Evidence for a tumor promoting effect of high-fat diet independent of insulin resistance in HER2/Neu mammary carcinogenesis

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Abstract The mechanism of the association between breast cancer and obesity remains unknown. To investigate this mice over-expressing HER2/Neu in the mammary gland (MMTV-HER2/Neu) were fed either a high-fat diet (45% of calories) (HFD) or low-fat diet (10%) (LFD) from 4 weeks of age and followed for up to 1 year, or sacrificed when a mammary tumor reached 1.5 cm. There was a small but significant increase in body weight on HFD ($P < 0.05$) and the HFD mice displayed a greater fat mass determined by MRI ($P < 0.01$). Mild glucose intolerance was observed from 3 months of age on HFD, but insulin levels were not elevated. While the time of onset of a first tumor and tumor growth rates were not altered, mice on HFD had an earlier onset of a second tumor and a twofold greater incidence (LFD 25%, HFD 54%) and a greater absolute number of multiple tumors (tumors/mouse, LFD 1.5 ± 0.25 vs. HFD 2.7 ± 0.23, $P < 0.01$). Consistent with a lack of hyperinsulinemia, immunoblotting of skeletal muscle lysates from mice injected with insulin showed no insulin resistance determined by the phosphorylation of Akt/PKB. Similarly, there was no difference in basal or maximum insulin-stimulated phosphorylation of IRS-1/2, Akt/PKB, or p70 S6K in tumor cell lysates from HFD and LFD groups. Immunohistochemistry revealed no difference in tumor tissue staining for the proliferative marker, Ki67, between diets. These data indicate that HFD, in the absence of significant insulin resistance, mediates a tumor promoting, but not a tumor growth effect in this model of mammary carcinogenesis.

Keywords Breast cancer · High-fat diet · HER2/Neu mice · Insulin resistance · Tumor promoter

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Introduction

Breast cancer is the most prevalent form of cancer, and the second leading cause of death from cancer in American women [1]. According to the American Cancer Society Statistics for the year 2009, it was estimated that approximately 200,000 women will be diagnosed with breast cancer, and approximately 40,000 will die from the disease [1]. This translates into one in every eight women expected to develop the disease at some point during her lifetime.

There are numerous risk factors that are known to influence the high incidence and mortality of breast cancer. These include both genetic and environmental factors [2, 3]. Of particular interest is the role that obesity plays in the development and progression of breast cancer. The high incidence of breast cancer among women in North America has been correlated to a major environmental influence consisting of a "western lifestyle," which is a combination of dietary excess energy intake along with a lack of exercise, and manifests itself as obesity [4, 5]. It has been previously noted that obesity is a risk factor for both the development and a poorer prognosis of breast cancer [5–9].

Obesity is a known risk factor for the development of the metabolic syndrome or the insulin resistance syndrome, which in general terms is defined as insulin resistance associated with glucose intolerance, dyslipidemia, and/or hypertension [10–12]. The increased prevalence of the metabolic syndrome has accompanied the increasing incidence of breast cancer, diabetes, and obesity worldwide [13, 14]. Type 2 Diabetes Mellitus (T2DM) is characterized by hyperglycemia, hyperinsulinemia, and insulin resistance. Epidemiological studies of the association between T2DM and risk of breast cancer have revealed a modest association between these two diseases [14–16]. This appears to be more consistent among postmenopausal than premenopausal women [15]. An evaluation of this link indicates that the risk is present prior to the onset of T2DM, namely at the stage of insulin resistance associated with compensatory hyperinsulinemia with normal or only impaired glucose concentrations [16].

The above data support the reported findings which correlated increased levels of insulin with an increased risk of the development of breast cancer, as well as increased mortality in women with the disease [17–19]. Similarly, Lawlor et al. observed a modest but linear increase of breast cancer risk between the lowest and the highest quartiles of insulin level, among postmenopausal, non-diabetic women [20]. Two studies in which careful adjustments were made to correct for other prognostic variables demonstrated that higher insulin levels increased the incidence of distant recurrence and death [18, 19]. Therefore, in human studies, elevated insulin appears to confer a small independent risk for the development of breast cancer, and more marked risk for disease progression and mortality.

Studies in rodent models further support the notion that insulin promotes tumor formation and/or increases mammary tumor growth. When the human insulin analog, AspB10, was given to normal rats, it resulted in an increase of spontaneous breast tumors after chronic administration [21]. Several studies of DMBA (7,12 dimethylbenz(a) anthracene)-induced breast cancer in rats have indicated that a high-fat diet increased tumor development [22–25]. In addition, transplanted tumor xenografts appear to grow more rapidly in rodents on a high-fat diet [25, 26]. However, the role of associated insulin resistance and elevated insulin as opposed to elevated circulating fatty acids is not clear. For example, in one model, a severely insulin resistant but non-obese "fatless" mouse was bred with a transgenic mouse mammary gland tumor model, C3(1)/T-Ag, and showed a higher tumor incidence and multiplicity, as well as decreased tumor latency [27]. Since these "fatless" mice lacked adipose tissue-derived hormones such as leptin, adiponectin, and resistin, a role for insulin was suggested, although elevated levels of free fatty acids and/or macrophage-derived inflammatory mediators could not be excluded as contributors.

In the present study, the effect of HFD-induced obesity, which is the most common cause of insulin resistance that is seen in human subjects, was examined in the mouse mammary tumor virus (MMTV) HER2/Neu transgenic mouse which develops mammary tumors that mimic a human subtype of breast cancer [28]. The presence of modest obesity without overt insulin resistance or hyperinsulinemia resulted in a decreased latency of appearance of a second tumor along with an increase in multiple tumors. In contrast, tumor growth was not altered. These data indicate an insulin-independent effect of HFD/obesity on tumor development.

Materials and methods

Reagents

Antibodies to phospho-p70 S6 kinase (Thr389), phospho-Akt/PKB (Ser473), Akt/PKB, and β-actin were obtained from Cell Signaling Technology Inc. (Beverly, MA). The anti-p70, agarose immunoprecipitation (IP) reagent, anti-phosphotyrosine (pY99), and anti-rabbit and anti-mouse IgG secondary antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Insulin enzyme-linked immunosorbent assay (ELISA) kits were from Linco Research Inc. (St. Charles, MO). Anti-IRS-1 and anti-IRS-2 antibodies were obtained from Upstate/Millipore (Billerica, MA). The enhanced-chemiluminescence (ECL) reagents were from KPL Inc. (Gaithersburg, MD). The biotinylated rabbit anti-rat IgG and the Elite ABC-horseradish peroxidase came from Vector Laboratories (Burlingame, CA). The Ki67 antibody was obtained from Dako (Glostrup, Denmark). Nitrocellulose membranes were from Invitrogen Canada Inc. (Burlington, ON).

Animals

The genetic model of breast cancer used was the murine mammary tumor virus (MMTV) long-terminal repeat WT

HER2/Neu transgenic mouse (FVB/N-Tg [MMTV-HER2/Neu] 202Mut/J (Stock No. 002376, Jackson Laboratories, Bar Harbor, ME). Focal mammary tumors begin to appear at approximately 5 months of age, with a median incidence of 205 days, and these usually metastasize to the lungs [28–30]. Mice were maintained on a 12-h light/dark cycle. All procedures were conducted according to the protocols and guidelines of the Canadian Council of Animal care and approved by the University Health Network Animal Care Committee.

Diet formulation and treatment

Forty female FVB/N HER2/Neu mice at the age of 4 weeks were randomly placed on a LFD which consisted of 10% kcal fat (n = 16), and a HFD which consisted of 45% kcal fat (n = 24) (Harlan Teklad, Madison, WI) (Table 1). Diets were stable for 6 months and stored at 4°C. Mice were allowed ad libitum access to food and water throughout the treatment period.

Body weights and magnetic resonance imaging (MRI)

Body weights were monitored and recorded weekly. For the assessment of fat vs. lean body mass, a mouse whole-body magnetic resonance analyzer was used (Echo Medical Systems, Houston, TX), and this was assessed monthly.

Intra-peritoneal glucose tolerance test and plasma insulin concentrations

Glucose tolerance tests (GTTs) were performed on mice fasted overnight using a glucose load of 1.5 g of glucose/kg body weight injected intra-peritoneally (i.p.). Blood glucose measurements were made at 0, 10, 20, 30, 60, 90, and 120 min after the injection using a Sure Step, One Touch Glucometer from Lifescan Inc. (Milpitas, CA). Tail vein blood samples were collected at 10 min after glucose administration in heparin-coated tubes. Plasma was separated from the whole blood by centrifugation at 3,000×g. Plasma insulin concentrations were assayed in duplicate by ELISA (Linco), according to the manufacturer's instructions.

Tumor detection and measurements

The mice were palpated twice weekly beginning at 4 months of age. Tumor size was assessed by measuring the perpendicular tumor diameters with a digital caliper. Tumor volume was calculated by the formula as follows: Tumor Volume = [((smallest diameter)^2 × (largest diameter)^2)/2)] [31] and values obtained from this calculation were then used to assess growth rates.

Tissue collection

The mice were euthanized when they reached 1 year of age or when the largest mammary tumor reached a diameter of 1.5 cm, in accordance with the Canadian Council on Animal Care Cancer Endpoint Guidelines. After induction of anesthesia, at 5 or 10 min prior to sacrifice, animals were injected i.p. with either saline (vehicle) or insulin at a dose of 2.0 U/kg body weight. The hindlimb (gastrocnemius) skeletal muscle and the mammary gland tumors were then excised, snap frozen in liquid nitrogen, and stored at −80°C until subsequent homogenization for immunoblot analysis. In addition, part of the tumor tissue was fixed in 10% buffered formalin for immunohistochemistry.

Immunoblotting and immunoprecipitation

For immunoblot analysis, mammary tumor samples were homogenized with a hand-held glass homogenizer in ice cold lysis buffer (50 mM Tris pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_4$, 1 μM

Table 1 Composition and breakdown of diets

LFD (10% kcal Fat)	kcal %	Detailed composition (kcal)		HFD (45% kcal Fat)	kcal %	Detailed composition (kcal)	
Protein	20	Casein, lactic	800	Protein	20	Casein, lactic	800
		L-Cystine	12			L-Cystine	12
Carbohydrate	70	Corn starch	1,260	Carbohydrate	35	Corn starch	291
		Maltodextrin	140			Maltodextrin	400
		Sucrose	1,400			Sucrose	691
Fat	10	Soybean oil	225	Fat	45	Soybean oil	225
		Lard	180			Lard	1,598
Total	100			Total	100		

LFD low-fat diet, *HFD* high-fat diet

okadaic acid, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cellular debris was removed by centrifugation at 10,000×*g* for 20 min at 4°C. Thirty μg of protein (Bradford assay, Biorad) were re-suspended in 2× Laemmli sample buffer (4% SDS, 20% glycerol, 125 mM Tris, pH 6.8, 0.2% bromophenol blue, 10% beta-mercaptoethanol), boiled for 5 min, separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were incubated for 1 h in Tris-Tween Normal Saline (TTNS) blocking buffer containing 5% BSA followed by sequential incubation with primary (1:1,000 except for *β*-actin 1:5,000) and secondary (1:5,000) antibodies. To correct the amount of phosphorylation for total protein content in each experiment the membranes were stripped after washing with TBST by incubation in a solution of 2% SDS, 62.5 mM Tris HCl, pH 6.8, 100 mM 2-mercaptoethanol for 30 min at 50°C following which the membranes were washed twice in TBST prior to reprobing. Antibody–protein complexes were visualized using Lumi-Glo ECL according to the manufacturer's instructions and band intensities were quantified by scanning laser densitometry.

For immunoprecipitation (IP), 500 μg of the tumor lysates were incubated with agarose-conjugated anti-IRS-1 antibody or with anti-IRS-2 antibody followed by incubation with Protein A/G-PLUS-Agarose immunoprecipitation reagent overnight at 4°C with rotation. Immune complexes were collected by centrifugation for 5 min and washed three times with 1.5 ml PBS. The precipitated proteins were separated by 8% SDS-PAGE and immunoblotted with anti-phosphotyrosine (pY99) (1:1,000) and IRS-1 or IRS-2 antibodies (1:1,000) and bands detected and quantified using ECL as above.

Immunostaining

Formalin-fixed paraffin-embedded tissue sections of breast tumors were cut into sections of 4 μm dewaxed in three changes of xylene and rehydrated through graded alcohols. After antigen retrieval in Tris–HCl buffer, pH 9.0 at 115°C for 10 min, followed by Avidin blocking in TTNS, sections were incubated with Ki67 antibody at a 1:25 dilution for 1 h at room temperature and subsequently washed with TTNS. After incubation with biotinylated rabbit anti-rat IgG (1:200) for 30 min, sections were washed and incubated with Elite ABC-horseradish peroxidase. Antibody binding was visualized with diaminobenzidine and counterstained with hematoxylin. Finally, sections were dehydrated through graded alcohol, cleared in xylene, and coverslipped. All of the slides were scanned at ×20 magnification by ScanScope XT (Aperio Technologies, Vista, CA) that provided high resolution images (0.5 microns/pixel). Images of the whole slides were analyzed using Positive Pixel Count algorithm available in ImageScope Software and each slide contained approximately 150,000–180,000 cells. Brown nuclear staining was considered to be positive for Ki67. The Ki67 labeling index was determined by counting of positively stained cells with the Aperio Image Scope v.8, positive pixel count algorithm software (Vista, CA). Scoring was performed on random tumor sections ($n = 7$) from each mouse.

Statistical analysis

All results are expressed as mean ± SE where applicable. Statistical significance was assessed by two-tailed Student's *t* test or ANOVA. Kaplan–Meier survival estimates were calculated for the time to the development of the first tumor, as well as second tumor in the various treatment groups (i.e., LFD vs. HFD). Median survival time analysis and Log-rank tests were performed to compare the survival curves of different groups. Mice which died from non-study related causes had the data censored at the time of death. Statistical calculations were performed using Statistica Software (Statistical Analysis System, Cary, NC). A $P < 0.05$ was considered statistically significant.

Results

Phenotype of HER2/Neu mice on a high-fat diet

To investigate the influence of diet on breast cancer, HER2/Neu mice were fed a HFD or LFD (45 vs. 10% kcal from fat, respectively). The diets were instituted at 4 weeks of age and were well tolerated. There were no statistically significant differences in grams of food consumed per day between the treatment groups. HER2/Neu mice showed a modest, but significant increase in body weight after 30 weeks on diet (LFD 24.4 ± 0.6 g vs. HFD 27.1 ± 1.3 g, $P < 0.05$) (Fig. 1a). To determine whether the HFD altered body fat content, MRI analysis was performed after 8, 13, 16, 21, and 25 weeks on diet. There was a significant increase in percent body fat in HFD compared to LFD mice at 13 weeks (LFD 15.8 ± 0.7% vs. HFD 22.9 ± 0.5%, $P < 0.01$), 21 weeks (LFD 15.6 ± 0.7% vs. HFD 22.6 ± 0.7%, $P < 0.01$), and 25 weeks (LFD 12.9 ± 0.4% vs. HFD 21.5 ± 0.8%, $P < 0.01$) (Fig. 1b).

To investigate the metabolic consequences of the HFD, i.p. GTTs were performed after an overnight fast, after 28 weeks on diet. The HFD-fed HER2/Neu mice showed no difference in fasting blood glucose levels, but a mild impairment of glucose tolerance with significantly increased values at 20, 60, 90, and 120 min after glucose loading compared to the LFD group ($P < 0.05$) (Fig. 2a). However, the mean insulin concentration in plasma samples

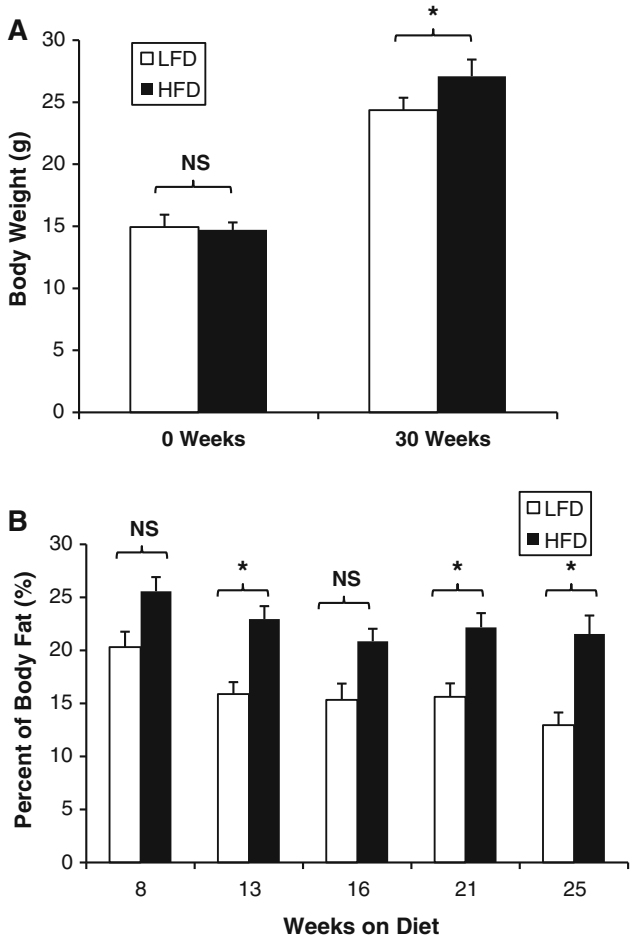

Fig. 1 Body weight and fat analysis. **a** Body weights of mice maintained on a low-fat diet (LFD $n = 16$) and on a high-fat diet (HFD $n = 24$) for 30 weeks. $^*P < 0.05$. **b** Body fat content was assessed by MRI in mice over a 25-week period. $^*P < 0.01$

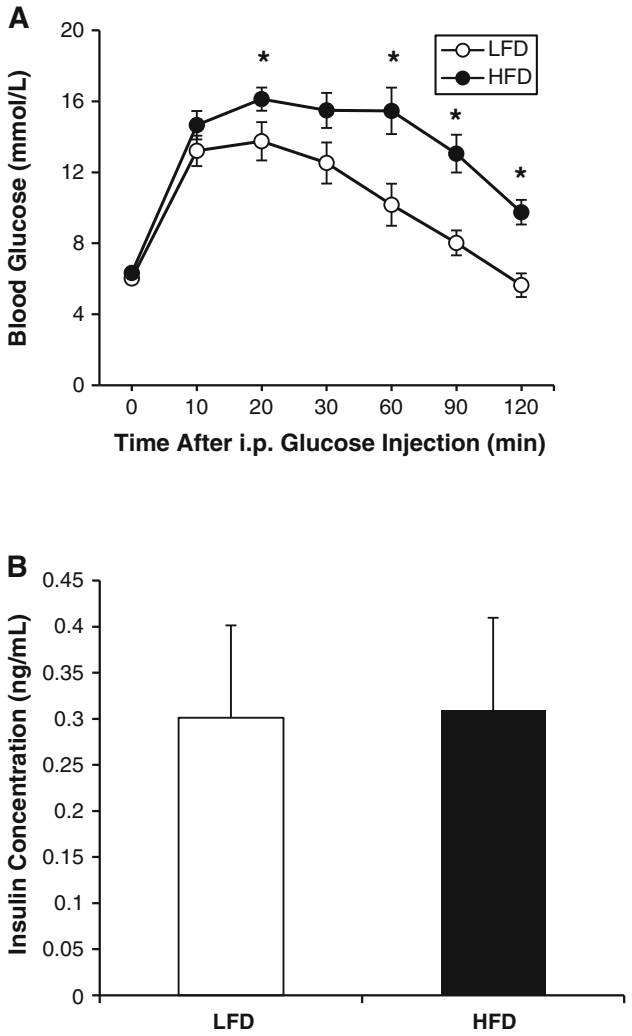

Fig. 2 Intraperitoneal glucose tolerance test and insulin concentrations. **a** Intraperitoneal glucose tolerance test (i.p. GTT) was performed after 28 weeks on diet. Following an overnight fast, glucose (1.5 g/kg body weight) was administered i.p. and tail vein blood glucose was measured at the times indicated (LFD $n = 16$, HFD $n = 24$). $^*P < 0.05$. **b** At the 10 min post-glucose time point serum insulin concentrations were measured (P = NS)

of mice on HFD was not different than that of mice on LFD 10 min after glucose administration (LFD 0.30 ± 0.01 ng/mL vs. HFD 0.31 ± 0.01 ng/mL) (Fig. 2b).

Assessment of tumor development and progression

To determine whether the HFD affected mammary gland tumor development and progression, the mice were palpated twice weekly and tumors were measured. The age of detection of the first mammary tumor in each mouse in both groups was plotted as a Kaplan–Meier "survival analysis." The median time of tumor-free survival was similar between LFD (243 days of age) and HFD (245 days of age) mice (Fig. 3a). Next, we examined the onset of second tumor formation since the HER2/Neu mice often develop multiple tumors. A similar analysis, namely the Kaplan–Meier "survival analysis" was performed. It should be noted that approximately twice as many mice on HFD developed a second tumor (61.1%) as those on LFD (36.4%), although this difference did not reach statistical significance. The median time of onset of a second tumor was not different, 325 days in LFD group vs. 337 days in HFD-fed mice (Fig. 3b). However, it can be seen from Fig. 3b that this may have been due to the smaller number of mice on LFD which developed second tumors combined with our predetermined 1 year maximum follow-up. Thus, if rather than examining the median time of second tumor appearance (which is close to the 365 day cut off), the time at which 66% of the mice remain free of a second tumor, i.e., when 1/3 of the mice developed a second tumor was determined, the HFD mice showed a decreased latency. Thus, the ages for the occurrence of a second tumor in 1/3

of the mice were 325 days for LFD and 274 days for HFD, 49 days earlier. One further measure of tumor promotion or development is the multiplicity of tumors. Thus, HER2/Neu mice often develop multiple tumors. Consistent with the above observation of an increased incidence of two tumors in the HFD group, the mean number of tumors/mouse was significantly greater in the HFD group (2.7 ± 0.23) vs. the LFD group (1.5 ± 0.25, $P < 0.01$) (Fig. 4).

To assess any differences in growth rates of tumors between the treatment groups, tumor growth rates were determined in three ways. First, we looked at the rate of tumor progression. This is the number of days between the first tumor measurement and the last tumor measurement (which is determined by the sacrifice cut-off point of 1.5 cm). There were no significant differences between the rates of tumor progression between the LFD- and HFD-fed mice (LFD 35 ± 7.4 days vs. HFD 38 ± 3.9 days) (Fig. 5a). Tumor growth rates in absolute terms (LFD 59.2 ± 13.3 mm^3/day vs. HFD 48.2 ± 6.3 mm^3/day) and percent daily relative growth rates, a more variable measure (LFD 204.8 ± 133.7%/day vs. HFD 67.5 ± 108.2%/day) were also not significantly different between the two groups (Fig. 5b, c).

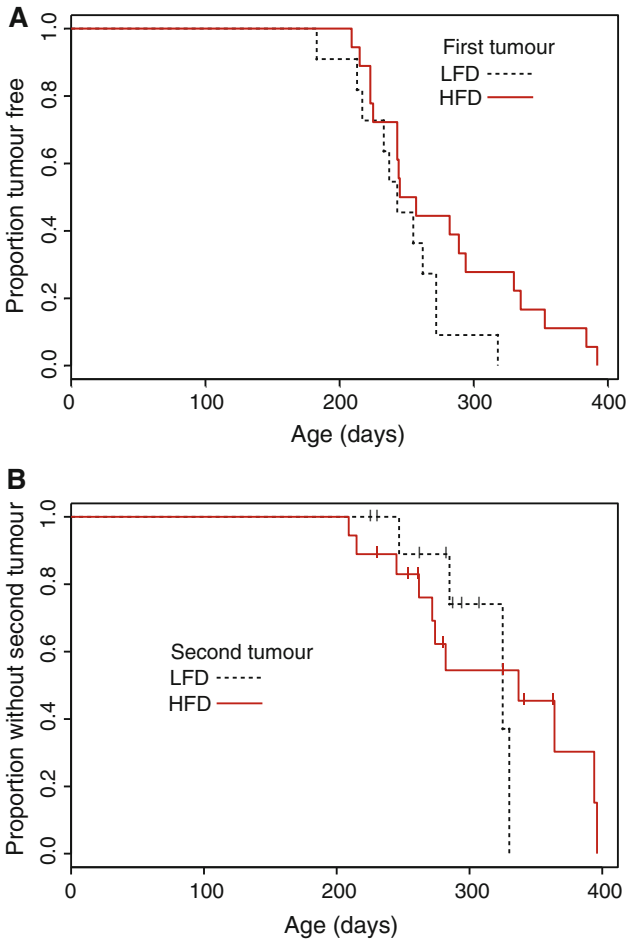

Fig. 3 Survival analysis of first and second tumor occurrence in HER2/Neu mice fed a low-fat (LFD) or high-fat (HFD) diet. **a** Kaplan–Meier survival analysis of the percent of mice which are tumor free. The median time of first tumor appearance was 243 days LFD and 245 days in HFD group. **b** Kaplan–Meier survival analysis of the percent of mice without two tumors, i.e., have 0 or 1 tumor. The time for 1/3 of the mice to develop a second tumor was 325 days in LFD and 274 days in HFD group. Data for mice reaching >365 days of age before the occurrence of the endpoint were censored

Assessment of insulin signaling in skeletal muscle and mammary tumors from HER2/Neu mice

It is well documented that changes in diet can influence insulin signaling in metabolic target tissues such as liver, muscle, and adipose cells. However, the effect if any, on mammary gland tumors has not been established. We investigated if the diet influenced signaling pathways in the mammary tumors as well as in the skeletal muscle from these mice in order to assess the extent of insulin resistance. Skeletal muscle lysates were prepared and there were no significant differences in the phosphorylation of Akt/PKB in the muscle between the LFD and HFD groups in either the basal state or 5 min after acute insulin stimulation, consistent with the absence of hyperinsulinemia and overt insulin resistance in this model (Fig. 6).

In the mammary tumor lysates, there were very low levels of phosphorylation of Akt/PKB and $p70^{S6K}$ in the basal state and no differences between the LFD and HFD groups (Fig. 7a). Similarly, maximum insulin-stimulated phosphorylation of Akt/PKB and $p70^{s6k}$ were similar in the mammary tumors from LFD and HFD groups (p-Akt/Akt; LFD 2.36 ± 0.04, HFD 3.22 ± 0.15: p-$p70^{s6k}$/$p70^{s6k}$, LFD 1.48 ± 0.08, HFD 1.82 ± 0.18) (Fig. 7). We noted that maximum insulin-stimulated phosphorylation was attained earlier in the HFD group (5 vs. 10 min). This "acceleration" of insulin signaling has been noted previously in some early obesity/insulin resistance models [32] (A. Marette, personal communication).

While the signaling proteins, Akt/PKB and $p70^{S6K}$ can be phosphorylated and activated downstream of insulin/IGF-1 receptor stimulation as well as by HER2/Neu, IRS proteins are more specifically tyrosine phosphorylated by insulin/IGF-1 receptors [33]. We thus examined the Tyr phosphorylation of IRS-1 and IRS-2 immunoprecipitated from the mammary tumor lysates. There were similar low levels of basal Tyr phosphorylation of IRS-1 and IRS-2 from tumors of both LFD and HFD mice. After in vivo insulin stimulation a pattern similar to Akt/PKB and $p70^{S6K}$ was observed. There was a significant increase in Tyr phosphorylation in both diet groups which was again, slightly greater in the HFD group for IRS-1 (LFD 1.91 ± 0.1, HFD 2.82 ± 0.2), but identical in the two

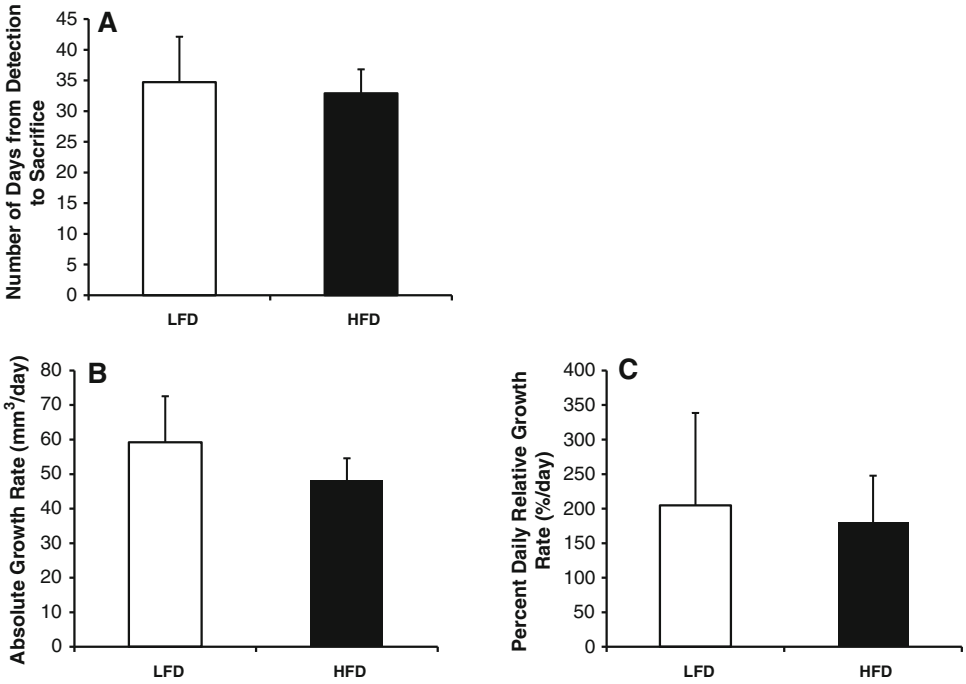

Fig. 4 Growth rates of mammary tumors in HER2/Neu mice fed a low-fat (LFD) or high-fat (HFD) diet. **a** The number of days from the detection of tumor to attainment of the sacrifice end-point size of 1.5 cm. **b** Absolute growth rate of tumors (mm^3/day) measured from tumor onset until sacrifice. Volumes are calculated from caliper measurements taken twice a week. c Percent daily relative growth rate (% change in volume/day). All values are mean ± SE (LFD $n = 10$, HFD $n = 18$; P = NS)

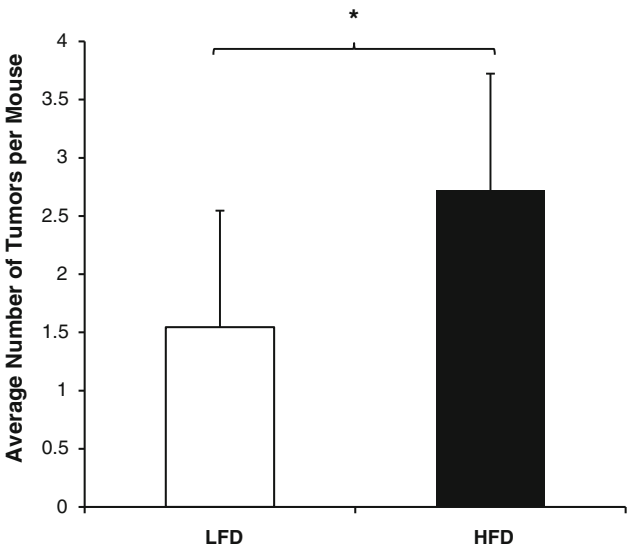

Fig. 5 Average number of tumors that developed per mouse in HER2/Neu mice fed a low-fat (LFD) or high-fat (HFD) diet. *$P < 0.01$

groups for IRS-2 (LFD 5.55 ± 0.3, HFD 5.77 ± 0.2) (Fig. 8).

Assessment of cellular proliferation in HER2/Neu mice

The lack of differences in basal and maximum insulin stimulation of growth signaling proteins, circulating insulin concentrations, and tumor growth rates in the two diet groups all suggested that the HFD did not affect tumor cell proliferation. To further test this notion, we studied Ki67; a marker of cellular proliferation. Positively stained cells were quantified using Aperio Image Scope software and expressed as the Ki67 Labeling Index. There was no difference in the percentage of positively stained nuclei in tumors from the LFD- and the HFD-treated groups of mice (LFD 5.55 ± 1.31% vs. HFD 5.77 ± 0.95%) (Fig. 9a, b).

Discussion

In this study, modest obesity was generated in the HER2/Neu mouse model of breast cancer without evidence of significant hyperinsulinemia or insulin resistance. It should be noted that these HER2/Neu mice are bred on a FVB background. Although some strains of mice develop insulin resistance on a 45% fat diet, a higher fat content (65% of calories) is usually required for insulin resistance to develop in the FVB/N strain (C. R. Kahn, personal communication). Previous studies using this strain demonstrated that it is somewhat obesity resistant when fed a calorie dense diet [34]. When fed the HFD we found that the HER2/Neu mice had an increased body weight and body fat content and a mild impairment in glucose tolerance, but there was no elevation in plasma insulin concentrations. We determined insulin concentrations at 10 min post-glucose administration in an attempt to detect small differences, since early and mild insulin resistance may be accompanied by glucose-stimulated hyperinsulinemia which is not seen in the basal state. Since there may be variability in the timing of peak insulin levels after glucose administration in the mice, we cannot completely

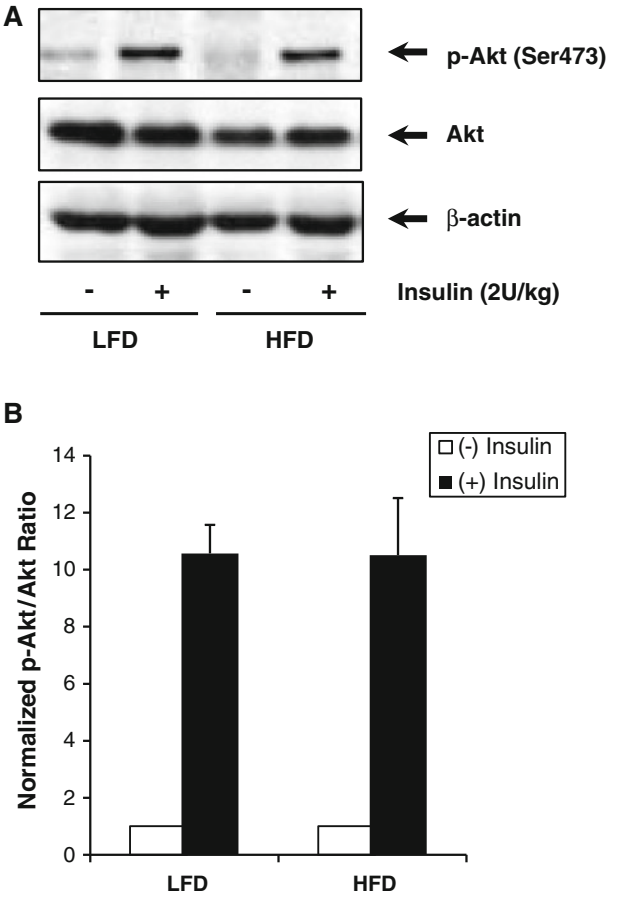

Fig. 6 Insulin stimulated phosphorylation of Akt in skeletal muscle in HER2/Neu mice fed a low-fat (LFD) or high-fat (HFD) diet. Insulin (2 U/kg) (+) or saline (−) was injected i.p. into anesthetized mice. Hindlimb (gastrocnemius) muscle was removed after 5 min and immediately frozen to −80°C. The tissues were thawed, homogenized, and lysates prepared in the presence of protease and phosphatase inhibitors. Equal amount of protein (30 μg) were separated by SDS-PAGE, transferred to membranes and immunoblotting performed as described in "Materials and methods." After blotting for pSer473 Akt, blots were stripped and reprobed for total Akt and β-actin as loading control. **a** Representative immunoblot. **b** Densitometric values (arbitrary units) are mean ± SE ($n = 3$ for each condition) and phospho-Akt corrected for total Akt in each experiment

exclude the possibility that a small increase could have occurred at 30 or 60 min post-glucose. At the same time, it is interesting to note the mild impairment of glucose tolerance observed. Combined with the lack of hyperinsulinemia and the absence of insulin resistance in the skeletal muscle, the data suggest a potential impairment of insulin secretion induced by the HFD. A somewhat similar observation was made in iNOS$^{-/-}$ mice fed a high-fat diet [35]. Thus, these iNOS knockout mice were protected from HFD-induced insulin resistance maintained normal serum insulin concentrations yet manifested impaired glucose tolerance. It is possible that subtle effects on β-cell function caused by the HFD are responsible.

With respect to the development and growth of mammary gland tumors, while the latency period of the appearance of the first tumor was not altered by the HFD, latency of the appearance of a second tumor appeared to be decreased. It was noted that second tumors in these mice occurred on average, between 11 and 12 months of age. Thus, due to our pre-specified maximum 1 year follow-up and the consequent censoring of mice surviving over 12 months, the median times of appearance of a second tumor were not felt to accurately reflect latency. However, comparing latencies at an earlier time, i.e., when 1/3 of animals developed second tumors, revealed a substantially shortened latency in the HFD group, 274 vs. 325 days in the LFD controls. In contrast, the growth rates of the mammary tumors were not altered by the HFD using three different measures; namely the absolute growth rate/day, the relative growth rate (% of initial size/day), and the time from detection to the pre-determined endpoint of 1.5 cm. Thus, the accelerated occurrence of a second tumor did not appeared due to more rapid growth of small undetectable tumors. This was confirmed by a lack of evidence of a difference in cellular proliferation in tumors between the LFD- and HFD-fed mice determined by Ki67 staining. The decreased latency observed in the occurrence of a second tumor; however, was accompanied by a twofold increase in the average number of tumors per mouse in the HFD-fed mice relative to the controls.

Together, these data suggest that it is the initiation of tumor formation that is being altered by the HFD rather than an alteration in tumor growth and proliferation. This tumor promoting effect appeared to be time dependent as it was not observed in the case of the first tumor. The diets were initiated at 4 weeks of age in these mice, which was 7 to 8 months before half the mice developed the first tumor and 10–11 months before half the mice developed a second tumor. This allowed, on average, about 3–4 months of additional exposure to the diet. Thus, there may either be a critical window of time or duration of exposure for the HFD to exert its effect. One other possibility which could contribute to these data is that the genetically predisposed breast cancer model we used is associated with a relatively weak HFD tumor promoting effect, i.e., in comparison to the HER2/Neu over-expression. Further experiments are required to sort out these factors.

Women in westernized countries who consume a high fat, calorie rich diet and are relatively sedentary, have an earlier onset of puberty [36]. A number of studies have also demonstrated that the earlier the onset of menarche, the earlier the onset of breast development as a result of the increase in serum estradiol levels which then persist into early adulthood [37, 38]. Thus, the onset of menarche before the age of 13 is associated with an increased risk of breast cancer [36–40]. However, these associations are

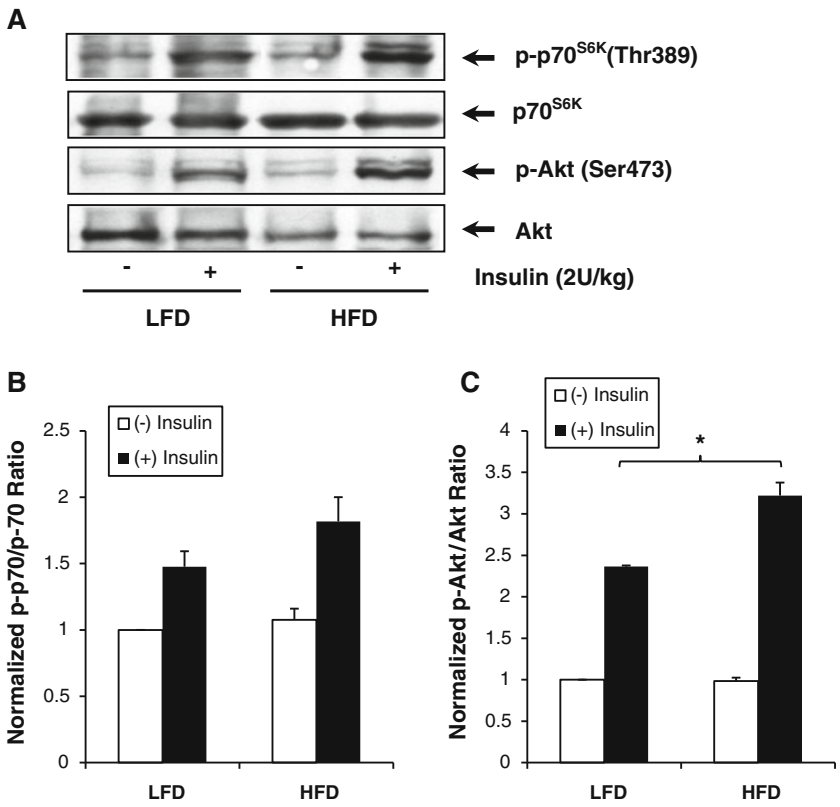

Fig. 7 Insulin-stimulated phosphorylation of Akt and $p70^{s6k}$ in mammary tumors in HER2/Neu mice fed a low-fat (LFD) or high-fat (HFD) diet. Insulin (+) or saline (−) was administered as in Fig. 6 and mammary tumors dissected free from stromal tissue, excised and immediately frozen to −80°C. Tissues were thawed, homogenized, and lysates prepared as described in "Materials and methods." Equal aliquots of proteins (30 μg) were separated by SDS-PAGE, transferred to membranes and immunoblotted with the antibodies indicated. **a** Representative immunoblot. **b**, **c** Densitometric values (arbitrary units) are mean ± SE ($n = 3$ for each condition) for phospho-$p70^{s6k}$ corrected for total $p70^{s6k}$ (**b**), and phospho-Akt corrected for total Akt (**c**)

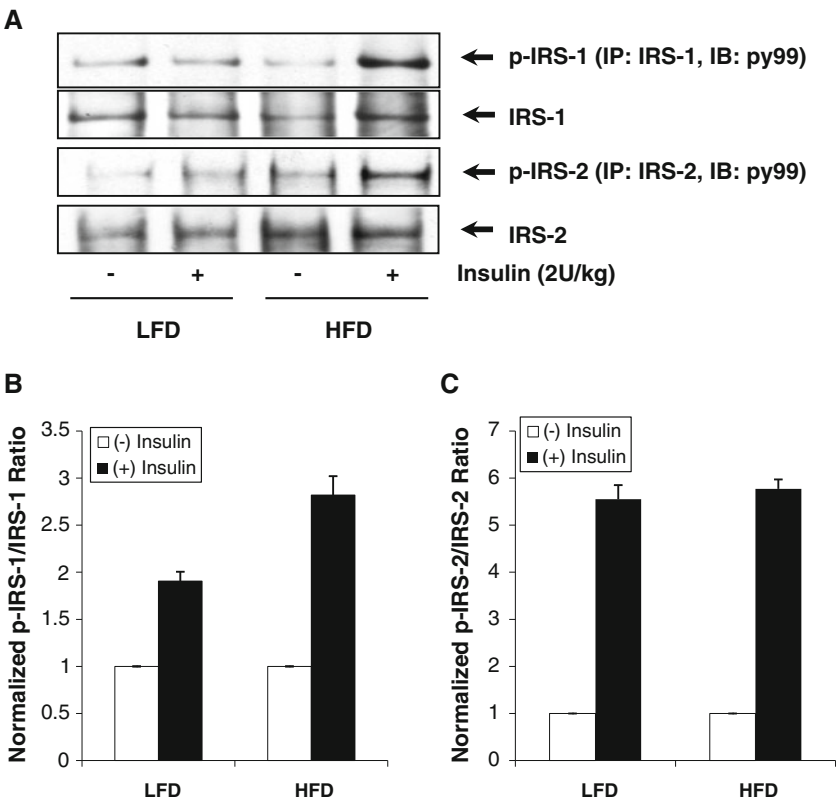

Fig. 8 Insulin-stimulated phosphorylation of IRS-1 and IRS-2 in mammary tumors in HER2/Neu mice fed a low-fat (LFD) or high-fat (HFD) diet. Insulin (+) or saline (−) was administered as in Fig. 6 and mammary tumor lysates prepared as in Fig. 7. IRS-1 and IRS-2 were immunoprecipitated from equal amount of protein (500 μg) as described in "Materials and methods" washed and proteins separated by SDS-PAGE. After transfer to membranes immunoblotting was performed with anti-phosphotyrosine (pY99) antibodies. Membranes were stripped and reprobed with anti-IRS-1 and anti-IRS-2 antibodies. **a** Representative immunoblot. **b**, **c** Densitometric values (arbitrary units) are mean ± SE ($n = 3$ for each condition). Tyr phosphorylated IRS-1 was corrected for total IRS-1 (**b**) and Tyr phosphorylated IRS-2 for total IRS-2 (c) in each experiment

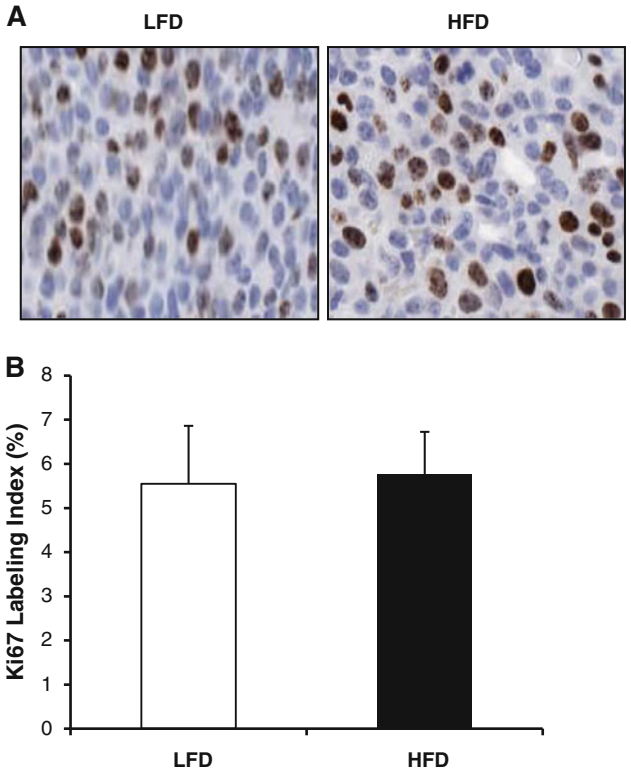

Fig. 9 Ki67 staining in mammary tumors from HER2/Neu mice fed a low-fat (LFD) or high-fat (HFD) diet. Mammary tumors were excised, fixed in 10% formalin, paraffin-embedded, sectioned and prepared as described in "Materials and methods," stained with anti-Ki67 antibody and counterstained with hematoxylin. After incubation with biotinylated secondary antibody, extent of Ki67 staining was visualized with ABC-HRP diaminobenzidine. **a** Representative tumor sections from LFD- and HFD-fed mice. **b** Ki67 labeling index (mean ± SD) was determined with Aperio Image scope pixel count algorithm software for seven separate tumors from different mice for each condition

unlikely to contribute to the findings here since it has been suggested that in the face of HER2/Neu overexpression, alterations in estrogen levels may not significantly influence tumors [41].

It is widely agreed that the western diet with its high saturated fatty acid content and high *n*-6/*n*-3 ratio of polyunsaturated fatty acids promotes obesity in individuals who are genetically predisposed and obesity is a risk factor for postmenopausal breast cancer [40, 42]. To study the effects of HFD and obesity, Cleary et al. used two mouse models of breast cancer. In MMTV-TGF-α mice with HFD-induced obesity the latency of mammary tumors was significantly shortened [43]. In contrast, when the same protocol was tested in the MMTV-HER2/Neu breast cancer model, mammary tumor latency, incidence, metastasis, and burden were similar in all groups [44]. It was concluded that obesity is not a risk factor for development of ER negative breast cancer, i.e., in the HER2/Neu model. This was based on the concept that hyperinsulinemia in the obese and HFD-fed state stimulates mammary carcinogenesis indirectly by elevating circulating levels of estrogen [45]. However, in human subjects the elevated risk associated with hyperinsulinemia was observed for both ER+ and ER− tumors [18] and in cell culture models insulin stimulates cell proliferation in both ER+ and ER− breast cancer cell lines [46]. Furthermore, there are some important points to note in the protocol and interpretation of the data of Cleary et al. In the MMTV-HER2/Neu study, the HFD-fed mice were divided into three groups at an early (19 weeks) age based on body weight. These were classified as obesity resistant, overweight, and obesity prone. While age at tumor detection, i.e., latency was not significantly decreased by obesity, a trend was observed and it is not clear whether this sub-grouping obscured a significant difference. Thus, the mean age of tumor detection in the chow-fed mice was 67.7 ± 4.9 weeks and those of the three HFD-fed groups were 63.8 ± 6.7 weeks, 61.7 ± 3.5 weeks, and 64.8 ± 5.9 weeks, all earlier. Similarly, the mean ages at which tumors reached 20 mm in size was 80.1 ± 2.6 weeks for chow fed and 75.7 ± 4.1 weeks, 72.7 ± 3.2 weeks, and 69.4 ± 4.9 weeks for HFD-fed groups, again all earlier. The incidence of mammary tumors showed a similar trend, chow fed 45% and 59, 56, and 67% in the three HFD groups. The number of tumors/mouse was not different, but in contrast to the calculation used in the present study in which this outcome is expressed for the entire population of mice. Cleary et al. used a denominator restricted to mice with at least one tumor, eliminating those in which tumors were not detected. This explains the discrepancy in tumor incidence we found in the present study, which was greater in the HFD group. While these trends in the HER2/neu mice in Cleary's studies were not as marked as those observed in the MMTV-TGF-α model, it is noteworthy that the latter model was bred on the C57Bl/6 background, while the MMTV-HER2/Neu was on the FVB background, similar to ours. In addition, the mice used by Cleary et al. were heterozygous for the transgene, while those used here were homozygotes. Finally, the HFD used by Cleary was 32.5% fat while that used in our study was 45%. Taken together, the data suggest that HFD does have an effect on latency and tumor development, but the magnitude of this effect depends on various factors, namely the model used, the background strain, the proportion of fat and perhaps, the fatty acid (FA) composition of the diet.

The potential contribution of the FA composition of the HFD is suggested by some in vitro data. In cell culture models, the saturated FA, palmitate, induces apoptosis, while the monounsaturated FA, oleate, can stimulate proliferation [47]. However, studies in women have not supported a significant effect [48]. This may be because the difference in dietary FA composition are not large enough

in free living human subjects to observe small effects. The FA composition of the diet used by Cleary was largely palmitic acid and stearic acid (approximately 20% of the 32.5%, in other words, 61% of the fat), similar to our HFD which was 86.7% lard oil. Previous studies have implicated some polyunsaturated fatty acids as tumor promoters in rodent models of mammary carcinogenesis [22, 23, 25, and reviewed in 49]. However, it appears that high saturated fat will also promote mammary carcinogenesis as long as it is accompanied by a small amount of polyunsaturated *n*-3 fatty acid [22]. On the other hand, tumor growth rates were not affected by altering the dietary fat content [23]. These data in DMBA-induced mammary tumors in rats are consistent with our results.

Insulin resistance and hyperinsulinemia have been postulated to contribute to tumor formation and growth via increased levels of estrogen and/or IGF-1 [50]. However, in the absence of hyperinsulinemia, it is unlikely that these factors contributed to our observations. Indeed, Cleary et al. found that even in the obesity resistant MMTV-TGF-α HFD-fed mice, the HFD decreased tumor latency without altering body weight [43]. The body fat content; however, was modestly increased. Furthermore, the same diet in the MMTV-HER2/Neu model did not cause any change in IGF-1 level and these tumors do not express ER [44]. Thus, our data indicating the absence of significant insulin resistance in skeletal muscle and the absence of hyperinsulinemia, combined with the results reported by Cleary et al. support the concept that HFD manifests a tumor promoting effect independent of overt insulin resistance.

Increases in adipose tissue mass, even in the presence of similar total body weight, may be associated with alterations in the synthesis and secretion of various "adipokines." Two circulating factors which have been suggested to influence breast cancer are the adipokines, leptin, and adiponectin. While leptin stimulates breast cancer cell growth in vitro [51], the results of associations with increased risk in human subjects are mixed and were not significant when corrected for other factors [52]. The other adipokine, adiponectin, is decreased in obesity and insulin resistance [53]. Recent clinical studies have shown that low serum levels of adiponectin are associated with an increased risk of breast cancer in postmenopausal women [54]. Wang et al. demonstrated that the inhibitory role of adiponectin on MDA-MB-231 cell growth might be attributed to its suppressive effects on the GSK-3β/β-catenin signaling pathway [55]. Their in vivo studies showed that both supplementation of recombinant adiponectin and adenovirus-mediated over-expression of this adipokine substantially reduced the mammary tumorigenesis of MDA-MB-231 cells in female nude mice. These data support a potential role of adiponectin as a negative regulator of breast cancer growth [56]. Due to inadequate volumes of blood and the lack of evidence of insulin resistance we did not measure circulating adiponectin and so cannot rule out a potential contribution.

Finally, tumor cells are known to exhibit an altered metabolism characterized by increased glucose uptake, elevated glycolysis and increased de novo FA synthesis [57–60]. The enzymes fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC), responsible for de novo FA synthesis are up-regulated and inhibition or knockdown by siRNA of FAS or ACC has been demonstrated to inhibit prostate and breast cancer growth [61, 62]. Of relevance, addition of palmitic acid was shown to circumvent the down-regulation of FAS and ACC and overcome the defect in FA synthesis. These findings raise the hypothesis that in some circumstances the dietary supply of FA may influence breast cancer growth and/or development. This offers an explanation of a dietary effect independent of obesity and insulin resistance.

In summary, the data in this study show that feeding a HFD to breast cancer-prone mice promotes tumor development in an apparent time-dependent manner independent of significant insulin resistance and overt hyperinsulinemia. However, tumor growth in this mild obesity phenotype is not affected. The precise mechanism of shortened tumor latency may involve an effect of increased adipose tissue mass, e.g., altered adipokines/cytokines, or more likely, a direct effect of increased exposure of mammary epithelial cells to FA. Further experiments are required to define the contribution of each of these mechanism.

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