Developmental Biology 359 (2011) 149–161

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

SoxE gene duplication and development of the lamprey branchial skeleton: Insights into development and evolution of the neural crest

Olga Lakiza, Sarah Miller, Ashley Bunce, Eric Myung-Jae Lee, David W. McCauley *

Department of Zoology, University of Oklahoma, 730 Van Vleet Oval, Norman, OK 73019, United States

ARTICLE INFO

Article history:
Received for publication 9 May 2011
Revised 6 August 2011
Accepted 19 August 2011
Available online 25 August 2011

Keywords:
Lamprey
SoxE
Neural crest
Chondrogenesis
Skeletogenesis
Vertebrate evolution

ABSTRACT

SoxE genes are multifunctional transcriptional regulators that play key roles in specification and differentiation of neural crest. Three members (*Sox8*, *Sox9*, *Sox10*) are expressed in the neural crest and are thought to modulate the expression and activity of each other. In addition to regulating the expression of other early neural crest marker genes, SoxE genes are required for development of cartilage. Here we investigated the role of SoxE genes in development of the neural crest-derived branchial skeleton in the sea lamprey. Using a morpholino knockdown approach, we show that all three SoxE genes described in lamprey are required for branchial basket development. Our results suggest that *SoxE1* and *SoxE2* are required for specification of the chondrogenic neural crest. *SoxE3* plays a morphogenetic role in patterning of the branchial basket and may be required for the development of mucocartilage, a tissue unique to larval lampreys. While the lamprey branchial basket develops primarily from an elastin-like major extracellular matrix protein that is specific to lampreys, fibrillar collagen is also expressed in developing branchial cartilage and may be regulated by the lamprey SoxE genes. Our data suggest that the regulation of Type II collagen by *Sox9* might have been co-opted by the neural crest in development of the branchial skeleton following the divergence of agnathan and gnathostome vertebrates. Finally, our results also have implications for understanding the independent evolution of duplicated SoxE genes among agnathan and gnathostome vertebrates.

Introduction

The presence of a supporting internal skeleton is a defining character of vertebrates. A second notable vertebrate character is the presence of the neural crest and it has been proposed that the appearance of the neural crest was a key in the emergence of vertebrates (Gans and Northcutt, 1983). The neural crest is a transitory population of cells that arises during vertebrate development and gives rise to numerous vertebrate-specific structures including neurons and glial cells of the peripheral nervous system, pigment cells, as well as making contributions to cranial ganglia in the vertebrate head (Le Douarin and Kalcheim, 1999). Importantly, the neural crest also makes a contribution to the head skeleton, giving rise to craniofacial bones and viscerocranial cartilage.

Because of their important phylogenetic position, lampreys are key species for understanding the evolution of many vertebrate developmental mechanisms, especially evolution of the neural crest and its derivatives (Gans and Northcutt, 1983). Knowledge about the evolutionary origins of neural crest developmental mechanisms will serve as a foundation for understanding the importance of the divergence of neural crest developmental mechanisms in diverse vertebrate models (Sauka-Spengler et al., 2007).

Among the key genes required for neural crest development are the SoxE genes (*Sox8*, *Sox9*, *Sox10*), HMG-box transcription factors that are required for specification of neural crest during early vertebrate development (Cheung and Briscoe, 2003). SoxE genes also function as survival factors. In the absence of *Sox9* expression, trunk neural crest cells undergo apoptosis (Cheung et al., 2005) while morpholino knockdown of *Sox10* in *Xenopus* results in increased apoptosis and the loss of neural crest precursors (Honore et al., 2003). Multiple studies have shown that SoxE genes also modulate the expression and function of each other (Maka et al., 2005; Reiprich et al., 2008; Suzuki et al., 2006). In addition to their cross-regulatory activity, *Sox9* and *Sox10* regulate differentiation of numerous neural crest derivatives. *Sox9* is known to directly regulate expression of Type II collagen, the major extracellular matrix protein in gnathostome vertebrate cartilage, and evidence from the chick also suggests a regulatory role for *Sox10* in chondrogenesis (Suzuki et al., 2006). *Sox10* also is known to regulate expression of genes required for additional neural crest derivatives including neurons, glia, and pigment (Britsch et al., 2001; Dutton et al., 2001).

We have recently described in the sea lamprey, *Petromyzon marinus*, development of the viscerocranial skeleton, the fused branchial basket that supports the lamprey pharynx (Martin et al., 2009); see also (Morrison et al., 2000; Yao et al., 2008). We showed that the skeletal rods of the branchial basket are comprised of

* Corresponding author. Fax: +1 405 325 6202.
E-mail address: dwmccauley@ou.edu (D.W. McCauley).

0012-1606/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2011.08.012

chondrocyte stacks while the subchordal, parachordal and trabecular cartilages form as aggregate condensations of polygonal cells. The subchordal and parachordal cartilage condensations form anchor points that tether the skeletal rods to the notochord. Several groups have also shown that SoxE genes are expressed in the developing branchial arches that will form the skeletal rods (McCauley and Bronner-Fraser, 2006; Ohtani et al., 2008; Zhang et al., 2006). It was suggested that chondrogenesis in the ancestral vertebrate likely involved SoxE regulation of cartilage effector proteins and that Type II collagen arose early in vertebrate evolution as an extracellular matrix protein in cartilage formation (Ohtani et al., 2008; Zhang et al., 2006).

Previously, we described the duplication of SoxE genes in the lamprey where we noted the presence of three SoxE paralogs that were named *SoxE1*, *SoxE2* and *SoxE3* (McCauley and Bronner-Fraser, 2006). Importantly, only one of these (*SoxE3*) showed sequence homology with gnathostome *Sox9* but there was little sequence similarity observed between the lamprey *SoxE1* and *SoxE2* paralogs with the remaining gnathostome SoxE paralogs, *Sox8* or *Sox10*. This result suggested that either multiple SoxE genes arose independently within the agnathan and gnathostome lineages, or that common duplication events have been masked by the deep divergence time between lampreys and gnathostomes. Further, we showed that each of the three lamprey SoxE genes is expressed in developing cartilage. These results suggest that in lampreys, multiple SoxE genes may be required for proper development of the viscerocranial skeleton (McCauley and Bronner-Fraser, 2006).

Here, we use a morpholino-knockdown approach and expression analysis to investigate the role of each of the SoxE genes in development of the branchial basket. We show that both *SoxE1* and *SoxE2* are required for specification of the chondrogenic neural crest, but our results suggest that the function of *SoxE3* is in patterning neural crest-derived chondrocyte stacks in the skeletal rods; following *SoxE3* knockdown, chondrocytes that form the skeletal rods have the appearance of the polygonal cells that form the subchordal and parachordal chondrocytes (Martin et al., 2009). To determine if the observed effects on formation of the viscerocranial skeleton are specific to the chondrogenic neural crest, or instead result from a failure of neural crest cells to form, we examined migratory and post-migratory neural crest stages of development following SoxE morpholino knockdown. We also examined the formation of pigment, another neural crest-derived cell type (Martin et al., 2009). Taken together, our results suggest that *SoxE1* and *SoxE2* are required for specification and migration of neural crest, but *SoxE3* plays a role in morphogenesis of the chondrocyte stacks in each branchial arch. Finally, we show that morpholino-mediated knockdown of each of the lamprey SoxE genes results in reduced expression of *Col2a1b*, confirming a regulatory link between SoxE genes and *Col2a1* expression as has been suggested (Ohtani et al., 2008; Zhang et al., 2006). We discuss the importance of our results in the context of functional divergence of duplicated SoxE genes in the agnathan and gnathostome lineages, and also with respect to evolution and development of the neural crest.

Materials and methods

Lamprey adult and embryo husbandry

To obtain embryos, gravid adult sea lampreys were collected from maturing cages submerged in the Ocqueoc River near Hammond Bay Biological Station, Millersburg, MI, and shipped to the University of Oklahoma where adult lampreys were housed at 14 °C in a recirculating water system. Eggs removed from females were either fertilized immediately in a beaker containing a small volume of water (200 ml) by expressing sperm from a male directly onto the eggs, or alternately eggs were expressed dry into a petri dish, sealed with parafilm, and stored at 8 °C for later in vitro fertilization. We have found that eggs held in this manner may still be fertilized for up to 4 days after collection. Embryos were reared in small Pyrex® dishes under a constant flow environment, in 0.05× Marc's Modified Ringers solution (MMR) chilled to 19 °C.

Pigment and skeletal staining and imaging

Month-old proammocoete larvae (wildtype, mismatch morpholino control, and experimental morpholino-injected embryos) were photographed for melanogenic effects and subsequently stained with Alcian Blue as described previously (Martin et al., 2009) to visualize the branchial basket. Alcian Blue-stained embryos were then photographed with transmitted light to visualize the mucocartilage and branchial basket elements, and the induced fluorescence of the branchial basket was then optically sectioned using a Zeiss Apotome as described (Martin et al., 2009). Z-series images of skeletons were collected and rendered as 3D reconstructions using Zeiss Axiovision software (v 4.8.1). Images of 3D reconstructions are shown as maximum intensity projections. Images of all Alcian Blue stained larvae are available upon request.

Morpholino injection

Zygotes or single blastomeres of two-cell stage embryos were injected with antisense morpholino (10–100 ng). We have shown previously that injection into one blastomere at the two-cell stage may result in morphant effects to one lateral half of an embryo, serving as an internal control for morpholino effects (McCauley and Bronner-Fraser, 2006; Nikitina et al., 2008; Sauka-Spengler et al., 2007). Five-base pair mismatch morpholinos were also injected to control for non-specific morpholino effects. Sequences, specificity and efficacy of *SoxE1* and *AP2* morpholinos used in this study have been established previously (McCauley and Bronner-Fraser, 2006; Nikitina et al., 2008; Sauka-Spengler et al., 2007). *SoxE2*MO; 5′-TCGATGACCCCGATTGCGACGACGA-3′. *SoxE3*MO; 5′-CTTTCGAGAGGAGAGCAGAGCCGCA-3′. Mismatch Control MOs; 5′-TCCATCACCCCCATTCCGACCACGA-3′, 5′- CTTTGGAGACGAGACCACAGCCCCA-3′. Numbers of embryos analyzed for morphant effects are shown in Results, and are also presented as Supplementary tables (Tables S1–S3).

In situ hybridization

Prior to in situ hybridization, embryos were fixed in 4% MEMFA, dehydrated in methanol, and stored at −20 °C (McCauley and Bronner-Fraser, 2002). Methods for in situ hybridization were followed as described previously (Nikitina et al., 2009).

Real time RT-PCR

To detect changes in embryonic expression of the lamprey Type II collagen gene *Col2a1b* following SoxE Morpholino injections as described above, we extracted and purified RNA from whole embryos (RNeasy Tissue Mini Kit;Qiagen) at embryonic day 15, st.26 of lamprey development (Tahara, 1988). To analyze changes in relative gene expression by quantitative RT-PCR, each sample was tested in triplicate (technical replicates) and two samples were obtained for each Morpholino tested from six independent injection experiments (biological replicates). For each experiment, 10 experimental or control morpholino-injected embryos (30 mg of embryonic tissues) were used per column for RNA isolation. Expression levels of 18S rDNA were used as an internal standard control. We performed 2 step RT-PCR. SuperScript™ was used for first-strand cDNA synthesis according to the manufacturer's instruction (Invitrogen™, Carlsbad, CA). For real-time quantitative RT-PCR, 5 μg of total RNA was treated with DNase I and reverse-transcribed using reverse transcriptase (Invitrogen) and primers combined with random hexamers. Quantitative RT-PCR reactions were performed with 1 μg cDNA/reaction