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Actin depolymerization enhances adipogenic differentiation in human stromal stem cells

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ABSTRACT

Human stromal stem cells (hMSCs) differentiate into adipocytes that play a role in skeletal tissue homeostasis and whole body energy metabolism. During adipocyte differentiation, hMSCs exhibit significant changes in cell morphology suggesting changes in cytoskeletal organization. Here, we examined the effect of direct modulation of actin microfilament dynamics on adipocyte differentiation. Stabilizing actin filaments in hMSCs by siRNA-mediated knock down of the two main actin depolymerizing factors (ADFs): Cofilin 1 (CFL1) and Destrin (DSTN) or treating the cells by Phalloidin reduced adipocyte differentiation as evidenced by decreased number of mature adipocytes and decreased adipocyte specific gene expression (ADIPOQ, LPL, PPARG, FABP4). In contrast, disruption of actin cytoskeleton by Cytochalasin D enhanced adipocyte differentiation. Follow up studies revealed that the effects of CFL1 on adipocyte differentiation depended on the activity of LIM domain kinase 1 (LIMK1) which is the major upstream kinase of CFL1. Inhibiting LIMK by its specific chemical inhibitor LIMKi inhibited the phosphorylation of CFL1 and actin polymerization, and enhanced the adipocyte differentiation. Moreover, treating hMSCs by Cytochalasin D inhibited ERK and Smad2 signaling and this was associated with enhanced adipocyte differentiation. On the other hand, Phalloidin enhanced ERK and Smad2 signaling, but inhibited adipocyte differentiation which was rescued by ERK specific chemical inhibitor U0126. Our data provide a link between restructuring of hMSCs cytoskeleton and hMSCs lineage commitment and differentiation.

1. Introduction

The cytoskeleton is a system of intracellular filaments crucial for cell shape, adhesion, growth, division and motility (Stossel, 1993; Wickstead and Gull, 2011). As one of the major component of cytoskeleton, actin filaments form a highly dynamic network composed of actin polymers and a large variety of associated proteins (Schmidt and Hall, 1998). The actin proteins exist within cells in either globular/monomer (G-actin) or filamentous (F-actin) form and thus in dynamic transitions between depolymerization and polymerization status (Ono, 2007). Actin depolymerization factor (ADF) cofilin is a family of actin binding proteins with actin filaments depolymerization function (Bamburg et al., 1980; Nishida et al., 1984). In mammals, the cofilin family consists of three highly similar paralogs: Cofilin-1 (CFL1, non-muscle cofilin, n-cofilin), Cofilin-2 (CFL2, muscle cofilin, m-cofilin) and destrin (DSTN). The activity of cofilins is regulated by phosphorylation, polyphosphoinositide interaction, pH and interaction with other actin binding proteins (Van Troys et al., 2008).

Human bone marrow-derived stromal (Skeletal) stem cells (hMSCs) are a group of clonogenic and multipotent cells capable of differentiation into mesoderm-type cells e.g. osteoblast and adipocyte (Abdallah and Kassem, 2008). During lineage specific differentiation, hMSCs exhibit significant changes in morphology and actin cytoskeletal organization (McBeath et al., 2004; Treiser et al., 2010; Yourek et al., 2007). Previous studies have suggested that RhoA-ROCK (Ras homolog gene family member A-Rho-associated protein kinase) signaling mediates changes in cell shape and cytoskeletal tension regulating hMSC lineage commitment (McBeath et al., 2004). A spheroidal morphology associated with a dispersed actin cytoskeleton with few focal adhesions encourages MSC differentiate into adipocytes whereas a stiff, spread actin cytoskeleton with greater numbers of focal adhesions drives MSC differentiate into osteoblasts (Mathieu and Loboa, 2012). The changes in cell shape are caused by cytoskeletal changes due to actin synthesis and reorganization (Antras et al., 1989; Spiegelman and Farmer, 1982), and thus modification by mechanical forces or regulation of relative kinases that change actin cytoskeletal dynamics, can regulate cell differentiation (Arnsdorf et al., 2009; Kanzaki and Pessin, 2001;

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McBeath et al., 2004; Noguchi et al., 2007). However, this approach has not been widely utilized in differentiation studies of hMSCs.

We have previously reported that interfering with actin assembly dynamics by knocking down cofilin-1 in hMSCs increased polymerized actin that promoted osteoblast cell differentiation through a mechanism of enhancing focal adhesion kinase (FAK), p38 and c-Jun N-terminal kinase (JNK) signaling (Chen et al., 2015). In the present study, we examined the effects of interfering in the actin cytoskeletal dynamics on the regulation of adipocyte differentiation of hMSCs.

2. Materials and methods

2.1. Cell culture and adipocyte differentiation of hMSCs

Primary hMSCs were isolated from human bone marrow aspirate or adipocyte as described (Stenderup et al., 2004). As a model for primary hMSCs, we employed the hMSCs-TERT cell (hMSCs here after) created in our laboratory by overexpression of human telomerase reverse transcriptase (hTERT) gene (Simonsen et al., 2002). Cells were cultured 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). For adipocyte differentiation, cells were induced by adipogenic induction medium containing 10% horse serum (Sigma), 100 nM dexamethasone (Sigma), 450μM IBMX (1-methyl-3-isobutylxanthine, Sigma), 1 μM BRL (Cayman Chemical), 3 μg/ml insulin (Sigma). Media were changed every two days. For chemical treatment experiment, before each medium change, the cells were pre-treated with Cytochalasin D (Sigma, C8273) (1–20 μM) for 1 h or Phalloidin (Sigma, P2141) (0-3 μM) for 3 h or DMSO as control. After treatment, cells were washed once and incubated in adipogenic differentiation medium. In some experiments, 1 μM LIM domain kinase inhibitor (LIMKi) (Calbiochem) or 10 μM and 25 μM ERK inhibitor U0126 (Cell Signaling, #9903) was used continuously with adipocyte induction medium during adipocyte differentiation, Oil red O staining for lipid droplets formation in mature adipocytes was performed as described (Abdallah et al., 2005).

2.2. Cell transfection

Small interfering RNA (siRNA) targeting DSTN (siR-DSTN), CFL1 (siR-CFL1) as well as non-targeting siRNA (siRNA negative control, siR-Ctrl) were purchased from Ambion (Life Technology Inc.). siRNAs were transfected into hMSCs-TERT at a final concentration of 10 nM by Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. 2–3 siRNAs for each gene were ordered and tested in the study to confirm the result.

2.3. Western blot analysis

Western blot analysis was performed as described previously (Chen et al., 2015). Antibodies (total or phosphor) specific for CFL1, LIMK1, Smad2, RhoA, Akt, JNK, p38, Smad1/5/8 were obtained from Cell Signaling Technology; antibodies for total and phosphor-ERK were purchased from Santa Cruz; antibodies for DSTN, α-tubulin and actin were bought from Sigma. All antibodies used at 1:1000 dilutions except α-tubulin and actin antibodies (1:5000) in this study.

2.4. G-actin/F-actin assay

G-actin and F-actin in cells were quantitated by G-actin/F-actin in vivo assay kit as manufacturer's instructions (Cytoskeleton® Inc.). Cells were harvested by scraping in lysis and F-actin stabilization buffer. The homogenates were incubated at 37 °C for 10 min, transferred to a pre-warmed (37 °C) ultracentrifuge (SORVALL/Thermo Scientific) and spun at 100,000*g* for 1 h at 37 °C to separate the globular (G)-actin (supernatant) and filamentous (F)-actin fractions (pellet). The pellets were re-suspended in ice-cooled depolymerizing buffer. All samples were diluted with appropriate loading buffer for Western blot analysis. The blots were scanned and the protein bands were subjected to intensity quantification in ImageJ® software. Ratios of F-actin or G-actin in cells were calculated according to the density.

2.5. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

For gene expression, total RNA was extracted with TriZoL reagent (Invitrogen), and cDNA was prepared using RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas). The PCR products were visualized in real-time using SYBR Green I Supermix (Bio-Rad) and an iCycle instrument (Bio-Rad) using standard curve protocols, normalized to beta-2-microglobulin (B2m). The quantitative data presented is an average of duplicate or triplicate per independent experiment.

2.6. Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical testing was performed using Student's *t*-test to detect differences between groups. Differences were considered statistically significant at $P < .05$ (*) or $P < .01$ (**).

3. Results

3.1. Actin cytoskeleton is dissembled during adipocyte differentiation of hMSCs

The success of adipocyte differentiation of hMSCs was evidenced by the upregulation of both early and late adipocyte gene markers including peroxisome proliferator activated receptor gamma 2 (PPARG2), Adiponectin (ADIPOQ), lipoprotein lipase (LPL) and fatty acid binding protein 4 (FABP4) (Fig. 1A) during a 13-day in vitro differentiation protocol. As shown in Fig. 1A, Oil-red O staining revealed the formation of mature lipid-filled adipocytes that increased in number throughout differentiation period. By determining the G-actin/F-actin ratio, we observed that monomeric G-actin was increased from 59% to 76% and assembled F-actin decreased from 42% to 24% during the adipocyte differentiation (day 0 and day 13 values, respectively) (Fig. 1B).

Since actin cytoskeleton is regulated by RhoA/LIMK1/CFL1 axis (Maekawa et al., 1999), we examined their expression and activity during adipocyte differentiation (Fig. 1C). We observed that the ratio of phosphor-CFL1 (p-CFL1) to total CFL1 indicating accumulation of the inactive form of CFL1 lacking the ability to disassemble actin fibres, decreased during adipogenic induction mostly and this was caused by highly increased expression of total CFL1 as soon as adipogenic induction started (Fig. 1C). The total and phosphorylation level of LIMK1, as well as the expression of RhoA, which are the upstream kinases responsible for CFL1 phosphorylation, were also reduced during adipocyte differentiation (Fig. 1C). The expression of another actin depolymerisation factor DSTN was unchanged (Fig. 1C). The results suggest that the observed decreased in assembled actin during adipocyte differentiation was associated with changes in RhoA/LIMK1/CFL1 signaling pathway.

3.2. Modulation of actin polymerization regulates adipocyte differentiation of hMSCs

To corroborate the importance of actin assembly for adipocyte differentiation, we examined the effects of cytochalasin D (CytoD) or Phalloidin, which have been shown to dissemble or stabilize the actin cytoskeleton in hMSCs, respectively (Chen et al., 2015), on adipocyte differentiation of hMSCs. We observed that CytoD inhibited but Phalloidin increased cell viability during adipocyte differentiation (Fig. 2A). Disruption of actin assembly by CytoD increased the