

Adiponectin Modulates the Glycogen Synthase Kinase-3 β / β -Catenin Signaling Pathway and Attenuates Mammary Tumorigenesis of MDA-MB-231 Cells in Nude Mice

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Abstract

Adiponectin is an adipokine that has pleiotropic beneficial roles in systemic insulin resistance and inflammation. Several recent clinical studies suggest that low serum levels of adiponectin are associated with increased risks of breast cancer. Here, we investigated the direct effects of adiponectin on breast cancer development *in vitro* and *in vivo*. Our results showed that adiponectin significantly attenuated the proliferations of two typical human breast cancer cells, MDA-MB-231 and T47D, in a cell type-specific manner. Further analysis revealed that adiponectin could induce apoptosis and arrest the cell cycle progression at G₀-G₁ phase in MDA-MB-231 cells. Prolonged treatment with adiponectin in this cell line blocked serum-induced phosphorylation of Akt and glycogen synthase kinase-3 β (GSK-3 β), suppressed intracellular accumulation of β -catenin and its nuclear activities, and consequently reduced expression of cyclin D1. Adiponectin-mediated suppression of cyclin D1 expression and attenuation of cell proliferation was abrogated by the GSK-3 β inhibitor lithium chloride. These results suggest that the inhibitory role of adiponectin on MDA-MB-231 cell growth might be attributed to its suppressive effects on the GSK-3 β / β -catenin signaling pathway. Furthermore, our *in vivo* study showed that both supplementation of recombinant adiponectin and adenovirus-mediated overexpression of this adipokine substantially reduced the mammary tumorigenesis of MDA-MB-231 cells in female nude mice. Taken together, these data support the role of adiponectin as a negative regulator of breast cancer development and also suggest that adiponectin might represent a novel therapeutic target for this disease. (Cancer Res 2006; 66(23): 11462-70)

Introduction

Breast cancer represents one of the leading causes of cancer death among women (1). Obesity is an independent risk factor for the development of breast cancer and is associated with late-stage disease and poor prognosis (2). Although the exact mechanisms underlying this relationship remain obscure, increasing evidence

suggests that adipose tissue (fat) of the mammary gland might play important roles in regulating cancer cell growth, invasion, and distant metastasis (3). The close relationship between adipocytes and mammary tumor growth has been shown by the finding that cotransplantation of tumor cells with adipocytes results in increased tumor growth and metastasis in mice (4). The conditioned medium derived from both preadipocytes (5) and mature adipocytes (6) has been shown to promote the growth of breast carcinoma cells *in vitro* and *in vivo*. In postmenopausal women, adipose tissue becomes the major source of estrogens through aromatization of C₁₉ steroid androstenedione. Elevated circulating levels of estrogens in obesity are causally associated with increased breast cancer risk and enhanced progression of estrogen receptor (ER)-positive breast cancers (7). In addition, the aberrant production of adipokines, which refer to a cluster of secreted polypeptides produced from adipocytes, might also play important roles in the pathogenesis of obesity-related breast carcinogenesis. Many proinflammatory adipokines, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), can induce insulin resistance, an independent risk factor for breast cancer (8). Some adipokines, including leptin, heparin-binding epidermal growth factor (EGF), and collagen VI, can directly act on breast cancer cells to regulate their proliferation, invasiveness, and malignancy (3, 9, 10).

Adiponectin is one of the major adipokines predominantly expressed from white adipose tissue (11). It belongs to the complement factor C1q-like protein superfamily and is composed of an NH₂-terminal collagenous domain and a COOH-terminal globular domain. Unlike most of the adipokines that are causally linked to obesity-related diseases, adiponectin has potent insulin-sensitizing, anti-inflammatory, and antiatherogenic activities. Plasma levels of adiponectin are decreased in obesity and various obesity-related pathologies (12). Replenishment of recombinant adiponectin in animal models can improve glucose/lipid homeostasis, increase insulin sensitivity, prevent atherosclerosis, and ameliorate fatty liver diseases (13–16).

Several recent case-control and prospective studies have shown the close association of low serum adiponectin with high incidence of obesity-related cancer diseases, including endometrial, breast, prostate, and gastric cancers (17–21). Although the correlation between adiponectin expression in local mammary tissue and the development of breast cancer has not been fully established, clinical data from different ethnic populations indicate that low adiponectin plasma level is an independent risk factor for breast cancer disease (18, 22–24). Moreover, tumors in women with low serum adiponectin levels are more likely to show a biologically aggressive phenotype (23). Notably, we and others have shown that

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adiponectin possesses inhibitory activities on the proliferation of various types of cells, including aortic smooth muscle cells, endothelial cells, and several types of cancer cells (25–28), although the underlying mechanisms are currently unclear. Taken together, these evidences suggest that adiponectin secreted from mammary adipose tissue might act as a growth-regulatory factor in the tumor microenvironment.

In this study, we have done a series of *in vitro* and *in vivo* experiments to test the effects of adiponectin on the growth of two typical human breast carcinoma cells, ER-negative MDA-MB-231 and ER-positive T47D cells. Our studies revealed that full-length adiponectin could significantly attenuate the proliferation of these cells stimulated by fetal bovine serum (FBS) or growth factors in a cell type-specific manner. We also found that adiponectin treatment could markedly inhibit the tumor growth and distant metastasis of the invasive MDA-MB-231 cells in nude mice. Accordingly, we further investigated the potential molecular mechanisms that underlie the inhibitory effects of adiponectin on breast cancer cell growth and tumor development.

Materials and Methods

Materials. Anti-total and phosphorylated (Ser³³/Ser³⁷/Thr⁴¹) β -catenin, anti-total and phosphorylated (Ser⁴⁷³) Akt, and anti-total and phosphorylated (Ser⁹) glycogen synthase kinase-3 β (GSK-3 β) antibodies were obtained from Cell Signaling (Beverly, MA). Anti-cyclin D1 rabbit monoclonal antibody was from Upstate (Lake Placid, NY). Anti- β -actin monoclonal antibody was from Sigma (St. Louis, MO). BioPlex phosphoprotein detection reagents were from Bio-Rad (Hercules, CA). SYBR Green real-time PCR reagents were from Applied Biosystems (Foster City, CA). The MDA-MB-231 and T47D cells were obtained from the American Type Culture Collection (Manassas, VA). Recombinant adiponectin was produced from *Escherichia coli* or HEK293 cells as previously described (15, 26, 29, 30). The endotoxin was removed by the Detoxi-Gel Endotoxin Removal kit (Pierce, Rockford, IL). The purity of the proteins was confirmed by silver staining analysis of SDS-PAGE gels, which detected only a single ~35-kDa protein species that correspond to adiponectin. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was also used to verify that there were no contaminations of FLAG peptide or other peptides in the purified protein solution. The purified adiponectin was stored at 5 μ g/ μ L in PBS at -80°C .

Cell proliferation and [³H]thymidine incorporation assays. Cells were seeded at a density of 4×10^3 per well in 96-well plates. After starving in DMEM with 0.5% FBS for 48 hours, cells were subsequently stimulated with various concentrations of FBS or different growth factors in the presence or absence of adiponectin (15 μ g/mL in PBS) as indicated. The viable cell numbers at different time points were manually counted by mixing with trypan blue dye. For [³H]thymidine incorporation experiment, cells seeded in 24-well plate (2×10^4 per well) were labeled with 1 μ Ci/mL [³H-methyl]thymidine for the last 6 hours of the treatment. After washing and trichloroacetic acid (TCA) precipitation, DNA was solubilized with 0.2 mol/L NaOH, neutralized with 0.2 mol/L HCl, and quantified with a liquid scintillation counter.

DNA fragmentation analysis and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. DNA fragmentation was quantified in breast cancer cells prelabeled with [³H-methyl]thymidine as previously described (31). Briefly, cells grown in 24-well dishes were prelabeled with 1 μ Ci/mL [³H-methyl]thymidine for 9 hours, treated with different stimulators for various periods, and then harvested in lysis buffer containing 10 mmol/L Tris (pH 7.4), 10 mmol/L EDTA, and 0.6% Triton X-100. After TCA precipitation, the cell lysates were centrifuged to separate double-stranded fragmented DNA from chromosomal DNA and quantified by liquid scintillation counting.

Apoptotic cells were also detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) *In situ* Cell Death

Detection kit (Roche, Indianapolis, IN). Briefly, cells were fixed in 4% paraformaldehyde and the assay was conducted according to the instructions from the manufacturer. Samples were viewed at 488 nm excitation/512 nm emission under a fluorescence microscope (Leica Microsystems, Bensheim, Germany). TUNEL-positive cells were manually counted in four to eight random areas of each coverslip to determine the percentage of apoptotic cells.

Cell cycle analysis. Cells fixed with 70% ethanol were resuspended in the staining buffer [100 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, 0.1% NP40, 2 μ g/mL propidium iodide] and incubated in the dark for 20 minutes on ice. The analysis was carried out with EPICS Elite ESP Flow Cytometer and EXPO software (Beckman Coulter, Miami, FL). Data were acquired from 1×10^4 cells per sample. Gating of sub-G₀-G₁, G₀-G₁, S, and G₂-M populations was subsequently done manually using WinList software (Purdue University Cytometry Laboratories, West Lafayette, IN).

Subcellular fractionation and Western blotting. Cells were resuspended in a hypotonic buffer [10 mmol/L Tris-HCl (pH 7.5), 25 mmol/L KCl, 2 mmol/L magnesium acetate, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, protease inhibitor cocktails]. After incubating on ice for 10 minutes, cell membranes were disrupted by 10 passes through a 23-gauge needle, and nuclear isolation was monitored under a microscope. After centrifugation for 5 minutes at $1,000 \times g$, the supernatant was saved as the "cytoplasmic fraction." The nuclear pellet was washed once with the hypotonic buffer and lysed with hypertonic buffer (hypotonic buffer plus 400 mmol/L KCl and 20% glycerol). The lysates were then centrifuged at $12,000 \times g$ for 5 minutes, and the supernatant was collected as the "nuclear fraction" and frozen at 80°C . Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce).

Total cell lysates and cytoplasmic or nuclear fractions were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then probed with various primary antibodies to determine the expression of the signaling proteins, including cyclin D1, phosphorylated and total β -catenin, extracellular signal-regulated kinase 1/2 (ERK1/2), GSK-3 β , and Akt in MDA-MB-231 and T47D cells. The antibody-antigen complexes were detected using an enhanced chemiluminescence kit from GE Healthcare (Uppsala, Sweden).

BioPlex phosphoprotein assay. Multisignaling phosphoprotein profiling was done on a BioPlex 100 suspension array system (Bio-Rad). The protein concentrations of total cell lysates collected at different time points were quantified using the BCA method, and equal amount of protein (50 μ g) was used for the BioPlex phosphoprotein assay following the instructions from the manufacturer.

β -Catenin/T-cell factor-lymphoid enhancer factor-1 transcription reporter assay. The nuclear activity of endogenous β -catenin in MDA-MB-231 cells was analyzed by using the TOPflash/FOPflash reporter system (Upstate, Charlottesville, VA). Cells grown in 24-well dishes were transfected with either the TOPflash or FOPflash plasmid using LipofectAMINE Plus reagent. To normalize transfection efficiency in the reporter assays, cells were cotransfected with pRL-TK plasmid, which contains a functional *Renilla luciferase* gene cloned downstream of a herpes simplex virus thymidine kinase promoter (Promega, Madison, WI). Four hours after transfection, the culture medium was replaced by DMEM supplemented with 0.5% or 10% FBS in the presence or absence of 15 μ g/mL adiponectin. Forty-eight hours after transfection, cells were washed with PBS and then lysed with the reporter lysis buffer (Promega). Luciferase activities in cell lysates were measured with the Promega Dual-Luciferase Reporter System and normalized to control *Renilla luciferase* signal.

Quantitative real-time PCR. Total RNA was isolated with RNeasy kit (Qiagen, Hilden, Germany). Total RNA (1 μ g) was used for the synthesis of first-strand cDNA using reverse transcriptase (Promega). Quantitative real-time PCR was done using the SYBR Green PCR Core Reagents kit (Applied Biosystems). The reactions were carried out with a 7000 Sequence Detection System (Applied Biosystems). Standard curves were generated for each gene of interest using 10-fold serial dilutions of a known amount of cDNA. Quantification was achieved using C_t values or values generated from standard curves that were normalized with β -actin mRNA levels. Primers for real-time PCR were listed in Supplementary Table S1.

Inoculation of breast cancer cells into nude mice and treatment with adiponectin. We applied three different approaches to investigate the effects of adiponectin on breast cancer development *in vivo*. First, MDA-MB-231 cells were pretreated with or without recombinant adiponectin (15 $\mu\text{g}/\text{mL}$) and 5×10^6 cells were then injected into the right thoracic mammary fat pad of 4- to 6-week-old female nude mice under anesthetic condition. Second, the recombinant adenovirus [10^8 plaque-forming units (pfu)] that expressed adiponectin (Ad-ADN) or luciferase (Ad-Luc; refs. 31, 32) was injected into the tumor of each mouse at the 14th day after initial implantation of the MDA-MB-231 cells. Third, 7 days after the initial inoculation of the MDA-MB-231 cells, recombinant adenoviruses (Ad-ADN or Ad-Luc) were administered into nude mice by tail vein injections. Note that the amount of injected adenovirus caused no toxicity in the mice as judged by their body weight gains, food and water intakes, liver functions, as well as other behavioral variables, such as fear or aggression, diarrhea, respiratory and cardiovascular signs, etc. Tumors were measured using digital vernier calipers, with tumor volume calculated using the formula [sagittal dimension (mm) \times cross dimension (mm)]² / 2 and expressed in cm^3 . All animals were sacrificed at 5 weeks after the initial implantation. Primary and secondary tumors, normal mammary fat pad, and lung and liver tissues were collected; fixed in 10% neutral-buffered formalin; and subjected to further analysis. All the experimental protocols were approved by the Animal Ethics Committee at the University of Hong Kong.

Analysis of lung tissues for metastasis. Formalin-fixed lungs were first assessed for surface white spots under a dissection microscope and then analyzed by H&E staining of the 4- μm sections. Six to 10 random fields of the stained tissue sections were analyzed under a light microscope. ImageJ Processing and Analysis software was used to quantify areas of abnormal tissue, which was classified as tissue that appeared as areas of solid mass compared with normal sparsely looking tissue (33). The percentage of abnormal lung tissue was calculated using the following formula: (area of abnormal tissue / total tissue area) \times 100. Three sections of at least 100 μm apart were used for each animal, and six to eight animals were used for each treatment group.

Transwell migration and invasion assays. For migration assays, cells pretreated with adiponectin for 24 hours were seeded at a density of 5×10^4 per well into the upper chamber of the 8- μm pore Transwell (Corning, Inc., Austin, TX), with the lower chamber containing DMEM with various concentrations of FBS. For invasion assays, cells at a density of 2×10^5 per well were seeded on the porous membranes precoated with 30 μL Matrigel (BD Biosciences, San Jose, CA). After incubating at 37°C for 5 hours (migration) or 24 hours (invasion), cells attached to the low surface of the membrane were fixed and stained with hematoxylin. Eight to 10 fields per Transwell were counted at a magnification of $\times 100$.

Statistics. All experiments were done with four to eight samples per group, and all results were derived from at least three independent experiments. Values are expressed as mean \pm SD. All the statistical calculations were done with the Statistical Package for the Social Sciences version 11.5 software package (SPSS, Inc., Chicago, IL). Comparison between groups was done using Student's unpaired *t* test. In all statistical comparisons, $P < 0.05$ was used to indicate a significant difference.

Results

Full-length adiponectin inhibits the proliferation of human breast cancer cells in a cell type-specific manner. We first investigated the direct effects of adiponectin on breast cancer cell proliferation in both ER-positive/luminal epithelial-like T47D cells and ER-negative/mesenchymal-like MDA-MB-231 cells. These two types of cells have been reported to have different oncogenic phenotypes and distinct gene expression profiles (34). Cell counting results showed that adiponectin inhibited the growth of MDA-MB-231 cells stimulated by various concentrations of FBS (Fig. 1A). At 48 hours after adiponectin treatment, the magnitude of 2.5% and 10% FBS-stimulated cell growth was inhibited by $\sim 34.5\%$ and 42.7% , respectively. [^3H]thymidine incorporation experiments also showed a significant decrease of DNA synthesis in adiponectin-treated cells, and this effect was observed as early as 24 hours after the treatment (Fig. 1B). Notably, the number of MDA-MB-231 cells cultured under a 2.5% FBS condition started to decrease after 72 hours, suggesting that the propagation of this cell line is dependent on higher concentrations of serum. Unlike MDA-MB-231 cells, we found that T47D cells could proliferate even in the medium containing 0.5% FBS (Supplementary Fig. S7), and adiponectin treatment had little effects on the cell growth stimulated by various concentrations of FBS in this cell line. On the other hand, adiponectin significantly decreased DNA synthesis and proliferation of T47D cells induced by several growth factors, including insulin (Supplementary Fig. S7), basic fibroblast growth factor, and heparin-binding EGF (data not shown). These results showed that adiponectin could inhibit the proliferation of both the mesenchymal-like and luminal epithelial-like breast cancer cells in a cell type-specific manner. In addition, we have also confirmed that the full-length recombinant adiponectin produced from other two commercial sources (R&D Systems, Minneapolis, MN, and BioVendor Laboratory Medicine, Modrice, Czech Republic) had similar

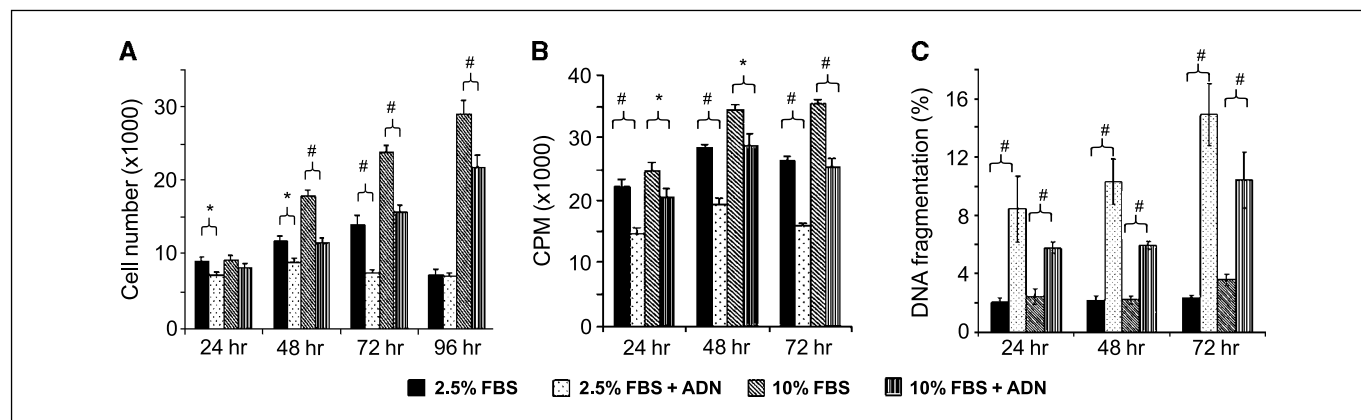
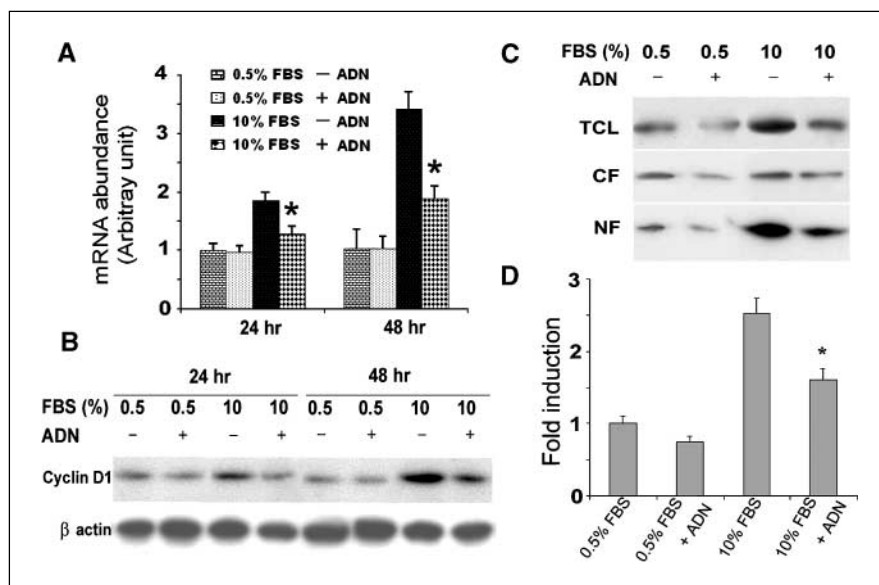


Figure 1. Adiponectin inhibits proliferation and induces DNA fragmentation in MDA-MB-231 cells. Cells were treated with various concentrations of FBS in the presence or absence of recombinant adiponectin (15 $\mu\text{g}/\text{mL}$ in PBS) for different periods as indicated. *A*, viable cell numbers were manually counted at different time points of treatment. *B*, DNA synthesis was determined by [^3H]thymidine incorporation assay. *C*, the fragmented DNA was quantified in cells prelabeled with [^3H]thymidine. ADN, adiponectin. *, $P < 0.05$; #, $P < 0.01$ ($n = 6$, from four independent assays).

Figure 2. Adiponectin treatment decreases serum-induced expression of cyclin D1, protein stabilization, and nuclear activities of β -catenin in MDA-MB-231 cells. **A**, real-time PCR analysis for quantification of the relative mRNA abundance of cyclin D1 after normalization against β -actin. **B**, Western blotting analysis for determination of cyclin D1 or β -actin protein levels under various treatment conditions. **C**, Western blotting analysis for β -catenin protein levels from 50 μ g samples derived from cell lysates (TCL), cytoplasmic fractions (CF), and nuclear fractions (NF). **D**, measurement of the nuclear β -catenin activities using TOPflash/FOPflash reporter system. The luciferase activity was measured at 48 hours after transfection as described in Materials and Methods. After normalization against the *Renilla* luciferase activities, the ratios of luciferase activities from cells transfected with the TOPflash reporter versus those transfected with FOPflash reporter were calculated, and the results were expressed as fold changes relative to the samples treated with 0.5% FBS. *, $P < 0.05$ versus cells treated with 10% FBS ($n = 3-6$, from three independent experiments).



inhibitory effects on the proliferation of these breast carcinoma cells. On the other hand, globular adiponectin that lacks the NH₂-terminal collagen-like domain did not possess such an activity (data not shown).

Adiponectin induces apoptosis in MDA-MB-231 but not T47D cells. We next tested whether adiponectin could induce apoptosis of these two breast carcinoma cells by measuring the fragmented DNA using cells that were prelabeled with [³H]thymidine. This analysis showed that adiponectin significantly induced DNA fragmentation in MDA-MB-231 cells treated with different concentrations of FBS (Fig. 1C). Consistently, TUNEL analysis also showed an increased population of apoptotic cells following adiponectin treatments (data not shown). These results suggest that the growth-inhibitory effects of adiponectin might be partly due to its ability to induce apoptosis in MDA-MB-231 cells. On the other hand, adiponectin could not increase DNA fragmentation and apoptosis in T47D cells (data not shown), suggesting that the proapoptotic effect of adiponectin is a cell type-specific event.

Adiponectin inhibits serum-induced cell cycle progression and cyclin D1 expression. To understand the detailed molecular events that underlie the antiproliferative actions of adiponectin, we further studied the effects of this adipokine on 10% serum-induced cell cycle progression of MDA-MB-231 cells by fluorescence-activated cell sorting (FACS) analysis. At 48 hours after the treatment, adiponectin significantly increased the population of cells in both sub-G₀-G₁ phase ($7.1 \pm 0.43\%$ versus $2.4 \pm 0.16\%$) and G₀-G₁ phase ($62.4 \pm 7.3\%$ versus $52.7 \pm 6.3\%$), whereas it decreased the percentage of cells that progressed into S phase ($7.3 \pm 0.86\%$ versus $10.9 \pm 1.5\%$) and G₂-M phase ($23.2 \pm 2.6\%$ versus $34 \pm 2.9\%$), comparing with those cells treated with serum only. These data indicate that adiponectin might inhibit the proliferation of MDA-MB-231 cells by arresting the cells at G₀-G₁ phase and promoting apoptosis.

Cyclin D1 is a key regulator that controls cell cycle progression from G₁ to S phase transition (35). We therefore tested the effects of adiponectin on expression of this protein. As shown in Fig. 2A, adiponectin treatment had no obvious effects on the mRNA abundance of cyclin D1 at the basal status (0.5% FBS) but significantly suppressed 10% FBS-stimulated cyclin D1 expression

by ~30% and ~45% at 24 and 48 hours, respectively. Western blotting analysis further confirmed that adiponectin could inhibit FBS-stimulated protein accumulation of cyclin D1 in MDA-MB-231 cells (Fig. 2B). In addition, we found that adiponectin treatment resulted in a decreased phosphorylation of retinoblastoma protein at Ser^{807/811} and a reduced expression of G₁-S cell cycle regulator cyclin E1 (data not shown).

Adiponectin decreases serum-induced stabilization and nuclear translocation of β -catenin. β -Catenin is a critical transcription factor that controls the expression of cyclin D1 and other growth-regulatory genes (35). Intracellular accumulation of β -catenin protein has been observed in a large portion of human breast tumors. We next investigated the effects of adiponectin on the expression and subcellular localization of this transcription factor in MDA-MB-231 cells. Real-time PCR analysis revealed that neither 10% FBS nor adiponectin had any effect on mRNA abundance of this gene (data not shown). On the other hand, the intracellular total protein concentrations of β -catenin were markedly increased following stimulation with 10% FBS (Fig. 2C). Subcellular fractionation analysis revealed that the majority of serum-induced β -catenin was accumulated in the nuclear fractions. Treatment of cells with adiponectin largely abrogated serum-induced increase in total β -catenin protein concentrations and its accumulation in the nuclei. On the other hand, neither serum nor adiponectin had any obvious effects on the intracellular level or nuclear translocation of β -catenin in T47D cells (data not shown).

We next investigated the effect of adiponectin on the transcriptional activities of β -catenin in MDA-MB-231 cells using the TOPflash/FOPflash reporter system. The TOPflash luciferase reporter plasmid contains three copies of the consensus T-cell factor (TCF) binding sites upstream of the *luciferase* gene, whereas its negative control version (FOPflash) carries mutations at these binding sites. The ratios of the luciferase activities of the TOPflash versus FOPflash were considered a measure of the relative transcriptional activity of the β -catenin/TCF complexes. As shown in Fig. 2D, stimulation with 10% serum increased the activity of the TOPflash reporter by ~2.5-fold, and this increase was markedly attenuated following treatment with adiponectin. These results suggest that the inhibitory effect of adiponectin on serum-induced

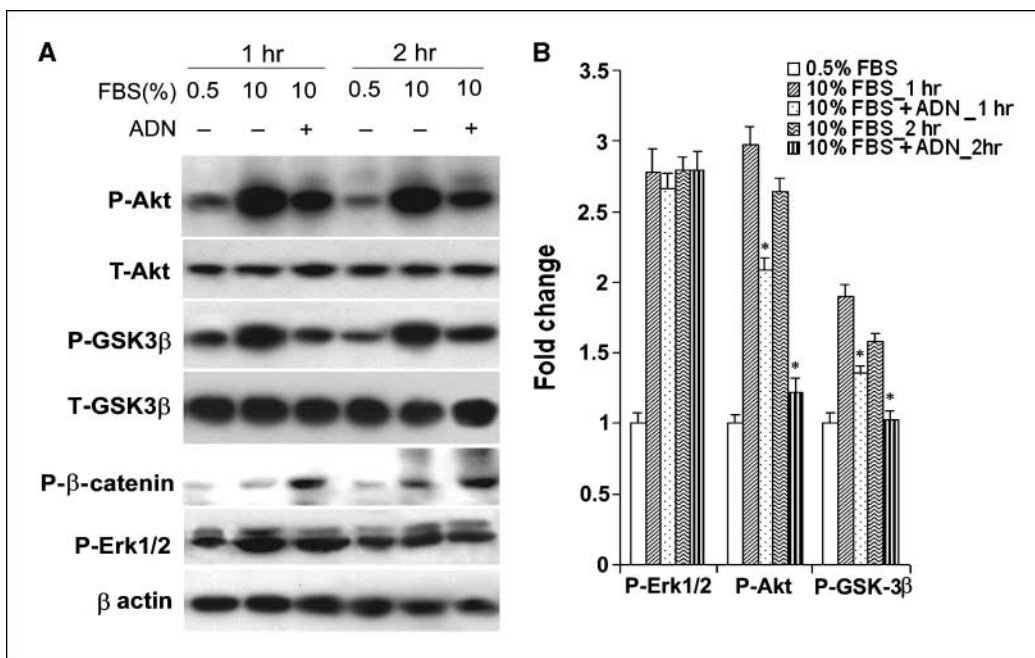


Figure 3. Chronic treatment with adiponec tin suppresses serum-stimulated Akt/GSK-3 β signaling pathway in MDA-MB-231 cells. Cells under different stimulations were harvested at various time points, as indicated, and solubilized in cell lysis buffer. An equal amount of total cell lysate (50 μ g) was used for the analysis. **A**, Western blotting analysis for phosphorylated Akt (P-Akt) and/or total Akt (T-Akt), phosphorylated GSK-3 β (P-GSK3 β), total GSK-3 β (T-GSK3 β), phosphorylated β -catenin (P- β -catenin), and phosphorylated ERK1/2 (P-Erk1/2) using their specific antibodies as specified in Materials and Methods. **B**, BioPlex phosphoprotein multiplex assays for measuring the phosphorylations of ERK1/2, Akt, and GSK-3 β . *, $P < 0.01$ versus serum-treated samples ($n = 4$, from three independent assays).

nuclear accumulation of β -catenin is correlated with the changes in β -catenin/TCF transcriptional activity.

Chronic treatment with adiponec tin suppresses serum-induced Akt/GSK-3 β signaling pathway in MDA-MB-231 cells.

It is well known that intracellular β -catenin levels are regulated through GSK-3 β -mediated phosphorylations on its several serine and threonine residues, which in turn facilitate the proteasome-mediated degradation of this protein by an ubiquitin-dependent mechanism (35). Inhibition of GSK-3 β activity results in β -catenin stabilization and its accumulation in the nucleus, where it associates with the transcription factor TCF/lymphoid enhancer factor (LEF) to activate certain genes that ultimately establish the oncogenic cellular phenotype (36). To investigate whether this pathway is involved in mediating the actions of adiponec tin in MDA-MB-231 cells, we examined the phosphorylation of GSK-3 β and its upstream kinase protein kinase B/Akt using both Western blotting and BioPlex phosphoprotein quantitative assays. As shown in Fig. 3, both types of analyses showed that serum-induced phosphorylations of Akt and GSK-3 β were significantly attenuated by pretreatment with adiponec tin for 24 hours in MDA-MB-231 cells. On the other hand, acute treatment with adiponec tin had no obvious effects on the phosphorylations of Akt and GSK-3 β (data not shown). Neither chronic nor acute treatment with adiponec tin modulates serum-induced phosphorylation and activation of ERK1/2. Treatment of cells with lithium chloride (LiCl), a selective GSK-3 β inhibitor, significantly attenuated the effects of adiponec tin on serum-stimulated cell proliferation, cyclin D1 expression, and β -catenin protein stabilization (Fig. 4). Similar results were obtained with another selective GSK-3 β inhibitor, SB-415286 (data not shown). FACS analysis revealed that adiponec tin-mediated inhibition of serum-stimulated cell cycle progression was also abrogated by the selective GSK-3 β inhibitors (Supplementary Fig. S8). Taken together, these results suggested that, in MDA-MB-231 cells, adiponec tin might elicit its growth-inhibitory effects through selective inhibition of Akt/GSK-3 β pathway, which resulted in the destabilization of β -catenin proteins, down-regulation of cyclin D1 expression, and cell cycle arrest at G₀-G₁ phase. In contrast, we

could not detect any obvious effects of adiponec tin on insulin- or serum-stimulated phosphorylations of Akt/GSK-3 β in T47D cells. However, the phosphorylation of ERK1/2 was significantly inhibited by adiponec tin in this cell line (data not shown).

Adiponec tin attenuates the tumor progression of MDA-MB-231 breast cancer xenograft in athymic nude mice.

To explore the physiologic relevance of the aforementioned *in vitro* findings,

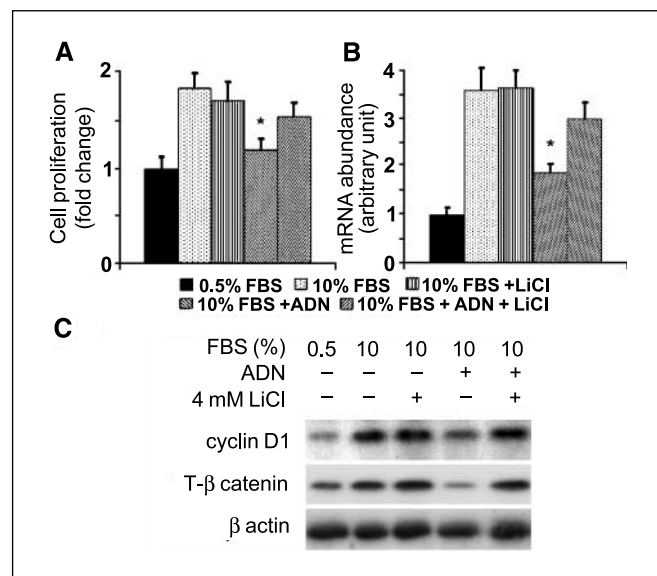


Figure 4. Pharmacologic inhibition of GSK-3 β attenuates the effects of adiponec tin on serum-induced cell proliferation, *cyclin D1* gene expression, and β -catenin stabilization. MDA-MB-231 cells were pretreated with 4 mmol/L LiCl for 24 hours and then incubated with 10% FBS in the presence or absence of adiponec tin for another 48 hours. **A**, viable cell numbers were manually counted after mixing with trypan blue dye. **B**, real-time PCR was done for quantitative analysis of the cyclin D1 mRNA. **C**, the protein levels of cyclin D1, β -catenin, and β -actin were monitored by Western blotting analysis. *, $P < 0.05$ versus LiCl-treated samples ($n = 4$, from three independent assays). Note that similar results were observed using SB-415286, another selective GSK-3 β inhibitor.

we next evaluated whether adiponectin has any suppressive effects on the development of breast carcinomas in nude mouse models. First, MDA-MB-231 cells were pretreated with or without recombinant adiponectin for 24 hours and then injected into fat pads. Second, equal amounts of recombinant adenoviruses that express either adiponectin or luciferase (as control) were introduced into the tumor xenograft at 14 days after initial implantation. As shown in Fig. 5, the visible tumors were developed at ~5 to 6 days after inoculation of cells into fat pads. In the experimental groups treated with recombinant adiponectin or the adenoviruses that express this adipokine, the rates of tumor growth were significantly attenuated and the tumor weights were reduced by 58% and 32%, respectively, compared with their respective controls.

To further investigate whether adiponectin can act in an endocrine manner to suppress the mammary tumor development, we also introduced the recombinant adenoviruses into nude mice through tail vein injection at 7 days after initial implantation of tumor cells. It was previously reported that i.v. administered adenoviruses were targeted predominantly to the liver tissue (37). Indeed, our PCR analysis showed a highly abundant mRNA expression of the adiponectin gene in the liver tissue of mice injected with the recombinant adenoviruses that encode adiponectin (Supplementary Fig. S9A). On the other hand, adiponectin mRNA expression was not detectable in the tumor tissues throughout the 5-week treatment period. Quantitative ELISA analysis revealed that single tail vein injection of adiponectin-expressing adenoviruses resulted in a sustained elevation of plasma adiponectin for up to 4 weeks (Supplementary Fig. S9B). Notably, the tumor development of MDA-MB-231 cells in mice was also markedly suppressed by adenovirus-mediated elevation of circulating adiponectin (Supplementary Fig. S9C and D). The results suggest that elevation of circulating adiponectin after implantation of breast carcinomas cells is still effective in suppressing the tumor growth in nude mice.

As MDA-MB-231 cells are highly invasive, we next investigated the metastasis status of these animals under various treatments. Macroscopic metastasis with a diffuse pattern was observed at the lung surfaces of the nude mice inoculated with cells alone or treated with Ad-Luc but not those mice treated with recombinant adiponectin or Ad-ADN (Supplementary Fig. S10). H&E staining analysis of lungs confirmed that the majority of the mice developed

microscopic metastasis along the alveolar septa. Treatment with recombinant adiponectin or local injection with the Ad-ADN recombinant adenoviruses substantially decreased metastasis of tumor cells into the lung tissue compared with their respective controls (Fig. 6A and B). In addition, there were much less secondary tumors developed at the mammary fat pads in adiponectin-treated animals. To examine whether adiponectin could directly act on MDA-MB-231 cells to attenuate their invasions *in vitro*, we have also done migration and invasion assays in the Transwell inserts. Our results showed that treatment of MDA-MB-231 cells with adiponectin markedly decreased 10% FBS-induced invasion as well as migration across the Transwell inserts (Fig. 6C-F).

Discussion

Several recent case-control studies have shown the close association between low serum adiponectin level (hypoadiponectinemia) and increased risk of breast cancer, independent of adiposity, insulin resistance, and other classic risk factors (18, 22–24). It has also been reported that breast cancers arising in women with low adiponectin levels are more likely to show a biologically aggressive phenotype (23). Nevertheless, whether hypoadiponectinemia is causally associated with this cancer disease remains to be established. In this study, we provide direct experimental evidence supporting the role of adiponectin as an inhibitory factor for breast cancer development. Our *in vitro* studies showed that adiponectin could attenuate the growth of MDA-MB-231 cells by inhibiting cell proliferation and inducing apoptosis (Fig. 1), a finding consistent with a recent report by Kang et al. (38). These *in vitro* data are further corroborated by our animal study showing that adiponectin supplement therapy could inhibit the breast tumor development in nude mice (Fig. 5). In addition, we observed a much lower degree of breast tumor metastasis in mice treated with adiponectin (Fig. 6). Further experiments are needed to discriminate whether the decreased metastasis is caused by the direct suppressive effects of adiponectin on cell migration and invasion or is simply secondary to the reduced sizes of primary tumors.

Hyperactivation of the canonical β -catenin/Wnt pathway is one of the most frequent signal abnormalities in many types of cancers

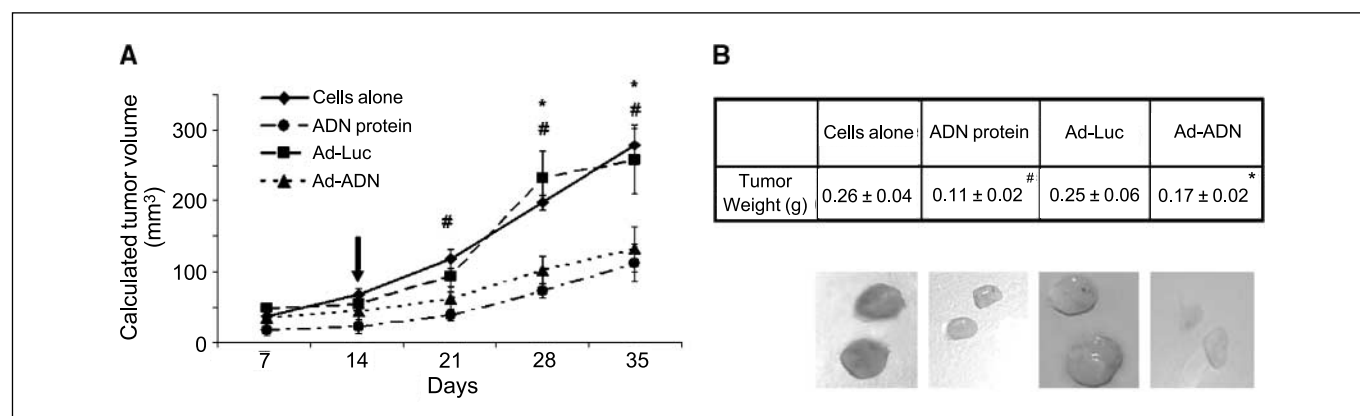


Figure 5. The inhibitory effects of adiponectin on the growth of MDA-MB-231 cell-derived tumor in nude mice. Cells pretreated without (*Cells alone*) or with (*ADN protein*) adiponectin were implanted into the mammary fat pad of nude mice as described in Materials and Methods. Alternatively, Ad-Luc or Ad-ADN (10^8 pfu) was locally injected into the tumor site at day 14 (arrow) after cell implantation. **A**, tumor growth was monitored by measuring the visible tumor sizes at various time points. At the end of experiment, tumors were collected from the mice and weighed. **B**, average weights and representative tumor images. #, $P < 0.05$, adiponectin protein versus cell alone; *, $P < 0.05$, Ad-ADN versus Ad-Luc at each time point ($n = 4-6$ mice per group, from four independent studies).

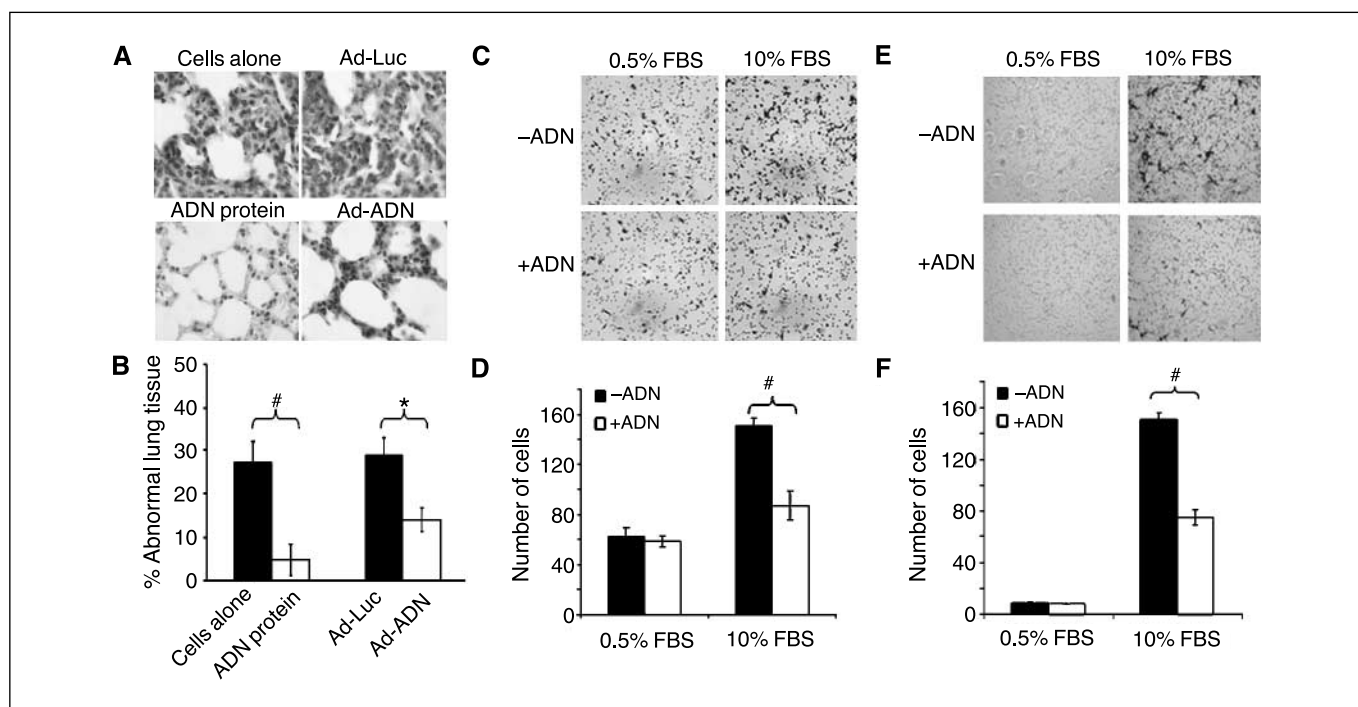


Figure 6. Effects of adiponectin on the invasion and migration of MDA-MB-231 cells *in vivo* and *in vitro*. Lung tissues from animals treated as in Fig. 5 were subjected to H&E staining (magnification, $\times 1,000$) to evaluate metastasis of tumor cells (A) and percentage of abnormal area as described in Materials and Methods (B). Cell migration (C and D) and invasion (E and F) were assessed *in vitro* in 8- μ m-pore Transwell inserts using FBS as chemoattractants. Cells located under the surface of the membrane were stained and counted as described in Materials and Methods. Magnification, $\times 100$. Top, typical images. #, $P < 0.01$; *, $P < 0.05$ ($n = 6-8$, from three independent experiments).

(39, 40). The central event in this pathway is the stabilization and nuclear translocation of β -catenin, where it binds to transcription factor TCF/LEF and consequently activates a cluster of genes that ultimately establish the oncogenic phenotype (36). Genetic mutations in the Wnt/ β -catenin pathway components, such as loss-of-function mutations in *APC* and *Axin* and gain-of-function mutations in β -catenin, have been observed in a wide range of human cancers (39). Despite the fact that such mutations are rare in breast cancer, stabilization of β -catenin protein and overexpression of cyclin D1 have been observed in >50% of human breast tumors (36). Furthermore, increased β -catenin activity was found to be significantly correlated with the poor prognosis of breast cancer patients (41). Consistent with these clinical data, numerous animal studies have shown that aberrant activation of the Wnt/ β -catenin signaling, either by overexpression of canonical Wnt proteins or by direct stabilization of β -catenin (42, 43), can lead to mammary tumorigenesis.

Our study suggests that the ability of adiponectin to modulate the GSK-3 β / β -catenin signaling pathway might play a critical role in mediating the inhibitory effects of adiponectin on mammary tumorigenesis. This conclusion is supported by our findings that chronic treatment of MDA-MB-231 cells with recombinant adiponectin markedly reduces serum-induced phosphorylation of Akt and GSK-3 β (Fig. 3), decreases intracellular accumulation and nuclear translocation of β -catenin, and down-regulates the cyclin D1 expression (Fig. 2). In addition, our results have shown that chronic treatment with LiCl and SB-415286, two selective chemical inhibitors of GSK-3 β , could attenuate the inhibitory effects of adiponectin on the expression levels of cyclin D1 and β -catenin as well as the proliferation of MDA-MB-231 cells (Fig. 4).

Although our results showed the inhibitory effects of adiponectin on serum-induced phosphorylation of Akt at Ser⁴⁷³, we do not have direct evidence supporting the involvement of this kinase in adiponectin-mediated inhibition of GSK-3 β / β -catenin pathway in MDA-MB-231 cells. Notably, a recent study on MC3T3-E1 osteoblast-like cells has shown that glucocorticoid-mediated inhibition on serum-induced phosphorylation of GSK-3 β and activation of nuclear β -catenin activity is markedly attenuated in the presence of dominant-negative forms of either phosphatidylinositol 3-kinase (PI3K) or Akt, suggesting the cross-talk between the PI3K/Akt and Wnt/ β -catenin signaling pathways in this cell line (44). The cross-talk between these two pathways was also supported by the finding that overexpression of the tumor suppressor gene *phosphatase and tensin homologue (PTEN)*, a lipid kinase that antagonizes the activity of PI3K, inhibits Wnt-1-induced β -catenin stabilization and mammary tumorigenesis in mice (45). Furthermore, inhibition of Wnt signaling by overexpression of Wnt inhibitory factor-1 has been shown to inhibit Akt activity in PTEN-mutated prostate cancer cells (46). Despite these evidences, further studies are needed to determine the role of Akt in regulating GSK-3 β / β -catenin pathway in our cell line. Two putative adiponectin receptors (adipoR1 and adipoR2) have recently been cloned by Kadowaki and Yamauchi (47). Our real-time PCR analysis revealed that both these receptors are expressed in MDA-MB-231 cells. However, RNA interference-mediated down-regulation of adipoR1 or adipoR2 or simultaneous down-regulation of both receptors had no effects on adiponectin-mediated inhibition of cell proliferation and β -catenin accumulation,⁷ suggesting that adipoR1

⁷ Y. Wang and A. Xu, unpublished observation.

and adipor2 might not be involved in mediating the growth-inhibitory activity of adiponectin in this cell line.

Another notable finding of this study is that the suppressive effects of adiponectin on the growth of breast carcinomas are cell type dependent. The inhibition of serum-induced proliferation and induction of apoptosis by adiponectin was observed in E-cadherin-negative and invasive MDA-MB-231 cells but not in noninvasive and E-cadherin-positive T47D cells (Figs. 1 and Supplementary Fig. S7). Although the detailed mechanism that accounts for the differential effects of adiponectin remains to be elucidated, it is worthwhile to note that these two breast cancer cell lines possess distinct Wnt/ β -catenin activities. A recent report by Bafico et al. (48) showed that the constitutive Wnt pathway activation, as evidenced by accumulation of active form of β -catenin in the nuclei, was only observed in a few invasive breast carcinoma cell lines, including MDA-MB-231 cells. Although the expression of β -catenin is detectable in T47D cells, this protein is mainly present in cytoplasm-bound, inactive form (49). The transcriptional activity of β -catenin in T47D cells is substantially lower than that in MDA-MB-231 cells. Furthermore, we have found that neither serum stimulation nor adiponectin treatment alters β -catenin levels in T47D cells (data not shown), suggesting that Wnt/ β -catenin signaling might not be the major player in determining the oncogenic phenotype of this cell line. The observed inhibitory effects of adiponectin on growth factor-induced proliferation of T47D cells might be attributed to its ability to sequester the growth factors at the prereceptor levels (26) or through other unknown mechanisms.

In summary, our present study showed, for the first time, that adiponectin could modulate the GSK-3 β / β -catenin pathway in certain types of breast cancer cells and suppress mammary tumorigenesis and cancer cell invasion in nude mice. Given the close proximity between mammary gland cells and adipocytes, our results raise the possibility that decreased adiponectin production might be causally linked to increased β -catenin accumulation and cyclin D1 overexpression observed in breast cancer patients. In addition to its direct suppressive effect on breast cancer formation, as an insulin-sensitizing hormone, adiponectin might ameliorate this disease indirectly by alleviating hyperglycemia and insulin resistance, the two established risk factors for breast cancers (8). Furthermore, adiponectin possesses anti-inflammatory activity and can inhibit the adipocyte production of several inflammatory factors involved in promoting mammary tumorigenesis, such as IL-6, TNF- α , and MCP-1 (50). Taken together, these evidences suggest that adiponectin might represent a novel therapeutic target for obesity-related cancerous diseases.

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