Potential Role of Leptin Signaling in DMBA-induced Mammary Tumors by Non-Responsive C57BL/6J Mice Fed a High-Fat Diet

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Abstract

Environmental carcinogens, High-Fat Diet (HFD) and elevated levels of leptin correlate to increase breast cancer incidence. To test whether these factors could affect the development of Mammary Tumors (MT) via DMBA (7,12-dimethylbenz[a]anthracene) challenge, we used C57BL/6J mice that are non-responsive to develop MT in absence of hormonal stimulation. C57BL/6J female mice without hormonal stimulation were fed HFD (55% cal-fat) and low-fat diets (10% cal-fat) and received DMBA (oral gavage: 1 mg/weekly) for 6 weeks. To test whether leptin signaling is involved in DMBA-MT development, a potent inhibitor, pegylated leptin peptide receptor antagonist (PEG-LPrA2; half-life 66 hours), was used for 30 weeks. As expected, irrespective of PEG-LPrA2 treatment, lean mice fed with low-fat diet did not develop MT. However, HFD induced obesity and significantly stimulated earlier onset (within 18 weeks) and marginally increased the incidence of MT (21%; 3/14) in DIO-mice (diet-induced-obesity). It appears that leptin signaling may be involved in DMBA-induced mammary carcinogenesis in obese mice because no evidence of MT was found in DIO-mice treated with PEG-LPrA2 (0% incidence; 0/14; Wilcoxon-Breslow test Ch2, p=0.03). Interestingly, PEG-LPrA2 treatment did not apparently affect body weight or food intake, but reduced protein levels of several proteins related to breast cancer [Aryl hydrocarbon Receptor (AhR), leptin receptor (OB-R), interleukin 1 receptor type I (IL-1R tI), hypoxia-induced factor 1 alpha (HIF-1α), Jagged1 (JAG1) and Notch1 activated (NICD1)] within the mammary glands. Our findings reinforce the idea that obesity induced by HFD is an additional risk factor for chemical-induced breast carcinogenesis. The present study reveals some potential mechanisms involving leptin in the effects of HFD and adiposity on mammary chemical-induced carcinogenesis. Overall, present data suggest that the inhibition of leptin signaling might be a new way to prevent breast cancer induced by chemical carcinogens, especially in obese individuals.

Keywords: High-fat diet; Leptin; Leptin-peptide receptor antagonist; PEG-LPrA2; DMBA; Obesity; Breast cancer prevention

Introduction

DMBA (7,12-dimethylbenz[a]anthracene), a Polycyclic Aromatic Hydrocarbon (PAH), is a proven carcinogen widely used for research in breast cancer [1]. PAH can induce several mutations promoting cancers of the breast, skin, pancreas, lung, immune system and others [2,3]. PAH-activated Aryl hydrocarbon Receptor (AhR) forms a complex with the nuclear translocator protein (ARNT) inducing phase-I detoxification enzymes (cytochrome P450: CYP1A1 and CYP1B1). These enzymes transform DMBA into toxic and mutagenic intermediates (i.e., pro-DNA adduct compound, epoxide intermediate), which induce carcinogenesis-promoting DNA mutations [1].

There is evidence that the development of DMBA-induced tumors require other factors that can act in a cell-context manner [4-6]. Breast cancer rates vary widely in different parts of the world. Environmental contaminants can be related to breast cancer incidence. Humans are exposed to thousands of naturally occurring and synthetic chemicals over a lifetime. Environmental Protection Agency (EPA) has suggested that exposure to environmental chemicals has been offered as possible explanation for higher incidence of breast cancer in certain regions. For example, various halogenated hydrocarbons and pesticides have been demonstrated to cause a variety of adverse health effects, including increase of breast cancer risk [7,8]. In addition, High-Fat Diet (HFD) or the Western diet and obesity are also positively correlated to breast cancer incidence. Obesity, characterized by high levels of leptin, is pandemic in the United States and associated with more than 100,000 incidents of cancer each year, particularly cancers of the breast, colon and endometrium. Obese breast cancer patients have increased mortality compared with non-obese [9,10].

Chemical carcinogenic challenge in rats has been the most successful model used to demonstrate the effects of pregnancy [11,12], lactation [13] and obesity in mammary tumorigenesis [14]. Intake of low-energy (caloric restriction) or low-fat diets reduces the growth of DMBA-mammary tumors (MT) in rats [15] suggesting a role of obesity/diet in chemical carcinogenic induced-MT. Reported data support the idea that rats fed a HFD and treated with DMBA have a higher positive response to the develop MT [14] when compared to mice under similar experimental conditions. This was true even in research that used mouse strains with high potential to develop MT induced by DMBA [16]. Therefore, the mouse model is more suitable to examine the conditions that can overcome the refractoriness to develop MT induced by carcinogens [13]. More common methods to induce MT in resistant mice after treatment with carcinogens include

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natural hormone stimulation (i.e., pregnancy, pseudopregnancy), and supply of hormones or implants of pituitary isografts [17]. Remarkably, C57BL/6J mice without hormonal stimulation are completely unresponsive to the development of DMBA-MT. Interestingly, caloric restriction reduces BW and increases life-span of C57BL/6 mice treated with DMBA, but without MT [18]. This would suggest that reduction of BW (adiposity) decrease DMBA damage in these mice.

Leptin, a key adipokine released from adipose tissue, serves as a major player in the basic homeostatic functions required to regulate appetite and energy expenditure for controlling body weight [19]. Leptin and its receptor, OB-R, are overexpressed in several cancer types, where they induce tumor growth through several actions (pro-angiogenic, pro-inflammatory and anti-apoptotic) [20-23]. Moreover, proving leptin’s oncogenic actions, the inhibition of leptin signaling with pegylated leptin receptor antagonist (PEG-LPrA2) significantly reduced tumor growth and, levels of pro-angiogenic and inflammatory molecules (i.e., VEGF/VEGFR-2, IL-1 system) in syngeneic and xenograft mouse models of breast cancer [20,22].

We hypothesize that obesity induced via HFD will accelerate DMBA-MT development in C57BL/6J mice without hormonal stimulation. Moreover, we postulate that leptin signaling could play a role in DMBA-MT development in obese mice fed HFD. Our results suggest that HFD intake shorten the onset and marginally increased incidence of DMBA-MT in female C57BL/6J mice. Notably, the inhibition of leptin signaling prevented MT development and reduced the levels of several molecules with potential oncogenic roles in breast cancer.

Materials and Methods

Reagents and antibodies

Polyclonal Notch1 (sc-373891), Notch4 (sc-56594), Jagged1 (sc-8303), OB-R-NH2 (sc-1834), leptin (sc-843), IL-1R α (sc-689), NF-kBp50 (sc-114) and -p65 (sc-109), Bcl-2 (sc-788), Flk-1 (VEGFR-2) (sc-6251), VEGF-A (sc-152) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Polyclonal NFkB-p105 (ab7971), Notch2 (ab-8926), Notch3 (ab-23426), DLL-4 (ab-7280) and monoclonal AhR (ab2770) antibodies were from Abcam Inc (Cambridge, MA, USA). Polyclonal Hey2 antibody (Ab5716) and insulin ELISA kit were from Millipore (Temecula, CA, USA), Polyclonal Survivin antibody (2808S) was from Cell Signaling (Danvers, MA, USA). Polyclonal anti-mouse and anti-rabbit antibodies horseradish peroxidase (HRP) conjugates were from Bio-Rad Lab. (Hercules, CA, USA). Recombinant mouse leptin, monoclonal HIF-1α (MAB153) and II-1 alpha/IL-1F1 and VEGF Quantikine ELISA Kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Estradiol ELISA kit was from Cayman Chemical Corporation (Ann Arbor, MI, USA). ECL Western blot stripping buffer was from Thermo Scientific (Rockford, IL, USA). 7.12-dimethylbenz[a]anthracene (DMBA), monoclonal Notch1 (N6786) and β-actin (A5316) antibodies, protease inhibitor, phosphatase inhibitor cocktails 1 and 2 and, other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Leptin peptide receptor antagonist (PEG-LPrA2)

The leptin receptor peptide antagonist 2 (LPrA2) with high binding affinity for the leptin receptor (OB-R; Kd = 0.6 × 10^-8 M) was synthesized and purified as described elsewhere [24]. To increase its solubility and half-life the peptide was coupled to polyethylene glycol (pegylated peptide: PEG-LPrA2; MW = 23000; half-life in mice via i.v.: unconjugated 1 hour versus pegylated 66 hours). In contrast to the unconjugated peptide, its derivative, PEG-LPrA2, is water-soluble [22].

Animals and experimental procedures

Female C57BL/6J mice seven-week old (Jackson Laboratories, Bar Harbor, ME, USA) were randomly allocated into two groups: lean (n=20) and DIO (n=30). Mice were housed three per cage in the animal facilities of Morehouse School of Medicine (MSM, Atlanta, GA, USA) in rooms maintained at 25°C with 10-15 air exchanges per hour. Artificial light was provided under a 12h/12h light/dark cycle. All housing materials, as well as food and water, were autoclaved prior to use. All experiments were performed according to the protocol approved by MSM-IACUC and NIH guide for the Care and Use of Laboratory Animals. The PDI-1 diet (low-fat diet: 10 % Kcal-fat; 4.1 cal/g), free of phytoestrogens (phyto-free; approximately 10–15 ppm of soy isoflavones; Ziegel Bros.; Gardner, PA, USA) was used to feed the lean control mice for 37 weeks. The PDI-1 (Phytoestrogen Reduced I 22.5-5) rodent diet is specifically formulated for studies where dietary concentrations of naturally occurring phytoestrogens, such as genistein, daidzein or glycitein, can influence experimental results. The formulation of this diet is modified according to the NIH-07 diet where ingredients such as soybean products and alfalfa meal, known to contain these hormones, are not used. Main components of the PDI-1 diet include: Corn, Wheat, Fish Meal, Wheat Middlings, Lactic Casein, Dried Skim Milk, Corn Gluten Meal, Corn Oil, Brewers Dried Yeast, Dry Molasses, Limestone, Salt, Dicalcium Phosphate, Vitamin D3 Supplement, dl-Alpha Tocopheryl Acetate (Vitamin E), Folic Acid, Nicin, Riboflavin, Thiamine, Vitamin B12 Supplement, Pyridoxine, Menadione, Sodium Bisulfite Complex (Vitamin K) Vitamin A Palmitate, Choline, Biotin Calcium, Pantothenate, Cobalt Carbonate, Copper Sulfate, Ferrous Sulfate, Manganese Oxide (for more details on PDI diet components see [25]). The PDI-1 diet was modified by adding 24.7 gm% corn oil (75.3g of PDI plus 24.7 gm of corn oil; total 55 % Kcal-fat; 5.2 cal/g). The high-fat diet was used to feed the mice in order to induce obesity (DIO) for 37 weeks. After one week of acclimatization the mice were fed either the lean or DIO diets at libitum. Lean control and DIO-diets were prepared in pellets. Each cage was provided with a supply of pre-weighted diet. Food intake was determined weekly by weighting the differences of remain diets in each cage divide by number of mice (n=3 mice/cage). Caloric intake was measured by multiplying the food intake by the calories per gm (i.e., lean control diet, 4.1 cal/gm; DIO-diet, 5.2 cal/gm). Obesity was identified in DIO-mice showing body weights (BW) greater than BW of lean control group (L-C) plus two standard deviation (SD) as follows: BW DIO > BW L-C + 2SD.

PEG-LPrA2 treatment

Half-life of PEG-LPrA2 is approximately 66 hour, therefore, to establish efficacy the pegylated antagonist was injected into lean and DIO-mice once (‘1’) or two times (‘II’) a week via tail vein (50ul/0.1mLm). Mice were randomly allocated into three subgroups each: Lean: control L-C (n=6) and, treated L-I (n=7) and L-II (n=7); and, DIO: control DIO-C (n=14) and, treated DIO-I (n=7) and DIO-II (n=7). PEG-LPrA2 treatment was initiated when obesity was detected after 7 weeks fed the DIO diet. PEG-LPrA2 treatment was applied to lean and DIO-mice for 30 weeks. L-C and DIO-C mice received two injections per week of saline (vehicle). Mice fed HFD that did not develop obesity were considered obesity-resistant (n=2; =7 %) and excluded from the studies.

DMBA challenge

Two weeks after initiated PEG-LPrA2 treatment all mice received...
Tumor detection

Mammary glands were palpated weekly for MT detection. Tumor onset, number (multiplicity) and growth were recorded when possible (caliper; 7/6 x width x length).

Blood collection and euthanasia

Blood samples from 8h-fasted mice were collected in tubes containing EDTA-citrate anticoagulant (Sigma) by cardiac or retro-orbital sinus puncures under anesthesia before euthanasia. Mice were euthanized at the end of the experimental period (37 weeks of feeding low-fat and HFD, which corresponds to 30 weeks of PEG-LPrA2 treatment and 28 weeks after the initiation of DMBA challenge). MTs were dissected and their weight/volume and multiplicity were determined after euthanasia. Mammary gland tissues without apparent MT development were also taken from mice.

Pathology

AP/CP and Cytopathology board certified pathologist (MP) evaluated the Hematoxylin and Eosin stained preparations of tumors in a blinded protocol.

Immunohistochemistry (IHC)

IHC was carried-out in paraffin embedded sections from mammary glands and MT (5 µm). Briefly, unmasking of tissue antigens was performed by heat treatment in sodium citrate buffer (pH 6, 10 mM) at 95°C for 15 minutes and/or partial digestion at 37°C for 10 minutes with protease (Sigma Inc.). After quenching endogenous peroxidase activity with H2O2 (3% water solution) and blocking (2.5% horse or rabbit normal serum), tissue sections were incubated for 1 hour at room temperature with the following primary antibodies diluted in PBS-0.1% BSA: anti AhR (1:100); OB-R (1:250); leptin (1:50); Flk-1 (VEGFR-2; 1:50); IL-1R tI (1:50); Notch1 (1:50); Notch2 (1:200); Notch3 (1:250); Notch4 (1:50); Jagged1 (JAG1; 1:50); Survivin (1:400); and Bcl-2 (1:50). The tissues were incubated with a streptavidin-biotin peroxidase system according to the manufacturer’s directions (Vectastain, ABC-AP kit; Vector, Burlingame, CA, USA), counterstained with hematoxylin (Dako Corp., Carpinteria, CA, USA), and mounted with VectaMount (Vector). Negative controls were also included in which the primary antibody was omitted or substituted with non-specific IgG. Positive cells were recorded and counted in randomly chosen fields. Staining intensity was assigned as weak, moderate, or strong.

Western Blot (WB)

Tissue lysates from MT and mammary glands from PEG-LPrA2 treated and untreated mice were prepared. Thirty µg of protein lysates were used for WB analysis of several leptin-targeted molecules: Notch/ NICD; Bcl-2; IL-1R tI and AhR as previously described [20]. β-actin serves as the experimental loading control. For quantitative evaluation of antigen expression, the blots were scanned and analyzed by the NIH Image program [22]. Protein concentrations from lysated tissues were determined by the Bradford method (Bio-Rad).

Immunoprecipitation (IP)

Fifty micrograms of proteins from tissue lysates were immunoprecipitated with specific antibodies and protein G-agarose beads for IP/WB analysis of VEGFR-2, OB-R, HIF-1α, and Survivin as described elsewhere [22].

Plasma and tissue determinations

ELISA (R&D System Inc) determined the levels of IL-1β, IL-1α, VEGF and leptin in plasma and tissues lysates. Plasma levels of insulin (Millipore), adiponectin (R&D Systems Inc) and estradiol (E2; Cayman Chem. Co.) were also determined by ELISA. Glucose levels were determined by glucometer (Accu-Chek Active, Roche Diagnostic, IN, USA).

Statistical Analysis

All continuous data were summarized using means ± standard deviation. An ANOVA model was implemented to analyze changes of body weight, food intake and caloric intake between the six subgroups (L-C, L-I, L-II, DIO-C, DIO-I and DIO-II). The effects of HFD and PEG-LPrA2 on MT incidence of DIO-mice were analyzed by the Pearson Chi2 and likelihood-ratio Chi-squared tests. The tumor-free times were plotted using a Kaplan–Meier curve [27] and the median times were compared using the Wilcoxon-Breslow test. Statistical significance was set at p<0.05. Within the lean group, an ANOVA model was also fitted to analyze plasma and mammary tissue parameters between treated and non-treated (control) groups. Within the obese (DIO) group, an ANOVA model was fitted to analyze the relative expression of antigens in mammary tissues and MT, and plasma parameters.

Results

Impact of diets and treatments on food/caloric intake and body weight

Figure 1 depicts weekly average of food (FI) (Figure 1A), caloric intake (CI) (Figure 1B) and, body weight (BW) (Figure 1C) in lean untreated control (L-C) and DIO-mice (DIO-C; only receiving saline), and treated mice receiving one (L-I and DIO-I) or two doses (L-II and DIO-II) of PEG-LPrA2. FI was similar between groups independently of diets or treatments (FI: 2-3g/day/mouse) (Figure 1A). However, because diets were not isocaloric, DIO-mice consumed 3-fold more calories during the 37-week experimental period (Figure 1B). Interestingly, DIO-C and DIO-I showed significantly higher CI than DIO-II at week 37 (p<0.01; see Figure 1B). Ninety three percent of mice fed a DIO diet (HFD; 55% Kcal-fat) were classified as obese (n=28/30) after 7 weeks. Obesity was determined using the criterion reported by Dogan et al. [28] as follows: mice fed the HFD with final BW ≥ 2 standard deviations of low-fat untreated (control) mice (BW≤ 141g (BW L-C + 2SD)). In contrast, only ≈ 7% (n=2/30) of HFD fed mice were classified as obesity-resistant (OR) using the following criterion: OR (BW<= BW L-C + 2SD). The OR mice were excluded from the trial. In comparison, the DIO-mouse group gained more weight (increase 199%) than the compared lean mouse group fed a low-fat diet during the experimental time (37 weeks) (Figure 1C and Table 1). However, due to age progression lean mice significantly increased its average BW (increase 140%) during the course of this study. However, no significant differences in BW were found between lean treated or untreated with PEG-LPrA2 at any dose (L-C vs L-I and L-II). Similar results were found in obese (DIO-C vs DIO-I) mice after PEG-LPrA2 treatments (Figure 1C and Table 1). Interestingly, DIO-mice receiving two doses of PEG-LPrA2 (DIO-II) showed significantly lower average BW than DIO-C (BW 100-105°C 30% BW 100,0°C P=0.01) (Figure 1C and Table 1).

Effects of HFD and PEG-LPrA2 on onset and incidence of MT

The effects of HFD and PEG-LPrA2 on DMB-A-1000132
as expected, lean mice fed low-fat diet treated or untreated with PEG-LPrA2 did not develop MT. Cumulative percentage of MT-free DIO-mice using a Kaplan–Meier curve is shown in figure 1D. Interestingly, the development of MT in C57BL/6J female mice (that are DMBA non-responsive without hormonal stimulation) [18,29] was dependent on HFD intake and obesity. MT development was only detected in DIO-C mice at weeks 3, 6 and 18. Obesity was defined as: BW > BWL-C + 2SD. (§) p<0.01 when comparing CI in DIO-C and DIO-I to DIO-II, L-C, L-I and L-II. (*) p<0.05 when comparing BW of lean to DIO-mice. (&) p<0.05 when comparing BW of DIO-C and DIO-I to DIO-II mice. (#) p=0.03 (likelihood-ratio Chi2 test) when comparing MT incidence in non-treated (DIO-C) to PEG-LPrA2 treated mice (DIO-I and DIO-II).

MT and other tumors induced by DMBA

Only one MT per mouse was found in each case. Pathological analysis showed that all DIO-MT were adenocarcinomas with squamous epithelial differentiation. Lymphomas and skin tumors (papilloma and neoplasia) were also found in several mouse subgroups irrespectively of diets fed or PEG-LPrA2 treatments (Table 2). However, L-C and DIO-I did not develop lymphoid tumors. These data suggest that HFD intake, obesity or leptin signaling were not significantly linked to DMBA-induced non-MTs in this mouse model.

DMBA-induced MTs overexpressed several leptin-targeted molecules

DMBA-MTs and mammary glands from DIO-mice untreated (DIO-C) and treated with PEG-LPrA2 were analyzed by IHC. Notably, several pro-angiogenic, inflammatory, anti-apoptotic molecules and AhR within were overexpressed in MT when compared to mammary glands from DIO-mice treated with PEG-LPrA2 (DIO-I or DIO-II) (Figure 2). AhR (Figure 2B), leptin (Figure 2F), Notch1 (Figure 2L), Notch2 (Figure 2M) and Survivin (Figure 2J) were strongly expressed in MT. In addition, OB-R (Figure 2D), VEGFR2 (Figure 2H), IL-1R tI (Figure 2), Notch4 (Figure 2R) and Bcl-2 (Figure 2Y) were moderately expressed in MT. In contrast, Notch2 (Figure 2M) and Survivin (Figure 2W) were weakly expressed in MT.

Mouse group n  
L-C 6 17.9±0.6 25.0±4.1 17.8±1.9  
L-I 7 17.2±1.4 24.7±4.9 17.2±3.6  
DIO-C 11 17.8±1.1 35.5±8.4 23.3±4.8  
DIO-I 7 18.1±0.8 34.9±9.7 19.4±3.4  
DIO-II 7 17.8±0.8 25.6±4.9 17.8±3.2  
DIO-MT 3 17.4±1.2 22.1±3.7 15.4±1.6

Term definitions:
BW: body weight (grams). g
DMIO-MT: DIO-mice hosting mammary tumors.

Table 1: Effects of leptin antagonist treatment on body weight of DMBA-mice.
Figure 2: Representative pictures from the immunohistochemical determination of several molecules expressed by mammary glands from mice treated with PEG-LPrA2 and mammary tumors (DMBA-MT) developed in DIO-C (untreated mice), respectively. (A, B) AhR; (C, D) OB-R; (E, F) Leptin; (G, H) VEGFR-2; (I, J) IL-1R tI; (K, L) Notch1; (M, N) Notch2; (O, P) Notch3; (Q, R) Notch4; (S, T) JAG1; (V, W) Survivin; (X, Y) Bcl-2; (Za, Zb) Negative control. Similar results were obtained from mammary glands without MT from the same animal (not included). Arrows indicate positive staining in MTs. Bar = 200 µm.

WB analysis further revealed that MT overexpressed several molecules with putative roles in breast cancer (i.e., Notch family: receptors Notch1-4; ligands: JAG-1, DLL-4 and targeted-molecules: Survivin and Hey2) when compared to mammary glands from untreated mice or treated with PEG-LPrA2 (Figure 3A). Additionally, MT showed higher levels of AhR, pro-angiogenic and inflammatory (HIF-1α, NFκB p105, VEGF/VEGFR-2 and IL-1R tI) and anti-apoptotic molecules (Bcl-2) when compared to mammary glands from untreated DIO-mice or treated with PEG-LPrA2 (Figure 3A). Remarkably, PEG-LPrA2 treatment to DIO-mice reduced the mammary gland levels of numerous molecules (i.e., AhR, OB-R, IL-1R tI, HIF-1α, JAG1 and NotchNICD1) when compared to mammary glands from untreated DIO-C mice without MT (Figure 3B).

Levels of cytokines and metabolic syndrome markers

In general, lean mice treated or untreated with PEG-LPrA2 showed no changes in the plasmatic or mammary tissue levels of leptin, VEGF, Adiponectin and IL-1 (Figures 4A and 4B). However, lean mice treated twice a week with PEG-LPrA2 (L-II) had lower levels of IL-1a in mammary tissues (Figure 4B). Similarly, DIO-mice without MT (untreated or treated with PEG-LPrA2) showed no differences in the plasmatic or mammary tissue levels of leptin, VEGF, Adiponectin and IL-1 (Figures 4C and 4D). However, DIO-mice hosting MT had significantly higher levels of VEGF in plasma and tumors tissues (Figures 4C and 4D). In addition, DIO-mice with MT had lower plasmatic levels of leptin and IL-1a in MT tissue (Figure 4D). Analysis of glucose, insulin and estradiol levels did not reveal differences between the groups of obese or lean mice (Table 3). However, DIO-II mice showed a trend of lower levels of glucose and insulin when compared to DIO-control (Table 3).

Discussion

According to the WHO, more than 30% of cancer deaths can be prevented by modifying or avoiding key risk factors [30]. Among these factors overweight/obesity, physical inactivity and consuming Western diets could play important roles for both cancer development and prognosis, increasing the risk for overall and breast cancer mortality.
by approximately 30% [31]. Obesity negatively impacts the survival of breast cancer patients regardless of menopausal status, as it has been positively associated with increased risk of recurrence and increased proportion of breast cancer unresponsive to estrogens [9].

Higher incidence and shorter onset of DMBA-induced mammary tumors are mainly found in rats compared to mice [14]. Moreover, in contrast to mice, carcinogen-induced MTs can be developed in rats without additional hormonal stimulation [14,15]. These data clearly suggest that DMBA-MT development is species-dependent.

Several mouse strains under hormonal stimulations have been used to study the effects of various compounds and diets on MT development [13,16-18,26,29,32-35]. However, scarce information on the effects and potential mechanisms involved in the relationships between HFD and carcinogen-induced MT in mice is available [16].

We purposely chose the C57BL/6J mice, which are non-responsive to DMBA-induced MT in absence of hormonal stimulation [18]. C57BL/6J mice are particularly resistant to developing DMBA-MT, even with the use of pituitary isografts (MT: 27-38% after 9 months) [29]. However, these mice are useful models to study the effects of obesity on cancer growth. C57BL/6J mice have a marked ability to gain weight and develop obesity when fed HFD [36].

In the present investigation C57BL/6J lean mice fed a low-fat diet were used as control. Ninety three percent of C57BL/6J–obesity prone mice (non-hormonal stimulated) fed HFD developed obesity. Later, these mice were challenged with the most effective reported dosage of DMBA (6-mg/6 weeks) [16]. It is known that C57BL/6J mice did not develop MT after 18 months of DMBA challenge when they are not hormonal stimulated [18]. Interestingly, present data show that only DIO-mice developed MT (21% incidence), suggesting positive, but marginal effects of HDF on obesity and incidence on the incidence of DMBA-MT. However, significantly earlier MT onset was detected
(3-18 weeks after DMBA), which sharply contrasts to previous data reported for hormonal-stimulated C57BL/6J mice (18-36 weeks; MT mean incidence: 32%) [29]. Present observations suggest that HFD and obesity accelerated the oncogenic transformation of the mammary glands initially elicited by DMBA. This is in agreement with previous observations showing dietetic caloric restriction increased survival (36%) of non-hormonal stimulated C57BL/6J mice treated with DMBA [18].

Some contradictory data on HFD and DMBA-MT have been reported in rodents, however. BALB/c female mice fed HFD (20% fat; 37% cal-fat) and treated with 6-mg DMBA dose showed 10% higher number of breast tumors than controls fed a low-fat diet (5% fat; 9.3% cal-fat), which was unrelated to lipid peroxidation [37]. In contrast, 75% of FVB mice (fed a low-fat diet by 8.5 months of age) had breast tumors, which suggested genetic background could play a role in DMBA-induced [38] and in spontaneous [39] cancer induced MT in DIO-C57BL/6J mice treated with DMBA [18].

Accumulating evidence suggests that leptin secreted by adipose and cancer tissues increases the development of breast cancer. A positive correlation between the levels of expression of leptin and its receptor, OB-R, and bad prognosis of breast cancer has been reported [42]. Here we provide data suggesting that leptin signaling may be essential for the development of DMBA-induced MT in DIO-C57BL/6J mice fed a HDF.

We found a trend for positive correlation between DMBA-MT and intact leptin-signaling axis (0% vs. 0; 0 of 14; Pearson Chi2, p=0.06; Likelihood-ratio Chi2, p=0.03). This was only detected in DIO-mice showing higher levels of leptin and treated with the leptin antagonist. No previous reported data on leptin actions is available from DMBA-MT mouse models. However, high levels of leptin were linked to azoxymethane (AOM)-induced colon carcinoma in mice [43]. In contrast, obese Zucker rats (that exhibit leptin resistance due to decreased functional OB-R) [44] were more susceptible to DMBA-induced tumorogenesis than lean controls [45]. In addition, adiposity and leptin levels were not associated in a carcinogenic-induced colon-cancer Sprague–Dawley rat model [46]. These disparate results derived from triplicate determinations.

Figure 4: Plasma and tissue levels of leptin and leptin-related cytokines in mice challenged with DMBA. Lean (n=20; fed low-fat diet: 10% cal-fat) and DIO-mice (n=28; fed high-fat diet, HFD: 55% cal-fat) were treated with PEG-LPrA2 (once (I) or twice (II) a week) or received saline injections for 32 weeks. Levels of leptin (ng/ml); VEGF (ng/ml); adiponectin (ng/ml x 10); and IL-1α and β (pg/ml x 10) in plasma (A and C) from lean and DIO-mice, respectively, as determined by ELISA. Levels of leptin (ng/ml/mg-protein); VEGF (ng/ml/mg-protein); and IL-1α and β (pg/ml/mg-protein x 10) in mammary and MT tissue homogenates (B and D) from lean and DIO-mice, respectively, as determined by ELISA. (a) p<0.05 when comparing levels to lean (L-C) and obese controls (DIO-C) without MT. Data (mean ± standard error) representative results derived from triplicate determinations.

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Notch signaling activation, a hallmark of breast cancer development [47] is also required for DMBA-induced MT [48]. Mammary glands from DIO-mice treated with PEG-LPrA2 showed lower levels of several leptin-related molecules (NotchNICD1, JAG1, IL-1r tI; OB-R, HIF-1a and Ahr) [49] than mammary glands from untreated mice. These results suggest that Notch signaling could be involved in DMBA-MT. Moreover, these data reinforce the idea that an intact leptin-signaling axis is needed for the increase of JAG1 (a Notch ligand) [47] in DMBA-induced MTs.

In addition, DMBA-induced pre-invasive MT requires angiogenesis via VEGF/VEGFR-2 mechanism [48]. Indeed, the inhibition of VEGF-R2 signaling abrogates the development of DMBA-MT in rats [48]. In line with these data, leptin induced a complex crosstalk of Notch-IL-1-leptin crosstalk outcome, that upregulates VEGF/VEGFR-2 [49]. Moreover, leptin can activate VEGF-R2 in absence of VEGF [49]. Therefore, current data suggest that the development of DMBA-MT in C57BL/6j obese mice probably involves leptin-induced activation of NILCO and upregulation of VEGF/VEGFR-2 [49].

Overall, present data suggest obesity and HFD accelerate DMBA-induced MT, and that leptin signaling may be essential for malignant transformation of mammary glands of C57BL/6j mice. Therefore, inhibition of leptin signaling may provide novel prevention strategy for carcinogenic-induced breast cancer, particularly, in overweight/obese individuals feeding high-fat diets.

**Limitation of the Study**

Although, we acknowledge the small sample size of the study, however, the data is compelling enough for hypothesis generation to support a bigger study to validate the findings of the present investigation.

**Disclosure of Potential Conflicts of Interest**

The authors declare that they have no conflicts of interest. RRGP is an inventor of the Boston Biomedical Research Institute’s patent Leptin Peptide Antagonists (US Patent 7407929, Application No. 10/841,218; International application No. PCT/US 05/15198). No financial benefits have been derived from this patent.

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**Table 2:** Incidence of mammary and non-mammary tumors in mice challenged with DMBA, treated and untreated with PEG-LPrA2, and fed with control or DIO-diets.

| Mouse group | n | Glucose | Insulin | E2 |
|-------------|---|---------|---------|----|
|             |   | Raw BW-N | Raw BW-N | Raw BW-N |
| L-C         | 6 | 173.7 ± 28.8 | 6.7 ± 1.7 | 78.2 ± 22.6 | 3.0 ± 1.1 | 2.4 ± 1.1 | 0.1 ± 0.0 |
| L-I         | 7 | 186.7 ± 21.7 | 7.1 ± 1.3 | 95.6 ± 15.4 | 4.3 ± 1.5 | 7.5 ± 7.8 | 0.3 ± 0.3 |
| L-II        | 7 | 162.5 ± 19.8 | 6.5 ± 0.9 | 96.1 ± 39.0 | 4.1 ± 1.6 | 4.1 ± 4.0 | 0.2 ± 0.2 |
| DIO-C       | 11| 228.8 ± 89.5 | 6.4 ± 2.8 | 85.1 ± 29.3 | 2.8 ± 1.1 | 6.2 ± 8.8 | 0.2 ± 0.0 |
| DIO-I       | 7 | 216.9 ± 58.6 | 7.0 ± 3.2 | 120.6 ± 48.1 | 4.0 ± 1.9 | 4.1 ± 2.1 | 0.1 ± 0.0 |
| DIO-II      | 7 | 174.9 ± 51.3 | 6.4 ± 1.5 | 106.7 ± 13.2 | 2.6 ± 0.4 | 4.3 ± 3.1 | 0.2 ± 0.1 |
| DIO-MT      | 3 | 333.9 ± 252.9 | 14.3 ± 11.7 | 70.6 ± 0.0 | 2.9 ± 0.2 | 4.7 ± 3.2 | 0.2 ± 0.2 |

Term definitions:

DIO-MT: DIO-mice hosting mammary tumors.

Raw: levels of plasmatic parameters not adjusted to body weight [(glucose, mg/dL); (insulin, pmol/L) and (E2, estradiol, pg/mL)].

BW-N: levels of plasmatic parameters normalized to body weight in grams [(glucose, mg/dL/g), (insulin, pmol/L/g) and (estradiol, pg/mL/g)].

**Table 3:** Effects of leptin antagonist treatment on plasmatic levels of glucose, insulin and estradiol in DMBA-mice.
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