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Demethylating agent, 5-azacytidine, reverses differentiation of embryonic stem cells☆

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Abstract

The de novo methylation activity is essential for embryonic development as well as embryonic stem (ES) cell differentiation, where the intensive and extensive DNA methylation was detected. In this study, we investigated the effects of a demethylating agent, 5-azacytidine (5-AzaC), on differentiated ES cells in order to study the possibility of reversing the differentiation process. We first induced differentiation of ES cells by forming embryoid bodies, and then the cells were treated with 5-AzaC. The cells showed some undifferentiated features such as stem cell-like morphology with unclear cell-to-cell boundary and proliferative responsiveness to LIF. Moreover, 5-AzaC increased the expressions of ES specific markers, SSEA-1, and alkaline phosphatase activity as well as ES specific genes, Oct4, Nanog, and Sox2. We also found that 5-AzaC demethylated the promoter region of H19 gene, a typical methylated gene during embryonic differentiation. These results indicate that 5-AzaC reverses differentiation state of ES cells through its DNA demethylating activity to differentiation related genes.

Keywords: ES; EB; 5-Azacytidine; H19; SSEA-1; Methylation; Demethylation; Differentiation

Epigenetic modification of the genome occurs during early development of embryos and DNA methylation of cytosine residues is a major cause of the modification [1–3]. To establish DNA methylation patterns during embryonic development, the embryo undergoes sequential events of demethylation and de novo methylation before and after implantation, respectively [4]. Therefore, de novo methylation activity is detected mainly in early postimplantation embryo [5], ES cells [6], and embryonic carcinoma cells [7], whereas in postgastrulation embryos, adult somatic cells, and differentiated EC cells its activity is low or undetectable [4,7,8].

In ES cells, four active enzymes of DNA cytosine methyltransferase, such as Dnmt1, Dnmt3a, Dnmt3b, and Dnmt3l, have been identified so far. Dnmt1 is a major DNA methyltransferase for maintaining methylation status during DNA replication, and inactivation of this enzyme in mice results in loss of genomic imprinting and leads to the early embryonic lethality [9,10]. Dnmt3a and Dnmt3b, which mainly catalyze de novo methylation, are highly active in ES cells and in vitro differentiation of ES cells is blocked in the absence of these enzymes [11]. Dnmt3l associates with both Dnmt3a and Dnmt3b, and regulates these activities [12]. These reports show the essential roles of methylation and demethylation in ES cell differentiation and developing embryos.

One of the demethylating agents, 5-AzaC, that is a cytosine analog, can cause extensive demethylation of 5-methylcytosine residues and reduce DNA methyltransferase activity in the cells [13,14]. It has been used as an effective chemotherapeutic agent for leukemia [15] and also as a useful experimental tool to study the

☆ *Abbreviations:* EB, embryoid body; ES, embryonic stem.

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roles of DNA methylation in the cell differentiation and gene activation mechanisms. For example, 5-AzaC could induce muscle cell differentiation from fibroblast cell lines [16] and cardiomyogenic cell differentiation from mesenchymal stem cells [17]. There are also reports on modulating activities of 5-AzaC to the embryonic development by influencing cell proliferation and differentiation [18–21].

In the present study, we investigated the effects of 5-AzaC on ES cell differentiation by forming embryoid body, which was often used as an in vitro model of embryonic development. We found that 5-AzaC reversed differentiation of ES cells on morphological, phenotypical, and genetic characteristics. We also indicated reversing effects of 5-AzaC through its demethylating activity by H19 gene analysis.

Materials and methods

Cell culture and 5-azacytidine treatment. The murine ES cell line, E14.1, was kindly provided by Dr. Kitamura (Tokyo University of Science, Japan). The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA) supplemented with non-essential amino acid, 15% heat-inactivated fetal bovine serum (FBS), and 10^{-4} M of 2-mercaptoethanol (2-ME) on gelatin-coated 10-cm culture plates. For an undifferentiated state, the cells were maintained with 10^3 U/ml LIF (Esgro, Gibco) on the feeder layers, mouse embryonic fibroblast (MEF) treated with mitomycin C. For induction of differentiation, 1×10^8 cells were subjected to spinner culture of embryoid body (EB) formation in 500 ml medium without MEF and LIF as previously described [22,23]. Every three days, the medium was freshly changed. After the days of 7 (EB7) and 11 (EB11), EBs were trypsinized to a single cell suspension and washed with culture medium. The cells (4×10^5 cells/90-mm dish) were treated with 1 μM of 5-azacytidine (5-AzaC, Sigma, MO, USA) for 6 h and were subsequently cultured without 5-AzaC for 3 days.

Flow cytometry analysis. Anti-SSEA-1 antibody was purchased from American Research Product (MA, USA) and anti-E-cadherin antibody was purchased from BD PharMingen (CA, USA). FITC labeled goat Anti-mouse Igs (Caltag Laboratories, CA, USA) for SSEA-1 and FITC labeled goat anti-rat Igs (Caltag Laboratories) for E-cadherin were used as second antibodies. The staining was performed and analyzed by flow cytometry (EPICS XL, Beckman–Coulter, FL, USA) as previously reported [24].

Alkaline phosphatase activity. Cell extracts were prepared by freezing/thawing the cells (1×10^5 cells/tube) and then centrifuging at 10,000*g* for 5 min. The supernatants were mixed with *p*-nitrophenyl-phosphate (SIGMA FAST *p*-nitrophenyl phosphate tablet set) as a substrate of alkaline phosphatase (ALP). After incubation for 30 min at 37 °C, the relative activities among the cells were measured by reading OD at 405 nm wavelength.

RT-PCR analysis. Total RNA was isolated from the cells with RNeasy (Qiagen, CA, USA). RT-PCR detection for Oct4, Nanog, and Sox2 genes was performed as previously described [25,26]. As a control, we used a housekeeping gene, HGPRT [24]. The primers used were: 5′ AGGGTCTGCTACTGAGATGCTCTG 3′ as a sense primer and 5′ CAACCACTGGTTTTTCTGCCACCG 3′ as an antisense primer for Nanog, 5′ CTGAGGGCCAGGCAGGAGCACGAG 3′ as a sense primer and 5′ CTGTAGGGAGGGCTTCGGGCACTT 3′ as an antisense primer for Oct4, and 5′ GGCAGCTACAGCATGATGCAGG AGC 3′ as a sense primer and 5′ CTGGTCATGGAGTTGTACTGC AGG 3′ as an antisense primer for Sox2. We determined semiquantitative conditions by changing PCR cycles for each gene (data not shown).

Bisulfite PCR for methylation analysis. Genomic DNA (10 μg) was digested with *Bam*HI and converted with bisulfite as previously reported [27,28]. Briefly, the digested genomic DNA (1–3.5 μg) was incubated in a solution of 3.2 M sodium bisulfate (Sigma) and 500 nM hydroquinone (Sigma) for 16 h at 55 °C. The reaction mixture was desalted using a DNA cleanup column (Promega, WI, USA) and eluted with H_2O as in manufacturer's instruction. The eluted DNA was incubated with 300 mM NaOH for 20 min at 37 °C and stored at −20 °C. The DNA was used as a template of bisulfite converted DNA for the methylation analysis. Bisulfite PCR were performed using AmpliTaq Gold (Perkin–Elmer, CA, USA). The primers used were: H19s 5′ GATTAGATAGTATTGAGTTTGTTTGGAGT 3′ (1089–1117 in H19 upstream sequence; GenBank Accession No. U19619) as a sense primer, H19a 5′ CCTAAAATACTAAACTTAAATAACCCAC AA 3′ (1481–1452) as an antisense primer, and the PCR conditions were used as described previously [29]. For determination of methylation on H19 promoter, the DNA after PCR amplification was digested with *Cla*I. This *Cla*I site was reported to be one of the target sequences of DNA methyltransferase in ES cells [29]. *Cla*I (recognition sequence, ATCGAT) could cut the methylated H19 sequence, which was not converted by bisulfite, however, *Cla*I could not cut the unmethylated H19 sequence, which was converted to ATTGAT by bisulfite. We confirmed the converted sequence by DNA sequence analysis (data not shown).

Results and discussion

The in vitro differentiation of murine ES cells to EB mimics events that occur after implantation in embryonic development in vivo. In the present study, we induced ES cell differentiation by forming EB clusters in a spinner culture method and prepared the cells from days 7 (EB7) and 11 (EB11) EBs after the differentiation induction. Each EB is supposed to contain cell lineages corresponding to the cells from three germ layers according to its differentiation progression. EB7 should contain primitive endoderm, primitive mesoderm, notochord, vasculature, and cardiogenic mesoderm, whereas EB11 should have more advanced cell lineage like neural tube [22]. We confirmed the fully differentiated features of our EBs by observing beating cells in an EB cluster and endoderm- and mesoderm-like tissues on EB histological sections. Therefore, we used these EB cells as a target of 5-AzaC treatments. Non-toxic dose (1 μM) of 5-AzaC was determined in a preliminary study (data not shown).

We first examined the effects of 5-AzaC in morphological aspects. As shown in Fig. 1A, the undifferentiated ES cells in culture exhibited a characteristic morphology, which was designated as a stem cell-like colony featuring clumped cells with unclear cell boundary [30]. Before the 5-AzaC treatments, adherent cells from EB7 and EB11 displayed fibroblast-like or epithelial-like morphology, which is distinct from that of ES cells (Figs. 1B and E). Once these cells were treated with 5-AzaC, the stem cell-like colony appeared again from the cultures of EB7 and EB11 (Figs. 1C and F).

In addition, these stem cell-like colonies induced by 5-AzaC were functionally similar to the original undifferentiated ES colonies because they were able to

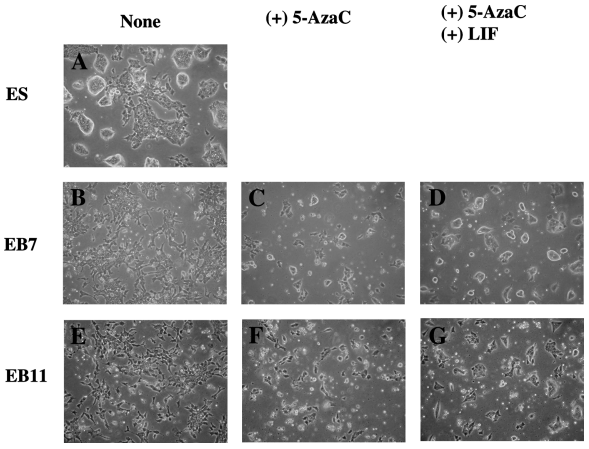

Fig. 1. Morphological changes of EB cells by 5-AzaC treatments. ES cells were induced first to form EB and then treated with 5-AzaC alone or 5-AzaC plus LIF. (A) ES cells, (B) EB7 cells, (C) EB7 cells with 5-AzaC, (D) EB7 cells with 5-AzaC and LIF, (E) EB11 cells, (F) EB11 cells with 5-AzaC, and (G) EB11 cells with 5-AzaC and LIF. A representative example of more than three experiments is shown.

respond to LIF and they proliferate well keeping the stem cell-like colony morphology (Figs. 1D and G). LIF increased the growth of 5-AzaC-treated EB7 cells more than twofold compared with non-LIF treatment (data not shown).

We further investigated the changes of two ES specific surface markers, SSEA-1 and E-cadherin, during the EB formation and 5-AzaC treatments. Both markers have been used to monitor the immature status of murine ES cells [23]. As shown in Fig. 2, expression of SSEA-1 and E-cadherin changed during EB formation. In particular, SSEA-1 expression became nearly undetectable in either EB7 or EB11 cells. Then, 5-AzaC treatments could restore the expression of SSEA-1 in these cells. The expression of E-cadherin shifted from low to high level during the EB formation, however, the expression in both EB7 and EB11 cells shifted to an ES levels by 5-AzaC treatments. We also examined another ES specific marker, alkaline phosphatase (ALP) activity [31]. As shown in Table 1, ALP activity decreased during differentiation from ES to EB11 cells and then increased again after 5-AzaC treatments to a higher level than in original ES cells.

We also analyzed the expressions of ES specific transcriptional factors, Oct4, Nanog, and Sox2. These genes have been reported to play essential roles in the maintenance of undifferentiated status of ES cells. Therefore, the expressions of these genes are highly correlated with undifferentiation status [25,26]. As shown in Fig. 3, Oct4, Nanog, and Sox2 expressions were detected at high levels in ES cells, but gradually reduced during EB formation. 5-AzaC treatment induced re-expression of Oct4 and Nanog genes in EB7 and Sox2 gene in EB11. Taken together, morphological/phenotypical analysis as well as gene expression analysis indicated

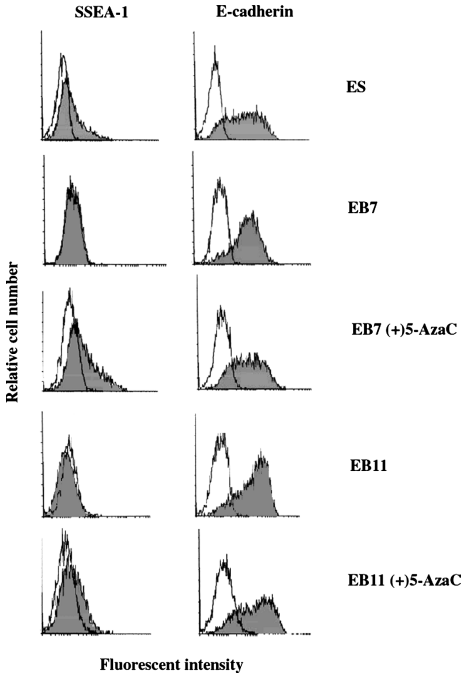

Fig. 2. Restoration of ES specific markers by 5-AzaC treatments. Surface expressions of SSEA-1 and E-cadherin were analyzed by flow cytometer. The cells were stained with an antibody specific for SSEA-1 or E-cadherin (shaded histogram). Staining with a negative control antibody is shown as open histograms. A representative example of more than three experiments is shown.

Table 1
Alkaline phosphatase (ALP) activity

	ALP activity/10^5 cells (OD 405 nm)
ES	0.835 ± 0.131
EB11	0.559 ± 0.082
EB11 + 5-AzaC	1.275 ± 0.127

ALP activities in ES, EB11, and 5-AzaC-treated EB11 cells were determined by measuring OD at 405 nm with *p*-nitrophenyl phosphate as a substrate. Data are given as means ± SD for three tubes and represent three separate experiments.

that 5-AzaC could reverse the differentiated status of EB cells back to ES cells.

To understand the mechanisms of differentiation reversing effects of 5-AzaC, we next examined the demethylating activity of this agent. One particular gene, H19, has been documented as a useful indicator for genome methylation status during ES cell differentiation [32–35]. As an imprinting gene, the promoter region of H19 gene locating approximately 2–4 kb upstream from the transcription start site has been designated a differentially methylated site depending on paternal and maternal origin during embryonic development [36]. Also, during ES cell differentiation, the promoter region of paternal H19 gene was de novo methylated and the methylation intensity was reduced when Dnmt1 was impaired [29]. Therefore, we analyzed DNA methylation

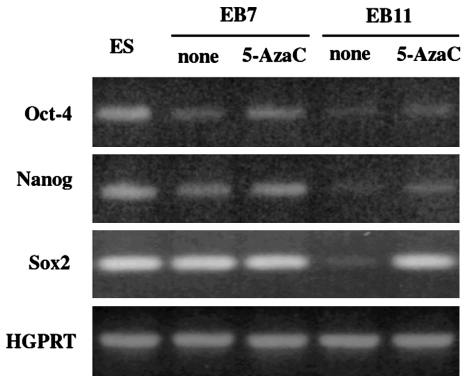

Fig. 3. Re-expression of ES specific regulatory genes after 5-AzaC treatments. RT-PCR analysis for Oct4, Nanog, and Sox2 genes was performed by preparing RNA from ES, EB, and 5-AzaC-treated EB cells. HGPRT was used as a control. A representative example from two independent experiments is shown.

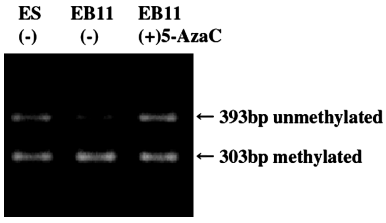

Fig. 4. Methylation analysis of H19 gene by bisulfite PCR. Genomic DNA was isolated from ES, EB11, and 5-AzaC-treated EB11 cells. Restriction digestions of bisulfite-treated and amplificated DNA were performed as in Materials and methods. The fragments of 303 and 393 bp indicate methylated and unmethylated H19 genes, respectively. A representative example of more than three experiments is shown.

level in the H19 promoter region by the bisulfate PCR method as previously described [29]. As shown in Fig. 4, we observed two PCR fragments of methylated (303 bp) and unmethylated (393 bp) H19 genes. In undifferentiated ES cells, there were both PCR bands at similar intensity between methylated and unmethylated genes, whereas in differentiated EB11 cells the methylated gene's band dominated over unmethylated one, showing that DNA methylation level increased during the cell differentiation. When EB11 cells were treated with 5-AzaC, unmethylated H19 gene increased again to the similar level to ES cells.

Reactivation of silenced genes by 5-AzaC has been reported for a number of specific genes, including VHL gene [37], E-cadherin gene [38], estrogen receptor gene [39], p16 gene [40], exogenously introduced genes [41], and entire genes on inactivated X-chromosome [42]. In our study, we could not identify the specific genes directly involved in reversing ES differentiation.

ES specific transcriptional factors, Nanog, Oct-4, and Sox2, were re-expressed after 5-AzaC treatments as shown in Fig. 3. We considered these genes as targets of 5-AzaC-induced DNA methylation. Very recently, Hattori et al. [43] demonstrated that there were correlations between DNA methylation status of Oct-4 and differentiation status of embryonic cells. They also observed that the treatment of a demethylating agent could reactivate Oct-4 gene in trophoblast stem cells. However, there is no report on the correlations between DNA methylation status of other genes, including Nanog and Sox2, and embryonic differentiation to our knowledge. STAT family genes are probable targets among candidate genes for demethylating agents. STAT molecules play key roles in maintaining an undifferentiated status and self-renewal activity of ES cells by transducing LIF signals [44]. Karpf et al. [45] observed that silenced STAT genes in colon tumor cells were activated by the treatment with 5-aza-2′-deoxycytidine, which resulted in a conversion of IFN-α resistant tumor cells into sensitive cells. Further experiments should be done to elucidate the target genes of DNA demethylation by 5-AzaC.

In this study, we demonstrated that 5-AzaC could reverse the differentiation of EB cells morphologically, phenotypically, and genetically. To our best knowledge, our study is the first to show the effects of 5-AzaC on ES cell differentiation, and indicates the correlation of both differentiation reversing activity and demethylating activity of 5-AzaC. Moreover, our findings will provide the demethylating agents as a useful tool for the analysis of embryonic development in vitro.

References

[1] F. Santos, B. Hendrich, W. Reik, W. Dean, Dynamic reprogramming of DNA methylation in the early mouse embryo, Dev. Biol. 241 (2002) 172–182.
[2] H. Lei, S.P. Oh, M. Okano, R. Juttermann, K.A. Goss, R. Jaenisch, E. Li, De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells, Development 122 (1996) 3195–3205.
[3] A.J. Peter, T. Daiya, The role of DNA methylation in mammalian epigenetics, Science 293 (2001) 1068–1070.
[4] R. Wolf, W. Dean, J. Walter, Epigenetic reprogramming in mammalian development, Science 293 (2001) 1089–1093.
[5] J.C. Gutierrez, S. Callejas, S. Borniquel, A. Martin-Gonzalez, DNA methylation in ciliates: implications in differentiation processes, Int. Microbiol. 3 (2000) 139–146.
[6] T.H. Bestor, The DNA methyltransferases of mammals, Hum. Mol. Genet. 9 (2000) 2395–2402.
[7] C. Stewart, H. Stuhlmann, D. Jahner, R. Jaenisch, De novo methylation, expression, and infectivity of retroviral genomes introduced into embryonal carcinoma cells, Proc. Natl. Acad. Sci. USA 79 (1982) 4098–4102.
[8] R.D. Palmiter, H.Y. Chen, R.L. Brinster, Differential regulation of metallothionein–thymidine kinase fusion genes in transgenic mice and their offspring, Cell 29 (1982) 701–710.
[9] D. Biniszkiewicz, J. Gribnau, B. Ramsahoye, F. Gaudet, K. Eggan, D. Humpherys, M.A. Mastrangelo, Z. Jun, J. Walter, R. Jaenisch, Dnmt1 overexpression causes genomic hypermethylation, loss of imprinting, and embryonic lethality, Mol. Cell. Biol. 22 (2002) 2124–2135.
[10] M.F. Chan, R. van Amerongen, T. Nijjar, E. Cuppen, P.A. Jones, P.W. Laird, Reduced rates of gene loss, gene silencing, and

gene mutation in Dnmt1-deficient embryonic stem cells, Mol. Cell. Biol. 21 (2001) 7587–7600.
[11] M. Okano, D.W. Bell, D.A. Haber, E. Li, DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development, Cell 99 (1999) 247–257.
[12] U. Aapola, R. Lyle, K. Krohn, S.E. Antonarakis, P. Peterson, Isolation and initial charasterization of the mouse Dnmt3l gene, Cytogenet. Cell Genet. 92 (2001) 122–126.
[13] T. Haaf, M. Schmid, Experimental condensation inhibition in constitutive and facultative heterochromatin of mammalian chromosomes, Cytogenet. Cell Genet. 91 (2000) 113–123.
[14] X. Cheng, DNA modification by methyltransferases, Curr. Opin. Struct. Biol. 5 (1995) 4–10.
[15] L.R. Silverman, J.F. Holland, R.S. Weinberg, B.P. Alter, R.B. Davis, R.R. Ellison, E.P. Demakos, C.J. Cornell, R.W. Carey, C. Schiffer, E. Frei III, O.R. McIntypr, Effects of treatment with 5-azacytidine on the in vivo and in vitro hematopoiesis in patients with myelodysplastic syndromes, Leukemia (Baltimore) 7 (1993) 21–29.
[16] S.M. Taylor, P.A. Jones, Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine, Cell 17 (1979) 771–779.
[17] K. Fukuda, Development of regenerative cardiomyocytes from mesenchymal stem cells for cardiovascular tissue engineering, Artif. Organs 25 (2001) 187–193.
[18] M. Vlahovic, F. Bulic-Jakus, G. Juric-Lekic, A. Fusis, S. Maric, D. Serman, Changes in the placenta and in the rat embryo caused by the demethylating agent 5-azacytidine, Int. J. Dev. Biol. 43 (1999) 843–846.
[19] F. Bulic-Jakus, M. Vlahovic, G. Juric-Lekic, V. Crenek, D. Serman, Gastrulating rat embryo in a serum-free culture model: changes of development caused by teratogen 5-azacytidine, Altern. Lab. Anim. 27 (1999) 925–933.
[20] E.L. Patkin, M.E. Kustova, P. Perticone, The influence of demethylating agents on preimplantation development of mice, Zygote 6 (1998) 351–358.
[21] M. Vlahovic, F. Bulic-Jakus, V. Crnek, D. Serman, S. Herman, N. Skreb, 5-Azacytidine can alter gene expression in cultivated mammalian embryos, Period. Biol. 96 (1994) 65–66.
[22] A. Leahy, J.W. Xiong, F. Kuhnert, H. Stuhlmann, Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation, J. Exp. Zool. 284 (1999) 67–81.
[23] V. Ling, S. Neben, In vitro differentiation of embryonic stem cells: immunophenotypic analysis of cultured embryoid bodies, J. Cell. Physiol. 171 (1997) 104–115.
[24] T. Otani, S. Nakamura, T. Inoue, Y. Ijiri, K. Tsuji-Takayama, R. Motoda, K. Orita, Erythroblasts derived in vitro from embryonic stem cells in the presence of erythropoietin do not express the TER-119 antigen, Exp. Hematol. 32 (2004) 607–613.
[25] K. Mitsui, Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda, S. Yamanaka, The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells, Cell 113 (2003) 631–642.
[26] M.V. Zappone, R. Galli, R. Catena, N. Meani, S. De Biasi, E. Mattei, C. Tiveron, A.L. Vescovi, R. Lovell-Badge, S. Ottolenghi, Sox2 regulatory sequences direct expression of a beta-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells, Development 127 (2000) 2367–2382.
[27] S.J. Clark, M. Frommer, Bisulfite genomic sequencing of methylated cytosines, in: G.R. Taylor (Ed.), Laboratory Methods for the Detection of Mutations and Polymorphisms in DNA, CRC Press, Boca Raton, FL, 1997, pp. 151–162.
[28] S.J. Clark, J. Harrison, C.L. Paul, M. Frommer, High sensitivity mapping of methylated cytosines, Nucleic Acids Res. 22 (1995) 2990–2997.
[29] P.M. Warnecke, D. Biniszkiewicz, R. Jaenisch, M. Frommer, S.J. Clark, Sequence-specific methylation of the mouse H19 gene in embryonic cells deficient in the Dnmt-1 gene, Dev. Genet. 22 (1998) 111–121.
[30] H. Niwa, J. Miyazaki, A.G.. Smith, Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells, Nat. Genet. 24 (2000) 372–376.
[31] S. Strickland, K.K. Smith, K.R. Marotti, Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP, Cell 21 (1980) 347–355.
[32] F. Poirier, C.T. Chan, P.M. Timmons, E.J. Robertson, M.J. Evans, P.W. Rigby, The murine H19 gene is activated during embryonic stem cell differentiation in vitro and at the time of implantation in the developing embryo, Development 113 (1991) 1105–1114.
[33] W. Dean, L. Bowden, A. Aitchison, J. Klose, T. Moore, J.J. Meneses, W. Reik, R. Feil, Altered imprinting gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes, Development 125 (1996) 2273–2282.
[34] R. Feil, J. Walter, N.D. Allen, W. Reik, Developmental control of allelic methylation in the imprinting mouse Igf2 and H19 genes, Development 120 (1994) 2933–2943.
[35] T.L. Davis, K.D. Tremblay, M.S. Bartolomei, Imprinted expression and methylation of the mouse H19 gene are conserved in extraembryonic lineages, Dev. Genet. 23 (1998) 111–118.
[36] K.D. Tremblay, K.L. Duran, M.S. Bartolomei, A 5′ 2-kilobase-pair region of the imprinted mouse H19 gene exhibits exclusive paternal methylation throughout development, Mol. Cell. Biol. 17 (1997) 4322–4329.
[37] J.G. Herman, F. Latif, Y. Weng, M.I. Lerman, B. Zbar, S. Liu, D. Samid, D.S. Duan, J.R. Gnarra, W.M. Linehan, S.B. Baylin, Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma, Proc. Natl. Acad. Sci. USA 91 (1994) 9700–9704.
[38] K. Yoshiura, Y. Kanai, A. Ochiari, Y. Shimoyama, T. Sugimura, S. Hirohashi, Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas, Proc. Natl. Acad. Sci. USA 92 (1995) 7416–7419.
[39] Y.L. Ottaviano, J.P. Issa, F.F. Parl, H.S. Smith, S.B. Baylin, N.E. Davidson, Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells, Cancer Res. 54 (1994) 2552–2555.
[40] C.M. Bender, M.M. Pao, P.A. Jones, Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines, Cancer Res. 58 (1998) 95–101.
[41] L. Broday, Y.W. Lee, M. Costa, 5-Azacytidine induces transgene silencing by DNA methylation in Chinese hamster cells, Mol. Cell. Biol. 19 (1999) 3198–3204.
[42] P.A. Jones, S.M. Taylor, T. Mohandas, L.J. Shapiro, Cell cycle-specific reactivation of an inactive X-chromosome locus by 5-azadeoxycytidine, Proc. Natl. Acad. Sci. USA 79 (1982) 1215–1219.
[43] N. Hattori, K. Nishino, Y.G. Ko, N. Hattori, J. Ohgane, S. Tanaka, K. Shiota, Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells, J. Biol. Chem. 279 (2004) 17063–17069.
[44] S. Rose-John, Gp130 stimulation and the maintenance of stem cells, Trend Biotechnol. 20 (2002) 417–419.
[45] A.R. Karpf, P.W. Peterson, J.T. Rawlins, B.K. Dalley, Q. Yang, H. Albertsen, D.A. Jones, Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells, Proc. Natl. Acad. Sci. USA 96 (1999) 14007–14012.