

Direct Role of Adiponectin and Adiponectin Receptors in Endometrial Cancer: *In Vitro* and *Ex Vivo* Studies in Humans

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Abstract

Low adiponectin levels are an independent risk factor for and mediate the effect of obesity on endometrial cancer in epidemiology studies. The direct or indirect mechanisms underlying these findings remain to be elucidated. We first examined the expression of adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) in normal human endometrium and in endometrial cancer tissues *ex vivo*. We then used KLE and RL95-2 human endometrial cancer cell lines *in vitro* to study relative expression of AdipoRs, to investigate the effect of adiponectin on activating intracellular signaling pathways, and to assess its potential to alter malignant properties. We report for the first time that the relative expression level of AdipoR1 is higher than AdipoR2 in human endometrial cancer tissue, but the expression of AdipoRs is not statistically different from nonneoplastic tissues. We also show for the first time in endometrial cancer cell lines *in vitro* that adiponectin suppresses endometrial cancer proliferation acting through AdipoRs. Adiponectin also increases the expression of the adaptor molecule *LKB1*, which is required for adiponectin-mediated activation of AMPK/S6 axis and modulation of cell proliferation, colony formation, adhesion, and invasion of KLE and RL95-2 cell lines. These novel mechanistic studies provide for the first time *in vitro* and *ex vivo* evidence for a causal role of adiponectin in endometrial cancer. *Mol Cancer Ther*; 10(12); 2234–43. ©2011 AACR.

Introduction

Adiponectin, one of the most abundant proteins in serum, is secreted mainly by white adipose tissue and acts through autocrine/paracrine and endocrine pathways (1). In obese subjects, adiponectin levels are significantly reduced and inversely correlated with body mass index and central obesity (2). Adiponectin improves insulin sensitivity in peripheral tissues (3), and low adiponectin levels lead to the development of the metabolic syndrome and eventually type-2 diabetes, atherosclerosis, and nonalcoholic fatty liver disease (1, 3). We and others have reported that reduced adiponectin levels may mediate the effect of obesity or risk for several malignancies including endometrial cancer (4, 5), with the association being stronger in premenopausal women (6).

The peripheral actions of adiponectin are mediated mainly through 2 distinct receptors, adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2; refs. 3, 7, 8). AdipoRs have been reported to have different affinity for lower versus higher molecular weight adiponectin complexes, and despite their structural similarity to 7-transmembrane receptors, they are not coupled to G proteins (9). Although epidemiology studies have reported associations between adiponectin and endometrial cancer, the presence of AdipoRs in endometrial cancer or the relative expression of AdipoR1 versus AdipoR2 in normal endometrium versus malignant endometrial tissue and/or endometrial cancer cell lines remains to be studied. Moreover, the importance of AdipoRs and/or the related adaptor molecule *LKB1* in activating signaling pathways and altering cell proliferation, adhesion, colony formation, and invasion of endometrial cancer cell lines have not yet been studied.

To address these questions, we first examined the expression of AdipoR1 and AdipoR2 in nonneoplastic and endometrial adenocarcinoma tissues *ex vivo* in humans. We also checked whether adiponectin activates intracellular signaling pathways and suppresses endometrial cancer proliferation acting through AdipoRs and whether reduction of AdipoR1 or AdipoR2 mRNA by short interfering RNA (siRNA) alleviates the adiponectin signaling in endometrial cancer cell lines *in vitro*. Because no previous study has evaluated whether the interaction of adiponectin with the adaptor molecule *LKB1* could alter malignant properties of endometrial cancer cell lines, we directly studied whether *LKB1* is required for

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adiponectin-mediated inhibition of cell proliferation, colony formation, adhesion, and invasion of endometrial cancer cells. The studies presented herein provide the first *in vitro* and *ex vivo* evidence that the association between adiponectin levels and endometrial cancer may be causal in nature.

Materials and Methods

Study subjects

Available for analysis were 23 tissue slides, Histo (TM)-arrays from U.S. patients, Imgenex Corp. To obtain supplementary information from a different population, we also studied 35 formalin-fixed, paraffin-embedded endometrial carcinoma specimens from Greek female subjects archived in the Department of Pathology of Athens University Medical School. Fifty-one nontumor endometrial specimens from Greek subjects were used as controls: 12 from early secretory tissue, 12 from proliferative tissue, 10 from endometrial tissue from pregnant subjects, 9 from hyperplastic tissue, and 8 from atrophic tissue from postmenopausal subjects. We did not conduct any grading of the carcinomas but used the generally established subclassification, as most of our specimens came from tissue microarrays. We selected our hyperplasia cases to be simple without severe atypia to avoid the high interobserver variability in diagnosing a complex hyperplasia with atypia versus a well-differentiated carcinoma. Control samples were not matched with tumor specimens.

Immunohistochemical analysis

The 4- μ m paraffin tissue sections were deparaffinized, rehydrated, and microwaved for 20 minutes in 10 mmol/L citrate buffer of pH 6.0 and incubated for 30 minutes in methanol containing 3% H₂O₂. After incubation in 16% normal goat serum for 1 hour at room temperature, the slides were incubated overnight with the primary antibodies at 4°C. Primary rabbit polyclonal antibodies (Phoenix Pharmaceuticals, Inc.) for human AdipoR1 (raised against amino acid residues 357–375) and human AdipoR2 (raised against amino acid residues 374–386) were used at 1:500. Then, slides were incubated with EnVision + anti-rabbit-horseradish peroxidase, a biotin-free visualization system (DAKO), for 30 minutes. The peroxidase reaction was developed with 3,3'-diaminobenzidine for 30 minutes, and the slides were then counterstained with hematoxylin.

Cell culture

RL95-2 and KLE endometrial cancer cell lines were purchased from American Type Culture Collection. The cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 medium, supplemented with 10% FBS and with or without 5- μ g/mL insulin (from bovine pancreas, Sigma), respectively. All cells were incubated at 37°C in an atmosphere of 5% CO₂ in air and subcultured beyond 80% confluency.

Real-time PCR

AdipoR expression was detected with real-time PCR (RT-PCR). RNA was extracted with TRIzol (Invitrogen), and first-strand cDNA synthesis was carried out by Superscript III (Invitrogen), according to the manufacturer's protocol. For RT-PCR, 100 ng of cDNA per 25- μ L reaction was amplified by TaqMan Gene Expression system (Applied Biosystems), specific primers and FAM tagged probes set (Applied Biosystems), and the standard real-time 7500 protocol (Applied Biosystems), with an initial polymerase activation step at 95°C for 10 minutes and 40 cycles. This included a 15 seconds melting step at 95°C and a 1 minute annealing–elongation step at 60°C. The analysis of relative gene expression was based on ΔC_t values obtained from RT-PCR. PCR products were separated in 2% agarose gels, in 0.5 \times 1 Tris borate EDTA, in a horizontal Mini-Sub cell GT electrophoresis apparatus (Bio-Rad), under a 100 V constant voltage and visualized by the Bio-Rad gel documentation system (Bio-Rad).

Introduction of siRNA

The siRNA of AdipoR1, AdipoR2, AMPK or LKB1, and scrambled siRNA (ssiRNA) were purchased from Santa Cruz. The cells were seeded onto 6-well tissue culture plates and grown to 60% confluency in the absence of antibiotic for 24 hours before transfection. Immediately before transfection, the culture medium was removed, and the cells were washed once with PBS, then transfected with either ssiRNA or siRNA with Fugene 6 transfection reagent in Opti-MEM medium according to the manufacturer's instructions (Roche). After transfection, cultures were incubated at 37°C for 5 hours and then placed in fresh culture medium. Cells were studied after an additional 36 hours.

Western blotting

The proteins were loaded in each lane. After SDS-PAGE, proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell, Inc.). The membranes were blocked for 1 hour in TBS containing 5% nonfat dry milk and 0.1% Tween-20. Incubation with primary antibodies was carried out in TBS containing 5% nonfat dry milk overnight and then incubated with horseradish peroxidase secondary antibodies for 2 hours. After incubation with antibodies, membranes were washed with TBS containing 0.1% Tween-20. Enhanced chemiluminescence was used for detection. Measurement of signal intensity on nitrocellulose membranes after Western Blotting with various antibodies was conducted with Image J processing and analysis software.

Proliferation assay

The cells were trypsinized and seeded in 96-well plates at a density of 5×10^3 cells per well. Cells were left to adhere overnight and then treated with adiponectin in serum-free medium for 24 hours. At the end of incubation period, the effect of adiponectin on cell proliferation was assayed with an MTT assay (Invitrogen). Cells were

washed with PBS and incubated with 100- μ L serum-free medium and 10- μ L Vybrant MTT solution for 2 hours. Formazan crystals were dissolved overnight at 37°C with the addition of 100 μ L of 10% SDS in 0.01 N HCl per well, and absorbance was measured at an optical density of 570 nm in a PowerWave XS (BioTek).

Clonogenic assay

The cells were grown in the media to 60% confluency and treated with adiponectin for 24 hours. After 24 hours, 500 cells were reseeded into a 100-mm culture dish and incubated for 12 days. Fresh media was changed every 3 days. At day 12, the media was removed, added to 2 mL of clonogenic reagent (50% ethanol + 0.25% 1,9-dimethylmethylene blue), and laved at room temperature for 45 minutes. After 45 minutes, the cells were washed with PBS, and the blue colonies were counted.

Adhesion assay

The cells were pretreated with 20 μ g/mL of adiponectin for 24 hours and plated (5×10^4 cells per well) in 10- μ g/cm² fibronectin-coated (Sigma) wells in 96-well plates, which were then incubated at 37°C (5% CO₂) for 60 minutes. Adherent cells were fixed with 3% paraformaldehyde for 10 minutes, washed with 2% methanol for 10 minutes, and stained with 0.5% crystal violet in 20% methanol for 10 minutes. The stain was eluted, and absorbance was measured at 540 nm.

Invasion assay

Matrigel invasion assay was conducted by using a Matrigel invasion chamber from BD BioCoat Cellware (BD Biosciences) according to the manufacturer's protocol.

Statistical analysis

Descriptive characteristics of patients providing tumor and nontumor specimens of endometrial cancer were summarized and compared using unpaired *t* tests and χ^2 tests for continuous and categorical measures accordingly. Case characteristics are presented as percentages. Unmatched analyses of expression of AdipoRs were conducted by unconditional, binomial, logistic regression

analysis in 2 different models: unadjusted and adjusted for age. A level of $\alpha = 0.05$ was set to determine statistical significance. All signaling data were analyzed using the Student *t* test and/or one-way ANOVA followed by *post hoc* tests (Bonferroni correction for multiple comparisons). All analyses were conducted with SPSS version 11.5 (SPSS Inc.) and Stata version 11.1 (Stata Corp.).

Results

AdipoR expression in benign endometrium versus endometrial cancer samples *ex vivo*

Descriptive characteristics of study subjects are presented in Supplementary Data S1. Pregnant subjects were on average younger than the individuals with colon cancer (26.6 ± 6.9 vs. 55.0 ± 13.7 , $P < 0.05$). Control samples reflecting different phases of the human endometrial cycle (proliferative and early secretory phases) were taken mainly from premenopausal women, whereas atrophic nontumor as well as tumor specimens were derived mainly from postmenopausal women. All tumors were staged according to the FIGO classification. Half (52.2%) of the endometrial cancer cases from U.S. subjects were stage I whereas 26.1% were stage III. In contrast, the majority of Greek adenocarcinoma samples were stage I (81.1%) and the remaining stage II (16.2%).

We first examined the expression of AdipoR1 and AdipoR2 in tumor tissues *ex vivo* by immunohistochemistry (Supplemental Data S2). We observed that tumor tissues were found to strongly express AdipoR1 and AdipoR2. We also found that the expression of AdipoR1 and AdipoR2 in human endometrial cancer tissue is not statistically different from that in nonneoplastic tissue (early secretory, proliferative, pregnant, hyperplasia, and atrophic phase) in the samples from the Greek individuals (Supplemental Data S3). Both receptors were detected in a patchy pattern mainly in the epithelial cells and to a lesser degree in the stromal cells. Also, we observed that endometrial cancer tissue was found to strongly express AdipoR1 (57.14% vs. 42.86% showing none or marginal staining) and AdipoR2 (65.71% vs. 34.29% showing none or marginal staining; Table 1).

Table 1. Comparison of AdipoR1 and AdipoR2 expression in immunohistochemical studies between neoplastic and nonneoplastic endometrial tissue of Greek subjects

	AdipoR1				AdipoR2			
	None or marginal	Positive or strong positive	<i>P</i> ^a	<i>P</i> ^b	None or marginal	Positive or strong positive	<i>P</i> ^a	<i>P</i> ^b
	<i>n</i> (%)	<i>n</i> (%)			<i>n</i> (%)	<i>n</i> (%)		
Neoplastic tissue (<i>n</i> = 35)	15 (42.86)	20 (57.14)	—	—	12 (34.29)	23 (65.71)	—	—
Nonneoplastic tissue (<i>n</i> = 51)	13 (25.49)	38 (74.51)	0.094	0.750	17 (33.33)	34 (66.67)	0.927	0.335

^a*P* from binomial logistic regression comparing neoplastic with nonneoplastic tissue, unadjusted model.

^b*P* from binomial logistic regression comparing neoplastic with nonneoplastic tissue, adjusted for age.

Table 2. Comparison of AdipoR1 and AdipoR2 expression in immunohistochemical studies between various stages of endometrial cancer of U.S. subjects

	AdipoR1				AdipoR2			
	None or Marginal	Positive or strong positive	<i>P</i> ^a	<i>P</i> ^b	None or Marginal	Positive or strong positive	<i>P</i> ^a	<i>P</i> ^b
	<i>n</i> (%)	<i>n</i> (%)			<i>n</i> (%)	<i>n</i> (%)		
FIGO stage I and II (<i>n</i> = 13)	10 (76.92)	3 (23.08)	—	—	7 (53.85)	6 (46.15)	—	—
FIGO stage III and IV (<i>n</i> = 8)	4 (50)	4 (50)	0.213	0.146	3 (37.5)	5 (62.5)	0.469	0.665

^a*P* from binomial logistic regression comparing FIGO stage I and II with FIGO stage III and IV, unadjusted model.

^b*P* from binomial logistic regression comparing FIGO stage I and II with FIGO stage III and IV, adjusted for age.

These results were confirmed in the samples of various stages of endometrial cancer derived from U.S. subjects (Table 2). Both AdipoR1 and AdipoR2 were expressed in all stages of endometrial cancer; however, this expression was not statistically different between the 2 groups (group 1: FIGO stages I and II and group 2: FIGO stages III and IV).

AdipoR expression in endometrial cancer cell lines *in vitro*

The expression of AdipoRs was tested by RT-PCR in 2 endometrial cancer cell lines, that is, KLE and RL95-2. The endometrial adenocarcinoma KLE and RL95-2 cell lines were found to express both AdipoR1 and AdipoR2 (data not shown). Also, we confirmed by Western blotting that both AdipoR1 and AdipoR2 are expressed in KLE and RL95-2 cell lines, respectively (Supplementary Data S4). In contrast, AdipoR1 expression levels were relatively higher than AdipoR2 in both cell lines.

Adiponectin reduced cell proliferation, colony formation, adhesion, and invasion of endometrial cancer cell lines *in vitro*

Treatment of KLE cells with adiponectin in low physiologic concentrations ranging from 5 to 10 µg/mL had no effect on cell proliferation (Fig. 1A). In contrast, 10 µg/mL of adiponectin administration in RL95-2 cells decreased cell proliferation by approximately 20%. Furthermore, cell proliferation was decreased by approximately 45% at high physiologic/pharmacologic doses, that is, 20 to 50 µg/mL of adiponectin, in both KLE and RL95-2 cell lines. We also examined the growth-inhibitory effects of adiponectin on endometrial cancer cells by a long-term colony formation assay showing that adiponectin treatment significantly decreased colony number (Fig. 1B). Cell adhesion assays were conducted using fibronectin as an adhesion substrate, and we observed that adiponectin-treated endometrial cancer cells had significantly reduced adhesion activity compared with controls (Fig. 1C). Adiponectin treatment also effectively inhibited invasion of endometrial cancer cells, as seen through the Matrigel invasion assay (Fig. 1D).

Antiproliferative effect of adiponectin is dependent on AdipoR1 and AdipoR2 in endometrial cancer cell lines *in vitro*

To examine whether the antiproliferative effect of adiponectin in endometrial cancer cell lines is mediated through AdipoRs, we repressed the expression of AdipoR1 or AdipoR2 via siRNA administration. When the levels of AdipoR1 or AdipoR2 mRNA were downregulated via siRNA, the inhibitory effect of adiponectin on endometrial cancer proliferation was reduced (Supplementary Data S5A). Under the same condition, adiponectin-activated 5'-AMP-activated protein kinase (AMPK) phosphorylation was disturbed by knockdown of AdipoR1 or AdipoR2 in endometrial cancer cells (Supplementary Data S5B). These data suggest that reduction of AdipoR1 or AdipoR2 mRNA alleviates adiponectin signaling in endometrial cancer cells and that adiponectin suppresses endometrial cancer proliferation through AdipoRs.

Depletion of *LKB1* abrogates adiponectin-mediated inhibition of cell proliferation, colony formation, adhesion, and invasion of endometrial cancer cell lines *in vitro*

As shown in Fig. 2A, adiponectin increased phosphorylation of the tumor suppressor *LKB1* in both KLE and RL95-2 cells. We also found that adiponectin-stimulated *LKB1* activation was blocked by *LKB1* siRNA administration in both cell lines (Fig. 2B). Hence, we next sought to determine the biological importance of depleting *LKB1* in the context of the effects of adiponectin on cell growth in endometrial cancer cells. As shown in Fig. 2C, *LKB1* siRNA administration increased cell proliferation when compared with control. Also, adiponectin treatment efficiently inhibited cell proliferation of both KLE and RL95-2 cells. However, these effects were diminished by *LKB1* siRNA administration. We also found that adiponectin-inhibited number and size of colonies in both KLE and RL95-2 cells is abolished by *LKB1* siRNA administration in long-term colony formation assays (Fig. 2D). In addition to examining the effect of adiponectin on cell proliferation and colony formation, we examined whether *LKB1* is required in terms of adiponectin-mediated inhibition of

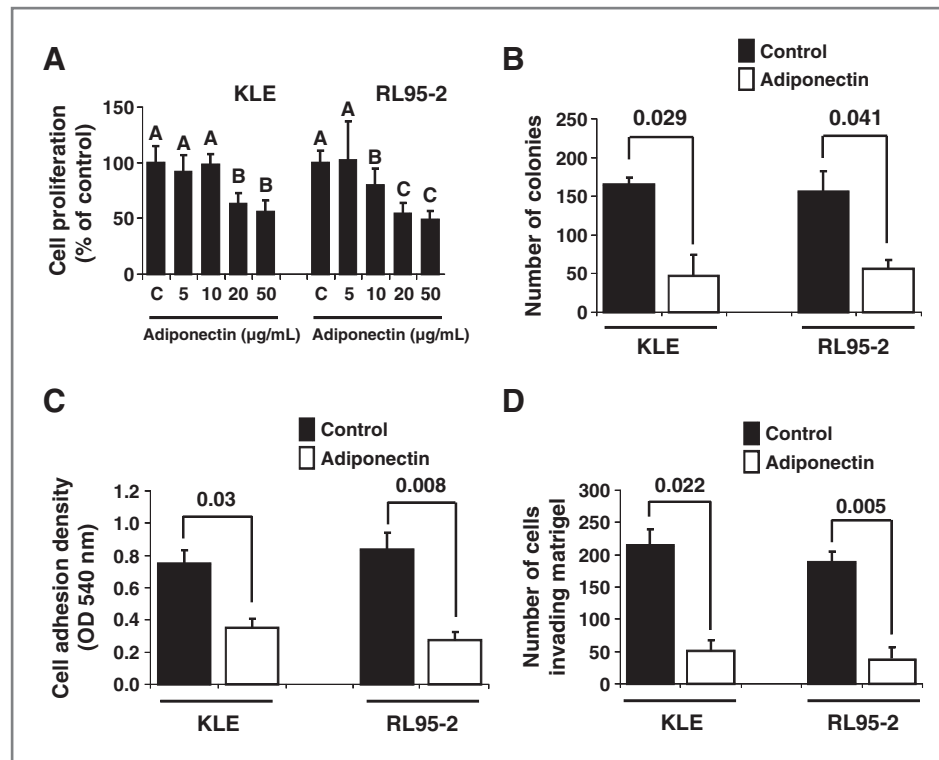


Figure 1. Antiproliferative effect of adiponectin on KLE and RL95-2 endometrial cancer cell lines *in vitro*. The cells were cultured as described in detail in Materials and Methods. A, the cells were incubated with adiponectin at indicated concentration for 24 hours, and cell viability was then measured by MTT assay as described in detail in Materials and Methods. B, the cells were incubated with adiponectin (20 $\mu\text{g}/\text{mL}$) for 24 hours, and the clonogenic assay was conducted as described in detail in Materials and Methods. C, the cells were incubated with adiponectin (20 $\mu\text{g}/\text{mL}$) for 24 hours, and the adhesion assay was conducted as described in detail in Materials and Methods. D, the cells were incubated with adiponectin (20 $\mu\text{g}/\text{mL}$) for 24 hours, and the invasion assay was conducted as described in detail in Materials and Methods. All data were analyzed using the Student *t* test and/or one-way ANOVA followed by *post hoc* test for multiple comparisons. Values are means ($n = 3$) \pm SD. Means with different letters are significantly different, $P < 0.05$.

cell adhesion and invasion of endometrial cancer cells. Similar to the results shown in Fig. 1C and D, adiponectin inhibited adhesion and invasion of both cells (Fig. 2E and F). However, these effects were diminished by *LKB1* siRNA administration. These results collectively show that adiponectin-induced *LKB1* activation is indeed a crucial component of the signaling machinery used by adiponectin in modulating cell proliferation, colony formation, cell adhesion, and/or invasion of endometrial cancer cells. Moreover, this effect is specific as adiponectin-mediated cell proliferation, colony formation, adhesion, and invasion were not regulated by siRNA administration when compared with control.

Antiproliferative effects of adiponectin are modified by PTEN activation in endometrial cancer cell lines *in vitro*

To understand the potential molecular mechanisms underlying the antiproliferative effects of adiponectin on endometrial cancer cells, we evaluated the signaling events induced by adiponectin in KLE, which contains the wild-type isoform of PTEN, and RL95-2, which is completely deficient in PTEN activity (10). We observed that adiponectin increases phosphorylation of AMPK and

acetyl-CoA carboxylase (ACC) within 15 minutes in both KLE and RL95-2 cells (Fig. 3A and B). Also, we found that adiponectin-stimulated AMPK phosphorylation was abolished by AMPK siRNA administration in both cell lines (Fig. 3C). In contrast, Akt was downregulated by adiponectin treatment in KLE but not in RL95-2 cells (Fig. 4A and B). Also, adiponectin decreased extracellular signal-regulated kinase (ERK) activation in RL95-2 but not in KLE cells (Fig. 4A and B). The signaling events diverged in these 2 cell lines in response to longer treatment (24 hours) with adiponectin. Accordingly, adiponectin treatment caused sharp reductions in cyclin D1 expression in KLE cells (Fig. 4C). During the same time frame of adiponectin treatment, the expression of cyclin E2 was unchanged in the KLE cells. In contrast, the expression of cyclin D1 did not change following treatment with adiponectin in RL95-2 cells, whereas the levels of cyclin E2 expression were suppressed by approximately 40%. Thus, the inhibitory effects of adiponectin on endometrial cancer cell proliferation were associated with distinct reductions of several key regulatory components of the cell cycle as well as progrowth kinases in the PTEN containing KLE cells versus in the PTEN-deficient RL95-2 cells. In contrast,

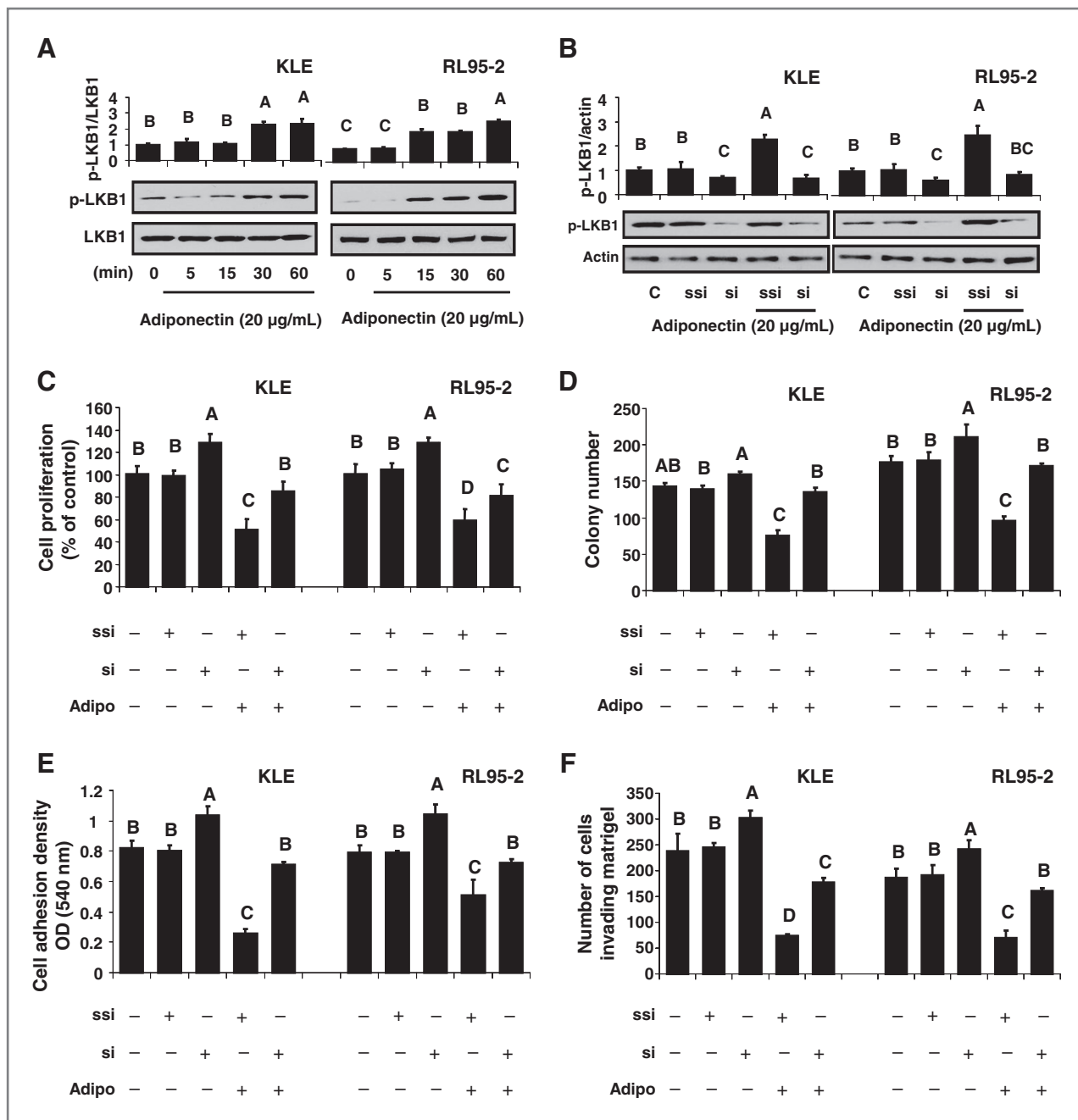


Figure 2. Effect of LKB1 on adiponectin-regulated cell growth in KLE and RL95-2 endometrial cancer cell lines *in vitro*. The cells were cultured as described in detail in Materials and Methods. A, the cells were treated with adiponectin (adipo; 20 μg/mL) at indicated times. B, the cells were transfected with LKB1 siRNA as described in detail in Materials and Methods and then stimulated with adiponectin (20 μg/mL) for 30 minutes. C, the cells were incubated with adiponectin at indicated concentrations for 24 hours, and cell viability was then measured by MTT assay as described in detail in Materials and Methods. D, the cells were incubated with adiponectin (20 μg/mL) for 24 hours, and the clonogenic assay was conducted as described in detail in Materials and Methods. E, the cells were incubated with adiponectin (20 μg/mL) for 24 hours, and the adhesion assay was conducted as described in detail in Materials and Methods. F, the cells were incubated with adiponectin (20 μg/mL) for 24 hours, and the invasion assay was conducted as described in detail in Materials and Methods. All data were analyzed using one-way ANOVA followed by *post hoc* test for multiple comparisons. Values are means ($n = 3$) \pm SD. Means with different letters are significantly different, $P < 0.05$. ssi, scrambled siRNA.

adiponectin-mediated expression of cell-cycle-regulatory genes, cyclin D1 in KLE and cyclin E2 in RL95-2, was largely abolished by LKB1 siRNA administration (Supplementary Data S6).

Discussion

We were the first to report that circulating adiponectin levels are associated with endometrial cancer (5), and our

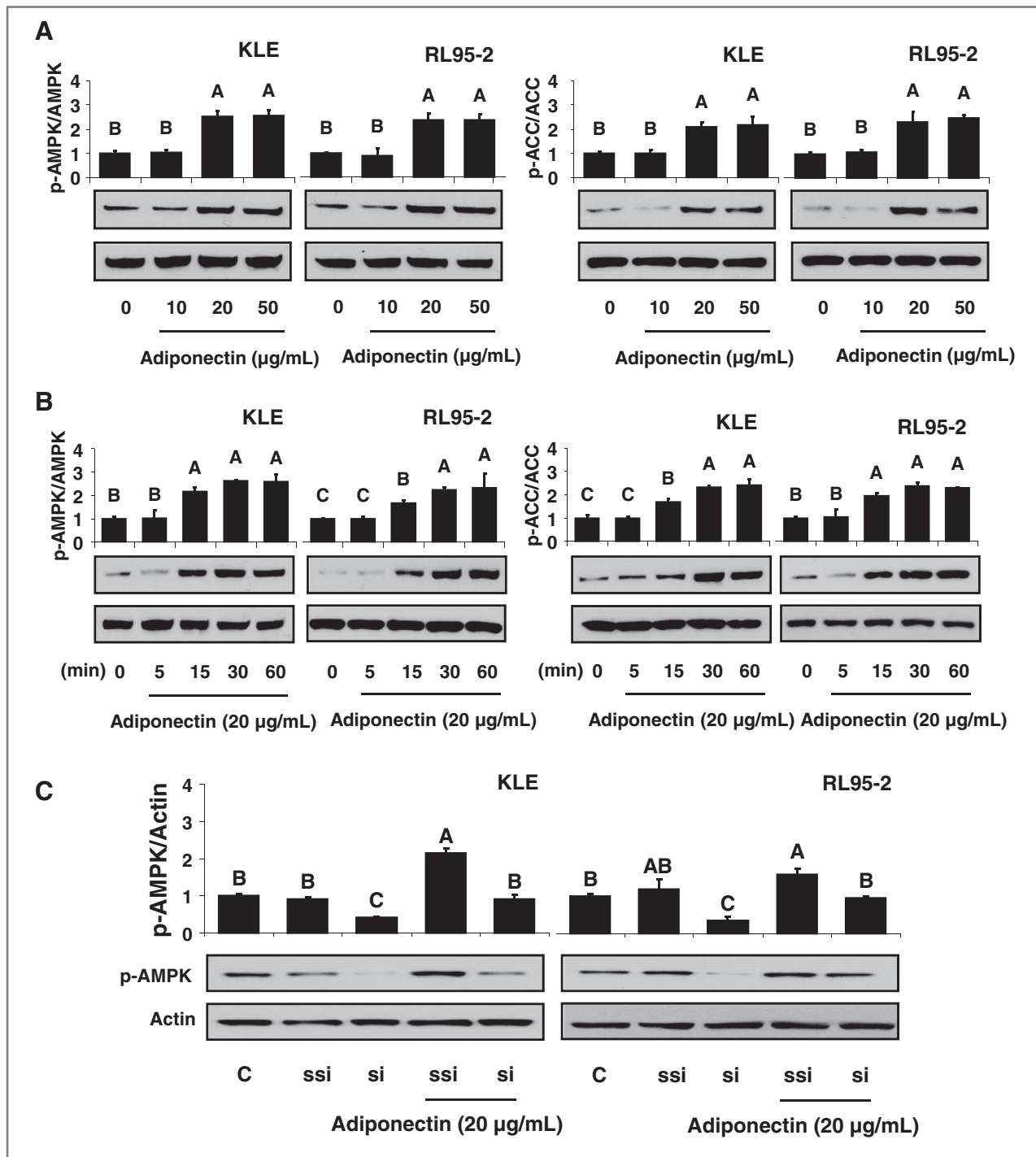


Figure 3. Adiponectin signaling in KLE and RL95-2 endometrial cancer cell lines *in vitro*. The cells were cultured as described in detail in Materials and Methods. A, the cells were treated with adiponectin at indicated concentrations for 30 minutes. B, the cells were treated with adiponectin (20 µg/mL) at indicated times. C, the cells were transfected with AMPK siRNA as described in detail in Materials and Methods and then stimulated with adiponectin (20 µg/mL) for 30 minutes. All data were analyzed using one-way ANOVA followed by *post hoc* test for multiple comparisons. Values are means ($n = 3$) \pm SD. Means with different letters are significantly different, $P < 0.05$. ssi, scrambled siRNA.

data have subsequently been confirmed by both case-control (6) and cohort studies (4). It has been shown that the leptin/adiponectin ratio was independently associated with an increased risk for endometrial cancer devel-

opment (11), and this result is consistent with our previous case-control study in multiple myeloma risk (12). Whether adiponectin is simply a marker of or whether it is causally implicated in endometrial carcinogenesis

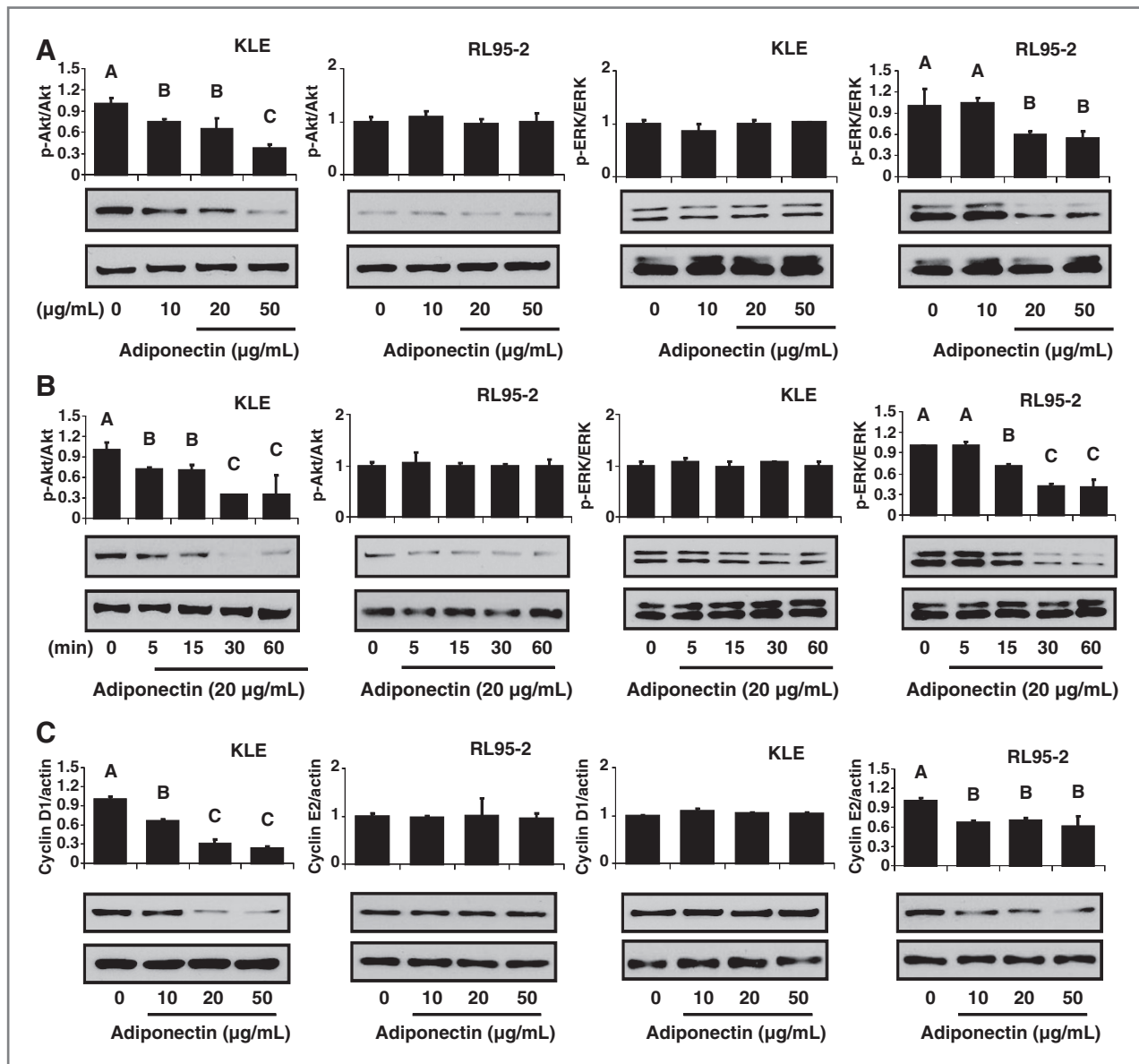


Figure 4. Adiponectin signaling in KLE and RL95-2 endometrial cancer cell lines *in vitro*. The cells were cultured as described in detail in Materials and Methods. A, the cells were treated with adiponectin at indicated concentrations for 30 minutes. B, the cells were treated with adiponectin (20 µg/mL) for indicated times. C, the cells were treated with adiponectin for 24 hours at indicated concentrations. All data were analyzed using one-way ANOVA followed by *post hoc* test for multiple comparisons. Values are means ($n = 3$) \pm SD. Means with different letters are significantly different, $P < 0.05$.

remains to be elucidated. Also, whether adiponectin may act not only by altering insulin resistance but also through direct effects on endometrial cancer cells has not yet been elucidated by published observational studies in humans.

We report herein that both AdipoR1 and AdipoR2 are expressed in endometrial cancer tissues, but their expression is not statistically different from that in non-neoplastic tissue. AdipoRs are expressed not only in human endometrial adenocarcinoma tissue samples but also in the KLE and RL95-2 endometrial cancer cell lines, both of which express relatively higher levels of AdipoR1 than AdipoR2. In addition to showing the presence of AdipoRs, we also report that adiponectin signaling is

mediated by AdipoRs in endometrial cancer cell lines. We also show that reduction of AdipoR1 or AdipoR2 mRNA by siRNA abrogates the adiponectin signaling in endometrial cancer cell lines and that adiponectin suppresses endometrial cancer proliferation through AdipoRs. We then hypothesized that antiproliferative effect of adiponectin on endometrial cancer cells could be mediated by *LKB1*, an adaptor molecule that has growth-suppressing effects on tumor cells by activating AMPK (13, 14). We found that *LKB1* gene knockdown results in abrogation of adiponectin-induced reduction of cellular activities including cell proliferation, colony formation, adhesion, and invasion in endometrial cancer cell lines.

Also, we observed that adiponectin-mediated AMPK activation and expression of cell-cycle-regulatory genes, cyclin D1 in KLE and cyclin E2 in RL95-2, are abolished by *LKB1* siRNA administration. Tumor suppressor effects of *LKB1* are due to its ability to activate the master metabolic regulator AMPK (14). In fact, *LKB1* is required for AMPK activation, as genetic depletion of *LKB1* in mouse embryonic fibroblasts results in a loss of AMPK activation following energy stresses that raise AMP (15). Our studies indicate for the first time that *LKB1* is important in adiponectin-mediated AMPK activation in endometrial cancer cell lines and that increased activation of *LKB1* in response to adiponectin treatment inhibits cell proliferation, colony formation, adhesion, and invasion properties of endometrial cancer cells.

We then focused on another tumor suppressor gene, *PTEN*, whose deficiency has been linked to several types of cancer including endometrial carcinoma (16). A major genetic difference between KLE and RL95-2 cells lies in that KLE cells contain the wild-type of *PTEN*, whereas RL95-2 cells are completely deficient in *PTEN* activity (10). To examine whether the *PTEN* signaling pathways could interact with adiponectin, we investigated the expression of ERK1/2 and Akt, both of which are known for their potent mitogenic actions (16). We observed that adiponectin treatment decreases ERK1/2 phosphorylation levels in the *PTEN*-deficient RL95-2 but had no effect in the *PTEN* containing KLE cell line. Also, we observed that adiponectin treatment had no effect on Akt phosphorylation levels in RL95-2, but it did suppress its activation in the KLE cell line. In agreement with these results, it has been previously shown that the inhibitory effects of adiponectin are associated with the reduction of different signaling proteins, ERK1/2 and Akt, depending on *PTEN* activity of the endometrial cancer cells (16). Unlike ERK1/2 and Akt signaling, the presence or absence of *PTEN* did not influence AMPK and/or adiponectin-mediated phosphorylation of its downstream target, ACC, in either KLE or RL95-2 endometrial cancer cell lines. Thus, adiponectin-induced activation of AMPK is likely an important signaling mechanism underlying suppressed growth in both KLE and RL95-2 cells, that is, regardless of *PTEN* activity.

We next tested whether adiponectin could influence cell-cycle-modulating molecules and whether these effects are modified by *PTEN* activity in endometrial cancer cell lines. It has been shown that adiponectin effects on cell proliferation are associated with distinct reductions of several key regulatory components of cell-cycle-regulatory genes, depending on the *PTEN* activity of the endometrial cancer cell lines (17). Consistently, we observed that adiponectin reduces the expression of cyclin D1 in the *PTEN* containing KLE cells and cyclin E2 in the *PTEN*-deficient RL95-2 cells. Cyclin D1 is a key regulatory element during cell cycle progression from G₁ to S-phase (18). It has been reported that overexpression of cyclin D1 not only exists in endometrial cancer but also correlates with its poor prognosis (19). Similarly, one of the molecular markers of endometrial cancer cells is the

elevated level of cyclin E2 (20), which also helps to drive the progression from G₁ to S-phase. Thus, adiponectin-induced reduction of cyclin D1 and/or cyclin E2 expression is potentially a critical step for the suppression of endometrial cancer cell growth.

In summary, we report for the first time herein that both AdipoR1 and AdipoR2 are expressed in endometrial cancer tissues, that the relative expression of AdipoR1 is higher than that of AdipoR2, and that the expression of both AdipoR1 and AdipoR2 in human endometrial cancer tissue is not statistically different from that in nonneoplastic tissues. Also, we show that the antiproliferative effect of adiponectin is dependent on AdipoRs in endometrial cancer cell lines. Importantly, our data show for the first time that adiponectin upregulates the tumor suppressor gene *LKB1* and that *LKB1* is required for adiponectin-mediated AMPK activation. Activation of intracellular signaling pathways by adiponectin leads to subsequent reduction of cell proliferation, colony formation, adhesion, and invasion of endometrial cancer cell lines. Moreover, depending on the presence of *PTEN* activity in endometrial cancer cell lines, antiproliferative effects of adiponectin are associated with the reduction of different progrowth regulators of cell cycle, cyclin D1 and E2, and signaling proteins ERK1/2 and Akt. These results suggest that *LKB1*-mediated adiponectin signaling may interact with *PTEN* activity in endometrial carcinogenesis, and this needs to be studied further by future investigations. These novel mechanistic studies provide evidence for a causal role of adiponectin in endometrial cancer and indicate that if the data are confirmed and extended by future studies, adiponectin could potentially prove to be a useful agent in the management of endometrial cancer. Further studies are needed to elucidate the relative importance of direct versus indirect actions of adiponectin (insulin-sensitizing effects *in vivo*) in endometrial carcinogenesis. Prospective studies are also needed to study the significance of not only adiponectin *per se* but also AdipoRs as prognostic markers and/or therapeutic targets in endometrial cancer. Finally, *in vivo* studies in rodents, and hopefully later in humans, could further elucidate the role of adiponectin's potential use as a therapeutic agent for endometrial cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

H.-S. Moon and C.S. Mantzoros wrote the manuscript. H.-S. Moon, J.P. Chamberland, K. Aronis, S. Tseloni-Balafouta, and C.S. Mantzoros participated in the study design, performance, and coordination. C.S. Mantzoros conceived the study. All authors read and approved the final manuscript.

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