Journal of Cellular Biochemistry 102:1472–1485 (2007)

Identification of Genetic Networks Involved in the Cell Growth Arrest and Differentiation of a Rat Astrocyte Cell Line RCG-12

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Abstract The purpose of the present study is to establish and characterize a conditionally immortalized astrocyte cell line and to clarify the genetic networks responsible for the cell growth arrest and differentiation. A conditionally immortalized astrocyte cell line, RCG-12, was established by infecting primary cultured rat cortical glia cells with a temperature-sensitive simian virus 40 large T-antigen. At a permissive temperature of 33°C, the large T-antigen was expressed and cells grew continuously. On the other hand, the down-regulation of T-antigen at a non-permissive temperature of 39°C led to growth arrest and differentiation. The cells expressed astrocyte-expressed genes such as glial fibrillary acidic protein. Interestingly, the differentiated condition induced by the non-permissive temperature significantly elevated the expression levels of several astrocyte-expressed genes. To identify the detailed mechanisms by which non-permissive temperature-induced cell growth arrest and differentiation, we performed high-density oligonucleotide microarray analysis and found that 556 out of 15,923 probe sets were differentially expressed 2.0-fold. A computational gene network analysis revealed that a genetic network containing up-regulated genes such as RB, NOTCH1, and CDKN1A was associated with the cellular growth and proliferation, and that a genetic network containing down-regulated genes such as MYC, CCNB1, and IGF1 was associated with the cell cycle. The established cell line RCG-12 retains some characteristics of astrocytes and should provide an excellent model for studies of astrocyte biology. The present results will also provide a basis for understanding the detailed molecular mechanisms of the growth arrest and differentiation of astrocytes. J. Cell. Biochem. 102: 1472–1485, 2007.

Key words: astroglia; temperature-sensitive simian virus 40 large T-antigen; immortalization; microarray; gene expression

Astrocytes are the most abundant cell type in the central nervous system (CNS). Astrocytes function to maintain the homeostatic environment of the CNS and also play an important role in immune regulation, acting as a source of chemokines, cytokines, and effector molecules. During development of the CNS, astrocytes play a role in guiding migration and axonal growth of neurons. The idea that astrocytes have active roles in the modulation of neuronal activity and synaptic neurotransmission is now widely accepted. The use of in vitro cell culture systems has been of central importance in the development of the cellular and molecular biology of tissues. Although primary cultures of rodent astrocytes are widely used in the study of the function of astrocytes, they are clearly heterogeneous and do not proliferate enough to permit single-cell cloning. Therefore, it needs to establish cell lines to understand the glial cell biology.

Some viral and cellular oncogenes have the ability to establish continuous proliferation in primary culture cells. Previous reports have

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

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Received 5 January 2007; Accepted 13 March 2007

DOI 10.1002/jcb.21369

indicated that a simian virus 40 (SV40) large T-antigen gene induces cell immortalization by inactivating several tumor suppressor molecules such as retinoblastoma binding protein (pRB) and p53 [Levine, 1997]. It has also been shown that the cell immortalization caused by a temperature-sensitive SV40 (tsSV40) large T-antigen gene accompanies more or less stable cell type-specific functions. Although tsSV40 large T-antigen forms complexes with pRB or p53 at a permissive temperature, the oncogene product is rapidly inactivated and degraded, and releases pRB or p53 from the complexes at non-permissive temperature [Jat and Sharp, 1989]. Thus, cell lines harboring the tsSV40 large T-antigen grow continuously at permissive temperatures, whereas inactivation of large T-antigen at non-permissive temperature is associated with growth arrest, apoptosis, and differentiation [Guenal and Mignotte, 1995]. To date, many kinds of conditionally immortalized cell lines have been developed by using the tsSV40 Large T-antigen, e.g., hepatocyte cells [Yanai et al., 1991], gastric surface mucus cells [Sugiyama et al., 1993], and epididymis caput epithelial cells [Tabuchi et al., 2005b]. Immortalized astrocyte cell lines have also been established by the tsSV40 large T-antigen gene [Groves et al., 1993; Whittemore et al., 1994; Kitamura et al., 1997; Tetsuka et al., 2001]. These reports have described that the cell lines retain some astrocyte-specific functions and characterizations, including the expression of astrocyte-expressed protein glial fibrillary acidic protein (GFAP), the ability to transport acidic amino acid [Tetsuka et al., 2001], and the induction of ATP-evoked Ca^{2+} mobilization [Whittemore et al., 1994]. It has also been reported that a non-permissive temperature causes cell growth arrest and differentiation in some astrocyte cell lines [Whittemore et al., 1994; Kitamura et al., 1997]. Because the differentiation of astrocytes occurs during brain development, clarification of the mechanisms of glial differentiation is of particular importance in understanding the developmental mechanisms of the CNS. However, the detailed mechanisms governing cell growth arrest and the differentiation of astrocytes have not been investigated in the previous studies [Groves et al., 1993; Whittemore et al., 1994; Kitamura et al., 1997; Tetsuka et al., 2001].

DNA microarray technology can provide a view of the expression profiles of many hundreds or many thousands of genes. In addition to this technology, pathway analysis technologies now allow the mapping of gene expression data into relevant pathway maps based on their functional annotation and known molecular interactions. These DNA microarray and pathway analysis technologies have been applied to biological experiments [Calvano et al., 2005; Tabuchi et al., 2006a,b]. The present study was undertaken to establish conditionally immortalized astrocyte cell lines by infecting primary culture rat embryonic cortical glial cells with the tsSV40 large T-antigen. As well, to clarify the detailed mechanisms by which non-permissive temperature induces cell growth arrest and differentiation of the astrocyte cell line, we performed microarray analysis using the Affymetrix GeneChip® oligonucleotide microarray platform with Rat Expression Array 230A in combination with the Ingenuity Pathway Analysis tool to examine the functional relationships between the candidate genes.

MATERIALS AND METHODS

Materials

The following antibodies were used: anti-GFAP (DakoCytomation, Glostrup, Denmark), anti-MAP2 and anti-α-tubulin (Sigma, St. Louis, MO), anti-CD11b (CHEMICON International, Inc., Temecula, CA), anti-oligodendrocyte and anti-$p21^{waf1}$ (M-19) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-actin (ICN Immuno-Biologicals, Irvine, CA), anti-T-antigen (Ab-1) (Oncogene Research Products, Cambridge, MA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Organon Teknika, Durham, NC).

Primary Cell Culture

Primary cultures of rat cortical glia cells were prepared from the cerebral cortices of 17–18-day-old Sprague–Dawley rat embryos as described previously [Tabuchi et al., 1998]. The experiments were carried out according to guidelines presented by the Animal Care and Use Committee of University of Toyama. Briefly, small pieces of cerebral cortex were dissected by enzymatic treatment (DNase I followed by trypsin) and mechanical dissociation, and the cells containing neuron and glia cells were seeded at 5×10^6 cells in a 60-mm culture dish. The cells were grown for 48 h in Dulbecco's modified Eagle's medium (DMEM;

Invitrogen, Tokyo, Japan) containing 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS).

Establishment of Immortalized Cell Lines

The tsSV40 large T-antigen gene (pSVtsA58 ori-) [Ohno and Yamaguchi, 1984] was obtained from RIKEN Bioresource Center DNA BANK (Tsukuba, Japan). A pcDNA3.1/tsSV40LT vector was constructed as follows; pSVtsA58 ori-*Bam*HI fragment was introduced downstream (*Bam*HI site) of the CMV promoter of pcDNA3.1 (Invitrogen). When immortalized cell lines were developed, cerebral cortex primary culture cells were transfected pcDNA3.1/tsSV40LT with Effectene Transfection Reagent (Qiagen, Valencia, CA) according to the manufacture's instructions. Stable transfectants were selected in DMEM containing 10% FBS and 0.5 mg/mL G418 (Nacalai Tesque, Tokyo, Japan), and were cloned by a single cell culture method. The cloned cells were maintained in DMEM containing 10% FBS and 0.5 mg/mL G418 in a culture dish at a permissive temperature of 33°C [Tabuchi et al., 2005b].

Measurement of Cell Growth

The cells were cultured in 24-well plate for 24 h at 33°C. For the temperature-sensitive growth experiment, the cells were then cultured in DMEM supplemented with 10% FBS for 0–5 days at 33°C or a non-permissive temperature of 39°C. When the effects of FBS were evaluated, the cells were incubated in DMEM containing FBS at concentrations of 0.5–10% for 5 days at 33°C. The number of cells was counted by using a hemocytometer. The cell size was measured by using NIH image software. Briefly, the cells in culture were photographed for each condition and the cell size of randomly chosen 50 cells was measured.

Immunocytochemistry

The cells were fixed in 4% paraformaldehyde at room temperature for 20 min. The cells were then rinsed three times with phosphate-buffered saline (PBS) containing 0.3% Triton X-100. After blocking with PBS containing 3% normal goat serum (NGS) and 0.3% Triton X-100 for 30 min at room temperature, the cells were incubated with primary antibodies in PBS containing 1% NGS and 0.3% Triton X-100 at 4°C for 16–18 h. After washed three times with PBS containing 0.3% Triton X-100, the cells were incubated with the appropriate secondary antibody conjugated with Alexa 488 or Alexa 546 in PBS containing 1% NGS and 0.3% Triton X-100. The fluorescence was detected with under a fluorescence microscope (BX50, Olympus, Tokyo, Japan) and photographed with a digital camera (DP70, Olympus). The fluorescent intensity was quantified by using NIH image software.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

Cellular materials were lysed in lysis buffer (150 mM NaCl, 1% NP-40, 10% glycerol, protease inhibitor mix (Nacalai Tesque), and 20 mM Tris–HCl (pH 7.4)). The lysate (10–20 μg total protein per lane) were subjected to SDS-PAGE and then were blotted onto PVDF membranes. The membranes were blocked with TBS (0.9% NaCl, and 10 mM Tris–HCl (pH7.4)) containing 4% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) at room temperature for 1 h. The membranes were then incubated with primary antibodies in TBS containing 0.8% Block Ace at 4°C for 16–18 h, washed three times with TBS containing 0.1% Tween 20, and exposed to peroxidase-conjugated secondary antibody in TBS containing 0.8% Block Ace at room temperature for 1–2 h. Immunoreactive proteins were visualized by a luminescent image analyzer (LAS-1000 plus, Fujifilm, Tokyo, Japan) using an enhanced chemiluminescence detection system (ECL plus, Amersham Biosciences, Tokyo, Japan). Bands of target proteins were quantified by using NIH image software. The intensity of each band was normalized by GAPDH.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the cells using an RNeasy Total RNA Extraction kit (Qiagen) and treated with DNase I (RNase-free DNase kit, Qiagen) for 15 min at room temperature to remove residual genomic DNA. Complementary DNA (cDNA) was synthesized from total RNA (1 μg) by using an oligo $(dT)_{16}$ primer and Omniscript Reverse Transcriptase (Qiagen). cDNA was then amplified with using Taq DNA polymerase (Applied Biosystems, Tokyo, Japan) and the specific primers listed in Supplementary Material (Table S1). The PCR conditions

were 94°C for 30 s, 60°C for 30 s, 72°C for 30 s. After PCR amplification, reaction products were separated on a 2% agarose gel and were stained with ethidium bromide.

Quantitative Real-Time PCR

Real-time PCR was performed by using a SYBR Premix Ex Taq Kit (Takara Bio, Kyoto, Japan) and Mx3000P QPCR systems (Stratagene, La Jolla, CA). The PCR conditions were 94°C for 10 s, 60°C for 40 s. Each mRNA expression level was normalized with respect to the mRNA of β-actin.

Microarray and Pathway Analyses

Total RNA was extracted from the cells using an RNeasy Total RNA Extraction Kit. Gene expression was analyzed using a GeneChip® system with Rat Expression Array 230A which was spotted with 15,923 probe sets (Affymetrix, Santa Clara, CA). In this study, a total of four arrays were used: two for the control conditions (33°C for 3 days) and two for the differentiated conditions (39°C for 3 days). Sample preparation for array hybridization was carried out following the manufacturer's instructions. In short, 3 μg of total RNA was used to synthesize double-strand cDNA with a GeneChip® Expression 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). Biotin-labeled cRNA was then synthesized from the cDNA using GeneChip® Expression 3′-Amplification Reagents for IVT Labeling (Affymetrix). After fragmentation, the biotinylated cRNA was hybridized to arrays at 45°C for 16 h. The arrays were washed, stained with streptavidin–phycoerythrin and scanned with a probe array scanner. The scanned chip was analyzed using the GeneChip Analysis Suite software (Affymetrix). Hybridization intensity data were converted into a presence/absence call for each gene, and changes in gene expression between experiments were detected by comparison analysis. The data were further analyzed using a GeneSpring software (Silicon Genetics, Redwood City, CA). In order to extract the significant genes, the GeneSpring "Filter on Volcano Plot" tool was applied to obtain the list of differentially expressed genes. A fold change value of greater than 2 (up-regulated) or less than 2 (down-regulated) was considered to be biologically important. The statistical significance of the fold change was calculated for two groups by performing a Student's *t*-test and *P* values less than 0.05 were regarded as significant.

To examine the gene ontology, including biological processes, cellular components, molecular functions and genetic networks, the data were analyzed using Ingenuity Pathway Analysis tools (Ingenuity Systems, Mountain View, CA), a web-delivered application that enables the discovery, visualization and exploration of molecular interaction networks in gene expression data. The gene lists identified by the GeneSpring containing Affymetrix gene ID and natural legalism were uploaded into the Ingenuity Pathway Analysis.

Statistical Analysis

Data are shown as means ± standard errors of mean (SEM). Statistical analysis was carried out using Student's *t*-test and *P* values less than 0.05 were regarded as significant.

RESULTS

Establishment of an Immortalized Rat Cortex Glial Cell Line RCG-12

Primary culture of cerebral cortex cells containing neurons and glia cells from rat embryo was placed in a culture dish. To establish rat cortical glia cell lines, we infected the primary culture cells with the pcDNA3.1 plasmid that contains the genes encoding for tsSV40 large T-antigen and resistance to neomycin. Cell colonies surviving in DMEM containing G418 (an analog of neomycin) at the permissive temperature of 33°C were isolated and cloned. In our series of several attempts, 12 cell lines have been established; they were named rat cortical glia (RCG)-01–12. Based on the ability to exhibit a glial morphology and gene expressions for astrocyte-specific protein GFAP, we selected a RCG-12 cell line (Fig. 1A).

The RCG-12 cells proliferated and had a stable homogeneous morphology at 33°C (Fig. 1A,B), whereas morphological changes were observed when the culture temperature was shifted from 33°C to the non-permissive temperature of 39°C, at which point the cell size was significantly larger than that at 33°C (Fig. 1C,D).

The gene product of the tsSV40 large T-antigen encoded by the tsA58 allele is thought to inactivate its immortalizing capabilities at non-permissive temperature [Jat and Sharp, 1989]. The RCG-12 cells proliferated at 33°C,

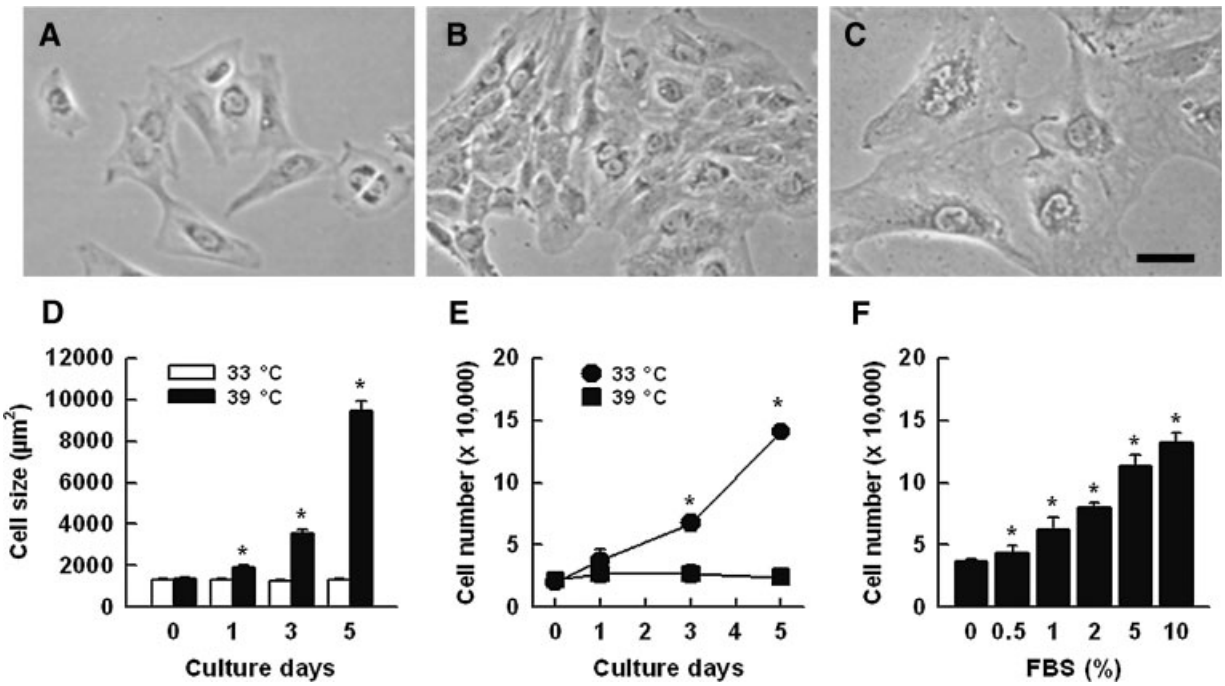

Fig. 1. Phase-contrast micrographs (**A**–**C**) and the cell growth characteristics (**E**,**F**) of RCG-12 cells. The cells were cultured for 0–3 days at permissive (33°C, A and B) or non-permissive (39°C, C) temperatures. A: 33°C day 0; B: 33°C day 3; C: 39°C day 3. Bar 50 μm. **D**: The cells were cultured for 0–5 days at 33 or 39°C, and the cell size was measured (n = 50). E: The cells were cultured for 0–5 days at 33 or 39°C, and the number of cells was counted by using a hemocytometer. F: Effects of FBS (0–10%) on cell growth. The cells were cultured for 5 days at 33°C. The data represent means ± SEM from three to four wells. D,E: $*P < 0.05$ versus control (day 0 at 33°C) (Student's *t*-test). E: $*P < 0.05$ versus control (FBS, 0%) (Student's *t*-test).

but the growth of the cells was markedly decreased at 39°C (Fig. 1E). Moreover, the effects of FBS on cell growth were evaluated at 33°C. FBS at concentrations from 0.5 to 10% elevated the cell number in a concentration-dependent manner (Fig. 1F), suggesting that the cells show serum-dependent growth. These results clearly indicated that the RCG-12 cells show temperature- and serum-dependent growth characteristics and that the non-permissive temperature induces growth arrest.

Expression of Large T-Antigen and $p21^{waf1}$

The expression of large T-antigen and $p21^{waf1}$ in the RCG-12 cells was determined by immunocytochemistry and Western blotting (Fig. 2). At 33°C, the large T-antigen was expressed in the nuclei of almost all cells (Fig. 2A). In contrast, at 39°C, the expression level of large T-antigen was markedly decreased (Fig. 2B) (the fluorescence intensity of large T-antigen in the nuclei; 33°C, 178.3 ± 4.6; 39°C, 60.4 ± 1.2 (n = 50)). Western blot analysis confirmed that the level of large T-antigen was approximately constant at 33°C. On the other hand, the expression level of large T-antigen gradually decreased in a time-dependent manner from days 0 to 5 at 39°C (Fig. 2E,F). To evaluate the post-transcription activities of p53, the level of $p21^{waf1}$, a cyclin-dependent kinase inhibitor regulated by p53, was measured. A low level of $p21^{waf1}$ was expressed in the nuclei at 33°C, whereas the expression level was significantly elevated (Fig. 2C,D) (the fluorescence intensity of $p21^{waf1}$ in the nuclei; 33°C, 50.3 ± 1.1; 39°C, 142.4 ± 6.2 (n = 50)). This observation was confirmed by Western blot analysis: the protein level of $p21^{waf1}$ was apparently increased at 39°C in a time-dependent manner (Fig. 2E,F).

It has been reported that inactivation of large T-antigen induces cell growth arrest and apoptosis in tsSV40LT-antigen-transformed cells [Guenal and Mignotte, 1995]. When apoptotic cell death of RCG-12 cells cultured at 39°C was determined by DNA fragmentation, no DNA ladder was observed (Supplementary Fig. 1). These finding indicates that inactivation of large T-antigen by the non-permissive temperature leads to the growth arrest and differentia-

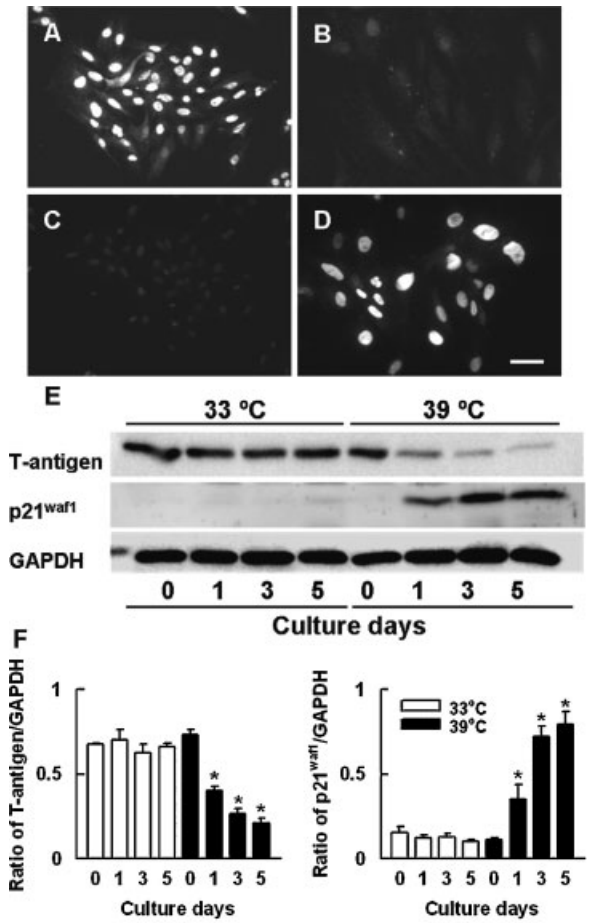

Fig. 2. Effects of culture temperatures on protein expression of large T-antigen and $p21^{waf1}$. The cells were cultured for 0–5 days at 33 or 39°C. **A–D**: Immunocytochemical analysis of Large T-antigen (A,B) and $p21^{waf1}$ (C,D). Bar 50 μm. **E**: SDS-PAGE and Western blot were performed. Signals were visualized by a luminescent image analyzer using an ECL system. **F**: Bands were quantified by using NIH image and the intensity was normalized by GAPDH. The data represent means ± SEM from three to four different experiments. $^*P < 0.05$ versus control (day 0 at 39°C) (Student's *t*-test).

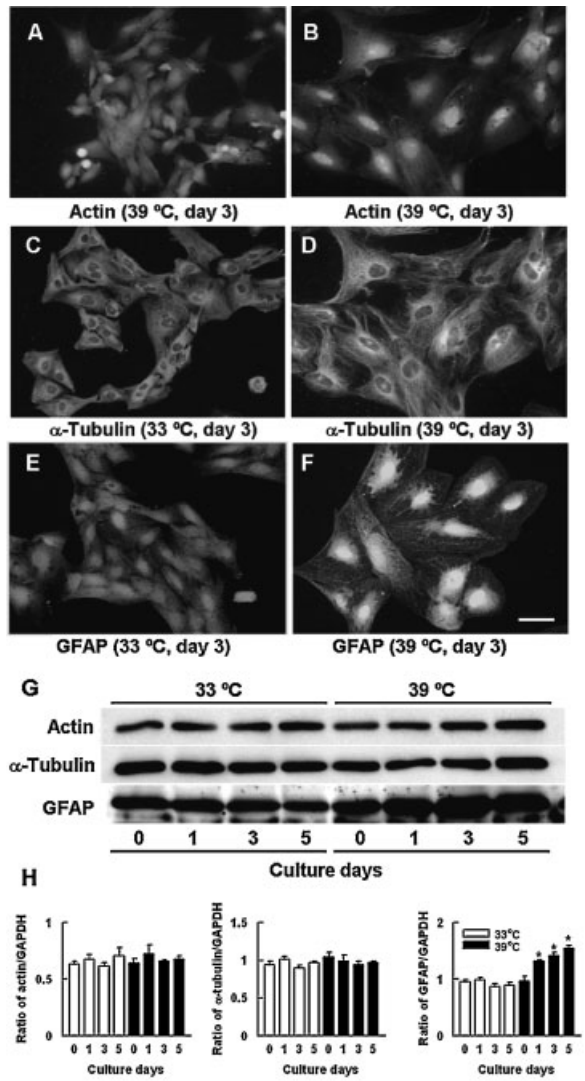

Fig. 3. Expression of actin, α-tubulin, and GFAP in the RCG-12 cells. **A–F**: Immunocytochemical analysis of actin, α-tubulin, and GFAP. The cells were cultured for 3 days at 33°C (A,C,E) or 39°C (B,D,F). Bar 50 μm. **G**: Western blot analysis of actin, α-tubulin, and GFAP. Signals were visualized by a luminescent image analyzer using an ECL system. **H**: Bands were quantified by using NIH image and the intensity was normalized by GAPDH. The data represent means ± SEM from three to four different experiments. $^*P < 0.05$ versus control (day 0 at 39°C) (Student's *t*-test).

tion without apoptotic cell death in RCG-12 cells.

Expression of Marker Proteins for Glial Cells

We examined the expression of astrocyte-expressed proteins using immunocytochemical and Western blotting methods with specific antibodies. In the RCG-12 cells, cytoskeletal protein including α-tubulin and actin was observed at 33 and 39°C (Fig. 3A–D). Expression of astrocyte-specific protein GFAP was observed in the RCG-12 cells at both temperatures (Fig. 3E,F). On the other hand, marker proteins of neuron (MAP-2), microglia (CD11b), and oligodendrocyte were not detected in the RCG-12 cells (data not shown). Although the expression levels of both α-tubulin and actin were not changed by the non-permissive temperature, Western blot analysis showed that the level of GFAP was significantly elevated on days 1–5 at 39°C (Fig. 3G,H).

Gene Expression and the Effects of Cell Growth Arrest on the Gene Expression

An established cell line RCG-12 was further characterized according to the production of the astrocyte-expressed genes using RT-PCR. In the RCG-12 cells, mRNAs for GFAP, L-glutamate/L-aspartate transporter (GLAST), glutamine synthase (GS), trkB T1, GFRα1, and RET were expressed at 33°C (Table I). In regard to P2 purinoreceptors, all subtypes of P2X and P2Y receptors were expressed in the RCG-12 cells. On the other hand, mRNAs of trkB FL and L-glutamate transporter (GLT-1) were not detected.

We investigated the effects of cell growth arrest on the gene expression of selected genes by quantitative real-time PCR (Fig. 4 and Table I). Although the mRNA level of P2Y2 was not altered (Fig. 4B) and mRNA for trkB FL was not detected (data not shown) at either temperature, the mRNA levels of P2Y1, GLAST, GLT-1, GS, trkB T1, GFRα1, and RET were significantly elevated at 39°C. These results suggest that RCG-12 cells show a differentiated phenotype under cell growth-restricted conditions.

Global Gene Expression Analysis and the Effects of Cell Growth Arrest on the Gene Expression

In order to understand the gene expression profiles in more detail and to identify the detailed mechanisms by which non-permissive temperature induces cell growth arrest and differentiation of RCG-12 cells, we carried out GeneChip analysis of cells cultured at either 33 or 39°C for 3 days. Genes that were up- or down-regulated by >2.0-fold were examined using the GeneSpring software. When the gene expression levels were compared, the total number of probe sets that were found to be differentially expressed by the non-permissive temperature was 556; that is, 3.5% of probe sets were affected by the non-permissive temperature. We found 295 probe sets with expression that was up-regulated and 261 probe sets with expression that was down-regulated.

TABLE I. Gene Expression in RCG-12 Cells

Genes	33°C	39°C
GFAP	+	+
trkB FL	−	−
trkB T1	+	+
GFRα1	+	+
RET	+	+
GLAST	+	+
GLT-1	−	+
GS	+	+
P2X1	+	+
P2X2	+	+
P2X3	+	+
P2X4	+	+
P2X5	+	+
P2X6	+	+
P2X7	+	+
P2Y1	+	+
P2Y2	+	+
P2Y4	+	+
P2Y6	+	+
P2Y12	+	+

The cells were cultured for 3 days at 33 or 39°C. mRNA expression was detected by RT-PCR. +, positive expression; −, negative expression.

Functional and Pathway Analyses

The functional and pathway analyses of the 295 up- and 261 down-regulated probe sets were explored using the Ingenuity Pathways Analysis Knowledge Base. Based on the significance and number of genes, the top 6 categories affected by the non-permissive temperature treatment are represented in Table II. The genes associated with these functional categories are listed in Supplementary Material Table S2 and S3.

Next, to determine the biologically relevant networks and pathways of the genes identified here, pathway analysis was carried out on the up- and down-regulated genes using the Ingenuity Pathways Analysis Knowledge Base. These networks describe the functional relationships between gene products based on known interactions reported in the literature. Several significant pathways were recognized in up- and down-regulated genes. Figure 5 shows the most significant network constructed with up-regulated genes. This network (score: 68; 35 genes) was associated with cellular growth and proliferation (*P*-value: 1.84E−13 to 1.23E−4; 28 genes), tissue morphology (*P*-value: 2.48E−12 to 9.08E−5; 20 genes), and cancer (*P*-value: 1.03E−11 to 1.23E−4; 28 genes). In this network, cell growth and proliferation-related genes including IL6, RB1, NOTCH1, and CDKN1A (whose nodes and edges are highlighted in blue) were observed (Fig. 5).

In down-regulated genes, a significant network (score: 70, 35 genes) was found to be associated with cell cycle (*P*-value: 1.65E−5 to 3.57E−3; 12 genes), cell morphology (*P*-value: 1.29E−6 to 3.57E−3; 16 genes), and DNA replication, recombination, and repair (*P*-value: 1.83E−5 to 3.57E−3; 10 genes). In this network,

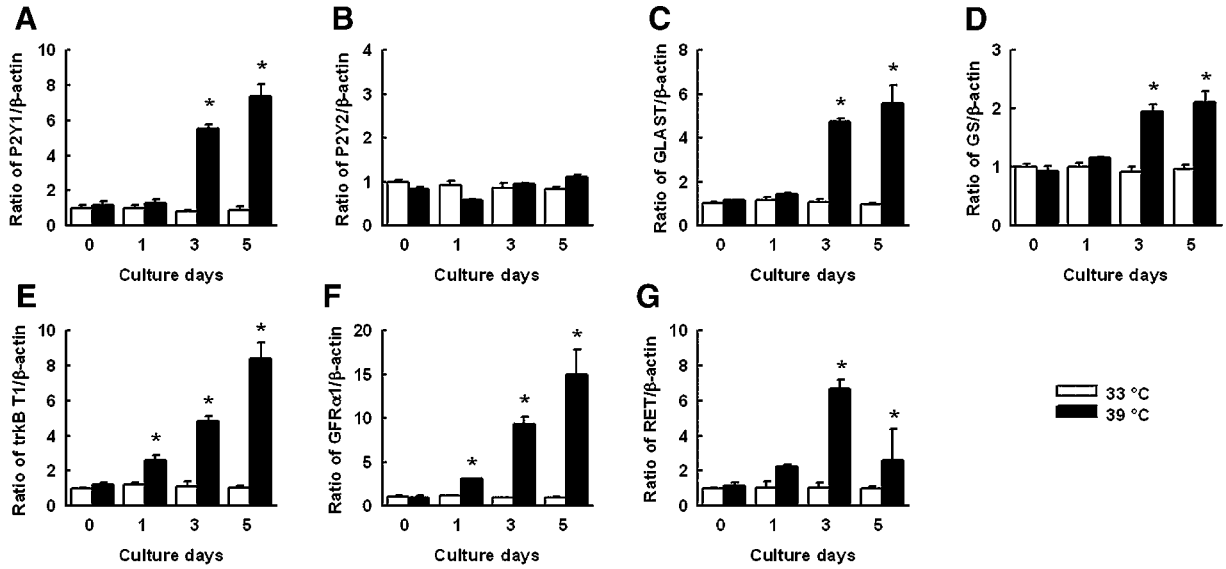

Fig. 4. The effects of cell growth arrest on gene expression for P2Y1 (**A**), P2Y2 (**B**), GLAST (**C**), GS (**D**), trkB T1 (**E**), GFRα1 (**F**), and RET (**G**). The cells were cultured for 0–5 days at 33°C (open columns) or 39°C (closed columns). Each expression level was normalized by β-actin. The data represent means ± SEM from three to four different experiments. $*P < 0.05$ versus control (day 0 at 39°C) (Student's *t*-test).

we observed cell cycle-related genes including MYC, IGF1, and CCNB1 (whose nodes and edges are highlighted in blue) (Fig. 6).

DISCUSSION

Immortalized cell lines retaining differentiated functions are required in order to study tissue functions at the cellular and molecular levels. It has been indicated that tsSV40 large T-antigen is useful for establishing conditionally immortal cell lines from many kinds of tissues. In this study, we succeeded in establishing the conditionally immortalized rat astrocyte cell line (RCG-12), which has astrocytes-specific functions, by using the tsSV40 large T-antigen. The RCG-12 cells exhibited fibroblast-like cell morphology at the permissive temperature and constitutively expressed the astrocyte-specific intermediate filament GFAP, suggesting that the cells are the type I astrocyte. Interestingly, the non-permissive temperature induced cell growth arrest, morphological changes, and an increase in the expression levels of GFAP; the cells appeared to have a flat and polygonal morphology. These results suggest that the non-permissive temperature induced cell differentiation and maturation in the RCG-12 cells.

Previous studies have shown that the tsSV40 large T-antigen induces immortalization by inactivating the function of several tumor suppressor molecules including p53 and pRB

TABLE II. Functional Analysis of the Genes Affected by Non-Permissive Temperature in RCG-12 Cells

Related function and diseases	Significance	Associated genes
Up-regulated genes		
Cellular growth and proliferation	1.50E−7 to 1.61E−2	67
Cancer	2.73E−7 to 1.61E−2	54
Cell death	2.21E−6 to 1.61E−2	52
Cell cycle	2.69E−6 to 1.61E−2	26
Connective tissue disorders	4.41E−6 to 2.62E−3	17
Cell morphology	2.35E−5 to 1.61E−2	28
Down-regulated genes		
Cell cycle	6.56E−5 to 2.68E−2	24
Cellular movement	6.56E−5 to 2.51E−2	15
Cellular assembly and organization	8.07E−5 to 2.68E−2	28
Cancer	1.11E−4 to 2.68E−2	38
Cellular growth and proliferation	2.34E−4 to 2.68E−2	41
Cell morphology	3.48E−4 to 2.68E−2	24

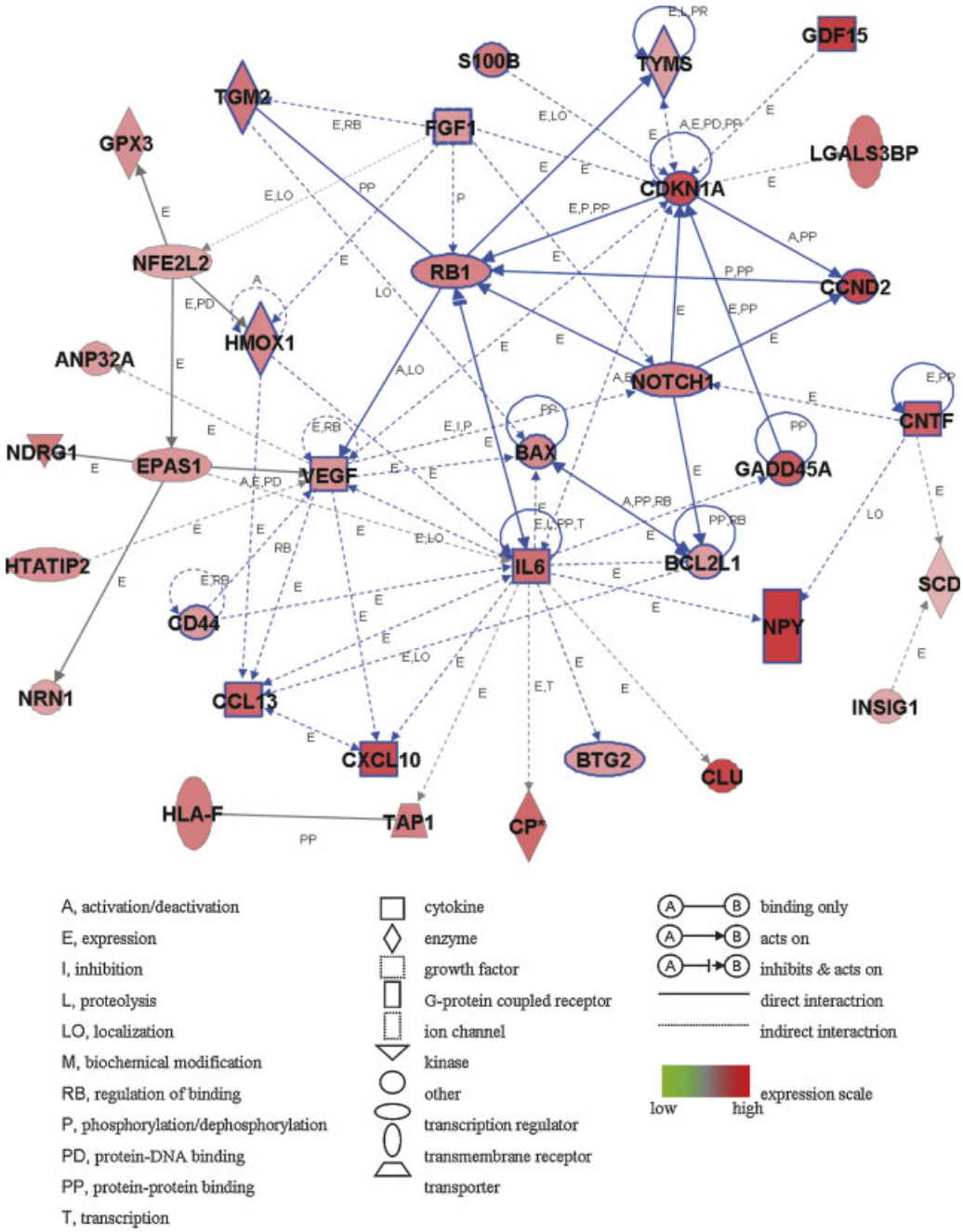

Fig. 5. A network of the genes that were up-regulated in the cells cultured at 39°C for 3 days. Probe sets were analyzed by the Ingenuity Pathway Analysis software. The network is displayed graphically as nodes (genes) and edges (the biological relationships between the nodes). The node color indicates the expression level of the genes. Nodes and edges are displayed with various shapes and labels that present the functional class of genes and the nature of the relationship between the nodes, respectively. The nodes and edges of genes associated with cell growth and the proliferation are highlighted in blue. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

at a permissive temperature, but is rapidly inactivated and degraded, and releases p53 or pRB from the complexes at non-permissive temperatures [Jat and Sharp, 1989; Levine, 1997]. The promoter of the cyclin-dependent kinase inhibitor $p21^{waf1}$ contains two elements recognizable by p53, and that binding of p53 to those regions up-regulates the transcription of $p21^{waf1}$ [el-Deiry et al., 1993]. In fact, it has been reported that inactivation of large T-antigen in

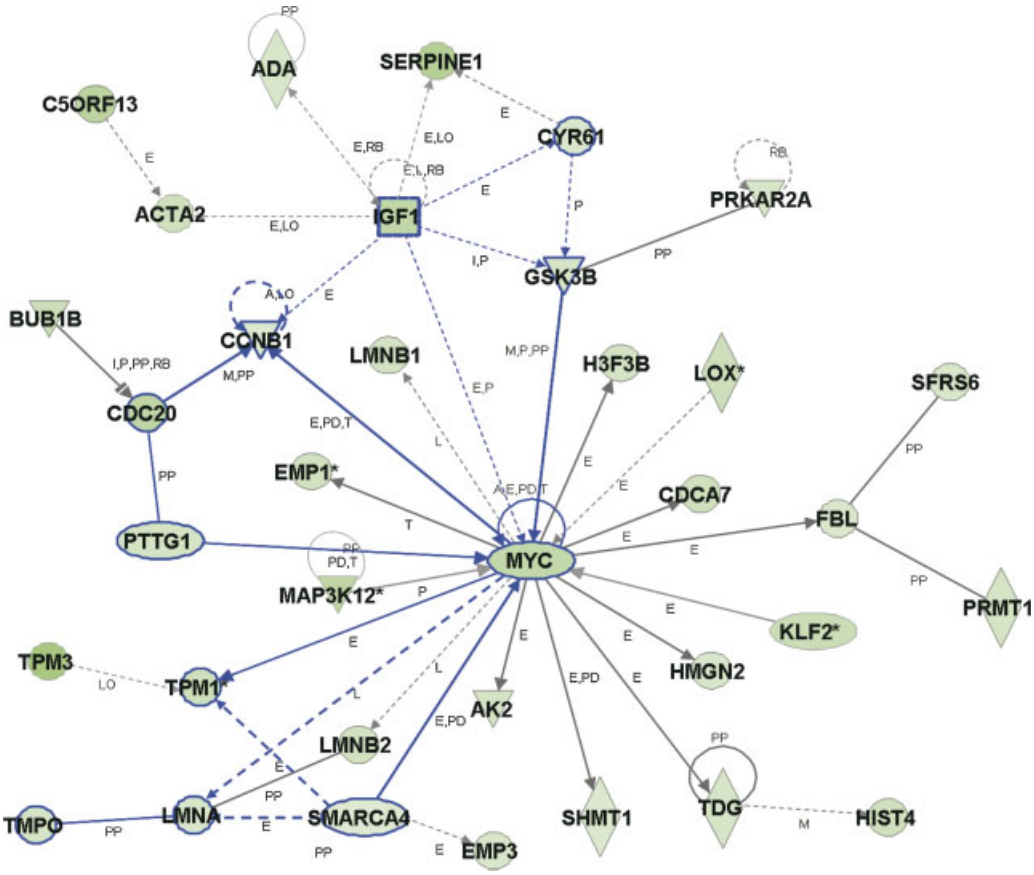

Fig. 6. A network of the genes that were up-regulated in the cells cultured at 39°C for 3 days. Probe sets were analyzed by the Ingenuity Pathway Analysis software. For the explanation of the symbols and letters, see Figure 5. The nodes and edges of genes associated with the cell cycle are highlighted in blue. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells immortalized by the tsSV40 large T-antigen is associated with growth arrest, apoptosis, and differentiation, and leads to the activation of p53 and the induction of $p21^{waf1}$ [Guenal and Mignotte, 1995]. As expected, the RCG-12 cells showed temperature-sensitive growth characteristics, as do other tsSV40 large T-antigen-expressed cell lines such as GSM06 [Sugiyama et al., 1993], TTE3 [Tabuchi et al., 2005a], and MEPC5 [Tabuchi et al., 2005b]. Although high expression level of large T-antigen was observed at 33°C in the RCG-12 cells, the level was gradually decreased at 39°C. In contrast, the expression level of $p21^{waf1}$ was significantly elevated at 39°C. These results suggest that the p53-$p21^{waf1}$ pathway is activated and leads to cell growth arrest at the non-permissive temperature in the RCG-12 cells.

It has been reported that GLT-1 and GLAST [Perego et al., 2000], GS [Low et al., 1992], P2X and P2Y receptors [James and Butt, 2002], trkB T1 [Rose et al., 2003; Ohira et al., 2005], GFRα1, and RET [Nicole et al., 2001] are expressed in astrocytes. In the RCG-12 cells, RT-PCR detected mRNAs for astrocytes-expressed genes such as GLAST, GS, P2X and P2Y receptors, trkB T1, GFRα1, and RET. Thus, the RCG-12 cells originating from the cortical glial cells retain some specific functions of astrocytes. However, RT-PCR failed to detect GLT-1 and trkB FL mRNAs in the RCG-12 cells at the permissive temperature of 33°C. GLT-1 and GLAST, a family of Na^+-dependent transporters expressed in astrocytes, control extracellular concentrations of glutamate in the CNS. They are expressed by morphologically distinct GFAP-positive astrocytes: GLAST is enriched in the flat polygonal cells (type 1 astrocytes) and GLT-1 is expressed in the process-bearing cells (type 2 astrocytes) [Perego et al., 2000]. Moreover, GLAST expression is high in embryonic rat brains, but the expression level of GLT-1 is low in both embryonic and neonatal brains [Perego et al., 2000]. These results therefore

strongly suggest that RCG-12 cells maintain the functions of astrocytes, especially type 1 astrocytes of the embryonic stage.

Several lines of evidence suggest that neurotrophin signaling plays an important role in the synaptic transmission between neuron and astrocytes [Elmariah et al., 2005]. There are three types of trkB receptor isoforms, which are generated by the differential splicing of trkB mRNA; full-length trkB (trkB FL) and truncated trkB (trkB T1 and T2). In the neurons, BDNF is well known to mediate various biological actions, including neural survival, differentiation, and plasticity, through mainly trkB FL receptors [Barbacid, 1995]. On the other hand, the actions of BDNF on astrocytes are not fully understood. It has been reported that astrocytes express trkB T1 but not trkB FL [Rose et al., 2003; Ohira et al., 2005]. BDNF evokes glial Ca^{2+} signaling [Rose et al., 2003] and changes glial cell morphology [Ohira et al., 2005] through truncated trkB T1 receptors. Consistent with these reports, in the present study, the RCG-12 cells were positive to trkB T1, but not trkB FL. ATP also plays an important role in the synaptic transmission between neuron and astrocytes [Fields and Burnstock, 2006]. ATP exerts its several functions via specific receptors (P2X and P2Y purinoreceptors). RT-PCR analysis revealed that almost all subtypes of ATP receptors were present in the RCG-12 cells (Table I). Among these receptors, P2Y1 and P2Y2 receptors are known to be involved in ATP-induced Ca^{2+} oscillation [Fam et al., 2000, 2003]. Actually, ATP elicited Ca^{2+} oscillation in the RCG-12 cells (unpublished data). These results suggest that the RCG-12 cells established here may be able to serve as an excellent model for studying the functions of BDNF and ATP in astrocytes.

It is interesting to note that the cell growth arrest at 39°C also induced an increase in the expression levels of GFAP, GLAST, trkB T1, GFRα1, RET, P2Y1 (Fig. 4), and GLT-1 (Table II). It has been reported that the expression of trkB T1, GLT-1, and GLAST is differentially regulated during development of the CNS [Allendoerfer et al., 1994; Furuta et al., 1997; Perego et al., 2000]; their expression increases with age and correlates with the states of neural maturation or differentiation. Especially, increased GFAP expression is clearly the most general indicator of astrocytic reactivity. Thus, the present results suggested that the RCG-12 cells show activated or differentiated phenotypes under cell growth-restricted conditions. In the present study, GFRα1, RET, and P2Y1 were newly identified genes whose relationship to the differentiation of astrocytes has not been reported. Further studies are required to clarify whether the up-regulated genes identified here are involved in cell differentiation or in the function of differentiated astrocytes.

Although it is important to identify individual genes that are differentially expressed, there is an increasing need to move beyond this level of analysis. Therefore, in order to further clarify the mechanisms by which non-permissive temperature induces cell growth arrest and the differentiation of glial cells, we performed high-density oligonucleotide microarray analysis. To this end, in the present study, we carried out pathway analysis using the Ingenuity Pathways Analysis as a way to understand the genetic networks. The present study successfully identified the increased and decreased genes including the cellular growth and proliferation-related genetic network and the cell cycle-related genetic network, respectively, in RCG-12 cells treated with non-permissive temperature. To the best of our knowledge, this is the first report of the identification of the genetic networks involved in the differentiation of glial cells using pathway analysis software. In the present study, 556 differentially expressed probe sets were identified in the RCG-12 cells to which had been applied non-permissive temperature. Interestingly, the cellular growth and proliferation-associated genetic network was composed of up-regulated genes (nodes and edges highlighted in blue) (Fig. 5). It has been reported that BTG12 [Kuo et al., 2003], HMOX1 [Pae et al., 2004], RB1 (pRB) [Paramio et al., 1998], CXCL10 [Rosenkilde et al., 1999], and CDKN1A [el-Deiry et al., 1993] are involved in the inhibition of cell proliferation. In addition, RB1 [Xu et al., 1997], NOTCH1 [Sriuranpong et al., 2001], CDKN1A [el-Deiry et al., 1993], BTG1 [El-Ghissassi et al., 2002] have been reported to be involved in cell growth arrest, and that IL6 [Taga and Fukuda, 2005] and CNTF [Park et al., 1999] increase differentiation of neuroepithelial cells to astrocytes. Together with these previous reports, the present results suggest that the up-regulation of genes involved in the inhibition of cell proliferation and the promotion of cell growth

arrest are closely associated with the cell growth arrest caused by the non-permissive temperature in the RCG-12 cells. As well as the Western blot analysis (Fig. 2), mRNA of CDKN1A ($p21^{waf1}$) was up-regulated. This up-regulation is attributed to the inactivation of the large T-antigen and activation of p53-$p21^{waf1}$ pathway by the non-permissive temperature. Therefore, we assumed that the genes that belonged to this network may be directly or indirectly influenced by the p53-$p21^{waf1}$ pathway. Additively, GDF15 [Li et al., 2000], GADD45 [Fan et al., 1999], RB1 [Fan et al., 1999], FGF1 [Johnson et al., 1998] and IL6 [Bellido et al., 1998] have been reported to positively stimulate the expression of CDKN1A, suggesting that the up-regulation of these genes also results in the differentiation of RCG-12 cells.

Of particular interest is our identification of the cell cycle-associated genetic network whose core contains MYC, CCNB1, and IGF1 (Fig. 6). The nodes and edges involved in the cell cycle are highlighted in blue. This network includes MYC (v-myc) at its center. MYC is well known to initiate cell cycle progression and promote cell proliferation [Grandori et al., 2000], suggesting that the down-regulation of MYC plays an important part in cell growth arrest. In this network, the decreased expression of CCNB1, a cell cycle-regulated transcript expressed predominantly during the G2/M phase, was observed (Fig. 6). It has been reported that the MYC protein increases the transcription of CCNB1 [Menssen and Hermeking, 2002], suggesting that a decrease in MYC resulted in the down-regulation of CCNB1. IGF1 is also known to increase the expression of MYC and CCNB1 [Conover and Bale, 1998; Stull et al., 2002], suggesting that a decrease in IGF1 resulted in the down-regulation of MYC and CCNB1. Therefore, the down-regulation of the genes of this genetic network resulted in the decrease of cell cycle progression and may play a central role in the process of the cell growth arrest of RCG-12 cells. However, in this study, how MYC expression is regulated and how MYC interacts with large T-antigen are unclear. These are important questions needed to be elucidated in the future study.

In conclusion, the RCG-12 cell, a rat astrocyte cell line, retains some of the characteristics of astrocytes, and should prove to be an excellent model for studies of gene expression and the physiological function of astrocytes. Global gene expression analysis and computational pathway analysis revealed that the differentially expressed genes and their genetic networks identified here are likely to be involved in cell growth arrest and differentiation. Moreover, the present results will provide additional novel insights into the detail of the molecular mechanisms of astrocyte differentiation.

ACKNOWLEDGMENTS

This study was supported by grants from Ministry of Education, Culture, Sports, Science and Technology of Japan.

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