Supporting Information

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SI Materials and Methods

Animals. Female SKH-1 hairless mice (6–7 wk old) were purchased from Charles River Breeding Laboratories and kept in our animal facility for 1 wk before use. Mice were maintained on a 12-h light/12-h dark cycle and provided food and water ad libitum, with fresh food replenished every day.

Chow Diet, 40%-kcal High-Fat Diet Rich in Omega-6 Fatty Acids, and a 60%-kcal Very-High-Fat Diet. Purina laboratory chow 5001 diet was purchased from Ralston Purina. The 40%-kcal high-fat diet rich in omega-6 fatty acids was purchased from Dyets and stored at −20 °C until use. This diet contains 20% by weight of mixed lipids premix. The mixed lipids premix was formulated to simulate the fat content of the American diet derived from hydrogenated soy bean oil (30%), corn oil (27%), beef tallow (16%), butter fat (12%), lard (10%), and peanut oil (5%) by weight. The mixed lipids premix contained 28% polyunsaturated omega-6, 24% monounsaturated omega-7/9, and 45% saturated fatty acids by weight as described by Reddy et al. (1). Our previous study showed that this high-fat diet rich in omega-6 fatty acids markedly increased UVB-induced skin carcinogenesis compared with a high-fat diet rich in omega-3 fatty acids (2). The 60%-kcal very-high-fat diet is AIN 76A modified Rodent OpenSource Diet and purchased from Research Diets. This diet contains 34.9% fat by weight and 54.4% of total kcal comes from lard as described in the data sheet from Research Diets.

Exposure of Mice to UVB and the Preparation of Skin Sections. The UV lamps (FS72T12-UVB-HO; National Biological) emitted UVB (280–320 nm; 75–80% of total energy) and UVA (320–375 nm; 20–25% of total energy). The dose of UVB was quantified with a UVB Spectra 305 dosimeter (Daevlin).

One hundred and sixty female SKH-1 mice were randomly equally divided into four groups (40 mice per group, 5 mice per cage). Two groups of mice were fed a regular low-fat chow diet, and the other two groups of mice were fed a high-fat diet rich in omega-6 fatty acids (40 mice/group). After 2 wk on each diet, one group of mice had their parametrial fat pads surgically removed, and the other group of mice served as a sham-operated control. After a 2-wk recovery, all of the mice were irradiated with 30 mJ/cm^2 of UVB twice a week for 33 wk.

The mice were killed after 33 wk of UVB irradiation, and the parametrial fat pads from sham-operated control mice or any residual tissue fat or compensatory fat around the uterus from partially lipectomized mice were removed and weighed. Part of the dorsal skin surrounding each grossly observed mass was taken, stapled flat to a plastic sheet, and placed in 10% phosphate-buffered formalin at 4 °C for 18–24 h. The skin samples were then dehydrated in ascending concentrations of ethanol, cleared in xylene, and embedded in Paraplast (Oxford Labware). Four-micrometer skin sections were made, deparaffinized, rehydrated with water, and used for regular hematoxylin-eosin (H&E) staining and immunohistochemical staining for caspase 3 (active form) and cell proliferation. Parts of the skin or tumor samples from partially lipectomized mice and sham-operated control mice were cut and used for measurement of adipokines with an antibody array.

Surgical Removal of Parametrial Fat Pads (Partial Lipectomy) in Mice. The mice were housed five mice per cage in a temperature- and humidity-controlled room with free access to food and water. The mice were injected intraperitoneally with ketamine HCl (120 mg/kg) and xylazine (10 mg/kg). The abdominal skin area of anesthetized mice was sterilized with 70% alcohol. About a 1-cm incision along the midline of skin in the abdomen was made. After finding the uterus, the parametrial fat pads together with the uterus were pulled out of the cavity. Both parametrial fat pads were dissected and removed. The uterus was returned to the peritoneal cavity, and the incision was sutured. Sham surgery was the same as for partial lipectomy except that the parametrial fat pads were left intact and placed back inside the peritoneal cavity. The animals were returned to their cages, and recovery from anesthesia (within 30–60 min postoperatively) was monitored. All experimental procedures were approved by the institutional animal care and use committee of Rutgers University.

Measurement of Body Fat Composition by Dual-Energy X-Ray Absorptiometry (DXA). The DXA instrument used was a GE PIXImus2 series densitometer. A quality control phantom mouse was scanned initially. The phantom and samples were mounted on an adhesive disposable plastic tray and placed on the imaging surface in a prostrate position with limbs extended away from the body. The tails of mice were not included in the scan analysis.

Characterization of Skin Tumors and Measurement of Tumor Size. Body weight and skin tumor growth (number of tumors per mouse, tumor volume per mouse) were measured every 2 wk. The counting and characterization of all tumors were performed blindly with respect to treatment group as previously described (3). Tumor volume was determined by measuring the 3D size (height, length, and width) of each mass. The average of the three measurements was used as the diameter. The radius (r) was determined, and the tumor volume was calculated by: Volume $= 4\pi r^3/3$.

Measurement of Caspase 3 (Active Form) Positive Cells in Tumors and in Areas away from Tumors. Affinity-purified polyclonal rabbit antibody that reacts with the mouse p20 subunit of caspase 3 but does not react with the precursor form was purchased from Cell Signaling Technology (cat. no. 9661). Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol. The sections were incubated with a protein block for 1 h at room temperature. The sections were incubated with caspase 3 primary antibody (1:300 dilution) overnight at 4 °C followed by incubation with a biotinylated antirabbit secondary antibody for 30 min, followed by incubation with conjugated-avidin solution (ABC Elite kit purchased from Vector Laboratory) for 30 min. Color development was achieved by incubation with 0.02% 3,3′-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide for 10 min at room temperature. The slides were then counterstained with hematoxylin and dehydrated. A positive reaction was shown as a brown precipitate in the cytoplasm and/or perinuclei of the cells (4). The percentage of caspase-3$^+$ cells in epidermis away from tumors and in tumors was calculated from the number of caspase-3 stained cells from the entire area of each section.

Bromodeoxyuridine (BrdU) Incorporation into DNA. All animals were injected with BrdU (50 mg/kg) i.p. and killed 1 h later. Endogenous peroxidase was blocked by incubating the tissue sections in 3% (vol/vol) hydrogen peroxide in methanol for 10 min at room temperature. The tissue sections were then incubated in a moist chamber with 0.125% trypsin for 10 min at 37 °C, rinsed in distilled water, and incubated at room temperature for 30 min with denaturing solution. The sections were incubated with blocking solution for 10 min at room temperature and covered with biotinylated mouse monoclonal anti-BrdU antibody (Life

Technologies) at room temperature for 90 min. Sections were rinsed with PBS and incubated with streptavidin-peroxidase for 10 min. Color development was achieved by incubation for 5 min at room temperature with a substrate solution containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide. The slides were weakly counterstained in Mayer's hematoxylin (Sigma) for 2 min, cleared with xylene, mounted with a coverslip, and scored under a light microscope. The percentage of BrdU-labeled cells in the epidermis and in the tumors was calculated from the number of stained $BrdU^+$ cells from the entire areas of each section.

Mouse Adipokine Antibody Array. Measurement of mouse adipokines (detection of 38 mouse adipokines) was performed using the Mouse Adipopkine Antibody Array kit (purchased from R&D Systems). The epidermal tissue lysate was prepared as described previously (5). Briefly, dorsal skin samples were removed and immediately placed in a buffer solution containing 50 mM potassium phosphate (pH 7.7) at 52 °C for 20 s. The skin samples were then immediately submerged in an ice bath containing the same buffer for 40 s and the epidermis was scraped from the dermis. The epidermis and tumors were placed in 1 mL of tissue lysis buffer (Cell Signaling; cat. no. 9803) containing 1 mM phenylmethylsulfonyl fluoride and sonicated five times (each for 5 s at 4 °C at 5-s intervals). Samples were centrifuged at 17,800 × *g* for 10 min at 4 °C. The antibody array was performed following the instructions provided by the manufacturer. Briefly, membranes were treated with 2 mL of blocking buffer and then incubated overnight with 1 mL of tissue lysates containing 150 μg protein at 4 °C. After washing, 1 mL of a mixture of biotin-conjugated antibodies that are specific to the different targets on the array were added for 1 h and then incubated with 2 mL of HRP-conjugated streptavidin at room temperature for 2 h. The membranes were then treated with 500 μL of detection buffer for 2 min and, finally, exposed to X-ray film using film developer. By comparing the signal intensities, relative expression levels of cytokines were determined and quantified by densitometry. Positive controls were used to normalize the results from the different membranes being compared.

Real-Time Quantitative RT-PCR. Total RNA was extracted by using RNeasy Lipid Tissue Mini kit (Qiagen) that was converted to cDNA by the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time RT-PCR was conducted in an ABI PRISM 7300 sequence detector (Applied Biosystems). The primers were purchased from Integrated DNA Technologies. RNA analysis was performed using the Syber Green PCR Master Mix reagents kit (Applied Biosystems) and quantification of gene expression was determined by the comparative $\Delta\Delta C_T$ method (6). The target gene expression in the test samples was normalized to the endogenous reference glyceraldehyde-3-phosphate dehydrogenase. (GAPDH) and was reported as the fold difference relative to the GAPDH gene expression. The primers used were as follows: MCP1, forward-CTTCTGGGCCTGCTGTTCA-3′ and reverse-CAGCCTACTCATTGGGATCA-3′; SerpinE1, forward-GACACCCTCAGCATGTTCATC-3′ and reverse-AGGGTTGCACTAAACATGTCAG-3′; TIMP1, forward -GGCATCCTCTTGTTGCTATCACTG-3′ and reverse-GTCATCTTGATCTCATAACGCTGG-3′; and GAPDH, forward-TTGTCTCCTGCGACTTCA-3′ and reverse-CACCACCCTGTTGCTGTA-3′.

Statistical Analysis. For the analyses of tumor-free distributions (percent of mice without tumors vs. time) between the two treatments, the Kaplan–Meier method was used for estimations, and the log-rank test was used to test homogeneity of the distributions between the two treatments (7). The repeated measurement models (8) were used for the analyses of the treatment effects on body weight, tumor number per mouse, and tumor volume per mouse between the two groups. To stabilize the variation, transformed responses were used in the analyses. More specifically, the cubic root for tumor volume per mouse were used in the analyses. Covariates included time (in weeks), time squared, and treatment together with the interactions. The treatment effects were assessed on the basis of the comparisons of slope (linear and quadratic trends over time) of the regression lines. Heterogeneous autoregressive covariance structures were used to account for the within-mouse correlation. Different covariance structure parameters were used for different treatment groups to account for the heterogeneity of the covariance between the two treatment groups. The comparisons were also performed at the last time point (week 33). A 5% significance level was used for all of the tests.

1. Reddy BS, Patlolla JM, Simi B, Wang SH, Rao CV (2005) Prevention of colon cancer by low doses of celecoxib, a cyclooxygenase inhibitor, administered in diet rich in omega-3 polyunsaturated fatty acids. *Cancer Res* 65:8022–8027.
2. Lou YR, et al. (2011) Effects of high-fat diets rich in either omega-3 or omega-6 fatty acids on UVB-induced skin carcinogenesis in SKH-1 mice. *Carcinogenesis* 32:1078–1084.
3. Lou YR, Lu YP, Xie JG, Huang MT, Conney AH (1999) Effects of oral administration of tea, decaffeinated tea, and caffeine on the formation and growth of tumors in high-risk SKH-1 mice previously treated with ultraviolet B light. *Nutr Cancer* 33: 146–153.
4. Lu YP, et al. (2002) Stimulatory effect of topical application of caffeine on UVB-induced apoptosis on mouse skin. *Oncol Res* 13:61–70.
5. Lu YP, et al. (2008) Effect of caffeine on the ATR/Chk1 pathway in the epidermis of UVB-irradiated mice. *Cancer Res* 68:2523–2529.
6. Bernard JJ, Gallo RL (2010) Cyclooxygenase-2 enhances antimicrobial peptide expression and killing of *Staphylococcus aureus*. *J Immunol* 185:6535–6544.
7. Lawless JF (1982) *Statistical Models and Methods for Lifetime Data* (John Wiley and Sons Inc, New York).
8. Lindy JK (1993) *Models for Repeated Measurements* (Clarendon Press, Oxford, UK).

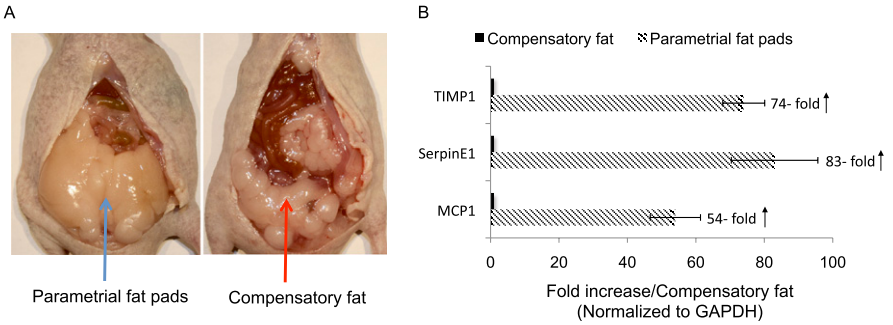

Fig. S1. mRNA expression for TIMP1, MCP1, and SerpinE1 is higher in the parametrial fat pads of sham-operated control mice than in the compensatory fat that appears after removal of the parametrial fat pads. The parametrial fat pads in sham-operated mice and the compensatory fat that returned after partial lipectomy at the end of the tumorigenesis study described in Fig. 1*A* are shown in *A*. Quantitative RT-PCR analysis of MCP1, SerpinE1, and TIMP1 mRNA on freshly pooled tissue fat from five animals in the parametrial fat pads from sham-operated control mice and in the compensatory fat from partially lipectomized mice was done at the end of the tumorigenesis study from mice fed the 40%-kcal high-fat diet and irradiated with UVB for 33 wk is shown in *B*. Each value is the mean ± SE from three separate measurements.

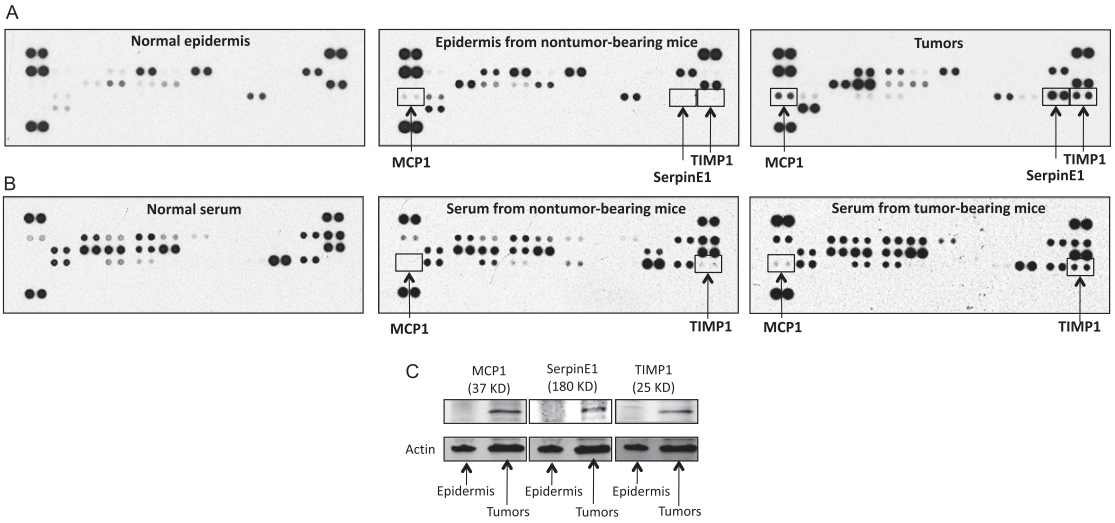

Fig. S2. Measurement of adipokines in the serum, epidermis, and tumors during a carcinogenesis study. An antibody array was used to measure the levels of 38 adipokines from five animals for normal epidermis without UVB exposure as well as from epidermis of UVB-treated nontumor-bearing animals and from squamous cell carcinomas from UVB-treated mice for 33 wk (*A*). An antibody array was used to measure the levels of adipokines in normal serum without UVB exposure as well as in serum from UVB-treated nontumor-bearing mice and in serum from UVB-treated tumor-bearing mice (*B*). The levels of MCP1, SerpinE1, and TIMP1 in normal epidermis without UVB exposure and in squamous cell carcinomas from UVB-treated mice by Western blot are shown in *C*. Representative arrays from pooled samples are presented.

Table S1. Effect of lipectomy on the size of histologically characterized skin tumors in SKH-1 mice treated with UVB light

		Squamous cell papillomas	Keratoacanthomas	Squamous cell carcinomas	Total tumors
Treatment	No. of mice	Tumor volume per mouse (mm^3)	Tumor volume per mouse (mm^3)	Tumor volume per mouse (mm^3)	Tumor volume per mouse (mm^3)
Sham-operated control	36	0.71 ± 0.49	51.63 ± 12.52	12.35 ± 5.33	66.12 ± 16.40
Lipectomy	39	0.46 ± 0.34 (35)	5.19 ± 2.18* (90)	1.51 ± 0.99* (88)	5.86 ± 1.98* (91)

Eighty female SKH-1 mice were given a high-fat diet rich in polyunsaturated omega-6 fatty acids for 2 wk. Mice were then equally divided into two groups on the basis of body weight. One group of mice had their parametrial fat pads removed and another group of mice was a sham-operated control. Two weeks after the surgery, all mice were treated with UVB (30 mJ/cm^2) once a day, twice weekly for 33 wk. The mice were killed at 24 h after the last irradiation with UVB, and all tumors were characterized by histopathology. Each value is the mean ± SE, and the numbers in parentheses represent percent decrease. Based on two-sample unequal variance *t* test with Satterthwaite's approximation, $*P < 0.00001$.