

Low-Intensity Pulsed Ultrasound (LIPUS) Influences the Multi-Lineage Differentiation of Mesenchymal Stem and Progenitor Cell Lines through ROCK-Cot/Tpl2-MEK-ERK Signaling Pathway*

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Running title: *Effects of ultrasound on MSC differentiation*

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Key words: Mesenchymal stem cells; Mechanotransduction; LIPUS; Cell differentiation; Adipogenesis; Osteogenesis; MAP kinases (MAPKs)

Background: Low-intensity pulsed ultrasound (LIPUS) is a mechanical stimulus clinically used to promote bone fracture healing.

Results: LIPUS suppress adipogenesis and promote osteogenesis of mesenchyme stem/progenitor cell lines by inhibiting PPAR γ 2 through ROCK-Cot/Tpl2-MEK-ERK pathway.

Conclusion: LIPUS influences multi-lineage differentiation of mesenchymal stem and progenitor cells.

Significance: LIPUS may be a new clinical approach to chronic bone metabolic disorders including osteoporosis.

ABSTRACT

Mesenchymal stem cells (MSCs) are pluripotent cells that can differentiate into multi-lineage cell types, including adipocytes and osteoblasts. Mechanical stimulus is one of the crucial factors in regulating MSC differentiation. However, it remains unknown how mechanical stimulus affects the balance between adipogenesis and osteogenesis. Low-intensity pulsed ultrasound (LIPUS) therapy is a clinical application of mechanical stimulus and

facilitates bone fracture healing. Here, we applied LIPUS to adipogenic progenitor cell and MSC lines to analyze how multi-lineage cell differentiation was affected. We found that LIPUS suppressed adipogenic differentiation of both cell types, represented by impaired lipid droplet appearance and decreased gene expressions of *peroxisome proliferator activated receptor gamma2 (PPAR γ 2)* and *fatty acid binding protein 4 (Fabp4)*. LIPUS also down-regulated the phosphorylation level of PPAR γ 2 protein, inhibiting its transcriptional activity. In contrast, LIPUS promoted osteogenic differentiation of MSC line, characterized by increased cell calcification as well as inductions of *runx-related transcription factor 2 (Runx2)* and *osteocalcin* mRNAs. LIPUS induced phosphorylation of Cancer Osaka thyroid oncogene/tumor progression locus 2 (Cot/Tpl2) kinase, which was essential for the phosphorylation of mitogen activated protein kinase 1 (MEK1) and p44/p42 extracellular signal-regulated kinases (ERKs). Notably, effects of LIPUS on both adipogenesis and osteogenesis were prevented by a

Cot/Tpl2-specific inhibitor. Furthermore, effects of LIPUS on MSC differentiation as well as Cot/Tpl2 phosphorylation were attenuated by the inhibition of Rho-associated kinase (ROCK). Taken together, these results indicate that mechanical stimuli with LIPUS suppress adipogenesis and promote osteogenesis of MSCs through ROCK-Cot/Tpl2-MEK-ERK signaling pathway.

Multipotent mesenchymal stem cells (MSCs) in bone marrow give rise to several cell lineages including adipocytes, osteocytes, and chondrocytes. Several studies have shown that the differentiating directions of MSCs into adipocytes and osteoblasts are controlled in a reciprocal fashion (1). The strict control of MSC differentiation is crucial for maintaining homeostasis of bone marrow between bone formation and fatty marrow, and the failed control of MSC differentiation often causes bone metabolic diseases (2). For example, biased MSC differentiation toward adipogenesis is strongly related to osteoporosis and other chronic bone loss diseases (3). Similarly, increased number of adipocytes and decreased number of osteoblasts are often found in age-related physiological bone reduction (4).

It has been well established that both osteogenic and adipogenic differentiations are regulated by critical master transcription factors. PPAR γ 2 is a master adipogenic transcription factor, which is related to age-related osteoporosis (5). Down-regulation of PPAR γ 2 promotes osteogenic differentiation of MSCs (6). On the other hand, up-regulation of Runx2 accelerates osteogenic differentiation of MSCs inducing increased bone mass (7). These findings suggest that transcriptional activities of these two master regulators may be regulated in inverse manners.

Mechanical stress is one of the effective regulators of MSC differentiation. Several kinds of mechanical stress, such as shear stress, cellular stretch and centrifugal force, are known to affect osteoblast differentiation in various ways (8). Low intensity pulsed ultrasound (LIPUS) is mechanical

stress which is already used as a clinical application to promote the healing of bone fracture (9). Several in vitro studies have shown that LIPUS facilitates osteoblast differentiation, represented by increased *osteocalcin* mRNA expression and extracellular calcification (10). We have previously reported that mRNA expression of several chemokines in mature osteoblasts is also induced with LIPUS treatment, which is mediated by angiotensin 2 type 1 receptor (11). In the site of bone fracture, MSCs are the cellular source of osteoprogenitor cells (12). Thus it seems reasonable to presume that the therapeutic advantage of LIPUS in bone fracture may be associated with the direct effects of LIPUS on not only osteoblasts but also MSCs. However, it remains mostly unknown how LIPUS affects the biological functions of MSCs.

Mitogen activated protein kinase (MAPK) cascades are well known signaling pathways controlling cellular proliferation, differentiation, and death in a variety of cell types (13). It has been reported that MAPK family proteins, including ERK, p38 MAPK, and c-jun N-terminal kinase (JNK) are activated by various mechanical stimuli (14). We and other groups have previously reported that ERKs are crucial signaling molecules in LIPUS-induced cellular responses (11,15). However, detailed signaling pathways of MAPK activation by mechanical stress are still poorly understood.

In this study, we have explored how LIPUS affects MSC differentiation. We have found that LIPUS treatment of MSCs is inhibitory to adipogenic differentiation, but promotive of osteogenic differentiation of MSCs. These effects of LIPUS on MSC differentiation are dependent on LIPUS-induced ERK activation. Through analyses with specific chemical inhibitors, it has been indicated that Cot/Tpl2 kinase, a serine threonine kinase known to be involved in LPS signaling, is essential for the LIPUS-mediated ERK activation. Moreover, Cot/Tpl2 activation by LIPUS is found to be mediated by a further upstream kinase, ROCK. Thus, these data indicate

that LIPUS effectively modulates MSC differentiation through ROCK-Cot/Tpl2-MEK-ERK signaling pathway.

EXPERIMENTAL PROCEDURES

Reagents and antibodies—TKI (Tpl2 kinase inhibitor), a specific Cot/Tp2 kinase inhibitor, Y-27632, a specific ROCK inhibitor, and U0126, a specific MEK inhibitor, were purchased from Merck KGaA (Darmstadt, Germany), WAKO (Osaka, Japan) and Funakoshi (Tokyo, Japan), respectively. Antibodies recognizing phosphorylated forms of ERKs, p38 kinases, and JNKs are purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against ERK1/2, p38 kinases, JNK1/2, Cot/Tpl2, IκBα, and PPARγ were also from Cell Signaling Technology. A phospho-specific antibody against Cot/Tpl2 was obtained from Bioss (Woburn, MA, USA). Antibodies against β-actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and phosphorylated form of PPARγ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture—3T3-L1, a mouse pre-adipocytic cell line, was obtained from DS Pharma Biomedical Co. Ltd. (Osaka, Japan), and maintained in Dulbecco's modified Eagle medium (DMEM) (WAKO) containing 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 mg/ml streptomycin. ST2, a mouse mesenchymal stem cell line, was obtained from RIKEN Cell Bank (Tsukuba, Japan) and maintained in RPMI 1640 (Roswell Park Memorial Institute 1640) (WAKO) containing 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. MC3T3-E1, a mouse osteoblastic cell line, was obtained from RIKEN Cell Bank and maintained in Eagle's α-minimal essential medium (αMEM) (WAKO) containing 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. 10T(1/2), a mouse mesenchymal stem cell line, was obtained from RIKEN Cell Bank and maintained in DMEM containing 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 mg/ml streptomycin. 10T(1/2) cells were

treated with 20 μM of 5'-azacytidine for 3 days to induce differentiation. Adipogenic differentiation of 3T3-L1 and ST2 were induced by the addition of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1.7 μM insulin, and 1 μM dexamethasone in the culture medium. Osteogenic differentiation of ST2 was induced by the addition of 280 μM L-ascorbic acid 2-phosphate trisodium and 5 mM β-glycerophosphate in the culture medium. The bi-lineage differentiation of 10T(1/2) cells were induced by the addition of 20 μM of 5'-azacytidine for 3 days.

Ultrasound application—Cells were stimulated using a LIPUS-generating system (Teijin Pharma Ltd., Tokyo, Japan), which was previously described (16). The LIPUS signal consisted of a series of 1.5-MHz, 200 μs burst sine waves at 1.0 kHz, and was delivered at an intensity of 30 mW/cm². The pattern and intensity of the LIPUS signal employed in this study were essentially the same as that used in clinical practice and in animal model experiments.

Oil Red O staining—Lipid droplet appearances were determined by Oil Red O staining. The cells were washed with Ca²⁺-free phosphate-buffered saline (PBS) twice and fixed in 10% formaldehyde in PBS for 1 h at 4°C. After three washes with distilled water and one wash with 60% isopropanol in distilled water, the cells were stained in 0.5% (w/v) Oil Red O in isopropanol for 15 min at room temperature. The remaining dye was washed out by three washes with distilled water.

Alizarin Red S staining—Matrix mineralization was visualized by alizarin red S staining. The cells were rinsed with Ca²⁺-free PBS twice and fixed in 10% formaldehyde in PBS for 20 min at 4°C. After three washes with distilled water, the cells were stained in 1% alizarin red S solution for 15 min at room temperature. The remaining dye was washed out by three washes with distilled water.

RNA interference of Cot/Tpl2 and ROCK1—3T3-L1 cells, MC3T3-E1 cells, and ST2 cells were transfected with small interference RNA (siRNA) duplexes specific for murine Cot/Tpl2:

r(GAGAACAUGCUGAGUUAU)dTdT and
r(AUAACUCAGCAAUGUUCU)dTdT or
ROCK1:

r(GYGGYAAAGGYAAYCGGCAT)dTdT and
Ur(GCCGAUUACCUUUACCAC)dTdT obtained
from Sigma Aldrich or nontargeting control siRNA
duplexes (Control siRNA-A; Santa Cruz
Biotechnology Inc.) using Hilymax (Dojondo)
according to the manufacturer's instructions.

Quantitative polymerase chain reaction (qPCR) analysis—Total RNA was isolated with TRI reagent (Molecular Research Center, Inc, Cincinnati, OH) and reverse-transcribed with reverse transcriptase (Toyobo, Osaka, Japan) in the presence of an oligo-(dT) primer and RNase inhibitor (TAKARA BIO INC., Otsu, Japan) at 37°C for 1 h. Real-time PCR was conducted using Step-One Plus (Life Technologies, Carlsbad, CA). The cDNA synthesized from 0.5 µg of total RNA was amplified in a 10 µl volume with 0.11 X SYBR Green I (CAMBREX, Rockland, ME), 0.1 mM dNTPs, 0.2 µM each primer, 0.1 µM ROX reference dye (Life Technologies, Carlsbad, CA) and 1 unit Blend Taq DNA polymerase (Toyobo, Osaka, Japan) under the following conditions: 95°C for 2 min, and then 55 PCR cycles at 95°C for 30 sec, 60°C for 20 sec, and 72°C for 20 sec. Fluorescent signals were measured in real time, and then each sample was quantified according to the manufacturer's instructions. To determine the absolute number of copies of the target transcript, PCR product dilutions ranging from 10³ to 10⁸ copies were used to generate standard curves. The primer sequences used in PCR are shown in Table 1.

Western blot analysis—Cells were lysed in RIPA lysis buffer (150 mM NaCl, 1.0% Nonident-P40, 0.5% Deoxycholic acid, 0.1% SDS, 50 mM Tris [pH 8.0], 0.1% Na₃VO₄, and protease inhibitor cocktail). Cell lysates were separated by SDS-PAGE and electro-transferred to Immobilon polyvinylidene difluoride membranes (Merck Millipore, MA, USA). The membranes were blocked for 1 h in 5% skim milk-TBST (TBST is 20 mM Tris-HCl [pH 7.6], 0.15 M sodium

chloride, and 0.1% Tween 20), washed three times with TBST, incubated for 2 h with primary antibodies in TBST, washed three times with TBST, and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or -rabbit immunoglobulin (Merck KGaA) diluted 1:5000 in 5% skim milk-TBST. After three washes in TBST, the blots were developed with the enhanced chemiluminescence substrate (PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions.

RESULTS

LIPUS induced ERK phosphorylation in preadipocytes, osteoblasts and MSCs—MSCs differentiate into precursors of osteoblasts and adipocytes in bone marrow (1). For the purpose of exploring the effects of LIPUS on these cell types, we stimulated a mouse preadipocyte line, 3T3-L1, a mouse osteoblast cell line, MC3T3-E1, and a mouse MSC line, ST2, with LIPUS and analyzed the induced intracellular signaling pathways (Fig. 1). LIPUS rapidly induced significant phosphorylation of ERK in 3T3-L1 (Fig. 1A), MC3T3-E1 (Fig. 1B) and ST2 cells (Fig. 1C). However, phosphorylation of p38 and JNK, as well as the degradation of IκBα were not induced by LIPUS treatment in any of the three cell lines (Fig. 1).

LIPUS stimulation suppressed adipogenic differentiation and promoted osteogenic differentiation—Previous studies have reported that osteogenic differentiation of osteoblasts is significantly promoted by daily LIPUS stimulation (10). However, the influence of LIPUS on adipogenic differentiation has never been elucidated. We thus induced adipogenic differentiation of 3T3-L1 cells with or without daily LIPUS stimulation. Adipogenic differentiation was evaluated by the appearance of lipid droplets in the cytoplasm visualized by Oil Red O staining. As a result, the lipid droplet appearance was significantly reduced by daily LIPUS treatment (Fig. 2A). We also examined the effect of LIPUS on the gene expression of

adipogenic marker proteins. Being consistent with the results of lipid staining, mRNA expression of the examined adipogenic marker genes, *Fabp4*, *PPAR γ 2*, *C/EBP α* , *C/EBP β* and *C/EBP δ* , were all significantly suppressed by LIPUS (Fig. 2B).

We also analyzed the effects of LIPUS treatment on adipogenic and osteogenic differentiation of a mouse MSC line, ST2 cells. ST2 cells were cultured in either adipogenic or osteogenic differentiation media with or without LIPUS stimulation. In the inducing condition of adipogenesis, lipid droplet appearances and expression of *Fabp4* and *PPAR γ 2* were significantly inhibited by LIPUS treatment (Fig. 3A, 3B). On the other hand, in the induction media of osteogenesis, the mRNA levels of two osteogenic marker genes, *Runx2* and *Osteocalcin*, were significantly increased by LIPUS (Fig. 3C). These results have indicated that LIPUS may be inhibitory to adipogenic differentiation but promotive of osteogenic differentiation of MSCs. Notably, in contrast to the above results with ST2 cells, LIPUS treatment had little effect on the induction of *Runx2* or *Oseocalcin* in osteogenic differentiation experiments using MC3T3-E1, a mouse osteoblast cell line, and mouse calvaria-derived primary osteoblasts (data not shown), indicating that the promotive effect of LIPUS on osteogenic differentiation may be dependent on the cell differentiation stage.

LIPUS-induced signals are mediated by Cot/Tpl2 kinase—As LIPUS efficiently phosphorylated ERKs among the three kinds of MAPKs (Fig. 1), we next explored the involvement of ERK in the LIPUS effects on ST2 cells. Cells were cultured in either adipogenic or osteogenic differentiation media with or without LIPUS treatment. Before the LIPUS stimulation, cells were treated with U0126, a specific MEK inhibitor for 60min. Cell culture media were changed to remove U0126 after each LIPUS stimulation. We found that LIPUS-induced inhibition of adipogenic marker mRNA expression was efficiently blocked by U0126 treatment in ST2 cells (Fig. 4A). On the other hand,

LIPUS-induced promotion of *Runx2* and *Osteocalcin* mRNA expression during osteogenic differentiation was significantly inhibited by U0126. (Fig. 4B).

We next explored the upstream signaling mechanisms controlling ERK phosphorylation. We examined the possible involvement of Cot/Tpl2, an essential upstream kinase for the LPS-induced ERK phosphorylation in a variety of cell types (17-21). We found that Cot/Tpl2 was rapidly phosphorylated in LIPUS-stimulated 3T3-L1, MC3T3-E1, and ST2 cells (Fig. 4C). Notably, pretreatment with TKI, a specific Cot/Tpl2 inhibitor, significantly decreased the level of LIPUS-induced ERK phosphorylation in each of these three cell lines (Fig. 4D). Furthermore, transient transfection with Cot/Tpl2-specific siRNA significantly suppressed the protein expression level of Cot/Tpl2 (Fig. 4E) and LIPUS-induced phosphorylation of ERK in all three cell lines (Fig. 4F).

We then explored the involvement of Cot/Tpl2 in LIPUS-induced suppression of adipogenic differentiation. 3T3-L1 cells were induced to differentiate into adipocytes with daily treatment by LIPUS in the presence or absence of TKI. As a result, LIPUS-induced decrease of lipid droplet appearance was significantly inhibited by the addition of TKI (Fig. 5A). Consistently, the induction of *Fabp4* and *PPAR γ 2* mRNA, which was inhibited by LIPUS, was recovered by TKI treatment (Fig. 5B). We further examined the role of Cot/Tpl2 in the LIPUS effects on a MSC line, ST2. ST2 cells were cultured in either adipogenic or osteogenic differentiation media with or without the addition of TKI and LIPUS. Being similar to 3T3-L1 cells, LIPUS-induced inhibition of lipid droplet appearance and adipogenic marker mRNA expression were partially blocked by TKI treatment in ST2 cells, when adipogenic differentiation was induced (Fig. 6A, B). We next examined the involvement of Cot/Tpl2 in the LIPUS-promoted osteogenic differentiation of ST2 cells. It was found that LIPUS-induced promotion of matrix mineralization was abrogated by the

addition of TKI (Fig. 6C). Consistently, the facilitation of *Runx2* and *Osteocalcin* mRNA expression by LIPUS was completely inhibited by TKI. (Fig. 6D). These findings have indicated that Cot/Tpl2 activation is essential for the LIPUS-induced promotion of osteogenic differentiation of ST2 cells.

ROCK is an essential upstream molecule regulating Cot/Tpl2-ERK activation by LIPUS—It has been reported that cytoskeletal organization is a key regulator of cell differentiation (22). ROCK is a serine/threonine kinase playing an important role in mediating cytoskeletal rearrangements (23). We therefore examined the involvement of ROCK in LIPUS stimulation. It was found that LIPUS-induced ERK phosphorylation was efficiently blocked by Y-27632, a specific ROCK inhibitor, in 3T3-L1, MC3T3-E1, and ST2 cells (Fig. 7A). We further examined the effects of this ROCK inhibitor on LIPUS-induced Cot/Tpl2 phosphorylation, and found that it was significantly suppressed with the treatment by Y-27632 in each of the examined three cell lines (Fig. 7B). We also used siRNA against ROCK in order to confirm its role in LIPUS-induced signal transduction. Transient transfection with ROCK-specific siRNA efficiently inhibited ROCK protein expression (Fig. 7C) and LIPUS-induced phosphorylation of Cot/Tpl2 and ERK in all three cell lines (Fig. 7D). Therefore, ROCK appears to be an essential upstream molecule in the activation of Cot/Tpl2 and ERK by LIPUS stimulation.

We subsequently examined the role of ROCK in LIPUS-induced regulation of cell differentiation. In adipogenic differentiation of 3T3-L1 cells, the LIPUS-induced inhibition of *Fabp4* and *PPAR γ 2* mRNA induction was blocked by Y-27632 (Fig. 8A), which was similar to the result of TKI (Fig. 5B). Similar effects of ROCK inhibitor were also observed in ST2 cells (Fig. 8B). On the other hand, the promotion of *Runx2* and *Osteocalcin* mRNA expression by LIPUS was inhibited by Y-27632 during osteogenic differentiation of ST2 cells (Fig. 8C). Taken together, these results have indicated that LIPUS suppresses adipogenic and promotes

osteogenic differentiation through ROCK-Cot/Tpl2-ERK signaling pathway.

LIPUS induces PPAR γ phosphorylation—A previous study has shown that PPAR γ 2 transcriptional activity is attenuated by the phosphorylation of Ser112 (24). We thus analyzed the possible effects of LIPUS on PPAR γ 2 phosphorylation, and found that PPAR γ 2 was rapidly phosphorylated by LIPUS, which lasted for at least 60 min (Fig. 9A). Notably, LIPUS-induced PPAR γ 2 phosphorylation was significantly inhibited by Y-27632, TKI, and U0126 (Fig. 9B). Additionally, Transient transfection with Cot/Tpl2-specific siRNA or ROCK-specific siRNA efficiently inhibited LIPUS-induced phosphorylation of PPAR γ 2 (Fig. 9C). These results have indicated that LIPUS-induced ROCK-Cot/Tpl2-MEK-ERK signaling pathway negatively regulates adipogenesis through regulating not only the expression but also the phosphorylation of PPAR γ 2.

LIPUS affects the cell fate determination for differentiation of a multipotent MSC cell line—Our data demonstrated that LIPUS exerted inhibitory and promotive effects on adipogenic and osteogenic differentiations, respectively. However, it remained ambiguous if LIPUS also influenced the cell fate determination of multipotent stem cell differentiation program. 10T(1/2), a mouse mesenchymal stem cell line, has a multipotency to differentiate into either adipocytes or myoblasts. Pretreatment by 5'-azacytidine for 3 days triggers the differentiation of 10T(1/2) cells, and some cells form lipid droplets and others show increased expression of myoblast-specific proteins at constant rates in regular D-MEM culture medium after a period of days (25). We thus used 10T(1/2) cells as our experimental model to evaluate the LIPUS effects on the cell fate determination of MSC differentiation. Following 3 day-treatment by 5'-azacytidine, 10T(1/2) cells were cultured for 14 days with or without daily treatment by LIPUS. As a result, the number of lipid droplets was significantly decreased by daily LIPUS

stimulation (Fig. 10A). Being consistent with this observation, the induction of *Fabp4* and *PPAR γ 2* mRNA was also significantly suppressed (Fig. 10B). Notably, the expression levels of myogenic marker genes, *myogenic differentiation 1* (*Myod1*) and *Myogenin*, were significantly promoted by LIPUS treatment (Fig. 10B). These findings have suggested that LIPUS influences the fate determination of MSC differentiation toward myoblasts rather than adipocytes.

DISCUSSION

LIPUS is micromechanical stress, which is already clinically applied in bone fracture healing (9). Thus, it is important to identify the types of cells in bone marrow, which are responsible for the clinical effectiveness of LIPUS. Bone marrow stroma contains MSCs, multi-lineage progenitor cells which can differentiate into various cell types including osteoblasts and adipocytes (26). Previous studies have shown that mechanical stress regulates osteogenic differentiation of osteoblasts (27), and osteoblasts are considered to be a major target cell type of LIPUS in bone marrow (28-31). Here, we have found that LIPUS induces intracellular signal transduction including ERK phosphorylation in preadipocyte (3T3-L1) and MSC (ST2) cell lines in similar manner to that for a osteoblast line (MC3T3-E1) (Fig. 1A and 1C), indicating that not only osteoblasts but also other cell types in bone marrow may mediate clinical effectiveness of LIPUS.

One of the significant novel findings of our present study is the inhibitory effect of LIPUS on adipogenic differentiation. When adipogenic differentiation was induced, both lipid droplet appearance and the gene expression of adipogenic differentiation markers were clearly inhibited by daily LIPUS treatment in 3T3-L1 (Fig. 2) and ST2 (Fig. 3) cell lines. Differentiation manners of MSCs are known to be regulated by various local and hormonal factors (1). For example, age-related osteoporosis is related to the biased differentiation of MSCs in bone marrow toward adipocytes rather than osteoblasts (3). The increased adipogenesis,

often referred as “fatty marrow”, impairs hematopoiesis in bone marrow, presumably due to the direct inhibitory effects of adipocytes on hematopoietic stem cells (32). The age-related fatty marrow has been proposed to be caused by numerous factors, including skeletal immobilization (33). Bone loss caused by skeletal immobilization has been demonstrated in human and experimental animal models. For example, hindlimb suspension of rats, an established animal model of skeletal unloading, inhibits long bone formation due to impaired recruitment and functions of osteoblasts (34). Conversely, skeletal immobilization is also known to increase adipogenesis in human bone marrow in addition to its negative effects on osteogenesis (26). These previous findings have indicated that mechanical loading of bone presumably affects MSC differentiation manners toward osteogenesis rather than adipogenesis in bone marrow. However, the detailed molecular mechanisms have not been elucidated.

It is known that adipogenic differentiation is regulated by some core regulatory proteins (35). *PPAR γ 2* is master transcriptional factor of adipogenesis. *C/EBP β* and *C/EBP δ* are co-regulatory transcription factors, and modulate adipogenic differentiation in association with *PPAR γ 2*. *Fabp4*, a representative adipogenic marker, is involved in the process of lipid droplet accumulation. LIPUS stimulation effectively decreased mRNA of *PPAR γ 2*, *C/EBP δ* , *C/EBP β* and *Fabp4* during adipogenic differentiation of both 3T3-L1 and ST2 cell lines (Fig. 2B). Among these four core regulatory proteins, we presumed that the down-regulation of *PPAR γ 2* is the decisive event for the LIPUS-induced inhibition of adipogenic differentiation, as *PPAR γ 2* is the only known factor that is necessary and sufficient for the induction of adipocyte differentiation (36). As a matter fact, it has been previously reported that the expressions of *C/EBP δ* , *C/EBP β* and *Fabp4* are all induced by the forced *PPAR γ 2* expression in fibroblasts (37). *PPAR γ 2* is a member of the nuclear receptor superfamily and forms a

heterodimer with retinoid X receptors (RXRs) to bind PPAR-responsive elements (PPREs). PPAR γ 2 is encoded by the *PPAR γ* gene, which also produces an alternative splicing variant, PPAR γ 1. PPAR γ 1 and PPAR γ 2 differ only in the N-terminal amino acid sequences, and PPAR γ 2 has a 5- to 6-fold increased transcriptional activity of the ligand-independent activation function-1 domain compared with PPAR γ 1 (38). These two variants are driven by distinct promoters. Although the expression of PPAR γ 2 is restricted to mature adipocytes, PPAR γ 1 is ubiquitously expressed, indicating distinct regulatory mechanisms for these two promoters (39,40). In our present study, daily LIPUS treatment inhibited the adipocyte differentiation-induced PPAR γ 2 mRNA increase (Fig. 2). In contrast, the level of PPAR γ 1 mRNA remained constant during adipogenic differentiation, and was not affected by LIPUS treatment (data not shown). These results indicated that LIPUS affects the PPAR γ 2 gene promoter in a specific manner.

Previous reports have revealed that the transcriptional activity of PPAR γ 2 is modulated by phosphorylation, sumoylation, ubiquitilation, and nitration (41). Phosphorylation of PPAR γ 2 is presumed to be the most significant negative posttranscriptional regulator among them (42). The Ser112 in PPAR γ 2 in the mouse and Ser114 in the human have been reported to be phosphorylated by each of the three MAPK groups (43,44). Being consistent with these reports, we found that Ser112 phosphorylation of PPAR γ 2 was induced by LIPUS through ERK activation in ST2 cells (Fig. 9A). These findings have indicated that LIPUS inhibits PPAR γ 2 through two distinct mechanisms: transcriptional repression, and phosphorylation-mediated inactivation. Interestingly, both mechanisms appear to require ERK activation. The relative contributions by these two mechanisms to the inhibition of adipogenesis remains unknown at present, and require further experiments.

In contrast to the negative effects on adipogenic differentiation, the promotive effects were induced

by LIPUS on osteogenic differentiation of ST2 cells, represented by facilitated mRNA expression of *Runx2* and *Osteocalcin* (Fig. 3). Being similar to the role of PPAR γ 2 in adipogenesis, Runx2 is considered as the master regulator of osteogenesis. Runx2 directly enhances the promoter activities of various osteogenic differentiation markers including Osteocalcin (45). Thus, it seems reasonable to presume that LIPUS promotes osteogenic differentiation of ST2 cells through the transcriptional activation of Runx2. It should be noted, however, that the expression of osteogenic differentiation marker genes was not altered by daily (20 min/day) LIPUS treatment in long-term (4 weeks) differentiation experiment of MC3T3-E1, an osteoblast cell line, and primary osteoblast isolated from calvaria of newborn C57BL/6 mice (data not shown). These seemingly incompatible results may have been caused by different differentiation stages of these two cell lines. PPAR γ 2 mRNA expression was detectable and gradually decreased during osteogenic differentiation in ST2 cells (Fig. 9). On the other hand, PPAR γ 2 expression could not be detected in MC3T3-E1 cells by either quantitative PCR or western blot analysis (data not shown). Reciprocal crossover regulation has been reported between PPAR γ 2 and Runx2 (46-48). As LIPUS effectively inhibits PPAR γ 2 activity through phosphorylation (Fig. 9A), one possible explanation is that LIPUS promotes osteogenesis through down-regulation of PPAR γ 2 and thus did not enhance osteogenic differentiation of MC3T3-E1 cells, which did not express PPAR γ 2.

Our results have also suggested that LIPUS affects the bi-lineage differentiation of 10T(1/2) cells toward myoblasts rather than adipocytes (Fig. 10). A previous study has reported that murine G8 myoblasts highly expressing PPAR γ and C/EBP α showed markedly reduced levels of MyoD and myogenin proteins under optimal conditions for muscle differentiation (49). In another study, a PPAR γ 2-overexpressing bovine embryonic fibroblast cell line grown in adipogenic differentiation media preferentially differentiated into adipogenic cells

even in the presence of ectopic MyoD expression(50). Thus, it seems reasonable to suppose that the LIPUS-induced reduction of PPAR γ 2 transcriptional activity enhances myogenic differentiation. LIPUS-induced alteration of PPAR γ 2 function might be an important regulator of MSC differentiation toward osteogenesis and myogenesis.

ERK phosphorylation is an important signaling event controlling differentiation of various cell types, including osteoblasts and adipocytes (13). We have found that ERK activation is crucial for the LIPUS-induced inhibitory effects on adipogenesis and promotive effects of osteogenesis (Fig.4A and 4B). This finding is consistent with several previous studies which showed inhibitory effects of ERK activation on adipogenic differentiation. For example, stimulation with oncostatin M inhibited C/EBP β -induced adipogenic differentiation through ERK signaling pathway in 3T3-L1 cells and mouse embryonic fibroblasts (51). Furthermore, apelin suppresses adipogenic differentiation through an ERK-dependent pathway in pre-adipocytes and mature adipocytes (52). In contrast, however, other previous studies have shown that ERK activation has promotive effects on adipogenic differentiation. Insulin, IBMX and dexamethasone-induced ERK phosphorylation enhanced the expressions of PPAR γ 2 and C/EBP α in 3T3-L1 cells (53). In another report, treatment with all-trans retinoic acid (RA) induced commitment of mouse embryonic stem cells into the adipocytic lineage by the ERK signaling pathway (54). We presume that the apparent discrepancies among reports including ours are due to the different degrees of adipogenic differentiation in the experimental cell systems. The treatment with the combination of insulin, IBMX and dexamethasone is widely used as the fundamental inducer to initiate adipogenesis. RA was also used as an initial factor to start the differentiation of MSCs into adipocytes. Thus, the activation of ERK appears to be essential in the initial step of adipogenic differentiation. We also found that 3T3L1 and ST2 cells could not be induced to differentiate with the treatment of U0126, a specific MEK inhibitor, in our experimental system (data not shown), indicating

that the inhibition of ERK alone does not induce adipogenic differentiation. In our study and the previous reports showing suppressive effects of ERK on adipogenesis (51,52), cells were stimulated by ERK activators after the initial induction of adipogenic differentiation by insulin, IBMX and dexamethasone. Thus it seems reasonable to presume that ERK activation exerts inhibitory effects on adipogenesis after the onset of differentiation.

Phosphorylation of ERK is induced by various extracellular stimuli including some types of mechanical stresses (14). Previous studies have demonstrated that FAK, Ras, Raf, and MEK are upstream signaling molecules of ERK phosphorylation by fluid shear stress (55). However, it has been reported that upstream activation cascades of ERK are varied depending on both cell types and the types of mechanical stress (56-59). In our present study, we have identified Cot/Tpl2 as an important signaling molecule in LIPUS-induced ERK activation (Fig. 4). Cot/Tpl2 has been known as an essential molecule in LPS-induced ERK activation in osteoblasts (17), mast cell (20), and macrophages (18,21), and our present study is the first report showing the involvement of Cot/Tpl2 in mechano-transduction.

Sensing receptors for some types of mechanical stress have been identified in several studies (11,60-62). However, it has never been clearly shown how cell differentiation is regulated by the signal transduction of a single mechanical stress-specific receptor (14). On the other hand, it has been reported that cytoskeletal organization is a key regulator of some types of cell differentiation (22). Especially, a previous study has reported that MSCs actively change their cytoskeleton (63) and cell membrane shape when their differentiation is induced (64). We found that ROCK, a major molecule involved in cytoskeletal rearrangements, is involved in LIPUS-induced signal transduction. ROCKs (ROCK1 and ROCK2) belong to the AGC family of serine-threonine kinases, and regulate a variety of fundamental cellular functions. Both isoforms, ROCK1 and ROCK2, are

ubiquitously expressed (65). Despite some functional differences, they share many downstream targets. ROCK is one of the important effectors of Rho, a small GTPase protein family (66). Following activation by Rho, ROCK functions as a regulator of cytoskeletal remodeling. ROCK induces actin filament stabilization, assembly of actin and actomyosin networks, and microtubule dynamics, through phosphorylation of its target proteins, directly contributing to a number of cytoskeleton-mediated processes, including adhesion, contraction, polarity, cytokinesis, motility, permeability, and phagocytosis (23).

Our present data have demonstrated that ROCK is an essential signaling component mediating ERK phosphorylation by LIPUS in MSC, osteoblast, and preadipocyte cell lines (Fig. 7). Recent reports have indicated that ROCK functions as an upstream activator of ERK in several cell types. For example, activation of ROCK induces smooth muscle cell proliferation through ERK phosphorylation (67). However, as ROCK is not considered as a direct upstream activator of MEK-ERK signaling pathway, it has remained ambiguous how activated ROCK can induce ERK phosphorylation. Our present data have indicated that Cot/Tpl2, which is an essential MEK kinase in the LPS signaling, mediates the ROCK-induced signal to ERK phosphorylation.

Activation of ROCK by mechanical stimuli other than LIPUS has been reported in several cell types (68,69). A quite recent report, which was published when our study was ongoing, has examined the role of ROCK in mechanical stress-induced MSC differentiation (70). In their report, inhibition of ROCK by Y-23672 suppressed stretch-induced tenogenic differentiation of human bone marrow-derived MSCs. Thus, taken together with our present report, some types of mechanical stress seem to affect differentiation of both human and mouse MSCs.

A previous study has shown that the cytoskeletal structure of SAOS-2, a human osteosarcoma cell line, is dramatically changed after LIPUS stimulation, especially with an enhancement of stress fiber formation (30). As Rho families and their effectors,

such as ROCK, are essential enzymes in the rearrangement of cellular architectures, their report raises the possibility that LIPUS induces dynamic alterations of actin fibers through activation of ROCK and its down-stream signaling molecules. The involvement of ROCK-mediated signaling in Cot/Tpl2 activation has not been previously reported. Two groups examined the involvement of ROCK in the reagents-induced phosphorylation of MEK, a downstream kinase of Cot/Tpl2. Treatment by transforming growth factor- α induced MEK activation through ROCK in rat chondrocytes (71). Cholinergic agonists induced activation of Rho and ROCK, which in turn activated MEK and ERK, in rat epithelial cells (72). Our present results suggest the possibility that Cot/Tpl2 might be an upstream kinase in the activation of MEK and ERK by ROCK in the previous reports.

In summary, our study has demonstrated that mechanical stimuli with LIPUS suppress adipogenesis and promote osteogenesis of mesenchyme stem and progenitor cell lines. These LIPUS-induced effects are mediated by ROCK-Cot/Tpl2-MEK-ERK signaling pathway and the modulation of PPAR γ 2 activity. These results possibly suggest new clinical approaches to chronic bone metabolic disorders such as osteoporosis using mechanical stimuli including LIPUS. This study also provides new insights into the molecular mechanisms of cellular effects by LIPUS as well as other mechanical stimuli.

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FOOTNOTES

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²The abbreviations used are: LIPUS, Low-intensity pulsed ultrasound; ROCK, Rho-associated kinase; Cot/Tpl2, Cancer Osaka thyroid oncogene/tumor progression locus 2; MEK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinases;

FIGURE REGENDS

FIGURE 1. LIPUS induces ERK phosphorylation in adipocytes and mesenchymal stem cells. (A) Preadipocytic 3T3-L1 cells were stimulated by LIPUS (30mW/cm²) for 20 min. Cells were lysed in RIPA lysis buffer at the indicated time after the LIPUS stimulation. Cell lysates were separated by SDS-PAGE and western blotting was performed with the indicated antibodies. (B) Osteoblastic MC3T3-E1 cells were stimulated by LIPUS and analyzed as in (A). (C) ST2, a mouse mesenchymal stem cell line, was stimulated by LIPUS and analyzed as in (A).

FIGURE 2. LIPUS suppresses adipogenic differentiation of 3T3-L1 cells. (A) 3T3-L1 cells were induced to differentiate by a combination of dexamethasone, insulin and IBMX for 12 days with or without the daily stimulation by LIPUS for 20 min. Cells were stained with Oil Red O to determine lipid droplet appearances. (B) 3T3-L1 cells were induced to differentiate by a combination of dexamethasone, insulin and IBMX for 12 days with or without the daily stimulation by LIPUS for 20 min. Total RNAs were isolated and reverse-transcribed. The gene expressions of adipogenic markers were analyzed by realtime PCR. Same experiments were performed at least 3 times. Relative mRNA expression levels are compared with RPL13a. Error bars represent s.d. Statistical significance was determined by Student's t-test ($p^* < 0.05$ or $**p < 0.01$).

FIGURE 3. LIPUS-induced effects on adipogenic and osteogenic differentiation of ST2 cells. (A) ST2 cells were cultured in adipogenic differentiation media (dexamethasone, insulin and IBMX) with or without daily 20 min stimulation by LIPUS. After 15 days, cells were stained with Oil Red O solution to determine lipid droplet appearances. (B) After the treatments as in (A), total RNAs were isolated and reverse-transcribed. The gene expressions were analyzed by realtime PCR. Each experiment was repeated at least 3 times. Relative mRNA expression levels compared with RPL13a are shown. Error bars represent s.d. Statistical significance was determined by Student's t-test ($p^* < 0.05$ or $**p < 0.01$). (C) ST2 cells were cultured in osteogenic differentiation media (280 μ M L-ascorbic acid 2-phosphate trisodium and 5 mM β -glycerophosphate) with or without the daily 20 min stimulation by LIPUS. After 15 days, the expression levels of osteogenic marker genes were compared with RPL13a. Error bars represent s.d. Statistical significance was determined by Student's t-test ($p^* < 0.05$ or $**p < 0.01$).

FIGURE 4. LIPUS-induced ERK phosphorylation is mediated by Cot/Tpl2 activation. (A) ST2 cells were cultured in adipogenic differentiation medium (dexamethasone, insulin and IBMX). Cells were treated with or without 2.5 mM U0126 for 60 min and stimulated by LIPUS for 20 min every day. After each LIPUS treatment, cell culture media were changed to remove U0126. After 15days, total RNAs were isolated, reverse-transcribed, and analyzed by realtime PCR. Each experiment was repeated at least 3 times with consistent results. Relative mRNA expression levels in comparison with RPL13a mRNA are shown. Error bars represent s.d. Statistical significance was determined by Student's t-test ($**p < 0.01$). (B) ST2 cells were cultured in osteogenic differentiation media (L-ascorbic acid 2-phosphate trisodium and β -glycerophosphate) with or without 2.5 mM U0126 for 60 min and stimulated by LIPUS for 20 min every day. After each LIPUS treatment, cell culture media were changed to remove U0126. The expressions of osteogenic marker genes were analyzed as in (A). (C) 3T3-L1, MC3T3-E1, and ST2 cells were stimulated by LIPUS for 20min. Cells were lysed in RIPA lysis buffer immediately after the stimulation. Cell lysates were separated by SDS-PAGE and levels of phosphorylated and total Cot/Tpl2 proteins were determined by western blotting. (D) 3T3-L1, MC3T3-E1, and ST2 cells were pretreated with 5 μ M TKI (a Cot/Tpl2-specific inhibitor) for 30 min, followed by LIPUS stimulation for 20 min. Cells lysates were prepared in RIPA lysis buffer and separated by SDS-PAGE. The levels of phosphorylated and total ERK proteins were determined by western blotting. (E) and (F) 3T3-L1, MC3T3-E1, and ST2 cells were transiently transfected with either Cot/Tpl2 siRNA or control siRNA. The effects of Cot/Tpl2 siRNA on Cot/Tpl2 protein expression levels (E) and LIPUS-induced ERK phosphorylation (F) were confirmed by western blotting.

FIGURE 5. Cot/Tpl2 is involved in the LIPUS-induced suppression of adipogenic differentiation of 3T3-L1 cells. (A) 3T3-L1 cells were cultured in adipogenic differentiation media (dexamethasone, insulin and IBMX) with or without 5 μ M TKI and stimulated by daily LIPUS for 20 min. After 12 days, cells

were stained with Oil Red O to determine lipid droplet appearances. (B) 3T3-L1 cells were induced to differentiate as in (A) for 12 days with or without 5 μ M TKI and daily 20 min LIPUS stimulation. Total RNAs were isolated and reverse-transcribed. The gene expressions of adipogenic markers were analyzed by realtime PCR. Each experiment was repeated at least 3 times with consistent results. Relative mRNA expression levels in comparison with RPL13a mRNA are shown. Error bars represent s.d. Statistical significance was determined by Student's t-test ($p^* < 0.05$ or $**p < 0.01$).

FIGURE 6. Cot/Tpl2 is an essential signaling molecule of LIPUS-induced suppression of adipogenesis and promotion of osteogenesis. (A) ST2 cells were cultured in adipogenic differentiation media (dexamethasone, insulin and IBMX) with or without 5 μ M TKI and stimulated by daily LIPUS for 20 min. After 15 days, cells were stained with Oil Red O to determine lipid droplet appearances. (B) ST2 cells were induced to differentiate as in (A). After 15days, total RNAs were isolated, reverse-transcribed, and analyzed by realtime PCR. Each experiment was repeated at least 3 times with consistent results. Relative mRNA expression levels in comparison with RPL13a mRNA are shown. Error bars represent s.d. Statistical significance was determined by Student's t-test ($p^* < 0.05$ or $**p < 0.01$). (C) ST2 cells were cultured in osteogenic differentiation media (280 μ M L-ascorbic acid 2-phosphate trisodium and 5 mM β -glycerophosphate) with or without 5 μ M TKI and stimulated by daily LIPUS for 20min. After 21days, cells were stained with alizarin red S for the detection for calcification. Calcified area was photographically measured, and mineralization ratio relative to control was expressed as mean \pm s.d. Statistical significance was determined by Student's t-test ($**p < 0.01$). (D) ST2 cells were induced to differentiate as in (C). Total RNAs were isolated and reverse-transcribed. The gene expressions of osteogenic markers were analyzed by realtime PCR. Each experiment was repeated at least 3 times with consistent results. Relative mRNA expression levels in comparison with RPL13a mRNA are shown. Error bars represent s.d. Statistical significance was determined by Student's t-test ($**p < 0.01$).

FIGURE 7. ROCK is an essential upstream molecule in LIPUS-induced Cot/Tpl2 and ERK activation. (A) 3T3-L1, MC3T3-E1, and ST2 cells were pretreated with 1, 2.5, 5 or 10 μ M of Y-27632 (a ROCK specific inhibitor) for 1h. followed by LIPUS stimulation for 20min. Cell lysates were separated by SDS-PAGE and levels of phosphorylated and total ERK proteins were determined by western blotting. (B) 3T3-L1, MC3T3-E1, and ST2 cells were pretreated with 5 μ M of Y-27632 for 1h. Levels of phosphorylated and total Cot/Tpl2 proteins were determined as in (A). (C) and (D) 3T3-L1, MC3T3-E1, and ST2 cells were transiently transfected with either ROCK1 siRNA or control siRNA. The inhibitory effects of ROCK1 siRNA on ROCK1 protein expression were confirmed by western blotting (C). Cot/Tpl2 and ERK phosphorylation was analyzed by Western blotting (D).

FIGURE 8. LIPUS suppresses adipogenesis and promotes osteogenesis through ROCK-Cot/Tpl2-ERK pathway. (A) Preadipocytic 3T3-L1 cells were induced to differentiate into adipocytes with a combination of dexamethasone, insulin and IBMX for 12 days with or without the addition of 5 μ M Y-27632 and stimulated with daily LIPUS stimulation for 20 min. Total RNAs were isolated and reverse-transcribed, and the gene expressions of adipogenic markers were analyzed by realtime PCR. Relative mRNA expression levels were calculated in comparison with the housekeeping RPL13a mRNA. Error bars represent the s.d. of triplicate values. Statistical significance was determined by Student's t-test (** $p < 0.01$). Each experiment was performed at least 3 times with consistent results. A typical result is shown. (B) Adipogenic differentiation of ST2 cells was induced to differentiate with a combination of dexamethasone, insulin and IBMX for 15 days with or without the addition of 5 μ M Y-27632 and/or daily 20 min LIPUS stimulation. Analyses of adipogenic gene marker mRNAs were performed as in (A). (C) Osteogenic differentiation of ST2 cells was induced to differentiate with a combination of L-ascorbic acid 2-phosphate trisodium and β -glycerophosphate for 23 days with or without 5 μ M Y-27632 and/or daily LIPUS stimulation for 20 min. The analysis of osteogenic gene marker mRNAs was performed by realtime PCR as in (A).

FIGURE 9. LIPUS induces PPAR γ 2 phosphorylation in ST2 cells. (A) ST2 cells were cultured in adipogenic differentiation medium for 5 days. Cells were stimulated by LIPUS for 20 min. Cells were lysed in RIPA lysis buffer at the indicated time after the stimulation. Cell lysates were separated by SDS-PAGE and levels of phosphorylated and total PPAR γ 2 proteins were determined by western blotting. (B) ST2 cells were pretreated with 5 μ M of Y-27632, 5 μ M of TKI or 2.5 μ M of U0126 for 1 h, followed by stimulation with LIPUS for 20 min. Western blotting analyses were performed as in (A). (C) ST2 cells were transiently transfected with Cot/Tpl2 siRNA, ROCK1 siRNA or control siRNA. The analysis of PPAR γ 2 phosphorylation was performed by western blotting as in (B).

FIGURE 10. LIPUS-induced effects on adipogenic and myogenic differentiation of 10T(1/2) cells. (A) 10T(1/2), a mouse mesenchymal stem cell line, was treated with 20 μ M of azacytidine for 3 days. After the treatment, cells were incubated in D-MEM with 10% FCS for 14 days. Cells were stained to determine lipid droplet appearances by Oil Red O. After the staining, the number of lipid droplets was counted under the microscope ($n=3$). Error bars represent s.d. Statistical significance was determined by Student's t-test ($p^{**} < 0.01$). (B) 10T(1/2) cells were treated with azacytidine as in (A) and cultured in D-MEM with 10% FCS in the presence or absence of daily LIPUS for 20 min. At day 14, the analysis of adipogenic and myogenic gene marker mRNAs was performed by realtime PCR. Relative mRNA expression levels in comparison with GAPDH mRNA are shown. Statistical significance was determined by Student's t-test ($p^* < 0.05$ or $p^{**} < 0.01$).

Table 1

Primers used in this study

Gene symbol	Primers [5' - 3']	Gene bank	Amplification length
<i>c/ebp-alpha</i>	ACAGAAGGTGCTGGAGTTGA CCTTGACCAAGGAGCTCTCA	NM_007678	125bp (1062-1186) nt
<i>c/ebp-beta</i>	CATGCACCGCCTGCTG CAGTCGGGCTCGTAGTAGAA	NM_001287738	98bp (107-205 nt)
<i>c/ebp-delta</i>	AGGCAGGGTGGACAAGC GTAGGCGCTGAAGTCGATG	NM_007679	112bp (120-231 nt)
<i>fabp4</i>	CGACAGGAAGGTGAAGAGCA ATTCCACCACCAGCTTGTCA	NM_024406	122bp (296-417 nt)
<i>gapdh</i>	TCAAGAAGGTGGTGAAGCAG GGTGGAAGAGTGGGAGTTGC	NM_008084	110bp (839-948 nt)
<i>myod1</i>	CTGCTCTGATGGCATGATGG CTTCCCTGGCCTGGACTC	NM_010866	112bp (811-922 nt)
<i>myogenin</i>	GTCCCAACCCAGGAGATCA CATGGTTTCGTCTGGGAAGG	NM_031189	128bp (591-718 nt)
<i>osteocalcin</i>	CTCACAGATGCCAAGCCCA CCAAGGTAGCGCCGGAGTCT	NM_007541	98bp (107-204 nt)
<i>ppar-gamma2</i>	TGAGCACTTCACAAGAAATTACCA TGTCAAAGGAATGCGAGTGG	NM_011146	117bp (113-229 nt)
<i>rpl13a</i>	GCTTACCTGGGGCGTCTG ACATTCTTTTCTGCCTGTTTCC	NM_009438	149bp (427-575 nt)
<i>runx2</i>	CCGTGGCCTTCAAGGTTGT TTCATAACAGCGGAGGCATTT	NM_009820	118bp (635-752 nt)

Figure 1

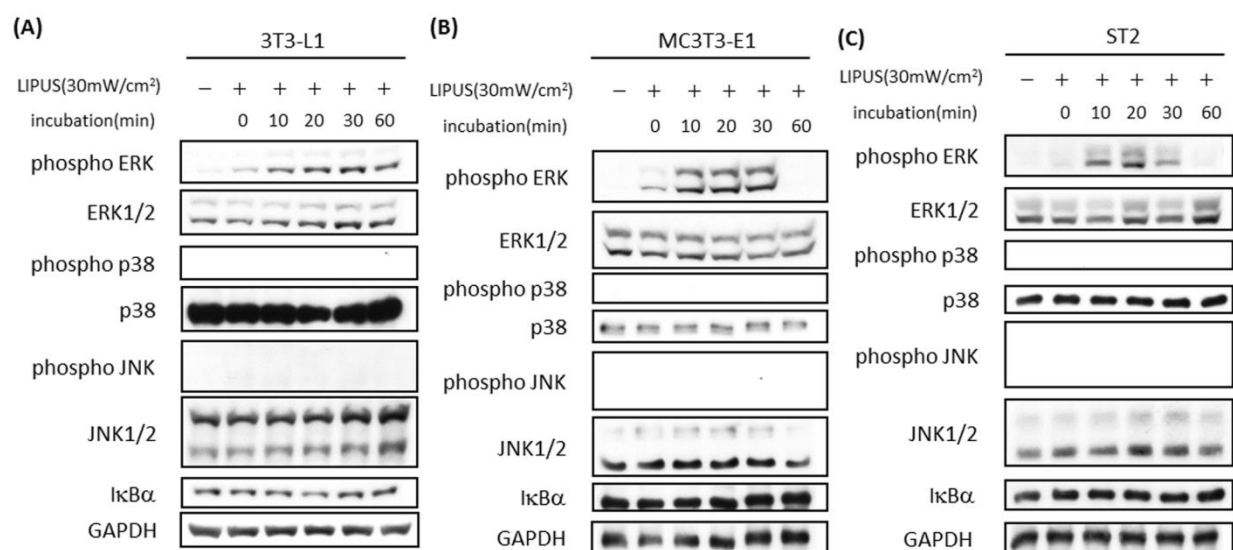


Figure 2

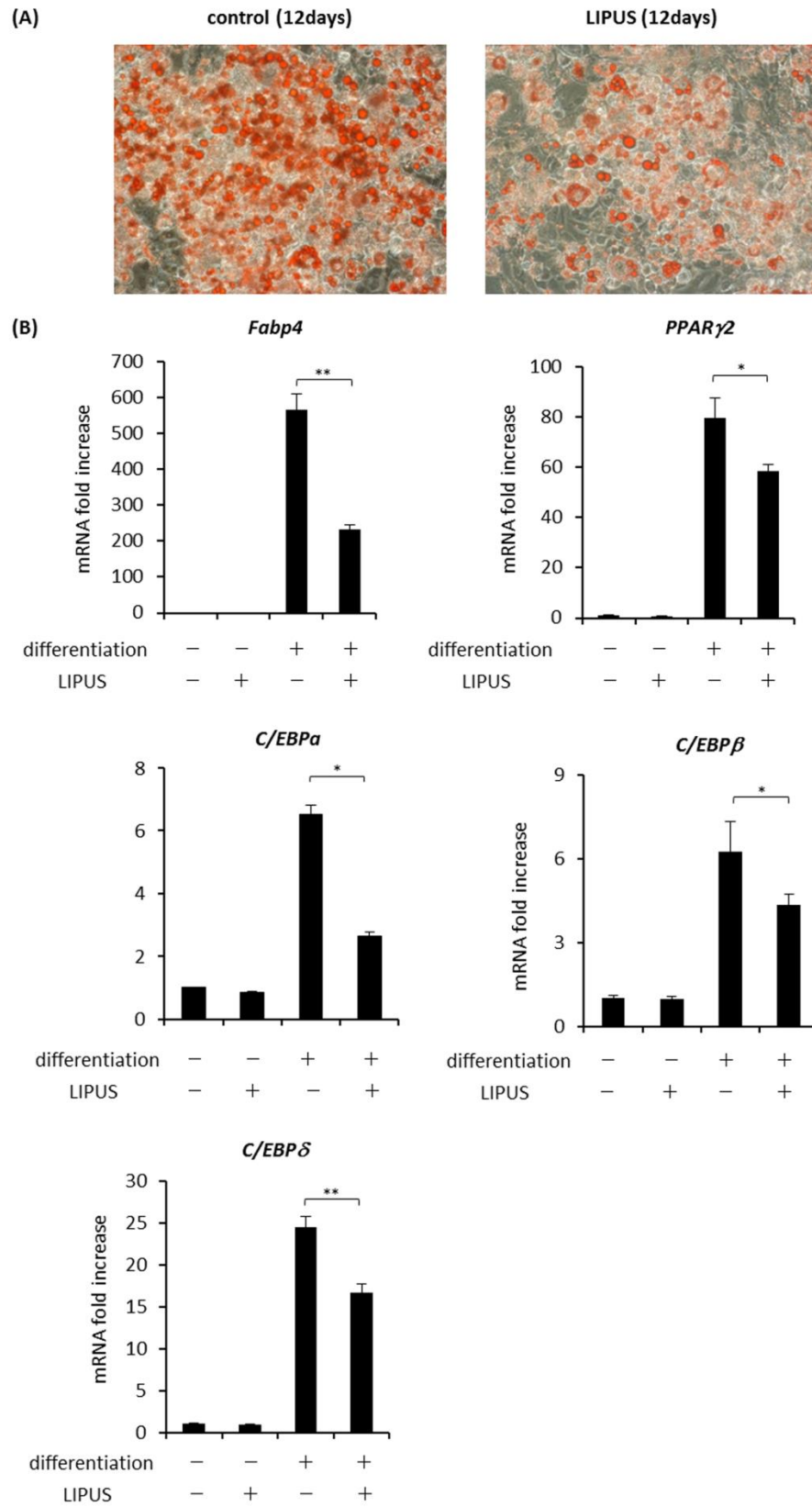


Figure 3

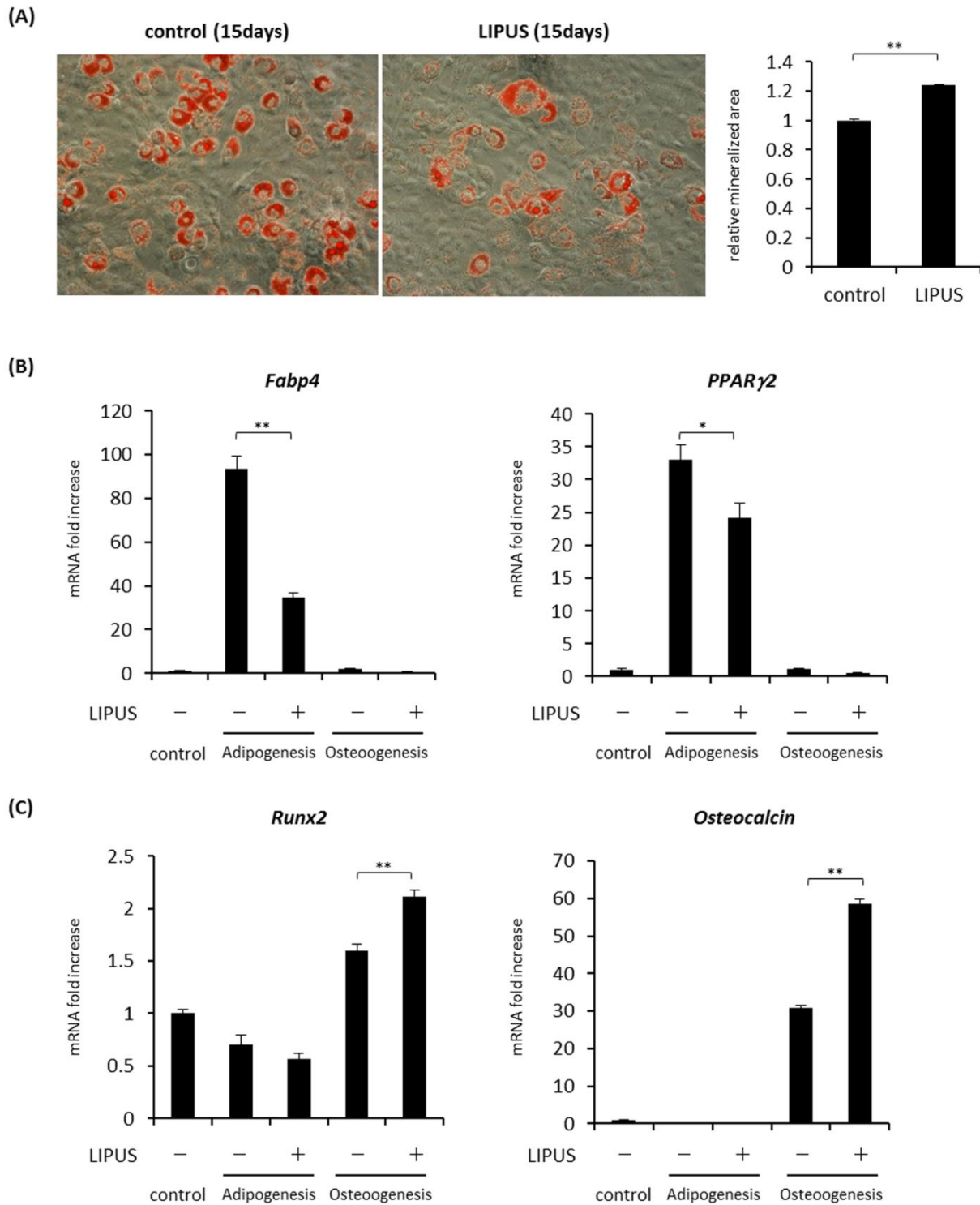


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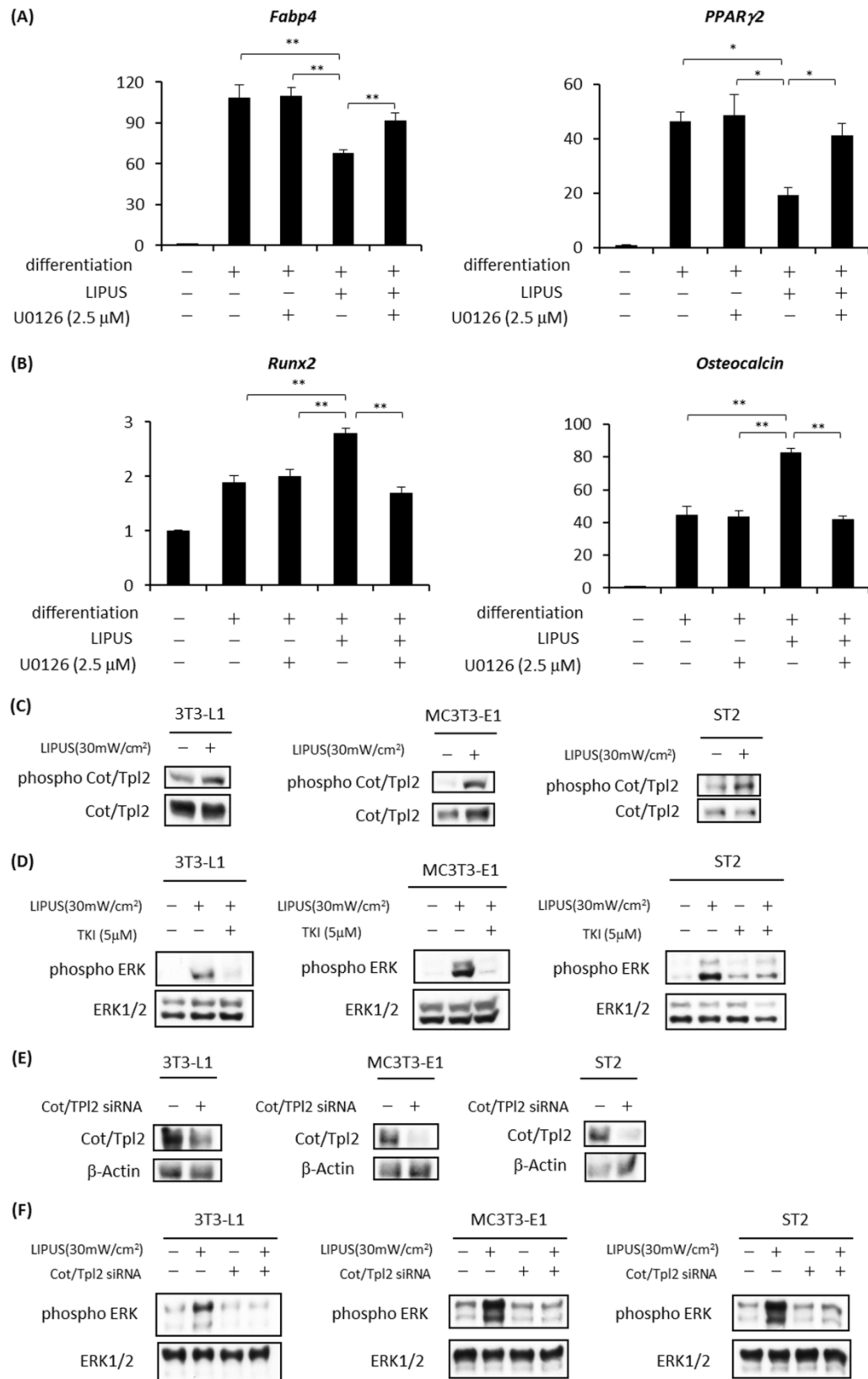


Figure 5

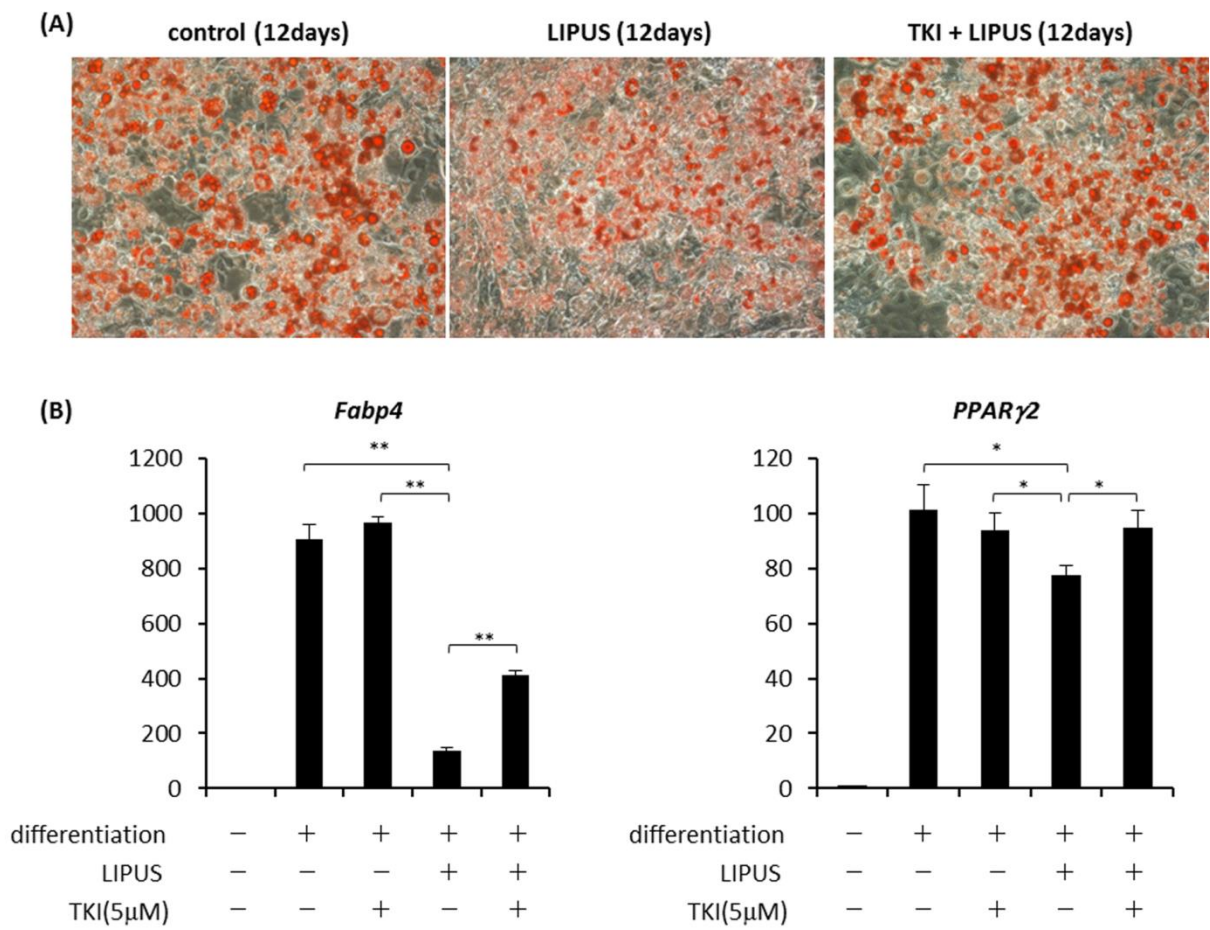


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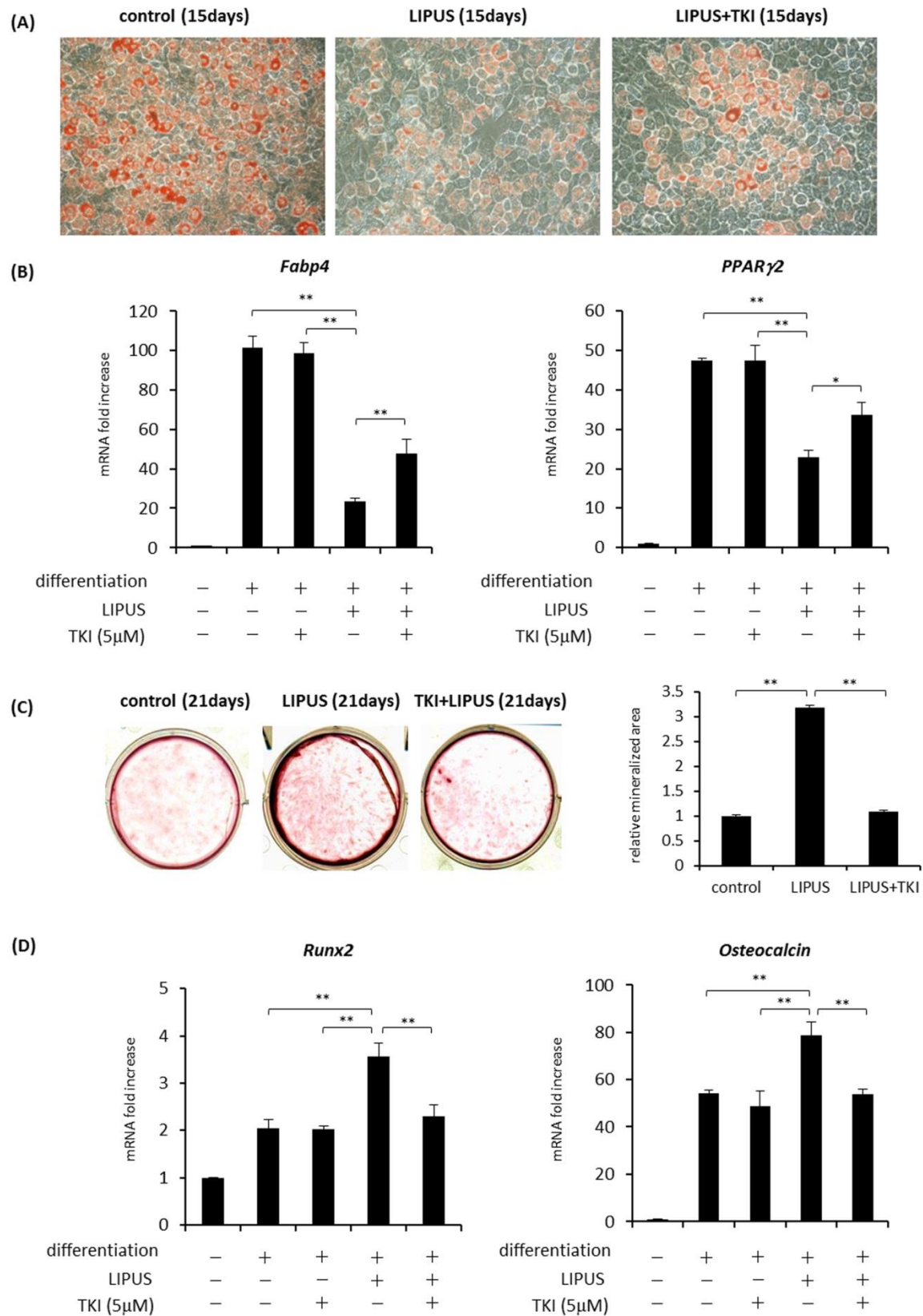


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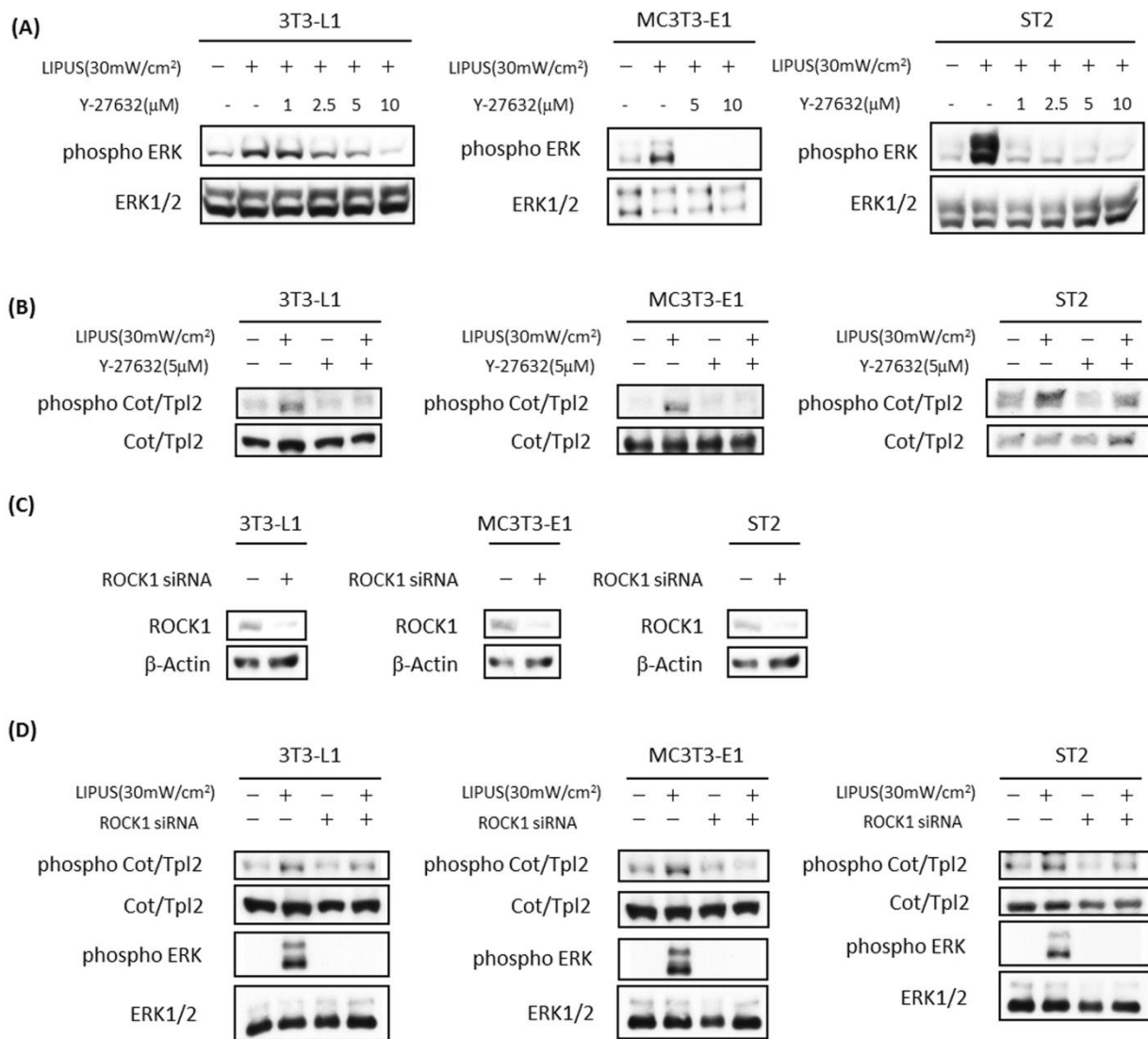


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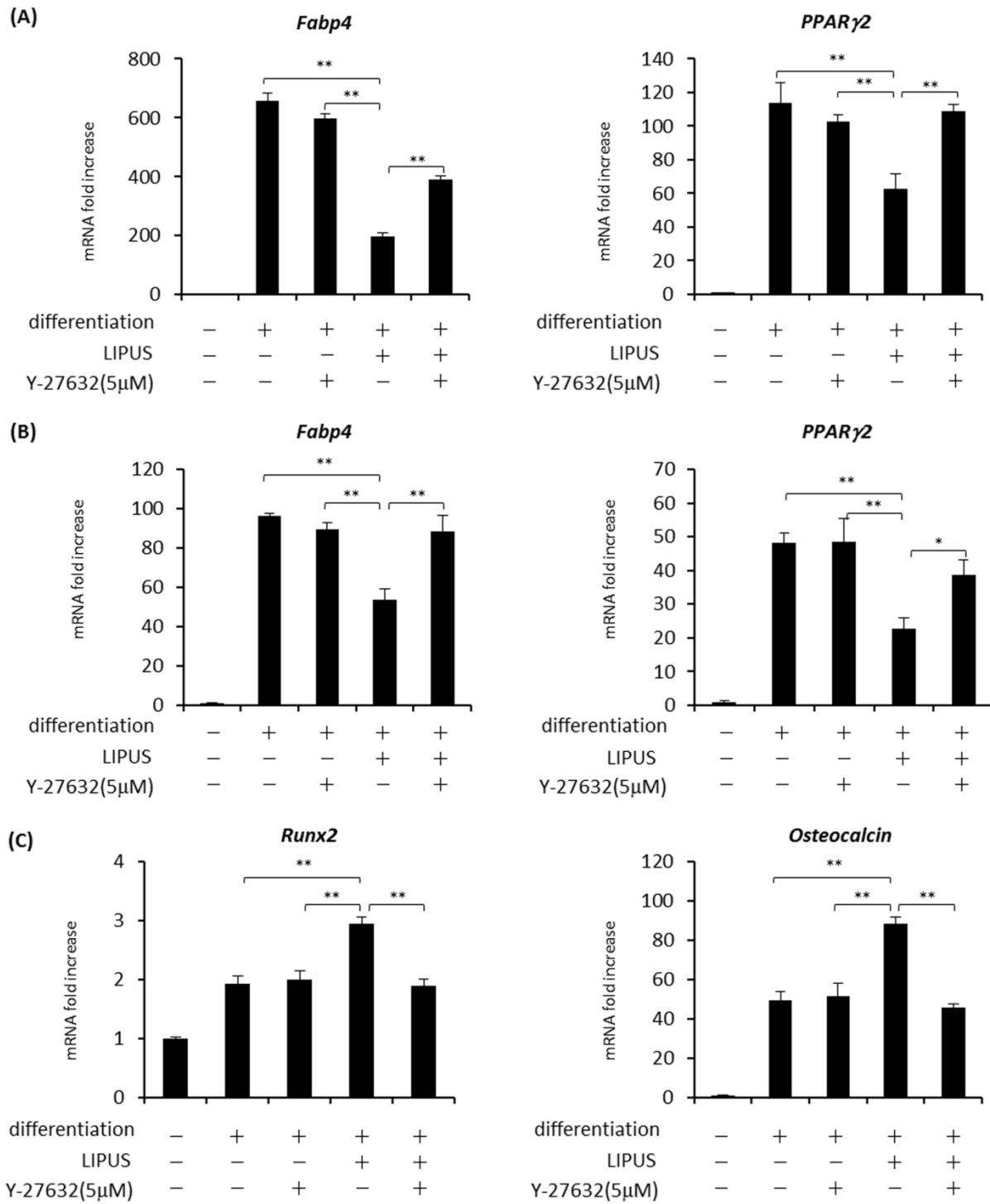


Figure 9

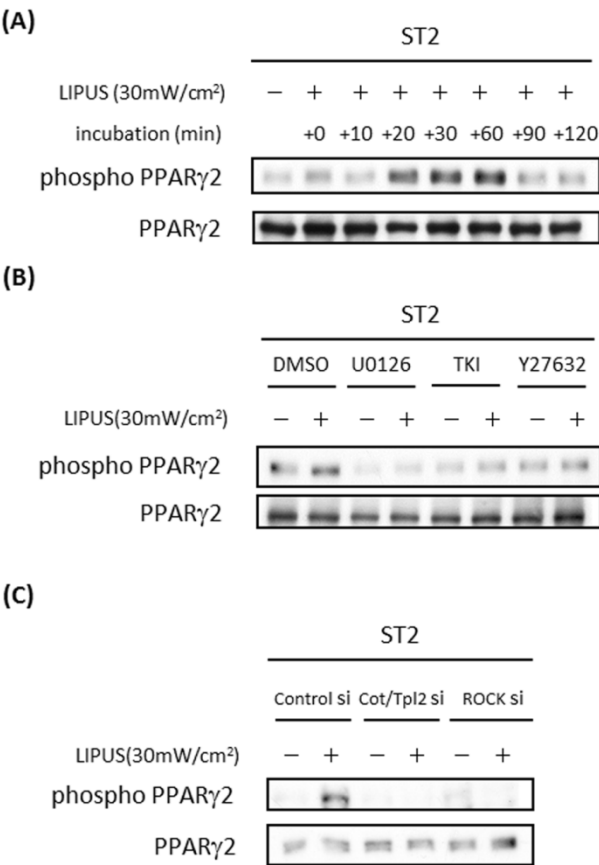
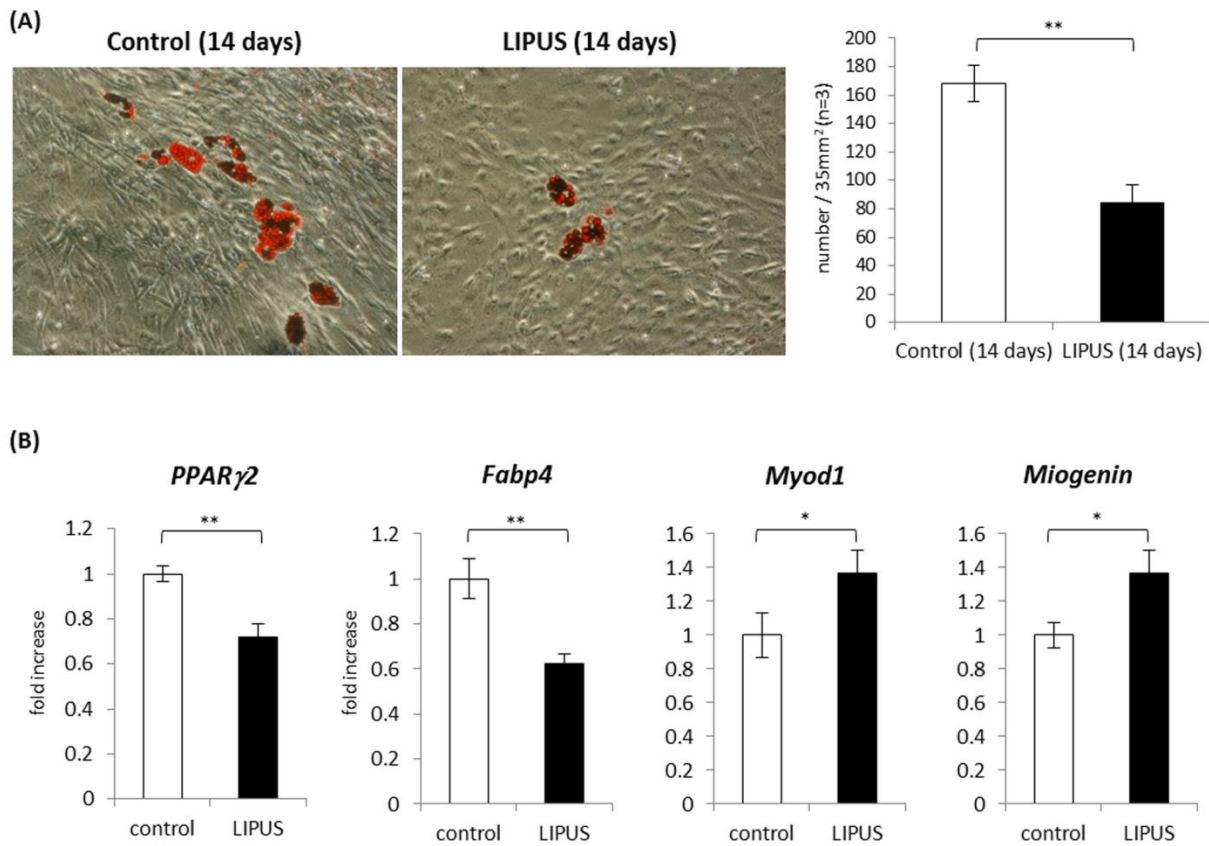


Figure 10



**Low-Intensity Pulsed Ultrasound (LIPUS) Influences the Multi-Lineage
Differentiation of Mesenchymal Stem and Progenitor Cell Lines through
ROCK-Cot/Tpl2-MEK-ERK Signaling Pathway**

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