

Voluntary physical activity abolishes the proliferative tumor growth microenvironment created by adipose tissue in animals fed a high fat diet

Christopher F. Theriau,^{1,2} Yaniv Shpilberg,¹ Michael C. Riddell,^{1,2} and Michael K. Connor^{1,2}

¹School of Kinesiology and Health Science and ²Muscle Health Research Centre, York University, Toronto, Ontario, Canada

Submitted 9 October 2015; accepted in final form 1 May 2016

Theriau CF, Shpilberg Y, Riddell MC, Connor MK. Voluntary physical activity abolishes the proliferative tumor growth microenvironment created by adipose tissue in animals fed a high fat diet. *J Appl Physiol* 121: 139–153, 2016. First published May 5, 2016; doi:10.1152/jappphysiol.00862.2015.—The molecular mechanisms behind the obesity-breast cancer association may be regulated via adipokine secretion by white adipose tissue. Specifically, adiponectin and leptin 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 whether 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 with 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 AMP-activated protein kinase (AMPK) and protein kinase B (Akt) signaling pathways, decreasing p27 protein levels via reduced phosphorylation at T198 and down-regulation of adiponectin receptor 1 (AdipoR1). PA can ameliorate these proliferative effects of HFD-CM on MCF7 cells, increasing p27^{T198} by AMPK, reducing pAkt^{T308}, 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 antiproliferative effects of PA on the HFD-CM. Thus voluntary PA represents a means to counteract the proliferative effects of adipose tissue on breast cancers in obese patients.

physical activity; obesity; adipokines; breast cancer

NEW & NOTEWORTHY

We hypothesized that voluntary physical activity (PA) would counteract the deleterious adipose-dependent growth microenvironment to which a breast cancer is exposed. 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.

OBESITY CONTINUES TO BE a growing concern not only within North America but also 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 body mass index have a more than twofold higher mortality rate compared with 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 or 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 and 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 with women without the disease (23, 29, 37). Decreased ADIPO signaling through adiponectin receptor 1 (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

Address for reprint requests and other correspondence: M. K. Connor, School of Kinesiology and Health Science, York Univ., 347 Bethune College, 4700 Keele St., Toronto, ON, M3J 1P3, Canada (e-mail: mconnor@yorku.ca).

by LEP (43a), 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 or intervention for the development of obesity and its associated effects on breast cancer (4, 9). Moderate physical activity >0.64 metabolic equivalent task (MET)-h/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 with 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 1 h/wk was associated with improved survival compared with sedentary women (17). These effects were more pronounced in women who engaged in moderately intense exercise between 3–5 h per wk (17). When exercise intensity was increased further (running >1.8 MET-h/day), breast cancer patients had an almost 90% lower risk of cancer mortality compared with women who walked (<1.07 MET-h/day) (46). Voluntary physical activity alters the production of both ADIPO and LEP in rats fed a high fat diet (HFD), lowering the levels of LEP and increasing the levels of ADIPO in the circulation compared with sedentary HFD fed animals (4, 24, 45, 50). Physical activity decreased pAkt⁴⁷³ and cyclin D1 and increased pAMPK¹⁷² and p27 within mammary carcinomas of those same animals (49). Furthermore, postmenopausal women exposed to 12 mo 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 the adipose-dependent tumor growth microenvironment remain unclear. We set out to determine whether a dose-dependent relationship between physical activity and the production or secretion of adipokines exists, and whether these effects can alter the deleterious adipose-dependent tumor growth microenvironment created in animals fed a 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 in the levels of pAMPK^{T172}, p27^{T198}, p27, and AdipoR1 and an increase in pAkt^{T308} in MCF7 cells grown in the conditioned media (CM) 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 activity completely abolishing the effects of the HFD-CM on MCF7 cell cycle regulation. In addition to physical activity, we show that overexpressing 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 wk of age) were purchased from Charles River Laboratories (Montreal, Canada) and were singly housed in standard clear, plastic cages. Male rats were used 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 12:12-h 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 h 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 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 makeup of 14% fat, 54% carbohydrate, and 32% protein (3.02 calories/g). The HFD (Harlan Laboratories, Madison, WI) had a caloric breakdown of 60% fat, 21% carbohydrate, and 18% protein (5.1 calories/g). HFD and CD fed animals were further subdivided into sedentary and physical activity (PA) groups designated as CD sedentary (CD; $n = 11$), CD low PA (CD+LPA; <3 km/day; $n = 4$), HFD sedentary (HFD; $n = 13$), and HFD PA (HFD+PA; $n = 12$) for 6 wk. Animals in the HFD PA group were further subdivided, depending on average wheel running distances, into animals that ran <3 km/day ($n = 6$) classified as the low PA group (HFD+LPA) while animals that ran >3 km/day ($n = 6$) were classed as the high PA group (HFD+HPA), to determine whether a dose response to physical activity was evident. Our physical activity cutoff of 3 km/day has also been used previously with Sprague-Dawley rats and wheel running to create low and high physical activity levels (21). Food intake and running distances were measured each day, and body weight was measured three times per week.

Tissue collection and CM. After the 6-wk 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- to 10-mg pieces, and immediately placed into 50-ml vented conical tubes containing Alpha Modification of Eagle's Medium (AMEM; 7.5 ml/g tissue; Wisent, St. Bruno, Canada) supplemented with 10% FBS (Hyclone, Thermo Fisher Scientific, Whitby, Canada), 2% antimicrobial/antibiotic (Wisent), 1 mM sodium pyruvate (Sigma, Oakville, ON), nonessential amino acids (Sigma), and 10 µg/ml insulin from human pancreas (Wisent) under sterile conditions and incubated for 24 h at 37°C with 5% CO₂. After 24 h, 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. To ensure that our mass-to-volume preparation of CM was not skewed by the presence of vast differences in adipose cellular content, we diluted 10- to 15-mg sections of adipose 30:1 in radioimmunoprecipitation assay (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 wt. The sequence in

which rats were killed was randomized across groups so as to minimize the likelihood that order effects would masquerade as treatment-associated effects. At time of death the gastrocnemius, soleus, and tibialis anterior were immediately excised, weighed, frozen, and stored at -84°C for future analyses.

Cytochrome-c oxidase activity assay. 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 midsection of the muscle belly), weighing roughly 20–30 mg, were diluted 80-fold (sedentary) or 160-fold (physical activity) in extraction buffer (100 mM Na-K-Phosphate, 2 mM EDTA, pH 7.2). Muscle extracts were prepared by homogenization with metal beads (2×30 s) 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).

Coculture adipokine measurement. The levels of ADIPO and LEP produced and secreted into the coculture 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 the manufacturer's instructions. ADIPO aliquots of CM were diluted 50-fold (HFD) and 100-fold (PA and CD; 100 μl total), while LEP ELISAs used a fivefold (HFD) or undiluted (PA and CD) CM (50 μl total) and were analyzed against standard curves. The levels of each adipokine were calculated in nanograms per milliliter values and converted to nanomolar values for stoichiometric comparison.

Cell culture. MCF7 cells were purchased from the American Tissue Culture Collection (Manassas, VA) and were maintained in AMEM, 10% FBS, 2% antimicrobial/antibiotic, 1 mM sodium pyruvate, nonessential amino acids, and 10 $\mu\text{g}/\text{ml}$ insulin from human pancreas at 37°C and 5% CO_2 .

Transformed DH5 α *E. coli* bacterial cells containing AdipoR1 plasmid vectors (OriGene, Rockville, MD) were obtained in glycerol stock. Plasmid vectors were driven by a cytomegalovirus (CMV) promoter with an *N*-terminal FLAG-tag (having the sequence motif DYKDDDDK) 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 the manufacturer's instructions (Fermentas, Burlington, ON). Transfected MCF7 cells were treated with G418 sulfate (400 $\mu\text{g}/\text{ml}$; Wisent), and G418 resistant colonies were transferred to a 24-well plate in AMEM. After 24 h, G418 was reduced to 200 $\mu\text{g}/\text{ml}$, 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 overexpressing and FLAG-expressing cells (p31-4-2-2) were seeded in six-well plates with AMEM for 24 h. At 70% confluence, cells were washed with PBS and incubated with CM produced from adipocytes for 24 h. 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 (gADIPO; Peprotech, Rocky Hill, NJ) or 300 nM human recombinant LEP (Peprotech) for 24 h.

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 polyvinylidene difluoride membranes (Bio-Rad, Mississauga, Canada), blocked for 2 h 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 (Pro-

mega, Madison, WI) and anti-goat (Santa Cruz) horseradish peroxidase secondary antibodies were used to visualize proteins using Immobilon enhanced chemiluminescence substrate (Millipore, Whitby, Canada) and detected or quantified on a Kodak In Vivo Pro imaging system (Marketlink Scientific, Burlington, Canada).

Cell cycle analyses. MCF7 cells isolated from six-well plates following trypsinization were washed in cold PBS and fixed by dropwise addition of ice-cold 70% ethanol. Cells were washed twice in PBS, resuspended in a propidium iodide/RNase solution, and subjected to fluorescence-activated cell sorting (FACS) analysis (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 \pm SEM of 5–13 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 hoc 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 values of $P \leq 0.05$ were considered to be significantly different.

RESULTS

HFD increases adiposity which is prevented by physical activity. Animals were fed either a HFD or standard CD and given access to running wheels as voluntary physical activity. Animals were placed in either 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 with 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 wt; Fig. 1B) in HFD fed sedentary animals compared with 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 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, HFD+LPA animals had no difference in total body wt or body weight adjusted epididymal fat mass compared with their sedentary counterparts (Fig. 1, A and B). The CD+LPA animals were 17% lighter and had 50% of the epididymal fat mass compared with HFD sedentary animals (Fig. 1, A and B).

To determine whether physical activity induced a training effect, we measured the weights of the gastrocnemius, soleus, and tibialis anterior muscles. Physical activity increased gastrocnemius, soleus, and tibialis relative weights in both HFD+LPA and HFD+HPA animals compared with their sedentary counterparts (Fig. 1, C, D, and E). Low physical activity and high physical activity resulted in an overall in-

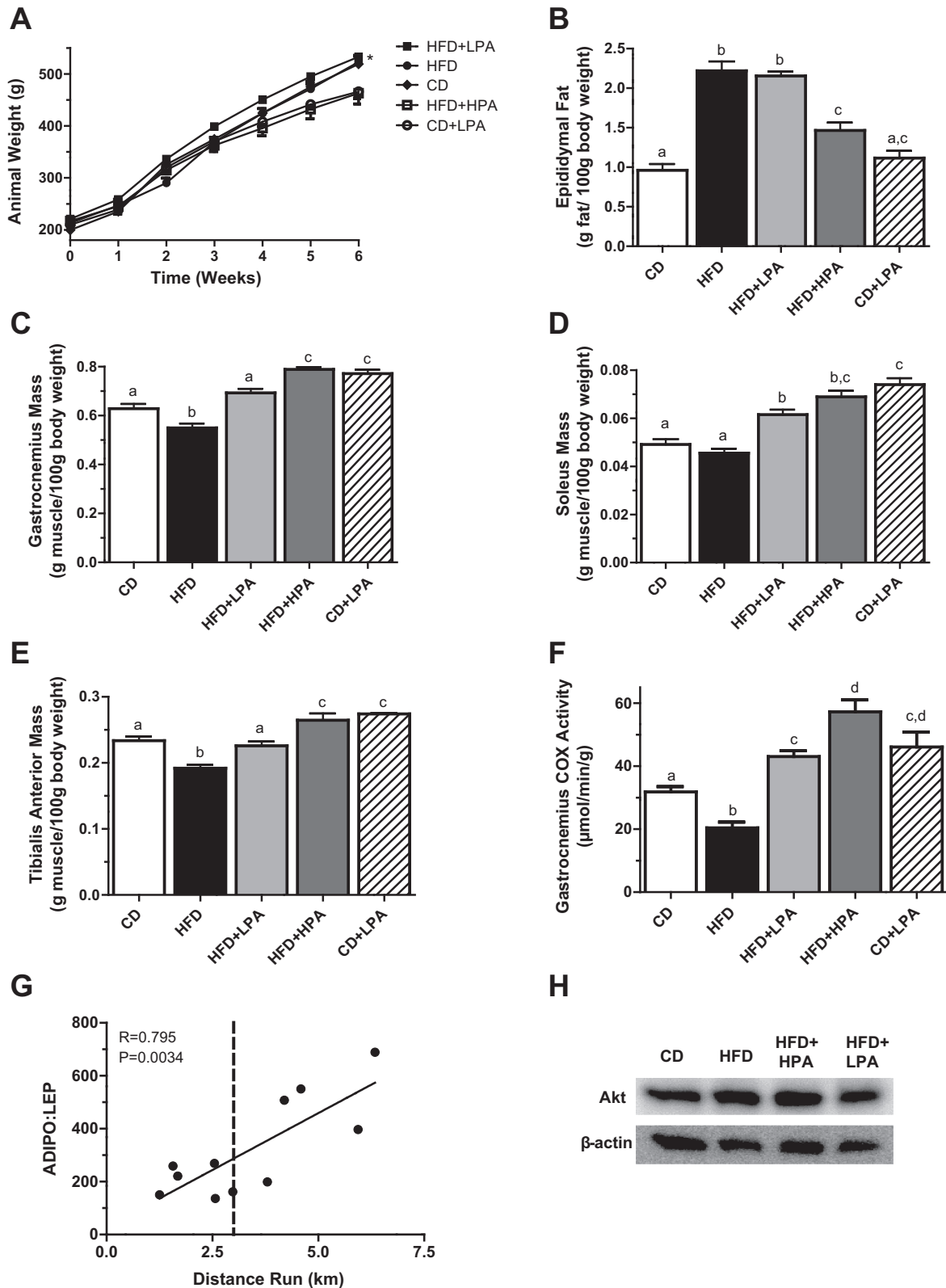


Fig. 1. High fat diet (HFD) increases epididymal fat and is ameliorated with physical activity (PA). *A*: body mass changes over the 6-wk protocol. *B*: body weight adjusted epididymal fat mass in chow diet (CD), HFD, HFD + low PA (HFD+LPA), HFD + high PA (HFD+HPA), and CD+LPA animals. Body weight adjusted muscle mass of the gastrocnemius (*C*), soleus (*D*), and tibialis anterior (*E*) muscles. *F*: physical activity alters cytochrome *c* oxidase (COX) enzyme activity in the gastrocnemius muscles of CD, HFD, HFD+LPA, HFD+HPA, and CD+LPA animals. *G*: plot of adiponectin (ADIPO) to leptin (LEP) ratio in conditioned media (CM) prepared from HPA and LPA animals vs. daily kilometers run. Dotted line, divider between HPA and LPA groups. *H*: Western blots showing levels of Akt and β -actin in adipose tissues from the indicated groups. * $P < 0.05$ (*A*), different from HFD, CD, and HFD+LPA animals. ^{a,b,c}Groups significantly different from each other ($P < 0.05$, $n = 6/\text{group}$).

crease of $27 \pm 3\%$ and $38 \pm 4\%$ 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). Low physical activity increased mixed gastrocnemius COX activity by 2.1-fold in HFD fed animals while high physical activity 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 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 with the HFD-CM (566.5 ± 197.2 vs. 122.1 ± 52.1 ng/ml; Table 1). High physical activity prevented this HFD-dependent decrease in the ratio of ADIPO:LEP. The HFD+HPA group had higher levels of ADIPO ($1,052.0 \pm 246.9$ ng/ml) and lower levels of LEP (1.17 ± 0.3 ng/ml) than 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 ($529.8 \pm 105.3:1$) compared with both HFD sedentary ($122.1 \pm 52.1:1$) and HFD+LPA ($199.7 \pm 57.7:1$) animals. The ratio of ADIPO:LEP in the CM prepared from HFD+HPA animals was not different from that prepared from both CD sedentary and CD+LPA. A linear relationship between the distance ran and the ratio of ADIPO:LEP in the CM was evident ($m = 85.94 \pm 21.83$, $R = 0.795$, $P = 0.0034$; Fig. 1G).

CM induces effects on cell signaling and cell cycle proteins in MCF7 cells. 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 whether 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). 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

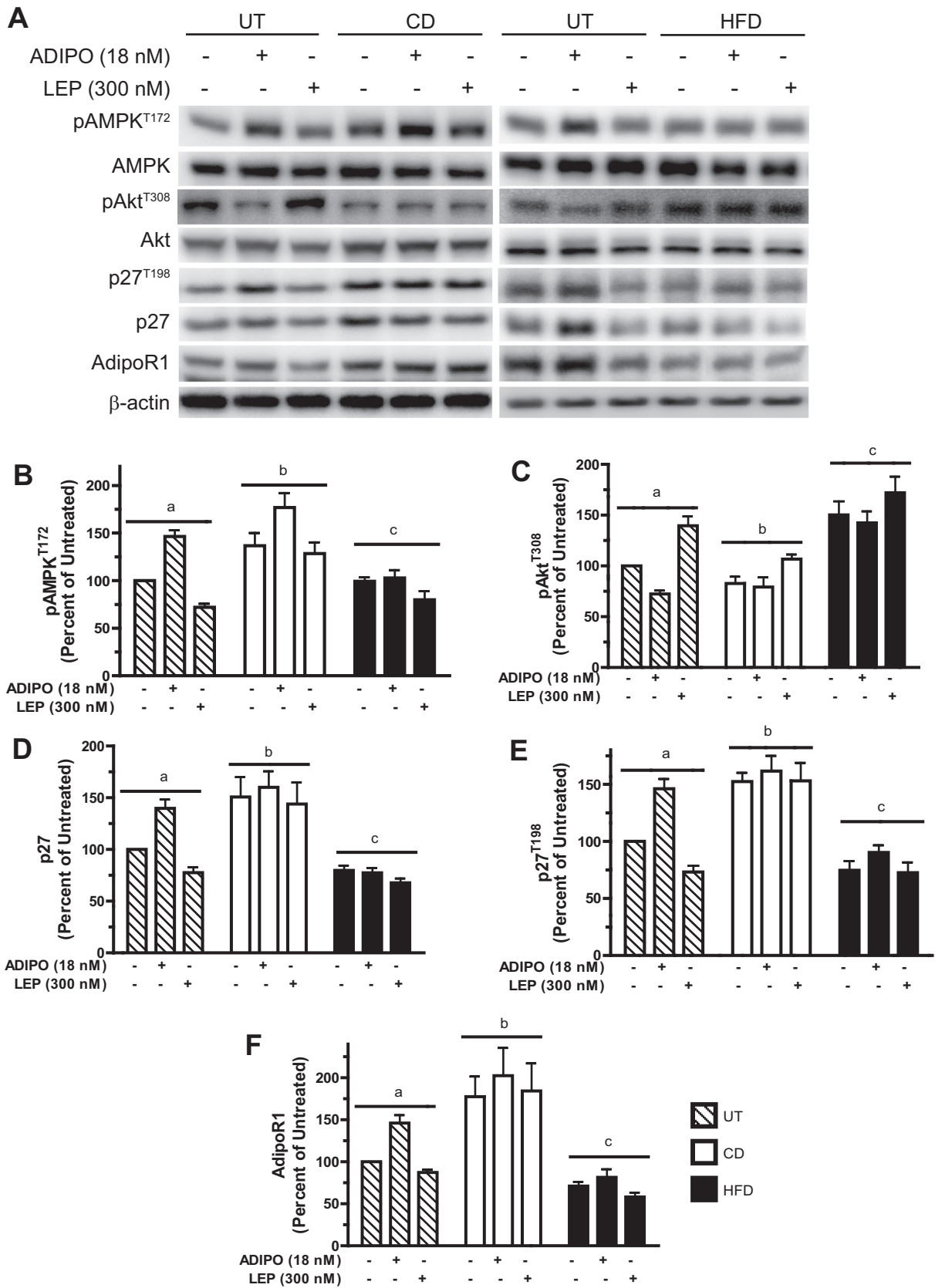
proteins loaded for any specific treatment (i.e., HFD had more protein than HFD+HPA), giving us confidence that the CM was not subjected to any preparation artifacts. However, we did see that CM treatment caused profound differences in MCF7 cells (Fig. 2). CD-CM increased pAMPK^{T172}, p27^{T198}, p27, and AdipoR1 while lowering pAkt^{T308} levels compared with 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. 2, A and B), p27 (Fig. 2, A and D), p27^{T198} (Fig. 2, A and E), or AdipoR1 (Fig. 2, A and F), or a decrease to pAkt^{T308} (Fig. 2, A and 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 and B). Antibiotic-resistant colonies were expanded (19 total), and two cell lines were chosen for their expression of the FLAG-tag and increased AdipoR1 expression compared with MockT cells (Fig. 3B). The level of overexpression of AdipoR1 in these cell lines amounted to 2.70 ± 0.46 (p31-3-2) and 2.64 ± 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. 3, A and B), p27 (Fig. 3, A and D), p27^{T198} (Fig. 3, A and E) and AdipoR1 (Fig. 3, A and F) compared with 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 with either UT or HFD-CM treated cells (Fig. 3, A and C). Unlike MockT cells, HFD-CM caused no effects on MCF7 cells compared with UT cells in all proteins except for p27 (Fig. 3, A and D). Most notably, HFD-CM treated cells caused no increase in pAkt^{T308} compared with CD-CM treated cells, again different from what was observed in MockT cells (Fig. 2, A and C vs. Fig. 3, A and C). Overall, unlike what was observed in MockT cells, the HFD-CM did not seem to cause the same effects compared with UT cells, seemingly eliminating the dominant effects of HFD-CM on MCF7 cell cycle regulation, compared with UT cells, that were apparent in MockT cells. As observed in MockT cells, addition of 18 nM gADIPO (lanes 2, 5, and 11) or 300 nM recombinant LEP (lanes 3, 6, and 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 with 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.

Table 1. ADIPO:LEP ratio for adipose-derived conditioned media

Group	ADIPO, ng/ml	LEP, ng/ml	ADIPO:LEP
HFD	558.9 ± 99.4	2.69 ± 0.8	122.1 ± 52.1
CD	$1,289.0 \pm 348.9^{*\dagger}$	$1.26 \pm 0.3^{*\dagger}$	$566.5 \pm 197.2^{*\dagger}$
HFD+LPA	622.0 ± 141.0	$1.71 \pm 0.4^{*}$	199.7 ± 57.7
HFD+HPA	$1,052.0 \pm 246.9^{*}$	$1.17 \pm 0.3^{*}$	$529.8 \pm 105.3^{*}$
CD+LPA	$1,618.2 \pm 873.3^{*\dagger}$	$1.20 \pm 0.6^{*\dagger}$	$704.2 \pm 258.9^{*\dagger}$

ADIPO, adiponectin; LEP, leptin; 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. *Significantly different from HFD, †significantly different from HFD+LPA ($P < 0.05$, $n = 6$ /group).



Voluntary physical activity ameliorates HFD-induced effects in a dose-dependent manner. Physical activity has been shown to counteract obesity and we wanted to observe whether the effects of voluntary physical activity which altered the adipokine profile (ADIPO:LEP ratio) that was created within the CM led to any changes within cocultured MCF7 cells. Voluntary physical activity elicited a dose-dependent response counteracting the effects of HFD on MCF7 cell cycle regulation (Fig. 4A, lane 3 vs. lanes 5 and 6). Specifically, HFD+HPA-CM increased pAMPK^{T172}, p27, and AdipoR1 by 93%, 67%, and 58%, respectively, compared with HFD-CM treated cells (Fig. 4 A, B, D, and F). In addition, pAkt^{T308} was decreased by 40% (Fig. 4, A and C). Furthermore, HFD+HPA-CM elicited the same effects on MCF7 cells as CD-CM (Fig. 4A, lane 2 vs. lane 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 physical activity on MCF7 cell growth displayed by the HFD+LPA-CM, increasing pAMPK^{T172} and AdipoR1 while decreasing pAkt^{T308} compared with the HFD-CM (Fig. 4, A, B, C, and F). Surprisingly, the effects of HFD+HPA observed were also similar to those elicited by CD+LPA-CM on MCF7 cell cycle regulation (Fig. 4A, lane 2 vs. lane 7). No changes in total AMPK and Akt were evident.

AdipoR1 overexpression enhances the effects of physical activity. 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, and p27^{T198}, while pAkt^{T308} levels were decreased compared with MockT cells (Fig. 4 vs. Fig. 5). While a dose response of physical activity 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} (Fig. 5, A and B), p27 (Fig. 5, A and D), and AdipoR1 (Fig. 5, A and F) by 58%, 27%, and 19%, respectively, compared with HFD-CM treated cells. Additionally, pAkt^{T308} was decreased by 25% (Fig. 5, A and C) compared with HFD-CM treatment. CD+LPA-CM increased pAMPK^{T172} by 37% above CD-CM (Fig. 5, A and B).

AdipoR1 overexpression abolishes effect of HFD and accentuates physical activity cell cycle effects in MCF7 cells. Despite the observed changes in cellular protein levels, we set out to determine whether HFD and physical activity caused corresponding overall cell cycle changes in the cultured MCF7 cells. Cell cycle status was determined using propidium iodide staining and computational analyses (Fig. 6, A and 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 with 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

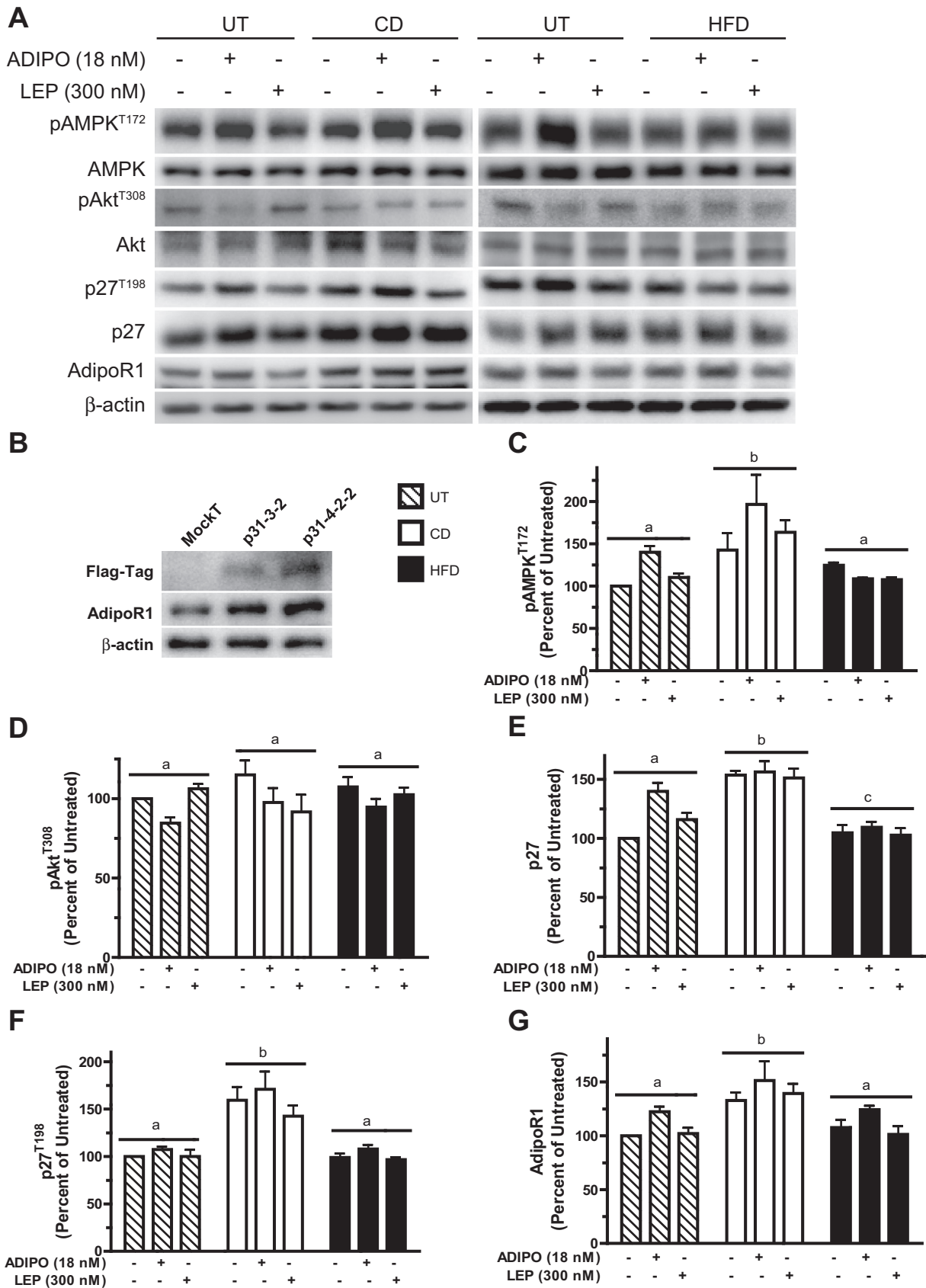
cells in S-phase in the HFD-CM compared with MockT HFD-CM treated cells (Figs. 6, C vs. D). The HFD-CM decreased the number of cells in G1/G0 by 8% and increased the number of cells in S-phase by 16% compared with CD-CM treated cells (Fig. 6D). Strikingly, AdipoR1 overexpression increased the number of cells in G0/G1 by 24% (52% vs. 41%) compared with MockT cells when exposed to HFD-CM (Fig. 6, C vs. 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 physical activity 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 physical activity. 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 with 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 with HFD-CM. The CD+PA elicited the same cell cycle effects compared with CD-CM. By overexpressing AdipoR1 within the MCF7 cells, there again was a dose response to physical activity 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 or secretion of over 400 adipose-derived proteins, with the most abundant being ADIPO and LEP (48). While each of these adipokines elicits 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 imposingly 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 the 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, because their levels are altered with obesity, and because 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.

Fig. 2. HFD-CM antagonizes the effects of CD-CM. A: representative Western blots for selected proteins showing the effects of treatment with CM (+/- ADIPO or LEP) prepared from FBS (untreated; UT), CD, or HFD animals on MockT MCF7 cells. Graphical representations of multiple experiments showing the effects of CM or CM plus ADIPO or LEP on pAMPK^{T172} (B), pAkt^{T308} (C), p27 (D), p27^{T198} (E), and ADIPO receptor 1 (AdipoR1; F) protein levels. β -Actin was used as a loading control. ^{a,b,c}Groups significantly different from each other ($P < 0.05$, $n = 6$ /group).



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 (43a). This suggests that the ADIPO:LEP ratio may represent a more reliable indicator of the tumor growth microenvironment and be a 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).

To induce adipose expansion, we employed 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 fat, we also determined whether there were combinatory effects of HFD and physical activity on adipose physiology. Because the association between obesity appears stronger in postmenopausal 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 the adipokine secretion profile with lower levels of ADIPO and higher levels of LEP compared with their lean CD fed counterparts (Table 1), a result similar to that seen in humans (41). Surprisingly, we observed no measureable 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 $17 \pm 3\%$ 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 CM 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 or prevention strategy for obesity-linked cancers. However, given that cancer patients often suffer from other comorbidities, implementing higher intensity exercise may not be an ideal option. Importantly, we demonstrate that lower intensity physical activity interventions, which 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^{T172} and decreases pAkt^{T308}, causing MCF7 breast cancer cells to arrest (43a). In corroboration with these results, altering these ratios by using CM prepared from adipocytes isolated from CD fed animals in vivo elicited identical effects compared with 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. 6C). We have previously shown that stabilizing AdipoR1 by constitutively overexpressing the receptor, enhances the effects of ADIPO present in the media and counteracts the effects of HFD on adipose-dependent alteration in the tumor growth environment (43a). 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 downregulated in preinvasive ductal carcinoma in situ (DCIS) (33). Thus we feel that destabilizing 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 with 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 or progression, interventions directed to counteract the effects of increased fat mass on the adipokine secretion profile and the accompanying promotion of breast cancer cell proliferation is an important observation to establish. The effects of physical activity 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 per 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

Fig. 3. Overexpression of AdipoR1 ameliorates the effects of the HFD-CM and LEP. A: representative Western blots for selected proteins showing the effects of treatment with CM (+/- ADIPO or LEP) prepared from FBS (UT), CD, or HFD animals on AdipoR1 stably transfected MCF7 cells. B: Western blots showing the expression of the FLAG-tag and AdipoR1 in stably transfected cell lines. Graphical representations of multiple experiments showing the effects of CM or CM plus ADIPO or LEP on pAMPK^{T172} (C), pAKT^{T308} (D), p27 (E), p27^{T198} (F), and AdipoR1 (G) protein levels. β -Actin was used as a loading control. ^{a,b,c}Groups significantly different from each other ($P < 0.05$, $n = 6$ /group).

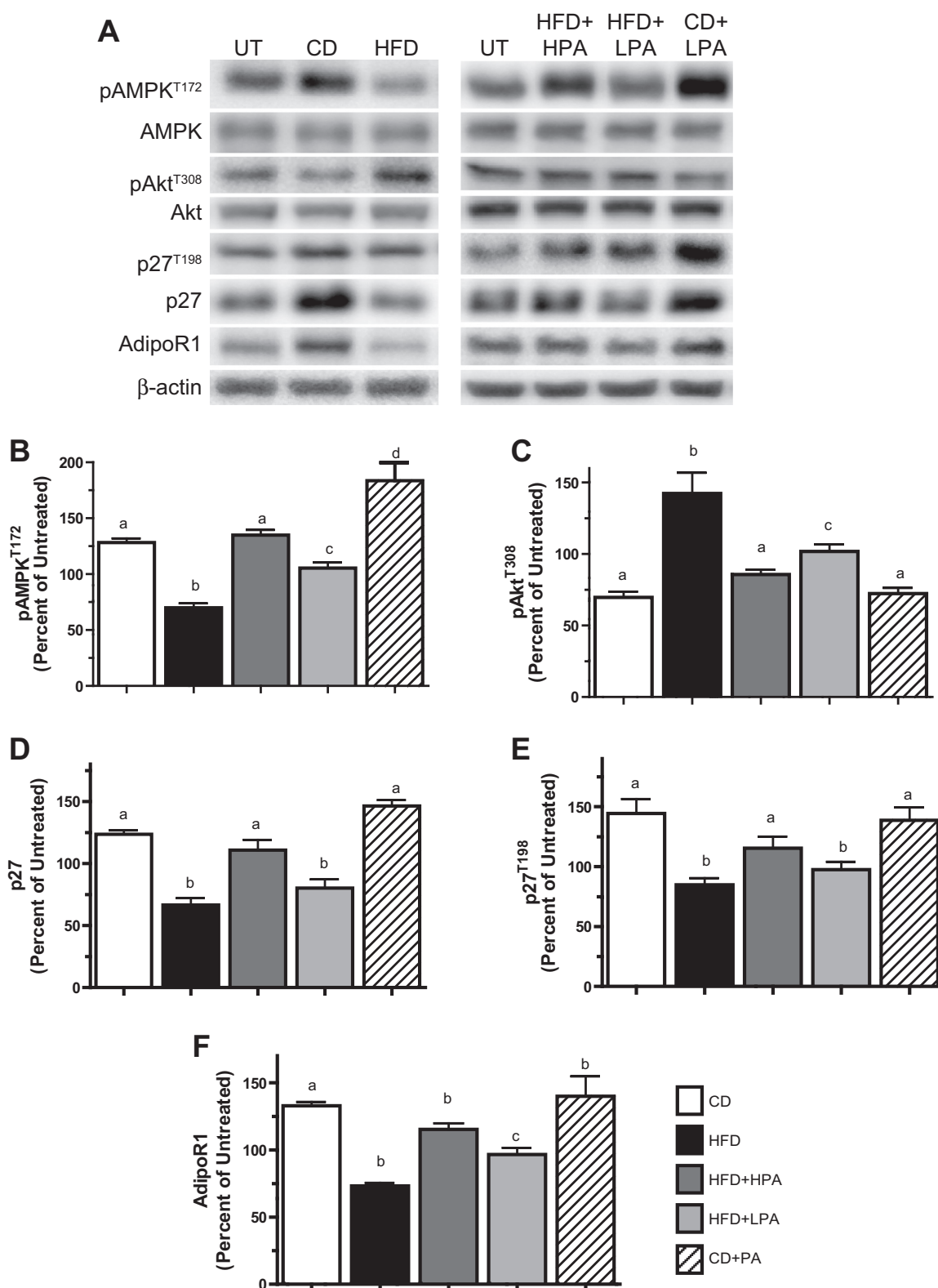


Fig. 4. Physical activity can abolish the effects of HFD on the adipose-dependent tumor growth microenvironment. *A*: representative Western blots for selected proteins showing the effects of treatment with CM prepared from CD, HFD, HFD+HPA, HFD+LPA, and CD+LPA animals on MockT MCF7 cells. Graphical representations of multiple experiments showing the effects of CM on pAMPK^{T172} (*B*), pAKT^{T308} (*C*), p27 (*D*), p27^{T198} (*E*), and AdipoR1 (*F*) protein levels. β-Actin was used as a loading control. ^{a,b,c}Groups significantly different from each other ($P < 0.05$, $n = 6/\text{group}$).

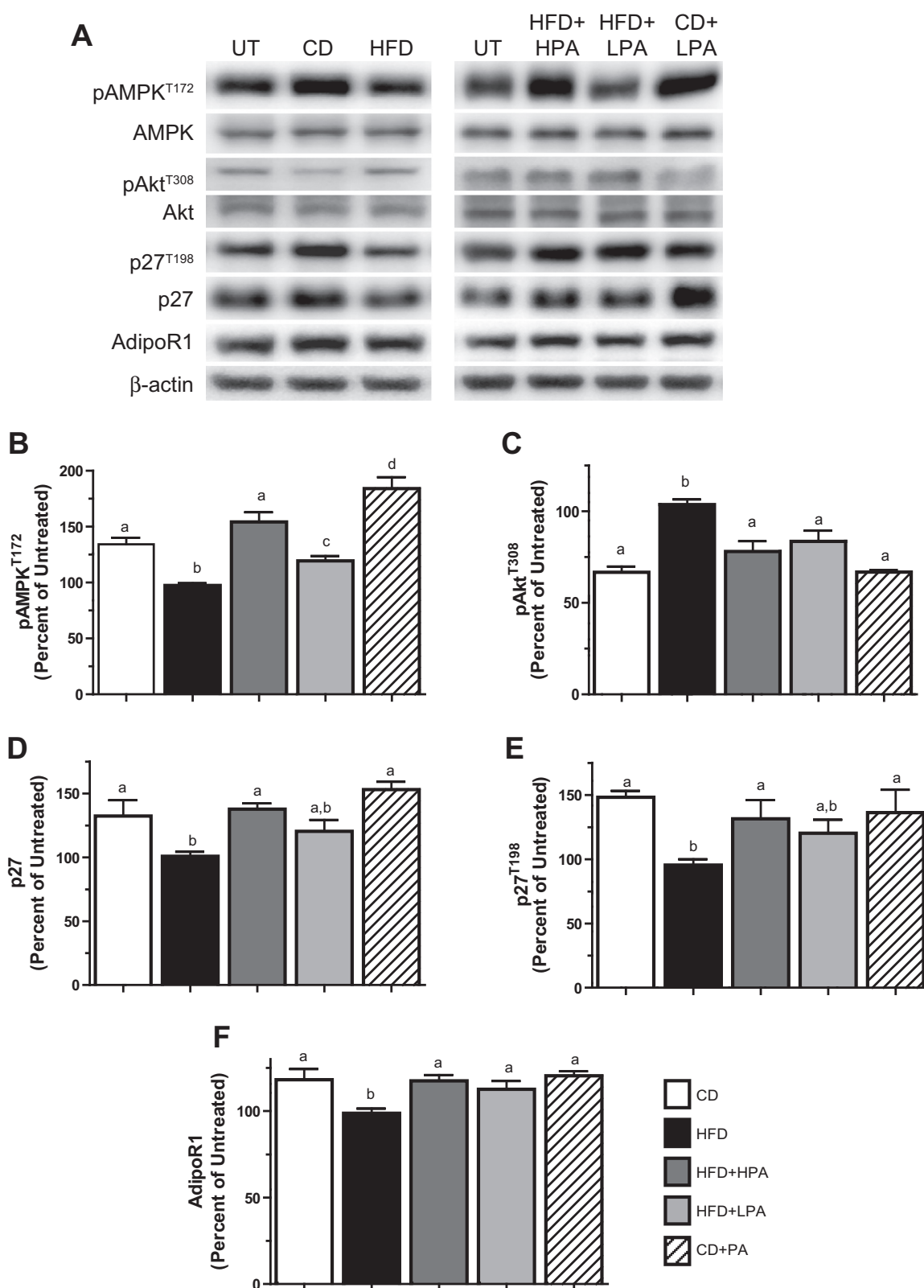


Fig. 5. Overexpression of AdipoR1 can counteract the effects of HFD. **A**: representative Western blots for selected proteins showing the effects of treatment with CM prepared from CD, HFD, HFD+HPA, HFD+LPA, and CD+LPA animals on AdipoR1 transfected (p31-4-2-2) MCF7 cells. Graphical representations of multiple experiments showing the effects of CM on pAMPK^{T172} (**B**), pAKT^{T308} (**C**), p27 (**D**), p27^{T198} (**E**), and AdipoR1 (**F**) protein levels. β-Actin was used as a loading control. ^{a,b,c}Groups significantly different from each other ($P < 0.05$, $n = 6$ /group).

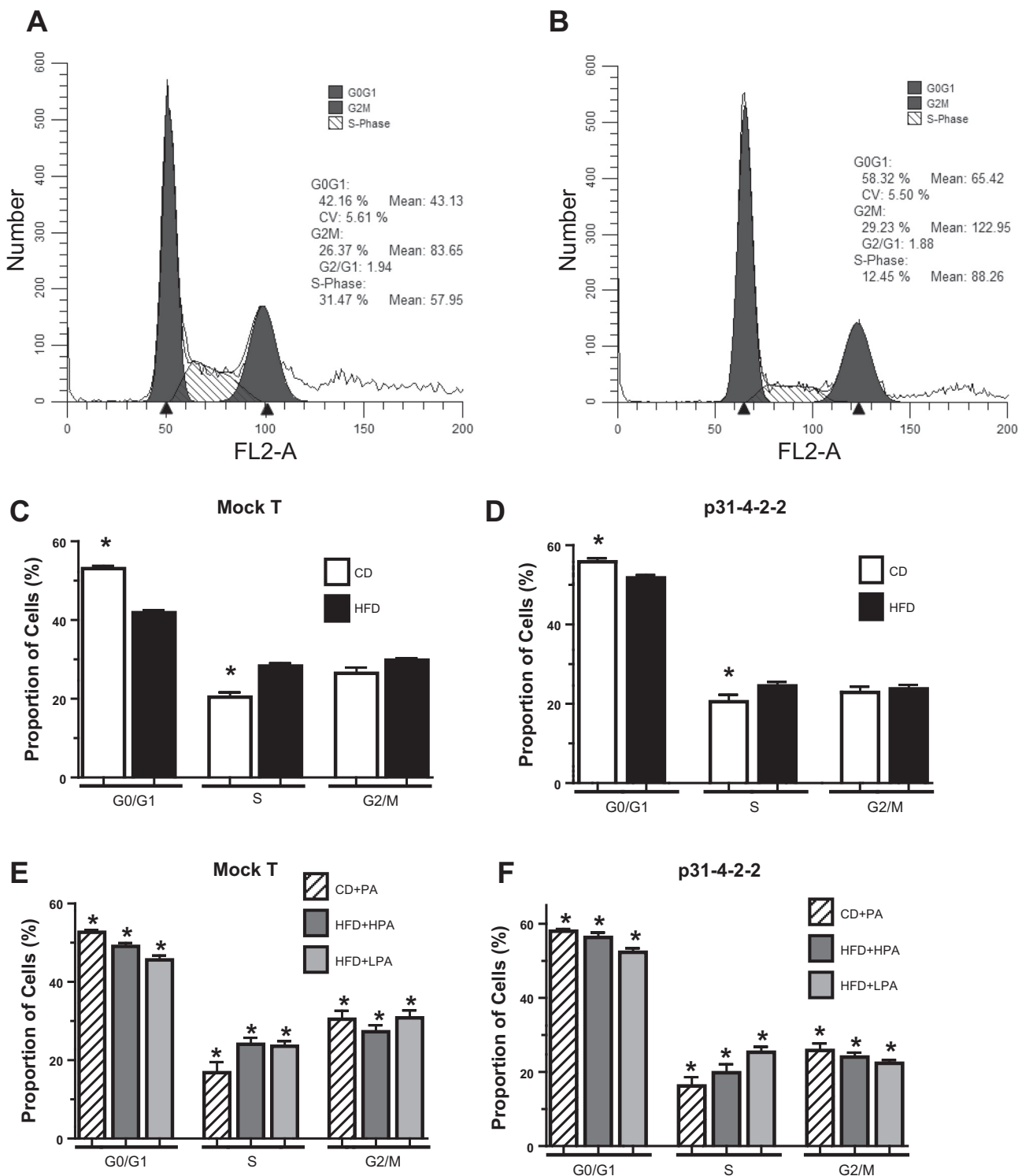


Fig. 6. Adipose-dependent growth environment causes cell cycle changes in CM experiments. Typical cell cycle profiles in MockT MCF7 cells (A) and stably transfected AdipoR1 overexpressing MCF7 cells (B). FL2-A, FL2-Area, total cell fluorescence. Graphical representation of multiple cell cycle profile experiments observing effects of diet on CM effects in CD and HFD 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, HFD+HPA, and HFD+LPA on MockT MCF7 cells (E) and MCF7 cells stably overexpressing AdipoR1 (F). *Groups significantly different from HFD treated cells ($P < 0.05$, $n = 6$ /group).

MCF7 cell cycle regulation response relative to daily kilometers run. By plotting the effects of kilometers run per day and effects on adipokine secretion, we showed linear correlations for the levels of ADIPO and LEP secreted into the culture

media (Fig. 1G). Although we were unable to specifically categorize the precise exercise performed (i.e., run, jog, walk), we showed that HFD fed animals that completed physical activity of over 3 km/day were lighter and had smaller visceral

fat depots compared with their sedentary counterparts, which was accompanied by a higher ADIPO:LEP ratio secreted into the CM (Fig. 1 and Table 1). Volume dependency was illustrated by the fact that HFD fed animals that performed physical activity that was <3 km/day had similar body and visceral fat masses as their sedentary counterparts, despite clear evidence of training adaptations in their hindlimb muscles (Fig. 1). Despite this lack of difference in fat mass, HFD+LPA-CM had lower levels of LEP compared with HFD-CM, with the ADIPO:LEP ratios being similar (Table 1). This altered LEP level may explain why we saw 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}, and 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 or secretion from adipose tissue, as this appears to be the major underlying contributor to adipose-dependent control of the tumor growth microenvironment. Although our work used ADIPO and LEP as markers of the adipokine secretion profile, we in no way suggest that these are the only adipokines of the more than 400 produced by adipocytes that underlie adipose-dependent effects. However, it is clear that their levels, relative to each other, are likely candidates for accurately predicting or estimating the growth microenvironment to which a breast cancer in an obese patient is exposed. Furthermore, therapies that alter the levels or ratio of these adipokines may represent interventions with a higher chance of success in obese breast cancer patients.

In the current study we demonstrate that increasing the volume of exercise (km/day) elicits greater protection against the deleterious effects of a 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., % $\dot{V}O_{2max}$) 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. To give a gross approximation of the relative workload that the daily kilometers 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/h), the longer duration in our study resulted in a higher total daily distance (1.71 km/day vs. LPA 2.47 km/day and HPA 7.21 km/day). Our daily distance physical activity 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 physical activity 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. (36) found that rats exercising at a treadmill exercise dose of 15 m/min at a 10% gradient for 1 h/day, 5 days/wk displayed a 1.8-fold increase in COX activity of the mixed gastrocnemius compared with their sed-

entary counterparts (36). LeBlanc et al. (25) showed that obese rats exercising at 20 m/min at a 10% gradient for 1 h/day, 5 days/wk 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 with CS in the same exercising muscle (18). Using this, we estimate that the 1.5-fold CS activity in the study by Leblanc et al. (25) 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 a workload greater than the 20 m/min at a 10% gradient used in this study. Using these two workload approximations, we can estimate a dose of exercise using a compilation of curves that approximate $\dot{V}O_{2max}$ from speed and grade of running (3). Using values contained in a study by Brooks and White (3), we estimate that our LPA group exercised at a rate roughly equivalent to a $\dot{V}O_2$ of 54 ml·kg⁻¹·min⁻¹. 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 $\dot{V}O_2$ of 60 ml·kg⁻¹·min⁻¹. Although the initial intent of our experiments was not to prescribe specific doses of exercise and we cannot 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., % $\dot{V}O_{2max}$), 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, because 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 (Theriau and Connor, unpublished). This is an important observation, because it highlights the potential of the tumor growth microenvironment that exists within obese breast cancer patients to act 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 of or 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.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

C.F.T., M.C.R., and M.K.C. conception and design of research; C.F.T. and Y.S. performed experiments; C.F.T. analyzed data; C.F.T. and M.K.C. interpreted results of experiments; C.F.T. prepared figures; C.F.T. drafted manuscript; C.F.T. and M.K.C. edited and revised manuscript; C.F.T., M.C.R., and M.K.C. approved final version of manuscript.

REFERENCES

- Abbenhardt C, McTiernan A, Alfano CM, Wener MH, Campbell KL, Duggan C, Foster-Schubert KE, Kong A, Toriola AT, Potter JD, Mason C, Xiao L, Blackburn GL, Bain C, Ulrich CM. Effects of individual and combined dietary weight loss and exercise interventions in postmenopausal women on adiponectin and leptin levels. *J Intern Med* 274: 163–175, 2013.
- Ashizawa N, Yahata T, Quan J, Adachi S, Yoshihara K, Tanaka K. Serum leptin-adiponectin ratio and endometrial cancer risk in postmenopausal female subjects. *Gynecol Oncol* 119: 65–69, 2010.
- Brooks GA, White TP. Determination of metabolic and heart rate responses of rats to treadmill exercise. *J Appl Physiol* 45: 1009–1015, 1978.
- Bradley RL, Jeon JY, Liu FF, Maratos-Flier E. Voluntary exercise improves insulin sensitivity and adipose tissue inflammation in diet-induced obese mice. *Am J Physiol Endocrinol Metab* 295: E586–E594, 2008.
- Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U. S. adults. *N Engl J Med* 348: 1625–1638, 2003.
- Chen DC, Chung YF, Yeh YT, Chaung HC, Kuo FC, Fu OY, Chen HY, Hou MF, Yuan SS. Serum adiponectin and leptin levels in Taiwanese breast cancer patients. *Cancer Lett* 237: 109–114, 2006.
- Chung H, Lee YS, Mayoral R, Oh DY, Siu JT, Webster NJ, Sears DD, Olefsky JM, Ellies LG. Omega-3 fatty acids reduce obesity-induced tumor progression independent of GPR120 in a mouse model of postmenopausal breast cancer. *Oncogene* 34: 3504–3513, 2014.
- Connor MK, Takahashi M, Hood DA. Tissue-specific stability of nuclear and mitochondrially encoded mRNAs. *Arch Biochem Biophys* 333: 103–108, 1996.
- Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 334: 292–295, 1996.
- Dieudonne MN, Bussiere M, Dos Santos E, Leneuve MC, Giudicelli Y, Pecquery R. Adiponectin mediates antiproliferative and apoptotic responses in human MCF7 breast cancer cells. *Biochem Biophys Res Commun* 345: 271–279, 2006.
- Dieudonne MN, Machinal-Quelin F, Serazin-Leroy V, Leneuve MC, Pecquery R, Giudicelli Y. Leptin mediates a proliferative response in human MCF7 breast cancer cells. *Biochem Biophys Res Commun* 293: 622–628, 2002.
- Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. *Cancer Res* 67: 10804–10812, 2007.
- Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ* 330: 1304–1305, 2005.
- Garofalo C, Koda M, Cascio S, Sulkowska M, Kanczuga-Koda L, Golaszewska J, Russo A, Sulkowski S, Surmacz E. Increased expression of leptin and the leptin receptor as a marker of breast cancer progression: possible role of obesity-related stimuli. *Clin Cancer Res* 12: 1447–1453, 2006.
- Grossmann ME, Nkhata KJ, Mizuno NK, Ray A, Cleary MP. Effects of adiponectin on breast cancer cell growth and signaling. *Br J Cancer* 98: 370–379, 2008.
- Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C; National Heart, Lung, and Blood Institute; American Heart Association. Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation* 109: 433–438, 2004.
- Holmes MD, Chen WY, Feskanich D, Kroenke CH, Colditz GA. Physical activity and survival after breast cancer diagnosis. *JAMA* 293: 2479–2486, 2005.
- Hood DA, Simoneau JA, Kelly AM, Pette D. Effect of thyroid status on the expression of metabolic enzymes during chronic stimulation. *Am J Physiol Cell Physiol* 263: C788–C793, 1992.
- Iyengar P, Combs TP, Shah SJ, Gouon-Evans V, Pollard JW, Albanese C, Flanagan L, Tenniswood MP, Guha C, Lisanti MP, Pestell RG, Scherer PE. Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization. *Oncogene* 22: 6408–6423, 2003.
- Jarde T, Caldefie-Chez F, Goncalves-Mendes N, Mishellany F, Buechler C, Penault-Llorca F, Vasson MP. Involvement of adiponectin and leptin in breast cancer: clinical and in vitro studies. *Endocr Relat Cancer* 16: 1197–1210, 2009.
- Jiang W, Zhu Z, Thompson HJ. Effects of limiting energy availability via diet and physical activity on mammalian target of rapamycin-related signaling in rat mammary carcinomas. *Carcinogenesis* 34: 378–387, 2013.
- Kampert JB, Blair SN, Barlow CE, Kohl HW III. Physical activity, physical fitness, and all-cause and cancer mortality: a prospective study of men and women. *Ann Epidemiol* 6: 452–457, 1996.
- Kim HS. Leptin and leptin receptor expression in breast cancer. *Cancer Res Treat* 41: 155–163, 2009.
- Krawczewski Carhuatanta KA, Demuro G, Tschop MH, Pfluger PT, Benoit SC, Obici S. Voluntary exercise improves high-fat diet-induced leptin resistance independent of adiposity. *Endocrinology* 152: 2655–2664, 2011.
- LeBlanc PJ, Mulligan M, Antolić A, MacPherson L, Inglis JG, Martin D, Roy BD, Peters SJ. Skeletal muscle type comparison of pyruvate dehydrogenase phosphatase activity and isoform expression: effects of obesity and endurance training. *Am J Physiol Regul Integr Comp Physiol* 295: R1224–R1230, 2008.
- Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL, Slingerland JM, Mills GB. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol* 9: 218–224, 2007.
- Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, Slingerland JM. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med* 8: 1153–1160, 2002.
- Mantzoros C, Petridou E, Dessypris N, Chavelas C, Dalamaga M, Alexe DM, Papadiamantis Y, Markopoulos C, Spanos E, Chrousos G, Trichopoulos D. Adiponectin and breast cancer risk. *J Clin Endocrinol Metab* 89: 1102–1107, 2004.
- Miyoshi Y, Funahashi T, Kihara S, Taguchi T, Tamaki Y, Matsuzawa Y, Noguchi S. Association of serum adiponectin levels with breast cancer risk. *Clin Cancer Res* 9: 5699–5704, 2003.
- Paffenbarger RS Jr, Lee IM, Wing AL. The influence of physical activity on the incidence of site-specific cancers in college alumni. *Adv Exp Med Biol* 322: 7–15, 1992.
- Parekh N, Chandran U, Bandera EV. Obesity in cancer survival. *Annu Rev Nutr* 32: 311–342, 2012.
- Park J, Euhus DM, Scherer PE. Paracrine and endocrine effects of adipose tissue on cancer development and progression. *Endocr Rev* 32: 550–570, 2011.
- Pfeiler G, Hudelist G, Wulffing P, Mattsson B, Konigsberg R, Kubista E, Singer CF. Impact of AdipoR1 expression on breast cancer development. *Gynecol Oncol* 117: 134–138, 2010.
- Rasmussen MS, Lihn AS, Pedersen SB, Bruun JM, Rasmussen M, Richelsen B. Adiponectin receptors in human adipose tissue: effects of obesity, weight loss, and fat depots. *Obesity (Silver Spring)* 14: 28–35, 2006.
- Rockhill B, Willett WC, Hunter DJ, Manson JE, Hankinson SE, Colditz GA. A prospective study of recreational physical activity and breast cancer risk. *Arch Intern Med* 159: 2290–2296, 1999.
- Samelman TR, Shiry LJ, Cameron DF. Endurance training increases the expression of mitochondrial and nuclear encoded cytochrome c oxidase subunits and heat shock proteins in rat skeletal muscle. *Eur J Appl Physiol* 83: 7–22, 2000.
- Schaffler A, Scholmerich J, Buechler C. Mechanisms of disease: adipokines and breast cancer - endocrine and paracrine mechanisms that

- connect adiposity and breast cancer. *Nat Clin Pract Endocrinol Metab* 3: 345–354, 2007.
38. **Schrauwen P, Westerterp KR.** The role of high-fat diets and physical activity in the regulation of body weight. *Br J Nutr* 84: 417–427, 2000.
39. **Sesso HD, Paffenbarger RS Jr, Lee IM.** Physical activity and breast cancer risk in the College Alumni Health Study (United States). *Cancer Causes Control* 9: 433–439, 1998.
40. **Shepherd RE, Gollnick PD.** Oxygen uptake of rats at different work intensities. *Eur J Physiol* 362: 219–222, 1976.
41. **Silha JV, Krsek M, Skrha JV, Sucharda P, Nyomba BL, Murphy LJ.** Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. *Eur J Endocrinol* 149: 331–335, 2003.
42. **Sneddon A, Steel JM, Strong JA.** Effect of thyroid function and of obesity on discriminant function for mammary carcinoma. *Lancet* 2: 892–894, 1968.
43. **Sutherland LN, Bomhof MR, Capozzi LC, Basaraba SA, Wright DC.** Exercise and adrenaline increase PGC-1 α mRNA expression in rat adipose tissue. *J Physiol* 587: 1607–1617, 2009.
44. **Thompson HJ, Wolfe P, McTiernan A, Jiang W, Zhu Z.** Wheel running-induced changes in plasma biomarkers and carcinogenic response in the 1-methyl-1-nitrosourea-induced rat model for breast cancer. *Cancer Prev Res* 3: 1484–1492, 2010.
45. **Thune I, Furberg AS.** Physical activity and cancer risk: dose-response and cancer, all sites and site-specific. *Med Sci Sports Exerc* 33: Suppl: S530–S550; S609–S610, 2001.
46. **Williams PT.** Significantly greater reduction in breast cancer mortality from post-diagnosis running than walking. *Int J Cancer* 135: 1195–1202, 2014.
47. **Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M.** Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. *Cancer Res* 66: 10269–10273, 2006.
48. **Zhong J, Krawczyk SA, Chaerkady R, Huang H, Goel R, Bader JS, Wong GW, Corkey BE, Pandey A.** Temporal profiling of the secretome during adipogenesis in humans. *J Proteome Res* 9: 5228–5238, 2010.
49. **Zhu Z, Jiang W, Sells JL, Neil ES, McGinley JN, Thompson HJ.** Effect of nonmotorized wheel running on mammary carcinogenesis: circulating biomarkers, cellular processes, and molecular mechanisms in rats. *Cancer Epidemiol Biomarkers Prev* 17: 1920–1929, 2008.
50. **Zhu Z, Jiang W, Zacher JH, Neil ES, McGinley JN, Thompson HJ.** Effects of energy restriction and wheel running on mammary carcinogenesis and host systemic factors in a rat model. *Cancer Prev Res (Phila)* 5: 414–422, 2012.

