Energy Balance Modulation Impacts Epigenetic Reprogramming, ERα and ERβ Expression, and Mammary Tumor Development in MMTV-neu Transgenic Mice

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Abstract

The association between obesity and breast cancer risk and prognosis is well established in estrogen receptor (ER)-positive disease but less clear in HER2-positive disease. Here, we report preclinical evidence suggesting weight maintenance through calorie restriction (CR) may limit risk of HER2-positive breast cancer. In female MMTV-HER2/neu transgenic mice, we found that ERα and ERβ expression, mammary tumorigenesis, and survival are energy balance dependent in association with epigenetic reprogramming. Mice were randomized to receive a CR, overweight-inducing, or diet-induced obesity regimen (n = 27/group). Subsets of mice (n = 4/group/time point) were euthanized after 1, 3, and 5 months to characterize diet-dependent metabolic, transcriptional, and epigenetic perturbations. Remaining mice were followed up to 22 months. Relative to the overweight and diet-induced obesity regimens, CR decreased body weight, adiposity, and serum metabolic hormones as expected and also elicited an increase in mammary ERα and ERβ expression. Increased DNA methylation accompanied this pattern, particularly at CpG dinucleotides located within binding or flanking regions for the transcriptional regulator CCCTC-binding factor of ESR1 and ESR2, consistent with sustained transcriptional activation of ERα and ERβ. Mammary expression of the DNA methylation enzyme DNMT1 was stable in CR mice but increased over time in overweight and diet-induced obesity mice, suggesting CR obviates epigenetic alterations concurrent with chronic excess energy intake. In the survival study, CR elicited a significant suppression in spontaneous mammary tumorigenesis. Overall, our findings suggest a mechanistic rationale to prevent or reverse excess body weight as a strategy to reduce HER2-positive breast cancer risk. Cancer Res; 77(9); 2500–11. ©2017 AACR.

Introduction

Breast cancer is the most frequently diagnosed noncutaneous neoplasm among women in the United States and is the leading cause of cancer-related death among women worldwide (1, 2). Approximately 30% of all breast cancers lack estrogen receptor α (ERα), which confers a worse prognosis in comparison with ERα-positive breast tumors (3). Furthermore, the expression of estrogen receptor β (ERβ), a putative tumor suppressor, is also lost in most ERα-negative breast tumors (4). ERβ is the more prevalent ER in normal mammary tissue, but its expression is reduced during tumor formation. Numerous studies have linked greater breast tumor ERβ expression with an improved prognosis (5, 6). However, the degree to which ERα and ERβ expression is impacted by dietary energy balance and/or controlled epigenetically in breast tumorigenesis remains unclear.

Obesity, a result of chronic positive dietary energy balance, is an established risk factor for postmenopausal breast cancer and may also enhance risk in premenopausal women with additional breast cancer risk factors, including type II diabetes (7, 8). In addition, excess energy intake and increased adiposity have been associated with greater breast tumor size, increased progression markers, and therapy resistance in both pre- and postmenopausal women (9–11). In contrast, the maintenance of a negative energy balance via calorie restriction (CR) prevents weight gain and inhibits the development of several types of cancer, including ER-positive and ER-negative breast cancers, in numerous animal models (12, 13). CR has also been associated with changes in...
several serum and tissue biomarkers in women causally linked with reduced breast cancer risk (14, 15).

Although links between energy metabolism, epigenetic regulation of gene expression, and several chronic diseases have been previously established, the relationship between dietary energy balance, epigenetics, and breast cancer is poorly understood (16, 17). DNA methylation levels are regulated in part by DNA (cytosine-5)-methyltransferase 1 (DNMT1), which predominantly serves to maintain genomic DNA methylation during DNA replication (17). Thus, during times of high cell proliferation, such as during development, DNMT1 is highly expressed. However, DNMT1 can become deregulated throughout the life course in response to metabolic, inflammatory, and environmental disturbances, and this dysregulation has been linked with aberrant DNA methylation and cancer (16, 18–20).

The effects of DNA methylation are dependent upon the location of methyl groups in the genomic landscape. In general, promoter methylation results in transcriptionally silent genes. However, methylation in the transcription region of genes is often positively correlated with gene expression (21, 22). In addition, methylation that prevents a repressor from binding DNA can correspond to increased gene expression (23). CCCTC-binding factor (CTCF) is an 11-zinc finger protein and highly conserved transcription factor with enhanced-blocking activity (24). DNA methylation at CpG (5′-C-phosphate-G-3′) dinucleotides is inversely correlated with CTCF occupancy (23, 25). We posit that the diet–methylation–CTCF axis may serve as a critical sensory system that regulates gene transcription via energy balance–associated changes in DNA methylation at or near CTCF-binding sites.

The genetically engineered mouse mammary tumor virus (MMTV)-neu mouse model is characterized by mammary gland overexpression of the oncogene HER2. MMTV-neu mice initially have histologically normal, ERα-positive mammary glands that subsequently develop regions of ductal carcinoma in situ with ERα expression lost in most cells. If untreated, these mice ultimately develop ERα-negative, HER2-positive mammary adenocarcinomas before 24 months of age (26). In this study, we tested the hypothesis that dietary energy balance modulation alters ERα and/or ERβ expression, DNA methylation, and tumor incidence in female MMTV-neu mice.

Materials and Methods

In vivo studies in MMTV-neu transgenic mice

All animal studies and procedures were approved and monitored by the University of Texas Institutional Animal Care and Use Committee (IACUC). Female 6- to 8-week-old MMTV-neu mice (JAX stock #002376, n = 86) were purchased from The Jackson Laboratory and fed a modified AIN-93G semipurified diet, defined as the overweight-inducing diet for this study (catalog #D12450, Research Diets, Inc.) ad libitum for 1 week of acclimation.

Baseline mice. Following acclimation, a subset of mice (n = 5) were fasted for 6 hours and then euthanized by CO2 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 × g for 5 minutes; serum was removed and stored at −80°C for subsequent analyses. Mammary tissues were collected for further molecular and pathologic analysis.

Time point study. A subset of 36 mice were singly housed and randomized (n = 12/diet group) to one of the following three diet treatment groups (each modified from the overweight diet, which is AIN-93G semipurified diet formulation) for a 5-month time point study: (i) CR regimen, a low-fat, low-carbohydrate diet (#D0302702); (ii) overweight diet, a high-carbohydrate, low-fat diet providing 3.8 kcal/g (#D12450B); or (iii) diet-induced obesity regimen, a high-carbohydrate, high-fat diet providing 5.2 kcal/g (#D12492), all from Research Diets, Inc. CR mice were administered their diet formulation as daily aliquots of food that provided 70% of the kcal but 100% of the vitamins, minerals, essential fatty acids and amino acids relative to the overweight group. Mice were weighed weekly. After 1, 3, and 5 months on diet, mice were analyzed for percent body fat using quantitative magnetic resonance spectroscopy (Echo Medical Systems). At each of these same time points, 4 mice per diet group were killed, and serum and nonmammary-bearing mammary tissue were collected as described above. No tumors developed in any mice in the time point study.

Survival study. The remaining subset of 45 mice was singly housed and randomized (n = 15/diet group) to the CR, overweight, or diet-induced obesity diet regimens and was followed for survival for up to 22 months. The survival curve for each diet group illustrates time-to-event data, with the event consisting of either death or the presence of a mammary tumor >1.0 cm in any direction, the IACUC-approved maximal tumor size (27). Nonmammary-tumor-related deaths were censored. Mice were palpated for mammary tumors weekly. Once detected, tumor diameters were measured in two dimensions twice weekly with electronic calipers. When tumor diameter reached 1.0 cm in either dimension (or after 22 months of study in the absence of tumor), mice were killed. Tumor and/or distal mammary tissue were collected and processed. One half of each collected tissue sample was 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 IHC analysis. The other half was placed in a cryotube, flash frozen in liquid nitrogen, and stored at −80°C for subsequent molecular analyses. Blood was collected by cardiac puncture, and serum was isolated for analysis.

Analyses of circulating energy balance–related hormones and 17β-estradiol

Serum samples from all 5 mice at baseline and all 4 mice per diet group at the 1-, 3-, and 5-month time points were collected after mice were fasted to reduce variability of metabolic hormones. Estrous cycle was not assessed. Serum samples were analyzed for leptin, insulin, insulin-like growth factor (IGF)-1, and adiponectin by Luminex-based bead array assay (Millipore) read on MagPix multianalyte detection system (Bio-Rad). The mean interassay coefficient of variation of multiplexed bead-based assays for metabolic hormone detection has been shown to be <15% in published studies (28). Serum 17β-estradiol was measured by ELISA (Alpha Diagnostics).

qRT-PCR analyses of ERα and ERβ

Total RNA was extracted using TRI-Reagent (Sigma-Aldrich) according to the manufacturer’s instructions from the flash-frozen mammary tissue samples collected at baseline (n = 5 mice) and each of the three time points (n = 4 mice/diet group/time point).
RNA was also extracted from nontumor-bearing, flash-frozen mammary tissue collected from mice in the survival study upon their termination (between 14 and 22 months). RNA concentration was spectrophotometrically determined using a NanoDrop (Thermo Fisher Scientific), and quality was confirmed using an Agilent 2100 Bioanalyzer. RNA was reverse transcribed with Multiscribe RT (Applied Biosystems), and resulting cDNAs were assayed in triplicate using TaqMan Gene Expression Assays for ERα, ERβ, and DNM1 (Applied Biosystems). PCR reactions were monitored by a ViiA 7 Real-Time PCR System (Applied Biosciences). Gene expression data were normalized to the housekeeping gene β-actin and analyzed using the ΔΔCt method.

**Histopathologic and IHC analyses**

Tumors were examined for histopathologic markers of tumor progression, including vascularity (presence of blood vessels) and proliferation (number of mitotic figures per field) in H&E sections by a board-certified veterinary pathologist. Vascularity was graded in a blinded fashion on a categorical score for the entire slide (0 = no intratumoral blood vessels present, 1 = low number of vessels present, 2 = medium number of vessels present, and 3 = high number of vessels present). Mitotic figures were counted in five nonoverlapping fields of view, and a mean number of mitotic figures was determined for each mouse. Values from each mouse were used to calculate mean vascularity score and mitotic figures for each diet group.

IHC staining of mammary tissue was performed (n = 4 mice/diet group) using a primary antibody for ERα (catalog #sc542, Santa Cruz Biotechnology) at 1:500 and ERβ (Abcam #3576) at 1:100. The secondary antibody was horseradish peroxidase-labeled anti-rabbit antibody (DakoCytomation).

**DNA methylation analysis**

DNA was extracted from a random sample (n = 3/group) of mammary tissues from baseline mice and CR and overweight mice in the 5-month time point and survival study using UltraPure phenol:chloroform:iSoamyl alcohol per manufacturer’s instructions (Life Technologies). Library preparation and sequencing for baseline, CR, and overweight in the 5-month time point and survival study were performed at UT MD Anderson Cancer Center’s DNA Methylation Analysis Core and Science Park Next Generation Sequencing Facility, according to published protocols as described previously (Supplementary Table S1; ref. 29). Samples from diet-induced obesity mice were not analyzed given the cost of reduced representation bisulfite sequencing (RRBS) and the similarities between overweight and diet-induced obesity mice in ERα and ERβ mRNA and protein expression. Gene promoter regions were calculated based on RefSeq gene annotations with regions starting 1 kb upstream of the annotated transcription start site (TSS) and extending 500 base pairs downstream of TSS. Differential methylation was calculated by filtering samples based on read coverage ≥ 20, then performed at the single base level. Methylkit R package was used to apply logistic regression and the likelihood ratio test. Observed P values were adjusted with the success likelihood index method. CpG dinucleotides that exhibited differential methylation patterns between CR and overweight groups were cross-referenced with annotated gene-regulatory regions within and surrounding ESR1 and ESR2 in Mus musculus outlined by Ensembl (30). To generate a heatmap, we identified CpG dinucleotides with significantly higher methylation in CR survival versus overweight survival mice that also had percent methylated DNA values available for baseline and CR and overweight mice at the 5-month time point. Differentially methylated CpG dinucleotides were clustered using hierarchical clustering with complete linkage and a Euclidian distance measure. Corresponding dendrogram and heatmaps for promoter, intron, exon, and other were produced using the heatmap2 function from the gplots package in R (version 3.3.1).

**Differential expression analysis using RNA sequencing and Ingenuity Pathway Analysis**

RNA was extracted as described above. RNA libraries were prepared using the Illumina TruSeq Stranded Total RNA Sample Preparation kit according to manufactures instructions. The libraries were sequenced using a 2 × 76 bases paired end protocol on the Illumina HiSeq 2000 instrument. The reads were mapped to mouse genome (mm10) by TopHat (version 2.0.7; ref. 31). The number of fragments in each known gene from RefSeq database (UCSC Genome Browser 2013; ref. 32) was enumerated using HTSeq-count from HTSeq package (version 0.5.3p9; HTSeq). The differential expression between conditions was statistically assessed by R/Bioconductor package EdgeR (version 1.10.1). Genes with FDR ≤ 0.05 were called significant. For pathway analysis, genes with differential expression in tissue from CR versus overweight mice after 5 months on diet and/or in survival study mice were integrated into Ingenuity Pathway Analysis (IPA) Path Designer (Qiagen) to draw connections of regulatory relationships using validated scientific findings.

**Statistical analyses**

Values are presented as mean ± SD. For all tests, GraphPad Prism software was used (GraphPad Software Inc.), and P < 0.05 was considered statistically significant. Differences between diet groups in body weight were analyzed by repeated measures ANOVA, followed by Tukey post hoc test. Differences between diet groups in insulin, leptin, adiponectin, IGF-1, 17β-estradiol, and mammary ERα and ERβ mRNA and protein expression at each time point were analyzed by one-way ANOVA followed by Tukey post hoc test. Kaplan–Meier survival curves were plotted, and the difference in overall survival between the groups was analyzed by the log-rank test.

**Results**

**Dietary energy balance modulation impacts body weight, body composition, and serum metabolic hormones**

Female MMTV-neu mice were randomized to receive dietary energy balance modulation via CR, overweight-inducing, or diet-induced obesity diet regimens and were monitored as part of a 1-, 3-, and 5-month time point study (n = 4 mice/diet for each time point) or for up to 22 months in a survival study (n = 15 mice/diet; hereafter referred to as ‘survival study mice’). CR, overweight, and diet-induced obesity diet regimens resulted in lean, overweight, and obese phenotypes, respectively. After 1 month of diet treatment, differences in average caloric intake (Supplementary Fig. S1) produced differences in mean body weight (Fig. 1A), with CR < overweight < diet-induced obesity. This continued for up to 15 months in the survival study mice, after which tumor development in overweight and diet-induced obesity mice made body weight measurements unstable. CR mice had significantly lower percent body fat (Fig. 1B) compared with diet-induced obesity mice after 1, 3, and 5 months on diet. Overweight mice...
Dietary energy balance modulation affects body weight and percent body fat in MMTV-neu mice. A, Body weight (mean ± SD) in mice receiving CR, overweight-inducing (OW), or diet-induced obesity (DIO) diet regimens over 15 months (n = 27 mice/diet). Heterogeneity in body weights after 15 months increased as mice became moribund; therefore, weight data beyond this point are not shown. Statistical differences in body weights between groups were determined by repeated measures ANOVA. B, Percent body fat assessed by quantitative magnetic resonance spectroscopy (mean ± SD) at 1, 3, and 5 months on diet (n = 4 mice/diet/time point). Statistical differences in body fat between groups were determined by one-way ANOVA. Overweight or diet-induced obesity versus CR: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Overweight versus diet-induced obesity: †, P < 0.05; ‡, P < 0.01; ‡‡‡, P < 0.001.

Figure 1.

Table 1. Energy balance impacts serum metabolic hormones

<table>
<thead>
<tr>
<th>Metabolic Hormone</th>
<th>Baseline (ng/mL)</th>
<th>CR</th>
<th>OW</th>
<th>DIO</th>
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<tr>
<td>Insulin</td>
<td>0.83 ± 0.40</td>
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<td>0.3 ± 0.1</td>
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<td>0.7 ± 0.2</td>
<td>1.8 ± 0.5***</td>
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<td>Leptin</td>
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<td></td>
<td>0.6 ± 0.5</td>
<td>4.1 ± 2.4*</td>
<td>6.0 ± 2.7*</td>
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<td></td>
<td>0.2 ± 0.2</td>
<td>3.2 ± 2.0</td>
<td>9.2 ± 6.1*</td>
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<tr>
<td>Adiponectin</td>
<td>2.02 ± 0.32</td>
<td>3.8 ± 0.5</td>
<td>3.1 ± 0.7</td>
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<tr>
<td></td>
<td></td>
<td>4.5 ± 0.9</td>
<td>3.1 ± 0.3*</td>
<td>2.4 ± 0.6*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.3 ± 0.9</td>
<td>2.6 ± 0.2**</td>
<td>2.5 ± 0.5**</td>
</tr>
<tr>
<td>IGF-1</td>
<td>463.2 ± 88.7</td>
<td>232.2 ± 96.7</td>
<td>396.5 ± 70.3*</td>
<td>425.4 ± 36.8*</td>
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<td>187.8 ± 26.5</td>
<td>344.4 ± 56.4*</td>
<td>503.9 ± 112.2***</td>
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<td>194.9 ± 26.4</td>
<td>376.2 ± 28.5***</td>
<td>452.0 ± 57.3***</td>
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<td>17-β estradiol</td>
<td>209.8</td>
<td>155.6 ± 50.9</td>
<td>180.4 ± 68.0</td>
<td>227.9 ± 36.7</td>
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<td>148.7 ± 25.2</td>
<td>262.4 ± 42.1*</td>
<td>260.1 ± 58.8*</td>
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<tr>
<td></td>
<td></td>
<td>117.4 ± 17.4</td>
<td>198.1 ± 22.3*</td>
<td>210.4 ± 54.5*</td>
</tr>
</tbody>
</table>

NOTE: Differences between diet groups in insulin, leptin, adiponectin, IGF-1, and 17β-estradiol at each time point were analyzed by one-way ANOVA followed by Tukey post hoc test. Overweight or diet-induced obesity vs. CR: *, P < 0.05; **, P < 0.01; †††, P < 0.001. Overweight versus diet-induced obesity: †, P < 0.05; ‡, P < 0.01; ‡‡‡, P < 0.001.

Abbreviations: DIO, diet-induced obesity; OW, overweight.

Dietary energy balance modulation impacts mammary ERα and ERβ expression

Mammary tissue was collected from time point study mice after 1, 3, and 5 months and from survival study mice between 14 and 22 months upon their termination. Tissues were analyzed for ERα and ERβ expression, and values are reported as relative to expression in mammary tissues collected from a baseline sample of 5 mice killed before initiation of dietary modulation. CR mice, compared with overweight and diet-induced obesity mice, had significantly higher mammary mRNA levels of ERα after 3 and 5 months on diet and in survival study mice (Fig. 2A). In addition, mammary ERα protein levels were decreased in overweight and diet-induced obesity mice, compared with CR mice, by 1 month and continued throughout study (Fig. 2B and F).

Relative to baseline, mammary ERβ mRNA levels were increased (at least 4-fold, on average) in tissues from CR mice, but decreased in tissues from overweight and diet-induced obesity mice (Fig. 2C). At the 1-month time point and every time point thereafter, CR mice had significantly greater levels of both ERβ mRNA and ERβ protein compared with overweight and diet-induced obesity mice (Fig. 2C, D, and F). No differences between overweight and diet-induced obesity mice in ERα expression, ERβ expression, or the ratio of ERα to ERβ were found. Significant diet-dependent differences in the ERα to ERβ ratio were detected at
each time point in tissues collected from CR versus overweight mice, as well CR versus diet-induced obesity mice (Fig. 2E).

Dietary energy balance modulation impacts DNA methylation within ESR1 and near ESR2

Using RRBS, we analyzed diet effects on the methylation status of DNA isolated from the mammary tissue of baseline, CR, and overweight mice at the 5-month time point and in survival study mice. Because of the similarities between overweight and diet-induced obesity mice in ERα and ERβ mRNA and protein expression, samples from diet-induced obesity mice were not analyzed.

Mammary DNA methylation was generally higher in CR mice than overweight mice, particularly in CpG dinucleotides at
CTCF-binding sites or flanking regions within ESR1 and near ESR2 (Supplementary Table S2). In mammary tissue collected in the survival study from CR mice, compared with overweight mice, the percentage of methylated DNA was significantly different (CR > overweight) at three distinct intronic CpG dinucleotides (Chr10:4710028, Chr10:4710036, and Chr10:4710084) at an annotated CTCF-binding site within ESR1, which encodes ERα. Diet-dependent effects on ERα DNA methylation were not observed in samples from the 5-month time point (Fig. 3A; Supplementary Table S3).

**Figure 3.**
Dietary energy balance modulation affects DNA methylation of the intron regions of ESR1 and upstream and downstream of ESR2. **A,** ESR1 intron methylation (mean percent DNA methylation ± SD) in the mammary tissue of CR and overweight mice at the 5-month time point and survival study mice. TF, transcription factor. DNA methylation at three distinct CpG dinucleotides (Chr 10: 4710028, Chr 10: 4710036, and Chr 10: 4710084), which all fall within a CTCF-binding site, are shown. **B,** Mean percent DNA methylation ± SD at CpG dinucleotides upstream (Chr 12: 76080926 and Chr 12: 76086754) and downstream (Chr 12: 76080926, Chr 12: 76086754, and Chr 12: 76086771) of ESR2 in the mammary tissue of mice maintained on CR or overweight diet regimens for 5 months or through survival are shown. These all fall within a CTCF-binding site or CTCF flanking region as indicated. Statistical differences determined by logistic regression and the likelihood ratio test; P values were adjusted with the success likelihood index method. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
In mammary tissue from CR mice compared with overweight mice, collected at either the 5-month time point or the survival study, 5 CpG dinucleotides near ESR2 (the gene encoding ERβ) had significantly higher methylation within or near a CTCF-binding site (Fig. 3B). Specifically, significant diet-dependent effects on DNA methylation were detected in samples from the survival study at three CpG dinucleotides near ESR2 (Chr 12:76080926, downstream of ESR2; and Chr 12:76184418 and Chr 12:76184436, both upstream) that all fell ± 1 kb of a CTCF-binding site, which we define as a CTCF flanking region. We also observed significantly higher methylation in CR mice at the 5-month time point at two other sites (Chr 12:76086754 and Chr 12:76086771, both downstream) that fell within an annotated CTCF-binding site (Fig. 3B; Supplementary Table S4).

Dietary energy balance modulation impacts ERα and ERβ expression in part through epigenetic mechanisms

We investigated global diet-dependent differences in mammary DNA methylation in CR versus overweight mice. After 5 months on diet, overweight mice had 511 CpG dinucleotides with significantly higher methylation and 248 CpG dinucleotides with significantly lower methylation compared with CR mice. Furthermore, survival study mice possessed more pronounced diet-dependent differences in mammary DNA methylation. Overweight survival study mice had 651 CpG dinucleotides with significantly higher methylation and 690 with significantly lower methylation compared with CR. Often, these genes with higher methylation in CR survival compared with overweight survival had higher methylation levels in baseline and in CR and overweight mice at the 5-month time point, supporting a deviance in overweight survival methylation (Fig. 4A and B).

Mammary gland RNA sequencing (RNA-seq) analysis demonstrated that DNMT1 expression was significantly higher in overweight compared with CR survival study mice (Supplementary Table S5). To validate RNA-seq results, we analyzed DNMT1 mRNA levels by qRT-PCR in the mammary tissue of CR, overweight, and diet-induced obesity mice at time points (Fig. 4C). DNMT1 mRNA levels were not significantly different among CR, overweight, and diet-induced obesity mice after 1, 3, and 5 months on diet. However, in survival study mice, mammary tissues from overweight and diet-induced obesity mice had significantly higher DNMT1 mRNA levels compared with mammary tissues from CR mice.

RNA-seq analysis in nontumor mammary tissue from the survival study also identified several components of the STAT3, NF-kB, E2F transcription factor (E2F), and insulin signaling pathways that can impact expression of DNMT1 and were significantly different in overweight than CR mice (Supplementary Table S5). Each of these pathways can impact expression of DNMT1 (33–35). Thus, the metabolic and inflammation-related perturbations measured in overweight and diet-induced obesity mice (relative to CR mice) may underlie the observed diet-dependent changes in expression of DNMT1.

CR inhibits MMTV-neu mammary tumor development

Compared with overweight and diet-induced obesity diet regimens, CR was associated with significantly increased survival (Fig. 5A). Survival was comparable between overweight and diet-induced obesity mice. After 22 months of study, only 1 overweight mouse and 2 diet-induced obesity mice, compared with 11 CR mice, remained alive and tumor free. Among the tumor-bearing mice, the mammary tumors from overweight (n = 12) and diet-induced obesity (n = 11) mice, compared with CR mice (n = 3), were generally more vascular and consisted of more proliferating cells (Fig. 5B).

Discussion

This study assessed whether dietary energy balance modulation, ranging from lean (CR) to overweight to obesity (diet-induced obesity), alters mammary ER expression, epigenetic reprogramming, and/or mammary tumor development in female MMTV-neu transgenic mice. We first characterized diet-dependent metabolic perturbations in subsets of diet-treated mice euthanized at baseline, 1, 3, and 5 months on study. CR mice, relative to overweight and diet-induced obesity mice, had decreased body weight, adiposity, and obesity-associated serum metabolic hormones, including insulin, leptin, IGF-1, and 17β-estradiol, as well as increased adiponectin, after 1 month of diet treatment, consistent with previous studies (36, 37).

We show for the first time that CR (but not overweight and diet-induced obesity regimens) preserves mammary ERα and ERβ expression in MMTV-neu mice. Loss of ERα and ERβ and increased ERα to ERβ, as observed in overweight and diet-induced obesity mice, have each been linked with poor breast cancer prognosis in clinical studies (38, 39). Our finding that the ERα to ERβ ratio, a prognostic indicator in breast cancer, can be manipulated by energy balance modulation has not, to our knowledge, been previously reported.

We and others have shown that obesity can induce aberrant DNA methylation of genes involved in growth factor and inflammatory signaling (29, 40). To assess whether the energy balance–dependent effects on ERα and ERβ expression are controlled epigenetically, we characterized epigenetic alterations in mammary tissue DNA from baseline mice, from CR and overweight mice after 5 months on diet, and from survival study mice after 14 to 22 months of diet. In survival study mice, differentially methylated CpG dinucleotides were observed in a CTCF-binding site within the ESR1 gene and in CTCF-binding sites or flanking regions (±1 kb of a CTCF-binding site) upstream and downstream of ESR2, consistent with sustained transcriptional activation of ERα and ERβ. Mammary expression of DNMT1 was stable in CR mice but increased over time in overweight and diet-induced obesity mice, suggesting CR prevented epigenetic reprogramming of DNMT1 that occurs with excess energy intake and weight gain. The effect of altered DNMT1 expression likely impacts the expression of many genes, including ESR1 and ESR2. However, a plausible mechanism for the sustained expression of the ESR1 and ESR2 genes in response to CR is the maintenance of DNMT1 expression and DNA methylation (Fig. 6). This can prohibit CTCF binding, thereby preventing allosteric repression and decreased interactions between enhancers and promoters (24). Previous studies have shown that DNA methylation can impede CTCF binding, positively influencing transcription via a loss of repression of specific genes, such as GAD1 (41) and XAF1 (42), and increased enhancer–promoter interactions in FGZ2 (43) and c-MYC (44).

We also assessed the effects of dietary energy balance modulation on mammary tumor development in a cohort of mice randomized to the three diets (n = 15/diet). CR, relative to the overweight and diet-induced obesity regimens, resulted in significantly increased survival in MMTV-neu mice in association with
Figure 4.
Differences in dietary energy balance impact genome-wide methylation patterns in the mammary tissue. A, Heatmap and clustering dendrogram of mammary tissue DNA methylation. CpG dinucleotides with significant differences in methylation [CR survival vs. overweight (OW) survival] are shown and clustered within genomic location, that is, promoter, exon, intron, and other. Baseline, CR, and overweight after 5 months on diet and CR and overweight survival study mice are each represented (n = 3/group, with one column per n shown). The methylation frequency percentage ranges from 0 to 100. A value of “0” is completely unmethylated and “100” is fully methylated. Statistical differences (CR vs. overweight survival) determined by logistic regression and the likelihood ratio test; P values were adjusted with the success likelihood index method. B, Representation of genomic locations of differentially methylated CpG dinucleotides; 6.5% mapped to a promoter region, 34.1% mapped to a gene exon, 25.6% mapped to an intron region, and 33.8% did not map to a promoter, exon, or intron and are classified as “other.” C, Mammary DNMT1 mRNA levels, measured by qRT-PCR, in mice maintained on CR, overweight, and diet-induced obesity (DIO) diet regimens for 1, 3, and 5 months or through survival. Data are presented as relative to baseline levels (n = 4 mice). Statistical differences in DNMT1 expression determined by one-way ANOVA. *, P < 0.05; **, P < 0.01.
obesity (determined by log-rank test. induced obesity mice) were censored. Statistical differences in survival rate period. Mice with nontumor-related deaths (1 CR, 2 overweight, and 2 diet-/C6 mean bears mice, the mammary tumors from overweight (group mean ¼ 3), were more vascular (group mean ± SD; vascularity scores: 2.6 ± 0.18, 2.2 ± 0.25, and 1.5 ± 1.5, respectively) and consisted of more proliferating cells (group mean ± SD; number of mitotic figures indicated by black arrowhead: 2.6 ± 0.5, 2.4 ± 0.3 and 0.5 ± 0.4, respectively). Statistical differences in vascularity and mitotic figures determined by one-way ANOVA. ***, P < 0.001.

increased mammary ERα and ERβ expression and DNA methylation at or near CTCF-binding sites. Specifically, of the 15 MMTV-neu mice fed the CR diet for up to 22 months, only 3 developed spontaneous mammary tumors, while the median survival of the overweight and diet-induced obesity groups was less than 15 months (Fig. 5A). To our knowledge, this study is the first to demonstrate the anticancer effects of a chronic CR regimen (compared with overweight or diet-induced obesity) in MMTV-neu mice. However, Mizuno and colleagues found that intermittent CR decreased mammary tumor incidence in MMTV-neu mice (45). Our findings of the anticancer effects of CR, compared with overweight or diet-induced obesity regimens, are consistent with reports of a link between dietary energy balance modulation and mammary tumorigenesis in other preclinical models of mammary cancer (46, 47).

Two previous publications compared diet-induced obesity versus chow diets (similar to our overweight regimen, Supplementary Table S6) on spontaneous mammary tumorigenesis in MMTV-neu mice. Cleary and colleagues reported that diet-induced obesity and chow-fed mice had similar mammary tumor development and survival, consistent with our observation of no significant difference in tumor development or survival in overweight versus diet-induced obesity mice (48). In contrast, Chen and colleagues reported a significant diet-dependent difference (chow > diet-induced obesity) in survival rates (49). Our findings with CR may help reconcile these apparently conflicting results, as we found highly significant differences in survival in CR mice relative to overweight and diet-induced obesity mice. Chen and colleagues linked the proancer effects of diet-induced obesity, relative to chow, in their study to increased signaling through the IKKβ, mTOR, and VEGF pathways, which stimulate proliferation and survival. We have previously established that diet-induced obesity increases, and CR decreases, circulating IGF-1, insulin, and their downstream signals through the IGF-1 and insulin receptor tyrosine kinases (36, 47). IGF-1 is a potent mitogen, which promotes signaling through the IKKβ, mTOR, and VEGF pathways, ultimately promoting growth and also inhibiting apoptosis. In women, circulating IGF-1 is positively associated with terminal duct lobular unit involution, mammographic density, and breast cancer risk (50, 51).

In this study, we found that after 1 month of diet treatment, CR mice, relative to overweight and diet-induced obesity mice, had decreased serum IGF-1 and insulin (Table 1). However, the diet-induced obesity and chow-fed mice in the Cleary study did not differ in IGF-1. Thus, one possible explanation for the observed differential tumor responses may involve the diet-dependent effect (or lack thereof) on systemic metabolism, particularly growth factors and their downstream signals. Differential tumor latencies across the studies may also contribute to the apparent study-specific differences in tumor responses to diet-induced obesity, as the median survival for the Cleary study chow-fed mice and our overweight mice were comparable (~18 and ~15 months, respectively), in contrast to the Chen study chow-fed mice (~7.5 months).

Interactions between dietary energy balance modulation and DNA methylation may influence the expression of key breast cancer–related genes, such as ERα, ERβ, via diet-induced changes in DNM1 expression and DNA methylation at or near CTCF-binding sites. Figure 6 integrates findings from RNA-seq analysis (Supplementary Table S5) using IPA to illustrate a proposed model of a diet-responsive network contributing to altered gene expression related to several transcription factor pathways (e.g., E2F, STAT3, and NF-kB) that serve as regulators of DNM1. Inflammation promotes DNM1 expression, which has been shown to positively correlate with IL6 expression in tumors and blood (52, 53). Overexpression of DNM1 may be a mechanism utilized by cancer cells to evade regulation by rendering tumor suppressors transcriptionally inactive (17, 54). Thus, mediators of obesity-associated inflammation may promote increased expression of DNM1, thereby contributing to aberrant DNA methylation and transcription of breast cancer–related genes such as ERα and ERβ.

To our knowledge, there are no previous reports regarding the maintenance of mammary ERα and ERβ positivity with a CR regimen. As mentioned previously, our CR mice had lower levels of energy balance–related metabolic factors, including IGF-1, insulin, and leptin, consistent with numerous studies in the literature (12, 15, 47). As illustrated in Fig. 6, these factors and
their downstream signaling pathways can also impact DNMT1, but our study is limited in the ability to establish their precise role. Future studies are planned to assess the possible links between systemic metabolic factors, such as insulin, IGF-1, leptin, and 17β-estradiol, and the increased mammary ERα- and ERβ-associated DNA methylation at or near CTCF-binding sites in CR mice. Alternatively, the decreased levels of 17β-estradiol observed in CR mice could contribute to prolonged survival, independent of...

Figure 6.
Proposed model of an energy balance–responsive network associated with DNMT1 regulation, DNA methylation, and transcriptional regulation of ERα and ERβ. Obesity and associated energy excess results in several metabolic perturbations that are ameliorated by calorie restriction. To identify regulatory relationships between DNMT1 and other genes that were differentially expressed in our RNA-seq analysis, we used the Path Designer function of IPA. We found transcription factors STAT3, NF-κB, and E2F to have direct relationships with DNMT1 activation. Upstream of these relationships, IPA also linked growth factors such as insulin and EGF, as well as cytokines, including TNF, IL1β, and IL8, to the E2F, STAT3, and NF-κB signaling pathways. We propose that one consequence of obesity-associated growth factor and cytokine signaling is increased DNMT1 activation, which in turn can modulate DNA methylation and impact transcription of important genes in breast cancer, such as ERα and ERβ. Red circles, methylated CpG dinucleotides; white circles, unmethylated CpG dinucleotides.
epigenetic events, as high levels of 17β-estradiol can promote cell proliferation and tumor progression (39). Additional study limitations include (i) restricting RRBS and RNA-seq analysis to CR and overweight mice (although overweight and diet-induced obesity mice were similar regarding ERα and ERβ mRNA and protein expression and survival); and (ii) not measuring physical activity (although we have previously shown that CR, relative to control or diet-induced obesity diets, does not increase locomotor activity in mice; ref. 55).

In conclusion, we found in MMTV-neu transgenic mice that mammary tumor development and ERα and ERβ expression are dietary energy balance dependent in association with epigenetic reprogramming. Specifically, a diet–DNMT1–methylation axis may serve as a primary regulator of gene transcription via diet-induced changes in DNA methylation at or near CTCF-binding sites. These preclinical findings suggest that interventions reducing the impact of excess weight on epigenetic dysregulation of ER may represent a new strategy for the prevention or control of HER2-positive breast cancer in overweight and obese women.

Disclosure of Potential Conflicts of Interest

P.H. Brown is a consultant/advisory board member for Susan G. Komen Foundation. No potential conflicts of interest were disclosed by the other authors.

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References


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