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Molecular and pharmacological characterization of zebrafish '*relaxant*' prostanoid receptors

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ABSTRACT

Prostanoids comprising prostaglandins (PGs) and thromboxanes have been shown to play physiological and pathological roles in zebrafish. However, the molecular basis of zebrafish prostanoid receptors has not been characterized to date. Here, we demonstrate that there exist at least six '*relaxant*' (Gs-coupled) prostanoid receptors in zebrafish; one PGI_2 receptor IP and five PGE_2 receptors comprising two EP2 (EP2a and EP2b), and three EP4 receptors (EP4a, EP4b and EP4c). In contrast, we failed to find a zebrafish PGD_2 receptor with any structure and/or character similarities to the mammalian DP1 receptor. [^{3}H]iloprost, a stable IP radioligand, specifically bound to the membrane of cells expressing zebrafish IP with a *Kd* of 42 nM, and [^{3}H]PGE_2 specifically bound to the membranes of cells expressing zebrafish EP2a, EP2b, EP4a, EP4b and EP4c with a *Kd* of 6.9, 6.0, 1.4, 3.3 and 1.2 nM, respectively. Upon agonist stimulation, the '*relaxant*' prostanoid receptors showed intracellular cAMP accumulation. The responsiveness of these zebrafish receptors to subtype-specific agonists correlated with their structural conservation to the corresponding receptor in mammals. RT-PCR analysis revealed that the six zebrafish prostanoid receptors show unique tissue distribution patterns; each receptor gene may hence be under unique transcriptional regulation. This work provides further insights into the diverse functions of prostanoids in zebrafish.

1. Introduction

Prostanoids comprising prostaglandins (PGs) and thromboxanes (TXs) are arachidonate metabolites synthesized by cyclooxygenase (COX) as the rate-limiting enzyme. The diverse actions of prostanoids are mediated by membrane-bound receptors on neighboring cells [1]. In mammals, there exist eight types and subtypes of prostanoid receptors; DP for PGD_2, FP for $PGF_{2\alpha}$, IP for PGI_2, TP for TXA_2, and four EP subtypes (EP1, EP2, EP3 and EP4) for PGE_2 [2–4]. The prostanoid receptors are sub-grouped into three clusters on the basis of their structure, signal transduction and actions: '*contractile*', '*relaxant*', and '*inhibitory*' receptors [5,6]. The '*contractile*' receptors consist of EP1, FP and TP, which mediate Ca^{2+} mobilization and induce smooth muscle contraction. The '*relaxant*' receptors, which consist of DP, IP, EP2 and EP4, mediate increase in cAMP and induce smooth muscle relaxation. EP3 is an '*inhibitory*' receptor that mediates decrease in cAMP and inhibits smooth muscle relaxation. Indeed, sequence homology among these functionally related receptors is higher than those between the receptors from the three separate clusters. Molecular evolution analysis suggested that the COX pathway was first initiated as a system composed of PGE and its receptor, and the PGE receptor subtypes then evolved from this primitive PGE receptor to mediate different signal transduction pathways, and subsequently the receptors for the other PGs and TXs evolved from functionally related PGE receptor subtypes by gene duplication [5,7].

Zebrafish is a vertebrate model organism that has been widely used for genetic and pharmacological analyses of embryogenesis because its fertilization and embryo development occur outside the maternal body under a transparent condition [8–10]. Furthermore, many disease models have been developed in zebrafish, and such models in combination with *in vivo* imaging of particular cells enabled the monitoring of specific pathological processes such as cardiovascular disease and cancer invasion [11,12]. Indeed, it has been demonstrated by using zebrafish as a model that prostanoids play critical roles in developmental processes such as gastrulation and hematopoietic stem cell expansion [13–16]. Moreover, it was recently suggested in a zebrafish model that leukocyte-derived PGs exert a trophic effect on tumor invasion [17].

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Nevertheless, there is no literature to date that systematically characterizes the pharmacological properties of zebrafish prostanoid receptors. Here, we characterized the pharmacological and signal transduction properties of the zebrafish '*relaxant*' prostanoid receptors. Such molecular knowledge will assist not only our understanding of the molecular evolution of prostanoid receptors, but also contribute towards the discovery of novel PG actions and mechanisms in embryogenesis and disease progression.

2. Materials and methods

2.1. Materials

The following materials were obtained from the sources indicated: [^{3}H]PGE_2 and [^{3}H]iloprost from PerkinElmer (Waltham, MA), PGD_2, PGE_2, $PGF_{2\alpha}$, iloprost, carbacyclin, cicaprost and U-46619 from Cayman Chemical (Ann Arbor, MI), cyclic AMP kit from Yamasa (Choshi, Japan). ONO-AE1-259 (an EP2-specific agonist), ONO-AE1-329 (an EP4-specific agonist) and ONO-AE3-208 (an EP4-specific antagonist) were generous gifts from Ono Pharmaceutical Co. (Osaka, Japan) [18,19]. All other chemicals were commercial products of reagent grade.

2.2. cDNA cloning

Total RNA was isolated from zebrafish embryos at 24 h post fertilization (h.p.f.), and cDNAs were synthesized using SuperScript III (Invitrogen, San Diego, CA) and an oligo (dT) primer, and used as a template for PCR. The coding regions of the '*relaxant*' prostanoid receptors were amplified and cloned into the pTA2 vector (TOYOBO, Osaka, Japan). Primer sequences used in the PCR are shown in Table S1. The cDNAs were then subcloned into the hemagglutinin- (HA-) tagged pcDNA3 expression vector. The resultant cDNA constructs were verified by dideoxy sequencing.

2.3. Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated (56 °C for 30 min) fetal bovine serum at 37 °C in a fully humidified 5% CO_2 atmosphere. Cells were transfected with each cDNA construct using FuGENE HD (Promega, Madison, WI) according to the manufacturer's instructions.

2.4. Binding assay

cDNA-transfected cells were harvested and homogenized in 0.25 M sucrose containing 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation at 100,000 × *g* for 1 h, the pellet was suspended in 20 mM 2-(N-morpholino) ethanesulfonic acid (pH 6.0) containing 10 mM $MgCl_2$, 1 mM EDTA and was used for the binding assay as crude membranes. The membranes (30 μg) were incubated with 20 nM [^{3}H]iloprost or 4 nM [^{3}H]PGE_2 with various concentrations of unlabeled ligands for 1 h. The reaction was terminated by addition of ice-cold K–P buffer (1.32 mM K_2HPO_4, 8.68 mM KH_2PO_4, 10 mM $MgCl_2$, 1 mM EDTA), and the mixture was rapidly filtered through a Unifilter-96-GF/C (Whatman, Florham Park, NJ). Radioactivity was measured using a LS6500 scintillation counter (Beckman, Miami, FL). The specific binding was calculated by subtracting the nonspecific binding from the total binding.

2.5. Measurement of cAMP formation

cDNA-transfected COS-7 cells were preincubated with HEPES-buffered saline consisting of 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, 15 mM HEPES (pH 7.4) and 100 μM indomethacin for 10 min at 37 °C. Reactions were started by the addition of test reagents along with 100 μM indomethacin and 100 μM Ro-20-1724 (an inhibitor of type IV phosphodiesterase). After incubation at 37 °C for 10 min, the reaction was terminated by the addition of ice-cold 10% trichloroacetic acid. The content of cAMP was measured by radioimmunoassay using a cAMP assay kit (Yamasa).

2.6. RT-PCR

Total RNA was extracted from various tissues in adult zebrafish with Sepasol RNA I Super G (Nacalai Tesque, Kyoto, Japan), subjected to the RT reaction with PrimeScript RT Master Mix (Takara Bio, Shiga, Japan), and subjected to PCR with Quick Taq HS DyeMix (Toyobo, Osaka, Japan). Primer sequences for each gene are shown in Table S2. The specificity of the PCR products was confirmed by gel electrophoresis.

2.7. Molecular phylogenetic analysis

A phylogenetic tree was constructed with amino acid sequences of zebrafish and human '*relaxant*' prostanoid receptors using the "AllAll program" at The Computational Server at Eidgenössische Technische Hochschule Zürich (ETHZ) (http://www.cbrg.ethz.ch/services/AllAll).

2.8. Statistical analysis

Data are represented as means ± SEM of at least three independent experiments. Two sets of data were compared by Student's *t* test. $P < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Molecular cloning of cDNAs for zebrafish 'relaxant' prostanoid receptors

To obtain functional cDNAs for Gs-coupled prostanoid receptors in zebrafish, we searched for zebrafish cDNA sequences showing high homology to the human '*relaxant*' prostanoid receptors from the NCBI database. We identified six different sequences as candidates for cDNAs encoding zebrafish Gs-coupled prostanoid receptors; GI:292621348, GI:82659770, GI:71834635, GI:88900452, GI:190358593 and GI:189515727. We amplified full-length cDNAs corresponding to GI:292621348, GI:82659770, GI:71834635, GI:88900452 and GI:190358593, cloned them into an expression vector, and named them as zPRS1 (zebrafish prostanoid receptor, Gs-coupled-1), zPRS2, zPRS3, zPRS4 and zPRS5, respectively (Table S3). The cDNA sequence deposited as GI:189515727 appears to lack the N-terminal region of the coding region, since the predicted amino acid sequence had only six hydrophobic regions. We therefore searched for an 'in-frame' ATG in the 5′-upstream adjacent region of the zebrafish gene, and found a 'likely' initiation codon. Indeed, we successfully amplified this hypothetically full-length cDNA from the first strand pool of the zebrafish embryo, cloned it into an expression vector, and named the cDNA as zPRS6 (Genbank accession No.: AB776993).

3.2. Ligand binding properties of zebrafish 'relaxant' prostanoid receptors

To identify their endogenous ligands, we transfected the full-length cDNA of each zebrafish '*relaxant*' prostanoid receptor (zPRS1–zPRS6) into COS-7 cells, and the membranes were subjected to a binding assay. The membranes of zPRS1-transfected cells showed specific binding only for [^{3}H]iloprost (a radio-labeled stable PGI_2 analogue). The *Kd* value of zPRS1-transfected cell membranes for [^{3}H]iloprost was 42.2 nM, which is comparable to the *Kd* values of mammalian IP receptors [20,21]. In contrast, cell membranes transfected with all five other receptor cDNAs showed specific binding activities to [^{3}H]PGE_2. The *Kd* values of zPRS2-, zPRS3-, zPRS4-, zPRS5-, and zPRS6-transfected cell membranes for [^{3}H]PGE_2 were 6.9, 6.0, 1.4, 3.3 and 1.2 nM, respectively. All these values are also comparable to the *Kd* values reported for mammalian EP receptors [22–25]. Only negligible levels of [^{3}H]iloprost or [^{3}H]PGE_2 binding were found in the membranes of the mock-transfected cells (data not shown). We next examined the ligand specificities of [^{3}H]iloprost binding (zPRS1) or [^{3}H]PGE_2 binding (zPRS2-6) (Fig. 1 and Fig. S1). The specific [^{3}H]iloprost binding to the zPRS1-transfected cell membranes was inhibited by unlabeled iloprost (IC_{50}, 1×10^{-8} M). The other PG analogues scarcely inhibited this binding even at 10^{-6} M (Fig. 1A). The specific [^{3}H]PGE_2 binding to zPRS2-transfected cell membranes was displaced most potently by PGE_2 (IC_{50}, 5×10^{-9} M), while the other PG analogues hardly inhibited this binding up to 10^{-6} M (Fig. 1B). The [^{3}H]PGE_2 binding activities to both zPRS3- and zPRS6-transfected cell membranes were inhibited most potently by PGE_2 (IC_{50} for zPRS3 and zPRS6, 5×10^{-9} and 1×10^{-8} M, respectively), and were also displaced by carbacyclin (a stable IP agonist) (IC_{50} for both, 3×10^{7} M), but they were hardly inhibited by the other PG analogues up to 10^{-6} M (Fig. 1, C and F). The specific [^{3}H]PGE_2 binding to zPRS4-transfected cell membranes was inhibited most potently by PGE_2 (IC_{50}, 3×10^{-9} M), modestly by carbacyclin (IC_{50}, 3×10^{-7} M), and weakly by $PGF_{2\alpha}$ (IC_{50}, 1×10^{-6} M), but hardly by PGD_2 and U-46619 (a stable TP agonist) (Fig. 1D). The specific [^{3}H]PGE_2 binding to zPRS5-transfected cell membranes was displaced most potently by PGE_2 (IC_{50}, 3×10^{-9} M), and modestly by carbacyclin (IC_{50}, 1×10^{-6} M), but was hardly inhibited by the other PG analogues up to 10^{-6} M (Fig. 1E). These results suggest that zPRS1 encodes the zebrafish IP receptor and zPRS2, zPRS3, zPRS4, zPRS5 and zPRS6 encode zebrafish EP receptors.

We next investigated whether IP agonists designed for the mammalian IP receptor are able to bind to zPRS1-transfected cell membranes, presumably expressing the zebrafish IP receptor. The

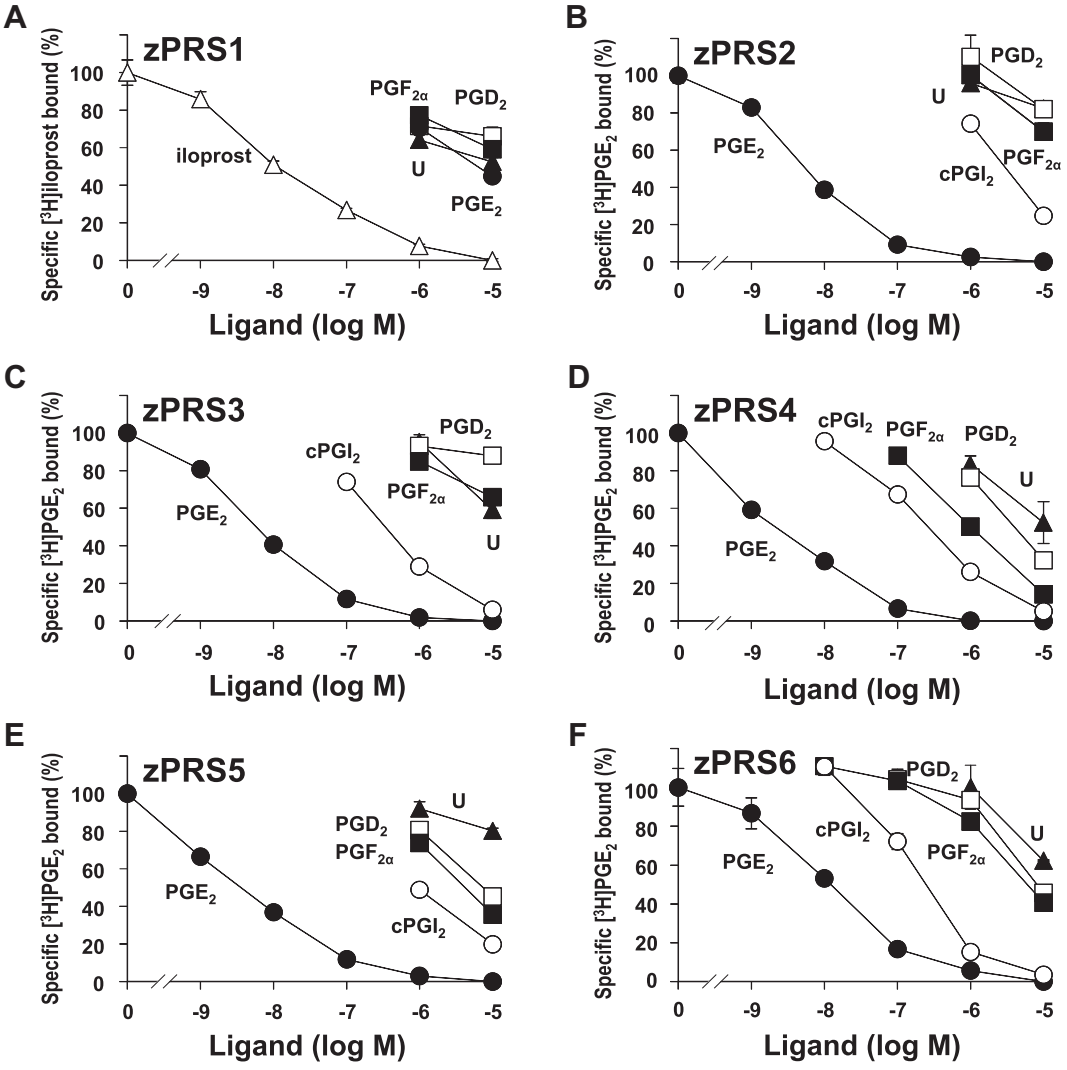

Fig. 1. Binding specificity of membranes expressing each full-length zebrafish '*relaxant*' prostanoid receptor. [^{3}H]iloprost (A) or [^{3}H]PGE_2 (B–F) binding to zPRS1- (A), zPRS2- (B), zPRS3- (C), zPRS4- (D), zPRS5- (E), or zPRS6-transfected cell membranes (F) was determined in the presence or absence of various concentrations of unlabeled PG analogues. *cPGI$_2$*, carbacyclin; *U*, U-46619. Values represent the means ± SEM ($n = 3$).

specific [^{3}H]iloprost binding to these membranes was also inhibited by carbacyclin and cicaprost (a stable IP agonist) (IC_{50}, 3×10^{-8}, 1×10^{-7} M, respectively) (Fig. S1A). These results show that the zPRS1 product possesses characteristics of the IP receptor. To identify the characteristics of the zebrafish EP receptors, we then investigated which EP-selective agonists are able to bind to each receptor. [^{3}H]PGE_2 binding to zPRS3-transfected cell membranes was inhibited modestly by ONO-AE1-259, an EP2 agonist, and weakly by butaprost, another EP2 agonist (IC_{50}, 3×10^{-7} and 3×10^{-6} M, respectively), indicating that the zPRS3 product possesses characteristics of the EP2 receptor (Fig. S1C). On the other hand, [^{3}H]PGE_2 binding to zPRS2-transfected cell membranes was inhibited only weakly by ONO-AE1-259 (IC_{50}, 3×10^{-6} M), but not by butaprost (Fig. S1B). Although the EP2 subtype was originally defined as butaprost-responsive EP receptors in mammals [1], ONO-AE1-259 has been shown to possess higher selectivity for the EP2 receptor. Therefore, since [^{3}H]PGE_2 binding of the zebrafish receptor encoded by zPRS2 was sensitive to ONO-AE1-259, we concluded that the zPRS2 product also possesses characteristics similar to EP2 receptors. The binding of [^{3}H]PGE_2 to zPRS4-, zPRS5- and zPRS6-transfected cell membranes were inhibited by ONO-AE1-329, an EP4-specific agonist (IC_{50}, 3×10^{-9} , 1×10^{-8}, 1×10^{-7} M, respectively) (Fig. S1, D–F). These results suggest that products of zPRS4, zPRS5 and zPRS6 all possess characteristics of the EP4 receptor.

3.3. Signal transduction properties of zebrafish 'relaxant' prostanoid receptors

Ligand binding studies suggest that zPRS1 encodes the IP receptor, zPRS2 and zPRS3 encode EP2 receptors, and zPRS4, zPRS5 and zPRS6 encode EP4 receptors. In mammals, IP, EP2 and EP4 receptors have been shown to elicit intracellular cAMP accumulation upon agonist stimulation [22,24,26]. To further confirm the receptor types and subtypes deduced from the binding studies, we investigated whether each receptor-bound agonist is able to induce cAMP accumulation in COS-7 cells (Fig. 2). In mock-transfected cells, both PGE_2 and iloprost failed to alter cAMP levels (data not shown). In zPRS1-transfected cells, iloprost, carbacyclin and cicaprost dose-dependently increased cAMP levels, and the agonist potency order was iloprost > carbacyclin > cicaprost (EC_{50}, 1×10^{-10}, 1×10^{-9}, and 3×10^{-8} M, respectively) (Fig. 2A). In zPRS3-transfected cells, PGE_2, ONO-AE1-259 and butaprost dose-dependently increased cAMP levels. The agonist potency order was PGE_2 >

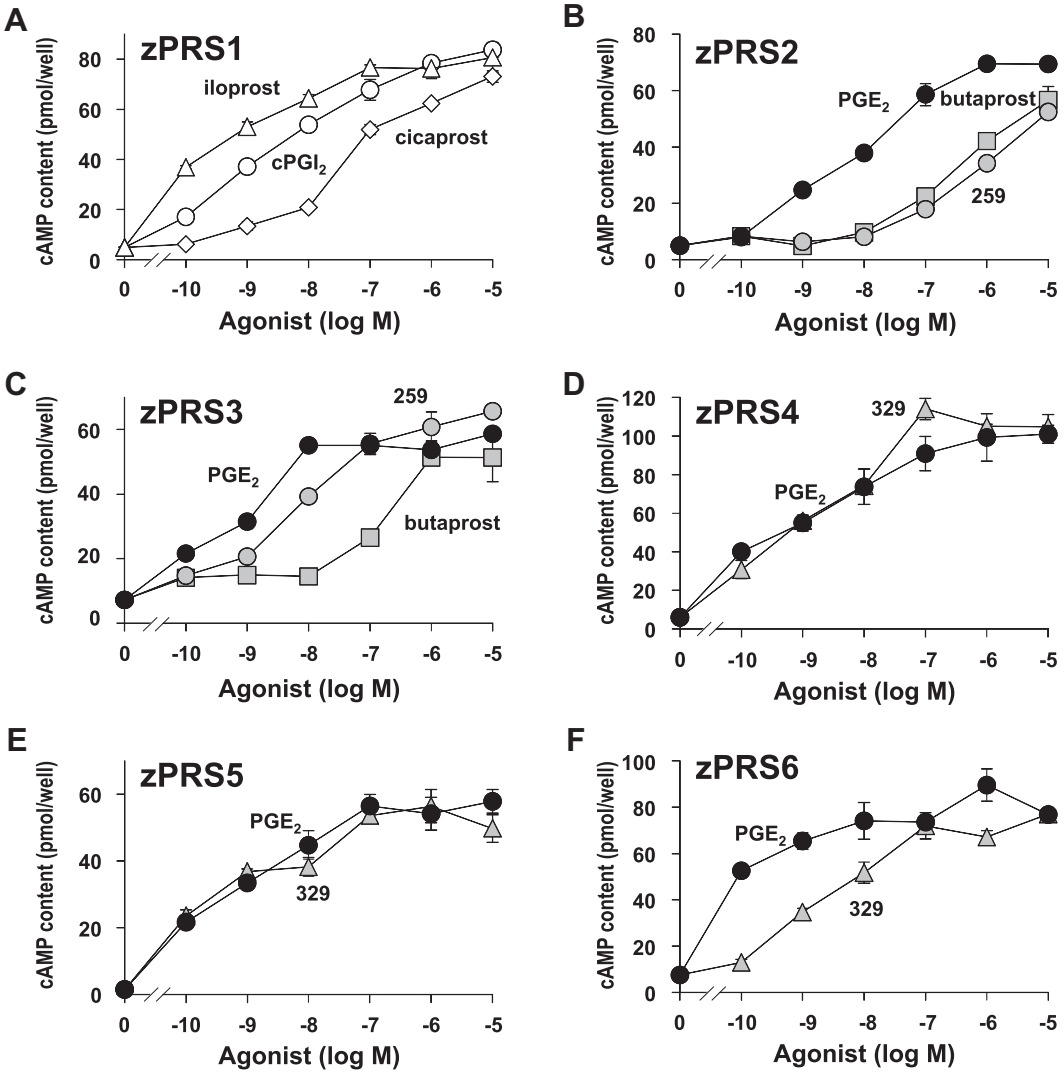

Fig. 2. Effects of IP- or EP-specific agonists on cAMP accumulation in cells transfected with each full-length '*relaxant*' prostanoid receptor cDNA. COS-7 cells transfected with zPRS1 (A), zPRS2 (B), zPRS3 (C), zPRS4 (D), zPRS5 (E) or zPRS6 (F) were stimulated with the indicated concentrations of IP- or EP-agonists, and the cAMP accumulation for 10 min was determined. *cPGI$_2$*, carbacyclin; *259*, ONO-AE1-259; *329*, ONO-AE1-329. Values represent the means ± SEM (n = 3).

ONO-AE1-259 > butaprost (EC_{50}, 1×10^{-9}, 3×10^{-9}, and 1×10^{-7} M, respectively) (Fig. 2C). On the other hand, in zPRS2-transfected cells, PGE_2 most potently increased cAMP levels (EC_{50}, 3×10^{-9} M), but ONO-AE1-259 and butaprost dose-dependently increased cAMP levels at doses higher than 10^{-7} M (EC_{50}, 3×10^{-7} M) (Fig. 2B). These results indicate that both zPRS2 and zPRS3 encode zebrafish EP2 receptors. In zPRS4- and zPRS5-transfected cells, PGE_2 dose dependently increased cAMP levels in similar manners (EC_{50}, 1×10^{-9} M) (Fig. 2, D and E). Moreover, in both cells, ONO-AE1-329 also raised intracellular cAMP in a manner similar to PGE_2 (EC_{50}, 1×10^{-9} M). On the other hand, in zPRS6-transfected cells, PGE_2 efficiently stimulated cAMP accumulation (EC_{50}, 1×10^{-10} M), and ONO-AE1-329 also increased cAMP levels with a slightly less agonist-dose efficiency (EC_{50}, 1×10^{-8} M) (Fig. 2F). These results support our conclusion that all three cDNAs, zPRS4, zPRS5 and zPRS6, encode zebrafish EP4 receptors. Based on these functional properties of the recombinant receptors, we renamed the zebrafish receptors encoded by zPRS1, zPRS2, zPRS3, zPRS4, zPRS5 and zPRS6 as IP, EP2a, EP2b, EP4a, EP4b and EP4c, respectively (Table S4).

We further investigated whether ONO-AE3-208, an EP4-specific antagonist, was able to block PGE_2-induced cAMP formation in the cells expressing zebrafish EP4a, EP4b or EP4c (Fig. S2). As to the EP4b receptor, ONO-AE3-208 suppressed PGE_2-induced cAMP formation in a dose-dependent manner (IC_{50}, 3×10^{-7} M). However, as to the EP4a and EP4c receptors, ONO-AE3-208, even at the dose of 10^{-5} M showed only partial inhibition of PGE_2-elicited responses. These results demonstrate that ONO-AE3-208 may act as an EP4b-selective antagonist in zebrafish.

3.4. Gene expression patterns of zebrafish 'relaxant' prostanoid receptors among adult tissues

We next investigated the gene expression patterns of the '*relaxant*' prostanoid receptors among zebrafish adult tissues by RT-PCR (Fig. 3). The mRNA for IP was detected in every tissue examined. Such ubiquitous expression may reflect the roles of PGI_2 in the nervous, vascular and immune systems, since IP receptor mRNA is expressed in peripheral neurons, arterial smooth muscle cells, and a specific population of lymphocytes [26,27]. It would be interesting to examine in the future whether zebrafish IP shows cellular distribution patterns similar to those in mice. The mRNAs for EP2a and EP2b were also detected in many tissues except for genital tissues. The amounts of EP2a mRNA were relatively constant among tissues, while the amounts of EP2b mRNA were variable. EP2b gene expression was quite low in the heart and kidney and remarkably high in the eye and gill. These results suggest that these two EP2 genes undergo quite different gene expression regulation. The mRNAs for EP2a and EP2b were also detected in similar levels in the brain, liver, spleen, muscle and intestine. In mice, PGE_2-EP2 signaling plays roles at least in the cardiovascular system [28], T cell regulation [29], and intestinal polyposis [30]. Whether such EP2 functions are conserved in zebrafish is an interesting point to be investigated. Three EP4 genes showed quite unique expression patterns; the EP4b mRNA was detected in all tissues except for the heart and testis, the EP4a mRNA was detected in the eye, heart and ovary, and the EP4c mRNA was seen in the brain and intestine. These results suggest that the EP4b gene may be regulated by a ubiquitous expression mechanism, while EP4a and EP4c genes may be under the control of tissue- or cell type-specific expression mechanisms.

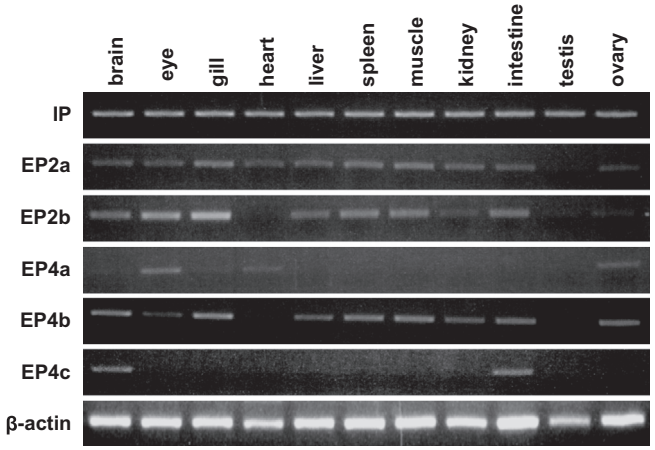

Fig. 3. Zebrafish '*relaxant*' prostanoid receptors show unique gene expression patterns among adult tissues. RT-PCR products were visualized in an ethidium bromide-stained agarose gel. β-Actin was amplified as an internal control for each sample (lowest panel).

3.5. Primary structure of zebrafish 'relaxant' prostanoid receptors and their molecular evolutions

Fig. S3 shows the alignment of amino acid sequences of human, mouse and zebrafish IP, EP2 and EP4 receptors. In all three types of receptors, the sequences within the transmembrane (TM) regions are highly conserved. Moreover, in addition to the TM regions, the sequences within the 2nd intracellular (i2) loop between TM3 and TM4 and the sequences within the 2nd extracellular (e2) loop between TM4 and TM5, which are considered to be important for Gs-coupling [31] and ligand binding [32], respectively, are also highly conserved. When we compared the sequence conservation among species, EP4 receptors showed the highest homology; for example, in addition to the regions described above, conserved sequences were found in the i1 and the e1 loops, and membrane boundaries of the i3, e3 loop and C-terminal region. Such high levels of sequence conservation may reflect the pivotal roles of EP4 compared with the other prostanoid receptors. Some potential N-linked glycosylation sites are also conserved among species: in IP receptors, a glycosylation site in the N-terminus is conserved among species. In human IP, glycosylation at this position was suggested to be important for appropriate membrane localization [33], and such a role of glycosylation in membrane targeting may be conserved in zebrafish prostanoid receptors. Also in EP2 receptors, two glycosylation sites (one in the N-terminus and one in the e1 loop) are completely conserved among species. On the other hand, one glycosylation site in the N-terminus is conserved in human, mouse and zebrafish EP4 receptors except for EP4a, which has a truncated N-terminus.

Fig. 4 shows the phylogenetic tree of human and zebrafish '*relaxant*' prostanoid receptors. It should be noted that the distance between human EP4 and zebrafish EP4a (or EP4b) is very close, compared to the distance between human and zebrafish IP receptors. Interestingly, the distance between human EP4 and zebrafish EP4c is not so close. Indeed, ONO-AE1-329, an agonist designed for human EP4, exhibits excellent agonist activity on zebrafish EP4a or EP4b, but this compound shows less potent activity on the EP4c receptor (Fig. 2, D–F). Moreover, ONO-AE3-208, an antagonist designed for human EP4, reveals the most effective antagonist activity on zebrafish EP4b (Fig. S2). Thus, the responsiveness of each form of zebrafish EP4 receptors to these compounds appears to be closely associated with its structural conservation with the human receptor. Compared to EP4 and IP clusters, the EP2 receptors showed higher divergence; the distance between human EP2 and zebrafish EP2a (or EP2b) is relatively higher. One of the remarkable findings in this study is the absence of a DP receptor in zebrafish. The phylogenetic tree indicates that the zebrafish EP2 receptor branched from its ancestor, and then the human DP receptor

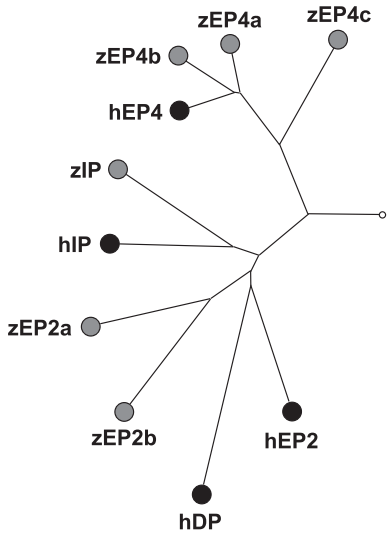

Fig. 4. Phylogenetic tree of zebrafish and human '*relaxant*' prostanoid receptors. Black circles indicate human '*relaxant*' prostanoid receptors, and gray circles indicate zebrafish receptors.

branched from the human EP2 receptor. This branching may have taken place as a result of the divergent ligand recognition nature of the EP2 receptor. If this is true, the roles played by the DP receptor in humans may be substituted by either EP2a or EP2b in zebrafish.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.017.

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