Supplementary Information

Real-time monitoring in three-dimensional hepatocytes reveals that insulin acts as a synchronizer for liver clock

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Supplementary Figure 1.

Insulin induces circadian rhythms of gene expression in H4IIE cells.

Temporal mRNA expression levels of *Per2* and *Dbp* were determined by northern blotting following a shift of H4IIE cells to medium containing 50 nM insulin for 1 h at T 0, washing with PBS, and incubation in serum-free medium.

Supplementary Figure 2.

Hepatocyte differentiation phenotypic function is related to the circadian gene expression of hepatocytes.

Differentially expressed genes related to liver differentiation (a) and circadian rythmicity (b) by using Affymetrix microarray analysis. Hepatocytes were cultured for 48 h on a TIC- and an EHS-gel-coated dish. cDNA microarray were performed to determine the transcripts abundance. Black and white bars indicate these gene expressions were changed significantly and not significantly, respectively.

Supplementary Figure 3.

Cell matrix contact is important for the maintenance of hepatic circadian gene expression.

Hepatocytes were plated on TIC- and EHS gel-coated dishes at a high density. Northern blotting was used to determine the *Dbp* mRNA level. Hepatocytes were collected at 4-h intervals. The open circles and filled circles represent TIC and EHS gel, respectively. The open and solid bars indicate light and dark conditions where the animals were kept before the preparation of hepatocytes.

Supplementary Figure 4.

The effects of extracelluar matrix and cell shape on circadian gene expression in rat primary hepatoxytes.

(a) Hepatocytes were cultured for 48 h (1400) and 56 h (2200) on dishes coated with TIC, TIVC, laminin, and EHS-gel at a high density. Northern blotting was used to determine the *Dbp*, *Per1*, *Per2*, and *Apo E* mRNA levels. (b) Hepatocytes were cultured for 48 h (1400) and 56 h (2200) on dished coated with TIC, PVLA, and EHS-gel at a high density. Northern blotting was used to determine the *Dbp*, *Per1*, and *Apo E* mRNA levels.

Supplementary Figure 5.

Hepatocytes do not need to communicate directly to generate autonomous circadian oscillations.

Hepatocytes were cultured on an EHS gel-coated dish at high and low densities. Northern blotting was used to determine the *Dbp* mRNA level in rat primary hepatocytes cultured on EHS gel at high and low densities.

Supplementary Figure 6.

Hepatic clock is sustained when the 3D structure of the cell is maintained.

Hepatocytes were cultured on dishes coated with TIC, PVLA, and EHS gel at a high density. Northern blotting was used to determine the *Dbp* mRNA level.

Supplementary Figure 7.

The effect of cytoskeleton on circadian gene expression in rat primary hepatoxytes.

Hepatocytes were cultured on TIC and an EHS-gel-coated dish for 45h, and cells were treated with colchicine (Col), paclitaxel (Pac), vinblastine sulfate (Vin), nocodazole (Noc), cytochalasin B (CB) and 3,3′-iminodipropionitrile (IDPN) for a further 12h. The *Dbp* mRNA level was shown as percentage relative to the value of hepatocytes cultured on EHS-gel at CT 14.

Supplementary Figure 8.

Effects of kinase inhibitors on insulin induced resetting of the rhythm in rat primary hepatocytes.

Hepatocytes derived from Per2-dLuc transgenic rats were isolated and plated on an EHS-gel coated dish in serum- and hormone-free medium. At the indicated circadian time, hepatocytes were treated for 2 h with 50 nM insulin or with vehicle or insulin + kinase inhibitor and bioluminescence levels measured.

Supplementary Figure 9.

Circadian profiles of serum glucose, total cholesterol, triglyceride, and phospholipids.

Values and bars represent means ± SEMs of 3-4 control (open circles) or STZ (filled circles) rats. Open and solid horizontal bars indicate light and dark periods, respectively.

Supplementary Figure 10.

A single insulin administration induces a phase shift in circadian gene expression in the STZ rat liver.

The phase response curve of circadian liver gene expression was obtained by recording

the circadian *Per1* mRNA after insulin injections into STZ rats at the indicated times.

Supplementary Figure 11.

STZ rats exposed to high-fat, high-cholesterol diets deteriorates hypercholesterolemia and hyperlipidemia.

Plasma levels of cholesterol (a) and triglyceride (b) in control rats fed a basal diet (○) , control rats fed a high-fat, high-cholesterol diet (●), diabetic rats fed a basal diet (□) and diabetic rats fed a high-fat, high-cholesterol diet (▲). Values are means ± SEMs of 3-7 rats at each time period.

Supplementary table 1

Category	P value	Gene number	Upregulated genes	Downregulated genes
Biofunctions[1]				
Amino Acid Metabolism	6.61E-13	42	38	4
Small Molecule Biochemistry	6.61E-13	190	157	33
Lipid Metabolism	1.74E-12	143	117	26
Vitamin and Mineral Metabolism	5.11E-12	56	47	9
Molecular Transport	1.51E-10	115	87	28
Carbohydrate Metabolism	9.91E-07	89	66	23
Cellular Movement	5.70E-06	117	52	65
Drug Metabolism	1.28E-05	28	26	2
Cellular Growth and Proliferation	3.05E-05	87	41	46
Cell-To-Cell Signaling and Interaction	8.51E-05	91	45	46
Cell Morphology	9.47E-05	67	22	45
Cell Signaling	9.47E-05	12	6	6
Cellular Development	9.47E-05	64	24	40
Protein Synthesis	2.34E-04	6	5	1
Protein Trafficking	2.66E-04	6	6	0
Antigen Presentation	4.52E-04	24	14	10
Cellular Assembly and Organization	6.83E-04	34	6	28
Cellular Function and Maintenance	6.83E-04	22	5	17
Energy Production	6.83E-04	12	9	4
Post-Translational Modification	1.69E-03	4	4	0
Cellular Compromise	3.14E-03	4	1	3
Nucleic Acid Metabolism	3.38E-03	17	17	0
Cell Death	5.26E-03	25	14	11
Cell Cycle	5.96E-03	11	5	6
Gene Expression	7.77E-03	4	4	0
KEGG pathways[2]				
Complement and coagulation cascades	3.15E-09	19	16	3
Retinol metabolism	1.06E-08	17	16	1
Metabolism of xenobiotics by cytochrome P450	6.88E-07	18	18	0
Drug metabolism	1.30E-06	19	19	0
PPAR signaling pathway	5.89E-06	15	15	0
Tryptophan metabolism	1.75E-04	10	10	0
Primary bile acid biosynthesis	5.28E-04	6	6	0
Nitrogen metabolism	6.28E-04	7	5	2
Glycine, serine and threonine metabolism	7.04E-04	8	8	0
Steroid hormone biosynthesis	9.29E-04	9	9	0
Fatty acid metabolism	3.72E-03	8	8	0
Tyrosine metabolism	5.34E-03	7	7	0
Prion diseases	6.19E-03	7	4	3
Arginine and proline metabolism	1.35E-02	8	7	1
Sulfur metabolism	1.41E-02	4	4	0
Drug metabolism	1.87E-02	7	7	0
ABC transporters	1.87E-02	7	6	1
Linoleic acid metabolism	2.04E-02	6	6	0
Cysteine and methionine metabolism	2.58E-02	6	5	1

Biosynthesis of unsaturated fatty acids	2.70E-02	5	4	1
Maturity onset diabetes of the young	3.52E-02	5	4	1

We compared EHS coating effect on gene expression with TIC coating culture condition in primary rat hepatocytes by using Affymetrix® microarray genechip Rat genome 230 2.0 .

1. Differentially expressed gene list from microarray experiment were imported in Ingenuity pathway analysis®, filtered by selecting only genes expressed in rat liver. Significance expressed as P-values were calculated using the right-tailed Fisher's exact test. Biofunctions significantly changed were obtained.
2. We conducted KEGG pathways analysis by importing differentially expressed gene list in DAVID (database for annotation, visualization, and integrated discovery). Significance expressed as P-values were calculated using modified Fisher exact probability (EASE score).

Yamajuku_ Supplementary table2

Estimation of peak times		
	ZT	
Genes	Control	STZ
Per1 *	14.00	12.83
Per2 *	17.67	15.50
Per3 *	13.83	12.17
DBP *	12.33	10.67
CYP7A1 *	14.00	12.33
Rev-erbAα (NR1D1) *	9.83	7.67
Rev-erbAβ (NR1D2) *	12.17	10.17
RORα (NR1F1)	arrhythmic	
RORγ (NR1F3)	18.83	17.33
Cry1 *	21.17	18.67
Dec1 (BHLHB2) *	18.33	15.00
Dec2 (BHLHB3) *	11.83	10.67
Clock *	4.17	2.50
Bmal1 (ARNTL or MOP3) *	1.83	1.00
Npas2 *	4.00	2.67

To assess the difference of circadian rhythm (phase) between the control and diabetic rat, we analyzed significance of the interaction between diabetic effect and circadian time effect by two-way ANOVA. Values with asterisk indicate a statistically significant difference ($P<0.05$).

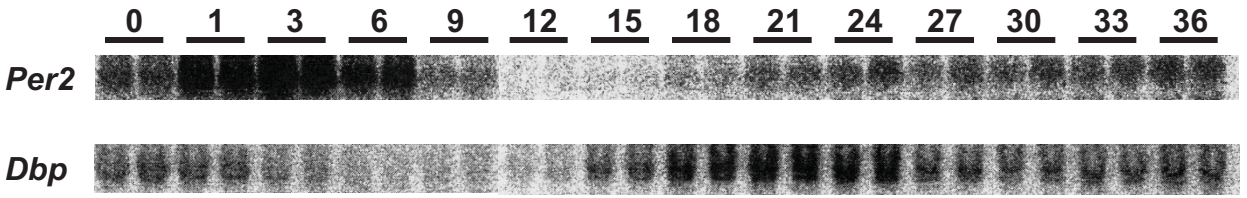
Composition of experimental diets

	(g/100g diet)
Casein	20
Vitamin mixture 1)	1
Mineral mixture 2)	3.5
Choline chloride	0.2
Corn oil	5
Cellulose	5
Starch	43.5
Sucrose	21.8

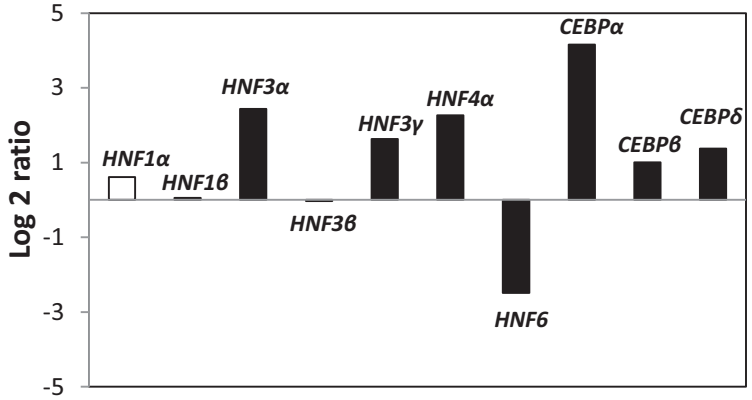
1) Vitamin mixture：AIN-93TM
2) Mineral mixture：AIN-93TM

Yamajuku_ Supplementary table4

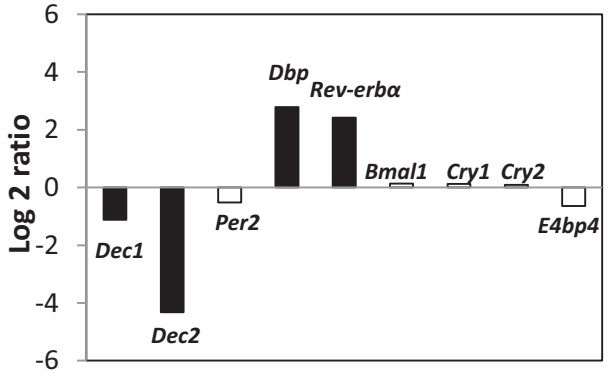
Gene	Forward primer	Reverse primer
rPer1	ACCAGCTCAAGGCTTAGGAGCT	TGGGATTTGGAGAGACCACTTC
rPer2	CAACCTTTGTCTGCCATATGAGG	CGTTAGAAACACAAGCTCTTCCAC
rPer3	CAGAAGGATTCAAGCCCGTG	TACTTGGCATGGTTCCTGCC
rDbp	CTCTAGGGACACACCCAGTCCT	AGGCTTCAATTCCTCCTCTGAGA
rCyp7a1	TGTGTGAGGGACCAGGTCTCT	AGCTCCAAAAGGTTGCAGGA
rRev-erbAα	GTCATTGTTCAACGTGAAGGACC	CTCCTCAGTAAGCGCCAGAGAG
rRev-erbAβ	CCAATGCATAGTTTTCTTCTATGGG	ACATATTTCCAAAGACGCAAACTG
rRORα	AAGAACTGAGAATGGAGCTGGC	CCGACTTGCTTCTTGCAACATA
rRORγ	GGAGACAAGAGTAATAGGATGCTG	ACAGGCTTGCAAAAACACTTTG
rCry1	GCTTCCCTGCAAAATATATCTACGA	GCTGCTGATAGATCTGCTTCATTCT
rDec1	GGGAAAAACTGTGTGCCAGTC	CGTGATCGCTCTTGAAGTAGGG
rDec2	AACCCCTTTGTCCCATGTCTC	GCCCAGAGCACTTTAACACCC
rClock	CCTATCCTACCTTTGCCACACAA	TCCTGGGAACTCTGCTGTGACT
rBmal1	GCCATGGCCACTGTAGACACTA	CAATGGCTCTGAGATGGCTTTTAT
rNpas2	ATGCAGCCACACAGCCTACAC	CCCCGTTTTCCATTGGTTAAA
rβactin	GGTCGTACCACTGGCATTGTG	GCTCGGTCAGGATCTTCATGAG
rGAPDH	GATACTGAGAGCAAGAGAGAGGCC	GATGGTATTCGAGAGAAGGGAGG

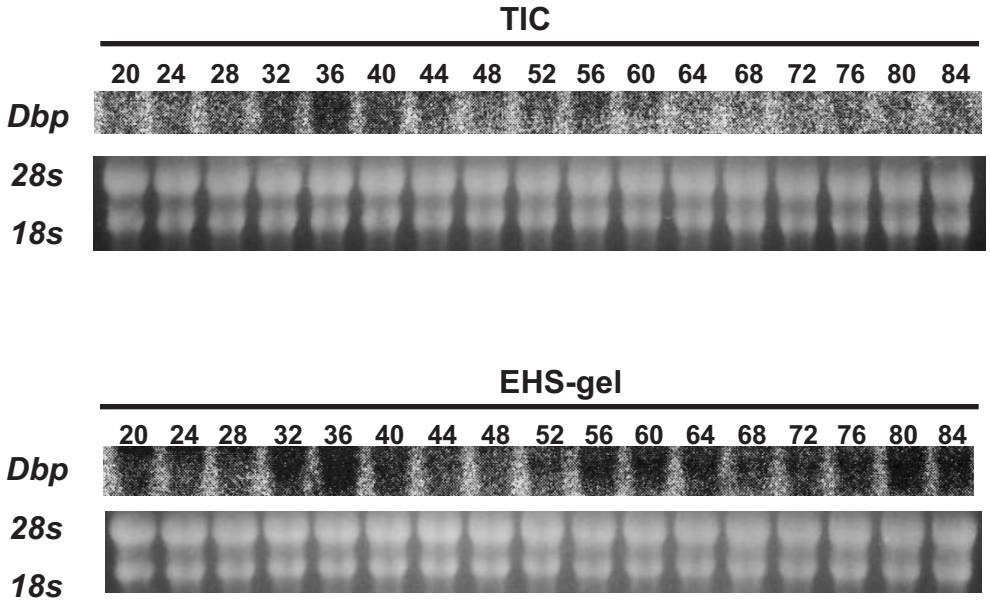

0
1
3
6
9
12
15
18
21
24
27
30
33
36
Per2
Dbp

a

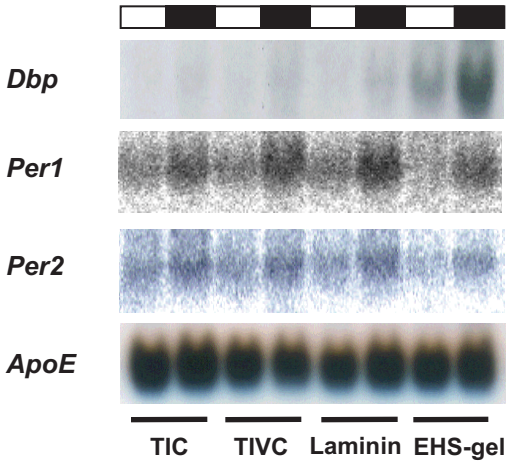

Log 2 ratio
5
3
1
-1
-3
-5
HNF1α
HNF1β
HNF3α
HNF3β
HNF3γ
HNF4α
HNF6
CEBPα
CEBPβ
CEBPδ

b

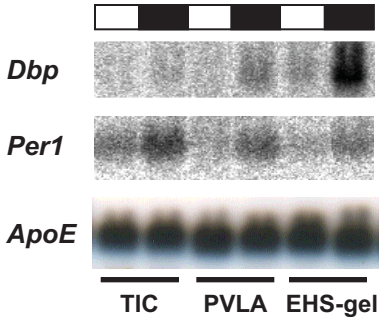

Log 2 ratio
6
4
2
0
-2
-4
-6
Dec1
Dec2
Per2
Dbp
Rev-erbα
Bmal1
Cry1
Cry2
E4bp4

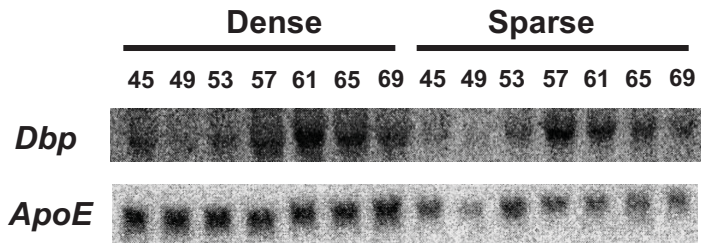

TIC
20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80 84
Dbp
28s
18s
EHS-gel
20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80 84
Dbp
28s
18s

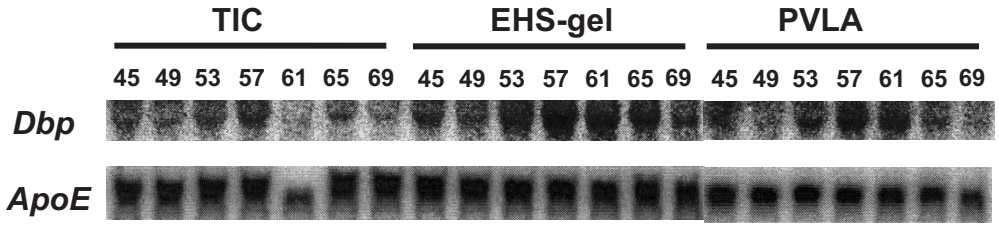
a

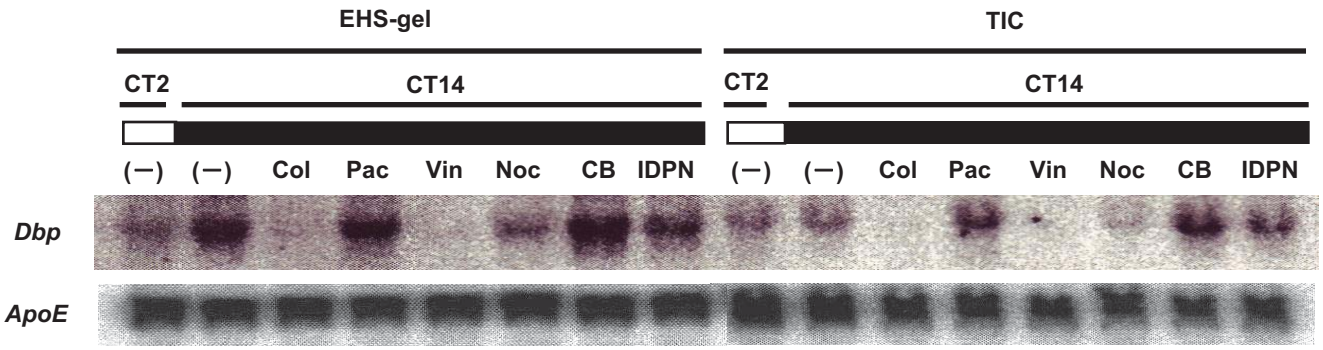

Dbp
Per1
Per2
ApoE
TIC
TIVC
Laminin
EHS-gel

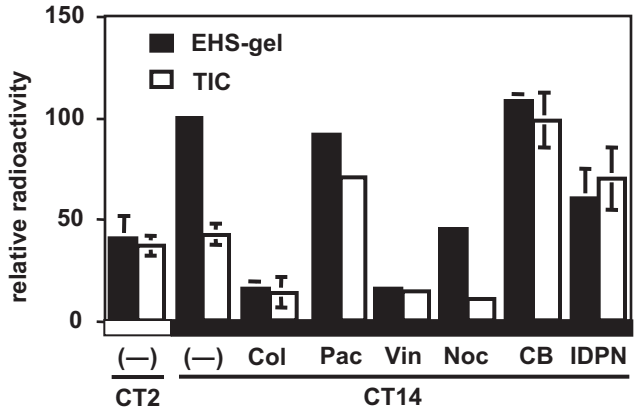
b

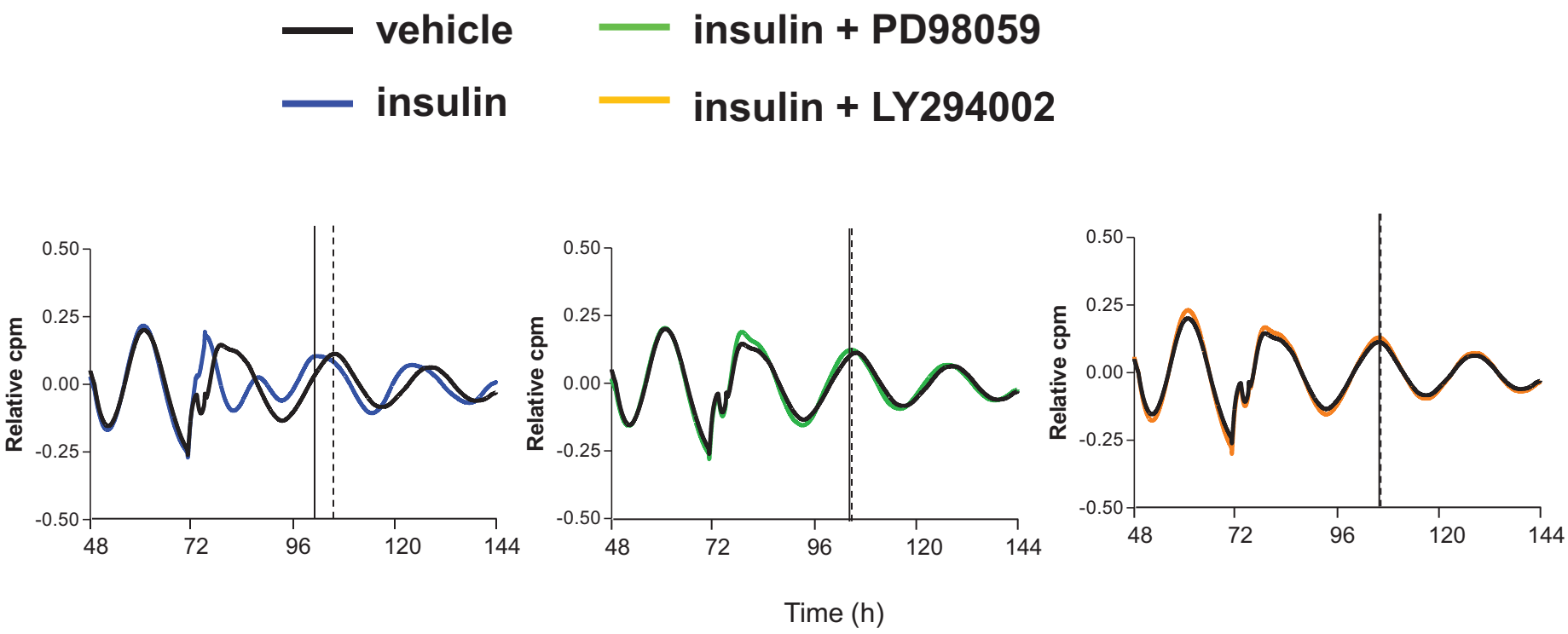

Dbp
Per1
ApoE
TIC
PVLA
EHS-gel

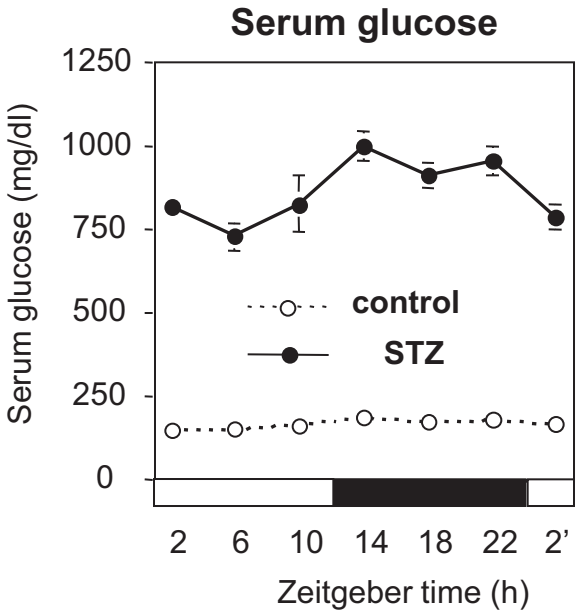

Dense
Sparse
45 49 53 57 61 65 69 45 49 53 57 61 65 69
Dbp
ApoE

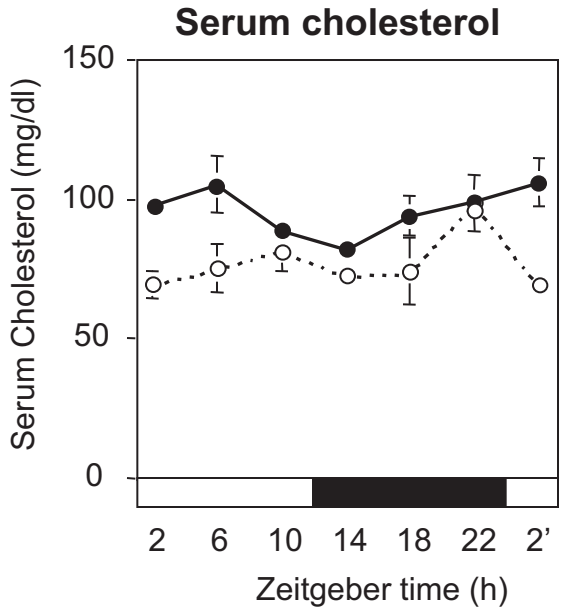

TIC
EHS-gel
PVLA
45 49 53 57 61 65 69 45 49 53 57 61 65 69 45 49 53 57 61 65 69
Dbp
ApoE

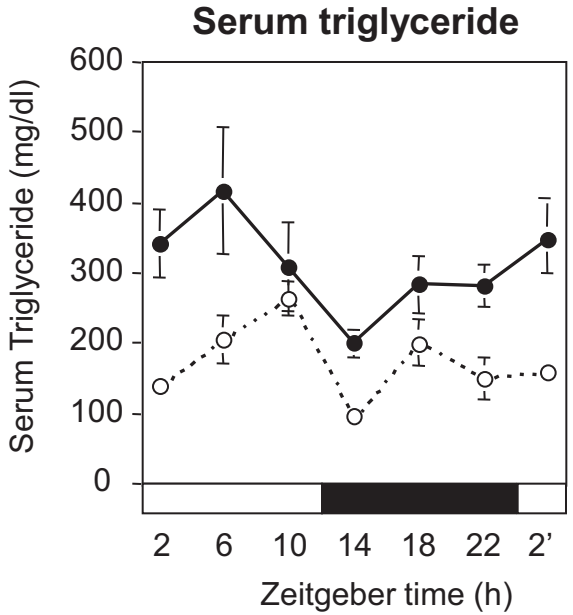

EHS-gel
TIC
CT2
CT14
CT2
CT14
(−) (−) Col Pac Vin Noc CB IDPN
(−) (−) Col Pac Vin Noc CB IDPN
Dbp
ApoE

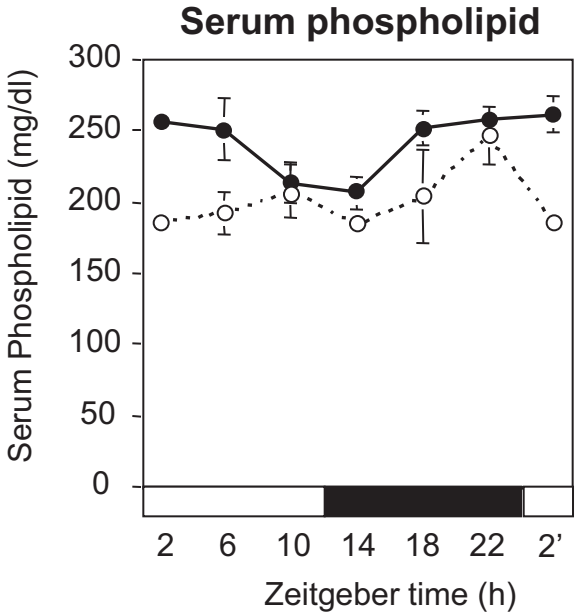

150
100
50
0
relative radioactivity
EHS-gel
TIC
(—) (—) Col Pac Vin Noc CB IDPN
CT2
CT14

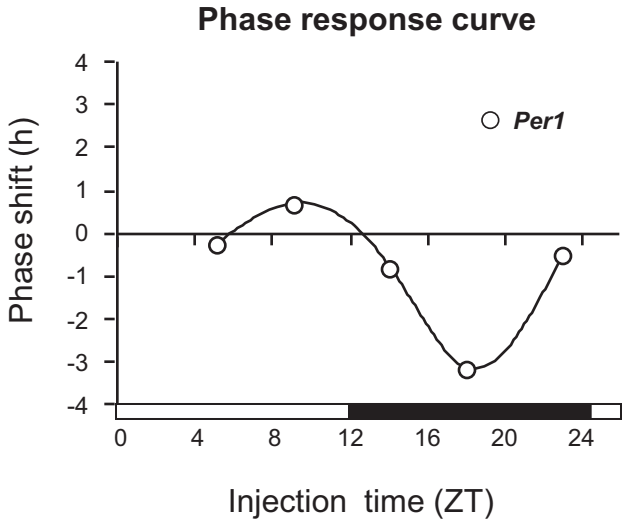

vehicle
insulin
insulin + PD98059
insulin + LY294002
Relative cpm
0.50
0.25
0.00
-0.25
-0.50
48
72
96
120
144
Time (h)


Serum glucose
Serum glucose (mg/dl)
1250
1000
750
500
250
0
control
STZ
2
6
10
14
18
22
2'
Zeitgeber time (h)

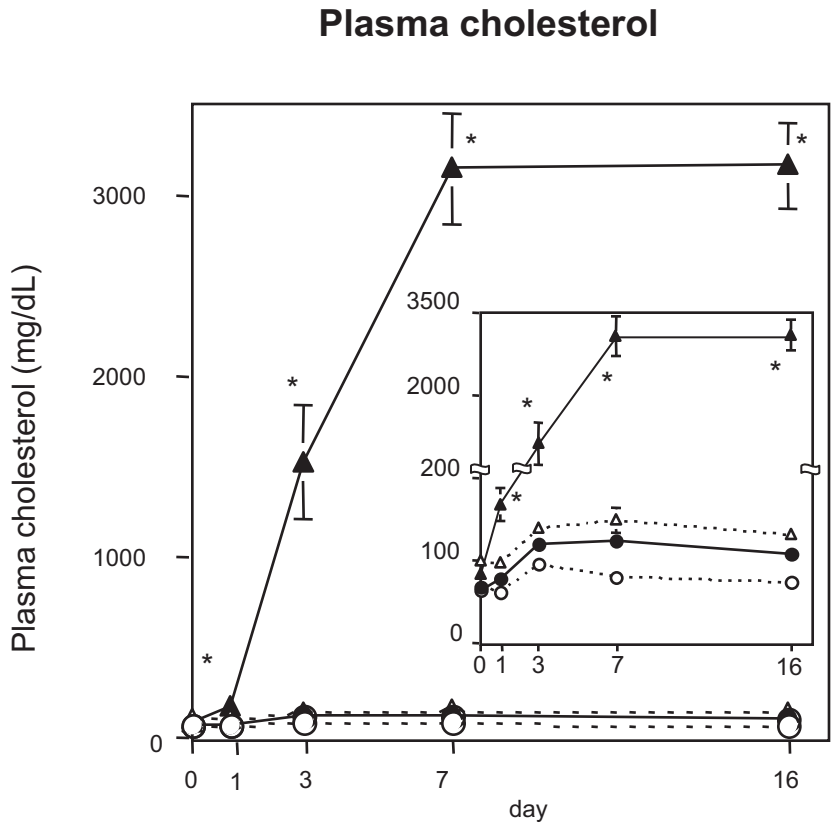

Serum cholesterol
Serum Cholesterol (mg/dl)
150
100
50
0
2
6
10
14
18
22
2'
Zeitgeber time (h)


Serum triglyceride
Serum Triglyceride (mg/dl)
600
500
400
300
200
100
0
2
6
10
14
18
22
2'
Zeitgeber time (h)

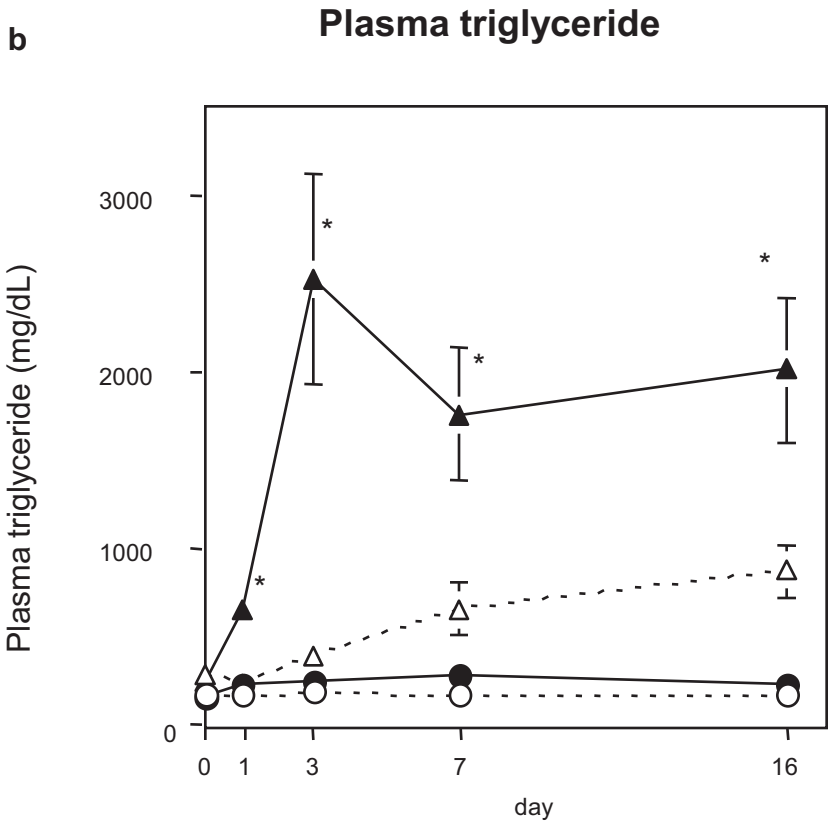

Serum phospholipid
Serum Phospholipid (mg/dl)
300
250
200
150
100
50
0
2
6
10
14
18
22
2'
Zeitgeber time (h)


Phase response curve
Per1
Phase shift (h)
4
3
2
1
0
-1
-2
-3
-4
0
4
8
12
16
20
24
Injection time (ZT)

a


Plasma cholesterol
Plasma cholesterol (mg/dL)
3000
2000
1000
0
3500
2000
200
100
0
0 1 3 7 16
day
*

b


Plasma triglyceride
Plasma triglyceride (mg/dL)
3000
2000
1000
0
0 1 3 7 16
day
*