Weight-Cycling Decreases Incidence and Increases Latency of Mammary Tumors to a Greater Extent Than Does Chronic Caloric Restriction in Mouse Mammary Tumor Virus-Transforming Growth Factor-α Female Mice[1]

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Abstract

Multiple periods of caloric restriction (or fasting)/refeeding in rodents have had inconsistent effects on mammary tumor (MT) development. In the present study, the consequence of intermittent caloric restriction/refeeding resulting in weight-cycling was evaluated using an oncogene-induced MT mouse model. Hybrid mouse MT virus-transforming growth factor α (MMTV-TGF-α)/*Lep*$^{+}$*Lep*^{ob} female mice were used. *Ad libitum*-fed mice ($n = 30$) were fed American Institute of Nutrition (AIN)-93M diet. Beginning at 10 weeks of age, weight-cycled mice ($n = 30$) were fed an AIN-93 modified diet (2-fold increase in protein, fat, vitamin, and mineral contents) at 50% of *ad libitum* for 3-week intervals followed by 3-week intervals of *ad libitum* feeding using AIN-93M diet. Pair-fed mice ($n = 33$), were fed a 2:1 mixture of AIN-93M:AIN-93 modified diets to match the caloric intake of weight-cycled mice for each 6-week age-matched caloric restriction/refeeding interval. Food intakes were determined daily and body weights weekly. Mice were euthanized when MTs exceeded 20 mm in length or at 80 weeks of age. Final body weights were similar, but cumulative food intake of *ad libitum*-fed mice was 21% greater than that of the other groups. *Ad libitum*-fed mice had a 77% MT incidence *versus* 3% for weight-cycled and 44% for pair-fed mice. MTs were detected earlier for *ad libitum*-fed mice, 64.1 weeks *versus* 73.5 weeks for pair-fed mice. The only MT in one weight-cycled mouse was excised at necropsy (80 weeks of age) and weighed only 0.063 g. Average MT weight for *ad libitum*-fed mice was 1.034 g and for pair-fed mice was 0.667 g. Intervals of caloric restriction/refeeding resulting in weight-cycling were protective against MT development in this mouse model. Future studies should address the application of this intervention to additional transgenic mice as well as other MT models.

Introduction

It is well documented that chronic caloric restriction decreases chemically induced, spontaneous, and transgene-induced MT[3] incidence and/or extends tumor latency in rodents (1–11). In contrast to these chronic caloric restriction studies, other studies have evaluated the effect of intermittent caloric restriction after chemical carcinogen treatment. Two basic types of protocols have been assessed: one period of caloric restriction at different times or multiple periods of caloric restriction followed by refeeding during the course of MT development.

Examples of studies involving single periods of restriction include Sylvester *et al.* (12), Kritchevsky *et al.* (13), and Engelman *et al.* (9). Sylvester *et al.* (12) reported that caloric restriction at 50% of *ad libitum* for 1 week prior to and during the first week after DMBA administration lowered the MT incidence rate by almost 70% in female rats. However, similar periods of caloric restriction, when started at either 1 or 3 weeks post-DMBA administration, had no effect on MT incidence (12). In a similar study, 25% caloric restriction for 16 weeks after DMBA treatment resulted in a 60% reduction in the incidence of MTs compared with *ad libitum*-fed rats (13). When 25% caloric restriction was used for only the first 4 weeks after DMBA administration followed by *ad libitum* feeding, MT incidence was not affected. Yet, this degree of caloric restriction for the first 8 weeks, the middle 8 weeks, or the last 8 weeks of the experiment offered various degrees of protection, *i.e.*, 20, 10, and 40%, respectively (13). Finally, in studies of C3H/HEOu mice, which develop spontaneous proviral associated MTs, mice chronically restricted to 70% of *ad libitum* for over 1 year had a MT incidence of 13% compared with 83% for *ad libitum*-fed mice (9). In this same study mice similarly restricted from 4–12 weeks of age had a MT incidence of 50% (9). Thus, in general, chronic caloric restriction appears to have a greater protective effect on MT development than do shorter periods of restriction in rodents.

The effect of intermittent caloric restriction/refeeding on MT development also has been evaluated. In two studies, rats with chemically induced MTs were used as the experimental

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[3] The abbreviations used are: MT, mammary tumor; AIN, American Institute of Nutrition; DMBA, 7,12-dimethyl-benz[*a*]anthracene; EGFR, epidermal growth factor receptor; IGF-I, insulin like growth factor 1; MMTV, mouse MT virus; NMU, nitrosomethylurea; TGF-α, transforming growth factor α.

model (14, 15). Mehta *et al.* (14) reported a 63% MT incidence for *ad libitum*-fed rats, 27% for chronic restricted (60% of *ad libitum*) rats, and 57% for intermittent restricted (48 h at 60% of *ad libitum* followed by 48 h of *ad libitum* feeding) rats indicating no effect of intermittent caloric restriction/refeeding. In the second study using rats with chemically induced MTs, Tagliaferro *et al.* (15) reported a 22% increase in MT incidence in intermittent restricted/refed rats compared with *ad libitum*-fed rats. In contrast to the preceding results, C3H/HeOu mice classified as "chronic energy intake restricted" had a significant delay in the appearance of spontaneous MTs compared with *ad libitum*-fed mice (16). However, examination of this experimental protocol suggests that these mice were actually intermittently fasted and refed as they were only fed twice a week. In an additional study, female rats that were fasted every other day had decreased spontaneous MT incidence and an extended period of tumor latency compared with rats that were fasted and refed every 3 or 4 days or that were *ad libitum* fed (17). There were, however, protective effects of the other fasting regimens, but they were not as robust as the every-other-day fasting protocol. Interestingly, fasting and refeeding initiated at 34 weeks of age had no effect on spontaneous MT development in mice (18). Taken together these results at this point do not provide a clear picture of the effect of intermittent caloric restriction/refeeding on the development of MTs.

In summary, rodent models have been used extensively to examine the effect of chronic caloric restriction on MT development. These studies, in general, support the notion that this dietary intervention has the potential to increase MT latency, and to significantly decrease MT incidence and burden. However, to date no consistent effect of intermittent caloric restriction/refeeding has been observed. This may be attributable to the use of different feeding regimens, *i.e.*, degree and length of restriction, and/or diet composition or differences in caloric intake and body weight. Even more importantly, carcinogen-treated rodents perhaps more accurately represent premenopausal rather than postmenopausal breast cancer development because MTs develop shortly after the administration of the compounds during the first 6 months of life. Here, we report the results of intermittent calorie restriction/refeeding on MT development later in life. In these studies, hybrid MMTV-TGF-α/$Lep^{+}Lep^{ob}$ mice were used. MMTV-TGF-α mice were originally described to have a 30% incidence of MTs by 16 months of age (19). These mice overexpress human TGF-α, and this factor is a component of EGFR/ErbB cascade known to play an important role in the development and progression of some human breast cancers (20–22). Specifically, expression of TGF-α is associated with 30–70% of human breast tumors (23–26). Furthermore, transgenic mice that overexpress TGF-α are considered to be good models to use for assessment of the breast cancer disease process (27, 28). We previously crossed the original MMTV-TGF-α mouse strain with the Lep^{ob} mouse strain to evaluate the effect of body weight on the development of MTs.[4] MMTV-TGF-α/$Lep^{ob}Lep^{ob}$ genetically obese mice died at much younger ages than did their lean transgenic counterparts and failed to develop MTs. Interestingly, heterozygous TGF-α/$Lep^{+}Lep^{ob}$ lean mice weighed more than did homozygous TGF-α/$Lep^{+}Lep^{+}$ lean mice over the 2 years of the study, and they had an MT incidence of 67% compared with 43% for the homozygous lean mice. In the present study, we used only TGF-α mice heterozygous for the Lep^{ob} gene because of the potential difference in incidence between the two lean genotypes. Interestingly, this genotype in humans is associated with an elevated body mass index (29). Experimental mice were intermittently calorie restricted and refed using a protocol previously shown to result in an overall reduction of caloric intake of 25% in female rats (30, 31). Despite reduced caloric intake, there was complete recovery of body weight after refeeding in restricted/refed rats. Thus, use of this model provides the opportunity to alter caloric intake independently of final body weight. This experimental protocol has been termed weight-cycling. Because of the anticipated decrease in overall caloric intake in the weight-cycled mice, we also included one control group of "pair-fed" mice that consumed the identical caloric and nutrient intake, as the weight-cycled mice, but in a chronic restricted regimen. Our results using an animal model that represents postmenopausal breast cancer provides support for the hypothesis that intermittent caloric restriction reduces the incidence and also delays the latency of MT development to a significantly greater extent than does chronic caloric restriction alone.

Table 1 Composition of experimental diets

	AIN-93[a] (g/kg)	AIN-93 modified[b] (g/kg)
Casein	140.0	280.0
L-Cystine	1.8	3.6
Corn starch	470.692	322.8
Maltodextrin	160.0	98.0
Sucrose	100.0	61.0
Soybean oil	40.0	80.0
Cellulose	40.0	59.584
Mineral mix (AIN-93M-MX)	35.0	70.0
Vitamin mix (AIN-93-VX)	10.0	20.0
Choline bitartrate	2.5	5.0
TBHQ[c] (antioxidant)	0.008	0.016

[a] Based on AIN-93 diet (33) designed for long-term maintenance of rodents.
[b] Based on modified AIN-93 diet but to be fed at one-half the amount consumed during *ad libitum* feeding. (See "Materials and Methods" for description.)
[c] TBHQ = tert-butylhydroguinone.

Materials and Methods

Experimental mice were obtained by mating nontransgenic heterozygous $Lep^{+}Lep^{ob}$ female mice with either heterozygous TGF-α/$Lep^{+}Lep^{ob}$ lean or homozygous TGF-α/$Lep^{ob}Lep^{ob}$ obese [treated with leptin to restore fertility (32)] male mice. Offspring were maintained with their mothers until 4 weeks of age. They were then housed with like-sexed pups, whereas genotypes (*TGF-α* and *Lep* status) were determined using DNA obtained from tail biopsies. Genotyping procedures have been described previously in detail.[4]

After genotyping, mice were assigned to one of three experimental groups; *ad libitum*-fed (n = 30), weight-cycled (n = 30), and pair-fed (n = 33). Experimental mice were housed individually in hanging stainless steel cages. Water was provided on an *ad libitum* basis. From 7–9 weeks of age, all of the mice were fed a purified diet based on AIN-93M recommendations for long-term maintenance of rodents (Table 1; Ref. 33). *Ad libitum*-fed mice were maintained on the AIN-93M diet throughout the experiment (all diets were from Harlan Teklad, Madison, WI). Beginning at 10 weeks of age weight-cycled mice were fed a modified version of the AIN-93 diet (AIN-93-mod; Table 1) for 3-week intervals at 50% of *ad libitum* intake.

[4] M. P. Cleary, F. C. Phillips, S. C. Getzin, T. L. Jacobson, M. K. Jacobson, T. A. Christensen, J. P. Grande, and N. J. Maihle. Genetically obese MMTV-TGF-α/$Lep^{ob}Lep^{ob}$ female mice do not develop mammary tumors. Breast Cancer Res. Treat., in press (2002).

AIN-93-mod had 2-fold increases in protein, vitamin, mineral, and fat contents and was formulated to be isocaloric with AIN-93M diet. After each calorie restriction period, weight-cycled mice were provided the AIN-93M diet for a 3-week interval of *ad libitum* feeding. On the basis of earlier work using the intermittent restriction/refeeding protocol in rats (30, 31), we anticipated that weight-cycled mice would have a decrease in caloric intake compared with the *ad libitum*-fed mice, and, therefore, we included an additional control group that was pair-fed to the weight-cycled mice. From 10 weeks of age, the mice assigned to the pair-fed group were fed a diet mixture of 2:1 of AIN-93M:AIN-93-mod at the average daily food intake for the corresponding age-matched 6-week food restriction/refeeding interval of the weight-cycled mice. This group in essence was chronically calorie-restricted and consumed similar calories and nutrients as the weight-cycled mice. For all of the mice, food was provided in small glass cups placed inside larger glass cups to collect spillage. The animal room was maintained at 22°C on a 12-hour light:12-hour dark cycle with humidity level of 50%. The Hormel Institute Animal Facility is accredited by the American Association for Accreditation of Laboratory Animal Care. The University of Minnesota Animal Care and Use Committee approved this study.

Food intakes were determined daily and body weights weekly. Mice were palpated weekly to determine whether MTs were present. Once MTs were detected, tumor growth was monitored with calipers. The majority of mice were killed when MT size exceeded 20 mm in length or they reached 80 weeks of age. This age was used because of the high incidence of MTs in the *ad libitum*-fed group by this time. For the weight-cycled mice this was 1 week into 12th refeeding period. Four weight-cycled mice were killed at 64 weeks of age because they were not cycling, *i.e.*; they failed to regain their lost weight.

When the mice were killed, a blood sample was obtained and serum prepared. Serum IGF-I concentrations were determined using rat IGF-I RIA kits obtained from Diagnostic Systems Laboratory (Webster, TX). Liver, kidneys, heart, spleen, and lungs were removed and weighed. In some cases, ovaries were removed. MTs, other tumors, and any other abnormal growth/tissue also were removed and weighed. Samples from organs and tissues were placed in 10% neutral buffered formalin for 24–48 h and then embedded in paraffin. Sections, 5-μm thick, were prepared, deparaffinized in xylene (2 $\times$ 5 min), rehydrated in a graded series of ethanol solutions, and rinsed in tap water. Sections were stained with H&E. Histopathological analysis was conducted in a blinded fashion without prior knowledge of group assignment. Retroperitoneal and parametrial fat pads also were removed and weighed.

Data are presented as mean $\pm$ SE. Comparisons of the body weight curves were done with one-way ANOVA for repeated measures. Comparisons among the three dietary groups were made by ANOVA followed by Neuman-Keul's test to determine statistical differences between specific groups. Comparisons of the *ad libitum*-fed and pair-fed mice with and without MTs were made by 2 $\times$ 2 ANOVA. In some cases comparisons between specific groups were made by Student's *t* test.

Results

During each weight loss/regain cycle, food intake for weight-cycled and pair-fed mice was 25–28% lower than that of *ad libitum*-fed mice (Table 2). However, cumulative food intakes of these first two groups decreased only 20% compared with the *ad libitum*-fed mice, because they lived longer than did the *ad*

Table 2 Food intake (grams) for *ad libitum*-fed, weight-cycled, and pair-fed TGF-α/$Lep^{+}Lep^{ob}$ female mice[a]

	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6	Cycle 7	Cycle 8	Cycle 9	Cycle 10	Cycle 11	Cycle[b] 12	Cum[c] intake
Ad libitum-fed	199.2 ± 2.5 (n = 30)	214.4 ± 3.5 (n = 30)	200.7 ± 7.1 (n = 30)	203.2 ± 3.7 (n = 30)	202.3 ± 3.7 (n = 30)	201.5 ± 4.1 (n = 30)	197.2 ± 4.1 (n = 30)	192.3 ± 5.7 (n = 29)	201.7 ± 5.5 (n = 24)	198.7 ± 6.6 (n = 22)	200.8 ± 8.7 (n = 20)	176.6 ± 9.8 (n = 17)	2145.5 ± 71.1 (n = 30)
Weight-cycled	141.8 ± 2.9 (n = 30)	149.6 ± 3.5 (n = 30)	148.7 ± 2.1 (n = 30)	151.3 ± 2.3 (n = 30)	155.4 ± 2.0 (n = 30)	155.8 ± 2.1 (n = 30)	156.0 ± 2.4 (n = 30)	158.9 ± 3.4 (n = 30)	158.3 ± 2.5 (n = 29)	161.3 ± 2.9 (n = 24)	163.7 ± 2.8 (n = 23)	100.2 ± 1.6 (n = 23)	1701.8 ± 39.9 (n = 30)
Pair-fed	138.9 ± 1.3 (n = 33)	150.3 ± 1.1 (n = 33)	140.2 ± 0.6 (n = 33)	146.8 ± 1.1 (n = 33)	148.6 ± 0.7 (n = 32)	151.0 ± 0.7 (n = 32)	155.8 ± 1.0 (n = 32)	157.7 ± 0.4 (n = 32)	154.7 ± 2.7 (n = 29)	155.9 ± 4.6 (n = 29)	162.8 ± 4.0 (n = 28)	111.3 ± 3.1 (n = 26)	1708.2 ± 31.1 (n = 33)

[a] Results are presented as mean $\pm$ SE. Number in parentheses equals number of mice in sample at that time. In each column, *ad libitum*-fed is significantly different from weight-cycled and pair-fed groups using Neuman-Keul's test after significant ANOVA calculation.
[b] Only 4 weeks in length. For the weight-cycled mice, this equaled 3 weeks of food restriction and 1 week of refeeding.
[c] Cum, cumulative food.

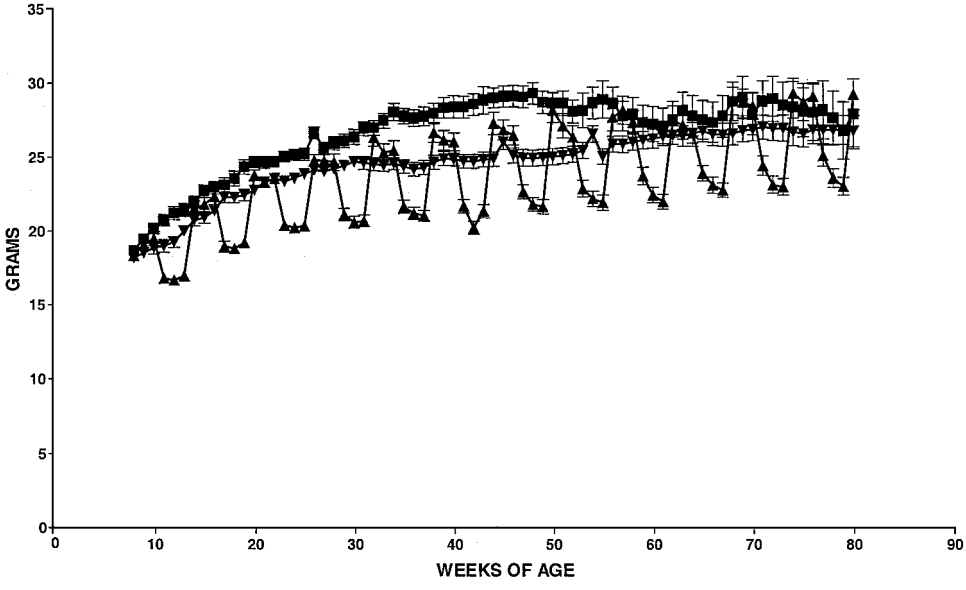

Fig. 1. Body weight curves of TGF-α/*Lep*$^{+}$*Lep*^{ob} female mice in the weight-cycling study. ■, *ad libitum*-fed mice (n = 12–30, depending on age); ▼, weight-cycled mice (n = 23–30, depending on age); ▲, pair-fed mice (n = 26–33, depending on age). ANOVA with repeated measures $P < 0.0001$; weight-cycled *versus ad libitum*-fed, $P < 0.001$; weight-cycled *versus* pair-fed, $P < 0.05$; and pair-fed *versus ad libitum*-fed, $P < 0.001$.

Table 3 Characteristics of MT development from *ad libitum*-fed, weight-cycled, and pair-fed TGF-α/*Lep*$^{+}$*Lep*^{ob} female mice[a]

	Age at MT detection (weeks)	% of mice in group with MT (no. with MT/total mice)	MT weight (g)	No. of MTs/mouse
Ad libitum-fed (n = 23)	63.8 ± 2.6[b]	77% (23/30)[c]	1.034 ± 0.187[b]	1.52 ± 0.25
Weight-cycled (n = 1)	80	3% (1/30)[c]	0.063	1.00
Pair-fed (n = 15)	73.5 ± 2.2[b]	44% (15/33)[c]	0.668 ± 0.127[b]	1.73 ± 0.31

[a] Values are means ± SE.
[b] *Ad libitum*-fed and pair-fed significantly different by Student's t test.
[c] Values are all different from each other by χ^2 analysis.

libitum-fed mice (Table 2). During each three-week refeeding period, weight-cycled mice consumed either similar amounts or significantly more food than did age-matched *ad libitum*-fed mice (results not shown). By design, food intakes of the weight-cycled and pair-fed groups were almost identical during each food restriction/refeeding period, as was cumulative food intake.

Body weights over the course of the experiment are presented in Fig. 1. In all cases, body weight curves for the three groups were significantly different from each other. *Ad libitum*-fed mice had a slow steady weight gain until 43–46 weeks of age when body weights peaked. Pair-fed mice had a more moderate weight gain than did the *ad libitum*-fed mice. Their body weight curve was characterized by long periods of weight stability. Pair-fed mice appeared to still be gaining weight at the termination of the experiment at 80 weeks of age. As expected, weight-cycled mice lost weight in each food restriction period. During the initial six cycles, most of the weight was lost in the first week of caloric restriction, and body weight was then maintained at 20–25% of that of *ad libitum*-fed mice for the remaining 2 weeks. In later cycles, weight-cycled mice lost small amounts of additional weight during the second and third weeks of restriction. Refeeding led to a rapid regain of lost weight with body weight recovering into the range of *ad libitum*-fed mice after 1 week of refeeding. Final body weights for the three groups were not statistically different from each other: *ad libitum*-fed, 29.10 ± 1.44 g; weight-cycled, 27.17 ± 1.07 g; and pair-fed, 26.86 ± 0.86 g (ANOVA; $P > 0.05$; Fig. 1).

Despite the absence of an effect of the dietary interventions on final body weights, there were statistically significant differences on the latency and incidence of MT development among these three groups. The average age of MT detection in *ad libitum*-fed mice was 64.1 weeks compared with 73.5 weeks for pair-fed mice (Table 3). Only 1 of 30 weight-cycled mice developed a MT, and this tumor was only detectable on necropsy (80 weeks of age). Of the *ad libitum*-fed mice 23 (77%) of 30 developed MTs detectable either by palpation (83%) or autopsy (17%; Table 3). In contrast, only 15 (44%) of 33 of the pair-fed mice developed MTs detectable either by palpation (60%) or necropsy (40%). The average tumor size also was significantly larger (55%) in *ad libitum*-fed than in pair-fed mice as determined by MT weight (Table 3). The single MT detected in the weight-cycled mouse was quite small, weighing only 0.063 g or 6% of the average tumor weight of the *ad libitum*-fed mice.

A summary of the histopathological properties of MTs from all of the mice is presented in Table 4. A total of 28 suspected MTs were detected in 23 *ad libitum*-fed mice; of these 27 of 28 were confirmed to be malignant. Twenty-three of these tumors were classified as low-grade adenocarcinomas, 1 as an adenocarcinoma with papillary features, 1 as an adenocarcinoma with desmoplastic features, 2 as high-grade adenocarcinomas, and 1 was determined to be only an atypical epithelium. Twenty-five MTs were identified in 15 pair-fed mice. Of these, 23 of 25 were identified as low-grade adenocarcinomas, 1 as a carcinoma *in situ*, and 1 as a high-grade adenocarcinoma. As indicated above, only one putative tumor was identified at autopsy in the weight-cycled group, and this

Table 4 Summary of histopathology from *ad libitum*-fed, weight-cycled, and pair-fed TGF-α/*Lep*$^{+}$*Lep*^{ob} female mice

	MT pathology				Other malignancies
	Total	Low-grade	High-grade	Other	
Ad libitum-fed	28	23	2	3[a]	1 possible myeloproliferative disorder based on liver pathology and 1 low-grade papillary adenocarcinoma of the ovary (both in mice with MTs)
Weight-cycled	1	1	0	0	
Pair-fed	25	23	1	1[b]	

[a] One, adenocarcinoma with papillary features; 1, atypical epithelium; and 1, adenocarcinoma with desmoplastic features.
[b] *In situ.*

Table 5 Final body weights, summed organ weights, carcass weights, fat pad weights, and IGF-I levels for *ad libitum*-fed, weight-cycled, and pair-fed TGF-α/*Lep*$^{+}$*Lep*^{ob} female mice[a]

	Carcass weight[b] (g)	Fat pad weight[c] (g)	IGF-I[d] (ng/ml)
Ad libitum-fed ($n = 30$)	26.13 ± 1.24	1.221 ± 0.194	586.9 ± 48.6
Weight-cycled ($n = 30$)	24.96 ± 1.03	1.129 ± 0.122	553.6 ± 37.5
Pair-fed ($n = 33$)	24.32 ± 0.813	1.105 ± 0.144	467.7 ± 22.5

[a] Values are means ± SE.
[b] Carcass weight equals body weight minus summed organ weights and weight of MTs or other tumors.
[c] Fat pad weight equals combined retroperitoneal and parametrial fat pads.
[d] For IGF-I, $n = 23$/group; $P < 0.07$.

Table 6 Comparisons of *ad libitum*-fed and pair-fed TGF-α/*Lep*$^{+}$*Lep*^{ob} mice with and without MTs[a]

	Final body weight (g)	Carcass weight (g)	Fat pad weight (g)
Ad libitum-fed			
No MT ($n = 7$)	20.36 ± 1.80[b]	18.729 ± 1.65	0.3264 ± 0.151
MT ($n = 23$)	31.76 ± 1.40[b]	28.379 ± 1.217[b]	1.5085 ± 0.218[b]
Pair-fed			
No MT ($n = 18$)	26.1 ± 1.47	23.710 ± 1.444	1.1895 ± 0.228
MT ($n = 15$)	27.78 ± 0.65[b]	25.060 ± 0.608[c]	1.1236 ± 0.164[b]

[a] Values are means ± SE. Data were analyzed by 2 × 2 ANOVA.
[b] Indicates a significant effect of the presence of MTs.

was identified as a low-grade adenocarcinoma of the mammary gland. The histopathology findings of these MTs are consistent with results previously reported for the original TGF-α mice (19, 34), as well as our earlier results for hybrid TGF-α/*Lep*$^{+}$*Lep*$^{+}$ and *Lep*$^{+}$*Lep*^{ob} mice.[4] Other than MTs, there were few notable pathological findings in these mice. In fact, none were detected in either the weight-cycled or pair-fed mice. In two tumor-bearing *ad libitum*-fed mice, additional pathologies were noted. One mouse had a possible myeloproliferative disorder (based on liver pathology), and a second developed a low-grade papillary adenocarcinoma of the ovary.

Correction of final body weight for organ weights, as well as any MTs or other abnormal growths indicated no significant differences in what was termed carcass weight among the dietary groups (Table 5). In addition, there were no differences in fat pad weights among the three groups (Table 5). Serum IGF-I concentrations were determined and these values also are presented in Table 5. Overall differences in serum IGF-I among the three groups approached, but did not reach, statistical significance ($P < 0.07$). The most obvious difference was the somewhat lower level of serum IGF-I in the pair-fed mice (468 ng/ml) compared with either the weight-cycled (554 ng/ml) or *ad libitum*-fed (587 ng/ml) groups.

Because only one weight-cycled mouse had a MT, comparisons for mice with and without MT are presented only for the *ad libitum*-fed and pair-fed mice (Table 6). There were significant effects of the presence of MTs on body weight, fat pad weight, and carcass weight. In general, these differences were primarily between the *ad libitum*-fed mice with and without MTs.

Discussion

In this study, we provide additional evidence in support of the hypothesis that decreased caloric intake has a protective effect on MT development. Specifically, we show that a decrease in caloric intake, whether administered as chronic caloric restriction or as intermittent caloric restriction/refeeding, protects against oncogene-induced MT development. However, an identical decrease in calories and nutrients consumed by intermittent caloric restriction/refeeding was more effective in both delaying the age of MT detection and in lowering overall MT incidence than were those consumed by chronic caloric restriction.

These new findings are in contrast to the results of two previous studies that assessed the effects of chemical carcinogen-induced MT development in rats subjected to two different intermittent caloric restriction/refeeding protocols (14, 15). In one of these studies (14), the degree of food restriction was substantially different between chronically calorie-restricted rats (60% of *ad libitum*) *versus* intermittent-restricted/refed rats (80% of *ad libitum*), and final body weights reflected these overall differences in caloric intake. In this same study, intermittent calorie restriction did not result in weight loss, *i.e.*, these rats had only a lower rate of weight gain relative to the *ad libitum*-fed rats. Only the chronically calorie-restricted rats had a decreased incidence of MTs when compared with the *ad libitum*-fed rats. In the second study (15), body weight loss also did not occur in the rats that were intermittently calorie restricted, and these rats had a 12% increase in MT incidence compared with their *ad libitum*-fed counterparts. Of further interest, both of these studies used high-fat diets with corn oil serving as the major source of dietary fat. Moreover, neither of these studies evaluated the effects of weight-cycling, but rather only the effects of intermittent caloric restriction regimens.

Studies using rodents that develop spontaneous MTs, however, are in good agreement with our results and provide further support for the concept that intermittent caloric restriction/refeeding has a protective effect. For example, mice fed either restricted amounts of high-fat or high-carbohydrate diets twice a week (in essence fasted and refed) exhibit a very low incidence of MT development, and those mice that did develop MTs had an extended latency [Chen *et al.* (16)]. Unfortunately, no body weight or food intake data were presented, so it is

difficult to compare the results of Chen *et al.* with those in the present study. In another study using female rats, fasting every other day also was reported to lower spontaneous MT incidence by 80% compared with *ad libitum* feeding or even with fasting every 3 days (17). MT latency, growth, and burden also were significantly impacted by fasting every other day. In contrast, fasting for 24 h twice a week initiated at 34 weeks of age did not inhibit spontaneous MT development in mice (18). It is important to note, however, that in that study, overall food intake was not different between the fasted/refed mice and the *ad libitum*-fed mice. In an additional study using female NZB × NZW mice, repeated periods of fasting/refeeding extended longevity of the mice compared with *ad libitum*-fed mice (35).

In contrast to the apparent protective effect of intermittent fasting/refeeding on spontaneous MT development and longevity and of intermittent calorie restriction/refeeding on oncogene-induced MT development, other studies suggest that single periods of fasting/refeeding may enhance chemical carcinogenesis in mammary tissue as well as in liver and colon. For example, Sesca *et al.* (36) reported that there was a significant decrease in MT latency and 100% MT incidence in rats that were fasted for 3 days (days 7–10) after DMBA administration and then refed, compared with nonfasted rats that had an 80% MT incidence. In liver, fasting/refeeding before carcinogen (dimethylnitrosamine) treatment at a level usually not considered to be carcinogenic was found to enhance the presence of γ-glutamyltransferase positive foci/nodules (37). However, the experiment was not carried out long enough to determine whether actual tumor formation occurred. In several other studies, one period of fasting/refeeding just before carcinogen administration also enhanced aberrant crypt foci or colon tumor formation in rats (38–39). With respect to the effects of fasting/refeeding on liver lesions, two or three periods had similar enhancing effects (40, 41). Three periods of fasting/refeeding after NMU administration was reported to increase the number of MTs per rat compared either with *ad libitum*-feeding or with one period of fasting/refeeding implemented prior to NMU administration (42). However, there was a 100% MT incidence in all three of the groups when the experiment was terminated 42 weeks after carcinogen administration.

How can we integrate these results? Because none of the studies cited above used similar protocols and the tumors were of different origins, comparison of the results is difficult. It is interesting to note that different cell kinetic alterations were reported for the refeeding phase after fasting/refeeding for transplanted *versus* spontaneous MTs in mice (43). Also, for carcinogen-induced tumor models that have a synchronized tumor induction, fasting/refeeding at initiation, and possibly also at the early promotion stage, may trigger tumor formation. However, when tumor development occurs over much longer time periods such as in the transgenic TGF-α mice or in rodents that develop spontaneous tumors, the effect of restricted caloric intake appears to override the growth promoting effect of refeeding. In this regard, two studies suggest that the degree and/or length of restriction may be critical component(s) of the response to caloric restriction (12, 13). For example, over the course of a 16-week experiment, neither 4 nor 8 weeks of caloric restriction at 75% of *ad libitum* feeding initiated at the time of DMBA administration (followed by *ad libitum* feeding) had a significant effect on palpable MT incidence compared with the effect in *ad libitum*-fed rats (13). However, rats restricted for the last 8 weeks had a similar MT incidence compared with rats that were calorie restricted for the whole 16 weeks (13). In a related study, 2 weeks of 50% caloric restriction, 1 week before and 1 week after DMBA administration was shown to dramatically decrease MT incidence (12). Integration of these results with the results presented here suggests that short-term periods of calorie restriction under some circumstances may have an impact on MT development if this restriction coincides with a critical period of tumor growth. If this hypothesis is correct, our results would suggest that this discrete period of MT development temporarily coincides with our intervals of caloric restriction and, in fact, that the severity of this restriction may be the determining factor in its antitumorigenic effect.

A "dose- dependent" protective effect of caloric restriction on the development of chemically induced MTs also has been reported (4, 44, 45). However, the results presented here suggest that this relationship may be more complex. In particular, the manner, *i.e.*, chronic caloric restriction or intermittent restriction, in which calories and/or nutrients are restricted, is clearly another important factor. In this regard, discrete periods of severe caloric restriction appear to provide far greater protection than do much longer periods of chronic caloric restriction.

How does caloric restriction mediate its protective effect on MT development? Chronic caloric restriction has been reported to increase urinary excretion of cortical steroids in a graded fashion in rats with chemically induced MTs (44). Chronic calorie restriction also has been documented to alter concentrations of serum growth factor-related components. For example, serum insulin and IGF-I levels were decreased in calorie-restricted DMBA-treated rats compared with *ad libitum*-fed rats (45). As expected, these calorie-restricted rats also had decreased MT incidence and burden. In addition, IGF-I binding was increased and insulin binding was decreased in the MTs of calorie-restricted rats (46). IGF-I is of particular interest because it has been implicated in human breast cancer development (47, 48). In the present study, IGF-I levels were slightly reduced in pair-fed, chronically restricted mice; however, after 1 week of refeeding, IGF-I levels in the weight-cycled mice were at levels comparable with those of the *ad libitum*-fed mice. Recent findings obtained from an ongoing study indicate that IGF-I levels at the end of the last restriction period (79 weeks of age) are comparable with those of the corresponding pair-fed mice.[5] Further study will also consider the effect of these interventions on serum IGF-binding proteins.

Chronic caloric restriction can also influence the expression of various oncogenes and tumor suppressors in mammary tissues and tumors. For example, Engelman *et al.* (49) have reported lower levels of *EGF* expression in the mammary tissues of chronically calorie-restricted C3H/HeOu mice when compared with *ad libitum*-fed mice. In another study, transgenic MMTV/v-Ha-*ras* mice calorically restricted to 60% of *ad libitum*-fed mice developed MTs that expressed significantly lower *ErbB2* levels, increased *p53*, and increased catalase activity (8). In weanling NMU-treated rats with accelerated MT growth, caloric restriction resulted in decreased MT incidence that was correlated with a decrease in cyclin D1 expression and an increase in p27 expression in tumor tissues (50). Studies on proto-oncogene and tumor suppressor expression have not yet been performed in intermittently calorie-restricted animals and should prove to be fertile ground for future mechanistic studies.

Together these results support a growing body of evidence, based on studies in animal models, that strongly supports

[5] M. P. Cleary, unpublished observations.

an important role for dietary factors in the development of mammary gland tumors. Are these results relevant to breast carcinogenesis in humans? In one recent study (51), weight cycling was defined as losing 20 pounds or more and gaining at least one-half of the lost weight back within 1 year. In this study, no differences in breast cancer risk were found for postmenopausal women reporting weight cycling *versus* women who reported no weight cycles. In addition, a similar percentage of breast cancer (18.6%) and control subjects (17.0%) reported a history of weight cycling (51). Given the fact that participants experienced only one cycle of weight loss (based on recall), it is not possible to compare the results of this study with our recent results. Also, it is unlikely that any human study would be undertaken with a protocol as rigid as that presented here. However, the magnitude of the protective effect revealed in our study, using a genetically defined mouse model that develops tumors in response to activation of an oncogene known to be relevant to human breast tumor development, should provide further impetus to conduct related studies in humans. In addition, determining how the protective effect of intermittent calorie restriction/refeeding is mediated may provide insight into the development of chemopreventive agents.

In summary, the results presented here indicate a greater protective effect of calorie restriction on MT development when it is implemented in an intermittent calorie-restriction/refeeding protocol rather than a protocol of chronic calorie restriction. Whether these results, which were obtained in a genetically determined animal model, *i.e.*, *MMTV-TGF-α* transgenic mice, are applicable to other models of breast cancer remain to be determined. Clearly, the experimental design used here cannot be applied directly in human studies. However, given that these mice develop MTs as a consequence of the constitutive activation of an EGFR/ErbB receptor signaling cascade, we can speculate that identifying the mechanism responsible for this protective effect may provide useful targets for clinical intervention.

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