Detection of RANKL-producing cells and osteoclastic activation by the addition of exogenous RANKL in the regenerating scales of goldfish

Tatsuki Yamamoto, Mika Ikegame, Umi Kawago, Yoshiaki Tabuchi, Jun Hirayama, Toshio Sekiguchi, Yukihiro Furusawa, Koji Yachiguchi, Hajime Matsubara, Makoto Urata, Atsuhiro Hattori and Nobuo Suzuki

Abstract

We have previously reported that microgravity promotes the activation of osteoclasts in cultured regenerating scales. This osteoclastic activation was induced by increased levels of receptor activator of nuclear factor-κB ligand (RANKL). Therefore, we determined that RANKL is an important factor in evaluating osteoclastogenesis in bone tissue. However, the role of RANKL in fish scales is poorly understood. In the present study, we prepared antiserum against goldfish RANKL in rabbits and detected RANKL-producing cells in regenerating goldfish scales. Furthermore, we studied osteoclastic activation by the addition of RANKL to examine exogenous RANKL on osteoclastogenesis in regenerating goldfish scales. As a result, RANKL immune-positive cells were detected in grooves of regenerating scales. In addition, treating the regenerating scales with mammalian RANKL for 3 h significantly increased the expression of the nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), which is essential for osteoclast differentiation. After 6 h of incubation with RANKL, the expression of cathepsin K, a functional osteoclastic gene, significantly increased. Furthermore, the molecules for osteoclast multinucleation and differentiation significantly increased following treatment with mammalian RANKL. Therefore, in fish scales as well as mammalian bone, we concluded that RANKL plays an important role in osteoclastogenesis.

Key words: RANKL, immunostaining, regenerating scale, osteoclasts, goldfish

Introduction

In osteoclasts, the receptor activator of nuclear factor-κB (RANK) and, in osteoblasts, the receptor activator of the nuclear factor-κB ligand (RANKL) play key roles in bone metabolism (Nagy and Penninger, 2015; Ono and Nakashima, 2018). Osteoclastic activation is induced by the binding of RANK to RANKL (Nagy and Penninger, 2015; Ono and Nakashima, 2018), and thus, RANKL has an important role in osteoclastogenesis which leads to bone resorption (Teitelbaum, 2000; Kondo et al., 2001). Additionally, RANK/RANKL signaling activates the nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), which is the master regulator of osteoclastogenesis and induces osteoclastogenic gene expression (Takayanagi et al., 2002; Asagiri et al., 2005; Yamashita et al., 2007). The activation of NFATc1 promotes the expression of various molecules that are involved in the differentiation of osteoclast precursor cells, the multinucleation of preosteoclasts, the bone resorption activity of osteoclasts, or the communication between osteoclasts and osteoblasts (Fig. 1) (Takayanagi et al., 2002; Asagiri et al., 2005; Zhao et al., 2006; Yang et al., 2008; Miyachi et al., 2010; Zhang et al., 2014).

Teleost fish have a unique hard tissue made of scales that contain osteoblasts, osteoclasts, and calcified bone matrix, which is made up of two layers, including a bony layer, which is a thin well-calcified external layer, and a fibrillary layer, which is a thick, partially calcified layer (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Suzuki et al., 2007; Ohira et al., 2008; Suzuki et al., 2008a). Therefore, we have previously used fish scales as a model of bone to assess the effects of bioactive substances and physical stimuli, including hypergravity and microgravity (Suzuki et al., 2008a; Suzuki et al., 2008b; Suzuki et al., 2016; Ikegame et al., 2016).
Furthermore, it is known that teleost scales regenerate after removal (Suzuki et al., 2009; Kakikawa et al., 2012). Our previous study indicated that osteogenesis in regenerating scales is very similar to that of calvarial bone (Yoshikubo et al., 2005; Thamamongood et al., 2012). Using these regenerating scales, we demonstrated that microgravity stimulated the multinucleation and resorption activity of osteoclasts in regenerating scales (Ikegame et al., 2019). The accelerated multinucleation and resorption activities of the regenerating scale osteoclasts in these microenvironments resembled the in vivo conditions of mammalian bone during space flight (Tamma et al., 2009; Gerbaix et al., 2017). Microgravity induces osteoclastic activation in regenerating scales by increasing the mRNA expression levels of RANKL (Ikegame et al., 2019). However, the location of the RANKL-producing cells in regenerating scales has not yet been investigated. Therefore, we prepared antiserum against goldfish RANKL and detected RANKL-producing cells in the regenerating scales of goldfish. To examine RANKL signaling molecules, including NFATc1, furthermore, we investigated the influence of exogenous RANKL on osteoclastogenesis in the regenerating scales of goldfish.

Materials and Methods

Animals
Male goldfish (Carassius auratus) (20-30 g) were purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) and used for our experiment. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Immunohistochemical analysis of RANKL in regenerating goldfish scales

The antiserum of goldfish RANKL was made by Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). Immunization was performed as follows. Two RANKL peptide sequences from Carassius auratus (YLRNHIDMEEAPARAPHC and CLASPQQSPNEEMHSETL, GenBank ID: AB894120) were conjugated with keyhole limpet hemocyanin and injected into rabbits. The antiserum was affinity-purified and used for immunohistochemical analysis.

Immunohistochemical staining was performed as follows. Goldfish were anesthetized with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich, Inc., St. Louis, MO, USA), and the normally developed scales were removed to allow scale regeneration. At day 14, goldfish were anesthetized, and
Exogenous RANKL activated osteoclasts in goldfish scales

Statistical analysis
All results are expressed as the means ± SEM. The statistical significance was assessed by paired t-test. The selected significance level was $p < 0.05$.

Results
Immunohistochemical analysis of RANKL in regenerating goldfish scales
In order to determine the localization of RANKL-expressing cells, antiserum against goldfish RANKL was prepared in rabbits. Immunohistochemical analysis showed that positive RANKL immunostaining was present in some mononucleated cells located in the grooves of the goldfish scales that were regenerated for 14 days (Fig. 2).

Effect of exogenous RANKL on osteoclastogenesis in the cultured regenerating scales of goldfish
Goldfish were anesthetized with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich, Inc.), and the scales were collected and cut in half. Half of each piece was then put into a 24-well microplate with 1 ml of Leibovitz’s L-15 medium (Thermo Fisher Scientific K.K., Tokyo, Japan) supplemented with antibiotic, 2% BSA, and mouse RANKL (150 ng/ml) (R&D Systems, Inc., Minneapolis, MN, USA). The other half of the scale was placed in another well of the microplate containing RANKL-free medium, as a control. The scales were incubated for 3 and 6 h in L-15 medium at 15°C. After incubation, the scales were frozen at -85°C until further mRNA analysis with the real time PCR. Expression of mRNA in the control and experimental scales from the same fish were compared.

Quantitative real-time PCR analysis
Total RNA was isolated from goldfish scales using a total RNA isolation kit for fibrous tissue, and complementary DNA synthesis was performed with a kit (RNase Easy Fibrous Mini-Kit, Qiagen GmbH, Hilden, Germany). PCR amplifications were analyzed using a real-time PCR apparatus (Mx3000pTM, Stratagene, La Jolla, CA, USA) with specific primers for Nfatc1, cathepsin K (Ctsk), osteoclast stimulatory transmembrane protein (Oc-stamp), dendritic cell-specific transmembrane protein (Dc-stamp), blymphocyte-induced maturation protein1 (Blimp1), EphrinB2, and elongation factor 1 alpha (Ef1α) (Table 1). Conditions for PCR was previously described (Suzuki et al., 2011; Sato et al., 2017; Ikegame et al., 2019).

| Target gene    | Forward sequence (5'→3') | Reverse sequence (5'→3') |
|----------------|---------------------------|--------------------------|
| Nfatc1         | CTGTCGTGGGTGTTTGGGAAAG    | GATGCTGGTGTTTGGGATACC    |
| Ctsk           | TGGAGGGGCTGGAAACTCAC      | CATGACCCTTGAAGCTTG      |
| Oc-stamp       | TGGTGTCGCTTTTGGCATTACC   | CATGCAGCGCTTGAAGCTTG    |
| Dc-stamp       | TTGGTTCGCTTTTGGCATTACC   | CATGCAGCGCTTGAAGCTTG    |
| Blimp1         | ATGGAAGGGGTTTGGCAGAGAGC  | ATGGAAGGGGTTTGGCAGAGAGC |
| EphrinB2       | AGAAAGGAAAGCAGAAAGAGGG   | CACACAGGGCAGAGGAGAAGAG |
| Ef1α           | ATGGTGAGGAGGAGGAGGAGGAG  | GGCAGGACTTTGCTTGGTGA    |

Fig. 2. Immunohistochemical detection of RANKL (A: Anti-RANKL serum; B: Control serum) in regenerating scales. RANKL immunostaining was performed overnight at 4°C using normal rabbit serum and overexpressed with DAPI staining (green) in scales after 14 days of regeneration (A). As a negative control, immunostaining was performed overnight at 4°C using normal rabbit serum and overexpressed with DAPI staining (green) in scales after 14 days of regeneration (B). Scale bar, 50 μm.
transcription factor, NFATc1 (Fig. 3A). After 6 h of incubation with RANKL, the mRNA expression of the osteoclastic-functional gene, Ctsk, significantly increased (Fig. 3B). In addition, the mRNA levels of the factors involved in osteoclast multinucleation, including OC-STAMP and DC-STAMP, increased following treatment with RANKL (Fig. 4). Significant differences in the mRNA levels of OC-STAMP and DC-STAMP were observed between the control and experimental groups after 6 h of incubation with RANKL (Fig. 4). After 3 h of incubation with RANKL, there was a statistically significant difference in the mRNA expression levels of OC-STAMP between the treated and control groups (Fig. 4). After 3 h of incubation with RANKL, the mRNA expression of BLIMP1 significantly increased in the treated group as compared to the levels in the control group (Fig. 5). The mRNA expression of EPHRINB2 also showed an increasing trend in the scales treated with RANKL for 3 h, as compared to the untreated control ($p < 0.07$, Fig. 5).
Discussion

In the present study, we detected RANKL-producing cells in the grooves of regenerating goldfish scales (Fig. 2). Several osteoclasts were also present in the grooves of the regenerating scales. Previous studies have shown that there was multinucleation in the grooves under microgravity, which was followed by bone resorption after 86 h of incubation under microgravity (Ikegame et al., 2019). These findings suggest that RANKL-producing cells interact with osteoclasts and induce osteoclastogenesis in the grooves of regenerating scales. One cause of this osteoclastic activation is due to an increase in RANKL expression, as Rankl mRNA expression remarkably increased under microgravity (Ikegame et al., 2019). Based on these findings, we aimed to examine the effects of exogenous RANKL on osteoclastogenesis in the cultured regenerating scales of goldfish.

We found that recombinant mouse RANKL effectively promoted osteoclastic activation in goldfish osteoclasts (Figs. 3-5). The mRNA expression of the transcription factor NFATc1, which is a master regulator of osteoclastogenesis, significantly increased after 3 h of incubation with RANKL, as compared to the levels in untreated control cells. In addition, the mRNA expression of the osteoclastic-functional gene, Ctsk, was significantly upregulated after 6 h of incubation with RANKL. After incubation with RANKL for 3 and 6 h, the mRNA expression of OC-STAMP and DC-STAMP, which are essential for the multinucleation of osteoclasts (Yang et al., 2008; Zhang et al., 2014), was also upregulated (Fig. 4). Additionally, the mRNA expression of BLIMP1, which is a factor involved in the differentiation of osteoclasts (Miyachi et al., 2010), increased with treatment of RANKL (Fig. 5). RANKL signaling was induced by exogenous RANKL in both the osteoclasts of fish scales and in mammalian bone. Furthermore, Ephrinb2 mRNA expression also increased (Fig. 5). After bone resorption, EPHRINB2 induces coupling and bone remodeling (Zhao et al., 2006; Matsuo and Otaki, 2012). Therefore, it is possible that RANKL treatment may promote bone remodeling in scales, as well as in mammalian bone. Future plans include the determination of the detailed RANKL signaling pathway using the regenerating scales of goldfish.

Teleost scales are a simple calcified tissue that coexists with osteoblasts, osteoclasts, and bone matrix coexists. As fish scales have bone-like features, they respond sensitively to microgravity (Ikegame et al., 2019) and hyper-loading (Suzuki et al., 2007; Suzuki et al., 2008b; Suzuki et al., 2009; Kakikawa et al., 2012). Additionally, we found that fish scales respond to low-intensity pulsed ultrasound (LIPUS) (Hanmoto et al., 2008b; Suzuki et al., 2009; Kakikawa et al., 2012). These findings suggest that fish scales respond sensitively to LIPUS stimuli.

Based on these previous studies and the results obtained in the present study, we concluded that RANKL plays an important role in fish bone metabolism, similar to that in mammals, and that fish scales are a suitable model for evaluating several physical stimuli or bioactive substances. We are planning to perform more detailed investigations of the mechanisms of RANKL-mediated osteoclastic activation with microgravity.

Acknowledgments

This study was supported in part by grants to N.S. (Grant-in-Aid for Scientific Research [C] No. 20K06718 by JSPS), to A.H. (Grant-in-Aid for Scientific Research [C] No. 18K11016 by JSPS), to T.S. (Grant-in-Aid for Scientific Research [C] No. 18K06312 by JSPS), to Y.T. (Grant-in-Aid for Scientific Research [C] No. 20K12619 by JSPS), and to J.H. (Grant-in-Aid for Scientific Research [B] No. 20H04565 and [C] No. 18KT0068 by JSPS). This work was partly supported by the cooperative research program of the Institute of Nature and Environmental Technology, Kanazawa University, Accept Nos. 2020, 2030, 2032, and 2033.

Declaration of Interests

The authors declare no competing interests.

References

Asagiri, M., Sato, K., Usami, T., Ochi, S., Nishina, H., Yoshida, H., Morita, I., Wagner, E.F., Mak, T.W., Serfling, E. and Takayanagi, H. (2005) Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. J. Exp. Med., 202, 1261-1269.

Bereiter-Hahn, J. and Zylberberg, L. (1993) Regeneration of teleost fish scale. Comp. Biochem. Physiol. Part A, 105, 625-641.

Gerbaix, M., Gnyubkin, V., Farlay, D., Olivier, C., Ammann, P., Courbon, G., Laroche, N., Genthial, R., Follet, H., Peyrin, F., Shenkman, B., Gauquelin-Koch, G. and Vico L. (2017) One-month spaceflight compromises the bone microstructure, tissue-level mechanical properties, osteocyte survival and lacunae volume in...
mature mice skeletons. Sci. Rep., 7, 2659.

Hamamoto, T., Tabuchi, Y., Ikegame, M., Kondo, T., Kitamura, K., Endo, M., Kobayashi, I., Mishima, H., Sekiguchi, T., Urata, M., Seki, A., Yano, S., Hattori, A. and Suzuki, N. (2017) Effects of low-intensity pulsed ultrasound on osteoclasts: Analysis with goldfish scales as a model of bone. Biomed. Res., 38, 71-77.

Hoffler, C.E., Hankenson, K.D., Miller, J.D., Bilikh, S.K. and Goldstein, S.A. (2006) Novel explant model to study mechanotransduction and cell-cell communication. J. Orthop. Res., 24, 1687-1698.

Ikegame, M., Hattori, A., Tabata, M.J., Kitamura, K., Tabuchi, Y., Furusawa, Y., Maruyama, Y., Yamamoto, T., Sekiguchi, T., Matsuoka, R., Hamamoto, T., Ikari, T., Endo, M., Omori, K., Nakano, M., Yashima, S., Ejiri, S., Taya, T., Nakashima, H., Shimizu, N., Nakamura, M., Kondo, T., Hayakawa, K., Takasaki, I., Kaminishi, A., Akatsuka, R., Sasayama, Y., Nishiguchi, T., Nara, M., Iseki, H., Chowdhury, V.S., Wada, S., Iijiri, K., Takeuchi, T., Suzuki, T., Ando, H., Matsuda, K., Somei, M., Mishima, H., Mikuni-Takagaki, Y., Funahashi, H., Takahashi, A., Watanabe, Y., Maeda, M., Uchida, H., Hayashi, A., Kambegawa, A., Seki, A., Yano, S., Shimazu, T., Suzuki, H., Hirayama, J. and Suzuki, N. (2019) Melatonin is a potential drug for the prevention of bone loss during space flight. J. Pineal Res., 67, e12594

Kakikawa, M., Yamamoto, T., Chowdhury, V.S., Satoh, Y., Kitamura, K., Sekiguchi, T., Funahashi, H., Omori, K., Endo, M., Yano, S., Yamada, S., Hayakawa, K., Chiba, A., Srivastava, A.K., Iijiri, K., Seki, A., Hattori, A. and Suzuki, N. (2012) Determination of calcium sensing receptor in the scales of goldfish and induction of its mRNA expression by acceleration loading. Biol. Sci. Space, 26, 26-31.

Kondo, Y., Irie, K., Ikegame, M., Ejiri, S., Hanada, K. and Ozawa, H. (2001) Role of stromal cells in osteoclast differentiation in bone marrow. J. Bone Miner. Metab., 19, 352-358.

Matsuo, K. and Otaki, N. (2012) Bone cell interactions through Eph/ephrin bone modeling, remodeling and associated diseases. Cell Adh. Migr., 6, 148-156.

Miyauuchi, Y., Ninomiya, K., Miyamoto, H., Sakamoto, A., Iwasaki, R., Hoshi, H., Miyamoto, K., Hao, W., Yoshida, S., Morioha, H., Chiba, K., Kato, S., Tokuhisa, T., Saitou, M., Toyama, Y., Suda, T. and Miyamoto, T. (2010) The Blimp1-Bcl6 axis is critical to regulate osteoclast differentiation and bone homeostasis. J. Exp. Med., 207, 751-762.

Nagy, V. and Penninger, J.M. (2015) The RANKL-RANK story. Gerontology, 61, 534-542.

Ohira, Y., Shimizu, M., Urak, K. and Takagi, Y. (2007) Scale regeneration and calcification in goldfish Carassius auratus: Quantitative and morphological processes. Fishery Sci., 73, 46-54.

Owan, I., Burr, D.B., Turner, C.H., Qiu, J., Tu, Y., Onyia, J.E. and Duncan, R.L. (1997) Mechanotransduction in bone: Osteoblasts are more responsive to fluid forces than mechanical strain. Am. J. Physiol., 273, C810-C815

Omori, K., Wada, S., Maruyama, Y., Hattori, A., Kitamura, K., Sato, Y., Nara, M., Funahashi, H., Yachiguchi, K., Hayakawa, K., Endo, M., Kusakari, R., Yano, S., Srivastava, A.K., Kusui, T., Ejiri, S., Chen, W., Tabuchi, Y., Furusawa, Y., Kondo, T., Sasayama, Y., Nishiuichi, T., Nakano, M., Sakamoto, T. and Suzuki, N. (2012) Prostaglandin E2 increases both osteoblastic and osteoclastic activities in the scales and participates in calcium metabolism in goldfish. Zool. Sci., 29, 499-504.

Ono, T. and Nakashima, T. (2018) Recent advances in osteoclast biology. Histochem. Cell Biol., 149, 325-341.

Sato, M., Yachiguchi, K., Motohashi, K., Yaguchi, Y., Tabuchi, Y., Kitani, Y., Ikari, T., Ogiso, S., Sekiguchi, T., Hai, T.N., Huang, D.T.T., Hoang, N.V., Urama, M., Mishima, H., Hattori, A. and Suzuki, N. (2017) Sodium fluoride influences calcium metabolism resulting from the suppression of osteoclasts in the scales of nibbler fish Girella punctate. Fisheries Sci., 83, 543-550.

Suzuki, N., Suzuki, T. and Kurokawa, T. (2000) Suppression of osteoclastic activities by calcitonin in the scales of goldfish (freshwater teleost) and nibbler fish (seawater teleost). Peptides, 21, 115-124.

Suzuki, N., Kitamura, K., Nemat, T., Shimizu, N., Wada, S., Kondo, T., Tabata, M.J., Sodeyama, F., Iijiri, K. and Hattori, A. (2007) Effect of vibration on osteoblastic and osteoclastic activities: Analysis of bone metabolism using goldfish scale as a model for bone. Adv. Space Res., 40, 1711-1721.

Suzuki, N., Somei, M., Seki, A., Reiter, R.J. and Hattori, A. (2008a) Novel bromomelanotin derivatives as potentially effective drugs to treat bone diseases. J. Pineal Res., 45, 229-234.

Suzuki, N., Omori, K., Nakamura, M., Tabata, M.J., Ikegame, M., Iijiri, K., Kitamura, K., Nemat, T., Shimizu, N., Kondo, T., Matsuda, K., Ando, H., Kasahara, H., Nagase, M., Nara, M. and Hattori, A. (2008b) Scale osteoblasts and osteoclasts sensitively respond to low-gravity loading by centrifuging. Biol. Sci. Space, 22, 3-7.

Suzuki, N., Kitamura, K., Omori, K., Nemat, T., Satoh, Y., Tabata, M.J., Ikegame, M., Yamamoto, T., Iijiri, K., Furusawa, Y., Kondo, T., Takasaki, I., Tabuchi, Y., Wada, S., Shimizu, N., Sasayama, Y., Endo, M., Takeuchi, T., Nara, M., Somei, M., Maruyama, Y., Hayakawa, K., Shimazu, T., Shigeto, Y., Yano, S. and Hattori, A. (2009) Response of osteoblasts and osteoclasts in regenerating scales to gravity loading. Biol Sci Space 23, 211-217.

Suzuki, N., Danks, J.A., Maruyama, Y., Ikegame, M., Sasayama, Y., Hattori, A., Nakamura, M., Tabata, M.J., Yamamoto, T., Furuya, R., Sajioh, K., Mishima, H., Srivastava, A.K., Furusawa, Y., Kondo, T., Tabuchi, Y., Takasaki, I., Chowdhury, V.S., Hayakawa, K. and Martin, T.J. (2011) Parathyroid hormone 1 (1-34) acts on the scales and involves calcium metabolism in goldfish. Bone 48, 1186-1193.
Suzuki, N., Kitamura, K. and Hattori, A. (2016) Fish scale is a suitable model for analyzing determinants of skeletal fragility in type 2 diabetes. Endocrine, 54, 575-577.

Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E.F., Mak, T.W., Kodama, T. and Taniguchi, T. (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev. Cell, 3, 889-901.

Tamma, R., Colaianni, G., Camerino, C., Di Benedetto, A., Greco, G., Strippoli, M., Vergari, R., Grano, A., Mancini, L., Mori, G., Colucci, S., Grano, M. and Zallone, A. (2009) Microgravity during spaceflight directly affects in vitro osteoclastogenesis and bone resorption. FASEB J., 23, 2549-2554.

Thamamongood, T.A., Furuya, R., Fukuba, S., Nakamura, M., Suzuki, N. and Hattori, A. (2012) Expression of osteoblastic and osteoclastic genes during spontaneous regeneration and autotransplantation of goldfish scale: A new tool to study intramembranous bone regeneration. Bone, 50, 1240-1249

Teitelbaum, S.L. (2000) Bone resorption by osteoclasts. Science, 289, 1504-1508.

Yang, M., Birnbaum, M.J., MacKay, C.A., Mason-Savas, A., Thompson, B. and Odgren P.R. (2008) Osteoclast stimulatory transmembrane protein (OC-STAMP), a novel protein induced by RANKL that promotes osteoclast differentiation. J. Cell. Physiol., 215, 497-505.

Yamashita, T., Yao, Z., Li, F., Zhang, Q., Badelli, I.R., Schwarz, E.M., Takeshita, S., Wagner, E.F., Noda, M., Matsuo, K., Xing, L. and Boyce, B.F. (2007) NF-κB p50 and p52 regulate receptor activator of NF-κB ligand (RANKL) and tumor necrosis factor-induced osteoclast precursor differentiation by activating c-Fos and NFATc1. J. Biol. Chem., 282, 18245-18253.

Yoshikubo, H., Suzuki, N., Takemura, K., Hosoi, M., Yashima, S., Iwamuro, S., Takagi, Y., Tabata, M.J. and Hattori, A. (2005) Osteoblastic activity and estrogenic response in the regenerating scale of goldfish, a good model of osteogenesis. Life Sci., 76, 2699-2709.

Watabe, H., Furuhama, T., Tani-Ishii, N. and Mikuni-Takagaki, Y. (2011) Mechanotransduction activates α5β1 integrin and PI3K/Akt signaling pathways in mandibular osteoblasts. Exp. Cell Res., 317, 2642-2649.

Zhao, C., Irie, N., Takada, Y., Shimoda, K., Miyamoto, T., Nishiwaki, T., Suda, T. and Matsuo, K. (2006) Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. Cell Metab., 4, 111-121.

Zhang, C., Dou, C.E., Xu, J. and Dong, S. (2014) DC-STAMP, the key fusion-mediating molecule in osteoclastogenesis. J. Cell. Physiol., 229, 1330-1335.