Evolutionarily conserved role for SoxC genes in neural crest specification and neuronal differentiation

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Abstract

Members of the Sox family of transcription factors play a variety of critical developmental roles in both vertebrates and invertebrates. Whereas SoxBs and SoxEs are involved in neural and neural crest development, respectively, far less is known about members of the SoxC subfamily. To address this from an evolutionary perspective, we compare expression and function of SoxC genes in neural crest cells and their derivatives in lamprey (Petromyzon marinus), a basal vertebrate, to frog (Xenopus laevis). Analysis of transcript distribution from reveals conservation of lamprey and X. laevis SoxC expression in premigratory neural crest, branchial arches, and cranial ganglia. Moreover, morpholino-mediated loss-of-function of selected SoxC family members demonstrates essential roles in aspects of neural crest development in both organisms. The results suggest important and conserved functions of SoxC genes during vertebrate evolution and a particularly critical, previously unrecognized role in early neural crest specification.

Keywords
SoxC; Neural crest; Neuronal differentiation; Lamprey; Evolution

Introduction

Members of the SRY-related high mobility group box (Sox) gene family are expressed in various cell lineages and are critical for cell fate determination and differentiation of many developing tissues (Bowles et al., 2000; Laudet et al., 1993). The Sox family is comprised of transcription factors that regulate gene expression through a single high-mobility group (HMG) DNA binding domain (Laudet et al., 1993). The family is divided into ten groups (A–J), based on the HMG sequence and other conserved domains. Sox transcription factors are thought to control transcription by altering chromatin conformation to allow formation of transcription enhancer complexes (Dy et al., 2008; Grosschedl et al., 1994; Kuroda et al., 2005). Because the family members sometimes contain trans-activation or trans-repression domains, they are able to interact with different partners to modulate gene expression in a variety of ways.

Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.09.022.
Sox genes have been shown to play crucial yet distinct roles during neural development. For instance, SoxB proteins Sox1–3 are expressed in neural precursors of the central nervous system, where they function in the maintenance of the progenitor pool population by inhibiting neuronal differentiation (Pevny and Placzek, 2005). Alternatively, SoxEs and SoxDs are critical players in neural crest development (Hong and Saint-Jeannet, 2005). SoxE factors Sox8–10 have been shown to be important for neural crest specification, migration, and diversification in chick (Bell et al., 2000; McKeown et al., 2005), frog (O'Donnell et al., 2006), zebrafish (Yan et al., 2005), and lamprey (McCauley and Bronner-Fraser, 2006). Additionally, the SoxD gene LSox5 is also expressed in neural crest progenitors, and it has been suggested that it cooperates with Sox9 to drive neural crest specification (Perez-Alcala et al., 2004).

The SoxC gene subfamily consists of three single exon genes: Sox4, Sox11, and Sox12 (also known as Sox22). Although best known for roles in cancer (Ikushima et al., 2009; Penzo-Mendez, 2010; Vegliante et al., 2013; Wasik et al., 2013), past studies have examined the effects of SoxC genes in neural development. Sox4, Sox11, and Sox12 are all expressed in the post-mitotic neurons of the central nervous system (Cheung et al., 2000; Dy et al., 2008) in a pattern that is complementary to SoxBs. SoxCs seem to be important for neuronal differentiation, driving expression of panneuronal proteins (Bergslund et al., 2006). This is consistent with data obtained from mouse knockout studies, which reveal a decrease in neuronal survival and loss of differentiated neurons (Bhattaram et al., 2010). Recently, Sox4 and Sox11 also have been shown to be crucial for survival of sympathetic ganglia (Potzner et al., 2010) and sensory neurons of the trigeminal and dorsal root ganglia (Lin et al., 2011). Thus, SoxCs seem to have an important role in development of neuronal derivatives of the neural crest.

While these results highlight a later role of SoxCs in neural crest diversification, little is known about the involvement of these factors in the early steps of neural crest formation. Similarly, there is no information regarding the expression or function of SoxCs in development of basal vertebrates, making it difficult to infer the pan-vertebrate role of this gene family. Although the expression pattern of other major Sox gene subfamilies have been described in the sea lamprey (Petromyzon marinus) (Uy et al., 2012), here we expand this analysis to include the SoxC subfamily and characterize the function of the lamprey SoxC orthologs in neural crest formation in a basal vertebrate. To this end, we have cloned and characterized the expression patterns and function of the lamprey SoxC gene family, termed SoxC1–C4, during neural crest development. To assess conservation of the roles of SoxCs in vertebrate development, we compared these to their homologs (Sox4, Sox11, and Sox12) in *Xenopus laevis*. We find, along with the *X. laevis* SoxCs, SoxC1, SoxC3, and SoxC4 are expressed in neural crest cells and/or their derivatives and play an important role in aspects of their development. Intriguingly, the results reveal a previously unknown early function for SoxC genes in neural crest specification that is conserved between lamprey and *X. laevis*. 
Methods and materials

Cloning and characterization of SoxC genes

We used a variety of methods to clone the SoxC genes from frog (*X. laevis*) and lamprey (*P. marinus*). The full-length clones of *X. laevis* Sox4 and Sox11 genes were obtained commercially through Open Biosystems while *X. laevis* Sox12 was PCR amplified from a cDNA library and TOPO cloned. Obtention of full-length lamprey SoxCs required a combination of heterospecific macro-array screening and 5' RACE as described below.

Heterospecific screening of an arrayed lamprey embryonic cDNA library

A high-quality directional full-length arrayed cDNA library (Sauka-Spengler et al., 2008) from embryonic day 2–12 lamprey embryos (an average efficiency of ~0.9 × 10⁸ transformants/μg of cDNA) was used for low-stringency screening. Nine individual nitrocellulose filters were screened using Sox heterospecific probes from *Gallus gallus* SoxC genes, yielding four different SoxC genes whose identities were confirmed by sequencing on both strands and by BLAST searching the database. The SoxC1 and SoxC4 clones were full length while SoxC2 and SoxC3 were truncated and thus required 5' RACE for the completion of the mRNA sequence.

RNA-ligated mediated 5' rapid amplification of cDNA ends (RLM-5' RACE)

Total RNA was extracted from 6, 8, 10, and 14 days old embryos using the Ambion RNAqueous kit. RLM-5' RACE was conducted on the total mRNA with the Invitrogen GeneRacer Kit. Total RNA was dephosphorylated through Calf Intestinal Phosphatase (CIP) treatment, decapped via Tobacco Acid Pyrophosphatase (TAP), ligated with the GeneRacer RNA oligo, and finally reverse-transcribed using random hexamer priming to form the cDNA template. *SoxC* fragments were amplified by touchdown PCR according to the Invitrogen GeneRacer Kit. The gene specific primers used were SoxC2: 5' ACGACGGGACGGATGACAAAGCA 3' and SoxC3: 5' GGGTGGCCCTCTCGCTTGCTC 3'.

Cloning the PCR product

Extraction of the PCR product was conducted corresponding to Qiagen:QIAquick Gel Extraction Kit and cloned with Invitrogen: TOPO TA Cloning. The clones were selected against the metabolism of X-gal and the production of β-galactosidase purified following the QIAprep spin miniprep kit and sent for sequencing. (Davis Sequencing, Davis, CA).

Embryo collection and maintenance

Mature or maturing *P. marinus* adults were obtained from Hammond Bay Biological Station, Millersburg, MI, USA. Mature animals were maintained in our lamprey facility in 12 °C chilled re-circulating water and used for spawning. Juveniles were kept at lower temperatures before the onset of maturation and then progressively induced to maturation by gradually augmenting the water temperature and expanding the daylight cycle. For *in vitro* fertilization, eggs are stripped manually from a single gravid female into a 500 ml crystallizing dish containing 100–200 ml of spring water, and milt from a spermiated male is
then applied directly onto the eggs. After 15 min, the fertilized eggs are washed through several changes of distilled 18 °C water and placed in spring water (Sparkletts Water) in the 18 °C incubator. After the first division, the embryos are transferred to 0.1X MMR (Marc's Modified Ringers) for long-term culture. The medium from each culture is replaced with fresh ringers every day to avoid fungal infection. Embryos were fixed in MEMFA (4% formaldehyde, 0.1 M MOPS (pH 7.4), 1 mM MgSO₄, 2 mM EGTA), dehydrated gradually, and stored in 100% methanol at −20 °C (Sauka-Spengler et al. 2007).

**Morpholino knockdown**

FITC-labeled morpholino antisense oligonucleotides (Gene Tools, Philomath, OR, USA) were designed to target the translation initiation site. The sequences of the oligonucleotides used were:

- 5’-AACGAGCTTATGGTCAGTAACAATA w/FITC-3' Control MO.
- 5’-TGGCCGTCCTGCGAGTTCCACGCTC w/FITC-3' SoxC1 MO.
- 5’-CGTGACGACCATGCTGCTAGCC w/FITC-3' SoxC3 MO.
- 5’-ATCGTGGGTGCCCCTGCTGCCGA w/FITC-3' SoxC4 MO.
- 5’-TGTTGTTGTTTTGTCACCATTGC w/FITC-3' Xl Sox4a/b MO.
- 5’-TGTCATGCTCTGCTGCTGCACCAT w/FITC-3' Xl Sox11a/b MO.
- 5’-CCCGTAGTCTTGTTCTGCACCATCC w/FITC-3' Xl Sox12 MO.

For lamprey embryo injections, approximately 10 ng was injected into a single blastomere at the two-cell stage as described (Sauka-Spengler et al., 2007). Embryos were raised until desired stages, fixed, and dehydrated as described above. Xenopus embryos were injected with 10 nL at 0.6 M with 30 ng/μL of lacZ at the two cell stage. For rescue experiments, we co-injected morpholinos with their respective SoxC mRNA containing mutations in the morpholino binding site. SoxC mRNAs were introduced at 25 ng/μL in lamprey and 40 ng/μL in Xenopus. mRNAs were synthesized using mMessage mMachne SP6 transcription kit (Life technologies).

**β-gal lineage tracing, in situ hybridization, and histology**

*X. laevis* embryos were injected with 0.3 ng of lacZ mRNA for lineage tracing. Embryos where fixed for 30 min in MEMFA and developed in 5 mM ferrocyanide, 5 mM ferrocyanide, 1 mg/mL X-gal, and 2 mM MgCl₂ solution at 37 °C for 15 min and re-fixed for 1 h at room temperature.

Whole-mount *in situ* hybridization of lamprey and *X. laevis* embryos were performed using digoxigenin or RNA probes according to Xu and Wilkinson (1998) with the following modifications: prior to treatment with Proteinase K, embryos equilibrated in the bleaching solution (0.5 × SSC, 5% formamide, 10% H₂O₂), according to Broadbent and Read (Broadbent and Read, 1999) were exposed to direct light a using light box for 10–15 min. The concentration and the length of Proteinase K treatment (~20 μg/mL, 10 min) was constant for embryos of all stages. Hybridization and subsequent washes were carried out at
70 °C in hybridization solution containing 50% formamide, 1.3 × SSC, 5 mM EDTA pH 8.0, 200 μg/mL yeast tRNA, 100 μg/ml heparin, 0.2% Tween-20, and 0.5% CHAPS. The hybridization signal was detected using BM Purple substrate (Roche, Indianapolis, IN) for early-stage embryos (E3–E10) or NBT/BCIP (Roche, Indianapolis, IN) for later stages. After photographing, embryos were post-fixed in 4% paraformaldehyde/PBS, rinsed in PBS, and cryo-protected in two subsequent steps: 15% sucrose/PBS and 7.5% gelatin/15% sucrose/ PBS, equilibrated and mounted in 20% gelatin/PBS and frozen with liquid nitrogen. 10 μm cryosections were collected on Super Frost Plus slides (Fischer Scientific, Pittsburgh, PA) (Sauka-Spengler et al., 2007).

**Morpholino efficiency assay**

To test for morpholino efficiency, we assembled GFP fusion constructs of each of the SoxC genes. These constructs contained ~100 bp of the 5'UTR and the first 30 bp of the coding region fused to the GFP coding sequence, thus encompassing the morpholino target site.

*X. laevis* embryos were injected as described (Sauka-Spengler et al., 2007) with the mRNA of the each SoxC gene fusion construct and each one of the morpholinos used. Constructs were injected at ~100 pg along together with either control or targeted morpholinos at the same concentrations as used for their respective experiments. Efficiency of the morpholinos was confirmed by absence of GFP protein. Wholemount immunostaining was conducted with anti-GFP rabbit IgG from Invitrogen at (1:200).

**Phylogenetic analysis**

The amino acid alignments and Neighbor Joining (NJ) tree were constructed using the ClustalW protocol from the DNA STAR package. The trees were visualized using Tree View v. 0.5.0.


**Embryo phenotype scoring**

*X. laevis* and lamprey embryos exhibiting a phenotype were scored at stage 25 and E8–10, respectively, by a strong phenotype (loss of one or all late stage structures), phenotype (exhibiting a difference in expression level) or no phenotype. Based on these numbers, statistical analysis was conducted using contingency tables ([http://www.physics.csbsju.edu/stats/](http://www.physics.csbsju.edu/stats/)) to obtain a chi-squared value.
Results

Overlapping expression of the SoxC family genes in lamprey embryos

We identified orthologs of the SoxC family genes in lamprey by conducting heterospecific screenings of a macroarrayed cDNA library (Sauka-Spengler et al., 2007), conducting bioinformatic analysis on the lamprey genome (Smith et al., 2013), and using rapid amplification of cDNA ends (5’ RACE). Four orthologs were identified in P. marinus: SoxC1–C4 (Fig. S1). Using in situ hybridization, we examined the distribution patterns of SoxC family members from gastrulation through early organogenesis, corresponding to embryonic days (E) 4–14, with emphasis on expression in the neural crest and its derivatives. Our results show that the lamprey SoxC genes, SoxC1, SoxC3, and SoxC4, are expressed in the neural plate border, migrating neural crest, as well as in some neural crest derived ganglia. We observed a high degree of overlap between the expression domains of the paralogs in the neural tube, cranial ganglia, and branchial arches.

Onset of SoxC1 initiates at low levels during gastrulation in the neural plate border and posterior mesoderm at E4 (Fig. 1A and Q), but increases by E5 in the dorsal neural tube where the neural crest progenitors are located (Fig. 1B and T). Sections reveal that SoxC1 is expressed in the neural tube, trigeminal and epibranchial ganglia, and endostyle (Fig. S2D’, D”, and E’). Later, it is expressed in the neural crest derived and mesenchymal portions of the branchial arches, where it persists through E14, the last stage examined (Fig. S2F, F’ and F”). Unlike the other paralogs, SoxC2, is barely detectable at the neural plate and neural plate border at stage E4. Transcripts are detected at E5 in the closing neural plate (Fig. 1F) and neural tube at stage E6 (Fig. 1G). In addition to the neural tube, SoxC2 is widely expressed in the notochord, otic vesicle, trigeminal ganglia, and endoderm derived portions of the branchial arches (Fig. S2L, L and L”).

Like SoxC1, SoxC3 is also expressed in the neural plate and neural plate border at E4 (Fig. 1I and R), prominently in the neural folds at E5 (Fig. 1J) dorsal neural tube at E6 (Fig. 1G and V), and in migrating neural crest cells (arrow in Fig. 1V). It is very strongly expressed in the dorsal portion of the neural tube and in the lateral portions of the branchial arches at E8 (Fig. 1L and Fig. S2P, P’, P”) corresponding to the ectoderm and neural crest. At E10, the otic vesicle and the branchial arches begins to express significant levels of SoxC3 (Fig. S2Q and Q”). At E14, SoxC3 is highly expressed in branchial arch-derived cartilage (Fig. S2R and R’). Finally, the expression domain of SoxC4 at E4 is broad but enhanced expression is detected in the neural plate and neural plate border (Fig. 1M and S). At E6, SoxC4 transcripts are detected in the neural tube, ectoderm, and endoderm layers (Fig. 1V and W). It is strongly expressed in migrating cranial neural crest cells of early embryos and in the branchial arches, particularly in the neural crest domain and mesodermal core of older embryos (Fig. S2W” and X”). In addition to the neural crest, it is expressed in the ectoderm and somites (Fig. 1P and Fig. S2T’ and V’), optic pit and vesicle (Fig. S2W’, X’, and X”).

Expression of Sox4, Sox11, and Sox12 in Xenopus laevis

Using in situ hybridization, we examined the distribution patterns of SoxC family members in X. laevis from gastrulation through early organogenesis (stages 14–26), focusing on
expression in the neural crest and derivatives. Similar to lamprey and mouse (Dy et al., 2008), there is large overlap between the expression patterns of Sox4, 11, and 12 in X. laevis. At approximately stage 14, Sox4, Sox11, and Sox12 are expressed broadly throughout the neural plate and later in migrating neural crest through stage 20 (Fig. 2A–C, F–H, and K–M). Expression of Sox4 and Sox11 is very widespread with expression in developing cranial ganglia and branchial arches. Elevated levels of expression in forming ganglia and branchial arches can be observed at stage 26 (Fig. 2D, I, and N). Later, the SoxCs are expressed in the otic vesicle, the lens, neural tube, and branchial arches. Sox4 and Sox12 both appear to be fairly ubiquitous in the head (Fig. 2E, J, and O). Thus, lamprey and X. laevis SoxC orthologs have similar expression domains, especially with respect to the neural crest and its derivatives.

**Early role of the SoxCs in the neural crest**

Expression of SoxC paralogs in the neural plate border of both lamprey and X. laevis embryos suggests a possible role for these genes in the specification of the neural crest. To investigate this possibility, we examined the early function of the SoxCs using morpholino-mediated gene knockdown in both model organisms. Phenotypic analysis focused on effects on early neural crest marker genes. For lamprey, we designed morpholinos targeted to SoxC1, C3, and C4, since they were the paralogs expressed in the neural plate border, neural crest and its derivatives. The morpholinos for X. laevis Sox4 and Sox11 were designed to eliminate both isoforms of the gene Sox4a/b and Sox11a/b respectively.

To test efficiency, we performed a control experiment in which either blocking morpholino or control morpholino was injected together with mRNA encoding ~100 bp of the 5’ UTR plus 10 amino acids of the target proteins fused to GFP into one cell stage frog embryos. The results show that function blocking morpholinos, but not control morpholinos, specifically knock-down GFP translation of their respective target construct, demonstrating that they efficiently eliminate production of the target protein (Fig. 3). Furthermore, the morpholinos do not affect translation of other SoxC–GFP fusion constructs that lack their full target sites (data not shown), highlighting their specificity.

We next examined the effects of morpholinos targeting SoxC genes in lamprey. To this end, a single blastomere of two-cell embryos was injected with the morpholino, and embryos were cultured until early stages of neural crest specification (E6), when they were fixed and processed for *in situ* hybridization. Lamprey embryos injected with SoxC1, C3, or C4 morpholino exhibited down-regulation of bona fide neural crest marker SoxE1 and FoxD3 on the injected side compared with the control side (Fig. 4A–F), as revealed by *in situ* hybridization. In contrast, there was no significant effect on embryos injected with control morpholino (Fig. 4J and K). Importantly, rescue experiments in which morpholinos were co-injected with mRNAs encoding their target protein, with mutations in the morpholino binding site, resulted in expression of the neural crest specifier gene FoxD3 at near normal levels, further confirming specificity of the morpholinos (Fig. 4G–L). These results support an important role for SoxCs in neural crest specification in the lamprey. Over-expression of SoxC genes resulted in neural tube closure defects and also decreased expression of neural crest specifiers (data not shown).
To assess whether the role of SoxCs in neural crest specification is conserved in other vertebrates, we performed similar loss-of-function experiments in *X. laevis* and examined the effects of SoxC loss of function at stage 14. Similar to lamprey, knockdown of Sox4a/b and Sox11a/b in *X. laevis* embryos results in loss of neural crest specifiers FoxD3, Sox10, and Twist (Fig. 5A–F and I–K). To demonstrate specificity, we performed rescue experiments in which morpholinos were co-injected with mRNA encoding their target protein. The results reveal very good rescue of neural crest specifier gene expression, further confirming specificity of the morpholinos (Fig. 5G, H, and L). Embryos injected with Sox12 MO were not viable and did not develop to neurulation (data not shown). Taken together, our data reveal a previously unknown role for some SoxC genes in the process of neural crest formation that appears to be conserved between gnathostomes and basal vertebrates.

**Loss of SoxC genes leads to a loss of neuronal fate in lamprey**

SoxCs play an important role in the establishment of sympathetic, trigeminal, and dorsal root ganglia in amniotes. To assess if this is evolutionarily conserved, we investigated the role of SoxC genes in later stages of neural crest development. Given that SoxC1, SoxC3, and SoxC4 are expressed not only in premigratory, but also migratory and neural crest-derived tissue, we examined the later effects of morpholino-mediated knockdown on the morphogenesis of neural crest derived ganglia. In lamprey, morpholinos (MO) targeted to SoxC1, SoxC3 and SoxC4 and in *X. laevis* Sox4 and Sox11 were injected into one blastomere of the two-cell stage embryo. We then we examined the subsequent effects on ganglion formation using *in situ* hybridization with neurogenin as a marker for neuronal differentiation at E8–10 in lamprey and stage 26 in *X. laevis*.

In E8.5 lamprey embryos, the number of neurogenin positive cells in the neural tube, acoustic ganglion, epibranchial ganglia, and dorsal root ganglia was greatly diminished after SoxC1 knockdown (Fig. 6A and B) when compared to the uninjected side (Fig. 6C). At E10 from a dorsal view, we observed a strong effect of the SoxC1 morpholino on the formation of the dorsal root ganglia and epibranchial ganglia (Fig. 6D–F) compared to the normal reiterative pattern of ganglia seen on the control side. Similar effects were seen after knockdown of SoxC3. At E8, neurogenin expression was down-regulated on the injected side in the cranial ganglia and forming branchial arches (Fig. 6G–I), and by E9, neurogenin was down-regulated in the acoustic ganglion and epibranchial ganglia (Fig. 6J–L). Similarly, SoxC4 loss-of-function drastically affects morphogenesis of the acoustic, trigeminal, and epibranchial ganglia. These structures are completely lost on the injected side at E8.5 (Fig. 6M–O) and E10 (Fig. 6P–R). Interestingly, neural tube expression of neurogenin was not affected by the SoxC4 knockdown.

Knockdown of SoxCs in *X. laevis* also resulted in strong effects on the formation of neural crest-derived structures. Sox4MO resulted in loss of neurogenin in a variety of structures including the branchial arches, otic vesicle, and optic lens (Fig. 7A–C). Sox11MO produced similar results (Fig. 7D–F) with the loss of neurogenin expression in the epibranchial ganglia and otic vesicle. Over-expression of the XISoxC genes resulted in increased Ngn-2 expression, which is a known bHLH factor downstream of the SoxCs. Taken together, our results point to an evolutionarily conserved dual role of SoxCs in the formation of neural crest.
crest-derived ganglia. First, SoxCs appear to be important for neural crest specification as shown by the loss of the neural crest specifiers SoxE1/Sox10 and FoxD3 in morphant embryos. This is a novel role for SoxC genes that has not been described previously. Second, our results reveal a requirement for SoxCs genes in the survival of neural crest-derived ganglia (Lin et al., 2011) that is conserved in lamprey and X. laevis, highlighting that this later role of SoxCs in neural crest development is shared with basal vertebrates.

Discussion

During development, SoxC genes are best known for their roles in neural precursor proliferation and neuronal survival in the central and sympathetic nervous system (Bhattaram et al., 2010; Cheung et al., 2000; Hoser et al., 2008; Potzner et al., 2010). In mice, SoxCs are functionally redundant, with double knockouts resulting in loss of differentiated neurons (Hoser et al., 2008) and heart defects (Hong and Saint-Jeannet, 2005). Triple Sox4/11/12 knockout animals die at mid-gestation with massive death of neural and mesenchymal progenitors (Bhattaram et al., 2010). SoxC genes are thought to act in conjunction with SoxBs during neuronal differentiation. SoxBs are expressed in neural precursors and function in maintaining those cells in a progenitor state. As neural precursors enter the post-mitotic differentiation program, they start expressing SoxC genes, which drive expression of proteins involved in neural fate commitment (Bergsland et al., 2011, 2006; Cheung et al., 2000; Hoser et al., 2008). SoxCs also have been shown to play key roles in the development of neural crest-derived ganglia. Sox4/11 knockout mice exhibit defective proliferation and survival in the sympathoadrenal lineage (Potzner et al., 2010).

Additionally, Sox11 knockout mice have a marked reduction in sensory neuron survival in the trigeminal and sensory ganglia (Lin et al., 2011). However, the role of SoxCs in early stages of neural development had not been investigated in depth.

In the present study, we describe a novel and evolutionarily conserved role for SoxC genes in neural crest development. We find that lamprey SoxC1–C4 family members exhibit a high degree of similarity in their expression patterns in relation to amniotes (Dy et al., 2008). In chicken and mouse, Sox4 and Sox11 are expressed in the pharyngeal arches, heart, and various ganglia, similar to lamprey SoxCs (de Martino et al., 2000; Hong and Saint-Jeannet, 2005; Maschhoff et al., 2003; Uwanogho et al., 1995). In both lamprey and X. laevis, we observed widespread expression of SoxC genes in the neural plate and neural tube. During later embryogenic development, SoxC genes are co-expressed in cranial ganglia and branchial arches. More importantly, most of the SoxC genes were expressed in the neural plate border, a finding which suggested a potential role for these transcription factors in neural plate and/or neural crest specification.

Consistent with this possibility, our results reveal that loss of lamprey SoxC1, SoxC3, and SoxC4 yield similar phenotypes, all resulting in down-regulation of the neural crest specifier genes SoxE1 and FoxD3 (Fig. 4). This role is conserved in X. laevis, as Sox4 and Sox11 knockdowns in this species also diminish expression of neural crest specifier genes Sox10, FoxD3, and Twist (Fig. 5). In addition, knockdown of SoxCs results in defects of neural crest-derived ganglia in both X. laevis and lamprey after neurulation (Fig. 6 and 7). One possible interpretation of these results is that the later knockdown effects in ganglia
formation are secondary to effects at the neural plate border or closing neural tube that lead to abnormal neural crest specification. However, data in other model organisms suggest otherwise. Bhattaram et al. (2010) have recently identified the transcription factor Tead2 as a direct target of SoxC in neural progenitors in the mouse. Tead2 is a transcriptional mediator of the Hippo signaling pathway, and it seems to play an important part in controlling survival of neural and mesenchymal progenitors during embryonic development (Bhattaram et al., 2010). Our results in lamprey and *X. laevis* highlight an early role for SoxCs at the neural plate border during neural crest specification, rather than changes in cell survival and proliferation. Thus, it is likely that SoxC genes may play multiple developmental roles in vertebrates, first acting in neural crest commitment at the neural plate and later ensuring progenitor cell survival in the neuronal lineages.

Studies scrutinizing the role of SoxCs in disease may provide important clues regarding the mechanisms through which these transcription factors drive neural crest specification. In addition to various roles in metazoan development and organogenesis, SoxC genes play critical roles in many types of cancer (Wasik et al., 2013). For example, Sox4 expression level is elevated in glioma, breast, prostate, small cell lung, and medulla blastoma cancer types. *In vitro* studies have shown that Sox4 is a direct target of TGF-b signaling and functions as an oncogene to maintain stemness of gliomas (Ikushima et al., 2009). Sox4 is known to stabilize b-catenin to activate TGF-b signaling, thereby maintaining Wnt signal transduction and play a role in the epithelial to mesenchymal transition *in vitro* (Vervoort et al., 2013; Zhang et al., 2012). The role of Wnt signaling in neural crest specification is well known (Garcia-Castro et al., 2002; Sauka-Spengler and Bronner-Fraser, 2008), and thus it is possible that SoxCs could be cooperating with this signaling pathway in instructing cells of the neural plate border to adopt a neural crest fate.

It has been suggested SoxCs to have redundant roles and even compete with one another for binding to their targets due to their similar spatial and temporal expression and conserved HMG domains (Dy et al., 2008). Surprisingly, while SoxCs have been shown to have a large degree of functional redundancy in mouse (Bhattaram et al., 2010), in lamprey we found that knockdown of each of the three lamprey orthologs was sufficient to affect both neural crest specification as well as neuronal survival in neural crest-derived ganglia. This suggests that lamprey SoxCs have lower functional redundancy than their amniote counterparts. Indeed, while lamprey SoxCs genes have similar expression patterns, we also observed expression domains unique to certain paralogs. For instance, SoxCs are found in either the mesenchymal or ectodermal portions of the branchial arches and various cranial ganglia, sometimes in different sub-regions of these structures (Figs. 1 and S2). Such differences may result from divergence of function accompanying independent gene duplication in jawless vertebrates (Green and Bronner, 2013).

Our results also point to a possible role of SoxCs in the evolution of the vertebrate neural crest. In contrast to the multiple family members observed in vertebrates, there is a single copy of SoxC in invertebrates such as drosophila, amphioxus, *C. elegans*, and ascidians (Cremazy et al., 2001). The drosophila SoxC gene is a dual exon gene involved in mesoderm survival and dendritic pruning (Kirilly et al., 2009; Osterloh and Freeman, 2009). The single amphioxus SoxC gene exhibits a broad expression pattern similar to the

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cumulative pattern of vertebrates Sox4, Sox11, and Sox12 (Lin et al., 2009). During gastrulation and neurulation, AmphiSoxC is expressed in the dorsal ectoderm, mostly in the neural ectoderm, and endomesoderm fates (Lin et al., 2009). The finding that lamprey has four SoxC orthologs indicates that the origin of neural crest cells in vertebrates coincided with more than one round of duplication of SoxC genes. Given the conserved role of SoxCs in multiple stages of neural crest development, it is tempting to speculate that duplications in the SoxC gene family may have contributed to the evolution of neural crest in vertebrates, and the elaboration of the vertebrate peripheral nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


Fig. 1.
SoxC1–C4 are expressed in the neural plate border, neural tube and neural crest derivatives in lamprey. Expression patterns of SoxC1 (A–D), SoxC2 (E–H), SoxC3 (I–L) and SoxC4 (M–P) in lamprey embryos from day (E) 4 to day 8 of development. SoxC1 (Q), SoxC3 (R) and SoxC4 (S) are expressed in the forming neural plate and neural plate border while SoxC2 transcript was not detected at the gastrula stage (E). (T–Y) Cross sections of embryos (A–P), showing SoxC expression in the dorsal neural tube (T and V), neural tube (U–W) and neural crest derivatives (U and Y). NT: neural tube, NP: neural plate, NPB: neural plate border.
Fig. 2.
*Xenopus laevis* SoxCs exhibit overlapping expression in the neural plate, neural crest, and facial region. Sox4 (St.14–28) is expressed broadly in the neural plate starting at stage 14/15 and in the forming neural tube and migrating neural crest (Dorsal: A–C). It is then strongly expressed in developing ganglia as well as weakly in the branchial arches, otic vesicle, and eye (D and E). Sox11 (St.14–28) is strongly expressed early in the neural plate border and neural folds (Dorsal:H). It is expressed in neural crest and ectodermal placodes (Dorsal:F–J). Expression is maintained in the neural tube, forming ganglia, and branchial arches (I and J). Sox12 (St.14–28) is expressed broadly and similarly to Sox4. At St 14–20, Sox12 is expressed throughout the neural plate and in the migrating neural crest (Dorsal:K–M). Expression is observed throughout the neural tube as well as in cranial ganglia, branchial arches, eye, and otic vesicle (N and O). Ot: Otic Vesicle; BA: Branchial Arches.
Fig. 3.
Morpholino antisense oligonucleotides efficiently disrupt target protein translation. Side by side comparison of *Xenopus laevis* embryos injected with 5'UTR-GFP mRNA (left column) and targeted morpholino versus 5'UTR-GFP mRNA and control morpholino (right column). (A) Embryos injected with either control or targeted FITC labeled morpholinos. (B–F) *X. laevis* embryos stained with anti-GFP antibody show abundance of GFP in embryos injected with control morpholino, while targeted morpholino efficiently prevents mRNA translation and GFP protein production.
Fig. 4.
Loss of SoxC1, C3, and C4 in lamprey results in loss of neural crest specifier genes Sox10 and FoxD3: At E6: Knockdown of SoxC1 (A and D) results in a loss of expression of SoxE1 and FoxD3. Loss of SoxC3 (B and E) causes loss of expression in SoxE1 and FoxD3. Loss of SoxC4 results in loss of expression in SoxE1 and FoxD3 (C and F). (J) Quantification of effects with SoxE1: SoxC1mo(n=24), SoxC3mo(n=43), SoxC4mo(n=38), and Controlmo(n=26) (χ²=50.2 with 3 degrees of freedom). (K) FoxD3: SoxC1mo(n=22), SoxC3mo(n=37), SoxC4mo(n=68), and Controlmo(n=76) (χ²=103 with 3 degrees of freedom). (G–H) SoxCmo injected embryos rescued with SoxC mRNA by FoxD3 expression. (L) Quantification of effects with FoxD3: SoxC1mo(n=23), SoxC3mo(n=27), and SoxC4mo(n=21); (χ²=1.97) with 2 degrees of freedom.
Fig. 5. Xenopus laevis Sox4 and Sox11 regulate neural crest specifier genes FoxD3, Sox10, and Twist. Functional knockdowns of Sox4 and Sox11 lead to the loss of neural crest specifiers on the injected size (shown by lacZ staining) of FoxD3 (A and B), Sox10 (C and D), and Twist (E and F). (I) Quantification of effects on FoxD3 expression: Sox4mo (n=36), Sox10mo (n=33), and Controlmo (n=23) $\chi^2=51.8$ with 3 degrees of freedom. (J) Quantification of effects on Sox10 expression: Sox4mo (n=43), Sox11mo (n=20), and Controlmo (n=22), $\chi^2=50.4$ with 4 degrees of freedom. (K) Quantification of effects on Twist expression: Sox4mo (n=73), Sox11mo (n=47), and Controlmo (n=24), $\chi^2=90.2$ with 4 degrees of freedom. (G–H) SoxCmo injected embryos rescued with SoxC mRNA by FoxD3 expression. (L) Quantification of effects with FoxD3: Sox4mo (n=67), and Sox11mo (n=30); $\chi^2=3.28$ with 2 degrees of freedom.
Fig. 6.
Loss of SoxC genes in lamprey causes defects in the cranial ganglia and branchial arches: Knockdown of SoxC1 results in defects in neurogenin expression (A–F). At E8.5, (Dorsal: A) showing loss of expression in cranial ganglia (red arrow) and in the forming branchial arches (black arrow) on the injected side (B) loss of rostral cranial ganglia (red arrow), branchial arch expression (black arrow), and forming epibranchial ganglia. (C) On the control side, both cranial ganglia and branchial arches appear normal. At E10 (D–F), (Dorsal: D) defects in expression of dorsal root ganglia (black arrows) on the injected side,
(E) absence of neurogenin expression in branchial arches (black arrows), (F) in contrast to their presence on the control side (black arrows). Knockdown of SoxC3 also results in defects in neurogenin expression (G–L). At E8.5, (Dorsal: G) loss of cranial ganglia expression (red arrow) on the injected side, (H) loss of cranial ganglia expression (red arrow) and no expression in forming branchial arches/epibranchial region (black arrow), (I) compared with the presence of both cranial ganglia (red arrow) and branchial arches/epibranchial ganglia (black arrow) on the uninjected side. At E9 [(Dorsal: J) and K] loss of expression in the acoustic ganglia (red), (K) loss of epibranchial ganglia, expression in both acoustic ganglia (red arrow), and branchial arches (black arrows). Knockdown of SoxC4 shows defects in neurogenin expression (M–R). At E8, (M, dorsal) loss of cranial ganglia expression (red) and acoustic ganglia/branchial arch stream expression (black arrow). At E10, [(Ventral: P) and Q] loss of branchial arch and epibranchial ganglia expression on the injected side (black arrows), (R) compared to retained expression in the branchial arches on the uninjected side. (S) Neurogenin: SoxC1mo(n=104), SoxC3mo(n=127), SoxC4mo(n=107) Control mo(n=28) (χ²=67.7 with 6 degrees of freedom). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 7. *Xenopus laevis* Sox4 and Sox11 knock-downs affect Neurogenin expression later in development. At stage 26, Sox4mo causes a loss of Neurogenin-2 expression in the branchial arches, otic vesicle, and eye (A–C). Sox11mo results in the loss of Neurogenin-2 expression in the branchial arches and otic vesicle (D–F). (H) Ngn1: Sox4mo (n=34), Sox11mo (n=52), and Controlmo (n=16).