Compensatory mammary growth following protein restriction during pregnancy and lactation increases early-onset mammary tumor incidence in rats

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Breast cancer incidence is increased in women with both high and low birth weight. The latter is also associated with hyperglycaemia, insulin resistance and type-2 diabetes, each of which independently increases breast cancer risk. We showed previously in our model of poor early-growth that pregnancy estradiol levels were raised while offspring developed type-2 diabetes. We hypothesized that nutritionally-induced poor early-growth influences breast cancer risk and investigated this in our model. Wistar rat dams were given either a control diet (20% casein) or an isocaloric low-protein (LP) diet (8% casein) throughout pregnancy and lactation. Offspring postnatal mammary gland development was assessed by morphometry. To identify potential growth mechanisms, we measured protein expression of receptors involved in insulin and hormone signaling, both in cleared mammary gland lysates and isolated epithelial cells. Mammary tumor incidence and latency (n = 96) was monitored after three weekly intraperitoneal nitrosomethylurea injections (50 mg/kg body wt). LP offspring displayed reduced postnatal ductal branching and epithelial invasion at 3 weeks, followed by compensatory mammary growth 1 week later coinciding with increased protein expression of receptors to insulin, IGF-1 and estrogen. Significantly, early-mammary tumor incidence (0−16 weeks post-treatment) was doubled in LP offspring [RR, 2.13 (1.02, 4.45); P = 0.046]. The data suggest that poor early nutrition has an important influence on the mammary primordium, and increases future susceptibility to breast cancer. Up-regulated growth factor and hormone signaling during compensatory mammary growth may mediate this increased susceptibility and present potential targets for intervention.

Introduction

Low birth weight is an anthropometric marker that has been linked to the increased risk of cardiovascular disease (1), insulin resistance, type-2 diabetes (2) and adult obesity (3). Low birth weight has also been linked to breast cancer (4–6). Most epidemiological studies to date report a U-shaped relation between birth weight and breast cancer risk (4–6), with both low and high birth weight associated with an increase in breast cancer incidence. Recent studies show that high birth weight increases mammary tumorigenesis in a rat model (7). In this model, offspring demonstrated a shortened latency to developing mammary tumors. The possible mechanisms mediating this include an observed increase in activated MAPK levels and a lower apoptotic response in the tumors.

The mechanistic basis of the relationship between low birth weight and subsequent disease risk is not known, however, human and animal studies suggest that the fetal and neonatal environment plays an important role [see review by Aerts and van Assche (8)]. In particular, early-nutrition has been identified as a critical component. The ‘Thrifty Phenotype’ hypothesis proposes that a conflict between poor early-nutrition and excess adult nutrition may provide a possible mechanism. It has been suggested in studies of individuals who were in utero during the Dutch Hunger Winter (9) and in other more recent studies, that the highest risk for development of diabetes or impaired glucose tolerance is seen when individuals experience poor fetal growth and rapid postnatal catch-up growth starting early in life (10) followed by adult obesity (9). Exposure to famine during pregnancy and early-life in this cohort has been shown to have wide ranging life-long effects on health depending on the timing of exposure (11).

Insulin resistance and type-2 diabetes are associated with an increased breast cancer risk. This association is thought to act via three mechanisms i.e. (i) activation of the insulin pathway, (ii) activation of the insulin-like growth factor-1 (IGF-1) pathway, and (iii) regulation of endogenous sex hormones (12). We hypothesize that the relationship between the background of low birth weight (i.e. poor fetal growth) and the distal outcome of breast cancer may be mediated by insulin resistance. An animal model of low birth weight that subsequently demonstrates diabetes would therefore be an extremely useful tool for studying the interacting mechanisms central to breast cancer. Our rat model of maternal dietary protein restriction during pregnancy and lactation results in offspring with low birth weight who grow slowly during lactation because of protein-restricted lactation nutrition as well. This has long-term effects in adulthood including the development of hyperinsulinemia, insulin resistance and type-2 diabetes (13,14), due at least in part, to fetal programming of specific defects in the insulin-signaling pathway (15). In more recent studies in mice we have shown that

Abbreviations: ERα/ERβ, estrogen receptor isoform alpha/beta; IGF-1, insulin-like growth factor-1; IGF-1Rβ, insulin-like growth factor-1 receptor beta subunit; IR, insulin receptor; LP, low-protein; NMU, nitrosomethylurea.
maternal protein restriction during fetal life followed by rapid catch-up growth during the lactation period leads to a permanent increase in appetite, excess weight gain and premature death (16). The known associations between breast cancer and type-2 diabetes and their common link to birth weight led us to hypothesize that maternal protein restriction could result in increased breast cancer risk in the offspring. Another observation supported this hypothesis: we showed that the maternal estradiol levels in the peripheral circulation of dams, fed a low-protein (LP) diet, are raised by 35% compared with controls in the last week of gestation (17), a risk factor for breast cancer in humans (18) and mammary tumors in a rodent model (19). The exposure of breast stem cells, which remain undifferentiated until puberty, to high concentrations of unbound maternal estrogens in utero is thought to be central to the development of breast cancer (20).

The objective of the current study was thus to investigate the effect of maternal protein restriction on mammary gland development and ultimately, susceptibility to mammary tumors. In this study, we report results from control and low-protein female offspring (LP group) from 3 to 7 weeks of age, including birth weights, body weights and prepared whole-mounts of mammary glands to assess gland development, as well as, measurements of protein expression of various growth factor and hormone receptors. We also administered the carcinogen nitrosomethylurea (NMU) and monitored the incidence of mammary tumors within the two groups.

METHODS

Ethical approval

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 were housed individually and maintained at 22°C on a 12 h light–dark cycle. They were mated overnight, at either 3, 4, 5 or 7 weeks of age. The following morning between 10.00 a.m. and 11.00 a.m., the rat was weighed and blood glucose measured. The rat was then anaesthetized and blood collected for EDTA plasma, aliquots of which were flash frozen in liquid nitrogen and then stored at −80°C for later plasma hormone analysis. The rat was then killed and both the thoracic (2nd and 3rd pairs) and inguinal (4th and 5th pairs) mammary glands were collected bilaterally. For western blotting analyses, mammary glands from one side of the animal were first cleared of lymph nodes before snap-freezing in liquid nitrogen and stored at −80°C until required for protein expression analysis. Mammary glands from the other side were collected for whole-mount morphometric analysis.

Western blot analysis

Mammary tissue was ground to a powder in a mortar and pestle on dry ice and ~250 mg of powder was homogenized in 1 ml lysis buffer [50 mM HEPES (pH 8), 150 mM NaCl, 1% Triton X-100, 1 mM Na2VO4, 30 mM NaF, 10 mM Na3P2O7, 10 mM EDTA and a protease inhibitor cocktail]. The total protein concentration in the lysates was determined by a Sigma copper/bichicinonic assay. Samples containing 10 μg of total protein (each animal being represented by one sample per well) were separated on 10% SDS–PAGE gels by electrophoresis and the proteins transferred onto PVDF Immobilon-P (Millipore) membrane. Each membrane was probed with antibodies either to: insulin receptor-beta subunit (IR-β) at 1:200 dilution (Santa Cruz); insulin-like growth factor-1 receptor beta subunit (IGF-1Rβ) at 1:200 dilution (Santa Cruz); ErbB2 (Her-2 or neu) at 1:500 dilution (Abcam, Cambridge, UK); estrogen receptor isofrom alpha (ERα) at 1:200 dilution (Novocastra); estrogen receptor isofrom beta (ERβ) at 1:1000 dilution (Amersham); and progesterone receptor (PR) at 1:1000 dilution (Novocastra). Horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham) were detected by chemiluminescence. Autoradiographed images were captured and spot-densitometry analysis carried out using the Alphaease software (Alphaimager).

A total of 16 samples could be run on a single gel at one time, with molecular weight markers and positive controls included. For each of the molecules analyzed, it was possible to fit all 16 samples for each time-point from both the control and LP groups onto a single gel, therefore no inter-gel comparisons were necessary. Equivalent total protein amounts were loaded and a positive control was also included in each gel. The analysis for each time-point containing eight controls and eight LP samples was therefore performed on separate gels and as such it was not possible to assess the impact of age.

Whole-mount analysis

Mammary fat pads from one side of the animal were dissected off the skin flap and spread onto glass slides, thus each slide represented the determination for one animal. These were fixed in Methacarn (1,1-trichloroethane, methanol and acetic acid) overnight, rehydrated in 70% ethanol and stained in carmine red. Following staining whole-mounts were dehydrated in 100% ethanol and finally cleared and kept in methyl salicylate. Images were captured with a Nikon Coolpix 4500 camera mounted on a dissecting microscope.
and LP inguinal glands was adapted from Imagawa et al. (22). The tissue was weighed and washed in Hanks balanced salt solution (HBSS) and then minced with a razor blade in a petri dish. The minced tissue was subsequently digested in 10 ml of HBSS containing 0.004% of deoxyribonuclease I (DNase I; Sigma) and 0.1% collagenase Type 3 (Worthington Biochemical Corporation, USA) for 2–3 h at 37°C with shaking at 100 r.p.m. After 2 h the digest was examined microscopically, and when smooth-bordered stroma-free organoids were observed, the digest was centrifuged at 100 g for 5 min to pellet the organoids. The pellet was then resuspended in fresh HBSS with DNase I added to prevent clumping. Epithelial cells/organoids were finally isolated on a pre-made Percoll gradient, washed in HBSS and centrifuged to pellet the organoids, and after removal of the solution, flash-frozen, in aliquots, in liquid N2 and kept at −80°C until required for protein extraction.

NMU administration
In this study 48 control and 48 LP female offspring were used, with 2 siblings coming from each litter. Hence a total of 24 control and 24 LP litters were used in all. All 96 rats were injected with NMU, freshly prepared in phosphate-buffered saline, pH 5.5, and injected within 5 min of preparation at a dose of 50 mg/kg body wt. The animals received in total three injections at 3, 4 and 5 weeks of age. Treated rats were closely monitored and palpated bi-weekly for mammary tumors and weighed weekly. The latency of appearance of the first tumor was recorded and the rat was killed when the largest tumor was ~2 cm in diameter or if limiting clinical signs were noted, whichever occurred first. We showed in pilot experiments that in saline-injected (pH 5.5) controls, no tumors were observed, therefore, negative controls were not included here.

Statistical analysis
Values are presented as mean ± standard error of the mean (SEM). All statistical calculations for the western blotting analyses were carried out using InStat (GraphPad Software, USA). As all eight controls and eight LP samples from cleared mammary gland lysates and isolated epithelial cells of a single time point were run on a single gel which included a positive control, inter-group comparisons were made separately for each time-point, and the significance of any difference between groups at each time-point was examined by two-tailed unpaired student’s t-tests unless stated otherwise. Results are presented as mean values with their standard errors or medians with inter-quartile ranges for non-parametric tests, and absolute probability (P) values. P-values ≤ 0.05 were considered to be statistically significant. The number of animals in each group is indicated by n. For the NMU tumor induction studies, the relative risk (RR) of incidence and its 95% confidence interval (CI) were estimated for 0–16 weeks and for 17–32 weeks, respectively, using EpiInfor (version 3.3). For 0–16 weeks, there were 48 rats in each group. For 17–32 weeks, the number of rats is the number of remaining non-tumor rats, i.e. there were 40 and 31 rats in control and LP groups, respectively.

Results
Birth weights and subsequent bodyweights of the LP group were lower than the controls at every time-point studied (two-tailed t-test, alpha = 0.05, P < 0.001; Table 1), suggesting that LP animals do not catch-up in terms of body weight. Mammary gland structural development was dramatically retarded in the LP offspring at 3 weeks of age, as evidenced by a lack of secondary branching in both the thoracic and inguinal regions (Figure 1). However, during the first week after weaning onto a normal diet, the LP mammary glands developed very rapidly such that they were visually indistinguishable from controls by 4 weeks of age (Figure 1). Morphometric analysis confirmed that ductal epithelial area was initially reduced in the LP thoracic glands at 3 and 4 weeks, but was equivalent to area measurements of controls at 5 and 7 weeks (Table II). In the inguinal glands also, we found LPs had a reduced ductal epithelial area at 3 weeks but by 4 weeks, epithelial area was comparable with controls. Inspection of the ducts at higher magnification suggests that there is greater lateral branching, as evidenced by a denser appearance. By 7 weeks there was no longer any difference in area between control and LP inguinal glands. The concentration of estradiol, progesterone and FSH were measured to relate changes in ductal area to cycling hormone levels, however, we observed no differences between the two groups (Table III).

At the molecular level, the rapid acceleration in mammary growth coincided with increased protein expression of both the insulin receptor (IR) and the insulin-like growth factor-1 receptor (IGF-1R) in whole thoracic glands of LP rats at 3 and 4 weeks of age and in LP inguinal glands at 5 weeks of age (Figure 2). As this model displays a metabolic phenotype later in life, we isolated mammary epithelium ex vivo to eliminate the possible contribution from changes related to insulin signaling in adipose tissue. We therefore chose the

### Table I. Body weights (obtained after overnight fast) of LP female offspring and controls

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Body weight (g)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 8)</td>
<td>LP (n = 8)</td>
</tr>
<tr>
<td>Birth</td>
<td>7.33 (0.19)</td>
<td>6.09 (0.20)</td>
</tr>
<tr>
<td>3</td>
<td>50.5 (1.82)</td>
<td>33.5 (1.57)</td>
</tr>
<tr>
<td>4</td>
<td>77.9 (2.34)</td>
<td>55 (1.65)</td>
</tr>
<tr>
<td>5</td>
<td>120.7 (2.58)</td>
<td>93.8 (3.06)</td>
</tr>
<tr>
<td>7</td>
<td>170.7 (2.93)</td>
<td>151.1 (4.22)</td>
</tr>
</tbody>
</table>

Values are means ± standard error of the mean. Student’s t-test, alpha = 0.05.

Plasticity and its 95% confidence interval. For the NMU tumor induction studies, the relative risk (RR) of incidence and its 95% confidence interval (CI) were estimated for 0–16 weeks and for 17–32 weeks, respectively, using EpiInfor (version 3.3).
time-point of 5 weeks for these extended studies using inguinal tissue because (i) this is where we saw the greatest difference of expression in the mammary gland lysates and (ii) this allowed us to verify whether the differences in protein expression were localized to the epithelial population and not due to adipose tissue. Additionally, with the epithelial cell marker cytokeratin 14, we were able to relate protein expression as a proportion of total epithelial cell content, thus any differences in expression can be directly related to cellular receptor content. We found that at 5 weeks of age, IR and IGF-1R protein levels were also increased in isolated mammary epithelial cells from LP inguinal glands (140 ± 8 and 122 ± 8%, respectively compared with controls [100 ± 6%] with the data expressed as mean percentage levels ± standard errors; \( P = 0.0011 \) and 0.047, respectively (two-tailed \( t \)-test and alpha of 0.05). These isolated epithelial cell preparations also over-expressed ER-\( \alpha \) and -\( \beta \) protein, as well as ErbB2 receptor (Table IV). We therefore specifically targeted this sensitive period of mammary gland development with carcinogen NMU treatment as described in the Methods. We observed a striking doubling of the incidence of early-onset tumors (weeks 0–16 after treatment).
in LP offspring \( [RR = 2.13, P = 0.036, 95\% \text{ CI (1.02, 4.45)}, \) (Table V). However, no differences in tumorigenesis were noted after Week 17.

**Discussion**

The data presented here show that maternal dietary protein restriction during pregnancy and lactation results in offspring with low birth weight. Prior to weaning on to a normal laboratory diet, these offspring displayed retarded mammary ductal development. This followed a period of rapid compensatory growth between 1 and 2 weeks after weaning, specifically in epithelial density. The molecular phenotype accompanying this rapid ductal/epithelial growth includes the over-expression of receptors to insulin, IGF-1, epidermal growth factor and estrogen. The increased expression of IR is consistent with that observed in other tissue (liver, muscle and adipose tissue) in young adult life (15).

After administration of the carcinogen and DNA methylating agent, NMU, the LP offspring were found to develop twice the number of early-mammary tumors compared with the control offspring. Possible explanations for this increased susceptibility in this model may include (i) an estrogenic **in utero** environment and (ii) rapid compensatory growth. **In utero** estrogen exposure has been linked to an increase in breast cancer risk (23) and can abnormally demethylate DNA sequences during organ development and possibly increase cancer risk later in life (24). Excess estrogen could also have permanent effects on hepatic monoamine oxidase, a phase II xenobiotic metabolizing enzyme (25), thereby influencing the potential for toxicity by xenobiotic or environmental carcinogen exposure in later life (26) or even its response to normal adult hormonal stimulation which occurs first at puberty and then during menstrual cycling. This suggests that LP offspring may have altered activities of hepatic phase I or phase II xenobiotic metabolizing enzymes due to an abnormal development of the liver and that inadequate detoxification and elimination may increase susceptibility. As yet however, there is no evidence relating **in utero** undernutrition to changes in the activities of these enzymes. Furthermore, it has been shown that the breast rather than the liver is the principal site of heterocyclic amine metabolic activation (27) and it would be interesting to measure these enzyme activities in LP mammary tissue in the future.

The rapid compensatory mammary epithelial growth following weaning may further expand the population of cells bearing transforming mutations, while the rate of cell division also contributes to cancer risk (28). Conceptually, compensatory or mammary epithelial ‘catch-up’ growth might enhance the rate of transformation in mammary cells including stem cells or ‘progenitor’ cells by exploiting the increased rates of mitogenesis and therefore replication errors. In human studies, proliferative changes and atypical hyperplasia of the breast were found to be strong predictors of subsequent breast cancers particularly in premenopausal women (29). A rapid growth rate during childhood is also thought to influence the risk of breast cancer (30,31). In culture, rapid growth of mammary epithelial cells accelerates replicative senescence, from which a subpopulation spontaneously escapes and acquires genomic changes including the loss of p16INK4a expression, a key cell cycle checkpoint regulator. The silencing of this gene is thought to be due to hypermethylation of the gene promoter (32). Compensatory mammary epithelial growth may also exert alterations in DNA methylation via the activation of endocrine and developmental signaling and thereby alter gene regulation to detrimental effect (33).

IGF-1R has been shown to have a role in mammary ductal growth, specifically in the proliferation of terminal end buds (TEBs) (34), and transgenic mice over-expressing activated IGF-1R have been found to develop salivary and mammary adenocarcinomas as early as 8 weeks of age (35). Furthermore, insulin receptor over-expression in human mammary epithelial cells induces a ligand-dependent phenotype (36). The over-expression of insulin and IGF-1 receptors in LP mammary glands, particularly during the post-weaning

**Table II.** Ductal epithelial area in control and LP animals at weaning and as at 4, 5 and 7 weeks of age

<table>
<thead>
<tr>
<th>Time-point</th>
<th>Ductal epithelial area (mm$^2$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>LP</td>
</tr>
<tr>
<td>3 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thoracic</td>
<td>2.69 (0.27)</td>
<td>1.31 (0.16)</td>
</tr>
<tr>
<td>Inguinal</td>
<td>4.84 (0.59)</td>
<td>4.84 (0.59)</td>
</tr>
<tr>
<td>4 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thoracic</td>
<td>5.42 (1.05)</td>
<td>3.37 (0.27)</td>
</tr>
<tr>
<td>Inguinal</td>
<td>8.09 (0.27)</td>
<td>7.35 (0.68)</td>
</tr>
<tr>
<td>5 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thoracic</td>
<td>5.61 (0.91)</td>
<td>6.89 (1.19)</td>
</tr>
<tr>
<td>Inguinal</td>
<td>11.28 (1.09)</td>
<td>9.98 (0.66)</td>
</tr>
<tr>
<td>7 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thoracic</td>
<td>5.84 (1.27)</td>
<td>8.78 (1.02)</td>
</tr>
<tr>
<td>Inguinal</td>
<td>22.14 (2.64)</td>
<td>27.42 (2.68)</td>
</tr>
</tbody>
</table>

Values are means (±SEM).

$n = 8$ except for 7 week: thoracic and inguinal areas where $n = 6$ for both groups.

Ductal epithelial area is reduced in LP animals at weaning but catch-up groups.

The data presented here show that maternal dietary protein restriction during pregnancy and lactation results in offspring with low birth weight. Prior to weaning on to a normal laboratory diet, these offspring displayed retarded mammary ductal development. This followed a period of rapid compensatory growth between 1 and 2 weeks after weaning, specifically in epithelial density. The molecular phenotype accompanying this rapid ductal/epithelial growth includes the over-expression of receptors to insulin, IGF-1, epidermal growth factor and estrogen. The increased expression of IR is consistent with that observed in other tissue (liver, muscle and adipose tissue) in young adult life (15).

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**Table III.** Effect of a control (200 g protein/kg body wt) or LP (80 g protein/kg body wt) maternal diet on plasma estradiol, progesterone and FSH concentrations of female offspring at 5 and 7 weeks of age

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Estradiol (pg/ml)</th>
<th>Progesterone (ng/ml)</th>
<th>FSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control LP</td>
<td>Control LP</td>
<td>Control LP</td>
</tr>
<tr>
<td></td>
<td>Mean (±SEM)</td>
<td>Mean (±SEM)</td>
<td>Median (inter-quartile range)</td>
</tr>
<tr>
<td>5</td>
<td>69.67 (5.50)</td>
<td>55.49 (6.03)</td>
<td>6.64 (1.57) 8.19 (1.80) 3.61 (1.54–6.56) 1.99 (1.74–3.85)</td>
</tr>
<tr>
<td>7</td>
<td>59.59 (9.57)</td>
<td>57.63 (6.63)</td>
<td>14.68 (2.16) 16.99 (2.64) 1.19 (1.09–1.57) 1.45 (1.07–4.41)</td>
</tr>
</tbody>
</table>

$n = 8$ for all groups and samples, except for 7 week control group where $n = 7$ due to sample hemolysis. Estradiol and progesterone measurements were analyzed by two-tailed t-tests, while FSH was analyzed by Mann–Whitney non-parametric tests. $P$-values for all three measurements at both time-points were >0.05.
period therefore, strongly points towards a period of susceptibility/sensitivity to mammary tumorigenesis. We suggest that poor maternal nutrition results in structural and molecular changes to the fetal and early-postnatal mammary gland such that early-development is impaired. Weaning onto normal nutrition reverses this trend and directs rapid growth through up-regulation of the insulin and IGF-1Rs for 2–3 weeks hence. Insulin and IGF-1R over-expression occurring at 3 weeks of age was significantly higher in the LP group at 5 weeks of age (P < 0.001 and P = 0.014, respectively). Clear bars indicate the control group; hatched bars indicate the LP group. LP protein levels are expressed as means percentage of controls. Error bars indicate SEM; n = 8 for both groups. *P < 0.05; **P < 0.01; and ***P < 0.001.

Fig. 2. Mammary glands of LP offspring express higher protein levels of IRβ and IGF-1Rβ during the catch-up growth window. (A) Western blotting analysis of thoracic mammary glands showed significantly increased IRβ expression in the LP group at 3 weeks (P < 0.001) and 4 weeks (P = 0.001) of age, whereas IGF-1Rβ protein levels were also raised in that group at 4 weeks of age (P = 0.048). (B) In inguinal mammary glands both IRβ and IGF-1Rβ expression were significantly higher in the LP group at 5 weeks of age (P < 0.001 and P = 0.014, respectively). Clear bars indicate the control group; hatched bars indicate the LP group. LP protein levels are expressed as means percentage of controls. Error bars indicate SEM; n = 8 for both groups. *P < 0.05; **P < 0.01; and ***P < 0.001.
Compensatory growth increases early-mammary tumor incidence

| Table IV. Effect of a control (200 g protein/kg body wt) or LP (80 g protein/kg body wt) maternal diet on protein expression levels of IR, IGF-1R, ER-α and β and ErbB2 in mammary epithelial cell preparations from inguinal mammary glands of female offspring at 5 weeks of age |
|----------------|----------------|----------------|----------------|----------------|
| IR (n = 8)     | IGF-1R (n = 8) | ER-α (n = 8)   | ErbB2 (n = 8)  |
| Control        | LP             | Control        | LP             | Control        | LP             |
| 100 (6)        | 140 (8)        | 100 (6)        | 122 (8)        | 100 (21)       | 230 (30)       |
| *P* = 0.001    |                | *P* = 0.047    |                | *P* = 0.003    | *P* = 0.034    |
| 100 (12)       | 176 (28)       | 100 (9)        | 164 (8)        |
| *P* = 0.0001   |                |                |                |

Values are means (± SEM); exact *P*-values derived from two-tailed student’s *t*-tests with alpha of 0.05. LP levels are expressed as a percentage of controls.

| Table V. Tumor incidence was analyzed in control and LP female offspring after NMU administration |
|----------------------------------------|----------------|----------------|----------------|
| Weeks after treatment (n = 48)         | Control (n = 48) | LP (tumor/non-tumor) | RR of incidence (95% CI) |
| 0–16                                   | 8/40            | 17/31           | 2.13 (1.015, 4.450) 0.046 |
| 17–32                                  | 17/23           | 15/16           | 1.14 (0.6, 1.90) 0.642 |

Relative risk of mammary tumor incidence was increased significantly in LP offspring between 0–16 weeks after treatment.

especially in the epithelial cell population as we have shown, would allow rapid cell proliferation of any transformed stem cells. Cross-talk between up-regulated estrogen receptors and IGF-1 signaling may further direct transformation (37–39).

We conclude that suboptimal fetal and early-postnatal nutrition results in under-development of the mammary epithelial tree, which then develops rapidly when adequate nutrition is provided postnatally. This leads to a substantial increase of the risk of early-onset mammary tumors. It is vital we understand the underlying mechanisms of breast development in low birth weight infants and the additive effects of insulin resistance in later life. Understanding such environmentally driven processes is critical as they provide a much more accessible target for intervention than those resulting from genetic alterations. The clinical features of our rodent model of low birth weight mirror those of low birth weight humans, i.e. hyperinsulinemia, type-2 diabetes in adulthood and an increased incidence of early-onset mammary tumors, makes it an extremely relevant and physiological tool for studying such processes and potential interventions.

Acknowledgements

The authors acknowledge the expert technical assistance of Ann Flack and Adrian Wayman. This work was supported by the NIH: grant number AG-20608-02, the British Heart Foundation, the European Union ‘Early Nutrition Programming Project- FOOD CT-2005-007036’, the Parthenon Trust and the 20608-02, the British Heart Foundation, the European Union ‘Early Nutrition Interventions.

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