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Profile of rhythmic gene expression

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Abstract

Although a number of genes expressed in most tissues, including the liver, exhibit circadian regulation, gene expression profiles are usually examined only at one scheduled time each day. In this study, we investigated the effects of obese diabetes on the hepatic mRNA levels of various genes at 6-h intervals over a single 24-h period. Microarray analysis revealed that many genes are expressed rhythmically, not only in control KK mice but also in obese diabetic KK-A^y mice. Real-time quantitative PCR verified that 19 of 23 putative circadianly expressed genes showed significant 24-h rhythmicity in both strains. However, obese diabetes attenuated these expression rhythms in 10 of 19 genes. More importantly, the effects of obese diabetes were observed throughout the day in only two genes. These results suggest that observation time influences the results of gene expression analyses of genes expressed circadianly.

Introduction

Many physiological and behavioral processes exhibit circadian, 24-h rhythmicity. Recent studies have revealed that these endogenous rhythms are generated at the cellular level by circadian core oscillators, which are composed of transcriptional/translational feedback loops involving a set of clock genes [1,2]. In mammals, rhythmic transcriptional enhancement by two basic helix-loop-helix Per-Arnt-Sim domain-containing transcription factors, CLOCK and brain and muscle Arnt-like protein 1 (BMAL1), provides the basic drive for the intracellular clock system; the CLOCK-BMAL1 heterodimer activates the transcription of various clock-controlled genes [3,4]. Given that some clock-controlled genes, including the albumin D-site binding protein (Dbp), also serve as transcription factors, the expression of numerous genes may be tied to the molecular clock [1,2]. In parallel, the CLOCK-BMAL1 heterodimer activates the transcription of the Period (PER) and Cryptochrome (CRY) genes [5-7]. When the PER and CRY proteins reach a critical concentration, they attenuate CLOCK-BMAL1 transactivation, thereby generating a circadian oscillation in their own transcription [4,5].

The molecular clock system resides not only in the hypothalamic suprachiasmatic nucleus, which is recognized as the mammalian central clock, but also in various peripheral

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tissues [8-10]. The suprachiasmatic nucleus is not essential for driving peripheral

oscillations but acts as to synchronize peripheral oscillators [10]. Therefore, the local

molecular clock may directly control the physiological rhythmicity in peripheral tissues.

Recent studies have suggested that malfunction of the molecular clock system is

involved in the development of metabolic syndrome, which is a constellation of metabolic

abnormalities including obesity, dyslipidemia, hypertension, and insulin resistance/type 2

diabetes [11]. In mice, inactivation of BMAL1 suppresses the diurnal variation in plasma

glucose and triglyceride concentrations and can lead to insulin resistance [12]. Moreover,

homozygous *Clock* mutant mice have an attenuated diurnal feeding rhythm, are

hyperphagic and obese, and develop hyperglycemia, hyperlipidemia, and hepatic steatosis

[13]. Furthermore, we have shown that the rhythmic expression of clock genes is

attenuated in the liver and visceral adipose tissue of KK-A[y] mice, a genetic model of severe

obesity and overt diabetes [14]. Liver and visceral adipose tissue have critical roles in the

development of metabolic syndrome/type 2 diabetes [15,16]. In the liver, approximately

10% of the genes are expressed circadianly, which may help maintain hepatic physiology

[8,9]. Therefore, the circadian expression of various genes appears to be dampened in the

livers of animals with metabolic syndrome/type 2 diabetes.

In general, the *in vivo* effects of metabolic abnormalities on gene expression are studied at only one scheduled time each day. The effects of obese diabetes on the hepatic mRNA levels of several clock genes were observed only at their peak times [14]. Therefore, differences in timing among experiments might cause diverse results, especially for genes expressed rhythmically. To test this hypothesis, we investigated the effects of obese diabetes on the hepatic mRNA levels of various genes at different times of day, using microarray and real-time quantitative PCR analyses.

Materials and methods

Mice

Female KK/Ta and KK-A^y/Ta mice ($n = 12$ for each strain) were obtained from

CLEA Japan (Tokyo, Japan) at 8 week of age and were maintained under specific

pathogen-free conditions with controlled temperature and humidity and a 12-h light (07:00-

19:00 h)/12-h dark (19:00-07:00 h) cycle. The mice were housed individually and were

given a standard laboratory diet (CE-2; CLEA Japan) and water *ad libitum*. After 2 weeks,

animals were sacrificed to obtain blood and liver samples at the following zeitgeber times

(ZT): 0, 6, 12, and 18, where ZT 0 is defined as lights on and ZT 12 as lights off. All

animal procedures were preformed in accordance with the Guidelines for Animal Research

of Jichi Medical University, Japan.

Measuring circulating glucose and insulin concentrations

The blood glucose concentration was measured using a Glutest Ace R (Sanwa

Kagaku Kenkyusyo, Nagoya, Japan). The radioimmunoassay for serum insulin was

performed using kits purchased from Linco Research (St. Charles, MO). The intra- and

interassay coefficients of variation were less than 10%.

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RNA isolation and microarray hybridization

Total RNA was isolated from the liver samples using an RNeasy Mini kit (Qiagen, Valencia, CA). The amount and quality of RNA were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

We used the samples obtained at ZT 0 and 12 for the microarray analysis because CLOCK-BMAL1 transcriptional activity peaks in the early dark phase and reaches a minimum in the early light phase [17]. Fragmented, biotin-labeled amplified cDNA was prepared from 85 ng of total RNA using the Ovation Biotin System (NuGEN Technologies, San Carlos, CA), which is powered by Ribo-SPIA technology [18], according to the manufacturer's instructions. The cDNA (2.2 µg) was then hybridized to the GeneChip Mouse Expression Array 430A (Affymetrix, Santa Clara, CA), which contains 22,626 probe sets primarily against well-annotated full-length genes, for 18 h at 45°C. The chips were washed, and the signal was detected using standard Affymetrix reagents and protocols.

Analysis of the microarray data

A scanned image was quantified using GeneChip operating software, version 1.2 (Affymetrix) with the default parameters, and these data were analyzed using GeneSpring, version 7.2 (Agilent Technologies). Initially, values less than 0.01 were set to 0.01. After global normalization, each transcript was normalized to the median expression level across the samples obtained from KK mice at ZT 0. Statistical comparisons between groups were made using one-way ANOVA, and the transcripts that showed a significantly ($P < 0.05$) greater than 2-fold or less than 0.5-fold change were subjected to GeneTree clustering.

Quantitative reverse transcription-PCR

Reverse transcription was performed with 1.2 µg of total RNA, random hexamer primers, and RevertAid M-MuLV reverse transcriptase (Fermentas, Hanover, MD). The real-time quantitative PCR was performed with the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA), as previously described [14,19]. All specific primer sets and TaqMan probes were obtained from Applied Biosystems, and their GenBank accession codes are shown in **Table 1**. The data were analyzed using the comparative threshold cycle method [20]. To control the variation in the amount of DNA

available for PCR in the different samples, the gene expression of the target sequence was

normalized in relation to the expression of an endogenous control, glyceraldehyde-3-

phosphate dehydrogenase.

Statistical analysis

Differences in circulating glucose and insulin concentrations between KK and KK-

A[y] mice were determined using the Mann-Whitney U test. The rhythmicity of each gene

was assessed using one-way ANOVA. Differences in the mRNA levels at each time point

between groups were evaluated using Student's *t*-test. The values are presented as the

means ± SEM, and $P < 0.05$ was considered significant. All calculations were performed

using StatView, version 5.0 (SAS Institute, Cary, NC).

Results

Compared with KK mice, the strain with the A^y allele (KK-A^y) developed severe

obesity (KK, 28.9 ± 0.5 g; KK-A^y, 43.5 ± 0.4 g; n = 12 in each strain; $P < 0.01$). Moreover,

in KK-A^y mice, marked hyperglycemia and hyperinsulinemia were observed throughout the

day (**Fig**. **1**). Therefore, the KK-A^y mice had developed obese type 2 diabetes by the time

of the study.

The CLOCK-BMAL1 transcriptional activity peaks in the early dark phase and

reaches a minimum in the early light phase [17]; therefore, microarray analyses were

performed using the samples obtained from both strains at ZT 0 and 12 ($n = 3$ in each of the

four groups). We identified 343 transcripts whose levels differed significantly, by > 2-fold,

among the groups ($P < 0.05$, one-way ANOVA) and subjected them to GeneTree clustering.

As expected, the 12 samples were divided correctly into the four groups (**Fig**. **2**). Note that

this analysis next grouped KK mice at ZT 12 and KK-A^y mice at ZT 12, rather than the

same strains. These results suggest that the mRNA expression of many genes exhibits daily

rhythmicity not only in KK mice but also in KK-A^y mice. Moreover, as a whole, the

observed gene expression was influenced more by the time at which the observation was

made than by obese diabetes.

Then, we investigated whether obese diabetes affects the circadian expression of

various genes. We selected those genes whose microarray expression levels differed by

> 5-fold between ZT 0 and 12 in KK and KK-A^y mice and analyzed their expression levels

at ZT 0, 6, 12, and 18, using real-time quantitative PCR. As shown in **Table 1**, 19 of the 23

selected genes showed significant rhythms of mRNA expression in both KK and KK-A^y

mice, suggesting that our microarray analyses were effective for detecting circadianly

expressed genes. Four of the 19 genes (*Dbp*, *Per3*, *Bmal1*, and *Npas2*) are well-known

clock and clock-controlled genes. Significant rhythmicity in the expression of three other

genes (*Cdkn1a*, *Pstpip2*, and *Psmc6*) was also observed in KK-A^y mice but not in KK mice.

Therefore, these results confirm that the mRNA expression of various genes exhibits 24-h

rhythmicity, even in obese diabetic mice. As shown in **Fig**. **3**, the phases of the daily

expression rhythms of all 19 circadianly expressed genes detected in this study did not

seem to differ between the strains. However, obese diabetes significantly affected the

mRNA levels of 13 rhythmically expressed genes at one or more observation time (**Fig**. **3**A,

B and C). In particular, the peak levels of ten genes were significantly attenuated in KK-A^y

mice compared with those in KK mice (**Fig**. **3**A and B). The differences between the

strains were observed throughout the day in only two of the 13 genes (*Por* and *Depdc6*)

(**Fig**. **3**A). Therefore, obese diabetes dampened the rhythmic expression of various genes in

the mouse liver, but these effects could be detected only at particular observation times in

most of the genes.

Discussion

Previously, we showed that the rhythmic expression of the clock genes is attenuated

in the liver of obese diabetic KK-A[y] mice compared with control KK mice [14]. Given that

the molecular clock consisting of clock genes is thought to regulate most circadian gene

expression [1,2], obese diabetes should attenuate the rhythmic expression of most genes. In

this study, we found that the peak mRNA levels of more than half of the genes examined

were reduced in the livers of KK-A[y] mice, whereas the transcript levels of about one-third

of the genes examined were hardly affected throughout the day. Therefore, the influence of

the molecular clock on rhythmic gene expression appears to vary among genes.

In concordance with previous results [14], the peak transcript levels of the clock

genes (*Bmal1*, *Per3*, and *Dbp*) in KK-A[y] mice were significantly lower than those in KK

mice (**Fig**. **3**A and B). By contrast, the mRNA level of neuronal PAS domain protein 2

(NPAS2), another clock gene, did not differ between the strains. NPAS2 is similar to

CLOCK in amino acid sequence, and these transcription factors share BMAL1 as an

obligate heterodimeric partner and bind to the same DNA recognition element [21]. The

NPAS2-BMAL1 heterodimer, like CLOCK-BMAL1, is reported to play a role in

maintaining circadian behaviors [22]. However, it remains unclear how NPAS2-BMAL1

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affects the rhythmic gene expression in peripheral tissues. Moreover, whether obese diabetes affects the NPAS2-BMAL1 activity, as well as the CLOCK-BMAL1 action, remains to be determined.

Our results demonstrate that obese diabetes impairs the rhythmic expression of various genes, including *Usp2*, *Upp2*, *Por*, and *Sphk2*. Ubiquitin-specific protease 2, a preproteasomal isopeptidase, has been reported to stabilize fatty acid synthase [23]. Hepatic uridine phosphorylase inversely regulates the circulating uridine level, and its circadian rhythmicity might be involved in the humoral control of sleep by uridine [24]. Cytochrome P450 oxidoreductase transfers electrons to all microsomal P450 enzymes, and its deficiency can affect steroidogenesis and drug metabolism [25]. Sphingosine kinase is a key enzyme modulating the cellular levels of sphingolipids, which are involved in regulating multiple cellular processes, including cell growth, apoptosis, and proliferation [26]. Therefore, obese diabetes probably dampens the circadian rhythmicity of various physiological functions. Further studies are needed to clarify the pathophysiological roles of these effects in obese diabetes.

It has been suggested that approximately 10% of the genes expressed in the liver and about 8% of the genes expressed in the heart exhibit circadian regulation [9]. As most

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tissues, including the liver and heart, have an intracellular clock system [10,27], the

expression of many genes in most organs is expected to exhibit rhythmicity. Our results

strongly suggest that gene expression analysis based on observations at only one time of

day would tend to overlook the effect of obese diabetes if the gene were to show rhythmic

regulation. Therefore, the observed effects of obese diabetes, and possibly those of the

other conditions, on many genes in various tissues might vary depending on the observation

time. We suggest that circadian variation be considered in the analysis of *in vivo* gene

expression.

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18.

Figure Legends

Fig. **1**. Daily profiles of the serum glucose and insulin concentrations in KK (solid circles)

and KK-A^y mice (open circles). Data are the means ± SEM of three mice at each time

point. *, $P < 0.05$ vs. KK mice.

Fig. **2**. GeneTree clustering analysis of gene expression profiles in the livers of KK and

KK-A^y mice obtained at ZT 0 and 12 ($n = 3$ for each time point in both mice). The

transcripts with significantly different levels ($P < 0.05$, one-way ANOVA), *i.e.*, > 2-fold,

among the four groups were used for the clustering. The increased expression is shown in

red; decreased expression is shown in blue.

Fig. **3**. Daily mRNA expression profiles of the circadianly expressed genes in the livers of

KK (solid circles) and KK-A^y mice (open circles). Liver samples were obtained from both

mice at ZT 0, 6, 12, and 18. Transcript levels were determined using real-time quantitative

reverse transcription-PCR. Data are the means ± SEM of three mice at each time point and

are expressed as values relative to the highest values in KK mice for each gene.

*, $P < 0.05$; **, $P < 0.01$ vs. KK mice.

Table 1 Rhythmicity in the expression of selected genes

Gene Name	Gene Symbol	GenBank accession code for microarray	GenBank accession code for real-time PCR	KK F	KK P	KK-Ay F	KK-Ay P
D site albumin promoter binding protein	Dbp	BC018323/BB550183	BC018323/NM_016974	25.7	< 0.01	65.1	< 0.01
period homolog 3 (Drosophila)	Per3	NM_011067	NM_011067	33.7	< 0.01	39.1	< 0.01
ubiquitin specific protease 2	Usp2	AI553394	AI553394	79.5	< 0.01	46.4	< 0.01
uridine phosphorylase 2	Upp2	BC027189	BC027189/NM_029692	16.9	< 0.01	17.9	< 0.01
P450 (cytochrome) oxidoreductase	Por	NM_008898	NM_008898	45.4	< 0.01	42.0	< 0.01
neuregulin 4	Nrg4	NM_032002	NM_032002	20.1	< 0.01	36.3	< 0.01
aryl hydrocarbon receptor nuclear translocator-like	Arntl/Bmal1	BC011080	BC011080/NM_007489	23.5	< 0.01	193.5	< 0.01
neuronal PAS domain protein 2	Npas2	BG070037	NM_008719	27.9	< 0.01	82.0	< 0.01
(Mus musculus transcribed sequences)	-	BB205273	BB205273	20.5	< 0.01	5.9	< 0.05
solute carrier family 34 (sodium phosphate), member 2	Slc34a2	NM_011402	AK004832	12.7	< 0.01	35.6	< 0.01
protein phosphatase 1, regulatory (inhibitor) subunit 3C	Ppp1r3c	BQ176864	NM_016854	25.9	< 0.01	9.6	< 0.01
RIKEN cDNA 1110067D22 gene	1110067D22Rik	BC019131	BC019131/NM_173752	51.7	< 0.01	49.2	< 0.01
caseinolytic protease X (E.coli)	Clpx	BF020441	NM_011802	33.3	< 0.01	34.0	< 0.01
N-myc downstream regulated 1	Ndrg1	AV309418	NM_010884	17.8	< 0.01	19.4	< 0.01
tubulin, beta 2	Tubb2	BC003475/M28739	BC003475/NM_009450	21.7	< 0.01	21.7	< 0.01
nuclear factor, interleukin 3, regulated	Nfil3	AY061760	AY061760/NM_017373	9.3	< 0.01	6.2	< 0.05
cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	AK007630	AK007630/NM_007669	3.9	0.06	13.0	< 0.01
proline-serine-threonine phosphatase-interacting protein 2	Pstpip2	BC002123	BC002123/NM_013831	3.7	0.07	14.7	< 0.01
(Mus musculus transcribed sequences)	-	BB530740	BB530740	51.4	< 0.01	26.6	< 0.01
DEP domain containing 6	Depdc6	BC004774	BC004774/NM_145470	13.2	< 0.01	6.0	< 0.05
sphingosine kinase 2	Sphk2	AK016616	NM_203280	11.1	< 0.01	13.4	< 0.01
proteasome (prosome, macropain) 26S subunit, ATPase, 6	Psmc6	AW208944	NM_025959	1.8	0.23	13.8	< 0.01
tumor necrosis factor	Tnf	NM_013693	NM_013693	1.6	0.27	1.6	0.27

Using the microarray analysis, genes whose expression levels differed markedly (> 5-fold, $P < 0.05$) between ZT 0 and 12 in KK or KK-A^y mice were selected, and their rhythmic mRNA expression was verified using the real-time PCR analysis. The rhythmicity of each gene was tested using one-way ANOVA.
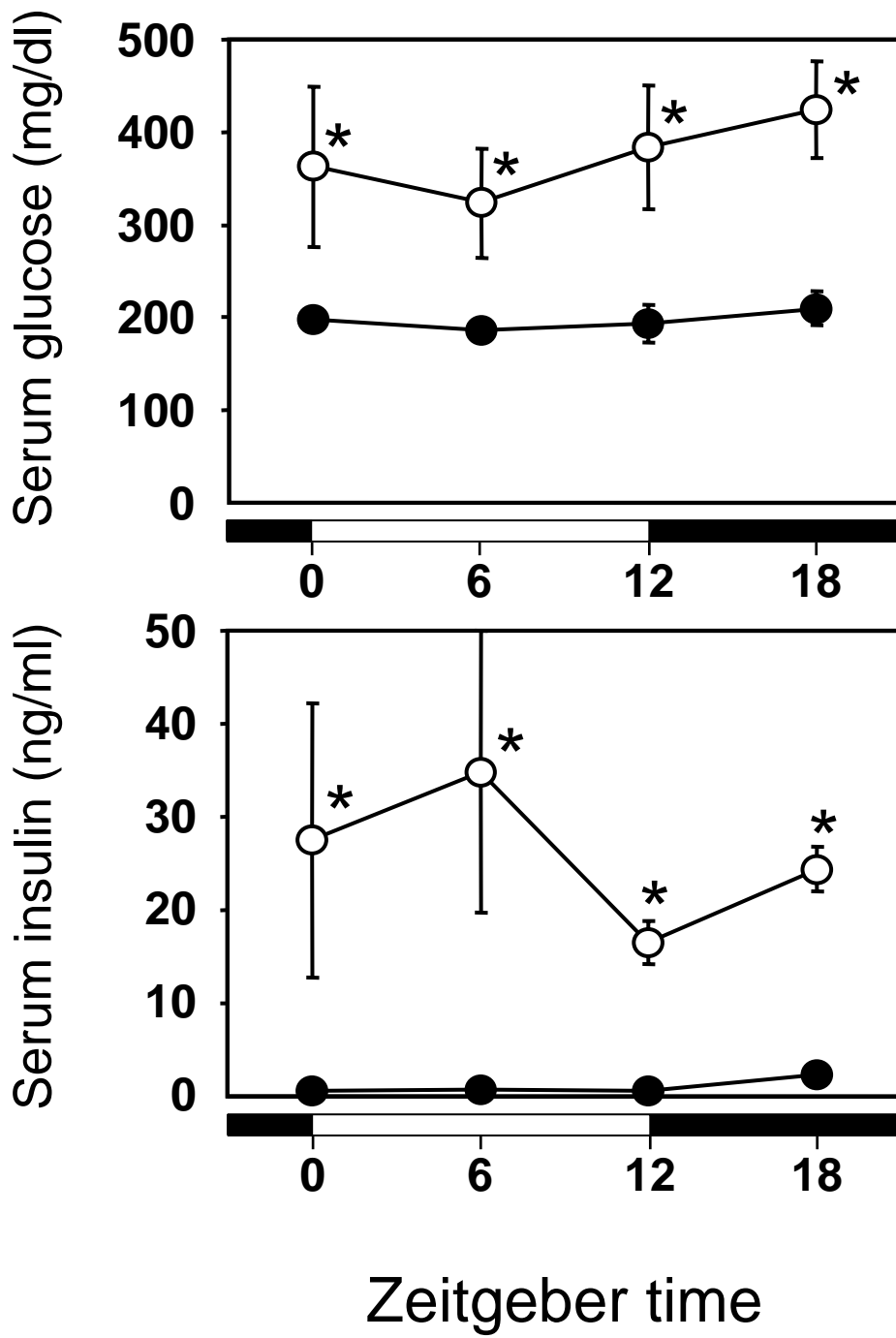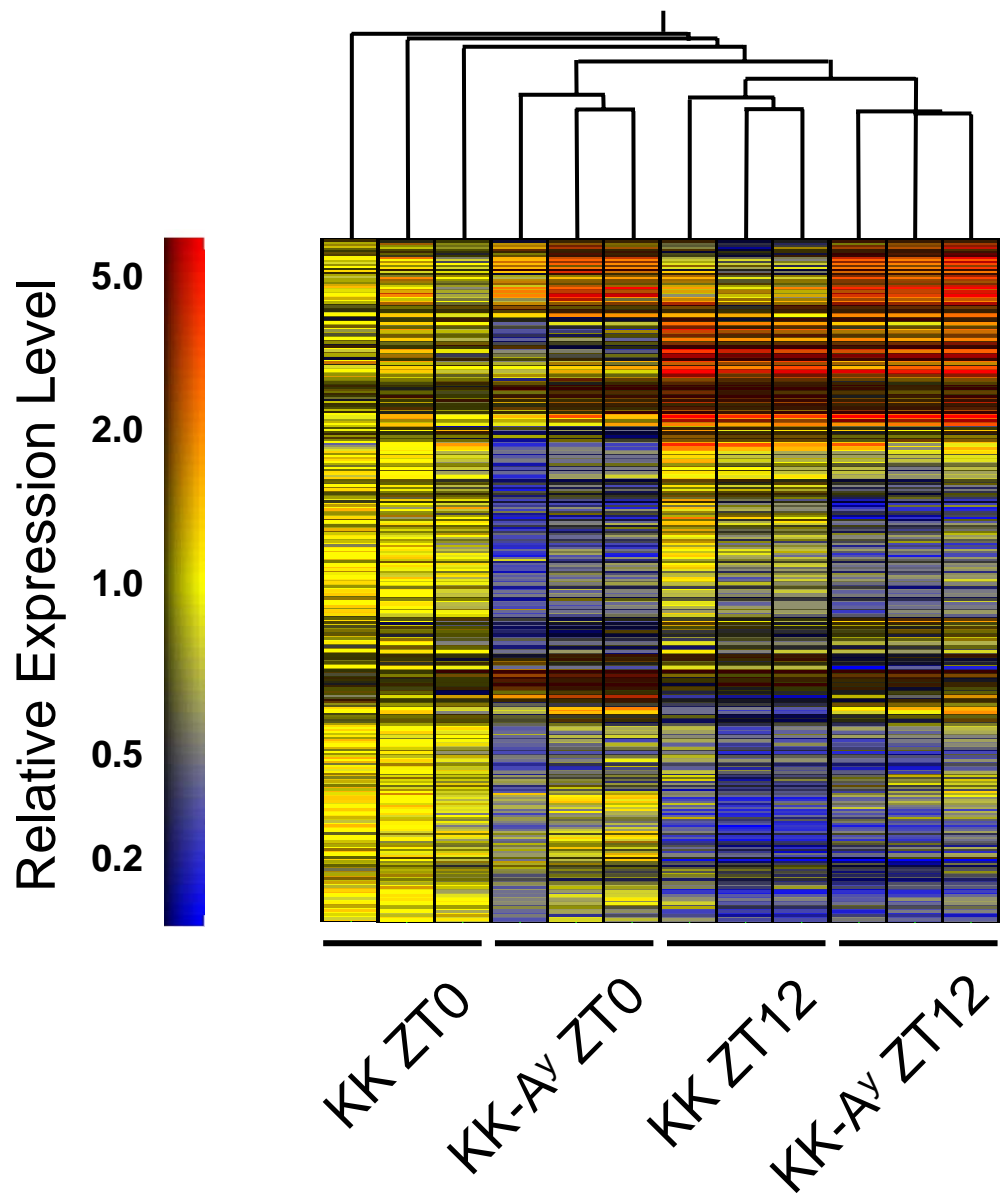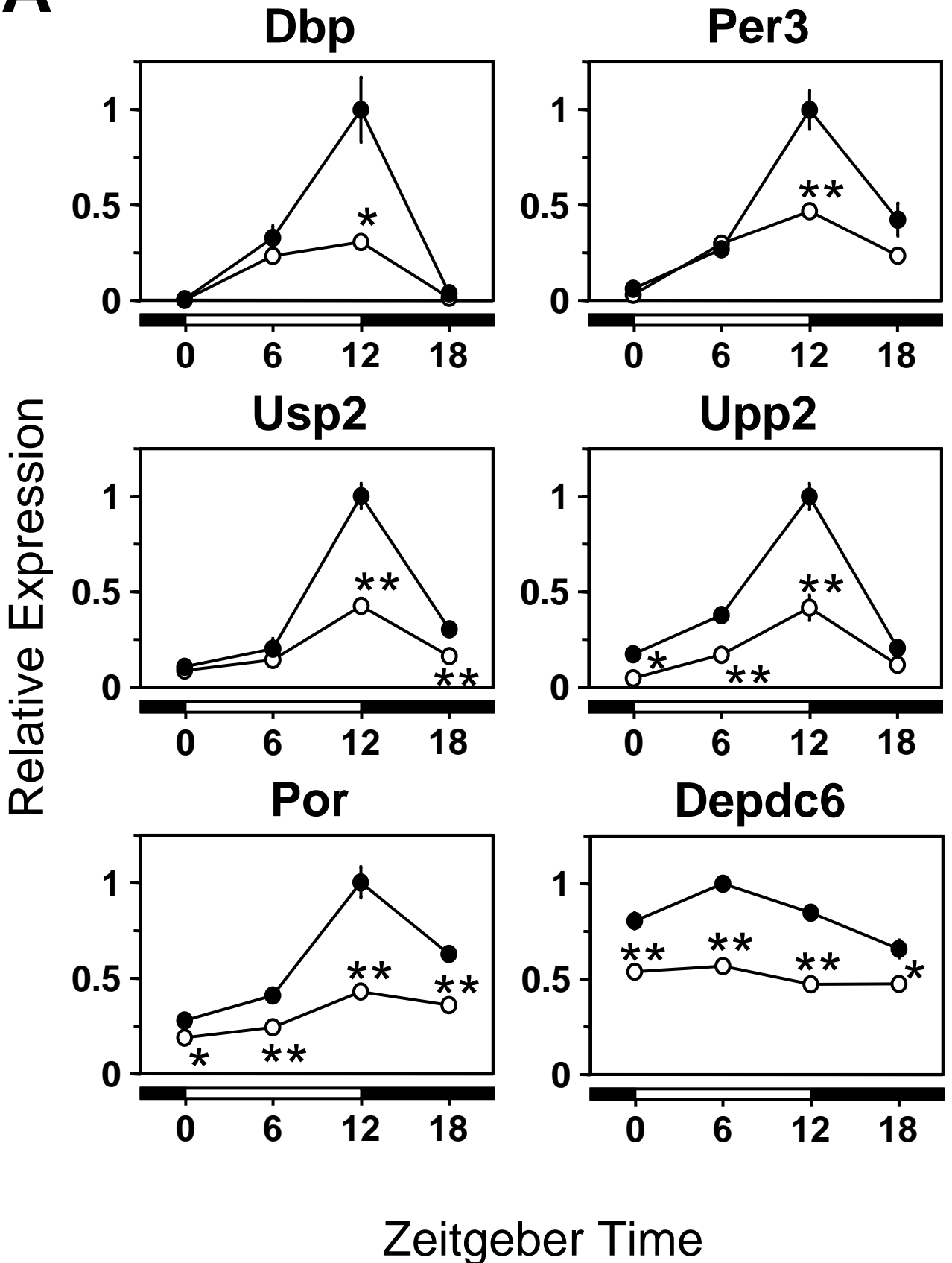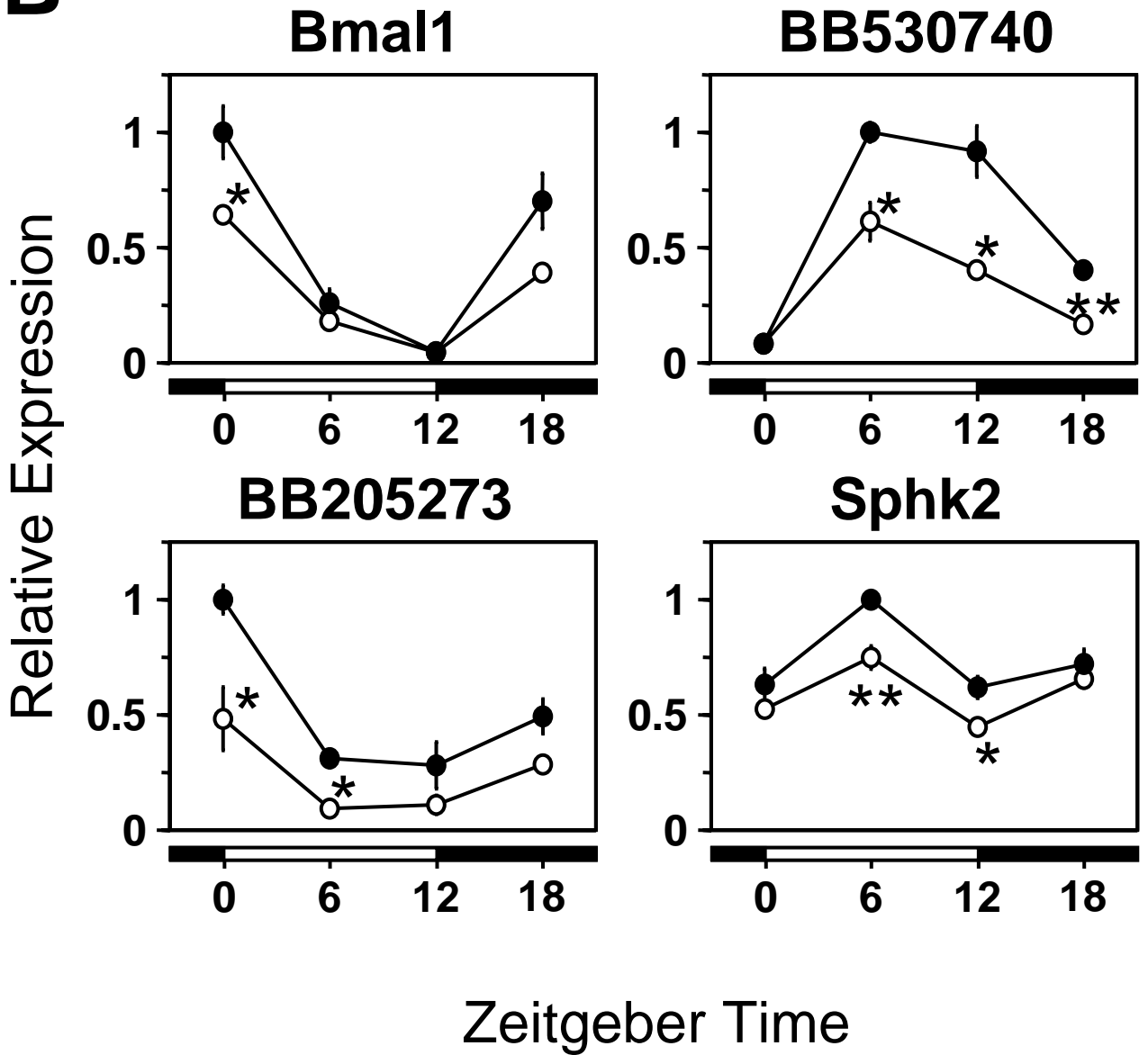
Fig. 1

Fig. 2

A

Dbp

Per3

Usp2

Upp2

Por

Depdc6

Relative Expression

Zeitgeber Time

Fig. 3A

B

Bmal1

BB530740

BB205273

Sphk2

Relative Expression

Zeitgeber Time

Fig. 3B

C

Clpx

Ndrg1

Tubb2

Relative Expression

Zeitgeber Time

Fig. 3C

D

Nrg4

Npas2

Nfil3

Slc34a2

Ppp1r3c

NM_173752

Relative Expression

Zeitgeber Time

Fig. 3D