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Identification of the neural crest-specific enhancer of *Seraf* gene in avian peripheral nervous system development

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ABSTRACT

In vertebrate embryos, trunk neural crest cells give rise to Schwann cells, along with other derivatives. In this study, to elucidate the molecular mechanisms for the Schwann cell specification, we aimed to identify enhancer elements responsible for the expression of the *Seraf* gene, the earliest marker for the Schwann cell precursors in the avian embryos. We first compared the genomic structure around the *Seraf* locus in various vertebrates, and found that, while mammals do not have a *Seraf* homolog, teleost fish species have it. However, the intergenic sequences around the *Seraf* locus are not conserved between zebrafish and chicken, consistent with the fact that fish *Seraf* expression is not Schwann cell precursor-specific. We thus compared the intergenic sequences around the *Seraf* locus among avian species, and identified a potential enhancer containing a cluster of Sox10-binding sites. Accordingly, the identified enhancer is activated in a neural crest-specific manner in transfected quail embryos. We also found that *Sox10* activated the enhancer in cultured cells. Thus, our results revealed a new role of *Sox10* in the earliest phase of the Schwann cell fate specification.

1. Introduction

In vertebrate embryos, trunk neural crest cells are formed on the dorsal top of the neural tube, and extensively migrate to give rise to a variety of cells types, including melanocytes, neurons and satellite glial cells in the dorsal root ganglia and sympathetic ganglia, as well as Schwann cells along the peripheral nerve [1]. While molecular mechanisms regulating these cell lineage segregations have been gradually uncovered, how Schwann cell lineage is established remains unclear.

Expression of the lineage-specific markers often indicates the timing of specification, and underlying regulatory mechanisms. Schwann cells express specific molecular markers, such as *Oct6*/*SCIP*, *Krox20*/*Egr2*, *myelin basic protein*, and *P0* (e. g. Ref. [2,3], see also [4] for a review). These markers are involved in the myelin formation, and therefore the timing of expression seems to be relatively late during the course of Schwann cell specification/differentiation. Many other genes expressed in the Schwann cell precursors are initially expressed in the neural crest cells, and are later restricted to the Schwann cells or Schwann cell/satellite glial lineages. This class of markers includes *Sox2* [5], *Sox10* [6], *cadherin7* [7], and *ErbB3* [8]. These genes, at least some of them, are known to regulate the differentiation of peripheral nervous system (PNS) glia and/or Schwann cells. In particular, *Sox10* gene, which encodes a member of group E HMG-box transcription factor protein, has important roles in various aspects of the neural crest development ([9,10], for reviews). *Sox10* positively regulates the expression of glial/Schwann cell markers such as *Krox20* [11], *P0* [11,12], and *S100β* [13], as well as *ErbB3* [14,15], which encodes a receptor for Neuregulin (Nrg) family ligands. The importance of *Sox10* for the Schwann cell development is further emphasized, as Nrg/ErbB3 signaling is known to be essential for the survival/proliferation/differentiation of Schwann cell precursors ([16], for a review).

Because the expression of the genes mentioned above in the Schwann cell lineage is either too late, or is not restricted to the Schwann cell lineage, they are not useful to elucidate the timing of the Schwann cell lineage segregation from the neural crest cells. *Schwann cell-specific EGF-repeat autocrine factor*, *Seraf*, seems to be the earliest marker for the Schwann cell precursors in avian PNS development [17]. This gene encodes a secreted protein with 5 EGF-

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like repeats, showing high homology to Wnt inhibitory factor 1 (WIF1) and Delta1 proteins [17], as well as the carboxy end of the vWDE (von Willebrand factor D and EGF domains) protein ([18], see also below). Function analysis has revealed that *Seraf* affects the peripheral nerve distribution [17]. As a marker, *Seraf* gene is expressed very early in a subset of the migrating neural crest cells, and subsequently in Schwann cell precursors along the peripheral nerve [17]. This Schwann cell precursor-specific expression of *Seraf* is transient, and in combination with the slightly later expression of *P0*, the early phase of Schwann cell development prior to myelin formation can be delineated. Another important feature of the *Seraf* expression is that the treatment of the primary cultured neural crest cells with Nrg1 could transiently induce *Seraf* expression, suggesting that the Nrg1 signaling is important for the very early phase of Schwann cell specification in avian embryos. These data indicate that studying the mechanisms for the Schwann cell precursor-specific expression of *Seraf* may provide important insights to understand how Schwann cell lineage segregates from the neural crest cells.

In this study, we analyzed the genome structure of *Seraf* locus, and have found a potential enhancer element contributing to the Schwann cell precursor-specific expression of this gene. We then analyzed the activity of the enhancer sequence in vivo, and showed that the enhancer is activated in the neural crest cells. We also showed that *Sox10* activates the identified enhancer. Our results uncovered a previously unknown role of *Sox10* in the earliest phase of the Schwann cell specification.

2. Materials and methods

2.1. Computational analyses of Seraf locus

Approximately 100 kb of genome sequence including *Tmem106b* and *vWDE* of human (*Homo Sapiens*, NC_000007), mouse (*Mus musculus*, NC_000072), rat (*Rattus rattus*, AC_000072), chicken (*Gallus gallus domesticas*, NC_006089), and zebrafish (*Danio rerio*, NC_007126) were retrieved from NCBI genbank (http://www.ncbi.nlm.nih.gov/genbank/). Annotations of exon/intro information were also taken from this browser. A pair-wise sequence alignments were performed with VISTA browser (http://genome.lbl.gov/vista/index.shtml), using chicken sequence as the baseline, measuring a 100 bp window and cutoff score of 60% identity, and alignments were visualized by VISTA plot.

Genome sequences of intergenic region between *Tmem106b* and *Seraf* of ostrich (*Struthio camelus*), common pigeon (*Columba livia*), common cuckoo (*Cuculus canorus*), adélie penguin (*Pygoscelis Adeliae*), and red-legged seriema (*Cariama cristata*) were also retrieved from NCBI genbank, and pair-wise sequence alignments were performed as mentioned above, using chicken sequence as the baseline, measuring a 100 bp window and cutoff score of 94% identity.

The intergenic region between *Tmem106b* and *Seraf* of chicken genome was searched for potential Sox10-binding sites with 95% threshold using JASPAR browser (http://jaspar.binf.ku.dk/cgi-bin/jaspar_db.pl).

2.2. Experimental animals

Fertilized Japanese quail (*Coturnix japonica*) eggs were obtained from local farms (Sendai Jun-ran). Embryos were staged according to Hamburger and Hamilton [19].

2.3. Expression vectors

Expression vectors of chicken *Sox10* (*pyDF30-cSox10*, [20]), quail *Sox2* (*pyDF30-qSox2*, [5]), chicken *Foxd3* (*pyDF30-cFoxd3*, [20,21]), *β-galactosidase* (*pmiwZ*, [22]) and *monomeric red fluorescent protein* (*pCAGGS-mRFP1*, [23]) were previously described. DNA fragment of chicken genome was PCR-amplified from a genomic library [24], and inserted into the multi-cloning site of *pEGFP-1* (Clontech) and *pGL3-basic* (Promega). As a transfection standard for luciferase assay, *pRL-tk* was obtained from Promega.

2.4. Immunological staining

Immuno-fluorescent staining of cryo-sections was performed as previously described [25,26]. 16A11 anti-HuC/D mouse IgG_{2b} (Molecular Probes [25,27]), and anti-β-galactosidase rabbit polyclonal (MP biomedicals) antibodies were used as described. Fluorochrome-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories. Sections treated with antibodies were also exposed to DAPI (Sigma) to visualize nuclei.

2.5. Electroporation into neural tube and whole embryo culture

Electroporation into the neural tube of stage 12 quail embryo was performed basically as described [5,20,28]. DNA solution (5 μg/μl in PBS containing 0.025% Fast Green) was injected into the lumen of the posterior neural tube (segmental plate level), and electrodes were laterally placed on both sides of the embryo. Electroporation was performed with CUY21 electroporator (BEX, condition: 25 V, 50 ms duration, 250 ms interval, and 5 pulses). Electroporated embryos were cultured ex ovo in the egg white-filled dishes at 38 °C for 24 or 48 h.

2.6. Luciferase assay

NIH3T3 cells were transfected with a *Luciferease* reporter and effector plasmid DNAs described above with LipofectAMINE Plus reagent (Invitrogen). *pRL-TK* was always co-transfected to normalize the transfection efficiency. Cell lysates were prepared for *Luciferase* activity 24 h after transfection with PicaGene Dual kit (Toyo Ink). Transfected cells were also exposed to the EGF domain fragment of Nrg1 (25 ng/ml, Heregulin-β EGF domain, Upstate biotechnology).

3. Results

3.1. Genomic structure of Seraf and vWDE loci in vertebrate species

To identify conserved cis regulatory elements, comparisons of genomic sequences nearby the gene of interest among different species have been practiced (e. g. Ref. [29–31]). Thus, we first looked for *Seraf* homologs in vertebrate species by searching the database. While we did not find a *Seraf* homolog in mammalian species, we could find *Seraf* of the trout origin [32]. We next examined the homology of the genomic sequences around chicken *Tmem106b*, *Seraf* and *vWDE* genes (chromosome 2), with those of syntenic positions in human (chromosome 7), and zebrafish (chromosome 15) visualized by VISTA plot analysis (Fig. 1A). While we could not identify a *Seraf* homolog in the syntenic position of the human genome, we could find homologous gene in the zebrafish genome (Fig. 1A, B). Interestingly, while entire Seraf protein sequence (211 a. a.) shows significant homology to zebrafish vWDE (210 a. a.), the chicken Seraf protein also showed a significant homology to the carboxy-terminal sequences of chicken vWDE (1845 a. a.), human vWDE (1590 a. a.), and zebrafish vWDE-like (1721 a. a.) (Fig. 1C). Considering the syntenic positions in the genomes and relatively short protein coding sequences, as well as the higher sequence homology, zebrafish *vWDE* gene seems to be a true

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