ONLINE APPENDIX- Supplemental data

Expanded - RESEARCH DESIGN AND METHODS

PMI-5011 preparation

PMI-5011 is a bioactive extract of Russian tarragon (*Artemisia dracunculus L.*) consisting of six potential components (1). It was prepared as previously described from hydroponically grown plants and purified as a resin (2-4).

Cell culture and reagents

The human adrenocortical carcinoma cell line NCI-h295R (ATCC, Manassas, VA), and mouse neuronal N38 (5) and GT1-7 (6) cell lines were cultured as previously described (7). PMI-5011 was dissolved in 70% ethanol and added in the media at the concentration of 40 µg/ml.

Whole brain primary cell cultures

Two whole mouse brains were removed and transferred immediately into 7 ml of dissection media (DM) (500 ml HBSS, Invitrogen, Carlsbad, CA), 1.19 g Hepes, 2.52 g Glucose, 50 µl Gentamycin (Invitrogen, Carlsbad, CA), 1.5 g BSA (Sigma, St. Louis, MO), 1.5 g Mg_2SO_4, 111 mg $CaCl_2$. Cells were dissociated and gradually attached to the bottoms of culture plates reaching approximately 90% confluency in 10-12 days.

AGRP promoter constructs

A 1107-bp *AGRP* promoter fragment (-796/+312) was amplified from human genomic DNA with Platinum Pfx polymerase (Invitrogen, Carlsbad, CA) using the PCR primers 5'- GTCAGCTAGCGTCTTAAACCCCTGGCCTTG -3' and 5'- CAGTCTCGAGCCTTGGAGTCCCCTCTAGG -3'. Amplicons was digested with NheI/MluI and cloned into the pGL3basic luciferase vector (Promega, Madison, WI). Site-directed mutagenesis was performed with the following oligonucleotides: mutA (+167/+168mut), 5'- ACTGTGCCCCCAGTCTCCAGGAAG -3' and 5'- GACCCTTCCTGGAGACTGGGGGCA-3'; mutB (-277/-283mut), 5'- TGGAGGGAGAATTGGAGGGGAGGACC -3' and 5'- TTCTGGTCCTCCCCTCCAATTCTCCC -3'. The double mutA+B construct was created using the mutA containing plasmid as a template and both primers for mutB to insert the second mutation. Plasmids were confirmed by digestion with restriction endonucleases and DNA sequencing. The numbering of DNA sequence cited in this manuscript (e.g. for the CACCC-box) was according to the sequence appearing in our previous publication (8) and GenBank accession number AF314194.

Transient transfections

Cells were transfected with Geneporter II (Gene Therapy systems, San Diego, CA) or Fugene 6 according to the manufacturers' protocols. Firefly and Renilla luciferase activities were measured using the Dual Luciferase Kit (Promega) and a single tube FB12 luminometer (Berthold Detection Systems, Oak Ridge, TN). *KLF4* human and mouse expression constructs were the kind gifts of Dr. Yet (Harvard Medical School, Boston, MA) and Dr. Ruppert (University of Alabama at Birmingham, Birmingham, AL). The SP1 expression construct was the kind gift of Dr. Robert Tjian (Howard Hughes Medical Institute, UC Berkeley, Berkeley, CA). All experiments were performed in triplicates, and data are shown as the average ± SEM of a representative experiment out of at least 3 independent replicate experiments.

For the *Klf4* silencing experiments, GT1-7 cells (1 x 10^6) were harvested and electroporated with 2 µg of *Klf4* siRNA using Nucleofector Kit V (Amaxa Biosystems). Silencer^R Pre-designed siRNA targeted to exon NM_010637 of the mouse *Klf4* gene (GGUACAGAACAUGUUCUAAtt) (Ambion) was used to knockdown *Klf4*. As a negative control Silencer Negative Control 5 siRNA (Ambion) was used. GT1-7 cells were cultured for 16 hours after electroporation in the presence or absence of PMI-5011 (40µg/ml) in complete medium.

Electrophoretic mobility shift assays

Nuclear extracts from N38 cells were prepared as described elsewhere (9). The following oligonucleotides were used in these experiments: human Wt, 5'-GGCCTGAACTGTGCCCCCACCCTCCAGGAAGGGTCCTTCA -3' and 5'-GCCGTGAAGGACCCTTCCTGGAGGGTGGGGGCACAGTTCA -3'; human mut A, 5'-GGCCTGAACTGTGCCCCCAGTCTCCAGGAAGGGTCCTTCA -3' and 5'-GCCGTGAAGGACCCTTCCTGGAGACTGGGGGCACAGTTCA -3'; mouse Wt, 5'-AGCCTGAATTGTGCCTCCATTTTCCAGGGAGGCACCTCATGC-3' and 5'-CAGGGCATGAGGTGCCTCCCTGGAAAATGGAGGCACAATTCA-3' and +30/66, 5'-TGTGGACCCTGGGCACCCTCTCTTGCTCCCAAATTTT-3' and 5'-GATTAAAATTTGGGAGCAAGAGAGGGTGCCCAGGGTC-3'. A known Klf4 binding site, the basic transcription element (BTE) of the CYP1A1 gene (10) was used in competition experiments.

Chromatin immunoprecipitation

NCI-h295R human adrenocortical cells were cultured in a 100-mm cell culture plate and transfected with *KLF4*-expressing vector or cotransfected with *KLF4* and -796/+373 *AGRP* promoter construct for 16h. The experimental procedure was according to the manufacturer's instructions (Upstate, Millipore, Billerica, MA). The DNA in the immunoprecipitate product was amplified in quantitative PCR reactions with the ChIP assay primers that are specific to the *AGRP* CACCCC box Site A: forward, 5'-AGGGAGTGAGGGCCTGAACT-3', and reverse, 5'- GCTTATATGGCAGGGCCTAATG-3'. Actin was used as an input control. The ChIP primers were: forward, 5'-TGCACTGTGCGGCGAAGC-3', and reverse, 5'-TCGAGCCATAAAAGGCAA-3' that amplify -980/-915 in the human actin gene promoter. Factor density from Chip assays are expressed as a signal ratio, R, using the following formula: $R=\exp_2(CT^{mock}-CT^{specific})$ (11), where CT^{mock} and $CT^{specific}$ are mean threshold cycles of PCR on DNA samples from specific and mock immunoprecipitations.

RNA extraction and real-time PCR

Total RNA was extracted from cultured cells or whole tissue using the RNeasy Mini Kit (Qiagen). Hypothalamic extracts were prepared as previously described (7). Quantitative PCR was performed using the TaqMan one-step RT-PCR core reagents kit (Applied Biosystems, CA, USA) as previously described (12). The primers and probes used were as follows: Agrp forward: 5'-GCTCCACTGAAGGGCATCA-3', Agrp reverse: 5'-GTGGATCTAGCACCTCCGC-3' and Agrp probe: 5'-6-FAM-TTCCCAGGTCTAAGTC-MGBNFQ-3'; Klf4 forward: 5'-CACACAGGCGAGAAACCTTACC -3', Klf4 reverse: 5'- CGGAGCGGGCGAATTT –3', and Klf4 probe: 5' FAM---CTGTGACTGGGACGGCTGTGGGT--- BHQ-1 3', and Cyclophilin forward: 5'-TAGAGGGCATGGATGTGGTAC-3', Cyclophilin reverse: 5'-GCCGGAGTCGACAATGATG-3', and Cyclophylin probe: 5'-6-FAM-

AGCCGGGACAAGCCACTGAAGGAT-BHQ-1-3'. The primers and probes for Neuropeptide Y (*Npy*), *Orexin*, Melanin Concentrating Hormone (*Mch*), Proopiomelanocortin (*Pomc*), and Cocaine and Amphetamine-Related Transcript (*Cart*) were as we have previously described (12). All experiments were performed in triplicates, and data are shown as the Mean±SEM of a representative experiment out of at least 3 independent experiments.

Western blotting

For Western blot analysis, total protein lysates (30–50 µg/lane) were separated on 15% or 10% SDS-polyacrylamide gels and blotted to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad). The membranes were incubated first with primary antibody to rat monoclonal anti-mouse Agrp (Alpha Diagnostic International San Antonio, TX) or rabbit polyclonal Klf4 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and then with anti-rat or anti-rabbit antibody respectfully as secondary antibody coupled to horseradish peroxidase. For loading control anti-GAPDH (AM4300, Ambion) or β-actin (Abcam, Cambridge, MA) antibody were used. The signal was detected with Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). Western blot images were quantified using Quantity One software (Bio-Rad, Hercules, CA).

Immunofluorescence

GT1-7 cells were plated on 12-mm glass coverslips coated with poly-D-lysine. Cells were washed in PBS and fixed in 4% paraformaldehyde. Immunocytochemistry was performed using Klf4 and Agrp antibodies (dilution 1:200) in a cocktail. This was followed by three washes in the presence of a secondary Alexa Fluor 488 goat anti-rat antibody and Alexa Fluor 594 goat anti-rabbit antibody (Invitrogen, Eugene, OR). Finally slides were mounted in Prolong Gold Antifade Reagent with DAPI (Invitrogen, Eugene, OR). Microphotographs of stained cells were taken on Axioscop 2 microscope (Zeiss) at 40x magnification. Color images were captured with a Zeiss Axiocam HRc CCD camera at 1300 x 1030 resolution.

In vivo bioluminescence

In vivo bioluminescence imaging was conducted on an IVIS 100 system (Xenogen Corp., CA, USA) using LivingImaging acquisition and analysis software (Xenogen). The rationale for the production of these mice and a full description of the constructs used is provided elsewhere (13). The mice used in this experiment contained the *Klf4*-binding CACCC-box and additional enhancer elements from downstream regions. Mice were anesthetized with isoflurane and received an i.p. injection of the substrate D-luciferin (100 mg/kg). An integration time of 3 min with a binning of 100 pixels was used for luminescence image acquisition. Signal intensity was quantified as the sum of all detected photon counts within the region of interest after subtraction of background luminescence. Mice were fed Chow diet (Chow 5001, 12.5% Kcal, Purina Lab diet, Framingham, MA) *ad libitum* and imaged the day before the start of feeding with PMI-5011 and imaged again one week later, at the end of the experiment.

Indirect calorimetry

The study protocols for all mouse feeding studies were approved by the Institutional Animal Care and Use Committee of the Pennington Biomedical Research Center (LSU System, Baton Rouge, LA). Mice were maintained on a 12h/12h, day/light cycle in a pathogen-free animal facility with lights coming on at 6:00am. Thirteen C57BL/6J (12-

week old) male mice were placed into Oxymax metabolic cages (Columbus Instruments, Columbus, OH) and allowed to adjust to their new environment for four days while receiving Chow diet (Labdiet 5001, 12.5% Kcal, Purina Lab diet, Framingham, MA). Afterwards, mice were randomized into two groups (N=6 in control group, and N=7 in PMI-5011 group) of matching mean body weights, to receive Chow or Chow+PMI-5011 diets *ad libitum*. PMI-5011 was dissolved in the food at the dose of 20 mg/mouse/day. After a further 3-day period of adjustment, the instrumentation was re-calibrated and metabolic measurements commenced and continued for four days. Data are shown for four consecutive light and dark cycles.

Body composition

Fat, free-body fluid, and fat-free mass were measured in a Nuclear Magnetic Resonance instrument (Bruker Optics Inc., The Woodlands, TX) as we have previously reported (14). Fat-free mass (i.e. muscle mass) was calculated by subtracting the fat-mass and fluid-mass from total body weight. Data are shown as means of grams of fat or grams of lean mass.

Mouse feeding studies with PMI-5011

For the 24-day Chow experiment, seven-week old C57BL/6J male mice were divided into two groups of equal mean body weights to receive Chow (N=6) or (Chow+PMI-5011) (N=7) diets. Chow (Labdiet 5001, 12.5% fat Kcal, Purina Lab diet, Framingham, MA) and Chow+PMI-5011 diets were provided *ad libitum* as described earlier for the metabolic cages experiment. Upon completion of the study hypothalami were dissected out. Food intake was monitored by weighing small quantities of pellets (25 grams) every five days. Body weight was also monitored every five days.

With regard to the effects of the High Fat Diet (HFD) (Cat #: DSD12331, 58% Kcal from fat, Research Diets, New Brunswick, NJ), three separate experiments were performed (HFD-1, HFD-2, and HFD-3). These studies had been performed to evaluate the effects of PMI-5011 on insulin sensitivity without measuring food intake (15). In HFD-1, 16 seven-week old mice were placed on HFD for eight weeks and then randomized (N=8 per group) to receive HFD or HFD+PMI-5011 by oral gavage. PMI-5011 was provided in a single dose at 15 mg/mouse/day dissolved in 2% DMSO for an additional eight weeks. Hypothalami were excised from semi-frozen brains for protein analysis. In HFD-2 (N=8 per group), the PMI-5011 was provided by gavage for eight weeks at 15 mg/mouse/day while mice were fed HFD ad libitum. In HFD-3 (N=8 per group), PMI-5011 was again provided in HFD at 15 mg/mouse/day *ad libitum* but for 40 days.

Food deprivation

Food deprivation was performed by removing all food pellets from mice (N=6 fed, N=7 food-deprived) at 17:00 and sacrificing all mice at the same time (10:00) the next morning. Hypothalami were excised and mRNA was prepared. Agrp and Klf4 mRNA levels were measured by qPCR as described above.

Peripheral and gut hormones

With regard to the measurements of insulin, leptin, and gut hormones, C57BL/6 male mice were fed HFD (N=8) or HFD+PMI-5011 (N=7) *ad libitum* for 11 days. PMI-5011 was dissolved in DMSO and then mixed with corn oil (5% DMSO). 0.1 ml per day at 16:00 h (i.e. 2 hours before lights out) of the corn-oil/DMSO solution was provided by gavage. Upon completion of the experiment, mice were partially anesthetized, and whole blood was collected. Mice were subsequently sacrificed. Hormones were

measured in plasma using a kit according to manufacturer's instructions (Lincoplex, Mouse Gut hormone panel, Millipore, MA).

Statistical analyses

Statistical significance was evaluated using one-way Analysis of Variance (ANOVA) and the Students T-test. The data were expressed as means ± SEM and calculated using variance analysis and the Newman-Keuls test for multiple comparisons among groups. Bonferroni adjustments were made for multi-testing. P-values less than 0.05 were considered to be statistically significant. The same methods were used for testing for differences in circulating hormone levels. Data analysis was carried out on SAS (SAS Version 9.1, 2003).

For the meta-analysis, the data from the three HFD studies were combined to examine whether there were statistically significant differences in body weight between the HFD- and PMI5011-fed groups. The random effects ANOVA meta-analysis model was use. The results represent the unweighted ANOVA analysis. The ANOVA analysis in a bootstrap fashion was also used and again the p-value was nearly identical. Data analysis was carried out on SAS (SAS Version 9.1, 2003).

Nomenclature

The gene names used were according to standard nomenclature (http://www.councilscienceeditors.org/publications/resources.cfm). Gene names of human genes are shown in upper case and italics, and gene names of mouse orthologs are shown with the first letter in upper case and the rest in lower case, all italics. References to protein products of genes are shown in regular font.

REFERENCES CITED

1. Schmidt B, Ribnicky DM, Poulev A, Logendra S, Cefalu WT, Raskin I: A natural history of botanical therapeutics. *Metabolism* 57:S3-9, 2008

2. Logendra S, Ribnicky DM, Yang H, Poulev A, Ma J, Kennelly EJ, Raskin I: Bioassay-guided isolation of aldose reductase inhibitors from Artemisia dracunculus. *Phytochemistry* 67:1539-1546, 2006

3. Ribnicky DM, Poulev A, O'Neal J, Wnorowski G, Malek DE, Jager R, Raskin I: Toxicological evaluation of the ethanolic extract of Artemisia dracunculus L. for use as a dietary supplement and in functional foods. *Food Chem Toxicol* 42:585-598, 2004

4. Ribnicky DM, Poulev A, Watford M, Cefalu WT, Raskin I: Antihyperglycemic activity of Tarralin, an ethanolic extract of Artemisia dracunculus L. *Phytomedicine* 13:550-557, 2006

5. Belsham DD, Cai F, Cui H, Smukler SR, Salapatek AM, Shkreta L: Generation of a phenotypic array of hypothalamic neuronal cell models to study complex neuroendocrine disorders. *Endocrinology* 145:393-400, 2004

6. Nelson SB, Lawson MA, Kelley CG, Mellon PL: Neuron-specific expression of the rat gonadotropin-releasing hormone gene is conferred by interactions of a defined promoter element with the enhancer in GT1-7 cells. *Mol Endocrinol* 14:1509-1522., 2000

7. Charbonneau C, Bai F, Smith Richards BK, Argyropoulos G: Central and Peripheral Interactions Between the Agouti Related Protein and Leptin. *Biochem Biophys Res Commun* 319:518-524, 2004

8. Brown AM, Mayfield DK, Volaufova J, Argyropoulos G: The gene structure and minimal promoter of the human agouti related protein. *Gene* 277:231-238., 2001
9. Andrews NC, Faller DV: A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 19:2499, 1991
10. Zhang W, Shields JM, Sogawa K, Fujii-Kuriyama Y, Yang VW: The gut-enriched Kruppel-like factor suppresses the activity of the CYP1A1 promoter in an Sp1-dependent fashion. *J Biol Chem* 273:17917-17925, 1998
11. Nelson JD, Denisenko O, Sova P, Bomsztyk K: Fast chromatin immunoprecipitation assay. *Nucleic Acids Res* 34:e2, 2006
12. Stutz AM, Staszkiewicz J, Ptitsyn A, Argyropoulos G: Circadian expression of genes regulating food intake. *Obesity (Silver Spring)* 15:607-615, 2007
13. Ilnytska O, Sozen MA, Dauterive R, Argyropoulos G: Control elements in the neighboring ATPase gene influence spatiotemporal expression of the human agouti-related protein. *J Mol Biol* 388:239-251, 2009
14. Redmann SM, Jr., Argyropoulos G: AgRP-deficiency could lead to increased lifespan. *Biochem Biophys Res Commun* 351:860-864, 2006
15. Wang ZQ, Ribnicky D, Zhang XH, Raskin I, Yu Y, Cefalu WT: Bioactives of Artemisia dracunculus L enhance cellular insulin signaling in primary human skeletal muscle culture. *Metabolism* 57:S58-64, 2008