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The interferon-inducible protein p205 acts as an activator in osteoblast differentiation of mouse BMSCs

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ABSTRACT

p205, an interferon-inducible protein, is induced in the course of osteogenesis in mouse bone marrow stromal cells (BMSCs). Knocking down p205 markedly impairs whereas overexpressing p205 enhances the osteoblast differentiation of BMSCs, as revealed by the altered expression of osteogenic genes, the change of ALP activity and the ARS-stained mineral nodules. The positive role of p205 in BMSC osteogenesis is probably due, at least in part, to the association of it with Id proteins. Further investigation indicated that p205 may disturb the formation of Runx2/Ids complex and free more Runx2 to induce the differentiation process. Taken together, our findings demonstrated for the first time that p205 functions as an activator in osteoblast differentiation.

1. Introduction

The bone is a living tissue throughout life, and bone remodeling is precisely coordinated by the interplay of osteoblasts and osteoclasts. The loss of equilibrium between osteoblastic and osteoclastic functions may result in bone diseases, such as osteoporosis or osteopetrosis (Zaidi, 2007). Osteoblasts in the bone derive from bone marrow stromal cells (BMSCs). The differentiation of uncommitted BMSCs to osteoblasts is a fundamental process, which is positively or negatively regulated by numerous activators and repressors (Imai et al., 2009; Gallagher and Sai, 2010). Among these, Runx2 (also known as Cbfa1, or Osf2) is an essential transcription factor for osteoblast differentiation, which regulates the expression of several bone-specific genes, and controls the deposition of bone extracellular matrix. Full knockout of Runx2 results in a complete lack of ossification in mice (Ducy et al., 1997).

Interferons are cytokines which extensively show their activities in cell growth and differentiation. The function of interferons is mediated by a variety of interferon-inducible proteins (Asefa et al., 2004; Kunzelmann et al., 2006; Caposio et al., 2007). The interferon- inducible p200 family of proteins (IFI-200 family) are defined by having at least one 200-amino-acid homology region, designated as either *a* or *b* domain, which is highly conserved among family members (Asefa et al., 2004). Murine IFI-200 family members include p202, p203, p204, p205, and AIM2. p202 and p204 are two most extensively studied family members, which inhibit the growth of many cell types, including NIH3T3, AKR2B, B6MEF, B/cMEF fibroblasts, and cancer cells (Lembo et al., 1995; Choubey, 2000; Wen et al., 2000; Luan et al., 2008a). They are also important regulators in myogenesis, cardiogenesis, chondrogenesis, osteogenesis, and adipogenesis (Datta et al., 1998; Liu et al., 2005; Ding et al., 2006; Zhang et al., 2008; Xiao et al., 2010). For example, p204 acts as a transcriptional coactivator of Runx2 and enhances the osteoblast differentiation process, which is due to p204 binding to inhibitor of differentiation (Id) proteins, and overcoming the inhibition of the Runx2 activity by the Id proteins (including Id1, Id2, and Id3) (Liu et al., 2005; Luan et al., 2008b). Id proteins belong to the helix-loop-helix (HLH) family, and function as regulators in cell growth and differentiation (Liu et al., 2002; Ruzinova and Benezra, 2003; Ding et al., 2006).

In contrast to p202 and p204, the involvement of p205 in cell growth and differentiation remains poorly characterized. p205 consists of 425 amino acid residues, which possesses only a single *a* domain (Asefa et al., 2004). As reported earlier, overexpression of p205 slows down the proliferation of hematopoietic cell lines and primary bone marrow cells (Dermott et al., 2004). p205 induces growth arrest in the human osteosarcoma cell lines U20s and Saos2 (Asefa et al., 2006). p205 is up-regulated in murine hematopoietic stem cells during myeloid cell differentiation, and

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plays a role in myeloid cell development (Weiler et al., 1999). Our recent publication indicated that p205 is involved in the adipogenic differentiation of mouse adipose-derived stem cells (mASCs) (Liu et al., 2014). These findings prompted us to test whether p205 also serves as a modulator in the course of osteoblast differentiation.

In this study, the time-course of p205 expression during mouse BMSC osteogenesis was examined. Next, the effects of knockdown and overexpression of p205 on osteoblast differentiation were determined, with regard to alkaline phosphatase (ALP) activity, mineralization, and expression of osteogenic marker genes, including Runx2, collagen type I (ColI), and osteocalcin (OCN). Furthermore, the molecular mechanisms underlying these processes were elucidated by using co-immunoprecipitation (Co-IP) assay, and a luciferase reporter assay for examining gene regulatory activity.

2. Material and methods

2.1. Isolation and culture of mouse BMSCs

Four-week-old female Kunming mice (SPF grade) were purchased from Center for New Drugs Evaluation of Shandong University. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals approved by the Animal Care and Use Committee of Shandong province, China. The mice were killed by cervical dislocation under sodium pentobarbital anesthesia. Mouse BMSCs were isolated from femur and tibia bone marrow as previously described (Li et al., 2010). The isolated cells were cultured and passaged as described. All the following experiments were performed using the third passage cells. Osteoblast differentiation of BMSCs was assessed in a complete culture medium supplemented with osteogenic stimuli (OS) consisting of 0.01 μM dexamethasone, 50 μg/mL L-ascorbic acid and 10 mM β-glycerophosphate.

2.2. Quantitative real time PCR (qRT-PCR)

After treating with experimental test agents, total cellular RNA was extracted using TRizol reagent (Invitrogen). cDNA was synthesized from 1 μg total RNA using a reverse transcription kit (Takara). cDNA samples were subjected to qRT-PCR amplification with primers as described in Table 1. qRT-PCR with SYBR Green was performed using a Bio-Rad real time PCR system following the manufacturer's protocol. Melt-curve analysis was conducted to verify that only one product was produced. The mRNA levels of specific genes were calculated relative to the GAPDH levels using the $2^{-\Delta\Delta Ct}$ method.

2.3. Western blotting

Total cellular protein was collected using RIPA Lysis Buffer according to the manufacturer's instruction (Beyotime, China). Aliquots of protein from each sample were used to assay the protein levels of p205, and Runx2, with GAPDH as the internal control. A rabbit p205 antibody (a 15-amino-acid sequence corresponding to the C-terminus of p205, KVTKAGKKKEASTVQ) was produced by Genescript Biotechnology (Nanjing, China). Other antibodies were purchased from Abcam or Santa Cruz Biotechnology. Proteins were subjected to SDS-PAGE on a 12% polyacrylamide gel and transferred onto a PVDF membrane (Millipore, MA). After blocking with 5% nonfat milk in TBS containing 0.05% Tween-20, the membrane was incubated overnight at 4 °C with the primary antibody. Subsequently, the appropriate secondary antibody (horseradish peroxidase-conjugated) was added, and bound antibody was visualized via chemiluminescence using an ECL kit (Amersham, Germany).

2.4. Construction of recombinant plasmids

A p205 expression plasmid was generated by inserting the entire coding region into the transfer vector pcDNA3.1(+), which was named as pcDNA-p205. To obtain luciferase reporter constructs, the appropriate DNA segment containing the promoter region of OCN was cloned into the pGL3-basic vector (pGL3-OCNp). The sequences of the primers with restriction enzyme sites were shown in Table 1. The correctness of the constructs was confirmed by sequencing.

2.5. Transfection procedures

The RNA interference technique was used to down-regulate p205 gene expression. The specific nucleotide sequences for silencing p205 (SiRNA-p205) and the control RNA duplex sequences (SiRNA-control) were presented in Table 1. BMSCs were cultured in an antibiotic-free medium for more than 24 h. Sub-confluent cells were exposed to Opti-MEM (Invitrogen) and were transfected with either SiRNA-p205 or SiRNA-control (120 nM) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Treatment with only Lipofectamine 2000 was also performed as a Mock sample in parallel. After 8 h, the induction medium containing OS was added to the culture plates (day 0). To overexpress the p205 protein, BMSCs were transfected with pcDNA-p205 (400 ng/mL) using Lipofectamine 2000. Vector pcDNA3.1(+) (pcDNA)-transfected sample was used as a control.

2.6. ALP activity and ARS staining assay

On day 4, treated cells were washed twice with PBS and lysed by two cycles of freezing and thaw. ALP activity in the cells was

Table 1
Sequences of the primers and siRNA duplexes used in this study. The restriction enzyme sites were labeled with bold.

Names	Forward (5′–3′)	Reverse (5′–3′)
p205	GCTGATGCTGGATTGGAC	GTGGCTTGTAGTTGATGTAGG
GAPDH	GACTTCAACAGCAACTCCCAC	TCCACCACCCTGTTGCTGTA
Runx2	CCGCACGACAACCGCACCAT	CGCTCCGGCCCACAAATCTC
ColI	CTGCCTGCTTCGTGTAAA	ACGTTCAGTTGGTCAAAGGTA
OCN	GAGCCCTTAGCCTTCCAT	GCGGTCTTCAAGCCATAC
SiRNA-p205	GAUCAAGGCAUCUGGGAAA	UUUCCCAGAUGCCUUGAUC
SiRNA-control	UUCUCCGAACGUGUCACGU	ACGUGACACGUUCGGAGAA
p205-fragment	CCC**AAGCTT**ATGGAGAATGAATATAAGAGACTTG	CCG**CTCGAG**TCACTGGACAGTTGATGCTTC
OCN-promoter	CCC**ACGCGT**ATCAAGCGGGCTCCTCAC	GGG**CTCGAG**ACCCTCCAGCGTCCAGTA