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Proteomic analysis of the effect of extracellular calcium ions on human mesenchymal stem cells: Implications for bone tissue engineering

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ABSTRACT

Human mesenchymal stem cells-bone marrow (BM-hMSCs) are considered as the most suitable seed cells for bone tissue engineering. Calcium ions (Ca^{2+}) forms an important component of a number of commercial bone substitutes and support materials. For efficient bone tissue engineering, it is crucial to explore the effect of extracellular Ca^{2+} on the growth and differentiation of BM-hMSCs, and to understand their molecular mechanisms. Therefore, in the present study, BM-hMSCs were cultivated in serum free growth medium or serum free growth medium with additional 4 or 6 mM Ca^{2+} for 3 weeks, following which, the proliferation and osteoblastic differentiation of these cells were evaluated. Differentially expressed proteins were established using iTRAQ labeling coupled with nano-LC–MS/MS. Our data revealed that Ca^{2+} significantly promoted the proliferation of BM-hMSCs in the early stage. Furthermore, Ca^{2+} showed osteoinduction properties. MAPKs signaling pathway might participate in the osteogenic differentiation of BM-hMSCs caused by Ca^{2+}. Certain newly found proteins could be potentially important for the osteogenic differentiation of BM-hMSCs and may be associated with osteogenesis.

1. Introduction

Mesenchymal stem cells (MSCs) are defined as multipotent cells capable of replicating extensively. They have the potential for multi-directional differentiation, and can differentiate into various types of cells *in vitro* under different stimulation. In recent years, researchers have focused considerably on expanding human mesenchymal stem cells (hMSCs), specifically derived from bone marrow (BM-hMSCs), and on using these cells in potential gene-based therapies or bone tissue engineering for bone tissue regeneration or repair [1–4]. However, a major challenge is to identify factors and pathways that promote proliferation and define osteogenic commitment of BM-hMSCs.

Calcium ion (Ca^{2+}), an essential constituent of bone tissues, is also a component of many commercial bone substitutes and support materials. For efficient bone tissue engineering, it is important to understand the influence of the composition of bone graft material on the biological behavior and function of seed cells. Zhang et al. found that modifying the microstructure of tricalcium phosphate (TCP) could increase its resorption rate and then induce the osteogenic differentiation of MSC [5]. Barradas et al. showed that TCP significantly increased genes expression of bone morphogenetic protein-2 (BMP-2), osteopontin (OP), osteocalcin(OC) and bone sialoprotein (BSP) in MSC than hydroxyapatite (HA) for high Ca^{2+} dissolution rate in medium [6]. Gene analysis found that Ca^{2+} might trigger the signaling pathway not only by L-voltage-gated Ca^{2+} channels (L-VGCCs) and Ca^{2+}-sensing receptor (CaSR), but also by some other unknown G protein-coupled receptor(GPCR) [7,8]. Several approaches were used to study the effect of Ca^{2+} on MSCs, and the higher mineralization was recorded at 6 mM calcium in the medium [9]. However, systemic approaches for identifying the effect of Ca^{2+} on BM-hMSCs have not yet to be investigated. Proteins, the real functional molecules in body to participate in all kinds of biological functions, should be translated from gene and then go through diversified decorated before acting as a functional protein, so the expression of molecules in gene level could not fully reflect the truth.

Proteomics, which involves direct identification and quantification of most proteins in the tissues or cells, provides a more holistic view of the various biological processes undergoing in cells. Therefore, proteomic analysis may determine the components and mechanisms that can be further used to stimulate osteogenic differentiation of BM-hMSCs.

Isobaric tags for relative and absolute quantification (iTRAQ) coupled with nano-liquid chromatography tandem mass

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spectrometry (nano-LC–MS/MS) is known to be a simple and efficient method for the detection of proteomic profiles, and makes simultaneous protein identification of up to eight different samples and relative quantification according to the reporter ions. Owing to the amine specificity of iTRAQ reagents for all primary amines, most peptides are labeled without loss of information during post-translational modifications, which is crucial for the scrutiny of signal transduction pathways [10]. The signal of the same peptide from all samples turns up in the same mass spectrometer (MS) peak for isobaric tags, thereby increasing the signal strength and quantification at the MS/MS is in the absence of chemical noise [11]. These features form a basis for the high sensitivity of iTRAQ coupled with nano-LC–MS/MS analysis.

HMSCs cultured in a serum free medium enables the study of the effect of specific molecules on the biological processes of cells, and investigation of the relevant signaling factors and pathways without serum interference [12]. Therefore, in our study, we cultured BM-hMSCs in serum free medium and analyzed the effect of Ca^{2+} on the proliferation and osteogenic differentiation of BM-hMSCs. In order to find the key signal transduction pathways and relevant proteins involved in osteogenic differentiation of BM-hMSCs, we further compared the proteomic profiles of BM-hMSCs between Ca^{2+} groups and the control by coupling iTRAQ with nano-LC–MS/MS.

2. Materials and methods

2.1. Preparation of serum free growth media with additional Ca^{2+}

Calcium chloride ($CaCl_2{\cdot}2H_2O$; Sigma, St. Louis, MO, USA) was dissolved in HEPES buffer solution to make Ca^{2+} stock solutions of concentrations 400 and 600 mM. The stock was then diluted with serum free growth medium (ScienCell, San Diego, CA, USA) to a concentration of 4 or 6 mM for further experiments.

2.2. BM-hMSC culture

BM-hMSCs (ScienCell) were grown in mesenchymal stem cell medium (ScienCell) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. The medium was changed every 2 days. On reaching confluence, the cells were detached with trypsin–EDTA (0.25% trypsin and 0.02% EDTA) (Gibco, Carlsbad, CA, USA) and subcultured at a split ratio of 1:3. Cells at passage 3 were used for further experimentation. The BM-hMSCs were divided into three groups: 4C group, cells cultured in serum free growth medium with additional 4 mM Ca^{2+}; 6C group, cells cultured in serum free growth medium with additional 6 mM Ca^{2+}; and GM group (control group), cells cultured in serum free growth medium, which consists of 500 ml of basal medium, 5 ml of mesenchymal stem cell growth supplement serum free, and 5 ml of penicillin/streptomycin solution, and the concentration of Ca^{2+} and Pi in it is 2 and 0.5 mM, respectively.

2.3. Cell proliferation assay

To evaluate the effect of extracellular Ca^{2+} on the growth of BM-hMSCs, cells were seeded onto 96-well culture plates at a subconfluence density of 10,000 cells/cm^2. After culturing under growth medium-serum free for 24 h, the cells were transferred to a growth medium-serum free or growth medium-serum free with additional 4 or 6 mM Ca^{2+} for 4, 7, 14, or 21 days. Each experiment had five repetitions. At a defined experimental time, the original medium was replaced by 150 μl of growth medium containing 10% Cell Counting Kit-8 (CCK8; Solarbio, Beijing, China). Cells were incubated at 37 °C for 5 h, and the visible light absorption of the solution was finally determined at 450 nm by microplate spectrophotometer (BioTek, Winooski, VT, USA). The absorbance measurements were compared between the Ca^{2+} groups and the GM group.

2.4. Calcium staining by Alizarin Red S

BM-MSCs were seeded at 10,000 cells/cm^2 in 24-well plates with serum free growth medium. After 24 h, the medium was replaced with growth medium-serum free or growth medium-serum free with additional 4 or 6 mM Ca^{2+} for 14 or 21 days. Each sample had three repetitions. Calcium deposition was analyzed using the Alizarin Red S staining method. Following 14 or 21 days of culture, cells were washed with phosphate buffered saline (PBS) and fixed in 95% alcohol solution for 10 min. The cells were then washed with PBS and stained in 0.5% Alizarin Red S (Sigma) for 10 min at room temperature. Finally, the cells were completely washed with PBS to remove any excess dye and then observed under the inverted microscope (Olympus, Japan).

2.5. Quantitative real-time PCR

To evaluate the expression of marker genes for osteogenic differentiation, alkaline phosphatase (ATP), OC, and collagen I (Col I), cells seeded at 10,000 cells/cm^2 in three groups were cultured for 7, 14, or 21 days. Each sample had three repetitions. RNA was isolated by Trizol RNA kit (Gibco). The primer sequences employed for the respective gene amplification were as follows:

ALP-F:5′-AACATCAGGGACATTGACGTG-3′, ALP-R:5′-GTATC TCGGTTTGAAGCTCTTCC-3′; COLI-F:5′-GAGGGCCAAGACGAAGAC-ATC-3′, COLI-R:5′-CAGATCACGTCATCGCACAAC-3′; OC-F:5′-CACTC CTCGCCCTATTGGC-3′, OC-R:5′-CCCTCCTGCTTGGACACAAAG-3′; GAPD H-F:5′-GAGTCAACGGATTTGGACGT-3′, GAPDH-R:5′-GACAAGCTTC CCGTTCTCAG-3′.

SYBR Green was used for emitting the fluorescence signal, while PCR analysis system (Bio-Rad, Hercules, CA, USA) was used to analyze the signals and quantity of the target genes.

2.6. Proteomic analysis by iTRAQ

BM-MSCs were seeded at 10,000 cells/cm^2 in 55 cm^2 plastic culture dish, and allowed to grow in serum free growth medium and in serum free growth medium with additional 4 or 6 mM Ca^{2+}. Each sample had three repetitions. After 14 days, cells were trypsinized and then lysed in 2 mL cell lysis buffer (8 M urea, 30 mM 4-(2-hydroxyerhyl)piperazine-1-erhanesulfonic acid (HEPES), 10 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 2 mM ethylene diamine tetraacetic acid (EDTA, pH 8.0–8.3). The concentration of proteins was measured using the Bradford Quant Kit (Amresco).

Each sample (100 μg) was digested with 3.3 μL of 1 μg/μL trypsin (Promega, Madison, WI, USA) at 37 °C for 24 h, and subsequently labeled with the iTRAQ tags as follows: GM group, iTRAQ 113; 4C group, iTRAQ 116; and 6C group, iTRAQ 114. The labeled samples were then pooled before analysis. The iTRAQ labeled peptides were desalted by C_{18} reversed-phase column (5 μm, 300A; Phenomenex). Each desalted product was further separated with the Dionex capillary/nano-HPLC system (Dionex, Sunnyvale, CA, USA) and then transported to and analyzed with Q-Exactive mass spectrometer (Thermo Fisher Scientific, Boston, MA, USA). The mass spectrum was acquired by the software Proteome Discoverer 1.3 in the data-dependent acquisition mode. The analytical cycle was composed of a single full-scan mass spectrum (350–2000 Da), followed by 15 data-dependent MS/MS scans, under which iTRAQ-labeled peptides were fragmented to produce reporter ions and fragment ions. The proportion of reporter ions