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Exchange protein activated by cyclic adenosine monophosphate regulates the switch between adipogenesis and osteogenesis of human mesenchymal stem cells through increasing the activation of phosphatidylinositol 3-kinase

Zihua Tang[a], Dongyan Shi[a], Bingbing Jia[a], Jiarong Chen[a], Chen Zong[a], Dan Shen[b], Qiang Zheng[c], Jinfu Wang[a,*], Xiangming Tong[b,**]

[a] Institute of Cell and Development Biology, College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China
[b] Laboratory of Bone Marrow, The First Hospital, Zhejiang University, Hangzhou, Zhejiang 310006, PR China
[c] Institute of Orthopedics, The Second Hospital, Zhejiang University, Hangzhou, Zhejiang 310009, PR China

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ABSTRACT

Epac, exchange protein activated by cyclic adenosine monophosphate (cAMP), could regulate the trans-differentiation between adipogenesis and osteogenesis of human mesenchymal stem cells (hMSCs). Epac activated by 8-pCPT-2′-*O*-Me-cAMP, a cAMP analog preferentially activating Epac, resulted in the increase of adipogenic gene expression and the decrease of osteogenic gene expression. The pro-adipogenic and anti-osteogenic effect of 8-pCPT-2′-*O*-Me-cAMP was attributed to that 8-pCPT-2′-*O*-Me-cAMP led to the activation of protein kinase B (PKB) and cAMP response element-binding protein (CREB) as well as the inhibition of Ras homolog gene family member A (RhoA), focal adhesion kinase (FAK), extracellular-signal-regulated kinase (ERK) and runt-related transcription factor 2 (Runx2) activities. Inhibition of Epac by a dominant-negative form of Epac1 resulted in the decrease of phosphatidylinositol 3-kinase (PI3K), PKB and CREB activities as well as down-regulation of peroxisome proliferator activated receptor-γ (PPARγ) expression. Inhibition of PI3K by a specific inhibitor or inhibition of Arf and Rho GAP adapter protein 3 (ARAP3, a phosphatidylinositol (PtdIns)(3,4,5)P_3 binding protein) by ARAP3 siRNA led to the recovery of RhoA and FAK activities. RhoA-V14, a constitutively active form of RhoA, could activate the MEK/ERK/Runx2 signaling. Therefore, we conclude that PI3K activated by Epac leads to the activation of PKB/CREB signaling and the up-regulation of PPARγ expression, which in turn activate the transcription of adipogenic genes; whereas osteogenesis is driven by Rho/FAK/MEK/ERK/Runx2 signaling, which can be inhibited by Epac via PI3K. These results should be helpful to provide new targets for treatment of osteoporosis and related bone-wasting diseases.

1. Introduction

Mesenchymal stem cells (MSCs), as a population of mesenchymal precursors, have the capability of self-renewing and the multipotentials of differentiation into a variety of mesenchymal lineages such as osteoblasts and adipocytes under a special culture condition (Pittenger et al., 1999). In bone marrow (BM), marrow adipocytes and osteoblasts are derived from a common mesenchymal progenitor (Robey and Bianco, 1999). The bone loss commonly seen during aging in both males and females is due in part to a reciprocal increase in the development of adipocytes and a decrease in osteoblast differentiation (Moore and Dawson, 1990; Kajkenova et al., 1997). It has been demonstrated that activation of peroxisome proliferator activated receptor-γ2 (PPARγ2) in marrow-derived mesenchymal progenitor cell line stimulates their differentiation into adipocytes and irreversibly blocks their ability to differentiate into osteoblasts (Lecka-Czernik et al., 1999). Thus, PPARγ2 is a potent suppressor of the osteoblast phenotype and critically involved in the differentiation of bone marrow mesenchymal progenitors toward adipocytes. Cyclic adenosine monophosphate (cAMP) has been implicated in the production of endogenous PPARγ ligand(s) occurring during the initial stages of differentiation (Tzameli et al., 2004). The cAMP-responsive element-binding protein (CREB), as a cAMP target whose transcriptional activity is stimulated by cAMP, is a central transcriptional activator of the adipocyte differentiation program (Gonzalez and Montminy, 1989). Activated CREB induces the expression of CCAAT/enhancer-binding protein β (C/EBPβ)

* Corresponding author at: Institute of Cell and Development Biology, College of Life Sciences, Zijingang Campus, Zhejiang University, Hangzhou 310058, PR China. Tel.: +86 571 88206592.
** Corresponding author at: Laboratory of Bone Marrow, The First Hospital, Zhejiang University, Hangzhou 310006, PR China.
E-mail addresses: wjfu@zju.edu.cn (J. Wang), xmtong2003@yahoo.com.cn (X. Tong).

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and thereby triggers the expression of a number of transcription factors, including PPARγ2, and adipocyte-specific genes such as leptin and adipsin (Tang et al., 2004; Zhang et al., 2004).

An exchange protein directly activated by cAMP (Epac) was discovered about 10 years ago. It functions as a guanine nucleotide exchange factor (GEF) for the Ras-like small GTPase Rap (Bos, 2006). It has now been accepted that Epac proteins are novel cAMP sensors that regulate several fundamental cellular processes such as cell proliferation, cell differentiation, cell maturation, cell migration, apoptosis, cytoskeletal rearrangements, and contraction (Takai et al., 2001; Mitin et al., 2005; Roscioni et al., 2007). Two isoforms of Epac, namely Epac1 and Epac2 have been identified so far. Epac1 and Epac2 exhibit a distinct expression pattern in mature and developing tissues. Epac2 mRNA is predominantly expressed in the brain and endocrine tissues and implicated in calcium-induced insulin secretion from pancreatic β-cells (Kang et al., 2003; Holz et al., 2006), whereas Epac1 mRNA is expressed ubiquitously (Roscioni et al., 2007). The recent study has demonstrated that Epac1, working via Rap, acts synergistically with PKA to promote adipogenesis (Petersen et al., 2008). The activation of Rap stimulates the activity of protein kinase B (PKB) via phosphatidylinositol 3-kinase (PI3K) (Mei et al., 2002). In serum-stimulated cells, PKB potently induces Ser-133 phosphorylation of CREB and thereby induces the transcription of PPARγ which is required for adipogenic gene expression (Du and Montminy, 1998).

Runt-related transcription factor 2 (Runx2), a bone-related transcription factor up-regulated by bone morphogenetic protein (BMP) (Ducy et al., 1997; Nakashima et al., 2002), is essential for the differentiation of osteoblasts from mesenchymal precursors and bone formation (Otto et al., 1997). Runx2 can directly stimulate transcription of osteoblast-related genes such as those encoding alkaline phosphatase (ALP), osteocalcin (OC) and type I collagen (COL I) by binding to specific enhancer regions containing the core sequence, PuCCPuCA (Ducy et al., 1997; Selvamurugan et al., 1998; Kern et al., 2001). However, the Runx2-dependent transcription is not simply regulated by levels of the Runx2 protein (Lee et al., 2000). This implies that this transcription factor should be regulated either by protein–protein interactions (Franceschi and Xiao, 2003). The activation of Runx2 may be affected by various factors. The production of extracellular matrix (ECM) dramatically increases the transcription of the OC gene. This matrix response requires Runx2 and its cognate DNA binding site osteoblast specific element 2 (OSE2) in the OC promoter (Xiao et al., 1997). The transcription of Runx2 may be important for its transcriptional activity. However, the Runx2-dependent increase in its transcriptional activity is not accompanied by a significant change in Runx2 mRNA or protein (Xiao et al., 1998). The activation of Runx2 by phosphorylation is also important for its transcriptional activity. The ECM signals to the differentiating preosteoblasts by binding to β1 subunit-containing integrins (Xiao et al., 1998; Takeuchi et al., 1997). As one of the primary transducers of integrin signals to the cell nucleus, the MEK/ERK branch of MAPK pathway provides a plausible link between cell surface integrin activation and subsequent stimulation of Runx2-dependent transcription (Xiao et al., 2000, 2002). The MAPK pathway is one of the principle signal transduction cascades associated with mechanotransduction signals. Integrins, which connects the cytoskeleton to the extracellular matrix and mediates a variety of signaling cascades, may convert mechanical stimuli into biochemical signals. This includes the activation of Ras homolog gene family member A (RhoA) and focal adhesion kinase (FAK), which finally leads to the activation of Runx2 by phosphorylation through MEK/ERK signaling (Schmidt et al., 1998; Ziros et al., 2002). Therefore, it is believed that the MAPK-mediated phosphorylation represents a crucial mechanism modulating the transcriptional activity of Runx2 (Franceschi and Xiao, 2003).

The present work was undertaken to determine whether Epac had any role in the lineage choice switch between adipogenesis and osteogenesis of hMSCs. We demonstrated that the activation of Epac promoted adipogenesis and inhibited osteogenesis of hMSCs. The activation of Epac led to increase of adipogenic gene expression and decrease of osteogenic gene expression. While the activity of Epac was stimulated by 8-pCPT-2′-*O*-Me-cAMP which was a cAMP analog preferentially activating Epac, activities of PI3K and CREB increased and activities of RhoA, FAK, ERK and Runx2 decreased. While Epac was activated, the inhibition of PI3K-kinase activity or the activation of RhoA by RhoA-V14 (a constitutively active RhoA) in hMSCs resulted in the increase of FAK, ERK and Runx2 activities and the decrease of CREB activation and PPARγ expression. We concluded that PI3K functioned as a switch between adipogenesis and osteogenesis of hMSCs. PI3K activated by Epac led to the activation of CREB which thereby up-regulated the expression of PPARγ while inhibited the activation of MEK/ERK/Runx2 signaling pathway through decreasing the activation of RhoA and FAK.

2. Materials and methods

2.1. Cell culture

Human bone marrow was kindly provided by the First People's Hospital of Zhejiang. Health donors, ranging in age from 55 to 65 years, gave written consent to the use of bone marrow for research purpose according to procedures approved by the human experimentation committee at Zhejiang Public Health Bureau. hMSCs were isolated and cultured following a previously described method with some modifications (Xiang et al., 2007). Briefly, hMSCs were cultured in the ordinary medium consisting of alpha minimum essential medium (α-MEM, Gibco-BRL, Hangzhou, China), 10% fetal bovine serum (FBS, Gibco-BRL), 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Beijing, China). Medium was changed every third day. hMSCs at passage 3 were used for the experiments.

2.2. Differentiation and detection

For general adipogenic differentiation, hMSCs with 70–80% confluence were induced into adipogenesis in DMEM (Gibco-BRL) supplemented with 10 ng/mL IGF-I, 100 μmol/L indomethacin, 1 μmol/L dexamethasone (DEX), 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX) (Sigma, Hangzhou, China) and 10% FBS (Sijiqing, Hangzhou, China) (short as DMII). For examination to the effect of Epac on adipogenic differentiation, the medium was changed (IBMX omitted) (short as DMI), and 50 μM 6-MB-cAMP and/or 10 μM 8-pCPT-2′-*O*-Me-cAMP (Sigma) was added. These doses of 6-MB-cAMP and 8-pCPT-2′-*O*-Me-cAMP used in experiments were determined as the lowest effective concentrations for adipogenic differentiation through the dose–response experiments. The media were then changed every other day and cells were harvested for analysis of adipogenesis at indicated days as previously described (Xiang et al., 2001). Briefly, after fixation in 10% formal calcium, induced hMSCs were stained in filtered Oil Red O for 2–3 h, then rinsed with 60% isopropyl alcohol. The optical density (OD) value of lipid vacuole accumulation was detected according to Green and Kehinde (1975).

For osteogenic differentiation, hMSCs were induced into osteocytes by the fluid shear stress (FSS) simulated by a perfusion culture system (Parallel-plate culture flow chamber) as described previously (Grellier et al., 2009). Briefly, a glass slide was seeded with hMSCs at a density of 5×10^4 cells/cm^2. FSS was simulated by the flowing medium consisting of DMEM and 10% FBS. hMSCs on the glass slide were exposed to FSS at a mean value of 4.2 dye/cm^2 for