Action of Vitamin D and the Receptor, VDRa, in Calcium Handling in Zebrafish (Danio rerio)

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Abstract

The purpose of the present study was to use zebrafish as a model to investigate how vitamin D and its receptors interact to control Ca2+ uptake function. Low-Ca2+ fresh water stimulated Ca2+ influx and expressions of epithelial calcium channel (ecac), vitamin D-25-hydroxylase (cyp2r1), vitamin D receptor a (vdrα), and vdrb in zebrafish. Exogenous vitamin D increased Ca2+ influx and expressions of ecac and 25-hydroxvitamin D3-24-hydroxylase (cyp24a1), but downregulated 1α-OHase (cyp27b1) with no effects on other Ca2+ transporters. Morpholino oligonucleotide knockdown of VDRA, but not VDRb, was found as a consequence of calcium uptake inhibition by knockdown of ecac, and ossification of vertebrae is impaired. Taken together, vitamin D-VDRα signaling may stimulate Ca2+ uptake by upregulating ECaC in zebrafish, thereby clarifying the Ca2+-handling function of only a VDR in teleosts. Zebrafish may be useful as a model to explore the function of vitamin D-VDR signaling in Ca2+ homeostasis and the related physiological processes in vertebrates.

Introduction

In vertebrates, one of physiological roles of Ca2+ is its involvement in bone formation. Decrease of renal Ca2+ reabsorption and intestinal Ca2+ absorption is an important factor causing osteoporosis [1]. Vanoevelen et al (2011) provided both genetic and functional evidence that transcellular epithelial Ca2+ uptake is vital to sustain life and enable bone formation [2]. Therefore, regulating Ca2+ uptake is highly essential to vertebrate life. The major source of Ca2+ in terrestrial vertebrates is from food. Fish, unlike terrestrial vertebrates, continually face ambient water with variable Ca2+ levels and absorb Ca2+ from the surrounding water. In adult fish, the predominant route of Ca2+ entry from the environment is across the gill epithelium, while in larvae, the body skin is the major route of Ca2+ uptake before full development of the gills occurs [3,4]. Both terrestrial and aquatic vertebrates share similar mechanisms of Ca2+ uptake in specific cells and organs. According to the current model in mammals and teleosts, active transcellular Ca2+ transport is carried out through the operation of apical epithelial Ca2+ channels (ECaC), and the basolateral plasma membrane Ca2+-ATPase (PMCA) and Na+/Ca2+ exchanger (NCX) [5-8].

Vitamin D is well documented as vital endocrine regulating Ca2+ uptake in mammals. A vitamin D precursor is initially synthesized in the skin. Through a series of reactions, vitamin D-25 hydroxylase (CYP2R1) converts the vitamin-D precursor into 25-hydroxyvitamin D3 (25(OH)D3), which is then converted to 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3), the active form of vitamin D, by renal 1α-OHase (CYP27B1) [9]. The 1α,25(OH)2D3 level is modulated by 25-hydroxyvitamin D3-24-hydroxylase (CYP24A1). CYP24A1, a mitochondrial enzyme in target cells, functions to degrade 1α,25(OH)2D3 [10]. Both the endocrine synthesis of 1α,25(OH)2D3 in the kidneys and degradation of this hormone at peripheral sites are associated with the homeostasis of 1α,25(OH)2D3 in mammals. Although 1α,25(OH)2D3 was also detected in the lamprey, one of the earliest vertebrate lacking a calcified skeleton and teeth, it was found to play a non-calcemic role there [11]. Those results imply that vitamin D α may initially have evolved a Ca2+ regulatory function in bony vertebrates. From evolutionary and physiological points of view, teleosts have been an important model to explore the hypothesis of whether vitamin D also has a calcemic function in bony vertebrates because Ca2+ uptake mechanisms of teleosts were demonstrated to be similar to those of mammals as described above. In teleosts 1α,25(OH)2D3 was also demonstrated to be produced by renal tissues and the liver [12-14], and CYP24A1, CYP2R1, and CYP27B1 were also identified in teleosts [15-17]. Vitamin D was reported to elevate the serum Ca2+ level in carp and cod [18,19]. Sea bream with a vitamin D-deficient diet showed reduced growth and lower Ca2+ turnover [20]. Changes in 1α,25(OH)2D3 concentrations and expressions of vitamin D receptor (VDR) were noted in Atlantic salmon undergoing smoltification and migrating from fresh water (low Ca2+ concentrations) to seawater (with high Ca2+ concentrations), suggesting that regulation of the synthesis of 1α,25(OH)2D3 and VDR is dependent upon ambient Ca2+ concentrations [21]. However, detailed mechanisms of how vitamin D regulates the Ca2+ uptake function in teleosts are still largely unclear.

The vitamin D receptor (VDR), a ligand-activated transcription factor, forms a vitamin D3-VDR complex upon binding with vitamin D. This complex could upregulate mammalian intestinal...
control of epithelial Ca²⁺ absorption, which is an important pathway for controlling Ca²⁺ homeostasis in mammals [9]. Recently in an ecac-defective zebrafish mutant, Vanoelen et al. [2] demonstrated the importance of ECaC in bone formation. In the ecac promoter region of zebrafish and fugu (Takifugu rubripes), putative VDREs were also identified by a bioinformatics analysis [17,23]. However, there is still no molecular physiological evidence to clarify target cells (ionocytes) or transporters (ECaC, NCX, and PMCA) that are regulated by vitamin D-VDR signaling pathway in teleosts. Telectosts have 2 paralogous VDR because of whole-genome duplication [24,25]. Although most gene-duplication events are non-functional and eventually result in gene loss, about 20%–50% of paralogous genes are conserved as one of the duplicates acquires a new function or subfunction [26]. Exploring if 2 paralogous VDRs have different roles in calcemic regulatory functions in teleosts has been a challenging issue. Mammalian cell lines expressing teleost VDRs were found to induce transcription of VDR-containing expression constructs with 1α,25(OH)₂D₃ [24,27,28]. On the other hand, 2 isoforms of medaka VDR showed different responses to 1α,25(OH)₂D₃ and induced different transcripts of VDR-containing expression constructs in cell lines [24]. These studies in vitro suggested that VDRs are activated by 1α,25(OH)₂D₃ and the 2 paralogous VDRs may undergo functional divergence in teleosts; however, these notions lack in vivo molecular/physiological evidence to support them.

Zebrafish with a well-established genomic database and advantages of morpholino gene knockdown technique is a competent model for research on ion regulation and related endocrine controls [3,17,29–31]. In zebrafish gills and, in embryonic stages, tent model for research on ion regulation and related endocrine advantages of morpholino gene knockdown technique is a competitive support them.

These notions lack in vivo molecular/physiological evidence to clarify the molecular physiological mechanisms of vitamin D control zebrafish Ca²⁺ uptake function [3,4,8,32,33], and this provides an excellent platform to further explore vitamin D’s control of Ca²⁺ uptake mechanisms. The purpose of the present study was to use zebrafish to clarify the molecular physiological mechanisms of vitamin D control of epithelial Ca²⁺ transport in teleosts. Experiments were designed to address 2 specific questions: (1) does the vitamin D system control zebrafish Ca²⁺ uptake function by regulating the Ca²⁺ transporters, ecac, ncx1b, and/or pma25; and (2) does vitamin D control zebrafish Ca²⁺ uptake function through 1 or both of the paralogous VDRs, V德拉 and VDRb? Hence, the effects of environmental Ca²⁺ levels and exogenous vitamin D on Ca²⁺ contents and influx, and the mRNA expressions of Ca²⁺ transporters (ecac, ncx1b, and pma25) and vitamin D-related genes (vdra, vдрb, cyp24a1, cyp2r1, and cyp27a1) were investigated. Moreover, the effects of knockdown of V德拉 or VDRb on Ca²⁺ contents/influx and the expression of Ca²⁺ transporters and Ca²⁺-regulatory endocinies in zebrafish embryos were also examined.

Methods

Experimental Animals

The wild-type AB strain of zebrafish (Danio rerio) were kept in local tap water (Ca²⁺) of 0.2 mM) at 28.5°C under a 14:10-h light-dark photoperiod at the Institute of Cellular and Organismic Biology, Academia Sinica, Taipie, Taiwan. Experimental protocols were approved by the Academia Sinica Institutional Animal Care and Utilization Committee (approval no.: RFI-ZOOH220782).

VDR and Ca²⁺ Regulation in Zebrafish

Acclimation Experiments

Artificial fresh water with high- (2 mM) or low-Ca²⁺ (0.02 mM) levels was prepared with double-deionized water (model Milli-RO60; Millipore, MA, USA) supplemented with adequate CaSO₄·2H₂O, MgSO₄·7H₂O, NaCl, KH₂PO₄, and KH₂PO₄. Ca²⁺ concentrations (total calcium levels measured by absorption spectrophotometry) of high- and low-Ca²⁺ media were 2 and 0.02 mM, respectively, but the other ion concentrations of the 3 media were the same ([Na⁺], 0.5 mM; [Mg²⁺], 0.16 mM; and [K⁺], 0.3 mM) as those in local tap water. Variations in ion concentrations were maintained within 10% of the predicted values. Fertilized zebrafish eggs were transferred to high- and low-Ca²⁺ media, respectively, and incubated thereafter until sampling at 3 or 5 d post-fertilization (dpf).

Vitamin D (1α,25(OH)₂D₃) Incubation Experiments

1α,25(OH)₂D₃ (cat. no. 32222-06-3, Sigma, St. Louis, MO, USA) was dissolved in 95% ethanol at 0 (control) and 20 µg/l (0.48 nM). Zebrafish embryos were incubated in 1α,25(OH)₂D₃-containing media immediately after fertilization, and were sampled at 3 dpf for subsequent analysis. Incubation media were changed with a new 1α,25(OH)₂D₃ solution every day to maintain constant levels of 1α,25(OH)₂D₃. During incubation, neither significant mortality nor abnormal behavior was found.

Whole-body Ca²⁺ Content

Three-dpf zebrafish embryos were anesthetized with 0.2% buffered MS-222 (Sigma) and then briefly rinsed in deionized water. Thirty individuals were pooled as 1 sample. HNO₃ (13.1 N) was added to samples for digestion at 60°C overnight. Digested solutions were diluted with double-deionized water, and the total calcium content was measured with a Z-8000 atomic absorption spectrophotometer (Hitachi, Tokyo, Japan). Standard solutions (Merck, Darmstadt, Germany) were used to make the standard curves.

Whole-body Ca²⁺ Influx

By following previously described methods [34] with some modifications, zebrafish embryos were dechorinated, rinsed briefly in deionized water, and then transferred to 2 ml of ⁴⁶Ca²⁺ (Amersham, Piscataway, NJ, USA; with a final working specific activity of 1–2 mCi/mmol)-containing medium for a subsequent 4-h incubation. After incubation, embryos were washed 4 times in fresh water without isotope. Six embryos were pooled into 1 vial, anesthetized with 0.2% buffered MS-222, and digested with tissue solubilizer (Solvable; Packard, Meriden, CT, USA) at 60°C for 8 h. The digested solutions were supplemented with counting solution (Ultima Gold; Packard), and the radioactivities of the solutions were counted with a liquid scintillation beta counter (LS6500; Beckman, Fullerton, CA, USA). The Ca²⁺ influx was calculated using the following formula: \[ \text{Ca}^{2+}\text{in} = \frac{Q_{\text{embryo}}}{X_{\text{out}}} \times \frac{1}{t} \] where \( Q_{\text{embryo}} \) is the influx (pmol/h), \( X_{\text{out}} \) is the specific activity of the incubation medium (cpm/pmol), \( t \) is the incubation time (h). The influx was expressed in pmol/mg⁻¹h⁻¹ by dividing \( Q_{\text{embryo}} \) by the embryo wet weight (mg). Both the data of \( Q_{\text{embryo}} \) and \( W \) were from pooled samples, and the averaged values were used during calculation.

RNA Extraction

After anesthetization with 0.03% MS222, appropriate amounts of zebrafish tissues or embryos were collected and homogenized in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA), then mixed
Reverse-transcription Polymerase Chain Reaction (RT-PCR) Analysis

For complementary (c)DNA synthesis, 1–5 µg of total RNA was reverse-transcribed in a final volume 20 µl containing 0.5 mM dNTPs, 2.5 µM oligo (dT)20, 250 ng random primers, 5 mM dithiothreitol, 40 units RNase inhibitor, and 200 units Superscript RT (Invitrogen) for 1 h at 50°C. For PCR amplification, 2 µl cDNA was used as a template in a 50-µl reaction volume containing 0.25 mM dNTPs, 2.5 units Taq polymerase (Takara, Shiga, Japan), and 0.2 µM of each primer (Table S1). Thirty cycles were performed for each reaction. All amplicons were sequenced to ensure that the PCR products were the desired gene fragments.

Quantitative Real-time (q)PCR

A qPCR was performed with a Light Cycler real-time PCR system (Roche, Penzberg, Germany) in a final volume of 10 µl, containing 5 µl 2x SYBR Green I Master Mix (Roche), 300 nM of the primer pairs, and 20–30 ng cDNA. The standard curve for each gene was checked in a linear range with β-actin as an internal control. The primer sets for the qPCR are shown in Table S1.

Bioinformatic Analysis

All protein sequences were obtained from the Ensembl and NCBI databases. The accession number of the sequence were as follows: zebrafish (Danio rerio) VDRa (NP_570994), zebrafish VDRb (NP_001153457), flounder (Paralichthys olivaceus) VDRa (BAA95016), flounder VDRb (BAA95015), frog (Xenopus laevis) VDR (NP_001079298), chicken (Gallus gallus) VDR (NP_990429), human (Homo sapiens) VDR (BAH02291), medaka (Oryzias latipes) VDRa (NP_001121988), medaka VDRb (NP_001121989), Nile tilapia (Oreochromis niloticus) VDRa (XP_003449167), Nile tilapia VDRb (XP_00341388), Atlantic salmon VDR (NP_001117029), stickleback (Gasterosteus aculeatus) VDRa (EN-SGACT00000006308), stickleback VDRb (EN-SGACT00000010601), seabass (Dicentrarchus labrax) VDRb (CBN80914), mouse (Mus musculus) VDR (NP_0033530), lizard (Anolis carolinensis) VDR (EN-SGACT00000013576), and lamprey (Petromyzon marinus) VDRA (AAP05810). Alignment of the amino-acid sequences was conducted using ClustalW via the SDSC Biology Workbench (http://workbench.sdsc.edu). Phylogenetic analyses were carried out using the Neighbor-joining method. Six hundred bootstrap replicate analyses were carried out with MEGA5.0 software.

In-situ Hybridization

Zebrafish eya3 (NM_0010018149, full length of the open reading frame) or inta (NW_003336067.1, nt114962~115564) fragments were obtained by a PCR and inserted into the pEGT-M easy vector (Promega, Madison, WI, USA). The inserted fragments were amplified with the T7 and SP6 primers by a PCR, and the products were used as templates for in vitro transcription with T7 and SP6 RNA polymerase (Roche) in the presence of digoxigenin (DIG)-UTP (Roche) to synthesize sense and antisense probes, respectively. Zebrafish embryos were anesthetized on ice and fixed with 4% paraformaldehyde (PFA) in a phosphate-buffered saline solution (PBS; 1.4 mM NaCl, 0.2 mM KCl, 0.1 mM Na2HPO4, and 0.002 mM KH2PO4; pH 7.4) at 4°C overnight. To perform the in situ hybridization, we followed a previous description [32]. For quantitative analysis, the numbers of eca-expressing cells in 12 randomly-selected areas (100×100 µm each) on the yolk-sac surface of an embryo were counted.

Morpholino Oligonucleotide (MO) Knockdown

The zebrafish VDRa MO (5’-ACGGGACTATTCTCCGTAAAGATC-3’) and VDRb MO (5’-AACGATCCGATGGAATCACCTGAGCAATCTGAGCAATCTGAC-3’) were prepared with 1x Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, and 5.0 mM HEPES; pH 7.6). A standard control MO (5’-CCTGTTACCTCAAGTTACACATTATA-3’) was used as the control. To analyze the physiological function of VDRa/b under normal development, we chose to inject 4 ng/embryo of the MO into embryos in the following experiments. At this dose, neither significant mortality nor abnormal behavior was found. The MOs (4 ng/embryo) were injected into embryos at the 1~2-cell stage using an IM-300 microinjector system (Narishige Scientific Instrument Laboratory, Tokyo, Japan). MO-injected embryos at 5 dpf were sampled for subsequent analyses.

Western Blot Analysis

Thirty embryos were pooled as 1 sample and homogenized. Protein at 50 µg/well was loaded onto 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 2 h. After separation, proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billericia, MA, USA) at 100 V for 2 h. After being blocked for 1.5 h in blocking buffer, blots were incubated with zebrafish VDRa or VDRb polyclonal antibodies overnight at 4°C, diluted 1:1000 with alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (diluted 1:2500, at room temperature; Jackson Laboratories, USA) for another 2 h. Blots were developed with 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium.

Vertebræ Staining

Zebrafish embryos were incubated in the staining solution (0.2% calcine, Sigma) for 10 min. After incubation, embryos were washed with fresh water, and then euthanized in MS-222. Observations were carried out using a microscope with a green-fluorescence filter set.

Cryosectioning

Fresh zebrafish gills were fixed with 4% PFA at 4°C for 3 h and then immersed in PBS containing 5%, 10%, and 20% sucrose for 15 min each at room temperature. Finally, gills were soaked in a mixed PBS solution (OCT compound; 20% sucrose at 1:2) overnight and embedded with OCT compound embedding medium (Sakura, Tokyo, Japan) at 20°C. Cryosections at 6 µm were made with a cryostat (CM 1900; Leica, Heidelberg, Germany), and these were placed onto poly-L-lysine-coated slides (EM, Hatfield, PA, USA).

Immunocytochemistry

Prepared slides were rinsed in PBS and blocked with 3% bovine serum albumin (BSA) for 30 min. Afterward, slides were first incubated with an α2 monoclonal antibody against the α subunit of avian Na,K-ATPase (NKA) (Hybrdioma Bank, University of Iowa, Ames, IA, USA; 1:600 dilution) overnight at 4°C. Slides were washed twice with PBS and incubated with an Alexa Fluor 568 goat anti-mouse IgG antibody (Molecular Probes, Carlsbad, CA, USA; 1:200 diluted with PBS) for 2 h at room temperature.
Results

Effects of Environmental Ca\(^{2+}\) Levels on Messenger (m)RNA Expressions of Ca\(^{2+}\)-Related Genes

After acclimation for 3 or 5 d in artificial fresh water containing different levels of Ca\(^{2+}\), zebrafish eac, vdra, and cyp24a1 mRNA expressions were significantly stimulated by low-Ca\(^{2+}\) water. On the contrary, pmca2, ncx1b, and cyp27b1 mRNA expressions were not affected by environmental Ca\(^{2+}\) levels. Environmental Ca\(^{2+}\) levels produced different effects on vdbb expression at 3 and 5 dpf. Low-Ca\(^{2+}\) water stimulated vdbb expression in 3-dpf embryo, but vdbb expression was not affected at 5 dpf (Fig. 1A, B). Furthermore, mRNA expressions of eac, pmca2, ncx1b, vdra, and vdbb were also analyzed in adult zebrafish acclimated to low-Ca\(^{2+}\) or high-Ca\(^{2+}\) water. After acclimation for 2d, branchial eac and vdra mRNA expressions of adult zebrafish were significantly stimulated by low-Ca\(^{2+}\) water (Fig. 1C). On the contrary, pmca2, ncx1b, and vdbb mRNA expressions were not affected by environmental Ca\(^{2+}\) levels (Fig. 1C).

Effects of Exogenous 1\(\alpha,25(\text{OH})_2\text{D}_3\) on Ca\(^{2+}\) Influx/contents and mRNA Expressions of Ca\(^{2+}\)-related Genes in Embryos

To test the hypothesis of whether vitamin D affects Ca\(^{2+}\) uptake, zebrafish embryos started to be treated with 1\(\alpha,25(\text{OH})_2\text{D}_3\) at the 1–2-cell stage and lasted 3 d. Compared to the control group (0 \(\mu\)g/l), 1\(\alpha,25(\text{OH})_2\text{D}_3\)-treated groups (20 \(\mu\)g/l) showed significant increases in Ca\(^{2+}\) contents and influxes in 3-dpf embryos (Fig. 2A, B). The qPCR analysis revealed differential effects of 1\(\alpha,25(\text{OH})_2\text{D}_3\) on mRNA expressions of Ca\(^{2+}\) transporters. mRNA expressions of zebrafish ncx1b and pmca2 were not affected by 1\(\alpha,25(\text{OH})_2\text{D}_3\) treatment (Fig. 2C); however, that of eac was significantly upregulated by 1\(\alpha,25(\text{OH})_2\text{D}_3\) in 3-dpf embryos (Fig. 2G). Exogenous 1\(\alpha,25(\text{OH})_2\text{D}_3\) also caused differential effects on mRNA expressions of cyp24a1 and cyp27b1 in zebrafish embryos. The qPCR analysis in 3-dpf embryos showed that exogenous 1\(\alpha,25(\text{OH})_2\text{D}_3\) significantly inhibited the mRNA expression of cyp27b1, but significantly stimulated cyp24a1 expression (Fig. 2C).

Characterization of Zebrafish (z)VDRa and VDRb

zVDRa (NP_570994) and zVDRb (NP_00113457) were identified from NCBI. The 2 VDRs have 453 (zVDRa) and 422 (zVDRb) amino acids (Fig. 3A), and calculated respective identities with medaka VDR\(\alpha\) and VDR\(\beta\), flounder V德拉 and VDRb, frog VDR, lizard VDR, chicken VDR, mouse VDR and human VDR, while zVDRb shared 85%, 87%, 85%, 87%, 67%, 69%, 70%, 69%, and 70% identities, respectively. A further analysis of important regions of the VDR among different species revealed that both zVDRa and zVDRb shared 93%~97% amino-acid sequence identities with others species at the DBD, and 79%~94% identities at the LBD (Table S2). According to the phylogenetic analysis of zVDRa and zVDRb sequences (Fig. 3B), the 2 zebrafish receptor sequences were classified into 2 clades representing teleost V德拉 and VDRb, respectively. However, the 2 teleost VDR groups diverged from those of terrestrial vertebrates (Fig. 3B).

mRNA Expressions of Vдра and Vдрb in Developing Embryos and Tissues

Both vdra and vдрb mRNA expressions were first detected by an RT-PCR at 0 h post-fertilization (hpf) and throughout development (Fig. 4A). The RT-PCR was also used to detect vdra and vдрb mRNA expressions in different tissues. Both vdra and vдрb expressions were detected in all of the tissues examined (Fig. 4B). On the other hand, vdra expression was more dominant than that of vдрb (over 2-fold higher) in all tissues except the testes and ovaries by the qPCR analysis (Fig. 4C).

Effects of V德拉/b Loss-of-function on Ca\(^{2+}\) Contents, Influx, and Transporters in Embryos

To block the endogenous vitamin D signaling pathway, V德拉 and VDRb MOs were used to respectively inhibit translation of zebrafish V德拉 and VDRb. A Western blot analysis was used to demonstrate MO specificity. As a result, V德拉 and VDRb MOs were respectively found to downregulate V德拉 and VDRb protein levels in 3-dpf zebrafish embryos (Fig. S1).

After specificity tests, respective MOs were injected into 1–2-cell-stage embryos. Compared to the control MO, the V德拉 MO caused significant decreases in the Ca\(^{2+}\) content and influx in 3-dpf zebrafish embryos, but the VDRb MO had no effects (Fig. 5A, B). The qPCR assay of mRNA expressions of Ca\(^{2+}\) transporters showed that the V德拉 MO significantly reduced the expression of eac, but did not affect ncx1b or pmca2 mRNA expressions in 3-dpf zebrafish embryos (Fig. 5C). In contrast with the V德拉 MO, the VDRb MO did not affect expressions of ncx1b, pmca2, or eac genes in 3-dpf zebrafish embryos (Fig. 5C). To further support these data, the intensity of eac mRNA signals and density of eac-expressing cells in the skin of zebrafish morphants were analyzed. In situ hybridization showed that the V德拉 MO, but not the VDRb MO, suppressed eac mRNA signals in embryonic skin. The density of eac-expressing cells also only significantly decreased with a V德拉 MO injection (Fig. 5D, E).

Vanoeveren et al. [2] demonstrated that an eac mutant resulted in delayed bone formation in zebrafish. In the present study, V德拉/b morphants showed different effects on eac expression and Ca\(^{2+}\) regulation (Fig. 5), and therefore subsequent experiments were designed to test if the V德拉 was involved in bone formation in zebrafish. According to results of vertebrae staining, the V德拉 MO delayed ossification of vertebrae in morphants at 5 dpf, but the VDRb MO did not show a significant effect (Fig. S2), supporting the above results of different functions of the 2 VDR paralogs.

Effects of V德拉/b Loss-of-function on Ca\(^{2+}\) Influx and Ecac mRNA Expression in Embryos Treated with 1\(\alpha,25(\text{OH})_2\text{D}_3\) or Exposed to Low-Ca\(^{2+}\) Medium

To precisely ascertain the different roles of zebrafish V德拉和VDRb, zebrafish were incubated with or without 1\(\alpha,25(\text{OH})_2\text{D}_3\) (20 \(\mu\)g/l) after injecting the MOs. Compared to the control group...
(control MO injection without \(1\alpha,25(\text{OH})_{2}\text{D}_3\), both groups of the control MO with \(1\alpha,25(\text{OH})_{2}\text{D}_3\) and the VDRb MO with \(1\alpha,25(\text{OH})_{2}\text{D}_3\) exhibited significantly higher \(\text{Ca}^{2+}\) influxes at 3 dpf, but VDRa MO-injected embryos with \(1\alpha,25(\text{OH})_{2}\text{D}_3\) showed no difference (Fig. 6A). Similarly, \(\text{ecac}\) mRNA expression in the control MO with \(1\alpha,25(\text{OH})_{2}\text{D}_3\) and the VDRb MO with \(1\alpha,25(\text{OH})_{2}\text{D}_3\) was significantly stimulated, while that of the VDRa MO-injected embryos was not affected by \(1\alpha,25(\text{OH})_{2}\text{D}_3\) (Fig. 6B).

Low-\(\text{Ca}^{2+}\) medium is known to stimulate \(\text{Ca}^{2+}\) influx and \(\text{ecac}\) expression in zebrafish [32,33] (Fig. 6C, D). Whether this \(\text{Ca}^{2+}\) influx and \(\text{ecac}\) expression were upregulated by low-\(\text{Ca}^{2+}\) medium as mediated by VDRa or VDRb was further clarified in the following experiments. Embryos at the 1–2-cell stage were respectively injected with the control MO, VDRa MO, and VDRb MO, and then incubated in 2.0 mM (high) or 0.02 mM (low) \(\text{Ca}^{2+}\) medium. Compared to the control MO in low-\(\text{Ca}^{2+}\) medium, VDRa morphants in low-\(\text{Ca}^{2+}\) medium had significantly lower \(\text{Ca}^{2+}\) influx and \(\text{ecac}\) mRNA expression at 3 dpf, but VDRb morphants in low-\(\text{Ca}^{2+}\) medium had similar values to the control group (Fig. 6C, D).

**Colocalization of VDRa with Na\(_K\)-ATPase-rich (NaR) Cells**

There are at least three subtypes of ionocytes, NaR (\(\text{Na}^+\)-\(\text{K}^+\)-ATPase-rich) cells, HR (\(\text{H}^+\)-ATPase-rich) cells, and NCC (\(\text{Na}^+\)/\(\text{Cl}^-\) cotransporter expressing) cells in zebrafish gill/skin ionocytes [4]. NaR cells, which expresses ECaC, PMCA2, and NCX1b, is mainly responsible for the \(\text{Ca}^{2+}\) uptake function in the skin of developing embryos and gills of adults [4]. To reinforce the above molecular physiological evidence for the involvement of vitamin D-VDRa signaling in zebrafish \(\text{Ca}^{2+}\) uptake function, we further tested the hypothesis of whether VDRa is expressed in NaR ionocytes by double in situ hybridization/immunocytochemistry.
for vdra mRNA and Na,K-ATPase (a marker of NaR cells) in zebrafish gills. As shown in Fig. S3, most of the vdra mRNA signals were colocalized in NaR ionocytes that were labeled with Na,K-ATPase.

Effects of VDRa/b Loss-of-function on mRNA Expressions of cyp27b1 and cyp24a1 in Embryos Treated with 1α,25(OH)2D3

The above experiments (Fig. 2) showed different effects of exogenous 1α,25(OH)2D3 on expressions of cyp27b1 and cyp24a1 in zebrafish embryos. A subsequent experiment was designed to further clarify if 1α,25(OH)2D3 regulates expressions of cyp27b1 and cyp24a1 through mediation by the VDR in zebrafish as in mammals [35–37]. Embryos at the 1–2-cell stage were injected with the control MO, VDRa MO, and VDRb MO, respectively, and then incubated without or with 20 μg/l 1α,25(OH)2D3. The VDRb MO could not neutralize effects of exogenous 1α,25(OH)2D3 on mRNA expressions of cyp27b1 and cyp24a1 in 3-dpf zebrafish embryos (Fig. 7A, B). On the contrary, the VDRa MO modulated cyp27b1 and cyp24a1 expressions (Fig. 7A, B). The VDRa MO neutralized both 1α,25(OH)2D3-downregulated cyp27b1 mRNA expression (Fig. 7A) and 1α,25(OH)2D3-upregulated cyp24a1 mRNA expression in zebrafish embryos (Fig. 7B).

Discussion

Besides the source from food and drinking, teleostean fish actively absorb Ca2+ from the aquatic environment with fluctuating in Ca2+ levels [5]. Therefore, body fluids Ca2+ homeostasis and bone formation (particularly in embryonic and larval stages) in freshwater teleosts must be strictly regulated to cope with a fluctuating environment. Vitamin D increased plasma calcium levels in cod [19] and caused dose-dependent hypercalcemia in carp [18]. Lock et al. (2007) suggested a crucial role of the vitamin D system in Ca2+ handling in Atlantic salmon because 1α,25(OH)2D3 concentrations and VDR mRNA expressions changed in salmon undergoing smoltification and migrating from freshwater (low calcium concentrations) to seawater (high calcium concentrations) [21]. Like other teleosts, zebrafish can enhance...
their Ca\textsuperscript{2+} uptake function by stimulating ECaC expression during acclimation to low-Ca\textsuperscript{2+} fresh water [32–34], and this functional regulation was further demonstrated to be mediated by vitamin D-VDR signaling in the present study. Low-Ca\textsuperscript{2+} fresh water stimulated mRNA expressions of \textit{ecac}, \textit{vdra}, \textit{vdrb}, and \textit{vitamin D-25hydroxylase} (\textit{cyp2r1}) in 3- and/or 5-dpf zebrafish embryos, implying a possible role of vitamin D in the functional control of Ca\textsuperscript{2+} uptake in zebrafish. To test this hypothesis, we treated zebrafish with exogenous 1\textalpha,25(OH)\textsubscript{2}D\textsubscript{3}, which was found to stimulate the mRNA expression of \textit{ecac} and Ca\textsuperscript{2+} influx and result in increased total calcium contents in the whole body. The following experiments from the molecular to the physiological level demonstrated the calciotropic effects of vitamin D in zebrafish.

Qiu et al., (2007) showed that 1\textalpha,25(OH)\textsubscript{2}D\textsubscript{3} stimulated branchial \textit{ecac} mRNA in trout, but they did not attempt to examine other Ca\textsuperscript{2+} transporters (NCX or PMCA) [38]. In zebrafish, a specific type of ionocyte that expresses ECaC, PMCA2, and NCX1b were identified to be responsible for the transepithelial Ca\textsuperscript{2+} uptake function [3,4,8,32,33], providing a suitable model to identify the exact target transporter(s) of vitamin D in Ca\textsuperscript{2+} uptake mechanisms. The present study first reports that 1\textalpha,25(OH)\textsubscript{2}D\textsubscript{3} only regulates the expression of \textit{ecac} but not that of \textit{ncx1b} or \textit{pmca2}. These results support a previous notion that ECaC is the major regulatory player in the epithelial Ca\textsuperscript{2+} uptake pathway in fish [4] as in mammals [39]. Similarly, both hypercalcemic cortisol and hypocalcemic stanniocalcin 1 (STC1) were also found to control Ca\textsuperscript{2+} influx by regulating the expression of \textit{ecac}, but neither affected that of \textit{ncx1b} or \textit{pmca2} in zebrafish [17,29]. \textit{ecac} appears to be the major regulatory target transporter gene not only in response to environmental Ca\textsuperscript{2+} but also in control pathways of related hormones in zebrafish.

Most physiological functions of vitamin D signaling are mediated by the VDR, which is a ligand-activated transcription factor [35]. Because of genome duplication, teleosts have 2 paralogous VDR forms [24,25]. Craig et al. (2008) immunohis-
tochemically demonstrated universal expression of the zebrafish VDR in most tissues; however, they did not try to identify the respective expressions of paralogous VDRs [40]. In the present study, 2 paralogous VDRs with a high degree of homology (86%) were also identified in zebrafish. In the phylogenetic analysis, the 2 paralogous VDRs of zebrafish were separated into 2 clades. The 2 paralogous VDRs were found to be expressed throughout the developmental stages and adult tissues of zebrafish, and VDRa showed a predominant expression over VDRb in most tissues. Although most gene duplicates are non-functionalized or the gene is eventually lost, some paralogous genes are preserved as one acquires new function or subfunction [26]. There has been no convincing evidence to answer the long-term challenging question: do the 2 paralogous VDRs in teleosts have divergent functions? In previous studies, exogenous 1,25(OH)2D3 treatment was found to stimulate transcription of VDRE-containing expression constructs in a mammalian cell line that overexpressed the teleost VDR [24,27,28]. Howarth et al (2008) further demonstrated in vitro that transcriptional regulation of 2 paralogous VDRs of medaka differed with 1,25(OH)2D3 treatment [24]. Those studies indicated that vitamin D can differentially activate teleost VDRs; however, it was unknown until the present study that vitamin D controls the Ca2+ uptake function through only one of the paralogous receptor genes, VDRa, in zebrafish based on the loss-of-function experiments of the 2 VDRs.

The teleost VDR simulated the transcript level of the VDRE-containing construct with 1,25(OH)2D3 treatment in a cell line experiment [24,27,28]. Furthermore, a putative VDRE was identified in the ecac promoter region of fugu and zebrafish [17,23]. Taken together, vitamin D-VDRa signaling is probably involved in controlling the expression and function of ecac in fish. In the present study, paralogous VDRs of zebrafish were differentially activated by vitamin D in the control pathways of Ca2+ uptake and ecac expression, implying some divergences in the functions between these two paralogous VDRs in zebrafish, as what was previously reported using in vitro experiments on the 2 medaka VDRs [24]. To ascertain functional information on the paralogous VDRs, Horwarth et al. (2008) constructed chimeric proteins containing the yeast Gal4 DNA-binding domain (DBD) fused with the VDR ligand-binding domain (LBD) of either medaka VDRα or VDRβ [24]. Activity of the medaka VDRα chimera exhibited little activation by 1,25(OH)2D3, but a stronger and more-specific response was observed in the VDRβ chimera [24]. In the present study, paralogous VDRs of zebrafish showed different activation extents by 1,25(OH)2D3, and this may have resulted from the difference of amino-acid sequence in the LBD. Comparison of amino-acid compositions between zebrafish VDRa and VDRb showed a higher degree of similarity in the DBD (97%) than LBD (92%), demonstrating that slight changes in the amino-acid composition may be associated with a significant difference in transactivation and thus physiological functions.

Figure 6. Effects of vitamin D receptor (VDRα) and VDRβ morpholino oligonucleotides (MOs) on zebrafish embryos with 1α,25(OH)2D3 (20 μg/l) or low Ca2+ (0.02 mM; LCa) treatment. Ca2+ influx (A, C) and ecac mRNA expression (B, D). mRNA expression of ecac was analyzed by a qPCR using β-actin as the internal control. Different letters indicate a significant difference (p<0.05) using one-way ANOVA followed by Tukey’s multiple-comparison test. Values are the mean ± SD. (n=6–8). High Ca2+ (HCa): 2.00 mM. doi:10.1371/journal.pone.0045650.g006

Vitamin D was found to directly stimulate the cyp24a1 transcript and inhibit the cyp27b1 transcript through its binding to the VDR in experiments on mammalian cell lines [24]. However, this regulation is still unclear in teleosts. In the present study, exogenous vitamin D (1α,25(OH)2D3) suppressed the mRNA expression of cyp27b1 and simultaneously stimulated that of cyp24a1 in 3-dpf zebrafish embryos, reflecting a feedback mechanism in homeostasis of vitamin D levels as found in mammals. In experiments on mammals or mammalian cell lines, exogenous 1α,25(OH)2D3 caused negative feedback which directly suppressed gene expression and activity of CYP27B1 [41–43]. On the other hand, exogenous 1α,25(OH)2D3 caused positive feedback to directly stimulate gene expression of CYP24A1 [43,44]. This regulation can be associated with the homeostasis of 1α,25(OH)2D3. Accordingly, stimulation of cyp24a1 and inhibition of cyp27b1 by exogenous 1,25(OH)2D3 treatment may provide feedback to control 1α,25(OH)2D3 levels in zebrafish. Taken together, 1α,25(OH)2D3 may regulate the function of the Ca2+ uptake mechanism in fish through feedback pathways, in which expressions of cyp27b1 and cyp24a1 are differentially modulated. Subsequent loss-of-function experiments further indicated that this
feedback control by the differential regulation of cyp27b1 and cyp24a1 expressions appears to be mediated by only one of the paralogous receptors, VDRa and VDRb. This differential regulation of the vitamin D receptor (VDR) and vitamin D receptor binding sites that mediate activation by 1,25-dihydroxyvitamin D3-induced hypercalcemia and hyperphosphatemia in male cyprinid Cyprinus carpio. Comp Biochem Physiol A Physiol 121(2): 257–263. 14. Takeuchi A, Okano T, Kobayashi T (1991) The existence of 25-hydroxyvitamin D3 1 alpha-hydroxylase in the liver of carp and bastard halibut. Life Sci 48: 169–212. 15. Cheng Y, Guo L, Zhang Z, Soo HM, Wen C, et al. (2006) HNF factors form a network to regulate liver-enriched genes in zebrafish. Dev Biol 294(2): 402–96. 16. Goldstone JV, McArthur AG, Kuhotha A, Zanette J, Parente T, et al. (2010) Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC Genomics 11: 643. 17. Lin CH, Tsai IL, Su CH, Tseng DY, Hwang PP (2011) Reverse effect of mammalian hypocalcemic cortical in fish: cortisol stimulates Ca2+ uptake via glucocorticoid receptor-mediated vitamin D3 metabolism. PLoS One 6(9): e23689. 18. Swarpuk K, Das VK, Norman AW (1991) Dose-dependent vitamin D3 and 1,25-dihydroxyvitamin D3-induced hypercalcemia and hyperphosphatemia in male cyprinid Cyprinus carpio. Comp Biochem Physiol A Physiol 100(2): 445–447. 19. Sundell K, Norman AW, Bjornsson BT (1993) 1,25(OH)2 vitamin D3 increases ionized plasma calcium concentrations in the immature Atlantic cod, Gadus morhua. Gen Comp Endocrinol 91(3): 344–351. 20. Abbonik W, Hang XM, Guerreiro PM, Spanings FA, Ross HA, et al. (2007) Parathyroid hormone-related protein and calcium regulation in vitamin D-deficient sea bream (Sparus aurata). J Endocrinol 193(3): 459–71. 21. Meyer MB, Watanuki M, Kim S, Shevde NK, Pike JW (2006) The human transient receptor potential vanilloid type 6 distal promoter contains multiple vitamin D receptor binding sites that mediate activation by 1,25-dihydroxyvitamin D3 in intestinal cells. Mol Endocrinol 20(6): 1447–61. 22. Qiu A, Hogstrang C (2004) Functional characterization and genomic analysis of an epithelial calcium channel (ECaC) from pufferfish, Fugu rubripes. Gene 342(1): 113–23. 23. Howarth DL, Law SH, Barnes B, Hall JM, Hinton DE, et al. (2008) Paradoxous vitamin D receptors in teleost: transition of nuclear receptor function. Endocrinology 149(5): 2411–22.
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