

Calorie restriction and rapamycin inhibit MMTV-Wnt-1 mammary tumor growth in a mouse model of postmenopausal obesity

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Abstract

Obesity is an established risk and progression factor for postmenopausal breast cancer. Interventions to decrease caloric intake and/or increase energy expenditure beneficially impact tumor progression in normoweight humans and animal models. However, despite the increasingly high global prevalence of obesity, the effects and underlying mechanisms of these energy balance modulating interventions are poorly characterized in obese individuals. The goal of this study was to better characterize the mechanism(s) responsible for the link between energy balance and breast cancer progression in the postmenopausal obesity context. We compared the effects of calorie restriction (CR), treadmill exercise (EX), and mammalian target of rapamycin (mTOR inhibitor) treatment on body composition, serum biomarkers, cellular signaling, and mammary tumor growth in obese mice. Ovariectomized C57BL/6 mice were administered a diet-induced obesity regimen for 8 weeks, then randomized into three treatment groups: control (semipurified diet fed *ad libitum*, maintained the obese state); 30% CR (isonutrient relative to control except 30% reduction in carbohydrate calories); and EX (control diet fed *ad libitum* plus treadmill exercise). Mice were implanted with syngeneic MMTV-Wnt-1 mammary tumor cells at week 12. Rapamycin treatment (5 mg/kg every 48 h) started at week 14. Tumors were excised at week 18. CR and rapamycin (but not EX) significantly reduced final tumor weight compared to control. In follow-up analysis, constitutive activation of mTOR ablated the inhibitory effects of CR on Wnt-1 mammary tumor growth. We conclude that mTOR inhibition may be a pharmacologic strategy to mimic the anticancer effects of CR and break the obesity–breast cancer progression link.

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Introduction

As the most frequently diagnosed female cancer in the USA, and the second leading cause of death in women, breast cancer claims more than 63 000 lives annually (American Cancer Society 2010). Obesity is associated with poor breast cancer prognosis, particularly in postmenopausal women (Petrelli *et al.* 2002). Over the last 40 years, the number of obese postmenopausal women increased dramatically in the USA (Flegal *et al.* 2010), putting more women at risk for the

detrimental effects of obesity on breast cancer outcome.

The most commonly recommended lifestyle-based strategies for preventing or reversing obesity are reduced calorie diet regimens and increased physical activity (AICR 2007). In experimental animals, calorie restriction (CR) refers to diet regimens formulated so that the amount of calories consumed is decreased (typically ~30%), while micronutrient levels are maintained constant relative to an *ad libitum*-fed

control group. CR effectively reduces tumor development, including spontaneous and transplanted mammary tumors, in multiple rodent models when begun in early life, but its effects in obese animals are poorly understood (Hursting *et al.* 2010). Exercise (defined as planned, structured, and repetitive bodily movements done to improve or maintain physical fitness) at intensities >70% of maximal aerobic capacity reduces mammary and colon tumorigenesis in normoweight rodent models (Thompson 1997). Physical activity in normoweight or overweight, but not obese, postmenopausal women improves prognosis of breast cancer, suggesting that obesity may impact the response to energy balance interventions such as exercise (McTiernan *et al.* 2003, Holmes *et al.* 2005).

Although the effects of CR (and to a lesser extent exercise) on body composition and mammary tumor growth have been established in normoweight animal models, the effects and underlying mechanisms of action of CR and exercise interventions on tumor burden in obese animal models have not been reported. Obesity-related serum hormones may contribute to breast cancer progression through regulation of cell growth. Insulin and leptin stimulate mammary tumor cell growth *in vitro* (Hursting & Berger 2010). Most obese humans have increased serum leptin levels, and elevated circulating leptin and/or increased expression of leptin receptors in breast cancer tissue is associated with poor prognosis (Ishikawa *et al.* 2004, Miyoshi *et al.* 2006). Conversely, adiponectin exerts antiproliferative effects in mammary tumor cells (Kang *et al.* 2005, Dieudonne *et al.* 2006). Circulating adiponectin levels negatively correlate with body weight, body mass index, and body fat (Ryan *et al.* 2003); and lower circulating adiponectin levels are associated with increased breast cancer risk (Tworoger *et al.* 2007). Intervention studies show that diet- or exercise-induced weight loss lowers serum levels of insulin and leptin and increases adiponectin levels (Ross *et al.* 2004, Weiss *et al.* 2006). Adiponectin levels also increase following bariatric surgery (Yang *et al.* 2001).

The energy balance-related hormones exert at least some of their effects through a common signaling intermediate, the mammalian target of rapamycin (mTOR). Elevated mTOR activity is common under conditions of obesity (Dann *et al.* 2007, Moore *et al.* 2008) and in the majority of human cancers (Menon & Manning 2008). Binding of insulin to its receptors activates the phosphatidylinositol-3 kinase (PI3K)/AKT pathway (Taniguchi *et al.* 2006), known to stimulate mTOR activity (Hay & Sonenberg 2004). Leptin activates both the PI3K/AKT and ERK pathways (Garofalo *et al.* 2004), which also lead to

mTOR activation (Roux *et al.* 2007). Adiponectin, on the other hand, activates AMPK (Goldstein & Scalia 2004, Luo *et al.* 2005), resulting in mTOR inhibition (Kimura *et al.* 2003). The major targets of mTOR are components of the translational machinery, with S6K1 and 4EBP1 as the best characterized downstream effectors (Hay & Sonenberg 2004). Rapamycin is a specific mTOR antagonist that inhibits mammary tumor cell growth *in vitro* and *in vivo* (Inoki *et al.* 2006, Namba *et al.* 2006). An analog of rapamycin, RAD001 (Afinitor; Novartis Pharmaceutical Corp.), is FDA-approved for use in patients with advanced renal cell carcinoma, and other mTOR inhibitors are being tested in various cancers. However, the effects of mTOR antagonists on breast tumor growth have not been studied in the context of postmenopausal obesity.

The purpose of this study was to directly compare mammary tumor growth and selected physiological and molecular changes in response to CR, treadmill exercise (EX), or rapamycin in a mouse model of postmenopausal obesity. To the best of our knowledge, this is the first study to compare the effects of these treatments in the context of obesity. Here we demonstrate that CR, but not EX, significantly reduced body weight, obesity-related serum markers, and mammary tumor burden in obese mice. Moreover, we provide evidence that rapamycin mimics the effects of CR, suggesting that components of the mTOR pathway represent mechanistic targets for blocking the effects of obesity on mammary cancer progression.

Materials and methods

The effects of CR, treadmill exercise, or rapamycin on body composition and transplanted Wnt-1 tumor growth

The University of Texas at Austin Institutional Animal Care and Use Committee approved all animal protocols. As shown in Fig. 1, ovariectomized 6–8 week old C57BL/6 mice ($n=105$) were administered a diet-induced obesity (DIO) regimen consisting of *ad libitum* access to a 60 kcal % fat diet (D12492; Research Diets, Inc., New Brunswick, NJ, USA) for 8 weeks. Mice were then randomized to receive a control diet ($n=45$), a treadmill exercise regimen (plus the control diet; $n=30$), or a 30% CR regimen ($n=30$) for 10 weeks. Our pilot studies showed that total calorie intake, body composition, and serum biomarkers do not significantly change in mice switched from the DIO to the control diet after they have become obese, at least in part because the mice consume more of the low fat/high carbohydrate control diet. Therefore, to control for

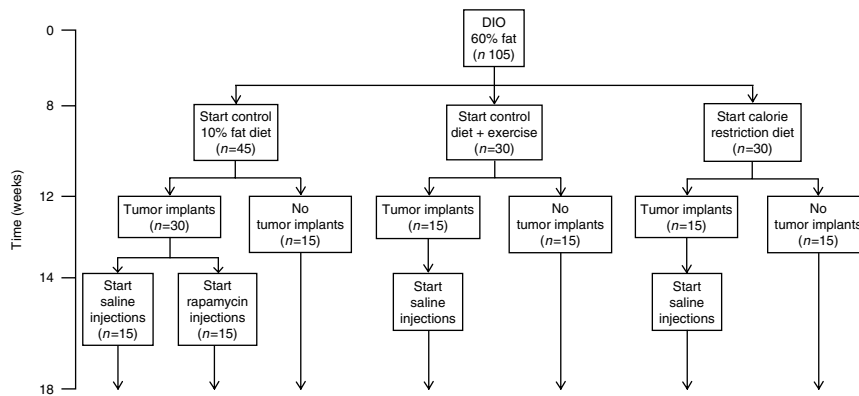


Figure 1 Experimental design. Diet-induced obesity (DIO) weight gain phase (weeks 1–8) followed by randomization to control, control + exercise, or calorie restriction (CR) diets. Some mice received MMTV-Wnt-1 mammary tumor cell suspension implants (week 12) which were allowed to grow for 6 weeks. A rapamycin treatment group started at week 14 with injections every 48 h. All other tumor implanted groups (control, control + exercise, and CR) received saline injections. A subgroup within each treatment did not receive tumors.

differential fat intakes between the diet treatments, all mice were switched to the control or CR diet (which provided the same amount of fat) after the initial 8 weeks of DIO. In this way, the mice switched to the control diet (without treadmill exercise) maintained the obese state and served as the reference group for the CR, EX, or rapamycin interventions. All diets were purchased from Research Diets, Inc. The control diet regimen was a modified AIN-76A diet (D12450B, 10 kcal % fat, administered *ad libitum*). The CR group received a diet regimen (D0302702, administered in daily aliquots) providing 30% fewer calories from carbohydrates compared to the control diet, with all other components, including fatty acids, being isonutrient when intake was limited to 70% of mean kilocalorie consumption of the control group (Yakar *et al.* 2006, Nunez *et al.* 2008). The exercise group received the control diet (fed *ad libitum*) and was also run on a variable speed treadmill 5 days/week on a 5% grade, beginning with 10 min/day at 12 m/min. Time and intensity were increased gradually over the next 2 weeks and were maintained at 40 min/day at a rate of 20 m/min ($\sim 70\%$ VO_2 max) for the duration of the experiment. Mice in the control and CR groups were placed on a stationary treadmill and remained stationary for the same time periods as the exercised group.

Four weeks after randomization to the control diet, CR regimen, or control diet plus EX regimen (week 12 on study), a subgroup of randomly selected mice ($n=30$ controls; $n=15$ exercised, $n=15$ CR) was injected with 5×10^4 syngeneic MMTV-Wnt-1 mammary tumor cells as previously described (Nunez *et al.* 2008). In brief, a cell suspension made from three

pooled MMTV-Wnt-1 mammary tumors was quantified using trypan blue and injected in the fourth mammary fat pad. Two weeks after tumor cell injection (week 14 on study), the control mice were further randomized to receive either 5 mg/kg rapamycin (0.1% DMSO in saline as vehicle; $n=15$) or vehicle ($n=15$), by i.p. injection every 48 h (Xing & Orsulic 2005). Starting at week 14 on study, mice injected with tumor cells in the CR and exercise groups also received i.p. vehicle injections every 48 h. Mammary tumors were palpated weekly until detected, and thereafter tumor growth was measured thrice weekly using electronic calipers.

Throughout the study, feed intake and body weights were measured weekly for all mice. Body composition was determined weekly using quantitative magnetic resonance imaging (Echo Medical Systems, Houston, TX, USA). At study termination (week 18), all mice were fasted for 8 h before blood samples were taken from the tail and analyzed for glucose concentration using an Ascencia Elite XL 3901G glucose analyzer (Bayer Corp.). The mice were then anesthetized with isoflurane for terminal blood collection via cardiac puncture prior to cervical dislocation. Mammary tissue and/or tumors were collected and either fixed in 10% formalin or flash frozen in liquid nitrogen and stored at -80°C until further analyses.

The impact of constitutively activated mTOR on *in vitro* Wnt-1 tumor cell proliferation and invasion

A clonal cell line derived from MMTV-Wnt-1 tumor tissue (M-Wnt cells) was transfected with either

wild-type (WT) mTOR or constitutively active (mTOR Δ) vectors (Fig. 2A). The mTOR Δ vector has a deletion in amino acids 2430–2450, which is the region responsible for mTOR repression. Gary G Chiang, PhD (Burnham Institute for Medical Research, La Jolla, CA, USA) generously donated the vectors. Cells were transfected through electroporation using a Bio-Rad GenePulser 2 (Bio-Rad). Transfected cells were selected as single clones using 800 μ g/ml of Geneticin (Invitrogen) until stable clones reached $\sim 1 \times 10^6$ cells. Transfection was confirmed by expression of the AU1 epitope tag (antibody from Bethyl Laboratories, Montgomery, TX, USA) using western blots.

Proliferation and invasion were assessed in M-Wnt cells with or without the constitutively active mTOR Δ construct in response to serum samples from tumor-free control and CR mice in the initial study. All the experiments were performed in triplicate. Proliferation was measured by seeding 2000 cells/well into 96 well plates. Cells were then serum-starved for 8 h to synchronize cell cycle and treated with either 1% control or CR serum for 24 or 48 h. Cells were

then treated with 50 μ l of 5 mg/ml thiazolyl blue tetrazolium bromide (Sigma–Aldrich) for 2 h. The stain was then dissolved in 100 μ l DMSO, and absorbance was read at 590 nm using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Invasion was measured with Matrigel Invasion Chambers (BD Biosciences, San Jose, CA, USA) by seeding 2500 cells/chamber, following the manufacturer's instructions, and using 1% control or CR serum as the chemotractant. After 18 or 30 h noninvading cells were wiped away from the upper part of the membrane and invading cells were fixed with 1% crystal violet in 70% methanol for 30 min before being assembled onto slides for counting.

The impact of constitutively activated mTOR on the inhibitory effects of CR on Wnt-1 tumor growth *in vivo*

In a separate animal study, 60 ovariectomized 6–8 week old C57BL/6 mice were administered (for 8 weeks) the same DIO diet (60 kcal % fat) as was used in the initial study (Fig. 2B). The mice were then randomized to receive for 10 weeks the same control diet ($n=30$) or CR diet ($n=30$) regimens as used in the initial study. Four weeks after randomization (week 12 of study), all mice were injected with 5×10^4 M-Wnt cells transfected (as described above) with either WT ($n=15$) or constitutively active mTOR (mTOR Δ). Mammary tumors were palpated weekly until detected, and thereafter tumor growth was measured thrice weekly using electronic calipers. At study termination (week 18), all mice were fasted for 8 h before being anesthetized with isoflurane for terminal blood collection via cardiac puncture. The mice were killed by cervical dislocation, and mammary tumors were collected and either fixed in 10% formalin or flash frozen in liquid nitrogen and stored at -80°C until further analyses.

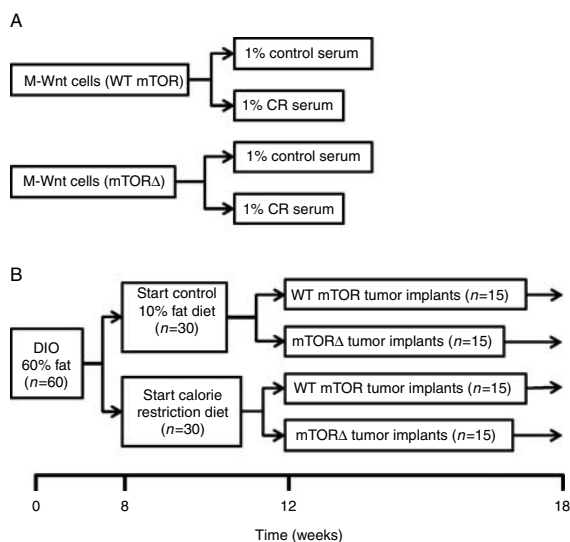


Figure 2 mTOR experimental design. (A) A clonal cell line derived from MMTV-Wnt-1 tumor tissue (M-Wnt cells) was transfected with either wild-type (WT mTOR) or constitutively active (mTOR Δ) vectors. Cells were serum-starved for 8 h to synchronize cell cycle and then treated with 1% serum from either control or CR mice for 24 or 48 h. (B) The effects of mTOR activity on tumor growth were investigated, *in vivo*. An 8-week DIO weight gain phase was followed by randomization onto control or calorie restricted diets. Twelve weeks into the study, mice were further randomized to receive MMTV-Wnt-1 tumor implants with WT mTOR or constitutively active mTOR (mTOR Δ) which were allowed to grow for 6 weeks.

Serum analyses

For samples obtained at study termination in both *in vivo* studies described above, serum leptin, adiponectin, and insulin were measured using mouse adipokine LINCOplex Multiplex Assays (Millipore, Inc., Billerica, MA, USA) analyzed on a BioRad Bioplex 200 analysis system (Bio-Rad, Inc.). Levels of fasting serum insulin and glucose were used to calculate quantitative insulin check index of insulin sensitivity ($\text{QUICKI} = 1/(\log \text{insulin ratio (mU/l)} + \log \text{baseline glucose (mg/dl)})$).

Tumor tissue analyses

Fixed tissue collected at termination from both *in vivo* studies described above ($n=4/\text{group}$) was embedded in paraffin and then cut into 4 μm thick sections for either hematoxylin and eosin (H&E) staining or immunohistochemical analysis. Slides were deparaffinized in xylene and rehydrated sequentially before being incubated with the primary antibody for immunohistochemistry analysis. Markers of mTOR activation (pMTOR, pS6, CyclinD1, VEGF), proliferation (Ki67, PCNA3), apoptosis (Cleaved Caspase-3), and angiogenesis (CD31) were evaluated using antibodies (Cell Signaling, Beverly, MA, USA) with 1:250 dilutions. Biotinylated Horse anti-goat IgG and SA-HRP (BioGenex, Fremont, CA, USA) were used for detection and a representative tumor section for each treatment is shown. For pMTOR, pS6, CyclinD1, VEGF, Ki67, and PCNA3 the intensity of positive immunostaining was graded as + + +, + +, +, and – for strong, moderate, weak, and negative results respectively. CD31-positive endothelial cells identified blood vessels that were counted and the mean number of vessels in five fields from each tumor section was determined. Cleaved Caspase-3-positive cells were counted in five randomly selected visual fields.

Statistical analyses

We determined that a minimum sample size of 13 animals per group was required to identify a 50% difference in tumor growth, using 90% power to detect a 20% difference between the groups with an α level equal to 0.05. Data are presented as mean with s.e.m., unless otherwise stated. Differences in serum hormones, glucose levels, and body composition were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. Kaplan–Meier survival analysis was used to evaluate differences in time to palpable tumors and Mann–Whitney statistical test was used for nonparametric comparison of final tumor weight between groups. Tumor latency was defined as the average time to palpable tumor. Significant differences between treatments for proliferation and invasion cell assays were analyzed by Student's *t*-test.

Results

CR, but not exercise, reverses physiologic markers of obesity

The initial *in vivo* study included a subset of mice that did not receive tumor implants but were fed a control diet ($n=15$), a 30% calorie restricted diet ($n=15$), or

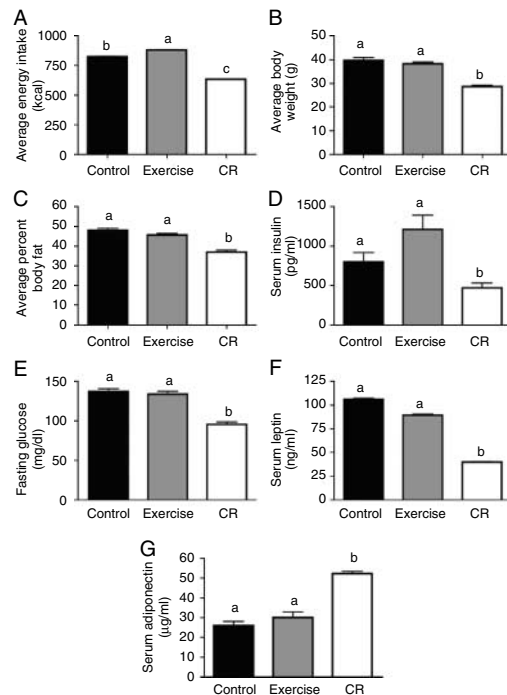


Figure 3 The effects of CR or EX on anthropometric and serum measurements. (A) Total calorie intake between weeks 8 (energy balance treatments start point) and week 18 (study endpoint). (B) Average body weight and (C) percent body fat at week 18, (D) serum insulin, (E) fasting blood glucose, (F) serum leptin, and (G) serum adiponectin levels at week 18. Significance was determined by one-way ANOVA and Tukey's *post hoc* test ($P<0.05$) and is denoted by different letters.

exercised ($n=15$) for 10 weeks (Fig. 1). Feed intake did not differ between the exercise group and control group in any time point throughout the study, but total calorie consumption calculated at the end of the study (week 18) was significantly higher in exercise than control (880 ± 3.8 vs 830 ± 3.2 kcal respectively) (Fig. 3A). At the end of the study, CR but not EX mice weighed significantly less (29 ± 0.6 and 38 ± 0.7 g respectively) than control mice (40 ± 1.2 g) (Fig. 3B). Also, percent body fat in the CR group was significantly less ($37 \pm 1\%$) than control ($48.1 \pm 1\%$), but exercised mice did not differ from control mice ($46 \pm 0.8\%$) (Fig. 3C).

Serum insulin levels were lower in CR mice (470 ± 63 pg/ml), but not in exercised mice (1200 ± 180 pg/ml), compared to control mice (800 ± 120 pg/ml) (Fig. 3D). Fasting glucose levels were also lower in CR (96 ± 3 mg/dl), but not in exercised mice (130 ± 3.8 mg/dl), compared to control mice (140 ± 3.1 mg/dl) (Fig. 3E). CR decreased serum leptin (4.0 ± 0.4 ng/ml), while exercise had no effect (9.0 ± 1.2 ng/ml), compared to control (11 ± 1.1 ng/ml) (Fig. 3F). Serum adiponectin levels were increased

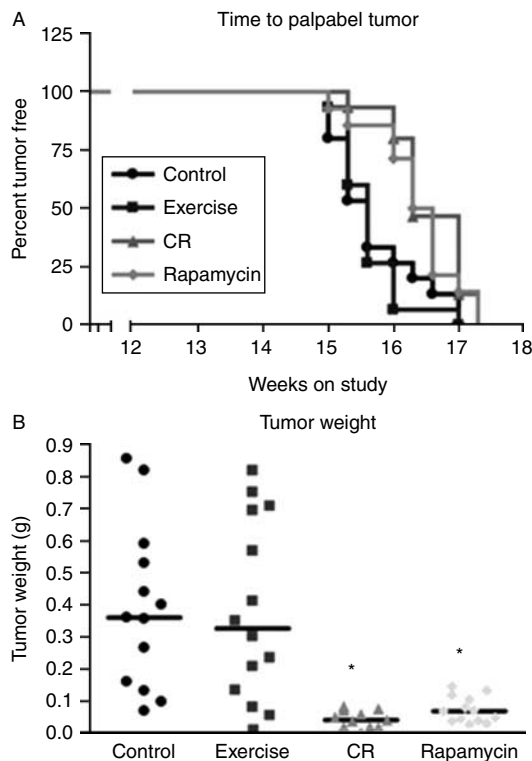


Figure 4 The effects of CR, EX or rapamycin on tumor latency and weight. Orthotopic transplant of MMTV-Wnt-1 mammary tumor cell suspension was used to assess treatment effects on tumor burden. (A) Kaplan–Meier survival curve with log-rank significance test for time to palpable tumor. (B) Weight of tumors extracted at study endpoint. Data shown are median \pm S.E.M. Significance was determined by Mann–Whitney nonparametric test and is denoted by asterisks.

in CR mice ($52 \pm 1.1 \mu\text{g/ml}$) compared to control mice ($26 \pm 2.0 \mu\text{g/ml}$), but exercise had no effect ($30 \pm 2.8 \mu\text{g/ml}$) (Fig. 3G). CR increased insulin sensitivity as indicated by a significantly increased QUICKI score compared to control and exercise (Supplementary Figure 1, see section on supplementary data given at the end of this article).

CR and rapamycin, but not exercise, reduce tumor burden in postmenopausal obese mice

The initial *in vivo* study investigated the effects of the interventions on tumor latency and final tumor weight in all tumor-bearing mice ($n=15$ per treatment) (Fig. 4). All mice developed tumors by week 18; hence, there was no difference in rate of tumor take between groups (data not shown). CR and rapamycin significantly increased tumor latency (16.3 and 16.5 weeks respectively, $P=0.006$), while exercise had no effect (15.6 weeks) compared to control (15.6 weeks) (Fig. 4A). Additionally, final tumor weights were

lower in the CR ($0.04 \pm 0.01 \text{ g}$) and rapamycin ($0.07 \pm 0.01 \text{ g}$) groups compared to control ($0.39 \pm 0.07 \text{ g}$), but exercise ($0.38 \pm 0.07 \text{ g}$) did not affect final tumor weight, $P=0.0002$ (Fig. 4B).

Rapamycin inhibits mTOR signaling in developed tumors

Based on immunohistochemistry analysis of tumors that developed during the initial *in vivo* study, phosphorylated mTOR (pmTOR) and its downstream effectors, phosphorylated S6 ribosomal protein (pS6) and cyclin D1, were down-regulated in the rapamycin, but not CR or EX groups, compared to control (Fig. 5 and Supplementary Figure 2, see section on supplementary data given at the end of this article). Also, rapamycin reduced angiogenesis, as identified through the blood vessel marker CD31, compared to control, while CR and exercise had no effects. VEGF, a downstream effector of mTOR that stimulates angiogenesis, followed the same pattern as CD31 (Fig. 5 and Supplementary Figure 2). Expression of the proliferation marker, Ki67 (Fig. 5 and Supplementary Figure 2); and the apoptosis marker, Cleaved Caspase-3 (Supplementary Figure 2) did not differ between groups.

Constitutively active mTOR ablates beneficial effects of CR on mammary tumor cells

In the *in vitro* study reported in Fig. 2, treatment with 1% serum from CR mice, relative to 1% serum from control mice, did not affect *in vitro* proliferation of M-Wnt cells derived from mouse MMTV-Wnt-1 mammary tumors (Fig. 6A). However, cell invasion was reduced in cells with the WT mTOR construct in response to 1% serum from CR mice, compared to 1% serum from control mice (380 ± 25 and 730 ± 25 cells invaded at 30 h, respectively). Constitutively active mTOR (mTOR Δ) ablated the effect of CR serum on cell invasion (790 ± 54 cells invaded at 30 h for CR compared with 710 ± 23 cells invaded at 30 h for control) (Fig. 6B).

We further demonstrate the role of mTOR in tumor growth in a follow-up *in vivo* study (Fig. 2). C57BL/6 mice that consumed either a control or CR diet were implanted with syngeneic orthotopic M-Wnt cells transfected with WT mTOR or mTOR Δ (Fig. 6C). In mice injected with WT mTOR, CR reduced weight of tumors ($0.29 \pm 0.03 \text{ g}$) compared with control ($0.62 \pm 0.12 \text{ g}$). CR did not affect tumor weight in mice injected with cells transfected with mTOR Δ ($0.38 \pm 0.05 \text{ g}$), compared to control ($0.55 \pm 0.08 \text{ g}$).

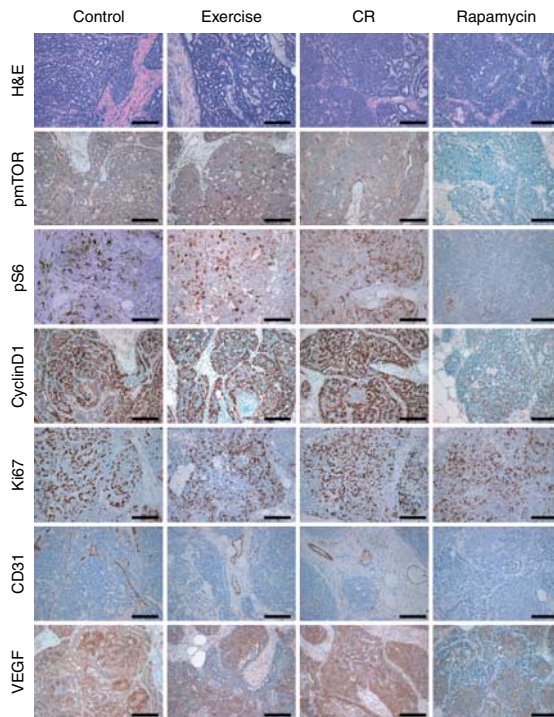


Figure 5 The effects of CR, EX and rapamycin on immunohistochemical staining of tumorigenic markers. Fixed tissue was embedded in paraffin and then cut into 4 μm thick sections for either hematoxylin and eosin (H&E) staining or immunohistochemical analysis. Markers of mTOR activation (mTOR, pS6, CyclinD1, VEGF), proliferation (Ki67, pHH3, PCNA3), apoptosis (Cleaved Caspase-3), and angiogenesis (CD31) were evaluated.

Discussion

Obesity is an established risk factor for postmenopausal breast cancer. Given that approximately one-third of the US population is obese; interventions to reverse the effects of obesity on breast cancer progression are urgently needed. Because mTOR modulates pathways regulating energy balance and cell growth, agents that target the mTOR pathway are promising for breaking the obesity–breast cancer link. Here, we directly compared the two most recommended strategies (CR diet and increased exercise) for reversing obesity (AICR 2007), on mammary tumor growth and metabolic profiles. Additionally, we compared the tumor inhibitory effects of these obesity reversing strategies with rapamycin, a well established mTOR inhibitor. We hypothesized that the beneficial effects of CR and exercise on mammary tumor burden are mediated by the mTOR pathway. The direct comparison of these energy balance regimens, as well as a pharmacologic intervention with the mTOR inhibitor rapamycin, in obese mice revealed the following novel findings: 1) CR, but not exercise,

reduced weight, modified obesity-related hormones, and reduced mammary tumor burden in obese mice; 2) rapamycin mimicked CR in decreasing mammary tumor burden in obese animals; and 3) constitutively active mTOR ablated the beneficial effects of CR on mammary tumor growth.

Previous reports suggest that decreased caloric intake or increased energy expenditure similarly affect body composition and metabolic parameters in normoweight humans and rodents (Frank *et al.* 2005, Brown *et al.* 2009, Vieira *et al.* 2009), although the effects of these interventions are not well established in the obesity context. We found that CR, but not EX, reduced weight and increased insulin sensitivity in obese mice (Fig. 3 and Supplementary Figure 1). Additionally, relative to controls, only CR positively altered obesity-related serum hormone levels, such as reducing leptin (62%) and increasing adiponectin (101%) (Fig. 3). Literature suggests that although exercise increases adiponectin receptor levels in the muscle (Bluher *et al.* 2006), exercise does not affect serum adiponectin levels in animals (Ziemke & Mantzoros 2010) or humans (Ryan *et al.* 2003), consistent with our findings (Fig. 3). Specifically, we found that a 10-week intervention of CR, but not exercise, effectively reversed weight gain and modulated obesity-driven biological markers.

CR and rapamycin, but not EX, decreased mammary tumor burden in our orthotopic Wnt-1 mammary tumor transplant model in obese C57BL/6 mice (Fig. 4). CR and rapamycin significantly reduced tumor weight by 89 and 82% respectively ($P=0.0002$). CR is a well established antitumor intervention in many different tumor models involving normoweight animals, but its effects in obese animals are not well characterized (Hursting *et al.* 2008). The effects of exercise on carcinogenesis are less clear and are affected by many factors, including age, gender, adiposity, as well as duration, frequency, and intensity of physical activity (Thompson 1997, McTiernan *et al.* 2008). While our novel studies on the effects of CR vs EX on obese rodents indicated that CR reduces tumor burden, it is important to note that our exercise regimen did not cause significant changes in circulating levels of obesity-related hormones or body composition. Exercise regimens that do not affect body composition may have a beneficial effect on reducing obesity-related, postmenopausal breast cancer incidence. In chemically induced models, mixed effects of exercise on tumor growth have been reported (Thompson *et al.* 1988, 1989, 1995a,b, Gillette *et al.* 1997). In a xenograft model involving human breast cancer cells in immunodeficient mice, exercise did not affect tumor

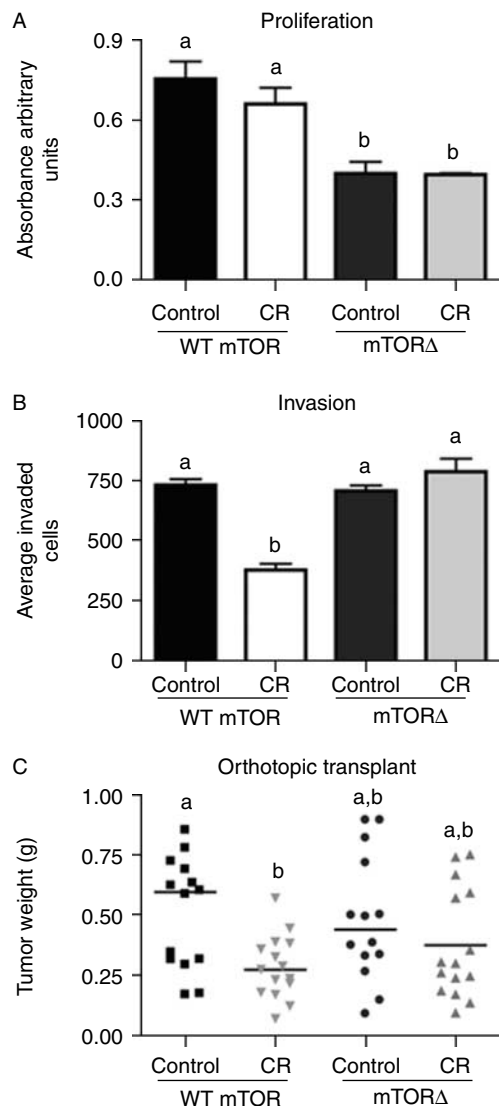


Figure 6 The effect of mTOR constitutive activation on MMTV-Wnt-1 mammary tumor cells exposed to CR or control treatments, *in vitro* and *in vivo*. (A) Proliferation and (B) invasion were assessed in M-Wnt cells with or without the constitutively active mTOR Δ construct in response to serum samples from control and CR mice. Proliferation was measured in response to treatment with either 1% control or CR serum for 24 or 48 h by MTT. To measure invasion, cells were plated in serum free media on an invasion chamber and allowed to invade for 18 h using control or CR serum as chemottractant. All experiments were performed in triplicate. (C) Sixty ovariectomized 6–8 week old female C57BL/6 mice were administered a diet-induced obesity diet (60 kcal % fat) for 8 weeks. The mice were then randomized to receive for 10 weeks control diet ($n=30$) or CR diet ($n=30$) regimens. Four weeks after randomization (week 12 of study), all mice were injected with 5×10^4 M-Wnt cells transfected with either wild-type (WT; $n=15$) or constitutively active mTOR (mTOR Δ). Tumors were removed and weighed at time of sacrifice. Significance was determined by one-way ANOVA and Tukey's post hoc test ($P < 0.05$) and is denoted by different letters.

burden (Jones *et al.* 2009). Use of immunodeficient mice (which are resistant to obesity) to understand the effects of dietary energy balance modulation or exercise on mammary tumor burden is not ideal because immune function/inflammatory processes are thought to be involved in the effects of both obesity and exercise on mammary tumors (McTiernan *et al.* 2008). We previously reported inhibitory effects of CR but detrimental effects of treadmill exercise compared to sedentary controls in p53-deficient Wnt-1 transgenic mice (Colbert *et al.* 2009). These findings suggest that CR and exercise exert very different responses. Taken together, reports in the literature are consistent with our findings that CR, more so than exercise, decreases mammary tumor burden. Additionally, we are the first to directly compare the effects of CR and exercise on mammary tumor burden in a mouse model of postmenopausal obesity that is particularly relevant to the rapidly growing population of obese women in the USA.

The use of mTOR inhibitors in cancer treatment is currently an intensive area of preclinical and clinical research (Dowling *et al.* 2011). In this study, rapamycin treatment decreased mammary tumor burden in obese mice (Fig. 4), suggesting that mTOR inhibitors may be particularly important for cancer treatment in the obese population. We evaluated activation of mTOR, which integrates extracellular and intracellular metabolic signals to control cell growth, cell division, and cell survival (Gwinn *et al.* 2008), in response to CR and exercise. Tumors with constitutive activation of PI3K (a positive upstream regulator of mTOR activity) are resistant to CR (Kalaany & Sabatini 2009) and it has been hypothesized that exercise may reduce tumor growth by targeting the mTOR pathway (Thompson *et al.* 2009). Our finding that established tumors from CR mice did not display decreased mTOR activation (Fig. 5) does not exclude mTOR pathway inhibition as a possible mechanism underlying the suppressive effect of CR on tumor growth. Instead, we hypothesize that energy balance-related growth factors greatly influence the early stages of tumor formation and progression.

We and others have shown that the mTOR pathway is regulated by CR in multiple epithelial tissues including the mammary epithelium (Moore *et al.* 2008). Thus, the Wnt-1 mammary tumors grow more slowly in CR mice due to reduced growth factor signaling through the mTOR pathway, and this is consistent with our observations in spontaneous and transplanted tumor models (Nunez *et al.* 2008). In contrast, advanced tumors can activate growth and survival pathways in a growth factor-independent fashion (Hanahan & Weinberg 2011). Hence, CR

may no longer modulate signaling in advanced tumors such as those we observed at tumor harvest. Consistent with previous studies evaluating the effects of mTOR activation in tumor growth *in vivo*, increased mTOR activity is only advantageous in the initial phase of tumor growth, but once tumors were detected, growth rate does not differ from controls (Kaper *et al.* 2006). Our findings are consistent with this report, as CR increased tumor latency, but once formed, CR tumors grew at similar rates relative to controls (Fig. 4).

To further elucidate the role of the mTOR pathway on the effects of CR on mammary tumor progression, we evaluated the growth of M-Wnt mammary tumor cells transfected with either a WT mTOR or constitutively active mTOR construct (mTOR Δ) *in vitro* (in response to CR vs control serum) and *in vivo* (when implanted in CR or control mice). CR inhibited cell proliferation by 13% and invasion by 49% *in vitro* (Fig. 6A and B) in WT mTOR cells. Although serum from CR mice did not significantly alter Wnt-1 tumor cell proliferation *in vitro* (Fig. 6A), CR serum decreased cell invasion in cells transfected with the WT mTOR construct compared to the control treatment (Fig. 6B). Processes associated with tumor invasion have previously been associated with changes in the mTOR pathway (Taliaferro-Smith *et al.* 2009), but not in the context of CR.

CR also inhibited tumor growth by 50% *in vivo* (Fig. 6C), when WT mTOR was present, but constitutively active mTOR Δ ablated this effect of CR. Signaling through mTOR promotes a negative feedback loop that represses insulin-mediated AKT activation (Haruta *et al.* 2000). Hence, while constitutively active mTOR would be predicted to drive tumor growth, the negative feedback loop would simultaneously restrain pro-growth signaling from AKT. Consistent with this hypothesis and with our results, previous studies have shown that mTOR activation in TSC2 heterozygous mice correlates with limited tumor growth (Ma *et al.* 2005). Our finding that constitutively active mTOR blocks (at least partially) the effects of CR serum on Wnt-1 mammary tumor cell invasion *in vitro* and tumor growth *in vivo* further supports an important underlying role of mTOR in the effects of CR on tumor progression.

We conclude that CR and rapamycin (at a dose of 5 mg/kg every 48 h), but not the exercise intervention, reduced transplanted Wnt-1 tumor burden in obese mice. In addition, constitutively active mTOR ablated the beneficial effects of CR on mammary tumor progression in obese mice. Hence, mTOR inhibitors should be considered in future studies for treatment or prevention of breast cancer in obese patients.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-11-0213>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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