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transfer using T cells isolated from cervical lymph nodes and spleen of *RbAp48*-Tg mice, no inflammatory lesions had developed in *Rag2*$^{-/-}$ mice (Table I). These data suggest that APCs besides T cells might be required for successful transfer of autoimmune exocrinopathy in *RbAp48*-Tg mice. Finally, to confirm that MHC class II expression on activated salivary or lacrimal gland cells of *RbAp48*-Tg mice can drive priming of purified T cells of cLNs from *RbAp48*-Tg mice to induce autoimmune lesions, the T cells of cLNs from *RbAp48*-Tg mice were transferred into Ovx-*Rag2*$^{-/-}$ mice. The autoimmune lesions of salivary and lacrimal glands from the recipient Ovx-*Rag2*$^{-/-}$ mice transferred with T cells of cLNs from *RbAp48*-Tg mice were observed, whereas no lesions were found in any organs of the recipient Ovx-*Rag2*$^{-/-}$ mice transferred with T cells of cLNs from WT mice (Fig. 7, E and G). These results demonstrate that the epithelial cells stimulated through increased RbAp48 because of estrogen deficiency could interact with T cells to induce autoimmunity via loss of local tolerance.

Table I. Induction of autoimmune lesions

Donor cells	Mice	Incidence
Spleen cells (5 × 10^6)	WT	0/4
	RbAp48-Tg	0/4
cLN cells (5 × 10^6)	WT	0/5
	RbAp48-Tg	4/5
cLN T cells (5 × 10^6)	WT	0/4
	RbAp48-Tg	0/5
cLN B cells (5 × 10^6)	WT	0/4
	RbAp48-Tg	0/4

Whole spleen, cLN cells, cLN T cells, or cLN B cells were transferred intravenously into *Rag2*$^{-/-}$ mice. The host mice were killed 6 wk after transfer. Inflammatory lesions of salivary or lacrimal glands were evaluated by pathological analysis.

IFN-γ and IL-18 expressions in human SS patients

Although it has been reported that immune cells express some cytokines, it is unclear whether IFN-γ or IL-18 together with RbAp48 in the epithelial cells of salivary glands from human SS patients are expressed. To confirm our hypothesis that autoimmunity is induced by a breakdown of local tolerance in salivary gland cells with up-regulated RbAp48 because of estrogen deficiency such as menopause, IFN-γ, IL-18, and RbAp48 expressions were detected by confocal microscopic analysis using human biopsy samples from SS patients and controls. Among 10 SS patients, RbAp48$^+$ and IFN-γ$^+$ epithelial cells were observed in three samples and RbAp48$^+$ and IL-18$^+$ epithelial cells were observed in four samples. A representative image of SS patients and controls is shown in Fig. 8. Although faint expressions of RbAp48 in the nucleus of salivary epithelial cells were detected in control samples, IL-18 or IFN-γ together with a prominent expression of RbAp48 was not observed (Fig. 8). Isotype-matched controls of staining for the mAbs were shown in Fig. S10 (available at http://www.jem.org/cgi/content/full/jem.20080174/DC1).

DISCUSSION

Although MHC class II molecules have been expressed aberrantly on epithelial cells in association with autoimmunity, it remains debatable whether class II molecules are the initiating event or the consequence of the autoimmune attack. For example, certain alleles of class II (mouse I-A^{g7}) might be particularly good at presenting glutamic acid decarboxylase–65 or insulin peptides to T cells in nonobese diabetic (NOD) mice, thus contributing to recognition and ultimate destruction of pancreatic β cells (10, 11). Some investigators have proposed that I-A^{g7}, because of its poor peptide-binding properties, enhances autoimmunity in NOD mice in a global fashion (42). In this case, the β cell specificity of autoimmunity in this strain and the switch to autoimmune thyroiditis when a class II molecule without these properties is exchanged for I-A^{g7} must be controlled by other genetic loci in NOD mice (43). It is possible that the most straightforward explanation for the effects of I-A^{g7} is that it predisposes to

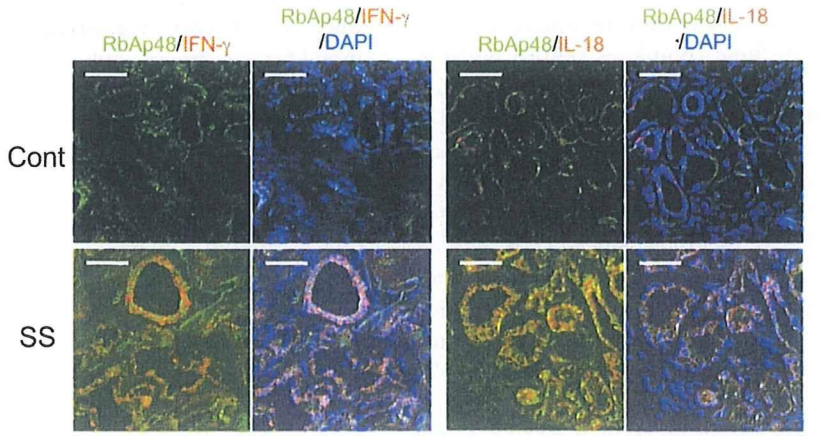

Figure 8. IFN-γ and IL-18 expressions together with RbAp48 in salivary glands from SS patients. The frozen sections of salivary glands from SS patients and controls were stained with IFN-γ or IL-18 (Alexa Fluor 568; red) and RbAp48 (Alexa Fluor 488; green) mAbs. The nuclei were stained with DAPI. The representative images in controls and SS patients were shown in three independent experiments.

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islet-specific autoimmunity and not system-wide reactivity. Another piece of evidence that the role of MHC class II is antigen-specific is that the class II alleles predisposing toward autoimmunity vary in one human disease to another, indicating that class II alleles act in autoimmunity via specific antigens rather than comprehensively.

We demonstrated in this study that autoimmune exocrinopathy resembling SS developed in almost all *RbAp48*-Tg mice, and that a high titer of serum autoantibodies against SS-A (Ro), SS-B (La), and 120-kD α-fodrin was detected in these Tg mice. We frequently found MHC class II molecule expression on the exocrine gland cells with autoimmune lesions in *RbAp48*-Tg mice. When we examined whether salivary epithelial cells could act on antigen presentation, we found a large proportion of MHC class II^+, $CD86^+$, $CD80^+$, and ICAM-1^+ cells primarily observed on cultured MSG cells from Tg mice. Moreover, CFSE-labeled purified $CD4^+$ (10^5) T cells from *RbAp48*-Tg mice were capable of responding to MSG cells from *RbAp48*-Tg mice, whereas anti–MHC class II antibody inhibited these responses. Although it has not been determined whether MHC class II–expressing epithelial cells can function as APCs, those data strongly suggest that the epithelial cells may function as APCs during development of autoimmune exocrinopathy. In *RbAp48*-Tg mice, a surprisingly prominent expression of epithelial IFN-γ was detected beside sporadically positive infiltrating cells. These findings were observed mainly in the MHC class II^+ ductal epithelium adjacent to lymphoid infiltrates. Epithelial IFN-γ expression in the exocrine glands of *RbAp48*-Tg mice was up-regulated during the course of autoimmune exocrinopathy. A previously unknown, multifaceted role of IFN-γ as regulator of the local immune system, which is termed here local tolerance, is disclosed. As to the mechanism of CIITA induction in *RbAp48*-Tg mice, our findings demonstrate the essential role of RbAp48-driven stimulation of IFN-γ production and signaling leading to up-regulation of IRF-1 and CIITA. RbAp48, initially identified as retinoblastoma-binding proteins (44), was characterized as a component of distinct nucleosome-modifying complexes, including the nuclear histone deacetylases (45, 46). Although the functions of the RbAp48-like proteins in these complexes remain undetermined, it was reported that E2F-1 and RbAp48 are physically associated in the presence of Rb and histone deacetylase (47), suggesting that RbAp48 could be involved in transcriptional repression of E2F-responsive genes. Several reports have demonstrated that estrogen may play an inhibitory role on apoptosis in endothelial cells, breast cancer cells, cardiac myocytes, prostate cells, and neuronal cells (48–51). It has been shown that the transcription factor IRF-1 mRNA expression is induced by ICI 182,780 as an antiestrogenic reagent and repressed by estrogens in antiestrogen-sensitive cells (52). We demonstrated the first evidence that IFN-γ–producing epithelial cells in the exocrine glands function as APCs through the IRF-1–CIITA pathway, resulting in the development of autoimmune exocrinopathy via loss of local tolerance. SS is known to have the most female predominance of >95% among all the autoimmune patients (15, 16). One of the key questions in respect to the pathophysiology of autoimmune diseases is how autoreactivity to particular autoantigens is initiated and maintained under an estrogen-deficient state. Although an important role for T cells in the development of autoimmune disease has been argued (53, 54), it is not known if disease is initiated by a retrained inflammatory reaction to autoantigen. We clarified that epithelial IFN-γ production is crucial for the initiation of autoimmune reactions between epithelial cells and autoreactive T cells with homeostatic expansion. Our previous study suggests that antiestrogenic actions have a potent effect on the proteolysis of α-fodrin autoantigen in the salivary gland through up-regulation of caspase 1 activity (22). These results strongly suggest that RbAp48-mediated activation of caspase 1 leads to the cleavage and activation of IL-18, which may act directly on IFN-γ production and on effector $CD4^+$ T cells by inducing migration and proliferation. Thus, aberrant expression of MHC class II in the exocrine glands facilitates loss of local tolerance before the development of autoimmune lesions, which is very similar to SS.

Evidence has been mounting that estrogen deficiency such as menopause is a proinflammatory state, which promotes osteoporosis and atherosclerosis, as well as autoimmunity (55, 56). In vivo and in vitro experiments here, including the induction of autoimmune lesions by T cell transfer from *RbAp48*-Tg mice into Ovx-*Rag2*$^{-/-}$ mice and in vitro antigen presentation of the Tg MSG cells to $CD4^+$ T cells, strongly suggests that estrogen deficiency stimulates salivary epithelial cells that are activated via the up-regulation of RbAp48 to present any endogenous autoantigen to $CD4^+$ T cells for the onset of autoimmune lesion in the salivary glands resembling human SS. Our data finds that transfer of cLN T cells from *RbAp48*-Tg mice into Ovx-*Rag2*$^{-/-}$ mice leads to autoimmune lesions, consistent with the conclusion that estrogen deficiency leads to the ability of salivary gland epithelial cells to express MHC class II and present any self-antigens. Most importantly, the salivary gland cells from human SS patients express RbAp48 together with IFN-γ or IL-18, as well as the findings of *RbAp48*-Tg mice in this work, suggesting that the molecules would be useful for any clinical application.

Collectively, our results demonstrate a direct molecular mechanism by which estrogen deficiency induces tissue-specific overexpression of RbAp48 (23), subsequently developing $CD4^+$ T cell–mediated autoimmunity through epithelial IFN-γ production. Thus, reducing the RbAp48 overexpression is a possible effective therapy in gender-based autoimmune exocrinopathy.

MATERIALS AND METHODS

Mice and histology. *RbAp48*-Tg mice have been previously described (23), and the RbAp48 gene is regulated by lacrimal and salivary gland-specific promoter (57). *Rag2*$^{-/-}$ mice were obtained from Taconic. All mice were reared in our specific pathogen–free mouse colony, and given food and water ad libitum. The experiments were approved by an animal ethics board of Tokushima University. All organs were removed from the mice, fixed with 4% phosphate-buffered formaldehyde (pH 7.2), and prepared for

histological examination. Formalin-fixed tissue sections were subjected to hematoxylin and eosin (H&E) staining, and three pathologists independently evaluated the histology without being informed of the condition of each individual mouse. Histological grading of the inflammatory lesions was done according to the method proposed by White and Casarett (58), as follows: 1 = 1–5 foci composed of >20 mononuclear cells per focus, 2 = >5 such foci, but without significant parenchymal destruction, 3 = degeneration of parenchymal tissue, and 4 = extensive infiltration of the glands with mononuclear cells and extensive parenchymal destruction. Histological evaluation of the salivary and lacrimal glands was performed in a blind manner, and a tissue section from each salivary and lacrimal gland was examined.

Measurement of fluid secretion. Analysis of the tear and saliva volume of WT and *RbAp48*-Tg mice was performed according to a previously described method (59).

Flow cytometric analysis. Lymphocytes in spleen, cLN, thymus, and MSG epithelial cells without immune cells (>95% of cells were keratin$^+$) were prepared. Surface markers were identified by mAbs with an EPICS flow cytometer (Beckman Coulter). Rat mAbs to FITC-, PE-, or PE-Cy5–conjugated anti-B220, CD4, MHC class II, CD86, CD80, ICAM1, and CD5 mAbs (eBioscience) were used. Appropriate isotype-matched controls were used, respectively. For detection of T cell activation markers, FITC-conjugated anti-CD25, CD44, CD62L, CD45RB, and CD69 mAbs (eBioscience) were used. Intracellular Foxp3 expression with an Intracellular Foxp3 Detection kit (eBioscience) was performed according to the manufacturer's instructions. Detections of intracellular IFN-γ or IL-18 were also performed by the same procedure. The data were analyzed with FlowJo FACS Analysis software (Tree Star, Inc.).

ELISA. The amount of mouse IL-2, IFN-γ, IL-4, and IL-10 in culture supernatants from CD4$^+$ T cells stimulated with anti-TCR mAb (~0–1 μg/ml) and anti-CD28 mAb (20 μg/ml; eBioscience), anti-SSA, anti-SSB, and anti–α-fodrin autoantibodies of sera from WT and *RbAp48*-Tg mice and human IL-18 and IFN-γ from cultured HSG and MCF-7 cells were analyzed by ELISA. In brief, plates were coated with a capture antibody or recombinant proteins (SSA, SSB, and α-fodrin), and washed with PBS/0.1% Tween 20. The plates were incubated with diluted culture supernatants or sera. After washing with PBS/0.1% Tween 20 and incubation of biotin-conjugated antibodies for cytokine detection, a horseradish peroxidase–conjugated detection antibody for autoantibody detection was added. After incubation with streptavidin-horseradish peroxidase for cytokine detection, plates were again washed with PBS/0.1% Tween 20 and o-phenylendiamine (Sigma-Aldrich) buffer added. Plates were then analyzed with a microplate reader reading at 490 nm.

Confocal microscopic analysis. Confocal microscopic analysis using anti-Thy1.2, B220, CD4, CD8, MHC class II, IFN-γ, CD4 (eBioscience), keratin (LSL CO., LTD), and IL-18 (MBL) antibodies was performed on the frozen sections of salivary glands from WT and *RbAp48*-Tg mice, and on the cultured cells using Confocal Laser Microscan (LSM 5 PASCAL; Carl Zeiss, Inc.). As the second antibodies, Alexa Fluor 488 anti–mouse IgG (H+L), Alexa Fluor 568 goat anti–rabbit IgG (H+L), Alexa Fluor 488 donkey anti–rat IgG (H+L), Alexa Fluor 488 chicken anti–goat IgG (H+L), and Alexa Fluor 568 rabbit anti–goat IgG (H+L; Invitrogen) were used. The nuclear DNA was stained with DAPI (Invitrogen).

Cell culture. For the co-culture of MSG with CD4$^+$ T cells, MSG cells were prepared by digestion of collagenase and hyaluronidase, and CD11c$^+$, CD11b$^+$, B220$^+$, NK1.1$^+$, and Thy1.2$^+$ cells were removed by the mAbs and magnetic bead–conjugated anti–rat IgG (Invitrogen). CD4$^+$ T cells from cLNs were purified by mAbs (anti-MHC class II, CD8, CD11b, CD11c, B220, and NK1.1) and magnetic bead–conjugated anti–rat IgG. CFSE-labeled CD4$^+$ T cells were co-cultured with MSG cells for 72 h. Cell division of CD4$^+$ T cells was analyzed by dilution of CFSE through flow cytometry.

As for the co-culture with DCs or MSG cells, DCs from *RbAp48*-Tg or WT mice were enriched using DC collection kit (Invitrogen). After DCs or MSG cells were irradiated (9 Gy), purified CD4$^+$ T cells of cLNs from RbAp48-Tg mice were co-cultured with the DCs or MSG cells for 72 h. The T cells were then pulsed with 0.5 μCi [^{3}H]thymidine per well for the last 12 h of the culture. [^{3}H]thymidine incorporation was evaluated using an automated β liquid scintillation counter. HSG and MCF-7 cells were cultured in DME containing 10% FBS at 37°C. Tam (Wako Pure Chemical), 17β-estradiol (Wako), 10 μM caspase 1 inhibitor (Sigma-Aldrich), and recombinant human IFN-γ (R&D Systems) were used for cell cultures. RbAp48 gene inserted into pCMV (2N3T) construct (a gift from D. Trouche, Centre National de la Recherche Scientifique, University of Tolouse, Tolouse, France) (47) was transfected into the cells using FuGENE6 Transfection Reagent (Roche). Small interfering RNA (siRNA) corresponding to coding sequence +136 to +156 of RbAp48 gene was synthesized by Hokkaido System Science: CGAGGAAUACAAAAUAUGGTT (sense), CCAUAUUUUGUAUUCCUCGTT (antisense). When the siRNA was transfected into HSG cells together with GFP plasmid, 73.4% of cells were found to be GFP$^+$ HSG cells by flow cytometric analysis. Furthermore, the relative protein expression of RbAp48 to β-actin was reduced to ~80% by the siRNA.

Real-time quantitative RT-PCR. Total RNA was extracted from cultured HSG and MCF-7 cells using ISOGEN (Wako Pure Chemical), and reverse transcribed. Transcript levels of IRF-1, CIITA, and β-actin were performed using PTC-200 DNA Engine Cycler (Bio-Rad Laboratories) with SYBR Premix Ex Taq (Takara). Primer sequences were as follows: IRF-1, forward 5′-ACCCTGGCTAGAGATGCAGA-3′ and reverse 5′-CCTTTTCCCCTGCTTTGTATCG-3′; CIITA, forward 5′-CAGGCAGCAGAGGAGAAGTTCACCATC-3′ and reverse 5′-CCGTGAGGATCCGCACCAGTTTGGGG-3′; β-actin, forward 5′-AAATCTGGCACCACACCTTC-3′ and reverse 5′-GAGGCGTACAGGGATAGCA-3′.

Caspase activity. Caspase activities were assayed using Caspase-Family Colorimetric Substrate Set (BioVision, Inc.). In brief, 100 μg cytoplasmic lysates from lacrimal glands, salivary glands, and spleen of WT and *RbAp48*-Tg mice were incubated with 200 μM Ac-YVAD-pNA (Caspase 1 substrate), at 37°C for 1 h. The absorbance of samples was read at 405 nm in a microplate reader.

Promoter assay. For the measurement of the transcriptional activity of IRF-1, IRF-1 luciferase reporter vector (IRF-1/Luc) was purchased from Panomics. HSG cells plated in a 48-well plate were transiently transfected with 0.1 μg of IRF-1/Luc and 0.1 μg of pCMV-*RbAp48* or mock plasmid and 0.05 μg of phRL-TK (Promega Corp.) as an internal control using the FuGENE6. The cells were incubated overnight and subsequently treated with IFN-γ. After 10 h, the cells were harvested and subjected to a luciferase assay by using a dual-luciferase reporter assay system (Promega Corp.) as per the manufacturer's instructions. Relative luciferase activity was expressed as the fold- increase relative to the activity of untreated controls after normalization to the relative background of Renilla luciferase activity.

Cell transfer. CFSE-labeled splenic and cLN T cells (5 × 10^6) from WT and *RbAp48*-Tg mice were intravenously transferred into irradiated (700 cGy) C57BL/6.Ly5.1 mice. On the seventh day after the transfer, spleen cells were analyzed to measure homeostatic proliferation via CFSE dilution by flow cytometry. For induction of autoimmune lesions, total cells, T cells, or B cells from spleen cells (5 × 10^6) or cLN cells (5 × 10^6) from WT and *RbAp48*-Tg mice were intravenously transferred into *Rag2*$^{-/-}$ mice. At 6 wk after the transfer, the pathology of all the organs, including salivary and lacrimal glands, was analyzed. In addition, *Rag2*$^{-/-}$ hosts were ovariectomized (Ovx) or sham operated (Sham). Adoptive cell transfer was performed on the next day after Ovx or Sham.

In situ hybridization. Mice were perfused transcardially with saline (0.9%) followed by 4% PFA. The salivary glands were collected and fixed in 4% PFA at 4°C for 3 h. 6 μm paraffin-embedded sections were prepared for ISH.

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The RNA probe (587 bp) of mouse IFN-γ was produced by RT-PCR using primers (T3, AATTAACCCTCACTAAAGGGACTGGCAAAAGG-ATGGTGAC; T7, TAATACGACTCACTATAGGGAGATACAACCC-CGCAATCAC). Digoxigenin (DIG)-labeled antisense and sense control riboprobes were generated using DIG RNA labeling mix (Roche). The sections were pretreated with 10 μg/ml proteinase K for 10 min at room temperature and then hybridized with 1 μg/ml DIG-labeled probes at 45°C for 16 h. DIG was immunodetected with alkaliphosphatase-conjugated anti-DIG antibody. For positive controls, sections of spleen from lipopolysaccaride-injected mice were used. The probe was confirmed with the positive control sections, as shown in Fig. S6 B.

Human samples. Immunostaining for RbAp48 and IL-18 or IFN-γ were performed using lip biopsy samples from human SS patients and controls. All samples were obtained from the Tokushima University Hospital, Tokushima, Japan. This study was approved by certification of the ethics board of Tokushima University Hospital. All subjects signed a written informed consent before enrollment. All patients with SS were female, had documented xerostomia and keratoconjunctivitis sicca, and fulfilled the criteria of the Ministry of Health, Labor, and Welfare of Japan for the diagnosis of SS. All patients with SS had focus scores of greater than two in their lip biopsy and all tested positive for autoantibodies against Ro. Analysis was performed under the certification of the ethics board of Tokushima University Hospital. Frozen sections were stained with anti–human RbAp48 mAb (BD) and Alexa Fluor 488 donkey anti–mouse IgG (H+L; Invitrogen) and Biotin-conjugated anti-human IL-18 (MBL) or IFN-γ (eBioscience) mAbs and Alexa Fluor 568–conjugated streptavidin and analyzed by confocal microscopy. The nuclear DNA was DAPI.

Statistics. Student's *t* test was used for statistical analyses.

Online supplemental material. Fig. S1 shows T cell phenotypes of thymus from *RbAp48*-Tg and WT mice. Fig. S2 shows T reg cells of thymus, spleen, and cLN from *RbAp48*-Tg and WT mice. Fig. S3 shows B1 cells in salivary glands and marginal B cells of spleen and cLN from *RbAp48*-Tg and WT mice. Fig. S4 shows the purified MSG cells, and images of control staining for the expressions of MHC class II, CD86, CD80, ICAM-1, IFN-γ, and IL18. Fig. S5 shows IRF-1 and CIITA mRNA of MCF-7 cells stimulated with Tam or transfected with pCMV-*RbAp48*. Fig. S6 shows IFN-γ concentration of tissue homogenates of lacrimal, salivary, and spleen from *RbAp48*-Tg and WT mice, and control sections for in situ hybridization of IFN-γ mRNA. Fig. S7 shows BAFF expression of salivary glands and spleen from *RbAp48*-Tg and WT mice. Fig. S8 shows the time courses of IL-18, IFN-γ, and HLA-DR expressions of HSG cells stimulated Tam or transfected with pCMV-*RbAp48*. Fig. S9 shows IFN-γ secretion from MCF-7 in response to IL-18. Fig. S10 shows control staining for RbAp48 expression together with IFN-γ or IL-18 in salivary glands from human SS patients and controls. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080174/DC1.

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Future alternatives in "3Rs": Learning from history

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Abstract
A large decrease in the number of experimental animals utilized in testing and research was reported in the last decade (Surveyed by Expt'l Animal Soc.[1]). For rats, the numbers used in experiments in Japan were 2.09 million in 1995, 1.53 million in 1998, and 1.24 million in 2001. Thus, there was a 40% decrease in the number of rats used from 1995 to 2001. For mice, a larger decrease (58%) was also observed, from 6.68 million in 1995 to 2.80 million in 2001. These decreases were clearly due not only to the development of 3Rs (i.e., Reduction, Refinement, and Replacement of animal use) in alternative research, but also to marked changes in the focus of experimental animal biology. In the academia, animal experiments using wild-type mice have decreased in number to a large extent relative to those using genetically modified mice because of the mechanistically much reliable outcomes obtained by genetically modified mice than those from wild-type animals. Yet, biological safety studies for pharmaceutical development as well as industrial chemical safety studies utilize conventional toxicological bioassays.

Keywords: 3Rs, Claude Bernard, Bruce N. Ames, Patric O. Brown

Introduction

Historically, three scientists are recognized in relation to the history of experimental animal use; the first, the initiator of experimental animal research; the second, the first contributor to the marked reduction of the number of experimental animals used; and the third, a potential contributor, who invented an ultimate method for reducing the number of animals for future research, the gene chip technology. The use of animals in experimental studies was initiated by Claude Bernard (1813-1878), originally who was trying to put an end to human vivisections common at that time; thus, he came to be regarded as "the devil of experimental animals." The most remarkable contribution to reducing the number of experimental animals used was made by Bruce N. Ames, who rescued innumerable animals that might have been used for genotoxic carcinogenicity studies. Another contribution may be attributed to Patrick O. Brown, who invented transcriptomics, which can be used to elucidate the underlying mechanistic background of phenotypes of experimental animals; the method is considered to have eventually led to the minimization of experimental animal use. Consequently, the most essential and powerful driving force for future alternatives may be minding the 3Rs but also the promotion of basic sciences and technologies.

1. Claude Bernard – An initiator of animal experiments

Claude Bernard (1813-1878)

Bernard was born in the village of Sain-Julien in 1813, and went to Paris at the age of twenty-one. As reported, he first wanted to be a play wright, but took up medical studies on the advice of a literary person. He learned medical science from the famous Françoise Magendie, and earned his PhD after pursuing the study of gastric acids. He was appointed as Magendie's deputy professor at the college in 1847, and made seminal discoveries such as those of hepatic glycogen, vasomotor neurons, and curare

narcosis. Any of these discoveries must have made him an accomplished medical scientist. Because of his scientific principles, he strictly defined *observers* and *experimenters*, critically. He called observers as those who do not alter "nature", but statically observe the ostensible world; whereas experimenters are those who purposely alter "nature" to obtain a reaction, and seek natural responses behind the phenomenal world. He strongly recommended the use of living organisms to obtain responses, and seek natural reactions behind the phenomenal world. This is the reason why he emphasized the use of vivisection in science throughout his life. In his major discourse on scientific methods, "An Introduction to the Study of Experimental Medicine" (1865), Claude Bernard described what makes a scientific theory good and what makes a scientist important and a true discoverer. Unlike many scientific writers of his time, Bernard writes about his own experiments and thoughts, and uses the first person[2].

Although Bernard was the first scientist who initiated the use of animals in experiments, his original aims at that time were to criticize physicians and to rescue humans from iatrogenic accidents due to poor and insufficient surgical treatments. However, his wife and daughter initiated the first "animal rights campaign" immediately after his death[3], because of their intense aversion to Bernard's animal studies without using anesthesia, namely, vivisection, although this is ironically the best and appropriate method of determining the response of experimental animals.

It is about a century since Bernard started a systematic education on animal experiments. Experimental studies using animals changed last decades because of not only a greater awareness about animal welfare, but also greater decreases in the need for conventional experiments. Accordingly, in 1984, the International Guiding Principles for Biomedical Research Involving Animals was established. Then, in 1985, the European Convention also established the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes.

In Japan as well also, laws for animal care were successively passed in the 1970's. The Act for Animal Welfare and Proper Administration was passed in 1973, and the Guidance Documents for Experimental Animal Maintenance and Proper Administration in 1980. The Guideline for Experimental Animal Use was established in the same year in 1980 by the Japanese Academy of Science, the Guideline for Proper Use of Experimental Animals in 1987 by the College Union, and the Extension of Animal Life and Ethics by the Japanese Academy of Science in 1996. Recently, the establishment of the Act for Animal Welfare and Proper Use in Experiment was issued in 1999. Despite these guidelines, we could not eliminate all the animal experiments at the moment. However, we are now at the turning point in the history of experimental animal use.

2. Bruce N. Ames – accomplished the most prominent alternative study –

Bruce N. Ames

We now introduce a scientist who developed the revertant mutagenesis assay, Bruce Ames[4]. Ames is now a Professor of Biochemistry and Molecular Biology at the University of California, Berkeley. He is a member of the National Academy of Sciences and he was on their commission on life sciences. His publications of more than 450 led to his being among the most cited scientists.

The idea of mutation induced by chemical compounds was first described in 1944[5-6]; this was about 30 years prior to Ames' development of revertant mutagenicity assay. Chemical mutagenesis became the focus of considerable attention, because large amounts of industrial chemicals started to be used in various industries in the mid-twentieth century. Afterwards, because Ames' test enabled the detection of most mutagenic compounds, it has contributed greatly to a large reduction of the number of experimental animals used for in vivo mutagenicity bioassays. What Bruce Ames originally proposed was to use an induced bacterial gene mutation as an evaluation tool for mammalian mutagenesis. He attempted to develop a system for incorporating mammalian microsomal metabolism to the assay also by him, which is presently known as the S9-mixture[7]. It took a very long-time to establish the test system after considerable debate, because scientists at that time had to learn the difference between direct genotoxic carcinogenesis and indirect genotoxic carcinogenesis, namely, epigenetic carcinogenesis. However, after the establishment of the test system, innumerable experimental rats and mice were saved from carcinogenicity bioassay. Although Ames did not intend to save experimental animals by his invention, knowing such common rule of mutagenicity in genetics between Salmonella and mammalians made innumerable number of reduction in experimental animal use possible. Thus, from the study of Ames, our conclusion on alternative studies, is, that an

essential strategy for reaching an alternative goal may be the "Development of True Sciences."

The current major interests of Bruce N. Ames are reported to be the determination of optimum micronutrient intake for minimizing human DNA damage as a preventive measure against cancer, and the study of other degenerative diseases associated with aging.[8-9]

3. Patric O. Brown – gene chip technology

Patrick O. Brown

The third person who we introduced is Patrick Brown, who invented a new methodology, that is, gene chip technology[10]. The gene chip technology and the consequent toxicogenomics[11] that he developed were supposed to rapidly minimize experimental animal use to a large extent[12] (Meeting proceedings from ECVAM-ICCVAM/NICEATM, 2006).

The establishment of the genome sequencing program in 2000 was supposed to be a strong driving force for the progress of alternative studies, particularly via toxicogenomics. All the information derived from animal experiments is incorporated in the genome expression database, that is, "computer mouse", which may be virtually used in the near future even without actual animal experiments.

The method established by Patrick Brown is "molecular microscopy", which enables the differentiation of patterns of gene expression profiles[13]. We showed sample expression profiles of genotoxic compounds studied by the consortium of International Life Science Institute (ILSI), which showed a short-term differential prediction of chemicals with DNA-binding affinity, such as cisplatin, methotrexate, mitomycin C, and chemicals with indirect genotoxicity, such as, taxol, hydroxiurea, and etoposide. Such a rapid and easy prediction may greatly contribute to the realization of essential purposes leading to the development of 3Rs.

Concerning the gene expression profiles, linear increase in dose-response relationship obtained by a conventional testing protocol may not be always applicable each other. In the presented example of microarray data after radiation exposure, because the expression levels of some genes increase with radiation dose and those of some genes decrease with increasing radiation dose, the dose-response relationship obtained by a conventional toxicological testing protocol can be assumed as the only phenomenologic outcome on the basis of one aspect. Rather, we recommend that the dose-response relationship should be considered complex, and that these combination profiles per se, may be essential biomarkers. The authors showed other sample data obtained after whole-body radiation in which one can observe dose-related expression profiles, on one hand, and dose-specific expression profilings, on the other.

Another issue that the authors introduced was age-related stochastic and probabilistic gene expression profilings, which can also be visualized in nontreated senescent mice when one focuses on their individual gene expression. By linear configuration for gene expression, one can clearly recognize that the divergent expression profiles of each individual mouse were not due to an error, but biological diversity with aging. Moreover, representative responsible genes showed clear differences between 2-month- and 21-month-old profiles, which elucidated the age-related responsible gene ontology, represented by the senescence-specific genes[14].

In the cases of experimental myeloid leukemias, spontaneous leukemias are differentiated from those of radiation-induced myeloid leukemias by their different responsible gene intensities in the line configuration of the expression gene profilings. They are also differentiated by the analysis of principal components, which are observed from the three dimensional expression. These databases are also supposed to be essential information for developing 3Rs supported by basic science.

Toxicogenomics sometimes makes the categorical border between physiology and toxicology ambiguous. Similar genes, such as those encoding apoptosis-related genes, caspases, participate simultaneously as physiologic and toxicologic parameters. Toxicogenomics sometimes changes a toxicologic paradigm. Depending on such fluctuating changes in the cell cycle genes, for example, and many other cellular functions, which may be mild or severe, the degree of oscillatory ranges differs from one another, which may be new risk factors.

Conclusion

Lastly, as we mentioned above, the use of experimental animals has, unfortunately, not been completely eliminated to date. Thus, in this regard, we would like to emphasize that "science should progress further". Certainly, one may not accept any risky drugs that have not undergone preclinical animal testing for use in one's children. On the other hand, no one may believe that animal studies will be continued for more than 4-500 years from now. We believe that experimental animals may be eventually replaced by other technical systems developed in the future, although such systems are still technologically immature to replace everything at this moment.

Table 1. Surveillance of experimental animals used in Japan.

	1995	1998	2001
rats*	2.09 (100)	1.53 (73)	1.24 (59)
mice *	6.68 (100)	[redacted]	2.80 (42)

* Million / (%)

Surveyed by the Society of Experimental Animals in Japan[1].

The authors emphasized that animal testing may be eventually replaced by other new technologies, and animal testing would eventually disappear. Some people, however, believe that animal testing should be replaced immediately by other technologies; hopefully today, if not today, maybe tomorrow! These gaps may be filled by nominal driving forces such as humane animal welfare, industrial economy, and politics. However, the essential driving force for this matter may be the development of science itself, particularly by the development of "genome sciences". In other words, an elimination of animal experiments may be l'oiseau bleu (blue bird) of each scientist for the development of future science.

A recent survey by the Experimental Animal Society of Japan showed marked decreases in the number of experimental animals used[15]. As shown in the **Table 1**, the numbers of rats and mice used decreased to 59% and 42%, respectively, since 1995. The possible reason for these decreases is the obtainment of considerably clear-cut experimental results using a relatively small number of genetically modified animals, whereas unreliable experimental results are obtained with a relatively larger number of wild-type animals. These data strongly suggest the future possible reduction in the use of experimental animals.

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FULL PAPER *Clinical Pathology*

Gene Expression Changes Induced by Type IV Allergy-Inducible Chemicals in Dendritic Cells

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ABSTRACT. In the present study, the changes of gene expression profile in dendritic cell (DC)-derived DC2.4 cells sensitized with two allergenic chemicals were analyzed by microarray analysis to develop a basis for an *in vitro* assessment system of type IV allergenic chemicals. Consequently, 26 genes were significantly up-regulated, and 53 were down-regulated in both groups. Interestingly, some of up-regulated genes were associated with the maturation process of DCs. A set of genes was further evaluated by real-time reverse transcription-polymerase chain reaction to identify the gene expression changes specifically induced by type IV allergy-inducible chemicals in DC2.4 cells, and 2 possible candidates, syndecan-1 (Sdc1) and smoothened (SMO) genes were identified. Thus, up-regulation of Sdc1 gene and down-regulation of SMO gene in DC2.4 cells may be diagnostic markers for the screening of type IV-allergenic chemicals.
KEY WORDS: chemical, dendritic cell, microarray analysis, type IV allergy.

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Allergic contact dermatitis (ACD), a type IV allergy, is one of the most common inflammatory diseases of the skin with unknown genetic basis and is often an occupationally related disorder in industrialized countries with an important socio-medical impact [14, 28]. At present, many chemicals are considered to have allergenic potency and thus risk assessment of such chemical substances is important. ACD has been intensively studied, and the development of an allergic hypersensitivity reaction in the skin is considered to be processes depending on the induction of specific T-lymphocyte responses [23]. At the initial step, chemical allergens exposed on the skin are recognized by Langerhans cells (LCs), the principal DC residing in the epidermis and known to play a key role in the development of ACD. Following an encounter with a chemical allergen, LCs are activated and subsequently migrate from the skin to the draining lymph nodes, undergoing a maturation process during the journey [20, 22].

For many years, guinea pigs have been applied for the hazard analysis of skin-sensitizing chemicals [9, 26]. Recently, the local lymph node assay (LLNA) was developed in mice as an alternative approach based on the characterization of initial proliferative responses in draining lymph nodes caused by chemicals exposure [16, 21, 34, 39], and this method is now widely used for estimation of the allergenic potency of chemical substances. Although these approaches are sensitive and reliable, more advantageous ways in terms of cost performance, safety, readiness and animal welfare are expected. More recently, *in vitro* assay for chemical substances with allergenic potency is extensively explored by using cultured cells, especially macrophages [5, 41]. In these experiments, up-regulation of several molecules including cell surface markers were reported to be induced by chemical exposure, suggesting that these markers may be candidates for evaluating chemicals with allergenic potency [5, 41]. However, as well documented, LCs, a family of DCs, are the main antigen presenting cell in the epidemis and have central roles on the induction of allergic skin disorders [4, 38]. In this regard, assessment of LC/DC responses to chemical exposures should be reasonable to know accurate mechanisms of ACD development. Although several studies evaluated the effect of chemicals on DCs by use of *in vitro* differentiated primary DCs, it is disadvantageous because of the limited numbers in the source, donor-to-donor variability and cost for obtaining these cells.

In the present study, the changes of gene expression profile in an established DC line sensitized with allergenic chemicals were analyzed by microarray analysis and real-time reverse transcription-polymerase chain reaction (RT-PCR) to develop a basis for an *in vitro* assessment system of type IV allergy-inducible chemicals.

MATERIALS AND METHODS

Chemicals: Chemical substances, 2,4-dinitrochlorobenzene (DNCB; Wako Pure Chemical Industries, Osaka, Japan), *p*-benzoquinone (BQ; Kanto Chemical, Tokyo, Japan), citral (Cit; Nacalai Tesque, Kyoto, Japan), trimellitic anhydride (TMA; Kanto Chemical) and dextran (Dex; Kanto Chemical) were dissolved in DMSO to a concentration of 10 $\mu g/ml$ as a stock solution and further diluted with cell culture medium for use. DNCB, BQ and Cit are known to be strong sensitizers and type IV allergy-inducible chemicals [15, 18, 24]. TMA is a respiratory sensitizer and a strong inducer of type I allergy. This chemical is also

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known as an irritant on the skin [10, 17]. Dex is reported to be a non-sensitizer on the skin [7, 39].

Cells: The cell line DC2.4, derived from C57BL/6 mice, was kindly provided by Dr. Kenneth L. Rock (Division of Lymphocyte Biology, Dana Farber Cancer Institute, Boston, MA, U.S.A.). The DC2.4 cells have a dendritic morphology, express DC-specific markers, MHC molecules and costimulatory molecules, and have phagocytic activity as well as antigen-presenting capacity [35]. The cells were maintained in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Cansera International Inc., Ontario, Canada), 4 mM L-glutamine and 50 μM 2-mercaptoethanol at 37°C under 5% CO_2 in air.

Sensitization of DC2.4 cells and RNA extraction: DC2.4 cells were plated at 1.0×10^5 cells/m*l* and incubated for 12 hr. The cells were treated with chemicals in non-toxic doses (0.1 μg/m*l* for DNCB, BQ and Cit, 1.0 μg/m*l* for TMA and Dex) and harvested after cultivation for 24 hr. Total RNA was extracted with RNeasy mini kit (QIAGEN K. K., Tokyo, Japan).

Microarray analysis: Total RNA from control and BQ-treated DC2.4 cells was reverse transcribed into cDNA using Fluorescent Direct Label Kit (Invitrogen, Carlsbad, CA). cDNA samples were labeled with Cy3-dCTP or Cy5-dCTP (AmershamPharmacia Biotech, Piscataway, NJ) and purified with MinElute Mini Kit (QIAGEN K. K.). Microarray was performed with Mouse cDNA Microarray Kit (Agilent Technologies, Palo Alto, CA), and the data was analyzed by Feature Extract software (Agilent Technologies). Microarray analysis of DNCB-treated DC2.4 cells was performed with Filgen Array Mouse 32K kit (Filgen Technologies), and the data was analyzed by GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA).

Quantification of the gene expression by real time RT-PCR: The real time RT-PCR was performed with the QuantiTect SYBR Green RT-PCR kit (QIAGEN K.K.) in accordance with the manufacture's instructions. Briefly, 25 μl of a PCR mixture in a tube containing each target gene-specific primer pair at 0.5 μM, 1 × QuantiTect SYBR Green RT-PCR Master Mix, 0.25 μl of QuantiTect RT Mix, and 0.2 μg of total RNA was subjected to reverse transcription at 50°C for 30 min. After inactivation of reverse transcriptase at 95°C for 15 min, real-time PCR amplification was performed with 40 cycles of denaturation (94°C for 15 sec), annealing (55°C for 30 sec), and polymerization (72°C for 30 sec). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was assessed by real-time RT-PCR. The target genes and their primer sequences addressed in this study were described in Table 1. For the compensation of accidental errors among samples, relative gene expression numbers were calculated as raw values divided by the amount of GAPDH in the same samples.

RESULTS

Sensitivity of DC2.4 cells to chemical exposures: To determine the dose of chemicals for *in vitro* sensitization to DC-derived DC2.4 cells, the cells were exposed to various concentrations (0–100 μg/m*l*) of chemicals for 24 hr, and cell viabilities were determined by trypan blue dye exclusion assay. The viability of DC2.4 cells was largely affected when exposed to DNCB, BQ and Cit at the concentration of 5–10 μg/m*l* although their cytotoxicity was low at 1–5 μg/m*l* and almost background level at 0–0.5 μg/m*l*. On the other hand, the effect of TMA or Dex on the viability of DC2.4 cells was minimal through the examined concentrations (0–100 μg/m*l*) (Fig. 1). Based on these findings, we decided to use these chemicals at the concentration of 0.1 μg/m*l* on DNCB, BQ and Cit, and 1.0 μg/m*l* on TMA and Dex for subsequent experiments.

Microarray analysis of gene expression changes in DC2.4 cells induced by chemical exposures: To determine the typical gene expression changes in DC2.4 cells induced by type IV allergenic chemical exposure, DC2.4 cells were exposed to DNCB or BQ, both of which are known as strong sensitizers of contact hypersensitivity, for 24 hr and subjected to cDNA microarray analysis. In the DNCB-treated DC2.4 cells, 957 genes were up-regulated with fold changes of +1.3 to +4.4 and 1933 genes were down-regulated with fold changes of –1.3 to –9.0 when compared with control samples. On the other hand, in the BQ-treated cells, 790 genes had fold increases in expression over control ranging from +1.3 to +3.9, and 726 genes showed a down-regulation with fold changes of –1.3 to –2.2. When we overlapped these two data, 26 genes were up-regulated, and 53 were down-regulated in both groups. The changed genes represented a wide range of cellular processes including signal transduction, protein modification/synthesis and transcription. A list of genes which were up-regulated in DC2.4 cells

Table 1. Primer sequenses used for real-time RT-PCR

Gene name	Gene symbol	Forward primer	Reverse primer
glyceraldehyde-3-phosphate dehydrogenase	GAPDH	5'-TGAACGGGAAGCTACACTGG-3'	5'-TCAGATCCACGACGGACACA-3'
Cytokine inducible SH2-containing protein 2	Socs2	5'-CAGTCAAACAGGATGGTACTGG-3'	5'-AGTCTTGTTGGTAAAGGCAGTC-3'
Syndecan-1 precursor	Sdc1	5'-TTCATTGTGGGGAGGTCTAC-3'	5'-AAGTTCTCACACAGGCTCTT-3'
Tubulin alpha-6 chain	Tuba6	5'-CTGATGGAGCGGCTCTCTGT-3'	5'-AAGCTGCTCATGGTAGGCTTTC-3'
Lymphocyte antigen 86 precursor (MD-1 protein)	LY86	5'GGAGAATATCAGCTCTTGCTGG-3'	5'-CCAGGCAACTTCAAGGAAGTG-3'
smoothened	SMO	5'-TTGGATGCAGACTCGGACTT-3'	5'-CCAGAAAAGGGCACTCATTGG-3'
Hairy/enhancer-of-split related with YRPW motif 1	Hey1	5'-CTTCGGACATCACCCACACA-3'	5'-GTCCCAACACACCTGGGATT-3'
phosphatidylinositol 3-kinase, C2 domain containing, alpha	Pik3c2a	5'-CCTTTGCTGGGTACATGATGAC-3'	5'-GGAAGGTTAACTGCTCGCTTT-3'
polypeptide epidermal growth factor receptor pathway substrate 8	Eps8	5'-AACTGGCCAGTTCGGTACTC-3'	5'-GGAGTTGACTTGAAAGGCATGG3'
exonuclease 1.	Exo1	5'-GCTGGCTGAAGATGACCTGTT-3'	5'-AGCTCAGATTGTGCATCCCATT-3'
N-acylsphingosine amidohydrolase 2; neutral/alkaline; neutral/alkaline ceramid	Asah2	5'-AACAACCATGTCGGGACGAA-3'	5'-GAGGAAGGTTTGATGGGTCTGG-3
carboxypeptidase D	Cpd	5'-CAACTTCACCCTTGTTCGATCT-3'	5'-CAGTTCCAACTGGAGCGTTT-3'

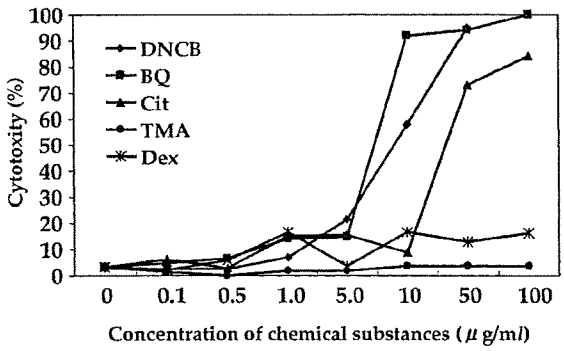

Fig. 1. Sensitivity of DC2.4 cells to chemical exposures. DC2.4 cells were exposed to chemicals at various concentrations (0–100 μg/ml) for 24 hr, and cell viabilities were determined by trypan blue dye exclusion assay.

after exposure to DNCB or BQ for 24 hr are presented in Table 2 along with their accession number and fold-change relative to the control cell cultures. The genes down-regulated are also shown in Table 3.

Real-time RT-PCR analyses of gene expression changes in DC2.4 cells induced by chemical exposures: In order to determine the reproducibility of the gene expression changes observed in microarray analysis, a set of genes was selected for evaluation by real-time RT-PCR. In the previous study, we assessed *in vivo* changes of gene expression in mouse ear sensitized with a type IV allergy-inducible chemical, BQ (data not shown). The microarray data from DNCB or BQ-treated DC2.4 cells were compared with that from type IV chemical-exposed mouse ears, and up- or down-regulated genes in all the experiments were explored. As the result, 3 up-regulated (Socs2, Sdc1 and Tuba6) and 8 down-regulated (Eps8, Exo1, Asah2, Cpd, LY86, SMO, Hey1 and Pik3c2a) genes showed strict changes in all the experiments,

Table 2. List of genes that were up-regulated by 24 hr exposure to DNCB and BQ

Accession no.	Gene name	Gene symbol	Fold-change	
			DNCB	BQ
Cytokine				
M27960	Interleukin-4 receptor alpha chain	Il4ra	1.3	1.5
AK078708	LPS-induced TNF-alpha factor	Litaf	1.6	1.3
Protein modification/synthesis				
AJ578468	defensin beta 14	NM_183026	1.7	1.4
U80019	proline dehydrogenase (oxidase) 2	Prodh2	1.3	1.4
AK054050	amine oxidase (flavin-containing)	Maob	1.3	1.5
L07645	Histidine ammonia-lyase	Hal	1.5	1.3
AK007058	L-lactate dehydrogenase C chain	Ldh3	1.8	2.7
AI323038	Cathelin-related antimicrobial peptide	Camp	1.4	1.4
NM_008185	Glutathione S-transferase theta 1	Gstt1	1.6	1.4
AK076002	proteasome 26S non-ATPase subunit 11	Psmd11	1.7	2.4
Siganal transduction				
AK036928	MAP kinase kinase 4	Map2k4	1.5	1.7
U88327	Cytokine inducible SH2-containing protein 2	Socs2	1.7	1.3
AK122347	Dedicator of cytokinesis protein 10	Dock10	2.7	1.5
Cell surface receptors/membrane proteins				
AF237914	membrane-spanning 4-domains.	Ms4a4c	1.7	1.4
NM_011519	Syndecan-1 precursor	Sdc1	1.6	1.3
BC057133	Chloride channel protein 3	Clcn3	1.4	1.4
Transcription factors/activators/repressors				
L09600	nuclear factor. erythroid derived 2	Nfe2	1.4	1.7
AK131183	Nuclear factor erythroid 2 related factor 1	Nfe2l1	1.7	1.7
Cell cycle/proliferation/death				
K014411	Synaptonemal complex protein 2	SCP2	1.6	1.5
Others				
BC040397	3 beta-hydroxysteroid dehydrogenase	Hsd3b2	2.1	1.9
U12147	Laminin alpha-2 chain	Lama2	1.4	1.4
AK081350	phosphatidylinositol 3-kinase. catalytic.	Pik3cb	2.3	1.7
M13441	Tubulin alpha-6 chain	Tuba6	1.3	1.6
BC010745	tubulointerstitial nephritis antigen	Tinag	1.4	1.7
L29468	Cofilin. muscle isoform (Cofilin 2)	Cfl2	1.6	1.3
XM 354745	Proliferin 1 precursor	Mrpplf3	1.3	1.4

Table 3. List of genes that were down-regulated by 24 hr exposure to DNCB and BQ

Accession no.	Gene name	Gene symbol	Fold-change	
			DNCB	BQ
Cell surface receptor/membrane proteins				
Y18365	CD97 antigen precursor.	Cd97	-1.6	-1.7
K027929	Lymphocyte antigen 86 precursor	LY86	-2.2	-2.1
K014556	epidermal growth factor receptor pathway	Eps8l1	-1.4	-1.6
AJ251594	CD44 antigen	CD44	-1.3	-1.3
F263247	macrophage erythroblast attacher	Maea	-1.4	-1.3
F033017	potassium channel. subfamily K. member 1	Kcnk1	-1.4	-1.6
F089721	smoothened	SMO	-1.4	-2.4
Transcription factors/activators/repressors				
AF172286	Hairy/enhancer-of-split related with YRPW	Hey1	-1.8	-1.8
F004295	myelin transcription factor 1-like	Myt1l	-1.4	-1.8
BC048845	inhibin beta-B	Inhbb	-1.4	-1.4
BC052697	splicing factor 3a. subunit 2	S3A2	-1.4	-1.6
BC068268	POU domain. class 5. transcription factor 1	Pou5f1	-1.4	-1.5
BC057126	Cbp/p300-interacting transactivator	Cited2	-1.3	-1.5
Y10926	binding protein suppressor of hairless-like	Rbpsuhl	-1.4	-1.6
AK049260	RNA polymerase II transcriptional coactivator	Rpo2tc1	-1.4	-1.5
U83630	nuclear antigen Sp100	Sp100	-1.4	-1.4
AK031025	enhancer of polycomb homolog 2	Epc2	-1.4	-1.4
Cell cycle/proliferation/death				
Z26580	Cyclin A2 (Cyclin A).	Ccna2	-1.6	-1.8
AK004355	cyclin-dependent kinase 2-interacting protein.	NM_026048	-1.8	-1.5
AF060246	zinc finger protein 106	Zfp106	-1.3	-1.3
AF316548	zinc finger protein 148	Zfp148	-1.4	-1.5
AF068780	geminin	GEMI	-1.4	-1.3
BC065144	meiotic recombination 11 homolog A	Mre11a	-1.3	-1.5
U52193	phosphatidylinositol 3-kinase. C2	Pik3c2a	-1.4	-1.8
Small molecule transport				
AK015400	ferritin heavy chain 3; mitochondrial ferritin.	NM_026286	-1.6	-1.6

and thus these genes were subjected to further real-time RT-PCR analysis. The real-time RT-PCR results showed that all up-regulated genes and 6 of 8 down-regulated ones corresponded to microarray analysis data although Sdc1 and Pik3c2a showed only limited changes when compared with microarray analysis data (Fig. 2).

To determine whether any of the significant gene changes observed were due to the reactive nature of the contact allergen, DC2.4 cells were exposed to 1.0 μg/ml DMSO (solvent used to dissolve chemicals as a control), 0.1 μg/ml BQ (a type IV allergy-inducible chemical), 0.1 μg/ml Cit (a type IV allergy-inducible chemical), 1.0 μg/ml TMA (an irritant on the skin and a type I allergy inducer) and 1.0 μg/ml Dex (a non-sensitizer on the skin) for 24 hr, and gene expression changes were determined by real-time RT-PCR. As shown in Fig. 2, all 3 up-regulated genes chosen were enhanced, and the 8 down-regulated genes were diminished their expression by BQ- or Cit-treatment for 24 hr although some genes such as Tuba6 and Cpd showed limited changes. When treated with TMA, enhanced expression of LY86 and SMO genes, which are down-regulated after type IV allergy-inducible chemicals, was observed (Fig. 2). Socs2 gene, which was up-regulated after sensitization by type IV allergy-inducible chemicals, was also up-regulated by the TMA-treatment, while the expression levels of other genes were scarcely affected by TMA (Fig. 2). In case of Dex-treated cells, the changes of gene expression levels were seen on some genes; up-regulation of SMO and down-regulation of Sdc1, Eps8, Exo1, Asah2, Hey1 and Pik3c2a (Fig. 2). From these results, up-regulation of Sdc1 gene and down-regulation of SMO gene in DC2.4 cells may be specific for type IV-allergy inducible chemicals.

DISCUSSION

In the present study, we focused on the induction phase of type IV allergy and attempted to identify the genes specifically changed by type IV allergy-inducible chemicals in DCs. For this purpose, changes of gene expression profile in DCs to hapten exposure were evaluated by microarray analysis, since LCs, a family of DCs, were shown to have central roles on the initiation of immune responses including allergic reactions in the skin [4, 38]. Recently, attempts have been made to develop *in vitro* sensitization tests using DCs derived from peripheral blood mononuclear cells or $CD34^+$ hematopoietic progenitor cells purified from cord

Table 3. Continued

Accession no.	Gene name	Gene symbol	Fold-change	
			DNCB	BQ
Signal transduction				
U85021	adenylate cyclase 8	Adcy8	-1.3	-1.4
BC012488	Rho guanine nucleotide exchange	Arhgef1	-1.3	-1.3
Y13346	adenosine A2a receptor	Adora2a	-1.4	-1.4
BC085270	RAB11B. member RAS oncogene family	Rab11b	-1.4	-1.5
L21671	epidermal growth factor receptor pathway	Eps8	-1.3	-1.6
Protein modification/synthesis				
D87521	protein kinase. DNA activated. catalytic	Prkdc	-1.4	-1.5
M95408	PTK2 protein tyrosine kinase 2	Ptk2	-1.4	-1.5
Others				
AF071316	COP9 (constitutive photomorphogenic)	Cops7a	-1.9	-1.5
AJ238213	exonuclease 1.	Exo1	-1.5	-1.7
BC056376	Myotubularin-related protein 1	Mtm1	-1.8	-1.6
AB037181	N-acylsphingosine amidohydrolase 2	Asah2	-1.7	-1.5
U94662	Trk-fused gene.	Tfg	-2.2	-1.6
AF123502	DNA polymerase epsilon. catalytic subunit A	Pole	-1.6	-1.7
BC068143	DNA-directed RNA polymerase III	Polr3b	-1.8	-1.5
M94584	chitinase 3-like 3; eosinophil chemotactic	Chi3l3	-1.9	-2.3
AK078888	interferon-related developmental regulator 1	Ifrd1	-1.4	-1.4
AF045252	tousled-like kinase 2 (Arabidopsis)	Tlk2	-1.3	-1.3
BQ928977	tumor protein D52	Tpd52	-1.3	-1.5
X84692	spermatid perinuclear RNA binding protein	Strbp	-1.3	-1.6
CA478631	metallothionein 1	Mt1	-1.3	-1.5
AF031939	RalBP1 associated Eps domain containing	Reps1	-1.4	-1.5
X97982	poly(rC) binding protein 2	Pcbp2	-1.4	-1.4
D85391	carboxypeptidase D	Cpd	-1.4	-1.8
NM_007622	Chromobox homolog 1 (Drosophila HP1 beta)	Cbx1	-1.4	-1.5
BC056376	myotubularin related protein 1	Mtm1	-1.4	-1.6
AF411253	EF hand calcium binding protein 2	Efcbp2	-1.4	-1.4
BC079642	abl-interactor 1	Abi1	-1.4	-1.4
BC011246	hemopexin	HEMO_MOUSE	-1.4	-1.3

blood or bone marrow [1, 36]. However, the use of *in vitro* differentiated primary DCs is difficult due to the nature of these cells such as low numbers in the source and donor-to-donor variability [2]. In addition, treatment with several cytokines is generally applied to obtain DCs from blood or bone marrow cells [3, 11, 30], and this process probably changes the cell reactivity to stimulations. Thus, established cell lines are preferable to standardize the condition among assay. A recently established DC line, DC2.4 cells, was applied as a target cell for this assay, and its reactivity to chemical exposures was addressed by microarray analysis. As the result, many gene expression changes were observed after treatment with two different allergenic chemicals, DNCB and BQ. Overall, the changes seemed to be not so noticeable. It is because of the nature of this cell line since the human monocyte-derived THP-1 cells with the same treatment extensively changed a large number of gene expression profile (data not shown). In addition, similar results to our data were reported in a recent study using primary DCs from peripheral blood after chemical treatment [31, 36, 37], suggesting that the effect of sensitization on the gene expression levels might be relatively mild in DC lineage. We analyzed the two data from DNCB- and BQ-sensitized cells and tried to line up the candidate genes specifically up- or down-regulated by type IV allergy-inducible chemicals. As the results, 26 genes were shown to be up-regulated, and 53 were down-regulated in both groups. Interestingly, some of up-regulated genes were associated with the maturation process of DCs. These include TNF-α (a maturation-inducing cytokine), Sdc-1 (a cell surface proteoglycan induced during the maturation process), Map2k4 (a member of MAP kinase kinase family associated with migration and maturation of DC) and Socs2 (a suppressor of cytokine signaling molecule induced during the maturation process) [12, 19, 40]. In addition, up-regulation was also detected on defensin and cathelin, which were formerly considered to work just as antimicrobial peptides [6, 8] and recently reported to have cell migration activity and to be associated with DC maturation process [25]. In contrast, down-regulation of CD44 was detected, which is reported to be expressed on mature DC and induce adhesion with T cell. This may be explained by that the time point at 24 hr after sensitization is still in the process of maturation. In the previous studies, many other molecules are reported to be associated with DC maturation process; for instance, up-regulation of transcripts for the co-stimulatory molecules

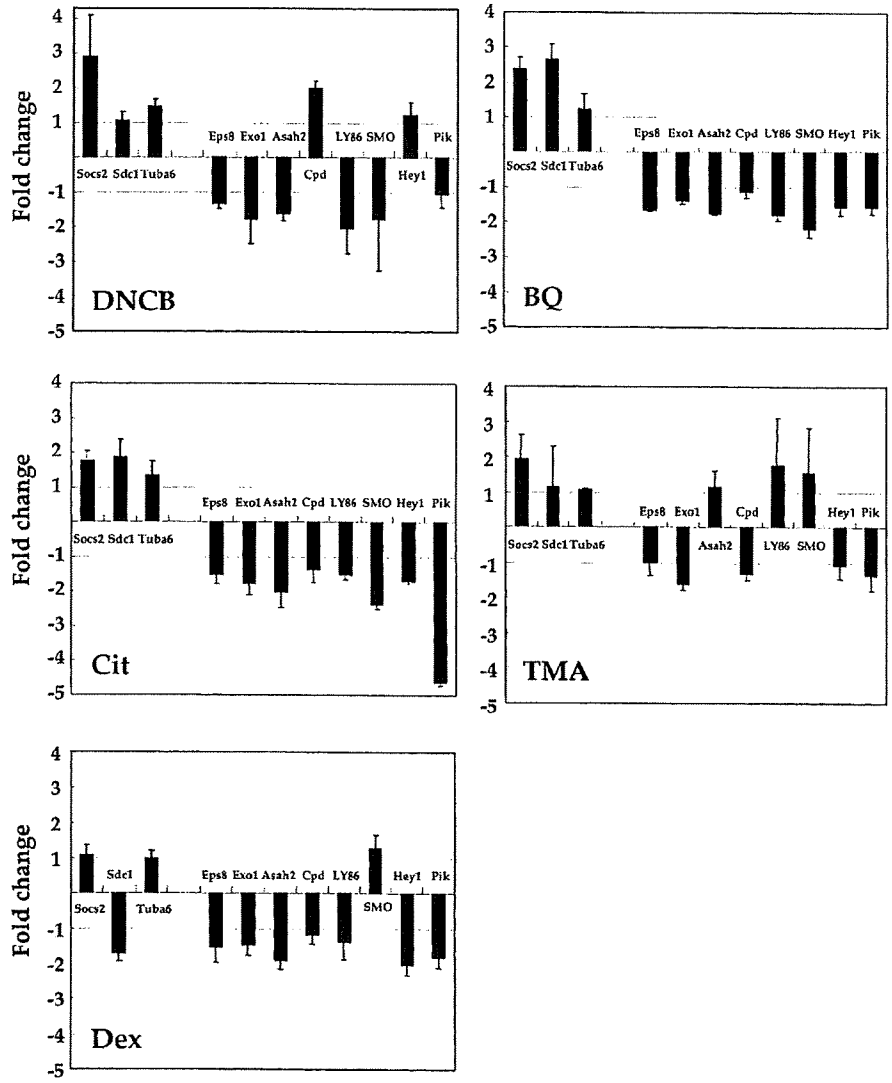

Fig. 2. Gene expression changes in DC2.4 cells induced by DNCB, BQ, Cit, TMA and Dex. DC2.4 cells were exposed to type IV allergy-inducible chemicals, DNCB, BQ or Cit (0.1 μg/m*l*), a type I allergy-inducible chemical, TMA (1.0 μg/m*l*) or a non-sensitizer, Dex (1.0 μg/m*l*) for 24 hr, and the changes of the gene expression were analyzed by real-time RT-PCR. Fold changes were determined based on the gene expression in the cells exposed to solvent DMSO used for solubilization of chemicals.

CD86 [29] and the constitutive chemokine receptor CXCR4 [32, 33], and down-regulation of genes encoding molecules involved in antigen uptake such as the high affinity IgE receptor [27], aquaporin 3 [13]. However, the changes of these molecules were not observed in our study. Assessments of gene expression changes in other time points may detect the up- or down-regulation of these genes. Alternatively, characteristics of DC2.4 cells may give rise to the results.

In order to determine the reproducibility of the gene expression changes observed by microarray analysis, the data from DNCB or BQ-treated DC2.4 cells were compared with that from type IV chemical-exposed mouse ears (data not shown), and 3 up-regulated (Socs2, Sdc1 and Tuba6) and 8 down-regulated (Eps8, Exo1, Asah2, Cpd, LY86, SMO, Hey1 and Pik3c2a) genes in all the experiments were selected for further evaluation by real-time RT-PCR. DC2.4 cells were treated with TMA, an irritant on the skin and type I allergy inducer, and Dex, a non-hazardous chemical on the skin, in addition to type IV allergy inducers, DNCB, BQ and Cit, to identify contact hypersensitivity-specific changes. Although DNCB-induced up-regulation of Sdc1 gene is

limited, other type IV allergy inducible chemicals, BQ and Cit, markedly up-regulated the gene expression as seen in microarray experiments in DNCB or BQ-exposed DC2.4 cells and DNCB-treated mouse tissues. TMA, an irritant on the skin and type I allergy inducer, also up-regulated Sdc1 gene expression in some experiment; however, the changes are neither significant nor reproducible. Thus, these results suggested that up-regulation of Sdc1 gene and especially, down-regulation of SMO gene in DC2.4 cells correlated with type IV allergic reaction (Fig. 2). In the experiment, Dex-treatment induced expression changes of Sdc1, Eps8, Exo1, Asah2, Hey1 and Pik3c2a genes (Fig. 2). Dex is known as a non-sensitizer on the skin; however, we suspected that it had some stimulatory effects on the cells when sensitized directly. Alternatively, uptake of such a high molecular compound with molecular mass of 60,000–90,000 probably initiated DC activation *in vitro*. At present, the function of the proteins derived from Sdc1 and SMO genes on DCs was not well documented although Sdc1 was shown to be a cell surface proteoglycan induced during the maturation process. Further functional analyses may bring interesting information about the role of these proteins on DC maturation and initiation of type IV allergic reaction.

In conclusion, we tried to identify the gene expression changes specifically induced by type IV allergy-inducible chemicals in DCs by microarray and real-time RT-PCR analyses, and 2 possible candidates, Sdc1 and SMO genes, were identified. Thus, up-regulation of Sdc1 gene and down-regulation of SMO gene in DC2.4 cells may be diagnostic markers for the screening of type IV-allergy inducible chemicals. Further analyses of the genes specifically changed by type IV allergy-inducible chemicals are required to clarify the gene expression profiles. The combination of expression changes on several candidate genes may promise reliable results for screening of the allergic chemicals.

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IDENTIFICATION OF GENES THAT RESTRICT ASTROCYTE DIFFERENTIATION OF MIDGESTATIONAL NEURAL PRECURSOR CELLS

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Abstract—During development of the mammalian CNS, neurons and glial cells (astrocytes and oligodendrocytes) are generated from common neural precursor cells (NPCs). However, neurogenesis precedes gliogenesis, which normally commences at later stages of fetal telencephalic development. Astrocyte differentiation of mouse NPCs at embryonic day (E) 14.5 (relatively late gestation) is induced by activation of the transcription factor signal transducer and activator of transcription (STAT) 3, whereas at E11.5 (mid-gestation) NPCs do not differentiate into astrocytes even when stimulated by STAT3-activating cytokines such as leukemia inhibitory factor (LIF). This can be explained in part by the fact that astrocyte-specific gene promoters are highly methylated in NPCs at E11.5, but other mechanisms are also likely to play a role. We therefore sought to identify genes involved in the inhibition of astrocyte differentiation of NPCs at midgestation. We first examined gene expression profiles in E11.5 and E14.5 NPCs, using Affymetrix GeneChip analysis, applying the Percellome method to normalize gene expression level. We then conducted *in situ* hybridization analysis for selected genes found to be highly expressed in NPCs at midgestation. Among these genes, we found that *N-myc* and high mobility group AT-hook 2 (*Hmga2*) were highly expressed in the E11.5 but not the E14.5 ventricular zone of mouse brain, where NPCs reside. Transduction of *N-myc* and *Hmga2* by retroviruses into E14.5 NPCs, which normally differentiate into astrocytes in response to LIF, resulted in suppression of astrocyte differentiation. However, sustained expression of *N-myc* and *Hmga2* in E11.5 NPCs failed to maintain the hypermethylated status of an astrocyte-specific gene promoter. Taken together, our data suggest that astrocyte differentiation of NPCs is regulated not only by DNA methylation but also by genes whose expression is controlled spatio-temporally during brain development. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

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Abbreviations: bHLH, basic helix–loop–helix; BMP, bone morphogenetic protein; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; DIG, digoxigenin; E, embryonic day; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GEO, Gene Expression Omnibus; *gfap*, glial fibrillary acidic protein; *Hmga2*, high mobility group AT-hook 2; JAK, janus kinase; LIF, leukemia inhibitory factor; NPC, neural precursor cell; SSC, sodium chloride sodium citrate; STAT, signal transducer and activator of transcription.

Key words: *N-myc*, *Hmga2*, epigenetics, Percellome method, differentiation.

The mammalian CNS is composed of neurons, astrocytes, and oligodendrocytes. Although these three cell types are derived from common multipotent neural precursor cells (NPCs), their differentiation is spatially and temporally regulated during development (Temple, 2001). Fetal telencephalic NPCs divide symmetrically in early gestation to increase their own numbers, and then undergo neurogenesis through mostly asymmetric divisions. Toward the end of the neurogenic phase, NPCs acquire multipotentiality to generate astrocytes and oligodendrocytes as well as neurons. It has recently become apparent that NPC fate determination is controlled by both extracellular cues, including cytokine signaling, and intracellular programs such as epigenetic gene regulation (Edlund and Jessell, 1999; Takizawa et al., 2001; Hsieh and Gage, 2004).

Interleukin (IL) -6 family cytokines such as cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) activate the janus kinase (JAK) –signal transducer and activator of transcription (STAT) signaling pathway and are known to induce astrocyte differentiation of NPCs (Bonni et al., 1997; Rajan and McKay, 1998). Gene knockouts of LIF (Bugga et al., 1998), LIF receptor β (Koblar et al., 1998), the common receptor component gp130 (Nakashima et al., 1999a) and STAT3 (He et al., 2005) all result in impaired astrocyte differentiation *in vivo*, emphasizing the contribution of JAK-STAT signaling to astrogliogenesis in the developing CNS. Bone morphogenetic proteins (BMPs) are another group of astrocyte-inducing cytokines. They synergistically induce astrocytic differentiation of NPCs via formation of a complex between STATs and BMP-activated transcription factor Smads, bridged by the transcriptional coactivators p300/CBP (Nakashima et al., 1999b).

In addition to these extracellular factors, intracellular programs and factors also play critical roles to regulate astrocytic differentiation of NPCs. We have previously shown that a CpG dinucleotide within a STAT3-binding element (TTCCGAGAA) in the astrocytic marker glial fibrillary acidic protein (*gfap*) gene promoter is highly methylated in NPCs at midgestation (embryonic day (E)11.5), when the cells differentiate only into neurons but not into astrocytes. Since STAT3 does not bind to the methylated cognate sequence, NPCs at midgestation do not express *gfap* even when stimulated by STAT3-activating cytokines such as LIF. As gestation proceeds, the STAT3-binding

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site becomes gradually demethylated in NPCs, enabling them to express *gfap* in response to LIF stimulation (Takizawa et al., 2001). Thus, we have proposed that DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation during brain development. However, the important question of how this astrocyte-specific gene promoter becomes demethylated in NPCs remains unanswered.

Neurogenic basic helix–loop–helix (bHLH) transcription factors have been also shown to regulate astrocyte differentiation during early neural development. Mice carrying mutations in *mash1* and *math3* (Tomita et al., 2000), or, to a lesser extent, *mash1* and *ngn2* (Nieto et al., 2001) exhibit decreased neurogenesis and premature astrogliogenesis. Conversely, overexpression of neurogenic bHLH factors, either *in vivo* during the gliogenic period (Cai et al., 2000) or in cultured NPCs exposed to CNTF (Sun et al., 2001), promotes neurogenesis at the expense of astrogliogenesis. A possible mechanism underlying the repressive effect on astrogliogenesis is that Ngn1 binds to p300/CBP and sequesters them away from STAT3, thereby preventing STAT3 from activating astrocytic gene expression (Sun et al., 2001). Such a mechanism may ensure the restriction of astrocyte differentiation in NPCs that would otherwise differentiate into neurons under the influence of high-level neurogenic bHLH factor expression during the neurogenic period.

Although these studies have provided us with an integrated insight into the mechanism of neurogenic-to-gliogenic switching in NPCs, they do not preclude the involvement of other, as yet unknown, factors. To identify such factors, we first in this study examined gene expression profiles of mid- and late-gestational NPCs by Affymetrix GeneChip analysis, which is widely used to obtain a complete picture of developmental stage-specific gene expression (Abramova et al., 2005; Ajioka et al., 2006). We then performed *in situ* hybridization experiments to investigate the spatio-temporal expression pattern of genes that were found to be highly expressed in midgestational NPCs. Two genes, *N-myc* and high mobility group AT-hook 2 (*Hmga2*), were highly expressed in the ventricular zone of E11.5 but not of E14.5 mouse brain. Transduction of *N-myc* and *Hmga2* into E14.5 NPCs resulted in suppression of astrocyte differentiation, even in the presence of LIF. However, the prolonged expression of these genes in E11.5 NPCs failed to preserve the hypermethylated status of the astrocyte-specific *gfap* promoter. These results suggest that the inhibition of astrocyte differentiation in midgestational NPCs is regulated not only by DNA methylation of astrocyte-specific gene promoters but also by transcription-regulating factors whose expression is controlled spatio-temporally during brain development.

EXPERIMENTAL PROCEDURES

NPC culture

Timed-pregnant ICR mice were used to prepare NPCs. The protocols described below were carried out according to the animal experimentation guidelines of Nara Instititute of Science and Technology that comply with National Institutes of Health Guide for Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. NPCs were prepared from telencephalons of E11.5 and E14.5 mice and cultured as described previously (Nakashima et al., 1999b). Briefly, the telencephalons were triturated in Hanks' balanced salt solution by mild pipetting with a 1-ml pipet tip (Gilson, Middleton, WI, USA). Dissociated cells were cultured in N2-supplemented Dulbecco's Modified Eagle's Medium with F12 (GIBCO, Grand Island, NY, USA) containing 10 ng/ml basic FGF (R&D Systems, Minneapolis, MN, USA) (N2/DMEM/F12/bFGF) on culture dishes (Nunc, Naperville, IL, USA) or chamber slides (Nunc) which had been precoated with poly-L-ornithine (Sigma, St. Louis, MO, USA) and fibronectin (Sigma).

Immunocytochemistry

E11.5 and E14.5 NPCs cultured on coated chamber slides were washed with PBS, fixed in 4% paraformaldehyde in PBS, and stained with the following primary antibodies: rabbit anti-SOX2 (1:1000, Chemicon, Temecula, CA, USA), mouse anti-βIII-tubulin (1:500, Sigma), rabbit anti-GFAP (1:2000, Dako, High Wycombe, UK). The following secondary antibodies were used: Alexa488-conjugated goat anti-rabbit IgG (1:500, Molecular Probes, Eugene, OR, USA), Cy3-conjugated goat anti-mouse IgG (1:500, Chemicon). Nuclei were stained using bisbenzimide H33258 fluorochrome trihydrochloride (Nacalai Tesque, Kyoto, Japan). All experiments were independently replicated at least three times.

Sample preparation and GeneChip analysis

These procedures were conducted according to the Percellome method (Kanno et al., 2006) to normalize mRNA expression values to sample cell numbers by adding external spike mRNAs to the sample in proportion to the genomic DNA concentration and utilizing the spike RNA quantity data as a dose-response standard curve for each sample. Cells cultured on coated dishes were washed with PBS, lysed in 500 ml of RLT buffer (Qiagen K.K., Tokyo, Japan) and transferred to a 1.5-ml tube. Two separate 10-μl aliquots were treated with DNAse-free RNase A (Nippon Gene, Tokyo, Japan) for 30 min at 37 °C, followed by proteinase K (Roche Diagnostics, Mannheim, Germany) for 3 h at 55 °C, and then transferred to a 96-well black plate. PicoGreen fluorescent dye (Molecular Probes) was added to each well, and then incubated for 2 min at 30 °C. The DNA concentration was measured using a 96-well fluorescence plate reader with excitation at 485 nm and emission at 538 nm. Lamda phage DNA (PicoGreen kit, Molecular Probes) was used as standard. The appropriate amount of spike RNA cocktail was added to the sample homogenates in proportion to their DNA concentration. Five independent *Bacillus subtilis* poly-A RNAs were included in the grade-dosed spike cocktail. Total RNAs were purified using an RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. First-strand cDNAs were synthesized by incubating 5 μg of total RNA with 200 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 100 pmol T7-$(dT)_{24}$ primer [5′-GGCCAG-TGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3′]. After second-strand synthesis, the double-stranded cDNAs were purified using a GeneChip Sample Cleanup Module (Affymetrix, Washington, DC, USA), according to the manufacturer's instructions, and labeled by *in vitro* transcription using a BioArray HighYield RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY, USA). The labeled cRNA was then purified using a GeneChip Sample Cleanup Module (Affymetrix) and treated with fragmentation buffer at 94 °C for 35 min. For hybridization to a GeneChip Mouse Genome 430 2.0 Array (Affymetrix), 15 μg of fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1× eukaryotic hybridization control (1.5 pM BioB, 5 pM BioC, 25 pM BioD and 100 pM Cre), 0.1 mg/ml herring sperm

DNA, 0.5 mg/ml acetylated BSA and 1× manufacturer-recommended hybridization buffer in a 45 °C rotisserie oven for 16 h. Washing and staining were performed in a GeneChip Fluidics Station (Affymetrix) using the appropriate antibody amplification, washing and staining protocols. The phycoerythrin-stained arrays were scanned as digital image files, which were analyzed with GeneChip Operating Software (Affymetrix). The expression data were converted to copy numbers of mRNA per cell by the Percellome method, quality controlled, and analyzed using Percellome software (Kanno et al., 2006). The GeneChip data have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO series accession number GSE 10796.

Quantitative real-time RT-PCR

Quantitative real-time PCR was performed to confirm the results of GeneChip analysis. RNAs from E11.5 and E14.5 NPCs were reverse transcribed using Superscript II (Invitrogen) and amplified by PCR, with a specific pair of primers for each gene, using the Mx3000P system (Stratagene, La Jolla, CA, USA). The expression of target genes was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The gene-specific primers were as follows: mouse N-myc: N-myc-S, 5′-aactatgctgcaccctcacc-3′; N-myc-AS, 5′-tagcaagtccgagcgtgttc-3′; mouse Hmga2: Hmga2-S, 5′-ggcagccgtccacatcag-3′; Hmga2-AS, 5′-taatcctcctctgcggactc-3′; mouse Sox11: Sox11-S, 5′-gagcctgtacgacgaagtgc-3′; Sox11-AS, 5′-tgaacaccaggtcggagaag-3′; mouse Bhlhb5: Bhlhb5-S, 5′-gttgcgcctcaacatcaac-3′; Bhlhb5-AS, 5′-acttttgcagaggctggac-3′; mouse Bcl11a: Bcl11a-S, 5′-gcatcaagctggagaaggag-3′; Bcl11a-AS, 5′-gagcttccatccgaaaactg-3′; mouse Gapdh: Gapdh-S, 5′-accacagtccatgccatcac-3′; Gapdh-AS, 5′-tccaccaccctgttgctgta-3′.

In situ hybridization

Digoxigenin- (DIG; Roche) labeled cRNA probes were synthesized for each gene, following the manufacturer's instructions. Cryosections were washed with PBS and fixed with 4% PFA. After fixation, sections were incubated in prehybridization solution (5× sodium chloride sodium citrate (SSC), 1% SDS, 50 μg/ml yeast transfer RNA, 50 μg/ml heparin in 50% formamide) at 70 °C for 1 h and hybridized with 500 ng/ml of DIG-labeled cRNA probes at 65 °C for 16 h. After three washes with wash solution 1 (5× SSC, 1% SDS in 50% formamide) and wash solution 3 (2× SSC in 50% formamide), sections were blocked with 10% normal sheep serum in TBST at room temperature for 1 h and then incubated with 1:1000 alkaline phosphatase-conjugated anti-DIG antibody (Roche) at 4 °C for 16 h. After four washes with TBST, hybridized probes were visualized with 5-bromo-4-chloro-3 indolylphosphate and nitro blue tetrazolium chloride.

Recombinant retrovirus construction and infection

Human *N-myc* and mouse *Hmga2* cDNAs were cloned into the expression vector pMYs, which contains an internal ribosome entry site followed by the region upstream of the *EGFP* gene (Morita et al., 2000). The Plat-E packaging cell line was transiently transfected with the retrovirus DNA by Trans-IT 293 (Mirus, Madison, WI, USA) (Morita et al., 2000). On the following day, the medium was replaced with N2/DMEM/F12/bFGF, and the cells were cultured in this medium for 1 day before virus was collected.

Fluorescence activated cell sorting

Virus-infected E11.5 NPCs were cultured for 4 days, after which GFP-labeled cells were sorted using a FACS Vantage (Becton Dickinson, Franklin Lakes, NJ, USA) at a flow rate of less than 1500 events/s; gating parameters were set by side and forward scatter to eliminate debris, dead and aggregated cells. After sorting, genomic DNA was extracted and used for bisulfite sequencing.

Bisulfite sequencing

Sodium bisulfite treatment of genomic DNA was performed using a Methylamp DNA Modification kit (Epigentek, Brooklyn, NY, USA), according to the manufacturer's instructions. The region in the *gfap* promoter containing the STAT-binding site of the bisulfite-treated genomic DNA was amplified by PCR using the following primers: GFmS (5′-GGGATTTATTAGGAGAATTTTAGAAGTAG-3′), GFmAS (5′-TCTACCCATACTTAAACTTCTAATATCTAC-3′). The PCR products were cloned into pT7Blue vector (Novagen, Madison, WI, USA) and at least 12 randomly selected clones were sequenced.

RESULTS

Preparation of NPCs from different developmental stages and comparison of their gene expression profiles by GeneChip analysis

E11.5 NPCs do not differentiate into astrocytes, even in the presence of the astrocyte-inducing cytokine LIF, in contrast to 4-day cultured E14.5 NPCs (Takizawa et al., 2001). As a first step toward identifying factors involved in the inhibition of astrocyte differentiation of NPCs at midgestation, we examined the gene expression profiles of E11.5 and E14.5 NPCs.

E11.5 and E14.5 NPCs were isolated from embryonic telencephalon and cultured as indicated in Fig. 1A. To evaluate the purity of NPCs in each cell population, the cells were stained with antibody against SOX2, an NPC marker (Graham et al., 2003). As shown in Fig. 1B and C, the majority of cells in both populations were positive for SOX2, indicating that NPCs were highly enriched. An Affymetrix mouse genome GeneChip array was chosen to compare expression profiles in the two populations, and we adopted the Percellome method to normalize gene expression from different samples (Kanno et al., 2006). The method enabled us to quantify mRNA molecules per cell based on the measurement of cell by adding a grade-dosed spike cocktail to the samples. We excluded genes whose transcript copy number was below six per cell. Scatter plots illustrating the differences between E11.5 and E14.5 NPCs are shown in Fig. 1D; 194 genes were expressed at >fivefold higher level in E11.5 NPCs than in E14.5 NPCs (Fig. 1D, light blue zone). Of these, 102 were known genes, and were classified by functional category (Fig. 1E). Since we wished to identify negative regulators of astrocyte differentiation, or factors involved in the epigenetic modification in midgestational NPCs, we focused on transcription-related genes (Fig. 1E, red). These 21 genes are listed in Table 1, and five (*N-myc*, *Hmga2*, *Bhlhb5*, *Sox11*, *Bcl11a*) were selected for further analysis because they have been reported to play roles in cell growth, differentiation, and chromatin remodeling in other types of stem cells (Sawai et al., 1990; Zhou et al., 1995; Saiki et al., 2000; Knoepfler et al., 2002; Brunelli et al., 2003; Sock et al., 2004).