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# Detection and localization of the thyroid hormone receptor beta mRNA in the immature olfactory receptor neurons of chum salmon

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## Abstract

Thyroid hormone (TH) plays an important role in regulating multiple cellular and metabolic processes, including cell proliferation, cell death, and energy metabolism, in various organs and tissues of vertebrates. It is generally accepted that anadromous Pacific salmon (*Oncorhynchus* spp.) imprint odorants from their natal stream during their seaward migration, and they then use olfaction to discriminate their natal stream during the spawning migration. Both serum TH levels and the specific binding values of TH in the salmon olfactory epithelium were markedly increased during the seaward migration. However, thyroid hormone receptor (TR) expression in the olfactory epithelium has not been confirmed in vertebrates. We investigated gene expression of TR isoforms in chum salmon (*O. keta*) by both molecular biological and histochemical techniques. Expression of TR $\beta$  mRNA was detected in the olfactory epithelium by reverse transcriptase polymerase chain reaction (RT-PCR). Nucleotide sequencing demonstrated the existence of a remarkable homology between the RT-PCR product and part of the ligand-binding domain of other teleost TR $\beta$  isoforms. By

in situ hybridization using a digoxigenin-labeled salmon olfactory TR $\beta$  cRNA probe, signals for salmon olfactory TR $\beta$  mRNA were observed preferentially in the perinuclear regions of immature olfactory receptor neurons (ORNs), as protein gene product 9.5 (PGP9.5)-immunopositive ORNs. Our results provide the first detection of TR $\beta$  gene expression in the olfactory epithelium, and suggested the possibility that TR $\beta$  may be involved in cell maturation and/or cell differentiation of the ORNs in Pacific salmon.

Keywords: Anatomy, Molecular biology, Neuroscience, Physiology, Zoology

## 1. Introduction

Thyroid hormones (THs) are important regulators of multiple cellular and metabolic processes in various organs and tissues of vertebrates (Mullur et al., 2014). Thyroxine (T4) is the main product secreted by the thyroid follicular cells and is a precursor of the bioactive hormone 3, 3', 5-triiodo-L-thyronine (T3), most of which is produced by outer ring deiodination of T4 in peripheral target tissues (Mullur et al., 2014). TH effects are mediated through thyroid hormone receptors (TRs). TRs comprise part of a superfamily of nuclear receptors that are characterized by distinct regions or domains for DNA binding (C domain) and ligand-binding (E/F domain), separated by “hinge” regions (D domain) that may target the receptor to the nucleus (Evans, 1988; Lazar and Chin, 1990). TRs bind to thyroid hormone-responsive elements in the promoter of their target genes to activate or repress transcription through interactions with other nuclear receptors such as retinoic acid receptor or retinoid X receptor (Rivas and Maranjo, 2007). TR isoforms,  $\alpha$  and  $\beta$ , are differentially expressed in tissues and have distinct roles in TH signaling (Cheng et al., 2010). Although THs are required for neuronal proliferation, migration, synaptogenesis, and myelination during brain development (Howdeshell, 2002; Horn and Heuer, 2010), understanding of the function of THs in the peripheral olfactory system remains limited. In humans, TH deficiency may contribute to olfactory dysfunction in patients with hypothyroidism (McConnell et al., 1975). Propylthiouracil-treated hypothyroidism in rodents was associated with behavioral olfactory dysfunction (Beard and Mackay-Sim, 1987; Brosvic et al., 1996) and reduced proliferation of olfactory epithelial cells including olfactory receptor neurons (ORNs) (Mackay-Sim and Beard, 1987; Paternostro and Meisami, 1991, 1994). However, it is unknown which TRs mediate these effects of THs on the peripheral olfactory system of mammals or other vertebrates.

Anadromous Pacific salmon (*Oncorhynchus* spp.) imprint odorants from their natal streams during their seaward migration (Stabell, 1992), and they then use olfaction to identify their natal stream during their spawning migration (Wisby and Hasler, 1954; Nevitt and Dittman, 1998). Blood T4 levels of salmon gradually increase

during the seaward migration and peak just before entering the sea (Dickhoff et al., 1982; Yamauchi et al., 1984). Our previous study indicated that T3-specific binding values markedly increased in the olfactory organs just before entering the sea during the seaward migration of masu salmon (*O. masou*) using *in vitro* autoradiography of  $^{125}\text{I}$ -T3 in frozen sections (Kudo et al., 1994). In addition, the olfactory epithelia of T3 intraperitoneally implanted juvenile coho salmon (*O. kisutch*) indicated high DNA synthesis as detected using a 5-bromo-2'-deoxyuridine cell birth-dating technique, and the activation of olfactory cellular proliferation was reported in these tissues (Lema and Nevitt, 2004). Just as in mammals, teleost fishes have multiple TR subtypes (Nelson and Habibi, 2009). Although the number of TRs identified in a given species varies, all teleost TRs are orthologous to either the TR alpha or TR beta of mammals (Johnson and Lema, 2011). The teleost TR alpha and TR beta forms can have distinct patterns of tissue expression and transcriptional regulation (Chen et al., 2014; Johnson and Lema, 2011; Manchado et al., 2009; Lema et al., 2009), suggesting functional differentiation between receptor types. In salmonid fish, TR genes have been detected in various organs (brain, intestine, gill, liver, muscle, kidney, ovary, skin, etc.) of rainbow trout (*O. mykiss*) (Jones et al., 2002; Raine et al., 2005, 2011), coho salmon (Harada et al., 2008), and Atlantic salmon (*Salmo salar*) (Marchand et al., 2001). However, TR gene expression in the peripheral olfactory system has not been widely studied in vertebrates, including teleost. Therefore, a critical gap remains in understanding the mechanism whereby THs promote cell proliferation in the peripheral olfactory system.

The main goal of the present study was to examine gene expression of the TRs in the peripheral olfactory system of Pacific salmon and their involvement in regulating the proliferation of ORNs. For this purpose, we performed cloning of a partial TR cDNA from the olfactory organs of chum salmon (*O. keta*). In addition, to presume the function of TRs in the olfactory organs, we localized TR mRNAs within the olfactory epithelium using *in situ* hybridization, and compared the localization of TR transcripts to the distribution of proliferating cell nuclear antigen (PCNA) - a marker of newly proliferated cells - and protein gene product 9.5 (PGP9.5) - a commonly used indicator of neuronal cells.

## 2. Material and methods

### 2.1. Animals

TR mRNA expression in the chum salmon olfactory epithelia were examined at feeding immature salmon. Fish were collected from the Northwest Pacific Ocean (43°N, 155°E) using a drift gill net deployed from the training ship “Oshoryo-Maru IV” (Hokkaido University) in early summer 2008 [Average body length (fork length)  $\pm$  standard error (S.E.):  $557 \pm 7.34$  mm, average body mass  $\pm$  S.E.:  $2092 \pm 95.12$  g, age: 3–5 years, n = 10]. Fish were immobilized in carbonated

water, and then the olfactory organs (i.e., olfactory rosettes) were dissected. The experiments were performed according to “National University Corporation Hokkaido University Provisions on Animal Experiments” which “Hokkaido University Animal Care and Use Committee” approved.

## 2.2. RNA preparation

Dissected tissues were immediately immersed in RNAlater (Ambion, Austin, TX, USA) and stored at  $-30^{\circ}\text{C}$  until RNA extraction. Total RNA was prepared from the olfactory rosette using a High Pure RNA Tissue Kit (Roche, Indianapolis, IN, USA) in accordance with the manufacturer’s instructions. Poly (A)<sup>+</sup> RNA was isolated using oligo (dT)-latex beads (Oligotex-dT30<Super>; Takara, Otsu, Japan) from total RNA.

## 2.3. Oligonucleotides

Oligonucleotides used as PCR primers are shown in **Table 1**. The degenerate fish TR primer pair (Fish TR-F and Fish TR-R) was designed from consensus sequences of TR found in several teleostean fish species (e.g., Kawakami et al., 2003, 2007, 2008).  $\beta$ -actin primers ( $\beta$ -actin-F and  $\beta$ -actin-R) were synthesized based on sequences of rainbow trout  $\beta$ -actin cDNA (GenBank accession no. AF254414) (Celiaus et al., 2000).

## 2.4. Cloning and sequencing of salmon TR cDNA

In brief, poly (A)<sup>+</sup> RNA from the olfactory rosettes of immature chum salmon were reverse transcribed using a Random Primer pd (N)<sub>6</sub> and PrimeScript Reverse Transcriptase (Takara, Tokyo, Japan) in accordance with the manufacturer’s instructions. The PCR conditions were: the first cycle, denaturation at  $94^{\circ}\text{C}$  for 3 min, annealing at  $55^{\circ}\text{C}$  for 5 min, and extension at  $72^{\circ}\text{C}$  for 5 min; 35 cycles of incubation, 30 sec at  $94^{\circ}\text{C}$ , 30 sec at  $56^{\circ}\text{C}$ , and 1 min at  $72^{\circ}\text{C}$ ; and finally extension at  $72^{\circ}\text{C}$  for 5 min for 3' A overhangs using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The amplicons were T-A ligated to pCRII-TOPO vector (TOPO TA Cloning Kit; Invitrogen, San Diego, CA) and sequenced.

**Table 1.** Primer sets used for RT-PCR for sequence and expression analysis of chum salmon TRs and  $\beta$ -actin.

Name	Primer sequence
Fish TR-F	5'-TGYTGYATGGAGATMATGTC-3'
Fish TR-R	5'-TGGGRCACTCSACYTTCATGTG-3'
$\beta$ -actin-F	5'-CACCGGTATCGTCATGGACT-3'
$\beta$ -actin-R	5'-CTCGTGGATAACCGCAAGACT-3'

DNA sequencing was conducted using a PRISM 3730 DNA analyzer and BigDye Terminator v3.1 (Applied Biosystems). Nucleotide sequence alignment was conducted using a nucleotide basic local alignment tool (blastn) offered by NCBI. The partial cDNA encoding chum salmon *trβ* was deposited in GenBank as accession no. AB669091.

## 2.5. In situ hybridization

After the plasmid vector was linearized, digoxigenin (DIG)-labeled antisense and sense cRNA probes were prepared using T7 and SP6 RNA polymerases with a DIG RNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany) in accordance with the manufacturer's instructions. Olfactory rosette tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 72 h, and then embedded in Histosec. Then, approximately 5-μm thick sections were prepared. Serial paraffin sections were digested with 20 μg/mL proteinase K in 10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0) for 20 min at 37 °C, and then treated with 0.2 mol/L HCl for 10 min and 0.25% acetic anhydride in 0.1 mol/L triethanolamine-HCl (pH 8.0) for 10 min. Hybridization and immunohistochemical detection for hybridized signals were performed using the previously reported method (Kudo et al., 1999). To assess the specificity of the in situ hybridization signals, two negative control procedures were performed. First, RNA was digested in randomly chosen sections with 20 μg/mL RNase A for 30 min at 37 °C prior to *in situ* hybridization. Second, digoxigenin-labeled sense RNA probes were hybridized in parallel to antisense RNA probes in all cases.

## 2.6. Immunohistochemistry

Sections semi-adjacent to those used for the above *in situ* hybridization were prepared. After deparaffinization and rehydration, the sections were blocked with 0.3% H<sub>2</sub>O<sub>2</sub> in absolute methanol for 20 min to deactivate endogenous peroxidases. After rinsing with phosphate-buffered saline (PBS), the sections were incubated with antigen-retrieval solution (L.A.B. Solution; Polysciences, Warrington, PA) for 10 min at room temperature, and then rinsed with PBS. Antigen-retrieved sections were incubated with 10% bovine serum albumin in PBS for 60 min at room temperature followed by incubation in a humid chamber with one of the following: rabbit anti-Nile tilapia (*Oreochromis niloticus*) PGP9.5 recombinant polyclonal antibody (Mochida et al., 2002) at a dilution of 1:1000 in PBS, or mouse anti-rat PCNA monoclonal antibody (PC10; Santa Cruz Biotechnology) at a dilution of 1:50 in PBS for 18 h at 4 °C. After rinsing in PBS, sections were reacted using the indirect immunoperoxidase method (Histofine Simple Stain MAX-PO Kit, Nichirei, Tokyo, Japan). PGP9.5 and PCNA localized the immature ORNs and the proliferating cells, respectively. The peroxidase complex was visualized by treatment with a freshly prepared

diaminobenzidine tetrahydrochloride (0.1 mg/mL) solution with 0.01% H<sub>2</sub>O<sub>2</sub> for 5 min at room temperature. The specificities of the above immunoreactivities were confirmed by replacing the primary antibody with a pre-immuno serum for PGP9.5, or a pre-absorption PCNA antibody with antigenic peptide for it. Sections semi-adjacent to those used for the above immunohistochemical analyses were counterstained with Delafield's hematoxylin and eosin staining.

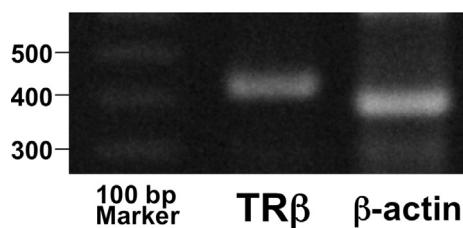
### 3. Results

#### 3.1. Characterization of a partial salmon TR gene in the olfactory organ

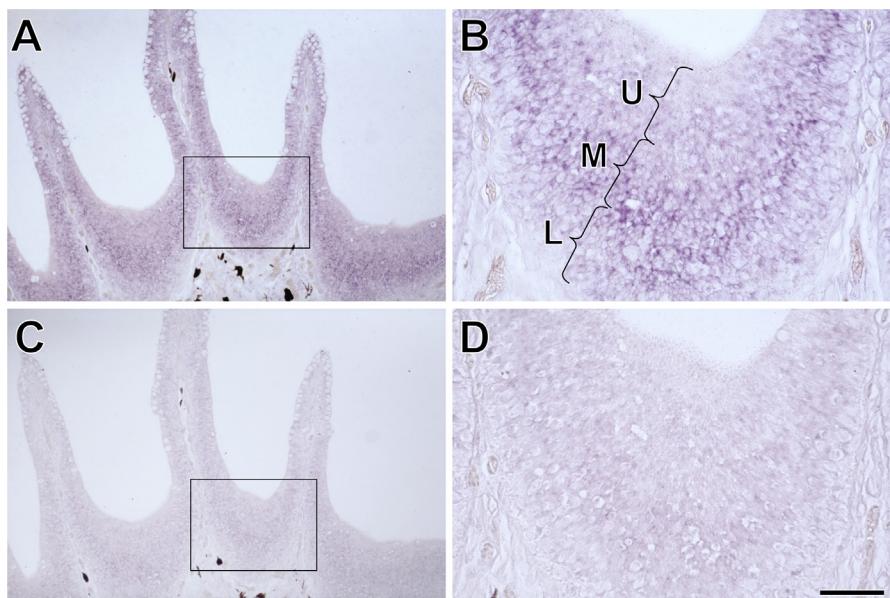
The amplified cDNA fragments of the TR and β-actin mRNAs of the expected sizes (422 and 385 bp, respectively) were detected in the olfactory organ of immature chum salmon using RT-PCR (Fig. 1; Supplemental data 1). The nucleotide sequence of the partial TR amplicon (AB669091) exhibited high homology (99% and 96%) with the corresponding region of TRβ2 (AB303987 and AF302252) previously examined in coho salmon and Atlantic salmon liver (Harada et al., 2008; Marchand et al., 2001), respectively. This amplicon included the sequence of the ligand-binding domain (E/F domain) in salmonid TR (Marchand et al., 2001). For this reason, the deduced amino acid sequence was identical to salmon TRβ. Sequencing resulted in identification of only a single TRβ cDNA from the salmon olfactory rosette; TRα in this tissue was not able to detect at the present study.

#### 3.2. Expression of TRβ mRNA in the olfactory epithelium

Signals of TRβ mRNA using a labeled cRNA antisense probe were seen preferentially in the perinuclear regions of the olfactory epithelial cells in the lower two-thirds of the middle layer and the upper area of the lower layer in the olfactory epithelium of immature chum salmon (Fig. 2A and B). In the RNase A pretreated control sections, signals were abolished in all cases (not shown). Hybridization



**Fig. 1.** Agarose gel electrophoresis showing TR and β-actin mRNA expression patterns in olfactory organs of immature chum salmon. PCR-amplified TR cDNA of approximately 422 bp and β-actin cDNA of approximately 385 bp. These amplicons were cloned, and both strands sequenced and confirmed as TRβ and β-actin cDNA fragments, respectively. Positions of 100 bp DNA markers, expressed in bases, are indicated on the left side of the figure. Non-adjusted electrophoresis image is shown in Supplemental data 1.

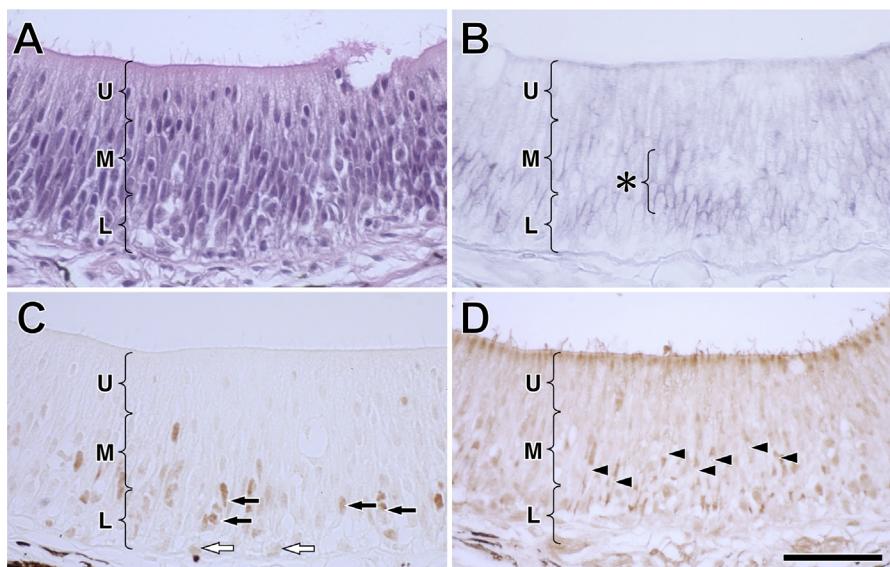


**Fig. 2.** *In situ* hybridization of TR $\beta$  mRNA in the olfactory epithelia of immature chum salmon by digoxigenin-labeled anti-sense probe for TR $\beta$  mRNA (A and B). Sections adjacent to those used for (A and B) represent the control signal intensity using a labeled sense control probe for TR $\beta$  mRNA (C and D, respectively). Signals of TR $\beta$  mRNA were observed preferentially in the perinuclear regions of olfactory epithelial cells in the lower two-thirds of the middle layer (M) and the upper area of the lower layer (L) in the olfactory epithelium. These signals were not observed in the upper layer (U). Bar: 50  $\mu$ m.

with a labeled sense control probe identified no detectable hybridized signals in adjacent sections to Fig. 2A and B of the olfactory epithelium (Fig. 2C and D). No clear differences in the localization or signal intensity for TR $\beta$  were observed between males and females in *in situ* hybridization examinations. Analysis of semi-serial sections of the olfactory epithelium showed signals of TR $\beta$  mRNA and immunoreactivities for PGP9.5 and PCNA in immature chum salmon (Fig. 3). Immunoreactivity for PCNA was mainly observed in the globose basal cells and part of horizontal basal cells (Fig. 3C). Immunoreactivities for PGP9.5 were observed in the perinuclear regions of the immature ORNs in the lower two-thirds of the middle layer and the upper area of the lower layer, and the dendrites of these neurons (Fig. 3D). Both signals of TR $\beta$  mRNA and immunoreactivity for PGP9.5 overlapped in the perinuclear regions of immature ORNs (Fig. 3B and D). Horizontal basal cells did not show either positive reaction for TR $\beta$  mRNA and PGP9.5 near the basal lamina. The above immunoreactivities were not detected with the pre-immuno antibody or the preabsorbed antibody with antigenic peptide.

#### 4. Discussion

Transcripts encoding a TR $\beta$  were identified for the first time in the peripheral olfactory system of a vertebrate. These results indicated that the olfactory organ was one of target tissues for THs in chum salmon. RT-PCR analysis in the present study



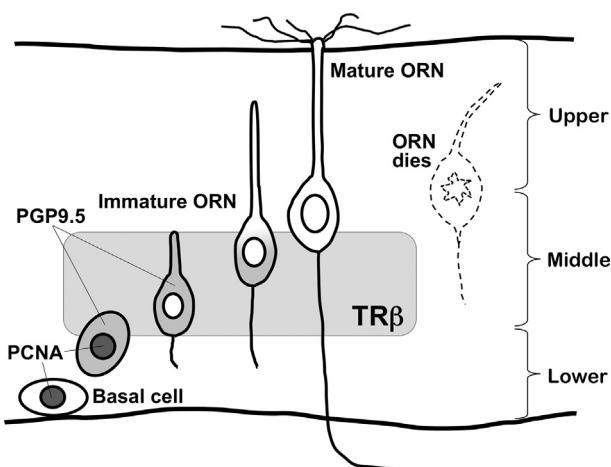
**Fig. 3.** Serial sections of the olfactory epithelium of immature chum salmon. Expression of TR $\beta$  mRNA (B), immunoreactivities for proliferating cell nuclear antigen (PCNA; C), protein gene product 9.5 (PGP9.5; D), and Delafield's hematoxylin and eosin staining (A). Asterisk indicates the signals of TR $\beta$  mRNA in the immature olfactory receptor neurons (ORNs). Black arrows indicate PCNA-immunoreactive globose basal cells. White arrows indicate the horizontal basal cells. Arrowheads indicate the nuclei of PGP9.5-immunoreactive ORNs. (L) lower, (M) middle, and (U) upper layers in the olfactory epithelium. Bar: 50  $\mu$ m.

revealed the presence of TR $\beta$  mRNA in the olfactory organs of chum salmon, but the expression of TR $\alpha$  mRNA was not detected. Previous studies indicated that TR $\beta$  was highly expressed in the brain and pituitary of teleostean fish, Japanese conger eel (*Conger myriaster*) (Kawakami et al., 2003), and Japanese eel (*Anguilla japonica*) (Kawakami et al., 2007). The present partial TR sequence from chum salmon olfactory organs exhibited high homology to coho salmon TR $\beta$  isoform (Harada et al., 2008), and was identified as an ortholog of fish TR $\beta$ . The TR $\alpha$  and  $\beta$  subtypes are the products of two distinct genes, whereas the two TR $\beta$  isoforms (TR $\beta$ 1 and TR $\beta$ 2) are splice variants from the same gene. In mammals, TR $\beta$ 1 is widely expressed and is particularly abundant in the liver and kidney (Lazar and Chin, 1990). Conversely, in mammals TR $\beta$ 2 is most highly expressed in the pituitary and hypothalamus (Hodin et al., 1989; Cook et al., 1992). However, the present partial TR $\beta$  amplicon was not able to distinguish TR $\beta$ 1 or TR $\beta$ 2 isoforms. Because, the deduced amino acid sequence of this amplicon was a region in which TR $\beta$ 1 and TR $\beta$ 2 hold the same amino acid sequence in coho salmon (Harada et al., 2008).

Histological examination revealed that a similar pattern of localization of TR $\beta$  mRNA and PGP9.5-immunoreactivity to in the lower two-thirds of the middle layer and the upper area of the lower layer of the olfactory epithelium. In mammals, PGP9.5 is expressed in ORNs over a broad range of developmental stages, including immature ORNs (Taniguchi et al., 1993). In teleosts, Nile tilapia ORNs expressed a

ubiquitin C-terminal hydrolase (EC 3.4.19.12) homologous to PGP9.5 (Mochida et al., 2002). Moreover, our previous immunohistochemical study revealed that the immunoreactivities for PGP9.5 were located in the cytosol of immature ORNs of lacustrine sockeye salmon (*O. nerka*) (Kudo et al., 2009). PCNA is an intranuclear polypeptide synthesized in the late G1 and S phase (Mathews et al., 1984). The localizations of TR $\beta$ -expressing cells and PCNA-immunoreactive basal cells differed in the olfactory epithelia. These findings strongly suggested that TR $\beta$  is expressed in immature ORNs in salmon olfactory epithelia (Fig. 4). The differentiation period from basal cell to mature ORN was reported to be about 1 week in mouse and salmon using *in vivo*  $^3\text{H}$ -thymidine (Graziadei and Monti-Graziadei, 1979) and 5-bromo-2'-deoxyuridine (Yanagi et al., 2004) labeling experiments, respectively. Based on the detection of TR $\beta$  only in the salmon olfactory rosette and the localization pattern of TR $\beta$  mRNA within the epithelium, it is possible that TR $\beta$  may mediate the effects of THs on cell maturation and/or cell differentiation of ORNs during this period in the olfactory organs of Pacific salmon. In fact, a previous report indicated that THs have an essential role in the maturation of ORNs in developing rats (Paternostro and Meisami, 1996). In mammalian brains, TH controls the expression of many genes encoding proteins with roles in cell differentiation, such as cytoskeletal proteins, neurotrophins, neurotrophin receptors, cell cycle regulators, and extracellular matrix proteins (Bernal, 2015). Presumably also in salmon ORNs, THs may regulate the transcription of these proteins via TRs as well as mammalian neurons, but direct support for this assumption was not available.

Filial imprinting is caused by visual stimuli, and it has been demonstrated that precocial chicks visually imprint on the first conspicuous moving object, and the visual stimulus then elevated THs in the chick's brain thereby initiating the imprinting phase (Yamaguchi et al., 2012). The relationship between the blood T4



**Fig. 4.** Schematic illustration of the localization of TR $\beta$  mRNA expression and marker molecules for basal cells and immature olfactory receptor neurons (ORNs) in the olfactory epithelium of chum salmon. PCNA: proliferating cell nuclear antigen, PGP9.5: protein gene product 9.5.

concentration peak and the onset of seaward migration (i.e., olfactory imprinting period) has been reported in juvenile chum salmon (Ojima and Iwata, 2007) and in parr-smolt transformation of coho salmon (Dittman and Quinn, 1996). More recently, thyrotropin-releasing hormone gene expression in hatchery-reared juvenile chum salmon brain increased immediately after release from a hatchery into the natal stream, and the expression of the essential NR1 subunit of the N-methyl-D-aspartate receptor increased during downstream migration (Ueda et al., 2016). In addition, oral T4 administration experiments in the same stage fish enhanced NR1 gene activation (Ueda et al., 2016). These results suggested that olfactory memory formation during juvenile seaward migration of chum salmon was controlled by THs in the central nervous system. THs that increase during downstream migration may also act on the peripheral olfactory system, and may be increasing the sensitivity of olfaction for their natal stream odorants.

The present study is the first to describe the detection and localization of TR $\beta$  mRNA in the vertebrate olfactory epithelium. These findings suggested the possibility that TR $\beta$  may be involved in cell maturation and/or cell differentiation of ORNs in Pacific salmon.

## Declarations

### Author contribution statement

Hideaki Kudo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Akihiro Eto, Takashi Abe, Kazuhiko Mochida: Performed the experiments; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

Data associated with this study (partial cDNA encoding chum salmon tr $\beta$ ) has been deposited at GenBank under the accession number AB669091.

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2018.e00744>.

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