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Adiponectin mediates an antiproliferative response in human MDA-MB 231 breast cancer cells

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Abstract. Numerous epidemiological studies have documented that obesity is a risk factor for breast cancer especially in post-menopausal women. However, the molecular basis of this association is not well known. In contrast to leptin, plasma levels of adiponectin, another major adipokine, are decreased in obese subjects. Therefore, we and others hypothesized that adiponectin may be a paracrine factor negatively controlling mammary tumor development. We recently demonstrated growth inhibition of the estrogen-sensitive breast cancer MCF-7 cell line by adiponectin. The purpose of the present study was to determine whether this anti-proliferative effect of adiponectin also applies to the MDA-MB 231 estrogen-insensitive breast epithelial cancer cell line. Our results demonstrate that i) the adiponectin-specific receptors AdipoR1 and R2 are expressed in these cells, and ii) the subphysiological concentrations of recombinant adiponectin inhibit MDA-MB 231 cell growth and concomitantly enhance the expression of Bax and p53, two pro-apoptotic genes. Moreover, the invalidation of AdipoR1 and R2 mRNA experiments demonstrated that the anti-proliferative and pro-apoptotic effects of adiponectin were partially mediated via AdipoR1 and R2. We describe, for the first time, that AdipoR mRNA expression was down-regulated by adiponectin and leptin in MDA-MB 231 cells. Taken altogether, these results strongly suggest that the two adipokines should be considered as i) additional factors of breast cancer risk, and ii) may therefore be potential targets in breast cancer therapy.

Introduction

It is well established that the risk of developing breast cancer is greater in post-menopausal women with upper body fat predominance than post-menopausal women with a normal weight (1,2). Although the biological mechanisms underlying this relationship between central obesity and breast cancer remain obscure, increasing evidence suggests that the adipose tissue of the mammary gland may play important roles in regulating cancer cell development (3). Besides estrogens, adipose tissue elicits significant endocrine functions, secreting a variety of bioactive peptides termed adipokines such as IGF-1, leptin and adiponectin (4,5). In obesity, altered adipokine secretion appears to contribute to an increased cancer risk. For example, leptin whose secretion is increased in obese patients promotes proliferation and suppresses apoptosis of different human breast epithelial cancer cell lines (6-8).

Adiponectin, a 30 kDa adipokine, circulates at relatively high concentrations in human plasma. In contrast to other adipokines, adiponectin levels are inversely correlated with central fat accumulation (9,10). Moreover, adiponectin improves insulin resistance and elicits anti-atherogenic and anti-inflammatory properties (11). Two types of adiponectin receptors (AdipoR1 and R2) have been described, both of which transduce an adiponectin signal via stimulation of the AMP- and MAP kinases, and PPARα signaling pathways (12-14). AdipoR1 is ubiquitously expressed and displays a high-affinity for globular adiponectin whereas AdipoR2 is predominantly expressed in the liver and has a higher affinity for full-length adiponectin (12).

In addition to its role in energy homeostasis, adiponectin exerts a protective action against cancer development. Circulating adiponectin levels are inversely correlated with an increased risk of gastric, prostate and breast cancers (15-17). In breast cancer, an inverse correlation was found between plasma adiponectin levels and the histological grade of the tumor (18). Different *in vitro* studies also reported that adiponectin reduces cell proliferation and promotes apoptosis of the human non-aggressive estrogen-responsive breast cancer cells, MCF-7 (7,19,20). We recently showed that a simultaneous exposure of these cells to adiponectin and 17ß-estradiol results in the total abolition of the mitogenic effect of 17ß-estradiol (20).

The aim of the present study was to determine whether adiponectin and its receptors are involved in the molecular mechanisms linking obesity and estrogen-independent breast cancer development. We thus tested *in vitro* the effects of this adipokine on the growth and apoptosis processes of the MDA-MB 231 breast cancer cell line.

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Materials and methods

Materials. Recombinant human adiponectin and leptin were provided by R&D Systems Europe Ltd. (Abingdon, UK), Superscript II Rnase H-RT by Gibco BRL (Grand Island, NY, USA), Taq polymerase and RNA guard by Pharmacia Biotechnology (Uppsala, Sweden). The *in situ* cell death detection kit was from Roche Molecular Biochemicals (Mannheim, Germany). [^{3}H]-thymidine was from Amersham.

Cell culture. The human breast cancer cells, MDA-MB 231, were obtained from the American Type Collection of Cell Collection (via Salisbury, UK). These cells were maintained in phenol red-free RPMI medium with 2% Hepes and 1% glutamin, 10% fetal calf serum (FCS), streptomycin (0.1 mg/ml) and penicillin (100 U/ml) at 37˚C in a 5% CO_2 atmosphere. The culture medium was removed 24 h later and replaced by phenol red-free RPMI supplemented with 5% charcoal-stripped FCS until the starting assays.

[^{3}H]-thymidine incorporation. MDA-MB 231 cells were suspended in 24-well plates in RPMI supplemented with 10% FCS. During the exponential phase of growth, the culture medium was replaced by RPMI containing 5% charcoal-stripped FCS for 48 h. The cells were exposed to various concentrations of human recombinant adiponectin (5-500 ng/ml) for 24 h. For the final six hours, [^{3}H]-thymidine (1 mCi/ml) was added to the culture medium and radioactivity was then counted.

Cell counting. The experimental design used was the one described above except that the adiponectin concentrations tested were 25 ng/ml. Cells were counted in a hemocytometer.

Apoptosis assay. After a 48 h incubation in RPMI supplemented with 5% charcoal-stripped FCS, cells were cultured in the presence of adiponectin (25 and 250 ng/ml) for 96 h. The validity of our experimental culture conditions was warranted by the well established pro-apoptotic response of MDA-MB 231 cells to camptothecin (6 μM) (21).

Attached and floating cells were fixed in 70% ethanol at -20˚C overnight and then labeled for DNA fragmentation by TUNEL (Terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling). The apoptotic index was calculated after counting a minimum of 5000 events by flow cytometry using an EPICS flow cytometer (Coulter Electronics, Miami, FL, USA).

AdipoR protein expression. Total extracts were resolved by SDS-PAGE (10% acrylamide), transferred to PVDF membrane and blocked in buffer A (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20) containing 2.5% gelatin for 2 h. PVDF membranes were incubated overnight at room temperature with rabbit polyclonal anti-AdipoR1 antibody (1:200 dilution, Santa Cruz Biotechnology, sc-46749) or anti-AdipoR2 antibody (1:200 dilution, Santa Cruz Biotechnology, sc-46756) in buffer A containing 2.5% gelatin. After washing, the membranes were incubated with the secondary antiserum coupled to peroxidase (1:10000 dilution in TBS-T) for 1 h. The membranes were then incubated with the enhanced chemiluminescence detection system and exposed to X-ray film.

The protein concentration was measured according to Bradford (22) with BSA as the standard.

Quantitative RT-PCR. Total RNA (0.5 μg) was reverse transcribed as previously described (23). Quantitative PCR was performed using a LightCycler® instrument (Roche Diagnostics) and primer sets as previously described in (9). The second derivative maximum method was used to automatically determine the Cp for the individual samples. For each sample, the concentration ratio (target/TBP gene, used as an internal standard) was calculated using the Roche software. Fold changes in expression were determined by calculating the normalized ratio which corresponds to concentration of the calibrator situation (without effector)/concentration of unknown situation (with effector) (relative quantification).

RNA interference for AdipoR. Two pairs of small-interfering RNAs (siRNAs) corresponding to different regions of each receptor gene were chemically synthesized by Qiagen. The sequences of the sense siRNAs were: for human AdipoR1: AAG GAC AAC GAC TAT CTG CTA and CTG GCT AAA GGA CAA CGA CTA and for human AdipoR2: ACC AAT TTA AGT GAA CAT TTA and CGG CTC TCC TTG AAT AAG AAA. A fluorescently labeled, non-silencing control siRNA was useful for the optimization of transfection conditions and as a control for non-specific silencing effects. A positive control, consisting of a siRNA directed against human MAP kinase RNA sequence, was used concomitantly in order to ensure that optimal conditions were maintained and confirm experimental results. For the knockdown experiments, MDA-MB 231 cells were plated in 12-well dishes at 1x10^5 cells/well and cultured for 24 h in medium without antibiotics. Cells were transfected with siRNAs (1 or 5 nM/well) using a HiPerfect transfection reagent (Qiagen) according to the manufacturer's instructions. After 72 h of culture, the [^{3}H]-thymidine incorporation or mRNA expression analyses were realized as described above.

Statistical analysis. All values were expressed as means ± SEM of 4-8 separate experiments, and statistical analysis was performed using the non-parametric paired Wilcoxon test.

Results

AdipoR expression and regulation by various cytokines. Using RT-PCR analysis, we found that the two adiponectin receptor AdipoR1 and R2 mRNAs are expressed in MDA-MB 231 cells (Fig. 1A). Moreover, AdipoR mRNA expression was comparable with that observed in another mammary epithelial cancer cell line, the MCF-7 cells used here as a positive control. Notably, in MDA-MB 231 cells, AdipoR1 mRNA was predominantly expressed (~10-fold higher) compared to AdipoR2 mRNA. Western blot analysis confirmed the presence of the two receptors in MDA-MB 231 cells (Fig. 1B).

Otherwise, exposure of these cells to recombinant adiponectin (25 ng/ml) resulted in a rapid (4 h) down-regulation of

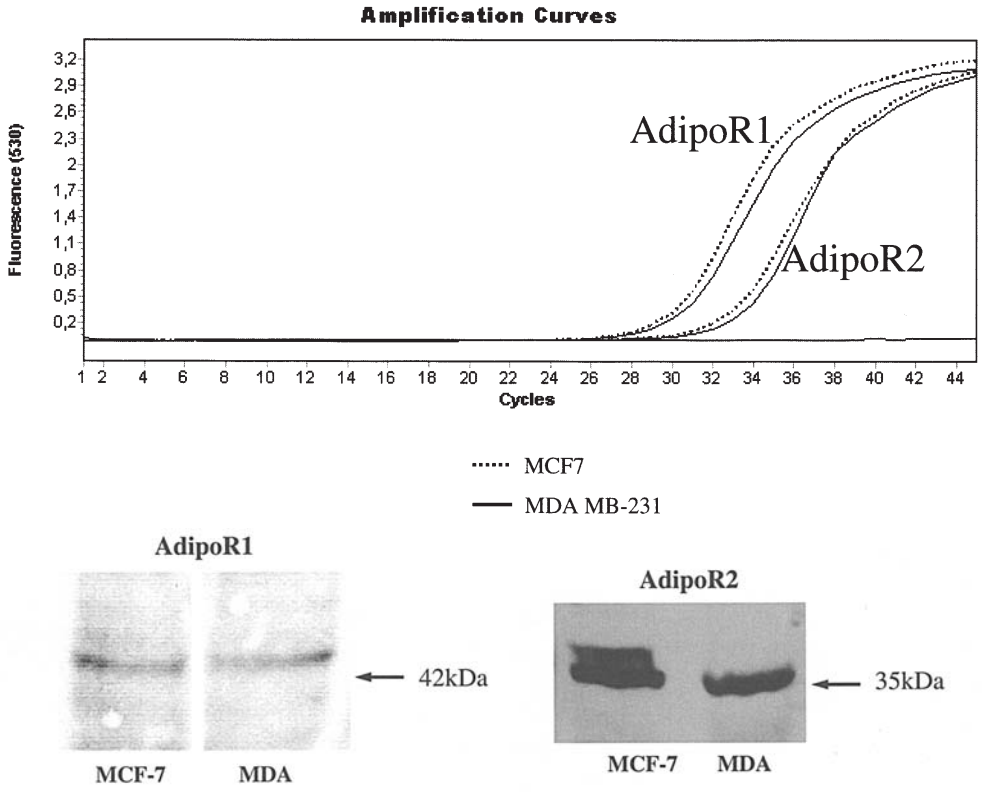


Figure 1. AdipoR expression in MDA-MB 231 cells. (A) AdipoR1 and R2 mRNA expression. Total RNA was extracted from MDA-MB 231 and MCF-7 cells and analyzed by quantitative RT-PCR. This figure shows representative amplification plots. (B) AdipoR1 and R2 protein expression. Cell lysates (100 μg) were subjected to Western blot analysis using anti-AdipoR1 or anti-AdipoR2 antibody. One representative experiment of three is shown.

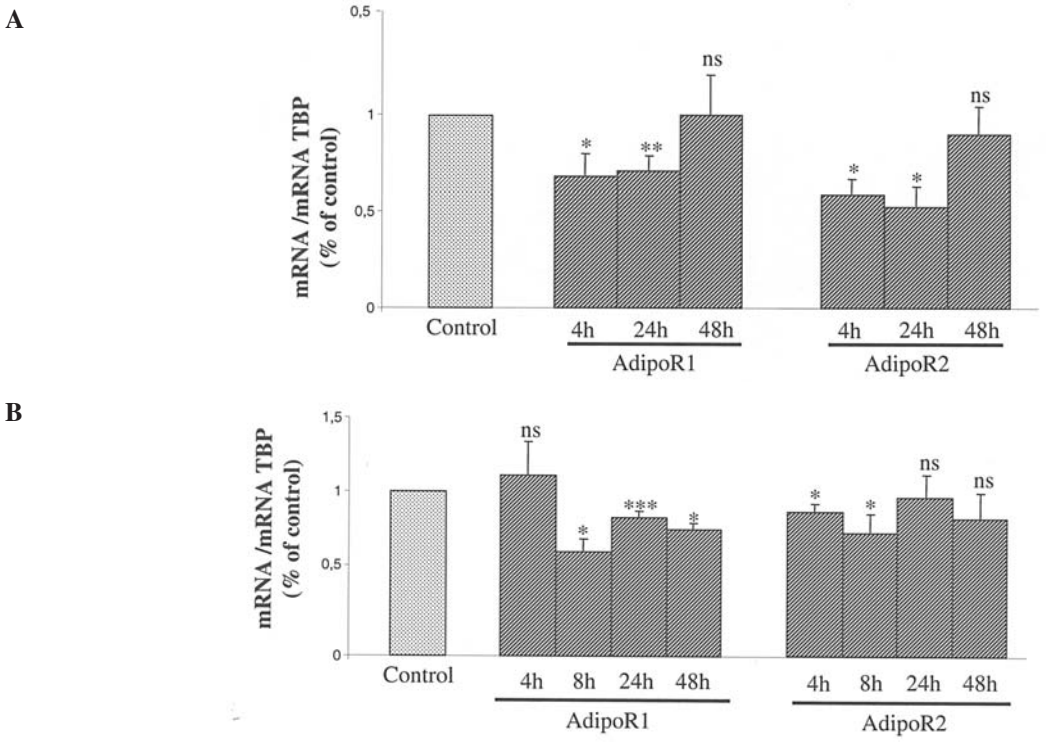


Figure 2. AdipoR mRNA regulation by adipokines in MDA-MB 231 cells. (A) AdipoR1 and R2 mRNA regulation by adiponectin. Cells were exposed to 25 ng/ml human recombinant adiponectin for different times as indicated in the figure. Total RNA was extracted and analyzed by RT-PCR. The values are the mean ± SEM obtained from 5-8 separate experiments and are expressed as percentages of the control value (without adiponectin); $^{*}p<0.05$ and $^{**}p<0.03$; ns, non-significant Wilcoxon test. (B) AdipoR1 and R2 mRNA regulation by leptin. Cells were exposed to 20 nM human recombinant leptin for different times. Total RNA was extracted and analyzed by RT-PCR. The values are the mean ± SEM obtained from 5-8 separate experiments and are expressed as percentages of the control value; $^{*}p<0.05$ and $^{***}p<0.01$; ns, non-significant, Wilcoxon test.

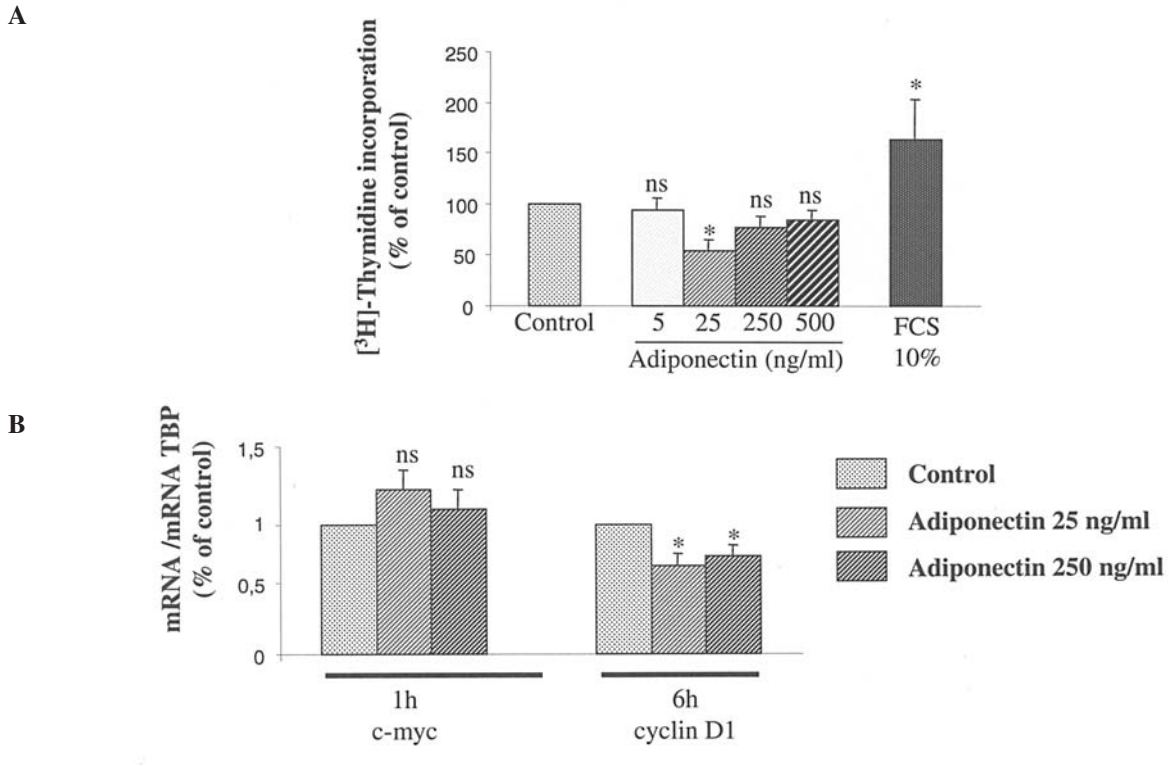


Figure 3. Effects of adiponectin on MDA-MB 231 cell proliferation. (A) Effects of adiponectin on DNA synthesis. Cells were exposed to different concentrations (5-500 ng/ml) of human recombinant adiponectin in the presence of [^{3}H]-thymidine as described in Materials and methods. Results are the means ± SEM of 5-8 experiments and are normalized as percentages of the control value; $^*p<0.05$; ns, non-significant Wilcoxon test. (B) Effects of adiponectin on cyclin D1 and c-myc mRNA expression. Cells were incubated during 1 or 6 h in the presence or absence of adiponectin (25 or 250 ng/ml) for c-myc and cyclin D1 expression, respectively. Total RNA was extracted and analyzed by RT-PCR. Results are the means ± SEM of eight experiments and are expressed as a percentage of the control; $^*p<0.05$; ns, non significant, Wilcoxon test.

the two AdipoR mRNA expression (-32±12 and -41±8% for AdipoR1 and R2, respectively) which still persisted after 24 h (Fig. 2A).

The regulation of AdipoR expression by leptin, another cytokine involved in breast tumorigenesis (24), was studied in MDA-MB 231 breast cancer cells. As shown in Fig. 2B, leptin (20 nM) significantly reduced the expression of AdipoR mRNAs. This effect was rapid for AdipoR1 and R2 (-37±8 and -25±12%, respectively, after 8 h of exposure) and persists until 48 h for AdipoR1.

Cell proliferation. Fig. 3A shows that adiponectin at 25 ng/ml significantly reduced [^{3}H]-thymidine incorporation (-47±12%) in MDA-MB 231 cells. Under the same experimental conditions, 10% FCS was used as a control and induced a 1.6±0.4-fold increase in [^{3}H]-thymidine incorporation. The anti-proliferative action of adiponectin was also confirmed by direct MDA-MB 231 cell counting (-43.5±4.8%).

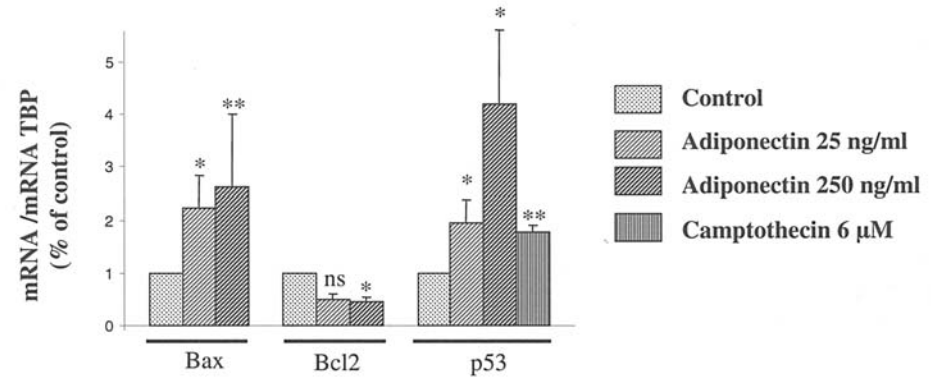
Expression of some cell cycle regulatory genes. C-myc and cyclin D1, two critical mediators of the cell cycle G1-S transition are convergent targets of mitogenic agents in breast cancer cells (25,26). We thus investigated, by RT-PCR, the influence of adiponectin on the expression of these two genes. As shown in Fig. 3B, cell exposure to adiponectin (25 and 250 ng/ml) decreased by ~30% cyclin D1 mRNA expression but did not affect the c-myc mRNA expression.

Cell apoptosis. Using quantitative RT-PCR analysis, we studied the influence of adiponectin on the expression of two pro-apoptotic genes (Bax and p53) and of one anti-apoptotic gene (Bcl2) in MDA-MB 231 cells.

As shown in Fig. 4A, cell exposure to adiponectin at 250 ng/ml for 24 h resulted in a significant stimulation of the Bax and p53 mRNA expression (2.5±1.2-fold change and 4.1±1.5-fold change, respectively). Under the same experimental conditions (24 h) and for comparison, camptothecin (an inhibitor of topoisomerases) was used as a positive control and enhanced (1.7±0.2-fold change) p53 mRNA expression. As shown in Fig. 4A, the anti-apoptotic gene, Bcl2, also appears to be a target for adiponectin as after 24 h of exposure, a -55±8% reduction of Bcl2 mRNA was observed.

Concomitantly, we tested the final step of apoptosis by using TUNEL analysis to determine the percentage of labeled apoptotic nuclei (Fig. 4B). Surprisingly, DNA fragmentation in MDA-MB 231 cells was apparently not affected by adiponectin whatever the concentration of hormone tested. Under the same conditions, however, camptothecin, an apoptotic agent used as a control, significantly increased the population of apoptotic cells (2.8±0.3-fold change).

AdipoR invalidation. In these experiments, we tested two different siRNA duplex for each receptor and finally chose those which induced a decrease of 79 and 76% in AdipoR1 and R2 expression, respectively, after a transfection of 72 h (Fig. 5A). Furthermore, as shown in Fig. 5B, the suppression



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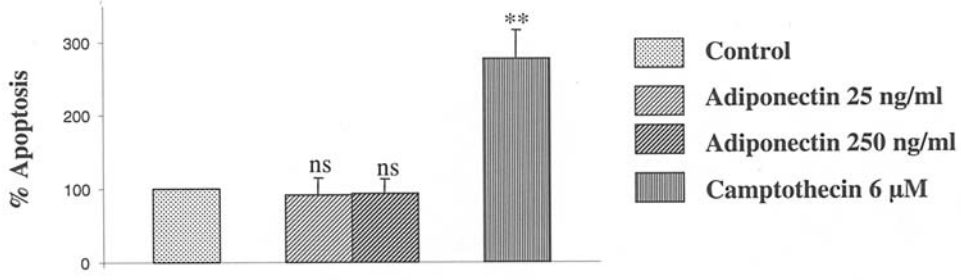

Figure 4. Effects of adiponectin on MDA-MB 231 cell apoptosis. (A) Effects of adiponectin on p53, Bax and Bcl2 mRNA expression. Cells were cultured for 24 h in the presence or absence of adiponectin (25 and 250 ng/ml) or camptothecin (6 μM). Total RNA was extracted and analyzed by RT-PCR. Results are the means ± SEM of 5-8 experiments and are expressed as a percentage of the control; $^{*}p<0.05$ and $^{**}p<0.03$; ns, non-significant Wilcoxon test. (B) Effects of adiponectin on DNA fragmentation. Cells were cultured for four days in the presence or absence of adiponectin (25 or 250 ng/ml) or camptothecin (6 μM). Cells were then analyzed by TUNEL staining and flow cytometry. Results are expressed as a percentage of the control. Each bar represents the mean ± SEM of four separate experiment; $^{**}p<0.01$; ns, non-significant, Wilcoxon test.

of AdipoR1 and R2 with siRNA inhibited the increasing Bax mRNA expression by adiponectin. These data indicated that the adiponectin-induced pro-apoptotic gene expression is mediated by AdipoR in MDA-MB 231 cells. In constrast, under these conditions, transfected MDA-MB 231 cells failed to elicit any significant change in the ability of adiponectin to reduce DNA synthesis after adiponectin treatment (Fig. 5C).

Discussion

Different *in vitro* studies recently described the direct anti-proliferative effects of adiponectin on the breast epithelial cancer MCF-7 cell line (20,27,28). However, the molecular mechanisms explaining these effects have yet to be fully elucidated. As most of these studies were conducted in ER-positive breast cancer cell lines, herein we provide evidence that adiponectin also inhibits the proliferation of the ER-negative breast cancer MDA-MB 231 cells. This finding suggests that adiponectin may exert an estrogen-independent protective effect *in vivo* against breast cancer development and that the cell growth actions of estrogens and adiponectin involve different transduction pathways.

For this purpose, we verified the presence of the two AdipoR isoform (AdipoR1 and R2) mRNA and protein levels in MDA-MB 231 cells. As with human adipose tissue and T47D mammary cancer cells (28,29), MDA-MB 231 cells predominantly express the AdipoR1 isoform which is consistent with the concept that AdipoR1 is ubiquitously expressed, whereas AdipoR2 is more restricted to the liver and skeletal muscle (12). In MDA-MB 231 cells, as described in the human placenta (30), adipose tissue (31) and prostate cancer cell lines (32), we observed that exposure to recombinant adiponectin causes a significant down-regulation of its own receptors. For the first time, we found that AdipoR mRNA expression is down-regulated by leptin. This adipokine which is considered to be a potential contributing growth factor leading to the development of breast cancer acts directly or indirectly through increased local estrogen production (4). Thus, this down-regulation of adiponectin signaling by leptin observed in the present study may be an additional mechanism amplifying the mitogenic and proliferative effects of leptin towards mammary epithelial cells.

Experiments testing the direct effect of adiponectin on MDA-MB 231 cells showed significant cell growth inhibition which was not adiponectin dose-dependent. The same situation was also reported in MCF-7 and osteoblastic cells (20,33). Thus, it cannot be excluded that the absence of adiponectin action on the cell number at concentrations >25 ng/ml is due to the rapid down-regulation of AdipoR by adiponectin.

In order to gain further insight into the mechanisms whereby adiponectin exposure decreases MDA-MB 231 cell growth, we investigated cell cycle and apoptosis regulatory genes. These experiments demonstrated that adiponectin significantly reduces the expression of cyclin D1 and of the anti-apoptotic gene, Bcl2, and conversely induces the expression of the pro-apoptotic genes p53 and Bax. Under our experimental conditions, however, adiponectin treatment failed to induce DNA fragmentation, the last step of apoptosis. These findings, which are consistent with those of another study on the T47D cell line (28), are in contrast with those we recently reported in MCF-7 cells where adiponectin was found not only to induce pro-apoptotic genes but also enhance

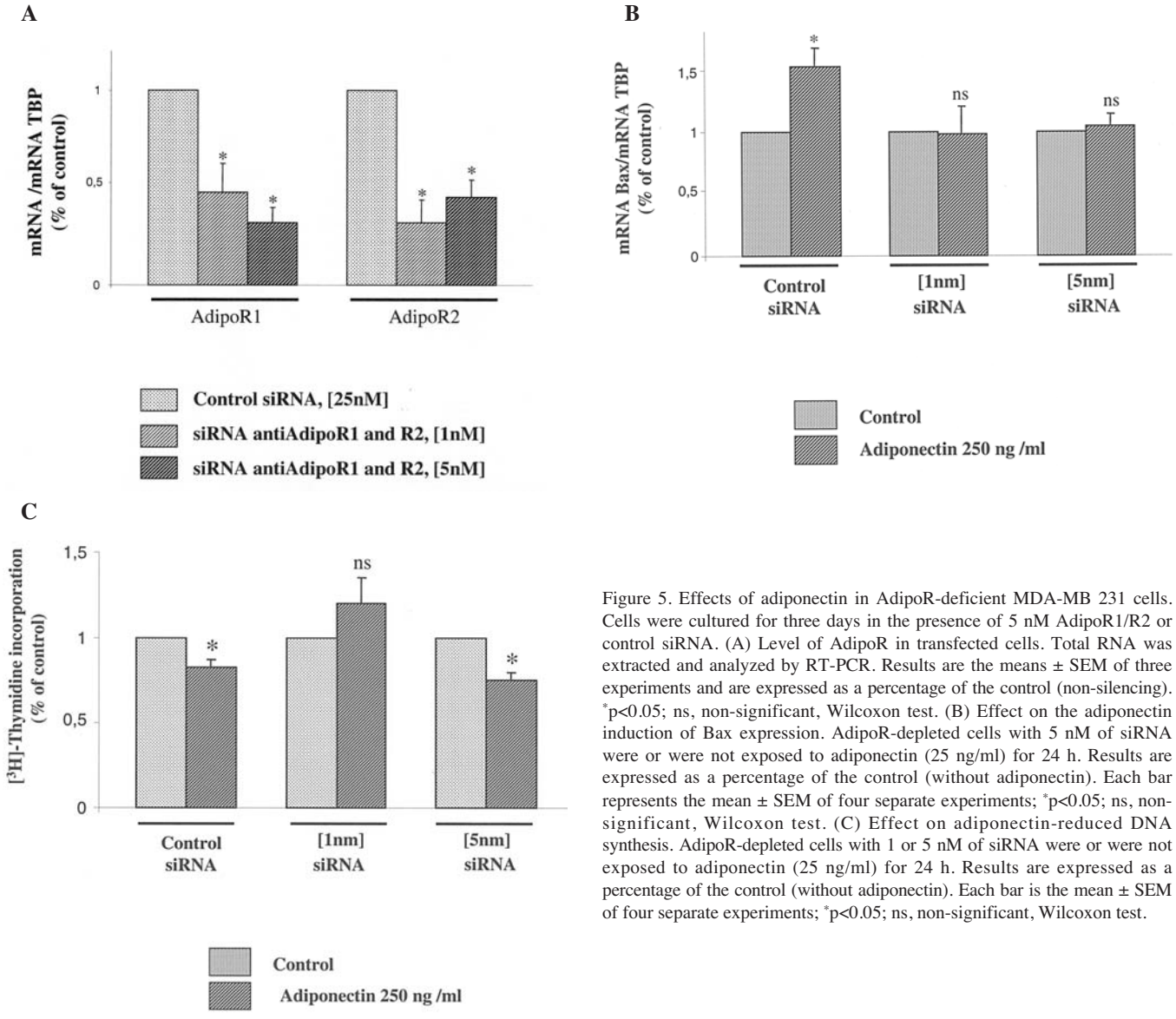

Figure 5. Effects of adiponectin in AdipoR-deficient MDA-MB 231 cells. Cells were cultured for three days in the presence of 5 nM AdipoR1/R2 or control siRNA. (A) Level of AdipoR in transfected cells. Total RNA was extracted and analyzed by RT-PCR. Results are the means ± SEM of three experiments and are expressed as a percentage of the control (non-silencing). *p<0.05; ns, non-significant, Wilcoxon test. (B) Effect on the adiponectin induction of Bax expression. AdipoR-depleted cells with 5 nM of siRNA were or were not exposed to adiponectin (25 ng/ml) for 24 h. Results are expressed as a percentage of the control (without adiponectin). Each bar represents the mean ± SEM of four separate experiments; *p<0.05; ns, non-significant, Wilcoxon test. (C) Effect on adiponectin-reduced DNA synthesis. AdipoR-depleted cells with 1 or 5 nM of siRNA were or were not exposed to adiponectin (25 ng/ml) for 24 h. Results are expressed as a percentage of the control (without adiponectin). Each bar is the mean ± SEM of four separate experiments; *p<0.05; ns, non-significant, Wilcoxon test.

DNA fragmentation (20). A possible explanation for these discrepancies is the variations in cell signaling between the cell lines or cell-specific effects of adiponectin on apoptosis. Several studies have reported a different response of the MDA-MB 231 and MCF-7 cell lines to different apoptotic agents including the soy isoflavone genistein (34). Moreover, the MCF-7 and MDA-MB 231 cells are distinguishable by their own characteristics: MCF-7 cells express functional ER, wild-type p53 and are caspase-3 deficient, whereas MDA-MB 231 cells are ER-negative and express functional caspase-3 and mutant p53 (35,36). The distinct apoptotic responses of MCF-7 and MDA-MB 231 cells to adiponectin are thus a novel illustration of the variability of mammary tumors *in vivo* and underline the complexity of breast cancer mechanisms.

As our data indicated that MDA-MB 231 cells expressed the two AdipoR, we investigated whether adiponectin affects these cells directly through AdipoR. Suppression of the two receptors by siRNA abolished adiponectin-induced pro-apoptotic Bax gene expression but not the adiponectin-reducing effect on cell proliferation. These data suggest that adiponectin action on MDA-MB 231 cells is presumably, in part, mediated by AdipoR. Persistence of the adiponectin anti-proliferative effect after a 70% invalidation of AdipoR raises the possibilities that i) the 30% remaining receptors after invalidation are sufficient to mediate the adiponectin effects and/or ii) the action of adiponectin is mediated via alternative molecular/signaling pathways. Additional receptors for adiponectin have been described, such as T-cadherin (37). Experiments are currently in progress in our laboratory in order to determine the eventual implication of T-cadherin in adiponectin effects in MDA-MB 231 cells.

Adiponectin signal transduction may involve different pathways including AMP-, p42/p44 MAP-, PI3-, and p38 MAP kinases (38). We recently demonstrated the involvement of AMPK and the p42/p44 MAP kinase activations in the anti-proliferative action of adiponectin in MCF-7 cells (20). Thus complementary experiments will be needed to describe more precisely the pathways that mediate adiponectin effects in MDA-MB 231 cells.

In conclusion, our data indicate that adiponectin acts as an inhibitory growth factor in the ER-negative MDA-MB 231 cell line. Thus, in obese women, we can hypothesize that the decreased protective effects of adiponectin on mammary epithelial cell growth facilitated mitogenic effects of leptin.

According to other *in vitro* studies, these two adipokines considered as molecular mediators linking adipose tissue to breast carcinogenesis, thereby constituting biomarkers for breast cancer risk in obese post-menopausal women.

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