Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 3764372, 10 pages
http://dx.doi.org/10.1155/2016/3764372

Research Article

Activation of the Extracellular Signal-Regulated Kinase Signaling Is Critical for Human Umbilical Cord Mesenchymal Stem Cell Osteogenic Differentiation

Chen-Shuang Li,[1,2] Zhong Zheng,[3] Xiao-Xia Su,[1,2] Fei Wang,[1,2] Michelle Ling,[4] Min Zou,[1,2] and Hong Zhou[1,2]

[1] *Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Research, College of Stomatology, Xi'an Jiaotong University, Xi'an, Shaanxi 710004, China*
[2] *Department of Orthodontics, College of Stomatology, Xi'an Jiaotong University, Xi'an, Shaanxi 710004, China*
[3] *Division of Growth and Development and Section of Orthodontics, School of Dentistry, University of California, Los Angeles, CA 90095, USA*
[4] *University of Chicago, Chicago, IL 60637, USA*

Correspondence should be addressed to Min Zou; zoumin@mail.xjtu.edu.cn and Hong Zhou; zhouhong@mail.xjtu.edu.cn

Received 22 October 2015; Revised 15 January 2016; Accepted 21 January 2016

Academic Editor: Martin Sebastian Staege

Human umbilical cord mesenchymal stem cells (hUCMSCs) are recognized as candidate progenitor cells for bone regeneration. However, the mechanism of hUCMSC osteogenesis remains unclear. In this study, we revealed that mitogen-activated protein kinases (MAPKs) signaling is involved in hUCMSC osteogenic differentiation *in vitro*. Particularly, the activation of c-Jun N-terminal kinases (JNK) and p38 signaling pathways maintained a consistent level in hUCMSCs through the entire 21-day osteogenic differentiation period. At the same time, the activation of extracellular signal-regulated kinases (ERK) signaling significantly increased from day 5, peaked at day 9, and declined thereafter. Moreover, gene profiling of osteogenic markers, alkaline phosphatase (ALP) activity measurement, and alizarin red staining demonstrated that the application of U0126, a specific inhibitor for ERK activation, completely prohibited hUCMSC osteogenic differentiation. However, when U0126 was removed from the culture at day 9, ERK activation and osteogenic differentiation of hUCMSCs were partially recovered. Together, these findings demonstrate that the activation of ERK signaling is essential for hUCMSC osteogenic differentiation, which points out the significance of ERK signaling pathway to regulate the osteogenic differentiation of hUCMSCs as an alternative cell source for bone tissue engineering.

1. Introduction

Although bone tissue has a high regenerative capacity, local endogenous cell numbers are often not adequate enough to reestablish tissue continuity or function in critical-sized defects [1–3]. Thus, there is a worldwide competition to develop engineered bone tissues to conquer this difficulty. However, after more than three decades of investigation, the success of bone tissue engineering is still limited [4]. One of the most critical obstacles is finding a suitable progenitor cell source. To date, human bone marrow mesenchymal stromal cells (hBMSCs) have been considered a native cell source and have been widely studied for osteogenic differentiation [5–7]. However several disadvantages, such as long derivation times, heterogeneous cell population, and variable potency [8, 9], markedly hinder the clinical application of hBMSCs for bone tissue engineering. Thus, alternative cell sources for bone tissue engineering are in high demand.

Human umbilical cord mesenchymal stem cells (hUCMSCs) isolated from Wharton's jelly of the umbilical cord have similar surface marker expression, high differentiation potential, low immunogenicity, and low tumorigenic risk as hBMSCs [10–17]. In contrast to hBMSCs that have to be harvested through invasive bone marrow aspiration, hUCMSCs are isolated from generally discarded tissue, umbilical cords, without ethical concerns [16, 18, 19], potential pain, and medical or surgical risks, such as bleeding and anesthesia [20]. Additionally, unlike hBMSCs and other stem cells isolated from adults, hUCMSCs share a high expansion capacity with fetal-derived stem cells [21]. These previous studies suggest that hUCMSCs may be a more suitable progenitor cell source than hBMSCs in a clinical setting [22], and thus multiple independent research groups have recruited hUCMSCs for various tissue regeneration, including bone tissue engineering [23–28]. However, the molecular mechanism of hUCMSC osteogenic differentiation has not been uncovered until now.

Mitogen-activated protein kinases (MAPKs) are a widely conserved family of serine-threonine protein kinases, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 [29]. Previous studies have indicated that distinct MAPK pathways independently modulate stem cell self-renewal and differentiation [30, 31]. For instance, Jaiswal et al. reported the regulatory role of the ERK pathway in hBMSCs osteogenic precursor commitment and differentiation [32]. However, conflicting results obtained from other investigations indicated whether activation of ERK signaling promotes stem cell osteogenic differentiation is a cell-type specific manner [32–36]. In this study, we intend to reveal the importance of MAPK signaling, especially the ERK pathway, in hUCMSC osteogenic differentiation.

2. Materials and Methods

2.1. Preparation of Human Umbilical Cord Mesenchymal Stem Cells. This study was ethically approved by the Xi'an Jiaotong University IRB. hUCMSCs were isolated and characterized in the manner previously described in the protocol [37]. Briefly, 15 cm long umbilical cord was rinsed with phosphate buffered saline (PBS) and cut into 1 mm^3 pieces and then digested with 0.1% type I collagenase (Sigma-Aldrich, USA) for 7–10 hours to form a homogeneous gelatinous solution. The gelatinous tissue solution was then mixed with 0.25% trypsin (Gibco, USA) at a ratio of 1 : 1 and incubated at 37°C for 30 min before being diluted in sterile PBS at a ratio of 1 : 10. After being centrifuged at 1200 rpm for 5 min, isolated hUCMSCs were resuspended in a maintenance medium consisting of DMEM/F12 (Hyclone, GE Healthcare Life Sciences, USA) supplemented with 15% fetal bovine serum (FBS; Hyclone, GE Healthcare Life Sciences, USA) and 1% penicillin and streptomycin (Gibco, USA) and seeded in cell culture dishes at a density of 1×10^4 cells/cm^2. Passage 3 hUCMSCs were characterized as $CD90^+/CD105^+/HLA\text{-}ABC^+/CD34^-/CD45^-/CD19^-/CD86^-/HLA\text{-}DR^-$ by cell flow cytometry [37]. The differentiation capacity of passage 3 hUCMSCs towards osteogenic, adipogenic, and chondrogenic lineages was verified accordingly [37].

2.2. Osteogenic Differentiation Induction of hUCMSCs. Passage 3 hUCMSCs were plated at $5 \times 10^4/cm^2$ and cultured in an osteogenic differentiation medium consisting of DMEM/F12 medium supplemented with 10% FBS, 10 nM dexamethasone (Sigma-Aldrich, USA), 10 mM β-glycerophosphate (Sigma-Aldrich, USA), and 50 μg/mL vitamin C (Sigma-Aldrich, USA) for 21 days. Medium was changed every three days.

2.3. Inhibit the Activation of ERK by U0126. To block the activation of ERK signaling, 25 μM U0126 (Calbiochem, Merck Millipore, USA), a specific inhibitor of ERK activation [38], was added to the osteogenic differentiation medium for the entire 21-day differentiation period. In a separate recovery experiment, hUCMSCs were only treated with 25 μM U0126 for the first 9 days, followed by a continual cultivation in osteogenic differentiation medium without U0126 until day 21.

2.4. Western Blot Analysis. 30 μg of protein lysates from hUCMSCs at days 0, 5, 9, 13, 17, and 21 were injected to 10% SDS-PAGE and transferred to PVDF membranes (Merck Millipore, USA), respectively. After blocking with 3% bovine serum albumin (BSA; Sigma-Aldrich, USA), membranes were probed with anti-phospho-ERK1/2 (1 : 500, Santa Cruz, CA, USA), anti-ERK1/2 (1 : 400, BIOSS, China), anti-phospho-JNK1 (1 : 500, Santa Cruz, CA, USA), anti-JNK1 (1 : 500, Santa Cruz, CA, USA), anti-phospho-p38 (1 : 100, Santa Cruz, CA, USA), anti-p38 (1 : 250, Santa Cruz, CA, USA), or anti-GAPDH (1 : 500, Santa Cruz, CA, USA) antibodies at 4°C overnight. After being washed three times with Tris Buffered Saline with Tween® 20 (TBST), the membranes were incubated with horseradish peroxidase- (HRP-) conjugated secondary antibodies (Donkey anti-Rabbit, 1 : 50,000, Abcam, USA, or Donkey anti-Mouse, 1 : 20000, Abcam, USA) at 25°C for 1 hour and developed with commercially available enhanced chemiluminescence reagent (Pioneer, China). Band intensities were determined using ImageJ software.

2.5. Quantitative Real-Time PCR. Total RNA were isolated by TRIzol® Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) followed by DNase treatment (Invitrogen, Life Technologies, Carlsbad, CA, USA). 1 μg RNA was used for reverse transcription with the SuperScript II Reverse Transcriptase Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) per the manufacturer's instruction. Real-time PCR was performed on the 7500 Real-Time PCR system (Applied Biosystems, USA) with Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas, USA). Primers used in this study are listed in Table 1. Concomitant *β-actin* was evaluated in separate tubes for each RT reaction as a housekeeping standard. Relative gene expression was analyzed by $\Delta\Delta$CT method [39].

2.6. Alkaline Phosphates Activity. Culture medium was collected and stored at −80°C until analysis. ALP activity in the culture medium was detected by a commercially available kit (Jiancheng biochemical, China) following the manufacturer's instructions. Briefly, each 30 μL culture medium was mixed with 500 μL buffer solution and 500 μL basic solution and then incubated at 37°C for 15 min with standards and a blank. After the incubation, 1500 μL of the chromogenic agent

Table 1: List of primers for quantitative real-time PCR.

Gene	Sequence
β-actin	5′-ATC GTG CGT GAC ATT AAG GAG AAG-3′ 5′-AGG AAG GAA GGC TGG AAG AGT G-3′
Collagen type I, α1 (COL 1A1)	5′-GTG AGA CAG GCG AAC AGG-3′ 5′-GAC CAG CAG GAC CAG AGG-3′
Osteocalcin (OCN)	5′-ACA CTC CTC GCC CTA TTG-3′ 5′-CAG CCA TTG ATA CAG GTA GC-3′
Osteopontin (OPN)	5′-GAA GTT TCG CAG ACC TGA CAT-3′ 5′-GTA TGC ACC ATT CAA CTC CTC G-3′
Bone Sialoprotein (BSP)	5′-CCC CAC CTT TTG GGA AAA CCA-3′ 5′-TCC CCG TTC TCA CTT TCA TAG AT-3′

was added to each sample. The absorbance at 520 nm was measured (OD value).

Additionally, ALP staining was performed as previously described [40]. In brief, hUCMSCs were fixed with an ice-cold 60% acetone-40% citrate solution and stained with diazonium salt with 4% naphthol AS-MX phosphate alkaline solution (Sigma-Aldrich, USA).

2.7. Alizarin Red Staining. After 21 days of cultivation, hUCMSCs were fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) for 15 min, washed with distilled water, and then stained with alizarin red solution (1% alizarin red and 2% ethanol in distilled water) for 15 min at room temperature. Excess stain was removed by washing with distilled water several times prior to photography.

2.8. Immunocytochemistry. After 21 days of cultivation, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) for 15 min and then washed with distilled water. After blocking with 3% BSA, the cells were incubated with anti-Osteocalcin (1 : 200, Santa Cruz, CA, USA) or anti-Osteopontin (1 : 100, Santa Cruz, CA, USA) antibodies at 4°C overnight. After being washed three times with PBS, the cells were incubated with FITC-conjugated Donkey anti-Rabbit secondary antibody (1 : 10,000, Abcam, USA) at 25°C for 1 hour. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, USA).

2.9. Imaging and Image Processing. Images were acquired at room temperature with the CellSens software (Olympus, America Inc., Center Valley, PA) on a fluorescence microscope (Olympus, America Inc., Center Valley, PA) using a 20x (dry HC Plan Apochromat, NA 0.17) objective lens.

2.10. Statistical Analysis. All experiments were performed for a minimum of six times. Statistical analysis was computed by SPSS 13.0 (IBM, USA). Statistical comparisons were performed using factorial analysis of variance (ANOVA), followed by an LSD test for comparing treatments between each two groups, and a p value less than 0.05 was considered statistically significant. Individual comparisons between two groups were determined by the Mann-Whitney test for nonparametric data.

3. Results

3.1. Osteogenic Differentiation of hUCMSCs. During the 21-day cultivation in the maintenance medium (Control group), hUCMSCs presented consistent levels of ALP activity (an early osteogenic commitment indicator) (Figure 1(a)) as well as transcription of *Osteocalcin* (a terminal osteogenesis marker) (Figure 1(b)). On the contrary, ALP activity of hUCMSCs cultured in the osteogenic differentiation medium (Osteogenic Stimulation (OS) group) significantly increased at day 5, peaked at day 9, and remained at high levels afterwards (Figure 1(a)). Meanwhile, gene expression of *Osteocalcin* of hUCMSCs continually increased in the OS group from day 9 to day 21 (Figure 1(b)). These data demonstrate that hUCMSCs have the capability of osteogenic differentiation; however, without suitable stimulation such as the osteogenic differentiation medium, hUCMSCs do not go through osteogenic differentiation spontaneously.

3.2. Diverse Activation of MAPK Signals during hUCMSC Osteogenic Differentiation. There are three major MAPKs in mammalians: ERK, JNK, and p38. Although all these three MAPKs are regulated by phosphorylation cascades [41], they may function differently in specific events [30, 31]. Particularly, during hUCMSCs osteogenic differentiation, activation of JNK and p38 was not induced by the osteogenic differentiation medium throughout the entire 21-day cultivation (Figure 2). This suggests that JNK and p38 signaling may not be essential for hUCMSC osteogenic differentiation. On the other hand, phosphorylation of ERK in hUCMSCs robustly increased from day 5 and peaked on day 9, followed by a decline stage thereafter (Figure 2).

3.3. Blocking hUCMSC Osteogenic Differentiation by ERK Activation Inhibitor. To reveal the significance of ERK activation in hUCMSC osteogenic differentiation, U0126, an inhibitor used to prevent ERK activation in BMSCs [42, 43], was added to the osteogenic differentiation medium through the entire 21-day cultivation period (Block group). In this

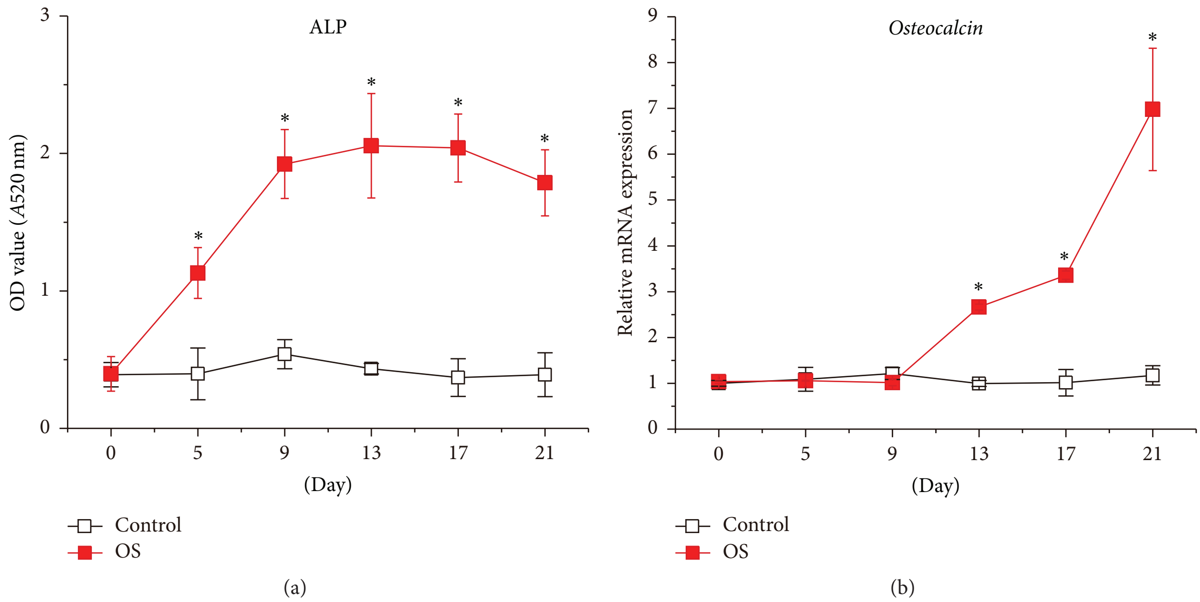

Figure 1: The osteogenic differentiation medium stimulates hUCMSC osteogenic differentiation. ALP activity (a) and transcription of *Osteocalcin* (b) were monitored in hUCMSCs during the 21-day cultivation in either the maintenance medium (Control) or the osteogenic differentiation medium (OS). Data were presented as Mean + SD; $^*p < 0.05$ ($N = 6$).

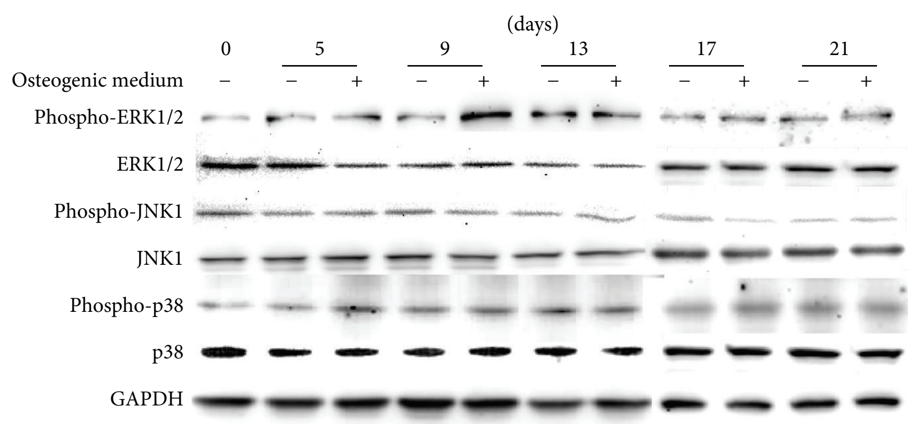

Figure 2: Western blotting revealed that the activation of MAPK signaling pathways was different during hUCMSC osteogenic differentiation. hUCMSCs were cultured in either the maintenance medium (−) or the osteogenic differentiation medium (+).

loss-of-function evaluation, U0126 completely eliminated the stimulation of the osteogenic differentiation medium on ERK activation in hUCMSCs (Figure 3).

As previously shown, ALP activity of hUCMSCs increased in the culture of osteogenic differentiation medium, which was completely prohibited by continuous inhibition of ERK activation by U0126 (Figure 4). In addition, transcription of osteogenic marker genes, such as *Type I Collagen*, *Osteopontin, Bone Sialoprotein*, and *Osteocalcin*, was also significantly induced in hUCMSCs by the osteogenic differentiation medium (Figure 5). However, this induction was fully abolished by continuous U0126 administration (Figure 5). Immunostaining against Osteopontin and Osteocalcin as well as Alizarin red staining for calcium deposition confirmed the inhibitory effects of the ERK activation inhibitor U0126 on hUCMSC osteogenic differentiation (Figure 6).

3.4. Rescuing hUCMSCs Osteogenic Differentiation by Removing U0126. A separate Recovery group, in which U0126 was removed from the osteogenic differentiation medium at day 9 of the cultivation, was employed to further confirm the importance of ERK activation in hUCMSC osteogenic differentiation. Western blotting showed that although ERK activation in hUCMSCs was effectively blocked by U0126 at day 9 (Figure 3(a)), the phosphorylation of ERK was induced by the osteogenic differentiation medium after removing the inhibitor in the Recovery group (Figure 3(b)).

Functionally, the activity of secreted ALP in the Recovery group was significantly higher than those of the Control or Block groups at the end of cultivation, even though it was not comparable to that of the OS group yet (Figure 4(b)). Similar trends were also detected in attached hUCMSCs by ALP staining (Figure 4(c)). Real-time PCR and immunostaining

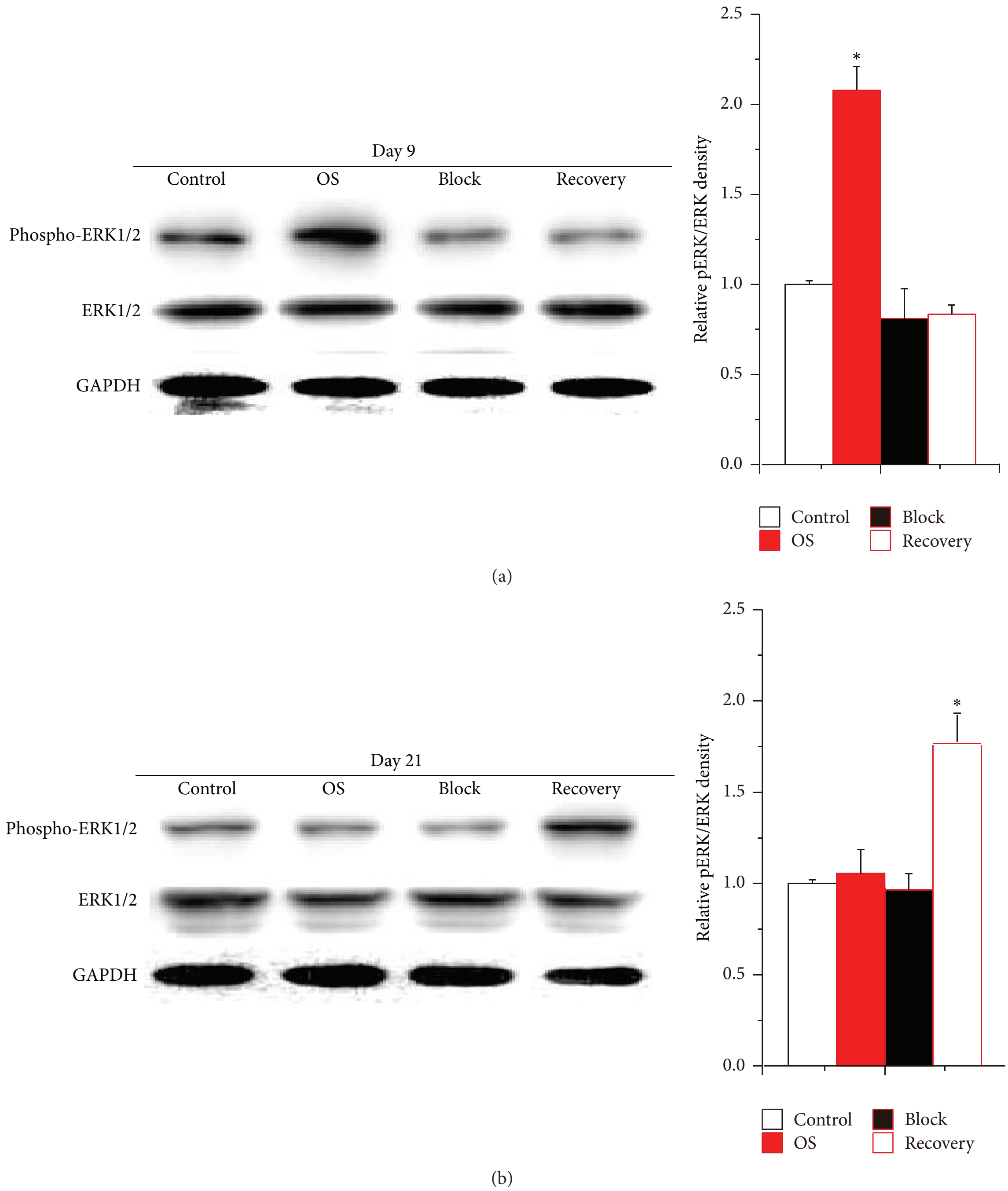

Figure 3: The activation of ERK signaling in hUCMSCs was blocked by U0126. Treated hUCMSCs with U0126 for 9 days completely prohibited the osteogenic differentiation medium-induced increase of ERK activation (a). After the inhibitor was removed, ERK activation of hUCMSCs of Recovery group was upregulated at day 21 (b). The relative densities were normalized to the Control group. Data were presented as Mean + SD; $^{*}p < 0.05$ ($N = 6$).

also showed the increase of osteogenic markers expression in the Recovery group at day 21, which indicated the osteogenic differentiation of hUCMSCs (Figures 5 and 6). However, the differentiation of the Recovery group was only partially characterized by lower levels of the osteogenic markers and less calcium accumulation than those of the OS group at the end of cultivation (Figures 5 and 6).

4. Discussion

Since its discovery, the MAPK family has been found to play important roles in controlling cellular behaviors. This includes, but is not limited to, cell differentiation induced by intracellular or extracellular stimulation [30, 41, 44, 45]. The subsets of MAPKs are characterized in mammalians:

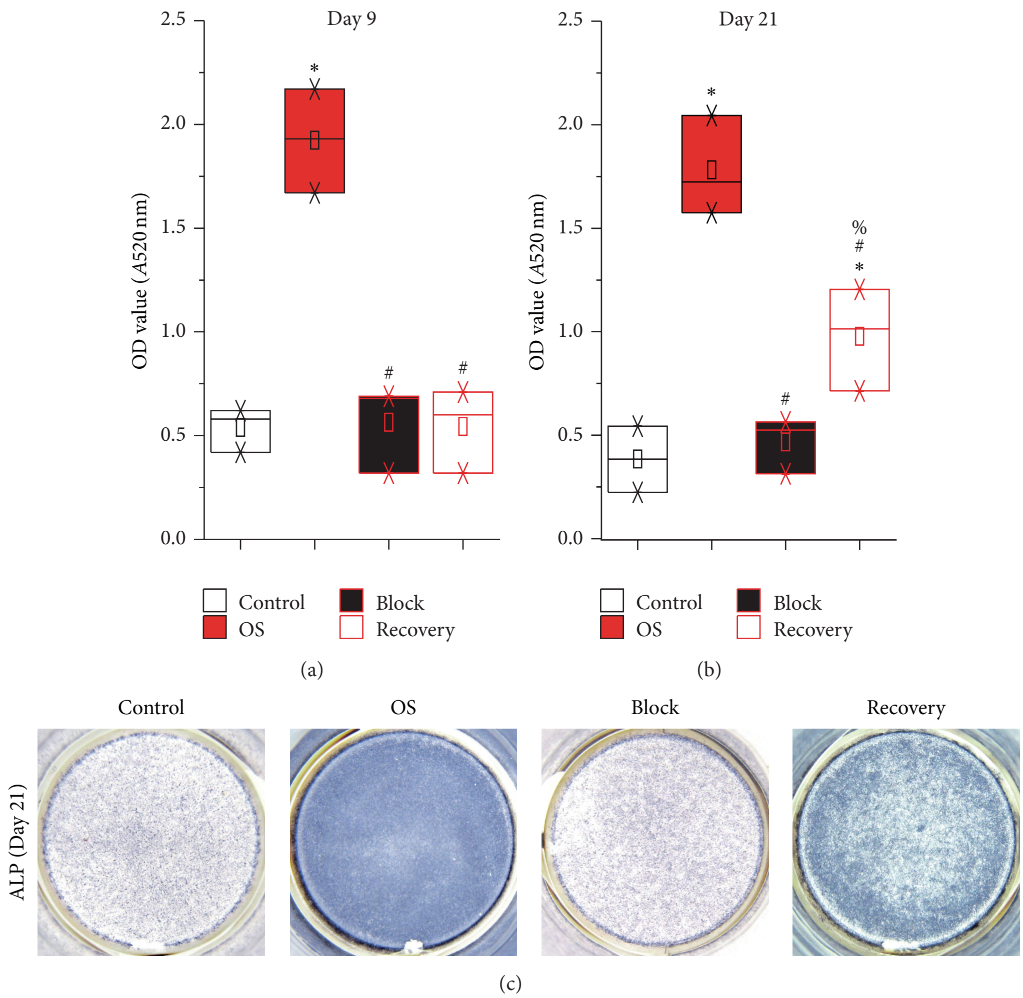

Figure 4: ALP activity was partially rescued in the Recovery group hUCMSCs at day 21. The secreted ALP activities in the hUCMSC culture media were analyzed at days 9 (a) and 21 (b). In addition, ALP staining of hUCMSCs was documented at day 21 (c). $^{*}p < 0.05$ compared with the Control group; $^{\#}p < 0.05$ compared with the OS group; $^{\%}p < 0.05$ compared with the Block group ($N = 6$).

ERK, JNK, and p38 [30]. Although all three MAPK subsets are regulated by phosphorylation cascades [41], they may function independently and distinctly [30, 31]. Previous studies indicate that p38 signaling is involved in stem cell neurogenic, adipogenic, and chondrogenic differentiation. In regard to osteogenesis, the enhancing role of p38 activation was widely described in mouse preosteoblastic cell line [46–49], mouse muscle-derived stem cells [50], human adipose-derived stem cells [33], and both mouse and human BMSCs [32, 43, 51]. Interestingly, since the p38 activation was not upregulated during the osteogenic differentiation of hUCMSCs, our current study implies that the p38 pathway may not be involved in this procedure.

With the osteogenic differentiation medium stimulation, JNK activation was found in the later stage of hBMSC osteogenic differentiation [32]. In addition, studies using a mouse preosteoblastic cell line suggested constitutive activation of JNK increased bone morphogenetic protein (BMP) 2-induced osteoblast differentiation and mineralization [52]. However, Sullivan et al. reported that the JNK inhibitor enhanced osteogenesis in neurofibromatosis type 1- (NF1-) deficient mouse osteoprogenitor cells, including primary neonatal calvarial cells and BMSCs [53]. Moreover, Doan et al. also described the repression effect of JNK on mouse BMSC osteogenic differentiation [43]. However, despite the conflicting observations in the influence of JNK on BMSC osteogenic differentiation, our data suggests that JNK signaling is not critical for hUCMSC osteogenic differentiation.

Meanwhile, the negative impact of ERK signaling on osteogenesis was also observed in the mouse preosteoblastic cell line [49, 54] and hBMSCs [43]. Actually, constitutive increases in activated ERK signaling were recognized as the reason for impaired osteogenesis in NF1-deficient patients [36]. Moreover, the blockade of the ERK activation in $Nf1^{-/-}$ mBMSCs could attenuate the increased cortical porosity observed in mutant pups [36, 55]. Conversely, in other studies, activation of ERK was thought to benefit mBMSC [33] and hBMSC [32, 45, 56] osteogenic differentiation. In this

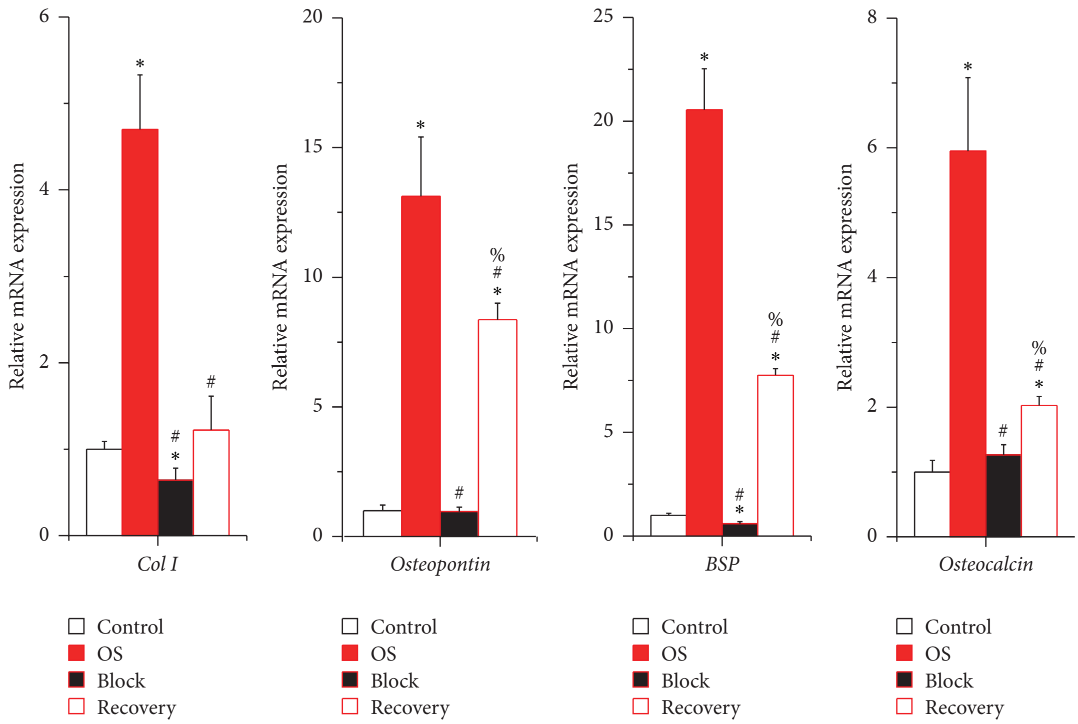

FIGURE 5: Expression of osteogenic marker genes in hUCMSCs was partially recovered by removing the ERK activation inhibitor U0126 at day 21. Data were presented as Mean + SD; $^{*}p < 0.05$ compared with the Control group; $^{\#}p < 0.05$ compared with the OS group; $^{\%}p < 0.05$ compared with the Block group ($N = 6$).

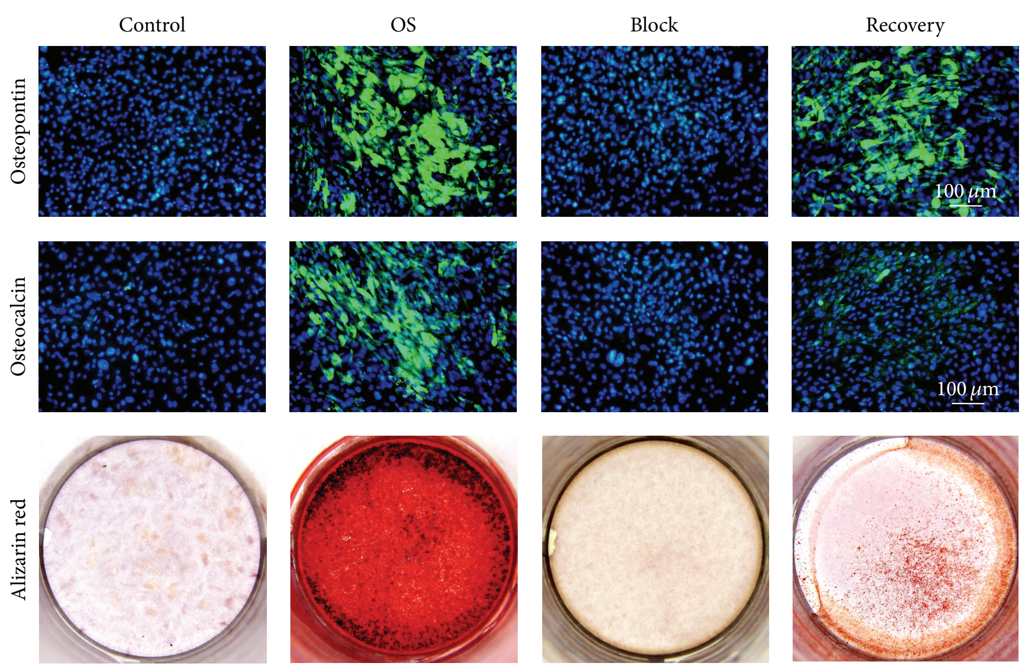

FIGURE 6: Immunocytochemistry staining of Osteopontin and Osteocalcin as well as the alizarin red staining confirmed the osteogenic differentiation of hUCMSCs in the Recovery group at day 21.

study, we found that the osteogenic differentiation medium strongly activated ERK, but not JNK and p38, in a time-dependent manner in hUCMSCs. By employing loss-of-function and recovery studies, we further confirmed that the activation of the ERK pathway critically regulates the osteogenic differentiation of hUCMSCs, another example of how hUCMSCs are not identical to hBMSCs [16, 17]. This discovery enriched our knowledge of underlying mechanisms behind the regulation of hUCMSC osteogenic differentiation and set up fundamental ideas to more effectively stimulate hUCMSCs conversion towards osteogenic lineage in cell therapy and tissue engineering strategy.

It is worth noting that several diverse techniques have been developed for the dissociation of tissues for primary cell isolation. To obtain hUCMSCs with high quantity and high stemness, especially with a higher capacity for osteogenic differentiation, a previously described collagenase/trypsin-based isolation method was used in this study [37, 57, 58]. Since Salehinejad et al. revealed that the isolation method could profoundly alter the cell harvesting and proliferation by comparing different methods for hUCMSC isolation from human umbilical cord Wharton's jelly [57], the osteogenic potential of our hUCMSCs used in this study was slightly different form that of the hUCMSCs reported by Bosch et al. [22].

In summary, our current results demonstrated that the activation of the ERK signaling pathway, but not JNK or p38, was necessary for the osteogenic differentiation of hUCMSCs, which deepened the understanding of the nature of hUCMSCs, a relatively new alternative stem cell source for tissue engineering. Moreover, our study significantly benefits the application of hUCMSCs, particularly in bone tissue engineering, by pointing out a potential regulatory direction to stimulate hUCMSC osteogenesis for engineered bone tissue generation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors would like to thank Chidiebele Amanda Enunwa at University of California, Los Angeles, for paper editing. This study was funded by University Innovation Research and Training Program of China (no. 101069868), Shaanxi Province, "13115" Technology Innovation Project, Major Scientific and Technological Special (2008ZDKG-65).

References

[1] M. N. Rahaman and J. J. Mao, "Stem cell-based composite tissue constructs for regenerative medicine," *Biotechnology and Bioengineering*, vol. 91, no. 3, pp. 261–284, 2005.

[2] K. Wieser, P. Zingg, and C. Dora, "Trochanteric osteotomy in primary and revision total hip arthroplasty: risk factors for non-union," *Archives of Orthopaedic and Trauma Surgery*, vol. 132, no. 5, pp. 711–717, 2012.

[3] X. Wang, Y. Wang, W. Gou, Q. Lu, J. Peng, and S. Lu, "Role of mesenchymal stem cells in bone regeneration and fracture repair: a review," *International Orthopaedics*, vol. 37, no. 12, pp. 2491–2498, 2013.

[4] A. R. Amini, C. T. Laurencin, and S. P. Nukavarapu, "Bone tissue engineering: recent advances and challenges," *Critical Reviews in Biomedical Engineering*, vol. 40, no. 5, pp. 363–408, 2012.

[5] M. Ohishi and E. Schipani, "Bone marrow mesenchymal stem cells," *Journal of Cellular Biochemistry*, vol. 109, no. 2, pp. 277–282, 2010.

[6] E. A. Jones, S. E. Kinsey, A. English et al., "Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells," *Arthritis and Rheumatism*, vol. 46, no. 12, pp. 3349–3360, 2002.

[7] D. Dallari, M. Fini, C. Stagni et al., "In vivo study on the healing of bone defects treated with bone marrow stromal cells, platelet-rich plasma, and freeze-dried bone allografts, alone and in combination," *Journal of Orthopaedic Research*, vol. 24, no. 5, pp. 877–888, 2006.

[8] S. Y. Lee, M. Miwa, Y. Sakai et al., "In vitro multipotentiality and characterization of human unfractured traumatic hemarthrosis-derived progenitor cells: a potential cell source for tissue repair," *Journal of Cellular Physiology*, vol. 210, no. 3, pp. 561–566, 2007.

[9] S. Bobis, D. Jarocha, and M. Majka, "Mesenchymal stem cells: characteristics and clinical applications," *Folia Histochemica et Cytobiologica*, vol. 44, no. 4, pp. 215–230, 2006.

[10] C. Zhou, B. Yang, Y. Tian et al., "Immunomodulatory effect of human umbilical cord Wharton's jelly-derived mesenchymal stem cells on lymphocytes," *Cellular Immunology*, vol. 272, no. 1, pp. 33–38, 2011.

[11] H. He, T. Nagamura-Inoue, A. Takahashi et al., "Immunosuppressive properties of Wharton's jelly-derived mesenchymal stromal cells in vitro," *International Journal of Hematology*, vol. 102, no. 3, pp. 368–378, 2015.

[12] L.-L. Lu, Y.-J. Liu, S.-G. Yang et al., "Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials," *Haematologica*, vol. 91, no. 8, pp. 1017–1028, 2006.

[13] M. B. Dehkordi, Z. Madjd, M. H. Chaleshtori, R. Meshkani, L. Nikfarjam, and A. Kajbafzadeh, "A simple, rapid, and efficient method for isolating mesenchymal stem cells from the entire umbilical cord," *Cell Transplantation*.

[14] M. L. Weiss, S. Medicetty, A. R. Bledsoe et al., "Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease," *STEM CELLS*, vol. 24, no. 3, pp. 781–792, 2006.

[15] M. T. Conconi, P. Burra, R. Di Liddo et al., "CD105(+) cells from Wharton's jelly show in vitro and in vivo myogenic differentiative potential," *International Journal of Molecular Medicine*, vol. 18, no. 6, pp. 1089–1096, 2006.

[16] A. Can and S. Karahuseyinoglu, "Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells," *Stem Cells*, vol. 25, no. 11, pp. 2886–2895, 2007.

[17] A. Subramanian, S.-U. Gan, K.-S. Ngo et al., "Human umbilical cord Wharton's jelly mesenchymal stem cells do not transform to tumor-associated fibroblasts in the presence of breast and ovarian cancer cells unlike bone marrow mesenchymal stem cells," *Journal of Cellular Biochemistry*, vol. 113, no. 6, pp. 1886–1895, 2012.

[18] R. Sarugaser, D. Lickorish, D. Baksh, M. M. Hosseini, and J. E. Davies, "Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors," *Stem Cells*, vol. 23, no. 2, pp. 220–229, 2005.

[19] D. Baksh, R. Yao, and R. S. Tuan, "Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow," *Stem Cells*, vol. 25, no. 6, pp. 1384–1392, 2007.

[20] N. Hjortholm, E. Jaddini, K. Hałaburda, and E. Snarski, "Strategies of pain reduction during the bone marrow biopsy," *Annals of Hematology*, vol. 92, no. 2, pp. 145–149, 2013.

[21] P. A. Sotiropoulou, S. A. Perez, M. Salagianni, C. N. Baxevanis, and M. Papamichail, "Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells," *Stem Cells*, vol. 24, no. 2, pp. 462–471, 2006.

[22] J. Bosch, A. P. Houben, T. F. Radke et al., "Distinct differentiation potential of 'mSC' derived from cord blood and umbilical cord: Are cord-derived cells true mesenchymal stromal cells?" *Stem Cells and Development*, vol. 21, no. 11, pp. 1977–1988, 2012.

[23] C. Leeb, M. Jurga, C. McGuckin, R. Moriggl, and L. Kenner, "Promising new sources for pluripotent stem cells," *Stem Cell Reviews and Reports*, vol. 6, no. 1, pp. 15–26, 2010.

[24] M. Latifpour, S. N. Nematollahi-Mahani, M. Deilamy et al., "Improvement in cardiac function following transplantation of human umbilical cord matrix-derived mesenchymal cells," *Cardiology*, vol. 120, no. 1, pp. 9–18, 2011.

[25] J. Sypecka and A. Sarnowska, "Mesenchymal cells of umbilical cord and umbilical cord blood as a source of human oligodendrocyte progenitors," *Life Sciences*, vol. 139, pp. 24–29, 2015.

[26] C. Nan, Y. Shi, Z. Zhao et al., "Monosialoteterahexosyl ganglioside induces the differentiation of human umbilical cord-derived mesenchymal stem cells into neuron-like cells," *International Journal of Molecular Medicine*, pp. 1057–1062, 2015.

[27] M. E. Klontzas, E. I. Kenanidis, M. Heliotis, E. Tsiridis, and A. Mantalaris, "Bone and cartilage regeneration with the use of umbilical cord mesenchymal stem cells," *Expert Opinion on Biological Therapy*, vol. 15, no. 11, pp. 1541–1552, 2015.

[28] Z.-Y. Guo, X. Sun, X.-L. Xu, J. Peng, and Y. Wang, "Human umbilical cord mesenchymal stem cells promote peripheral nerve repair via paracrine mechanisms," *Neural Regeneration Research*, vol. 10, no. 4, pp. 651–658, 2015.

[29] R. A. Hipskind and G. Bilbe, "MAP kinase signaling cascades and gene expression in osteoblasts," *Frontiers in Bioscience*, vol. 3, pp. d804–d816, 1998.

[30] N. Blüthgen and S. Legewie, "Systems analysis of MAPK signal transduction," *Essays in Biochemistry*, vol. 45, pp. 95–107, 2008.

[31] L. Fu, T. Tang, Y. Miao, S. Zhang, Z. Qu, and K. Dai, "Stimulation of osteogenic differentiation and inhibition of adipogenic differentiation in bone marrow stromal cells by alendronate via ERK and JNK activation," *Bone*, vol. 43, no. 1, pp. 40–47, 2008.

[32] R. K. Jaiswal, N. Jaiswal, S. P. Bruder, G. Mbalaviele, D. R. Marshak, and M. F. Pittenger, "Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase," *The Journal of Biological Chemistry*, vol. 275, no. 13, pp. 9645–9652, 2000.

[33] Q. Liu, L. Cen, H. Zhou et al., "The role of the extracellular signal-related kinase signaling pathway in osteogenic differentiation of human adipose-derived stem cells and in adipogenic transition initiated by dexamethasone," *Tissue Engineering Part: A*, vol. 15, no. 11, pp. 3487–3497, 2009.

[34] F.-H. Lin, J. B. Chang, and B. E. Brigman, "Role of mitogen-activated protein kinase in osteoblast differentiation," *Journal of Orthopaedic Research*, vol. 29, no. 2, pp. 204–210, 2011.

[35] A. Suzuki, J. Guicheux, G. Palmer et al., "Evidence for a role of p38 MAP kinase in expression of alkaline phosphatase during osteoblastic cell differentiation," *Bone*, vol. 30, no. 1, pp. 91–98, 2002.

[36] A. Schindeler and D. G. Little, "Ras-MAPK signaling in osteogenic differentiation: friend or foe?" *Journal of Bone and Mineral Research*, vol. 21, no. 9, pp. 1331–1338, 2006.

[37] F. Wang, Y.-C. Zhang, H. Zhou, Y.-C. Guo, and X.-X. Su, "Evaluation of in vitro and in vivo osteogenic differentiation of nano-hydroxyapatite/chitosan/poly(lactide-co-glycolide) scaffolds with human umbilical cord mesenchymal stem cells," *Journal of Biomedical Materials Research Part: A*, vol. 102, no. 3, pp. 760–768, 2014.

[38] J. V. Duncia, J. B. Santella III, C. A. Higley et al., "MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products," *Bioorganic and Medicinal Chemistry Letters*, vol. 8, no. 20, pp. 2839–2844, 1998.

[39] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[40] J. Shen, A. W. James, J. Chung et al., "NELL-1 promotes cell adhesion and differentiation via Integrinβ1," *Journal of Cellular Biochemistry*, vol. 113, no. 12, pp. 3620–3628, 2012.

[41] A. Plotnikov, E. Zehorai, S. Procaccia, and R. Seger, "The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation," *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*, vol. 1813, no. 9, pp. 1619–1633, 2011.

[42] H. S. Jung, Y. H. Kim, and J. W. Lee, "Duration and magnitude of extracellular signal-regulated protein kinase phosphorylation determine adipogenesis or osteogenesis in human bone marrow-derived stem cells," *Yonsei Medical Journal*, vol. 52, no. 1, pp. 165–172, 2011.

[43] T. K. P. Doan, K. S. Park, H. K. Kim, D. S. Park, J. H. Kim, and T. R. Yoon, "Inhibition of JNK and ERK pathways by SP600125-and U0126-enhanced osteogenic differentiation of bone marrow stromal cells," *Tissue Engineering and Regenerative Medicine*, vol. 9, no. 6, pp. 283–294, 2012.

[44] J. Caverzasio and D. Manen, "Essential role of Wnt3a-mediated activation of mitogen-activated protein kinase p38 for the stimulation of alkaline phosphatase activity and matrix mineralization in C3H10T1/2 mesenchymal cells," *Endocrinology*, vol. 148, no. 11, pp. 5323–5330, 2007.

[45] A. K. Kundu, C. B. Khatiwala, and A. J. Putnam, "Extracellular matrix remodeling, integrin expression, and downstream signaling pathways influence the osteogenic differentiation of mesenchymal stem cells on poly(Lactide-co-glycolide) substrates," *Tissue Engineering Part: A*, vol. 15, no. 2, pp. 273–283, 2009.

[46] A. Suzuki, Á. Raya, Y. Kawakami et al., "Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 27, pp. 10294–10299, 2006.

[47] Y. Hu, E. Chan, S. X. Wang, and B. Li, "Activation of p38 mitogen-activated protein kinase is required for osteoblast differentiation," *Endocrinology*, vol. 144, no. 5, pp. 2068–2074, 2003.

[48] J. Guicheux, J. Lemonnier, C. Ghayor, A. Suzuki, G. Palmer, and J. Caverzasio, "Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation," *Journal of Bone and Mineral Research*, vol. 18, no. 11, pp. 2060–2068, 2003.

[49] O. Kozawa, D. Hatakeyama, and T. Uematsu, "Divergent regulation by p44/p42 MAP kinase and p38 MAP kinase of bone morphogenetic protein-4-stimulated osteocalcin synthesis in osteoblasts," *Journal of Cellular Biochemistry*, vol. 84, no. 3, pp. 583–589, 2001.

[50] K. A. Payne, L. B. Meszaros, J. A. Phillippi, and J. Huard, "Effect of phosphatidyl inositol 3-kinase, extracellular signal-regulated kinases 1/2, and p38 mitogen-activated protein kinase inhibition on osteogenic differentiation of muscle-derived stem

cells," *Tissue Engineering Part A*, vol. 16, no. 12, pp. 3647–3655, 2010.

[51] S. Peng, G. Zhou, K. D. K. Luk et al., "Strontium promotes osteogenic differentiation of mesenchymal stem cells through the Ras/MAPK signaling pathway," *Cellular Physiology and Biochemistry*, vol. 23, no. 1–3, pp. 165–174, 2009.

[52] H. Liu, Y. Liu, M. Viggeswarapu, Z. Zheng, L. Titus, and S. D. Boden, "Activation of c-Jun NH2-terminal kinase 1 increases cellular responsiveness to BMP-2 and decreases binding of inhibitory Smad6 to the type 1 BMP receptor," *Journal of Bone and Mineral Research*, vol. 26, no. 5, pp. 1122–1132, 2011.

[53] K. Sullivan, J. El-Hoss, D. G. Little, and A. Schindeler, "JNK inhibitors increase osteogenesis in Nf1-deficient cells," *Bone*, vol. 49, no. 6, pp. 1311–1316, 2011.

[54] C. Higuchi, A. Myoui, N. Hashimoto et al., "Continuous inhibition of MAPK signaling promotes the early osteoblastic differentiation and mineralization of the extracellular matrix," *Journal of Bone and Mineral Research*, vol. 17, no. 10, pp. 1785–1794, 2002.

[55] W. X. Wang, J. S. Nyman, K. Ono, D. A. Stevenson, X. Yang, and F. Elefteriou, "Mice lacking Nf1 in osteochondroprogenitor cells display skeletal dysplasia similar to patients with neurofibromatosis type I," *Human Molecular Genetics*, vol. 20, no. 20, pp. 3910–3924, 2011.

[56] L. R. Chaudhary and L. V. Avioli, "Activation of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) by FGF-2 and PDGF-BB in normal human osteoblastic and bone marrow stromal cells: differences in mobility and in-gel renaturation of ERK1 in human, rat, and mouse osteoblastic cells," *Biochemical and Biophysical Research Communications*, vol. 238, no. 1, pp. 134–139, 1997.

[57] P. Salehinejad, N. Banu Alitheen, A. M. Ali et al., "Comparison of different methods for the isolation of mesenchymal stem cells from human umbilical cord Wharton's jelly," *In Vitro Cellular and Developmental Biology—Animal*, vol. 48, no. 2, pp. 75–83, 2012.

[58] R. C. Schugar, B. M. Deasy, S. M. Chirieleison et al., "High harvest yield, high expansion, and phenotype stability of CD146 mesenchymal stromal cells from whole primitive human umbilical cord tissue," *Journal of Biomedicine and Biotechnology*, vol. 2009, Article ID 789526, 11 pages, 2009.