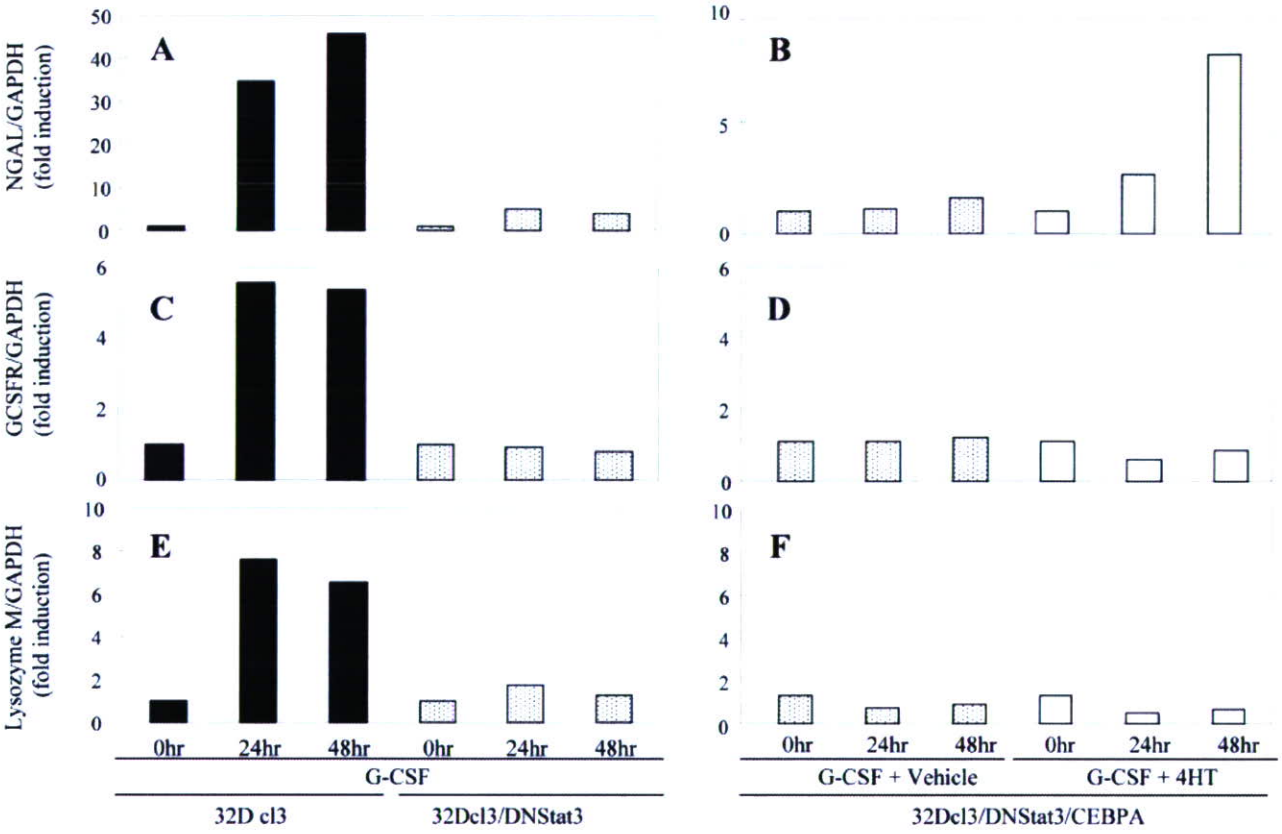

FIG. 8. **Granulocyte-specific gene expressions after C/EBPα induction.** The time course of NGAL (*A* and *B*), G-CSF receptor (*G-CSFR*) (*C* and *D*), and lysozyme M (*E* and *F*) mRNA expression following G-CSF stimulation in 32Dcl3 and 32Dcl3/DNStat3 cells (*A*, *C*, and *E*) or by G-CSF stimulation with 4-HT or vehicle in 32Dcl3/DNStat3/CEBPA cells (*B*, *D*, and *F*) is shown. Cells maintained in IL-3 were starved of cytokines for 8 h and stimulated with G-CSF, G-CSF, plus 4-HT and G-CSF plus vehicle. Total RNA was isolated at the indicated times after the stimulation and transcribed to cDNA, which was subjected to real-time PCR. The *numbers* given on the *vertical axis* represent the fold induction of ratios of average GAPDH-normalized expression values when compared with those before stimulation. Three independent experiments were performed, and similar results were obtained and shown data are the representative of them.

The CEBP family of transcription factor is expressed in multiple cell types, including hepatocytes, adipocytes, keratinocytes, enterocytes, and cells of the lung (30, 31). C/EBPα transactivates the promoters of hepatocyte- and adipocyte-specific genes, which are important for energy homeostasis (32, 33), and *C/EBPα*-deficient mice lack hepatic glycogen stores and die from hypoglycemia within 8 h of birth (34). In the hematopoietic system, C/EBPα is exclusively expressed in myelomonocytic cells (35, 36). C/EBPα expression is prominent in mature myeloid cells, and previous investigations found that C/EBPα is critical for early granulocytic differentiation. Mice with a targeted disruption of the *C/EBPα* gene demonstrate an early block in granulocytic differentiation, but they develop normal monocytes (19). Conditional expression of C/EBPα is sufficient to induce granulocytic differentiation (17). In contrast to the essential role of C/EBPα in granulocytic differentiation, the role of Stat in granulopoiesis is controversial. Stat3 is the principle Stat protein activated by G-CSF, with Stat5 and Stat1 also activated to a lesser degree (8, 10). In mice lacking *Stat5a* and *Stat5b*, the number of colonies produced in response to G-CSF was reduced 2-fold despite normal circulating numbers of neutrophils (9). Myeloid cell lines expressing dominant-negative forms of Stat3 (11, 37, 38) and transgenic mice with a targeted mutation of the G-CSF receptor that abolishes G-CSF-dependent Stat3 activation (12) demonstrate that Stat3-activation is required for G-CSF-dependent granulocytic proliferation and differentiation.

In the present study, we clearly demonstrate that the expression of C/EBPα mRNA is up-regulated through the activation of Stat3 in response to G-CSF, and the Stat3-C/EBPα signaling cascade plays an important role in G-CSF-induced differentiation. Contrary to these data, however, we and others showed that mice conditionally lacking Stat3 in their hematopoietic progenitors developed neutrophilia, and bone marrow cells were hyper-responsive to G-CSF stimulation (23, 39). Additionally, mice with tissue-specific disruption of *Stat3* in bone marrow cells die within 4–6 weeks after birth with Crohn's disease-like pathogenesis (40). These mice exhibit phenotypes with dramatic expansion of myeloid cells, leading to massive infiltration of the intestine with neutrophils, macrophages, and eosinophils. Cells of the myeloid lineage also demonstrate autonomous proliferation. These apparently disparate results may be explained by the need for molecules in addition to Stat3 to regulate C/EBPα expression *in vivo*, the *in vivo* functional redundancy among C/EBPα regulators, or the absence of the abrogation of SOCS3 induction by G-CSF in 32Dcl3/DNStat3 cells. In 32Dcl3 cells, the Stat3-C/EBPα pathway might be favored, and other pathways may contribute little to granulocytic differentiation in response to G-CSF.

Among C/EBP family, C/EBPε is important for late phase of granulocytic differentiation, and its expression is up-regulated by G-CSF independent of Stat3 (11). A previous report showed that C/EBPε is a transcriptional target of C/EBPα in 32Dcl3 cells (41). From these reports and our results, we speculated that a small amount of C/EBPα is enough for the induction of the transcription of C/EBPε by G-CSF or that there are multiple signaling steps except for Stat3-C/EBPα to induce the transcription of C/EBPε by G-CSF.

Induction of C/EBPα led to not only morphologic differentiation but also expression of granulocyte-specific genes (17). In 32Dcl3/DNStat3 cells, the induction of the G-CSF receptor, lysozyme M, and NGAL in response to G-CSF was abrogated (Fig. 8). Restoration of C/EBPα in these cells led to expression of only the *NGAL* gene, and thus, 32Dcl3/DNStat3 cells differentiated by the induction of C/EBPα may not be functional as mature neutrophils. In these cells, therefore, activation of C/EBPα is not sufficient for the induction of lysozyme M or G-CSF receptor genes, and the presence of other molecules appears to be required for their expression.

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〈抄録〉第25回 日本臨床薬理学会年会 2004年9月17～18日 静岡
シンポジウム2（安全性分野）：トキシコゲノミクス―現状と臨床薬理学への応用―

4．日本人組織を用いたトキシコゲノミクス研究

大島康雄* 藤村昭夫*

我々の研究室では日本人由来の組織を用いたトキシコゲノミクス研究を行っている。現状では日本人組織を商業ベースで合法的に入手することはできないために、我々は自治医科大学附属病院で手術を受ける患者さんにご協力いただき、手術時に病変部位と同時にやむを得ず切除される正常組織を研究に利用することとした。動物実験で得られた情報だけではヒトへの外挿が必ずしも十分ではなく、ヒトで最終的に確認できればより好ましい。一方、臨床検体の入手には困難な点が多く、研究計画の自由度は低い。動物実験・細胞株を用いた研究と我々の様にヒト組織を用いた研究は相補的な位置づけとされ、それぞれの役割を担うことが期待される。

1．倫理評価ワーキンググループ：
治療を目的として我々の病院を訪れる患者さんから、研究のための組織を提供していただくにあたり、我々は倫理評価ワーキンググループを立ち上げ、研究計画の審査・インフォームドコンセント取得の手順・検体採取後の病理組織の評価・匿名化の手順・関係書類の保管などにつき詳細な検討を行った。倫理評価ワーキンググループの中には、宗教家・法律家などの学外委員も含まれている。彼らとの討論の中で、医療関係者以外の第三者から誤解を受けやすい点が一つ浮かび上がった。今回我々が研究に用いる検体は、従来の手術方法で切除されてしまう非病変部組織であり、通常廃棄処分される組織部分を研究へ利用させていただく計画であった。しかし、非医療関係者は、病変の治療のためには必要もないのに研究目的のためだけに正常組織を切除するものと誤解されるようであった。このような誤解を受けやすい部分を今後も啓発することによって、日本人組織を研究・開発に利用しやすい社会環境を形成してゆく必要がある。

2．臨床検体取得の現場：
患者さんよりインフォームドコンセントをいただき、附属病院の手術室から臨床検体を取得し、プライマリーカルチャーを作成することができた。我々が試みた範囲ではディスパーゼとトリプシンを併用する方法が安定した良好な結果をもたらすものと思われた[1]。得られた細胞は、上皮性の細胞として矛盾のない形態を示し、腎臓皮質由来の細胞の多くはGlut-2抗原及びγGTP活性を示し、尿細管由来であることが示された。また、肝臓由来の細胞はアルブミン産生能及びCyp3A4抗原の存在から、主に肝細胞であると判断した。

3．臨床検体を用いた遺伝子発現解析の問題点：
出版された論文とともに公開されたデータベース(http://www.ncbi.nlm.nih.gov/geo/)をレビューした結果、171の臨床検体を用いたGeneChipデータのうち29 Chip (17%)ではRNAの質が不良であると判定された。一方で63の非臨床検体を用いたGeneChipデータではRNAの質がすべて良と判定された。このようにすでに公開・出版されているデータですら臨床検体のデータには問題点があることが示された。これは、臨床検体の取り扱いの難しさを示している。同時に、臨床検体を用いた網羅的遺伝子発現解析の実行・データの解釈にはRNAの質に注意すべきであることも示している。
網羅的遺伝子発現解析のもう一つの問題点は、個別の遺伝子発現全てにつきそのデータの信頼性とそれが意味するところを研究者自身が検証しながら研究を進めていくことが困難であることがあげられる。このためチップデータ全体の質を管理することが必要と考えられる。我々が使用している

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Affymetrix 社の GeneChip システムでは、こうした実験の質の管理を行うために用いることのできる様々なパラメータを実験結果の一部として得ることができる。このようなパラメータにはバックグラウンドノイズ・ハウスキーピングジーンの 3'/5'比・パーセントプレゼントなどがある。こうしたパラメータを活用し、研究の質をコントロールしつつ臨床検体の処理・プライマリーカルチャーの作成を行うことによって、我々はより良質のプライマリーカルチャーを作成することができたと考えている。また、既知濃度のスパイク RNA を用いて検出系の定量性・ダイナミックレンジの検討も行った。その結果、1.5 pM~100 pM での範囲での RNA 濃度の読みとりの直線性は良好であった。

4. 日本人組織を用いる必要性～人種差の克服：

海外で調整済みのプライマリーカルチャーを用いることに比較して、日本人組織を用いた遺伝子発現解析の利点として、理論的には研究結果について人種差を懸念する必要がないこと、海外調整済みのプライマリーカルチャーの利用にかかわる資金や場合によっては知的所有権などが海外へ流出する心配がないこと等がある。もちろんヒト組織であるから、種差も存在しない。さらに、自前でヒト組織を調整するため、組織の質を制御することが可能である。

我々の研究室ではこれまでのところ 11 名分の組織を研究に使用している。この 11 名につきそれぞれ3回、薬物等の刺激に未曝露の状態で発現解析を行ったところ、約 44,000 のトランスクリプトの内、統計学的に有意に個人差がある（t-test p 値が 0.01 未満）トランスクリプトは 100 に満たなかった。大多数の遺伝子発現は、薬物未曝露の状態では有意な個人差が見られないと判断された。

5. 現状と限界：

これまで 20 以上の薬物をプライマリーカルチャーへ曝露し、遺伝子発現解析を行った。腎障害をしばしば起こすことによって臨床的に問題になる薬物を用いて、有意に誘導や抑制される遺伝子を同定した。現在その遺伝子発現の確認を進めるとともに、その細胞内での働きを解析している。また、クラスタリングされた遺伝子群に有意に高頻度に出現する転写因子（DNA binding protein）認識配列を検索するシステムを構築した。[2]

本稿で記載した、個人差の研究により得られた情報は、重要な基礎的検討である。先行している実験動物や培養細胞株の研究に追従する形で曝露化合物の数を増加することよりも、データの質を管理するプロセスや、未刺激の日本人プライマリー腎細胞にどのような遺伝子に個人差があるのかを明らかにすることは、日本人の臨床検体を取り扱っている我々が取るべき、より優先順位の高い課題である。これらこそが限られたリソースと与えられた条件のなかで今後の日本人組織を用いた研究を活かし、示唆に富む情報を提供するもである。

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[2] Yasuo Oshima, Yusuke Ishida, Ayumi Shinohara, Hiroyuki Mano, Akio Fujimura. Expression Profiling of Gene with Upstream Aml1 Recognition Sequence in Hematopoietic Stem Cell-Like Fractions from Individuals with the M2 Subtype of Human Acute Myeloid Leukemia. Annual Meeting for International Society of Experimental Hematology, New Orleans, LA. USA. Jul 16-20, 2004

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Profile of rhythmic gene expression in the livers of obese diabetic KK-A^y mice

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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.

Keywords: Circadian rhythm; Gene expression; Type 2 diabetes; Obesity; Molecular clock; Clock gene; Liver

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 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

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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 weeks 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%.

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 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

Table 1
Rhythmicity in the expression of selected genes

Gene name	Gene symbol	GenBank Accession Code		KK		KK-Ay	
		For microarray	For real-time PCR	*F*	*P*	*F*	*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 (*Escherichia 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	Nfi3	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.

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. 3A–C). In particular, the peak levels of ten genes were significantly attenuated in KK-A^y mice compared with those in KK mice (Fig. 3A and B). The differences between the strains were observed throughout the day in only two of the 13 genes (*Por* and *Depdc6*) (Fig. 3A). 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.

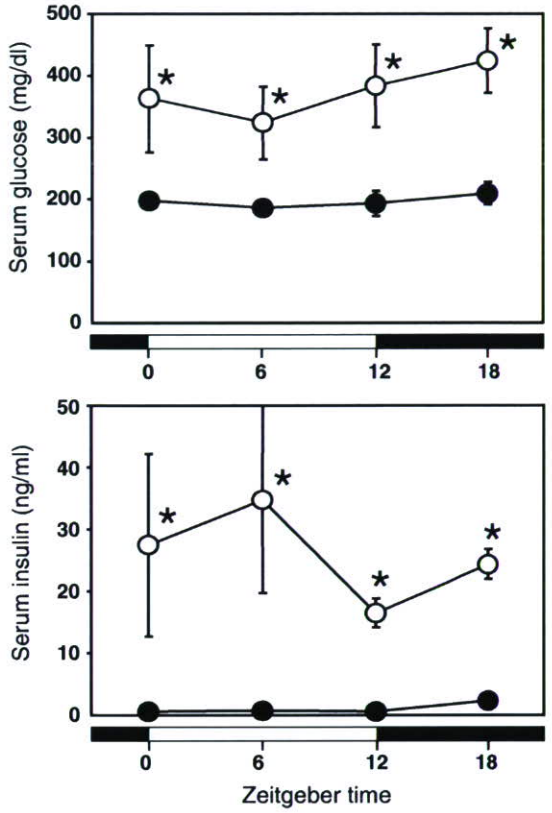

Fig. 1. Daily profiles of the serum glucose and insulin concentrations in KK (solid circles) and KK-A^y mice (open circles). Data are means ± SEM of three mice at each time point. $^*P < 0.05$ vs. KK mice.

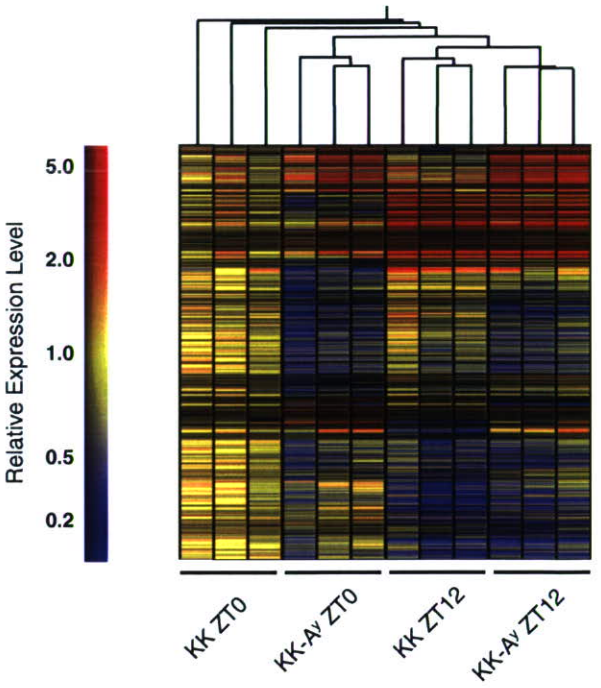

Fig. 2. GeneTree clustering analysis of gene expression profiles in the livers of KK and KK-A^y mice obtained at ZT 0 and 12 ($n = 3$ for each time point in both mice). The transcripts with significantly different levels ($P < 0.05$, one-way ANOVA), i.e., >2-fold, among the four groups were used for the clustering. The increased expression is shown in red; decreased expression is shown in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

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. 3A 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 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 pre-proteasomal 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 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.

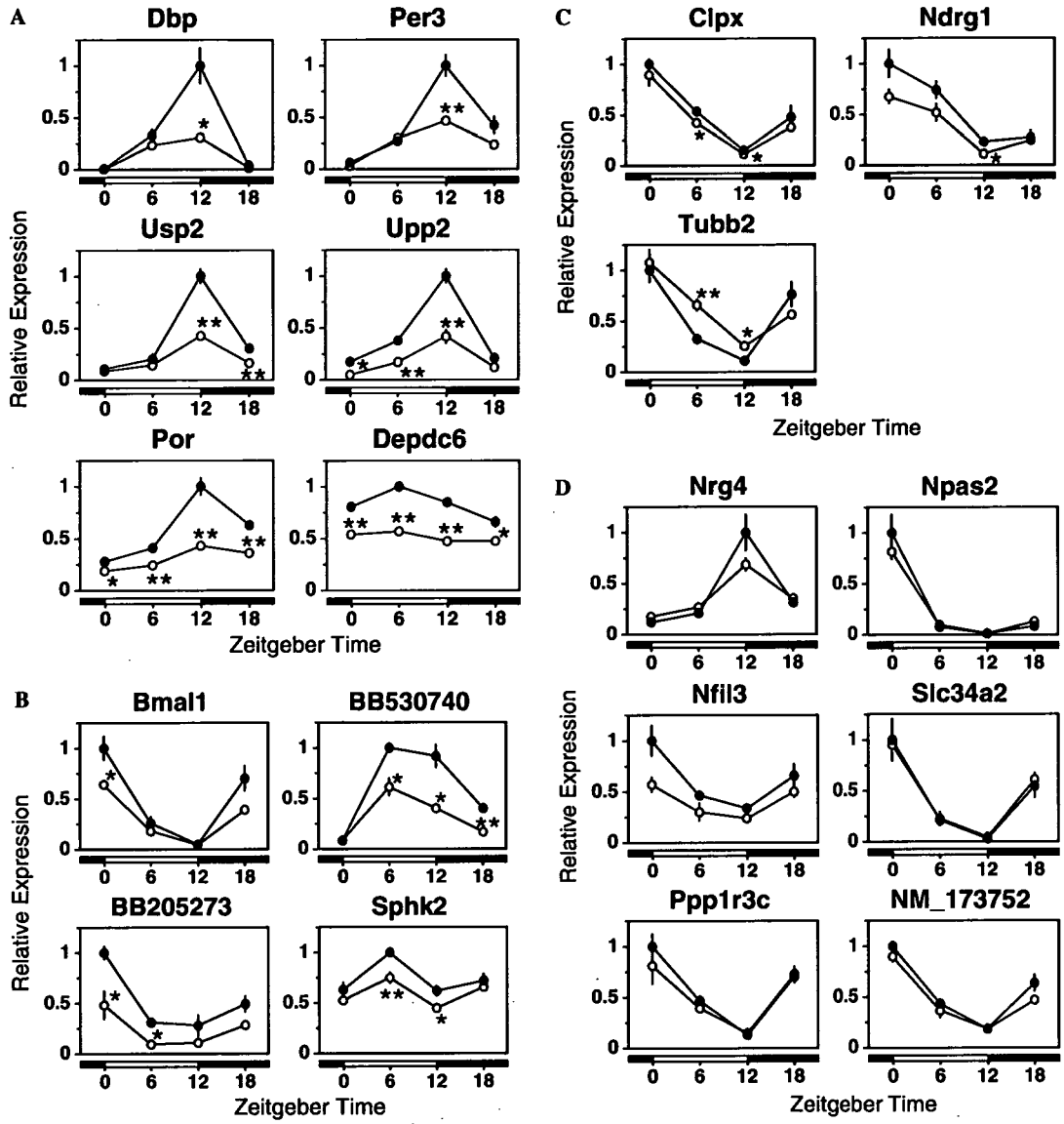

Fig. 3. Daily mRNA expression profiles of the circadianly expressed genes in the livers of KK (solid circles) and KK-A^y mice (open circles). Liver samples were obtained from both mice at ZT 0, 6, 12, and 18. Transcript levels were determined using real-time quantitative reverse transcription-PCR. Data are 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.

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Original Paper

Individual Differences in Gene Expression in Primary Cultured Renal Cortex Cells Derived from Japanese Subjects

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We used microarrays to examine individual-based differences in gene expression in primary cultures of renal tubular cells derived from Japanese subjects. The subjects had solitary tumors in the kidney or urinary tract, which were diagnosed pathologically as renal cell carcinoma or transitional cell carcinoma. Renal tissue samples collected from a non-tumorous portion of the tissue were regarded as normal tissues, as there were no abnormal microscopic findings and no evidence of renal dysfunction from the clinical laboratory data. The genome-wide gene expression profiles of nine human renal cell cultures were analyzed using the Affymetrix GeneChip HG-U133A and HG-U133B arrays. Approximately 8,500 transcripts exhibited significant differential expression ($p < 0.05$) among the subjects, and the coefficients of variation for 1,338 transcripts were greater than 50%. Some of these transcripts encode drug-metabolizing enzymes (e.g., *UGT1A8* and *UGT1A9*) or sodium/phosphate cotransporters (e.g., *PDZK1*). These data provide the basis for toxicogenomic studies using primary cultured renal cortical cells from Japanese subjects.

1. Introduction

We use toxicogenomics to clarify toxicity mechanisms and to identify biomarkers that predict kidney toxicity. Toxicogenomics involves the application of microarrays to analyzing gene expression patterns after exposure to toxic compounds, which can contribute to establishing the functional profile of the genome and the discovery of useful markers of toxicity [1),2)]. In addition, the information derived from toxicogenomic studies may improve predictions of the toxicity of new compounds and provide clues to the mechanisms of toxicity. The current trend in toxicogenomic studies is to analyze gene expression profiles after exposing animals, such as rodents, to toxic chemicals or medicines in order to examine toxicity-related gene expression. However, adverse events in humans are often not predicted from animal studies conducted during the drug development process. Therefore, research techniques that employ human tissues are necessary. However, due to the scarcity and size of suitable human tissue samples, it is difficult to repeat exposure experiments using the same batch of tissue. Thus, it is necessary to perform such studies with a small number of human samples. While it is known that inherited and environmental individual differences occur in humans, it is unclear whether the data obtained using small numbers of human samples are applicable to general toxicity-related gene expression research. Information is also lacking on the extent of individual differences in gene expression. To understand gene expression patterns using human tissues that originate from few individuals, it is important to examine the individual differences in gene expression of cultured human tissues. The use of human tissues collected during surgical procedures is restricted in Japan. It is also prohibited to use human organs removed for organ transplantation from a brain dead donor but not transplanted into a recipient, even if the donor had expressed a willingness to donate the organ to research and the organ was kept in very good condition post-mortem. Japanese tissues are not available from any commercial source. Moreover, the National BioResource Center of Japan (http://www.nbrp.jp) does not supply Japanese human tissues that have been harvested by surgery. Therefore, to use primary Japanese human tissues, researchers must prepare themselves. In addition, it is becoming more and more difficult to obtain primary tissues from celiotomy incision, as the current trend in the treatment of solitary renal or urinary tract tumors is laparoscopy, which is not suitable for the harvesting of tissues for biochemical research. In this study, we used the cultured cells because warm

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ischemic time was often more than 30 minutes and thereby the quality of RNA extracted from the kidney was defective without cell viability recovery by culturing for a few weeks. The kidney has many functions, such as the excretion of soluble waste substances, homeostatic maintenance, endocrine secretion, and metabolism. It is also known that some of these functions have genetic polymorphisms, which result in individual differences. Therefore, for our toxicogenomic study, it is important to collect gene expression data on all subjects, to understand these individual differences. In this study, we analyzed the gene expression patterns of primary renal tubular cells, and searched for differential gene expression among individuals. This is the first study to examine individual-based differences in the gene expression of primary cultured cells from Japanese subjects.

2. Materials and Methods

2.1 Subjects and Sampling of Renal Tubular Cells

Japanese patients of at least 18 years of age, who were admitted to the Jichi Medical School Hospital for renal resection of a confirmed solitary tumor in one kidney, the renal pelvis or the urethra, were eligible for this study. Patients with renal dysfunction (i.e., serum creatinine levels > 2.0 mg/ml or abnormalities in serum levels of sodium, potassium, or chloride) were excluded. Our institutional review boards, including the Bioethics Committee of Jichi Medical University and the Bioethics Committee for Human Gene Analysis, approved the study protocol. All patients or their legally authorized representatives gave written informed consent before enrolment in the study, which was carried out in accordance with the principles of the Declaration of Helsinki (as revised, 1996). The preparation and cryopreservation of the primary cultured cells were conducted using previously described methods, and the function and formation of these cells were confirmed previously [3]. In brief, a few grams of tissue were chopped and washed with Euro-Collins solution (Kobayshi Pharmaceutical Co., Ltd., Osaka, Japan). After 60 min of continuous agitation in an intracellular-like solution with 1,500 U/ml dispase (Godo Shusei Co., Ltd., Tokyo, Japan), the cells were incubated in 0.05% trypsin and 0.53 mM sodium ethylenediamine tetraacetate (EDTA) (Invitrogen Corp., Carlsbad, CA, USA) at room temperature. The cells showed a uniform morphology of epithelial cells, which suggested that the purified cells were of uniform origin and characteristics. Expression analysis revealed that five kidney-specific genes-KL, SLC17A3, AQP2, SLC22A2, and KCNJ–were present in these cells. The cells exhibited gamma-GTP enzymatic activity and Glut2 antigen expression, which suggests renal tubular origin. Thus, the majority of the purified and cultured cells originated from the proximal renal tubule and retained at least some of the characteristics of the original tissue. Details of the individuals and sample preparation are described in the supplemental data.

2.2 Gene Expression Analysis

Primary renal cortical cell cultures were derived from 11 individuals ($n = 3$ cultures for each individual) and grown to confluence before harvesting. Total RNA samples were extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The purified RNA samples were quantified on a U-2000 spectrometer (Hitachi Instruments Service Co., Ltd., Tokyo, Japan). Double-strand cDNA was synthesized from 20 μg of purified RNA and used to prepare biotin-labeled complementary RNA for hybridization on the Test3, HG-U133A, and HG-U133B microarray chips of the GeneChip system (Affymetrix, Santa Clara, CA, USA), which contain 45,000 oligonucleotide probe sets that correspond to approximately 39,000 transcripts. Hybridization and washing of the arrays and detection of the signals were performed using the GeneChip system according to the manufacturer's instructions (Affymetrix). We measured the 3'-end to 5'-end ratios of housekeeping genes, such as β-actin and GAPDH. This ratio gives an indication of the integrity of the starting RNA, efficiency of first-strand cDNA synthesis, and *in vitro* transcription of cRNA. The signal obtained for each probe set reflects the probe sequences and their hybridization properties (GeneChip Expression Analysis Technical Manual, Affymetrix). When the 3'-end to 5'-end ratio was > 3.0, the data were excluded from analysis. Details of the cDNA, cRNA preparation, labeling, hybridization and scanning procedures are described in the supplemental data.

2.3 Normalization

Normalization was performed using the GeneSpring® version 7.1 software (Silicon Ge-

Table 1 Patient characteristics.

UPN	Age	Gender	Prior Treatment	Prescribed Medication	Histological Diagnosis	Histological Findings other than tumor
#01	78	M	No	cefcapene	TCC	normal
#02	66	M	No	No	RCC	normal
#03	57	M	No	tamsulosin, brotizolam	TCC	normal
#04	35	M	No	No	RCC	normal
#05	66	F	No	No	RCC	normal
#06	47	M	No	No	RCC	normal
#07	77	M	No	No	RCC	normal
#08	67	M	No	No	TCC	normal
#11	85	F	No	brotizolam	RCC	normal

M, male; F, female; TCC, transitional cell carcinoma; RCC, renal cell carcinoma; UPN, unique patient number

Table 2 Clinical laboratory findings for subjects included in the analysis.

UPN	BUN (mg/dl)	Cr (mmole/l)	Na (mmole/l)	K (mmole/l)	Cl (mmole/l)
#01	16	0.86	142	3.8	104
#02	13	0.73	139	4.4	103
#03	13	0.84	144	4.1	106
#04	14	0.80	139	4.1	101
#05	15	0.91	139	3.9	102
#06	13	0.83	140	4.1	105
#07	18	0.78	140	4.1	105
#08	14	0.79	140	4	103
#11	13	0.55	138	3.8	102
Normal range	8-20	0.63-1.03	136-148	3.6-5.0	96-108

UPN, unique patient number; BUN, blood urea nitrogen; Cr, serum creatinine; Na, sodium; K, potassium; Cl, chloride

netics, Redwood, CA, USA) and was applied in two steps: 'per chip normalization' and 'per gene normalization'. For per chip normalization, all of the expression data on a chip were normalized to those of the housekeeping genes using the subset list 'betterHK.txt'[4]. For per gene normalization, each transcript was normalized to the median expression level across the samples.

2.4 Statistical Analysis

The transcripts were accepted only if 60% or more of the samples indicated 'present' or 'marginal' flags, and the data were further refined using Cross Gene Error Modeling[5]. Transcripts with control signals that were lower than the calculated base/proportion value were removed from the analysis. The fold-changes and C.V. values among the individuals were calculated as follows:

$$C.V.\,(\%) = \frac{\text{standard deviation}}{\text{mean of gene expression}} \times 100$$

Based on the null hypothesis that there was no difference on the average of the gene expression between individuals, p value was calculated using a non-parametric test and the Benjamini and Hochberg false discovery rate procedure for multiple testing[6].

2.5 Comparison of the Gene Expression with Cancer Genome Anatomy Project (CGAP) Resource

Among gene expression data from 27 samples in total, the genes with 'positive' or 'marginal' flag in at least 60% of samples were listed in 'high expressing gene list'. Rests of them was listed in 'negative expressing gene list'. On the other hand, the gene expression data originated from normal kidney was obtained from CGAP resource. Based on the null hypothesis that there was no relation between our experimental data and CGAP expression data, Fisher exact test was conducted.

3. Results

3.1 Subjects

We analyzed the comprehensive gene expression profiles of primary cultures of renal cortical cells derived from nine Japanese subjects who were admitted for kidney resection. **Table 1** and **Table 2** summarize the patients' characteristics and the clinical laboratory find-

Table 3 The number of genes exhibiting differential expression in the primary cultured renal cortex cells of one or more individuals. *Fold-change in expression level compared with the mean expression level for all subjects. **Number of individuals that exhibit differential expression for the same gene.

Number of individuals**	Up or down regulation	Fold change* × 2	× 3	× 4	× 5
1	Up	2,885	456	85	32
	Down	566	799	404	264
2	Up	649	36	12	5
	Down	620	127	42	23
3	Up	138	6	1	5
	Down	209	1	10	4
4	Up	7	1	0	0
	Down	24	3	2	1
5	Up	0	0	0	0
	Down	1	0	0	0

Up, up-regulation; Down, down-regulation.

ings for the nine subjects (seven males and two females). None of the subjects received chemotherapy or radiation prior to surgery. The prescribed medications are listed in Table 1. No patient suffered from hypertension or diabetes mellitus and none of the patients required medication that would affect renal function. Six patients had renal cell carcinoma and three had transitional cell carcinoma. All of the clinical laboratory tests were within the normal range, with the exception of serum creatinine for UPN #11.

3.2 Microarray Analysis

Using microarray analysis, we classified the genes according to the fold-change in expression as compared to the mean expression level, to identify individual differences. Of approximately 39,000 transcripts analyzed, 2,885 exhibited two-fold up-regulation, while 566 exhibited two-fold down-regulation in at least one subject. Similarly, 456, 85, and 32 transcripts exhibited three-, four-, and five-fold up-regulation of expression, respectively, while 799, 404, and 264 transcripts exhibited three-, four-, and five-fold down-regulation, respectively, for at least one subject in comparison with the mean expression level. Two-fold up-regulated differential expression was detected in 2, 3, 4, and 5 subjects for 649, 138, 7, and 0 transcripts, respectively, while 620, 209, and 24 transcripts, respectively, were down-regulated (**Table 3**). We also classified the genes according to the calculated p-values, which indicate significantly different expression levels among the subjects: $p < 0.05$, 8,596 transcripts; $p < 0.01$, 4,319 transcripts; $p < 0.001$, 1,212 transcripts (**Table 4**). The estimated numbers of false-positive transcripts, which by chance give values less than the indicated p-value, were: 748 for $p < 0.05$; 149 for $p < 0.01$; and 15 for $p < 0.001$ (Table 4).

Table 4 Classification of genes by p-value indicating significantly different expression levels among the subjects.

p-value	Number of genes	Number of false-positive genes
$p < 0.05$	8,596	748
$p < 0.01$	4,319	149
$p < 0.001$	1,212	15

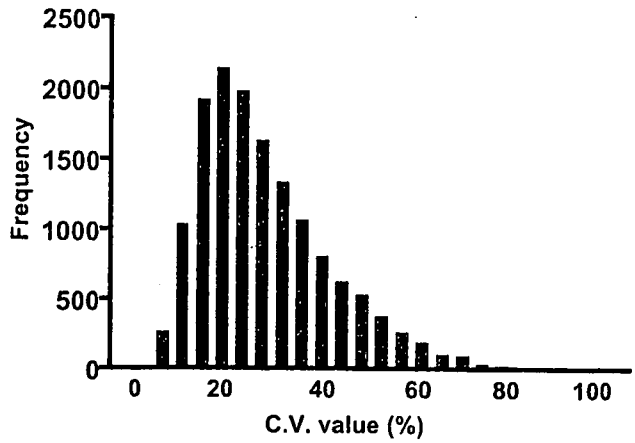

Fig. 1 Classification of genes according to the coefficient of variation (C.V.) among the subjects. Histogram showing the number of transcripts with the indicated C.V. levels

Table 5 The number of transcripts with C.V. values that exceeded the indicated thresholds.

C.V. value (%)	Number of genes
> 50	1,338
> 70	212
> 80	96
> 90	44
> 100	27

We examined the coefficient of variation (C.V.) for the gene expression levels among the subjects (**Fig. 1**). The C.V. was > 50% for 1,338 transcripts, > 70% for 212 transcripts, and > 90% for 44 transcripts (**Table 5**). Included among the transcripts with C.V. values > 90% were *PDZK1*, *UGT1A8*, and *UGT1A9*, which are associated with drug metabolism.

3.3 Comparison of the Gene Expression with CGAP Resource

According to Section 2 Fisher exact test was conducted. p-value was below 0.0001 and the null hypothesis was rejected. Therefore, it was considered that there was a significant correlation between our experimental data and CGAP expression data.

4. Discussion

In this study, we used microarray analysis to investigate individual differences in the gene expression of primary cultured renal cortical cells from Japanese subjects. The current social structure and environment in Japan are not favorable to the use of human tissues collected during surgical procedures in biochemical research. Therefore, Japanese tissues are not available from commercial sources or from the National BioResource Center. Primary Japanese human tissues can be used only for limited projects in limited facilities. It has become more and more difficult to obtain kidney samples from surgery, due to recent changes in operational procedures. Therefore, studies such as the present one, using Japanese primary tissues, are rare. Primary cultures were prepared from the non-tumor portions of extirpated kidneys. The non-tumor portions showed no pathological abnormalities. All of the subjects had normal Na, K, and BUN values and none of the subjects exceeded the upper limit for creatinine (Cr) content. There was no evidence to suggest any underlying disease, such as hypertension or diabetes mellitus, which might lead to renal dysfunction. No patient was prescribed renal-toxic medicines before the operation. Thus, the renal tissues that we harvested were regarded as normal. However, it is possible that these tissues had an unknown genetic factor(s) and they may have been exposed to environmental factors related to carcinogenesis, as the samples were from the non-tumor tissues of cancer patients. As primary cultured renal cortical cells consist mainly of renal tubular cells, the gene expression profiles in this study were expected to reflect those of renal tubular cells [3]. We analyzed the gene expression patterns of nine primary cultures of renal tubular cells. Of the 39,000 transcripts analyzed by the microarray system, 8,569 (approximately 22%) exhibited significantly different expression levels $p < 0.05$ among the subjects. In pharmacokinetics, high inter-subject variability is considered present when the C.V. of a pharmacokinetic parameter (such as the maximum plasma concentration, the area under the plasma concentration-time curve, or clearance) is $> 50\%$ [7),8)]. When we classified the genes based on their C.V. values, we found that 44 transcripts, including the *PDZK1*, *UGT1A8*, and *UGT1A9* transcripts, had C.V. values $>$ 90%. *PDZK1* encodes a protein that is regulated by dietary phosphate, increases phosphate uptake through a sodium/phosphate cotransporter, and plays an important role in cellular phosphate regulation [9)]. The *UGT1A8* and *UGT1A9* transcripts belong to the *UGT1A* glycosyltransferase gene family and catalyze the conjugation of glucuronic acid to various endobiotics and xenobiotics [10)]. Thus, some individual differences are seen for genes that encode drug-metabolizing enzymes or transporters. These genes may be associated with individual differences in drug metabolism, distribution, and excretion.

We analyzed the gene expression profiles of primary cultures of cells from Japanese individuals, to determine individual differences using various statistical methods. Caution is necessary in the identification of these genes, which showed individual-based differences or were listed as outliers form the data obtained from primary renal cell cultures. Thus, these data are useful in interpreting and understanding the toxicogenomics of primary cultured renal cortical cells from Japanese subjects.

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An approach to elucidate potential mechanism of renal toxicity of arsenic trioxide

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Objective. **To investigate arsenic trioxide's renal toxicity, we analyzed the gene-expression patterns of primary renal and human kidney cells (HEK293 cell line) following exposure to arsenic trioxide. Moreover, we examined a potential renal toxic mechanism(s) of arsenic trioxide by using a toxicity-related gene and investigated potential treatments to reduce the renal toxicity of arsenic trioxide.**

Materials and Methods. **Arsenic trioxide was exposed to primary renal and HEK293 cells, and the gene-expression analysis was conducted using DNA microarray. Then, reactive oxygen species inhibitors or α-lipoic acid were added to HEK293 cells exposed arsenic trioxide and cell viability was determined.**

Results. **Expression of *HMOX1* mRNA increased in a time- and dose-dependent manner, and translation of heme oxygenase 1 protein was also induced. Arsenic trioxide-induced cytotoxicity was inhibited by reactive oxygen species inhibitors. Moreover, superoxide anion was detected in arsenic trioxide-treated HEK293 cells. α-Lipoic acid ameliorated arsenic trioxide-induced cytotoxicity and reduced superoxide anion production in HEK293 cells, whereas it had no effect in promyelocytic leukemia cells (HL-60 cells and NB4 cells) and myeloma cells (KMS12BM cells and U266 cells).**

Conclusions. **Arsenic trioxide-induced renal toxicity is strongly associated with the increased expression of *HMOX1*, and the cytotoxic mechanisms of arsenic trioxide involves reactive oxygen species production as well as another pathway. These preliminary results suggest that α-lipoic acid may be a suitable agent for prevention or treatment of arsenic trioxide-induced renal toxicity.** © **2007 International Society for Experimental Hematology Published by Elsevier Inc.**

Microarray technology, an inclusive gene-expression analysis tool, can be applied to new drug development [1], gene mutation analysis of diseases [2,3], and toxicity evaluation following chemical exposure [4,5]. This new technology can measure the expression levels of thousands of transcripts in a single experiment and lead to a new field of study, toxicogenomics, which brings together the sciences of genomics and toxicology [6–8]. We used toxicogenomics to clarify renal toxicity mechanisms and to identify biomarkers that predict renal toxicity. Human primary renal cortical cells (PRCC) were exposed to different drugs or chemicals that cause human renal toxicity, and the associated gene-expression patterns were analyzed by microarray technology.

Arsenic is a ubiquitous environmental element; its ingestion can cause adverse health effects, including skin and lung cancer [9,10], neurotoxicity [11], and peripheral vascular disease [12,13]. Arsenic is eliminated by urinary excretion, therefore, it can accumulate in the kidneys. Keith et al. [14] reported that arsenic compounds are cytotoxic to renal tissue at high concentrations. Recently, arsenic trioxide has been used for treatment of relapsed or refractory acute promyelocytic leukemia and developed for treatment of multiple myeloma, and was reported to cause renal injury [15–19], such as hypourea, elevated serum creatinine and blood urea nitrogen, and proteinuria, in clinical studies. Arsenic trioxide-induced cell death may be caused by one or more mechanisms, including activation of caspase, enhanced generation of reactive oxygen species (ROS),

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opening of mitochondrial permeability transition pores, suppression of apoptosis inhibitory Ras/mitogen-activated protein kinase cascade, and enhanced translocation of promyelocytic leukemia protein to nuclear bodies [20].

Antioxidants have been shown to reduce sister chromatin exchange, micronuclei, apoptosis, and cytotoxicity in arsenite-exposed cells [21], and the antioxidant α-lipoic acid has been used as a detoxification agent for heavy metal poisoning and has been implicated in age-associated cognitive decline [22]. The precise mechanism of arsenic trioxide-induced cell death is still unclear. In the present study, we used toxicogenomics to investigate gene-expression patterns after exposure of PRCC and HEK293 cells to arsenic trioxide in order to identify the genes related to its renal toxicity. Moreover, we elucidated a potential renal toxic mechanism(s) of arsenic trioxide by using a toxicity-related gene and investigated potential treatments to reduce the renal toxicity of arsenic trioxide.

Materials and methods

Chemicals

Arsenic trioxide, hypoxanthine, hydrogen peroxide, and superoxide dismutase were obtained from Wako Pure Chemical Industries (Osaka, Japan). α-Lipoic acid, diphenylene iodonium (DPI), 4,5-dihydro-1,3-benzene disulfonic acid (Tiron), xanthine oxidase, and catalase were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

PRCC from Japanese subjects were prepared according to a previously reported method and were cultured in William's medium E supplemented with 20% fetal bovine serum [23], 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human embryonic renal cell line, HEK293 cells (RIKEN BioResource Center, Tsukuba, Japan), human acute promyelocytic leukemia cell line NB4 and HL-60 cells (American Type Culture Collection, Manassas, VA, USA), human multiple myeloma cell line U266 cells (IgE, λ-light chain-producing; American Type Culture Collection) and KMS12BM (immunoglobulin nonproducing, kindly provided by Prof. T. Otsuki, Kawasaki Medical School, Japan) were each cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C.

Arsenic trioxide treatment

For DNA microarray analyses, subconfluent PRCC and HEK293 cells were treated for 10 minutes and 1, 6, and 24 hours with 0.1 μM arsenic trioxide in the appropriate culture media described above. For cell viability testing, cells were treated for 48 hours with various concentration of arsenic trioxide in the appropriate culture media.

Superoxide anion treatment

Superoxide anion was produced by adding hypoxanthine and xanthine oxidase to the culture medium. HEK293 cells were treated for 24 hours with 1 mM hypoxanthine and 0.01 to 2 mU/mL xanthine oxidase in the culture media.

Hydrogen peroxide treatment

HEK293 cells were treated for 24 hours with 1 to 200 μM hydrogen peroxide in the culture medium.

Gene-expression analysis

Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Double-stranded cDNA was synthesized from 20 μg purified RNA and was used to prepare biotin-labeled cRNA for hybridization on the Test3, HG-U133A, and HG-U133B DNA microarray chips (GeneChip System; Affymetrix, Santa Clara, CA, USA). The hybridization and washing of the microarray chips and detection of the signals were performed using the GeneChip system according to manufacturer's instructions. We measured the ratio of the 3′ to 5′ ends of housekeeping genes, such as β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an indicator of the integrity of the starting RNA and the efficiency of first-strand cDNA synthesis and in vitro transcription of cRNA. The signal obtained for each probe set reflected the probe sequences and hybridization properties (*GeneChip Expression Analysis Technical Manual*; Affymetrix). The data from experiments in which the 3′- to 5′-end ratio was >3 were excluded from analysis.

Quantitative polymerase chain reaction

Double-stranded cDNA was used for quantitative polymerase chain reaction (PCR) with the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). The specific primer sets were purchased from Applied Biosystems, and detection and quantification were performed according to manufacturer's instructions. To control for variations in the amount of DNA available for PCR in the different samples, gene expression of the target sequence was normalized to the expression of an endogenous control, GAPDH.

Western blot analysis

Cells were lysed in CelLytic MT reagent an the proteins (50 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10–20% Tricine gel; Invitrogen, Carlsbad, CA, USA) and then transferred to polyvinylidene difluoride membranes (Invitrogen). Nonspecific binding was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. The membrane was subsequently incubated for 1 hour at room temperature with anti-human heme oxygenase 1 goat polyclonal antibody (Heme Oxygenase 1 [C-20]; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 100-fold in Tris-buffered saline containing 0.1% Tween 20 and 3% skim milk. After incubation with alkaline phosphatase-conjugated anti-goat immunoglobulin (Invitrogen), the blots were visualized using the Western Breeze chemiluminescent substrate (Invitrogen). The signals were quantified by densitometry using the 1D Imaging Analysis software (Eastman Kodak, Rochester, NY, USA).

Cell viability

Cell viability was measured with a Premix WST-1 cell proliferation assay system (Takara Bio, Otsu, Japan). Cells were treated with the different compounds and incubated for 20 or 44 hours. After incubation, 10 μL WST-1 reagent were added to each

well, and plates were incubated for 4 hours. The optical density of each well was determined at 440 to 600 nm.

Transfection

The HMOX-1 cDNA prepared from the total RNA of HEK293 cells treated with arsenic trioxide was amplified by PCR using the primer pair 5′-TACTCGAGAGCACGAACGAGCCCAG-CAA-3′ and 5′-ATGGATCCACGGTAAGGAAGCCAGCCAA-GAG-3′. These primers created *Xho*I and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively, of the cDNA. The PCR product was subcloned using a TOPO TA cloning kit (Invitrogen), and the sequence was confirmed. To insert the *HMOX1* cDNA into the pCI mammalian expression vector (Promega, Madison, WI, USA), the PCR product was excised by digestion with *Xho*I and *Bam*HI (Takara Bio), gel-purified, and ligated into *Xho*I- and *Bam*HI-digested pCI, using T4 DNA ligase (Takara). The ligated plasmid was transfected into HEK293 cells, using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. After 48 hours, cell viability testing and Western blotting were performed.

Superoxide anion measurement

The cells were suspended in 0.9% NaCl and were treated with 10 μM arsenic trioxide, 10 μM α-lipoic acid, or both. Superoxide anion was detected at 5 minutes after the addition of 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazine-3-one (MCLA, final concentration, 1 μM; Tokyo Kasei Kogyou, Tokyo, Japan) with the Xenogen In Vivo imaging system (IVIS; Xenogen, Alameda, CA, USA).

Statistical analysis

Data analysis was performed using GeneSpring version 7.1 software (Silicon Genetics, Redwood, CA, USA) and included normalization, Cross Gene Error Modeling, and K-means clustering. Initially, values <0.01 were set to 0.01. For normalization within each chip, all expression data on a chip were normalized to those of the housekeeping genes. For normalization per gene, each transcript was normalized to the median expression level across the samples at time zero. Transcripts were accepted only if 60% or more of the samples indicated present or marginal flags, and the data were further refined using Cross Gene Error Modeling. Transcripts with control signals lower than the calculated base/proportion value were removed from the analysis. Genes that showed a greater than twofold or less than 0.5-fold change in expression at one condition were subjected to K-means clustering.

All numerical data are expressed as mean ± standard errors. Statistical analysis was performed by Student's *t*-test. A value of $p < 0.05$ was considered significant. Statistical calculations were performed using the computer program StatView (version 5.0; SAS Institute, Cary, NC, USA).

Results

HMOX1 is involved in arsenic trioxide-induced renal toxicity

Genes whose expression changed in both PRCC and HEK293 cells after treatment with arsenic trioxide were chosen for cluster analysis. Based on the similarity of their expression patterns, 73 genes were grouped into five clusters. Twenty-four genes in one of the clusters showed increased expression in a time-dependent manner. The functions of 17 of the 24 genes have been well established (Table 1). In these genes, *HMOX1* was confirmed as an arsenic trioxide-induced gene by quantitative PCR (Fig. 1A). The relative expression of *HMOX1* at 0.1, 0.5, and 2 μM arsenic trioxide was increased 2.2-, 11.7-, and 33.5-fold, respectively, in PRCC and 1.2-, 8.3-, and 224.9-fold, respectively, in HEK293 cells (Fig. 1B). These results clearly show that the induction of *HMOX1* by arsenic trioxide was exposure time- and dose-dependent in both PRCC and HEK293 cells.

Table 1. Gene clusters upregulated in a time-dependent manner by arsenic trioxide treatment in both primary renal cortical cells and HEK293 cells

Gene symbol	Biological process	Molecular function
SEPT11	Cytokinesis, cell cycle	GTP binding
HMOX1	Heme oxidation, stress response	Heme oxygenase (decyclizing) activity
CLTB	Intracellular protein transport	Calcium ion binding
AP1S1	Intracellular protein transport	Protein binding, protein transport activity
GSR	Electron transport, glutathione metabolism	Glutathione-disulfide reductase activity
MAPK1	Signal transduction, response to stress, induction of apoptosis, cell cycle	MAP kinase activity, protein serine/threonine kinase activity
BAX	Induction of apoptosis	
FHL1	Cell growth, cell differentiation	Zinc ion binding
MYO6	Striated muscle contraction	Motor activity, actin binding
FGFR1	MAPKKK cascade, skeletal development	Protein serine/threonine kinase activity
H41	Cell proliferation	
ROD1	Nuclear mRNA splicing, morphogenesis	Nucleic acid binding, RNA binding
FN1	Acute-phase response, cell adhesion	Extracellular matrix structural constituent
SEP6	Protein complex assembly	Phospholipids binding
FLJ20986	Cation transport, metabolism	Magnesium ion binding, ATP binding
CLIC4	Ion transport, chloride transport, apoptosis	Voltage-gated chloride channel activity
LOC51762	Small GTPase-mediated signal transduction	GTPase activity, GTP binding

ATP = adenosine triphosphate; GTP = guanosine triphosphate; MAP = mitogen-activated protein; MAPKKK = MAP Kinase Kinase Kinase.

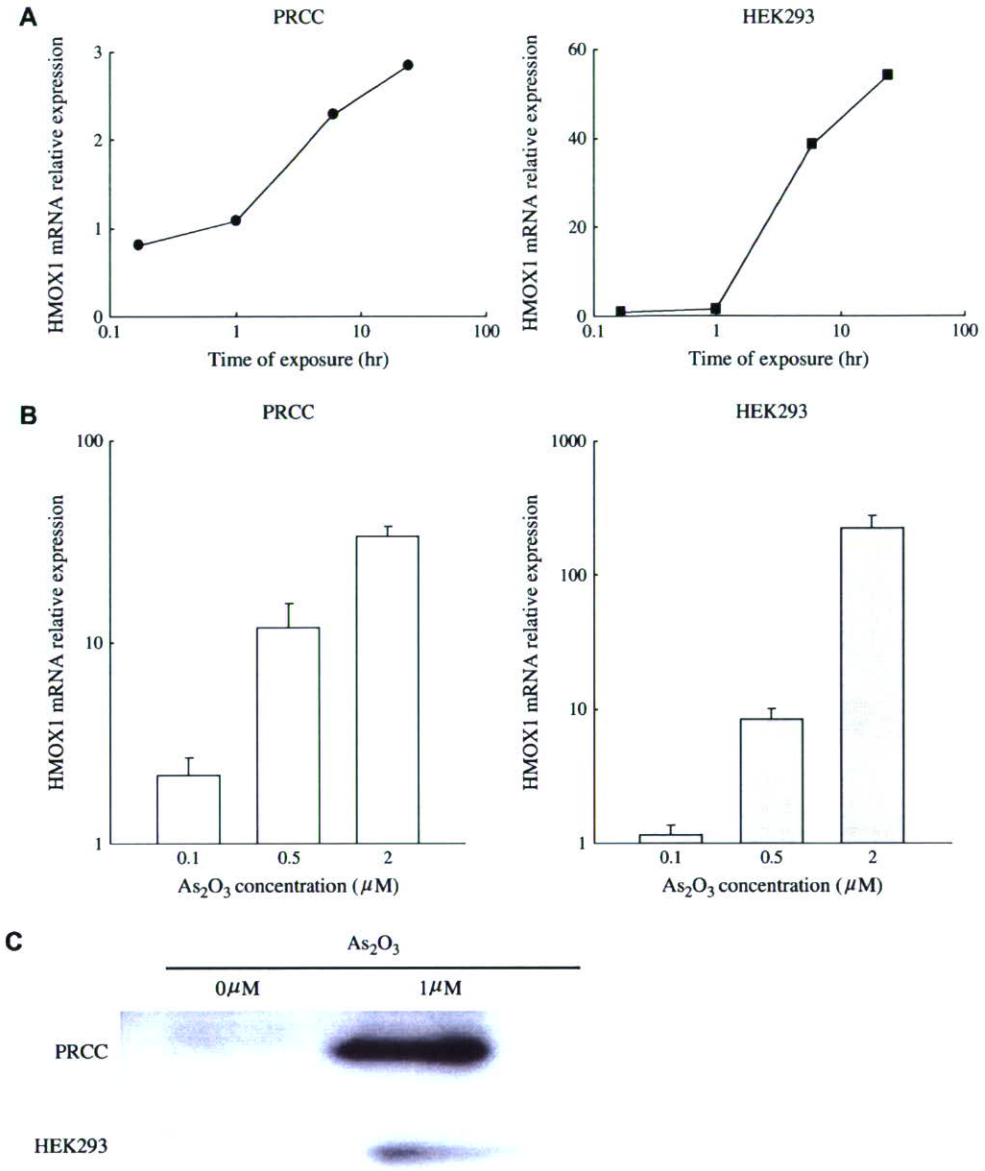

Figure 1. *HMOX1* gene expression and induction of heme oxygenase 1 protein expression after arsenic trioxide (As_2O_3) exposure in primary renal cortical cells (PRCC) and HEK293 cells. **(A)** PRCC were treated for 10 minutes and 1, 6, and 24 hours with 0.1 μM As_2O_3, and HEK293 cells were treated for 10 minutes, and 1, 6, and 24 hours with 1 μM As_2O_3. mRNA expression level of *HMOX1* was determined by quantitative polymerase chain reaction. **(B)** PRCC or HEK293 cells were treated for 24 hours with 0.1, 0.5, and 2 μM As_2O_3, and the mRNA expression level of *HMOX1* was determined by quantitative PCR. The assay was performed in quadruplicate. Data are presented as mean ± standard error and are expressed relative to the sample treated for 24 hours with medium. **(C)** PRCC or HEK293 cells were treated for 24 hours with 1 μM As_2O_3. Total cell extracts (50 μg/lane protein) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blot analysis was performed with anti-heme oxygenase 1 antibody.

As *HMOX1* mRNA was induced by arsenic trioxide exposure, heme oxygenase 1 protein expression in PRCC and HEK293 cells was assessed by Western blotting. Heme oxygenase 1 protein was clearly detected from PRCC and HEK293 cells exposed to arsenic trioxide, whereas only faint expression of heme oxygenase 1 protein was detected without arsenic trioxide treatment (Fig. 1C). Therefore, arsenic trioxide induced heme oxygenase 1 protein in PRCC and HEK293 cells. To examine the role of *HMOX1*, cells overexpressing *HMOX1* (HEK293-*HMOX1*) were treated with different concentrations of arsenic trioxide for 48 hours, and cell viability was compared with that of mock-transfected HEK293 cells. The viability of both cell populations decreased with increasing arsenic trioxide concentration (Fig. 2). The viability of HEK293-*HMOX1* cells exposed to 0.5, 1, 2, and 5 μM arsenic trioxide was 67.8%,