Original Article

Characterization of Peripheral Circadian Clocks in Adipose Tissues

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First described in the suprachiasmatic nucleus, circadian clocks have since been found in several peripheral tissues. Although obesity has been associated with dysregulated circadian expression profiles of leptin, adiponectin, and other fat-derived cytokines, there have been no comprehensive analyses of the circadian clock machinery in adipose depots. In this study, we show robust and coordinated expression of circadian oscillator genes (*Npas2*, *Bmal1*, *Per1-3*, and *Cry1-2*) and clock-controlled downstream genes (*Rev-erbα*, *Rev-erbβ*, *Dbp*, *E4bp4*, *Stra13*, and *Id2*) in murine brown, inguinal, and epididymal (BAT, iWAT, and eWAT) adipose tissues. These results correlated with respective gene expression in liver and the serum markers of circadian function. Through Affymetrix microarray analysis, we identified 650 genes that shared circadian expression profiles in BAT, iWAT, and liver. Furthermore, we have demonstrated that temporally restricted feeding causes a coordinated phase-shift in circadian expression of the major oscillator genes and their downstream targets in adipose tissues. The presence of circadian oscillator genes in fat has significant metabolic implications, and their characterization may have potential therapeutic relevance with respect to the pathogenesis and treatment of diseases such as obesity, type 2 diabetes, and the metabolic syndrome. *Diabetes* 55:962–970, 2006

Organisms throughout the phylogenetic tree display some form of circadian rhythm. These cyclic patterns of gene expression synchronize various physiological processes with the daily changes in the external environment, allowing the organism to anticipate, adapt, and respond to varying physiological challenges (1).

Circadian gene expression is maintained through the coordinated action of basic helix-loop-helix/Per-Arnt-Simpleminded (bHLH-PAS) domain proteins encoded by *Clock* (or its paralog *Npas2*), *Bmal1*, *Period* (*Per*), and *Cryptochrome* (*Cry*) genes (1). CLOCK heterodimerizes with BMAL1 to drive the rhythmic expression of *Per* and *Cry* (2,3). After accumulating in the cytoplasm, PER and CRY proteins heterodimerize, translocate to the nucleus, and regulate the activity of CLOCK:BMAL1, completing a transcriptional/translational feedback loop (4,5). Therefore, the peak expression of these two distinct sets of genes occurs in antiphase of one another. CLOCK:BMAL1 dimers also drive the expression of circadian effector genes, such as those encoding transcription factors albumin D-element–binding protein (DBP) and REV-ERBα, implicated in a multitude of physiological functions (6,7).

Recent work using the *mPer2* promoter:*Luciferase* mice has demonstrated a persistent oscillatory *Luciferase* profile for >20 days ex vivo, not just in the core circadian oscillator in the suprachiasmatic nucleus (SCN) but also in liver and muscle explants (8). These peripheral oscillators continue to operate in animals in which the SCN has been surgically ablated, demonstrating that independent circadian oscillators operate within peripheral tissues. In vitro studies of fibroblast cell lines further support these findings, where exposure to dexamethasone, high serum concentrations, or glucose induces the circadian expression of *Clock*, *Per*, *Dbp*, and *Bmal1* (9).

Adipose tissue function in several disease states is associated with altered circadian rhythms. Patients suffering from bipolar disorders exhibit abnormal sleep patterns and disordered circadian function; when treated with pharmacological agents such as lithium chloride, they gain weight and become obese (10). Adipose tissue is a source of tumor necrosis factor-α, interleukin-6, adiponectin, leptin, and plasminogen activator inhibitor-1 (PAI-1), whose circulating levels display a strong circadian pattern (11–13). The peak incidence of myocardial infarction, sudden death, and heart failure occurs in the early morning hours, coinciding with the peak circulating levels of PAI-1 (14). The PAI-1 promoter contains DNA response elements recognized by the CLOCK:BMAL1 dimers (13). Interestingly, patients diagnosed with obesity and type 2 diabetes fail to display circadian variability in the incidence of myocardial infarction (14). Several epidemiological studies show that night-shift workers, whose activity period is chronically reversed, show an increased incidence of the metabolic syndrome (15). Recently, two murine studies have demonstrated relationships between circadian mech-

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Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

BAT, brown adipose tissue; bHLH-PAS, basic helix-loop-helix/Per-Arnt-Simpleminded; DBP, albumin D-element–binding protein; eWAT, epididymal adipose tissue; iWAT, inguinal adipose tissue; PAI-1, plasminogen activator inhibitor-1; SCN, suprachiasmatic nucleus.

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anism dysfunction and metabolic abnormalities. One group demonstrated impaired glucose metabolism in mice with *Bmal1* or *Clock* mutations (16). A second group found that *Clock* mutant mice increased their caloric intake and total body weight relative to wild-type controls. This was accompanied by significant changes in the diurnal rhythms of locomotor activity, feeding, and metabolic rate (17).

Although circadian dysfunction and disease pathogenesis are clearly linked, the role of circadian genes in adipose tissue physiology remains unexplored in a comprehensive manner. We find that a significant percentage of genes in adipose tissue depots display robust and coordinated circadian expression profiles and that a subset of these genes is conserved among adipose depots and liver.

RESEARCH DESIGN AND METHODS

In vivo circadian studies. Protocols were approved by the institutional animal care and use committee and used 8- to 10-week-old male AKR/J mice (The Jackson Laboratories, Bar Harbor, ME). Animals were acclimated to a regular Chow diet (Purina 5015) ad libitum and were under a strict 12-h-light/12-h-dark cycle for 2 weeks. During this period, the staff handled all animals frequently to reduce the stress introduced by human contact. After the acclimation period, animals were killed in groups of three (December 2003) or five (September 2004) every 4 h over a 48-h period. Animals in the temporally restricted feeding study were divided into a control cohort with ad libitum access to food and a restricted feeding cohort with food access during the 12-h-light period only. Individual body weight and food intake were monitored daily during the 7-day restricted feeding period, and animals were killed in groups of three every 4 h over a 24-h period. Animals were killed by CO_2 asphyxiation and cervical dislocation and harvested for serum, inquinal adipose tissue (iWAT), epididymal adipose tissue (eWAT) brown adipose tissue (BAT), and liver.

Quantitative real-time RT-PCR. Total RNA was purified from tissues using TriReagent (Molecular Research Center). RNA (~2 μg) was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega), with oligo dT at 42°C for 1 h in a 20-μl reaction. Primers for genes of interest (listed in supplemental Table 1, which is detailed in the online appendix [available at http://diabetes.diabetesjournals.org]) were designed using Primer Express (Applied Biosystems). Quantitative RT-PCR was performed on diluted cDNA samples with SYBR Green PCR Master Mix (Applied Biosystems) using the 7900 Real-Time PCR system (Applied Biosystems) under universal cycling conditions (95°C for 10 min; 40 cycles of 95°C for 15 s; and then 60°C for 1 min). All results were normalized relative to a *Cyclophilin B* expression control. To control for possible circadian changes in *Cyclophilin B* expression, we also normalized gene expression relative to *β-actin* and *Gapdh*. Results shown in supplemental Fig. 1, which is detailed in the online appendix indicate that our choice of expression control gene did not alter the experimental outcome.

Serum analysis. ELISA kits for melatonin (RE54021; Research Diagnostics, Flanders, NJ) and leptin (EZML-82K; Linco Research, St. Louis, MO), and radioimmunoassay kit for corticosterone (07-120102; Mp Biomedicals, Orangeburg, NY) were used according to the manufacturer's protocols. Assays were performed on serum samples pooled from $n = 3$–5 animals harvested at individual time points. Corticosterone assays were performed in triplicate on pooled samples.

Periodicity analysis. Periodicity of the circadian data obtained by quantitative RT-PCR was tested with Time Series Analysis-Single Cosinor v. 6.0 software (Expert Soft Technologie) (18).

Affymetrix oligonucleotide microarray gene expression analysis. RNA integrity was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Double-stranded cDNA was synthesized from ~9 μg total RNA using a Superscript cDNA Synthesis kit (Invitrogen, Carlsbad, CA) in combination with a T7-$(dT)_{24}$ primer. Biotinylated cRNA was transcribed in vitro using the GeneChip IVT Labeling kit (Affymetrix, Santa Clara, CA) and purified using the GeneChip Sample Cleanup Module. Ten micrograms of purified cRNA was fragmented by incubation in fragmentation buffer (200 mmol/l Tris-acetate, pH 8.1, 500 mmol/l potassium acetate, and 150 mmol/l magnesium acetate) at 94°C for 35 min and chilled on ice. Fragmented biotin-labeled cRNA (6.5 μg) was hybridized to the Mouse Genome 430A 2.0 Array (Affymetrix), interrogating >14,000 substantiated mouse genes. Arrays were incubated for 16 h at 45°C with constant rotation (60 rpm), washed, and stained for 10 min at 25°C with 10 μg/ml streptavidin-R phycoerythrin (Vector Laboratories, Burlingame, CA) followed by 3 μg/ml biotinylated goat anti-streptavidin antibody (Vector Laboratories) for 10 min at 25°C. Arrays were stained once again with streptavidin-R phycoerythrin for 10 min at 25°C, washed, and scanned using a GeneChip Scanner 3000. Pixel intensities were measured, expression signals were analyzed, and features were extracted using the commercial software package GeneChip Operating Software v.1.2 (Affymetrix). Data mining and statistical analyses were performed with Data Mining Tool v.3.0 (Affymetrix) algorithms. Arrays were globally scaled to a target intensity value of 2,500 to compare individual experiments. The absolute call (present, marginal, and absent) of each gene expression in each sample and the direction of change and fold change of gene expressions between samples were identified using the above-mentioned software.

Spectral analysis of microarray data. Series of microarray expression values for gene x with N samples of the form $x_0, x_1, x_2, \ldots x_{n-1}$ were converted from time domain to a frequency domain using discrete Fourier transform algorithm:

$$I(\omega) = \frac{1}{N}\left|\sum_{t=0}^{N-1} x_t e^{(-i\omega t)}\right|^2, \qquad \omega \in [0,\pi]$$

Time series with a significant sinusoidal component with frequency $\omega \in [0,\pi]$ showed a peak (periodogram) at that frequency with a high probability, unlike the purely random series whose periodogram approaches a flat line (19). The significance of the observed periodicity was estimated by Fisher's g-test, as recently recommended (20). To account for multiple testing problems, we used the false discovery rate method as a multiple comparison procedure (21). This method is adaptive to the actual data (20) and has been shown to control the false discovery rate (21).

RESULTS

Adipose tissues express circadian oscillator mechanism genes. We used a quantitative RT-PCR approach to examine the circadian gene expression patterns in BAT, iWAT, and eWAT of 8-week-old AKR/J mice. We detected robust cyclic expression of the majority of circadian oscillator genes examined (Fig. 1). *Npas2* and *Bmal1* cycled in synchrony, reaching their zenith (highest levels) around zeitgeber time (ZT) 0 (0, 24, and 48 h or the end of the 12-h-dark period) and their nadir (lowest levels) around ZT 12 (12 and 36 h or the end of the 12-h-light period). Their expression patterns were consistent among BAT, iWAT, eWAT, and liver, with minor differences in the amplitudes. In contrast, *Clock* expression did not follow a consistent circadian pattern in any of these tissues (Fig. 1). *Per1*, *Per2*, and *Per3* expression demonstrated synchronized 24-h oscillations, reaching zenith around ZT 12 (12 and 36 h) and nadir around ZT 0 (0, 24, and 48 h) (Fig. 1). Although we observed some inconsistencies in the *Cry2* expression, the overall gene expression of *Cry1* and *Cry2* followed a circadian profile, with a zenith around ZT 20 (20 and 44 h) and a nadir around ZT 8 (8 and 32 h) (Fig. 1).

To confirm these findings, we repeated the study several months later (September 2004), and as shown in supplemental Fig. 2, which is detailed in the online appendix, we obtained results that display a close similarity to those in Fig. 1. We also demonstrated the periodic nature of the observed gene expression patterns by fitting the data in Fig. 1 to cosine curves as mathematical models of periodic oscillatory patterns (supplemental Fig. 3, which is detailed in the online appendix).

Circadian-controlled gene oscillations in adipose tissues. The presence of active circadian clocks in BAT, iWAT, and eWAT was further investigated by examining the expression levels of several genes known to be circadian controlled. The expression of *Rev-erbα* and *Rev-erbβ* oscillated in phase with the *Per* genes in BAT, iWAT, and eWAT; these reflected the pattern observed in liver (Fig. 2). The expression of *Dbp* showed an oscillatory pattern similar to *Per* and *Rev-erb* genes, whereas the expression

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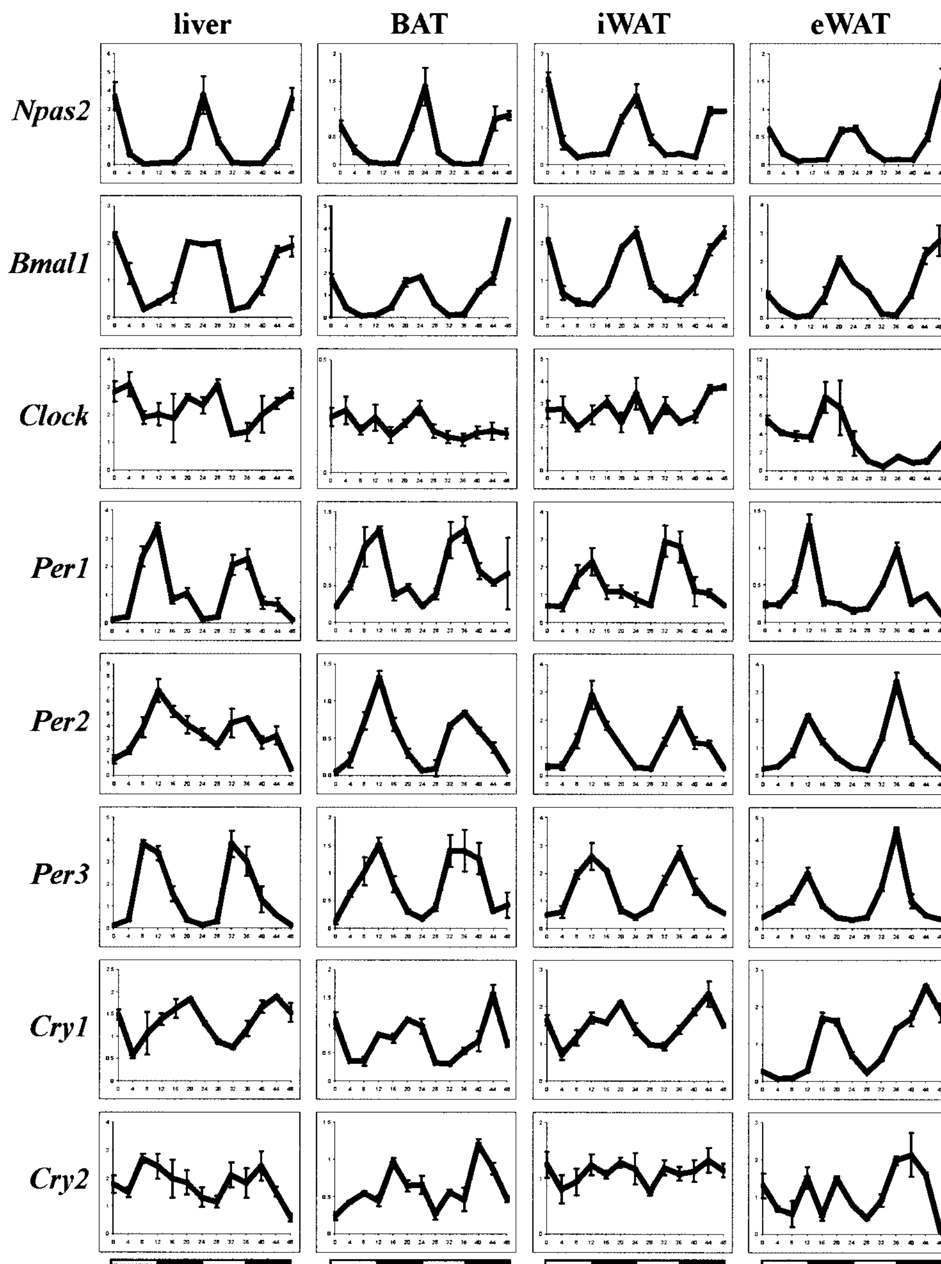

FIG. 1. Circadian oscillator gene expression patterns in peripheral tissues. Liver, BAT, iWAT, and eWAT were harvested from three male, 8-week-old, AKR/J mice every 4 h over a 48-h period (13 time points, n = 3 in each time point). Total RNA was extracted from collected tissues and used for quantitative RT-PCR analysis of gene expression, normalized with the corresponding *Cyclophilin B* levels. All values are reported as averages ± SD.

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of *E4bp4* followed a circadian profile approximately in phase to *Npas2* and *Bmal1* and out of phase with *Dbp* (Fig. 2). *Stra13* expression in fat tissues, especially in iWAT, showed a strong oscillatory trend but did not follow a specific circadian pattern. Although *Arnt* gene expression did not fluctuate significantly, we observed a circadian pattern of *Id2* expression in all tissues (Fig. 2).

Serum measures of circadian rhythm. Serum corticosterone levels served as a systemic control and showed a circadian profile with a zenith at the end of the 12-h-light period, similar to that of *Per* and *Cry* genes (Fig. 3). However, measurements of circadian oscillations for both melatonin and leptin serum levels did not achieve significance.

Microarray analysis reveals a large number of periodically expressed genes in adipose tissues. To determine the extent of circadian gene expression in adipose tissues, we performed an Affymetrix microarray gene expression analysis on the samples previously examined by quantitative RT-PCR (Fig. 1). A large number of genes showed oscillatory expression patterns in iWAT (4,398 genes), BAT (5,061 genes), and liver (5,386 genes) (Fig. 4). Of these, 650 genes showed a conserved circadian expression pattern in BAT, iWAT, and liver, representing 14.8, 12.8, and 12% of the tissue-specific oscillatory transcriptome, respectively (Fig. 4; supplemental Table 3, which is detailed in the online appendix). Although this group of genes was predominantly composed of those involved in basic metabolism and "housekeeping" functions, it also contained the circadian clock oscillator genes *Npas2*, *Bmal1* (*Arntl*), *Per1*, *Per2*, *Per3*, and *Cry1* as well as *Dbp* (supplemental Table 3), consistent with our quantitative RT-PCR studies (Figs. 1 and 2) and confirmed by cosine-fit analysis (supplemental Table 2). Furthermore, several genes involved in adipose function (*Cebpα*, *Cebpγ*, *Lpl*, *Pparα*, *Pgc1β*, and *Stat5A*) also oscillated in these three tissues (supplemental Table 3).

Another feature of the circadian transcriptome was that

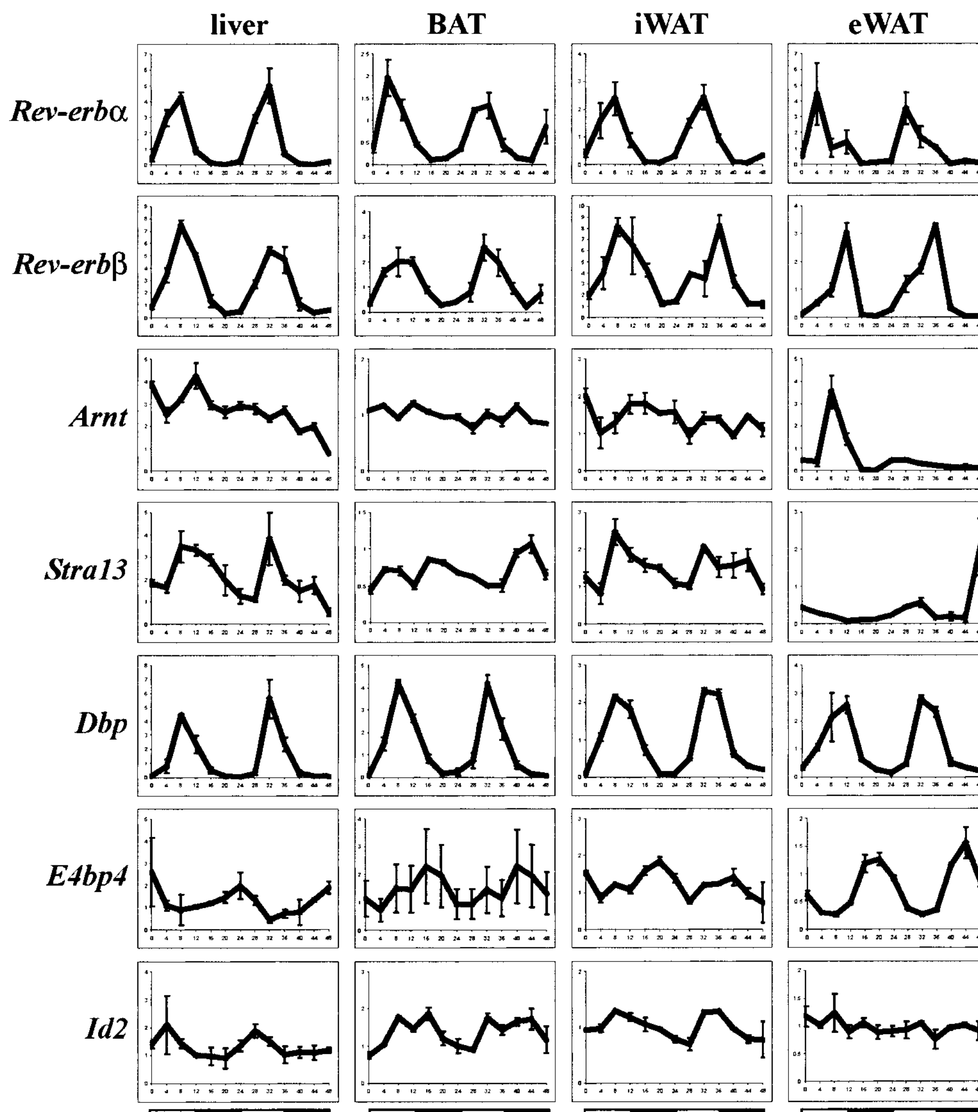

FIG. 2. Circadian-controlled gene expression patterns in peripheral tissues. Expression patterns of candidate genes from liver, BAT, iWAT, and eWAT were determined and reported as described in Fig. 1.

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oscillating genes cycle in distinct temporal groups, suggesting another level of transcriptional coordination. In supplemental Fig. 4, which is detailed in the online appendix, genes were grouped based on the zenith of their oscillatory phase, at ZT 0, 4, 8, or 16, demonstrating that this pattern is detectable among shared oscillatory genes in BAT, iWAT, and liver.

Temporally restricted feeding regimen alters the circadian expression profile in adipose depots. To determine whether the oscillatory patterns of gene expression in BAT, iWAT, and eWAT could be experimentally phase-shifted, we temporally restricted food availability to the 12-h-light period in the experimental animal cohort (restricted feeding), whereas the control animal cohort ate ad libitum. In this 24-h study (Figs. 5 and 6), the control animals (dotted lines) displayed circadian patterns of gene expression comparable with those seen in Figs. 1 and 2. However, the animals whose food access was temporally restricted (solid lines) showed phase shifts in gene expression relative to control animals.

As additional controls, the serum corticosterone, body weight, and food consumption were monitored during the 7-day temporal restricted feeding study (Fig. 7). The temporally restricted feeding regimen led to a phase shift and amplitude dampening in the circadian pattern of corticosterone serum levels. There was no significant difference, past the initial adjustment period, in the food intake between the control and restricted feeding animals. Body weight was not significantly different between the groups, although we did observe a trend toward a body weight increase in the restricted feeding animals.

DISCUSSION

Previously published gene expression profiles have determined, through global transcriptomic approaches, that an estimated 9% of the expressed genes in SCN, heart, and liver follow a circadian expression profile (22–25). Only a further subset of 8–10% of these circadian genes was shared between the SCN and individual peripheral tissues (25). This suggests that the circadian expression profile of a large population of genes is tissue specific.

Our studies clearly demonstrate the presence of active peripheral circadian clocks in BAT, iWAT, and eWAT. The robust cyclic expression of the circadian oscillator genes examined (*Npas2*, *Bmal1*, *Per1-3*, and *Cry1-2*) was consistent among these tissues and mirrored those in liver, whose circadian clock has been independently characterized (25,26), making it a suitable control for this study. However, the expression of *Clock* did not follow a consis-

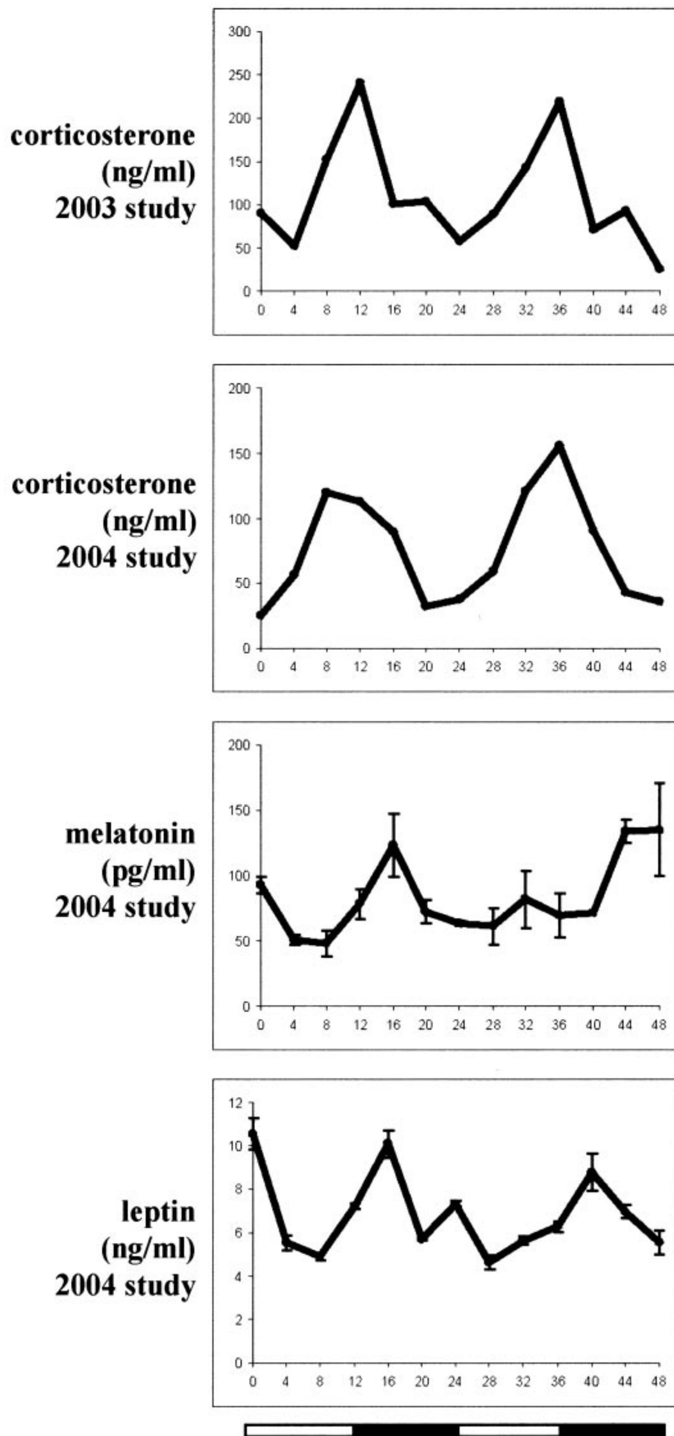

FIG. 3. Circadian oscillation of serum biomarkers. Blood samples were collected from male, 8-week-old, AKR/J mice every 4 h over a 48-h period (13 time points) in two independent studies. After centrifugation, sera from each time point were pooled and used for determination of serum proteins by radioimmunoassay (corticosterone) or ELISA (melatonin and leptin). All studies were performed on pooled samples (within a single time point). Values are reported as averages ± SD.

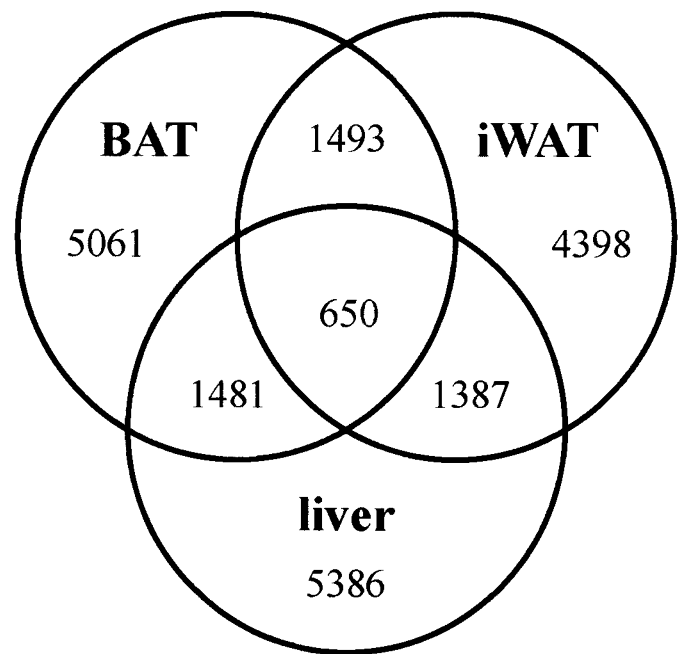

FIG. 4. A large number of periodically expressed genes in adipose tissues are revealed by microarray analysis. Total RNA was extracted from liver, BAT, and iWAT as described in Fig. 1 and used for Affymetrix microarray analysis to determine the expression levels of individual genes. After standard normalization, periodicity of gene expression was detected by discrete Fourier transform, and significance of the circadian period was confirmed by Fisher's g-test.

tent circadian pattern in any of these tissues. Others have shown that *Clock* expression, at least in the SCN, appears to be constitutive rather than cyclic and that in peripheral tissues and forebrain, CLOCK actions can also be carried out by its orthologs, such as NPAS2 (27). It is important to state that so far, no studies have conclusively shown that the apparent lack of an oscillatory expression profile for *Clock* excludes this gene as a component of the circadian oscillator in adipose tissue. Likewise, the oscillatory pattern of *Npas2* does not prove that it is a critical component of the core oscillator in adipose tissue.

The slight lag observed between *Cry* and *Per* expression phase has been previously documented in the SCN and in peripheral tissues (28). However, the daily oscillations of all *Per* and *Cry* genes occurred in a synchronous manner in all tissues examined. Moreover, the oscillations of *Npas2* and *Bmal1* occurred in antiphase to those of *Per* and *Cry*, recapitulating the autoregulatory mechanisms of an active circadian clock, as previously identified in other mammalian tissues (1). This notion was further supported by our cosine-fit analyses, clearly showing that gene expression follows the harmonic trends of the cosine curve and the antiphase oscillations within the circadian clock. Together, these findings not only illustrate the presence of active circadian clock mechanisms in adipose tissues but also confirm their periodic nature.

The activity of peripheral circadian clocks is most evident through their effects on the expression patterns of several circadian-controlled genes. REV-ERBα and REV-ERBβ are orphan nuclear hormone receptors that act as negative transcriptional regulators by binding RORE response elements in gene promoters, thus preventing the binding of a positive transcription regulator, RORα. They have also been shown to directly regulate the expression of *Bmal1*, *Clock*, and *Cry1* through this mechanism (29). The expression of *Rev-erbα* and *Rev-erbβ* is positively regulated by CLOCK:BMAL1 and negatively regulated by PER:CRY dimers in agreement with the expression profile observed in this study. Expression of *Rev-erbα* has been shown to correlate with adipogenesis (30), and its ectopic expression enhances adipocyte differentiation in vitro and

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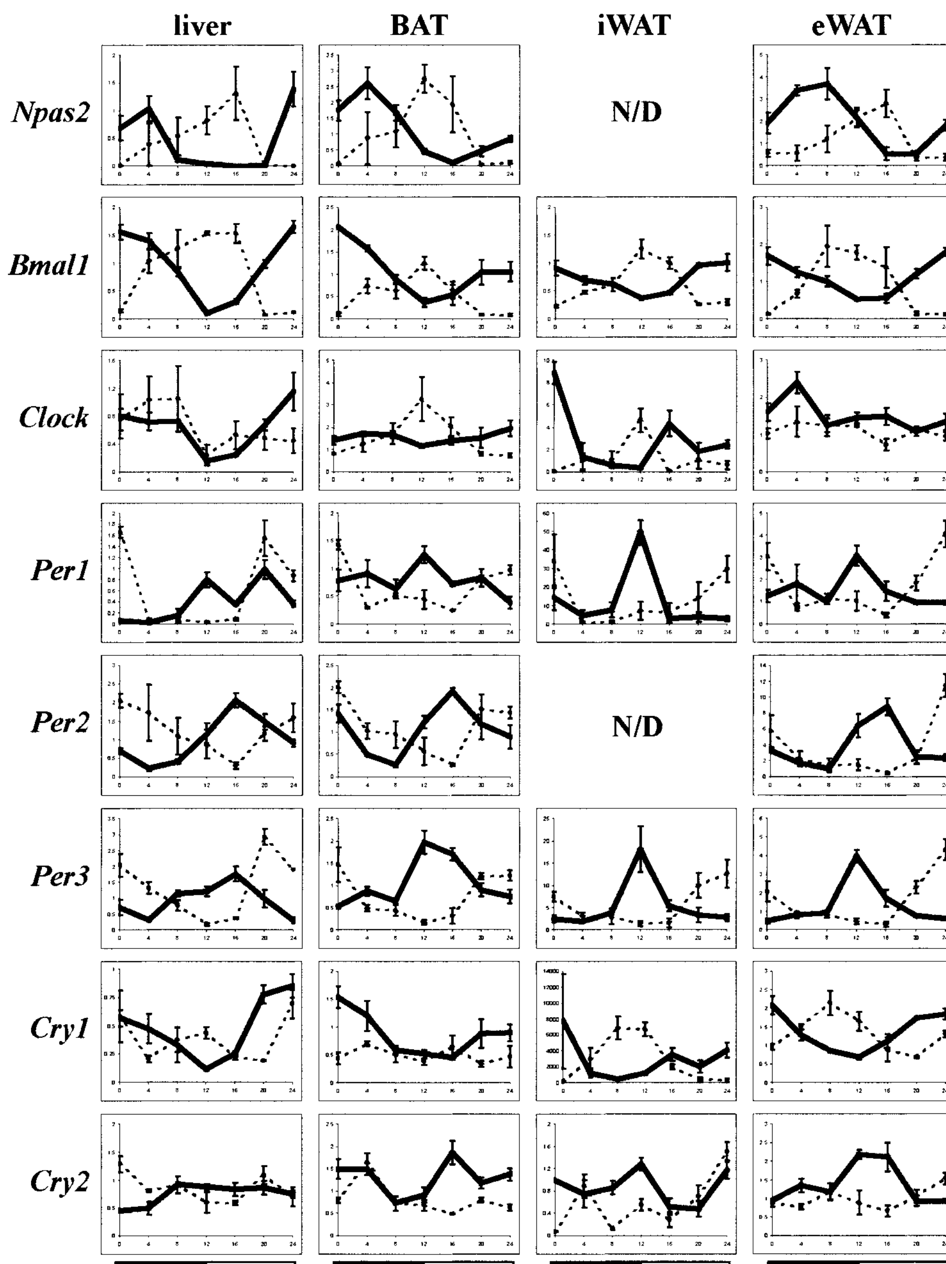

FIG. 5. Temporal restricted feeding alters circadian oscillator gene expression patterns. Forty-two male, 8-week-old, AKR/J mice were divided into two experimental groups, restricted feeding (solid line) and control (dashed line), and maintained single housed for 7 days before they were killed. Liver, BAT, iWAT, and eWAT were harvested from three animals from each group every 4 h over a 24-h period (seven time points, two groups per time point, n = 3 in each group per time point). Expression patterns of candidate genes from liver, BAT, iWAT, and eWAT were determined and reported as described in Fig. 1. N/D, not determined.

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in vivo (31). Thus, the circadian-regulated expression of these genes may prove to play an important role in the adipocyte differentiation program.

DBP is a PAR-domain transcription factor whose expression is under circadian control (32). Our findings are consistent with prior reports that *Dbp* transcription can be driven by the CLOCK:BMAL1 and suppressed by PER:CRY dimers (7). Emerging evidence suggests that *Dbp* may not only be a circadian output gene but also an integral component of the circadian oscillator. Studies have revealed that DBP-deficient mice exhibit altered activity periods, suggesting a role of *Dbp* in the control of the circadian oscillator (32). Furthermore, DBP may have a regulatory effect on the circadian oscillator mechanism because it can stimulate *Per1* transcription (33).

The E4BP4 protein is closely related to DBP. Its promoter contains a RORE element, making it susceptible to transcriptional suppression by REV-ERBs (24). Hence, although the *E4bp4* expression follows an oscillatory pattern, its phase is opposite to that of *Dbp* (34). Our results are consistent with these findings.

Stra13 (*Dec1*) encodes a circadian-controlled transcriptional repressor/regulator of multiple genes, including several downstream circadian genes (35). *Stra13* transcription is activated by CLOCK:BMAL1, whereas the STRA13 protein acts as a repressor of CLOCK:BMAL1 activity (35,36). Maximal levels of *Stra13* mRNA in liver have been reported to coincide with the peak of CLOCK: BMAL1 transcriptional activity (25). Although we observed similar oscillations in liver, we were not able to detect circadian expression of *Stra13* in adipose tissues. These inconsistencies may stem from the relatively low expression of *Stra13* in these tissues; STRA13 exerts an autologous negative feedback control on its own promoter transcription, thus keeping the gene expression low (37).

ARNT is a bHLH-PAS domain protein, structurally similar to PER proteins (1). ARNT protein levels have been shown to follow a circadian oscillatory trend in liver, lung,

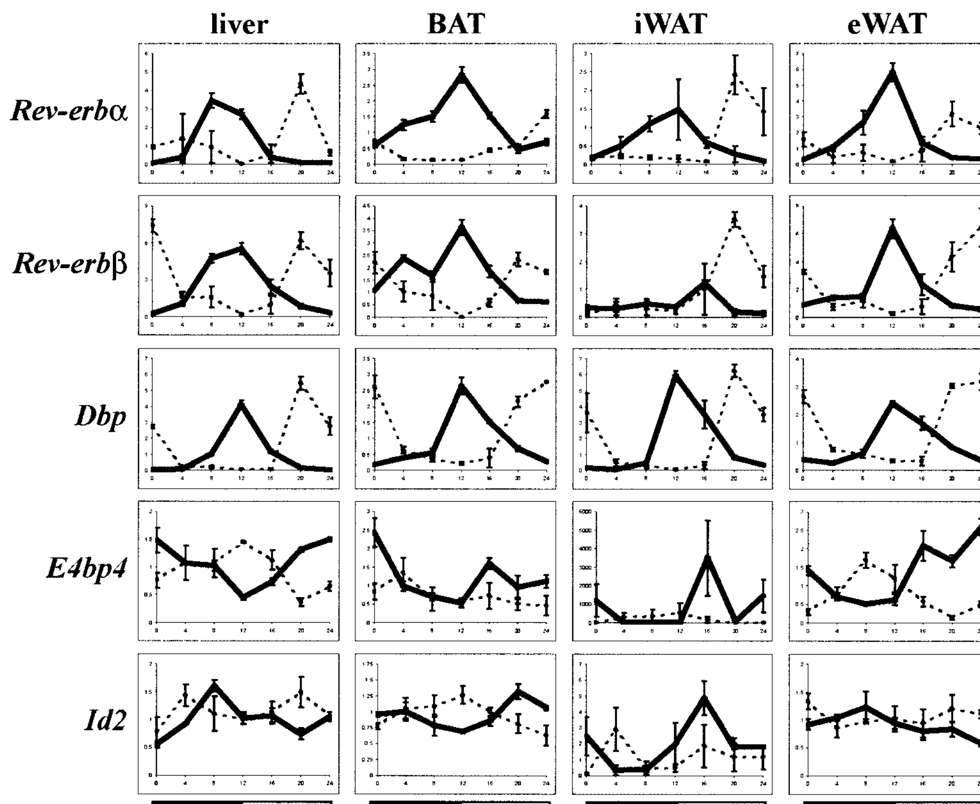

FIG. 6. Temporal restricted feeding alters circadian-controlled output gene expression patterns. Expression patterns of candidate genes from liver, BAT, iWAT, and eWAT were determined and reported as described in Fig. 5.

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and thymus but not in spleen (38). However, we were not able to detect any significant fluctuations of *Arnt* gene expression in any of the tissues we examined, including liver. This may be indicative of diurnal changes in protein level through a posttranslational rather than a transcriptional control mechanism.

Id2 gene encodes a HLH protein lacking a DNA-binding domain. ID2 proteins dimerize with other bHLH proteins, thereby inhibiting their DNA-binding activities (39). E-box sequences in the *Id2* promoter are potential regulatory targets for circadian bHLH-PAS transcription factors (39). In a recent microarray analysis of SCN and liver, *Id2* provided a prototype for a large cluster of circadian-regulated genes (24). Consistent with these findings, we observed a strong oscillatory pattern in *Id2* expression, with phase similar to that of other CLOCK-regulated genes, implying the involvement of the positive circadian regulators in *Id2* expression.

Corticosterone levels display a characteristic circadian rhythmicity (40) and served as controls in our study. Although melatonin and leptin serum levels in our cohorts of five animals showed trends of an oscillatory profile, they did not achieve significance. Because circadian oscillations in these serum markers have been reported (9,11,12), we postulate that significance could be reached with larger cohorts.

Our Affymetrix microarray gene expression analysis revealed a large number of genes with oscillatory expression patterns in iWAT, BAT, and liver. Although our detection of >5,300 genes exceeds previously reported values in the liver (23,41), we believe our analysis is a valid description of the temporal gene expression in these tissues. As described in RESEARCH DESIGN AND METHODS, we used a frequency conversion approach to ensure that we detect all periodically expressed genes, regardless of their amplitude and "noise" level. A companion manuscript will describe the details of our mathematical analysis (A.A.P., unpublished data).

Only 650 of these genes showed a circadian expression pattern that was conserved among all of the tissues examined. Dominated by basic metabolism and housekeeping genes, the list also contained the circadian clock oscillator genes *Npas2*, *Bmal1* (*Arntl*), *Per1*, *Per2*, *Per3*, and *Cry1* as well as *Dbp*, and several genes involved in adipose function (*Cebpα*, *Cebpγ*, *Lpl*, *Pparα*, *Pgc1β*, and *Stat5A*). Together, these findings suggest an overall coordination between the metabolic activities of these different tissues. This finding is further supported by the observation that oscillating genes cycle in distinct temporal groups, as is evident among the oscillatory genes in BAT, iWAT, and liver. Recent studies by Ueda et al. (42) begin to provide a systems approach to understanding this phenomenon. Their work highlights the importance of coordinate regulation through E/E′ box element points in establishing signaling circuits and posttranscriptional mechanisms (protein translation, phosphorylation, and proteasomal targeting) required for circadian gene oscillations.

Feeding and restrictive meal timing have been shown to serve as potent regulators of circadian rhythm in peripheral tissues, generating pronounced phase shifts in circadian gene expression in liver, skeletal muscle, heart, and kidneys (26,28,43–46). In this study, individual genes showed consistent phase shifts between liver, BAT, iWAT, and eWAT. However, the phase shifts observed were not uniform among all of the genes within individual tissues. Because the individual circadian clock components have unique regulatory mechanisms, restricted feeding may modulate each gene's phase differently. Similar observations have been made in previous studies on liver (44,45). Most importantly, we observed that the antiphase relationship between the circadian oscillator genes (in liver, BAT,

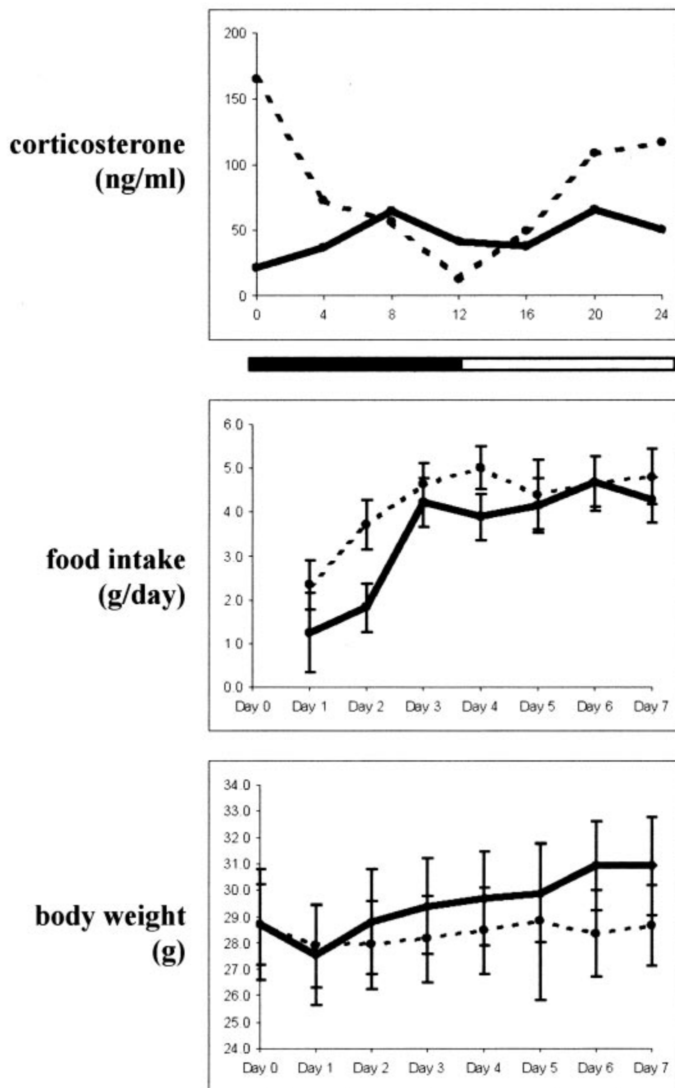

FIG. 7. Temporal restricted feeding does not affect food intake or animal body weight but does alter daily oscillation pattern of serum corticosterone levels. Blood samples were collected from animals as described in Fig. 5. After centrifugation, sera from each group, within a given time point, were pooled and used for a single determination of serum corticosterone by radioimmunoassay. Individual animal's food intake and body weights were measured daily until killed and reported as averages ± SD.

iWAT, and eWAT) was not affected by the temporal restricted feeding regimen. Instead, this relationship adjusted itself to the phase shifts of the individual genes, implying that these peripheral clocks possess mechanisms to adapt to entraining stimuli and varied physiological demands without compromising the oscillatory actions of the clock itself. Likewise, the temporal food restriction phase-shifted the output genes in a manner consistent with the oscillator genes regulating their expression (Fig. 2), thereby recapitulating the connection between the oscillator function and output gene expression.

As suggested by neuroanatomical studies (47,48), the SCN regulates peripheral circadian clocks by direct neuronal input. However, sympathetic inputs from the SCN alone cannot account for all circadian activities. Although hepatic sympathectomy has been shown to disrupt the liver's control of oscillatory serum glucose levels, this was not accompanied by any disruption of the oscillatory pattern of hepatic circadian gene mRNA levels (49). It is possible that circulating hormones like corticosterone may mediate central SCN regulation of circadian genes in peripheral tissues in rodent models. The current findings are consistent with reports that restricted feeding changes the oscillatory phase of serum corticosterone concentrations (45); however, these observations do not exclude the possibility of SCN-independent effects of restricted feeding, as described in liver (26).

The presence of an active circadian clock in adipose tissue depots suggests that there is a temporal component to the regulation of adipose tissue function. Recent evidence linking circadian dysfunction to obesity and the metabolic syndrome (16,17) strongly supports this notion. Metabolism and maintenance of energy homeostasis require functional coordination among individual adipose depots and other metabolically active tissue sites, to insure proper nutrient/energy flux and substrate use by the organism. Asynchrony, brought about by feeding or alternative entrainment mechanisms, may lead to defective substrate use, resulting in the disruption of metabolic pathways leading to hepatosteatosis, intramyocellular lipid accumulation, and insulin resistance. Therefore, further investigations of circadian rhythms in adipose tissues will provide insight into the physiology of energy homeostasis and the etiology of metabolic diseases.

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While this work was under revision, another manuscript (50) was published reporting confirmatory findings.

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