Molecular Physiology of the Hypocalcemic Action of Fibroblast Growth Factor 23 in Zebrafish (Danio rerio)

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Fibroblast growth factor 23 (FGF23), a hormone required for phosphorus metabolism, was recently proposed to act on Ca2+ uptake; however, the available evidence of how FGF23 controls the body fluid Ca2+ homeostasis needs to be further clarified. The use of zebrafish as a model system revealed that FGF23 is specifically expressed in the corpuscles of Stannius (CS), an organ involved in Ca2+ homeostasis in fish, and that its expression is stimulated by ambient water with a high Ca2+ level. The overexpression of FGF23 inhibited Ca2+ uptake by downregulating the messenger RNA (mRNA) expression of epithelium calcium channel. Calcium-sensing receptor (CaSR), which senses changes in extracellular Ca2+ levels and modulates calcitropic hormones in organs controlling Ca2+ homeostasis in vertebrates, was found to be coexpressed with FGF23 in the CS. In addition, upregulated expression of FGF23 mRNA was detected in morphants of stanniocalcin 1 (stc1, another hypocalcemic factor synthesized in the CS), and knockdown of CaSR suppressed such upregulation and enhanced Ca2+ uptake. Taken together, our data indicate that FGF23 functions as a hypocalcemic hormone in zebrafish and that the CaSR/STC1-FGF23 axis is involved in body fluid Ca2+ homeostasis in vertebrates. (Endocrinology 158: 1347–1358, 2017)

Changes in body fluid Ca2+ levels are required for diverse physiological activities, such as neurotransmission, bone remodeling, and muscle contraction (1, 2). Systemic Ca2+ homeostasis is maintained by complex hormonal systems that regulate Ca2+ uptake in the kidney and intestine and Ca2+ storage or release from the bone (3). Fibroblast growth factor 23 (FGF23) is primarily expressed and secreted from the bone and is related to phosphorus regulation in mammals. Phosphorus homeostasis involves a close interaction among FGF23, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], and parathyroid hormone (PTH) (3). Systemic homeostasis of phosphorus and calcium are closely associated, and both 1,25(OH)2D3 and PTH affect body fluid Ca2+ regulation (3). As such, FGF23 may also be involved in body fluid Ca2+ homeostasis; however, more studies are required to clarify this issue.

Previous studies have indicated that FGF23 messenger RNA (mRNA) expression in osteoblasts or the circulation of mice is increased by the administration of extra Ca2+ (4). In vitamin D receptor (VDR) knockout (KO) mice, Ca2+ supplementation stimulates FGF23 mRNA expression in the bone and enhances the circulating level of FGF23 (5). In addition, intraperitoneal injection of calcium gluconate elevated FGF23 levels in PTH KO mice (6), and low Ca2+ and 1,25(OH)2D3 diets cause hypocalcemia, low circulating levels of FGF23, and high circulating levels of 1,25(OH)2D3 and PTH in rats (7). The low level of circulating FGF23 was suggested to arise from hypocalcemia (7). These studies imply that FGF23 modulation in animal models through administration of extra Ca2+ may be independent of 1,25(OH)2D3 or PTH feedback regulation; however, FGF23 promoter activity was not stimulated in ROS17/2.8 osteoblasts treated with...

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in USA
Copyright © 2017 Endocrine Society
Received 30 November 2016. Accepted 23 February 2017.
First Published Online 1 March 2017

doi: 10.1210/en.2016-1883
Endocrinology, May 2017, 158(5):1347–1358  https://academic.oup.com/endo

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5 mM Ca^{2+} in vitro (8). On the other hand, FGF23 promoter activity was upregulated by 6 mM Ca^{2+} treatment, but not modulated by 1, 2, 4, and 8 mM Ca^{2+} in MC3T3-E1 osteoblast experiments (4). Nifedipine (an L-type Ca^{2+} channel blocker) treatment blocked the effect of extracellular Ca^{2+} and resulted in decreased FGF23 promoter activity in MC3T3-E1 osteoblast (4). In addition, the FGF23 promoter activity was mediated by the increased intracellular Ca^{2+} level in osteoblast (4, 9). As such, the effects of extracellular Ca^{2+} on FGF23 expression is yet to be comprehensively elucidated.

Stanniocalcin 1 (STC1) is a homodimeric glycoprotein; its encoding gene was first cloned from fish (10, 11). STC1 functions as a hypocalcemic hormone in fish, but acts in a paracrine and autocrine manner in mammals (11). STC1 suppresses intestinal Ca^{2+} uptake and renal phosphate excretion, but stimulates intestinal phosphate uptake (11–13). Renal STC1 mRNA expression was significantly increased in mice lacking klotho (KL; the coreceptor of FGF23), and KL has been suggested to regulate STC1 mRNA expression through controlling the circulation of Ca^{2+} and PO_4^{3–} levels (14). In addition, two STC1 transgenic mice exhibited dwarfism (15, 16), and skeletal developmental was inhibited in transgenic mice expressing human STC1 (17). STC1 expression has been detected in both osteoblast progenitors and mature osteoblasts and is associated with osteoblast differentiation (18, 19). FGF23 expression and secretion are potentially linked to osteoblast differentiation (3, 4), and STC1 may be able to regulate FGF23. However, there were previously no published reports elucidating the relationship between STC1 and FGF23 in vertebrates in terms of body fluid Ca^{2+} homeostasis.

Calcium-sensing receptor (CaSR) expressed in the parathyroid gland and thyroid C cells can sense extracellular Ca^{2+} levels and modulate the secretion of PTH and calcitonin (20). CaSR is also expressed in osteoblasts, and high Ca^{2+} can induce cell proliferation in rat calvarial osteoblasts; however, such proliferation is inhibited in cells transfected with dominant-negative CaSR (21). High Ca^{2+} supplementation can stimulate rat and mouse osteoblast differentiation, but administration of CaSR antagonists attenuates this phenomenon (22). In addition, impaired skeletal development is observed in mice with a specific CaSR KO in osteoblasts (23). Hence, CaSR may be able to sense extracellular Ca^{2+} and then regulate the expression and/or secretion of FGF23. However, FGF23 levels in PTH and PTH-CaSR KO mice were not affected by intraperitoneal injection of calcium gluconate, suggesting that CaSR is not involved in the regulation of serum FGF23 levels (6). As such, the effect of CaSR on FGF23-mediated control of body fluid Ca^{2+} homeostasis remains a puzzle.

In contrast to the observations in mammals, fish FGF23, STC1, and CaSR are dominantly coexpressed in the corpuscles of Stannius (CS) (24–29). Fish absorb Ca^{2+} from ambient water, and the adult gills (the skin in embryonic stages), which function as an external kidney, are required for Ca^{2+} uptake. Surgical excision of CS in fish was reported to cause hypercalcemia and a concomitant increase of branchial Ca^{2+} uptake (30, 31), whereas injection of CS extract was reported to inhibit branchial Ca^{2+} uptake (31–33). STC is one of the major hypocalcemic factors of the CS (24–26, 29), in which fgf23 is also highly expressed (27, 28). In some mammalian models, fgf23 mRNA expression is associated with extracellular Ca^{2+} levels (4–7). Taken together, these earlier findings suggest that FGF23 may contribute to the hypocalcemic functions of the CS, but this hypothesis is yet to be tested.

Examining the hormonal control of transepithelial Ca^{2+} transport in mammals is challenging because TPV5 and TRPV6, the major transporters for Ca^{2+} reabsorption in the kidney, preferentially form heteromultimers that exhibit functional redundancy and compensation (34–36). Zebrafish, on the other hand, possess only a single ortholog of mammalian TRPV5/6: the epithelium Ca^{2+} channel (ECaC) (37). ECaC is dominantly expressed in ionocytes of the embryonic skin or adult gills and functions as the major transporter for Ca^{2+} uptake in zebrafish (37, 38). Previous studies have revealed that ECaC is the main target of calciotropic hormones for regulating Ca^{2+} uptake in zebrafish (26, 29, 39–43). In addition, zebrafish is a common animal model for biomedical research, and FGF23, STC1, and CaSR are dominantly coexpressed in the CS (26, 28, 29). As such, we used zebrafish to test the hypothesis that FGF23 affects body fluid Ca^{2+} homeostasis through regulating Ca^{2+} uptake by answering the following questions. 1) Is fgf23 expression regulated by environmental (and thus extracellular) Ca^{2+} levels? 2) Does FGF23 regulate Ca^{2+} absorption? 3) Does STC1 modulate fgf23 expression? 4) Does CaSR regulate fgf23 expression? Addressing these questions promises to enhance our understanding of the actions of FGF23 on body fluid Ca^{2+} homeostasis.

Materials and Methods

Experimental animals

The wild-type AB strain of zebrafish (Danio rerio) was obtained from the Taiwan zebrafish core facility and reared in a freshwater (FW) fish-rearing system—[Ca^{2+}] of 0.2 mM—at 28.5°C under a 14:10-hour light-dark photoperiod at the Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan. Embryos were collected after fertilization and then kept in Petri dishes. The experiments were conducted by following the experimental protocols described in the following.
Ca$_2^+$ (LCa) media was 2 and 0.02 mM, respectively; [Na$^+$] (0.5 mM), [Mg$^{2+}$] (0.16 mM), and [K$^+$] (0.3 mM) were the same.

During time-course experiments, fertilized embryos were treated with LCa media for sampling at 2 or 4 days postfertilization (dpf). Zebrfish eggs were incubated in either HCa or LCa media until maintained within 10% of the predicted values. Fertilized eggs were treated with LCa media for 2 days and were then transferred to HCa or LCa. After transfer, larvae were sampled at 3, 6, 12, 24, and 48 hours.

**Acclimation experiments**

Artificial FW was prepared by dissolving the desired amounts of CaSO$_4$·2H$_2$O, MgSO$_4$·7H$_2$O, NaCl, K$_2$HPO$_4$, and KH$_2$PO$_4$ in double-deionized water (model Milli-RO60; Millipore, Billerica, MA). The final [Ca$^{2+}$] in the high Ca$^{2+}$ (HCa) and low Ca$^{2+}$ (LCa) media was 2 and 0.02 mM, respectively; [Na$^+$] (0.5 mM), [Mg$^{2+}$] (0.16 mM), and [K$^+$] (0.3 mM) were the same in HCa and LCa. Z-8000 atomic absorption spectrophotometry (Hitachi, Japan) was used to examine ion levels in artificial FW and ensure that any variations in ion concentrations were maintained within 10% of the predicted values. Fertilized zebrafish eggs were incubated in either HCa or LCa media until sampling at 2 or 4 days postfertilization (dpf). During time-course experiments, fertilized embryos were treated with LCa media for 2 days and were then transferred to HCa or LCa. After transfer, larvae were sampled at 3, 6, 12, 24, and 48 hours.

**Microinjection of capped mRNA and antisense MO**

A previously described, an expression construct for STC1 (26) was used in the current study. In addition, full-length zebrafish FGF23 was amplified by polymerase chain reaction (PCR) with gene-specific primers (Supplemental Table 1) and then cloned into the pCS2+ expression vector. This construct, which expresses GFP, was used to examine MO specificity. The specificity and effectiveness of the MO were confirmed by coinjection with zebrafish FGF23 translation in this study. Embryos were micro-injected with 5 ng fgf23 MO/embryo, 4 ng stc1 MO/embryo, or 4 ng casr MO/embryo. The same doses of MO were used for the double knockdown experiment. At this dose, neither substantial mortality nor abnormal behavior was observed. To confirm the specificity of fgf23 MO, we used PCR with gene-specific primers to amplify full-length FGF23 containing the MO-targeted sequences (Supplemental Table 1), and then cloned it into the pCS2+ green fluorescent protein (GFP) XL7 vector. This construct, which expresses GFP, was used to examine fgf23 MO specificity. The specificity and effectiveness of the fgf23 MO were confirmed by coinjection with zebrafish fgf23 capped mRNAs. Zebrafish embryos injected with only capped mRNAs (with GFP fusion) revealed GFP translation signals [Supplemental Fig. 1(A)]. On the other hand, embryos co-injected with the fgf23 MO showed no green fluorescence [Supplemental Fig. 1(B)], indicating that the MO specifically and effectively knocked down the translation of the target gene. The capped mRNA and/or MOs were injected into embryos at the one- to two-cell stage using an IM-300 microinjector system (Narishige Scientific Instrument Laboratory, Tokyo, Japan); injected embryos at 2 and 4 dpf were sampled for subsequent analyses.

**RNA extraction**

Zebrafish larvae were anesthetized using 0.03% MS222 (Sigma-Aldrich, St. Louis, MO), and 20 larvae were pooled into a single vial. One milliliter of Tri Rol was added into each vial before homogenization (Invitrogen, Carlsbad, CA). Next, 0.2 mL chloroform was added into each vial, which was then shaken thoroughly. The mixed and homogenized solutions were centrifuged at 4°C and 12,000g for 30 minutes. Supernatants were mixed with an equal volume of isopropanol and then stored at −20°C overnight. Thereafter, pellets were precipitated by centrifugation at 4°C and 12,000g for 30 minutes, and then washed with 70% alcohol. Finally, pellets were dissolved in diethylpyrocarbonate water and stored at −20°C until use.

**Table 1. Antibody Used**

| Peptide/Protein Target | Name of Antibody | Manufacturer, Catalog #, and/or Name of Individual Providing the Antibody | Species Raised in; Monoclonal or Polyclonal | Dilution Used | RRID |
|------------------------|------------------|-------------------------------------------------|------------------------------------------|--------------|------|
| Na$^+$, K$^+$-ATPase, alpha subunit | α5 | DSHB Cat# α5 | Mouse, monoclonal | 2.5 μg/mL in PBS | AB_2166869 |

Abbreviation: RRID, research resource identifier.

Figure 1. Expression of FGF23 mRNA in zebrafish larvae. In situ hybridization was used to detect fgf23 signals at the indicated developmental stages (a) 1 dpf, (b) 2 dpf, (c) 3 dpf, and (d) 5 dpf. Arrows indicate the fgf23 mRNA signal.
Supplemental Table 2. The calculation of relative mRNA levels was based on the comparative cycle threshold method (43).

**Complementary DNA synthesis**

Superscript RT (Invitrogen) was used for complementary DNA (cDNA) synthesis. First, 3 to 5 μg of total RNA was preincubated with 0.5 mM deoxynucleotide triphosphates, 2.5 μM oligo (dT)20, and 250 ng random primers at 60°C for 5 minutes. Thereafter, reaction buffer, 5 mM dithiothreitol, 40 units RNase inhibitor, and 200 units of reverse transcription were added to preincubated solution for 1 hour at 50°C. The reaction was stopped by heating the mixture at 70°C for 5 minutes. Thereafter, Hyb was removed and Hyb containing 500 μg/mL yeast transfer RNA was used to treat samples at 65°C for 4 hours before hybridization. Subsequently, 100 ng/mL digoxigenin-labeled antisense or sense RNA probes were used for hybridization. After hybridization, samples were washed with 0.1% Tween 20 and then incubated with an Alexa Fluor 488 goat anti-mouse immunoglobulin G antibody (Molecular Probes, Carlsbad, CA) for 2 hours at room temperature. The washed samples were then immunoreacted with an alkaline phosphatase-coupled anti-Dig antibody (1:8000), and stained with nitro blue tetrazolium (Roche) and 5-bromo-4-chloro-3-indolyl phosphate (Roche) for the alkaline phosphatase reaction.

**Immunocytochemistry**

Prepared embryos were rinsed in PBS and blocked with 3% bovine serum albumin for 30 minutes. Afterward, the embryos were incubated with a 5 mouse anti-Na+–K+–ATPase (2.5 μg/mL in PBS) overnight at 4°C. The embryos were washed twice with PBS and then incubated with an Alexa Fluor 488 goat antimouse immunoglobulin G antibody (Molecular Probes, Carlsbad, CA; 1:200 diluted with PBS) for 2 hours at room temperature. Images were acquired with an Axioplan 2 imaging microscope (Zeiss) (Table 1).

**Whole-body Ca2+ influx**

We modified the method of Chen et al. (45) for whole-body Ca2+ influx. Zebrafish larvae were rinsed briefly in deionized water and then transferred to 2 mL of 44Ca2+ (Amersham, Piscataway, NJ), with a final working specific activity of 1 to 2 mCi/mmol-containing medium for a subsequent 4-hour incubation. After the incubation period, larvae were washed with isotope-free FW. Six washed larvae were pooled into one vial, anesthetized with MS222, and then digested with tissue solubilizer (Solvable; Packard, Meriden, CT) at 65°C for 8 hours. Next, counting solution was added into the vial with digestion solution (Ultima Gold, Packard Instrument Co., Meriden, CT). The radioactivities of the solutions were then counted with a liquid scintillation beta counter (LS6500; Beckman, Fullerton, CA). The Ca2+ influx was calculated using the following formula: $J_{in} = Q_{lava}X_{out}^{-1}t^{-1}$, where $J_{in}$ is the influx (pmol/h⁻¹), $Q_{lava}$ is the radioactivity of the larva (counts per minute per individual) at the end of incubation period, $X_{out}$ is the specific activity of the incubation medium (counts per minute/pmol), and $t$ is the incubation time (hours). The influx was expressed in pmol/mg⁻1/h⁻¹ by dividing $J_{in}$ by the embryo wet weight (mg). The $Q_{lava}$ and W data were both obtained from pooled samples, and the averaged values were used during calculation.
Statistical analysis

Group data sets were confirmed to be normally distributed by the Anderson Darling Normality Test ($P > 0.05$). Student $t$ test was used to compare the two groups. Significance was set at $P < 0.05$. Data are presented as the mean ± standard deviation (SD).

Results

FGF23 expression and its role in Ca$^{2+}$ uptake in zebrafish

Although in situ hybridization did not reveal fgf23 mRNA signals in the CS of zebrafish at 1 dpf, strong fgf23 mRNA signals were detected from 2 dpf onward (Fig. 1). In addition, zebrafish acclimated to artificial FW containing HCa (2.0 mM Ca$^{2+}$) expressed enhanced levels of fgf23 mRNA at 2 and 4 dpf [Fig. 2(a)]. Quantified expression levels, as determined by qPCR, are shown in Fig. 2(b).

To clarify the effect of FGF23 on Ca$^{2+}$ homeostasis, we injected zebrafish embryos at the one- to two-cell stage with fgf23 MO, which enhanced both Ca$^{2+}$ uptake and ecac expression in 2 and 4 dpf zebrafish larvae [Fig. 3(a) and (b)]. On the other hand, injection of FGF23 capped mRNA into one- to two-cell embryos caused substantial decreases in Ca$^{2+}$ uptake and ecac expression in 2 and 4 dpf zebrafish larvae [Fig. 3(c) and (d)]. To reinforce this molecular physiological evidence for the effect of FGF23 on ecac expression, we further examined whether KL and fibroblast growth factor receptor 1 (FGFR1), the coreceptor and receptor for FGF23 separately, are expressed in ecac-expressing cells by double in situ hybridization/immunocytochemistry against kl and fgfr1mRNA and Na,K-ATPase (NKA), the marker of ecac-expressing cells, respectively, in yolk sac skin of zebrafish larvae. As shown in Fig. 4, kl and fgfr1 mRNA signals were localized with NKA.

Knockdown of CaSR decreases expression of stc1, but not fgf23, in zebrafish

In situ hybridization revealed fgf23, stc1, and casr mRNA signals in the CS [Fig. 5(a)]. To determine whether CaSR affects expression of stc1 and fgf23, we microinjected zebrafish embryos at the one- to two-cell stage with casr...
MO. Such knockdown resulted in a substantial decrease of stc1 expression at 2 dpf, but not at 4 dpf [Fig. 5(b)]. In contrast, expression of fgf23 mRNA was unaffected by CaSR knockdown in 2 and 4 dpf zebrafish [Fig. 5(b)].

**Knockdown of CaSR influences the effect of HCa transfer on stc1 and fgf23 expression in zebrafish**

To further elucidate the effect of CaSR on stc1 and fgf23 expression, we injected zebrafish embryos at the one- to two-cell stage with control or casr MO, and then incubated the embryos in LCa (0.02 mM Ca2+) for 2 days. Thereafter, zebrafish control or casr morphants were transferred to either HCa or LCa. In control morphants, stc1 and fgf23 expression exhibited substantial stimulation by 3 and 12 hours after transfer to HCa, respectively, and such stimulation persisted thereafter (Fig. 6). On the other hand, whereas stc1 expression was still stimulated in casr morphants following transfer to HCa, the relative increase (LCa-HCa to LCa-LCa group) was attenuated compared with that of control morphants [Fig. 6(a)]. Conversely, fgf23 expression was stimulated earlier (at 3 hours) in casr morphants after HCa transfer and exhibited a greater relative increase (LCa-HCa to LCa-LCa group) than in control morphants [Fig. 6(b)].

**CaSR knockdown decreases fgf23 expression in zebrafish stc1 morphants**

We observed that HCa-induced stc1 expression was attenuated, but fgf23 expression was elevated in zebrafish casr morphants. We therefore proceeded to investigate whether STC1 affects fgf23 expression. Injection of stc1 MO induced substantial upregulation of fgf23 at 2 and 4 dpf [Fig. 7(b)]. In contrast, fgf23 expression was reduced in zebrafish injected with stc1 capped mRNA [Fig. 7(a)]. To determine whether FGF23 also regulates stc1 expression, we microinjected zebrafish embryos with fgf23 capped mRNA. Overexpression of fgf23 was found to inhibit stc1 expression at 2 dpf, but not 4 dpf [Fig. 7(c)], whereas fgf23 MO injection resulted in upregulated stc1 expression at 2 dpf, but not 4 dpf [Fig. 7(d)].

CaSR knockdown decreases stc1 expression, and this decrease has a counterbalancing effect on fgf23 expression in zebrafish larvae (Fig. 7). To further investigate the effect of CaSR on FGF23, we coinjected casr and/or stc1 MOs into zebrafish embryos at the one- to two-cell stage. Thereafter, zebrafish embryos were incubated in either FW or HCa. Our qPCR results revealed that casr and stc1 MOs caused differential effects on the mRNA expression.
of STC1 and ECaC on 2 and 4 dpf zebrafish larvae following FW treatment. Compared with stc1 morphants, fgf23 expression was significantly inhibited in 2 and 4 dpf in zebrafish stc1-casr morphants [Fig. 8(a)]. On the contrary, ecac expression and Ca2+ uptake were significantly upregulated at 4 dpf (but not 2 dpf) in zebrafish stc1-casr morphants [Fig. 8(a) and (b)]. Furthermore, the same phenomenon was observed in stc1-casr morphants treated with HCa [Fig. 8(c) and (d)].

**Discussion**

It was previously reported that Ca2+ supplementation increases fgf23 mRNA expression in the femurs, and the circulating FGF23 level, in mice (4). Such an effect was observed even in VDR and PTH KO mice (5, 6). These studies revealed that FGF23 expression and/or secretion is stimulated by high extracellular Ca2+ levels in mammals. The skeleton is composed of acellular bone in teleost fish (46), and present and previous studies have shown that FGF23 is primarily expressed in the CS from 2 dpf onward by *in situ* hybridization in zebrafish (Fig. 1) (26, 27). We further revealed that FGF23 mRNA expression is stimulated by an HCa environment (2.0 mM Ca2+) (Fig. 2), a condition that is known to enhance the Ca2+ content of the body (45). The present results are consistent with the earlier finding that FGF23 expression is stimulated by high extracellular Ca2+ levels in mammals, and imply that FGF23 has hypocalcemic effects. Our subsequent experiments provided molecular physiological evidence that also support these findings.
Removal of the CS has hypercalcemic effects, whereas injection of CS extract has hypocalcemic effects in fish (30–33). In fish, STC1 is mainly synthesized in the CS, and its expression is stimulated by high environmental Ca\(^{2+}\) treatment (25, 26). Exogenous STC1 inhibits branchial Ca\(^{2+}\) uptake (47, 48), whereas knockdown of STC1 increases Ca\(^{2+}\) uptake (26), indicating that STC1 is an important hypocalcemic hormone in the CS. Here, we have revealed that FGF23 is also a hypocalcemic hormone with similar characteristics to STC1. FGF23 is dominantly expressed in the CS and stimulated by HCa treatment (25, 26). Exogenous STC1 inhibits branchial Ca\(^{2+}\) uptake (47, 48), whereas knockdown of STC1 increases Ca\(^{2+}\) uptake (26), indicating that STC1 is an important hypocalcemic hormone in the CS. Here, we have revealed that FGF23 is also a hypocalcemic hormone with similar characteristics to STC1. FGF23 is dominantly expressed in the CS and stimulated by HCa treatment (Fig. 2); gain- and loss-of-function of FGF23 resulted in respective inhibition and stimulation of both ECaC mRNA expression and Ca\(^{2+}\) uptake in zebrafish (Fig. 3). Furthermore, mRNA signals of the coreceptor KL and receptor FGFR1 of FGF23 were colocalized with Na\(^{+}\)-K\(^{-}\)-ATPase, the marker of ecac-expressing cells, in yolk sac skin of zebrafish larvae (Fig. 4) (38). This result implied that FGF23 had a direct effect on ECaC expression. As such, it appears that FGF23 controls body fluid Ca\(^{2+}\) homeostasis mainly by modulating the expression and function of ECaC in a similar manner to other zebrafish calcitropic hormones (e.g., STC1, cortisol, 1,25(OH)\(_2\)D\(_3\), PTH, calcitonin) (26, 29, 39–44). TRPV5 and TRPV6 are orthologous of ECaC and essential for Ca\(^{2+}\) reabsorption in renal distal tubules of in mammals; nonetheless, a recent study showed that FGF23 treatment stimulated TRPV5 protein abundance in the plasma membrane of isolated distal tubules in a mouse model (49). In a pharmacological study, ruthenium red (a potential inhibitor for TRPV5 and TRPV6), 8 hours after injection of FGF23, increased Ca\(^{2+}\) reabsorption through stimulating the activity of TRPV5, but not that of TRPV6, in the renal distal tubular cells of mouse (49). In the current

Figure 7. STC1 and FGF23 have mutual stimulatory effects in 2 and 4 dpf zebrafish larvae. (a) Effect of stc1 capped mRNA (cRNA) injection on fgf23 expression. (b) Effect of stc1 MO injection on fgf23 expression. (c) Effect of fgf23 cRNA injection on stc1 expression. (d) Effect of fgf23 MO injection on stc1 expression. Gene expression was analyzed by qPCR, and the values were normalized to those of β-actin. Student t test, *P < 0.05; **P < 0.01; ***P < 0.001. Values are the mean ± SD (n = 6).
study, we found FGF23 had a hypocalcemic effect in zebrafish by decreasing ECaC mRNA expression in zebrafish (Fig. 3). The properties of zebrafish ECaC are closer to mammalian TRPV6 than to TRPV5 according to the phylogenetic and pharmacological analyses (37, 50). TRPV6 is the vital transporter for Ca\(^2+\) uptake in mammalian intestine (35); therefore, the present result provides us another viewpoint to understand the role of FGF23 in systemic Ca\(^2+\) regulation in vertebrates.

Both FGF23 and STC1 (two hormones mainly synthesized in the CS) exert hypocalcemic effects, and derivation of how these two hormones cooperate to achieve body fluid Ca\(^2+\) homeostasis is a question of physiological importance. The current study has provided some clues toward answering this question. The stimulation of stc1 mRNA expression was initiated earlier (3 vs 12 hours) and showed a higher increase (fivefold vs twofold) than that of fgf23 after HCa treatment (Fig. 6), suggesting that STC1 is more sensitive and responsive to changes in environmental Ca\(^2+\) levels (and thus extracellular Ca\(^2+\) concentrations). CaSR, a receptor that modulates the secretion of calciotropic hormones (20), appears to mediate the differences in the sensitivity and response to HCa between FGF23 and STC1, because CaSR loss-of-function diminishes the observed differences in timing and strength of responses (Fig. 6). Further elaboration of the differences revealed that translational knockdown of CaSR accelerated and intensified the response of FGF23, with a concomitant impairment of that of STC1 (Fig. 6). This implies the existence of mutual effects between FGF23 and STC1. The effects of knockdown and overexpression of FGF23 and STC1 (Fig. 7) supported this hypothesis. FGF23 and STC1 negatively regulate each other, but STC1 has a stronger effect on fgf23 expression; hence, upregulated fgf23 expression in casr morphants upon HCa acclimation may reflect the decrease in stc1 expression. However, the identity of STC receptor(s) is still unknown, making it difficult to explore the direct effect of STC1 on FGF23 expression in the CS. On the other hand, FGF23 is unable to regulate STC1 expression directly because the expression of KL, an essential coreceptor for FGF23, was not found in the CS in the present and previous studies (data not shown) (28).

Although the direct regulation between STC1 and FGF23 is unclear, an indirect and mutual regulation between STC1 and FGF23 appears to be developed—at least in zebrafish. Mutual counterbalance between calciotropic hormones in zebrafish was previously reported:

Figure 8. (a, c) Effects of CaSR-STC1 knockdown on gene expression and (b, d) Ca\(^2+\) influx in 2 and 4 dpf zebrafish larvae following exposure to (a, b) FW or (c, d) HCa. Gene expression was analyzed by qPCR, and the values were normalized to those of \(\beta\)-actin. Student t test, \(*P < 0.05; **P < 0.01; ***P < 0.001\). Values are the mean ± SD (n = 6-8).
overexpression of hypocalcemic calcitonin upregulated the expression of PTH receptor and VDR in 4 dpf zebrafish larvae with a resultant increase of Ca\(^{2+}\) content (40). Furthermore, the expression of \emph{cyp24a1} and \emph{cyp27b1}, the genes encoding the enzymes for 1,25(OH)\(_2\)D\(_3\) synthesis and degradation, were stimulated and inhibited, respectively, in zebrafish treated with exogenous 1,25(OH)\(_2\)D\(_3\), and such feedback regulation was suggested to modulate the impact of exogenous 1,25(OH)\(_2\)D\(_3\) (42). As such, systems of mutual counterbalance between calcitropic hormones for the maintenance of body fluid Ca\(^{2+}\) homeostasis appear to be conserved among vertebrates. Herein, we reported that both FGF23 and STC1 have redundant functions in terms of hypocalcemic action in zebrafish. When expression of either \emph{fgf23} or \emph{stc1} is abnormal, expression of the other hormone may be modulated to compensate for the loss in hypocalcemic activity. In the current study, 1,25(OH)\(_2\)D\(_3\) and PTH may be involved in mutual regulation between FGF23 and STC1. 1,25(OH)\(_2\)D\(_3\) treatment was reported to stimulate expression of \emph{fgf23} and \emph{stc1} in mammals, and FGF23 has an inhibitory effect on 1,25(OH)\(_2\)D\(_3\) synthesis (3, 14). In addition, CYP27B1, the gene encoding an enzyme for the final step of 1,25(OH)\(_2\)D\(_3\) synthesis, expression was upregulated in KL KO mice (14). Moreover, STC1 and KL are associated with the control of circulating calcium and phosphorous levels, and the renal \emph{stc1} expression is increased in KL KO mice (14). Hence, it is possible that 1,25(OH)\(_2\)D\(_3\) is involved in the mutual regulation between STC1 and FGF23. However, the effect of 1,25(OH)\(_2\)D\(_3\) on FGF23 and STC1 is still unclear in zebrafish and should be further explored. On the other hand, PTH is known to

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**Figure 9.** Proposed model of CaSR/STC1-FGF23 axis in regulation of zebrafish Ca\(^{2+}\) homeostasis. The activation of CaSR by extracellular Ca\(^{2+}\) enhances the expression (and/or secretion) of STC-1 and FGF23, thereby inhibiting \emph{ecac} expression and Ca\(^{2+}\) uptake. The gene expression of STC1 is more sensitive and responsive than that of FGF23 to increased extracellular Ca\(^{2+}\) level. Further, increased STC-1 expression exerts its inhibitory effect on FGF23 expression and hence interferes in the effect of CaSR on FGF23. The details refer to the text. NCX, Na\(^{+}\)/Ca\(^{2+}\) exchanger; PMCA, plasma membrane Ca\(^{2+}\)-ATPase; STCR, STC receptor.
stimulate fgf23 expression in mammals (3). In zebrafish, PTH1 overexpression results in the upregulation of stc1 expression and vice versa (29). Furthermore, the PTH may also be connected to the mutual regulation between STC1 and FGF23.

CaSR, STC, and FGF23 are all highly expressed in the CS of fish (24–29). Injection of calcimimetics stimulated STC1 secretion in fish (24, 25), whereas translational knockdown of CaSR resulted in decreased STC1 expression in zebrafish (29) [Fig. 5(b)]. Taken together, these findings suggest that CaSR can sense extracellular Ca^{2+} to modulate STC1 expression and/or secretion in the CS of fish. On the contrary, we observed that fgf23 expression is not affected in zebrafish casr morphants exposed to FW. In mammalian experiments, FGF23 circulating levels were observed to be similar between PTH and PTH-CaSR KO mice under both baseline and circulating levels were observed to be similar between exposed to FW. In mammalian experiments, FGF23

Ca2+ supplementation conditions (6), suggesting that PTH and PTH-CaSR KO mice under both baseline and

RESULTS, however, indicate that FGF23 may not be regulated by CaSR. The present results, however, indicate that fgf23 expression is affected by STC1 in casr morphants. To exclude the effects of STC1 interference, we further compared fgf23 expression between zebrafish stc1 and stc1-casr morphants, and observed that fgf23 expression was lower in stc1-casr morphants than in stc1 morphants. On the contrary, ecac expression and Ca^{2+} uptake were upregulated in stc1-casr morphants; these hypercalcemic effects may partially reflect the effects of decreased fgf23 expression. A similar situation was observed for Ca^{2+} uptake and expression of fgf23 and ecac in stc1-casr morphants treated with HCa. Taking all of these findings into consideration, we suggest that CaSR senses external Ca^{2+} levels and then modulates fgf23 expression through a process that may be modulated by STC1. In mammalian osteoblast models, additional Ca^{2+} supplementation stimulated osteoblast differentiation, and FGF23 expression was found to be positively correlated with high extracellular Ca^{2+} and osteoblast differentiation (3, 4, 22). Furthermore, osteoblast differentiation and/or bone formation was inhibited in osteoblast-specific CaSR KO and STC1-transgenic mice (15–17, 23). Therefore, the functional relationship among CaSR, FGF23, and STC1 in fish may be conserved in mammalian bone cells. However, this is yet to be confirmed.

In summary (Fig. 9), the current study has clarified the relationship among FGF23, STC1, and CaSR, demonstrating the involvement of CaSR in the regulation of FGF23 expression. FGF23 was shown to inhibit Ca^{2+} uptake through the downregulation of ecac expression, and CaSR and STC1 were shown to be specifically colocalized with FGF23 in the organ controlling Ca^{2+} homeostasis. We observed that fgf23 expression was upregulated in stc1 morphants, but this effect was suppressed by knockdown of casr. Based on these findings, we propose the existence of a CaSR/STC1–FGF23 axis, which regulates body fluid Ca^{2+} homeostasis in vertebrates.

Acknowledgments

We thank the Institute of Cellular and Organismic Biology Core Facility and the Taiwan Zebrafish Core Facility for technical support during the experiments and Ms. Y.C. Tung and Mr. J.Y. Wang for their assistance during the experiments.

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Disclosure Summary: The authors have nothing to disclose.

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