Comprehensive molecular characterization of surgical vs. dietary weight loss: impact on mammary tumor burden

by

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I. Abstract

**Background:** Obesity is a widespread health concern and established risk factor for basal-like breast cancer. However, studies are conflicted on the benefits of weight loss relevant to breast cancer prevention. Recent studies suggest that certain methods of robust weight loss with long-term maintenance, such as bariatric surgery, might in fact be able to reverse obesity-associated breast cancer risk.

**Hypothesis:** We hypothesize that weight loss by sleeve gastrectomy will generate sufficient metabolic normalization to reverse obesity-driven mammary tumor burden more effectively than weight loss by diet alone.

**Methods:** Mice were fed a low-fat control (CON) or high-fat diet-induced obesity (DIO) regimen for 15 weeks to model chronic obesity. DIO mice were randomized to continue on a high-fat diet (Obese) or undergo weight loss by either sleeve gastrectomy (~70% excision of the stomach) in combination with switching to a low-fat diet or by switching to a low-fat diet alone, resulting in formerly obese (FOB)-Surg and FOB-Diet mice, respectively. Additionally, a subset of normal weight (NW) Control mice (Con, n=25) was maintained on a low-fat diet throughout the study. NW Control, FOB-Diet, and Obese mice underwent a sham procedure to control for the insult of surgery. FOB-Surg and FOB-Diet mice did not lose a significantly different amount of weight and body fat; both groups had significantly lower weight and percent body fat than Obese mice. Eight weeks after surgical procedures and diet switches, all mice on study were orthotopically injected with E0771 mammary tumor cells, which model BLBC.
**Results:** At the end of study, *ex vivo* tumor volume in FOB-Surg mice was not significantly different from NW Control mice and significantly different from Obese mice. However, tumor volume in FOB-Diet mice was significantly different from NW Control mice and not significantly different from Obese mice. In addition, FOB-Surg mice had levels of serum tumor necrosis factor-alpha (TNF-α), insulin, and mammary adipocyte size that were not significantly different from NW Control mice and significantly different from FOB-Diet and Obese mice. Lastly, pathway analysis of mammary tissue gene expression revealed redundant upregulation of genes in extracellular matrix remodeling and growth factor signaling in Obese vs. FOB-Surg and FOB-Diet vs. FOB-Surg mice.

**Conclusions:** Our results demonstrate that surgical weight loss imparted a plurality of metabolic advantages, functional genomic changes, and successful reversal of obesity-associated mammary tumor burden that were not similarly achieved by dietary weight loss.
II. Introduction

The predominance of obesity persists in the United States, with recent epidemiologic studies reporting a prevalence of 37.7% in the adult population (1). A robust body of evidence demonstrates that obesity significantly increases the risk of developing breast cancer in postmenopausal women (2). Furthermore, obese women experience poorer breast cancer outcomes by virtue of increased tumor grade at clinical presentation, disease recurrence, and substandard response to certain therapies (3). Accordingly, rigorous scientific investigation has been dedicated to interrogating the mechanisms complicit in this obesity-cancer link.

Obesity is attendant to a spectrum of physiological aberrations resulting from chronic positive energy intake (4). In obesity, excess energy is converted to triacylglycerol and stored in various adipose tissue depots throughout the body. The obese state is thus associated with profound expansion of the adipose tissue, which occurs by adipocyte hyperplasia (increase in adipocyte number) or hypertrophy (increase in adipocyte size). In obese adults, hypertrophy predominates as the mechanism to support the growth in adipose tissue (5). Adipocyte hypertrophy, and the associated fatty acid spill over, results in insulin resistance, elevated serum triglycerides, local tissue hypoxia, inflammation, and altered sex hormone levels (6). In consequence, obesity is a strong risk factor for a host of diseases including cardiovascular disease, type II diabetes, and a variety of cancers including those of the breast, pancreas, colorectum, endometrium, esophagus, and kidney (7).

Several key obesity-driven physiological disruptions have been identified as critical mediators between host energy status and breast cancer risk. Namely, these
include growth factor signaling—particularly insulin-like growth factor 1 (IGF-1)—and inflammatory cytokines and adipokines secreted from immune cells and adipocytes (8). Obesity is known to induce insulin resistance, resulting in compensatory hyperinsulinemia, which causes enhanced synthesis of IGF-1. IGF-1 is a potent mitogen that activates a receptor-tyrosine kinase signaling cascade resulting in cell growth, survival, proliferation, and evasion of apoptosis; thus, IGF-1 and intracellular growth factor signaling are heavily implicated in breast cancer risk and progression (9). Obesity confers its pro-inflammatory effects in part as a result of adipose tissue remodeling. Positive energy balance and triglyceride storage causes adipocyte hypertrophy. In consequence, adipocytes can outgrow supporting vasculature, resulting in hypoxia, and expand the margins permitted by the outer cellular membrane, resulting in apoptosis (7). Moreover, it is well-documented that adipose tissue in obese subjects exhibits increased secretions of several pro-inflammatory cytokines, including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) (7). These cellular phenomena contribute to substantial recruitment of immune cells to the adipose tissue as well as proliferation of macrophage populations native to adipose depots (6, 7). In this inflammatory milieu, activated macrophages supply significant secretions of cytokines and engage in phagocytosis of necrotic engorged adipocytes, forming visible histopathological signatures known as crown-like structures (CLS) (10). This cycle can self-perpetuate by way of cytokine-induced lipolysis, which results in release of fatty acids that activate toll-like receptors on immune cells to sustain elevated cytokine production (11).
Tissues proximal to adipose tissue, such as the mammary epithelium, are vulnerable to this obesity-associated inflammatory cascade, which promotes carcinogenic events and cancer progression through several molecular levers. For instance, increased IL-6 secretions promote cell proliferation through the Janus Kinase (JAK)/ Signal transducer and activator of transcription 3 (STAT3) pathway (12), and TNF-α secretions promote cell proliferation, angiogenesis, and evasion of apoptosis via nuclear factor-kappa B (NF-κB) signaling (13). In addition, reactive oxygen and nitrogen species originating from adipose tissue macrophages jeopardize genomic integrity by inducing structural DNA alterations (7). Therefore, it is critical to target the progression of obesity-associated adipose dysregulation in order to reduce the barrage of survival signals from adipose tissue coterminous with potential sites of malignant disease.

Given the indisputable positive associations between obesity and breast cancer, it would follow that obesity reversal can achieve corresponding reversal of obesity-associated cancer risk. However, the scientific community has failed to reach a consensus on the efficacy of weight loss on cancer risk and outcomes. Despite a handful of studies demonstrating intermediate reductions in biomarkers of cancer risk with intermittently promising outcomes, results are often inconsistent (14), thereby precluding an evidence-based recommendation of certain weight loss strategies for targeted breast cancer risk reduction. This is largely due to the low success rate of achieving significant weight loss and an even lower rate of long-term weight loss maintenance. Indeed, we and others have explored obesity-reversal in preclinical mouse models, and formerly-obese mice often exhibit persistent dysregulation in inflammation and adipose tissue homeostasis characteristic of obesity (15, 16).
Although weight loss, in particular weight loss exclusively by low-fat diet, has not demonstrated success as a comprehensive risk reduction method, weight loss by bariatric surgery has shown auspicious results for reversal of obesity-associated tumor burden. Christou et al. showed that in comparison to morbidly obese control subjects, individuals who underwent bariatric surgery had a remarkable five-fold reduction in 5-year incidence of all cancers and breast cancer (17). Bariatric surgery describes a variety of surgical procedures that modify the anatomy of the digestive tract in an effort to impart weight loss and resolve insulin resistance and hyperglycemia, for which it is often exceptionally effective. The two most common bariatric surgery procedures are Roux-en Y gastric bypass (RYGB) and vertical sleeve gastrectomy (VSG) (18). RYGB is regarded as the “gold standard” bariatric surgery procedure due to its extensive documented success in generating long-term weight loss and mitigation of obesity-associated comorbidities. Until recently, RYGB was the most common bariatric operation, but due to the inherent complexity of constructing multiple anastomoses for each procedure and the associated surgical complications, VSG surpassed RYGB as the most common procedure in 2013, boasting a straightforward surgical protocol, low complication rate, and impressive clinical outcomes (19, 20). Weight loss achieved by VSG is comparable to RYGB, and both procedures give rise to greater weight loss and better hemoglobin A1c (HbA1c) outcomes, a diagnostic tool for diabetes, than standard medical therapy (21, 22). Furthermore, VSG does not pose a risk of vitamin and mineral absorption posed by RYGB, an important consideration for obese women of childbearing age (20).
A variety of mechanisms are under investigation to explain the efficacy of bariatric surgery with respect to endocrine health and cancer outcomes; top candidates include caloric restriction due to sensitive food reward signals, caloric malabsorption in the small intestine, gut hormone alterations, and bile acid and gut microbiota changes (18, 23). Furthermore, bariatric surgery has been shown to drastically modulate the epigenome, in particular DNA methylation. Studies have demonstrated profound remodeling of methylation profiles in gene clusters relevant to obesity caused by bariatric surgery. In one study comparing DNA methylation profiles in subcutaneous adipose tissue of women before and after undergoing bariatric surgery, researchers reported differential methylation in genes with established involvement in obesity-associated adipose tissue dysfunction (24). Additional research has revealed that bariatric surgery can reverse obesity-associated DNA methylation signatures to levels comparable to never-obese subjects in skeletal muscle (25), and of particular importance to obesity-breast cancer link, adipose tissue (26). Given the potency of weight loss, metabolic improvements, and epigenetic modulation achieved exclusively by bariatric surgery, we sought to compare VSG and dietary weight loss as obesity-reversal interventions and characterize their respective cancer-protective effects in a model of premenopausal basal-like breast cancer.
III. Hypothesis, Specific Aims

**Hypothesis:** Considering the robust degree of longitudinal weight loss, endocrine health resolution, and epigenetic remodeling achieved by bariatric surgery, we hypothesize that weight loss by sleeve gastrectomy will generate sufficient metabolic normalization to reverse obesity-driven mammary tumor burden more effectively than weight loss by diet alone.

**Aim 1:** Compare the effectiveness of bariatric surgery and weight loss by diet alone to reduce obesity-associated metabolic perturbations, adipose tissue dysfunction, inflammation, and tumor burden.

**Aim 2:** Characterize the DNA methylation profiles in the mammary fat pad to determine exclusive regulation by weight loss intervention and test concordance with obesity-associated methylation in the Normal Breast Study.

**Aim 3:** Profile the transcriptome of the mammary fat pad and conduct unbiased pathway analysis to identify key signaling pathways that are associated with favorable mammary tumor burden outcomes.
IV. Methods

Animal Study Design: Husbandry and Diet

All animal study protocols were approved and coordinated in compliance with guidelines issued by the University of North Carolina at Chapel Hill Institutional Care and Use Committee (IACUC). Ninety four female 6-8 week old C57BL/6 mice (a well characterized energy balance-responsive mouse model) were purchased from Charles River Laboratories International, Inc. (Wilmington, MA). Upon arrival, mice were housed two per cage on a 12-hour light/dark cycle and offered food and water ad libitum. A diet of standard chow was administered to all mice for a one week acclimation period. Next, mice were randomized to two groups, receiving either a control (CON; 10% kcal from fat) diet (n=22; Product # D12450J; Research Diets, Inc.) or a diet-induced obesity (DIO; 60% kcal from fat) regimen (n=72; Product # D12492; Research Diets, Inc.) to generate a normal-weight (NW) control or obese phenotype, respectively. Body weight and food intake were measured weekly. After 15 weeks on diet, with the weights of NW CON and DIO mice significantly different from each other (p<0.001), obese mice were then randomized to continue the DIO diet (Obese) or receive a surgical or diet weight loss intervention, resulting in formerly obese (FOB)-Surg and FOB-Diet groups, respectively. FOB-Surg mice were subject to sleeve gastrectomy (~80% excision of the stomach), and NW Control, Obese, and FOB-Diet mice received a sham procedure. Three days post-operation, both FOB-Surg and FOB-Diet began the same low-fat control diet as the NW Control group. Eight weeks after surgery when body weights of mice were relatively stable, all mice were orthotopically injected with $3.5 \times 10^4$ E0771 mammary tumor cells into the 4th mammary fat pad (Figure 1A), a model of basal-like
breast cancer originally isolated from a spontaneous medullary breast adenocarcinoma in a C57BL/6 mouse (27). In vivo tumor growth was measured two times per week with skinfold calipers and in vivo tumor area was determined using the formula \( \pi r^2 \). Four weeks following orthotopic injection, tumors in 50% of Obese mice (the fastest growing group) reached the requisite size defined by the IACUC protocol; therefore, all mice on study were sacrificed. Mammary tumors, tumor-adjacent and tumor-distal mammary fat pad were excised and sectioned to either be formalin fixed or flash frozen in liquid nitrogen and stored at -80°C until further analysis. Ex vivo tumor volume was calculated using the formula \( 1/6\pi \times D1 \times D2 \times D3 \) (where D is equal to ex vivo diameter of the tumor). End of study blood was collected by cardiac puncture, allowed to clot at room temperature for 30 minutes and centrifuged for 10 minutes at 1000 x g to isolate serum, and stored at -80°C.

**Sleeve Gastrectomy and Sham Procedures**

Sleeve gastrectomy and sham procedures were performed by trained animal surgeons according to a validated protocol (28). Briefly, vertical sleeve gastrectomy (VSG) involved excision of ~80% of the lateral stomach. The sham procedure, performed on NW Control, Obese, and FOB-Diet mice to control for the physiological insult of surgery, was executed by first isolating the stomach and then applying manual pressure with forceps for five seconds. The excision and pressure were applied along a line continuous with the esophagus and pylorus. All surgeries occurred within a four day window, and mice within all study groups were randomized to the day of operation. Pre-operation fasting, exposure to isofluorane and administration of analgesics were
consistent across all groups. Additionally, all mice received a three-day liquid diet (Osmolite OneCal) before being reintroduced to solid food. Antibiotics were given to all mice three days post-op. All mice were weighed daily and food intake was quantified for one week post-op.

Quantitative Magnetic Resonance Imaging Analysis

Quantitative magnetic resonance imaging (qMRI) (Echo Medical Systems, Houston, TX) was used to measure the body composition for all groups (n=6-9 mice/group) at the end of study. Lean body mass, fat body mass, and free water were quantified. Body fat percentage was calculated by dividing the fat body mass by the body weight measured with a digital scale.

Metabolic Analysis of Serum Hormones, Cytokines, and Adiponectin

One week prior to tumor injection, serum was collected from mice fasted 4-6 hours by submandibular bleed. Serum hormones, cytokines, and adipokines including insulin, leptin, adiponectin, IL-6, TNFα, and resistin, were measured using Milliplex Mouse Metabolic Hormone Magnetic bead Panel (MMHMAG-44K), Bio-Plex Pro™ Mouse Adiponectin Assay, and Mouse Cytokine Panel A 6-Plex, respectively (Bio-Rad Laboratories; Hercules, California). Insulin-like growth factor 1 (IGF-1) concentrations were measured using R&D Systems IGF-1 Bead-Based Single-plex Luminex assay (Minneapolis, MN).
**Mammary Fat Pad Adipocyte Size and Crown Like Structure Analysis**

Hematoxylin and eosin (H&E) staining on 4-micron thick sections from formalin-fixed, paraffin embedded distal mammary fat pad tissue was processed, scanned and imaged using Aperio CS2 Digital Pathology Scanner (Leica Biosystems, Wetzlar, Germany) at 40X magnification. Representative snapshots (n=9-11 mice/group; 3 snapshots were sample) were randomly selected from whole tissue images zoomed in at 8.8X (300 μM) utilizing ImageScope Viewing Software Version 12.0 (Leica Biosystems). Mammary fat pad average adipocytes size and number of adipocytes were quantified using ImageJ Version 1.51e (National Institute of Health, Bethesda, Maryland). An adipocyte tool macro (MRI Adipocyte Tools.txt) was downloaded from (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Adipocytes_Tool) and imported into ImageJ. Furthermore, the number of crown-like structures (CLS) (10) was quantified from whole tissue H&E stained distal mammary fat pad sections (n=10-15 mice/group). Briefly, the number of CLS were counted in a blinded fashion, and CLS density measures were achieved by dividing the number of CLS by the total slide area eligible for analysis using ImageScope Viewing Software Version 12.0 (Leica Biosystems).

**DNA methylation analysis**

Genome-wide methylation profiles for the distal mammary fat pad were determined by reduced representation bisulfite sequencing (RBBS). DNA was extracted from a random sample (n=4 mice/group) of distal mammary tissues using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Library preparation and sequencing were performed at the University of North Carolina at Chapel Hill High-Throughput
Sequencing Facility. Alignment and differential methylation analysis were conducted as previously described (15).

**RNA-Seq**

Total RNA was extracted from the flash-frozen tumor-adjacent and tumor-distal mammary fat pad samples collected at the end of the study using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. RNA libraries were prepared using the Illumina TruSeq Stranded Total RNA Sample Preparation kit according to manufactures instructions. The libraries were sequenced using a 2x76 bases paired end protocol on the Illumina HiSeq 2000 instrument. The reads were mapped to mouse genome (mm10) by TopHat (version 2.0.7). The number of fragments in each known gene from RefSeq database (UCSC Genome Browser 2013) was enumerated using HTSeq-count from HTSeq package (version 0.5.3p9). Differential expression was performed using DESeq2.

**Pathway Analysis**

WebGestalt (www.webgestalt.org) (29) over representation enrichment analysis (ORA) of KEGG pathways and gene ontology biological process curated gene sets was performed for selected pairwise comparisons of RNA-Seq and RRBS data.

**Statistical Analysis**

All values are represented as mean ± standard deviation (STDEV). One-way analysis of variance (ANOVA) using Tukey's post hoc multiple comparisons correction was used to
assess the effects of diet and weight loss on body weight and fat percentage, tumor volume, serum hormone and cytokine concentrations, and mammary fat pad adipocyte size and CLS density. Results were analyzed using GraphPad Prism software (Graphpad Software Inc., La Jolla, CA) and p ≤ 0.05 was considered statistically significant.

V. Results

**Weight Loss Interventions by Surgery (FOB-Surg) or Diet Alone (FOB-Diet) are Equally Effective at Reducing Body Weight and Fat Mass**

Mice were fed a low fat control or a diet-induced obesity regimen for 15 weeks in order to establish a normal weight control or obese phenotype, respectively. Obese mice had significantly higher body weight at the time of sleeve gastrectomy or sham procedure relative to control mice (p < 0.0001). Eight weeks following the operations and diet switch, body weight between FOB-Surg and FOB-Diet mice was not significantly different (Figure 1A), and neither group was significantly different from NW Control mice, however all were significantly different from Obese mice prior to tumor cell injection (p < 0.0001 for all comparisons, NW Control vs. Obese, FOB-Surg vs. Obese, FOB-Diet vs. Obese). At end of study, body fat percentage (Figure 1B) was not statistically different among NW Control, FOB-Surg and FOB-Diet mice, and all groups were significantly lower than Obese mice (p < 0.0001 for all comparisons, NW Control vs. Obese, FOB-Surg vs. Obese, FOB-Diet vs. Obese), indicating successful and equivalent reversal of the obese phenotype in both weight loss groups.
Figure 1. Surgical and dietary weight loss generate comparable reductions in body weight and body fat percentage. (A) Body weight of mice throughout the course of study. (B) Combined violin and box plots of body fat percentage at end of study. Differences in significance denoted by different letters (a,b) p-value <0.05.

Surgical Weight Loss in Mice More Effectively Reduces Circulating Growth Factors and Pro-Inflammatory Mediators in Serum

Seven weeks after surgical procedures and diet switch when weights were stabilized, serum was collected from mice (n=10-12 mice/group) by submandibular bleed for multiplex metabolite analyses. For all metabolites measured except adiponectin, levels in NW Control mice were significantly lower than Obese mice. Insulin levels in FOB-Surg mice were significantly different from both FOB-Diet and Obese mice and not significantly different from NW Control mice; insulin levels in FOB-Diet exhibited intermediate reductions from Obese levels but were not significantly different. For Insulin-like growth factor-1 (IGF-1) and resistin, FOB-Surg mice displayed significantly lower levels than Obese mice, whereas FOB-Diet mice displayed intermediate, non-significant reductions from Obese levels. For measures of leptin, leptin to adiponectin
ratio, interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), both FOB-Surg and FOB-Diet mice displayed significantly lower levels relative to Obese mice. Lastly, FOB-Surg mice exhibited significantly lower levels of tumor necrosis factor alpha (TNF\(\alpha\)) than both FOB-Diet and Obese mice; levels of TNF\(\alpha\) in FOB-Diet mice were not statistically different from only Obese mice (Table 1).

### Table 1. Serum hormones and cytokines

<table>
<thead>
<tr>
<th></th>
<th>NW Control</th>
<th>FOB-Surg</th>
<th>FOB-Diet</th>
<th>Obese</th>
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<tbody>
<tr>
<td><strong>Hormones</strong></td>
<td></td>
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<tr>
<td>Insulin (ng/mL)</td>
<td>0.74 ± 0.25(^a,b)</td>
<td>0.56 ± 0.22(^a)</td>
<td>1.04 ± 0.35(^b,c)</td>
<td>1.35 ± 0.49(^c)</td>
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<tr>
<td>IGF-1 (ng/mL)</td>
<td>42.2 ± 13.8(^a)</td>
<td>39.0 ± 15.9(^a)</td>
<td>51.4 ± 18.6(^a,b)</td>
<td>76.7 ± 29.6(^b)</td>
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<tr>
<td>Leptin (ng/mL)</td>
<td>2.41 ± 0.89(^a)</td>
<td>2.16 ± 1.20(^a)</td>
<td>4.77 ± 4.21(^a)</td>
<td>11.5 ± 0.05(^b)</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>12.0 ± 2.99</td>
<td>11.1 ± 5.01</td>
<td>12.6 ± 2.91</td>
<td>9.68 ± 5.13</td>
</tr>
<tr>
<td>Leptin:Adiponectin</td>
<td>2.22E-06 ± 1.34E-06(^a)</td>
<td>2.34E-06 ± 1.29E-06(^a)</td>
<td>4.95E-06 ± 5.29E-06(^a)</td>
<td>3.50E-05 ± 1.30E-05(^b)</td>
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<tr>
<td>Resistin (ng/mL)</td>
<td>11840 ± 4143(^a)</td>
<td>6732 ± 2237(^a)</td>
<td>12528 ± 4717(^a,b)</td>
<td>20214 ± 10323(^b)</td>
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<td><strong>Cytokines</strong></td>
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<tr>
<td>TNF(\alpha) (pg/mL)</td>
<td>11.5 ± 7.31(^a)</td>
<td>18.8 ± 14.1(^a)</td>
<td>41.2 ± 14.1(^b)</td>
<td>56.9 ± 18.2(^b)</td>
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<td>IL-6 (pg/mL)</td>
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<td>39.5 ± 17.5(^a)</td>
<td>50.8 ± 24.0(^a)</td>
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<td>MCP-1 (pg/mL)</td>
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<td>102.2 ± 22.4(^a)</td>
<td>136.8 ± 50.4(^a)</td>
<td>192.3 ± 51.9(^b)</td>
</tr>
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</table>

Data presented as mean ± std dev. One-way ANOVA and Tukey’s post hoc multiple comparisons were used to test statistical differences between study groups. Results from pairwise comparisons are presented as letters, where common letters indicate statistical equivalence and different letters indicate statistical difference according to p<0.05.
Surgical Weight Loss in Mice Reverses Adipocyte Hypertrophy and Crown-Like Structure Density in the Mammary Tissue

Adipocyte area in the mammary fat pad displayed significant fluctuation by phenotype. Both surgical and dietary weight loss resulted in reduced adipocyte area, with the most profound differences relative to Obese mice occurring at the 50th and 90th percentile of adipocyte area (Figure 2A). More precisely, FOB-Surg mice exhibited an average adipocyte area not significantly different from NW Control mice and significantly different from both FOB-Diet and Obese Mice (p<0.0001), while average adipocyte area of FOB-Diet mice displayed significant reduction relative to Obese mice (p<0.0001) but remained significantly different from both NW Control (p<0.0001) and FOB-Surg (p<0.05) (Figure 2B). Furthermore, density of crown-like structures in the mammary fat pad was lowest in FOB-Surg mice and significantly lower than that of FOB-Diet and Obese mice. Of note, crown-like structure density in FOB-Diet mice was not statistically different from Obese mice (Figure 2C). Representative images of H&E stained mammary fat pad depicting adipocyte size and CLS are displayed (Figure 2D).

Surgical Weight Loss, but not Weight Loss by Diet Alone, Reverses the Pro-Tumorigenic Effects of Obesity

Intriguingly, *ex vivo* tumor volume of FOB-Surg mice was not statistically different from NW Control mice and significantly different than Obese mice. However, FOB-Diet mice had tumors that were not significantly different from Obese mice and significantly different than NW Control mice (Figure 3). Therefore, FOB-Surg mice, but not FOB-Diet mice, achieved reversal of obesity-associated mammary tumor burden.
Figure 2. Surgical weight loss results in more robust reductions in adipocyte hypertrophy and crown-like structure density in the mammary fat pad. (A) Panel displaying density functions for 10th, 50th, and 90th percentiles of adipocyte area across all groups; dashed red line indicates mean of distribution. (B) Average adipocyte area. (C) Crown-like structure density. (D) Representative images of H&E stained mammary fat pad sections depicting adipocyte size and CLS. Differences in significance denoted by different letters (a,b,c) p-value <0.05.
Figure 3. *Ex vivo* tumor volume reveals unique cancer-protective effects of surgical versus dietary weight loss. Differences in significance denoted by different letters (a,b,c) p-value <0.05.

The Methylation of Genes in the Mammary Tissue of Mice that Lost Weight via Surgery Displays a Pattern that is Distinct from FOB-Diet and Obese mice

The complete RRBS dataset was filtered by pairwise comparisons between all study groups with p<0.0001 and false discovery rate (FDR)<0.0001 as filtering criteria for differential methylation. Genes with differential methylation according to these criteria at any gene feature (e.g. promoter, intron) were included. Gene lists were entered into WebGestalt for overrepresentation enrichment analysis (ORA) in curated KEGG pathways (Table 2). The genes harboring differential methylation between Obese and FOB-Surg mice were highly enriched for a variety of pathways implicated in mammary carcinogenesis and a tumorigenic microenvironment. Interestingly, there were markedly fewer genes displaying differential methylation according to the criteria above in Obese vs. FOB-Diet mice relative to Obese vs. FOB-Surg mice (2011 and 4258 genes, respectively), suggesting that Obese mice have a global DNA methylation profile more similar to FOB-Diet mice than FOB-Surg mice. Lastly, there was considerable
redundancy in pathways represented by differentially methylated genes between FOB-Diet vs FOB-Surg mice and Obese vs FOB-Surg mice, suggesting that Obese and FOB-Diet mice share a significant number of similar DNA methylation features that explain overlapping pathway enrichment relative to FOB-Surg mice.

The Expression of Genes in the Mammary Tissue of Mice that Lost Weight via Surgery Displays a Pattern that is Distinct from FOB-Diet and Obese mice

RNA Sequencing data was filtered to create gene lists for all pairwise comparisons between groups; differential expression with group-specific directionality was achieved by selecting genes with log₂(fold change) > 0.58 (which is equivalent to fold change > 1.5) for each inter-group comparison. Gene lists were entered into WebGestalt for overrepresentation enrichment analysis (ORA) in curated KEGG pathways. There was significant overlap between pathways upregulated in Obese vs. FOB-Surg mice and FOB-Diet vs FOB-Surg mice, pointing to critical gene clusters that display robust
activation by obesity and persist despite weight loss by diet alone (Figure 4A,B). These pathways are well-characterized in the context of obesity and breast cancer risk and suggest broad activation of canonical cell signaling cascades involved in cell proliferation, growth, and extracellular matrix function. Next, comparisons of the two weight loss groups to Obese mice reveals significant overlap in pathway characterization reflecting upregulated genes in FOB-Surg (Figure 4C) and FOB-Diet (Figure 4E) mice vs. Obese mice. There were remarkably few upregulated genes in Obese vs. FOB-Diet mice relative to other comparisons. The resulting ORA revealed a

<table>
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<th>Pathway</th>
<th>Observed Genes in Pathway</th>
<th>Percent of All Genes in Pathway</th>
<th>P-value</th>
<th>FDR</th>
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<td>Differentially methylated Obese vs FOB-Surg (n = 4258)</td>
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<td>Signaling pathways regulating pluripotency of stem cells*</td>
<td>47</td>
<td>33.6</td>
<td>1.34E-07</td>
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<td>32.4</td>
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Respective gene sets were subjected to over representation enrichment analysis (ORA) for specific KEGG pathways as compared to the mouse genome by applying a hypergeometric test and threshold minimum of five genes represented in the pathway. Asterisk indicates redundant pathway among the comparisons. FDR: false discovery rate.
correspondingly small number of enriched pathways (Figure 4D), perhaps telling of latent obesity-associated gene expression patterns in FOB-Diet but not FOB-Surg mice. Lastly, the genes that were upregulated in FOB-Surg vs. FOB-Diet mice were enriched for pathways implicated in a spectrum of inflammatory processes (Figure 4F). Considering the potential inconsistencies between this pathway analysis and the serum metabolite data in Table 1 showing FOB-Surg mice having lower levels of inflammatory markers relative to FOB-Diet mice, gene-level analyses were pursued. Interestingly, explorations of redundant pathways upregulated in both FOB-Diet and FOB-Surg mice revealed group-exclusive expression profiles of distinct molecular functions. For example, the “Cytokine-cytokine receptor interaction” KEGG pathway was shown to be enriched for genes upregulated in FOB-Diet vs. FOB-Surg mice and vice versa. In this pathway, Lep, the gene encoding the adipokine leptin, was upregulated in FOB-Diet vs. FOB-Surg mice, whereas Lepr, the gene encoding leptin receptor, was upregulated in FOB-Surg vs. FOB-Diet mice, perhaps suggesting resolution of leptin resistance. The proinflammatory chemokine Ccl7 and chemokine receptor Ccr1 (30) were both upregulated in FOB-Diet vs. FOB-Surg, and the anti-inflammatory cytokine interleukin-10 receptor Il10ra (31) and anti-obesigenic chemokine Cxcr4 (32) were both upregulated in FOB-Surg vs. FOB-Diet.
**Figure 4.** Pathway analysis of differentially expressed genes in the mammary fat pad. Selected KEGG pathways showing enrichment for differentially expressed (fold change > 1.5) genes with corresponding p-values from hypergeometric test for overrepresentation enrichment analysis (ORA). Pathways displayed for genes (A) upregulated in Obese vs FOB-Surg mice, (B) upregulated in FOB-Surg vs Obese mice, (C) upregulated in FOB-Diet vs FOB-Surg mice, (D) upregulated in Obese vs FOB-Diet mice, (E) upregulated in FOB-Surg vs FOB-Diet mice, and (F) upregulated in FOB-Diet vs Obese mice.

**DNA Methylation and Gene Expression Profiles of Genes Altered After Surgical Weight Loss Displays Partial Concordance with Human Data Sets**

Genes exhibiting significant hyper- and hypomethylation in Obese vs NW Control, FOB-Surg, or FOB-Diet mice were selected by calculating the difference in average percent methylation of the groups and attributing significance according to false discovery rate < 0.05 and p-value < 0.05. These differentially methylated features were compared to DNA methylation data obtained from mammary tissue cataloged in the Normal Breast Study showing concordant hyper- or hypomethylation in obese subjects. Displayed are results of concordance analysis (Table 3) showing robust alignment of genes implicated in a variety of pathways relevant to carcinogenesis and regulation of cell proliferation. Additionally, gene ontology (GO) and KEGG pathways regulated by differential gene expression were compared to a microarray gene expression study of white adipose tissue (WAT) of obese vs. lean subjects and subjects after vs. before undergoing bariatric surgery performed by Henegar et al. (33). For concordance analysis, Obese vs. NW Control mice were used in comparison with obese vs. lean subjects. Our data exhibited overlap in up- and down-regulated pathways, with six out of eleven GO and five out of ten KEGG pathways showing identical regulation for comparisons between obese vs. lean subjects and Obese vs. NW Control mice. Furthermore, Obese vs. FOB-
Surg mice were used in comparison with analysis of samples after vs. before bariatric surgery. Again, our data displayed unique similarity with directional regulation of GO and KEGG pathways, with eight out of fourteen GO and five out of 12 KEGG pathways showing identical regulation.

VI. Discussion

The results of our preclinical study using a mouse model of premenopausal basal-like breast cancer demonstrate that weight loss by bariatric surgery, but not weight loss by diet alone, is able to reverse obesity-driven transformations in metabolism, inflammation, DNA methylation, gene expression, and mammary tumor burden. Consistent with established trends in the literature, Obese mice, relative to NW Control mice, exhibited significantly higher levels of the following obesity-associated cancer promoting perturbations: serum hormones (insulin, IGF-1, leptin:adiponectin, resistin), circulating inflammatory markers (TNF-α, IL-6, MCP-1), body weight, body fat percentage, adipocyte size, and CLS density in the mammary fat pad. FOB-Surg mice, which achieved an obese phenotype by consuming a high fat diet for 15 weeks and underwent a vertical sleeve gastrectomy procedure followed by a switch to a low fat diet, displayed significant reductions in the obesity-associated cancer promoting perturbations from Obese mice and not significantly different from NW Control mice in all of these measures. FOB-Diet mice, which likewise achieved an obese phenotype and received a sham procedure followed by a switch to a low-fat diet to lose weight, displayed significant reductions from Obese mice in measures of body weight, body...
Table 3. DNA Methylation Comparisons Between Obese vs. Nonobese Women and Obese vs. NW Control, FOB-Surg, and FOB-Diet mice

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  - Methylation %: 7.1E-06
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  - Methylation %: 7.1E-06

- **Fgfr1**
  - chr8:1934 Promoter
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  - Methylation %: 7.1E-02
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  - Methylation %: 7.1E-02

**Pathways in cancer**

- **Bcr**
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- **Adcy7**
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**MAPK signaling pathway**

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Table 3. DNA Methylation Comparisons Between Obese vs. Nonobese Women and Obese vs. NW Control, F08-Surg, and F08-Diet mice

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Pathways regulating pluripotency of stem cells

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Differences in methylation calculated by taking the average DNA methylation for obese and subtracting the average DNA methylation for nonobese (women) or control (mice).
percentage, leptin:adiponectin, IL-6, and MCP-1, although mammary adipocyte size remained significantly higher than NW Control mice. FOB-Surg mice, but not FOB-Diet mice, exhibited average adipocyte size, CLS density, circulating TNF-α, and *ex vivo* mammary tumor volume was not significantly different from NW Control mice, while the same measures in FOB-Diet mice, (with the exception of adipocyte size, which was intermediate), were not significantly different from Obese mice. Therefore, we conclude that surgical weight loss imparted a plurality of metabolic advantages and successful reversal of obesity-associated mammary tumor burden that were not similarly achieved by dietary weight loss.

In order to provide a comprehensive molecular comparison of surgical and dietary weight loss relevant to breast cancer risk and progression, we performed parallel mRNA sequencing and reduced representation bisulfite sequencing of the mammary fat pad to delineate functional changes in the transcriptome and epigenome. Intriguingly, there were many more genes with differentially methylated features in Obese vs. FOB-Surg mice (n=4258 genes) than Obese vs. FOB-Diet mice (n=2011 genes), suggesting that surgical weight loss was more effective at generating a DNA methylation profile distinct from Obese mice. Differentially methylated genes in Obese vs. FOB-Surg mice and FOB-Diet vs. FOB-Surg mice were enriched for similar canonical signaling pathways, pointing to DNA methylation as a potential mechanism by which obesity-driven cellular changes remain active in FOB-Diet mice but not FOB-Surg mice.

Next, differentially expressed genes were subjected to unbiased pathway analysis to highlight coherent gene clusters in the context of curated signaling pathways. Similar to trends observed in pathway analysis of differentially methylated
genes, pathways upregulated in Obese vs. FOB-Surg mice and FOB-Diet vs. FOB-Surg mice exhibited substantial redundancy, suggesting that obesity-associated gene expression profiles in the mammary fat pad are responsible for characterizing differential gene expression between mice that underwent dietary vs. surgical weight loss. Genes and pathways that were upregulated by the weight loss interventions (that is, upregulated genes and pathways in FOB-Diet vs. Obese mice and FOB-Surg vs. Obese mice) showed considerable similarity in their biological function. Namely, the majority of the pathways involved macronutrient metabolism and metabolic machinery of core anabolic and catabolic pathways, such as fatty acid metabolism, tricarboxylic acid (TCA) cycle, insulin signaling, pyruvate metabolism, and peroxisome proliferator-activated receptor (PPAR) signaling. These results provide robust evidence for the centrality of metabolic control in obesity-reversal interventions. Interestingly, genes upregulated in FOB-Surg vs. FOB-Diet mice are heavily enriched for pathways implicated in a variety of inflammatory processes, despite powerful anti-inflammatory effects of surgical weight loss substantiated by circulating cytokine levels and CLS density in the mammary fat pad. However, curated signaling pathways often assemble genes that both positively and negatively regulate a given process under a common term. Therefore, further gene-level analysis is warranted to understand the distinct molecular mechanisms of inflammation that are regulated by the two weight loss interventions respectively.

Lastly, the sequencing results of our animal study demonstrate considerable similarity with similar molecular investigations in humans. The results of our RRBS studies were compared to DNA methylation in normal breast tissue from the Normal
Breast Study. This study generated correlation coefficients between methylation levels and obesity, and accordingly each methylation probe can be observed for up- or downregulation in obesity. Concordance analysis between these two datasets revealed a number of gene features that exhibit concordant reduction or enrichment of CpG methylation in obese vs. nonobese women in comparisons to Obese vs. NW Control mice, Obese vs. FOB-Surg mice, and failure to reverse these trends in FOB-Diet mice. The resulting genes are therefore critical targets under epigenetic control that in part mediate the differential outcomes between the two weight loss groups.

This is the first study to compare the effects of surgical vs. dietary weight loss on mammary tumor burden in a preclinical mouse model of basal-like breast cancer. In light of a recent study by our group that discovered persistent obesity-associated inflammation, DNA methylation, and tumor outcomes in formerly-obese mice that lost weight by diet (15), we report that similar obesity-associated measures are successfully reversed via weight loss by sleeve gastrectomy alone. Leveraging the power of next-generation sequencing platforms, RNA-Seq revealed upregulation of macronutrient metabolic machinery as a consistent transcriptomic phenomenon between both weight loss groups. However, we observed an upregulation of certain molecular processes implicated in the innate immune and inflammatory response in FOB-Surg mice compared to FOB-Diet, which are perhaps at play in the exclusive cancer-protective effects observed in FOB-Surg mice. Lastly, genome-wide DNA methylation profiles are distinct between the two weight loss groups, highlighting the importance of epigenetic reprogramming in response to ambient nutrient load and whole-organism physiology. Further studies combining dietary weight loss and targeted inhibition of gene products
with persistent obesity-associated expression are needed to obviate the burden of undergoing bariatric surgery while mimicking the procedure's unique cancer-protective effects.

VII. References