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Pubertally Initiated High-Fat Diet Promotes Mammary Tumorigenesis in Obesity-Prone FVB Mice Similarly to Obesity-Resistant BALB/c Mice[1]

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Abstract

Premenopausal breast cancer is associated with increased animal fat consumption among normal-weight but not overweight women. Our previous findings in obesity-resistant BALB/c mice showed that a diet high in saturated animal fat (HFD) promotes mammary tumorigenesis in both DMBA carcinogenesis and *Trp53-null* transplant models. Having made these observations in BALB/c mice, which have very modest HFD weight gain, we determined the effects of HFD in FVB mice, which gain significant weight on HFD. Three-week-old FVB mice fed a low-fat diet or HFD were subjected to 7,12-dimethylbenz[*a*]anthracene-induced carcinogenesis. Like BALB/c mice, HFD promoted mammary tumorigenesis. Development of tumors largely occurred prior to mice becoming obese, indicating the role of animal-derived HFD rather than resulting obesity in tumor promotion. Also similar to BALB/c mice, early-occurring adenosquamous mammary tumors were abundant among HFD-fed FVB mice. Tumors from HFD mice also had increased intra-tumor M2 macrophages. Prior to tumor development, HFD accelerated normal mammary gland development and increased mammary M2 macrophages, similarly to BALB/c mice. The promotional effects of puberty-initiated HFD on carcinogen-induced mammary cancer are thus largely weight gain-independent. Like BALB/c mice, HFD promoted adenosquamous tumors, suggesting a role for early age HFD in promoting this subtype of triple negative mammary cancer. M2 macrophage recruitment was common to both mouse strains. We speculate that a similar effect of HFD on immune function may contribute to epidemiological findings of increased breast cancer risk in young, premenopausal, normal-weight women who consume a diet high in saturated animal fat.

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Introduction

Many case–control studies identify a positive association between fat intake and risk of breast cancer, but recent meta-analyses and pooled analyses of cohort studies fail to support this association [1]. These studies are based on the diet reported from a single time point, and neither account for changes in diet over time nor the potential differential effects of a diet with life stage. One compelling study based on the Nurses' Study II cohort examined adolescent diet through a food frequency questionnaire and found an association between total fat intake and breast cancer risk, but this association was not evident with individual types of fat [2]. Consumption of a high-fat diet (HFD) is also associated with increased BMI and obesity

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[3]. Therefore, distinguishing between the effects HFD versus weight gain/obesity is challenging. Recent updates on the Nurses' Health Study II cohort identify a positive association of high red meat intake in early adulthood [4] and adolescence [5] with premenopausal breast cancer risk, and also identify an association of high animal fat intake in early adulthood with premenopausal breast cancer risk [6]. Strikingly, the increase in premenopausal breast cancer risk by high animal fat was only significant in normal-weight women. These latter findings are in accord with our recent findings in a DMBA-induced tumorigenesis model in obesity-resistant BALB/c mice, where we found that a lifelong HFD initiated at puberty [7] or a puberty-restricted HFD [8] promotes mammary cancer development. Most recently, we found that both puberty- and adult-restricted HFD promoted mammary cancer development in *Trp53-null* transplanted BALB/c mice [9]. Having made these observations in BALB/c mice, which have very modest weight gain on HFD, we sought to determine the effects of HFD-induced weight gain in the FVB mouse strain, which is reported to gain significant weight on HFD [10].

Material and Methods

Mice and Diets

Three-week-old female FVB mice were purchased from Charles River Laboratories (Portage, MI). After 1 day of acclimatization, they were assigned to either a low fat diet (LFD) or HFD. LFD (Research Diets, New Brunswick, NJ; D11012202) had 10% calories from fat; HFD (Research Diets; D11012204) had 60% calories from fat. The additional source of fat in the HFD is lard (Table 1). Mice were maintained on their respective diets until the end of the experiments. Food and water were provided ad libitum, and mice were housed in standard facilities with a 12:12 h light–dark cycle. Body weight was measured twice weekly. Sexual maturity was monitored by daily observation for vaginal opening between post-natal day (PND) 25 and PND 35. For assessment of the temporal effects of diet, mice were sacrificed after 1, 2, 3, or 4 weeks on either LFD or HFD. All mice were sacrificed at estrus. 5-bromo-2′-deoxyuridine (BrdU) (70 μg/g body weight; Sigma-Aldrich, St. Louis, MO) was administered via intraperitoneal injection two hours prior to sacrifice for analysis of cellular proliferation. Plasma was obtained via cardiac puncture. All animal experimentation was conducted in accord with accepted standards of humane animal care and approved by the All University Committee on Animal Use and Care at Michigan State University.

Table 1. Diet Composition.

Ingredients (g/100 g)		Low Fat	High Fat
Fat	Corn Oil	2.369	16.1498
	Lard	1.8957	31.6537
Carbohydrate	Corn Starch	54.407	8.888
	Maltodextrin	11.848	16.1498
Protein	Casein	18.987	25.8397
	L-cysteine	0.2843	0.3876
Fiber	Cellulose	4.7393	6.4599
Vitamins	Vitamin Mix V10001	0.9479	1.2919
	Choline Bitartrate	0.1896	0.2584
Minerals	Mineral Mix S10026	0.9479	0.1286
	DiCalcium Phosphate	1.2322	1.6795
	Calcium Carbonate	0.5213	0.7106
	Potassium Citrate, 1 H_20	1.5639	2.1318
Energy kcal density/g		3.8	5.2
% kcal	Fat	10	60
	Carbohydrate	70	20
	Protein	20	20

Tumorigenesis

3-week-old mice were randomly assigned to LFD and HFD groups (LFD, n = 60; HFD, n = 80). Beginning at 5 weeks of age, mice in both groups were treated by oral gavage once a week for four weeks with 7,12-dimethylbenz[a]anthracene (DMBA) dissolved in vegetable oil (50 mg/kg body weight/mouse). At 13 weeks of age (10 weeks on diet) and at 19 weeks of age (16 weeks on diet), 3 to 5 mice from each group were randomly selected and sacrificed at estrus to examine the early effects of HFD prior to the development of palpable tumors. The remaining mice were palpated twice weekly for tumor development until the end of the experiment at 52 weeks of age. Tumors were harvested at 1 cm in diameter. At termination, portions of tumors and mammary tissues were either formalin-fixed and processed as whole mounts [11], or paraffin-embedded for hematoxylin and eosin staining and immunohistochemistry [12]. Whole mount preparations of glands and hematoxylin and eosin sections were scored for overall morphology, the presence of hyperplasia, and neoplasia [13]. All lesions and tumors were reviewed and classified, as previously described [14].

Whole Mount Analysis

Formalin-fixed inguinal mammary glands were assessed for longitudinal ductal growth measured by the distance between the most distal terminal duct and the lymph node. Terminal end buds (TEBs) were defined as enlarged multilayered ductal tips with a diameter greater than 100 μm that were surrounded by adipocytes and located in the periphery of the gland.

Immunohistochemistry

BrdU was detected using a mouse monoclonal antibody (1:100; Cat #: RPN202; GE Healthcare, Little Chalfont, Buckinghamshire, UK) with incubation at room temperature for 2 hours followed by Alexa 488–labeled goat anti-mouse secondary Ab (1:200; Invitrogen Molecular Probes, Grand Island, NY). CD31 was detected with rabbit polyclonal anti-CD31 (1:50; Cat #: AP15436PU-N; Acris Antibodies, Inc., San Diego, CA) with incubation at room temperature for 2 hours followed by secondary swine anti-rabbit Ab (DAKO, Carpinteria, CA), and ABC reagent (Cat #: PK-7100; Vector Laboratories, Inc., Burlingame, CA), as described previously [7]. Double staining of F4/80 and Arg1 has been described previously [7] using monoclonal rat anti-F4/80 (1:75; Cat #: MCA497R; AbD Serotec, Raleigh, NC) and goat anti-Arg1 (1:200; Cat #: sc-18,354; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). As described previously [7], estrogen receptor (ER) was detected with mouse anti-ERα (1:10; Cat #: NCL-ER-6 F11; Novocastra Laboratories Ltd., Novocastra Laboratories, Ltd., Newcastle upon Tyne, UK) and progesterone receptor (PR) was detected with rabbit anti-PR (1:200; Cat #: A0092; DAKO). Images were captured with a Nikon Eclipse TE2000-U fluorescence microscope (Nikon, Inc., Melville, NY) using a 40x objective lens. At least 1000 cells and 3 sections per animal were analyzed. For CD31 analysis, the images were overlaid with grids containing 240 squares (324 μm^2/square). Blood vessel density is expressed as the percentage of CD31-positive squares. Macrophage density is expressed as number of macrophages per tumor image. Tumors were considered to be ERα positive (ER+) if

10% or more of the total cells counted were ER+ [15]. Mammary tissue sections stained for macrophages, cellular proliferation, and blood vessel density were analyzed by mammary gland epithelial structure: small ducts, large ducts, TEBs, or hyperplastic foci as previously described [7].

Metabolic Parameters

Mice were fasted for 4 hours prior to blood collection and sacrifice. Plasma glucose and insulin levels were sampled via cardiac puncture and coagulation prevented with EDTA. Plasma glucose levels were determined by OneTouch UltraMini (Lifescan, Milpitas, CA), and insulin levels were determined with the rat/mouse insulin ELISA kit (Millipore, Billerica, MA), according to the manufacturer's instructions.

Statistical Analyses

Results are shown as mean ± standard deviation (SD) for body weight, and mean ± standard error of the mean (SEM) for immunohistochemistry analyses. Differences were considered significant at $P < .05$ using Student's t-test. Mammary tumor-free and overall survival were determined from Kaplan–Meier plots by log-rank tests. Tumor incidence was analyzed by the Chi-square test.

Results

Tumor Development and Characteristics

Over a time course of 52 weeks, only 4% of mice (2 mice; n = 50) fed LFD developed mammary tumors, compared to 15.7% of mice fed HFD (11 mice; n = 70) (Figure 1*A*). The majority of the tumors were ER and PR negative (2/2 LFD tumors; 11/12 HFD tumors) (Table 2). Of the two tumors that developed in LFD-fed mice, one was of adenosquamous and one was of epithelial histopathology. Half of the HFD tumors were of epithelial histopathology (6/12; glandular, papillary, cribriform, solid), while the remainder were of adenosquamous (4/12) and spindle cell (2/12) histopathologies (Table 2). HFD also promoted the development of tumors in other organ systems, producing skin and liver tumors and lymphomas that resulted in significantly worse overall survival (Figure 1*B*).

Table 2. Properties of Tumors.

Tumor	Diet	Latency (Weeks)	Histopathology	Receptor Status
M11	HFD	17	Epithelial	ER–/PR-
M21	HFD	19	Adenosquamous	ER–/PR-
M45	HFD	19	Adenosquamous	ER–/PR-
M20	HFD	20	Adenosquamous	ER–/PR-
M27	HFD	22	Epithelial	ER–/PR-
M72	HFD	24	Epithelial	ER–/PR-
M26	HFD	25	Adenosquamous	ER–/PR-
M37	HFD	28	Spindle cell	ER–/PR-
M37	HFD	28	Epithelial	ER–/PR-
M47	HFD	28	Spindle cell	ER+/PR+
M80	HFD	34	Epithelial	ER–/PR-
M84	HFD	47	Epithelial	ER–/PR-
M46	LFD	28	Adenosquamous	ER–/PR-
M34	LFD	42	Epithelial	ER–/PR-

The tumors that developed in mice fed HFD had a mean latency of 25.9 ± 2.4 weeks compared to 35 weeks for LFD tumors. Additionally, there was a subset of HFD tumors that developed before the earliest tumor in LFD-fed mice (28 weeks of age) (Table 2). This early subset had a predominance of adenosquamous tumors (4/6). The early tumors had a mean latency of 20.9 ± 1.1 weeks and late developing HFD tumors had a mean latency of 33 ± 3.7 weeks.

We previously observed that HFD promoted tumor development in BALB/c mice through increased proliferation, angiogenesis, and recruitment of alternatively activated M2 macrophages [7,8]. To determine the basis for HFD promotion of tumorigenesis in this study, tumors were analyzed for proliferation, angiogenesis and macrophage recruitment. Tumors that developed in FVB mice fed LFD and HFD had similar levels of proliferation (HFD = 8.3 ± 1.0%, LFD = 8.7 ± 2.4%), angiogenesis (HFD = 16.2 ± 1.2%, LFD = 19.1 ± 4.6%), and similar levels of total macrophages within the tumors (HFD = 31.8 ± 5.7, LFD 31 ± 18.4. However, there

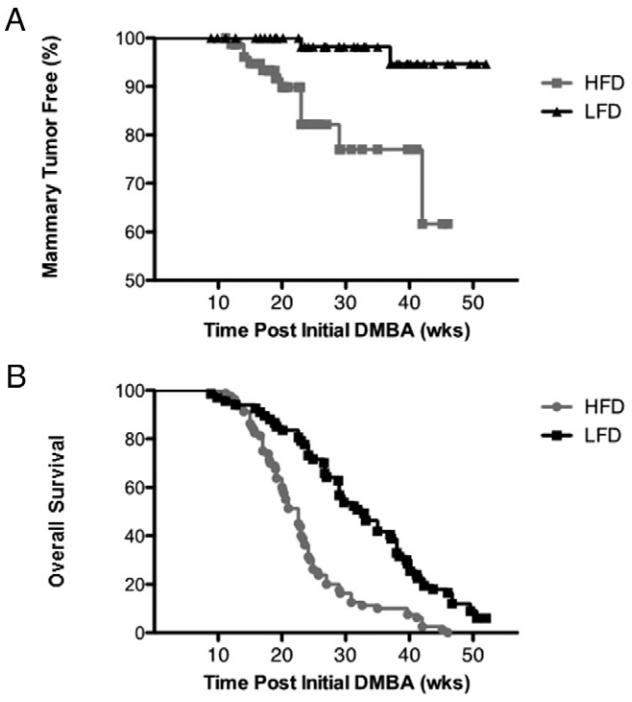

Figure 1. High-fat diet promotes DMBA-induced mammary tumorigenesis. (A) A Kaplan–Meier plot indicated that the number of tumor-free mammary glands decreased over time to a greater extent on high-fat diet (n = 70) than on low fat diet (n = 50). (B) A Kaplan–Meier plot indicated that high-fat diet diminished overall survival.

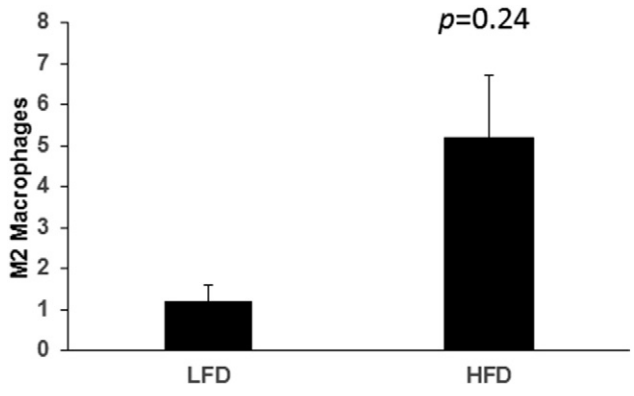

Figure 2. High-fat diet elicited a trend toward increased intra-tumor M2 macrophages as measured by F4/80-Arg1 double staining ($P = .24$). LFD (n = 2), HFD (n = 8).

was a trend toward increased numbers of intra-tumor M2 macrophages in tumors that developed on HFD (5.2 ± 1.5) compared to LFD (1.2 ± 0.4) (P = .24) (Figure 2).

HFD Promotes Pubertal Ductal Development and Epithelial Cell Proliferation

Having established that HFD promotes carcinogen-induced tumor development, we sought to examine early HFD effects on mammary gland development. After only 1 week on diet, HFD increased the number of TEBs, the highly proliferative structures found at the tips of growing ducts during puberty (Figure 3*A*). After 2 weeks on diet, HFD-fed mice had enhanced ductal elongation (Figure 3, *B* and *C*). By 3 weeks on diet, ductal growth in mice fed LFD and HFD were indistinguishable, with similar levels of ductal elongation and number of TEBs (Figure 3, *A* and *B*). By 4 weeks on diet, distal ductal elongation reached the limit of the inguinal fat pad for both LFD- and HFD-fed mice, and TEBs were similarly reduced in number in both groups.

Consistent with the pattern of enhanced ductal elongation, ductal proliferation was significantly increased in HFD-fed mice at the height of pubertal growth after 2 weeks on diet (Figure 4). The accelerated mammary gland development in HFD-fed mice was not the result of early onset of puberty, as there was no significant difference in the mean age of vaginal opening in LFD and HFD-fed mice (29.3 ± 1.8 days and 29.6 ± 2.3 days, respectively).

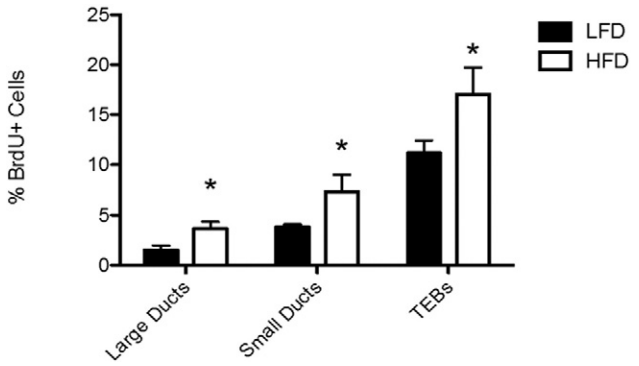

Figure 4. Mice fed high-fat diet for 2 weeks exhibited increased cellular proliferation in normal epithelial structures, as measured by 5-bromo-2′-deoxyuridine (BrdU) incorporation (*, $P < .05$). LFD (n = 5), HFD (n = 5).

Macrophages participate in the remodeling of the mammary gland during pubertal development [16]. The majority of macrophages for both diets were alternatively activated M2 macrophages (i.e., Arginase

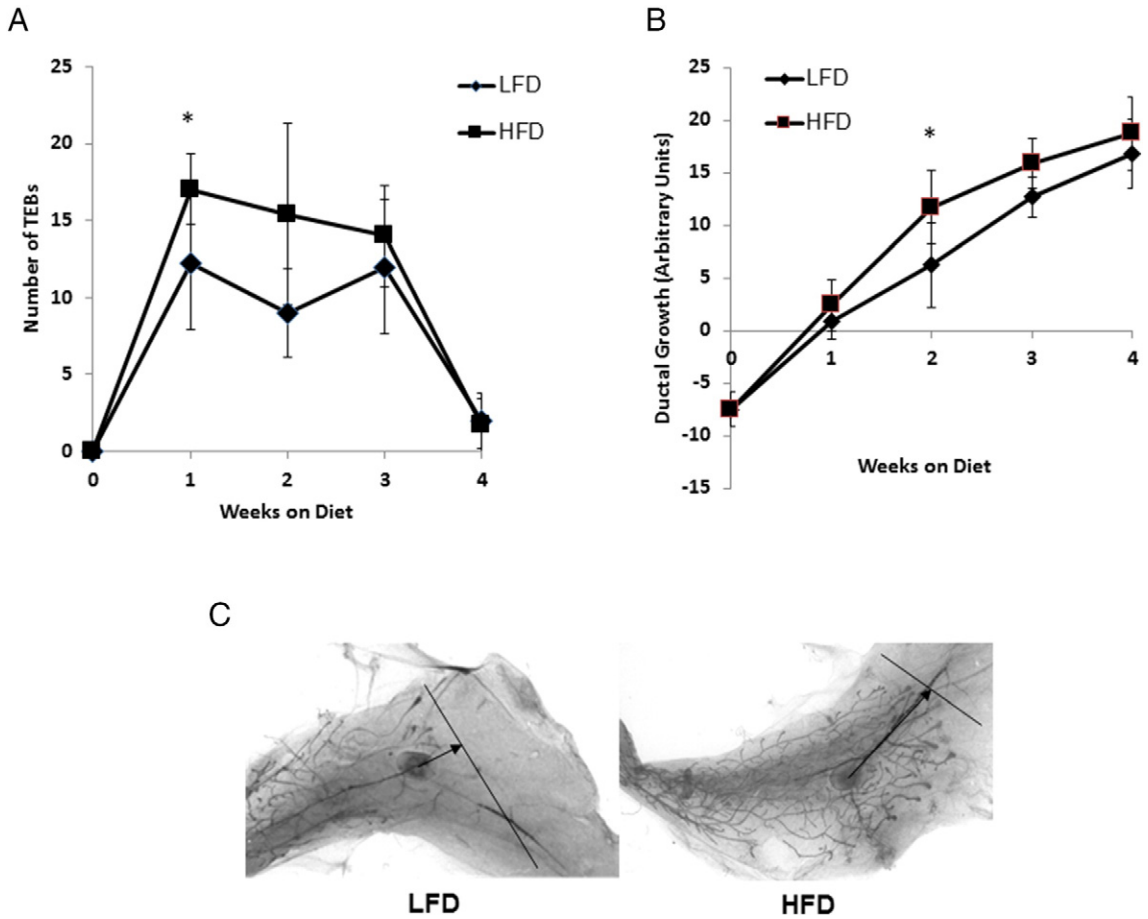

Figure 3. High-fat diet promotes pubertal ductal development and epithelial cell proliferation. (A) High-fat diet increased the number of terminal end buds (TEBs) (*, $P < .05$). LFD (n = 5), HFD (n = 5). (B) High-fat diet enhanced ductal elongation (*, $P < .05$). LFD (n = 5 for 0, 1, 2, and 3 weeks on diet; n = 15 for 4 weeks on diet); HFD (n = 5 for 0, 1, 2, and 3 weeks on diet; n = 15 for 4 weeks on diet). (C) A representative whole mount at 2 weeks on diet shows high-fat diet enhancement of ductal elongation. Longitudinal growth was measured by the distance between the most distal terminal duct (black line) and the lymph node.

1 positive), and there was no difference in the total number of macrophages recruited to the mammary peri-epithelium or the proportion of classically activated M1 versus alternatively activated M2 macrophages between diets after 2 weeks on diet (data not shown).

Analysis of Dietary Effects on Carcinogen-Treated Mammary Glands Prior to Tumor Development

To assess the early effects of HFD on tumor progression, we examined DMBA-treated mammary glands at 10 weeks and 16 weeks on diet, and prior to the development of palpable tumors. There was a trend toward increased hyperplastic lesions per gland at 10 weeks on diet for HFD mice (P = .065), but this effect was absent by 16 weeks on diet (Figure 5*A*). Analysis of proliferation at 10 weeks on diet showed increased proliferation in hyperplastic lesions compared to normal tissue in both LFD- and HFD-fed mice with a trend toward greater proliferation of lesions on HFD (P = .14) (Figure 5*B*). Large ducts showed a significant increase in proliferation among the HFD-fed mice. There were no dietary differences in proliferation among the various mammary gland structures and hyperplasias at 16 weeks on diet, or blood vessel density at either 10 or 16 weeks on diet (data not shown).

Tumor-associated macrophages can play several, sometimes opposing roles in tumor development. M1 macrophages can promote anti-tumor immunity, while M2 macrophages can provide a tumor-promoting microenvironment (reviewed in Mills et al., 2016). A significantly increased number of total macrophages (M1 and M2) was recruited to the peri-epithelial region of hyperplastic lesions in HFD-fed mice at 10 weeks on diet; a trend toward increased total macrophages was observed at 16 weeks on diet (P =.062) (Figure 6*A*). There were significantly increased M1 (F4/80 + Arg1-) macrophages associated with hyperplastic lesions at 10 weeks on HFD (Figure 6*A*), while M2 (F4/80 + Arg1+) macrophages were significantly increased at 16 weeks on HFD diet (Figure 6*B*).

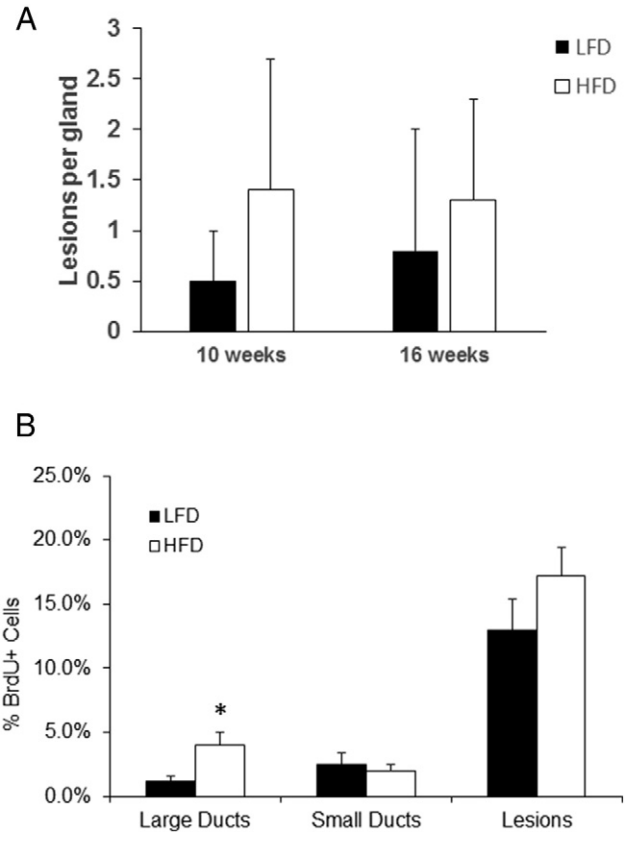

Figure 5. Lesions and cellular proliferation at 10 weeks on diet. (A) Mice fed high-fat diet for 10 weeks showed a trend toward increased hyperplastic lesions per gland (P = .065). (B) Mice fed high-fat diet for 10 weeks showed increased proliferation in large ducts (*, P < .05). Hyperplastic lesions showed a trend toward greater proliferation on HFD (P = .14).

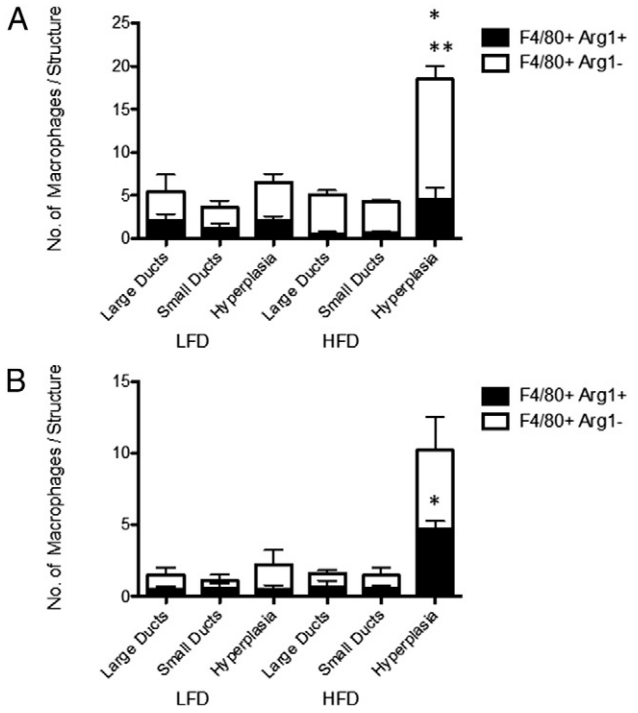

Figure 6. Recruitment of macrophages to mammary gland structures in mice fed low fat and high-fat diets. (A) Mice fed high-fat diet for 10 weeks showed significantly increased total and M1 (F4/80 + Arg1-) macrophages in the peri-epithelial region of hyperplastic lesions (*, P < .05 and **, P < .01, respectively). LFD (n = 3), HFD (n = 3). (B) Mice fed high-fat diet for 16 weeks showed significantly increased M2 (F4/80 + Arg1+) macrophages in the peri-epithelial region of hyperplastic lesions (*, P < .05). LFD (n = 3), HFD (n = 3).

Effects of Diet on Weight and Metabolic Parameters

HFD caused a significant increase in body weight by 10 days on diet, early in the peripubertal period (Figure 7*A*). The overall increase in mean body weight by 28 days on HFD was 11% over the mean body weight of LFD-fed mice, and did not produce an obese state. After the pubertal increase in body weight between 10 and 28 days on HFD, a significant weight loss occurred because of the DMBA treatments (Figure 7*B*). Mice on both LFD and HFD regained weight, but HFD-fed mice did not exhibit a significant increase in body weight compared with LFD until 14 weeks on diet (Figure 7*B*). HFD-fed mice reached a 24% increase in body weight by 32 weeks on diet. Thus, the HFD-fed mice only reached an obese state near the end of the experiment, whereas the majority of tumors developed prior to this time.

To determine the effects of diet on metabolic state, fasting plasma glucose and insulin levels were determined at 10 and 16 weeks on diet. HFD had no effect on either glucose or insulin levels at 10 weeks

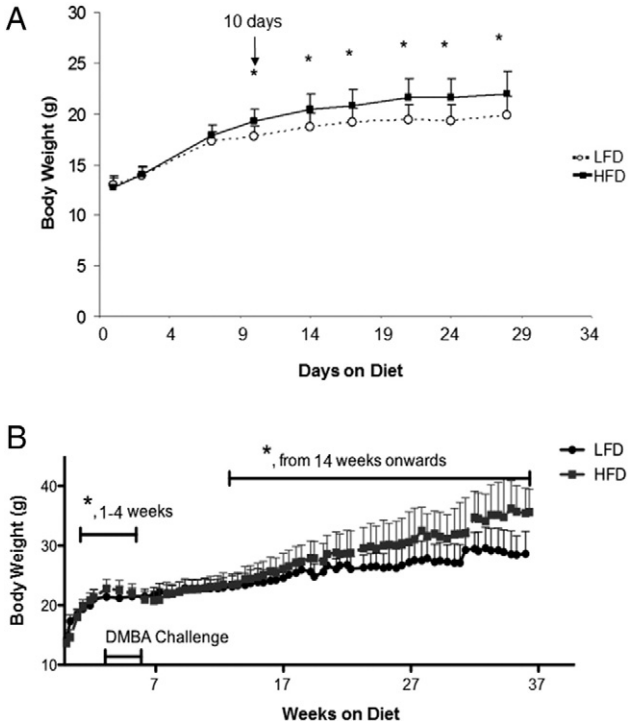

Figure 7. Body weight over time. (A) High-fat diet caused a significant increase in body weight by 10 days on diet. (B) A significant weight loss occurred because of the DMBA treatments, then mice on both low and high-fat diets regained weight. High-fat diet-fed mice did not exhibit a significant increase in body weight compared with low fat diet-fed mice until 14 weeks on diet (*, P < .05). LFD (n = 80), HFD (n = 101).

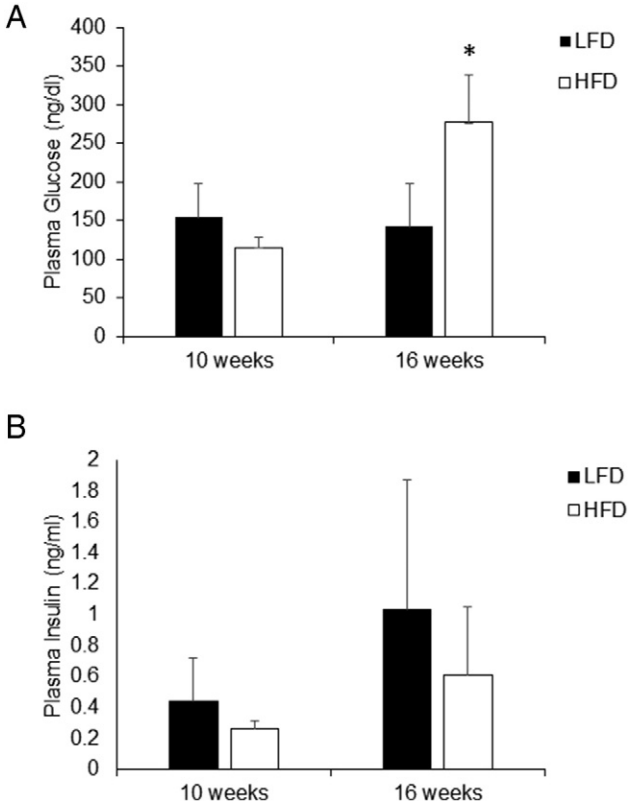

Figure 8. Effects of diet on plasma levels of glucose and insulin. No significant differences were found between non-fasting plasma glucose (A) and insulin (B) levels in mice fed low and high-fat diets after 10 weeks on diet, but high-fat diet increased glucose after 16 weeks on diet (*, *P* < .05). LFD (n = 5), HFD (n = 5).

on diet. HFD led to increased glucose after 16 weeks on diet, but did not alter insulin levels (Figure 8, *A* and *B*).

Discussion

HFD Promotion of Tumorigenesis

This study shows that HFD initiated at puberty in FVB mice significantly promoted DMBA-induced mammary tumorigenesis compared with LFD. The development of tumors largely occurred prior to mice becoming obese, indicating the role of an animal-derived HFD rather than resulting obesity in tumor promotion. This is consistent with our earlier studies in obesity-resistant BALB/c mice [7–9]. The DMBA regimen employed produced a tumor incidence of 15.7% in HFD-fed versus 4% in LFD-fed mice, clearly demonstrating the tumor promotional effects of HFD. The low number of tumors developing on LFD (n = 2) precluded an extensive comparative histopathological analysis of tumors arising on LFD versus HFD. Thus, our tumor analysis focused on HFD tumors.

DMBA carcinogenesis produces various tumor phenotypes, including ER + PR+ tumors. The majority of the tumors arising on HFD in the present study were ER- PR-. A subgroup of early occurring HFD tumors that were ER-PR- had an adenosquamous histopathology. These adenosquamous mammary tumors were similar to early tumors that developed in DMBA-treated, HFD-fed, obesity-resistant BALB/c mice [7,8]. Adenosquamous mammary carcinomas are similar to a sub-type of human basal-like breast cancer [17]. The occurrence of basal-like breast cancer in humans has similarly been associated with an earlier age of onset and increased abdominal adiposity [18]; although obesity is not a causative factor in this study, obesity is associated with HFD. While epidemiological data support an association between high consumption of animal fat in young adulthood and premenopausal breast cancer risk [6], data is lacking for the association of HFD consumption with specific premenopausal breast cancer subtypes. A previous study reported a positive association between HFD and receptor positive disease, but not receptor negative disease [19]. However, a number of confounding variables, such the numbers of pre- versus post-menopausal women analyzed and the age at HFD exposure could preclude an accurate assessment of early life HFD and breast cancer subtype. In this regard, we found a significant association of pubertal, but not adult, HFD with increased early development of DMBA-induced ER-PR- tumors in normal-weight BALB/c mice [8].

We previously showed that HFD promotion of mammary tumor development in normal-weight, obesity-resistant BALB/c mice was associated with increased proliferation of normal and hyperplastic mammary epithelium and tumor cells, increased angiogenesis, and recruitment of pro-tumorigenic M2 macrophages [7–9]. The limited occurrence of mammary tumors in LFD-fed mice makes comparison with tumors from HFD-fed mice speculative. Interestingly herein, we found no differences in tumor cell proliferation or angiogenesis between LFD and HFD tumors. However, tumors from HFD mice had a trend toward increased abundance of intra-tumor M2 macrophages. A similar pubertally initiated HFD increased macrophage recruitment in the BALB/c 4T1 tumor transplant model, while

also increasing tumor burden and metastasis [20]. The relationship between HFD and increased tumor-associated macrophages warrants further investigation for understanding the mechanistic basis for HFD promotion of tumorigenesis, and development of therapeutic and preventive strategies for the reduction of breast cancer risk and breast cancer treatment.

HFD Effects on Normal Pubertal Mammary Gland Development

Mouse strains vary significantly in their response to HFD with regard to pubertal mammary gland development. We found that HFD-fed FVB mice exhibited accelerated pubertal mammary gland development. This is in contrast to the inhibition of pubertal mammary gland development observed in HFD-fed, obesity-prone C57BL/6 mice [21]. In FVB mice, it is difficult to distinguish between the effects of weight gain and those that are a direct consequence of animal fat ingestion, but increased pubertal mammary gland development was also reported in HFD-fed, normal-weight, obesity-resistant BALB/c mice [21], suggesting that some of the proliferative effects of HFD involve pathways independent of weight gain.

Estrogen is the predominant driver of ductal elongation in puberty [22,23]. Although these studies did not directly measure estrogen levels, estrogen drives vaginal opening and can serve as a surrogate for estrogen activity. In this regard, there was no difference in age of vaginal opening between HFD- and LFD-fed mice. Previously, we found that estrogen levels were not altered in either obesity-resistant BALB/c mice or obesity-prone C57BL/6 mice fed HFD [21].

Pre-Tumor Effects of HFD in DMBA-Treated Mammary Glands

DMBA-induced mammary tumors are preceded in time by the development of hyperplastic lesions. There was a trend toward increased numbers of lesions in HFD mammary glands at 10 weeks on diet (13 weeks of age and 8 weeks post 1st DMBA treatment). There was also enhanced proliferation in large ducts and a trend toward enhanced proliferation in hyperplastic lesions in HFD-fed mice at 10 weeks on diet. At this time point, there was no difference in weight between HFD- and LFD-fed mice. This again supports the conclusion that HFD evokes proliferative effects that are independent of weight gain. These results contrast with the significant 2 to 3-fold HFD-induced increases in proliferation in both normal and hyperplastic epithelium in BALB/c mice at 10 weeks on diet [7,8]. In BALB/c mice, lifelong HFD additionally enhanced angiogenesis [7,8]. Perhaps, the proliferative effects of HFD are more profound in animals that have limited potential for weight gain. As is the case in BALB/c mice [7,8], HFD in FVB mice induced greater macrophage recruitment to hyperplastic lesions than that observed with LFD. But unlike the case with BALB/c mice, this enhanced macrophage recruitment did not extend to normal structures, again highlighting the diminished effects of HFD in FVB mice. We also observed a progression of macrophage subtypes in recruitment to hyperplastic lesions. At 10 weeks on diet, the increased recruitment of macrophages to HFD hyperplastic lesions was predominantly M1 (i.e., Arg1-) macrophages. By 16 weeks on diet, increased M2 (i.e., Arg1+) macrophages were evident. This differs from BALB/c mice, which showed recruitment at 10 weeks HFD to consist predominantly of M2 macrophages [7]. The M2 phenotype is associated with tumor-associated macrophages that can promote the growth of tumors through support of angiogenic and tissue remodeling processes, as well as immune suppression [24]. As with the proliferative response to HFD, macrophage recruitment is likely independent of weight gain, and more robust in mice with a more limited potential for weight gain (i.e., BALB/c versus FVB). At the same time, weight gain also seems to influence the polarization of macrophages in the mammary gland. Obesity induces a proinflammatory M1 phenotype in adipose tissue macrophages, while macrophages in lean mice have an alternatively activated M2 phenotype [25]. Our results in BALB/c mice reiterate findings for adipose macrophages in lean mice, while our results in FVB mice that show modest weight gain seem intermediate between BALB/c and the C57BL/6 mice used in the cited obesity study. The association of the tumor-promoting M2 phenotype with HFD in lean animals is reminiscent of the association of animal HFD with premenopausal breast cancer risk in normal-weight women found in Nurses' Health Study II [4–6]. This association warrants further investigation.

Relationship Between HFD and Weight Gain on Tumor Promotion

The effect of HFD on body weight produced a complex pattern in the FVB strain. Diets were initiated at 3 weeks of age and the first significant HFD-induced increase in body weight was noted at 10 days on diet. By 4 weeks on diet, HFD-fed mice weighed 14% more than LFD-fed mice; this level of weight gain does not constitute obesity. Significant weight gain continued during DMBA treatment, 2 through 5 weeks on diet. The greater number of lesions in HFD-fed mice at 10 weeks on diet may have resulted from the increased bioavailability of the lipophilic carcinogen DMBA and its activated metabolites in the context of HFD, with resulting increases in DNA damage events. HFD increases expression of the aryl hydrocarbon receptor [26], which is a receptor for DMBA and its metabolites, and thus may increase DMBA carcinogenicity. Between 6 and 14 weeks on diet, the mice fed HFD and LFD both initially lost weight and then re-gained weight; there was no difference in body weight between the diet groups until 14 weeks on diet. It is difficult to assess the effects of HFD in itself versus weight gain at 10 and 16 weeks on diet, when pre-tumor analyses were performed, since this was a period of no or minimal weight gain preceded by a period of pubertal HFD-induced weight gain and followed by a period of adult HFD-induced weight gain. Finally, weight gain continued in HFD-fed mice to the termination of the experiment, at which time these mice were obese with a maximum weight gain that was 24% above LFD weight. Most of the HFD tumors arose before significant weight gain was attained. Taken together, these results suggest either that the mechanism of the HFD promotional effect is independent of weight gain, or that the modest weight gain during puberty and/or early adulthood plays a role. As plasma glucose or insulin levels at 10 and 16 weeks on diet did not indicate that HFD produced significant metabolic changes, it is unlikely that the modest weight gains associated with the period of tumorigenesis are driving tumor promotion. The modest weight gain may have even attenuated the degree of tumor-promoting M2 macrophage polarization, as already discussed.

Conclusion

The present studies extend our earlier findings of HFD tumor promotion in obesity-resistant BALB/c mice [7–9] to FVB mice, which do gain weight in response to HFD. These results indicate that the promotional effects of HFD initiated at puberty on carcinogen-induced mammary cancer are largely independent of weight gain. Furthermore, HFD promoted a subset of early adenosquamous tumors, also observed in HFD-fed BALB/c mice, suggesting that early age exposure to HFD may be promotional for this specific tumor subtype of triple negative mammary cancer.

It is interesting that the incidence of mammary tumors in the FVB mouse strain fed LFD was much lower than that of the BALB/c strain, while FVB and BALB/c mice fed HFD showed more similar tumor incidence. This underscores the potent influence of HFD in tumor promotion. Contributing factors to increased incidence of mammary cancers, such as increased proliferation and angiogenesis differed between HFD-fed FVB and BALB/c mice. However, notably, HFD-associated increase in pro-tumorigenic M2 macrophage recruitment was a common factor in both strains. Thus, we speculate that a similar effect of HFD on immune function may provide clues to the basis for the epidemiological findings of increased breast cancer risk in young, premenopausal, normal-weight women who consume a diet high in saturated animal fat.

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