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Differential and overlapping expression pattern of SOX2 and SOX9 in inner ear development

Angel C.Y. Mak^a, Irene Y.Y. Szeto^{a,b}, Bernd Fritzsche^c, and Kathryn S.E. Cheah^{a,b,*}

^a Department of Biochemistry, The University of Hong Kong, Li Ka Shing Faculty of Medicine Building, 21 Sassoon Road, Pokfulam, Hong Kong, China

^b Centre for Reproduction, Development and Growth, The University of Hong Kong, Li Ka Shing Faculty of Medicine Building, 21 Sassoon Road, Pokfulam, Hong Kong, China

^c Department of Biology, College of Liberal Arts and Sciences, 143 Biology Building, Iowa City, IA 52242-1324, USA

Abstract

The development of the inner ear involves complex processes of morphological changes, patterning and cell fate specification that are under strict molecular control. SOX2 and SOX9 are SOX family transcription factors that are involved in the regulation of one or more of these processes. Previous findings have shown early expression of SOX9 in the otic placode and vesicle at E8.5–E9.5. Here we describe in detail, the expression pattern of SOX9 in the developing mouse inner ear beyond the otocyst stage and compare it with that of SOX2 from E9.5 to E18.5 using double fluorescence immunohistochemistry. We found that SOX9 was widely expressed in the otic epithelium, periotic mesenchyme and cartilaginous otic capsule. SOX2 persistently marked the prosensory and sensory epithelia. During the development of the sensory epithelia, SOX2 was initially expressed in all prosensory regions and later in both the supporting and hair cells up to E15.5, when its expression in hair cells gradually diminished. SOX9 expression overlapped with that of SOX2 in the prosensory and sensory region until E14.5 when its expression was restricted to supporting cells. This initial overlap but subsequent differential expression of SOX2 and SOX9 in the sensory epithelia, suggest that SOX2 and SOX9 may have distinct roles in molecular pathways that direct cells towards different cell fates.

Keywords

SOX2; SOX9; Inner ear; Otocyst; Hair cells; Sensory epithelia; Spiral ganglion

1. Results and discussion

The mammalian inner ear is an intricate organ responsible for the perception of sound and balance. The mouse inner ear arises from a thickening of the surface ectoderm called otic placode located adjacent to rhombomeres 5 and 6 of the hindbrain (reviewed in Barald and Kelley, 2004). At E9.0, the otic placode invaginates to form the otic vesicle. Neuroblasts delaminate from the ventral thickening of the otic vesicle and form the otic ganglion which will become the sensory innervation of the inner ear (Rubel and Fritzsche, 2002). The otic

*Corresponding author: Department of Biochemistry, 3/F, Laboratory Block, Li Ka Shing Faculty of Medicine, 21 Sassoon Road, Pokfulam, Hong Kong. Tel.: +852 2819 9240; fax: +852 2855 1254. hrmbdkc@hkusua.hku.hk (K.S.E. Cheah).

vesicle also undergoes a series of morphological changes until it reaches its mature shape by E17 (Morsli et al., 1998).

The inner ear consists of six sensory organs: the three cristae in the semi-circular canals and the maculae in the utricle and saccule are responsible for vestibular function; the organ of Corti is responsible for auditory function. The sensory patches in these organs consist of hair and supporting cells. The development of sensory patches in the inner ear requires complex processes of prosensory cell specification and cell fate determination (reviewed in Fritzsche et al., 2006; Kelley, 2007), in which SOX2 and SOX9 are likely to be involved (see below).

SOX2 and SOX9 are SOX family transcription factors characterized by a high mobility group (HMG) DNA-binding domain. Mutations in human *SOX2* results in anophthalmia, a severe eye malformation, and some patients showed sensorineural hearing loss (Fantès et al., 2003; Hagstrom et al., 2005). *SOX2* interacts with *EYA1* for prosensory specification (Zou et al., 2008). Mouse mutants with no and reduced expression of *SOX2* in the developing inner ear failed to establish a prosensory domain and formed an abnormal sensory epithelium with disorganized and fewer hair cells, respectively (Kiernan et al., 2005). The expression pattern of *Sox2*/*SOX2* in the inner ear has been described in chick (Uchikawa et al., 1999; Neves et al., 2007) and mouse (Wood and Episkopou, 1999; Kiernan et al., 2005; Hume et al., 2007). In chick, *SOX2* expression is restricted to the supporting cells during differentiation of the sensory epithelia (Neves et al., 2007). This is in contrast to that in the mouse in which *SOX2* is expressed in both hair and supporting cells of all the sensory epithelia until early neonatal stage (P2) when its expression in hair cells is lost (Kiernan et al., 2005; Hume et al., 2007; Dabdoub et al., 2008).

Mutations in human *SOX9* result in skeletal abnormality and XY sex reversal syndrome, campomelic dysplasia (CD), which has a high lethality rate. Sensorineural hearing loss has been reported in the surviving CD patients, suggesting a role of *SOX9* in inner ear development (Savarirayan et al., 2003). Various studies in *Xenopus* (Saint-Germain et al., 2004; Taylor and LaBonne, 2005), zebrafish (Yan et al., 2005) and mouse (Barrionuevo et al., 2008) have described the expression of *SOX9* at the otic placode and/or vesicle stage and they have focused on uncovering the early role of *SOX9* in inner ear development. In *Xenopus* and zebrafish, the otic placodes and vesicles were absent upon *SOX9* depletion (Saint-Germain et al., 2004; Yan et al., 2005). In addition, over-expression of *SOX9* in *Xenopus* results in enlarged or ectopic otic vesicles (Taylor and LaBonne, 2005). In mouse, *SOX9* was found to be important for otic placode invagination (Barrionuevo et al., 2008). However, the dynamics of *SOX9* expression in the different cell types beyond early stages (>E9.5) of inner ear development has not been described.

Both *SOX2* (Bylund et al., 2003; Graham et al., 2003; Ferri et al., 2004; Okubo et al., 2006; Taranova et al., 2006; Que et al., 2007; Holmberg et al., 2008) and *SOX9* (Huang et al., 1999; Bi et al., 2001; Sahar et al., 2005; Seymour et al., 2008) operate in a dose dependent manner in cell fate specification. The expression of *SOX2* and *SOX9* has been previously studied in relation to each other in the mouse developing retina, adult pituitary gland and brain (Sottile et al., 2006; Fauquier et al., 2008; Poche et al., 2008). These studies have suggested cell populations expressing either *SOX2* or *SOX9*, or both together may represent different populations of pluripotent progenitors or cells undergoing different cell fate decisions. To facilitate the interpretation of its role in inner ear development, we present for the first time a comprehensive description of the temporal and spatial expression profile of *SOX9* in the developing inner ear beyond the otocyst stage in relation to the expression of *SOX2*.

1.1. E9.5–E10.5 otocyst

In E9.5 otocyst, SOX2 was expressed in the proneural region in which neuroblast will delaminate to form the statoacoustic ganglion (Fig. 1A). It was also expressed in the adjacent hindbrain. SOX9 was faintly expressed in the periotic mesenchyme and prominently in the entire otic epithelium (Fig. 1B).

The expression signal of SOX9 was stronger on the medial side while that of SOX2 was stronger on the ventral side of E9.5 otic vesicle (Fig. 1). This variation in SOX2 and SOX9 expression was also the results of a smaller percentage of cells expressing SOX2 in the dorsal side and SOX9 in the lateral side of the otic vesicle, respectively. The expression pattern of SOX2 and SOX9 overlapped most strongly at the ventro-medial side which has been predicted to give rise to the saccule, cochlea and part of the vestibular ganglion in chick (Wu and Oh, 1996) and mice (Farinas et al., 2001). Fig. 1C shows a gradient of expression signal in the otic vesicle which varied from the dorsal–medial side with predominant SOX9 expression signal, to the ventral–medial side with overlapping SOX2 and SOX9 signal, and finally to the lateral side with predominant SOX2 signal.

In the E10.5 otocyst, SOX9 was expressed in the otic epithelium and periotic mesenchyme. Regions with relatively stronger and weaker signal for SOX9 in the otic epithelium were observed (Fig. 2A'–C'). In the most anterior transverse sections of the E10.5 otocyst, SOX2 was expressed and overlapped with SOX9 on the ventro-lateral side of the otocyst (Fig. 2A–A"). In subsequent sections (Fig. 2B–B"), two domains of SOX2 expression were detected in the otocyst – one on the lateral side and one on the ventral side. SOX2 was also detected in the delaminating neuroblasts that surround the vestibular ganglion. In more posterior sections (Fig. 2C–C"), SOX2 was expressed and overlapped with SOX9-expressing cells found on two domains on the medial side of the otocyst. However, SOX9 expression signal was relatively weaker in the ventral–medial SOX2-expressing region than in the rest of the otocyst.

1.2. E12.5

At E12.5, SOX2 expression marked the sensory primordia of the cristae (Fig. 3A, B and E), maculae (Fig. 3D) and cochlear duct (Fig. 3C). SOX9 was expressed in the periotic mesenchyme and the entire otic epithelium. In the lateral and posterior cristae and vestibular ganglion, relatively weak SOX9 expression signal than in the rest of the otic epithelium was detected (Fig. 3A' and E). This difference was less obvious in the cochlea (Fig. 3C'). In the utricular macula (Fig. 3D), SOX2 was expressed in the entire sensory epithelia while SOX9 expression became restricted to the supporting cells.

1.3. E14.5, E16.5, E18.5

By E14.5, the sensory epithelia of the utricle, saccule and cristae have initiated differentiation into hair and supporting cells. At E14.5–E18.5, SOX2 was expressed in both cell types while SOX9 expression became restricted to supporting cells only (Figs. 4–6). SOX9 was also expressed throughout the non-sensory otic epithelium and periotic mesenchyme of the developing inner ear.

In the cochlea, hair cells start to differentiate at E13.5–E14.5. Hair cell cycle exit starts in the apex at E11.5 (Matei et al., 2005) whereas differentiation starts in the mid-basal region of the cochlear duct and progresses in the apical direction (Chen et al., 2002). A more differentiated cochlear sensory epithelium is thus expected at the basal end. The gradient developmental pattern of the organ of Corti allows analysis on the graded developmental changes of the expression pattern of SOX2 and SOX9 in a single cochlea.

At E14.5, an obvious degree of differentiation of the sensory epithelia was noticeable when comparing the basal and apical cochlea (Fig. 4E and F). A clearer demonstration of the relationship between the differentiation gradient along the cochlear duct and the expression pattern of SOX2 and SOX9 can be seen at E16.5 and E18.5 (Figs. 5A–D, 6A–D). In the undifferentiated cochlear duct (Fig. 6A), SOX9 expression signal was found throughout the entire otic epithelium and overlapped with that of SOX2 at the sensory epithelium. As differentiation continued, SOX9 became restricted to the supporting cells including the inner and outer pillar cells, inner phalangeal supporting cells and Deiters' cells (Fig. 6C). SOX2 maintained its expression in both the hair and supporting cells. At E18.5, SOX9 continued to be excluded from the hair cells but was expressed on the other part of the otic epithelium including the supporting cells, stria vascularis, the epithelial layer of the Reissner's membrane, interdental cells, spiral limbus, spiral prominence (Fig. 6C and D). In particular, SOX9 expression signal in the interdental cells and spiral limbus was noticeably stronger than that in other parts of the otic epithelium.

The differentiation of the spiral ganglion progresses in a basal to apical manner. A temporal change of expression of SOX2 and SOX9 was observed in this process. SOX9 was not detected in the less differentiated spiral ganglion (Figs. 4E–F and 5E), but in more differentiated spiral ganglion cells (Figs. 5F–H and 6E–F). Likewise, SOX2 immunostaining was initially faint and diffuse but was eventually up-regulated in many cells of the spiral ganglion. At E16.5, only SOX2 was detected at the apical end of the spiral ganglion (Fig. 5E). SOX9 was detected at the basal end of the spiral ganglion and was co-expressed with SOX2 (Fig. 5F–H). Expression of SOX2 and SOX9 was more prominent and in more spiral ganglion cells at E18.5 (Fig. 6E–F). TUJ1 is a neuron-specific marker. In the developing chick inner ear, SOX2 was not co-expressed with TUJ1 in the ganglion (Neves et al., 2007). Similarly, expression of SOX9 did not overlap with that of TUJ1, suggesting these cells are not neurons (Fig. 6G–H). SOX2 protein can be very stable, persisting in the absence of transcript and can be localized in the cytoplasm (Avilion et al., 2003). The developmental pattern of SOX2 protein expression reported here is consistent with the recent description of localization of *Sox2* mRNAs in the inner ear by whole mount *in situ* hybridization (Nichols et al., 2008), suggesting concordance between transcript and protein stability in the developing inner ear.

The expression profiles of SOX2 and SOX9 relative to each other from E9.5 to E18.5 mouse inner ear development are summarized in Fig. 7A and B. This study of the expression pattern of SOX9 in relation to SOX2 facilitates the understanding of its roles in inner ear development. A compartment-boundary model of inner ear development has been proposed in which the otocyst is subdivided into lineage-restricted compartments based on regional gene expression (Fekete, 1996; Brigande et al., 2000; Fekete and Wu, 2002). Within these compartments, unknown molecular mechanisms govern the specification of different inner ear structures and their corresponding sensory patches. Fig. 7A summarizes the expression of SOX2 and SOX9 in an E9.5 otic vesicle and lists the inner ear structures that have been proposed by Fekete and Wu (2002) to arise from these compartments, demonstrating that our data are consistent with this model. Using SOX2 as a marker for the prosensory and sensory epithelium, the initial overlap but subsequent differential expression of SOX2 and SOX9 in the sensory epithelium suggests that SOX2 and SOX9 may have distinct roles in molecular pathways that direct cells towards different cell fates.

2. Experimental procedures

2.1. Mouse husbandry

Pregnancies of F1 (C57BL/6N × CBA/Ca) mice were timed from the day of the vaginal plug which was designated as embryonic day 0.5 (E0.5). Animal care and sacrifice were

conducted according to methods authorized by licences from the Department of Health of the Government of the Hong Kong Special Administrative Region.

2.2. Immunohistochemistry

Staged mouse embryos were fixed and processed using standard procedures (Hogan et al., 1994). Paraffin embedded mouse embryos were cut into 5–6 μ m sections. Fluorescence immunohistochemistry was carried out using the following antibody dilutions with 10% heat-inactivated donkey serum (SIGMA D9663), 0.5% Triton X-100, in PBS as diluent: 1:1000 for rabbit anti-SOX9 (Chemicon AB5535), 1:600 for goat anti-SOX2 (Neuromics GT15098), 1:500 for mouse anti-TUJ1 (COVANCE MMS-435P), 1:1000 for CyTM3-conjugated donkey anti-rabbit IgG (Jackson 711-166-152), 1:500 for Alexa Fluor® 488-conjugated donkey anti-goat IgG (Molecular Probes A-11055), 1:500 for CyTM3-conjugated donkey anti-mouse IgG (Jackson 715-166-150), 1:500 for Alexa Fluor® 647 donkey anti-rabbit IgG (Molecular Probes A-31573). Sections were incubated in primary antibody at 4 °C overnight and in secondary antibody in dark at room temperature for 2 h. Nucleus staining was performed at room temperature for 15 min with 1:2000 TOTO@3 iodide (Invitrogen T3604) in PBS. Specimens were mounted in VECTASHIELD mounting media (Vector Labs). Images were captured using ZEISS LSM510 META confocal microscope and exported using LSM Image Browser (version 4.0.2.121).

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Fig. 1. SOX2 (green) and SOX9 (red) expression pattern at E9.5 (24 somites) mouse otic vesicle (ov). The labels, orientation and scale bar of C also apply to A and B. The signal of SOX9 expression was stronger on the medial side of the otic vesicle (B). The signal of SOX2 expression was stronger on the ventral side (A). SOX2 and SOX9 were coexpressed most strongly on the ventral–medial domain of the otic vesicle (C). hb, neural tube of hindbrain; D, dorsal; L, lateral. Scale bar = 50 μ m.

Fig. 2. SOX2 (green) and SOX9 (red) expression pattern at E10.5 mouse otocyst showing sections from anterior (A–A'') to posterior (C–C''). The scale bar and orientation of A'' apply to all pictures in this figure. SOX2 marked the proneural and prosensory domains of the developing inner ear (A–C). It was also expressed in delaminating neuroblasts that stayed on the boundary of the vestibular ganglion (B, asterisk). SOX9 was expressed in the entire otic epithelium and periotic mesenchyme. There were regions with relatively stronger (arrowhead) and weaker (arrow) signal for SOX9 (A'–C'). ed, endolymphatic duct; sg, spiral ganglion; vg, vestibular ganglion; hb, hindbrain; D, dorsal; L, lateral. Scale bar = 100 μ m.

Fig. 3. SOX2 (green) and SOX9 (red) expression pattern at E12.5 mouse developing inner ear. SOX2 marked the prosensory domains of the cristae (A, B and E), utricular macula (D) and the organ of Corti (C). SOX9 continued to be expressed in the periotic mesenchyme and otic epithelium. In the lateral crista, sections closer to the dorsal boundary have relatively weaker SOX9 signal (A'). This was shown in A'' as a predominant green signal (SOX2). Similar observations were obtained in the posterior crista (E) and the cochlear duct (C''). The SOX9 detection signal in the anterior crista was as prominent as other regions of the otic epithelium. It was apparent that SOX2 and SOX9 expression were partially overlapping which is shown in B'' as a mosaic of co-expressing (yellow) and SOX2 only (green) signal. In the utricular macula (D), SOX9 expression was limited to the supporting cells while SOX2 was expressed in both hair and supporting cells. ed, endolymphatic duct; cd, cochlear duct; vg, vestibular ganglion; ac, anterior crista; lc, lateral crista; lsc, lateral semi-circular canal; cp, otic capsule; pc, posterior crista; u, utricle; A, anterior; L, lateral. Scale bar = 100 μ m.

Fig. 4. SOX2 (green) and SOX9 (red) expression pattern at E14.5 mouse developing inner ear. SOX2 was expressed in both the hair and supporting cells of the cristae (A and B), utricular (C) and saccular (D) maculae. In the developing cochlear duct, SOX2 marked the prosensory region of the organ of Corti and its expression overlapped with that of SOX9 (E and F). SOX9 continued to be expressed in the periotic mesenchyme and most part of the otic epithelium. SOX9 was not expressed in the hair cells but its expression overlapped with SOX2 in the supporting cells of the cristae and the maculae (A'', B'', C and D). ac, anterior crista; cd, cochlear duct; lc, lateral crista; pc, posterior crista; s, saccule; sg, spiral ganglion; u, utricle; A, anterior; D, dorsal. Scale bar = 50 μ m. The orientation in A applies to A–A''. The scale bar in A applies to A–A'', B–B''. The scale bar in C applies to D–F.

Fig. 5. SOX2 (green) and SOX9 (red) expression pattern at E16.5 mouse developing inner ear. SOX2 marked all the sensory epithelia in the vestibule (I–K) and the organ of Corti (A–D), as well as some cells in the cochleovestibular ganglion (E–H). In the vestibular sensory epithelia – the cristae (K'), maculae (I and J), SOX9 was only expressed in the supporting cells but not in the hair cells. In the cochlear duct (A–D), expression of SOX9 initially overlapped with that of SOX2 in the prosensory epithelium (A) but later became restricted to supporting cells in the more differentiated sensory epithelium at the basal end (C and D). In the spiral ganglion (sg), only SOX2 was expressed in the sg of the least mature end of the cochlear duct (E). SOX9-expressing cells appeared next to SOX2 positive cells in the sg of the basal end (H). Cells strongly positive for both SOX2 and SOX9 below the basilar membrane are red blood cells in the spiral artery whose autofluorescence is very strong for these excitation wavelengths. ac, anterior crista; lc, lateral crista; dc, Deiters' cells; hc, Hensen cells; ihc, inner hair cell; ipc, inner phalangeal cells; ohc, outer hair cells; pc, pillar cell; u, utricle; s, saccule. Scale bar = 50 μ m. Scale bar in A applies to B–D, E applies to F–H, K applies to I, J, K–K''.

Fig. 6.

SOX2 (green) and SOX9 (red) expression pattern at E18.5 mouse developing inner ear. SOX2 marked all the sensory epithelia in the organ of Corti (A–C) and the vestibular organs (I–L). In the utricular macula (K), saccular macula (L) and cristae (I and J), SOX9 was not expressed in the hair cells but expressed in the supporting cells. Along the cochlear duct from the apex to the base, SOX9 expression initially overlapped with that of SOX2 but later became restricted to supporting cells in more differentiated sensory epithelium at the basal end (A–D). SOX9 was expressed in the epithelial layer of the Reissner's membrane (D). In the spiral ganglion, expression of SOX2 and SOX9 was dispersed but in discretely positive cells of non-neuronal identity (E–H). There is no overlap of expression between SOX9 and TUJ1 (G and H). ac, anterior crista; sg, spiral ganglion; dc, Deiters' cells; hc, Hensen cells; id, interdental cells; ihc, inner hair cell; is, inner sulcus cells; lc, lateral crista; ohc, outer hair cells; r (ep/me), Reissner's membrane (epithelial/mesenchymal layer); ipc, inner phalangeal cells; pc, pillar cell; s, saccule; sl, spiral limbus; sp, spiral prominence; sv, stria vascularis; u, utricle. Scale bar = 50 μ m. Scale bar in A applies to B–D, E applies to F, G applies to H, I applies to J, K applies to L.

Fig. 7. A summary on the expression pattern of SOX2 and SOX9 in E9.5–E18.5 mouse developing inner ear. (A) Diagrammatic representation of SOX2 and SOX9 expression pattern in an E9.5 otic vesicle and the inner ear structures that will arise from different compartments. The color gradient depicts the expression gradient of SOX2 and SOX9 observed in the E9.5 otic vesicle. This gradient is due to variation in expression signal as well as a variation in the numbers of cells expressing SOX2 and SOX9. Cell fate of different compartments of the otic vesicle is based on works on chick by Fekete and Wu (2002). (B) A summary on SOX2 and SOX9 expression in E9.5–E18.5 mouse developing inner ear. D, dorsal; L, lateral; M, medial; V, ventral.