Maternal metabolic perturbations elicited by high-fat diet promote Wnt-1-induced mammary tumor risk in adult female offspring via long-term effects on mammary and systemic phenotypes

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Many adult chronic diseases are thought to be influenced during early life by maternal nutrition; however, the underlying mechanisms remain largely unknown. Obesity-related diseases may be due partly to high fat consumption. Herein, we evaluated mammary tumor risk in female mouse mammary tumor virus-Wnt-1 transgenic (Tg) offspring exposed to high-fat diet (HFD) or control diet (CD) (45% and 17% kcal from fat, respectively) during gestation and lactation, with CD provided to progeny at weaning. In Tg offspring, maternal HFD exposure increased mammary tumor incidence and decreased tumor latency without affecting tumor volume. Tumor risk was associated with higher tumor necrosis factor-α and insulin and altered oxidative stress biomarkers in sera and with early changes in mammary expression of genes linked to tumor promotion [interleukin 6 (Il6)] or inhibition [phosphatase and tensin homolog deleted on chromosome 10 (Pten), B-cell lymphoma 2 (Bcl2)]. Corresponding wild-type progeny exposed to maternal HFD displayed accelerated mammary development, higher mammary adiposity, increased insulin resistance and early changes in Pten, Bcl2 and Il6, than CD-exposed offspring. Dams-fed HFD showed higher serum glucose and oxidative stress biomarkers but comparable adiposity compared with CD-fed counterparts. In human breast cancer MCF-7 cells, sera from maternal HFD-exposed Tg offspring elicited changes in Pten, Bcl2 and Il6 gene expression, mimicking in vivo exposure; increased cell viability and mammosphere formation and induced measures [insulin receptor substrate-1 (IRS-1), IRS-2] of insulin sensitivity. Serum effects on IRS-1 were recapitulated by exogenous insulin and the PTEN-specific inhibitor SF1670. Hyperinsulinemia and PTEN loss-of-function may thus, couple maternal HFD exposure to enhanced insulin sensitivity via increased mammary IRS-1 expression in progeny, to promote breast cancer risk.

Introduction

Breast cancer is the most commonly diagnosed malignancy in women in the USA and other highly developed countries and is the second leading cause of cancer mortality worldwide (1,2). The mechanisms of breast cancer remain elusive; genetics and the environment individually contribute to disease susceptibility and their interactions may exacerbate disease development and progression (3). Obesity is a major health risk for many chronic diseases and has been linked to advanced breast cancer with poor prognosis in both pre- and postmenopausal women (4). Poor diet and a sedentary lifestyle contribute to an obesogenic state and hence, are widely considered to promote breast cancer. Nevertheless, a direct association between poor nutrition and obesity leading to increased breast cancer susceptibility remains lacking, given that factors such as low-grade inflammation, increased oxidative stress, hyperglycemia and insulin resistance, all of which have been linked to breast cancer risk, can occur in addition to and/or independent of obesity (4,5).

There is growing evidence and hence, acceptance for the concept of the early origins of adult chronic diseases (6). The seminal work of Prof. Barker et al. (7) demonstrating that adult men born of mothers during the famine years in the Netherlands manifested increased susceptibility to glucose dysfunction and cardiovascular diseases, has since been supported by additional epidemiological and animal studies (8-9). For instance, in both humans and rodents, babies (pups) born from diabetic mothers (dams) and/or from those consuming high-fat diet (HFD) during pregnancy and lactation were found to be at increased risk of glucose intolerance and diabetes in adult life (10,11). In the context of breast cancer, animal studies have shown that offspring exposed to maternal HFD (HFDO) had higher incidence, earlier onset and larger 7,12-dimethylbenz[a]anthracene-induced mammary tumors (12,13). Hilakivi-Clarke et al. (13,14) have suggested that the increased breast cancer risk in the offspring of dams-fed HFD may relate to the high estrogenic environment in utero elicited by high fat consumption and which compromises developing mammary glands exposed to this environment. Nevertheless, given that the developing mammary gland is subject to numerous stimuli beginning from the early primordial stage (15), that the connection between obesity and breast cancer has been attributed in part to adipose tissue dysfunction, which may occur locally within mammary fat pads (16); and that fetal mammary stem cells whose frequency peak at late embryogenesis (17) may constitute specific in utero targets of the maternal environment, there are undoubtedly additional mechanisms that may underlie mammary dysfunctions in offspring that are elicited by early maternal perturbations due to poor nutrition and/or obesity.

In the present study, we tested the hypothesis that early exposure to maternal metabolic stress due to poor nutrition has long-term consequences on progeny’s mammary and systemic phenotype and mammary tumor risk and evaluated a potential mechanism underlying these maternal effects.

Materials and methods

Animals and diets

Animal experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee, University of Arkansas for Medical Sciences. Mice were housed in polycarbonate cages under conditions of 24°C, 40% humidity and a 12 h light, 12 h dark cycle and were provided food and water ad libitum. Male mouse mammary tumor virus-Wnt-1 transgenic (MMTV-Wnt1-Tg) mice (B6SJL-Tg(Wnt1)1Hev/J) and wild-type (WT) females of the same strain were obtained from Jackson Laboratories (Bar Harbor, ME). MMTV-Wnt1-Tg female mice develop spontaneous mammary tumors within 5–6 months of age (18). To generate the offspring used for the experiments, WT females were randomly assigned to one of two American Institute of Nutrition-93G-based pelleted diets: (i) Control diet (CD) containing casein as the major protein source and 17% total kcal from fat (Harlan), and wild-type (WT) females of the same strain were obtained from Jackson Laboratories (Bar Harbor, ME). MMTV-Wnt1-Tg female mice develop spontaneous mammary tumors within 5–6 months of age (18). To generate the offspring used for the experiments, WT females were randomly assigned to one of two American Institute of Nutrition-93G-based pelleted diets: (i) Control diet (CD) containing casein as the major protein source and 17% total kcal from fat (Harlan), and wild-type (WT) females of the same strain were obtained from Jackson Laboratories (Bar Harbor, ME).
individual diets. Food and water were provided ad libitum. WT female mice were exposed to the diets beginning at weaning (postnatal day (PND) 21) and maintained on the same diet 12 weeks prior to mating and throughout pregnancy and lactation (Figure 1). Mating with Tg males generated pups which were either WT and Tg; the genotype of each mouse pup was confirmed for presence of the transgene (Wnt1-transgene by genomic DNA as described previously (20)). Female pups of either genotype were weaned to CD and used for subsequent analyses (Figure 1A). At killing coinciding with the weaning of pups, dams were analyzed for body and adipose depot weights and fasting glucose, and sera were collected (Figure 1A).

**Mammary tumor analysis**

Wnt1-Tg female offspring were weaned (PND21) to CD and followed for spontaneous mammary tumor formation as described previously (20,21). Briefly, mice were palpated twice weekly starting from 4 weeks until 6 months of age. Tumors were measured at initial detection using a caliper for length, width and height and 2 weeks later were excised, weighed and remeasured. Percent tumor growth was calculated based on tumor volume changes as described previously (20,21). Tumors were divided into two portions: one portion was immediately frozen in liquid nitrogen for real-time quantitative PCR (QPCR) or metabolite analyses, whereas another portion was fixed in formalin for histopathological analysis and immunohistochemistry. Tumor sections (5 μm) were stained with hematoxylin–eosin and examined by an experienced Veterinary Pathologist (Dr L. Hennings, Experimental Pathology Core Laboratory, UAMS). Supplementary Table 2 (available at Carcinogenesis Online) is a summary of the histopathological report of the tumor types for the diet groups. To evaluate tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) expression, paraffin-embedded tumor sections (n = 5) with adnexa were stained for specific protein presence. (Cell Signaling, Danver, MA) following the manufacturer’s protocols as described in our previous studies (20). Pictures were acquired and analyzed for nuclear staining using Aperio ImageScope (Vista, CA). Percent of nuclear PTEN immunopositive epithelial cells (relative to the total number of epithelial cells counted per ductal structure) was determined from 10 randomly selected fields per slide per mouse using Aperio-associated software. Mice which did not develop mammary tumors at 6 months of age were euthanized.

**Mammary tissue collection and analyses**

Mammary gland pairs #3 and #4 were harvested from PND30 WT and Tg offspring of CD-fed and HFD-fed dams. For WT offspring, the left mammary gland #4 was fixed for whole mounts analyses of fat pads as described previously (20). The total numbers of terminal end buds (TEBs), located at the leading edge of the fat pad, and the numbers of branching points within a box of fixed dimensions were counted from 4–5 mice of each diet group, each representing an individual dam. To determine mammary adipocyte size, right mammary gland #3 of WT offspring were cut into sections (5 μm) and stained with hematoxylin–eosin. Adipocyte areas were measured in 2–3 random fields per slide (n = 200–300 cells per field) from four individual mice per diet group, using Axiovision software (Carl Zeiss AG, Oberkochen, Germany), as described previously (22). For both WT and Tg mice, mammary gland #4 was homogenized in Trizol (Invitrogen, Carlsbad, CA) for gene expression analyses (below).

**Measurement of serum parameters**

Glucose levels of mice fasted for 2h were measured from tail vein blood by glucometer (One Touch; Lifescan, Milpitas, CA) using glucose strips (One Touch Blue; Lifescan). Blood samples for all other analyses were collected at killing and centrifuged at 7000 r.p.m. for 1 h at 4°C. Serum insulin, triglyceride, adiponectin, leptin, estradiol and tumor necrosis factor-α (TNFα) were measured using individual ELISA kits (n = 4–6 individual mice per group, each representing an individual dam) following the manufacturer’s instructions. The sources of these kits were as follows: (i) Rat/Mouse Inulin ELISA kit (Millipore Corp., Billerica, MA); (ii) Triglyceride Assay kit (Cayman Chemical Company, Ann Arbor, MI); (iii) Mouse Adiponectin ELISA kit (Millipore); (iv) Mouse Leptin ELISA Kit (Millipore); (v) Estradiol ELISA kit (Cayman) and (vi) Mouse TNF-α ELISA kit (R&D Systems, Minneapolis, MN).

**Oxidative stress biomarker analysis**

The concentrations of free aminothiol (glutathione reduced (GSH)/oxidized (GSSG); cysteine reduced/oxidized) and cellular methylation biomarkers S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) were determined as measures of redox/metabolic status in tumor or sera by using the high-performance liquid chromatography (HPLC) with coulometric Electrochemical Detection (HPLC-ED) system (MCM, Tokyo, Japan). The methodological details of these assays are previously published (23,24). Briefly, 100 μl of 10% meta-phosphoric acid was added to 200 μl of plasma or 100 μl tissue homogenate (prepared at a ratio of 10 mg wet tissue/100 μl of phosphate-buffered saline solution) to precipitate protein; the solution was mixed well and incubated on ice for 30 min. After centrifugation for 15 min at 18 000g at 4°C, supernatants were passed through a 0.2 μm nylon membrane filter and 20 μl were injected into the HPLC-ED system. All plasma or tissue metabolites were quantified using the associated HPLC-ED software.

In vitro studies—sera

To examine effects of sera from offspring of the two diet groups on mammary epithelial parameters, the human breast cancer cell line MCF-7 (American Type Culture Collection, Manassas, VA) was treated with sera (equally pooled from 4–5 individual PND30 Tg offspring, each representing a different dam-fed CD or HFD) at 2.5% (vol/vol) final concentration. The propagation and culture of MCF-7 cells in 5% CO2–95% air at 37°C followed previously described protocols (22). Cell proliferation/viability was quantified 48h after serum treatment using the trypan blue exclusion method and the Vi-Cell cell viability analyzer (Beckman Coulter, Atlanta, GA). The formation of mammospheres, a surrogate marker of stem/progenitor cell expansion (25), by MCF-7 cells was evaluated in 24-well low attachment plates as described previously (26). Mammospheres that formed 5 days post-plating were counted and mammosphere-forming units were expressed as a percent of total number of initially plated cells (2.5 × 104 cells per well). Gene expression of treated cells (2.5% sera; 48h) was evaluated by QPCR (below). All assays were repeated at least twice using the same pooled sera for treatments, with each experiment performed in duplicates (cell viability) or quadruplicates (mammosphere formation).

**Insulin and PTEN inhibitor treatments**

MCF-7 cells were plated at a density of 2.0 × 104 cells per 6-well plates, and 24h later were treated with 2 μM insulin (Sigma, St Louis, MO) or vehicle (phosphate-buffered saline). For treatments with the PTEN-specific inhibitor SFI1760 (Echelon Biosciences, Salt Lake, UT), cells were treated with pooled sera from CD offspring (CDO) [2.5% (vol/vol) final concentration] with and without added inhibitor (2 μM). Treated cells were collected 48h later and evaluated for gene expression by QPCR (below). Each assay was repeated twice, with each experiment performed in quadruplicates.

**Real-time quantitative PCR**

RNA isolation from mouse tissues and MCF-7 cells, complementary DNA preparation and QPCR analyses were performed following previously published protocols (26,27). Mouse and human primers (Supplementary Table 2A and B, available at Carcinogenesis Online, respectively) were synthesized by Integrated DNA Technologies (Coralville, IA) using Primer Express software (Applied Biosystems, Foster City, CA). Target messenger RNA expression was calibrated to a standard curve using pooled complementary DNA stocks and normalized to a factor that was derived from the geometric mean of expression for Tubulin-1 and binding protein (Tbp), beta-actin (Actb) and cyclophilin A (CypA), using GeNorm excel file software as described previously (28). QPCR was performed using SYBR Green and the ABI Prism 7000 Detection System (Applied Biosystems).

**Data analysis**

Data are presented as the mean ± standard error of the mean. Statistical significance between diet groups was evaluated by Student’s t-test using SigmaStat version 3.5 for Windows. A P value < 0.05 was considered to be statistically significant, with tendency for significance at 0.05 ≤ P ≤ 0.1.

**Results**

In utero and lactational exposure to HFD predisposed female progeny to increased mammary tumor risk

Epidemiological and animal studies from many laboratories including our own have demonstrated the ability of diet and dietary factors to modify breast cancer risk (12,20,21,29,30). However, the developmental period at which diet is most effective in exerting its effects (positive or negative) on mammary tumor formation remains largely unknown. To investigate the effect of exposure to maternal HFD alone on breast cancer risk, we used the MMTV-Wnt-1-Tg female offspring of dams fed either CD or HFD, the latter containing 45% of total kcal derived from fat, typical of standard ‘Western diet’. The MMTV-Wnt-1-Tg mouse model recapitulates deregulated mammary Wnt1 expression, a feature of >50% of human breast carcinoma (31). Dams were provided their respective diets for 12 weeks prior to mating and then throughout pregnancy and lactation. Female Tg offspring were subsequently weaned at PND21 to CD and followed...
Fig. 1. In utero and lactational HFD exposure increases mammary tumor incidence and shortens tumor latency in Wnt1-Tg female progeny. (A) Dietary regimen. WT dams were fed with AIN-93G-based diets (casein as sole protein source) containing 17% kcal from fat (CD) or 45% kcal from fat (HFD) beginning at postnatal day 21 (PND21; weaning) and were continued on these diets throughout pregnancy and lactation. Dams were mated with Wnt-1 Tg males to yield either WT or Tg progeny. At the cessation of lactation (weaning of pups), dams were killed and sera were collected for various endocrine and oxidative stress marker analyses. WT and Tg female offspring were subsequently weaned to CD. Body weight and serum hormones were analyzed in both WT and Tg progeny, whereas mammary gland parameters were analyzed in WT progeny, and the rest of Tg littermates were followed for mammary tumor formation. (B) Mammary tumor incidence of Tg female offspring of 7–8 dams fed either CD or HFD. Mammary tumor formation was observed weekly from 4 weeks to 6 months of age by palpation. *P < 0.05. (C) Age of tumor onset was calculated as the age of initial tumor appearance (by palpation) prior to 6 months of age. (D) Tumor weight was measured and normalized to body weight. (E) Change in tumor growth (%) was calculated as the difference in tumor volume from the time of initial detection to time of sac (2 weeks post-tumor detection). Data are presented as mean ± standard error of the mean (SEM); *P < 0.05 between diet groups.
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For mammmary tumor formation until 6 months of age. Mammary tumor incidence (Figure 1B) was significantly higher (42.9%; 6 of 14 mice) in HFD0 than in CDO (26.1%; 6 of 23 mice). Further, the mean age of tumor onset (Figure 1C) was significantly reduced in HFD0 (3.76±0.42 months) compared with CDO (5.22±0.28 months). Tumor growth rates (within 2 weeks from initial detection to sac) and tumor weights at sac of HFD0 and CDO did not differ (Figure 1D and E). Histopathological analysis revealed that while the same histological types of mammary tumors (solid and papillary carcinoma) were found for offspring of the two maternal diet groups, mammary tumors from HFD0 displayed a propensity to develop mammary tumors with metastatic potential (described as angiolymphatic invasion), which was not seen in CD counterparts (Supplementary Table 2, available at Carcinogenesis Online).

Maternal HFD reduced mammary tumor suppressor expression and promoted inflammation and oxidative stress biomarkers in Tg progeny

To determine the effects of maternal HFD exposure on the expression of select genes involved in tumorigenesis, transcript levels of tumor suppressor Pten, inflammatory mediator interleukin 6 (Ih6) and antiapoptotic B-cell lymphoma 2 (Bcl2) in mammary tumors of CDO and HFD0 were quantified by QPCR. The selection of these genes was based on our previous demonstration of their regulation by diet (21,27,32). Maternal exposure to HFD decreased mammary tumor Pten gene expression (Figure 2A) which was confirmed by immunohistochemistry as a ~20-fold reduction in nuclear protein levels (Figure 2B). Tumors from HFD0 showed lower expression of the antiapoptotic gene Bcl2 but comparable Ih6 expression when compared with CDO tumors. HFD0 tumors tended to have lower GSH to GSSG ratios (P = 0.06) and higher SAM/SAH ratios (P = 0.07) (Figure 2C), indicating increased oxidative stress and greater global methylation status, respectively (24,33). Further, sera from Tg offspring of dams-fed HFD0 showed modest but significant elevation in levels of TNFα, a proinflammatory cytokine associated with promotion of breast cancer risk (34).

Maternal exposure to HFD elicited early effects on mammary gene expression and promoted insulin resistance in Tg progeny

To determine if maternal HFD elicited early effects on mammary and endocrine parameters in HFD0 that may contribute to their increased mammary tumor risk as adults (Figure 1B and C), body weights, fasting blood glucose and systemic E levels were measured in prepubertal (PND30) Tg HFD0 and corresponding CDO. Body weights, blood glucose and serum E levels did not differ between CDO and HFD0 (data not shown). Mammary glands of PND30 Tg HFD0 showed lower Pten and Bcl2 gene expression, similar to HFD0 mammary tumors and significantly higher Ih6 gene expression, when compared with CDO tissues (Figure 2E). The gene expression changes were accompanied in HFD0 by a 2-fold increase in serum insulin levels (Figure 2F).

Maternal exposure to HFD altered mammary gland phenotype and influenced systemic parameters in prepubertal WT progeny

Limited exposure to high fat through maternal diet altered various body, endocrine and mammary epithelial parameters in WT female progeny. Although weaning body weights (at PND21) did not differ between WT HFD0 and CDO, the mean body weights of HFD0 exceeded that of CDO by PND30 and remained higher throughout adulthood (PND110) (Figure 3A). The increased adiposity of adult HFD0 was further supported by their higher abdominal depot mass and higher serum leptin and correspondingly lower serum adiponectin levels (Figure 3B). Prepubertal WT HFD0 showed higher blood glucose, elevated serum insulin and distinct mammary Pten, Bcl2 and Ih6 gene expression, relative to WT CDO (Figure 3C and D).

Although studies suggest that diet alters mammary gland morphogenesis (20,35), there is little information on the magnitude of effects due to early and limited exposure to specific diets. To address maternal HFD effects on early mammary gland development in progeny, mammary glands of PND30 (prepubertal) WT female CDO or HFD0 were evaluated by whole mount and by measurement of fat pad adipocyte sizes. Representative carmine-stained whole mounts of mammary fat pads for each dietary group are shown (Figure 3E). HFD0 showed significantly increased TEBs but comparable branching density in mammary glands when compared with CDO. The adipocyte sizes in mammary glands of HFD0 and CDO were quantitatively analyzed; representative pictures of hematoxylin and eosin-stained sections of the mammary fat pads for each dietary group are shown (Figure 3F). A majority (~80%) of mammary adipocytes in the HFD0 was in the size range of >2500 μm²; in contrast, only ~30% of the adipocytes in the CDO fell within this range (Figure 3F).

Dietary high fat promotes systemic oxidative stress and higher fasting blood glucose in dams

The striking effects of the limited exposure to maternal HFD on mammary phenotype (WT offspring), mammary tumor risk (Tg offspring) and systemic parameters (WT and Tg offspring) suggest diet-induced changes in dams that elicited these long-term modifications in offspring. To address this, we evaluated body and fat depot weights, fasting glucose and serum hormone, triglyceride and oxidative stress biomarkers of dams on CD and HFD at the time their pups were weaned (Figure 1A). The HFD-fed dams did not differ in body weights from those of CD-fed dams at all the time points evaluated (Figure 4A). The latter was reflected in the comparable depot mass (abdominal, retroperitoneal) between dams of the two diet groups at killing (Figure 4B). The duration of pregnancy (18–19 days), mean litter size (5–7 pups/litter) and ratio of male to female pups did not differ between the two groups (data not shown). Blood glucose was significantly elevated in HFD0-fed when compared with CD-fed dams (Figure 4C), but serum insulin, estradiol and leptin (Figure 4C) and serum triglyceride (data not shown) did not differ between dams of the two diet groups. However, serum levels of GSSG and cysteine differed for dams fed CD and HFD, with the latter group showing lower GSH/GSSG ratio and higher cystine/cysteine ratio than those fed CD (Figure 4D and F).

HFD0 sera mimic insulin- and PTEN-specific effects on mammary carcinoma cells

To begin to identify mechanism(s) underlying the long-term effects of maternal HFD on progeny’s mammary health, we hypothesized that systemic factors altered by maternal HFD in offspring could function to mediate mammary epithelial changes leading to increased tumor risk. We utilized the human breast cancer MCF-7 cell line as an in vitro model since similar to Wnt-Tg tumors, these cells are well differentiated and contain a small population of mammary stem/progenitor cells (within the basal epithelial subpopulation) that grow in suspension as spheres (mammospheres) and can give rise to mammary tumors (25,26). Sera pools from HFD0 and CDO (n = 4–5 PND30 Tg mice per diet group; each mouse representing an individual dam) were added to MCF-7 cells at 2.5% (vol/vol) final concentrations. Cells treated with HFD0 sera showed greater cell viability (Figure 5A) and increased numbers of mammosphere-forming units (Figure 5B) than corresponding cells treated with CDO sera. Since maternal gene expression differed in tumors from HFD0 and CDO in vivo, we measured PTEN, BCL2 and IL6 transcript levels in treated cells. The downward trend in PTEN and BCL2 and the higher IL6 transcript levels in cells treated with HFD0 relative to CDO sera (Figure 5C) are consistent with those found for HFD0 tumor and PND 30 mammary tissues (Tg, WT) (Figures 2 and 3). We also evaluated the expression of downstream effectors of insulin action namely insulin receptor substrate-1 (IRS-1) and IRS-2 since reduction in PTEN expression characterized HFD0 mammary tissue and tumors, we treated cells with CDO sera alone and with added PTEN-specific inhibitor SFI670 (2 μM), which binds to the PTEN active site, resulting in elevated phosphatidylinositol (3,4,5) triphosphate signaling (36). We found that inhibition of PTEN activity
Fig. 2. Maternal HFD exposure modifies tumor phenotype in adult Wnt1-Tg progeny and mammary and systemic phenotypes in PND30 (prepubertal) Wnt1-Tg progeny. (A) Transcript levels of tumor suppressor (Pten), antiapoptotic marker (Bcl2) and proinflammatory cytokine (Il6) were quantified by QPCR in mammary tumors from Wnt1-Tg mice exposed to CD or HFD via their respective dams. Data are mean ± SEM of fold-change in normalized expression between CDO and HFDO (n = 5 offspring per maternal diet group, with each offspring representing an individual dam). (B) Percentage of tumor suppressor PTEN nuclear-staining cells in mammary tumors. Magnification = ×20; scale bars = 100 μm. Designations of high, medium and low refer to relative staining intensities, quantified by Aperio software as described under Materials and methods. (C) Tissue levels of GSH and GSSG, SAM and SAH are presented as calculated ratios of GSH/GSSG and SAM/SAH in mammary tumors of Wnt1-Tg offspring. (D) TNFα levels were quantified in sera of HFDO or CDO with tumors, at killing. (E) Transcript levels for Pten, Bcl2 and Il6 were evaluated by QPCR in PND30 (prepubertal) mammary glands of Wnt1-Tg CDO or HFDO. Data are mean ± SEM of fold-change in normalized expression to CD offspring. (F) Serum insulin levels were quantified in sera of PND30 Wnt1-Tg HFDO or CDO as described in Materials and methods. For C–F, results (mean ± SEM) are from n = 5–6 offspring/maternal diet group, with each offspring representing an individual dam. *P < 0.05 between diet groups.
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Fig. 3. Maternal HFD exposure alters metabolic phenotype and normal mammary gland development in PND30 (prepubertal) WT offspring. (A) Body weights of WT offspring of the two maternal diet groups with increasing age. Results (mean ± SEM) are from n = 12–15 mice/diet group, representing at least five independent dams for each diet group. *P < 0.05 between diet groups. (B) Abdominal fat of individual mouse (at PND 110) for each maternal diet group were dissected, weighed and normalized to body weight. Serum leptin and adiponectin (AdipoQ) levels were subsequently quantified; ratios of leptin to AdipoQ were calculated from the obtained values. Results shown are expressed as mean ± SEM from n = 5–7 mice/diet group, with each mouse representing an individual dam. *P < 0.05 between diet groups; #P = 0.07. (C) Fasting blood glucose and serum levels of insulin were quantified at killing from PND30 WT female offspring of the two maternal diet groups (n = 5–6 mice/diet group, with each offspring representing an individual dam). *P < 0.05 between diet groups. (D) Transcript levels for Pten, Bcl2 and Il6 were evaluated by QPCR in mammary glands of PND30 WT CDO or HFDO. Data are mean ± SEM of fold-change in normalized expression to CDO (n = 10 offspring/diet group, with each offspring representing an individual dam). *P < 0.05 between diet groups. (E) Representative whole mounts of mammary glands from PND30 WT pups upon exposure (in utero through lactation) to maternal CD or HFD. Scale bar = 300 μm. Mammary branching density and total numbers of TEBs were quantified. Data are mean ± SEM from analyses of whole mounts from n = 5 offspring per diet group, with each offspring representing an individual dam. *P < 0.05 between diet groups. (F) Representative hematoxylin-eosin-stained sections of mammary fat pads of PND30 WT mice from the two maternal diet groups. Magnification = ×40. Distribution of mammary adipocyte sizes (expressed as percent from total numbers of adipocytes counted) differed between offspring of the maternal diet groups (n = 5 mice/diet group, with each offspring representing an individual dam). *P < 0.05 between diet groups.
Fig. 4. Exposure of dams to HFD from weaning through first pregnancy and lactation increases systemic oxidative stress and fasting blood glucose in the absence of overt obesity. (A) Maternal body weights recorded at different ages during dietary exposure. (B) Fat pad depot weights (abdominal and retroperitoneal) were measured for dams of the two diet groups. (C) Serum levels of various hormones and fasting blood glucose were measured for dams of the two diet groups. *P < 0.05 between diet groups. (D and E) Concentrations of aminothiols glutathione (reduced and oxidized) and cysteine (reduced and oxidized) were measured by HPLC-ED in sera of dams exposed to either CD or HFD. Inset, GSH/GSSG and cystine/cysteine ratios for each diet group. Data (mean ± SEM) are from n = 5–8 dams per diet group. *P < 0.05 between diet groups.
Fig. 5. Serum factors from HFDO modify growth, mammosphere-forming ability and gene expression of human MCF-7 breast cancer cells. (A) Addition of pooled sera from PND30 (prepubertal) Tg HFDO at 2.5% (vol/vol) final concentration to MCF-7 cells increased the numbers of viable cells when compared with treatment with pooled sera from same-aged CDO. Results (mean ± SEM) are from two independent experiments, each experiment carried out in duplicates, *P < 0.05 between diet groups. (B) Sera from offspring exposed to maternal HFD, when added at 2.5% (vol/vol) final concentration to plating medium, increased mammosphere formation of MCF-7 cells relative to CDO sera. Passage 1 (P1) spheres were manually counted after 5 days. Data (mean ± SEM) represent the number of P1 mammospheres (per 2500 plated cells) from n = 2 independent experiments, with each experiment carried out in quadruplicates. *P < 0.05 between diet groups. (C) Transcript levels for PTEN, BCL2, IL6, IRS-1 and IRS-2 were evaluated by QPCR in MCF-7 cells treated with sera from PND 30 Tg offspring. Data are mean ± SEM of fold-change in normalized expression to CD offspring sera treatment. #P = 0.08; *P < 0.05, between diet groups. (D) Transcript levels for PTEN, BCL2, IL6, IRS-1 and IRS-2 were analyzed in MCF-7 cells treated with sera from CDO in the absence or presence of 2 μM PTEN-specific inhibitor SF1670. Data are mean ± SEM of fold-change in normalized expression to CDO sera treatment alone. *P < 0.05 between treatment groups. (E) The expression of the same set of genes (D, above) was determined by QPCR in MCF-7 cells without or with 2 μM insulin treatment. Data are mean ± SEM of fold-change in normalized expression to MCF-7 cells treated with vehicle. *P < 0.05 between treatment groups.
(recapitulating reduced PTEN expression in HFDO tumor and mammary tissue) resulted in lower BCL2, higher IL6 and IRS-1 and unaltered IRS-2 expression, relative to control (CDO sera) (Figure 5D). To mimic the hyperinsulinemia in HFDO, we treated MCF-7 cells with 2 μM insulin. Treatment with insulin for 48 h had no effect on PTEN and IL6 transcript levels, but resulted in a significant increase in both IRS-1 and IRS-2 transcript levels (Figure 5E). These results suggest that maternal HFD-elicted systemic changes in offspring may underlie increased mammary tumor risk and implicate PTEN loss-of-function and hyperinsulinemia as contributing to these maternal effects.

Discussion

We report here that exposure (in utero and lactation) to maternal HFD, in the absence of overt obesity in dams, modified mammary phenotype, enhanced mammary tumor risk and altered systemic profiles, in offspring. Using WT and Tg littersmates, we exploited spontaneous mammary tumor formation in Tg offspring (18) and normal mammary gland development in corresponding WT littersmates to identify common pathways that may be perturbed by early (maternal) metabolic deregulation. Further, using the well-differentiated MCF-7 breast cancer cells as in vitro model, we evaluated a potential mechanism by which systemic changes in offspring conferred by perturbations in maternal metabolic status and glucose homeostasis may promote breast cancer risk. Our collective data suggest a model (Figure 6) in which hyperinsulinemia and PTEN loss-of-function couple maternal HFD exposure to increased tumor insulin sensitivity via upregulation of IRS-1 expression. Although it is presently unclear how impaired maternal metabolic status may directly elicit the systemic changes in the absence of dramatic weight gains in offspring, one possibility is that changes in maternal metabolites, the latter acting as cellular substrates for many enzymes including DNA methylation and histone-modifying enzymes to epigenetically control chromatin modification patterns (37,38), may alter the transcripational output of endocrine and inflammatory networks during fetal development. Our findings that IRS-1 constitutes a potential target of systemic changes in progeny exposed to maternal HFD are consistent with insulin and insulin-like growth factors as major promoters of breast cancer (39,40).

The experimental paradigm utilized in these studies differed from previous studies that examined maternal high-fat dietary effects on mammary gland biology and pathology, in several ways. First, we utilized a mouse model of breast cancer (MMTV-Wnt-1-Tg mice) which develops spontaneous mammary tumors (18), rather than 7,12-dimethylbenz(a)anthracene-induced rat models of mammary tumorigenesis (13). Second, HFD was provided to female mice destined to become dams, from weaning through mating, pregnancy and lactation to simulate the population of women who experience a lifetime of poor nutrition based on the regular consumption of a ‘Western diet’. Third, the amount of high fat provided in the diet (45% kcal from fat) is more akin to the fat content in ‘Western diets’ (19), in contrast to the higher amounts (60% kcal from fat) used in many animal studies (13). Finally, the simultaneous analyses of WT and Tg littersmates from the same dams of the two diet groups allowed for evaluation of dietary effects on common pathways involved in normal mammary biology and tumorigenesis.

Our data demonstrate that maternal HFD elicited similar effects on WT and Tg progeny, suggesting that changes in mammary and systemic phenotypes may have been established early during development leading to increased tumor risk as adults. Notably, we found that key genes associated with tumor promotion (IL6) and inhibition (Pten) were comparably altered in WT and Tg prepubertal (PND30) mammary glands and that hyperinsulinemia is a feature of maternal HFD progeny, regardless of genotype. The decrease in expression of the antiapoptotic gene Bcl2 with HFD is unexpected, given our previous demonstration that diet with documented health benefits (e.g. soy proteins, blueberry) attenuated Bcl2 transcript levels (20,32). However, this present observation may be related to the enhanced oxidative stress status observed with HFD exposure, since reactive oxygen species can sensitize cells to apoptosis by decreasing BCL2 expression (41). The early changes in mammary gland phenotype observed at prepuberty (PND30) with HFD exposure, which include increased TEB numbers, enhanced adipogenesis and reduced tumor suppressor Pten expression, constitute early markers of tumor development in adults. TEBs are highly sensitive to malignant transformations (42) and are the site of localization of basal-like epithelial subpopulations (mammary stem/progenitor cells), which when deregulated, can give rise to tumors (43,44). Moreover, adipocyte size is positively associated with insulin resistance, subclinical inflammation, higher leptin and lower adiponectin expression (45,46), all of which have been linked to increased risk of breast cancer. Finally, PTEN loss-of-expression is a hallmark of breast tumorigenesis (47).

Chronic consumption of HFD has been shown to induce body weight gain resulting in significant increases in insulin, blood glucose and insulin resistance (48). Interestingly, dams-fed HFD in this study were neither hyperphagic, obese nor hyperinsulinemic after 12 weeks of high fat intake. Nevertheless, the higher fasting blood glucose levels, suggestive of insulin resistance and pancreatic beta-cell dysfunction in HFD dams, imply that their offspring were persistently exposed to high glucose and which may account for the latter’s metabolic syndrome-like phenotype (i.e. higher serum insulin) as adults. High concentrations of oxidant molecules in the mother’s blood may directly affect fetal DNA (49) and may be passed on to offspring by fetomaternal placental transfer (50). Indeed, the lower GSH/GSSG and higher SAM/SAH ratios in mammary tumors of HFDO are indicative of enhanced pro-oxidative and compromised global methylation status, the latter suggesting potential epigenetic underpinnings. A limitation to the present study is the restricted assessment of metabolic/oxidative stress biomarkers to post-lactation in dams. Thus, we may have missed the ‘window’ (e.g. pregnancy, lactation) at which more dramatic changes in maternal metabolic status may have occurred and which may be more highly associated with the striking phenotypic changes noted in offspring. Future studies will address these important questions.
Our results suggest the novel concept that the negative long-term consequences of maternal HFD in offspring may converge on the regulation of sensitivity to insulin/insulin-like growth factor-1 signaling. The mechanisms inducing IRS-1 expression in tumor cells with early exposure to HFD are currently unknown but possibly involve epigenetic mechanisms. In a previous study, insulin acting via its cognate receptor was reported to inhibit PTEN transcription and protein levels in MDA-MB-231 breast cancer cells (51). We found that while HFDO sera inhibited PTEN expression, insulin which is present at higher levels in sera of HFDO than CDO, had no effect on PTEN transcript levels after acute (1 h; data not shown) or long-term (48 h) exposure, implying that other serum-associated factors may regulate PTEN expression in the more differentiated MCF-7 cells. Alternatively (or in addition), insulin sensitivity may differ for well-differentiated versus highly invasive mammary tumor cells. The limited exposure (48 h) at less than full-strength (2.5%) serum concentration compared with chronic systemic exposure may account for the noted tendency for significant (rather than highly significant) differences between CDO and HFDO serum effects in treated cells. Although PTEN loss-of-function (via SF1760) and hyperinsulinaemia similarly reduced BCL2 and IRS-1 expression, PTEN is not likely a direct target of insulin since increased IL6 transcript levels were specific to the PTEN inhibitor while augmented IRS-2 expression is solely altered by the hyper-insulinemic dose. We suggest that cross-talk between hyperinsulinaemia and PTEN dysfunction in the promotion of breast cancers may converge at IRS-1, raising the latter’s potential as a candidate therapeutic target for addressing breast cancer. Interestingly, PTEN was previously reported to upregulate IRS-2 expression in the highly invasive MDA-MB-238 cells, albeit the functional outcome is decreased Akt signaling (52), concordant with the tumor suppressor function of PTEN. Moreover, ectopic expression of WT PTEN in MCF-7 cells inhibited insulin action by hindering IRS-1 phosphorylation in the absence of effects on IRS-1 expression (53). The mechanistic promotion of IRS-1 expression by hyperinsulinaemia and PTEN loss-of-function as a consequence of maternal HFD warrants further investigation.

In summary, our studies utilizing prepubertal and adult WT and Tg HFDO provide strong support to the early origins of adult breast cancer. Maternal metabolic perturbations and an impaired redox system elicited by HFD and occurring in the absence of overt obesity can induce myriad mammary-specific and systemic changes in progeny that may converge at the level of dysfunctional growth factor signaling. Data from this study highlight the importance of proper maternal nutrition as a tractable strategy to break the cycle of breast cancer and have important implications for developing novel strategies for the prevention of maternal HFD-induced breast cancer.

Supplementary material

Supplementary Tables 1–3 can be found at http://carcin.oxfordjournals.org/

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