Direct role of adiponectin and adiponectin receptors in endometrial cancer: *in vitro* and *ex vivo* studies in humans

Hyun-Seuk Moon¹, John P. Chamberland¹,², Konstantinos Aronis¹, Sofia Tseleni-Balafouta³, Christos S. Mantzoros¹,²

**Running title:** Adiponectin signaling in endometrial cancer

**Key words:** adiponectin, adiponectin receptor, intracellular signaling, LKB1, AMPK, endometrial adenocarcinoma

¹Division of Endocrinology, Diabetes and Metabolism, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; ²Section of Endocrinology, Boston VA Healthcare System, Harvard Medical School, Boston, Massachusetts 02130, USA; ³Department of Pathology, Faculty of Medicine, University of Athens, Athens, Greece.

**Correspondence should be addressed to:** Christos S. Mantzoros, MD, DSc, Professor of Medicine, Harvard Medical School; Professor in Environmental Health, Harvard School of Public Health; FD-876, 330 Brookline Avenue, Boston, MA 02215, Tel: (617) 667-8630, Fax: (617) 667-8634, Email: cmantzor@bidmc.harvard.edu

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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 utilized 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 non-neoplastic tissues. We also demonstrate 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.
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 non-alcoholic 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 pre-menopausal women (6).

Adiponectin’s peripheral actions are mediated mainly through two distinct receptors, AdipoR1 and AdipoR2 (3, 7-8). AdipoRs have been reported to have different affinity for lower vs. higher molecular weight adiponectin complexes, and despite their structural similarity to seven-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 vs. AdipoR2 in normal endometrium vs. 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 non-neoplastic 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 siRNA alleviates the adiponectin signaling in endometrial cancer cell lines in vitro. Since no previous study has evaluated whether adiponectin’s interaction with the adaptor molecule LKB1 could alter malignant properties of endometrial cancer cell lines, we directly studied whether LKB1 is required for 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 US patients, Imgenex Co. (San Diego, CA). 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 non-tumor 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 post-menopausal subjects. We did not perform any grading of the carcinomas, but used the generally established sub-classification since most of our specimens came from TMs. We selected our hyperplasia cases to be simple without severe atypia in order to avoid the high inter-observer variability in diagnosing a complex hyperplasia with atypia vs.
a well differentiated carcinoma. Control samples were not matched with tumor specimens.

**Immunohistochemical analysis** - The 4 μm paraffin tissue sections were deparaffinized, rehydrated, microwaved for 20 min in 10 mM citrate buffer of pH 6.0, and incubated for 30 min in methanol containing 3 % H₂O₂. After incubation in 16 % normal goat serum for 1 hr at room temperature, the slides were incubated overnight with the primary antibodies at 4°C. Primary rabbit polyclonal antibodies (Phoenix Pharmaceuticals, Inc., Belmont, CA) 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-HRP, a biotin-free visualization system (DAKO, Carinteria, CA), for 30 min. The peroxidase (POD) reaction was developed with diaminobenzidine for 30 min, 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 (ATCC, Manassas, VA). The cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, supplemented with 10 % fetal bovine serum and with or without 5 μg/ml insulin (from bovine pancreas, Sigma, St. Louis, MO), respectively. All cells were incubated at 37°C in an atmosphere of 5 % CO₂ in air, and sub-cultured beyond 80 % confluency.

**Real-time polymerase chain reaction (RT-PCR)** - Adiponectin receptor expression was detected with RT-PCR. RNA was extracted with Trizol® (Invitrogen, Carlsbad, CA) and first-strand cDNA synthesis was performed using Superscript III® (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. For RT-PCR, 100 ng of cDNA per 25 μl reaction were amplified using TaqMan Gene Expression system (Applied Biosystems, Foster City, CA), specific primers and FAM tagged
probes set (Applied Biosystems, Foster City, CA), and the standard real-time 7500 protocol (Applied Biosystems, Foster City, CA), with an initial polymerase activation step at 95°C for 10 min and 40 cycles. This included a 15 sec melting step at 95°C and a 1 min annealing-elongation step at 60°C. The analysis of relative gene expression was based on ΔCt values obtained from RT-PCR. PCR products were separated in 2 % agarose gels, in 0.5× TBE, in a horizontal Mini-Sub Cell GT electrophoresis apparatus (BIORAD, Hercules, CA), under a 100 V constant voltage and visualized using the BIORAD gel documentation system (BIORAD, Hercules, CA).

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

**Western Blotting** - The proteins were loaded in each lane. After SDS-polyacrylamide gel electrophoresis, proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The membranes were blocked for 1 hr in TBS containing 5 % nonfat dry milk and 0.1 % Tween-20. Incubation with primary antibodies was performed in TBS containing 5 % nonfat dry milk overnight and then incubated with horseradish peroxidase secondary antibodies for 2 hr. 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 performed using Image J processing and analysis software.

**Proliferation assay** - The cells were trypsinized and seeded in 96-well plates at a density of $5 \times 10^3$ cells/well. Cells were left to adhere overnight and then treated with adiponectin in serum-free medium for 24 hr. At the end of incubation period, the effect of adiponectin on cell proliferation was assayed with an MTT assay (Invitrogen, Carlsbad, CA). Cells were washed with PBS and incubated with 100µl serum-free medium and 10 µl Vybrant MTT solution for 2 hr. Formazan crystals were dissolved overnight at 37°C with the addition of 100 µl 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, Winooski, VT).

**Clonogenic assay** - The cells were grown in the media to 60 % confluency and treated with adiponectin for 24 hr. After 24 hr, 500 cells were re-seeded 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-dimethyl-methylene blue), and laved at room temperature for 45 min. After 45 min, the cells were washed with PBS and the blue colonies were counted.

**Adhesion assay** - The cells were pretreated with 20 µg/ml of adiponectin for 24 hr and plated ($5 \times 10^4$ cells per well) in 10 µg/cm² fibronectin-coated (Sigma, St Louis, MO, USA) wells in 96-well plates which were then incubated at 37°C (5 % CO₂) for 60 min. Adherent cells were fixed with 3 % paraformaldehyde for 10 min, washed with 2 % methanol for 10 min and stained with 0.5 % crystal violet in 20 % methanol for 10 min. The stain was eluted and absorbance was measured at 540 nm.
Invasion assay - Matrigel invasion assay was performed by using a Matrigel invasion chamber from BD BioCoat Cellware (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s protocol.

Statistical analysis - Descriptive characteristics of patients providing tumor and non-tumor specimens of endometrial cancer were summarized and compared using unpaired $t$-tests and $X^2$ tests for continuous and categorical measures accordingly. Case characteristics are presented as percents. Unmatched analyses of expression of adiponectin receptors were conducted using unconditional, binomial, logistic regression analysis in two 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 Student’s $t$-test and/or one-way ANOVA followed by post-hoc tests (Bonferroni correction for multiple comparisons). All analyses were performed using SPSS version 11.5 (SPSS Inc., Chicago, IL) and stata version 11.1 (Stata Corp. College Station, TX).

Results

Adiponectin receptor expression in benign endometrium vs. endometrial cancer samples ex vivo: Descriptive characteristics of study subjects are presented in Supplementary data 1. 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 pre-menopausal women, while atrophic non-tumor as well as tumor specimens were derived mainly from post-menopausal women. All tumors were staged according to the FIGO classification. Half (52.2 %) of the endometrial cancer cases from US subjects were Stage I, while 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 \textit{ex vivo} by immunohistochemistry (Supplemental data 2). 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 non-neoplastic tissue (early secretory, proliferative, pregnant, hyperplasia and atrophic phase) in the samples from the Greek individuals (Supplemental data 3). 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). These results were confirmed in the samples of various stages of endometrial cancer derived from US subjects (Table 2). Both AdipoR1 and AdipoR2 were expressed in all stages of endometrial cancer; however this expression was not statistically different between the two groups (group 1: FIGO stages I & II, group 2: FIGO stages III & IV).

\textbf{Adiponectin receptor expression in endometrial cancer cell lines \textit{in vitro}:} The expression of AdipoRs was tested using RT-PCR in two endometrial cancer cell lines i.e. KLE and RL95-2. The endometrial adenocarcinoma KLE and RL95-2 cell lines were found to express in both AdipoR1 and AdipoR2 (data not shown). Also, we confirmed by Western Blotting that the both AdipoR1 and AdipoR2 are expressed in KLE and RL95-2 cell lines (Supplemental data 4). By 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 physiological concentrations ranging from 5 to 10 μg/ml had no effect on cell proliferation (Fig. 1A). By contrast, 10 μg/ml of adiponectin administration in RL95-2 cells decreased cell proliferation by ~20 %. Furthermore, cell proliferation was decreased by ~45 % at high physiological/pharmacological doses, i.e., 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 using a long-term colony formation assay showing that adiponectin treatment significantly decreased colony number (Fig. 1B). Cell adhesion assays were performed using fibronectin as an adhesion substrate and we observed that adiponectin-treated endometrial cancer cells had significantly reduced adhesion activity compared to controls (Fig. 1C). Adiponectin treatment also effectively inhibited invasion of endometrial cancer cells, as seen through the Matrigel invasion assay (Fig. 1D).

Anti-proliferative effect of adiponectin is dependent on AdipoR1 and AdipoR2 in endometrial cancer cell lines in vitro: To examine whether the anti-proliferative 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 down-regulated via siRNA, the inhibitory effect of adiponectin on endometrial cancer proliferation was reduced (Supplemental data 5A). Under the same condition, adiponectin-activated AMPK phosphorylation was disturbed by knockdown of AdipoR1 or AdipoR2 in endometrial cancer cells (Supplemental data 5B). 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 adiponectin’s effects on cell growth in endometrial cancer cells. As shown in Fig 2C, LKB1 siRNA administration increased cell proliferation when compared to 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 the 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 cell adhesion and invasion of endometrial cancer cells. Similar to the results shown in Fig. 1C and 1D, adiponectin inhibited adhesion and invasion of both cells (Fig. 2E and 2F). 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 since adiponectin-mediated cell proliferation, colony formation, adhesion and invasion were not regulated by ssiRNA administration when compared to control.
Anti-proliferative effects of adiponectin are modified by PTEN activation in endometrial cancer cell lines in vitro: To understand the potential molecular mechanisms underlying the anti-proliferative 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 ACC within 15 min in both KLE and RL95-2 cells (Fig. 3A and 3B). Also, we found that adiponectin-stimulated AMPK phosphorylation was abolished by AMPK siRNA administration in both cell lines (Fig. 3C). By contrast, Akt was down-regulated by adiponectin treatment in KLE, but not in RL95-2 cells (Fig. 4A and 4B). Also, adiponectin decreased ERK activation in RL95-2, but not in KLE cells (Fig. 4A and 4B). The signaling events diverged in these two cell lines in response to the longer treatment (24 hr) with adiponectin. Accordingly, adiponectin treatment caused sharp reductions in Cyclin D1 expression in KLE cells (Fig. 4C). During the same timeframe 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 ~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 pro-growth kinases in the PTEN containing KLE cells vs. in the PTEN deficient RL95-2 cells. By contrast, 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 (Supplemental data 6).
Discussion

We were the first to report that circulating adiponectin levels are associated with endometrial cancer (5), and our data have subsequently been confirmed by both case-control (6) and cohort studies (4). It has been demonstrated that the leptin/adiponectin ratio was independently associated with an increased risk for endometrial cancer development (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 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 in human endometrial cancer tissue is not statistically different from that in non-neoplastic tissue. Adiponectin receptors 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 demonstrating the presence of adiponectin receptors, we also report that adiponectin signaling is mediated by AdipoRs in endometrial cancer cell lines. We also demonstrate 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 anti-proliferative 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 knock-down 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 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 the 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 demonstrated 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 i.e. 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 over-expression 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 non-neoplastic tissues. Also, we demonstrate that the anti-proliferative effect of
adiponectin is dependent on AdipoRs in endometrial cancer cell lines. Importantly, our data demonstrate for the first time that adiponectin up-regulates the tumor suppressor gene \textit{LKB1}, and that \textit{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, anti-proliferative effects of adiponectin are associated with the reduction of different pro-growth regulators of cell cycle, Cyclin D1 and E2, and signaling proteins, ERK1/2 and Akt. These results suggest that \textit{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 adiponectin’s direct vs. indirect actions (insulin sensitizing effects \textit{in vivo}) in endometrial carcinogenesis. Prospective studies are also needed to study the significance of not only adiponectin \textit{per se} but also adiponectin receptors as prognostic markers and/or therapeutic targets in endometrial cancer. Finally, \textit{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.
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Conflict of Interest: All authors state that there is no conflict of interest related to this research paper.
References


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

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P^1 from binomial logistic regression comparing neoplastic with non-neoplastic tissue, unadjusted model.
P^2 from binomial logistic regression comparing neoplastic with non-neoplastic tissue, adjusted for age.
Table 2. Comparison of AdipoR1 and AdipoR2 expression in immunohistochemistry studies between various stages of endometrial cancer of US subjects.

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P₁ from binominal logistic regression comparing FIGO stage I&II with FIGO Stage III&IV, unadjusted model. P² from binominal logistic regression comparing FIGO stage I&II with FIGO Stage III&IV, adjusted for age.
Figure legends

**Fig. 1.** Anti-proliferative effect of adiponectin on KLE and RL95-2 endometrial cancer cell lines *in vitro* - The cells were cultured as described in detail in the “Materials and Methods” section. **(A)** The cells were incubated with adiponectin at indicated concentration for 24 hr and cell viability was then measured by MTT assay as described in detail in the “Materials and Methods” section. **(B)** The cells were incubated with adiponectin (20 μg/ml) for 24 hr and the clonogenic assay was performed as described in detail in the “Materials and Methods” section. **(C)** The cells were incubated with adiponectin (20 μg/ml) for 24 hr and the adhesion assay was performed as described in detail in the “Materials and Methods” section. **(D)** The cells were incubated with adiponectin (20 μg/ml) for 24 hr and the invasion assay was performed as described in detail in the “Materials and Methods” section. All data were analyzed using student t-test and/or one-way ANOVA followed by post-hoc test for multiple comparisons. Values are means (n=3) ± SD. Means with different letters are significantly different, p<0.05.

**Fig. 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 the “Materials and Methods” section. **(A)** The cells were treated with adiponectin (20 μg/ml) at indicated times. **(B)** The cells were transfected with LKB1 siRNA as described in detail in the “Materials and Methods” section and then stimulated with adiponectin (20 μg/ml) for 30 min. **(C)** The cells were incubated with adiponectin at indicated concentrations for 24 hr and cell viability was then measured by MTT assay as described in detail in the “Materials and Methods” section. **(D)** The cells were incubated with adiponectin (20 μg/ml) for 24 hr and the clonogenic assay was performed as described in detail in the “Materials and Methods” section. **(E)** The cells
were incubated with adiponectin (20 μg/ml) for 24 hr and the adhesion assay was performed as described in detail in the “Materials and Methods” section. (F) The cells were incubated with adiponectin (20 μg/ml) for 24 hr and the invasion assay was performed as described in detail in the “Materials and Methods” section. All data were analyzed using one-way ANOVA followed by post-hoc test for multiple comparisons. Values are means (n=3) ± SD. Means with different letters are significantly different, p<0.05.

**Fig. 3. Adiponectin signaling in KLE and RL95-2 endometrial cancer cell lines in vitro** - The cells were cultured as described in detail in the “Materials and Methods” section. (A) The cells were treated with adiponectin at indicated concentrations for 30 min. (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 the “Materials and Methods” section and then stimulated with adiponectin (20 μg/ml) for 30 min. All data were analyzed using one-way ANOVA followed by post-hoc test for multiple comparisons. Values are means (n=3) ± SD. Means with different letters are significantly different, p<0.05.

**Fig. 4. Adiponectin signaling in KLE and RL95-2 endometrial cancer cell lines in vitro** - The cells were cultured as described in detail in the “Materials and Methods” section. (A) The cells were treated with adiponectin at indicated concentrations for 30 min. (B) The cells were treated with adiponectin (20 μg/ml) for indicated times. (C) The cells were treated with adiponectin for 24 hr at indicated concentrations. All data were analyzed using one-way ANOVA followed by post-hoc test for multiple comparisons. Values are means (n=3) ± SD. Means with different letters are significantly different, p<0.05.
Figure 1

A

Cell Proliferation (% of Control) vs. Adiponectin (µg/ml)

B

Number of Colonies vs. Adiponectin (µg/ml)

C

Cell Adhesion Density (OD 540nm) vs. Adiponectin

D

Number of cells Invading Matrigel vs. Adiponectin
Figure 2

A

KLE

RL95-2

p-p-LKB1/Actin

p-LKB1/Actin

LKB1

p-LKB1

Adiponectin (20µg/ml) Adiponectin (20µg/ml)

B

KLE

RL95-2

Adiponectin (20µg/ml) Adiponectin (20µg/ml)

C

KLE

RL95-2

Cell Proliferation (% of Control)

D

KLE

RL95-2

Colony Number

E

KLE

RL95-2

OD (540 nm)

F

KLE

RL95-2

Number of Cells Invading Matrigel
Figure 3

A

![Graph showing p-AMPK/AMPK and p-ACC/ACC levels in KLE and RL95-2 cells treated with Adiponectin at different concentrations of 0, 10, 20, and 50 µg/ml.](image)

B

![Graph showing p-AMPK/AMPK and p-ACC/ACC levels in KLE and RL95-2 cells treated with Adiponectin over time (in minutes) from 0 to 60.](image)

C

![Graph showing p-AMPK/Actin expression in KLE and RL95-2 cells treated with Adiponectin at 20 µg/ml.](image)
Figure 4

A

KLE

RL95-2

Adiponectin

Adiponectin

(µg/ml) 0 10 20 50

0 10 20 50

0 10 20 50

0 10 20 50

B

KLE

RL95-2

Adiponectin (20 µg/ml)

Adiponectin (20 µg/ml)

(min) 0 5 15 30 60

0 5 15 30 60

0 5 15 30 60

0 5 15 30 60

C

KLE

RL95-2

Adiponectin (µg/ml)

Adiponectin (µg/ml)

Adiponectin (µg/ml)

Adiponectin (µg/ml)
Molecular Cancer Therapeutics

Direct role of adiponectin and adiponectin receptors in endometrial cancer: in vitro and ex vivo studies in humans

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