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Extracellular acidification activates ovarian cancer G-protein-coupled receptor 1 and GPR4 homologs of zebra fish

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ABSTRACT

Mammalian ovarian G-protein-coupled receptor 1 (OGR1) and GPR4 are identified as a proton-sensing G-protein-coupled receptor coupling to multiple intracellular signaling pathways. In the present study, we examined whether zebra fish OGR1 and GPR4 homologs (zOGR1 and zGPR4) could sense protons and activate the multiple intracellular signaling pathways and, if so, whether the similar positions of histidine residue, which is critical for sensing protons in mammalian OGR and GPR4, also play a role to sense protons and activate the multiple signaling pathways in the zebra fish receptors. We found that extracellular acidic pH stimulated CRE-, SRE-, and NFAT-promoter activities in zOGR1 overexpressed cells and stimulated CRE- and SRE- but not NFAT-promoter activities in zGPR4 overexpressed cells. The substitution of histidine residues at the 12th, 15th, 162th, and 264th positions from the N-terminal of zOGR1 with phenylalanine attenuated the proton-induced SRE-promoter activities. The mutation of the histidine residue at the 78th but not the 84th position from the N-terminal of zGPR4 to phenylalanine attenuated the proton-induced SRE-promoter activities. These results suggest that zOGR1 and zGPR4 are also proton-sensing G-protein-coupled receptors, and the receptor activation mechanisms may be similar to those of the mammalian receptors.

1. Introduction

Mammalian ovarian cancer G-protein-coupled receptor 1 (OGR1) and GPR4 were originally reported to be activated by lysolipids, such as sphingosylphosphorylcholine (SPC) and lysophosphatidylcholine (LPC), which act as ligands [1]; however, the direct binding of the lipids to the receptors has not been proven. On the other hand, Ludwig reported that the human receptors sense extracellular protons and activate intracellular signaling pathways through trimeric G proteins [2]. Thus, human OGR1 stimulation causes phospholipase C (PLC) activation and subsequent intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization through $G_{q/11}$ proteins, and human GPR4 stimulates adenylyl cyclase activation through G_s proteins, in response to the extracellular acidification. Later, we showed that human OGR1 is also coupled to G_s/cAMP and G_{13}/Rho signaling pathways [3,4], and GPR4 is coupled to G_{13}/Rho and $G_{q/11}$/PLC signaling pathways [4,5], when the receptors were overexpressed in HEK293T cells. Site mutagenesis studies show that the specific histidine residues at the extracellular surface of the receptors are responsible for proton sensing [2,5].

OGR1 and GPR4 expressions are widely detected in many tissues [1]. The receptor expressions are also reported in vascular endothelial and smooth muscle cells [6–19]. Under an acidic pH condition, OGR1 mediates COX-2, MKP-1, IL-6, CTGF, VCAM-1, and ICAM-1 expressions and PGI_2 production in human vascular smooth muscle and airway smooth muscle cells [15–19]. GPR4 mediates VCAM-1, ICAM-1, COX-2, and a number of inflammatory gene expressions [9,10]. The physiological and pathophysiological

Abbreviations: [1]z, zebra fish (*Danio rerio*); OGR1, ovarian cancer G-protein-coupled receptor 1; GPCR, G-protein-coupled receptor.

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roles of OGR1 and GPR4 are examined using OGR1-and GPR4-deficient mice. However, the molecular mechanism by which the receptors are concerned with the phenotype is largely unknown.

Zebra fish can provide a useful vertebrate model system to elucidate the molecular mechanism of the receptor functions in vivo. The embryo is transparent, and development takes place outside the maternal body. These characteristics make them suitable to use for in vivo imaging. Indeed, zebra fish have been used in especially studies of blood vessel formation [20] and cancer invasion [21].

We found zebra fish OGR1 and GPR4 homologs (zOGR1 and zGPR4) in the genome database; however, their characterizations have not yet been reported. In this study, we characterized the functions of the homologs and focused on their ligand specificity and signaling pathways by expressing them in HEK293T cells. We found that these receptors sense protons like the mammalian receptors and activate multiple signaling pathways.

2. Materials and methods

2.1. Materials

A dual luciferase kit was purchased from Promega (Tokyo, Japan); Fura2 AM from Dojindo (Tokyo, Japan); fatty acid-free BSA from Calbiochem-Novabiochem Co. (San Diego, CA); and Lipofectamine 2000 Reagent from Life Technologies (Tokyo, Japan). The sources of all other reagents were the same as described previously [4,5].

2.2. Preparation of receptor cDNA plasmids and expression

The entire coding regions of zOGR1 (1032 bp, GenBank accession No. XM_001339552), zGPR4 (1122 bp, GenBank accession No. XM_687123), zOGR1-H4F (the 12th, 15th, 162th, and 264th positions of histidine from the N terminus were substituted with phenylalanine), GPR4-H78F (the 78th position of histidine from the N terminus was substituted with phenylalanine), and GPR4-H84F (the 84th position of histidine from the N terminus was substituted with phenylalanine) were synthesized and cloned into a pBo-CMV vector (Takara, Japan) with a Kozak sequence (CCACC) in front of the 1st methionine codon.

The wild-type or the substituted constructs were transfected into HEK293T cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) and plated onto 12 multiplates, as described previously [4].

2.3. Cell cultures

HEK293T cells were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The cells were cultured in DMEM containing 10% (v/v) FBS (Life Technologies) in a humidified air/CO_2 (19:1) atmosphere.

2.4. Measurement of intracellular calcium

The change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using a fura-2 method as described previously [22,23]. The changes in the intensities of 540 nm fluorescence obtained by 340 nm and 380 nm excitations were monitored by an FP-8200 spectrofluorometer (JASCO, Tokyo, Japan).

2.5. Dual luciferase reporter assay

cAMP response element (CRE)-, serum response element (SRE)-, or nuclear factor of activated T-cells (NFAT)-driven promoter activity was assayed using the PathDetect Signal Transduction Pathway cis-Reporting Systems (Agilent Technologies, Santa Clara, CA) as described in the previous paper [4].

2.6. Reverse transcriptase (RT) polymerase chain reaction (PCR)

Total RNA was extracted from each 10 embryos or baby fishes at 3, 24, 48, 72, and 96 h post fertilization (hpf). RT-PCR was carried out as follows: preheat at 95 °C for 4 min, then proceed to 34 cycles (OGR1 and GPR4) or 30 cycles (β-actin) at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. The forward primers were CGGGACTGCAACTTCATTGAG for zOGR1, GAAGTGAGACCATGTGCAAC for zGPR4, and GTGATGGACTCTGGTGATGGTGT for zβ-actin. The reverse primers were AGTGGAGTGTGTGTTGAACCTTC for zOGR1, AGAGGTCTGCTATCGAGAGGTTC for zGPR4, and TGAAGCTGTAGCCTCTCTCGGTC for zβ-actin. The expected size of each product was 204 bp for zOGR1, 201 bp for zGPR4, and 148 bp for zβ-actin.

2.7. Data presentation

All the experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the mean ± SE from more than three different batches of cells unless otherwise stated. Statistical significance was assessed by ANOVA; values were considered significant at $p < 0.05$ (*).

3. Results

3.1. Zebra fish OGR1 and GPR4 homologs sense protons to activate multiple signaling pathways

Transient expression of wild-type zebra fish OGR1 (zOGR1) and GPR4 (zGPR4) homologs in HEK293T cells induced CRE-, SRE-, and NFAT-driven transcriptional activation when the extracellular pH was reduced from 7.6 to 6.3, indicating that these homologs sense protons to activate multiple signaling pathways (Fig. 1A, B). The activated signaling pathways were different between zOGR1 and zGPR4: zOGR1 activates all three (CRE, SRE, and NFAT) pathways. On the other hand, zGPR4 activates CRE and SRE pathways but not the NFAT pathway.

In the previous study [4], we showed that CRE, SRE, and NFAT promoters were activated through the G_s-protein/adenylyl cyclase/cAMP signaling pathway, $G_{12/13}$-protein/Rho signaling pathway, and G_q-protein/phospholipase C–Ca^{2+} signaling pathway in HEK293T cells, respectively. In agreement with this, the $[Ca^{2+}]_i$ was increased, which reflects phospholipase C activation when extracellular pH was reduced from 7.6 to 6.3 in the zOGR1 expressed cells (Fig. 1C). On the other hand, the $[Ca^{2+}]_i$ concentration in the zGPR4 expressed cells was not significantly increased from that in vector-transfected HEK293 cells at pH 6.3 (Fig. 1C). The significant activation of CRE and SRE promoters was observed in the zOGR1 and zGPR4 expressed cells, even at a neutral pH of 7.4 (Fig. 1A, B). Thus, zOGR1 and zGPR4 are stimulated under neutral pH as if the receptors had constitutive activity.

3.2. The similar position of histidine residue in zOGR1 and zGPR4 to that of human receptors is involved in the receptor activation

The histidine residues in the extracellular surface of human OGR1 and GPR4 are crucial for sensing protons [2,5]. The amino acid identity between human and zebra fish OGR1 is 57%, and that between human and zebra fish GPR4 is 73%. The four residues of the five crucial histidine residues (depicted as H in bold type in Fig. 2A) to sense protons in human OGR1 are conserved in zOGR1. To clarify that the histidine residues of zOGR1 play a role to sense protons, we made a construct in which the four histidine residues of the 12th,

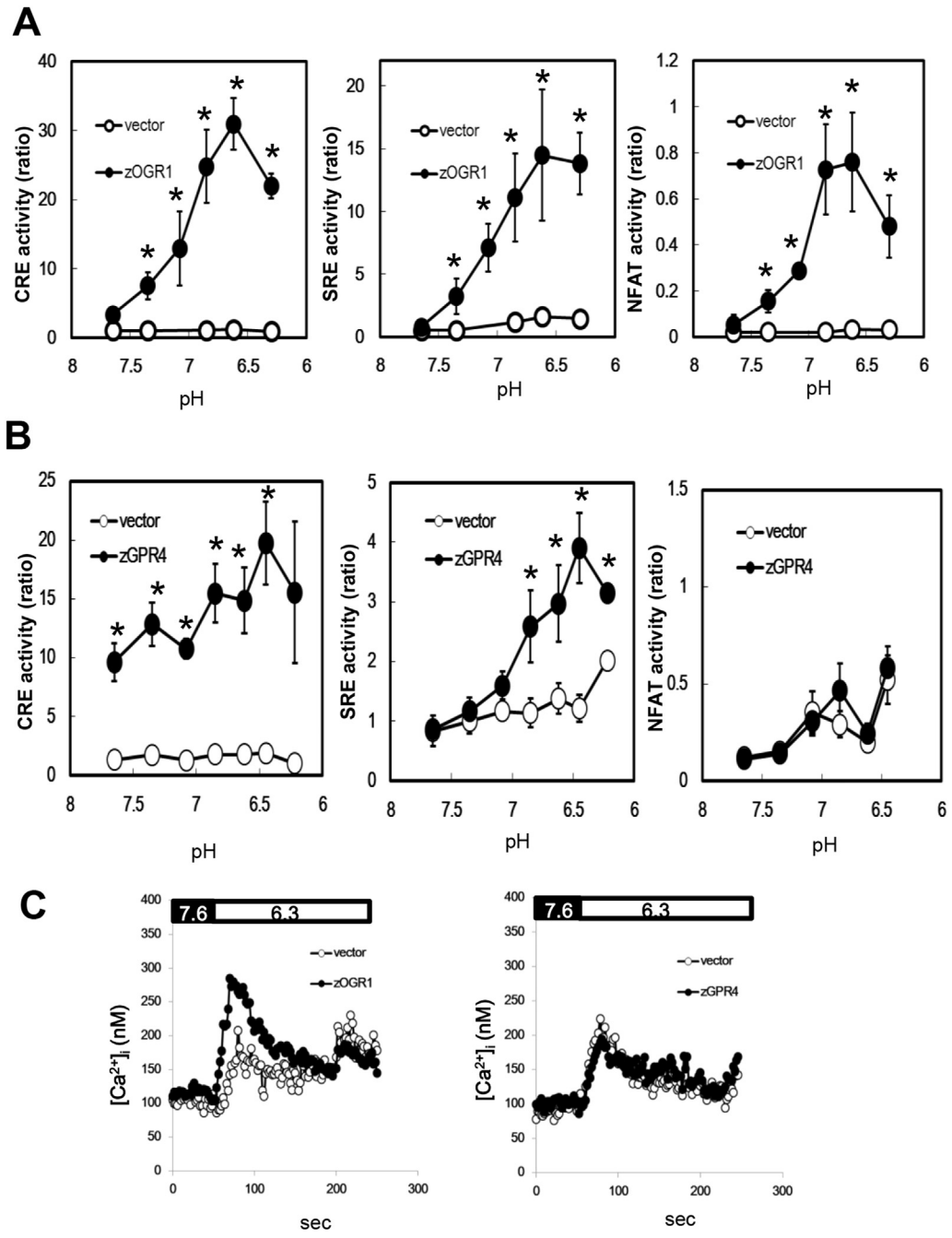

Fig. 1. Extracellular acidic pH-induced CRE-, SRE-, and NFAT-driven transcriptional activation (A, B) and an increment of $[Ca^{2+}]_i$ (C) in zOGR1-and zGPR4-transfected HEK293T cells. In A and B, HEK293T cells were transiently transfected with wild-type zOGR1 and zGPR4 (closed circle) expression plasmids and a pBo-CMV vector (open circle) together with pRL-TK and pCRE-, pSRE-, or pNFAT-luc. The cells were incubated for 6 h at the indicated pH to measure CRE-, SRE-, and NFAT-promoter activities. See "Materials and methods" for more detail. Results are means ± SE. The asterisk (*) indicates that the effects of zOGR1 and zGPR4 were significant under the indicated pH. In C, the cells were harvested from the dishes, and $[Ca^{2+}]_i$ was measured. The typical trace of $[Ca^{2+}]_i$ change by acidic pH (pH 6.3) was shown in the vector-(open circle), zOGR1-, and zGPR4-(closed circle) transfected cells. Two other experiments yielded similar results.

15th, 162th, and 264th positions from the N terminus of zOGR1 were substituted with phenylalanine (H4F) and examined the SRE-promoter activities upon reducing extracellular pH from 7.6 to 6.3. As shown in the left panel of Fig. 2C, the mutant showed little activity. Thus, the histidine residues, which are crucial for sensing protons in human OGR1, are conserved in zOGR1. Next we examined whether the similar position of histidine residue is responsible for proton sensing. Based on the result of human GPR4 [5], the histidine residue of the 78th amino acid from the N terminus of zGPR4 is supposed to play a role to sense protons. On the other hand, the histidine residue of the 84th amino acid may be not to play that role. We made two different mutants in which the histidine residues at the 78th and 84th positions were substituted with phenylalanine (H78F and H84F, respectively) and examined their activity. As expected, the H78F mutant showed reduced SRE-promoter activity compared with the activity of wild-type zGPR4; however, the activity of the H84F mutant was comparable to the activity of the wild type (Fig. 2C, right panel). This suggests that the histidine position for proton sensing is conserved between the human and zebra fish receptors.

A

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H.sapiens       MGNITADNSSMSCTIDHTIHQTLAPVVYVTVLVVGFPANCLSLYFGYLQIKARNELGVYL 60
D.rerio         -----MSEEQINCTINHDIHQYLFSVAYLLVLLVGLPANAYSLYHAWLQLKARNELGVYL 55

H.sapiens       CNLTVADLFYICSLPFWLQYVLQHDNWSHGDLSCQVCGILLYENIYISVGFLCCISVDRY 120
D.rerio         LNLTISDLLYLGSLPLWLQYIFQGDNWSGSEWLCQLCGFLLYENIYVSIGFLCCISIDRY 115

H.sapiens       LAVAHPFRFHQFRTLKAAVGVSVVIWAKELLTSIYFLMHEEVIEDENQHRVCFEHYPIQA 180
D.rerio         LAVVYPFRFSAFRTVRAATLVSTVVWLKELAVGVVFFLHKELSRDKHNQSVCFEHYPMKT 175

H.sapiens       WQRAINYYRFLVGFLFPICLLLASYQGILRAVRRSHGTQKSRKDQIQRLVLSTVVIFLAC 240
D.rerio         WEYQINYYRFYIGFLFPLGILSVSYFRVLRAVGKSAGTQTSQKIRIKYLVTSTIVIFLVC 235

H.sapiens       FLPYHVLLLVRSVWEASCDFAKGVFNAYHFSLLLTSFNCVADPVLYCFVSETTHRDLARL 300
D.rerio         FSPYHIFLLVRTIFERDCNFIESIFNYYHFSLLLTSFNCVADPALYCFISESAQKGIQKA 295

H.sapiens       RGACLAFLTCSRTGRAREAYPLGAPEASGKSGAQGEEPELLTKLHPAFQTPNSPGSGGFP 360
D.rerio         HDACTRVFCCCSKSHGRFNTHSTELAVTNDNVTGTSVVTLLQQIKTEV---------- 343

H.sapiens       TGRLA 365
D.rerio         -----
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B

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H.sapiens       ---------MGNHTWEGCHVDSRVDHLFPPSLYIFVIGVGLPTNCLALWAAYRQVQQRNE 51
D.rerio         MASLRGSETMCNITTS-CNVDSNIDQFFQPTLYIIVIVLGFPTNCMALWAAYMQVKQKNE 59

H.sapiens       LGVYLMNLSIADLLYICTLPLWVDYFLHHDNWIHGPGSCKLFGFIFYTNIYISIAFLCCI 111
D.rerio         LGIYLMNLSIADLLYITTLPLWIDYFVHHDNWIHGQVSCKLFGFIFYTNIYVSIAFLCCI 119
                                          78    84

H.sapiens       SVDRYLAVAHPLRFARLRRVKTAVAVSSVVWATELGANSAPLFHDELFRDRYNHTFCFEK 171
D.rerio         SVDRYLAVAHPLKFAKVRRVKTALLVSAIVWLTEIVANSAPLFHDELFQDRFNHTFCFEK 179

H.sapiens       FPMEGWVAWMNLYRVFVGFLFPWALMLLSYRGILRAVRGSVSTERQEKAKIKRLALSLIA 231
D.rerio         YPMEPWVAGMNLYRTFLGFLAPWGIMLAAYRGILRAVRGNVSTERQEKAKIKRLALSLIL 239

H.sapiens       IVLVCFAPYHVLLLSRSAIYLGRPWDCGFEERVFSAYHSSLAFTSLNCVADPILYCLVNE 291
D.rerio         IVLLCFAPYHVLLLWRSVLFLINPCDCGGEENLFGAYHVTLALTSLNCVADPILYCFVNE 299

H.sapiens       GARSDVAKALHNLLRFLASDK-PQEMANASLTLETPLTSKR-NSTAKAMTGSWAATPPSQ 349
D.rerio         GARHDVGRALATLLGLFQRGKSPETLMGASITVETPLAVKKPDFYSEVKTNAYKNDIEVL 359

H.sapiens       GDQ-VQLKMLPPAQ 362
D.rerio         KDECLQMTILSVKK 373
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C

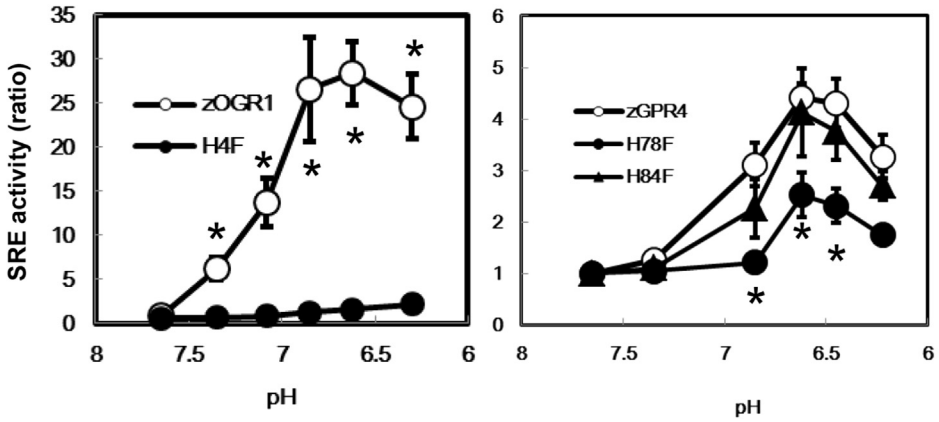

Fig. 2. Amino acid sequence alignment between OGR1 (A) and GPR4 (B) of humans (H.sapiens) and zebra fish (*D. rerio*) and the effects of the substitution of histidine with phenylalanine in zOGR1 and zGPR4 on proton-induced SRE-promoter activity (C). The histidine (H), which is concerned with the proton sensing of human OGR1 and GPR4, is depicted in bold type. All the corresponding histidines of zOGR1 were substituted with phenylalanine (H4F). The number in B indicates the mutant, whose histidine was substituted with phenylalanine in zGPR4 (H78F or H84F). HEK293T cells were transiently transfected with wild-type zOGR1, zGPR4 together with pRL-TK, and pSRE-luc. The cells were incubated for 6 h at the indicated pH to measure SRE-promoter activity. Results are means ± SE. The asterisk (*) indicates that the activities of H4F and H78F were significantly different from those of the wild-type receptors under the indicated pH.

3.3. LPC and SPC do not seem to mediate SRE activation in the zOGR1 and zGPR4 overexpressed cells

LPC and SPC modulate the mammalian OGR1-and GPR4-mediated cell responses [3,6,11,24–28]. We next examined whether these lysolipids modulate the zOGR1-and/or GPR4-mediated SRE-promoter activation. As shown in the right panels of Fig. 3A and B, SPC at 10 μM induced SRE-promoter activation; however, similar activation was also detected in the vector-transfected cells. Thus, SPC does not modulate zOGR1-and zGPR4-mediated SRE-reporter activation. LPC at 10 μM did not stimulate SRE-promoter activation (Fig. 3A and B, left panels).

3.4. zOGR1 and zGPR4 were expressed in zebra fish

We finally examined the gene expression of zOGR1 and zGPR4 at different developmental stages of zebra fish embryogenesis (Fig. 4). Both receptors were expressed in zebra fish; however, the expression pattern was different, i.e., the transcript of zOGR1 was detected in the stages from 24 to 96 hpf. On the other hand, the transcript of zGPR4 was present in all the analyzed stages from 3 to 96 hpf.

4. Discussion

In the present study, we show for the first time that OGR1 and GPR4 homologs of zebra fish sensed protons and activated the multiple signaling pathways (Fig. 1A and B). Thus, zOGR1 and zGPR4 are proton-sensing GPCRs, as are human OGR1 and GPR4; however, the NFAT-reporter activation profile was different between zebra fish and human GPR4. In the previous study [4,5], extracellular acidification induced NFAT-driven transcriptional activity of human GPR4; however, the transcriptional activity of zGPR4 was not attenuated at any pH tested (Fig. 1B). It may be due to a difference of coupling efficiency to human $G_{q/11}$ between human and zebra fish GPR4. It should be investigated in the future. The high CRE-reporter activation of zGPR4 was detected even at pH 7.6 and was further enhanced by extracellular acidification (Fig. 1B). The increment of cAMP accumulation is also detected even at pH 7.6 in the original report [2]. This is not due to the effect of overexpression of GPR4. The high cAMP accumulation at pH 7.8 is reported in the kidney cells [29].

The specific histidine residues of human OGR1 and GPR4 play a crucial role in sensing extracellular protons [2,5,30]. Five histidine residues (depicted as H in bold type in Fig. 2A) are responsible for sensing and activating the signaling pathways in human OGR1 [14,31]. Four of the five corresponding positions of histidine residues are conserved in zOGR1 (Fig. 2A). In this study, the mutant that was substituted in these four histidine residues with phenylalanine failed to stimulate the SRE-reporter (Fig. 2C, left panel). This result suggests that the activation mechanisms are similar between human and zebra fish OGR1. We confirmed the similarity of the receptor activation between human and zebra fish using the zGPR4 mutants (Fig. 2B). As shown in Fig. 2C, the mutant of the 78th but not of 84th position attenuated acidic pH-induced SRE-reporter activation. This result is the same as the result of human GPR4 [5]. The similarity between the human and zebra fish receptors

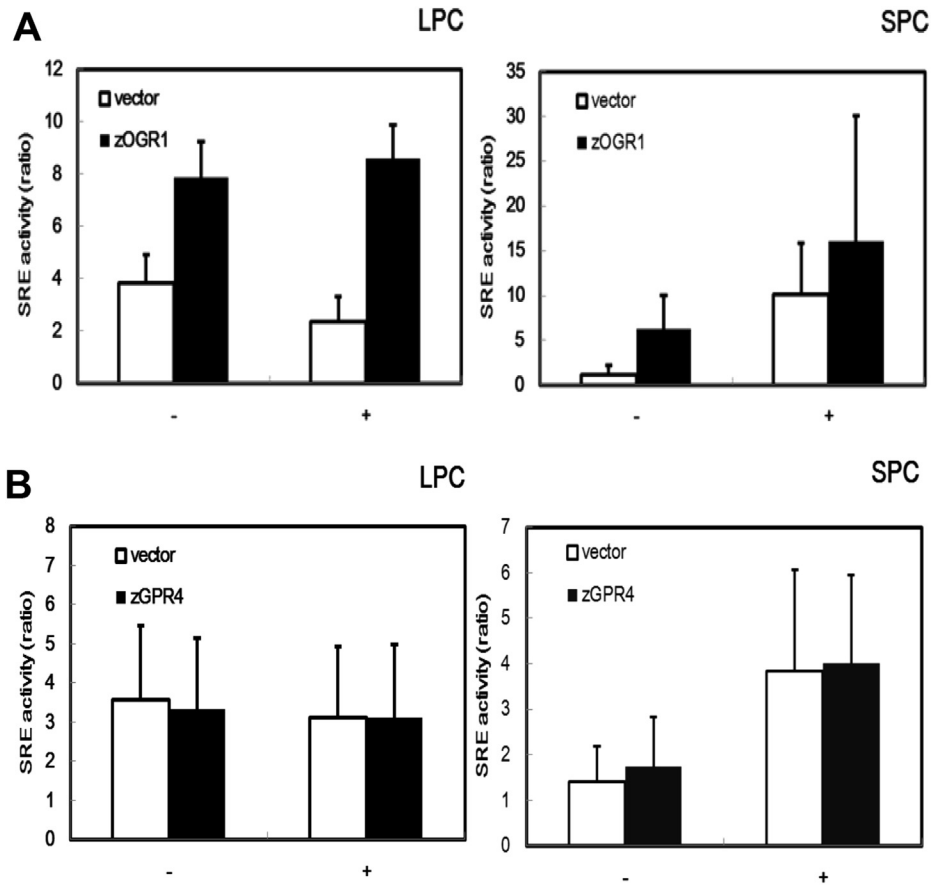

Fig. 3. Effect of SPC and LPC on SRE-promoter activity in zOGR1 (A) and zGPR4 (B) overexpressed HEK292T cells. HEK293T cells were transiently transfected with wild-type zOGR1 and with zGPR4 together with pRL-TK and pSRE-luc. The cells were incubated for 6 h in the presence (+) or absence (−) of 10 μM LPC or 10 μM SPC to measure SRE-promoter activity. Results are means ± SE of six determinations from two separate experiments.

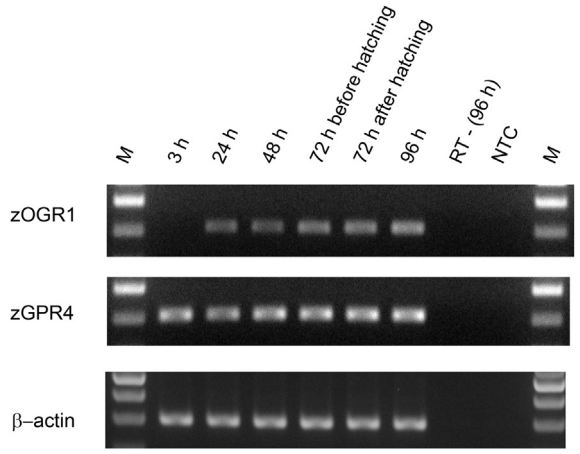

Fig. 4. Expression pattern of zOGR1 or zGPR4 transcripts during zebra fish embryogenesis. RT-PCR reactions were performed at different stages of embryogenesis. The developmental stage was depicted as hours post fertilization (h) on top of the figure. β-actin was used as a control of RT-PCR. RT-indicates a negative PCR control reaction without reverse transcriptase reaction. NTC also indicates a negative control reaction without RNA. M indicates a 100 bp molecular weight marker. The expected size of zOGR1 is 204 bp, and that of zGPR4 is 201 bp.

suggests that we can use zebra fish for the screening of chemical compounds that will have agonist and/or antagonist activity with human OGR1 and GPR4.

Human OGR1 and GPR4 were originally reported as the receptors for SPC and LPC; however, the report was retracted. In this study, we examined the possibility of whether these lysolipids stimulate the zOGR1-and zGPR4-mediated pathways. As shown in Fig. 3, LPC did not stimulate the zOGR1-or zGPR4-induced reporter activity. On the other hand, SPC stimulated SRE-promoter activation; however, similar activation was also detected in vector-transfected cells. This zOGR1-and zGPR4-independent SPC action may be partly mediated by endogeneous S1P receptors [4].

zOGR1 and zGPR4 were expressed in various developmental stages of zebra fish (Fig. 4). These receptors may play physiological and pathophysiological common roles in vertebrates. The physiological and pathophysiological roles of OGR1 and GPR4 have been examined using OGR1-and GPR4-deficient mice. OGR1-deficient mice show tumor growth inhibition [32], enhanced proton extrusion in the proximal tube of the kidney [14], and decreased insulin secretion from the islet [33]. GPR4-deficient mice show vascular abnormality [12], reduced angiogenesis and tumor growth [34], decreased acid secretion from the kidney [29], and improved glucose tolerance and insulin sensitivity [35]. However, the molecular mechanism by which the receptors are concerned with the phenotype is largely unknown.

Zebra fish have been extensively used for in vivo imaging, especially in the studies of blood vessel formation [20] and cancer invasion [21]. Since OGR1-and GPR4-deficient mice show the receptors' effect on tumor growth and vascular formation, as described above, zebra fish studies will provide useful information for the physiological and pathophysiological roles of OGR1 and GPR4 in the future.

Disclosure statement

The authors have declared no conflicts of interest.

Acknowledgments

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