Low-intensity pulsed ultrasound induces apoptosis in osteoclasts: Fish scales are a suitable model for the analysis of bone metabolism by ultrasound

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Running title: Effect of LIPUS on fish osteoclasts and osteoblasts

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Abstract Using fish scales in which osteoclasts and osteoblasts coexist on the calcified bone matrix, we examined the effects of low-intensity pulsed ultrasound (LIPUS) on both osteoclasts and osteoblasts. At 3 hours of incubation after LIPUS treatment, osteoclastic markers such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K mRNA expressions decreased significantly while mRNA expressions of osteoblastic markers, osteocalcin, distal-less homeobox 5, runt-related transcription factor 2a, and runt-related transcription factor 2b, increased significantly. At 6 and 18 hours of incubation, however, both osteoclastic and osteoblastic marker mRNA expression did not change at least present conditions. Using GeneChip analysis of zebrafish scales treated with LIPUS, we found that cell death-related genes were upregulated with LIPUS treatment. Real-time PCR analysis indicated that the expression of apoptosis-related genes also increased significantly. To confirm the involvement of apoptosis in osteoclasts with LIPUS, osteoclasts were induced by autotransplanting scales in goldfish. Thereafter, the DNA fragmentation associated with apoptosis was detected in the osteoclasts using the TUNEL (TdT-mediated dUTP nick-end labeling) method. The multi-nuclei of TRAP-stained osteoclasts in the scales were labeled with TUNEL. TUNEL staining showed that the number of apoptotic osteoclasts in goldfish scales was significantly elevated by treatment with LIPUS at 3 hours of incubation. Thus, we are the first to demonstrate that LIPUS directly functions to osteoclasts and to conclude that LIPUS directly causes apoptosis in osteoclasts shortly after exposure.

Key words: LIPUS, osteoclasts, osteoblasts, fish scales, apoptosis, GeneChip analysis

Abbreviations: ALP, alkaline phosphatase; Bcl-2, B-cell lymphoma 2; CTSK, Cathepsin K; Dlx5, distal less homeobox5; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; LIPUS, low-intensity pulsed ultrasound; OCN, osteocalcin; PMAIP, phorbol-12-myristate-13-acetate-induced protein 1; SAFHS, sonic accelerated fracture healing system; pNP, para-nitrophenol; TRAP, tartrate-resistant acid phosphatase; TUNEL, TdT-mediated dUTP nick end labelling; TNFRSF10A, tumor necrosis factor receptor superfamily, member a; Runx2a, runt related transcription factor 2a; Runx2b, runt related transcription factor 2b

1. Introduction

Low-intensity pulsed ultrasound (LIPUS) provides noninvasive therapeutic treatment to accelerate fracture repair and distraction osteogenesis (see a review, Warden et al., 2000). However, most studies regarding the influence of LIPUS on bone metabolism have used osteoblast cell lines (Bandow et al., 2007; Katiyar et al., 2014; Manaka et al., 2015). Therefore, details of the direct effect of LIPUS on osteoclasts are still not fully understood because of the difficulty in handling osteoclasts. Bone consists of osteoblasts, osteoclasts, and a bone matrix, and cell-to-cell and cell-to-matrix interactions are critical for cell response to physical stress (Harter et al., 1995; Owan et al., 1997; Hoffler et al., 2006). Consequently, co-culture systems that include bone matrix and both osteoclasts and osteoblasts are required to accurately assess the effect of ultrasound stimuli on bone formation and resorption. However, few techniques for a system of culturing bone cells that include bone matrix have been developed.

Teleost scale is calcified tissue that contains osteoclasts, osteoblasts, and the bone matrix of two layers (a bony layer: a thin, well-calcified external layer; a fibrillary layer: a thick, partially calcified layer) (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Azuma et al., 2007; Suzuki et al., 2007; Ohira et al., 2007; Suzuki et al., 2008). Its bone matrix, which includes type I collagen (Zylberberg et al., 1992), bone γ-carboxyglutamic acid protein (Nishimoto et al., 1992), osteonectin (Lehane et al., 1999; Redruello et al., 2005), and hydroxyapatite (Onozato and Watabe, 1979), is similar to that of mammalian bone. In light of these findings, we recently developed an *in vitro* assay system

with teleost scales (Suzuki et al., 2000; Suzuki and Hattori, 2002). With this system, we examined the effect of LIPUS on the osteoclasts and osteoblasts of zebrafish scales. To analyze their detail mechanism, we examined mRNA expression in the scales treated by LIPUS using a GeneChip system (Affymetrix). Furthermore, using osteoclasts induced by the autotransplantation of goldfish scales, we demonstrated that LIPUS treatment significantly elevated the number of apoptotic osteoclasts.

The present study is the first report that LIPUS directly functions in osteoclasts and promotes apoptosis in osteoclasts shortly after the exposure.

2. Materials and Methods

2.1 Animals

Zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*) were purchased from a commercial source and used to analyze mRNA expression and apoptotic cells, respectively. All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University, Japan.

2.2 Ultrasound conditions

Ultrasounds were generated by the Sonic Accelerated Fracture Healing System (SAFHS) 4000J (Teijin Pharma Ltd., Tokyo, Japan) through a transducer (effective area: 3.88 cm^2) at a frequency of 1.5 MHz with a pulsed-wave mode (pulse-burst width: 0.2 s; pulse repetition frequency: 1 kHz; and intensity: 30 mW/cm^2). This apparatus is the same as Exogen's SAFHS

apparatus (Exogen Inc., Piscataway, NJ, USA).

2.3 LIPUS treatments and RNA isolation

Scales were collected from zebrafish under anesthesia (ethyl 3-aminobenzoate, methanesulfonic acid salt, Sigma-Aldrich, Inc., St. Louis, MO, USA) and then treated with LIPUS. After LIPUS treatment (20 min), the scales were incubated at 15°C for 3, 6, and 18 hours with Leibovitz's L-15 medium (Invitrogen, Grand Island, NY, USA) and then frozen at -80°C for mRNA analysis. Scales not treated with LIPUS were incubated under the same conditions as the experimental group and compared with LIPUS-treated scales. Before incubation (0 hour), the scales were also frozen at -80°C and compared with incubated scales. Total RNAs were prepared from zebrafish scales using a total RNA isolation kit for fibrous tissue (Qiagen GmbH, Hilden, Germany) and treated with DNase I (RNase-Free DNase kit, Qiagen) for 15 min at room temperature to remove residual genomic DNA.

2.4 Real-time quantitative PCR assay

Real-time quantitative PCR (qPCR) assay was performed on a Real-Time PCR system (Mx3000P, Stratagene Japan, Tokyo) using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) or qPCR MasterMix (for the use of TaqMan probes; Eurogentec, Seraing, Belgium) in accordance with the manufacturer's protocols. Reverse transcriptase reaction (Omniscript Reverse Transcriptase, Qiagen) was carried out with DNase-treated total RNA using an oligo (dT)16 primer. Real-time qPCR assay was performed using the specific primers listed in

Table 1. Each mRNA expression level was normalized to the mRNA expression level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.5 GeneChip analysis

Gene expression was analyzed using a GeneChip system with a zebrafish array (Affymetrix, Santa Clara, CA, USA). Samples for array hybridization were prepared as described in the Affymetrix GeneChip Expression Technical Manual. The data were further analyzed using GeneSpring (Silicon Genetics, Redwood City, CA, USA) to extract the significant genes (Tabuchi et al., 2008).

2.6 Analyses of apoptotic osteoclasts

To examine the possible involvement of apoptosis in the inhibition of osteoclastic activity by LIPUS, osteoclasts were induced by the autotransplantation of scales in goldfish (Takahashi et al., 2008). The collected scales were intramuscularly autotransplanted, and the fish were kept in fresh water containing an antibiotic (Green F Gold, Sanei Co. Ltd., Tokyo, Japan) at 25°C for 7 days. Thereafter, the transplanted scales were removed under anesthesia with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich), cut into halves, and the halves treated with LIPUS as described above and compared with each other. After 3 hours of incubation with L-15 medium (Invitrogen), the scale halves were fixed with 4% paraformaldehyde-phosphate buffer, and TRAP staining was performed by the methods of Cole and Walters (1987). After TRAP staining, the DNA fragmentation associated with

apoptosis was detected by the TdT-mediated dUTP nick-end labeling (TUNEL) method of Gavrieli et al. (1992) using an *in situ* cell death detection kit (Roche, Tokyo, Japan). The details of the methods are described in Takahashi et al. (2008). The TRAP-stained samples were washed twice in 100 mM Tris-HCl buffer, pH 7.6, containing 150 mM sodium chloride and 0.1% Tween 20 (TBST), and fixed in methanol at -20°C for ≥ 24 hours. To rehydrate the samples following methanol fixation, each scale was washed in TBST four times at room temperature for 15 min. The samples were microwave irradiated to enhance signals for TUNEL staining. Then, the samples were transferred to TUNEL buffer (25 mM Tris-HCl, pH 7.6, containing 200 mM sodium cacodylate, 5 mM cobalt chloride, and 0.25% bovine serum albumin) and incubated overnight at 4°C. After washing in TUNEL buffer for 30 min at room temperature, the scales were incubated with TdT and fluorescein-labeled dUTP at 37°C for 4 hours. The reaction was terminated by transferring the scales to TBST for 15 min, which were then mounted on a glass slide with a coverslip. The specimens were observed under a fluorescence microscope (BX51, Olympus Corporation, Tokyo, Japan).

2.7 Assay of osteoclastic activity

We detected osteoclastic activity in individual goldfish scales by transferring each scale into a well of a 96-well microplate for incubation (Suzuki et al., 2000; Suzuki and Hattori, 2002; Suzuki et al., 2011). This method was used to examine the effect of LIPUS on osteoclasts.

Osteoclasts were induced by the autotransplantation of scales in goldfish. Thereafter, the

transplanted scales were removed under anesthesia with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich), cut into halves, and treated with LIPUS as described above.

After LIPUS treatment, osteoclastic (TRAP) activity was measured. The method for measuring TRAP activity was as follows (Yachiguchi et al., 2014). An aliquot of 100 μl of an acid buffer (0.1 M sodium acetate, including 20 mM tartrate, pH 5.3) was added to each well. Then, each scale was put into its own well in a 96-well microplate. This microplate was immediately frozen at -80°C and then kept at -20°C until analysis. An aliquot of 100 μl of 20 mM para-nitrophenyl phosphate in an acid buffer was then added to a melted solution in each well of the microplate. This plate was incubated at 23°C for 20 min while being shaken. After incubation, the reaction was stopped by adding 50 μl of 3 N NaOH. One hundred fifty μl of a colored solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced para-nitrophenol (pNP) using a standard curve for pNP.

After measuring TRAP activities, the scales were measured with Image J. Afterward, the TRAP activities were normalized to the surface area (mm^2) of each scale (Suzuki et al., 2009).

2.8 Statistical analysis

All results are expressed as the means ± SEM (n = 6 - 8). The value from the control group was compared with that in the experimental group. The data were assessed using the paired *t*-test, and the significance level chosen was $p < 0.05$.

3. Results

3.1 Analysis of real-time quantitative PCR assay

The effects of LIPUS on osteoclasts and osteoblasts were examined with zebrafish scales. At 3 hours of incubation after LIPUS treatment, osteoclastic markers such as TRAP and cathepsin K (CTSK) mRNA expressions decreased significantly (Figure 1), while mRNA expressions of osteoblastic markers, osteocalcin (OCN), distal-less homeobox 5 (Dlx5), runt-related transcription factor 2a (Runx2a), and runt-related transcription factor 2b (Runx2b), increased significantly (Figure 2). At 6 and 18 hours of incubation, however, the changes disappeared, and both osteoclastic and osteoblastic marker mRNA expression did not change at least present conditions (Figures 1 and 2).

3.2 GeneChip analysis

The GeneChip analysis of zebrafish scales treated with LIPUS was examined. In molecular and cellular function, GeneChip analysis showed that cell death was a top molecular and cellular function. Cell death-related genes were upregulated with LIPUS treatment as compared with untreated control scales (Table 2). Real-time PCR analysis indicated that the expression of apoptosis-related genes such as phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1) and tumor necrosis factor receptor superfamily member 10a (TNFRSF10A) also increased (Figure 3).

3.3 Analyses of apoptotic osteoclasts

To examine the possible involvement of apoptosis in osteoclasts by LIPUS, we induced osteoclasts by the autotransplantation of scales in goldfish.

At 3 hours of incubation after LIPUS treatment, osteoclastic activity (TRAP activity) decreased significantly (Figure 4A). LIPUS treatment also significantly elevated the number of apoptotic osteoclasts with 3 hours of incubation (Figure 4B).

Actually, DNA fragmentation associated with apoptosis was detected in the osteoclasts using the TUNEL method. The multi-nuclei of TRAP-stained osteoclasts in the scales (Figure 5A) were labeled using TUNEL for DNA breaks (Figure 5B).

4. Discussion

There was little information regarding the influence of LIPUS on osteoclasts. Using zebrafish scales, we demonstrated that osteoclastic markers such as TRAP and CTSK mRNA expressions decreased significantly at 3 hours of incubation after LIPUS treatment. Using GeneChip analysis of zebrafish scales treated with LIPUS, we found that cell death-related genes were upregulated. Real-time PCR analysis indicated that the expression of apoptosis-related genes increased. Furthermore, the multi-nuclei of TRAP-stained osteoclasts in the scales were labeled using TUNEL for DNA breaks. Treatment with LIPUS also significantly elevated the number of apoptotic osteoclasts after 3 hours of incubation. In addition, osteoclastic activity in the LIPUS-treated scales decreased significantly. With short incubation times, thus, we conclude that LIPUS directly functions in osteoclasts and promotes apoptosis

in osteoclasts.

In the case of osteoblasts, the mRNA expression of osteoblastic markers such as OCN, Dlx5, Runx2a, and Runx2b increased significantly at 3 hours of incubation after LIPUS treatment. In mammalian osteoblasts, the anti-apoptotic protein Bcl-2 (B-cell lymphoma 2) was upregulated from very low baseline levels (Watabe et al., 2011). This suggests that the anti-apoptotic protein protects apoptosis in osteoblasts of zebrafish scales as well as in mammalian osteoblasts.

Recently, we analyzed the effect of several degrees of acceleration (0.5, 1, 2, 4, and 6G) loading by vibration on osteoblasts and osteoclasts using a goldfish scale *in vitro* assay system (Suzuki et al., 2007). As a result, scales of both osteoclasts and osteoblasts responded to low-gravity acceleration loading. Particularly, the response of acceleration loading in osteoclast scales was very sensitive compared with that in osteoblasts. Osteoclastic activity decreased under low acceleration (0.5- and 1-G) as LIPUS suppressed osteoclastic activity. Fish scales contain osteoblasts, osteoclasts, and a bone matrix similar to that in bone (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Azuma et al., 2007). Bone matrix plays an important role in the response to physical stress (Owan et al., 1997; Hoffer et al., 2006). In addition, Watabe et al. (2011) reported that $\alpha_5\beta_1$ integrin, which participates in cell adhesion with the bone matrix, plays an important role in mechanotransduction by LIPUS. Therefore, we strongly believe that scales having a bone matrix responded to low-gravity acceleration loading as LIPUS did in the present study. Moreover, in an *in vivo* experiment with rats, LIPUS inhibited the resorption of tooth roots

(Inubushi et al., 2013). This result agreed with our data obtained with fish scales. Thus, as a next step, we plan to conduct an *in vivo* experiment with goldfish to further elucidate the detail mechanism of influence in both osteoclasts and osteoblasts of the scales.

Our scale assay system is a suitable assay for physical stimuli such as ultrasound and hypergravity as described above. Using this system, we first demonstrated that calcitonin, a hypocalcemic hormone, suppressed osteoclastic activity in teleosts as well as in mammals (Suzuki et al., 2000; Sekiguchi et al., 2009) and that parathyroid hormone, a hypercalcemic hormone, increased the function in both osteoclasts and osteoblasts (Suzuki et al., 2011). Estrogen acts on osteoblasts of scales as it does in mammals (Yoshikubo et al., 2005). Also, prostaglandin E_2 acts on osteoblasts and then increases the osteoclastic activity in the scales of goldfish as it does in the bone of mammals (Omori et al., 2011). In addition, we discovered a new function of prolactin—to reduce osteoclastic activity *via* osteoclast apoptosis—after 6–18 hours of incubation (Takahashi et al., 2008). Considering these results together with our present results, we can conclude that our scale assay system will be useful for analyzing bone metabolism.

In conclusion, we are the first to demonstrate that LIPUS directly functions in osteoclasts and promotes apoptosis with short incubation times, which strongly suggests that fish scales are a suitable model for ultrasound stimuli.

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Figure Legends

Figure 1 The effects of LIPUS on osteoclastic markers such as TRAP (tartrate-resistant acid phosphatase) and CTSK (cathepsin K) before incubation (0) and after 3, 6, and 18 hours of incubation. ** indicates statistically a significant difference at $p < 0.01$ from the values in the control scales of zebrafish.

Figure 2 The effects of LIPUS on osteoblastic markers such as OCN (osteocalcin), Dlx5 (distal-less homeobox 5), Runx2a (runt-related transcription factor 2a), and Runx2b (runt-related transcription factor 2b) before incubation (0) and after 3, 6, and 18 hours of incubation. * and ** indicate statistically significant differences at $p < 0.05$ and $p < 0.01$, respectively, from the values in the control scales of zebrafish.

Figure 3 The effects of LIPUS on apoptosis-related genes such as PMAIP1 (phorbol-12-myristate-13-acetate-induced protein 1) (A) and TNFRSF10A (tumor necrosis factor receptor superfamily member a) (B) after 3, 6, and 18 hours of incubation. *, **, and *** indicate statistically significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, from the values in the control scales of zebrafish.

Figure 4 Analyses of TRAP (tartrate-resistant acid phosphatase) activity produced pNP (nmol/mm^2 scale/h) (A) and the percentage of apoptotic osteoclasts (apoptotic osteoclasts/ total osteoclasts x 100 (%)) (B) using the autotransplantation of scales in goldfish at 3 hours of incubation after LIPUS treatment. * and *** indicate statistically significant differences at $p < 0.05$ and $p < 0.001$, respectively, from the values in the control scales of goldfish.

Figure 5 TRAP (tartrate-resistant acid phosphatase) (A) and TUNEL (the TdT-mediated dUTP nick end labeling) (B) staining using the autotransplantation of scales in goldfish at 3 hours of incubation after LIPUS treatment.

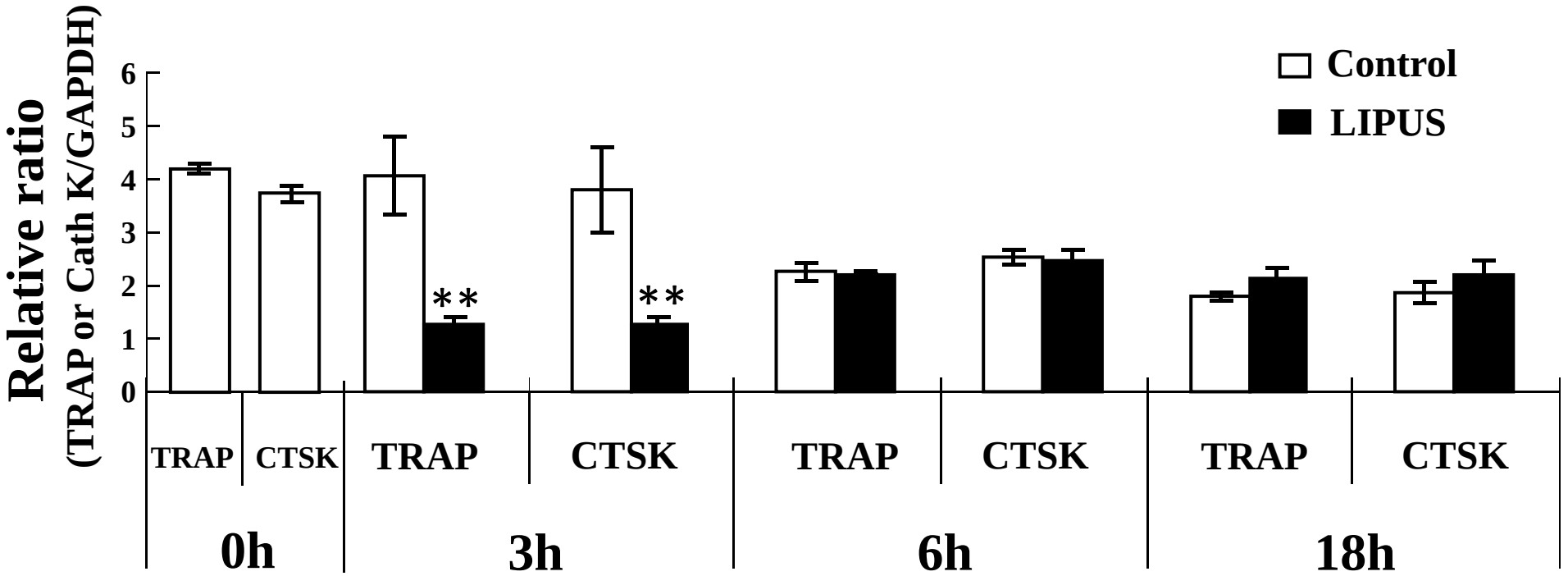

Figure 1 Suzuki et al.

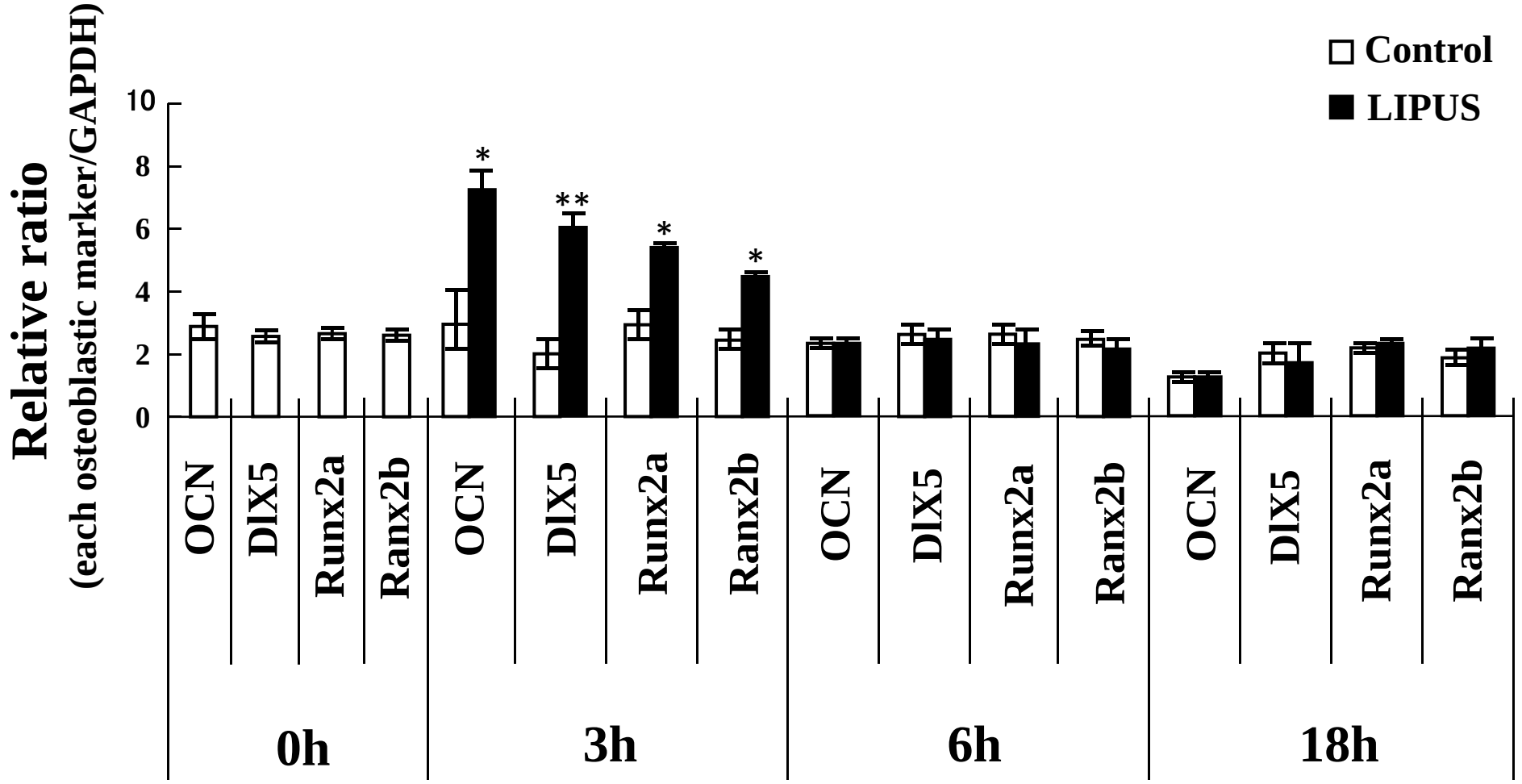

Figure 2 Suzuki et al.

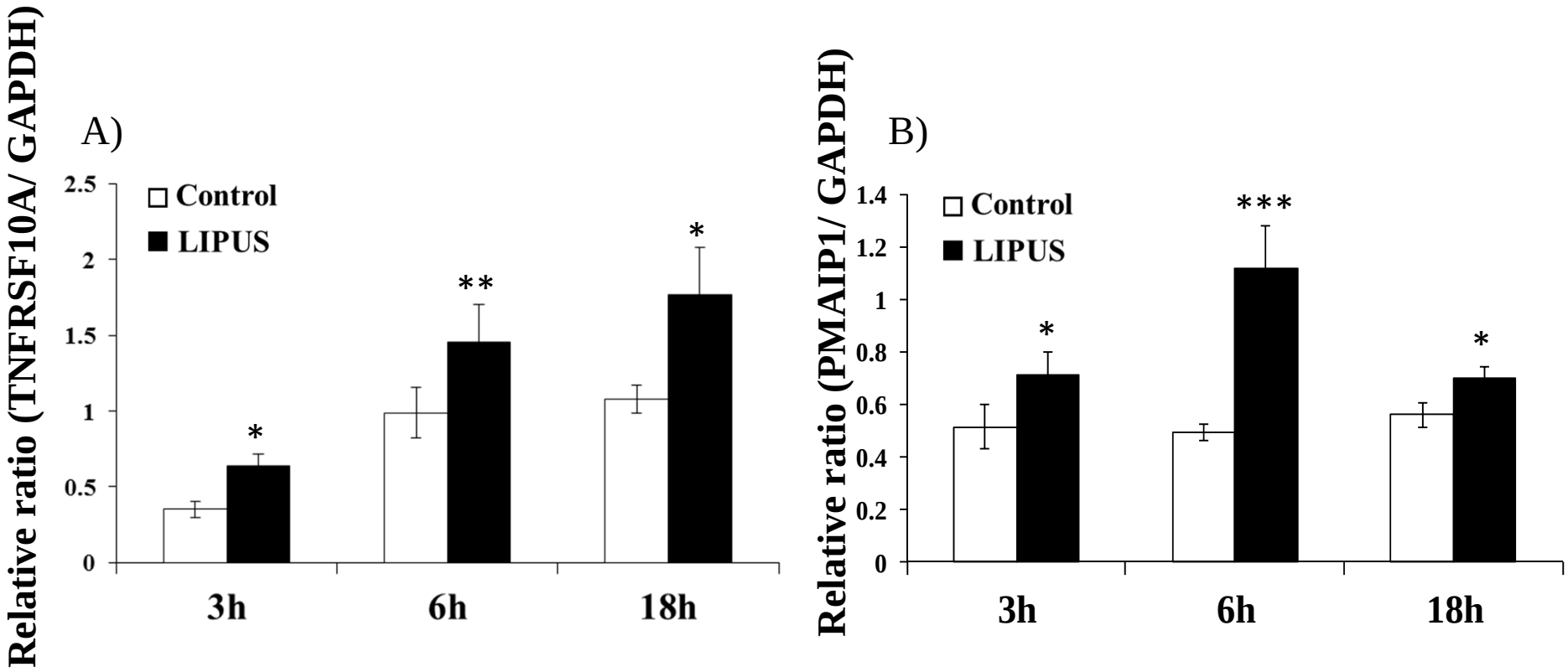

Figure 3 Suzuki et al.

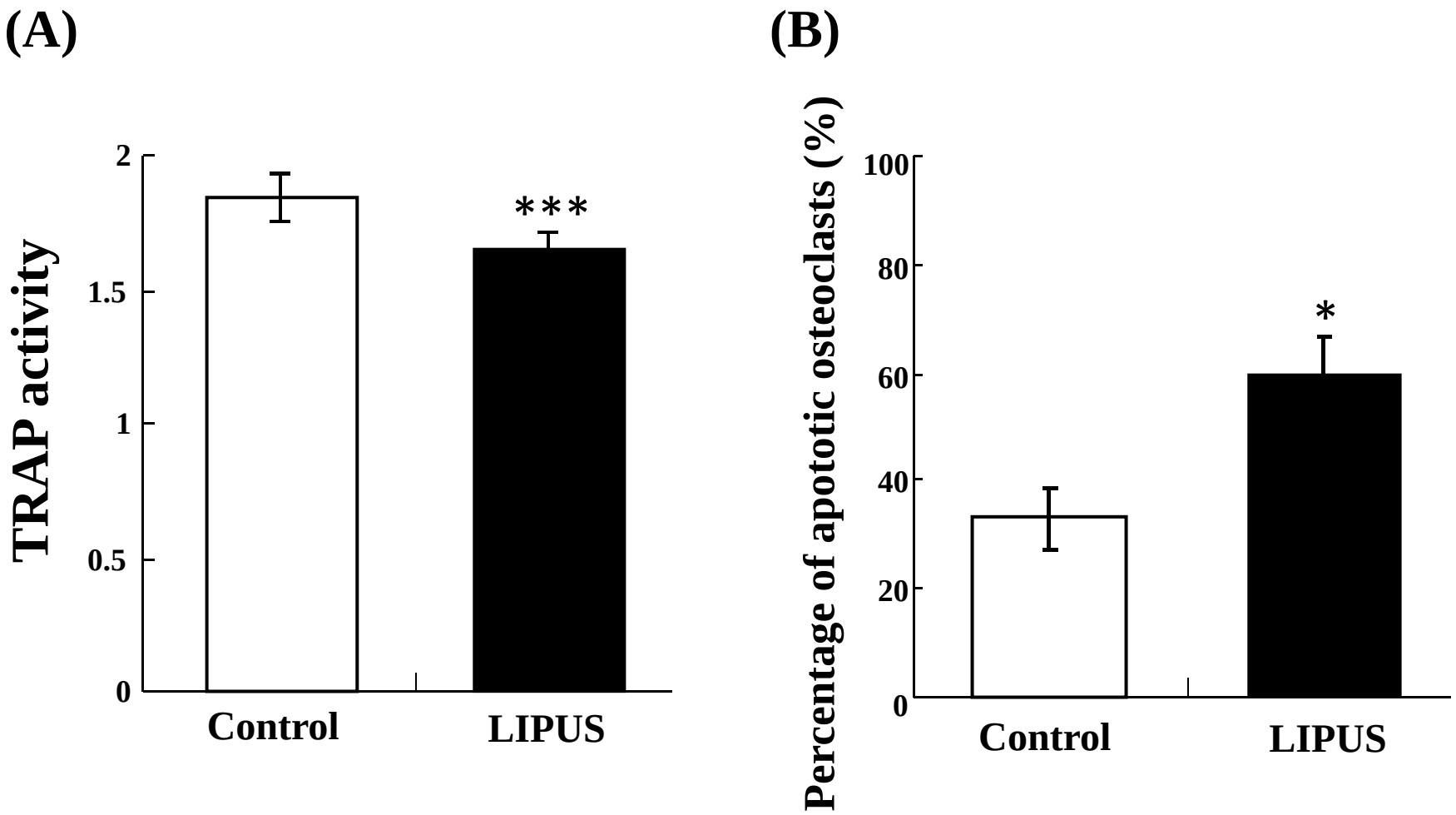

Figure 4 Suzuki et al.

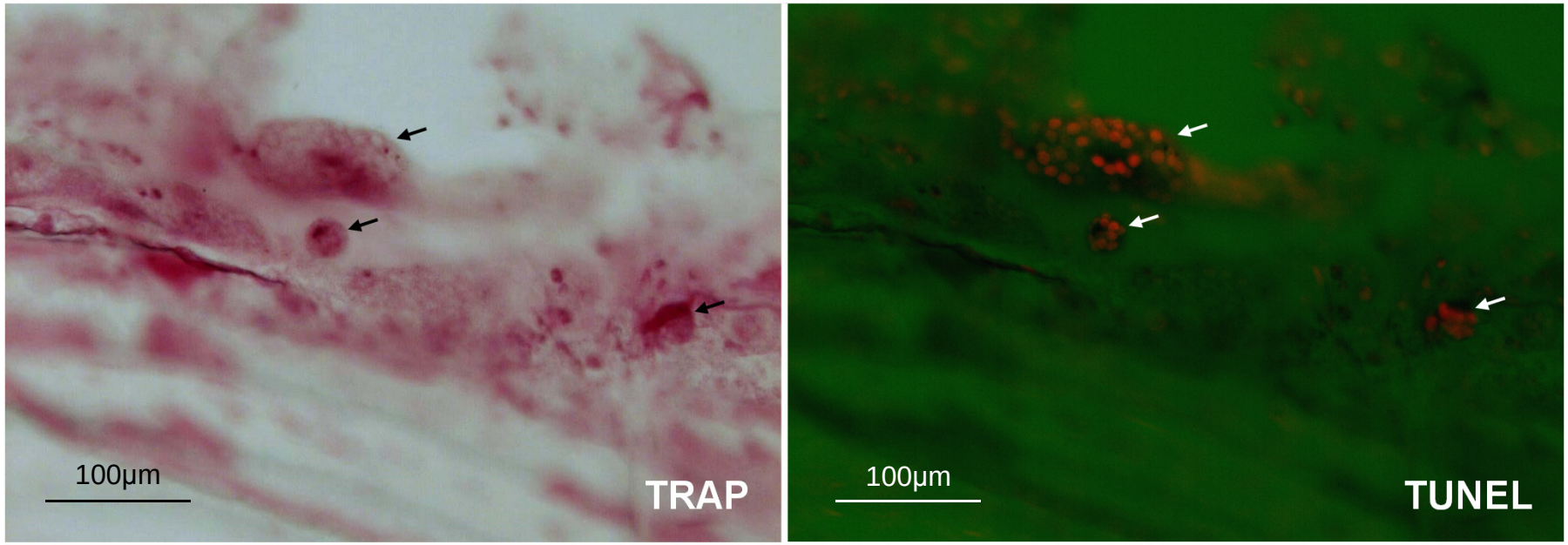

Figure 5 Suzuki et al.

Table 1 Primer sequences for real-time quantitive PCR

Gene	Orientation	Nucleotide sequence (5’ to 3’)
TRAP	Sense	TGTCATCGTGGTTGGTCACT
	Antisense	CTCAACACCAGCTCCACTGA
CTSK	Sense	ATGATCTGGGCATGAACCAT
	Antisense	CCGAAGTGACGTATCCCAGT
OCN	Sense	TGACGTGGCCTCTATCATCA
	Antisense	TTTATAGGCGGCGATGATTC
Dlx5	Sense	GCCACGGATTCTGGCTATTA
	Antisense	TGAGCCGTAATCAGGGTAGG
Runx2a	Sense	GCTGTGCAAACCCAGAGTCC
	Antisense	CATAGGACCAGGGCGGAGAG
Runx2b	Sense	TGGCCAGTTTGAGCGCCAGT
	Antisense	AGCGCTGCCCAATGACATGC
TNFRSF10A	Sense	ATGCTCGCCCTGTGAGAAGGGCACG
	Antisense	GCCTTCTGTCGGGTAAGAGGGGCGC
PMAIP1	Sense	GGCGAAGAAAGAGCAAACCGCTG
	Antisense	GTTCAACAGATCTCCAATGTTGCGC
GAPDH	Sense	CCCCCAATGTCTCTGTTGTG
	Antisense	ACCAGCGTCAAAGATGGATG

Table 2 Cell death related genes (up-regulation) by LIPUS

Gene symbol	Gene name	Fold 3hr	Fold 6hr	Fold 18hr	Entrez Gene ID
ACSL4	acyl-CoA synthetase long-chain family member 4a	2.45	2.09	1.50	393622
CDKN1B	cyclin-dependent kinase inhibitor 1b (p27 Kip1)	2.53	1.98	2.50	368329
CTH	cystathionase (cystathionine gamma-lyase)	2.28	1.58	1.30	322055
EIF4G2	eukaryotic translation initiation factor 4, gamma 2a	2.53	1.61	1.18	323453
HSP90AA1	heat shock protein 90-alpha 2	2.66	3.08	0.99	565155
HSPA4	heat shock protein 4, like	2.82	1.99	1.46	335865
KRT18	keratin 18	2.46	2.08	1.63	352912
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein b	2.03	1.20	2.03	405809
MDK	midkine-related growth factor	2.33	1.40	1.78	30277
NDEL1	nudE nuclear distribution gene E homolog (*A. nidulans*) like 1 B	2.57	2.05	1.13	333957
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	2.32	1.83	1.79	751765
PTGIS	prostaglandin I2 (prostacyclin) synthase like	3.50	2.32	1.82	559148
SOCS3	suppressor of cytokine signaling 3b	2.35	1.61	1.14	406596
SOX4	SRY-box containing gene 4a	2.33	3.44	1.93	336346
TNFRSF10A	tumor necrosis factor receptor superfamily, member 10a	2.70	1.01	1.54	114461