Regulation of the osteogenic and adipogenic differentiation of bone marrow-derived stromal cells by extracellular uridine triphosphate: The role of P2Y2 receptor and ERK1/2 signaling

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Abstract. An imbalance in the osteogenesis and adipogenesis of bone marrow-derived stromal cells (BMSCs) is a crucial pathological factor in the development of osteoporosis. Growing evidence suggests that extracellular nucleotide signaling involving the P2 receptors plays a significant role in bone metabolism. The aim of the present study was to investigate the effects of uridine triphosphate (UTP) on the osteogenic and adipogenic differentiation of BMSCs, and to elucidate the underlying mechanisms. The differentiation of the BMSCs was determined by measuring the mRNA and protein expression levels of osteogenic- and adipogenic-related markers, alkaline phosphatase (ALP) staining, alizarin red staining and Oil Red O staining. The effects of UTP on BMSC differentiation were assayed using selective P2Y receptor antagonists, small interfering RNA (siRNA) and an intracellular signaling inhibitor. The incubation of the BMSCs with UTP resulted in a dose-dependent decrease in osteogenesis and an increase in adipogenesis, without affecting cell proliferation. Significantly, siRNA targeting the P2Y2 receptor prevented the effects of UTP, whereas the P2Y6 receptor antagonist (MRS2578) and siRNA targeting the P2Y4 receptor had little effect. The activation of P2Y receptors by UTP transduced to the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. This transduction was prevented by the mitogen-activated protein kinase inhibitor (U0126) and siRNA targeting the P2Y2 receptor. U0126 prevented the effects of UTP on osteogenic- and adipogenic-related gene expression after 24 h of culture, as opposed to 3 to 7 days of culture. Thus, our data suggest that UTP suppresses the osteogenic and enhances the adipogenic differentiation of BMSCs by activating the P2Y2 receptor. The ERK1/2 signaling pathway mediates the early stages of this process.

Introduction

Osteoporosis is a systemic disease characterized by low bone mass, increased bone fragility and susceptibility to fracture (1). Although the detailed pathological mechanisms remain unknown, previous studies have suggested that the structural abnormalities associated with osteoporotic bones are attributable to the dysfunction of bone marrow-derived stromal cell (BMSC) differentiation (2-6). BMSCs are self-renewable, multipotent stem cells with the capacity to differentiate into lineages of mesenchymal tissues, such as chondrocytes, osteoblasts and adipocytes, when cultivated under appropriate conditions using specific hormonal inducers or growth factors (7,8). Bone regeneration i s a complex process mediated by the close association between the activities of osteogenic and adipogenic progenitor cells, which both derive from BMSCs (7,8). The balance between the osteogenesis and adipogenesis of BMSCs can be disrupted. BMSCs have a reduced capacity to differentiate into osteoblasts, but an increased capacity for adipocyte differentiation. The increase in the proportion of fat in bone marrow subsequently induces the apoptosis of osteoblasts and the proliferation of osteoclasts, which results in further bone resorption and overall bone loss (9,10).

Mechanical loads on bone tissue increase bone formation and improve bone strength (11). The removal of mechanical stimuli during immobilization or in microgravity results in a rapid loss of bone mass, whereas the application of exogenous mechanical loading leads to increased bone formation in the modeling skeleton (12). The molecular mechanisms mediating the conversion of mechanical stimuli into biochemical signaling remain poorly understood. Previous studies have suggested that extracellular nucleotides, such as adenosine 5'-triphosphate (ATP) and uridine triphosphate (UTP), are soluble factors released in response to mechanical stimulation in different cell

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Abbreviations: UTP, uridine triphosphate; BMSCs, bone marrow-derived stromal cells; ERK1/2, extracellular signal-regulated kinase 1/2

Key words: uridine triphosphate, mesenchymal stem cells, osteogenesis, adipogenesis, purinergic P2Y2 receptor, extracellular signal-regulated kinase 1/2

types (13-15). Once released, extracellular nucleotides stimulate plasma membrane-localized nucleotide receptors: P2 receptors play a significant role in bone remodeling (16-18). Based on their molecular structure and activated signaling pathways, the P2 receptor family is divided into 2 subfamilies: the P2X and P2Y receptors (19). Currently, 7 P2X (P2X1-7) and 8 P2Y (P2Y1, 2, 4, 6, 11, 12, 13 and 14) receptors have been identified, each of which has been cloned, characterized and assigned distinct tissue expression patterns and pharmacology. P2XRs are ligand-gated ion channels, whereas P2YRs are G protein-coupled receptors (20). The pattern of expression of different P2R subtypes on cell membranes influences the activity and the effects of nucleotides (20).

In particular, UTP stimulates the P2Y2 and P2Y4 receptors. In addition, UTP is hydrolyzed to uridine diphosphate (UDP), which acts on the P2Y6 receptor (21). UTP has been implicated in the regulation of osteogenesis in a number of cell types, including rat osteoblasts and human BMSCs. However, these results appear to demonstrate certain discrepancies: for example, UTP, but not ADP or UDP, promotes alkaline phosphatase (ALP) activity and bone mineralization, and increases the mRNA levels of ALP, bone morphogenetic protein (BMP)-2, BMP-4, BMP-5 and bone sialoprotein (BSP) through the P2Y2 receptor in rat primary ostoblasts (22). UTP and UDP facilitate the osteogenic differentiation of cells which is indicated by an increase in ALP activity through the activation of UDP-sensitive P2Y6 receptors, but not through P2Y2 and P2Y4 receptors, in the BMSCs of post-menopausal women (17). A component of the inhibitory action of ATP and UTP on bone mineralization could thus be mediated directly by PPi, independently of P2 receptors (23). UTP, signaling via the P2Y2 receptor on osteoblasts, blocks bone mineralization and bone formation (24,25). The various physiological effects of UTP and P2Y receptors have also been studied in adipocytes. UTP and UDP have been shown to increase intracellular Ca^{2+} levels in brown adipocytes (26). UTP has also been shown to effectively elevate the intracellular calcium levels in white adipocytes via the P2Y2 receptor, and the activation of the P2Y11 receptor inhibited leptin production and stimulated lipolysis (27). Based on the above-mentioned evidence, we hypothesized that UTP and P2Y receptors play a critical role in the osteogenic and adipogenic differentiation of BMSCs. Thus far, little is known about the expression of P2Y receptor subtypes and the potential effects of UTP on the differentiation process of rat BMSCs. Thus, in the present study, we aimed to determine whether UTP regulates the osteogenic and adipogenic differentiation of BMSCs and if so, to identity which of the P2Y receptors mediate such a response, and to elucidate the underlying mechanisms.

Materials and methods

Reagents. Unless otherwise stated, all cell culture reagents were purchased from Gibco (Paisley, UK). TRIzol reagent was supplied by Invitrogen (Grand Island, NY, USA). ALP kits, alizarin red S, Oil Red O, UTP and MRS2578 (P2Y6 receptor antagonist; 1 μM was added to the cell cultures 1 h prior to UTP treatment) were obtained from Sigma-Aldrich (St. Louis, MO, USA). U0126 [a mitogen-activated protein kinase inhibitor (MAPK; 5 μM were added to the cell cultures 1 h prior to UTP treatment)] was procured from Beyotime Institute of Biotechnology (Shanghai,

China). Lipofectamine 3000 was purchased from Invitrogen. Rat mesenchymal stem cell (MSC) adipogenic and osteogenic differentiation medium were purchased from Cyagen Biosciences Inc. (Santa Clara, CA, USA).

BMSC culture. Rat BMSCs were isolated from 4-week-old male Sprague-Dawley (SD) rats and expanded in accordance with previously published techniques (28). All animal experiments were approved by the Animal Care and Use Committee for Teaching and Research, of Huazhong University of Science and Technology (Wuhan, China). The cells were maintained in expansion medium, consisting of Dulbecco's modified Eagle's medium/F12 (1:1) and 10% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin and 100 U/ml streptomycin, in a humidified atmosphere containing 5% CO_2. Upon reaching confluence, the cells were detached with 0.25% trypsin (Boster Inc., Wuhan, China) and passaged at a ratio of 1:2. BMSCs of passages 3-5 were used in the experiments. Cells maintained in expansion medium were used as undifferentiated cells. To induce osteogenic differentiation, the cells were cultured in osteogenic differentiation medium (10 nM dexamethasone, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate in expansion medium) supplemented with UTP for 21 days. The medium was changed every 3 days.

CCK-8 proliferation assay. The cells were seeded in 96-well plates at a density of $2x10^3$ cells/well, and divided into 4 groups as follows: the control (without any treatment), and the cells treated with 5, 25 and 125 μM UTP, respectively. Each group comprised 5 sub-wells. Cell proliferation was assessed by CCK-8 (Beyotime Institute of Biotechnology) assay, after processing for 0, 24, 48 and 72 h. Briefly, 10 μl CCK-8 solution were added to each well followed by incubation in the dark for 1.5 h and the absorbance was then read using a microplate reader (Sunrise RC; Tecan, Mannedorf, Switzerland) at 450 nm.

Total RNA extraction and quantitative PCR (qPCR). Total RNA was extracted using TRIzol reagent. The purity and concentration of the RNA samples were determined spectroscopically. First-strand cDNA was synthesized from 3 μg RNA, using an EasyScript First-Strand cDNA synthesis super mix kit (TransGen Biotech Co., Ltd., Beijing, China) and used for qPCR. The expression of runt-related transcription factor 2 (RUNX2), ALP and osteopontin (OPN) was quantified using a Bio-Rad MyiQ2 sequence detection system and TransStart Eco Green qPCR SuperMix (TransGen Biotech Co., Ltd.). The primers were synthesized by Invitrogen, and their sequences are listed in Table I. The reactions were incubated at 95°C for 30 sec, followed by 40 cycles of 94°C for 5 sec and 60°C for 35 sec. The relative expression of gene-specific products was analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to the corresponding GAPDH values.

ALP staining and quantification. The cells were seeded at a density of 10^5 cells/well in 35-mm plastic dishes with or without UTP (125 μM) in osteogenic medium. After 7 days, ALP staining was performed using ALP kits. Briefly, after discarding the medium, the cells were gently washed with PBS 3 times and fixed with 4% paraformaldehyde for 15 min at 4°C. The cells were washed with deionized water and then

Table I. List of specific primers used in the present study.

Gene	Forward (5'→3')	Reverse (5'→3')
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
RUNX2	GCACCCAGCCCATAATAGA	TTGGAGCAAGGAGAACCC
PPARγ	CCTTTACCACGGTTGATTTCTC	GGCTCTACTTTGATCGCACTTT
ALP	CAAGGACCAACTACAACCA	AGGGAAGGGTCAGTCAGGTT
OPN	CCTGGACCTCATCAGCATTT	GGAGAC AGGAGGCAAGG
Adipsin	CACGTGTGCGGTGGCACCCTG	CCCCTGCAAGTGTCCCTGCGGT
Fabp4	GCGTAGAAGGGGACTTGGTC	TTCCTGTCATCTGGGGTGATT

RUNX2, runt-related transcription factor 2; ALP, alkaline phosphatase; OPN, osteopontin; PPARγ, peroxisome proliferator-activated receptor γ; FABP4, fatty acid binding protein 4

stained with naphthol AS-MX phosphate for 30 min in the dark and washed 3 times with PBS. Images were acquired using a light microscope (Eclipse Ti; Nikon, Tokyo, Japan). Image-Pro Plus 5.0 was used to analyze the quantity of the dyed areas.

Alizarin red S staining and quantification. The cells were cultured in osteogenic medium in 35-mm plastic dishes for 21 days with or without UTP (125 μM). Briefly, the cells were washed with PBS and fixed with 4% paraformaldehyde, for 30 min at room temperature. After washing with deionized water, the fixed cells were stained with 2% alizarin red S (pH 4.2) in deionized water. After 20 min, the cells were washed with deionized water and observed under a light microscope (Eclipse Ti; Nikon). Image-Pro Plus 5.0 was used to quantify the nodule areas.

Oil Red O staining and quantification. To induce adipogenic differentiation, the BMSCs were seeded at 2×10^4 cells/cm^2 on 35-mm plastic dishes and grown for 3 days in adipogenic induction medium (Cyagen Biosciences, Inc.) containing additional SD rat MSC adipogenic differentiation basal medium A, FBS, insulin, glutamine, rosiglitazone, dexamethasone, 3-isobutyl-1-methylxanthine and penicillin/streptomycin, followed by 1 day in adipogenic maintenance medium containing SD rat MSC adipogenic differentiation basal medium A, FBS, insulin, glutamine and penicillin/streptomycin (1 cycle). Both steps were repeated up to day 21 (indicated as the 5th cycle), when the cell culture was terminated for Oil Red O staining. UTP (125 μM) was added to the culture medium every 3 days. Briefly, the cells were washed with PBS and fixed with 4% paraformaldehyde, for 30 min at room temperature. After washing with PBS, the fixed cells were stained with Oil Red O in deionized water. After 20 min, the cells were washed with PBS twice and observed under a light microscope (Eclipse Ti; Nikon). Image-Pro Plus 5.0 was used to quantify the nodule areas.

Western blot analysis. The cells were lysed using the protein extraction reagent RIPA supplement, with protease and phosphatase inhibitor and phenylmethylsulfonyl fluoride (all from Beyotime Institute of Biotechnology). Cell homogenates were sonicated for 5 min and protein concentrations from the lysates were determined by BCA protein assay (Boster Inc.). Prior to loading, total protein samples were denatured by incubation at 95°C for 5 min in the presence of 5X reducing sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 10% β-mercaptoethanol and 0.1% bromophenol blue). Thirty micrograms of protein sample were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. Following incubation in 5% BSA at room temperature, the membranes were incubated with rabbit anti-total-ERK1/2 antibody (1:1,000; Cat. no. 4695P; Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit anti-p-ERK1/2 antibody (1:1,000; Cat. no. 4377S; Cell Signaling Technology, Inc.), rabbit anti-total-JNK antibody (1:1000; Cat. no. 9252S; Cell Signaling Technology, Inc.), rabbit anti-p-JNK antibody (1:1,000; Cat. no. 4668T; Cell Signaling Technology, Inc.), rabbit anti-total-p38 antibody (1:1,000; Cat. no. 8690P; Cell Signaling Technology, Inc.), mouse anti-p-p38 antibody (1:1,000; Cat. no. 9216S; Cell Signaling Technology, Inc.), mouse anti-GAPDH antibody (1:5,000; Cat. no. BM1623; Boster Inc.), mouse anti-RUNX2 antibody (1:400; Cat. no. ab76596; Abcam, Cambridge, UK), rabbit anti-ALP antibody (1:1,000; Cat. no. ab133602; Abcam), mouse anti-OPN antibody (1:500; Cat. no. ab69498; Abcam), rabbit anti-PPARγ antibody (1:400; Cat. no. ab133602; Abcam), rabbit anti-FABP4 antibody (1:1,000; Cat. no. ab92501; Abcam), goat anti-adipsin antibody (1:400; Cat. no. sc12403; Santa Cruz Biotechnology, Inc., CA, USA) at 4°C overnight. Anti-mouse horseradish peroxidase (HRP)-conjugated IgG (1:5,000; Cat. no. 7076P2) and anti-rabbit HRP-conjugated IgG (1:5,000; Cat. no. 7074P2; both from Cell Signaling Technology, Inc., Beverly, MA, USA) were used as the secondary antibodies. The immunostained protein bands were detected by chemiluminescence. Protein levels were determined by normalizing to GAPDH.

Gene silencing by small interfering RNA (siRNA). Scrambled siRNA, P2Y2 siRNA and P2Y4 siRNA were designed and synthesized by RiboBio (Guangzhou, China). According to the manufacturer's instructions (RiboBio and Invitrogen), BMSCs were seeded on 6-well plates at a density of 5×10^4 cells/well in normal medium and grown to 70-90% confluence prior to transfection. A transfection mixture containing Lipofectamine 3000 (7.5 μl/well; Invitrogen) and 100 nM siRNA targeting P2Y2 or P2Y4 receptor or scrambled siRNA

(all from RiboBio) sequence was prepared in 250 μl Opti-MEM (Invitrogen) and incubated for 5 min at room temperature. After being washed with Opti-MEM, the cells were incubated with the transfection mixture in 1,750 μl Opti-MEM for 4 days at 37°C. The effects of gene silencing were determined by qPCR at 24, 48, 72 and 96 h following transfection.

Immunofluorescence staining. Rat BMSCs were seeded onto sterile 1-cm-diameter discs in 24-well trays at 2.5×10^4 cells/disc for 5 days. The discs were removed and fixed with 4% para-formaldehyde for 15 min at room temperature; after washing 3 times with PBS, the cells were blocked for 1 h with PBS containing 5% FBS. The samples were then incubated overnight at 4°C with anti-P2Y2 (1:200; Cat. no. ab10270; Abcam) primary antibody, washed 3 times with PBS and incubated for 1 h at room temperature with the goat anti-rabbit Cy3-labelled secondary antibody solution (1:500; Cat. no. AB6939; Abcam) diluted in blocking solution. The cells were further counterstained with DAPI (1:3,000; Cat. no. AR1176; Boster Inc.). Fluorescent images were obtained with an inverted fluorescent microscope (Eclipse Ti; Nikon). Cy3 absorbance and emission at 552 and 565 nm, and DAPI absorbance and emission at 360 and 460 nm, respectively were assessed.

Statistical analysis. The results are expressed as the means ± SD (n=3). Statistical comparisons were performed using one-way ANOVA, followed by Tukey's post hoc test, which was carried out using SPSS 19.0 software. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

UTP decreases the expression of osteogenic-related markers and increases the expression of adipogenic-related markers in undifferentiated BMSCs. BMSCs were cultured in expansion medium, with various concentrations of UTP (5-125 μM), for 7 days. The expression levels of osteogenic- and adipogenic-related markers were measurd by qPCR and western blot analysis. As shown in Fig. 1A and B, UTP decreased RUNX2, ALP and OPN mRNA and protein expression and increased peroxisome proliferator-activated receptor γ (PPAR γ), fatty acid binding protein 4 (FABP4) and adipsin mRNA and protein expression in a dose-dependent manner.

The number of cells was measured using a CCK-8 kit, in order to examine the effects of UTP on the proliferation of BMSCs. As shown in Fig. 1B, no significant differences in the numbers of cells were observed (Fig. 1C), indicating that UTP affected the differentiation potential of BMSCs and was not cytotoxic. The concentration of 125 μM UTP did not affect the proliferation of the BMSCs, but had the maximum effect on differentiation, and was therefore used in the following experiments.

UTP inhibits the osteogenic and enhances the adipogenic differentiation of stimulated BMSCs. To determine the effects of UTP on the differentiation of BMSCs into osteoblasts, the cells were cultured in osteogenic medium, with or without UTP (125 μM) treatment for 7 days. The expression levels of osteogenic- and adipogenic-related markers were measured by qPCR and western blot analysis. As shown in Fig. 2A and B,

the osteogenic medium-induced upregulation of RUNX2, ALP and OPN mRNA and protein expression was significantly reduced by UTP treatment.

Extracellular matrix mineralization was measured using alizarin red staining when the BMSCs were treated with UTP in osteogenic medium for 21 days. The BMSCs formed abundant characteristic nodules in the control cultures, and the number of these nodules was markedly decreased in the UTP-treated cultures (Fig. 2C). Thus, our data suggest that ALP plays a key role in bone mineralization. The effects of UTP on ALP expression were also examined at 7 and 21 days of culture in osteogenic medium. UTP inhibited ALP expression compared to the controls both at 7 and 21 days of culture (Fig. 2C).

We then examined the effects of UTP on the adipogenic differentiation of BMSCs. The BMSCs were cultured in adipogenic medium with or without UTP. As shown in Fig. 2D and E, UTP increased the mRNA and protein expression levels of PPAR, FABP4 and adipsin compared to the control on day 7. After 3 weeks of adipogenic differentiation, numerous lipid drops were observed in the intracellular spaces of the differentiated cells. The lipid content of the cells was demonstrated by Oil Red O staining; lipid accumulation was more evident in the UTP-treated cells compared to the untreated cell cultures (Fig. 2F).

UTP regulates the osteogenic and adipogenic differentiation of BMSCs via the P2Y2 receptor. UTP stimulates the P2Y2 and P2Y4 receptors. In addition, UTP is hydrolyzed to UDP, which acts on the P2Y6 receptor (28). Thus, to identify which of the P2Y receptor subtypes is associated with the effects of UTP on BMSCs, we added the selective P2Y6 receptor antagonist, MRS2578 (1 μM) (29,30), to the cell cultures 1 h prior to UTP treatment. We observed that the effects of UTP + MRS2578 on osteogenic- and adipogenic-related gene expression were similar to those observed wtih UTP treatment (Fig. 3A), suggesting that the effects of UTP on BMSCs are mediated via P2Y2 or P2Y4 receptors rather than the P2Y6 receptor.

A number of previous studies have reported that UTP inhibits bone mineralization *in vitro* via the P2Y2 receptor in rat primary osteoblasts (23-25,31). To determine whether the effects induced by UTP are mediated through the P2Y2 receptor, we employed siRNAs targeting the P2Y2 and P2Y4 receptor genes. The P2Y2 and P2Y4 siRNA silencing efficiency were both 85% at 2 days following transfection (data not shown). To examine the effects of P2Y2 and P2Y4 siRNA on BMSC differentiation, the cells were incubated with the transfection mixture for 2 days. Subsequently, the transfection mixture was replaced with osteogenic or adipogenic medium with UTP (125 μM) and the cells were cultured for 5 days. Total RNA extraction and qPCR were then performed to determine the expression of osteogenic- and adipogenic-related genes. We found that P2Y2 siRNA prevented the downregulation of osteogenic-related gene expression and the upregulation of adipogenic-related genes induced by UTP, whereas P2Y4 receptor siRNA did not have a marked effect on the expression of these genes (Fig. 3B).

Effect of BMSC differentiation on the expression of P2Y2 receptor. We first confirmed that the P2Y2 receptor was expressed by rat BMSCs using immunofluorescence
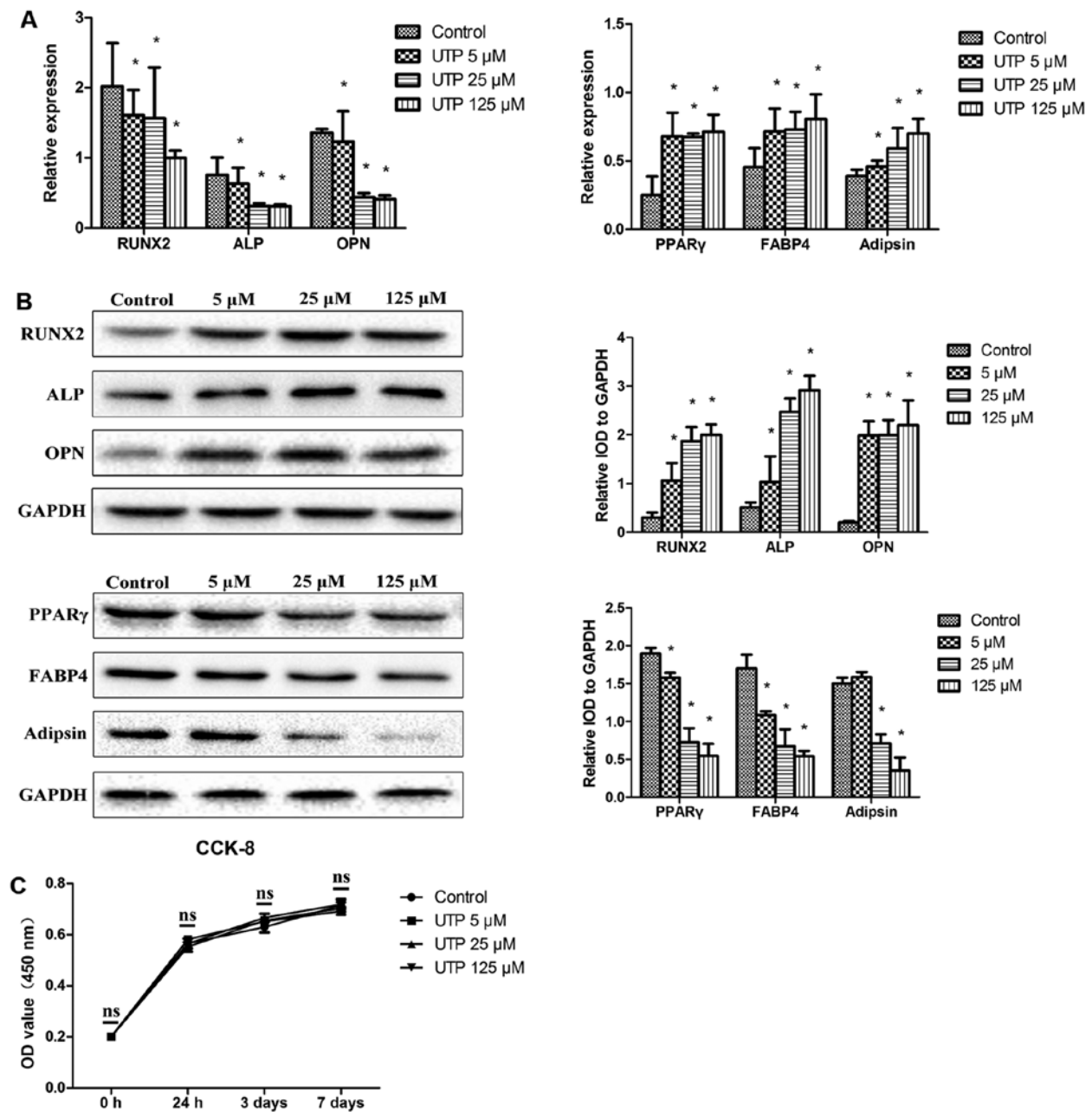
Figure 1. Effect of uridine triphosphate (UTP) on osteogenic- and adipogenic-related gene expression in undifferentiated bone marrow-derived stromal cells (BMSCs). UTP decreased osteogenic-related (A) gene and (B) protein expression and increased adipogenic-related (A) gene and (B) protein expression in a dose-dependent manner. Results are expressed as percentages with respect to GAPDH expression. Data represent the means ± SD, n=3, *P<0.05 vs. control. (C) A CCK-8 assay was used to measure the proliferation of BMSCs at different time points (0 and 24 h, and 3 and 7 days). UTP did not affect the proliferation of BMSCs. Data represent the means ± SD, n=3, not significant (ns) P>0.05 vs. control. IOD, integrated optical density.

staining (Fig. 4A). P2Y2 receptor expression was evaluated by qPCR, in both stimulated (osteogenic and adipogenic medium) and unstimulated (expansion medium) BMSCs, with or without UTP treatment, on days 0, 3 and 7. As shown in Fig. 4B, in the cells cultured in adipogenic medium, the mRNA expression of the P2Y2 receptor increased on days 3 and 7 compared to day 0. However, the mRNA expression of the P2Y2 receptor in the cells cultured in osteogenic medium decreased on days 3 and 7 compared to day 0. Furthermore, UTP treatment failed to affect the expression of the P2Y2 receptor in both the osteogenic and adipogenic media. P2Y2 receptor expression remained relatively unaltered in the unstimulated cells.

UTP activates the ERK1/2 signaling pathway in BMSCs. MAPKs have been shown to be important in the differentiation of a number of cell types, including BMSCs (32-38). Thus, in this study, we examined the effects of treatment with UTP on the phosphorylation of 3 members of the MAPK family in rat BMSCs. As shown in Fig. 5A, of the 3 MAPK isoforms, only ERK1/2 was significantly phosphorylated following treatment with UTP. Maximal ERK1/2 activation was observed at 5 min and remained activated for up to 60 min. Furthermore, U0126 (5 μM), a selective MAPK inhibitor, completely abolished the phosphorylation of ERK1/2 induced by UTP (Fig. 5B). P2Y2 receptor siRNA was also used to suppress
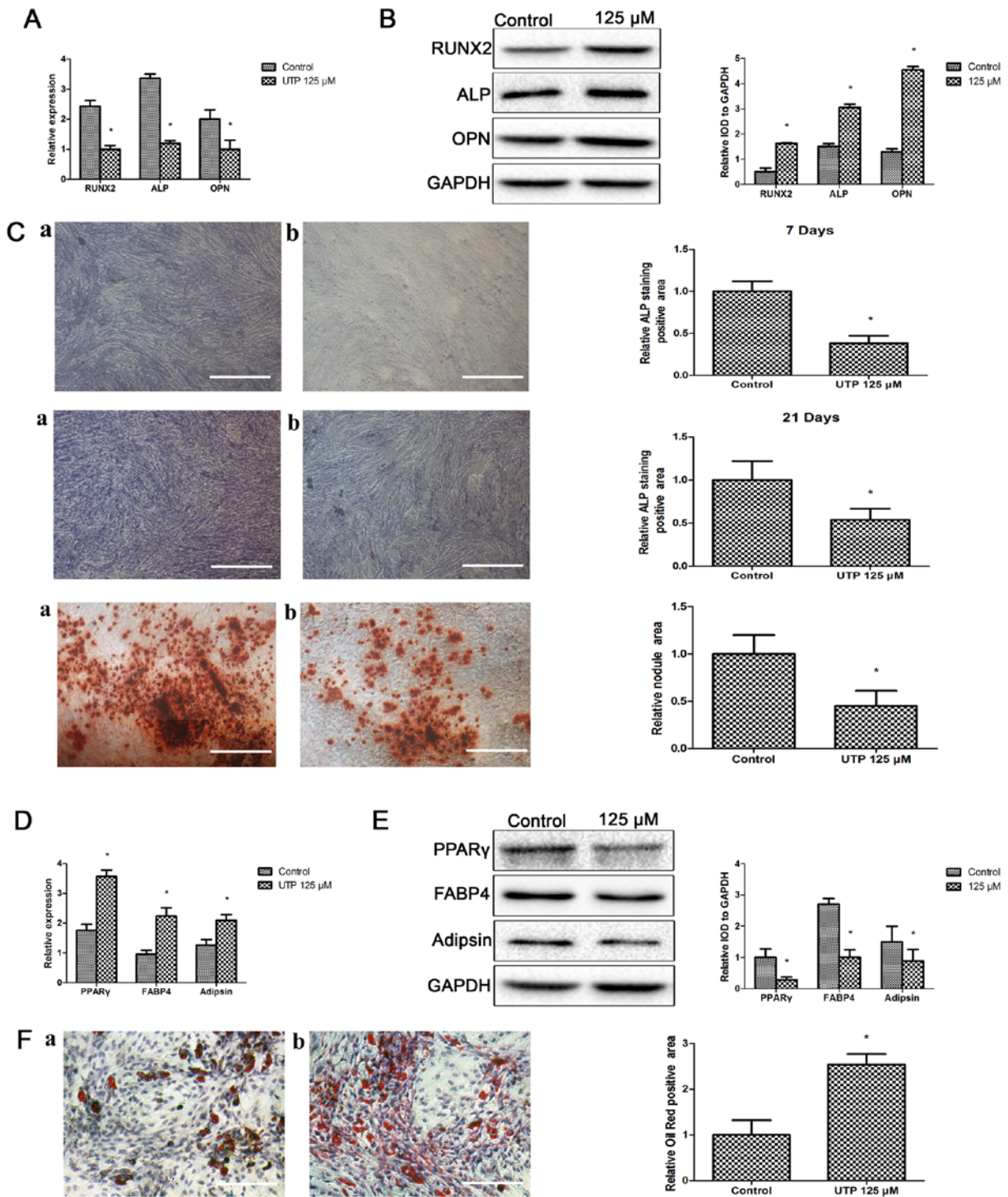
Figure 2. Uridine triphosphate (UTP) inhibits the osteogenic and enhances the adipogenic differentiation of stimulated bone marrow-derived stromal cells (BMSCs). Osteogenic medium-induced upregulation of osteogenic-related (A) mRNA and (B) protein expression was significantly reduced by UTP (125 μM) treatment. Results are expressed as percentages with respect to GAPDH expression. Data represent the means ± SD, n=3, *P<0.05 vs. control. (C) Representative images and semi-quantitative analysis of alkaline phosphatase (ALP) staining [7 days (upper panel) and 21 days (middle panel)] and alizarin red S staining (lower panel). Bar, 50 μm; panels a, control; panels b, UTP 125 μM. Data represent the means ± SD, n=3, *P<0.05 vs. control. UTP increased adipogenic-related (D) mRNA and (E) protein expression compared to control in BMSCs cultured in adipogenic medium. Results are expressed as percentages with respect to GAPDH expression. Data represent the means ± SD, n=3, *P<0.05 vs. control. (F) Representative images and semi-quantitative analysis of Oil Red O staining. Bar, 50 μm; panel a, control; panel b, UTP 125 μM. Data represent the means ± SD, n=3, *P<0.05 vs. control.

receptor expression in order to examine its role in ERK1/2 phosphorylation. UTP-induced ERK1/2 phosphorylation was attenuated by approximately 90% when the cells were treated with P2Y2 siRNA (Fig. 5B).

Role of the ERK1/2 signaling pathway in the osteogenic and adipogenic differentiation of BMSCs induced by UTP. To determine the role of ERK1/2 in the differentiation of BMSCs, we assessed the effects of UTP on osteogenic- and adipogenic-
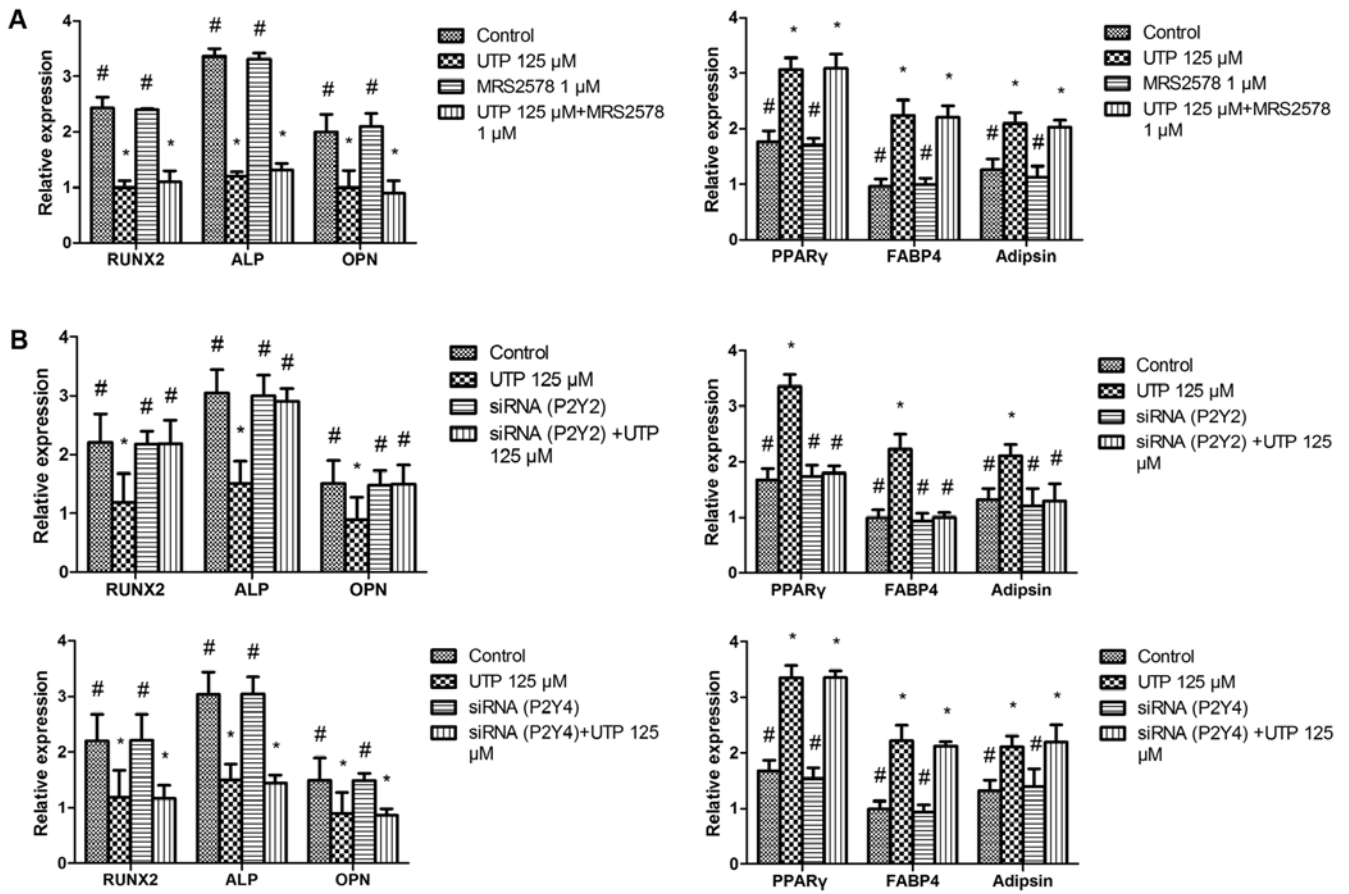
Figure 3. Uridine triphosphate (UTP) regulates the osteogenic and adipogenic differentiation of bone marrow-derived stromal cells (BMSCs) via the P2Y2 receptor. (A) Effects of UTP + MRS2578 on osteogenic- and adipogenic-related gene expression were similar to those observed with UTP treatment. Results are expressed as percentages with respect to GAPDH expression. Data represent the means ± SD, n=3, *P<0.05 vs. control, #P<0.05 vs. UTP (125 μM). (B) siRNA suppressed P2Y2 receptor expression, and prevented the downregulation of osteogenic gene expression and upregulation of adipogenic-related genes induced by UTP, whereas P2Y4 receptor siRNA did not have a marked effect on the expression of these genes. Results are expressed as percentages with respect to GAPDH expression. Data represent the means ± SD, n=3, *P<0.05 vs. control, #P<0.05 vs. UTP (125 μM).
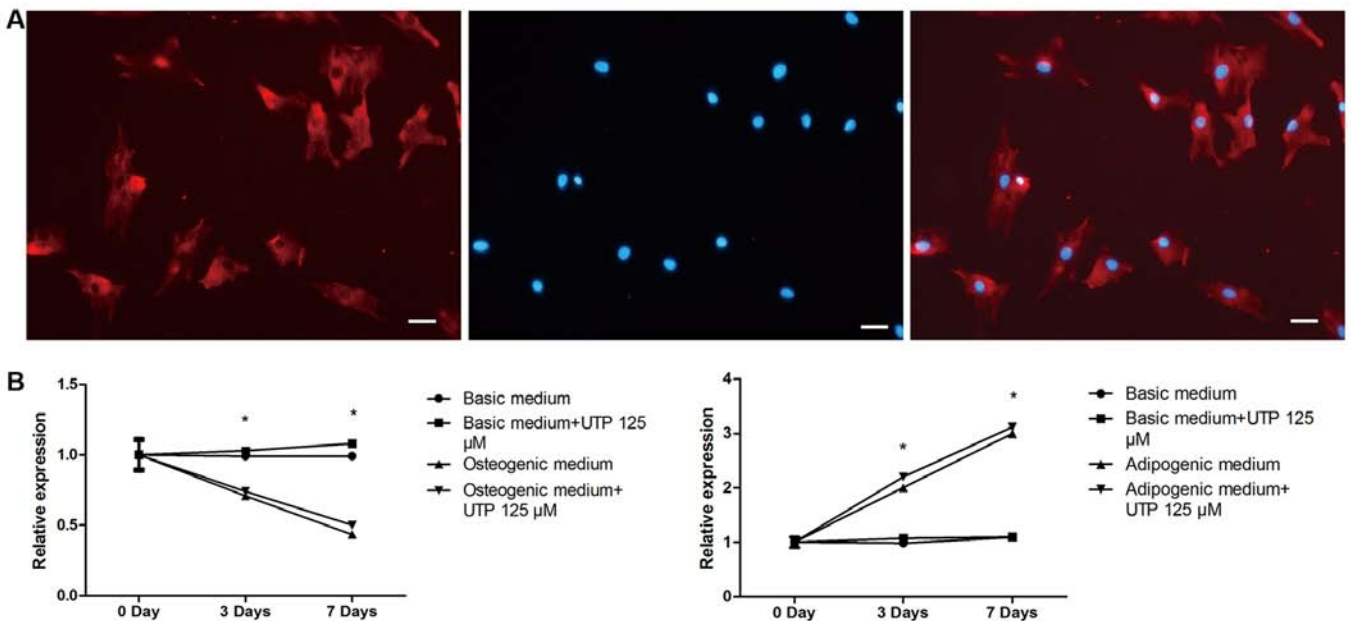

Figure 4. Effect of bone marrow-derived stromal cell (BMSC) differentiation on the expression of P2Y2 receptor. (A) Expression of the P2Y2 receptor by rat BMSCs. Cells were fixed and stained with anti-P2Y2 conjugated-secondary antibodies (red), and cell nuclei were counterstained with DAPI (blue). Scale bar, 50 μm. (B) qPCR of P2Y2 receptor expression during osteogenic and adipogenic differentiation of BMSCs. Results are expressed as percentage with respect to GAPDH expression. Data represent the means ± SD, n=3, *p<0.05 vs. expansion medium.
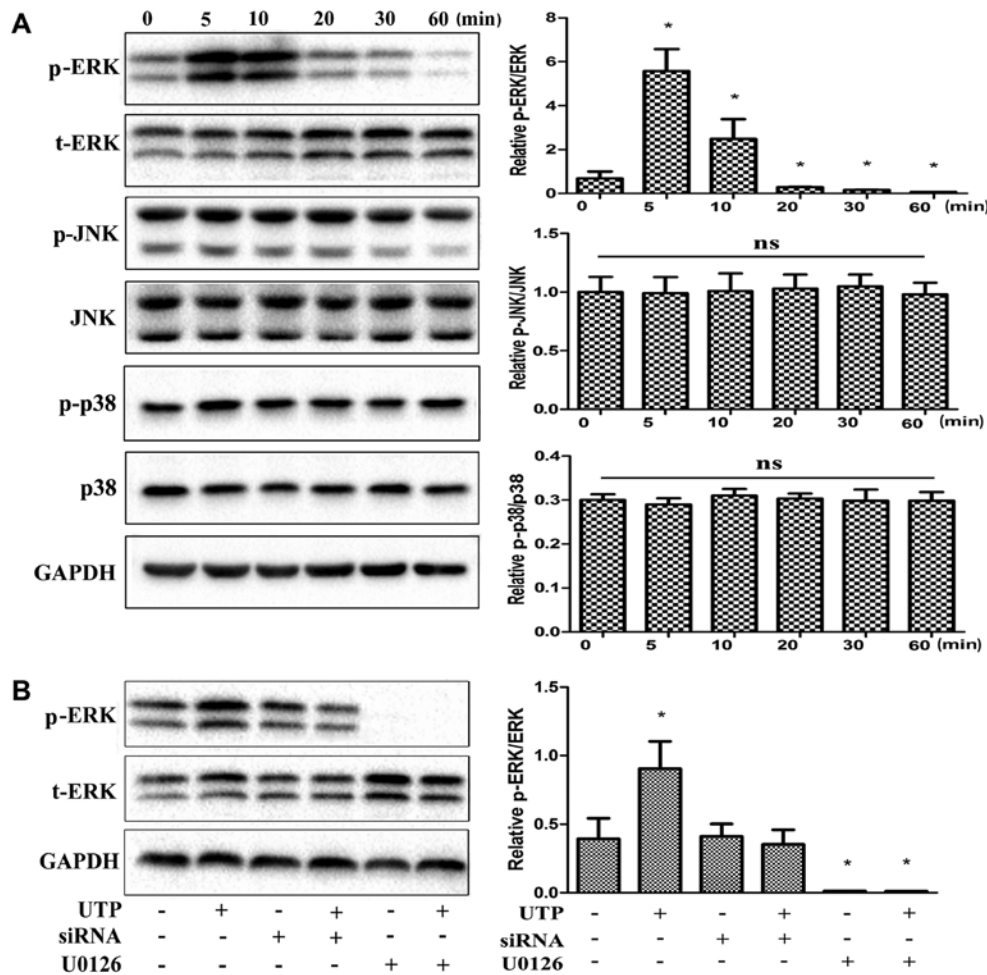
Figure 5. Representative images and semi-quantitative analysis of uridine triphosphate (UTP)-induced activation of MAPK signaling pathway. (A) Of the 3 MAPK isoforms, only extracellular signal-regulated kinase 1/2 (ERK1/2) was significantly phosphorylated following treatment with UTP. Data represent the means ± SD, n=3, *P<0.05 vs. 0 min, not significant (ns), P>0.05 vs. 0 min. (B) The UTP-induced ERK1/2 phosphorylation was blocked by U0126 and P2Y2 receptor siRNA. Data represent the means ± SD, n=3, *P<0.05 vs. (UTP-, siRNA- and U0126-).

related gene expression in BMSCs in the presence or absence of U0126 (5 μM). UTP significantly inhibited osteogenic-related gene expression and increased adipogenic-related gene expression. These effects were significantly attenuated by U0126 in the cells cultured for 24 h (Fig. 6A). However, U0126 failed to prevent the effects induced by UTP on 3- and 7-day-old cell cultures (Fig. 6B). In addition, U0126 and U0126 + UTP induced the downregulation of osteogenic-related genes and the upregulation of adipogenic-related genes compared with the controls on days 3 and 7.

Discussion

This study demonstrated that, in relation to BMSCs, UTP inhibited osteogenesis and enhanced adipogenesis, without affecting cell growth. Furthermore, we confirmed that these effects induced by UTP acted via P2Y2 receptors. We also demonstrated that ERK1/2 signaling played differential roles in the differentiation of BMSCs. These findings help to broaden our understanding of the role of purinergic receptors, particularly the P2Y2 receptor, in the functional differentiation of BMSCs.

It has previously been demonstrated that extracellular UTP exerts profound inhibitory effects on the bone mineralization

mediated by P2Y receptors in primary osteoblasts (23-25). In the present study, we analyzed the molecular pathway activated by extracellular UTP during the BMSC differentiation process. Our findings revealed that the ability of UTP to modulate the differentiation of BMSCs into the osteoblastic lineage is relevant at a physiological level, as the number of mineralized nodules and ALP expression induced by culture in osteogenic medium were markedly reduced in UTP-treated cell cultures. Furthermore, UTP also decreased osteogenic-related mRNA and protein expression in unstimulated BMSCs, indicating that UTP may prevent precursor cells from differentiating into osteoblasts, and also inhibited extracellular matrix mineralization in osteoblasts differentiated from BMSCs. Few studies have, however, reported different results. It has previously been noted that UTP stimulated BMP gene expression and mineralization in rat primary osteoblasts (22), and enhanced the osteogenic differentiation of BMSCs obtained from post-menopausal women (17). We suggest that differences between cell types, osteogenic culture systems and variable methods for detecting osteogenic differentiation contribute to explaining the discrepancies. For example, first, osteoblasts from Wistar rats and BMSCs from post-menopausal women were used in these studies, whereas in the present study, we used BMSCs from
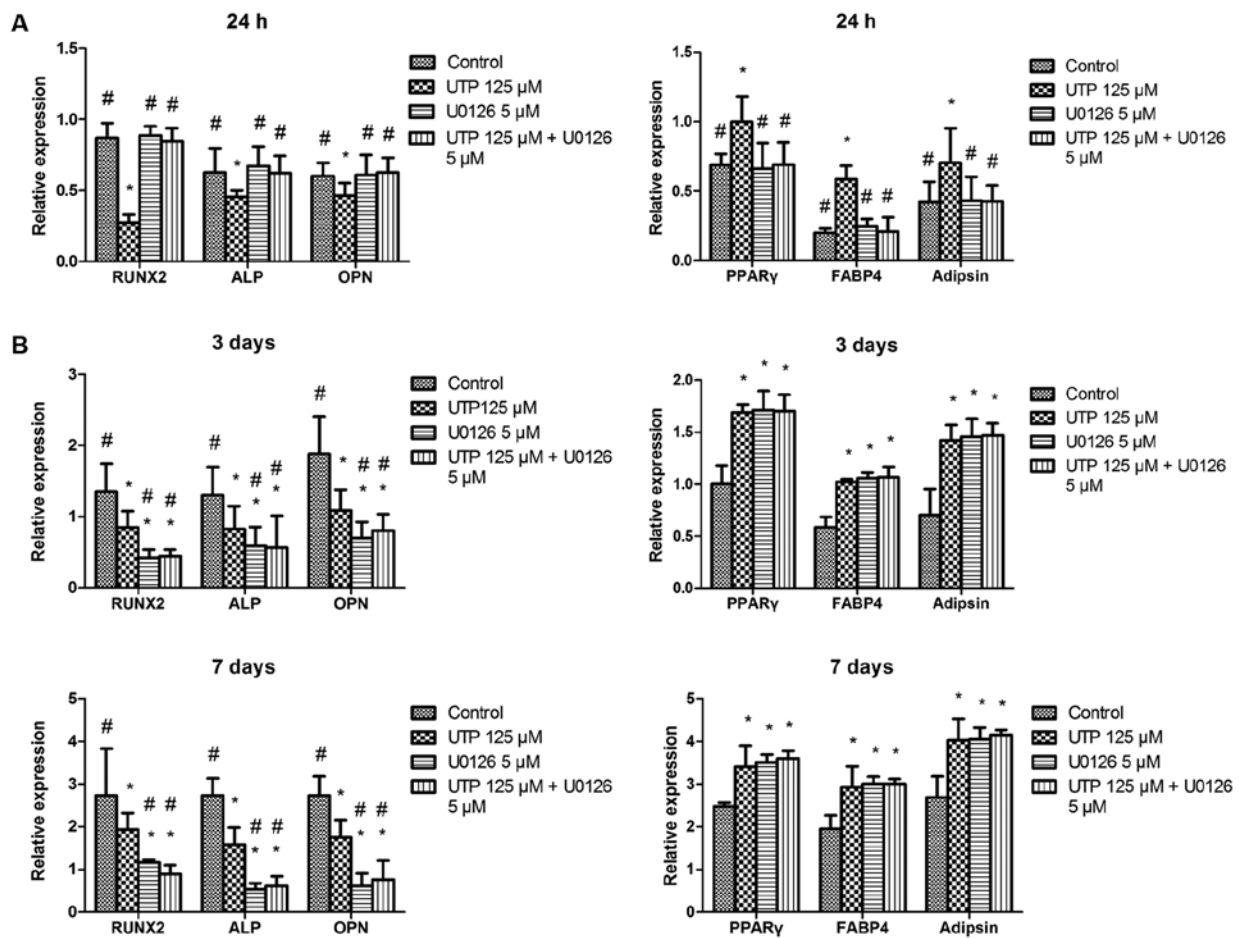
Figure 6. Role of extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in osteogenic and adipogenic differentiation of bone marrow-derived stromal cells (BMSCs) induced by uridine triphosphate (UTP). (A) Effect of UTP on osteogenic- and adipogenic-related gene expression was significantly attenuated by U0126 following 24 h of culture. Results are expressed as percentages with respect to GAPDH expression. Data represent the means ± SD, n=3, *P<0.05 vs. control, #P<0.05 vs. UTP (125 μM). (B) U0126 failed to prevent the effect induced by UTP on 3- and 7-day-old cultures. Results are expressed as percentages with respect to GAPDH expression. Data represent the means ± SD, n=3, *P<0.05 vs. control, #P<0.05 vs. UTP (125 μM).

SD rats. Second, in this previous study, dexamethasone was not added to the osteogenic medium during osteoblast differentiation (22). It has been noted that dexamethasone downregulates Runx2, a transcription factor required for osteoblast differentiation (39), and inhibits Wnt/β-catenin, a signaling pathway involved in the osteoblast differentiation of mesenchymal progenitor cells (40). Third, the detection indices and methods used in these studies differed from those used in our research. Any one or a combination of these factors explains the differences in these studies.

The differentiation of BMSCs into the osteoblastic or adipogenic lineages is not an independent process: molecular components promoting one cell fate inhibit the mechanisms governing the differentiation of the alternative lineage (5,41). This is also the case for UTP. In the present study, UTP increased the expression of adipogenic-related markers in a dose-dependent manner. Furthermore, the formation of lipid droplets, another specific characteristic of adipogenic differentiation, was also increased by UTP treatment. Consistent with our study, Ciciarello et al reported that extracellular UTP increased the mRNA expression of PPAR in human BMSCs (42). However, a previous study reported that ATP and adenine compounds, but not other nucleotides (UTP, UDP,

CTP, GTP, ITP and diadenosine tetraphosphate), stimulated lipogenesis in adipocytes (43). In this previous study, the lipogenesis of adipocytes derived from the epididymal fat pads of male Wistar rats was detected by measuring the incorporation of D-[3-^{3}H]glucose in toluene extractable lipids, but without mRNA or protein data on any key adipogenic related genes, such as PPARγ. Adipogenesis consists of integrated cascades that involve several transcription factors. The initial step of adipogenesis is the lineage commitment of MSC followed by the expansion of preadipocytes. PPARγ is a critical component in adipogenesis, as indicated by the fact that loss of PPARγ expression in murine embryonic fibroblasts leads to a complete absence of adipogenic capacity (44). MSCs differentiate into adipocytes when they express PPARγ, which enhances the expression of adipogenic genes (45). Although it is not clear whether the apparent discrepancy is a matter of the detection method or of the different cell types used in experiments, we consider that the results of qPCR and immunohistochemical staining in our study strongly indicate that UTP is a positive stimulus of adipogenesis in rat BMSCs.

UTP stimulates the P2Y2 receptor and, following degradation to UDP, also acts on the P2Y6 receptor (21). In this study, we demonstrated that UTP regulated the differentiation of

BMSCs into adipogenic cells, but not osteoblasts by activating the P2Y2 receptor rather than P2Y4 or P2Y6 receptors. Studies have previously reported the effects of P2Y receptors on the osteogenic and adipogenic differentiation of BMSCs, with varying results. For example, Ciciarello *et al* reported that ATP stimulated adipogenic differentiation of human BMSCs, mainly acting through P2Y1 and P2Y4 subtypes (42). Conversely, adenosine resulting from ATP degradation increased BMSC osteogenic differentiation, by activating the A2B adenosine-specific receptor subtype (42). Zippel *et al* reported that ATP, but not UTP partially compensated for the potent inhibitory effects on matrix mineralization induced by suramin and PPADS (P2 receptor antagonists) in human BMSCs, indicating that P2Y2 and P2Y4 receptors had no effect on osteogenesis (18). UTP, but not ATP, partly compensated for the decrease in formation of lipid droplets induced by PPADS, thus suggesting the involvement of P2Y4 receptor (18). In these two studies, the authors used several agonists and antagonists of P2 receptors to identify which receptors were activated in the processes of osteogenic and adipogenic differentiation. Indeed, many of the P2 receptor subtypes are still lacking potent and selective synthetic agonists and antagonists. These reagents are considered effective stimulators and inhibitors of P2 receptors. Thus, to delineate the role of each P2 receptor in osteogenesis and adipogensis of BMSCs, several issues need to be addressed, including specific agonists and antagonists of P2 receptors, and gene knockout models.

In the present study, pharmacological approaches revealed that UTP enhanced the differentiation of BMSCs into adipocytes, but not osteoblasts by stimulating the ERK1/2 signaling pathway in a P2Y2R-dependent manner. In agreement with our data, it has been previously reported that the activation of P2Y2 receptors by extracellular nucleotides is responsible for the phosphorylation of ERK1/2 in osteoblasts. For example, Costessi *et al* reported that extracellular ATP and UTP stimulate the ERK1/2-dependent activation of the transcription factor Runx2 via the P2Y2 receptor in the osteoblast-like HOBIT cell line (46). Katz *et al* reported that P2Y2 receptor stimulation by ATP in osteoblasts sensitizes mechanical stress-activated calcium channels, leading to calcium influx and fast activation of the ERK1/2 and p38 MAPK pathways (47). However, in studies on MAPK signaling and its role in the differentiation of BMSCs, the ERK1/2 pathway is widely reported as a positive regulator of osteogenesis (32,33,35,36) and a negative regulator of adipogenesis (37,38). To investigate the differences between our observations and previous studies, in the present study we employed U0126, a MAPK inhibitor. Consistent with these studies, we observed that the inhibition of ERK1/2 activation by U0126 inhibited osteogenic-related gene expression, and enhanced adipogenic-related gene expression in 3- and 7-day-old cultures. However, U0126 significantly attenuated the effects of UTP on these genes at 24 h of incubation. Our study on ERK1/2 modulation by UTP showed that purinergic activation rapidly stimulated MAPK phosphorylation in BMSCs. UTP-induced ERK1/2 activation reached a peak at 5 min and was weaker at 60 min. In view of this finding, we speculate that ERK1/2 is a branch or a feedback loop of the complex signaling pathways involved in the onset of differentiation of BMSCs. In other words, we suggest that UTP mediates the early stage of osteogenic and adipogenic

differentiation of BMSCs via activation of the ERK1/2 signaling pathway. We believe that the reverse effect of ERK1/2 signaling may be a possible interpretation for contrary views widely reported in the literature and may provide new insight into the molecular regulation of the osteogenic differentiation of rat BMSCs. The exact downstream components of the signaling pathway remain unknown. Thus, we have great interest in researching them in future studies.

Acknowledgements

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