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萌芽的先端医療技術推進研究総事業（トキシコゲノミクス分野）
遺伝子発現の網羅的解析によるワクチンの新しい安全性評価に関する研究
分担研究報告書

研究課題：DNA マイクロアレイクラスター解析による百日せきワクチンの毒性関連遺伝子の同定

分担研究者　渡辺慎哉　東京医科歯科大学　助教授
分担研究者　野村信夫　産業技術総合研究所・生物情報解析研究センター・副センター長

研究要旨
百日せきワクチンの安全性評価法の確立を目指して、従来の動物を用いた安全性試験にかわる、感度が高く、科学的根拠が得られる新しい方法の開発のために、ワクチンの毒性に関連する遺伝子を同定する目的で DNA マイクロアレイクラスター解析をおこなった。ワクチン接種ラットより摘出した合計１９２臓器についてクラスター解析を行なったところ、ワクチン接種１日目で肝臓に高発現する AGP、Lbp、Hpx の３遺伝子が毒性に密接に関連していることを明らかにした。

A. 研究目的

DNA マイクロアレイはサンプル中に発現する遺伝子をハイブリダイゼーション法の原理を応用することにより検出するものである。この方法を用いてワクチン接種に伴う遺伝子発現変化を検出することにより、毒性関連遺伝子を網羅的に解析することができると考えられる。今日マイクロアレイは医薬品の副作用解析のために盛んに用いられているが、ワクチンの副反応の原因となる毒性の評価についても、新たな知見を得ることができるとともに理想的な評価法となりうると思われる。ワクチンの安全性評価法の確立を目指して、従来の動物を用いた安全性試験にかわる、感度が高く、科学的根拠が得られる新しい方法の開発のために、ワクチンの毒性に関連する遺伝子をDNA マイクロアレイで取得した遺伝子発現プロファイルを基にしたクラスター解析より同定する。

B. 研究方法

１）動物

８週齢の Wister 雄ラットを SLC より購入して使用した。

2）ワクチンおよび毒素

毒性参照用ワクチン（RE）は国立感染症研究所に準備されている標準品を使用した。粉末標準品を 12 ml の生理食塩水で融解し、5 ml を腹腔内接種した。百日咳毒素（PT）は Wako より購入し、精製百日せきワクチン(PV)は化学及び血清療法研究所より供与された。PT および精製百日せきワクチンは PT 含有量を 5 μg/ml に調整し、5 ml を腹腔内に接種した。生理食塩水（SA）をコントロールとして 5 ml 腹腔内に接種した。

3）RNA 抽出

ワクチンおよび毒素が投与されたラットから肝臓左外葉、左肺、左腎臓、脳を接種後１～４日に採取した。臓器は即座に液体窒素中で凍結させ、ISOGEN 試薬（Nippon Gene）中で溶解させた。Total RNA を抽出し、Ambion 社の Poly(A) RNA Purist kit を用いて Poly(A)+RNA を精製した。

4）DNA マイクロアレイ解析

Poly(A)+RNA から逆転写酵素を用いた逆転写反応を行なう際に、共通リファレンスは Cyanine3 を、サンプル接種した RNA は Cyanine5 を取り込ませてサンプルをラベルした。ラベルされたサンプルをスライドグラス上に固定された 11,464 個の遺伝子特異的配列オリゴ DNA（８０ mer）と結合（ハイブリダイゼーション）させた。各スポットの蛍光強度の比率（Cyanine3 と Cyanine5 の比率）をスキャナーおよび解析ソフトで数値化することにより、共通リファレンスに対する各遺伝子の発現量比を検出した。

C．研究結果

1）DNA マイクロアレイ解析

百日せきワクチンの毒性に関して網羅的遺伝子発現解析を行なうために、接種後１、２、３、４日目に肝臓、肺、脳、腎臓を摘出した。動物実験は繰り返し２度行ない、合計１９２臓器から poly(A)+RNA を抽出した。逆転写酵素を用いた逆転写反応を行なう際に、共通リファレンスを Cyanine3 で、サンプル接種した RNA を Cyanine5 でラベルした。その後それらを混合し、スライドグラス上に固定された 11,464 個の遺伝子特異的配列オリゴ DNA（８０ mer）と結合（ハイブリダイゼーション）させた。各スポットの蛍光強度の比率（Cyanine3 と Cyanine5 の比率）をスキャナーおよび解析ソフトで数値化することにより、共通リファレンスに対する各遺伝子の発現量比を検出した。

取得した各サンプルの遺伝子発現プロファイルを基にして二次元階層クラスター解析を行なった。この結果それぞれの臓器ごとにクラスターを形成した。４つの臓器のうち、サンプル（RE、PT、PV、SA）接種による遺伝子発現の変化が最も鮮明であったのは肝臓であった。これらの 192 サンプルのクラスター解析から肝臓が遺伝子発現解析をさらに進めるのに適していると考えられた。(図７)

2）ワクチン毒性に関連した遺伝子群

肝臓での遺伝子発現をさらに詳細に解析した。遺伝子発現量比に基づくクラスター分類では、RE 接種群は明らかに、他のサンプル接種群と大きな違いが認められた。（図８）接種日からの時間経過に伴う発現様式の変化について解析したところ、１日目、２日目には PT 接種群は生理食塩水を接種した群と大差なかったが、３日目、４日目になると RE 接種群の示す遺伝子発現様式に類似してくることが明らかになった。RE と PT 接種群が類似した結果を得ることは、動物を用いた白血球増加試験の結果とも一致し、包括的遺伝子発現解析がワクチンの安全性試験の指標になる可能性が示唆された。

次に RE 接種群と生理食塩水接種群とで著明な発現量変化をきたす 150 個の遺伝子を指標になる候補遺伝子として抽出した。このうち、61 遺伝子が代謝に関連しており、21 遺伝子がシグナル伝達系に関連したものであった。他に毒性反応性関連遺伝子、炎症関連遺伝子、ヒト疾患関連遺伝子が存在した。150 個の遺伝子の約３分の１は機能が未解明の遺伝子であった。（図９）

3）ワクチン毒性に関連した遺伝子の同定

RE 群と SA 群との差に基づく 150 の遺伝子に追加して、PT 接種群と PV 接種群とで著明な変化を認める遺伝子をさらに抽出し、その中でとくにワクチンの毒性に関連している可能性のある９遺伝子を特定した。これらの遺伝子は S100a8、Acmsd (2-amino-3-carboxymuconate-6-semialdehyde decarboxylase)、Sterol-12-alpha hydroxylase、AGP (alpha 1-acid glycoprotein)、Tat (tyrosine aminotransferase)、Lbp (lipopolysaccaride binding protein)、Phyh (Phytanoyl-CoA hydroxylase)、Hpx (hemopexin)、Got1 (glutamate oxaloacetate transaminase 1)の９遺伝子である。これらの遺伝子はすべて、RE 接種群で強発現し、PT 接種群では RE 接種群よりも発現は劣るものの PV や SA 接種群よりも有意に発現が亢進していた。これらの結果からこれら９個の遺伝子はワクチンの毒性に関連した遺伝子と考えられる。

これらの遺伝子のうちマイクロアレイ解析で毒性を特異的かつ再現よく検知できる３つの遺伝子 AGP、Lbp、Hpx を特定した。（図１０）

D．考察

ラット 10,490 遺伝子の網羅的解析から RE 接種により発現が大きく変化する 150 遺伝子を特定した。これらの遺伝子の中には、FABP や NaCT といった代謝関連遺伝子が含まれていた。これらの遺伝子は脂肪組織からエネルギーが産生される際の脂肪酸合成に関連する。また分担研究者、浜口による病理検査でみられた RE 接種による PAS 陽性グリコーゲン顆粒の

減少（図６）はグリコーゲン分解にともなうエネルギー産生によるものであると考える。これまでに毒性物質がグリコーゲン分解を誘導し、肝臓で PAS 陽性細胞の減少がおこることは報告されており、この可能性は高い。また MIF、AGP、IL-1βといった炎症関連遺伝子の発現が RE 接種により上昇していた。解毒に関する P450 分子の発現も上昇していた。これらの結果から RE 接種により肝臓で急性炎症が起こっていると考えられる。

アレイのクラスター分類で候補遺伝子として挙げられたもののうち、とくに毒性に密接に関連していると考えられるものとして、S100a8、Acmsd、Sterol-12-alpha hydroxylase、AGP、Tat、Lbp、Phyh、Hpx、Got1 に注目した。これらの９遺伝子はすべてワクチンの毒性と関連していると考えられるが、PT の濃度の変化に伴う影響を鋭敏に検出できる、AGP、Lbp、Hpx を特定した。AGP は 41-43kDa の糖蛋白で、様々な免疫反応に関連した機能、薬剤結合能などが報告されている。また血中 AGP が急性炎症期に５、６倍に上昇することが分かっている。Lbp も急性炎症反応物質で肝臓から主に産生され、炎症に伴い血中濃度が劇的に上昇する。Lbp は明らかに LPS と結合し、CD14 および TLR に結合する。これまでの報告から LPS が負荷されると Lbp の発現は横ばいから上昇する。Hpx は肝臓で産生される糖蛋白でヘムに高い接着能を示す。また急性炎症反応物質である。これら３つの遺伝子はみな急性炎症関連物質で RE、PT に含まれる毒性に特異的なものと考えられる。すなわちこれらの遺伝子は百日せきワクチンの毒性を検知するためのバイオマーカーとなりうると考えられる。

E. 結論

百日せきワクチンを接種したラット肝臓を用いて 11,464 個の遺伝子をクラスター分類した結果、AGP、Lbp、Hpx の３遺伝子が毒性に密接に関連していることを明らかにした。

F. 健康危険情報

該当なし

G. 研究発表

1) 論文発表

Fujita N, Miyamoto T, Imai JI, Hosogane N, Suzuki T, Yagi M, Morita K, Ninomiya K, Miyamoto K, Takaishi H, Matsumoto M, Morioka H, Yabe H, Chiba K, Watanabe S, Toyama Y,Suda T. CD24 is expressed specifically in the nucleus pulposus of intervertebral discs.
Biochem Biophys Res Commun.,2005, 338: 1890-1896.

Sakamoto A, Imai J, Nishikawa A, Honma R, Ito E, Yanagisawa Y, Kawamura M, Ogawa R, Watanabe S. Influence of inhalation anesthesia assessed by comprehensive gene expression profiling.
Gene., 2005, 356:39-48.

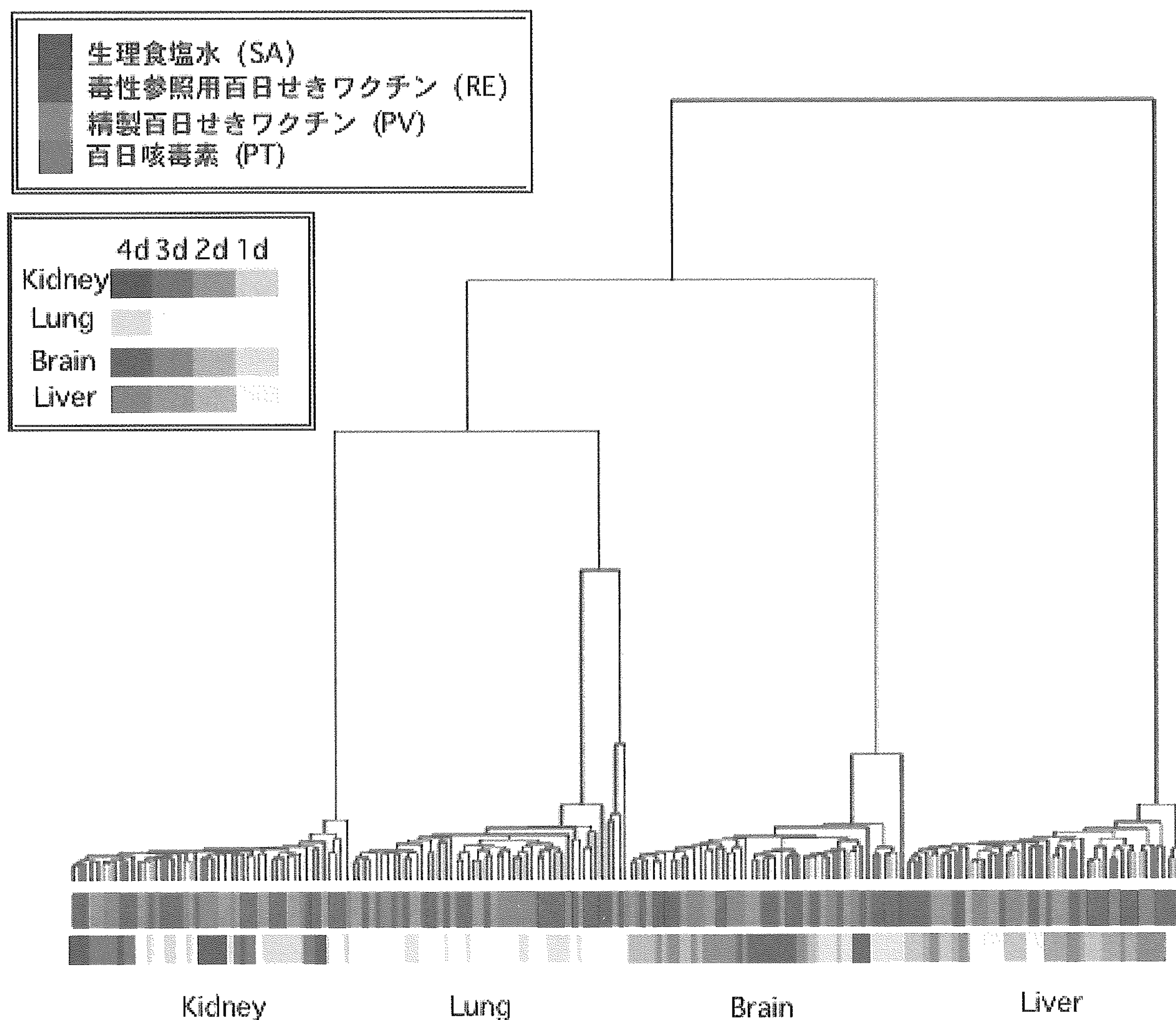

図７．ワクチン接種ラットの各臓器における遺伝子発現様式のクラスター解析

ラット腹腔に SA（生理食塩水）、RE（毒性参照用百日せきワクチン）、PV（精製百日せきワクチン）および PT（百日咳毒素）を接種した。肝臓、脳、肺、腎臓を 1d-4d で摘出し、遺伝子発現様式についてクラスター解析を行なった。

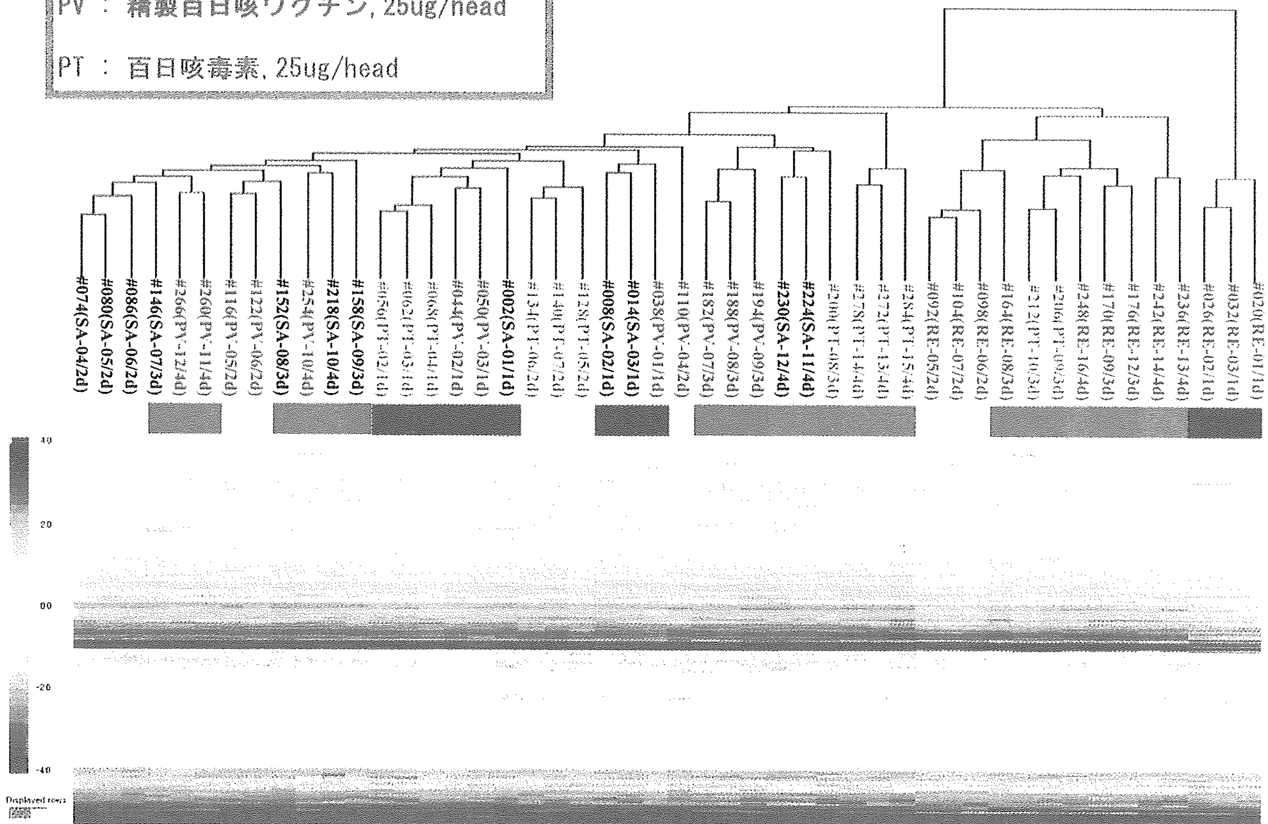

図８．肝臓における遺伝子発現様式のクラスター解析

ラット腹腔に SA（生理食塩水）、RE（毒性参照用百日せきワクチン）、PV（精製百日せきワクチン）および PT（百日咳毒素）を接種し、接種後１日～４日に肝臓を摘出し、遺伝子発現様式をクラスター解析した。赤および青の色調の強さでそれぞれ発現の増加及び減少を表す。

a

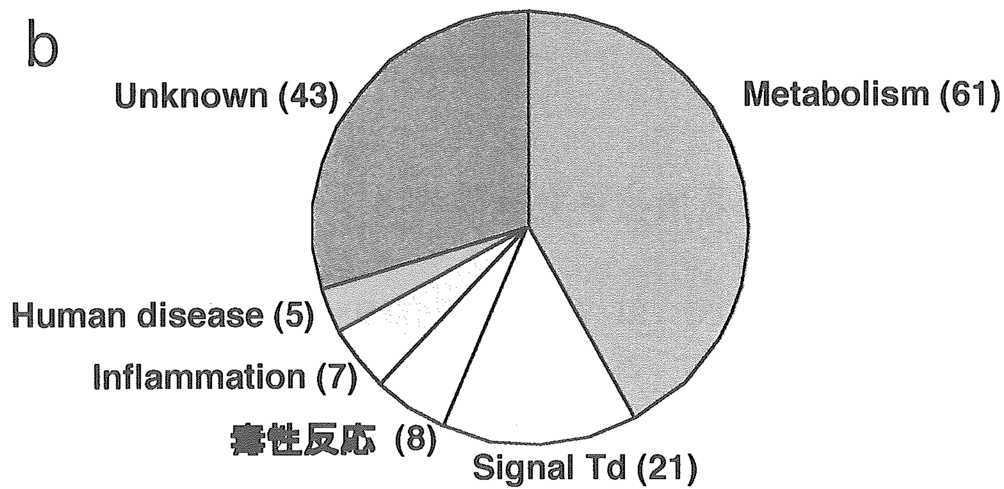

図９．毒性参照用ワクチン（RE）特異的遺伝子とその特徴

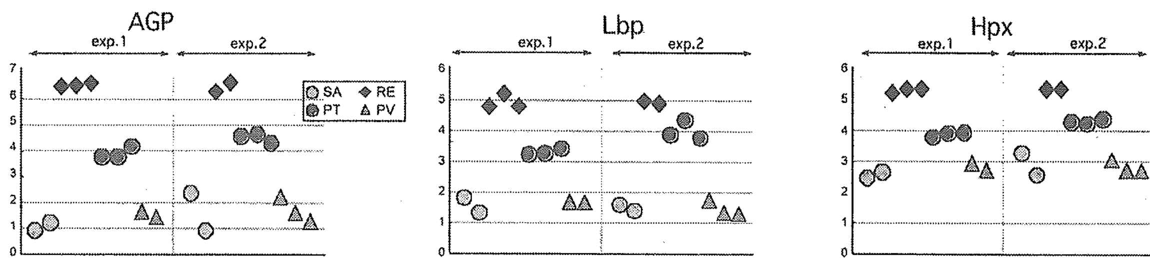

図１０．百日せきワクチンの毒性に関連した遺伝子の同定

ラット腹腔に SA（生理食塩水）、RE（毒性参照用百日せきワクチン）、PV（精製百日せきワクチン）および PT（百日咳毒素）を接種し、接種後１日目に肝臓を摘出し、AGP、Lbp、Hpx の遺伝子発現をマイクロアレイ解析による相対的遺伝子発現比として解析した。それぞれ実験１と実験２の２回のデータを示す。

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	頁	出版年
A. Sakamoto, J-I. Imai, A. Nishikawa, R. Honma, E. Ito, Y. Yanagisawa, M. Kawamura, R. Ogawa, S. Watanabe	Influence of inhalation anesthesia assessed by comprehensive gene expression profiling	Gene	356	39-48	2005
N. Fujita, T. Miyamoto, J-I. Imai, N. Hosogane, T. Suzuki, M. Yagi, K. Morita, K. Ninomiya, K. Miyamoto, H. Takahashi, M. Matsumoto, H. Morioka, H. Yabe, K. Chiba, S. Watanabe, Y. Toyama, T. Suda	CD24 is expressed specifically in the nucleus pulposus of intervertebral discs	Biochem. Biophys. Rec. Commun.	338	1890-1896	2005

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Influence of inhalation anesthesia assessed by comprehensive gene expression profiling☆

Atsuhiro Sakamoto[a,*], Jun-ichi Imai[b,c], Akira Nishikawa[c,d], Reiko Honma[b], Emi Ito[b], Yuka Yanagisawa[b], Mika Kawamura[c], Ryo Ogawa[a], Shinya Watanabe[b,*]

[a]*Department of Anesthesiology, Nippon Medical School, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan*
[b]*Department of Clinical Informatics, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*
[c]*Medicrome, Inc., Sendagaya, Shibuya-ku, Tokyo 151-0051, Japan*
[d]*Nippon Gene, Co., Ltd, Kandanishiki-cho, Chiyoda-ku, Tokyo 101-0054, Japan*

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Abstract

Although general anesthesia is routinely used as an essential surgical procedure and its harmlessness has been evaluated and endorsed by clinical outcomes, little is known about its comprehensive influence that is not reflected in mortality and morbidity. In this paper, we have shown that inhalation anesthesia affected the expression of $<1.5\%$ of $>10,000$ genes, by analyzing the expression profiles for multiple organs of rats anesthetized with sevoflurane. The small number of transcripts affected by the inhalation anesthesia comprised those specific to single and common in multiple organs. The former included genes mainly associated with drug metabolism in the liver and influenced by agents such as amphetamine in the brain. The latter contained multiple circadian genes. In the brain, we failed to detect the alteration of the clock gene expression with the exception of *Per2*, assuming that anesthesia perturbs circadian rhythms. Our findings provide the first assessment for the influence of inhalation anesthesia by approaches of experimental biology and genome science.
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Keywords: General anesthesia; Gene expression profile; DNA microarray; Circadian gene; Endothelin1; *Per2*

1. Introduction

Inhalation anesthetics induce general anesthesia that results in unconsciousness, insensitivity to pain, and lack of memory of pain. Although general anesthesia has been routinely used as an essential surgical procedure for approximately 150 years and its harmlessness has been evaluated and endorsed by clinical outcomes (Forrest et al., 1990; Levy, 1984; Brown and Frink, 1993), little is known with regard to its comprehensive influence, which is not reflected in mortality and morbidity. A few studies were reported on the results of inhalation anesthesia on cells and tissues at the molecular level. This anesthetic acted upon an extremely restricted number of genes including those that control the expression of ligand-gated ion channels and G-protein-coupled receptors (Franks and Lieb, 1994; Harris et al., 1995).

The recent progress in genomics enables us to comprehensively describe and analyze the alteration in cells and tissues at the gene expression level by hybridization with DNA microarrays representing genome-wide or subgenome-wide species of transcripts. Therefore, we attempted to comprehensively analyze the effects of

Abbreviations: Arc, activity-regulated cytoskeleton; Bmal1, Brain-Muscle-Arnt-Like-protein 1; Cry2, Cryptochrome2; Dbp, albumin site d-binding protein; Egr1, early growth response gene-1; NGFI-B, nerve growth factor-induced gene B; PCR, polymerase chain reaction; Per2, Period2; SD, standard deviation; Tef, thyrotroph embryonic factor.

☆ Data from all the arrays used in this paper will be available at DDBJ via CIBEX (http://www.cibex.nig.ac.jp/cibex/HTML/index.html) under accession nos.: for the array design, CBX4; for the experiments, CAR4.

* Corresponding authors. Tel.: +81 3 5414 6010; fax: +81 3 5775 1352.
E-mail addresses: no1-saka@nms.ac.jp (A. Sakamoto) swata@mvc.biglobe.ne.jp (S. Watanabe).

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inhalation anesthesia at the molecular level by obtaining gene expression profiles from rats under inhalation anesthesia.

In this study, we have shown that inhalation anesthesia affected expression of an extremely limited number of genes. This was done by analyzing expression profiles for multiple organs of rats at different time points after anesthetizing with sevoflurane. Our findings could provide a basis for exploring comprehensive influence of inhalation anesthesia and to endorse the safety of anesthesia in the future. The expression profiling analyses also demonstrate the possible association between the anesthetic status and alteration in modulation of the circadian gene expression that were previously identified in addition to nominating novel candidates for the circadian gene.

2. Materials and methods

2.1. Animals and tissue collection

Six-week-old male rats (Wister; Charles River Japan, Inc., Atsugi, Japan) were purchased and adapted to a 12-h light/12-h dark cycle starting at 06:00 and 18:00 for a week before the experiments. The anesthesia experiment was replicated twice with an interval of several weeks between the two experiments (Exp. 1 and Exp. 2). Anesthesia was used at 09:00 on nine rats in Exp. 1 and on 10 rats in Exp. 2. Rats were housed in a plastic box supplied with sevoflurane (4.5% air mixture gas) at the rate of 6 L/min. As the 0-h control, rats ($n=3$, in Exp. 1; $n=4$, in Exp. 2) were subjected to tracheal intubation immediately after induction of anesthesia in order to maintain the anesthetic status and sacrificed to obtain organs. The organs were obtained in the order of the blood, the spleen, the kidneys, the liver, the lungs, the heart, and the brain within 5–10 min per animal after the intubation. As the 2-h and 6-h samples, the anesthetized rats were picked up from the plastic box supplied with sevoflurane, subjected to the intubation, and sacrificed to similarly obtain the organs 2 h and 6 h after induction of anesthesia, respectively. Three rats were used for each time point of each experiment with the exception of the time point of 0 h in Exp. 2, in which four rats were used; eventually, a total of 19 rats were used in the two replicated experiments.

2.2. RNA preparation

The blood obtained was immediately mixed with an ISOGEN-LS reagent (NIPPON GENE, Tokyo, Japan) after dilution with an equal volume of water. The other organs (the whole brain, the whole heart, the left lung, the lateral left lobe of the liver, the whole spleen, and both the kidneys) were immediately frozen in liquid nitrogen and lysed with an ISOGEN reagent (NIPPON GENE). Total RNA was prepared from the lysate in accordance with the manufacturer's instructions. Poly(A)+ RNA was prepared from total RNA with a Poly(A) Purist Kit (Ambion, TX, USA), in accordance with the manufacturer's instructions.

2.3. Microarray preparation and expression profile acquisition

A set of synthetic polynucleotides (80-mers) that represented 11,464 rat transcripts derived from 10,490 independent genes, including most of the RefSeq clones deposited in the NCBI database (MicroDiagnostic, Tokyo, Japan), was arrayed on a slide glass (S9115; Matsunami, Kishiwada, Japan) with a custom-made arrayer (Kobayashi et al., 2004; Ito et al., 2003). Poly(A)+ RNA (2 μg) was labeled with SuperScript II (Invitrogen, CA, USA) and Cyanine 5-dUTP for each sample or Cyanine 3-dUTP (PerkinElmer, MA, USA) for a rat common reference RNA (MicroDiagnostic). Labeling, hybridization, and subsequent washes of microarrays were performed with a Labeling and Hybridization Kit (MicroDiagnostic), in accordance with the manufacturer's instructions. The rat common reference RNA was purchased as a single batch and labeled as an aliquot with Cyanine-3 for a single microarray side by side with each sample which was labeled with Cyanine-5. Hybridization signals were measured using a GenePix 400A scanner (Axon Instruments, CA, USA) and then processed into primary expression ratios ([Cyanine 5-intensity obtained from each sample]/[Cyanine 3-intensity obtained from the rat common reference RNA], which are indicated as 'median of ratios' in GenePix Pro 3.0 software (Axon Instruments)). Normalization was performed for the median of ratios (primary expression ratios) by multiplying normalization factors calculated for each feature on a microarray by the GenePix Pro 3.0 software. All the data in accordance with the MIAME guideline were deposited at DDBJ via CIBEX (http://www.cibex.nig.ac.jp/cibex/HTML/index.html) under accession numbers CBX4 (for the array design) and CAR4 (for the experiments).

2.4. Data analysis

Data processing and subsequent hierarchical clustering analysis were performed with an Excel program (Microsoft, WA, USA) and an MDI gene expression analysis software package (MicroDiagnostic). The primary expression ratios were converted into $\log_2$ values ($\log_2$ Cyanine-5 intensity/Cyanine-3 intensity) (designated log ratios) and compiled into a matrix (designated primary data matrix).

We conducted the following operations to extract genes, from the primary data matrix, in which the expression levels altered specifically on inhalation anesthesia. (i) The mean average of log ratios for each 0-h data set of each organ (designated 0-h averages) was calculated. (ii) The relative ratios against the respective 0-h average values for all the log ratios (designated relative log ratios) were generated. This enabled us to compare all data as expression differences that deviated from the mean average

of each gene and to introduce a single threshold value (=0.75) to filter the genes in which the expression levels altered. (iii) All the relative log ratios were arranged into a matrix (designated secondary data matrix). (iv) In order to filter out the genes in which the expression levels deviated from the mean average in an individual data set for each gene, each time point, and each organ, we calculated standard deviation of relative log ratios for each time point and each organ, using the data from all the animals (Exp. 1 and Exp. 2). The SD values calculated by every organ showed similar distributions and there was no obvious correlation between the SD values and the fluorescent intensities initially detected in each microarray. This enabled us to introduce a single SD value (=1.0) as a cutoff threshold for all the genes. The calculated value should reflect the differences between independently repeated anesthetic experiments and the responses of individual rats. (v) The genes with standard deviation of the relative log ratios greater than 1 for at least one time point for each organ were detected from the secondary data matrix. (vi) The genes with relative log ratios commonly greater than 0.75 or uniformly smaller than −0.75 in each organ among at least five individual rats, which had an identical anesthetic period (2 h or 6 h), were selected from the secondary data matrix.

Next, we extracted genes, in which expression patterns were specific to a single organ or common to multiple organs from the secondary data matrix by the following operations. (i) The mean average of log ratios for 2 h or 6 h for each organ (designated 2-h and 6-h averages) was calculated. (ii) The relative ratios of 2-h and 6-h averages against 0-h average for each organ (designated relative 2-h and 6-h averages) were generated. (iii) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in a single organ were selected. (iv) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in any two organs were selected. (v) Genes with relative 2-h average or relative 6-h average was greater than 0.75 or lower than −0.75 in any three organs were selected. (vi) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in any four organs were selected. (vii) Genes with relative 2-h average or relative 6-h average greater than 0.75 or lower than −0.75 in more than four organs were selected.

3. Results

In order to comprehensively evaluate the influences of general anesthesia at the gene expression level, we obtained seven major organs from rats under general anesthesia with sevoflurane, an inhalation anesthetic, at 0 h, 2 h, and 6 h after induction of anesthesia. We performed the animal experiment with sevoflurane twice with an interval of several weeks between the two experiments (designated as Exp. 1 and Exp. 2). In Exp. 1, we used three rats for each time point and obtained for the seven organs but failed to draw peripheral blood from a rat assigned for 2 h. In Exp. 2, we used four rats for 0 h and three rats for 2 h and 6 h, respectively, and failed tissue lysate preparation from the lungs and liver of a rat assigned for 0 h. Eventually, we obtained 130 independent tissue samples for the three time points. We labeled poly(A)+ RNA purified from the samples and a rat common reference RNA with Cyanine-5 and Cyanine-3, respectively, and hybridized to microarrays representing 11,464 transcripts derived from 10,490 individual genes. Hybridization signals were processed into expression ratios as $\log_2$ values (designated log ratios) and compiled into a matrix designated as the primary data matrix (see Materials and methods). Data from all the arrays used in this paper are available at DDBJ via CIBEX (http://www.cibex.nig.ac.jp/cibex/HTML/index.html) under accession numbers CBX4 (for the array design) and CAR4 (for the experiments).

3.1. Overview of gene expression patterns in rat tissues after being subjected to general anesthesia

First, we sought to seize an overview of the experiments conducted in this study by two-dimensional clustering analysis of log ratios calculated from primary expression ratios against the common reference RNA (primary data matrix), prior to extracting genes that were affected by inhalation anesthesia at the expression level. The primary data matrix should enable us to relatively compare all samples one another without conducting direct comparison by hybridization on an identical microarray. We predicted that the most obvious differences obtained from the clustering analysis of the primary data matrix should be those reflecting tissues examined in this study, represented as sample clusters consisting of each organ only (tissue clusters) in a single dendrogram. Furthermore, we presumed that we might be able to compare gene expression levels that were associated with different experimental conditions within each tissue cluster in the dendrogram.

We extracted genes with log ratios over 1 or under −1 in at least one sample from the primary data matrix and subjected them to two-dimensional hierarchical clustering analysis for samples and genes (Fig. 1) (Schena et al., 1996; Lyons et al., 2000). When the clustering analysis for the samples was performed, the greatest seven clusters corresponding to the individual organs were obtained as predicted. The clusters representing the lungs, heart, liver, and blood comprised two smaller clusters completely corresponding to differences between Exp. 1 and Exp. 2, reflecting experimental errors. The clusters representing the kidney and brain consisted of two smaller clusters that incompletely corresponded to the experiment differences with some exceptions. With the exception of the smaller cluster in Exp. 1, representing the liver, in which three

Fig. 1. Gene expression profiles obtained from seven organs of rats under inhalation anesthesia. Rows and columns represent genes (10,796) and samples (130), respectively. The genes and samples are assembled in the order obtained from the results of the two-dimensional hierarchical clustering analysis. Dendrogram at the top of the figure indicates the relationship among the samples after clustering analysis; the y-axis of the dendrogram depicts Euclid square distance as the dissimilarity coefficient. The color bar on the left side of the figure shows expression ratio against the common reference RNA in $\log_2$; red and blue indicate increase and decrease of the expression ratios, respectively. Solid circles indicate individual rats assigned for 0 h, 2 h, and 6 h by black, green, and red, respectively. Red and blue bars show two independent anesthesia experiments, i.e., Exp. 1 and Exp. 2, respectively. Color bars in pink, light blue, orange, violet, dark blue, light green, and gray represent the lungs, spleen, heart, kidney, brain, liver, and blood, respectively.

different time points formed the three smallest clusters, the smaller clusters by the differences in experiment were not divided by differences of anesthetic periods but by the individual differences among rats. These results obtained from the clustering analysis for the 130 samples indicate that differences among anesthesia periods with regard to the expression profiles of over 10,000 genes are much smaller than differences among individual rats, independent experiments, and organs.

3.2. Selection of genes in which expression patterns were affected by inhalation anesthesia

In order to extract genes in which expression levels altered specifically due to inhalation anesthesia, from the primary data matrix, we conducted the operations described in the Materials and methods and generated the secondary data matrix comprising relative log ratios. From the secondary data matrix, we successfully obtained 177 transcripts originating from 167 genes that satisfied the conditions described in the Materials and methods and subsequently subjected the data of the 177 transcripts to clustering analysis for genes (Fig. 2). Clustering analysis demonstrated that the 167 genes comprised those genes specific to a single organ and common in different organs. It also demonstrated that among the organs tested in this study, expression alteration of 114 genes was predominantly detected in the liver and that of 42 genes was predominantly detected in the lungs and the expression alteration of a few genes was predominantly detected in the blood, brain, heart, kidney, and spleen. Moreover, we noticed that the alteration of expression levels was detected mainly in the longer period but rarely in the shorter period. These results confirm that the inhalation anesthesia affected expression of a small number of genes except for the liver. This result was also confirmed by the two-dimensional clustering analysis for the primary data matrix of over 10,000 genes.

3.3. Expression profiles for genes that are immediately affected by anesthesia

Next, we extracted genes whose expression levels were immediately affected by the inhalation anesthesia, particularly in the shorter period by filtering operations similar to those described above. The reason was the following. Although we noticed that the predominant alteration appeared at the time point of 6 h, we expected the possibility that the data obtained at 2 h might provide information on genes that may specifically regulate the expression of the

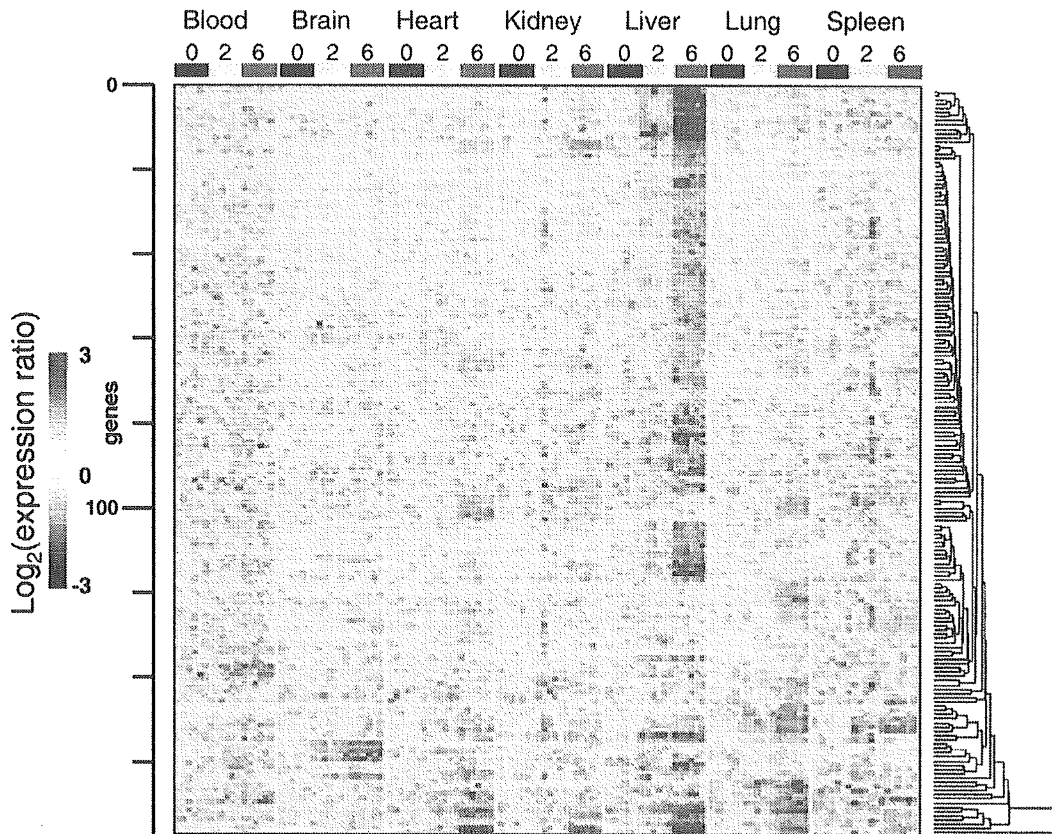


Fig. 2. Clustering analysis of genes in which expression levels were obviously altered in an inhalation anesthesia-specific manner. Rows and columns represent genes and samples, respectively. Rows are assembled in the order derived from clustering analysis. Columns are assembled in the order of organs and time points; black, green, and red bars exhibit 0-h, 2-h, and 6-h samples, respectively. Dendrogram on the right side of the figure indicates the relationship of genes after clustering. The color bar on the left side of the figure shows expression ratio against the common reference RNA in $\log_2$; red and blue indicate increase and decrease of the expression ratios, respectively. Color bars in pink, light blue, orange, violet, dark blue, light green, and gray represent the lungs, spleen, heart, kidney, brain, liver, and blood, respectively.

genes affected in the later phase. We obtained only 20 transcripts derived from 18 genes, as genes affected immediately by the inhalation anesthesia (Fig. 3). These results indicate that in the early phase, an extremely small number of genes are affected by inhalation anesthesia (approximately 0.17% of the total genes tested). Among the 20 transcripts, 18 exhibited single organ-specific expression patterns but only two represented those common in multiple organs. One of the two genes common to the multiple organs was a gene previously known as the circadian gene, *Rev-ErbA-alpha* (Leloup and Goldbeter, 2003). We notice that the gene expressing vasoconstrictor endothelin 1 (Yanagisawa et al., 1988) was upregulated as an early responsive gene in the lungs. This may be because inhalation anesthetics are initially exposed at the highest concentration to the lungs as compared to the otherorgans.

3.4. Extraction of genes in which expression was influenced by inhalation anesthesia in an organ-specific manner

We extracted genes in which expression patterns were specific to a single organ or common in different organs by the operations described in the Materials and methods. Subsequently, we selected genes that satisfied the conditions described in the Materials and methods from the genes shown in Fig. 2. We classified the selected genes in which the expression patterns were influenced by the inhalation anesthesia, as shown in Fig. 4a–e.

In the liver, we detected the maximum number of genes in which the expression was influenced by the inhalation anesthesia (99 transcripts; approximately 56% of the total genes influenced). These liver-specific genes include those characterized as the drug metabolism-associated or the drug response-associated genes such as cytochrome *P*450s (Lu, 1998), epoxide hydrolases (Cannady et al., 2002), UDP-glucuronosyl transferases (Mackenzie et al., 1997), and glutathione sulfotransferases (Snyder and Maddison, 1997). We obtained the second highest number of influenced genes in the lungs (15 transcripts). Since anesthetics act on the brain and induce unconsciousness, we focused on the genes in which the expression specifically influenced the brain. We did not observe an elevation of expression levels of any genes in the brain but detected four genes in which expression levels decreased after the induction of the inhalation

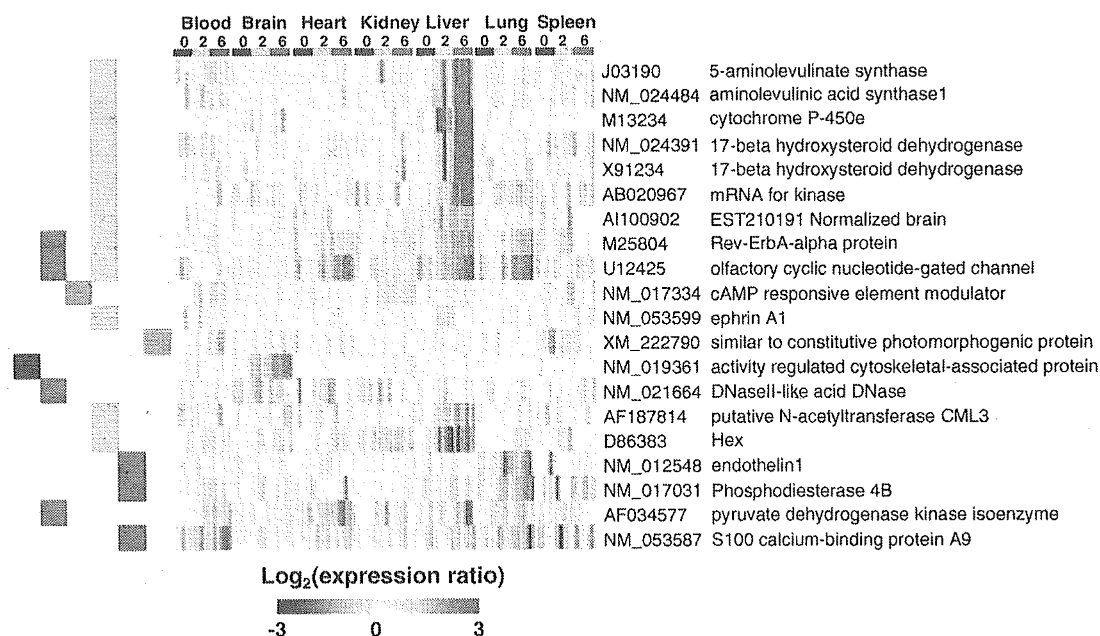


Fig. 3. Genes in which expression levels altered in the early phase (2 h) of inhalation anesthesia. The genes are assembled in the order obtained from the results of hierarchical clustering analysis. The color bar at the bottom of the figure shows expression ratio against the common reference RNA in $\log_2$; red and blue indicate increase and decrease of the expression ratios, respectively. Color bars in pink, light blue, orange, violet, dark blue, light green, and gray represent the lungs, spleen, heart, kidney, brain, liver, and blood, respectively.

anesthesia (*Arc* (Link et al., 1995), *NGFI-B* (Maruyama et al., 1998), *Krox20* (Bhat et al., 1992), and *Egr1* (Liu et al., 1996)). These four genes were previously reported to encode transcription factors and to be those in which the expression levels were differently affected by treatment with the agents acting on the central nervous system (CNS), such as amphetamine (Gonzalez-Nicolini and McGinty, 2002), cocaine (Freeman et al., 2002), pentobarbital (Ryabinin et al., 2000), and antidepressants (Pei et al., 2003).

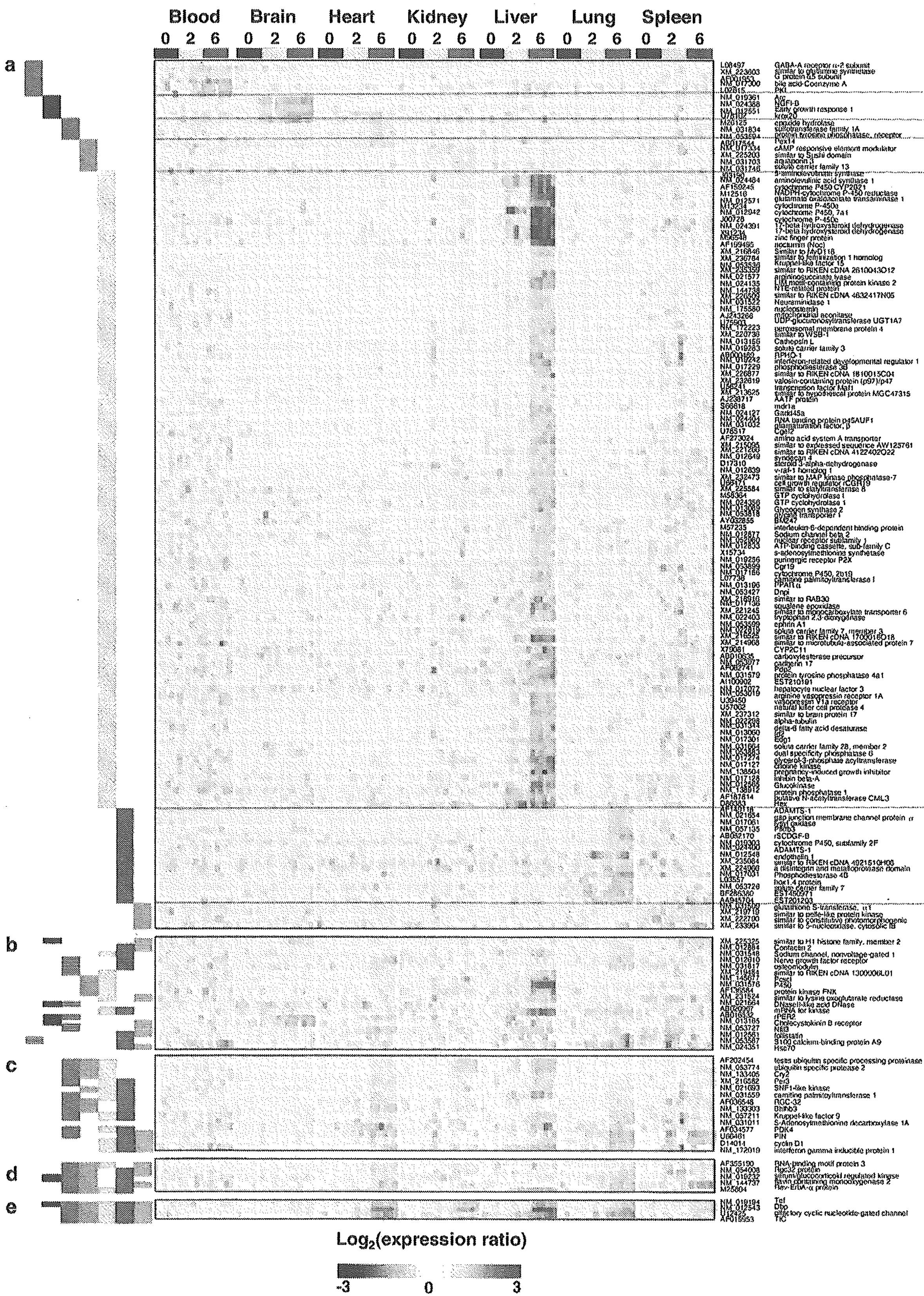
3.5. Evaluation of genes in which expression was altered during inhalation anesthesia in a multiple organ-common manner

As described above, we notice that several genes were affected during anesthesia in a similar manner among multiple organs. These included the previously reported circadian genes in which the expression level alters with an oscillating rhythm based on whether it is day or night. We selected expression data for the representative circadian genes from the secondary data matrix (Fig. 5). Most temporal expression patterns for known circadian genes obtained in this study were consistent with those reported previously with the exception of the genes from the brain (Ueda et al., 2002; Storch et al., 2002; Panda et al., 2002). In the brain, we observed no alteration of the expression of the known circadian genes during anesthesia with the exception of *Per2*. The *Per2* gene provided an expression pattern contradictory to those reported previously, representing decreased expression levels during the day. Furthermore, we compared expression patterns obtained in this study with those recently reported for the mouse circadian genes using the microarray technology (Ueda et al., 2002). The rat orthologues of the mouse circadian genes in the brain exhibited no alteration of expression during the inhalation anesthesia. The genes compared will be listed in Supplementary Information Table 6 (http://www.cibex.nig.ac.jp/cibex/HTML/index.html; under accession no. CAR4). These findings indicate that under inhalation anesthesia, the circadian rhythm may differ from normal circadian rhythms.

4. Discussion

By analyzing expression profiles obtained from rats under general anesthesia,we have shown that inhalation anesthesia affected expression of a small number of genes.

Fig. 4. Classification of genes that showed alteration in expression levels during anesthesia by the number of the organs in which the alteration was detected. a, genes in which expression levels altered in a single organ-specific manner. The genes of each gene set for a specific organ are assembled in the order derived from clustering. b, c, d, e, and f, genes in which expression levels altered commonly in two, three, four, and more than four organs, respectively. The color bar at the bottom of the figure shows expression ratio against the common reference RNA in $\log_2$; red and blue indicate increase and decrease of the expression ratios, respectively. Color bars in pink, light blue, orange, violet, dark blue, light green, and gray represent the lungs, spleen, heart, kidney, brain, liver, and blood, respectively.


Blood
Brain
Heart
Kidney
Liver
Lung
Spleen
0 2 6
a
b
c
d
e
Log2(expression ratio)
-3
0
3

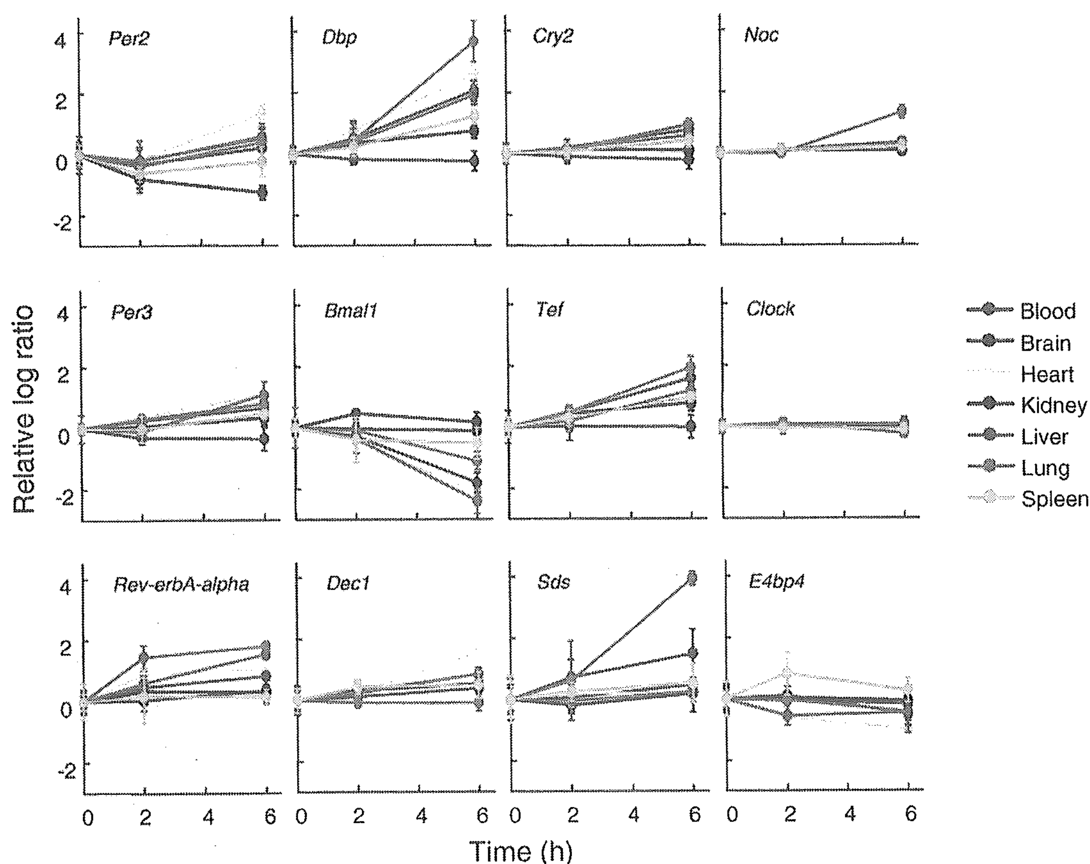


Fig. 5. Expression kinetics of the rat orthologues for the previously identified mouse circadian genes in rats under inhalation anesthesia. Relative log ratios were extracted from the secondary data matrix for the following genes: *Clock*, *Per2*, *Cry2*, *Per3*, *Noc*, *Tef*, *Rev-erbA-alpha*, *Dbp*, and *Bmal1*. The extracted relative log ratios were subjected to calculation of mean average (solid circle) and standard deviation (error bar) for each time point of individual organs. The x and y axes show time (h) and relative log ratio against the 0-h control, respectively. Graphs in pink, light blue, orange, violet, dark blue, light green, and gray represent the lungs, spleen, heart, kidney, brain, liver, and blood, respectively.

Our study should provide the first comprehensive assessment for the influence of inhalation anesthesia with regard to experimental biology and genome science. Although we did not observe obvious alterations for the majority of genes tested, we successfully detected genes in which expression levels were influenced in an inhalation anesthesia-dependent manner.

Among the genes in which expression levels altered in an anesthesia-dependent manner, the most predominant gene cluster was found in the samples obtained from the liver. The genes will be listed in Supplementary Information Table 4 (http://www.cibex.nig.ac.jp/cibex/HTML/index.html; under accession no. CAR4). These liver-specific genes include many cytochrome *P*450s, UDP-glucuronosyl transferases, and glutathione sulfotransferases, representing involvement and activation of drug-metabolizing enzyme systems by hydroxylation, glucuronide conjugation, and glutathione conjugation, respectively (Lu, 1998; Cannady et al., 2002; Mackenzie et al., 1997; Clarke et al., 1997). This indicates that almost all the genes in the enzyme systems previously identified to be involved in drug metabolism in the liver are engaged (Gerhold et al., 2001). However, it is rather predictable that an inhalation anesthetic induces the expression of the drug metabolism-related genes as a toxicological response. On the other hand, as novel findings, our data propose that many transcripts (thus far uncharacterized) may be regulated by a common set of trasncription factors, in addition to being involved in the metabolism of the anesthetic in a similar manner to the genes for drug-metabolizing enzymes that were previously identified. Moreover, we presume that the induction of endothelin at the early phase of anesthesia in the lungs represents one of the toxicological responses and that endothelin possibly induces some effects in the vascular system, particularly in the early phase of the anesthetic period. This is presumed because the induction of the endothelin gene expression has been previously reported as responses to toxins (Sonin et al., 1999; Baveja et al., 2002). It needs to be investigated whether other anesthetics affect the expression of the genes in a manner similar to sevoflurane, as demonstrated in this study. Such a comparative study would provide information that is necessary to assess the side effects of the currently available anesthetics and to develop safe tools in anesthesiology.

We should mention that the findings presented here may not provide direct evidence to suggest that the anesthetic causes the anesthetic status by inducing alteration in the expression of certain genes since we did not detect many regulated genes in the brain, which should comprise the main target of the anesthetic. Despite the low number of regulated genes (3 up, 6 down), these genes encode transcription factors and circadian rhythm genes that were previously reported to be increased by amphetamine (Gonzalez-Nicolini and McGinty, 2002), cocaine (Freeman et al., 2002), and morphine withdrawal (Ammon et al., 2003), whereas their expression decreases by anesthesia with an intravenous anesthetic (Ryabinin et al., 2000).

One of the interesting findings of this study is that several circadian genes exhibited alteration of expression during an anesthetic period in a multiple organ-common manner. However, in the brain, we obtained several findings that were inconsistent with those reported previously (Ueda et al., 2002). We did not detect an alteration in several genes in which expression exhibited the circadian rhythm and we observed contradictory kinetics in the expression of *Per2*. The hypothesis that inhalation anesthesia perturbs the circadian rhythms requires further investigations. We assume that one of the reasons for these differences may be due to the tissue used for analysis. Most previous studies focused on the center of the circadian rhythm, the suprachiasmatic nucleus (Ueda et al., 2002; Panda et al., 2002). On the other hand, we used the whole brain as a target. The suprachiasmatic nucleus occupies an extremely small region of the whole brain. Therefore, the dilution of mRNA of the suprachiasmatic nucleus with mRNA of the whole brain might have affected the detection of the expression of the circadian genes mainly regulated in the suprachiasmatic nucleus. Furthermore, to obtain conclusive evidence for the association of anesthetic periods and the expression of circadian genes, future studies should scrutinize expression profiles for circadian genes at more multiple time points with a shorter interval than those used in this study. In order to further analyze the circadian gene expression, it would be useful to perform a non-microarray approach such as real-time PCR for the limited number of target genes as shown in this study.

In this study, we independently repeated the identical anesthetic experiment twice with rats. Clustering analysis shown in Fig. 1 exhibits that with the exception of the brain and kidney, in the other five organs smaller clusters are generated for each, which correspond to the two independent anesthetic experiments, reflecting experimental errors. Only the brain and kidney do not exhibit such smaller clusters that completely corresponded to the two independent experiments, indicating that in these two organs, experimental errors between the two experiments were smaller than the differences among individual rats and/or experimental conditions for anesthesia. These results clearly indicate that experimental errors among independent anesthetic experiments are not negligible and that one should be careful while drawing conclusions from a single experiment with animals under mildly different experimental conditions such as inhalation anesthesia. We successfully avoided highlighting genes that reflected the experimental errors by repeating the identical anesthetic experiments and conducting filtering operations for a combined gene expression data set. These findings provide experimental evidence that endorses the significance of repeating an independent experiment with animals under experimental conditions with mild differences and of data processing to reduce noises that reflect the differences among the repeated experiments.

As described above, our study demonstrates that the experimental errors observed between the independently repeated anesthetic experiments provided the second highest difference that follows the differences among the individual organs tested, which generated the most distinguishable clusters. This finding led us to use threshold-based approaches with strict cutoff values to extract genes in which expression was specifically affected by inhalation anesthesia and to avoid influence of the experimental errors and the differences among individual rats assigned for the identical conditions. These relatively strict analytical approaches may result in insufficient extraction of specifically affected genes, particularly for those in which expression was altered in a subtle manner. One of the analytical approaches that may enable us to obtain a greater number of genes specifically affected by inhalation anesthesia should be to apply lower thresholds in the filtering operation described in the Materials and methods for the data set compiled in this study. The approach may be effective to obtain as much information as possible from our primary data set. However, this would require additional confirmation for the individual values through non-microarray approaches.

We have provided a large and powerful data set for exploring the influence of inhalation anesthesia in rats. The entire data set will be available in a public database. The results presented here were derived from the data set by relatively conservative approaches due to the experimental differences greater than those expected prior to data analysis. Therefore, apart from conducting additional more fine-tuned experiments, another approach may be helpful for the further understanding of comprehensive influence of anesthesia by means of utilizing advanced statistical methods such as analysis of variance (ANOVA). Application of the more sophisticated approaches would enable those who use the data set to explore additional categories of genes that are modulated under anesthesia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2005.03.022.

References

Ammon, S., Mayer, P., Riechert, U., Tischmeyer, H., Hollt, V., 2003. Microarray analysis of genes expressed in the frontal cortex of rats chronically treated with morphine and after naloxone precipitated withdrawal. Brain Res. Mol. Brain Res. 112, 113–125.

Baveja, R., Keller, S., Yokoyama, Y., Sonin, N., Clemens, M.G., Zhang, J.X., 2002. LPS-induced imbalanced expression of hepatic vascular stress genes in cirrhosis: possible mechanism of increased susceptibility to endotoxemia. Shock 17, 316–321.

Bhat, R.V., Worley, P.F., Cole, A.J., Baraban, J.M., 1992. Activation of the zinc finger encoding gene krox-20 in adult rat brain: comparison with zif268. Brain Res. Mol. Brain Res. 13, 263–266.

Brown Jr., B.R., Frink, E.J., 1993. The safety of sevoflurane in humans. Anesthesiology 79, 201–202.

Cannady, E.A., Dyer, C.A., Christian, P.J., Sipes, I.G., Hoyer, P.B., 2002. Expression and activity of microsomal epoxide hydrolase in follicles isolated from mouse ovaries. Toxicol. Sci. 68, 24–31.

Clarke, H., et al., 1997. Alpha-gluthathione *S*-transferase (alpha-GST) release, an early indicator of carbon tetrachloride hepatotoxicity in the rat. Hum. Exp. Toxicol. 16, 154–157.

Forrest, J.B., et al., 1990. Multicenter study of general anesthesia: I. Design and patient demography. Anesthesiology 72, 252–261.

Franks, N.P., Lieb, W.R., 1994. Molecular and cellular mechanisms of general anaesthesia. Nature 17, 607–614.

Freeman, W.M., et al., 2002. Changes in rat frontal cortex gene expression following chronic cocaine. Brain Res. Mol. Brain Res. 104, 11–20.

Gerhold, D., Lu, M., Xu, J., Austin, C., Caskey, C.T., Rushmore, T., 2001. Monitoring expression of genes involved in drug metabolism and toxicology using DNA microarrays. Physiol. Genomics 5, 161–170.

Gonzalez-Nicolini, V., McGinty, J.F., 2002. Gene expression profile from the striatum of amphetamine-treated rats: a cDNA array and in situ hybridization histochemical study. Gene Expr. Patterns 1, 193–198.

Harris, R.A., Mihic, S.J., Dildy-Mayfield, J.E., Machu, T.K., 1995. Actions of anesthetics on ligand-gated ion channels: role of receptor subunit composition. FASEB J. 9, 1454–1462.

Ito, E., et al., 2003. A tetraspanin-family protein, T-cell acute lymphoblastic leukemia-associated antigen 1, is induced by the Ewing's sarcoma–Wilms' tumor 1 fusion protein of desmoplastic small round-cell tumor. Am. J. Pathol. 163, 2165–2172.

Kobayashi, S., et al., 2004. Dynamic regulation of gene expression by the Flt-1 kinase and Matrigel in endothelial tubulogenesis. Genomics 84, 185–192.

Leloup, J.C., Goldbeter, A., 2003. Toward a detailed computational model for the mammalian circadian clock. Proc. Natl. Acad. Sci. U. S. A. 100, 7051–7056.

Levy, W.J., 1984. Clinical anaesthesia with isoflurane. A review of the multicentre study. Br. J. Anaesth. 56, 101S–112S.

Link, W., et al., 1995. Somatodendritic expression of an immediate early gene is regulated by synaptic activity. Proc. Natl. Acad. Sci. U. S. A. 92, 5734–5738.

Liu, C., Calogero, A., Ragona, G., Adamson, E., Mercola, D., 1996. EGR-1, the reluctant suppression factor: EGR-1 is known to function in the regulation of growth, differentiation, and also has significant tumor suppressor activity and a mechanism involving the induction of TGF-beta1 is postulated to account for this suppressor activity. Crit. Rev. Oncog. 7, 101–125.

Lu, A.Y., 1998. Drug-metabolism research challenges in the new millennium: individual variability in drug therapy and drug safety. Drug Metab. Dispos. 26, 1217–1222.

Lyons, T.J., Gasch, A.P., Gaither, L.A., Botstein, D., Brown, P.O., Eide, D.J., 2000. Genome-wide characterization of the Zap1p zinc-responsive regulon in yeast. Proc. Natl. Acad. Sci. U. S. A. 97, 7957–7962.

Mackenzie, P.I., et al., 1997. The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. Pharmacogenetics 7, 255–269.

Maruyama, K., Tsukada, T., Ohkura, N., Bandoh, S., Hosono, T., Yamaguchi, K., 1998. The NGFI-B subfamily of the nuclear receptor superfamily (review). Int. J. Oncol. 12, 1237–1243.

Panda, S., et al., 2002. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109, 307–320.

Pei, Q., Zetterstrom, T.S., Sprakes, M., Tordera, R., Sharp, T., 2003. Antidepressant drug treatment induces Arc gene expression in the rat brain. Neuroscience 121, 975–982.

Ryabinin, A.E., Wang, Y.M., Bachtell, R.K., Kinney, A.E., Grubb, M.C., Mark, G.P., 2000. Cocaine- and alcohol-mediated expression of inducible transcription factors is blocked by pentobarbital anesthesia. Brain Res. 877, 251–261.

Schena, M., Shalon, D., Heller, R., Chai, A., Brown, P.O., Davis, R.W., 1996. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc. Natl. Acad. Sci. U. S. A. 93, 10614–10619.

Snyder, M.J., Maddison, D.R., 1997. Molecular phylogeny of glutathione-*S*-transferases. DNA Cell Biol. 16, 1373–1384.

Sonin, N.V., Garcia-Pagan, J.C., Nakanishi, K., Zhang, J.X., Clemens, M.G., 1999. Patterns of vasoregulatory gene expression in the liver response to ischemia/reperfusion and endotoxemia. Shock 11, 175–179.

Storch, K.F., et al., 2002. Extensive and divergent circadian gene expression in liver and heart. Nature 417, 78–83.

Ueda, H.R., et al., 2002. Transcription factor response element for gene expression during circadian night. Nature 418, 534–539.

Yanagisawa, M., et al., 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411–415.

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CD24 is expressed specifically in the nucleus pulposus of intervertebral discs

Nobuyuki Fujita [a,b,1], Takeshi Miyamoto [a,b,*,1], Jun-ichi Imai [c], Naobumi Hosogane [a,b], Toru Suzuki [a,b], Mitsuru Yagi [a,b], Kozo Morita [a,b], Ken Ninomiya [a,b], Kana Miyamoto [a,d], Hironari Takaishi [b], Morio Matsumoto [b], Hideo Morioka [b], Hiroo Yabe [b], Kazuhiro Chiba [b], Shinya Watanabe [c], Yoshiaki Toyama [b], Toshio Suda [a,*]

[a] *Department of Cell Differentiation, The Sakaguchi Laboratory, Japan*
[b] *Department of Orthopedic Surgery, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[c] *Department of Clinical Informatics, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*
[d] *Department of Medicine and Biosystemic Science, Graduate School of Medicine, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582, Japan*

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Abstract

Intervertebral disc (IVD) consists of a soft gelatinous material in its center, the nucleus pulposus (NP), bounded peripherally by fibrocartilage, annulus fibrosus (AF). Despite the number of patients with IVD degeneration, gene expression analysis has not been undertaken in NP and therefore little is known about the molecular markers expressed in NP. Here, we undertook a microarray screen in NP with the other nine tissues to identify the specific cell surface markers for NP. Five membrane associating molecules out of 10,490 genes were identified as highly expressing genes in NP compared with the other tissues. Among them, we identified CD24, a glycosylphosphatidylinositol (GPI) anchor protein as a cell surface marker for NP. CD24 expression was also detected in the herniated NP and chordoma, a malignant primary tumor derived from notochordal cells, while it was absent in chondrosarcoma. Therefore, CD24 is a molecular marker for NP as well as the diseases of IVD.
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Keywords: Intervertebral disc; Nucleus pulposus; CD24

Intervertebral disc (IVD) degeneration and concomitant herniation are the distinguishing anatomical features associated with lumbar disc disease (LDD). The molecular mechanisms leading to the onset of IVD degeneration are not well understood. Recent genetic studies in humans and mice indicate that genetic factors play an important role in the etiology and pathogenesis associated with LDD [1–3].

The IVD is composed of two discrete components termed the nucleus pulposus (NP) and the annulus fibrosus (AF). The interior structure, NP, is a soft gelatinous avascular cartilage-like tissue, derived from notochord, containing extracellular matrix proteins (ECM) rich in large proteoglycans such as aggrecan and collagens. ECM proteins play a central role in chondrocyte metabolism through regulation of growth factors and appear to be crucial for maintaining IVD homeostasis and integrity [4,5]. A recent genetic study demonstrated the role of cartilage intermediate layer protein (CILP), an ECM that acts as a modulator of LDD susceptibility through deregulation of TGFβ signaling [3].

The AF, a fibrous cartilage composed of an inner and outer coaxial lamella, is bound to the periphery of the NP. The onset of degenerative disc disease is marked by mechanical stress-induced apoptosis in the AF resulting

* Corresponding authors. Fax: +81 3 5363 3475.
E-mail addresses: miyamoto@sc.itc.keio.ac.jp (T. Miyamoto), sudato@sc.itc.keio.ac.jp (T. Suda).
[1] These authors contributed equally to this work.

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