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14. ABSTRACT

Obesity increases postmenopausal breast cancer risk and increases mortality in pre- and postmenopausal women. While the majority of breast cancers in obese women are estrogen receptor (ERα)-positive, ERα-negative tumors confer a much worse prognosis. The mechanism by which obesity affects ER-negative breast cancer risk and prognosis is not clear, and strategies for offsetting the negative effects of obesity are urgently needed. In this study, we utilized the MMTV-neu mouse model of luminal-type breast cancer to test the hypothesis that *energy balance modulation, through diet-induced obesity (DIO) or calorie restriction (CR) regimens, alters mammary tumor development and progression through regulation of ER in the mammary epithelium*. MMTV-neu mice form spontaneous mammary tumors that progress from an ERα-positive hyperplasia to aggressive ERα-negative ductal adenocarcinomas. Female MMTV-neu transgenic mice and non-transgenic host strain (FVB) mice (6-8 weeks old; n=90/genotype) were randomized (30/group) to receive: control diet (modified AIN-76A); a 30% CR regimen (isonutrient); or a DIO regimen. A subset of mice (n=4 per group) was killed at 1, 3, and 5 months following diet initiation, and tissues were collected for analysis; remaining animals were followed for a 60-week survival study. We found that, relative to control diet, the DIO regimen significantly increased body weight, percent body fat ($p<0.0001$), and obesity-associated serum hormones and growth factors (IGF-I, insulin, leptin, and estradiol; $p<0.01$ for all) in both MMTV-neu and FVB controls. Conversely, CR significantly decreased body weight, percent body fat ($p<0.0001$) and decreased serum hormones/ growth factors, while increasing adiponectin (each at $p<0.01$). Gene expression (qRT-PCR) and protein expression (immunohistochemistry) analysis revealed that loss of mammary ERα expression, known to occur by 8 weeks of age in control MMTV-neu mice, was accelerated by DIO and delayed by CR. Additionally, we found that CR (relative to control diet) significantly increased mammary ERβ expression ($p<0.0001$) and delayed the onset of hyperplasia ($p<0.001$) in both MMTV-neu and FVB mice. Importantly, we found that after 60 weeks of feeding, CR significantly increased tumor-free survival of MMTV-neu mice ($p=0.01$). CR tumor samples showed a decrease in vascularity and presence of mitotic figures. In conclusion, dietary energy balance modulation impacts spontaneous MMTV-neu mammary tumor development and ERα and ERβ levels in normal and tumor tissue. In addition, increased mammary ERβ expression may represent a novel mechanism underlying the anticancer effects of CR.

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INTRODUCTION:

Breast cancer is the leading cause of cancer death in women in the United States who are aged 20 to 59 years (*1*). In 2010, an estimated 207,090 new cases of breast cancer were identified in the United States (*1*). Although breast cancer risk increases with age, approximately 35% of breast cancers occur during the reproductive and perimenopausal years (*2*). Of the premenopausal breast cancers diagnosed, approximately 20% are both ER and PR negative (*3*), and tumors negative for estrogen receptor (ER-) confer a much worse prognosis (*4*). The HER-2/neu/erbB2 proto-oncogene is amplified in 25-30% of human primary breast cancers, and increased levels of HER-2/neu expression in tumors negatively impact prognosis regardless of menopausal status (*5*).

Obesity is associated with increased tumor size (*6*), progression markers (*7*) and therapy resistance (*8*), especially in ER- tumors, in both pre- and postmenopausal women (*6*). Although obesity is typically associated with increased risk of breast cancer for postmenopausal women (*9*), new data shows that obesity may also be a risk factor for premenopausal women 35 and older, who have additional risk factors for breast cancer, including Type-2 diabetes (*10*). Perhaps most importantly, data shown here, as well as decades of calorie restriction research (*9*) show that avoiding weight gain after age 30 is increasingly being recognized as a simple way to reduce risk of breast cancer (*11*). Energy balance modulation (diet-induced obesity and calorie restriction) modifies serum levels of many growth factors and hormones (*9*). However, the specific mechanisms by which dietary energy balance modulation affects ER- breast cancer risk, progression or prognosis are not clearly understood.

BODY:

In our original proposal, we hypothesized that: ***Obesity-induced increases in circulating IGF-1 levels promote IGF-1/ER crosstalk in the mammary epithelium, leading to a reduction in the chemopreventive efficacy of tamoxifen.*** In Specific Aim 1, we outlined the **characterization of the effects of dietary energy balance modulation on metabolic hormones and mammary tumor development, growth, and progression in MMTVerbB2 mice (referred to as MMTVneu mice in the rest of the report).** The model in which we have chosen to test this hypothesis is the MMTVneu transgenic mouse model, in which mice were fed a standard diet-induced obesity regimen. **Task 1** was to modulate diets in MMTVneu mice and measure effects on tumor. The first **Milestone** was to receive IACUC approval, which was accomplished on 13 August 2008, renewed on 06 July 2009, and on 03 August 2010, and as needed for personnel and minor modifications. The USAMRMC has been notified of all major and minor protocol modifications. **Task 1.a** was accomplished when 90 MMTVneu and 90 age-matched non-transgenic control mice were mice ordered and put on diet regimens. Blood samples were taken for fasting glucose measurements and for serum hormone analysis (**Task 1.b**). As tumors became palpable in the transgenic mice, tumor weight measurements were taken (**Task 1.c**). Tissues were harvested from the mice at the time of sacrifice, including tumor, mammary fat pad, liver, visceral white adipose tissue, skin, and skeletal muscle (**Task 1.d**). The following results section details our findings.

Results:
Dietary energy balance modulation regulates ERα/β mRNA expression.
Generation of DIO, control, and CR phenotypes

MMTVneu and non-transgenic FVB/NJ mice administered DIO, control or CR diet regimens manifested three distinct phenotypes: obese, overweight and lean, which has been published in several models by our laboratory and others (*9*). After 15 months on diet, body weights in MMTVneu mice were significantly heavier (44.4 ± 0.56 g) in DIO mice and significantly lighter in CR fed mice (18.8 ± 0.13 g) relative to control fed mice (36.8 ± 0.67 g) (*P*<0.01) and similarly FVB/NJ mice on the DIO regimen were significantly heavier (39.2 ± 0.18 g) and the CR fed mice were significantly lighter (18.8 ± 0.12 g) compared with control fed mice (34.5 ± 0.22 g) (*P*<0.01) (**Figure 1A**). This was due to a significant difference in caloric intake over time between diet groups in both MMTVneu and FVB/NJ (*P*<0.01 both genotypes). After 15 months, MMTVneu mice consumed on average 107 ± 5.57 kcal per week on the DIO

regimen, 99.7 ± 3.83 kcal per week when fed the control diet, and 69.2 ± 2.43 kcal per week when fed the CR diet and FVB/NJ mice consumed on average 106 ± 1.84 kcal per week on the DIO regimen, 105 ± 4.83 kcal per week when fed the control diet, and 69.8 ± 2.00 kcal per week in when fed the CR diet (**Figure 1B**). Fasting blood glucose was reduced in CR mice (MMTVneu 42.5 ± 6.22mg/dl; FVB/NJ 48.3 ± 5.71mg/dl), relative to Control mice at all timepoints measured (MMTVneu 129 ± 26.8mg/dl; FVB/NJ 142 ±10.4 mg/dl) but DIO did not further increase fasting serum glucose ($P<0.05$ for both genotypes). Specific readings given for the 5 month timepoint, but significance was found at 1, 3, and 5 months on diet (**Figure 1C**). Percent body fat was modulated by the DIO regimen (MMTVneu 43.14% ± 1.29%; FVB/NJ, 40.0% ± 1.51%) and was significantly reduced by the CR diet (MMTVneu 16.4% ± 1.60%; FVB/NJ 16.2% ± 1.27%) compared with control fed mice (MMTVneu 28.3% ± 2.90% ; FVB/NJ 30.3% ± 2.54%) ($P<0.05$) at all timepoints measured (data points stated are after 6 months of dietary regimens) (**Figure 1D**).

Significant differences in serum levels of energy balance-related hormones and growth factors were seen at all timepoints tested. Serum IGF-1 significantly increased in DIO mice and significantly decreased in CR mice ($P<0.01$ for both genotypes). Additionally, we saw a significant increase in IGF-1 levels after 5 months on diet in FVB/NJ mice ($P<0.01$). Similarly, serum insulin increased in response to the DIO regimen and significantly decreased in response to the CR diet ($P<0.01$; both genotypes). Calorie restriction significantly reduced circulating serum leptin relative to Control and DIO ($P<0.01$; both genotypes). As expected, CR mice had significantly higher levels of adiponectin relative to Control and DIO mice ($P<0.01$; both genotypes). Also, CR mice had significantly lower levels of circulating 17-b estradiol versus Control and DIO mice (MMTVneu $P<0.01$; FVB/NJ $P=0.02$) (**Figure 2**).

Regulation of ERα/β mRNA expression

MMTV mice have a well-documented tumor progression phenotype transitioning from normal mammary glands (ERα-positive) to DCIS (ERα-mixed) to ductal adenocarcinoma (ERα-negative) (*12*). We confirmed that in non-tumor bearing mammary fat pad of Control MMTVneu mice, ERα mRNA levels decreased over time versus Baseline ($P<0.05$ vs Baseline; at every timepoint). Interestingly, over time CR significantly increased ERα mRNA levels in MMTVneu mice relative to Baseline ($P<0.01$) but did not significantly increase ERα gene expression in the FVB/NJ model. In contrast, DIO significantly accelerated loss of ERα mRNA levels ($P<0.01$ vs Baseline; both genotypes) Epidemiological studies have shown that higher levels of ERβ have been correlated with lower breast tumor grade and better prognosis (*13*). We found that CR significantly upregulated the putative tumor suppressor ERβ mRNA levels in both MMTVneu and FVB/NJ non-tumor bearing mammary fat pad relative to baseline expression ($P<0.01$).

Calorie restriction prevents loss of ERα protein expression and decreases mammary gland hyperplasia.

Regulation of ERα protein expression

In order to confirm that changes in mRNA levels resulted in ERα protein expression changes, we performed immunohistochemistry on MFP samples from the same MMMTVneu and FVB/NJ mice used in the gene expression analysis. In mammary glands, we saw a significant loss of ERα protein in DIO animals compared with Baseline (MMTVneu $P<0.01$; $P=0.03$ FVB/NJ). In MMTVneu mice, CR protected against loss of ERα protein expression (no difference vs. Baseline; at every timepoint). CR also protected against loss of ERα protein expression In the FVB/NJ animals, although the effect was not as pronounced (at every timepoint). As anticipated, we also saw a significant loss of ERα protein expression in mammary glands over time ($P<0.01$ both genotypes) (**Figure 4**).

Changes in mammary gland hyperplasia

In order to determine the effect of dietary energy balance modulation on mammary gland hyperplasia over time, we histopathologically scored sequential H&E sections from the same MMTVneu and FVB/NJ mice. We found that at all timepoints examined (1, 3, and 5 months) in both MMTVneu and FVB/NJ mice CR

completely prevented mammary gland hyperplasia, with the exception of one sample at 3 months (FVB/NJ). Similar levels of hyperplasia were seen in Control and DIO mice of both genotypes. The pattern of hyperplasia was variable and included diffuse (symmetrical thickening of the epithelium), solid, papillary, and cribriform. The most common pattern of hyperplasia was diffuse. No DCIS were seen in samples examined (**Supplementary Table 1**).

Calorie restriction significantly increases survival and decreases tumor vascularity and proliferation.
Impact of energy balance modulation on survival
Calorie restriction has been well documented to increase longevity in many species and models (*9*). As anticipated, a 30% reduction in calorie consumption (in CR mice) significantly increased tumor-free survival in MMTVneu mice (*P*=0.01). In this model of luminal ductal adenocarcinoma, DIO did not significantly increase tumor initiation, as previously reported (*14*). FVB/NJ mice did not present any spontaneous mammary tumors, as expected; therefore, no effect of diet was seen in these mice (**Figure 5A**).

Impact of energy balance modulation on tumor progression
Tumors excised from MMTVneu animals on DIO, Control, and CR diets were examined for histopathological markers of tumor progression in H&E sections including vascularity (presence of blood vessels), proliferation (# of mitotic figures per field), nuclear atypia (# per field), inflammation (inflammatory cell infiltration), and necrosis. We observed that tumors in MMTVneu CR mice had less vascularity and fewer mitotic figures per field. However, after 15 months on diet, CR was so effective at preventing tumor initiation, there were only two tumors to examine; therefore, no statistical analysis was performed. No difference was seen in other markers of tumor progression between diet groups (**Supplementary Table 2**).

Dietary energy balance modulation regulation of ERβ mRNA expression is dependent on Her2/neu positivity.
In vitro treatment with BMI stratified human sera
In order to validate the finding that dietary energy balance modulation regulates ERβ mRNA expression levels, we treated two human cell lines (SK-BR3, MCF-7) and two mouse cell lines (MMTVneu E18-9A-42, ZR-75) with human sera stratified by BMI (<25 kg/m^2 vs >30 kg/m^2). In SK-BR3 cells, which are a Her2/neu-positive model of human Luminal B breast cancer (*15, 16*), human sera collected from women with a BMI<25 significantly upregulated ERβ expression (*P*<0.05), while sera from obese women (BMI>30) did not affect ERβ mRNA expression levels. In contrast, using MCF-7 cells, which are a Her2/neu-negative model of human Luminal A breast cancer (*15, 16*), showed no difference between sera treatment groups. Similarly, in the mouse cell line derived from the MMTVneu spontaneous mouse model (MMTVneu E18-9A-42), which is HER2/neu-positive model of Luminal mammary cancer (*16*), BMI<25 sera significantly upregulated ERβ mRNA expression relative to sera treatments from women with a BMI greater than 30 kg/m^2 (*P*<0.05). We also saw that in the ZR-75 cells, a Her2/neu-negative model of Luminal mammary cancer (*16*), there was no significant difference between sera treatment groups (**Figure 5B**).

Materials and Methods:
All animal studies and procedures were approved and monitored by the University of Texas at Austin Institutional Animal Care and Use Committee and the USAMRMC.

Effect of dietary energy balance manipulation on MMTVneu spontaneous tumor development.
Animals and study design. Upon arrival after purchase (Jackson Laboratory, Bar Harbor, ME), 90 naïve, 6- to 8-week-old female MMTVneu (JAX stock #002376, FVB/N-Tg(MMTVneu)202Mul/J) mice and 90 naïve, 6-8-week old female FVB/NJ (JAX stock #001800), a subset of mice (n=5 mice/genotype) were euthanized for all "Baseline" readings within 48 hours of arrival. Mice were fasted for 6 hours and then euthanized (by

CO_2 asphyxiation followed by cervical dislocation). Blood was collected by cardiac puncture, allowed to coagulate for 30 minutes at room temperature, and centrifuged at 10,000 x g for 5 minutes; serum was removed and stored at -80^oC for subsequent analyses. Mammary fat pad were collected for further molecular and pathological analysis. Remaining mice were singly housed and fed a control diet (AIN-76A based diet; catalog #D12450B, Research Diets, Inc., New Brunswick, NJ) ad libitum for one week of acclimation. Mice were then randomized (n=30/diet group/genotype) to receive one of three dietary regimens for the duration of the study (all diets purchased as pellets from Research Diets, Inc.): 1) control diet, fed ad libitum, providing 3.8 kcal/g; 2) 30% CR diet (catalog #D0302702); 3) DIO diet, fed ad libitum (catalog #D12492), providing 5.2 kcal/g with 60% kcal from fat. CR mice received a modified formulation of control diet such that daily aliquots of food provided 70% of the mean caloric consumption (and 100% of the vitamins, minerals, essential fatty acids and amino acids) of control mice. Mice were weighed weekly.   After 2, 4, and 6 months on diet, all mice were analyzed for percent body fat using quantitative magnetic resonance (Echo Medical Systems, Houston, TX). After 1, 3, and 5 months on diet a subset of non-tumor bearing mice were sacrificed (n=4/diet group/genotype). Mice were fasted for 6 hours and then euthanized (by CO_2 asphyxiation followed by cervical dislocation). Blood was collected by cardiac puncture, and mammary fat pad were collected for further molecular and pathological analysis. Mice not sacrificed at 1, 3, or 5 months were used for survival analysis (n = approximately 15/dietary group/genotype); non-tumor related deaths were screened. Mice were palpated for tumors weekly. Once detected, tumors were measured twice weekly in two perpendicular dimensions using electronic calipers, and cross-sectional area was calculated (maximal length x width, mm^2). When tumor diameter reached 1.0 cm in either dimension mice were fasted for 6 hours and then euthanized (by CO_2 asphyxiation followed by cervical dislocation), and tumor was excised, measured and weighed. Blood was collected by cardiac puncture, and mammary fat pad were collected for further molecular and pathological analysis.

Mammary fat pad, and tumor processing and storage. After being excised, mammary tumors and mammary fat pads were equally divided into 2 portions that were either: 1) fixed in 10% neutral buffered formalin for 24 hours, transferred to 70% ethanol for at least 24 hours, embedded in paraffin, and cut into 4 µm thick sections for hematoxylin and eosin (H&E) staining or immunohistochemical analysis; 2) placed in a cryotube, flash frozen in liquid nitrogen and stored at -80^oC for subsequent molecular analyses.

Serum biomarker, molecular, and pathological analysis.
Serum biomarkers. All 4 animals per diet and genotype group were used for analyses of blood glucose (by Ascencia Elite Glucometer, Bayer, Minnneapolis, MN); serum leptin, insulin, insulin-like growth factor (IGF)-1, and adiponectin (by Luminex-based LINCOplex bead array assay, Millipore, Billerica, MA; read on multianalyte detection system, BioRad, Hercules, CA); and 17-b estradiol (by ELISA; Alphay Diagnostics, San Antonio, TX).

Real-time quantitative reverse transcription (qRT)-PCR of ERα/β. Total RNA was extracted from cell lines using a RNeasy Mini kit (Qiagen, Valencia CA) and from mammary fat pad tissue samples using a FastRNA Pro Green Kit (MP Biomedicals, Solon, OH) (n = 4 per diet and genotype group). RNA concentration was spectrophoretically determined using a nanodrop (Thermo Scientific, Logan, UT) and quality was confirmed by an Agilent 2100 Bioanalyzer (Santa Clara, CA). RNA was reverse transcribed with Multiscribe RT (Applied Biosystems, Carlsbad, CA). Resulting cDNA from tissue samples were assayed in triplicate for PCR using Taqman® Gene Expression Assays for ERα and ERβ (Applied Biosystems). Resulting cDNA from cell line samples were assaying in triplicate using human (SK-BR3, MCF-7) or mouse (MMTVneu E18-9A-42, ZR-75) ERβ primers (Origene, Rockville, MD) and the QuantiFast SYBR Green PCR kit (Qiagen).   PCR reactions were monitored by a ViiATM7 Real time PCR system (Applied Biosciences). Gene expression data were normalized to the housekeeping gene b-actin and analyzed using the delta delta Ct method.

Immunohistochemistry. Immunohistochemical staining was performed as previously described (n = 4 per diet and genotype group) (*17*) using a primary antibody for estrogen receptor (ER)-α (Catalog #sc542, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500. The secondary antibody was horseradish peroxidase-labeled anti-rabbit antibody (DAKO Cytomation). All photomicrographs were acquired using the ScanScope XT (Aperio Technologies, Vista, CA) and quantification was done with the Aperio Digital Pathology platform (Aperio Technologies) after generating the appropriate algorithm to identify positive nuclear staining. Positive staining for ERα was further stratified by 1+, low; 2+, moderate; and 3+, intense staining.
Pathological analysis. All pathological analyses were performed by Donna F. Kusewitt, DVM, PhD, ACVP, veterinary pathologist at the University of Texas MD Anderson Cancer Center Department of Carcinogenesis Histopathology Core Facility (Smithville, TX). Mammary fat pads excised at 1, 3, and 5 months on dietary regimens were analyzed for the presence of hyperplasia (symmetrical thickening of the epithelium, solid, papillary, and cribriform were all included). Percentages were calculated as the number of animals per group with any hyperplasia present in a single cross-section H&E slide. Spontaneous tumors in MMTVneu mice were analyzed for vascularity (graded on a 0-3 categorical scale for the entire slide) and for the presence of mitotic figures (counted in 5 non-overlapping fields of view). Statistical analysis could not be performed because the CR group only had 2 tumors form in the 15 month study.

Effect of serum energy balance manipulation on ERβ expression in vitro.
Isolation of MMTVneu cell line. The MMTVneu cell line was derived from the normal mammary fat pad of an MMTVneu transgenic mouse, as previously described (*17*). In brief, excised mammary fat pads were dissected, mechanically dissociated into small pieces, and plated in 10ml of media in 100mm plates, grown at 37°C in 5% CO_2 in DMEM media, 10% FBS, 1% penicillin/streptomycin and 1% Fungizone (all media components from HyClone, Waltham, MA) overnight. After 24 hours, the supernatant was removed and floating cells/mammary fat pad pieces were pelleted at 850 RPM for 5 minutes. The resulting cell pellet was resuspened in 10ml of complete media in a new 100mm plate. The media was also replaced on the original plate. Cell lines were cultured for a minimum of 4 weeks. The cell line resulting from animal E18-9A-42 was used for this experiment. Its cellular morphology is similar to other cell lines derived from non-tumor bearing, untreated MMTVneu mammary fat pads. This line is denoted as MMTVneu E18-9A-42.

In vitro treatments. Serum was collected from postmenopausal breast cancer patients under an Institutional Review Board (IRB) approved biorepository collection protocol at the Cancer Therapy and Research Center at the University of Texas Health Science Center at San Antonio (UTHSCSA). Approval for this study was obtained from the IRB of UTHSCSA (HSC20070684H). Serum was pooled, aliquoted, and stored at -80°C according to the BMI category of the patient (normal weight: 18.5-24.9 kg/m^2; obese: ≥30 kg/m^2). SKBR3, MCF-7, and ZR75 cells (ATCC) were maintained in IMEM (GIBCO Life Technologies) supplemented with 10% FBS. The MMTVneu E18-9A-42 cells were grown in DMEM (GIBCO Life Technologies) containing 10% FBS and 2 mM L-glutamine. These cell lines were all serum-starved for 18 hours, then exposed to 2% pooled human sera or 2% FBS (in appropriate medium) for 48 hours prior to cell harvest and ERβ expression was analyzed by qRT-PCR. In vitro serum treatments were performed in triplicate on separate days.

Statistical Analyses.
Summarized data are expressed as means ± standard error of the mean, and analyses were performed using SAS 9.3 (Cary, NC). Survival data was analyzed by the Kaplan-Meier estimator. Body weight and feed intake were assessed by repeated measures analysis of variance (ANOVA) followed by Tukey's post hoc analysis. Fasting glucose, body composition, serum adipokines, mammary fat pad ERa and ERb gene expression and ERa protein were analyzed by two-way ANOVA followed by Tukey's post hoc test. When significant interactions were detected (ERa gene expression), analysis of simple effects was completed. In vitro ERb gene expression data were analyzed by one-way ANOVA followed by Tukey's analysis. When

test assumptions were not met (serum insulin and leptin), data were transformed by natural log. Differences were considered statistically significant at $P \leq 0.05$.

KEY RESEARCH ACCOMPLISHMENTS:
- DIO, Control, and CR diet regimens caused significant differences in body weights, caloric intake, and body composition of MMTV-erbB2 mice.
- CR significantly increases lifespan in MMTV-neu mice.
- CR maintained baseline levels of ERα expression (mRNA and protein) and significantly increased ERβ expression in the normal mammary fat pad of both MMTVneu and non-transgenic mice.
- Regulation of ERβ expression in tumors by diet is Her2/neu dependent in both human and mouse cell lines in vitro.

REPORTABLE OUTCOMES:
No reportable outcomes have yet resulted from this research. However, a manuscript will be submitted in February 2013 entitled, "Calorie Restriction Prevents Basal-like Mammary Cancer in MMTV-Neu Mice Through Modulation of Estrogen Receptor-α and β."

CONCLUSION:
We have accomplished all tasks outlined in the original proposal for the first year of the study. Here we characterize the effects of dietary energy balance modulation on body weight, body composition, and metabolic hormones/growth factors, as well as on mammary tumor development and progression in MMTVneu (HER-2/neu overexpressing, ERα- model of luminal mammary cancer) mice. Our data demonstrates that calorie restriction significantly decreases tumor initiation through upregulation of the putative tumor suppressor ERβ, prevention of the loss of ERα, and a reduction in mammary gland hyperplasia. The finding that ERβ expression is protective and modulated by caloric intake is directly applicable to the patient population at high risk for recurrence of ERα- breast cancer, both for protection and prognostic value. Significantly, we found that DIO did not further increase the risk of developing HER-2/neu positive, ERα-negative luminal breast cancer. Therefore, Specific Aims 2 and 3 were not applicable in this subtype of breast cancer. Instead, we more performed a more comprehensive study of the mechanism of the protective effects of CR.

The prevention of obesity, through caloric restriction, has been shown in many models over many years to be the standard method for prevention of many types of cancer (*9*). Key serum hormones and growth factors, like IGF-1, have been demonstrated to be the upstream effectors for prevention. However, many of the subsequent gene expression changes in normal target tissue, and how this affects cancer risk, remain unknown. In this study, we demonstrate that caloric restriction has immediate and long term effects on the expression of ERβ, which leads to a significant reduction of mammary cancer development in a model of luminal breast cancer. Conversely, high energy regimens, in Control and DIO feeding, confer an increased risk for mammary cancer. The mechanism for differential risk does not lie solely with ERβ. In these animals, we see that DIO significantly increases the speed of loss of ERα in the normal mammary fat pad, and CR prevents the loss. Interestingly, we see the regulation of ERα and ERβ expression in both transgenic and age-matched wild-type animals, driving home the broad implications of dietary energy balance.

This study will lay the foundation for larger translational/clinical studies investigating the role of IGF-1 in promoting the adverse effects of obesity on breast tumor development and progression, and validating suppression of IGF-1 pharmacologically as an effective chemopreventive approach. Specifically, we evaluated the crosstalk of IGF-1/HER-2/ER in the normal mammary fat pad. The publication of our findings will highlight the importance of maintaining a healthy body weight over a lifetime in order to prevent obesity and decrease risk for many types of cancer, including breast cancer.

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13. Y. K. Leung, M. T. Lee, H. M. Lam, P. Tarapore, S. M. Ho, Estrogen receptor-beta and breast cancer: translating biology into clinical practice. *Steroids* **77**, 727 (Jun, 2012).
14. M. P. Cleary, J. P. Grande, S. C. Juneja, N. J. Maihle, Diet-induced obesity and mammary tumor development in MMTV-neu female mice. *Nutrition and cancer* **50**, 174 (2004).
15. K. Subik *et al.*, The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines. *Breast cancer : basic and clinical research* **4**, 35 (2010).
16. J. I. Herschkowitz *et al.*, Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome biology* **8**, R76 (2007).
17. S. M. Dunlap *et al.*, Dietary energy balance modulates epithelial-to-mesenchymal transition and tumor progression in murine claudin-low and basal-like mammary tumor models. *Cancer Prev Res (Phila)* **5**, 930 (Jul, 2012).

APPENDICES: Curriculum Vitae for Sarah M. Dunlap, Ph.D.

Sarah M. Dunlap, Ph.D.

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Email: sarah.dunlap@austin.utexas.edu

Education

Postdoctoral Fellow, Department of Nutritional Sciences, *July 2008-present*
Dell Pediatric Research Institute, University of Texas at Austin, TX, Advisor: Dr. Stephen Hursting

Postdoctoral Fellow, Department of Pathology, *December 2007- May 2008*
University of Texas M.D. Anderson Cancer Center, Houston, TX, Advisor: Dr. Wei Zhang

Ph.D. in Cancer Biology, *August 2003- December 2007*
Graduate School of Biomedical Sciences University of Texas Health Science Center, Houston, Texas
Advisor: Dr. Wei Zhang

B.S. Genetics, *August 1999- May 2003*
Texas A&M University, College Station, Texas

Research Experience

Postdoctoral Fellow- Energy Balance and Cancer Prevention
> Department of Nutritional Sciences, *July 2008-present*
> Dell Pediatric Research Institute, University of Texas Austin (Dr. Stephen Hursting)

- Design and implement specifically formulated dietary regimens for pre-clinical animal trials (calorie-restriction, Atkins, low-carbohydrate, nutraceutical enriched)
- Determined the effects of energy balance modulation on epithelial-to-mesenchymal transition and autophagy in clinically relevant murine models of claudin-low, basal-like, and HER2/neu positive breast cancer
- Elucidated the role of specific receptor/ligand interactions (Leptin receptor, IGF1-receptor, Insulin receptor, etc) and their role in regulating cell growth, differentiation, or malignant transformation in the context of dietary alterations in mammary cancer prevention
- Utilized receptor/ligand interaction assays, epigenetic and genomic screens, Luminex-based western blotting and serum protein/cytokine analysis, ELISA for serum protein levels, flow cytometry for stem cell population and cell cycle analysis, invasion/migration chambers, immunofluorescence and immunohistochemistry for tumor proteins, in vivo techniques (tumor injections, drug delivery, surgeries, necropsy, cardiac puncture), and statistical analysis

Postdoctoral Fellow- NeuroOncology
> Department of Pathology, *December 2007- May 2008*
> University of Texas MD Anderson Cancer Center (Dr. Wei Zhang)

- Published translational research describing the role of integrin binding and NFκB signaling in IGFBP2-driven glioma progression in vivo and in human tumor samples
- Determined the role of Ink4a/Arf deletion, a common gene locus lost in human patients, in glioma progression in vitro and in preclinical models of human brain tumor subtypes
- Accessed human tumor banks to build custom core biopsy arrays, correlated human RNA and protein expression with mechanistic murine studies, and recommended new clinical strategies for specific subtypes of human glioma, performed epigenetic and genomic screens

- Developed stem cell assays in the laboratory for examination of the role of progenitor cells in the origin of glioma (primary cell harvest from mouse brains, cell line development, tumorsphere formation, flow cytometry)

Graduate Research Assistant- Cancer Biology
Department of Pathology, *August 2003- December 2007*
University of Texas MD Anderson Cancer Center (Dr. Wei Zhang)
- Designed Ph.D. project entitled, "Elucidation of the role of Insulin-like Growth Factor Binding Protein-2 (IGFBP-2) in glioma progression using a glial-specific transgenic mouse model."
- Examined the mechanism of protein-protein interactions between IGFBP2 and its known and unknown binding partners, performed si/shRNA knock-down screens
- Manager of murine colony (bred, genotyped, and maintained a 2000+ animal colony), trained with Chief of Neuropathology of MD Anderson, Dr. Greg Fuller, in histopathological grading of human and mouse brain tumors, performed preclinical screens of chemotheraputic agents for glioblastoma, and developed many in vitro protocols for protein expression analysis (transfection, viral infection), CHIP-assays, DNA and RNA expression microarrays, laser capture microdissection

Undergraduate Research Assistant- Protein Chemistry
Laboratory of Biological Mass Spectrometry, *January 2000-May 2003*
Department of Chemistry, Texas A&M University (Dr. David H. Russell)
- Analyzed the effect of detergents on peptide resolution using matrix-assisted laser desorption ionization (MALDI), using time-of-flight (TOF) as the main mass analyzer
- Pioneered a joint research project with Dr. Dickson Varner in the College of Veterinary Medicine that analyzed the semen protein content of sub-fertile stallions that have good sperm quality and motility versus the protein content of fertile stallions
- Analyzed the bacterial proteome of *E. coli* using HPLC and MALDI-TOF MS

Undergraduate Research Assistant- Immunology
Laboratory for Antigen Processing, *May 2002-August 2002*
Department of Immunology, Memorial Sloan-Kettering Cancer Center, NYC (Dr. Lisa Denzin)
- Developed the protocol to generate H2-Oα and H2-Oβ (mouse homologues of DOα and DOβ) His-tagged fusion proteins

Welch Summer Scholar- Inorganic Chemistry
Welch Summer Scholar Program, *June 1998-July 1998*
University of Texas, Arlington (Dr. Frederick M. MacDonnell)
- Developed the protocol to synthesize a chiral ruthenium dimmer, then connect the repeating unit to form a polymer

Laboratory Assistant- Virology
Department of Carcinogenesis, Veterinary Division, *May 1997-August 1999*
University of Texas MD Anderson Cancer Center (Dr. Tahir Rizvi)
- Research focused on the regulation of viral gene expression and viral RNA packaging using human and simian immunodeficiency viruses (HIV/SIV) and Mason-Pfizer monkey virus (MPMV) as model systems.

Journal Publications

Also published as Sarah M. Smith.

McDonald JM*, **Dunlap SM***, Cogdell D, Dunmire V, Wei Q, Starzinski-Powitz A, Sawaya R, Bruner J, Fuller GN, Aldape K, and Zhang W. The SHREW1 Gene, Frequently Deleted in Oligodendrogliomas, Functions to Inhibit Cell Adhesion and Migration. *Cancer Biology & Therapy* 5(3):300-4, 2006**.
*Co-first author.
**Publication resulted in cover image for this edition.

Dunlap SM, Celestino J, Wang H, Jiang R, Holland E, Fuller G, and Zhang W. Insulin-like Growth Factor Binding Protein 2 promotes glioma development and progression. *Proceedings of the National Academy of the Sciences* 104(28):11736-41, July 2007.

Moore LM, Holmes KM, **Smith SM**, Wu Y, Tchougounova E, Uhrbom L, Sawaya R, Bruner JM, Fuller GN, and Zhang W. IGFBP2 is a candidate biomarker for *Ink4a-Arf* status and a therapeutic target for high-grade gliomas. *Proceedings of the National Academy of the Sciences* 106(39):16675-9, September 2009.

Hursting SD**, Smith SM**, Lashinger LM, Harvey AE and SN Perkins. Calories and *Carcinogenesis:* Lessons Learned from Thirty Years of Calorie Restriction Research. *Carcinogenesis* 31(1):83-9, January 2010.

Ping J, **Smith SM**, Song SW, Jiang R, Li B, Sawaya R, Bruner JM Kuang J, Yu H, Fuller GN and Zhang W. Inhibition of gliomagenesis and attenuation of mitotic transition by MIIP. *Oncogene* 29(24):3501-8, June 2010.

De Angel RE, **Smith SM**, Glickman RD, Perkins SN, Hursting, SD. Antitumor effects of ursolic acid in a mouse model of postmenopausal breast cancer. *Nutrition and Cancer* 62(8):1074-86, 2010.

Zheng Q, **Dunlap SM**, Zhu J, Downs-Kelly E, Hursting SD, and Reizes O. Leptin deficiency suppresses MMTV-Wnt-1 mammary tumor growth in obese mice and abrogates tumor initiating cell survival. *Journal of Enodcrine-Related Cancer* 18(4):491-503, 2011.

Wong AW, **Dunlap SM**, and Nunez NP. Alcohol promotes mammary tumor development via the estrogen pathway in estrogen receptor alpha-negative HER2/neu mice. *Alcoholism: Clinical and Experimental Research* 36(4):577-87, 2012.

Nogueira LM, **Dunlap SM**, Ford NA, and Hursting SD. Calorie restriction and rapamycin inhibit MMTV-Wnt-1 mammary tumor growth in a mouse model of postmenopausal obesity. *Journal of Enodcrine-Related Cancer* 19(1):57-68, 2012.

Holmes KM, Annala M, **Dunlap SM**, Liu Y, Hugen N, Moore LM, Cogdell D, Hu L, Nykter M, Hess K, Fuller GN, Zhang W. IGFBP2-driven glioma progression is prevented by blocking a clinically significant network of integrin, ILK, and NFκB. *Proceedings of the National Academy of the Sciences* 109(9):3475-80, 2012.

Dunlap SM*, Chiao LJ, Nogueria LM, Usary J, Perou CM, Varticovski L, and Hursting SD. Dietary energy balance modulates epithelial-to-mesenchymal transition and tumor progression in murine claudin-low and basal-like mammary tumor models. *Cancer Prevention Research* 5(7):930-42, 2012.
*Publication resulted in cover image for this edition.

Hursting SD and **Dunlap SM**. Obesity, metabolic dysregulation and cancer: A growing concern and an inflammatory issue. *Annals of the New York Academy of Sciences* 1271:82-7, 2012.

De Angel, Blando J, Hogan M, Sandoval M, Lansakara D, **Dunlap SM**, Hursting SD, and Cui Z. Stearoyl gemcitabine nanoparticles overcome obesity-induced cancer cell resistance to gemcitabine in a mouse postmenopausal breast cancer model. *Cancer Biology and Therapy* (Accpeted, January 2013).

Hursting SD, **Dunlap SM**, Ford NA, Hursting MJ, and Lashinger LM. Calorie Restriction and Cancer Prevention: A Mechanistic Perspective. *Cancer & Metabolism* (invited review, submitted December 2012).

Dunlap SM, Chiao LJ, Lim J, Rasmussen AJ, Malone L, Hursting SD. Calorie restriction significantly prevents spontaneous tumor formation through modulation of estrogen receptor-β in the MMTV-neu mouse model of human luminal-A type breast cancer. (In preparation January 2013).

Dunlap SM, Lashinger LM, White E, Hursting SD. Calorie restriction synergizes with autophagy inhibition to suppress K-Ras driven oncogenesis through substrate deprivation. (In preparation January 2013).

Book Chapter

Hursting SD, **Smith SM**, Nogueira L, DeAngel R, Lashinger L, and SN Perkins. "Dietary Energy Balance, Calorie Restriction, and Cancer Prevention." <u>Bioactive Food Components and Cancer</u>. Pages 147-164. Ed. Milner JA and DF Romagin. Humana Press/Springer: New York, 2010.

Academic Awards and Honors

1999 Presidential Endowed Scholarship, Texas A&M University
1999 Brown Foundation Scholarship, Texas A&M University
1999 Dallas Morning-Star Scholar-Athlete Award
1999 Houston-Livestock Show and Rodeo Go-Texan Scholarship
1999 Robert C. Byrd Scholarship, State of Texas
1999 Lower Colorado Valley River Authority (LCRA) Scholarship
2003 Sigma Xi Honorable Mention poster presentation (Immunology Division), National Conference
2003 First Place poster presentation (Immunology Division), Student Research Week, Texas A&M University
2003 N.R. Bottino Outstanding Senior Student Research Award, Department of Biochemistry and Biophysics, Texas A&M University
2005-2007 Keck Center Pharmacoinformatics Training Program of the Gulf Coast Consortia (National Institutes of Health Grant 5 T90 DK070109-02) 3-year Fellowship, Houston, TX
2005 Unconditional pass on both written and oral portions of candidacy exam, Graduate School of Biomedical Sciences Cancer Biology Program, Houston, TX
2006 "Scholar-in-training" award for American Association of Cancer Research National Meeting, sponsored by Aflac Inc., Washington, DC
2006 GSBS Post-candidacy Travel Award for American Association of Cancer Research National Meeting, Washington, DC
2006 Third Place Platform Talk Cancer Biology Program Retreat, Graduate School of Biomedical Sciences Cancer Biology Program, Houston, TX
2006 Second Place abstract award winner (Basic Science Research Category, Graduate Division), Trainee Recognition Day, MD Anderson Cancer Center, Houston, TX
2006 American Legion Auxiliary Fellowship, Graduate School of Biomedical Sciences, Houston, TX
2007 Third Place Platform Talk Cancer Biology Program Retreat, Graduate School of Biomedical Sciences Cancer Biology Program, Houston, TX
2007 GSBS Post-candidacy Travel Award for American Association of Cancer Research National Meeting, Los Angeles, CA
2007 First place abstract award winner (Clinical/Translational Research Category, Graduate Division), Trainee Recognition Day, MD Anderson Cancer Center, Houston, TX
2007 NIH Travel award to the National Graduate Student Research Festival, Washington DC
2007 American Legion Auxiliary Fellowship, Graduate School of Biomedical Sciences, Houston, TX
2008 Trainee Excellence Award, MD Anderson Cancer Center, Houston, TX
2009 Department of Toxicology NIH Training Grant (T32-ES007247) 1-year Fellowship, University of Texas, Austin, TX

2009 Department of Defense (DOD) Breast Cancer Research Program (BCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP) FY08 Postdoctoral Award (3-years), Department of the Army, Fort Detrick, MD
2010 Department of Nutritional Sciences, Postdoctoral Fellow Research Award, University of Texas, Austin, TX
2011 Betty Hay Travel Award to the 5th International Epithelial-Mesenchymal Transition Meeting in Singapore, awarded by The EMT International Association, Sydney, Australia
2011 American Association for Cancer Research Travel Award to the San Antonio Breast Cancer Symposium, San Antonio, TX

Conference Presentations

2003 Sigma Xi National Conference, Poster presentation, Galveston, TX "The isolation and purification of H2-Oα and H2-Oβ (mouse homologues of DOα and DOβ) His-tagged fusion proteins"
2003 Student Research Week, Poster presentation, Texas A&M University, College Station, TX. "The isolation and purification of H2-Oα and H2-Oβ (mouse homologues of DOα and DOβ) His-tagged fusion proteins"
2005 American Association of Cancer Research Annual Meeting, Poster presentation, Anaheim, CA. "The putative tumor suppressor Shrew-1 gene on chromosome 1p36 inhibits adhesion and migration of glioma cells"
2005 M.D. Anderson Trainee Recognition Day, Poster presentation, Houston, TX. "The putative tumor suppressor Shrew-1 gene on chromosome 1p36 inhibits adhesion and migration of glioma cells"
2005 2nd Annual Conference for Tumor Progression and Therapeutic Resistance, Platform presentation, Boston MA. "IGFBP2 causes glioma progression *in vivo*"
2005 15th Annual Keck Center Research Conference, Poster presentation, Houston, TX. "IGFBP2 causes glioma progression *in vivo*"
2005 Graduate School of Biomedical Sciences Annual Poster Competition, Poster presentation, Houston, TX. "IGFBP2 Actively Contributes to Glioma Initiation and Progression in the RCAS-tva Glial-specific Transgenic Mouse Model System"
2006 United States and Canadian Society of Pathology Annual Meeting, **Platform presentation**, Atlanta, GA. "IGFBP2-Associated Diffuse Glioma Initiation and Progression Demonstrated in the RCAS-tva Mouse Model System"
2006 Graduate School of Biomedical Sciences Cancer Biology Program Retreat, **Platform presentation**, Houston, TX. "IGFBP2-Associated Diffuse Glioma Initiation and Progression Demonstrated in the RCAS-tva Mouse Model System"
2006 American Association of Cancer Research Annual Meeting, **Platform presentation**, Washington D.C. "Tissue-specific transgenic mouse model experiments demonstrate that IGFBP2 actively contributes to glioma development and progression"
2006 M.D. Anderson Genomics Mini-Symposium, **Platform presentation**, Houston, TX. "IGFBP2: From genomic marker to functional characterization."
2006 M.D. Anderson Trainee Recognition Day, Poster presentation, Houston, TX. " Tissue-specific transgenic mouse model experiments demonstrate that IGFBP2 actively contributes to glioma development and progression."
2006 16th Annual Keck Center Research Conference, Poster presentation, Houston, TX. "Tissue-specific transgenic mouse model experiments demonstrate that IGFBP2 actively contributes to glioma development and progression."
2006 AACR Special Conference on Mouse Models of Cancer, Poster presentation, Cambridge, MA. "Tissue-specific transgenic mouse model experiments demonstrate that IGFBP2 actively contributes to glioma development and progression."

2006 Society for Neuro-Oncology 11th Annual Meeting, **Platform presentation**, Orlando, FL. "The *SHREW1* gene, frequently deleted in oligodendrogliomas, functions to inhibit cell adhesion and migration."

2006 Society for Neuro-Oncology 11th Annual Meeting, **Platform presentation**, Orlando, FL. "Tissue-specific transgenic mouse model experiments demonstrate that IGFBP2 actively contributes to glioma development and progression."

2007 Graduate School of Biomedical Sciences Cancer Biology Program Retreat, **Platform presentation**, Houston, TX. "IIp45 attenuates IGFBP2-driven glioma progression and sensitizes glioma cells to DNA damage-induced cell cycle arrest and apoptosis."

2007 Graduate School of Biomedical Sciences Annual Poster Contest, Poster presentation, Houston, TX. "IGFBP2 activates the Akt pathway and collaborates with K-Ras or PDGFB in gliomagenesis and glioma progression"

2007 American Association of Cancer Research Annual Meeting, Poster presentation, Los Angeles, CA. "IIp45 attenuates IGFBP2-driven glioma progression and sensitizes glioma cells to DNA damage-induced cell cycle arrest and apoptosis"

2007 M.D. Anderson Trainee Recognition Day, **Platform presentation**, Houston, TX. "IGFBP2 activates the Akt pathway and collaborates with K-Ras or PDGFB in gliomagenesis and glioma progression"

2007 The National Institutes of Health (NIH) National Graduate Student Research Festival, Poster presentation, Washington D.C. "Insulin-like Growth Factor Binding Protein 2 promotes glioma development and progression via Akt pathway activation"

2007 The University of Texas Health Science Center Houston Annual Research Day, Poster presentation, Houston, TX. "Insulin-like Growth Factor Binding Protein 2 promotes glioma development and progression via Akt pathway activation"

2008 American Association of Cancer Research Annual Meeting, Poster presentation, San Diego, CA. "Integrin binding is essential for IGFBP2-driven glioma progression"

2009 American Association of Cancer Research Annual Meeting, Poster presentation, Denver, CO. "Wnt-1 mammary tumors are enriched in CD44+/CD24- cells with cancer stem cell characteristics"

2009 Aspen Cancer Conference, Invited Trainee/Poster presentation, Aspen, CO.

2009 American Association of Cancer Research Frontiers in Cancer Prevention Research Meeting, Houston, TX. "Aggressiveness of MMTV-Wnt-1 mammary tumors corresponds with enrichment of a $CD44^+/CD24^-$ putative cancer stem cell population"

2010 American Association of Cancer Research Annual Meeting, Poster presentation, Washington, D.C. "Enrichment of a $CD44^+/CD24^-$ putative cancer stem cell population drives tumor progression and growth factor independence in a mouse model of post-menopausal breast cancer."

2011 American Association of Cancer Research Annual Meeting, Poster presentation, Orlando, FL. "Obesity drives EMT and tumor progression in a novel Wnt-1 mammary cancer model."

2011 Era of Hope Conference- Military Health Research Forum, Poster presentation, Orlando FL. "Calorie restriction significantly prevents spontaneous tumor formation in the MMTV-neu mouse model of human luminal-A type breast cancer."

2011 5th International Epithelial-Mesenchymal Transition Meeting, **Platform presentation**, Biopolis, Singapore. "Obesity Promotes Epithelial-To-Mesenchymal Transition and Tumor Progression in a Syngeneic Mouse Model of Claudin-Low Breast Cancer."

2011 San Antonio Breast Cancer Symposium, **Platform presentation**, San Antonio, TX. "Obesity drives epithelial-to-mesenchymal transition and tumor progression in a novel murine claudin-low model."

2012 American Association of Cancer Research Annual Meeting, Poster presentation, Chicago, IL. "Calorie restriction activates autophagy during tumor growth suppression."
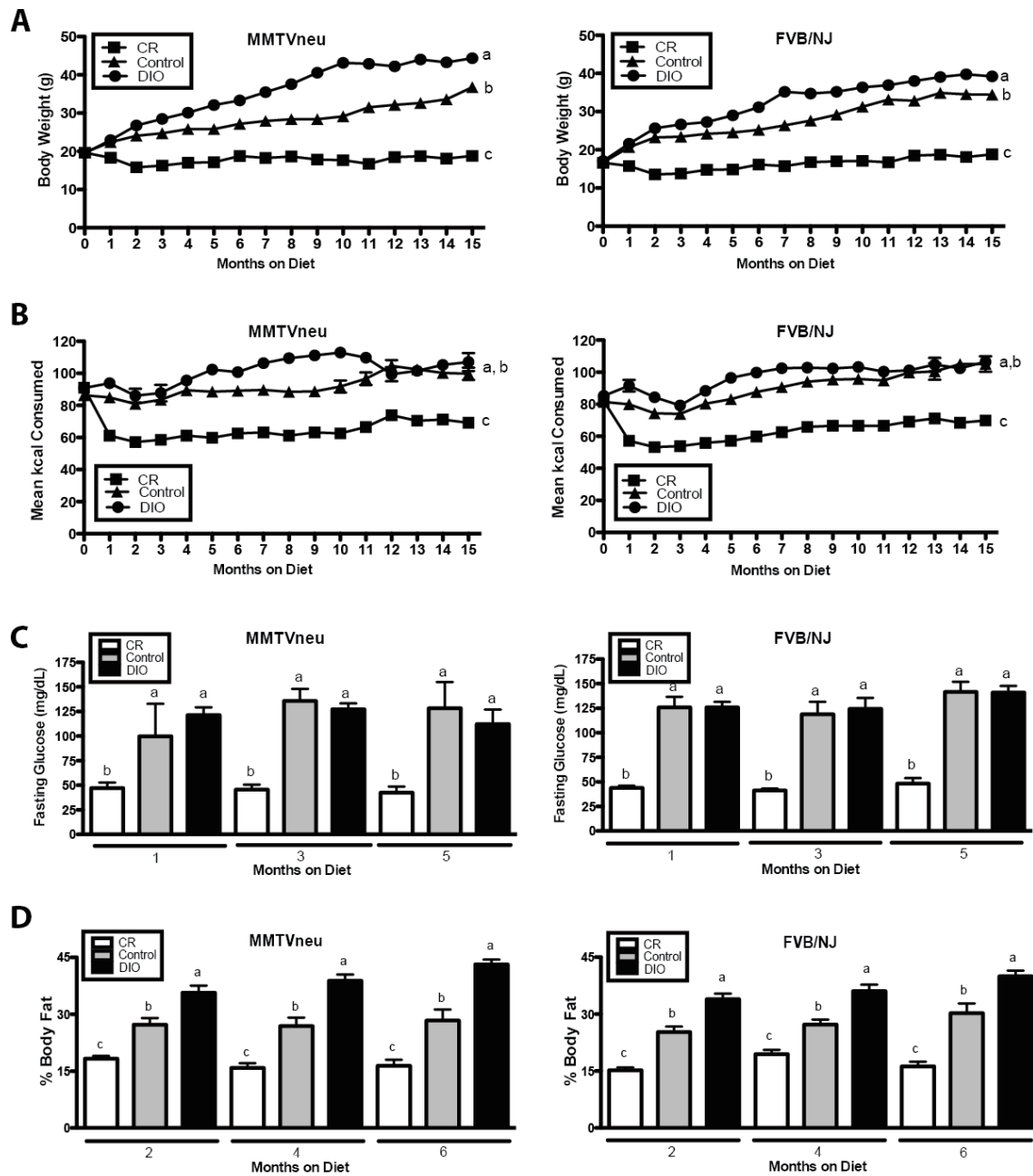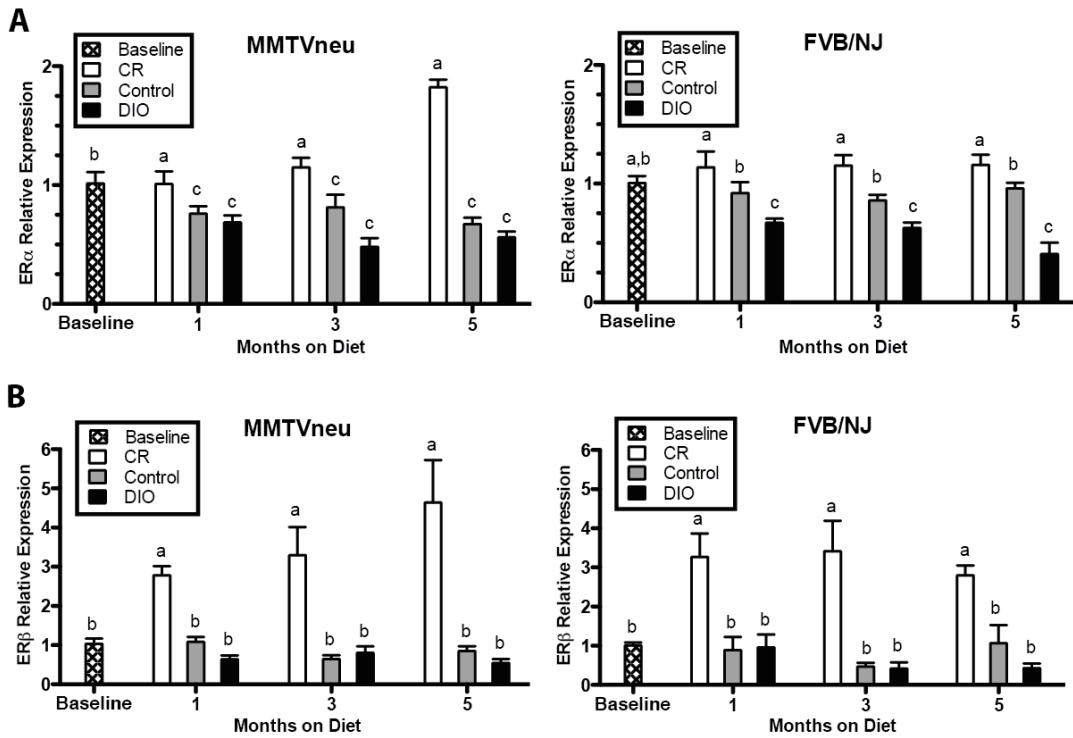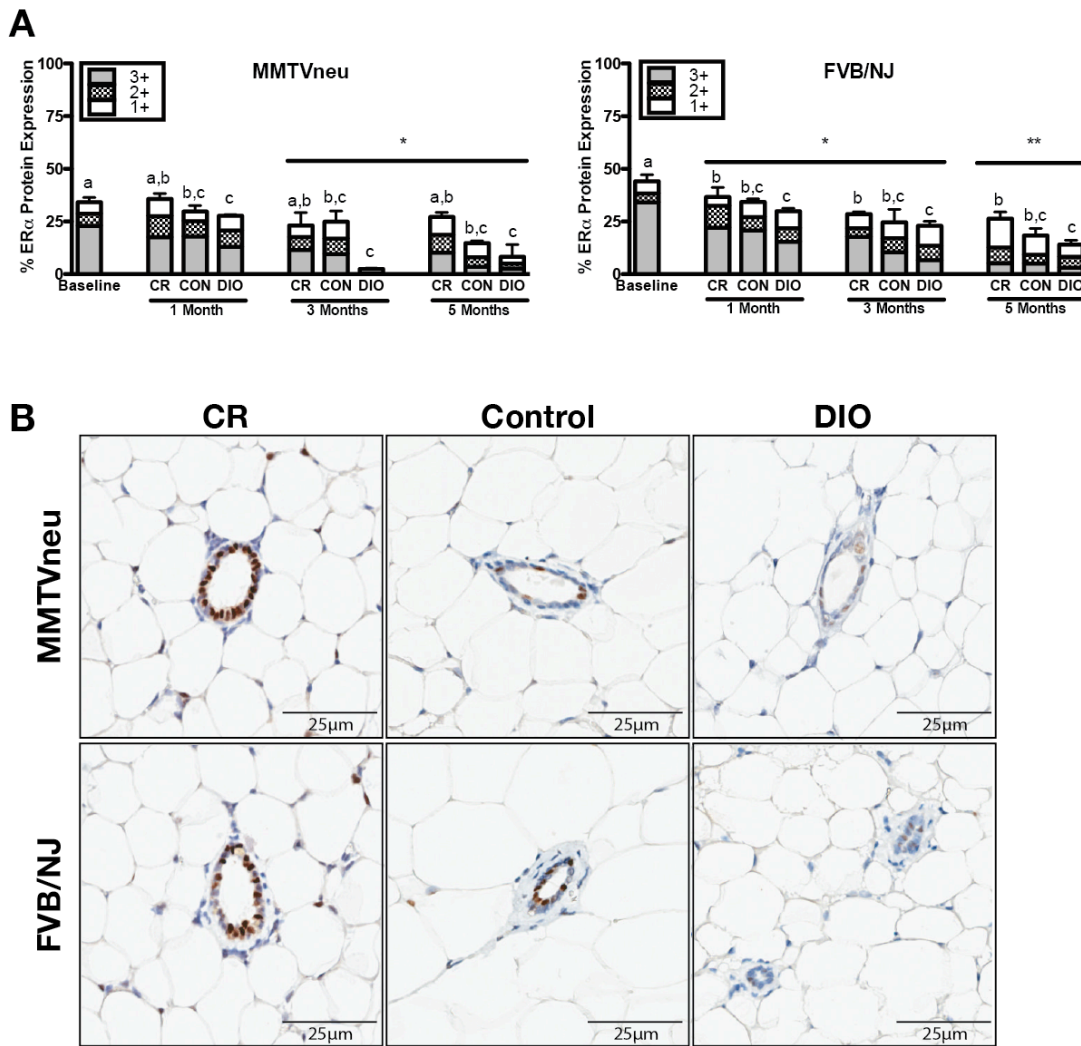
Figure 1. Effect of dietary energy balance modulation on body weights, caloric intake, fasting glucose, and body composition. (A) Body weights and (B) caloric intake for MMTVneu and age-matched non-transgenic (FVB/NJ) controls (n=30 mice/diet/genotype at 0 months) The CR mice consumed 30% less calories daily, per study design. (C) Fasting glucose readings were collected (n=4 mice/diet /genotype) and analyzed after a 6 hour fast at 1, 3, and 5 months on diet. (D) Body composition was analyzed at 4, 6, and 8 months on diet. Data are presented as means ± SEM. Different letters represent a significant effect of the dietary intervention ($P \leq 0.05$).
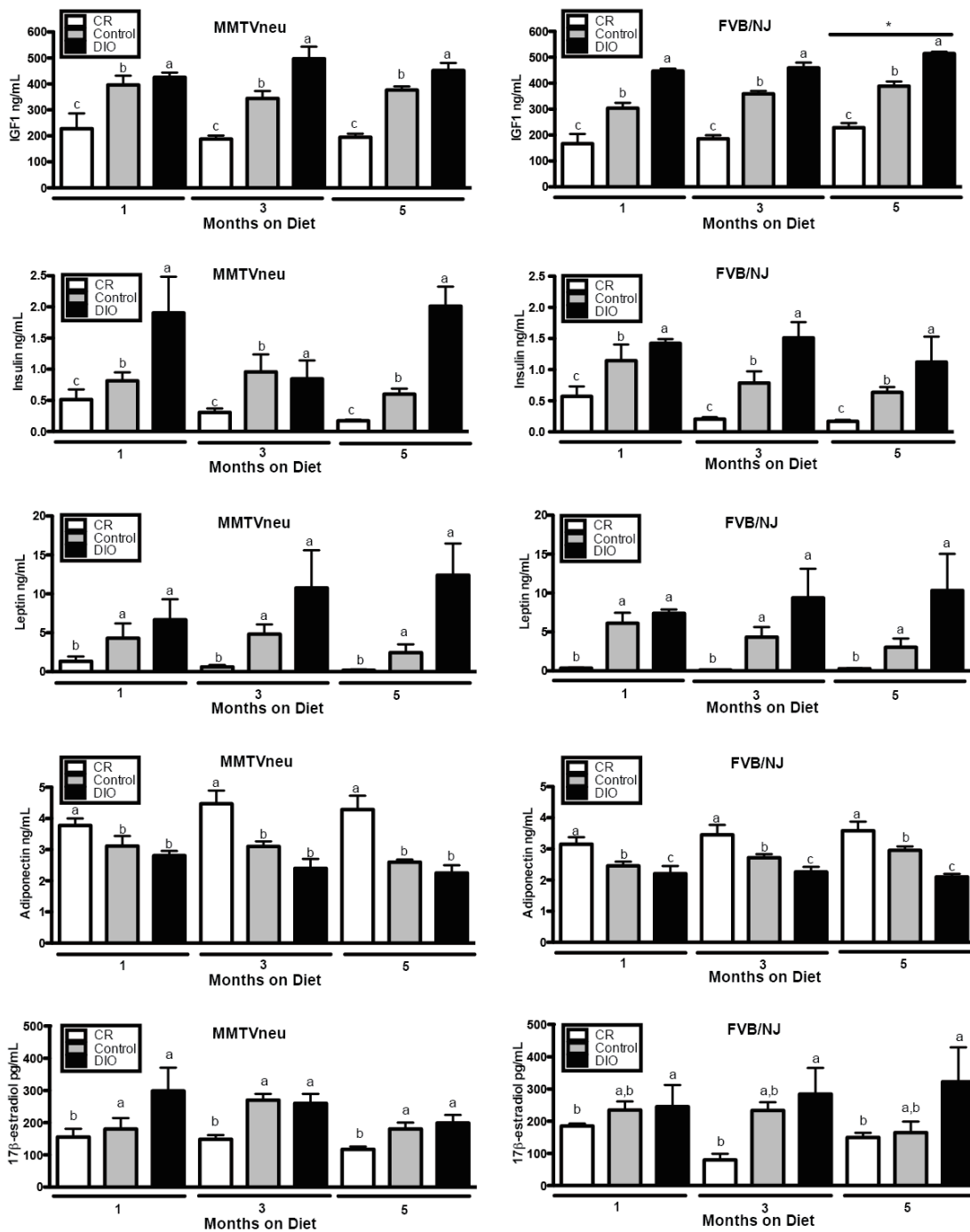
Figure 2. Effect of dietary energy balance modulation on metabolic growth factors and hormones.
Fasting serum levels of IGF-I, insulin, leptin, adiponectin, and 17b-estradiol were measured after 1, 3, and 5 months of dietary intervention (n=4 mice/diet/genotype) for MMTVneu and age-matched non-transgenic (FVB/NJ) controls. Data are presented as means ± SEM. Different letters represent significant differences between dietary groups ($P \leq 0.05$); serum insulin and leptin data were log transformed to meet test assumptions. Bars represent significant changes of serum factors over time ($P \leq 0.05$).

Figure 3. Impact of dietary energy balance on ERα/β mRNA expression in MFP of MMTVneu and FVB/NJ mice. (A) ERα and (B) ERβ relative expression at Baseline and 1, 3, and 5 months on CR, Control or DIO diets (n=4/timepoint/ diet/genotype). Data are presented as means ± SEM. Different letters represent significant differences between diet groups ($P \leq 0.05$ versus baseline; Mean ± SEM). Due to a significant interaction between diet and time in MMTVneu ERa relative expression, analysis of simple effects was performed and significant impact of diet is indicated by different letters within each time point.

Figure 4. The effect of dietary energy balance on ERα protein expression in mammary fat pad tissue of MMTVneu and FVB/NJ mice. (A) Quantification of ERα-positive cells and (B) Representative photomicrographs of mammary ducts stained using an antibody against ERα and counterstained with hematoxylin. Scale bar=25mm. Data are expressed as Mean ± SEM (n=3-4 slides/diet/genotype; 5 representative fields of view/slide). Positive staining for ERα was further stratified by 1+, low; 2+, moderate; and 3+, intense staining. Different letters represent significant differences between diet groups ($P \leq 0.05$). Bars represent significant changes in expression over time and the number of asterisks depict differences by group ($P \leq 0.05$).
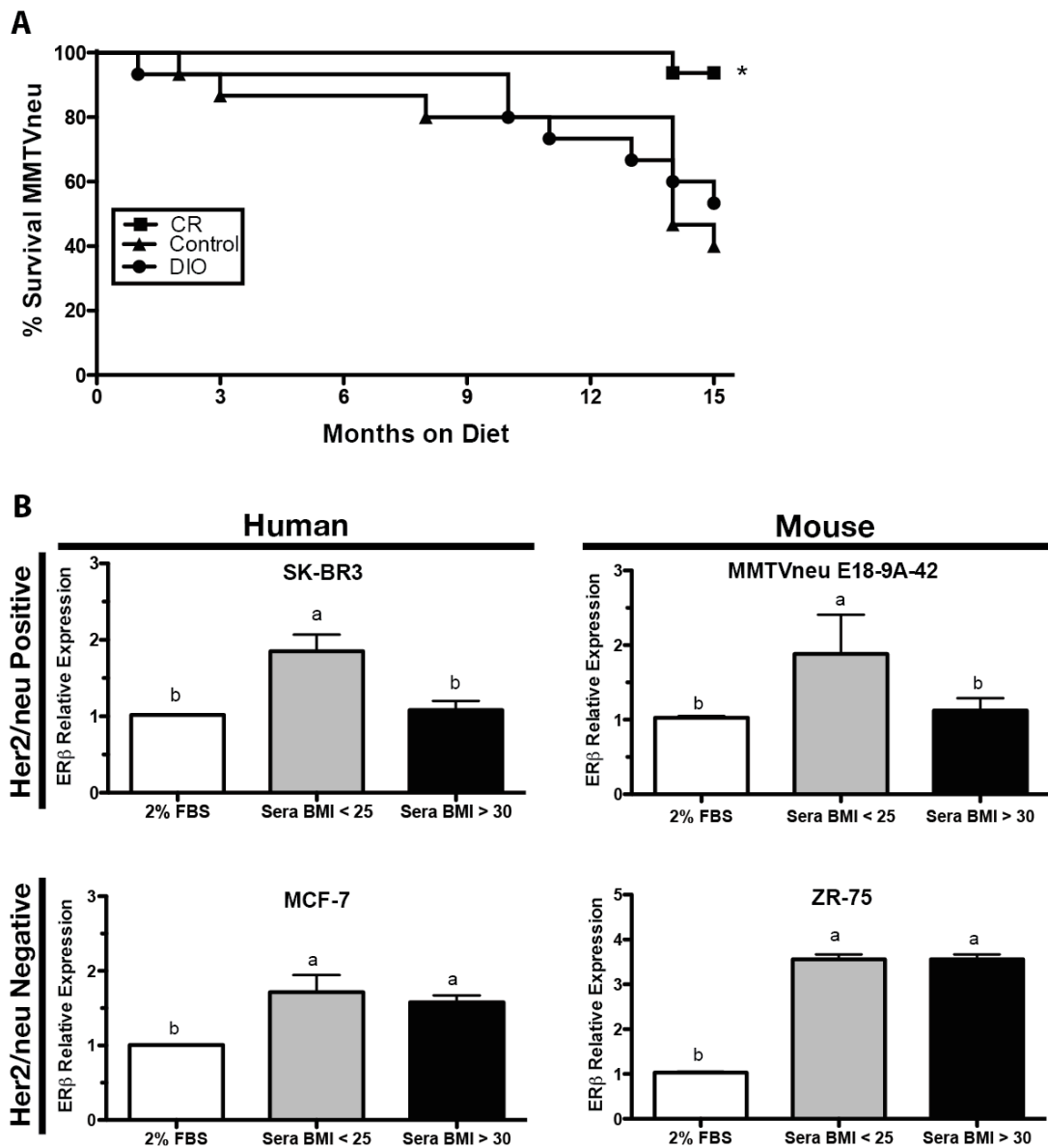
Figure 5. The impact of energy balance regimens on overall survival of MMTVneu mice and HER2/neu dependent ER-β mRNA expression alterations in vitro. (A) MMTVneu mice (n=30 mice/diet) were placed on a DIO regimen (60% kcal from fat), Control diet which induces a slightly overweight phenotype (10% kcal from fat), and 30% calorie restriction (CR; 30% reduction in calories from Control ad-libitum diet, lean phenotype) for 15 months. Data are presented as mean ± SEM and significant effects on survival are noted by an asterisk ($P \leq 0.05$)(B) ER-β mRNA expression in human (SK-BR3, MCF-7) and mouse (MMTVneu E18-9A-42, ZR-75) cell lines treated with pooled female human sera (BMI <25 or BMI >30). A 2% fetal bovine serum (FBS) treatment was used as a positive control. Different letters represent significant differences between treatment groups (Performed in triplicate, $P<0.05$).

Genotype	Diet	Months on Diet	Hyperplasia present (n=4/group)
MMTVneu	**CR**	**1**	**0%**
MMTVneu	Control	1	67%
MMTVneu	HF	1	80%
MMTVneu	**CR**	**3**	**0%**
MMTVneu	Control	3	0%
MMTVneu	HF	3	0%
MMTVneu	**CR**	**5**	**0%**
MMTVneu	Control	5	100%
MMTVneu	HF	5	33%
FVB/NJ	**CR**	**1**	**0%**
FVB/NJ	Control	1	0%
FVB/NJ	HF	1	33%
FVB/NJ	**CR**	**3**	**33%**
FVB/NJ	Control	3	50%
FVB/NJ	HF	3	80%
FVB/NJ	**CR**	**5**	**0%**
FVB/NJ	Control	5	60%
FVB/NJ	HF	5	75%

Supplementary Table 1. The impact of calorie restriction and a DIO regimen on mammary gland hyperplasia in MMTVneu and age-matched FVB/NJ mice. Data are presented as percentage of mice presenting with hyperplasia (n=4 mice/diet/genotype).

Tumor #	Genotype	Diet	Vascularity	Mitotic figures					
				Field of view: 1	*Field of view: 2*	*Field of view: 3*	*Field of view: 4*	*Field of view: 5*	*Mean*
1	MMTVneu	CR	3	1	2	0	1	0	0.8
2	MMTVneu	CR	0	0	0	1	0	0	0.2
		Avg Score	**1.5**						**0.5 +/- 0.4**
1	MMTVneu	Control	3	6	4	2	5	2	3.8
2	MMTVneu	Control	3	6	4	3	4	4	4.2
3	MMTVneu	Control	3	0	2	2	3	5	2.4
4	MMTVneu	Control	3	0	4	2	3	3	2.4
5	MMTVneu	Control	2	0	1	0	0	1	0.4
6	MMTVneu	Control	2	0	1	1	0	2	0.8
7	MMTVneu	Control	3	2	6	7	5	12	6.4
8	MMTVneu	Control	2	2	3	6	0	1	2.4
9	MMTVneu	Control	2	0	1	0	2	1	0.8
		Avg Score	**2.6**						**2.6 +/- 0.5**
1	MMTVneu	DIO	3	5	7	1	0	1	2.8
2	MMTVneu	DIO	2	0	1	0	3	3	1.4
3	MMTVneu	DIO	3	0	0	1	1	1	0.6
4	MMTVneu	DIO	1	4	2	5	6	2	3.8
5	MMTVneu	DIO	1	0	5	1	1	1	1.6
6	MMTVneu	DIO	3	2	3	10	2	5	4.4
7	MMTVneu	DIO	2	4	3	3	2	3	3
8	MMTVneu	DIO	3	1	0	1	4	3	1.8
9	MMTVneu	DIO	2	3	2	3	3	2	2.6
10	MMTVneu	DIO	2	4	2	1	2	3	2.4
		Avg Score	**2.2**						**2.4 +/- 0.3**

Supplementary Table 2. The impact of energy balance regimens on Calorie vascularity and mitotic figures in MMTVneu tumors. Key for vascularity: 0=none; 1=mild; 2=moderate; 3=marked. Key for mitotic figures: Numbers represent actual number of mitotic figures in each field of view. No *P*-value obtained due to the small number of CR tumors.