Response of osteoblasts and osteoclasts to gravity : analysis by goldfish scales as a model of bone

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学 位 論 文 要 旨

Dissertation Abstract

Response of osteoblasts and osteoclasts to gravity：analysis by goldfish scales as a model of bone
重力に対する骨芽細胞及び破骨細胞の応答：キンギョのウロコを骨モデルとした解析

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Abstract

There are many points needing clarification about the detailed mechanisms of bone disease under microgravity due to the lack of a suitable bone model system to analyze the responses of gravity unloading. Bone is an active connective tissue composed of three types of cells: osteoblasts, osteoclasts, and osteocytes. In my study, in order to validate goldfish scales as a bone model for the analysis of gravity stimuli, I performed following three experiments. First, I forced on receptor activator of NF-κB ligand (RANKL), which is important for osteoclastogenesis and examined the localization of RANKL-positive cells in the regenerating scales of goldfish immunohistochemically. Furthermore, I investigated the induction of signal transduction pathways for osteoclast formation by the addition of exogenous RANKL. Next, the localization of osteocyte-like cells in the regenerating scales of goldfish was investigated. Subsequentially, I evaluated the effect of microgravity by space flight on the mRNA expression of *Sost*, which is known to be important in responding to physical stimuli, in the regenerating scales of goldfish. Finally, the response of osteoblasts and osteoclasts to the physical stimuli of hypergravity and microgravity was analyzed simultaneously for the first time in molecular biology and morphology using the regenerating scales of goldfish. This study provides a significant evidence to prove that teleost scales are an excellent model system for analyzing the effects of physical stimuli on bone metabolism.

Under microgravity conditions, astronauts suffer from bone disease caused by a decline in bone mass. For example, it has been reported that bone mass in the hip and spine decrease by about 1% per month under microgravity conditions during space flight. This disease is one of the main problems during space flight. There are many points needing clarification about the detailed mechanisms of bone disease under microgravity due to the lack of a suitable bone model system to analyze the responses of gravity unloading.

Bone is an active connective tissue composed of three types of cells: osteoblasts, osteoclasts, and osteocytes (Fig. 1). Osteoblasts are bone-forming cells that secrete bone matrix to form hard bone, and osteoclasts are bone-absorbing cells that elute the bone matrix by several enzymic actions. Osteocytes are derived from fully differentiated and mature osteoblasts and are buried in the bone matrix secreted by osteoblasts. Osteocytes act as a leading commander of bone remodeling, sensing and integrating mechanical and chemical signals from the environment to regulate bone formation and bone resorption. However, the breakdown of bone homeostasis causes osteoporosis due to the predominance of bone resorption over bone formation, and, conversely, the predominance of bone formation such as calcification causes bone diseases such as osteopetrosis. Therefore, it is quite important to keep well-balanced bone homeostasis.

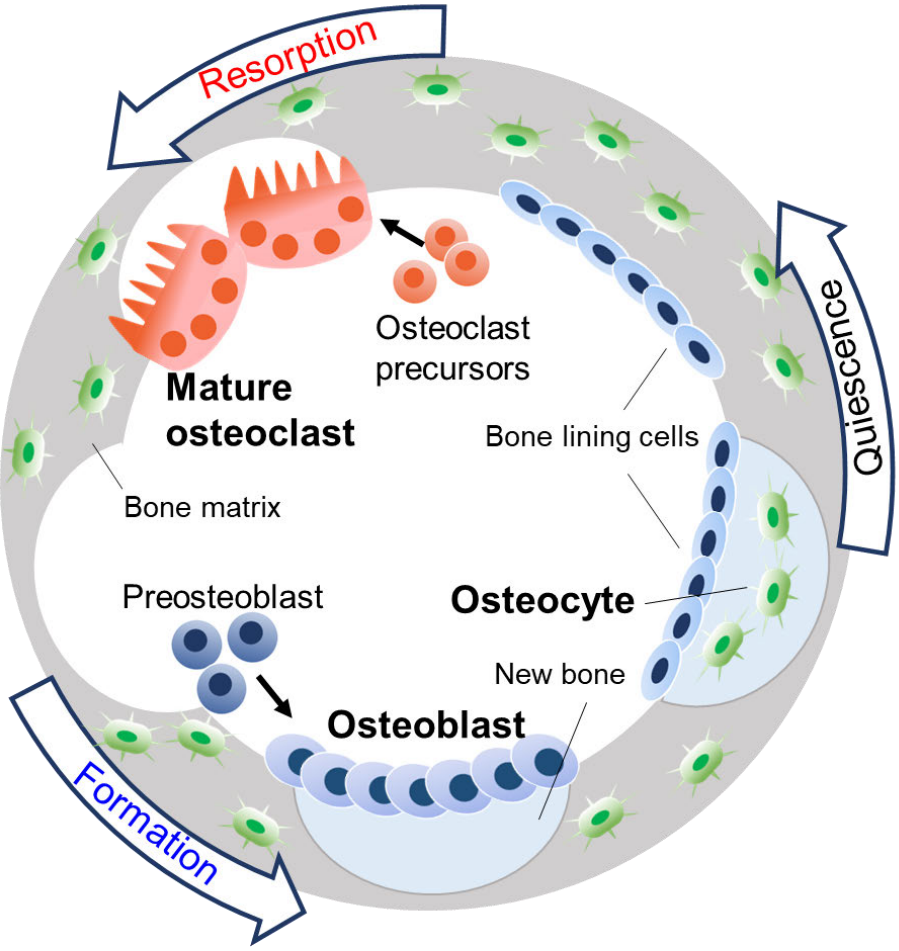

Fig. 1. Bone remodeling

Space experiments using rats and simulated unloading caused by the suspension of the hind limbs with rats have been conducted. However, the results are inconsistent, and many points remain unclear. Under *in vivo* conditions, bone homeostasis is affected by some bioactive substances (e.g., growth factors, cytokines, chemokines) and sexual hormones (estrogen). Therefore, an *in vitro* culture system under coexisting conditions with osteoclasts, osteoblasts, and bone matrix is desired in order to accurately analyze the bone metabolic response to physical stimuli such as hypergravity and microgravity. However, unlike osteoblasts with established cell lines, osteoclasts need to be induced to a multinucleated active form by cell fusion and are not easy to culture. It has been elucidated that the interaction between the osteoclast differentiation factor (receptor activator NF-κB ligand: RANKL) expressed on the cell membrane of osteoblasts and the receptor for RANK expressed on the cell membrane of precursor osteoclasts is important for the differentiation of osteoclasts (Fig. 2). Therefore, a co-culture of osteoblasts and osteoclasts is necessary for investigating bone metabolism.

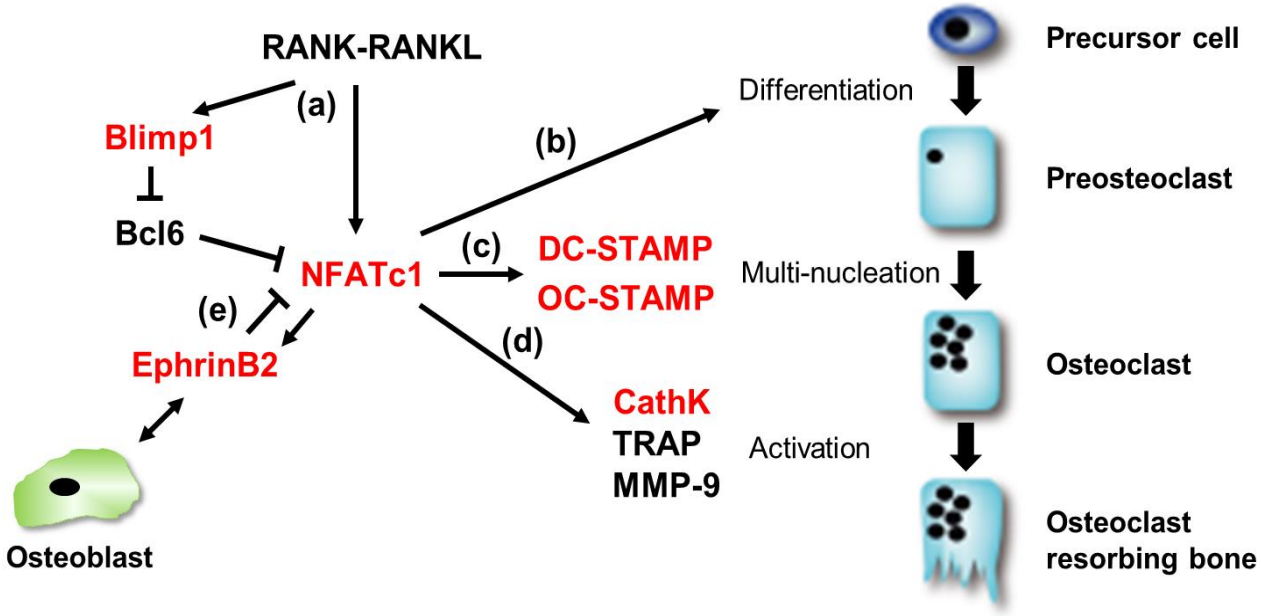

Fig. 2. RANK / RANKL-dependent signaling pathway regulating osteoclastogenesis

On the other hand, teleost fish have a unique hard tissue, fish scale that consists of osteoclasts, osteoblasts, and calcified bone matrix including a fibrillary layer (a thick, partially calcified layer) and a bony layer (a thin, well-calcified external layer). The morphological features of fish scales are very similar to those of mammalian membrane bone. Fish scales as a bone model have been shown to respond sensitively to bioactive substances and physical stimuli including hypergravity and microgravity. Furthermore, fish scales have a characteristic feature regeneration after the removal of ontogenic scales and the regenerating scales have higher cell activity and higher responsiveness to hormones than do normal scales. In my study, in order to validate goldfish scales as a bone model for the analysis of gravity stimuli, I performed following three experiments.

1. **Detection of RANKL-producing cells and the activation of osteoclasts by adding exogenous RANKL to goldfish scales**

First, the localization of RANKL-producing cells in the regenerating scales of goldfish was examined. RANKL-induced osteoclast differentiation and the subsequent enhancement of bone resorption activity is regulated by the master regulator of osteoclast formation (nuclear factor of activated T-cell c1: NFATc1). It has been reported that microgravity exposure enhances bone resorption activity and increases the mRNA expression of *Rankl* of the regenerating scales. However, the localization of RANKL-expressing cells in the regenerating scales of teleost fish was still unknown. Therefore, the localization of RANKL-positive cells in the regenerating scales of goldfish was examined immunohistochemically. As a result, RANKL-producing cells were detected in the grooves of regenerating goldfish scales (Fig. 3). These findings suggest that

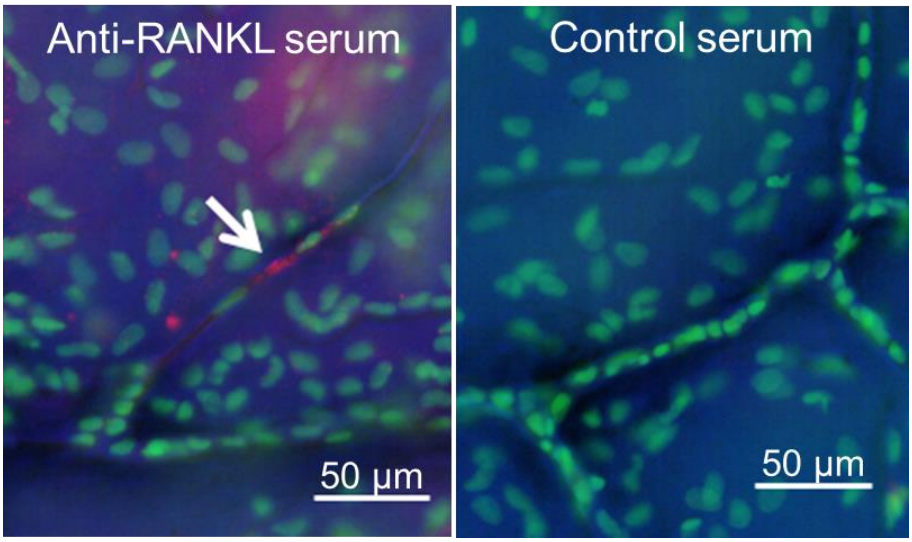

Fig. 3. Immunohistochemical detection of RANKL in regenerating. RANKL immunostaining (red) overexposed with DAPI staining (green).

RANKL-producing cells interact with osteoclasts and induce osteoclastogenesis in the grooves of regenerating scales. Furthermore, I investigated the induction of signal transduction pathways for osteoclast formation by the addition of exogenous RANKL. I found that recombinant mouse RANKL effectively promotes osteoclast activation in goldfish osteoclasts. The mRNA expression of the transcription factor *Nfatc1*, the master regulator of osteoclast formation, significantly increased after 3 hours of incubation with RANKL as compared to the expression level of untreated control cells. In addition, the mRNA expression of the osteoclast function gene, *Ctsk*, was significantly upregulated after 6 hours of incubation with RANKL. After incubating with RANKL for 3 and 6 hours, respectively, the mRNA expressions of *Dc-stamp* and *Oc-stamp*, which are essential for osteoclast multinucleation, were also upregulated. In addition, the mRNA expression of *Blimp1*, a factor involved in osteoclast differentiation, increased with RANKL treatment. RANKL signaling was induced by extrinsic RANKL in the osteoclasts of goldfish scales as in mammalian bone. Also, the expression of *Ephrinb2* mRNA increased significantly. After bone resorption, I estimate that EPHRIN B2 induces coupling and bone remodeling, as it does in mammalian bone. Therefore, I conclude

that RANKL plays an important role in osteoclastogenesis in fish scales as well as mammalian bone.

2. SCLEROSTIN expression in the regenerating scales of goldfish and its increase due to microgravity during space flight

Next, the localization of osteocyte-like cells in the regenerating scales of goldfish was investigated. SCLEROSTIN, a protein encoded by the *Sost* gene, is expressed mainly in osteocytes in mammalian bone. SCLEROSTIN, a negative regulator of bone formation, acts as an inhibitor of the *Wnt/β*-catenin signaling pathway that positively regulates bone formation. SCLEROSTIN produced by osteocytes is known to be an important in responding to physical stimuli. However, the localization of SCLEROSTIN-positive cells in the scales of teleost fish was unknown. In my study, whole-mount ISH and IHC were performed using regenerating goldfish scales to investigate the expression of *Sost* mRNA and SCLEROSTIN proteins in the cells of fish scales. I found that cells located along the grooves and ridges express *Sost* mRNA and its proteins on regenerating goldfish scales (Fig. 4). Next, ISH and IHC were performed using frozen sections of

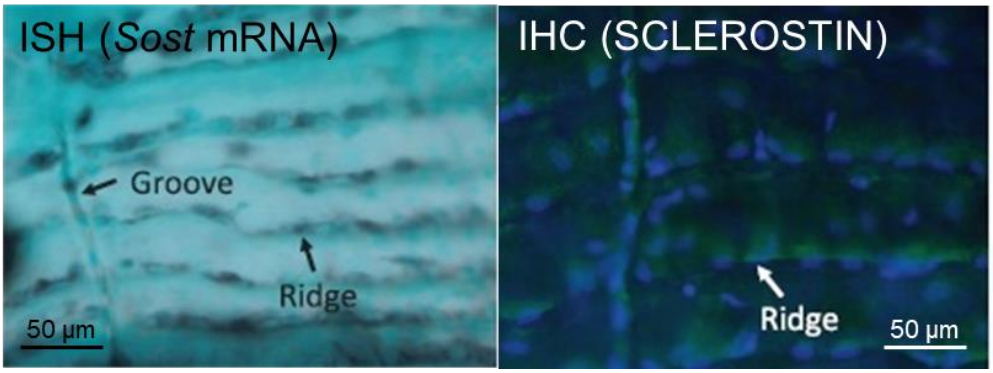

Fig. 4. Whole-mount detection of *Sost*/sclerostin in the peripheral area of the regenerating scales by ISH and IHC. Detection of *Sost* mRNA with an antisense probe by ISH (dark purple). Detection of sclerostin with anti-sclerostin antibody by IHC (green color). Nuclei were stained with methyl green in ISH (light green) and with DAPI in IHC (blue).

regenerating scales. Signals were detected in cells covering the fibrous layer in both the central and peripheral regions of scales. Cells with ISH or IHC signals in the fiber layer were found to have a rounded shape, suggesting that they produced a matrix of scales. In addition to round cells, ISH or IHC signals were detected in the flat cells lining both the bone layer along the surrounding ridge and the central mineralization matrix surface. Therefore, these results show that scale osteoblasts expressed *Sost* mRNA. In addition, a *Sost* /*sclerostin* signal was detected in cells half-embedded in the groove or calcification matrix. Furthermore, using a transmission electron microscope, two types of semi-embedded cells were found in the bone layer (Fig. 5). One type was observed at the bottom of the groove in contact with the collagen bundle in the fibrous layer. The other type was mostly surrounded by a mineralization matrix. Therefore, it is speculated that these half-embedded cells are osteocyte-like cells that eventually

differentiate from osteoblasts and become implanted in the bone matrix, as mammalian osteocyte does.

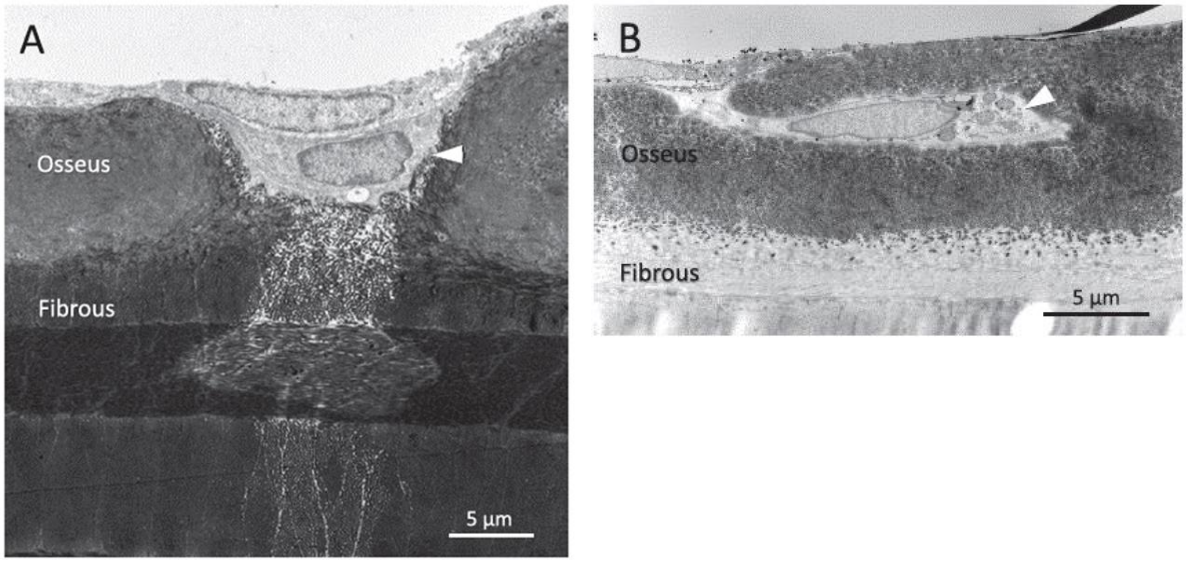

Fig. 5. Electron microscopic observation of the half-embedded cells in osseous layer of regenerating scale. A: A decalcified scale. B: An un-decalcified scale.

Next, I evaluated the effect of microgravity by space flight on the mRNA expression of *Sost* in the regenerating scales of goldfish. Outer space microgravity (F-μg) exposure increased *Sost* mRNA expression but did not affect the expression of *Dkk1* and *Wif1* mRNA. These results show for the first time that the expression of *Sost* in fish scales is stimulated by microgravity, suggesting that SCLEROSTIN is involved in the mechanisms of bone loss in outer space.

3. Response of osteoblasts and osteoclasts to hypergravity and microgravity using goldfish scales as a bone model

Finally, the influence of bone metabolism in response to both hypergravity and microgravity was examined using the regenerating scales of goldfish. In mammalian bone, the mechanisms of converting physical stimuli into biological responses (called *mechanotransduction*) remain unknown due to the lack of an appropriate *in vitro* model system. In addition, there is a lack of agreement regarding the responsiveness of osteoclasts in the *in vitro* cell culture studies of simulated microgravity on the ground and in space experiments. Osteocytes are known to be the major sensor of mechanical loading in mammalian bone. Therefore, the scales of teleost fish, in which osteocyte-like cells coexist on the bone matrix, in addition to osteoblasts and osteoclasts were used to evaluate the mechanical stimulation. A simulated microgravity environment was made using a three-dimensional clinostat, and a comparison between the simulated microgravity environment and the space flight (microgravity) environment was carried out. Results indicate that the mRNA expression of marker in osteoclasts and osteoblasts was changed to induce bone formation

under 3G loading, but to induce bone resorption under simulated g-μG (Fig. 6). These results were

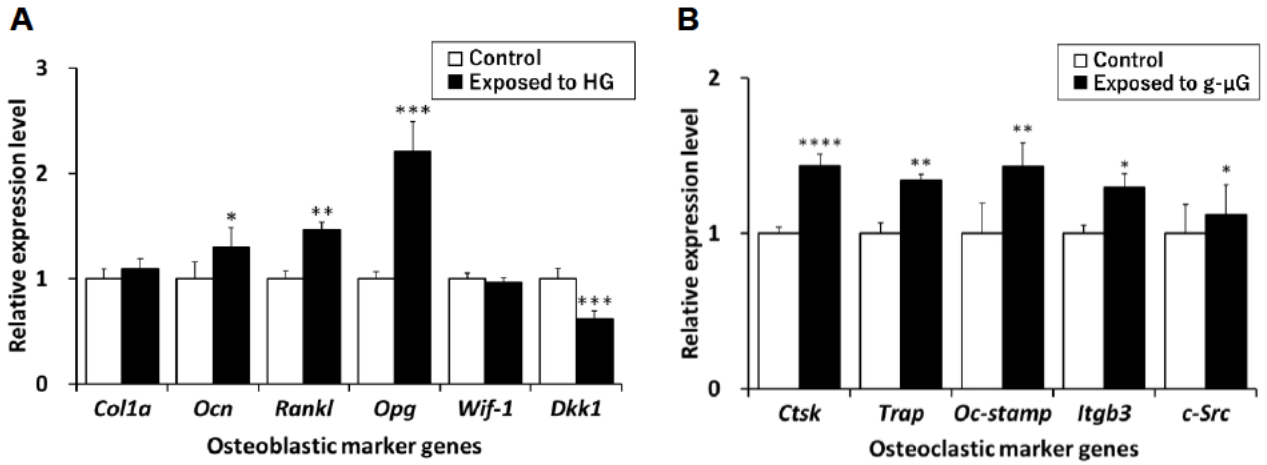

Fig. 6. Effects of gravity on expression of osteoblastic or osteoclastic marker genes. A: Hypergravity (HG). B: Ground microgravity. The value for the control sample was set as 1 for each gene. Data represent the mean ± S.E.M. ($n = 10$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ (Paired t-test).

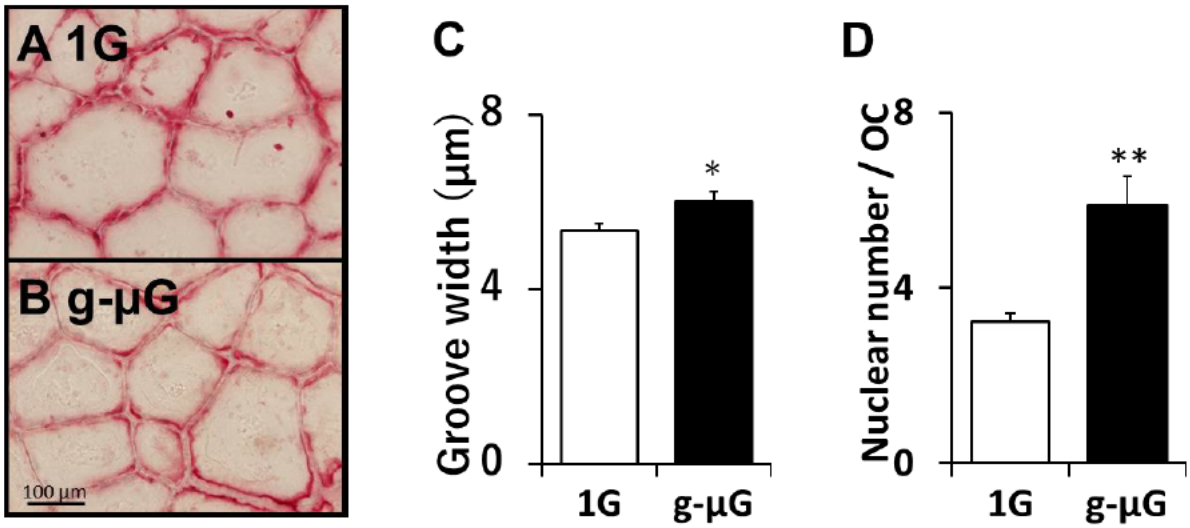

Fig. 7. Histomorphometric analysis of the osteoclasts treated with simulated ground-microgravity (g-μG). A and B: Light microscopy images of regenerating scales stained for TRAP activity under control (1G) (A) or simulated g-μG (B). C: Impact of simulated g-μG on the groove width. D: The average number of nuclei per multinucleated osteoclast. Values are mean ± S.E.M. of ten independent experiments. * $p < 0.05$, ** $p < 0.01$ (Paired t-test).

supported by morphological observations (Fig. 7). The obtained findings using goldfish scales were consistent with those of mammalian *in vivo* studies. In my study, the *Rankl* / *Opg* ratio significantly decreased with 3G loading, while the ratio increased under simulated g-μG. It was suggested that inhibitors of the *Wnt*/β-catenin signaling pathway (*Wif1* and *Dkk1*), which are important signaling pathways

regulating *Opg* expression, are involved in this phenomenon. Both hypergravity and simulated g-μG treatments function to increase *Rankl* expression. These results indicate that the *Rankl* gene may be generally sensitive to changes in gravity. During space flight, the expression of osteoclast marker genes increased, and several genes involved in the *Wnt* / β -catenin pathway were downregulated. These results were in agreement with the simulated g-μG results.

Under the simulated g-μG, the size of osteoclasts was enlarged, and the number of multinucleated osteoclasts increased significantly. Most of the activated osteoclasts were distributed along the edges of the scale grooves. This strongly suggests that osteoclasts activated by RANKL secreted from RANKL-producing cells are present in grooves. This is the first morphological demonstration of the response of osteoclasts to hypergravity and g-μG using the same method.

Current research results, along with the above findings using fish scales, provide evidence that an organ culture system of fish scale in which osteoclasts and osteoblasts coexist on an intact bone matrix and sensitively respond to changes in gravity, is an excellent experimental system for studying the response of bone cells to changes in gravity loading or unloading.

In my study, the response of osteoblasts and osteoclasts to the physical stimuli of hypergravity and microgravity was analyzed simultaneously for the first time in molecular biology and morphology using the regenerating scales of goldfish. The present study provides evidence to prove that teleost scales are an excellent model system for analyzing the effects of physical stimuli on bone metabolism.

令和　4年　7　月26　日

学位論文審査報告書（甲）

1．学位論文題目（外国語の場合は和訳を付けること。）

重力に対する骨芽細胞及び破骨細胞の応答：キンギョのウロコを骨モデルとした解析

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3．審査結果の要旨（600～650字）

山本樹氏は、魚類のウロコを骨モデルとして用いて、物理的刺激（加重力及び微小重力）の影響を評価した。学位論文の第一章では、物理的刺激に対する応答に関連して破骨細胞を活性化する因子（RANKL）に注目して、RANKL産生細胞を特定して、さらに擬似微小重力により、RANKL産生細胞が活性化することを証明した。一方、物理的刺激のメカノセンサーは、骨細胞にあること事が知られている。しかしながら、ウロコには骨細胞の存在は確認されていなかった。そこで第二章では、ウロコに骨細胞様細胞があることを山本氏は証明して、その骨細胞のマーカーが宇宙空間で活性化することを見出した。次に第三章では、遠心機による過重力応答と、3次元クリノスタットによる擬似微小重力により骨芽細胞及び破骨細胞のマーカー遺伝子を解析して、Wntシグナル系が重要な働きを示すことを見出した。Wntシグナル系の発現解析を宇宙実験においても行って、RANKLのインヒビターであるOPGの発現がWntシグナルにより低下することを証明した。これらの結果は、First Authorの論文3報として発表しており、日本宇宙生物科学会から奨励賞を受賞した。審議の結果、博士（理学）の学位に値すると判断された。

4．審査結果　(1) 判　　定（いずれかに○印）　(合　格)　・　不合格

(2) 授与学位　博　士（　理学　）