Voluntary Physical Activity Abolishes the Proliferative Tumor Growth Microenvironment

Created by Adipose Tissue in Animals Fed a High Fat Diet

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Running Head: Exercise counteracts obesity/breast cancer association

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The molecular mechanisms behind the obesity-breast cancer association may be regulated via adipokine secretion by white adipose tissue. Specifically, adiponectin (ADIPO) and leptin (LEP) are altered with adiposity and exert antagonistic effects on cancer cell proliferation. We set out to determine whether altering adiposity in-vivo via high fat diet (HFD) feeding changed the tumor growth supporting nature of adipose tissue and if voluntary physical activity (PA) could ameliorate these HFD-dependent effects. We show that conditioned media (CM) created from the adipose tissue of HFD fed animals caused an increase in the proliferation of MCF7 cells compared to cells exposed to CM prepared from the adipose of lean chow diet fed counterparts. This increased proliferation was driven within the MCF7 cells by an HFD-dependent antagonism between AMPK and Akt signaling pathways, decreasing p27 protein levels via reduced phosphorylation at T198 and downregulation of AdiporR1. PA can ameliorate these proliferative effects of HFD-CM on MCF7 cells, increasing p27T198 by AMPK, reducing pAktT308 and increasing AdipoR1, resulting in cell cycle withdrawal in a manner that depends on the PA intensity. High physical activity (>3 km/day) completely abolished the effects of HFD feeding. In addition, AdipoR1 overexpression mimics the effects of exercise, abolishing the proliferative effects of the HFD-CM on MCF7 cells and further enhancing the anti-proliferative effects physical activity on the HFD-CM. Thus, VPA represents a means to counteract the proliferative effects of adipose tissue on breast cancers in obese patients.

Keywords: Physical Activity, obesity, adipokines, breast cancer
We hypothesized that voluntary physical activity (PA) would counteract the deleterious adipose-dependent growth microenvironment that a breast cancer is exposed to. We show that PA altered the adipokine secretion profile of adipose in a volume-dependent manner. This alteration resulted in growth inhibition of estrogen receptor positive breast cancer cells in culture. Furthermore, stabilizing adiponectin receptor 1 expression in the cancer cells made them resistant to the cell cycle entry effects that accompany obesity.
INTRODUCTION

Obesity continues to be a growing concern not only within North America but around the world. It has been linked with several detrimental health issues such as insulin resistance, hyperglycemia, dyslipidemia and hypertension (16). Growing evidence now supports the notion of several cancers being associated with obesity including breast, renal, esophageal, gastrointestinal and reproductive cancers. Numerous clinical and preclinical studies have demonstrated that increased adiposity increases the risk of cancer incidence, morbidity and mortality while imparting a poorer response to therapy (5, 31, 42). Specifically, obese breast cancer patients in the highest quintile of BMI have a more than two-fold higher mortality rate compared to their lean counterparts (5).

To elucidate the mechanism behind the association between obesity and breast cancer investigations have focused on the role of adipose-derived cytokines, termed adipokines. There are more than 400 adipokines released from adipocytes which are also dysregulated in obese individuals and exert endocrine effects on numerous different body tissues (48). Given that the human breast is composed primarily of epithelial cells surrounded by adipose tissue, the potential exists for these adipokines to exert their effects directly on breast cancers. These adipokines can play a crucial role in shaping the growth microenvironment that a breast cancer is exposed to within the body. In addition to the adipocytes, the stromal compartments of adipose tissue have been shown to elicit effects on breast cancer proliferation (32). Of the adipokines identified to date, adiponectin (ADIPO) and leptin (LEP) represent major potential contributors to adipose-dependent effects. They are among the most abundant adipokines produced/secreted, both are altered by obesity and they exert antagonistic cell cycle regulatory effects on breast cancer cells (10, 11, 20). ADIPO is a 30 kDa protein whose production/secretion decreases with obesity and
induces cell cycle exit by activating AMPK, which directly phosphorylates p27 at T198, increasing p27 stability and inducing G1 arrest (10, 15, 26). LEP (16 kDa) production increases with adiposity and its cell cycle effects directly oppose those of ADIPO (10, 11). LEP induces cell cycle entry by activating Akt, which phosphorylates p27 at T157, denying p27 entry into the nucleus, thereby preventing it from inhibiting cyclin E/cdk2 and inducing cell cycle entry (11, 14, 27). The decreased ADIPO and increased LEP in the circulation of obese individuals creates a microenvironment that promotes tumor growth by accelerating cell cycle entry, causing a greater incidence of detectable tumor formation and more advanced tumors in obese women than in lean women (6, 28). Independent of adiposity, serum ADIPO has also been found to be reduced while LEP is increased in women with breast cancer compared to women without the disease (23, 29, 37). Decreased ADIPO signaling through AdipoR1 has been shown to be associated with higher tumor grade and poorer patient outcomes (33). We have previously demonstrated in cell culture and in-vivo that increasing AdipoR1 levels increases the cell cycle effects of ADIPO via AMPK signaling and can counteract the antagonism of ADIPO by LEP (51), a condition that exists in obese breast cancer patients and may underlie the association with poor prognosis and a less favorable response to therapy.

An increasingly sedentary lifestyle is a major contributor to the increase in obesity and its associated disorders (38). This highlights increased physical activity as a potential prevention/intervention for the development of obesity and its associated effects on breast cancer (4, 9). Moderate physical activity (>0.64 MET-hours/day) reduces the incidence of breast cancer, with women who are physically active exhibiting a 20-30% reduction in the relative risk of developing breast cancer compared to their sedentary counterparts (22, 30, 35, 39, 45). The effect of physical activity is also important in improving patient survival in breast cancer as seen
by an up to 40% reduction in cancer-related death and cancer recurrence in physically active women (17). In addition, there are dose-dependent (intensity and duration) relationships among physical activity, cancer risk and overall survival in breast cancer patients (46). Breast cancer patients participating in physical activity consisting of walking as little as one hour/week was associated with improved survival compared to sedentary women (17). These effects were more pronounced in women who engaged in moderately intense exercise between 3-5 hours per week (17). When exercise intensity was increased further (running >1.8 MET-hours/day) breast cancer patients had an almost 90% lower risk of cancer mortality compared to women who walked (<1.07 MET-hours/day) (46). Voluntary physical activity alters the production of both ADIPO and LEP in rats fed a high fat diet, lowering the levels of LEP and increasing the levels of ADIPO in the circulation compared to sedentary high fat diet fed animals (4, 24, 45, 50). Physical activity decreased pAkt and cyclin D1 and increased pAMPK and p27 within mammary carcinomas of those same animals (49). Furthermore, postmenopausal women exposed to 12 months of consuming a calorie restricted diet and moderate physical activity caused an increase (9.5%) in plasma ADIPO and a decrease (40.1%) in LEP (1). Thus, there are clear positive dose-dependent effects of diet and the amount of physical activity and its benefits to breast cancer patient prognosis.

The exact mechanisms behind the effects of physical activity on regulating adipose-dependent tumor growth microenvironment remain unclear. We set out to determine whether a dose-dependent relationship between physical activity and the productionSECRETION of adipokines exists and whether these effects can alter the deleterious adipose-dependent tumour growth microenvironment created in animals fed a high fat diet (HFD). We show that HFD fed animals decreased the ADIPO:LEP ratio secreted by isolated adipose tissue into culture media. We also
found a decrease the levels of pAMPK$^{T172}$, p27$^{T198}$, p27, AdipoR1 and an increase pAkt$^{T308}$ in MCF7 cells grown in the conditioned media prepared from the adipose of HFD animals resulting in cell cycle entry. A dose-dependent effect of physical activity was observed on the adipokine profile by increasing the ratio of ADIPO:LEP. Physical activity counteracted the effects of the HFD with high physical completely abolishing the effects of the HFD conditioned media on MCF7 cell cycle regulation. In addition to physical activity, we show that over expressing AdipoR1 in the MCF7 cells also counteracts the effects of HFD, highlighting the importance of AdipoR1 signaling on overcoming the positive growth promoting microenvironment that is present in obese breast cancer patients.

**METHODS**

**Animals.** All animal experiments were approved by York University Animal Care Committee in accordance with Canadian Council for Animal Care guidelines. Forty four male Sprague-Dawley rats (7 weeks of age) were purchased from Charles River Laboratories (Montreal, QC, Canada) and were singly housed in standard clear, plastic cages. Male rats were used in order to create an estrogen free environment and allow for the delineation of the effects of diet and exercise on the microenvironment created by the adipose tissue adipokine secretion profile from additional estrogen-dependent effects. All animals had a 7 day habituation period to a 12 hour light-dark cycle (lights on at 0600) in a temperature (22°C) and humidity (50-60%) controlled room.

After the habituation period, animals were randomly selected and given free access to a running wheel (wheel circumference, 106 cm; Harvard Apparatus, Holliston, MA) in their cage. A magnetic counter was mounted to each wheel which detected the revolutions after 24 hours of
use. The animals were given a 7 day acclimation period to the wheels. After this period, animals were then divided into two groups: chow diet (CD; n=19) and high fat diet (HFD; n=25) with both groups given access to food and water (*ad libitum*). The CD (no. 5012 Lab Chows, Ralston Purina, St. Louis, MO) had caloric make-up of 14% fat, 54% carbohydrate, 32% protein (3.02 calories/g). The HFD (Harlan Laboratories, Madison, WI) had a caloric breakdown of 60% fat, 21% carbohydrate, 18% protein (5.1 calories/g). HFD and CD fed animals were further subdivided into sedentary and physical activity (PA) groups designated as chow diet-sedentary (CD; n=11), chow diet-low physical activity (CD+LPA; <3 km/day; n=4), HFD-sedentary (HFD; n=13), and HFD-physical activity (HFD+PA; n=12) for 6 weeks. Animals in the HFD-PA group were further sub-divided depending on average wheel running distances into animals that ran less than 3km/day (n=6) classified as “low physical activity” group (HFD+LPA) while animals that ran more than 3km/day (n=6) were classed as “high physical activity” (HFD+HPA) to determine if a dose response to physical activity was evident. Our physical activity cutoff of 3km/day has also been used previously with Sprague-Dawley rats and wheel running to create a low and high PA level (21). Food intake and running distances were measured each day, and body weight was measured three times per week.

*Tissue Collection and Conditioned Media.* After the 6 week protocol, visceral (epididymal) adipose tissue was quickly removed from CD, CD+PA, HFD, HFD+LPA and HFD+HPA animals and cultured as previously described (38). Briefly, the epididymal fat was weighed, minced into ~5-10mg pieces and immediately placed in 50 ml vented conical tubes containing AMEM (7.5 ml/g tissue; Wisent, St.Bruno, QC) supplemented with 10% fetal bovine serum (Hyclone, Thermo Fisher Scientific, Whitby, ON), 2% antimotic/antibiotic (Wisent), 1 mM sodium pyruvate (Sigma, Oakville, ON), non-essential amino acids (Sigma), and 10 µg/ml
insulin from human pancreas (Wisent) under sterile conditions and incubated for 24 hours at 37°C with 5% CO₂. After 24 hours, conditioned media (CM) was then collected and stored at -84°C. We have conducted numerous experiments using this type of model to establish the efficacy of combining rat adipose tissue and human epithelial cells. In addition, many xenograft models grow human cells in mice, further illustrating the benefits of using a rodent/human tumor growth model. In order to ensure that our mass:volume preparation of CM was not skewed by the presence of vast differences in adipose cellular content, we diluted 10-15 mg sections of adipose 30:1 in RIPA buffer for protein extraction. Equal volumes of lysate (25 μl) were subjected to SDS-PAGE using 12% gels and membranes were probed for total Akt and β-actin to evaluate equivalency of specific protein content between groups. Weights of all tissues collected were measured and normalized per 100 g of body weight. The sequence in which rats were sacrificed was randomized across groups so as to minimize the likelihood that order effects would masquerade as treatment-associated effects. At time of sacrifice the gastrocnemius, soleus and tibialis anterior were immediately excised, weighed, frozen and stored at -84°C for future analyses.

**Cytochrome-c Oxidase Activity Assay.** In order to confirm that the physical activity protocol elicited a training effect, we measured cytochrome-c oxidase (COX) activity in the gastrocnemius muscles. COX activity was determined according to a modification of a method previously described (8). Briefly, cross-sections of mixed gastrocnemius muscles (from the mid-section of the muscle belly) weighing roughly 20-30 mg were diluted 80-fold (sedentary) or 160-fold (PA) in extraction buffer (100mM Na-K-Phosphate, 2 mM EDTA, pH 7.2). Muscle extracts were prepared by homogenization with metal beads (2X30 sec) at a frequency of 30 Hz in a magnetic homogenizer (Mixer Mill MM 400, Retsch, Germany). These homogenates were then
used for the analysis of the maximum rate of oxidation of fully reduced cytochrome c at 30°C as indicated by changes in absorbance (550 nm).

**Co-Culture Adipokine Measurement.** The levels of ADIPO and LEP produced and secreted into the co-culture media by adipocytes was determined using a rat adiponectin sandwich ELISA kit (BioVision, Milpitas, CA) and a mouse/rat leptin quantikine sandwich ELISA kit (R&D Systems, Minneapolis, MN), respectively, as per manufacturer instructions. For ADIPO aliquots of conditioned media were diluted 50-fold (HFD) and 100-fold (PA and CD; 100 µl total), while LEP ELISAs used a 5-fold (HFD) or undiluted (PA and CD) conditioned media (50 µl total) and were analysed against standard curves. The levels of each adipokine were calculated in ng/ml values and converted to nM values for stoichiometric comparison.

**Cell Culture.** MCF7 cells were purchased from the American Tissue type Culture Collection (ATCC, Manassas, VA) and were maintained in AMEM, 10% FBS, 2% antimicotic/antibiotic, 1 mM sodium pyruvate, non-essential amino acids, and 10 µg/ml insulin from human pancreas at 37°C and 5% CO₂.

Transformed DH5α *E.coli* bacterial cells containing AdipoR1 plasmid vectors (OriGene, Rockville, MD) were obtained in glycerol stock. Plasmid vectors were driven by a CMV promoter with an N-terminal FLAG-tag and contained neomycin and kanamycin resistance regions. AdipoR1 cDNAs (5 µg) were transfected into MCF7 cells using ExGen 500 *in-vitro* transfection reagent according to manufacturer instructions (Fermentas, Burlington, ON). Transfected MCF7 cells were treated with G418 sulfate (400 µg/ml, Wisent) and G418 resistant colonies were transferred to a 24 well plate in AMEM. After 24 hrs G418 was reduced to 200 µg/ml, allowing for growth but maintaining selection pressure. Viable colonies were subsequently tested for FLAG-tag expression and ADIPOR1 protein levels.
Mock transfected (MockT) MCF7 cells and stably transfected AdipoR1 over-expressing and FLAG-expressing cell (p31-4-2-2) were seeded in 6 well plates with AMEM for 24 hrs. At 70% confluence, cells were washed with PBS and incubated with CM produced from adipocytes for 24 hours. MCF7 cells grown in AMEM supplemented with 10% FBS served as untreated controls (UT). For initial experiments, CD-CM and HFD-CM were further supplemented with either 18 nM human globular adiponectin (Peprotech, Rocky Hill, NJ) or 300 nM human recombinant LEP (Peprotech) for 24 hrs.

**Immunoblotting.** The effects of adipokines on specific cellular proteins were measured using standard SDS-PAGE protocols using 12% polyacrylamide gels. Proteins (25 µg) were transferred to PVDF membranes (Bio-rad, Mississauga, ON, CAN), blocked for 2 hours in 10% skim milk and subsequently incubated overnight with primary antibodies: p27^{Kip1} (BD Biosciences); p27^{T198} (R&D Systems); pAkt^{T308}, Akt, pAMPK^{T172} and AMPK (Cell Signaling); AdipoR1 (Santa Cruz Biotech, Santa Cruz, CA) and β-actin (Abcam, Cambridge, MA). Anti-mouse and anti-rabbit (Promega, Madison, WI) and anti-goat (Santa Cruz) horseradish peroxidase secondary antibodies were used to visualize proteins using Immobilon enhanced chemiluminescence substrate (Millipore, Whitby, ON, CAN) and detected/quantified on a Kodak In Vivo Pro imaging system (Marketlink Scientific, Burlington, ON, CAN).

**Cell cycle analyses.** MCF7 cells isolated from 6-well plates following trypsinization were washed in cold PBS and fixed by drop wise addition of ice-cold 70% ethanol. Cells were washed twice in PBS, re-suspended in a propidium iodide/RNAse solution and subjected to FACS analyses (Gallios Flow Cytometer, Beckman Coulter Mississauga, Canada). Cell cycle profiles were determined using Mod-fit software (Verity Software House, Topsham, ME), by
fitting curves to profiles and measuring the areas under the curve to determine relative numbers of cells in G1, S and G2/M phases.

Statistical Analyses. All values are expressed as means ± SEM of five to thirteen separate experiments (as indicated) and statistical analyses were performed using a one-way ANOVA with Tukey’s post-hoc tests conducted when significant main effects were found. A two-way ANOVA with Bonferroni post-test comparisons was used for the CM plus adipokine experiments. Individual t-tests were used to identify differences in FACS analysis between groups. Group means value p≤0.05 were considered to be significantly different.
RESULTS

HFD increases adiposity which is prevented by physical activity. Animals were fed either a high-fat diet (HFD) or standard chow diet (CD) and a given access to running wheels as voluntary physical activity. Animals were placed in either a low physical activity (HFD+LPA, CD+LPA) or high physical activity (HFD+HPA) groups. The CD+LPA and HFD+LPA animals ran similar distances (2.2±0.3 vs. 2.5 ±0.6 km/day) but both ran less than the HFD+HPA (7.21 ±3.1 km/day). HFD-fed animals showed no difference in total body mass compared to sedentary CD fed animals (Fig. 1A). Despite the lack of difference in total body mass there was a 2.31-fold higher relative epididymal fat mass (2.22 ±0.26 vs. 0.96 ±0.23 g/100 g body weight; Fig. 1B) in HFD fed sedentary animals compared to CD fed sedentary animals. There was no difference in overall weekly calorie intake between the CD and HFD sedentary animals, indicating that the increased epididymal adiposity in HFD animals was a result of increased fat content and not increased caloric intake.

High intensity voluntary physical activity (HPA) counteracted the HFD-dependent increase in epididymal adiposity as demonstrated by the HFD+HPA animals being 16% lighter than their sedentary counterparts (452.9 ±19.1 g vs. 525.2 ±12.0 g; Fig. 1A). Consistent with these results we observed a 52% decrease in epididymal fat mass between HFD+HPA and HFD sedentary animals (Fig. 1B). In contrast, LPA-HFD animals had no difference in total body weight or body weight adjusted epididymal fat mass compared to their sedentary counterparts (Figs. 1A,B). The LPA-CD animals were 17% lighter and had 50% of the epididymal fat mass compared to HFD sedentary animals (Figs. 1A,B).

In order to determine if PA induced a training effect, we measured the weights of the gastrocnemius, soleus and tibialis anterior muscles. PA increased gastrocnemius, soleus and
tibialis relative weights in both HFD+LPA and HFD+HPA animals compared to their sedentary counterparts (Figs. 1C,D,E). LPA and HPA resulted in an overall increase of 27±3% and 38±4% in muscle mass above HFD animals, respectively. This volume-dependent effect of training was further evident from the changes in oxidative capacity of gastrocnemius muscles (Fig. 1F). LPA increased mixed gastrocnemius COX activity by 2.1-fold in HFD-fed animals while HPA further increased COX activity to levels that were 2.8-fold above those in sedentary HFD-fed animals.

Adipose tissue was excised from animals in each of the experimental groups and used to prepare conditioned media (CM). We measured the levels of ADIPO and LEP within the CM created from the secretome of the adipose tissue. In agreement with epididymal fat mass, the ratio of ADIPO:LEP was higher in the CD-CM compared to the HFD-CM (566.5 ± 197.2 vs.122.1 ± 52.1 ng/ml; Table 1). HPA prevented this HFD-dependent decrease in the ratio of ADIPO:LEP. The HFD+HPA had higher levels of ADIPO (1052.0 ± 246.9 ng/ml) and lower levels of LEP (1.17 ± 0.3 ng/ml) then both the HFD sedentary (ADIPO: 558.9 ± 99.4 ng/ml, LEP: 2.69 ± 0.8 ng/ml) and the HFD+LPA (ADIPO: 622.0 ± 141.0 ng/ml, LEP: 1.71 ± 0.4 ng/ml) groups, respectively. The overall stoichiometric ratio between ADIPO:LEP within the CM was increased in the HFD+HPA (529.8 ± 105.3:1) compared to both HFD (122.1 ± 52.1:1) and HFD+LPA (199.7 ± 57.7:1) animals. The ratio of ADIPO:LEP in the CM prepared from HFD+HPA animals was not different than those of both CD sedentary and CD+LPA. A linear relationship between the distance ran and the ratio of ADIPO:LEP in the CM was evident (m= 85.94 ± 21.83, R=0.795, p= 0.0034; Fig 1G).

**Conditioned media induces effects on cell signaling and cell cycle proteins in MCF7 cells.** In order to determine whether these changes in ADIPO and LEP elicit any effects on the breast cancer tumor growth environment, we treated MCF7 cells with CM created from the
adipose tissue of HFD and CD fed animals. Additionally, we also wanted to determine if adding additional ADIPO or LEP to CM would elicit any further effects on MCF7 cell cycle regulation. CM was created using equivalent dilutions of adipose tissue across treatment groups (7.5 ml of media/g of tissue). In order to determine whether approximately equal amounts of protein were being used in our CM preparation, we conducted western blot analyses using proteins isolated from the adipose tissue used in our CM treatments and measured the levels of Akt and β-actin proteins that are often used as loading controls in various experimental treatments (Fig 1H). We found that there was no specific pattern that suggested inequivalence of proteins loaded for any specific treatment (i.e. HFD had more protein than HFD+HPA), giving us confidence that the CM was not subjected to any preparation artifacts. However, we did see that CM treatment caused profound differences in MCF7 cells (Fig. 2). CD-CM increased pAMPK\(^{T172}\), p27\(^{T198}\), p27 and AdipoR1 while lowering pAkt\(^{T308}\) levels compared to HFD-CM treated MCF7 cells (Fig. 2 A-F). The addition of 18 nM gADIPO to CD-CM caused no further increases in pAMPK\(^{T172}\) (Fig. 2A,B), p27 (Fig. 2A,D), p27\(^{T198}\) (Fig. 2A,E), AdipoR1 (Fig. 2A,F) or decrease to pAkt\(^{T308}\) (Fig. 2A,C). Similarly, the addition of exogenous 300 nM LEP to CD-CM caused no effects on measured proteins. As was the case in CD-CM treated cells, LEP caused no further decrease in pAMPK\(^{T172}\), p27\(^{T198}\), p27 and AdipoR1 or increase in pAkt\(^{T308}\) while ADIPO could not rescue the levels of the proteins measured in HFD-CM treated MCF7 cells. Thus, the CM created from adipose tissue of HFD and CD fed animals was the driving force in creating the growth environment for the MCF7 cells. No changes in total AMPK and Akt were evident.

*AdipoR1 overexpression ameliorates the effect of HFD-CM.* We next determined whether AdipoR1 overexpression would ameliorate the effects of HFD-CM(Fig 3 A,B). Antibiotic-resistant colonies were expanded (19 total) and 2 cell lines were chosen for their expression of
the FLAG-tag and increased AdipoR1 expression compared to MockT cells (Fig. 3B). The level of overexpression of AdipoR1 in these cell lines amounted to $2.70 \pm 0.46$ (p31-3-2) and $2.64 \pm 0.51$ (p31-4-2-2) fold above MockT cells. Pilot experiments showed that each cell line elicited identical results so one cell line (p31-3-2) was used for the AdipoR1 overexpression experiments. The CD-CM caused an increase in pAMPK$^{T172}$ (Fig. 3A,B), p27 (Fig. 3A,D), p27$^{T198}$ (Fig. 3A,E) and AdipoR1 (Fig. 3A,F) compared to both UT and HFD-CM cells. This result was similar to what was seen in MockT cells (Fig. 2). Unlike what was observed in MockT cells, CD-CM was found to cause no decrease in pAkt$^{T308}$ compared to either UT or HFD-CM treated cells (Fig. 3A,C). Unlike MockT cells, HFD-CM caused no effects on MCF7 cells compared to UT cells in all proteins except for p27 (Fig. 3A,D). Most notably, HFD-CM treated cells caused no increase in pAkt$^{T308}$ compared to CD-CM treated cells, again different than what was observed in MockT cells (Fig. 2A,C vs. Fig. 3A,C). Overall, unlike what was observed in MockT cells, the HFD-CM did not seem to cause the same effects compared to UT cells, seemingly eliminating the dominant effects of HFD-CM on MCF7 cell cycle regulation, compared to UT cells, that were apparent in MockT cells. As observed in MockT cells, addition of 18 nM gADIPO (lane 2, 5 & 11) or 300 nM recombinant LEP (lane 3, 6 & 12) to either CD-CM or HFD-CM caused no additional effects in MCF7 cells. Unlike what was observed in MockT cells, addition of recombinant LEP had no effects compared to UT cells. This suggests that AdipoR1 overexpression can overcome any cell cycle entry effects of increasing LEP, as is seen with increased adiposity, in MCF7 cells. No changes in total AMPK and Akt were evident. These results highlight the importance of available AdipoR1 and show that increasing the available binding sites for ADIPO can override the cell cycle control regardless of the external growth environment.
Voluntary physical activity ameliorates HFD induced effects in a dose-dependent manner. PA has been shown to counteract obesity and we wanted to observe whether the effects of voluntary PA which altered the adipokine profile (ADIPO:LEP ratio) that was created within the CM led to any changes within co-cultured MCF7 cells. Voluntary PA elicited a dose-dependent response counteracting the effects of HFD on MCF7 cell cycle regulation (Fig. 4A lane 3 vs 5 & 6). Specifically, HFD+HPA-CM increased pAMPK$^{T172}$, p27 and AdipoR1 by 93%, 67% and 58%, respectfully, compared to HFD-CM treated cells (Figs. 4 A,B,D,F). In addition, pAkt$^{T308}$ was decreased by 40% (Figs. 4 A,C). Furthermore, HFD+HPA-CM elicited the same effects on MCF7 cells as CD-CM (Fig. 4A lane 2 vs. 5). HFD+LPA-CM caused effects that were intermediate to those observed in HFD and HFD+HPA CM treated cells. This indicates dose-dependent effects of PA on MCF7 cell growth displayed by the HFD+LPA-CM, increasing pAMPK$^{T172}$ and AdipoR1 while decreasing pAkt$^{T308}$ compared to the HFD-CM (Fig. 4 A,B,C,F). Surprisingly, the effects of HFD+HPA observed were also similar to those elicited by CD+LPA-CM on MCF7 cell cycle regulation (Fig. 4 A, lane 2 vs. lane 7). No changes in total AMPK and Akt were evident.

AdipoR1 overexpression enhances the effects of PA. Given that the CM elicits regulatory effects on AdipoR1, we overexpressed AdipoR1 to determine any absolute/synergistic effects of augmented AdipoR1 signaling in MCF7 cell cycle regulation. AdipoR1 overexpression increased the levels of pAMPK$^{T172}$, p27, p27$^{T198}$ while pAkt$^{T308}$ levels were decreased when compared to MockT cells (Fig. 4 vs 5). While a dose-response of PA was observed in MockT cells, the effects of the HFD+LPA-CM were further amplified with AdipoR1 overexpression such that the effects were no different from those elicited by CD-CM or HFD+HPA-CM. The HFD+HPA-CM was found to cause an increase in pAMPK$^{T172}$ (Figs. 5A,B), p27 (Figs. 5A,D) and AdipoR1 (Figs.
5A,F) by 58%, 27% and 19%, respectively compared to HFD-CM treated cells. Additionally, pAkt\(^{T308}\) was decreased by 25% (Figs. 5A,C) compared to HFD-CM treatment. CD+LPA-CM increased pAMPK\(^{T172}\) by 37% above CD-CM (Fig. 5A,B).

**AdipoR1 overexpression abolishes effect of HFD and accentuates PA cell cycle effects in MCF7 cells.** Despite the observed changes in cellular protein levels we set out to determine whether HFD and PA caused corresponding overall cell cycle changes in the cultured MCF7 cells. Cell cycle status was determined using propidium iodide staining and computational analyses (Figs. 6A,B). When cells were exposed to HFD-CM there was a 27% decrease in the number of cells in G1/G0 (53% vs. 42%) and a 34% increase in the number of cells in S-phase (20% vs. 28%) compared to CD-CM in MockT MCF7 cells (Fig. 6C). Elevating AdipoR1 expression increased the percentage of cells in G0/G1 in both CD-CM and HFD-CM while decreasing the number of cells in S-phase in the HFD-CM when compared to MockT HFD-CM treated cells (Figs. 6C vs. D). The HFD-CM decreased the number of cells in G1/G0 by 8% and increased the number of cells in S-phase by 16% compared to CD-CM treated cells (Fig. 6D). Strikingly, AdipoR1 overexpression increased the number of cells in G0/G1 by 24% (52% vs. 41%) compared to MockT cells when exposed to HFD-CM (Figs. 6 Cvs.D). This highlights the overall proliferative effects of the microenvironment created by the adipose tissue from HFD-fed animals and the powerful inhibitory effects that maintaining AdipoR1 protein expression elicits on the HFD-dependent effects of adipose tissue on cell cycle regulation.

In addition, we evaluated whether voluntary PA was able to counteract the HFD-dependent overall cell cycle effects on MCF7 cells. As observed with individual proteins there were dose-dependent effects evident depending on the intensity of PA. The HFD+HPA-CM caused a 17% increase in G1/G0 cells (49% vs. 41%) and a 15% decrease in the number of S-
phase cells (24% vs. 28%) compared to HFD-CM (Fig. 6E). LPA caused intermediate effects with HFD+LPA-CM increasing the percentage of G1/G0 cells by 9% (46% vs. 41%) and decreasing the number of S-phase cells by 17% (23% vs. 28%) compared to HFD-CM. The CD+PA elicited the same cell cycle effects compared to CD-CM. By overexpressing AdipoR1 within the MCF7 cells, there again was a dose-response to PA but to a much lesser extent (Fig. 6F).

**DISCUSSION**

It is clear that adipose tissue elicits proliferative effects on breast cancer cells, in large part due to the production/secretion of over 400 adipose-derived proteins, with the most abundant being adiponectin (ADIPO) and leptin (LEP; 48). While each of these adipokines elicit effects individually, emerging evidence suggests that the ADIPO:LEP ratio may be a more reliable predictive indicator of the adipose-dependent proliferative effects on breast cancer cells (2, 6). Given the inherent genetic variability and instability that are characteristic of cancers in general, it is likely that individual breast cancer patients likely possess unique and specific carcinomas making tumor-directed therapies an impossibly difficult therapeutic avenue. However, the overall tumor growth microenvironment that a tumor is exposed to and that exists among patients is regulated by far more stable and predictable physiological mechanisms. Adipose is one of the most important tissues that contributes to this growth microenvironment and alterations in adipokine secretion profile that accompany obesity may represent the molecular link between obesity and cancer. ADIPO and LEP have emerged as prime candidates as master regulators of this phenomenon because of their relatively high abundance, their levels are altered with obesity and they have been shown to elicit numerous effects on breast cancer cell...
cycle regulation (10, 11, 19). In addition, ADIPO and LEP activate unique intracellular signaling pathways (AMKP vs. Akt) which directly antagonize each other and elicit opposite effects on proliferation, with AMPK promoting cell cycle exit and AKT leading to cell cycle entry (51). This suggests that the ADIPO:LEP ratio may represent a more reliable indicator of tumor growth microenvironment and better predictor of cancer aggressiveness and patient outcome in breast cancer patients than either adipokine alone (6). In support of this, in obese breast cancer patients the serum ADIPO:LEP ratio is decreased and this is associated with more aggressive tumors and a poorer prognosis (9).

In order to induce adipose expansion we employed high-fat diet (HFD, 60% calories from fat) feeding as a means to induce obesity and observe the resultant effects on ADIPO and LEP production/secretion. Given that physical activity is a definitive means to manage body fatness, we also determined whether there were combinatory effects of HFD and physical activity (PA) on adipose physiology. Since the association between obesity appears stronger in post-menopausal women and studies have shown that HFD feeding can promote mammary tumor progression in ovariectomized mice, we conducted our interventions in male animals to simulate an estrogen-free environment without the potential surgical complications associated with ovariectomizing mice (7). Adipose tissue from HFD fed animals demonstrated alterations in adipokine secretion profile with lower levels of ADIPO and higher levels of LEP compared to their lean chow diet (CD) fed counterparts (Table 1), a result similar to that seen in humans (41). Surprisingly, we observed no measurable difference in total body weight between the HFD- and CD-fed animals. This may be due to the fact that the CD and HFD animals had identical specific daily caloric intake. Despite this lack of difference in total body weight, there was an evident 2.3±0.3-fold HFD-dependent increase in visceral fat mass and a 17±3% decrease in measured
muscle mass (gastrocnemius, soleus and tibialis anterior; Fig. 1). Thus, although body weight did not change, there was a definitive redistribution of body mass within the HFD-fed animals. Furthermore, given that these animals were in a rapid growth phase, adipose mass difference between our CD and HFD-fed animals may have been masked by rapid overall increases in body size (Fig 1A). The decrease in ADIPO:LEP ratio that was induced by HFD feeding resulted in a reduction in protein levels of certain cell cycle inhibitory regulators (Fig. 2) and caused S-phase entry in MCF7 cells exposed to conditioned media prepared from the adipose of HFD-fed animals (Fig. 6C). Our results point to HFD-dependent effects being completely abolished by high levels of physical activity, illustrating exercise as a powerful intervention/prevention strategy for obesity-linked cancers. However, given that cancer patients often suffer from other co-morbidities, implementing higher intensity exercise may not be an ideal option. Importantly, we demonstrate that lower intensity physical activity interventions, ones that do not induce weight loss, are still effective in counteracting the adipose-dependent deleterious growth microenvironment that a breast cancer is exposed to. This also suggests that the effects of diet and exercise that are protective against breast cancer depend on the alterations in the adipokine secretion profile from adipose tissue rather than the loss of fat mass itself.

Previous work in our lab has shown that increasing the ratio of ADIPO:LEP using recombinant proteins in cell culture subsequently increases the levels of pAMPK<sup>T172</sup> and decreases pAkt<sup>T308</sup>, causing MCF7 breast cancer cells to arrest (51). In corroboration with these results, altering these ratios by using conditioned media (CM) prepared from adipocytes isolated from CD fed animals in-vivo elicited identical effects when compared to cells treated with HFD-CM (Fig. 2). In addition, the growth environment created by the CM basically rendered the MCF7 cells unresponsive to the addition of exogenous ADIPO and LEP, which highlights the
powerful nature of the control on MCF7 cell cycle regulation exerted by the adipose-created growth microenvironment. HFD feeding decreased the ADIPO:LEP ratio in the CM which increased MCF7 cell proliferation by activating AKT and inhibiting AMPK, ultimately reducing the cell cycle inhibitory effects of p27 and fostering S-phase entry (Fig. 6 C). We have previously shown that stabilizing AdipoR1 by constitutively overexpressing the receptor we enhance the effects of ADIPO present in the media and counteract the effects of HFD on adipose-dependent alteration in tumor growth environment (51). AdipoR1 overexpression is also able to overcome the effects of addition of recombinant LEP (300 nM) to HFD-CM (Fig. 3), further highlighting the importance of maintaining ADIPO signaling in obese cancer patients. This observation has clinical relevance, since AdipoR1 protein levels are decreased in subcutaneous and visceral adipose tissue of obese women (34) and also are down regulated in pre-invasive DCIS (33). Thus, we feel that de-stabilizing the level of AdipoR1 within mammary carcinomas and in healthy breast tissue may be one of the important factors driving the increased rate and aggressiveness of breast cancers in obese women compared to lean women, in conjunction with the decreased levels of ADIPO. Increasing AdipoR1 protein levels increases the possible binding sites available for ADIPO and the cell cycle inhibitory effects of ADIPO can then be enhanced, thereby suppressing tumor growth without specifically altering individual components within the growth microenvironment. This also highlights AdipoR1 stabilization as a target for novel breast cancer pharmacological therapeutics.

Given the strong association between obesity and breast cancer development/progression, interventions directed to counteract the effects of increased fat mass on adipokine secretion profile and the accompanying promotion of breast cancer cell proliferation is an important observation to establish. The effects of PA appear to be dose-dependent and do not follow
threshold characteristics, meaning that there seems to be an effect of increasing the volume of exercise/day rather than a simple “response or no response” effect. With increased numbers we may have been able to establish a linearity of overall endocrine effects of adipose tissue on MCF7 cell cycle regulation response relative to daily km run. By plotting the effects of km run/day and effects on adipokine secretion we show linear correlations for the levels of ADIPO and LEP secreted into the culture media (Fig. 1G). While we are unable to specifically categorize the precise exercise performed (i.e. run, jog, walk) we do show that HFD-fed animals that completed PA of over 3 km/day were lighter and had smaller visceral fat depots compared to their sedentary counterparts which was accompanied by a higher ADIPO:LEP ratio secreted into the CM (Fig. 1, Table 1). Volume dependency was illustrated by the fact that HFD fed animals who performed PA that was less than 3 km/day had similar body and visceral fat masses as their sedentary counterparts, despite clear evidence of training adaptations in their hind limb muscles (Fig. 1). Despite this lack of difference in fat mass, HFD+LPA-CM had lower levels of LEP compared to HFD-CM with the ADIPO:LEP ratios being similar (Table 1). This altered LEP level may explain why we see an intermediate effect of the HFD+LPA-CM on MCF7 cells. The HFD+LPA-CM treated cells were found to have higher levels of pAMPK T172, AdipoR1 and lower levels of pAkt T308 (Fig. 4). In addition, HFD+LPA-CM increased and decreased the percentage of MCF7 cells in G0/G1 and S-phase, respectively, with respect to HFD-CM treated cells, but failed to completely abolish all of the effects of the HFD-CM treated cells (Fig. 6). Taken together these results suggest that any interventions designed to counteract the effects of obesity on breast cancers do not necessarily have to alter absolute adiposity, but do need to elicit effects on adipokine production/secretion from adipose tissue, as this appears to be the major underlying contributor to adipose-dependent control of tumor growth microenvironment.
Although our work used ADIPO and LEP as markers of adipokine secretion profile, we are in no way suggesting that these are the only adipokines of the more than 400 produced by adipocytes that underlie adipose-dependent effects. However, it is clear their levels, relative to each other, are likely candidates for accurately predicting/estimating the growth microenvironment that a breast cancer in an obese patient is exposed to. Furthermore, therapies that alter the levels/ratio of these adipokines may represent interventions with a higher chance of success in obese breast cancer patients.

In the current study we demonstrate the increasing the volume of exercise (km/day) elicits greater protection against the deleterious effects on high-fat diet in cancer cell cycle regulation. Our voluntary exercise wheel model of physical activity does not allow for specific work rates (i.e. %VO$_2$ max) to be determined, only the overall amount of work performed per day. This can be problematic when trying to prescribe a specific dose of exercise for therapeutic intervention. In order to give a gross approximation of the relative workload that the daily km completed by the animals in our study would equate to, we have tried to use literature values for an exercise protocol that would elicit adaptations in mixed gastrocnemius oxidative enzyme activities akin to what we observed. Evaluation of studies which used motorized wheel running (40) showed that despite our animals exercising at a much lower intensity (km/hour), the longer duration in our study resulted in a higher total daily distance (1.71 km/day vs. LPA 2.47 km/day & HPA 7.21 km/day). Our daily distance PA division was based on where the gap in distance covered appeared to lie and is in line with previously published data for creating low and high PA groups (21). The COX activity in mixed gastrocnemius muscle for the HFD+LPA group was 2.1-fold higher than HFD sedentary animals (Fig. 1F). A paper by Samelman et al. (2000) found that rats exercising at a treadmill exercise dose of 15m/min at a 10% gradient for 1hr/day, 5
days/wk displayed a 1.8-fold increase in COX activity of the mixed gastrocnemius compared to their sedentary counterparts (36). *LeBlanc et al.* (2008) showed that obese rats exercising at 20m/min at a 10% gradient for 1hr/day, 5 days/week had a gastrocnemius citrate synthase activity (CS) that was 1.5-fold higher than their sedentary obese counterparts (25). Previous work has shown that in rats changes in gastrocnemius COX activity in response to increased physical activity is approximately 1.5-fold higher compared to CS in the same exercising muscle (18). Using this we estimate that the 1.5-fold CS activity in Leblanc et al. would likely be accompanied by a 2.3-fold increase in COX activity. Using these changes as a guide, we estimate that the 2.8-fold increase in COX activity exhibited by our HPA group was suggestive of workload greater than the 20m/min at a 10% gradient used in this study (25). Using these two workload approximations, we can estimate a dose of exercise using a compilation of curves that approximate VO2max from speed and grade of running (3). Using figures contained in *Brooks and White* (1978) we estimate that our LPA group exercised at a rate roughly equivalent to a VO2 of 54 ml/kg/min. Using similar analyses, we estimate that the volume of exercise that our HPA group completed caused changes greater than if they were exercised at a VO2 of 60 ml/kg/min. Although the initial intent of our experiments were not intended to prescribe specific doses of exercise and we can’t prescribe specific dose rates of exercise for human patients from our study due to species and age differences and the lack of a clear absolute work rate (i.e. %VO2max), it is evident that increasing the volume of exercise, whether it be walking, jogging or running, provides a protective effect on the endocrine tumor growth microenvironment created by adipose tissue. It may also be suggestive of volume of exercise being as important as intensity of exercise. This has important ramifications clinically as older cancer patients may not
be capable of performing high-intensity (shorter duration) exercise due to numerous disease-related pathologies.

Previous work in our lab has shown that activation of AMPK signaling decreases AdipoR1 degradation, increasing AdipoR1 protein levels in a positive feedback manner (51). This is an important observation as it highlights the potential of the tumor growth microenvironment that exists within obese breast cancer patients acting through a two-pronged mechanism. Obese patients will have lower levels of circulating ADIPO which will reduce the direct growth inhibitory effects on breast cancer cells. In addition, this reduced ADIPO content will result in decreased activation of AMPK within the cancer cells, which will subsequently result in a secondary destabilization/reduction in AdipoR1 protein levels. This will reduce the number of available receptor sites for ADIPO binding at the cell surface. This mechanism may explain, in part, why obese breast cancer patients have more aggressive tumors and express lower levels of AdipoR1. This AMPK-dependent mechanism may explain why anti-diabetic medications that activate AMPK (i.e. metformin) have been associated with improvements in cancer patient prognosis (12, 13, 47). Furthermore, it may highlight the potential of nutritional supplements that activate AMPK (i.e. resveratrol) as possible augmentations to existing cancer therapies, potentially eliciting few harmful side-effects but at the same time counteracting the deleterious effects of the tumor growth microenvironment that exists in obese breast cancer patients.
REFERENCES


REFERENCES IN REVISION
Table 1. *ADIPO*:LEP ratio for adipose-derived conditioned media

<table>
<thead>
<tr>
<th>Group</th>
<th>ADIPO (ng/ml)</th>
<th>LEP (ng/ml)</th>
<th>ADIPO:LEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFD</td>
<td>558.9 ± 99.4</td>
<td>2.69 ± 0.8</td>
<td>122.1 ± 52.1</td>
</tr>
<tr>
<td>CD</td>
<td>1289.0 ± 348.9* ***</td>
<td>1.26 ± 0.3* ***</td>
<td>566.5 ± 197.2* ***</td>
</tr>
<tr>
<td>HFD+LPA</td>
<td>622.0 ± 141.0</td>
<td>1.71 ± 0.4*</td>
<td>199.7 ± 57.7</td>
</tr>
<tr>
<td>HFD+HPA</td>
<td>1052.0 ± 246.9* ***</td>
<td>1.17 ± 0.3* ***</td>
<td>529.8 ± 105.3* ***</td>
</tr>
<tr>
<td>CD+LPA</td>
<td>1618.2 ± 873.3* ***</td>
<td>1.20 ± 0.6* ***</td>
<td>704.2 ± 258.9* ***</td>
</tr>
</tbody>
</table>

HFD, high fat diet; CD, chow diet; HFD+LPA, high fat diet + low physical activity; HFD+HPA, high fat diet + high physical activity; CD+LPA, chow diet + low physical activity; * indicates significantly different from HFD, ** indicates significantly different from HFD+LPA (p<0.05, n=6/group).
FIGURE LEGENDS

Fig 1. HFD increases epididymal fat and is ameliorated with PA. Body mass changes over the 6 week protocol (A). Body weight adjusted epididymal fat mass in CD (open bar), HFD (black bar), HFD+LPA (light grey bar), HFD+HPA (dark grey bar) and CD+LPA (hatched bar) animals (B). Body weight adjusted muscle mass of the gastrocnemius (C), soleus (D) and tibialis anterior (E) muscles. Physical activity alters cytochrome C oxidase (COX) enzyme activity in the gastrocnemius muscles of CD, HFD, HFD+LPA, HFD+HPA and CD+LPA animals (F). Plotting of ADIPO:LEP ratio in CM prepared from HPA and LPA animals vs. daily km run (G). Dotted line indicates divider between HPA and LPA groups. Western blots showing levels of Akt and β-actin in adipose tissues from the indicated groups (H). Fig. A * indicates different from HFD, CD and HFD+LPA animals (p<0.05). Different letters (Figs. B-F) indicate groups that are significantly different from each other (p<0.05, n=6/group).

Fig. 2. HFD-CM antagonizes the effects of CD-CM. Representative western blots for selected proteins showing the effects of treatment with CM (+/- ADIPO or LEP) prepared from FBS (hatched bar), or CD (open bar) or HFD (black bars) animals on MockT MCF7 cells (A). Graphical representations of multiple experiments showing the effects of CM or CM plus ADIPO or LEP on pAMPK\textsuperscript{T172} (B), pAKT\textsuperscript{T308} (C), p27 (D), p27\textsuperscript{T198} (E) and AdipoR1 (F) protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other (p<0.05, n=6/group).

Fig. 3. Overexpression of AdipoR1 ameliorates the effects of the HFD-CM and LEP. Representative western blots for selected proteins showing the effects of treatment with CM (+/-
ADIPO or LEP) prepared from FBS (hatched bars), or CD (open bars), HFD (black bars) animals on AdipoR1 stably transfected MCF7 cells (A). Western blots showing the expression of the FLAG-tag and AdipoR1 in stably transfected cell lines (B). Graphical representations of multiple experiments showing the effects of CM or CM plus ADIPO or LEP on pAMPK<sup>T172</sup> (C), pAKT<sup>T308</sup> (D), p27 (E), p27<sup>T198</sup> (F) and AdipoR1 (G) protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other (p<0.05, n=6/group).

Fig. 4. Physical activity can abolish effects of a HFD on adipose-dependent tumor growth microenvironment. Representative western blots for selected proteins showing the effects of treatment with CM prepared from CD (open bar), HFD (black bar), HFD+HPA (dark grey bar), HFD+LPA (light grey bar) and CD+LPA (hatched bar) animals on MockT MCF7 cells (A). Graphical representations of multiple experiments showing the effects of CM on pAMPK<sup>T172</sup> (B), pAKT<sup>T308</sup> (C), p27 (D), p27<sup>T198</sup> (E) and AdipoR1 (F) protein levels. β-actin was used as a loading control. Different letters indicate groups that are significantly different from each other (p<0.05, n=6/group).

Fig. 5. Overexpression of AdipoR1 can counteract the effects of HFD. Representative western blots for selected proteins showing the effects of treatment with CM prepared from CD (open bar), HFD (black bar), HFD+HPA (dark grey bar), HFD+LPA (light grey bar) and CD+LPA (hatched bar) animals on AdipoR1 transfected (p31-4-2-2) MCF7 cells (A). Graphical representations of multiple experiments showing the effects of CM on pAMPK<sup>T172</sup> (B), pAKT<sup>T308</sup> (C), p27 (D), p27<sup>T198</sup> (E) and AdipoR1 (F) protein levels. β-actin was used as a
loading control. Different letters indicate groups that are significantly different from each other (p<0.05, n=6/group).

Fig. 6. Adipose-dependent growth environment causes cell cycle changes in CM experiments. Typical cell cycle profiles in MockT MCF7 cells (A) and stably transfected AdipoR1 overexpressing MCF7 cells (B). Graphical representation of multiple cell cycle profile experiments observing effects of diet on CM effects in CD (open bar), HFD (black bar) animals in MockT MCF7 cells (C) and in MCF7 cells stably overexpressing AdipoR1 (D). Graphical representation of multiple cell cycle profiles showing the effects of exercise and diet CD+LPA (hatched bar), HFD+HPA (dark grey bar) and HFD+LPA (light grey bar) on MockT MCF7 cells (E) and MCF7 cells stably overexpressing AdipoR1 (F). * indicate groups that are significantly different from HFD treated cells (p<0.05, n=6/group).
Figure 1
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Figure 6