



HOKKAIDO UNIVERSITY

Title	Molecular characterization and histochemical demonstration of salmon olfactory marker protein in the olfactory epithelium of lacustrine sockeye salmon (<i>Oncorhynchus nerka</i>)
Author(s)	Kudo, H.; Doi, Y.; Ueda, H. et al.
Citation	Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology, 154(1), 142-150 https://doi.org/10.1016/j.cbpa.2009.05.123
Issue Date	2009-09
Doc URL	https://hdl.handle.net/2115/38841
Type	journal article
File Information	CBP_A_ms17027_Kudo.pdf



Molecular characterization and histochemical demonstration of salmon olfactory marker protein in the olfactory epithelium of lacustrine sockeye salmon (*Oncorhynchus nerka*)

H. Kudo ^{a,*}, Y. Doi ^b, H. Ueda ^c, M. Kaeriyama ^a

^a Laboratory of Strategic Studies on Marine Bioresource Conservation and Management, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan

^b Department of Anatomy, University of Occupational and Environmental Health, School of Medicine, Kitakyushu 807-8555, Japan

^c Laboratory of Aquatic Ecosystem Conservation, Field Science Center for Northern Biosphere, Hokkaido University, Sapporo 060-0809, Japan

Key words: olfactory marker protein - olfactory receptor neuron – cloning – *in situ* hybridization – immunocytochemistry – Pacific salmon – Teleostei

*** Correspondence to:** Hideaki Kudo, PhD, Laboratory of Strategic Studies on Marine Bioresource Conservation and Management, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan.

Tel & Fax: +81-138-40-5602, E-mail: hidea-k@fish.hokudai.ac.jp

Abstract

Despite the importance of olfactory receptor neurons (ORNs) for homing migration, the expression of olfactory marker protein (OMP) is not well understood in ORNs of Pacific salmon (Genus *Oncorhynchus*). In this study, salmon OMP was characterized in the olfactory epithelia of lacustrine sockeye salmon (*O. nerka*) by molecular biological and histochemical techniques. Two cDNAs encoding salmon OMP were isolated and sequenced. These cDNAs both contained a coding region encoding 173 amino acid residues, and the molecular mass of the two proteins was calculated to be 19,581.17 and 19,387.11 Da, respectively. Both amino acid sequences showed marked homology (90%). The protein and nucleotide sequencing demonstrates the existence of high-level homology between

salmon OMPs and those of other teleosts. By *in situ* hybridization using a digoxigenin-labeled salmon OMP cRNA probe, signals for salmon OMP mRNA were observed preferentially in the perinuclear regions of the ORNs. By immunohistochemistry using a specific antibody to salmon OMP, OMP-immunoreactivities were noted in the cytosol of those neurons. The present study is the first to describe cDNA cloning of OMP in salmon olfactory epithelium, and indicate that OMP is a useful molecular marker for the detection of the ORNs in Pacific salmon.

1. Introduction

Pacific salmon (Genus *Oncorhynchus*) exhibits the most representative anadromous migration between the natal stream and the sea. The homing migration of salmon to the natal stream is one of the most interesting phenomena in fish biology. It is generally accepted that salmon imprint some odorants of their natal streams on downstream migration (Stabell, 1992), and use their olfaction to identify those streams during spawning migration. Despite the importance of the olfactory system for the olfactory imprinting, the neurological and molecular biological mechanisms of these phenomena are not well understood in the salmon. Olfactory stimuli are transmitted to the brain through the olfactory receptor neurons (ORNs) in salmon as well as other vertebrates. For histological and cytological approaches, the straightforward detection of ORNs is important in salmon migration research. Previous studies demonstrated that glutathione *S*-transferase (GST; EC 2.5.1.18) class pi (salmon olfactory GST; soGST), as a phase II xenobiotic metabolizing enzyme, was one of the molecular markers of ORNs in the lacustrine sockeye salmon (*O. nerka*; Shimizu et al., 1993; Kudo et al., 1996, 1999), and in the rainbow trout (*O. mykiss*; Starcevic and Zielinski, 1995, 1997). In a recent study, GST activities in the olfactory epithelia of rainbow trout significantly increased in the presence of an environmentally realistic pesticide mixture (Tierney et al., 2008). These results indicated that salmon ORNs were related to both olfaction and xenobiotic metabolism in olfactory organs. It is essential to investigate more direct molecular markers of olfaction in salmon ORNs.

Olfactory marker protein (OMP) is an abundant 19 kDa cytoplasmic protein that is almost exclusively expressed in mature, but not immature, ORNs of vertebrates (e.g., Keller and Margolis, 1975; Farbman and Margolis, 1980), and only in small numbers in other neuronal populations (Baker et al., 1989). The important role of OMP in ORN function was confirmed by generating OMP knockout mice (*Mus musculus*) whose electrophysiological

responses to odorants are impaired (Buiakova et al., 1996; Ivic et al., 2000) and whose detection threshold for odors is increased 50-100 fold (Youngentob and Margolis, 1999; Youngentob et al., 2001), demonstrating a modulatory function in olfactory signal transduction. In fish, some previous studies have demonstrated this through immunohistochemical analyses and molecular cloning (Celik et al., 2002; Yoshida et al., 2002; Yasuoka et al., 1999, Ferrando et al., 2007). However, in salmonid fish, immunohistochemistry using antibodies against mammalian OMP has only been conducted in ORNs of rainbow trout (Riddle and Oakley, 1992). The biochemical and molecular biological characters of salmon OMP remain to be fully elucidated, although OMP-like substances exist in the ORNs of rainbow trout.

In this study, salmon OMP was characterized in the olfactory epithelia of lacustrine sockeye salmon by employing molecular biological and histochemical techniques, showing that OMP is a useful molecular marker for ORNs in Pacific salmon. In addition, we conducted immunohistochemical analyses of soGST and protein gene product 9.5 (PGP9.5), as broad developmental ORN marker, to compare localization with OMP and other ORN marker molecules.

2. Materials and methods

2.1. Animals

One-year-old lacustrine sockeye salmon of both sexes used in this study were reared at Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University, in outdoor FRP tanks supplied with a continuous flow of spring water at an ambient temperature and under natural photoperiod conditions (body length [fork length] 130 ± 1.10 mm and body weight 21 ± 0.5 g; mean $\pm$ SE). Fish were anesthetized with 2-phenoxyethanol (200 mg/l), and the olfactory rosettes, including the olfactory epithelia, were surgically isolated.

Male Wistar rats (*Rattus norvegicus*) aged 8 weeks and weighing 250 ± 30 g (Seac Yoshitomi, Fukuoka, Japan) were provided for the present study. The care and use of animals followed 'The Guiding Principles for the Care and Use of Animals', approved by our university in accordance with the principles of the Declaration of Helsinki. Rats were deeply anesthetized with an intraperitoneal injection of 5 mg of pentobarbital per 100 g body weight and perfused intracardially with physiologic saline. After perfusion, the nasal cavities, including the olfactory epithelia, were carefully dissected and removed for OMP-

immunohistochemistry. Paraffin sections and soluble extracts were prepared according to the procedure described by Kudo et al. (2000) and Nishino et al. (2001), respectively.

2.2. RNA preparation

Total RNA was prepared from the olfactory epithelia of lacustrine sockeye salmon by the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) using ISOGEN (Nippongene, Tokyo, Japan). Poly(A)⁺ RNA was isolated by oligo(dT)-Latex beads (Oligotex-dT30<Super>; Takara, Otsu, Japan).

2.3. Oligonucleotides

Oligonucleotides used as PCR primers are shown in Table 1. The degenerate primer pair (OMPdn-F and OMPdn-R) was designed against highly conserved regions of OMP cDNA of zebrafish (*Danio rerio*) 1 (GenBank accession no. **AB073551**), *Xenopus laevis* (**XLA010978**), humans (*Homo sapiens*; **HSU01212**), rats (**RNM26926**), and mice (**MMU01213**). Gene-specific primers were synthesized based on our partial sequences of salmon OMP cDNA.

2.4. Cloning of salmon OMP cDNA

Described briefly as above, five hundred nanograms of poly (A)⁺ RNA from the olfactory epithelium was reverse transcribed using an oligo (dT) primer and Super ScriptTM II RNaseH⁻ Reverse Transcriptase (Gibco BRL, Rockville, MD) according to the manufacturer's instructions. PCR was performed using degenerate primers OMPdn-F and OMPdn-R) designed from a highly conserved region of known OMP sequences. The PCR conditions were: the first cycle, denaturation at 94°C for 3 min, annealing at 55°C for 5 min, and extension at 72°C for 5 min; 35 cycles of incubation, 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C; and finally extension at 72°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.

Based on the sequence information of the presumptive OMP cDNA amplicon, rapid amplification of cDNA ends (RACE) procedures were performed to isolate the 5' and 3' ends of the cDNA (SMART RACE cDNA Amplification Kit; Clontech Laboratories, Mountain View, CA). In each RACE procedure, the initial PCR amplification was followed by nested amplification. Gene-specific primer sets (sp series) in combination with adaptor primers

were used, respectively, for 5'- and 3'-RACE. RACE PCR cycling conditions were 35 cycles with 5 sec at 94°C, 10 sec at 70°C or 68°C, and 2 min at 72°C.

2.5. DNA sequencing analysis

The resulting plasmid DNA was alkaline-denatured and both strands were sequenced using a Dye Terminator Cycle Sequencing Kit and ABI Prism Model 3100 Auto Sequencer (Applied Biosystems, Foster City, CA). Sequence analysis and comparison were carried out using DNASIS software (Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The sequences of salmon OMP1 and OMP2 were deposited in GenBank (accession numbers **AB490250** and **AB490251**, respectively). The alignment of multiple protein sequences was performed using the ClustalW multiple sequence alignment program, and homology values (percent of amino acid sequence identity) were calculated by pair-wise alignment. A phylogenetic tree was constructed using the Neighbor-joining method (Saitou and Nei, 1987) and viewed with TreeViewX (Ver. 0.5.0). Program settings are detailed in Figure legends.

2.6. Polyclonal antibody production

For antigen of the salmon OMP antibody, synthetic peptide was synthesized at Genenet Co., Ltd. (Fukuoka, Japan) and coupled to bovine serum albumin (BSA). The peptide chain was designed against highly conserved regions of OMP amino acid sequences of salmon and other teleosts (Fig. 2; 92-110; SQLWTPDLTHLMTRQLLEP-C). A rabbit was injected with 150 µg BSA-conjugated synthetic peptide in Freund's complete adjuvant and boosted at 2-week intervals by the subcutaneous injection of 300 µg of the antigen. The rabbit was bled one week after the fifth injection. Antiserum was purified with affinity peptide column coupling using the same peptide as above. The titer and specificity of purified antibody was tested by ELISA and western blotting analyses.

2.7. Western blot analysis

Soluble extracts from lacustrine sockeye salmon olfactory rosettes and rat nasal cavities containing 20 µg protein, as determined by Lowry's method (Lowry et al., 1951), were heated at 100°C for 2 min in 3% sodium dodecyl sulfate (SDS) with a reduction with 10% 2-mercaptoethanol. They were separated using 15% SDS- polyacrylamide gel electrophoresis (PAGE), and the separated proteins were stained with 0.1% Coomassie brilliant blue R-250 in a mixture of 40% ethanol, 10% acetic acid, and 50% DDW. Protein molecular weight

markers (low MW set) were obtained from Pharmacia (Uppsala, Sweden). Proteins resolved by SDS-PAGE were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) with a Semidry Trans-blot SD (Bio-Rad, South Richmond, CA). The membrane was incubated for 1 h with 5% skim milk in TBS to reduce non-specific protein absorption, and allowed to react overnight with purified salmon OMP polyclonal antibody at a dilution of 1:500 in Solution 1 of Can Get Signal (Toyobo, Osaka, Japan). After rinsing with Tris-buffered saline (TBS), the membrane was incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) at a dilution of 1:1000 in TBS for 3 h, developed in 0.06% 4-chloro-1-naphthol solution in TBS containing 0.01% H₂O₂ between 3 and 5 min, and air-dried. The specificity of the above immunoreactivities was confirmed by replacing the primary antibody with pre-immuno serum.

2.8. Immunohistochemistry

Serial paraffin sections of lacustrine sockeye salmon olfactory epithelia of both sexes were prepared according to the procedure described by Kudo et al. (1996). After deparaffinization and hydration, sections were blocked with 0.3% H₂O₂ in absolute methanol for 20 min to remove endogenous peroxidase. After rinsing with phosphate-buffered saline (PBS), they were incubated with 10% BSA in PBS for 15 min followed by incubation in a humid chamber with one of the following: the purified rabbit anti-salmon OMP polyclonal antibody, rabbit anti-salmon olfactory GST (soGST) polyclonal antibody (anti-N24; Shimizu et al., 1993; Kudo et al., 1999), or rabbit anti-protein gene product 9.5 (PGP9.5) polyclonal antibody (UltraClone, Cambridge, UK) at a dilution from 1:200 to 1:2000 in PBS for 16 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). The specificity of the above antibody was confirmed by a preabsorption test with antigenic peptide for it. The peroxidase complex was visualized by treatment with a freshly prepared diaminobenzidine tetrahydrochloride (0.1 mg/mL) solution with 0.01% H₂O₂ for 5 min at room temperature. The specificity of the above immunoreactivities was confirmed by replacing the primary antibody with pre-immuno serum.

The double immunofluorescence method was performed in order to compare the localization between OMP and GST in the same sections. Two-step immunoreactions for primary antibodies were carried out as described below because both antibodies were rabbit polyclonal antibodies. Sections were treated with 10% normal donkey serum in PBS for 30

min, and first immunoreacted with purified rabbit anti-salmon OMP antibody at a dilution of 1:100 in PBS for 16 h at 4°C. After PBS washing, sections were reacted with Alexa Fluor 488 donkey anti-rabbit IgG conjugate (Molecular Probes, Eugene, OR) at a dilution of 1:100 in PBS for 2 h at room temperature. All incubations were carried out in a humid and dark chamber. Sections were coverslipped with 90% nonfluorescent glycerol containing 7.5 µg/mL of 4', 6-diamino-2-phenylindole dihydrochloride (DAPI) in PBS, and digitally captured on a fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan) equipped with a Canon EOS 5D camera (Tokyo, Japan). Decoverslipped sections were treated with the antibody-removal solution (0.2 mol/L glycine in DDW; pH 2.8) for 16 h at room temperature under stirring. After washing with 0.5 M Tris-HCl buffer (pH 7.8), sections were checked for residual fluorescence using fluorescence microscope. Secondary immunoreaction was performed by replacing OMP antibody and Alexa Fluor 488 with rabbit anti-salmon olfactory GST antibody and Alexa Fluor 546, respectively. The captured images of both OMP- and GST-immunoreactions were superimposed based on the DAPI-counterstained nuclei using Adobe Photoshop software (Adobe System Inc., San Jose, CA).

2.9. In situ hybridization

After the partial salmon OMP1 cDNA (389 bp; homology vs. salmon OMP2 is 94.3%) was linearized, digoxigenin (DIG)-labeled antisense and sense cRNA probes were prepared with T7 and SP6 RNA polymerase using a DIG RNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Sections adjacent to those used for the above immunohistochemistry were digested with 5 µg/mL proteinase K in 10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0) for 20 min at 37°C, and 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 method of Kudo et al. (1999). In control experiments, the sense RNA probe was hybridized to tissue sections as described for the antisense RNA probe. Alternatively, the sections were treated with 20 µg/mL RNase A for 30 min at 37°C prior to *in situ* hybridization.

3. Results

3.1. Nucleotide sequence of salmon OMP cDNA

The degenerate RT-PCR generated an amplicon of expected size (155 bp) from the olfactory epithelium. This amplicon was sequenced, and the nucleotide sequence was that of OMP. 3'-RACE reactions using the sense primer OMP1sp-3 in combination with the adapter amplified the entire 3' terminus of cDNA (including the poly-A tail). On the other hand, 5'-RACE reactions using the antisense primer OMP1sp-5 or OMP2sp-5 were conducted to amplify the remainder of the 5' end, a portion of the untranslated region. Two salmon OMP1 and -2 cDNAs contained 1818 and 1750 nucleotides with a putative 519-bp open reading frame (ORF) and polyadenylation signal, AATAAA, located 23 and 17 bp upstream of the poly (A)⁺ tail, respectively (Fig. 1). These cDNAs contained a coding region encoding 173 amino acid residues, and the molecular mass of the protein was calculated to be 19,581.17 and 19,387.11Da, respectively.

3.2. Sequence comparisons

Alignment of the deduced amino acid sequences is shown in Figure 2. They showed a 90.17% (sockeye salmon2), 62.99% (zebrafish1: GenBank accession no. **AB073551**), 64.52% (fugu, *Takifugu rubripes*: Ensembl Translation ID. **SINFRUP00000150379**), 52.53% (*Xenopus*: XLA010978), 50.62% (human: GenBank accession no. **HSU01212**), and 51.23% (rat and mouse: **RNM26926** and **MMU01213**, respectively) similarity at the amino acid level with the present sequencing. Medaka (*Oryzias latipes*) and Japanese loach (*Misgurnus anguillicaudatus*) OMPs underwent partial sequencing (Yasuoka et al., 1999 and Irie-Kushiya et al., 2004, respectively). The present sequence of salmon and other vertebrate OMPs demonstrated a marked conservation of the two domains (Fig. 2). These regions, ranging from positions 92 to 110 and from 130 to 152, were highly conserved and demonstrated a higher sequence identity of 84 and 78% within sOMP and other OMPs. Phylogenetic analysis of the full-length salmon OMP1 and -2 in comparison with other reported OMPs indicated that the salmon OMPs could be clearly segregated into the teleost OMP branch (Fig. 3).

3.3. Expression of OMP in olfactory tissues

Soluble extracts from lacustrine sockeye salmon olfactory rosettes and rat nasal cavities were resolved by SDS-PAGE (lanes 2 and 3 in Fig. 4). On western blot analysis, a new polyclonal antibody to OMP produced in this study recognized a band with a molecular mass of 20 and 19 kDa in the salmon olfactory rosettes and rat nasal cavities, respectively (lanes 4

and 5 in Fig. 4). Control immunoreaction using pre-immuno serum showed no detectable positive immunoreactivities in all cases.

Analysis of serial sections of the lacustrine sockeye salmon olfactory epithelium showed immunoreactivities for OMP in the perinuclear region, dendric cytosol, and axons of the ORNs (Fig. 5A), and signals for OMP mRNA using labeled cRNA antisense probes in the perinuclear regions of these ORNs (Fig. 5B). Hybridization with the labeled sense control probe identified no detectable hybridized signals in adjacent sections. In addition, immunoreactivities for OMP were also found in the perinuclear region, dendric cytosol, and axons of rat ORNs (Fig. 5D).

No clear differences in the localization and the signal intensity for OMP were observed between males and females in both immunohistochemistry and *in situ* hybridization examinations.

3.4. Comparisons of immunoreactivities for OMP and other marker molecules

Analysis of serial sections of the lacustrine sockeye salmon olfactory epithelium showed immunoreactivities for OMP, soGST, and PGP9.5 in the ORNs (Fig. 6). The number of ORNs positive for OMP was markedly less than those for other molecules. Furthermore, some ORNs near the basal region in the olfactory epithelia showed immunoreactivities for PGP9.5 (Fig. 6C). No distinct immunohistochemical differences for these three molecules in ORNs between males and females were detected.

Double immunofluorescence labeling of OMP and soGST in the same section of a single specimen of olfactory epithelium from lacustrine sockeye salmon showed immunoreactivities in a certain population of ORNs for either soGST (red signals) or the colocalization (yellow or yellow green signals) of both molecules (Fig. 7D). ORNs that were independently immunoreactive to OMP (green signals) were not detected in this section. Before immunoreaction for soGST, fluorescence signals were abolished in all cases. Even when the order of the primary antibody was changed, the same result was obtained.

4. Discussion

The present study isolated two subtypes of cDNA clone for a salmon OMP gene expressed in the olfactory epithelium of lacustrine sockeye salmon. These OMPs, which show high-level homology, may reflect ancestral tetraploidy in salmonid fish (Johnson et al., 1987).

Regarding this, some genes encoding salmon-expressed protein have sequences of two

subtypes (e.g., Ono et al., 1988; Harada et al., 2008). Sequence comparison demonstrated a moderate overall homology with highly conserved OMPs from other vertebrates; however, two domains emerged that are identical in all OMP subtypes. These two domains were equivalent to the “Eph2B receptor-like loop” and “ β -7 domain”, clarified by X-ray crystallography (Smith et al., 2002; Baldisseri et al., 2002). The EphB2 receptor is a tyrosine kinase that is activated by interaction with B-type ephrins, a family of closely related axonal guidance factors (Himanen et al., 1998; Cramer et al., 2006). In invertebrates, several ORN-specific marker proteins have been identified from the olfactory organs (e.g., lobster, *Homarus americanus*, Hollins et al. 2003; land snail, *Eobania vermiculata*, Mazzatenta et al. 2004). However, these amino acid and nucleotide sequences indicated that these molecules of the invertebrates were not homologous to vertebrate OMP including salmon OMP. It is likely that OMP functions in only vertebrate ORN, and it is conserved phylogenically in vertebrate. Our *in situ* hybridization and immunohistochemistry studies demonstrated the gene and protein expression of OMP in ORNs of the lacustrine sockeye salmon using specific probes and antibodies for salmon OMP, respectively. The present immunoreactivities of OMP was similar to that in rainbow trout using antibodies against mammalian OMP (Riddle and Oakley, 1992). In teleosts, two morphological distinct ORN types, ciliated and microvillous ORNs, exist according to the ultrastructure of the olfactory knob in the dendrite of the ORN (Zeiske et al., 1992). Our previous study indicated that the immunoreactivities of soGST exist in the cytoplasm of both ORN types in the lacustrine sockeye salmon by post-embedding immunoelectron microscopy (immuno-gold method; Kudo et al., 1996). The ultrastructure of the olfactory knob in the dendrite of the microvillous ORN (Yamamoto and Ueda, 1977) is very similar to that of the mammalian vomeronasal neurons (Takami et al., 2005), although functional information on the microvillous ORN is scarce in salmon (Sato and Suzuki, 2001). At the same time, the mammalian vomeronasal neurons indicated the expression of OMP (Johnson et al., 1993; Takami et al., 1993). However, OMP of zebrafish was expressed only in the ciliated ORNs, and not in the microvillous ORNs (Sato et al., 2005). Therefore, it is suitable to consider that OMP is expressed in ciliated ORN type in salmon.

The comparison among OMP, GST, and PGP9.5 in salmon olfactory epithelium demonstrated that PGP9.5-immunoreactive ORNs were the largest in number, and OMP-immunoreactive ORNs were the least common. Furthermore, double immunofluorescence labeling of OMP and GST revealed that ORNs in the olfactory epithelia could be divided into

two groups based on their distinct immunoreactivities: i) ORNs immunoreactive for both OMP and GST; and ii) ORNs immunoreactive for only GST. In mammals, OMP is abundantly expressed in mature ORNs (Farbman and Margolis, 1980), and PGP9.5 is expressed in ORNs of broad developmental stages, including immature ORNs (Taniguchi et al., 1993). In teleosts, Nile tilapia (*Oreochromis niloticus*) ORNs expressed an ubiquitin C-terminal hydrolase (EC 3.4.19.12) homologous to PGP9.5 (Mochida et al., 2002). An immunohistochemical study of GST during ontogeny indicated that GST started to be synthesized in embryonic ORNs of lacustrine sockeye salmon after the projection of olfactory nerve axons to the olfactory bulb (Yanagi et al., 2004). These findings strongly suggest that salmon OMP is expressed as well as mammalian OMP in mature ORNs, and GST is expressed following the appearance of PGP9.5 in semi-mature ORNs.

Several ORN-related proteins except for OMP and GST have been identified and applied as molecular markers to study olfactory function, including olfactory receptors (ORs: Morinishi et al., 2007), and calretinin (calcium-binding protein: Castro et al., 2008) in the olfactory system of several other salmonid fish. However, a single ORN expresses only one allele of a given OR gene (Chess et al., 1994) although the regulation of OR gene expression is unclear in the salmon ORN at present. It is difficult to discuss the condition of all ORNs based on the analyses of only a few ORs. Calretinin is promising as an ORN marker molecule in salmonid olfactory epithelia although biochemical and molecular biological information is insufficient in salmon ORNs. However, calretinin is widely distributed in various other neurons and chemosensory cells of the teleosts (Levanti et al. 2008). It seems reasonable to suppose that our salmon OMP is the most suitable as an ORN specific detectable marker because its gene expression has been proven in salmon olfactory epithelium.

In conclusion, we performed molecular cloning and verified the expression of salmon OMP in salmon olfactory epithelium. Salmon OMP has been confirmed to be a useful molecular marker for the detection of ORNs in the salmon olfactory system.

Acknowledgements

This study was supported in part by Grant-in-Aids for Scientific Research (C) from the Japan Society for the Promotion of Science (JSPS; No. 2058018808), and for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT; No. 17710049).

References

- Baker, H., Grillo, M., Margolis, F.L., 1989. Biochemical and immunocytochemical characterization of olfactory marker protein in the rodent central nervous system. *J. Comp. Neurol.* 285, 246-261.
- Baldisseri, D.M., Margolis, J.W., Weber, D.J., Koo, J.H., Margolis, F.L., 2002. Olfactory marker protein (OMP) exhibits a β -clam fold in solution: Implications for target peptide interaction and olfactory signal transduction. *J. Mol. Biol.* 319, 823-837.
- Buiakova, O.I., Baker, H., Scott, J.W., Farbman, A.I., Kream, R., Grillo, M., Franzen, L., Richman, M., Davis, L.M., Abbondanzo, S., Stewart, C.L., Margolis, F.L., 1996. Olfactory marker protein (OMP) gene deletion causes altered physiological activity of olfactory sensory neurons. *Proc. Natl. Acad. Sci. USA.* 93, 9858-9863.
- Castro, A., Becerra, M., Anadón, R., Manso, M.J., 2008. Distribution of calretinin during development of the olfactory system in the brown trout, *Salmo trutta fario*: Comparison with other immunohistochemical markers. *J. Chem. Neuroanat.* 35, 306-316.
- Celik, A., Fuss, S.H., Korsching, S., 2002. Selective targeting of zebrafish olfactory receptor neurons by the endogenous OMP promoter. *Eur. J. Neurosci.* 15, 798-806.
- Chess, A., Simon, I., Cedar, H., Axel, R., 1994. Allelic inactivation regulates olfactory receptor gene expression. *Cell*, 78, 823-834.
- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.
- Cramer, K.S., Cerretti, D.P., Siddiqui, S.A., 2006. EphB2 regulates axonal growth at the midline in the developing auditory brainstem. *Dev. Biol.* 295, 76-89.
- Farbman, A.I., Margolis, F.L., 1980. Olfactory marker protein during ontogeny: immunohistochemical localization. *Dev. Biol.* 74, 205-215.
- Ferrando, A., Bottaro, M., Gallus, L., Giroi, L., Vacchi, M., Tagliafierro, G., 2007. First detection of olfactory marker protein (OMP) immunoreactivity in the olfactory epithelium of a cartilaginous fish. *Neurosci. Lett.* 413, 173-176.
- Harada, M., Yoshinaga, T., Ojima, D., Iwata, M., 2008. cDNA cloning and expression analysis of thyroid hormone receptor in the coho salmon *Oncorhynchus kisutch* during smoltification. *Gen. Comp. Endocrinol.* 155, 658-667.

- Himanen, J.P., Henkemeyer, M., Nikolov, D.B., 1998. Crystal structure of the ligand-binding domain of the receptor tyrosine kinase EphB2. *Nature* 396, 486-491.
- Hollins, B., Hardin, D., Gimelbrant, A.A., McClintock, T.S., 2003. Olfactory-enriched transcripts are cell-specific marker in the lobster olfactory organ. *J. Comp. Neurol.* 455, 125-138.
- Irie-Kushiyama, S., Asano-Miyoshi, M., Suda, T., Abe, K., Emori, Y., 2004. Identification of 24 genes and two pseudogenes coding for olfactory receptors in Japanese loach, classified into four subfamilies: a putative evolutionary process for fish olfactory receptor genes by comprehensive phylogenetic analysis. *Gene* 325, 123-135.
- Ivic, L., Pyrski, M.M., Margolis, J.W., Richards, L.J., Firestein, S., Margolis, F.L., 2000. Adenoviral vector-mediated rescue of the OMP-null phenotype *in vivo*. *Nat. Neurosci.* 3, 1113-1120.
- Johnson, E.W., Eller, P.M., Jafek, B.W., 1993. An immuno-electron microscopic comparison of olfactory marker protein localization in the supranuclear regions of the rat olfactory epithelium and vomeronasal organ neuroepithelium. *Acta Oto-laryngol.* 113, 766-771.
- Johnson, K.R., Wright, J.E., May, B., 1987. Linkage relationships reflecting ancestral tetraploidy in salmonid fish. *Genetics* 116, 579-591.
- Keller, A., Margolis, F.L., 1975. Immunological studies of the rat olfactory marker protein. *J. Neurochem.* 24, 1101-1106.
- Kudo, H., Ueda, H., Yamauchi, K., 1996. Immunocytochemical investigation of a salmonid olfactory system-specific protein in the kokanee salmon (*Oncorhynchus nerka*). *Zool. Sci.* 13, 647-653.
- Kudo, H., Ueda, H., Mochida, K., Adachi, S., Hara, A., Nagasawa, H., Doi, Y., Fujimoto, S., Yamauchi, K., 1999. Salmonid olfactory system-specific protein (N24) exhibits glutathione S-transferase class pi-like structure. *J. Neurochem.* 72, 1344-1352.
- Kudo, H., Doi, Y., Nishino, T., Nara, S., Hamasaki, K., Fujimoto, S., 2000. Dietary zinc deficiency decreases glutathione S-transferase expression in the rat olfactory epithelium. *J. Nutr.* 130, 38-44.
- Levanti, M.B., Montalbano, G., Laurà, R., Ciriaco, E., Cobo, T., Garcia-Suarez, O., Germanà, A., Vega, J.A., 2008. Calretinin in the peripheral nervous system of the adult zebrafish. *L. Anat.* 212, 67-71.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265-275.
- Mazzatenta, A., Pelosi, P., Cellerino, A., 2007. Cloning of an olfactory sensory neuron-specific protein in the land snail (*Eobania vermiculata*). *Proc. R. Soc. Lond. B* 271 (Suppl.), S46-S49.
- Mochida, K., Matsubara, T., Kudo, H., Andoh, T., Ueda, H., Adachi, S., Yamauchi, K., 2002. Molecular cloning and immunohistochemical localization of ubiquitin C-terminal hydrolase

- expressed in testis of a teleost, the Nile Tilapia, *Oreochromis niloticus*. J. Exp. Zool. 293, 368-383.
- Morinishi, F., Shiga, T., Suzuki, N., Ueda, H., 2007. Cloning and characterization of an odorant receptor in five Pacific salmon. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 148, 329-336.
- Nishino, T., Kudo, H., Doi, Y., Maeda, M., Hamasaki, K., Morita, M., Fujimoto, S., 2001. Immunocytochemistry of glutathione S-transferase in taste bud cells of rat circumvallate and foliate papillae. Chem. Senses 26, 179-188.
- Ono, M., Wada, C., Oikawa, I., Kawazoe, I., Kawauchi, H., 1988. Structures of two kinds of mRNA encoding the chum salmon melanin-concentrating hormone. Gene 71, 433-438.
- Riddle, D.R., Oakley, B., 1992. Immunocytochemical identification of primary olfactory afferents in rainbow trout. J. Comp. Neurol. 324, 575-589.
- Saitoh, N., Nei, M., 1987. The neighbor-joining method: new method for reconstructing phylogenetic tree. Mol. Biol. Evol. 4, 406-425.
- Sato, K., Suzuki, N., 2001. Whole-cell response characteristics of ciliated and microvillous olfactory receptor neurons to amino acids, pheromone candidates and urine in rainbow trout. Chem. Senses 26, 1145-1156.
- Sato, Y., Miyasaka, N., Toshihara, Y., 2005. Mutually exclusive glomerular innervation by two distinct types of olfactory sensory neurons revealed in transgenic zebrafish. J. Neurosci. 25, 4889-4897.
- Shimizu, M., Kudo, H., Ueda, H., Hara, A., Shimazaki, K., Yamauchi, K., 1993. Identification and immunological properties of an olfactory system-specific protein in kokanee salmon (*Oncorhynchus nerka*). Zool. Sci. 10, 287-294.
- Smith, P.C., Firestein, S., Hunt, J.F., 2002. The crystal structure of the olfactory marker protein at 2.3 Å resolution. J. Mol. Biol. 319, 807-821.
- Stabell, O.B., 1992. Olfactory control of homing behaviour in salmonids. In: Hara, T.J. (Ed), Fish Chemoreception, Chapman and Hall, London, pp. 249-270.
- Starcevic, S.L., Zielinski, B.S., 1995. Immunohistochemical localization of glutathione S-transferase pi in rainbow trout olfactory receptor neurons. Neurosci. Lett. 183, 175-178.
- Starcevic, S.L., Zielinski, B.S., 1997. Glutathione and glutathione S-transferase in the rainbow trout olfactory mucosa during retrograde degeneration and regeneration of the olfactory nerve. Exp. Neurol. 146, 331-340.
- Takami, S., Getchell, M.L., Chen, Y., Monti-Bloch, L., Berliner, D.L., Stensaas, L.J., Getchell, T.V., 1993. Vomeronasal epithelial cells of the adult human express neuron-specific molecules. Neuroreport 4, 375-378.

- Takami, S., Iwai, T., Hasegawa, R., Nishiyama, F., 2005. Ultrastructural localization of alpha-galactose-containing glycoconjugates in the rat vomeronasal organ. *J. Neurocytol.* 34, 123-133.
- Taniguchi, K., Saito, H., Okamura, M., Ogawa, K., 1993. Immunohistochemical demonstration of protein gene product 9.5 (PGP9.5) in the primary olfactory system of the rat. *Neurosci. Lett.* 156, 6-24.
- Tierney, K.B., Sampson, J.L., Ross, P.S., Sekela, M.A., Kennedy, C.J., 2008. Salmon olfaction is impaired by an environmentally realistic pesticide mixture. *Environ. Sci. Technol.* 42, 4996-5001.
- Yamamoto, M., Ueda, K., 1977. Comparative morphology of fish olfactory epithelium Salmoniforms. *Bull. Jpn. Soc. Sci. Fish.* 43, 1163-1174.
- Yanagi, S., Kudo, H., Doi, Y., Yamauchi, K., Ueda, H., 2004. Immunohistochemical demonstration of salmon olfactory glutathione S-transferase class pi (N24) in the olfactory system of lacustrine sockeye salmon during ontogenesis and cell proliferation. *Anat. Embryol.* 208, 231-238.
- Yasuoka, A., Endo, K., Asano-Miyoshi, M., Abe, K., Emori, Y., 1999. Two subfamilies of olfactory receptor genes in medaka fish, *Oryzias latipes*: Genomic organization and differential expression in olfactory epithelium. *J. Biochem.* 126, 866-873.
- Yoshida, T., Ito, A., Matsuda, N., Mishina, M., 2002. Regulation by protein kinase A switching of axonal pathfinding of zebrafish olfactory sensory neurons through the olfactory placode-olfactory bulb boundary. *J. Neurosci.* 22, 4964-4972.
- Youngentob, S.L., Margolis, F.L., 1999. OMP gene deletion causes an elevation in behavioral threshold sensitivity. *Neuroreport* 10, 15-19.
- Youngentob, S.L., Margolis, F.L., Youngentob, L.M., 2001. OMP gene deletion results in an alteration in odorant quality perception. *Behav. Neurosci.* 115, 626-631.
- Zeiske, E., Theisen, B., Breuker, H., 1992. Structure, development and evolutionary aspects of the peripheral olfactory system. In: Hara, T.J. (Ed), *Fish Chemoreception*, Chapman and Hall, London, pp. 13-39.

Table 1. Primer Sets Used for RT-PCR, and 5' - and 3' -RACE for Sequence Analyses of Lacustrine Sockeye Salmon, *Oncorhynchus nerka*, OMP1 and -2.

Primer name	Sequence	Nucleotide position in OMP1
OMPdn-F	5'-TTNATGACNCGNCAGCT-3'	391-407
OMPdn-R	5'-AGRAARTACATVACYTTKC-3'	527-545
OMP1sp-5	5'-GTACATGACCTTGCGGACCTTGGCCAGCTC-3'	511-540
OMP1sp-3	5'-GATGACCCGGCAGCTTCTAGAACCCGTCGG-3'	393-422
OMP2sp-5	5'-GTTACCCATAGACTTCCAGTCATTGCCCCGA-3'	814-843 ^a

^a Nucleotide position in OMP2, OMP: olfactory marker protein, RACE: rapid amplification of cDNA ends.

Captions to figures

Fig. 1. Nucleotide and deduced amino acid sequences of the lacustrine sockeye salmon, *Oncorhynchus nerka*, cDNA of OMPs. The putative polyadenylation signal is in bold. The positions of degenerate PCR primers are underlined.

Fig. 2. Alignments of amino acid sequences of the OMPs of the lacustrine sockeye salmon, *Oncorhynchus nerka*, and those of other vertebrates (Zebrafish, *Danio rerio*; Fugu, *Takifugu rubripes*; Medaka, *Oryzias latipes*; Loach, *Misgurnus anguillicaudatus*; Xenopus, *Xenopus laevis*; Human, *Homo sapiens*; Rat, *Rattus norvegicus*; Mouse, *Mus musculus*). Sequences are positioned to yield maximum homology. Amino acids identical between salmon OMP1 and other OMPs are shown by dashes (-). The synthesized peptide sequence for the antigen of salmon OMP antibody is shaded in the salmon OMP1 (92-110). The region of Eph2B receptor-like loop is boxed (94-110).

Fig. 3. Evolutionary relationships between the known OMPs. The phylogenetic tree was constructed using the neighbor-joining method. Comparisons were made with amino acid sequences of salmon, *Oncorhynchus nerka*, 1 (GenBank accession no. **AB490250**) and 2 (**AB490251**), zebrafish, *Danio rerio*, 1 (GenBank accession no. **AB073551**) and 2 (**AF457189**), fugu (*Takifugu rubripes*, Ensembl Translation ID. **SINFRUP00000150379**), *Xenopus laevis* 1 (GenBank accession no. **XLA010978**) and 2 (**XLA010979**), human (*Homo sapiens*, **HSU01212**), rat (*Rattus norvegicus*, **RNM26926**), and mouse (*Mus musculus*, **MMU01213**). The values are bootstrap probabilities estimated by 1000 replications. The horizontal line indicates the genetic distance.

Fig. 4. SDS-PAGE (1-3) and western blotting (4 and 5) of soluble extracts in lacustrine sockeye salmon, *Oncorhynchus nerka*, olfactory rosettes (2 and 4), and rat, *Rattus norvegicus*, nasal cavities (3 and 5). Anti-salmon OMP antibody (1:500) recognizes a band with a molecular weight of 20 kDa (upper arrow) in the salmon olfactory rosettes, and 19 kDa (lower arrow) in the rat nasal cavities. Positions of low-molecular-weight markers (1), expressed in kDa, are indicated on the left side of the figure.

Fig. 5. Immunohistochemical localization of OMP in the olfactory epithelium of lacustrine sockeye salmon, *Oncorhynchus nerka* (A). Adjacent sections show expressions of OMP mRNA demonstrated by *in situ* hybridization (B) and Delafield's hematoxylin and eosin staining (C). Immunohistochemical localization of OMP using anti-salmon OMP antibody in rat, *Rattus norvegicus*, olfactory epithelium (D). Bars: 20 μ m.

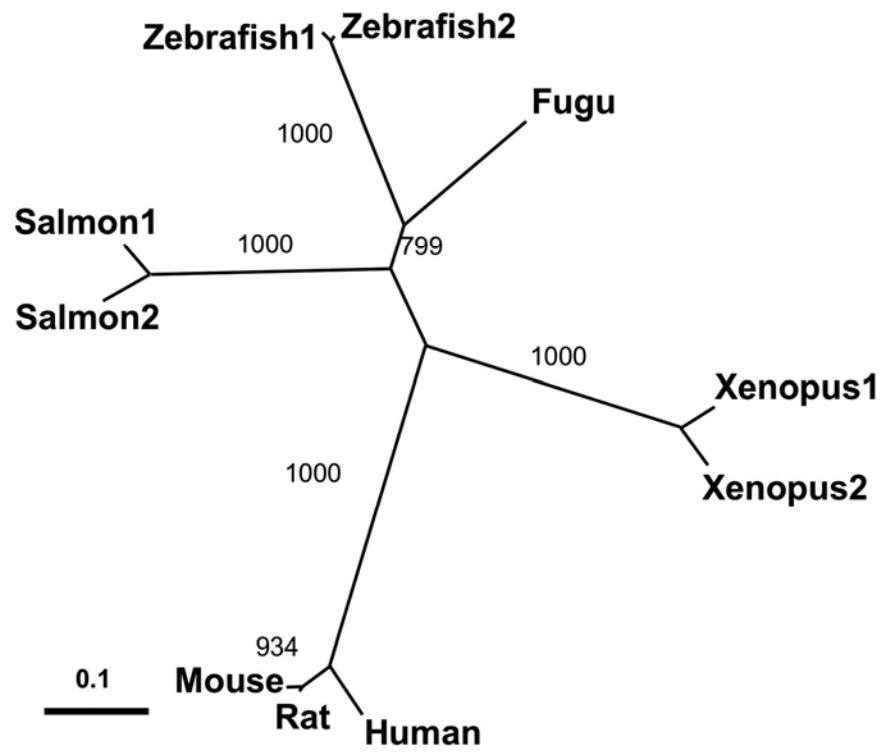
Fig. 6. Serial sections of the olfactory epithelium of lacustrine sockeye salmon, *Oncorhynchus nerka*. Immunoreactivities for OMP (A), salmon olfactory glutathione S-transferase pi (soGST; B), and protein gene product 9.5 (PGP9.5; C). Delafield's hematoxylin and eosin staining (D). Bar: 50 μ m.

Fig. 7. Double immunofluorescence labeling of soGST (red signal; A) and OMP (green signal; B) in the olfactory epithelium of lacustrine sockeye salmon, *Oncorhynchus nerka*. Blue signals indicate nuclei by DAPI staining (C). Combined double-labeled image of OMP and soGST shown in (B) and (A). The yellow or yellow green signal reflects overlapping of immunoreactivities for OMP and soGST (D). Bar: 50 μ m.

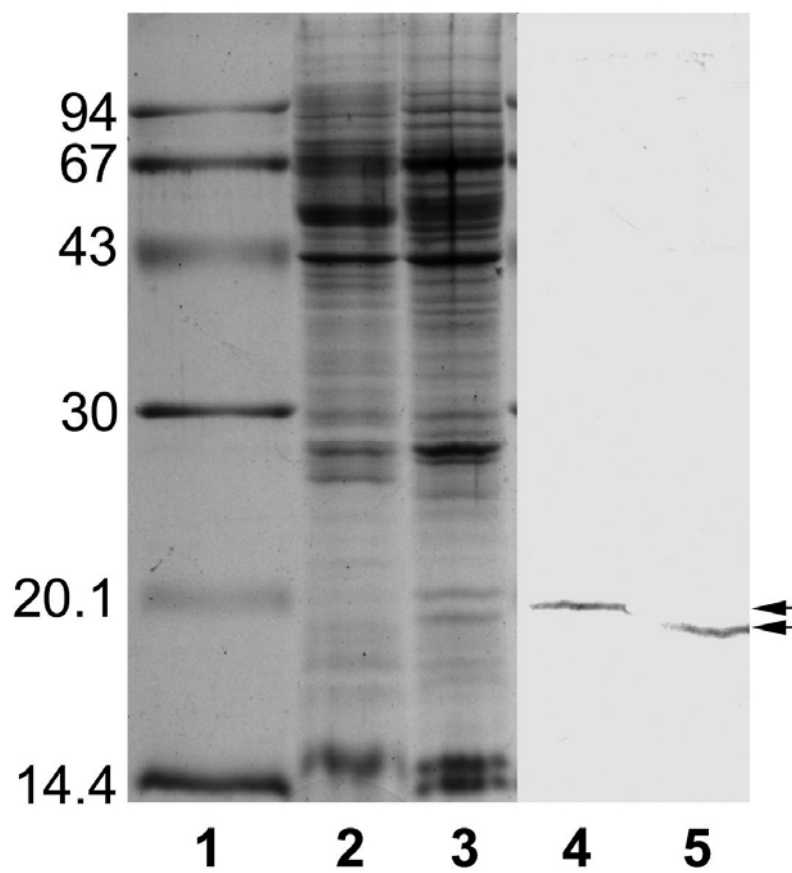
Kudo *et al.*
Fig. 1

		10	20	30	40	50	
Salmon1	1	MSSQMYTELS	AAPSSGSALE	LRFAEDTSLT	EVMRLRVQSL	QRSGQRRQEG	50
Salmon2	1	----TG-DPA	-----M-	-----K-----	-----K---D-		50
Zebrafl	1	M-	--	-T-NP-VQ--	-M-----	-QR--K---D-	50
Fugu	1		--E -Q	-P-RP-NQ--	-----	-QR--KK-D-	50
Medaka	1						50
Loach	1						50
Xenopus	1	MA SET-EM	-	-P-I---Q--	KC--I---T-	-QKNAKP---	50
Human	1	MA EDRPQQPQ-D	MPLVL-QG--	RQ-----E--	KQR-EK--D-		50
Rat	1	MA EDGPQKQQ-D	MPLVL-QD--	KQ-----E--	KQR-EKK-D-		50
Mouse	1	MA EDGPQKQQ--	MPLVL-QD--	QQ-----E--	KQR-EKK-D-		50
		60	70	80	90	100	
Salmon1	51	ERLLLPHAEV	YRLDFANQE	LTFVHWSVSL	IGNGRVTVTG	ISQLWTPDLT	100
Salmon2	51	-----	-C---S--K	-----	-----I--	-----	100
Zebrafl	51	----KSN-H-	-S---SE-A	-H-TC-NICI	SSP--LNIIA	T-----	100
Fugu	51	----R-N---	-----PT-V	-R-SR-M-R-	ARS--LTI-A	T-----	100
Medaka	51					-----	100
Loach	51						100
Xenopus	51	-M--RAN-YI	--V--SK-K	-R-LW-K-H-	KSP-K-MI--	T--H-----	100
Human	51	-K--Q-A-S-	---N-TQ-QR	-Q-ER-N-V-	DKP-K--I--	T--N-----	100
Rat	51	-K--R-A-S-	-----IQ-QK	-Q-D--N-V-	DKP-K--I--	T--N-----	100
Mouse	51	-K-IR-A-S-	-----IQ-QK	-Q-D--N-V-	DKP-K--I--	T--N-----	100
		110	120	130	140	150	
Salmon1	101	HLMTRQLLEP	VGTFWRNAGD	PEDTPLKCLE	ADIQEFGERI	AELAKVRKVM	150
Salmon2	101	-----	-----	L-----	-----	-----	150
Zebrafl	101	-----	T-L---S- -	D-NIQ -Y-	--A-----	-----	150
Fugu	101	N-----	A-S---GQ-E	ACG-VVQ-Y-	--A-----	-----	150
Medaka	101	N-----	I-----E-	--ES-L----	--M-----	-----I----	150
Loach	101	-----	I-Q-----	---S-I----	-----	--M--I----	150
Xenopus	101	N-----	SAV-YKKDAN	D-VE -N-	--A-----	-----I----	150
Human	101	N-----D-	TAI---KEDS	DAID WN-	--AL-----L	SD---I----	150
Rat	101	N-----D-	AAI---KEDS	DAMD WN-	--AL-----L	SD---I----	150
Mouse	101	N-----D-	AAI---KEDS	DAMD WN-	--AL-----L	SD---I----	150
		160	170	180	190	200	
Salmon1	151	YFLFAFKEGA	EADKVSISVE	FNQA			200
Salmon2	151	---L-----	-----M-	----			200
Zebrafl	151	-----ED-L	SPES-EC-I-	-QTSK			200
Fugu	151	-----ED-C	SPETVDSCIT	-			200
Medaka	151	--					200
Loach	151	-					200
Xenopus	151	--VIT-LD--	DPATIEC-IG	-RA			200
Human	151	---VT-G--V	-PANLKA--V	---L			200
Rat	151	---IT-G--V	-PANLKA--V	---L			200
Mouse	151	---IT-G--V	-PANLKA--V	---L			200

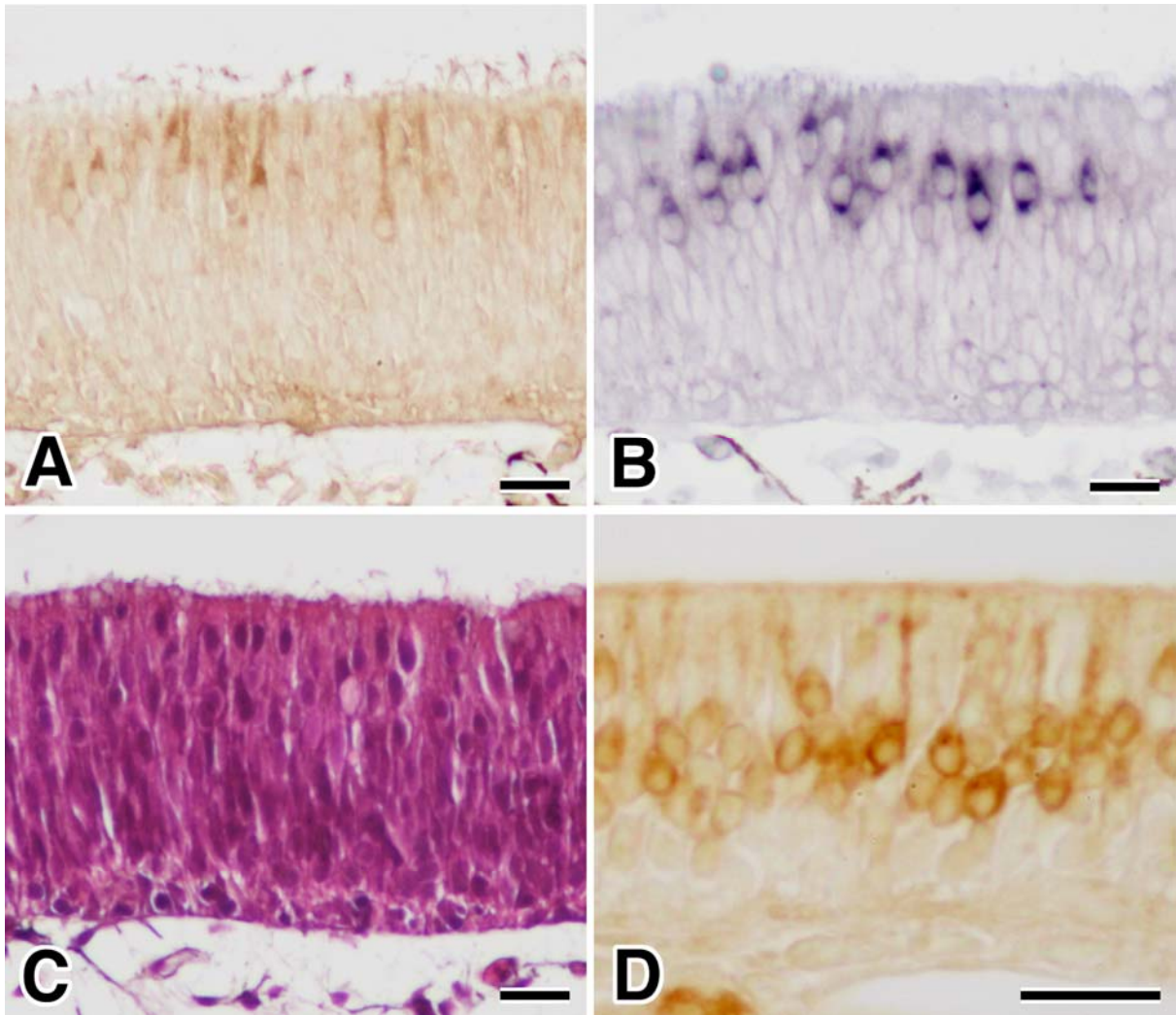
Kudo et al.
Fig. 2



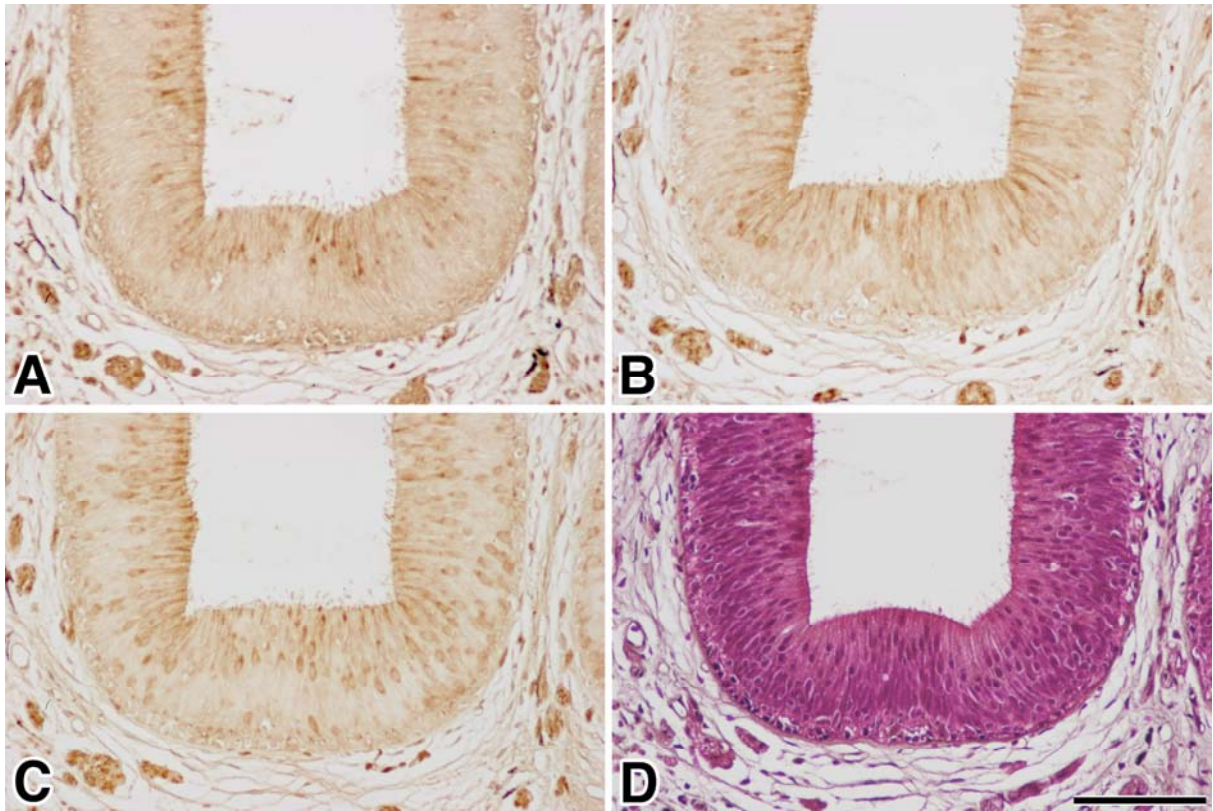
Kudo *et al.*
Fig. 3



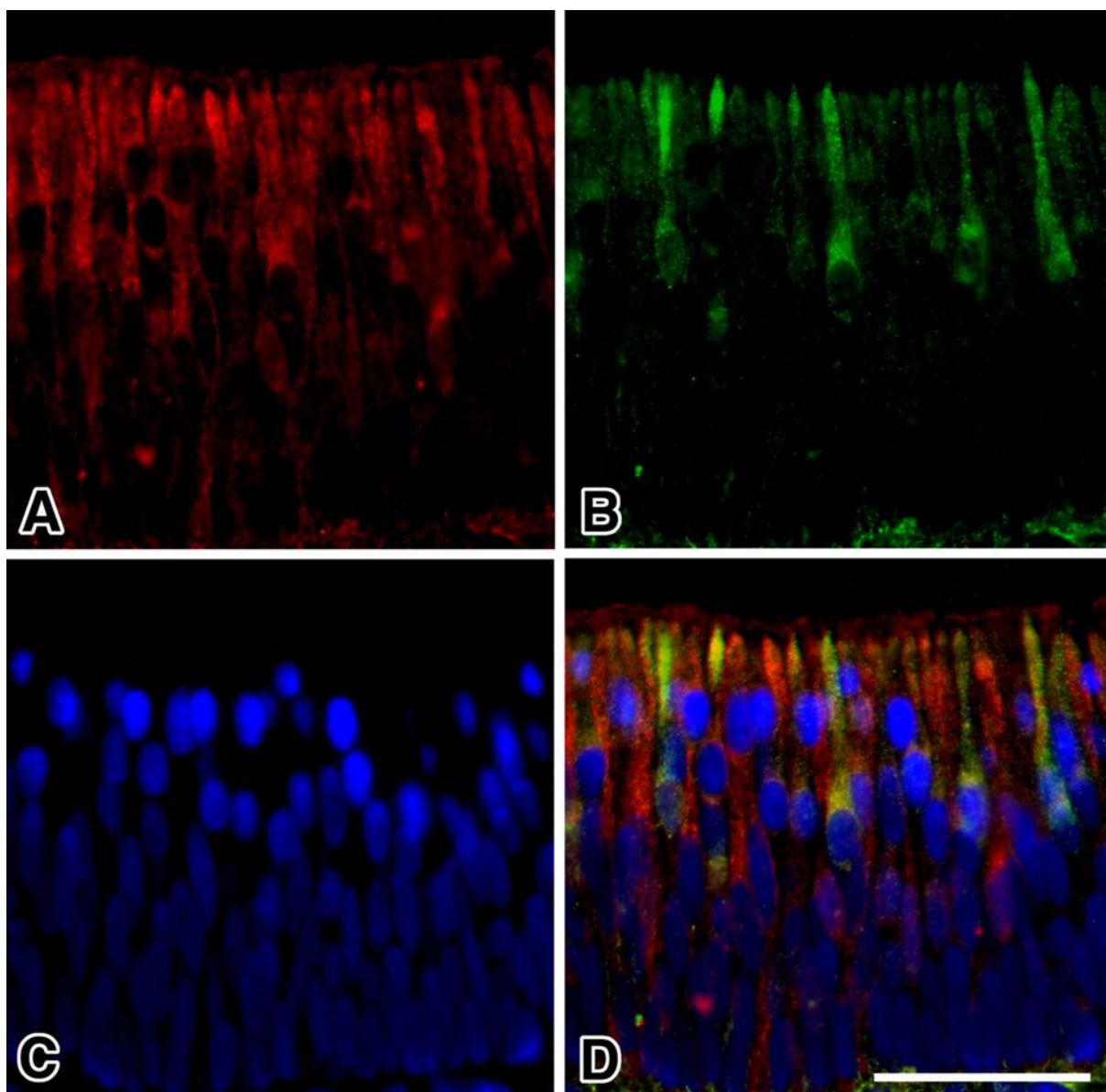
Kudo *et al.*
Fig. 4



Kudo *et al.*
Fig. 5



Kudo *et al.*
Fig. 6



Kudo *et al.*
Fig. 7