Expression of sclerostin in the regenerating scales of goldfish and its increase under microgravity during space flight

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ABSTRACT

Osteocytes, osteoblasts (bone-forming cells), and osteoclasts (bone-resorbing cells) are the primary types of cells that regulate bone metabolism in mammals. Sclerostin produced in bone cells activates osteoclasts, inhibiting bone formation; excess production of sclerostin, therefore, leads to the loss of bone mass. Fish scales have been reported to have morphological and functional similarities to mammalian bones, making them a useful experimental system for analyzing vertebrate bone metabolism *in vitro*. However, whether fish scales contain cells producing sclerostin and/or osteocytes has not been determined. The current study demonstrated, for the first time, that sclerostin-containing cells exist in goldfish scales. Analysis of the distribution and shape of sclerostin-expressing cells provided evidence that osteoblasts produce sclerostin in goldfish scales. Furthermore, our results found that osteocyte-like cells exist in goldfish scales, which also produce sclerostin. Finally, we demonstrated that microgravity in outer space increased the level of sclerostin in the scales of goldfish, a finding suggesting that the induction of sclerostin is the mechanism underlying the activation of osteoclasts under microgravity.

INTRODUCTION

Osteoblasts and osteoclasts are the two primary cell types that regulate bone metabolism. Osteoblasts are responsible for the formation of new bone, while osteoclasts are involved in the resorption of aged bone (Henry and Bordonni 2020). Sclerostin, a protein encoded by the *Sost* gene, is a negative regulator of

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bone formation. Mutations in *Sost* are associated with bone diseases characterized by high bone mass, such as sclerosteosis and Van Buchem disease (Balemans *et al.* 2001; Brunkow *et al.* 2001; Balemans *et al.* 2002; Staehling-Hampton *et al.* 2002). In bone, *Sost* is primarily expressed in osteocytes; however, osteoblasts and osteoclasts have been found to have low levels of *Sost* (Collette *et al.* 2013). At the molecular level, sclerostin bound to LRP5/6 receptors inhibited the Wnt/ β -catenin signaling pathway and then decreased bone formation (Li *et al.* 2005; Poole *et al.* 2005; Ellies *et al.* 2006). It has been reported that both *Sost*^{-/-} and osteocyte-ablated mice showed resistance to bone loss induced by hindlimb unloading *via* tail suspension (Tatsumi *et al.* 2007; Lin *et al.* 2009). These previous studies have provided evidence that sclerostin produced by osteocyte has an important role in the response of mechanical stimuli (Pajevic *et al.* 2013; Delgado-Calle *et al.* 2017; Hinton *et al.* 2018).

Osteoblastic cells regulate the differentiation and activation of osteoclasts in mammalian bone (Kondo *et al.* 2001; Kearns *et al.* 2008; Lacey *et al.* 2012). Some stromal cells, such as osteoblasts and bone marrow cells, produce the receptor activator for nuclear factor κ B ligand (RANKL). RANKL binds to the receptor activator for nuclear factor κ B (RANK) on osteoclasts and promotes osteoclast differentiation and activation (Kondo *et al.* 2001; Kearns *et al.* 2008). The effect of RANKL on osteoclasts is inhibited by osteoprotegerin (OPG), a decoy receptor produced in stromal cells. Mammalian sclerostin suppresses OPG production by inhibiting Wnt signaling in stromal cells, thus facilitating osteoclastogenesis (Kubota *et al.* 2009; Silva and Branco 2011; Pajevic *et al.* 2013; Delgado-Calle *et al.* 2017).

Several groups, including ours, have reported that fish scales are morphologically and functionally similar to mammalian bones in some ways. Fish scales contain osteoblasts, osteoclasts, and mineralized matrix (osseous layer, on epithelial side) underlined with collagen rich matrix (fibrous layer, on dermis side) (Bereiter-Hahn and Zylberberg 1993; Azuma *et al.* 2007; Ohira *et al.* 2007; Suzuki *et al.* 2007; Suzuki *et al.* 2008b; de Vrieze *et al.* 2014). We established fish scale as a useful experimental system for analyzing vertebrate bone metabolism *in vitro* (Suzuki *et al.* 2000; Suzuki and Hattori 2002). In this system, the expression of genes involved in bone metabolism and the activities of osteoblasts or osteoclasts show high sensitivity to a variety of mechanical stimuli, including static hypergravity by centrifugation (Suzuki *et al.* 2008a), dynamic hyper-

gravity by vibration (Suzuki *et al.* 2007; Suzuki *et al.* 2009), ultrasound (Kitamura *et al.* 2010; Suzuki *et al.* 2016; Hanmoto *et al.* 2017), and microgravity in outer space (Ikegame *et al.* 2019). These responses provide evidence that fish scale is a suitable experimental model for analyzing the responses of bone metabolism to mechanical stimuli.

The current study demonstrated for the first time that sclerostin is produced in goldfish scales at the mRNA and protein levels. We also found evidence that osteocyte-like cells in a calcified matrix and osteoblasts in scales are responsible for sclerostin production. Finally, we found that microgravity in outer space increased the expression of *Sost* in scales, a possible mechanism of osteoclast activation.

MATERIALS AND METHODS

Animals. One female and one male goldfish (*Carassius auratus*) were purchased from Higashikawa Fish Farm (Yamatokoriyama, Japan). Artificial fertilization was carried out at Tokyo University of Marine Science and Technology to obtain fertilized eggs. The eggs were grown to adult fish with body lengths of 12–15 cm. Then, adult fish were transferred to Kanazawa University and the Tokyo Medical and Dental University and used for *in vitro* experiments. Fish were fed a commercial pellet diet for puffer fish every morning and were maintained in fresh water at 26°C until used. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Kanazawa University and the Tokyo Medical and Dental University.

Preparation of regenerating goldfish scales. Fully grown scales were removed from the goldfish, which were anesthetized with 0.03% ethyl 3-aminobenzoate methanesulfonic acid salt (Sigma-Aldrich, Inc., St. Louis, MO, USA), to allow for the regeneration of scales. On day 15 post removal, the regenerating scales were removed from the goldfish and were used for the experiments.

In situ hybridization. Digoxigenin (DIG)-labeled sense and antisense single-stranded RNA probes for goldfish *Sost* were prepared using DIG RNA Labeling Kits (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions. The cDNA sequence used for the preparation of probes was a 293 bp fragment (AB970730).

The regenerating scales were fixed in 4% paraformaldehyde (PFA) solution in phosphate buffered sa-

line (PBS). After rinsing in RNase-free PBS, the scales were used as whole-mount samples or embedded in Tissue-Tek[®] Optimal Cutting Temperature Compound for the preparation of cryosections. Whole-mount and cryosection samples were pretreated with 0.2 N HCl, proteinase K, and 0.25% acetic anhydride. The samples were then hybridized with the probes at a final concentration of 1 µg/mL in a hybridization solution composed of 50% deionized formamide, 2% blocking reagent, 5 × saline-sodium citrate (SSC), 0.02% SDS, 0.1% N-lauroylsarcosine, and 200 µg/mL tRNA at 55°C overnight. After hybridization, the sections were washed with 0.1 × SSC and 2 × SSC and treated with ribonuclease. The probe signals were detected with anti-DIG antibody conjugated to alkaline phosphatase in accordance with the manufacturer's protocol (DIG Detection Kit; Boehringer Mannheim, Mannheim, Germany). The samples were counterstained with methyl green to visualize the nuclei and examined by light microscopy.

Immunohistochemistry. The rabbit anti-human sclerostin (N-terminal 12-42) (ab63097; Abcam, Cambridge, UK) was used as the primary antibody. The regenerating scales were fixed in 4% PFA solution in PBS, and whole-mount or cryosection samples of scales were prepared as described above. The samples were incubated in blocking solution containing 0.1% Tween 20, 0.3% glycine, 10% normal goat serum, and 1% bovine serum albumin (BSA) for 1 h at room temperature. Subsequently, they were incubated with the primary antibody (×100) overnight at 4°C, then rinsed in PBS, and incubated with Alexa Fluor[®] 488-labeled anti-rabbit IgG (A11034, Molecular Probes; ×1000) for 1 h at room temperature. They were then rinsed in PBS and stained with 4',6-diamidino-2-phenylindole to visualize the nuclei. To prepare negative control samples, the above-mentioned procedures were carried out using normal rabbit serum instead of anti-sclerostin as the primary antibody. A fluorescence microscope (BX51; Olympus, Tokyo, Japan) was used to observe the samples.

Transmission electron microscopy. The regenerating scales were fixed in 4% PFA and 4% glutaraldehyde in 0.1 M phosphate buffer at 4°C overnight. The samples were rinsed thoroughly in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide (Merck KGaA, Darmstadt, Germany) for 1 h at 4°C, and dehydrated in graded ethanol. After 5 min treatment with propylene oxide, the samples were infiltrated

with Epon 812 (TAAB Laboratories, Berks, U.K.), to which accelerator DMP-30 was added. The samples were then cured at 60°C for 2 days. Ultrathin sections perpendicular to the scale surface were cut using a diamond knife. Sections were then stained with aqueous lead citrate. Some of the samples were decalcified with 5% EDTA for 4 days after fixation, then treated as described above. The ultrathin sections obtained from the decalcified samples were stained with 1% tannic acid, alcoholic uranyl acetate and aqueous lead citrate. All ultrathin sections were observed under a transmission electron microscope (H-7100; Hitachi, Tokyo, Japan) at a 100 kV accelerating voltage.

Space experiment on the International Space Station. Goldfish were anesthetized with 0.03% ethyl 3-aminobenzoate methanesulfonic acid salt (Sigma-Aldrich Inc.; neutralized using NaHCO₃), and scales were removed one by one using sharpened forceps, as shown in Suzuki *et al.* (2009). Subsequently, goldfish were maintained at 26°C. On day 12 after scale removal, goldfish were bred in water with the anti-infection reagent Green F Gold (Japan Pet Design Co. Ltd., Tokyo, Japan). On the following day, a few regenerating scales were collected from each goldfish. The scales with similar cell activities were selected as previously described (Ikegame *et al.* 2019). Subsequently, the regenerating scales were sampled on day 14 from goldfish on ice that were sterilized using hypochlorous and fungizone solutions and immersed in Leibovitz's L-15 medium containing 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 200 µg/mL kanamycin. Thereafter, the sterilized scales were packed into a culture chamber (Cell Experiment Small Chamber; Chiyoda Corporation, Yokohama, Japan). The culture chambers were kept at 2.5–4.0°C and sent to the International Space Station (ISS) by Space Shuttle Atlantis (STS-132) (Yano 2011). After their arrival on the ISS, they were incubated for 86 h under microgravity at the Cell Biology Experiment Facility (CBEF) (Yano 2011). The CBEF is equipped with a centrifuge to treat samples with one gravity [in-flight artificial microgravity (F-1g)] (Yano *et al.* 2012). This facility has the capability of culturing bio-specimens at a temperature range of 15–40°C (Yano *et al.* 2012). The differences among the sensors ranged from 0.1°C to 0.2°C (Yano *et al.* 2012). During incubation in the CBEF, culture chambers installed in the microgravity section (F-µg) and 1G section (F-1g) of the CBEF were incubated at 21.9–22.0°C in the Measurement Experiment Unit (MEU) (Yano

Table 1 Primer sequences used for quantitative real-time PCR

Target gene	Forward sequence	Reverse sequence
<i>Sost</i>	5'-CCGATTACCGCTGCATTCT-3'	5'-CGTCCGATTGGTTGTGATGG-3'
<i>Dkk1</i>	5'-AAGCACAAGAGGAAAGGCACCCAT-3'	5'-TTAGTGTCTCTGGCAAGTGTGCAG-3'
<i>Wif1</i>	5'-TGCCGAAGAGCAAGGAAGC-3'	5'-TCCTGAAGTCGTGTGTGAATGGGG-3'
<i>Efla</i>	5'-ATTGTTGCTGGTGGTGTGG-3'	5'-GGCACTGACTTCCTTGGTGA-3'

2011, 2012). The culture chambers were treated with 1 g gravity in the 1G section, whereas they were kept without the gravity treatment in the microgravity section. After incubation, culture chambers were removed from the MEU. Then, the culture medium in the culture chamber was replaced with RNAlater (Sigma-Aldrich, St. Louis, MO, USA) for gene expression analysis. Scales treated with RNAlater were stored at -96°C until the return of STS-132 to the Kennedy Space Center in Florida.

Quantitative real-time PCR. Isolation of total RNA and cDNA synthesis were performed using kits (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions (Ishizu *et al.* 2018). Each quantitative real-time PCR reaction was performed using real-time PCR apparatus (Mx3000p™; Stratagene, La Jolla, CA, USA). For the PCR reaction, a cDNA template mixed with the appropriate primers was combined with SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) (Ikegame *et al.* 2019). The elongation factor 1 α (*Efla*) gene was used for normalization. PCR primer sequences used in the current study are listed in Table 1.

Statistics. All results are expressed as means $\pm$ SEM ($n = 4$). The values of the control scales were compared with those of the experimental scales. Data were assessed using Student's *t*-tests, and the significance level chosen was $P < 0.05$.

RESULTS AND DISCUSSION

Detection of cells expressing mRNA of Sost (sclerostin) and its protein in the regenerating scales of goldfish

To obtain insight into whether the cells in the fish scales expressed the mRNA of *Sost*, whole-mount *in situ* hybridization (ISH) was conducted using the regenerating scales of the goldfish (Figs. 1 and 2). First, we focused on the peripheral area of the scales, where the ridges as well as the grooves exist (Fig. 1A). The ISH with the antisense *Sost* probe detected the signals in numerous cells located along the ridges and in a few cells in the grooves

(Fig. 1B), whereas that with the sense *Sost* probe did not (Fig. 1C), confirming their specificity. We next performed immunohistochemistry (IHC) with the sclerostin antibody. As shown in Figure 1D, IHC detected the signals in cells that were located mainly along the inner slope of the ridges and in a few cells in grooves, which were not detected by IHC with normal IgG (Fig. 1E). Taken together, we found that cells located in grooves and along ridges express the mRNA of *Sost* (sclerostin) and its protein in the regenerating scales of goldfish.

Next, we focused on the central area of the scales, where the grooves form a mesh-like structure (Fig. 2A). The ISH with the antisense *Sost* probe detected signals in cells located in the grooves (Fig. 2B), whereas that with the sense *Sost* probe did not (Fig. 2C). We next performed IHC with the sclerostin antibody to evaluate whether cells containing the sclerostin protein exist in the scales. As shown in Figure 2D, IHC detected signals in cells located in the grooves, which were not detected by IHC with normal IgG (Fig. 2E). IHC signals were also detected in cells outside the groove (Fig. 2D), although *Sost* signals of ISH were very weak in them. The reason why the different expression profiles exist between *Sost* mRNA and sclerostin protein have not been identified. A possible explanation is the different half-life between *Sost* mRNA and sclerostin protein. In future study, we will test this possibility by evaluating the time-dependent expression of *Sost* mRNA and sclerostin protein during the regeneration of scales.

It should be noted that several types of cells that regulate bone metabolism, such as osteoclasts and osteoblasts, were reported to exist in grooves and ridges of the regenerating scales of goldfish (Yoshikubo *et al.* 2005; Suzuki *et al.* 2007, 2008b; Yachiguchi *et al.* 2014; Ikegame *et al.* 2019; Yamamoto *et al.* 2020). Accordingly, we further examined the sclerostin-producing cells using cryosections in addition to whole-mount analysis, as described below.

Fig. 1 Whole-mount detection of *Sost*/sclerostin in the peripheral area of the regenerating scales by *in situ* hybridization (ISH) and immunohistochemistry (IHC). **A:** Binocular view of regenerating scale on day 15. The parts corresponding to the area in the yellow square are magnified and shown in B to E. **B:** Detection of *Sost* mRNA with an antisense probe by ISH. Positive staining is indicated by a dark purple color. **C:** Negative control for ISH with *Sost* sense probe. **D:** Detection of sclerostin with anti-sclerostin antibody by IHC. Positive staining is indicated by a green color. **E:** Negative control for IHC with normal rabbit IgG. Arrows indicate grooves or ridges. Nuclei were stained with methyl green in B and C (light green) and with 4',6-diamidino-2-phenylindole (DAPI) in D and E (blue). The images are representative data obtained from repeated experiments using five or more scales from different goldfish. The images were taken from the epithelial side (osseous layer) of scale.

Fig. 2 Whole-mount detection of *Sost*/sclerostin in the central area of the regenerating scales by *in situ* hybridization (ISH) and immunohistochemistry (IHC). **A:** Binocular view of regenerating scale on day 15. The parts corresponding to the area in the yellow square are magnified and shown in B to E. **B:** Detection of *Sost* mRNA with an antisense probe by ISH. Positive staining is indicated by a dark purple color, which is observed in the cytoplasm of 6 to 8 mononuclear cells in the groove. **C:** Negative control for ISH with *Sost* sense probe. **D:** Detection of sclerostin with anti-sclerostin antibody by IHC. Positive staining is indicated by a green color. **E:** Negative control for IHC with normal rabbit IgG. Arrows indicate grooves. Nuclei were stained with methyl green in B and C (light green) and with DAPI in D and E (blue). The images are representative data obtained from repeated experiments using five or more scales from different goldfish. The images were taken from the epithelial side (osseous layer) of scale.

Fig. 3 Detection of *Sost*/sclerostin in the peripheral area of the regenerating scales with cryosections by *in situ* hybridization (ISH) and immunohistochemistry (IHC). **A:** Binocular view of a regenerating scale on day 15. The parts corresponding to the area in the yellow square in cryosections are magnified and shown in B to E, in which the epidermis and dermis sides are shown on the upper and lower sides, respectively. The cryosections were cut along the red line. **B:** Detection of *Sost* mRNA with an antisense probe by ISH. Positive staining is indicated by a brownish-purple color (arrows). Osseous and fibrous layers are indicated by black and white curly braces respectively. **C:** Negative control for ISH with a *Sost* sense probe. Osseous and fibrous layers are indicated by black and white curly braces respectively. **D:** Detection of sclerostin with anti-sclerostin antibodies by IHC. Positive staining is indicated by a green color (arrows). **E:** Negative control for IHC with normal rabbit IgG. Nuclei were stained with methyl green in B and C (light green) and with DAPI in D and E (blue). The images are representative data obtained from repeated experiments using five or more scales from different goldfish.

Cells expressing sclerostin are half-embedded cells, bone lining cells and osteoblasts

To evaluate the distribution of cells expressing *Sost* mRNA separately in the osseous and fibrous layers of the scales, ISH was conducted using cryosections of the regenerating scales (Figs. 3A and 4A). In both the central and peripheral areas of the scales (Figs. 3B and 4C), ISH with the antisense *Sost* probe detected signals in the cells covering the fibrous layers, but not in the cells of the osseous layers except for a few cells close to the peripheral end of the scale (Fig. 3B). This result is inconsistent with our finding that the signals were detected in the cells on the osseous layers of grooves and along ridges by whole mount ISH (Figs. 1 and 2). We speculate that the ISH signals in the cells of the osseous layers are too weak to be detected on the thin cryosections. The specificity of the signals was confirmed by ISH with the sense *Sost* probe, which did not detect the signals (Figs. 3C and 4D). We next performed IHC with the sclerostin antibody (Figs. 3D and 4E), whose specificity was confirmed by IHC with nor-

mal IgG (Figs. 3E and 4F). The distribution of cells containing sclerostin protein was similar to that of cells expressing *Sost* mRNA covering the fibrous layers. IHC signals were also detected in cells of the osseous layers. Flat lining cells and half-embedded cells in the mineralized matrix expressed sclerostin (Figs. 3D and 4E).

Of note, cells with ISH or IHC signals on fibrous layers were found to have rounded shapes, suggesting that they are producing the matrix of scale (Yoshikubo *et al.* 2005; Suzuki *et al.* 2007, 2008b; Ikegame *et al.* 2019; Yamamoto *et al.* 2020). In addition to round cells, ISH or IHC signals were detected in flat cells lining the osseous layer along peripheral ridges and on the mineralized matrix surface in the center (Figs. 1D, 2B, 2D, 3D and 4E). The cell shape and situation correspond to inactive osteoblasts—lining cells in bone tissue. Therefore, our results indicate that osteoblasts in scales express *Sost* mRNA.

In addition, *Sost*/sclerostin signals were detected in half-embedded cells in a groove or in the calci-

Fig. 4 Detection of *Sost*/Sclerostin in the central area of regenerating scales with cryosections by *in situ* hybridization (ISH) and immunohistochemistry (IHC). **A**: Binocular view of a regenerating scale on day 15. The parts corresponding to the area in the yellow square are magnified in cryosections and shown in B to F. The cryosections were cut along the red line. **B**: Bright-field observation of the cryosection. The upper side is the epidermis side and is composed of a thin osseous layer covered with flat lining cells. Some cells were half embedded in the calcified matrix. The lower side is the dermis side and is composed of a thick fibrous layer covered with scale osteoblasts. The inset is a magnified view of the area in the square. The arrowheads indicate half-embedded cells in a groove or in the calcified matrix. Osseous and fibrous layers are indicated by black and white curly braces respectively. **C**: Detection of *Sost* mRNA with an antisense probe by ISH. Positive staining is indicated by a brownish-purple color. The inset is a magnified view of the area in the square. The arrowhead indicates half-embedded cells in a groove. Osseous and fibrous layers are indicated by black and white curly braces respectively. **D**: Negative control for ISH with a *Sost* sense probe. The inset is a magnified view of the area in the square. The arrowhead indicates half-embedded cells in a groove. Osseous and fibrous layers are indicated by black and white curly braces respectively. **E**: Same field of view as B, observed with fluorescent microscopy. Sclerostin was detected with anti-sclerostin antibodies by IHC. Positive staining is indicated by a green color. The inset is a magnified view of the area in the square. The arrowheads indicate half-embedded cells in a groove or in the calcified matrix. **F**: Negative control for IHC with normal rabbit IgG. The inset is a magnified view of the area in the square. The arrowhead indicates half-embedded cells in a groove or in the calcified matrix. Nuclei were stained with methyl green in C and D (light green) and with DAPI in B (pale blue), E (blue), and F (blue). The nuclei appear pale blue in B due to the fact that the blue fluorescent color of DAPI and the visible light image are superimposed. The images are representative data obtained from repeated experiments using five or more scales from different goldfish.

fied matrix (Fig. 4C, E). Judging from observation by transmission electron microscopy, we found two types of half-embedded cells in the osseous layer. One type was observed at the bottom of the groove, in contact with the collagen bundles of the fibrous layer (Fig. 5A). The other type was almost surrounded by the mineralized matrix (Fig. 5B). Thus, we speculate that these half-embedded cells are osteocyte-like cells, which terminally differentiate from osteoblasts and become embedded in the bone matrix (Sawa *et al.* 2019).

The basis for this speculation is not only the cell

localization (half embedded in the mineralized matrix) and *Sost*/sclerostin expression. Osteoclasts expressing tartrate-resistant acid phosphatase have been reported to exist in the osseous layers, especially along grooves, but not in fibrous layers (Ikegame *et al.* 2019). In addition, RANKL expression has been found mainly in cells in the grooves (Yamamoto *et al.* 2020). Accordingly, it is speculated that half-embedded osteocyte-like cells in the osseous layers promote osteoclast differentiation and activation by producing RANKL (Yamamoto *et al.* 2020), as those cells do in mammalian bone (Nakashima *et al.*

Fig. 5 Electron microscopic observation of the half-embedded cells in osseous layer of regenerating scale. **A:** A decalcified scale. A half-embedded cell (arrowhead) in a groove observed between the mineralized matrices. A part of fibrous layer is shown in the bottom half of the panel; plywood-like collagen fibers are stained by tannic acid. **B:** An un-decalcified scale. A half-embedded cell (arrowhead) observed in the mineralized matrix.

2011).

Increased Sost expression in the regenerating scales of goldfish under microgravity in outer space

Our previous experiments in space aboard the ISS demonstrated that osteoclast activity along grooves and the ratio of RANKL : OPG expression in goldfish scale increase under microgravity conditions (Ikegame *et al.* 2019). It has been reported that sclerostin stimulates RANKL production in osteocytes (Wijenayaka *et al.* 2011) and inhibits OPG production in osteoblasts/osteocytes by disturbing the Wnt signaling pathway to activate osteoclasts (Kubota *et al.* 2009; Kobayashi *et al.* 2016; Delgado-Calle *et al.* 2017; Figurek *et al.* 2020). This fact, together with our

finding of the presence of *Sost*-expressing cells in goldfish scales, indicates that sclerostin may contribute to the microgravity-induced activation of osteoclasts in goldfish scales. We compared the expression levels of *Sost*, as well as genes encoding suppressors of the Wnt signaling pathway, *Dkk1* and *Wif1*, in goldfish scales kept under microgravity conditions in outer space (F- μ g) with those of goldfish scales treated with 1 g gravity in outer space (F-1g). Microgravity was found to increase the expression of *Sost*, whereas it did not have any effect on *Dkk1* or *Wif1* (Fig. 6).

Sost/sclerostin expression is known to be upregulated by mechanical unloading and plays a critical role in bone mechano-response (Robling *et al.* 2008; Lin *et al.* 2009). Our results showed for the first time that the expression of *Sost* in fish scales is stimulated by microgravity. In support of our idea, it was reported that the *Sost* expression level in mouse calvariae was significantly higher than that of ground control after 30 days of spaceflight (Macaulay *et al.* 2017). These suggest a mechanism underlying the osteoporosis-like loss of bone mass in outer space: microgravity increases sclerostin in osteocytes and osteoblasts by facilitating the expression of the *Sost* gene. The increased sclerostin then excessively activates osteoclasts, leading to the loss of bone mass.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

Fig. 6 Expression analyses of Wnt inhibitors *Sost*, *Dkk1*, and *Wif1* in the regenerating scales of goldfish under microgravity by quantitative RT-PCR. *Ef1α* gene was used for normalization of respective gene expression. * $P < 0.05$ versus control; $n = 4$

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