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

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

Academic Editor: Martin Sebastian Staegge

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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 mm² 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 isolated hUCMSCs were resuspended in a maintenance 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

2.2. Osteogenic Differentiation Induction of hUCMSCs. Passage 3 hUCMSCs were plated at 5 × 10⁴/cm² 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 instructions. 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 ΔΔCT method [39].

2.6. Alkaline Phosphates Activity. Culture medium was collected and stored at approximately 8°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'-ATCGTGCGTGACATTAAAGGAG-3'           |
|                          | 5'-AGGAAAGGAGGACCGTGGAAAGG-3'          |
| Collagen type I, α1 (COL Iα1) | 5'-ACAAGAAAGGACCGTGGAAAGG-3'         |
|                          | 5'-GAGCAGCAGCAGCAGGAG-3'              |
| Osteocalcin (OCN)        | 5'-GAAAGGACCGGCAACGAC-3'              |
|                          | 5'-GAAAGGACCGGCAACGAC-3'              |
| Osteopontin (OPN)        | 5'-GAAAGGACCGGCAACGAC-3'              |
|                          | 5'-GAAAGGACCGGCAACGAC-3'              |
| Bone Sialoprotein (BSP)  | 5'-CCCACCTTGGAGAACCAACA-3'            |
|                          | 5'-TCCCGCTTCCTCTTCTCCTGTA-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 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 (+).

3.4. Rescuing hUCMSC 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
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 mammals:
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 [32]. 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 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).
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
of bone tissue engineering, 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. Nakavarapu, "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 unfactured 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. Kajbażadeh, "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. Halaburda, and E. Snarski, "Strategies of pain reduction during the bone marrow biopsy," Annals of Hematology, vol. 92, no. 2, pp. 145–149, 2013.
[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. Vigeswarapu, 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 I 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.