# Osteogenic activities of human fracture haematoma-derived progenitor cells are stimulated by low intensity pulsed ultrasound in vitro

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# Osteogenic activities of human fracture haematoma-derived progenitor cells are stimulated by low intensity pulsed ultrasound *in vitro*

# 多分化能を持つ骨折血腫細胞の骨分化能への低出力超音波パルス(LIPUS)の効果

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Key words: low intensity pulsed ultrasound, haematoma, progenitor cells, fracture

**Abstract**

Haematoma occurring at a fracture site is known to play an important role in bone healing. Recently, we discovered, for the first time, progenitor cells exist in human fracture haematoma and demonstrated that these cells have the capacity for multilineage mesenchymal differentiation. Meanwhile, there have been numerous reports showing low intensity pulsed ultrasound (LIPUS) treatment stimulating differentiation of a variety of cells. However, there have been no reports investigating the effects of LIPUS in cells derived from actual human fracture tissue including human fracture haematoma-derived progenitor cells (HCs). In this *in vitro* study, we investigated the effects of LIPUS on osteogenic activity of HCs. The alkaline phosphatase (ALP) activity, the osteocalcin (OCN) secretion, the expression of osteoblast related genes and the mineralization of HCs were shown to be significantly higher in the LIPUS treatment group than in the control group. Our results demonstrated osteogenic differentiation of HCs was increased by LIPUS treatment.

## Introduction

In the process of fracture healing, a haematoma is initially formed at the fracture site. The bone is formed by two different mechanisms, intramembranous bone formation and endochondral bone formation. Haematoma occurring at a fracture site at the initial stage is known to play an important role in fracture healing. Mizuno et al. reported that fracture haematoma has an inherent osteogenic potential which significantly contributes to fracture healing.[1] Grundnes et al. also reported that removal of an organized haematoma some days after fracture impaired fracture healing.[2] Recently, we discovered for the first time that progenitor cells exist in human fracture haematoma and demonstrated that those cells have the capacity for multilineage mesenchymal differentiation.[3] This finding was a revolutionary because it showed human fracture haematoma-derived progenitor cells (HCs) play a significant and dynamic role in fracture healing.

Millions of fractures occur annually as a result of human activity, morbidity and from bone fragility.[4,5] In an effort to reduce the substantial associated disabilities and socioeconomic costs, a variety of interventions have been attempted, including the use of low-intensity pulsed ultrasound (LIPUS).[6] Following confirmation of the positive effects of LIPUS on fractures[6-9], LIPUS has already been used at many facilities due to the clinical benefits of accelerated fracture healing and the shortening of durations of hospital stays. In an effort to determine optimum signal parameters, Duarte demonstrated as for back as 1983 that ultrasound signals successfully accelerate cortical bridging after fibular osteotomy.[7] However, studies into the effects of LIPUS have advanced clinically rather than basically, and the mechanism underlying the ultrasound effect is not yet well understood. There have been numerous reports showing LIPUS treatment stimulating differentiation of a variety of cells such as bone marrow stromal cells, mesenchymal stem cells, chondrocyte and osteoblasts in vitro[10-22]. However, there have been no reports

investigating the effects of LIPUS in cells derived from actual human fracture tissue including HCs.

The purpose of this study is to investigate the effect of LIPUS on the progenitor cells derived from actual human fracture haematoma. In this *in vitro* study, we hypothesized that the osteogenic activity of HCs derived from human fracture haematoma would be increased by LIPUS treatment, which led us to investigate the effect of LIPUS on the osteogenic activity of HCs.

## Materials and Methods

*Patient characteristics*

Fracture haematomas were obtained from 8 consecutive patients with a mean age of 24.6 years (16 to 41) during osteosynthesis a mean of 6 days (2 to 10) after fracture. The fracture sites involved were tibia (5 patients), clavicle (1 patient) and fibula (2 patient). Patients taking anticoagulants, steroids or non-steroidal anti-inflammatory drugs in the three months prior to injury were excluded. Informed consent was obtained from all patients. The project was approved by the Ethics of Human Experiments at the Faculty of Medicine, Kobe University.

*Isolation and culture of HCs*

Haematoma which had formed fibrin-clots, was removed manually before any manipulation or irrigation and placed in sterile polypropylene containers, to avoid contamination during the operation. The wet weight of haematoma obtained ranged from 0.4 to 2.0g (mean, 1.1g). Specimens were minced with a scalpel into small pieces with original medium, α-Modified Minimum Essential Medium (α-MEM) (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Sigma), 2mM L-glutamine (Gibco BRL, Grand Island, NY, USA) and antibiotics on 100mm culture dish. The cultures were incubated at 37°C with 5% humidified $CO_2$. Seven days after initial incubation, the dish was washed with phosphate-buffered saline (PBS) (Wako, Osaka, Japan) to remove nonviable cells and debris and thereafter the culture medium was changed twice weekly. Approximately two to three weeks later, the adherent cells were harvested with 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) (Wako) and passaged into non-coated 75 $cm^2$ culture flasks with a density of approximately $2\times10^5$cells/75 $cm^2$-flasks for further expansion. Cells that had undergone one to three passages were used in the following assays.

*Low intensity pulsed ultrasound treatment*

We used a LIPUS exposure device (TEIJIN PHARMA LIMITED, Tokyo, Japan) which is adapted to 6-well tissue cell culture plate in the vitro experiment. This device was set at 1.5 MHz, 200μs burst width sine wave, with a repeating pulse at 1kHz, and intensity of 30 $mW/cm^2$. This wave is equal to the wave conditions of sonic accelerated fracture healing system (SAFHS) (TEIJIN PHARMA LIMITED). Briefly, $5 \times 10^4$ HCs per well were seeded into a six-well plate until they reached subconfluence. The medium was replaced with a fresh osteogenic medium consisting of the original medium, 10 mM β-glycerophosphate (Sigma), and 50 μg/ml ascorbic acid (Wako). The culture plate was placed on the ultrasound transducer with a thin layer of water to maintain contact. LIPUS was given through the bottom of the culture plates for 20 minutes daily at 37°C for 2, 4, 7, 14 and 28 days. Cells without LIPUS treatment acted as controls. The cells were subjected to the following biochemical assays and reverse transcription polymerase chain reaction (RT-PCR) analysis after the treatment. Three wells were used for each treatment group and the average was calculated (n=8).

*Cell count*

$5 \times 10^4$ HCs per well were seeded into a six-well plate and stationary cultured for 2 days. LIPUS was applied for 2, 4 and 7 days. An original medium was used for all group. HCs were detached with 0.05% trypsin-0.02% EDTA (Wako). The number of HCs was counted twice using a Hemacytometer and the average was calculated. Cell viability was >99% by the trypan blue dye (Gibco BRL, Grand Island, NY, USA) exclusion technique.

*Alkaline phosphatase (ALP) activity assay*

LIPUS was applied for 2, 4, 7 and 14 days. At 24 hours after the LIPUS

treatment of each group, ALP activities of extracted samples were assayed. The cell layer from each well was washed twice with phosphate buffered saline, sonicated with a Microson Ultrasonic Cell Diaruptor XL2000 (Misonix, Farmingdale, New York) and stored at -20°C until assay. ALP activity was assayed as the release of p-nitrophenol from p-nitrophenylphosphate, pH 9.8, and the p-nitrophenol release was monitored by optical density at 405 nm using SenoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec Corporate Headquarter, San Jose, CA). Protein concentration in the sonicate was measured by BCA Protein Assay Kit (Pierce Chemical Co, Rockford, IL). The results are expressed as p-nitrophenol produced in nmol/min/mg of protein.

*Osteocalcin (OCN) secretion assay*

LIPUS was applied for 2, 4, 7, 14 and 28 days. After removing the medium, 2 ml original medium plus $10^{-8}$ mol/l $1,25(OH)_2$ vitamin $D_3$ was added and incubated at 37°C for 24 hours. The medium in each well was collected and OCN secretion was quantified using Gla-OC Competitive enzyme immunoassay (EIA) Kit (TaKaRa, Shiga, Japan). For the Gla-OC Competitive EIA Kit, on the 96-well plate coated with monoclonal antibody recognizing γ-carboxylated glutamic acid residue (Gla) at position 17, Gla-OC in sample competes with biotinylated Gla-OC. After washing, a color development reaction was performed using avidin-POD conjugate and the amount of Gla-OC present in the sample was quantified by measuring the absorbance. Absorption at 450nm was measured with a spectrophotometer.

*Total RNA extraction and RT-PCR*

LIPUS was applied for 2, 4, 7, 14 and 28 days. At one hour after the LIPUS treatment of each group, expression of osteoblast-related genes, bone sialoprotein (BSP), osteopontin (OPN), runt-related gene 2 (Runx2), Osterix (OSX) and parathyroid hormone receptor (PTH-R) were also measured by RT-PCR. To detect

expression levels of osteoblast-related genes, differentiated cells were harvested. Total ribonucleic acid (RNA) was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. From each sample, approximately 1 μg of total RNA was reverse-transcribed using oligo (dT) primer, dNTP, 10×PCR buffer, $MgCl_2$, RNase inhibitor, and Mulv Reverse Transcriptase (All are from Applied Biosystems, Branchburg, NJ, USA). The converted complementary deoxyribonucleic acid (cDNA) samples were amplified by PCR using Taq Gold DNA polymerase (Applied Biosystems).[3,22,23] In all RT-PCR assays, the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed to monitor RNA loading. Primers used for amplification are listed as following, BSP : sense (S) ; 5'-ATTTCCAGTTCAGGGCAGTAG-3', antisense (AS) ; 5'-ACACTTTCTTCTTCCCCTTCT-3', OPN: S ; 5'-GGCTAAAC CCTGACCCATCTC-3', AS ; 5'-TCATTGCTCTCATCATTGGCT-3', OSX: S ; 5'-AACCCCCAGCTGCCCACCTACC -3', AS ; 5'-GACGCTCCAGCTCATCCG AACG -3', Runx2: S ; 5'-AGTTCCCAAGCATTTCATCC-3', AS ; 5'-TCAATAT GGTCGCCAAACAG-3', PTH-R: S ; 5'-AGGCCAGCCAGCATAATGGAA-3', AS ; 5'-CTCCCGTTCACGAGTCTCAT-3', GAPDH : S ; 5'-CCACCCATGGCAA ATTCCATGGCA-3' , AS ; 5'-TCTAGACGGCAGGTCAGGTCCACC-3'. PCR products were visualized by the luminescence of ethidium bromide by ultraviolet rays after electrophoresis in 2% agarose gel. The expression levels of the genes were quantified using a densitometric program (Scion Image for Windows, Scion Corporation, USA). After normalizing by the band intensity of GAPDH, percentage changes of the genes were determined. Primers used for amplification are listed in Table 1.

*Mineralization assay*

The cells were treated with LIPUS for 20 minutes daily for 4 weeks in a six-well plate and then fixed for 1 hour at room temperature in 95% ethanol. The

plate was stained with 1% Alizarin Red S (Hartman Leddon, Philadelphia, PA, USA) at pH 4.0 (Sigma) for 5 minutes, washed with water, and dried. The positive result is red. Alizarin Red S staining was released from the cell matrix by incubation in 10% etylpyridinium chloride for 15 minutes. The amount of dye released was quantified by spectrophotometer at 562 nm.[24]

*Statistical analysis*

Stat View-J 4.5 software (HULINKS Inc., Tokyo, Japan) was used for statistical analysis. Data were presented as mean ± standard error (SE). To assess differences between treated and control cells, signed Wilcoxon's rank sum test was performed. A value of $p < 0.05$ was considered to be statistically significant.

## Results

*Proliferation*

There was no significant difference in the total number of cells with LIPUS treatment and that of the control at 2, 4 and 7 days of treatments (Figure 1).

*ALP activity*

ALP activity in LIPUS-treated cells was significantly higher than that in the control group at 2, 4, 7 and 14 days of treatments ($p < 0.05$). The percentage of change in ALP activity after the 14 day treatment was highest among all groups (Figure 2).

*OCN secretion*

OCN secretion of LIPUS-treated cells was significantly higher than that of the control group at 4, 7, 14 and 28 days except for 2 days ($p < 0.05$). The percentage of change in OCN secretion after the 28 days treatment was highest among all groups (Figure 3).

*Gene expression*

Osteogenic activities up regulated by LIPUS treatment were further

confirmed by RT-PCR analysis, showing the expressions of BSP, OPN, Runx2, OSX and PTH-R after the LIPUS treatment at 2, 4, 7, 14 and 28 days. The expressions after LIPUS treatment were significantly higher than in the control group at 2, 4, 7, 14 and 28 days ($p < 0.05$). (Figure 4).

*Mineralization*

Alizarin Red S staining of LIPUS-treated cells was significantly higher than in the control group in the 4 week treatment group ($p < 0.05$) (Figure 5).

## Discussion

The current study shows for the first time that the osteogenic activity of HCs is increased by LIPUS treatment.

The ALP activity, the OCN secretion, the expression of osteoblast related genes and the mineralization of HCs were shown to be significantly higher in the LIPUS treatment group than the control group. These results indicate that osteogenic differentiation of HCs is promoted by LIPUS treatment. The effect of LIPUS on fracture healing has been reported in clinical and laboratory studies[6-22, 26, 27]. One in vitro report has suggested that LIPUS treatment had a stimulatory effect on rat calvaria osteoblasts with increased ALP activity[20]. Some reports have shown an increase in transcription of OCN messenger RNA (mRNA) in osteocytes after mechanical stimulation[28, 29]. Additionally, LIPUS have been shown to increase mRNA levels for the bone matrix proteins ALP and OCN in a rat osteosarcoma cell line[21] and a mouse oseteoblastic cell line[16]. LIPUS has also been shown to increase mRNA levels for OP and Runx2 in CD-1 mice osteoblast.[30] There are numerous reports investigating the stimulatory effect of LIPUS in a variety of cells in vitro[10-22]. However, the cells in these reports were not derived from a human fracture site and thus it has remained unclear whether the osteogenic activity on a cellular level of a human fracture site is really increased by LIPUS treatment. In this study, we

investigated cells derived from actual human fracture tissue i.e. HCs and confirmed osteogenic differentiation of HCs was promoted by LIPUS treatment.

The mechanism underlying the ultrasound effect is not yet well understood. Azuma et al investigated the relationship between the timing of partial LIPUS treatment and the efficiency of accelerating the action of healing[31]. They reported that LIPUS accelerated fracture healing and that at an inflammatory phase, macrophages contain a stretch-sensitive potassium channel, the activity of which is modified by mechanical stress.[32, 33] This may be one mechanism that explains the effects of LIPUS on fracture healing. Runx2, a member of the runt family of transcription factors, has a crucial role in the early determination stage of the osteoblast lineage, whereas OSX, a zinc finger-containing transcription factor encoded by SP7, regulates the later stage of osteoblast differentiation and bone formation[34-36]. In this study, we revealed one mechanism for osteogenic differentiation of HCs was promoted by the high expressions of Runx2 and OSX after LIPUS treatment.

It is widely agreed that the presence of growth factors, specifically the TGF-β, IGF, PDGF, aFGF, and bFGF, within the fracture haematoma is a prerequisite substance for the regulation of the process that occur during fracture healing.[37-44] Some reports have described that mesenchymal stem cells are differentiated into osteoblasts and/or chondrocytes under the influence of several growth factors.[45-47] Recently we reported one possible mechanism for fracture healing suggesting these growth factors may act on the HCs at different stages of fracture healing, and the HCs may then differentiate into osteoblasts and/or chondrocytes in an autocrine and/or paracrine manner.[3] We speculated that LIPUS treatment stimulated HCs directly and/or several growth factors are released from HCs in the inflammatory phase of fracture healing, which, as a result, accelerates fracture healing at all phases. In the future, further investigation is needed to discover whether growth factors released from HCs are increased by LIPUS

treatment.

There was no significant difference in the total number of cells after LIPUS treatment and in the control group. These results indicate that LIPUS did not affect cell proliferation in this study. Some reports have indicated LIPUS affected cell proliferation in rat calvaria osteoblast[20], CD-1 mice osteoblast[30], and periosteal cell[22]. In contrast, some reports have indicated that LIPUS affects mainly cell differentiation rather than cell proliferation in human mesenchymal stem cells[10], rabbits chondrocytes[48], rat femoral fracture cells [49-51] and rat fracture callus cells [52]. In this study, we investigated progenitor cells derived from actual human fracture haematoma, which may indicate that LIPUS affects cell differentiation in HCs more than cell proliferation on HCs.

In this *in vitro* study, we investigated the effect of LIPUS on the osteogenic activity of HCs. Osteogenic differentiation of HCs was increased by LIPUS treatment. We demonstrated for the first time that progenitor cells existing in actual human fracture haematoma were affected by LIPUS treatment. This study provides significant evidence for the clinical application of LIPUS for fracture treatment.

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## Figure legends

Fig.1: The total number of cells with LIPUS treatment and the control group.

There was no significant difference in the total number of cells with LIPUS treatment and those in the control at 2, 4 and 7 days of treatments.

Fig.2: ALP activity in LIPUS-treated cells and the control group.

ALP activity in LIPUS-treated cells was significantly higher than that in the control group at 2, 4, 7 and 14 days of treatments ($p < 0.05$). The percentage of change in ALP activity after the 14 day treatment was highest among all groups.

Fig.3: OCN secretion in LIPUS-treated cells and the control group.

OCN secretion of LIPUS-treated cells was significantly higher than that of the control group at 4, 7, 14 and 28 days except for 2 days ($p < 0.05$). The percentage of change in OCN secretion after the 28 days treatment was highest among all groups.

Fig.4: Gene expression after LIPUS treatment and the control group.

The expressions after LIPUS treatment by RT-PCR analysis of BSP, OPN, Runx2, OSX and PTH-R were significantly higher than in the control group at 2, 4, 7, 14 and 28 days ($p < 0.05$).

Fig.5: Alizarin Red S staining of LIPUS-treated cells and the control group.

Alizarin Red S staining of LIPUS-treated cells was significantly higher than in the control group in the 4 week treatment group (x 40) ($p < 0.05$).

Table.1:Primers used for amplication

bone sialoprotein, BSP, osteopontin, OPN, runt-related gene 2, Runx2, Osterix, OSX, parathyroid hormone receptor, PTH-R, glyceraldehyde-3-phosphate dehydrogenase, GAPDH

Figure 1

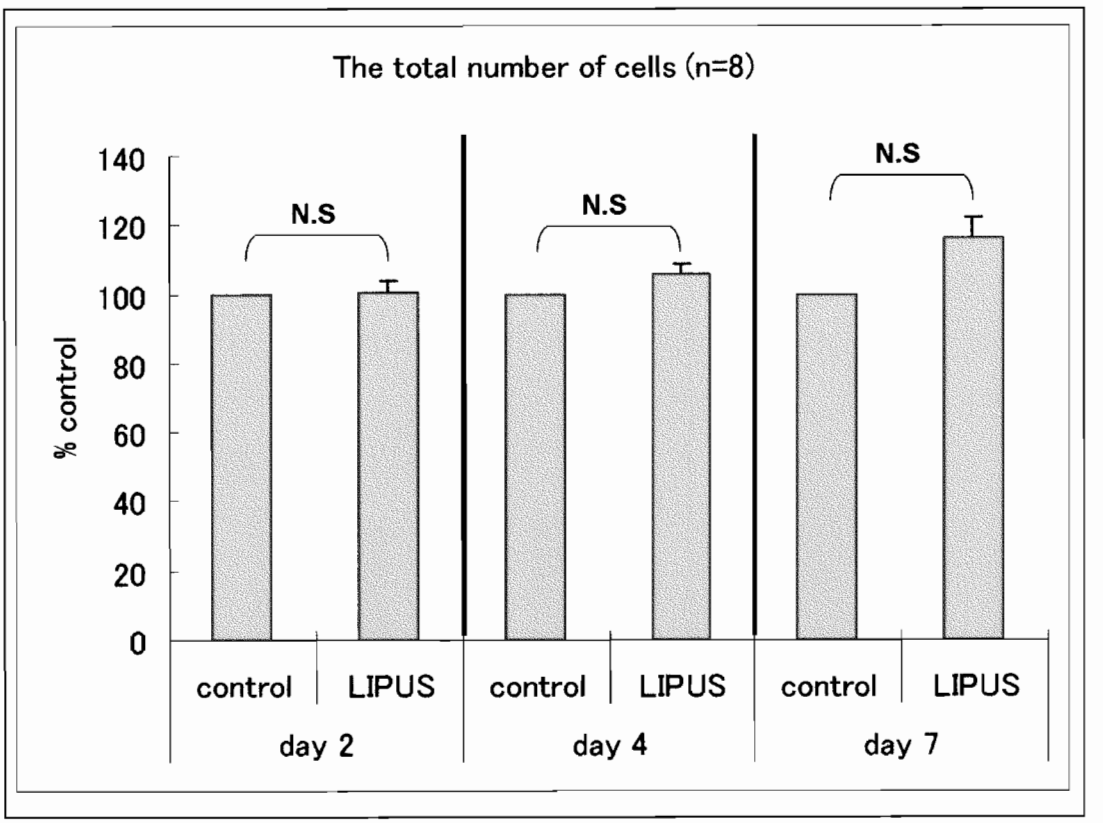


Figure 2

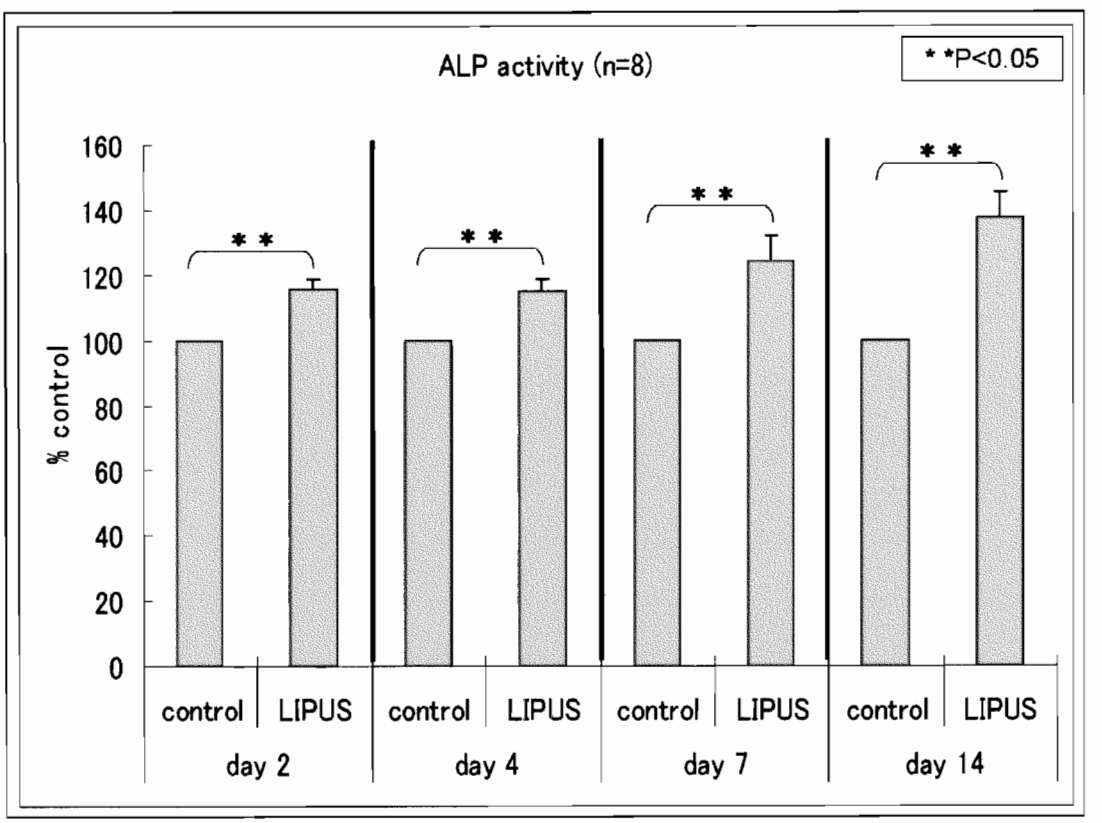

Figure 3

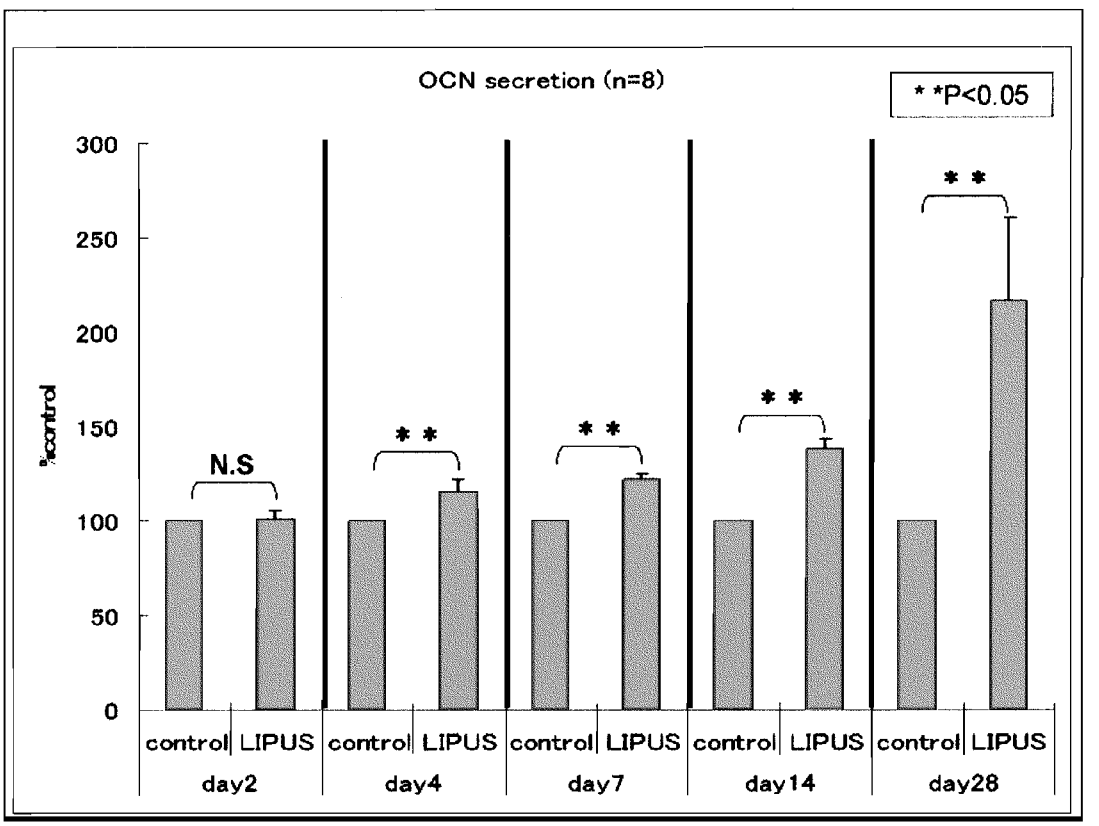
OCN secretion (n=8)
* *P<0.05
%control
300
250
200
150
100
50
0
N.S
* *
* *
* *
* *
control
LIPUS
day2
day4
day7
day14
day28

Figure 4

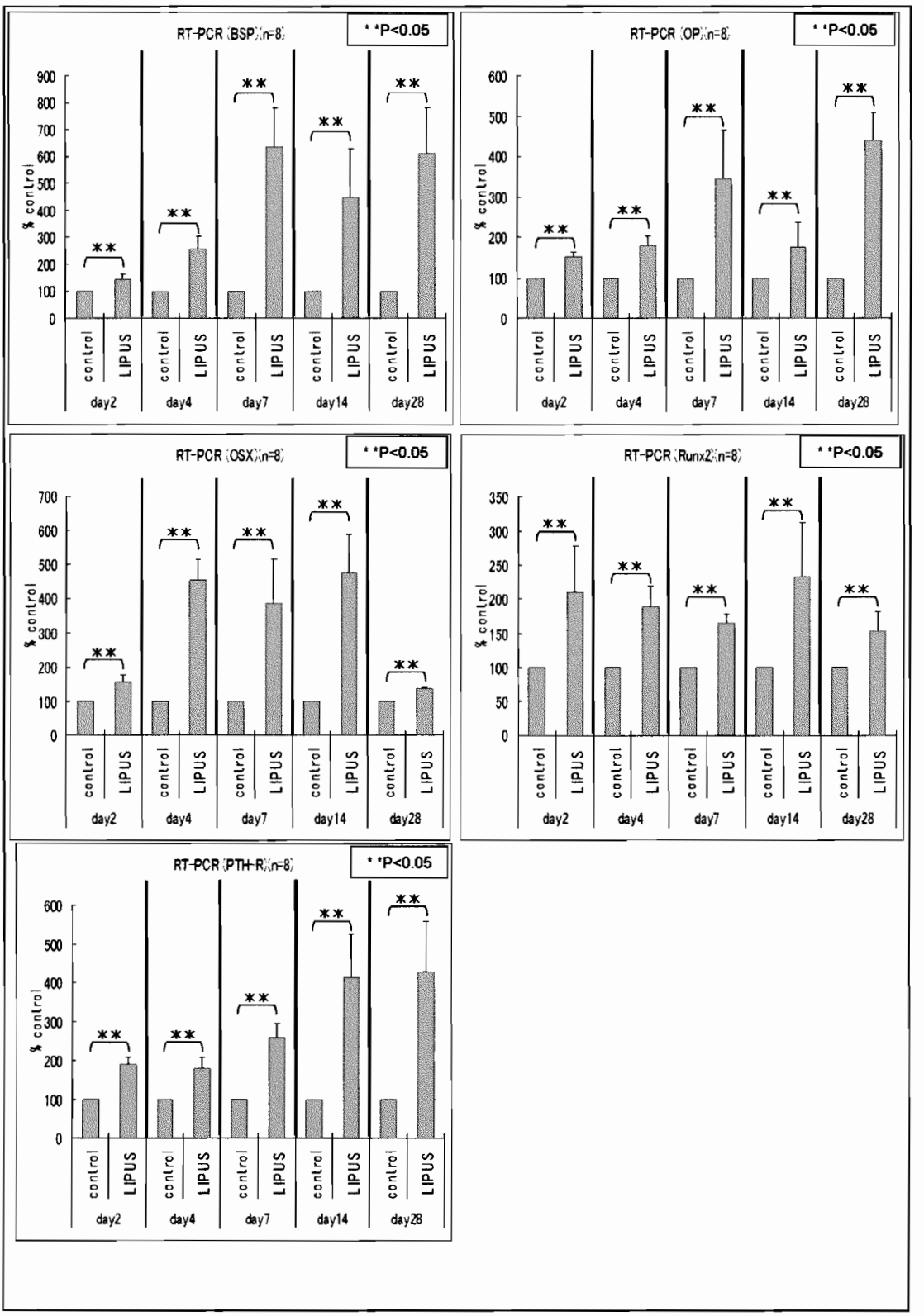
RT-PCR (BSP) (n=8)
* *P<0.05
RT-PCR (OP) (n=8)
* *P<0.05
RT-PCR (OSX) (n=8)
* *P<0.05
RT-PCR (Runx2) (n=8)
* *P<0.05
RT-PCR (PTH-R) (n=8)
* *P<0.05
% control
control
LIPUS
day2
day4
day7
day14
day28

Figure 5

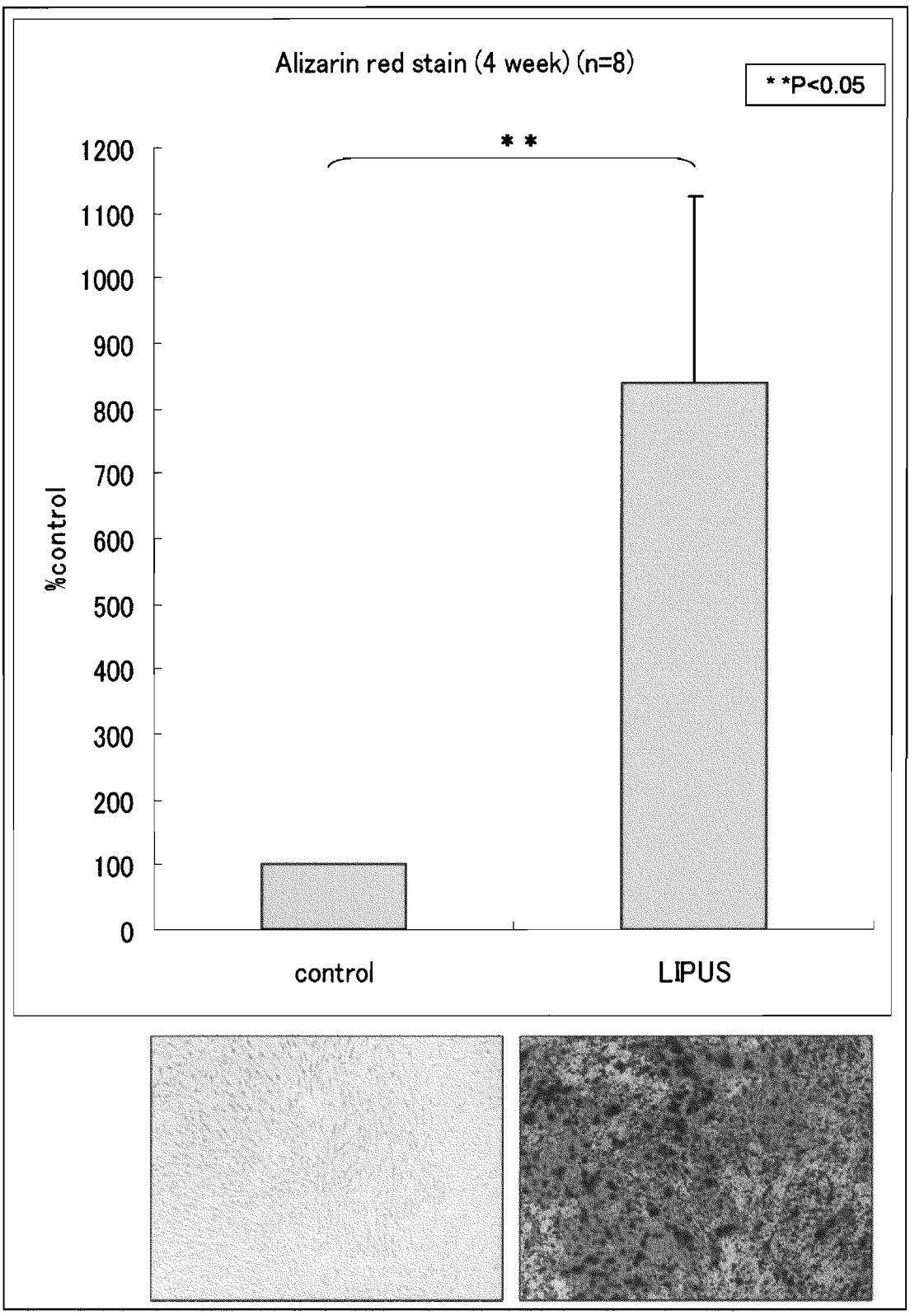
Alizarin red stain (4 week) (n=8)
* *P<0.05
* *
%control
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
control
LIPUS

Table 1

| Gene | Primer Sequences (5`-3`) (sense/antisense) | Product Size (bp) | Annealing Temperature($^{O}$C) | No. of Cycles |
|---|---|---|---|---|
| GAPDH | CCACCCATGGCAAATTCCATGGCA<br>TCTAGACGGCAGGTCAGGTCCACC | 593 | 55 | 25 |
| BSP | ATTTCCAGTTCAGGGCAGTAG<br>ACACTTTCTTCTTCCCCTTCT | 447 | 57 | 35 |
| OPN | GGCTAAACCCTGACCCATCTC<br>TCATTGCTCTCATCATTGGCT | 640 | 60 | 35 |
| OSX | AACCCCCAGCTGCCCACCTACC<br>GACGCTCCAGCTCATCCGAACG | 457 | 60 | 30 |
| Runx2 | AGTTCCCAAGCATTTCATCC<br>TCAATATGGTCGCCAAACAG | 421 | 57 | 34 |
| PTH-R | AGGCCAGCCAGCATAATGGAA<br>CTCCCGTTCACGAGTCTCAT | 374 | 60 | 35 |