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Inhibiting actin depolymerization enhances osteoblast differentiation and bone formation in human stromal stem cells☆

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ABSTRACT

Remodeling of the actin cytoskeleton through actin dynamics is involved in a number of biological processes, but its role in human stromal (skeletal) stem cells (hMSCs) differentiation is poorly understood. In the present study, we demonstrated that stabilizing actin filaments by inhibiting gene expression of the two main actin depolymerizing factors (ADFs): Cofilin 1 (CFL1) and Destrin (DSTN) in hMSCs, enhanced cell viability and differentiation into osteoblastic cells (OB) *in vitro*, as well as heterotopic bone formation *in vivo*. Similarly, treating hMSC with Phalloidin, which is known to stabilize polymerized actin filaments, increased hMSCs viability and OB differentiation. Conversely, Cytocholasin D, an inhibitor of actin polymerization, reduced cell viability and inhibited OB differentiation of hMSC. At a molecular level, preventing Cofilin phosphorylation through inhibition of LIM domain kinase 1 (LIMK1) decreased cell viability and impaired OB differentiation of hMSCs. Moreover, depolymerizing actin reduced FAK, p38 and JNK activation during OB differentiation of hMSCs, while polymerizing actin enhanced these signaling pathways. Our results demonstrate that the actin dynamic reassembly and Cofilin phosphorylation loop is involved in the control of hMSC proliferation and osteoblasts differentiation.

1. Introduction

Cytoskeleton is a cellular scaffolding contained within cytoplasm. It maintains the cell shape, provides mechanical strength, directs locomotion, regulates chromosome separation in mitosis and meiosis and intracellular transport of organelles in cells (Doherty and McMahon, 2008; Van Troys et al., 2008). Actin microfilaments are the major structure of the cytoskeleton. The actin protein exists within cells in either globular/monomer (G-actin) or filamentous (F-actin) forms and thus in highly dynamic transitions of depolymerization and polymerizationin. During depolymerization, polymerized actin (F-actin) is severed/depolymerized and turns to monomer actin (G-actin). G-actin can be recycled, transferred back into filament form by an ADP-to-ATP exchange. ATP-actin becomes available for assembly and polymerization to F-actin (Ono, 2007). The process of assembly and disassembly of actin filaments in cells is regulated by actin depolymerizing factors (ADFs) that in mammals include: Cofilin1 (CFL1, non-muscle Cofilin), Cofilin2 (CFL2, muscle Cofilin), and Destrin (DSTN, also called ADF or Corn1). CFL1 is ubiquitously expressed, while CFL2 is only expressed in muscles. DSTN also exhibits ubiquitous expression, and its levels are about 5% to 10% of CFL1 levels (Vartiainen et al., 2002). Cofilin binds to actin monomers and filaments, causing depolymerization of actin filaments preventing their reassembly (Ghosh et al., 2004). Phosphorylation and dephosphorylation regulate Cofilin's binding and associating activity with actin (Lappalainen and Drubin, 1997; Maekawa et al., 1999). DSTN is a component protein in microfilaments. It severs actin filaments (F-actin) and binds to G-actin, thereby, sequestering actin monomers and preventing polymerization (Hawkins et al., 1993).

During lineage specific differentiation, human stromal (skeletal) stem cells (hMSCs) exhibit significant changes in morphology and actin cytoskeletal organization (McBeath et al., 2004; Yourek et al., 2007; Treiser et al., 2010). For example, during adipocyte differentiation, the cells undergo a morphological change from fibroblastic to spherical cells filled with lipid droplets (Fan et al., 1983). The change in cell shape takes place early in the differentiation process prior to the up-regulation of many adipocyte specific genes and in association with cytoskeletal changes including decreased actin synthesis and actin reorganization (Antras et al., 1989). Altered actin organization influences cytoskeletal tension which has been demonstrated to play a

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role in adipogenesis in cultured MSCs (McBeath et al., 2004). Similarly, during chondrocyte differentiation of hMSCs, significant changes in cell shape that transform from a fibroblast-like to a circular morphology leading to a significant increase in cell volume (Xu et al., 2008). Similarly, the regulatory role of actin dynamics in chondrocyte differentiation has been supported by a number of experiments. Actin-disrupting compounds, such as cytochalasin D, stimulate chondrogenesis (Loty et al., 1995). In addition, intracellular kinases induced by adhesion signaling from extracellular matrix (ECM) proteins, regulate chondrocyte differentiation through changes in actin cytoskeleton (Woods et al., 2005, 2007; Nurminsky et al., 2007).

We became interested in the potential role of ADF genes and actin dynamics during osteoblast (OB) differentiation of hMSCs for several reasons. First, actin and microtubules are abundant in osteoblasts (Aubin et al., 1983; Arena et al., 1991). During OB differentiation, cell morphology is found to alter from fibroblast-like to cuboidal morphology; this is accompanied by changes in actin cytoskeleton (Lomri et al., 1987). Second, bone is a tissue that is sensitive to mechanical stimulation. The responses of osteoblastic cells to the applied mechanical forces are transmitted through plasma membrane adhesion molecules and induce alterations in actin cytoskeletal organization (Sakai et al., 2011). Third, some studies have reported that the upstream regulator of ADFs, such as Rho GTPase/Rho-Associated kinase (Rock), extracellular matrix (ECM) components, and adhesion kinases, act as regulators for OB differentiation (Harmey et al., 2004; Hamidouche et al., 2009; Mathews et al., 2012). However, evidence for the direct role of ADFs and dynamic organization of actin in MSCs biology and OB differentiation are not clear (Kawano et al., 2013; Shuang et al., 2013).

Thus, we examined the regulatory role of ADFs and actin cytoskeletal changes on lineage specific differentiation of hMSCs. We demonstrated that G-actin (monomer) and F-actin (polymerized) ratio was altered after induction of OB differentiation of hMSCs and was associated with significant changes in expression of ADFs. Lowering the expression levels of ADFs enhanced the polymerization of actin filaments, enhanced cell viability, and OB differentiation of hMSCs.

2. Materials and methods

2.1. Cell culture and OB differentiation of hMSCs

Primary hMSCs were isolated from human bone marrow aspirate as described previously (Stenderup et al., 2004). As a model for primary hMSCs, we employed the hMSC-TERT cell created in our laboratory by overexpression of human telomerase reverse transcriptase (hTERT) gene (Simonsen et al., 2002). hMSC-TERT exhibits all the characteristics of primary MSC *in vitro* and form normal heterotopic bone when implanted *in vivo* (Simonsen et al., 2002). Cells were grown in Minimal Essential Media (MEM) without Phenol red and L-glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM Glutamax, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco-Invitrogen, Carlsbad, CA), referred to as standard growth medium. *In vitro* OB differentiation of hMSC was carried out using OB induction medium containing standard growth medium supplemented with 10^{-8} M dexamethasone, 0.2 mM L-ascorbic acid, 10 mM β-glycerophosphate (Sigma), and 10 mM 1.25-vitamin-D3 (Leo® pharma).

2.2. Alkaline phosphatase (ALP) and Alizarin red S (AZR) staining

ALP cytochemical staining was performed on cultured cells by rinsing the cell layer with PBS followed by fixation in acetone/citrate (1.5:1, v:v) buffer (pH 4.2) for 5 minutes at room temperature. The cells were incubated with buffer containing 0.2 mg/ml naphthol AS-TR phosphate (Sigma). After incubation for one hour at 37 °C, the cell layer was washed with deionized water. Alizarin red S staining was employed to assess the presence of in vitro formed mineralized matrix. The medium was removed, and the cell layer was rinsed with PBS and fixed in 70% ethanol for 1 hour at -20 °C. The cell layer was washed with deionized water. The fixed cells were stained with 40 mM Alizarin red S pH 4.2 (Sigma) for 10 minutes at room temperature. The cell layer was washed with deionized water. For quantitative assessment of the degree of mineralization, the color was eluted by 10% (wt/vol) cetylpyridinium chloride (Sigma) for 1 h and quantified by spectrophotometric absorbance measurements at OD570nm (Gregory et al., 2004).

2.3. ALP activity assay

ALP activity assay was performed in the96-well plate. Cell number (i.e. the number of viable cells) was determined by adding the CellTiter-Blue solution (Promega) for 1 hour and measured the fluorescence at wavelength of $579_{Ex}/584_{Em}$. The cells were then rinsed with PBS and fixed in 3.7% formaldehyde-90% ethanol for 30 seconds at room temperature. A reaction mixture containing 100 μl 50 mM $NaHCO_3$, 1 mM $MgCl_2$ (Sigma) and 1 mg/ml of p-nitrophenyl phosphate (Sigma) was added into each well and incubated at 37 °C for 20 minutes. The reaction was stopped by adding 50 μl of 3 M NaOH. Absorbance was determined at 405 nm in an ELISA microplate reader. ALP enzymatic activity was normalized to cell number in each well.

2.4. Cell transfection

Small interfering RNA (siRNA) duplex oligos targeting DSTN, CFL1, CFL2, LIMK1, as well as non-targeting duplex oligo (siRNA negative controls) were purchased from Ambion (Life Technology Inc.). These reagents were transfected into hMSC-TERT at a final concentration of 12 nM for siRNA. All transfections were performed using Lipofectamine2000 Transfection Reagent (Invitrogen), according to the manufacturer's instructions.

2.5. Treatment with Cytochalasin D and Phalloidin during OB differentiation in hMSCs

hMSCs were seeded in 24-well plate or 96-well plate and induced to osteogenic differentiation as described above. The media was changed every third day. Before media change, the cells were pre-treated with Cytochalstin D (Sigma, C8273) (1-20 uM) for 1 h or Phalloidin (Sigma, P2141) (0-6 uM) for 3 h. After treatment, cells were washed and incubated in differentiation medium. Equal volumes of DMSO solution was added into the corresponding controls wells.

2.6. Actin staining and cell shape detection by high content cellomic analysis

Cells were seeded at a density of 2000 cells per well into clear bottom 96-well CellCarrier™ microtiter plates (PerkinElmer), the Operetta® High Content Imaging System was used to study the cellular changes. Cells were washed with PBS, fixed with 4% formaldehyde for 30 min, and permeabilized with 0.1% Triton™-X-100 for 10 min. Subsequently, the cells were stained with a dye solution containing 10 μg/mL 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich®, D8417) and 5 μg/mL Phalloidin-TRITC (Sigma-Aldrich®, P1951). Images were acquired on an Operetta® high content image System (Perkin Elmer) using a 10X high NA (numerical apertures) objective in wide-field mode. All pictures were acquired with the same contrast and brightness parameters. For quantitative analyses, individual cells were segmented based on the DAPI nuclear stain using the Find Nuclei building block in the Harmony® High Content Imaging and Analysis Software. The following parameters were determined per cell: cell size (area) (μm2), cell length (μm), cell width (μm), cell roundness, cell ratio (width to length), nuclear size (μm2), and nuclear roundness. The staining intensity of TRITC was measured in each well and normalized to the cell number.