Parathyroid hormone 1 (1-34) acts on the scales and involves calcium metabolism in goldfish

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Parathyroid hormone 1 (1-34) acts on the scales and involves calcium metabolism in goldfish

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Abstract

The effect of fugu parathyroid hormone 1 (fugu PTH1) on osteoblasts and osteoclasts in teleosts was examined with an assay system using teleost scale and the following markers: alkaline phosphatase (ALP) for osteoblasts and tartrate-resistant acid phosphatase (TRAP) for osteoclasts. Synthetic fugu PTH1 (1-34) (100 pg/ml to 10 ng/ml) significantly increased ALP activity at 6 h of incubation. High-dose (10 ng/ml) fugu PTH1 significantly increased ALP activity even after 18 h of incubation. In the case of TRAP activity, fugu PTH1 did not change at 6 h of incubation, but fugu PTH1 (100 pg/ml to 10 ng/ml) significantly increased TRAP activity at 18 h. Similar results were obtained for human PTH (1-34), but there was an even greater response with fugu PTH1 than with human PTH. *In vitro,* we demonstrated that both the receptor activator of the NF-κB ligand in osteoblasts and the receptor activator NF-κB mRNA expression in osteoclasts increased significantly by fugu PTH1 treatment. In an *in vivo* experiment, fugu PTH1 induced hypercalcemia resulted from the increase of both osteoblastic and osteoclastic activities in the scale as well as the decrease of scale calcium contents after fugu PTH1 injection. In addition, an *in vitro* experiment with intramuscular autotransplanted scale indicated that the ratio of multinucleated osteoclasts/mononucleated osteoclasts in PTH-treated scales was significantly higher than that in the control scales. Thus, we concluded that PTH acts on osteoblasts and osteoclasts in the scales and regulates calcium metabolism in goldfish.

Introduction

Parathyroid hormone (PTH) increases plasma calcium level in mammals and is secreted from the parathyroid gland in all tetrapods, but not in fish. The first animal to evolve parathyroid glands is the amphibian [1]. Recently, the PTH gene has been discovered in *Fugu rubripes*, and 80 amino acids of the protein-coding region were determined [2]. Fugu PTH1 as well as human PTH specifically bound to the human parathyroid hormone receptor (PTHR) and promoted cyclic AMP formation in a mammalian cell line [2]. In addition, it was found that zebrafish had two PTHs [3] and that zebrafish PTH1 and zebrafish PTH2 mRNAs and PTH1 protein were detected in the neuromasts of the lateral line and in the central nervous system during embryogenesis [4]. These results suggest that PTH synthesized in the neural tissues has a physiological significant role in teleosts.

The teleost scale is a calcified tissue that contains osteoblasts and osteoclasts [5-7]. The scales are a functional internal calcium reservoir during periods of increased calcium demand, such as sexual maturation and starvation [5, 7-11]. In the scale, as in mammalian bone, type I collagen [12], bone γ-carboxyglutamic acid protein [13], osteonectin [14, 15], and hydroxyapatite [16] are present. Thus, the teleost scale has a number of features in common with mammalian membranous bone.

Recently, we developed a new *in vitro* assay system using fish scale [17, 18], which can be used to detect the activities of scale osteoblasts and osteoclasts simultaneously using the alkaline phosphatase (ALP) and the tartrate-resistant acid phosphatase (TRAP) assay, respectively. This assay is an original assay system to detect the respective enzyme activity from a single scale by transferring each scale to a 96-well

microplate. These markers (TRAP and ALP) have been shown to be affected by a number of hormones and other factors in osteoclasts and osteoblasts [19-21] in mammals. In the scales of carp, de Vrieze et al. [22] also demonstrated that ALP and TRAP are valid markers for osteoblasts and osteoclasts, respectively.

Using this system, we demonstrated that calcitonin suppressed osteoclastic activity in scale osteoclasts as it does in mammalian osteoclasts [17]. Moreover, we were the first to find that melatonin, a major hormonal product of the pineal gland, functioned negatively in both osteoclasts and osteoblasts [18]. The action of melatonin on bone has also subsequently been reported in *in vivo* studies in the rat [23]. Moreover, we indicated that osteogenesis in the regenerating scale is very similar to that seen in mammalian membranous bone and that regenerating scales have estrogen receptors which respond to estrogen in the same manner as mammalian osteoblasts [24]. Persson et al [25] reported that estrogen specific binding was detected in the scales of rainbow trout. We suggest that this fish scale culture system is useful for the evaluation of the effect of PTH on bone.

In the present study, we examined the effect of fugu PTH1 on goldfish scale osteoblastic and osteoclastic activities and compared the actions of fugu PTH1 with those of human PTH. To confirm the effect of fugu PTH1 on plasma calcium and the scale (osteoblasts and osteoclasts), *in vivo* experiments were carried out. In teleosts, three types of receptors for PTH have been identified [26] and it has been reported that zebrafish PTH1 binds to both PTH1R and PTH3R [3]. In order to confirm that PTH's action on the scales of goldfish was *via* the PTH receptors, we established their presence in the scales by reverse transcription-polymerase chain reaction (RT-PCR) with primers

based on the conserved regions for both mammalian and zebrafish PTHR sequences. In addition, expression analyses of both the receptor activator of NF-κB (RANK) and the receptor activator of the NF-κB ligand (RANKL) in the fugu PTH1-treated scales were performed because the RANK-RANKL pathway is necessary for osteoclast differentiation [27-29]. Moreover, the induction of mononucleated osteoclasts to multinucleated osteoclasts was investigated using goldfish scales autotransplanted to muscle.

Materials and Methods

Animals

A previous study [17] indicated that the sensitivity for calcemic hormones was higher in mature female than in mature male teleosts. Therefore, female goldfish (*Carassius auratus*) (30-40 g) were purchased and used for all of the *in vitro* experiments and mRNA expression analyses. To examine the effect of fugu PTH1 on the calcium metabolism, immature goldfish (4-6 g), in which the endogenous effects of sex steroids are negligible, were used for the *in vivo* study.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Kanazawa University.

Effects of fugu PTH1 (1-34) and human PTH (1-34) on ALP and TRAP activities in the cultured scales of goldfish

A 1% penicillin-streptomycin mixture (ICN Biomedicals Inc., OH, USA) was added to Eagle's modified minimum essential medium (MEM; ICN Biomedicals, Inc.).

HEPES (Research Organics Inc., OH, USA) (20 mM) was added to MEM and adjusted to pH 7.0. After filtration, MEM was used in the experiments. Scales were collected from goldfish under anesthesia with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich, Inc., MO, USA) and incubated for 6 and 18 h in MEM supplemented with: 1) fugu PTH1 (1-34) (1 pg/ml to 10 ng/ml); 2) human PTH (1-34) (Bachem AG, Bubendorf, Switzerland) (1 pg/ml to 10 ng/ml); 3) a PTH-free medium as a control. Fugu PTH1 synthesis was carried out as described previously [2]. After incubation, ALP and TRAP activities were measured using the same methods described in Suzuki and Hattori [18]. The results are shown as means ± SEM (n=8). The value of EC50 was calculated using SigmaPlot software (Systat Software Inc.).

Effects of fugu PTH1 (1-34) on scale ALP and TRAP activities, plasma calcium level, and scale calcium content in the goldfish (in vivo experiment)

In the experimental group, goldfish (body weight: 4-7 g) were anesthetized in the same manner as above and then fugu PTH1 (500 ng/g body weight) was injected intraperitoneally. The goldfish in the control group were injected with saline (0.9% NaCl) in the same manner as experimental goldfish. These goldfish were kept in the aquarium for 1, 2, 3, and 4 days (each n=10). During the experimental periods, these goldfish were fasted to exclude intestinal calcium uptake from diets. Each day after injection, blood samples were collected from the gill using a heparinized capillary from individual, anesthetized goldfish. After centrifugation at 15,000 rpm for 3 min, the plasma was immediately frozen and kept at –80 °C until use. The plasma total calcium level (mM) was determined using an assay kit (Calcium C, Wako Pure Chemical

Industries, Ltd., Osaka, Japan). At the highest plasma calcium level in goldfish after PTH injection, we measured the ALP and TRAP activity and calcium content in the scale. The scale calcium content (mg/ dry weight (mg) of scale) was determined using the Calcium C kit (Wako) after the scale was dissolved in nitric acid and then neutralized by NaOH.

Cloning of PTHR cDNA from the goldfish scales by RT-PCR

Total RNA was prepared from goldfish scales using a total RNA isolation kit (Nippon Gene, Tokyo, Japan). RT-PCR was performed using Oligotex-dT 30 Super (Takara Bio, Inc., Otsu, Japan) as an oligo dT primer to prevent genomic DNA contamination [30]. The primers were designed on the basis of mammalian PTHRs (mouse PTH1R: P41593; mouse PTH2R: Q91V95; human PTH1R: NP_000307; humanPTH2R: NP_005039); and zebrafish (PTH1R: AF132084; PTH2R: AF132082; PTH3R: AF132085). The primer sequences were sense-1: 5'-TAYRTNTAYGAYTTYAAYCAY-3'; sense-2: 5'-CAYTGYACNMGNAAYTAYATHCAY-3'; antisense-1: 5'-GYYTGNACYTCNCCRTTRCA-3'; and antisense-2: 5'-ACYTCNCCRTTRCARWARCARTA-3'. The first and second PCR (first PCR: sense-1/ antisense-1 primer set; second PCR sense-2/ antisense-2 primer set) were performed using Taq polymerase (Nippon Gene). The PCR parameters were 35 cycles of denaturation for 0.5 min at 96 °C, annealing for 1 min at 45 °C, and extension for 2 min at 72 °C, followed by a single cycle at 72 °C for 30 min. PCR products obtained by the nested PCR were gel-purified and cloned into pT7blue-T vector (EMD Biosciences,

Inc., Novagen Brand, WI, USA). Inserts were sequenced by an automated DNA sequencer (3100 type, Applied Biosystems, CA, USA).

Comparison of PTH1R and PTH2R mRNA expression in the scales

Total RNA was prepared from goldfish scales using a total RNA isolation kit for fibrous tissue and complementary DNA synthesis was performed (RNase Easy Fibrous Mini-Kit, Qiagen GmbH, Hilden, Germany). The PCR amplification was analyzed with a real-time PCR apparatus (Mx3000p^{TM}, Stratagene, CA, USA) using the primers for PTH1R (sense: 5'-GCCACTCTTGCTGACACCGAGT-3'; antisense: 5'-ATCTGGATTTGCCACGGCACCC-3') and for PTH2R (sense: 5'-GGCAACGCTGGCGGATGTAA-3'; antisense: 5'-TGTGTCGTATCGCCCCGCAT-3'). The annealing temperature of PTH1R and PTH2R was 60 °C. The detailed conditions of PCR were described in our previous study [31].

To confirm the expression level of PTH1R and PTH2R in the goldfish scales, we used a different method in which the PCR conditions for each receptor cDNA would be specifically amplified using the primers for PTH1R (sense: 5'-AATGGAGAGCATCACTGTGGAAG-3'; antisense: 5'-ACGGCACCCCAGACACTTCT-3') and for PTH2R (sense: 5'-CAACATCAGCACCGTCTCCATT-3'; antisense: 5'-CCCAGGCCAGACCTGTGAA-3'). Amplification of β-actin cDNA used the primer set: sense: 5'-CACTGTGCCCATCTACGAG-3'; antisense: 5'-CCATCTCCTGCTCGAAGTC-3' (32). The conditions for PCR amplification were

denaturation for 0.5 min at 96 °C, annealing for 1 min at 60 °C, and extension for 2 min at 72 °C followed by a single cycle at 72 °C for 30 min. The PCR products were analyzed on a 2.5% NuSive GTG agarose gel (FMC BioProducts, ME, USA) and stained with ethidium bromide. The band densities were estimated using a computer program (NIH Image J).

Expression analyses of RANK and RANKL mRNAs in fugu PTH1 (1-34)-treated scales

Scales collected from goldfish under anesthesia as described above and incubated for 6 and 18h in MEM supplemented with fugu PTH1 (10 ng/ml) and a PTH-free medium as a control. After incubation, the scales were immediately frozen and kept at -80 °C until use.

Total RNA was prepared from goldfish scales using the method set out above and complementary DNA was synthesized. The primers (RANK-sense:5'-AAGTGGACAGATTGTAAAGCTAT-3'; RANK-antisense: 5'-GCCACCTGATGAGGTTTCAGCAC-3'; RANKL-sense: 5'-GCGCTTACCTGCGGAATCATATC-3'; RANKL-antisense: 5'-AAGTGCAACAGAATCGCCACAC-3') were designed using the zebrafish RANK sequence (Accession No. XM_001918948) and the RANKL sequence in goldfish (Accession No. AB459540). The PCR amplification was analyzed by a real-time PCR apparatus (Stratagene). Amplification of β-actin was performed using primers (sense: 5'-CGAGCGTGGCTACAGCTTCA-3' ; antisense: 5'-GCCCGTCAGGGAGCTCATAG-3') [33]. The PCR amplification was analyzed by real-time PCR. The detailed conditions of PCR were described in our previous study

[31]. The annealing temperature of RANK, RANKL, and β-actin was 60 °C. The RANK and RANKL mRNA levels were normalized to the β-actin mRNA level.

Effect of fugu PTH1(1-34) on the differentiation of mononucleated osteoclasts into multinucleated osteoclasts using the intramuscular autotransplanted scales

Goldfish were anesthetized and to induce mononucleated osteoclasts into the surface of the scale, the scales collected from goldfish were intramuscularly autotransplanted [34]. Thereafter, the goldfish were kept in tap water containing an antibiotic (Green F Gold, Sanei Co., Ltd., Tokyo, Japan) at 25 °C for 3 days. The goldfish were again anesthetized. The implanted scales were taken out and cut into two pieces. The half was put into each well of a microplate and pre-incubated with MEM for 6 h. One piece of scale was then incubated with fugu PTH1 (10 ng/ml), and the other piece was incubated without hormone. After the incubation, the pieces of the scales were fixed in 10% formalin in a 0.05 M cacodylate buffer (pH 7.4). TRAP staining of the scales was performed by the methods of Cole and Walters [35]. After TRAP staining, the specimens were counterstained with 4', 6-diamino-2-phenylindole (DAPI) (Molecular Probes, Inc., Eugene, OR, USA). Then, the numbers of mono- and multinucleated osteoclasts were counted.

To examine the fine structure of multinucleated osteoclasts, the fugu PTH1-treated scales were fixed with 2.5% glutaraldehyde (Nacalai Tesque, Inc., Kyoto, Japan) in a 0.1M cacodylate buffer at pH 7.4 for 1 h. After fixation, the specimens were decalcified with 5% EDTA for 3 days. Following post-fixation with 1% osmium tetroxide (Merck KGaA, Darmstadt, Germany), the specimens were dehydrated and embedded in Epon

812 (TAAB Laboratories, Berks, U.K.). Ultrathin sections were obtained from the Epon blocks and stained with uranyl acetate and lead citrate. These sections were observed under an electron microscope (H-500, Hitachi, Tokyo, Japan) operated at 100 kV.

Statistical analysis

In the experiments involving RT-PCR and osteoclast differentiation with intramuscular autotransplanted scale, the data were analyzed using the paired *t*-test. In other experiments, statistical significance was analyzed by one- or two-way ANOVA followed by the Dunnett test. The significance level was $P<0.05$.

Results

Effects of fugu PTH1 (1-34) and human PTH (1-34) on ALP and TRAP activities in the cultured scales of goldfish

Fugu PTH1 (100 pg/ml to 10 ng/ml) significantly increased ALP activity in 6 h incubations (Fig. 1A). After 18 h of incubation, the ALP activity remained significantly increased only at 10 ng/ml (Fig. 1B).

ALP activity was also increased by human PTH with 6 h of incubation; however, by two-way ANOVA analysis, there was a significantly greater ($P<0.05$) response with fugu PTH1 than with human PTH at 6 h of incubation (Fig. 1A). The EC50 values of fugu PTH1 and human PTH were 18 and 76 (pg/ml), respectively. After 18 h of incubation, the activation of ALP remained high only at the 10 ng/ml dose of both fugu PTH1 and human PTH (Fig. 1B).

Neither fugu nor human PTH treatment altered TRAP activity after 6 h incubation

(Fig. 2A), but, at 18 h TRAP activity increased in scales treated with either PTH (Fig. 2B), with fugu PTH1 being more potent than human PTH.

Effects of fugu PTH1 (1-34) on scale ALP and TRAP activities, plasma calcium level, and scale calcium content in the goldfish (in vivo experiment)

After a single injection of fugu PTH1 into immature goldfish, the plasma calcium level increased for 1 to 3 days, with the highest level at day 2 after injection. At that time, the scale calcium content was decreased significantly (Fig. 3B), and both ALP and TRAP activities in the scale were significantly increased (Figs. 3C and 3D).

Partial amino acid sequences of goldfish PTHRs

Sequence analysis indicated that there were two different cDNA fragments amplified. One fragment had a high degree (96.6%) of amino acid identity to zebrafish PTH1R (AF132084), while the identity of the other fragment to zebrafish PTH2R (AF132082) was 84.8%. The amino acid sequence identity of both fragments to zebrafish PTHR3 (AF132085) was 60.3 and 50.0%, respectively. Therefore, it was concluded that these goldfish fragments are goldfish PTH1R (AB497045) and PTH2R (AB497046), respectively.

Comparison of PTH1R and PTH2R mRNA expressions in the scales

Since it appeared from semi-quantitative PCR that PTH1R was more abundant than PTH2R, we carried out quantitative RT-PCR. The band strength of RT-PCR indicated that expression of PTH1R mRNA in the scale was greater than that of PTH2R

mRNA (Fig. 4).

Expression analyses of RANK and RANKL mRNAs in fugu PTH1 (1-34)-treated scales

Figure 5 shows RANK and RANKL mRNA expression with and without fugu PTH1 treatment. After 6 h incubation of goldfish scales *in vitro*, RANK mRNA expression in fugu PTH-treated scales was significantly increased compared with control (Fig. 5), and this difference was further enhanced at the 18 h incubation time point. RANKL mRNA expression in the PTH-treated scales increased significantly and to approximately the same extent at both 6 and 18 h of incubation.

Effect of fugu PTH1 (1-34) on the differentiation of mononucleated osteoclasts into multinucleated osteoclasts using the intramuscular autotransplanted scales

Mononucleated osteoclasts were induced on the surface of the scales at 3 days after intramuscular autotransplantation of the goldfish scales. Using these scales, an *in vitro* experiment was performed. After 24 and 48 h of incubation, the ratio of multinucleated osteoclasts/mononucleated osteoclasts in PTH-treated scales was significantly higher than that in the control scales (Fig. 6A). The number of mono- and multinucleated osteoclasts in the fugu PTH1-treated scales was significantly higher than that in the control scales at both 24 and 48 h (Fig. 6B). Results of TRAP staining in the PTH-treated scale and control scale are indicated in figure 7. The edges of the PTH-treated scale were strongly stained by TRAP. Typical mono- and multi-nucleated osteoclasts in the edges of the scales are indicated in figure 8. In addition, the fine structure of the multinucleated osteoclasts is shown in Fig. 9. The multinucleated

osteoclasts had several nuclei, a ruffled border, a clear zone, abundant mitochondria, and perinuclear Golgi complexes and were very similar to the mammalian multinucleated osteoclasts.

Discussion

Using an *in vitro* assay system with goldfish scales, we have shown that fugu PTH1 (1-34) increased both ALP and TRAP activities in the scales. The *in vitro* data were supported by our *in vivo* findings, in which hypercalcemia was induced by fugu PTH and was associated with a decline of scale calcium content, consistent with mobilization of scale calcium. In addition, we have shown that the ratio of multinucleated osteoclasts/mononucleated osteoclasts in PTH-treated scales was significantly higher than that in the control scales using intramuscular autotransplanted scales *in vitro*. The osteoclasts in the goldfish scale were found to be almost identical to mammalian osteoclasts, judging from their electron microscopic appearance. In teleosts, thus, PTH appears to act directly on the scales and may influence the plasma calcium level through those cellular actions, as is the case in mammalian bone.

In the reproductive period of female teleosts, the plasma calcium level increases remarkably [36-38]. This calcium is bound to vitellogenin, which is a major component of egg protein and a calcium-binding protein [39, 40]. In this period, calcitonin, which has a hypocalcemic action through inhibiting scale osteoclasts, plays an important role. In fact, the plasma calcitonin level of female teleost increases in the reproductive period [38, 41]. Using an *in vitro* assay system, we found that goldfish calcitonin suppressed osteoclastic activity in the goldfish scales [17]. In our present *in vivo* study,

hypercalcemia was induced by fugu PTH1 injection. In the vitellogenesis of female teleosts, we believe that PTH functions to remove calcium from the scales. However, there is no data concerning the involvement of PTH in bone metabolism in fish during reproductive seasons. Further work to follow up this study will include examining PTH and PTHR mRNA expression in the scales during reproduction, and, thereby, elucidating the physiological role of PTH in teleosts.

In mammals, RANKL produced by cells in the osteoblast lineage binds to RANK in mononuclear hemopoietic precursors and promotes the formation and activity of multinucleated osteoclasts [27]. In mammals, PTH1R is located in the osteoblast lineage and increases osteoclastic formation and activity *via* the RANK-RANKL pathway [27-29]. Our present data demonstrates that fugu PTH1 can increase osteoblastic activity and then activate osteoclasts in the goldfish scale. The increased RANKL mRNA in response to PTH treatment in scales (Fig 5) most likely reflects an action of PTH on the osteoblasts of scales, whereas the increased RANK production is the result of increased osteoclast generation, as confirmed in Figs 6-9. Since PTH1R is mainly expressed in the scale, this hormone seems likely to bind to PTH1R in osteoblasts as well in mammals.

In the present study, we cloned both PTH1R and PTH2R cDNAs from the goldfish scales and found a greater abundance of PTH1R than PTH2R. A ligand of PTH2R is a tuberoinfundibular peptide (TIP) isolated from bovine hypothalamus [42, 43]. As both TIP and PTH2R are strongly expressed in the hypothalamus, it was considered that TIP acts on the nervous system in mammals [43, 44]. In tilapia, however, TIP mRNA was expressed in excretory organs, such as the gill and kidney [45]. In addition, the

expression of TIP mRNA in the gill and kidney of seawater-acclimated fish was higher than that of freshwater-acclimated fish, suggesting that the TIP as well as PTH influences the mineral metabolism in teleosts.

Rotllant et al. [46] found that the parathyroid hormone-related protein increased osteoclastic activity in seawater fish scales *via* cAMP/AC, and Canario et al. [47] found that PTH1 or PTH2 failed to activate the cAMP/AC pathway or stimulate calcium transport in seawater fish scales. Notwithstanding these differences, it is known that the exchange of calcium in the scales of freshwater teleosts is faster than that in marine teleosts because freshwater teleosts live in a low-calcium environment. In fact, the response of estrogen and calcitonin in the scales of freshwater teleosts was higher than that in those of marine teleosts [17, 48-50]. In a freshwater teleost, such as goldfish, we can clearly show dose-dependent effects of PTH on osteoblastic and osteoclastic parameters *in vitro,* and effects of PTH on scale and plasma calcium *in vivo*. Therefore, further studies are needed to elucidate the signaling pathway and physiological role of PTH in the scale.

In teleosts, plasma PTH level has not yet been measured by homologous assay. In trout and goldfish, plasma PTH levels have been detected by heterologous RIA system with bovine PTH (1-84) antibody. The plasma level of goldfish was around 1.5 (ng/ml) [51]. This might indicate that plasma level of PTH in goldfish is at least one order of magnitude higher than in mammals, but specific, homologous assays will be needed to establish this. In the future, we will determine the PTH sequence in goldfish and develop specific antibody to measure the plasma level of PTH in goldfish.

We recently sequenced osteocalcin from goldfish scales. Using this sequence, we

preliminarily examined the effect of PTH on osteoblasts by real-time PCR method. We found that the mRNA expression of osteocalcin in PTH-treated scales was twice higher than that in control scales. We strongly believe that the action of PTH on scale osteoblasts is direct, and most likely indirect on scale osteoclasts, resemble those in mammalian bone. In addition, we are planning for future experiment with prelabeling mononucleated-osteoclasts or transgenetic mononucleated-osteoclasts to demonstrate the differentiation from mononucleated-osteoclasts to multinucleated-osteoclasts.

In the past, our laboratory has sequenced TRAP and cathepsin K from the goldfish scale [33]. In the scale osteoclasts, the osteoclast markers TRAP and cathepsin K were expressed which were detected by *in situ* hybridization [33]. In osteoblasts as well, type I collagen [12], osteocalcin [13], and osteonectin [14, 15] are present in the scales. In fact, it was reported that teleost scale was more important in these animals as a store of calcium than vertebra, jaw, and otolith, judging from the study of $^{45}Ca^{2+}$-prelabeled scale, vertebra, jaw, and otolith of goldfish and killifish [9]. Considering these facts together with the present study, we conclude that the teleost scale is a functional calcium source analogous to the skeleton in mammals.

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Figure legends

Fig. 1. Effects of fugu PTH1 and human PTH on ALP activity in cultured goldfish scales after 6 h (A) and 18 h (B) of incubation. * and ** indicate statistically significant differences at $P<0.05$ and $P<0.01$, respectively, from the values in the control scales. ALP activity was calculated as nmol pNP produced/mg scale/h (n=8).

Fig. 2. Effects of fugu PTH1 and human PTH on TRAP activity in cultured goldfish scales after 6 h (A) and 18 h (B) of incubation. * and ** indicate statistically significant differences at $P<0.05$ and $P<0.01$, respectively, from the values in the control scales. TRAP activity was calculated as nmol pNP produced/mg scale/h (n=8).

Fig. 3. Effects of fugu PTH1 on plasma calcium (mM) (A), scale calcium content (mg/ dry weight (mg) of scale) (B), and scale ALP (C) and TRAP (D) activities (nmol pNP produced/mg scale/h) in an *in vivo* experiment using immature goldfish. *, **, and *** indicate statistically significant differences at $P<0.05$, $P<0.01$, and $P<0.001$, respectively, from the values in the control. The graph shows the means ± SEM (n=10).

Fig. 4. Comparison of PTH1R and PTH2R mRNA expression in goldfish scales. In the RT-PCR analysis, the PTH1R and PTH2R mRNA levels were normalized to the β-actin mRNA level (n=8). *** indicates statistically significant difference at $P<0.001$ from the values in the control scales.

Fig. 5. Expression analysis of RANK and RANKL mRNAs in the fugu PTH1 (10 ng/ml)-treated scale. The RANK and RANKL mRNA levels were normalized to the β-actin mRNA level. * and ** indicate statistically significant differences at $P<0.05$ and $P<0.01$, respectively, from the values in the control scales (n=8).

Fig. 6. Relative ratio of multinucleated osteoclasts/ mononucleated osteoclasts (A) and number of mono- and multinucleated osteoclasts (B) in the fugu PTH1-treated scales. After 3 days after intramuscular autotransplantion, the scales were removed and cut into half. After pre-incubation with a PTH-free culture medium for 6 h, one half of scale was cultured for 48 hours with fugu PTH1 (10 ng/ml) and then fixed and stained for TRAP. The other half was cultured for 48 hours without fugu PTH1 and then fixed and stained for TRAP. After 4',6-diamino-2-phenylindole (DAPI) staining, the number of mono- and multinucleated osteoclasts was counted. * and ** indicate statistically significant differences at $P<0.05$ and $P<0.01$, respectively, from the values in the control scales (n=8).

Fig. 7. The whole mount TRAP staining of the intramuscular autotransplanted scale. At 3 days after intramuscular autotransplantion, the scales removed and bisected. After pre-incubation with a PTH-free culture medium for 6 h, one half of scale was cultured for 48 hours with fugu PTH1 (10 ng/ml) and the other half was cultured for 48 hours without fugu PTH1. Both halves were fixed and stained for TRAP.

Fig. 8. Microscopic views of autotransplanted scales stained for TRAP (A) and TRAP-DAPI (B). At 3 days after intramuscular autotransplantion, the scales were removed and bisected. After pre-incubation with a PTH-free culture medium for 6 h, one half of the scales were cultured for 48 h with fugu PTH1 (10 ng/ml), and subsequently fixed and stained for TRAP and counterstained with 4',6-diamino-2-phenylindole (DAPI). Arrow heads: mononucleated osteoclasts. Arrows: multinucleated osteoclasts.

Fig. 9. Fine structure of a multinucleated osteoclast in the fugu PTH1-treated scales. At 3 days after intramuscular autotransplantion, the intramuscular autotransplanted scales were removed and cut into half. After pre-incubation with a PTH-free culture medium for 6 h, one half scale cultured for 48 h with fugu PTH1 (10 ng/ml) was then fixed and observed using electron microscopy. The inset is a higher magnification of the square. Nu: nucleus, RB: ruffled border, asterisk (*): clear zone.

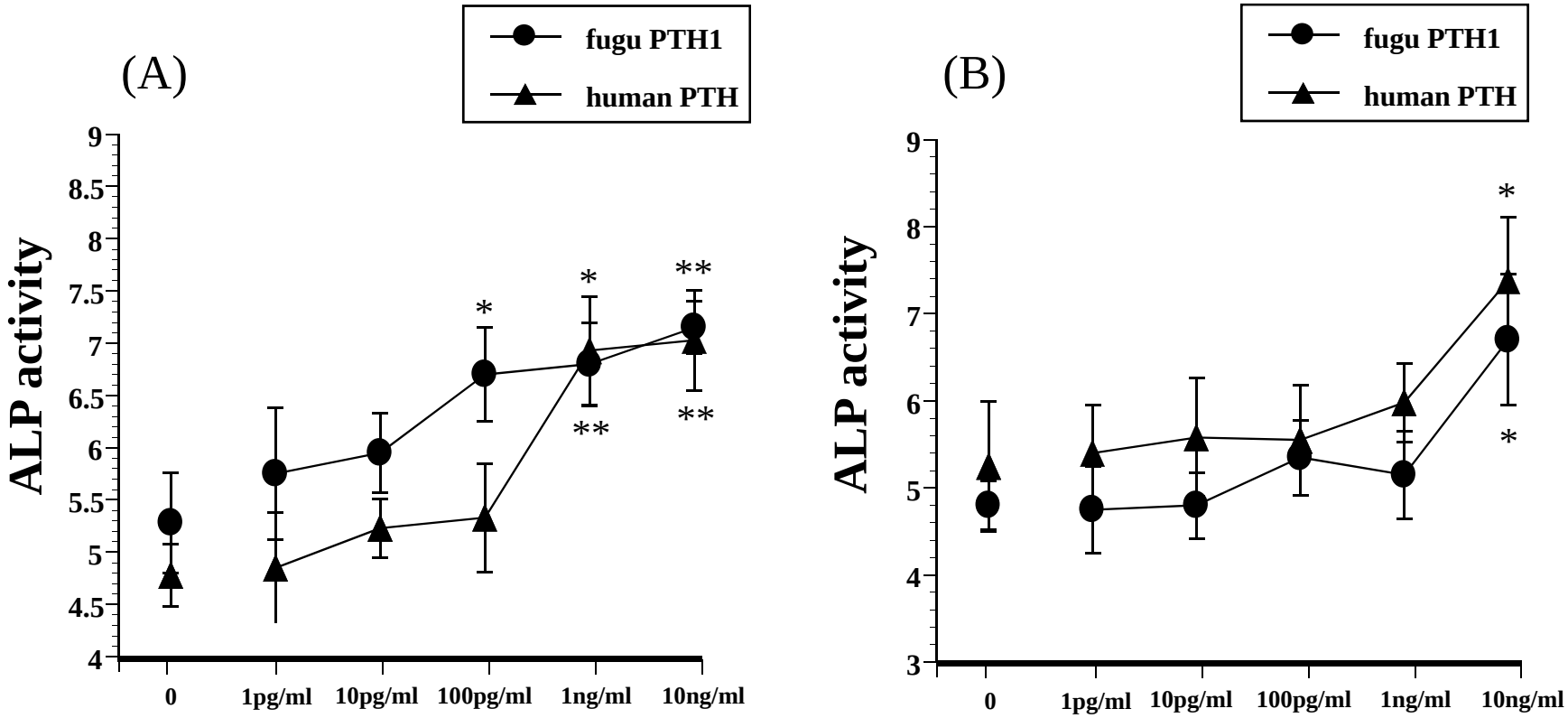
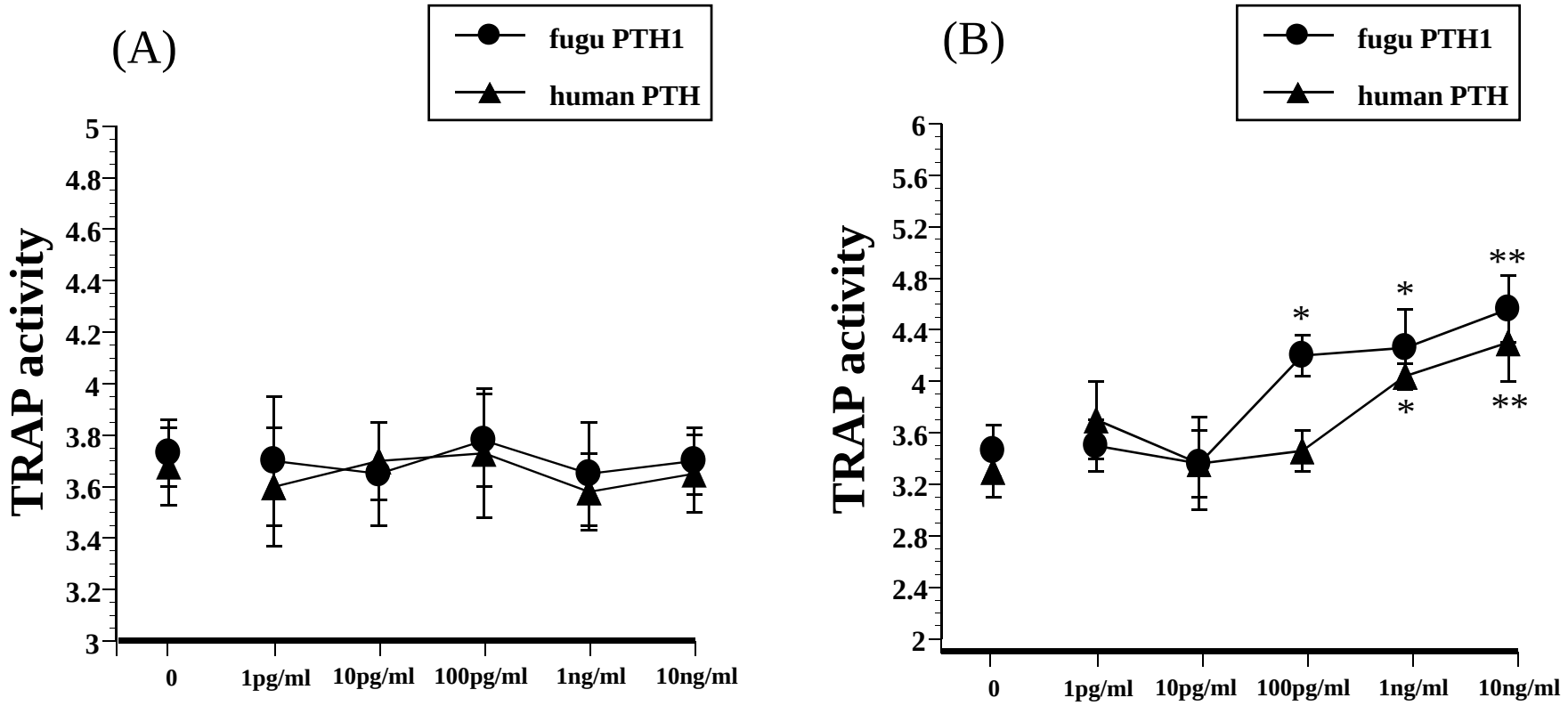


Figure 1 Suzuki et al.



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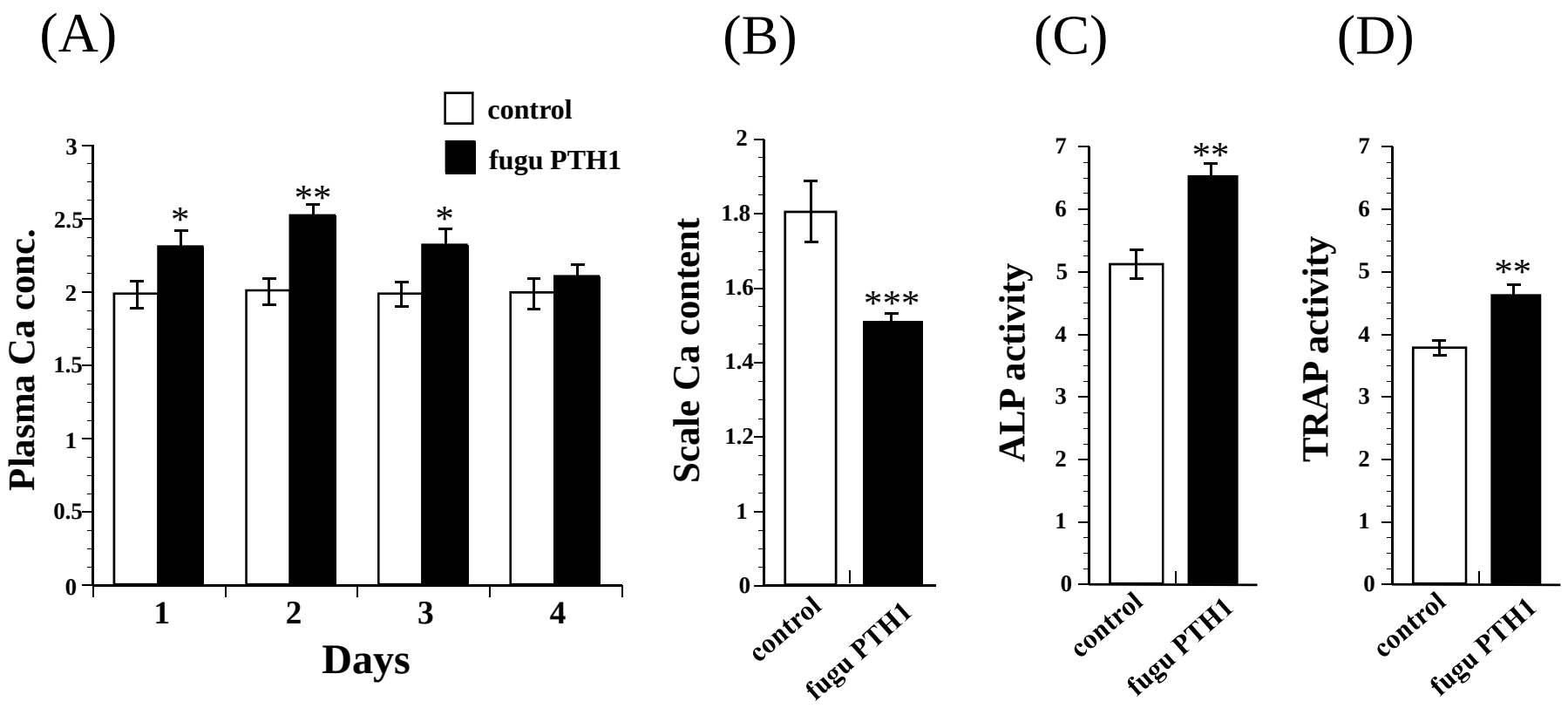

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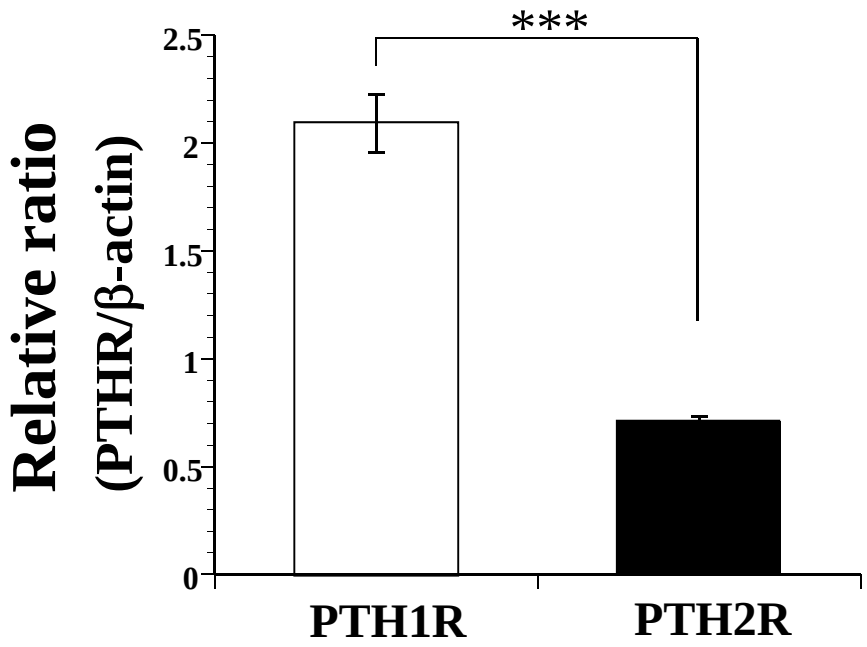

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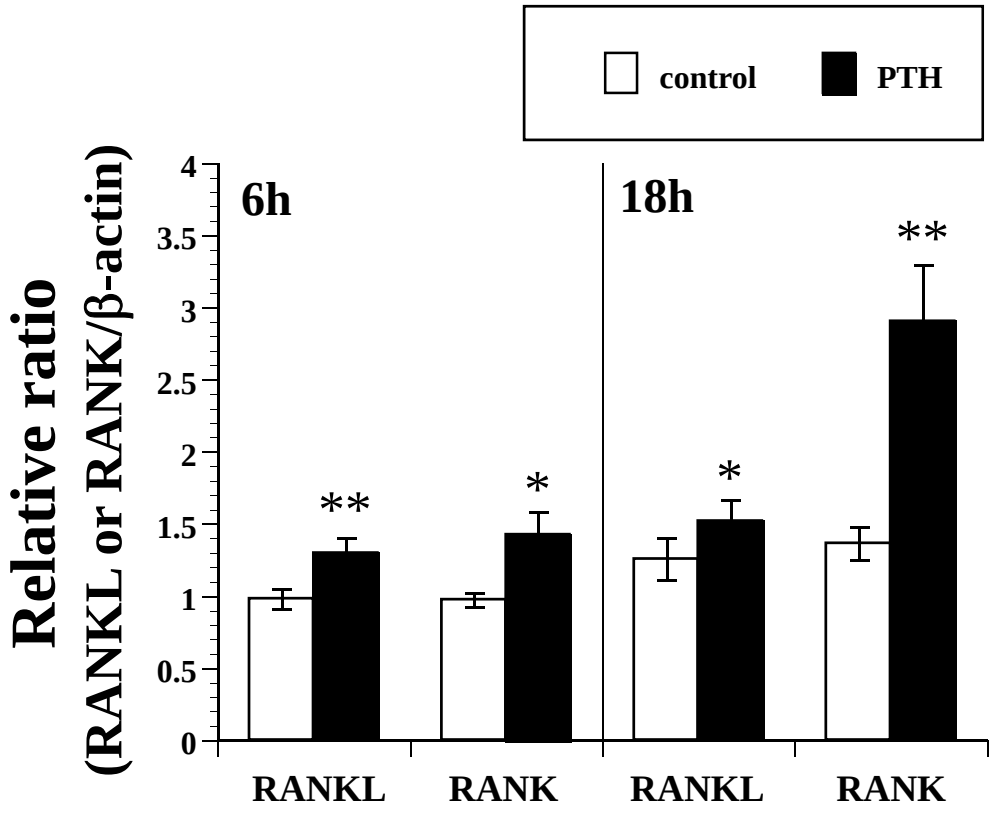


Figure 5 Suzuki et al.

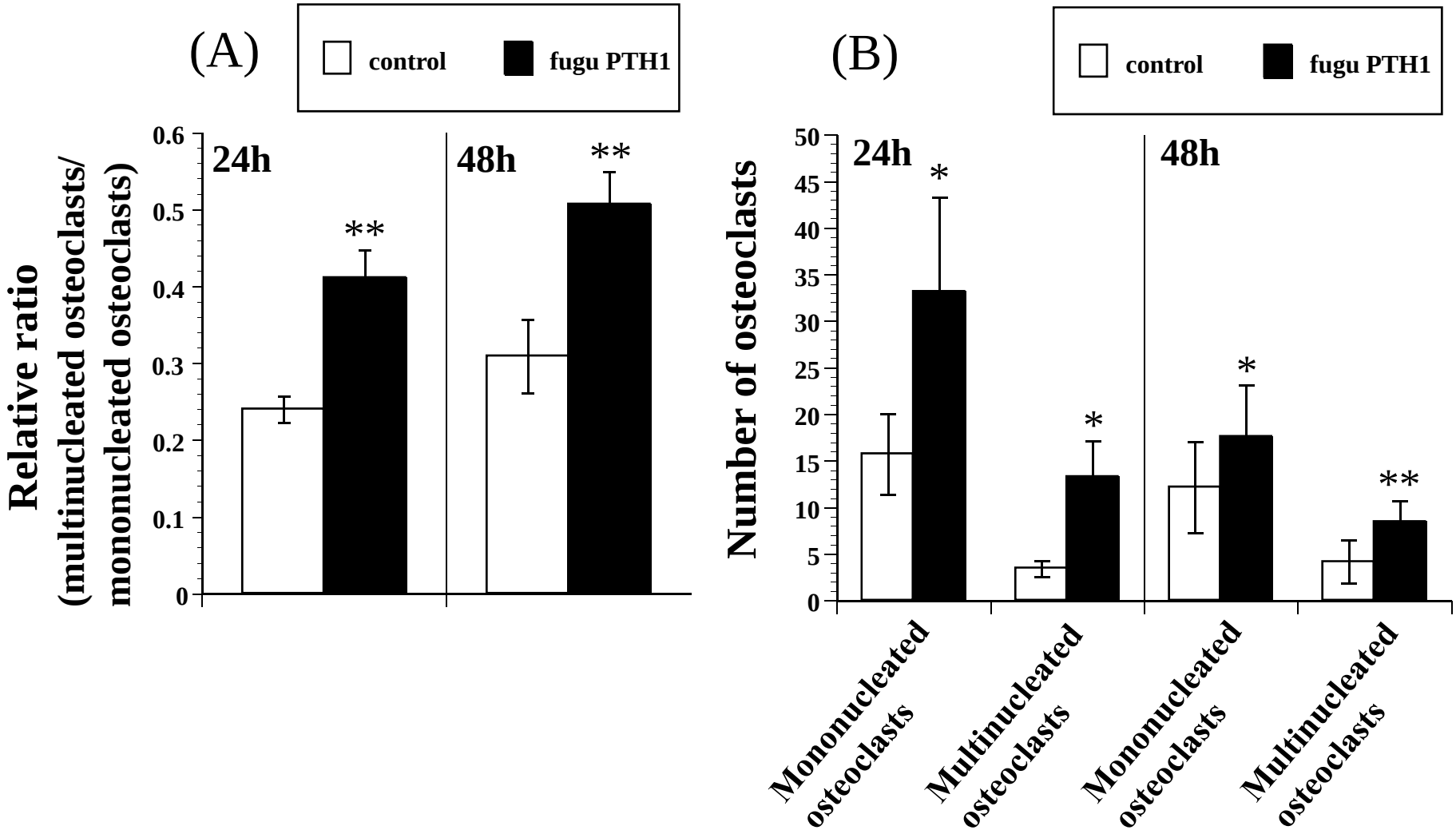
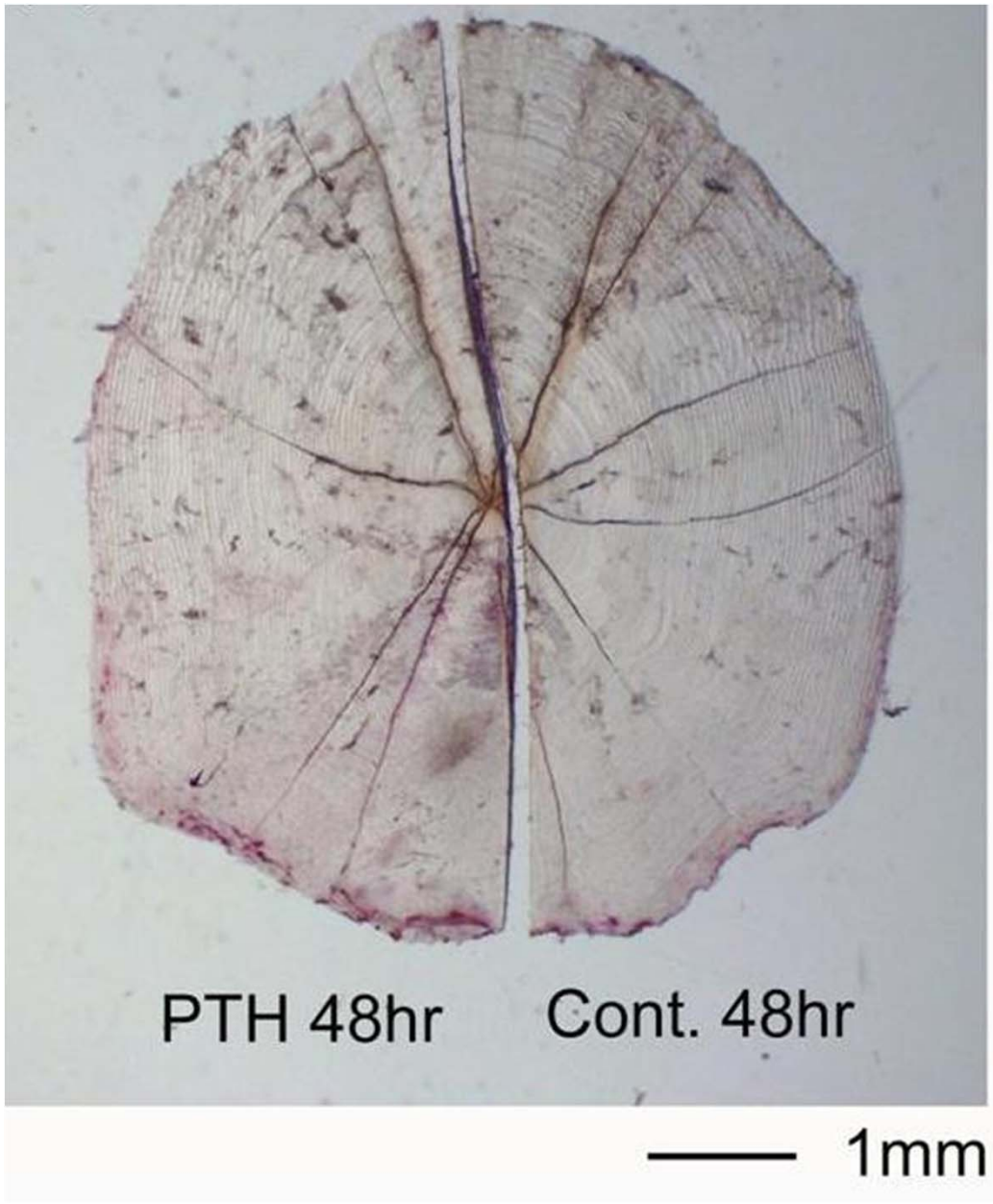


Figure 6 Suzuki et al.



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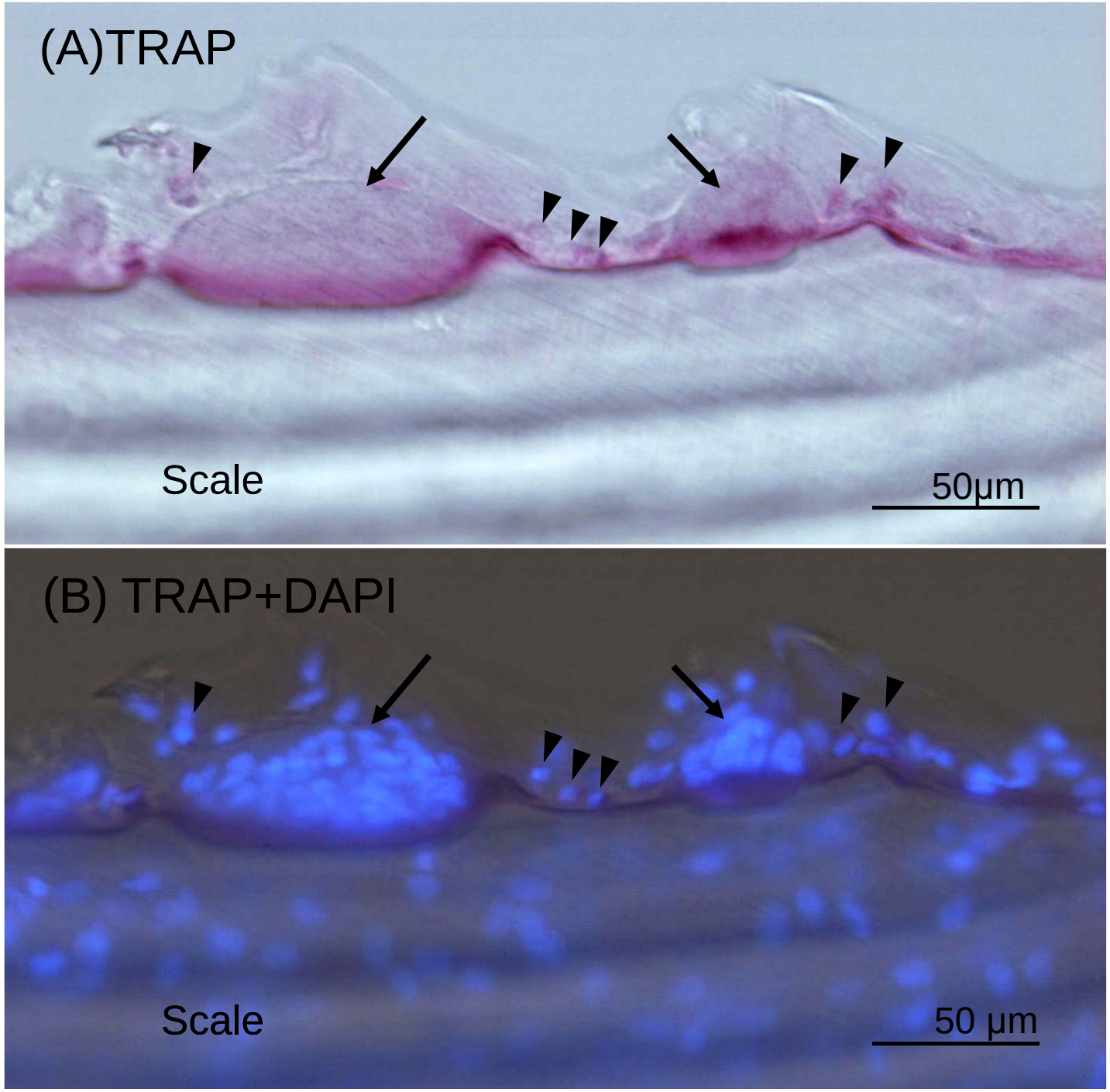

Figure 8 Suzuki et al.

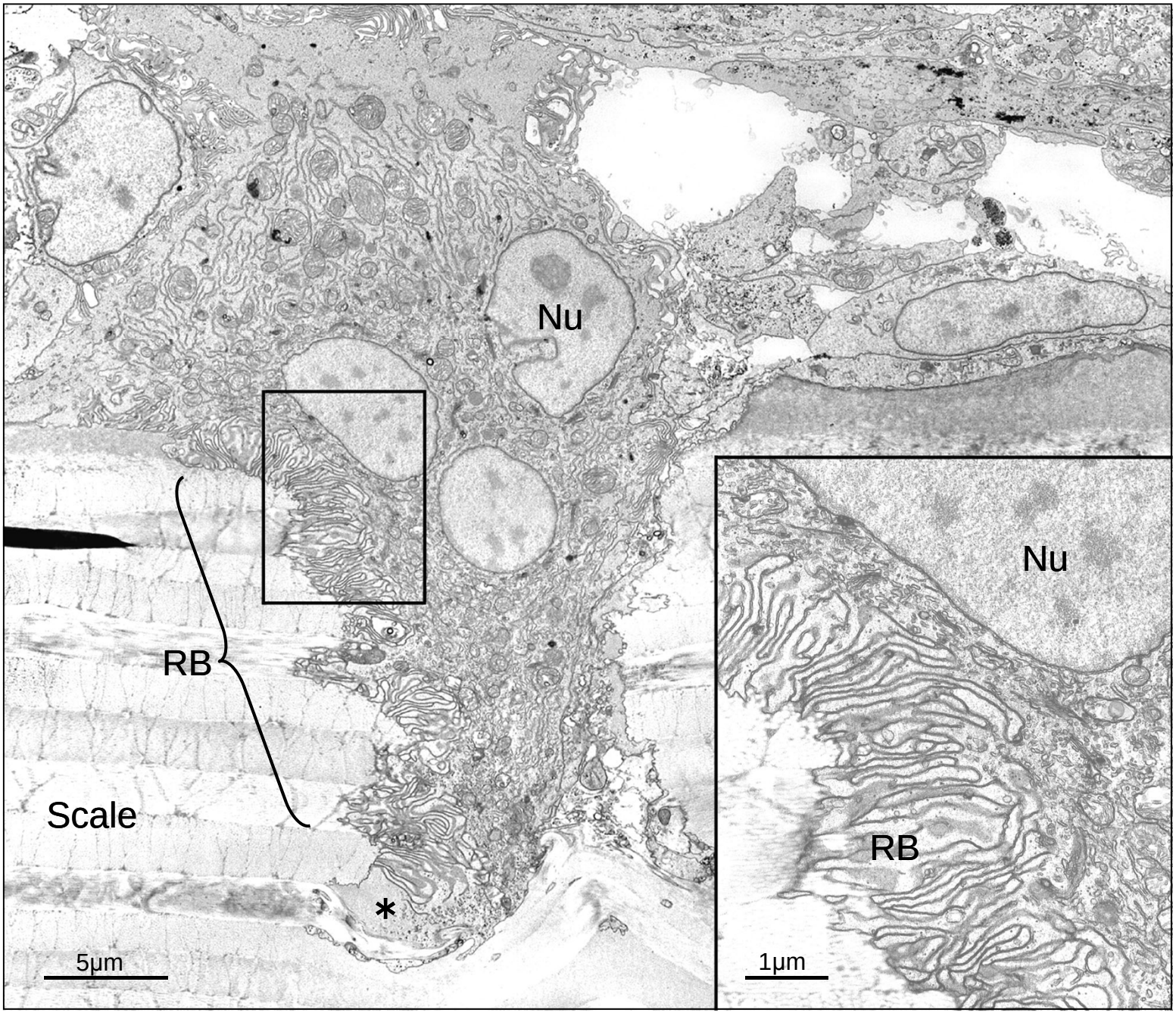

Figure 9 Suzuki et al.