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Time-restricted Feeding Attenuates High-fat Diet-enhanced Spontaneous Metastasis of Lewis Lung Carcinoma in Mice

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Abstract. *Background/Aim: Obesity is a risk factor for cancer. Disruption of the daily feeding and fasting rhythm can contribute to obesity. This study tested the hypothesis that time-restricted feeding (TRF) attenuates obesity-enhanced metastasis. Materials and Methods: In a spontaneous metastasis model of Lewis lung carcinoma (LLC), male C57BL/6 mice were fed the standard AIN93G diet or a high-fat diet (HFD) with or without dark-phase restricted feeding (12 h per day) for 10 weeks. Pulmonary metastases from a subcutaneous tumor were quantified. Results: The number and size of lung metastases were greater in the HFD group than in the AIN93G group, but did not differ between the TRF and AIN93G groups. TRF prevented HFD-induced increases in plasma concentrations of glucose, insulin, proinflammatory cytokines (leptin, monocyte chemotactic protein-1, plasminogen activator inhibitor-1), and angiogenic factors (angiopoietin-2, hepatic growth factor, vascular endothelial growth factor). Conclusion: TRF attenuates the HFD-enhanced spontaneous metastasis of LLC in mice.*

Circadian rhythms, which cycle approximately every 24 hours, control physiological processes of daily functions, *e.g.* feeding and fasting, rest and activity, and sleep and wake. Altering the finely-tuned circadian rhythms by an erratic lifestyle (*e.g.* irregular eating or sleeping pattern) may lead to various metabolic disorders, including obesity (1, 2).

Being overweight or obese at the time of diagnosis of cancer can be predictive of early recurrence and metastasis (3, 4), which in turn directly affects the prognosis and disease-free interval of cancer patients. For example, obese breast cancer patients are at a greater risk of recurrence (5) and have a shorter disease-free interval compared to patients with normal body weights (4). Obese prostate cancer patients are at an increased risk of recurrence after radical prostatectomy compared to those with normal body weights (6, 7). Laboratory studies show that obesity, induced by consumption of an obesogenic diet, enhances primary tumorigenesis (8-10) and distant metastasis (10-14) in rodent models of cancer.

Human trials show that meal timing alters the rhythm of energy metabolism. For example, early meal eaters generally have better metabolic homeostasis than the late eaters (15-17). Rodent studies show that there are differences in weight gain and circadian rhythms in mice with food available in different times of the day, *e.g.* the light phase (rest phase) *versus* the dark phase (active phase) (1, 18, 19). Mice with unrestricted access to a high-fat diet (HFD) in both light and dark phases exhibit adipose accumulation, disruptions in energy metabolism, and disruption in expression of circadian genes (1, 19, 20).

Time-restricted feeding (TRF) is the restriction of timing of food intake to certain hours of the active phase of the day (*e.g.* the dark phase for nocturnal rodents), rather than the quantity of food intake. The restriction synchronizes feeding and fasting with normal circadian rhythms, restores daily oscillations in energy metabolism, and reduces metabolic disturbance (1, 21). We (20) and others (1, 21) found that restricted feeding of an HFD to the dark phase for 12 hours, compared to feeding *ad libitum*, reduces body adiposity and restores diurnal rhythms of energy expenditure and circadian gene expression. This reduction in body adiposity is accompanied with decreases in pro-inflammatory cytokines with carcinogenic potential (20, 22). We hypothesized that restriction of feeding during the dark phase reduces obesity-enhanced metastasis. This hypothesis was tested by determining the effect of dark phase restricted feeding of HFD on spontaneous metastasis using the mouse model of Lewis lung carcinoma (LLC).

Materials and Methods

Animals and diets. Three to four-week-old male C57BL/6 mice were obtained from Envigo (Madison, WI, USA). Mice were maintained in a pathogen-free room with a 12:12-h light/dark cycle and a temperature of 22±1˚C for the duration of the study. The standard AIN93G diet (23) and a modified AIN93G diet (22) containing 45%

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energy from soybean oil [high-fat diet (HFD)] were used in this study (Table I). Gross energy of each diet was quantified using an oxygen bomb calorimeter (Model 6200, Parr Instrument, Moline, IL, USA) (Table I). Both diets (powder diets) were stored at –20˚C; fresh diets were provided to mice every other day.

Lewis lung carcinoma cell line. The LLC cell line, a variant that metastasizes to the lungs (24), was obtained from Dr. Pnina Brodt (McGill University, Montreal, Quebec, Canada). The cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum and maintained in a humidified atmosphere of 5% CO_2 in air at 37˚C. Cells used for animal studies were *in vivo*-selected once (25). Cells were free of mycoplasma based on Hoechst DNA staining and direct culture tests performed by American Type Cell Collection (Manassas, VA, USA).

Experimental design. This study (YAN32) was approved by the Institutional Animal Care and Use Committee of the Grand Forks Human Nutrition Research Center. The study procedures followed the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (26).

After acclimation with the AIN93G diet for one week, mice were randomly assigned to two groups and fed the AIN93G diet (n=28) or the HFD (n=56) *ad libitum*. Three weeks later, mice fed the HFD were further assigned to two groups of 28 each; one remained on unrestricted access to the HFD during both light and dark phases, the other had feeding restricted to the dark phase for 12 h between Zeitgeber times (ZT) 12 and 24 (ZT 0, light on). The duration of the restricted feeding was 10 weeks (Figure 1). Mice of the restricted group were transferred to cages containing water only at ZT 0 and returned to cages containing both diet and water at ZT 12 daily for the duration of the study. To ensure consistent mouse handling, mice with unrestricted access to their diets were transferred between cages containing both diet and water daily at ZT 0 and 12. Mice were housed individually and weighed weekly. Three weeks following the restricted feeding, food intake (6 mice per group) was recorded seven days per week for three consecutive weeks. Body composition was determined using an Echo Whole Body Composition Analyzer (Model 100, Echo Medical Systems, Houston, TX, USA) one week before the subcutaneous injection of LLC cells.

Seven weeks after the initiation of the restricted feeding, mice were injected subcutaneously with 2.5×10^5 viable LLC cells per mouse into the lower dorsal region. Ten days later, the resulting subcutaneous tumor was resected when it was approximately 1 cm in diameter. Following surgery, mice were maintained on their respective feeding regimen for an additional 10 days. An additional 25 mice fed the AIN93G diet but not injected with cancer cells served as controls to assess changes in plasma concentrations of cytokines and related biomarkers due to metastasis in LLC-bearing mice fed the AIN93G diet.

At termination, mice were fasted for six hours before being euthanized by injecting a mixture of ketamine and xylazine intraperitoneally. Lungs were harvested and fixed with Bouin's solution for analysis of pulmonary metastasis. The number of metastases per mouse and the cross sectional area and average diameter of each metastasis were analyzed using a camera-equipped stereomicroscope and ImagePro-Plus software (Media Cybernetics, Silver Spring, MD, USA). The cross-sectional area of a metastasis was the surface area of the metastasis. The volume was estimated by assuming that the metastasis was spherical and using its average diameter (27). The average diameter was the average measured at two-degree intervals joining two outline points and passing through the centroid. Plasma was collected for quantitation of cytokines, angiogenic factors, and related metabolic markers. Liver and primary tumor were harvested for measuring the expression of circadian genes.

Quantitation of cytokines, angiogenic factors, and metabolic markers in plasma. Milliplex MAP mouse adipokine (catalog no. MADKMAG-71K) and angiogenesis/growth factor (catalog no. MAGPMAG-24K) magnetic bead panels (MilliporeSigma, Burlington, MA, USA) were used to quantify plasma concentrations of adipokines [leptin, monocyte chemotactic protein-1 (MCP-1), and plasminogen activator inhibitor-1 (PAI-1)] and angiogenesis factors [angiopoietin-1, hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF)]. Adiponectin (R&D Systems, Minneapolis, MN, USA), glucose (Cayman Chemical, Ann Arbor, MI, USA), and insulin (Mercodia, Winston-Salem, NC, USA) were quantified using sandwich enzyme-linked immunosorbent assays. The accuracy of analyses was confirmed with controls provided in each kit. All samples analyzed were within the linear range of the assay. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula [glucose (mg/dl) × insulin (ng/ml)/405] to predict insulin resistance (28, 29).

RNA isolation and real-time quantitative PCR. Total RNA from frozen liver and primary tumor was isolated using the QIAzol Lysis reagent with DNAse treatment following the manufacturer's protocol (RNeasy Lipid Tissue Mini Kit mRNA, Qiagen, Germantown, MD, USA). The quality and quantity of the RNA were analyzed using NanoDrop 8000 (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized using the High capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA) following the manufacturer's protocol. Real-time qPCR of circadian locomotor output cycles kaput (*Clock*), aryl hydrocarbon receptor nuclear translocator-like protein 1 (*Bmal1*/*Arntl*), period-1 (*Per1*) and *Per2*, cryptochrome 1 (*Cry1*), nuclear receptor subfamily 1 group D member 1 (*Nr1d1*/*1Rev-erbα*) was analyzed and normalized to the 18s rRNA using the TaqMan Assay of Demand primers on the ABI QuantStudio 12K-Flex Real-time PCR system (Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative changes in gene expression (30).

Analysis of BMAL1 protein. Proteins were extracted from frozen liver (31, 32). Total protein was quantified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein expression of BMAL1 was analyzed using a capillary-based SallySue instrument (ProteinSimple, San Jose, CA, USA). The ProteinSimple system control protein served as an internal control and was used to normalize protein expression as previously described (31, 32). The anti-BMAL1 antibody (#14020) was purchased from Cell Signaling Technology (Danvers, MA, USA). Additional antibodies, including CLOCK (#AB2203) from MilliporeSigma or CLOCK (#5157) from Cell Signaling Technology, Rev-Erbα (#13418) from Cell Signaling Technology or Rev-Erbα (#ab174309) from Abcam (Cambridge, MA, USA), PER1 (#ab136451) from Abcam, PER2 (#AB2202) from MilliporeSigma or PER2 (#PER21-A) from Alpha Diagnostic International (San Antonio, TX, USA), and CRY (#21414) from SAB Biotech (College Park, MD, USA), were examined.

Table I. *Composition of diets*.

Ingredient	AIN93G g/kg	High-fat g/kg
Corn starch	397.5	42.5
Casein	200	239.4
Dextrin	132	239.4
Sucrose	100	119.7
Soybean oil	70	239.4
Cellulose	50	59.8
AIN93 mineral mix	35	41.9
AIN93 vitamin mix	10	12
L-Cystine	3	3.6
Choline bitartrate	2.5	3
t-Butylhydroquinone	0.014	0.017
Total	1000	1000
Energy		
Protein	20%	20%
Fat	16%	45%
Carbohydrate	64%	35%
Analyzed gross energy kcal/g[a]	4.3±0.1	5.2±0.1

[a]Values are means±SEM of three samples analyzed from each diet.

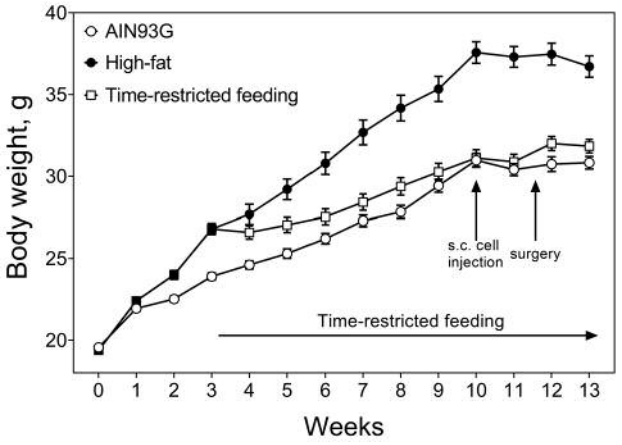

Figure 1. *Body weight of mice fed the AIN93G, the high-fat diet (HFD), or with the time-restricted feeding (TRF) of the HFD. Mice fed the HFD were heavier than those fed the AIN93G diet; the difference was significant two weeks after the initiation of the HFD ($p<0.05$) and continued throughout the study. Body weight of the TRF group was lower than that of the HFD group and remained similar to that of the AIN93G group. The difference between the TRF and the HFD groups was significant two weeks after the initiation of TRF ($p<0.05$) and continued throughout the study. Values are means±SEM (n=28 per group).*

Statistical analyses. One-way analysis of variance (ANOVA) and Tukey contrasts were performed to compare differences among the groups. A mixed model ANOVA with 'mouse' as the random blocking factor was used to compare differences in size of metastases (cross-sectional area and volume) among the groups. Pearson's correlation was performed to examine the relationship between *Bmal1* mRNA and BMAL1 protein expressions. All results are presented as means±standard error of the mean (SEM). Differences with a *p*-value of 0.05 or less are considered significant. All analyses were performed using the SAS software (version 9.4, SAS Institute, Cary, NC, USA).

Results

Body weight, body composition, and energy intake. Mice fed the HFD were heavier than those fed the AIN93G diet; the difference was significant two weeks after the initiation of the HFD ($p<0.05$) and continued throughout the study (Figure 1). Body weight of the TRF group was lower than that of the HFD group, but did not differ from the AIN93G group; the difference between the TRF and the HFD groups was significant two weeks after the initiation of the TRF ($p<0.05$) and continued throughout the study (Figure 1).

The percentage body fat was 52% greater (Figure 2A) and the percentage body lean mass 15% lower (Figure 2B) in the HFD group than the AIN93G group. The percentage body fat in the TRF group was 25% lower than that of the HFD group (Figure 2A). The percentage body lean mass of the TRF group did not differ from the AIN93G group (Figure 2B). The lean mass of the HFD group was slightly but significantly greater than that of the AIN93G group (22.0±0.3 *versus* 21.1±0.2 g); there was no difference in lean mass between the TRF (20.6±0.2 g) and AIN93G groups (Figure 2C). The energy intake of the HFD group was 12% lower than that of the AIN93G group (15.5±0.3 *versus* 17.5±0.7 kcal/day); there was no significant difference in energy intake between the TRF (14.6±0.7 kcal/day) and the HFD groups (Figure 2D).

Lung metastasis. All mice subcutaneously injected with LLC cells developed a primary tumor at the injection site and metastases in the lungs. The number of lung metastases of the HFD group was 45% greater than that of the AIN93G group (63 *versus* 44 metastases; Figure 3A). The number of metastases of the TRF group (49 metastases) was 22% lower than that of the HFD group but did not differ from the AIN93G group (Figure 3A). Compared to the AIN93G diet, the HFD increased metastatic cross-sectional area by 47% (1.04±0.06 *versus* 0.71±0.04 mm^2; Figure 3B) and volume by 72% (1.14±0.10 *versus* 0.66±0.05 mm^3; Figure 3C). The metastatic cross-sectional area and volume of the TRF group were 22% (0.81±0.05 mm^2; Figure 3B) and 30% (0.80±0.07 mm^3; Figure 3C) lower than those of the HFD group, respectively, but did not differ from the AIN93G group.

Concentrations of glucose and insulin in plasma and HOMA-IR. Plasma concentrations of glucose were not different between LLC-bearing and non-tumor bearing mice fed the AIN93G diet (Table II). In LLC-bearing mice, the HFD increased plasma glucose by 26% compared to the

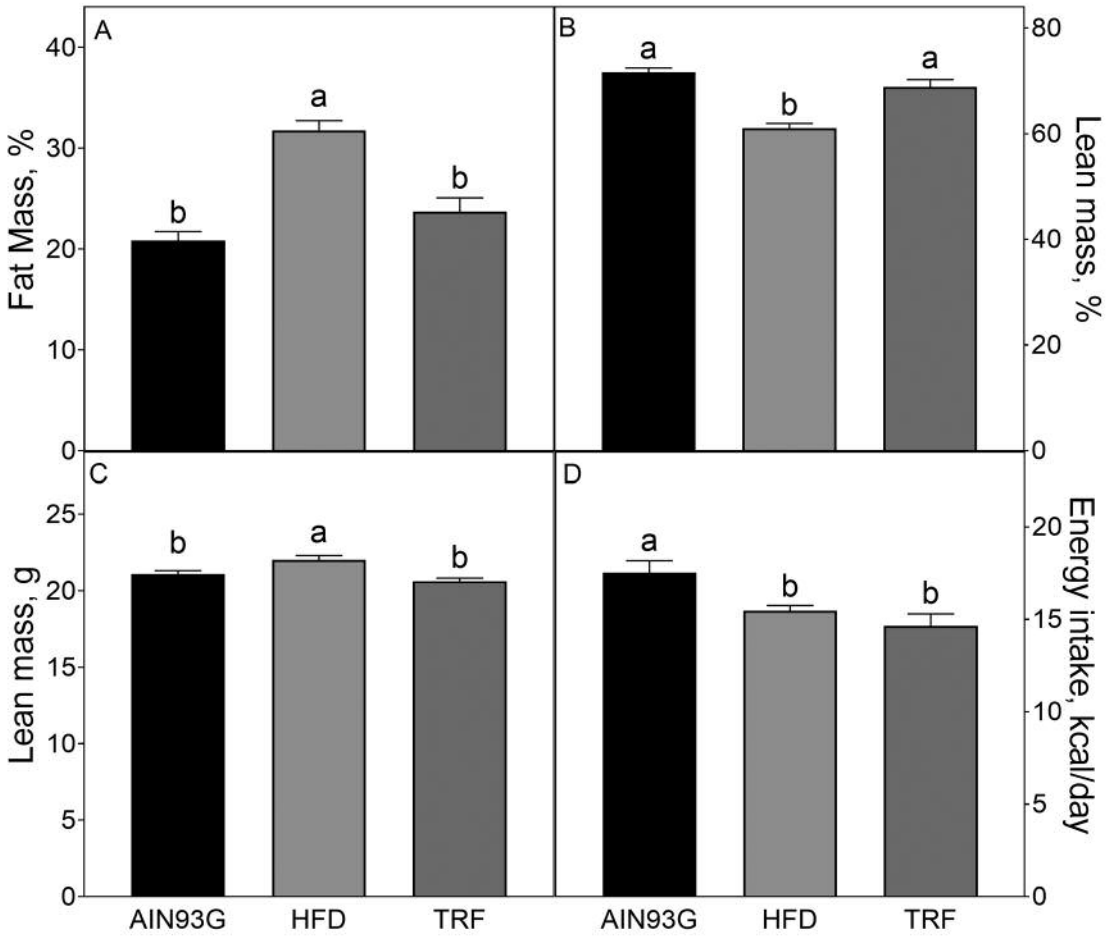

Figure 2. *Fat mass:body mass ratio (A), lean mass:body mass ratio (B), lean mass (C), and energy intake (d) of mice fed the AIN93G, the high-fat diet (HFD), or with the time-restricted feeding (TRF) of the HFD. Values (means±SEM) with different letters are different at p<0.05 (n=28 per group for A, B, C; n=6 per group for D).*

AIN93G diet (Table II). The glucose concentration of the TRF group was not different from that of the AIN93G group (Table II).

Plasma concentration of insulin was 10% lower in LLC-bearing mice than in non-tumor bearing mice fed the AIN93G diet (Table II). In LLC-bearing mice, the HFD increased insulin by 14% compared to the AIN93G diet (Table II). Plasma insulin of the TRF group was not different from that of the AIN93G group (Table II). The presence of LLC decreased HOMA-IR by 15% compared to non-tumor-bearing mice (Table II). The HOMA-IR was 41% higher in LLC-bearing mice fed the HFD than in their AIN93G-fed counterparts; HOMA-IR of the TRF group did not differ from that of the AIN93G group (Table II).

Concentrations of adipokines and angiogenic factors in plasma. The concentration of adiponectin in the plasma of LLC-bearing mice was 24% lower than that of the non-tumor-bearing controls (Table II). In LLC-bearing mice, adiponectin concentration of the HFD group was 99% lower than that of the AIN93G group (Table II). Restricted feeding to the dark phase elevated adiponectin compared to the HFD group; there was no significant difference in adiponectin between the TRF and the AIN93G groups (Table II).

In mice fed the AIN93G diet, plasma leptin concentration of LLC-bearing mice was 84% lower than that of non-tumor-bearing controls (Table II). In LLC-bearing mice, leptin concentration was 1400% greater in the HFD group than in the AIN93G group (Table II). Plasma leptin of the TRF group did not differ from that of the AIN93G group (Table II).

There were no significant differences in plasma concentrations of MCP-1 and PAI-1 in AIN93G-fed mice with or without LLC (Table II). In LLC-bearing mice, plasma

Table II. *Concentrations of glucose, insulin, adipokines and angiogenic factors in plasma and homeostasis model assessment of insulin resistance (HOMA-IR).*

	Control	AIN93G	High-Fat	TRF
Glucose, mg/dl	260.77±7.50^{b}	240.68±13.81^{b}	302.85±10.15^{a}	257.50±8.59^{b}
Insulin, ng/ml	0.31±0.01^{ab}	0.28±0.01^{c}	0.32±0.01^{a}	0.30±0.01b^{c}
HOMA-IR	0.20±0.01^{b}	0.17±0.01^{c}	0.24±0.01^{a}	0.19±0.01b^{c}
Adiponectin, μg/ml	5.69±0.34^{a}	4.34±0.44^{b}	0.08±0.01^{c}	3.31±0.31^{b}
Leptin, ng/ml	1.20±0.21^{b}	0.19±0.05^{c}	2.68±0.30^{a}	0.25±0.05^{c}
MCP-1, pg/ml	41.94±5.12^{b}	29.87±2.55^{b}	74.85±4.27^{a}	36.26±5.83^{b}
PAI-1, ng/ml	1.15±0.15^{b}	1.16±0.11^{b}	1.76±0.14^{a}	1.22±0.10^{b}
Angiopoietin, ng/ml	3.13±0.20^{b}	3.07±0.24^{b}	4.36±1.44^{a}	3.24±0.30^{b}
HGF, ng/ml	0.40±0.04^{b}	0.37±0.03^{b}	0.57±0.04^{a}	0.35±0.05^{b}
VEGF, ng/ml	0.18±0.01^{b}	0.24±0.02^{b}	0.34±0.02^{a}	0.23±0.03^{b}

Control: non-tumor-bearing mice fed the AIN93G diet; TRF: time-restricted feeding. Values (means±SEM) with different letters are different at $p<0.05$ (n=19 per group; n=16 per group for glucose, insulin, and HOMA-IR).

Table III. *Expression of circadian genes in liver and primary tumor of Lewis lung carcinoma and the expression of BMAL1 protein in liver.*

	Control	AIN93G	High-Fat	TRF
Liver, mRNA		Relative expression		
Clock	1.00±0.28^{a}	0.52±0.08^{ab}	0.41±0.05^{b}	0.48±0.07^{b}
Bmal1	1.00±0.30^{b}	1.48±0.17^{a}	0.89±0.08^{ab}	1.52±0.20^{a}
Per1	1.00±0.10	1.00±0.23	1.01±0.31	0.88±0.15
Per2	1.00±0.20^{a}	0.19±0.04^{b}	0.53±0.08^{a}	0.26±0.05^{b}
Rev-erbα	1.00±0.20^{a}	0.09±0.02^{c}	0.54±0.08^{ab}	0.15±0.04^{bc}
Cry1	1.00±0.24	1.46±0.33	0.75±0.08	1.41±0.25
Liver, Protein				
BMAL1	1.00±0.14^{b}	2.50±0.32^{a}	1.78±0.24^{ab}	2.20±0.30^{a}
Primary tumor, mRNA				
Clock		1.00±0.07	1.19±0.10	0.92±0.1
Bmal1		1.00±0.07^{c}	3.46±0.29^{a}	2.38±0.18^{b}
Per1		1.00±0.08^{b}	2.11±0.46^{a}	0.98±0.08^{b}
Per2		1.00±0.10^{b}	1.54±0.12^{a}	1.06±0.13^{b}
Rev-erbα		1.00±0.17^{b}	2.53±0.41^{a}	0.98±0.22^{b}
Cry1		1.00±0.07	1.04±0.11	0.85±0.06

Control: non-tumor-bearing mice fed the AIN93G diet; TRF: time-restricted feeding. Values (means±SEM) with different letters are different at $p<0.05$ (n=10 per group).

concentrations of MCP-1 and PAI-1 were 151% and 52% greater, respectively, in the HFD group than in the AIN93G group (Table II). Concentrations of MCP-1 and PAI-1 in the TRF group were not different from those in the AIN93G group (Table II).

There were no significant differences in plasma concentrations of angiopoietin-2, HGF, and VEGF in AIN93G-fed mice with or without LLC (Table II). In LLC-bearing mice, concentrations of angiopoietin-2, HGF, and VEGF were 42%, 54%, and 42%, respectively, greater in the HFD group than in the AIN93G group (Table II). Concentrations of these angiogenic factors in the TRF group were not different from those in the AIN93G group (Table II).

Expression of circadian genes and proteins. The presence of lung metastasis affected hepatic expression of circadian genes. The largest differences were observed in *Bmal1, Per2*, and *Rev-erbα* expression when comparing LLC-bearing mice to non-tumor-bearing mice fed the AIN93G diet (Table III). BMAL1 migrated to 78 kDa, the expected size for BMAL1, and was normalized to the system control (26 kDa) (Figure 4A). The rabbit primary system control antibody also reacted with a non-specific protein of 16 kDa in liver (Figure 4A). Furthermore, metastasis elevated the expression of BMAL1 protein in liver (Table III, Figures 4A and B). The expression of *Bmal1* mRNA was positively correlated with that of BMAL1 protein ($r=0.49$, $p<0.01$) (Figure 4C). Protein

expression of CLOCK, PER1, PER2, CRY, and REV-ERBα in liver were either below the level of detection or the antibody cross-reacted with multiple non-specific proteins that prevented accurate analysis (data not shown).

The HFD elevated the expression of *Bmal1, Per1, Per2*, and *Rev-erbα* mRNAs in LLC primary tumor compared to the AIN93G diet (Table III). Time-restricted feeding reduced the expression of these circadian genes compared to the HFD (Table III).

Discussion

Consistent with our previous reports (12-14), feeding mice the HFD *ad libitum* enhanced metastasis. The present study showed that the number and size of lung metastases of the TRF group were lower than those of the HFD group and did not differ from those of the AIN93G control group. Fewer metastases indicate reduction in malignant spread, and smaller sizes of metastases indicate an inhibition of metastatic growth. These findings show that TRF attenuates the HFD-enhanced spontaneous metastasis of LLC and support our hypothesis that restriction of feeding to the dark phase reduces HFD-enhanced metastasis in mice.

Adipose tissue produces inflammatory cytokines. A major finding from this study was the lower body adiposity and plasma concentrations of pro-inflammatory cytokines (*e.g.* MCP-1, PAI-1, and leptin) in mice receiving TRF. Both MCP-1 and PAI-1 have prognostic values; cancer patients with elevated MCP-1 (33) or PAI-1 (34, 35) show poor prognosis and early recurrence. Lower concentrations of pro-inflammatory cytokines in plasma, as a result of TRF, may have contributed to the observed attenuation in lung metastasis. In rodents, elevated expression of MCP-1 and PAI-1 occurs in HFD-enhanced primary tumorigenesis (8, 9) and metastasis (12-14), while knocking-out MCP-1 (13) or PAI-1 (12) reduces spontaneous metastasis in mice. A recent study showed that mitigation of HFD-enhanced mammary tumorigenesis by TRF is accompanied by lower plasma concentrations of pro-inflammatory cytokines in a mouse model of mouse mammary tumor virus-polyomavirus middle T-antigen (22). A reduction in plasma concentration of leptin may also contribute to the observed protective effect of TRF because leptin can be tumorigenic (36). Our results suggest that TRF resulting in lower body adiposity decreases pro-inflammatory cytokine production that contributes to the reduction in the spread of LLC.

Consistent with our previous report (22), TRF mice exhibited lower plasma concentrations of angiogenic factors angiopoietin-2, HGF, and VEGF. Angiogenesis plays an important role in malignant progression. Elevated angiogenic expression (37-39) in patients with invasive breast cancer is associated with poor survival outcomes. Greater concentrations of angiogenic factors, including angiopoietin, HGF, and VEGF, are found in diet-enhanced primary tumorigenesis (9, 22) and metastasis (12, 13). Lower plasma concentrations of angiogenic factors in TRF mice indicate that dark-phase restricted feeding down-regulates angiogenesis, which may contribute to the observed attenuation of LLC metastasis in the present study. Furthermore, the lower concentrations of MCP-1 and PAI-1 by TRF may also contribute to the down-regulation of angiogenesis because both are angiogenic in tumorigenesis (40, 41).

In the present study, TRF elevated the plasma concentration of adiponectin in mice fed the HFD. Adiponectin is an adipokine with demonstrated anticancer potential in both humans (42) and laboratory rodents (43).

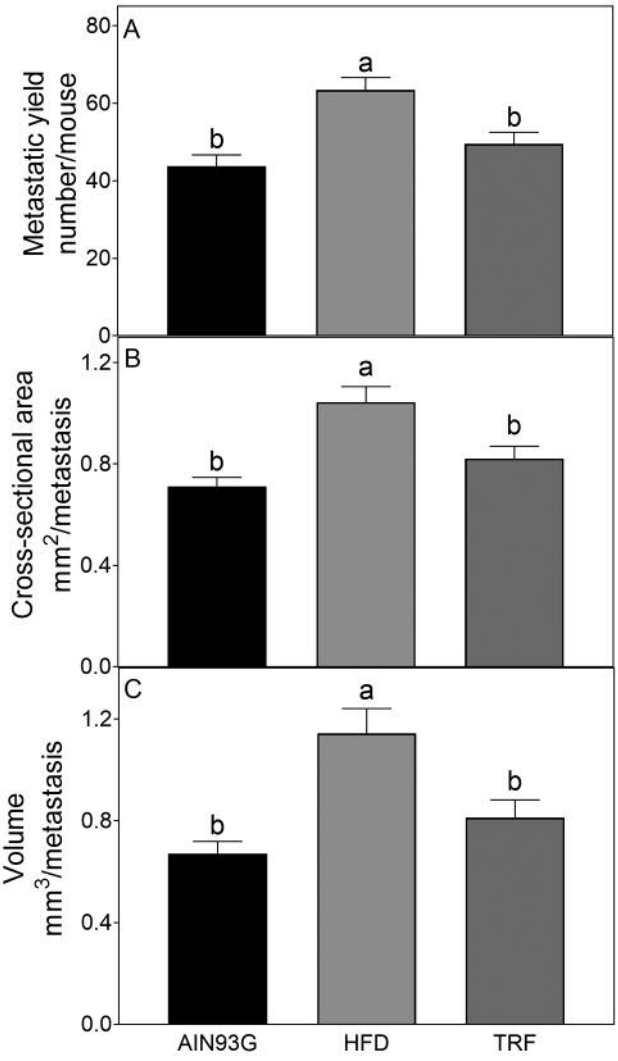

Figure 3. *The number (A), cross-sectional area (B), and volume (C) of lung metastases in mice fed the AIN93G, the high-fat diet (HFD), or with the time-restricted feeding (TRF) of the HFD. Values (means±SEM) with different letters are different at p<0.05 (n=28 per group).*

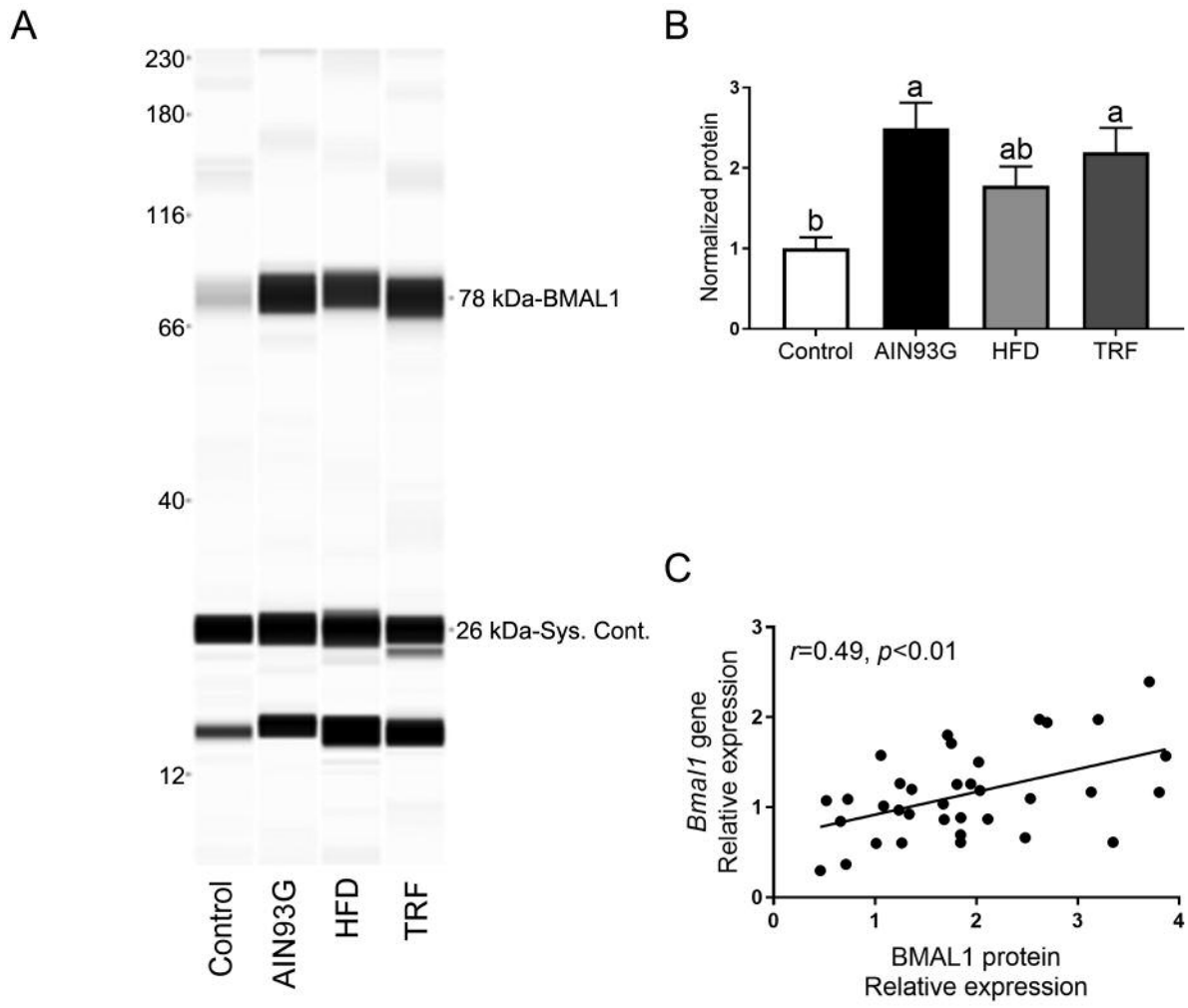

Figure 4. *Representative SallySue image of hepatic BMAL1 expression (A), normalized BMAL1 protein expression (B), and Pearson's correlation between hepatic Bmal1 mRNA and BMAL1 protein (C). Values (means±SEM) with different letters are different at p<0.05 (n=10 per group). Control: non-tumor-bearing mice fed the AIN93G diet; HFD: high-fat diet; TRF: time-restricted feeding.*

Adiponectin participates in glucose and lipid homeostasis and is an insulin sensitizer in liver and skeletal muscles (44). Our findings of elevation in adiponectin, together with reductions in glucose, insulin, and leptin, in TRF mice indicate that TRF restores metabolic homeostasis, which may contribute to the anti-metastatic effects of dark-phase restricted feeding.

The protective effect of TRF on LLC metastasis is likely through its entrainment of circadian genes and metabolic regulators to a fixed feeding time. Feeding mice an HFD dampens the respiratory exchange ratio (a measurement of the balance between carbohydrates and fats metabolized for energy utilization) (20) and oscillations of circadian gene expression including *Clock, Bmal1, Per1, Per2, Cry1*, and *Rev-erbα* in liver (1). Restricted feeding of the same diet to the dark phase restores the diurnal oscillations of respiratory exchange ratio (20) and circadian gene expression (1) and improves metabolic homeostasis (1, 20). Evidenced by improvement in insulin sensitivity, elevation in adiponectin and reduction in leptin, and alterations in hepatic circadian gene expression, the present study supports the concept that temporal regulation of food intake mitigates HFD-induced metabolic disturbance and adipogenesis.

The presence of LLC in mice, even under the control, AIN93G-fed condition, altered circadian gene expression in liver. The impact of the HFD in some cases was to reverse these changes (*e.g. Rev-erbα* and *Per2*), while TRF mice were similar to the AIN93G-fed mice, even under the HFD-fed condition. These findings point to LLC-induced signaling that impacts hepatic circadian rhythmicity and that intake of the HFD further modifies the hepatic responses to this signaling. More studies are needed to determine (a) the

mechanisms of this circadian disruption, (b) whether these changes in hepatic circadian expression lead to changes in hepatic metabolism, or (c) whether these changes are pro-tumorigenic *versus* anti-tumorigenic. These findings support the published work of disturbed expression of circadian genes in humans (45) and rodents (46) with cancer.

Changes in circadian gene expression in primary tumors indicate that LLC exhibits its own circadian rhythmicity which responds to dietary and eating pattern modifications in the host. In the present study, the primary tumor, was a reservoir of cancer cells from where the malignant cells spread to the lungs. Thus, it remains an interest to further investigate whether changes in circadian regulation affect the growth of the primary tumor and aggressiveness of malignant cells originated from it.

A limitation of this study is that we were not able to assess the effect of TRF on rhythmic changes in hepatic circadian gene expression in the presence of lung metastasis. Nevertheless, our findings indicate that metastatic progression may disrupt circadian rhythms and their metabolic regulations in peripheral organs and TRF may reverse the malignancy-mediated disruptions. These results warrant further investigation.

In summary, the present study demonstrated that time-restricted feeding to the dark phase attenuates the HFD-enhanced spontaneous metastasis of LLC in mice. The protective effect is likely through the temporal regulation of food intake to a fixed feeding time during the active phase, which restores metabolic homeostasis and reduces body adiposity and related cancer promoting factors. The present study provides laboratory evidence that having a fixed feeding time during the active phase of the day reduces malignant spread. It suggests that maintaining a healthy eating pattern may be useful as an adjuvant in cancer prevention.

Conflicts of Interest

The Authors have declared that no competing interests exist regarding this study.

Authors' Contributions

LY, SS, and MP conceived and designed the study. LY, SS, and AM performed the experiments and data analysis and interpreted results. All Authors contributed to the preparation, review, and revision of the manuscript and agreed to be accountable for the content of the work.

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