

Cyclic food restriction, insulin and mammary cell proliferation in the rat

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We reported recently that weight cycling significantly increased the incidence of mammary cancer in virgin female rats that were pretreated with *N*-methyl-*N*-nitrosourea. The present study investigated the effect of weight cycling on mammary epithelial cell proliferation and its relationship to changes in plasma insulin, estrogen, progesterone and urinary corticosterone in 30 female virgin Sprague–Dawley rats. Animals were fed a modified AIN-76A diet containing 24.6% corn oil by weight. Weight-cycled (WC) rats were food restricted daily by either 33% or 50% of non-restricted controls for 1 week followed by 3 weeks compensatory refeeding and weight recovery over 18 weeks or 4.5 weight cycles. WC rats consumed 6–10% less food than controls ($P = 0.01$) but showed a 71–89% greater efficiency of food utilization for growth ($P < 0.0001$) than controls. There were no differences in total weight gain during treatment. Mammary lobuloalveolar and ductal cell proliferation of WC rats, measured by 5-bromo-2'-deoxyuridine labelling, increased in a dose–response fashion, $P = 0.03$, $P = 0.06$ respectively in comparison to controls. Energy and substrate utilization measured by indirect calorimetry indicated WC animals expended less energy ($P = 0.005$) and utilized less glucose ($P = 0.0001$) and protein ($P = 0.006$) during restriction, and less lipid during recovery ($P = 0.05$) than controls. There were no significant differences in hormone levels between groups. Multiple regression analysis with plasma insulin, estrogen, progesterone and urinary corticosterone as independent variables ($r = 0.947$, $r^2 = 0.897$, $P = 0.003$) showed that plasma insulin was the only significant predictor ($P < 0.01$) of mammary cell proliferation. In accord with this observation, tyrosine-phosphorylated activation of insulin receptor substrate-1, detected by immunoprecipitation and Western immunoblot analysis in mammary tumors of WC rats from our previous study, was 3–5 times greater than in non-restricted controls ($P < 0.01$). Present findings suggest that weight cycling in rats increases risk of breast cancer development via insulin stimulated mammary cell proliferation.

*Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; IRS-1, insulin receptor substrate-1; BRDU, 5-bromo-2'-deoxyuridine; DMBA, 7,12-dimethylbenz[*a*]anthracene; EGF, epidermal growth factor.

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Introduction

We reported recently that weight cycling abolished the protective effects of chronic restriction against mammary carcinogenesis (1). Rats that were administered *N*-methyl-*N*-nitrosourea (MNU*) and food restricted by 33% for 1 week, and then re-fed for 3 weeks (i.e. weight cycled) repeatedly for 18 weeks, consumed significantly less food, but showed a greater incidence of breast cancer than *ad libitum*-fed animals. Although body weight gain of the weight-cycled animals was less than the *ad libitum* animals, their efficiency for growth (i.e. total weight gain/total food intake $\times 100$) was consistently greater. Measurement of substrate utilization by indirect calorimetry indicated that the weight-cycled animals used more glucose and less fatty acid for energy than the non-restricted animals during their recovery from food restriction.

Several hormones have been associated with mammary cell proliferation (2). In particular, insulin is both a regulator of energy metabolism and a mitogenic stimulus of peripheral tissue growth. It has been shown to be essential for mammary gland development (3) and a promoter of mammary tumorigenesis in laboratory rats (4–6). Similarly, mammary gland growth (7) and tumorigenesis are directly affected by circulating ovarian sex hormones (8). On the other hand, corticosterone, a counter-regulatory hormone of insulin, has been reported to inhibit mammary cancer development (9).

Therefore, the loss of protection against mammary cancer that we observed in response to weight cycling could be related either to an increased stimulation of mammary cell proliferation by insulin and estrogen and/or an inhibition of adrenal corticosteroid function.

In the present study, the effects of weight cycling on mammary epithelial cell proliferation were examined in relation to plasma concentration of insulin, estrogen, progesterone and urinary corticosterone. Related to that experiment, we also measured tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), a cytoplasmic marker of insulin stimulation, in tumor tissue of weight-cycled and control animals from our previous study (1). Also, in the present experiment, the effects of weight cycling were studied using two levels of food restriction and a regimen of *ad libitum* and meal feeding. The rationale for including these additional treatment conditions was to determine whether the physiological effect of weight cycling on mammary tissue may vary with feeding regimen and intensity of food restriction.

We report herein, that weight cycling stimulated mammary lobuloalveolar cell proliferation in a dose–response fashion. Furthermore, the increase in mammary cell proliferation could be explained primarily by circulating levels of plasma insulin. Phosphorylated IRS-1 levels in mammary tumors of weight-cycled animals were several fold greater than in tumors of non-restricted control animals. Estrogen, progesterone and corticosterone were not found to be major predictors of mammary cell proliferation.

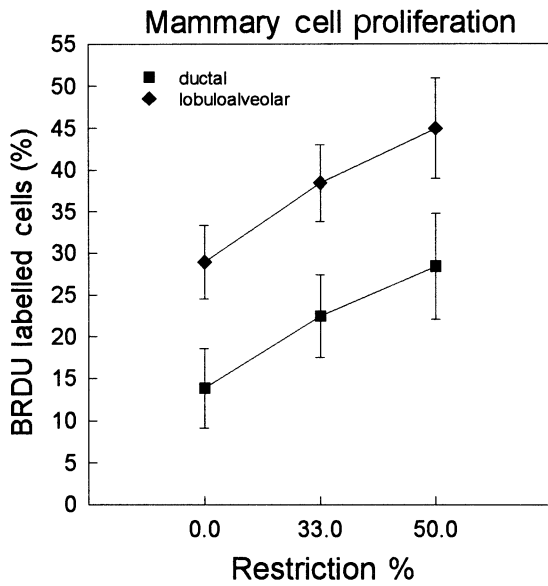


Fig. 2. Percentage of mammary epithelial cell proliferation among non-restricted control and weight-cycling animals that were restricted by either 33% or 50%. Cell proliferation measured by percentage BRDU labelled cells during week 18 (first refeed week of cycle 5). Values are mean \pm SE. Multiple regression analysis: lobuloalveolar cells $P = 0.029$; ductal cells $P = 0.06$. Restriction treatment: 0, non-restricted controls ($n = 12$); 33, WC animals ($n = 12$) that were restricted by 33%; 50, WC animals ($n = 6$) that were restricted by 50%.

Table I. Mammary epithelial cell proliferation (% BRDU cells labelled)

Group	(n)	Lobuloalveolar	Ductal
Ad lib-C	6	29.51 \pm 6.2 ^a	11.70 \pm 6.62
Ad lib-WC ₃₃	6	39.31 \pm 6.8	23.46 \pm 7.25
ME-C	6	28.29 \pm 6.8	16.44 \pm 7.25
ME-WC ₃₃	6	37.59 \pm 6.8	21.42 \pm 7.25
ME-WC ₅₀	6	44.97 \pm 6.2	28.44 \pm 6.62

^aMean \pm SE of BRDU-labelled proliferation of mammary epithelial lobuloalveolar and ductal cells of animals that were either restricted or non-restricted and fed either *ad libitum* or in two meals daily for 18 weeks. Ad lib-C (*ad libitum*-fed control); Ad lib-WC₃₃ (*ad libitum*-fed and 33% restricted); ME-C (non-restricted control fed two meals); ME-WC₃₃ (33% restricted and fed two meals); ME-WC₅₀ (50% restricted and fed two meals). Treatment effects analyzed by multiple regression: meal-eating vs *ad libitum* NS; weight cycling vs non-restricted $P < 0.05$.

were diluted with NP-40 buffer to normalize protein concentrations to 0.6 mg/ml. Homogenates were precleared by shaking with protein A-agarose beads (Pierce), agitated at 4°C for 20–30 min and centrifuged. Aliquots of precleared homogenates were added to IRS antibody (polyclonal rabbit, Upstate Biotechnology Inc., Lake Placid, NY) and incubated on ice. Fresh Protein A-agarose beads were added to each sample, which was then shaken, centrifuged and washed 5 times with NP-40/EDTA buffer. The samples were boiled in SDS buffer containing 5% mercaptoethanol, then centrifuged. Samples were loaded on SDS-polyacrylamide gels for resolution by electrophoresis according to procedure reported by Laemmli (16) using a 5% acrylamide lower gel. Samples were transferred to nitrocellulose in transfer buffer at 200 V for ~12 h. For assessing levels of phosphorylation of IRS-1, an anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology) was used as the primary antibody after multiple washes with PBS/Tween. Following incubation with primary antibody, the nitrocellulose membrane was washed and a secondary antibody (horseradish peroxidase conjugated sheep antimouse IgG) was added. The membrane was washed and tyrosine-phosphorylated IRS-1 was detected using Enhanced Chemiluminescence (ECL) (Pierce) on Hyperfilm-ECL (Kodak). Quantification of immunoblot density was measured by Arcus II transparency scanner (Agfa) and NIH Image 1.54 software for digitizing the image and quantifying its density in square pixels.

Effects of weight cycling and feeding regimen on cell proliferation were

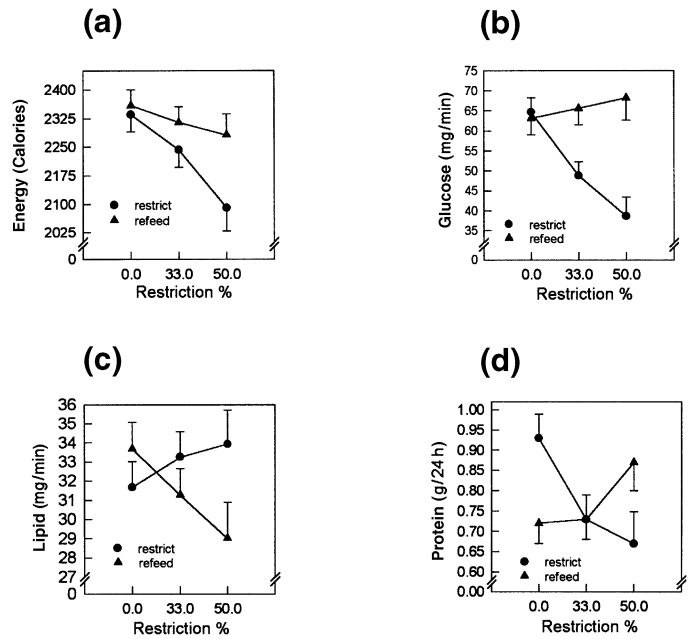


Fig. 3. (a) Mean \pm SE rate of energy expenditure during 24 h of non-restricted and weight-cycled animals during week of restriction and weeks of recovery from food restriction. Multiple regression analysis: restriction week (●) $P = 0.015$; refeed weeks (▲) NS. (b) Mean \pm SE rate of glucose utilization during 24 h of non-restricted and weight-cycled animals during week of restriction and weeks of recovery from food restriction. Multiple regression analysis: restriction week (●) $P < 0.0001$; recovery weeks (▲) NS. (c) Mean \pm SE rate of lipid utilization during 24 h of non-restricted and weight-cycled animals during week of restriction and weeks of recovery from food restriction. Multiple regression analysis: restriction week (●) NS; recovery weeks (▲) $P = 0.05$. (d) Mean \pm SE rate of protein utilization during 24 h of non-restricted and weight-cycled animals during week of restriction and weeks of recovery from food restriction. Multiple regression analysis: restriction week (●) $P = 0.006$; recovery weeks (▲) NS.

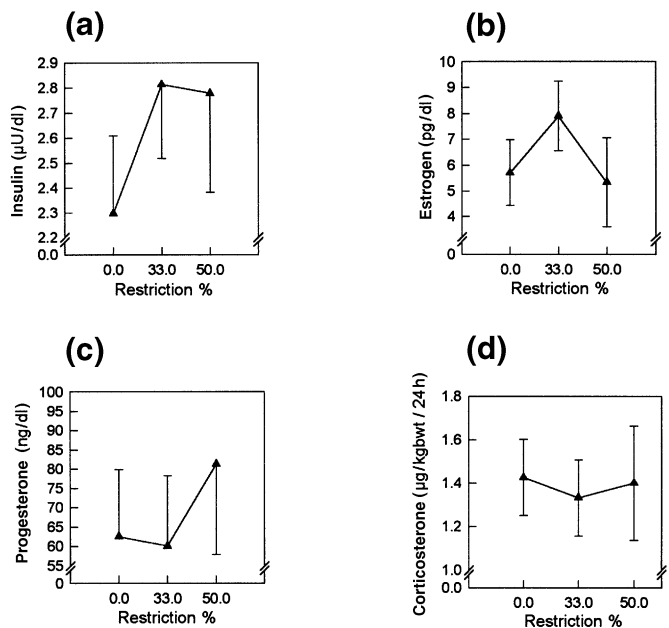


Fig. 4. (a) Levels of plasma insulin (µU/dl), (b) estrogen (pg/dl), (c) progesterone (ng/dl), and urinary (d) corticosterone (µg/kg body wt/24 h) of non-restricted and weight-cycled rats. Hormones were measured in week 18 (first refeed week of the fifth weight cycle). Values are mean \pm SE.

Table II. Food intake, body weight and efficiency

Group	(n)	Total food intake (g)	Body weight gain (g)	Efficiency ^a restrict (%)	Refeed (%)
Non-restricted	12	1316 ± 30 ^b	127 ± 8	10.9 ± 1.1	7.7 ± 1.0
Weight-cycled ₃₃	12	1225 ± 32	128 ± 8	-3.2 ± 1.2	13.2 ± 1.0
Weight-cycled ₅₀	6	1189 ± 41	125 ± 11	-18.6 ± 1.5	14.6 ± 1.3

^aEfficiency of food utilization for growth (total weight gain/total food consumption×100). During restriction (weeks 1, 5, 9, 13, 17), efficiency of food utilization among weight-cycled animals was less than for non-restricted controls ($P < 0.0001$); conversely, during weeks of refeeding (weeks 2–4, 6–8, 10–12, 14–16, 18), efficiency of food utilization among weight-cycled animals was greater than among non-restricted animals ($P < 0.0001$).

^bAll values are mean ± SE. Cumulative food intake, body weight gain and efficiency of food intake for growth of non-restricted and weight-cycled groups. Cumulative food consumed ($P = 0.01$); body weight gain (NS) over 18 weeks. Effects of treatment analyzed by multiple regression.

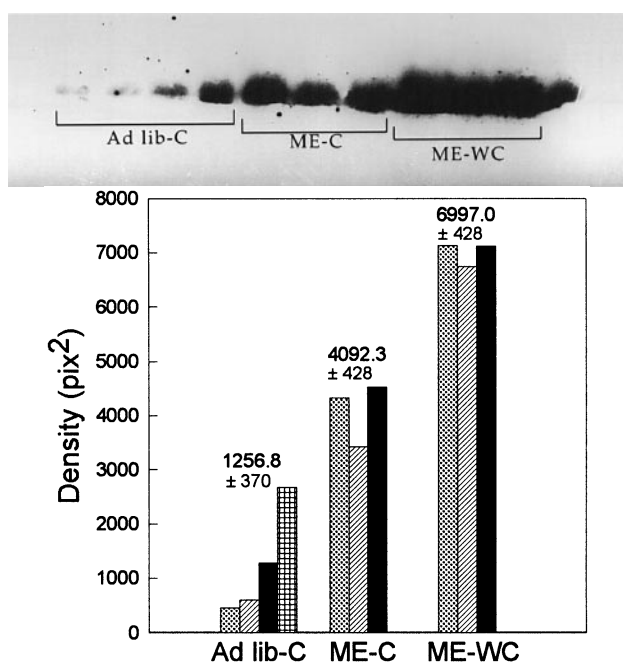


Fig. 5. Phosphorylated IRS-1 was detected by immunoprecipitation with an IRS-1 antibody followed by Western immunoblot analysis using a monoclonal antiphosphotyrosine primary antibody and horseradish peroxidase conjugated sheep antimouse secondary antibody. Samples were diluted with NP-40 to normalize protein concentration. Shown in gel are tumors from ($n = 4$) non food-restricted animals fed *ad libitum* (Ad lib-C); from non food-restricted animals ($n = 3$) fed in two 3 h meals (ME-C); and from animals ($n = 4$) that were food restricted by 33% for 1 week, then refeed for 3 weeks over an 18 week period (ME-WC). For ME-WC, immunoblots in the first three lanes were used for statistical comparison because the blot in the fourth lane was not complete. Density of blots was quantified using digitized image analysis. Density values are expressed as mean ± SE squared pixels. Diet effect on IRS-1 phosphorylation determined by ANOVA ($P < 0.0001$) and Tukey HSD for between group differences. ME-WC>Ad lib-C, $P < 0.001$; ME-C>Ad lib-C and ME-WC>ME-C, $P < 0.01$.

analyzed by multiple regression using the General linear model (Glm). Effects of weight cycling, substrate utilization, hormone concentration and the relationship between hormones and mammary lobuloalveolar cell proliferation were analyzed by multivariate or univariate regression analyses using the Glm. Tumor levels of tyrosine-phosphorylated IRS-1 were analyzed by analyses of variance and Tukey HSD for between group comparisons. All statistical analyses were performed using Systat for Windows: Statistics (version 5, 1992; Systat Inc., Evanston, IL) (17). Statistical significance of each analysis is reported in the text and in relevant figures.

All experimental methods and procedures were approved by the University's Animal Care and Use Committee.

Results

Analysis of variance of mammary epithelial cell proliferation in response to *ad libitum* versus meal eating and food restriction versus no restriction, indicated that only weight cycling had a significant effect on cell proliferation ($P < 0.05$) (Table I). Therefore, groups that were not food restricted (i.e. Ad lib-C and ME-C) and weight-cycled groups (Ad lib-WC₃₃ and ME-WC₃₃) were combined (as indicated in Figure 1) for all further statistical comparisons. Upon further analysis, weight cycling was found to increase mammary lobuloalveolar cell proliferation in a linear fashion ($P = 0.029$). Proliferation of mammary ductal epithelial cells showed a similar trend that approached statistical significance ($P = 0.06$) (Figure 2). There were no differences in estrous cyclicity among groups during restriction or refeeding weeks. The mean (± SD) length of an estrous cycle among non-restricted and weight-cycled animals was 4.3 ± 0.6 and 4.5 ± 0.6 days respectively.

Weight-cycled groups consumed 6–10% less total food than the non-restricted groups ($P = 0.01$). During food restriction, efficiency of food utilization for growth among the weight-cycled animals was lower than for non-restricted animals ($P < 0.0001$). On the other hand, efficiency of food utilization during weeks of refeeding from food restriction was ~71–89% greater than controls ($P < 0.0001$). Hence, there was no difference in body weight gain or final body weights between non-restricted and weight-cycled groups (Table II).

During weeks of food restriction, energy expenditure ($P = 0.005$), glucose ($P < 0.0001$), and protein ($P = 0.006$) utilization of the weight-cycled animals were significantly less than the non-restricted control animals (Figure 3). There were no differences in lipid utilization. During weeks of recovery from food restriction, weight-cycled animals used less lipid for energy ($P = 0.05$) than the non-restricted controls. There were no differences in energy, glucose or protein utilization between groups. High within-group variability precluded our detecting any statistical differences in plasma insulin, estrogen, progesterone, or urinary corticosterone between weight-cycled and non food-restricted animals. From inspection, the plasma insulin, and to a lesser extent, estrogen and progesterone, varied non linearly in response to weight cycling (Figure 4). In an initial attempt to determine whether the effects of weight cycling on mammary lobuloalveolar cell proliferation were related to variations in hormone levels, univariate regression analyses were performed using both a linear and squared expression of each hormone as the independent variables and mammary lobuloalveolar cell proliferation as the dependent variable. Insulin and estrogen, separately, were both linearly

and quadratically related to mammary cell proliferation ($P < 0.01$); insulin: $r = 0.71$, $r^2 = 0.5$; estrogen: $r = 0.55$, $r^2 = 0.3$. There was no relationship between either progesterone or corticosterone and mammary cell growth. When the linear and quadratic components of all four hormones were entered together as independent variables in a multivariate regression model, the model had a multiple $r = 0.947$ and an $r^2 = 0.897$ ($P = 0.003$), and insulin (both linear and quadratic components) was the only significant predictor ($P \leq 0.01$) of mammary lobuloalveolar cell proliferation.

In mammary tumors of weight-cycled animals (Experiment 2), phosphotyrosine activation of IRS-1 was five times greater than that of *ad libitum*-fed, non-restricted animals ($P < 0.0001$) and almost 3-fold greater than meal-fed, non-restricted animals ($P < 0.01$). Phosphorylation of IRS-1 (Figure 5) in mammary tumors of meal-fed controls also was greater than *ad libitum*-fed controls ($P < 0.01$).

Discussion

Energy intake has been found to be a sensitive modulator of mammary carcinogenesis in laboratory animals (18–22). A 12% restriction in dietary energy was found to reduce both incidence and development of mammary cancer in rats pretreated with 7,12-dimethylbenz[*a*]anthracene (DMBA) (23). Furthermore, a 14–25% restriction of dietary energy of young virgin female mice dramatically reduced mammary lobuloalveolar and ductal cell proliferation (24). The inhibitory action of energy restriction on mammary cell proliferation and tumorigenesis has been reported to be effected at least in part by the suppression of insulin (6,25) and estrogen (26). Engleman *et al.* (27) have shown that caloric restriction suppresses local growth stimuli, such as epidermal growth factor (EGF). In addition, the positive status of corticosterone, through mechanisms that are less apparent, is necessary for inhibition of tumorigenesis by chronic caloric restriction (28).

Recently, we reported female rats (weight cycled for 18 weeks) decreased total food intake by 14% but increased the incidence of MNU-induced mammary cancer in comparison to *ad libitum*-fed controls. Furthermore, tumors of the weight-cycled animals were heavier ($P = 0.09$) and tumor number was similar between groups (1). The present investigation was a follow-up to that experiment. Weight-cycled animals that were food restricted by 33% and 50% consumed significantly less food (6–10%) than the non-restricted controls. However, rate of mammary lobuloalveolar cell proliferation among weight-cycled animals was 33–55% greater than the controls. Although weight-cycled animals consumed less food than the controls, the former had a 71–89% greater efficiency of food utilization for growth during recovery weeks that resulted in a total body weight gain that was similar to the non-restricted controls. Only plasma insulin was a significant predictor of mammary lobuloalveolar cell proliferation. Although univariate regression analysis indicated that plasma estrogen also was highly related to mammary lobuloalveolar cell growth, it was not found to be significant when it was included with insulin, progesterone, and urinary corticosterone in a multivariate regression model that explained 90% of the variation in mammary cell growth. This finding indicated that the effects of estrogen on mammary cell growth were not independent of insulin. Insulin has been reported to increase estrogen binding to mammary cells (4). Hence, we suspect that most of the hormonal effects on mammary cell proliferation may have

been the result of mitogenic stimulation by insulin and its secondary effects via an enhancement of estrogen binding.

Cell proliferation is a high risk condition for genomic insult by a carcinogen and/or the promotion of a neoplastic growth (28,29). In the present study, weight cycling stimulated mammary lobuloalveolar cell proliferation which could be explained, to a great extent, by plasma insulin. Insulin-like growth factor 1 (IGF-1) also has been reported to be a promoter of mammary cell proliferation (31). Since IGF-1 was not measured, we cannot rule out its involvement in the effects reported here. Stimulation of DNA synthesis and cell proliferation by insulin require tyrosine phosphorylation of IRS-1 which is dependent on direct stimulation by either insulin and/or IGF-1 and their respective membrane receptors (32). Tyrosine phosphorylation of IRS-1 in mammary tumors of weight-cycled rats, in the present study, was several fold greater than non-restricted controls.

The link between breast cancer and insulin is not new. Earlier reports have shown that normal insulin secretion was required for development of mammary cancer in rats pretreated with DMBA (5,33) and promoted breast cancer in genetically obese, hyperinsulinemic LN/A-cp rats in comparison to their lean littermates (6). Examination of glucose oxidation *in vitro*, showed that the dependency of mammary tumor cell proliferation on insulin was not related to glucose metabolism, which indicated indirectly, that the primary cellular pathway of insulin action was on mitogenesis (33). On the other hand, chronic caloric restriction has been shown to suppress plasma insulin (34) and IGF-1 levels (34) and DMBA-induced cancer in rats. There is growing speculation that insulin may be an important etiological factor in both human breast and colon cancers (35–37).

To our knowledge, the evidence of weight cycling, mammary epithelial cell proliferation, and their relationship to insulin stimulation has not been reported. We hypothesize that weight cycling may increase the development of a subpopulation of cancer in breast as well as other metabolically active tissues that are exposed to frequent or chronic insulin stimulation. Since weight cycling is a common method of weight control among women, further study of this dietary practice and insulin stimulation on regulatory mechanisms of mammary cell proliferation and tumor development warrant further investigation.

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