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***Xenopus laevis* Nkx5.3 and sensory organ homeobox (SOHo) are expressed in developing sensory organs and ganglia of the head and anterior trunk**

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Abstract

Nkx5 family members are homeobox transcription factors important for sensory organ development. Several members of the Nkx5 family are expressed in the eye, brain, developing ear, and lateral line. Members of this family have been previously identified in medaka, chick, and mouse. Here, we characterize two members of the Nkx5 family, Nkx5.3 and SOHo, in *Xenopus laevis*. We verify the identity of *X. laevis* Nkx5.3 and SOHo by phylogenetic comparison to chicken, medaka, and zebrafish orthologs. Both Nkx5.3 and SOHo are expressed in the developing eye, ear, lateral line system, and cranial neurons as determined by in situ hybridization.

Keywords

Nkx5.3; Sensory organ homeobox; *Xenopus laevis*; Retina; Cranial ganglia; Lateral line

Results

Homeobox genes play numerous essential roles in embryonic development. In addition to organization of the body plan and other early developmental events, homeobox genes are involved in regulation of expression of genes specific to differentiated cell function. For example, transcription of the rhodopsin gene, expressed in differentiated rod cells of the mature neural retina, is regulated by the homeobox genes Rax, Otx2, and Crx/Otx5b (Mitton et al. 2000; Whitaker and Knox 2004). Nkx5 family members are generally expressed in developing sensory organs (Wang et al. 2000; Adamska et al. 2001). The Nkx5 family members Nkx5.3 and SOHo (sensory organ homeobox) are expressed in the developing retinas of zebrafish, medaka, and chick, (Deitcher et al. 1994; Stadler and Solursh 1994; Wang et al. 2000; Adamska et al. 2001). Additionally, Nkx5.3 is expressed in the developing mouse and human retina (Schorderet et al. 2008). Loss-of-function of Nkx5.3 results in microphthalmia in zebrafish, mice, and humans. To further our investigations of homeobox

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genes involved in retinal development, we sought to investigate the expression of Nkx5.3 and SOHo during *Xenopus laevis* embryonic development.

Sequence comparison

X. laevis Nkx5.3 and SOHo were most related to chicken Nkx5.3 and SOHo in BLAST analysis of these sequences (not shown). *X. laevis* Nkx5.3 was aligned to medaka, zebrafish, and chicken Nkx 5.3 and SOHo (Fig. 1). Frog Nkx5.3 is 40.9, 49.6, and 59.9 % identical to medaka, zebrafish, and chicken Nkx5.3 on the amino acid level, respectively. There is near identity throughout the homeodomain (aa 151–212) and the adjacent HMXSD1 and SD2 domains (aa 213–237) (Adamska et al. 2001) (Fig. 1a). Additionally, there is a conserved N-terminal domain (aa 20–30). This degree of identity is noticeably greater than that to *X. laevis* Nkx2.2 (26.8 % identity). Similarly, *X. laevis* SOHo exhibits a higher degree of amino acid identity to chicken, medaka, and zebrafish SOHo than to *X. laevis* Nkx2.2, used to root the tree. (40.0, 43.4, 75.0 % identity to chicken, medaka, and zebrafish SOHo, respectively, vs 28.9 % identity to Nkx2.2). Again, there is near identity throughout the homeodomain (aa 127–187), although there is lower identity through the HMXSD1-SD2 domain than in the case of Nkx5.3 (Fig. 1b). Phylogenetic tree analysis reveals that *X. laevis* Nkx5.3 is more closely related to other Nkx5.3 than to *X. laevis* SOHo and vice versa (Fig. 1c). *X. laevis* Nkx5.3 and SOHo are more closely related to each other than to Nkx2.2 (Saha et al. 1993), used to root the tree.

Eye expression

We used whole mount in situ hybridization (WISH) and section in situ hybridization (SISH) to visualize Nkx5.3 and SOHo expression. Nkx5.3 and SOHo are expressed in the developing optic vesicle; however, Nkx5.3 is apparent earlier than SOHo, at stages 23 and 25, respectively (Figs. 2, 3, a).

The expression of both genes continues in the developing retina through differentiation and stratification (Figs. 2, 3, g–i). We did not observe the same anterior retinal regionalization reported in medaka and mouse for Nkx5.3 or in chick for SOHo (Deitcher et al. 1994; Stadler and Solursh 1994; Wang et al. 2000; Adamska et al. 2001). However, both Nkx5.3 and SOHo have slightly stronger expression in the dorsal retina at stage 32 (Fig. 2d, 3d inset compare yellow and green arrows). Dorsal differential expression was reported in chick for Nkx5.3 (Stadler and Solursh 1994). This asymmetry becomes less apparent as development progresses. Nkx5.3 is strongly expressed throughout the retina at stage 35 (Fig. 2e). SOHo is expressed evenly nasal to caudal and dorsal to ventral, in coronal sections of stage 41 embryos (Fig. 3j). Nkx5.3 is expressed throughout the ganglion cell layer and inner nuclear layer, but is absent from the photoreceptor layer (Fig. 2g–i). Nkx5.3 is also expressed in zones 2–4 of the ciliary marginal zone (CMZ) (Perron et al. 1998), by comparison to expression of Notch1 (Fig. 2j, j') and NeuroD (Fig. 2k, k'), markers for zones 2 and 3 of the CMZ, respectively. SOHo is expressed in the ganglion cell layer and weakly and unevenly in a portion of the inner nuclear layer (Fig. 3g–i, g'–i'), but is absent from the photoreceptor layer and CMZ. Both Nkx5.3 and SOHo are strongly expressed in the developing lens placode (Fig. 2c, 3c). Expression of both Nkx5.3 and SOHo persists in the lens placode and mature lens throughout lens development, in areas of proliferation (Fig. 2h, 3h).

Other expression

Nkx5.3 expression starts in a confined region of the common posterior placode area where the lateral line, otic, and epibranchial placodes originate (Fig. 2a) (Schlosser 2014). At stage 25, this expression becomes clear in the developing trigeminal nerve, just caudal to the eye (Fig. 2b). This expression is consistent with medaka and mouse (Wang et al. 2000; Adamska et al. 2001). Expression also begins in the otic vesicle at stage 25, as the ear begins to develop (Fig. 2b). Expression in the developing ear continues through tadpole stages (Fig. 2c–e). This differs from its expression in medaka, where it is expressed in the medial wall of the ear, but is absent from the otic vesicle (Adamska et al. 2001). At stage 29, Nkx5.3 continues to be expressed in the trigeminal nerve, and expression starts in the placode for the anterodorsal lateral line, adjacent to the otic vesicle (Fig. 2c). Nkx5.3 expression starts in the middle lateral line placode, developing cranial nerve IX, and placode of anteroventral lateral line by stage 32 (Fig. 2d). Interestingly, Nkx5.3 was not detected in the lateral lines in medaka (Adamska et al. 2001). At stage 35, Nkx5.3 expression extends ventrally in the anteroventral lateral line placode and cranial nerve IX and is detectable in cranial nerves VII and the termini of cranial nerve VII (Fig. 2e). The expression in cranial nerve VII is also seen in mouse and chick (Stadler and Solursh 1994; Wang et al. 2000).

SOHo expression is first detected outside the eye in the otic vesicle at stage 25 (Fig. 3a). Like medaka and chick, the expression of SOHo is asymmetrical with the strongest expression at the most anterior part of the otic placode (Fig. 3a). Like Nkx5.3, SOHo expression remains strong in the ear throughout development (Fig. 3b–e). At stage 29, SOHo expression starts in the trigeminal nerve, adjacent to the optic vesicle, and the anterodorsal lateral line placode rostral to the ear (Fig. 3c). By stage 32, SOHo expression is detectable in the middle lateral line placode (Fig. 3d). Weak expression of SOHo in the lateral line was also described in medaka (Adamska et al. 2001). At stage 35, SOHo expression persists in the trigeminal nerve, strengthens in the middle lateral line placode, begins in cranial nerves VII and IX including the termini of cranial nerve VII, and is expressed diffusely throughout the brain (Fig. 3e). Expression in the middle lateral line placode extends posteriorly as development progresses. Expression in the trigeminal nerve and brain anlage has also been described in chick (Deitcher et al. 1994).

In summary, we found that the expression of Nkx5.3 and SOHo in *X. laevis* is largely similar to expression reported in other species. One notable exception is that the anterior regionalization of expression in the retina of some species was not observed in *X. laevis*. Instead, there is a dorsal ventral variability noticeable at stage 32 similar to Nkx5.3 expression observed in chick. This may correspond to the difference in maturation rate of the dorsal and ventral regions of the retina in these species, as it is eventually resolved as the retina matures. Interestingly, we observed strong expression of Nkx5.3 in the middle lateral line nerve and weak expression in the anterodorsal lateral line and anteroventral lateral line placodes, but that weak expression of SOHo, but not Nkx5.3, was reported in the lateral line system of medaka. This could point to potential differences in lateral line development in amphibians and fishes.

Materials and methods

Nkx5.3 and SOHo cDNA clones

Nkx5.3 complementary DNA (cDNA) was a kind gift from B. Knox. *X. tropicalis* SOHo was identified by searching genomic sequence using chicken SOHo as query and subsequently used as query to search *X. laevis* genomic sequence. Putative *X. laevis* SOHo sequence was used to design primers for 5'- and 3'-RACE which were carried out using the SMARTer Kit (Clontech) and cDNA prepared from total RNA isolated from st 41 tadpole heads (Trizol). *X. laevis* SOHo sequence has been deposited into GenBank (accession number KT318280). Sequences were aligned using ClustalW 1.8 at the EMBL-EBI webpage (URL: <http://www.ebi.ac.uk/clustalw/index.html>) and then formatted using BOXSHADE at the EMBnet website (URL: http://www.ch.embnet.org/software/BOX_form.html). Pairwise percent amino acid conservation among vertebrate Nkx5.3 and SOHo gene products was calculated using a pairwise alignment program (MacVector). Accession numbers for sequences used are as follows: chick Nkx5.3—Q9DE09, medaka Nkx 5.3—AAL04487.1, zebrafish Nkx5.3—ABY60841.1, chick SOHo—NP_990717, medaka SOHo—AAL04488, zebrafish SOHo—translated from AI658291, and *X. laevis* Nkx2.2—AAB28271.

In situ hybridization

In situ hybridization was performed using antisense riboprobes labeled with digoxigenin. Template DNA was linearized and transcribed in vitro. Nkx5.3 probe was prepared from pCS2/Nkx5.3 plasmid linearized with BamHI and transcribed with T7 RNA polymerase. SOHo was prepared from TA-SOHo plasmid linearized with BamHI and transcribed with T7 RNA polymerase. NeuroD and Notch probes were prepared as described previously (Perron et al. 1998). Whole mount in situ hybridization (WISH) was performed using fixed *X. laevis* embryos described previously (Sive et al. 2000). Embryo stages were determined by the accepted standard (Nieuwkoop and Faber 1994). WISH was performed twice per probe using at least four embryos per desired stage (see Figs. 2, 3). Section in situ hybridization (SISH) was performed using 8- μ M sections of fixed, paraffin-embedded st 38, 41, and 45 tadpoles (Vicgian et al. 2003). SISH was performed at least twice (twice for Nkx5.3, thrice for SOHo) using at least four embryos per stage.

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Fig. 1.

Sequence comparison of *Xenopus laevis* Nkx5.3 and SOHo (a, b). Predicted amino acid sequence of *X. laevis* Nkx5.3 (a) and SOHo (b) aligned with orthologs from chick, medaka, and zebrafish. *Solid line* indicates homeodomain and *dashed line* indicates HMXSD1 and SD2 domains. c. Phylogenetic tree visualization of relatedness of Nkx5.3 and SOHo from different species. *Gg Gallus gallus* (chick), *Xl Xenopus laevis* (frog), *Dr Danio rerio* (zebrafish), *Ol Oryzias latipes* (medaka)

Fig. 2.

Spatial and temporal expression of Nkx5.3 in *Xenopus laevis*. **a–f** Lateral view of *X. laevis* embryos that have been subjected to in whole mount in situ hybridization using antisense riboprobes for Nkx5.3 (**a–e**) or sense Nkx 5.3 riboprobe (**f**). **a** Stage 23 tailbud. **b** Stage 25 tailbud. **c** Stage 29. **d, f** Stage 32. **e** Stage 35 tadpole. **g–l** Transverse sections of paraffin-embedded *X. laevis* tadpoles subjected to section in situ hybridization using antisense riboprobes for Nkx5.3 (**g–i, h'**), Notch1 (**j, j'**), NeuroD (**k, k'**), and sense Nkx5.3 riboprobe (**l**). **g** Stage 38. **h, j–l, j', k'** Stage 41, *black arrowheads* indicate expression in the lens. **i** Stage 45. **h'–k'** Higher magnification (digital) of pictures of **h–k**, respectively, to compare expression in zones 1–4 (*numbered segments*) in the CMZ (*right of vertical orange line*). *Yellow and green arrowheads* indicate dorsal and ventral eye, respectively (**d inset**). *Scale bars* (*black bars*) in **d** (**a–f**), **d inset**, **h** (**g–l**), and **h'** (**h'–k'**) are 1 μ M. Abbreviations: *B* brain, *epVII* termini of nerve VII, *G* ganglion cell layer, *I* inner nuclear layer, *IX* cranial nerve IX, *L* lens, *LP* lens placode, *M* nerve of middle lateral line, *OT* otic vesicle, *OV* optic vesicle, *P* photoreceptor layer, *pAD* placode of anterodorsal lateral line, *pAV* placode of anteroventral lateral line, *PP* posterior placode, *R* retina, *T* trigeminal nerve, *VII* cranial nerve VII

Fig. 3.

Spatial and temporal expression of SOHo in *Xenopus laevis*. **a–f** Lateral view of *X. laevis* embryos that have been subjected to in whole mount in situ hybridization using antisense riboprobes for SOHo (**a–e**) or sense SOHo riboprobe (**f**). **a** Stage 25 tailbud. **b** Stage 26 tailbud. **c** Stage 29. **d, f** Stage 32. **e** Stage 35 tadpole. **g–i, k** Transverse sections of paraffin-embedded *X. laevis* tadpoles subjected to section in situ hybridization using antisense riboprobes for SOHo (**g–i**), and sense SOHo riboprobe (**k**). **j** Coronal section of paraffin-embedded *Xenopus* tadpole subjected to in situ hybridization for SOHo. **g, g'** Stage 38. **h, h', j, k** Stage 41, **black arrowheads** indicate expression in the lens. **i, i'** Stage 45. **g'–i'** Higher magnification (digital) of **g–i**, layers separated by **black dotted lines**, **black arrowheads** highlight expression of SOHo. Scale bars are black bar in **d (a–f)**, **d inset**, **h (g–i, k)**, **h' (g'–i')**, and **j** are set to 1 μ m. **Yellow and green arrowheads** indicate dorsal and ventral eye, respectively (**d inset**). Abbreviations: *D* dorsal, *N* nasal. See Fig. 2 for further abbreviations