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Protein tyrosine phosphatase profiling studies during brown adipogenic differentiation of mouse primary brown preadipocytes

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There is a correlation between obesity and the amount of brown adipose tissue; however, the molecular mechanism of brown adipogenic differentiation has not been as extensively studied. In this study, we performed a protein tyrosine phosphatase (PTP) profiling analysis during the brown adipogenic differentiation of mouse primary brown preadipocytes. Several PTPs, including PTPRF, PTPRZ, and DUSP12 showing differential expression patterns were identified. In the case of DUSP12, the expression level is dramatically downregulated during brown adipogenesis. The ectopic expression of DUSP12 using a retroviral expression system induces the suppression of adipogenic differentiation, whereas a catalytic inactive DUSP12 mutant showed no effect on differentiation. These results suggest that DUSP12 is involved in brown adipogenic differentiation and may be used as a target protein for the treatment or prevention of obesity by the regulation of brown adipogenic differentiation. [BMB Reports 2013; 46(11): 539-543]

INTRODUCTION

Brown adipose tissue (BAT) is a specialized form of adipose tissue in mammals. It is mainly localized around the neck and large blood vessels of the thorax. BAT can generate body heat by uncoupling the respiratory chain of oxidative phosphorylation in mitochondria. Energy expenditure for thermogenesis in BAT serves either to maintain the body's temperature against cold exposure or to waste food energy, indicating its role in both thermal and energy balance. Until recently, BAT has been considered to be limited to human infants. However, mounting evidence of the existence of BAT in human adults has been reported (1, 2). Furthermore, a strong correlation between obesity and the amount of BAT in the body has been reported by many research groups (1-3). Therefore, a deep understanding of molecular mechanisms for brown adipogenesis is critical with regard to the treatment and prevention of obesity. Research on the molecular mechanisms and signal transduction activities related to brown adipogenic differentiation has not been as extensive as that pertaining to white adipogenic differentiation because it was only recently identified in adult humans (3, 4).

Reversible phosphorylation on the tyrosine residue of proteins is one of the most common and typical regulatory mechanisms by which organisms control key cellular processes, such as cell adhesion, the cell cycle, cell survival, as well as cell proliferation and differentiation (5). The levels of phosphotyrosine are tightly regulated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). It has long been considered that PTPs fulfill a housekeeping function associated with the maintenance of the basal state. However, there is mounting evidence that PTPs play a more active role in various important cellular key processes (4-6). Presently, it is generally accepted that PTPs act as both positive and negative regulators for many signaling pathways, including those related to cell differentiation. However, their relationship to brown adipogenesis has not been extensively investigated until now. Recently, several studies regarding the involvement of PTP1B in brown adipogenesis have been reported (7, 8).

There are a total of 107 PTPs in the human genome. Of these, 38 encode classical, phosphotyrosine-specific enzymes (receptor type PTPs and non-receptor type PTPs), whereas the other 43 are defined as dual-specificity phosphatases (DUSPs) that can dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues in proteins (MKPs, PTENs, PRLs and atypical PTPs etc). DUSPs have been identified as the main modulators of key signaling pathways that are dysregulated in many diseases (9). DUSP12 is an evolutionary conserved atypical DUSP whose function is poorly explored. DUSP12 was reported as a pro-survival phosphatase and is known to be related to cancer (9). Additionally, DUSP12 plays a role in ribosome biogenesis and in cell cycle progression

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(10, 11). In this study, we suggest that DUSP12 is involved in the brown adipogenic differentiation of mouse primary brown preadipocytes.

RESULTS AND DISCUSSION

As an initial step, we isolated brown preadipocytes from mouse interscapular brown adipose tissue and induced differentiation into brown adipocytes. As shown in Fig. 1A, the cells showed increasing numbers of Oil-red O-stainable lipid drops with an increase in the culture time. In addition, the expression levels of the brown adipocyte-specific genes of UCP-1, PGC-1α, and PRDM16 were significantly upregulated during brown adipogenesis (Fig. 1B). These results strongly indicated that brown preadipocytes from mice were successfully isolated and differentiated into mature brown adipocytes. Next, to determine the PTPs involved in brown adipogenesis, we performed a reverse transcriptase (RT) PCR analysis on samples obtained from both preadipocytes and mature brown adipocytes. Among a total of 107 PTPs, various PTPs showed differential expression patterns during brown adipogenesis (Fig. 2A). In particular, the number of receptor-type PTPs was decreased in mature brown adipocytes compared to that in preadipocytes. These PTP profiling results using mouse primary brown preadipocytes were significant different from those obtained from the brown preadipocyte cell line HIB1B (12). The exact cause of this is not known, but we believe that data obtained from primary brown adipocytes is more valuable for studying the mechanisms of brown adipogenesis. Among the PTPs showing differential expression patterns dur-

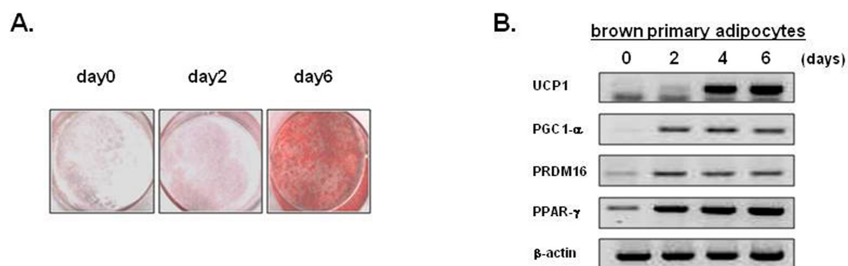

Fig. 1. Brown adipogenic differentiation of mouse primary preadipocytes. (A) The accumulation of lipid droplets in the cells was measured using Oil-red O staining. (B) The mRNA expression levels of brown adipocyte-specific markers, in this case UCP-1, PGC-1α, PRDM16 and PPARγ, were analyzed in a RT-PCR analysis. The total RNA was extracted on the indicated days of differentiation. β-actin was used as a loading control.

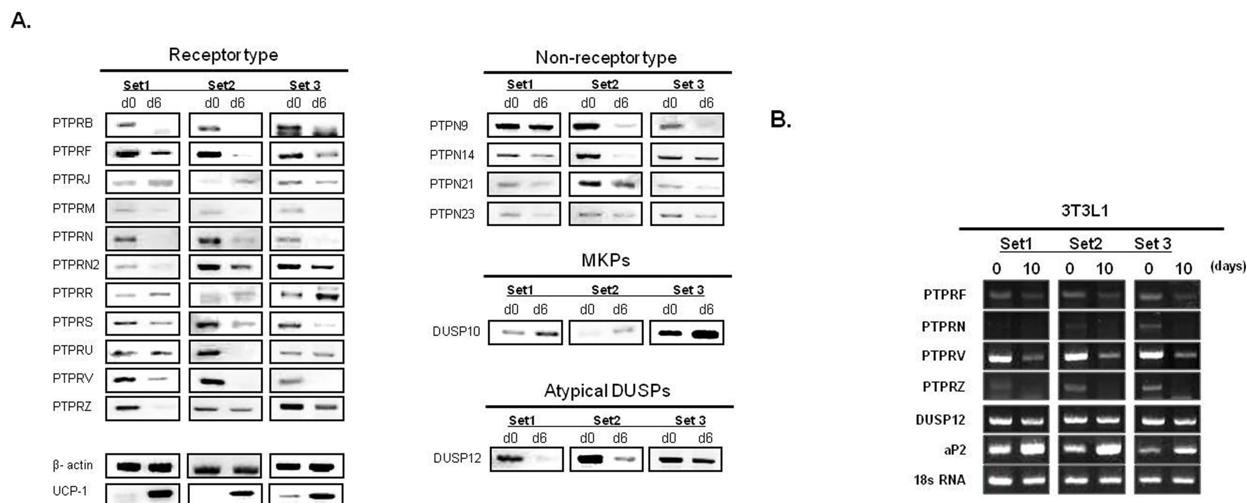

Fig. 2. PTP mRNA profiling analysis during adipogenesis. (A) PTP mRNA profiling analysis during the brown adipogenesis of primary mouse brown preadipocytes. We performed the experiments three times using independent samples. The PTPs showing differential expression patterns are displayed. (B) mRNA profiling analysis of PTPRF, PTPRN, PTPRV, PTPRZ, and DUSP12 during the white adipogenesis of 3T3-L1 preadipocytes. The mRNA expression levels of PTPs were measured by RT-PCR.

ing brown adipogenesis, DUSP12 showed dramatic expression change and there have been no reports about its involvement in adipogenesis or the insulin signaling pathway. Furthermore, the brown adipogenesis-specific differential expression pattern was detected only in the case of DUSP12 (Fig. 2B). Therefore, we focused on DUSP12. As shown in Fig. 2, a marked reduction in the mRNA levels of DUSP12 was detected in mature brown adipocytes. The protein levels of DUSP12 were also measured by a western blot analysis using the anti-DUSP12 antibody (Fig. 3A). The protein levels of DUSP12 also dramatically decreased from the early stages of brown adipogenesis. This result suggests that the reduced expression of DUSP12 at the early stages of adipogenesis is important. Next, to clarify the functional role of DUSP12 in brown adipogenesis, we infected primary brown adipocytes with DUSP12 using a retroviral expression system (DUSP12-IRES-GFP). A catalytically inactive mutant DUSP12 (DUSP-CS) and a control vector were used as negative controls. Infected preadipocytes were enriched using a FACS sorter. Most of the cells were found to be GFP-positive according to fluorescence microscopy results, and the overexpression of the wild-type and mutant DUSP12 was examined by a western blot analysis (Fig. 3B). Then, preadipocytes were induced to differentiate into mature brown adipocytes, after which Oil-red O staining was performed to measure the lipid accumulation after six days of differentiation (Fig. 3C). The ectopic expression of DUSP12 was continuously detected in mature brown adipocytes (data not shown). The ectopic expression of DUSP12 induced a significant suppression of brown adipocyte differentiation compared to that of the DUSP12-CS mutant. This result indicates that the phosphatase activity of DUSP12 is essential in brown adipogenesis. Next, we checked the mRNA changes of brown adipocyte-specific marker proteins, specifically UCP-1 and PGC-1α, upon DUSP12 ectopic expression (Fig. 3E). The ectopic expression of DUSP12 led to a slight decrease in the mRNA levels of UCP-1 and PGC-1α, indicating that DUSP12 may induce a reduction of brown adipogenesis by decreasing the thermogenic program during differentiation.

The DUSPs are members of the PTP family, and they dephosphorylate serine, threonine, and tyrosine residues. In addition, they are important regulators of multiple signaling pathways that modulate cell processes, such as proliferation, apoptosis, and differentiation (13). The DUSP family can be subdivided into subgroups based on the existence of specific domains and on the degree of sequence similarity. A poorly explored subgroup, atypical DUSPs, does not fit into any of the better characterized subgroups. DUSP12 is an atypical DUSP that has not been extensively investigated in relation to its functional role in mammals (9-11). DUSP12 is ubiquitously expressed, especially highly in the spleen, testes and ovaries (from GeneCard®,

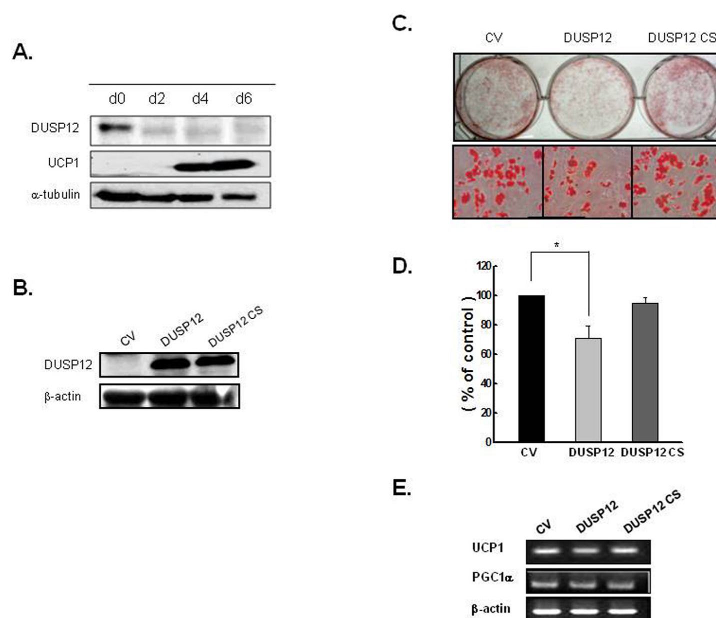

Fig. 3. Overexpression of DUSP12 suppresses brown adipogenic differentiation. (A) The protein level of DUSP12 was checked by western blot analysis. (B) The expression of DUSP12 was confirmed by western blot analysis using the anti-DUSP12 antibody. (C) Brown preadipocytes expressing DUSP12 or a DUSP12-CS mutant were induced to differentiate into mature brown adipocytes for six days after culturing with a differentiation medium. Then, the samples were stained with Oil-red O to visualize the lipid droplets. (D) Quantification of the stained cells was performed using a dye extraction buffer. Data represent the mean percentage levels ± s.d. values compared with a control vector (n = 3; *P < 0.05). (E) The expression levels of brown-specific adipogenic markers (UCP-1 and PGC-1α) were analyzed using RT-PCR after DUSP12 overexpression.

www.genecards.org). In particular, DUSP12 is an interaction protein with glucokinase whose activity is controlled by phosphorylation (14). Thus, it may participate in the glycolysis pathway by the dephosphorylation of glucokinase in the glucokinase-expressed tissues (e.g., the liver and pancreas). In addition, it has been reported that adipocyte-derived factors induce glucokinase gene transcription (15). However, there has been no report demonstrating the role of DUSP12 in adipose tissues. In future studies, we aim to determine the intracellular substrate(s) of DUSP12 and investigate the detailed functional role of DUSP12 in brown adipogenesis.

Similar expression pattern changes were detected during white adipogenesis in relation to PTPRF, PTPRN, PTPRV and PTPRZ (Fig. 2B). We already reported the functional roles of PTPRF as a negative regulator in white adipogenesis in a previous work (16). However, there has been no report on the role of PTPRN, PTPRV, and PTPRZ in adipogenesis. Therefore, further investigations of the roles of PTPRN, PTPRV and PTPRZ are necessary.

In conclusion, our PTP profiling analysis data together with the further characterization of PTPs showing a differential expression pattern should provide valuable information leading to a deeper understanding of brown adipogenesis.

MATERIALS AND METHODS

Isolation and differentiation of mouse primary brown preadipocytes

Brown preadipocytes were obtained from the interscapular brown adipose tissue (BAT) of mice (age: late fetal to post-natal days 1-2) and were enriched by collagenase dispersion, as described previously (17). Enriched cells were cultured in a growth medium [high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 1% antibiotic-antimycotic solution and 20% fetal bovine serum; Gibco-Invitrogen] at 37°C in a humidified atmosphere with 5% CO_2. The brown preadipocytes were induced to differentiate into mature brown adipocytes using a method described in the literature (17).

Oil-red O staining

Lipid droplets of differentiating or mature brown adipocytes were stained by an Oil-red O staining method, as described previously (17-20). For a quantification analysis, the Oil-red O staining dye was extracted and quantified, as previously described (18, 19).

Quantification of PTPs during brown adipogenesis

Total RNA was extracted from cultured cells using the QIAzol lysis reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized using total RNA (2 μg) as a template, random primers (500 ng), and cDNA synthesis kit components (Promega) in a total volume of 25 μl in accordance with the manufacturer's recommendations (21-24). The targeted fragment of cDNA for each of the brown adipocyte differentiation-associated genes was amplified by PCR with 2 μl of the reverse transcription (RT) product, 10 pmol of each primer, and a PCR premix (Nanohelix, Daejeon, Korea). In three sets of experiments, PTPs displaying significant changes were defined as differentially expressed.

DUSP12 overexpression using retroviral expression system

To express the DUSP12 in primary preadipocytes, a retrovirus-mediated infection system was used. The gene encoding DUSP12 was inserted into the multi-cloning site of the pRetroX-IRES-ZsGreen1 vector (Clontech). Then, retroviruses were generated by transiently co-transfecting GP2-293 cells with a retroviral vector and the VSV-G plasmid using Lipofectamine 2000 (Gibco-Invitrogen). At 48 h after transfection, media containing retroviruses were collected, filtered with 0.45-μm filters, and used to infect cells in the presence of polybrene (8 μg/ml). Infected cells were selected by means of fluorescence-activated cell sorting (FACS; FACSAria cell sorter, BD Biosciences) and were further maintained in a growth medium as described previously (18-20).

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