Published OnlineFirst April 14, 2009; DOI: 10.1158/0008-5472.CAN-08-2641

Cancer Research

Adiponectin-Activated AMPK Stimulates Dephosphorylation of AKT through Protein Phosphatase 2A Activation

Kun-yong Kim, Ahmi Baek, Ji-Eun Hwang, et al.

Cancer Res 2009;69:4018-4026. Published OnlineFirst April 14, 2009.

Published OnlineFirst April 14, 2009; DOI: 10.1158/0008-5472.CAN-08-2641

Research Article

Adiponectin-Activated AMPK Stimulates Dephosphorylation of AKT through Protein Phosphatase 2A Activation

Kun-yong Kim,[1] Ahmi Baek,[1] Ji-Eun Hwang,[1] Yeon A. Choi,[1] Joon Jeong,[2] Myeong-Sok Lee,[1] Dea Ho Cho,[1] Jong-Seok Lim,[1] Keun Il Kim,[1] and Young Yang[1]

[1]Research Center for Women's Diseases, Department of Life Science, Sookmyung Women's University; and [2]Department of Surgery, College of Medicine, Yonsei University, Seoul, Korea

Abstract

Low serum levels of adiponectin are a high risk factor for various types of cancer. Although adiponectin inhibits proliferation and metastasis of breast cancer cells, the underlying molecular mechanisms remain obscure. In this study, we show that adiponectin-activated AMPK reduces the invasiveness of MDA-MB-231 cells by stimulating dephosphorylation of AKT by increasing protein phosphatase 2A (PP2A) activity. Among the various regulatory B56 subunits, B56γ was directly phosphorylated by AMPK at Ser[298] and Ser[336], leading to an increase of PP2A activity through dephosphorylation of PP2Ac at Tyr[307]. We also show that both the blood levels of adiponectin and the tissue levels of PP2A activity were decreased in breast cancer patients and that the direct administration of adiponectin into tumor tissues stimulates PP2A activity. Taken together, these findings show that adiponectin, derived from adipocytes, negatively regulates the invasiveness of breast cancer cells by activating the tumor suppressor PP2A. [Cancer Res 2009;69(9):4018–26]

Introduction

Obesity is an independent risk factor for the development of breast cancer. Increasing evidence suggests that adipose tissue may play an important role in regulating breast cancer progression and distant metastasis (1). The aberrant production of adipokines, adipose tissue secreting cytokines, plays an important role in the pathogenesis of obesity-related breast carcinogenesis. Some adipokines, including leptin, heparin-binding epidermal growth factor, and collagen VI act directly on breast cancer cells to stimulate their proliferation, invasiveness, and malignancy (2). Several recent studies have shown that low serum adiponectin is highly associated with obesity-related cancers, including endometrial, breast, prostate, and gastric cancers. In addition, breast tumors in women with low serum adiponectin levels are more likely to show an aggressive phenotype and adiponectin suppresses the metastasis of breast cancer cells (3).

Many reports show that inactivation of AKT by dephosphorylation, which plays a key role in tumor suppression, occurs concomitantly with the activation of AMPK. For example, the two AMPK-activating drugs, phenformin and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), inactivate AKT by dephosphorylation (4). A mammary carcinogenesis inhibitor, 2-deoxyglucose, also activates AMPK and inactivates AKT in MDA-MB-468 human breast cancer cells (5). Deguelin, a lung cancer chemopreventive agent, induces activation of AMPK and inactivation of AKT (6). Although many reports show concomitant AMPK activation and AKT inactivation, little is known about how the activated AMPK is able to inactivate AKT.

Protein phosphatase 2A (PP2A) is a major cellular serine/threonine phosphatase that plays important roles in cell proliferation and cell transformation (7–10). For example, PP2A blocks entry into mitosis (11), inactivates the antiapoptotic protein Bcl-2 in Jurkat cells (12), and destabilizes the c-Myc oncoprotein by dephosphorylating Ser[62] (13). The evidence of an antitumor role for PP2A was supported by the identification of alterations in PP2A subunits in human cancers. Many alterations in the gene encoding the Aβ subunit have been identified, primarily in human lung and colorectal cancers (14, 15). In addition, mutations (16) and reduced expression of the Aα subunit have been reported in human tumors (17) and in the MCF-7 breast cancer cell line (18). In addition to alterations of the A subunits, many alterations to the tumor suppressor activity mediated by the PP2A B56 subunit have been reported. A truncated B56γ1 isoform has been identified in a metastatic clone of the mouse B16 melanoma cells, BL16 (19). This truncation prevents a critical interaction between B56γ-containing PP2A and p53, resulting in a decrease in tumor suppressor activity (20). B56γ-containing PP2A also inhibits Wnt signaling through β-catenin degradation in *Xenopus* (21). B56α directs PP2A holoenzymes to c-Myc, resulting in a reduction of c-Myc levels (22). In this study, we examine the ability of AMPK, activated by adiponectin to activate PP2A by phosphorylation of its B56 subunit, which in turn suppresses invasiveness by inactivating AKT.

Materials and Methods

Reagents and plasmids. Plasmids encoding AMPK-DN were obtained from Dr. Juhun Ha (Kyunghee University). Plasmids encoding AMPK-WT and AMPK-CA were obtained from Dr. David Carling (MRC Clinical Sciences Centre). B56α, B56β, B56γ, and B56δ were amplified by pfu PCR from a human brain cDNA library and subcloned into the pCMV-tag2B expression vector. The S261A, S298A, S336A, and S298A/S336A B56γ1 mutants were generated using a mutation primer and subcloned into the pCMV-tag2B expression vector.

Invasion and proliferation assay. The ability of cells to migrate through Matrigel-coated filters was determined using modified 24-well Transwell cell culture chambers. Cells were seeded at a density of 1×10^4 per well and treated with 20 μg/mL adiponectin for the indicated times. Cells that subsequently invaded the lower chamber of each plate were labeled with calcein-AM (Molecular Probes) and measured with a Wallace 1420 Victor3 plate reader (Perkin-Elmer) at an excitation of 485 ± 10 nm and an emission of 520 ± 10 nm. For the proliferation assay, cells were seeded in 12-well plates at a density of 1×10^5 per well and subsequently

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Young Yang, Research Center for Women's Diseases, Department of Life Science, Sookmyung Women's University, Seoul 140-742, Korea. Phone: 82-2-710-9590; Fax: 82-2-2077-7322; E-mail: yyang@sookmyung.ac.kr.

doi:10.1158/0008-5472.CAN-08-2641

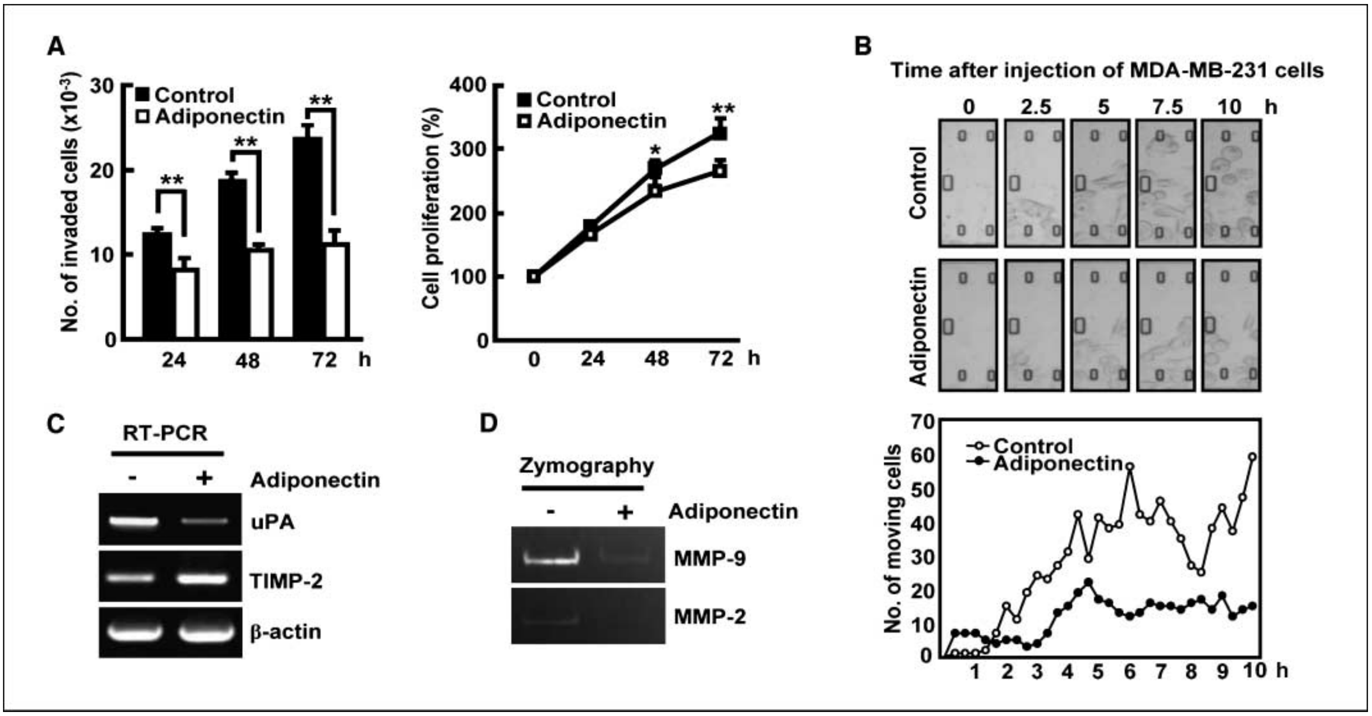

Figure 1. Effects of adiponectin on the invasiveness of MDA-MB-231 cells. *A,* cells were plated in the top chamber of Matrigel-coated Transwell plates and incubated with 20 μg/mL adiponectin for the indicated time. Fluorescence of the migrated cells was measured after treatment with calcein-AM solution. The number of viable cells was manually counted at different time points of treatment. Mean ± SE of three independent experiments done in triplicate. *, $P < 0.05$; **, $P < 0.01$ versus control. *B,* cells were pretreated with 20 μg/mL adiponectin for 18 h. Migrating cells were recorded with a charge-coupled device camera. Photographs were taken at the indicated time points. *Bottom,* number of cells that migrated for the indicated periods. *C,* cells were incubated in the presence of 20 μg/mL adiponectin for 24 h and reverse transcription-PCR was done for uPA and TIMP-2. *D,* cells were cultured for 24 h in serum-free DMEM in the absence or presence of 20 μg/mL adiponectin. Matrix metalloproteinase (*MMP*) activity in the culture supernatant was measured by gelatin zymography.

treated with 20 μg/mL adiponectin. The numbers of viable cells at different time points were determined by manually counting cells that did not stain with trypan blue dye.

Horizontal migration assay. The EZ-TAXIScan (Effector Cell Institute) was used to detect real-time horizontal migration of MDA-MB-231 cells. Adiponectin-pretreated MDA-MB-231 cells were placed into the single hole and DMEM containing 10% fetal bovine serum was placed into the contra-hole. A charge-coupled device camera was used to record the migration of the cells toward the DMEM containing 10% fetal bovine serum. Moving cells in a fixed gate were counted using the TAXIScan Analyzer.

Preparation of conditioned medium and zymography. Subconfluent cells were washed three times with serum-free medium and cultured for 24 h in the absence or presence of 20 μg/mL adiponectin. The conditioned medium was collected and clarified by centrifugation and then concentrated by a Centricon centrifugal filter (Millipore). Gelatinolytic activity in the conditioned medium was analyzed by zymography.

Immunoblot analysis. Analyses of cell lysates were done as described previously (23). Phospho-AMPK, phospho-AKT (Cell Signaling), and phospho-PP2Ac (Abcam) antibodies were used. Levels of phosphorylation were quantified relative to β-actin or α-tubulin.

AMPK, phosphatidylinositol 3-kinase, and PP2A phosphatase activity assay. Transfected cells were lysed and incubated with anti-PP2Ac antibody for 2 h. After the addition of 30 μL agarose A-beads, lysates were further incubated for 2 h at 4°C. Immunoprecipitates were washed three times with AMPK kinase reaction buffer (Cell Signaling). The immunoprecipitates were resuspended in 50 μL AMPK kinase reaction buffer containing 50 ng recombinant AMPK kinase, 0.16 μCi ^{32}P-labeled ATP, and 250 μmol/L ATP followed by incubation for 30 min at room temperature. The reactions were analyzed by SDS-PAGE. Autoradiographs were visualized with a FLA-3000G Three-Laser Imaging System (Fujifilm). Phosphatidylinositol 3-kinase (PI3K) activity was assayed with the PI3K ELISA kit (Echelon Biosciences) in accordance with the manufacturer's instructions. PP2A activity was determined using a serine/threonine phosphatase assay system in accordance with the manufacturer's protocols (Promega). Cells were briefly lysed with a phosphatase lysis buffer [20 mmol/L HEPES (pH 7.4), 10% glycerol, 0.1% NP-40, 30 mmol/L β-mercaptoethanol, 1 mmol/L EGTA] and measured for phosphatase activity using a PP2A-type specific buffer [50 mmol/L imidazole (pH 7.2), 0.2 mmol/L EGTA, 0.03% β-mercaptoethanol, 0.1 mg/mL bovine serum albumin]. Free phosphate, generated from a synthetic phosphothreonine peptide RRA(pT)VA specific for PP2A, was quantified by measuring molybdate/malachite green/phosphate complex at 600 nm. EGTA and EDTA were included in the lysis buffer to inhibit PP2B and PP2C, respectively. Okadaic acid was used to confirm the specificity of these reaction conditions.

Subcellular fractionation. Cells were treated with 20 μg/mL adiponectin for 30 min and membrane fractions were obtained as described previously (24).

Tumor formation. Female BALB/c mice (6 weeks old; Dae Han Biolink) were subcutaneously injected with 1.2×10^4 4T1 adenocarcinoma cells in the right anterior mammary region. BALB/c mice bearing 4T1 murine mammary tumors were intratumorally injected with 100 μg recombinant adiponectin and 0.2 mg of the AMPK activator AICAR on day 14 after initial implantation of the 4T1 cells. Primary tumors were collected and subjected to further analysis 8 h after the injection.

Small interfering RNA preparation. The following small interfering RNA (siRNA) sequences were designed and purchased from Samchully Pharmaceuticals. siRNA-B56α forward 5′-CUGCACCGAAUUUAUGGGAAA-3′ and reverse 5′-UUUCCCAUAAAUUCGGUGCAG-3′, siRNA-B56β forward 5′-CCGUCUACCCAGACAUCAUCC-3′ and reverse 5′-GGAUGAUGUCUGG-GUAGACGG-3′, siRNA-B56γ forward 5′-GCCUAUUUACCCAGAAGUAGU-3′ and reverse 5′-ACUACUUCUGGGUAAAUAGGC-3′, siRNA-B56δ forward 5′-GCUGCCACCUUCAUCGAAUCC-3′ and reverse 5′-GGAUUCGAUGAAG-GUGGCAGC-3′, and siRNA-GFP was used as control.

Subjects. Tumor and normal tissue samples were collected from patients with newly diagnosed breast cancer with the patients' consent. Patients were surgically treated at the Young Dong Severance Hospital.

Statistical analysis. Data (mean ± SE) were statistically analyzed using an unpaired *t* test. $P < 0.05$ was considered a significant difference.

Results

Adiponectin suppresses metastasis of MDA-MB-231 cells. To determine the direct effects of adiponectin on the invasiveness of breast cancer cells, we performed invasion assays with MDA-MB-231 cells. The invasiveness of MDA-MB-231 cells was significantly reduced 24 h after treatment with adiponectin, but statistically significant inhibition of proliferation was not observed at the same time (Fig. 1*A*). One caveat to this study is that previous work has shown that adiponectin induces apoptosis of MDA-MB-231 cells. We therefore tested if adiponectin-mediated apoptosis is responsible for the observed decrease in invasiveness. Our data show that adiponectin induces apoptosis in only 3% of the cells (Supplementary Fig. S1) but suppresses invasiveness by 33%, indicating that a reduction in proliferation and an increase in apoptosis induction are responsible for only a small percent of the decrease in invasiveness induced by adiponectin. We next tested whether adiponectin could induce migration. The migration of cells in the presence of adiponectin was directly visualized using the EZ-TAXIScan, which monitors the real-time horizontal movement of cells. This analysis showed that the migration of adiponectin-pretreated MDA-MB-231 cells was dramatically inhibited compared with control cells (Fig. 1*B*).

To understand the detailed molecular events that underlie the inhibition of invasiveness by adiponectin, we examined the expression levels of metastasis-associated genes. Treatment with adiponectin greatly reduced the expression levels of the

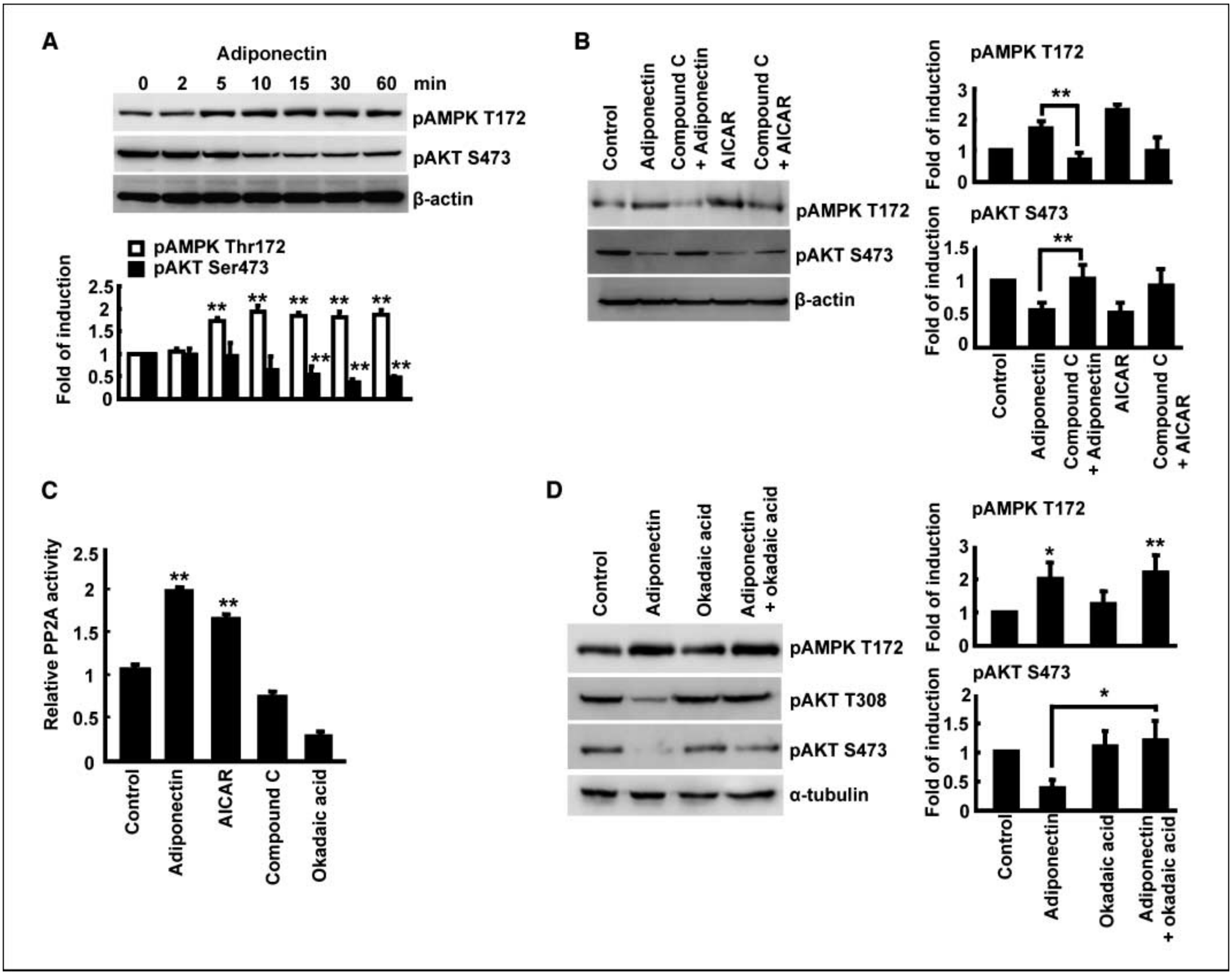

Figure 2. Effect of adiponectin on AMPK, AKT, and PP2A activities. *A,* phosphorylation levels of AMPK and AKT were examined in MDA-MB-231 cells treated with 20 μg/mL adiponectin for the indicated time. *B,* cells were treated with 20 μg/mL adiponectin, 250 μmol/L AICAR, and 5 μmol/L compound C for 30 min. Phosphorylation levels of AMPK and AKT were then examined using immunoblot analysis. *C,* cells were treated with 20 μg/mL adiponectin, 250 μmol/L AICAR, 5 μmol/L compound C, and 250 nmol/L okadaic acid for 30 min. PP2A activity was subsequently measured. Mean ± SE of three independent experiments in triplicate. **, $P < 0.01$ versus control. *D,* cells were pretreated with okadaic acid for 30 min and then with adiponectin for an additional 30 min. Phosphorylation levels of AMPK and AKT were examined. *A, B*, and *D,* mean ± SE. Blots are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ versus control or as indicated otherwise.

Published OnlineFirst April 14, 2009; DOI: 10.1158/0008-5472.CAN-08-2641

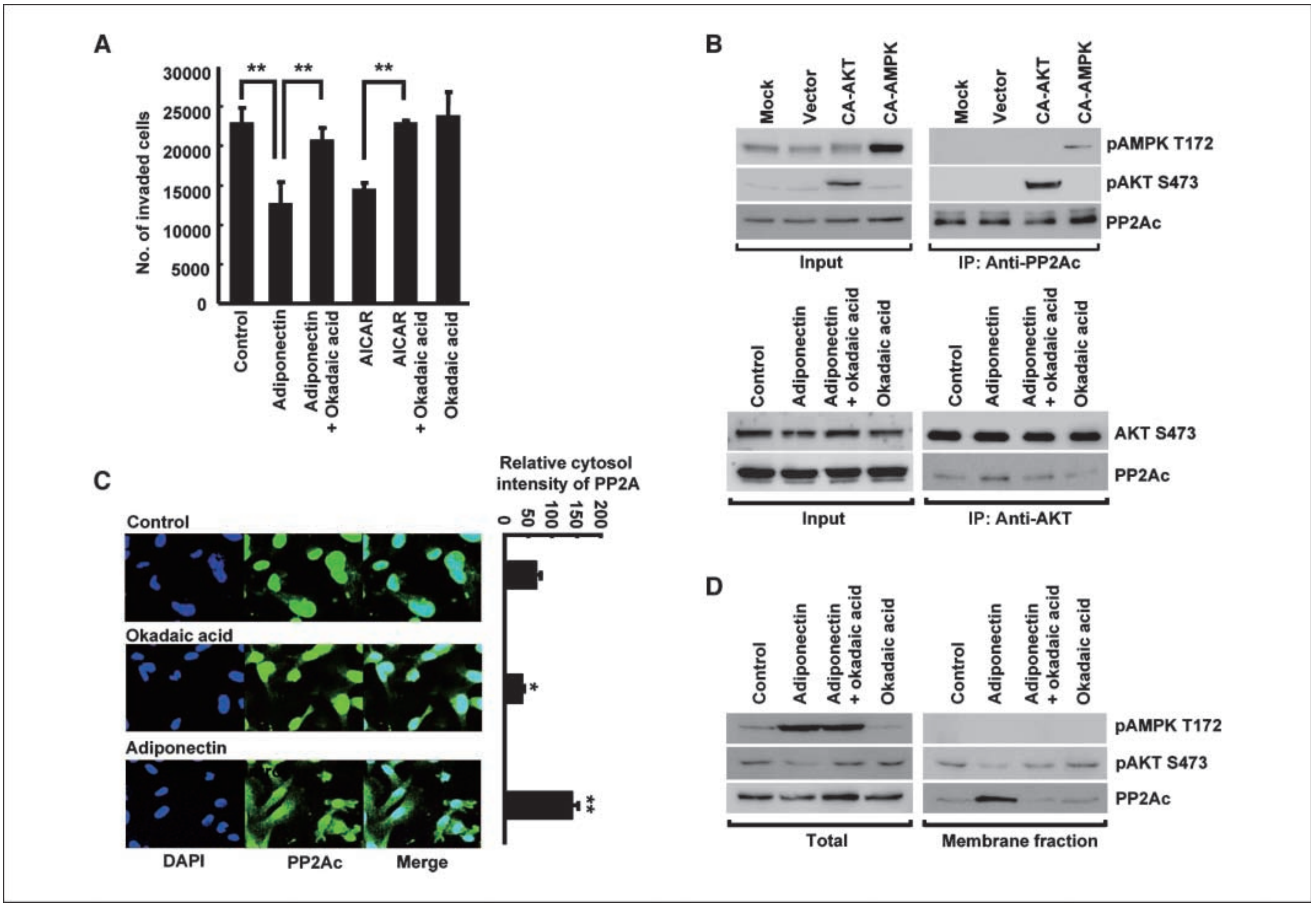

Figure 3. Effect of adiponectin on the subcellular localization of PP2A. *A,* cells were pretreated with 250 nmol/L okadaic acid and an invasion assay was done in the presence of 20 μg/mL adiponectin and 250 μmol/L AICAR. Mean ± SE of three independent experiments. **, $P < 0.01$. *B,* cells were transfected with 3 μg constitutively active AKT (*CA-AKT*) or constitutively active AMPK (*CA-AMPK*) expression plasmids. Immunoprecipitations using an anti-PP2Ac antibody were done on cell lysates 24 h after transfection. Interactions between PP2A and AKT or AMPK were analyzed using immunoblot analysis (*top*). Cells treated with adiponectin and okadaic acid for 30 min were lysed and then subjected to an immunoprecipitation using anti-AKT antibody. Interactions between AKT and PP2Ac were examined by immunoblot analysis using 2% input and 20% immunoprecipitation samples (*bottom*). *C,* cells were treated with okadaic acid or adiponectin for 30 min and endogenous localization of PP2A was examined by confocal microscopy. Images were quantified by FV300 analysis. *, $P < 0.05$, **, $P < 0.01$ versus control. *D,* cells were treated with adiponectin, okadaic acid, or both. Membrane fraction was obtained 30 min after treatment and subjected to immunoblot analysis.

metastasis-promoting gene uPA, whereas the mRNA expression of TIMP-2, a metastasis-suppressing gene, was significantly increased (Fig. 1*C*). Zymography analysis also showed that the activities of matrix metalloproteinase-2 and -9 were markedly inhibited by treatment with adiponectin (Fig. 1*D*).

Adiponectin stimulates dephosphorylation of AKT through AMPK. To decipher the signal transduction pathway involved in the inhibition of invasion, changes in phosphorylation patterns of kinases were analyzed. Phosphorylation of p38 was slightly increased, whereas phosphorylation of AKT1/2 and GSK3β was significantly inhibited by adiponectin treatment (Supplementary Fig. S2). In addition, adiponectin time-dependently increased AMPK phosphorylation and decreased AKT phosphorylation (Fig. 2*A*). To determine whether AKT dephosphorylation is due to AMPK activation, AKT phosphorylation was examined after treatment with AICAR, an AMPK activator, and compound C, an AMPK inhibitor. Like adiponectin, AICAR stimulated dephosphorylation of AKT, whereas compound C blocked AMPK phosphorylation and AKT dephosphorylation by adiponectin (Fig. 2*B*). These results indicate that AMPK activation is associated with adiponectin-induced AKT dephosphorylation.

To test whether adiponectin-activated AMPK inhibits AKT phosphorylation through the inhibition of PI3K (the upstream activator of the AKT signaling pathway), PI3K activity was measured in cells treated with adiponectin, AICAR, and compound C. Adiponectin and AICAR failed to inhibit PI3K activity, indicating that AMPK-induced AKT dephosphorylation is not mediated by the suppression of PI3K activity (Supplementary Fig. S3).

Adiponectin-activated AMPK increases PP2A activity. To examine whether PP2A is associated with AKT dephosphorylation, PP2A activity was measured. PP2A activity was greatly increased in cells treated with adiponectin or AICAR for 30 min (Fig. 2*C*). Next, cells were treated with the PP2A inhibitor okadaic acid to examine whether PP2A activation is indeed involved in AKT dephosphorylation. Interestingly, okadaic acid completely blocked adiponectin-mediated AKT dephosphorylation of Ser^{308} and Ser^{473}, whereas it did not affect phosphorylation of AMPK (Fig. 2*D*). To investigate whether the increase in PP2A activity is indeed involved in the inhibition of invasion, cells were pretreated with okadaic acid

30 min before adiponectin treatment. Figure 3*A* shows that PP2A blocked the inhibitory effect of adiponectin on invasiveness. These results imply that PP2A is an important signaling molecule involved in the adiponectin-mediated suppression of invasiveness.

Because adiponectin-activated AMPK increases the activity of PP2A and the resulting PP2A causes the dephosphorylation of AKT, we examined whether AMPK and AKT directly interact with PP2A. An immunoprecipitation using an anti-PP2Ac antibody was done in lysates from cells overexpressing constitutively active AMPK and AKT. The precipitates were then analyzed by immunoblot. Phospho-AMPK and phospho-AKT were detected in the immunoprecipitates (Fig. 3*B, top*). To examine whether adiponectin increases the interaction between endogenous AKT and PP2Ac, a pull-down was done on adiponectin-treated cell lysates with an anti-AKT antibody. The level of PP2Ac in the precipitate was then determined. Adiponectin increased the interaction between endogenous AKT and PP2Ac, which could be inhibited with okadaic acid (Fig. 3*B, bottom*).

Adiponectin regulates membrane localization of PP2A activity. Because PP2A function is regulated by localization, PP2A localization was examined. Adiponectin treatment leads to the translocation of PP2A from the nucleus to the cytosol and plasma membrane, whereas PP2A remains in the nucleus with okadaic acid treatment (Fig. 3*C*). This result indicates that the translocation of PP2Ac to the cytosol may substantially contribute to the enhancement of the AKT dephosphorylation caused by adiponectin.

To further characterize the effect of adiponectin on the differential localization of PP2A, membrane fractions were examined in cells treated with adiponectin in the presence or absence of okadaic acid. The treatment of adiponectin markedly increased membrane localization of PP2A. However, treatment with adiponectin and okadaic acid prevented the adiponectin-induced membrane localization of PP2A (Fig. 3*D*).

AMPK stimulates the dephosphorylation of the PP2A catalytic subunit leading to increased activity. Phosphorylation of Tyr^{307} on PP2Ac decreases its phosphatase activity (25). Therefore, it was expected that adiponectin-activated AMPK might stimulate the dephosphorylation of PP2Ac at Tyr^{307}, resulting in an increase of phosphatase activity. To test this possibility, the level of

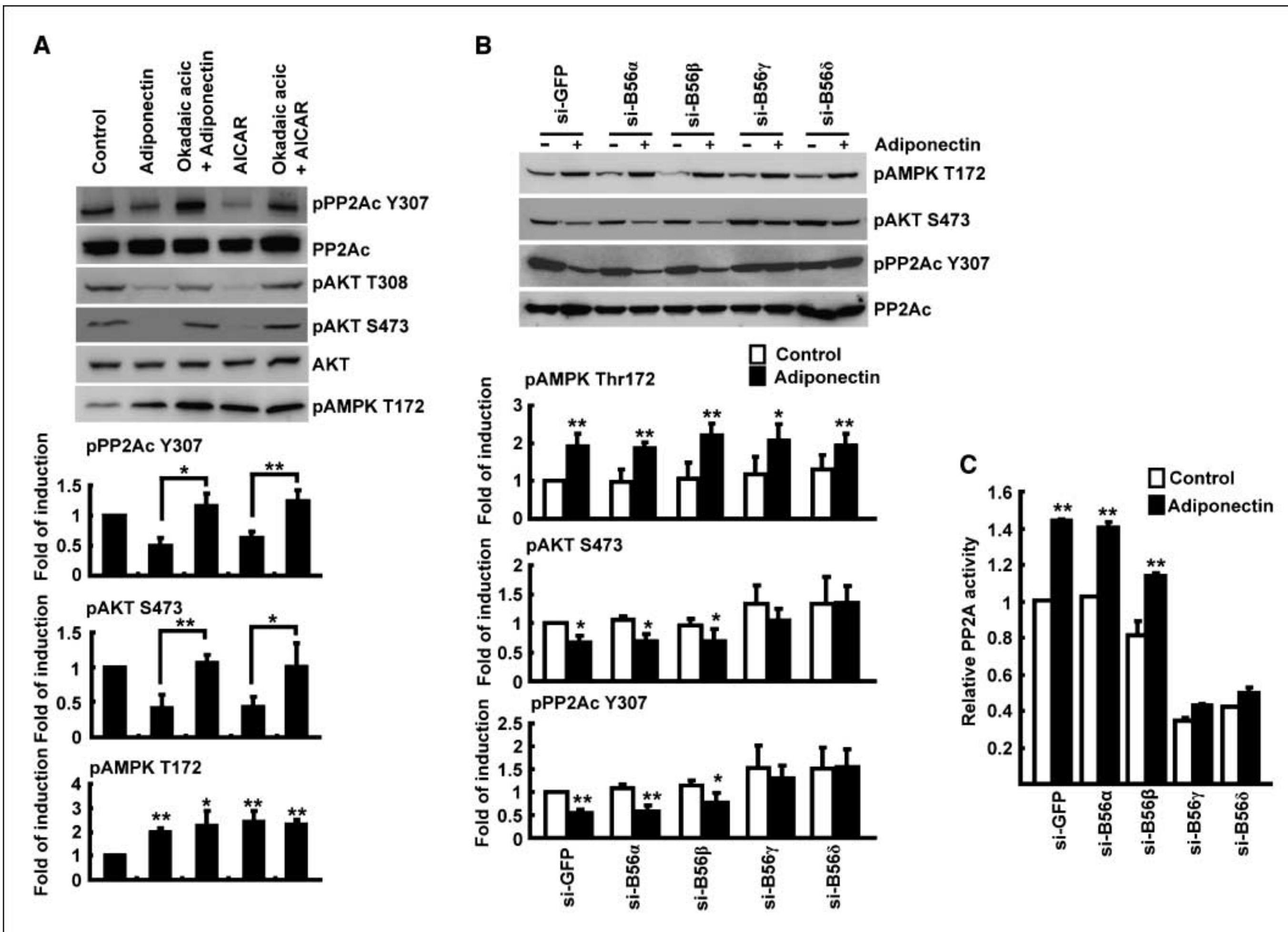

Figure 4. AMPK activates phosphatase activity of PP2A through the dephosphorylation of PP2A at Tyr^{307}. *A,* cells were treated with 20 μg/mL adiponectin, 250 μmol/L AICAR, adiponectin, and 250 nmol/L okadaic acid for 30 min, and phosphorylation levels of PP2A, AKT, and AMPK were measured using immunoblot analysis. *B,* cells were transfected with 100 nmol/L siRNA for each of the B56 isoforms and then treated with 20 μg/mL adiponectin for 30 min. Cell lysates were subjected to immunoblot analysis. *A* and *B,* mean ± SE of three independent experiments. *, $P < 0.05$; **, $P < 0.01$, comparison of treatment with adiponectin to control or as indicated otherwise. *C,* following transfection with siRNAs for each B56 isoform, PP2A activity was measured 30 min after treatment with adiponectin. Mean ± SE of three independent experiments. **, $P < 0.01$, comparison of treatment with adiponectin to control.

Published OnlineFirst April 14, 2009; DOI: 10.1158/0008-5472.CAN-08-2641

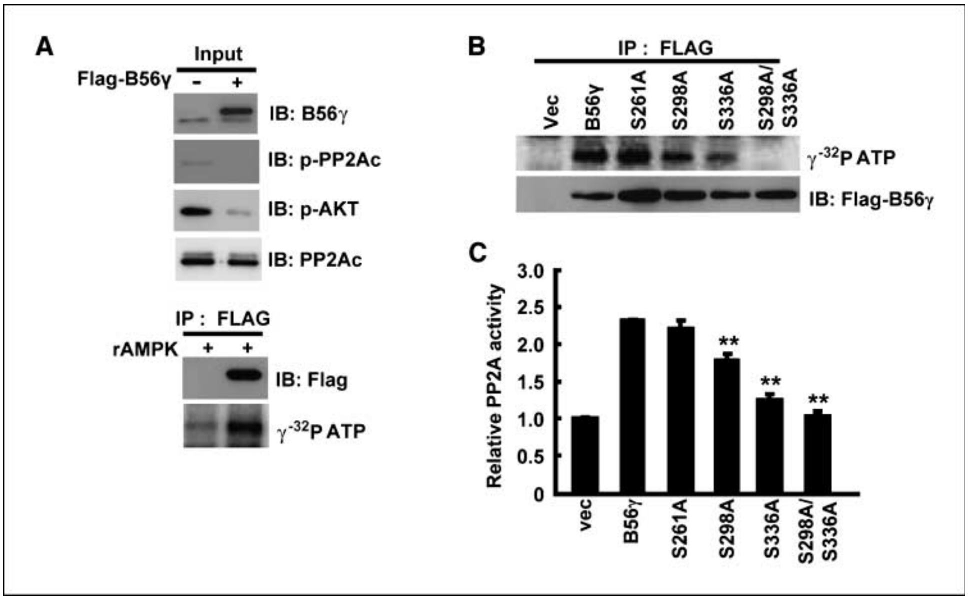

Figure 5. B56γ isoform is phosphorylated by AMPK. *A,* cells were transfected with 3 μg FLAG-tagged B56γ-expressing vector. Phosphorylation levels of PP2Ac and AKT were examined using immunoblot analysis (*top*). Anti-FLAG antibody immunoprecipitates were treated with 50 ng recombinant AMPK and ^{32}P-labeled ATP to examine phosphorylation of B56γ (*bottom*). *B,* cells were transfected with various B56γ mutants and immunoprecipitated using anti-FLAG antibody. Recombinant AMPK and ^{32}P-labeled ATP were added to examine phosphorylation levels of B56γ. *C,* phosphatase activity of PP2A was measured using cell lysates transfected with various B56γ mutants. Mean ± SE of three independent experiments done in triplicate. **, $P < 0.01$ versus B56γ.

phospho-PP2Ac was measured in adiponectin-treated cells. As expected, adiponectin stimulated the dephosphorylation of PP2Ac. Cotreatment of cells with okadaic acid and adiponectin inhibited dephosphorylation of PP2Ac and also blocked AKT dephosphorylation, but AMPK activation was not affected by the phosphorylation status of PP2Ac (Fig. 4*A*). These results indicate that adiponectin-induced dephosphorylation of PP2Ac increases PP2A phosphatase activity, resulting in dephosphorylation of AKT.

Adiponectin-mediated increase in phosphatase activity is impaired by the knockdown of B56β, B56γ, and B56δ isoforms of PP2A. PP2A is a trimer composed of a catalytic C subunit, a scaffold A subunit, and multiple regulatory B subunits that are thought to influence enzyme activity, substrate specificity, and subcellular localization. It is therefore possible that AMPK regulates PP2A activity through modification of the regulatory B subunit. Among the B subunits, the B56 subunit is known to play an antioncogenic role in cancer (26), so we determined which B56 isoform was related to adiponectin-mediated PP2A activation. siRNAs to various B56 isoforms were designed, and their efficiency was determined by reverse transcription-PCR (Supplementary Fig. S4). Next, cells were treated for 30 min with adiponectin after the transfection of each B56 isoform siRNA. Adiponectin-mediated AMPK phosphorylation was not impaired in the absence of all B56 isoforms, whereas adiponectin-mediated dephosphorylation of AKT and PP2Ac was significantly impaired in the absence of B56γ and B56δ expression (Fig. 4*B*).

To examine if impaired phosphorylation by PP2Ac also affected its activity, phosphatase activity was measured after treatment with adiponectin in cells treated with siRNA to the B56 isoforms. Knockdown of B56γ and B56δ showed no resulting increase in PP2A activity with adiponectin, whereas knockdown of B56α and B56β expression did not affect adiponectin-increased PP2A activity. Knockdown of B56γ and B56δ expression decreased even basal PP2A activity (Fig. 4*C*). This result implies that B56γ and B56δ expression is necessary for adiponectin-mediated PP2A activation.

AMPK directly phosphorylates B56 isoforms. We hypothesized that AMPK may phosphorylate the B56γ and B56δ isoforms, which in turn stimulate PP2A activity. To test this possibility, AMPK phosphorylation sites on the B56 isoforms were predicted using a the NetPhos 2.0 server. Several AMPK consensus sequences (27) were present in B56β, B56γ, and B56δ, whereas the B56α subunit does not have an AMPK phosphorylation site. The B56β subunit has two predicted potential sites, and the B56γ and B56δ subunits have three sites (Supplementary Table S1). To determine whether these predicted sites are indeed phosphorylated by AMPK, overexpressed FLAG-tagged B56γ was immunoprecipitated and incubated with recombinant AMPK and isotope-labeled ATP. As predicted, B56γ was phosphorylated by AMPK (Fig. 5*A*). To predict the exact phosphorylation site of B56γ, S261A, S298A, and S336A mutants were generated and subjected to an *in vitro* phosphorylation assay. S298A and S336A mutants of B56γ were partially phosphorylated. Thus, a S298A/S336A double mutant was generated. The level of phosphorylation in the S298A/S336A double mutant was completely blocked, indicating that AMPK phosphorylates both S298 and S336 residues (Fig. 5*B*). To determine if PP2A activity is affected by B56γ mutants, PP2A activity was measured in cells overexpressing B56γ mutants. Expression of the S298A/S336A double mutant failed to increase phosphatase activity compared with expression of the wild-type form (Fig. 5*C*).

Adiponectin increases PP2A activity *in vivo*. We wondered whether adiponectin levels and PP2A activities were decreased in human breast tumor tissues. The level of adiponectin was significantly decreased in the serum of patients (Fig. 6*A*), and PP2A activities were dramatically reduced compared with that of adjacent normal tissues (Fig. 6*B*). Next, we wondered whether the direct administration of adiponectin into a tumor mass would increase PP2A activity in a murine mammary tumor model. Thus, intratumoral administration of adiponectin was done 14 days after initial implantation of 4T1 murine mammary tumor cells into syngeneic BALB/c mice. PP2A activity within the adiponectin-injected tumor mass was measured 8 h after the intratumoral administration. PP2A activity increased within the adiponectin-injected tumor masses (Fig. 6*C*), consistent with the result seen in the breast cancer cell line. The phosphorylation levels of PP2Ac and AMPK were also evaluated. The phosphorylation level of PP2Ac decreased and the phosphorylation level of AMPK increased in adiponectin-injected tumor mass (Fig. 6*D*), indicating that adiponectin was indeed able to increase PP2A activity through AMPK activation *in vivo*.

Discussion

Although it has not yet been determined if the decreased production of adiponectin is a cause or a result of breast cancer growth, it is conceivable that tumor cells may grow well and gain metastatic potential more easily in an environment in which adiponectin is decreased because adiponectin suppresses the metastasis and proliferation of cancer cells through AKT inactivation. However, the molecular mechanism underlying this process is not fully understood. Recently, it was shown that AMPK, activated by energy depletion, phosphorylates IRS-1 at Ser^{794}, leading to the inhibition of AKT through suppression of PI3K (28). In this study, however, our data clearly showed that AMPK activates PP2A, which directly inactivates AKT by the dephosphorylation of Thr^{308} and Ser^{473} without affecting PI3K activity, indicating that AMPK is able to suppress AKT function by either an IRS-1-dependent or a PP2A-dependent pathway. In addition to AKT suppression by AMPK-activated PP2A, there is another possible mechanism by which AMPK affects AKT activity. The mammalian target of rapamycin complex (mTORC) 2 phosphorylates AKT at Ser^{473} (29, 30), and the TSC2 complex is required for AKT phosphorylation by mTORC2 (31). Because AMPK phosphorylates and activates TSC2, it is possible that adiponectin-activated AMPK affects the activity of AKT through the regulation of mTORC2 activation. Therefore, it remains to be determined if adiponectin-activated AMPK is able to regulate mTORC2 activity through TSC2 phosphorylation.

In contrast to the AMPK-mediated regulation of AKT activity, AKT is also able to regulate AMPK function. AKT increases the production of ATP through accelerated aerobic glycolysis in tumors (32, 33), leading to the increase in intracellular ATP level, which in turn decreases AMPK activity. Moreover, AKT and AMPK reciprocally regulate mTORC1 activity through the differential phosphorylation of TSC2. In the presence of growth factors, AKT phosphorylates TSC2, leading to an increase in mTORC1 activity, resulting in the activation of the ATP-consuming pathway. In low nutritional conditions, AMPK phosphorylates TSC2 at a different site, leading to the suppression of mTORC1 activity, resulting in the activation of the ATP-producing pathway. The bidirectional communication between AMPK and AKT plays an important role in the regulation of cellular energy balance. Therefore, the disruption of AMPK and AKT crosstalk may play a critical role in the deregulation of cell cycle or tumor progression. The complete understanding of bidirectional communication between AMPK and AKT may uncover a potential therapeutic target.

Our data show that the phosphorylation of B56γ by AMPK increases dephosphorylation of PP2Ac at Tyr^{307}, leading to the up-regulation of PP2A activity. This result may be explained by the autodephosphorylation of PP2A (25, 34). That is, AMPK-mediated

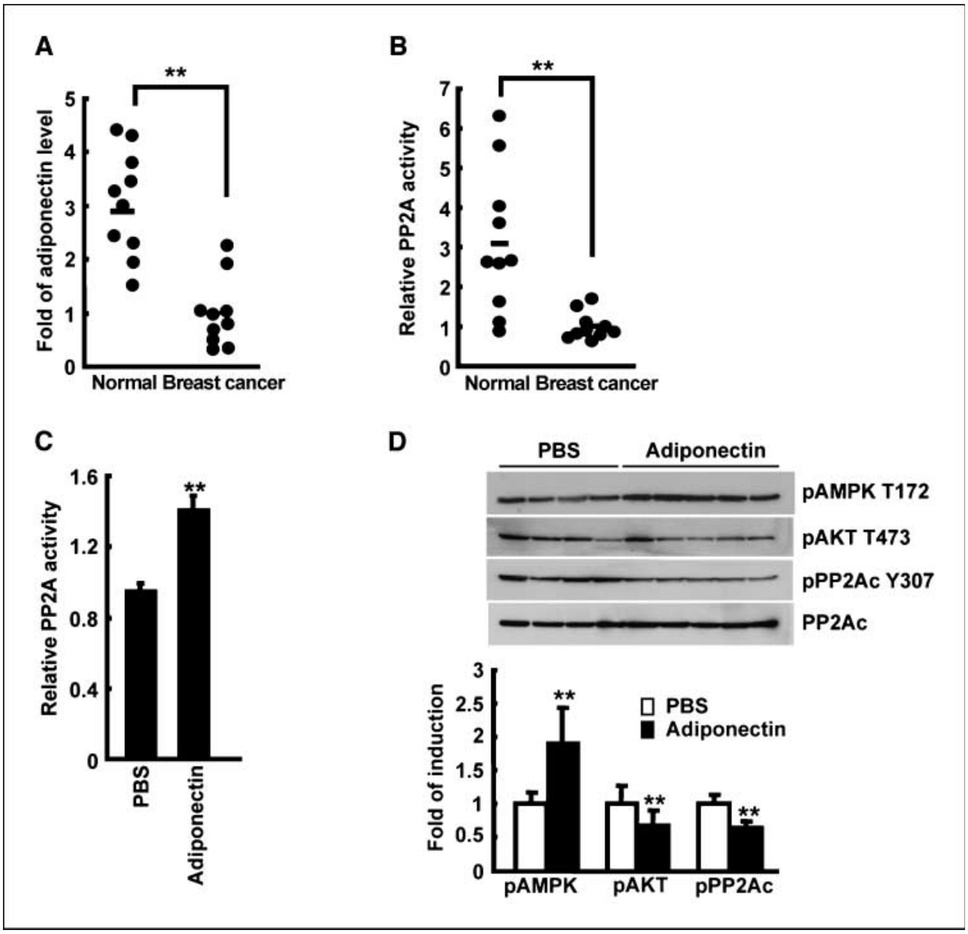

Figure 6. AMPK and PP2A are activated by intratumoral injection of adiponectin. *A,* level of adiponectin was measured in the sera of normal healthy individuals and breast cancer patients using immunoblot analysis (n = 10). **, $P < 0.01$ versus healthy individuals. *B,* PP2A activity was measured using tumor lysates and adjacent normal tissue lysates (n = 10). **, $P < 0.01$ versus healthy individuals. *C* and *D,* BALB/c mice (n = 5 per group) bearing 4T1 murine mammary tumors were intratumorally injected with adiponectin on day 14. PP2A activity and phosphorylation levels of AMPK and PP2Ac were measured using tumor lysates 8 h after administration of 100 μg adiponectin. Mean ± SE of three independent experiments. Blots are representative of three independent experiments. **, $P < 0.01$ versus PBS.

phosphorylation of B56γ affects phosphotyrosyl phosphatase activity, causing an increase in the basal level of protein tyrosine phosphatase activity of PP2A (35–38). However, we cannot completely exclude the possibility that phosphorylated B56 recruits a tyrosine phosphatase or phosphotyrosyl phosphatase activator into the entire PP2A complex, which is responsible for the dephosphorylation of PP2A at Tyr^{307}. A complete picture of the mechanism of PP2A autodephosphorylation remains to be elucidated. In addition to the regulation of PP2A activity by the modification of catalytic subunits, it has also been reported that PP2A activity can be regulated by the modification of regulatory subunits. Various kinases including extracellular signal-regulated kinase, protein kinase A, and calcein-AM kinase II have been shown to phosphorylate PP2A regulatory subunits (39–43). Our data show that AMPK phosphorylates Ser^{298} and Ser^{336} residues of the B56γ isoform. Among B56 subunits, B56α has no AMPK consensus site, and B56β has no consensus site corresponding to Ser^{298} residue of B56γ isoform. Moreover, the level of B56β expression is much lower than that of other subunits in MDA-MB-231 cells (Supplementary Fig. S3). This may explain why knockdown of B56α and B56β does not markedly affect adiponectin-mediated increase of PP2A activity (Fig. 4*C*). In addition to AMPK sites, B56γ has additional phosphorylation sites that could be simultaneously phosphorylated. Elucidating the effect of multiple phosphorylations by various kinases on B56 isoforms will be important for understanding the physiologic role of PP2A as a signal integrator of various stimuli, including growth factors and nutrients in different cellular contexts. Several reports support the tumor-suppressive role of the B56γ isoform. Overexpression of B56γ partially reverses the tumorigenic phenotype of lung cancer cells (26) and reduces the abundance of β-catenin protein through inhibition of APC-axin formation, leading to destabilization of the β-catenin protein (44). B56γ1- and B56γ3-containing PP2A holoenzymes also directly dephosphorylate p53 at Thr^{55}, leading to the inhibition of cell proliferation and transformation (20). The work presented here lends additional support to a tumor-suppressive role of B56γ by showing that adiponectin-activated AMPK phosphorylates B56γ, leading to the reactivation of the tumor suppressor PP2A, which in turn reduces the metastasis of breast tumor cells.

In summary, we show for the first time that adiponectin-activated AMPK directly phosphorylates a B56γ regulatory subunit, leading to the activation of PP2A, and that AMPK-activated PP2A is required to suppress AKT. Therefore, we propose that reactivation of PP2A tumor suppressor activity by adiponectin is a promising novel therapeutic strategy for treating cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/11/08; revised 2/2/09; accepted 2/12/09; published OnlineFirst 4/14/09.

Grant support: SRC Program of KOSEF R11-2005-017-04002-0(2008), Korea Research Foundation grant KRF-2007-313-C00519 funded by the Korean Government, and Rural Development Administration Biogreen 21 Project 20070301034031.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Harvie M, Howell A. Energy balance adiposity and breast cancer—energy restriction strategies for breast cancer prevention. Obes Rev 2006;7:33–47.
2. Iyengar P, Espina V, Williams TW, et al. Adipocyte-derived collagen VI affects early mammary tumor progression *in vivo*, demonstrating a critical interaction in the tumor/stroma microenvironment. J Clin Invest 2005;115:1163–76.
3. Wang Y, Lam JB, Lam KS, et al. Adiponectin modulates the glycogen synthase kinase-3β/β-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. Cancer Res 2006;66:11462–70.
4. King TD, Song L, Jope RS. AMP-activated protein kinase (AMPK) activating agents cause dephosphorylation of Akt and glycogen synthase kinase-3. Biochem Pharmacol 2006;71:1637–47.
5. Jiang W, Zhu Z, Thompson HJ. Modulation of the activities of AMP-activated protein kinase, protein kinase B, and mammalian target of rapamycin by limiting energy availability with 2-deoxyglucose. Mol Carcinog 2008;47:616–28.
6. Jin Q, Feng L, Behrens C, et al. Implication of AMP-activated protein kinase and Akt-regulated survivin in lung cancer chemopreventive activities of deguelin. Cancer Res 2007;67:11630–9.
7. Purev E, Giordano A, Soprano DR, Soprano KJ. Interaction of PP2A catalytic subunit with Rb2/p130 is required for all-*trans* retinoic acid suppression of ovarian carcinoma cell growth. J Cell Physiol 2006;206:495–502.
8. Van Hoof C, Goris J. PP2A fulfills its promises as tumor suppressor: which subunits are important? Cancer Cell 2004;5:105–6.
9. Arroyo JD, Hahn WC. Involvement of PP2A in viral and cellular transformation. Oncogene 2005;24:7746–55.
10. Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. Trends Mol Med 2008;14:152–60.
11. Lee TH, Turck C, Kirschner MW. Inhibition of cdc2 activation by INH/PP2A. Mol Biol Cell 1994;5:323–38.
12. Santoro MF, Annand RR, Robertson MM, et al. Regulation of protein phosphatase 2A activity by caspase-3 during apoptosis. J Biol Chem 1998;273:13119–28.
13. Yeh E, Cunningham M, Arnold H, et al. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nat Cell Biol 2004;6:308–18.
14. Takagi Y, Futamura M, Yamaguchi K, Aoki S, Takahashi T, Saji S. Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers. Gut 2000;47:268–71.
15. Wang SS, Esplin ED, Li JL, et al. Alterations of the PPP2R1B gene in human lung and colon cancer. Science 1998;282:284–7.
16. Ruediger R, Pham HT, Walter G. Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the Aα subunit gene. Oncogene 2001;20:10–5.
17. Colella S, Ohgaki H, Ruediger R, et al. Reduced expression of the Aα subunit of protein phosphatase 2A in human gliomas in the absence of mutations in the Aα and Aβ subunit genes. Int J Cancer 2001;93:798–804.
18. Suzuki K, Takahashi K. Reduced expression of the regulatory A subunit of serine/threonine protein phosphatase 2A in human breast cancer MCF-7 cells. Int J Oncol 2003;23:1263–8.
19. Ito A, Kataoka TR, Watanabe M, et al. A truncated isoform of the PP2A B56 subunit promotes cell motility through paxillin phosphorylation. EMBO J 2000;19:562–71.
20. Li HH, Cai X, Shouse GP, Piluso LG, Liu X. A specific PP2A regulatory subunit, B56γ, mediates DNA damage-induced dephosphorylation of p53 at Thr^{55}. EMBO J 2007;26:402–11.
21. Li X, Yost HJ, Virshup DM, Seeling JM. Protein phosphatase 2A and its B56 regulatory subunit inhibit Wnt signaling in *Xenopus*. EMBO J 2001;20:4122–31.
22. Arnold HK, Sears RC. Protein phosphatase 2A regulatory subunit B56α associates with c-myc and negatively regulates c-myc accumulation. Mol Cell Biol 2006;26:2832–44.
23. Kim KY, Kim JK, Han SH, et al. Adiponectin is a negative regulator of NK cell cytotoxicity. J Immunol 2006;176:5958–64.
24. Zuluaga S, Alvarez-Barrientos A, Gutierrez-Uzquiza A, Benito M, Nebreda AR, Porras A. Negative regulation of Akt activity by p38α MAP kinase in cardiomyocytes involves membrane localization of PP2A through interaction with caveolin-1. Cell Signal 2007;19:62–74.
25. Chen J, Martin BL, Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. Science 1992;257:1261–4.
26. Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC, Hahn WC. Identification of specific PP2A complexes involved in human cell transformation. Cancer Cell 2004;5:127–36.
27. Hardie DG, Scott JW, Pan DA, Hudson ER. Management of cellular energy by the AMP-activated protein kinase system. FEBS Lett 2003;546:113–20.
28. Tzatsos A, Tsichlis PN. Energy depletion inhibits phosphatidylinositol 3-kinase/Akt signaling and induces apoptosis via AMP-activated protein kinase-dependent phosphorylation of IRS-1 at Ser-794. J Biol Chem 2007;282:18069–82.
29. Guertin DA, Stevens DM, Thoreen CC, et al. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCα, but not S6K1. Dev Cell 2006;11:859–71.

30. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005;307:1098–101.
31. Huang J, Dibble CC, Matsuzaki M, Manning BD. The TSC1-2 complex is required for proper activation of mTOR complex 2. Mol Cell Biol 2008;28:4104–15.
32. Elstrom RL, Bauer DE, Buzzai M, et al. Akt stimulates aerobic glycolysis in cancer cells. Cancer Res 2004;64:3892–9.
33. Hahn-Windgassen A, Nogueira V, Chen CC, Skeen JE, Sonenberg N, Hay N. Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. J Biol Chem 2005;280:32081–9.
34. Guo H, Damuni Z. Autophosphorylation-activated protein kinase phosphorylates and inactivates protein phosphatase 2A. Proc Natl Acad Sci U S A 1993;90:2500–4.
35. Chao Y, Xing Y, Chen Y, et al. Structure and mechanism of the phosphotyrosyl phosphatase activator. Mol Cell 2006;23:535–46.
36. Leulliot N, Vicentini G, Jordens J, et al. Crystal structure of the PP2A phosphatase activator: implications for its PP2A-specific PPIase activity. Mol Cell 2006;23:413–24.
37. Cayla X, Goris J, Hermann J, Hendrix P, Ozon R, Merlevede W. Isolation and characterization of a tyrosyl phosphatase activator from rabbit skeletal muscle and *Xenopus laevis* oocytes. Biochemistry (Mosc) 1990;29:658–67.
38. Goris J, Pallen CJ, Parker PJ, Hermann J, Waterfield MD, Merlevede W. Conversion of a phosphoseryl/threonyl phosphatase into a phosphotyrosyl phosphatase. Biochem J 1988;256:1029–34.
39. Fukunaga K, Muller D, Ohmitsu M, Bako E, DePaoli-Roach AA, Miyamoto E. Decreased protein phosphatase 2A activity in hippocampal long-term potentiation. J Neurochem 2000;74:807–17.
40. Xu Z, Williams BR. The B56α regulatory subunit of protein phosphatase 2A is a target for regulation by double-stranded RNA-dependent protein kinase PKR. Mol Cell Biol 2000;20:5285–99.
41. Ahn JH, McAvoy T, Rakhilin SV, Nishi A, Greengard P, Nairn AC. Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56δ subunit. Proc Natl Acad Sci U S A 2007;104:2979–84.
42. Letourneux C, Rocher G, Porteu F. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. EMBO J 2006;25:727–38.
43. Rocher G, Letourneux C, Lenormand P, Porteu F. Inhibition of B56-containing protein phosphatase 2As by the early response gene IEX-1 leads to control of Akt activity. J Biol Chem 2007;282:5468–77.
44. Polakis P. Wnt signaling and cancer. Genes Dev 2000;14:1837–51.