Characterization of the Trunk Neural Crest in the Bamboo Shark, *Chiloscyllium punctatum*

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ABSTRACT

The neural crest is a population of mesenchymal cells that after migrating from the neural tube gives rise to structure and cell types: the jaw, part of the peripheral ganglia, and melanocytes. Although much is known about neural crest development in jawed vertebrates, a clear picture of trunk neural crest development for elasmobranchs is yet to be developed. Here we present a detailed study of trunk neural crest development in the bamboo shark, *Chiloscyllium punctatum*. Vital labeling with dioctadecyl tetramethylindocarbocyanine perchlorate (DiI) and in situ hybridization using cloned Sox8 and Sox9 probes demonstrated that trunk neural crest cells follow a pattern similar to the migratory paths already described in zebrafish and amphibians. We found shark trunk neural crest along the rostral side of the somites, the ventromedial pathway, the branchial arches, the gut, the sensory ganglia, and the nerves. Interestingly, *C. punctatum* Sox8 and Sox9 sequences aligned with vertebrate SoxE genes, but appeared to be more ancient than the corresponding vertebrate paralogs. The expression of these two SoxE genes in trunk neural crest cells, especially Sox9, matched the Sox10 migratory patterns observed in teleosts. Also of interest, we observed DiI cells and Sox9 labeling along the lateral line, suggesting that in *C. punctatum*, glial cells in the lateral line are likely of neural crest origin. Although this has been observed in other vertebrates, we are the first to show that the pattern is present in cartilaginous fishes. These findings demonstrate that trunk neural crest cell development in *C. punctatum* follows the same highly conserved migratory pattern observed in jawed vertebrates. J. Comp. Neurol. 521:3303–3320, 2013.

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INDEXING TERMS: shark embryo; sox8; sox9; neural crest; *Chiloscyllium punctatum*

Neural crest cells in vertebrates are a population of cells that originate in the dorsal neural tube, but delaminate and migrate throughout the embryo to give rise to a significant portion of the peripheral nervous system, consisting of neurons and glia, craniofacial structures, and even endocrine organs (Northcutt and Gans, 1983; Baker, 2005; Le Douarin et al., 2007). Because they are such a versatile group of cells, neural crest cells are involved in critical embryonic "breakthroughs" in vertebrate embryogenesis such as jaw and peripheral sensory ganglia formation (Gans and Northcutt, 1983; Northcutt, 2005). Understanding their diversity is critical for a better understanding of the key components in vertebrate evolution (Baker and Schlosser, 2005; Holland et al., 2008; Sauka-Spengler and Bronner-Fraser, 2008a).

The cartilaginous fishes (chondrichthians) belong to one of two extant lineages of gnathostomes (jawed vertebrates). The bamboo shark, *Chiloscyllium punctatum*, stems from the basal lineage of gnathostomes and offers insight into the evolution of the ancestral condition for gnathostomes. The comparison between their developmental genetic mechanisms and those in the

Additional Supplementary Material may be found in the online version of this article.

The first three authors contributed equally to this work.

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sister lineage, osteichthyans (bony fishes), can also provide essential insight into the developmental events that progressed into the common ancestor of jawed vertebrates (Gans and Northcutt, 1983; Northcutt, 2005). The common ancestor of cartilaginous and bony fishes lived some 400 million years ago (Cole and Currie, 2007).

In the 1800s, chondrichthyans held a pre-eminent position in the field of comparative embryology. However, in the 20th century chondrichthyans were replaced by model organisms, i.e., frogs, mice, and zebrafish. These model organisms sustained life better in laboratory environments and responded better to genetic manipulations (Balfour, 1876; Cole and Currie, 2007). Although scientists have concluded that chondrichthyans have trunk neural crest cells due to the appearance of dorsal root ganglia and melanocytes, most of the past research focused on the development of the head and placodes, where little is known about trunk neural crest (Horigome et al., 1999; Kuratani and Horigome, 2000; Kuratani, 2005; Freitas et al., 2006; Ota et al., 2007; Adachi et al., 2012; Gillis et al., 2012). From these studies, we learned that the migratory pathways of cranial neural crest cells and lateral line placode are highly conserved among amniotes but that none have been studied in the development of trunk neural crest cells.

Trunk neural crest cells have been studied extensively in more recent amniotes, especially in avian and mammals (Le Douarin, 2004; Sauka-Spengler and Bronner-Fraser, 2008aa; Kulesa and Gammill, 2010; Minoux and Rijli, 2010). Their migratory behavior has been divided into two main pathways: 1) ventromedial along the rostral portion of the somites, where these cells will give rise to sensory ganglia and other tissues; and 2) dorsolateral between the somites and ectoderm for the cells that will make future melanocytes.

In the last 20 years, the field of evolution and development exploded with new techniques attributed to orthologous gene identification and in situ hybridization. However, there have been few molecular studies on the early development of the nervous system in sharks, specifically research focused on the expression of some key orthologous, transcription factors such as Otx (Sauka-Spengler et al., 2001), Pax, NeuroD, and Phox2B (Derobert et al., 2002; O’Neill et al., 2007), and FoxD (Wotton et al., 2008). These studies demonstrated that a majority of sharks show similar patterns in the formation of their nervous systems and are highly conserved among agnathans and other gnathostomes. Furthermore, the roles of high-mobility group (HMG) domain transcription factors in brain regionalization are highly conserved across vertebrate evolution (Derobert et al., 2002). The members of SoxE are among the best described group of transcription factors because of their critical role in glia development but also in neural crest development. The expression patterns of SoxE family members (Sox8, Sox9, and Sox10) have been well studied across teleosts (Cheng et al., 2000; Dutton et al., 2001; Kim et al., 2003). However, although Sox8 has been cloned in the spotted catshark Scyliorhinus canicula (Freitas et al., 2006), we still do not know whether chondrichthyes neural crest cells express SoxE genes, as is known for teleosts (Kuhlbrodt et al., 1998; Cheng et al., 2000; Freitas et al., 2006; Lakiza et al., 2011).

In order to characterize in detail the development of elasmobranch trunk neural crest, in particular in shark, we took advantage of vital labeling techniques of neural crest progenitor cells as well as molecular biology tools to clone neural crest SoxE transcription factors. Vital labeling with fluorescent dyes has been a preferred approach for years to study the migration of neural crest cells during their development (Serbedzija et al., 1992; Kulesa and Fraser, 1998). This method has been used successfully in fish, amphibians, lampreys (Collazo et al., 1993; Raible and Eisen, 1994; McCauley and Bronner-Fraser, 2003; Epperlein et al., 2007), and more recently in snakes (Reyes et al., 2010). However, although the method has recently been used successfully to follow lateral line development in the little skate, Leucoraja erinacea (Gillis et al., 2012), it has never been used in shark embryos to look at the neural crest. Here we show for the first time the migration pattern of trunk neural crest cells in the bamboo shark C. punctatum by observing dioctadecyl tetramethylindocarbocyanine perchlorate (Dil) cells and Sox8 and Sox9 expression patterns.

MATERIALS AND METHODS

Collection and staging of embryos

Bamboo shark, C. punctatum (Müller and Henle, 1838), egg cases, or mermaid’s purses were harvested from the Long Beach Aquarium (kindly provided by Chris Plante), reared at 25°C in sea water, and collected at different developmental stages. Embryos were removed from egg cases and staged according to the developmental table of Ballard et al. (1993) when feasible, or by their length in centimeters. The youngest embryos collected were stage 23, 3 cm long, with most of their forebrain in full development. The oldest embryos were 10 cm long, a stage at which they showed much physical activity and looked similar to their adult counterparts. Embryos were fixed in either Carnoy’s solution (70% ethanol, 20% formaldehyde, and
10% glacial acetic acid) or in 4% paraformaldehyde (PFA) overnight at 4°C and kept in 70% ethanol at −20°C until histological preparation. For sufficient paraffin penetration, embryos needed extensive dehydration steps (about 1 day per alcohol grade) and 2 full days in Histosol (National Diagnostics, Atlanta, GA) for clearing. The tissues were then immersed in hot paraffin (McCormick Scientific [Maryland Heights, MO] Paraplast Plus) and placed in a vacuum oven for 2 days before preparation of the blocks and sectioning. Embryos were sectioned (7–12 μm) with a microtome, collected on Super-Frost slides (Fisher Scientific, Pittsburgh, PA), and dried overnight at 37°C on a slide warmer. Dil-injected embryos were cryoprotected in 15% sucrose, 30% sucrose overnight, then embedded in gelatin for 3 hours at 38°C with slow freezing in liquid nitrogen, and sectioned at 12 μm. These experiments with C. punctatum sharks were approved by the Institutional Animal Care and Use Committee at California State University Northridge.

Dil vital labeling

For live labeling, stages 23–29 shark embryos were partially immobilized with tricaine and injected with Dil (cell tracker CM-Dil, C-7001, Invitrogen/Molecular Probes, Carlsbad, CA) diluted in ethanol (1:10) and 10% sucrose. Vital labeling was performed by injecting the Dil from the hindbrain region until the Dil reached the tail end. Embryos were placed in a Petri dish after a thorough rinsing in sterile seawater and incubated with 5 ml of Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37°C for 12 hours or by placing the egg cases with the labeled embryos in a humidified chamber overnight at 25°C. A total of 10 embryos survived and were fixed first by immersing the case in PFA for 1 hour after which embryos were removed and fixed further in 4% PFA overnight at 4°C.

Scanning electron microscopy

Embryos were treated with dispase for 30 minutes in order to loosen the ectoderm, rinsed in phosphate-buffered saline (PBS), and fixed in 4% PFA overnight. The ectoderm was removed from embryo pieces with fine needles and postfixed in Karnovsky’ fixative (5 ml 8% PFA, 2 ml 25% glutaraldehyde, 1 ml 0.2 M/2N PBS, and 3 ml distilled water). After this step, embryos were postfixed again in 4% osmium–tetroxide fixative for 1 hour and then washed in PBS. Dehydrated embryos were coated in propylene oxide and resin mixtures by gradually increasing the concentration of resin and cured at 60°C for 1 day before scanning.

Production of cDNA

RNA from a prehatching embryo (≈ 7 cm) was used to make cDNA under RNase-free conditions following the Ambion (Austin, TX) Poly(A)Purist mRNA Purification Kit protocol. The reverse transcriptase (RT) reaction was performed by using Invitrogen’s directions. Briefly, 10 μl of cDNA library was mixed with 0.5 μg random hexamers and 1 μl of SuperScript II RT, and incubated at 42°C for 50 minutes and then at 70°C for 15 minutes. RNase H was added, and the mixture was incubated at 37°C for 20 minutes to remove all remaining RNA.

Primer design, PCR, and sequencing

Degenerate primers were designed manually from sequence alignments (5’-GAY AAR AGR CCN TTY ATH and 3’-CC DAT RTC NAC RTT NCC). The amplified fragments were purified by running the entire polymerase chain reaction (PCR) product on a 1.5% agarose gel, excising the bands using a clean scalpel, and purifying the DNA by using a Qiagen (Gaithersburg, MD) MinElute Gel Extraction kit. Then 4 μl of purified cDNA fragments were ligated into the TOPO vector (Invitrogen), according to the manufacturer’s instructions. The ligations were then used to transform TOPO into electrocompetent E. coli. Plasmids were purified by using a Qiagen Miniprep kit. Sequence results were analyzed by using BLAST to determine cloned sequence identities.

Isolation of neural crest markers Sox8 and Sox9

From our C. punctatum cDNA library, we specifically targeted and amplified two neural crest markers, Sox8 and Sox9, by using degenerate PCR primers. The fragments were sequenced, and then their identities were verified by a BLAST search for the presence of the HMG protein motif of KRPMNAFMVWAQAARRK. To determine the open reading frame (ORF) from the C. punctatum sequenced clones, we used the software from Sequence Manipulation Suite (Stothard, 2000), and the dataset was built by using complete protein sequences selected from bilaterian vertebrates from GenBank (Table 1).

Whole-mount in situ hybridization

Antisense RNA probes were transcribed by using T7 or SP6 RNA polymerases (Roche, Branchburg, NJ) in conjunction with digoxigenin- or biotin-conjugated dUTPs (Roche or Fermentas/Thermo Scientific, West Palm Beach, FL) by using the corresponding commercial protocols. For in situ hybridization, C. punctatum embryos were removed from the eggs, stripped of their
membranes and fixed in 4% PFA overnight before being stored in 100% methanol at −20°C. Embryos were prepared for hybridization by slow rehydration in PBS-Tween (0.5%) series and pretreatment with Proteinase K (10 μg/ml) for 15 minutes. After the embryos were postfixed to prevent damage during hybridization for 20 minutes in PFA/0.5% glutaraldehyde, they were prehybridized at 65°C. After a minimum of 2 hours, of prehybridization, embryos were hybridized with Sox8 or Sox9 probe overnight at 65°C. On the following day, after extensive hot and then cold long washes in hybridization buffer and maleic acid buffer/Tween 20 (MABT),

<table>
<thead>
<tr>
<th>Organism of interest</th>
<th>Common name</th>
<th>Clone ID no.</th>
<th>HMG domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chiloscyllium punctatum</td>
<td>Bamboo shark</td>
<td>A2.7</td>
<td>Sox 8a</td>
</tr>
<tr>
<td>Chiloscyllium punctatum</td>
<td>Bamboo shark</td>
<td>A2.16</td>
<td>Sox 8b</td>
</tr>
<tr>
<td>Chiloscyllium punctatum</td>
<td>Bamboo shark</td>
<td>A2.5</td>
<td>Sox 9</td>
</tr>
</tbody>
</table>

**Species name** | **Common name** | **Protein accession no.** | **HMG domain**
---|---|---|---
Scyliorhinus canicula | Small-spotted catshark | ABA10785 | Sox 8 |
Trachemys scripta | Red-eared slider turtle | AAP59791 | Sox 8 |
Dicentrarchus labrax | European sea bass | CBN81184 | Sox 8 |
Sparus aurata | Gilt-headed sea bream | AEV53629 | Sox 8 |
Tetraodon nigroviridis | Green spotted pufferfish | AAT42231 | Sox 8 |
Oryzias latipes | Japanese medaka | NP_001158342 | Sox 8 |
Epinephelus coioides | Orange spotted pufferfish | AFF57873 | Sox 8 |
Mogurnda anguillicaudatus | Oriental weatherfish | ACZ65966 | Sox 8 |
Anolis carolinensis | Green anole lizard | XP_000224806 | Sox 8 |
Salmo salar | Salmon | NP_001117071 | Sox 8 |
Gallus gallus | Chicken | NP_990062 | Sox 8 |
Mus musculus | Mouse | NP_035577 | Sox 8 |
Xenopus laevis | African clawed frog | NP_001083964 | Sox 8 |
Danio rerio | Zebrafish | NP_001020636 | Sox 8 |
Takifugu rubripes | Pufferfish | NP_001072112 | Sox 8 |
Paralichthys olivaceus | Olive flounder | ACO0490 | Sox 9 |
Xenopus laevis | African clawed frog | AFK08429 | Sox 9 |
Mus musculus | Mouse | NP_035578 | Sox 9 |
Gadus morhua | Atlantic cod | ADV03670 | Sox 9 |
Trachemys scripta | Red-eared slider turtle | ACG70782 | Sox 9 |
Aspidoscelis inornata | Little striped whiptail | ABQ44208 | Sox 9 |
Epinephelus coioides | Orange spotted pufferfish | ACZ51153 | Sox 9 |
Alligator mississippiensis | American alligator | AAD17974 | Sox 9 |
Lepidochelys olivacea | Olive ridley sea turtle | ACT82009 | Sox 9 |
Crocodylus palustris | Mugger crocodile | ACJ2296 | Sox 9 |
Oreochromis aureus | Blue tilapia | ABY6377 | Sox 9 |
Scyliorhinus canicula | Small-spotted catshark | ABY71239 | Sox 9 |
Oryzias latipes | Japanese medaka | NP_001098555 | Sox 9 |
Gallus gallus | Chicken | NP_989612 | Sox 9 |
Danio rerio | Zebrafish | NP_571718 | Sox 9 |
Takifugu rubripes | Pufferfish | AF329945_3 | Sox 9 |
Oryctolagus cuniculus | European rabbit | XP_002723578 | Sox 10 |
Mus musculus | Mouse | NP_035567 | Sox 10 |
Danio rerio | Zebrafish | NP_571950 | Sox 10 |
Gallus gallus | Chicken | NP_990123 | Sox 10 |
Xenopus laevis | African clawed frog | NP_001082358 | Sox 10 |
Paramusigurnus dabryanus | Carp | AF97051 | Sox 10 |
Mogurnda anguillicaudatus | Oriental weatherfish | AF97052 | Sox 10 |
Oryzias latipes | Japanese medaka | NP_001158343 | Sox 10 |
Epinephelus coioides | Orange spotted pufferfish | AFF57872 | Sox 10 |
Ambystoma mexicanum | Salamander | ABI97016 | Sox 10 |
Xenopus tropicalis | Western clawed frog | AAJ36048 | Sox 10 |
Cynoglossus semilaevis | Tongue sole | ABW87298 | Sox 10 |

**Outgroup** | **Common name** | **Protein accession no.** | **HMG domain**
---|---|---|---
Mus musculus | Mouse | CAA49779 | Sox 4 |
Xenopus laevis | African clawed frog | NP_001165672 | Sox 4 |
Takifugu rubripes | Japanese medaka | AAQ18501 | Sox 4 |
Danio rerio | Zebrafish | CAE18168 | Sox 4 |

1Protein sequences used for phylogenetic analysis of Sox8, Sox9, and Sox10 HMG domains were compared with the three C. punctatum Sox8 and Sox9 clones. The outgroup used was from the Sox4 subfamily.
embryos were blocked with a special blocking agent from Boehringer Mannheim (Indianapolis, IN) for 2 hours minimum, and then anti-digoxigenin antibodies (Roche) conjugated with alkaline phosphatase (1:2,000) were added overnight at 4°C. On the next day, embryos were extensively washed in MABT and then Tris buffer; they were then visualized by adding 5-bromo-4-chloro-3′-indoly phosphate/nitroblue tetrazolium (BCIP/NBT) developers (Roche). A detailed protocol is publicly available at http://neuro.bcm.edu/groveslab/ (Henrique et al., 1995).

In situ hybridization on slides

In situ hybridization of RNA probes to *C. punctatum* sections was performed as described above, after adapting the method to sections. This time embryos were fixed in modified Carnoy’s solution and then dehydrated in an ethanol series, followed by two changes of Histosol, and then paraffin and sectioning. Prior to in situ hybridization, slides were dewaxed in Histosol, rehydrated by passing them through a series of ethanol rinses, and then rinsed in water, PBS, and 2X standard saline citrate (SSC). Hybridization was performed by using 1.5 ng/µl of ShSox8 and ShSox9 probes from two different clones.

Immunohistochemistry on tissue sections

*C. punctatum* tissue sections were dewaxed in Histosol, rehydrated in a graded series of ethanol washes, and then equilibrated in PBS before blocking in PBS containing 10% FBS and 1% Triton X-100. Primary antibodies were added at 1:500 dilution in PBS overnight at 4°C followed by extensive PBS washes. Secondary antibody Alexa fluoroprobes (Invitrogen) were added for 30 minutes with 4′,6-diamidino-2-phenylindole (DAPI; to label the nuclei), and then the sections were washed in PBS 3 times for 5 minutes and coverslipped with Permount. Pictures of sections were taken by using Axiovision LE software (Zeiss, Thornwood, NY) with an AxioCam camera attached to a Zeiss AxioimagerA1 upright fluorescent microscope and assembled into figures with Adobe (San Jose, CA) Photoshop 7 by adjusting each color channel level (increasing contrast and reducing background to make images clearer) and reducing image size to a 300-dpi and 3 × 3-inch size.

Antibody characterization

Antibodies used are as follows (Table 2):

1. Monoclonal TuJ1 (cat. #MMS-435P, Covance, Princeton, NJ) was raised against microtubules derived from rat brain and recognizes a 50-kDa protein on western blot. It is well characterized and highly reactive to neuron-specific class III β-tubulin (βIII) (Bronner-Fraser, 1986) and lateral line neuromast (Ghysen and Dambly-Chaudiere, 2004).

2. Monoclonal HNK1 (cat. #3H5, Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA), is derived from the VC1.1 hybridoma, which recognizes the HNK-1 epitope, an N-linked carbohydrate. It is a well-known avian neural crest marker (Bronner-Fraser, 1986) and lateral line neuromast (Ghysen and Dambly-Chaudiere, 2004).

3. Monoclonal 7B3/transitin (a kind gift from Jim Weston, University of Oregon, Corvallis) recognizes a 300-kDa nestin-like intermediate filament in stem cells (Wakamatsu et al., 2007); it can now be purchased from DSHB, #A2B11.

4. FoxD3 polyclonal (a gift from David Raible, University of Washington, Seattle, WA) was raised against purified zebrafish FoxD3 and its specificity characterized it recognized on western blots after immunoprecipitation of translated protein against control (Lister et al., 2006).

Multiple Sequence Alignment and Phylogeny of Sox8, Sox9 and Sox10.

We performed a multiple sequence alignment (MSA) to confirm that the three unknown *C. punctatum* clones

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Immunogen (what the antibody was raised against; full sequence and species)</th>
<th>Manufacturer, species antibody was raised in, mono- vs. polyclonal, cat. or lot no.</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>TuJ1</td>
<td>Raised against microtubules derived from rat brain. It is well characterized and highly reactive to neuron-specific class III β-tubulin (βIII)</td>
<td>Covance Research Products (San Diego, CA), purified mouse IgG2a monoclonal antibody, unconjugated, clone TUJ1, #MMS-435P-100</td>
<td>1:500</td>
</tr>
<tr>
<td>HNK1</td>
<td>E10 chick optic nerve</td>
<td>Developmental Studies Hybridoma Bank (Iowa City, IA), mouse monoclonal VC1.1 hybridoma</td>
<td>1:500</td>
</tr>
<tr>
<td>7B3/transitin</td>
<td>E7 chick retinal cells. Genbank accession no. X80877</td>
<td>Developmental Studies Hybridoma Bank, rat monoclonal, #A2B11</td>
<td>1:500</td>
</tr>
<tr>
<td>FoxD3</td>
<td>Purified in vitro translated FoxD3</td>
<td>Laboratory of David Raible (Univ. of Washington, Seattle), raised in rabbit</td>
<td>1:500</td>
</tr>
</tbody>
</table>

**TABLE 2.**

Primary Antibodies Used

<table>
<thead>
<tr>
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</tr>
</tbody>
</table>
belonged to the Sox 8 and Sox9 subfamilies rather than the Sox10 subfamily. We used Sox4 as the MSA outgroup: mouse Sox4, Xenopus laevis Sox4, Takifugu rubripes Sox4, and Danio rerio Sox4. The MSA (Supplementary Fig. 1) was performed by using MUSCLE with its default parameters (Edgar, 2004). Only full-length HMG box domains were included in the alignment of the three C. punctatum clones. For the rooted phylogenetic tree, the MUSCLE “profile–profile alignment” algorithm was used between our three C. punctatum clones and the 43 BLAST-verified protein sequences against the Sox4 outgroup protein sequences.

The distance matrix FastTree was carried out on the MSA, which uses the maximum-likelihood (ML) method and nearest-neighbor interchanges (NNIs) (Price et al., 2010). FastTree creates trees by using 1,000 replicates and utilizes the Jones–Taylor–Thornton (JTT) and/or the Whelan Goldman (WAG) models to determine amino acid evolution as well as the unbiased Shimodaira–Hasegawa test (SH) (Shimodaira, 2002). Both phylogenetic trees were viewed by using FigTree (http://tree.bio.ed.ac.uk/software/figtree). The rooted tree was aligned and analyzed against the established Sox4 outgroup: mouse Sox4, Xenopus laevis Sox4, Takifugu rubripes Sox4, and Danio rerio Sox4.

RESULTS

Vital labeling of trunk neural crest

In our experiments, we used the classic chicken vital labeling method in C. punctatum by injecting Dil into the neural tubes of live C. punctatum shark embryos and then incubating them for 24 hours in their own egg white. Due to the scarcity of shark embryos and the low survival rate with this method (~50%), we had positive results from relatively few embryos (n = 10).

We performed these experiments in embryos ranging between stages 23 and 29. From the best labeled Dil-injected embryos (now at stages 25–26, n = 7/10), we observed two major trends. First, one set of labeled cells migrated outside the neural tube following the classic segmental pattern of trunk neural crest cells in other species (Gammill and Roffers-Agarwal, 2010) (Fig. 1). In some instances we observed Dil cells in the developing heart (Fig. 1D) and branchial arches (Fig. 1F). Sections through these embryos confirmed the presence of Dil cells migrating along the classic ventromedial pathway of trunk neural crest cells, reaching the lateral sides of the aorta (Fig. 2).

In addition, another set of Dil cells was observed along what would correspond to the lateral line (a group of sensory neurons of placodal origin used for detecting water movements) at the tail end (Fig. 1A–C,H, at higher magnification). This observation suggested that some neural crest cells were able to migrate within 24 hours into the developing lateral line. Interestingly, it seemed that once this line of trunk Dil-positive cells reached the tailmost end, it continued migrating rostrally along the lateral line (arrowhead in Figs. 1G, 3A–C). We observed this pattern in 6/10 Dil-labeled embryos.

Although most of the embryos were already too old for complete labeling of the cranial neural crest, we observed Dil-positive cells in cranial structures. We found that at the level of the forebrain and hindbrain, a few Dil-positive cells had migrated under the ectoderm as well as into cranial ganglia (data not shown). Interestingly, we also observed Dil-positive cells migrating along the branchial arches (6/10), around the eye (2/10), and in the heart (2/10). More importantly, we observed in some embryos (n = 4/10) a large number of Dil-positive cells in the developing gut (red arrow in Fig. 3I).

After counterstaining with HNK1, which labels lateral line sensory neurons, and then sectioning these embryos, we were able to corroborate that these medial Dil-positive cells were along the lateral line at both the hindbrain and trunk levels (arrows in Figs. 2A–C, 3F,G). However, the total numbers of Dil-positive cells migrating into the lateral line were trivial, as shown by whole-mount double labeling with HNK1 in stage 26 embryos (Fig. 3F). Furthermore, whereas we observed a few Dil cells along the lateral line in conjunction with FoxD3, which labels glial cells (Fig. 3G), we never saw overlap of Dil with TuJ1, which labels placodal sensory cells along the lateral line.

Dil labeling of an older embryo (stage 27) corroborated the presence of migrating trunk neural crest cells as a broad wave (n = 6/10), indicating a classic ventral pathway (Fig. 3D). More importantly, we observed Dil-positive cells coming out of the dorsal part of the neural tube and migrating along the rostral side of the somites (arrowhead in Fig. 3E).

Scanning electron microscopy of neural crest

Because neural crest cells migrate under the ectoderm, it is easy to observe this group of cells after removing the tissue. We performed scanning electron microscopy (SEM) of C. punctatum embryos at stages 25, 27, and 29. The results showed that in early embryos (stages 25 and 27), there is a population of cells that migrate beneath the ectoderm and over the rostral neural tube, similar to cranial neural crest cells migrating in amphibians, fish, birds, and mammals.
stage 25 embryos, we observed migrating cells over the hindbrain rostrally, with other cells migrating ventrally (Fig. 4A). SEM of a stage 27 embryo showed cells delaminating from the neural tube and along the ventral pathway at the junction of the hindbrain and vagal region (Fig. 4B). Finally, SEM in the caudal trunk of an

Figure 1. Dil labels migrating neural crest in stages 25–27 C. punctatum embryos. Shark embryo neural tubes were vitally labeled with Dil for 24 hours. A–C: Stage 25 embryo. Tail end (A) and caudal trunk (B,C) show segmental migration of neural tube–derived cells (arrows) as well as a ventral line of Dil cells (arrowheads). D: Two sets of migrating Dil cells in the out-tract of the heart (arrows). E–H: Stage 27 embryo. This older embryo shows Dil-labeled cells in neural tube and segmentally migrating cells (arrows in E,G,H), ventral locations (red arrows in H), branchial arches (arrows in F) and turning rostrally (arrowhead in G). Abbreviations: D, dorsal; V, ventral; A, anterior; P, posterior. Scale bar = 500 μm applies to A, B and 200 μm applies to E, F, G.
older, stage 29 embryo showed a significant number of cells migrating outside the neural tube ventrally (Fig. 4C and pseudo-colored cell in D).

**Isolation, alignment, and phylogenetic analysis of *C. punctatum* neural crest markers Sox8 and Sox9**

The protein sequence motif RPMNAFMVWAQ is conserved in all Sox8, Sox9, and Sox10 sequences, and some sequence similarity is seen in the Sox4 outgroups (Yusuf et al., 2012). From a cDNA library of *C. punctatum*, we isolated fragments of two neural crest markers, Sox8 and Sox9, by using degenerate PCR primers. The sequencing of three *C. punctatum* clones and posterior BLAST showed 43 complete coding HMG domain sequences from the Sox8, Sox9, and Sox10 subfamilies, as illustrated in the colored alignment (Supplementary Fig. 1). The HMG domain is where the transcription factors for Sox8, Sox9, and Sox10 bind onto the DNA minor groove (Bowles et al., 2000).

*C. punctatum* Sox clones (termed here Sox8a, Sox8b, and Sox9) were compared with known Sox8 and Sox9 sequences. Sequences were aligned by using MUSCLE, and phylogenies were inferred with FastTree. The tree was rooted by using Sox4 as an outgroup, which is considered reliable because Sox4 is part of the SoxC family and contains the HMG protein domain. *C. punctatum* sequences clustered with metazoan Sox8, Sox9, and Sox10 subfamilies, demonstrating that they are true members of the metazoan Sox8 and Sox9 subfamilies (Fig. 5). Clustering *C. punctatum* Sox8a, Sox8b, and...
Sox9 genes with their respective groups in the rooted tree showed that *C. punctatum* Sox8 and Sox9 are true orthologs in their subfamilies (see also Supplementary Fig. 2). The SH test of alternative topologies showed strong support of these groupings (Shimodaira, 2002; Shimodaira and Hasegawa, 2001).

**Sox8 and Sox9 expression in *C. punctatum* embryos**

At stages 25 and 27, *C. punctatum* embryos were injected with Dil, and then in situ hybridization was performed with Sox8 and Sox9 probes. We observed that Sox8 colocalized robustly with Dil along the lateral line (Fig. 6A–C). Importantly, it colocalized with transitin/7B3, which labels neural crest and glial precursors along the lateral line (Rotenstein et al., 2009) (Fig. 6A). During double labeling with Tuj1, which marked neurons, we did not observe colocalization of Tuj1 with Sox8 along the lateral line and did not observe Sox8 cells in the developing gut. In contrast, we did observe Tuj1-labeled ventral motor axons where both Tuj1 and Sox8 colocalized and robustly labeled sensory ganglia (Fig. 6D–F). Sox8 labeled a different set of cells located more laterally than the Tuj1-positive axons (white arrow in Fig. 6D). Sox9 partly colocalized with Dil-labeled neural crest cells along the lateral line (white arrow in Fig. 6G and inserts) and weakly with peripheral nerves (red arrow in Fig. 6H). Importantly, Sox9 was observed in the dorsal neural tube, gut, and developing kidneys (Fig. 6H).

Whole-mount in situ hybridization for Sox8 of *C. punctatum* stage 27 embryos only labeled the lateral line and notochord (Fig. 7A). However, in older *C. punctatum* stage 29 embryos, some cells migrated beneath the ectoderm on the rostral side of the somites and had the appearance of true neural crest cells (Fig. 7B). At stage 32, when the *C. punctatum* embryos displayed the least morphological and physical resemblance to other vertebrate species. We observed Sox8 in pharyngeal cleft cells (Fig. 7C), differentiating neurons in the young spinal cord (Fig. 7D), spinal nerves (Fig. 7E), cells surrounding the lumen, enteric neurons (Fig. 7F), and developing cartilage (data not shown).

Whole-mount in situ hybridization of Sox9 at stage 26 embryos showed a more definite neural crest pattern (Fig. 8). We observed cells migrating into branchial arches, around the eye and nasal pit (Fig. 8A,C,E,G). Along the trunk, we observed two groups of cells with Sox8 in stage 29 *C. punctatum* embryos. The first group was the most caudal, along the lateral line and 20 somites up. The second group was a set of cells migrating on the rostral side of the somites beneath the ectoderm (Fig. 8B,D,F). Sections through an older embryo, stage 28, tested positive with Sox9 cells in sensory ganglia especially in the spinal nerves; there were also a few cells positive for Sox9 along the lateral line (Fig. 8H,I) and in the branchial arches (Fig. 8J).

**DISCUSSION**

Identification of neural crest cells that have delaminated and migrated away from the neural tube requires the use of both vital labeling and classical markers within the developing embryo. By using a combination of these techniques, we are the first to describe the migration pattern of trunk neural crest cells in a shark embryo.

Classic vital labeling of trunk neural tube cells in developing *C. punctatum* embryos was accomplished by injecting Dil into the neural tubes between stages 25 and 29. During this period, trunk neural crest cells actively developed and expanded. Although survival was low, some Dil-labeled embryos at stages 25–29 (*n* = 10) persisted. The remaining embryos showed migrating neural crest cells along the ventromedial pathway and into the lateral line. These migratory patterns of neural crest cells are unique in teleosts and elasmobranchs (Gillis et al., 2012).

The striking similarity of migration patterns detected by using vital labeling and results obtained from Sox8 and Sox9 in situ hybridization underscores an emerging paradigm, specifically, that neural crest cell development is highly conserved across vertebrate lineages (Holland and Holland, 2001; Meulemans and Bronner-Fraser, 2004). Past studies have shown that Sox8 and Sox9 sequences are highly conserved among fish, amphibians, reptiles, and mammals. Even more striking than sequence conservation, however, is the conserved patterns of migration and expression in vertebrate neural crest cells and their derivatives (Horigome et al., 1999; Bell et al., 2000; Sauka-Spengler and Bronner-Fraser, 2008bb; Guth et al., 2010; Reyes et al., 2010). This was especially remarkable in the trunk regions of *C. punctatum* embryos, because segmental migration through the rostral portion of the somites is practically indistinguishable from that of other vertebrates. We also observed a reduced number of migrating trunk neural crest cells in comparison with the large number of amniotes. This finding is consistent with observations in other aquatic vertebrates (fishes and amphibians) (dutton et al., 2001; Carmona-Fontaine et al., 2008).

Our present study demonstrates that, in *C. punctatum*, glial cells in the lateral line are likely of neural
crest origins. Although this has been observed in other vertebrates, we are the first to show that the pattern is present in cartilaginous fishes. Previous studies using DiI labeling in *Xenopus* (Collazo et al., 1993) and zebrafish neural tubes (Collazo et al., 1994) showed that neural crest cells contributed to both lateral line organs.

**Figure 3.**
and other structures. The Nusslein-Volhard and Eisen laboratories have demonstrated that neuromasts are of placodal origin, whereas the Schwann cells along the lateral line are of neural crest origin (Kelsh et al., 2000; Gilmour et al., 2002). Here we demonstrate conservation of these patterns in *C. punctatum* (O’Neill et al., 2007; Gillis et al., 2012). Although we only double-labeled lateral line DiI cells with the glia-specific markers Sox8, Sox9, and FoxD3, we hypothesize them to be glia in the light of our past research showing the presence of glial markers along the lateral line in *C. punctatum* shark (Rotenstein et al., 2009). Although Baker and co-workers extensively studied the shark’s placodal development in the lateral line, they did not focus on the glial component in the ganglia (O’Neill et al., 2007; Baker et al., 2008; Gillis et al., 2011, 2012). Our current findings strongly support a neural crest contribution to the lateral line in *C. punctatum*.

In addition to observing DiI cells in segmented streams along the lateral line in the trunk, we also

**Figure 3.** Dil labels migrating neural crest in stages 26 and 29 *C. punctatum* embryos. Shark embryo neural tubes were vitally labeled with Dil for 24 hours. **A, B:** Stage 26 shark embryo shows Dil cells as two bright lines (arrows in A) and as a sheet at tail end (arrow in B). **C–E:** Stage 29 shark embryo shows Dil cells as two bright lines (arrows in C) and as a sheet at the tail end (arrow in magnified area in D). Pseudo-longitudinal section shows delaminated Dil cells on top of the neural tube (arrow in E) and on one portion (rostral) of the somite (arrowhead in E). **F:** Whole-mount image of the embryo after HNK1 immunostaining of lateral line placodal neurons (arrow). **G:** FoxD3 antibody stain (green arrow) colocalized with Dil cells (white arrow). **H:** Anti-β III tubulin labeling (Tuj1) neurons (green arrow) along the lateral line did not overlap with Dil cells (white arrow). **I:** The developing gut had a line of Dil cells (arrow). **J:** Cartoon of shark embryo stage 26. Abbreviations: NT, neural tube; D, dorsal; V, ventral; A, anterior; P, posterior. Scale bar = 100 μm in A (applies to A–E), F, and G (applies to G–I).

**Figure 4.** Scanning electron microscopy of *C. punctatum* embryo neural crest. SEM of *C. punctatum* embryos at stages 25, 27, and 29 shows that in early embryos there is a population of cells that migrated beneath the ectoderm and over the rostral neural tube, such as cranial and trunk neural crest cells do in amphibians, fish, birds, and mammals. **A:** In stage 25 embryos, we observed migrating cells over the hindbrain, some migrating rostrally (arrow) and others migrating ventrally (arrowhead). **B:** SEM of a stage 27 embryo shows cells delaminating from the neural tube (arrowhead) along the ventral pathway and at the junction of hindbrain and trunk (arrows). **C, D:** SEM in the caudal trunk of older, stage 29 embryo, shows a good number of cells migrating outside the neural tube ventrally (arrows in C and pseudo-colored arrow in D). Abbreviations: R, rostral; C, caudal. Scale bar = 50 μm in B (applies to A, B); 10 μm in C; XX μm in D.
Figure 5. Rooted phylogenetic tree for Sox8, Sox9, and Sox10 HMG domains. The rooted phylogenetic tree was obtained from the Nearest-Neighborhood Interchanges program performed on FastTree. The Sox8, Sox9, and Sox10 HMG domain protein sequences were found in various vertebrate species and used in construction of the tree. The bar range located on the bottom of the rooted phylogenetic tree represents the branch length of 0.4 units. The branch length is proportionally related to the evolutionary distances and reflects the divergence among the Sox8, Sox9, and Sox10 genes. The unbiased SH test value suggests that the branch nodes represent events similar in size between the clades. The outgroup used was Sox4, which is part of the SoxC subfamily. *C. punctatum* Sox8 and Sox9 clones were positioned at the beginning of the phylogenetic tree, indicating that our *C. punctatum* clones are more ancient than the rest of the vertebrates with Sox8, Sox9, and Sox10 subfamilies.
observed a group of Dil cells at the tail end migrating as a sheet and a smaller number as a stream migrating rostrally on the ventral portion of the tail. Although we do not know the ultimate destiny of these “returning” populations, the same neural crest migratory pattern has been observed in fish (Collazo et al., 1994) and amphibians (Collazo et al., 1993) but not in reptiles (Reyes et al., 2010) or mammals (Kuhlbrodt et al.,

Figure 6. *C. punctatum* Sox8 and Sox9 follow neural crest path and locations. Mid-trunk sections were observed through a Dil-injected embryo after Sox8 (A–F) and Sox9 (G–I) whole-mount in situ hybridization. These mid-trunk sections, Sox8 and Sox9, colocalized with Dil cells. A–C: Sections were immunostained with 7B3, which labels neural crest cells. Arrows point to cells in the ventromedial path and costained for all three markers, 7B3, Sox8, and Dil. D–F: Both Sox8 and TuJ1 (red) staining. E,F: Single channel of the same section. Sox8-labeled myotome (arrow) and notochord. In F, arrowheads point to TuJ1. G: Section shows both Sox9 and Dil staining. G–I: Single channel of the same section. White arrows point to cells positive for both Sox9 and Dil along the ventromedial path (higher magnification in inserts). In G, the white arrowhead points to Sox9 on the dorsal neural tube, where premigratory neural crest cells reside. In H, the red arrow points to Sox9-positive cells along the ventral spinal nerve. Red arrowhead marks Sox9 cells in the developing gut. Scale bar = 100 μm in D (applies to A–I).
This suggests that the migratory path became lost in nonaquatic gnathostomes.

It is well known that, in vertebrates, the neural crest gives rise to the enteric nervous system (Le Douarin and Teillet, 1973; Young and Newgreen, 2001; Newgreen and Young, 2002; Elworthy et al., 2005; Kuhlman and Eisen, 2007). Our observation of neural crest cells in the developing gut strongly supports what has been, until now, suspected based on the development of other vertebrates: that neural crest cells are responsible for generating the elasmobranch enteric nervous system. Thus, this neural crest pathway is very ancient and developmentally shares SoxE genes among gnathostomes (Elworthy et al., 2005; Hoff et al., 2008).

Neural crest cells are known to migrate on the rostral side of somites while avoiding caudal portions that have a set of repellants (e.g., ephrins, Slits, Sema; Gammill and Roffers-Agarwal, 2010; Kulesa and Gammill, 2010). We observed that trunk neural crest cells avoided the caudal portion of the somites, suggesting that this migratory route/behavior is very old and likely due to a conserved structure in the somites. This observation has also been made in chordates such as lampreys and hagfish (Gostling and Shimeld, 2003; Meulemans and Bronner-Fraser, 2007; Ota et al., 2007; McCauley, 2008).

Evolution of the Sox genes is of keen interest given their role in stem cell fate and cancer progression (Scott et al., 2010). The presence of Sox8 and Sox9 in trunk neural crest in *C. punctatum* stands in contrast to current findings of SoxE phylogeny for lampreys (Lakiza et al., 2011) and amphioxus (Yu et al., 2008). *C. punctatum* Sox8, Sox9, Sox10, Sox4, and MSA sequences did not cluster with teleost SoxE genes, nor did they group with SoxE genes from lamprey (data not shown). The clade containing *C. punctatum* Sox8a, Sox8b, and Sox9 sequences nested squarely between *Scyliorhinus canicula* Sox8 and Sox9 genes rather than grouping with the rest of the vertebrate Sox8, Sox9, and Sox10 sequences. This arrangement provides strong phylogenetic support to the hypothesis that shark Sox8 and Sox9 genes can be considered paralogs of amniotes Sox8 and Sox9 rather than orthologs (Cui et al., 2011).

In other words, phylogenetic analysis of these sequences suggests that Sox8 and Sox9 genes appeared first in a common ancestor of chondrichthyan and teleosts rather than in the common ancestor of teleosts.

More importantly, we showed that the expression of these two SoxE genes in trunk neural crest cells was highly conserved. *C. punctatum*’s Sox9 expression matched the Sox10 migratory patterns observed in teleosts. Expression profiles were particularly similar in delaminated migrating neural crest cells along the trunk and around the developing eye, branchial arches, gut, and peripheral nerves (Horigome et al., 1999; Bell et al., 2000; Sauka-Spengler and Bronner-Fraser, 2008bb; Guth et al., 2010; Reyes et al., 2010). Expression patterns seen in this study differ from observations in *Xenopus*, zebrafish, chicken, and mammals. In these systems, Sox9 expression has not been observed in the peripheral nervous system (Li et al., 2002; Hong and Saint-Jeannet, 2005). However, hagfish trunk neural crest cells appear to express Sox9, although these cells do not enter the somites, as was observed in *C. punctatum* and osteichthyans (Ota et al., 2007). Although...
Sox8 and Sox9 expression has been fully characterized in the lamprey cranial neural crest, little is known about expression in the trunk (Lakiza et al., 2011; Uy et al., 2012). Preliminary results indicate that lamprey trunk neural crest migration is segmental, similar to our observations in shark (Dr. Marianne Bronner, personal communication).

Sox8 expression in the C. punctatum neural crest has been previously characterized. (O’Donnell et al., 2006). Our observations matched expectations based on these studies. However, we also observed Sox8 expression in developing chondrocytes (Schmidt and Patel, 2005), which was similar to Sox9 patterns observed in the mouse (Mori-Akiyama et al., 2003) and chicken dermomyotome (Bell et al., 2000; Hall and Gillis, 2013). Although we were not able to clone Sox10 from C. punctatum cDNA, there is strong evidence that it exists (Dr. Clare V. Baker, personal communication). Therefore, we conclude that C. punctatum has a full set of osteichthyan Sox8, Sox9, and Sox10 genes. This stands in contrast to earlier vertebrates, such as lampreys, which contain SoxE1–3 (Meulemans and Bronner-Fraser, 2004; Uy et al., 2012).

In summary, we describe the migration of trunk neural crest cells in C. punctatum through vital labeling and in situ hybridization. To our knowledge, this is the first

Figure 8. C. punctatum Sox9 expression at different stages of development. A–G: Whole-mount of stage 26 embryo shows Sox9 along classic neural crest cells pathways along branchial arches (arrowheads in B,G; arrows in C), along the somites (arrows in B,D,F), and around the eye (arrows in A,E) and otic vesicle (G). H–J: Sections through a stage 26 embryo highlights Sox9 cells along the ventromedial path (arrowhead in H), spinal nerves (arrows in H), cranial ganglia (arrow in I), and branchial arch (arrow in J). Scale bar = 1 mm in A (applies to A–G); 50 μm in J (applies to H–J).
time that this approach has been utilized to study the trunk neural crest in elasmobranchs. Sox8 and Sox9 migration through C. punctatum’s neural crest and derivatives suggests that, once these genes were co-opted by neural crest cells, they proved to be highly conserved across vertebrates (Martinez-Morales et al., 2007). In addition, our observations with Dil labeling indicate that the migration of trunk neural crest cells has not changed much during evolution.

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CONFLICT OF INTEREST STATEMENT

The authors have no competing interests or conflicts.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. MJ: performed Sox8 and Sox9 cloning; RM: oversaw bioinformatics analysis; performed sectioning; DM and MJ: performed Sox8 and phylogenetic figures, performed bioinformatics analysis, tions; TC: performed DNA/protein analyses, designed data and the accuracy of the data analysis. MJ: performed the manuscript.

LITERATURE CITED


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