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Isoform-specific regulation of adipocyte differentiation by Akt/protein kinase Bα

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ABSTRACT

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway tightly regulates adipose cell differentiation. Here we show that loss of Akt1/PKBα in primary mouse embryo fibroblast (MEF) cells results in a defect of adipocyte differentiation. Adipocyte differentiation *in vitro* and *ex vivo* was restored in cells lacking both Akt1/PKBα and Akt2/PKBβ by ectopic expression of Akt1/PKBα but not Akt2/PKBβ. Akt1/PKBα was found to be the major regulator of phosphorylation and nuclear export of FoxO1, whose presence in the nucleus strongly attenuates adipocyte differentiation. Differentiation-induced cell division was significantly abrogated in Akt1/PKBα-deficient cells, but was restored after forced expression of Akt1/PKBα. Moreover, expression of $p27^{Kip1}$, an inhibitor of the cell cycle, was down regulated in an Akt1/PKBα-specific manner during adipocyte differentiation. Based on these data, we suggest that the Akt1/PKBα isoform plays a major role in adipocyte differentiation by regulating FoxO1 and $p27^{Kip1}$.

The transition from preadipocyte proliferation to the differentiated state is controlled by the interplay of both cell cycle regulators and differentiation factors, ultimately generating a series of events that lead to the commitment of cells to the adipocyte phenotype. Progression to the adipocyte phenotype has been divided into several steps, such as first cell cycle arrest, clonal expansion, and second cell cycle arrest [1]. Two factors that are thought to be master regulators of adipocyte differentiation are the transcription factors C/EBPα and PPARγ. These molecules coordinately activate many adipocyte genes that are necessary for acquiring the mature adipocyte phenotype [2].

Several lines of evidence have implicated important roles of PI3K-Akt/PKB signaling pathway(s) in adipocyte differentiation. Addition of PI3K inhibitors completely blocks the differentiation process, suggesting that PI3K is necessary for adipogenesis [3,4]. Expression of a constitutively active version of Akt1/PKBα causes spontaneous adipocyte differentiation of 3T3-L1 cells [5]. Recently, it has been reported that mice lacking Akt1/PKBα have diminished size [6], and histological analysis reveals a moderate reduction in subcutaneous fat [7]. Unlike the Akt1/PKBα isoform, mice lacking Akt2/PKBβ or Akt3/PKBγ have a diabetes-like syndrome and reduction in brain size, respectively [8,9]. The phenotypic differences among these Akt/PKB knock-out mice indicate distinct roles of Akt/PKB isoforms in many cellular processes, which may result from isoform-specific capacities for signaling.

In previous studies, we found fibroblasts derived from *Akt1/PKBα*$^{(-/-)}$ mice were unable to carry out adipocyte differentiation [10]. In the current report, we investigate the isoform-specific roles of Akt/PKB in adipocyte differentiation. We show that Akt1/PKBα specifically regulates adipocyte differentiation, probably *via* FoxO1 phosphorylation, and $p27^{Kip1}$-dependent cell cycle regulation.

Materials and methods

Preparation of mouse embryo fibroblast (MEF) cells. MEF cells were established from E13.5 embryos [10]. Embryos were dissected from pregnant *Akt1/PKBα* $^{+/-}$; *Akt2/PKBβ* $^{+/-}$ females that had been bred to *Akt1/PKBα* $^{+/-}$; *Akt2/PKBβ* $^{+/-}$ males. The yolk sacs, heads, and internal organs were isolated and used for genotyping by RT-PCR. Carcasses were utilized for the preparation of immortalized fibroblasts by continuous culturing for 30 passages as described previously [10].

Retroviral infection. The generation of viral supernatant was performed as described previously [10]. Briefly, ecotropic BOSC23 cells were transiently transfected with pVSV/G and pCgp, pantropic retroviral packaging constructs, and retroviral vector containing PPAR-γ2, Akt1/PKBα, Akt2/PKBβ [10], FoxO1, or a constitutively active FoxO1 (FoxO1-CA). Cell-free viral supernatants were harvested at 24 and 48 h, mixed with one volume of complete media in the presence of 8 μg/ml polybrene and used to infect immortalized MEF cells. Cells expressing PPAR-γ2 were selected by culturing cells with complete medium containing 10 μg/ml of puromycin. Cells infected with FoxO1, FoxO1-CA or Akt/PKB retrovirus were enriched by sorting for GFP using a flow cytometer (BD biosciences).

Adipocyte differentiation. Adipose differentiation was induced by treatment of cells with 2 μg/ml insulin, 0.4 μg/ml dexamethasone, 0.5 mM isobutyl-methyl xanthine, and 0.1 mg/ml of Troglitazone (BIOMOL) as described [11]. Cells were incubated with differentiation cocktail for 4 days, which was subsequently replaced

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with complete medium for 4 days. Adipose differentiation was verified either by Oil Red O staining or Western blotting with adipose cell marker proteins, aP2 (FABP4) and perilipin.

Ex vivo differentiation assay. To distinguish injected cells from surrounding cells, MEF cells were infected with retrovirus carrying the pMIGR vector, which expresses both the gene of interest and green fluorescence protein (GFP) simultaneously. A total of 1×10^7 cells were subcutaneously injected into athymic BALB/c-nu mice (Japan, SLC Inc.). After 10 days, the skin of the injected area was excised and fixed with 4% formaldehyde in PBS at 4 °C overnight. Tissue fragments were embedded in paraffin and frozen in liquid nitrogen. For histological analysis, 7 μm sections were cut with a microtome (MICROM, Germany) and stained with Hematoxylin followed by Oil Red O or anti-perilipin antibody. Sections were visualized with bright field or fluorescent microscopy (Axiovert 200, Germany).

Clonal expansion assay. Confluent cells were incubated with either complete medium or differentiation cocktail (MDI). One day later, 5-bromo 2-deoxyuridine (BrdU, 10 μM, Amersham Biosciences) was added for 1 day. Cells were fixed with 4% paraformaldehyde, and stained with anti-BrdU antibody and DAPI. Both DAPI- and BrdU-positive cells were visualized using a fluorescence microscope. Images were taken at 20× magnification.

Biochemical fractionation of nuclear protein. Cells were washed with ice-cold PBS, scraped into 150 μl of scraping buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulphonylfluoride (PMSF), and incubated on ice for 15 min. After addition of Nonidet P-40 (NP-40, 0.8%), extracts were vortexed and subsequently centrifuged at 13,000*g* for 2 min to separate nuclei from the cytosol. Supernatants were discarded, and remnants were washed with washing buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% NP-40) three times. Pellets were resuspended in 150 μl of scraping buffer, and 20 μl of samples were subjected to Western blotting.

Western blotting. Cell lysates were subjected to 7–16% gradient polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were incubated with the indicated primary antibody and HRP-conjugated secondary antibody. Protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

Reagents. All culture media were purchased from Gibco-BRL Inc. Rabbit polyclonal antibodies against HA epitope and $p27^{Kip1}$ were obtained from Santa Cruz Biotechnology. Anti-phospho-FoxO1 was purchased from Cell Signaling Technology. Anti-BrdU antibody was purchased from Invitrogen and anti-aP2 antibody was generous gift from Dr. David A. Bernlohr (University of Minnesota). Anti-perilipin antibody and all other high quality reagents were purchased from Sigma–Aldrich unless indicated elsewhere.

Results

Requirement of Akt1/PKBα in adipose differentiation of MEF cells in vitro

To further clarify the isoform-specific role of Akt/PKB in adipocyte differentiation, we have generated mouse embryo fibroblasts (MEFs) from the following combinations of genotype: WT, wild type; 1KO, *Akt1* / *PKBα*$^{(-/-)}$; 2KO, *Akt2* / *PKBβ*$^{(-/-)}$; DKO, *Akt1* / *PKBα*$^{(-/-)}$; *Akt2*/*PKBβ* $^{(-/-)}$. In primary MEF cells, adipocyte differentiation, as assessed by accumulation of neutral lipid, occurred in about 30% in WT and 2KO cells. However, cells lacking *Akt1*/*PKBα* were completely defective in adipocyte differentiation (Fig. 1A). To verify the effect of the Akt1/PKBα deficiency in adipocyte differentiation, we expressed the adipogenic transcription factor, PPAR-γ2, in immortalized MEF cell. As shown in Fig. 1B, staining the cells with anti-PPAR-γ2 antibody showed that almost all the cells expressed PPAR-γ2, and equivalent expression of PPAR-γ2 was ascertained by Western blotting. Over-expression of PPAR-γ2 strongly enhanced the adipocyte differentiation of WT and 2KO

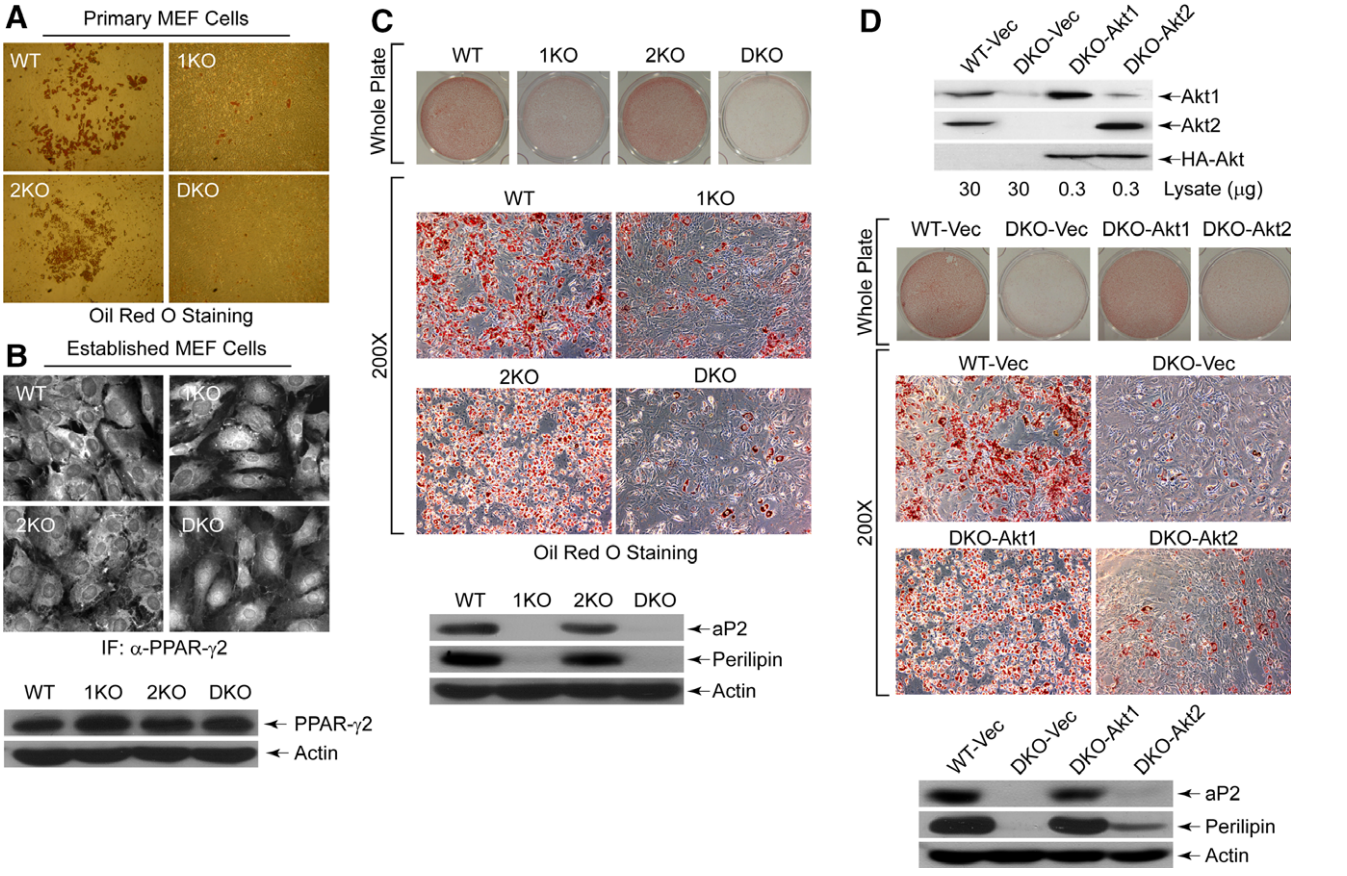

Fig. 1. Defect of adipocyte differentiation in MEF cells lacking *Akt1*/*PKBα in vitro*. (A) Primary MEF cells were induced to differentiate for 8 days. Differentiation was assessed by staining neutral lipid with Oil Red O. (B) PPAR-γ2 was over-expressed using retroviral infection in immortalized MEF cells. Expression of PPAR-γ2 was ascertained by immunocytochemistry (*top*) or Western blotting (*bottom*) against PPAR-γ2. (C) Immortalized MEF cells expressing PPAR-γ2 were differentiated into adipocytes followed by staining with Oil Red O (*top*) or Western blotting with anti-aP2 and anti-perilipin antibodies (*bottom*). (D) PPAR-γ2 over-expressing MEF cells were infected with viruses containing either HA-tagged Akt1/PKBα or Akt2/PKBβ as described in 'Materials and methods'. Expression of each HA-tagged Akt1/PKBα or Akt2/PKBβ was verified by Western blotting with anti-HA, anti-Akt1/PKBα, and Akt2/PKBβ antibodies (*top*), and the differentiation was visualized by Oil Red O staining (*middle*) or determined by Western blotting with anti-aP2 and anti-perilipin antibodies (*bottom*).

cells, however, over-expression of PPAR-γ2 did not rescue impaired adipocyte differentiation in 1KO and DKO cells (Fig. 1C). Over-expression of C/EBPα was also unable to restore adipocyte differentiation in 1KO and DKO cells (data not shown). We next examined whether the impairment of adipocyte differentiation in 1KO and DKO cells was specifically due to the absence of Akt1/PKBα. Cells were infected with retroviruses carrying either HA epitope-tagged Akt1/PKBα or Akt2/PKBβ. As shown in Fig. 1D, an equivalent ectopic expression level of Akt/PKBs was confirmed by Western blotting and each ectopic expression of Akt1/PKBα and Akt2/PKBβ was about 100-fold higher than that of endogenous WT levels. In this context, re-expression of Akt1/PKBα restored adipocyte differentiation in DKO cells as ascertained by Oil Red O staining for neutral lipid and Western blotting against aP2 or perilipin. However, re-expression of Akt2/PKBβ in DKO cells did not fully restored adipocyte differentiation.

Akt1/PKBα is necessary for adipocyte differentiation in an ex vivo context

Green and Kehinde have reported that injection of 3T3-F442A preadipocytes into athymic mice gave rise to fat pads indistinguishable from normal adipose tissue [12]. We have exploited this approach to assess the Akt/PKB isoform-specific function in adipocyte differentiation *ex vivo*. PPAR-γ2 over-expressing MEF cells were tagged by retroviral transduction with the pMIGR vector, which expresses the gene of interest and GFP simultaneously [10]. Injected cells were easily identified by fluorescence microscopy (Fig. 2A). In this experimental system, subcutaneous injection of WT and 2KO cells resulted in significant adipocyte differentiation as judged by both Oil Red O and perilipin staining. In contrast, cells lacking *Akt1/PKBα*, such as 1KO and DKO, displayed impaired adipocyte differentiation *ex vivo*. Moreover, re-expression of Akt1/PKBα in DKO cells restored adipocyte differentiation capability of MEF cells (Fig. 2B). However, expression of Akt2/PKBβ in DKO cells resulted in a reduced Oil Red O staining and a smaller lipid droplet size.

Phosphorylation and localization of FoxO1 are regulated in an Akt1/PKBα-specific manner

Transcriptional activity of FoxO is regulated by phosphorylation and nuclear exclusion, which is mediated by Akt/PKB [13]. As shown in Fig 3A, phosphorylation of FoxO1 at Ser^{256} was significantly impaired in the *Akt1/PKBα*-deficient cells, such as 1KO and DKO. However, Ser^{256} of FoxO1 was normally phosphorylated in cells lacking Akt2/PKBβ. Over-expression of the constitutively active form of FoxO1 significantly reduced adipocyte differentiation (Fig. 3B and C). Consistent with these data, nuclear exclusion of FoxO1 by IGF-1 stimulation, which is normally present, was not observed in *Akt1/PKBα*-deficient cells (Fig. 3D). Finally, IGF-1 rapidly induced translocation of Akt1/PKBα to the nucleus where it remained for at least 30 min (Fig. 3E).

Akt1/PKBα-specific regulation of clonal expansion during adipocyte differentiation

Over-expression of FoxOs causes growth suppression in a variety of cell lines. The FoxO-induced cell cycle arrest depends on the transcriptional up-regulation of the cell cycle inhibitor, $p27^{Kip1}$ [14]. Since cell cycle progression during adipocyte differentiation (Clonal Expansion) is a critical step in adipogenesis *in vitro*, we examined clonal expansion in MEF cells. As shown in Fig. 4A, addition of differentiation inducers (MDI) caused BrdU incorporation in WT and 2KO cells. However, cells lacking *Akt1/PKBα* such as 1KO and DKO did not show MDI-induced BrdU incorporation. Also, re-expression of Akt1/PKBα, but not Akt2/PKBβ, restored MDI-induced BrdU incorporation (Fig. 4B). Finally, expression of $p27^{Kip1}$ was down regulated in WT and 2KO cells after treatment with MDI. However, the expression level of $p27^{Kip1}$ was not down regulated by MDI treatment in cells lacking Akt1/PKBα (Fig. 4C). Consistent with these data, the high basal level of $p27^{Kip1}$ in DKO cells was reduced by re-expression of Akt1/PKBα but not by Akt2/PKBβ. Also, the MDI-induced down regulation of $p27^{Kip1}$ in DKO cells was occurred by re-expression of Akt1/PKBα but not by Akt2/PKBβ.

Discussion

Despite a high degree of sequence similarity, targeted disruption of Akt/PKB isoforms has been shown previously to result in different *in vivo* phenotypes [6–9]. Involvement of each isoform of Akt/PKB in adipocyte differentiation was evaluated by looking at mice with only one isoform of Akt/PKB. For example, mice expressing only *Akt3/PKBγ* ($Akt1^{-/-}Akt2^{-/-}$) or *Akt2/PKBβ* ($Akt1^{-/-}Akt3^{-/-}$) showed defects in skin development as well as

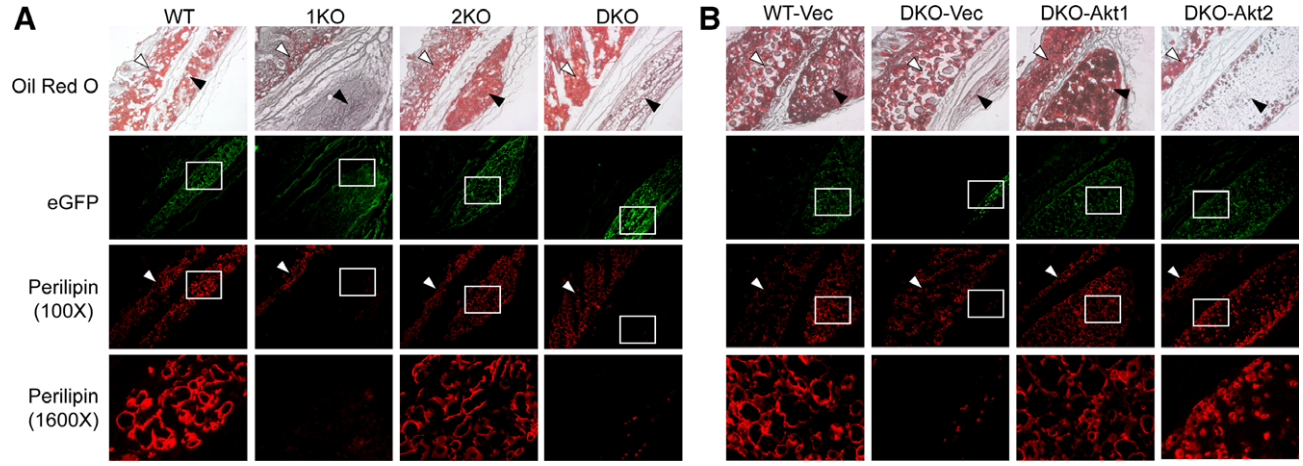

Fig. 2. Restoration of adipocyte differentiation by re-expression of Akt1/PKBα *ex vivo*. (A) Cells lacking either Akt1/PKBα or Akt2/PKBβ were labeled with retroviral gene transduction of GFP, and subcutaneously injected into athymic nude mice for 10 days. Tissue sections showing green fluorescence (*solid arrows*) were stained with either Hematoxylin/Oil Red O or anti-perilipin antibody. Subcutaneous fat was also indicated by open arrows. Images were visualized at 100× magnification; the indicated area (*rectangle*) was enlarged a further 16-fold *in silico* (AxioVision). (B) DKO cells were infected with retrovirus containing GFP as well as either Akt1/PKBα or Akt2/PKBβ, and subcutaneously injected into athymic nude mice for 10 days. Adipocyte differentiation *ex vivo* was verified essentially same as above. Data are the representative images of four independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

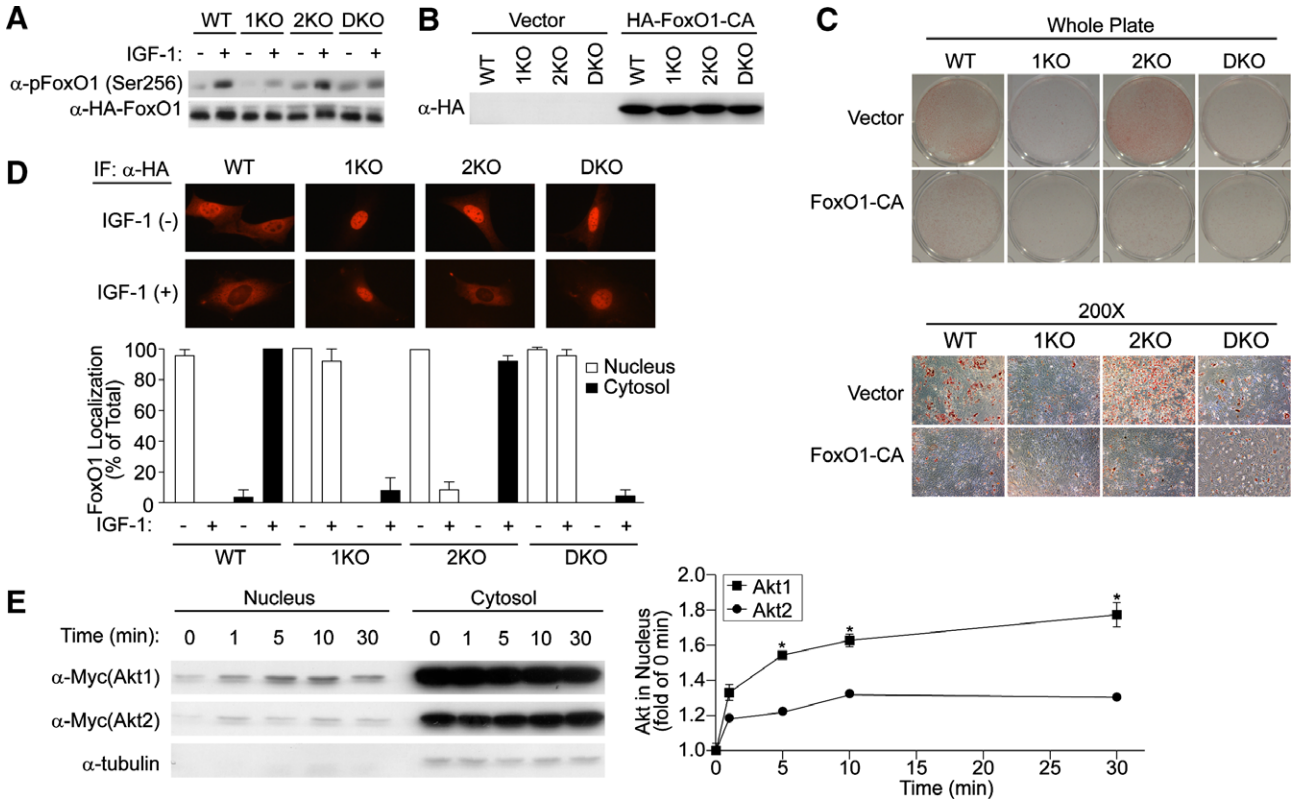

Fig. 3. Regulation of FoxO1 phosphorylation and nuclear exclusion by Akt1/PKBα. (A) Cells were infected with retrovirus containing HA-tagged FoxO1 followed by stimulation with IGF-1 (50 ng/ml) for 5 min. FoxO1 was immunoprecipitated with anti-HA antibody and phosphorylation at specific site was assessed by Western blotting with phospho-specific antibodies. (B) Cells were infected with an HA epitope-tagged constitutively active form of FoxO1 (HA-FoxO1-CA). Expression of HA-FoxO1-CA was verified by Western blotting with anti-HA antibody or adipocyte differentiation was induced for 8 days followed by staining with Oil Red O (C). (D) Cells were transiently infected with HA-FoxO1. IGF-1-induced re-localization of FoxO1 was verified by immunocytochemistry. Images were visualized at 40× magnification, and cells showing nuclear or cytosolic FoxO1 were counted. Data are means ± SE of three independent experiments. (E) WT cells expressing either myc-tagged Akt1/PKBα or Akt2/PKBβ were stimulated with IGF-1 (50 ng/ml) for the indicated times. Samples were separated into nuclear and cytosolic fractions followed by immunoprecipitation with anti-Myc antibody as described in 'Materials and methods'. Relative translocation of each isoform was determined by measuring the ratio of nuclear Akt/PKB to total Akt/PKB (Science Lab 2005). Akt/PKB in the nucleus was expressed as fold increase compared to 0 min. Data are means ± SE of three independent experiments. Asterisk indicates statistically meaningful translocation of Akt1/PKBα, $p < 0.01$.

adipocyte differentiation [7,15]. In contrast, mice with a single isoform of *Akt1/PKBα* (*Akt2*$^{-/-}$*Akt3*$^{-/-}$) showed normal skin development and adipocyte differentiation [16]. Hence, it is likely that Akt1/PKBα rather than Akt2/PKBβ or Akt3/PKBγ is more important in the regulation of adipocyte differentiation and normal development *in vivo*. The goal of this study was to uncover potential molecular mechanisms by which Akt1/PKBα dictates specificity in adipocyte differentiation. In this study, we have provided evidence that the capacity of individual Akt/PKB members causes specific physiological responses, *e.g.*, adipocyte differentiation.

PPAR-γ2 induces a variety of adipocyte-specific genes, and is the most critical transcriptional factor required for adipocyte differentiation. It is likely that many signaling pathways responsible for adipocyte differentiation converge on PPAR-γ2. Recently, Peng et al. have shown that cells lacking Akt1/PKBα displayed impairment of adipocyte differentiation and over-expression of PPAR-γ in Akt1/PKBα and Akt2/PKBβ double knock-out cells partially restored adipocyte differentiation [7]. Likewise, our results also showed that ectopic expression of PPAR-γ2 enhanced adipocyte differentiation of wild type and *Akt2*/*PKBβ*$^{-/-}$ MEF cells, and partially restored impairment of adipocyte differentiation caused by Akt1/PKBα deficiency (Fig. 1). However, our results show that an additional Akt1/PKBα signaling pathway(s) is required for PPAR-γ2-enhanced adipocyte differentiation. For instance, re-expression of *Akt1/PKBα* into *Akt1*/*PKBα*$^{-/-}$ cells fully restored PPAR-γ2-dependent adipocyte differentiation both *in vitro* and *ex vivo* (Figs. 1 and 2). It is also worthy to emphasize that Akt2/PKBβ, when over-expressed 100-fold, did not fully rescue the adipocyte differentiation both *in vitro* and *ex vivo*, demonstrating that existence of Akt1/PKBα isoform is required for adipocyte differentiation regardless of expression level. In these regards, it is likely that Akt1/PKBα-dependent signaling pathway in addition to PPAR-γ2 expression might be involved in the adipocyte differentiation.

Akt/PKB regulates gene expression by the forkhead family of transcription factors, which is a transcription factor negatively regulated by Akt/PKB. Recently, it has been reported that FoxO1 negatively regulates adipocyte differentiation through the control of a clonal expansion [17]. Particularly, adipocyte differentiation required existence of Akt1/PKBα even in the presence of PPAR-γ2 (Fig. 1) and over-expression of a constitutively active form of FoxO1 (FoxO1-CA) completely blocked PPAR-γ2-enhanced adipocyte differentiation (Fig. 3B and C), indicating that inactivation of FoxO1 is required for the induction of adipocyte differentiation. Since inactivation of FoxO1 was specifically mediated by Akt1/PKBα (Fig. 3A and D), it is likely that the signaling specificity from Akt1/PKBα relay to FoxO1 during adipocyte differentiation. In contrast to previous report that adenoviral gene delivery (transient expression) of a dominant negative form of FoxO1 (FoxO1-DN) enhanced adipocyte differentiation [17], we found that retroviral gene delivery (permanent expression) of FoxO1-DN inhibited adipocyte differentiation but showed neoplastic morphology (data not shown). Therefore, transient inhibition of FoxO1 activity might

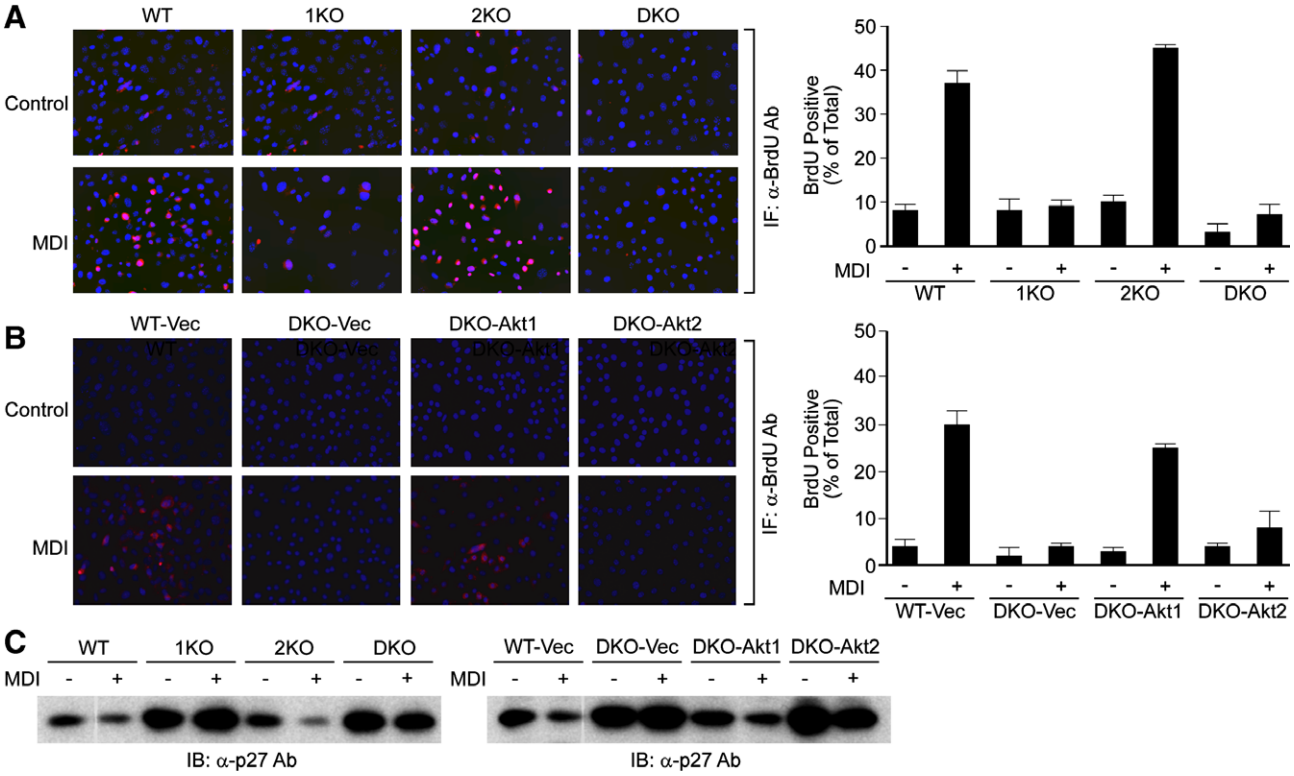

Fig. 4. Control of mitotic clonal expansion by Akt1/PKBα.(A, B) Confluent cells were incubated with differentiation inducers (*MDI*) for 1 day followed by addition of BrdU. Cells undergoing mitotic clonal expansion and nuclei were detected by anti-BrdU antibody and DAPI, respectively. Images were taken at 20× magnification. Data are means ± SD of three duplicated independent experiments. (C) Expression of $p27^{Kip1}$ during the adipocyte differentiation was determined by Western blotting with anti-$p27^{Kip1}$ antibody.

be necessary to coordinate exit from the cell cycle in order to activate the terminal differentiation program. In this context, transient inhibition FoxO1 might be exclusively regulated by specific translocation of Akt1/PKBα to the nucleus (Fig. 3).

FoxO1 regulates a variety of genes at the transcriptional level including not only death-related genes but also cell cycle regulators. The period of cell cycle progression, referred to as mitotic clonal expansion, is a prerequisite for adipocyte differentiation [18]. During the clonal expansion periods, several cell cycle regulators are dynamically changed in expression level either by transcriptional regulation or proteasomal degradation. For instance, expression of $p21^{Waf1/Cip1}$ or $p27^{Kip1}$ is reduced during the clonal expansion [19]. Correlating with this, loss of $p21^{Waf1/Cip1}$ and/or $p27^{Kip1}$ produced adipocyte hyperplasia and obesity [20]. FoxO1 also regulates the cell cycle through transcriptional regulation of $p27^{Kip1}$ [14]. In this study, we demonstrated that inactivation of FoxO1, which is required for PPAR-γ2-enhanced adipocyte differentiation (Fig. 3B and C), is acquired by phosphorylation and nuclear exclusion in an Akt1/PKBα-specific manner (Fig. 3A and D). Likewise, we demonstrated that the expression of $p27^{Kip1}$ and clonal expansion, which are mediated by FoxO1, were also regulated by Akt1/PKBα-specific manner (Fig. 4). Therefore, it may account that signaling specificity of Akt1/PKBα in the regulation of adipocyte differentiation is relayed to FoxO1, $p27^{Kip1}$, and clonal expansion.

Although it is evident that FoxO1 is the key player in mediating Akt1/PKBα-dependent adipocyte differentiation, recent evidence supports that other members of the forkhead family of transcriptional factors, Foxa-2 and FoxC2, are phosphorylated by Akt/PKB and regulate adipocyte differentiation [21,22]. It also has been reported that Akt/PKB regulates gene expression of Kruppel-like factor 15 (KLF 15) [23], which plays key roles in adipocyte differentiation [24]. In this regard, it is likely that Akt1/PKBα can affect adipogenesis at multiple levels. Therefore, further studies delineating the exact molecular mechanisms by which Akt1/PKBα exerts its effect on these genes might be worthy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.04.029.

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