J Hum Genet (2008) 53:185–191
DOI 10.1007/s10038-007-0228-0

SHORT COMMUNICATION

Genome-wide demethylation during neural differentiation of P19 embryonal carcinoma cells

Izuho Hatada · Sumiyo Morita · Mika Kimura · Takuro Horii · Riu Yamashita · Kenta Nakai

Received: 24 July 2007 / Accepted: 11 November 2007 / Published online: 17 January 2008

Abstract Epigenetic regulation including DNA methylation plays an important role in several differentiation processes. We profiled global DNA methylation in the neural differentiation of P19 embryonic carcinoma cells using a microarray-based method called MIAMI. We found a genome-wide demethylation of genes. This suggests demethylation rather than methylation is important in neural differentiation.

Keywords Methylation · Epigenetics · Demethylation · Genome-wide · Profiling

Introduction

5-Methylcytosine is the only covalent DNA modification known in vertebrates (Jeltsch 2002). This epigenetic modification is essential for differentiation, embryonic development (Li et al. 1992), genomic imprinting (Li et al. 1993), and X-chromosome inactivation (Heard et al. 1997). Changes of DNA methylation are also important in human diseases, including cancer (Egger et al. 2004).

In mammals, DNA methylation mainly occurs at CpG dinucleotides, with approximately 60–90% of cytosines at these sites methylated (Razin et al. 1984). CpG-rich DNA fragments, or CpG islands (Bird 1987), are preferentially located at the transcription start site of genes. It has been thought that most CpG islands remain unmethylated, even in cell types that do not express genes (Bird 2002). However, changes in DNA methylation linked to tissue-specific gene expression have been seen sporadically in CpG-rich promoters (Ohgane et al. 1998; Song et al. 2005).

Previously, we developed a genome-wide DNA methylation analysis called MIAMI using a microarray (Hatada et al. 2006). With this method, we detected DNA methylation using the methylation-sensitive restriction enzyme *Hpa*II and its methylation-insensitive isoschizomer *Msp*I. Although *Hpa*II cleavage differences are usually related to methylation differences of two samples, restriction-site polymorphisms and/or incomplete digestion of one of the sample DNA results in false positives. We utilized methylation-insensitive restriction enzyme *Msp*I to judge the false positive results for *Hpa*II cleavage differences. If two samples have a restriction site polymorphism at a *Hpa*II site and/or one of the samples has incomplete digestion at a *Hpa*II site, they will differ in *Hpa*II cleavage. However, in this case, the methylation-insensitive *Msp*I cleavage at this site will also differ between samples because both enzymes recognize the same recognition site: CCGG. Therefore, we can treat such changes as false positives based on *Msp*I cleavage difference. We detected both *Hpa*II and *Msp*I cleavage difference by polymerase chain reaction (PCR) amplification of both *Hpa*II and *Msp*I cleavable DNA. Detection of both *Hpa*II and *Msp*I cleavage difference was

Electronic supplementary material The online version of this article (doi:10.1007/s10038-007-0228-0) contains supplementary material, which is available to authorized users.

I. Hatada (✉) · S. Morita · M. Kimura · T. Horii
Laboratory of Genome Science, Biosignal Genome Resource Center, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-machi, Maebashi 371-8512, Japan
e-mail: ihatada@showa.gunma-u.ac.jp

R. Yamashita · K. Nakai
Laboratory of Functional Analysis in silico, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

performed using two identical oligonucleotide microarrays made by Agilent inkjet technology.

Both DNA methylation and demethylation occur during development (Kremenskoy et al. 2003). However, it is unclear which is important in differentiation. To clarify this, we studied DNA methylation in a model of neural differentiation using P19 embryonic carcinoma cells. These cells differentiate into neural cells when exposed to retinoic acids (Runnicki and Mcbruney 1987). To study the changes in DNA methylation with neural differentiation, we profiled the methylation changes during neural differentiation of P19 cells using MIAMI.

Materials and methods

Methylation profiling by MIAMI

The MIAMI method was performed using 1 μg of genomic DNA as previously described (Hatada et al. 2006). The complete experimental procedure can be obtained at http://grc.dept.med.gunma-u.ac.jp/~gene/image/MIAMI%20Protocol%20V4.pdf. The analysis of microarray data was improved by using a new threshold to remove spots and employing an additional model for judging changes in methylation. The previous threshold used was 0.001% for the sum of all the spot signals. This definition cannot be applied to microarrays with different numbers of spots. Therefore, we adopted a new definition: a fixed ratio of the average of the larger half of all spot signals. This definition is applicable to microarrays with different spot numbers. The reason we do not use a fixed ratio of the average of all spot signals is that it results in too low a threshold when there are many spots with weak signals. We used 5% of the average of the larger half of all spot signals and found that it worked well (Fig. 1, and data not shown).

For judging changes in methylation, we used the difference in methylation-sensitive *Hpa*II cleavage and methylation-insensitive *Msp*I cleavage between the samples. Previously, we only used a model in which spots with methylation changes had different values for *Hpa*II and *Msp*I cleavage. However, this model had the risk of false positives in cases of large *Msp*I cleavage differences. So we also adopted an additional model in which spots with methylation changes have a large *Hpa*II cleavage difference (more than five) and a small *Msp*I cleavage difference (less than two). We confirmed that the spots fit both models and found that all 15 spots that fit the models actually had methylation changes (Fig. 1, and data not shown). The reproducibility of the experiment was analyzed with 885 triplicated probes. These triplicated probes showed high R values ranging from 0.96 to 0.99.

Bisulfite genomic analysis

Bisulfite treatment of genomic DNA was performed using a CpGenome DNA modification kit (INTERGEN). The modified DNA was amplified with the primers described in electronic supplementary material (ESM) 1.

Combined bisulfite restriction analysis (COBRA)

Combined bisulfite restriction analysis (COBRA) was performed as described (Xiong et al. 1997). PCR primers and restriction enzymes used were described in ESM 1. DNA fragments were separated on a 5% polyacrylamide gel.

Real-time quantitative PCR

Real-time quantitative PCR was performed using CYBR Green system using ABI PRISM 7700 (Applied Biosystems). The primers used are described in ESM 1.

Expression microarray analysis

Expression microarray analysis was performed using the Agilent mouse whole genome array and the procedure provided by Agilent technologies. A signal ratio of more than two with a P value of less than 0.01 was judged as upregulated. A signal ratio of less than 0.5 with a P value of less than 0.01 was judged as downregulated.

Neuronal differentiation of P19 cells

The P19 EC cells were monodispersed and seeded at a density of 10^5 cells/ml in medium containing 0.3 μM retinoic acid in 60 mm bacteriological grade Petri dishes. After 4 days, the aggregates were transferred into 100 mm tissue-culture-grade Petri dishes in medium without retinoic acid. After 2 days, cells were collected.

Gene ontology (GO) annotation

The information on gene ontology (GO) (Harris et al. 2004) was obtained from the loc2go table in the National Center for Biotechnology Information (NCBI). The GO slim information in the European Bioinformatics Institute (EBI) was used to simplify the annotation by obtaining top-level GO terms. We calculated a P value for each term when a hypergeometric distribution was assumed.

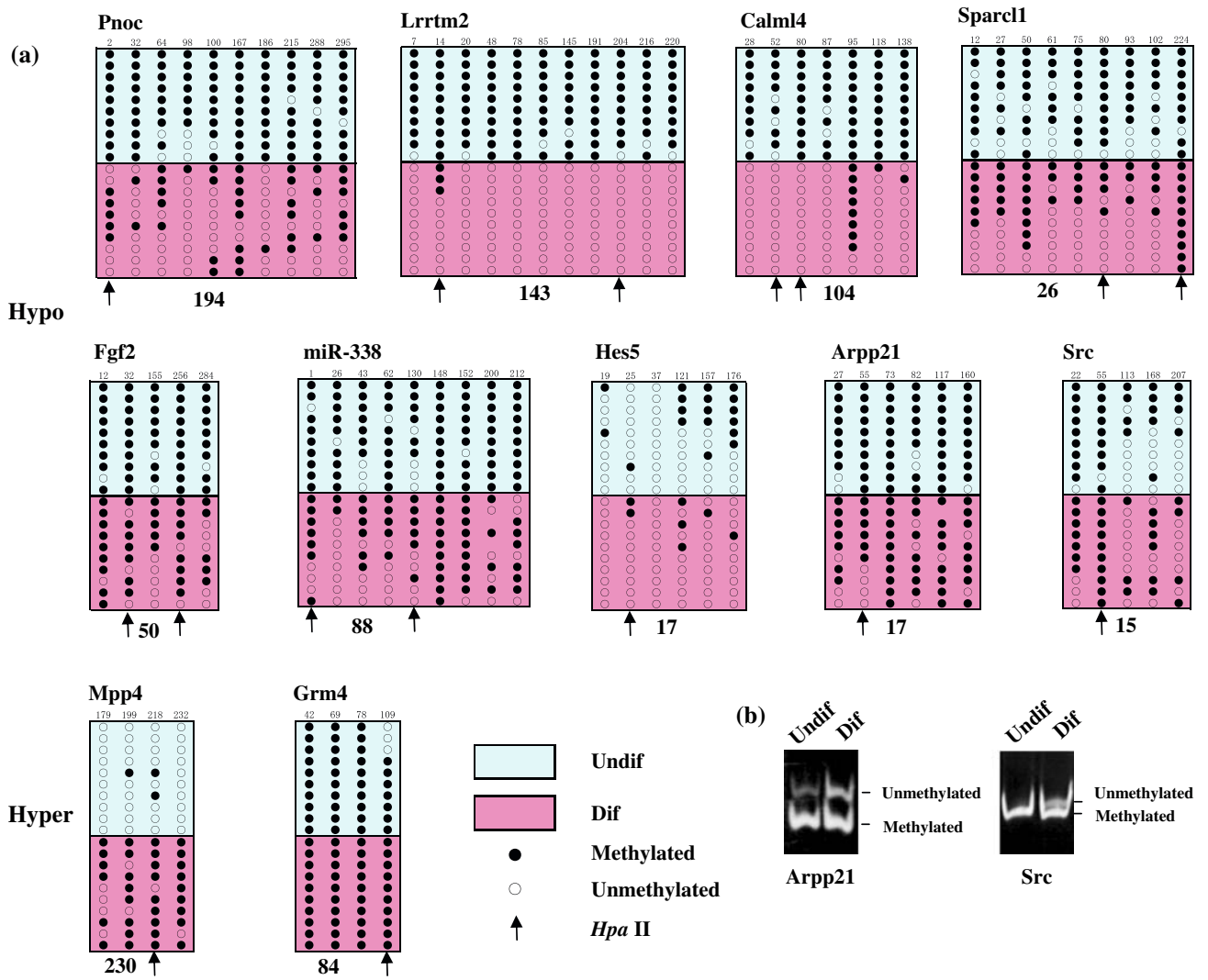

Fig. 1 **a** Bisulfite sequencing of nine demethylated genes in undifferentiated and differentiated P19 cells. *Closed circles* indicate methylated CpG, and *open circles* indicate unmethylated CpG. *Circles* in the *blue* areas indicate methylation of undifferentiated P19 cells, and *circles* in the *pink* areas indicate methylation of differentiated P19 cells. *Hpa*II sites are indicated by *arrows*. Higher *Hpa*II intensity of each gene (Cy3 for hypermethylated genes and Cy5 for hypomethylated genes) is indicated in the *bottom* of the bisulfite sequence pattern. **b** Combined bisulfite restriction analysis (COBRA) analysis of *Hpa*II sites of Arpp21 and Src

Results

Genome-wide profiling of changes in DNA methylation during the neuronal differentiation of P19 cells

Changes in methylation during neuronal differentiation were analyzed by comparing undifferentiated and differentiated P19 cells using the MIAMI method. The microarray used consisted of probes chosen from the Agilent promoter array using an eArray system (http://earray.chem.agilent.com/earray/). The probes are located on *Hpa*II fragments of less than 1 kb and cover 14,543 genes. The changes mostly involved demethylation in differentiated P19 cells: 414 probes (390 genes) were demethylated, and ten probes (ten genes) were hypermethylated in differentiated P19 cells (Fig. 2a, b, ESM 2). Reliability of the analysis was examined for nine hypomethylated genes with various *Hpa*II signal intensities by bisulfite sequencing (Fig. 1a) and six hypomethylated genes by COBRA (data not shown). All genes except for Arpp21 and Src clearly showed demethylation. Demethylation levels of Arpp21 and Src seems not to be obvious by bisulfite sequencing method; however, slight demethylation of these genes could be confirmed by COBRA (Fig. 1b). *Hpa*II signal intensities of Arpp21 and Src are closed to the threshold level (indicated in the bottom of bisulfite sequence pattern). One the other hand, those of other genes such as Lrrtm2 and Calml4 are high. As shown in Fig. 1a, the extent of methylation changes seems to be related to the *Hpa*II signal intensity of each gene. This could mean that the reliability of the data

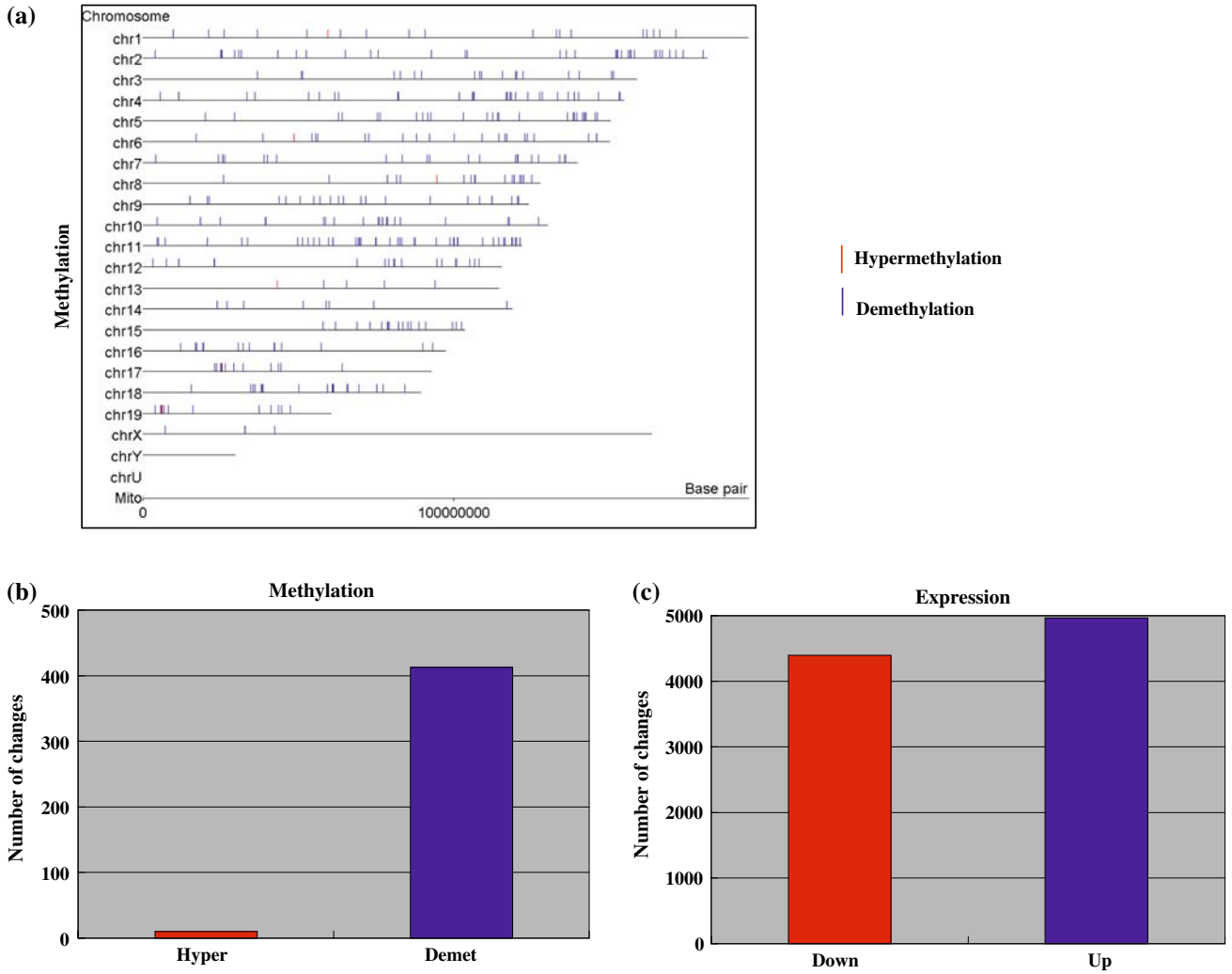

Fig. 2 Difference in DNA methylation and expression between undifferentiated and differentiated P19 cells. **a** Chromosomal location of the changes in methylation in differentiated P19 cells. *Red bars* indicate hypermethylation, and *blue bars* indicate demethylation. **b** Methylation during neural differentiation analyzed by MIAMI. The number of hypermethylated and demethylated sequences in differentiated P19 cells compared with undifferentiated P19 cells. **c** Expression during neural differentiation analyzed with a microarray. The number of downregulated and upregulated genes in differentiated P19 cells compared with undifferentiated P19 cells

depends on the signal intensity. Thus, we concluded that 15 of the 15 hypomethylated genes actually have methylation changes. We also performed bisulfite sequencing the hypermethylated genes and found the methylation status showed good agreement with that of MIAMI results. To see whether the CpG methylation changes in *Hpa*II and non-*Hpa*II sites are different or not, we calculated the number of changes in both sites and found that there is no difference between them ($P = 0.96$).

The correlation between demethylation and upregulation of expression of the nine genes was analyzed by real-time quantitative PCR and with the expression microarray (Fig. 3). A microRNA, miR-338, could not be used for this experiment because the expression microarray we used did not contain this gene. The real-time quantitative PCR analysis revealed all of the nine demethylated genes to be upregulated in differentiated P19 cells (Fig. 3a). Expression microarray analysis failed to show upregulation of three genes (Fig. 3b). This discrepancy can be explained by the detection limit of the expression microarray because two demethylated genes whose upregulation was not detected in the expression microarray analysis had weak signals (Fig. 3b).

Expression changes during neuronal differentiation of P19 cells

Changes in expression during neuronal differentiation were analyzed by comparing undifferentiated and differentiated P19 cells in an expression microarray analysis (Fig. 2c, ESM 3). Neuron-specific genes, such as Map2 and Tubb3,

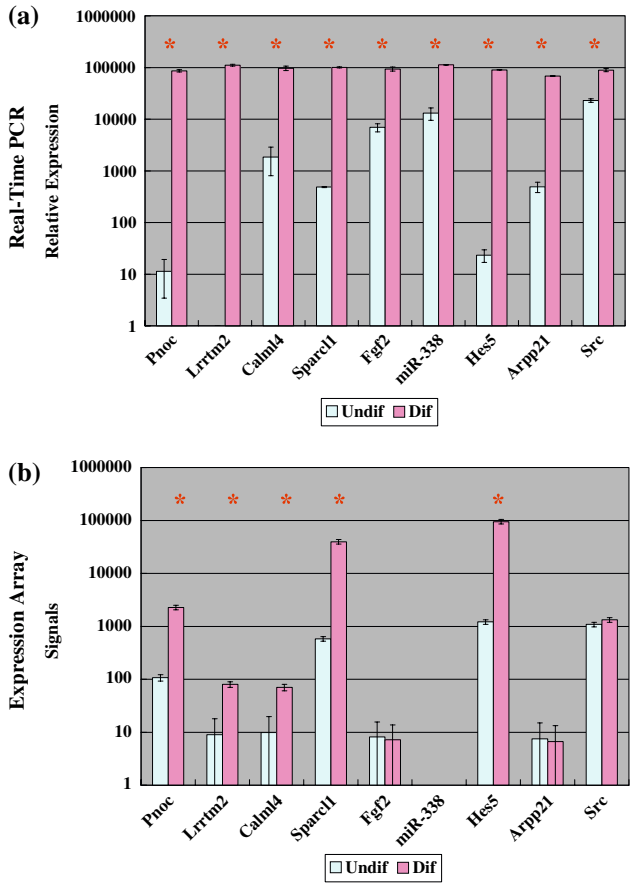

Fig. 3 Expression of nine demethylated genes in differentiated P19 cells analyzed using real-time polymerase chain reaction (PCR) or the expression microarray. *Blue bars* indicate undifferentiated P19 cells, and *pink bars* indicate differentiated P19 cells. **a** Real-time PCR analysis. Gapdh was used for standardization of the results. *P* values less than 0.01 are indicated by *asterisks*. **b** Expression microarray analysis. *P* values less than 0.01 are indicated by *asterisks*

were upregulated in differentiated P19. On the other hand, an astrocyte-specific gene, Gfap, was not upregulated. Thus, neuronal differentiation of P19 cells was confirmed. In contrast with the methylation analysis, upregulation and downregulation were equally observed. The correlation between demethylation and upregulation at various positions was highest in the demethylated sequence located near the transcription start site (Fig. 4). Probes located between 0 and 1,000 base pairs from the transcription start sites showed a significant *P* value ($P = 0.01$). Thus, demethylation near the transcription start site is better correlated to changes in expression.

Ontology of demethylated genes in differentiated P19 cells

An ontology-based analysis of demethylated genes using 4,057 GO terms revealed no significant enrichment of any

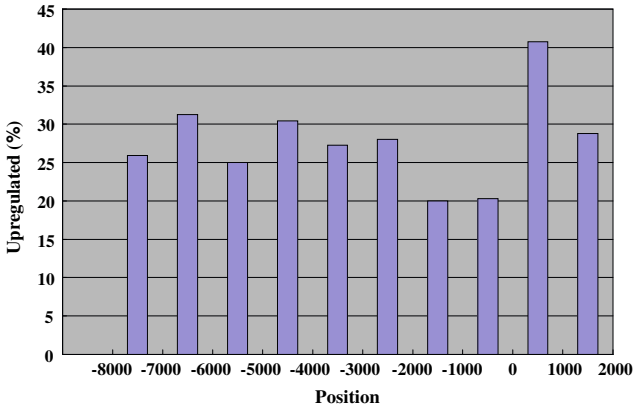

Fig. 4 Correlation of demethylated sequences in differentiated P19 cells with the position from the transcription start site

terms; however, "intercellular junction" was relatively overrepresented, and "nucleus" was relatively underrepresented (Table 1). Next, we used the annotation GO slim, which is a cutdown version of the GO ontology containing only a subset of the terms. It gives a broad overview of the ontology without details of specific terms. The analysis using 74 GO slim terms revealed no significant overrepresentation; however, terms such as "membrane" and "signal transducer activity" were relatively overrepresented (Table 1). On the other hand, terms such as "nucleus" and "metabolism" were significantly underrepresented. Taken together, terms related to the interaction between cells were relatively overrepresented and terms related to housekeeping processes such as metabolism were significantly underrepresented.

Discussion

A genome-wide comparison of DNA methylation between undifferentiated and differentiated P19 embryonal carcinoma cells revealed that most changes involved demethylation (Fig. 2b). We also observed global demethylation and only a few hypermethylated genes during neural differentiation of P19 cells by a methylcytosine antibody-based microarray method (unpublished data). Thus, our results were confirmed by another method. These suggest that DNA demethylation rather than methylation is important for neural differentiation. Some of the demethylated genes found in our study could have important roles in inducing neural differentiation or maintaining the neural phenotype (ESM 2). Embryonal carcinoma cells are thought to resemble methylation status at the blastula stage, which is followed by the differentiation stage with global demethylation (Yeivin et al. 1996). From this point of view, it is reasonable that embryonal carcinoma cells are globally demethylated during differentiation.

Table 1 Overrepresented and underrepresented gene ontology (GO) and GO slim terms

GO all	*P* value
Overrepresented	
GO term	
Intercellular junction	7.95E-04
Underrepresented	
GO term	
Nucleus	4.07E-05
GO slim	
Overrepresented	
GO term	
Membrane	1.44E-02
Signal transducer activity	2.27E-02
Enzyme regulator activity	2.48E-02
Underrepresented	
GO term	
Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism	9.83E-06*
Nucleus	2.42E-05*
Cellular physiological process	6.60E-05*
Metabolism	1.47E-04*
Physiological process	1.59E-04*

* Significant with Bonferroni correction

On the other hand, the expression microarray analysis revealed numerous genes whose expression was upregulated or downregulated (Fig. 2c). This discrepancy could be explained by the fact that the number of changes in expression was ten times larger. Also, the upregulated genes slightly outnumbered the downregulated genes, suggesting that this difference contributes to the upregulated demethylated genes. Therefore, most genes are not regulated by DNA methylation but by other mechanisms. However, the small number of genes that are regulated by DNA methylation might be more stable in terms of expression and play an important role in differentiation because they include relatively many in signal transducer genes, which might play an important role in differentiation (Table 1).

Comparison of the expression of demethylated genes using real-time PCR and expression microarray revealed that the latter was unable to detect changes in the expression of some demethylated genes, whereas real-time PCR detected the upregulated expression of all genes examined (Fig. 3). The methylation microarray analysis could be better for detecting changes in low-expression genes than the expression microarray analysis. In fact, no upregulated gene discovered with the expression microarray showed a signal of less than 10; however, 17% of the demethylated genes in the differentiated P19 cells showed a signal of less than 10 in the expression microarray analysis (data not shown). This advantage could be valuable in detecting changes in upstream regulator genes whose expression is weak or human samples such as cancers whose mRNA is sometimes degraded.

Genes involved in the interaction between cells were relatively overrepresented, whereas housekeeping genes were significantly underrepresented (Table 1). It is reasonable that housekeeping genes are underrepresented because they function in every cell all the same time. It is also reasonable that genes involved in the interaction between cells are relatively overrepresented because interaction via signal transducer molecules is an important aspect of differentiation.

Although term cell differentiation was not overrepresented in GO analysis, several genes belonging to this category were found to be demethylated in differentiated P19 cells. Some of these genes, such as Ctnb1 and Fyn, are known to be involved in differentiation of neurons. Beta-catenin (Ctnnb1) signaling is required for neural differentiation of embryonic stem cells (Otero et al. 2004). Fyn tyrosine kinase is involved in regulation of dendritic branching and spine maturation by semaphorin3A-Fyn signaling pathway. This gene is also required for migration of cortical neurons through the Reelin signaling pathway (Yuasa et al. 2004).

In conclusion, genome-wide demethylation was observed during the neural differentiation of P19 embryonal carcinoma cells, suggesting that demethylation rather than methylation is important to the differentiation process. Overrepresented gene criteria in demethylated sequences, such as signal transducers, could have important roles in neural differentiation and maintaining the neural phenotype.

Acknowledgments This study was supported in part by grants from the Japanese Science and Technology Agency, the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labor, and Welfare of Japan.

References

Bird AP (1987) CpG islands as gene markers in the vertebrate nucleus. Trends Genet 3:342–347

Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16:6–21

Egger G, Liang G, Aparicio A, Jones PA (2004) Epigenetics in human disease and prospects for epigenetic therapy. Nature 429:457–463

Harris MA, Clark J, Ireland A, Lomax J, Ashburner M, Foulger R, Eilbeck K, Lewis S, Marshall B, Mungall C, Richter J, Rubin GM, Blake JA, Bult C, Dolan M, Drabkin H, Eppig JT, Hill DP, Ni L, Ringwald M, Balakrishnan R, Cherry JM, Christie KR, Costanzo MC, Dwight SS, Engel S, Fisk DG, Hirschman JE,

Hong EL, Nash RS, Sethuraman A, Theesfeld CL, Botstein D, Dolinski K, Feierbach B, Berardini T, Mundodi S, Rhee SY, Apweiler R, Barrell D, Camon E, Dimmer E, Lee V, Chisholm R, Gaudet P, Kibbe W, Kishore R, Schwarz EM, Sternberg P, Gwinn M, Hannick L, Wortman J, Berriman M, Wood V, de la Cruz N, Tonellato P, Jaiswal P, Seigfried T, White R (2004) The Gene Ontology (GO) database and informatics resource. Nucleic Acids Res 32:D258–D261

Hatada I, Fukasawa M, Kimura M, Morita S, Yamada K, Yoshikawa T, Yamanaka S, Endo C, Sakurada A, Sato M, Kondo T, Horii A, Ushijima T, Sasaki H (2006) Genome-wide profiling of promoter methylation in human. Oncogene 25:3059–3064

Heard E, Clerc P, Avner P (1997) X-chromosome inactivation in mammals. Annu Rev Genet 31:571–610

Kremenskoy M, Kremenska Y, Ohgane J, Hattori N, Tanaka S, Hashizume K, Shiota K (2003) Genome-wide analysis of DNA methylation status of CpG islands in embryoid bodies, teratomas, and fetuses. Biochem Biophys Res Commun 311:884–890

Jeltsch A (2002) Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. Chembiochem 3:274–293

Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69:915–926

Li E, Beard C, Jaenisch R (1993)Role for DNA methylation in genomic imprinting. Nature 366:362–365

Ohgane J, Aikawa J, Ogura A,Hattori N, Ogawa T, Shiota K (1998) Analysis of CpG islands of trophoblast giant cells by restriction landmark genomic scanning. Dev Genet 22:132–140

Otero JJ, Fu W, Kan L, Cuadra AE, Kessler JA (2004) Beta-catenin signaling is required for neural differentiation of embryonic stem cells. Development 131:3545–3557

Razin A, Webb C, Szyf M, Yisraeli J, Rozanthal A, Naveh-Many T, Sciaky-Gallili N Cedar H (1984) Variation in DNA methylation during mouse cell differentiation in vivo and in vitro. Proc Natl Acad Sci USA 81:2275–2279

Runnicki, Mcbruney (1987) Cell culture methods and induction of differentiation of embryonal carcinoma cell line. In: Roberson EJ (ed) Teratocarcinomas and embryonic stem cells. IRL Press, pp 19–49

Song F, Smith JF, Kimura MT, Morrow AD, Matsuyama T, Nagase H, Held WA (2005) Association of tissue-specific differentially methylated regions (TDMs) with differential gene expression. Proc Natl Acad Sci USA 102:3336–3341

Xiong Z, Laird PW (1997). COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 25:2532–2534

Yeivin A, Levine A, Razin A (1996) DNA methylation patterns in tumors derived from F9 cells resemble methylation at the blastula stage. FEBS Lett 395:11–16

Yuasa S, Hattori K, Yagi T (2004) Defective neocortical development in Fyn-tyrosine-kinase-deficient mice. Neuroreport 15:819–822