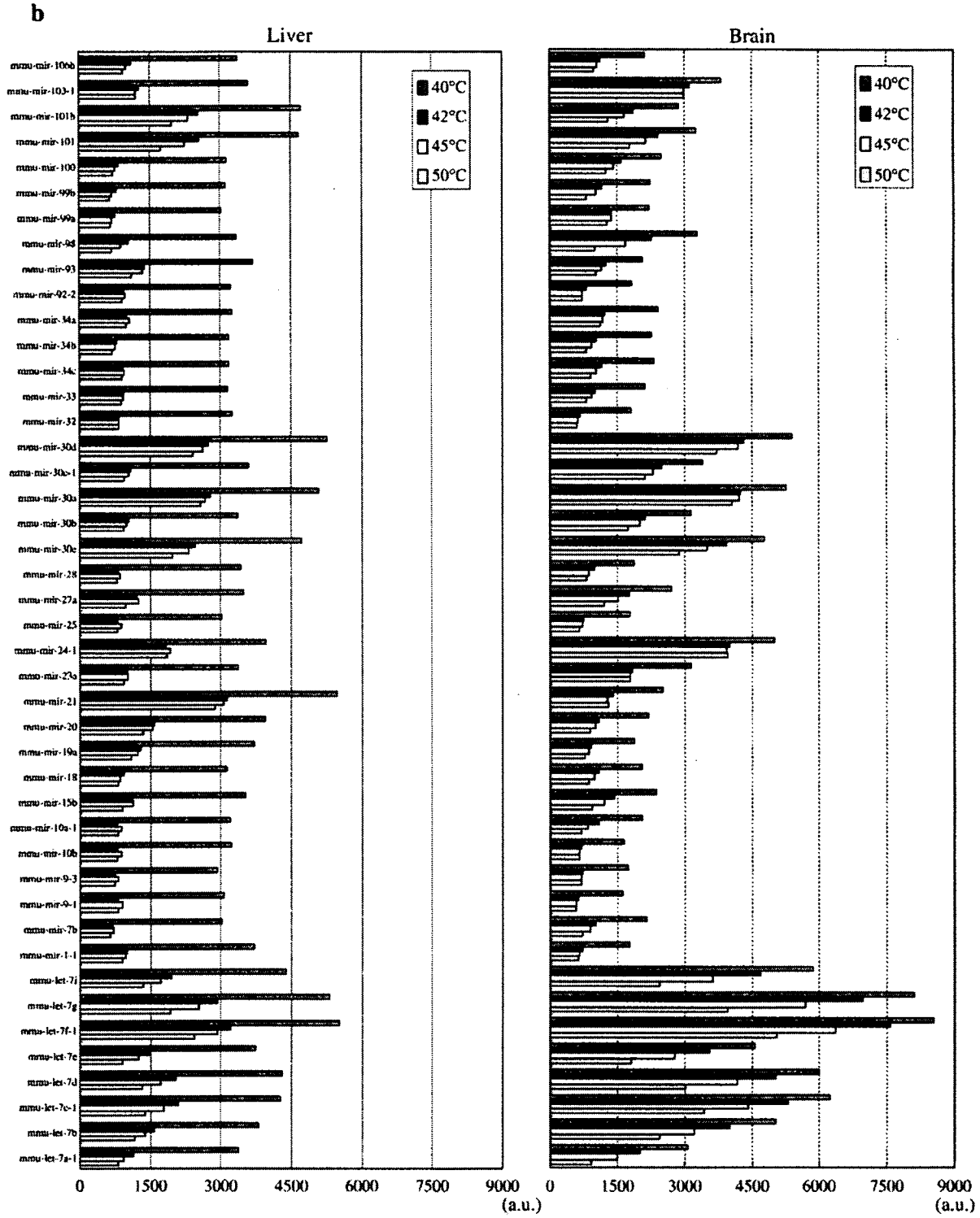

Fig. 1 (*continued*).

standard deviation [SD(nc)] were calculated, and background intensity (BI) was estimated using the following formula: $BI = AVE(nc) + 3 \times SD(nc)$.

Detected hybridization signals that were higher than the background intensity (BI) were regard as 'positive'.

2.4. Reverse transcription- (real-time) polymerase chain reaction

In order to examine the expression levels of miRNAs, total RNA was extracted from mouse brain and was subjected to

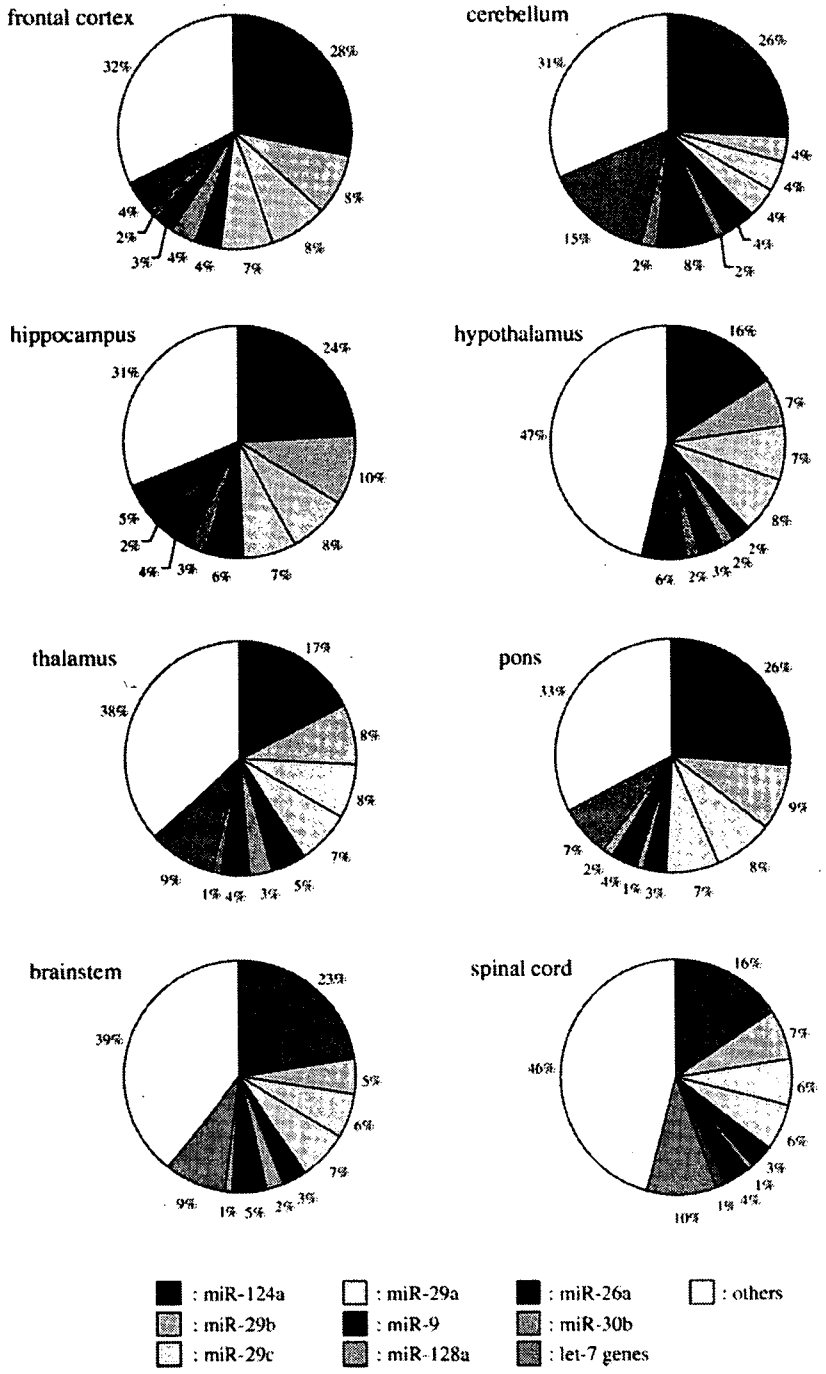

Fig. 2. Expression profile of miRNA in the mouse central nervous system (CNS). Small-sized RNA containing miRNAs was prepared from total RNA extracted from the indicated CNS subregion, labeled with Cy5 and subjected to hybridization with a DNA chip. After hybridization, the chip was washed in 2 × SSC containing 0.2% SDS at 40 °C, 42 °C, 45 °C, and 50 °C for 20 min each, and hybridization signals then examined at every step of washing as in Fig. 1. Based on the data after the 45 °C washing, relative expression ratios of detected miRNAs were estimated: the percentage (%) of signal intensity (expression level) of each positive miRNA against the sum of signal intensities of all the positive miRNAs was calculated and plotted onto a pie graph. Major miRNAs expressed in the CNS are indicated and shown in different colors.

reverse transcription- (real-time) polymerase chain reaction [RT-(real-time) PCR] using the mirVana qRT-PCR detection kit (Ambion) according to the manufacturer's instructions. Real-time PCR was performed using the ABI PRISM 7300 sequence detection system (Applied Biosystems) with SuperTaq polymerase (Ambion). The mirVana qRT-PCR primer sets used were as follows: has-miR-16, has-miR-34a, has-miR-138, has-miR-195, and 5S RNA.

End-point PCR analysis after RT reaction was also performed using the GeneAmp PCR system 9700 (Applied Biosystems) according to the manufacturer's instructions. The PCR products were electrophoretically separated on 12%

polyacrylamide gels, and visualized by ethidium bromide staining.

3. Results and discussion

3.1. Detection of miRNAs with DNA chips

We examined 182 mouse miRNAs (see supplementary Table S1) in this study. Small-sized RNAs containing miRNAs were prepared, directly labeled with Cy3 or Cy5 fluorescent substrate, and used in hybridization. Note that no ligation of the prepared RNAs with oligonucleotide anchors and no reverse transcription followed by PCR was performed during fluorescent labeling of the isolated RNAs; thus, there was no biased nucleotide labeling resulting from ligation and amplification efficiencies in this system for detection of miRNAs.

Since hybridization signals can be examined with the chips soaking in a washing buffer, the present system allows for the real-time detection of hybridized signals on the chip at every step of washing (Fig. 1), through which we can determine the most suitable conditions for precise detection of miRNAs. A drawback of this system is that there is a limitation in the number of probes installed onto the DNA chip: up to 200 probes can be installed onto the chip.

Because some miRNA genes are known to be expressed in a tissue-specific manner, we investigated whether our system with Genopal could detect such miRNAs and provide the tissue-specific expression profiles of the miRNAs. Small-sized RNAs extracted from mouse brain, liver, and heart were labeled with Cy3 or Cy5 and then used in hybridization. A merged image of expression profiles, as well as dual-color hybridization with different RNA samples, detected tissue-specifically expressed miRNAs, in which miRNAs known to be expressed in a tissue-specific manner (Lagos-Quintana et al., 2002; Krichevsky et al., 2003; Babak et al., 2004) were consistently detected: miR-124a, -125, -128 and -9 were specifically expressed in the brain; miR-1, -133a, and -133b in the heart; and miR-122a in the liver (Fig. 1a). In addition, dual-color hybridization further showed reliable data which agreed with the data obtained using separated single-color hybridizations with the same RNA samples (data not shown).

3.2. Expression profiles of miRNA in the central nervous system

The central nervous system (CNS) is composed of seven subregions: spinal cord, medulla oblongata, pons, cerebellum, midbrain, diencephalons, and cerebral hemispheres; these subregions appear to be specialized for different functions.

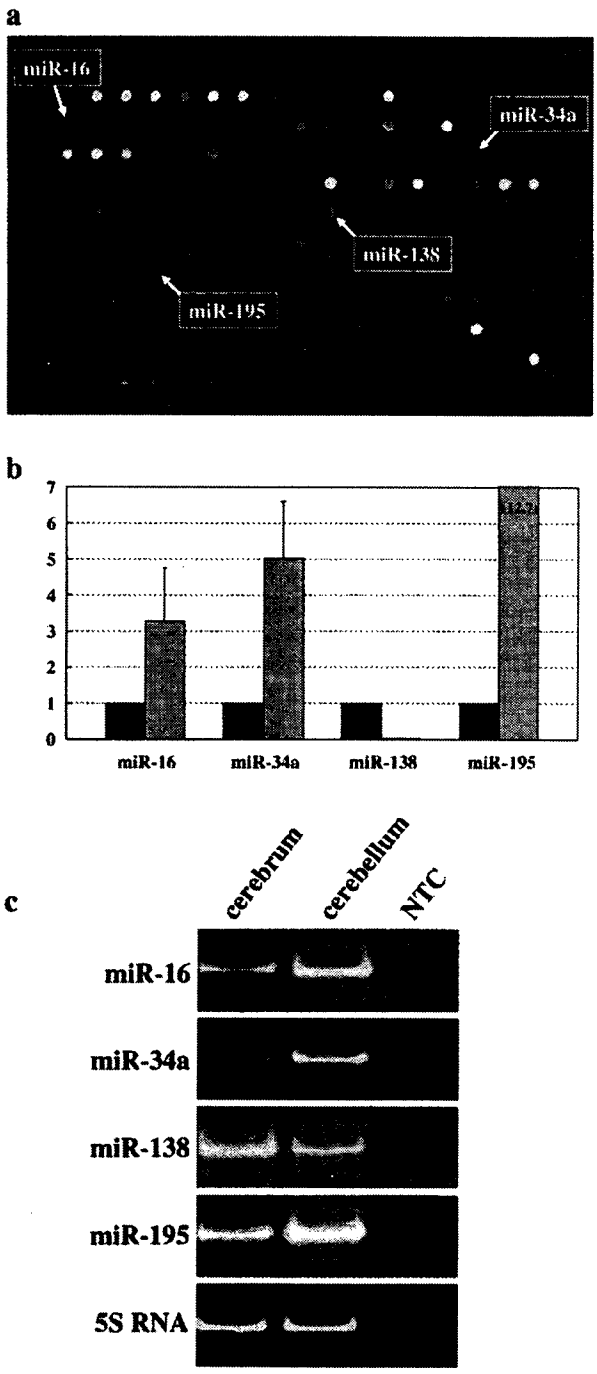

Fig. 3. Expression levels of miR-16, -34a, -138 and -195 in the cerebrum and cerebellum. (a) Merged image of miRNA expression profiles of the mouse cerebrum and cerebellum. Expression profile analysis with small-sized RNA isolated from the mouse cerebrum and cerebellum was performed as in Fig. 1. The cerebral and cerebellar signals are indicated in green and red, respectively. The signals of miR-16, -34a, -138 and -195 are indicated. (b) Expression profiles of miR-16, -34a, -138 and -195 in the cerebrum (wine-red bars) and cerebellum (blue bars). The levels of expression of mir-16, -34a, -138, -195, and 5S RNA as controls were examined by RT-(real-time) PCR with total RNA extracted from the mouse cerebri and cerebelli. The expression levels of the miRNAs were normalized to that of the 5S RNA and plotted when the expression level of each miRNA in the cerebrum was 1. The figure in parentheses in the cerebellar miR-195 indicates the average expression level, which is over the plotted area. Data are averages of three independent experiments. Error bars represent standard deviations. (c) End-point PCR analysis of miR-16, -34a, -138, -195 and 5S RNA (indicated). Total RNA (25 ng) extracted from mouse cerebrum and cerebellum (indicated) was subjected to cDNA synthesis. End-point PCR with the cDNAs was performed according to the manufacturer's instructions (Ambion). The resultant PCR products were examined by electrophoresis through 12% polyacrylamide gels followed by ethidium bromide staining. NTC: no template control.

Because different tissues have different expression profiles of miRNA, and because our present system for detection of miRNA appeared to be able to detect such expression profiles between different tissues (Fig. 1), we examined the expression profiles of miRNA in various CNS subregions using our system, i.e., we investigated whether the CNS subregions specialized for different functions show distinct expression profiles of miRNA. RNAs extracted from mouse frontal cortex (cerebral hemispheres), cerebellum, hippocampus (cerebral hemispheres), hypothalamus (diencephalons), thalamus (diencephalons), pons, brainstem (midbrain, pons, and medulla oblongata) and spinal cord were examined. Fig. 2 shows the results of relative miRNA expression ratios in the CNS subregions examined. As shown in the figure, although the CNS subregions appear to express similar miRNA genes, the relative expression ratios of the major miRNAs vary among the subregions; e.g., the difference in the expression ratios of the miR-124a and let-7 genes among the subregions is marked.

Other than the major miRNAs expressed in the CNS, some minor miRNAs also displayed marked differences in their expression among the CNS subregions. In particular, the cerebellum appeared to have intriguing expression of miRNAs, different from those in the other CNS subregions, i.e., the expression levels of the miR-16, -34a, and -195 genes in the cerebellum appeared to be higher than those in the other subregions, and in contrast, the expression of miR-138 looked markedly lower in the cerebellum (Fig. 3a). To further confirm these observations, we carried out quantitative RT-PCR to examine the levels of expression of such miRNAs with total RNAs extracted from mouse cerebelli and cerebri. As shown in Fig. 3b and c, the data consistently supported the observations described above.

Together, the data presented here suggest that different mouse CNS subregions most likely possess different expression patterns of miRNAs, which leads to the possibility that the different functions of the CNS subregions are reflected in the differing expression of miRNAs, i.e., different expression patterns of miRNAs may confer different regulation of expression of various genes, by which the CNS subregions might exert their different functions. More extensive studies are required to examine this possibility and the association of miRNAs with CNS function.

3.3. Conclusion

The features of the miRNA detection system presented here may be summarized as follows: (1) It uses direct labeling of cellular RNAs with fluorescent substrates, which, unlike cDNA-based labeling, appears to result in little or no nucleotide bias; and (2) it allows for real-time detection of hybridization signals during washing. These features appear to be important for the precise detection of small RNAs such as miRNAs, and allow us to obtain highly reproducible miRNA expression profiles. Finally, the expression profile analyses of miRNA in various mouse CNS subregions by means of the system suggested that different CNS subregions which are specialized for different functions possessed different expression profiles of miRNAs.

Acknowledgments

We would like to thank Y. Okazaki for her helpful cooperation. This work was supported in part by research grants from the Ministry of Health, Labor and Welfare in Japan.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2006.11.018.

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Marked change in microRNA expression during neuronal differentiation of human teratocarcinoma NTera2D1 and mouse embryonal carcinoma P19 cells

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Abstract

MicroRNAs (miRNAs) are small noncoding RNAs, with a length of 19–23 nucleotides, which appear to be involved in the regulation of gene expression by inhibiting the translation of messenger RNAs carrying partially or nearly complementary sequences to the miRNAs in their 3′ untranslated regions. Expression analysis of miRNAs is necessary to understand their complex role in the regulation of gene expression during the development, differentiation and proliferation of cells. Here we report on the expression profile analysis of miRNAs in human teratocarcinoma NTere2D1, mouse embryonic carcinoma P19, mouse neuroblastoma Neuro2a and rat pheochromocytoma PC12D cells, which can be induced into differentiated cells with long neuritic processes, i.e., after cell differentiation, such that the resultant cells look similar to neuronal cells. The data presented here indicate marked changes in the expression of miRNAs, as well as genes related to neuronal development, occurred in the differentiation of NTera2D1 and P19 cells. Significant changes in miRNA expression were not observed in Neuro2a and PC12D cells, although they showed apparent morphologic change between undifferentiated and differentiated cells. Of the miRNAs investigated, the expression of miRNAs belonging to the miR-302 cluster, which is known to be specifically expressed in embryonic stem cells, and of miR-124a specific to the brain, appeared to be markedly changed. The miR-302 cluster was potently expressed in undifferentiated NTera2D1 and P19 cells, but hardly in differentiated cells, such that miR-124a showed an opposite expression pattern to the miR-302 cluster. Based on these observations, it is suggested that the miR-302 cluster and miR-124a may be useful molecular indicators in the assessment of degree of undifferentiation and/or differentiation in the course of neuronal differentiation.

Keywords: MicroRNA; miR-302; miR-124a; Embryonic carcinoma cell; Undifferentiation; Neuronal differentiation; Neuron

MicroRNAs (miRNAs) are small noncoding RNAs, typically 19–23 nt in length, which are processed from primary miRNA transcripts forming stem-loop structures by digestion with a microprocessor complex containing Drosha and Pasha in the nucleus and Dicer in the cytoplasm [1–3]. After Dicer processing, the resultant miRNA duplexes undergo strand selection, and the single-stranded mature miRNA elements are incorporated into the RNA-induced silencing complex (RISC) and function as mediators in the suppression of gene expression [4]. MicroRNAs are thought to play an important role in the regulation of gene expression by inhibiting translation of messenger RNAs (mRNAs), which are partially complementary to the miRNAs, and by digestion of mRNAs which are nearly complementary to the miRNAs, such as RNA interference (RNAi), during development, differentiation and proliferation [5–9]. In addition, recent studies have further suggested significant association of miRNA with various cancers [10–13].

Hundreds of miRNA genes have been found in plants and animals [3,5,14]. They appear to be expressed by

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RNA polymerase II [15], and tissue-specific or organ-specific expression of miRNAs has been detected [8,14,16], suggesting their participation in tissue and organ-specific functions. Additionally, certain miRNAs appear to be correlated with the maintenance of pluripotent cell state during early mammalian development [17]. Comprehensive analysis of miRNA expression is helpful for understanding the complex regulation of gene expression involving miRNAs and is also necessary for the characterization of miRNAs. DNA microarray is a powerful tool for analysis of the expression profiles of miRNAs, although the shortness of miRNA, at ~22 nt, appears to make such analyses difficult [5,8,16,18]. In a previous study, we established a detection system for the expression profiles of miRNAs with a new type of DNA chip, which allowed real-time detection of hybridization signals at every wash step and resulted in highly reproducible miRNA expression profiles [19]. Using the system for detection of miRNAs, we investigated miRNA expression profiles in the mouse central nervous system (CNS), which is composed of seven subregions specialized for different functions, suggesting differences in miRNA expression among the CNS subregions.

NTera2D1 (a human teratocarcinoma cell line), P19 (a mouse embryonic carcinoma cell line), Neuro2a (a mouse neuroblastoma cell line), and PC12 D (a rat pheochromocytoma cell line) cells can be induced to differentiate into neurons or neuron-like cells which exhibit differentiated morphology with long neuritic processes (Fig. 1). Since miRNAs appear to be involved in the development and differentiation of cells and also in the maintenance of pluripotent cell state, it is of interest to see how miRNA expression is associated with neuronal differentiation of these cells. We investigated the expression of miRNAs and also protein-coding genes associated with the development of neuron in the course of their neuronal differentiation. The results indicated expression profiles of miRNAs in NTera2D1 and P19 cells dramatically changed during their neuronal differentiation; however, such a marked change in the expression of miRNAs was hardly seen in Neuro2a and PC12D cells.

Materials and methods

Cell culture and induction of differentiation. NTera2D1 and Neuro2a (N2a) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Wako) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), as previously described [20,21]. PC12D cells were grown in DMEM supplemented with 10% fetal bovine serum, 5% horse serum and the antibiotics mentioned above. P19 cells were grown in α-MEM (Wako) supplemented with 10% fetal bovine serum and the antibiotics listed above. All the cells were cultured at 37 °C in 5% CO_2-humidified chamber.

Induction of cell differentiation was carried out as follows.

Differentiation of NTera2D1 cells. NTera2D1 cells were cultured in the presence of 1×10^{-5} M all-*trans*-retinoic acid (RA) (Sigma) for three weeks. The treated cells were trypsinized, diluted with the fresh medium lacking RA and seeded into poly-D-lysine (PDL) coated 12-well culture plates (BD Bioscience). After 24-h incubation, medium was replaced with the fresh medium containing 10 μM cytosine arabinoside (Ara-C) (Sigma), and further incubated at 37 °C.

Differentiation of P19 cells. P19 cells were cultured in the presence of 5×10^{-7} M RA for 4 days in Ultra low cluster plates (Costar). After 4 days incubation, aggregated cells were collected, trypsinized, diluted with the fresh medium lacking RA and seeded into PDL-coated 12-well culture plates (BD Bioscience). After three-day incubation, the medium was changed to the Neurobasal (Invitrogen) medium containing B27 supplement (Invitrogen) and 10 μM Ara-C, and then returned to the incubator.

Differentiation of N2a cells. N2a cells were trypsinized, diluted with the fresh medium and seeded into PDL-coated 12-well culture plates (BD Bioscience). After one-day incubation, the medium was changed to the OPTI-MEM I (Invitrogen) medium containing 5×10^{-7} M RA, and then incubated at 37 °C.

Differentiation of PC12D cells. PC12D cells N2a cells were trypsinized, diluted with the fresh medium, and seeded into collagen I-coated 12-well culture plates (BD Bioscience). After one-day incubation, the medium was changed to the DMEM/F12 (Invitrogen) containing 1× ITS-X supplement (Invitrogen), and the cells were cultured in the presence and absence of 100 ng/ml Murine 2.5S Nerve Growth Factor (Promega).

Preparation of small-sized RNAs and fluorescent labeling. Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen). For preparation of cellular miRNAs, small-sized RNAs containing miRNAs were isolated from total RNA using the RNeasy MinElute Cleanup kit (Qiagen), as described previously. The isolated small-sized RNAs (~1 μg) were subjected to direct labeling with a fluorescent dye using the Platinum*Bright* 647 Infrared nucleic acid labeling kit (KREATECH), according to the manufacturer's instructions. After labeling, the labeled RNAs were purified from free fluorescent substrates using KREA*pure* columns (KREATECH) according to the manufacturer's instructions, and used in hybridization.

Hybridization with DNA chips. Hybridization was carried out with the Genopal®-MICM and -MICH DNA chips (Mitsubishi Rayon), where 180 and 127 oligonucleotide DNA probes are installed for detection of mouse and human miRNAs, respectively, in 150 μl of hybridization buffer [2× SSC, 0.2% SDS and ~1 μg of heat-denatured labeled RNAs] at 50 °C overnight. After hybridization, the DNA chips were washed twice in 2× SSC containing 0.2% SDS at 50 °C for 20 min followed by washing in 2× SSC at 50 °C for 10 min, and then hybridization signals were examined and analyzed using a DNA chip image analyzer according to the manufacturer's instructions (Mitsubishi Rayon). The Chip analysis was repeated at least two times, and hybridized signal intensities were analyzed as described previously [19].

Reverse transcription-polymerase chain reaction (RT-PCR). In order to examine the expression of genes related to neuronal differentiation, total RNAs isolated from cultured cells were subjected to cDNA synthesis using oligo(dT) primers and a Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The resultant cDNAs were examined by polymerase chain reaction (PCR) with the ABI GeneAmp PCR system 9700 (Applied Biosystems) followed by agarose gel electrophoresis and ethidium bromide staining. The PCR primer sets targeting for the following genes were purchased from TaKaRa Bio Inc.: the human *POU5F1*, *ASCL1*, *MAP2*, *NEFL*, *GFAP* and *GAPDH* genes; the mouse *Nefl* and *Gapdh* genes; the rat *Map2* *Nefl*, *Gfap*, and *Gapdh* genes. The synthesized oligonucleotides for PCR primers were as follows:

Mouse *Pou5f1*-F; 5′-AAGCTGCTGAAGCAGAAGAGGATC-3′
Mouse *Pou5f1*-R; 5′-ACCTCACACGGTTCTCAATGCTAG-3′
Mouse *Ascl1*-F; 5′-CCAACAAGAAGATGAGCAAGGTG-3′
Mouse *Ascl1*-R; 5′-AACACTAAAGATGCAGGATCTGCTG-3′
Mouse *Map2*-F; 5′-TTAAACAGGCGAAGGATAAAGTCAC-3′
Mouse *Map2*-R; 5′-TGATTGCAGTTGATCCAGGGGTAG-3′

RT—real time PCR analysis to see the expression levels of the mouse miR-124a, miR-302c, and U6RNA as a control was carried out by means of the TaqMan MicroRNA assay using the ABI 7300 Real Time PCR system (Applied Biosystems) according to the manufacturer's instructions. The TaqMan MicroRNA assays used (Assay name and Part number) were as follows: mmu-miR-124a; 4373295, mmu-miR-302c; 4381036, RNU6B (U6); 4373381.

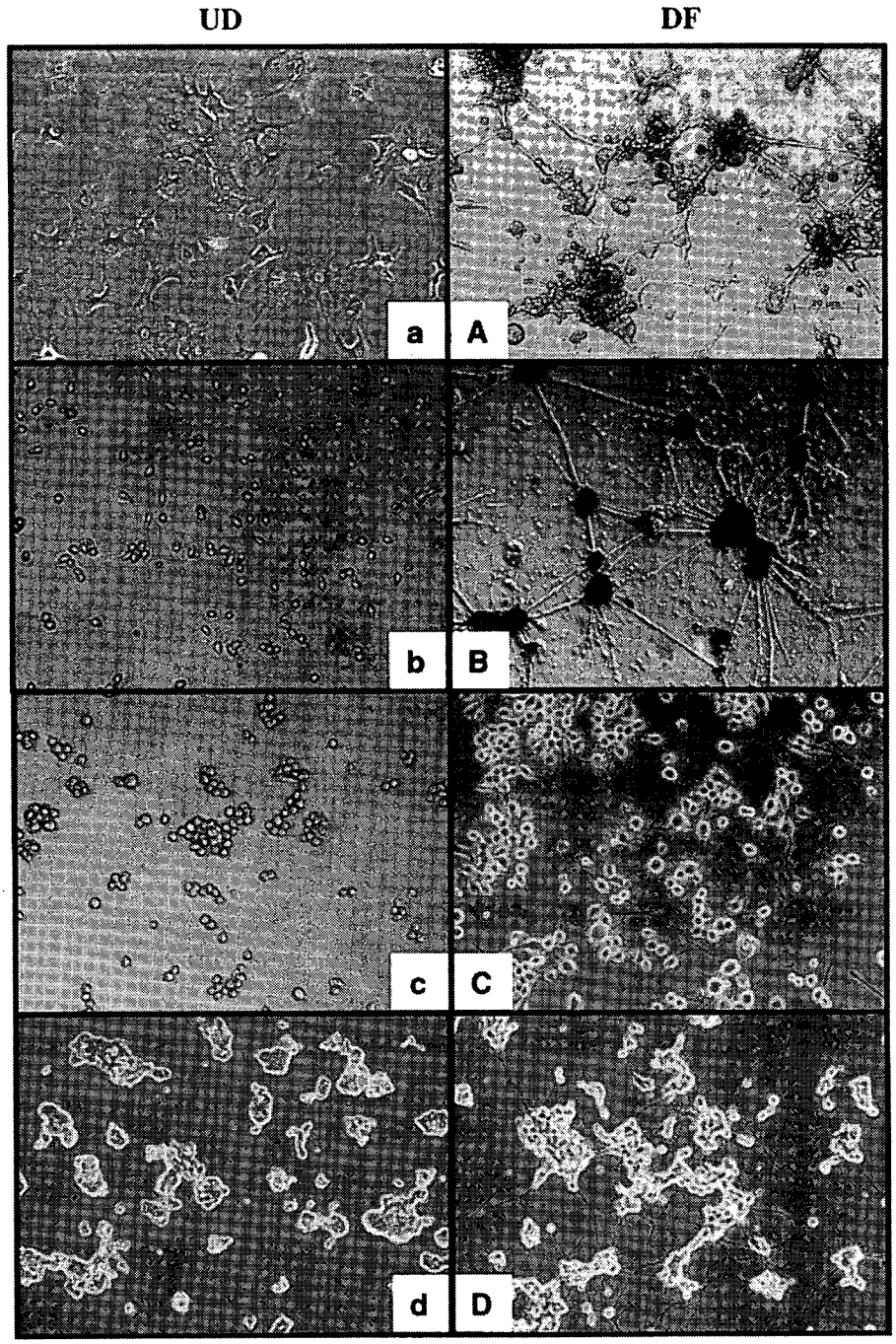

Fig. 1. Morphological differentiation of NTera2D1 (a,A), P19 (b,B), Neuro2a (c,C) and PC12D (d,D) cells. Differentiation of the cells was performed as described in the Materials and methods. Undifferentiated (UD) and completely differentiated (DF) cells are indicated by small and capital letters, respectively.

Results and discussion

Marked change in miRNA expression during neuronal differentiation of NTera2D1 and P19 cells

NTera2D1, P19, Neuro2a (N2a) and PC12D cells can be induced into differentiated cells with long neuritic processes (Fig. 1) which look very similar to neuronal cells. To see the relationship between the differentiation and miRNAs, we carried out expression profile analysis of miRNAs during neuronal differentiation of the cells by means of the Genopal-MICH and -MICM DNA chips (Mitsubishi Rayon). The Genopal is a new type of DNA chip composed of plastic hollow fibers, and oligonucleotide DNA

probes are attached to a gel within the three-dimensional space of each hollow fiber. The detection system for miRNAs with the Genopal allows real-time detection of hybridization signals at every step of washing, resulting in highly reproducible miRNA expression profiles [19].

Fig. 2 and Supplementary Fig. s1 show the results of the expression profiles of miRNAs in undifferentiated and differentiated cells. A marked difference in the expression profiles between undifferentiated and differentiated cells was seen in NTera2D1 and P19 cells. Note that the expression of miRNAs belonging to the miR-302 cluster [22], i.e., miR-302a, -302b, -302c, -302d and -367, which were specifically expressed in embryonic stem (ES) cells [23], was detected in a higher level in the undifferentiated NTera2D1 and P19 cells, but hardly in the differentiated neurons (Figs. 2 and 3A). This was in contrast to the expression of the brain-specific miRNA miR-124a [24]; miR-124a was increased in its expression level after the differentiation (Figs. 2 and 3A), and this was consistent with the previous study [24]. In relation to the miR-302 cluster, the miR-290–295 cluster composed of miR-290, -291a, -292, -291b, -293, -294, and -295 is also known as the ES-specific miRNAs [17]. The expression levels of miR-291, -292, -293 and -295, whose probes were installed into the chip, were consistently decreased during the differentiation of P19 cells (Supplementary Fig. s2), although those of the miRNAs were detected at much lower levels than that of the miR-302 cluster in undifferentiated cells. Accordingly, it is possible that the miR-302 cluster could be correlated with maintenance of pluripotency versus the miR290–295 cluster in such embryonic carcinoma cell lines and perhaps neuronal stem cells.

When N2a and PC12D cells were investigated, although their marked morphologic changes were seen (Fig. 1), they barely indicated qualitative differences in the expression of several miRNAs between undifferentiated and differentiated cells (Supplementary Fig. s1); and in PC12D cells some miRNAs appeared to be changed in their expression levels in some degree between undifferentiated and differentiated cells. In addition to the observations, we should mention that

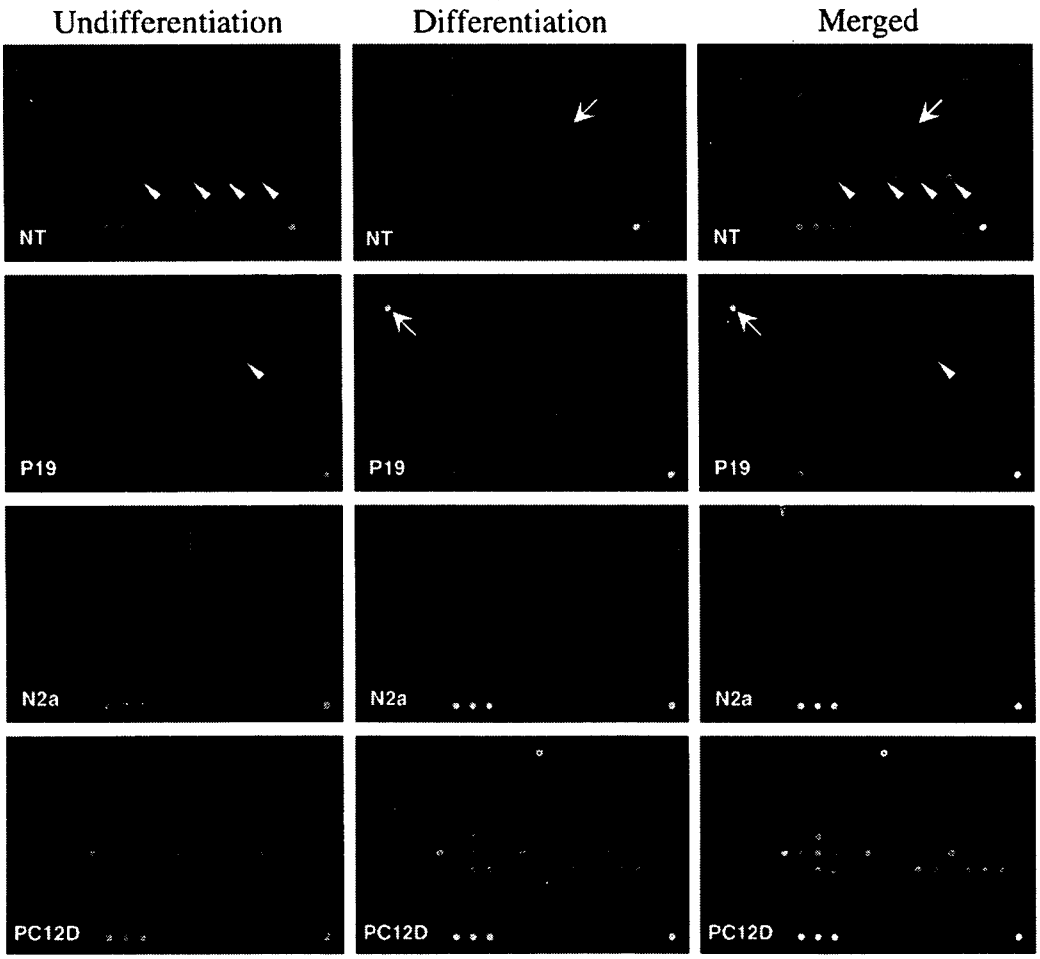

Fig. 2. Expression profiles of miRNAs between undifferentiated and differentiated cells. Undifferentiation, differentiation and merged images of miRNA expression profiles of each cell are indicated. NTera2D1 (NT) cells were examined by the MICH DNA chips for detection of human miRNAs. P19, N2a and PC12D cells (indicated) were examined by the MICM DNA chips for detection of mouse miRNAs. The miRNAs belonging to the miR-302 cluster and miR-124a are indicated by arrowheads and arrows, respectively.

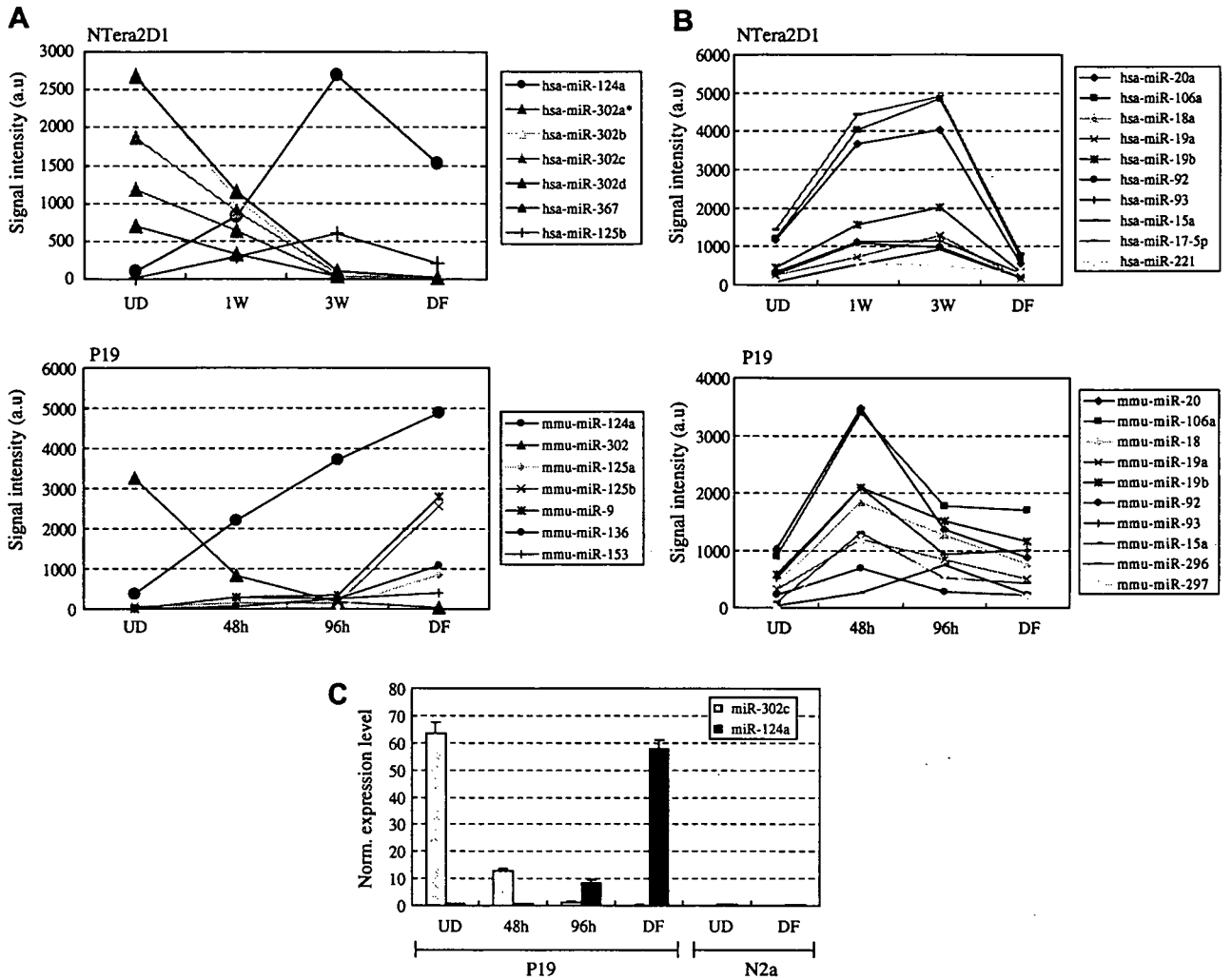

Fig. 3. Expression profiles of miRNAs during neuronal differentiation of NTera2D1 and P19 cells. Cells were induced by all-*trans*-retinoic acid (RA) to differentiate into neuronal cells. RNA samples were prepared from the cells at the indicated time point after RA treatment [1 and 3 weeks (W) in NTera2D1; 48 and 96 hours (h) in P19] and examined together with the samples prepared from undifferentiated (UD) and differentiated (DF) cells. (A) Expression profiles of the brain- and ES-specific miRNAs during neuronal differentiation. The expression levels of the brain- and ES-specific miRNAs indicated were examined and displayed. (B) Expression profiles of miRNAs specifically enhanced during RA treatment. The expression data of miRNAs indicated are shown as in (A). (C) Expression profiles of miR-124a and miR-302c during neuronal differentiation of P19 cells. The levels of expression of miR-124a and -302c and U6RNA as a control were examined by means of RT- (real time) PCR with total RNA extracted from the cells. The expression levels of the miRNAs were analyzed by the cycle threshold (Ct) method, and plotted when the expression level of U6RNA was 1. As a negative control, undifferentiated and differentiated N2a cells were also examined. Data are averages of three measurements by real time PCR analyses. Error bars represent standard deviations.

little or no expression of the ES-specific miRNAs was detected even in undifferentiated N2a and PC12D cells, and also that significantly increased expression of the miRNAs specific for neuron and/or the brain tissue, e.g., miR-124a, -125a, -125b. -136, and miR-9, was not detected in differentiated N2a and PC12D cells (Supplementary Fig. s1).

Since dramatic morphologic change in either NTera2D1 or P19 cells took place in the normal media in the absence of RA after a certain period of RA treatment, we investigated whether the marked change in the expression of miRNAs described above occurred during the treatment of RA or in the course of their morphological change after RA treatment. We examined the expression of miRNAs in NTera2D1 and P19 cells in the presence of RA. As shown in Fig. 3 and Supplementary Fig. s3, it was found that the expression profile of miRNAs in either NTera2D1 or P19 cells was dramatically changed during RA treatment. Note that the expression of the miRNAs belonging to the miR-302 cluster markedly decreased in the presence of RA in both NTera2D1 and P19 cells, and that miR-124a, miR-9a and miR-125b, which are brain-specific miRNAs, began to increase in their expression by the treatment of RA (Fig. 3A). Of the brain-specific miRNAs investigated, miR-124a was greatly increased in its expression during

the neuronal differentiation of either NTera2D1 or P19 cells (Fig. 3A). The mouse brain-specific miRNAs such as miR-125a, miR-125b, miR-136 and miR-9 appeared to be expressed, especially in differentiated P19 neurons (Fig. 3A), suggesting increase in the expression of these miRNAs during the morphological change of P19 cells after RA treatment. Other than the increased or decreased miRNAs during the neuronal differentiation, the miRNAs which appeared to significantly increase in their expression in the presence of RA were also detected in both the cells (Fig. 3B). In support of the observations, RT- (real time) PCR analysis with the TaqMan microRNA probes against the mouse miR-124a and -302c was carried out, and the results compatible with the data obtained from DNA chips were observed (Fig. 3C). Taken together, the observations suggest that regulation of miRNA expression in NTera2D1 and P19 cells was promptly changed by RA treatment. As a whole, the expression profiles of miRNAs during the neuronal differentiation of NTera2D1 and P19 cells appeared similar, suggesting comparable regulation of the expression of miRNAs in the course of neuronal differentiation. To further evaluate this, subsequent studies are necessary.

Expression profile of protein-coding genes related to neuronal differentiation

It is of interest and importance to see any relationships between the expression of miRNAs and protein-coding genes specific for neuronal development and/or differentiation during the differentiation of the cells examined here. For this purpose, we carried out RT-PCR analysis with total RNAs prepared from undifferentiated and differentiated cells. The investigated genes were as follows: *POU Domain, Class 5, Transcription Factor 1* (*POU5F1*); *Achaete-Scute Complex, Drosophila, homolog of, 1* (*ASCL1*), *Microtubule-Associated Protein 2* (*MAP2*); *Neurofilament Protein, Light Polypeptide* (*NEFL*); *Glial Fibrillary Acidic Protein* (*GFAP*); and *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*) as a control. Fig. 4 shows the results of RT-PCR analysis. The data of NTera2D1 and P19 cells

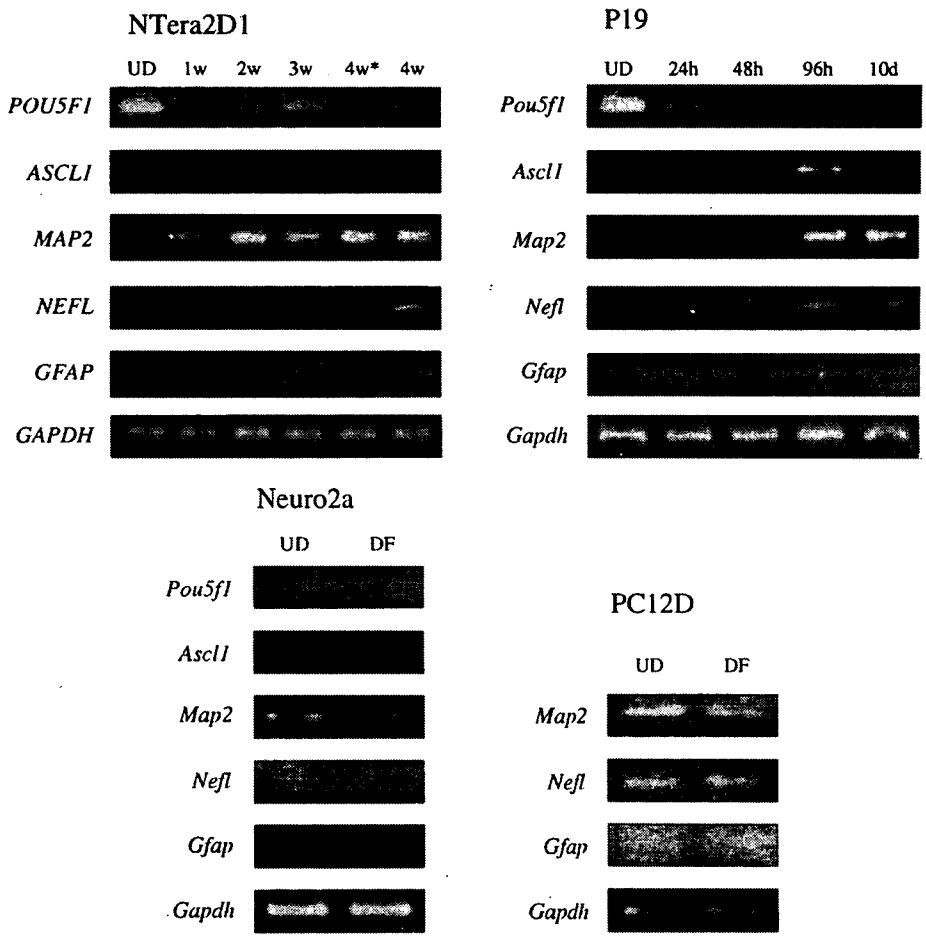

Fig. 4. Expression profiles of protein-coding genes related to neuronal differentiation. Total RNA was extracted from undifferentiated (UD) and differentiated (DF) cells. As for NTera2D1 and P19 cells, RNAs were prepared from the cells at various periods (indicated) after induction of differentiation: 1–4 weeks (1–4 w) in NTera2D1 cells and 24 h–10 days (24 h–10 d) in P19 cells. The RNAs were subjected to RT-PCR analysis to examine the expression of genes indicated. The expression of *Gapdh* was examined as a control. Asterisk indicates the NTera2D1 neurons grown on normal cultured plates instead of PDL-coated culture plates.

indicate: (i) the expression of *POU5F1* was detected in both of the undifferentiated cells, but rapidly reduced by RA treatment, (ii) an acute expression of *ASCL1* was detected after the RA treatment, (iii) *MAP2* and *NEFL*, which are specific to the neuron, appeared to increase in their expression after RA treatment and their potent expression was detected in the differentiated neurons, and (iv) little or no expression of *GFAP* was detected; meaning this could be due to the treatment of Ara-C during neuronal differentiation of the cells. Together with the expression profiles of the miR-302 cluster and miR-124a described above, it is likely that both the miRNAs and protein-coding genes were properly controlled during the differentiation of either NTera2D1 or P19 cells, and that the gene expression may be largely similar to authentic regulation of gene expression during neuronal development *in vivo*. Thus, we propose that expression of the miR-302 cluster and miR-124a could be molecular indicators for the degree of undifferentiation or differentiation in the course of neuronal differentiation in this kind of embryonic carcinoma or stem cells.

When N2a and PC12D cells were examined (Fig. 4), the expression of the neuron-specific *Map2* gene was detected in both the undifferentiated and differentiated cells. Interestingly, PC12D cells further expressed the *Nefl* gene at a state of either undifferentiation or differentiation. These observations suggest undifferentiated N2a and PC12D cells may be found more in neuronal differentiation than NTera2D1 and P19 cells. This indicates that they might stop differentiation before their morphologic changes, maintaining their undifferentiated state. Taken together, it is possible that a major change in the expression of genes, including miRNAs, would occur prior to morphologic changes with neurite outgrowth in the course of neuronal differentiation and/or development. To further evaluate this, more extensive studies must be conducted.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.189.

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Molecular Psychiatry (2007) 12, 519

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IMAGE

The possible association between epigenetic aberration in DNA methylation in *RELN* and psychiatric disorders

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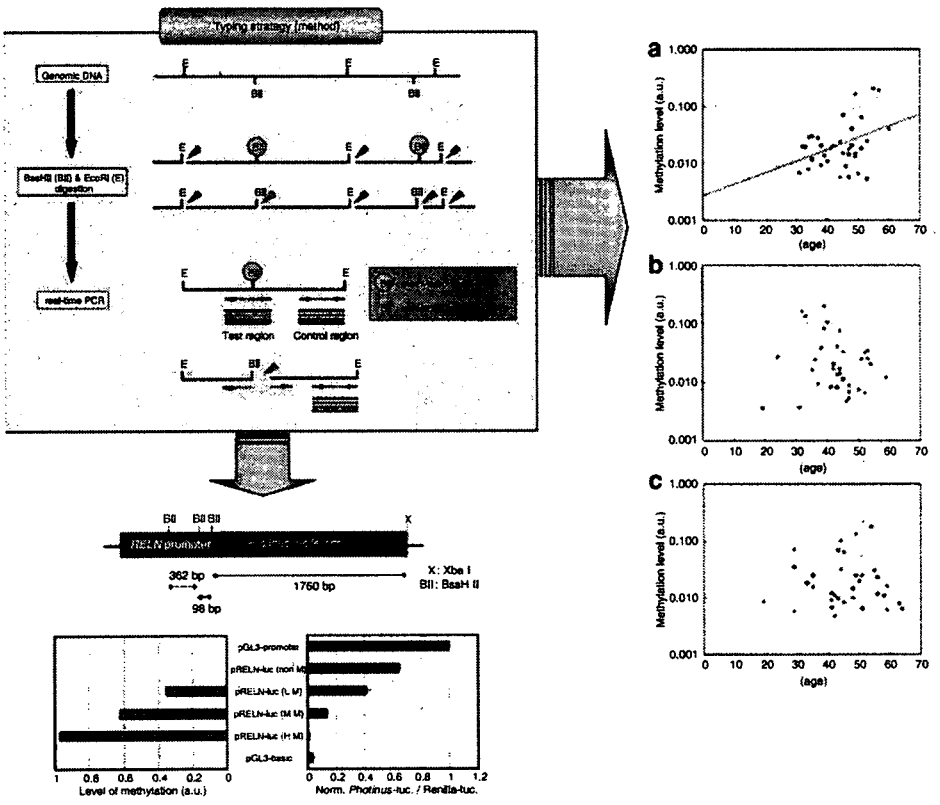

Molecular Psychiatry (2007) **12,** 519; doi:10.1038/sj.mp.4002014

Typing strategy (method) for determination of DNA methylation status: genomic DNA is digested with *Bss*HII, a methylation-sensitive restriction enzyme, and then subjected to real-time PCR such that a target region carrying a *Bss*HII site of interest can be amplified. In addition, regions adjacent to the target region, where no restriction enzyme sites are present, are also amplified and used as controls for normalization of the amplified target region. Using the method, the levels of DNA methylation in the human *REELIN* (*RELN*) gene in postmortem brain genomic DNA from schizophrenic, bipolar and healthy individuals were examined. The scatter graphs (the right panels) show that a statistically significant correlation between the levels of DNA methylation in *RELN* and age was detected in healthy individuals (**a**), no such correlations were seen in either schizophrenic or bipolar patients (**b** and **c**, respectively). The method can also be used for determination of DNA methylation status against the *RELN* reporter plasmids that are partially methylated *in vitro* (the downward arrow). The *in vitro* expression assay with the resultant reporter plasmids with various DNA methylation levels demonstrated a reverse correlation between the levels of DNA methylation and expression in *RLEN*. For more information on this topic, please refer to the article by Tamura *et al.* on pages 593–600.

Molecular Psychiatry (2007) 12, 593–600

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ORIGINAL ARTICLE

Epigenetic aberration of the human *REELIN* gene in psychiatric disorders

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Epigenetic genome modifications such as DNA methylation appear to be involved in various diseases. Here, we suggest that the levels of DNA methylation at the *BssH*II methylation-sensitive restriction enzyme sites in the human *REELIN* (*RELN*) gene in the forebrain vary among individuals. Interestingly, although a statistically significant correlation between the levels of DNA methylation in *RELN* and age was detected in healthy individuals, no such correlations were seen in either schizophrenic or bipolar patients. In addition, reverse correlations between DNA methylation levels and *RELN* expression were also detected in postmortem brain RNA and on *in vitro* assay. These data suggest the possibility that epigenetic aberration from the normal DNA methylation status of *RELN* may confer susceptibility to psychiatric disorders.
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Keywords: epigenetics; DNA methylation; REELIN; promoter activity; human postmortem forebrains

Introduction

DNA methylation is an epigenetic modification on the mammalian genome, and cytosine residues in CpG dinucleotides appear to be favorable targets for methylation, resulting in C^mpG. The process of DNA methylation and resultant C^mpG appear to be involved in tissue-specific gene expression, chromatin modification and genomic imprinting, which play essential roles in development, cell proliferation and differentiation.[1,2] DNA methylation is also involved in various diseases, including cancer,[3] and may be associated with psychotic disorders such as schizophrenia.[4–8] To better understand the implications of DNA methylation in disease susceptibility, it is necessary to examine both the qualitative and quantitative status of DNA methylation within the genome.

In this study we developed a method by which relative levels of DNA methylation at methylation-sensitive restriction enzyme sites within the genome can be determined. The method depends on methylation-sensitive restriction enzymes and real-time polymerase chain reaction (real-time PCR). We investigated this method in order to determine any associations between DNA methylation and psychotic disorders by measuring the levels of DNA methylation in genomic DNA from schizophrenic, bipolar and healthy subjects using samples kindly provided by the Stanley Medical Research Institute.

The results presented here suggest that the levels of DNA methylation at methylation-sensitive restriction enzyme sites in the human *REELIN* (*RELN*) gene vary among individuals, and that a possible correlation between the levels of DNA methylation in *RELN* and age occurs in healthy individuals, but not in either schizophrenic or bipolar patients. The present study suggests the possible association between epigenetic aberration in DNA methylation in *RELN* and psychiatric disorders.

Materials and methods

DNA and RNA samples

All genomic DNA and RNA samples examined here were extracted from human postmortem forebrains and were kindly provided by the Stanley Medical Research Institute. Samples were blinded (coded) during our experiments (determination of DNA methylation and examination of expression levels), and after reporting all experimental data to the Institute, we obtained information regarding the diagnoses of the coded subjects, and then analyzed our typing data using subject information (Table 1).

Determination of relative DNA methylation levels

Genomic DNA (0.5 μg) was digested with *Eco*RI and *BssH*II (methylation-sensitive restriction enzyme) at 37°C overnight, collected by ethanol precipitation, and dissolved in 50 μl of TE. Digested DNA (60 ng/

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Table 1 Data of the subjects examined in this study

	Schizophrenia (n = *35*)	*Bipolar* (n = *35*)	*Control* (n = *35*)
Gender	26 males, nine females	17 males, 18 females	26 males, nine females
Age range (years at death)	19–59	19–64	31–60
Mean age (years)	43	46	45
Age at disease onset (years)			
⩽15	5	4	NA
16–20	14	9	NA
21–25	9	9	NA
26–30	3	4	NA
⩾31	4	9	NA
Lifetime alcohol use			
Little or no	10	4	18
Social	7	8	12
Moderate (past or present)	6	9	3
Heavy (past or present)	12	13	2
No information	0	1	0
Lifetime drug use			
Little or no	14	11	30
Social	4	4	4
Moderate (past or present)	6	9	1
Heavy (past or present)	9	11	0
No information	2	0	0
Suicide victims	7	15	0
Postmortem interval (hours)	9–80	12–84	9–58

Abbreviation: NA, not applicable.

test) was used as a template and was examined by means of real-time PCR using the following PCR primer sets:

For amplification of the control region of *RELN*;
RC-F1, 5′-GAACAGTCCGGCGAAGAGAG-3′
RC-R1, 5′-CAGAGCCTCATCTGTAGAGGATTT-3′
For the test region of *RELN*, we carried out real-time PCR with a *RELN* probe that we designed:
RC-F3, 5′-CGGCGTCTCCAAAACTGAATGA-3′
RC-R3, 5′-GTGGGGTTGCCCGCAATATGCAG-3′
RELN probe, 5′-(FAM)-CTAGCGCTGTTGCTGGGGGC GACGCTG-(TAMRA)-3′

Real-time PCR was carried out using the ABI PRISM 7000 or 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) with SYBR GREEN PCR Master Mix (for control region) or TaqMan Universal PCR Master Mix (for test region) according to the manufacturers' instructions and was repeated at least three times. Two individuals' samples were examined as controls for every PCR in order to normalize test data.

Cell culture
NTera2D1 cells were grown as described previously.[9] Cells ($\sim 7 \times 10^4/cm^2$) were treated with 10^{-5} M all-*trans*-retinoic acid (RA) (SIGMA-ALDRICH, St Louis, MO, USA) in Dulbecco's modified eagle medium (SIGMA-ALDRICH) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen) for 3 weeks and reseeded ($\sim 5 \times 10^4/cm^2$), and further culture was carried out in the absence of RA.

Reverse transcription (RT)-(real-time) PCR
Total RNAs isolated from the same postmortem forebrain specimens were also provided by the Stanley Medical Research Institute, and each RNA sample ($\sim$0.5. μg) was examined by means of reverse transcription (RT) – real-time PCR (RT-PCR) with TaqMan Gene Expression Assays (Applied Biosystems) using TaqMan probes specific for *RELN* (assay ID: Hs00192449_m1) and *S18 rRNA* (assay ID: Hs99999901_s1) as a control. The assay RT-PCR) was carried out using the ABI PRISM 7000 or 7300 sequence detection system (Applied Biosystems) with TaqMan One Step RT-PCR Master Mix Reagents kit (Applied Biosystems) according to the manufacturer's instructions and was repeated at least three times.

Construction of reporter plasmid and partial methylation
In order to construct a reporter plasmid carrying a putative *RELN* promoter linked to the *Photinus luciferase* reporter gene, the *RELN* promoter region from positions −525 to +333 relative to the *RELN*

transcription start site was amplified by PCR with human genomic DNA and the following PCR primer set:

RELN-532F; 5′-GTTCTAGATCTTCCCAGGAAAAAC AGGGCACACTG-3′
RELN + misR; 5′-AATATCCATGGTGGCGAGCACCTC GCCCTGC-3′

The resultant PCR product was digested with *Bgl*II and *Nco*I and was subjected to ligation with pGL3-control treated with the same restriction enzymes. The resultant plasmid possessing the putative human *RELN* promoter was designated 'pRELN–Luc'.

Partial methylation was carried out as follows: ten micrograms of the pRELN–Luc plasmid was treated with 4 U of CpG methylase (M. Sss I) (New England BioLabs, Inc., Beverly, MA, USA) in the presence of 2×, 1× and 0.2× provided *S*-adenosyl methionine at 37°C for 6, 3 and 2 h, respectively. The treated plasmids were purified with a Wizard SV Gel and PCR clean-up system (Promega, Madison, WI, USA), and aliquots of the purified plasmids were subjected to digestion with *BamH*I and *BssH*II followed by real time PCR (1.25 ng each/test) for determination of DNA methylation levels. The PCR primer sets used were as follows:

For amplification of control region in the vector;
GL3-F1044, 5′-TTTGATATGTGGATTTCGAG-3′
GL3-R1194, 5′-ATCGTATTTGTCAATCAGAG-3′

For test region in the partially methylated *RELN*, the same PCR primers, RC-F3 and RC-R3, and the *RELN* probe described above were used. Real-time PCR and analyses of methylation levels were also carried out as described above.

Transfection

The day before transfection, NTera2D1 cells treated with RA for 3 weeks were trypsinized, diluted with fresh medium without RA and antibiotics, and seeded into 24-well culture plates (approximately 0.5×10^5 cells/well). Cotransfection of partially methylated pRELN–Luc plasmid with phRL–TK plasmid (Promega) as a control was carried out using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Before cotransfection, the culture medium was replaced with 0.4 ml of OPTI-MEM I (Invitrogen), and to each well, 0.2 μg of

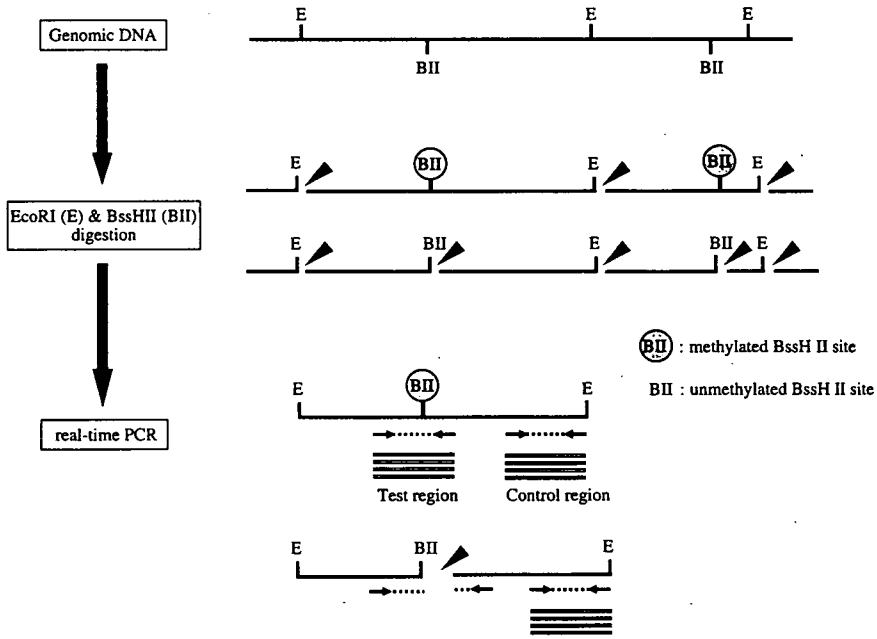

Figure 1 Outline of typing strategy for determination of DNA methylation status in the genome. Genomic DNA was digested with *EcoR*I and *BssH*II (methylation-sensitive restriction enzyme), and was subjected to real-time PCR such that a target region carrying *BssH*II site(s) of interest could be amplified. In addition, a region adjacent to the target region, where no *BssH*II sites were present, was also amplified and used as a control to normalize the amplified target region. When the *BssH*II site of interest in the target region was methylated, digestion of the site with the enzyme was inhibited, resulting in successful PCR amplification of the target region during real-time PCR. In contrast, when the *BssH*II site was not methylated, complete digestion of the site by the enzyme occurred, resulting in little or no PCR amplification. Accordingly, the level of DNA methylation at the *BssH*II site of interest in the target region is quantitatively reflected by the efficiency of real-time PCR amplification, and the observed amount of target region was subjected to normalization against that of the control region. A schematic representation is shown. *EcoR*I, unmethylated *BssH*II and methylated *BssH*II sites are indicated by 'E', 'BII', and 'BII', respectively. Arrow heads indicate restriction enzymes. PCR primers and amplified products are indicated by arrows and bars, respectively.

pRELN–Luc plasmid and 0.05 μg of phRL–TK plasmid were applied. Cells were incubated for 4 h at 37°C. After the 4-h incubation, 1 ml of the fresh culture medium without RA and antibiotics was added, and further incubation at 37°C was carried out. Forty-eight hours after transfection, cell lysate was prepared and expression levels of luciferase were examined by the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Results and discussion

Typing strategy for determination of DNA methylation status

Figure 1 shows our typing strategy (method) for determination of DNA methylation status in the genome. Briefly, genomic DNA is digested with a methylation-sensitive restriction enzyme, and is then subjected to real-time PCR such that a target region carrying methylation-sensitive restriction enzyme site of interest can be amplified. In addition, regions adjacent to the target region, where no restriction enzyme sites are present, are also amplified and used as controls for normalization of the amplified target region. Compared with the method with bisulfite-modification of genomic DNA, the present method appears to have little or no bias from insufficient chemical modification of genomic DNA and biased PCR amplification with such a modified DNA in determination of methylation levels of target genes (or regions). Furthermore, this method, unlike a conventional Southern blot analysis with methylation-sensitive restriction enzymes, does not need a large amount of genomic DNA. Although the present system for determination of methylation levels appears to have the benefits that are not in conventional methods, the system profoundly depends upon methylation-sensitive restriction enzymes, by which investigation is usually restricted to the restriction enzyme sites; accordingly, this may be a major drawback of this method.

By using the method we investigated the levels of DNA methylation in genomic DNA from schizophrenic, bipolar and healthy subjects to determine any associations between DNA methylation and psychotic disorders. The genomic DNA examined in this study was extracted from human postmortem forebrains of schizophrenic, bipolar and healthy individuals (35 samples each) (Table 1), and was kindly provided by the Stanley Medical Research Institute.

DNA methylation status in RELN

We examined the human *REELIN* (*RELN*) gene, as its putative promoter region (including exon 1) includes a GC-rich sequence containing several methylation-sensitive restriction enzyme sites,[5] and because possible associations between this gene and psychotic disorders have been reported.[10–14] The methylation status at the *BssH*II sites (GCGCGC) in the promoter region was investigated using the above-described

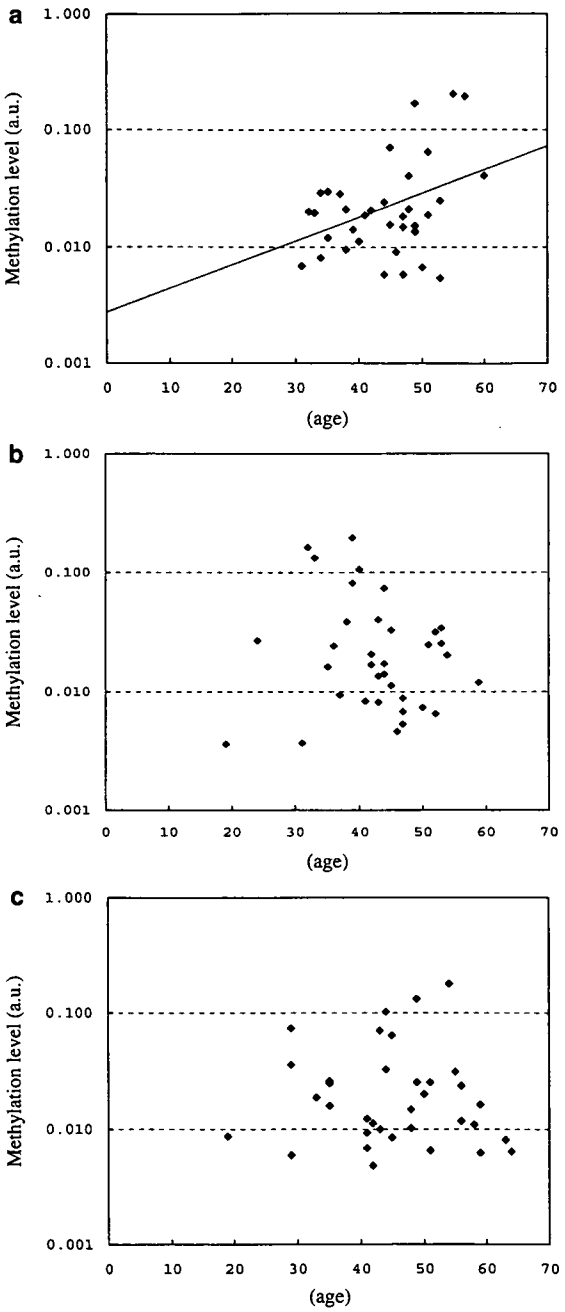

Figure 2 Scatter graphs of DNA methylation levels in *RELN* and age. All genomic DNA samples (35 samples each from healthy (**a**), schizophrenic (**b**) and bipolar (**c**) individuals) extracted from human postmortem forebrains were kindly provided by the Stanley Medical Research Institute. Relative levels of DNA methylation at *BssH*II sites in *RELN* were examined as described in Figure 1. Data regarding the levels of the DNA methylation are averages of three independent examinations and are given in arbitrary methylation units (a.u.). Tests for equal variance were carried out to determine whether there were any correlations between DNA methylation levels and aging.

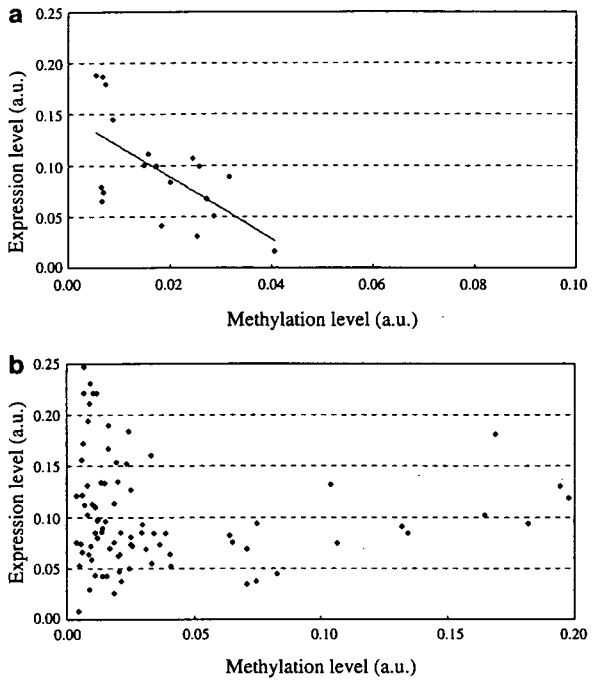

Figure 3 Relationship between DNA methylation levels and expression of *RELN*. Levels of expression of *RELN* and *18S rRNA* (control) among RNA samples extracted from the same postmortem forebrains as described in Figure 2 were examined by means of RT-PCR with TaqMan Gene Expression Assays (Applied Biosystems). Levels of expression of *RELN* were normalized against those of the control (18S rRNA). Data for DNA methylation and RNA expression are averages of three independent examinations and are given in arbitrary (methylation or expression) units (a.u.). Data for samples prepared within 18 h of death or beyond 18 h after death are shown in **a** and **b**, respectively. Tests for equal variance were performed to determine whether there were any correlations between the levels of DNA methylation and expression.

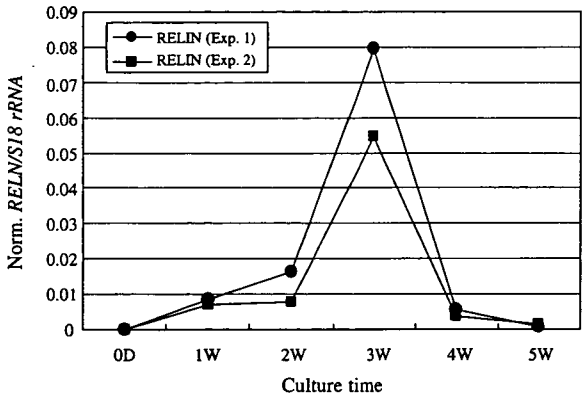

Figure 4 Expression profiles of *RELN* during neuronal differentiation of NTera2D1 cells. To investigate the endogenous expression of *RELN* during neuronal differentiation of NTera2D1 cells, expression profile analysis was carried out. Cells were treated with 10^{-5} M all-RA for 3 weeks and reseeded ($\sim 5 \times 10^4/cm^2$), and further culture was carried out in the absence of RA. Total RNA was extracted from cells before RA treatment (0 day: 0 D) and cells at the indicated time point after RA treatment (1 week $\sim$ 5 weeks: 1 W $\sim$ 5 W). Extracted RNAs were subjected to RT-PCR with TaqMan Gene Expression Assay as in Figure 3. Levels of *RELN* expression were normalized against those of *S18 rRNA*. Expression profile analysis was repeated twice independently (Exp.1 and Exp. 2). The results indicate that the expression of endogenous *RELN* is induced by RA treatment and that *RELN* expression peaks at around 3 weeks after RA treatment. Accordingly, we decided to use NTera2D1 cells treated with RA for 3 weeks host cells in the transient expression assay using partially methylated pRELN–Luc plasmids (Figure 5).

method. The results indicated that the relative levels of the DNA methylation varied among the schizophrenic, bipolar and healthy individuals. Because differences in the average levels of DNA methylation among the three groups did not reach statistical significance, further analyses stratified by age, gender, age at disease onset, life time alcohol and drug use and suicide status (Table 1) were carried out In this series of analyses, we identified an intriguing association; when DNA methylation levels and subject age were plotted in scatter graphs (Figure 2), a correlation between DNA methylation and age was seen in healthy individuals ($r=0.436$, $P<0.01$). It should be noted that no such correlation was seen in either schizophrenic or bipolar patients. In addition, it was observed that several patients under the age of 50 had higher levels of DNA methylation >0.03 (a.u.) at the level of DNA methylation shown in Figure 2: nine schizophrenia (25.7%) and seven bipolar (20.0%) cases met these criteria, but only three healthy individuals (8.6%) met these criteria. The difference in percentages between healthy individuals and either schizophrenic or bipolar patients, however, did not reach statistical significance owing to the small number of samples examined in this study. As for the other stratification analyses, we could not see their significant associations with the DNA methylation status in *RELN*.

Grayson *et al.* showed significant association of methylated cytosine at positions −139(CpApG) and −134(CpTpG) in *RELN* with schizophrenia by means of bisulfite-modification of genomic DNA followed by PCR amplification and sequence determination.[4] Our present study focused on the *BssH*II sites at positions +131, +227 and +229 in *RELN*. Therefore, different results between the previous and present studies may be attributable to the different positions examined.

Reverse correlation between the levels of DNA methylation and expression of the RELN *gene*

A previous study with completely methylated *RELN* promoter by CpG methylase (M.Sss I) suggested that DNA methylation of the *RELN* promoter *in vitro* decreased its promoter activity;[5] but correlations between DNA methylation levels in *RELN* and *RELN*

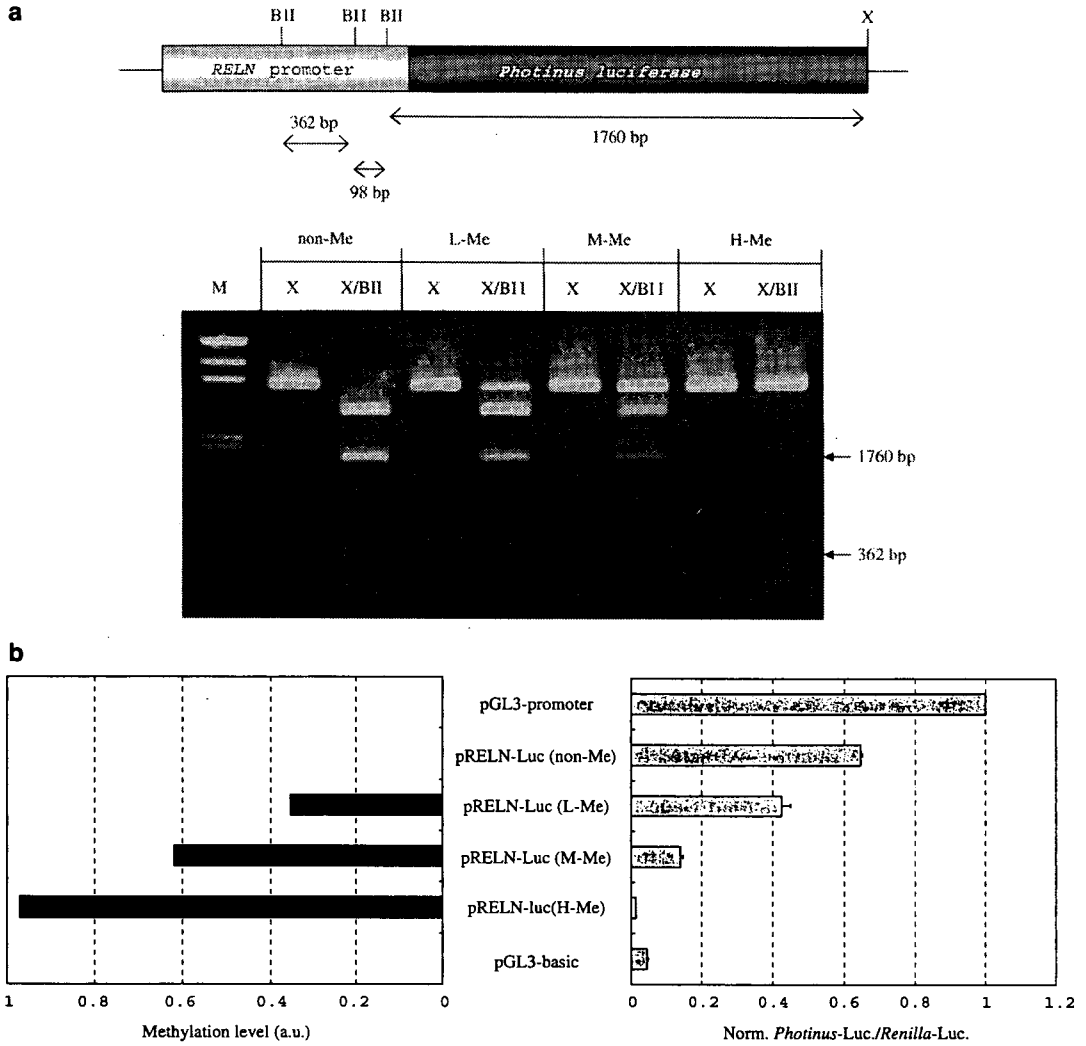

Figure 5 Reverse correlation between DNA methylation levels and transcriptional expression of *RELN*. (**a**) Digestion profiles of partially methylated plasmids with restriction enzymes. The pRELN–Luc plasmid carrying *Photinus luciferase* driven by the human *RELN* promoter was partially methylated using a CpG methylase (M. Sss I), after which the levels of DNA methylation were determined as in Figure 1. The resultant plasmids with various levels of DNA methylation, high (H-Me)-, moderate (M-Me)- and low (L-Me)-level methylated pRELN–Luc, were further confirmed by restriction enzyme digestion with *BssH* II (BII) and *Xba* I (X) followed by agarose gel electrophoresis. A schematic restriction enzyme map of pRELN–Luc is shown. Non-methylated plasmids and size markers (*Hind*III-cut λ DNA) are indicated by 'non-Me' and 'M', respectively. Note that the data also indicate that our typing method properly worked. (**b**) Reverse correlation between the levels of DNA methylation and expression of *RELN*. pRELN–Luc plasmids with various levels of DNA methylation were subjected to transfection together with phRL–TK encoding *Renilla luciferase* (control) into human teratocarcimona NTera2D1 cells treated with RA for 3 weeks, and in which endogenous *RELN* was being expressed (Figure 4). In addition, unmethylated SV40 promoter (in pGL3-Promoter) and no promoter (in pGL3-Basic) were also examined as positive and negative controls, respectively. Forty-eight hours after transfection, luciferase activities were examined. Test (*Photinus*) luciferase activity normalized against control (*Renilla*) luciferase activity and is given in arbitrary units (a.u.). Levels of DNA methylation are indicated as in Figure 2 and are given in arbitrary methylation units (a.u.).

expression remained unanswered. It is of interest and importance to determine whether there is any association between DNA methylation levels and expression in *RELN*. Total RNAs isolated from the same postmortem forebrains were also provided by the Stanley Medical Research Institute, and the expression levels of *RELN* and *18S rRNA* (control) were examined by means of RT-PCR. In the samples prepared within 18 h of death (eight schizophrenia and four bipolar cases, and seven healthy controls), a reverse correlation between DNA methylation and *RELN* expression was detected ($r=-0.63$, $P<0.01$), but no such correlation was seen in the samples prepared beyond 18 h after death (Figure 3). These

observations suggest that the level of DNA methylation in *RELN* likely influences its expression; and the observations also suggest the possibility that the postmortem DNA and/or RNA might undergo substantial degradation within 18 h of death. To address the possibility, more extensive studies must be carried out.

To further evaluate the correlation between DNA methylation levels and expression in *RELN*, we constructed a reporter plasmid carrying the *RELN* promoter region linked to the *Photinus luciferase* gene. The reporter plasmid was subjected to partial methylation with CpG methylase (M.Sss I), after which the level of DNA methylation was determined using the above-described method. The resultant reporter plasmids with various levels of DNA methylation (Figure 5a) and phRL–TK carrying *Renilla luciferase* (control) were cotransfected into NTera2D1 cells where endogenous *RELN* was expressed (Figure 4), that is, in which transacting (transcription) factors necessary for the proper *RELN* expression could occur. After incubation, luciferase expression levels in the transfected cells were examined. As shown in Figure 5b, *Photinus luciferase* expression decreased with increased levels of DNA methylation in the *RELN* promoter; thus, the results indicate a reverse correlation between the levels of DNA methylation and expression of the *RELN* gene. Together with the results shown in Figure 3a, the data strongly suggest that the level of DNA methylation in *RELN* is significantly associated with its expression. The previous study using the *RELN* promoters which were completely methylated *in vitro* with various bacterial methylases suggested that DNA methylation participated in downregulation of *RELN* expression.[5] Therefore, it is most likely that DNA methylation in *RELN* is a key element functioning in regulation of the expression of *RELN*.

Our present data appear to be compatible with those of the previous studies using bisulfite-modification of genomic DNA,[4,6] both suggesting the aberrant DNA methylation status of *RELN* in psychiatric disorders. In addition, recent studies also indicated that the mouse *DNA methyltransferase 1* (*Dnmt1*) gene knockdown was accompanied by increased expression of *Reln*[15] and that protracted administration of L-methionine, a precursor of the methyl donor *S*-adenosyl-methionine in Dnmt1 catalytic activity, into mice led to decreased *Reln* expression,[7] suggesting possible inhibitions of the expression of *Reln* involving DNA methylation *in vivo*. Altogether, it is conceivable that *RELN* may undergo various levels of epigenetic modifications of DNA methylation, and that aberrant DNA methylation status in *RELN* leading to aberrant expression may confer susceptibility to psychiatric disorders.

Genetic and epigenetic factors conferring susceptibility to psychiatric disorders

The human *RELN* gene is mapped to chromosome 7q22, where few associations with human genetic diseases, except for a possible association with Finnish schizophrenia,[16] have been reported, whereas significant reductions in *RELN* transcripts and polypeptides in the brains of patients with psychiatric disorders have been repeatedly observed.[10–14] The present study indicates the possible association between epigenetic aberration in DNA methylation in *RELN* and psychiatric disorders; such aberration is probably undetectable using conventional genetic analyses. It is conceivable that even though genes were negative for associations with diseases on conventional genetic analysis, genes such as *RELN* may be involved in disease susceptibility through aberrant epigenetic modifications. Therefore, to identify the culprits conferring susceptibility to diseases, extensive studies focusing on both genetic factors and epigenetic factors are required.

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