

## Effect of energy restriction on tissue size regulation during chemically induced mammary carcinogenesis

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**Energy restriction (ER) has documented beneficial effects on numerous diseases including cancer, yet the mechanism(s) that accounts for these effects is unknown. Experiments were designed to determine the effect of ER: (i) on the growth and development of the mammary gland; (ii) on the growth of carcinomas induced in the mammary gland by treatment with 1-methyl-1-nitrosourea (MNU); (iii) on rates of cell proliferation and apoptosis in pre-malignant and malignant mammary lesions. Mammary carcinogenesis was induced in female Sprague–Dawley rats by the i.p. administration of MNU (50 mg MNU/kg body wt) at 21 days of age. Rats were randomized to one of four dietary treatment groups: *ad libitum* fed or restriction of calorie intake to 90, 80 or 60% of *ad libitum* intake. ER reduced the ductal extension of the mammary gland into the fat pad in proportion to its effect on growth measured as body weight, however, the reduction in ductal branching, breast density and carcinoma volume by ER was greater than its effect on body weight. An animal's breast density was predictive of its carcinogenic response, irrespective of the level of ER imposed. While ER inhibited cell proliferation and induced apoptosis in pre-malignant and malignant mammary gland lesions, the magnitude of these effects make it unlikely that they fully account for the protective effects of ER against mammary carcinogenesis.**

### Introduction

Carcinogenesis is characterized by a failure in the regulation of tissue size homeostasis in which a clone(s) of transformed cells achieves a growth advantage due to an increased rate of cell proliferation and/or a decreased rate of cell death in comparison with neighboring populations of cells (1,2). The net result of this process in epithelial tissues is the development of a tumor. One of the most potent physiological inhibitors of the carcinogenic process in multiple epithelial target organs is energy restriction (3,4). We have recently reported a model for mammary carcinogenesis in which the effect of an agent on both the pre-malignant and malignant stages of the disease process can be investigated in a period of 5 weeks (5). Using this model, we observed that mammary carcinogenesis was inhibited in a dose-dependent manner by energy restriction (6). In order to extend these findings, tissue from the experiment reported in Zhu *et al.* (6) was used to address the following questions: (i) is the effect of energy restriction on the growth

of the mammary gland proportional to its effect on overall growth measured as body weight; (ii) is the effect of energy restriction on the growth of mammary carcinomas proportional to its effect on the growth of the animal; (iii) does energy restriction alter the rates of cell proliferation and/or cell death due to apoptosis in pre-malignant and malignant mammary gland lesions?

### Materials and methods

#### *Carcinogen administration and diets*

Female Sprague–Dawley rats were obtained from Taconic Farms (Germantown, NY) at 20 days of age. At 21 days of age, animals were administered 50 mg 1-methyl-1-nitrosourea (MNU)/kg body wt (i.p.) as previously described (5) and randomly divided into each of the following groups (25 rats/group): *ad libitum* fed (control); restricted-fed (RF) 90% of control; RF 80% of control; RF 60% of control. The formulation of the diet (AIN-93G) and the feeding protocols that were used have been described (6). The diets fed to energy-restricted animals were designed to ensure an equivalent intake of all nutrients, while limiting total dietary calories by reducing the carbohydrate content of the diet. All rats were meal fed and given 2 meals/day (6:00–9:00 a.m. and 2:00–5:00 p.m.), 7 days/week, in order to avoid possible confounding due to inter-group variation of meal timing, meal number or duration of fasting. The amount of diet provided to the RF rats was calculated every 2 days based on the amount consumed by the control group during the previous 2 day time frame. Rats were housed individually in stainless steel metabolic cages with wire mesh bottoms. The cages were equipped with adjustable width external tunnel feeders that permitted accurate quantification of food intake. Rooms were maintained at  $22 \pm 1^\circ\text{C}$  with 50% relative humidity and a 12 h light/12 h dark cycle. All rats were weighed three times per week. The animal facility in which the rats were housed is AAALAC accredited. This experiment was reviewed and approved by the AMC Cancer Research Center Institutional Animal Care and Use Committee.

#### *Necropsy*

All rats were killed at 5 weeks post-carcinogen using a stratification sequence such that time from last meal and time of day at which death occurred were equally represented in all groups. The abdominal–inguinal mammary glands from all animals were carefully excised and prepared as whole mounts as previously described (5).

#### *Histopathological classification*

Lesions identified in the whole mounts were processed for histopathological classification. Representative lesions are shown in Figure 1. The primary criteria used for diagnosing intraductal proliferation was an increase in the layers of epithelial cells lining the acini and ducts. Combined cytological and architectural criteria were employed in diagnosing ductal carcinoma *in situ* (DCIS). Keeping in mind that these lesions were dissected out at a very early stage post-carcinogen (35 days), we required that at least one expanded ductal structure be completely replaced by neoplastic cells that had uniform, monotonous, round, hyperchromatic and non-overlapping nuclei. In distinguishing DCIS from adenocarcinoma (AC), the earliest evidence of invasion was observed as a breach of the basement membrane of the ducts. Rat mammary ACs do not tend to invade as single cells; rather, they invade on a broad front with clusters of neoplastic acini.

#### *Measurement of mammary gland growth and adenocarcinoma volume*

All whole mounts of the abdominal–inguinal mammary gland chains were photographed and the images obtained were digitized. Measurements of ductal extension of the mammary gland into the fat pad and of the amount of area occupied by mammary epithelium were performed on the digitized images of the entire abdominal–inguinal mammary gland chain using IMAGE-PRO PLUS software (Media Cybernetics, Silver Spring, MD). As shown in Figure 2, the length of mammary gland between the superior lymph node in mammary gland 4 and the mammary branch border was quantified as a measure of ductal extension. Images were then further processed to remove the lymph

**Abbreviations:** AC, adenocarcinoma; BrdU, 5-bromo-2'-deoxyuridine; DAB, 3,3'-diaminobenzidine; DCIS, ductal carcinoma *in situ*; IDP, intraductal proliferation; MNU, 1-methyl-1-nitrosourea; RF, restricted-fed.

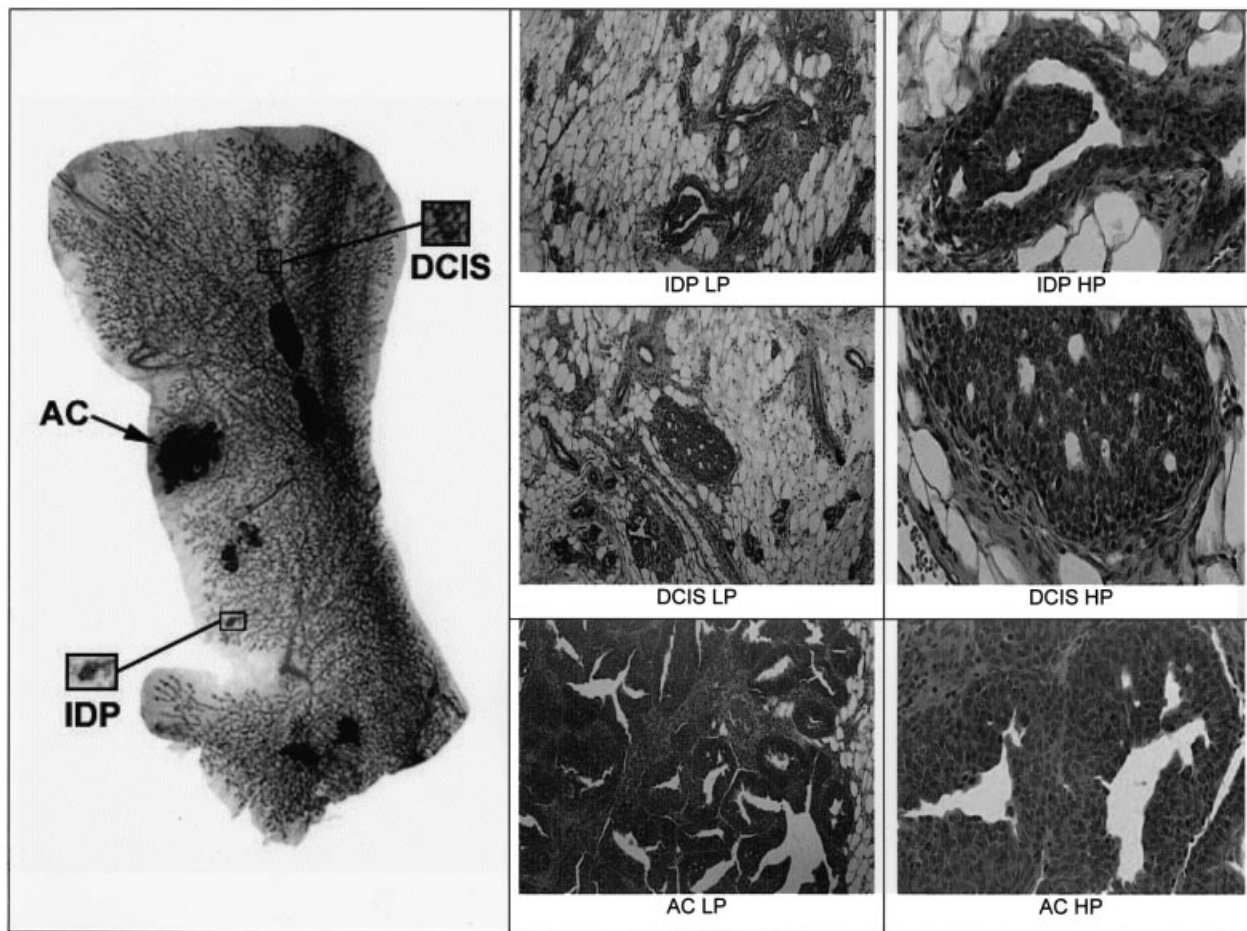


Fig. 1. Representative illustrations of IDP, DCIS and AC. Low power (LP) 100×; high power (HP) 400×.

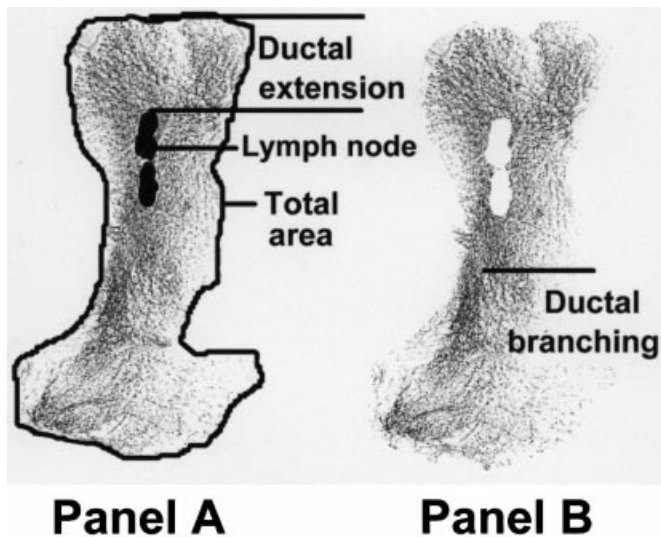


Fig. 2. Illustration of mammary gland growth measurements. Ductal extension is the distance between the uppermost lymph node and the mammary branch border. Total area is the area of fat pad into which mammary ductal epithelium extended. Ductal branching is the area occupied by mammary ductal epithelium.

nodes and lesions from the mammary gland. This processed image was then evaluated for total area of the mammary gland fat pad occupied by mammary epithelium as well as total area of the fat pad encompassed by the mammary ductal tree. Area occupied by mammary epithelium divided by total area encompassed by the mammary ductal tree was calculated and is comparable

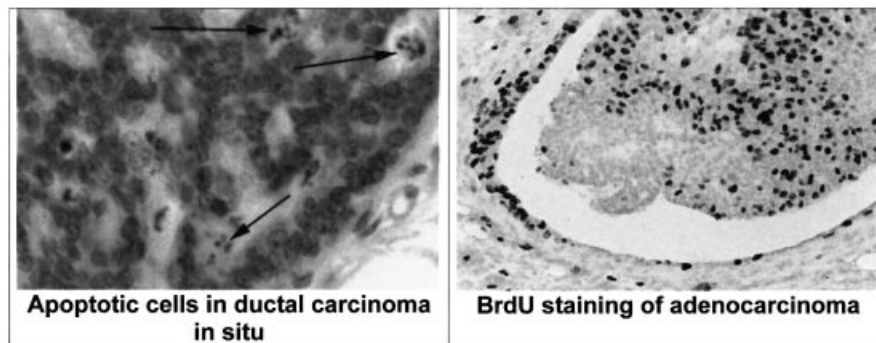
with breast density determined from a mammogram. The size of mammary gland ACs was measured on the whole mount images and volume was calculated using the formula  $V = (\text{length} \times \text{width}^2) / 2$  (2).

*5-Bromo-2'-deoxyuridine (BrdU) labeling and counting*

A pulse labeling technique was used to assess the rate of cell proliferation. Rats were injected i.p. with 50 mg/kg body wt BrdU (Sigma Chemical Co., St Louis, MO) exactly 3 h prior to killing. The 3 h period of labeling was selected since evidence indicates that this is also the timespan represented in the quantification of cell death by apoptosis using morphological criteria to identify apoptotic cells (7). Pre-malignant and malignant mammary gland lesions were stained for immunohistochemical analysis. Anti-BrdU antibody (1:40) (Becton-Dickinson, Lincoln Park, NJ) was used to detect BrdU-labeled nuclei. Representative staining of nuclei is shown in Figure 3. Labeled and unlabeled cells were imaged and counted at 400× with a computer-assisted image analyzer (CAS-200), using the Quantitative Nuclear Antigen Program v.3.0 (Becton-Dickinson/Cellular Imaging Systems, San Jose, CA) (8). Two cameras with two bandpass filters: one at 620 nm, which measures all nuclei stained with hematoxylin, with or without 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co.) staining; the other at 500 nm, which measures only nuclei stained with DAB, allowing for excellent spectral discrimination between the brown DAB and blue hematoxylin chromagens. Both nuclear and antibody thresholds were set with a negative control antibody cocktail slide. The nuclear threshold was set to the value that best discriminated between nuclei and cytoplasm. The antibody threshold was set to the value at which no stain could be detected in the nuclei of the negative control slide. Standardization was established through the use of control tumor tissue in each assay, which was treated in the same manner as the sample tissue. The Quantitative Nuclear Antigen software application was used to measure the percentage of cell nuclei in the tissue sections that contained BrdU-labeled antigen. The proliferation index, the percentage of labeled cells divided by total cells counted, was determined by counting 20 fields/slide (~2000 cells). Ten slides were evaluated per lesion type in each experimental group.

*Apoptotic cell counting*

Apoptotic cells were identified using morphological criteria: pyknosis and karyorrhexis characterized by cell shrinkage, nuclear condensation, fragmenta-



**Fig. 3.** Representative apoptotic cells and BrdU-positive nuclei of mammary tumor epithelial cells. The arrows in the left panel indicate the apoptotic cells in which the nuclei have broken into several apoptotic bodies (400 $\times$ ). The dark spots on the right panel indicate the BrdU-positive nuclei (100 $\times$ ).

**Table I.** Effect of energy restriction on the average number of pre-malignant and malignant mammary gland lesions per rat<sup>a</sup>

Lesions	Control	90% RF <sup>b</sup>	80% RF	60% RF
IDP	0.98 $\pm$ 0.17	0.88 $\pm$ 0.23	0.96 $\pm$ 0.22	0.63 $\pm$ 0.16
DCIS	0.37 $\pm$ 0.09	0.16 $\pm$ 0.08	0.04 $\pm$ 0.04	0.13 $\pm$ 0.07
AC	3.88 $\pm$ 0.34	2.40 $\pm$ 0.47	1.56 $\pm$ 0.44	0.29 $\pm$ 0.11

<sup>a</sup>Values are means  $\pm$  SEM. Data were analyzed by multivariate analysis of variance. The overall Hotelling–Lawley *F* statistic for an effect of energy restriction on lesion occurrence was 17.6,  $P < 0.001$ . The univariate *F* tests for the effects of energy restriction on occurrence was: of IDP 1.2,  $P = 0.27$  (*F* value probability); of DCIS 7.0,  $P < 0.009$ ; of AC 52.1,  $P < 0.001$ .

<sup>b</sup>All rats were meal fed. Animals in the control group were fed *ad libitum* at each meal; RF rats were fed 90, 80 or 60% of the *ad libitum* intake.

tion, presence of apoptotic bodies and lack of inflammatory components (9–13; illustrated in Figure 3). The apoptotic index, the percentage of apoptotic cells divided by total cells counted, was determined on the same 20 microscopic fields subjected to analysis for cell proliferation.

#### Statistical analyses

Unless otherwise stated, data are reported as means  $\pm$  SEM. Differences among groups were analyzed by analysis of variance with *post hoc* comparisons by the method of Tukey (14), analysis of covariance (14), multivariate analysis of variance (14) and the Kruskal–Wallis rank test (15). Tests for trend were by linear or Poisson regression analyses (14).

## Results

### Effect of energy restriction on the carcinogenic response in the abdominal–inguinal mammary gland chain

Table I shows the effect of energy restriction on the neoplastic response in the abdominal–inguinal chain of the mammary gland. This is the mammary gland chain on which the measurements of mammary gland size (Table II), carcinoma volume (Table III), cell proliferation (Table IV) and apoptosis (Table V) were made. Whereas energy restriction had only a modest effect on the number of intraductal proliferations (IDPs) (36% reduction at the highest level of energy restriction), the effect of energy restriction in reducing the occurrence of DCIS (65% reduction at the highest level of restriction) and ACs (93% reduction at the highest level of energy restriction) was dose dependent and highly significant statistically ( $P < 0.009$  and  $P < 0.001$ , respectively).

### Effect of energy restriction on mammary gland size

As shown in Table II, energy restriction resulted in a dose-dependent reduction in overall growth measured as body weight ( $P < 0.001$  by regression analysis). Total body weight

gain over the course of the study was 89, 67, 58 and 42 g for control, 90% RF, 80% RF and 60% RF, respectively. Figure 4 shows whole mount preparations that were representative of the effects of energy restriction on the size and morphology of the abdominal–inguinal mammary gland chain. In order to quantify and evaluate the differences reflected in Figure 4, the abdominal–inguinal mammary gland chains of all animals in this study were digitized and the digital images analyzed as described in Materials and methods. The results of these analyses are shown in Table II. An inverse relationship between ductal extension of the mammary gland into the fat pad and degree of energy restriction was observed ( $P < 0.001$  by regression analysis). We next asked whether the effect of energy restriction was greater on ductal extension than on body weight. To examine this question the ductal extension data were evaluated using covariate analysis of variance with level of energy restriction as the independent variable and body weight as a covariate. The results of this analysis indicated that the magnitudes of the effects of energy restriction on final body weight gain, a reflection of weight gain over the course of the experiment, and on ductal extension were similar. This is shown in Table II by the expression of ductal extension (mm/100 g body wt). The resulting ratios were essentially the same irrespective of the level of energy restriction, i.e. the reduction in ductal extension was proportional to the change in body weight.

We next extended the analysis of mammary gland images to the measurement of the total area of the mammary gland occupied by mammary epithelium (Table II). The total area of mammary gland occupied by mammary epithelium was reduced by energy restriction and the decrease was proportional to the degree of energy restriction ( $P < 0.01$ ). These data were then evaluated by covariate analysis of variance with level of energy restriction as the independent variable and body weight as a covariate. The results of that analysis indicated that the magnitude of the effect of energy restriction on total area occupied by mammary epithelium was significantly greater than its effect on body weight. This is shown in Table II by expression of the area data (cm<sup>2</sup>/100 g body wt). An energy restriction-dependent reduction in the amount of mammary epithelium per 100 g body wt was observed with increasing energy restriction; a 37% reduction was observed at the highest level of energy restriction. The effect of energy restriction on breast density was also determined; a dose-dependent reduction in breast density was observed with increasing levels of energy restriction ( $P < 0.01$ ).

**Table II.** Effects of energy restriction on mammary gland development of the rat<sup>a</sup>

	Control	90% RF <sup>b</sup>	80% RF	60% RF
Body weight (g)	149 ± 2 <sup>d</sup>	127 ± 2 <sup>e</sup>	118 ± 1 <sup>f</sup>	102 ± 1 <sup>g</sup>
Ductal extension (mm)	12.35 ± 0.25 <sup>d</sup>	10.64 ± 0.36 <sup>e</sup>	10.32 ± 0.38 <sup>e</sup>	8.02 ± 0.43 <sup>f</sup>
Ductal extension per 100 g body wt <sup>c</sup>	8.63 ± 0.14 <sup>d</sup>	8.63 ± 0.20 <sup>d</sup>	8.69 ± 0.26 <sup>d</sup>	8.31 ± 0.41 <sup>d</sup>
Total mammary gland area (cm <sup>2</sup> )	7.01 ± 0.22 <sup>d</sup>	6.85 ± 0.21 <sup>d</sup>	6.57 ± 0.14 <sup>d</sup>	4.92 ± 0.15 <sup>e</sup>
Total mammary gland area per 100 g body wt	4.70 ± 0.14 <sup>d</sup>	5.41 ± 0.14 <sup>e,f</sup>	5.56 ± 0.12 <sup>e</sup>	4.82 ± 0.16 <sup>d,f</sup>
Total area occupied by mammary epithelium (cm <sup>2</sup> )	3.93 ± 0.10 <sup>d</sup>	2.92 ± 0.08 <sup>e</sup>	2.49 ± 0.10 <sup>f</sup>	1.72 ± 0.07 <sup>g</sup>
Area occupied by mammary epithelium/100 g body weight	2.65 ± 0.08 <sup>d</sup>	2.30 ± 0.05 <sup>e</sup>	2.11 ± 0.09 <sup>e</sup>	1.68 ± 0.07 <sup>f</sup>
Breast density (%)	57 ± 1 <sup>d</sup>	43 ± 2 <sup>e</sup>	38 ± 1 <sup>f</sup>	35 ± 1 <sup>f</sup>

<sup>a</sup>Values are means ± standard error. Data were analyzed by analysis of variance or covariance. *Post hoc* comparisons were made using the Tukey multiple range test. Values with different superscript letters d–g are significantly different ( $P < 0.05$ ).

<sup>b</sup>All rats were meal fed. Animals in the control group were fed *ad libitum* at each meal; RF rats were fed 90, 80 or 60% of the *ad libitum* intake.

<sup>c</sup>Ductal extension of the mammary gland into the fat pad, total area occupied by mammary epithelium and breast density were quantified as described in Materials and methods.

**Table III.** Effects of energy restriction on mammary AC volume of rat<sup>a</sup>

	Control	90% RF <sup>b</sup>	80% RF	60% RF
Volume (mm <sup>3</sup> ) per rat	433 ± 153 <sup>c</sup>	52 ± 15 <sup>d</sup>	35 ± 16 <sup>d,e</sup>	4 ± 2 <sup>e</sup>
Volume (mm <sup>3</sup> ) per 100 g body weight	299 ± 112 <sup>c</sup>	41 ± 12 <sup>d</sup>	31 ± 14 <sup>d,e</sup>	3.7 ± 2.0 <sup>e</sup>
Volume (mm <sup>3</sup> ) per carcinoma	81 ± 22 <sup>c</sup>	17 ± 5 <sup>d</sup>	9 ± 3 <sup>d,e</sup>	4 ± 2 <sup>e</sup>

<sup>a</sup>Values are means ± SEM. Data were analyzed by the Kruskal–Wallis rank test. Values with different superscript letters c–e are significantly different ( $P < 0.05$ ).

<sup>b</sup>All rats were meal fed. Animals in the control group were fed *ad libitum* at each meal; RF rats were fed 90, 80 or 60% of the *ad libitum* intake.

**Table IV.** Effects of energy restriction on cell proliferation in mammary gland ducts and in pre-malignant and malignant mammary lesions<sup>a</sup>

	Control	90% RF <sup>b</sup>	80% RF	60% RF
IDP	25.4 ± 2.3	19.6 ± 4.8	19.2 ± 1.7	17.4 ± 1.1
DCIS	16.6 ± 1.2	16.2 ± 7.1	15.7 ± 5.2	15.6 ± 3.7
AC	33.8 ± 1.4	29.2 ± 3.8	26.0 ± 3.2	24.3 ± 1.1

<sup>a</sup>The measurements reflect the percentage of cells that were BrdU-positive. Values are means ± SEM. Each data point is the mean of 10 independent observations from 10 different rats. Data were evaluated by regression analysis for trend. Cell proliferation tended to be lower with increasing level of energy restriction ( $P < 0.03$ ).

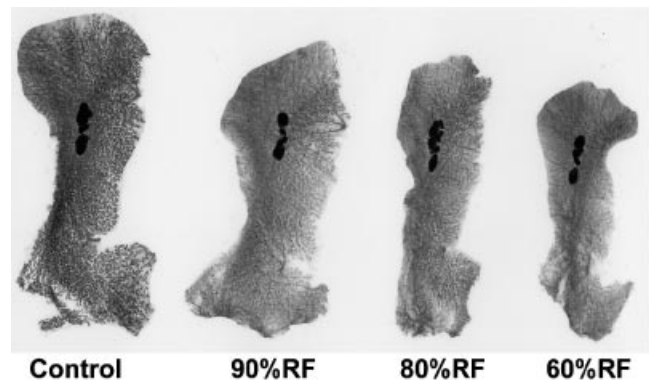
<sup>b</sup>All rats were meal fed. Animals in the control group were fed *ad libitum* at each meal; RF rats were fed 90, 80 or 60% of the *ad libitum* intake.

**Table V.** Effects of energy restriction on apoptosis in mammary gland ducts and in pre-malignant and malignant mammary lesions<sup>a</sup>

	Control	90% RF <sup>b</sup>	80% RF	60% RF
IDP	0.16 ± 0.02	0.19 ± 0.01	0.22 ± 0.06	0.25 ± 0.04
DCIS	0.44 ± 0.09	0.45 ± 0.13	0.48 ± 0.07	0.49 ± 0.26
AC	0.77 ± 0.08	0.80 ± 0.17	0.82 ± 0.20	0.93 ± 0.28

<sup>a</sup>The measurements reflect the percentage of cells that were apoptotic. Values are means ± SEM. Each data point is the mean of 10 independent observations from 10 different rats. Data were evaluated by Poisson regression analysis for trend. Apoptosis tended to be higher with increasing level of energy restriction ( $P < 0.05$ ).

<sup>b</sup>All rats were meal fed. Animals in the control group were fed *ad libitum* at each meal; RF rats were fed 90, 80 or 60% of the *ad libitum* intake.



**Fig. 4.** Whole mounts of abdominal–inguinal mammary gland chains. MNU-administered rats were fed either *ad libitum* (control) or were RF 90, 80 or 60% of control intake. Whole mounts of the abdominal–inguinal mammary glands were prepared at 35 days post-carcinogen as described in Materials and methods. A representative whole mount from each experimental group is shown.

#### Effect of energy restriction on mammary carcinoma volume

Table III shows the effect of energy restriction on AC volume. Carcinoma volume per tumor-bearing rat was significantly reduced with increasing level of energy restriction. When carcinoma volume was expressed per 100 g body wt, a significant inverse relationship with level of energy restriction remained; in fact, at the highest level of restriction, there was a 20-fold reduction in carcinoma volume.

#### Effect of energy restriction on cell proliferation and apoptosis in the mammary gland

Tables IV and V show the effects of energy restriction on the rates of cell proliferation and apoptosis of mammary epithelium in IDP, DCIS and AC. Because of the small number of lesions available for analysis in some groups, the evaluation of rates of proliferation and apoptosis was limited to 10 specimens/lesion type at each level of energy restriction. As a consequence of the relatively low rates of apoptosis observed and the variance associated with these values, statistical power was limited. Therefore, in our statistical analyses the proliferation or apoptosis data from all lesions types across all levels of energy restriction were subjected to trend analyses; evidence was found in support of the hypothesis that cell proliferation was reduced ( $P < 0.03$ ) and that apoptosis was increased ( $P < 0.05$ ) by energy restriction. In IDP, a pre-malignant mammary gland lesion, apoptosis was increased 56% at the highest level of energy restriction, while cell proliferation was

decreased 31%. Cell proliferation rates were somewhat lower and rates of apoptosis were higher in DCIS versus IDP, however, energy restriction was without effect on either cell proliferation or apoptosis within DCIS. Rates of cell proliferation and apoptosis were higher in AC than in any other lesion category in *ad libitum* fed animals. The rate of cell proliferation was reduced by 28% and the rate of apoptotic cell death increased by 21% at the highest level of energy restriction.

## Discussion

Consistent with our previous report, energy restriction was observed to inhibit the carcinogenic response in the mammary gland when the analysis was limited to the mammary gland chains in the abdominal–inguinal region (Table I; 6). This re-analysis of the carcinogenesis data presented in Zhu *et al.* (6) was critical since assessment of the effects of energy restriction on ductal extension of the mammary gland into the fat pad, total area occupied by mammary epithelium, breast density, cell proliferation and apoptosis in the experiments reported in this study was limited to these glands. The reason for this limitation is that a band of muscle tissue is interspersed between mammary glands 2 and 3 in the cervical–thoracic mammary gland chain (16). The presence of this muscle tissue prevented us from performing the digital image analyses described in Materials and methods on the cervical–thoracic mammary gland chain.

While dose-dependent inhibition of mammary carcinogenesis by energy restriction has been observed previously (17), we are unaware of other reports in which the degree of inhibition of lesion occurrence was shown to increase across lesion types from IDP to DCIS to AC, i.e. 36, 65 and 93% inhibition, respectively. We speculate that these data indicate that energy restriction blocks one or more steps in the progression of foci of initiated cells prior to their emergence as carcinomas such that the greatest effect of energy restriction was its inhibition of carcinoma occurrence.

The data shown in Table II demonstrate that overall size of an animal, measured as body weight, was reduced in a manner directly proportional to the degree of energy restriction imposed. This observation is consistent with a large body of literature, part of which was reviewed by Freedman *et al.* (17). However, to our knowledge no one has ever extended this line of inquiry to ask how the growth and development of the mammary gland is affected by energy restriction relative to its effect on body weight. As shown in Table II, energy restriction decreased ductal extension of the mammary gland into the fat pad, an effect comparable in magnitude with its effect on growth measured as body weight. However, the effect of energy restriction on the total amount of mammary epithelium was greater than its effect on body weight. We interpret these data to indicate that energy restriction exerts a specific effect on branching morphogenesis of the mammary ductal tree rather than on its linear extension. In the light of the fact that ductal extension is dependent on estrogen (18,19), this interpretation is consistent with evidence that estrogen levels are unaffected by moderate dietary restriction in women and animals and that underfed female rats retain the ability to respond to elevated concentrations of estrogen (20–23). Nonetheless, we emphasize the need for caution in interpreting these data, since the mammary glands were undergoing extensive development during the period over which energy restriction was imposed.

To further explore potential relationships between the carcinogenesis data reported in Table I and the mammary gland size data reported in Table II, we performed a series of regression analyses. Both total number of pre-malignant and malignant lesions and the number of AC were observed to be significantly correlated with both total area per 100 g body wt occupied by mammary epithelium ( $r^2 = 0.36$ ,  $P < 0.01$ ) and breast density ( $r^2 = 0.48$ ,  $P < 0.001$ ), but not with ductal extension expressed per 100 g body wt ( $r^2 = 0.09$ ,  $P > 0.1$ ). Thus energy restriction-mediated effects on area occupied by mammary epithelium, a reflection of branching morphogenesis, and on breast density were predictive of whether or not an animal was likely to develop a malignancy. This implies a relationship between the mechanisms that account for the effects of energy restriction on branching morphogenesis and breast density and those inhibiting the carcinogenic process.

As shown in Table III, energy restriction also affected carcinoma volume. The inverse relationship between degree of energy restriction and carcinoma volume was observed irrespective of the manner in which these data were expressed, i.e. average volume per carcinoma, carcinoma volume per tumor bearing rat or carcinoma volume per 100 g body wt. Whether a relationship exists between factors that determine breast density and those that account for the profound effect of energy restriction on tumor volume is unclear, but the fact that tumor volume was reduced 20-fold at the highest level of restriction suggests a specific effect that exceeds in magnitude other effects of energy restriction on the mammary gland as documented in this investigation. To further explore this issue, we considered the effects of energy restriction on rates of proliferation and death. Overall, the data presented in Tables IV and V indicate that energy restriction inhibited cell proliferation and induced apoptosis, a finding consistent with several reports about the effects of energy restriction on cell proliferation and apoptotic cell death in other model systems (24–27). However, given the magnitude of these effects, it is difficult to envision how a reduction in cell proliferation or an increase in cell death alone could account for the profound inhibitory effects of energy restriction on either the carcinogenic response (Table I) or on tumor volume (Table III). We speculate that some other mechanism is likely to be operative; one attractive candidate mechanism is inhibition of angiogenesis. Blocking angiogenesis could result in profound inhibitory effects on tumor development consistent with the data reported in this paper. Moreover, we and others have reported that energy restriction induces a glucocorticoid response and suppresses circulating levels of insulin-like growth factor-1 (6,27–29). This pattern of hormonal changes would be expected to inhibit angiogenesis based on data reported using other model systems (30–34).

In conclusion, energy restriction reduced breast density, an effect that was predictive of its effect on the carcinogenic response. Clues to the specific locus of this effect(s) may emerge from the observations that: (i) events that affect branching morphogenesis, rather than ductal extension of the mammary gland, appear to be preferentially affected by energy restriction; (ii) that carcinoma volume is profoundly reduced by energy restriction. Ongoing studies are evaluating the effects of energy restriction on cell cycle regulatory mechanisms and on the process of angiogenesis, in an effort to explain the profound protection against carcinogenesis that is attributed to energy restriction.

## Acknowledgement

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