Original Article

Circadian modulation of hepatic transcriptome in transgenic rats expressing human growth hormone

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ABSTRACT — The secretory profile of growth hormone (GH) is sexually dimorphic in rats. In male transgenic (TG) rats expressing human GH (hGH) that we generated, the circulating levels of both hGH and endogenous GH are flattened with no male-type pulsatility. To elucidate the regulatory role of episodic GH profile on the liver, the hepatic transcriptome of male TG rats at the middle of the light and dark phases was characterized by genome-wide analyses as compared with that of male wild-type (WT) rats. Transcripts commonly up- or down-regulated regardless of the lighting conditions in TG rats were mainly enriched in the metabolism of xenobiotics. In TG rats, the gene expression profile was functionally feminized, verifying that the sexually dimorphic profile of GH rather than genetic sexuality is a stronger sex-determining factor on the hepatic transcriptome. The common transcripts which fluctuated during the day in both TG and WT rats were enriched in circadian rhythm signaling, and physiological rhythmicity was considered to be finely interconnected with liver metabolism via sexually dimorphic GH secretion. In contrast, some genes were differentially regulated in TG rats at only one of two time points measured, and others were fluctuated daily in only one genotype. In particular, some genes involved in the GH signaling pathway were included, suggesting the signal transduction is circadian-modulated depending upon the GH profile. Our transcriptome analyses clarified the regulatory role of episodic GH profile on the liver and strengthen the functional link between sexually dimorphic GH secretion, liver metabolism, and its circadian regulation.

Key words: Transcriptome, Growth hormone, Liver, Transgenic, Sex, Circadian

INTRODUCTION

Growth hormone (GH) is secreted from the anterior pituitary gland in a sex-dependent manner, particularly in rodents but also in humans (Eden, 1979; Lichanska and Waters, 2008; Shapiro *et al.*, 1995; Tannenbaum and Martin, 1976). In adult male rats, GH secretion is characterized by intermittent bursts of high amplitude spaced approximately 3.5 hr apart, whereas GH secretion in adult female rats is characterized by irregular and more frequent GH releases of low amplitude with a higher GH

nadir. The regulatory role of episodic GH profile is not fully characterized.

The liver is one of the primary target organs for GH. The role of GH on hepatic transcriptome regulation has been previously investigated in male rats treated with GH by a 7-day continuous infusion (Ahluwalia *et al.*, 2004), but the slower feminization of male hepatic gene expression following a continuous GH infusion via epigenetic regulation has been reported in mice (Holloway *et al.*, 2006; Waxman and Holloway, 2009). These experiments indicate the impact of subacutely infused GH on hepat-

ic function is inherently distinct from that of permanently secreted GH as a transgene product. Additionally, the role of GH on hepatic transcriptome regulation has been investigated in hypophysectomized male rats treated with human GH (hGH) by a 3-week continuous infusion (Flores-Morales *et al.*, 2001). However, hypophysectomy induces deficiencies of multiple hormones including GH but also other pituitary hormones, e.g. prolactin, adrenocorticotrophic hormone, thyroid stimulating hormone, follicle stimulating hormone, and luteinizing hormone; and therefore the resulting hormonal homeostasis is essentially unique to this animal model.

Transgenic (TG) rats carrying a chimeric gene comprising a regulatory portion of murine whey acidic protein and a structural portion of hGH genes have been previously generated in our laboratory (Furuhata *et al.*, 2002; Ikeda *et al.*, 1994, 1998). Moreover, we have shown that the circulating levels of both hGH and endogenous GH are continuously low and flattened with no male-type pulsatility in male TG rats which constitutively express hGH gene (Ikeda *et al.*, 1994). The low GH levels devoid of pulsatile secretion in the peripheral circulation are sufficient to support normal body growth, although TG rats develop insulin resistance and a type of obesity that is responsive to simulation of pulsatile GH secretion (Ikeda *et al.*, 1998). Male TG rats, therefore, are considered to be a useful animal model for elucidating the feminizing effects of masculine GH profile on the liver.

We have previously reported that the daily profile of hepatic gene expression in rats is strongly influenced by the circadian components related to feeding cycle (Hirao *et al.*, 2006). Ultradian GH profile is associated with the daily rhythm of hepatic P450 monooxygenase activities (Furukawa *et al.*, 1999), and the circadian clock components, *Cryptochrome* genes, have been reported to be necessary to sustain sex dimorphism in mouse liver metabolism via the GH profile (Bur *et al.*, 2009). On the basis of the evidence, it is essential to take the circadian effects into consideration to fully characterize the regulatory role of episodic GH on the liver.

To clarify the circadian modulation of the regulatory mechanism of GH in the liver, a genome-wide analysis of liver genes expression at 2 time points of the day-night cycle was performed in male TG rats as compared to male wild-type (WT) rats. The integration of the differentially expressed genes into known biological pathways is a versatile tool for understanding the biological complexity of gene expression (Curtis *et al.*, 2005; Jimenez-Marin *et al.*, 2009; Werner, 2008). Therefore, biological pathway analyses were also performed using commonly or differentially regulated gene lists for comparison.

MATERIALS AND METHODS

Experimental animals and sample preparation

TG rats expressing low levels of hGH under the control of murine whey acidic protein promoter were generated as previously described (Ikeda *et al.*, 1994). The resulting male TG (heterozygotes) and WT littermates were used in the present study. The animals were acclimatized in an animal room controlled at a room temperature of $23 \pm 1^\circ\text{C}$ with 12 hr light/dark cycles (ZT, *zeitgeber* time; ZT0, lights on; ZT12, lights off). All the animals were allowed free access to water and laboratory chow. TG and WT rats at 16 weeks of age, each weighing 410 to 610 g, were decapitated at ZT6 and ZT18 (the middle of the light and dark phases). The left lateral lobes of the liver were dissected from each animal, and the obtained liver samples were immediately frozen in liquid nitrogen and stored at -80°C until further use. All experiments were conducted in accordance with the Guideline for the Care and Use of Laboratory Animals, the University of Tokyo and the Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

Microarray analyses

The total RNA was isolated from a thawed liver sample ($n = 4$ for each genotype) using an RNeasy mini kit (QIAGEN, Valencia, CA, USA). The RNA samples were individually prepared from each rat and the cDNA was synthesized using a One-Cycle cDNA Synthesis Kit (Affymetrix Inc., Santa Clara, CA, USA). The biotin-labeled cRNA was transcribed using GeneChip Expression 3'-Amplification Reagents for IVT Labeling (Affymetrix Inc.). The labeled cRNA was hybridized to GeneChip Rat Genome 230 2.0 Array (Affymetrix Inc.) at 45°C for 16 hr. The array was washed and stained using a Fluidics Station (Affymetrix Inc.) and scanned with a GeneArray Scanner (Hewlett-Packard, Palo Alto, CA, USA). The microarray imaging data was analyzed using Microarray Suite ver. 5.0 (Affymetrix Inc.), followed by Spotfire (Spotfire, Somerville, MA, USA). To investigate the regulatory role of sexually dimorphic GH secretion on hepatic gene expression, the gene expression in TG rats at ZT6 or ZT18 was firstly compared with the time-matched gene expression in WT rats. The threshold criteria were set as follows: average expression ratio ≥ 1.5 (or ≤ 0.66) and a statistical significance of $P < 0.05$. And then, the gene expression at ZT18 in each genotype was compared with those at ZT6 to identify the daily fluctuated transcripts under the above threshold criteria.

Biological pathways analyses

Differentially regulated and overlapped gene lists in the above comparisons were used for the biological pathways analyses. The canonical pathways analyses were performed using Ingenuity Pathway Analysis (IPA) software ver. 8.0 (Ingenuity Systems, Inc., Redwood City, CA, USA). The significance of the association between the data set and the canonical pathway was determined by two parameters: (1) the ratio of the number of genes from the data set that map the pathway divided by the total number of genes that map the canonical pathway and (2) a Fisher's exact test *P*-value (threshold value: 0.05) determining the probability that the association is not due to chance alone. Regarding the threshold value, the $-\log(P\text{-value})$ is equal to 1.30 on the condition that the cutoff of the *P*-value is 0.05, and the $-\log(P\text{-value})$ increases with statistical significance. Unless stated otherwise, official gene symbols were used to represent molecules related to biological pathways.

Quantitative real-time PCR (RT-PCR)

To verify the microarray data with a central focus on circadian rhythm signaling, TaqMan RT-PCR (Gibson *et al.*, 1996; Heid *et al.*, 1996) was conducted for four representative clock or clock-controlled genes which were diurnally expressed, i.e. P450 oxidoreductase (*Por*), Period homolog 2 (*Per2*), cytochrome P450, family 7, subfamily a, polypeptide 1 (*Cyp7a1*), and basic helix-loop-helix domain containing, class B2 (*Bhlhb2*). The total RNA was treated with DNase I (Takara, Shiga, Japan) in the manufacturer's buffer to which an RNase inhibitor (Toyobo, Osaka, Japan) was added for 20 min at 37°C. After the phenol-chloroform purification, the DNA-free total RNA was reverse-transcribed into first-strand cDNA using Superscript II (Invitrogen, Carlsbad, CA, USA) in the manufacturer's buffer supplemented with 10 mM dithiothreitol, 0.5 mM deoxyribonucleoside triphosphates, and 40 U RNase inhibitor (Toyobo). The qPCR reactions were performed using qPCR Mastermix Plus (Eurogentec, Philadelphia, PA, USA) on an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). The following probes and primers were designed using ABI Primer Express software (Applied Biosystems): for *Por*, 5'-CCCCTTCGATGCTAAGAATCC-3' (forward), 5'-CGCTCAGTGCCTTGGTTCA-3' (reverse), and 5'-TTCCTGGCTGCTGTCACCGCC-3' (probe); for *Per2*, 5'-AGCATCCAGTCCTGTTTCTTTAGA-3' (forward), 5'-ATCGCACAACTCTGAGAGCTTATT-3' (reverse), and 5'-GGTGTCTGAGACTTTCTCTATTTGAAACT-3' (probe); for *Cyp7a1*, 5'-CGCCCTAGCGACTGGATTAG-3' (forward), 5'-GGCCCAGCTATGTGAACAC-3' (reverse),

and 5'-AGAACTTTGTTCTCGCTGCCCACATTCC-3' (probe); and for *Bhlhb2*, 5'-GCTTCCAGGAAACCATTGGA-3' (forward), 5'-GGCTAGGAAGCTGGGCTTCT-3' (reverse), and 5'-TCAGTCCCAAACCCGTGGAC-3' (probe). All probes were 5'-labeled with FAM, i.e. 6-carboxyfluorescein, and 3'-labeled with TAMRA, i.e. 6-carboxytetramethylrhodamine. The program was 50°C for 2 min and 95°C for 10 min, with amplification in 40 cycles of 95°C for 15 sec and 60°C for 1 min. GAPDH (glyceraldehyde-3-phosphate dehydrogenase), of which the primers and probes were from Applied Biosystems, was used as an endogenous control.

Immunoblot analyses

The thawed liver samples were mixed with 1.15% potassium chloride with a three-fold volume of the sample and homogenized on ice. The homogenate was centrifuged at 9,000 g for 20 min at 4°C, and the supernatant fraction was further centrifuged at 105,000 g for 1 hr at 4°C. The obtained supernatant was used as the cytosolic fraction. The resultant precipitate was suspended with 1.15% potassium chloride of a volume equal to each supernatant and centrifuged at 105,000 g for 1 hr at 4°C again. The precipitate was resuspended with 1.15% potassium chloride containing 20% glycerol of a volume equal to each supernatant and used as the microsomal fraction. The protein concentrations in the cytosolic and microsomal fractions were determined by the methods of Lowry *et al.* (1951).

The hepatic gene expression profile in male TG rats was functionally feminized and demasculinized with a central focus on Phase I drug-metabolizing enzymes. To confirm demasculinization of the liver in TG rats at two time points measured, immunoblots were conducted for some cytochrome P450 enzymes which included representative male-specific isoforms (CYP2C11 and CYP3A2) and a diurnally expressed isoform CYP7A1. The microsomal proteins prepared from the liver samples of both TG and WT rats collected at ZT6 and ZT18 were size-fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis with 7.5% acrylamide slab gel. After the electrophoresis, the proteins were electrophoretically transferred to an Immobilon-P transfer membrane (Millipore Corporation, Billerica, MA, USA). The blotted membrane was incubated with antibodies against CYP2C11 (Fitzgerald Industries International, Inc., Concord, MA, USA); CYP2E1 (GE Healthcare Bio-Sciences KK, Tokyo, Japan); CYP3A1, CYP3A2, and CYP7A1 (Chemicon International Inc., Billerica, MA, USA); and POR (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan).

Measurement of glutathione S-transferase (GST) activity

The gene expression levels of Phase II drug-metabolizing enzymes, i.e. GST, class mu 1, 2, and 5, were down-regulated in TG rats as compared to WT rats. To verify the results, GST activity in the cytosolic fraction was measured using 1-chloro-2,4-dinitrobenzene (GST-C activity) or 1,2-dichloro-4-nitrobenzene (GST-D activity) as a substrate by the method of Habig *et al.* (1974). GST-C shows the broad activity of GST, whereas GST-D is the activity specific to mu class GST. Enzymatic activity was measured spectrophotometrically.

Statistical analyses

For microarray expression data and GST activity, the homogeneity of variance was estimated by *F*-test; and any significant difference of the mean value was evaluated by Student's *t*-test (for homogeneous data) or Aspin-Welch's *t*-test (for heterogeneous data). Statistical analysis was performed with SAS System ver. 6.1.2 (SAS Institute Inc., Cary, NC, USA). The *P*-values less than 0.05 were considered to be significant.

RESULTS

Daily profiles of differentially expressed genes in TG versus WT rats

The hepatic gene expression in TG rats at ZT6 or ZT18 was compared with that in WT rats. In TG rats, 931 up-regulated and 978 down-regulated transcripts were identified at ZT6 as compared with WT rats (Supplementary Table 1). Moreover, 835 up-regulated and 842 down-regulated transcripts were identified in TG rats at ZT18 as compared with WT rats (Supplementary Table 2). Co-occurrences of these differentially expressed transcripts determined between the up-regulated and down-regulated genes (Fig. 1A) indicated that 542 up-regulated transcripts (proportion: 58% at ZT6; 65% at ZT18) and 480 down-regulated transcripts (proportion: 49% at ZT6; 57% at ZT18) were overlapped regardless of the lighting conditions. Biological pathways analysis identified that these overlapping transcripts were mainly involved in the metabolism of xenobiotics, fatty acid, steroid hormone, pyruvate, and a number of amino acids (Table 1). Additionally, the other 2 overlapping transcripts were up-regulated at ZT6, whereas these were down-regulated at ZT18. Interestingly, these transcripts corresponded to a negative regulator [cytokine inducible SH2-containing protein (*Cis*)] on GH signaling (Karlsson *et al.*, 1999; Leroith and Nissley, 2005), i.e. Janus kinase (Jak)-signal transducer and activation of transcription (Stat) signaling.

Transcripts altered in TG rats at ZT6 or ZT18 only were related to the pathways shown in Supplementary Table 3. Transcripts differentially regulated in TG rats at ZT6 only were enriched in biological pathways including IGF-1 signaling.

Comparison of daily fluctuated gene expression between genotypes

The hepatic gene expression that fluctuated during the day was compared between genotypes. In TG rats, the number of transcripts which fluctuated daily was smaller than that in WT rats. In TG rats, 299 up-regulated and 266 down-regulated transcripts were identified at ZT18 *versus* ZT6 (Supplementary Table 4); while 460 up-regulated and 506 down-regulated transcripts were identified in WT rats at ZT18 *versus* ZT6 (Supplementary Table 5). Co-occurrences of these differentially expressed transcripts in TG and WT rats were determined between the up-regulated and down-regulated transcripts (Fig. 1B), demonstrating that 117 up-regulated transcripts (proportion: 39% in TG rats; 25% in WT rats) and 118 down-regulated transcripts (proportion: 44% in TG rats; 23% in WT rats) were overlapped in both genotypes. Subsequently, the categorized gene lists were analyzed for enrichment of the biological pathways. For the transcripts which fluctuated daily in both genotypes, the most significant gene network ($-\log(P\text{-value}) = 5.85$) was circadian rhythm signaling, which included core clock-related genes (Table 2). Other enriched pathways were involved in glucide and amino acid metabolism, steroid biosynthesis, and stress responses (Table 2). Interestingly, the expression of *Stat5b* is down-regulated at ZT18 *versus* ZT6 in the TG, as well as in WT rats. Furthermore, the other 3 overlapping transcripts were differentially regulated between genotypes. These were hepatocyte nuclear factor 6 (*Hnf6*), ATPase inhibitor factor 1, and dapper homolog 2, antagonist of beta-catenin. The expression of *Hnf6*, a female-predominant GH-regulated nuclear factor (Waxman and O'Connor, 2006), was down-regulated at ZT18 *versus* ZT6 in TG rats, whereas it was up-regulated at ZT18 *versus* ZT6 in WT rats. The transcripts which fluctuated daily only in TG or WT rats were related to the pathways shown in Supplementary Table 6. The transcripts which fluctuated daily only in TG rats were enriched in biological pathways including IGF-1 signaling.

Verification of microarray expression data

The relative expression levels for *Per2*, *Bhlhb2*, *Cyp7a1*, and *Por* were quantified by RT-PCR analyses to verify the microarray expression data with a central focus on circadian rhythm signaling. The relative gene expres-

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Fig. 1. Commonly or differentially expressed transcripts between genotypes. The number of transcripts classified in each region is indicated. A, Venn diagrams showing up- and/or down-regulated transcripts in TG *versus* WT rats at ZT6 and ZT18. B, Venn diagrams showing up- and/or down-regulated transcripts at ZT18 *versus* ZT6 in TG and WT rats.

sion levels calculated from the RT-PCR data were in line with microarray results and verified the results (Fig. 2).

Characterization of drug-metabolizing enzymes

Fig. 3A illustrates the translational levels for CYP2C11,

CYP2E1, CYP3A1, CYP3A2, and POR. The levels in TG rats were lower than those in WT rats at ZT6 and ZT18. In particular, the male-specific isoforms CYP2C11 and CYP3A2 were undetectable in TG rats. The expression of CYP7A1 was observed to fluctuate daily in both gen-

Table 1. Biological pathways differentially regulated at both ZT6 and ZT18 in TG rats vs. WT rats

Pathway	-Log (P-value)	Molecules - increased expression (descending order in fold change)	Molecules - decreased expression (descending order in fold change)
LPS/IL-1 Mediated Inhibition of RXR Function	9.10	SULT2A2, ALDH1B1, GST-Yc2, FABP5L2, ACS5, FABP, ALDH1, CYP4A1, PAPSS2, GSTkappa, CYP2A1, ACS3, GSTA4	FXR, SR-B1, GSTM2, TRAF6, CPT-1a, GSTM5, CD14, SULT1C1, GSTM1, OATPIA4, MAP3K1, CAR, CYP2CII, ESTSUL, CYP3A2
C21-Steroid Hormone Metabolism	8.12	EBP	CYP2D18, CCA2, HSD11B1, AKR1C18, AKR1D1, AKR1C1, HSD3B
Metabolism of Xenobiotics by Cytochrome P450	8.05	CYP2C12, GST-Yc2, ADH7, UGT2B1, GSTkappa, CYP2A1, GSTA4	GSTM2, CYP2D18, GSTM5, GSTM1, CYP2D13, AKR1C18, AKR1C1, CYP4A8, CYP3A18, CYP2CII, CYP3A2
Fatty Acid Metabolism	6.75	CYP2C12, ALDH1B1, ACS5, ADH7, ALDH1, CYP4A1, ALDH1A4, ACAT2, CYP2A1, ACS3	ACADSB, CPT-1a, ACAA2, CYP2D18, CYP2D13, CYP4A12, CYP3A18, CYP2CII, CYP3A2
Pyruvate Metabolism	6.14	ALDH1B1, MOD1, ALDH1, PDC-E2, ACAC, AKR1B10, PK1, ALDH1A4, MDL1, ACAT2, ACS3, NUDT7	CACH
Bile Acid Biosynthesis	5.65	ALDH1B1, SRD5A1, ADH7, ALDH1, ALDH1A4	ACAA2, CCA2, AKR1D1, AKR1C1, CYP3A2
TR/RXR Activation	5.54	FASN, SRG1, MOD1, LPPG, ACAC, LDLRA, F10	SR-B1, TBL1XR1, 5DI, DCIP2, PIK3C2G, COL6A3, AKR1C18
Xenobiotic Metabolism Signaling	5.32	SULT2A2, ALDH1B1, GST-Yc2, ALDH1, UGT2B1, PPP2R1B, PKCA, GSTkappa, NADPH-D, GSTA4	GSTM2, GSTM5, SULT1C1, GSTM1, CES3, MRG1, CES1, PPP2R5E, MAP3K1, RFMO1A, PIK3C2G, CAR, CYP2CII, ESTSUL, CYP3A2
Androgen and Estrogen Metabolism	4.96	SULT2A2, SRD5A1, UGT2B1, HSD17B9, EBP	CCA2, HSD11B, AKR1D1, AKR1C1, ESTSUL, HSD3B
Glutathione Metabolism	4.17	G6PDX, GST-Yc2, GSTkappa, GSTA4	GSTM2, GSTM5, OPLAH, GSTM1, IDH2, GLCLR
Retinol Metabolism	3.61	ADH7, ALDH1, UGT2B1, ALDH1A4, RDH11	RDH2
Glycerolipid Metabolism	3.51	ALDH1B1, GPAT, ADH7, DGAT2, ALDH1, AKR1B10, ALDH1A4, AGPAT3, DGK4	AGPAT6
Linoleic Acid Metabolism	3.42	CYP2C12, FADS5D6, CYP2A1	CYP2D18, PLA2G12A, CYP2D13, CYP4A12, CYP3A18, CYP2CII, CYP3A2
Biosynthesis of Steroids	3.29	CYP26, LSS, EBP, SC5D, FDPS, NADPH-D	
Arachidonic Acid Metabolism	3.06	CYP2C12, CYP4A1, GSTkappa, CYP2A1	CYP2D18, PLA2G12A, CYP2D13, AKR1C18, CYP4A8, CYP3A18, CYP2CII, CYP3A2
Glycolysis/Gluconeogenesis	2.91	ALDH1B1, ADH7, ALDH1, PDC-E2, PK1, ALDH1A4, ACS3, UAE1	
Histidine Metabolism	2.82	ELOVL6, ALDH1B1, ALDH1, ELOVL2, HAL, ALDH1A4	PRPS2
FXR/RXR Activation	2.80	FASN, PON1, UGT2B1, MTP, PK1, SYND1, IGIF	FXR, SR-B1, PPARG1, IBAT

Official gene symbols were used to represent molecules.

Table 1. (Continued)

Pathway	-Log (P-value)	Molecules - increased expression (descending order in fold change)	Molecules - decreased expression (descending order in fold change)
PXR/RXR Activation	2.79	SCD1, ALDH1, PAPSS2, ALDH1A4	GSTM2, CPT-1a, CAR, CYP2C11, CYP3A2
Tryptophan Metabolism	2.78	CYP2C12, ALDH1B1, ADH7, ALDH1, ALDH1A4, ACAT2, CYP2A1	CYP2D18, CYP2D3, CYP4A8, CYP3A18, CYP2C11, CYP3A2
Valine, Leucine and Isoleucine Degradation	2.72	ELOVL6, ALDH1B1, ADH7, ALDH1, ELOVL2, ALDH1A4, ACAT2	ACADSB, ACAA2
LXR/RXR Activation	2.46	FAT, FASN, SCD1, ACAC, APOA4, LDLRA, IGIF	FXR, CDI4
Sulfur Metabolism	2.33	SULT2A2, PAPSS2	SULT1A2, ESTSUL
NRF2-mediated Oxidative Stress Response	2.30	GST-Yc2, PKCA, GSTkappa, NADPH-D, GSTA4	DNAJA5, SR-B1, FKBP5, GSTM2, GSTM5, GSTM1, MAP3K1, GLCLR, RFMO1A, PIK3C2G
Aryl Hydrocarbon Receptor Signaling	2.26	ALDH1B1, FASN, GST-Yc2, CHK1, ALDH1, GSTkappa, NADPH-D, GSTA4	GSTM2, GSTM5, GSTM1, RB1, HSP25
Ascorbate and Aldarate Metabolism	1.95	ALDH1B1, CYP26, ALDH1, ALDH1A4	
Butanoate Metabolism	1.89	ELOVL6, ALDH1B1, ALDH1, ELOVL2, AACS, ALDH1A4, ACAT2	
Type II Diabetes Mellitus Signaling	1.84	FAT, ACS5, IRS3, CCA1, PK1, PKCA, ACS3	PPARG1, MAP3K1, PIK3C2G
Fatty Acid Biosynthesis	1.84	FASN, ACAC, ACS3	
Propanoate Metabolism	1.83	ALDH1B1, ALDH1, ACAC, ALDH1A4, ACAT2, ACS3	ACADSB
RAR Activation	1.69	TRIM24, CYP26, ADH7, ALDH1, ALDH1A4, CRBP1, PKCA, RDH11	IGFBP3, MRG1, MAP3K1, RDH2
Arginine and Proline Metabolism	1.68	ALDH1B1, PRODH2, ALDH1, rOAT, ALDH1A4	TIFF66, CTBP
p53 Signaling	1.63	FASN, CHEK1, PRKDC, CYCG	RB1, STAG1, PIK3C2G
Cell Cycle Regulation by BTG Family Proteins	1.58	CCR4, PPP2R1B	PPP2R5E, RB1
Tight Junction Signaling	1.46	PPP2R1B, PRP4	MLCK, AF-6, JAM3, SPNA2, NECTIN3, PPP2R5E, CLDN3, CRB3
Glycerophospholipid Metabolism	1.46	ELOVL6, GPAT, ELOVL2, AGPAT3, DGK4	GPLD1, AGPAT6, PLA2G12A
Nicotinate and Nicotinamide Metabolism	1.42	ADH7, NAPRT1, DAPK	QPRT, NNNMT, ATX, TIFF66
EGF Signaling	1.41	PKCA	RASA, ERPP, MAP3K1, PIK3C2G
Nitrogen Metabolism	1.31	HAL	PTPG, TIFF66, CA3

Official gene symbols were used to represent molecules.

Table 2. Biological pathways fluctuated during the day in both TG and WT rats.

Pathway	-Log (P-value)	Molecules - increased expression (descending order in fold change)	Molecules - decreased expression (descending order in fold change)
Circadian Rhythm Signaling	5.85	PER2, CRY1, PER3, CRY2	NR1D1
Glycine, Serine and Threonine Metabolism	3.58	ELOVL6, ALAS1	CTH, AGXT, CHKA
Biosynthesis of Steroids	2.71	HMGCR, LSS	CYP8B1
Nitrogen Metabolism	2.39	ALAS1	CTH, CA3
Starch and Sucrose Metabolism	1.88	GCK, GYS2	G6PC
Glycolysis/Gluconeogenesis	1.85	GCK, DLAT	G6PC
NRF2-mediated Oxidative Stress Response	1.60	MAP2K3, GCCLC, DNAAJ4	MAFG, DNABJ9
Glycerolipid Metabolism	1.59		LPIN1, GK, LPL
Methionine Metabolism	1.58	ALAS1	CTH
Valine, Leucine and Isoleucine Degradation	1.54	ELOVL6, ACAT2, ACAA2	
Alanine and Aspartate Metabolism	1.49	DLAT	AGXT
Galactose Metabolism	1.41	GCK	G6PC
Lysine Degradation	1.41	ELOVL6, ACAT2	CLPX, KYNU
HIF1 α Signaling	1.36	EGLN3, HSP90, ARNT	
Aryl Hydrocarbon Receptor Signaling	1.32	HSP90, CHEK1, ARNT	AHR
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.31	WEE1, CHEK1	

Official gene symbols were used to represent molecules.

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Fig. 2. RT-PCR verification of microarray data. Data represent the mean $\pm$ S.D. ($n = 4/\text{group}$) where the value in WT rats at ZT6 was defined as 1. Probe sets for each selected gene in microarray were as follows: *Per2*, 1368303_at; *Bhlhb2*, 1369415_at; *Cyp7a1*, 1368458_at; and *Por*, 1368213_at (left) and 1387109_at (right).

otypes, and the level was slightly higher in TG rats compared to WT rats.

GST-C and GST-D activities in TG rats were significantly lower than those in WT rats at both ZT6 and ZT18 (Fig. 3B).

DISCUSSION

Using male TG rats that constitutively express hGH gene, the hepatic transcriptome was analyzed to elucidate the regulatory role of episodic GH profile on the liver. The daily gene expression profiles in TG rats were quite different from those in WT rats. There were transcripts commonly up- or down-regulated regardless of the lighting conditions in TG *versus* WT rats. These transcripts were mainly enriched in the metabolism of xenobiotics, fatty acid, steroid hormone, pyruvate, and a number of amino acids. In general, the hepatic gene expression profile in male TG rats was functionally feminized and demasculinized as follows.

Representative female-specific genes such as *Cyp2c12* and alpha-1-B glycoprotein (*A1bg*) (Wauthier *et al.*, 2007; Waxman and Holloway, 2009) were dramatically up-regulated in male TG rats compared to WT rats. In contrast, representative male-specific genes such as *Cyp2a2*, *Cyp2c13*, and *Cyp3a2* (Wauthier *et al.*, 2007; Waxman and Holloway, 2009) were dramatically down-regulated in TG rats. Consistent with a previous report (Takahashi *et al.*, 1999), the translational levels of the male-specific isoforms (CYP2C11 and CYP3A2) were lowered and undetectable in TG rats along with downregulation of CYP2E1 when compared to WT rats. A GH interpulse interval has been reported to be required for the full expression of CYP2C11 in male rat liver (Waxman *et al.*, 1991). Moreover, continuous GH infusion in intact male rats has been reported to induce CYP2C12 (Sundseth and Waxman, 1992). These findings suggest that hGH chronically secreted as a transgene product feminized the Phase I drug-metabolizing enzymes by abolishing a GH

Fig. 3. Characterization of drug-metabolizing enzymes in the liver. A, Immunoblot analyses of some P450 enzymes and POR. The microsomal proteins were prepared from the liver samples ($n = 4/\text{group}$) collected at ZT6 and ZT18 in both genotypes. Thereafter, those were analyzed individually by immunoblotting with each antibody. B, Measurements of GST-C and GST-D activities in cytosolic fractions of the liver. The values are depicted as the mean $\pm$ S.D. ($n = 6/\text{group}$). **, $P < 0.01$ (Student's t -test); ##, $P < 0.01$ (Aspin-Welch's t -test); significant difference from WT rats at the same sampling time point.

interpulse interval. Additionally, the gene expression levels of Phase II drug-metabolizing enzymes, i.e. GST, class mu 1, 2, and 5, were down-regulated in TG rats as compared to WT rats. The enzyme activity of mu class GST in TG rats were significantly lower than those in WT rats at both ZT6 and ZT18. The expression levels of *Gstm1* and *Gstm2* in females are known to be lower than those in males (Laz *et al.*, 2004; Mugford and Kedderis, 1998; Srivastava and Waxman, 1993), implying that the phenotype of GST in TG rats is also feminized. Taken together with the above information, the present results veri-

fy that the sexually dimorphic profile of GH rather than genetic sexuality is a stronger sex-determining factor on the hepatic transcriptome in rats. Since sexually dimorphic patterns of GH release are imprinted by the action of gonadal hormones on the hypothalamus during the neonatal period (Jansson *et al.*, 1985; Waxman and Holloway, 2009), gonadal hormones are suggestive of indirect sex-determining factors on the hepatic transcriptome via the regulation of GH.

Some genes were up- or down-regulated at only one time point (ZT6 or ZT18) in TG rats, and others were

fluctuated during the day in only one genotype (TG or WT rats). Interestingly, the genes involved in GH signaling fluctuated daily and/or were regulated differentially between genotypes. GH binding to GH receptor (GHR) phosphorylates the GHR-related tyrosine kinases of Jak family, and thereafter activated Jaks phosphorylate a number of signaling molecules including transcription factors of Stat family (Argetsinger and Carter-Su, 1996; Waxman and O'Connor, 2006). In the present study, the gene expression of *Stat5b* showed daily fluctuations with higher values at ZT6 and lower values at ZT18 in both genotypes. Additionally, the gene expression of *Cis*, i.e. a negative regulator of the Jak-Stat pathway (Karlsson *et al.*, 1999; Leroith and Nissley, 2005), showed daily fluctuations with lower values at ZT6 and higher values at ZT18 in WT rats. The gene expression of *Cis* was higher at ZT6 in TG rats compared to WT rats, whereas it was lower at ZT18. The gene expression of *Hnf6*, i.e. a female-predominant GH-regulated nuclear factor (Waxman and O'Connor, 2006), was lower at ZT18 *versus* ZT6 in TG rats, whereas it was higher at ZT18 *versus* ZT6 in WT rats. The gene expression of *Hnf6* was higher at ZT6 in TG rats compared to WT rats. The biological pathways analyses revealed that transcripts differentially regulated in TG rats at ZT6 only and transcripts which fluctuated daily only in TG rats were enriched in biological pathways including IGF-1 signaling. Collectively, these results suggest that GH signaling in the liver is circadian-modulated, depending upon the GH profile. Exogenous hGH is expressed constitutively in TG rats only (Ikeda *et al.*, 1994), and serum hGH level at ZT6 was comparable to that at ZT18 in the present study (data not shown). Endogenous GH secretion in adult rats is most likely to occur in association with the sleep-wakefulness cycle (Kimura and Tsai, 1984; Obal and Krueger, 2004), although contradictory data have been published (Willoughby *et al.*, 1976). The possible correlation is supported by the findings that GH-releasing hormone promotes REM sleep in rats (Obal and Krueger, 2004) and sleep deprivation without influencing corticosterone is effective in preventing high-level GH pulses in rats (Kimura and Tsai, 1984). The difference of circadian modulation between exogenous and endogenous GH secretion might, in part, elicit these variances.

The common transcripts which fluctuated daily in both genotypes were enriched in biological pathways such as circadian rhythm signaling, glucide and amino acid metabolism, steroids biosynthesis, and stress responses. Many of them coincided with transcripts entrained by the circadian components related to feeding cycle (Hirao *et al.*, 2006), suggesting that these are generally expressed

in a circadian fashion. In particular, the most significant gene network was circadian rhythm signaling which included main circadian clock genes such as *Period* genes (*Per2* and *Per3*), *Cryptochrome* genes (*Cry1* and *Cry2*), and *Nr1d1*. The liver clock in TG rats is therefore considered to be properly ticking, although the day/night fluctuations of *Per2*, *Bhlhb2*, *Cyp7a1*, and *Por* expression levels appear slightly reduced. Circadian oscillators exist not only in the suprachiasmatic nucleus, i.e. the central pacemaker, but also in peripheral organs such as the liver, heart, and kidney (Balsalobre *et al.*, 1998; Yamazaki *et al.*, 2000). In homozygous double mutant *Cry1* and *Cry2* male mice that lack functional circadian oscillators, the liver metabolism is reportedly functionally feminized along with a feminized pattern of circulating GH (Bur *et al.*, 2009). The present study clarified that the circadian oscillations of *Cry1* and *Cry2* are sustained in the liver of TG rats as well as WT rats, indicating that the hepatic *Cry* genes are not involved in sexual dimorphism of the liver metabolism and the GH secretory profile is the main cause for the dimorphism in TG rats. Taken together, circadian oscillators in the central pacemaker are suggested to control the liver metabolism by pacing the secretion of pituitary GH.

Our transcriptome analyses using adult male TG rats clarified the regulatory role of episodic GH profile on the liver and strengthen the functional link between sexually dimorphic GH secretion, liver metabolism, and its circadian regulation. The sexually dimorphic GH profile in rats shows age-dependent changes; and the low GH nadir is eliminated in senescent male rats, followed by feminization and demasculinization of the sex-dependent isoforms of P450 (Dhir and Shapiro, 2003; Lee *et al.*, 2008; Mori *et al.*, 2007). The secretory profile of GH is likely to play a key role on the liver as a hub in the complex networks among inter- and intra-individual variation factors such as aging, sex, and circadian rhythm. These confounders can cause inter- and intra-individual differences in xenobiotic detoxification and can alter the balance between effectiveness and toxicity of xenobiotics. The complex interrelationships might be more clearly understood by further studies which focus on circadian regulatory mechanisms for GH through the lifespan of the animals.

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