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The effect of obesity on postmenopausal mammary tumor growth and differentiation is p53-dependent

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The effect of obesity on postmenopausal mammary tumor growth and differentiation is p53-dependent

by

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Abstract

The effect of obesity on postmenopausal mammary tumor growth and differentiation is p53-dependent

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The University of Texas at Austin, 2011

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The adult prevalence of obesity in the United States exceeds 30% and obesity is associated with increased cancer risk and poor prognosis, including postmenopausal breast cancer. p53 is a tumor suppressor gene that responds to diverse cellular stress including DNA damage, oxidative stress and hypoxia. p53 is mutated in most human cancers, including postmenopausal breast cancer, and is involved in the regulation of lipogenic enzymes. However, the links between p53 and obesity in postmenopausal breast cancer are poorly understood. Here we test the hypothesis that the effect of obesity on mammary tumor growth is impacted by p53 status. The aim of this study was to determine how p53-deficient mammary tumor cells (relative to p53 wild-type cells) respond to obesity-driven tumor growth. To test this hypothesis, we used ovariectomized (OVX) C57BL/6 mice randomized to a control diet (n=40) or a diet-induced obesity (DIO) regimen (n=40) for 10 weeks. At the time, DIO mice were approximately 40% heavier ($p<0.001$) and had 45% greater adiposity ($p<0.001$) than

control mice. Mice were then injected (in the 4th mammary fat pad) with either p53-deficient (p53+/-) or p53 wild-type (p53+/+) MMTV-Wnt-1 mammary tumor cells. Mice were monitored for tumor growth, killed when moribund, and tumors were collected at study end point. We found an interaction between diet and p53 status, with p53+/+ Wnt-1 tumors grown in DIO mice developing the more aggressive morphology compared to p53+/+ Wnt tumors in control mice while the observation was not seen in p53+/- Wnt tumors. From histopathological analysis we also discovered that the DIO regimen promotes local invasion of mammary tumor cells and alters the morphology of MMTV-Wnt-1 p53+/+ mammary tumors. Specifically, p53+/+ Wnt tumors grown in DIO mice displayed disorganized ductal structures characteristic of p53+/- tumors grown in control mice, and DIO exacerbated this aberrant morphology in p53+/- Wnt tumors. Moreover, immunohistological analyses showed that DIO reduces p53 protein expression while elevating Ki-67 expression only in the p53+/+ Wnt mammary tumors. These results suggest that p53 and DIO have interactive effects on mammary tumor growth, as p53+/+ Wnt tumors growing in DIO mice resulted in higher tumor grade similar to p53+/- Wnt tumors.

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Chapter 1: Introduction

Breast cancer is the most commonly occurring noncutaneous cancer among US women and the second leading cause of cancer-related death in women following lung cancer [1]. An estimated 261,100 new cases of invasive and non-invasive (in situ) breast cancer were diagnosed among women in the US in 2010 [2]. Etiologic factors associated with breast cancer are varied and include age, reproductive events (menarche, menopause, pregnancy, and breastfeeding), exogenous hormone exposure, and genetic factors (for example, mutation in BRCA1, BRCA2 and p53) [3]. However, there are modifiable lifestyle factors that suggest women can manipulate their risk.

Obesity is a modifiable risk factor that significantly increases the development and progression of breast cancer, most notably among postmenopausal women. Unfortunately, there has been a sharp rise in the prevalence of obesity over the past 40 years, with 35.5% of adult U.S. women currently considered obese (body mass index (BMI) 30 kg/m2 or greater) [4]. The relationship between obesity and breast cancer has been investigated widely. In fact, it is reported that for every 5 kg of weight gained, breast cancer risk increases by 8% [5]. In addition to increasing risk for development of breast cancer, studies show that women with increased body size have a worsened prognosis [6].

Previous data suggests that the amount of estrogen exposure over the lifetime can affect risk of developing breast cancer [7]. Estrogen is a steroid hormone produced predominantly by the ovaries but to a lesser extent by adipocytes. After menopause, adipocytes become the chief supplier of systemic estrogen; therefore, obesity can extend the exposure to estrogen beyond menopause [8]. In fact, studies have reported that postmenopausal women with elevated levels of circulating estrogen had higher breast

cancer risk [9]. Estrogen receptor alpha (ERα), one of the two nuclear receptors that bind estrogen, plays an important role in normal breast development and is also involved with the pathogenesis of breast cancer. Loss of functional ERα is associated with a reduction in mammary tumorigenesis in transgenic mice expressing different oncogenes in mammary epithelial cells [10], and overexpression of ERα in transgenic mice is associated with an increased rate of mammary adenocarcinoma development [11]. Frech *et al.* demonstrated that increased ERα expression in mammary epithelial cells of transgenic mice increased rates of mammary epithelial cell proliferation and induced the development of ductal carcinoma *in situ* [12].

p53 was the first tumor suppressor gene linked to hereditary breast cancer. Localized on chromosome 17p13, p53 is a 53 kDa nuclear phosphoprotein that regulates cell cycle, gene transcription, DNA repair, genome stability and apoptosis. The fate of a cell in response to injury or mutation is largely determined by the ability of p53 to inhibit cell cycle progression or initiate cell death [13][14]. The biological outcome is determined by the cellular stress involved and the extent of cellular damage. Regardless of the outcome, expression of p53 minimizes transfer of damaged DNA to daughter cells and transcription of mutated genes. Because of its crucial role in DNA repair, p53 is inactivated as a direct result of mutations in TP53 gene in approximately half of all human cancers [15].

Recent studies have shown that p53 can regulate ER expression; however, both positive and negative effects have been reported. Shirley *et al.* demonstrated that p53 regulates ER expression through transcriptional control of the ER promoter [16]. They observed that ER expression was significantly higher in mammary tumors arising in p53 wild-type (p53+/+) mice relative to tumors from p53-deficient (p53+/-) mice, indicating the presence of a functional link between the tumor suppressor p53 and growth-

promoting ER signaling pathways. However, Akaogi *et al.* indicated that the accumulation and activation of p53 abrogates the transcriptional activity of ER [17], which is consistent with the earlier reports in which p53 was shown to interfere with the ability of ER to bind estrogen-response elements (EREs) and thereby inhibit ER-dependent transcription [18].

Donehower *et al.* have reported that homozygous p53-knockout (p53-/-) mice appeared developmentally normal; however, they were highly susceptible to spontaneous tumorigenesis at an early age (between 10 to 20 weeks of age) [19]. In addition, most heterozygote p53-deficient (p53+/-) mice developed tumors by 9 months of age while p53 wild-type (p53+/+) mice failed to develop any tumor or illness. Other findings also demonstrated that the average growth rates of both p53-/- and p53+/- tumors are accelerated compared to p53+/+ tumors, and this accelerated tumor growth rate resulted from an increase in cell proliferation [20]. Our previous studies concluded that although p53 status strongly influences the rapidity with which spontaneous tumors develop, reducing caloric intake in both p53+/+ and p53+/- mice increases the latency of tumor development, indicating that the antiproliferative effects of calorie restriction are p53-independent [21]. However, there are not many studies showing the effect of p53 status on tumorigenesis in obese mice. Therefore, in this report, we compared the effects of diet-induced obesity (DIO) diet on p53+/+ and p53+/- mammary tumor growth to investigate whether p53 plays a role in the tumor growth-promoting effects of obesity. Our findings suggested that at least some of the proliferative effects of obese diet may be mediated through a p53-dependent pathway.

Chapter 2: Materials and Methods

MICE AND DIETS

The animal study conducted for the purposes of this project was approved by the University of Texas at Austin Institutional Animal Care and Use Committee and carried out in compliance with all guidelines and regulations. The 6-wk-old ovariectomized female C57BL/6 mice (n = 80) were purchased from Taconic Farms, Inc. (Germantown, NY) and placed on a chow diet (Harlan Diets #2018, Madison, WI) for one week. After delivery to the Animal Resource Center at University of Texas at Austin, mice were individually housed on a 12 hour light/dark cycle and consumed food and water ad libitum. One week following arrival, mice were randomly assigned (n = 40 per group) to receive either a control diet (CON; 70% carbohydrate, 20% protein, 10% fat; # D12450B) or a diet-induced obesity regimen (DIO; 20% carbohydrate, 20% protein, 60% fat; #D12492), fed ad libitum over a 10 week period. Body weights and caloric intake were analyzed weekly. Mice received assigned diets for the remainder of the study. Diets were formulated by Research Diets, Inc. (New Brunswick, NJ).

MOUSE MAMMARY TUMOR VIRUS (MMTV)-WNT-1 TUMOR CELL LINES

The p53 wild-type (p53+/+) or p53-deficient (p53+/-) mammary tumor cells used in these studies were obtained directly from MMTV-Wnt-1 transgenic mice on a C57BL/6 background. To generate tumor cells with these genotypes, MMTV-Wnt-1 transgenic mice were bred with p53+/+ or p53+/- mice. For isolation and transplantation of tumor cells from these crosses into wild type C57BL/6 female mice, we used a procedure developed for harvesting and transplanting tumors from genetically engineered mice [22]. Briefly, mice were euthanized with CO_2, and tumors were collected

aseptically from donor mice using blunt dissection, trimmed of extraneous tissues, mechanically dissociated by mincing and passage through a 40-micron mesh sterile screen, and suspended in serum-free RPMI 1640 (Gibco Laboratory, Grand Island, NY). Cells were further dissociated by serial passage through a syringe with 18-25 gauge needles. The cell suspension was washed twice and resuspended in serum-free RPMI 1640 medium, and viable cell counts were determined by hemocytometer counting following 0.4% trypan blue staining. Cells were resuspended at 2×10^6 cells per mL in 10% dimethylsulfoxide cell freezing medium and cryopreserved using stepped rate freezing.

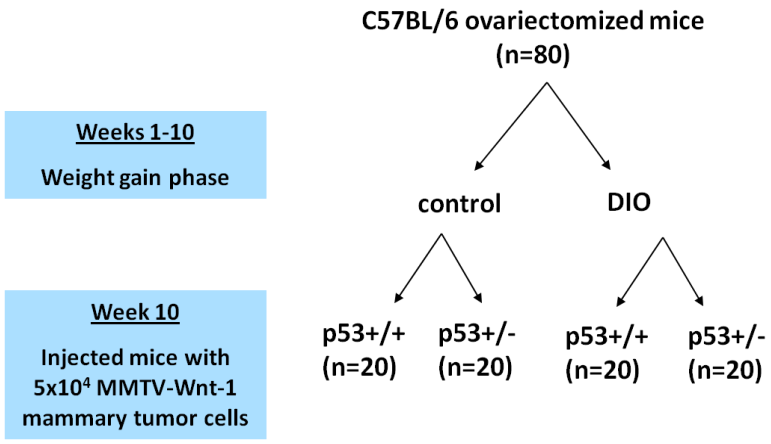

Figure 1: Study Design

6-wk-old ovariectomized female C57BL/6 mice fed with control or DIO diet for 10 weeks and received p53+/+ or p53+/- MMTV-Wnt-1 mammary tumor cells.

MMTV-WNT-1 TUMOR CELL LINES AND INJECTION

After 10 wks on the diet treatments, all mice were orthotopically injected with $5x10^4$ p53+/+ (n=20 per diet group) or p53+/- (n=20 per diet group) MMTV-Wnt-1 murine mammary tumor cells. Prior to transplantation, cells were cultured in RPMI-1640 medium (Gibco Laboratory, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), penicillin-streptomycin (10000U/mL and 0.1mg/mL, respectively; Sigma-Aldrich, St. Louis, MO), and 2mM L-glutamine (Invitrogen, Carlsbad, CA) and maintained in a humidified incubator with 5% CO_2 at 37°C. Cells were trypsinized, washed with PBS, and 5×10^4 cells per 100µL were resuspended in RPMI-1640 for injection into the 4^{th} mammary fat pad. Once palpable, tumors were measured twice weekly using electronic Vermeer™ calipers by measuring maximal length and perpendicular width. Tumor volume was approximated using the formula $4/3\pi$ $(r_1^2 r_2)$ $(r_1 < r_2)$ where radius (r) $_1$ and r_2 represent half of the measured diameters in two separate dimensions. To derive a single tumor volume for each mouse with multiple tumors, volume measurements for each single tumor were summed. When one or more tumors reached 1.0cm in either length or width, all mice from that group were fasted for 10 hours, anesthetized with isofluorane then underwent cardiac puncture for blood collection. Finally, mice were killed by cervical dislocation and tumors were excised and either fixed in 10% neutral-buffered formalin or flash frozen in liquid nitrogen and stored at -80˚C until further analysis.

MEASURE PERCENT BODY FAT

Percent body fat was assessed on each mouse carcass by dual-energy X-ray absorptiometry (DEXA) (GE Lunar PIXImus II, Madison, WI). The head of each mouse

was eliminated from the scan by using the region of interest exclusion option provided in the software.

MEASUREMENT OF SERUM HORMONES AND ADIPONECTIN

Whole blood from each mouse (n = 20 per diet group) collected using cardiac puncture was allowed to incubate at room temperature for 1 hour then spun at 5000 rpm for 10 minutes. Final supernatant (serum) was collected and then stored at −80°C until use. We assayed 9 randomly selected serum samples using the mouse adipokine LINCOplex®Multiplex Assays (Millipore, Inc., Billerica, MA), according to the manufacturer's instructions, analyzed on a BioRad Bioplex 200 analysis system (Biorad, Inc. Hercules, CA) to determine serum levels of insulin, leptin, and adiponectin. Total circulating levels of insulin-like growth factor (IGF)-1were measured using Mouse/Rat IGF-I Quantikine ELISA Kit (R&D System, Inc., Minneapolis, MN) analyzed on a plate reader. One extracted aliquot of serum from each mouse was analyzed in duplicate.

FASTING BLOOD GLUCOSE

To determine the effects of tumor p53 genotype on glucose homeostasis, we conducted a fasting blood glucose test at the end of study. Mice were fasted for 10 hours with ad libitum access to water before being sacrificed. Blood samples were taken from the tail and analyzed for glucose concentration using an Ascencia Elite XL 3901G glucose analyzer (Bayer Corporation, Mishawaka, IN).

IMMUNOHISTOCHEMICAL STAINING

Tissues (tumor and surrounding mammary fat pad) fixed in formalin were embedded in paraffin, then cut into 4 μm thick sections for either hematoxlin and eosin (H&E) staining or immunohistochemical analysis. Briefly, slides were deparaffinized in xylene and rehydrated sequentially in ethanol to water then incubated in 3% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was performed in 10mM citrate buffer pH 6.0 (DAKO Cytomation, Carpinteria, CA) for 15 minutes in a microwave oven. Nonspecific antibody binding was blocked by incubating slides with Biocare Blocking Reagent (Biocare, catalog #BS977M, Concord, CA) for 10 minutes. Slides were washed and primary antibodies were added at indicated concentrations at 4°C overnight. Slides were then incubated with Dako EnVision™ labeled polymer for 30 minutes at room temperature, followed by incubation with Dako diaminobenzidine and counterstained with hematoxylin.

Immunostaining was performed for Ki-67 (1:200 for overnight at 4°C; DAKO Cytomation, Carpinteria, CA), CD31 (1:400 for overnight at 4°C; Clone MEC 13.3, BD Biosciences, San Jose, CA), p53 (1:500 for 1 hour at room temperature; Navocastra Laboratories Ltd, Newcastle, UK), Progesterone Receptor (PR) (1:100 for overnight at 4°C; Abcam Inc, Cambridge, MA), and estrogen receptor-alpha (ERα) (1:500 for 1 hour at room temperature; Santa Cruz Biotechnology, Santa Cruz, CA).

Images were captured using a light microscope equipped with a Leica digital color camera (Leica Camera Inc, Allendale, NJ). Three different tumors were randomly chosen in each group in order to quantify Ki-67 and p53 expression. A determination of the number of p53- and Ki-67-positive cells in tumor samples was performed by counting the number of visible p53- and Ki-67-positive cells in three random fields using a 40X objective. Data are presented as mean ± SD. ERα expression was determined by 4

random tumors per group. The approximate level of ERα expression in each tumor is indicated by the number of pluses, and we use the slide which has the strongest staining as positive control. +++, 70-100% staining compared to control; ++, 40-70% staining compared to control; +, 0-40 staining compared to control.

STATISTICS

All statistical analyses were conducted using GraphPad Prism version 4.0 (GraphPad Software Inc, La Jolla, CA) with students t-test. Data are presented as mean ± SD. Significance was considered at $p \leq 0.05$.

Chapter 3: Results

DIO REGIMEN INCREASES BODY WEIGHT AND BODY FAT

Dietary intervention effectively generated two different weight phenotypes in C57BL/6 mice. After 10 weeks on respective diet, the mean body weight for DIO mice (45.4g ± 3.8g; $p<0.0001$; Figure 2) was significantly heavier than control mice (31.9g ± 4.5g). Also, DIO mice had a significantly higher percent body fat (57.1% ± 1.9%; $p<0.0001$) compared to control mice (39.3% ± 4.4%). These data showed that the DIO regimen resulted in increased body weight.

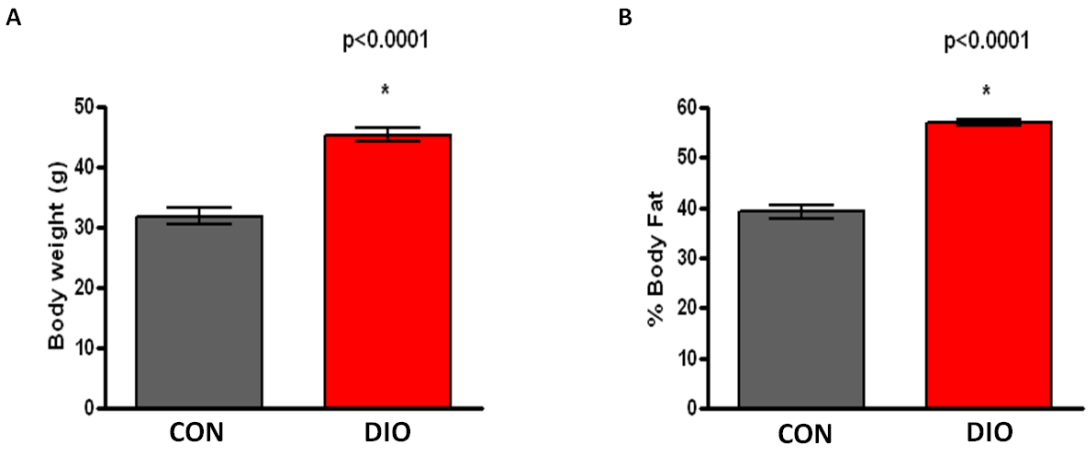

Figure 2: Effect of diet on body weight and percent body fat in female C57BL/6 mice

Ovariectomized C57BL/6 female mice were fed either a control or DIO diet (n=20/group) for 10 weeks. **A.** Final body weights of DIO mice were significantly heavier (45.4 g ± 3.8; $p<0.0001$) than control (31.9 g ± 4.5) mice. **B.** At week 10, average % body fat of DIO mice was significantly greater (57.1% ± 1.9; $p<0.0001$) than control (39.3% ± 4.4) mice. Data represent mean ± SD. Significant difference is indicated by *. CON = control; DIO = diet-induced obesity.

p53 HETEROZYGOSITY ACCELERATES MMTV-WNT-1 MAMMARY TUMOR GROWTH AND INCREASES PERCENT OF TUMOR FORMATION IN RESPONSE TO THE DIO REGIMEN

We observed a rapid onset of palpable p53+/- MMTV-Wnt-1 mammary tumors irrespective of diet (Figure 3A). Mice fed either diet had notable p53+/- MMTV-Wnt-1 mammary tumors only 13 days after tumor cell injection while 30 and 32 days elapsed before there were detectable p53+/+ MMTV-Wnt-1 mammary tumors in the DIO and control mice, respectively. Study endpoint for the mice with p53+/- MMTV-Wnt-1 and p53+/+ MMTV-Wnt-1 tumors occurred 23 and 52 days after injection, respectively. Actually, 100% of DIO and 90% of control mice injected with p53+/- MMTV-Wnt-1 mammary tumor cells had palpable tumors at study endpoint while only 80% of DIO and 55% of control mice had palpable p53+/+ MMTV-Wnt-1 tumors.

At final measurement, DIO mice with p53+/- MMTV-Wnt-1 mammary tumors were significantly larger ($471.6mm^3 \pm 287.5mm^3$; p=0.005; Figure 3B) than control mice with p53+/- tumors ($220.8mm^3 \pm 212.5mm^3$). The DIO mice injected with p53+/+ MMTV-Wnt-1 mammary tumor cells also exhibited a significantly larger final tumor volume ($214.3mm^3 \pm 308.5mm^3$; p=0.01) than the control mice with p53+/+ tumors ($28.6mm^3 \pm 54.1mm^3$).

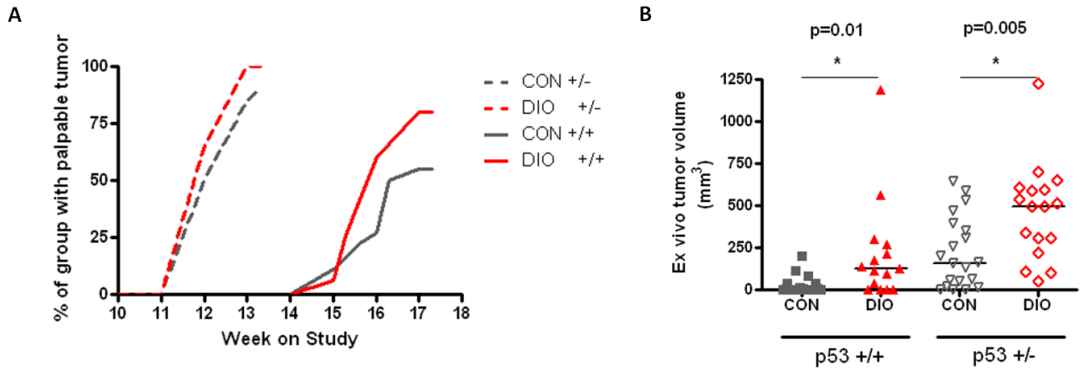

Figure 3: Effect of diet on MMTV-Wnt-1 mammary tumor growth rate and volume

A. Percentage of palpable tumor. 3.3 weeks after injection, 100% of DIO and 90% of control mice had palpable p53 +/- tumors. 7.3 weeks after injection, 80% of DIO and 55% of control mice had palpable p53 +/+ tumors. **B.** Tumor volume measured ex vivo at study termination. DIO mice with p53 +/- tumor had significantly higher tumor volume (471.6 mm^3 ± 287.5, n=17; p=0.005) than control mice (220.8 mm^3 ± 212.5, n=20). DIO mice with p53 +/+ tumor also had significantly higher tumor volume (214.3 mm^3 ± 308.5, n=15; p=0.01) than control mice (28.6 mm^3 ± 54.1, n=18). Data represent mean ± SD. Significant difference is indicated by *. CON = control; DIO = diet-induced obesity; p53 +/+ = p53 +/+ MMTV-Wnt-1 mammary tumor; p53 +/- = p53 +/- MMTV-Wnt-1 mammary tumor.

DIO REGIMEN INCREASES IGF-1 AND LEPTIN AND FASTING BLOOD GLUCOSE LEVELS WHILE DECREASE ADIPONECTIN LEVEL

Consistent with previous findings, the DIO mice had significantly increased circulating levels of IGF-1 (413.9ng/mL ± 38.7ng/mL; $p=0.02$; Table 1) and leptin (12.0ng/mL ± 0.6ng/mL; $p<0.0001$) relative to control mice (311.2ng/mL ± 17.7ng/mL; 6.4ng/mL ± 0.5ng/mL, respectively). Although it did not approach significance ($p=0.07$), DIO treatments resulted in moderately elevated insulin levels (1.7ng/mL ± 0.4ng/mL) compared to control mice (0.9ng/mL ± 0.3 ng/mL). Conversely, DIO mice had significantly decreased levels of adiponectin (17.3µg/mL ± 1.4µg/mL; $p<0.01$) than control mice (22.6µg/mL ± 1.1µg/mL). Also, DIO mice had significantly higher levels of fasting blood glucose (181.3mg/dL ± 8.6mg/dL; $p=0.05$) than control mice (155.9mg/dL ± 8.5mg/dL).

Table 1: Effect of diet on serum hormones in mice

	CON	DIO	p-value
IGF-1 (ng/mL)	311.2±17.7	413.9±38.7*	p=0.02
Leptin (ng/mL)	6.4±0.5	12.0±0.6*	p<0.0001
Insulin (ng/mL)	0.9±0.3	1.7±0.4	p=0.07
Adiponectin (µg/mL)	22.6±1.1	17.3±1.4*	p<0.01
Fasting Blood Glucose (mg/dL)	155.9±8.5	181.3±8.6*	p=0.05

Serum hormones IGF-1, leptin and fasting blood glucose levels are significantly higher and adiponectin level is significantly lower in DIO mice than control mice. Data represent mean ± SD. Significant difference is indicated by *. CON = control; DIO = diet-induced obesity.

OBESITY ENHANCED PATHOLOGICAL PROGRESSION IN P53 +/+ MMTV-WNT-1 TUMORS

Because our studies demonstrated significant increases in both p53+/+ and p53+/- MMTV-Wnt-1 mammary tumor size in mice fed with DIO diet, we excised these tumors and examined histopathological features by H&E staining. Histopathological examination revealed that p53+/+ MMTV-Wnt-1 mammary tumors in control mice were well-differentiated ductal adenocarcinomas with clearly defined margins and no central necrosis (Figure 4). Alternatively, p53+/+ MMTV-Wnt-1 mammary tumors in DIO mice were poorly differentiated ductal adenocarcinomas with disorganized ductal structures, papillary and cystic structures, less fibrosis, and wide spread central necrosis. On the other hand, diet exerted no effect on p53+/- tumors. These tumors displayed disorganized structures with consistent presence of cystic and papillary features. In fact, DIO mice displayed p53+/+ MMTV-Wnt-1 mammary tumors with similar histopathologic features comparable to either group with p53 +/- MMTV-Wnt-1 tumors.

It is reported that white adipose tissue cells are recruited by tumors and promote cancer progression; therefore, we examined the margin of tumors and mammary fat pads. p53 +/+ MMTV-Wnt-1 mammary tumors in control mice displayed a clearly defined, encapsulated border between tumor and mammary fat pad (Figure 5); however, p53+/+ MMTV-Wnt-1 tumor cells in DIO mice infiltrated surrounding mammary fat pads. p53+/- MMTV-Wnt-1 tumors did not display a distinct boundary between tumor and surrounding mammary fat pad in both diet groups. p53+/- MMTV-Wnt-1 mammary tumor cells in both DIO and control mice displayed marked adipocyte infiltration, and even higher infiltration levels were observed in DIO mice with p53+/- tumor.

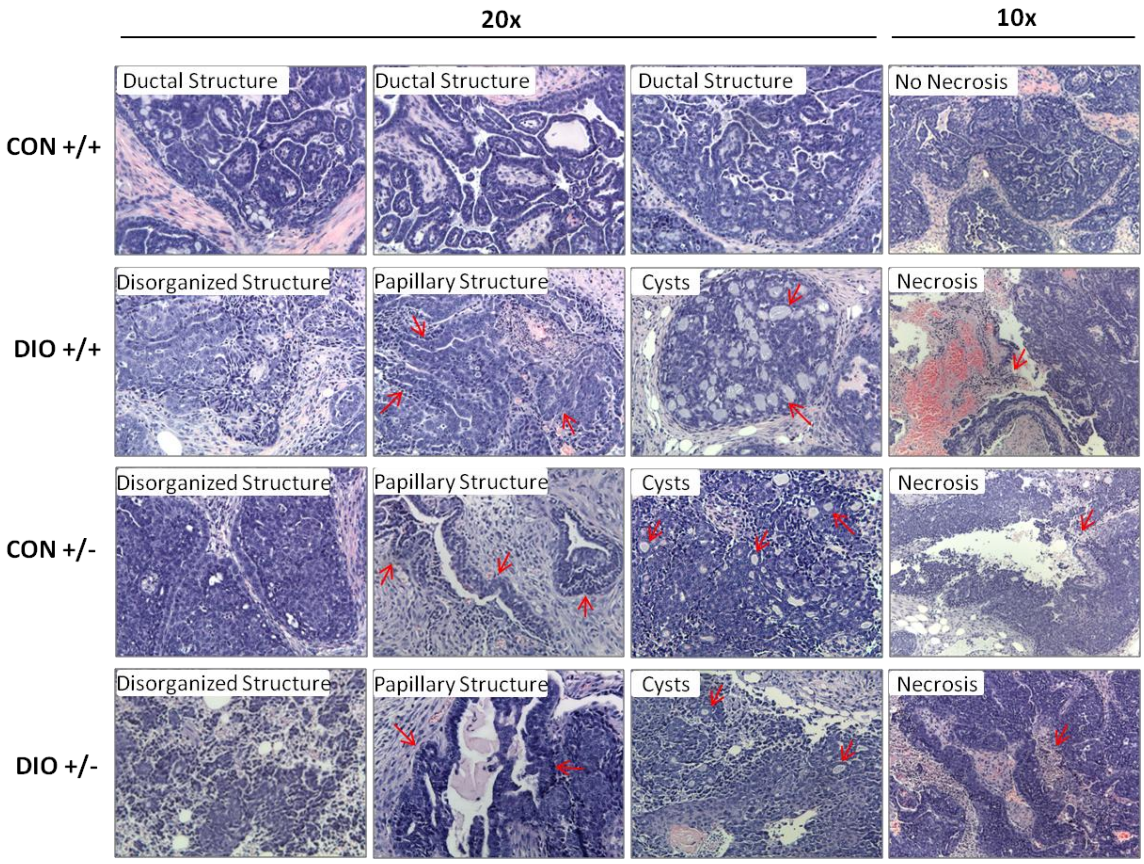

Figure 4: H & E staining for MMTV-Wnt-1 mammary tumor histology

H & E staining on p53 +/+ tumors from mice in control group showed well differentiated adenocarcinoma without necrosis. Disorganized, papillary structure, cysts and central necrosis were seen in MMTV-Wnt-1 tumors from other three groups of mice. CON = control; DIO = diet-induced obesity; p53 +/+ = p53 +/+ MMTV-Wnt-1 mammary tumor; p53 +/- = p53 +/- MMTV-Wnt-1 mammary tumor. Arrows point to indicated structures.

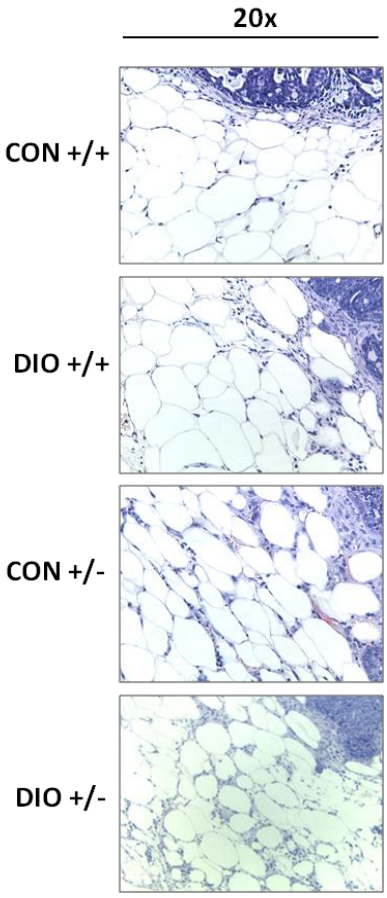

Figure 5: H & E staining for MMTV-Wnt-1 mammary tumor cell local invasion

p53 +/+ tumors in control group displayed a distinct border of tumor and adipocytes of local mammary fat pad. p53 +/+ tumors in DIO group showed migration of tumor cells into mammary fat pad. Infiltration was also seen in p53 +/- tumors fed with either diet. CON = control; DIO = diet-induced obesity; p53 +/+ = p53 +/+ MMTV-Wnt-1 mammary tumor; p53 +/- = p53 +/- MMTV-Wnt-1 mammary tumor.

DIET RESPONSIVE CHANGES IN P53 EXPRESSION AND TUMOR PROLIFERATION

To confirm the levels of p53 expression in tumor cells engineered to have more or less p53 , we performed immunohistochemistry on paraffin-embedded sections taken from p53+/+ MMTV-Wnt-1 and p53+/- mammary tumors. As expected, more p53 protein was expressed in p53+/+ MMTV-Wnt-1 tumors compared to p53 +/- tumors (Figure 6). In fact, p53 expression in control p53+/- tumors was about 40% of that seen in control p53+/+ tumors. We observed that p53+/+ MMTV-Wnt-1 tumors in DIO mice had fewer p53-positive cells (71 ± 23 cells under a 40X objective field) compared to p53+/+ tumors in control mice (94 ± 40) (Table 2). In contrast, diet exerted no effect on p53+/- MMTV-Wnt-1 tumors. The amount of p53-positive cells in p53+/- tumors in DIO mice (40 ± 12) were closed to p53+/- tumors in control mice (37 ± 6). We also found that p53+/- MMTV-Wnt-1 mammary tumors had higher cellular proliferation levels than p53+/+ tumors, as measured by Ki-67 staining. Actually, in control p53+/- tumors, Ki-67 expression was increased 1.8-fold relative to control p53+/+ tumors. Also, p53+/- tumors in DIO mice expressed 1.4-fold higher Ki-67 levels relative to p53+/+ tumors in DIO mice. DIO mice with p53+/+ MMTV-Wnt-1 mammary tumors had more Ki-67 staining (207 ± 13 cells under a 40X objective field) compared to control p53+/+ tumors (165 ± 58). However, DIO treatment did not elevate Ki-67 expression in mice with p53+/- tumors (281 ± 37) relative to control p53+/- tumors (291 ± 58). No quantitative differences were apparent between different diets and p53 -status groups in CD-31 staining, a marker of endothelial cells lining blood vessels formation. However, bigger blood vessels were observed in p53+/- MMTV-Wnt-1 mammary tumors in control mice.

Immunohistochemical analysis for ER antibody revealed that p53+/- MMTV-Wnt-1 tumors displayed higher level of ER staining than p53+/+ Wnt-1 tumors (Figure

7). We observed that the DIO regimen increased ER expression in both p53+/+ MMTV-Wnt-1 and p53+/- mammary tumors relative to the same tumors in control mice (Figure 8). Moreover, the effects of DIO on ER expression were exhibited to a greater extent in mice with p53+/+ tumors compared to p53+/- tumors. We found no change in progesterone receptor (PR) expression in either p53+/+ MMTV-Wnt-1 or p53+/- mammary tumors regardless of diet.

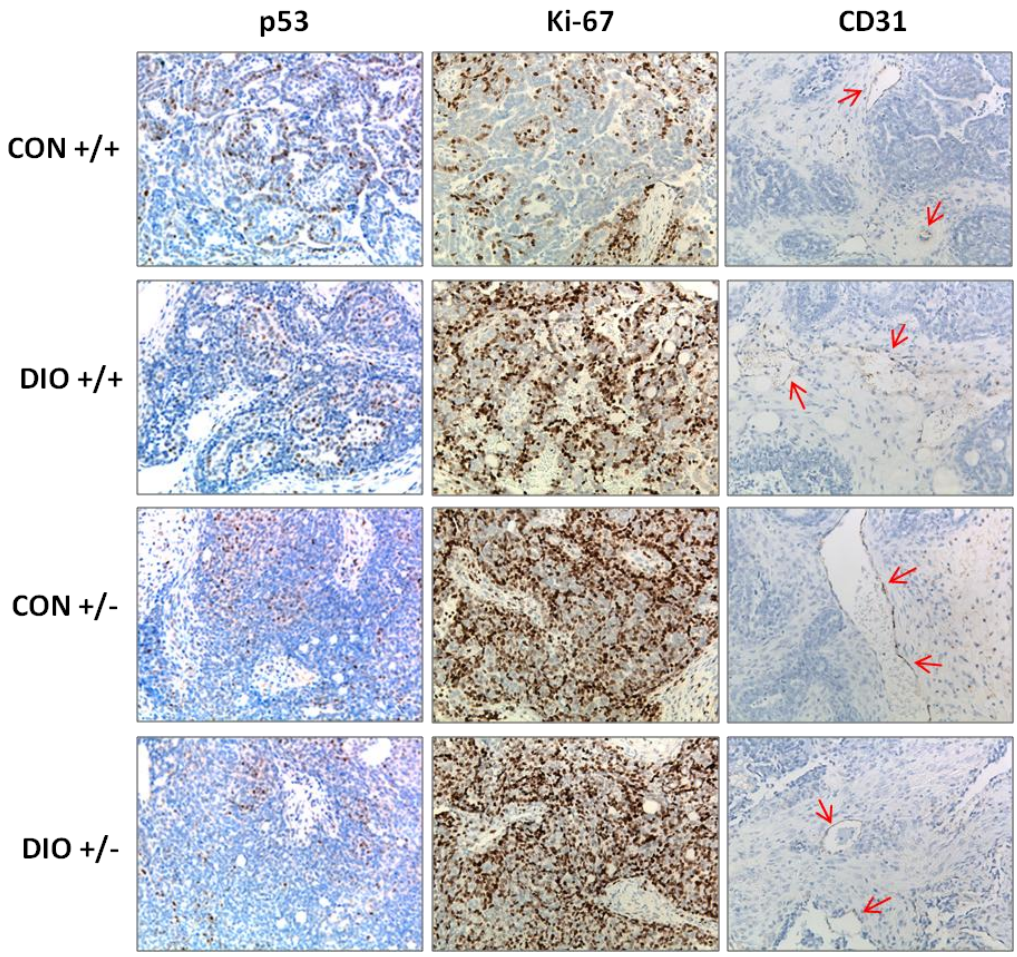

Figure 6: Immunohistochemical staining for p53 , Ki-67 and CD31 of MMTV-Wnt-1 mammary tumors

Immunohistological staining of p53 +/+ tumors in DIO mice showed a decrease in p53 expression and an increase in cellular proliferation by Ki67 staining compare to p53 +/+ tumors in control mice. p53 +/- tumors displayed low p53 expression and widespread Ki-67 expression relative to p53 +/+ tumors. CD31 staining for endothelial cells lining blood vessels showed no difference between 4 groups. CON = control; DIO = diet-induced obesity; p53 +/+ = p53 +/+ MMTV-Wnt-1 mammary tumor; p53 +/- = p53 +/- MMTV-Wnt-1 mammary tumor. Arrows point to indicated structures.

Table 2: Quantification of immunohistochemiscal staining for p53 , Ki-67

	p53			Ki-67		
	CON	DIO	**DIO/CON**	CON	DIO	**DIO/CON**
p53 +/+	94	71	0.75	165	207	1.25
p53 +/-	37	40	1.08	291	281	0.97

The average antibodies p53 and Ki-67 staining-positive cells in three tumors per group and three fields each tumor under high magnification (40X). More p53 -positive cells and less Ki-67-positive cells staining were seen in control mice with p53 +/+ tumors relative to DIO mice. Both staining in p53 +/- tumors remain closed regardless of diet. CON = control; DIO = diet-induced obesity; p53 +/+ = p53 +/+ MMTV-Wnt-1 mammary tumor; p53 +/- = p53 +/- MMTV-Wnt-1 mammary tumor.

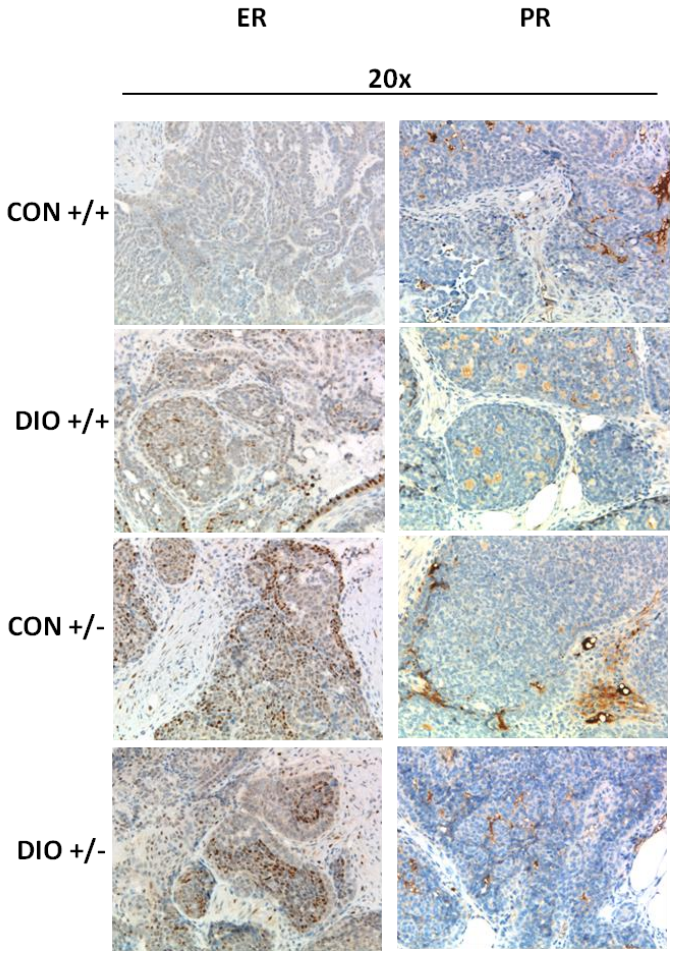

Figure 7: Immunohistochemical staining for ER and PR of MMTV-Wnt-1 mammary tumors

Immunohistological staining of p53 +/+ tumors showed low ER expression in control mice and an increase ER level in DIO mice, similar to ER level displayed in p53 +/- tumors. No difference for PR staining between 4 groups. CON = control; DIO = diet-induced obesity; p53 +/+ = p53 +/+ MMTV-Wnt-1 mammary tumor; p53 +/- = p53 +/- MMTV-Wnt-1 mammary tumor.

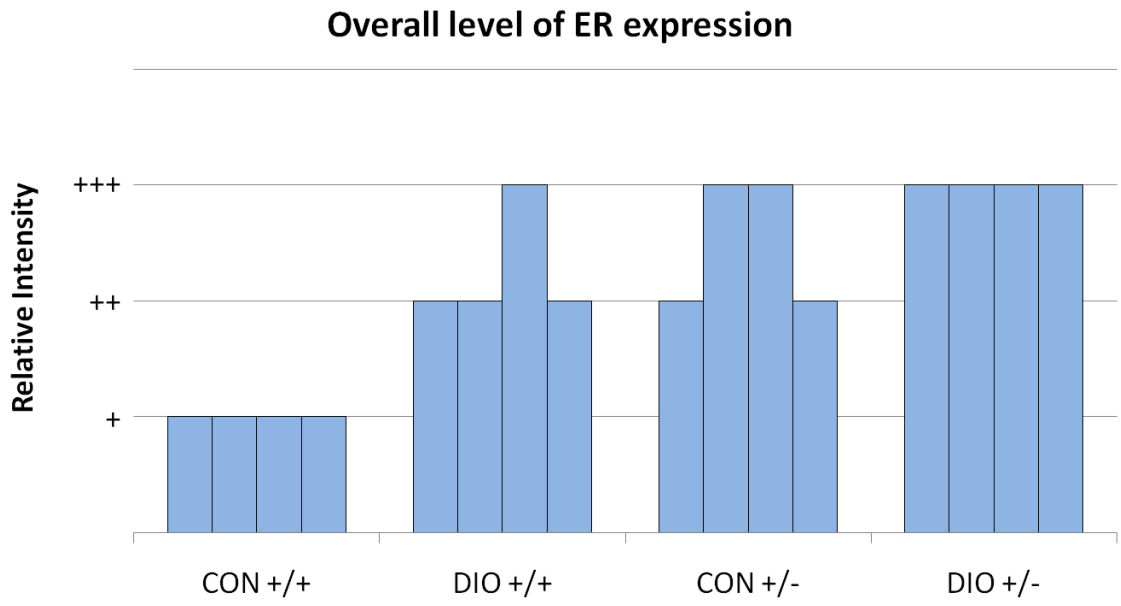

Figure 8: Relative intensity of ERα expression in MMTV-Wnt-1 mammary tumors

Quantify immunohistological staining for ERα expression. Each column represents a separate tumor. +++, 70-100% staining compared to control; ++, 40-70% staining compared to control; +, 0-40 staining compared to control. CON = control; DIO = diet-induced obesity; p53 +/+ = p53 +/+ MMTV-Wnt-1 mammary tumor; p53 +/- = p53 +/- MMTV-Wnt-1 mammary tumor.

Chapter 4: Discussion

In our studies, we compared the effects of obesity on the growth of p53+/+ MMTV-Wnt-1 or p53+/- MMTV-Wnt-1 mammary tumors in a C57BL/6 mouse model of postmenopausal breast cancer. After 10 weeks on diet, mice fed the DIO diet had significantly greater body weight, percent body fat, and circulating levels of insulin, IGF-1, and leptin; all biomarkers indicative of an obese phenotype. As expected, p53 status exerted the greatest effect on tumor latency with p53+/- mammary tumors appearing 17 days earlier than p53+/+ tumors. However, within the genotypes, DIO mice exhibited significantly larger tumors, relative to their respective controls, with increased expression of the proliferation marker, Ki-67. Moreover, the DIO diet resulted in p53+/+ tumors that appeared histologically similar to the p53+/- tumors of either group.

Mutations in tumor suppressor p53 are frequently related to human malignancies, including 40% of breast cancers [15]. It acts as a DNA damage sensor, responding to various severe damages by inducting cell-cycle arrest or apoptosis-related genes [23]. Many studies have investigated and reported that overall and disease-free survival rates were significantly poorer in breast cancer cases with somatic mutations in p53 [24]. It is also documented that cells with inactivated p53 have an elevated proliferation rate [25] and loss of the G_1-S check point [26]. In fact, our studies showed that p53-deficiency accelerated growth of MMTV-Wnt-1 tumors and had a higher percent of tumor formation, which is consistent with previous studies.

The histopathological features of p53+/+ MMTV-Wnt-1 and p53+/- mammary tumors in control mice suggested that biological differences exist between those tumors expressing more p53-positive cells and those expressing less. As expected, p53+/+ MMTV-Wnt-1 mammary tumors in control mice were well-differentiated and organized

adenocarcinomas (lower tumor grade) while p53+/- tumors displayed disorganized ductal structures with wide spread central necrosis (higher tumor grade). However, DIO regimen altered the morphology of p53+/+ MMTV-Wnt-1 mammary tumors into p53 +/- tumor-like properties. Thus, the p53+/+ MMTV-Wnt-1 mammary tumors in control mice represented a more benign stage of mammary tumor progression while the p53+/+ tumors in DIO and p53+/- tumors in both dietary groups are modeled as more invasive stages of mammary carcinomas.

Emerging evidence has suggested that p53 can specifically regulate cancer cell migration and its role is extended beyond apoptosis and cell cycle arrest [23]. Gadea *et al*. reported that blocking p53 in tumors is sufficient to promote motility and invasion, and further contributing to metastasis [27]. In this report, histopathological data displayed that a distinct boundary can easily be distinguished between p53 +/+ MMTV-Wnt-1 mammary tumor cells and surrounding adipocytes in control mice. In addition, p53 +/- MMTV-Wnt-1 tumor cells in both DIO and control mice tended to invade into surrounding adipocytes, which is in agreement with previous data. However, in DIO mice, p53+/+ MMTV-Wnt-1 mammary tumor cells apparently separated from their neighbor cells and migrated into surrounding adipocytes. These findings indicated that although the tumor p53 suppresses cancer cell invasion, the benefit may be interfered by diet-induced obesity. Actually, recent studies have demonstrated that adipose stromal cells, a resident cell population in adipose tissues, stimulate migration and invasion of breast cancer cells both *in vivo* and *in vitro* [28], which are consistent with our findings.

In Western women, the incidence of ER positive breast cancer and expression of ER in breast cancer tissue has increased in the past 40 years [29]. A positive correlation has been shown between the presence of obesity and ER positive breast cancer, especially in postmenopausal women [30]. In addition to the cross-talk between obesity and ER,

other observations also suggested that there is another potential link between p53 and ERα. Liu *et al*. previously reported that ERα physically binds to p53, resulting in inhibition of transcriptional regulation by p53 [31]. Recently, Konduri *et al*. provided evidence that ERα recruits corepressors when bound to p53 and showed that ER and p53 interaction is relevant to normal mammary cells regulation and to breast cancer patient response to antiestrogen therapy [32]. In addition to the direct inhibition of ERα transcriptional activity by p53, Akaogi *et al.* also discovered another pathway of cross-talk between p53 and ERα that involves a transcription factor, KLF4 [17]. Although many studies have investigated the interaction between ER and obesity or ER and p53 separately, no report has focused on examining the effects of obesity and p53 together on ER expression. We showed that p53+/+ MMTV-Wnt-1 mammary tumors in control mice have lower levels of ER expression relative to p53+/- MMTV-Wnt-1 mammary tumors in control mice, which is in agreement with the previous literatures. Moreover, we discovered that DIO mice had an increase in ER expression in p53+/+ mammary tumors, indicating that obesity may silence p53 expression in an epigenetic manner.

In summary, our finding that p53+/+ MMTV-Wnt-1 mammary tumors in DIO mice increases the percent of tumor burden, enhances pathological progression, promotes local invasion of tumor cells, and elevates ER expression compare to p53+/+ tumors in control mice. These observations that DIO altered the features of p53+/+ mammary tumors in a manner similar to p53+/- like mammary tumors, suggests that obesity may influence expression of p53 as well as determine histological progression of mammary tumors. In the future, further studies are needed to elucidate diet-gene interactions underlying the cancer-promoting effects of obesity and to determine which DIO-responsive genes are related to attenuate p53 expression.

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