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Molecular characterization of the prostaglandin E receptor subtypes 2a and 4b and their expression patterns during embryogenesis in zebrafish

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ABSTRACT The molecular expression profiles of zebrafish *ep2a* and *ep4b* have not been defined to date. Phylogenetic trees of EP2a and EP4b in zebrafish and other species revealed that human EP4 and zebrafish EP4b were more closely related than EP2a. Zebrafish EP2a is a 281 amino acid protein which shares high identity with that of human (43%), mouse (44%), rat (43%), dog (44%), cattle (41%), and chicken (41%). Zebrafish EP4b encoded a 497 amino acid precursor with high amino acid identity to that of mammals, including human (57%), mouse (54%), rat (55%), dog (55%), cattle (56%), and chicken (54%). Whole-mount *in situ* hybridization revealed that *ep2a* was robustly expressed in the anterior four somites at the 10-somites stages, but was absent in the somites at 19 hpf. It was observed again in the pronephric duct at 24 hpf, in the intermediate cell mass located in the trunk, and in the rostral blood island at 30 hpf. *Ep2a* was also expressed in the notochord at 48 hpf. During somitogenesis, *ep4b* was highly expressed in the eyes, somites, and the trunk neural crest. From 30 to 48 hpf, *ep4b* could be detected in the posterior cardinal vein and the neighboring inner cell mass. From these data we conclude that *ep2a* and *ep4b* are conserved in vertebrates and that the presence of *ep2a* and *ep4b* transcripts during developmental stages infers their role during early zebrafish larval development. In addition, the variable expression of the two receptor isoforms was strongly suggestive of divergent roles of molecular regulation.

KEY WORDS: *ep2a, ep4b, expression, whole-mount in situ hybridization, zebrafish*

Prostaglandin E2 (PGE2) is an important arachidonate metabolite that regulates an array of physiological processes in vertebrates, including immune responses (Kutyrev *et al.,* 2017), gastrointestinal function (Takeuchi and Amagase, 2018), testicular homeostasis (Rey-Ares *et al.,* 2018), and ovulation (Baker and Van Der Kraak, 2019). PGE_2 binds to four specific G-protein-coupled receptors (EP1, EP2, EP3, and EP4) in humans and mice (Ball *et al.,* 2013; Kimple *et al.,* 2013; Zhu *et al.,* 2018). EP1 is coupled to Gq to elevate intracellular Ca^2+, EP3 couples to Gi to inhibit adenylyl cyclase (AC) and cyclic AMP (cAMP), and both EP2 and EP4 couple to Gs to stimulate AC and increase cAMP (Samuchiwal *et al.,* 2017; Wang *et al.,* 2010), and have been identified as crucial mediators of PGE_2 in both physiological and pathophysiological processes, including closure of the ductus arteriosus (Sakuma *et al.,* 2018; Yokoyama *et al.,* 2014), bone formation (Graham *et al.,* 2009), ovulation and fertilization (Niringiyumukiza *et al.,* 2018) and salt sensitive hypertension (Kennedy *et al.,* 1999). Knockdown of EP2a results in smaller embryonic livers in zebrafish (Nissim *et al.,* 2014). In medaka, impairment of the EP4b receptor *in vitro* using a competitive antagonist resulted in anovulation (Fujimori *et al.,* 2012).

Though both EP2 and EP4 were identified in mammals (Kershaw-Young *et al.,* 2009; Kowalewski *et al.,* 2008; Reitmair *et al.,* 2010) and chickens (Kwok *et al.,* 2008), their expression has not been investigated in fish. Zebrafish (Danio rerio) are a vertebrate model organism that is widely used for genetic and pharmacological analysis of embryogenesis due to their high fecundity and translucency (Akhter *et al.,* 2016; Ellertsdottir *et al.,* 2010). In addition, a number of disease models have been developed in zebrafish that can be combined with *in vivo* imaging approaches to monitor specific pathological processes, including cardiovascular disease and cancer metastasis (Bournele and Beis, 2016; Tulotta *et al.,* 2016).

Abbreviations used in this paper: ICM, inner cell mass; PGE2, prostaglandin E2.

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In this regard, it has been shown that prostaglandin receptors play a key role in zebrafish development, including ovulation and T cell precursor development (Baker and Van Der Kraak, 2019; Villablanca *et al.*, 2007). However, the expression of zebrafish *ep2a* and *ep4b* has not been systematically defined in the literature.

Here, we report the expression patterns of *ep2a* and *ep4b* during

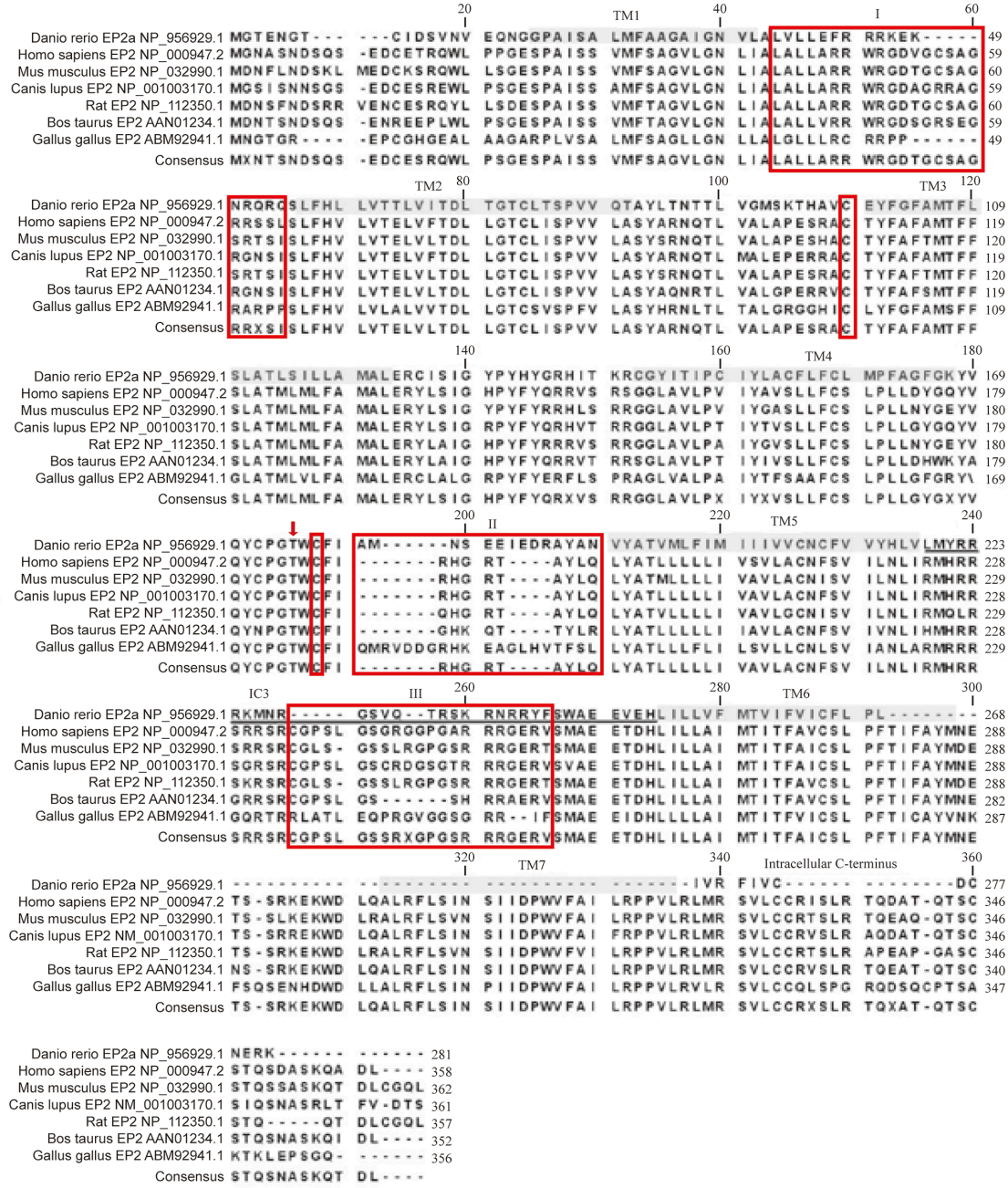

Fig. 1. Sequence alignment of zebrafish EP2a (Danio rerio EP2a: NP_956929.1) to human (Homo sapiens EP2: NP_000947.2), mouse (Mus musculus EP2: NP_032990.1), rat (Rat EP2: NP_112350.1), dog (Canis lupus EP2: NP_001003170.1), cattle (Bos taurus EP2: AAN01234.1) and chicken (Gallus gallus EP2: ABM92941.1). *The seven putative transmembrane domains are shaded and labeled. Sequences underlined and in bold represent the third intracellular loop (IC3). Three highly variable regions (I, II, and III) between zebrafish and other species are boxed and labeled. Arrow heads indicate the conserved threonine. Two cysteine residues forming disulphide bonds are boxed. Dots indicate amino acids that are not present within the sequence.*

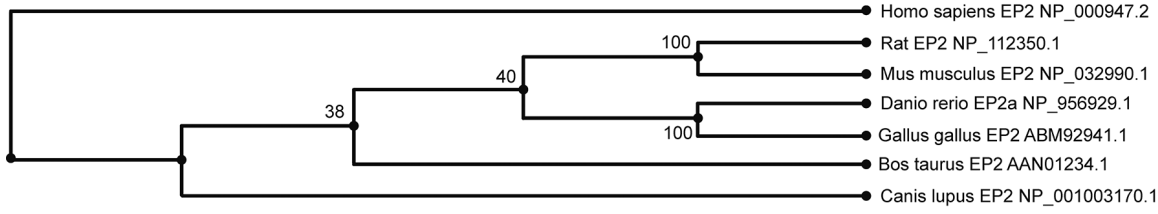

Fig. 2. Phylogenetic tree of EP2 across different species constructed using the Neighbor-joining method. *An evolutionary relationship of EP2 in different species, including human, mouse, rat, dog, cattle, chicken and zebrafish were observed. Numbers adjacent to the branch points indicate bootstrap values.*

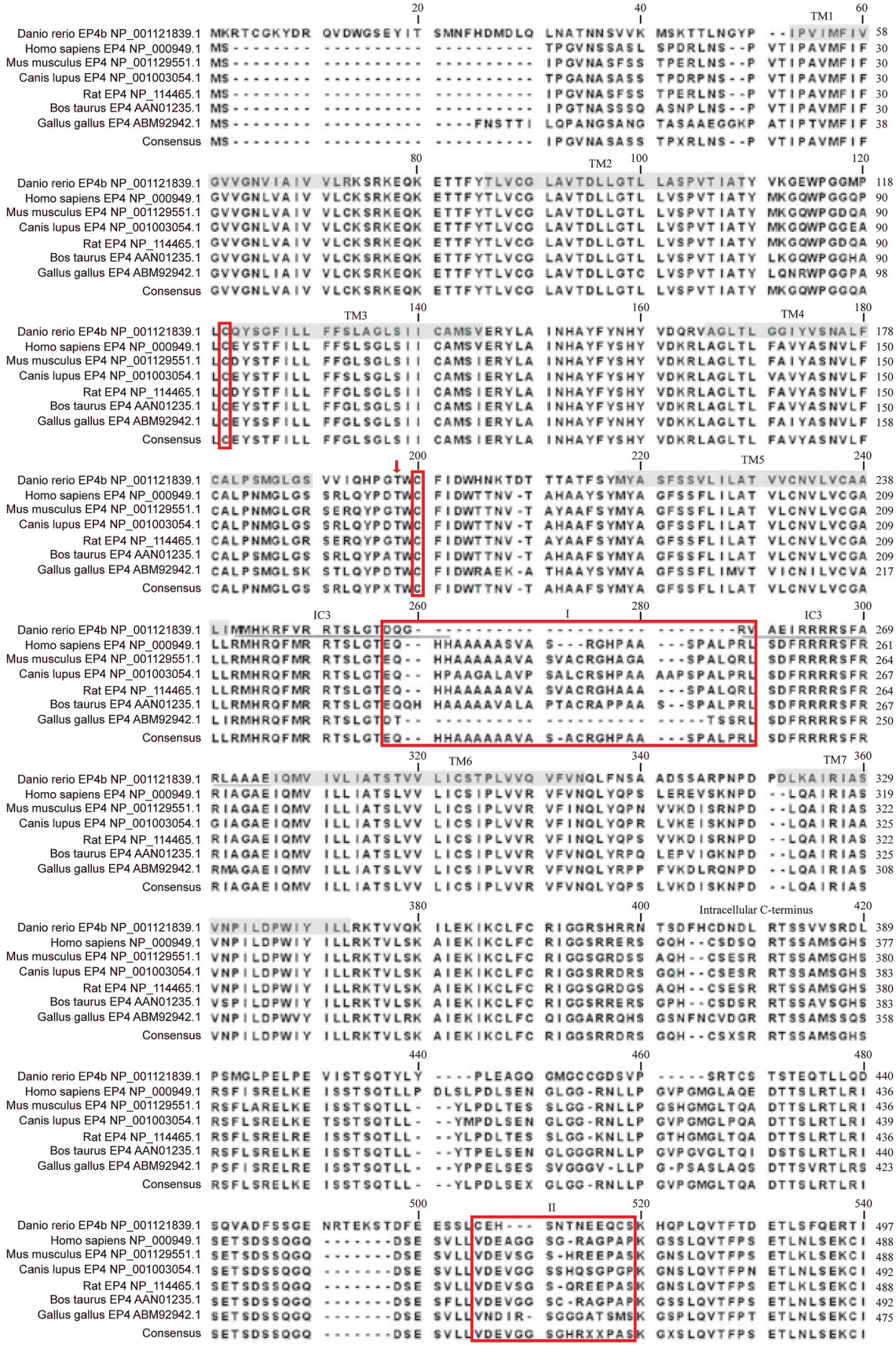

Fig. 3. Sequence alignment of zebrafish EP4b (Dario rerio EP4b: NP_001121839.1) to that of human (Homo sapiens EP4: NP_000949.1), mouse (Mus musculus EP4: NP_001129551.1), rat (Rat EP4: NP_114465.1), dog (Canis lupus EP4: NP_001003054.1), cattle (Bos taurus EP4: AAN01235.1), and chicken EP4 (Gallus EP4: ABM92942.1). *The seven putative transmembrane domains are shaded and labeled. Sequences underlined and in bold represent the third intracellular loop (IC3). Two highly variable regions (I and II) of EP4 between zebrafish and other species are boxed. The two cysteine residues proposed to form disulphide bonds are also boxed. Arrow heads indicate the conserved threonine residue of EP4. Dots indicate amino acids that are absent within the sequence.*

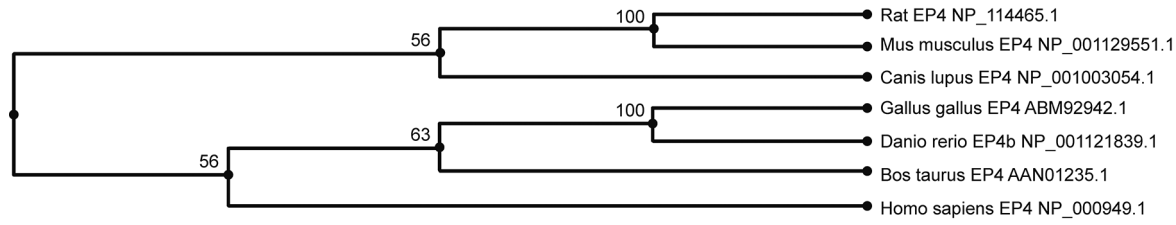

Fig. 4. Phylogenetic tree of EP4 across the indicated species. *The phylogenetic tree was constructed using the Neighbor-joining method. Evolutionary relationships of EP4 in different species, including human, mouse, rat, dog, cattle, chicken and zebrafish. Numbers adjacent to the branch points indicate bootstrap values.*

zebrafish embryogenesis. We show that *ep2a* is robustly expressed in the somites, the pronephric duct, ICM, and notochord, whilst *ep4b* is highly expressed in the somites, the trunk neural crest, the posterior cardinal vein and the neighboring ICM. These data reveal important information regarding the expressional characteristics of zebrafish *ep2a* and *ep4b* during developmental stages. Differences in the expression of these transcripts during development indicate differential molecular regulatory patterns.

Results

Sequence alignment of EP2a from various vertebrate species

Full-length cDNAs for zebrafish *ep2a* and *ep4b* were cloned and the sequences were verified by blast searches. Full-length cDNA of zebrafish *ep2a* was 2366 bp (GenBank accession No: NP_956929.1), encoded a 281 amino acid protein (Tsuge *et al.,* 2013). As expected, zebrafish EP2a exhibited high amino acid identity to that of human (Homo sapiens) (43%), mouse (Mus musculus) (44%), rat (Rattus norvegicus) (43%), dog (Canis lupus familiaris) (44%), cattle (Bos taurus) (41%), and chicken (Gallus gallus) (41%) E2a. The protein possessed seven putative transmembrane domains. In contrast, the amino acid identity of EP2 significantly differed between zebrafish and other species. In the ORF region of EP2, three highly variable regions were identified, located in intracellular loop 1, extracellular loop 2, and intracellular loop 3 (Fig. 1).

Phylogenetic tree of EP2a in different species

As shown in Fig. 2, a close distance between chicken EP2 and zebrafish EP2a was observed. Interestingly, the distance between human EP2 and zebrafish EP2a was less close. Analysis of the phylogenetic tree indicated that the zebrafish EP2a receptor branched from its ancestor. Zebrafish EP2a receptors similarly diverged from their ancestors. This branching may have occurred due to the different ligand recognition properties of the EP2 receptors.

Sequence alignment of EP4b proteins from various vertebrate species

The full-length cDNA of zebrafish *ep4b* was 1494 bp (Genbank accession NP_001121839.1) encoding a protein of 497 amino acids (Fig. 3). Zebrafish EP4b showed high sequence homology to human (57%), mouse (54%), rat (55%), dog (55%), cattle (56%), and chicken (54%) EP4 (Fig. 3). Despite the high homology, the 3rd intracellular loop and intracellular C-terminus significantly differed between zebrafish and other species (Fig. 3).

Phylogenetic tree of EP4b across different species

Fig. 4 shows the phylogenetic tree of EP4 in zebrafish and other species. The distance between chicken EP4 and zebrafish EP4b was close. Interestingly, human EP4 and zebrafish EP4b were also closely related compared to EP2a. ONO-AE3-208, an antagonist for human EP4, effectively blocks zebrafish EP4b activity (Tsuge *et al.,* 2013). Thus, the response of each zebrafish EP4 receptor to

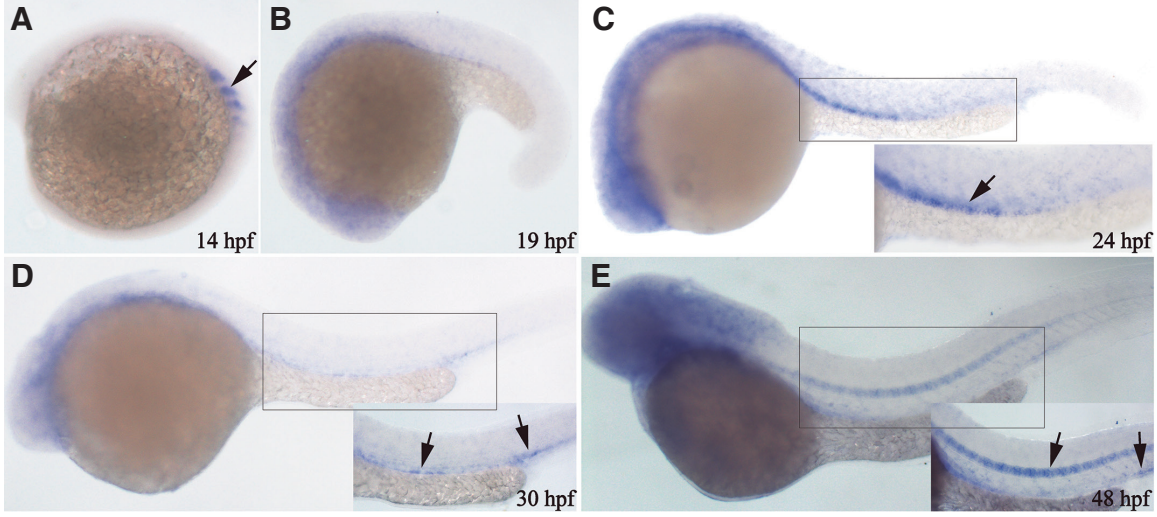

Fig. 5. Ep2a expression during zebrafish development. (A) *ep2a was expressed in the anterior four somites at 14 hpf.* **(B)** *ep2a was ubiquitously expressed in the anterior region of the emybros at 19 hpf.* **(C)** *At 24 hpf, ep2a expression was observed in the pronephric duct and diffusely in the posterior region of the trunk.* **(D)** *At 30 hpf, ep2a expression was maintained in the ICM located in the trunk and in the rostral blood island.* **(E)** *At 48 hpf, ep2a was strongly expressed in the notochord, but weakly expressed in the blood. In* **(A)** *embryos are orientated in the lateral view with the anterior side up and the dorsal side to the right. Embryos in* **(B-E)** *are orientated in the lateral view with the anterior to the left and the dorsal side up. ep2a expression is indicated with arrows. Scale bar, 200 μm.*

these compounds was closely related to their structural conservation with the human receptor.

Expression of zebrafish ep2a during early embryogenesis

Whole mount *in situ* hybridization was used to determine the temporal and spatial expression patterns of *ep2a*. At the 10-somites stage (14 hpf), robust *ep2a* expression was observed in the anterior four somites (Fig. 5A). Interestingly, at 19 hpf, *ep2a* could no longer be detected in the somites, although its expression was diffuse in the anterior region of the embryo (Fig. 5B). At 24 hpf, *ep2a* expression was observed in the pronephric duct, and diffusely in the posterior region of the trunk (Fig. 5C). At 30 hpf, *ep2a* was maintained in the ICM of the trunk and in the rostral blood island (Fig. 5D). At 48 hpf, *ep2a* was strongly expressed in the notochord, but weakly expressed in the blood (Fig. 5E).

Zebrafish ep4b expression during early embryogenesis

Whole mount *in situ* hybridization was used to investigate the temporal and spatial expression pattern of *ep4b*. During the shield stage, *ep4b* was present in the germ ring, showing peak expression in the embryonic shield (Fig. 6A). At the end of gastrulation, *ep4b* became more localized to the posterior region of the embryo (Fig. 6B). The dorsal view showed that the expression was arranged in two sides (ellipse) of the prechordal plate hypoblast (Fig. 6C), in which the cells contribute to the posterior trunk, somites, and neural (Kimmel *et al.,* 1995). During somitogenesis, *ep4b* was robustly expressed in the eyes, somites (Fig. 6D), but it doesn't seem to be a strong space constraint. From 19 to 24 hpf, *ep4b* remained highly expressed in the somites and the trunk neural crest (Fig. 6E and F). *Ep4b* expression in the trunk neural crest continued to the 30 hpf stage (Fig. 6G). From 30 to 48 hpf, *ep4b* could be detected in the posterior cardinal vein and the neighboring ICM (Fig. 6G and H), as reported for *ptger4a* (Baker and Van Der Kraak, 2019).

Discussion

In this study, we show that *ep2a* is robustly expressed in the anterior four somites at 14 hpf, but is absent in the somites at 19 hpf. At later stages, *ep2a* could be observed in the pronephric duct, ICM, and in the rostral blood island between 24 to 30 hpf. Robust expression was observed in the notochord at 48 hpf. Interestingly, the expression of *ep2a* and *ep4b* differed during zebrafish development. During somitogenesis, *ep4b* was present in the eyes, somites, and the trunk neural crest. From 30 to 48 hpf, *ep4b* was present in the posterior cardinal vein and the neighboring ICM. Variations in the expression of these transcripts during developmental stages suggested different modes of molecular regulation.

Species comparison of EP2 and EP4 showed that EP2a and EP4b showed high homology across mammalian and non-mammalian vertebrate species, including human (43%; 57%, respectively), mouse (44%; 54%, respectively), rat (43%; 55%, respectively), dog (44%; 55%, respectively), cattle (41%; 56%, respectively),

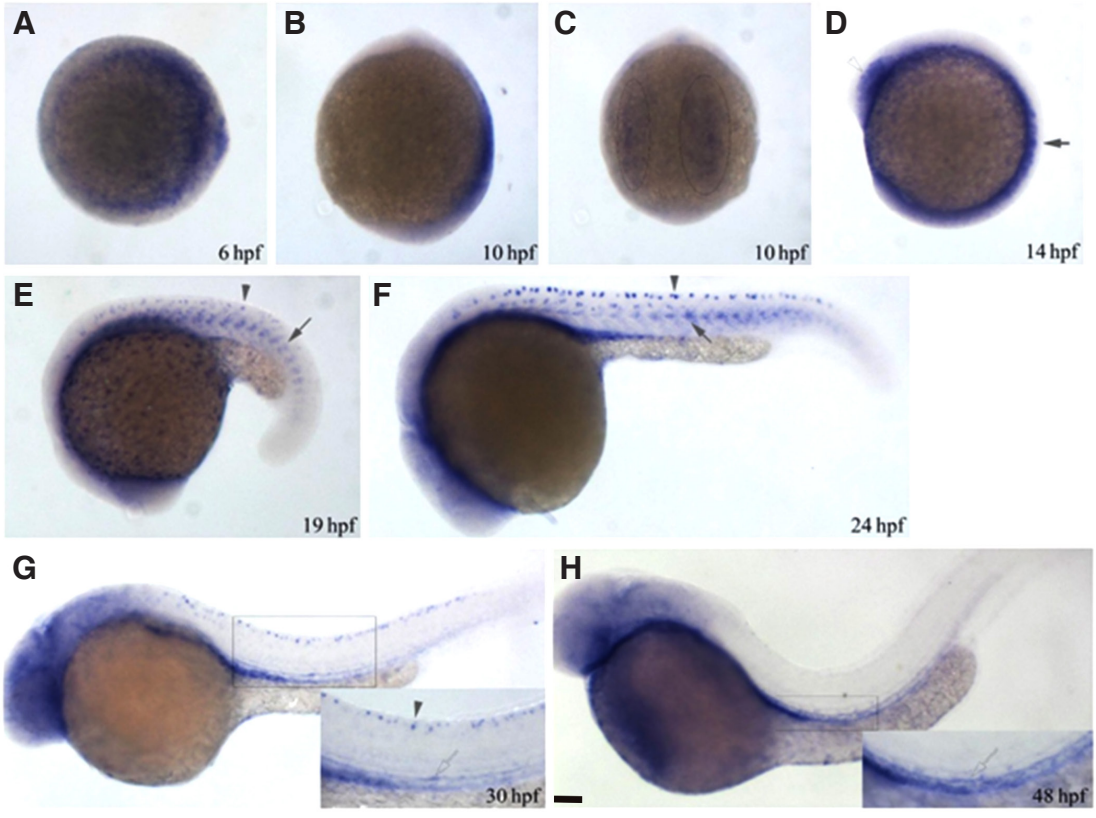

Fig. 6. Ep4b expression during zebrafish development. **(A)** *Embryonic animal polar diagrams at 6 hpf show that ep4b displayed a graded pattern of expression in the embryonic ring, with the highest expression levels observed in the embryonic shield.* **(B)** *Lateral views (anterior side up and the dorsal to the right) of the embryo show that ep4b was expressed in the posterior region of the emybro at 10 hpf.* **(C)** *Dorsal view (anterior side up) showing that the expression was arranged in two sides (ellipse) of the prechordal plate hypoblast.* **(D)** *Lateral views (anterior side up and the dorsal to the right) of the embryo at 14 hpf show that ep4b was expressed in the somites (black arrows), and the eyes (white arrowheads).* **(E-H)** *Lateral views (anterior to the right and the dorsal side up) of the embryos. At 19 hpf* **(E)** *and 24 hpf* **(F)**, *black arrows in* **(E, F)** *indicate that ep4b was expressed in the somites, the trunk neural crest (black arrowhead).* **(G,H)** *show that ep4b was expressed in the trunk neural crest (black arrowhead) at 30 hpf* **(G)**, *in the posterior cardinal vein, and in the neighboring ICM (white arrow) at 30 hpf and at 48 hpf* **(H)**. *Insets represent magnification of the boxes in* **(G,H).** *Scale bar, 200 μm.*

and chicken (41%; 54%, respectively), as shown in Fig. 1 and 3. Of note, the distance between human EP4 and zebrafish EP4b was close compared to human and zebrafish IP receptors. Interestingly, the distance between human EP4 and zebrafish EP4c was less close (Tsuge *et al.,* 2013). Consistent with these data, ONO-AE1-329, an agonist specifically designed for human EP4, showed high activity against zebrafish EP4b but low activity against the EP4c receptor (Stillman *et al.,* 1998). Thus, zebrafish EP4 receptors and their interaction with these compounds were closely related to the structural conservation of the human receptor. Compared with EP4 and IP clusters, EP2 receptors showed higher differentiation. The distance between human EP2 and zebrafish EP2a was relatively large.

EP2 and EP4 belong to the GPCR superfamily and can be subdivided into rhodopsin receptor members. Both share common characteristics. Phylogenetic trees with bootstrap values for EP2 and EP4 were constructed using the Neighbor-joining method (Fig. 2 and 4), and indicated that both EP2 and EP4 are closely related to their corresponding counterparts in mammalian species and chicken. This suggested that the cloned receptors are potential EP2 and EP4 receptors in zebrafish. Two highly conserved cysteines present amongst GPCRs (Stillman *et al.,* 1998) with proposed roles in disulphide bond formation were located as Cys110 and Cys188 in EP2, and Cys122 and Cys200 in EP4 (Fig. 1 and 3). A threonine residue that is conserved amongst EP2 and EP4 has also been suggested to be important for ligand binding (Stillman *et al.,* 1998), and was identified in the cloned receptors (Thr186 in EP2 and Thr198 in EP4) (Fig. 1 and 3). Despite these similarities, EP2 and EP4 showed several distinct differences. As in mammals (Desai *et al.,* 2000), EP4 was longer than EP2 (497 a.a. vs. 281 a.a.), with the major differences observed in the lengths of the 3rd intracellular loops and intracellular C-termini (Fig. 1 and 3). The longer C-terminus of EP4 possesses a number of putative phosphorylation sites that undergo short term ligand-induced receptor internalization and desensitization. This was suggestive of potential functional differences in signal transduction between EP2 and EP4 in response to their ligands (Davidson and Zon, 2004; Sugimoto and Narumiya, 2007).

Using whole mount *in situ* hybridization, *ep2a* and *ep4b* expression were analyzed in the zebrafish embryos. Robust expression of *ep2a* was observed in the anterior four somites at 14 hpf (Fig. 5A), indicating its relationship to the development stages of somatic cells. In zebrafish, inhibition of Wnt signaling results in a posterior shift of the somite boundary. COX-2/PGE2 facilitates fracture healing by activating the Wnt/β -catenin signaling pathway (Velloso *et al.,* 2021). Between 14 and 19 hpf, Wnt/β -catenin signaling pathway may regulate the expression of *ep2a*. At 24 hpf, *ep2a* expression was observed in the pronephric duct, mainly and in the posterior region of the trunk with diffuse but ubiquitous expression (Fig. 5C). At 30 hpf, *ep2a* is expressed in the ICM and is located in the trunk and in the rostral blood island (Fig. 5D), whilst definitive hemopoiesis occurred in the DA-PCV joint region, which is equivalent to the AGM region ((Taylor *et al*., 2010; Hui *et al*., 2020; Villablanca *et al.,* 2007). Similarly, *pges* was expressed in the endothelium, and *cox-2* was expressed in the wall of the aortic arch ((Patel-Hett and D'Amore, 2011; Pini *et al.,* 2005). The expression patterns of *ep2a* and *cox-2* and *pges-1* in zebrafish highlight their potential during vascular development and ability to modulate key components of these developmental stages, including VEGF. During somitogenesis, *ep4b* was robustly expressed in the eyes, somites (Fig. 6D), but it doesn't seem to be a strong space constraint. From 19 to 24 hpf, *ep4b* remained highly expressed in the somites and the trunk neural crest (Fig. 6E and F). In addition, at 30 hpf, *ep4b* was expressed in the trunk neural crest (Fig. 6G). These data suggest that *ep4b* plays an important role in the development of somites and the trunk neural crest. From 30 to 48 hpf, *ep4b* expression could be detected in the posterior cardinal vein and the neighboring ICM (Fig. 6G and H), and was similar to that observed for *ep4b* (Sun *et al*., 2017; Baker and Van Der Kraak, 2019). In addition, *cox-1* and *cox-2* were expressed in the carotid arteries and the vasculature of the pharyngeal arches at 96 hpf (Ishikawa *et al.,* 2007; Pini *et al.,* 2005). This highlights a novel role for the PGE_2 / EP4 axis during vascular development.

Materials and Methods

Zebrafish models

Embryos of AB wild type zebrafish were raised and staged as described by Kimmel *et al.,* (Kimmel *et al.,* 1995). Embryos were maintained in E3 solution at 28.5 °C with 0.003% 1-phenyl-2-thiourea (Sigma) to inhibit pigmentation. Embryos were staged according to somite number or hours post-fertilization (hpf).

Cloning of ep2a and ep4b

Total RNA was extracted from zebrafish embryos at 0.5-24 hpf stages using commercially available RNeasy mini-kits (Qiagen) according to manufacturer's protocols. First-strand cDNA was generated from normalized input RNA using random hexamers and commercial Superscript II kits (Invitrogen). RT-PCRs were performed with specific primer pairs: *ep2a* (846 bp fragment, forward: 5'-CGGATCCGATGGGCACTGAAAATGGGACCTGTA-3', BamHI; reverse: 5'-CATCGATTTATTTTCTTTCGTTACAATCACAT-3', ClaI), *ep4b* (1494 bp fragment, forward: 5'-CATCGATATGAAACGCACGTGTG-GAAAGTATG-3', ClaI; reverse: 5'- CGGAATTCATATGGTTCTCTCTT-GAAAGCTC-3', EcoRI). PCR parameters were as follows: 95 °C for 3 min, (95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s) x 35, 72 °C for 5 min. Full-length PCR products were cloned into the pGEM-T easy vector (Promega) and used for antisense digoxigenin labeled probe synthesis with SP6 RNA polymerase.

Sequence alignment and phylogenetic analyses

EP2 and EP4 orthologs were retrieved from GenBank and included Danio rerio EP2a (Accession Nos.: NP_956929.1), homo sapiens EP2 (NP_000947.2), Mus musculus EP2 (NP_032990.1), Canis lupus EP2 (NP_001003170.1), Rat EP2 (NP_112350.1), Bos taurus EP2 (AAN01234.1), Gallus EP2 (ABM92941.1), Dario rerio EP4b (NP_001121839.1), Homo sapiens EP4 (NP_000949.1), Mus musculus EP4 (NP_001129551.1), Rat EP4 (NP_114465.1), Canis lupus EP4 (NP_001003054.1), Bos taurus EP4 (AAN01235.1), and Gallus EP4 (ABM92942.1). Alignments were performed based on derived amino acid sequences using MUSCLE in MEGA version 5.

A phylogenetic tree was constructed using the sequences of zebrafish, human, mouse, rat, dog, cattle, and chicken EP2 and EP4 via the "AllAll program". Analysis was performed using the Computational Server at Eidgenössische Technische Hochschule Zürich (ETHZ) (http://www.cbrg.ethz.ch/services/AllAll).

Whole mount in situ hybridization

Antisense probes for *ep2a* and *ep4b* were synthesized using DIG RNA labeling kit (Roche, US). Standard procedures were performed as per the manufacturer's recommendations. Whole mount *in situ* hybridizations was performed as described by Thisse *et al.,* (Thisse and Thisse, 2014). Embryos were imaged on a Zeiss confocal microscope. *In situ* experiments were performed on a minimum of three independent occasions using 20 embryos.

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