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PI3K/Akt is involved in brown adipogenesis mediated by growth differentiation factor-5 in association with activation of the Smad pathway ☆

Eiichi Hinoi, Takashi Iezaki, Hiroyuki Fujita, Takumi Watanabe, Yoshiaki Odaka, Kakeru Ozaki, Yukio Yoneda *

Laboratory of Molecular Pharmacology, Division of Pharmaceutical Sciences, Kanazawa University Graduate School, Kanazawa, Ishikawa 920-1192, Japan

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ABSTRACT

We have previously demonstrated promotion by growth differentiation factor-5 (GDF5) of brown adipogenesis for systemic energy expenditure through a mechanism relevant to activating the bone morphological protein (BMP) receptor/mothers against decapentaplegic homolog (Smad)/peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α) pathway. Here, we show the involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in brown adipogenesis mediated by GDF5. Overexpression of GDF5 in cells expressing adipocyte protein-2 markedly accelerated the phosphorylation of Smad1/5/8 and Akt in white and brown adipose tissues. In brown adipose tissue from heterozygous *GDF5*^{Rgsc451} mutant mice expressing a dominant-negative (DN) GDF5 under obesogenic conditions, the basal phosphorylation of Smad1/5/8 and Akt was significantly attenuated. Exposure to GDF5 not only promoted the phosphorylation of both Smad1/5/8 and Akt in cultured brown pre-adipocytes, but also up-regulated *Pgc1a* and *uncoupling protein-1* expression in a manner sensitive to the PI3K/Akt inhibitor Ly294002 as well as retroviral infection with DN-Akt. GDF5 drastically promoted BMP-responsive luciferase reporter activity in a Ly294002-sensitive fashion. Both Ly294002 and DN-Akt markedly inhibited phosphorylation of Smad5 in the nuclei of brown pre-adipocytes. These results suggest that PI3K/Akt signals play a role in the GDF5-mediated brown adipogenesis through a mechanism related to activation of the Smad pathway.

1. Introduction

Growth differentiation factor-5 (GDF5), which is also known as cartilage-derived morphogenetic protein-1, is a member of bone morphogenic protein (BMP)/GDF subfamily [1,2]. Mutations of GDF5 are shown to lead to skeletal dysplasia and osteoarthritis in humans and mice [3,4]. We have recently demonstrated that transgenic mice overexpressing GDF5 in adipose tissues (=adipocyte protein-2 (*aP2*)-*GDF5* mice) showed a lean phenotype with a protective property against the high fat diet (HFD)-induced obesity in association with increased systemic energy expenditure [5]. Marked impairment of both energy expenditure and thermogenesis was seen in mutant mice harboring dominant-negative (DN) form of GDF5 (=*DN-GDF5* mice) during breeding under obesogenic conditions [5]. Moreover, exposure to GDF5 promoted cellular differentiation and maturation through a mechanism related to the successive signaling from surface BMP receptor (BMPR) to intracellular mothers against decapentaplegic homolog-5 (Smad5)

Abbreviations: AMPK, AMP-activated protein kinase; aP2, adipocyte protein-2; BAT, brown adipose tissue; BMP, bone morphogenic protein; BMPR, bone morphogenic protein receptor; BRE-Luc, bone morphogenic protein-responsive luciferase reporter plasmid; DMEM, Dulbecco's modified Eagle medium; DN, dominant negative; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDF5, growth differentiation factor-5; HFD, high fat diet; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; PGC-1α, peroxisome proliferator-activated receptor gamma co-activator 1α; PI3K, phosphatidylinositol 3-kinase; Smad, mothers against decapentaplegic homolog; sWAT, subcutaneous white adipose tissue; UCP1, uncoupling protein-1; vWAT, visceral white adipose tissue; WT, wild-type.

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* Corresponding author. Address: Laboratory of Molecular Pharmacology, Division of Pharmaceutical Sciences, Kanazawa University Graduate School, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan. Fax: +81 (0)76 234 4471.

E-mail address: yyoneda@p.kanazawa-u.ac.jp (Y. Yoneda).

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and nuclear peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α) in cultured brown adipocytes [5]. Activation of these BMP/GDF signals leads to facilitation of a variety of intracellular signals in a manner dependent on the type I and type II serine/threonine kinase [6]. The BMP/GDF signaling pathway involves the transcription factors Smad1/5/8, which are all capable of interacting with the universal co-Smad, Smad4, to form heterodimers for recognition of the Smad binding element at upstream promoter regions of different target genes [7]. In addition to this Smad pathway, BMP/GDF family members utilize the phosphatidylinositol 3-kinase (PI3K)/Akt pathway to regulate a variety of biological activities [8]. BMP2 promotes osteogenesis by directly stimulating PI3K and subsequent Akt via BMPR [9], for example, while BMP7 suppresses granulosa cell apoptosis in conjunction with activation of the PI3K/3-phosphoinositide-dependent protein kinase-1/Akt signaling pathway [10]. In the present study, therefore, we have attempted to demonstrate the possible involvement of PI3K/Akt in mechanisms underlying brown adipogenesis mediated by GDF5 *in vivo* and *in vitro*.

2. Materials and methods

2.1. Mice

aP2-GDF5 mice were generated as previously described [5]. *GDF5*^{Rgsc451} mice (M100451) were generously provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan [11]. Mice were maintained on a 12 h light/dark cycle with free access to food and water. Male mice were used throughout experiments. The protocol employed here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University.

2.2. Materials

Brown pre-adipocyte cell lines derived from newborn wild-type (WT) mice were kindly provided by Dr. C.R. Kahn (Joslin Diabetes Center, Boston, MA, USA) [12]. PLAT-E cells were generously provided by Dr. T. Kitamura (Tokyo University, Tokyo, Japan) [13]. 1053 pBabe puroL Akt K179M T308A S473A (Addgene plasmid 9013) was obtained from Addgene (Cambridge, MA, USA). Recombinant mouse GDF5 was purchased from R&D Systems (Minneapolis, MN, USA). LY294002 was obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies were purchased from different companies as follows: anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-phospho Smad1/5/8, anti-phospho Smad2 and anti-phospho Akt (T308) from Cell Signaling Technology; anti-Smad5 and anti-β-Actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-Lamin B1 from Zymed laboratories (South San Francisco, CA, USA). THUNDERBIRD SYBR qPCR Mix was supplied by TOYOBO (Osaka, Japan). Other chemicals used were all of the highest purity commercially available.

2.3. Cell culture and luciferase assay

Brown pre-adipocyte cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) to confluence, followed by further culture in DMEM-based growth medium containing 20 nM insulin and 1 nM triiodothyronine in either the presence or absence of GDF5 for an additional 7 days. For luciferase assay [14], cells were transfected with reporter vectors, followed by preparation of cell lysates and subsequent determination of luciferase activity using specific substrates in a luminometer (ATTO, Tokyo, Japan).

2.4. Retroviral transfection

Retroviral vectors were transfected into PLAT-E cells using the calcium carbonate method. Virus supernatants were collected 48 h after transfection, and then cells were infected with virus supernatants for 72 h in the presence of 4 μg/ml polybrene. Cells were then subjected to selection by culture with 1 μg/ml puromycin for 3 days before usage for experiments [15].

2.5. Real-time based quantitative polymerase chain reaction (PCR)

Total RNA was extracted from cells or tissues, followed by synthesis of cDNA with reverse transcriptase and oligo-dT primer. The cDNA samples were then used as templates for real-time PCR analysis, which was performed on an MX3005P instrument (Agilent Technologies, Santa Clara, CA, USA), by using specific primers for each gene [5]. Expression levels of the genes examined were normalized by using *36b4* expression levels as an internal control for each sample.

2.6. Immunoblotting analysis

Tissues and cultured cells were solubilized in lysis buffer containing 1% Nonidet P-40. Samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by transfer to a polyvinylidene fluoride membrane and subsequent immunoblotting assay [14]. Quantification was performed by densitometry using ImageJ (NIH, Bethesda, MD, USA).

2.7. Statistical analysis

Results are all expressed as the mean ± standard error of the mean (SEM) and the statistical significance was determined by the two-tailed and unpaired Students' *t*-test or the one-way analysis of variance with Bonferroni/Dunnett post hoc test.

3. Results

3.1. Phosphorylation by GDF5 of Smad1/5/8 and Akt in adipose tissues in vivo

To evaluate the possible involvement of the PI3K/Akt pathway *in vivo*, we used gain and loss of function model animals for GDF5. The former is *aP2-GDF5* mice which are transgenic mice with overexpression of GDF5 in adipose tissues expressing aP2 [5], while the latter is heterozygous *GDF5*^{Rgsc451} mice characterized as DN-GDF5 mutant mice [11]. Constitutive phosphorylation of Akt as well as Smad1/5/8 was seen in inguinal subcutaneous white adipose tissue (sWAT), epididymal visceral WAT (vWAT) and interscapular brown adipose tissue (BAT) from WT mice (Fig. 1A), while a statistically significant increase was found in the phosphorylation of Smad1/5/8 (Fig. 1B) and Akt (Fig. 1C) in these three different adipose tissues from *aP2-GDF5* mice. Since GDF5 is selectively upregulated in BAT, but not in sWAT or vWAT, in mice with inborn and acquired obesity [5], next experiments were done with phosphorylation of Smad1/5/8 and Akt in BAT from *DN-GDF5* mice fed with HFD for 6 months. Under these obesogenic conditions with HFD, markedly decreased levels of phosphorylated Smad1/5/8 and Akt in BAT were found in *DN-GDF5* mice compared with those in WT mice (Fig. 1D and E).

3.2. Promotion by GDF5 of brown adipogenesis in vitro

Brown pre-adipocytes were cultured with GDF5 for subsequent determination of phosphorylation of Smad and Akt *in vitro*. In cells treated with 100 ng/mL GDF5 for 30–60 min, marked

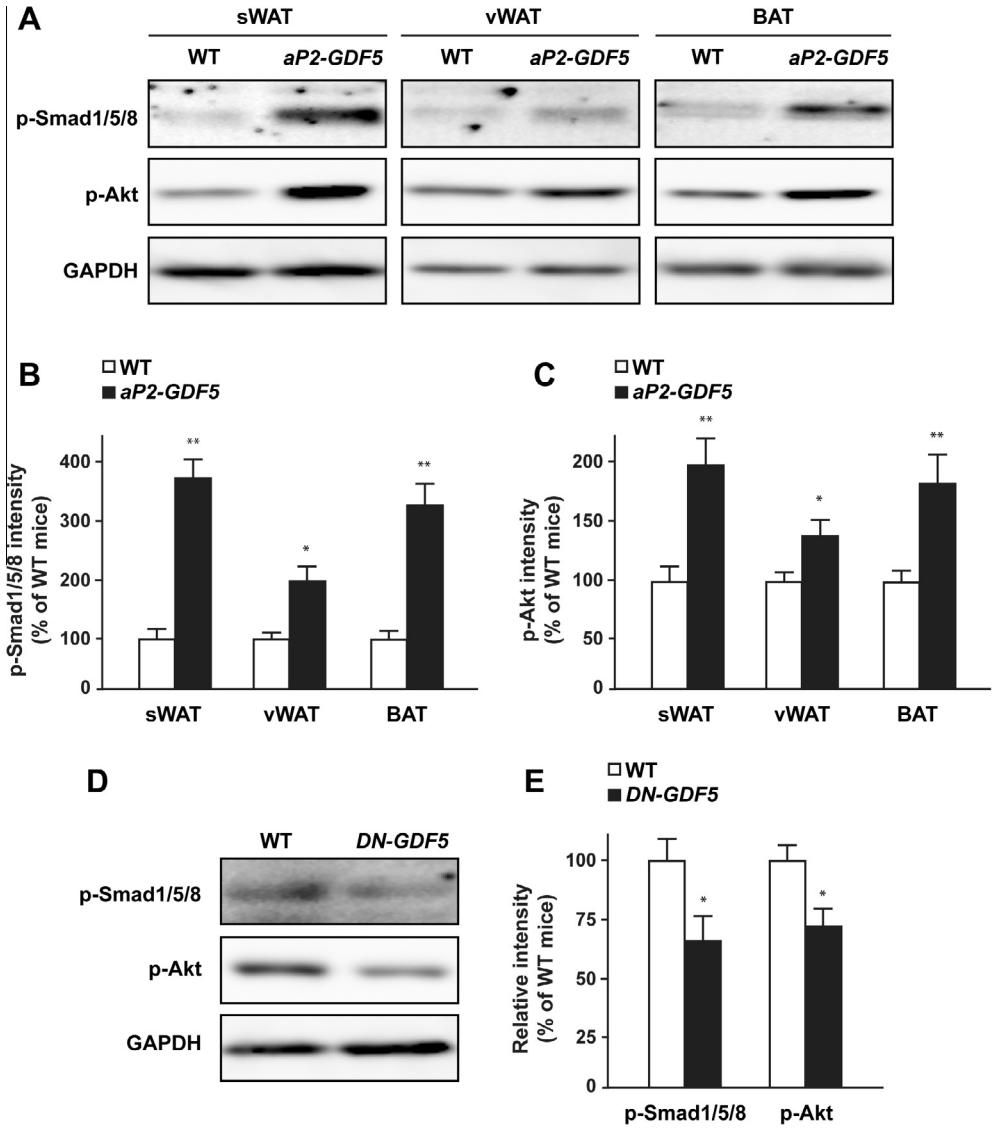

Fig. 1. Akt and Smad1/5/8 are phosphorylated in adipose tissues of *aP2-GDF5* mice. sWAT, vWAT and BAT were isolated from *aP2-GDF5* mice, followed by determination of phosphorylated Smad1/5/8 (p-Smad1/5/8) and Akt (p-Akt) levels by immunoblotting. Representative pictures are shown in the panel A, while quantitative data for p-Smad1/5/8 and p-Akt are shown in the panel B and C, respectively. In addition, BAT was isolated from *DN-GDF5* mice fed with HFD for 6 months for determination of both p-Smad1/5/8 and p-Akt levels. Representative pictures are shown in the panel D, while quantitative data are shown in the panel E. $^{**}P < 0.01$, $^{*}P < 0.05$, significantly different from each control value obtained in WT mice.

phosphorylation was seen for Smad1/5/8, but not for Smad2, along with markedly facilitated phosphorylation of Akt (Fig. 2A). Brown pre-adipocytes were then cultured with GDF5 for 7 days to monitor expression profiles of *Pgc1a* and *uncoupling protein-1* (*Ucp1*) in either the presence or absence of a PI3K-Akt inhibitor. Although GDF5 significantly up-regulated mRNA expression of both brown adipocyte markers, the selective inhibitor of PI3K/Akt, Ly294002, significantly inhibited the GDF5-induced upregulation of *Pgc1a* (Fig. 2B) and *Ucp1* (Fig. 2C) expression in a concentration-dependent manner at concentrations of 1–10 μM without significantly affecting constitutive expression in the absence of GDF5. Furthermore, retroviral infection with DN-Akt led to significant inhibition of the upregulation of both *Pgc1a* (Fig. 2D) and *Ucp1* (Fig. 2E) expression in cells exposed to GDF5.

3.3. Involvement of PI3K/Akt in GDF5-mediated brown adipogenesis

We next investigated the possible signal crosstalk between PI3K/Akt and Smad pathways in brown adipogenesis mediated by GDF5. Brown pre-adipocytes were transfected with the BMP-responsive luciferase reporter plasmid (BRE-Luc), followed by exposure to GDF5 in either the presence or absence of a PI3K/Akt inhibitor. Exposure to GDF5 markedly promoted BRE-Luc activity in a LY294002-sensitive manner (Fig. 3A). In immunoblotting analysis using the anti-Smad5 antibody, phosphorylated Smad5 is usually detected at a molecular weight position higher than that of dephosphorylated Smad5 on the gel as seen in Figs. 3B and C. In nuclear fractions of brown pre-adipocytes, similarly, the PI3K/Akt inhibitor Ly294002 was effective in significantly inhibiting the GDF5-induced phosphorylation of Smad5 without affecting the basal phosphorylation (Fig. 3B). Moreover, retroviral transfection with DN-Akt significantly inhibited the phosphorylation of Smad5 in the presence of GDF5, without altering that in its absence, in nuclear fractions of brown pre-adipocytes (Fig. 3C).

4. Discussion

The essential importance of the present findings is that both Smad1/5/8 and Akt were highly phosphorylated in adipose tissues

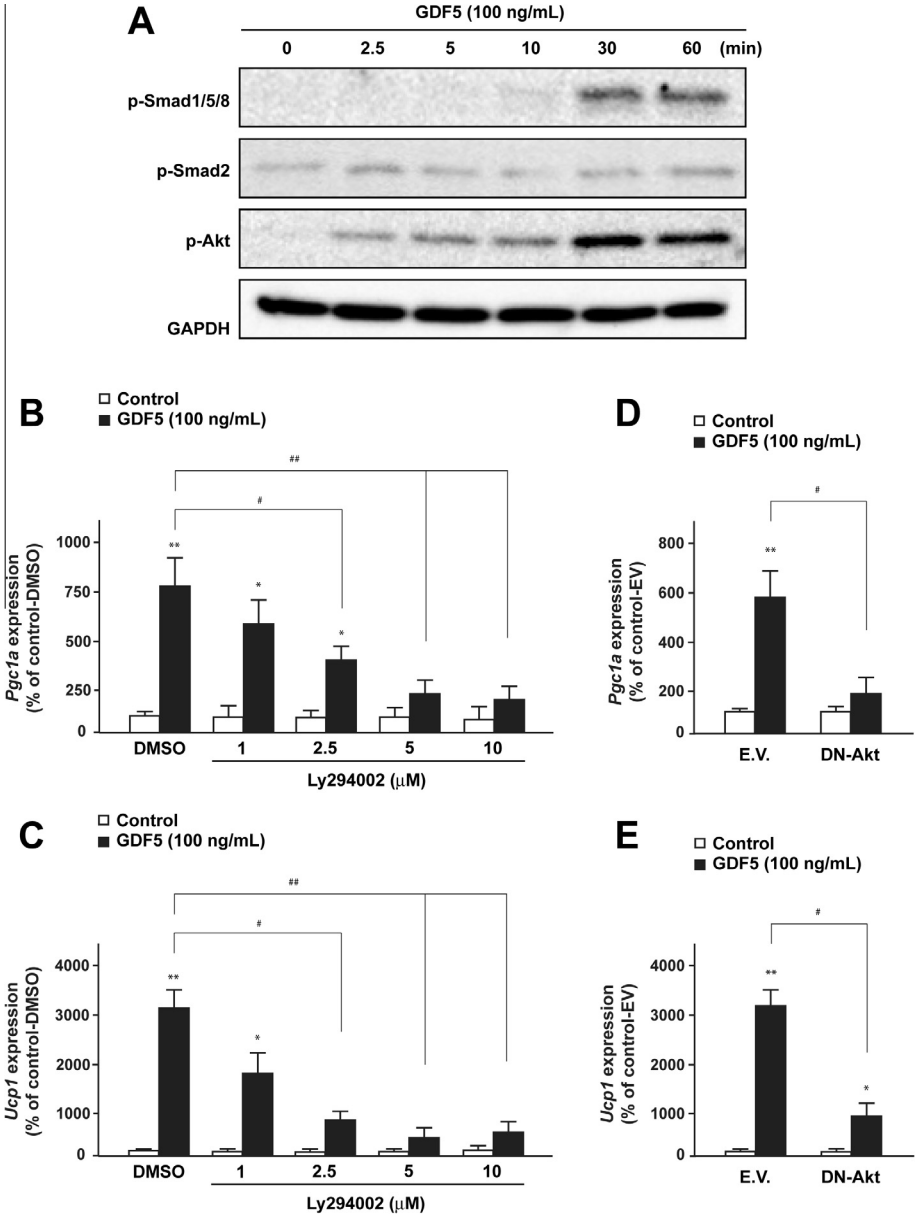

Fig. 2. PI3K/Akt pathway is involved in GDF5-induced brown adipogenesis. (A) Brown pre-adipocytes were exposed to 100 ng/mL GDF5 for different periods indicated, followed by determination of the phosphorylation of downstream mediators of the Smad and Akt. Representative images are shown. In addition, brown pre-adipocytes were cultured with 100 ng/mL GDF5 in either the presence or absence of Ly294002 at 1–10 μM for 7 days, followed by determination of (B) *Pgc1a* and (C) *Ucp1* expression. Brown pre-adipocytes were retrovirally infected with DN-Akt, followed by culture with 100 ng/mL GDF5 for 7 days and subsequent determination of (D) *Pgc1a* and (E) *Ucp1* expression. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from each control value obtained in cells not treated with GDF5. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the value obtained in (B and C) cells treated with GDF5 alone or (D and E) EV-infected cells treated with GDF5.

overexpressing GDF5 *in vivo*, along with promoted dephosphorylation in BAT from *DN-GDF5* mice fed HFD. Moreover, exposure to GDF5 led to upregulation of *Pgc1a* and *Ucp1* expression, in addition to facilitated phosphorylation of both Smad1/5/8 and Akt, in cultured brown pre-adipocytes *in vitro*. To our knowledge, this is the first direct demonstration of the involvement of Smad1/5/8 and Akt in mechanisms underlying upregulation of *Pgc1a* and *Ucp1* expression toward brown adipogenesis. The present findings that nuclear phosphorylated Smad5 levels were increased in brown pre-adipocytes exposed to GDF5 in a manner sensitive the inhibition of PI3K/Akt activity give rise to an idea that Akt could mediate phosphorylation and/or subsequent nuclear translocation of Smad5 as an upstream signal during brown adipogenesis mediated by GDF5. The PI3K/Akt pathway is required for nuclear translocation of Smad5 by BMP2 in osteoblasts [9], in fact, while Smad5 is phosphorylated by Akt on *in vitro* kinase assay [16]. The possibility that Smad1/8, besides Smad5, would additionally participate in the GDF5-mediated activation of the PI3K/Akt pathway in brown adipocytes, however, is conceivable so far.

In addition, the p38 mitogen-activated protein kinase (MAPK) pathway is responsible for the regulation mediated by the BMP/GDF family of cellular differentiation and function in brown adipocytes. BMP7 accelerates a full program of brown adipogenesis through activation of p38 MAPK [12], for example, while BMP8B centrally or peripherally enhances BAT thermogenesis along with the activation of either AMP-activated protein kinase (AMPK) or p38 MAPK [17]. A p38 inhibitor is shown to completely block BMP7-induced UCP1 expression in brown pre-adipocytes [12], whereas several p38 inhibitors failed to significantly affect the upregulation of UCP1 expression in spite of marked phosphorylation

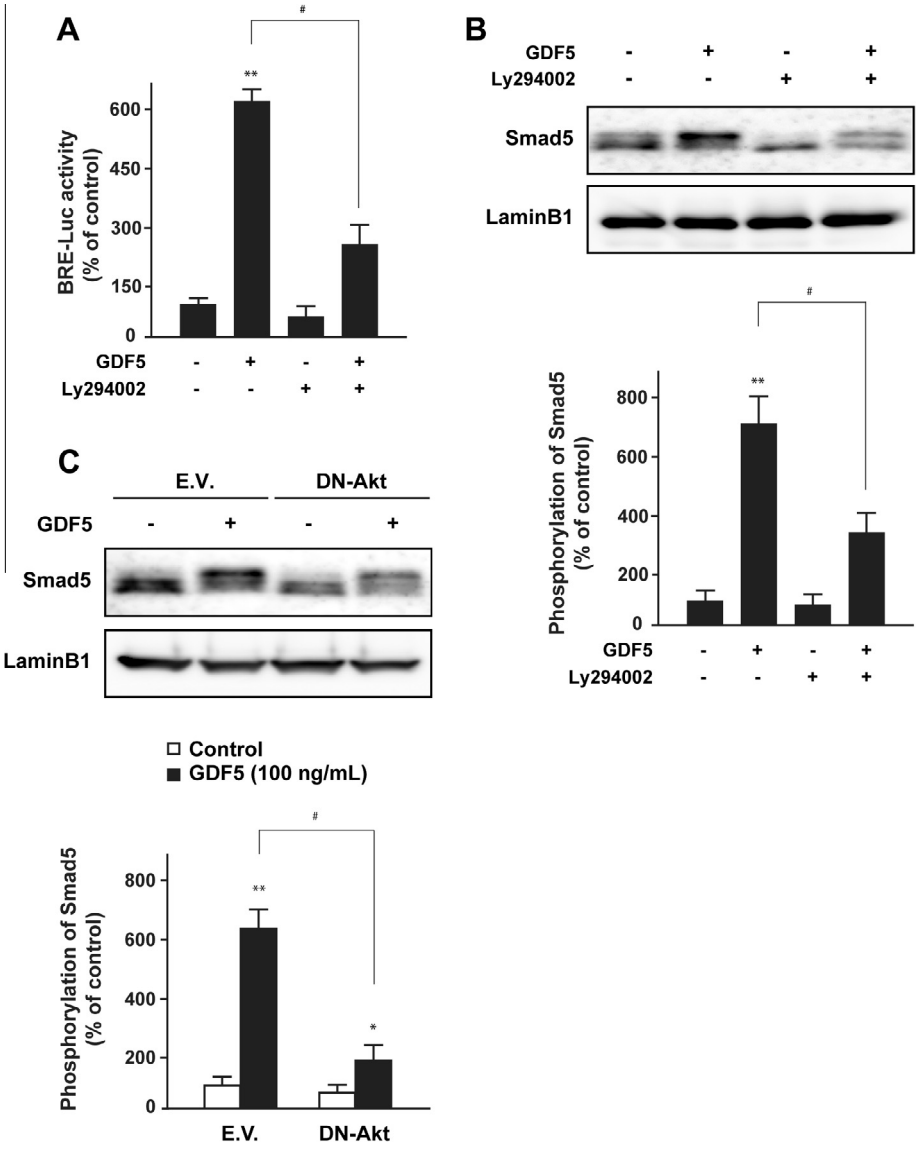

Fig. 3. PI3K/Akt pathway is involved in GDF5-induced phosphorylation of Smad5. (A) Brown pre-adipocytes were transfected with BRE-Luc, followed by culture with 100 ng/mL GDF5 in either the presence or absence of 10 μM Ly294002 for 48 h and subsequent determination of luciferase activity. (B) Brown pre-adipocytes were cultured with 100 ng/mL GDF5 in either the presence or absence of 10 μM Ly294002 for 1 h, followed by determination of Smad5 expression by immunoblotting. Representative images are shown in the upper panel, while quantitative data are shown in the lower panel. (C) Brown pre-adipocytes were retrovirally infected with DN-Akt, followed by culture with 100 ng/mL GDF5 for 1 h and subsequent determination Smad5 expression. Representative images are shown in the upper panel, while quantitative data are shown in the lower panel. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from each control value obtained in cells not treated with GDF5. $^{\#}P < 0.05$, significantly different from the value obtained in (B) cells treated with GDF5 alone or (C) EV-infected cells treated with GDF5.

of p38 in brown pre-adipocytes exposed to GDF5 in our preliminary experiments. Taken together, the PI3K/Akt pathway would play a pivotal role in brown adipogenesis mediated by GDF5, but the p38 MAPK pathway could be responsible for the signal transduction mediated by BMP7 and BMP8b in brown adipocytes. Future analysis is undoubtedly required for clarification of the involvement of P38 MAPK in brown adipogenesis mediated by GDF5.

Since GDF5 expression is similarly up-regulated in sWAT, vWAT and BAT in *aP2-GDF5* transgenic mice [5], the possibility that endogenous BMP antagonistic factors [18] would compensate the stimulatory property of GDF5 on cellular differentiation mediated by the Smad/Akt signaling pathway toward a brown adipocyte phenotype in vWAT from *aP2-GDF5* mice is not rule out. Moreover, several stimuli are implicated as an activator of the development of brown-like adipocytes in WAT. These include β-adrenergic agonists [19], peroxisome proliferator-activated receptor-γ agonists [20], prostaglandin [21] and fibroblast growth factor-21 [22]. Modulation the GDF5/BMPR/Akt/Smad pathway in brown adipocytes could provide a clue for the discovery and development of innovative drugs beneficial for the prophylaxis and the treatment of patients suffering from obesity and obesity-related metabolic dysfunctions such as type-2 diabetes.

Conflict of interest

All authors have no conflict of interest.

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