Poor early growth and excessive adult calorie intake independently and additively affect mitogenic signaling and increase mammary tumor susceptibility


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We previously showed that offspring of rat dams receiving a protein-restricted (low protein) diet throughout pregnancy and lactation develop mammary tumors more quickly. Rapid post-weaning mammary growth and mammary tissue overexpression of insulin receptor, insulin-like growth factor-1 receptor (IGF-1R), estrogen receptor isoform alpha and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2), correlated with this risk. The objectives of this study were therefore (i) to identify underlying mechanisms of increased risk through candidate and global approaches; (ii) to determine if excessive calorie intake further increased risk and if so, (iii) to identify the molecular mechanisms mediating this. We provide evidence for transcriptional upregulation of IGF-1R by Sp1 in LP mammary tissue (P < 0.01). Cell cycle control and DNA damage repair gene cyclin-dependent kinase inhibitor 1A (CDKN1A) (p21Waf1) was also up-regulated (P < 0.05) as was transcription factor nuclear factor of kappa light polypeptide gene enhancer in B-cell (P < 0.05) and adhesion gene CDH1 (P < 0.05). Invasion and metastasis markers matrix metalloproteinase 9 and serpin peptidase inhibitor, clade E, member 1 (SERPIN1) were upregulated (both P < 0.05), whereas metastasis suppressor gene NME1 was downregulated (P < 0.01). Feeding a highly palatable diet (HPD) to increase calorie intake from puberty, additively and independently increased early mammary tumor risk, which correlated with increased serum insulin and triglyceride concentrations (P < 0.05). PTEN gene expression was reduced both by early protein restriction (P < 0.05) and HPD (P < 0.01), which may induce Akt in cell survival pathways. Progesterone receptor and ERBB2 (both P < 0.05) expression increased as an effect of an interaction between maternal diet and adult nutrition, with subsequent downstream activation of the mitogen-activated protein kinase pathway. We conclude that poor early growth and excessive calorie intake exert independent and additive effects on mitogenic growth factor signaling to influence mammary tumor susceptibility.

Introduction

Breast cancer remains the most common cancer found in women. Understanding its pathogenesis and identifying key risk factors is therefore of great social and therapeutic importance. Recent studies have suggested that there is a relationship between breast cancer and raised fasting glucose and peripheral insulin resistance (characteristics of type-2 diabetes) (1–3). Although adult obesity has also been associated with increased risk of breast cancer for post-menopausal women (4), it is also associated with a decreased risk of pre-menopausal breast cancer (5,6). More recent studies have found, however, that a childhood and adolescent diet high in fat may contribute to increased risk of breast cancer (7,8) and there is evidence to suggest that this risk is specifically associated with central obesity (9). Other studies have found that a greater waist-to-hip ratio, a robust indicator of central/abdominal obesity, is associated with increased risk of breast cancer regardless of menopausal status (10). The association of waist to hip ratio with type-2 diabetes is well established. The clustering of obesity, insulin resistance and breast cancer (11–13) has led us to suggest that these pathologies may share a common origin.

One possibility is poor early growth. Low birth weight has been linked to the increased risk of insulin resistance, type-2 diabetes (14), central adiposity (15) and breast cancer (16–18). Although a number of studies have correlated high birth weight with an increased breast cancer risk (16–19), some of these studies have shown that low birth weight (<2500g) is also positively associated with early-onset (pre-menopausal) breast cancer (16–18). Thus, both high and low birth weights appear to be risk factors for breast cancer. Recent epidemiological studies suggest that children who are born small for gestational age have a predisposition to accumulating fat mass, particularly intra-abdominal fat (20). There is also data on adiposity rebound in cohorts from the Avon Longitudinal Study of Parents and Children study (21), which is reportedly due to overfeeding through milk formula intake. The children in this study are, however, still very young and long-term consequences on breast cancer incidence are yet unknown. There is also data suggesting that maternal dairy protein intake influences birth weight in a contemporary Danish cohort (22). However, again, this cohort is still too young to study for breast cancer incidence.

The mechanistic basis of the relationships between low birth weight and adult diseases is not known though human and animal studies suggest that the fetal/early postnatal environment is important. In particular, early nutrition has been identified as a critical component, particularly in relation to low birth weight. The ‘Thrifty Phenotype’ hypothesis proposes that a conflict between poor early nutrition and excess adult nutrition may provide the basis. Studies of individuals who were in utero during the Dutch Hunger Winter (23) and more recent reports suggest that the highest risk for diabetes or impaired glucose tolerance is seen where poor early growth in utero is followed by accelerated postnatal growth starting early in life (24) and with adult obesity (25). Most recent studies confirm an increased breast cancer risk in this same Dutch cohort, supporting the suggestion that poor early nutrition followed by adequate/excess nutrition in adulthood increases breast cancer risk (25,26).

The association between diabetes and breast cancer is postulated to act via three mechanisms, i.e. (i) activation of the insulin pathway, (ii) activation of the insulin-like-growth-factor (IGF) pathway and (iii) regulation of endogenous sex hormones (27). We have used the model of maternal protein restriction during pregnancy and lactation [low protein (LP) model] to dissect the molecular mechanisms of these associations because the offspring of LP mothers have low birth weight (28,29) and they develop hyperinsulinemia with age (30). In a longitudinal study, we found that an early retardation of mammary gland development was followed by rapid compensatory structural development in the week following weaning (31) characterized by increased epithelial density and terminal end bud number and therefore an increased number of targets for environmental or other carcinogens. At the molecular level, both insulin receptor (IR) and IGF-1

Abbreviations: CI, confidence interval; ERx, estrogen receptor isoform alpha; ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; HR, hazard ratio; HPD, highly palatable diet; HPRT, hypoxanthine guanine phosphoribosyl transferase; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor-1 receptor; IR, insulin receptor; JAK2, Janus kinase 2; LP, low protein; mRNA, messenger RNA; NMU, N-nitroso-N-methyleurea; PCNA, proliferating cell nuclear antigen; PR, progesterone receptor.

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receptor protein was overexpressed in whole tissue and in enriched epithelial cell fractions (31). Furthermore, we showed that female LP offspring administered with N-nitroso-N-methylurea (NMU) demonstrated an increased susceptibility to mammary tumors compared with control offspring reared by mothers fed a standard laboratory chow during pregnancy and lactation (31).

Insulin and IGF-1 are both involved in early mammmogenesis. IGF-1 has been shown to be important in normal mammary gland development, in particular terminal end bud development and ductal morphogenesis (32). More significantly, it is involved in the transition from normal mammary development to preneoplastic mammary lesions (32) and transgenic expression of IGF-1 receptor results in tumour development (33). Experimental overexpression of the IGF-1 receptor also induces transformation (34), whereas transgenic mouse models of breast cancer induced by the oncogenes Wnt-1, Neu and Ret all demonstrate an elevated IR content (35).

In the current study, our aims were to determine the upstream mechanisms responsible for the protein overexpression of our primary candidates insulin-like growth factor-1 receptor (IGF-1R) and IR during the period of rapid mammary growth. IGF-1R was found to be overexpressed at the transcript level, potentially through transcriptional activation by Sp1, which was overexpressed. Downstream of IGF-1, Janus kinase 2 (JAK2) transcript overexpression coincided with increased protein levels of the proliferative marker proliferating cell nuclear antigen (PCNA). We also determined that increased IR protein levels might be controlled by increased transcription of both the IR-A and IR-B messenger RNA (mRNA) isoforms. A global screen identified cancer pathway genes altered in LP offspring mammary tissue suggestive of an oncogenic signature.

We further postulated that feeding a highly palatable diet (HPD) would induce hyperinsulinemia, thus adding to mammary tumor risk in LP offspring by increasing the bioavailability of ligands such as insulin and/or IGF-1 thereby promoting ‘metabolic carcinogenesis’ through increased signaling in the mitogenic pathway. Indeed, when challenged by a HPD, Control-HPD and LP-HPD rats gained excess weight and both groups demonstrated higher susceptibility to chemically induced mammary tumors compared with their Chow-fed siblings, with the LP-HPD group showing highest susceptibility. The effects of poor early growth and excess calorie intake on mammary tumor risk were therefore independent and additive.

Materials and methods

Animal model

All experimental procedures involving animals were approved by the Local Ethical Review Committee and carried out under the British Home Office Animals (Scientific Procedures) Act, 1986 and under the guidance of The United Kingdom Coordinating Committee on Cancer Research’s ‘Guidelines for the Welfare of Animals in Experimental Neoplasia’ (Second Edition, 1997). Virgin female Wistar rats weighing 240–260 g, housed individually and maintained at 22°C on a 12 h light–dark cycle, were mated and Day 1 of gestation taken as the day on which vaginal plugs were expelled. Pregnant dams were randomized into two dietary groups, Control and LP, which were fed ad libitum. The control dams received a 20% protein diet and the LP group received an isonenergetic diet containing 8% protein, as described by Snoeck et al. (36), throughout gestation and lactation.

At birth, litters were standardized to four males and four females, which were left to suckle for 21 days. At 21 days, offspring of both Control and LP groups (n = 8) were weaned onto laboratory chow containing 20% protein (rodent diet LAD 1 from Special Diets Services, Witham, UK) fed ad libitum. To investigate mechanisms of increased mammary tumor risk arising from maternal protein restriction, animals were studied at 5 weeks of age, prior to any previously observed tumor development (31).

Effects of excessive calorie intake

To establish the combined effects of maternal protein restriction and adult excessive calorie intake, Control and LP offspring groups were set up as before. However, from 7 weeks of age (postpuberty), half of the female offspring from both Control and LP groups were fed a HPD (n = 8 per maternal dietary group). This HPD was formulated by combining 330 g/kg powdered chow, 330 g/kg Nestle’s full-fat sweetened condensed milk, 70 g/kg sucrose and 270 g/kg water. The other half of each litter remained on chow. The energy content of both chow and HPD diets is detailed in supplementary Table 1 (available at Carcinogenesis Online). Animal body weights were measured weekly until the end of the study (16 weeks).

Mammary gland isolation

Female rats were killed by rising concentration of CO2 and the fourth pair of mammary glands excised, taking care to exclude the lymph nodes, and the tissue flash frozen in liquid nitrogen and stored at –80°C until further use for RNA and protein expression analysis.

RNA isolation

Total RNA was isolated by Trizol (Sigma-Aldrich, Poole, UK) extraction, precipitation in isopropanol with subsequent DNase treatment (QIAEN, Crawley, UK) and purification using QIAGEN RNeasy columns (QIAGEN). RNA concentration was determined by spectrophotometry (NanoDrop, Wilmington, DE) and 500 ng total RNA from individual sample preparations reverse transcribed using random hexamers and MMTV Reverse Transcriptase (Promega, Southampton, UK).

Polymerase chain reaction array

The expression of 84 genes implicated in rat cancer was analyzed using the RT² Profiler Rat Cancer PathwayFinder PCR Array (SuperArray Bioscience, Frederick, MD). Complementary DNA was prepared from 2 μg pooled RNA isolated from 5 week mammary glands of Control and LP rats (n = 8 per group) according to manufacturer’s instructions (SuperArray Bioscience) then incubated with RT² Real-Time PCR master mix and then each sample applied to one of two identical batch controlled polymerase chain reaction (PCR) arrays containing primer sets for 84 pathway-specific genes and five housekeeping genes as well as controls to verify the linear dynamic range of the assay. Thermal cycling was carried out for 40 cycles and threshold cycle (Ct) values entered and fold-changes in gene expression calculated by a pair-wise comparison using the ΔΔCt method.

Quantitative real-time PCR

Primer sets used for reverse transcription–PCR validation of mRNA expression as an effect of maternal protein restriction are listed in supplementary Table 2 (available at Carcinogenesis Online), whereas the effects of maternal protein restriction and postpubertal HPD diet on gene expression were studied using primers listed in supplementary Table 3 (available at Carcinogenesis Online). All primers were designed (ProbeFinder, Roche Applied Science, Burgess Hill, UK) to yield a single amplicon between 75–150 bp when subject to PCR with primers spanning an intron. Quantitative real-time PCR was performed with the SYBR Green detection system (Applied Biosystems, Carlsbad, CA) using an ABI Prism 7900 sequence detector and under thermal cycling conditions of preincubation (50°C, 2 min); DNA polymerase activation (95°C, 1 min) and 40 PCR cycles for 15 s at 95°C, 1 min at 95°C and 1 min at 60°C. Standard curves were generated by serial dilution of pooled total complementary DNAs prepared from all groups. Each sample was tested in duplicate, and mRNA levels were normalized to hypoxanthine phosphoribosyl transferase (HPRT), cyclophilin and porphobilinogen deaminase (PBGD) as housekeeping genes.

Tumor susceptibility

Ninety-six control (n = 24 litters) and 96 LP offspring (n = 24 litters) were bred as described previously. Forty-eight from each group (half of each litter) were randomized to receive either standard chow or HPD from 7 weeks of age. All groups received NMU as described previously (31). Briefly, NMU (50 mg/kg in saline) was administered by intraperitoneal injection at 3, 4 and 5 weeks of age. Three doses were required due to the low incidence rate of the Wistar strain. Rats were then monitored for tumors weekly. Tumors were allowed to grow to no > 2 cm diameter, at which point the animal was killed and both tumor and non-tumor tissue collected for histological and molecular analysis.

Fasting plasma analysis

Blood glucose was measured from tail blood at postmortem using a blood glucose analyser (Hemocue, Shefield, UK). Plasma insulin and IGF-1 were measured using ELISA kits from Merodia and GroPep, respectively (distributed by Immunodiagnostic Systems Limited, Boldon, UK). Lipid analysis was carried out by the Core Biochemical Assay Laboratory, Department of Clinical Biochemistry, Addenbrooke’s Hospital, Cambridge, UK.

Histology

Tumor samples were fixed in formalin for 24–48 h before transferring to 70% ethanol. Samples were then processed to wax and sectioned for hematoxylin and eosin staining. Sections were examined and classified for tumor type and grade.
Western blot analysis

Mammary tissue was ground to a powder in a mortar and pestle on dry ice and ~250 mg powder homogenized in 1 ml lysis buffer [50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (pH 8), 150 mM NaCl, 1% Triton X-100, 1 mM Na3VO4, 30 mM NaF, 10 mM Na2P2O7 and 10 mM ethylenediaminetraacetic acid with protease inhibitors (Calbiochem, Cambridge, UK)]. Total protein concentration in lysates was determined by a copper/bicinchoninic assay (Sigma-Aldrich, Poole, UK). Twenty micrograms of each sample (each animal and each litter being represented by one sample per well) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and proteins transferred onto polyvinylidene difluoride Immobilon-P (Millipore, Billerica) membrane. Primary antibodies included: P-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (Abcam); estrogen receptor isoform alpha (ERα) (Novocasa, Newcastle Upon Tyne, UK); estrogen receptor isoform beta (ERβ) (Abcam) and progesterone receptor (PR) (Novocasa, Newcastle upon Tyne). This was followed by incubation with horseradish peroxidase-linked secondary antibodies (Jackson ImmunoResearch, Strathe, UK). Immunoreactivity was detected by chemiluminescence (Super West Pico; Pierce, Rockford, IL) and protein expression assessed by spot-densitometry using AlphaEase (AlphaImager, Santa Clara). All 24 samples from both maternal diet groups and postpubertal diet groups (n = 6 samples for each of the Control and LP groups fed chow or HPD), molecular weight markers and positive controls were loaded on the same gel. This allowed direct comparisons between each of the four groups.

Statistical analysis

Statistical analyses for western blotting were carried out using Statistica (StatSoft, Tulsa, OK). The significance of any difference between groups was examined by two-tailed unpaired Student’s t-tests (5 weeks samples) or by two-way analysis of variances followed by Duncan’s post-hoc tests where appropriate (16 weeks samples) and results presented as mean values and standard errors. Data that was not normally distributed or for which standard deviations were statistically different were analyzed by non-parametric tests and presented as medians and inter-quartile ranges. Body weights over time were analyzed by repeated measures analysis of variance. Absolute probability (P) values <0.05 were considered statistically significant. The number of animals in each group (all from separate litters) is indicated by n.

PCR array gene expression of LP tissue was expressed as fold expression of Controls. Individual gene expression in 5 weeks and 16 weeks samples was normalized to housekeeping genes and plotted as relative expression. Protein levels by western blotting were expressed as a percentage relative to Controls for the 5 weeks analysis and as a percentage relative to Chow-fed Controls for the 16 weeks analysis.

For NMU tumor induction studies, mammary tumor incidence was expressed as percentage of rats developing mammary tumors 0–16 weeks post-NMU treatment. The hazard ratio (HR) of incidence [95% confidence interval (CI)] was calculated using EpInfo (version 3.3).

Results

Five weeks mammary gland protein and gene expression analysis

IGF-1R transcript levels were increased in LP mammary glands compared with Controls (Figure 1A, P < 0.05). This was accompanied by a significant increase in transcriptional expression of Sp1 (P < 0.01) but not of either bcral or p53 (Figure 1A). Increased IGF-1R and Sp1 transcript were related to increased protein expression of both Sp1 and IGF-1R (Figure 1B). Neither p53 nor Wilm’s tumor-1 (WT-1) protein expression was affected by maternal protein restriction (Figure 1B). PCNA and mTOR protein expression was increased in LP mammary gland (P < 0.01 and P < 0.001, respectively; Figure 1C). Non-canonical signaling downstream of IGF-1 via the JAK/STAT pathway was measured by JAK2 and STAT5a mRNA expression. JAK2 transcript was upregulated in LP offspring by 40% (Figure 1D; P < 0.05), but there was no difference in STAT5a expression between the two groups (Figure 1D; P > 0.05).

PCR arrays were analyzed by selecting 1.4-fold as a threshold for determining altered expression. Of the five housekeeping genes, four showed no difference in expression, namely large ribosomal protein I (RLBP 1), HPRT, lactate dehydrogenase A (LDHA) and ribosomal protein, L13A (RPL13a) and these were therefore selected as normal-

Protein and mRNA expression of signaling molecules in the insulin, progesterone and ERBB2 signaling pathways. Mammary gland protein expression of IR was increased in LP offspring compared with controls as an effect of maternal protein restriction (Figure 4a; \( P < 0.05 \)). PR expression was also increased in LP offspring (\( P < 0.01 \)) and there was a significant interaction between maternal and postpubertal diets on the expression of PR (\( P < 0.05 \); Figure 4d). This reflected the protein expression of PR (\( P < 0.05 \)) being highest in LP-HPD group at 4 months of age. The pattern of ERBB2 protein expression was similar to that of PR, with highest expression observed for the LP-HPD group, which was significantly increased when compared with LP-chow and Control-HPD groups (\( P < 0.05 \); Figure 4d).

Neither maternal diet nor postpubertal diet had any effect on the protein expression of IGF-1R (Figure 4b) or ER\(_a\) (Figure 4c). Significantly, Akt1 protein expression was very robustly and significantly increased in LP offspring solely as an effect of maternal protein restriction (Figure 4f; \( P < 0.001 \)).

Gene expression of insulin/IGF-1 and steroid receptors as well as the ERBB2/ERBB3 receptors was measured by reverse transcription–PCR to identify if transcript levels directly influenced protein levels. IR mRNA expression tended to be upregulated in parallel with protein expression, as an effect of maternal protein restriction; however, this was not significant (Figure 5a; \( P > 0.05 \)). PTEN transcript was downregulated as an effect of maternal protein restriction (Figure 5b;
There was also a significant interaction with HPD diet such that PTEN was reduced in HPD fed controls (P < 0.01), although this effect was not observed in the LP group.

PR transcript paralleled protein expression with the highest levels observed for the LP-HPD group, approximately double the levels seen in the Control-chow, Control-HPD and LP-chow groups (Figure 5c; P < 0.01). This suggested an interaction between maternal protein restriction and postpubertal diet (P < 0.05).

ERBB2 mRNA expression (Figure 5d), did not parallel protein expression and likewise, ERBB3 transcript was not affected either by maternal protein restriction nor postpubertal diet (Figure 5e; P > 0.05). However, a very significant interaction between maternal protein restriction and postpubertal diet was observed for dual specificity mitogen-activated protein kinase kinase 2 (MAP2K2) mRNA expression, which provided a read-out for both PR and ERBB2/ERBB3 signaling. dual specificity mitogen-activated protein kinase kinase 2 was significantly downregulated by the effect of maternal protein restriction (P < 0.001; Figure 5f) and by HPD diet only in Controls (P < 0.001), whereas the HPD feeding in LP offspring resulted in an upregulation of this gene (P < 0.01; LP-chow compared with LP-HPD).

We also investigated the effects of phospho-ERK1/2 protein expression in healthy mammary tissue adjacent to tumor tissue in Control and LP offspring and found both pERK1 (P < 0.05) and pERK2 (P < 0.01) to be more highly expressed in LP tissue (supplementary Figure 2 is available at Carcinogenesis Online).

Table I. RT2 rat cancer PathwayFinder array results

<table>
<thead>
<tr>
<th>UniGene</th>
<th>RefSeq</th>
<th>Symbol</th>
<th>Description</th>
<th>Gene name</th>
<th>Fold upregulation or downregulation</th>
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<tbody>
<tr>
<td>Rn.10668</td>
<td>NM_017059</td>
<td>Bax</td>
<td>Bcl2-associated X protein</td>
<td>Bax</td>
<td>LP/Control</td>
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<td>Rn.10089</td>
<td>NM_080782</td>
<td>Cdkn1a</td>
<td>Cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>Cip1/Waf1</td>
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<td>Rn.48717</td>
<td>NM_031550</td>
<td>Cdkn2a</td>
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<td>Rn.107896</td>
<td>NM_017025</td>
<td>Ldhaa</td>
<td>Lactate dehydrogenase A</td>
<td>Ldhaa</td>
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</tr>
</tbody>
</table>

*Housekeeping genes.

Table II. Latency and multiplicity of adenocarcinomas as an effect of maternal and postpubertal obesity-inducing diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mean latency (weeks, SEM)</th>
<th>Mean multiplicity (weeks, SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-chow</td>
<td>19.7 (6.0)</td>
<td>0.9 (1.1)</td>
</tr>
<tr>
<td>Control-HPD</td>
<td>14.5 (1.3)</td>
<td>1.1 (0.9)</td>
</tr>
<tr>
<td>LP-chow</td>
<td>17.7 (1.0)</td>
<td>1.8 (0.2)</td>
</tr>
<tr>
<td>LP-HPD</td>
<td>14.0 (1.0)</td>
<td>1.7 (1.1)</td>
</tr>
</tbody>
</table>

Figure 5f and by HPD diet only in Controls (P < 0.001), whereas the HPD feeding in LP offspring resulted in an upregulation of this gene (P < 0.01; LP-chow compared with LP-HPD).

We also investigated the effects of phospho-ERK1/2 protein expression in healthy mammary tissue adjacent to tumor tissue in Control and LP offspring and found both pERK1 (P < 0.05) and pERK2 (P < 0.01) to be more highly expressed in LP tissue (supplementary Figure 2 is available at Carcinogenesis Online).
**Discussion**

In our previous studies (31), we showed that rapid compensatory growth of LP mammary tissue occurring at 5 weeks of age correlated with increased expression of IR, IGF-1R and ERBB2 (Her2). This molecular phenotype was accompanied by an increase in early-onset mammary tumor incidence compared with controls. Overexpression of IR has been shown to induce malignant transformation in breast cancer cell lines (34). Conversely, the inactivation of p53 or activation of oncogenes WNT1, ERBB2 and RET can lead to overexpression of IR in breast cancer (35,37).

IGF-1R transcription is controlled by a number of tumor suppressor genes, including Wilm’s tumor-1, p53 and BRCA1. These normally suppress IGF-1R promoter activity, by binding to the promoter directly, by physical interaction with a stimulatory transcription factor Sp1 in the case of BRCA1 (38); or as for Wilm’s tumor-1, via protein–protein interaction with ERx (39). Here, we found that IGF-1R protein overexpression correlated with transcriptional upregulation. That BRCA1 and p53 were not different from control offspring while Sp1 transcript was upregulated by 44% is consistent with findings in cell lines. In human breast cancer-derived cell lines T47D, MCF-7 and MDA-MB-231, BRCA1 has been shown not to bind specifically to the IGF-1R promoter, but rather, exerts its action by preventing Sp1 binding to the promoter (38). As p53 DNA binding and activity is regulated by phosphorylation, any further study on its regulatory activity on IGF-1R should consider this. However, we did show components regulating the p53 pathway, e.g. CDKN1A (p21) to be upregulated, which may induce repression of p53 (40) thus allowing escape from cell cycle arrest. The IGF-1R promoter is highly responsive to Sp1 and transcriptional control by Sp1 occurs primarily through a cluster of four GC boxes in the 5'-flanking region (41). Our data is consistent with overexpressed Sp1 driving increased IGF-1R transcription and protein expression in LP mammary tissue. Recent studies suggest, in addition to the canonical IGF-I signaling pathways through mitogen activated protein kinases and PI3K-Akt, IGF-I also signals through the JAK/STAT pathway (42). During normal mammary gland development, JAK2 and its main substrate, STAT5, are critical for the growth and differentiation of alveolar progenitors as well as the survival of secretory mammary epithelial cells. Genetic studies in mouse models support a role for the Stat5 transcription factor as a proto-oncogene in mammary tumor initiation (43). Our observation of JAK2 overexpression is therefore consistent with activation of this pathway.
Although STAT5 transcription was not different, it is noted that activity can only be measured by phosphorylation. We demonstrated that LP mammary tissue at 5 weeks of age is engaged in a program of proliferation and DNA repair, as demonstrated by increased PCNA protein expression (44), potentially facilitated by the activation of the nutrient-sensing pathway via mTOR. Our experiments also suggest that LP mammary tissue is engaged in activities involving: (i) upregulation of cell cycle control genes CDKN1A (p21Waf1) and RB1; concurrent with a downregulation of the apoptotic signal, BAX. As these are early events, G1/S checkpoint is very probably to be activated in response to DNA damage arising from increased proliferation. Loss of BAX would therefore imply withdrawal from the apoptotic cascade. NME23A (gene product of NME1) was first identified because of its reduced mRNA transcript levels in highly metastatic cells. Mutations in this gene have been identified in aggressive neuroblastomas (45). The loss of NME23A in LP tissue suggests a movement toward a metastatic phenotype. This is consistent with an upregulation of invasion and metastasis gene expression (MMP9 and SERPIN1) as well as those concerned with adhesion (NCAM1) and angiogenesis (EGFR, HGF and THBS1) seen in the array data and with the observation that only LP-HPD animals developed mammary tumors of DCIS type. These molecular events occur within a framework of rapid compensatory prepubertal growth, which is immediately prior to hormonal surges experienced during estrus cycling. They may therefore mediate the effects of poor early growth on increased mammary tumor risk.

There is ample evidence linking obesity to breast cancer risk, however, most of this evidence has been associated with post-menopausal status. More recent case–control studies have found waist circumference in Taiwanese women (46) and body mass index in a Swedish cohort (47) are significant predictors of breast cancer in both pre- and post-menopausal women. Both studies also found that higher leptin concentrations associated with increased risk, whereas the latter study found HbA1c to influence tumor initiation. In another study, increased triglycerides with unchanged HDL-cholesterol in both pre-menopausal and post-menopausal phases has been shown to correlate with breast cancer risk (48).

We therefore investigated if weight gain just after puberty would have an impact on breast cancer risk in our model of increased mammary tumor risk resulting from poor early growth and rapid compensatory mammary growth (31). This has proven an appropriate model for studying the increased breast cancer risk seen in women born with a low birth weight (26). Consequently, HPD alone was found to raise plasma insulin and triglycerides. This was accompanied by increased IR, PR and ERBB2 protein expression resulting from poor early growth and excessive calorie intake. These molecular events correlated with increased early-onset mammary tumor incidence in a separate cohort treated with NMU carcinogen. Poor early growth combined with excessive calorie intake in adulthood...

Fig. 5. Effect of poor early growth and postpubertal HPD on mRNA expression of signaling molecules in insulin, progesterone and ERBB2 pathways. RNA was isolated from mammary glands of Control and LP offspring at 16 weeks of age (n = 8 per group). Gene expression was quantified by real-time PCR using primers shown in Supplementary Table 2. All data were normalized to four housekeeping genes as described in Materials and Methods. Open bars (Chow diet) and filled bars (HPD diet). Data expressed as means ± SEMs; **P < 0.001; *P < 0.01.
significantly downregulated PTEN transcript and upregulated Akt1 protein expression, implying Akt activation, with potential implications for cell survival (49), cell motility and invasion (50) and angiogenesis (51).

The role of PR action in breast cancer has been little studied until recently, when it was shown that PR functions as a ligand-activated transcription factor and initiator of c-Src kinase and mitogen-activated protein kinase signaling (52). It was therefore interesting to find that dual specificity mitogen-activated protein kinase 2 transcript, though downregulated in LP offspring compared with controls, was upregulated as an effect of feeding the HPD diet. This was accompanied by an increased phosphorylation and thus activation of extracellular signal-regulated kinase 1/2 and phospho-p38 mitogen-activated protein kinase (Supplementary Figure 2), which were more highly expressed in healthy surrounding tissue. This active mitogenic stimulus would then eventually have detrimental effects on chromatin integrity and DNA damage resulting in mutations and transformation.

Just as others have observed using mouse breast models, e.g. MMTV-TGF-alpha mice exposed to high-fat feeding (53), we have also shown that high calorie intake independently and additively to contribute to breast cancer risk. This is the first report of increased mammary tumor risk due to excessive calorie intake in a non-genetically modified rodent model. These findings are highly significant as they identify and underscore the role of pivotal mechanisms by which metabolic pathways can be hijacked in the process of carcinogenesis.

Supplementary material

Supplementary Figures 1–2 and Tables 1–4 can be found at http://carcin.oxfordjournals.org/.

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References


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