) and auditory (l) sensory patches. Tuj1 labeled neurons of the vestibular (vg) and cochlear ganglion (cg) and the fibers innervating the prosensory domains (d–f–i–l). SOX2 in posterior crista (b,i), superior crista (b,h), lateral crista (l), macula utriculi (c,d,j), macula sacculi (e,k), and basilar papilla (f,l). pc, Posterior crista; sc, superior crista; lc, lateral crista; mu, macula utriculi; ms, macula sacculi; bp, basilar papilla; ml, macula lagena; vg, vestibular ganglion; cg, cochlear ganglion. Orientation: D, dorsal; M, medial; A, anterior. Scale bars = 100 μm in b (applies to b–f); 100 μm in h (applies to h–l).
between SOX2 expression and the sensory patches continued in E5, as illustrated in Figure 2h–l, which shows an experiment processed as described above for SOX2 and Tuj1. Again, the expression of SOX2 was restricted to the sensory patches. From dorsal to ventral, SOX2 expression is shown in cristae (Fig. 2h,i), maculae (Fig. 2j,k), and basilar papilla (Fig. 2l). The diagrams in Figure 2a–g show the location of sensory patches as drawn from gene expression patterns and were modified from Sanchez-Calderon et al. (2005).

To analyze better the correspondence between SOX2 expression and cell fate acquisition in sensory patches, we performed a comparison between SOX2 expression and MyoVIIa, which is a well-established cellular marker for the incipient differentiation of hair cells (Sahly et al., 1997; Wolfrum et al., 1998). Serial alternate sections of E5 embryos were immunostained in parallel for SOX2/Tuj1 (Fig. 3a–c) and MyoVIIa/Tuj1 (Fig. 3d–f). The experiment illustrates that the expression of SOX2 and MyoVIIa overlapped in sensory patches. To analyze this correspondence further at the cellular level, selected patches were examined at higher resolution and are shown in Figure 3g,h. As shown, SOX2 was concentrated in the basal cell layer, whereas MyoVIIa was restricted to the luminal layer occupied by hair cells. At these early stages of hair cell production, SOX2 was sometimes detected in the hair cell layer, but, later in development, SOX2 was not expressed in hair cells (see below).

Interestingly, and in contrast to what was observed in previous stages, SOX2 was now expressed in the cochleovestibular ganglion. In E5 embryos, SOX2 was distinctively expressed in a spotted pattern that intermingled with neurons (Fig. 3i). The staining was nuclear, as confirmed at high magnification with DAPI counterstaining (Fig. 3j). These cells corresponded well to Schwann glial cells that populate the ganglion and that surround the neurons and nerve fibers. Note that the large nuclei of Tuj1-positive neurons (Fig. 3j) are negative for SOX2, suggesting that SOX2 was not expressed in postmitotic otic neurons.

Islet1 is known to be expressed in nascent hair and supporting cells, and it persists until later stages of development in differentiating sensory cells (Li et al., 2004). The correspondence between SOX2 and Islet1 expression in E5 otocysts is shown in Figure 4a–c (superior and posterior cristae, Fig. 4a; basilar papilla, Fig. 4b). Both in crista and basilar papilla there was a restricted and overlapping expression of SOX2 and Islet1. At the cellular level, sensory patches showed cells that were positive for SOX2, for Islet1, and for both (Fig. 4e). Generally, the SOX2 domain was contained within the Islet1 patch (Fig. 4f). Taking Islet1 as an early marker for sensory cell commitment, this suggests that SOX2 expression persisted transiently during early stages of cell fate acquisition (see below). Double-labeling experiments with SOX2 and the proliferation marker PCNA showed that most SOX2 expression in sensory patches of the E5 otocyst was related to proliferating cells (Fig. 4d–f). Double labelling with SOX2 and PCNA showed a very intense proliferative activity in the otocyst at this stage and a great extent of overlap (Fig. 4d–f). At the cellular level, Figure 4f illustrates that most SOX2-positive cells also expressed PCNA. This would indicate that there is a transition state in which sensory progenitors are committed but still proliferative (Doetzlhofer et al., 2006).

**SOX2 is expressed in differentiation stages (E8 and E17)**

Expression of SOX2 was followed further in development and studied at stages E8 and E17, well beyond the period of cell specification and when hair and supporting cells are, respectively, in the process of early and late differentiation. The results in Figure 5show the expression of SOX2 and Tuj1 in a stage E8 otocyst. SOX2 was, as before, restricted to the sensory organs, which were now clearly identified by morphological criteria and by the expression of Tuj1 that labelled both hair cells and the innervating fibers. SOX2 expression was intense in all sensory organs, some of which are shown as examples in Figure 5: posterior crista (Fig. 5b), macula utriculi (Fig. 5c), macula sacculi (Fig. 5d), and basilar papilla (Fig. 5e).

A detailed analysis with cellular resolution is shown in Figure 5f for the macula sacculi and in Figure 5g for the basilar papilla. It can be seen that SOX2 expression was low or absent in hair cells but remained intense in supporting cells (Fig. 5f,g).

The results obtained from stage E17 otocysts are shown in Figure 6. Again, SOX2 was expressed in all sensory organs. Examples are shown of the posterior cristae (Fig. 6a,b), macula utriculi (Fig. 6c), macula sacculi (Fig. 6d), and basilar papilla (Fig. 6e,f, inset shows the macula lagena). High magnifications in Figure 6b,e show that SOX2 was not expressed in hair cells of vestibular and auditory organs, but it persisted at high levels in the layer of supporting cells. This was clearly distinguishable by the ordered position of nuclei and the negative staining for Tuj1.

Further confirmation of this pattern of expression was obtained by double labelling E17 otocysts with SOX2 and Islet1. The latter is typically expressed in supporting cells at this stage (Fig. 6g,h; Li et al., 2004). This is seen at high resolution in Figure 6i–k. Islet1 (green) was coexpressed with SOX2 in the supporting cells of macula utriculi (Fig. 6i) and in the basilar papilla (Fig. 6j,k). Note also that hair cells in the macula (Fig. 6i) still expressed Islet1, whereas it was down-regulated in the papilla (Fig. 6j,k; see also Li et al., 2004). SOX2 was absent from hair cells in both sensory organs.

Examination of E17 otocysts with SOX2 and PCNA revealed that some, but not all, of the SOX2-positive supporting cells were actively proliferating. As shown in Figure 6l,m, SOX2 labelling concentrated in the ordered supporting cell layer, which was spotted with PCNA-positive cells (yellow-green, arrows). At higher magnification, double-labelled cells were clearly visible in the supporting cell layer. Some of them are indicated with arrows in the examples shown in Figure 6o–q.

**SOX2 but not SOX3 is expressed in prosensory patches in E5**

Sox3 is known to be expressed in the neurogenic placodes (Abu-Elmagd et al., 2001), and we had some evidence of its early role in the specification of the proneural domain of the otic placode (Abelló et al., 2007). We were interested in exploring the possibility of SOX2 and SOX3 playing specific roles in proneural commitment by comparing their expression in the proneural domain of the early otic cup and in the sensory patches of E5 otocysts. Figure 7a–d shows the expression of SOX3 in the otic cup
Fig. 3. Expression of SOX2 and MyoVIIa in E5 embryos and SOX2 expression in Schwann cells. Alternate coronal sections of an E5 otocyst were immunostained for SOX2 (red) and Tuj1 (green; a–c) or MyoVIIa (red) and Tuj1 (green; d–f), respectively. SOX2 and MyoVIIa staining in vestibular prosensory patches, in a dorsal-ventral sequence: posterior crista and superior crista (a,d), lateral crista (b,e), macula utriculi and macula sacculi (c,f). High magnifications of superior crista show MyoVIIa (g) and SOX2 (h) in alternate sections. Note that SOX2 was expressed in some cells corresponding to the hair cell (HC) layer but not in the most apical ones (arrowheads). SOX2 expression in Schwann cells of the cochleovestibular ganglion (i,j). SOX2 was expressed in small nuclei typical of Schwann cells and not in large nuclei of Tuj1-positive neurons. pc, Posterior crista; sc, superior crista; lc, lateral crista; mu, macula utriculi; ms, macula sacculi; vg, vestibular ganglion. Brackets in g,h indicate the position of the row of hair cells. A, anterior; M, medial. Scale bars = 100 μm in a (applies to a–f); 20 μm in g (applies to g–i); 10 μm in j.
SOX3 was expressed in the proneural domain of the otic cup (Fig. 7a,b), in a manner complementary to HNK1 (a–c). Figure 7e–j shows the expression of SOX3 in an E5 embryo. Parallel, equivalent sections were analyzed for SOX2 and SOX3 expression in vestibular (Fig. 7e,h) and auditory (Fig. 7f,i) domains. No SOX3 immunoreactivity was detected in the otocyst, either in the vestibular or in the auditory domains. The positive control of the SOX3 was its strong positive reaction in the ventricular zone of the neural tube (Fig. 7j), which was very similar to that of SOX2 (Fig. 7g). A similar difference in the expression pattern between Sox2 and Sox3 was observed when comparing the mRNA expression of both genes at E5. This is shown in Figure 7k–n. In situ hybridization is shown as a blue precipitate and immunostaining Tuj1 in brown. Sox2 was expressed in the basilar papilla, macula utriculi, and lateral crista (Fig. 7k), whereas Sox3 expression was absent from the otocyst (Fig. 7l). A detail of the differential expression in the papilla is shown in Figure 7m,n. Sox2 and Sox3 were strongly coexpressed in the ventricular zone of the hindbrain (Fig. 7k,l). These results suggest the possibility that the neurogenic potential of otic progenitors may require the expression of both SOX2 and SOX3, whereas the prosensory potential would be specifically associated with that of SOX2 (Uchikawa et al., 1999).

**DISCUSSION**

**SOX2 and the state of commitment of sensory progenitors**

SOX2 is a HMG box transcription factor that is expressed in multipotent neural stem cells at all stages during mouse ontogeny (Wegner and Stolt, 2005). Sox2 expression in the early embryonic CNS is pieced together by separate enhancers with distinct spatiotemporal specificities, and enhancers for Sox2 expression in the lens and nasal/otic placodes have been identified (Uchikawa et al., 2003). SOX2 belongs to the stem-cell cassette that maintains the self-renewal state and pluripotency of progenitors (Takahashi and Yamanaka, 2006). Sox1–3 interact with various partner transcription factors and participate in defining distinct cell states that depend on the partner factors, Pax6 for lens differentiation, Oct3/4 for establishing the epiblast/ES cell state, and Brn2 for the neural primordia. All three factors are coexpressed in proliferating neural progenitors of the embryonic and adult CNS. The SoxB2 subgroup of Sox factors, including Sox14 and Sox21, are very similar to SoxB1 in their HMG-DNA binding domain but act as transrepression domains. A key common feature of SoxB1, SoxB2, and SoxE, however, is their ability to maintain neural progenitor or stem cell identity (for review see Wegner and Stolt, 2005).
As discussed by Fritzsch et al. (2006), the vertebrate sensory organ requires a mechanism for rapidly expanding the basic sensory unit, so that placodal epithelial cells bear characteristics of stem cells. Therefore, it is expected that they express typical genes of the stem cell cassette (Takahashi and Yamanaka, 2006). Our experiments show that the expression of SOX2 in the ear is reminiscent of this general stem-cell function but restricted to neural committed progenitors. Early in development, during otic vesicle stages, SOX2 is found in proliferating cells but only within the proneural domain of the early otocyst. The proneural domain of the otic vesicle is associated with neuron generation and is characterized by the expression of proneural genes such as Ngn1, NeuroD, and NeuroM, and Notch signalling elements such as Dll1, Hes5, and Lfng (Adam et al., 1998; Cole et al., 2000; Alsina et al., 2004). Null mutations of Ngn1 and NeuroD in mice show that these genes are necessary for neuronal determination and differentiation (Ma et al., 2000; Kim et al., 2001; Matei et al., 2005). Cell communication signals are also expressed in this domain, including several FGFs, which have been shown to be required for neuronal production (Alsina et al., 2004; Sanchez-Calderon et al., 2004, 2005). Other genes are expressed in the complementary posterior and dorsal domains of the otic cup and vesicle (Alsina et al., 2004; Abelló et al., 2006). Our results show that SOX2 expression is temporally and spatially restricted to the domain expected for the activity of proliferating cells that are committed to generate neurons and sensory cells.

Fig. 5. SOX2 expression in the sensory patches of the E8 embryo. Diagram representing an otocyst from E8 chicken embryo (a). Sensory patches are represented in red (adapted from Sánchez-Calderón et al. 2005). Sections (b–e) were immunostained for SOX2 (red), Tuj1 (green), and DAPI (blue). b–e: Serial coronal sections of an E8 otocyst are shown in a dorsal-ventral sequence. SOX2 expression was detected in vestibular (b–d) and auditory (e) sensory organs (pink for double staining with SOX2 and DAPI). SOX2-labelled cells in posterior crista (b), macula utriculi (c), macula sacculi (d), and basilar papilla (e). High magnification of macula sacculi (f). In this sensory patch, hair cells, indicated with arrows, did not express SOX2, which was detected in supporting cells (pink nuclei). High magnification of basilar papilla shown in e, illustrating SOX2 staining in supporting cells (g), pc. Posterior crista; mu, macula utriculi; ms, macula sacculi; bp, basilar papilla; cg, cochlear ganglion. Orientation in a: D, dorsal; M, medial; A, anterior. Orientation in b applies to b–e: M, medial; A, anterior. Scale bars = 100 µm in b (applies to b–e); 50 µm in f (applies to f,g).
Fig. 6. SOX2 expression is detected at postdifferentiation stages in supporting cells of E17 sensory organs. Sections (a–f) were immunostained for SOX2 (red) and Tuj1 (green). Sections (g–k) were immunostained for SOX2 (red) and Islet1 (green) and sections (l–q) were immunostained for SOX2 (red) and PCNA (green). DAPI staining is shown in (b,e,g,h,j,l,m) and (p). Transverse sections of E17 otocysts showing vestibular (a,c,d) and auditory (f) sensory organs. SOX2-labelled cells in posterior crista (a), macula utriculi (c), macula sacculi (d), and basilar papilla (f). High-magnification details of posterior crista and basilar papilla (b,e). SOX2 was restricted to the supporting cells (red nuclei) and it was excluded from the hair cell layer (blue nuclei), that was also labelled with Tuj1. Vestibular (g, macula utriculi) and auditory (h, basilar papilla) sensory patches of an E17 embryo otocyst doubly labelled for SOX2 and Islet1. High magnification of macula utriculi (i) and basilar papilla (j,k). SOX2 expression occurred in the supporting cell row. Islet1 expression in supporting cells in vestibular (i) and auditory (j,k) sensory organs and in hair cells of the macula utriculi (i). Macula utriculi (i) and cristae (m) organs of an E17 embryo otocyst labelled with SOX2 and PCNA. (p–q). SOX2 expression was restricted to the supporting cell layer. Proliferating cells were detected with PCNA (yellow nuclei with arrows). No PCNA-positive cells were detected in the hair cell layer, with the exception of one cell in the macula utriculi (l,o, green nucleus). pc, Posterior crista; mu, macula utriculi; ms, macula sacculi; bp, basilar papilla; ml, macula lagina; HC, hair cells, SC, supporting cells. Brackets in b,e,i,k,o,q indicate rows of hair and supporting cells. Scale bars = 100 \( \mu \text{m} \) in a (applies to a,c,d,f); 20 \( \mu \text{m} \) in b (applies to b,e,i–k,o–q); 50 \( \mu \text{m} \) in g (applies to g,h,l,m).
Fig. 7. SOX2 and SOX3 expression during ear development. SOX3 expression in the E2 otic cup. Double immunostaining of parasagittal sections of E2, HH14, otic cup for SOX3 protein, red, and the HNK1 epitope (green; a). SOX3 expression was detected in the nuclei of the cells located in the anterior domain (b,d) and HNK1 expression in a complementary fashion in the posterior domain (c). Sections were oriented with anterior to the right and dorsal to the top. SOX2 and SOX3 expression in sensory organs of E5 embryos (e–j). Coronal sections of an E5 otocyst, processed for SOX2 expression (red) and Tuj1 (green; e–g). SOX2 expression in the macula sacculi and macula utriculi (e), basilar papilla (f), and ventricular zone of the neural tube (g). Corresponding alternate sections, but processed for SOX3 expression (h–j). Macula sacculi and macula utriculi (h), basilar papilla (i), and neural tube (j). In situ hybridization for mRNA of Sox2 and Sox3 in sensory organs of E5 embryos (k–n); sections were counterstained for Tuj1. mu, Macula utriculi; ms, macula sacculi; bp, basilar papilla; nt, neural tube. D, dorsal; M, medial; A, anterior. Scale bars = 50 μm in a (applies to a–d); 100 μm in e (applies to e–n).
2006). Other genes, such as BMP4, and neurotrophin factors foreshadow the sensory domains (Wu et al., 1998; Farinas et al., 2001; Pirvola and Ylikoski, 2003), but it is not until E4 when Atho1 (Cath1)-positive cells are clearly identifiable at the sensory patches (Pujades et al., 2006). Serrate1 is probably accompanying the prospective sensory domain from very initial steps of specification onward, and it has been recently shown to be required for sensory development (Cole et al., 2000; Daudet and Lewis, 2005). Because SOX2 is expressed in both early neurogenic and prosensory domains, it appears that SOX2 may be used by all proneural-neurogenic and prosensory progenitors as a common requirement for neural commitment. Otic neurons and hair cells are neural cell types in a strict sense and both are specified upon activation of proneural basic helix-loop-helix (bHLH) genes into neurons by NeuroD or hair cells by Atho1 (Alsina et al., 2004).

In the neural tube, SOX2 is responsible for proneural competence, but it maintains repression of the activity of proneural genes until the expression of the SoxB2 gene group, which counteracts this effect (Bylund et al., 2003). SOX2 may play a similar function in the ear and maintain the state of proneural commitment and the capacity for self-renewal, its activity being down-regulated upon terminal division. This would be consistent with the observation that SOX2 is expressed not in neurons and hair cells but in supporting cells.

Identification of otic proneural progenitors

Otic progenitors undergo several cycles of cell division and progressive specialization to generate committed cells, first neurons and then sensory cells. It has been shown that neurons and sensory cells can derive from a common progenitor cell that resides in the otic placode (Fekete et al., 1998; Satoh and Fekete, 2005). Cell progenitors may change their properties because of intrinsic mechanisms related to the rounds of cell divisions, because they are exposed to different signals, or because of both. On the other hand, it may be that an initially common proneural progenitor located within the proneural domain of the otic placode becomes further specified by its position within the proneural domain. Studies in the vertebrate retina indicate that progenitors undergo a series of competence states, progressively changing their responsiveness to instructive extracellular signals that influence the type of cells that they will become (Livesey and Cepko, 2001; Cayouette et al., 2003). Little is known about how these mechanisms operate in the determination of otic lineages. It has been suggested that coevolutionary history of eyes and ears shows conserved cellular developmental programs (Fritzsch et al., 2006).

Two allelic mouse mutants, light coat and circling (Lcc) and yellow submarine (Ysb), show hearing and balance impairment. These phenotypes are due to the absence (in Lcc mutants) or reduced expression (in Ysb mutants) of the transcription factor SOX2, pointing toward a requirement for SOX2 in sensory progenitor development (Kiernan et al., 2005). Our results are consistent with this notion, because they show that SOX2 may be traced back to early stages of sensory progenitor specification. However, both SOX2 and SOX3 are expressed during the generation of neurons, but only SOX2 remains during sensory organ formation. This suggests the possibility that, at a given stage of development, SOX3 expression would be switched off, and the persistent SOX2 expression would result in sensory cell generation. The possibility of a phenotypic switch of cycling neural progenitors from neuron to hair cell fate has been suggested to occur in the Ngn1 null mice (Matei et al., 2005).

The results also show the expression of SOX2 in the Schwann glial cells of the cochleovestibular ganglion. Expression of Sox2 is down-regulated in the neural plate when neural crest segregates from dorsal neural tube and remains low during crest cell migration. Sox2 expression is subsequently up-regulated in some crest-derived cells in the developing peripheral nervous system and is later restricted to glial sublineages. This is interesting in connection to the neural crest origin of the glial cells of the cochleovestibular ganglion as described by (D’Amico-Martel and Noden, 1983; Matei et al., 2005). SOX2 expression in Schwann cells of the auditory and vestibular ganglion may then reflect its neural crest origin.

Significance of SOX2 expression in differentiation and postdifferentiation stages

In addition to the mouse mutants defective for SOX2, which show hearing and balance impairment, a nonsense mutation of the Sox2 gene has been recently reported in one patient suffering from bilateral clinical anophthalmia, absence of optic pathways, and sensorineural deficit (Matei et al., 2005; Hagstrom et al., 2005). This illustrates the potential importance of Sox2 in human development and disease. The persistent expression of SOX2 in the supporting cells of the sensory organs during postdifferentiation stages may be important in connection to the regenerative properties of the avian inner ear. This is a well known ability in birds and other vertebrates that seems to rely on the capacity of supporting cells to reactivate cell division. Damaged cells reactivate cell division and a dormant developmental program that results in the generation of a cluster of Atho1-positive cells and the singling out of hair and supporting cells via the Delta-Notch pathway (Cotanche et al., 1994; Stone and Rubel, 2000; Birmingham-McDonogh and Rubel, 2003). Our data show that SOX2 is maintained in differentiated supporting cells, which may suggest a mechanism to enable them to reenter the cell cycle in case of damage.

In conclusion, the present study illustrates the pattern of expression of SOX2 protein throughout development of the inner ear. We show that SOX2 is initially expressed along with SOX3 in the neurogenic domain. As otic development proceeds, SOX3 is lost, and SOX2 is maintained in progenitor cells of the prosensory region. At postdifferentiation stages, SOX2 withdraws from hair cells and remains expressed in supporting cells from the sensory patches. Our data suggest that SOX2 may have a dual critical function in ear development, first, conferring the proneural potential to otic progenitors and, second, retaining the regenerative capacity of sensory supporting cells.

ACKNOWLEDGMENTS

We are grateful to Thomas Schimmang, Matías Hidalgo-Sánchez, Cristina Pujades, and Isabel Varela-Nieto for comments on the manuscript. We thank Marta Linares for excellent technical assistance. Islet-1 monoclonal antibody was 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). The anti-SOX3 antibody was a kind gift of Thomas Edlund and Jonas Muhr. Probes for the chick Sox2 and Sox3 genes were kindly provided by Paul Scotting, University of Nottingham.

LITERATURE CITED


Alsina B, Abello G, Khatri S, Giraldez F, Alsina B. 2007. Early regionalization of the chick Sox2 and Sox3 genes were kindly provided by the University of Iowa Department of Biological Sciences (Iowa City, IA). The anti-SOX3 antibody maintained by the University of Iowa Department of Biology.


