Obesity and the Associated Mediators Leptin, Estrogen and IGF-I Enhance the Cell Proliferation and Early Tumorigenesis of Breast Cancer Cells

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Breast cancer continues to be a major cause of cancer deaths in women. Estrogen, which is also produced by the adipose tissue, is held responsible for the elevated risk of breast cancer in obese women. However, the adipose tissue secretes hormones and adipokines such as leptin and IGF-I and these substances could also contribute to an increased breast cancer risk for obese women. In this study, the impact of obesity on cell proliferation was investigated. The carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) was administered to normal weight and diet-induced obese female Sprague-Dawley rats. Cell proliferation was evaluated by immunohistological staining of BrdU-incorporation. In the mammary glands and inguinal lymphatic nodes of the obese rats, cell proliferation was significantly increased, indicating a significant influence of obesity on breast cancer. Effects of leptin, estrogen, and IGF-I on the proliferation of MCF-7 cells in vitro were assessed using an MTT assay. Cell culture experiments demonstrated a mitogenic role of these three mediators on cell proliferation. Our data demonstrate a stimulative effect of substances produced by the adipose tissue on breast cancer. Body weight specific cell proliferation suggests that obesity-related adipokines and mediators enhance cell proliferation and increase the risk for breast cancer.

INTRODUCTION

In both developed and developing countries, breast cancer is the most common cancer in women, with higher death rates in developed countries (1) and it is responsible for most common cancer deaths (2). One in eight women will develop breast cancer, and 30% of patients will die of this malignancy. The median survival after diagnosis is only two to four years in cases of advanced breast cancer (3). Risk factors include early menarche (4), late menopause (5), family disposition (6), genetic disposition (7), as well as postmenopausal hormone substitution (8) and obesity, especially in postmenopausal women (9,10). The prevalence of overweight and obesity in most developed countries has been markedly increased over the past two decades. In 2000, there were 300 million obese adults worldwide. Results from epidemiological studies have indicated that obesity contributes to the increased incidence and risk of death in many types of cancer including breast cancer (in postmenopausal woman). Estrogen is mainly thought to be responsible for this elevated risk for breast cancer in obese women, especially because adipose

Submitted 17 March 2008; accepted in final form 30 October 2008.
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tissue is a major source of extragonadal estrogen production (4). Studies have shown approximately twofold increases in breast cancer risk in postmenopausal women and a central role of estrogen in regulating cellular differentiation, proliferation, and apoptosis induction (11,12). However, obesity is also associated with an enhanced secretion of other mediators such as the insulin-like growth factor-I (IGF-I) and the adipokine leptin as well as various cytokines such as TNF-α and IL-6 (1,13).

Leptin, a hormone mainly generated by adipocytes, acts centrally in the hypothalamus to regulate body weight and peripheral energy expenditure (10). Circulating levels of free/unbound leptin are strongly correlated to body fat content and are significantly elevated in obese compared to normal weight humans and rodents (14,15). Furthermore, leptin has been reported to stimulate the proliferation of various cell types such as monocytes, respiratory epithelium, glomerular endothelial cells, vascular endothelium, and adrenal cells (16,17). In vitro experiments showed a stimulative effect of leptin on breast cancer cells (18). The third mediator, mainly produced by the liver, is IGF-I acting as an essential factor for regular growth processes by increasing protein synthesis, stimulating cell proliferation, or inhibiting apoptosis (19). Frystyk and colleagues (20) reported an association between adiposity and high levels of free IGF-I, and elevated serum concentrations of IGF-I have been linked to risk of premenopausal breast cancer (21). Animal studies have also shown reduced tumor growth after inactivation of the IGF-I receptor or after manipulations to reduce circulating or tissue IGF-I levels. Blood levels of the IGF-I associated insulin-like growth factor binding protein 1 (IGFBP1) are reduced in obesity, which leads to an enhanced bioavailability of IGF-I. IGF-I promotes cellular proliferation and inhibits apoptosis in many tissue types so that these effects might contribute to tumorigenesis (12).

The aim of this study was to test the hypothesis that different adipose tissue derived mediators such as leptin, estrogen, and IGF-I play a role in the development and progression of breast cancer; and thereby, adipose tissue as a hormone-producing organ may be responsible for the elevated breast cancer risk in obesity. The influence of obesity on cell proliferation and early tumorigenesis in the mammary glands and the early changes of cell proliferation and immunocytes in their draining lymph nodes were investigated in female Sprague Dawley (SD) rats by immunohistochemical staining of BrdU-incorporation in cells. In addition, to evaluate a direct stimulative effect of adipose tissue derived mediators on breast cancer growth the cell proliferation of a human breast cancer cell line (MCF-7) was compared after treatment with estrogen, leptin, and IGF-I alone or in different combinations at different time points.

MATERIALS AND METHODS

Animals

Female SD rats (CD IGS rat) at the same stage of the estrus cycle were obtained from the Central Animal Facility (Hannover Medical School, Hannover, Germany) at the age of 8 wk and individually housed in plastic-based cages (40 × 26 × 15 cm), in sound-proofed, air-conditioned, and artificially lighted rooms (lights on at 0700 h to 1900 h) at an ambient temperature of 24.0 ± 0.5°C. The animals were kept under specific pathogen-free conditions according to the FELASA list (www.felasa.eu). Animals were randomized into two groups and fed as follows during the whole period of experiments. One group received standard rat chow containing 6.0% carbohydrate, 19.0% protein, 4.0% fat, and 2.1 kcal/kg (Altromin1234, Altromin GmbH & Co KG, Lage, Germany). The other group was fed a high-calorie diet containing 7.5% carbohydrate, 24.5% protein, 60% fat, and 5.2 kcal/g (Altromin C1057, Altromin GmbH & Co KG). Body weight was measured once a week, and diet-induced obese animals gained continuously more body weight than lean animals until the termination of the experiments. Tap water was available ad libitum. After 5 wk, experiments started with the normal chow-diet fed animals (mean body weight 272 g ± 10 g) and the diet-induced obese littermates (mean body weight 322 g ± 15 g). All research and animal care procedures were approved by the Lower Saxony district government in Hannover, Germany (AZ 01/481).

Experimental Design for the In Vivo Experiments and BrdU Application

For tumor induction, obese (n = 6) and lean (n = 6) SD rats received an oral application of 10 mg 7,12-dimethylbenz[a]anthracene (DMBA; Sigma, Steinheim, Germany) dissolved in 0.5 ml peanut oil (10 mg/0.5 ml). Lean (n = 3) and obese (n = 3) control animals were treated with the vehicle alone. After 7 wk, experiments were finalized. To measure cell proliferation in breasts and lymphatic nodes, 5-bromo-2-deoxyuridine (BrdU, Sigma, Steinheim, Germany) in phosphate-buffered saline (PBS) was administered by intraperitoneal injection (50 mg/kg body weight). Normal weight animals were treated with 13.8 mg dissolved in 0.7 ml PBS, and obese animals were treated with 16.3 mg BrdU dissolved in 1 ml PBS in adjustment to the higher body weight. Two hours after BrdU injection, animals were anesthetized via intramuscular injection (0.35 ml 10% ketamine hydrochloride; Albrecht GmbH, Aulendorf, Germany and 0.15 ml 2% xylazin hydrochloride; Bayer, Leverkusen, Germany) and perfused transcardially with 120 ml phosphate-buffered saline (PBS) followed by 150 ml of 4% paraformaldehyde in PBS. The mammary glands and lymph nodes were harvested, immediately frozen in liquid nitrogen, and stored at −80°C.

Immunohistochemistry

Cryosections (7 μm) were air dried and stored at −20°C. Sections were fixed for 10 min in equal parts of methanol and acetone and thoroughly washed in Tris-buffered saline (TBS) containing 0.05% Tween 20 (Serva, Heidelberg, Germany). To detect BrdU, DNA was denatured with formamide (Sigma,
Steinheim, Germany) and NaOH. Formamide (190 ml) and NaOH (1 N, 10 ml) were separately warmed to 70°C and then mixed and incubated for 8 min. The sections were immersed in this solution for 30 s, washed with TBS, and immersed in formamide and 0.15% 0.15 M trinatrium citrate (70°C) for 15 min. Thereafter, sections were washed in ice water and fixed in 1% formaldehyde (30 min) and 0.2% glutaraldehyde (10 min). Sections were incubated overnight (4°C) with a mouse monoclonal anti-BrdU antibody dissolved in TBS/Tween (Becton Dickinson; Heidelberg, Germany). After washing with TBS, the sections were incubated with a secondary antibody (rabbit anti-mouse, Z259; DakoCytomation, Hamburg, Germany) and an APAAP (Alkaline phosphatase-anti-alkaline phosphatase; DakoCytomation, Hamburg, Germany) complex for 30 min. After repetition of the last two steps (each one for 15 min), a color reaction was induced by incubation of with 2 mg Fast Blue (Sigma, Steinheim, Germany) mixed with 4 ml APAAP substrate for 25 min. The sections were counterstained with Mayer's Hemalaun (Hematoxylin, Merck, Darmstadt, Germany) and mounted in glycergel (C0563 Glycergel Mounting Medium, DakoCytomation, Hamburg, Germany).

Quantification of Proliferating Cells In Situ
Each experimental group consisted of 6 rats, and each control group consisted of 3 rats. For the quantitative analysis, the sections were examined under light microscopy (Axiophot, Zeiss, Jena, Germany) by one observer blind to the treatment conditions. In 10 visual fields, the number of positive stained cells was counted. Five sections of each organ of an animal were assessed, resulting in 40 mm²/organ/animal.

Cell Line and Culture Conditions
To evaluate the impact of substances produced by the adipose tissue on tumor cell proliferation in vitro, MCF-7 cells (DSMZ, Braunschweig, Germany) were used. Cells were routinely cultured in RPMI (RPMI Medium 1640, Gibco®, Karlsruhe, Germany) with 10% fetal calf serum (FCS GOLD, PAA Laboratories GmbH, Pasching, Austria), 1% amino acids, insulin (10 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C with 5% CO₂ atmosphere and subcultured every 5 days after detachment with 0.05% trypsin until experiments were performed. All products were obtained from Gibco®, Karlsruhe, Germany unless otherwise noted.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) Assay
Trypsinized cells were washed with PBS twice. Insulin-transferrin-selenium ITS-enriched medium (supplement, Invitrogen, Karlsruhe, Germany with 1 μg insulin/ml medium, 0.55 μg transferrin/ml medium, 0.5 ng sodium selenite/ml medium) was added to remove all endogenous growth factors and cytokines from the medium. The addition of these substances permits one to work in a serum-free environment to avoid the variability of bovine cytokines. Cells at a concentration of 10⁵ cells/ml were plated in 96-well culture microtiter plates and incubated for 24 h. All experiments were performed in triplicate. Cells were stimulated with estrogen (β-estradiol, Sigma, Steinheim, Germany), leptin (human leptin, NatuTec GmbH, Frankfurt, Germany), IGF-I (NatuTec), as well as different combinations of them to induce cell proliferation.

Estrogen was used in concentrations of 50 nM, 100 nM, and 200 nM; leptin in concentrations of 3.67 nM, 36.7 nM, and 360.7 nM; and IGF-I in concentrations of 0.065 nM, 0.13 nM, and 0.261 nM. These dosages were chosen after initial experiments, and other groups have also effectively worked with those (18, 22). In combination, leptin and estrogen were used in concentrations of 3.67 nM + 100 nM, 36.7 nM + 100 nM, and 367 nM + 100 nM. Leptin and IGF-I were used in concentrations of 3.67 nM + 0.13 nM, 36.7 nM + 0.13 nM, and 367 nM + 0.13 nM; and estrogen and IGF-I as a combined stimulus were chosen in a concentration of 100 nM + 0.13 nM, respectively. Controls were stimulated with ITS medium only. One hundred microliters of the stimulants were added to each well followed by a further incubation for 6, 24, or 48 h. Afterwards 100 μl of supernatant from each well was removed and 10 μl of MTT (3-[4.5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium) was added. After 2 h, crystal formation was visible, and the reaction was stopped by adding 100 μl of stop solution (50 g sodium dodecyl sulfate with 500 ml dimethyl formamide in 1 l aqua dest.; Fluka, Buchs, Switzerland). The microtiter plates were stored at –80°C until read out. After finishing all experiments, all microtiter plates were incubated overnight to dissolve formazan crystals and then evaluated by using an ELISA reader (TECAN, Magellan V2.22, Tecan Deutschland GmbH, Germany) at 570 nm.

Statistical Evaluation
Data are expressed as means ± SEM (standard error of the mean). To analyze the effect of the body weight or different treatments on the cell proliferation in vivo and in vitro, 2-way ANOVA was applied. LSD post hoc analysis was implemented to determine significant differences in the case of main treatment effects. Differences were considered significant if P value < 0.05.

RESULTS
Cell Proliferation in Mammary Glands of Obese Tumor Animals Is Significantly Enhanced
For tumor induction, the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) was administered to normal weight and diet-induced obese female Sprague-Dawley rats. According to preliminary studies, animals were sacrificed at an early time point of tumorigenesis, 7 wk after carcinogen application. Animals were sacrificed, and cell proliferation in the mammary glands and the draining lymph nodes was evaluated by BrdU
OBESITY AND ASSOCIATED MEDIATORS ENHANCE CELL PROLIFERATION AND EARLY TUMORIGENESIS OF BREAST CANCER

Fig. 1. A: Representative immunohistological sections of 5-bromo-2-deoxyuridine (BrdU) incorporation in mammary glands of a female obese control rat and B: a female obese rat after tumor induction by DMBA treatment ×200 magnification. BrdU incorporation represents proliferating cells. C: Quantitative evaluation of BrdU incorporation in the breast tissue of female normal weight and obese rats after tumor induction or vehicle treatment. Significant post hoc effects of obese and normal weight tumor animals vs. their corresponding controls are indicated by an asterisk; *p < 0.05. Significant post hoc effects of obese tumor animals vs. normal weight tumor animals are indicated by a rhomb; #p < 0.05. nw-c = normal weight control animal; ob-c = obese control animal; nw-tu = normal weight tumor animal; ob-tu = obese tumor animal.

incorporation. Because the animals were sacrificed at an early time point, tumors in the mammary glands were not palpable in most experimental animals with one exception. One obese tumor animal developed palpable tumors in two mammary glands after 7 wk. BrdU incorporation was used as reliable indicator of cell proliferation.

As expected, the cell proliferation in the mammary glands of both lean and obese tumor animals was significantly higher compared to their corresponding untreated control group (Fig. 1). In lean tumor animals, the cell proliferation was significantly increased by 132% and in obese tumor animals by 330% as compared to control animals. The difference in increase of cell proliferation between lean and obese tumor animals was also significant, suggesting an enhancing effect of obesity on tumor cell growth in the mammary gland. There was no difference in the cell proliferation rate between the mammary glands within one animal. The amount of BrdU incorporation in the mammary gland of lean and obese control animals was similar.

Cells of the Draining Lymph Node of Obese Tumor Animals Proliferate at a Higher Rate Than Those of Lean Tumor Animals

To investigate the paracrine effects of the tumorous cells in the mammary glands, the cell proliferation in the draining lymph nodes was investigated. It is known that cancer cells release various cytokines and growth factors into their surroundings and recruit other types of cells in order to establish a tumor environment (23). In the draining lymph nodes, similar cell proliferation results as compared to the mammary glands were found (Fig. 2). Lean and obese tumor animals displayed higher rates of BrdU incorporation (predominantly in the T-cell region) than the healthy control animals. By comparing lean tumor animals with lean control animals, an increase in proliferation of 132% was detectable. Comparing obese control animals with obese tumor animals, 139% more proliferation was seen in the latter. Additionally, obese tumor animals showed a significantly higher proliferation rate, by 21%, than lean tumor animals. Comparison of BrdU incorporation in inguinal lymph nodes of control
FIG. 2. A: Representative immunohistological sections of BrdU-incorporation in inguinal lymph node of a female obese control rat and B: a female obese rat after tumor induction by DMBA treatment × 200 magnification. BrdU incorporation represents proliferating cells. C: Quantitative evaluation of BrdU incorporation in the inguinal lymph node of female normal weight and obese rats after tumor induction or vehicle treatment. Significant post hoc effects of obese and normal weight tumor animals vs. their corresponding controls are indicated by an asterisk; *p < 0.05. Significant post hoc effects of obese tumor animals vs. normal weight tumor animals are indicated by a rhomb: #p < 0.05. nw-c = normal weight control animal; ob-c = obese control animal; nw-tu = normal weight tumor animal; ob-tu = obese tumor animal.

animals showed no body weight dependent differences. The carcinogen did not affect the cell proliferation in various other organs such as the liver, spleen, and the colon (data not shown).

Cell Culture Experiments Showed Antiproliferative Effects of Leptin Alone and Stimulatory Effects of Leptin in Combination With Estrogen and IGF-I

The following three substances highly secreted in rodent and human obesity were investigated in a human breast cancer cell line to at least partially explain the higher cell proliferation rate in the rat model: Different concentrations of leptin, estrogen, and IGF-I had time- and concentration-dependent effects on tumor cell proliferation. In a first set, different concentrations of leptin, estrogen, and IGF-I were tested, and their possible effects were observed 6, 24, and 48 h later (Fig. 3A–3C). In a second set, time-dependent effects on tumor cell proliferation were detected with different combinations of leptin, estrogen, and IGF-I (Fig. 4A, 4B). The application of 3.67 nM leptin had no effect on tumor cell proliferation (Fig. 3A). However, at higher dosages (36.7 nM and 360.7 nM), leptin treatment resulted in a time-dependent decreased tumor cell proliferation. Estrogen (50 nM, 100 nM, and 200 nM) treated cells showed more than 20% increase of cell proliferation after 48 h compared to control cells. 50 nM estrogen treatment resulted in a significantly enhanced cell proliferation already after 24 h (increase of 23%; Fig. 3B). IGF-I (0.261 nM) treated cells showed a similar significant increase of proliferation after 48 h (increase of 23%). Low-dose treatment with IGF-I (0.065 nM) resulted in a dramatic time-dependent decrease of MCF-7 cell proliferation (Fig. 3C). The combination of 3.67 nM leptin with 100 nM estrogen (Fig. 4) induced a significant increase of 19% cell proliferation after 48 h compared to the untreated control cells. The combination of 3.67 nM leptin with 0.13 nM IGF-I (Fig. 4) revealed significant results in tumor cell growth after 24 (>20%) and 48 h (>30%). Higher doses of leptin (36.7 nM) combined with IGF-I (0.13 nM) revealed comparable results after 48 h (Fig. 4). A combined treatment with IGF-I (0.13 nM) with estrogen (100 nM)
FIG. 3. MTT proliferation assay. Proliferation of MCF-7 cells (human breast adenocarcinoma cancer cell line) treated with different dosages of leptin (A), estrogen (B), and IGF-I (C) after 6, 24, and 48 h. Significant differences as compared to the control medium (ITS-medium = insulin-transferrin-selenium supplement; 100%) are indicated by an asterisk; *p < 0.05.

enhanced MCF-7 cell proliferation significantly after 48 h (increase of 40%; Fig. 4).

DISCUSSION

Several studies have demonstrated that obesity in women is associated with a higher risk of breast cancer. So far, this phenomenon has mainly contributed to higher serum estrogen levels in obese women. Adipose tissue is the primary extragonadal source of estrogen. However, nowadays the adipose tissue is recognized as an immuno-endocrine organ producing a variety of hormones, adipokines, and cytokines (13). The most prominent adipokine is leptin, which among other functions also influences cell proliferation (24,15). Thus, it is important to investigate whether leptin itself or its combination with other hormones and cytokines released by the adipose tissue plays a crucial role in the genesis of breast cancer.

In the present study, we observed that obese SD rats are much more susceptible to DMBA-induced tumorigenesis than their lean littermates. Calorie intake among the groups was at least twofold higher in diet-induced animals until the termination of the experiments. The cell proliferation of obese rats was significantly increased compared to lean rats. Specifically, obese rats showed about 90% more tumor cell proliferation in the breast tissue than lean animals. Several studies have investigated the influence of body weight on tumor cell proliferation. The results of our present study oppose findings in lean and obese Zucker (fa/fa) rats, which were investigated for their susceptibility to MNU-induced mammary cancer. Lee and colleagues (25) showed that obesity in female rats does not render these animals more susceptible to mammary gland carcinogenesis and that lean and obese rats developed tumors at the same rate. In line with our study is an investigation performed by Hakkak and colleagues (26) using female fa/fa rats. Obese fa/fa rats developed significantly more DMBA-induced mammary tumors compared to their lean littermates. Furthermore, their tumor latency was shorter (66 days vs. 118 days in lean littermates), the first mammary tumor was detected earlier (49 days vs. 86 days for lean rats), and their median tumor-free time was significantly lower. After 4 mo, 68% of obese rats had developed mammary tumors as compared with only 32% of the lean rats. The study supports the hypothesis that obesity and some of its mediators play a significant role in carcinogenesis. Studies by Trayhurn and Wood (27) and Hakkak and colleagues (26) have supported the hypothesis that adipose tissue associated hormones and adipokines are mediators of enhanced carcinogenesis in obese subjects.

Most studies exploring the impact of adipokines on tumor growth and carcinogenesis are based on investigations of genetically obese animals such as fa/fa rats and ob/ob mice. Importantly, in the present study, we chose a model of diet-induced obesity, as it recapitulates more realistic conditions in humans. Furthermore, we were the first evaluating the proliferation index in the draining lymph nodes, the primary site of metastatic spread. Importantly, the BrdU-incorporation rate was likewise enhanced in the inguinal lymph nodes of the obese animals, with 21% more proliferating cells in the draining lymph nodes compared to lean littermates. Cell culture experiments with MCF-7 cells treated with different concentrations of estrogen, leptin, and IGF-I clearly showed that leptin itself had slightly antiproliferative effects on the cell proliferation. Both estrogen and IGF-I mono-treatments had a time dependent proliferative effect on cancer cells. But the most potent stimulation of cell proliferation was achieved by the synergistic combination of estrogen and IGF-I at intermediate dosages followed by the combination of leptin and IGF-I. Leptin combined with estrogen had slightly proliferative effects. Various reports have focused on leptin’s role on breast cancer risk and carcinogenesis. At
FIG. 4. MTT proliferation assay. A: Proliferation of MCF-7 cells (human breast adenocarcinoma cancer cell line) treated with different combinations of leptin and estrogen, leptin and IGF-I and IGF-I and estrogen after 6, 24, and 48 h. B: Significant differences as compared to the control medium (ITS-medium = insulin-transferrin-selenium supplement; 100%); n.s. = not significant.

Present, there are several publications of case-control studies demonstrating that relatively high leptin serum concentrations were associated with an increased risk of breast cancer (28–30). But conflicting results are shown in another two case-control studies (31,2): all have shown the antiproliferative effects of leptin. Concerning leptin and its influence on cell proliferation, our results have to be added to the actual discussion about the pathogenesis of increased tumor growth in obese humans (32). Obesity-related increase in adipokine production and a reduction in adiponectin were suggested to affect breast cancer outcome by their angiogenesis-related activities. We could show for the first time that leptin has mitogenic effects only in combination with other peptides and cytokines. The mechanism underlying this combined proliferation stimulus may be explained by the effects of leptin on estrogen receptor \(\alpha (\text{ER}\alpha)\) in MCF-7 cells (33). Leptin induces functional transactivation of \(\text{ER}\alpha\) via ERK 1/2. Therefore, it is capable to potentiate the effects of estrogen. Recent investigations of our group showed the abrogation of the JAK-2, PK B and AMPK\(\alpha\) signaling pathways in NK cells from obese rats (15), resulting in leptin resistant NK cells in obese subjects. Since NK cells are an integral component of the innate immune system, both in the production of cytokines to stimulate other immune cells and in the direct destruction of infected or transformed cells, the fact that NK cells of obese subjects cannot be activated by leptin may be another explanation for the increased tumorigenesis in obesity.

In conclusion, we have demonstrated that obesity increases the susceptibility of female SD rats to the development of DMBA-induced mammary tumors. Investigating the influence of the adipokines leptin, estrogen, and IGF-I on tumor cell proliferation, in vivo and in vitro experiments have shown comparable results. Leptin treatment alone had antiproliferative effects, whereas leptin combined with estrogen or IGF-I had stimulatory effects on tumor cell growth.

Acknowledgments

The excellent technical assistance of I. Dressendörfer is very much appreciated. This work was supported by a grant from the Danone Foundation, Haar Germany. The authors declare that there is no conflict of interest that would prejudice the impartiality of this work.

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