Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 345 (2006) 271–279
BBRC
www.elsevier.com/locate/ybbrc

Adiponectin mediates antiproliferative and apoptotic responses in human MCF7 breast cancer cells ☆

Marie-Noelle Dieudonne, Marianne Bussiere, Esther Dos Santos, Marie-Christine Leneveu, Yves Giudicelli *, René Pecquery

Service de Biochimie et Biologie Moléculaire, UPRES-EA 2493, Faculté de Médecine Paris-Ile de France Ouest, Université Versailles-St Quentin, Centre Hospitalier de Poissy, 78303 Poissy Cedex, France

Received 14 April 2006
Available online 27 April 2006

Abstract

It is well established that obesity is a risk factor for breast cancer and that blood levels of adiponectin, a hormone mainly secreted by white adipocytes, are inversely correlated with the body fat mass. As adiponectin elicits anti-proliferative effects in some cell types, we tested the hypothesis that adiponectin could influence human breast cancer MCF-7 cell growth. Here we show that MCF-7 cells express adiponectin receptors and respond to human recombinant adiponectin by reducing their growth, AMPkinase activation, and p42/p44 MAPkinase inactivation. Further, we demonstrate that the anti-proliferative effect of adiponectin involves activation of cell apoptosis and inhibition of cell cycle. These findings suggest that adiponectin could act in vivo as a paracrine/endocrine growth inhibitor towards mammary epithelial cells. Moreover, adipose adiponectin production being strongly reduced in obesity, this study may help to explain why obesity is a risk factor of developing breast cancers.

Keywords: Adiponectin; Adiponectin receptors; MCF-7 proliferation; Signal transduction

Obesity is a serious health problem, as it is not only associated with a variety of metabolic disorders but also with an increased risk of developing cancer. Various epidemiological studies documented that obesity is a risk factor for postmenopausal breast cancer [1,2]. Moreover, in normal weighed women, the frequency of breast cancer increases with menopause, while, at the same time, important cellular rehandlings occur within the mammary gland with progressive reduction of the glandular tissue and increase in the surrounding fat mass. However, the cellular and molecular mechanisms that could establish a link between obesity and breast cancer are poorly understood. Among these, higher circulating estrogen levels that have been attributed to elevated aromatase activity in expanded adipose tissue depots have been considered as a potential contributing factor. Besides estrogens however, adipose tissue secretes other products such as TNFα, interleukins, leptin, and adiponectin [3]. Recently, it was shown that leptin stimulates the mammary epithelial cell proliferation [4–6].

Adiponectin, a hormone mainly produced in adipose tissue, is abundantly present in human plasma and has been described as an insulin-sensitizing adipocytokine [7,8]. However, unlike TNFα and leptin, circulating adiponectin levels are strongly decreased in obese individuals [9,10].

Several studies suggest that adiponectin may influence cancer pathogenesis. As a matter of fact, circulating adiponectin levels have been reported to be inversely associated with an increased risk of breast [11,12] and endometrial [13,14] cancer. Furthermore, adiponectin was also shown to control cell number by inhibiting cell proliferation and by inducing apoptosis of leukemia and endothelial cells

☆ *Abbreviations:* MAPkinase, mitogen-activated protein kinase; AMPkinase, 5'-AMP-activated protein kinase; RT-PCR, reverse transcription-polymerase chain reaction; FCS, fetal calf serum; TNFα, tumor necrosis factor α.

* Corresponding author. Fax: +33 1 39 27 44 20.
E-mail address: biochip@wanadoo.fr (Y. Giudicelli).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.04.076

[15,16]. The latter observations have led us to investigate the hypothesis that adiponectin may play a negative role in the development and progression of breast cancer by altering cell proliferation and/or apoptosis processes. Cell proliferation is tightly controlled at the nuclear level by transcriptional factors like the protooncogene c-myc which stimulates, through cyclin D1 activation, the G1-S phase transition of the cell cycle [17]. Otherwise, apoptosis, which is characterized by several morphological features such as nuclei fragmentation, is regulated by the expression of a number of specific genes. In particular, Bcl2 is known to protect cells from apoptosis and conversely Bax and p53 expressions accelerate cell death [18]. In the present study, we have thus tested the influence of human recombinant adiponectin, in vitro, on the human breast cancer MCF-7 cells, by following cell density variations and the expression of some mitogenic genes as well as of key pro- and anti-apoptotic genes. Biological effects of adiponectin are initiated by the two transmembranous adiponectin receptor subtypes, AdipoR1 and AdipoR2, inducing the activation of protein kinases, mainly the AMPkinase and the MAPkinase [19]. After having characterized the presence of functional AdipoR1 and AdipoR2 receptors in MCF-7 cells, we clearly demonstrate that adiponectin inhibits proliferation and induces apoptosis in these breast cancer cells.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, Hepes, leupeptin, aprotinin, phenylsulfonylfluoride (PMSF), 5-aminoimidazole-4-carboxamide 1β-D-ribofuranosyl-5′-monophosphate (AICAR) and bovine serum albumine (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagenase and "in situ cell death detection KIT fluorescein" were from Roche Molecular Biochemicals (Mannheim, Germany). Recombinant human adiponectin was provided by R&D Systems Europe Ltd (Abingdon, UK), Superscript II Rnase H-RT by Gibco BRL (Grand Island, NY, USA), and RNA guard by Pharmacia Biotechnology (Uppsala, Sweden). Origins of the different antibodies used are described in the following paragraphs.

Cell culture. The human breast cancer cells, MCF-7, were obtained from European Collection of Cell Cultures (Salisbury, UK). These cells were maintained routinely in phenol-red-free DMEM with Hepes (20 mM), 10% fetal calf serum (FCS), streptomycin (0.1 mg/ml), and penicillin (100 U/ml) at 37 °C under 5% CO_2 atmosphere. After 48 h, medium was removed and replaced by a phenol-red free DMEM supplemented with 8% charcoal-stripped FCS until starting assays.

[^{3}H]Thymidine incorporation. MCF-7 cells were propagated in 24-well plates in DMEM supplemented with 8% charcoal-stripped FCS. During the exponential phase of growing, cells were exposed to various concentrations of human recombinant adiponectin for 24 h. For the 6 last hours, [^{3}H]thymidine (1 mCi/ml) was added to the culture medium. After washing three times with saline, cells were lysed during 5 min with 1% SDS and treated with 10% trichloroacetic acid for 45 min at 4 °C. After filtration on GF/C filters (Whatman, Clifton, NY), radioactivity was counted.

Apoptosis assay. After 48 h in DMEM supplemented with 8% charcoal-stripped FCS, cells were cultured in the presence of various concentrations of adiponectin (25 ng/ml and 250 ng/ml) during 96 h. The validity of our experimental culture conditions was warranted by the well-established pro-apoptotic response of MCF-7 cells to camptothecin (5.7 μM) or to tamoxifen (7 μM) [20,21].

Attached cells were harvested by trypsinization, combined with floating cells, and suspended in PBS at a density of 10^6 cells/ml. Then, cells were fixed in 70% ethanol at −20 °C overnight and washed twice with PBS. Cells were labeled for DNA fragmentation by TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) according to the instructions provided by the manufacturer. Apoptotic index was calculated after counting a minimum of 5000 events by flow cytometry using an EPICS flow cytometer (Coulter Electronics, Miami, USA).

MAPkinase and AMPkinase activation. Cells were maintained in a serum-free medium overnight for AMPkinase studies or maintained in the presence of 8% charcoal-stripped FCS for MAPkinase. Then, cells were exposed during 2, 5, 15, and 30 min to human recombinant adiponectin (25 ng/ml) or 5 min to 10% FCS or 30 min to AICAR (500 μM). Thereafter, cells were scrapped and sonicated on ice in buffer containing 50 mM Tris, 120 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.5 mM desoxycholate, 0.1% sodium dodecyl sulfate, 1 mM sodium vanadate, 0.57 mM PMSF, 30 mM β-glycerophosphate, 5 μg/ml aprotinin and 12.5 μg/ml leupeptin. After centrifugation at 100,000*g* for 10 min at 4 °C, supernatants were diluted in Laemmli's buffer (vol/vol). Equal amounts (10 μg) of cellular extracts were subjected to SDS–PAGE (12.5%). Proteins were transferred to PVDF membrane and blocked in buffer A with 2.5% gelatin during 2 h. Then membranes were incubated overnight at room temperature with rabbit polyclonal anti-phosphorylated p42/p44 MAPkinase antibody (1:7000 dilution, Promega, Charbonnières, France) or with mouse monoclonal anti-total ERK (pan-ERK) antibody (1:500 dilution, Transduction Laboratories, Lexington, KY, USA) or with rabbit polyclonal anti-phospho-AMPK-α antibody (1:700 dilution, Cell Signaling Technology, St. Quentin Yvelines, France) or with rabbit polyclonal anti-AMPK-α antibody (1:1000 dilution, Cell Signaling Technology, St. Quentin Yvelines, France). Finally, an enhanced chemiluminescence kit from Pierce (Interchim) was used for signal detection. Control experiments with various protein amounts (10–100 μg) were performed to ensure that the densitometric signal intensity was proportional to the loaded amount of protein.

AdipoR1 protein expression. At confluence, MCF7 cells were scraped and sonicated in cold buffer containing 10 mM Tris (pH 7.4), 0.25 M sucrose, 5 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 25 μg/ml aprotinin, and 105 μM leupeptin. After centrifugation at 21,000*g* for 20 min at +4 °C, the pellet was resuspended and denatured with Laemmli buffer (vol/vol) and stored at −20 °C. Membrane extracts (50–100 μg) were resolved by SDS–PAGE (7.5% acrylamide). Proteins were transferred to PVDF membrane and blocked in buffer A with 2.5% gelatin during 2 h. Then membranes were incubated overnight at room temperature with rabbit polyclonal anti-AdipoR1 antibody (1:600 dilution, Phoenix Pharmaceuticals, Belmont, CA) in buffer A with 2.5% gelatin. Incubation with the secondary antiserum and signal detection were performed as described above.

Quantitative RT-PCR. Total RNA (0.5 μg) was reverse transcribed as previously described [22]. Quantitative PCR was performed using a LightCycler® instrument (Roche Diagnostics). Primer sets used are indicated in Table 1. cDNA calibrators were prepared by PCR amplification run to saturation (35 cycles) with the appropriate primers. The resulting cDNAs were purified by QIAquick PCR purification Kit (Qiagen). The samples showed a unique band in agarose electrophoresis. Numbers of cDNA copies were calculated from the absorbance at 260 nm. Calibrators were defined to contain arbitrary units of p53, Bax, Bcl2, cmyc, cyclin D1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, and all calculated p53, Bax, Bcl2, c-myc, and cyclin D1 mRNA concentrations are relative to GAPDH mRNA concentrations. Separate calibration curves for human p53, Bax, Bcl2, c-myc, cyclin D1, and GAPDH were constructed from serial dilutions from 10^8 copies to 100 copies of cDNA calibrators. Calibration curves were log-linear over the quantification range with correlation coefficient (r^2) Σ0.99 and slopes ranging from −3.5 to −3.8. The intra-assay variability of duplicate crossing point (Cp) values never exceeded 0.2 cycle and the interassay variability (CV value) ranged from 1% to 5% CV values for the three or four runs of each transcript.

Real-time PCR was performed in a total reaction volume of 20 μl per capillary for the LightCycler format. Each provided cDNA preparation

Table 1
List of primers used for PCR

Primer sets	Sequence	PCR product (bp)	Reference
p53			
Sense	5′-ACT AAG CGA GCA CTG CCC AA-3′	231	None
Antisense	5′-ATG GCG GGA GGT AGA CTG AC-3′		
Bax			
Sense	5′-CAA ACT GGT GCT CAA GGC C-3′	188	None
Antisense	5′-GCA CTC CCG CCA CAA AGA T-3′		
Bcl2			
Sense	5′-ATG TGT GTG GAG AGC GTC AAC C-3′	196	None
Antisens	5′-TGA GCA GAG TCT TCA GAG ACA GCC-3′		
GAPDH			
Sense	5′-ACC CAC TCC TCC ACC TTT G-3′	178	[46]
Antisense	5′-CTC TTG TGC TCT TGC TGG G-3′		
c-Myc			
Sense	5′-GAC GCG GGG AGG CTA TTC TG-3′	236	[47]
Antisense	5′-GAC TCG TAG AAA TAC GGC TGC ACC GAG TC-3′		
CyclinD1			
Sense	5′-CCT CCT CCT CGC ACT TCT GT-3′	69	None
Antisense	5′-CCG TCC ATG CGG AAG ATC-3′		
AdipoR1			
Sense	5′-TTC TTC CTC ATG GCT GTG ATG T-3′	71	[19]
Antisense	5′-AAG AAG CGC TCA GGA ATT CG-3′		
AdipoR2			
Sense	5′-ATA GGG CAG ATA GGC TGG TTG A-3′	76	[19]
Antisense	5′-GGA TCC GGG CAG CAT ACA-3′		
TBP			
Sense	5′-TGC ACA GGA GCC AAG AGT GAA-3′	132	[48]
Antisense	5′-CAC ATC ACA GCT CCT CAC CA-3′		

(50 ng/μl) was diluted 1:10 in water. The reaction buffer contained 10 μl of 2X QuantiTect SYBR Green PCR Master Mix (Quiagen) (including HotStar *Taq* DNA polymerase, reaction buffer, desoxynucleotide triphosphate mixture and SYBR Green I), 0.5 μM of each primer and 4 μl of diluted cDNA or calibrator. To verify that fluorescence generated by SYBR green incorporation into double strand DNA was not over-estimated by contaminations resulting from residual genomic DNA amplification and/or from primer dimer formation, controls without reverse transcriptase and without DNA template or reverse transcriptase were included in each experiment.

After PCR, a melting curve was constructed by increasing the temperature from 65 °C to 95 °C with a transition rate of 0.1 °C/s to verify the specificity of the desired PCR products and the absence of primer–dimers. To validate the melting curve results, representative samples of PCR products were separated by 2% agarose gel electrophoresis.

The Second Derivative Maximum Method was used to automatically determine the Cp for the individual samples. For each sample, ΔCp values were determined (Cp of the target gene minus Cp of the GAPDH gene). Fold changes in expression were calculated according to the transformation: fold increase $= 2^{-\text{difference in } \Delta Cp}$.

AdipoR1–R2 and TATA binding protein (TBP) (used as internal standard) PCR products were analyzed on a 2% agarose gel in 90 ml Tris–borate, 2 mM EDTA buffer (TBE), pH 8, and visualized by staining with ethidium bromide and ultraviolet transillumination.

Protein concentration was measured according to Bradford [23] with BSA as standard.

Statistical analysis. All values were expressed as means ± SEM of four to eight separate experiments and statistical analysis was performed using the nonparametric paired Wilcoxon test.

Results

1-Adiponectin receptor expression in MCF-7 cells

Using two different primer sets in RT-PCR analysis, we found that adiponectin receptor AdipoR1 and AdipoR2 mRNAs are expressed in MCF-7 cells as they are in human preadipocytes and mature adipocytes shown as controls (Fig. 1A). The result of RT-PCR showed a 71 bp fragment specific to the AdipoR1 and a 76 bp fragment specific to the AdipoR2.

Interestingly, two other human breast cancer cell lines, namely the MDA-MB-231 and the T47-D cells, also express both AdipoR mRNAs (Fig. 1A).

Western blot analysis confirmed the presence of AdipoR1 in MCF7 cells as well as in mature adipocytes (Fig. 1B).

2-Modulation of AMPkinase and MAPkinase pathways by adiponectin

As the phosphorylation-dependent activation of AMPkinase is the major transduction pathway reported for adiponectin signaling [19], expression of phosphorylated AMPkinase was investigated in cellular extracts

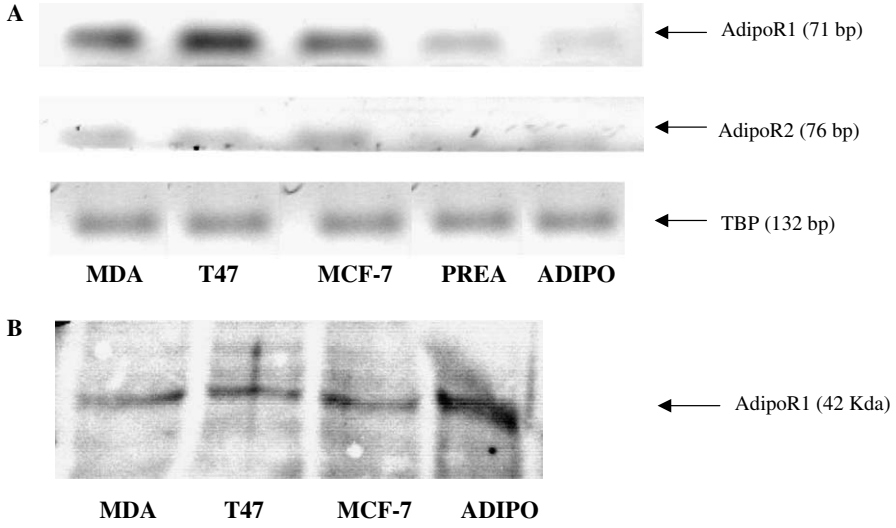

Fig. 1. (A) AdipoR1 and AdipoR2 mRNA expressions in MCF-7 cells. Total RNA was extracted from MCF-7, T47D, MDA-MB-231 cells, human adipocytes and preadipocytes, and analyzed by RT-PCR with the primers as described in Table 1. PCR products were separated by 2% agarose gel electrophoresis. This figure shows one RT-PCR representative of three separate experiments. (B) AdipoR1 protein expression in MCF-7 cells. Cell lysates (100 μg) were subjected to Western blot analysis using anti-AdipoR1 antibody as described under Materials and methods. One experiment representative of three is shown.

from MCF-7 cells immediately after their in vitro exposure to adiponectin. Data in Fig. 2A show the kinetics of AMPkinase activation resulting from adiponectin (25 ng/ml) exposure. This dosage was chosen because dose–response experiments (0.25–2500 ng/ml) revealed that 25 ng/ml was the lowest adiponectin concentration giving the maximal AMPkinase activation and MAPkinase inactivation (data not shown). As can be seen, MCF-7 cell exposure to adiponectin induced a rapid increase in the phosphorylated AMPkinase form. This activation was maximal after 2 min and then started to decrease after 30 min exposure to adiponectin. It is important to notice that the maximal effect of adiponectin was of similar magnitude as that found after MCF-7 cell exposure to AICAR (500 μg/ml). Stripping of the immunoblots and reprobing with anti-AMPkinase antibody confirmed the presence of equal amounts of AMPkinase in each lane (Fig. 2A).

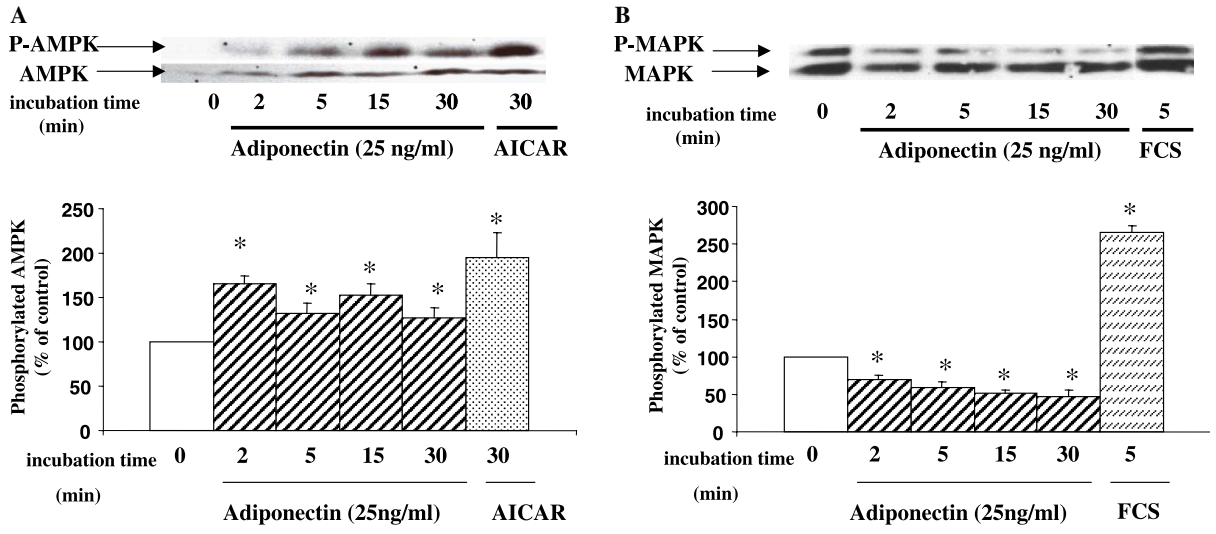

Fig. 2. Phosphorylation of AMPkinase and MAPkinase by adiponectin in MCF-7 cells. Cells were maintained in a serum-free culture medium overnight (for AMPkinase studies) or maintained in 8% charcoal-stripped FCS (for MAPkinase studies). Then cells were exposed to human recombinant adiponectin (25 ng/ml) or 10% FCS or AICAR (500 μg/ml). At the indicated times, cellular extracts were prepared and immunoblotted with either anti-p42/p44MAPkinase active or anti-ERK (pan-ERK) antibodies or anti p-AMPkinase or anti-total AMPkinase. (A) Western-blot analysis from one representative experiment and densitometric analysis of AMPkinase immunoblots. (B) Western-blot analysis from one representative experiment and densitometric analysis of MAPkinase immunoblots. Values are means ± SEM obtained from four to six separate experiments and are expressed as percentages of control value without adiponectin (0). *$p < 0.05$, Wilcoxon test.

Previous reports have shown that adiponectin binding to AdipoR also leads to the phosphorylation-dependent activation of the MAPkinase pathway [9,19,24]. Therefore, the phosphorylated form of p42/p44 MAPkinase was investigated in cytosolic extracts of MCF-7 cells following their in vitro exposure to adiponectin or 10% FCS in the culture medium. As shown in Fig. 2B, exposure to adiponectin (25 ng/ml) induced a time-dependent decrease in the phosphorylated p42/p44 MAPkinase isoforms starting after 2 min and reaching maximal reduction (−50%) after 30 min.

3-Cell proliferation

Cell proliferation was studied by measuring changes in the rate of DNA synthesis ([^{3}H]thymidine incorporation) and in the cell number. As shown in Fig. 3, adiponectin, in vitro, decreased [^{3}H]thymidine incorporation (−40%) whatever the concentrations tested (25–250 ng/ml). For the following experiments, we have thus decided to use the intermediate adiponectin concentration of 25 ng/ml. Used as a control, 100 nM 17β-estradiol induced a 40% increase in [^{3}H]thymidine incorporation. Importantly, when MCF-7 cells were exposed to both adiponectin and 17β-estradiol, the positive effect of 17β-estradiol "per se" on [^{3}H]thymidine incorporation was completely suppressed. The adiponectin-inhibited proliferation of MCF-7 cells was also confirmed by direct cell counting (×0.645 ± 0.03).

Breast cancer cell proliferation is the result of the balance between cell division and cell apoptosis. To gain more informations about the molecular basis underlying the adiponectin-inhibited cell proliferation, expressions of some cell cycle and cell apoptosis key regulatory genes were studied in MCF-7 cells.

4-Expression of c-myc and cyclin D1

Two critical mediators of cell cycle activation, the transcription factor c-Myc and cyclin D1, represent points of convergence in the action of mitogenic agents in MCF-7 cells [25]. Therefore, we have measured by RT-PCR the influence of adiponectin on c-myc and cyclin D1 expressions in MCF-7 cells. As shown in Fig. 4, adiponectin (25 ng/ml) decreased by half c-myc and by 30% cyclin D1 mRNA expressions.

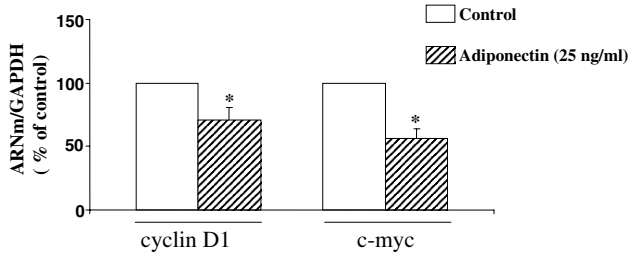

Fig. 4. Effects of adiponectin on cyclin D1 and c-myc expressions. MCF-7 cells were incubated during 2 h or 6 h in the presence of adiponectin (25 ng/ml) for c-myc and cyclin D1 expression, respectively. Total RNA was extracted from these cells and analyzed by RT-PCR with the primers described in Table 1. Results are means ± SEM of eight experiments and are expressed as percentage of control (without adiponectin). *$p < 0.05$, Wilcoxon test.

5-Cell apoptosis

To test the influence of adiponectin on cell apoptosis, we have at first used the TUNEL assay which allows to determine the percentage of labeled apoptotic nuclei. As shown in Fig. 5, adiponectin had to be raised to the concentration of 250 ng/ml to induce a significant apoptosis in MCF-7 cells (+39%) after four days exposure. Under the same conditions, MCF-7 cell exposure to camptothecin (apoptotic agent) and tamoxifen (an antagonist of estrogen receptor), used as positive controls, increased apoptosis by 60% and 90%, respectively.

To further investigate the apoptotic action of adiponectin, we used quantitative RT-PCR to study the influence of adiponectin on Bcl2, Bax, and p53 mRNA expressions.

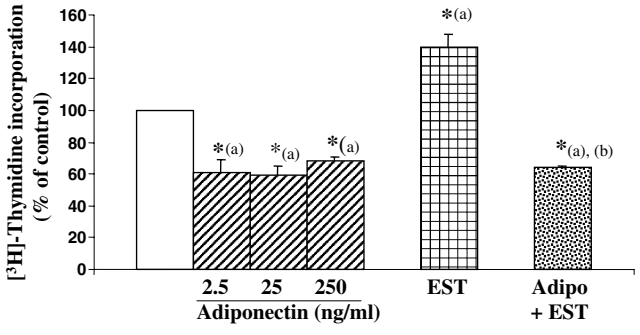

Fig. 3. Effects of adiponectin on DNA synthesis. Cells were exposed to 2.5, 25 or 250 ng/ml human recombinant adiponectin or to 100 nM 17β-estradiol with or without 25 ng/ml adiponectin in the presence of [^{3}H]thymidine as described under Materials and methods. Results are means ± SEM of five to eight experiments and are normalized as percentages of the control value (without adiponectin) (a) versus control, (b) adiponectin + 17β-estradiol versus 17β-estradiol. *$p < 0.05$, Wilcoxon test.

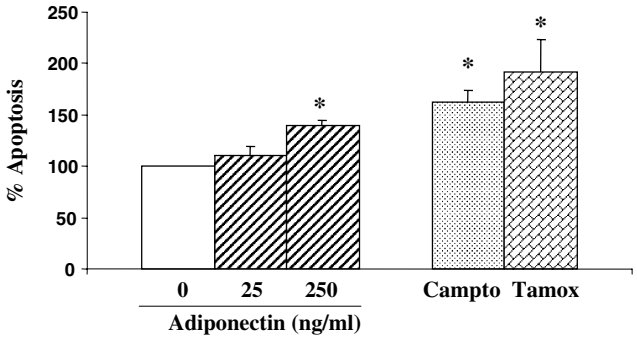

Fig. 5. Effects of adiponectin on DNA fragmentation. MCF-7 cells were cultured for four days in the presence of adiponectin (25 ng/ml or 250 ng/ml) or camptothecin (5.7 μM) or tamoxifen (7 μM). Cells were then analyzed by TUNEL staining and flow cytometry. Results are expressed as a percentage of control (without adiponectin). Each bar represents means ± SEM of four separate experiment. *$p < 0.05$, Wilcoxon test.

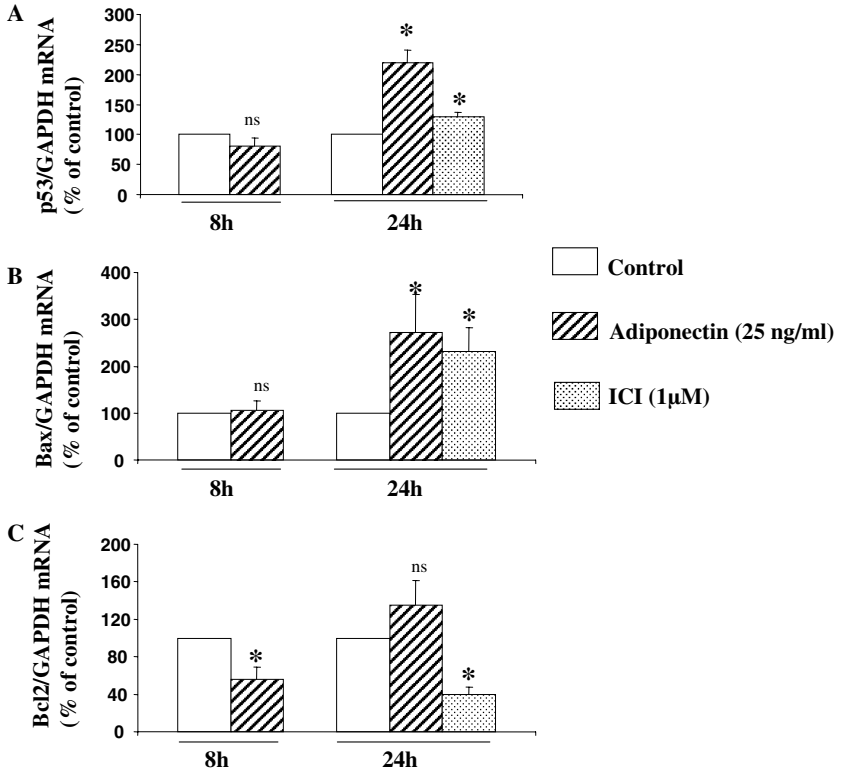

Fig. 6. Effects of adiponectin on p53 (A), Bax (B), and Bcl2 (C) mRNA expressions. MCF-7 cells were cultured for 8 h or 24 h in the presence of adiponectin (25 ng/ml) or ICI182780 (1 μM). Total RNA was extracted from these cells and analyzed by RT-PCR with the primers described in Table 1. Results are means ± SEM of five to eight experiments and are expressed as percentage of control (without adiponectin). ns, non-significant, $*p < 0.05$, Wilcoxon test.

As shown in Fig. 6, adiponectin (25 ng/ml) stimulated the expression of the apoptotic genes p53 and Bax (×2.2 ± 0.2 and ×2.73 ± 0.8, respectively) after 24 h exposure. However, a shorter incubation time (8 h) with adiponectin had no influence on p53 and Bax mRNA expressions. Under the same conditions (24 h) and for comparison, ICI 182780 (a specific antagonist of estrogen receptor) used as a positive control enhanced p53 (×1.3 ± 0.075) and Bax (×2.31 ± 0.52) mRNA expressions. Expression of the anti-apoptotic gene Bcl2 was also a target for adiponectin as after 8 h exposure, a 40% reduction of Bcl2 mRNA expression was observed. This effect, however, was only transient, since after 24 h, it was no longer seen, in contrast with ICI 182780 which still induced 60% inhibition of Bcl2 expression.

Discussion

Several studies have identified obesity as a risk factor for breast cancer in postmenopausal women [1,2]. Until now, the pathogenesis of the relationship between obesity and breast cancer has not been clearly delineated. Higher levels of estrogens, insulin/IGF-1, and leptin are associated with obesity and have been considered as potential contributing growth factors leading to the development of breast cancer [2,26–28]. Serum adiponectin levels are negatively related to obesity [9,10]. In addition to its function as a metabolic hormone, there are growing evidences suggesting that adiponectin plays a regulatory role in cell growth, angiogenesis, and tissue remodeling. Particularly, adiponectin has been shown to inhibit proliferation of aortic smooth muscle [9], hepatic [29], endothelial [16], and prostate cancer [30] cells.

In this study, we identified adiponectin as a novel growth inhibitor of MCF7 breast cancer cells. At first, we have shown that these cells express both adiponectin receptors (AdipoR1 and AdipoR2). At second, these adiponectin receptors are functional since we observed both AMPkinase activation and MAPkinase inhibition following MCF-7 cell exposure to adiponectin concentrations (25–250 ng/ml) much lower than those found in normal human blood [9].The human recombinant adiponectin used in the present study contains mainly the high molecular weight (HMW) adiponectin form [30] previously reported to specifically activate the AMPKinase signaling cascade [31]. Moreover, like in endothelial and osteoblast cells [16,32] and in prostate and colonic epithelial cancer cells [30,33], we found that human recombinant adiponectin reduces MCF-7 cell growth at subphysiological concentrations. Cancer cell proliferation is the result of the balance between cell division and cell apoptosis. In order to explain the inhibitory effect of adiponectin on MCF-7 cell proliferation, we have tested the hypothesis that the expression of some specific cell cycle and apoptotic genes could be under the control of adiponectin.

Estrogens, which are major mitogens for breast cancer cells, induce G1-S phase cell cycle progression through increased expression of c-myc and cyclin D1 genes [17]. In our study, we have shown that adiponectin represses c-myc and cyclin D1 mRNA expressions which strongly suggests that the inhibitory effect of this hormone on MCF-7 cell growth is due to a direct blockade of G1-S phase cell cycle progression. In addition, we have observed that a simultaneous exposure to adiponectin and 17β-estradiol leads to suppress the mitogenic effect of 17β-estradiol on MCF-7 cells. The latter finding provides an additional argument to support the involvement of c-myc and cyclin D1 repressions in the antimitogenic action of adiponectin on MCF-7 cells.

As previously reported in other cell types [15,16,29,34], we have also observed increased MCF-7 cell DNA fragmentation after prolonged exposure to adiponectin. This effect was preceded by changes in the expression profile of some pro- and anti-apoptotic genes: the pro-apoptotic Bax and p53 mRNAs were increased while the anti-apoptotic Bcl2 mRNA was reduced.

Adiponectin, via its specific receptors, mediates several intracellular signaling pathways such as AMPkinase [19], JAK/STAT3 [35] and MAPkinase (p38 MAPK, ERK, JNK) [19,24]. In MCF-7 cells, we have shown that adiponectin stimulates AMPkinase pathway which has recently been implicated in the negative control of cell cycle progression [24,36–38]. Moreover, in various cell types [39–41], AMPkinase activation was reported to induce pro-apoptotic responses. These observations together with our present results strongly suggest that both the antiproliferative and pro-apoptotic effects of adiponectin that we have observed in MCF-7 cells are mediated via AMPkinase activation.

AMPkinase activation is probably not the unique mechanism explaining the influence of adiponectin on MCF-7 cell proliferation and apoptosis. As a matter of fact, inhibition of the MAPkinase signaling pathway is associated with a decrease of cell proliferation but also with increased apoptosis in human osteoblast [24]. Moreover, inhibition of MAPkinase phosphorylation by a specific inhibitor, PD98059, was reported to stimulate p53 and Bax expressions in MCF-7 cells [42]. In our study, we have demonstrated that adiponectin induces also an inhibition of MAPkinase pathway which could thus be involved in both the inhibition of cell proliferation and the increase in apoptosis. To gain further insight into the mechanisms of MCF-7 cell proliferation inhibition by adiponectin, knock-down of AdipoR1, AdipoR2, AMPkinase and MAPkinase expressions with siRNA are currently in progress in our laboratory. This experimental approach has been prefered because our preliminary studies with different specific inhibitors of the intracellular AMPkinase and MAPkinase pathways were not compatible with MCF-7 cell survival.

Another mechanism whereby adiponectin could directly or indirectly modulate cell proliferation is a reduction in the bio-availability of some growth factors. In fact, in human aortic vascular smooth muscle cells, two recent studies reported that the anti-proliferative effect of adiponectin could be explained, at least in part, by specific interactions of adiponectin with several growth factors (PDGF, FGF, and EGF) [9,43]. These interactions preclude growth factor binding to their respective membrane receptors thereby attenuating cell proliferation and occur with or without post-receptor modifications.

In addition to adiponectin, leptin is another adipose-derived endocrine factor which could also play a significant role in breast cancer development. In vitro, leptin has been shown to stimulate proliferation of different human breast cancer cell lines [4,5]. In the breast, leptin is produced by epithelial and tumor cells as well as by the surrounding adipocytes. This adipokine enhances the cell proliferation directly through their specific receptors or indirectly by increasing local estrogen production through aromatase stimulation [44]. Two recent studies have demonstrated that an increased expression of leptin and leptin receptors was related to breast cancer progression [28,45]. The present experiments delineate an inverse picture for adiponectin which, being produced only by adipocytes, elicits antimitogenic effects towards MCF-7 cells. Thus, adiponectin and leptin with their specific receptors have opposite but significant effects in the control of breast cancer cell proliferation and they could represent new markers to evaluate the risk and/or the progression to develop a breast cancer.

In conclusion, our results, together with the previously reported proliferative action of leptin, (i) strengthen the role of mammary adipose cells and of adipose cells in general as a producer of signals modulating the growth of mammary epithelial cells and (ii) contribute to explain why the obese state characterized by high leptin and low adiponectin productions is an important risk factor of breast cancer.

Acknowledgments

We are grateful to V. Rincheval (Université de Versailles/St. Quentin-en-Yvelines, CNRS FRE-2445, Laboratoire de Génétique et Biologie cellulaire) for assistance in flow cytometry analysis.

References

[1] R. Ballard-Barbash, A. Schatzkin, C.L. Carter, W.B. Kannel, B.E. Kreger, D.A. RB, G.L. Splansky, K.M. Anderson, W.E. Helsel, Body fat distribution and breast cancer in the Framingham Study, J. Natl. Cancer Inst. 82 (1990) 286–290.

[2] G.D. Stephenson, D.P. Rose, Breast cancer and obesity: an update, Nutr. Cancer 45 (2003) 1–16.

[3] R.S. Ahima, J.S. Flier, Adipose tissue as an endocrine organ, Trends Endocrinol. Metab. 11 (2000) 327–332.

[4] K. Laud, I. Gourdou, L. Pessemesse, J.P. Peyrat, J. Djiane, Identification of leptin receptors in human breast cancer: functional activity in the T47-D breast cancer cell line, Mol. Cell. Endocrinol. 188 (2002) 219–226.

[5] M.N. Dieudonne, F. Machinal-Quelin, V. Serazin-Leroy, M.C. Leneveu, R. Pecquery, Y. Giudicelli, Leptin mediates a proliferative

response in human MCF7 breast cancer cells, Biochem. Biophys. Res. Commun. 293 (2002) 622–628.
[6] X. Hu, S.C. Juneja, N.J. Maihle, M.P. Cleary, Leptin–a growth factor in normal and malignant breast cells and for normal mammary gland development, J. Natl. Cancer Inst. 94 (2002) 1704–1711.
[7] A.H. Berg, T.P. Combs, X. Du, M. Brownlee, P.E. Scherer, The adipocyte-secreted protein Acrp30 enhances hepatic insulin action, Nat. Med. 7 (2001) 947–953.
[8] T. Yamauchi, J. Kamon, H. Waki, Y. Terauchi, N. Kubota, K. Hara, Y. Mori, T. Ide, K. Murakami, N. TsuboyamaKasaoka, O. Ezaki, Y. Akanuma, O. Gavrilova, C. Vinson, M.L. Reitman, H. Kagechika, K. Shudo, M. Yoda, Y. Nakano, K. Tobe, R. Nagai, S. Kimura, M. Tomita, P. Froguel, T. Kadowaki, The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity, Nat. Med. 7 (2001) 941–946.
[9] Y. Arita, S. Kihara, N. Ouchi, M. Takahashi, K. Maeda, J. Miyagawa, K. Hotta, I. Shimomura, T. Nakamura, K. Miyaoka, H. Kuriyama, M. Nishida, S. Yamashita, K. Okubo, K. Matsubara, M. Muraguchi, Y. Ohmoto, T. Funahashi, Y. Matsuzawa, Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity, Biochem. Biophys. Res. Commun. 257 (1999) 79–83.
[10] C. Weyer, T. Funahashi, S. Tanaka, K. Hotta, Y. Matsuzawa, R.E. Pratley, P.A. Tataranni, Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia, J. Clin. Endocrinol. Metab. 86 (2001) 1930–1935.
[11] C. Mantzoros, E. Petridou, N. Dessypris, C. Chavelas, M. Dalamaga, D.M. Alexe, Y. Papadiamantis, C. Markopoulos, E. Spanos, G. Chrousos, D. Trichopoulos, Adiponectin and breast cancer risk, J. Clin. Endocrinol. Metab. 89 (2004) 1102–1107.
[12] Y. Miyoshi, T. Funahashi, S. Kihara, T. Taguchi, Y. Tamaki, Y. Matsuzawa, S. Noguchi, Association of serum adiponectin levels with breast cancer risk, Clin. Cancer Res. 9 (2003) 5699–5704.
[13] E. Petridou, C. Mantzoros, N. Dessypris, P. Koukoulomatis, C. Addy, Z. Voulgaris, G. Chrousos, D. Trichopoulos, Plasma adiponectin concentrations in relation to endometrial cancer: a case-control study in Greece, J. Clin. Endocrinol. Metab. 88 (2003) 993–997.
[14] L. Dal Maso, L.S. Augustin, A. Karalis, R. Talamini, S. Franceschi, D. Trichopoulos, C.S. Mantzoros, C. La Vecchia, Circulating adiponectin and endometrial cancer risk, J. Clin. Endocrinol. Metab. 89 (2004) 1160–1163.
[15] T. Yokota, K. Oritani, I. Takahashi, J. Ishikawa, A. Matsuyama, N. Ouchi, S. Kihara, T. Funahashi, A.J. Tenner, Y. Tomiyama, Y. Matsuzawa, Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages, Blood 96 (2000) 1723–1732.
[16] E. Brakenhielm, N. Veitonmaki, R. Cao, S. Kihara, Y. Matsuzawa, B. Zhivotovsky, T. Funahashi, Y. Cao, Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis, Proc. Natl. Acad. Sci. USA 11 (2004) 1263–1269.
[17] O.W. Prall, E.M. Rogan, E.A. Musgrove, C.K. Watts, R.L. Sutherland, c-Myc or cyclin D1 mimics estrogen effects on cyclin E-Cdk2 activation and cell cycle reentry, Mol. Cell. Biol. 18 (1998) 4499–4508.
[18] J.S. Fridman, S.W. Lowe, Control of apoptosis by p53, Oncogene 22 (2003) 9030–9040.
[19] T. Yamauchi, J. Kamon, Y. Ito, A. Tsuchida, T. Yokomizo, S. Kita, T. Sugiyama, M. Miyagishi, K. Hara, M. Tsunoda, K. Murakami, T. Ohteki, S. Uchida, S. Takekawa, H. Waki, N.H. Tsuno, Y. Shibata, Y. Terauchi, P. Froguel, K. Tobe, S. Koyasu, K. Taira, T. Kitamura, T. Shimizu, R. Nagai, T. Kadowaki, Cloning of adiponectin receptors that mediate antidiabetic metabolic effects, Nature 423 (2003) 762–769.
[20] P. Diel, K. Smolnikar, H. Michna, The pure antiestrogen ICI 182780 is more effective in the induction of apoptosis and down regulation of BCL-2 than tamoxifen in MCF-7 cells, Breast Cancer Res. Treat. 58 (1999) 87–97.
[21] M. Gorka, M.M. Godlewski, B. Gajkowska, U. Wojewodzka, T. Motyl, Kinetics of Smac/DIABLO release from mitochondria during apoptosis of MCF-7 breast cancer cells, Cell Biol. Int. 28 (2004) 741–754.
[22] F. Machinal-Quelin, M.N. Dieudonne, M.C. Leneveu, R. Pecquery, Y. Giudicelli, Proadipogenic effect of leptin on rat preadipocytes in vitro: activation of MAPK and STAT3 signaling pathways, Am. J. Physiol. Cell Physiol. 282 (2002) C853–C863.
[23] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[24] X.H. Luo, L.J. Guo, L.Q. Yuan, H. Xie, H.D. Zhou, X.P. Wu, E.Y. Liao, Adiponectin stimulates human osteoblasts proliferation and differentiation via the MAPK signaling pathway, Exp. Cell Res. 309 (2005) 99–109.
[25] A. Mawson, A. Lai, J.S. Carroll, C.M. Sergio, C.J. Mitchell, B. Sarcevic, Estrogen and insulin/IGF-1 cooperatively stimulate cell cycle progression in MCF-7 breast cancer cells through differential regulation of c-Myc and cyclin D1, Mol. Cell. Endocrinol. 229 (2005) 161–173.
[26] P.F. Bruning, J.M. Bonfrer, P.A. van Noord, A.A. Hart, M. de Jong-Bakker, W.J. Nooijen, Insulin resistance and breast-cancer risk, Int. J. Cancer 52 (1992) 511–516.
[27] E.E. Calle, R. Kaaks, Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms, Nat. Rev. Cancer 4 (2004) 579–591.
[28] C. Garofalo, E. Surmacz, Leptin and cancer, J. Cell. Physiol. 207 (2006) 12–22.
[29] X. Ding, N.K. Saxena, S. Lin, A. Xu, S. Srinivasan, F.A. Anania, The roles of leptin and adiponectin: a novel paradigm in adipocytokine regulation of liver fibrosis and stellate cell biology, Am. J. Pathol. 166 (2005) 1655–1669.
[30] J.D. Bub, T. Miyazaki, Y. Iwamoto, Adiponectin as a growth inhibitor in prostate cancer cells, Biochem. Biophys. Res. Commun. 340 (2006) 1158–1166.
[31] H. Kobayashi, N. Ouchi, S. Kihara, K. Walsh, M. Kumada, Y. Abe, T. Funahashi, Y. Matsuzawa, Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin, Circ. Res. 94 (2004) e27–e31.
[32] H.S. Berner, S.P. Lyngstadaas, A. Spahr, M. Monjo, L. Thommesen, C.A. Drevon, U. Syversen, J.E. Reseland, Adiponectin and its receptors are expressed in bone-forming cells, Bone 35 (2004) 842–849.
[33] O.O. Ogunwobi, I.L. Beales, Adiponectin stimulates proliferation and cytokine secretion in colonic epithelial cells, Regul. Pept. (2006), Epub.
[34] M. Neumeier, J. Weigert, A. Schaffler, G. Wehrwein, U. Muller-Ladner, J. Scholmerich, C. Wrede, C. Buechler, Different effects of adiponectin isoforms in human monocytic cells, J. Leukoc. Biol. 79 (2006) 803–808.
[35] T. Miyazaki, J.D. Bub, M. Uzuki, Y. Iwamoto, Adiponectin activates c-Jun NH2-terminal kinase and inhibits signal transducer and activator of transcription 3, Biochem. Biophys. Res. Commun. 333 (2005) 79–87.
[36] M. Igata, H. Motoshima, K. Tsuruzoe, K. Kojima, T. Matsumura, T. Kondo, T. Taguchi, K. Nakamaru, M. Yano, D. Kukidome, K. Matsumoto, T. Toyonaga, T. Asano, T. Nishikawa, E. Araki, Adenosine monophosphate-activated protein kinase suppresses vascular smooth muscle cell proliferation through the inhibition of cell cycle progression, Circ. Res. 97 (2005) 837–844.
[37] R. Rattan, S. Giri, A.K. Singh, I. Singh, 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside inhibits cancer cell proliferation in vitro and in vivo via AMP-activated protein kinase, J. Biol. Chem. 280 (2005) 39582–39593.
[38] A. Woods, S.R. Johnstone, K. Dickerson, F.C. Leiper, L.G. Fryer, D. Neumann, U. Schlattner, T. Wallimann, M. Carlson, D. Carling, LKB1 is the upstream kinase in the AMP-activated protein kinase cascade, Curr. Biol. 13 (2003) 2004–2008.
[39] Y. Dagon, Y. Avraham, E.M. Berry, AMPK activation regulates apoptosis, adipogenesis, and lipolysis by eIF2alpha in adipocytes, Biochem. Biophys. Res. Commun. 340 (2006) 43–47.

[40] M. Saitoh, K. Nagai, K. Nakagawa, T. Yamamura, S. Yamamoto, T. Nishizaki, Adenosine induces apoptosis in the human gastric cancer cells via an intrinsic pathway relevant to activation of AMP-activated protein kinase, Biochem. Pharmacol. 67 (2004) 2005–2011.

[41] D.G. Hardie, The AMP-activated protein kinase pathway - new players upstream and downstream, J. Cell Sci. 117 (2004) 5479–5487.

[42] E.A. Ostrakhovitch, M.G. Cherian, Inhibition of extracellular signal regulated kinase (ERK) leads to apoptosis inducing factor (AIF) mediated apoptosis in epithelial breast cancer cells: the lack of effect of ERK in p53 mediated copper induced apoptosis, J. Cell. Biochem. 95 (2005) 1120–1134.

[43] Y. Wang, K.S. Lam, J.Y. Xu, G. Lu, L.Y. Xu, G.J. Cooper, A. Xu, Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner, J. Biol. Chem. 280 (2005) 18341–18347.

[44] S. Catalano, S. Marsico, C. Giordano, L. Mauro, P. Rizza, M.L. Panno, S. Ando, Leptin enhances, via AP-1, expression of aromatase in MCF-7 cell line, J. Biol. Chem. (2003).

[45] M. Ishikawa, J. Kitayama, H. Nagawa, Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer, Clin. Cancer Res. 10 (2004) 4325–4331.

[46] H. Dotzlaw, E. Leygue, P.H. Watson, L.C. Murphy, Expression of estrogen receptor-beta in human breast tumors, J. Clin. Endocrinol. Metab. 82 (1997) 2371–2374.

[47] K. Specht, M. Kremer, U. Muller, S. Dirnhofer, M. Rosemann, H. Hofler, L. Quintanilla-Martinez, F. Fend, Identification of cyclin D1 mRNA overexpression in B-cell neoplasias by real-time reverse transcription-PCR of microdissected paraffin sections, Clin. Cancer Res. 8 (2002) 2902–2911.

[48] I. Girault, C. Andrieu, S. Tozlu, F. Spyratos, I. Bieche, R. Lidereau, Altered expression pattern of alternatively spliced estrogen receptor beta transcripts in breast carcinoma, Cancer Lett. 215 (2004) 101–112.