Cellular Signalling 28 (2016) 1270–1282

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

AMPK promotes osteogenesis and inhibits adipogenesis through AMPK-Gfi1-OPN axis

Yu-gang Wang [a], Xin-hua Qu [a], Ying Yang [a], Xiu-guo Han [a], Lei Wang [a], Han Qiao [a], Qi-ming Fan [a,*], Ting-ting Tang [a,*], Ke-rong Dai [a,b,**]

[a] Shanghai Key Laboratory of Orthopedic Implants, Department of Orthopedic Surgery, Shanghai Ninth People's Hospital, Shanghai JiaoTong University School of Medicine, 639 Zhizaoju Road, Shanghai 200011, People's Republic of China

[b] The Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai JiaoTong University School of Medicine (SJTUSM) & Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS), Shanghai 200025, China

ARTICLE INFO

Article history:
Received 3 April 2016
Received in revised form 27 May 2016
Accepted 5 June 2016
Available online 06 June 2016

Keywords:
AMP-activated protein kinase
Osteopontin
Growth factor independence-1
Osteogenesis
Adipogenesis

ABSTRACT

Several metabolic, genetic and oncogenic bone diseases share the common pathological phenotype of defective bone marrow stromal cell (BMSC) differentiation. Many reports in bone science in the past several years have suggested that the skeleton also has an endocrine role. The role of AMP-activated protein kinase (AMPK) as an energy metabolism sensor and how it regulates BMSC differentiation is largely unknown. In the current study, we used AMPK agonists to activate AMPK in MC3T3-E1 cells to investigate the functional roles of AMPK in osteogenesis. However, metformin and AICAR failed to activate AMPK consistently. Therefore, we established MC3T3-E1 and 3T3-L1 cell models of AMPK α subunit overexpression through lentivirus vector, in which AMPK was overactivated. AMPK hyperactivation stimulated MC3T3-E1 cell osteogenesis and inhibited 3T3-L1 cell adipogenesis. Osteopontin (OPN) mediated AMPK regulation of osteogenesis and adipogenesis. Furthermore, we provided evidence that the transcriptional repressor growth factor independence-1 (Gfi1) was downregulated and disassociated from the OPN promoter in response to AMPK activation, resulting in the upregulation of OPN. Overexpression of wild-type and dominant-negative Gfi1 modulated MC3T3-E1 osteogenesis and 3T3-L1 adipogenesis. Further evidence suggested that AMPK enhanced ectopic bone formation of MC3T3-E1 cells through the AMPK-Gfi1-OPN axis. In conclusion, AMPK was sufficient to stimulate osteogenesis of MC3T3-E1 cells and inhibit adipogenesis of 3T3-L1 cells through the AMPK-Gfi1-OPN axis. These findings helped elucidate the molecular mechanisms underlying AMPK regulation of osteogenesis and adipogenesis.

1. Introduction

Several metabolic, genetic and oncogenic bone diseases, including diabetes, osteoporosis, fibrous dysplasia and osteosarcoma, share a common pathological phenotype of defective bone marrow stromal cell (BMSC) differentiation [1–3]. BMSCs are characterized by their phenotype and ability to differentiate into at least three lineages (chondrocytes, osteoblasts and adipocytes) when cultured under defined *in vitro* conditions [4–6]. In healthy humans, BMSC differentiation is tightly balanced [7]. This balance is disrupted in various physiological and pathological conditions, including aging, immobility, corticosteroid overuse and progressive osseous hyperplasia [8–11]. The balance between osteogenesis and adipogenesis might provide a therapeutic target with which to prevent or treat conditions where there is inadequate bone formation and excessive marrow adipogenesis [12].

The onset of osteogenic differentiation is marked by a decrease in Twist expression and an increase in Runt related transcription factor 2 (Runx2) and Osterix (Osx) expression. Alkaline phosphatase (ALP) is expressed during the early stages of osteogenesis, and bone sialoprotein (BSP) and osteocalcin (OC) are expressed by more mature osteoblasts during matrix maturation and mineralization [13]. Similar to osteogenesis, adipogenic BMSC differentiation is initiated with the commitment to pre-adipocytes, which express adipocyte-specific genes and mature into terminally differentiated, functional adipocytes, in which several

Abbreviations: BMSC, bone marrow stromal cell; AMPK, AMP-activated protein kinase; OPN, osteopontin; Gfi1, growth factor independence-1; Runx2, runt-related transcription factor 2; Osx, Osterix; ALP, alkaline phosphatase; BSP, bone sialoprotein; OC, osteocalcin; PPARγ, peroxisome proliferator activated receptor-γ; C/EBP, CAAT/enhancer binding protein; aP2, adipocyte Protein 2; GLUT4, glucose transporter 4; AICAR, 5-Aminoimidazole-4-carboxamide ribonucleoside; ACC, acetyl-CoA carboxylase; GS, glycogen synthase; PGC1α, peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α.

* Corresponding authors.
** Correspondence to: K. Dai, Shanghai Key Laboratory of Orthopedic Implants, Department of Orthopedic Surgery, Shanghai Ninth People's Hospital, Shanghai JiaoTong University School of Medicine, 639 Zhizaoju Road, Shanghai 200011, People's Republic of China.
E-mail addresses: chillow@163.com (Q. Fan), tingtingtang@hotmail.com (T. Tang), krdai@163.com (K. Dai).

http://dx.doi.org/10.1016/j.cellsig.2016.06.004
0898-6568/

factors are involved, most notably peroxisome proliferator activated receptor-γ (PPARγ) and the CAAT/enhancer binding protein (C/EBP) family. They then direct the expression of adipocyte Protein 2 (aP2), fatty acid synthase (FAS), lipoprotein lipase (LPL), and glucose transporter 4 (GLUT4), all of which induce changes in extracellular matrix (ECM) production, metabolism, and lipid accumulation associated with adipocyte maturation.

Several factors and signaling pathways, including PPARγ [14–16], C/EBPα [7], Notch [17,18] and Wnt [19] pathways, have been reported to play important roles in the balance between BMSC osteogenesis and adipogenesis. However, BMSC fate determination is a complicated process. The aforementioned target pathways are not sufficient to completely elucidate the underlying transcriptional mechanisms. The identification of new target genes and pathways involved in regulating the balance between BMSC osteogenesis and adipogenesis is required.

Recent reports have identified several important hormones secreted by bone cells that control energy balance and mineral ion homeostasis, highlighting an endocrine role for the skeleton. The elucidation of molecular mechanisms underlying how energy metabolism and hormone production in the skeleton are regulated will be of biological and clinical importance and provide deeper insights into the pathogenesis of many diseases. In eukaryotic cells, AMP-activated protein kinase (AMPK) monitors changes in intracellular ATP levels and couples this change to phosphorylation of downstream substrates, leading to an increase in the rate of ATP-producing pathways and/or a decrease in the rate of ATP-utilizing pathways [20]. Mammalian AMPK comprises three subunits, termed α, β and γ, in a heterotrimeric complex. The phosphorylation of α subunit Thr172 by upstream kinases is required for AMPK activation.

The definitive function of AMPK in bone has remained elusive despite several *in vitro* and *in vivo* studies [21]. In previous reports aimed at the role of AMPK in bone metabolism, some AMPK agonists, such as metformin and 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), were employed to activate AMPK in mammalian cells [21–24]. However, the use of these AMPK agonists is somewhat problematic. For example, they have several AMPK-independent effects that cause off-target effects, and they are difficult to consistently activate AMPK in some cell types, *e.g.*, MC3T3-E1, which has hindered the elucidation of AMPK function in bone metabolism. Therefore, we attempted to establish a cell model in which AMPK could be specifically and continuously activated. Lentivirus vector was employed to stably overexpress AMPK α subunit in MC3T3-E1 and 3T3-L1 cells. Using these cell models, we showed that the AMPK-growth factor independent 1 (Gfi1) -osteopontin (OPN) axis regulated osteogenesis and adipogenesis.

2. Materials and methods

2.1. Cell culture

MC3T3-E1 cells were cultured in α-minimal essential medium (MEM) (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Hyclone). Cultures were incubated in a humidified atmosphere at 37 °C and 5% CO_2. Culture medium was changed every 3 days. MC3T3-E1 cells were treated with rhBMP-2 (R&D, Minneapolis, MN, USA) at a final concentration of 100 ng/ml for osteogenesis induction. The medium was changed every 3 days.

3T3-L1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. The cultures were incubated in a humidified atmosphere at 37 °C and 5% CO_2. Culture medium was changed every 3 days. Adipogenic differentiation was induced as described previously [7]. Briefly, when cells reached confluence, they were fed with complete adipogenic hormone cocktail: DMEM supplemented with 10% FBS, 10 μg/ml insulin (Sigma, St. Louis, MO, USA), 0.5 mM methylisobutylxanthine (MIX) (Sigma) and 1 μM Dex (Sigma). The start point of differentiation was considered day 0. At day 3, cells were fed with DMEM containing only insulin and 10% FBS. At day 6, complete adipogenic hormone cocktail was added a second time.

2.2. Reverse transcription (RT)-PCR and quantitative RT-PCR

Total cellular RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. For RT-PCR, single-stranded cDNA was reverse transcribed from 1 μg total RNA using reverse transcriptase with oligo-dT primer. All PCR reactions were carried out using 1 μl of each cDNA using the following cycling parameters: 94 °C, 40 s; 60 °C, 40 s; and 72 °C, 40 s for 30 cycles. PCR samples were analyzed by DNA agarose gel electrophoresis. Quantitative PCR analysis was performed on a 96-well plate ABI Prism 7500 Sequence Detection system (Applied BioSystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (Takara Bio Inc., Otsu, Japan). Cycling conditions was as follows: 94 °C, 5 s; 60 °C, 34 s; and 72 °C for 40 cycles. The comparative $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of each target gene as described previously [1]. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as an internal control for adipogenic genes, including PPARγ, C/EBPα and aP2. β-Actin was used as an internal control for the other genes. Primer sequences are listed in Supplementary Table 1.

2.3. Western blot

Cells were lysed on ice for 30 min in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors (10 g/ml leupeptin, 10 g/ml pepstatin A and 10 g/ml aprotinin). Proteins were fractionated by SDS-PAGE, transferred to nitrocellulose membrane and detected using the following antibodies: anti-p-AMPKα (Thr172) (#2535, Cell Signaling Technology, Danvers, MA, USA), anti-AMPKα (#5831, CST), anti-AMPKα1 (#2795, CST), anti-p-ACC (Ser79) (#11818, CST), anti-ACC (#3676, CST), anti-p-GS (Ser641) (#3891, CST), anti-GS (#3886, CST), anti-PPARγ (#2435, CST), anti-C/EBPα (#8178, CST), anti-HPRT (ab10479, Abcam, Cambridge, MA, USA), anti-Gfi1 (ab107589, Abcam), anti-HA tag (#5017, CST) and anti-β-actin (#4970, CST). Proteins were detected using an enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ, USA).

2.4. Lentivirus

Lentiviral vectors containing the coding sequences for AMPKα, Gfi1 and siRNA against OPN were purchased from Genecopoeia™. Lentiviral particles were generated as described previously [3]. Briefly, 1.3–1.5 × 10^6 293 T cells were plated in a 10 cm dish until they reached 70–80% confluence. The transfection complex was directly added to each dish. The cells were then incubated in a CO_2 incubator at 37 °C overnight. Lentivirus particle-containing culture medium was collected 48 h post-transfection.

2.5. Alizarin red and oil red O staining

Mineralization nodules were stained with alizarin red S (AR-S). After fixation, cells were washed in PBS and soaked in 40 mM AR-S (pH 4.2) for 30 min at 37 °C. The cells were then washed with PBS and photographed.

Adipogenesis was evaluated through oil red O staining as described previously [2]. Briefly, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 2 h at 4 °C. After two washes in PBS, cells were stained for 2 h in freshly diluted oil red O solution (6 parts oil red O stock solution and 4 parts H_2O; oil red O stock solution is 0.5% oil red O in isopropanol) at 4 °C. The stain was removed, and the cells were washed twice with PBS. Oil red O staining was examined with an inverted microscope.