Investigation of osteogenesis changes in medaka larvae reared in normal gravity, simulated-microgravity and hypergravity environments

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Abstract

Bones are important organs for body resistance against force produced by gravity, though the influence of gravity on bone development is unclear. To examine the effects of gravity on osteogenesis, medaka larvae were reared in water or gel under various conditions. For determining the effects on bone development in a state of motion, larvae were reared in water under normal gravity (*1g*) or hypergravity (*5g*) conditions. Also, to examine the direct effect of gravity on bone mineralization, larvae were embedded in low melting agarose gel containing alizarin complexone (ALC) and reared for three days under a normal gravity (*1g*), simulated-microgravity (*s-μg*) with use of a clinostat device, or *5g* condition. Medaka reared in water under the *5g* condition showed forward protruding jaws and spreading of the mineralized area of jaw teeth as compared to those reared under the *1g* control condition. In addition, the direction of growth of the notochord in the fin region was changed upward in those reared under the *5g* condition, accompanied by a part of acetylated tubulin-positive nerves also localized upward, while positive signals for DsRed, expressed by an *osterix* promoter, in osteoblasts were increased in the fin region. On the other hand, in medaka reared in gel, ALC signals in the fin ray of those in the *s-μg* condition were increased as compared to those in the *5g* condition. Changes noted in medaka larvae over three days indicated osteogenesis adaptation to the specific gravity environment. The present results obtained with an experimental system

are considered useful for examinations in the future regarding changes of osteogenesis, which will be needed to clarify the mechanism of the effects of gravity on bone development.

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Introduction

During space flight, changes in bones under a microgravity condition are dependent on the skeletal-site position relative to the gravitational vector. (Stavnichuk et al. 2020). For the various vertebrates present on Earth, bones are important organs that support body weight produced by gravity, though it not clears how gravity affects bone development.

Small fish, such as medaka and zebrafish, provide a good animal model for examining hard tissues under various gravity environments, such as microgravity in a space environment, simulated-microgravity (*s-μg*) generated by a clinostat device, and hypergravity. In 1994, medaka were transported to space in a space shuttle, and the first investigations of mating and rearing under microgravity were performed (Ijiri 1995). In 2012, osteoclast- and osteoblast-double transgenic medaka lines were reared for two months at the International Space Station (ISS) for a study of bone metabolism under microgravity (Chatani et al. 2015), while changes in fluorescent signals in bone-related transgenic medaka larvae embedded in gel at the ISS were also observed (Chatani et al. 2016). On the other hand, clinostat devices have been used for examinations of the effects of microgravity on earth, as it has an ability to cancel the sum of the gravity vector as a time average by changing its direction (Borst and Van Loon 2009). Another study reported bone changes in zebrafish larvae reared in clinostats in water rather than gels (Aceto et al. 2016), while in our previous examination of hypergravity, young adult medaka were reared under a *5g* condition for six months, which resulted in findings showing spine curvature and otolith formation defects (Chatani et al. 2019). More recently, it was reported that hypergravity (*6g*) induced abnormal chondrocyte formation in zebrafish larvae (Lawrence et al. 2021). However, the involvement of gravity in bone development remains to be elucidated.

The present study was conducted to assess bone morphology in larvae reared in water under *5g* and *1g* (control) conditions. In addition, signals showing uptake of alizarin complexone (ALC) in larvae reared in gel under *s-μg*, hypergravity (*5g*), control (*1g*) conditions were also examined. The results showed several differences in bone formation depending on the gravity condition. We consider that this study will be useful for providing base research findings regarding gravity-related bone biology.

Materials and Methods

Egg collection

Eggs were obtained from Cab or *TRAP* promoter-GFP/*osterix* promoter-DsRed double transgenic medaka lines, and placed in 30-mm dishes (SUMILON Dish, Sumitomo Bakelite Co., Ltd.) filled with egg water, and

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then shaken for seven days on a shaker (Labo Shaker BC-730, Bio Craft) in a 28°C incubator (MIR-154S-PJ, Panasonic).

Rearing larvae in water

Hatched medaka larvae from Cab or TRAP-GFP/Osterix-DsRed double transgenic lines were placed in bottles (Nunc EasyFlask 75 cm², Thermo Scientific) containing 200 ml of rearing water, then in an incubator for three days with light blocked. For the 5g condition, that was generated by spinning with a centrifuge (LIX-140SP, Tomy Seiko Co., Ltd) at 160 rpm (Chatani et al. 2019).

Alizarin Red stain

Medaka were fixed in 4% Paraformaldehyde Phosphate Buffer Solution (PFA; Fujifilm Wako Pure Chemical Corporation) overnight at 4°C, then washed with PBS and placed into 0.1N KOH. Next, 50 µl of Alizarin Red solution saturated in ethanol was added to 1 ml of 0.1N KOH, mixed gently, and allowed to stand overnight at 4°C. After rinsing with 0.1N KOH, the samples underwent stepwise replacement with Glycerin (Fujifilm Wako Pure Chemical Corporation) 70%/0.1N KOH and photographed under a microscope.

Nerve stain

Larvae were fixed with 80% methanol/20% DMSO overnight at 4°C. After washing three times with 1 ml of PBST (0.1% Tween), 10% sheep serum/PBST at 200 µl was used as the blocking liquid. Next, monoclonal anti-acetylated tubulin antibodies (Sigma, T7451) at 0.1 µl were added as the primary antibodies and the larvae were left overnight at 4°C, then washing with 1 ml of PBST was performed three times and Donkey Anti-Mouse IgG H&L (Alexa Fluor® 568, Abcam, ab175472) at 0.1 µl was added and again they were left overnight at 4°C. Finally, fins were washed using PBS.

Rearing larvae in agarose gel

Medaka larvae were anesthetized at two days after hatching by placing them on ice and then into a 30-mm dish that had been previously filled with 4 ml of low melting point agarose gel (Agarose-LM Sieve, Nacalai Tesque). Next, another 4 ml of liquid low melting point agarose gel was added and the specimen was oriented with tweezers. The agarose gel was prepared in autoclaved rearing water containing 1% alizarin complexone (ALC; 348-00093, Wako Pure Chemical Corporation).

Each dish was set under the following gravity conditions with no light. The 1g control group was placed stationary on a desk. For the 5g (5.29 times greater than normal gravity) group, that was generated by centrifuge spinning at 160 rpm (Chatani et al. 2019), while the s-µg group was rotated in a clinostat device (Zeromo CL-100, Kitagawa). Larvae were removed from the agarose gel after three days and signals indicating calcification were observed using a fluorescence microscope (M205FA, Leica).

Measurements

All images were captured using LAS X software (LAS

X 3.3.169258.3, Leica) and body part lengths were measured with another LAS X software package (LAS X 3.7.21655.1, Leica). Area and intensity of fluorescence in the images of medaka larvae were determined by use of Fiji ImageJ software (ImageJ 1.53c, NIH) (Schneider, Rasband, and Eliceiri 2012). All graphs were constructing with RStudio Desktop, v. 1.4.1717.

For quantitative analysis of mandibular protrusion to the maxilla (lateral) (Fig. 2C), measurement values were calculated using an original formula for normalization, as shown following. Head=p, Mandibular=q, Body length=L,

$$\text{Mandibular protrusion to maxilla (lateral)} = \frac{q}{L}.$$

For quantitative analysis of mandibular protrusion (dorsal) (Fig. 2F), measurement values were calculated using an original formula for normalization, as shown following. Head=x, Mandibular=y, Body length=L,

$$\text{Mandibular protrusion (dorsal)} = \frac{y}{L}.$$

For quantitative analysis of cross eye distance (Fig. 2F), measurement values were calculated using an original formula for normalization, as shown following. Right eye=v, Left eye=w, Eye distance=z, Body length=L,

$$\text{Cross eye distance} = \frac{z}{\frac{(v+w)}{2}}.$$

For quantitative analysis of the aspect ratio of the spine (Fig. 2J), measurement values were calculated using an original formula for normalization, as shown following. 13th Width=l, 13th Length=m, 14th Width=n, 14th

$$\text{Length=o, Body length=L, Aspect ratio of the spine} = \frac{\frac{l}{m} + \frac{n}{o}}{2}.$$

For quantitative analysis of mandibular protrusion (dorsal) (Fig. 5C), measurement values were calculated using an original formula for normalization, as shown following. Head=x, Mandibular=y, Body length=L,

$$\text{Mandibular protrusion (dorsal)} = \frac{y}{L}.$$

For quantitative analysis of the aspect ratio of the spine (Fig. 5E), measurement values were calculated using an original formula for normalization, as shown following. 14th Width=l, 14th Length=m, 15th Width=n, 15th

$$\text{Length=o, Body length=L, Aspect ratio of the spine} = \frac{\frac{l}{m} + \frac{n}{o}}{2}.$$

For quantitative analysis of the growth area of the

spine (Fig. 5G), measurement values were calculated using an original formula for normalization, as shown following. Upper front=p, Upper rear=q, Lower front=r, Lower rear=s, Body length=L, Growth area of the spine= $\frac{p+q+r+s}{L}$. (Total area from 15th to 18th.)

Statistics

Calculated values for the *1g*, *s-μg*, and *5g* conditions were compared, and p-values were estimated using Tukey's test. Graph data are mean±s.d.

Results

Medaka larvae are relatively small in size and bone formation through the whole body can be easily observed. To examine the effects of gravity in a state of motion while in water, hatched larvae were reared in water for three days under the *1g* or *5g* condition, then bones were stained with Alizarin Red (Fig. 1A, B). Those results showed a significant difference in body length between the gravity conditions (Fig. 2A, B). Observations of heads from the lateral or dorsal side showed that the mandible protruded forward in the *5g* group as compared to the *1g* group (control) (Fig. 2C-F). Furthermore, observations in those two directions revealed increases in mandibular teeth and bone areas in the *5g* group (Fig. 2C, F-H), while there was no difference in distance between the eyes (Fig. 2F, I), suggesting that the mandible specifically was deformed as a result of higher gravity. The ratio of length to width of the vertebrae in the lateral view (Fig. 2J, K) was unchanged. In the fin region, the notochord was bent upward at a greater angle in larvae reared in the *5g* environment as compared to the control group (Fig. 2L, M). Also, acetylated tubulin staining showed that some of the nerves were localized upward along the notochord in medaka reared in the higher gravity condition (Fig. 3A). In the *5g* group, the number of cells positive for TRAP, a known osteoclast marker, was decreased (Fig. 3E, G),

whereas the intensity of *osterix*-DsRed signals in osteoblasts was increased (Fig. 3B, D). There were no differences between the groups regarding fluorescence areas of *osterix*-DsRed and TRAP-GFP (Fig. 3C, F). These findings indicated that the properties of cells constituting fin tissue were changed according to the gravitational environment.

Next, to examine the direct effects of gravity on bone growth, larvae were embedded in low-melting-point agarose gel containing alizarin complexone (ALC), which marks calcium uptake on the bone surface during bone formation (Fig. 4A). Thereafter, larvae at two days after hatching were reared for three days in a *1g* (normal gravity), *s-μg* (Fig. 4B), or *5g* (Fig. 4C) environment. Our results showed no significant differences in morphology, such as body length, ratio of jaw protrusion, spine shape, or bending of the notochord in the fin region (Fig. 5A-J). At the end of the spine, the area with ALC fluorescence signals tended to be greater under the *s-μg* condition as compared to the others, indicating increased uptake. Although, though the differences were not significant, a comparison between the *s-μg* and *5g* groups showed a p-value of 0.173 (Fig. 5G, H). Furthermore, while the area of ALC fluorescence signals in the fin ray did not change, the intensity of ALC fluorescence signals there was increased under the *s-μg* as compared to the *5g* condition (Fig. 5K-M).

Discussion

Using a novel approach to better understand the involvement of gravity in osteogenesis, some striking changes were noted in larvae. We have investigated bone formation under two conditions. One is rearing in water, the condition that is affected by floating power. The other is rearing in a gel, which we consider as a kind of organ culture, which is not affected by floating power. A clinostat is a device that uses rotation to cancel the sum of gravity vectors over a period of time. The condition in this device

Figure 1. Overview of bone development analysis using larvae reared in water under *1g* and *5g* conditions. (A) Schedule for bone staining of larvae reared for three days in *1g* and *5g*. (B) Bottles placed in centrifuge.

Figure 2. Hypergravity in state of motion in water affects jaw protrusion and fin formation.

Alizarin Red staining of medaka larvae reared under 1g and 5g conditions for three days. (A) Lateral views of whole body. (B) Comparison of body length. (C) Lateral view of head region (upper) and measured sites (lower). p, q: measured length. Yellow arrows show lower jaw teeth. (D) Comparison of mandibular protrusion to maxilla (lateral). (E) Comparison of mandibular protrusion (dorsal). (F) Dorsal view of head region (upper) and measured sites (lower). v, w, x, y, z: measured length. Yellow arrows show jaw teeth. (G) Comparison of calcified area (lateral). (H) Comparison of calcified area (dorsal). (I) Comparison of cross eye distance. (J) Lateral view of vertebral body (upper). Enlarged views show 13th and 14th vertebrae in dotted squares indicated in upper images (lower). l, m, n, o: measured length. (K) Comparison of aspect ratio of spine. (L) Lateral view of fin region (upper). θ in lower images show angle of bending notochord. (M) Comparison of angle of notochord. P-values were determined using Tukey's test. n.s.: not significant difference, ** $p < 0.01$. (B, D, E, G, H, I: 1g, n=42; 5g, n=45. K: 1g, n=30; 5g, n=35. M: 1g, n=76; 5g, n=77)

Figure 3. Distribution of nerve and osteoblast changes in larvae reared in water under hypergravity. Comparison of fin region in larvae reared for three days under 1g and 5g conditions. (A) Acetylated tubulin staining. (B-G) Analysis of *osterix*-DsRed/*TRAP*-GFP double-stained transgenic medaka line. (B) Merged images of optical and *osterix*-DsRed/*TRAP*-GFP (upper), and *osterix*-DsRed (middle and lower) signals. Area circled in yellow in lower images shows measurement area. (C) Comparison of areas of fluorescent *osterix*-DsRed signals. (D) Comparison of fluorescent intensity of *osterix*-DsRed signals. (E) *TRAP*-GFP. Area circled in yellow in lower images shows measurement area. (F) Comparison of areas of fluorescent *TRAP*-GFP signals. (G) Comparison of areas of fluorescent intensity of *TRAP*-GFP signals. P-values were determined using Tukey's test. n.s.: not significant difference, * $p < 0.05$. (C, D: 1g, n=9; 5g, n=10. F, G: 1g, n=8; 5g, n=11.)

Figure 4. Overview of ALC uptake analysis using larvae embedded in agarose gel under $1g$, $s\text{-}\mu g$, and $5g$ conditions. (A) Schedule for bone staining and gravity change. Larvae were stained with alizarin complexone (ALC) and kept under one of the three gravity conditions for three days. (B) Dish placed in clinostat device for $s\text{-}\mu g$ condition. (C) Dish placed in centrifuge for $5g$ condition.

was defined as a simulated-microgravity condition ($s\text{-}\mu g$), and the effect on bone formation in larvae embedded in gel was investigated.

For medaka kept in water, changes were observed in two different areas. First, the jaw protruded forward and the calcified area was increased in those reared in the $5g$ environment. Other studies have reported that mandibles in mice and rats changed under various gravity conditions, indicating that mandibular formation may be dependent on gravity (Ghosh et al. 2016; Mednieks and Hand 2019; Simmons et al. 1983). In addition, as dentistry is considered to be an important issue for astronauts living in space (Zaitzu et al. 2014), it is expected that the related mechanism will be clarified in the future. Additionally, the present results showed that the fins grew upward in medaka exposed to the hypergravity environment. The tail fin has been shown to have an optimal shape (Zhiqiang and Chuijie 2013), with its structure remodeled according to specific types of fin motion (Enny et al. 2020) and the angle of the caudal fin having an effect on thrust production (Matta et al. 2019). Thus, the present finding of an upward-facing fin phenotype may indicate that the morphology of the medaka fin has an ability to change to adapt to a hypergravity environment.

Together, the gravity preferentially affected structure of the jaw and the fin. The mandible is located below the maxilla and is the only bone in the skull that can be moved. Since the mandible is located where it needs to resist the direction of gravity, the mandible may be the sensitive tissue to the effects of gravity. It is thought that floating power may be insufficient for larvae to swim in a $5g$ environment, and it is inferred that the fin structure was changed to move it in order to increase the lift force. As for the body length, the jaws were slightly longer in $5g$, but the body length was shorter because the fins were bent farther upward.

When reared in gel, medaka in the $s\text{-}\mu g$ condition tended to show greater amounts of uptake of ALC into the vertebral body and fin ray as compared to those in the $5g$ condition. Other studies have noted changes in osteoblast activity under clinostat conditions (Granet et al. 2002;

Ulbrich et al. 2014), suggesting that gravity vector diffusion stimulates medaka osteoblasts and promotes bone formation.

The results of our experiments with medaka reared in water indicate that the composition of fins and teeth changed as a result of adaptation to movement in a hypergravity environment. On the other hand, results of those reared in gel seem to show that gravity had direct effects on calcification, because the medaka were unable swim. These results provide an initial step in understanding gravity-dependent osteogenesis. We did not confirm any changes in the osteoclasts on vertebrae because we observed them at an early developmental stage. In future studies, detailed analysis of related mechanisms is anticipated to provide new discoveries, including changes in gene expression levels under various gravity conditions.

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Authors' contributions

M.C. designed the research contents, supported the

Figure 5. ALC uptake increased in spine and fin ray under *s-μg* condition.

Larvae were kept under 1g, *s-μg*, and 5g conditions, and stained with alizarin complexone (ALC). (A) Whole body. (B) Comparison of body length. (C) Enlarged view of head region. x, y: measured lengths. (D) Comparison of ratio of mandibular protrusion. (E) Lateral view of vertebral body (upper). Enlarged views show 14th and 15th vertebrae in dotted squares indicated in upper images (lower). l, m, n, o: measured lengths. (F) Comparison of aspect ratio of spine. (G) ALC signals in vertebral body. Enlarged view shows measured area to determine epiphyseal bone growth. (H) Growth area of spine. A comparison of the *s-μg* and 5g conditions showed a p-value of 0.173. (I) Optical image of fin region. θ in lower images shows angle of bending notochord. (J) Comparison of angle of notochord. (K) ALC signals in fin region. Area circled in yellow in lower images shows measurement area. (L) Comparison of ALC positive areas of fins. (M) Comparison of fluorescent intensity in fins. P-values were determined using Tukey's test. n.s.: not significant difference, *p<0.05. (B, D, F, H, J, L, M: 1g, n=8; *s-μg*, n=10; 5g, n=8.)

experiments, and wrote the manuscript. N.T. performed the experiments, summarized the results, and performed quantitative analysis. M.T. provided the research environment and advised regarding the content of the paper.

Declaration of Interests

The authors have no competing financial interests to declare.

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