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Increased expression of prion protein gene is accompanied by demethylation of CpG sites in a mouse embryonal carcinoma cell line, P19C6

Wuyun DALAI[1)], Eiko MATSUO[1)], Natsumi TAKEYAMA[2)], Junichi KAWANO[1)] and Keiichi SAEKI[1)]*

[1)]Laboratory of Microbiology and Immunology, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657-8501, Japan

[2)]Research Department, Nippon Institute for Biological Science, 9-2221-1 Shinmachi, Ome, Tokyo 198-0024, Japan

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ABSTRACT. Elucidation of the processes regulating the prion protein gene (*Prnp*) is an important key to understanding the development of prion disorders. In this study, we explored the involvement of DNA methylation in *Prnp* transcriptional regulation during neuronal differentiation of embryonic carcinoma P19C6 cells. When P19C6 cells were differentiated into neuronal cells, the expression of *Prnp* was markedly increased, while CpG methylation was significantly demethylated at the nucleotide region between −599 and −238 from the transcription start site. In addition, when P19C6 cells were applied in a DNA methyltransferase inhibitor, RG108, *Prnp* transcripts were also significantly increased in relation to the decreased methylation statuses. These findings helped to elucidate the DNA methylation-mediated regulation of *Prnp* expression during neuronal differentiation.

KEY WORDS: CpG methylation, epigenetics, neuronal differentiation, prion protein gene

The expression of the prion protein gene (*Prnp*) that is encoded by mammalian chromosomes is highly regulated during development [14, 17] and plays critical roles in the neurodegeneration of prion disorders [21]. Although *Prnp* is abundantly expressed in neurons, the epigenetic regulation including DNA methylation on *Prnp* during neuronal differentiation is still unknown. Methylation of cytosines is an essential epigenetic modification in mammalian genomes, which influences gene expression during development and disease [11]. Cytosine methylation occurs mostly in presence of CpG dinucleotides and is low at the promoter regions. Recently, we reported that the *Prnp* CpG island (CGI, nucleotide positioned at −218 to +152 from the transcriptional start site) including the putative core promoter region (−62 to −1) was completely unmethylated in adult mouse tissues [8]. Furthermore, the 5′-shore region (−599 to −238) from CGI showed tissue- and site-specific methylation frequency patterns so-called "differentially methylated regions, DMRs" [10, 18, 20]. In this study, we explored the DNA methylation changes of *Prnp* in P19C6 cells during neuronal differentiation as well as the methylation patterns in several mouse cell lines.

Mouse neuroblastoma Neuro-2a, hepatoma HePa1-6 and macrophage RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific, Wilmington, DE, U.S.A.) in a humidified incubator with 5% CO_2 at 37°C. Neuroblastoma C-1300 and B cell lymphoma WEHI-231 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI1640, Nacalai) supplemented with 10% FBS. Embryonal carcinoma P19C6 cells were obtained from RIKEN Bioresource Center Cell Bank (Tsukuba, Japan) and maintained in DMEM supplemented with 15% FBS. All cells were plated at 5×10^5 cells/100-mm cell culture dish and grown for 48 hr before the extraction of genomic DNA and total RNA.

Mouse neuroblastoma Neuro-2a cells are wildly used as a model for finding prion properties, because this cell line is a suitable host for infection with prions [4]. However, DNA methylations on *Prnp* in clonal cell lines have little understanding. Herein, we report epigenetic studies with several different mouse cell lines. To investigate the methylation frequency of individual CpG sites in *Prnp*, we performed bisulfite sequencing PCR and the calibration of each CpG methylation status according to the procedure described previously [8]. The targeted region (−599 to +286) contains a total of 46 CpG sites numbered CpG 1 to CpG 46, and the CGI spans between nucleotides −218 and +152 contained 32 CpG sites (CpGs 9 to 40) (Fig. 1). The *Prnp* first exon and putative

*Correspondence to: Saeki, K., Laboratory of Microbiology and Immunology, Graduate School of Agriculture, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657-8501, Japan. e-mail: Saeki@pegasus.kobe-u.ac.jp

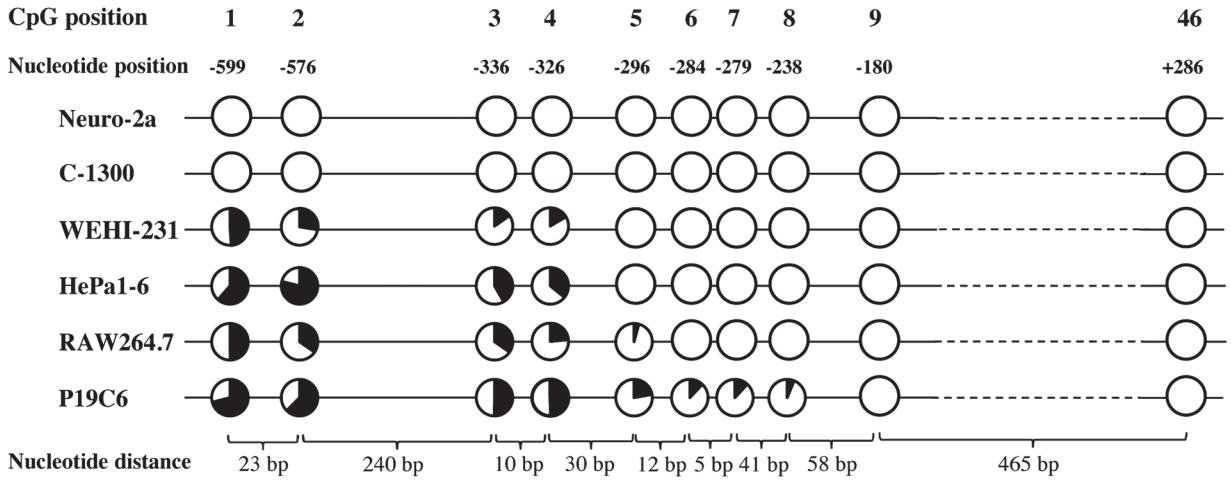

Fig. 1. Methylation frequencies of *Prnp* in mouse cultured cells. The methylation percentage at each CpG position (1 to 46) is shown by the black region on pie charts and represents the average of eight independent experiments. Pie charts of CpG sites 10 to 45 are not indicated, because of unmethylated sites. Nucleotide positions are numbered in association with the transcription initiation site of the *Prnp* gene. Nucleotide distances between CpGs are shown by base pairs (bp).

core promoter region are localized in the nucleotide regions from +1 to +67 (CpGs 25 to 33) and −62 to −6 (CpGs 20 to 24), respectively [2]. The methylation analysis of the 46 different CpG sites showed that CpGs 9 to 46 (−180 to +286) were completely unmethylated in all cell lines, whereas CpGs 1 to 8 (−599 to −238) were differentially methylated depending on the cultured cells. Methylation was completely absence in CGI (CpGs 9 to 40) including the putative core promoter region (CpGs 20 to 24) in all cell lines in addition to the results from mouse tissues, demonstrating that *Prnp* was a typical house-keeping gene [8, 11]. Moreover, the mouse neuroblastoma cell lines, Neuro-2a and C-1300, were hypomethylated even at CpGs 1 to 8. In WEHI-231, HePa1-6, RAW264.7 and P19C6 cells, the partial methylation levels at CpGs 1 to 4 were in the range of 49–71% (CpG 1), 28–79% (CpG 2), 15–50% (CpG 3) and 17–49% (CpG 4), respectively. At CpG 5, RAW264.7 and P19C6 cells had methylation frequencies of 4.2 ± 1.0% and 22.2 ± 0.8%, respectively. In P19C6 cells, additional CpG site methylations were observed at CpG 6 (12.1 ± 0.3%), CpG 7 (11.8 ± 0.5%) and CpG 8 (6.0 ± 0.6%). The methylome demonstrated in mouse pluripotent embryonic stem cells and in neuronal progenitors reveals that DMRs or low-methylation regions are defined by locally reduced levels of methylation in the range of 10–50% [20]. In addition, this characteristic shows 4.1% of all CpGs in the mouse genome and is not randomly distributed but clustered locally. Moreover, a large number of DMRs are found within the enhancer regions [11, 18, 20]. Our observations suggest that the region between −599 and −238 is one of DMRs and may share a putative enhancer/repressor region to lead *Prnp* regulation of cell-type-specific expression.

To analyze the correlation between CpG site methylation and *Prnp* expression, we performed quantitative real-time RT-PCR (qRT-PCR) using the total RNA from each cell sample according to the procedure as described previously [8]. The highest level of *Prnp* mRNA was found in Neuro-2a (9.3 ± 2.6) followed by C-1300 (7.3 ± 2.2), WEHI-231 (1.6 ± 1.5), Hepa1-6 (0.7 ± 0.3), RAW264.7 (0.5 ± 0.6) and P19C6 (0.5 ± 0.3) (Fig. 2A). However, *Prnp* expression in these cell lines was much lower than in an 8-week-old normal mouse cerebrum (=100.0). We performed Pearson's correlation analysis based on these results from qRT-PCR and the methylation statuses for individual CpG sites (Table 1). The analysis revealed that cell-specific expressions of *Prnp* were inversely correlated to the levels of methylation at CpG positions from 1 to 4 ($r > -0.3$ and $P<0.05$), but no significant correlations were observed at CpGs 5 to 8 ($P>0.05$). Our previous study demonstrated that the methylation at only the CpG 2 site localized in the N-box sequence (CACGAG) following an inverted CTCF insulator element (GAGGG) was negatively correlated with *Prnp* expressions in normal mouse tissues [8]. To define more precisely which CpG site plays most important role in *Prnp* expression in cultured cells, a greater variety of cell lines should be analyzed and classified according to each cell line, because the aberrational methylations at promoters and enhancers are caused by various tumor cells [1, 11, 16].

To better understand the relation between *Prnp* expression and CpG methylation during neuronal differentiation, we investigated the alteration of CpG methylation in differentiated and undifferentiated P19C6 cells. P19C6 cells were subcloned from the pluripotent embryonal carcinoma cells, P19, which have been used as a model of neurogenesis [9, 12]. P19C6 cells were differentiated into neuronal cells in the presence of all-*trans*-retinoic acid (ATRA) as described by Gao and co-workers [9]. To induce differentiation, cells were seeded at a density of 3×10^5 cells/90-mm bacterial grade dish (Koryo Chemical, Nara, Japan) in alpha minimal essential medium (αMEM, Nacalai) supplemented with 10% FBS and 1.0 μM ATRA (Sigma-Aldrich, St. Louis, MO, U.S.A.) and were allowed to aggregate. After 4 days, aggregated cells were treated by 0.25% Trypsin/1 mM EDTA (Nacalai) and were replated in a culture dish coated by ploy-L-lysine (Iwaki, Shizuoka, Japan) at a density of 4×10^5 cells/60-mm dish in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA, U.S.A.) containing N-2 supplement (Thermo Fisher Scientific) and 1 μg/m*l* fibronectin (Thermo Fisher Scientific). The cells were then cultured for 7 days with replacement of the medium every 2 days.

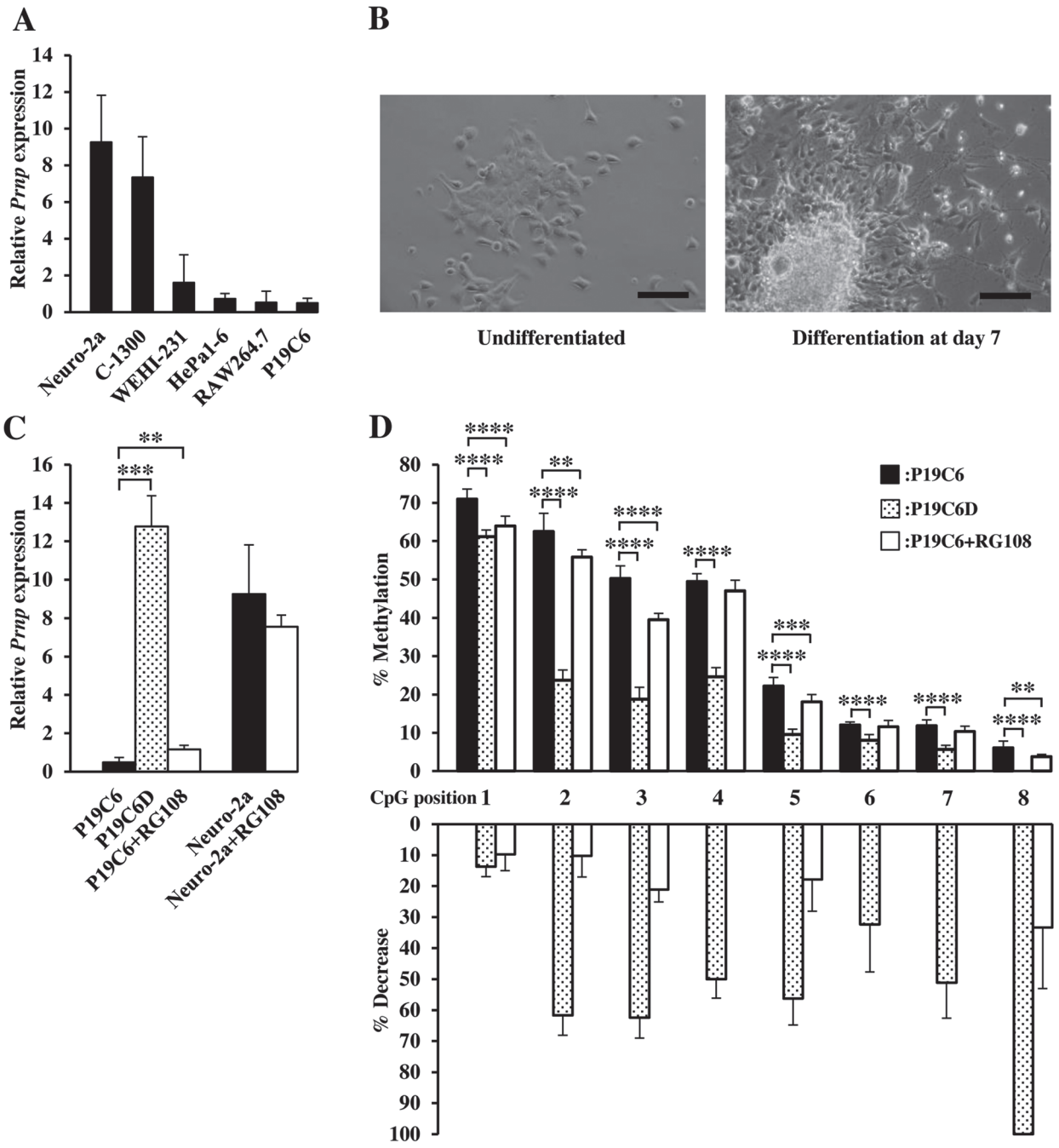

Fig. 2. (A)*Prnp* gene expression in each cell line. The *Prnp* mRNA level in the cerebrum is presented as 100. Each value represents the mean ± SD (n=3). (B) P19C6 cell morphology. P19C6 cells were differentiated into neuronal cells in DMEM/F12 medium containing N-2 supplement and 1 μg/m*l* fibronectin. The cells were then cultured for 7 days with the medium replaced every 2 days. Bar scale: 100 μm. (C) *Prnp* expression in untreated (P19C6 and Neuro-2a), differentiated (P19C6D) and RG108-treated cells (P19C6+RG108 and Neuro-2a+RG108). Each value represents the mean ± SD (n=3). Statistical analysis was performed using Student's *t*-test for 2-group comparisons. Asterisks indicate statistical significance (***P*<0.01, ****P*<0.001). (D) Methylation frequency of *Prnp* in untreated (P19C6), differentiated (P19C6D) and RG108-treated cells (P19C6+RG108). Each methylation percentage at CpG positions (1 to 8) is shown as the mean ± SD (n=8). The percentage decrease was calculated relative to the values of untreated cells. Values were analyzed using the one-way ANOVA with Tukey-Kramer post-hoc test for 3-group comparisons. Asterisks represent statistical significance (***P*<0.01, ****P*<0.001, *****P*<0.0001).

P19C6 cells extended neurite projections within 7 days before the extraction of genomic DNA and total RNA (Fig. 2B). In differentiated P19C6 cells (P19C6D), the *Prnp* mRNA level was dramatically increased by 26-fold compared to that in undifferentiated cells (Fig. 2C). In the meantime, CpG methylations after differentiation were significantly reduced at CpGs 1 to 8 (Fig. 2D). Demethylations at CpGs 2–5, 7 and 8 were particularly marked with decreases of more than 50%. The

Table 1. Correlations between each CpG methylation and *Prnp* expression

	CpG position							
	1	***2***	***3***	***4***	5	6	7	8
Pearson's *r*-values	***–0.963***	***–0.837***	***–0.882***	***–0.848***	–0.428	–0.354	–0.354	–0.354
P-value	***0.002***	***0.038***	***0.020***	***0.033***	0.398	0.491	0.491	0.491

Pearson's *r*-values ≥ ± 0.3 and *P*-values ≤0.05 are considered to indicate significant correlations. CpGs 1 to 4, where methylation was negatively correlated with *Prnp* expression, are indicated in bold and italic.

unmethylated status at positions CpGs 9 to 46 remained unchanged. The normal cellular isoform of the prion protein, PrP^C, is produced beginning in the early stages of embryonic stem cell differentiation, suggesting its participation in cell pluripotency and neurogenesis [13, 15, 17, 19]. Our observations might support the active involvement of CpG demethylations in up-regulating expression of *Prnp* during neuronal differentiation.

To further investigate the role of demethylation on *Prnp* expression, P19C6 and Neuro-2a cells were treated with DNA methyltransferase inhibitor, RG108 [3]. Before the extraction of genomic DNA and total RNA, the cells were plated at 5×10^5 cells/100-mm cell culture dish and grown for 48 hr in the presence of 0.1 mM RG108 (Abcam plc, Cambridge, U.K.). We observed a 2.4-fold increase in *Prnp* transcripts in RG108-treated P19C6 cells (P19C6+RG108), whereas a DNA demethylating agent had no impact on *Prnp* expression in Neuro-2a cells (Neuro-2a+RG108) (Fig 2C). Furthermore, the results of methylation analysis in P19C6+RG108 cells showed that the demethylations significantly occurred at CpGs 1–3, 5 and 8 with 9.8%, 10.3%, 21.1%, 17.9% and 33.4% decreases, respectively (Fig. 2D). On the other hand, the unmethylated status at CpGs 1 to 46 in Neuro-2a stayed unchanged against RG108 (data not shown). These results suggest that *Prnp* expression remains unaffected by DNA methyltransferase inhibitor under the complete lack of methylation at the 5′-shore region of CGI, such as in Neuro-2a cells. Together with our results from the differentiation experiment, these results imply that the methylations at nucleotide positions −599 to −238 play an important role in *Prnp* regulation.

Cabral and co-workers [5] demonstrated that the histone deacetylase inhibitor, trichostain A, highly increased *Prnp* expression and PrP^C levels in rat PC-12 and C6 cells, indicating that *Prnp* regulation depends on chromatin conformation. Although the crosstalk control between DNA methylation and histone modification is involved in establishing patterns of gene expression, the mechanisms regulating DNA methylation at promoters and enhancers are largely unknown [6, 7]. In general, enhancers/repressors are situated at variable distances from the promoter and tend to have DMRs, but the exact role of DNA methylation at enhancers/repressors still needs to be elucidated [6, 7, 11].

Using the quantification of DNA methylation, we identified the epigenetic relationship between DNA methylation on CpGs and *Prnp* gene expression in cultured cells. Furthermore, this study is the first to describe reduced levels of methylation on *Prnp* DMR including a possible enhancer region during neuronal differentiation. Although DNA-binding factors and histone modifications in the *Prnp*-regulatory region must still be identified, our findings provide novel information for the epigenetics of the *Prnp* gene and could contribute to elucidating the physiological roles of PrP^C and the molecular pathogenesis of prion diseases.

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